EMERGING ROLES AND MECHANISMS OF STROMAL CELLS IN CARCINOMAS AT THE MOLECULAR LEVEL

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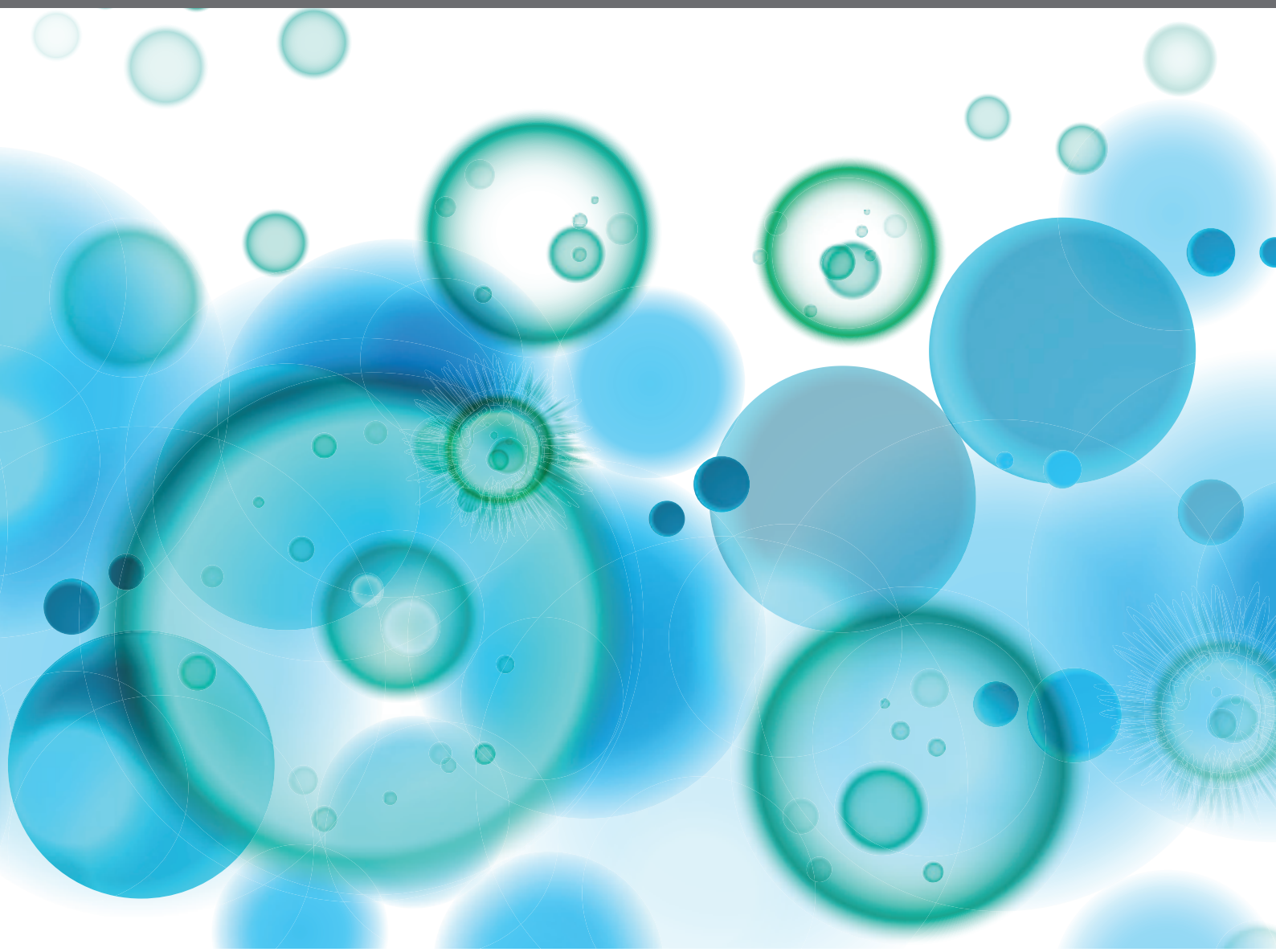

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Editorial: Emerging roles and mechanisms of stromal cells in carcinomas at the molecular level

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Editorial on the Research Topic
Emerging roles and mechanisms of stromal cells in carcinomas at the molecular level

For an extended period, the tumor microenvironment (TME) has not been focused on in the field of cancer biology before Stephen Paget's "seed and soil" hypothesis (1). The representative characters of cells involved in TME are high plasticity and continuous phenotypic and functional change. For example, the desmoplastic reaction in pancreatic cancer is a critical histological observation, tightly associated with significantly increasing the interstitial fluid pressure within the tumor niche. Furthermore, the desmoplastic stroma and compressed vessel delay or block the circulating therapeutic agents' target location. Inflammation is well-known as a critical factor in developing TME enhancing tumorigenesis and cancer promotions in carcinomas.

Stromal and immune cells usually surround and harmonize with cancer cells or mass, forming the inflammatory TME. Interactions between tumor cells and tumor-associated stromal cells (TASCs) have critical roles in tumor growth and progression. In this context, among stromal cells, fibroblast-like cells, mesenchymal stromal cells,

and carcinoma-associated fibroblasts can be considered the main players involved in either pro- or anti-tumorigenic effects. The complexity of stroma-tumor interaction shows remarkable heterogeneous tumor mass formation even though this process has high similarity with normal wound healing processes such as neoangiogenesis, fibroblast, and immune cell infiltration.

Ten articles were contributed to this specific Research Topic and classified into the following categories: three original research (Gao et al., Joo et al., and Peng et al.) and six reviews (Hwang et al., Tagirasa and Yoo, Kim et al., Shim et al., Mun et al., Kim et al., and Koppensteiner et al.).

Gao et al. revealed the upregulation of PD-L1 expression in colorectal cancer (CRC) by cancer-associated fibroblasts (CAFs) is mediated by Akt phosphorylation. Since CAFs are one of the major components of TME and exert as immune regulators to generate immune suppression in TME. In this study, the upregulation of PD-L1 expression in CRC by CAFs through the activation of Akt was confirmed with colorectal cancer cell lines and also with human CRC patients in correlation with the disease-free survival. Koppensteiner et al. reviewed the immune regulating effects of CAFs in anti-cancer T cell therapy. In this review, the authors suggested the interplay of T cells and CAFs by bidirectional crosstalk plays a significant role in TME. They discussed various mechanisms by the interplay and crosstalk of CAFs and T cells that leads to the negative anti-cancer immune responses.

We have two review articles addressing specific molecular families affecting TME originating from stromal environments. First, Tagirasa and Yoo reviewed an exciting point of view on the tumor-stroma interface. They focused on the enzymatic activities of serine proteases, while most other contributors focused more on cellular effects on TME. They dealt with stromal serine proteases such as fibroblast-activation protein, urokinase-type plasminogen activator, kallikrein-related peptidases, and granzymes that led to the tumor progression and discussed the therapeutic applications. Secondly, Shim et al. discussed the IL-32 subfamily affecting TME. Each IL-32 subtype has a different role on cancer cells, and the stromal IL-32 is still unclear to the TME yet, but they discuss IL-32 as a possible regulatory role in cancers.

Two original articles revealed the novel mechanisms of TME affected by the stromal environments. Joo et al. told a new subset of CAF using single-cell RNA sequencing from frozen skin tissue with adult T-cell leukemia/lymphoma (ATLL) patients. Their study identified a novel CAF subset with enhanced EGR1 and EGR2 expression. These cells can highly proliferate CD4 T cells *via* FGF7-FGF1 and PDGFA-PDGFRA/B signaling. They are also associated with the CD8 and NKT subset expansions, which can be a new therapeutic target in the future. Peng et al. state the evasion of NK cell immune surveillance *via* cytoskeleton remodeling. They discovered cancer cells resistant to the immune responses through enhanced vimentin and actin reorganization. This was also observed from human tumor samples, which may have clinical value in terms of cancer diagnostics.

Kim et al. discussed the role of stroma in specific cancer subsets such as an endometrial tumor. They were focusing on stromal tumors rather than carcinomas. Endometrial stromal tumor is a rare subset of cancer, and they categorized the disease categories according to the genetic alteration and suggested possible therapeutic approaches. Indeed, the discovery of drugs to treat stromal tumor may represent a powerful approach to affect the pro-tumorigenic effect of stromal cells in carcinomas.

Mun et al. reviewed the interplay of immune cells and stromal cells in the tumor microenvironment. They discussed about the positive and negative relationships from the point of view of tumor development for use in research applications and therapeutic strategies. Kim et al. reviewed the solid tumor and TME. They discuss the adaptation process of tumors to adverse environments *via* communication with neighboring cells to overcome unwanted growth conditions.

This Research Topic focuses on enlightening the current and recent findings on the interplay between cancer inflammation and TME to understand the obstacles of cancer therapy for epithelial cancers. Cancer immunotherapy is one of the powerful strategies to cure cancer since engineered cell therapy has been popular recently, such as oncolytic viruses, antibody therapies, and CAR-T therapy (2). However, most current immunotherapies have limitations in targeting solid tumors like carcinomas. Tumor microenvironment-targeting therapy for carcinomas has the unmeasurable potential to synergize the current immunotherapy if we overcome the current hurdles (3). The etiologies of immunotherapy resistance are multi-layered, not only the issue of tumor cells but also the complexity of the interplay between carcinomas and the microenvironment in their solid mass. One reason to look at the stromal cells in carcinomas in the aspect of inflammation is that TASCs secrete many inflammation-related molecules, including IL-6, IL-8, stromal-derived factor-1 alpha VEGF, besides TGFβ. These molecules can trigger carcinoma cell growth and subsequent metastasis through epithelial-mesenchymal transition improving the pro-tumorigenic properties of TME. Solving the puzzles of tumor microenvironment and inflammation in carcinomas at the molecular levels will enable us to address currently unsolved problems in understanding malignant carcinomas' mechanisms and therapeutic directions.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Cancer-Associated Fibroblasts Promote the Upregulation of PD-L1 Expression Through Akt Phosphorylation in Colorectal Cancer

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Upregulation of immune checkpoint proteins is one of the main mechanisms for tumor immune escape. The expression of programmed death ligand-1 (PD-L1) in colorectal cancer (CRC) is higher than in normal colorectal epithelial tissue, and patients with higher PD-L1 expression have a poorer prognosis. Additionally, PD-L1 expression in CRC is affected by the tumor microenvironment (TME). As a major component of the TME, cancer-associated fibroblasts (CAFs) can act as immune regulators and generate an immunosuppressive tumor microenvironment. Therefore, we speculated that CAFs may be related to the upregulation of PD-L1 in CRC, which leads to tumor immune escape. We found that CAFs upregulate PD-L1 expression in CRC cells through AKT phosphorylation, thereby reducing the killing of CRC cells by peripheral blood mononuclear cells. The ratio of CAFs to CRC cells was positively correlated with AKT phosphorylation and the expression of PD-L1 in CRC *in vitro*. Consistent with the *in vitro* results, high CAF content and high expression of PD-L1 were negatively correlated with disease-free survival (DFS) of CRC patients. These results indicate that the upregulation of PD-L1 expression in CRC by CAFs through the activation of Akt is one of the molecular mechanisms of tumor immune escape. Thus, targeted anti-CAF therapy may help improve the efficacy of immunotherapy.

Keywords: colorectal cancer, cancer-associated fibroblasts, PD-L1, Akt phosphorylation, immune escape

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies around the world, ranking third in overall incidence and fourth in terms of cancer-related mortality (1, 2). In spite of continuous improvements in CRC therapy, the average 5-year survival of cancer patients at all stages is 45-60% (2, 3). Recent studies have found that the tumor immune microenvironment, infiltration of immune

cells, inflammatory cytokines, and expression of immune checkpoint proteins greatly impact the survival of CRC patients (4–9).

As one of the most important immune checkpoint proteins, programmed death ligand-1 (PD-L1) is upregulated in CRC and its high expression is corelated with a poor prognosis, suggesting that PD-L1 may be involved in the progression of CRC (4, 8, 10). The binding of PD-L1 to PD-1 inhibits T cell function and promotes tumor evasion (11). The upregulation of PD-L1 is one of the most important and most widely studied mechanisms of immune escape (12). Blocking the PD-1/PD-L1 axis can improve the efficacy of immunotherapy, and improve the survival of patients (4, 13–15). Therefore, it is warranted to study the mechanism of PD-L1 upregulation in CRC, which may provide new therapeutic strategies.

Upregulation of PD-L1 expression in tumor cells may be affected by the tumor microenvironment (TME) (16–18). As one of its most abundant components, cancer-associated fibroblasts (CAFs) can affect the TME by secreting a variety of chemokines, cytokines, and growth factors, as well as regulating the composition of the extracellular matrix, leading to immunosuppression and tumor progression (19, 20). Previous studies have shown that CAFs can promote the growth and survival of tumor cells as well as tumor angiogenesis in CRC, leading to a poor prognosis (21–23). CAFs are proven to upregulate PD-L1 expression in tumor cells, but the underlying mechanism remains insufficiently clear (16–18). In our previous study, we also found that the expression of α-smooth muscle actin [α-SMA, a marker gene of CAFs (24)] is positively correlated with PD-L1 expression in clinical CRC samples. Consequently, we further explored the mechanism by which CAFs regulate the expression of PD -L1, thus elucidating a possible mechanism through which CAFs promote the immune escape of CRC.

MATERIALS AND METHODS

Isolation and Culture of Human Adipose Tissue Derived Mesenchymal Stem Cells (hAD-MSCs)

Human adipose tissue was collected from the plastic surgery department of Peking Union Medical College Hospital (PUMCH, Beijing, China) with the donors' informed consent. Then, hAD-MSCs were isolated and cultured according to the method described in our previous study (25). The adipose tissue was washed with D-Hanks' buffer and centrifuged at 1,000 g for 3min. The adipose tissue below the liquid surface was transferred to a fresh centrifugal tube, washed, digested with 0.2% collagenase P (Life Technology Corporation, USA) and incubated at 37°C for 30min. The undigested adipose tissue was removed with a 100-μm cell strainer. Then, an appropriate amount of D-Hanks' buffer was added and centrifuged at 1500g for 10min. The supernatant was discarded and the pellet was washed twice with phosphate buffered saline (PBS). Then, the cells were collected through centrifugation. Finally, 1×10^6 cells were seeded into the culture medium and incubated at 37°C in a humidified atmosphere comprising 5% CO_2. The culture medium was changed every 2-3 days. The cells were passaged or cryopreserved after reaching 80% confluence.

Flow Cytometry

Flow cytometry was used to identify the immunophenotypes of hAD-MSCs according to a published method (25). Approximately 2×10^5 hAD-MSCs were harvested, washed with PBS, and incubated with primary antibodies (CD31, CD34, CD106, CD29, CD44, CD73, CD90; BD Pharmingen, USA) at 4°C for 1h. After washing off the primary antibodies, the hAD-MSCs cells were incubated with a fluorescence-labeled secondary antibody (BD Pharmingen, USA) at 4°C for 30min. The immunopositive cells were quantified using an Accuri C6 Flow Cytometer (BD Biosciences, USA) and the data were analyzed using Cflow Plus Software (BD Biosciences, USA).

Cultivation of CRC Cell Lines

Human CRC cell lines HCT116, HCT8 and LOVO were obtained from the Cell Resource Center, IBMS, CAMS/PUMC (Beijing, China). The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Gibco, USA) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA) at 37°C in a humidified atmosphere comprising 5% CO_2.

Extraction of Exosomes From CRC Cell Lines (CRC-Exosomes)

The HCT116/HCT8/LOVO cells were cultured in FBS-free DMEM/high glucose medium for 36-48 hours before exosome extraction. Then, the culture supernatants were collected and centrifuged at 3000g for 30min to remove cell debris and dead cells. The residual cell debris and large vesicles were removed by filtered through a 0.22-μm pore-size membrane. The filtered supernatant was sequential centrifuged at 120,000g for 2 hours at 4°C in an ultracentrifuge (Optima XPN-100, Beckman Coulter, USA). The sample was washed twice with moderate D-Hanks' buffer. The remaining liquid was filtered through a 0.2-μm pore-size membrane, aliquoted into 1.5-ml sterile EP tubes and stored at -80°C (26).

Transmission Electron Microscopy

The ultrastructure of exosomes was analyzed by transmission electron microscopy (TEM). The exosomes were collected and suspended in PBS. Then, the CRC-exosomes were fixed, dehydrated, embedded, sliced, stained with uranium acetate and lead citrate, and observed under a TEM as described previously (27).

CRC-Exosome-Induced Differentiation of hAD-MSCs Into CAFs

When the hAD-MSCs adhered to the dish after cell passaging, the medium was replaced with FBS-free DMEM/F-12 medium (Gibco, USA) and changed every other day. CRC-exosomes were added to the medium to a final concentration of 50 mg/L on d0, d2, d4, d6 and d8, respectively. HAD-MSCs were confirmed to be induced to differentiate into CAFs on the 9th day.

Adipogenic and Osteogenic Differentiation of hAD-MSCs and CAFs

When hAD-MSCs/CAFs grew to 90% confluence, the medium was replaced with adipogenic differentiation medium. The new culture medium was replaced at intervals of two days. Oil red O staining was performed on the 11th day of induction to detect the formation of lipid droplets in cells as described previously (25).

When hAD-MSCs/CAFs grew to 80% confluence, the spent medium was discarded, after which osteogenic differentiation medium was added and replaced at an interval of two days. Alizarin red staining was carried out on the 14th day of induction to detect extracellular mineralization. After incubating for 30 min at 37°C, the staining results were observed under a conventional optical microscope (25).

Uptake of Exosomes by hAD-MSCs

CRC-exosomes were labeled with 1μM 1′-Dioctadecyl-3,3,3′,3-tetramethylindocarbocyanine perchlorate (Dil) (ThermoFisher, USA) and incubated for 10min. The labeled exosomes were centrifuged at 700,000 × g at 4°C for 40min. After discarding the supernatant, the exosomes were then added into hAD-MSCs and co-incubated in dark. The cells were washed 3 times with PBS after 4 hours, fixed in 4% paraformaldehyde for 10min, and stained with Hoechst 33342 (ThermoFisher, USA). After washing 3 times with PBS, the uptake of exosomes was observed under fluorescence microscope.

Treatment of CRC Cells With CAF-CM

When the hAD-MSCs were induced to differentiate into CAFs after CRC-exosomes treatment on day9, the culture medium of CAFs was removed and the cells were rinsed with D-Hanks' buffer. Then, the cells were cultured in DMEM/high glucose medium containing 10% FBS for 24 hours, and the resulting supernatant, also known as CAFs-conditioned medium (CAF-CM), was removed and aliquoted into 1.5ml EP tubes for cryopreservation at -80°C.

CRC cells (HCT116/HCT8/LOVO) were cultured in CAF-CM for 1h, 3h, 6h, 24h and 48h, respectively. Another group of CRC cells in high glucose medium was set as a control group. The cells were suspended in neutral Radio Immunoprecipitation Assay (RIPA) lysis buffer containing 1mM phenylmethanesulfonylfluoride with/without AKT phosphorylase inhibitor (pAKTi, MK-2206 2HCI, Celleck, USA) and collected into 1.5ml EP tubes. After centrifugation, the supernatant was collected and cryopreserved.

In Vitro PBMCs Cytotoxicity Assay

Approximately 5×10^3 CRC cells (HCT8, HCT116, LOVO) were seeded into 96-well plates and co-cultured with different proportions of peripheral blood mononuclear cells (PBMCs) from healthy donors for 3 days. Firstly, the target cells were added to the wells with culture medium and incubated for 4 hours so that the cells became adherent. Then, the effector cells were mixed in the wells according to the indicated effector: target (E:T) ratios. Wells containing only target cells were used as the positive control. The blank well was used as the background control. The cells were washed with PBS for 3 times, then, 20μl of MTS [3- (4, 5-dimethylthiazol-2-yl)-5- (3-carboxymethoxyphenyl)-2- (4-sulfophenyl)-2H-tetrazolium] (Promega, USA) was added to each plate and incubated at 37°C for 1h. The absorbance at 490nm (A_{490}) was measured for each plate using a microplate reader (BioTek Epoch, USA). Each group was replicated in 6 wells. The survival of CRC cells was calculated based on the A_{490} value of each well. The cytotoxicity was calculated based on the survival of CRC cells. To further investigate the effect of Akt and PD-L1, pAKTi and PD-L1 blocker (αPD-L1) atezolizumab biosimilar (R&D System, USA) were used to pre-block pAkt and PD-L1 respectively. The cells were incubated with pAKTi/αPD-L1 for 72h and washed with PBS for 3 times before the cytotoxicity assay.

$$\%\ Cytotoxicicty = \left[1 - \frac{A_{490}Experimental\ \ well - A_{490}Background}{A_{490}Positive\ \ control - A_{490}Background}\right] \times 100$$

Analysis of Tumor Cell Apoptosis

Tumor cell apoptosis rate was detected according to the protocol of the Annexin V-FITC/PI Apoptosis Detection Kit (YEASEN, CHN). HCT116 cells were cultured with CAF-CM or normal medium for 48h. PBMCs were then added and co-cultured with tumor cells for 2 days. After HCT116 cells were treated with standard protocol, cell apoptosis rate was detected and analyzed using flow cytometry.

Western Blot Analysis

Western blot analysis was done using the same protocol as in our previous study (28). Protein concentrations of cell lysates were determined using a BCA Protein Assay Kit (Beyotime, USA). Protein samples were separated by 10% acrylamide sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA). The membranes were blocked with skim milk in Tris-buffered saline with Tween 20 (TBST) for 1 hour and incubated overnight at 4°C with primary antibodies against PD-L1 (Abcam, UK), p-Akt (Cell Signaling Technology, USA), α-SMA (Cell Signaling Technology, USA), PD-1(Cell Signaling Technology, USA), and GAPDH (Cell Signaling Technology, USA). After washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1h at room temperature. The results were recorded using an ImageQuant LAS 4000 mini imaging system (GE Healthcare, USA).

Patients and Samples

Colorectal cancer samples were retrospectively collected with informed consent from patients undergoing surgery at PUMCH from November 2014 to December 2015. All the patients underwent R0 resection and were histologically diagnosed as having CRC. The last follow-up date for patients with no progression was December 2020. The study was approved by the Ethics Committee of the Chinese Academy of Medical Sciences and Peking Union Medical College. All patients signed written informed consent forms.

Immunohistochemistry

Immunohistochemistry was performed using a standard protocol (29). The specimens were fixed with 10% formaldehyde, embedded in paraffin and sectioned into slides with 4μm thickness. The expression of PD-L1 (Abcam, UK), p-Akt (Cell Signaling Technology, USA), and α-SMA (Cell Signaling Technology, USA) were detected by immunohistochemistry (IHC). The slides were dewaxed and rehydrated, followed by antigen retrieval using microwaving in 0.01 mol/L citric acid buffer (pH 6.0). The endogenous peroxidase activity was blocked with 3% hydrogen peroxide at room temperature for 10 minutes. Goat serum was added as sealant and incubated for 20 minutes. The slides were incubated with primary antibodies at 4°C overnight. On the next day, the slides were incubated with the secondary antibody (Cell Signaling Technology, USA) at room temperature for 2 hours. Finally, the sections were observed under a microscope.

The results of IHC of all slides were reviewed independently by two pathologists who were blinded to the clinical data. If the results were inconsistent, a third pathologist was called to make the final decision. The percentage and intensity of PD-L1, p-AKT and α-SMA expression in tumor cells was scored. The membrane staining of tumor cells ≥ 1% was defined as PD-L1 positive (30). The expression of p-AKT and α-SMA were assessed using a previously published semiquantitative method (31).

Statistical Analysis

The statistical analysis was conducted using SPSS 25.0 (IBM, Armonk, NY, USA) and GraphPad Prism 8(California, USA). The correlation between the expression of PD-L1, p-AKT, α-SMA and clinicopathological features was tested using the χ^2 test. The relationship between the expression of PD-L1, p-AKT, α-SMA and disease-free survival (DFS) time was tested using the Kaplan-Meier method and the survival curve was plotted. DFS refers to the time from the R0 resection to disease recurrence demonstrated by imaging. Cytotoxicity to tumor cells was analyzed by two-way ANOVA. The two-sided probability test was adopted, with a significance level of P =0.05. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

CRC-Exosomes Induced the Differentiation of hAD-MSCs Into CAFs

To investigate the effect of CAFs on immune escape of CRC cell lines, we induced the differentiation of hAD-MSCs into CAFs *in vitro* using exosomes from CRC cell lines. We isolated hAD-MSCs from human adipose tissue according to our previous protocol (25). The hAD-MSCs expressed CD29, CD44, CD73, and CD90 (**Supplementary Figure 1A**), and had the ability of adipogenic and osteogenic differentiation (**Figure 1A**).

CRC-exosomes were purified from HCT8, HCT116 and LOVO cell lines, respectively. Western blot analysis showed that CRC-exosomes expressed Hsp70, Hsp90, and CD63 (**Figure 1B**). Exosomes purified from HCT116 cells presented as vesicles with a bilayer membrane structure with an average size of 40-100nm under TEM (**Figure 1C**). To examine whether CRC-exosomes can be taken up by hAD-MSCs, exosomes from HCT116 cells were stained with Dil and incubated with the hAD-MSCs. The results of fluorescence microscopy indicated that the exosomes were taken up into the cytoplasm (**Figure 1D**). After induction by CRC-exosomes, the expression of the marker proteins α-SMA and FAPα in hAD-MSCs was upregulated in a time-dependent manner (**Figure 1E**). The morphological changes of MSCs induced by CRC-exosomes over time were shown in **Supplementary Figure 1B**. As shown in **Figure 1A**, the pluripotency of hAD-MSCs was significantly decreased after they were induced to differentiate into CAFs. Therefore, it was demonstrated that CRC-exosomes can induce the differentiation of hAD-MSCs into CAFs.

CAFs Promote the Immune Escape of CRC Cell Lines Through PD-L1 Upregulation

To verify that CAFs promote the immune escape of tumor cells, we collected the conditioned medium of CAFs (CAF-CM) from HCT116 cells. We cultured HCT116 cells using CAF-CM and normal medium respectively, and then analyzed the killing rate of PBMCs with CAF-CM-treated HCT116 cells and control group after 3 days. Our data demonstrated that HCT116 cells treated with CAF-CM were significantly more resistant to killing by PBMCs compared with the control group (**Figure 2A**). We conducted the same experiment using HCT8 and LOVO cells, and the results were consistent (**Figures 2B, C**). To further confirm the result, we performed flow cytometry to detected the apoptosis ratio of CRC cells. We cultured HCT116 with CAF-CM or normal medium respectively for 48h. PBMCs were then added and co-cultured with tumor cells. The apoptosis kit was used for detection after 2 days. The results indicated that the apoptosis of tumor cells cultured using CAF-CM was less than that of control group (**Supplementary Figure 2**) which was consistent with the results of cytotoxicity assay. These results indicated that CAFs promoted the immune escape of CRC cell lines.

After finding that CAFs can promote the immune escape of CRC cells, we inferred that this may be mediated by the upregulation of the immune checkpoint protein PD-L1, similar to previous studies (16–18). First, we detected the effect of CAF-CM on the expression of PD-L1 in HCT8, HCT116 and LOVO cells, and found that the expression of PD-L1 was significantly upregulated 3 hours after CAF induction (**Figures 2D–F** and **Supplementary Figure 3**). While the expression of PD-1 on PBMCs did not change significantly after co-culture with CRC cell for 2 days (**Supplementary Figure 4**).

CAFs Upregulated PD-L1 in CRC Cells Through Akt Phosphorylation

Since the upregulation of PD-L1 in CRC cell lines was fast, we inferred that the upregulation might be due to phosphorylation of proteins in signaling pathways. Previous studies have shown that CAFs promote the progression of various tumors by activating the Akt signaling pathway, so we detected the expression and phosphorylation of Akt in CRC cells treated

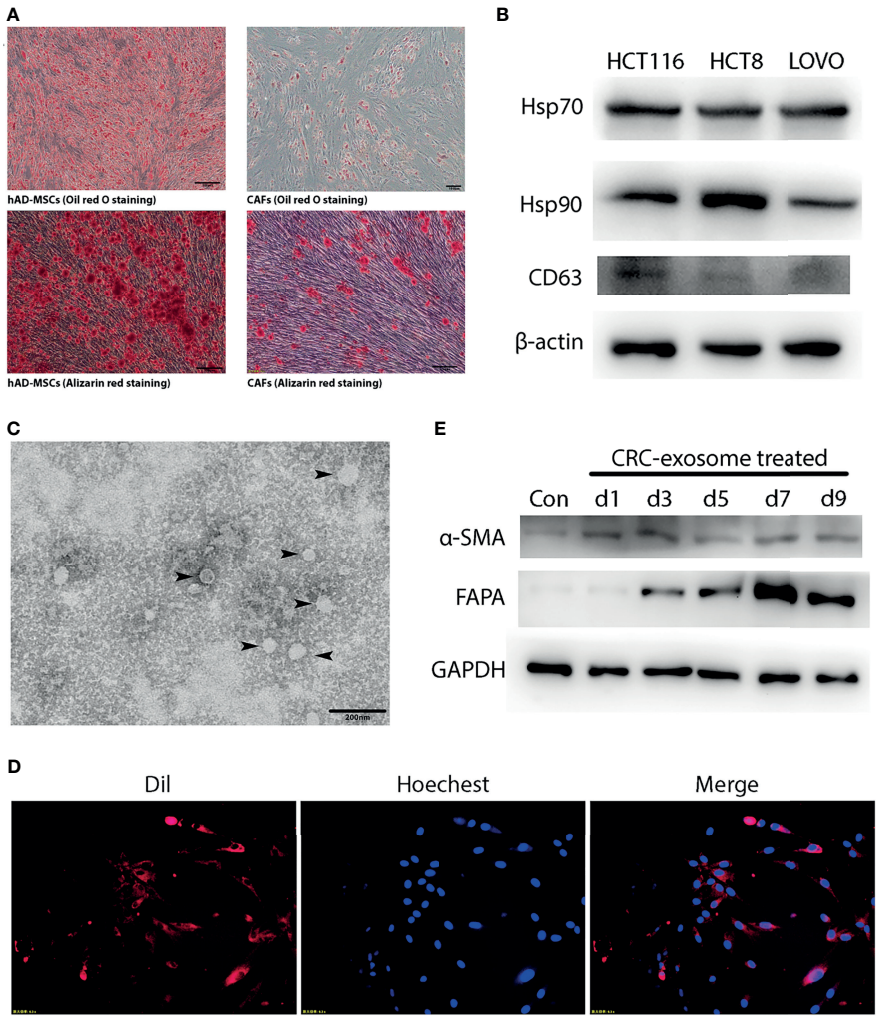

FIGURE 1 | CRC-exosomes induced differentiation of hAD-MSCs into CAFs. **(A)** Comparison of adipogenic (Oil red O staining, day11) and osteogenic (Alizarin red staining, day14) differentiation ability between hAD-MSCs and CAFs. **(B)** Detection of CRC-exosomes specific marker proteins by Western Blot. **(C)** Ultrastructure of exosomes (black arrows) purified from HCT116 under TEM. **(D)** Exosomes from HCT116 (Dil-labeled) ingested by hAD-MSCs were verified with fluorescence microscopy after incubating for 4 hours. Red: Exosomes (Dil). Blue: Nucleus (Hoechst 33342). **(E)** Western Blot analysis showed that the expression of CAFs-specific proteins was upregulated after induction with CRC-exosomes.

with CAF-CM (32–35). We found that the phosphorylation of Akt in HCT8, HCT116 and LOVO cells was significantly increased at 1h, while the total Akt protein level was practically unchanged (**Figures 2D–F**). To prove the relationship between Akt phosphorylation and PD-L1 upregulation, we added the Akt phosphorylation inhibitor (pAKTi) MK-2206 2HCl into the medium. After inhibiting Akt phosphorylation, the upregulation of PD-L1 expression by CAF-CM was markedly weakened (**Figures 3D–F**). These results demonstrated that Akt phosphorylation is necessary for the CAF-induced upregulation of PD-L1 expression in CRC cell lines.

To further verify that CAFs promote the immune escape of colorectal cancer cells by upregulating the expression of PD-L1 through Akt phosphorylation, we cultured CRC cells with CAF-CM, CAF-CM + pAKTi, and normal medium, and the rate of killing by PBMCs was detected after 3 days. The results showed that the killing rate of CRC cells by PBMCs was significantly increased after inhibiting Akt phosphorylation in CRC cells compared with the CAF-CM group, which was similar to the CRC cells cultured in normal medium (**Figures 3A–C**). These results indicated that CAFs may promote the immune escape of CRC cells by upregulating Akt phosphorylation.

To investigate the exact contribution of PD-L1 to immune escape, we performed PD-L1 pre-blockade on HCT116 before cytotoxic assay using PD-L1 blocker(αPD-L1) atezolizumab biosimilar with/without pAKTi. We detected the blocking efficiency of αPD-L1 against PD-L1 by flow cytometry according to a published method (36), and then selected the concentration of αPD-L1 with the blocking efficiency of 90% for the following experiment. When PD-L1 was blocked, the killing

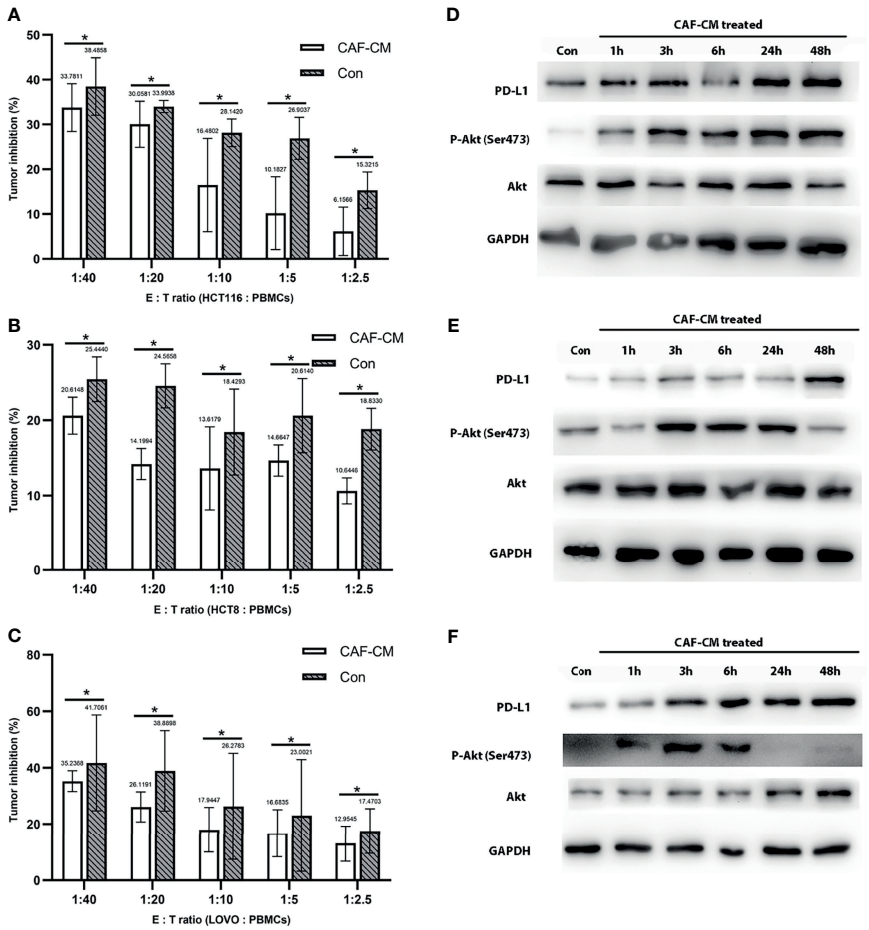

FIGURE 2 | CAFs promote the immune escape of CRC cell lines through PD-L1 upregulation. **(A-C)** The killing rate of HCT116, HCT8, and LOVO cultured with CAF-CM for 72h was significantly lower than cells cultured with normal culture medium (Con). **(A)** HCT116; **(B)** HCT8; **(C)** LOVO. **(D–F)** The expression of PD-L1 and p-Akt on HCT116 **(D)**, HCT8 **(E)** and LOVO **(F)** was upregulated after treatment with CAF-CM. Each group was replicated in 6 wells. *P < 0.05.

rate was significant higher in CAF-CM + αPD-L1 group compared with CAF-CM + human IgG1 group (**Figure 3G**). Consistent with above results, tumor cells in CAF-CM + pAKTi group were significant more vulnerable to killing by PBMCs than CAF-CM + DMSO group (**Figure 3G**). When both AKT and PD-L1 were blocked, the killing rate was similar to the CAF-CM + human IgG1 + DMSO group (**Figure 3G**). There was no significant difference between the CAF-CM + αPD-L1 group with CAF-CM + pAKTi group, suggesting they could be in the same signaling pathway. In the meanwhile, the results of killing assay for CAF-CM + pAKTi + αPD-L1 group, CAF-CM + pAKTi group and CAF-CM + αPD-L1 group were similar. The results further manifested that the phosphorylation of AKT acted upstream of PD-L1, mainly affecting PD-L1 expression. When PD-L1 expression was neutralized, AKT phosphorylation blocking would not work.

Clinical Characteristics of Patients

After *in vitro* experiments confirmed that CAFs can promote the phosphorylation of Akt in CRC cell lines, thereby upregulating the expression of PD-L1 and leading to immune escape of CRC, we investigated the correlations of PD-L1, CAFs, and p-Akt with the prognosis of CRC patients. A total of 102 postoperative patients with colorectal cancer were enrolled, including 49 males and 53 females. The clinical characteristics of the patients are summarized in **Table 1**. We determined the expression of PD-L1, p-Akt, and the commonly used marker protein α-SMA using IHC. Among 102 enrolled patients, 40 patients (39.2%) were positive for PD-L1 expression in the membrane of tumor cells, 20 (19.6%) were positive for p-AKT in the cytoplasm of tumor cells, and 25 (24.5%) had high α-SMA expression in the stromal cytoplasm (**Figure 4A**). Positive PD-L1 expression was associated with inferior tumor stage (χ^2 = 7.808, P=0.005). There was no significant correlation of PD-L1, p-AKT or α-SMA expression with age, gender, tumor location and tumor differentiation (P>0.05; **Table 1**). Correlation analysis suggested that the expression of PD-L1 was positively correlated with the p-AKT (Spearman R=0.213, P=0.031) and α-SMA levels (Spearman R=0.246, P=0.012). These data demonstrated that the expression of PD-L1 in CRC tissues was correlated with p-AKT and α-SMA.

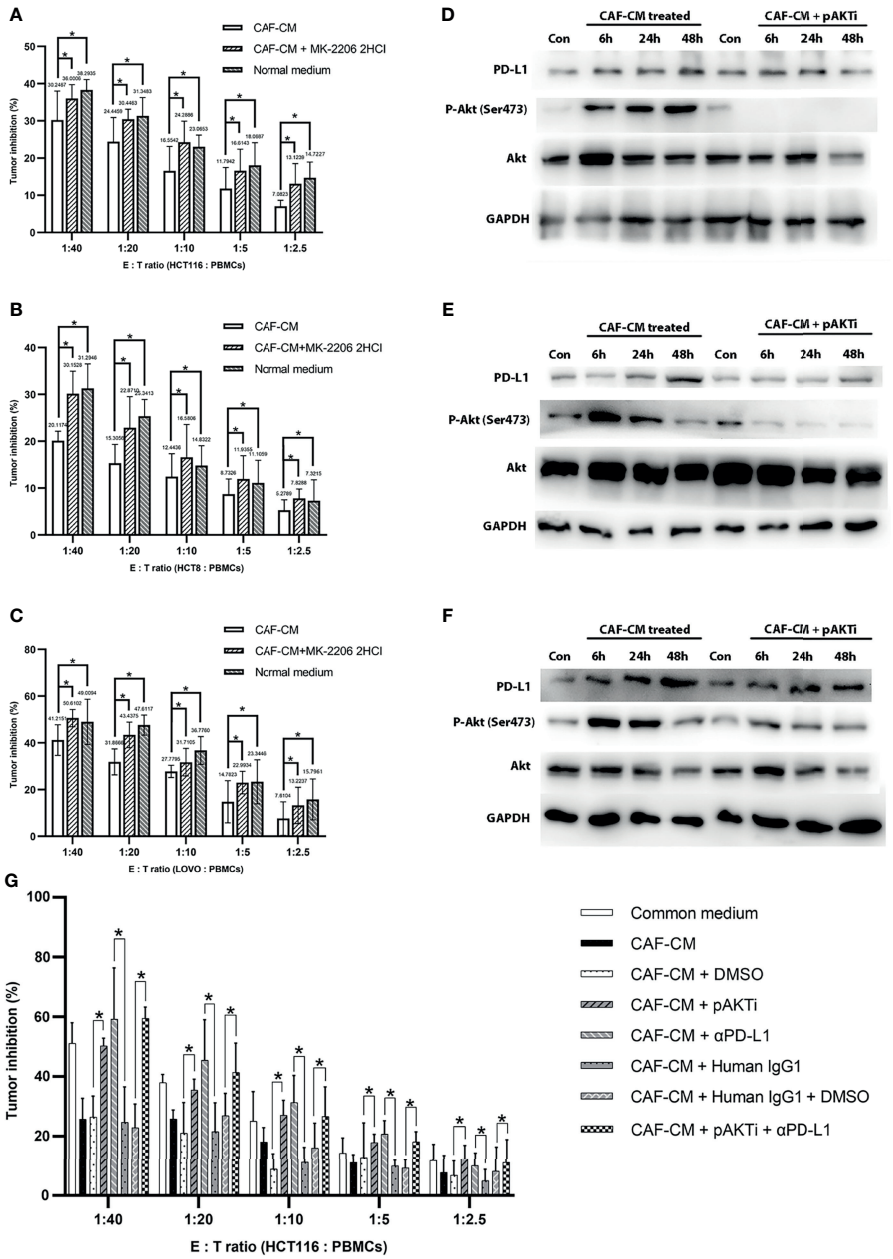

FIGURE 3 | CAFs upregulated PD-L1 in CRC cells through Akt phosphorylation and promoted immune escape. **(A–C)** The killing rate of HCT116, HCT8 and LOVO cells cultured with CAF-CM was significant lower than these cells cultured with CAF-CM + pAKTi or normal medium after 72h. **(A)** HCT116; **(B)** HCT8; **(C)** LOVO. **(D–F)** The upregulation of PD-L1 expression on HCT116 **(D)**, HCT8 **(E)** and LOVO **(F)** was weakened compared with control groups after pAKTi (MK-2206 2HCl) was added. **(G)** After blocking of PD-L1, the killing rate was significant higher in CAF-CM + αPD-L1 group compared with CAF-CM + human IgG1 group. When both AKT and PD-L1 were blocked, the killing rate was similar to the CAF-CM + human IgG1 + DMSO group. There was no significant difference between the CAF-CM + pAKTi + αPD-L1 group with CAF-CM + pAKTi group or CAF-CM + αPD-L1 group. Each group was replicated in 6 wells. *P < 0.05. Con means cells treated with normal medium.

Correlation of PD-L1, p-AKT, and α-SMA Levels With Disease-Free Survival (DFS) of CRC Patients

After our data showed a correlation between PD-L1, CAFs, and p-Akt, we further explored their correlations with the DFS of CRC patients using Kaplan-Meier curve analysis. As shown in **Figures 4B–E**, the DFS of patients with positive PD-L1 expression [27.8 months, 95% confidence interval (CI)=23.3-32.4] was significantly shorter than that of patients with negative expression (36.5 months, 95%CI=33.2-39.9, P=0.011) (**Figure 4B**). Patients with high α-SMA expression (28.0months, 95%CI=21.7-34.3) had shorter DFS than those with low expression (35.2

TABLE 1 | Relationship between PD-L1, p-AKT and α-SMA expression and clinicopathological characteristics in 102 patients with CRC.

	n	PD-L1 expression		χ2	P	p-AKT expression		χ2	P	α-SMA expression		χ2	P
		Positive (n)	Negative (n)			Positive (n)	Negative (n)			High (n)	Low (n)		
Gender				0.525	0.469			1.695	0.193			0.208	0.648
Male	49	21	28			7	42			13	36		
Female	53	19	34			13	40			12	41		
Age (years)				0.458	0.498			1.494	0.222			0.836	0.361
≤60	32	11	21			4	28			6	26		
>60	70	29	41			16	54			19	51		
Location				5.284	0.071			0.786	0.675			1.581	0.454
Right colonic carcinoma	77	26	51			14	63			17	60		
Left colonic carcinoma	24	14	10			6	18			8	16		
Right and left	1	0	1			0	1			0	1		
Differentiation				1.975	0.578			7.657	0.054			2.371	0.499
Highly differentiated	22	11	11			0	22			3	19		
Moderately differentiated	70	26	44			17	53			19	51		
Poorly differentiated	5	2	3			2	3			2	3		
Others	5	1	4			1	4			1	4		

months, 95%CI=32.1-38.4, P=0.025) (**Figure 4C**). The DFS of p-Akt positive patients tended to be worse than that of negative patients, but the difference was not significant (27.8 vs. 34.6 months, P=0.132) (**Figure 4D**). The DFS of patients with combined PD-L1^{+}, p-AKT^{+} and α-SMA^{high} status (15.8 months, 95%CI=11.5-20.1) was remarkably shorter than that of patients with triple negative expression (36.7 months, 95%CI=32.8-40.6, P<0.001) (**Figure 4E**). These results showed that high expression of PD-L1 and α-SMA had a negative effect on the DFS of CRC patients.

DISCUSSION

As a vital element of the TME, CAFs play an essential role in cancer progression by releasing various immunosuppressors and regulating the composition of the extracellular matrix (19, 20). Notably, CAFs promote immune escape through various mechanisms, including enhancing the activity of myeloid-derived suppressor cells and regulatory T cells, upregulating the expression of Fas ligand (FasL) and PD-L1, as well as downregulating the expression of the major histocompatibility complex (MHC) (18). In this study, we found that CAF-CM could decrease the killing of cultured CRC cell lines by PBMCs, which suggested that CAFs may promote the immune escape of CRC.

CAFs were found to upregulate the expression of PD-L1 in cancer cells and promote tumor development in previous studies (17, 21, 23). The α-SMA protein is a commonly used marker of CAFs (24). Li et al. confirmed that the expression of PD-L1 in colorectal and melanoma tissues was positively correlated with the expression of α-SMA (17). It has also been reported that α-SMA^{+} CAFs from human colon cancer can express PD-L1 and significantly inhibit T cell proliferation (16). To validate the expression of PD-L1 and its role in CAFs, we detected the expression of PD-L1 and α-SMA in CRC cell lines and CRC samples. The IHC results showed that PD-L1 was expressed in CRC tumor cells. Among the enrolled patients, 39.2% had tumors with positive PD-L1 expression and 24.5% exhibited high α-SMA expression. The correlation analysis suggested that the expression of PD-L1 was correlated with the high expression of α-SMA. We confirmed that the expression of CAF and PD-L1 was positively correlated in CRC. Our cell culture experiments showed that CAFs can continuously upregulate PD-L1 expression in CRC cell lines, indicating that CAFs may promote the expression of PD-L1 in CRC.

In addition, many previous studies have shown that PD-L1 was upregulated and acted as an independent predictive factor of worse prognosis in a variety of tumors, including non-small cell lung cancer, breast cancer, melanoma and renal cancer (37–40). Our results showed that the expression of PD-L1 (P = 0.011) and high expression of α-SMA (P=0.025) were associated with a significant reduction in the DFS of the patients with resectable colorectal cancer, similar to the results reported previously (41–45). We inferred that downregulating the expression of PD-L1 may reduce immune escape and increase the killing of tumor cells by PBMCs. These findings indicate new potential targets for antitumor therapy related to the regulation of PD-L1 expression by the TME.

We further explored the mechanism by which CAFs promoted the upregulation of PD-L1 expression in CRC. According to the current literature, the expression of PD-L1 is regulated by multiple mechanisms, including (1) transcriptional regulation: MYC was reported to bind to the promoter region of PD-L1, increasing the expression of PD-L1 mRNA and protein (46, 47). It has been found that hypoxia–inducible factor–1 (HIF-1) could bind with hypoxic response element (HRE) and upregulate the PD-L1 expression, simultaneously cause T-cell apoptosis and function inhibition (48, 49). STAT3 and NF-κB were also important transcription factors upregulating PD-L1 (4). (2) Phosphorylation regulation of signal pathway: The hyperactive oncogenic pathways that regulate the expression of PD-L1 mainly include the phosphoinositide 3-kinase (PI3K)/Akt pathway, mitogen-activated protein kinase (MAPK) pathway, Janus protein tyrosine kinase/signal transducer and activator of transcription (JAK/STAT) pathway and NF-κB

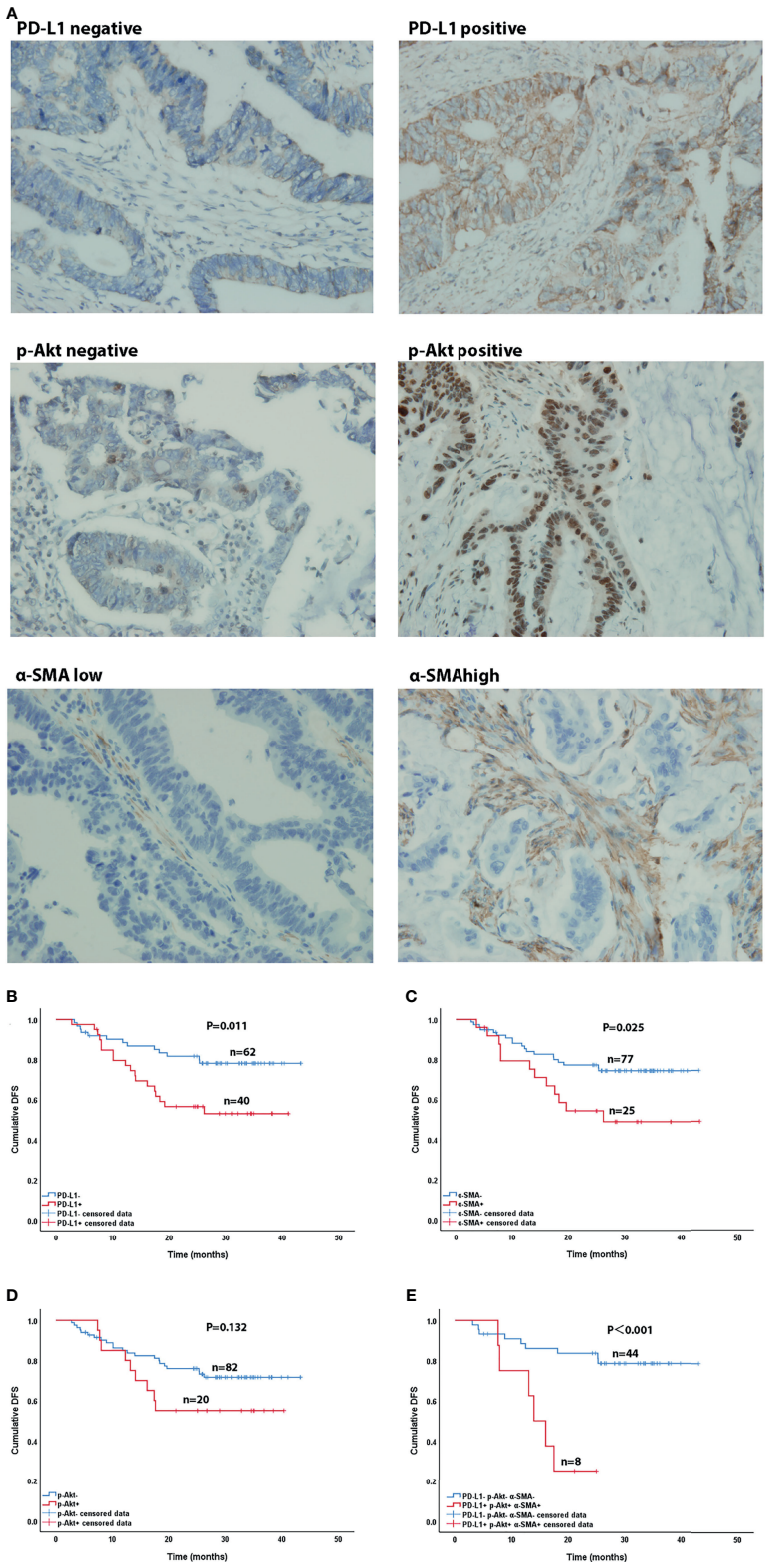

FIGURE 4 | Immunohistochemical (×400) and Kaplan-Meier curves of CRC patients. **(A)** Immunohistochemical staining of PD-L1, p-AKT and α-SMA expression. **(B–D)** Effect of PD-L1 expression **(B)**, p-AKT expression **(C)**, and α-SMA high expression **(D)** on DFS in CRC patients. **(E)** Effect of co-expression of PD-L1, p-AKT and α-SMA high and triple-negative expression on DFS in CRC patients.

pathway (4, 50–52). (3) Epigenetic regulation: MicroRNAs (miRNAs), such as miR513 (53), miR570 (54), miR142 (55) and miR200 (56), were also pivotal regulators of PD-L1 at epigenetic level. As for CAFs, it has been shown to promote the expression of PD-L1 by secreting CXCL5 in mice cancer cells (57). Dou D. et al. found high level of miR92 in CAFs-derived exosomes and the expression of PD-L1 was regulated after treating with the exosomes (58). But the mechanisms were not entirely clear due to the complex composition of CAF-CM.

Previous studies have demonstrated that CAFs activate the Akt signaling pathway in tumor cells to promote the progression of various cancers, including CRC, gastric cancer and lung cancer (32–35). Moreover, the expression of PD-L1 was significantly upregulated in CRC cell lines within a short period of time under the action of CAFs in our study. Consequently, we analyzed the phosphorylation status of the Akt signaling pathway and found that the levels of Akt and p-Akt were increased after treatment of CRC cell lines with CAFs. Conversely, blocking Akt phosphorylation with the specific inhibitor MK-2206 2HCI markedly reduced PD-L1 expression and significantly improved the killing rate of CRC cells by PBMCs. These results indicated that, in addition to the reported mechanisms, CAFs may induce immune escape of CRC by upregulating PD-L1 expression through Akt phosphorylation.

Although this study confirmed the correlation of PD-L1 and p-AKT expression with clinical characteristics and patient prognosis, it still has limitations due to a lack of enrolled patients in stage I and stage IV. The conclusions of this study should be confirmed by enrolling more patients in the future.

CONCLUSIONS

In conclusion, we found that CAFs may promote the expression of PD-L1 in tumor cells *via* the Akt signaling pathway, leading to immune escape in CRC. Furthermore, the expression of PD-L1 was correlated with higher TNM stage and shorter DFS in CRC patients. PD-L1 and p-AKT may be potential targets for combined therapy in CRC.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Chinese Academy of Medical Sciences and Peking Union Medical College. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YG, ZS, JG, ZL, XX, CX, and XL conducted the experiments, acquired the data, and wrote the manuscript. LZ and JZ contributed to the collection and analysis of clinical data. CB, QH, and RC designed the experiments and supervised the study. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.748465/full#supplementary-material

Supplementary Figure 1 | Phenotype and morphology of hAD-MSCs. **(A)** Phenotype of hAD-MSCs detected by flow cytometry. **(B)** Morphology changes of MSCs over time after treating with CRC-exosomes.

Supplementary Figure 2 | Apoptosis of HCT116 cells co-cultured with PBMCs after treatment with CAF-CM or normal medium. **(A)** The apoptosis of HCT116 cultured using CAF-CM was significant less than that of control group. **(B, C)** The results of HCT116 apoptosis detected by flow cytometry. *P<0.05

Supplementary Figure 3 | Western Blot analysis showed that the upregulation of PD-L1 was maintained until 72h after treatment with CAF-CM.

Supplementary Figure 4 | The expression of PD-1 on PBMCs didn't change significantly after co-culture with CRC cell.

