BiTE secretion by adoptively transferred stem-like T cells improves FRα+ ovarian cancer control

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ABSTRACT

Background Cancer immunotherapies can produce complete therapeutic responses, however, outcomes in ovarian cancer (OC) are modest. While adoptive T-cell transfer (ACT) has been evaluated in OC, durable effects are rare. Poor therapeutic efficacy is likely multifactorial, stemming from limited antigen recognition, insufficient tumor targeting due to a suppressive tumor microenvironment (TME), and limited intratumoral accumulation/persistence of infused T cells. Importantly, host T cells infiltrate tumors, and ACT approaches that leverage endogenous tumor-infiltrating T cells for antitumor immunity could effectively magnify therapeutic responses.

Methods Using retroviral transduction, we have generated T cells that secrete a folate receptor alpha (FRα)-directed bispecific T-cell engager (FR-B T cells), a tumor antigen commonly overexpressed in OC and other tumor types. The antitumor activity and therapeutic efficacy of FR-B T cells was assessed using FRα+ cancer cell lines, OC patient samples, and preclinical tumor models with accompanying mechanistic studies. Different cytokine stimulation of T cells (interleukin (IL)-2+IL-7 vs IL-2+IL-15) during FR-B T cell production and the resulting impact on therapeutic outcome following ACT was also assessed.

Results FR-B T cells efficiently lysed FRα+ cell lines, targeted FRα+ OC patient tumor cells, and were found to engage and activate patient T cells present in the TME through secretion of T cell engagers. Additionally, FR-B T cell therapy was effective in an immunocompetent in vivo OC model, with response duration dependent on both endogenous T cells and FR-B T cell persistence. IL-2/IL-15 preconditioning prior to ACT produced less differentiated FR-B T cells and enhanced therapeutic efficacy, with mechanistic studies revealing preferential accumulation of TGF-1+CD39+CD69– stem-like CD8+ FR-B T cells in the peritoneal cavity over solid tumors.

Conclusions These findings highlight the therapeutic potential of FR-B T cells in OC and suggest FR-B T cells can persist in extratumoral spaces while actively directing antitumor immunity. As the therapeutic activity of infused T cell therapies in solid tumor indications is often limited by poor intratumoral accumulation of transferred T cells, engager-secreting T cells that can effectively leverage endogenous immunity may have distinct mechanistic advantages for enhancing therapeutic responses rates.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Despite promise, current immunotherapies (including adoptive T cell therapy; ACT) are ineffective for patients with ovarian cancer. While folate receptor alpha (FRα) is considered a promising target antigen in ovarian cancer, no broadly effective FRα-targeted therapies have been realized clinically.

WHAT THIS STUDY ADDS

⇒ Engineered T cells secreting FRα-targeted T cell engagers (FR-B T cells) can be employed to effectively target ovarian cancer. Further, FR-B T cells can preferentially persist in extratumoral locations outside of solid tumors and secrete engagers to redirect host T cells for tumor attack, thereby leveraging endogenous immunity to control tumor growth.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ ACTs that incorporate strategies for redirecting host T cells for tumor attack have the potential to magnify therapeutic responses in tumor indications that are refractory to immunotherapy, including ovarian cancer.

BACKGROUND

Cancer immunotherapies, including adoptive T-cell transfer (ACT), have demonstrated impressive clinical activity, however their benefit in ovarian cancer (OC) has generally been limited. While effective in hematological cancers, ACT has shown modest clinical impact when treating solid tumors, with the notable exception of autologous tumor-infiltrating T cells (TILs) where clinical responses have been achieved in melanoma and less frequently in other cancers. In OC, the limited impact of ACT likely arises from the immunosuppressive tumor microenvironment (TME). Additionally, although TIL abundance correlates with improved survival in OC, recent evidence suggests most CD8+ TILs in OC patient tumors do not recognize cancer cells, instead comprised predominantly of
bystander TILs. Importantly, bystander TILs do not upregulate inhibitory receptors and persist as functional effector T cells. Therefore, ACT-based approaches that effectively engage and mechanistically redirect bystander TILs for antitumor immunity are likely to overcome local immune suppression and enhance tumor attack.

Specific T-cell engagers or BiTEs (a trademark of Amgen) can redirect T cells for antigen-specific targeting and are currently in development for OC. However, conventional BiTEs have an intrinsically short half-life, necessitating repeated or continuous infusion to achieve therapeutic BiTE exposure, in addition to a prerequisite for adequate intratumoral T cell availability to elicit responses. To overcome these limitations, generating BiTE-secreting T cells (referred to here as BiTE-T cells) has emerged as a promising modality, where unlike conventional chimeric antigen receptor (CAR)-engineering or T cell receptor (TCR)-engineering strategies, BiTE-T cells secrete BiTEs to redirect both BiTE-T cells and host T cells, thereby magnifying therapeutic responses.

When assessing target antigens for ACT with broad expression in OC that can be targeted without severe risk of on target/off tumor toxicity, folate receptor alpha (FRα) has emerged as an optimal target. FRα is expressed by most epithelial OC cells with restricted normal tissue expression and has been associated with OC relapse and chemotherapy resistance. Further, targeting FRα using multiple therapeutic approaches have been or are being tested clinically, collectively demonstrating encouraging clinical responses and a generally favorable safety profile as highlighted by the recent Food and Drug Administration accelerated approval of the FRα-targeted antibody drug conjugate mirvetuximab soravtansine (MIRV). However, durable and/or broadly curative therapies targeting FRα in OC have not been identified, suggesting innovative strategies that integrate multiple approaches to enhance FRα targeting are needed to improve outcome.

