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ORIGINAL RESEARCH

Modulation of CD8$^+$ memory stem T cell activity and glycogen synthase kinase 3β inhibition enhances anti-tumoral immunity in gastric cancer

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ABSTRACT

The potential contributions of CD8$^+$ memory stem T cells to anti-tumor immunity and immunotherapy responses in gastric cancer has not been demonstrated. We found that CD8$^+$ memory stem T cell frequencies were increased in the peripheral blood of gastric cancer patients compared to healthy donors and declined in frequency with disease progression. Despite minimal *in vitro* cytotoxic activity, the adoptive transfer of CD8$^+$ memory stem T cells into Rag1$^{-/-}$ tumor bearing mice enhanced tumor regression compared to CD8$^+$ central or effector memory T cell counterparts. This effect was associated with an increase in splenic, draining lymph node and tumor infiltrating CD8$^+$ T cell numbers and the development of an altered CD8$^+$ T cell phenotype not seen during homeostasis. GSK-3β inhibition is known to promote memory stem T cell accumulation by arresting effector T cell differentiation *in vivo*. Surprisingly however, GSK-3β inhibition conversely increased the cytotoxic capacity of CD8$^+$ memory stem T cells *in vitro*, and this was associated with the induction of effector T cell-associated effector proteins including FasL. Finally, FasL neutralization following GSK-3β inhibition directly attenuated the anti-tumoral capacity of CD8$^+$ memory stem T cells both *in vitro* and *in vivo*. Altogether, our findings identify the therapeutic potential of modulating CD8$^+$ memory stem T cells for improved anti-tumoral responses against gastric cancer.

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Introduction

CD8$^+$ T cells play a critical role in the adaptive immune response to cancer. T cells are phenotypically categorized into naïve precursors (Tn), central memory (Tcm), effector memory (Tem), tissue-resident memory (Trm) and effector cells (Te). A rare subset of CD8$^+$ memory T cells capable of self-renewal and the multipotent reconstitution of effector and central and effector memory T cell subsets was recently identified in a mouse model of graft-versus-host disease.[1,2] Termed memory stem T cells (Tscm), these cells exhibit a naive-like phenotype but also co-express stem cell antigen-1 (Sca-1), their prototypic marker in mice, high levels of the anti-apoptotic molecule B cell lymphoma 2 (Bcl-2), the β chain of the IL-2 and IL-15 receptors (IL-2Rβ) and the chemokine (C-X-C motif) receptor CXCR3.[1] Tscm cells were also identified in humans and share phenotypic similarities with naïve T cells (CD45RO$^-$, CCR7$^+$, CD45RA$^+$, CD62L$^+$, CD27$^+$, CD28$^+$ and IL-7Rα^+) whilst also highly expressing CD95, IL-2Rβ and CXCR3.[3] Due to their ability to self-renew and continually differentiate into functional effector T cells, Tscm cells may represent a valuable tool in the development of more effective adoptive T-cell therapies against cancer. Although previously demonstrated in mice,[2,3] the potential importance of Tscm cells to anti-tumoral immunity and immunotherapy responses remains to be established in cancer patients.

Gastric cancer (GC) is one of the leading causes of cancer death in developing countries.[4] The prognosis of GC is frequently very poor partly due to its late diagnosis.[5] The potential long-term functional maintenance of tumor-specific CD8$^+$ Tscm cells in GC patients has not been formally established or quantitatively monitored. The putative anti-tumoral mechanism of CD8$^+$ Tscm cells in GC also remains to be elucidated.

The serine/threonine kinase, glycogen synthase kinase 3 (GSK-3), is constitutively active in resting T cells.[6] Two isoforms of GSK-3 (α and β) exist which have similar kinase domains but divergent N and C termini. TCR-CD28 signaling

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phosphorylates and inactivates GSK-3 whilst constitutively active GSK-3β inhibits $CD8^+$ T cell proliferation and IL-2 production, implying that GSK-3β inhibition mediates stimulatory responses following T cell priming.[7] Induction of Wnt-β-catenin signaling by GSK-3β inhibition, however, has also been established to promote the generation of $CD44^-CD62\ L^+Sca\text{-}1^+CD122^+Bcl\text{-}2^+CD8^+$ Tscm cells whilst arresting the development of $CD8^+$ T effector cells.[2] In $CD4^+$ T cells, GSK-3β inhibition is also reported to enhance iTreg differentiation.[8]

Herein, we demonstrate that $CD8^+$ Tscm cells are present in the peripheral blood, but not tumor tissue, of GC patients and decline in frequency with disease progression. Although cytotoxic responses were predominately generated by $CD8^+$ Tem cells *in vitro*, the adoptive transfer of $CD8^+$ Tscm cells into $Rag1^{-/-}$ tumor bearing mice enhanced tumor regression compared to other $CD8^+$ T cell subsets. This effect was associated with an increase in splenic, draining lymph node and tumor infiltrating $CD8^+$ T cell numbers and the development of an altered $CD44^-CD62\ L^-$ T cell phenotype not seen during homeostasis. GSK-3β inhibition conversely increased the cytotoxic capacity of $CD8^+$ Tscm cells *in vitro*, and this was associated with the induction of effector T cell-associated effector proteins like granzyme B and FasL. FasL neutralization following GSK-3β inhibition attenuated the anti-tumoral capacity of $CD8^+$ Tscm cells selectively *in vitro* and *in vivo*. In summary, our findings suggest that immunotherapy approaches incorporating $CD8^+$ Tscm cells modulation and/or GSK-3β inhibition may be of therapeutic interest in GC patients.

Results

$CD8^+$ Tscm cell frequencies are increased in the peripheral blood of GC patients and decline with disease progression

To evaluate the potential role of $CD8^+$ Tscm cells in human GC, we first phenotyped $CD8^+$ T cells immediately after isolation from the peripheral blood of GC patients. $CD8^+$ Tscm cells were defined as $CD45RA^+CCR7^+CD27^+CD95^+CD3^+CD8^+$ cells in our study (Fig. 1A). The percentage of $CD8^+$ Tscm cells within the total population of $CD8^+$ T cells was analyzed in samples from GC patients at various stages of disease progression. Peripheral blood samples from healthy donors were used as controls. GC patients showed a higher percentage of $CD8^+$ Tscm cells in the peripheral blood compared to healthy donors whereas no differences were detected for either $CD8^+$ Tn or Te cell subsets (Fig. 1B). Tumor progression was associated with a decrease in the frequency of peripheral blood $CD8^+$ Tscm cells in GC patients (Fig. 1C). Variations in $CD8^+$ Tscm cell percentages were not associated with age or gender (Supplementary Fig. S1 and S2). In line with previous reports,[9,10] we found that aging was associated with a gradual decline in naïve $CD8^+$ T cells and an increased proportion of $CD8^+$ memory T cells (Supplementary Fig. S2A).

We next characterized the distribution of different $CD8^+$ T cell subsets in GC patient tumor tissues. $CD8^+$ Tscm cells were not detected in neither tumor nor matched non-tumor tissues (Fig. 1D). In contrast, the percentage of $CD8^+$ Tcm cells was higher in tumor compared to non-tumor tissues, while $CD8^+$ Te cell percentages were lower in tumor compared to non-tumor tissues (Fig. 1E). Overall, we found that GC patients displayed higher frequencies of peripheral blood $CD8^+$ Tscm cells compared to healthy donors independent of age or gender and that peripheral blood $CD8^+$ Tscm cell frequencies declined with GC tumor progression.