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Platelet CLEC2-Podoplanin Axis as a Promising Target for Oral Cancer Treatment

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Cancer tissues are not just simple masses of malignant cells, but rather complex and heterogeneous collections of cellular and even non-cellular components, such as endothelial cells, stromal cells, immune cells, and collagens, referred to as tumor microenvironment (TME). These multiple players in the TME develop dynamic interactions with each other, which determines the characteristics of the tumor. Platelets are the smallest cells in the bloodstream and primarily regulate blood coagulation and hemostasis. Notably, cancer patients often show thrombocytosis, a status of an increased platelet number in the bloodstream, as well as the platelet infiltration into the tumor stroma, which contributes to cancer promotion and progression. Thus, platelets function as one of the important stromal components in the TME, emerging as a promising chemotherapeutic target. However, the use of traditional antiplatelet agents, such as aspirin, has limitations mainly due to increased bleeding complications. This requires to implement new strategies to target platelets for anti-cancer effects. In oral squamous cell carcinoma (OSCC) patients, both high platelet counts and low tumor-stromal ratio (high stroma) are strongly correlated with increased metastasis and poor prognosis. OSCC tends to invade adjacent tissues and bones and spread to the lymph nodes for distant metastasis, which is a huge hurdle for OSCC treatment in spite of relatively easy access for visual examination of precancerous lesions in the oral cavity. Therefore, locoregional control of the primary tumor is crucial for OSCC treatment. Similar to thrombocytosis, higher expression of podoplanin (PDPN) has been suggested as a predictive marker for higher frequency of lymph node metastasis of OSCC. Cumulative evidence supports that platelets can directly interact with PDPN-expressing cancer cells *via* C-type lectin-like receptor 2 (CLEC2), contributing to cancer cell invasion and metastasis. Thus, the platelet CLEC2-PDPN axis could be a pinpoint target to inhibit interaction between platelets and OSCC, avoiding undesirable side effects. Here, we will

review the role of platelets in cancer, particularly focusing on CLEC2-PDPN interaction, and will assess their potentials as therapeutic targets for OSCC treatment.

Keywords: platelets, tumor cell-induced platelet aggregation (TCIPA), CLEC2, PDPN, ezrin/radixin/moesin (ERM), oral cancer

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most prevalent type of head and neck malignancies that occur in oral cavity, salivary gland, pharynx, larynx, nasal cavity, thyroid, and bone (1). Unlike the other types of cancers, OSCC usually arises from the body part that is easily accessible for visual examinations. Despite this advantage in detection of precancerous lesions, most of the OSCC patients are not diagnosed until the advanced stages with metastasis, which is attributed to low overall survival rates (2). Oral mucosa contains a connective tissue enriched with type I collagen that is synthesized by stromal cells (3). Desmoplasia, a status of the excessive growth of the stromal tissue, is closely associated with OSCC (4, 5). In OSCC patients, stroma-rich tumors are more aggressive and metastatic than stroma-poor tumors, finally contributing to the poor survival rates (6, 7). The activated tumor stroma can supply a variety of growth factors and cytokines that induces cancer cell proliferation as well as extracellular matrix (ECM) remodeling (5, 8). In support of the tumor stroma, OSCC cells tend to invade adjacent tissues, such as bones, and spread to the lymph nodes (9). This locoregional characteristic of OSCC is the primary cause of treatment failure (10). Thus, how to control the local and distal metastasis is crucial for successful treatment and better prognosis in OSCC patients.

Platelets, the smallest cells in blood circulation, play a major role in blood coagulation and hemostasis (11–13). In addition to their primary physiological functions, platelets are profoundly involved in cancer promotion and progression (14, 15). Recently, it has been reported that platelets can infiltrate into the tumor stroma in colorectal and pancreatic cancer patients (16–18). As a part of the tumor stromal components, platelets crosstalk with cancer cells either directly or indirectly, promoting invasion and metastasis (19–21). For the physical interaction, C-type lectin-like receptor 2 (CLEC2) and podoplanin (PDPN) are suggested as the key molecular links expressed in platelets and tumors, respectively (22). Moreover, cancer cells activate and educate platelets, thus the bilateral interaction between platelets and cancer can further promote tumorigenesis, creating a positive feedback loop (23). Notably, OSCC patients often show increased platelet counts, which is strongly associated with poor prognosis (24, 25). Thus, platelets are emerging as an important target for chemotherapy in OSCC patients.

Aspirin, a representative antiplatelet agent, is well known to protect against carcinogenesis (26–28). Aspirin irreversibly inhibits both cyclooxygenase-1 (COX-1) and COX-2, reducing synthesis of prostaglandins and thromboxanes responsible for inflammation and platelet aggregation (27). Despite its chemopreventive effect, a daily use of low-dose aspirin frequently causes adverse complications, primarily increased bleeding risk (29, 30). Thus, instead of using traditional antiplatelet agents, the pinpoint targeting of the platelet-tumor cell interaction would be a more precise and effective strategy for OSCC treatment, avoiding undesirable harmful effects. In this regards, we will highlight the role of platelets in carcinogenesis and OSCC, particularly focusing on the physical interaction between platelets and tumors *via* the CLEC2-PDPN axis.

ROLES OF PLATELETS IN CANCER

Thrombocytosis in Cancer Patients

Platelets are anucleated cells originated from megakaryocytes in the bone marrow and abundant in healthy individual 150,000~400,000 per microliter of blood (11–13). In spite of lack of genomic DNA, platelets release plenty of granular ingredients, such as platelet-derived growth factor (PDGF), transforming growth factor β (TGFβ), stromal cell-derived factor-1 (SDF-1), and serotonin, which contributes to signal transduction in nearby cells (31). Cancer is often associated with thrombocytosis, a status of an abnormal elevation of platelet counts, which shows a positive correlation with worse outcomes in many types of cancers (24, 32–34). High platelet counts are involved with development of venous thromboembolism (VTE) in cancer patients, the second leading cause of cancer death (35–38). Besides an increased risk of VTE, thrombocytosis is associated with cancer mortality by accelerating tumor promotion and progression as well (39–41). In mice bearing tumors, platelet transfusion induced the blood platelet counts as well as tumor growth, while reducing the survival rates (37, 42). Thus, the platelet counts have long been considered as a valuable prognostic marker in cancer patients.

It has been reported that inflammatory cytokines, such as interleukin-6 (IL-6), are highly associated with thrombocytosis in cancer patients (33, 43). IL-6 can stimulate platelet production through inducing thrombopoietin (33, 44). In murine colitis model, colitis-induced wild type (WT) mice showed thrombocytosis and platelet aggregation, which were absent in IL-6-deficient mice (45). Moreover, neutralization of IL-6 led to reduction of platelet counts and tumor growth in the mouse ovarian cancer model (33). Thus, IL-6 inhibitors might be utilized to mitigate cancer-associated thrombocytosis (46). However, anti-IL-6 treatments need meticulous assessment, regarding that IL-6 pleiotropically functions in immune system (47, 48).

Platelets as a Part of Stromal Components in Tumor Microenvironment

Tumor tissues are not just simple masses of malignant cells, but rather complex and heterogeneous collections of cellular and

even non-cellular components, referred to as tumor microenvironment (TME) (49). The multiple players in the TME develop dynamic interactions with each other, which determines the characteristics of the tumor (50). The non-cellular parts of the TME comprise primarily the ECM, a three-dimensional scaffold that contains collagens, proteoglycans, and fibronectins (51). The acellular ECM is crucial for providing mechanical (structural) and biochemical (nutritional) supports to cellular components in the TME (52). The cellular players in the TME can be largely divided into stromal cells and tumor-infiltrating immune cells. The tumor stroma is a heterogeneous population of distinct types of cells, including fibroblasts and endothelial cells (53). Among them, cancer-associated fibroblasts (CAFs) are the most abundant type of the stromal cells in TME that display enhanced expression of the signature proteins, including α-smooth muscle actin and PDGF receptors (54). Moreover, the TME contains a broad spectrum of immune cells, such as tumor-associated macrophages, tumor-associated neutrophils, and regulatory T cells. Notably, the infiltration of platelets into the tumor stroma has been observed in cancer patients (18, 55, 56), along with increased blood platelet counts (24, 32–34). Tumor-infiltrating platelets can interact with other stromal players of TME, contributing to tumor promotion and progression (57). Miyashita et al. have found that CAFs were surrounded by platelets in almost half of the pancreatic cancer patients (58). Platelet-derived factors, like TGFβ, PDGF, and SDF-1, can stimulate recruitment and activation of CAFs in the TME (59–62). Platelets also accommodate various angiogenesis regulators, which can turn on local angiogenesis in the TME (63). Depletion of tumor-infiltrating platelets showed impaired tumor blood vessel structures in mice (64). Moreover, it has been reported that fusion between platelets and endothelial cells promotes cancer metastasis by facilitating adhesion of tumor and endothelial cells (65). In consistent, the intratumoral accumulation of platelets are related to tumor progression (18, 55, 56). These investigations support that platelets function as a crucial stromal component in the TME through vigorous interplay with other members.

Platelets in Cancer Invasion and Metastasis

Metastasis is a multi-step process, including local invasion, intravasation, and colonization at the distal sites (66). Invading cancer cells undergo dramatic alterations in their morphology and phenotypes, such as epithelial-to-mesenchymal transition (EMT), which is accompanied by remodeling of the ECM (66). As a poor prognostic indicator, thrombocytosis is associated with lymph node metastasis and invasion in cancer patients (24, 67). In consistent, platelet transfusion significantly enhanced metastasis of cancer cells in the murine experimental models (15, 68). However, platelet decoys bound to tumor cells as effectively as normal intact platelets and inhibited thrombosis and metastatic tumor formation, further supporting the role of platelets in metastasis (69). Of note, platelets are frequently detected at the invasive front where both EMT and ECM remodeling occur actively (70). Platelets contain about 40% of TGFβ found in the peripheral blood plasma, which plays a crucial role in cancer cell invasion (71). Co-culture with platelets remarkably enhanced invasiveness and EMT process of cancer cells in a TGFβ-dependent manner (72, 73). Platelet-specific *Tgfb1*-deficient mice showed reduction in tumor growth and platelet extravasation, compared to WT mice (74). Moreover, various types of matrix metalloproteinases (MMPs) responsible for ECM degradation are stored in the resting platelets and released upon stimulation, such as cancer cell-induced aggregation (35, 75, 76). Platelets upregulate production of MMPs in cancer cells as well as fibroblasts, accelerating invasion of cancer cells (77–79). These data suggest that platelets can change TME through their releasates, such as TGFβ and MMPs, conferring cancer cells invasive capability and metastatic potential. In addition, direct contact with platelets can promote invasion and metastasis of cancer cells *in vitro* and *in vivo* (19, 80).

Platelets can promote metastasis through interaction with other cells in the bloodstream as well, like in the TME. Platelets rapidly adhere to circulating cancer cells in the blood, protecting tumors from immune surveillance (41, 81). Natural killer (NK) and CD8 T cells are cytotoxic lymphocytes that play a central role in cancer immunosurveillance (82). Once tumors are coated by platelets, platelets inhibit NK cell-mediated antitumor activity through downregulating tumor cell NK2D expression by TGFβ and inducing pseudoexpression of immunomodulating molecules, such as MHC I and GITR (83–85). Moreover, platelet-derived factors, such as TGFβ and programmed death-ligand 1 (PD-L1), suppressed the cytotoxic antitumor T cell immunity in the mouse cancer models (86–88). Taken together, these data suggest that platelets facilitate tumor immune escape by surrounding cancer cells in the bloodstream, thus, the platelet-camouflaged cancer cells safely migrate to the metastatic sites. Of note, co-incubation with platelets protected cancer cells against anoikis, implying that platelets enhance anchorage-independent survival of circulating tumor cells in the bloodstream (15).

Platelets as a Potential Target for OSCC Treatment

Similar to other types of cancer patients, increased platelet counts are significantly correlated with poor prognosis in OSCC patients (25, 89). Based on the analysis of relationship between platelet counts and disease progression in a total of 253 OSCC patients, thrombocytosis was associated with lymph node metastasis as well as distant metastasis (90). Along with metastasis, advanced OSCC often shows invasion into the facial bones, due to close anatomical relationship (91, 92). The bone invasion causes severe pains, greatly lowering the quality of life and the survival rates in OSCC patients (91, 93). Notably, platelet aggregation plays a critical role in tumor-associated bone destruction (94). In line with that, the pharmacological inhibition of platelet aggregation reduced bone metastasis in the murine cancer model (95). Platelet-secreted lysophosphatidic acid is thought to be one of the primary mediators in platelet-promoted bone invasion and metastasis (96, 97). Taken together, platelets can facilitate bone invasion through direct contact with

tumor as well as their releasates. In OSCC, bone destruction and invasion are closely related to TGFβ signaling pathway (98, 99). Considering that platelets store most of the plasma TGFβ, it is plausible that platelets aggravate invasion of OSCC, and thus further pre-clinical and clinical investigations will shed light on a noble would be novel strategies for OSCC treatment.

INTERACTION BETWEEN PLATELETS AND CANCER: CLEC2-PDPN-ERM AXIS

PDPN in Cancer and Platelet Aggregation

PDPN is a type I transmembrane glycoprotein expressed in kidney podocytes, skeletal muscles, lungs, hearts, myofibroblasts, osteoblasts, mesothelial cells, and lymphatic endothelial cells (100). PDPN knockout mice die shortly after birth due to an impaired respiratory system (101). These mice also show defects in the lymphatic vasculature, disorganization of spleen, and lack of lymph nodes (102, 103). PDPN is thus an important regulator in the normal organogenesis and development processes.

Upregulation of PDPN has been observed in a variety of human cancers, including brain cancer, breast cancer, lung cancer, and mesothelioma, which is associated with poor prognosis (104–107). In athymic nude mice, injection of PDPN-overexpressing cancer cells generated bigger tumors, while silencing of PDPN suppressed tumor growth (108). Moreover, PDPN-high tumors exhibited increased peritumoral lymphangiogenesis, invasiveness, migratory ability, and metastasis, implying a pro-tumorigenic role of PDPN (109–112). Notably, it has been reported that PDPN expression is elevated at the leading edge of tumor tissues, which promotes cell surface extension and cell motility in keratinocytes (111, 113). In the two-stage skin carcinogenesis model, epidermal ablation of PDPN reduced tumor growth and invasion (109). Overall, these data suggest that PDPN confers cancer cells survival benefits, promoting tumor growth, invasion, and metastasis.

Interestingly, PDPN-overexpressing cancer cells evoke platelet aggregation, also known as tumor cell-induced platelet aggregation (TCIPA) (108, 114). PDPN-positive human glioblastoma Gli16 cells were able to markedly induce platelet aggregation, whereas not detected by PDPN-negative cells (115). In tumor-bearing mouse models, either ablating *PDPN* gene or blocking PDPN by monoclonal antibody (mAb) injection effectively suppressed platelet aggregation, supporting that PDPN is crucial for TCIPA formation (116, 117). The PDPN-mediated TCIPA was strongly associated with an increased incidence of VTE in cancer patients (115, 118). Moreover, PDPN overexpression is also involved in TCIPA-induced tumor promotion and progression. The platelet-tumor aggregates are readily arrested in the microvasculature, facilitating tumor metastasis (20). PDPN neutralization significantly inhibited TCIPA occurrence, tumor growth, and metastasis in nude mice injected with human melanoma or lung cancer cell lines (108, 116, 119). Moreover, platelet-derived TGFβ upregulated PDPN expression in human bladder cancer cells, which induced EMT process and cancer cell invasion (120). Taken together, PDPN is considered as a 'pinpoint' that interconnects between tumor and platelets, regulating VTE as well as tumor progression.

Platelet CLEC2-PDPN Axis: A Pinpoint of Platelet-Tumor Cell Interaction

PDPN consists of an extracellular domain, a transmembrane domain, and a cytoplasmic domain (121). The extracellular domain of PDPN carries four platelet aggregation-stimulating (PLAG) domains with a plenty of potential O-glycosylation sites, crucial for interaction with platelets (121). The PLAG domain of PDPN has been reported to bind to CLEC2 that is abundantly expressed on the surface of platelets (122). Interestingly, CLEC2-deficient mice phenocopy PDPN-knockout mice, like prenatal lethality and impaired lymphatic vasculature (123). Either platelet-specific deletion of CLEC2 or inhibition of PDPN was associated with reduced thrombosis in a murine deep vein thrombosis model of inferior vena cava stenosis (124). Similarly, cancer cell lines with high endogenous PDPN expression levels, such as LN319 and Colon-26, showed induced platelet aggregation, which was attenuated by pre-incubation with an anti-CLEC2 antibody (125). Tsukiji et al. have found that cobalt hematoporphyrin (Co-HP) directly binds to PDPN-binding sites of CLEC2, functioning as an inhibitor of the CLEC2-PDPN axis (126). Both Co-HP administration and CLEC2 neutralization significantly inhibited CLEC2-dependent platelet aggregation in tumor-bearing mice (126, 127). Taken together, these data support that PDPN is interdependent with CLEC2, thus, the platelet CLEC2-PDPN axis is crucial for platelet-tumor cell interaction (**Figure 1**).

In conjunction with TCIPA formation, the platelet CLEC2-PDPN axis mediates cancer promotion and progression. In mice inoculated with PDPN-expressing B16F10 melanoma cells, CLEC2 depletion by anti-CLEC2 mAb 2A2B10 injection reduced plasma levels of inflammatory cytokines and lung metastasis, resulting in prolonged survival compared to control mice (127). Treatment with a CLEC2 inhibitor Co-HP suppressed lung metastasis of PDPN-expressing melanoma cells, but not that of PDPN-negative lung cancer cells (126). In platelet-depleted mice, platelet transfusion induced much more lung colonization as well as bone metastasis of PDPN-expressing osteosarcoma cells, while CLEC2 mAb injection reduced lung colonization (68). Likewise, injection of PDPN mAb (MS-1) remarkably suppressed platelet aggregation as well as lung metastasis in the murine cancer metastasis model (128). Therefore, the platelet CLEC2-PDPN axis is considered as a pinpoint for platelet-tumor interaction that promotes tumor progression (**Figure 1**). It has been demonstrated that CLEC2 deficiency is not significantly related to bleeding tendency (123, 129). In this regard, the platelet CLEC2-PDPN axis could be a promising target to inhibit TCIPA-induced tumor progression without bleeding risk, a major complication of the traditional antiplatelet agents.

PDPN-ERM Axis: An Executor in Cancer Progression

PDPN has a short cytoplasmic tail associated with ezrin/radixin/moesin (ERM) proteins that primarily bridge between plasma

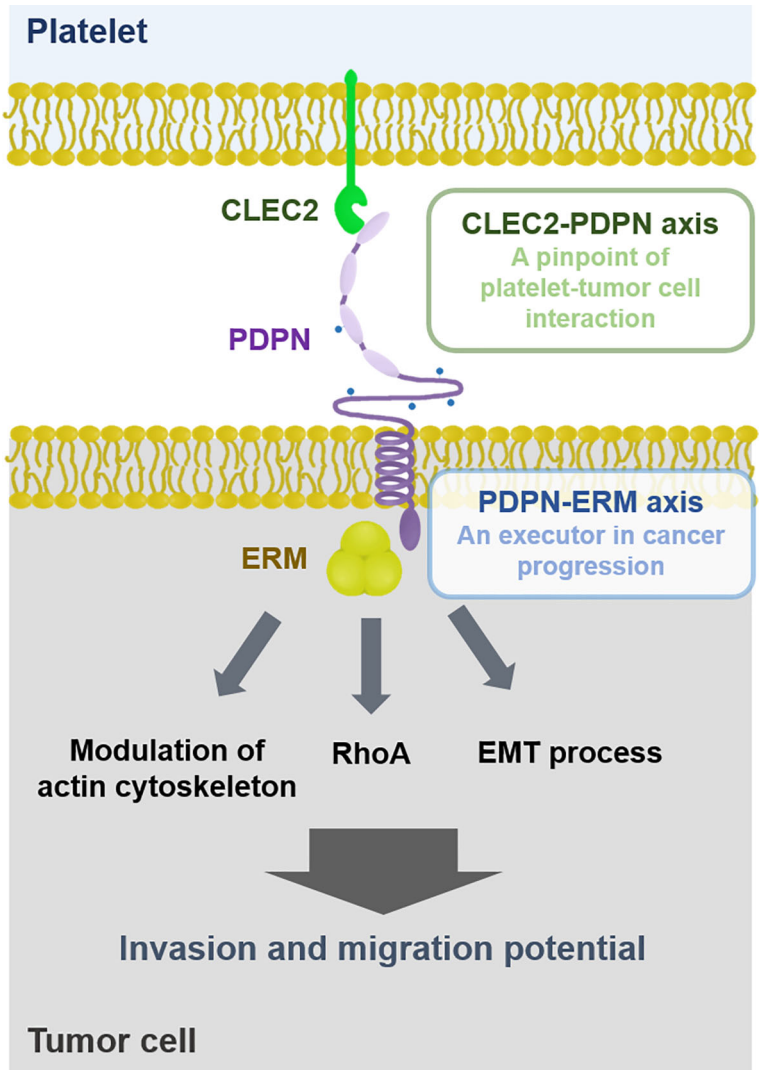

FIGURE 1 | Interaction between platelet and tumor cell. Platelets can physically interact with tumor cells *via* the CLEC2-PDPN axis. PDPN is associated with ERM proteins that promote cancer cell migration and invasion through modulating actin cytoskeleton, RhoA, and EMT process. Thus, the CLEC2-PDPN-ERM axis is a crucial target for chemotherapy.

membrane proteins and F-actin filaments of the cytoskeleton (100, 130). It is well documented that cells and tissues utilize this ERM crosslink system to maintain the architectures necessary for their own biological functions (131). In particular, ERM proteins are crucial regulators for epithelial morphogenesis and integrity, mitosis, cell polarity, and cell adhesion (132, 133). Among the ERM protein members, ezrin-null mice displayed much more severe phenotypes compared to moesin- or radixin-deficient mice (134). Ezrin-deficient mice showed defects in intestinal villus morphogenesis and epithelial cell organization (135). In addition, ERM proteins regulate the cell-cell and cell-matrix interactions, particularly in cancer cells (136). Thus, PDPN is engaged in cell adhesion, migration, and invasion through association with the ERM proteins, as illustrated in **Figure 1** (113, 133).

PDPN expression is upregulated peculiarly in the growing edge of tumors and commonly co-localized with ERM proteins (100, 106). Similar to PDPN, overexpression of ERM proteins has been detected in various types of cancers: ezrin overexpression in breast, hepatocellular, colon, ovarian, and pancreatic cancers (137–141); radixin overexpression in pancreatic cancer with lymph node metastasis (142); moesin overexpression in skin cancer, colorectal carcinoma, endometrial adenocarcinoma, and glioma (143–146). Moreover, upregulation of ERM proteins is associated with poor prognosis in cancer patients (140, 147–150). In athymic nude mice, intracranial injection of moesin-overexpressing glioblastoma cells significantly reduced the survival rates compared to the control group (146). Moreover, ERM proteins were frequently mislocalized during tumor progression, from plasma

membrane to cytoplasm (136). Thus, dysregulation of ERM proteins takes part in cancer promotion and progression, possibly in an interdependent manner with PDPN.

It has been reported that PDPN mediated TCIPA-induced EMT process in human cancer cell lines (120). In non-cancerous experimental settings, PDPN can bind to ERM proteins through its cytoplasmic domain, promoting the EMT process as well as cell migration (130, 151). Silencing of radixin, one of the ERM protein members, suppressed the EMT process as well as migration and invasion in human gastric carcinoma SGC-7901 cells (152). Moreover, PDPN can induce migration ability in cancer cells that bypass the EMT process *via* filopodia formation (106). Instead of the EMT process, PDPN recruits ERM proteins to modulate the actin cytoskeleton in a RhoA-dependent manner, consequently promoting cancer cell migration and invasion. Taken together, the PDPN-ERM axis can promote migratory capability and invasiveness of tumor cells, through either EMT process or cytoskeletal rearrangement.

It has been reported that the CLEC2-PDPN axis can regulate cell contractility and migration through activation of ERM proteins in non-cancerous settings (153–155). In this regard, it is plausible that the PDPN-ERM axis could be recruited by tumors bound to platelets *via* CLEC2-PDPN interaction, conferring cancer cells metastatic potentials (**Figure 1**). Further investigation is necessary to clarify the role of the platelet CLEC2-PDPN-ERM axis in cancer progression.

Platelet CLEC2-PDPN and PDPN-ERM Axes in OSCC

According to the Cancer Genome Atlas analysis, head and neck cancer patients present much higher PDPN expression levels compared to other types of cancer patients. While PDPN expression is rarely detected in normal oral epithelial cells, OSCC patients show upregulation of PDPN in tumors, which contributed to poor prognosis (156–159). In the xenograft mouse model, PDPN-overexpressing OSCC cells promoted tumor growth and intratumoral platelet accumulation, implying that PDPN mediates TCIPA formation in OSCC (160). Similar to high platelet counts (90), elevated PDPN expression was often found at the invasive front and correlated with lymph node metastasis in OSCC patients (156, 161). In line with that, silencing of *PDPN* gene expression attenuated migration and invasion in human OSCC cell lines (160, 162–164). Considering that platelet CLEC2 is crucial for PDPN-dependent TCIPA formation, the platelet CLEC2-PDPN axis would be a feasible target for successful local control in OSCC patients.

In OSCC patients, overexpression of ezrin and moesin has been detected in advanced staged tumors and significantly associated with worse overall survival rates (149, 164, 165). Kobayashi et al. have reported that cytoplasmic expression of moesin shows a strong correlation with lymph node metastasis in OSCC patients (166). Of note, PDPN expression was positively related to ezrin expression, particularly in the cytoplasm of the odontogenic tumors (167). Moreover, this co-expression between PDPN and ezrin was frequently detected in the invasive front and possibly involved with lymph node metastasis in the lip cancer (168). These data suggest that the PDPN-ERM axis may contribute to increased metastatic potential in OSCC. In consistent, PDPN has been reported to enhance cell motility and invasiveness through interaction with ERM binding partners, such as membrane type 1 MMP, Cdc42, and CD44, in humans OSCC cell lines (162, 164). These data suggest that ERM proteins function as an intracellular executor of the CLEC2-PDPN axis in invasion and metastasis of OSCC.

TARGETING PLATELET-TUMOR INTERACTION FOR CHEMOTHERAPY

Aspirin

Considering pro-tumorigenic activities of platelets, antiplatelet agents could be promising chemotherapeutics, as shown in **Table 1**. A classical antithrombotic drug aspirin has been used for chemoprevention. The meta-analysis and retrospective cohort study showed that a regular use of aspirin is associated with reduced risk of cancers in liver, stomach, colorectum, lung, pancreas, and oesophagus (26, 169). In head and neck cancer patients, evaluation of aspirin as a chemopreventive agent is still controversial. A hospital-based case control study revealed that aspirin use can reduce head and neck cancer risk (170), whereas the other investigations demonstrated that there was no significant correlation between aspirin intake and head and neck cancer (171, 172). Moreover, the risk of gastrointestinal bleeding could limit the use of aspirin for cancer prevention and/or treatment (29, 30).

Platelet P2Y12 Receptor Antagonists

Platelet P2Y12 receptor is involved in ADP-stimulated activation of glycoprotein IIb/IIIa (GPIIb/IIIa) responsible for platelet aggregation (197). It has been reported that GPIIb/IIIa mediates platelet-tumor interaction and cancer metastasis (198–200). In conjunction with GPIIb/IIIa, stimulation of P2Y12 receptor can promote platelet-tumor crosstalk and cancer metastasis (**Figure 2**), suggesting P2Y12 receptor antagonists as anticancer drugs (73, 201). Clopidogrel, the most widely used P2Y12 receptor antagonist, markedly inhibited tumor growth in mouse ovarian and liver cancer models (176, 177). Another P2Y12 inhibitor ticagrelor suppressed proliferation of ovarian cancer cells *in vivo* and *in vitro*, which was not detected in absence of platelets (176). Moreover, treatment with ticagrelor attenuated TCIPA formation and cancer metastasis in the murine experimental models (178–180). These pre-clinical data suggest platelet P2Y12 receptor as a target for cancer treatment by controlling platelet-tumor aggregation. However, a population-based cohort study showed that the use of clopidogrel has no huge impact on cancer mortality in colorectal, breast, and prostate cancer patients (181). Even worse, the clinical trial-based analyses revealed that ticagrelor increased cancer risks (183, 184). In another patient-level meta-analysis of randomized clinical trials, a long-term intake of clopidogrel was associated with bleeding risk and hemorrhage (182). Overall, the use of P2Y12 receptor antagonists for

TABLE 1 | Strategies to target platelet-tumor interaction for chemotherapy.

Agent	TCIPA	Cancer risk/metastasis	Bleeding	References
Classical antiplatelet drug				
Aspirin	Inhibit TCIPA *in vitro* and *in vivo*	Inhibit metastasis *in vivo* Cancer preventive effect in human subjects (controversial in head and neck cancer) Reduce metastasis in cancer patients	Increased gastrointestinal bleeding	(26, 29, 30, 169–175)
P2Y12 receptor antagonism				
Clopidogrel	Inhibit TCIPA in mice	Inhibit tumor metastasis in mice No impact on cancer motility in human colorectal, breast, and prostate cancer patients	A long-term use can increase bleeding risk	(176–182)
Ticagrelor	Inhibit TCIPA	Increase cancer risks in human	More major bleeding compared to clopidogrel in patients with acute coronary syndrome	(180, 183–185)
GPVI antagonism				
Anti-GPVI mAb (JAQ1)	Inhibit TCIPA	Inhibit cancer cell extravasation *in vitro* Inhibit metastasis in mice Induce intratumoral hemorrhage and accumulation of co-administrated anticancer drugs in mice	No impact on bleeding time	(129, 186–188)
Revacept	Inhibit TCIPA in mice and human	Inhibit EMT marker expression *in vitro*	No impact on bleeding time in mice and human	(189–191)
Targeting CLEC2-PDPN axis				
Anti-CLEC2 mAb (2A2B10 and INU1)	Inhibit intratumoral thrombus formation in mice	Inhibit metastasis in mice	No impact on bleeding time	(68, 127, 129)
Anti-PDPN mAb (NZ-1, MS-1, and SZ-168)	Inhibit platelet aggregation in mice	Inhibit metastasis in mice Inhibit VET in mice		(119, 128, 192–194)
2CP	Inhibit TCIPA in mice	Inhibit metastasis in mice	No impact on bleeding time	(195)
Co-HP	Inhibit platelet aggregation	Inhibit metastasis in mice Inhibit VET in mice	No impact on bleeding time	(126)
Polysaccharide extracted from *Artemisia argyi* leaves	Inhibit TCIPA			(196)

chemotherapy is controversial, in spite of the compelling pre-clinical evidence.

Platelet GPVI Antagonism

GPVI is the major platelet-activating receptor exclusively expressed on platelets and megakaryocytes (202). GPVI-null mice showed lack of thrombus formation and defective platelet activation without severe bleeding tendency (203, 204). Moreover, these GPVI-deficient mice developed less metastatic tumors by injection of lung cancer or melanoma cells than WT mice (205). Notably, platelet GPVI can bind to galectin-3 on tumor cells, provoking platelet-tumor cell interaction and metastasis (**Figure 2**) (186, 189). These pre-clinical data suggest that GPVI antagonism is a conceivable strategy to block TCIPA-mediated tumor progression without adverse effects. In line with this notion, platelets preincubated with an anti-GPVI antibody (JAQ1) were less able to form aggregates with human breast cancer cells and eventually reduced cancer cell extravasation in the transendothelial migration assay (187). Moreover, treatment with JAQ1 reduced tumor metastasis in the murine lung metastasis models, further supporting antitumor effects of GPVI antagonism *via* blocking TCIPA formation (186). Interestingly, JAQ1 Fab2 fragment induced intratumoral hemorrhage that led to accumulation of co-administrated chemotherapeutics without systemic bleeding complications, thus allowing to maximize anticancer effects (188). Revacept, a competitive GPVI inhibitor comprising a soluble Fc fusion protein, decreased platelet-tumor interaction and metastatic potential *in vitro* (189). In atherosclerotic mice and healthy human subjects, Revacept reduced platelet aggregation with no impact on bleeding times (190, 191). Based on this drug safety assurance, the antitumor efficacy of GPVI antagonists must be further evaluated in human cancer patients.

Targeting Platelet CLEC2-PDPN Axis

As described in **Figure 1**, the platelet CLEC2-PDPN axis is emerging as a pinpoint to control the platelet-tumor interaction and subsequent tumor progression. In order to disconnect the platelet CLEC2-PDPN axis, diverse approaches have been made, including mAbs against CLEC2 or PDPN and pharmacological inhibitors. PDPN mAbs, such as NZ-1 and MS-1, can bind to the PLAG domain of PDPN and neutralize interaction with platelet CLEC2 (**Figure 2**) (192, 193). These PDPN mAbs specifically inhibited PDPN-mediated platelet aggregation and cancer metastasis in the murine experimental models (128, 192, 193). Moreover, anti-PDPN antibody SZ-168 reduced the incidence of VTE in mice (194). Similar to PDPN mAbs, anti-CLEC2 antibody 2A2B10 suppressed intratumoral thrombus formation as well as metastasis in mice (68, 127). These investigations suggest that mAbs neutralizing either CLEC2 or

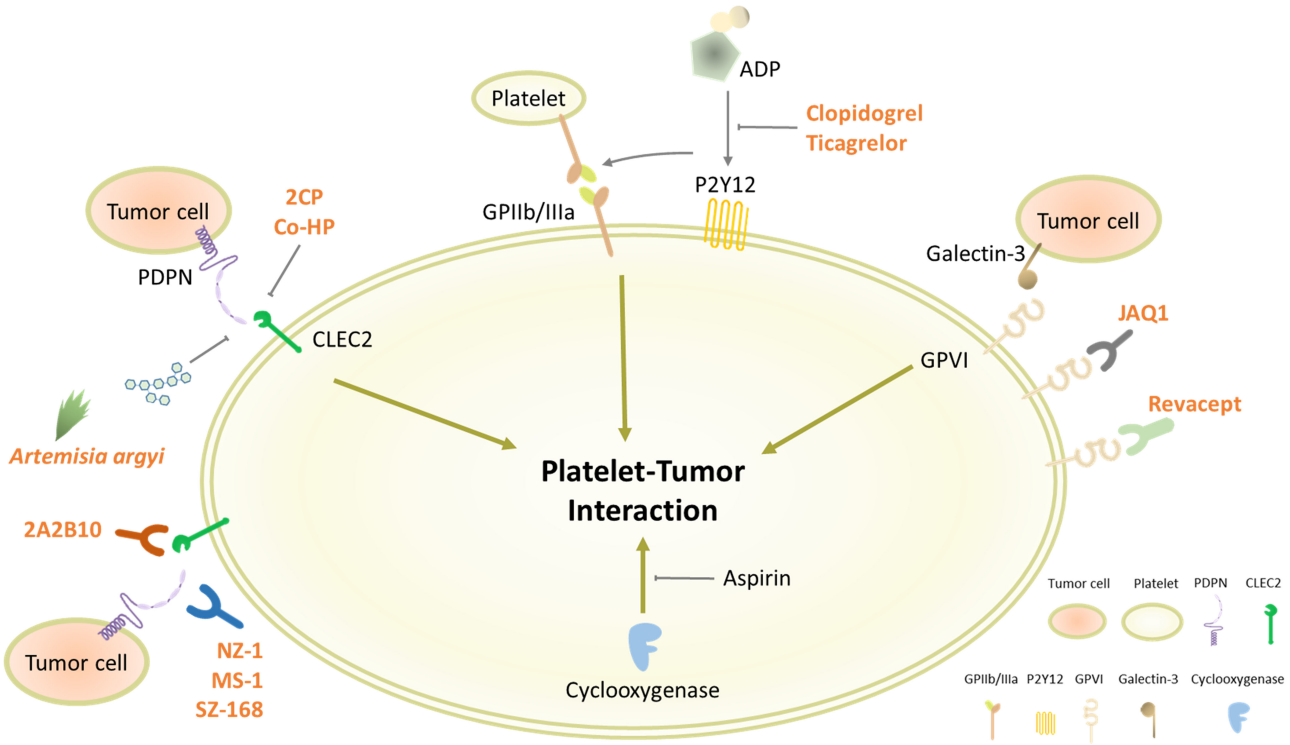

FIGURE 2 | Platelet receptors involved in platelet-tumor interaction. Platelets contain various types of receptors on the cell surface for diverse physiological functions, including cell adhesion and aggregation. Some of the surface molecules, such as CLEC2, P2Y12, and GPVI, can promote the interaction between platelets and cancer cells, which could be plausible targets for blocking TCIPA formation.

PDPN specifically inhibit platelet-tumor interaction and tumor metastasis. Although the influence of CLEC2 deficiency on bleeding is conflicting in CLEC2-null mice, CLEC2 mAb-treated mice had no sign of prolonged bleeding compared to control mice (123, 129, 206, 207). Overall, CLEC2 neutralization seems not to affect bleeding time profoundly.

In addition to neutralizing antibodies, pharmacological inhibitors display potent inhibitory effects on the CLEC2-PDPN axis. Chang et al. have newly synthesized a non-cytotoxic 5-nitrobenzoate compound 2CP that specifically inhibits the CLEC2-PDPN interaction (195). 2CP selectively blocked PDPN-induced TCIPA formation and lung metastasis in the xenograft model, whereas bleeding time was not affected by 2CP (195). Co-HP can directly bind to CLEC2 at PDPN-binding sites and potently block CLEC2-PDPN interaction (126). Co-HP injection significantly reduced tumor metastasis and the incidence of VTE in mice, but not affecting the bleeding time (126). Moreover, a bioactive polysaccharide extracted from *Artemisia argyi* leaves inhibited CLEC2-PDPN interaction and PDPN-dependent TCIPA formation (196).

Taken together, inhibition of the platelet CLEC2-PDPN axis is a promising chemotherapeutic strategy by suppressing TCIPA formation and metastasis (**Table 1**). In particular, targeting the CLEC2-PDPN axis seems to be a relatively safer approach to block platelet-tumor interaction without severe adverse effects, such as increased bleeding risk. Further clinical studies are needed to validate their anti-thrombotic and anti-metastatic effects in human subjects. Although targeting the CLEC2-PDPN axis is relatively harmless, it still requires caution to be clinically applied, since CLEC2- or PDPN-deficient mice showed abnormal lymphatic vessel formation (123).

CONCLUSION

Despite advances in surgical techniques and therapeutic strategies including radiotherapy and immunotherapy, the survival rate of OSCC has not been improved for the past decade due to failure of local control of primary tumor (2, 208). Currently, platelets are well recognized as a stromal member of the TME and an important prognostic index in OSCC patients (25, 57, 89). In particular, platelets directly interact with cancer cells *via* CLEC2-PDPN binding, fortifying metastatic potentials of cancer cells. Regarding that PDPN is the only known endogenous ligand for CLEC2, the platelet CLEC2-PDPN axis is a pinpoint target to control TCIPA formation-mediated metastasis without undesirable complications. Thus, blockade of the CLEC2-PDPN axis could be a prospective strategy for successful local control and improvement of survival in OSCC patients, which merits further pre-clinical and clinical investigations.

AUTHOR CONTRIBUTIONS

N-YS contributed to study conception. S-YP, B-OH, ESC, XZ, SKL, H-JA, K-SC, W-YC, and N-YS performed literature review and analysis and revised the manuscript. N-YS, S-YP, and B-OH drafted the manuscript, figures, and tables. All authors contributed to the article and approved the submitted version.

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Role of Serine Proteases at the Tumor-Stroma Interface

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During tumor development, invasion and metastasis, the intimate interaction between tumor and stroma shapes the tumor microenvironment and dictates the fate of tumor cells. Stromal cells can also influence anti-tumor immunity and response to immunotherapy. Understanding the molecular mechanisms that govern this complex and dynamic interplay, thus is important for cancer diagnosis and therapy. Proteolytic enzymes that are expressed and secreted by both cancer and stromal cells play important roles in modulating tumor-stromal interaction. Among, several serine proteases such as fibroblast activation protein, urokinase-type plasminogen activator, kallikrein-related peptidases, and granzymes have attracted great attention owing to their elevated expression and dysregulated activity in the tumor microenvironment. This review highlights the role of serine proteases that are mainly derived from stromal cells in tumor progression and associated theranostic applications.

Keywords: tumor-stromal interaction, serine protease, fibroblast activation protein, urokinase plasminogen activator, kallikrein, granzyme, extracellular matrix remodeling, signaling pathways

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INTRODUCTION

The tumor microenvironment (TME) is a highly complex system that is comprised of a heterogenous population of cancer cells and associated stromal cells, and the extracellular matrix (ECM). The ECM not only provides structural support in the extracellular space but also regulates multiple cellular signaling in tumor tissues (1, 2). The tumor stroma, as a critical component of the TME, actively contributes to cancer proliferation, angiogenesis, invasion and metastasis, immune evasion, and resistance to cancer therapy (3, 4). The function of stromal cells and their interaction with cancer cells within the TME are modulated by expression and secretion of various signaling molecules such as growth factors (5–7), chemokines (8–10), cytokines (11–14), and proteolytic enzymes (15–17). Although once thought to be limited to the degradation of ECM, the role of proteases in tumors is now better understood to be significantly more complicated and critical. In addition to cancer cells, stromal cells including fibroblasts, endothelial cells and infiltrating immune cells all contribute proteases in developing tumors such as matrix metalloproteinases (MMPs), cysteine cathepsins, and serine proteases. Proteases are involved in proteolytic networks, remodeling of ECM, regulation of growth factor and cytokine signaling, and modulation of inflammatory responses and immunosuppressive effects (**Figure 1**). Depending on the cellular context, activity and interaction of these proteases can have either tumor-promoting or -suppressing effects. Among, serine proteases, which make up approximately one-third of human proteases, are important class of proteases in carcinomas. Dysregulated expression and activity of several serine proteases that are derived from stromal cells have been associated with tumor development

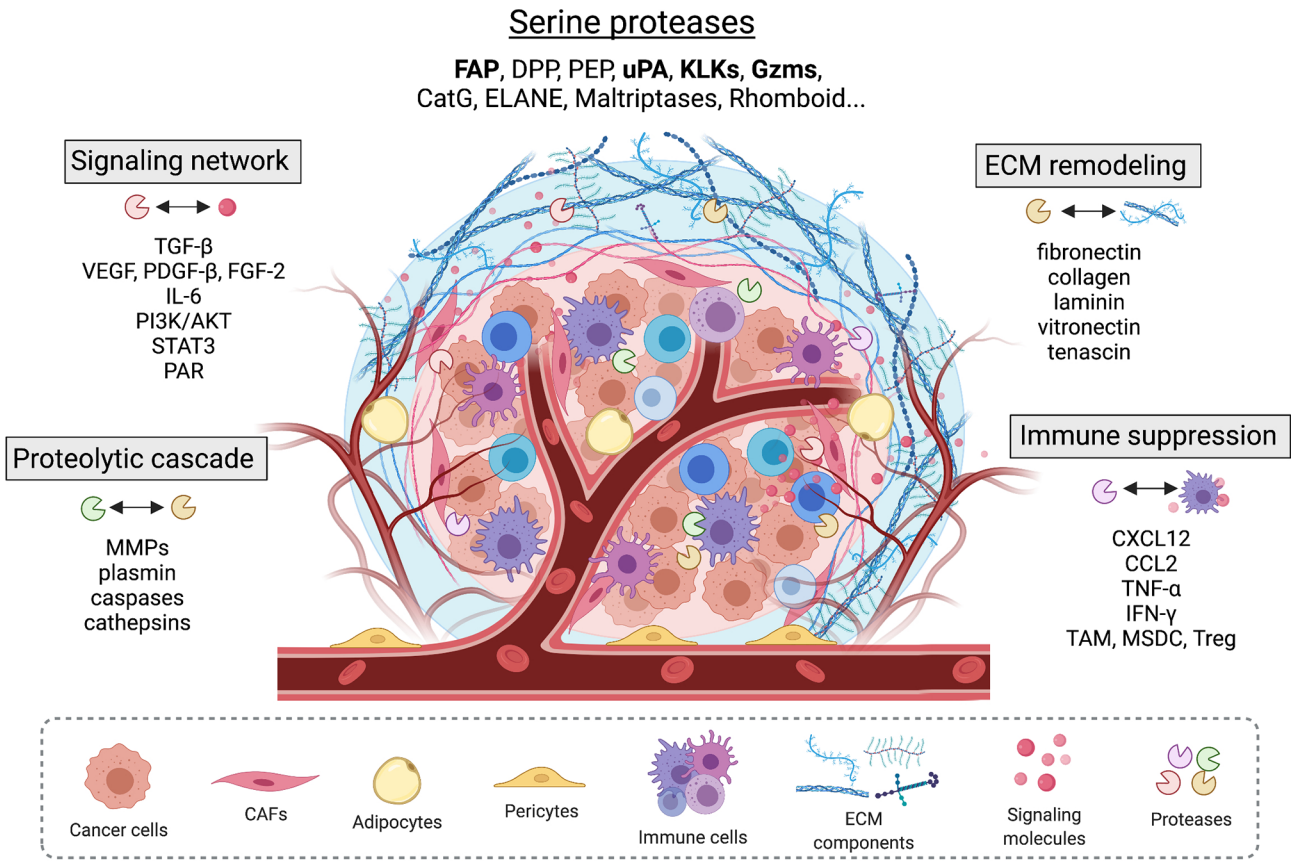

FIGURE 1 | Serine proteases found in the tumor microenvironment modulate tumor-stromal interplay by interacting with other proteases, extracellular matrix proteins, and important signaling pathways that involve growth factors, cell surface receptors, cytokines and chemokines.

and metastasis. In this review, we aim to summarize our current understanding of functional roles that key serine proteases play in the transformed stroma and discuss their theranostic potential.

FIBROBLAST ACTIVATION PROTEIN α (FAPα, FAP OR SEPRASE)

Cancer-associated fibroblasts (CAFs), which are perpetually activated and distinct from normal fibroblasts in their morphological and functional features, are extremely abundant in the tumor microenvironment including breast, prostate, and pancreatic carcinomas with potent tumorigenic effects (18). CAFs are highly heterogeneous, proliferative, and are resistant to apoptotic cell death. Among several surface bound markers identified in CAFs, fibroblast activation protein α (FAP) is shown to be selectively upregulated on reactive stromal fibroblasts of more than 90% of human epithelial carcinomas (19). It has been demonstrated that altered tumor microenvironment or inflammation induces FAP expression through stimulation of cytokines such as TGF-β1 and TNF-α (20, 21), chemical substances (22), or physical stimulants (23). FAP is a type II transmembrane glycoprotein that displays both dipeptidyl peptidase and endopeptidase activities, removing two amino acids from the N-terminus of the substrate as well as hydrolyzing peptide bonds of nonterminal amino acids after a proline residue. The postprolyl peptidase activity of FAP requires homodimerization of the protein and is carried out by the catalytic triad consisting of Ser, Asp, and His residues (24). Physiological substrates of FAP include gelatin, denatured type I collagen, α-antitrypsin, α2-antiplasmin, fibroblast growth factor 21, fibrillin-2, extracellular matrix protein 1, C-X-C motif chemokine 5, tumor necrosis factor related protein 6, lysyl oxidase homolog 1, and several neuropeptides (25–27). Protease-independent activity of FAP has been associated with activation of MMP2/9, phosphoinositide 3-kinases (PI3Ks), and STAT signaling pathways (28, 29).

Given its high expression on CAFs and ability to degrade major ECM proteins, the role of FAP in remodeling and patterning of ECM, which in turn affects cellular response and tumorigenesis, has been investigated. In both syngeneic transplant and endogenous mouse tumor models, genetic deletion of FAP or pharmacologic inhibition of its enzymatic activity led to excessive accumulation and disorganization of collagen and decrease in myofibroblast content and blood vessel

density in tumors, thus inhibiting tumor growth (30). Utilizing an *in vivo*-like 3-dimensional matrix system, Lee et al. demonstrated that FAP overexpression on fibroblasts modifies architecture and composition of ECM through inducing tumor stromal-like parallel organization of fibronectin and collagen I fibers and modulating protein levels of tenascin C, collagen I, fibronectin and α-smooth muscle actin (31). Enhanced velocity and directionality of pancreatic cancer cells invading through FAP^+ matrices were observed, which was effectively reversed by inhibition of FAP enzymatic activity. *In vitro*, a study showed that compared to control transfectants that do not express FAP and form slow growing tumors, cells expressing not only wild type FAP but also catalytic mutant of FAP degrade fibronectin matrices more extensively, accumulate higher levels of MMP-9, invade type I collagen gels to a significantly higher degree, and have altered pattern of tyrosine phosphorylated proteins, suggesting the functions of FAP independent of its enzymatic activity (32). FAP is found to form a protease complex with dipeptidyl peptidase IV (DPP4), which is another type II transmembrane protein with serine protease activity, at the endothelial cells of capillary-like micro-vessels within invasive breast ductal carcinoma. *In vitro* experiments showed that gelatin-binding domain of DPP4 brings this DPP4-FAP complex together with gelatin substrates at the migratory endothelial cells facilitating the local degradation of the extracellular matrix and subsequent cell migration and invasion (33). Analysis of gastric cancer (GC) patient tissue samples showed that FAP expression is positively correlated with micro-vessel density indicating the role of FAP expression in angiogenesis and metastasis (34). Many studies have demonstrated that FAP expression regulates signaling pathways that control cell cycle, proliferation, migration, and invasion (29, 35–37).

Growing evidence has also suggested that FAP^+CAFs can promote the immunosuppressive tumor microenvironment. When FAP^+ cells were depleted in lung or pancreatic cancers, it caused immediate growth arrest of immunogenic tumor through TNF-α- and IFN-γ-mediated mechanism (38). The study by Feig et al. showed that production of chemokine (C-X-C motif) ligand 12 (CXCL12) by FAP^+CAFs mediates the immune suppressive activity and accounts for the failure of T cell checkpoint inhibitors in pancreatic ductal adenocarcinoma (PDA) (39). Combining with administration of α-CTLA-4 or α-PD-L1, depletion of FAP^+ cells or inhibiting CXCR4 (CXCL12 receptor) diminished PDA growth. FAP also induces inflammatory CAFs by STAT3 activation leading to increased expression of CCL2, which promotes the tumor recruitment of myeloid-derived suppressor cells (MDSCs) and immunosuppression (40, 41). In murine models of pancreatic adenocarcinoma, inhibition of FAP proteolytic activity resulted in decreased macrophage recruitment, and genetic knockout of FAP enhanced T cell infiltration and cytotoxicity (42). However, in another study in non-small cell lung cancer (NSCLC), high density of FAP^+CAFs was found to be associated with improved prognosis in patients with high expression of CD8 and CD3 T lymphocytes (43, 44). This result is contrary to previous finding in a small study (n= 59) that higher levels of FAP expressing stromal cells are associated with worse overall survival and increased peripheral neutrophil and lymphocyte count ratio (NLR) in NSCLC patients (45). In invasive ductal carcinoma of the breast, FAP^+CAFs have been associated with longer survival (46), while in pancreatic adenocarcinoma and rectum they are found to be associated with worse clinical outcome (47–49). Thus, the cellular context and mechanism of FAP on the antitumor immune responses in the TME would require further investigation.

UROKINASE PLASMINOGEN ACTIVATOR (UROKINASE-TYPE PLASMINOGEN ACTIVATOR, UROKINASE, UPA)

Urokinase plasminogen activator (uPA) is a serine protease that is extracellularly localized and involved in the plasminogen activator system. It was first found in the urine (50), but later identified also in plasma, seminal fluid, and the extracellular matrix (51). It is synthesized and secreted as an inactive zymogen known as pro-uPA and different proteases such as cathepsin B and L, trypsin, kallikrein, and mast cell tryptase convert pro-uPA into an active uPA (52, 53). Binding of uPA to uPA receptor (uPAR), which is a glycosyl-phosphatidylinositol (GPI)-anchored cell membrane receptor highly expressed in most types of solid tumors such as breast, prostate, brain, and head and neck cancers, localizes the active uPA to the cell surface and converts its major substrate plasminogen into plasmin. Once activated, non-specific protease plasmin is involved in degradation of collagen IV, laminin, fibronectin, vitronectin, fibrin, and several blood clotting factors either directly or through activation of other proteases such as MMPs (54). The inhibitory proteins, plasminogen activator inhibitor-1 (PAI-1) and PAI-2, regulate the activity of uPA (55).

The role of uPA in tumor invasion and metastasis has been widely investigated. Depending on cancer type, uPA and uPAR are expressed both by cancer and stromal cells. uPA is mainly expressed by tumor-associated macrophages (TAMs) and CAFs, and on tumor endothelial cells (TECs) to lesser extent. Through interaction between uPAR and integrins as well as ECM components such as vitronectin, uPA system regulates cell adhesion and migration. uPA supports tumor cell proliferation by proteolytically activating various growth factors that include epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2) and hepatocyte growth factor/scatter factor (HGF-SF) (56). TGF-β, which is a predominant and multifunctional cytokine found in the TME, regulates the expression of uPA in several types of transformed cells (57–59). uPA-activated plasmin, in turn, activates the secreted TGF-β precursor by a proteolytic cleavage within the N-terminal region of latency-associated peptide (60–62). This loop contributes to tumor growth, cancer cell migration, epithelial to mesenchymal transition (EMT), and metastasis. uPA also mediates the effects of vascular endothelial growth factor (VEGF), a key factor in

angiogenesis (63). A study showed that downregulation of uPA/uPAR inhibits angiogenesis in glioblastoma and endothelial cells by regulating tissue inhibitors of metalloproteinase-1 (TIMP-1) secretion, subsequently enhancing secretion of sVEGFR1, a known scavenger of VEGF (64). *In vitro*, uPA derived from CAFs was found to promote esophageal squamous cell carcinoma (ESCC) cell proliferation, migration, and invasion by activating PI3K/AKT and ERK signaling pathways (65). In multiple myeloma, increased expression and activity of both uPA and uPAR on CAF cells with higher proliferative rate and invasion potential were observed, suggesting the potential role of uPA/uPAR system in promoting metastasis of malignant plasma cells (66). uPA/uPAR system has been implicated in suppression of apoptotic cell death. RNAi-mediated downregulation of uPA and uPAR led to dephosphorylation of focal adhesion kinase (FAK), p38 MAPK, janus kinase (JNK) and ERK1/2 which in turn activates caspase-8, cytochrome c release, PARP cleavage, and subsequent apoptosis of human glioma cells (67). The uPA/uPAR system also plays a critical role in macrophage infiltration (68). TAMs, one of most abundant types of tumor-infiltrating immune cells found in the TME, exhibit important functions in tumor growth, metastasis, angiogenesis, and immune regulation. For example, by producing cytokines, chemokines, growth factors, and triggering the inhibitory immune checkpoint proteins release in T cells, TAMs promote immunosuppression (69). In addition, expression of uPAR and PAI-1 in TAMs has been correlated with vessel remodeling and node status and tumor grade, indicating that TAMs have an important role in the expression and regulation of uPA system for establishing the vascular network in tumors (70).

KALLIKREIN RELATED PEPTIDASES (KLKS)

Kallikrein or kallikrein related peptidases are a family of secreted serine proteases that play important roles in ECM remodeling, angiogenesis, skin homeostasis, innate immunity, male reproduction, tooth enamel formation, and neural development. In humans, there are 15 secreted KLKs (KLK1-15). KLK1-2, KLK4-6, KLK8, and KLK10-15 have trypsin-like and KLK3, KLK7, and KLK9 have chymotrypsin-like activities. Aberrant expression of KLKs has been associated with a variety of malignancies, thus the potential of KLKs as cancer markers has been suggested for several members of this protease family. In particular, because of the restricted expression in prostate, KLK3, also known as a prostate-specific antigen (PSA), has been widely employed as a clinical biomarker for prostate cancer. Laser cell microdissection analysis and immunochemistry in human breast cancer surrounding stromal cells showed significant upregulation of KLK4 but downregulation of all other KLKs (71). Immunohistochemistry analysis of tissue sections of ovarian and melanoma patients found the overexpression of KLK6 in tumor associated stromal cells and keratinocytes (72, 73). In pancreatic ductal adenocarcinoma, immunostaining analysis of epithelial tumor cells and the surrounding stroma and immune cells showed that high KLK6 protein levels in the tumor and immune cells are significantly associated with shorter survival compared to low protein levels (74). mRNA analysis of colorectal cancer (CRC) tissue samples from 136 patients showed upregulated KLK10 expression (75). Significantly increased expression of KLK10 has been also found in ovarian cancer tissues (76).