In this study, we have combined BiTE-based technologies and the therapeutic potential of targeting FRα to develop a novel ACT approach for OC that uses engineered FRα-targeted BiTE-T cells (FR-Bh T cells; human, FR-B T cells; mouse). FR-B(h) T cells were highly effective against both FRα+ OC patient samples and in immunocompetent preclinical tumor models. Moreover, mechanistic studies revealed that improved therapeutic efficacy was accompanied by preferential accumulation of less differentiated stem-like FR-B T cells in the extratumoral peritoneal OC TME over solid tumor lesions. This suggests that FR-B T cells in remote locations can promote tumor destruction in OC (by secreting BiTEs and engaging endogenous T cells) without an absolute requirement for direct accumulation in solid tumors. These findings have important implications for future ACT therapies used to treat solid tumors, including OC, where limited tumor reactivity from endogenous T cells can create therapeutic challenges.

**METHODS**

**Cell culture**

SKOV-6 (cervical), SKOV-3 (ovarian), OV167 (ovarian), OVCAR8 (ovarian), OVCAR3 (ovarian), K562 (leukemia), IE9-mp1 (ovarian), IE9-mp1-human FRα (hFRα) (ovarian), Pan02-hFRα (pancreatic) cancer cell lines were grown in complete Roswell Park Memorial Institute media (RPMI; cRPMI) containing 10% fetal bovine serum (FBS), 25 mM HEPES, 2 mM L-glutamine, 100 IU/mL Pen/Strep, 1 mM sodium pyruvate, 1x nonessential amino acids, and 0.05 mM β-mercaptopethanol. 293T, PG13, and PLAT-E cell lines were grown in complete Dulbecco’s Modified Eagle Medium (DMEM; cDMEM) containing 10% FBS and 100 IU/mL Pen/Strep. Cell lines were IMPACT tested and/or confirmed Mycoplasma negative prior to use.

**Generation of hFRα-expressing cell lines, BiTE constructs and retroviral vectors**

An aggressively growing and immunotherapy-resistant IE9-mp1 variant recovered at disease relapse following immunotherapy was used to generate the hFRα-expressing IE9-mp1-hFRα cell line using the Sleeping Beauty Transposon system as detailed in the online supplemental methods. As a second murine model, Pan02-hFRα cells were also produced. FR-Bh binds hFRα via a single chain variable fragment (scFv) derived from the M0v19 antibody and human CD3ε via an scFv derived from the UCHT1 antibody. FR-B binds hFRα as above and mouse CD3ε via an scFv derived from the 145–2C11 antibody. The design/ construction of all engager sequences, production of retroviruses, and testing for engager binding have been described in the online supplemental methods.

**T-cell activation and transduction**

Human or mouse T cells were activated using anti-CD3ε and anti-CD28 antibodies (Bio X Cell) prior to retroviral transduction. Specific activation/culture conditions and retroviral transduction protocols have been detailed in the online supplemental methods.

**In vitro co-cultures**

Human or mouse T cells were cultured for no less than 8 days post activation before assay set-up. T cells were co-cultured with target cells at the indicated effector to target (E:T) ratios in cRPMI for 24 or 48 hours. For serial stress test studies involving repeated and prolonged co-culture of mouse T cells with target cells, T cells were harvested, counted, and resuspended in fresh cRPMI–cytokine support (interleukin (IL)-2+IL-7 or IL-2+IL-15 as indicated) at the start of each new 3-day co-culture period. Additional details have been included in the online supplemental methods.

**OC patient samples and targeting using BiTE-T cells**

Cryopreserved OC patient ascites samples (online supplemental table 1) containing both immune cells and tumor cells were obtained from the Roswell Park Gyn Onc Tissue
Bank and were collected from patients with OC undergoing care at Roswell Park and processed for banking. Thawed cells were washed, counted to determine tumor cell number (viable tumor cells defined as large mononuclear cells using trypan blue exclusion), and plated in 6-well plates at 10^5 tumor cells/well in cRPMI. Patient samples were cultured with FR-Bh T cells or T cells secreting a control engager (CONT-ENG T cells) that were prelabeled with CellTrace Violet and added at a BiTE:T cell: tumor cell ratio of 4:1. OC patient ascites samples±FR-Bh/CONT-ENG T cells were co-cultured for 48 hours prior to harvest. Additional details related to these studies have been included in the online supplemental methods.

Preclinical mouse models and therapeutic delivery of T cells
FR-Bh T cell evaluation in the SKOV-6 human xenograft model has been described in the online supplemental methods. For studies using immunocompetent mice, 6–8 weeks old female C57BL/6j and Rag1 KO (B6.129S7-Rag1<sup>−/−</sup>Menom/J) mice were purchased from the Jackson Laboratory and housed in the Roswell Park Comparative Oncology Shared Resource. 5×10<sup>6</sup> E9-mp1-hFRα cells (intraperitoneal (IP) in 500 µL phosphate buffered saline (PBS)) or 2×10<sup>6</sup> Pan02-hFRα (subcutaneous (SQ) in 100 µL PBS) were injected to establish tumors, with ACT commenced 5 days later. Mice received 8.33×10<sup>3</sup>–3×10<sup>6</sup> FR-B T cells or an equal number of unarmored control T cells (luciferase (Luc)/green fluorescent protein (GFP) transduced or mock transduced) delivered by locoregional injection (IP or intratumoral delivery for SQ tumors), with timing/dosing as indicated. FR-B T cell accumulation in the blood, peritoneal TME, or solid tumors was assessed 5-19 days post ACT. Additional details related to in vivo studies, tissue collection, processing, and analysis have been included in the online supplemental methods.