$CD8^+$ Tem cells are the predominant mediators of tumor cell cytotoxicity in vitro

Since $CD8^+$ Tscm cells are relatively rare in human peripheral blood samples, C57 BL/6 mice injected subcutaneously with the fore stomach carcinoma cell line (MFC) were established as previously described[11] to confirm the relationship between $CD8^+$ Tscm cells and tumor progression. 4×10^6 MFC cells were subcutaneously injected into the mouse flank and tumor growth was monitored daily. $CD8^+$ Tscm cell analysis was conducted at day 10 post-MFC cell injection. $CD8^+$ Tscm cells were detected in the spleen and draining lymph node (DLN) of tumor-bearing mice. Similar to GC patient samples, $CD8^+$ Tscm cells were not identified in the tumors themselves. All $CD8^+$ T cells present in the tumors were $CD62\ L^-CD44^+$ (Supplementary Fig. S3), further indicating that $CD8^+$ Tscm cells did not traffic to the tumor site.

We next investigated the functional properties of different $CD8^+$ T subsets using our MFC tumor model. $CD8^+$ Tn, Tscm, Tcm and Tem cells from the spleens of MFC tumor-bearing C57 BL/6 mice were sorted and co-cultured with mitomycin C treated MFC cells (Fig. 2A). $CD8^+$ Tem cells were highly cytotoxic against MFC cells and there was a linear increase in killing activity across effector/target (E/T) ratios of 5:1 to 40:1. In contrast, the cytotoxic effects of $CD8^+$ Tn, Tscm or Tcm cells were modest and not enhanced by increasing E/T ratios (Fig. 2B). Only $CD8^+$ Tem cells produced IFN-γ when co-incubated with MFC cells (Fig. 2B; upper panel). We then analyzed for changes in the expression of CD44 and CD62 L in these co-cultured $CD8^+$ T cell subsets. During T cell differentiation, CD44 expression is known to increase whilst CD62 L expression is lost.[12] After MFC cell co-culture, a significant proportion of both Tn and Tscm cells became $CD62\ L^-$, potentially suggesting the acquisition of elements of *in vitro* activation. In contrast, $CD8^+$ Tcm cells either retained a central memory phenotype ($CD62\ L^+CD44^+$) or differentiated into Tem cells ($CD62\ L^-CD44^+$), but did not dedifferentiate into Tscm cells ($CD62\ L^+$). Consistent with their advanced differentiation state, $CD8^+$ Tem cells did not reacquire CD62 L expression or dedifferentiate into Tcm or Tscm cells *in vitro* (Fig. 2B; lower panel).

$CD8^+$ Tscm cell adoptive transfer enhances tumor regression in MFC tumor bearing $Rag1^{-/-}$ mice

To determine the potential contributions of $CD8^+$ Tscm cells to tumor progression, splenic $CD8^+$ Tscm, Tcm and Tem cells were purified from day 10 MFC injected C57BL/6 mice by FACS-based cell sorting and adoptively transferred into MFC tumor bearing $Rag1^{-/-}$ mice. $Rag1^{-/-}$ mouse tumors were then analyzed at day 10 post-adoptive $CD8^+$ T cell transfer. Interestingly in this scenario, adoptive transfer of $CD8^+$ Tem cells was less effective compared to Tcm cells at suppressing solid tumor growth in $Rag1^{-/-}$ mice (Fig. 2C). $CD8^+$ Tem cells have been shown to exhibit a shorter half-life after transfer into