During the tumor progression, KLKs from cancer and stromal cells are released into the TME, where they can exert their proteolytic activity, mainly activating signaling networks and modulating the expression of genes and proteins important to tumor growth and invasion. For example, KLKs activate EGFR and protease-activated receptor (PAR), resulting in the stimulation of ERK1/2 signaling and enhanced subsequent cell proliferation (77–79). Analyzing the secretome of endothelial cells, a study showed that KLK12 can catalyze the release of PDGF-β from ECM components and cell surface. The released PDGF-β then mediates secretion of VEGF and angiogenesis. In addition, KLK12 has been shown to cleave the ECM proteins fibronectin and tenascin (80). KLK12-mediated remodeling of fibronectin matrix led to an increase in endothelial cell migration which was inhibited by a polyclonal antibody directed against the KLK12 cleavage site (81). An *in vitro* study showed that KLK14 recognizes and hydrolyzes pro-MMPs to active MMPs, especially the membrane-type MMPs (82). Combined expression of KLK4-7 in ovarian cancer has been associated with increased level of TGF-β1, neural cell adhesion molecule L1 (L1CAM), and other tumor-associated factors such as keratin 19 and moesin, indicating the impact of KLK proteases on the secreted proteomes shaping tumor microenvironment (83, 84). KLK4, highly expressed in prostate cancer, promotes CAF differentiation. Through PAR1 activation, KLK4 regulates FGF-1, tasgelin, and lysyl oxidase, and several soluble factors in the prostate stromal cell secretome (85). KLK4 can also stimulate uPA/uPAR system and activate MMPs, leading to the ECM degradation. Overexpression of KLK7 in melanoma cells was shown to induce a decrease in cell proliferation and colony formation but an increase in cell motility and invasion possibly through modulating cell adhesion molecules such as E-cadherin and MCAM/CD146 (86). Kallikreins have been also associated with infiltration of immune cells (87). Although many more studies are needed to better define the pathophysiological functions of each KLK in cancer, emerging evidence suggest that KLKs have significant effect on the tumor-microenvironment interaction and targeting specific KLK may provide therapeutic benefits in cancer therapy.

GRANZYMES (GZMS)

Granzymes are cell death-inducing serine proteases primarily known for their role in eliminating infected and transformed cells through cytotoxic T cells and natural killer cells. Among five granzymes identified in humans (A, B, H, K, and M), GzmA and GzmB have been most widely studied. The expression of granzymes is regulated by many factors including receptor

engagement and stimulation with cytokines. Granzymes are expressed as an inactive pro-enzyme that requires cleavage of N-terminal dipeptide inside secretory granules by cathepsin C (88). As granzyme is optimally active at neutral pH, granzymes stored in acidic granules are quiescent and become enzymatically active following release from the granules into the cytoplasm. In addition, the presence of protein inhibitors such as serpins regulates the proteolytic activity of granzymes (89).

Infiltrating immune cells within the TME significantly contribute to tumor suppression (immune surveillance) or tumor promotion (inflammation and angiogenesis) either directly or through the interplay with other stromal components. In regard to anti-tumor immunity, the intracellular role of granzymes is well characterized and appreciated. Upon activation, the pore-forming protein perforin helps deliver GzmB into the cytosol of target cells, where it induces apoptosis by caspase-dependent and -independent mechanisms. Activation of gasdermin B (GSDMB) by GzmA (90) or GSDME by GzmB (and indirectly by caspase-3) initiates pyroptotic cell death in tumors (91). Mounting evidence suggests that granzymes are also active players in immune regulatory cells and tumor cells. Regulatory T cells (Tregs), MDSCs, dendritic cells (DCs), mast cells, and Bregs are found to express granzymes. If not controlled, granzymes can cause self-inflicted damage of expressing cells. It has been suggested that GzmB is involved in Treg-mediated suppression and elimination of activated CTL/NK cells and antigen-presenting cells, indicating Treg cells utilize GzmB to suppress immune responses and tumor clearance, thus depending on the relative abundance of these cells in the tumor, GzmB can have either detrimental or protective function in antitumor immunity (92, 93).

In addition, extracellular (perforin-independent) functions of granzymes are emerging. Although it remains unclear what stimuli and signaling pathways regulate granzyme release, studies have long shown that patients with infectious diseases and certain proinflammatory conditions have elevated levels of extracellular GzmA/B. GzmB is also found to be constitutively released from CTL/NK cells *in vivo* (94). Once released, they can mediate the cleavage of extracellular matrix, cell surface receptors, cytokines, and act as proinflammatory proteases. The ECM substrates of GzmB include fibronectin, vitronectin, aggrecan, laminin, and decorin. D'Eliseo et al. showed that GzmB expressed in bladder cancer cell lines and urothelial carcinoma tissues is active in catalyzing vitronectin cleavage, and inhibition of GzmB activity suppresses bladder cancer cell invasion (95). A recent study also demonstrated that active GzmB released from migrating CTLs contribute to extravasation and homing of CTLs *via* basement membrane cleavage and remodeling. GzmB-null CTLs exhibited impaired homing and decreased transmigration through the vessel wall in mouse models of viral infection and inflammation. *In vitro* migration assays using Matrigel or Madin-Darby canine kidney (MDCK) cell basement membrane showed that active GzmB released from migrating CTLs enabled chemokine-driven movement and cleavage of basement membrane components (96). GzmM expressed in carcinomas has been implicated in promoting tumor growth, metastasis, and EMT dependent on STAT3 signaling (97). GzmM expression is positively related to IL-6 and VEGF release from cancer cells. Recently, extracellular GzmA was found to be engaged in gut inflammation in colorectal cancer by inducing NF-kB-dependent IL-6 production in macrophages leading to STAT3 activation (98). In mouse models, GzmA knockout or inhibition reduced inflammation and CRC development, suggesting that development of effective GzmA inhibitors could offer therapeutic benefits treating gut inflammation and CRC.

TARGETING SERINE PROTEASES IN TUMOR STROMA

With respect to the clinical applications in cancer diagnosis and therapy, a number of tools that either detect the protein expression or harness and leverage the specific protease activity have been developed (**Table 1**).

Given their high and selective expression on tumor-associated stromal cells, inhibition of serine proteases by active-site targeting small molecules has been extensively investigated in cancer treatment (30, 42, 102, 103, 115–121, 126–130). Antibody-based approaches that can inhibit the activity of serine proteases aim to achieve better selectivity. For example, a humanized version of monoclonal antibody F19, sibrotuzumab has been developed to target the cell surface bound FAP on tumor stromal fibroblasts and explored for its anti-tumor response (99). Protease biology in cancer offers opportunities for diagnostic profiling as well. Radiolabeled antibodies targeting FAP (106), uPA (122, 123), and KLK3 (135, 136) have been evaluated in single photon emission computed tomography (SPECT) and positron emission tomography (PET) imaging of primary and metastatic tumors. *In vivo* imaging tools exploit the cleavage activity of serine proteases. Incorporating the optimal peptidic sequence, substrate-based, activatable probes are designed either as quenched probes or as FRET probes that produce a fluorescent signal only after cleavage as a measure for protease activity (112, 113, 124, 142, 144–148). On the other hand, activity-based probes contain an electrophilic warhead that reacts with catalytic serine of the protease and a reporter group that generates a signal upon covalent interaction with the protease (137, 141, 149–152). In that case not only peptides but also non-peptidic small molecules can serve as a recognition motif targeting the protease active site (153–156).

Taking advantage of upregulation of specific serine protease activity in tumors, protease-activatable prodrug approach has been widely used in cancer therapy for selective delivery of drugs while minimizing toxicity to normal tissues. Chemotherapeutic compounds such as doxorubicin are conjugated to cleavable linkers sensitive to active serine proteases (104, 105, 131–134). For example, the FAP-cleavable GP linker and KLK3-cleavable SSKYQSL linker have been successfully used in prodrug approach. Antibody-drug conjugates that incorporate tumor-targeting antibodies into protease-activatable prodrug format further enhance tumor-specific activation. A new technology termed as Probody masks antibody binding using linkers cleaved

TABLE 1 | Theranostic targeting of serine proteases.

Target	Agent	Function	Cancer model tested	Ref.
FAP	Sibrotuzumab	humanized monoclonal antibody (mAbF19)	metastatic colorectal cancer	(99)
	FAP5-DM1	monoclonal antibody	epithelial cancer xenograft model	(100)
	OS4 TTS	bispecific antibody	fibrosarcoma cell line	(101)
	PT-100 (Val-BoroPro)	FAP and DPP inhibition	metastatic colon cancer	(102)
	PT-630 (Glu-BoroPro)	FAP and DPP4 inhibition	metastatic colorectal cancer	(30)
	M83	FAP and PREP inhibition	lung and colon cancer xenograft	(103)
	UAMC-1110 (SP-13786)	FAP and PREP inhibition	pancreatic adenocarcinoma mouse model	(42)
	FTPD	FAP-targeting prodrug of doxorubicin	breast cancer mouse model	(104)
	ASGPAGP-A12ADT	FAP-targeting prodrug of thapsigargin	breast cancer xenograft	(105)
	^{131}I-mAbF19	SPECT imaging	breast adenocarcinoma and prostate cancer xenograft	(106)
	^{99m}Tc-FAPI-34	SPECT imaging	metastasized ovarian and pancreatic cancer	(107)
	^{68}Ga-FAPI-04, FAPI-74	PET/CT imaging	28 different cancers, gastric and lung cancer	(108–110)
	^{177}Lu-FAP-2286	peptide-targeted radionuclide therapy	pancreatic, breast, rectal and ovarian cancer	(111)
	ANP_{FAP}	NIR fluorescent imaging	glioblastoma xenograft	(112)
	HCFP	NIR fluorescent imaging	breast cancer mouse model	(113)
uPA	ATN-291	monoclonal antibody	prostate cancer	(114)
	Amiloride, HMA	uPA inhibition	metastatic lung and pancreatic cancer xenograft, cervical cancer	(115, 116)
	B-428, B-623	uPA inhibition	fibrosarcoma, prostate and breast cancer mouse models	(117, 118)
	WX-671, WX-UK1	uPA inhibition	breast and cervical cancer, head and neck squamous cell carcinoma	(119, 120)
	UK122	uPA inhibition	pancreatic cancer	(121)
	AF680-U33 IgG	NIR fluorescent imaging	prostate cancer xenograft	(122)
	^{111}In-U33 IgG	SPECT/CT imaging	prostate cancer xenograft	(122)
	^{89}Zr-Df-ATN-291	PET imaging	glioblastoma xenograft	(123)
	P-Dex	NIR fluorescent and photoacoustic imaging	breast cancer xenograft	(124)
	PB1	Antibody prodrugs	lung cancer xenograft	(125)
KLKs	FE999024	KLK1 inhibition	breast cancer cell line	(126)
	MDPK67b	KLK2 inhibition	prostate cancer xenograft	(127)
	SFTI-FCQR (SFTI)	KLK4 inhibition	ovarian cancer cell line	(128)
	APPI-4M	KLK6 inhibition	breast cancer cell line	(129)
	DKFZ-251	KLK6 inhibition	pharynx carcinoma cell line	(130)
	Ac-GKAFRRL-12ADT	KLK2-targeting prodrug of thapsigargin	prostate cancer cell line	(131)
	L-377,202	KLK3-targeting prodrug of doxorubicin	prostate cancer	(132, 133)
	KCC-TGX	KLK3-targeting prodrug of TGX-221 (PI3Kβ) inhibitor	prostate cancer cell line	(134)
	^{89}Zr-labeled 5A10 mAb	PET imaging	prostate cancer xenograft	(135)
	^{111}In-DTPA-11B6 mAb	SPECT/CT imaging	prostate cancer xenograft	(136)
	KLK2/3/14_fABP	fluorescent imaging	prostate cancer cell line	(137)
Gzms	NOTA-GZP	PET imaging	colon cancer mouse model	(138, 139)
	^{64}Cu-GRIP B	PET/CT imaging	colorectal cancer mouse model	(140)
	SK15.5	fluorescent imaging	breast cancer cell line	(141)
	qTJ71	fluorescent imaging	breast cancer cell line	(142)
	GzmB probe 1	chemiluminescent imaging	breast cancer mouse model	(143)
	GNR (nanoreporter)	NIR fluorescent imaging	colon carcinoma mouse model	(144)
	$CyGbP_F$	NIR fluorescent imaging	breast cancer mouse model	(145)
	SPNP	NIR fluorescent and photoacoustic imaging	breast cancer mouse model	(146)

by extracellular proteases. EGFR Probody PB1 only binds EGFR following cleavage by matriptase, uPA, or legumain (125).

While cancer immunotherapies have shown significant clinical outcomes, only a small subset of patients respond to the treatment, calling for reliable biomarkers and therapeutic strategies to maximize the benefits of the immunotherapy. Targeting the immunomodulatory, tumor stroma-associated serine proteases may provide a potential therapeutic option that complements and/or synergizes with the currently available immuno-oncology therapeutics. In cancer patients that high FAP expression in CAFs restricts T cell distribution and promotes immune checkpoint blockades (ICBs) resistance (41, 157, 158), treatment with FAP inhibitor could neutralize the immunosuppressive function of CAFs and reverse anti-PD1 drug resistance. Conditional depletion of FAP^+CAFs could result in enhanced T cell infiltration and better response to anti-CTLA4 and anti-PD-L1 treatment. Injection of chimeric antigen receptor (CAR) T-cells constructed with anti-FAP mAb effectively

depleted FAP^{hi} stromal cells and inhibited tumor growth. More importantly, FAP-specific CAR T cells augmented the antitumor responses of endogenous $CD8^+$ T cells (159). The FAP-specific CAR T cell therapy lately advanced into a phase I clinical trial for treatment of patients with malignant pleural mesothelioma (NCT01722149).

Tumor-specific protease-activation can also be exploited to limit systemic exposure of immunotherapeutic agents and minimize unwanted immune toxicity and side effects. Recently, a dual variable domain (DVD) immunoglobulin of anti-CTLA4 antibody, that an outer domain (tumor targeting) is connected by serine protease cleavable linkers to an inner domain (CTLA4 targeting), has been developed (160). During systemic circulation, the CTLA4-binding site is shielded by the outer domain. Upon reaching the tumor, the outer domain is cleaved by membrane type-serine protease 1 (MT-SP1) present in the tumor microenvironment, leading to enhanced localization of anti-CTLA4 without causing significant treatment-associated toxicity. To deliver functionally active cytokines, that are found to be critical for establishing and maintaining the immune response to tumors, preferentially at the tumor site, the Frelinger group developed an activatable IL-2 fusion protein consisting of IL-2 joined to a specific IL-2 binder that blocks its function connected by a KLK3 cleavage sequence (161). This PACman (for *p*rotease *a*ctivated *c*ytokine) approach has been also applied to the generation of IL-12 fusion protein (162). Tumor-specific protease-activation strategy should be applicable to other TME proteases and immunomodulators allowing site-specific activation in immunotherapy.

A granule-associated serine protease GzmB has attracted significant attention as an early predictive biomarker for monitoring of immunotherapy responses. In particular, *in vivo* imaging approaches with activatable probes utilizing GzmB-cleavable IEPD or IEFD linker allow to directly report functional readouts of immune cell (e.g. CTLs and NK cells) infiltration, activation, and cytotoxicity in tumors after immunotherapy treatments (138–140, 143–146). Advanced protease-responsive technologies would enable more comprehensive exploration of proteolytic networks in cancer and provide the next generation of clinical modalities for cancer chemotherapy and immunotherapy.

CONCLUSION

Understanding molecular mechanisms that orchestrate the complex and dynamic interplay between tumor cells and stromal microenvironment is important to provide insight into cancer biology. During malignant progression, several serine proteases appear to be key players at the tumor-stroma interface. These serine proteases function in multidirectional way by interacting not only with other proteases but also with important signaling pathways that involve activation or inactivation of cytokines, chemokines, growth factors, and kinases. The functions of serine proteases described in this review are not intended to be exhaustive but rather representative examples of recent discoveries. It is necessary to continue investigating the multifaceted roles proteases play within the tumor microenvironment in addition to their effects on the degradation of extracellular matrix to find more relevant diagnostic markers and therapeutic targets. Especially, in the context of anti-tumor immunity either naturally occurring or induced by immunotherapy, elucidating how these serine proteases on stromal cells are involved in modulating immune responses will help advance pharmaceutical strategies. Given the functional redundancy of proteases and cellular heterogeneity within the TME, advanced technologies that allow to dissect and manipulate specific serine protease with a spatiotemporal control will be highly required.

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RT and EY made substantial contributions to conception and design of the review, contributed to the manuscript writing and revision, read and approved the submitted version.

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High-Grade Endometrial Stromal Sarcoma: Molecular Alterations and Potential Immunotherapeutic Strategies

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Endometrial stromal tumor (EST) is an uncommon and unusual mesenchymal tumor of the uterus characterized by multicolored histopathological, immunohistochemical, and molecular features. The morphology of ESTs is similar to normal endometrial stromal cells during the proliferative phase of the menstrual cycle. ESTs were first classified into benign and malignant based on the number of mitotic cells. However, recently WHO has divided ESTs into four categories: endometrial stromal nodules (ESN), undifferentiated uterine sarcoma (UUS), low-grade endometrial stromal sarcoma (LG-ESS), and high-grade endometrial stromal sarcoma (HG-ESS). HG-ESS is the most malignant of these categories, with poor clinical outcomes compared to other types. With advances in molecular biology, ESTs have been further classified with morphological identification. ESTs, including HG-ESS, is a relatively rare type of cancer, and the therapeutics are not being developed compared to other cancers. However, considering the tumor microenvironment of usual stromal cancers, the advance of immunotherapy shows auspicious outcomes reported in many different stromal tumors and non-identified uterine cancers. These studies show the high possibility of successful immunotherapy in HG-ESS patients in the future. In this review, we are discussing the background of ESTs and the BCOR and the development of HG-ESS by mutations of BCOR or other related genes. Among the gene mutations of HG-ESSs, BCOR shows the most common mutations in different ways. In current tumor therapies, immunotherapy is one of the most effective therapeutic approaches. In order to connect immunotherapy with HG-ESS, the understanding of tumor microenvironment (TME) is required. The TME of HG-ESS shows the mixture of tumor cells, vessels, immune cells and non-malignant stromal cells. Macrophages, neutrophils, dendritic cells and natural killer cells lose their expected functions, but rather show pro-tumoral functions by the matricellular proteins, extracellular matrix and other complicated environment in TME. In order to overcome the current therapeutic limitations of HG-ESS, immunotherapies should be considered in addition to the current surgical strategies. Checkpoint inhibitors, cytokine-based immunotherapies, immune cell therapies are good candidates to be considered as they show promising results in other stromal cancers and uterine cancers, while less studied because of the rarity of ESTs. Based on the advance of knowledge of immune therapies in

HG-ESS, the new strategies can also be applied to the current therapies and also in other ESTs.

Keywords: BCOR sarcoma, rare cancer, stromal sarcoma, tumor microenvironment, inflammation

INTRODUCTION

Endometrial stromal tumors (ESTs) are an uncommon, unique, and complicated subset of uterine mesenchymal cancers. ESTs show heterogeneous microscopic and genetic characters (1). The morphology of ESTs resembles normal proliferative endometrial stromal cells, so in most cases, an EST has to be identified by the genetic analysis and lesions (2).

ESTs can be classified into four groups along the criteria announced by the World Health Organization (WHO), i.e., endometrial stromal nodule (ESN), low-grade endometrial stromal sarcoma (LG-ESS), high-grade endometrial stromal sarcoma (HG-ESS), and undifferentiated uterine sarcoma (UUS). Molecular analysis of the tumor tissue is a promising method to classify ESTs. The number of members of UUS has been decreased as the technology of genetic analysis has been advanced. For example, NTRK-sarcomas were classified as a UUS, but this has been re-categorized as HG-ESS as the molecular mechanism has revealed (3).

The current therapeutic strategy of ESTs is surgical removal. For lesions limited in the uterus, en bloc removal of the affected and intact area is suggested. For HG-ESS patients with advanced-stage, adequate cytoreduction by metastasectomy is standard therapeutic protocol, while it is unclear that the cytoreduction improves the patient survival. Additionally, aggressive cytoreduction such as pelvic and para-aortic lymphadenectomy is not suggested with LG-ESS patients (4). Because the efficiency of adjuvant radiotherapy or chemotherapy is controversial, new medical strategies such as immunotherapy may have to be considered (5).

BCOR (BCL6 corepressor) gene resides on chromosome X, in the Xp11.4, and it has 16 alternative exons coding several proteins, with principal isoform encoded by 14 exons, giving rise to 1775 amino acids (6, 7). The nuclear protein of molecular mass ~190Kda is ubiquitously expressed in various tissues. However, the BCOR protein expression in adult human tissue is unknown (8). BCOR includes BCL-6-and MLLT3-binding domains, ANK repeats, and PUFD domain. The function of BCOR is mainly mediated by the BCL-6 binding domain, which interacts with the transcriptional repressor BCL-6, and the RAWUL domain, to which PCGF binds (7). BCOR genetic variation causes several carcinomas, and Gene fusions relating to it are associated with a diverse range of human neoplasms. BCOR mutation is directly related to cancer development by changing the protein's usual RNA recognition preference by various alternation splicing at the pre-mRNA level (9, 10).

In recent findings, BCOR mutation induces HG-ESS in several clinical cases. BCOR ESS shows a broad range of clinical cases complicating the diagnosis and therapeutic strategies. We aim to enlighten the complicity of BCOR-ESS *via* the viewpoint of genetic alterations and the tumor microenvironment (TME) formations.

HG-ESS

HG-ESS is a rare tumor officially recognized as a malignant tumor of the endometrial stroma in the 2014 WHO classification. HG-ESS is a stromal neoplasm displaying unclear uniform features intermediate between classic LG-ESS and UUS. They have characteristic genetic abnormalities t(10;17)(q22;P13), chiefly associated with the YWHAE-NUTM2 A/B fusion and often associated with a morphologically low-grade component. Morphological spectra vary according to genetic abnormalities. Recently, another subtype of a ZC3H7B-BCOR gene fusion-induced HG-ESS was discovered in new studies (11–14). Significant genetic alterations of HG-ESS show distinct characters and patterns histologically and mechanistically. The direct mechanistic evidence is not sufficient yet, but many of HG-ESS cases show the mutation of BCOR or other genes as the molecular analysis techniques advances (**Table 1**). YWHAE, NUTM2, EPC1, SUZ12, BCOR, BRD8, PHF1, ZC3H7B, TPR, NTRK1, LMNA, TPM3, RBPMS, NTRK3, EML4, COL1A1, PDGFB, STRN mutations are already reported mutations and other mutations may be revealed as the Next-generation Sequencing technique is being advanced.

YWHAE-NUTM2 Fusion

The YWHAE gene belongs to a broad family of proteins that mediate signaling by binding to phosphoserine-containing proteins (22). FAM22A/B was renamed NUTM2A/B due to sequence homology with NUT (NUTM1), famous for its role in nut midline carcinoma (22, 23). YWHAE-NUTM2 fusion tumors consisted of high-grade round and low-grade spindle cell components. The morphology is consisted of round cell sheets with intermediate size ovoid to round nuclei. Chromatin is open, and the staining pattern is scant to moderate eosinophilic cytoplasm. These sheets are adjacent to the fascicles of spindle cells resembling fibroblastic LG-ESS (16). Immunohistochemically, YWHAE-NUTM2 HG-ESS cells were perhaps immunoreactive to Cyclin D1 and BCOR.

ZC3H7-BCOR Fusion

A retrospective molecular reanalysis of uterine sarcoma patients with BCOR gene rearrangements confirmed that ZC3H7B was the most common partner of gene rearrangement by fusion at either front or back of BCOR. In addition to ZC3H7B, ten other BCOR gene rearrangement partners have been identified, which are EP300-BCOR, BCOR-L3MBTL2, BCOR-RALGPS1, BCOR-NUTM2G, BCOR-MAP7D2, ING3-BCOR, RGAG1-BCOR,

TABLE 1 | HG-ESSs with molecular alterations frequently reported.

Genes involved	Reported fusions/gene rearrangements/alterations	Translocations	References
YWHAE	*YWHAE/NUTM2*	t(10;17)(q22;p13)	(12)
NUTM2A/B/E	*EPC1-BCOR*	t(10;X)(p11;p11)	(15, 16)
EPC1	*EPC1-SUZ12*	t(10;17)(p11;q11)	(15, 16)
SUZ12	*BRD8-PHF1*	t(5;6)(q31;p21	(15, 16)
BCOR	*BCOR* alteration	none	(3)
BRD8			
PHF1			
ZC3H7B	*ZC3H7B-BCOR*	t(22;X)(q13;p11)	(14, 17)
BCOR	*BCOR-ZC3H7B*	t(X;22)(p11;q13)	
BCOR	*BCOR ITD*	none	(14)
TPR	*TPR-NTRK1*	1q31.1-1q23.1	(18)
NTRK1	*LMNA-NTRK1*	1q22-1q23.1	(18)
LMNA	*TPM3-NTRK1*	1q21.3-1q23.1	(18, 19)
TPM3	*RBPMS-NTRK3*	t(8;15)(p12;q25.3)	(18)
RBPMS	*EML4-NTRK3*	t(2;15)(p21;q25.3)	(19)
NTRK3	*COL1A1-PDGFB*	t(17;22)(q21.33;q13.1)	(18, 20)
EML4	*STRN-NTRK3*	t(2;15)(p22.2;q25.3)	(21)
COL1A1	*TPR-NTRK1*	1q31.1-1q23.1	(18)
PDGFB	*LMNA-NTRK1*	1q22-1q23.1	(18)
STRN	*TPM3-NTRK1*	1q21.3-1q23.1	(18, 19)

KMT2D-BCOR, BCOR-NUGGC, and CREBBP-BCOR (24). Molecular biologically, endometrial stromal sarcomas with BCOR rearrangement bear common MDM2 amplification and activation of the cyclin D1-CDK4 pathway by CDK4 amplification by cyclin D1 protein overexpression or CDKN2A deletion (25).

In tumors with BCOR gene rearrangements demonstrated sarcomas. Most cases were portrayed by spindle cells and various amounts of small round cell or epithelioid cell morphology. These cells usually have uniform nuclei, clear cytoplasm, and mild to moderate atypia. However, more minor cases exhibited moderate to severe atypia, illustrated by condensed chromatin, prominent nucleoli, and nuclear enlargement, often with an associated epithelioid or small round cell component. Additionally, some cases with BCOR rearrangements newly discovered sarcomas with epithelioid, spindle, or small round cell components and variable degrees of fascicular growth with collagenous or myxoid stromal change (24).

BCOR Internal Tandem Duplication

HG-ESS often contains an oncogenic fusion, and however, some tumors have morphological overlap with HG-ESS even without gene fusion. This kind of genetic alteration is also found in some pediatric primitive sarcomas, including undifferentiated circular cell sarcoma and clear cell sarcoma of the kidney in infancy. This same subset of pediatric sarcomas lacks the oncogenic fusion but instead has an internal tandem duplication (ITD) associated with exon 15 of BCOR, the so-called BCOR ITD. According to previous studies, BCOR ITD was confirmed in 3 cases out of 26 HG-ESS, 2 cases were undifferentiated uterine sarcoma with uniform nuclear characteristics, and 1 case was diagnosed as YWHAE-NUTM2-negative HG-ESS. All three mutations have resulted in tandem replication of varying sizes of exon 15, which is the 3' end of BCOR encoding the C-terminal end of BCOR protein (26).

Other Alterations

EPC1-BCOR, EPC1-SUZ12, BRD8-PHF1, BCOR-ZC3H7 also show HG-ESS progression in the uterus (2, 15, 16). As a newer type of HG-ESS is being discovered, histopathological observation is not enough to identify a specific type of genetic alteration. This is why genetic analysis of ESS samples is required to distinguish the kind of ESS, including HG-ESS, such as the next-generation sequencing or even Sanger sequencing of the RT-PCR product (**Table 1**).

TUMOR MICROENVIRONMENT OF SARCOMAS

The tumor microenvironment (TME) of stromal sarcomas comprises non-malignant stromal cells, blood vessels, immune cells, and tumor cells. Matricellular proteins and extracellular matrix (ECM) proteins arising from stromal cells are crucial for cellular movement *via* structural support and signal transduction. Immune cells in TME of sarcomas are composed of innate immune cells such as neutrophils, tumor-associated macrophages (TAM), tumor-associated dendritic cells (TADCs), and natural killer (NK) cells, and adaptive immune cells like B-cells and T-cells. TAMs, TANs and TADCs show protumoral functions leading to metastasis and cell invasion, ECM remodeling and angiogenesis by suppressing immune surveillance for antitumoral effect (27). TAMs share much of the characters of M2 macrophages, which is protumoral. As EST turns malignant, M2 is being dominant over M1 TAMs. Dominant M2 portion in malignancy is because angiogenic environment such as VEGF, hypoxia and epigenetic derangements (28). TANs are mostly consisted with protumoral N2 cells rather than antitumoral N1 cells like the case of TAMs. TADCs lose the antigen-presenting cell (APC) function and obtain protumoral effect (29). The composition of

individual subsets of these immune cells differs significantly depending on the prior treatment, primary tumor location, sarcoma subtype, and genetic background. TAM is one of the four major subgroups of tumor-associated myeloid cells (TAMCs), which also include tumor-associated neutrophils (TANs), myeloid-derived suppressor cells (MDSCs), and Tie2-expressing monocytes (TEMs) (**Figure 1A**) (30–32). Several activated and antigen-specific T-cell therapies have been tested for sarcomas, which showed exhaustion of T-cells by immune regulation by TME such as mesenchymal stem cells (MSCs), regulatory T cells (Tregs), and TAMs.

The strong correlation of macrophages and sarcomas shows TAMs may have to be therapeutically targeted to overcome tumor progression and patient survival in sarcoma patients (33–36). Even though TAMs phagocytose necrotic tumor cells, TAMs show tumor-promoting functions and immunosuppression in sarcomas. For example, TAMs increased number of TAMs in TME induced the decrease of the effect of chimeric antigen receptor (CAR) T-cell immunotherapy (37). Meanwhile, the entire mechanism of the number and density of TAMs in sarcomas should still be investigated to promote tumor progression and immune cell profiles.

IMMUNE SUPPRESSION IN ENDOMETRIAL CANCERS

CIBERSORT is a retrospective in silico analysis. CIBERSORT enables profiling immune cells through the deconvolution of gene microarray data sets (35, 38). The deconvolution reconstructed relative quantity, and the immune cell subsets residing in tumor tissue. The cell-type determination is made from the gene expression dataset by matching the information of 547 markers of 22 known peripheral immune cells (38). This method has the advantage of enabling detecting functionally

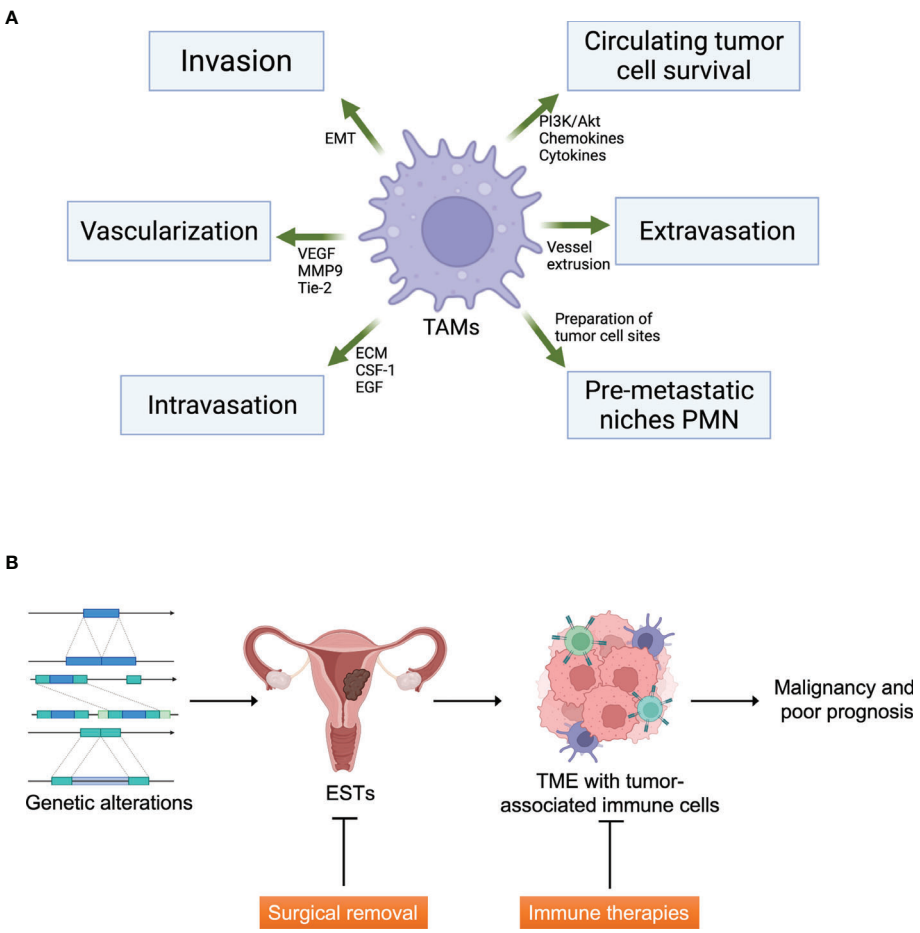

FIGURE 1 | Tumor microenvironment and ESTs. **(A)** The functions of tumor-associated macrophages (TAMs) in tumor microenvironment formation. TAMs affect tumor cell metastasis including invasion, vascularization, intravasation, formation of pre-metastatic niches and protection of circulating tumor cells. EMT, epithelial-mesenchymal transition; VEGF, vascular endothelial growth factor; MMP9, matrix metallopeptidase-9; ECM, extracellular matrix; CSF-1, colony-stimulating factor-1; EGF, epithelial growth factor. Created with Biorender.com. **(B)** The graphical abstract of ESTs and therapeutic strategies. Immune therapies can be an additive strategy to cure malignant ESTs targeting TME. Created with Biorender.com.

distinct and rare immune cell types such as Tregs, γδ T cells, mast cells, and memory B cells (35). Flow cytometry has effectively confirmed this advanced technology and is used to decide the composition of infiltrated immune cells in many different malignant tumors like colon cancer and breast cancer (39, 40).

The immunological aspect of the endometrial TME has been less studied, unlike ovarian and other solid tumors. Furthermore, ESS has been even less studied than endometrial adenocarcinomas. In this aspect, it is worthwhile to learn the TME of adenocarcinomas to apply to ESSs. Especially TAMs and antitumor adaptive immune responses, $FoxP3^+$ Tregs in endometrial cancers is not the only issue of adenocarcinomas as they are the most abundant immune cells in stroma (41).

POSSIBLE IMMUNOTHERAPEUTIC STRATEGIES

The possible involvement of the immune system in regulating cancer was first observed in patients with sarcoma when Wilhelm Busch of Germany reported tumor regression in 1866 of a sarcoma patient who developed an erysipelas infection (42). Immunotherapy to treat sarcoma can be traced back to at least 1891. At the time, William Coley, a noticeable orthopedic surgeon at New York Memorial Hospital, currently Memorial Sloan Kettering Cancer Center, developed what was known as "Coley's Toxin" to treat a series of osteosarcomas (43, 44). He found that injection of a streptococcal organism (originally a live bacterium, a mixture that was later killed by heat containing Serratia marcescens) could induce remission in some patients with inoperable sarcoma. His use of the toxin was controversial and eventually lost popularity, but many consider it today as a forerunner of modern anticancer immunotherapy (45).

Maybe the best definition of modern immunotherapy is from Paul Ehrlich's early 1900's description of "Magic bullet" - a specific drug that only attacks and kills diseased cells, leaving no surrounding normal cells (46). The increased frequency of lymphoid malignancies in immunocompromised patients suggests that the immune system plays an essential role in carcinogenesis (47). In addition, the development of sarcoma is well known in allograft recipients, and the risk of developing it in non-immunocompromised patients more than doubles (48). As the cancer treatment adapts personalized and tailored medicine, personally tailored immunotherapies may be an additional plus to the traditional immunotherapeutic strategies. In this section, we want to mention currently available immunotherapies that may treat HG-ESS.

Targets of immune checkpoint inhibitors include programmed cell death protein-1 (PD1), and its ligands, PDL1/PDL2, mucin protein 3 (Tim3), and its ligand galectin-9, T cell immunoglobulin, and CTL-associated antigen 4 (CTLA-4). Blocking CTLA-4 while priming has been reported to the CD8+T cell accumulation leading to the production of effector cytokines like INF-γ. For example, CTLA-4 blockade successfully showed decreased tumor size in the mouse stromal cancer model (49). Numbers of tumoral CD4+ and CD8+ T-cells or CD8/Treg ratio in tumors are not affected by CTLA-4 blockade. Still, the increased production of IFN-γ from tumoral CD8+ T-cells shows the benefit of CTLA-4 inhibition (50).

PD1 is exclusively expressed in immune cells such as T-cells. Meanwhile, PD-L1 is widely expressed by different cells, especially tumor cells. The interaction of PD1 and PD-L1 is one of the significant immune escape mechanisms of tumor cells. PD-L1 mRNA expression is highly upregulated in stromal cancer cells as well as heterogeneously expressed across tumor tissue (51). One confusing point of PD-L1 is that patients with low PD-L1 expression may have higher metastatic risk than patients with high PD-L1 expression. The analysis of immune checkpoint molecules from stromal cancers found PD1, lymphocyte activation gene 3 (LAG3), and T-cell immunoglobulin mucin 3 (TIM3) upregulation on tumor-infiltrating T-cells compared matched control blood cells (52). Although PD-L1 expression in ESTs has not been studied systemically, PD-L1 has been positively expressed in primary tumors of 77% patients and 30-40% in metastatic lesions from 88 cases of uterine cancer (53). Although this report revealed PD-L1 or PD-1 expression is not directly associated with the prognosis, the origin of cells was not categorized, so further detail study from EST patients is required. Considering the prognostic value of PD-L1 in other stromal cancers, the importance of analyses in ESTs seems to be required.

The next considered immune checkpoint in stromal cancers are TIM3 and its binding partner, galectin-9. TIM3 is expressed on immune cells, while galectin-9 is expressed on cancer cells. The pathway of TIM3 and galectin-9 has been known for their role in cancer malignancies. Blocking this pathway is being actively investigated in the meaning of immune checkpoint inhibition (54, 55). Studies on TIM3 revealed NK cells and tumor tissues show the expression of TIM3 and galectin-9, respectively (55, 56). This shows TIM3/galectin-9 may also have an essential role in HG-ESS, even though the direct evidence is low. Currently, Nivolumab which inhibits PD-1 is under clinical trials with or without the combination of Ipilimumab, which inhibits CTLA-4 (57). It is not easy to expect how the outcome of the trials will be, but immune checkpoint inhibitors can be an excellent candidate in HG-ESS therapy in the future.

Cytokine-based immunotherapy can be the following strategy for HG-ESS. Type I interferons such as IFNα are a physiological danger signal that promotes Th1 responses and memory T-cell differentiation. IFNα treatment combined with imatinib remarkably achieved complete responses in stromal cancer patients (58). IFNγ is well-known for its innate and adaptive immunity role and is produced by activated NK cells, NKT cells, CD4+ T-cells, and CD8+ T-cells. IFNγ was decreased in stromal cancer cell patients, but IFNγ producing cell subsets increased significantly after IFNα treatment. Furthermore, tumor-infiltrating leukocytes (TILs) of the patients actively expressed IFNγ after IFNα treatment (58).

Immune cell therapy is a highly focused area in cancer therapy. For example, the chimeric antigen receptor (CAR) T-cells are genetically modified specific cancer-specific T lymphocytes produced against a particular tumor antigen of a specific cancer cell. T-cells are isolated from a patient's peripheral blood and then

activated. Retroviral or lentiviral transduction with a CAR lets the T-cell express the surface receptor, explicitly recognizing the tumor cells with the cognate antigen. Several studies observed CAR-T-cells are effective in stromal cancers and uterine cancers, which is also a promising therapeutic future of HG-ESS (59, 60). These results show that CAR T-cells have a substantial role in the immunotherapy against stromal cancers, including ESS. Additionally, a recent finding shows active neutrophils drive unconventional T cells to mediate resistance to sarcomas in mouse and some human cancer (61). With the help of neutrophils, T cells are polarized to UTCαβ and type 1 immunity is strongly activated, which may lead to the resistance to the stromal tumors including HG-ESS and other ESTs.

The strategies stated in this section, such as checkpoint inhibitors, cytokine therapy, and immune cell therapy, are not being vigorously studied for the treatment of HG-ESS currently. However, the potential of immunotherapy is highly promising considering the successful results from the other stromal cell tumors and uterine cancers. The need of promising survival of patients still exists with advanced HG-ESS patients with cytoreduction surgery. Because the complete removal of the tumor is not available in patients with advanced cancer, uncertain radiotherapy and chemotherapy have been the only options for those patients. So, the development of immune therapy will be the next therapeutic option for patients with advanced HG-ESS.

CONCLUSIONS

Recent advances in Next Generation Sequencing (NGS) have made tremendous efforts to incorporate the immunohistochemical and morphological EST classifications. This new change enabled molecular subclassifications, making HG-ESS identification clearer than before. The purpose of the molecular classification of ESTs is to understand the molecular biology to the development of specific-targeted therapies for each subcategory. This is very important for planning treatment strategies for metastatic disease. Therefore, we will observe the discovery of new individuals and gradually replace histopathological classification with molecular classification based on genetic analysis.

Scientific and medical evidence highly supports the perception that host immunity can be suppressed in many different mechanisms to eradicate and control stromal cancers. It is always complicated to fight rare cancers with various genetic mutations. However, new results configure the actions, types, and prognostic significance of TILs and clarify the mechanisms. TILs can be handled directly or in combination with other molecular therapeutic strategies to optimize tumor cell death. However, limiting the toxicity of this strategy should be considered. Immunotherapies are still in very early steps in experiments and development, but their potential in cancer therapy is tremendous and must be explored robustly to cure patients with ESS.

HG-ESS patients with advanced-stage have few therapeutic options when the tumor cannot be surgically removed ideally. However, adjuvant radiotherapy and chemotherapy have a prominent disadvantage, which shows inconsistent and controversial therapeutic efficiency. Personalized and tailored immunotherapy such as cytokine therapies, immune checkpoint inhibitors, and immune cell therapies remarkably succeeded in several advanced cancers, including stromal cancers and uterine cancers. This is why immune therapy should be considered in HG-ESS patients. Furthermore, based on the advance of knowledge of immune therapies in HG-ESS, the new strategies can also be applied to en bloc resection-available HG-ESS, LG-ESS, and UUS in the future (**Figure 1B**).

AUTHOR CONTRIBUTIONS

YK and DK wrote the original draft. WJS and JH supervised and finished the manuscript. All authors contributed to the article and approved the submitted version.

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A Paradoxical Effect of Interleukin-32 Isoforms on Cancer

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IL-32 plays a contradictory role such as tumor proliferation or suppressor in cancer development depending on the cancer type. In most cancers, it was found that the high expression of IL-32 was associated with more proliferative and progression of cancer. However, studying the isoforms of IL-32 cytokine has placed its paradoxical role into a wide range of functions based on its dominant isoform and surrounding environment. IL-32β, for example, was found mostly in different types of cancer and associated with cancer expansion. This observation is legitimate since cancer exhibits some hypoxic environment and IL-32β was known to be induced under hypoxic conditions. However, IL-32θ interacts directly with protein kinase C-δ reducing NF-κB and STAT3 levels to inhibit epithelial-mesenchymal transition (EMT). This effect could explain the different functions of IL-32 isoforms in cancer. However, pro- or antitumor activity which is dependant on obesity, gender, and age as it relates to IL-32 has yet to be studied. Obesity-related IL-32 regulation indicated the role of IL-32 in cancer metabolism and inflammation. IL-32-specific direction in cancer therapy is difficult to conclude. In this review, we address that the paradoxical effect of IL-32 on cancer is attributed to the dominant isoform, cancer type, tumor microenvironment, and genetic background. IL-32 seems to have a contradictory role in cancer. However, investigating multiple IL-32 isoforms could explain this doubt and bring us closer to using them in therapy.

Keywords: interleukin-32, tumor microenvironment, stromal tumor, hypoxia, metastasis

INTRODUCTION

The human interleukin-32 (IL-32) is a novel cytokine that exerts both pro and anti-inflammatory roles. IL-32 gene is found in higher primates, and it is located in chromosome 16 at p13.3 encoding for various isoforms. IL-32 plays an essential role in innate and adaptive immune responses, and it induces various cytokines such as tumor necrosis factor (TNF)-α, IL-1β, IL-6, and IL-8 (1). After its

identification, it has been studied in inflammatory disorders including autoimmune diseases and cancers (2, 3).

In cancer, inflammatory tumor microenvironment such as cytokines, IL-32 plays a crucial role in its progression (4). Therefore, IL-32 has been studied for its tumor control direction in several cancer types. However, paradoxical effects have been reported regarding IL-32 on cancers, which may be attributed to the dominant isoform, cancer type, and genetic background. On the one hand, IL-32 was reported to augment cancer progression, proliferation, invasion, and metastasis in many tumors including acute myeloid leukemia (AML), hepatocellular carcinoma (HCC), and breast, lung, colon, pancreatic, and gastric cancers (5–12). On the other hand, it was also reported to have anticancer activity in different cancers including acute and chronic myeloid leukemia (AML and CML) and breast, lung, and colon cancers (13–19).

IL-32 gene was found to have several isoforms based on different alternative splicing sites. It has eight exons in which the first exon does not translate into amino acids. Mainly, seven isoforms were depicted and were identified separately which are IL-32α, IL-32β, IL-32γ, IL-32δ, IL-32ε, IL-32ζ, and IL-32θ (3). IL-32α, IL-32β, IL-32γ, and IL-32δ were primarily detected in IL-2-stimulated human NK cells. While IL-32ε and IL-32ζ were observed to be expressed in the activated T cells (20), and IL-32θ was found within dendritic, Jurkat, human leukemia T cells (21). Structural characteristics of the seven IL-32 isoforms were reviewed based on the IL-32 eleven protein domains (3). However, a lot of knowledge is waiting to be revealed regarding IL-32 isoforms, such as their specific receptors. These isoforms displayed distinctive roles and consequences in different conditions although they are deficient in signal peptides. Therefore, a functional comparison between these isoforms as well as specific antibodies to detect IL-32 isoforms is considered necessary.

Nevertheless, what has been discovered so far still lacks explicit knowledge about IL-32 function in cancers. It is known that many factors can affect the disease outcome, especially in cancer, yet this much contradiction was not reported to any cytokine other than IL-32. This contradiction is mainly due to not considering IL-32 isoforms in most of the studies. In this review, we aim to analyze previous reports to address the most probable functions of IL-32 on different cancers to provide recommendations for further studies and unravel possible therapeutic options.

IL-32 IN CANCER PROLIFERATION AND APOPTOSIS

IL-32 was found to play two contradictory roles in cancer development among various cancer types, one role as a critical proliferation and growth factor and the other as a tumor suppressor. Higher expression of IL-32 was found to be associated with more proliferative and progression in the following cancers, AML, cutaneous T-cell lymphoma (CTCL), gastric B-cell lymphoma (GBCL), multiple myeloma (MM), HCC, and breast, lung, colon, pancreatic, gastric, and esophageal cancers (5–12, 22–25).

In acute leukemia peripheral blood of patients, IL-32 was closely related to the disease development (5). Recently, AML-derived mesenchymal stem cells (AML-MSCs) when cocultured with K562/K562 ADM cells, showed changes in the expression of IL-6 and IL-32 cytokines. These data suggested its effect on proliferation, invasion, metastatic, and drug resistance through dysregulation of bone morphogenetic protein-4 (BMP4) pathway as well as increased the connective tissue growth factor (cTGF) in K562 ADM cells (**Figure 1A**) (6). BMP pathways modulate the expression of target genes, and it was found to inhibit the expression of IL-6, suggesting a similar effect on IL-32 (26, 27). Therefore, dysregulation of BMP4 seems to have the opposite effect and thus increase the expression of cytokines. Moreover, a recent study has revealed a cancer suppressor effect when the BMP4 signaling pathway is activated (28). On the other hand, cTGF promotes the spindle shape transformation that is responsible for the invasion and metastatic thus, contributing to the disease progress.

Although studies mentioned above indicated the enhancement role of IL-32 in AML survival, an inhibitory effect of this cytokine was also reported, specifically IL-32θ isoform, by regulating TNF-α production in AML (13). In this study, they divided AML patients into two groups based on the presence of IL-32θ and found that IL-32θ inhibits the increment of TNF-α. They then confirm that IL-32θ inhibited phosphorylation of p38 mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) *in vivo*. In addition, IL-32θ attenuated TNF-α promoter activity and the binding of NF-κB with the TNF-α promoter (**Figure 1B**). Moreover, another inhibitory effect of IL-32 was reported in CML cells through enhancing natural killer (NK) cell-mediated killing (14). Here, the NK killing activity is achieved through stimulation of both the Fas receptor and UL16-binding protein (ULBP), ligands of NKG2D in NK. The performance of more IL-32 experiments in the absence of specific IL-32 isoform characterization may show vast contradictions. The wide range of activities can be confusing at this moment, but studying its isoforms in depth may shed light on this seemingly paradoxical function.

IL-32α induces this stimulation through activation of p38 MAPK. IL-32α also inhibits B-cell CLL lymphoma through regulation on epigenetic posttranslational modifications. B-cell lymphoma-6 (Bcl-6) has been associated with progression of lymphomas and is considered a master regulator of cellular processes (29). Bcl-6 was found to be inhibited by IL-32α *via* the production of IL-6 and PKCε-mediated cell adhesion (30). PKCε is known to have two major roles that are inhibition of apoptosis and promotion of cell survival as one of its regulated pathways in the activation of STAT3 (31, 32). IL-32 regulates this activation and induces apoptosis (**Figure 2**).

Active PKCε crosstalks to multiple signal transduction pathways result in the following two major cellular effects: (1) inhibition of apoptosis and (2) promotion of cell survival. PKCε-regulated cell survival pathways include Stat3 activation, expression of growth-stimulating cytokines (TNF-α, GM-CSF, and G-CSF), and growth factors (e.g., EGFR). PKCε mediates

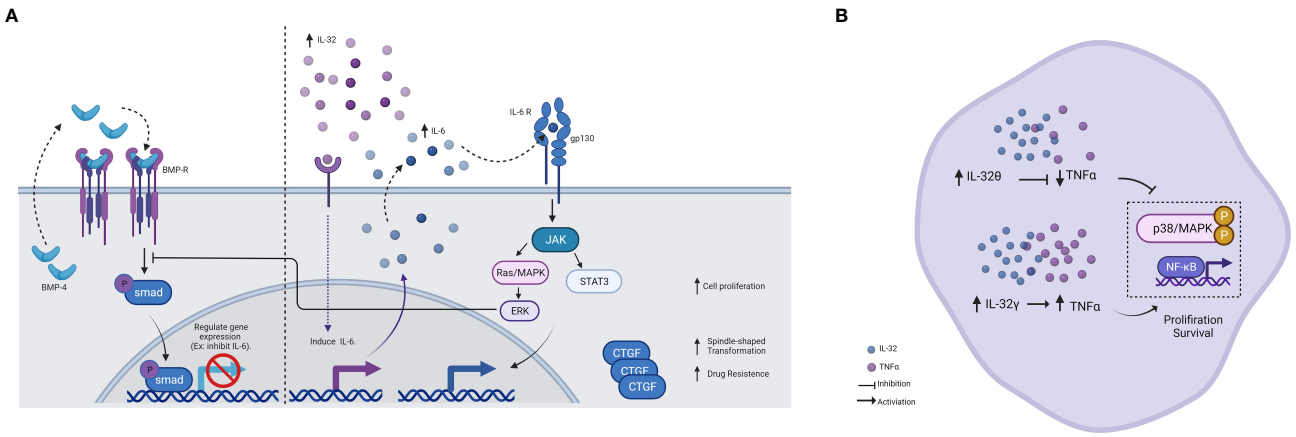

FIGURE 1 | Schematic illustration showing the effect of IL-32. **(A)** Schematic illustration showing the effect of IL-32 on the BMP pathway and IL-6 induction. In the presence of BMP4, it feeds the loop and binds to the BMP receptor, activating SMAD and thus regulating gene expression. IL-6 is inhibited by this regulation. On the other hand, in the presence of IL-32, IL-6 is induced and activates several pathways. One of these pathways is ERK, which in turn inhibits SMAD. Therefore, IL-6 induced by IL-32 acts as negative feedback for the BMP pathway, as results of cell proliferation increased. IL-32 was found to increase the expression (either directly or indirectly) of the connective tissue growth factor (cTGF), as results of spindle-shape transformation increased, and thus invasion and metastasis occurred. **(B)** Schematic illustration showing the different effects of IL-32 isoform in AML. IL-32γ was shown to induce TNF-α production and activate NF-κB and MAPK signaling pathways and therefore, increased proliferation and survival. Whereas, IL-32θ was shown to inhibit TNF-α and phosphorylated p38 MAPK and NF-κB, thus, reducing cancer progression. This makes IL-32θ to be considered a potent inhibitor of TNF-α in patients with AML. Figure created by BioRender App.

inhibition of apoptosis *via* inhibition of FADD expression. All these pathways in fact constitute a network.

THE EFFECT OF CYTOKINE SIGNAL PATHWAYS IN THE ROLE OF IL-32 IN CANCER

The influence of IL-32 in tumor growth through the inactivation of NF-κB and signal transducer and activator of transcription 3 (STAT3) pathways have been mentioned earlier (33). Moreover, IL-32γ downregulates vital cancer progression proteins including antiapoptotic, cell proliferation, and tumor-promoting genes, while upregulating the apoptotic genes. On the contrary, IL-32γ isoform is shown to diminish the levels of cytokines that promote tumor growth such as TNF-α, IL-1β, and IL-6, whereas the levels of IL-10 cytokine, a tumor growth-inhibiting cytokine, were elevated. The anticancer activity of IL-32γ was found in several cancer cells but not in melanomas like colon, prostate, liver, and lung. It also induces the activation of cytotoxic T cells and NK cells to the tumor site to expand the cancer eradication effect (33, 34) as well as recently, it showed better immunotherapy response (35).

Later, IL-32β has been found to play an antitumor activity role as it downregulates vital cancer progression proteins including antiapoptotic proteins, proliferation, and cell growth regulatory proteins through the same pathways, NF-κB and STAT3. In addition, IL-32β was found to induce the expressions of proapoptotic proteins and regulate the release of cytokines in colon and prostate cancer cells (15). Nevertheless, higher expression of IL-32α has been found to activate NF-κB and STAT3 pathways and induce the production of IL-6, thus supporting the cancer proliferation and progression in MM patients (25). Therefore, finding the exact function of the IL-32 isoform is still a sensitive consideration and may be influenced not only by its isoform but also with cancer type as well as the whole tumor microenvironment.

We have mentioned the anticancer activity of IL-32γ in colon cancers, which is considered through activation of p38 MAPK pathways (16). Moreover, IL-32α and IL-32θ have been found to suppress the effect on colon cancer, as well (17, 18, 36). In the case of the expression IL-32α, the expression of TNF receptor 1 and the production of reactive oxygen species was increased, thus facilitating apoptosis and prolonged JNK activation. At the same time, several studies have mentioned the contradictory role of IL-32 in colon cancer (7, 37, 38) whereas IL-32 was found to be upregulated and associated with poor survival. In this regard, it is worth mentioning a finding that provides evidence on the contribution of IL-32α in the development of obesity-associated colon cancer by favorably remodeling cytokine for tumor growth (39). According to the currently available data, we can suppose that in colon cancer, IL-32α has both pro- and antitumor activity depending on other factors such as obesity, gender, and/or age-related factors which have not been studied yet. However, obesity-related IL-32 manipulation indicates that IL-32 could play a role in cancer metabolism as well as inflammation.