Antibodies and flow cytometry staining/analysis
Antibodies for flow cytometry were purchased from BioLegend or BD Biosciences and have been listed in online supplemental table 2. Antibodies were titrated for optimal staining for 30 min at 4°C in fluorescence activated cell sorting (FACS) buffer (2% FBS in PBS), BD Horizon Brilliant Staining Buffer, or intracellular staining buffer as required. Additional details related to sample staining and analysis have been included in the online supplemental methods.

Statistical analysis
Two-tailed, unpaired and paired t-tests were used to compare data between two groups. One-way and two-way analysis of variances were used for data analysis of more than two groups and/or across multiple time points and a Tukey’s or Sidak’s multiple comparisons test was used to determine significant differences between groups. Survival data was compared using a log-rank test. Results were generated using GraphPad Prism software.

RESULTS

Human BiTE-secreting T cells have specificity for FRα+ cancer cells and actively target OC
To target FRα+ OC, we generated an FRα-specific BiTE by linking a human CD3ε-specific scFv (UCHT1) and an MOV19-derived FRα-specific scFv using optimized linker sequences (online supplemental figure 1A, left panel). This BiTE, hereafter referred to as FR-Bh, was confirmed to bind FRα+ cancer cells and human T cells (online supplemental figure 1A, right panel). BiTE-secreting FR-Bh T cells were efficiently produced using retroviral transduction of activated primary human T cells (figure 1A), and in proof-of-concept studies, FR-Bh T cells (but not CONT-ENG T cells secreting an engager that binds FRα, but lacks CD3 binding; online supplemental figure 1A, right panel) effectively lysed FRα<sup>hi</sup> SKOV-6 target cells in vitro at even low E:T ratios (figure 1B, left panel and online supplemental figure 1B). Tumor cell lysis was accompanied by interferon (IFN)-γ production by FR-Bh T cells (figure 1B, right panel), consistent with antigen-driven effector function. FR-Bh T cells were confirmed to actively engage bystander T cells (via secreted BiTEs) using a transwell co-culture assay, where FR-Bh T cells plated in the upper chamber led to robust FRα+ target cell killing and effector function by untransduced (UTD) T cells in the lower chamber (online supplemental figure 2A). Furthermore, BiTE secretion by FR-Bh T cells was enhanced following acute or repeat antigen stimulation using FRα+ SKOV-6 cells, where BiTE production increased from 355 pg/10<sup>6</sup> FR-Bh T cells at baseline (measured over 3 days prior to antigen stimulation) to >2000 pg/10<sup>6</sup> FR-Bh T cells following antigen stimulation (measured over 3 days following co-cultures), an increase of >5-fold (online supplemental figure 2B) and suggesting that FRα+ tumor cell targeting by FR-Bh T cells led to increased BiTE availability. Therapeutic delivery of FR-Bh T cells to SKOV-6 tumor-bearing NSG mice produced robust tumor regressions not observed with CONT-ENG T-cell infusion (online supplemental figure 2C), confirming therapeutic activity of FR-Bh T cells against growing tumors.

To test if FR-Bh T cells could target clinically-relevant OC, FR-Bh or CONT-ENG T cells were co-cultured at a T cell: tumor cell ratio of 4:1 with OC patient specimens (isolated from peritoneal ascites at the time of surgery, online supplemental table 1) containing tumor cells and the patient’s own immune cells (online supplemental figure 3A). The frequency of FRα+ tumor cells (CD45–EpCAM+cells) across patients with OC was variable, ranging from 3.36% to 91.8% (figure 1C, online supplemental figure 3B, and online supplemental table 1), highlighting the heterogeneity of FRα positivity in OC. Patients
Figure 1  FR-Bh T cells target FRα+ tumor cells and initiate antitumor immune responses against patient with OC specimens. (A) Representative fluorescence activated cell sorting plots demonstrating efficient production of FR-Bh T cells via retroviral transduction. (B) % SKOV-6 target cell lysis (left) and IFN-γ production (right) following 24 hours co-culture with FR-Bh or CONT-ENG T cells at specified E:T ratios (n=3/condition). (C) % FRα+ cancer cells across tested patients with OC (n=10). (D–F) FRα+ and FRα− tumor cell number and corresponding IFN-γ production from 48 hour OC patient co-cultures following the addition of CONT-ENG or FR-Bh T cells. Baseline tumor cell number and IFN-γ (co-cultures containing endogenous TALs only) shown for comparison. Individual patients and FRα status as shown. (G) Heatmap showing relative changes and average log10 fold change for inflammatory factors following addition of CONT-ENG or FR-Bh T cells to patient co-cultures (n=4 patients). Data presented as mean±SEM. Data in (B) is from one representative experiment (three independent studies for target cell lysis, two independent studies for IFN-γ production), (D–F) was conducted once for each individual patient with OC. Data in (B) two-way analysis of variance and (G) paired t-test (two-tailed), p<0.05, ***p<0.001. BiTEs, bispecific T-cell engagers; CONT-ENG, control engager; E:T, effector to target; FC, Fold Change; FR-Bh, folate receptor alpha bispecific T-cell engager-secreting T cell; FRα, folate receptor alpha; GFP, green fluorescent protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; hFRα, human FRα; IFN, interferon; IL, interleukin; IP, intraperitoneal; MIP-1, macrophage inflammatory protein 1; OC, ovarian cancer; TALs, tumor associated lymphocytes; TNF, tumor necrosis factor.
with <20% FRα+ tumor cells were considered FRα- (n=3),
20%–50% FRα+ tumor cells FRα+ (n=3), and >50% FRα+ tumor cells FRαhi (n=4), respectively. Following 48 hours
culture, the FRα+ tumor cell number was reduced in the
majority of OC patient co-cultures when FR-Bh T cells
were added compared with cultures containing endoge-
nous tumor-associated lymphocytes alone (patient T cells
present in ascites; TALS only) or where CONT ENG
T cells were added (figure 1D–F and online supplemental
figure 3C), which was particularly evident for OC patients
with FRαim or FRαhi tumor cell frequencies. The reduc-
tion in FRαhi tumor cells was accompanied by increased
IFN-γ production in co-cultures containing FR-Bh T cells
for 9/10 patients (figure 1D–F and online supplemental
figure 3C, Blue Lines), with the only exception being a
patient with FRαhi OC (patient 1), where the addition of
CONT ENG or FR-Bh T cells produced a similar increase
in IFN-γ, possibly due to alloreactivity of the engineered
T cells to the OC patient cells (figure 1D). These studies
were conducted using engineered FR-Bh T cells (or
CONT ENG T cells) generated from two different healthy
donors (online supplemental figure 3D) and immune
reactivity to FRα+ OC patient specimens following addition
of FR-Bh T cells was observed for both donors (online
supplemental figure 3E).