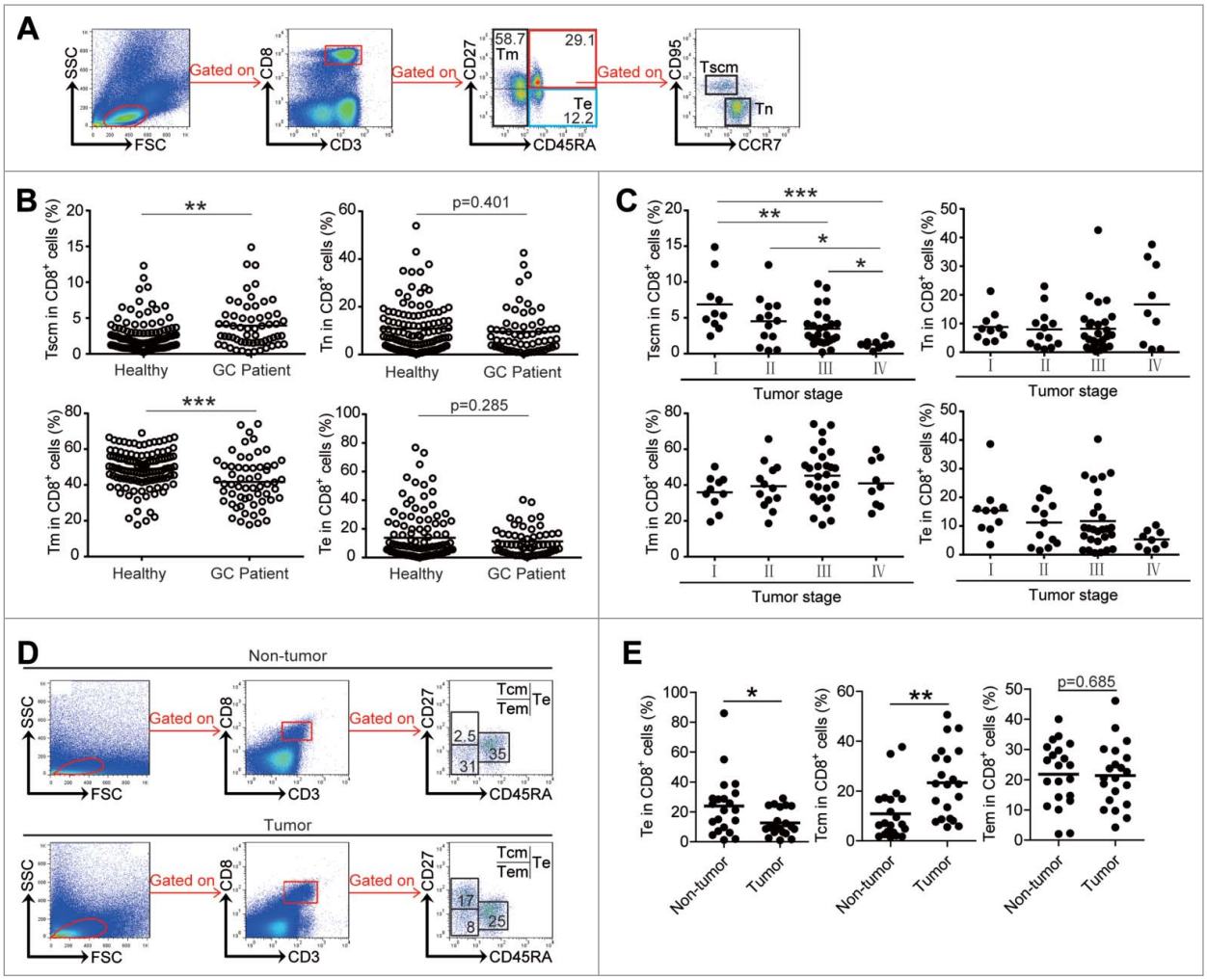

Figure 1. Tscm cells accumulate in peripheral blood of GC patients and decrease with disease progression. A. Flow cytometry analysis of a peripheral blood sample from a GC patient. Dot plots show the gating strategy used to identify Tscm cells. B. Percentages of circulating $CD8^+$ T cell subsets in GC patients compared to healthy donors. C. Percentages of circulating $CD8^+$ T cell subsets from GC patients at all TNM stages. D. Flow cytometry analysis of tumor tissue and non-tumor tissue from a GC patient. E. Percentages of $CD8^+$ T cell subsets in tumor and non-tumor tissue of GC patients. $CD8^+$ T cell subsets were defined as follows: Tn, $CD3^+CD8^+CCR7^+CD45RA^+CD27^+CD95^-$; Tscm, $CD3^+CD8^+CCR7^+CD45RA^+CD27^+CD95^+$; Tm, $CD3^+CD8^+CD45RA^-$; Te, $CD3^+CD8^+$ $CD45RA^+CD27^-$. Each dot represents an individual patient. $^* = P < 0.05$; $^{**} = P < 0.01$; $^{***} = P < 0.001$.

lymphopenic recipients,[13] which may explain their decreased cytotoxic capacity in contrast to Tscm cell counterparts *in vivo*. Overall, adoptive transfer of $CD8^+$ Tscm cells exerted the strongest anti-tumoral effects compared to even Tem and Tcm cell subsets (Fig. 2C).

Interestingly, $CD8^+$ T cell phenotyping of $Rag1^{-/-}$ recipients showed that the transferred $CD8^+$ Tscm cells acquired a Tem-like phenotype ($CD62L^-CD44^+$) in both the spleen and DLN, potentially because of homeostatic proliferation and differentiation. Within the tumor, however, originator Tscm cells now displayed an advanced CD62 L^-CD44^- phenotype rarely found during homeostasis (Fig. 2D). These respective phenotypes were also observed following the adoptive transfer of $CD8^+$ Tcm or Tem cells, suggesting that the $CD62L^-CD44^-$ phenotype may represent an advanced Tem cell differentiation phenotype selective to the tumor microenvironment and/or tumor mouse model (Fig. 2D). Notably, the overall number of $CD8^+$ T cells in $CD8^+$ Tscm cell transferred $Rag1^{-/-}$ mice was significantly higher compared to $CD8^+$ Tem cell transferred $Rag1^{-/-}$ mice both in the tumor and spleen (Fig. 2E). Overall, these results indicate that $CD8^+$ Tscm cells conferred superior anti-tumoral responses *in vivo* compared to Tem and Tcm cell subsets, most likely through their enhanced replicative and/or survival capacities following adoptive transfer.

GSK-3β inhibition increases Tscm cell-mediated tumor cell cytotoxicity in vitro

Tscm cells can be effectively generated *in vitro* through the induction of Wnt signaling during T cell priming using either Wnt3 A or GSK-3β inhibitors.[2] We therefore first assessed the level of intracellular GSK-3β phosphorylation (p-GSK-3β) in all $CD8^+$ T cell subsets in normal C57BL/6 mice and humans. Contrary to our expectations, $CD8^+$ Tscm cells exhibited the highest levels of p-GSK-3β amongst all $CD8^+$ T cell subsets studied in both mice and humans. No differences in p-GSK-3β levels were observed between $CD8^+$ Tn, Tm and Te cell subsets in mice or humans (Fig. 3A and B).