IL-32 IN BREAST CANCER

In breast cancer, its metabolism regulation was found to be influenced by IL-32β expression. IL-32β was stimulated due to hypoxia and found to increase glycolysis and Src (proto-oncogene

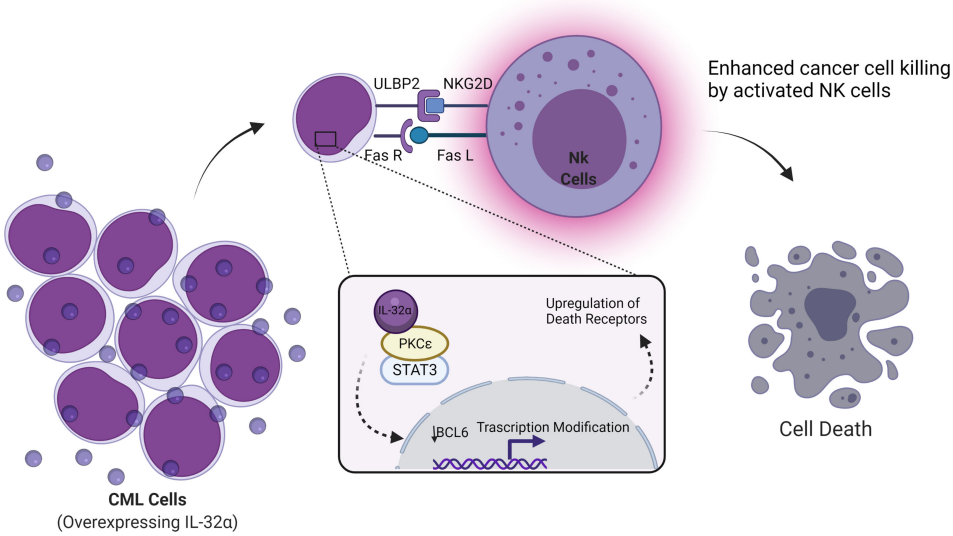

FIGURE 2 | Schematic illustration showing cancer cell death by IL-32α in CML and lymphoma. Cancer cell death was reported when the IL-32α isoform is expressed in CML or lymphoma. This cancer inhibitory effect occurs through enhancing natural killer (NK) cell-mediated killing. PKCε inhibits apoptosis and the promotion of cell survival, by regulating several pathways, one of PKCε regulations is the activation of STAT3. IL-32α binds to PKCε and inhibits its functions and regulations. As a result, transcriptional modifications occurred including the downregulation of Bcl-6 and the upregulation of death receptors (ULBP2 and Fas receptor) resulting in NK cell-mediated killing by the stimulation of both Fas receptor and ULBP. ULBP is a ligand of NKG2D in NK. Figure created by BioRender App.

tyrosine-protein kinase) activation by activating lactate dehydrogenase and inhibiting Src dephosphorylation, respectively (40). This metabolic change is achieved through lactate dehydrogenase activation when IL-32β translocated into mitochondria due to its accumulation. Moreover, the inhibition of hypoxia-induced IL-32β impairs tumor cell growth, making it a potential drug target (8, 40). Interestingly, when both mRNA and protein levels were evaluated, IL-32 demonstrated isoform switching and self-regulation, as at mRNA levels IL-32β and IL-32γ were detected. However, at the protein level, through Western blot, only IL-32β was detected (41). Another study has also reported that elevated IL-32 promoted growth, stemness, and progression in breast cancer (42). In addition, because IL-32 was found to be highly expressed in cancer tissue of triple-negative breast cancer patients, it was suggested as a probable therapeutic target (9).

Moreover, the elevation of IL-32β expression under hypoxic conditions was also found in ovarian cancer cells by reducing its degradation. They found that IL-32β interacts with protein kinase Cδ (PKCδ) thus promoting antiapoptotic function under oxidative stress, which is almost the case in breast cancer. However, more recently, IL-32θ isoform was found to utilize antiproliferative effects in breast cancer cells and initiate senescence (43, 44). Intriguingly, it was revealed that IL-32θ interacts directly with PKCδ and subsequently reduces NF-κB and STAT3 levels and thus inhibits epithelial-mesenchymal transition (EMT). This effect could provide a clue regarding the different functions of IL-32 reported in cancer. Although PKCδ is known for its proapoptotic function in cancer cells (45), it seems that PKCδ when interacting with a different isoform of IL-32 exhibits different signal therefore different effect (**Figure 3**).

Also, when IL-32 has been reviewed, the difference between these two isoforms (IL-32β and IL-32θ) was revealed to be only one motif consisting of 20 amino acids (DDFKEGH LETVAAYYEEQHP) (3). In another word, both isoforms shared the binding site for PKC, but this motif is mainly responsible for its furthered role. Therefore, it can be suggested that this motif found within isoform β but not θ (DDFKEGHLETVAAYYEEQHP) can activate PKC function and therefore enhance cancer progression. Extended physical interaction and functional studies are required to prove this conclusion. IL-32 altered the same pathway among several types of cancer; when the isoform is changed, the final effect is also changed. Therefore, it is very crucial to introduce some regulations when studying this IL-32 cytokine. It is necessary to detect the isoforms and their levels in the same study case. Isoforms should be determined in both mRNA and protein levels and recognize their specific cellular localization such as cytoplasm, extracellular, or nucleus.

IL-32 IN GI, ESOPHAGEAL, GASTRIC, LIVER, AND PANCREATIC CANCERS

Most GI cancers include esophageal, gastric, liver (e.g., HCC), and pancreatic cancers, were found to express higher levels of IL-32, and mostly exhibit a facilitating cancer progression role. IL-32 was highly expressed in tissue and serum of patients with HCC and was associated with disease progression (46–48). The only isoform studied in this cancer type was IL-32α, and its expression was correlated with antiapoptotic signals, mainly Bcl-2 regulator protein,

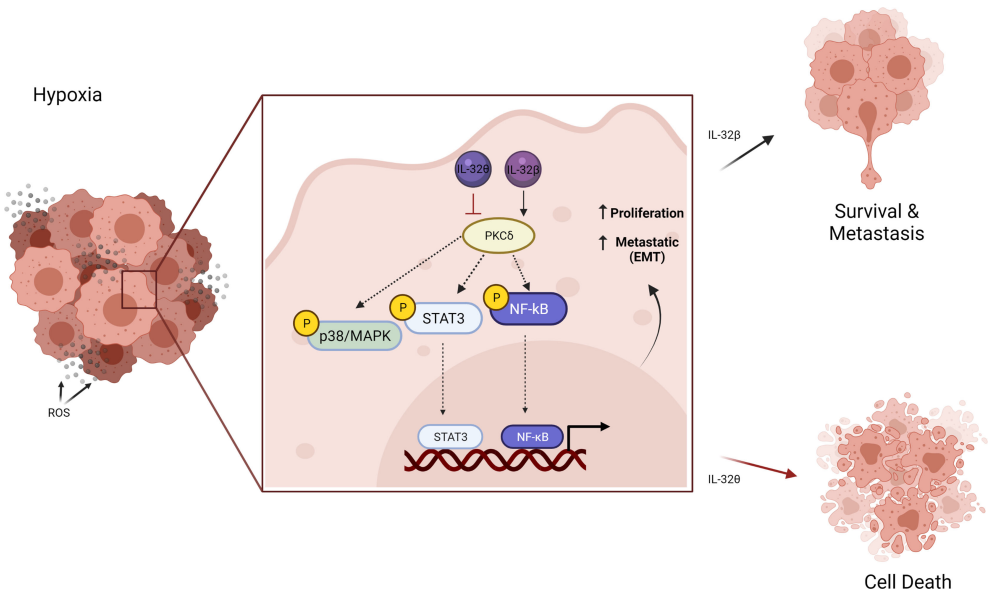

FIGURE 3 | Schematic illustration showing the different effects of IL-32 isoform in cancer cells under hypoxic conditions. The elevated IL-32β interacts with PKCδ in tumor-promoting antiapoptotic signaling that increased cancer progression. On the contrary, IL-32θ interacts with PKCδ inhibiting its antiapoptotic effect and reduces NF-κB and STAT3, thus inhibiting epithelial-mesenchymal transition (EMT). Figure created by BioRender App.

p38-MAPK, and NF-κB pathways. Moreover, similar activity for IL-32 in promoting cancer growth and survival was reported in pancreatic cancer (11, 49). Furthermore, its induction was facilitated through phosphatidylinositol 3–kinase/protein kinase B (PI3K/Akt) pathway-dependent NF-κB/AP-1 activation.

As IL-32 is highly expressed in serum and tissues of GI cancers, it was found with 99.5% accuracy in detected gastroesophageal cancers as a biomarker (12, 22, 37, 50–57). In both cancers, gastric and esophagus, IL-32 upregulation was coupregulated with proinflammatory cytokines such as TNF-α, IL-1β, and IL-6, suggesting its induction *via* NF-κB and STAT3 signaling pathways was linked to poor-prognosis cases. It was found that IL-32β was the dominant isoform expressed in gastric tissues with 90%, and the remaining 10% was IL-32ε with no detection for any other isoforms. However, their total sample was only 20, which signifies the need for further investigation into a wider cohort. The recent publication evaluated the expression of IL-32 in different immune cells from esophageal squamous cell carcinoma (ESCC) by single-cell RNA sequencing found that IL-32 may have a paradoxical effect (22). They found that IL-32 stimulates the expression of IFN-γ in $CD8^+$ T cells which is responsible for the antitumor role, while in $CD4^+$ T cells it induces Foxp3 expression, which accounts for the suppressor role.

IL-32 IN CANCER ANGIOGENESIS, INVASION, AND METASTASIS

IL-32 numerous roles in angiogenesis, EMT, and metastasis are summarized in **Figure 4**. Angiogenesis invasion and metastatic both are features established in more aggressive tumors. Therefore, IL-32 involvement in these two processes was evaluated in several studies. Angiogenesis occurs as a response due to diminishing oxygen and nutrients, the new vessels formed provide a crucial pathway for metastasis. IL-32 was found to influence angiogenesis in glioblastoma, yet the underlying mechanism remains to be defined (58). In this study, it was found that IL-32 controls angiogenesis through integrin αVβ3, that usually expressed in new vessels and is considered the most important integrin for angiogenesis (59, 60). The expression of IL-32 was significantly increased and colocalized with integrin αVβ3. Vascular endothelial growth factor (VEGF) is a well-known critical factor for metastatic and angiogenesis and is the most expressed in advanced cancers (61). The tube formation was found to be increased in a dose-dependent manner as well. Besides, αVβ3 inhibitor reduced IL-32, and induced IL-8 (one of the advocates of angiogenesis), therefore blocking the angiogenetic effect.

Interestingly, they found that the reduction of IL-32 affects IL-8, nitric oxide, and matrix metalloproteinases 9 (MMP9), whereas levels of VEGF and TGFβ were not affected. Thus, it was concluded that the angiogenetic activity conducted by IL-32, specifically IL-32γ, was not mediated by VEGF. Since IL-32 induced IL-8, which could be the indirect way of promoting angiogenesis. IL-8 plays a role in invasion, metastasis, and angiogenesis (62). VEGF expression was found to be correlated with the expression of IL-32 in cancers with invasion and migration ability such as lung, breast, and gastric cancers although it was indicated that IL-32γ pro angiogenetic activity was not mediated by VEGF (8, 10, 50).

Matrix metalloproteinases family (MMPs) of endopeptidases having proteolytic activity play a critical role in the invasion and

metastasis of tumors through their function of extracellular matrix degradation (63–65). In gastric and lung cancer, not only VEGF and IL-8 were found to be coexpressed with IL-32 but also MMP2 and MMP9 (10, 50). IL-32 significantly increased in metastatic patients of both cancer types (10, 51, 66). As mentioned above, IL-32 was highly associated with gastric cancer progression mainly due to its stimulation of cell elongation and in turn enhanced invasion and migration. This effect occurs through activation of AKT, β-catenin, and hypoxia-inducible factor 1α (HIF1- α) signaling pathways.

It was noted that the expressed IL-32 isoforms were α, β, and γ in gastric cancer samples, while the dominant isoform was IL-32β. Since IL-32γ was found to be spliced into IL-32 α and β, they evaluate the effect of IL-32γ on the gastric carcinoma cell line (TSGH9201). As a result, they found that cells overexpressing IL-32 show elongated spindle-like morphology compared to the control cells (50). Invasion stimulation in cancer cells *via* the Akt pathway was also reported within osteosarcoma cells mediated by the expression and secretion of MMP13 (67). On the other hand, in lung cancer cells MMP 2 and 9 were also found to be induced by IL-32 but *via* NF-kB (10).

The overexpression of IL-32 was found to be correlated with metastasis in ESCC and colorectal cancer (37, 38). However, one study revealed that IL-32 isoform could play an opposite migratory role in colon cancer cells (18). It was found that isoform IL-32θ represses the invasion and migration of colon cancer cells by preventing EMT. This was achieved by the interaction of IL-32θ with STAT3 to suppress ZEB1 and Bmi1 transcription which in turn avoids stemness and EMT.

Moreover, this inhibitory effect of IL-32θ was addressed in breast cancer as well, as it suppresses the binding of CCL18, a chemotactic cytokine involved in the several cancer pathogenesis and progression and associated with poor prognosis (68–70), to its receptor and therefore inhibited the further cascade of activation/phosphorylation of STAT3 (44). Phosphorylation of STAT3, regardless of its upstream activation, leads to dimerization and translocation into the nucleus. Following that, STAT3 binds to its target gene promoters and regulates their expression (71–73). MMPs are among its target genes, which in this way STAT3 is involved in regulating cancer cell migration (74, 75). In addition, STAT3 regulates VEGF and HIF1-α that are well known for their role in angiogenesis (76–78).

Taken together, STAT3 signaling pathways play a key role in cancer metastasis (73) and are found to be regulated by IL-32. The upregulation of MMPs (MMP2, MMP9, and MMP13) was also

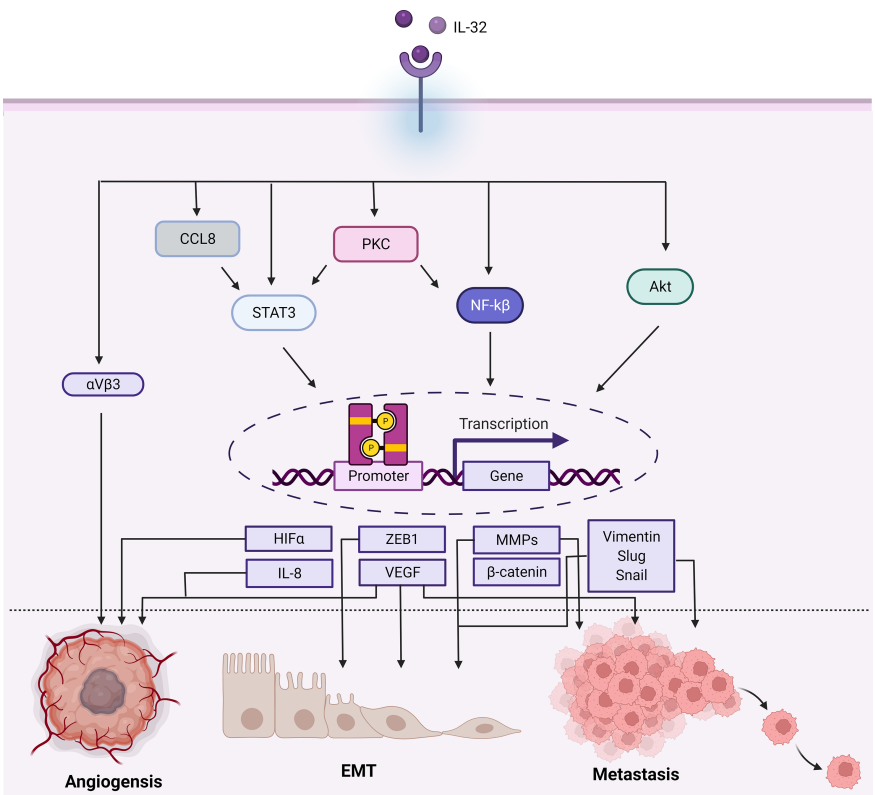

FIGURE 4 | Schematic illustration showing the range of signaling pathways that are activated by IL-32 and promoting cancer progression. In terms of angiogenesis, EMT, and metastasis. In brief, IL-32 promotes the Akt, NF-kB, STAT3 (which can be activated by PKC/CCL8), and integrin αVβ3 signaling cascades, each having different transcription modifications. Therefore, regulating the activity of several transcription factors play a role in cancer such as angiogenesis, EMT, and metastasis as well as αVβ3, VEGF, and HIF-α enhance angiogenesis. The ZEB1 or B-catenin enhances EMT. VEGF is also associated with EMT and metastasis. Additionally, the transcription of MMPs (like MMP2 and MMP9), Vimentin, Slug, and Snail promotes metastasis. Figure created by BioRender App.

reported in cancers overexpressing IL-32 along with other EMT markers including vimentin, Slug, Snail, and ZEB1, as well as they are well known for their contribution to cancer metastatic.

IL-32 IN THE TUMOR MICROENVIRONMENT AND STROMAL TUMOR

The tumor microenvironment refers to the surrounding ecosystem that includes extracellular matrix, blood vessels, and an array of cells such as fibroblasts, immune cells, and heterogeneous tumor cells. These components influence one another and thus, contribute to tumor progression and metastasis in either a positive or negative way. Therefore, a better understanding of the tumor microenvironment offers new insights for improving cancer therapies (79, 80). Cytokines are one of the key mediators for interactions between immune and nonimmune cells in the tumor microenvironment (TME) (81). It has been shown to have a different role that is isoform dependent since many cells express IL-32. However, it is not clear yet how IL-32 contributes to the different tumor types including stromal tumor microenvironment.

In a study investigating the IL-32 effect in the pathogenesis of endometriosis as an example of stromal cancer, IL-32 showed a correlation in cancer progression. This study revealed that the IL-32 concentration in the peritoneal fluid was drastically greater in patients of advanced-stage endometriosis as compared with the controls. Moreover, they showed that IL-32α and IL-32γ significantly increased cellular viability, proliferating cell nuclear antigen expression, and invasive ability (82).

Several studies showed that the overexpression of IL-32, specifically α, β, and γ were able to reduce tumor growth through inducing apoptosis in tumor cells, which led to $CD8^+$ T-cell responses (15, 17, 33). Nevertheless, other than the antitumor effect, IL-32 demonstrates a monocyte differentiation stimulator as well as cytokine production. Moreover, it has been reported for its ability to activate T cells and therefore stimulate antigen presentation utilizing dendritic cells (DCs). On the contrary, functional studies demonstrated that IL-32γ induced PD-L1 expression on monocytes but not tumor cells, which may contribute to local immunosuppression and therefore are candidates for cotargeting in combination treatment regimens. IL-32γ expression correlates with a treatment-resistant dedifferentiated genetic signature and genes related to T-cell infiltration. This was reported in melanoma cells, suggesting it influences nonmelanoma cells in the tumor microenvironment, such as myeloid cells (83).

More recently, IL-32γ potentiates antitumor immunity in melanoma as the antitumor microenvironment. This result is shown to be enriched in mature DC and M1 macrophages resulting in enhancing the recurrence of activated tumor-specific $CD8^+$ T cells to generate antitumor immunity. Therefore, IL-32 resulted in reducing tumor growth and rendering immune checkpoint blockade resistance (35). On the other hand, IL-32β stimulates the activation-induced apoptosis of T cells, NK cell cytotoxicity toward tumor cells like IL-32γ in the activation of monocyte differentiation. In addition, IL-32α is shown to be a stimulator of NK cell cytotoxicity, whereas IL-32θ has been shown as an inhibitory effect on monocyte differentiation and cytokine production (36, 84–88). However, better characterization of the tumor microenvironment is needed to understand how different cell types in the tumor microenvironment are influenced by IL-32.

Moreover, how IL-32 isoforms implicated each other is another key factor in overall response to cancer. As we mentioned above, the possibility of IL-32 in exhibiting an isoform switching and self-regulation between IL-32β and IL-32γ was reported (41). Likewise, isoform δ of IL-32 was found to modulate another isoform, IL-32β, by interacting with it and thus inhibiting its production of IL-10 (89). Both observations suggest that IL-32 performs its feedback regulation through its isoforms.

IMPLICATIONS OF IL-32 POLYMORPHISMS IN CANCER

Changes in the genetic material provide different effects within individuals and populations. Recently, several studies have demonstrated the impact of IL-32 polymorphisms on cancer progression. Moreover, IL-32 SNPs were studied and reviewed with their association to disease outcome (90–95), and by 2021, one review performed a meta-analysis to evaluate the SNPs in malignancy (96). Up to now, three polymorphisms of IL-32 were found to be associated with the progression of several cancers that are rs28372698, rs12934561, and rs2015620 (**Table 1**).

SNP rs28372698 was found in many cancers including thyroid carcinoma and lung, endometrial, ovarian, gastric, bladder, and

TABLE 1 | IL-32 polymorphisms and their associated cancers.

IL-32 SNP	Chromosome location[a]	Type	Associated cancer/s	SNP interaction	Ref
Rs28372698	3,065,110	Noncoding/ upstream variant	Thyroid carcinoma, lung, endometrial, ovarian, gastric cancer, bladder cancer, and colorectal cancer	rs4073 (IL-8)–gastric cancer	(97–103)
Rs12934561	3,068,864	Noncoding/Intron variant	Squamous carcinoma, and bladder cancer		(98, 102)
Rs2015620	3,063,897	Noncoding	Gastric cancer	rs917997 (IL-18RAP), rs1179251 (IL-22)	(103)

[a]*Based on human genome build 38: GRCh38.*

colorectal cancers that are related to the higher expression of IL-32 resulting in cancer progression (96–104). In thyroid carcinoma, this polymorphism revealed higher expression of isoform IL–32γ that increased the risk of tumor development (104). In a study to evaluate cytokine polymorphisms and their association with gastric cancer, this SNP (rs28372698) of IL-32 has shown no association. However, when the patient has another SNP, IL-8 rs4073, there was an interaction between both SNPs and thus suggested increased gastric cancer risk (103).

Interestingly, another study on the Chinese population revealed that IL-32 SNP rs2015620 is highly associated with the risk of gastric cancer by interacting with two more SNPs, IL-18RAP rs917997 and IL-22 rs1179251 (101). However, these studies were subjected to two different populations, Chinese and Chilean; the reason why IL-32 SNP has a different effect. Although studies on IL-32 SNPs are not dispersed in the world, yet according to the published data, SNP rs28372698 showed high cancer influence on the Chinese population.

Moreover, this SNP was linked to colorectal cancer in the Swedish cohort but not reported in the Chinese colorectal cancer patients (99). Both IL-32 SNPs of rs28372698 and rs12934561 have been correlated with bladder cancer processes (102). However, only rs12934561 was related to poor survival status in squamous carcinoma (98). Overall, these association studies were subjected to some limitations due to the limited population and selected population. A large-scale study must include more than one kind of population and ethnicity to discover the role of IL-32 SNPs in cancers.

CONCLUSION

It conflicts in targeting therapy for IL-32 in cancer because IL-32 roles remain unclear, thus there is no specific direction for IL-32 in cancer therapy. However, some isoforms showed an inhibitory effect that can be administered exogenously to stop or reverse cancer progression such as IL-32θ for cytokine-based immunotherapy. Moreover, it was found that patients with higher expression of IL-32 demonstrated more aggressive cancers. In these cases, IL-32 can be targeted precisely to stop its progression role. There is a great gap in this matter even after selecting the IL-32 isoform for cancer therapy. A lot more studies are needed before this knowledge can be used clinically. This difficulty regarding IL-32 was addressed in a recent review considering interleukins in improving cancer therapies (4). Again, this is due to IL-32 showing no clear effect on cancer which differs based on IL-32 isoforms, cancer type, and genetic background.

AUTHOR CONTRIBUTIONS

Conceptualization: SS, SL, YH, SK, TN, AT, JH, HJ, YL, SY, Y-GK, and SHK. Funding acquisition: HJ, SHK. Supervision: SHK. Writing—original draft: SS, SL, YH, and SHK. Writing—review and editing: AT and SHK. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Dual Relationship Between Stromal Cells and Immune Cells in the Tumor Microenvironment

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The tumor microenvironment (TME) plays a critical role in tumorigenesis and is comprised of different components, including tumor cells, stromal cells, and immune cells. Among them, the relationship between each mediator involved in the construction of the TME can be understood by focusing on the secreting or expressing factors from each cells. Therefore, understanding the various interactions between each cellular component of the TME is necessary for precise therapeutic approaches. In carcinoma, stromal cells are well known to influence extracellular matrix (ECM) formation and tumor progression through multiple mediators. Immune cells respond to tumor cells by causing cytotoxicity or inflammatory responses. However, they are involved in tumor escape through immunoregulatory mechanisms. In general, anti-cancer therapy has mainly been focused on cancer cells themselves or the interactions between cancer cells and specific cell components. However, cancer cells directly or indirectly influence other TME partners, and members such as stromal cells and immune cells also participate in TME organization through their mutual communication. In this review, we summarized the relationship between stromal cells and immune cells in the TME and discussed the positive and negative relationships from the point of view of tumor development for use in research applications and therapeutic strategies.

Keywords: tumor microenvironment, immune cells, stromal cells, cancer-associated fibroblast (CAF), tumor endothelial cell, cancer-associated adipocyte, T cell, NK cell

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INTRODUCTION

The tumor microenvironment (TME), a highly heterogeneous environment composed of many different types of cells and many molecules produced or released by tumor cells, stromal cells, and immune cells, is now widely recognized (1). The TME is composed of cellular components such as cancer-associated fibroblasts (CAFs), tumor-endothelial cells (TECs), cancer-associated adipocytes (CAAs), mesenchymal stem cells (MSCs), T cells, B cells, natural killer (NK) cells, and tumor-associated macrophages (TAMs) (2). In addition, the TME is rich in hypoxic, acidic, and immune/inflammatory cells known to play important roles in tumor development, growth, progression, and resistance to treatment (3–6). In the past, cancer therapies were designed to target cancer cells directly. Recently, they are designed to destroy networks formed by tumors. Immunotherapy, in

addition to surgery, chemotherapy, and radiation therapy, has emerged as a breakthrough treatment modality for cancer patients (7). Besides therapies that directly target tumor cells, promising therapies targeting stromal cells present in the TME are also attracting attention. Targeting immunomodulatory pathways in the TME is considered a central step in cancer treatment (8–10). Indeed, the TME is currently in the spotlight as a new target for cancer therapeutics with many ongoing studies.

Treatments for cancer patients include surgical resection and chemotherapy or radiation therapy (11). With recent advances in onco-immunological studies, the use of immune checkpoint inhibitors (ICIs) taking advantage of the antitumor activity within the TME is considered as an effective therapeutic modality for cancer patients. Among them, inhibitors of the cytotoxic T lymphocyte antigen-4 (CTLA-4) blockade have shown remarkable efficacy in clinical trials. Both ipilimumab and tremelimumab are human antibodies to CTLA-4 (12, 13). In recent studies, both drugs have achieved considerable clinical success for patients with advanced malignant melanoma. A phase 3 study has shown that ipilimumab, a CTLA-4 blockade, can significantly improve the overall survival of melanoma patients. Based on results of these studies, ipilimumab, a T-cell potentiator, is considered useful as a treatment for advanced melanoma patients (14, 15). In addition, it has been suggested that PD-1/PD-L1 expression plays an important role in immune evasion by cells other than tumor cells present in the TME. Nivolumab is an ICI targeting PD-1/PD-L1. It is being used to treat melanoma, non-small cell lung cancer, kidney cancer, hepatocellular carcinoma, urinary tract cancer, gastric cancer, and triple-negative breast cancer (TNBC) (16–18). In addition to immunotherapy-based ipilimumab and nivolumab, a variety of FDA-approved ICI treatments such as cemiplimab and pembrolizumab are also being actively implemented for cancer treatment (19).

The expression of a modified T-cell receptor or chimeric antigen receptor (CAR) to enhance antigen specificity can be engineered using a patient's own T cells. Thus, it is possible to efficiently generate tumor-targeting T cells by solving the fundamental problem without requiring the patient to activate *de novo* T cells (20, 21). Clinical trials conducted on patients with acute lymphocytic leukemia have shown a complete recovery in up to 92% of patients with very meaningful results (20). CAR-T cell therapy is being actively implemented for treating large B-cell lymphoma and B-cell precursor acute lymphoblastic leukemia (22, 23). Although this CAR-T cell therapy has achieved clinical success in some hematologic cancer patients (24), it has two main problems that need improvement. First, it is very difficult to obtain enough T cells from cancer patients to isolate CAR-T cells because of lymphocytopenia due to prior treatments. Second, there is not enough time to implement CAR-T cell therapy for rapidly advancing cancers (25). Tumor-infiltrating lymphocyte (TIL) therapy can be proposed as a form of therapy to solve these problems. TIL therapy is a treatment involves removing T cells infiltrating a patient's tumor, proliferating them to a large amount in the laboratory, and injecting them back into the patient to help the patient's immune system kill cancer cells (26). It has the advantage of being able to locate and destroy the patient's tumor directly because it secures T cells that have already penetrated the patient's tumor and reinjects them into the patient. Activated NK cells can also directly lyse tumor cells by releasing cytotoxic granules (including perforin and granzymes) in a manner similar to that of activated cytotoxic T cells. Since NK cells can eliminate tumors, immunotherapy based on NK cells has been developed. It is currently being used strategically. In addition, CAR-NK cells, like CAR-T cells, are genetically modified to express CARs that can recognize specific antigens that are characteristically overexpressed by target cells. Preclinical studies of CAR-NK cells have been performed on hematologic cancers and some solid cancers (27). These CAR-NK cells, along with T cell-based therapies, could be proposed as an improved therapy for solid tumors.

Strategies using drugs that can specifically target stromal cells within the TME have been proposed to further enhance patient survival and therapeutic effects. Therapeutic drugs that target stromal cells within the TME are under investigation (28). These stromal cells are important components of the TME as they express specific markers that can be targeted for tumor treatment (29). Of note, treatment with microsomal prostaglandin E synthase-1 inhibitor compound III targeting CAF-derived prostaglandin E2 (PGE2) can reduce tumor growth, suppress CAF migration/infiltration, and increase M1 macrophage ratio in neuroblastoma tumor studies (30). Furthermore, studies using a mouse model of cholangiocarcinoma induction have confirmed that navitoclax (BCl-2 inhibitor) treatment can induce CAF apoptosis, reduce the expression of tenascin C, and suppress tumor growth. This suggests that navitoclax may strategically target and destroy CAFs within TMEs to attack tumors (31). As an inhibitor of fibroblast growth factor receptors (FGFR), PD173704 can reduce the growth of both CAFs and endothelial cells (ECs), thereby inhibiting stromal cell-mediated FGFR pathway in a co-cultured environment of head and neck squamous cell carcinoma (HNSCC) cells. These inhibitor molecules can inhibit tumor cell growth, thus playing a crucial role in tumor-matrix interaction. They have been proposed as potential therapeutic agents for HNSCC (32). Phosphodiesterase (PDE), a class of enzymes that can hydrolyze cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), are composed of 11 different subtypes. A lung cancer study has shown that phosphodiesterase-4 (PDE4) as a type of PDE can promote lung cancer proliferation and angiogenesis by having a crosstalk with hypoxia-inducible transcription factors (HIFs) factor (33). CC-5079 is an analog of these PDE4 inhibitors. It can inhibit the proliferation and migration of fibroblasts, bladder cells, and EC cells, stimulate mitogen-activated protein kinase phosphatase 1 (MKP1) expression, and inhibit micro-angiogenesis through its upregulation. It has been demonstrated that CC-5079 is a candidate for treating colon cancer by targeting ECs within the TME (34). However, further studies on the detailed mechanism of action of CC-5079 are needed. Although the TME component targeting therapy such as

immune cell targeting therapy and stromal targeting therapy has been applied to treat cancer patients in various studies mentioned above, it has some limitations because the TME is heterogeneous, showing different characteristics in each patient (29).

Although the role of the TME in cancer processes has been extensively investigated, its contribution to the crosstalk between stromal cells and immune cells, which are components of the TME based on the tumor, is only partially known. Previous therapeutic approaches have focused on cancer cells and established relationships between cancer cells and immune cells or stromal cells. Based on an understanding of the relationship, it is more effective to consider treatment methods in a direction that can restore the relationship between stromal cells and cancer cells and the self-dependent role in the TME by understanding the relationship between cells in the TME. Communication between TME constituent cells should be understood and therapeutically targeted. Characteristic parts of cells must be considered. In this respect, unlike cancer cells, the somatic cells that make up our body are controllable. The need to find ways to overcome cancer treatment by properly regulating the communication between immune cells has emerged. In particular, exploring interactions and intercellular relationships in the TME will improve our understanding of cancer treatment (35, 36). Therefore, in this review, we described and summarized interactions, roles, and importance of the relationship between stromal cells and immune cells within the TME.

THE PRO-TUMORIGENIC EFFECT OF THE RELATIONSHIP BETWEEN STROMAL CELLS AND IMMUNE CELLS IN THE TME

CAFs Interact With Myeloid Cell-Derived Immune Cells in the TME to Enhance Tumorigenesis and Immune Evasion

CAFs are the most dominant cell type in the TME. They are known to crosstalk with immune cells (37). CAFs may play a pivotal role in tumor development and survival as they are involved in TME composition and participate in mechanisms that promote tumor growth and invasion and subvert defense system (38–40). Recently, studies have attempted to clarify this point. CAFs can secrete components such as cytokines and chemokines known to be continuously activated in the TME through various signaling mechanisms and function as primary immunosuppressive mediators (41, 42). CAF-derived cytokines and chemokines are attracting attention not only for their roles in tumor progression, but also for their ability to regulate the recruitment and function of immune cells.

TAMs are macrophages that participate in the formation of the TME by producing cytokines, chemokines, and growth factors. TAMs are divided into two types, M1 and M2 macrophages (43, 44). Characteristically, M1 macrophages produce large amounts of pro-inflammatory cytokines and regulate the Th1 antitumor immune response, whereas M2 macrophages play an important role in tumor progression. Gokyavuz et al. have shown that CAFs can secrete monocyte chemotactic protein-1 (MCP-1) and stromal cell-derived factor-1 (SDF-1) and effectively recruit monocytes (45). MCP-1 is a chemokine that contributes to the recruitment of monocytes to the site of an inflammatory response. It is expressed in a variety of cancer types such as prostate and ovarian cancers (46, 47). SDF-1 is expressed in stromal fibroblasts in organs including the brain, breast, and lung. It is involved in cancer survival, proliferation, and metastasis (48, 49). Specifically, monocytes recruited by CAFs *via* MCP-1 and SDF-1 can reduce the secretion of pro-inflammatory cytokine interleukin (IL)-12 and increase the production of anti-inflammatory cytokine IL-10. CAF-educated monocytes can increase the motility and invasiveness of breast cancer cells and the expression of epithelial-mesenchymal transition (EMT)-related genes and vimentin proteins, eventually exerting their immuno suppressive role in breast cancer (45). In prostate cancer, CAFs can also promote the differentiation to the M2 macrophage phenotype *via* SDF-1. Analysis of patients with prostate cancer has confirmed a clear increase in the M2/M1 macrophage ratio, which is correlated with clinical prognosis. Thus, CAFs and M2-polarized macrophages actively contribute to the promotion of the invasive ability of prostate cancer cells. They are correlated with the aggressiveness of cancer *via* the infiltration of M2 macrophage (50). Chemokine (C-C motif) ligand 2 (CCL2; MCP-1) secreted by CAF can induce blood monocyte recruitment and differentiate into TAMs in breast cancer (51). Additional research has confirmed that the increase in monocyte migration to breast tumor spheroids is associated with CAF-derived CCL2 and that the CCR2A/2B pathway and the CCL2 receptor play important roles in monocyte recruitment (52). These findings suggest that CAF-derived factors can affect a wide range of processes, leading to monocyte recruitment and M2 macrophage differentiation.

Among various cancer types, pancreatic cancer has a low response to immunotherapy. Thus, it is necessary to understand the antitumor immune response in pancreatic stromal cells. Stromal cells in the pancreatic cancer microenvironment can generate numerous factors that support the growth and survival of tumor cells. They are being studied to increase the understanding and relevance of immune cells (53). Pancreatic stellate cells (PSCs) are resident cells in the pancreas under quiescent conditions. They are isolated from the human pancreas as fibroblast-like cells (54). These cells display some characteristics of activated myofibroblasts such as α-smooth muscle actin (α-SMA) expression and ECM proteins synthesis (55). Much evidence has confirmed the importance of PSCs in pancreatic ductal adenocarcinoma (PDAC) development. Importantly, myeloid-derived suppressor cells (MDSCs) are identified as immature myeloid cells that can induce immunosuppression, mediate multiple signaling pathways, and interact with immune cells and mediators (56, 57). It has been shown that MDSCs can promote cancer progression, angiogenesis, and metastasis and disrupt the efficacy of therapeutic agents (58, 59). In particular, the number of

MDSCs is highly correlated with the staging of pancreatic cancer patients, indicating that an increase in MDSC levels could be an indicator of disease progression (60). Thus, MDSCs could be targeted in the treatment of PDAC patients. Mace et al. have reported that soluble factor IL-6 generated from patient PSCs with confirmed phenotypic α-smooth muscle actin and glial fibrillary acidic protein expression can promote the differentiation of MDSCs (61). PSCs in PDAC can modulate MDSC differentiation *via* IL-6 and STAT3 signal transduction pathways (61). Targeting PSCs in the TME by promoting the generation of immunosuppressive cells that suppress the innate or acquired immune response against pancreatic cancer may reduce MDSC levels, thereby enhancing the effectiveness of immunotherapy. Dendritic cells (DCs) represent immune cells that link innate and adaptive immunity. They are derived from hematopoietic bone marrow precursor cells (62). DCs are essential antigen-presenting cells (APCs) that can induce the activation of naïve T cells. Levels of peripheral blood DCs are decreased in cancer patients than in normal controls (63–65). By expressing high levels of immunoregulatory cytokines, they can induce the differentiation of regulatory T cells (Tregs) and help tumor cells evade the immune response. Cheng et al. have shown that CAF-derived IL-6 can induce the activation of the STAT3 pathway, leading to immunosuppressive cell types (66). Especially, STAT3 activation by CAF-derived IL-6 plays a pivotal role in indoleamine-2,3-dioxygenase (IDO) production and induced regulatory DC recruitment (66). Cheng et al. have evaluated CAF-regulated neutrophil function and the activation of hepatocellular carcinoma (HCC) *via* the IL-6/STAT3/PD-L1 signaling cascade (67). They found that IL-6 derived from CAFs could recruit PD-L1$^+$ neutrophils and impair T-cell function *via* PD1/PD-L1 signaling (67). Cho et al. have reported that IL-6 and granulocyte-macrophage colony-stimulating factor (GM-CSF) released from cancer cell-activated CAFs through co-culture of monocytes and CAFs can increase TAM infiltration and metastasis and direct monocytes to differentiate into M2 TAMs (68). Thus, the development of inhibitors or neutralizing antibodies targeting IL-6, IDO, GM-CSF, and STAT3 may lead to a new cancer immunotherapeutic approach that can induce tumor immune evasion of CAFs *via* the cell network in the TME. In the TME, secreted chemokines including IL-8 play important roles in tumorigenesis. These chemokines could be secreted by CAF (69). According to a study on colorectal cancer-derived CAFs isolated from human colorectal cancer tissue, the CAF-derived IL-8 can be secreted to attract monocytes and promote polarization from anti-tumorigenic/pro-inflammatory M1 macrophages to pro-tumorigenic/anti-inflammatory M2 (70). These findings indicate that CAFs can also inhibit NK cell function and promote CRC cell progression by increasing TAM infiltration.

CAFs Interact With Lymphocytes in the TME to Enhance Tumorigenesis and Progression

Transforming growth factor-β (TGF-β) is a representative cytokine secreted from CAF with a strong immunomodulatory function. It is involved in the suppression of immune responses. It also plays an important role in tumor initiation, invasion, and metastasis (71–73). CAFs-derived TGF-β can regulate various types of immune cells through the paracrine signaling pathway with growth factors and cytokines. TGF-β can suppress IL-2 production and T cell proliferation. It plays an important role in CD4$^+$CD25$^+$ Treg production and function (74). Furthermore, it has been proposed that transcriptional inactivation of TGF-β by suppressing T-box expressed in T cells (T-bet) and GATA-binding protein 3 (GATA-3) expression can control the differentiation of murine CD4$^+$ T cells (75, 76). According to immunological studies conducted in HCC cells, IL-10, TGF-β, and IL-4 secreted by stromal cells in the TME can increase polarization to M2 macrophages. It is known that M2 macrophages can enhance tissue remodeling, angiogenesis, tumor progression, and ultimately IL-10, increasing PD-L1 and human leukocyte antigen (HLA)-DR expression to induce immunosuppression (77–79).

Since stromal cells in the TME express cytokines and immunological factors, it is critical to consider stromal cells to rescue tumor-reactive CD8$^+$ T cells from immune evasion mechanisms (80). According to a study by Lakins et al., CAF-educated T cells can induce death of T cells by PD-L2 and Fas ligand (FasL) engagement in the lung tumor stroma (81). These results indicate that CAFs can enhance tumor viability by inducing the dysfunction of encountered CD8$^+$ T cells, suggesting that CAFs can directly contribute to the pro-tumor T cell immune response.

Wu et al. have investigated the heterogeneity of stromal cell population of TNBC patients using single-cell RNA sequencing technology and confirmed the existence of an inflammatory CAF (iCAF) subpopulation (82). These iCAFs showed upregulation of CXCL12 (SDF-1)-CXCR4 chemoattractant pathway genes and suggested a strong association of CD8$^+$ T cell dysfunction with iCAF presence and exclusion (82). In breast cancer studies, four different CAF subsets of human breast cancer have been identified, among which the CAF subset 1 is characterized as immuno suppressive cells. CAF subset 1 can secrete CXCL12 to attract T cells, increase the survival of CD4$^+$CD25$^+$ T cells, and promote differentiation into CD25^{high}FOXP3^{high} Treg cells *via* B7H3, CD73, and DPP4 (83, 84). These studies indicate that the CAF-S1 fibroblast subset contributes to immunosuppression in breast cancer. Takahashi et al. have shown that IL-6, CXCL8, tumor necrosis factor (TNF), and vascular endothelial growth factor (VEGF)-α are detected more in CAFs than in normal fibroblasts in the TME of HNSCC. These CAFs can modulate effector T cell function by expressing co-regulatory molecules B7H1 and B7DC and enhance T cell apoptosis and the induction of Treg cells (85). Thus, CAFs have been implicated in the proliferation of CD4$^+$FOXP3$^+$ Treg cells, tumor progression, angiogenesis, and metastasis (86, 87). These cells play an important role in angiogenesis, invasion, and metastasis. They contribute to the immunosuppressive process that promotes tumor evasion by the T cell network within the TME of HNSCC. Moreover, CAFs can release different factors including chemokines, cytokines, and growth factors that can promote immunosuppression through recruitment of immunosuppressive cells such as Tregs and myeloid cells, upregulation of immune checkpoint molecules on T

cells, and regulation of T cell migration (88). Notably, in PDACs, T cells can be inhibited by tumor stroma, suggesting that CAF-derived factors may regulate T cell function and phenotype (89–91). Most T cells in the TME are exhausted, leading to cancer immune evasion. Restoring exhausted T cells appears to be an excellent strategy in cancer immunotherapeutic therapy (92). PD-1, a critical inhibitory receptor regulating T cell exhaustion, can attenuate its ability to clear cancer *via* high expression in T cells (91). Reversing this T cell exhaustion represents a major strategy for cancer treatment (93).

Candidate factors that can interfere with signal transduction between CAF and NK cells have been suggested as potential strategies for cancer treatments (94). NK cells are cells that can produce cytokines to communicate with other cells. They have the ability to kill tumor cells (95, 96). NK cell activation releases perforin, granzymes, inflammatory cytokines, and chemokines toward target cells (97). PGE2 is a major product of arachidonic metabolism. It is synthesized *via* cyclooxygenase-2 (COX2) and prostaglandin synthase pathways (98). According to Balsamo et al., PGE2 released by melanoma-derived fibroblasts can suppress the expression of NK receptors, perforins, and granzymes, meaning that they can interfere with the phenotype and function of NK cells (99, 100). PGE2 secreted from HCC-associated fibroblasts can inhibit the expression of NK receptors exhibiting immune function, including NKp30, NKp44, and NKG2D, thus impairing NK cell-mediated cytolytic activity (101). In addition, it has been confirmed that blocking the activity of PGE2 and IDO in activated HCC-related fibroblasts can restore the function of NK cells and promote the progression of HCC (102). Thus, metastatic melanoma-derived fibroblasts and HCC-associated fibroblasts can release PGE2 and IDO to affect NK cell function and exert strong immunosuppressive activity (99–101).

TECs, CAAs, and MSCs Interact With Immune Cells in the TME to Induce Immune Evasion and Reduce Anti-Tumor Functions

Endothelial cells (ECs) are a major type of cells found inside the lining of blood vessels, lymph vessels, and heart. They fulfill many physiological processes in the body (103). ECs are included in stromal cells. They represent an important interface between tissue and blood (104). TECs found in most tumors can also form an essential vascular inner layer in tumors (105). TECs play an important role in orchestrating the TME. TECs are known to be particularly important for T cell recruitment and activation. Previous studies have shown that the interaction between T cells and ECs plays an important role in the regulation of the immune system during chronic inflammation (106). TECs in the tumor microenvironment are particularly relevant to circulating immune cells. They may influence anti-tumor cell immune responses. TECs are APCs with an inhibitory activity. They express MHC class II and PD-L1. They can impair the production of pro-inflammatory cytokines including IL-2, TNF-α, and IFN-γ in $CD8^+$ T cells (107, 108). TECs are also known to play a critical role in tumor cell growth and invasion (109). Vascular cell adhesion molecule 1 (VCAM1) induction in endothelial cells can regulate tumor progression, provide angiogenic factors, promote neutrophil infiltration and tumor cell adhesion to the endothelium, and promote metastasis by sustaining vascular Notch1 signaling (110).

In gliomas, penetration of T cells into tumor tissue as TILs is extremely low. Although this feature is controversial for the correlation with TIL-induced tumor prognosis in glioma, it has a useful aspect in the evaluation of factors that reduce the presence of T cells in tumor tissues. Moreover, FasL is well-known as a pro-apoptotic cell surface protein that plays an important role in confirming T cell depletion in tumor tissues. This has been demonstrated by flow cytometric analysis, showing that FasL levels expressed in the endothelium of brain tumors are inversely correlated with the $CD8^+/CD4^+$ TIL ratio (111). That is, FasL expression indicates not only the $CD8^+/CD4^+$ TIL ratio, but also the tumor contribution by immune avoidance in brain tumors due to decreased T cell presence. In melanoma, it has been confirmed that TECs are APCs that express MHC class II and PD-L1 with an inhibitory activity. These TECs can inhibit the proliferation of $CD8^+$ T cells *via* inhibitory cytokines including IL-10 and TGF-β. They can also attenuate the antitumor effect of antigen-specific $CD8^+$ T cells. Experimental results have shown that TECs can induce immune responses of tumor antigen-specific $CD8^+$ T cells through the PD-1/PD-L1 pathway and evade tumor immunity by regulating immunosuppressive $CD4^+$ T cells in an antigen-specific manner (112). Common lymphatic endothelial and vascular endothelial receptor (CLEVER-1/stabilin-1) are also expressed on lymphatic vessels, high endothelial venules, and non-continuous endothelium (113). CLEVER-1/stabilin-1 identified in HCC endothelium can recruit $FOXP3^+$ Treg cells (114). These findings make it possible to confirm the tumorigenic effect of TECs under the influence of various mechanisms and factors.

CAAs play a central role in tumorigenesis, tumor growth, and metastasis (115). CAAs can help cancer cells by storing energy as triacylglycerol and directly providing lipids. They can release pro-inflammatory cytokines such as IL-8, CCL2, VEGF, TGF-β, and cathepsin S that can recruit bone marrow cells to the TME, thus regulating the differentiation of M2/MDSC and promoting the angiogenesis process (116–119). A breast cancer study by Arendt et al. has shown that adipocytes in human and mouse breast tissues can activate and recruit macrophages *via* the CCL-2/IL-1β/CXCL12 signaling pathway (117). These activated macrophages are sufficient to promote angiogenesis and accelerate stromal vascularization and breast cancer formation. Understanding major interactions between immune cells and CAAs in the TME can be an effective way to improve the effectiveness of existing therapies. PD-L1 can regulate anti-tumor immunity as described above. It is the main target of checkpoint-blocking immunotherapy. Previous studies on breast cancer have shown that the contribution of PD-L1 expression to adipogenesis remains an issue that merits further investigation. During the adipogenesis process, the expression of PD-L1 in primary human adipose stromal cells and adipocytes is highly induced. It is known that PD-L1 expression in breast cancer adipocytes is significantly elevated and that adipocyte-derived PD-L1 can inhibit the activity of important antitumor functions of $CD8^+$ T cells (120). Fatty acids (FAs) as CAA-derived

metabolites can influence immune cell homeostasis and differentiation (121), leading to immune evasion and tumor progression. Based on this, FAs are necessary for promoting proper differentiation of neutrophils. They can potentially promote the function of tumor-associated neutrophils (TANs). Besides FAs, CAA-derived IL-8 can recruit neutrophils to the TME. In PDAC, neutrophils are recruited by CAA-derived IL-1β to promote tumor progression and further activate PSCs (122).

In the TME, MSCs are multipotent stromal stem cells found in most cancers that play a central role in cancer cell growth, invasion, and metastasis by interacting with the tumor and immune cells in the TME (123, 124). MSC-derived TGF-β can increase the frequency of Treg cells, protect breast cancer cells, and support the growth of breast cancer (125). These MSCs can induce IL-10^+ regulatory B (Breg) cells involved in the production of immunosuppressive environments through SDF-1α and CXCR7 (126). IL-10^+ Breg cells are a subset of immunosuppressive cells. The frequency or the function of Breg cells is involved in the tumorigenesis of some cancers (127–130). It has been shown that PD-L1 in stromal cells in the TME can be induced by TNF-α signals to promote the progression of colorectal cancer by suppressing the antitumor immune response of $CD8^+$ T cells (131). Recently, research and development for antitumor treatments targeting CAAs has progressed rapidly. Because CAAs can interact with TME component cells in a complex cell network, they can be combined with a variety of therapies, including targeted or immunotherapies, which can selectively eliminate tumor-promoting CAAs.

Immune Cells Contribute to Immune Suppression With Stromal Cells in the TME

In the TME formed by tumor cells, the interaction between immune cells and stromal cells plays an important role in both cancer progression and anticancer activity. Among various types of immune cells in the TME, neutrophils have received less attention than other immune cell types. IL-1β is a cytokine secreted by neutrophils that can activate ECs. Treatment with IL-1 receptor antagonists can reduce *in vitro* endothelial cell migration, where IL-1β secreted by $Ly6G^+$ neutrophils can directly activate endothelial cells and MMP-9, thus effectively increasing metastasis capability (132, 133). This demonstrates the distinct metastatic role of $Ly6G^+$ neutrophils and confirms that they are beneficial for promoting tumors (132, 134). These increases of MMPs not only contribute to local invasion and metastasis-related cascades, but also contribute to the intravascular invasion process (135–137). Further research is needed to determine the role of neutrophils in the TME of various cancer types.

Among various immune cells, TAMs exist in the vicinity of CAFs and constitute the most abundant innate immune cell type (138). TAMs are classified as pro-tumorigenic macrophages that can promote the development of malignant tumors and activate CAFs to aid in tumor progression (139, 140). Tokuda et al. have shown that osteopontin is a key molecule involved in cancer-CAF-TAM interactions and that increased osteopontin can promote malignant tumors (141). This characterizes the importance of cancer cell-TAM-CAF interactions in HCC. Lung cancer is developed in the region of major fibrosis. Particularly, idiopathic pulmonary fibrosis case has been reported to be associated with increased risk of lung cancer (142, 143). Wnt7a secreted by M2 macrophages in pulmonary fibrosis can interact with Frizzled-1 to activate Wnt/β-catenin signaling and promote differentiation of MSCs into myofibroblasts. In this way, myofibroblasts activated in idiopathic pulmonary fibrosis can lead to accumulation of ECM, resulting in an acute exacerbation of lung disease such as lung cancer (144, 145). In addition, TAMs can regulate communication with cancer cells and other stromal cells in the TME through nanovesicle secretion, which carries various molecules, including microRNAs (146). Exosomes containing microRNAs can affect cellular processes and promote tumor progression and angiogenesis. Macrophage-derived exosomes containing miR-155-5p and miR-221-5p can be transmitted to ECs *via* an E2F Transcription Factor 1 (E2F1)-dependent manner, thus promoting EC proliferation and PDAC growth (147, 148).

Human decidual NK cells are $CD56^{superbright}CD16^-$ that can increase tumor growth by angiogenic activity *via* the production of VEGF, placental growth factor, and IL-8 (149). In non-small cell lung cancer patients, pro-angiogenic factors such as VEGF and placental growth factor are released from NK cells and defined NK cell subsets to promote human umbilical vein endothelial cell migration and capillary-like structure formation (150, 151). Mast cells in the TME can regulate adaptive immunity to tumors. Recent studies have shown that the infiltration of mast cells into tumors can indicate a poor patient prognosis (152, 153). A study of neurofibromas by Yang et al. has shown that $Nf1^{+/-}$ mast cells can secrete TGF-β, thus promoting fibroblast proliferation *via* TGF-β (154). In a human prostate cancer microtissue model, mast cells can release tryptase to enhance CAF-induced transformation of epithelial cell morphology, thus playing an important role in prostate cancer progression (155). In the TME of PDAC, mast cells are essential for tumorigenesis. Mast cells can secrete cytokines IL-13 and tryptase and promote the proliferation of PSCs (156). This suggests that mast cell infiltration and activation can contribute to the formation of dense fibrotic stromal formation characteristics of PDACs and that mast cells can promote PSC proliferation in the TME. Thus, targeting mast cells could be a way to improve PDAC therapy. **Table 1** describes tumorigenic effects of various mediators in TME on the relationship between stromal cells and immune cells.

ANTI-TUMORIGENIC ASSOCIATION BETWEEN STROMAL CELLS AND IMMUNE CELLS IN THE TME

Crosstalk Between Stromal Cells and Immune Cells in the TME Induces Anti-Tumor Immunity

Although numerous studies have suggested that CAFs can exert a tumorigenic effect, other studies have suggested that they are also involved in tumor suppression. According to *Ozdemir*'s study, CAF-depleted tumors are associated with increased CTLA-4

TABLE 1 | Tumorigenic effect of the relationship between stromal cells and immune cells in TME.

Cell type	Mediator	Effect function	Type of Tumor	Ref.
CAFs	MCP-1, SDF-1 production → IL-10↑, IL-12↓	Monocyte recruitment M2-like macrophage differentiation	Breast cancer	(45)
TEC				
CAA	IL-6, SDF-1 production	M2 macrophage differentiation	Prostate cancer	(50)
	CCL-2 (MCP-1) production → CCR2A/2B pathway activation	Monocyte recruitment for TAM differentiation	Breast cancer	(51, 52)
	IL-6/STAT3 pathway activation	Promotion of MDSC differentiation	Pancreatic cancer	(61)
	IL-6/STAT3 activation → IDO production↑	Induction of DC cell	Hepatocellular carcinoma	(66)
	IL-6, STAT3, PD-L1 signaling pathway	Activation of neutrophil Impairment of T cell function	Hepatocellular carcinoma	(67)
	IL-6, GM-CSF production	Induction of TAM infiltration	Colon cancer	(68)
	IL-6, IL-8 production	Attraction of monocyte recruitment for TAM differentiation, NK cell function inhibition	Colorectal cancer	(70)
	TGF-β production → IL-4↑, IL-10↑, IL-12↓ → PD-L1, HLA-DR↑	M2 macrophage polarization	Hepatocellular carcinoma	(78, 79)
	PD-L2, FASL engagement↑	Induction of $CD8^+$ T cell death	Lung cancer	(81)
	CXCL12 (SDF-1)-CXCR4 expression	$CD4^+CD25^+$ T cell proliferation	Breast cancer	(82–84)
	CXCL12 → *via* B7H3, CD73, DPP4↑	Attraction of $CD4^+CD25^+$ T cell, Increase T cell survival, differentiation		
	PD-L1(B7H1), B7DC expression	Induction of T cell apoptosis and $FOXP3^+$ Treg proliferation	Head and neck squamous cancer	(85)
	IL-6, CXCL8, TNF, TGFB1, VEGFA↑			
	PGE2 production → inhibition of NK receptor (NKp44, NKp30), perforins, granzymes	Inhibition of NK cell function	Melanoma	(99, 100)
	PGE2 expression, IDO production	Suppression of NK cell activation	Hepatocellular carcinoma	(101, 102)
	Notch1-induced VCAM1 expression	Promotion of Neutrophil infiltration	Ovarian, Lung carcinoma, melanoma	(110)
	FasL production → Fas/FasL death signaling activation	Suppression of $CD8^+$ T cell	Glioma	(111)
	TGF-β, IL-10 production, via PD1/PD-L1 pathway	Attenuation of $CD8^+$ T cell function	Melanoma	(112)
	CLEVER-1/stabilin-1 production	$FOXP3^+$ Treg recruitment	Hepatocellular carcinoma	(114)
	CCL-2 production → IL-1β/CXCL12 activation	Induction of macrophage recruitment	Breast cancer	(117)
	PD-L1 expression	Inhibition of $CD8^+$ T cells	Breast cancer	(120)
	IL-8 production	Induction of Neutrophil recruitment	Pancreatic ductal adenocarcinoma	(122)
MSC	TGF-β production	Induction of Treg cell	Breast cancer	(125)
	SDF-1/CXCR7 axis	Induction of Breg cell	Non-cancerous	(126)
	TNF-α production → induction PD-L1↑	Suppression of $CD8^+$ T cell	Colon cancer	(131)
Neutrophil	IL-1β production → MMP-9 activation	EC activation → metastasis ability↑	Pancreatic ductal adenocarcinoma	(132–134)
Monocyte/ Macrophage	OPN production	CAF proliferation → promoting malignancy↑	Hepatocellular carcinoma	(141)
	Wnt7a expression → Wnt/β-catenin signaling	Myofibroblasts differentiation of MSC → fibrosis↑	Non-cancer (Pulmonary fibrosis)	(144)
	miR-155-5p, 221-5p in MDE	TEC proliferation → promoting growth↑	Pancreatic ductal adenocarcinoma	(147, 148)
NK cell	VEGF, PIGF production	HUVECs migration, formation↑ → tumor growth↑, angiogenesis↑	Non-small cell lung cancer	(150)
Mast cell	TGF-β production	Myofibroblasts differentiation induction, proliferation↑	Neurofibromas	(154)
	Tryptase production	Promoting the transformation of prostate ECs morphology	Prostate cancer	(155)
	IL-13, Tryptase production	Stimulation of PSC proliferation	Pancreatic ductal adenocarcinoma	(156)

CAFs, Cancer-associated fibroblasts; TECs, Tumor-endothelial cells; CAAs, Cancer-associated adipocytes; PSC, Pancreatic stellate cell; TAN, Tumor-associated neutrophil; CM, Conditioned medium; EC, Endothelial cells; FasL, Fibroblast associated ligand; OPN, Osteopontin; MDE, Macrophage-derived exosomes; VEGF, Vascular endothelial growth factor; PIGF, Placental growth factor; HUVEC, Human umbilical vein endothelial cells.