To gain insights into the breadth of inflammatory
changes driven by FR-Bh T cell therapy against clinical OC
specimens, we selected four OC patients that responded
to FR-Bh T cells from the FRαim and FRαhi cohorts and
broadly analyzed immunological changes in co-cul-
tures containing CONT ENG T cells or FR-Bh T cells
using the Isoplex Human Adaptive Immune Codeplex
Secretome Panel (figure 1G). Analysis revealed robust
inflammatory changes beyond IFN-γ (log10 FC=1.83, p=0.0026),
including increased production of granulocyte-macrophage
colony-stimulating factor (GM-CSF) (log10 FC=3.21, p=0.0006), granzyme B (log10
FC=2.29, p=0.0035), macrophage inflammatory protein 1
(MIP-1)α/β (log10 FC=1.53, p=0.0191 and log10 FC=1.783,
p=0.0213, respectively), as well as upregulation of type-1
cytokines including IL-5 (log10 FC=1.705, p=0.0039) and the
upper panel and online supplemental figure 4B). In
contrast, upregulation of these activation markers by
FR-Bh T cells was limited/variable in co-cultures with
FRαhi specimens (figure 2B lower panel and online
supplemental figure 4B) and were nearly absent when
FR-Bh T cells were cultured alone (online supplemental
figure 4C), demonstrating FRα-dependent FR-Bh T cell
activation. Activation of UTD bystander CD8+ and
CD4+ T cells was also observed in FRα+ OC patient samples after
FR-Bh T cell addition, although the effects were modest
compared with transduced FR-Bh T cells (online
supplemental figure 4D). Notably, activation of endogenous OC
patient CD8+ TALs was readily observed following the
addition of FR-Bh T cells but not CONT ENG T cells to
FRα+ OC patient samples, with significant upregulation
of surface CD25 and CD137 (figure 2C,D), demonstrating
effective activation and redirection of endogenous T cells
present in the OC TME of human cancer by FR-Bh T cells.
Activation of OC patient CD4+ TALs by addition of
FR-Bh T cells was also observed, although the effects
were modest and variable across patients with FRα+ OC
(online supplemental figure 4E). No correlation between
the frequency of endogenous patient CD8+ or CD4+
TALs and the magnitude of response to FR-Bh T cells
(measured as IFN-γ production) was observed across
patients (online supplemental figure 3A,B, left), nor was
a clear threshold identified for the frequency of endoge-
nous T cells required to elicit a response. Furthermore,
the frequencies of endogenous CD8+ or CD4+ TALs did
not differ significantly between patients with FRαhi and
FRα+ OC (online supplemental figure 5A,B, right). Of
note, the majority of OC patient endogenous CD8+ TALs
were negative for CD39 at baseline (online supplemental
figure 5C), consistent with bystander T cells lacking
tumor-specificity10 and suggesting OC patient TALs acti-
vated via BiTE secretion by FR-Bh T cells had limited
intrinsic tumor reactivity. These findings suggest that

**FR-Bh T cells effectively engage OC patient T cells present in
the peritoneal TME**

Based on these data, we reasoned that both FR-Bh T
cells and endogenous patient T cells present in the
OC TME may be actively engaged following delivery of
FR-Bh T cells, thereby contributing to the BiTE-driven
T-cell response. To permit separate interrogation of
BiTE-T cell versus host T-cell activation in co-cultures
containing patient OC specimens, exogenously added
T cells (comprised of engineered FR-Bh/CONT-ENG-
producing and bystander non-transduced T cells) were
labeled with CellTrace Violet (CTV) prior to addition to
co-cultures, permitting discrete assessment of transferred
(CTV+; transduced (green fluorescent protein (GFP)+)
and UTD bystander (GFP−) T cells) and endogenous
(CTV–GFP−) T cells (online supplemental figure 4A).
As we had observed similar inflammatory responses and
accompanying reduction in FRα+ tumor cells for FRαhi
and FRαhi OC patients following addition of FR-Bh T
cells, these patients were grouped as FRα+ patients
(>20% FRα+ tumor cells) to evaluate T-cell activation
and compared with patients with FRαhi OC (<20% FRα+
tumor cells) where only modest responses were observed.
Following co-culture, CD8+ and CD4+ FR-Bh T cells (but
not CONT ENG T cells) were highly reactive to FRα+ OC
specimens, leading to robust upregulation of multiple
activation markers including CD25, CD69, CD137, and
Programmed cell death protein 1 (PD-1) (figure 2A,B
upper panel and online supplemental figure 4B). In
contrast, upregulation of these activation markers by
FR-Bh T cells was limited/variable in co-cultures with
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figure 5C), consistent with bystander T cells lacking
tumor-specificity10 and suggesting OC patient TALs acti-
vated via BiTE secretion by FR-Bh T cells had limited
intrinsic tumor reactivity. These findings suggest that
the antitumor response driven by FR-Bh T cells in OC involves activation of BiTE-producing FR-Bh T cells and engagement of endogenous OC patient T cells present in the peritoneal TME.

