Expression of inhibitory receptors on intratumoral T cells modulates the activity of a T cell-bispecific antibody targeting folate receptor

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#ABSTRACT

T-cell bispecific antibodies (TCBs) are a novel therapeutic tool designed to selectively recruit T-cells to tumor cells and simultaneously activate them. However, it is currently unknown whether the dysfunctional state of T-cells, embedded into the tumor microenvironment, imprints on the therapeutic activity of TCBs. We performed a comprehensive analysis of activation and effector functions of tumor-infiltrating T-cells (TILs) in different tumor types, upon stimulation by a TCB targeting folate receptor 1 and CD3 (FolR1-TCB). We observed a considerable heterogeneity in T-cell activation, cytokine production and tumor cell killing upon exposure to FolR1-TCB among different FolR1-expressing tumors. Of note, tumors presenting with a high frequency of PD-1hi TILs displayed significantly impaired tumor cell killing and T-cell function. Further characterization of additional T-cell inhibitory receptors revealed that PD-1hi TILs defined a T-cell subset with particularly high levels of multiple inhibitory receptors compared with PD-1int and PD-1neg T-cells. PD-1 blockade could restore cytokine secretion but not cytotoxicity of TILs in a subset of patients with scarce PD-1hi expressing cells; in contrast, patients with abundance of PD-1hi expressing T-cells did not benefit from PD-1 blockade. Our data highlight that FolR1-TCB is a promising novel immunotherapeutic treatment option which is capable of activating intratumoral T-cells in different carcinomas. However, its therapeutic efficacy may be substantially hampered by a pre-existing dysfunctional state of T-cells, reflected by abundance of intratumoral PD-1hi T-cells. These findings present a rationale for combinatorial approaches of TCBs with other therapeutic strategies targeting T-cell dysfunction.

Introduction

Though early clinical trials have yielded mixed results with ambiguous clinical benefits, cancer immunotherapy is now emerging as a broadly applicable therapeutic option for cancer treatment. Several strategies can be employed to overcome T-cell dysfunction and (re-)activate T-cells to fight cancer. Aside from blockade of immune checkpoints, such as cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1), recombinant antibody-based technologies, which redirect T-cells against tumor cells, represent a promising clinical approach to harness and empower T-cells with the capacity to effectively combat cancer. These dual specific antibodies target the T-cell receptor by binding to CD3ε on T-cells with one arm, while the other arm engages a tumor cell-selective surface antigen. Cross-linking of effector and target cells via such T-cell bispecific antibodies (TCBs) induces formation of an immunological synapse and polyclonal T-cell activation, which ultimately results in potent killing of the bound tumor cells. T-cell activation upon TCB engagement is independent of the T-cell receptor specificity and does not require costimulatory signals. In addition, due to agonistic engagement of CD3ε, sub-optimal TCR affinity and T cell avidity are less likely to hamper T cell function during the antitumor immune response. Hence, TCBs are a highly attractive approach to activate intratumoral T-cells regardless of their antigen specificity; of note, both CD8+ and CD4+ T-cells mediate TCB-mediated tumor cell killing.

The development of TCBs has been pioneered by blinatumomab, which targets the CD19 antigen on the surface of B cells. Its therapeutic activity has been demonstrated for patients with B-cell lymphoma as well as B lineage acute lymphoblastic leukemia. Blinatumomab has been FDA approved for the treatment of relapsed or refractory B-cell precursor acute lymphoblastic leukemia. Catumaxomab, EMA approved for intraperitoneal administration, is a trifunctional TCB, in which a mouse IgG2a anti-Epithelial cell adhesion molecule (EpCAM) hemi-antibody is
co-cultured with the FolR1+ ovarian cancer cell line Skov3 (Fig. S2A). Upon exposure to increasing concentrations of FolR1-TCB ranging from 0.6pM to 2nM for 24h we observed a strong activation of CD8+ T-cells with upregulation of CD25, CD137, and ICOS. In addition, T-cells secreted IL-2, IFNγ, and TNF. Exposure to DP47-TCB, a TCB directed against an irrelevant antigen, did not induce any T-cell activation (Fig. S2B and C).