↑, increase; ↓, decrease.

expression with a reduced Teff/Treg cell ratio in the PDAC model. In this study, the cytotoxic Teff/Treg ratio was decreased in the myofibroblast-depleted tumor and associated with a significant elevation in CTLA-4. Thus, CAF depletion can induce immunosuppression, reduce survival, and further accelerate PDAC (157). Suppression of the checkpoint blockade using CAF depletion and anti-CTLA-4 antibody can improve mouse PDAC tumors and increase overall survival (157). CAF depletion has breakthrough efficacy with significant changes in major contributors to cancer development within the TME.

McAndrews et al. have demonstrated that depletion of αSMA$^+$ CAFs is associated with increased Lgr5$^+$ cancer stem cells and the generation of an immunosuppressive TME with increased frequency of Foxp3$^+$ Treg cells and suppression of CD8$^+$ T cells. Thus, αSMA$^+$ CAFs in CRC can promote an anti-tumor effect *via* BMP4/TGF-β signaling (158). Interestingly, infiltrated CD8$^+$ T cell accumulation in non-small cell lung cancer with a high proportion of fibroblasts is associated with the expression of CCL19 identified in the lungs and tumors of patients (159). Fibroblasts expressing CCL19 can form perivascular niches and promote the accumulation of CD8$^+$ T cells. These results can be proposed as a new marker for immune tumor treatment by targeting CAFs that produce CCL19 (159). A study by Kamata et al. using G12DKRAS and V600EBRAF-driven mouse models that develop lung adenocarcinoma and adenoma has confirmed that stanniocalcin1 secreted by tumor-associated fibroblasts can inhibit TAM differentiation. The secreted stanniocalcin1 can inhibit TAM differentiation by sequestering the binding of glucose regulatory protein 94 (GRP94), an autocrine macrophage differentiation-inducing factor, to the scavenger receptor (160).

A variety of cytokines have been shown to be either pro- or anti-inflammatory depending on the cell type and disease model. IL-33 is an inflammatory cytokine released during necrotic cell death (161). CAFs can release IL-33, induce metastasis *via* EMT, and promote cell migration and invasion. In cancer immune response, IL-33 exhibits both pro-tumoral functions and antitumor functions (162). IL-33 can also induce IL-33 gene expression in HNSCC cells through a positive feedback process (163). The production of IL-33 is responsible for the antitumor response as CD8$^+$ T cell infiltration. In a colon cancer model, IL-33 can increase interferon (IFN)-γ production by tumor-invasive CD4$^+$ and CD8$^+$ T cells. This has been confirmed by the accumulation of infiltrated CD8$^+$ T cells, which exerts an antitumor effect (164). These results demonstrate the synergistic increase in IFN-γ, and IL-12 release, along with increases in proliferation, infiltration, and the number of cytotoxic NK cells activated by IL-33 (165). IL-33 administered to a mouse breast cancer model can potently suppress lung metastasis and increase the number of NK cells recruited within the TME (166). Furthermore, it has been shown that NK cell depletion in IL-33/ST2-deficient mice is associated with tumor growth promotion (167).

Interestingly, inhibitor of κB kinase beta (IKKβ)-depletion in intestinal mesenchymal cells (IMCs) can decrease immune cells infiltration and the expression of several pro-inflammatory mediators. Supernatant of IKKβ-depleted IMCs mouse model also shows decreased secretion of chemokine MIP2, cytokines IL-6, TNF, FOX2, and MMP9 in organ culture. Therefore, IKKβ in IMCs of inflammation-associated colorectal cancer might have a tumor-suppressive effect (168).

Several studies have reported that the sirtuin 1 (SIRT1) signaling pathway can regulate vascular inflammation. The role and molecular interaction of SIRT1 and Toll-like receptor 2 (TLR2) in monocyte adhesion to the vascular endothelium have been found to be important. These results suggest potential therapeutic targets for a variety of vascular inflammation, including atherosclerosis (169–171), although these results are not about cancer. Recent studies have shown that extracellular vesicles (EVs) derived from immune cells can perform various roles in immune responses (172). EVs can deliver not only proteins, but also biomolecules such as nucleic acids and lipids. They play a very essential role in the cell-to-cell communication process. Activated CD8$^+$ T cells in a mouse model can temporarily release cytotoxic EVs and prevent the progression, invasion, and metastasis of fibroblast stroma-mediated tumors (173).

TABLE 2 | Anti-tumorigenic effect of the relationship between stromal cells and immune cells in TME.

Cell type	Mediator	Effect function	Type of Tumor	Ref.
CAF	Regulation of CTLA4 expression	Balance of Teff/Treg ratio	Pancreatic adenocarcinoma	(157)
TEC	BMP/TGF-β signaling → Lgr5$^+$ CSCs↓	Suppression of FOXP3$^+$ Treg Induction of CD8$^+$ T cell	Colorectal cancer	(158)
	CCL19 production	Intratumoral accumulation of CD8$^+$T cell infiltration↑	Lung carcinoma	(159)
	STC1 production → Inhibition of GRP94 binding on TAM	Inhibition of TAM differentiation	Lung adenocarcinoma Lung adenoma	(160)
	IL-33 production → IL-12↑, IFN-γ↑	Promotion of CD8$^+$ T cell infiltration	Colon cancer	(163, 164)
		Induction of cytotoxic NK cell proliferation	Breast cancer	(165–167)
	NF-kb-IKKβ signaling in intestinal mesenchymal cells (IMCs)	Induction of T cell infiltration	Inflammation-associated colorectal cancer	(168)
	SIRT1/TLR2 interaction↑	ECs-monocyte adhesion Inflammation↑	Non-cancer (Vascular inflammation)	(170)
T cell	Activated CD8$^+$ T cell derived EVs	CAF progression, invasion, and metastasis inhibition	Pancreatic cancer	(173)
	Regulation of GSH/Cystine Metabolism in CAF	Diminished fibroblast mediated platinum resistance	Ovarian cancer	(174)
DC	Induction of TNF-α, IL-1β, IL-6, and IL-12p70	Fusion with CAF and induction of T cell stimulation	Hepatoma	(175)
NK cell	DNAM-1 activation	Suppression of EC and induction of NK cell cytotoxicity	Multiple myeloma	(176)

CSCs, Cancer stem cells; CCL19, Chemokine (C-C motif) ligand 19; STC1, Stanniocalcin-1, GRP94, Glucose-regulated protein 94; IKK, Inhibitor of nuclear factor-kB kinase; SIRT1, Sirtulin1; TLR2, Toll like receptor 2; EV, Extracellular vesicles; GSH, Glutathione.; DNAM1, DNAX accessory molecule (CD226).
↑, increase; ↓, decrease.

In platinum-based chemotherapy for ovarian cancer cells, IFN-γ-producing $CD8^+$ T cells show altered glutathione (GSH)/cystine metabolism of fibroblasts and reduced fibroblast-mediated platinum resistance (174). Thus, IFN-γ-producing $CD8^+$ T cells can eliminate chemoresistance of ovarian tumors and offer a combined treatment method that utilizes immune and stromal cell relationships in cancer treatment. In addition to effector T cells, other immune cells can also influence tumors by controlling fibroblast function. Interestingly, when DCs as potent APCs are fused with CAFs, they can stimulate T cells to attack cancer cells. These DC/CAF fusion cells can produce TNF-α, IL-1β, IL-6, and IL-12p70 and stimulate T cells to produce IFN-α and IFN-γ. T cells activated by the fusion of DCs and CAFs can induce a strong cytotoxic T cell response that has emerged as a new antitumor response through tumor growth inhibition (175). A multiple myeloma study has analyzed the activity of cytokine-stimulated NK cells on tumor-associated endothelial cells. IL15-activated-NK cells can enhance the killing of multiple myeloma

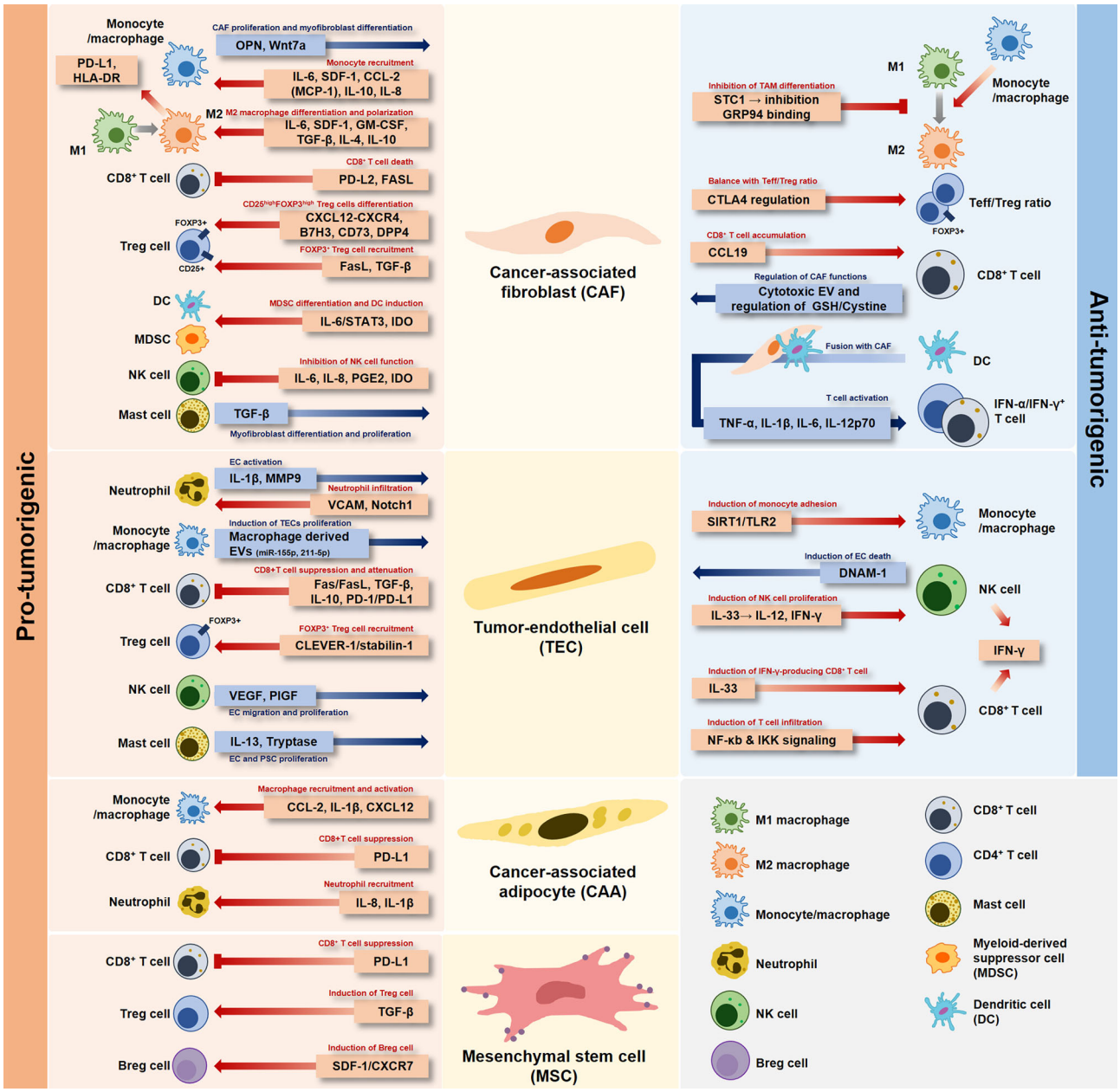

FIGURE 1 | Dual role of stromal cells in the tumor microenvironment. Each stromal cell developed in the tumor-specific microenvironment has a pro-tumorigenic or anti-tumorigenic role depending on the interaction with immune cells.

patient's endothelial cells *via* DNAX accessory molecule 1 (DNAM-1) (176). **Table 2** describes anti-tumorigenic effects of various mediators in TME on the relationship between stromal cells and immune cells. It is necessary to understand the complexity between stromal cells and immune cells and carefully consider their targeting and use in drug development to overcome cancer treatment resistance.

CONCLUSIONS AND PROSPECTION

Early studies have focused on "tumor cells" to explain anticancer drug resistance and progression of tumors by specific signaling mechanisms, rather than heterogenic TME characteristics. Successful cancer treatment strategies need to be developed based on understanding the relationship between stromal cells and immune cells in the TME. Recently, research on cancer cells has continued to transition toward studying the network of stromal cells and inflammatory immune cells in the TME. Ultimately, the organic relationship between stromal cells and immune cells present in the TME not only involves pro-tumorigenic and anti-tumorigenic cells, but also shows a mixed environment of these aspects in the TME (**Figure 1**).

Since the TME is heterogeneous, the relationship between stromal cells and immune cells, which are components of the TME, is complex. However, additional mechanisms that control the interactions between immune cells and stromal cells in the TME remain unknown. Therefore, rather than interpreting a one-way relationship between two cells, it is necessary to understand and approach two-way relationships. The relationship between immune cells and stromal cells in the TME merits further investigation.

In particular, the most interesting aspect of the two orientations proposed in this review is the association of stromal cells with immune cells in controlling tumor formation and development. The network requires more attention when developing drugs that target their relationships. Increasing the understanding of the network between stromal cells and immune cells within the TME will ultimately improve the development and efficacy of cancer therapies. Finally, understanding the complex interactions between stromal cells and immune cells within the TME is necessary to identify potential strategies for cancer treatment.

AUTHOR CONTRIBUTIONS

HK contributed to study conception. J-YM, S-HL, and JL performed literature review and analysis and revised the manuscript. J-YM and HK drafted the manuscript, figures, and tables. All authors contributed to the article and approved the submitted version.

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Deconvolution of Adult T-Cell Leukemia/Lymphoma With Single-Cell RNA-Seq Using Frozen Archived Skin Tissue Reveals New Subset of Cancer-Associated Fibroblast

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Adult T-cell Leukemia/Lymphoma (ATLL) is a rare aggressive T-cell malignancy caused by human T-cell leukemia virus type 1 (HTLV-1) infection. However, little is known about the underlying activated molecular pathways at the single cell level. Moreover, the intercellular communications between the tumor microenvironment (TME) and tumor cells in this malignancy are currently unknown. Difficulties in harvesting fresh tissue in a clinical setting have hampered our deeper understanding of this malignancy. Herein, we examined ATLL using archived fresh frozen tissue after biopsy using single-cell RNA sequencing (scRNA-seq) with T-cell receptor (TCR) clonal analysis. Highly clonal tumor cells showed multiple activating pathways, suggesting dynamic evolution of the malignancy. By dissecting diverse cell types comprising the TME, we identified a novel subset of cancer-associated fibroblast, which showed enriched epidermal growth factor receptor (EGFR)-related transcripts including early growth response 1 and 2 (EGR1 and EGR2). Cancer associated fibroblasts (CAFs) of ATLL play an important role for CD4 T-cell proliferation *via* FGF7-FGF1 and PDGFA-PDGFRA/B signaling, and CAFs, particularly EGR-enriched, are also associated with CD8 and NKT expansion by EGFR. These findings suggest a potential targeted therapeutic pathway to better treat this neoplasm.

Keywords: adult T-cell leukemia/lymphoma, single-cell RNA-seq, cancer-associated fibroblast, frozen tissue, epidermal growth factor receptor pathway

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INTRODUCTION

Adult T-cell Leukemia/Lymphoma (ATLL) is an aggressive mature T-cell neoplasm caused by human T-cell leukemia virus type 1 (HTLV-1) infection (1, 2). Since HTLV-1 infection is endemic in southwestern Japan, ATLL has been mainly reported in the same region (3, 4). Recent advances in next-generation sequencing (NGS) technology have provided more detailed information on the unique pathogenesis of ATLL compared to other subtypes of peripheral T-cell lymphoma (PTCL) (5–7). Copy number abnormalities (CNAs) of ATLL are comparable to the PTCL-GATA3 subgroup (5), and gene expression profiling has been used to define distinct diagnostic and prognostic subtypes of PTCL (8). However, investigations using single-cell RNA sequencing (scRNA-seq) technology with T-cell receptor (TCR) clonal analysis have been lacking, making it difficult to understand the dynamics of the immune response during ATLL progression. HTLV-1 not only infects T-cells, but also various cell types including B-cells, myeloid cells, and fibroblasts (9, 10). Accordingly, the interaction with the tumor microenvironment (TME) surrounding the neoplasm is important for its regulation and growth. ScRNA-seq has provided a useful cell atlas to better understand the intra-tumoral diversity of the TME and disease-specific cellular crosstalk with malignant cells (11). In this study, we used scRNA-seq and TCR clonal analysis to dissect the malignant tumor cells and TME of ATLL. With this approach, we were able to identify distinct T-cell subpopulations that likely represent the malignant clones in ATLL *via* an integrated analysis of the transcriptome and T-cell clonal repertoire. We also examined non T-cell components of the ATLL TME including myeloid cells and stromal cells, especially cancer associated fibroblast (CAF) subtypes and their potential communications with T-cells. In this study, we sought to identify a new possible target for ATLL treatment, while also considering clonal malignancy and their TME interactions.

2 METHODS

Patient Information

Human skin samples were obtained using remnants of biopsy tissue taken for diagnostic purposes under an Institutional Review Board (IRB)-approved protocol (IRB# 2020-03-060). For this study, we used a fresh frozen tissue sample from a 69-year-old man who presented to our clinic with newly developed erythematous nodules of his bilateral axillae, inguinal areas, and flexural surfaces of the arms. At the time of tissue profiling, he had not been diagnosed nor underwent any treatment. The samples were obtained by 4 mm skin punch biopsies of the right axilla. In laboratory investigations, lactate dehydrogenase (LDH) was elevated to 392 U/L (normal range <225 U/L) and B2-microglobulin was elevated to 3.47 μg/mL (normal range < 2.4 μg/mL).

Tissue Collection and Dissociation

Specimens were placed in phosphate-buffered saline (PBS) on ice. Biopsy samples were cryopreserved in optimal cutting temperature (OCT) compound and stored at -80°C. For cell dissociation, cryopreserved tissue was thawed in a 37°C water bath and transferred to freshly prepared dissociation solution composed of 200 μL of Liberase TL (2 mg/mL; Sigma Aldrich) and 1800 μL PBS, and incubated at 37°C for 15 min. The tissue was manually disaggregated, using a 1 mL pipette with a wide bore and gently pulling the solution up and down 10 times. The cells were collected through a 70-μm cell strainer (#352340, Corning) and stored on ice. The tissue was transferred to a dissociation solution for a second round of dissociation as noted above, followed by dissociation in Trypsin solution (350 μL PBS, 50 μL 0.25% Trypsin). Cells were washed once and re-suspended in 100 μL of freshly prepared PBS-bovine serum albumin (BSA; 1 x PBS and 0.04% BSA) and processed on the 10x Genomics platform.

Library Construction for Single Cell Gene Expression and TCR Profiling and NGS Sequencing

Single cell dissociates were loaded into the Chromium system (10x Genomics, USA) to encapsulate into a single droplet targeting approximately 25,000 cells. The Chromium Single Cell 5' Kit (10x Genomics, USA) was used to generate scRNA-seq and TCR libraries, according to the manufacturer's instructions. Briefly, single Cell 5' Kit enables the measurement of gene expression and the immune repertoire from the same cells, profiling the full-length of 5' UTR and paired TCR transcripts from individual cells. Chromium Controller™ splits the cells into nano-scale Gel Beads-in-emulsion (GEM), where barcoded cDNA was generated. The TCR library was constructed by PCR amplification of GEM with TCR region specific primers, whereas the gene expression library was made without V(D)J segment amplification. Each library was loaded on a NovaSeq 6000 platform (Illumina, USA) with pair-end reads of 150 bp to generate the sequencing data.

Data Processing

ScRNA-seq and TCR-seq data were pre-processed and aligned to the human reference genome (GRCh38) using the CellRanger 4.0.0 pipeline (https://support.10xgenomics.com/single-cell-vdj/software/pipelines/latest/what-is-cell-ranger). Raw sequence base call (BCL) files were converted into FASTQ files using the "mkfastq" command. For scRNA-seq, the "count" command was used to align the reads to the genome, annotate with transcripts, and count UMI with correction steps. For TCR-seq, the "vdj" command was used to assemble the reads into contigs and annotated with V, D and J segments and CDR3 regions. Gene expression matrix from the CellRanger count was filtered, normalized using the Seurat 3.1.4 in R 4.0.5 software (R Foundation for Statistical Computing, Vienna, Austria) and selected according to the following criteria: cells with >200 genes; and <20% of mitochondrial gene expression in unique molecular identifier (UMI) counts. We used "filtered_contig_annotations" determined by Cell Ranger vdj, which contained the alpha chain, beta chain and CDR3 nucleotide sequences by each barcode. Following QC, scRNA-seq and TCR-seq data were merged on the Seurat objects.

Dimensionality Reduction, Clustering, and Differential Expression Analysis

Dimensionality reduction and clustering were done as recommended by the Seurat developers (12). Briefly, gene expression counts were LogNormalized, 2,000 variable features were selected and scaled to the expression level for Principal Components Analysis (PCA). PCA allowed reduction of the high variable gene expression data set into a low-dimensional space for characterizing transcriptional profiles (13). Clustering was performed using louvain algorithm based on PCs, and Uniform Manifold Approximation and Projection (UMAP) was used to visualize clustering results into two dimensions (14). Each cluster was manually annotated with canonical cell type features. Differentially expressed genes across the clusters were performed using Model-based Analysis of Single-cell Transcriptomics (MAST) test (15).

Copy Number Variation Analysis

To identify malignant tumor cells with chromosomal copy number changes, we used inferCNV 1.6.0 (https://github.com/broadinstitute/inferCNV). The raw gene expression data were extracted from the Seurat object and public single-cell data derived from a healthy donor (16) were included as a normal control reference.

TCR Repertoire Analysis

We analyzed outputs of CellRanger vdj (10x Genomics) that included the CDR3 sequences and clonotype of assembled alpha and beta chains of TCR. We also analyzed the V-gene usage and the frequency of both alpha and beta chains to investigate the variation across the clonotype. Weblogo plots were generated using WebLogo 3.7.4 (https://weblogo.berkeley.edu). Sequence logos are a graphical representation for conservation of amino acid sequence alignment and height of the symbol indicates the relative frequency of each amino at that position.

Cell-Cell Interaction Analysis

To investigate the potential cell-cell communication between ATLL T-cells and other cell types including stromal cells, we applied CellChat 1.1.2 (https://github.com/sqjin/CellChat) (17) with scRNA-seq data. CellChat inferred the potential cell-cell ligand-receptor interaction between assigned cell types and visualized into a diagram.

Resource Availability - Data and Code Availability

10x Genomics peripheral blood mononuclear cells (PBMCs) datasets from a healthy donor are available at the 10x Genomics website (16).

RESULTS

Patient Characteristics

A 69-year-old man with a history of hypertension presented with multiple erythematous papules and nodules of the bilateral axillae, inguinal areas, and flexural surfaces of the arms. At the 2-week follow-up visit, the skin lesions had increased both in number and size (**Figure 1A**). A biopsy of a palpable mass of the neck favored T-cell lymphoma. Chest and abdominal computed tomography showed bilateral neck as well as abdominal and pelvic lymph node enlargement, suspicious for lymphoma involvement (**Figure 1D**). Diagnostic bilateral bone marrow biopsies were performed, and no lymphoma was found. Positron emission tomography/computed tomography done for staging was consistent with stage III lymphoma. Skin biopsy showed dense infiltration of atypical lymphocytes. Immunohistochemical studies demonstrated predominance of CD4+ over CD8+ T cells, and CD30, CD20, CD56, CD123 were all negative in the lymphocytic infiltrate (**Figure 1B**). Lymphocytic component stained positive for Ki-67 in 95% of tumor cells. The patient was initially diagnosed with peripheral T-cell lymphoma, not otherwise specified. After discussion with the hematology/oncology team, the patient was started on combination chemotherapy consisting of cyclophosphamide, doxorubicin, vincristine, and prednisone. After six courses of chemotherapy, the patient was free of new skin lesion. The patient's biopsy specimen of a neck lymph node showed CD4+ and CD25+ non-cytotoxic mature T-cell lymphoma (**Figure 1C**). Due to suspicion for ATLL, HTLV-1 polymerase chain reaction (PCR) was done, and the result was positive. Thus, the patient ultimately received a diagnosis of ATLL.

Annotation of the Multiple Cell Types Comprising ATLL Skin by scRNA-Seq

We performed scRNA-seq analysis to better investigate the cellular composition of ATLL (**Figure 2A**). After filtering-out for quality assessment, a total of 15,550 cells were obtained. Unbiased clustering followed by UMAP dimension reduction revealed 10 distinct cell clusters according to gene expression pattern (**Figure 2D**). Each cluster was well characterized by the transcriptional profile representing specific cell types. We annotated cell type identity in each cluster with highly expressed canonical markers (**Figure 2B**): CD3D for T-cells, ALF1 and CD1C for macrophages and dendritic cells (DCs), COL1A1 for fibroblasts, RGS5 for pericytes, VWF for endothelial cells, SCGB1B2P for eccrine gland/duct cells, and DEFB1 and KRT1 for keratinocytes. Among $CD3D^+$ T-cells, we found the following subtypes according to surface markers: $CD4^-/CD8^-$ T-cells (double negative, dnT), $CD4^+$ T-cells, proliferating $CD4^+$ T-cells with TOP2A, and $CD8^+$ T-cells. We found that 62% of ATLL cells were T-cells (**Figure 2C**), suggesting significant expansion of malignant T-cells as a characteristic of ATLL. Other cell type proportions were as follows: 9% for macrophages/DCs, 7% for fibroblasts, 14% for endothelial cells, 5% for pericytes, 2% for keratinocytes, and 1% for gland cells.

Heterogeneity of T-Cells and Tumor Identification Within ATLL

To investigate the heterogeneity within T-cells, we sub-analyzed 9,625 T-cells and revealed 9 sub-clusters (**Figure 3A**). Each sub-cluster was annotated based on relative expression of functional genes related to immune status (**Figure 3B**). CD4+ T-cells were separated into 3 sub-populations: CD4 effector memory T-cells (Tem)

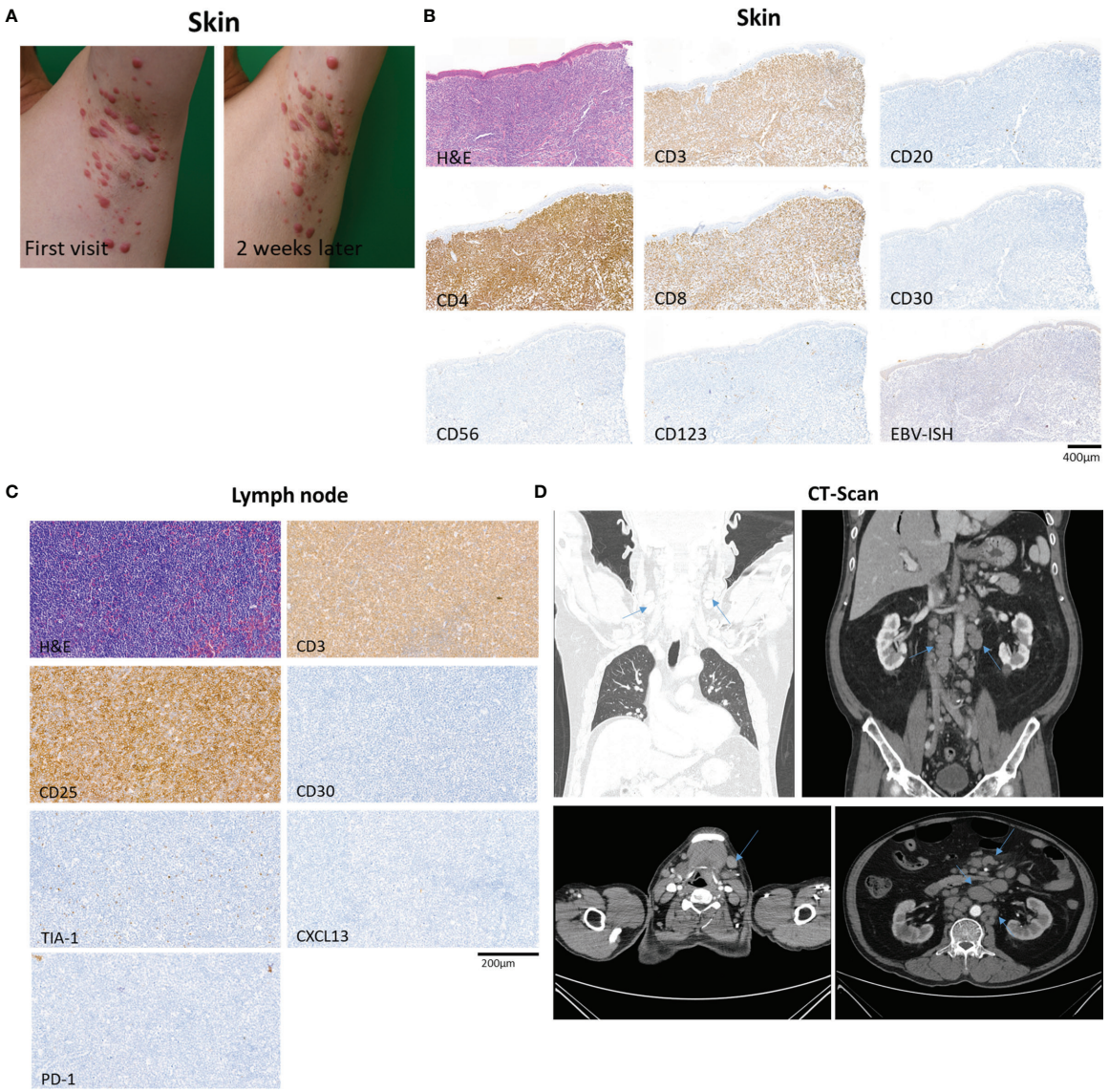

FIGURE 1 | Overview of a patient with adult T-cell leukemia/lymphoma involving skin and lymph node. **(A)** The patient's skin lesions at first visit and 2 weeks after the first visit, showing rapidly progressing erythematous papules and nodules of the left axilla. **(B)** Biopsy specimen of skin showing dense infiltration of atypical lymphocytes with predominance of CD4+ over CD8+ T cells, and negative expression of CD30, CD20, CD56, and CD123. In situ hybridization of EBV was negative. **(C)** Excisional biopsy specimen of neck lymph node demonstrating CD4+ and CD25+ non-cytotoxic mature T-cell lymphoma involvement. **(D)** Computed tomography of chest, abdomen and pelvis showing abnormal lymph node enlargement (arrows) in the abdomen, pelvis, and bilateral neck, highly suspicious of lymphoma involvement.

expressing MAL and RORA (18) (**Supplementary Figure S1**), CD4 regulatory T-cells (Treg) expressing IL2RA, CCR4 and GATA3, and proliferating T-cells expressing MKI67 and TOP2A (**Supplementary Figure S1**). CD8 T-cells were separated into 3 sub-populations: CD8 naive T-cells expressing CCR7 and NOSIP with less expression of effector genes compared to other CD8 T-cells, CD8 Tem expressing cytotoxic effectors such as NKG7, GZMA, and GZMK, and CD8 exhausted T-cells expressing immune checkpoint genes such as LAG3, CTLA4, TIGIT, and HAVCR2. NKT was identified by CD3D, NKG7, GNLY, and KLRB1 expression. dnT-cells rarely expressed functional genes, whereas dnT proliferating cells mainly expressed cell-cycle related genes such as STMN1, TOP2A, UBE2C, and MKI67 (**Supplementary Figure S1**).

To differentiate the tumor and non-tumor immune cells, we utilized the ESTIMATE (19) algorithm to compare predicted immune proportion and tumor purity across T-cell subtypes (**Figure 3C**, upper). CD4+ cells (CD4 Tem, CD4 Treg, and CD4 proliferating) and dnT-cells (dnT and dnT proliferating) showed lower immune score but higher tumor purity compared to CD8 T-cells and NKT. Further, we computed module scores using

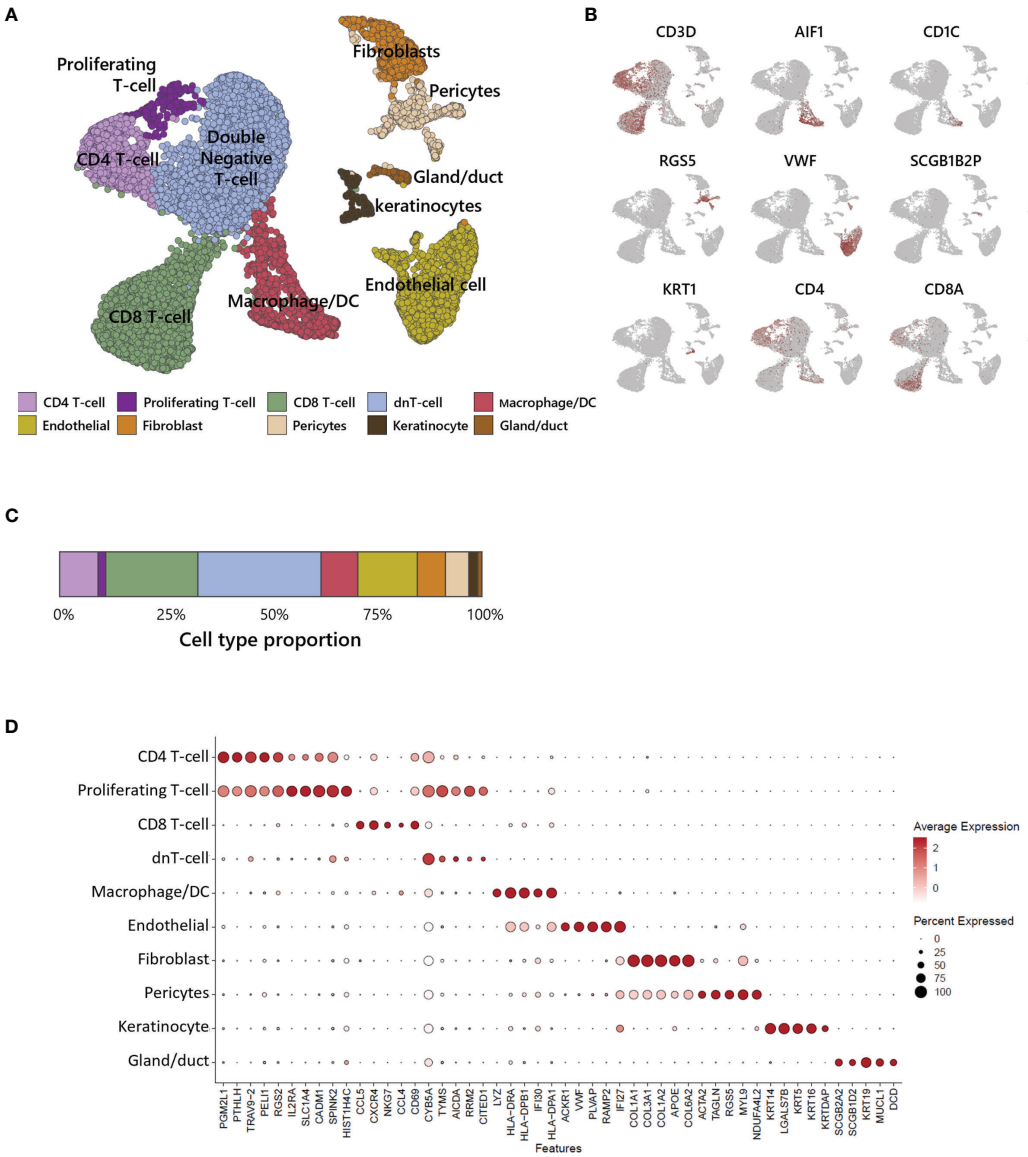

FIGURE 2 | Single-cell transcriptomic analysis of ATLL. **(A)** UMAP plot for 15,550 skin cells clustered by unsupervised Seurat clustering and annotated with 10 cell types. **(B)** Each cluster is identified by canonical cell type marker expression. **(C)** Proportion of each cell type in ATLL skin sample. **(D)** Dot-plot showing scaled average gene expression of the top 5 differentially expressed genes in each cluster of **(B)**.

previously suggested ATLL signatures by Sasaki (20) and Iqbal (8) (**Figure 3C**, bottom). All three types of CD4+ T-cells and all dnT-cell types exhibited marked scores for ATLL signatures, but CD8+ T-cell types and NKT scored low, suggesting tumor malignancy of ATLL is more associated with CD4+ and dnT-cells than CD8+ and NKT cells.

Next, to evaluate the genomic variation within T-cells, we used inferCNV to identify the copy number variation (CNV) between each cell subtype with T-cells from a healthy donor as a normal control (**Figure 3D**). CD4+ T-cells and dnT-cells were found to have multiple chromosomal changes (gain of 1q, 2p, 7p, 17q, 18p and 18q, and loss of 6p, 7q, 13q, and 14q) compared to CD8+ T-cells, which suggests that CD4+ T-cells accumulated more CNV abnormalities compared to normal T-cells. In a previous genomic study, Heavican observed the same pattern of CNV aberration mainly in GATA3 T-cell lymphoma (5). Consequently, CD4+ T-cells and dnT-cells can be defined as a malignant tumor within ATLL, based on transcriptomic characteristics and either type of CNV abnormalities.

Single-Cell V(D)J Recombination Repertoire Analysis of T-Cell Receptors

V(D)J recombination sequencing revealed clonally expanded T-cell subtypes with scRNA-seq. We identified 3 largely expanded

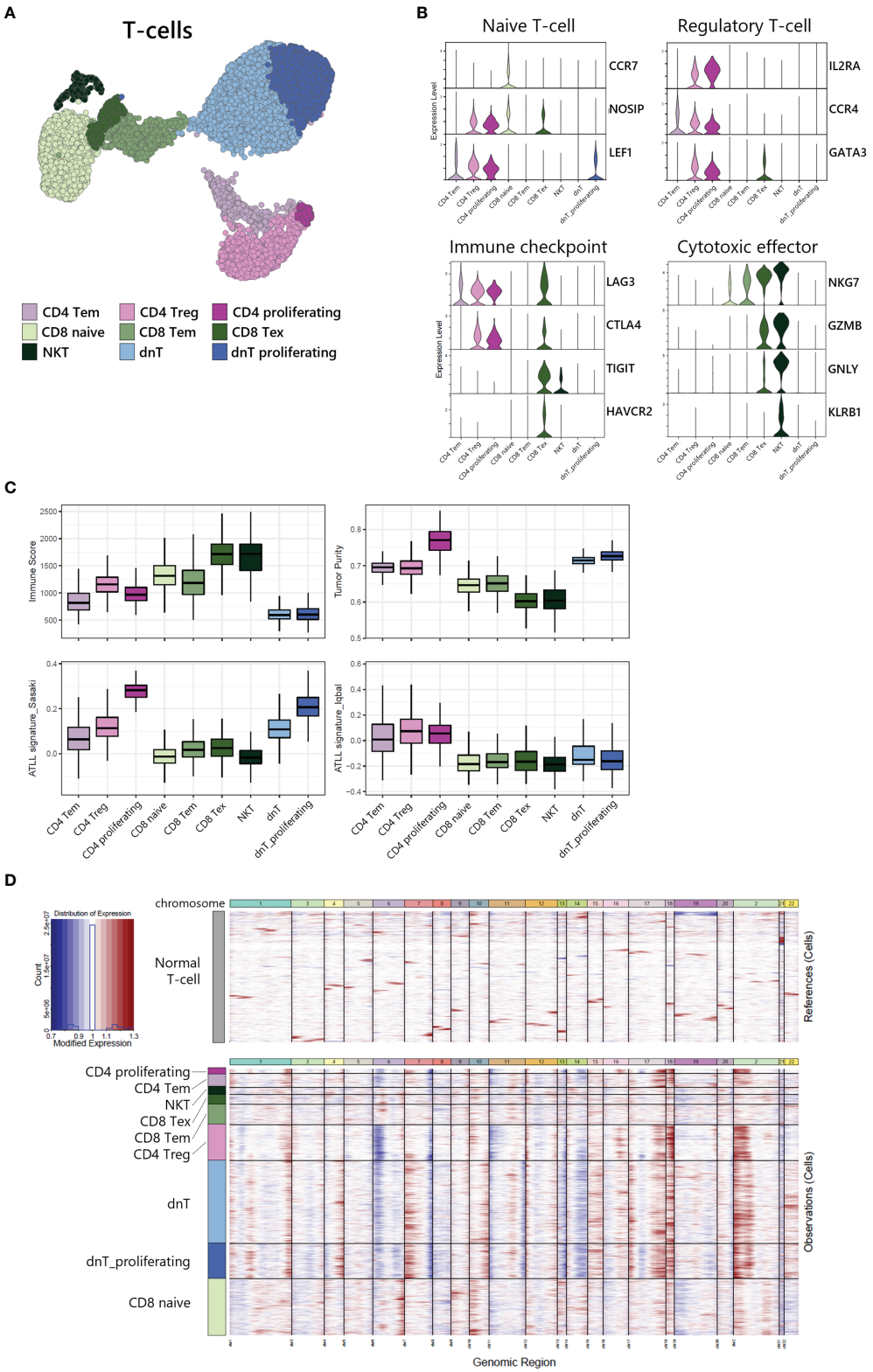

FIGURE 3 | Tumor identification within T-cell subpopulation. **(A)** UMAP of the 9,625 cell T-cell population reveals 9 distinct clusters. **(B)** Functional gene expression of T-cell subtypes. **(C)** Estimated Immune Score and Tumor Purity of T-cell subtypes. **(D)** Heatmap of inferred copy number changes (infercnv) compared to T-cells from healthy donors.

clones (clonotypes 1, 2, and 3), which accounted for 53% of TCR-expanded cells, and other polyclonal clones (clonotypes 4~1686) by overlapping each clonotype onto UMAP plot of T-cells (**Figure 4A**). Largely expanded clones were mainly detected in CD4 Treg and proliferating T-cell populations, while polyclonal T-cells were observed in CD8 naive, CD8 Tem, and CD8 Tex populations (**Figure 4B**), suggesting that malignancy is originating in the CD4 T-cell compartment. Further, variable gene (V gene) usage in T-cell receptor alpha and beta (TRA and TRB) showed that largely expanded clones had uniform repertoires using the same V gene, TRAV9-2 for TRA of clonotypes 1 and 3, and TRBV19 for TRB of clonotypes 1 and 2 (**Figure 4C**). We also analyzed the CDR3 amino acid sequence conservation of TRA and TRB according to TCR clonality (**Figure 4D**). In the case of TCRs in CD4 T-cells and dnT-cells, sequence prevalence was strongly conserved to CALTGTASKLTF for TRA and CASSIGGLCGNTIYF for TRB. In the case of TCRs in CD8 T-cells and NKT, there was no representative sequences due to the variation in the middle of the sequence (5 to 10).

Gene Expression Profiles of Clonally Expanded T-Cells and Polyclonal T-Cells in ATLL

To investigate the transcriptomic differences between clonally expanded T-cells and polyclonal T-cells, we analyzed differentially expressed genes (DEGs) in ATLL T-cells compared to healthy T-cells (**Supplementary Figure S2**). We discovered 117 genes up-regulated in T-cells from ATLL compared to T-cells from a healthy donor (**Supplementary Figure S2A**). Up-regulated genes in ATLL were related to metabolic pathways, phosphorylation, cytokine production, cell differentiation, and

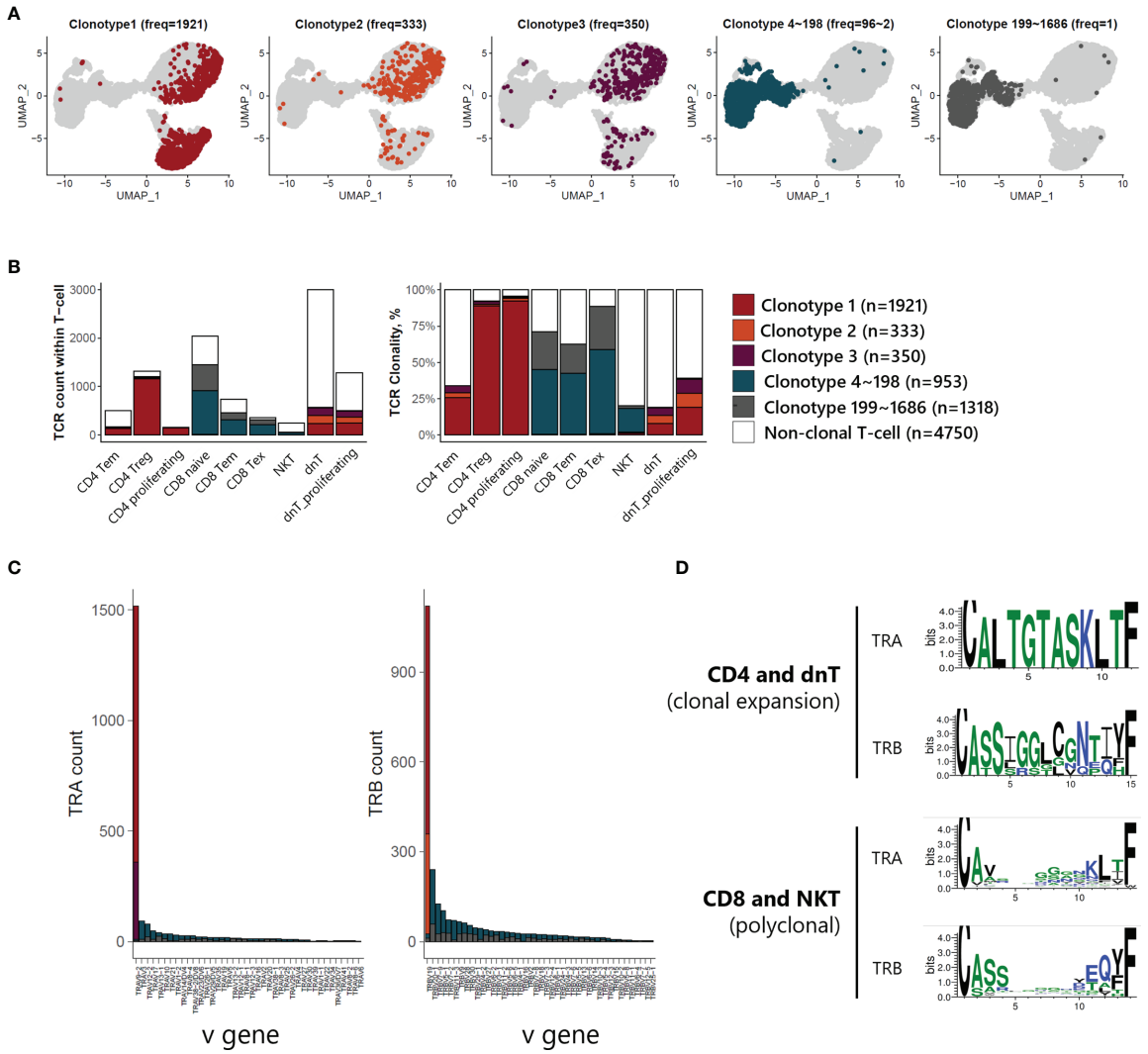

FIGURE 4 | TCR analysis of T-cells of ATLL **(A)** TCR clonotype expansion within T-cells **(B)** Proportional expansion of each TCR clonotype within the different T-cell subtypes **(C)** Composition of variable gene of TRA and TRB **(D)** Weblogo plot showing conserved amino acid sequence of TRA and TRB according to T-cell subtypes.

growth factor stimuli, suggesting that ATLL is a metabolically active cancer type. Down-regulated genes in ATLL were mainly related to lymphocyte activation, immune system development, and hematopoiesis, suggesting the loss of normal lymphocytic differentiation (**Supplementary Figure S2B**). Among 117 ATLL-specific DEGs, we identified 61 genes that were up-regulated in the clonally expanded T-cell group, and 26 in the polyclonal T-cell group (**Table 1** and **Supplementary Figure S2C**). Clonally expanded T-cells had up-regulation of genes related to metabolism (ENO1 and PKM), immunity (CADM1), differentiation (CITED1 and TCF4), oxidoreductase (PRDX1 and TECR) and ATLL pathways (CAV1, CD99 and PTHLH). In contrast, polyclonal T-cells had up-regulation of genes related to HTLV-1 infection (NFKBIA and EGR1), cytokine interaction (CCL4 and IL2RG), apoptosis, and inflammatory response, as well as genes down-regulated in angioimmunoblastic T-cell lymphoma (AILT). Our finding of polyclonal T cells, and not clonal T cells, showing a HTLV-1 infection signature suggests the role of HTLV-1 infection in mediating the initial process of malignancy rather than clonal expansion.

Heterogeneity of Myeloid Cells in ATLL and Characterization of Tumor-Associated Macrophages (TAMs)

Myeloid cells, including macrophages and DCs, are closely associated with survival in T-cell lymphoma patients, and the presence of TAMs has been used as a predictive biomarker (21). We found a large proportion of myeloid cells in our ATLL sample (1,181 of 15,550 total cells) and identified 4 subtypes (**Figure 5A, C**): macrophages with FTL, proliferating macrophages with STMN1, TAMs with MRC1 and MSR1, and DCs with CD1C. Further, the computed TAM signature suggested by Bagaev (22) was dominant in the TAM cluster of ATLL (**Figure 5B**). TAMs of ATLL showed a distinct tumor-associated gene expression signature, sharing innate immunity-associated genes (C1QA, C1QB, and C1QC) with the macrophage cluster (**Figure 5D**). Proliferating macrophages exhibited depleted innate immunity gene expression. Instead, NPM1, which is involved in non-Hodgkin lymphoma and acute myelogenous leukemia (23), and CCTs (CCT4, CCT5, and CCT7) were up-regulated. DCs of ATLL showed a similar transcriptional pattern to that of TAMs, and their correlation was relatively high (**Figure 5D** and **Supplementary Figure S3A**). The high expression of CD1C, CD14 and FCER1 suggests that they are closely related to monocyte-derived DCs (moDCs) (24). The expression of several cytokine genes (CD40, CXCR4 and IL1RN) in DCs characterizes inflammatory DCs (iDCs) as well (**Supplementary Figure S3B**). We observed that some of the TAMs and DCs of ATLL exhibited inflammatory properties, with moDCs sharing similar characteristics to TAMs.

Heterogeneity of Stromal Cells in ATLL and Characterization of CAFs

Stromal cells, including fibroblasts and pericytes, play an important role in cancer initiation and progression. Pericyte-fibroblast transition has often been associated with tumor invasion and metastasis (12). We analyzed 1,212 stromal cells and identified two vascular cell subtypes, pericytes and vascular smooth muscle cells (vSMC) and two CAF subgroups (**Figure 6A**). While all stromal cell types expressed COL1A1, only fibroblast cell types expressed DCN, and only vascular cell types expressed RGS5 (**Figure 6C**). Both fibroblast cell types exhibited a CAF signature (22) as seen in the UMAP of stromal cells (**Figure 6B**). Within the CAF-related gene set, ACTA2, PDGFRB, and FN1 were not associated with CAFs of ATLL (**Figure 6D**). Rather, LUM, FBLN1, LRP1, COL5A1, MMP2, FAP, and PDGFRA were strongly expressed only in CAFs of ATLL. Since MMP2 is important for extracellular matrix digestion, we speculate that tumor cells promote fibroblast to secret matrix digestion products to facilitate metastasis. However, the cellular mechanism for how the cancer cells regulate the gene expression pattern of other cell types merits further investigation.

CAFs of ATLL were separated into two subgroups: CAF/EGR_{high} exhibited relatively higher expression of epidermal growth response (EGR) genes such as EGR1, EGR2, EGR3, and ICAM1, while CAF/EGR_{low} showed relatively lower expression of these genes (**Figure 6C**). The CAF/EGR_{low} subgroup had relatively increased expression of CAF-related genes compared to the CAF/

TABLE 1 | Differentially expressed genes in T-cells of ATLL compared to healthy donor.

Clonality	Number of unique genes	Functional category	Gene symbols
CD4 and dn T-cell (clonally expanded)	61	Metabolic	NDUFV2, NME1, NME2, ADA, COX5A, ENO1, GAPDH, HPGDS, PKM, RRM2, TYMS, TPI1
		HTLV-1 infection	RAN, SLC25A5
		Immunity	CADM1, HMGB1, HMGB2, ISG20, MIF, PTMS
		Differentiation	CITED1, NME1, CADM1, GTSF1, STMN1, TCF4
		Oxido reductase	NDUFV2, COX5A, GAPDH, PRDX1, PRDX3, RRM2, TECR
		ATLL-related	TYMS, TUBB, UBE2C, NME1, PRDX1, CD99, HMGB2, SLC25A46, ISG20, HPGDS, CAV1, CADM1, PTHLH
CD8 and NKT (polyclonal)	26	HTLV-1 infection	FOS, NFKBIA, ZFP36, CREM, EGR1, IL2RG
		Cytokine-cytokine receptor interaction	CCL4, CXCR4, IL2RG
		Apoptosis	ARL6IP1, TNFAIP3, PPP1R15A, SRGN
		Inflammatory response	CCL4, TNFAIP3, ANXA1
		Down-regulated genes in angioimmunoblastic T-cell lymphoma (AILT)	PPP1R15A, TNFAIP3, ZFP36, FOSB, NR4A2, TSC22D3, RGCC, CREM, YPEL5

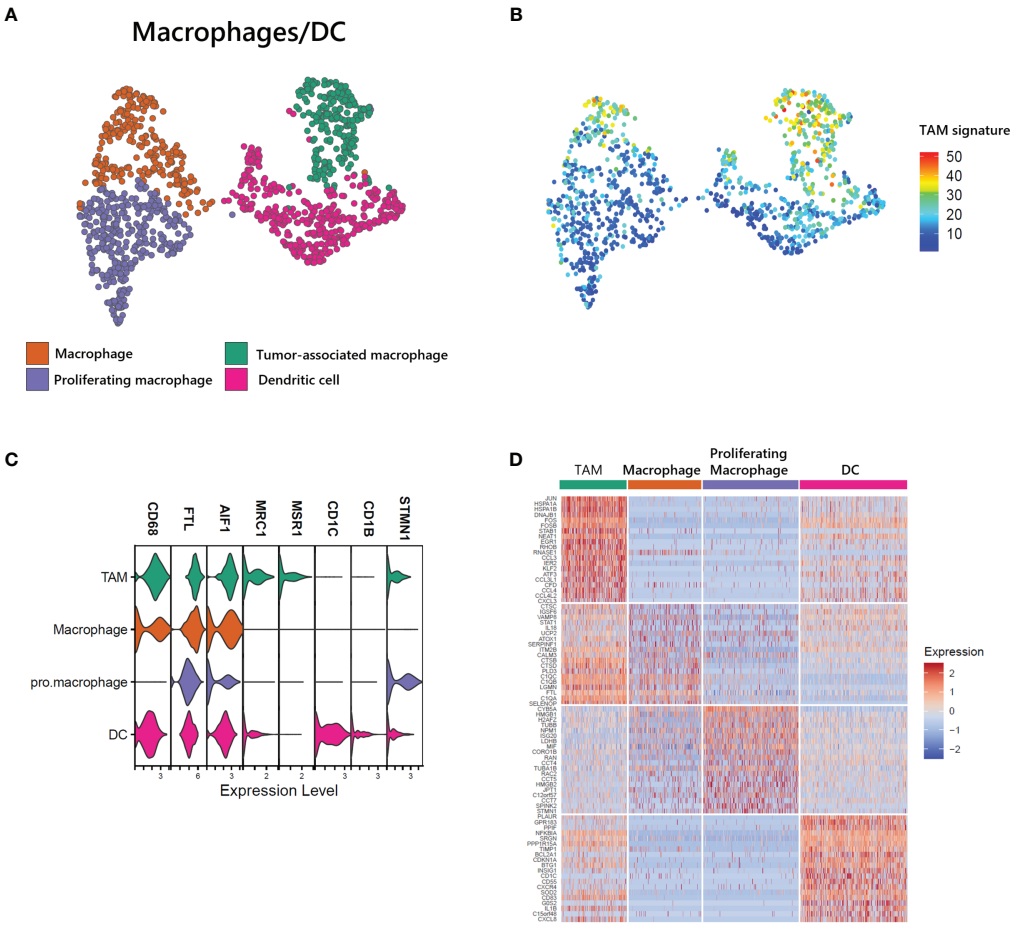

FIGURE 5 | Identification of tumor-associated macrophages (TAMs) in ATLL **(A)** Subtypes of myeloid cells **(B)** TAM score calculated with the gene set suggested by Bagaev (17) within myeloid cells **(C)** Cell-type markers of myeloid cells **(D)** Top DEGs of myeloid cell subtypes.