**Therapeutic delivery of BiTE-secreting T cells improves tumor control and survival in an immunocompetent OC model**

Given that FR-Bh T cells engaged/activated endogenous T cells in OC patient samples, we next tested the therapeutic delivery of FRα-directed BiTE-T cells in an immunocompetent OC mouse model. To do so, an aggressively growing and immunotherapy-resistant variant of the IE9-mp1 OC cell line was engineered to stably express hFRα (IE9-mp1-hFRα) and a chimeric BiTE specific for hFRα and mouse CD3ε was generated (hereafter referred to as FR-B) (online supplemental figure 6A). FR-B was confirmed to bind to both IE9-mp1-hFRα target cells and mouse T cells (online supplemental figure 6B). FR-B-secreting T cells (FR-B

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**Figure 2** FR-Bh T cells and endogenous OC patient T cells are activated by bispecific T-cell engagers when directed against FRα+ OC patient samples. (A) Representative fluorescence activated cell sorting (FACs) plots showing surface expression of activation markers (CD25, CD69, CD137, PD-1) for CD8+ CONT ENG or FR-Bh T cells following 48 hours co-culture with a FRα+ OC patient sample. (B) Graphical representation of data in (A) across all FRα+ patients (upper, n=7) and FRα− patients (lower, n=3). (C) Data as in (A) for endogenous CD8+ tumor associated lymphocytes (TALs). (D) Graphical representation of data in (C) across all FRα+ patients (n=7). Baseline activation (endogenous T cells only) was used for comparison. For data in (B) and (D), connected data points and unique colors correspond to individual patients with OC. Data in (B) unpaired and (D) paired t-test (two-tailed), *p<0.05, **p<0.01, ***p<0.001. CONT-ENG, control engager; FR-Bh, folate receptor alpha bispecific T-cell engager-secreting T cell; FRα, folate receptor alpha; OC, ovarian cancer; PD-1, Programmed cell death protein 1.
T cells (FR-B T cells) were generated with high efficiency from activated mouse splenocytes by retroviral transduction (figure 3A) and demonstrated robust killing and antigen-driven effector function in co-culture assays with IE9-mp1-hFRa, but not FRα- parental IE9-mp1 target cells (figure 3B). Like human FR-Bh T cells, transduced CD8+ and CD4+ FR-B T cells and accompanying UTD bystander T cells were activated in the presence of hFRα+ target cells (figure 3C), consistent with FR-B-mediated redirection of bystander T cells.

To evaluate FR-B T cells therapeutically, IE9-mp1-hFRα tumor-bearing mice were treated with FR-B or unarmed control T cells (either UTD or T cells engineered to express a Luc-GFP fusion protein) and monitored for tumor progression and survival (figure 3D). As localized delivery of adoptively transferred CAR-T cells directly into the peritoneal OC TME can effectively control OC progression,30-32 tumor-bearing mice were treated by IP injection of FR-B T cells. Locoregional delivery of FR-B T cells significantly delayed OC progression compared with control T cells (figure 3D, median survival for unarmed control T cells of 36.5 days compared with 51 days for FR-B T cells) and this effect was confirmed in the subcutaneous Pan02-hFRα tumor model (online supplemental figure 6C). Consistent with data from patients with OC demonstrating endogenous T-cell activity in response to secreted BiTEs, lymphodepletion of mice prior to implanting IE9-mp1-hFRα tumors and infusing FR-B T cells led to early tumor progression similar to treatment with unarmed T cells (online supplemental figure 6D), confirming endogenous T cells are required for optimal tumor control following ACT with FR-B T cells. Analysis of TILs (solid tumor) and TALs (ascites) from IE9-mp1-hFRα tumor-bearing mice at experimental endpoint (due to progressive disease) revealed limited persistence of FR-B T cells (online supplemental figure 6E), suggesting that
tumor outgrowth was associated with clearance of FR-B T cells.