To comprehensively dissect the immune-modulatory capacity of FolR1-TCB on TILs, we used fresh tumor resections and malignant effusions from 25 newly diagnosed patients submitted to surgery or drainage, respectively. The samples consisted of 15 tumor derived single cell suspensions and 10 malignant effusions from patients with NSCLC (n = 16), EOC (n = 7), and RCC (n = 2). Detailed patients’ characteristics are available in Table S1. The proportion of CD3+ T-cells and FolR1+ tumor cells varied substantially between patients (CD3+: mean 33.9% ± standard deviation of 16.6%, FolR1+: 17.1 ± 16.8%) Table S1). To determine FolR1-TCB-induced activation of CD8+ T-cells, tumor samples were cultured in the presence and absence of 2nM FolR1-TCB for 24h. After TCB-stimulation CD8+ T-cells were characterized by multicolor flow cytometry for expression of activation markers. CD8+ T-cells from healthy donors co-cultured with the FolR1+ Skov3 tumor cell line served as a correlate for a ‘fully functional’ T-cell repertoire. Notably, a broad heterogeneity in FolR1-TCB-induced T-cell activation was found among patients. In particular, we observed upregulation of CD25, CD137, and ICOS (43.3 ± 23.5%, 17.6 ± 16.5%, and 20.5 ± 4.2%, respectively; Fig. 1A). Notably, the upregulation of CD25 and ICOS induced by FolR1-TCB stimulation was significantly stronger in peripheral CD8+ T-cells from healthy donors than for tumor-derived CD8+ cells (p = 0.002 and P < 0.001, respectively; Fig. 1A). The secretion of T-cell effector cytokines IFNγ, IL-2, and TNF upon FolR1-TCB stimulation was largely diminished among TILs in the majority of tumors compared with PBMCs (p = 0.0047, P < 0.001, and p = 0.006, respectively; Fig. 1B). FolR1-TCB-induced perforin secretion was highly variable in TILs, and severely impaired in a subset of patients (Fig. 1B).

To assess whether the abundance of intratumoral T-cells or the level of FolR1 expression might impact on the activity of the TCB, the increase in T-cell activation was correlated to the E:T ratio (E: effector CD45+ CD3+ T-cells; T: FolR1+ cells) and to the percentage and level of tumor antigen expression of FolR1+ cells. The latter was determined by the mean fluorescence intensity of FolR1 on tumor cells (CD45+ EpCAM+) using flow cytometry (Fig. S2). However, neither of these parameters did correlate with T-cell activation. Even low levels of FolR1 expression or poor T-cell infiltration were sufficient for efficient upregulation of activation and functional markers. In addition, the presence of potentially immune-suppressive cell populations such as regulatory T-cells or immature myeloid cells did not influence T-cell activation or T-cell function (Fig. S4).

**Results**

**Activation of T-cells from healthy donors and cancer patients by FolR1-TCB**

To assess the effect of FolR1-TCB on T-cell activation peripheral blood mononuclear cells (PBMCs) from healthy donors were paired with a rat IgG2b anti-CD3 hemi-antibody. It has been demonstrated to reduce tumor cell burden in the peritoneal fluids of EpCAM positive carcinoma patients.

In this study we utilized an IgG-based TCB targeting folate receptor 1 (FolR1), with the FolR1 specific binding moieties derived from the anti-human FolR1 antibody Mov19, and a monovalent anti-human CD3 antibody fused to an Fc part without any FcgR function (FolR1-TCB). FolR1 is a glycosphatidylinositol-anchored membrane protein that binds folic acid with high affinity, mediating its cellular uptake via receptor-mediated endocytosis. While FolR1 exhibits limited distribution in healthy tissues, with expression only being detected on the apical surfaces of a few epithelial cells including lung, kidney and choroid plexus, it is overexpressed on the surface of cancer cells in a spectrum of epithelial malignancies, including ovarian, lung, renal and other solid cancers.

This tumor cell-selective expression pattern makes FolR1 an attractive candidate for targeted therapy.