EGR_{high} subgroup (**Figures 6B, D**), suggesting that the CAF/EGR_{low} subgroup is the primary contributor to tumorigenesis among the two CAF subgroups. Next, we analyzed DEGs between the two CAF subgroups to characterize their functional differences. Forty-two genes were up-regulated in the CAF/EGR_{high} subgroup, but none exceeded the cut-off in the CAF/EGR_{low} subgroup (**Figure 6E**). We performed an enriched signaling pathways analysis using the clusterProfiler package (V3.18.1) (25) using up-regulated genes in CAF/EGR_{high}. CAF/EGR_{high} was considered highly related to interleukin signaling such as IL18, IL4, IL13, and IL6 (**Figure 6F**), suggesting that the CAF/EGR_{high} subgroup plays a key role in inflammatory responses, cytokine induction, and proliferation of fibroblasts.

Cell-Cell Interactions Between T-Cells and CAFs in ATLL Mediated by Growth Factors

To gain further insight into the potential interaction between T-cells and CAFs of ATLL, we analyzed cell-cell interaction acting through ligands and their suggested receptors. By analyzing the types and expression level of known membrane-bound factors in each cell type, we can infer signaling crosstalk between the cell types (17). We found that proliferating CD4 T-cell and CD4 Tregs abundantly expressed FGFR1 as a receptor for FGF7 from stromal cells (**Figure 7A**, left). Additionally, proliferating CD4 T-cells strongly expressed PDGFA as a cognate ligand of PDGFRA and PDGFRB, which were abundantly expressed on CAFs and pericytes (**Figure 7A**, right). For NKT and CD8 naive T-cells, there was abundant expression of AREG (amphiregulin), and CAF/EGR_{high} exclusively expressed EGFR as a cognate receptor of AREG (**Figure 7B**). Potential communications between T-cells and CAFs suggest that CD4 T-cell expansion is closely affected by CAF activity mediated by FGFR1. Moreover, both CD4 T-cells and CD8 T-cells contribute to CAF development through distinct signaling pathways.

Further, we focused on the canonical genes involved in FGFR signaling and its downstream pathways such as PI3K-AKT-mTOR, IKK, PDK1-PKN, NFkB and JAK-STAT signaling in different T-cell subtypes (**Figure 7C**). ATL-related genes were up-regulated in both CD4 T-cells dnT-cells, but receptor tyrosine kinases (RTKs) such as IGF1R, KDR (VEGFR), and FGFR1 were

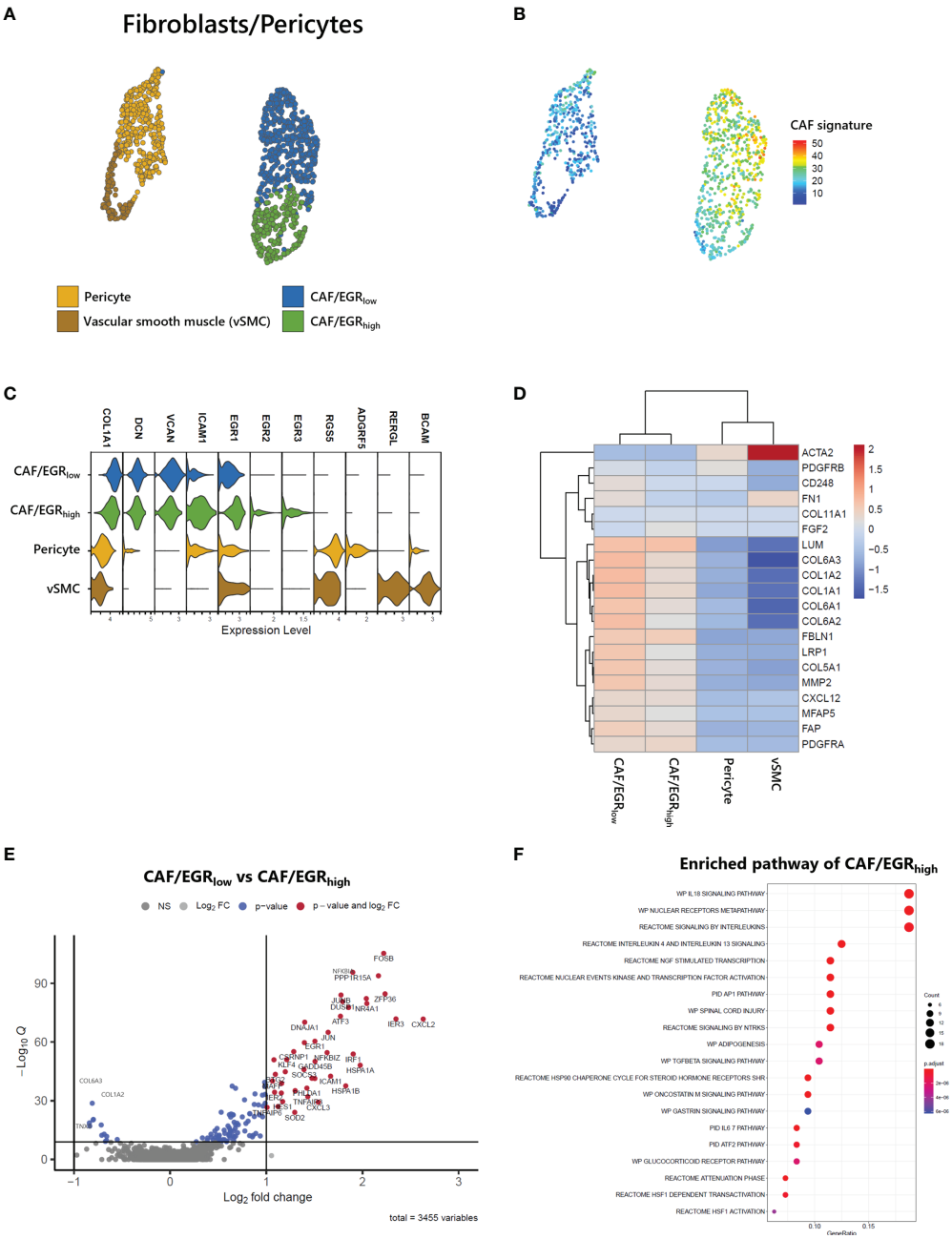

FIGURE 6 | Identification of CAFs in ATLL **(A)** Subtypes of stromal cells **(B)** CAF score calculated with the gene set suggested by Bagaev (17) within stromal cells **(C)** Cell-type markers of stromal cells **(D)** Scaled gene expression related to CAFs **(E)** Volcano plot showing DEGs of CAF subgroups **(F)** Enriched pathway of CAF/EGR_{high} subtype.

mainly expressed in CD4 T-cells. Also, RTKs demonstrated a potential network with up-regulated genes of clonally expanded T-cells in **Table 1** (**Supplementary Figure S4**). PI3K-AKT-mTOR signaling genes, which are frequently up-regulated in lymphomas including ATLL, were primarily up-regulated in CD4 Tem and CD4 proliferating T-cells. However, NFkB and JAK-STAT signaling genes were up-regulated in CD8 T-cells and NKT. Since ATLL is thought to originate primarily from CD4 T cells, we posit that IGF1R, KDR (VEGFR), and FGFR1 may serve as potential therapeutic targets for future treatments of ATLL.

DISCUSSION

In this study, we propose new therapeutic targets for this rare, aggressive malignancy using clinically feasible sample archiving,

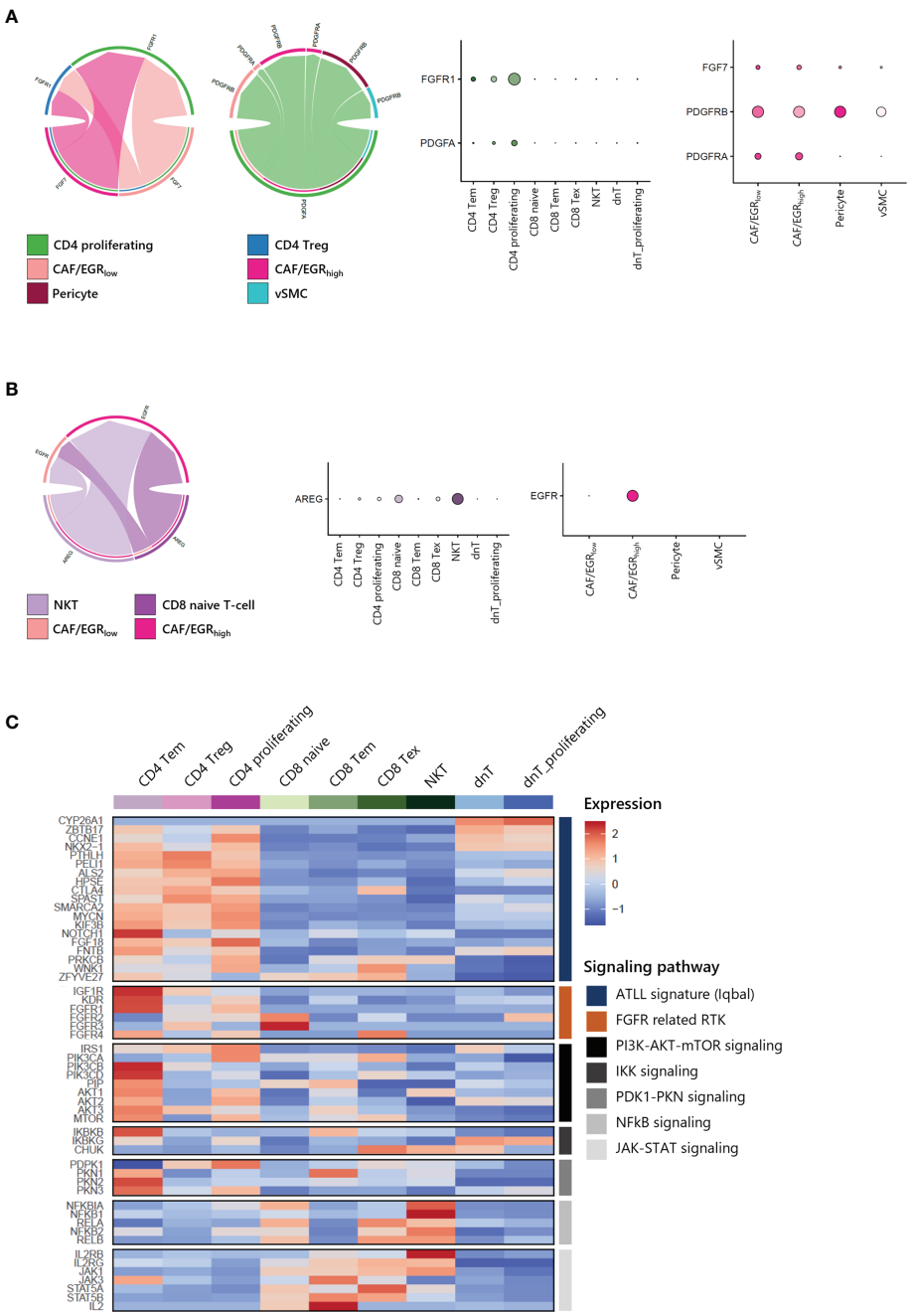

FIGURE 7 | Cell-cell interactions within ATLL **(A)** FGF and PDGF signaling between CD4 T-cells and CAFs **(B)** EGF and AREG signaling between CD8 T-cells and CAF/EGR$_{high}$ **(C)** Signaling pathways in T-cell subpopulations.

processing, profiling, and analysis pipelines. Indeed, majority of investigators using RNAseq consider fresh frozen tissue to not be suitable for single 'cell' gene expression profiling with TCR analysis given the technical difficulty of the experiments. However, we successfully profiled more than 10,000 cells from metabolically active skin tumors that were vulnerable to tissue processing with the 10X Genomics platform.

ATLL is typically characterized by proliferation of CD4+ and CD25+ T-cells since HTLV-1 mainly infects CD4+ T cells and induces proliferation of this cell subset (1). Clonal proliferation contributes to increasing the number of HTLV-1-infected cells and thus development of ATLL (26), and a recent study demonstrated a strong correlation between the clonality pattern and tumor progression (27). In the patient

studied herein, we observed the malignant clonal expansion of CD4+ cells.

The molecular features of ATLL are mostly induced by HTLV-1 infection (28). HTLV-1 induced Th2/Treg-related chemokine receptor CCR4 is frequently expressed in ATLL (2, 29). Moreover, CCR4 is known as a GATA3 target gene that is responsible for FOXP3 expression and controlling Treg function (30). In malignancy, CCR4-expressing Treg interacts with CCL17 and CCL22-secreting tumor cells, with resultant impairment of host antitumor immunity (31). Increase of CCR4 on the cell surface activates the PI3K/AKT signaling pathway to promote cell survival (26, 32). In this context, the anti-CCR4 monoclonal antibody mogamulizumab is already approved in Japan for ATLL treatment. However, there are concerns that mogamulizumab could induce adverse events, and that immunological statuses of patients may also affect treatment outcome (33). Therefore, alternative drug targets for ATLL are needed and are undergoing active investigation (**Supplementary Table 1**).

HTLV-1 Tax protein can infect and transform not only T-cells, but also various cell types including epithelial cells and fibroblasts (28, 34). In this regard, the microenvironment may contribute to survival and drug response of ATLL. Little is known about the role of stromal cells in ATLL, whereas in Hodgkin lymphoma (HL), it is suggested that the secretion of extracellular vesicles from HL changes the phenotype of fibroblasts to support tumor growth (35). In the case of T-cell lymphoma and leukemia, FGFR fusion genes are frequently found (36). During cancer progression, FGFR mediates crosstalk of CAFs with cancer cells and related target signaling pathways (37). Moreover, as a transmembrane growth factor, FGFR can activate the PI3K/AKT pathway that is closely related to ATLL (38).

In this study, we observed that the clonally expanded malignant tumor cells in ATLL are CD4 T-cells through scRNA-seq combined with TCR clonal analysis. We also identified the characteristics of CAFs within ATLL, including minimal expression of ACTA2 and PDGFRB, but high expression of FAP and PDGFRA. In particular, we identified a novel subgroup of CAFs characterized by high expression of EGR genes that may play an important role in the conditioning of the TME. We found that malignant T-cells and CAFs contribute to each other bidirectionally in ATLL, with CAFs promoting the clonal expansion of CD4 T-cells mediated by FGF7-FGFR1 signaling, and proliferating CD4 T-cells contributing to the growth of CAFs *via* PDGFA-PDGFRA/PDGFRB signaling.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE195674.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by IRB# SMC 2020-03-060. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EHJ and JHB contributed equally. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.856363/full#supplementary-material

Supplementary Figure 1 | Functional gene expression of T-cell subtypes.

Supplementary Figure 2 | Differentially expressed gene (DEG) analysis of T-cells of ATLL compared to healthy donors **(A)** Volcano plot represents ATLL-specific DEGs. Cut-off of p-value < 0.05 and Log2 fold-change value > 1. **(B)** Gene ontology terms enriched in ATLL are highly related to tumor metabolic process. **(C)** Diagram of DEG analysis steps for clonal-specific gene expression within T-cells of ATLL.

Supplementary Figure 3 | Characteristics of DC in ATLL **(A)** Correlation plot showing correlation coefficient between each myeloid cell type using variable feature genes (Spearman's rank correlation coefficient of TAM between DC = 0.51). **(B)** Expression level of inflammatory genes in DC.

Supplementary Figure 4 | Network of FGFR-related tyrosine kinase inhibitor (TKI) target genes between ATLL marker genes.

Supplementary Table 1 | Candidates of precision medicine.

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REVIEW
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Cancer Associated Fibroblasts - An Impediment to Effective Anti-Cancer T Cell Immunity

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The presence of functionally efficient cytotoxic T lymphocytes (CTL) in the Tumour nest is crucial in mediating a successful immune response to cancer. The detection and elimination of cancer cells by CTL can be impaired by cancer-mediated immune evasion. In recent years, it has become increasingly clear that not only neoplastic cells themselves, but also cells of the tumour microenvironment (TME) exert immunosuppressive functions and thereby play an integral part in the immune escape of cancer. The most abundant stromal cells of the TME, cancer associated fibroblasts (CAFs), promote tumour progression *via* multiple pathways and play a role in dampening the immune response to cancer. Recent research indicates that T cells react to CAF signalling and establish bidirectional crosstalk that plays a significant role in the tumour immune response. This review discusses the various mechanisms by which the CAF/T cell crosstalk may impede anti-cancer immunity.

Keywords: cancer-associated fibroblast (CAF), T cell exhaustion, targeting CAFs, mechanisms of immune evasion, tumour microenvironment

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INTRODUCTION

The tumour stroma plays a critical role in shaping the immune landscape in cancer. The most abundant stromal cells of the TME are cancer- associated fibroblasts (CAFs). Fibroblasts are typically activated during wound healing and revert to their quiescent state after exerting their function. However, in cancer, they remain perpetually activated by a number of factors including the presence of cancer cells, as indicated by their expression of activation markers [e.g., α smooth muscle actin (αSMA), fibroblast activation protein (FAP)] and promote tumour progression *via* multiple pathways. CAFs secrete angiogenic factors [e.g. vascular endothelial growth factor (VEGF)], factors degrading the basal membrane [matrix metallopeptidases (MMPs)], which promotes metastasis, and even alter their metabolic profile to produce energy metabolites (lactate, pyruvate) useful for cancer cells ("reverse Warburg effect") (1). Furthermore, a growing body of research shows that CAFs are implicated in cancer immunotherapy failure across cancer types, and inhibiting CAFs revives the antitumour immune response in preclinical studies (2, 3).

CAFs employ immunosuppressive functions that involve various immune cells and stages of antitumoral immunity. In this review, we aim to establish a framework to understand the part CAFs play in inhibiting an efficient T cell response. An efficient T cell response relies on a number of

orchestrated steps involving many cell types in the TME and results in cancer cell killing by cytotoxic T cells. In an ideal scenario, antigen presenting cells (APCs) present tumour antigen to naive T cells within tumour draining lymph nodes, inciting their activation, differentiation into cytotoxic T cells and travel to the tumour site. Here supported by a Th-1 mediated response of CD4+ T helper cells, primed activated cytotoxic T cells recognize their cognate antigen on the surface of cancer cells leading to clonal expansion of tumour specific effector T cells. This is followed by secretion of cytotoxic granules containing perforin and granzymes and killing of target cells in an antigen directed manner (4, 5). Successful T cell infiltration and the immune landscape of the TME are determining factors in the failure or success of the anti-cancer response.

HOW CAN CAFs BE CLASSIFIED?

Various cell types can give rise to CAFs if exposed to environmental triggers such as TGF-β, platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) (6). The absence of lineage markers such as cytokeratin and CD31 helps identify fibroblasts and while there is no specific marker to stringently differentiate CAFs from fibroblasts in adjacent non-cancerous tissue, CAFs typically show enhanced expression of surface markers αSMA, fibroblast specific protein (FSP), PDGF and FAP (6).

It is now widely recognised that CAFs display high heterogeneity (7, 8). Known activation markers of CAFs have been investigated by multiple groups and found to display difference expression levels within different CAFs, which have been characterised as subtypes (9–11). Common markers used to distinguish these subtypes include FAP, CD29, αSMA, podoplanin (PDPN) and platelet-derived growth factor receptor beta (PDGFRβ). The subtypes are defined by the expression levels of these markers, as no single specific marker for CAFs exists (12). Studies have defined subtypes of CAFs present in cancers such as breast cancer, ovarian cancer and pancreatic cancer. The markers used to define these CAF subsets are shown in **Table 1**.

The most notable subsets investigated are the CAF-S1 and CAF-S4 as they have been found to be associated with cancer cell invasion and poor prognosis in breast cancer (10, 13). CAF-S1 were found to promote cancer cell migration and epithelial-mesenchymal transition (EMT) by the CXCL12 and TGFβ pathways whereas CAF-S4 were found to promote cancer cell invasion, particularly in three-dimensional models through NOTCH signalling. These more aggressive subsets of CAFs show the utility of being able to identify the level of heterogeneity within patient CAFs to determine which treatment mechanisms will be effective and to predict prognosis.

Studies have shown that these different CAF subtypes also play different roles in immunosuppression, and these roles would be a consideration when designing new therapies (7, 14). Illustrating the magnitude of differences between CAF subtypes, Costa et al. show CAF S1 ($CD29^{Med}$ FAP^{Hi} $FSP1^{Low-Hi}$ αSMA^{Hi} $PDGFRb^{Med-Hi}$ $CAV1^{Low}$) promotes the activation and differentiation of $CD25^+$ T cells to $FoxP3^+$ Treg, whereas CAF S4 ($CD29^{Hi}$ FAP^{Neg} $FSP1^{Low-Med}$ αSMA^{Hi} $PDGFRb^{Low-Med}$ $CAV1^{Neg-Low}$) does not, even though both are αSMA^{high} and could therefore be classified as CAFs. Significant differences in their inhibitory capacity were also seen regarding cytokine production and migration of T cells (10). Therefore, merely identifying CAFs by their expression of e.g., αSMA, might include CAF subtypes with very different functions in the TME. In fact, a more recent study further divided Costa et al.'s CAF S1 subtype into 8 clusters based on single cell RNA sequencing of 19000 CAF S1 breast cancer fibroblasts. Two of those clusters were associated with immunomodulation, one of which also promoted $FoxP3^+$ T cell frequency within $CD4^+CD25^+$ T cells whereas others did not, further highlighting the heterogeneity of the population (15).

In some tumours, such as neuroblastoma, certain mesenchymal stromal cells also harbour immunomodulatory properties, and while these also express the potential fibroblast marker CD90 it is not known whether they align with a specific CAF subtype found in other types of cancer (16).

CAF heterogeneity poses a challenge in CAF research, as separate studies use different classifications for CAF subsets illustrating the need for a unified approach. Considering different CAF classifications in the current literature, we do not restrict this review to a specific subcategory of CAF.

HOW DO CAFs RESTRICT T CELL MIGRATION?

ECM and Stromal Density

The immune landscape of cancer is classified in three distinct immunophenotypes. Inflamed, "hot" (1), tumours are

TABLE 1 | Definitions of CAF subsets identified in breast, ovarian and pancreatic cancer showing the differences in classifications.

CAF Subset	Origin	Markers	Reference
CAF-S1	Breast cancer	FAP^{High}, $CD29^{Med-High}$, αSMA^{High}, $PDPN^{High}$, $PDGFR\beta^{High}$	Costa et al. (10)
CAF-S2		FAP^{Neg}, $CD29^{Low}$, $\alpha SMA^{Neg-Low}$, $PDPN^{Low}$, $PDGFR\beta^{Low}$	
CAF-S3		$FAP^{Neg-Low}$, $CD29^{Med}$, $\alpha SMA^{Neg-Low}$, $PDPN^{Low}$, $PDGFR\beta^{Low-Med}$	
CAF-S4		$FAP^{Low-Med}$, $CD29^{High}$, αSMA^{High}, $PDPN^{Low}$, $PDGFR\beta^{Med}$	
CAF-S1	Ovarian Cancer	$CD29^{Med-High}$, FAP^{High}, $\alpha SMA^{Med-High}$, $FSP1^{Med-High}$, $PDGFR\beta^{Med-High}$, $CAV1^{Low}$	Kanzaki and Pietras (8)
CAF-S2		$CD29^{Low}$, FAP^{Neg}, $\alpha SMA^{Neg-Low}$, $FSP\text{-}1^{Neg-Low}$, $PDGFR\beta^{Neg-Low}$, $CAV1^{Neg}$	
CAF-S3		$CD29^{Med}$, FAP^{Low}, αSMA^{Low}, $FSP1^{Med-High}$, $PDGFR\beta^{Med}$, $CAV1^{Neg-Low}$	
CAF-S4		$CD29^{High}$, FAP^{Low}, αSMA^{High}, $FSP1^{High}$, $PDGFR\beta^{Med-High}$, $CAV1^{Neg-Low}$	
myCAF	Pancreatic Cancer	αSMA^{High}, $IL\text{-}6^{Low}$	Öhlund et al. (11)
iCAF		αSMA^{Low}, $IL\text{-}6^{High}$	

characterised by T cell infiltration and associated with enhanced response to checkpoint inhibition (17). It is well understood that the infiltration of cytotoxic T cells into the tumour nest is a prerequisite for T cell mediated killing of cancer cells (18). As a result, "cold" tumours are associated with poor response to checkpoint inhibition and a failed immune response. Here, $CD8^+$ T cells are either absent ["deserted" (2)] or do not effectively infiltrate the tumour islands, as they are either restricted to the invasive margins or the stromal regions of the tumour, unable to be in physical contact with cancer cells ["excluded" (3)]. Factors determining the immunophenotype of a tumour include tumour mutational burden (TMB) and MHCI expression and recent research indicates a crucial role of CAFs (19).

Main mechanisms of CAF mediated effects on T cells are depicted in **Figure 1**. One mechanism of CAF mediated immunotherapy failure is the CAF-induced increase in ECM density. Firstly, this results in reduced drug penetration of the tumour tissue (1). Secondly, the tight matrix and increased interstitial fluid pressure in CAF rich stroma can also promote T cell exclusion from the tumour nest. Salmon et al. used live cell imaging to investigate the localisation and migration of T cells in tumours. Fluorescently dyed freshly isolated TILs were added on top of human lung tumour slices *in vitro*. Added TILs accumulated at 5x higher numbers in the tumour stroma compared to tumour islets and travelled along linear tracks parallel to stromal fibres. They further showed that T cell counts were negatively correlated with ECM density and that fibronectin rich regions, such as areas immediately surrounding tumour islets, inhibited T cell motility (38). Interestingly, Blair et al. report that pharmacologically degrading stromal hyaluronan resulted in an increase in effector memory $CD8^+$

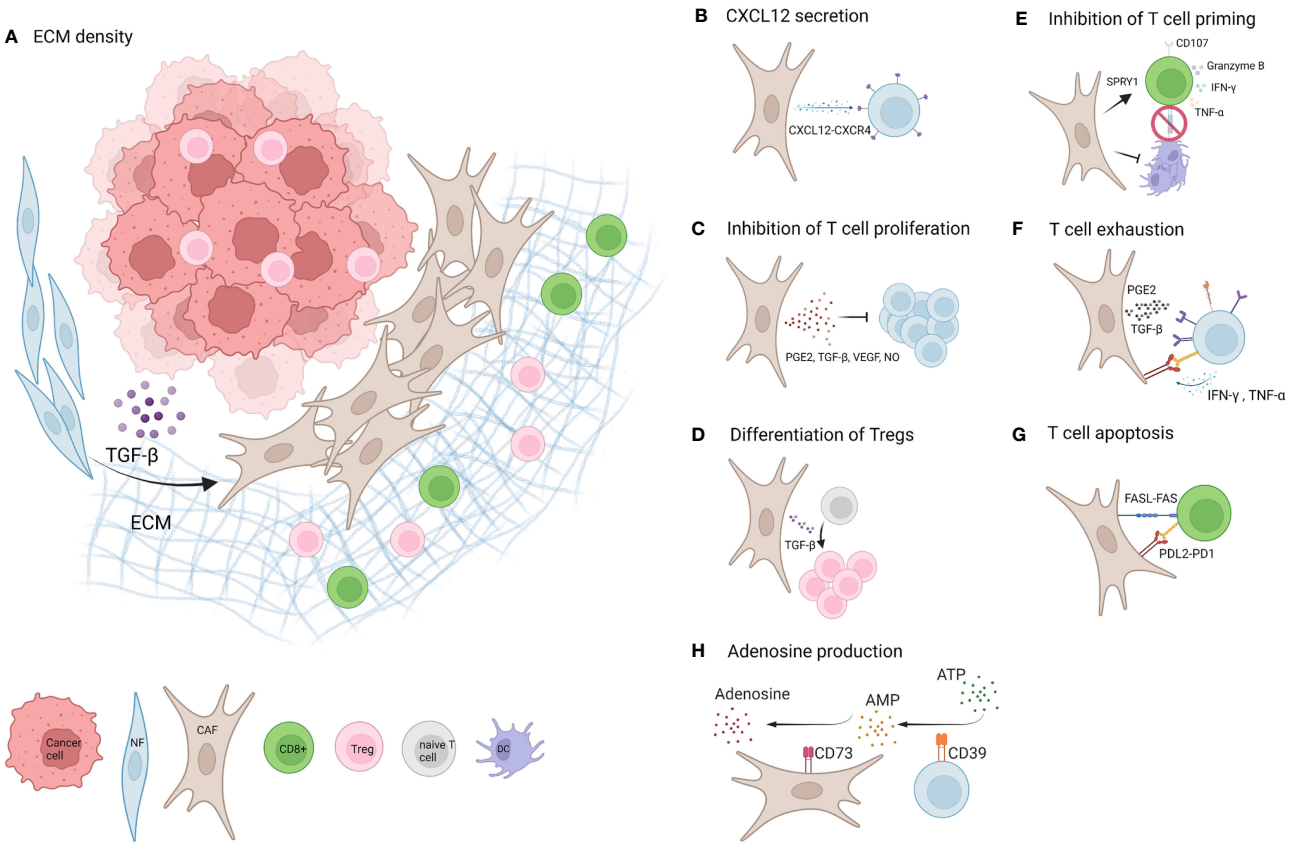

FIGURE 1 | Mechanisms of CAF mediated T cell inhibition. **(A)** Factors including TGF-β and ECM promote the development of CAFs in the TME of solid tumours. A dense network of CAF secreted ECM restricts T cell mobility and entry of $CD8^+$ T cells to the tumour nest (1). **(B)** CXCL12 secreted by CAFs binds to CXCR4 on T cells and likely contributes to T cell restriction to the tumour stroma (20–22). **(C)** CAFs limit T cell proliferation by factors including PGE2, TGF-β, VEGF and NO (23–27). **(D)** One study suggests CAF secreted TGF-β promotes differentiation of naive $CD4^+$ T cells into $FOXP3^+$Tregs (28). **(E)** CAFs inhibit dendritic cell (DC) differentiation and maturation and thereby limit T cell priming (7, 29). Additionally CAFs upregulate SPRY1 in T cells associated with reduced activation and downregulate CD107, and cause a reduction in secretion of Granzyme B and TNF-α. Opposing studies report an increase or decrease of IFN-γ secretion by T cells (7, 30–32). **(F)** CAFs upregulate exhaustion markers PD1, Lag3, TIGIT, Tim3and CD39 on T cells (*via* e.g. PGE2 and TGF-β) and T cells upregulate PD-L1 and PD-L2 expression on CAFs *via* IFN-γ and TNF-α (15, 22, 23, 33–36). **(G)** CAFs promote apoptosis of T cells *via* high levels of FASL and PDL2 expression and upregulation of FAS and PD1 on T cells (35). **(H)** CAFs upregulate CD39 on T cells and T cell secreted IFN-γ and TNF-α upregulate CD73 expression on CAFs, which could potentially increase the production of immunosuppressive Adenosine (37). Created with BioRender.com.

TIL and improved antitumour immunity in a murine model of pancreatic ductal adenocarcinoma (39).

Multiple studies have reported reduced CTL infiltration in CAF rich tumours compared to their CAF low counterparts (33, 40). For example, Kato et al. report $CD8^+$ T cells to be located in peritumoral rather than intratumoral tissue in CAF-rich oesophageal cancer, while in CAF-low tumours, $CD8^+$ T cells were found to be distributed across both sites (40).

CAFs expressing high levels of FAP and αSMA are prominently implicated in $CD8^+$ T cell exclusion. In line with Salmons study, Gorchs et al. describe dense stroma surrounding tumour nests in pancreatic cancer and report that these areas express αSMA (23). Gene analysis of CAFs and normal fibroblasts (NF) in ovarian cancer showed nine differentially expressed genes linked to $CD8^+$ T cell infiltration. The upstream regulator of three of the genes, preselin1 (PS1), co-localises with CAF activation markers FAP and αSMA and was associated with low TIL counts. PS1 silencing reduced the expression of FAP and αSMA on CAFs and reduced tumour burden in an ovarian tumour mouse model *via* increased CTL infiltration, indicating that PS1 has a significant role in CAF mediated T cell exclusion from the tumour nest, likely upstream *via* promoting CAF activation (41). Using a whole tumour cell vaccine genetically modified to express FAP in a murine model of melanoma and lung cancer, resulted in reduced tumour growth and prolonged survival that was dependent on enhanced $CD8^+$ TIL infiltration. This effect was significantly higher than that of unmodified whole tumour cell vaccine and was attributed to directly inhibiting CAFs, as it resulted in reduced FAP – and collagen type I expression in these tumours (2).

Ford et al. investigated how CAFs confer immune checkpoint inhibition (ICI) resistance using CAF rich murine tumour models. They previously reported that the downstream target of TGF-β1, NADPH oxidase 4 (NOX4), which generates reactive oxygen species (ROS), can regulate fibroblast differentiation into myofibroblasts (42). Using single cell RNA sequencing, NOX4 expression was correlated to CAF markers including FAP, Thy1, decorin, and collagen type I and VI (43). Targeting NOX4 in murine models of CAF rich tumours (either by silencing or pharmacologically inhibition) suppressed the TGF-β mediated differentiation into myofibroblasts and additionally downregulated functional markers including αSMA and collagen 1 of fully differentiated CAFs resulting in a more "quiescent" state and a rescued $CD8^+$ TIL response by redistribution of $CD8^+$ T cells into the tumour (33). These studies suggest that the activation of CAFs and the resulting increased synthesis of matrix components such as hyaluronan are implicated in CTL exclusion. Pharmacologically targeting CAFs to reduce ECM density could improve T cell trafficking to the tumour nest which could increase efficacy of checkpoint inhibition therapy as well as improving drug penetration of agents that directly target cancer cells.

Fibroblastic reticular cells (FRCs) employ chemotactic strategies to modulate T cell trafficking in the healthy lymph node by expression of lymphocyte attractants CCL19 and CCL21 which bind to CCR7 on naïve T cells (34). Similarly, in cancer, aside from the physical restrictions of dense ECM, it has come to light that CAFs secrete factors which directly influence T cell migration and function in the TME. The best characterised of these mechanisms is the CXCL12-CXCR4 chemokine axis which contributes to CTL exclusion. CXCL12, produced by FAP^+CAFs in the TME binds to its receptor CXCR4, expressed by T cells, thereby trapping TIL in the tumour stroma and restricting their access to tumour areas containing cancer cells (20). Feig et al. demonstrated the significance of this pathway in a pancreatic ductal adenocarcinoma mouse model by showing that inhibition of CXCR4 caused a redistribution of T cells within the tumour tissue, improved CTL activity and decelerated tumour growth (21). Notably first clinical data shows CXCR4 inhibition in human pancreatic ductal adenocarcinoma also increased $CD8^+$ T cell infiltration into the tumour (44).

Another factor attenuating CD8 T cell infiltration is Fibroblast growth factor-β (FGF2) a cytokine released by cancer cells that activates quiescent fibroblasts and upregulates CAF marker expression like αSMA and FAP (45). In a mouse model of pulmonary metastasis inhibition of FGF Receptor signalling resulted in a dose dependent increase in CD8 T cell infiltration and significantly delayed tumour growth. Mechanistically, gene expression data of urothelial carcinoma cells treated with a different FGFR inhibitor revealed FGFR inhibited T cell chemoattractant CXCL16 and CD8 T cell infiltration and effects were attributed to inhibiting FGFR on cancer cells. CAFs were not investigated in this study but it could be hypothesised that the delay in tumour growth and increase in $CD8^+$ T cell infiltration could be partly mediated by a negative regulation of CAFs *via* FGFR inhibition (46).

Production of TGF-β by CAFs also suppresses anti-tumour immunity. CAFs produce significantly more TGF-β than normal fibroblasts from non-cancerous tissue and TGF-β is particularly high in immunosuppressive $PDPN^{high}$ CAFs (47, 48). Similarly, single cell sequencing of breast cancer fibroblasts, identified a subtype of CAFs expressing high levels of TGF-β which correlates with resistance to immunotherapy (15).

Recently, Desbois et al. demonstrated, using a combined IHC and transcriptome analysis approach of a large ovarian cancer cohort, that a key hallmark defining T cell excluding tumours is the upregulation of TGF-β and activated stroma. Mechanistically, the majority of TGF-β elicited changes in the transcriptional programme consists of ECM related genes, glycoproteins, and reactive stroma markers, reinforcing the idea that the main mechanism of T cell exclusion is ultimately the establishment of a physical barrier by activated stroma (49). Similarly, TGF-β plays a significant role in urothelial cancer, where blocking TGF-β allowed T cell entry to the centre of the tumour, followed by tumour regression (50). Furthermore, Desbois et al. described that while immune deserted tumours had a slightly lower neoantigen load, they did not differ from infiltrated tumours in neoantigen load or TMB. Rather the main difference, was a downregulation of antigen presenting genes and low MHC-I expression mainly in the tumour compartment. While deserted tumours had overall low MHC-I and infiltrated tumours showed strong homogenous MHC-I on tumour cells, it

seems T cells were trapped in the MHC-I expressing stroma (49). While cancer cells downregulate MHC-I to avoid CTL killing, Phipps et al. have seen an upregulation of MHC-I on CAFs in response to IFN-γ and in line with this we have seen the same effect induced by activated T cells (37, 51). It could be postulated that this is an additional mechanism of retaining passing T cells in FAP^+ CAF rich stroma on their way to the tumour nest. Furthermore, gene expression analysis of TGF-β activated fibroblasts shows upregulation of genes encoding immunomodulatory cytokines IL11, TNF-AIP6 and IL-6 (49). CAFs produce significantly more IL-6 than normal fibroblasts and thereby promote epithelial to mesenchymal transition in NSCLC (52). Interactions of CAFs with cancer cells additionally results in a dramatic increase in IL-6 production *in vitro* (53). Notably, FRC derived IL-6 is known to be able to affect the fate of T cells during T cell priming in the lymph node suggesting this as a possible mode of T cell modulation by CAFs in cancer (34). Interestingly, high IL-6 secretion is significantly associated with a particularly immunosuppressive FAP^+ CAF phenotype (CAF S1) in breast cancer and a dominant feature of an inflammatory CAF phenotype in pancreatic ductal adenocarcinoma (10, 11). Single cell RNA sequencing of this inflammatory CAF subtype showed upregulation of other inflammatory pathways including IFN-γ, TNF and NFKβ. Notably hyaluronan synthases as well as matrix proteins were specific to this subset, suggesting this subset is active in producing dense extracellular matrix (54). Ohno et al. report that IL-6 deficient mice showed significantly decreased tumour growth of colon cancer compared to wildtype mice. This observation co-occurred with increased numbers of IFN-γ producing T cells, increased PDL1 and MHC1 expression on cancer cells and was dependent on $CD8^+$ T cells (55). Using a colon cancer mouse model, Kato et al. showed that co-transferring fibroblasts together with cancer cells resulted in slightly increased tumour growth in immunodeficient nude mice, and that this effect was more pronounced in immunocompetent mice, suggesting CAFs support tumour growth *via* modulating the immune response. Notably, they established that $CD8^+$T cell exclusion caused by CAFs was dependent on IL-6, as tumours regularly injected with IL-6 mirrored the effects of CAFs and IL-6 blockade caused a significant shift in the TIL population from $FoxP3^+$ to $CD8^+$ T cells (40). Additionally, blocking IL-6 together with PD1-PDL1 blockade caused a significantly improved T cell response (56). Similarly, co-administering TGF-β antibody and anti-PDL1 in a mouse model of urothelial cancer caused a reduction in TGF-β signalling in stroma and allowed T cell infiltration into the tumour centre, suggesting that these two cytokines secreted by CAFs might offer targets to relieve the immunosuppressive effects of CAFs on immune cells and promote efficacy of ICI (50).

Multiple studies have shown that CAF high tumours have low CD8 T cell infiltration, while Treg infiltration is actually increased in these tumours indicating that active stroma may affect cytotoxic T cells and Treg differently. A recent *in vitro* study modelling matrix stiffness in a 3D culture system, offers some insight into possible underlying mechanisms, and reports that the viability of $CD4^+$ T cells exceeded that of $CD8^+$ T cells in ECM with high rigidity, which could indicate that $CD8^+$ T cells are more sensitive to mechanical pressure (57). Further studies are needed to understand the specific mechanisms by which CAFs selectively exclude CTL and promote Treg.

WHAT HAPPENS TO T CELLS IN THE STROMA?

CAFs Inhibit T Cell Proliferation

As T cells are sequestered in the tumour stroma, their phenotype and function are directly affected by the influence of surrounding CAFs. Multiple *in vitro* studies show that the presence of CAFs significantly reduces the proliferation of both $CD4^+$ and $CD8^+$ T cells in a contact-independent manner, suggesting this as a possible explanation for reduced TIL frequencies in CAF high tumours (23–27). In fact, using a pancreatic cancer cell line, Gorchs et al. demonstrate that CAFs have higher inhibitory potential on T cell proliferation than cancer cells do (23). In terms of the underlying mechanisms, multiple factors have been reported to mediate this inhibition, such as CAF-derived prostaglandin E2 (23). and AKT3, a protein kinase with a role in immunosuppressive activity of CAFs (58). Having established that PS1 is an upstream regulator of genes differentially expressed in CAFs compared to normal fibroblasts and a factor promoting CAF expression of the activation markers FAP and αSMA, Zhang et al. report that silencing PS1 reversed the anti-proliferative effects of CAFs on T cells (41). Takahashi et al. investigated PD-L1 and PD-L2, co-inhibitory ligands expressed by a subset of CAFs which inhibit T cell activation and - function *via* PD-1 binding. The authors report that blocking PD-L1 and PD-L2 normalised T cell proliferation and additionally, a similar effect was shown by neutralising CAF-secreted VEGF and TGF-β, possibly due to a loss of the CAF stimulating effect of these signals (24). Conversely, Gorchs et al. see no significant changes in proliferation following TGF-β blocking (23). It is important to consider that study designs varied in terms of cancer type and model and that CAFs comprise a particularly heterogenous population of cells and without further discrimination of their specific phenotype, results are likely to vary. Cremasco et al. looked into nitric oxide (NO) production by CAFs as a mechanism of inhibiting T cell proliferation in cancer, as this has been found to be a mechanism employed by FRCs after sensing T cell secreted IFN-γ and TNF-α to limit their proliferation in the healthy lymph node. Underlining the functional heterogeneity of CAFs, they report a similar mechanism in breast cancer where $PDPN^+$ CAFs significantly inhibited the proliferation of $CD4^+$ and $CD8^+$ T cells in co-culture *via* production of NO while $PDPN^-$ CAFs do not (34, 59). More drastically, CAFs can limit the cytotoxic T cell pool by inducing apoptosis in CD8 T cells *via* FAS ligand and PD-L2 engagement (35).

DO CAFs TRULY DRIVE REGULATORY T CELL DIFFERENTIATION?

While restricting $CD8^+$ T cell infiltration, recent research indicates that specific CAF subtypes can selectively attract and

retain $CD4^+$ $CD25^+$ T cells (15). Tumours high in FAP^+CAFs are positively associated with an increase in $FoxP3^+$ Treg infiltration (40, 60–62). Notably, Givel et al. report that mesenchymal high-grade serous ovarian cancer which is associated with poor survival showed high stromal density of fibroblasts and an enrichment for the CAF-S1 subtype, which was associated with a significant increase in $FOXP3^+$ cells compared to tumours enriched with CAF-S4 fibroblasts. This was attributed to high expression of the CXCL12β isoform of this subtype, as *in vitro* functional experiments showed an increased migration of $CD4^+CD25^+$ T cells, but not $CD4^+CD25^-$ T cells in the presence of CAF-S1 that was dependent on CXCL12β. In addition to the enhanced attraction of $CD4^+CD25^+$ T cells, co-culture of CAF-S1 fibroblasts and $CD4^+CD25^+$ T cells demonstrated an increase in $CD25^+FOXP3^+$ T cells and enhanced their survival in a contact dependent manner (9) Similarly, in breast cancer, the above mentioned immunosuppressive CAF S1 subtype correlates with an increase in $FOXP3^+$ regulatory T cells. *In vitro* co-culture of CAF S1 and T cells revealed a shift towards $FOXP3^+$ Tregs that was dependent on immune checkpoint molecules B7H3, CD73 as well as DPP4, a membrane bound enzyme closely related to FAP that is known to cleave the effector T cell chemoattractant CXCL10 (10). Furthermore, multiple studies report an increased frequency of $FOXP3^+$ cells after co-culture of PBMCs from healthy donors with CAFs compared to normal fibroblasts. This shift has previously been attributed to CAFs driving Treg differentiation, could however also result from differences in proliferation amongst $FOXP3^+$ cells and other T cells or increased survival of existent Tregs rather than an induction of FOXP3 amongst naïve T cells (23, 24). Notably however, Kinoshita et al. exposed purified naïve conventional $CD4^+$ T cells ($CD4^+$25-$CD45RA^+$) from healthy donor PBMCs to supernatant from CAFs from Treg-high lung tumours and did see a significant increase in $FOXP3^+$ cells compared to supernatant from CAFs from Treg-low tumours that was mirrored by treatment with TGF-β suggesting true induction of Tregs *via* CAF secreted TGF-β (28). Furthermore, a murine fibroblast cell line transfected with FAP induced the differentiation of primary cultured murine splenocytes to $CD4^+CD25^+$T cells, however the frequency of $FOXP3^+$ amongst this pool is not reported and mediating factors are unknown (63).

Notably, when exposing stimulated T cells to CAFs, differentiation into effector- ($CD45RA^-CCR7^-$) and central ($CD45RA^-CCR7^+$) – memory T cells amongst proliferating T cells is significantly reduced. Instead a larger pool of T cells remains in their naïve state ($CD45RA^+CCR7^+$) (23). In addition to possibly promoting regulatory T cells, TGF-β could also negatively affect cytotoxic T cell differentiation, as a recent study on oral squamous cell carcinoma illustrates the significance of TGF-β in attenuating the cell cycle of CTL, inhibiting their proliferation during effector phase as well as their differentiation into TEM, promoting apoptosis induction, and ultimately causing a decrease in the $CD8^+$ T cell/Treg ratio (64). In line with this, inhibiting TGF-β in a mouse model of pancreatic cancer in combination with gemcitabine caused an increase in naïve Treg markers (CD62L, CCR7) and downregulation of markers associated with an effector/memory Treg phenotype. Additionally, inhibiting TGF-β caused a reduction of Treg-mediated suppression of $CD8^+$ T cells. This suggests that TGF-β could promote the development of effector memory Tregs with suppressive activity against CTL, further highlighting the role of TGF-β in indirectly impairing CTL function in cancer (30). To conclude, CAFs could potentially mediate induction of Tregs and suppress memory T cells, however, current evidence is not conclusive.

CAFs Attenuate T Cell Activation and Prevent Effective T Cell Priming

In the lymph node, FRCs upregulate immunostimulatory factors (ICOS ligand, CD40 and IL-6) in response to signals from activated T cells which enhances IL-2 and TNF-α production by activated CD8 T cells. Conversely, in cancer, some studies indicate that CAFs reduce CTL activation. The presence of melanoma CAFs during $CD8^+$T cell activation reduces the percentage of cells exhibiting the early T cell activation marker CD69. Additionally, in PBMCs co-culture, CAFs promote an increase in cytokines typical for Tregs, such as IL-10 and TGF-β in line with their positive influence on Treg differentiation, and thereby add to the consequent anti-inflammatory environment. In fact, when pre-exposed to the CAF subtype CAF-S1, $CD25^{High}CD127^{low}CD45RA^{low}$ T cells increased the ability to inhibit the proliferation of effector T cells. CAFs thereby equip Tregs with increased suppressive activity against effector T cells (10).

It has been shown that CAFs also affect other immune cells in the TME and mediate T cell suppression indirectly [reviewed (7)]. For example, DCs conditioned with supernatant of CAFs, induce a TH2 cytokine response from $CD4^+$ T cells during co-culture (29). Additionally, CAFs have recently been shown to suppress DC differentiation, maturation and enhance $CD11c^+$ inhibitory phenotypes ultimately inhibiting $CD8^+$ T cell priming, likely *via* the WNT catenin signalling pathway (31) Furthermore, CAFs interact with tumour-associated macrophages (TAMs) in a reciprocal fashion, promoting the development of an M2 TAM phenotype with pro-tumour functions such as expression of PD1-ligands which ultimately impairs cytotoxic T cell function [reviewed (65, 66)]. Consequently, CAFs might reduce CTL activation directly as well as indirectly, either *via* modulation of the environment or through the modulation of their interaction with other immune cells.

A recent study in oesophageal cancer shines some light on how CAFs facilitate inhibition of T cell activation. In response to FGFR signalling by αSMA^+ fibroblasts, T cells upregulate the FGF2 antagonist SPRY1. SPRY1 reduces NfKB, NFAT, Ras MAPK signalling and limits T cell activation. FGF2 causes a significant reduction of IFN-γ, TNF-α and granzyme B production by *in vitro* stimulated CD8 T cells and decreased their efficiency in killing target cancer cells (67). Similarly, a subset of $FGF2^+$ CAFs that secrete WNT2 are correlated with a high ratio of $Foxp3^+CD4^+$ T cells/$CD4^+$ T cells and show reduced ratio of IFN-γ producing $CD8^+$ T cells (31). Additionally, using a mouse model of systemic infection, Shehata et al. have shown

that T cells negative for SPRY1 have enhanced survival (32). Furthermore, compared to NF, CAFs express higher levels of FASL, and additionally upregulate FAS expression on TIL, leading to the suppression of CTL activity (35). Notably, research suggests that CAFs also mediate suppression of CTL cytotoxicity *via* decreasing the expression of CD107a as well as granzyme B in T cells (22, 23, 68). Similarly, low expression of overall CXCR4 in tissue slides of pancreatic cancer was significantly associated with increased granzyme A and perforin, marking cytotoxic capacity of CTL. This highlights potentially increased T cell cytotoxicity when they are not attracted and retained to the stroma *via* the CXCR4-CXCL12 axis (39). In line with this, Zhang et al. report that silencing PS1, which as indicated above reduces FAP and αSMA expression on CAFs, causes an increase in the activity of CTL, here shown in an increased IFN-γ release (41). Other studies also report reduced levels of TH1 cytokines in the presence of CAFs (60). Using a 3D scaffold *in vitro* model of breast cancer, Phan-Lai et al. observed CAF-mediated suppression of TNF-α release by tumour reactive T cells (69). Again, reduced CXCR4 gene expression was also correlated with improved IL-17a cytokine production by $CD8^+$TIL, likely due to increased CTL infiltration, and increase IFN-γ levels amongst isolated $CD8^+$TIL (39). A recent study by Li et al. showed TGF-β inhibition caused a temporary shift from myCAF, which were located tightly around the tumour islands to iCAF which were loosely connected interspersed. Inhibiting TGF-β in combination with gemcitabine, caused an increase in IFN-γ production by $CD8^+$ T cells as well as increased T cell activation markers 4-1BB (CD137) and OX40 and markers of cytotoxicity (granzyme and perforin) (30).

However, while Nazareth et al. report a suppressive effect of CAFs on T cell activation in 3/8 NSCLC tumours, in the other five tumours, CAFs surprisingly produced IFN-γ, and induced the activation of T cells, which increased their response to TCR stimulation, a phenomenon that could be partially reversed by TGF-β (47). Interestingly, a study by Barnas et al. revealed that fibroblasts from lung tumours, non-cancerous lung tissue or even skin fibroblasts increased the secretion of IFN-γ and IL-17 by lung cancer TILs (70). This effect was only observed in the presence of activation stimuli and was in part mediated by a common CAF cytokine, IL-6, which was in turn increased by T cell conditioned media (70). The authors therefore attribute CAFs an immunostimulatory role in the TME. Similarly, we saw increased T cell production of IFN-γ in co-culture with CAFs. IFN-γ from T cells in turn, together with TNF-α, upregulates MHC I and - II on CAFs and in line with Barnas et al., increased CAF-production of IL-6 (37). While IFN-γ does activate cytotoxic T cells, it also negatively regulates TILs by upregulating PD1 ligand expression on CAFs and promoting production of IDO, (71). and therefore might not be purely immunostimulatory in this context. Additionally, T cells induce IL-27 secretion by CAFs, which again promotes PD1 ligand expression as well as inducing Tim3 expression and IL10 production by T cells (72). At the same time, IL-27 signalling supports granzyme B expression and proliferation of cytotoxic T cells, illustrating that CAFs may not rigidly act as either immunosuppressive- or stimulatory, but rather adjust their immunomodulatory activity depending on the extent of the T cell response (73).

T Cells Find CAFs Tiring

As a final barrier to T cells that have overcome these hurdles, CAFs dampen the remaining CTL response to cancer cells by promoting the development of functionally exhausted CTL through the upregulation of co-IR expression. As a physiological regulatory mechanism to limit T cell cytotoxicity, T cell exhaustion is characterised by a progressive loss of effector function, expression of multiple co-IR and a common transcriptional and epigenetic program (36). Given that CAFs co-localise with PD-1^+TIL in pancreatic cancer (23), researchers have investigated the effect of CAFs on the expression of other co-IR on TIL. Indeed, CAFs upregulate expression of Tim3, CTLA4, Lag-3 on both $CD4^+$ and $CD8^+$ TIL (23, 33, 35). Moreover, melanoma derived CAFs elicit TIGIT and BTLA expression in $CD8^+$T lymphocytes, mediated *via* l-arginase (22). Deletion of stromal cells *via* targeting FAP using a vaccine significantly lowered PD-1 levels in preclinical models of melanoma (74). The underlying mechanisms are not fully understood; however, first reports show that Tim-3 and PD-1 upregulation was enabled by CAF derived PGE2 (23). Remarkably, in response to the T cell cytokine IFN-γ, CAFs react with an upregulation of PD-L1 and -2 expression (37, 47).

In a recent study investigating the modulation of phenotype and function of TIL by CAFs in NSCLC, in addition to upregulated PD-1 and TIM3, our group also observed an upregulated expression of CD39 on T cells when co-cultured with CAFs, that was mediated *via* TGFβ (37). Another co-IR, CTLA-4, is involved in adhesion and migration of T cells. In their murine models, Ford et al. saw an increase in CTLA-4 expression amongst $CD8^+$TIL in CAF rich tumours using RNA sequencing and flow cytometry. Immunohistochemistry of human HNSCC tumours confirmed CTLA4 expression of on average 15.3% of excluded $CD8^+$T cells. Blocking CTLA-4 in their murine lung tumour model increased $CD8^+$T cell infiltration and reduced tumour growth (33). Taken together, these studies demonstrate that CAFs promote the progression of an exhausted phenotype of cytotoxic T cells.