**Stem-like FR-B T cells can be produced through cytokine preconditioning and improve antitumor immunity following ACT**

Based on our in vivo findings, we reasoned that employing strategies to improve FR-B T cell persistence following infusion would improve therapeutic efficacy. As IL-15 stimulation has been shown to promote a less-differentiated stem cell memory phenotype, increase mitochondrial metabolic fitness, improve T-cell persistence following infusion of CAR-T cells, and can enhance the activity of BiTE-T cells, we tested whether IL-15 preconditioning prior to ACT would impact FR-B T cell efficacy and response durability against OC. As FR-B T cells were produced in the presence of IL-2 and IL-7 (FR-B 2/7) in prior experiments, we directly compared this approach to FR-B T cells produced using IL-2 and IL-15 stimulation (FR-B 2/15). FR-B 2/7 and FR-B 2/15T cells were generated with similar efficiency by retroviral transduction (figure 4A), with FR-B 2/15T cells having increased TCF-1 expression (figure 4B) and an elevated usage of mitochondrial metabolism (figure 4C) compared with FR-B 2/7T cells, consistent with previous data. FR-B 2/15T cells produced more than 10-fold less IFN-γ than FR-B 2/7T cells (figure 4D, left panel) and had a reduced capacity to kill IE9-mp1-hFRa cells in co-culture assays (figure 4D, right panel), consistent with a less differentiated T cell phenotype. However, when tested in an in vitro serial co-culture ‘stress test’ of chronic antigen exposure (online supplemental figure 7A), the capacity of FR-B 2/15T cells to promote durable antitumor activity emerged. While FR-B 2/7T cells dramatically expanded (>5-fold) prior to abrupt contraction, FR-B 2/15T cells demonstrated limited expansion in response to antigen stimulation over the entire co-culture period (figure 4E). However, while both FR-B 2/7 and FR-B 2/15T cells cleared all tumor cells in the first two serial co-cultures, FR-B 2/7T cells developed a reduced ability to lyse IE9-mp1-hFRa tumor cell targets by the third co-culture, while FR-B 2/15T cell lytic function was maintained (figure 4F), suggesting that FR-B 2/15T cells have a greater capacity to sustain antitumor activity over a prolonged period. When evaluated therapeutically, adoptive transfer of a single dose of FR-B 2/15T cells 5 days post tumor implantation significantly improved tumor control and long-term survival of IE9-mp1-hFRa tumor-bearing mice compared with FR-B 2/7T cells (figure 4G).

Similar to data generated following lymphodepletion of IE9-mp1-hFRa tumor-bearing host mice (online supplemental figure 6D), the therapeutic activity of FR-B 2/15T cells was almost completely abrogated in IE9-mp1-hFRa-bearing RAG1 KO mice lacking mature T cells and B cells (online supplemental figure 7B), consistent with a key mechanistic role for endogenous T cells in promoting the therapeutic activity of FR-B T cells. Together, these data suggest that generation of FR-B T cells that effectively persist following chronic tumor antigen stimulation led to improved therapeutic efficacy compared with engineered T cells with a heightened capacity for short-term effector function.

**IL-2/IL-15 preconditioning improves FR-B T cell persistence in the extratumoral OC peritoneal TME**

To better understand the improved antitumor effects of FR-B 2/15T cells, we compared the tissue localization of FR-B 2/7 and FR-B 2/15T cells following infusion. FR-B CD4+T cells demonstrated limited accumulation in the blood, peritoneal TME (TALs), as well as solid tumor lesions (TILs), although a trend towards increased accumulation of FR-B 2/15 CD4+ TALs compared with FR-B 2/7 CD4+ TALs was observed (online supplemental figure 8A). FR-B CD8+ T cells had limited accumulation in the blood, with a modest increase in abundance in solid tumor lesions (figure 5A and online supplemental figure 8B), consistent with antigen-driven FR-B T cell accumulation at tumor sites. However, there was no difference in the accumulation of FR-B CD8+TILs between 2/7 and 2/15 conditioned T cells, with the majority of CD8+TILs (~90%) comprised of GFP-endogenous and/or bystander T cells (figure 5A). Further analysis revealed increased activation (CD69+) and proliferation (Ki67+) of endogenous/bystander CD8+ TILs present within solid OC tumor lesions in response to either FR-B 2/7 or FR-B 2/15 T cell delivery (online supplemental figure 8), consistent with data from human patients with OC and supporting a mechanistic role for these T cells in the antitumor response.

In contrast to the blood and solid OC tumors, the frequency of FR-B 2/15 CD8+TALs in the peritoneal cavity was elevated more than threefold compared with FR-B 2/7 TALs (figure 5B) and comprised an increased proportion of the total CD45+ immune infiltrate in the peritoneal TME (online supplemental figure 8), suggesting an overall improved capacity of FR-B 2/15 CD8+T cells to persist in the extratumoral peritoneal OC TME. Moreover, increased Ki67+FR-B 2/15 CD8+TALs were observed compared with FR-B 2/7 CD8+TALs (figure 5C, online supplemental figure 8E), suggesting ongoing T-cell proliferation. Further phenotypic analysis revealed increased accumulation of stem-like CD39–CD69– CD8+ T cells among the FR-B 2/15 CD8+TALs versus more differentiated CD39+CD69+ T cells present in the FR-B 2/7 CD8+ TALs (figure 5D, online supplemental figure 8F). Consistent with this finding, FR-B 2/15 CD8+ TALs also maintained elevated TCF-1 following ACT when compared with the FR-B 2/7 CD8+ TALs (figure 5E, online supplemental figure 8G). Follow-up studies revealed that the stem-like CD39–CD69– phenotype of the FR-B 2/15 CD8+ TALs diminished over time, but was maintained in a proportion (38%) of the FR-B 2/15 CD8+ TALs at 19 days post ACT (figure 5F, online supplemental figure 9). Additionally, the frequency of TCF-1+FR-B 2/15 CD8+ TALs was similar 5 and 12 days post ACT and was further elevated
in the FR-B 2/15 CD8+ TALs persisting 19 days following infusion (figure 5G, online supplemental figure 9).