Tumor-specific T-cells residing within the tumor microenvironment are chronically exposed to immune-suppressive factors as well as high antigen load. Consequently, bona fide human self-antigen-specific T-cells within the tumor microenvironment are often characterized by a severely impaired inflammatory and cytotoxic capacity as well as sustained expression of inhibitory receptors and a transcriptional state distinct from that of functional effector or memory T-cells. The loss of effector functions, which has been initially described in chronic murine lymphocytic choriomeningitis virus (LCMV) infection, typically occurs in a hierarchical manner: while IL-2 production, proliferation and cytotoxicity perish early, TNF, IFNγ and beta-chemokine secretion follow at later stages. The dysfunctional state of these T-cells is regulated, at least in part, by the cell surface expression of inhibitory receptors including PD-1, T cell immunoglobulin and mucin domain-containing molecule 3 (Tim-3), CTLA-4, lymphocyte activation gene-3 (Lag-3) and B and T cell lymphocyte attenuator (BTLA). Importantly, the pattern of inhibitory receptor expression and the number of receptors, which are simultaneously present on the same T-cell, can influence the severity of T-cell dysfunction. Thus, blockade of immune checkpoints on tumor-infiltrating T-cells (TILs) by therapeutic antibodies targeting CTLA-4 or the PD-1/PD-L1 axis, has become an important therapeutic strategy in cancer immunotherapy to overcome T-cell dysfunction in multiple cancer types.

Here, we explored whether the dysfunctional state of intratumoral T-cells may pose an obstacle to an efficient, TCB-mediated T-cell activation, which is critically required for its therapeutic efficacy. To this end, we dissected T-cell activation and tumor cell killing induced by FolR1-TCB utilizing primary tumor lesions from patients with non-small cell lung cancer (NSCLC), epithelial ovarian carcinoma (EOC) and renal cell carcinoma (RCC). Our data demonstrate for the first time the impact of inhibitory receptor expression on TILs in human tumors on the efficacy of TCB induced T-cell activation and tumor cell killing.

**FolR1-TCB-induced tumor killing is impaired in tumor samples**

TCB-induced killing is highly variable between tumor samples and depends on the E:T ratio and on the constitution of tumor cells, e.g. the PD-L1 expression level. To compare the FolR1-TCB-induced killing capacity of T-cells between tumors samples, and to exclude
additional factors suppressing T-cell functionality, such as expression of PD-L1 on the tumor cells, we exogenously added CFSE-labeled FolR1^C^Skov3 cells to the tumor digests and adjusted the E:T ratio to 1:1. We then measured the FolR1-TCB-induced killing of CFSE-labeled Skov3 cells, which allowed us to also include FolR1^- tumor samples into the analysis. As some tumors from the initial cohort could not be used to characterize TCB-mediated tumor cell killing due to a very low amount of effector cells, a separate cohort of 12 tumor digests and 5 malignant effusions from 15 NSCLC and 2 EOC patients was analyzed. All samples were characterized for their CD3^C^ effector and FolR1^C^ target cell content (Table S1). Tumor cell killing of CD3^C^ T-cells from patients was compared with PBMC-derived T-cells from healthy donors. A substantial heterogeneity in tumor cell killing between individual patients was observed (26 ± 11.8%) after 24h (Fig. 2). Of note, CD3^C^ T-cells from healthy donors induced a significantly better killing than TILs (42.8 ± 9.7%, p = 0.013). Exposure to a control TCB with no binding to a tumor antigen (DP47-TCB) did not induce any tumor cell killing (data not shown).