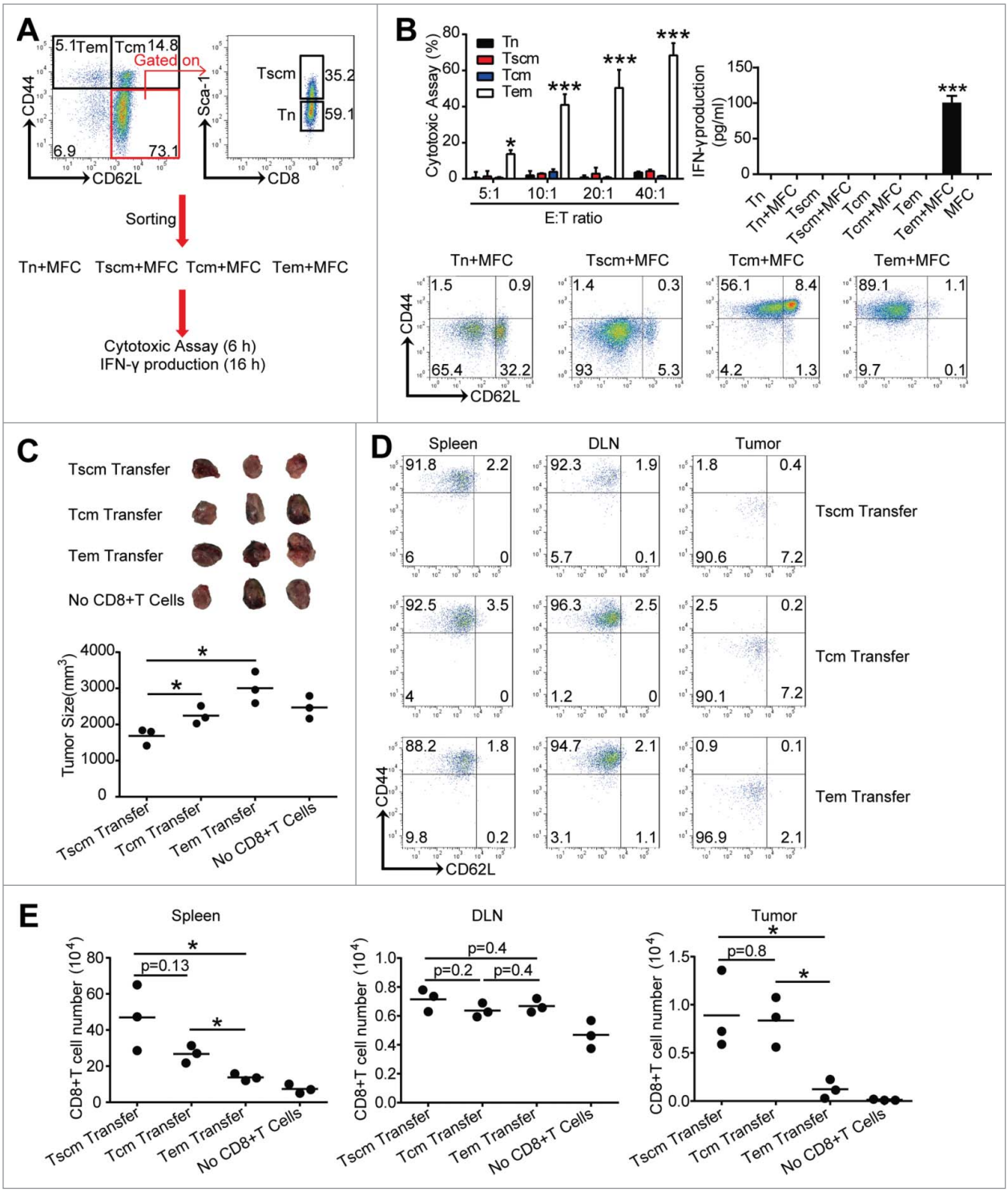

Figure 2. Comparison of tumor cytotoxicity capacities differ between mouse $CD8^+$ T cell subsets in vitro and in vivo. $CD8^+$ Tn, Tscm, Tcm or Tem cells from the spleen of tumor-bearing mice were sorted by cell surface phenotype (Tn, $CD3^+CD8^+CD44-CD62L^+Sca^{-1-}$; Tscm, $CD3^+CD8^+CD44-CD62L^+Sca\text{-}1^+$; Tcm, $CD3^+CD8^+CD44^+CD62L^+$; Tem, $CD3^+CD8^+CD44^+CD62L-$). For in vivo experiments, $Rag1^{-/-}$ mice were adoptively transferred 5 × 105 sorted MFC-primed Tscm, Tcm or Tem cells intravenously and then subcutaneously injected with MFC. A. Diagram summarizing the $CD8^+$ Tn, Tscm, Tcm and Tem cell sorting procedure. B. Comparison of the cytotoxic activity of $CD8^+$ T cell subsets 6h post-MFC co-cultures (E/T = 5:1 to 40:1; upper left panel), supernatant IFN-γ levels (E/T = 10:1; upper right panel) and flow cytometry analysis (E/T = 10:1; lower panel) showing CD62L and CD44 expression 16h post-MFC co-culture. Cells are gated as $CD3^+CD8^+$ events for flow cytometry analysis. C. Tumor sizes in $Rag1^{-/-}$ recipient mice bearing MFC tumors following the adoptive transfer of 5 × 105 sorted MFC-primed $CD8^+$ Tscm, Tcm or Tem cells. Mice are sacrificed at day 10 post-adoptive cell transfer. D. Flow cytometry analysis of spleen, DLN and dissected tumors from all respective groups. Cells are gated as $CD3^+CD8^+$ events. E. Total number of $CD8^+$ T cells recovered in the spleen, DLN and tumor of respective $Rag1^{-/-}$ recipient mice. $^* = P < 0.05$; $^{**} = P < 0.01$; $^{***} = p < 0.001$; Data are represented as mean ± SEM. (n = 3). All data shown are representative of two independently performed experiments.

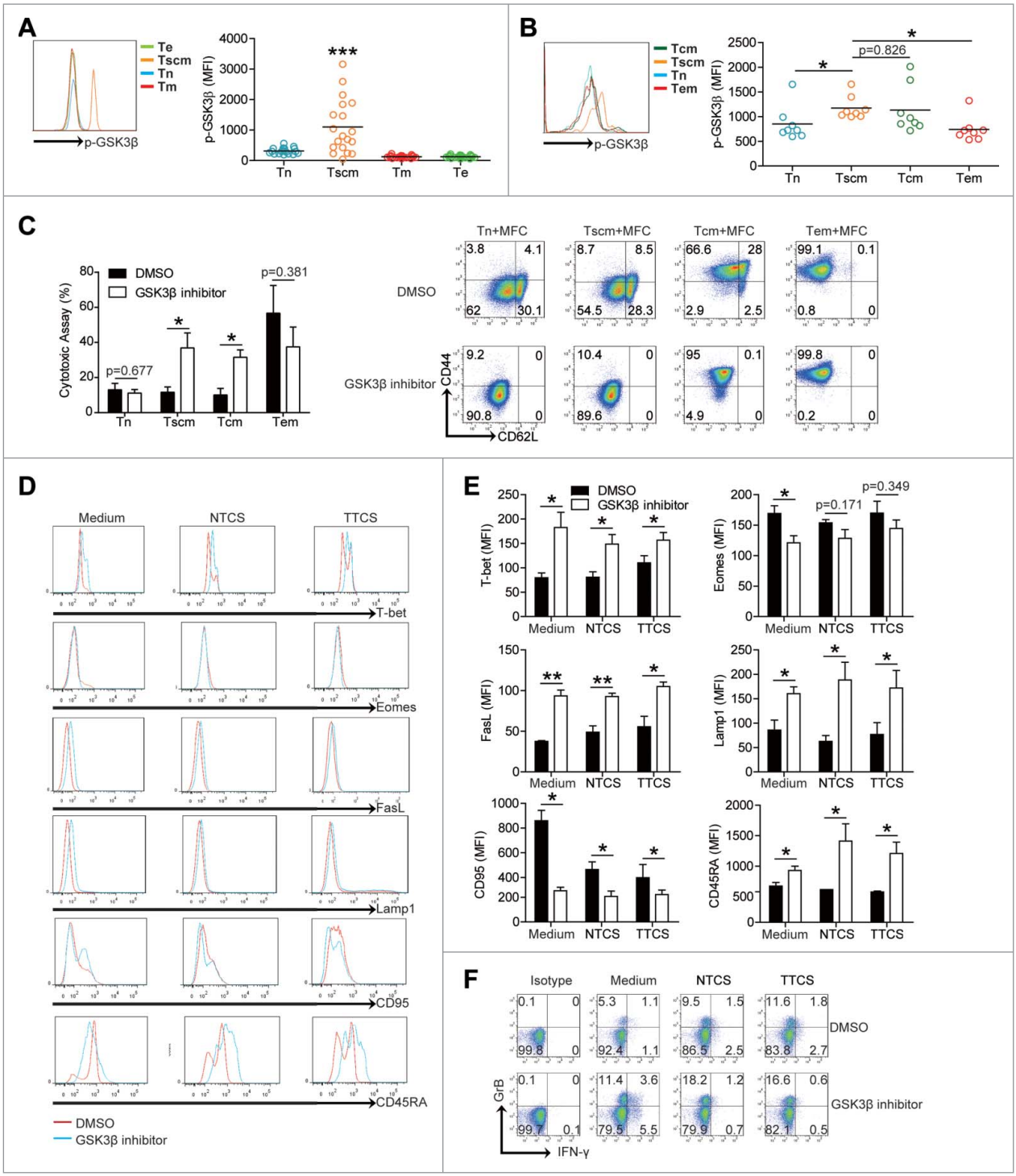

Figure 3. In vitro CD8$^+$ T cell GSK-3β inhibition increases Tscm cell-induced tumor cell cytotoxicity and the upregulation of CD8$^+$ effector T cell markers. A. Flow cytometry analysis of p-GSK-3β expression in respective human CD8$^+$ T cell subsets. B. Flow cytometry analysis of p-GSK-3β expression in respective mouse CD8$^+$ T cell subsets. C. Cytotoxic activity of mouse CD8$^+$ T cell subsets with GSK-3β inhibitor or DMSO control 6h post-MFC co-culture (left panel). Flow cytometry analysis (right panel) showing CD62L and CD44 expression 16h post-MFC co-culture. D. CD8$^+$ T cells from PBMC of healthy donors stimulated with anti-CD3/CD28 and co-cultured with either 25% TTCS, 25% NTCS or RPMI-1640 medium alone. Overlaid histogram plots showing the expression levels of CD8$^+$ effector T cell markers from CD8$^+$ T cells in a representative healthy donor. E. Statistical analysis of marker expression levels in NTCS, TTCS or media cultured human CD8$^+$ T cells. F. Flow cytometry analysis of IFN-γ and GrB levels. Cells are gated as all CD3$^+$CD8$^+$ events. Data are represented as mean ± SEM. * = P < 0.05; ** = P < 0.01; *** = p < 0.001. All data shown are representative of three independently performed experiments.