Transcriptional profiling of flow cytometry-sorted CD8+FRB 2/15 and FR-B 2/7 TALs isolated 5 days post ACT suggested limited differences in effector function or expression of checkpoint pathways between the transferred T cells (online supplemental figure 10A). However, hierarchical clustering of differentially expressed genes revealed FR-B 2/15 and FR-B 2/7 CD8+TALs to have highly distinct transcriptional profiles (online supplemental figure 10), with differences in genes associated with multiple cellular processes (figure 5H). CD8+ FRB
2/15 TALs had upregulated expression of genes associated with cell proliferation (E2F8, Ercc6l, Cenph, Cdc7, Trip13) and cell survival (Ift3, Egr1), consistent with improved in vivo persistence observed at the cellular level. Additional upregulated genes associated with T-cell activation and IFN response (Cstad, Ifit1), as well as cellular metabolism and energy homeostasis (Gstm5, Bco1, Ckb) were observed, suggesting that CD8+ FRB 2/15 TALs can...
persist as activated T cells, potentially through changes in cellular metabolism. In contrast, CD8+ FRB 2/7 TALs upregulated genes related to apoptotic signaling (Rai14) and negative regulation of transcription and NF-kB signaling (Zscan10, Ppm1n), consistent with poor in vivo persistence and limited T-cell activity. Additionally, FR-B 2/7 TALs upregulated genes associated with fatty acid metabolism (Acox1) and while transcriptional modeling using an established metabolic pipeline did not reveal significant metabolic pathway alterations between FR-B 2/7 and FR-B 2/15 T cells, the most highly upregulated pathways for FR-B 2/7 CD8+ TALs were fatty acid associated pathways (online supplemental figure 11A). Modeling of mitochondrial long chain fatty acid beta oxidation pathway usage by FR-B T cells revealed multiple transcripts, including ACOT4 and ACSL6, to be upregulated in FR-B 2/7 T cells (inversely, suppressed in FR-B 2/15 T cells) (online supplemental figure 11B), suggesting cellular changes in FR-B 2/7 T cells may result in alterations to both peroxisomal function and mitochondrial respiratory capacity. Furthermore, FR-B 2/7 T cells were also enriched for genes associated with regulation of endocytic process (Ston2), increased inflammatory response (CSF2), collagen binding (Coch), extracellular matrix adhesion (TINAGL1), as well as responses to extracellular signaling (Pde4c, Pibh4), consistent with interactions between T cells and tumor stroma. Further, upregulation of CXCR5 and CCR6 by CD8+ FRB 2/7 TALs suggested an increased capacity for tissue homing by FR-B 2/7 TALs. Pathway analysis revealed key differences between preconditioning strategies, with FR-B 2/15 TALs enriched for pathways associated with cell replication and T-cell function, whereas FR-B 2/7 TALs were enriched for transforming growth factor beta (TGF-B) responsiveness, chemokine signaling, and extracellular matrix (ECM) interaction (online supplemental figure 12). Collectively, these data suggest that IL2/IL-15 preconditioned CD8+ FRB TALs have improved persistence and an increased capacity to maintain a less differentiated phenotype, while upregulating cellular processes that serve to maintain FR-B T cell proliferation, survival, and BiTE-driven tumor attack from within the peritoneal OC TME.

**DISCUSSION**

In the present study, the robust activity of FR-B(h) T cells was accompanied by engagement of endogenous T cells in the OC TME, thereby overcoming limited endogenous immunoreactivity or local tumor immunosuppression. Delivery of T cells by IP injection has been shown to result in accumulation of infused T cells in solid tumors in the peritoneal cavity, which is in line with our data for FR-B T cells. However, FR-B T cells comprised only a small fraction of the TILs found in solid OC and the improved therapeutic effects of FR-B 2/15 over FR-B 2/7 T cell therapy correlated with differences in FR-B T cell accumulation outside of solid tumors (figure 6). Of note, although the majority of the FR-B 2/15 T cells that accumulated in the peritoneal TME were CD8+ TALs, we also noted a modest increase in the frequency of FR-B 2/15 CD4+TALs compared with FR-B 2/7 CD4+TALs, suggesting IL-2/IL-15 preconditioning can improve the accumulation/persistence of both FR-B T cell subsets. While IL-2/IL-15 preconditioning produced stem-like FR-B T cells with a predominantly TCF-1+CD39– CD69– phenotype and improved persistence, we also recently reported on engineering T cells to stably express TCF-1 to enhance T-cell persistence and this approach could be incorporated when generating BiTE-T cells. Importantly, unlike conventional ACT approaches (eg, engineering tumor reactive CAR-T or TCR-T cells) where therapeutic failure can stem from limited tumor infiltration following T-cell infusion, the capacity of BiTE-T cells to mediate tumor attack from remote location(s) outside of solid tumors suggests strategies for generating durable responses may differ among these approaches.

A recent report demonstrated that tumor-specific CD8+ T cells that infiltrate and remain in tumors for at least 72 hours upregulate checkpoint receptors and can rapidly develop an exhausted phenotype, emphasizing that (i) limiting BiTE-T cell infiltration into tumors may be beneficial for prolonging BiTE-T cell activity and (ii) the activation of endogenous T cells by secreted BiTEs suggests the presence of newly infiltrating (and not yet exhausted) tumor-specific T cells or activation of bystander T cells that remain functional in the TME. Importantly, although we did not see clear correlation between the level of endogenous OC patient TALs and FR-Bh T cell-mediated responses in co-culture assays, endogenous patient T cells are engaged by secreted BiTEs, suggesting that the presence of endogenous T cells in the OC TME is likely to enhance the response to BiTE-T cells, in line with data from our immunocompetent mouse studies. Additionally, our data suggest that effector-like FR-B 2/7 CD8+ TALs increase fatty acid/lipid metabolism within the OC TME, metabolic reprogramming that has been associated with PD-1 signaling and suggesting FR-B T cells can also be impacted by inhibitory cues in the broader peritoneal OC TME that may promote early T-cell clearance. In light of our data, further studies will be needed to effectively unravel the complex relationship between the phenotype of FR-B T cells, accompanying metabolic alterations, and how these potentially intersecting and dynamic features can be leveraged to improve FR-B T cell persistence and antitumor immunity following infusion. Moreover, a recent report demonstrated that CD39-expressing CD8+ T cells can directly suppress the antitumor activity of tumor-specific T cells, suggesting the predominantly CD39+FRB 2/7 FR-B TALs may actually limit tumor attack in the OC TME within the first few days post infusión.