**Tumor-infiltrating CD8^+ T-cells are highly diverse in the expression of inhibitory receptors**

As tumor-resident T-cells frequently display a highly dysfunctional phenotype^{24-26,35} the observed heterogeneity in T-cell activation among different patients after FolR1-TCB stimulation may be due to an impaired TIL functionality. A hallmark of dysfunctional T-cells in both chronic viral infections and in tumors is the overexpression of inhibitory receptors.^{25,26,31-33}

Figure 1. Activation of CD8^+ T-cells in tumor samples and peripheral blood T-cells from healthy donors upon exposure to FolR1-TCB. FolR1^+ tumor digests and malignant effusions were cultured for 24h in the presence or absence of FolR1-TCB. As comparison, PBMC from healthy donors were co-cultured with the Skov3 tumor cell line and stimulated with FolR1-TCB. (A) The expression of activation markers on CD8^+ T-cells upon FolR1-TCB stimulation was determined by flow cytometry. The FACS plots show FolR1-TCB-induced T-cell activation in a representative patient. The graphs represent the increase in marker expression after FolR1-TCB treatment with mean and standard deviations. (B) IFNγ, IL-2, TNF and perforin in the cell culture supernatants was determined by Cytometric Bead Array or ELISA and normalized to the amount of 1x10^5 CD3^+ T-cells (IFNγ, TNF, IL-2) or CD3^+ CD8^+ T-cells (perforin) in the culture. The P-values were calculated using the unpaired Mann—Whitney test.

Figure 2. FolR1-TCB-induced tumor cell killing varies largely in tumor digests and malignant effusions. FolR1 positive and negative tumor digests, malignant effusions or PBMCs from healthy donors were co-cultured with exogenously added fluorescently labeled FolR1^+ Skov3 cells at an E:T ratio of 1:1 for 24 h in the presence or absence of FolR1-TCB. The FolR1-TCB-induced specific killing of the Skov3 cells was determined by flow cytometry by measuring activated caspase 3 and the live/dead marker Live/Dead-near-IR. FolR1-TCB-mediated killing was calculated as follows: % specific killing = 100 - [(% of Skov3 live cells in FolR1-TCB treated sample / % of Skov3 live cells in untreated sample) × 100]. FACS plots show FolR1-TCB-induced killing in a representative patient. The p-value was calculated using the unpaired Mann—Whitney test.
To this end, we determined the expression of the immune checkpoints PD-1, Tim-3, CTLA-4, Lag-3, and BTLA on tumor-infiltrating CD8^+ T-cells in all tumor samples. We observed a high diversity in frequency and combined expression of these receptors among different tumors: PD-1 was found to be the most prominent inhibitory receptor with the highest percentage of expression (60.2 ± 30%), followed by Tim-3 (29.5 ± 24.4%), CTLA-4 (24.6 ± 17.6%), Lag-3 (7.0 ± 5.9%), and BTLA (3.9 ± 2.6%) (Fig. 3A). As described previously,^37 the PD-1^hi population could be divided into a PD-1^hi and a PD-1^int expressing subgroup (Fig. 3B) Analysis of additional inhibitory receptors expressed on these particular subsets showed a significantly higher expression of all other inhibitory receptors, including Tim-3, CTLA-4, Lag-3, and BTLA, in the PD-1^hi subpopulation as compared with the expression of these receptors in the PD-1^int and PD-1^neg subsets (Fig. 3C) Therefore, we used the percentage of PD-1^hi T-cells in the CD8^+ subset as a surrogate marker for the cumulative expression of inhibitory receptors. The tumor samples were divided according to the frequency of PD-1^hi cells into two groups with high (PD-1^hi abundant tumors) and low frequencies of PD-1^hi expressing T-cells (PD-1^hi scarce tumors), respectively. A cut-off value of 30% PD-1^hi expression was chosen to separate the two groups. The percentage of PD-1^hi cells ranged from 39.1% to 60.5% in the PD-1^hi abundant (49.5 ± 7.9%) and from 2.6% to 19.5% in the PD-1^hi scarce group (8.4 ± 5.7%; Fig. 3D). The cut-off value was validated in a second cohort of 14 NSCLC and 2 ovarian cancer patients with a similar distribution in the frequency of PD-1^hi cells, where we observed comparable results upon polyclonal stimulation by anti-CD3/anti-CD28 antibodies (Fig. S1).