ATP competitive and non-competitive inhibitors of GSK-3 kinase exist.[14] GSK-3β inhibitor VI (hereafter shortened and referred to as GSK-3β inhibitor) acts as a non-ATP competitor of GSK-3β and does not affect protein kinase A (PKA) activity. We first examined the effects of GSK-3β inhibition on the cytotoxic capacity of different CD8$^+$ T cell subsets *in vitro*. Mouse MFC-primed splenic CD8$^+$ Tn, Tscm, Tcm and Tem cell subsets were sorted and co-cultured with MFC in the presence of the GSK-3β inhibitor or DMSO control. Interestingly, we found that GSK-3β inhibition increased the cytotoxic

capacity of Tscm and Tcm cells, but not that of Tn or Tem cells *in vitro* (Fig. 3C; left panel). The rate of spontaneous cell death, however, was also increased in the presence of the GSK-3β inhibitor (Supplementary Fig. S4). Contrary to previous reports, the presence of GSK-3β inhibitor either directly or indirectly induced the differentiation of a subset of CD62$^+$ Tn and Tscm cells into a more effector-like CD62L$^-$ phenotype *in vitro* (Fig. 3C; right panel). This suggested that GSK-3β inhibition promoted at least partial changes suggestive of CD8$^+$ Tscm and Tn cell differentiation, and directly or indirectly enhanced the tumor cell cytotoxicity of CD8$^+$ Tscm cells *in vitro*.

GSK-3β inhibition increases the expression of T-bet, CD45RA, Fas-ligand, Lamp1 and granzyme B in human CD8$^+$ T cells in vitro

We sought to further investigate the factors associated with the GSK-3β inhibition dependent enhancement of CD8$^+$ Tscm cell cytotoxicity *in vitro* and also whether this was dependent on signals from the tumor microenvironment. Purified peripheral blood CD8$^+$ T cells from healthy human donors were primed with anti-CD3/CD28 and co-cultured with tumor tissue culture supernatant (TTCS), non-tumor tissue culture supernatant (NTCS) or medium in the presence of GSK-3β inhibitor or DMSO control. GSK-3 inhibition has been reported to selectively block CD8$^+$ T cell PD-1 expression and consequently increase CD8$^+$ T cell cytotoxicity.[15] However, we did not observe changes in CD8$^+$ T cell PD-1or PDL-1 expression levels in our experiments (Supplementary Fig. S5). The transcription factors T-bet and Eomes play additive roles in inducing IFN-γ production and CD8$^+$ effector T cell cytotoxicity *in vitro*.[16] Surprisingly, GSK-3β inhibition enhanced the expression of T-bet in TTCS, NTCS or medium-treated activated CD8$^+$ T cells *in vitro*. The expression of Eomes was not altered in TTCS or NTCS-treated CD8$^+$ T cells following GSK-3β inhibition (Fig. 3D and E). We then evaluated the expression levels of naïve T cell markers (CD45RA, CD27 and CCR7) in each activated CD8$^+$ T cell condition. CD27 and CCR7 expression levels were unaffected by GSK-3β inhibition (Supplementary Fig. S5). However, an increase in CD45RA expression irrespective of TTCS or NTCS culture conditions was observed and suggested that GSK-3β inhibition was indeed capable of promoting partial aspects of human effector CD8$^+$ T cell differentiation *in vitro* (Fig. 3D and E). Consistent with enhanced mouse Tn and Tscm tumor cell cytotoxicity responses observed *in vivo* (Fig. 3C), both FasL and lysosomal-associated membrane protein 1 (Lamp1; CD107a) expression levels, measured as changes in mean fluorescent intensities (MFI), were increased in TTCS, NTCS and medium-treated activated CD8$^+$ T cells *in vitro* (Fig. 3D and E). The frequency of CD8$^+$ T cells expressing GrB (Granzyme B) was also notably enhanced following GSK-3β inhibition under all conditions (Fig. 3F). Interestingly, the frequency of CD8$^+$ T cells co-expressing IFN-γ and GrB was increased in medium-treated CD8$^+$ T cells alone following GSK-3β inhibition (Fig. 3F). Altogether, these results indicate that GSK-3β inhibition can increase human CD8$^+$ T cell T-bet, CD45RA, FasL, Lamp1, GrB and to a lesser extent IFN-γ expression levels following CD8$^+$ T cell activation *in vitro*.

GSK-3β inhibition induces FasL-dependent enhancement of CD8$^+$ T cell cytotoxicity in vivo

FasL is a well-established effector of CD8$^+$ T cell-mediated cytotoxicity in addition to granzyme B and IFN-γ.[17] FasL can also directly mediate tumor growth and[18] T cell co-stimulation[19] and differentiation.[20] Thus, we postulated whether the observed FasL upregulation functionally contributed to GSK-3β inhibition-induced CD8$^+$ T cell tumor cell cytotoxicity *in vivo* and/or induced GSK-3β inhibition-mediated CD8$^+$ effector-like T cell activity *in vitro*. To test the first hypothesis, C57BL/6 mice were injected with MFC cells in the presence of GSK-3β inhibitor or DMSO control, and a cohort additionally administered anti-FasL blocking antibodies. The spleen and DLN was then harvested at day 10 post-MFC injection. GSK-3β inhibition reduced p-GSK-3β levels in mouse CD8$^+$ T cells, whilst anti-FasL antibody blocking did not re-activate CD8$^+$ T cell p-GSK-3β signaling (Fig. 4A). GSK-3β inhibition induced significant tumor regression compared to both DMSO control ($p < 0.05$) and anti-FasL antibody administered mouse cohorts (Fig. 4B). Consistent with our *in vitro* observations, GSK-3β inhibition increased FasL, Lamp1 and IFN-γ expression levels on mouse splenic and DLN CD8$^+$ T cells and this was partially decreased upon anti-FasL addition (Fig. 4C and D). Meanwhile, GSK-3β inhibition caused a significant attrition of Tscm cells and accumulation of CD62L$^-$CD44$^-$CD8$^+$ T cells both in spleen and DLN. In the presence of anti-FasL, the induced differentiation by GSK-3β inhibition was limited, as evidenced by an increased percentage of Tscm cells (Fig. 4E). Altogether, these experiments indicate that FasL is functionally required for both the GSK-3β inhibition-mediated induction of CD8$^+$ T cell tumor cytotoxicity and upregulation of CD8$^+$ effector T cell markers *in vivo*.

The downstream effects of GSK-3β inhibition mediated FasL induction are potentially not confined to CD8$^+$ T cells alone. To additionally investigate whether FasL induction by GSK-3β inhibition directly modulated the anti-tumoral activity of CD8$^+$ Tscm cells (the CD8$^+$ T cell subset with the highest p-GSK-3β levels), purified MFC-primed mouse Tscm cells pre-treated with GSK-3β inhibitor in the presence or absence of anti-FasL blocking antibody and co-cultured with MFC cells. As anticipated, GSK-3β inhibition increased the cytotoxic capacity of CD8$^+$ Tscm cells, and this was markedly reduced by antibody-mediated FasL blocking (Fig. 5A).