DO CAF- T CELL INTERACTIONS AFFECT ADENOSINE LEVELS?

An area of interest in CAF mediated immunosuppression, with a promising therapeutic target, is their role in the production of adenosine. Adenosine signals *via* P1 receptors on immune cells and exerts immunosuppressive functions. It is produced by hydrolysis of pro-inflammatory extracellular ATP (eATP), *via* the cell-surface enzyme CD39, into adenosine monophosphate (AMP), which is further converted into adenosine by CD73 (75). Expression of CD39 and P1 receptors is typically upregulated in response to tissue damage, tissue remodelling, hypoxia/oxidative stress and chronic inflammation as a means to protect the

surrounding tissue from immune-mediated tissue damage. In recent years, it has become evident that CD39 is also expressed in the often chronic inflammatory, hypoxic environment of tumours. In cancer, adenosine has detrimental effects, as it promotes tumour growth and progression *via* suppressing the immune response. It enhances the immunosuppressive effects of tumour associated macrophages (TAM), myeloid derived suppressor cells (MDSC) and regulatory T cells while dampening immunostimulatory effects of neutrophils, NK cells and inhibits T cell priming by inhibiting the activation of dendritic cells (DCs) (75). Furthermore, reduction of available eATP and intracellular accumulation of cAMP in CTL significantly impairs their effector function.

We have recently shown that CAFs upregulate expression of CD39 on T cells and in turn, T cells upregulate CD73 expression on CAFs (37). They could thereby establish a feedforward loop to sustain adenosine in the TME, that is sensitive to the extent of T cell infiltration sustaining a local immunosuppressive environment. CD39 can also be expressed by CAFs, and upregulated, much like other CAF immunosuppressive factors (TGF-β, ARG, IDO, CXCL12, PGE2, PD-L1), during hypoxic stress (76). CAFs could therefore theoretically produce adenosine from ATP on their own, however, it has previously been shown that mesenchymal stromal cells are able to produce significantly more adenosine in the presence of activated $CD39^+$ T cells than each cell type alone and additionally upregulate CD73 expression on $CD4^+$ T cells (77). A recent study reported CD73 expression in the TME is mainly attributed to CAFs, as 75-90% of CD73 immunofluorescence staining of human colorectal cancer tissues was found on αSMA^+cells (78). Interestingly, in the previously mentioned study by Costa et al., CD73 expression was particularly high in the highly immunosuppressive CAF S1 subset (10), which further implies that CD73 activity contributes to CAF mediated immunosuppression in the TME. Notably, Yu et al. demonstrate that the levels of adenosine in the TME regulates CD73 expression on CAFs *via* A2B receptors on CAFs in a feedforward loop amplifying immunosuppression in the presence of adenosine (78). CD73 expression levels are also affected by the cytokine milieu of the TME. A previous study has shown that IL-6 can upregulate CD73 on nasopharyngeal cancer cells (79), and Hu et al. reported similar observations for the effect of IL-6 on CD73 expression on γδ T cells (80). Importantly, they report that $CD73^+$γδ T cells can in turn promote IL-6 production by CAFS. In line with this observation, Barnas et al. saw a synergistic increase in IL-6 production when activated T cells were co-cultured with CAFs derived from human NSCLC tumours (70). Furthermore, TGF-β, which is expressed more in CAFs compared to NF (24). has been shown to sustain CD73 expression on T cells (81). and promote IL-6 production by pericytes in NSCLC (82). IL-6 and IL27 are key factors in upregulating CD39 expression on TILs (83–85). IL-27 is prevalent in the TME and notably, our group has shown that the presence of TIL elicit IL-27 secretion by CAFs (37). And while we saw an upregulation of CD39 on T cells in the presence of CAFs, we found this was dependent on TGF-β. These studies suggest that in response to tumour infiltrating T cells, CAFs might promote the upregulation of factors needed for adenosine production in a complex interplay of IL-6, IL27, TGF-β and CD39 and CD73.

DO CAFs DIRECTLY INTERACT WITH TIL?

As discussed above, CAF inhibitory effects can be mediated *via* soluble factors (23, 24, 26, 70). However, since TIL and CAFs colocalize in the tumour stroma, researchers have been curious as to whether they engage in direct cell-cell interactions. Indeed, CAFs have been shown to have the ability to uptake, process and present antigen *via* MHCII, thereby further increasing their potential to interact with TIL (54). Furthermore, using the antigen ovalbumin, Lakins et al. show CAFs were similarly efficient in processing antigen as FRCs, however unlike FRCs and normal fibroblasts, CAFs displayed delayed endosome mediated processing, like APCs, resulting in enhanced cross-presentation to T cells (35). Notably, CD74, involved in the formation of MHC II, is highly expressed on iCAF, a subcluster of CAF-S1, which is unlike the other subclusters ecm-myCAF and TGF-β-myCAF not associated with immune modulation (15). It remains to be seen whether the ability to present antigen *via* MHCII is a general CAF trait or if it is reserved for immunosuppressive CAFs. CAF-S1 additionally increase their contact with TIL by expressing the PD-1 co-IR ligands PD-L1 (47). and PD-L2 (10, 35, 47), the adhesion molecule JAM2, and OX40L (10), which have been confirmed to co-localise with $CD4^+CD25^+$ TIL in breast cancer (10). In fact, the presence of TIL increases the expression of MHC I and MHC II on CAFs, as well as their expression of PDL1 and PDL2, possibly in an effort to increase their interaction with TIL (37). Additionally, CAFs interact with other immune cell types which ultimately also affects TILs. Rodriguez recently uncovered a role of CAFs in the establishment of tertiary lymphoid structures (TLS), lymphoid formations in the tumour microenvironment that share similarities with secondary lymphoid organs and are correlated to enhanced survival and response to immunotherapies. *Via* secretion of CXCL13, CAFs drive expansion of tumour associated TLS by attracting B cells with the cognate receptor CXCR5. It is furthermore important to consider the stage of tumour progression as the immune landscape is highly dynamic and recent research shows three distinct functionally diverse stromal populations at different timepoints over the course of tumour progression. These three clusters, identified by single cell sequencing of $CD31^-$ stromal cells of murine melanoma tumours sampled at different time points, differed in combined expression of mesenchymal markers and pathways indicating their function including cytokine, chemokines, complement and genes regulating ECM. All three populations were present in all timepoints, however dynamic differences were observed regarding the dominant stromal cluster. Cluster S1, described as "immune" stromal cells, were found early in tumour progression and showed high CXCL12 levels suggesting this might be a driver of early CAF T cell interaction during tumour progression. Similarly, S1 had high CD34 expression

with recruitment of macrophages *via* direct crosstalk of C3 and C3aR and low expression of αSMA, whereas the αSMA^{high} Cluster S3 dominated later stage tumours and consisted of "contractile" stromal cells with high expression of genes regulating actin (86).

Targeting CAFs

Various strategies have been employed to target characteristics of immunosuppressive CAFs such as high FAP expression. (**Figure 2**). CAFs offer a good CAR T cell target as they have powerful multifaceted protumour effects and are more genetically stable than cancer cells. Multiple studies report FAP specific CAR-T cells cause an inhibition of tumour growth in multiple mouse models that was dependent on the immune response (87). and mediated mainly *via* the CD8$^+$ T cell response (88) (**Figure 2A**). However, an earlier study by Roberts et al demonstrated that systemic FAP ablation in mice can also have severe adverse effects as it is not only expressed in tumour environments but rather in most tissues of the mouse including skeletal muscle and adipose tissue. Ablation of FAP expressing cells resulted in cachexia and a reduction of erythropoiesis, suggesting that FAP$^+$ cells in healthy tissues contribute to essential physiological functions (89). Using a nanoparticle-based photoimmunotherapy method, Zhen et al. conjugated a FAP specific antibody to the nanoparticle ferritin that using photoirradiation allowed local, direct and selective elimination of FAP$^+$ CAFs leading to tumour suppression in mice. While this treatment had minimal direct effect on cancer

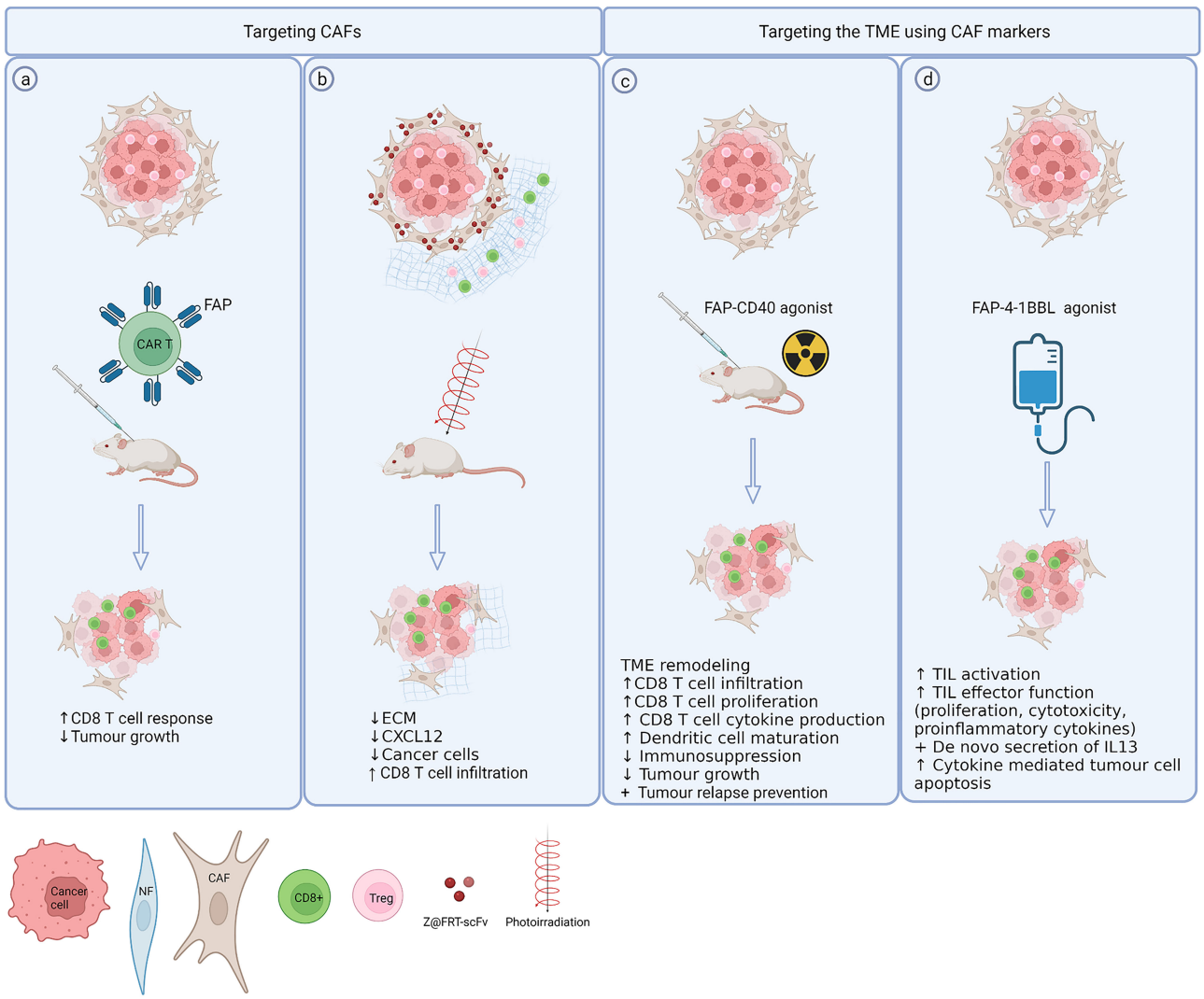

FIGURE 2 | Exploiting CAF markers to selectively target CAFs and the TME. **(A)** Directly targeting CAFs using FAP specific CAR T cells results in reduced tumour growth depending on increased CD8 T cell responses (81, 82). **(B)** A FAP specific single chain variable fragment conjugated to ferritin (Z@FRT-scFv) allows nanoparticle based photoimmunotherapy of CAFs resulting in tumour suppression *via* ECM, CXCL12 reduction and CD8 T cell infiltration (84). **(C, D)** Expression of FAP can be exploited to selectively deliver co-stimulatory agents [CD40 (87), 4-1BBL (88)] to the TME thereby avoiding systemic toxicity. Created with BioRender.com.

cells, it led to a reduction of serum levels of IL-6 and EGF, known CAF secreted factors and reduced tumour growth was mediated by a reduction of CXCL12 and destruction of ECM (90) (**Figure 2B**).

It is unknown whether targeting CAFs affects their beneficial functions such as TLS formation. Again, CAF heterogeneity must be considered, as TLS formation is driven by FAP^- CAFs suggesting that targeting FAP may not interfere with this beneficial function of CAFs (91). It is currently unknown whether depleting FAP affects TLS formation in tumours but a murine model of autoimmune disease demonstrated that genetic depletion of FAP abolishes TLS formation (92).

In addition to inhibiting FAP, the fact that FAP is restricted to the tumour tissue has been exploited to specifically deliver therapies to the TME. As such, FAP has been used as a co-target to deliver the T cell co-stimulatory 4-1BB ligand selectively to the tumour thereby circumventing systemic side effects of 4-1BB ligand such as cytokine release syndrome (93) (**Figure 2D**). Similarly a ligand for a different co-stimulatory receptor CD40 was linked to a bispecific FAP antibody to ensure activation of CD40 was only induced around FAP-expressing cells in an experimental model of murine head and neck cancer that synergised with radiotherapy causing tumour regression and long term survival (94) (**Figure 2C**).

The above evidence suggests that while targeting FAP systemically in mice can have severe adverse effects preclinical studies show that local inhibition of FAP in the TME of tumour bearing mice can be a potent therapeutic tool to allow redistribution of CD8 T cells into the tumour. This illustrates the need for further investigation into underlying mechanisms and suggests that FAP might offer an attractive target to locally modulate immune responses in cancer.

CONCLUSION

In conclusion, it has become evident that CAFs significantly impede effective cytotoxic T cell immunity across cancer types. Current knowledge paints the picture that the TME establishes an environment, that promotes the development of hyperactive fibroblasts, CAFs, that perpetually secrete ECM thereby producing a dense web of collagen with high interstitial pressure to protect cancer cells from infiltrating T cells. T cells are trapped in the stroma, physically and *via* chemokines (e.g. CXCL12), CTLA4 and MHC expression, where they are in bidirectional crosstalk with CAFs. This results in reduced proliferation, activation, and differentiation of cytotoxic T cells in an environment that instead nurtures immunosuppressive cells. The reduced number of cytotoxic T cells that do remain are incapacitated by upregulation of co-IR, while their presence causes CAFs to upregulate the corresponding ligands, likely to limit their residual function further.

CAFs affect a multitude of changes in T cell biology, and it seems T cells in turn elicit changes in CAF secretome and surface marker expression. These feedback mechanisms highlight the complex bidirectional crosstalk between CAFs and TILs, illustrating the need for further investigation. However, variable definitions of CAFs and their subtypes remain a challenge in the field as they cause a lack of comparability between studies. Increased understanding of CAF subtypes and whether they are found across cancer types is key to disentangle CAF – TIL interactions. The significant role of CAFs in all steps of the tumour immunity cycle as well as the fact that CAF inhibition resulting in delayed tumour growth was entirely dependent on CTL, underscore that CAFs might present a target to relieve environmental pressures on cytotoxic T cells to increase the efficacy of therapies aimed to revive the cytotoxic T cell response.

AUTHOR CONTRIBUTIONS

LK and LM drafted the initial version of the review. All authors edited and revised the review and approved the final manuscript.

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Interplay between Solid Tumors and Tumor Microenvironment

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Over the past few decades, basic studies aimed at curing patients with cancer have been constantly evolving. A myriad of mechanistic studies on physiological changes and related factors in tumor growth and metastasis have been reported. Recently, several studies have been considerate to how tumors adapt to unfavorable environments, such as glucose deprivation, oxidative stress, hypoxic conditions, and immune responses. Tumors attempt to adapt to unfavorable environments with genetic or non-genetic changes, the alteration of metabolic signals, or the reconfiguration of their environment through migration to other organs. One of the distinct features in solid tumors is heterogeneity because their environments vary due to the characteristics of colony growth. For this reason, researchers are paying attention to the communication between growing tumors and neighboring environments, including stromal cells, immune cells, fibroblasts, and secreted molecules, such as proteins and RNAs. During cancer survival and progression, tumor cells undergo phenotype and molecular changes collectively referred to as cellular plasticity, which result from microenvironment signals, genetics and epigenetic alterations thereby contributing to tumor heterogeneity and therapy response. In this review, we herein discuss the adaptation process of tumors to adverse environments *via* communication with neighboring cells for overcoming unfavorable growth conditions. Understanding the physiology of these tumors and their communication with the tumor environment can help to develop promising tumor treatment strategies.

Keywords: tumor microenvironment, stromal cell, metastasis, tumor heterogeneity, extracellular matrix

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INTRODUCTION

Understanding of the physiology of solid tumors has changed significantly over the past 30 years. Cancer research has typically focused on the growth and inhibition of primary tumors, but recently more research has focused on the growth and malignancy of tumors through their genetic and non-genetic modification (1, 2). Primary tumors are exposed to various stressful environments, such as oxidative stress, hypoxia, and acidosis, with rapid growth, thereby accelerating their heterogeneity (3, 4). This not only changes the metabolism or genetic modification of the tumor, but also changes

the neighboring tumor microenvironment (TME). Conversely, stimulation of the TME promotes changes in tumor development and aggressiveness (**Figure 1**). For this reason, it is necessary to understand communication between tumors and the TME, which includes blood vessels, immune cells, fibroblasts, stromal cells, the extracellular matrix (ECM), and secreted molecules that exist around primary tumors. In this mini-review, we briefly summarize how the interplay between tumors and the TME impacts tumor cell physiology and adaptation for overcoming unfavorable environments.

EXTRACELLULAR MATRIX

The ECM is a complex ecosystem of various components, such as fibrous proteins (collagen and elastin) or glycoproteins (fibronectin 1, laminins, and tenascin), proteoglycans (chondroitin sulfate and heparan sulfate), and polysaccharides, which includes several growth factors and creates rigid interactions with cancer cells in the TME (5). In the TME, the ECM functions as a framework for the tumor cells and plays an active role in tumor progression, particularly as a vital mediator of invasive processes (6). The ECM performs a tumor-suppressing role in healthy tissues, but it performs a tumor-promoting role in solid tumors. However, numerous effective components in tumor-stimulating roles in the ECM are produced in the TME (7, 8), and they affect cancer cells during interconnection with integrins (9). According to Glasner et al. (10), INF-γ released from intratumoral natural killer (NK) cells alter primary tumor structure by induction of fibronectin 1 in the tumors resulting in restriction of metastases formation. Regulate cancer metastasis formation through stimulating the tumor structure by regulating fibronectin 1 secretion, which is a key component of the ECM. ECM proteins can be formed by numerous stromal cell types and tumor cells, while cancer-associated fibroblasts (CAFs) are a major source for synthesis,

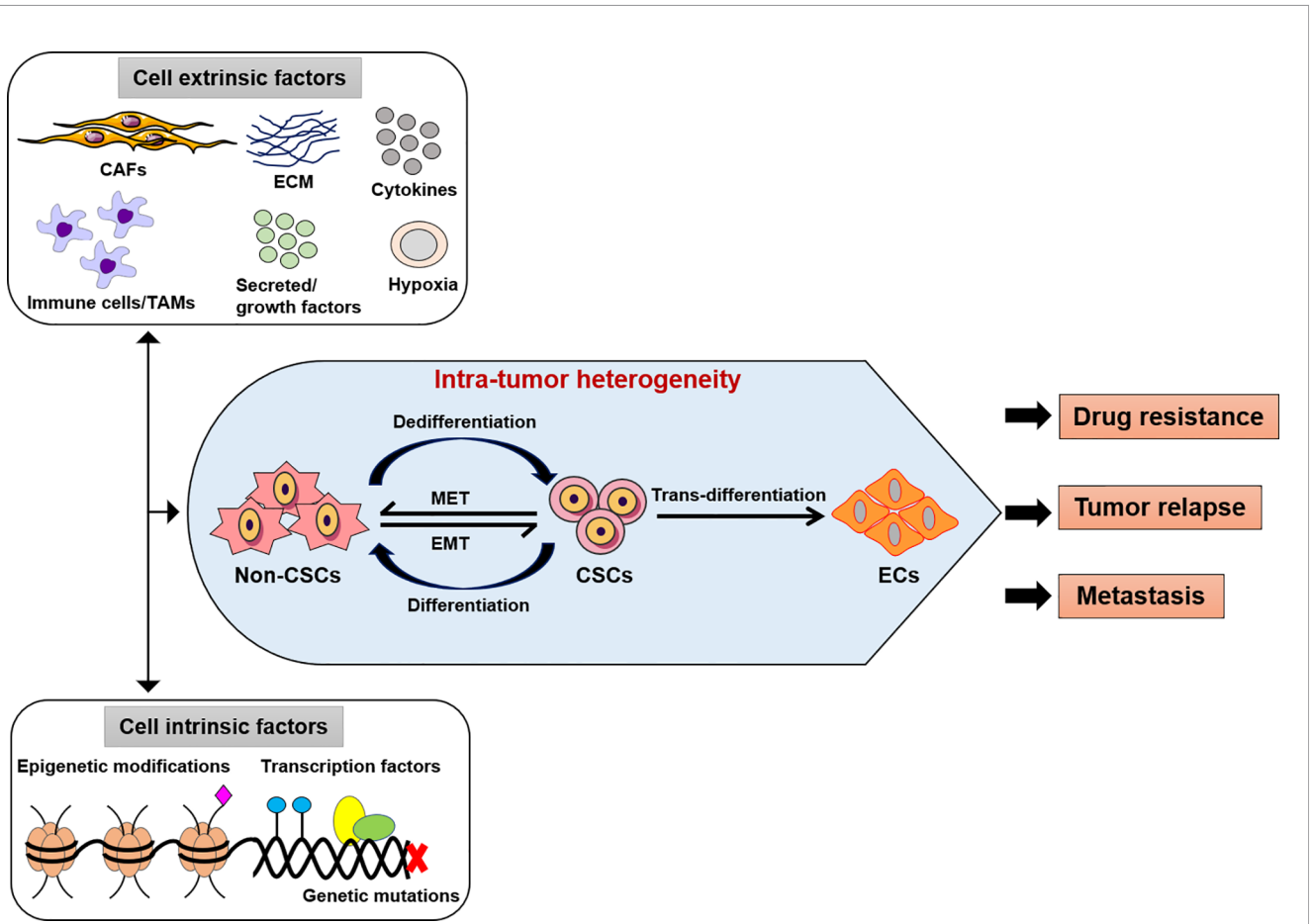

FIGURE 1 | Intra-tumor heterogeneity by extrinsic or intrinsic factors. The modifications of cancer cells such as genetic, epigenetic alterations, and microenvironment perturbations. CSCs show an induced EMT system, which mostly exhibit an intermediate condition. This activity depends on both genetic mutations, epigenetic alterations, and transcriptional modification of cancer cells and signals provided by TME (CAFs or TAMs, immune cells, ECM, cytokines, secreted or growth factors). Thus, the intra-tumor heterogeneity might be play a potential role in the development of effective therapeutic approaches as drug resistance, tumor relapse and metastasis. CSCs, cancer stem-like cells; EMT, epithelial-to-mesenchymal transition; MET, mesenchymal-to-epithelial transition; ECs, endothelial-like cells, CAFs, cancer-associated fibroblasts; TAMs, tumor-associated macrophages; ECM, extracellular matrix.

assembly, secretion, and alteration of ECM development (11, 12). Besides the intermolecular covalent cross-linkages of ECM, the biophysical characteristics include its rigidity, topography, molecular density, and tension. Thus, the ECM is extremely versatile causing it to experience cellular remodeling under the effect of tumors or tumor stromal cells (13, 14). As the dynamic crosstalk is facilitated by chemokines and growth factors, metastatic circulating tumor cells are secured to and released from the ECM in addition to metabolic changes of the tumor cells. During enlarged tissue rigidity and desmoplasia, the ECM might act as a barrier for drug delivery or gate for opening the basement membrane to promote metastasis (15, 16). Moreover, the ECM of remote tissues or organs could be somewhat formed into permissive soils by circulating tumor cells, soluble factors, or exosomes from primary tumors to mediate the sowing of metastasizing tumor cells (17).

STROMAL CELLS

Stromal cells are connective tissue cells, which are one of the key components of cancer progression and regression involved in the TME. They are engaged by tumor cells, and then involve metastasis initiation through the regulation of tumor cells and themselves (18). Glucose deprivation, reactive oxygen species (ROS), hypoxia, and inflammatory signals create unfavorable environments, leading to epithelial-mesenchymal transition (EMT), tumorigenesis, and tumor metastasis (19, 20). These signals are generally accepted that tumor cells alter their microenvironments through the regulation of stromal cells (18). Stromal cells include mesenchymal stem cells (MSCs), fibroblasts, macrophages, endothelial cells (ECs), lymphocytes, and pericytes in tumors, which contribute toward tumor progression and regression (6). The characteristics of cancer are replicative ability, continued angiogenesis, invasion, and metastasis, which are regulated by the interactions within genetically altered cancer and stromal cells. A previous study showed that stromal cells also undergo metabolic changes in the TME, reforming TME metabolism, and translating nutrients into forms that can be absorbed by tumor cells (21).

In the stromal environment, CAFs are the foremost stromal factor of various solid tumors and are also the best-known phenotypic transformers (22). CAFs are a vastly heterogeneous stromal cell population that participates in drug resistance, proliferation, and metastasis in tumor cells *via* the secretion of cytokines and matrix metalloproteinases (MMPs) (18, 22, 23). They promote angiogenesis, ECM remodeling, wound healing, and cancer progression through the regulation of immune systems in immune cells (24). Several key markers are used to identify CAFs, such as fibroblast-specific protein 1, α-smooth muscle actin (α-SMA), platelet-derived growth factor (PDGF) receptor α, and fibroblast activation protein α (FAP-α). Although they include a heterogeneous cell population, the degree of diversity has hardly been studied (25). Therefore, fibroblasts are separated into quiescent fibroblasts and myofibroblasts/CAFs on the basis of distinct expression. In particular, quiescent fibroblasts are less carcinogenic and mostly found in non-malignant tissues, and myofibroblasts or CAFs encourage tumors and trigger tumor relapse along with tumor resistance and are intensely enhanced in metastatic or malignant tumors. Both fibroblast types secrete an exceptional range of elastins and collagens that maintain the ECM, resulting in desmoplasia (26, 27). However, quiescent fibroblasts secrete low levels of collagens (particularly *Col13a1* and *Col14a1*) and high levels of elastins. In addition, myofibroblasts/CAFs are completely derived from tumor tissues and primarily enhanced in collagens and low levels of elastins. CAFs promote angiogenesis, tumorigenesis, and metastasis by secreting pro-inflammatory cytokines and growth factors and enhancing TME remodeling *via* the secretion of ECM components, MMPs, and other molecules (22, 25).

For immune action, CAFs inhibit the activity of recruited lymphocytes and cytotoxic T lymphocytes that form the inflammatory signals to advance tumor progression, and CAFs can rebuild into a pro-metastatic TME from the post-metastatic TME (27). In the context of the TME, the subtypes of CAFs have shown distinct mechanisms of activation, i.e., the stimulation of transforming growth factor (TGF)-β1 or IL-11 and the treatment of IL-1β or IL-6 that activate the upregulation of inflammatory CAF-associated marker genes (28). Furthermore, the differentiation of CAF-related specific markers can result in α-SMA, also called ACTA2, FAP, S100A4, desmin, collagen, and circulating pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, TGF-β, and CXCL12 (29). CAFs can directly secrete vascular endothelial growth factor (VEGF) in addition to the other growth factors that regulate angiogenesis by suppressing the angiogenesis-blocking role of TSP1 (22). CAFs are additionally typified based on different cellular sources, such as vascular CAFs that originate from perivascular areas, cycling CAFs, matrix CAFs, and developmental CAFs, which are the product of native fibroblasts found in the TME of the genetically engineered MMTV-PyMT breast cancer mouse model (30). According to Brown et al. (31), In a human PDAC model, CAFs are also derived to be immunomodulatory presenting MHCII genes that regulate antigen-specific ligation with $CD4^+$ T helper cells by expressing CD74 (32). Despite this, CAFs deviate in metastatic tumors from early-stage tumors, including high metabolic synthesis and released transcriptional profiling. Correspondingly, CAFs release ECM factors that facilitate collagen crosslinking and regulate the survival signals of tumor cells, which immunomodulate the TME avoidance tumor surveillance (22).

MSCs are derived from the umbilical cord, bone marrow, adipose tissue, etc., and form a fibro-vascular network in fibroblasts and vascular pericytes *via* the formation of tumor barrier differentiation. Emerging evidence has strongly suggested that MSCs can be activated by exosomes and participate in the communication of the transfer of proteins in the tumor cells as well as in the stromal cells (24).

Tumor-associated macrophages (TAMs) are the key cells in several types of solid tumors, which can promote tumor progression by generating pro-inflammatory mediators, include cytokines or chemokines, growth factors that alter the tumor-supportive TME and encourage tumor cell proliferation,

multidrug resistance and plasticity (33, 34). For instance, NF-κB-mediated factors (TNF-α, IL-1β, IL-6, CCL2, CXCL8 and CXCL10) can protect against apoptosis, and pro-angiogenic growth factors (such as VEGF or PDGF, TGF-β and FGF) that adapt tissue architecture and support tumor cell migration, invasion and metastasis (34, 35). In addition, TAMs destabilize local immune surveillance as they can directly decrease T cell and natural killer cell (NK) activities by releasing soluble factors or by expressing cell surface proteins that exhibit the immunosuppressive functions [e.g. arginase 1 (ARG1), indoleamine 2,3-dioxygenase (IDO), programmed death ligand 1 (PD-L1) and TGF-β] or they can indirectly suppress the activities of T cell through the engagement of other immune suppressive cells i.e. regulatory T cells (36, 37). In general, TAMs are a major component of TME that play mutually a significant role as tumor promoters and immune suppressors because they could promote tumor initiation, and act as the fundamental drivers of the immunosuppressive TME, which control the recruitment and function of multiple immune cells.

Adipocytes are the most abundant cells to compose adipose tissue and they play key roles in energy storage and homeostasis in the body. Cancer-associated adipocytes are key players in cancer progression and migration (38). They highly express matrix remodeling- and EMT-related factors, produce free fatty acids (FFAs) through lipolysis and insulin-like growth factor binding protein 2 (IGFBP-2), and participate in the development of the TME and metastasis (38, 39) (**Figure 2**).

IMMUNE CELLS

In the TME, all immune cells aim to protect the whole body but can ultimately turn into a tumor-supporting cell population (40). Immune cells are remarkably complex and include several

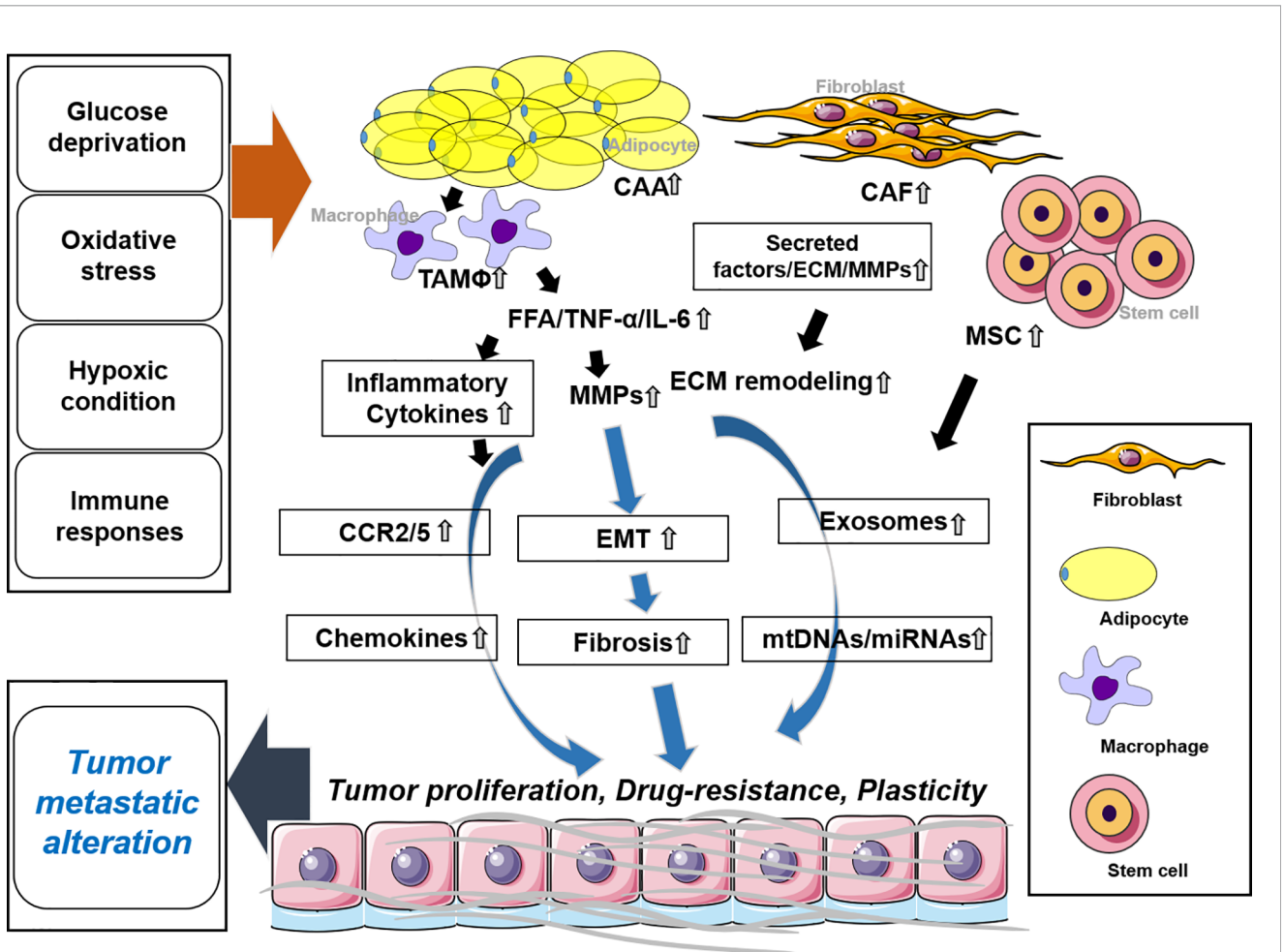

FIGURE 2 | Stromal cells and the tumor microenvironment. CAA regulates EMT by secreting tumor necrosis factor (TNF)-α, IL-6, and FFA along with MMPs. Inflammatory cytokines are secreted by TAM and trigger chemokines. In CAF release, secreted factors and MMPs promote ECM remodeling. MSCs secrete exosomes along with mtDNAs and microRNAs (miRNAs). These molecules synergistically or individually promote tumor proliferation, drug resistance, and plasticity and affect tumor metastatic alteration. CAA, cancer-associated adipocytes; CAF, cancer-associated fibroblast; MSC, mesenchymal stem cell; TAM, tumor-associated macrophage.

different lineages that make them tough to study and target. Depending upon the stage of cancer, both lymphoid and myeloid lineage cells play roles in pro- or anti-tumoral activity. For example, macrophages encourage the activation of T cells to clear tumor cells at early stages but inhibit T cells from even identifying the tumor cells as a tumor growth (41). However, immune cells lead each other to control the mechanisms related to tissue homeostasis and change the survival rate (42). Cellular secretions of molecules from immune cells also influence the activity within the TME. The secretion of cellular molecules, such as CCL5 and XCL1, from NK cells targets antigen-presenting dendritic cells (DCs). Moreover, IFNγ secretion stimulates macrophage polarization and Th1 cell hyperactivation that eventually activates the immune microenvironment against cancer cells (28, 43). In response, cancer cells secrete molecules, i.e., pro-inflammatory cytokines, such as IL-8 and CXCL-1, 2, and 8, that target neutrophils. Despite this, neutrophils generate neutrophil extracellular traps, which protect the cancer cells from NK and cytotoxic $CD8^+$ T cells and reduce the influence of immunotherapies (44, 45). Understanding the indispensable role of each immune cell should facilitate the control of immunosuppressive responses and improvement of immunostimulatory functions in secondary tumor proliferation. Considering heterogeneity of immune cells, scRNA-Seq is an advanced technique, which is able to examine the immune cells that show distinct phenotypes *in vivo* models (46).

Monocytes and macrophages are the major phenotypic markers of the aggressive TME (26). In humans, monocytes are subdivided into three largest clusters; namely, classical ($CD14^{++}$, $CD16^-$), intermediate ($CD14^+$ $CD16^+$), and non-classical ($CD14^+$ $CD16^{++}$). In tumor cells, recent studies have reported new monocytic markers, such as CD68, CSF1-R, CSF2-R, CD11C, CD1C, CD141, and HLA-DR surface markers (47, 48). TIE2 is a subset of monocytes expressing the angiopoietin receptor that play an important role in tumor angiogenesis, and its expression is highly increased in response to hypoxia (49). Monocytes are absorbed to the TME by chemo-attractants (CCL2 or CCL4), which further differentiate into TAMs. Macrophages are conservatively divided into two main clusters: classical macrophages (CD14+ $S100A8/9^+$ M1-like) with antitumor functions and alternate macrophages [$CD16^+$(FCGR3A) M2-like] with pro-tumorigenic phenotypes (50). M2 macrophages exhibit the phenotypes of aggressive tumor growth, immune evasion, angiogenesis, and cancer stemness. Furthermore, they assist tumor initiation and the mutagenic microenvironment by releasing circulating pro-inflammatory cytokines (IL6, TNF-α, and IFN-γ), growth factors (VEGF and EGF), ROS, and proteases (51).

The T cell population is usually organized by the cell surface markers ($CD3^+CD4^+CD8^+CD25^+$). The complication of tumor-infiltrating T cells indicates a powerful impact of tumors on the T cell transcriptome (52). Conventionally, T cells are categorized into naive, effector, and memory T cells. In lung TME study, single-cell sequencing separated the clusters of T cells into regulatory ($FOXP3^+$), $CD4^+$ ($CD4^+$), $CD8^+$ ($CD8^+$, naive, effector, memory, or exhausted), NK ($FGFBP2^+$), and lesser γδ T cells (26). Naive T cells can be separated into effector T cells following infiltration and further stimulated into cytotoxic memory T cells (53). Mostly, primary tumors are augmented with subtypes of effector T cells that are differentiated by the high expression of chemokine receptors or cytotoxic gene markers (CD28, CD40L, CD137, ICOS, and OX40) and exhibit decreased T cell expression. The expression of co-inhibitory receptors (PD-1, CTLA-4, CD160, LAG3, TIM-3, and TIGIT) leads to progressive T cell dysfunctions with tumor progression from primary to metastatic sites (54). The cells expressing co-inhibitory receptors are immunosuppressive and originate from several sources induced by the TME, including by migration from circulatory systems, effector T cell translation, and separation caused by the inhibition of Antigen Presenting Cells (55).

B cells are adaptive immune cells that infiltrate the TME through CXCL13 secretions from tumor cells (56). In solid tumor tissues, B cells are comparatively plentiful compared to non-tumor tissues (51), and what's more a relatively rare number of B cells compared to T cells in the TME (28). B cells can be separated into five groups, i.e., plasma B cells expressing IgG (MZB1 and CD138); follicular B cells expressing CD20, CXCR4, and HLA-DRs; mucosa-associated lymphoid tissue-derived plasma B cells expressing IgA ($CD38^+$); germinal center B cells; and granzyme B-secreting B cells (26). Although migrating through the germinal center, follicular B cells individually contain mature or naive B cells ($CD27^-$, CD72, and IGHM) that result in memory B cells ($CD27^+$ and IGHG1) (51). Compared to B cells in the non-tumorigenic environment, B cells residing in the TME are characterized by less protein secretion and the reduction of mTOR or Myc pathways (26). B cells encourage antitumor immunity by motivating complement activation, stimulating cytotoxic immune reactions, antibody-dependent cellular cytotoxicity, phagocytosis, and T cell activation, and releasing granzyme B or TRAIL factors (57). In addition, B cells have immunosuppressive subsets of pro-tumorigenic regulatory B cells ($CD1d^+CD5^+CD19^+$ and $CD5^+CD19^+$) and $CD5^+$ B cells (58) that modulate the production of immunomodulatory cytokines (IL10 and TGFβ), which may enhance metastatic ability by transition of CD4+ T cells into T-reg cells (59).

With the help of a unique set of receptors, NK cells belong to an innate lymphoid cell group and have a cytotoxic or cytokine-producing ability and can recognize tumor cells. However, NK cells are different from the immune cell population as they have diverse cell surface markers ($CD3^-CD16^+$ or $CD3^-CD56^+$). Thus, NK cells are mainly subdivided into distinct subsets depending upon the expression of CD16 and CD56 markers with their different phenotypic properties (60). Tumor-specific NK cells in lung carcinoma reveal the upregulation of CD69 and NKp44 markers and downregulated NKp30, NKp80, DNAM-1, CD16, and ILT2 expression against the peripheral blood and NK cells of normal lung (61). Likewise, DCs have many specific subtypes (DC1, DC2, and CD3) present in the TME that play a significant role in adaptive immune responses, antigen

presentation, and phagocytosis. For the other immune cells, the distinguishing markers of DC subsets are HLA DR^+ $lineage^-$ cells, including $CD11C^+$ conventional DCs, which are also differentiated into either $CD141^+$ or $CD1C^+$ cells, and $CD123^+$ plasmacytoid DCs (47). DCs are classified based on their presence in lymph nodes or tumor cells. The clusters of tumor cDC1 express Cd103 as a dermal DC marker, while the lymph node population expresses the CD8a marker specific to dendritic populations of the lymph nodes (29).

ENDOTHELIAL CELLS

Depending on the metabolic needs or requirements of growing tumors, ECs are in a coefficient mode of activation or reactivation and quiescence. The phenotypes of ECs are mostly subdivided into tip and stalk cells that show the different genotypes. Consistent with the tumor requirements, these individual cells adopt distinct phenotypes and functions (62). From the rest of the tumor cells, the first parameter to differentiate ECs is a division through $CD45^-$, as a pan-hematopoietic marker that combines with CD31, CD144 (VE-Cadherin), and vWF (von Willebrand Factor). However, CD31 is a transmembrane glycoprotein that develops intercellular intersections. Similarly, CD144 is an endothelial adhesion molecule and vWF is a glycoprotein that mediates platelet adhesion in the endothelium (63). These are the preliminary markers to disconnect the EC population. In contrast, other EC markers in different cancer types comprise tip genes (CLDN5, DLL4, EDNRB, ESM1, KCNE3, NID2, and RAMP3), capillary markers (CA4 and CD36), arterial markers (FBLN5 and GJA5), and ACKR1 gene expression by high endothelial venules, non-myeloid specific marker AIF1, lymphatic markers (PROX1 and PDPN), and pericyte marker RGS5 (64). According to Lambrechts et al. (26), tumor ECs in distinct clusters based on the marker genes are lymphatic ECs ($PDPN^+$ and $PROX1^+$), tumor-derived blood ECs ($FLT1^+$, $IGFBP3^+$, and $SPRY1^+$), and malignant or non-malignant ECs. In the TME, the dysregulation of epigenetic and transcriptional factors triggers the production of these angiogenic candidates and their subtypes from healthy blood ECs. The subtypes of tumor ECs directly damage the vascular integrity and structure of leaking blood vessels and migration of immune cells, thereby contributing to the growing tumor's complexity (65).

A previous study investigated the development of the heterogeneity of ECs by determining functionally validated endothelial phenotypes through patients with cancer as well as *in vivo* and *in vitro* models. Compared to aggressive tumors, non-malignant lung tissues have a relatively high profusion of alveolar type II, postcapillary, scavenging capillary, and lymphatic ECs. Even though the phenotypes of the tumor ECs were primarily immature ECs or human-specific lymphatic tumor ECs and tip cells, in tumor or non-tumor tissues, alveolar type II, activated postcapillary vein, and arterial phenotypes are common (66). Goveia et al. also classified the top-ranked marker genes and their specified significant roles in tumor progression as well as in regulating immune surveillance, matrix remodeling, EC migration, and angiogenesis by modulating growth factors and chemical stimuli that activate the angiogenic cascade within the TME, involving fibroblast growth factor (FGF), VEGF, PDGF, TGF-β, TNF, insulin-like growth factor, and MMP (67). In tumor ECs, blood vessels discharge the interconnecting tight junctions, which are complex with high interstitial pressure and are irregularly shaped. However, tumor ECs produce pro-angiogenic growth factors (FGF, VEGF, and PDGF) that exhibit chromosomal abnormalities, which function against cancer therapies (68). In addition, tumor EC-originating cadherin 2 activates VEGF-associated angiogenesis by controlling MAPK/ERK and MAPK/JNK signaling pathways (69).

SECRETED MOLECULES

Secreted molecules are major factors in the TME function and ECM remodeling, such as cytokines, proteases, integrins, and miRNAs (70). Cytokines are types of proteins that mediate the interaction between cells in the TME, including TNF, interleukins, chemokines, and growth factors, and regulate tumor progression and stromal cells. Moreover, the roles of cytokines in inflammation, apoptosis, tumorigenesis, proliferation, and migration depend on the maintenance of their anti-targets (71). In the TME, extracellular proteolysis acts as a key role that facilitates the proteolysis of the ECM and MMPs among other proteinases, having the nearest connection with tumor progression (72, 73). According to Kessenbrock et al. (73), the degradation of the ECM is mediated by MMPs that promote tumor invasion and metastasis. Additionally, MMPs stimulate tumor growth and angiogenesis as well as regulate apoptosis, whereas the functions of certain MMPs include tumor suppression. Therefore, MMPs are also a set of proteins with inconsistent roles in the TME (74).

Among secreted factors, integrins are essential membrane proteins and cell surface receptors that have an important role in the signaling and transfer of cellular information among cells or between cells and the ECM. In addition, integrins are central to the control of cell-matrix adhesions and play a critical role in the adhesion of circulating tumor cells to original sites leading formation of secondary tumors in TME. However, irregular cell-cell adhesions are a sign of tumors being triggered by disturbed integrins. The expression of metastasis-assisting integrins in the TME is induced, whereas those suppressing proliferation, migration, and survival are inhibited (75). Therefore, integrin expression is usually dysregulated in many solid tumors and play key roles in signaling as well as promotion of tumor cell invasion and migration. Recently, it has emerged that integrins are expressed not only in cells but also in exosomes, which are fundamental units of extracellular vesicles secreted from cells. Numerous studies are concerned with exosome originating integrins as the exploration on exosomes are increasing, in addition integrins are notified to influence the interior actions of tumors, as nucleus alteration. Most of research

efforts have focused on supporting incorporation of exosomes by target cells and facilitating exosome-mediated transfer of the membrane proteins and associated kinases to target cells in premetastatic niches. Moreover, integrins have demonstrated the ability to encouraging stem cell-like properties in tumor cells as well as drug resistance (76, 77). miRNAs are endogenous and small non-coding RNAs that negatively regulate specific target mRNAs or post-transcriptionally activate by disordering transcription or translation (78). miRNAs are involved in various pathways and functions in the regulation of distinct constituents of the TME (79). In addition to miRNAs, long non-coding RNAs (lncRNAs) are also effective components that are secreted in the TME. Among lncRNAs, some serve in the interaction between the TME and stromal cells as the transforming fibroblasts that are tumor-promoting (80).

CONCLUSION

Despite attempts to discover new anticancer drugs, multidrug resistance and the risk of recurrence remain. In particular, the TME in late-stage tumors is very complex and diverse, thus, it is essential to study the interplay between tumors and the TME for new drug discovery and validation. It is expected that endeavors to understand how tumor cells are reprogramed by communication with adjacent cells and molecules will support the development of new strategies to treat cancers.

AUTHOR CONTRIBUTIONS

S-JK designed and wrote the manuscript. DK wrote and reviewed the manuscript. JS supervised the whole project, wrote and reviewed the manuscript. All authors substantially contributed to the article and approved the submitted version.

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Evasion of NK cell immune surveillance *via* the vimentin-mediated cytoskeleton remodeling

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Cancer immunotherapy uses the immune system to achieve therapeutic effects; however, its effect is still limited. Therefore, in addition to immune checkpoint-based treatment, the development of other strategies that can inhibit cancer cells from resisting immune cytotoxicity is important. There are currently few studies on the mechanism of tumors using cytoskeletal proteins reorganization to participate in immune escape. In this study, we identified cancer cell lines that were sensitive or resistant to natural killer cells in urothelial and lung cancer using the natural killer cell sensitivity assay. We found that immunoresistant cancer cells avoid natural killer cell-mediated cytotoxicity by upregulation of vimentin and remodeling of actin cytoskeleton. Immunofluorescence staining showed that immune cells promoted the formation of actin filaments at the immune synapse, which was not found in immunosensitive cancer cells. Pretreatment of the actin polymerization inhibitors latrunculin B increased the cytotoxicity of natural killer cells, suggesting that cytoskeleton remodeling plays a role in resisting immune cell attack. In addition, silencing of vimentin with shRNA potentiated the cytotoxicity of natural killer cells. Interestingly, the upregulation and extension of vimentin was found in tumor islands of upper tract urothelial carcinoma infiltrated by natural killer cells. Conversely, tumors without natural killer cell invasion showed less vimentin signal. The expression level of vimentin was highly correlated with natural killer cell infiltration. In summary, we found that when immune cells attack cancer cells, the cancer cells resist immune cytotoxicity through upregulated vimentin and actin reorganization. In addition, this immune resistance mechanism was also found in patient tumors, indicating the possibility that they can be applied to evaluate the immune response in clinical diagnosis.

KEYWORDS

natural killer cell, immune surveillance, vimentin, intracellular cytoskeleton remodeling, immunotherapy

Introduction

For the upper tract urothelial carcinoma (UTUC), the incidence was less than 10% in the United Sates, whereas it was very high in Taiwan, being up to 30% of urothelial carcinomas (1). UTUC and bladder cancer belong to the same urothelial cell type, but their clinical prognoses are very different. Tumors invade the muscular layer in 60% of UTUC while invasion is about 10–20% in bladder cancers. Although most tumors can be completely removed, a high percentage of patients die from disease progression and distant metastasis (2). UTUC is often accompanied by renal insufficiency, which makes it difficult for the patient to receive an adequate dose of chemotherapy (3). Therefore, invasive or metastatic UTUC has a very poor clinical prognosis. At present, effective biomarkers are still quite limited, and it is necessary to identify markers to predict tumors and prognoses. There is an urgent need to develop more effective treatment strategies and improve prognosis with chemotherapy (4).

The increase in cancer mortality affects human health and social development, and finding safe and effective treatments is a challenge for cancer researchers worldwide. Traditional cancer treatments, such as surgical, radiotherapy and chemotherapy, do not ensure patient outcomes. In recent years, the development of gene targeted therapy has extended the survival of tumor patients, but the recurrence after treatment is still high. Immunotherapy, which aims to activate the patient's immune system to kill cancer cells, has been studied most at immune checkpoint inhibitors, while others include chimeric antigen receptor T cell therapy (CAR-T). Tumor cells can inhibit the function of natural killer cells (NK) and cytotoxic T lymphocytes (CTLs) through cytokines, chemokines and metabolites, thus helping tumor cells escape recognition and attack by the immune system (5). Among these immune cells, NK cells play a key role in the first-line immune response and are characterized by rapid response. The main approaches to NK cell-based cancer treatment include cytokines, antibodies, and adoptive transfer of NK cells by increasing the persistence, activation, number, or targeting of tumor cells (6)

Recent literature reveals that treatment of TGF-β blocking agent can enhance immune cell response and infiltration (7). Upregulation of TGF-β1 mediated vimentin is one of the key hallmarks of epithelial–mesenchymal transition (EMT) progression (8). Vimentin is an intermediate filament protein and a member of cytoskeletal proteins. TGF-β1 drives vimentin activation, which plays a key role in the cytoskeleton remodeling and mobility during EMT (9). Recent studies have found that vimentin can be detected in many types of cancers, including lung, colon, cervical and prostatic cancers, and the expression of vimentin is highly correlated with extent of these tumors (8, 10–12). vimentin is originally expressed in mesenchymal cells, such as fibroblasts, chondrocytes, macrophages and endothelial cells, rather than in epithelial cells (13). The determination of vimentin was thought to be as a predictive factor of poor prognosis in patients with gastric cancer (14), but this result is still controversial in other cancers (15). Thus, exploring the mechanism and the role of TGF-β or vimentin mediated signaling in the immune cell response can provide a clearer definition in patient samples.

Mechanotransduction in cancer cells contain variable processes of physical structure rearrangement that force chemical stimulation and cause signal transduction of cell function (16, 17). Our results show that vimentin upregulation and cytoskeleton remodeling are the novel tumor suppression strategy for enhancing immune cell-mediated cytotoxicity. Both silencing of vimentin and inhibiting the formation of actin filaments increased cytotoxicity of NK cells. Excitingly, these results are even more obvious in tumor tissue from patients with UTUC. It is currently unclear how vimentin and cytoskeleton remodeling affect the immune cell response in the primary and invasive tumors. Here, we uncover a novel strategy for improving clinical diagnosis by regulating vimentin and cytoskeleton remodeling to modulate the immune response of cancer cells.

Materials and methods

Cell lines and materials

Human cells, HT24 (bladder cancer, HTB-4, ATCC), J82 (bladder cancer, HTB-1, ATCC), BFTC909 (colorectal cancer, 60069, BCRC), H292 (lung cancer, CRL-1848, ATCC), A549 (lung cancer, CCL-185, ATCC) and NK-92MI (lymphoma, CRL-2408, ATCC) were obtained from the American Type Culture Collection (ATCC) or Bioresource

Collection and Research Center (BCRC). Cells were cultured in DMEM medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% (volume/volume; v/v) fetal bovine serum (FBS), sodium bicarbonate and 1% (v/v) antibiotic-antimycotic (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in an incubator with a humidified atmosphere of 5% CO2. NK-92MI cells were cultured in alpha minimum essential medium (Thermo Fisher Scientific) supplemented with 12.5% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis MO, USA), 2 mM L-glutamine (Thermo Fisher Scientific), 1.5 g/L sodium bicarbonate (Thermo Fisher Scientific), 0.2 mM inositol; (I7508, Sigma-Aldrich), 0.1 mM 2-mercaptoethanol; (M6250, Sigma-Aldrich), 0.02 mM folic acid; (F8758, Sigma-Aldrich), 12.5% horse serum. Latrunculin B (L5288, Sigma-Aldrich). Antibodies, CD161/NK1.1 was from Novus Biologicals (NB100-77528SS, Littleton, CO, USA), vimentin was from abcam (ab92547, Cambridge, UK). Phalloidin staining reagent was from Abcam (ab112125, Cambridge, UK).

Natural killer cell cytotoxicity assay

Cells were seeded at 2 x 10^4 or 4 x 10^4 cells per well in a 96-well plate (Jet Biofil, Guangzhou, China). Next day, NK-92MI cells were added into well in a serial dilution for 24 or 48 h (dilution 1:0.25, 1:0.5, 1:1, 1:3, 1:6 in culture media, #356234, Corning, Bedford, MA, USA). The fixed cells were stained with crystal violet (0.1 mg/mL) for 2 h, and the images were photographed under an inverted microscope (IX51, Olympus, Japan). The crystal violet staining cells were quantitatively analyzed and modified according to the study of Cvetannova et al. (18). The crystal violet-stained cells were then dissolved in 250 µL 20% acetic acid. Absorbance (O.D. 595 nm) was measured using an ELISA reader (Varioskan LUX Multimode Microplate Reader, Thermo Fisher Scientific, USA). The plots of NK cell cytotoxicity were analyzed using GraphPad Prism 8 software (GraphPad, San Diego, CA, USA).

shRNA and lentivirus infection

RNA was knocked down using lentivirus containing the shRNA for empty vector (shEV) or vimentin (shVimentin), virus was obtained from National RNAi Core in Taiwan. Cells were seeded at 1 x 10^5 cells per well in a 12-well plate and infected with shEV and shVimentin with MOI 2 for 48 h prior to puromycin selection. The shRNA targeting sequences of vimentin were shVimentin#1: GCTAACTACCAAGACACTATT and shVimentin#2: GCAGGATGAGATTCAGAATAT. PCR was performed using PFU Turbo polymerase (Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions.