While we have focused our analysis on FR-B T cells residing broadly in the peritoneal cavity, it is possible that FR-B T cells may localize to other sites in the peritoneal space, including tumor-draining lymph nodes or the spleen. A small frequency of FR-B T cells was also
observed in circulation, raising the possibility that locoregional delivery of FR-B T cells could lead to antitumor immunity at distant metastatic sites. Importantly, as hFRα expression was restricted to the tumor in the current study, future studies will need to explore whether FR-B T cell antitumor activity can occur in the absence of unwanted reactivity when antigen is expressed in normal tissues. As such, we are also exploring incorporation of a switchable ON/OFF system to finely tune BiTE secretion by FR-B(h) T cells. Alternatively, FRα targeting using T-cell engagers that use a bivalent FRα-specific binding arm could improve safety and tumor selectivity in OC by eliciting low affinity/high avidity binding specifically to FRα high-expressing cells.15

Of note, we determined the frequency of FRα+ tumor cells by flow cytometry, which may yield differing results from established immunohistochemistry (IHC) assays,26 including the VENTANA FOLR1 companion diagnostic assay approved for use with MIRV.28 Therefore, it will be necessary to compare FRα positivity by flow cytometry and IHC, particularly as MIRV was found to be most effective for FRα+ patients (defined as >75% positive membrane staining, where a modest improvement over chemotherapy was observed).28 In contrast, we observed robust T cell activity in OC patient samples with >20% FRα+ tumor cells. As such, careful consideration of how OC patient FRα status is determined/defined may be essential in evaluating the clinical impact of different FRα targeting agents.

Intriguingly, in addition to elevated IFN-γ levels, we also noted increased production of Th2-associated cytokines (in particular IL-5) in response to FR-Bh T cells in OC patient samples, suggesting BiTE-driven activity of diverse T-cell subsets. Engaging multiple T-cell subsets, whether CD8+T cells or differentially polarized CD4+T cells (which can include regulatory T cells)11 may impact therapeutic response, particularly as T cells present in the TME at the time of ACT may exist in multiple heterogeneous states.42 Notably, we also observed a modest
reduction in IL-6 in patient samples treated with FR-Bh T cells, which contrasts with CAR-T cell therapy where elevated IL-6 has been associated with increasing severity of cytokine release syndrome.43

One potential limitation of BiTE-T cells is a lack of encoded co-stimulatory signal, which may promote early or rapid clearance following infusion. However, it was recently reported that CD19-specific BiTE-T cells could outperform CD19-specific CAR-T cells in a murine B-ALL patient-derived xenograft model,44 suggesting this limitation may be effectively balanced by engaging bystander T cells for cytolytic activity. Further, BiTE-T cell therapy can be augmented by engineering T cells to express co-stimulatory molecules or cytokines20 34 and given that soluble BiTEs effectively combine with blockade of checkpoint receptors including PD-1 and Cytotoxic T-Lymphocyte Associated Protein 4 (CTLA-4),17 it is likely that FR-B(h) T cells can additionally synergize with checkpoint blockade for treating OC.

Recent data suggests engaging CD3 using BiTEs may serve as a new strategy for generating ‘Off the Shelf’ T-cell therapies, whereby BiTEs delivered from BiTE-T cells can down regulate both CD3ε and TCR α/β on BiTE-engaged T cells.30 Additionally, multiarming T cells, for example, with CARs and BiTEs,21 46 can target multiple tumor antigens to overcome tumor heterogeneity and/or elicit immune attack on multiple target cell subsets. As we have noted heterogeneous FRα levels among OC patients, such strategies may be needed to overcome incomplete tumor targeting and outgrowth of antigen-loss-variants. In this regard, a recent report suggests engineering T cells with a tandem CAR targeting both FRα and mesothelin can enhance antitumor activity compared with single antigen targeting,47 although careful selection of multiple target antigens as to not increase toxicity needs to be carefully considered.

In conclusion, our findings demonstrate the potent effects of FR-B(h) T cells for ACT in OC, which can effectively redirect endogenous T cells to amplify antitumor immunity. Our data further highlight a unique attribute of FR-B T cells in OC to persist and direct antitumor activity from solid tumor-adjacent or extratumoral locations in the peritoneal TME, which may have distinct mechanistic advantages for enhancing response duration following ACT.

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Competing interests AJRM, TT, and KO are inventors on provisional patents pertaining to the development and use of BiTE-secreting T cells in cancer. KO is a co-founder of Tactical Therapeutics. All other authors have no financial conflicts of interest to disclose.

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Data availability statement Data are available upon reasonable request. Raw and processed RNA-seq data derived from FR-B 2/7 (n = 2) or FR-B 2/15 (n = 3) T cells supporting the findings of this study have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (NCBI-GEO) under accession number GSE218730. All other data will be made available upon reasonable request made to the corresponding author.

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