Finally, to directly assess the impact of CD8$^+$ Tscm cell selective GSK-3β inhibition on tumor regression *in vivo*, MFC-primed CD8$^+$ Tscm cells were adoptively transferred into Rag1$^{-/-}$ mice bearing MFC tumors and treated with either the GSK-3β inhibitor or DMSO control. In line with our expectations, administration of the GSK-3β inhibitor increased tumor regression in Rag1$^{-/-}$ recipient mice (Fig. 5B). Altogether, our study confirms the therapeutic potential of utilizing CD8$^+$ Tscm cells and CD8$^+$ T cell selective GSK-3β inhibition to enhance immunotherapy responses against GC.

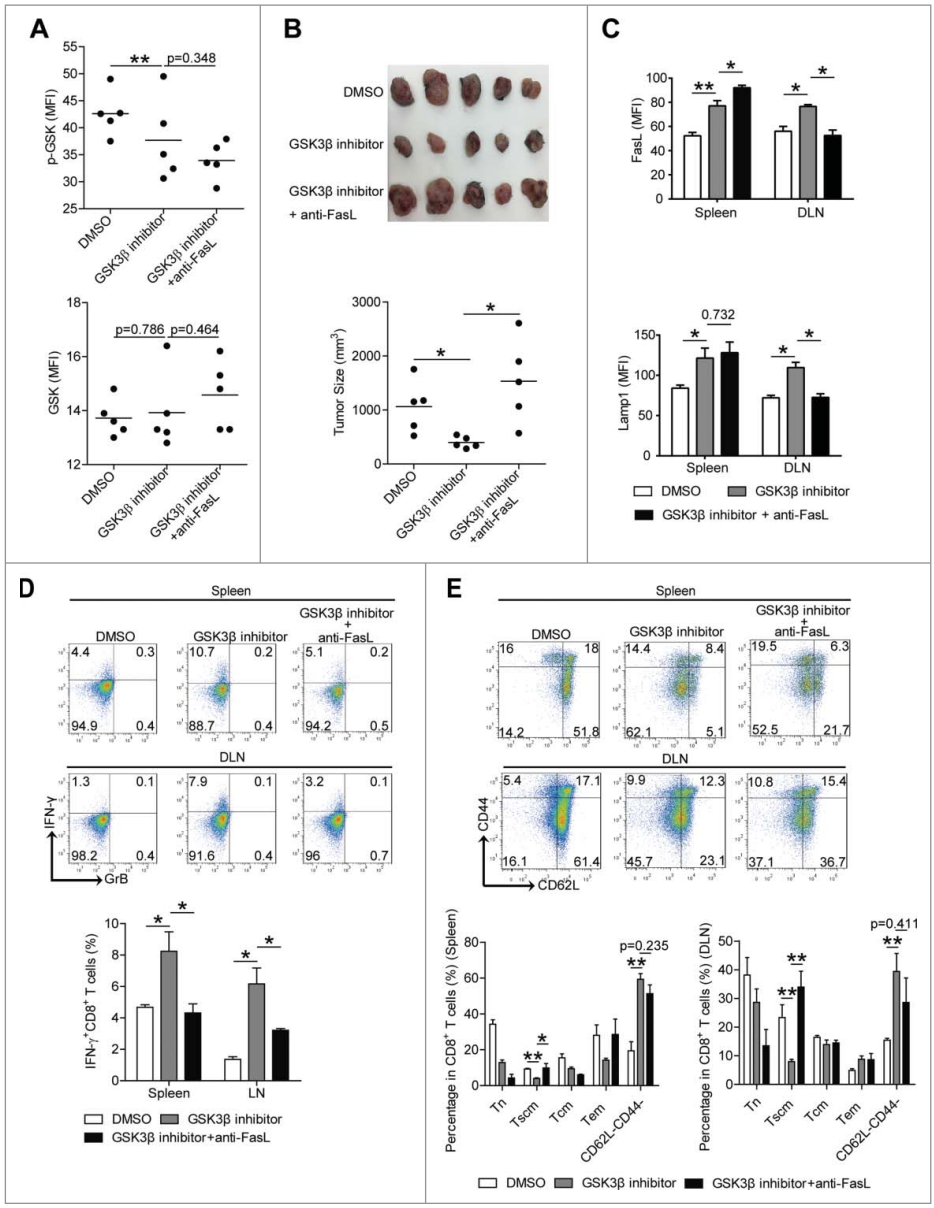

Figure 4. GSK-3β inhibition induces FasL-dependent enhancement of CD8$^+$ T cell cytotoxicity and aspects of CD8$^+$ effector T cell differentiation in vivo. C57BL/6 mice are subcutaneously injected with MFC in the presence of GSK-3β inhibitor or DMSO control, and a cohort additionally administered anti-FasL antibody. Mice were then taken day 10 post-MFC injection. A. MFI values for p-GSK-3β (upper panel) and GSK-3β (lower panel) expression in splenic CD8$^+$ T cells from each respective treatment group. Cells are gated as all CD3$^+$CD8$^+$ events. B. Tumors isolated from each respective treatment group. C. MFI values for FasL and Lamp1 expression of splenic and DLN CD8$^+$ T cells from each respective treatment group. Cells are gated as all CD3$^+$CD8$^+$ events. D. Percentages of IFN-γ^+ and GrB$^+$ splenic and DLN CD8$^+$ T cells from each respective treatment group. Cells are gated as all CD3$^+$CD8$^+$ events. E. Flow cytometry depicting CD62L and CD44 expression of splenic and DLN CD8$^+$ T cells from each respective treatment group. Lower graph represents the percentages of different CD8$^+$ T cell subsets (n = 4). Cells are gated as all CD3$^+$CD8$^+$ events. Data are represented as mean ± SEM. * = P < 0.05; ** = P < 0.01. All data shown are representative of two independently performed experiments.

Discussion

Tscm cells are attractive candidates for adoptive T cell therapies due to their long-term capacity for self-renewal and cellular multipotency. Tscm cells are a clonally expanded primordial memory T subset which arises following antigenic stimulation and exhibit significantly enhanced proliferative and reconstitution capacities.[3] Tscm cell contributions to human cancer progression, however, are still currently unclear. In this study, we have shown that CD8$^+$ Tscm cell frequencies are increased in the peripheral blood of GC patients compared with healthy donors, and that CD8$^+$ Tscm cell frequencies are negatively associated with GC progression.