Quantitative real-time PCR

RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and cDNA was synthesized using SuperScript™ III Reverse Transcriptase (Thermo Fisher Scientific). qRT-PCR was performed using SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and ABI StepOnePlus sequence detection system (Thermo Fisher Scientific, Waltham, MA, USA). The real-time PCR primers were as follows: Vimentin forward, 5′-GACGCCATCAACACCG AGTT-3′; Vimentin reverse primer, 5′-CTTTGTCGTTGGT TAGCTGGT-3′; GAPDH forward, 5′-TGAAGGTCGGAGTC AACGGATT-3′; GAPDH reverse primer, 5′-CCTGGAA GATGGTGATGGGATT-3′.

Immunofluorescence staining

The cancer cell lines (2 x 10^4 cells) were seeded on coverslips in a 12-well plate overnight. The next day, the NK-92MI cells were stained with CellTracker Red CMTPX dye (C34552, Thermo Fisher Scientific) for 30 min and washed twice with PBS. Cancer cells were treated with CMTPX-stained NK-92MI cells for 2, 6, and 24 h (dilution, 1:0.5). Cells were fixed in 4% paraformaldehyde for 20 min and parametrization was performed in 0.1% Triton X-100 for 15 min. Blocking buffer (1% BSA, 0.1 M glycine) was added for 30 min and then reacted with first antibodies (1:200) in blocking buffer overnight. The samples were washed with PBST (0.1% Tween-20) three times and then secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594 (1:200) in blocking buffer were added for 2 h. Samples were stained with DAPI and mounted with Prolong Gold antifade reagent (Thermo Fisher Scientific, Waltham, MA, USA). The samples were stored at 4°C in the dark. Fluorescent cells were observed using a confocal laser scanning microscope (FluoView FV10i, Olympus, Tokyo, Japan). Ten fields for each experiment were examined at 100-fold magnification and up to 630-fold magnification to obtain detailed images. Quantification of β-tubulin and vimentin was performed after the cells were selected, and the intensity of the fluorescent protein was analyzed with and without NK cell interaction. Quantitative analysis of actin responses in front regions of the immune synapse bound to NK cells, marked with dotted rectangles (F), and unbound regions at the rear, marked with dotted rectangles (R), was performed. Fluorescence intensity (unit) = F-R in the immune synapse, showing the level of F-actin aggregation. Fluorescence intensity and quantification were analyzed using the FV10-ASW software (version 4.0) and ImageJ software (version 1.8). For analysis of immunofluorescence staining of tumor sections, a Vectra Polaris Automated Quantitative Pathology

Imaging System (PerkinElmer, Boston, MA, USA) was used to scan the slides. Fluorescence intensity and quantification were analyzed using inForm software (version 2.3, PerkinElmer).

Immunohistochemistry staining

Tumors were fixed in 10% formalin overnight and embedded in paraffin for sectioning. The sections were cut at 4-5 μm. Tumor sections were deparaffinized in xylene and antigen retrieval reagent was added for 30 min (Agilent Dako, Santa clara, CA, USA). Tissue sections were added blocking buffer (1% BSA, 0.1% Tween-20) for 30 min and then reacted with first antibodies (1:200) in blocking buffer overnight. The samples were washed with PBST for three times and then added secondary antibodies-conjugated with horseradish peroxidase (HRP) (1:200) in blocking buffer for 2 h. Samples were counterstained with hematoxylin and stained with 3,3'-Diaminobenzidine (DAB). For analysis of tumor sections, a Vectra Polaris Automated Quantitative Pathology Imaging System (PerkinElmer, Boston, MA, USA) was used to scan the slides. Intensity and quantification were analyzed using inForm software (version 2.3, PerkinElmer).

Western blot assay

Cells were washed in ice-cold PBS and lysed in a RIPA buffer containing 50 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40, 0.25% Na deoxycholate, 1 mM DTT, 1 mM PMSF, 1 mM EDTA, 1 mM NAF, 1 mM Na_3VO_4, 2 mg/mL aprotinin, and 2 mg/ml leupeptin. Cellular debris were removed by centrifugation at 13 krpm at 4°C for 15 min. The protein lysates were quantified by BCA assay (Thermo Fisher Scientific, Waltham, MA, USA) and equal amounts of protein (20 μg) was resolved on SDS polyacrylamide gels, transferred to PVDF membranes (Schleicher & Schuell, Dassel, Germany), and then incubated with primary antibodies overnight. After reaction with HRP-conjugated secondary antibody (1:2000 dilution; Cell Signaling Technology, Beverly, MA, USA) for 2 h, each membrane was scanned using a UVP ChemStudio PLUS instrument (UVP Inc., Upland, CA, USA) and analyzed with the ImageJ software (version 1.8).

Statistical analysis

Unless otherwise stated, all experiments were conducted at least three times. Data from the NK cytotoxicity assay, real-time qPCR, immunohistochemistry staining in this study was expressed as mean ± s.d. Statistical significance between different experimental groups was analyzed using the Student's t-test (two-tailed), 1-way with Dunnett's multiple comparisons test or 2-way ANOVA with Tukey's multiple comparisons test. P values less than 0.05 were considered significant. Statistical analyses were performed using GraphPad Prism 8 (GraphPad software, San Diego, CA, USA).

Results

Identifying cancer cell groups that were sensitive or resistant to immune cells

Currently, few studies have investigated the mechanism of the involvement of cytoskeleton-associated proteins of cancer cells in immune cell tolerance (19, 20). First, a NK cell cytotoxicity assay was performed to explore the mechanism by which cancer cells resist cell death caused by NK cells. The purpose of this analysis was to identify the sensitivity of cancer cells to the cytotoxicity of immune cells. Cancer cells used in the analysis were urothelial cancer cells (J82, T24, and BFTC909). Since the immune response regulated by NK cells had been widely studied in lung cancer, the lung cancer cells (H292 and A549) were also studied. Cancer cells were seeded into 96-well plates and incubated for 24 h. Next day, different proportions of NK cells were added (NK-92MI). The ratios of cancer cells to NK cells were 1:0.25, 1:0.5, 1:1, 1:3, and 1:6. The cancer cells and immune cells were co-incubated for 24 or 48 h and then fixed and stained with crystal violet dye (Figure 1A). To quantify the number of cells, acetic acid was used to dissolve the crystal violet dye and absorbance was measured. T24 cells were found to be more sensitive to NK cells than the other two cell lines. Over 90% of the cells died at an NK cell ratio of 1:1. In contrast, J82 and BFTC909 cell lines were resistant to NK cells, with approximately 20% of cell death occurring after incubation with NK cells at a ratio of 1:3 for 48 h. The crystal violet-stained cells in Figure 1A were observed using a microscope to observe the morphology of cancer cells after treatment with NK cells (Figure 1B). The shape of the T24 cells changed from round to elongated at a ratio of 1:1 (Figure 1B). Only some J82 and BFTC909 cells appeared to shrink at a ratio of 1:6, while the remaining cells showed normal morphology. The crystal violet-stained cells from the plate in Figure 1A were quantitatively analyzed and modified according to the study of Cvetanova et al. (18). The fixed cells were dissolved in 20% acetic acid, and the absorbance at 595 nm was measured using an ELISA reader (Figure 1B, right panel). Statistical quantification of cells revealed that when treated with NK cells at a ratio of 1:3, the rate of T24 cell death was 95.8%, whereas that of J82 and BFTC909 cells were 22.4% and 15.4%, respectively (Figure 1B). This result showed that T24 cells were sensitive to NK cells, while J82 and BFTC909 cells were resistant to cancer cells.

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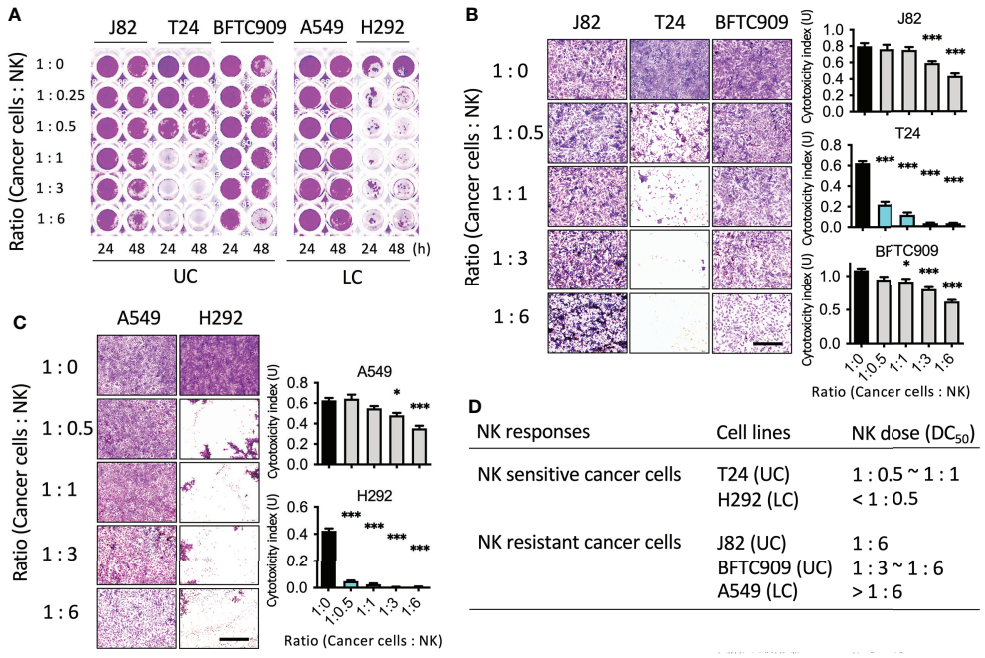

FIGURE 1
Identification of the cancer cell lines susceptible or resistant to NK cell-mediated cell death. **(A)** NK cytotoxicity assay using cancer cells from urothelial cancer (UC) and lung cancer (LC). Cells were seeded in the 96-well plate and incubated with NK cells for 24 or 48 h. **(B)** J82, T24 and BFTC909 cells were co-cultured with NK cells for 48 h and stained with crystal violet. The stained cells were observed by microscope. **(C)** A549 and H292 cells were co-cultured with NK cells for 48 h and stained with crystal violet. The stained cells were observed by microscope. **(D)** The NK sensitive or resistant cancer cells and the dose of NK ratio are listed. Scale bars, 1 mm. *$P < 0.05$; ***$P < 0.001$. Data are presented as mean ± s.d. (one-way ANOVA with Dunnett's multiple comparisons test) and experiments were repeated three times (n = 3).

We then investigated the cytotoxicity of NK cells on lung cancer cells. After adding NK cells at different ratios, cancer and immune cells were incubated for 24 and 48 h, respectively, and the cells were then fixed and stained with crystal violet dye (Figure 1C). H292 cells were found to be more sensitive to NK cells than A549 cells. Over 90% of cells died when incubated with NK cells at a ratio of 1:0.5. In contrast, A549 cells were resistant to NK cells, with approximately 20% cell death occurring in the presence of NK cells at a ratio of 1:3, indicating that A549 cells had low sensitivity to NK cells. The morphology of cancer cells after treatment with NK cells was observed with a microscope, and H292 cells were found with round shape in the presence of NK cells a ratio of 1:0.5 (Figure 1C). However, at a ratio of 1:6, only a few A549 cells shrank and the rest of the cells had normal morphology. Statistical quantification of the cells showed that when treated with NK cells at a ratio of 1:3, the rate of H292 cell death was 96.2%, while that of A549 cells was 23.6% (Figure 1C). This result showed that H292 cells were immunosensitive to NK cells, while A549 were resistant to cancer cells. To summarize, these cells were classified as sensitive or resistant urothelial or lung cancer cells based on their sensitivity to NK cells (Figure 1D). The immunosensitive cancer cells were T24 and H292, and the immunoresistant cancer cells were J82, BFTC909, and A549.

Accumulation of actin filaments was formed at the junction of immune cells and resistant cancer cells

After categorizing the immune sensitive and resistant cancer cells, we then investigated whether they responded differently when attacked by NK cells. In recent years, immunosuppression-related research has mostly focused on immune checkpoint proteins, PD-1 and PD-L1 (21). Although both BFTC909 and A549 were highly resistant to NK cells, A549 was known to express lower levels of PD-L1. This shows that there were other reasons for the immunoresistance of cancer cells. Mariathasan et al. and Tauriello et al. found that the use of TGF-β1-blocking antibody significantly promoted immune cell invasion (7, 22). TGF-β1 has been found to trigger many deteriorating reactions in cancer cells. Most of these reactions result in the reorganization of cytoskeletal proteins and fibrin. Therefore, we further explored whether cytoskeletal proteins of cancer cells affected immune cell

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attack. The three most important cytoskeletal proteins, namely β-tubulin, vimentin, and actin filaments were screened to observe any significant changes due to immune cell attack. The immunoresistant cancer cell line BFTC909 in Figure 1 was used to detect changes in these cytoskeletal proteins and fibrin. The experimental design involved co-culture of the resistant cancer cells and NK cells for 6 h (the ratio of cancer cells to NK cells was 1:0.5), followed by fixing of the cells for immunofluorescence staining.

The staining results for β-tubulin, vimentin, and actin filaments showed that NK cells did not cause changes to β-tubulin until 6 h. It was unexpectedly observed in the staining of the other two cytoskeletal proteins that NK cells significantly caused the upregulation of vimentin and actin response (Figure 2A). Quantification of β-tubulin and vimentin was performed after the cells were selected, and the intensity of the fluorescent protein was analyzed with and without NK cell interaction. Quantitative analysis of actin responses was performed as described by Al Absi et al. (19). Front regions of the immune synapse bound to NK cells are marked with dotted rectangles (F), and unbound regions at the rear are marked with dotted rectangles (R). Mean fluorescence intensity (unit/μm^2) = F-R in the immune synapse, showing the

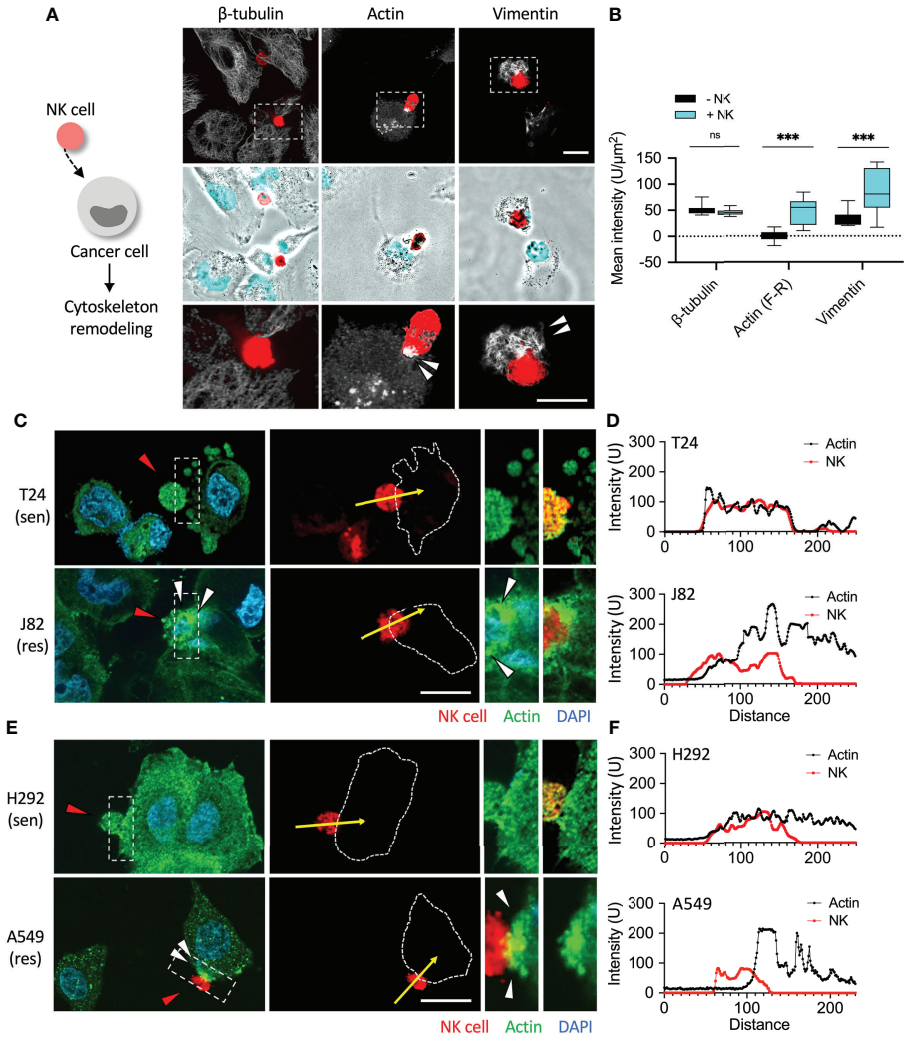

FIGURE 2
Induction of cytoskeleton remodeling by NK cells. **(A, B)** Comparison of the expression of β-tubulin, vimentin and actin filaments in NK cell-mediated cell lysis. BFTC909 cells were incubated with NK cells for 6 h and the expression β-tubulin, vimentin, and actin filaments was detected by immunofluorescence assays. **(C)** T24 or J82 cells were co-cultured with NK cells for 6 h and stained with a β-actin antibody. The stained cells were observed using a fluorescence microscope. **(E)** H292 and A549 cells were co-cultured with NK cells for 6 h and stained with a β-actin antibody. The stained cells were observed using a fluorescence microscope. **(D, F)** The intensity of prominent fibrous actin near the immunologic synapse was measured and quantified by ImageJ. ns, not significant; ***P < 0.001. Data are presented as means ± s.d. (two-way ANOVA with Sidak's multiple comparisons test). Red arrowhead, NK cell; white arrowhead, actin aggregation. Scale bars, 20 μm. Experiments were repeated at three times (n = 3).

level of F-actin aggregation (Supplementary Figure 1). After 6 h of NK cell treatment, the expression of β-tubulin, vimentin, and actin filaments was quantified. We performed the quantification of fluorescence intensity in 10 fields (2–3 cells per field) after cells were selected and analyzed, and the results showed that after adding NK cells, the mean intensity of β-tubulin was NK-:NK+ = 50.75:45.48 (P = not significant, ns), mean intensity of actin response was NK-:NK+ = 0.05:47.21 (P < 0.001), and the mean intensity of vimentin was NK-:NK+ = 33.69:83.87 (P < 0.001) (Figure 2B). The expression of the fluorescence signal was obvious at 6 h after treatment with NK cells, indicating that vimentin and actin filaments of cancer cells may reflect NK cell attack.

We wanted to further compare whether actin filaments responded differently in sensitive and resistant cancer cells when attacked by NK cells. First, the urothelial cancer cells T24 and J82 were co-cultured with NK cells for 6 h, and no change was observed in actin filaments of T24 cells (Figure 2C, and Supplementary Figure S2A). However, 6 h after addition of NK cells to the resistant cell line J82, aggregations of actin filaments were clearly observed following the contact between cancer cells and immune cells (Figure 2C and Supplementary Figure S2B). Quantitative analysis showed that the expression of actin filaments at the immune synapses was high in J82 cells and low in T24 cells (Figure 2D).

We then investigated whether actin filaments of sensitive and resistant cancer cells responded differently upon attack by NK cells in lung cancer cells H292 and A549. After co-incubation with NK cells for 6 h, no change in actin filaments was observed in H292 cells (Figure 2E, and Supplementary Figure S2C). As expected, 6 h after adding NK cells to A549 cells (resistant cells), accumulation of actin filaments was clearly detected (Figure 2E and Supplementary Figure S2D). We concluded that cancer cells with high immunoresistance produced and aggregated of actin filaments in response to immune cell attack (Figure 2F).

To further confirm the effect of NK cells on the organization and expression of cytoskeletal proteins after binding to cancer cells, we co-cultured NK cells with cancer cells and performed immunofluorescence staining in a time-dependent manner. The fluorescence was quantified in 10 fields (2–3 cells each field). The mean fluorescence intensity (F-R) of the actin response at the immune synapse in T24 cells co-cultured with NK cells for 2, 6, and 24 h were NK-:NK+ = 0.01:5.56, 5.96:8.72, and 0.25:-2.53, respectively (all P = ns) (Figures 3A, B). The mean fluorescence intensity (F-R) of the actin response at the immune synapse in J82 cells after 2, 6, and 24 h with NK cell co-culture was NK-:NK+ = -1.34:22.12, 1.87:39.56, and 3.84:37.36, respectively (P < 0.05, < 0.001, and < 0.01, respectively) (Figures 3C, D). In J82 cancer cells, the mean intensity of actin response was higher after adding NK cells at 6 and 24 h than at 2 h (2, 6 and 24 h; 22.12, 39.56, and 37.36, respectively) (Figure 3D).

We performed the same experiment on lung cancer cells and quantified proteins in 10 fields. The mean fluorescence intensity (F-R) of the actin response at the immune synapse in H292 cells co-cultured with NK cells for 2, 6, and 24 h was NK-:NK+ = 1.23:5.55, -1.13:8.85, and -3.38:0.1, respectively (all P = ns) (Figures 3E, F). The mean fluorescence intensity (F-R) of the actin response at the immune synapse in A549 cells after 2, 6, and 24 h with NK cell co-culture was NK-:NK+ = -0.54:12.52, -0.36:30.01, and -0.68:37.55, respectively (P = ns, < 0.01, and < 0.001, respectively) (Figures 3G, H). Since vimentin can directly interact with actin filaments through its C-terminal tail (23, 24), and mutually affect actin stress fiber assembly and actin-dependent processes such as cell adhesion and migration (9, 25–29), we subsequently explored whether vimentin participates in the resistance response against immune cells.

Attack by NK cells upregulated vimentin

Jiu et al. demonstrated that one of the functions of vimentin was to facilitate the aggregation and localization of actin. A lack of vimentin disables the cancer cells to promote actin polymerization (25). The results in Figure 2A confirmed that the expression of vimentin was significantly increased after NK cell attack. Therefore, a further comparison of the response of vimentin was made between sensitive and resistant cancer cells when attacked by NK cells. Urothelial cancer cells T24 and J82 were used, and after co-cultured with NK cells for 6 h, no change was observed in the vimentin content of T24 cells (Figure 4A and Supplementary Figure S3A). However, expression of vimentin was clearly detected in BFTC909 cells along with obvious polymerized morphology (Figure 4B and Supplementary Figure S3B). The quantitative results showed that the vimentin expression after exposure to immune cells was high in J82 and low in T24 (Figure 4B).

We then examined whether the vimentin responded differently within sensitive and resistant lung cancer cells upon attack by NK cells. After co-culture of the lung cells H292 and A549 with NK cells for 6 h, no change in the vimentin expression was observed in H292 cells (Figure 4C and Supplementary Figure S3C). Conversely, an expansion of vimentin network was detected in A549 cells (Figure 4C and Supplementary Figure S3D). Therefore, we found that vimentin was upregulated in highly immunoresistant cancer cells in response to immune cell attack (Figure 4D).

The mean fluorescence intensity of vimentin in T24 cells co-cultured with NK cells for 2, 6, and 24 h was NK-:NK+ = 16.02:18.39, 17.6:17.76, and 19.4:21.78, respectively (all P = ns) (Figures 5A, B). The mean fluorescence intensity of vimentin in J82 cells after 2, 6, and 24 h with NK cell co-culture was NK-:NK+ = 28.85:50.09, 33.68:71.39, and 46.48:91.96, respectively (P = ns, < 0.001, and < 0.01, respectively) (Figures 5C, D). In J82 cancer cells, the mean intensity of vimentin was higher at 24 h than at 2 h after

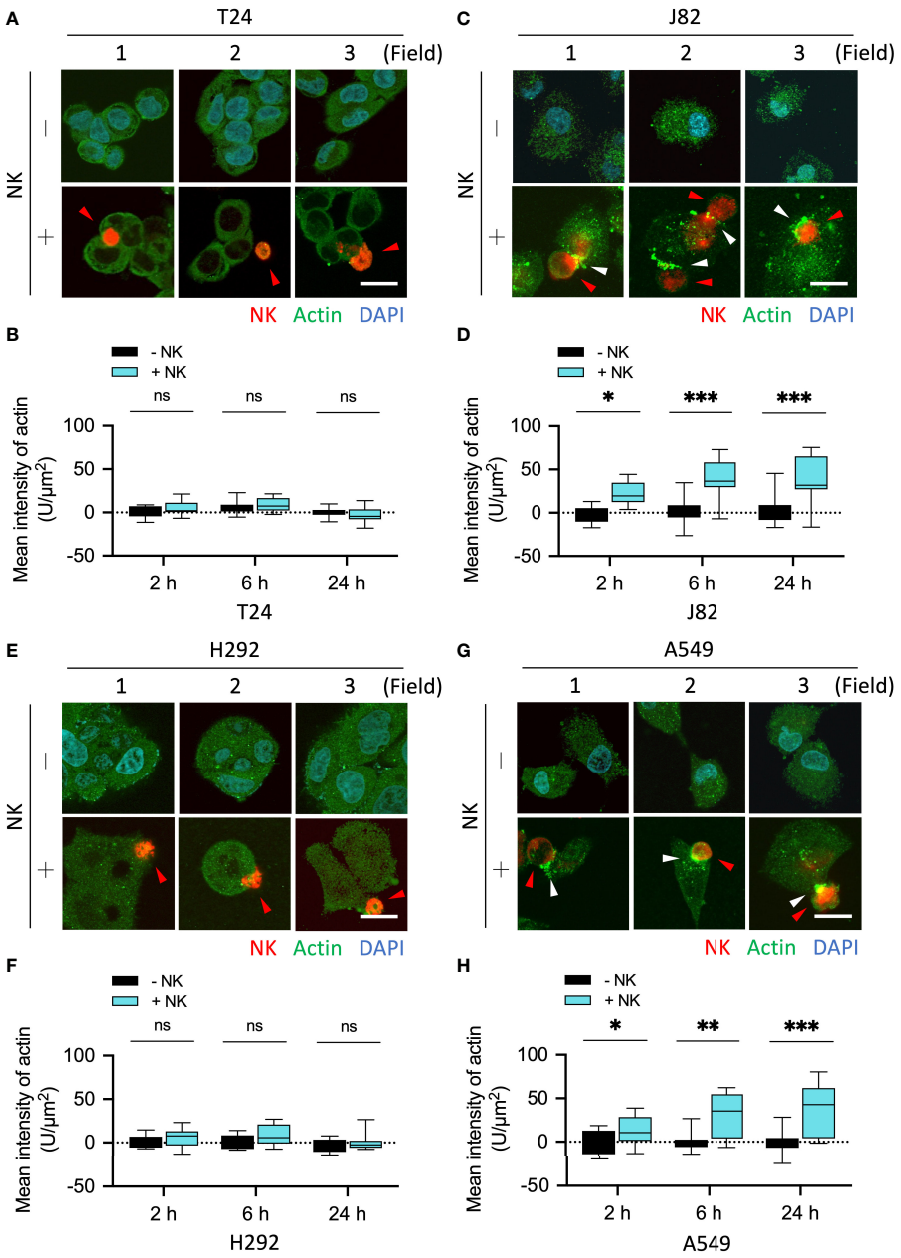

FIGURE 3
NK cells induced actin responses at the immune synapses in the immune-resistant cancer cells. **(A, C)** Comparison of the expression of actin filaments in NK cell-interacted cancer cells. T24 and J82 cells were incubated with NK cells for 2, 6 and 24 h, and the expression of actin filaments was detected using immunofluorescence assays. **(B, D)** The intensity of actin's response at the immune synapses was measured and quantified by ImageJ. **(E, G)** H292 and A549 cells were incubated with NK cells for 2, 6 and 24 h, followed by the detection of actin filaments expression. **(F, H)** Quantification of the intensity of actin response at the immune synapses using ImageJ. ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001. Data are presented as mean ± s.d. (two-way ANOVA with Sidak's multiple comparisons test). Red arrowhead, NK cell; white arrowhead, actin filaments. Scale bars, 20 μm. Data derived from 10 fields, each including 2–3 cells.

adding NK cells (2 and 24 h; 50.09 and 81.96, respectively, $P < 0.01$) (Figure 5D).

The mean fluorescence intensity of vimentin in H292 cells co-cultured with NK cells for 2, 6, and 24 h was NK-:NK+ = 12.3:15.66, 12.87:16.8, and 14.74:13.7, respectively (all P = ns) (Figures 5E, F). The mean fluorescence intensity of vimentin in A549 cells after 2, 6, and 24 h with NK cells co-culture was NK-:NK+ = 33.46:49.28, 49.75:70.64, and 37.16:55.85, respectively (P < 0.05, < 0.01, and < 0.01, respectively) (Figures 5G, H). In summary, the accumulation of actin filaments and the upregulation of vimentin were involved in the response of cancer cells to immune cells. However, the

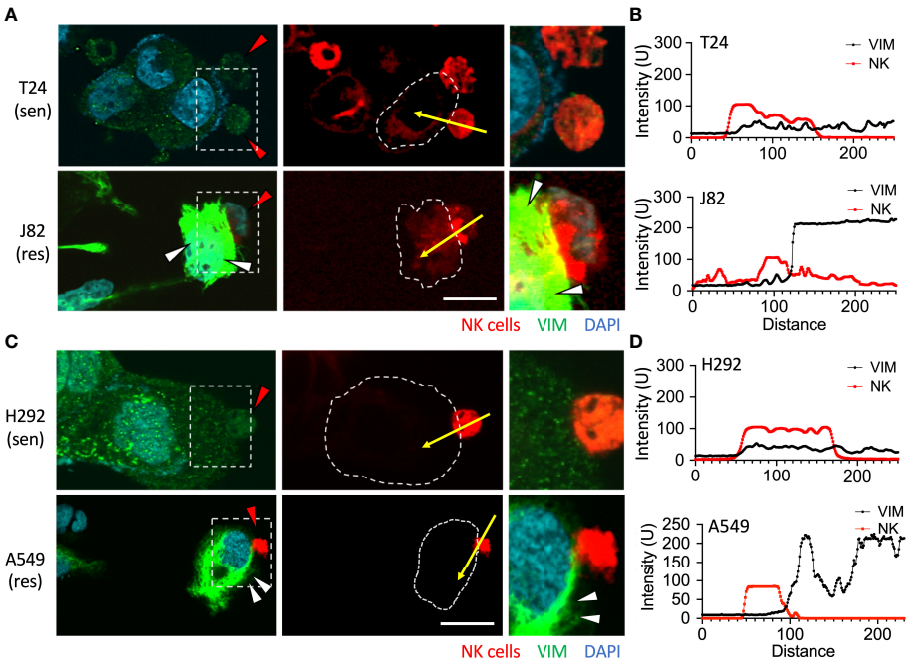

FIGURE 4
Upregulation of vimentin upon NK cell interaction. **(A)** T24 or J82 cells were co-cultured with NK cells for 6 h and stained with a vimentin antibody. The stained cells were observed using a fluorescence microscope. **(C)** H292 and A549 cells were co-cultured with NK cells for 6 h and stained with a vimentin antibody. The stained cells were observed using a fluorescence microscope. **(B, D)** The intensity of upregulated vimentin in the cancer cells was measured and quantified by ImageJ. Red arrowhead, NK cell; white arrowhead, vimentin upregulation. Scale bars, 20 μm. Experiments were repeated at three times (n = 3).

presence of such phenomena in UTUC tumors is what we want to explore next.

Invasion of NK cells in UTUC upregulated vimentin in cancer cells

First, we investigated whether changes in vimentin of cancer cells caused by NK cells could be detected in cancer patients. Staining of immune cells and vimentin was performed in stage III tumors of UTUC. Tumors were fixed and cut into slices for immunofluorescence and immunohistochemistry staining. The NK1.1 is expressed primary on NK cells and also found on NKT cells, a subset of $CD4^+$ T cells and dendritic cells. The specificity of NK1.1 and vimentin antibodies was verified using an IgG antibody (Supplementary Figure S4). In the cold areas for the immune responses, i.e., the tumor without invasion by NK cells, less vimentin signal was detected either.

Interestingly, in tumor islands invaded by a small number of NK cells, vimentin was significantly detectable in the cancer cells surrounding the NK cells (Figure 6A). This was more apparent in tumors invaded by a large number of NK cells (hot areas), wherein strong vimentin signals were detected in the cancer cells around the NK cells (Figure 6A). Vimentin was also detected by immunohistochemistry assay, and hematoxylin staining was used to visualize cell morphology of the tissue (Figure 6A). Quantification of these results showed a high level of correlation between the NK cells in tumor and vimentin in cancer cells (Figure 6B). In tumors where no NK cell invasion was detected, the vimentin signal was extremely low (mean intensity 7.1 – 20.9), while in tumor islands with high level of invasion by NK cells, the mean intensity of vimentin was 79.1 – 113.7 (Figure 6B). These results showed that vimentin expression in the cancer cells caused by invasion of NK cells was indeed upregulated in UTUC tumors (Figure 6C). In summary, the expression of vimentin was stimulated in tumors invaded by NK cells.

Interference with actin filament polymerization promoted the cytotoxicity of NK cells

Reorganization of actin cytoskeleton plays a role in promoting the migration and metastasis of malignant tumors. Our previous study reported that inhibiting actin reorganization limited tumor progression and metastasis (30). The results in Figures 1 and 2 demonstrated that NK cells promoted the

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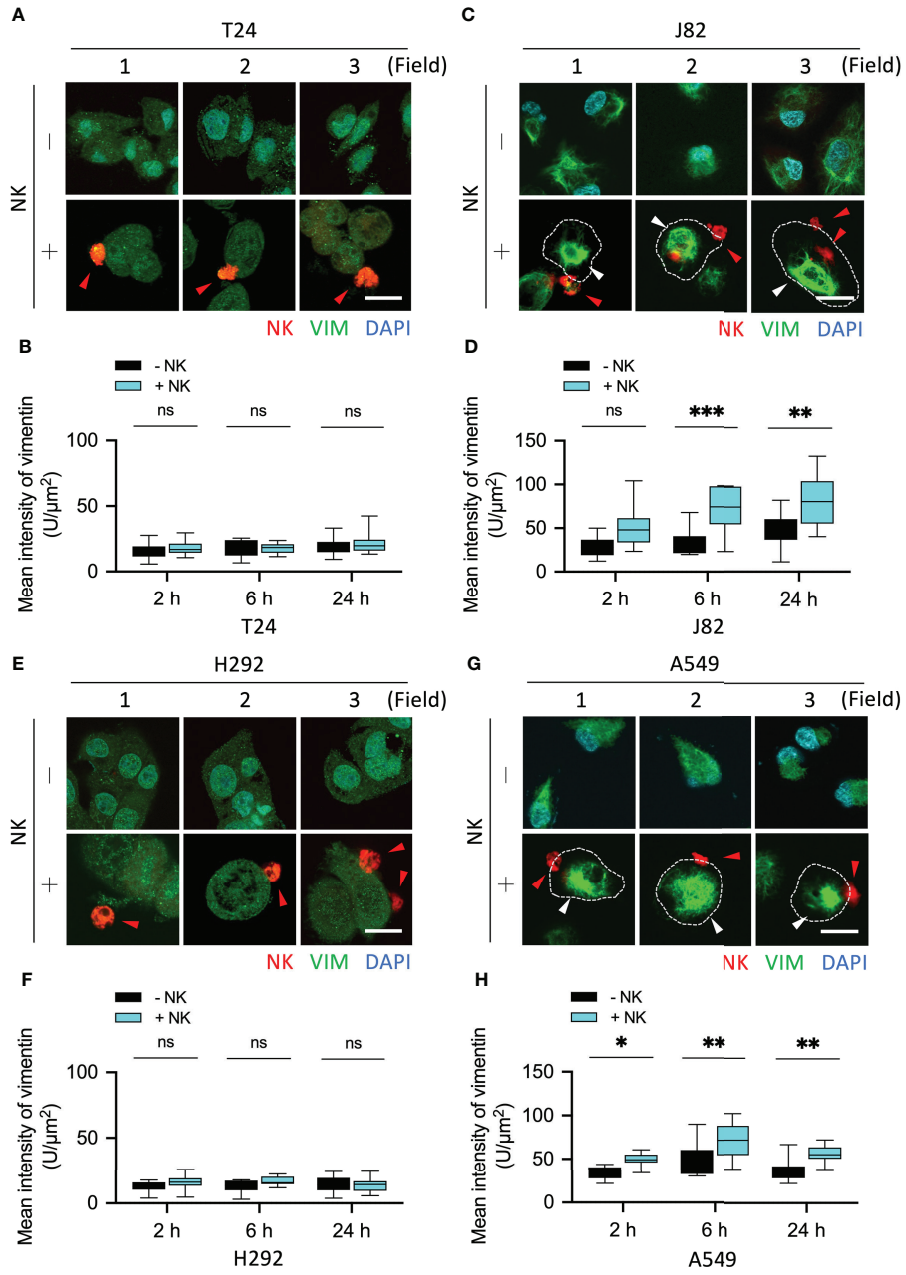

FIGURE 5
NK cells induced vimentin expression in the immune-resistant cancer cells. **(A, C)** Comparison of the expression of vimentin in NK cell-interacted cancer cells. T24 and J82 cells were incubated with NK cells for 2, 6 and 24 h, and the expression of vimentin was detected using immunofluorescence assays. **(B, D)** The intensity of vimentin was measured and quantified by ImageJ. **(E, G)** H292 and A549 cells were incubated with NK cells for 2, 6 and 24 h, followed by the detection of vimentin expression. **(F, H)** Quantification of the intensity of vimentin using ImageJ. ns, not significant; *P < 0.05; **P < 0.01; ***P < 0.001. Data are presented as mean ± s.d. (two-way ANOVA with Sidak's multiple comparisons test). Red arrowhead, NK cell; white arrowhead, vimentin. Scale bars, 20 μm. Data derived from 10 fields, each including 2–3 cells.

accumulation of actin filaments in resistant cancer cells. Therefore, we further investigated whether actin filament polymerization affected the cytotoxicity of immune cells (Figure 7A). Immunoresistant cells (BFTC909 and A549) were pretreated with an actin polymerization inhibitor (latrunculin B), which could inhibit actin polymerization (Figure 7B). After pretreatment with latrunculin B for 6 h, the inhibitor was removed, and NK cells were added for another 24 h (the ratios of cancer cells to NK cells were 1:0.25; 1:0.5, 1:1, 1:3, and 1:6). Subsequently, cells were fixed and stained with crystal violet dye (Figures 7C, D), and the morphology after treatment with NK cells was observed under a microscope. The result demonstrated

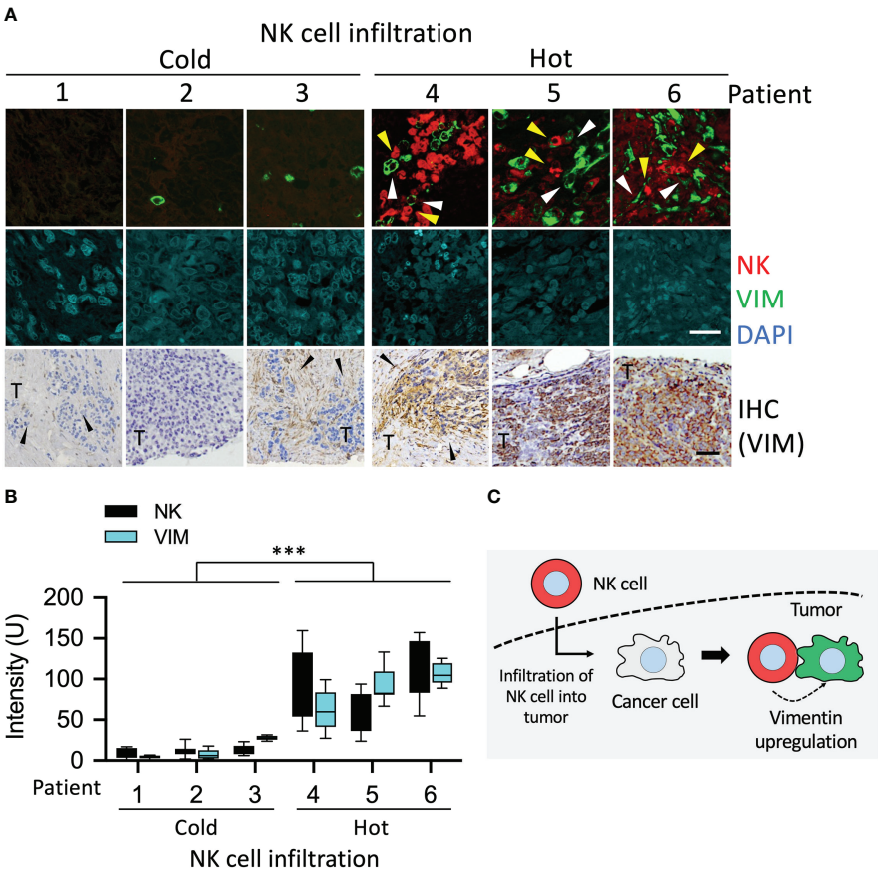

FIGURE 6
Upregulation of vimentin in NK cell infiltrated tumor of UTUC. **(A)** Different levels of NK cell-infiltrated tumors from patients with UTUC were collected. The tissue sections of UTUC were co-stained with NK1.1 and vimentin antibodies followed by detection of fluorescence signals using a confocal fluorescence microscope. Vimentin was also detected using immunohistochemistry staining. **(B)** The fluorescence intensity of NK1.1 and upregulated vimentin in the tumor was quantified by ImageJ. ***$P < 0.001$. Data are presented as means ± s.d. (two-way ANOVA) from three fields (n = 3). **(C)** Schematic represented the upregulation of vimentin induced by NK cells. Yellow arrowhead, NK cell; white arrowhead, vimentin. White scale bars, 50 μm, Black scale bars, 100 μm.

that when BFTC909 cells were treated at a ratio of 1:6, the morphology of the cells was normal. By contrast, after the NK cells were co-incubated with cancer cells pre-treated with latrunculin B at a ratio of 1:3 for 24 h, the cancer cells changed shape from round to elongated type. We speculated that pretreatment with latrunculin B altered the regulation of cancer cell cytoskeletal proteins to respond to NK cell attack (Figure 7C). The results revealed that BFTC909 cells were not sensitive to NK cells before treatment with latrunculin B, with 39.7% of cell death at a ratio to NK cells of 1:6. In contrast, cells became sensitive to NK cells after treatment with latrunculin B, with approximately 84.6% of cells dying after co-culture with NK cells at a ratio of 1:3. Up to 47.2% of cells died after co-culture with NK cells a ratio of 1:1 for 24 h (Figure 7D).

Similar results were obtained using another resistant cancer cell line, J82. Statistical quantification of the cells showed that the rate of cell death was 43.2% at a ratio to NK cells of 1:6, while the rate reached > 90% in cells treated with latrunculin B after co-culture with NK cells a ratio of 1:3 for 24 h (Figure 7C). This result showed that in urothelial cancer cells, pretreatment with actin polymerization inhibitors changed the response of BFTC909 and J82 cells to NK cells from resistant to sensitive cells. Our results show that the inhibition of actin reorganization in resistant cancer cells may enhance immune cell attack.

Silencing vimentin promoted the cytotoxicity of NK cells

Previously, the vimentin expression was found to be increased in many cancers, such as colorectal and lung cancer. However, the correlation between vimentin and tumor progression remained controversial (14). As shown in Figures 2 and 3, high vimentin expression was found when

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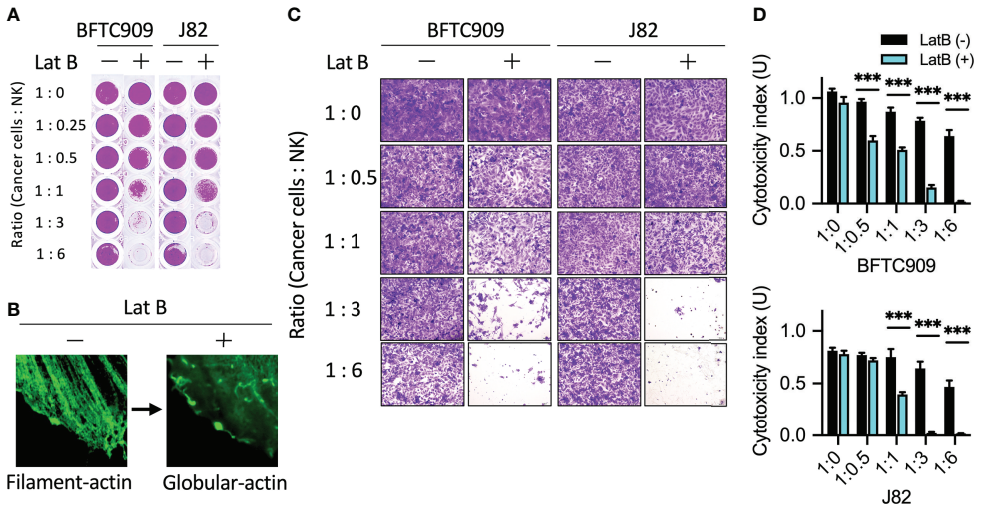

FIGURE 7
Disruption of actin filaments formation enhanced NK cell-mediated cell death. **(A)** Pretreatment of latrunculin B, an actin filaments inhibitor, increased NK cytotoxicity using urothelial cancer cells. Cells were seeded in the 96-well plate and pretreated with 0.5 μM latrunculin B for 6 h following which the inhibitor was removed. The cancer cells were then incubated with NK cells for 24 h. **(B)** Representative images of latrunculin B showing reduced actin filaments formation. **(C)** J82 and BFTC909 cells were co-cultured with NK cells for 24 h and stained with crystal violet. The stained cells were observed using a microscope. **(D)** The stained cells were counted and statistically analysis was performed using the Prism software. ***$P < 0.001$. Data are presented as means ± s.d. (two-way ANOVA with Tukey's multiple comparisons test) and experiments were repeated three times (n = 3). Latrunculin B, LatB.

cancer cells were attacked by NK cells. Such phenomena were also verified in patient tumor tissues (Figures 6A, B). Based on this, we further explored if vimentin expression affected the cytotoxicity of immune cells. The experiment was designed to suppress the expression of vimentin in the immunoresistant cells (BFTC909) by using shRNA, which inhibited the expression of vimentin (Figure 8A). A stable cell line was selected from cells with knocked-down vimentin and was then co-cultured with NK cells for 24 h (the ratios of cancer cells to NK cells were 1:0.25, 1:0.5, 1:1, 1:3, and 1:6). Cells were then fixed and stained with crystal violet dye (Figure 8B). The morphology of cancer cells treated with NK cells was observed using a microscope, and shrinkage of some cells was observed in BFTC909 cells at a ratio of 1:6, while other cells showed normal morphology. After silencing vimentin, cancer cells were co-cultured with NK cells at a ratio of 1:3 for 24 h and the cancer cells were sensitive to immune cells (Figure 8C). Statistical quantification of the cells found that BFTC909 cells were not sensitive to NK cells, with 46.1% of dead cells observed at an NK cell ratio of 1:6. In contrast, after silencing vimentin, BFTC909 cells became sensitive to NK cells, and 77.9% - 89% of cells died after co-incubation with NK cells at a ratio of 1:3 for 24 h. After co-culture with NK cells at a ratio of 1:1 for 24 h, 32.1% – 49.1% of the cells were dead (Figure 8D). These results demonstrated that silencing vimentin in urothelial cancer cells transformed the response of BFTC909 cells to NK cells from being resistant to being sensitive. It is implied from this conclusion that inhibiting the expression of vimentin in resistant cancer cells may enhance the cytotoxicity of immune cells.

Discussion

Tumor evasion of immune surveillance is the main reason for the inefficiency of immunotherapy. Here, we demonstrated that actin cytoskeleton reorganization and vimentin expression in urothelial cancer cells were resistant to NK cell-induced cytotoxicity. Using the NK cytotoxicity assay, we found that in urothelial and lung cancer cell lines, two types of cells can be distinguished, which differ in their sensitivity and resistance to NK cell attack (Figure 1). Notably, when resistant cancer cells were attacked by NK cells, obvious actin filaments aggregation occurred at the immune synapse, whereas this phenomenon was not evident in sensitive cells (Figure 2). The same phenomenon was observed when detecting vimentin expression, and resistant cells exhibited vimentin upregulation after NK cell attack (Figure 3). Tissue staining provided direct evidence that tumors with high NK cell infiltration exhibited more vimentin expression (Figure 4). Furthermore, in highly resistant cells, pretreatment with latrunculin B promoted the cytotoxicity of NK cells (Figure 5). Inhibition of vimentin expression by shRNA was also sufficient to convert the resistant cancer cell lines to a sensitive phenotype (Figure 6). Taken together, these findings

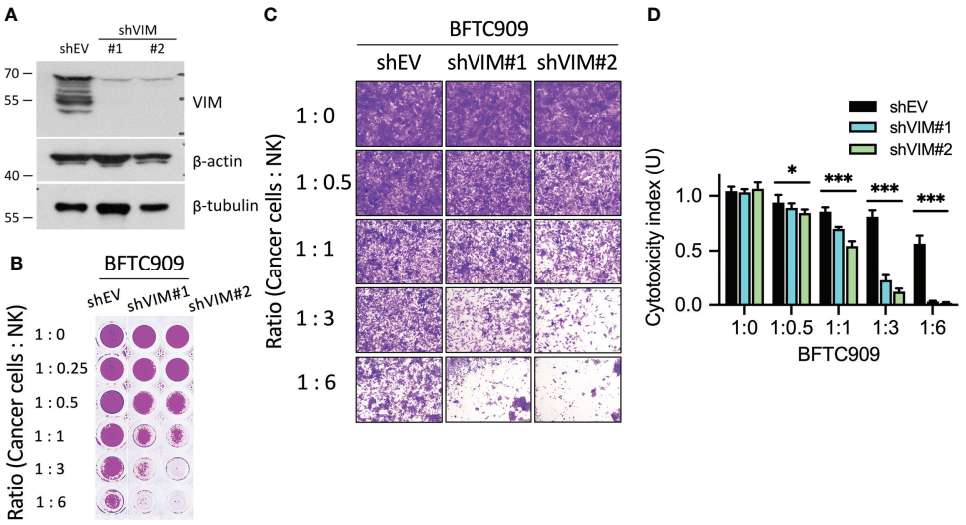

FIGURE 8
Silencing of vimentin enhanced NK cell-mediated cell death. **(A)** Silencing efficacy of shVIM#1 and shVIM#2 by western blot. **(B)** Silencing of vimentin in urothelial cancer cells increased NK cytotoxicity. BFTC909 cells were silenced with shEV, shVIM#1 or shVIM#2 and stable cells were screened. The stable cells were seeded in the 96-well plate 24 h and then incubated with NK cells for 24 h. **(C)** BFTC909-shEV, shVIM#1 or shVIM#2 cells were co-cultured with NK cells for 24 h and stained with crystal violet. The stained cells were observed using a microscope. **(D)** The stained cells were counted and statistically analysis was performed using the Prism software. $^{*}P < 0.05$; $^{***}P < 0.001$. Data are presented as means ± s.d. (two-way ANOVA with Tukey's multiple comparisons test) and experiments were repeated three times (n = 3).

demonstrated that actin reorganization and vimentin expression of cancer cells affected NK cell cytotoxicity.

NK cell cytotoxicity is regulated by a multistep process. NK cells interact with intercellular adhesion molecule 1 (ICAM1) and activating receptors, such as NCR, NKG2D, and DNAM1 *via* CD2, selectin CD62L, and adhesion integrin receptors (LFA1 and Mac1) (31, 32). These processes cooperate with integrin signaling to promote the formation of NK cell immunological synapses (NKIS). The formation of adhesion ring junctions after NK cell contact with cancer cells requires signaling mediated by cytoskeleton remodeling (33). F-actin accumulates in NKIS and is important for promoting the aggregation of various activating receptors. F-actin organization is a major driver of immune synapse formation and lipid raft polarization between NK and target cells (34–36). Actin retrograde flow (ARF) regulates NK cell signaling and NK cell activation and inhibition. This mechanotransduction process is regulated by the dynamics of actomyosin (37–39).

The stiffness of the matrix affected the cytotoxicity of NK cells and promoted the secretion of the NK cytokine interferon-gamma (IFN-γ). Cytokine release increases when NK cells interact with stiffer substrates. Cell stiffness changes continuously during the tumor process, with primary tumor cells being stiffer than healthy cells, and highly metastatic cells being less stiff (40). Additionally, viral infections can increase stiffness by inducing cortical actin rearrangement (41). Therefore, we speculated that the stiffness of the cells could affect the responsiveness of NK cells to infiltrate tumors.

Al Absi et al. found that actin cytoskeleton remodeling drives cancer cells to escape NK cell-mediated cytotoxicity (19). For example, after breast cancer cells encounter NK cells, actin accumulates at the immune synapses, which in turn limits the accumulation of the cytotoxic molecule granzyme B (GrzB) in cancer cells. Thus, cancer cells are protected from lysis and apoptosis (42, 43). The urothelial and lung cancer cell lines used in this study also exhibited actin accumulation at the immune synapses in resistant cancer cells but not in NK-sensitive cancer cells (Figure 2). This suggests that sensitive cancer cells may have a higher capacity to accumulate actin filaments at the immune synapses than resistant cancer cells. This reveals that differences in the accumulation of actin filaments in cancer cells at immune synapses may affect NK cell killing and cancer cell resistance. Most studies so far have focused on actin reorganization regulating NK cell function, and our study showed that interference with actin reorganization and vimentin expression affected the ability of NK cells to kill cancer cells (Figures 2, 3). Our findings reveal the molecular and biophysical mechanisms by which dynamic cytoskeletal networks within cancer cells regulate NK cytotoxicity. The expression of vimentin, which coordinates lytic granule trafficking, MTOC polarization and actin dynamics at the immune synapses, remains unclear. An in-depth exploration of this information will help classify cancer cell sensitivity to NK cells and provide opportunities for future cancer treatment.

Al Absi et al. demonstrated that actin accumulation significantly protects cancer cells from NK-mediated cytotoxicity (19). A possible reason why cancer cells escape death is the creation of a physical barrier that renders lytic particles ineffective. Another escape mechanism can be the aggregation of the inhibitory checkpoint ligands. We speculated that actin reorganization and vimentin expression may induce PD-L1 coalescence at NKIS to evade NK cell surveillance. Studies from Al Absi et al. also showed that the inhibition of actin nucleation factors, such as N-WASP or Cdc42, in cancer cells restored NK cell-mediated cytotoxicity (19). In our study, interference with the remodeling of actin cytoskeleton and inhibition of vimentin can effectively promote NK cytotoxicity. Therefore, approaches aiming to modulate cytoskeletal proteins of cancer cells may have therapeutic potential.

Periodic exposure to the tumor microenvironment may induce NK cell exhaustion (44–48). Such exhausted NK cells typically express one or more inhibitory checkpoint receptors, such as programmed cell death protein 1 (PD-1), TIM-3, or TIGIT, which limits their cellular activity. Exhausted NK cells show reduced proliferation, cytokine release and degranulation. Actin cytoskeleton remodeling of target cells affects CTL recognition and lysis efficiency of NK cells. Changes in the actin dynamics in cancer cells result in immune evasion by inhibiting immune synapse formation or interfering with effector functions (19, 49–51). Decreased actin on the surface of cancer cell membranes may not only prevent strong adhesion of immune cells but also reduce the strength of mechanoreceptor signaling, resulting in insufficient NK activation signaling at the immune synapse. Enhanced actin dynamics in cancer cells reduce the cytotoxic enzymes perforin and GrzB (19). The actin cytoskeleton flanking the immune synapse determines lymphocyte cytotoxic attack and cancer cell resistance.

An interesting finding of our study was that urothelial cancer cell lines contain two types of cells, one of which was immunoresistant cells that responded to NK cell attack by regulating actin reorganization and vimentin expression while surviving this attack. Another group of immune-sensitive cells lacked this regulatory function and was highly sensitive to lysis by NK cells. Therefore, when studying cytotoxic immune responses against tumors, the immunoresistance caused by reorganization of cytoskeletal proteins in response to immune cell attack should be considered. We propose that targeting cytoskeleton remodeling in cancer cells may improve the efficacy of immunotherapy.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Committee of Chang-Gung Medical Foundation (IRB number: 201504731B0 on 14 September 2015; 202101713B0 on 4 October 2021). The patients/participants provided their written informed consent to participate in this study.

Author contributions

Conceived and designed the experiments: J-MP, H-YL, Y-LC, and H-LL. Performed the experiments: J-MP, J-WL, and Y-TW. Contributed reagents/materials/analysis tools: C-FC, J-HC, and H-LL. Wrote the manuscript: J-MP, J-WL. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.883178/full#supplementary-material

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