**FolR1-TCB-induced T-cell activation largely depends on the level of PD-1 expression on CD8^+ T-cells**

In a next step, we analyzed whether the expression of inhibitory receptors could be correlated with a diminished T-cell functionality upon FolR1-TCB treatment. FolR1-TCB-induced T-cell activation, as exemplified by CD25, CD137, and ICOS expression (p = 0.028; P < 0.001, and p = 0.008, respectively), and T-cell effector functions, indicated by IFNγ, IL-2, TNF, as well as perforin secretion, were significantly impaired in PD-1^hi abundant tumors compared with PD-1^hi scarce tumors (p = 0.019; p = 0.007; p = 0.028, and p = 0.029, respectively; Fig. 4A and B) Similarly, PD-1^hi abundant tumors displayed a significantly reduced cytotoxicity upon FolR1-TCB stimulation whereas a strong tumor cell killing could be observed in the majority of PD-1^hi scarce tumors (p = 0.021; Fig. 4C)

**PD-1 blockade restores FolR1-TCB-induced T-cell function only in PD-1^hi scarce tumors**

As the level of PD-1 expression on TILs correlates with the efficacy of FolR1-TCB, we analyzed whether blockade of the PD-1/PD-L1 axis in combination with FolR1-TCB treatment might be able to restore T-cell function. We found that upon combined treatment with FolR1-TCB and the PD-1 blocking antibody nivolumab (MDX5C4) secretion of the effector cytokines IFNγ, TNF, and IL-2 as well as perforin could be increased only in some of the PD-1^hi scarce tumors. In contrast, in PD-1^hi abundant tumors PD-1 blockade failed to elicit any response (Fig. 5A) Of note, cytotoxic tumor cell killing could neither be improved in T-cells from PD-1^hi scarce nor from PD-1^hi abundant tumors by the additional PD-1 blockade (Fig. 5B).

**Discussion**

T-cell bispecific approaches that combine activating and recruiting T-cells to the tumor cell surface represent an increasingly attractive addition to the immunotherapeutic arsenal available to treat cancer. We here report on the immuno-modulatory capacity of a CD3 × FolR1-specific TCB in primary cancer lesions from patients with NSCLC, EOC, and RCC. Compared with fully functional peripheral T-cells from healthy donors, we observed a substantial heterogeneity in FolR1-TCB-induced tumor cell killing and T-cell activation among different human tumor samples, resulting in partial or complete impairment of T-cell function in the majority of patients. Comprehensive analysis of inhibitory receptor expression on the cell surface of intratumoral T-cells revealed that the efficacy of T-cell activation by FolR1-TCB inversely correlated with the expression levels of PD-1. Patients with PD-1^hi abundant tumors displayed impaired T-cell activation and effector function upon FolR1-TCB treatment; additionally, these patients did not respond to PD-1 blockade in contrast to their PD-1^hi scarce expressing counterparts. Thus, the bioactivity of bispecific antibodies is considerably hampered by T-cell dysfunction, which is orchestrated, at least in part, by the sustained and highly diverse expression of inhibitory receptors.