Mouse Tscm cell generation and the arrestment of T cell proliferation and differentiation is triggered through the induction of Wnt signaling pathway during T cell priming using Wnt3 A or GSK-3β inhibitors.[2] Interestingly, in our hands, CD8$^+$ Tscm cells showed the highest intracellular levels of phosphorylated GSK-3β (p-GSK-3β activity) among all CD8$^+$ T cell subsets studied in both mice and humans. In our study, GSK-3β inhibition also conferred enhanced anti-tumoral activity following CD8$^+$ Tscm adoptive transfer through increased CD8$^+$ T cell proliferation and/or survival as well as the latent activation of a more CD8$^+$ effector T cell-like phenotype, which was unexpected. Although the GSK-3/Wnt pathway is considered critical for the generation and maintenance of memory

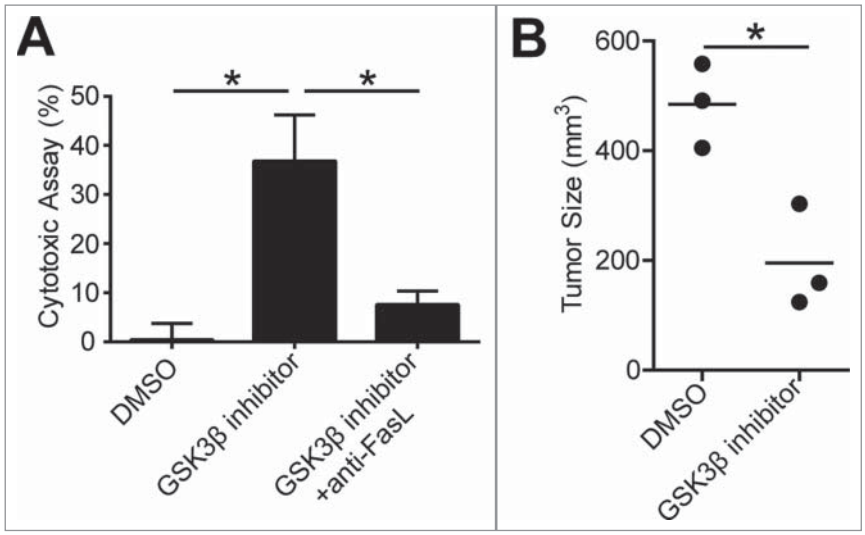

Figure 5. GSK-3β inhibition induces FasL-dependent enhancement of CD8$^+$ Tscm cell-mediated cytotoxicity. A. Mouse CD8$^+$ Tscm cell MFC cell cytotoxicity levels post-GSK-3β inhibitor treatment in the presence or absence of anti-FasL antibody addition. B. Rag1$^{-/-}$ recipient mice are adoptively transferred 5 $\times$ 105 sorted MFC-primed CD8$^+$ Tscm cells, and then subcutaneously injected with MFC in the presence of GSK-3β inhibitor or DMSO control. Tumor sizes were measured at day 10 post-transfer. Data are represented as mean $\pm$ SEM. * = P $<$ 0.05. All data shown are representative of two independently performed experiments.

and stem cell-like properties, Wnt-dependent genes are not upregulated themselves following GSK-3β inhibition.[21] This may indicate the existence of post-transcriptional regulation mechanisms or alternative signaling pathways responsible for Tscm stemness per se. It is also possible that the GSK-3β inhibitor VI separately regulates CD8$^+$ effector T cell differentiation through non-Wnt dependent pathways.

GSK-3 inactivation has previously been reported to enhance T-bet expression and suppress Pdcd1 (encodes PD-1) transcription in CD8$^+$ T cells, which is turn enhances CD8$^+$ T cell cytotoxicity.[15] In line with these findings, GSK-3β inhibition increased the cytotoxic capacity of human CD8$^+$ Tscm cells and Tcm cells, but not Tn cells or Tem cells, *in vitro*. CD8$^+$ Tem cells expressed the lowest levels of p-GSK-3β, which may explain why GSK-3β inhibition did not alter their cytotoxic capacities. Paralleling our *in vitro* observations, GSK-3β inhibition also induced tumor regression in MFC tumor bearing Rag1$^{-/-}$ mice. Interestingly in our study, expression of the TNF superfamily member FasL was upregulated upon GSK-3β inhibition. CD95 and FasL are well established pro-apoptotic factors, although the precise cellular outcome of the FasL-CD95 interaction depends on the differentiation state of individual cells.[22] CD95 triggers apoptosis in terminally differentiated neurons through the formation of a death-inducing complex, but conversely promotes cell proliferation in neural progenitors and cancer stem cells by inducing TCF/β-catenin signaling.[23] GSK-3β inhibition-induced FasL upregulation may therefore promote different downstream responses in the Tscm cells compared to terminally differentiated Tem cells. We also demonstrated that GSK-3β inhibition-induced FasL upregulation functionally contributed to anti-tumoral immunity, since tumor regression was attenuated by antibody-mediated FasL neutralization *in vivo*. FasL induction occurred alongside the increased expression of other markers of CD8$^+$ effector T cell differentiation, such as T-bet, GrB, Lamp1 and to a lesser extent, IFN-γ. GSK-3 inactivation is known to enhance T-bet transcription,[15] and T-bet binding sites exist in GrB and IFN-γ promoter regions.[24] Enhanced GrB and IFN-γ expression levels may therefore be a downstream consequence of T-bet upregulation following GSK-3β inhibition.

FasL-induced Tn cell differentiation is associated with an increase in Akt signaling.[20] Fas signaling also induces migration and invasion through the PI3K/AKT/GSK3β/β-catenin/MMP pathway in neoplastic cells[18] and induces the Src/PI3K/AKT/mTOR signaling pathway in neuronal stem cells.[25] As Akt signaling is involved in effector CD8$^+$ T cell differentiation, we measured p-Akt levels following GSK-3β inhibition.[26] GSK-3β inhibition did not alter p-Akt expression in TTCS, NTCS or medium-treated CD8$^+$ T cells, however, although p-S6 levels were significantly decreased (Supplementary Fig. S6). How p-S6 attenuation is mediated by GSK-3β inhibition in CD8$^+$ T cells, through Akt independent or dependent pathways, will be required for future investigation. We surprisingly also found minimal differences in TTCS, NTCS or medium-treated CD8$^+$ T cells following GSK-3β inhibition, as we anticipated the TTCS microenvironment to be more immunosuppressive. Further work will be required to dissect whether the effects of GSK-3β inhibition on CD8$^+$ T cell activity can override specific tumor-mediated immunosuppressive signaling pathways, a feature which may be ideal for immunotherapy agents.

Although adoptive transfer of CD8$^+$ Tscm cells mediated the greatest degree of tumor regression in Rag1$^{-/-}$ mice, CD8$^+$ Tscm cells themselves were not phenotypically preserved in the spleen, DLNs or tumors. This suggests that transferred CD8$^+$ Tscm cells exerted anti-tumoral effects most likely through at least a partial differentiation into terminally differentiated CD8$^+$ T cells. Tscm cells have been previously demonstrated to express IFN-γ following PMA/ionomycin stimulation.[3] However, in our study, only CD8$^+$ Tem cells secreted IFN-γ in MFC cell co-cultures, suggesting that Tem cell subsets were most likely to be the end stage mediators of anti-tumoral cytotoxicity whilst CD8$^+$ Tscm cells indirectly increased anti-tumoral immunity through *in vivo* differentiation into a more Tem-like effector cell. Overall, our study indicates that the

modulation of $CD8^+$ Tscm cells combined with GSK-3β inhibition may be a new strategy to boost anti-tumoral immunity in human patients.

In conclusion, we found that $CD8^+$ Tscm cells accumulated in peripheral blood of GC patients and decreased in frequency with disease progression. The adoptive transfer of $CD8^+$ Tscm cells mediated superior tumor regression responses compared with other $CD8^+$ T cell subsets *in vivo*. Mechanistically, $CD8^+$ Tscm cells most likely indirectly mediated tumor cytotoxicity through enhanced proliferative and/or differentiation into a more effector-like phenotype. This was supported by *in vitro* experiments showing that GSK-3β inactivation upregulated FasL, Lamp1, GrB and IFN-γ expression, in activated human $CD8^+$ T cells. Our findings further identify GSK-3β as a mediator of $CD8^+$ T cell FasL expression and demonstrate the applicability of $CD8^+$ Tscm cells in combination with GSK-3β inhibition for the modulation of anti-tumoral immunity in GC.

Materials and methods

Patient and specimens

Fresh gastric tumor and matched non-tumor (at least 5cm away from the tumor site) tissue samples and autologous peripheral blood samples were obtained from GC patients who underwent surgical resection at the Southwest Hospital of Third Military Medical University, Chongqing, China. All patients had not received chemotherapy or radiotherapy prior to surgery. Patients with infectious diseases, autoimmune diseases or multi-primary cancers were excluded. Peripheral blood samples from 115 healthy donors were used as control. The clinical stages of tumors were determined according to the TNM classification system of the International Union Against Cancer (7th edition). The study was approved by the Ethics Committee of the Southwest Hospital of Third Military Medical University and written informed consent was obtained from each patient. Antibodies and other reagents used for patient sample analyses are listed in Supplementary Table S1. The clinical characteristics of patients with gastric cancer in this study are listed in Supplementary Table S2.

Mice and cell line

C57 BL/6 mice and $Rag1^{-/-}$ mice were purchased from Jackson Laboratories. 8 to 10 week old female mice were used and bred under specific pathogen-free conditions at the Third Military Medical University. All mice were maintained in accordance with institutional guidelines for animal welfare.

Mouse fore stomach carcinoma (MFC), a mouse gastric cancer cell line, was purchased from the Shanghai Cell Biology Institutes, Chinese Academy of Sciences (Shanghai, China). MFC cells were cultured in RPMI-1640 medium containing 10% FCS, at 37°C in a humidified incubator with a 5% CO_2 atmosphere prior to *in vitro* experiments or subcutaneous injection.

Isolation of single cells from GC tissues

Fresh GC tissues were washed 3 times with Hank's solution containing 1% FCS before being cut into small pieces. The specimens were then collected into RPMI-1640 medium containing 1mg/ml collagenase IV and 10mg/ml DNase I, and mechanically dissociated using the gentle MACS Dissociator (Miltenyi Biotec). Dissociated cell suspensions were further incubated for 1h at 37°C under continuous rotation. The cell suspensions were then filtered through a 70 μm cell strainer (BD Labware). Cell viability, as determined by trypan blue exclusion staining, was typically >90%.

Preparation of TTCS and NTCS

Tumor tissue culture supernatants (TTCS) or non-tumor tissue culture supernatants (NTCS) were prepared by plating autologous tumor or non-tumor gastric tissues in 1ml RPMI-1640 medium for 24h. The suspensions were then centrifuged and the supernatants harvested.

MFC tumor mouse model

To create syngeneic subcutaneous tumors, MFC cells were injected into the rear flanks of C57BL/6or $Rag1^{-/-}$ mice using 4×10^6 cells/mouse. Tumor growth was monitored by daily measurements of tumor diameter. Mice were sacrificed and tumor, spleen, and draining lymph node (DLN) tissue removed and processed for analysis. Tumors were digested by collagenase IV and 10mg/ml DNase I and then isolated by centrifugation using 30/70 Percoll (GE Healthcare Life Sciences) gradients.

T-Cell adoptive transfer

Spleen lymphocytes from tumor-bearing mice were isolated at day 10 post-MFC injection. Single cell suspensions were labeled with anti-CD3, anti-CD8, anti-CD62L, anti-CD44, and anti-Sca-1 antibodies, and $CD8^+$ Tn, Tscm, Tcm and Tem cell subsets purified using the fluorescence activating cell sorter (FACS, FACSAria II; BD Biosciences). Respective T cell subsets were injected intravenously (5×10^5 cells/mouse) into tumor bearing $Rag1^{-/-}$ recipient mice. In some experiments, the GSK-3β VI inhibitor was then administered intraperitoneally (2 μg/mouse). To block FasL *in vivo*, 10 μg/mouse of anti-mouse FasL antibody (RD) was then injected intraperitoneally every 2 days.

In vitro *human* $CD8^+$ *T cell assay*

Bead-purified $CD8^+$ T cells from the PBMC of healthy donors were stimulated with coated anti-CD3 (2μg/ml), soluble anti-CD28 (1μg/ml), and cultured with GSK-3β inhibitor VI (10 μM)/Akt inhibitor VIII (10 μM) or DMSO control. In the supernatant co-culture system, 25% TTCS or NTCS or media control was then added. Cells were then harvested for flow cytometry analysis 24h after.

CD8$^+$ T cell cytotoxicity assay

For *in vitro* cytotoxicity assays, sorted CD8$^+$ Tn, Tscm, Tcm and Tem cells from the spleens of mice bearing MFC tumors (harvested at day 10 post-MFC injection) were plated in 96-well plates with mitomycin C (Sigma-Aldrich, 10 μg/ml) treated MFC cells. Unless specifically mentioned, the effector/target cell ratio used was 10:1. For GSK-3β inhibition experiments, sorted CD8$^+$ Tn, Tscm, Tcm and Tem cells from the spleens of mice bearing MFC tumors were pre-treated with GSK-3β Inhibitor VI (10 μM) in the presence or absence of anti-FasL blocking antibody for 1h prior to MFC co-culture. Cytotoxicity was assayed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) according to manufacturer's instructions.

$$\text{Cytotoxicity \%} = \frac{\text{Experimental} - \text{Effector Spontaneous} - \text{Target Spontaneous}}{\text{Target Maximum} - \text{Target Spontaneous}} \times 100$$

Cell culture supernatants were collected for IFN-γ measurements and cells analyzed by flow cytometry.

Flow cytometry, ELISA and apoptosis assays

Assays were conducted as described in Supplementary Methods.

Statistical analysis

Results are expressed as mean ± SEM. The Student *t* test was used to analyze the differences between two groups except when the variances differed, in which case the Mann-Whitney U test was used. For multigroup data analysis, the one-way ANOVA test was used. The SPSS statistical software (version 13.0) was used for all statistical analysis. All data were analyzed using 2-tailed tests, and $p < 0.05$ considered to be statistically significant.

Abbreviations

CTL	cytotoxic T lymphocyte
DLN	draining lymph nodes
GC	gastric cancer
GrB	granzyme B
GSK	glycogen synthase kinase
MFC	mouse fore stomach carcinoma
NTCS	non-tumor tissue culture supernatant
Sca-1	stem cell antigen-1
Tcm	central memory T cells
Tem	effector memory T cells
Te	effector cells
Tn	naïve T cells
Tscm	memory stem T cells
TTCS	tumor tissue culture supernatant

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Ethics approval

The biopsy specimens were obtained under protocols approved by the ethics committees of Southwest Hospital of Third Military Medical University and informed consent was obtained from all patients. All animal experiments were undertaken with approval from the Animal Ethical and Experimental Committee of Third Military Medical University.

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