It has been convincingly demonstrated that polyclonal T-cell activation by TCBs has therapeutic activity and leads to tumor cell death.6,8,11 The capability of TCBs to successfully target T-cells to tumor cells, thereby overcoming the low clonal frequency of tumor antigen-specific T-cells present within the tumor, has been demonstrated in multiple preclinical studies targeting a large variety of tumor antigens including CD19, EpCAM, CD33 and human epidermal growth factor receptor 2 (Her2).9,10,13,14 Although more than 40 different bispecific antibodies have been described, the promising results obtained from preclinical studies do not easily translate into antitumoral activity in clinical trials.6 To exert their full activity TCBs need to meet and bridge both T-cells and tumor cells at the same time. However, the probability that all three components, T-cells, target cells, and TCB, encounter each other is limited by several factors. Particularly, the administration of TCBs in solid tumors is hampered by multiple local constraints such as low levels of target antigen expression, concentration of the antibody in the tumor, local frequencies of effector and target cells and insufficient T-cell activation.6 In particular, a dysfunctional T-cell state may greatly impact on the therapeutic activity of TCBs. Yet, our current understanding is limited as the majority of proof-of-concept studies utilize T-cells obtained from healthy donors.9,11,12 In agreement with these data we observed a strong upregulation of T-cell activation markers, effector cytokine secretion and tumor cell killing upon FolR1-TCB stimulation in PBMCs from healthy donors. In stark contrast, however, T-cell effector functions largely varied and were generally diminished in intratumoral T-cells. Particularly, killing capacity and effector cytokine production was significantly lower.
Figure 3. Pattern of inhibitory receptor expression on intratumoral CD8⁺ T-cells. (A) The expression level of the inhibitory receptors PD-1, Tim-3, CTLA-4, Lag-3, and BTLA was determined by flow cytometry on CD8⁺ T-cells from tumor digests or malignant effusions. (B) Shows the gating strategy for identification of PD-1⁺, PD-1⁻, and PD-1⁻ CD8⁺ subsets of T-cells from two representative patients and their distribution in the tumor samples analyzed. (C) Co-expression of Tim-3, CTLA-4, Lag-3, and BTLA on PD-1⁺, PD-1⁻, and PD-1⁻ CD8⁺ T-cells. The P-values were calculated using one-way ANOVA with Bonferroni post-hoc-test. (D) FolR1⁺ tumor samples were divided according to the percentage of PD-1⁺ expressing CD8⁺ cells in two groups with PD-1⁺ scarce and abundant expression, respectively.
in TILs with complete loss of IL-2 production and severely impaired TNF and IFNγ secretion in the majority of tumors. These findings are in line with previous observations from chronic viral infections where progressive T-cell exhaustion with hierarchical loss of effector functions has been described.\textsuperscript{28,30,31} Using a malignant peritoneal effusion ex vivo model system, Goere et al. could recently document a heterogeneous T-cell activation upon exposure to catumaxomab, which likely reflects functional hyporesponsiveness. Furthermore, and in line with our own findings, the lack of T-cell activation was not related to the T-cell to tumor cell ratio or the level of tumor-antigen expression on tumor cells.\textsuperscript{38}

Sustained expression of immune checkpoints is a hallmark of exhausted T-cells and co-regulates their dysfunctional state.\textsuperscript{31-33} We documented the expression of the inhibitory receptors PD-1, Tim-3, CTLA-4, Lag-3, and BTLA on

Figure 4. FolR1-TCB-induced T-cell functions depend on the PD-1 expression level of CD8\textsuperscript{+} T-cells. FolR1\textsuperscript{+} tumor digests and malignant effusions were cultured for 24h in the presence or absence of FolR1-TCB. The increase in the expression of activation markers on CD8\textsuperscript{+} T-cells (A) and the increase in the effector cytokines IFNγ, IL-2, TNF, and perforin (B) was determined in PD-1\textsuperscript{hi} scarce and abundant tumors. (C) Both FolR1 positive and negative tumor samples were adjusted by addition of the FolR1\textsuperscript{+} Skov3 cell line to an E:T ratio of 1:1 and killing was compared in PD-1\textsuperscript{hi} scarce and abundant tumors. P-values were calculated using the unpaired Mann–Whitney test.
intratumoral CD8^+ T-cells. In agreement with recent data from our group in NSCLC, PD-1 displayed the broadest expression of all analyzed inhibitory receptors. Observations from chronic murine LCMV infections by Blackburn and colleagues suggest the presence of functionally distinct PD-1 positive T-cell subsets, which can be separated on the basis of MFI levels, using flow cytometry. Of note, PD-1^hi T-cell subsets displayed a high co-expression of Tim-3 and CTLA-4 and to a lesser extent of Lag-3 and BTLA, while their PD-1^int counterparts expressed only low levels of other inhibitory receptors, comparable to PD-1^neg T-cells. The frequency of PD-1^hi CD8^+ T-cells differed largely between patients and allowed us to discriminate between PD-1^hi abundant and scarce tumors. In contrast to patients with a PD-1^hi scarce phenotype, FoR1-TCB-mediated T-cell activation and tumor cell killing was significantly impaired in tumors displaying a PD-1^hi abundant phenotype. These data extend and confirm previous observations that the activation and effector function of CD8^+ T-cells correlates with the co-expression of multiple immune checkpoints. The frequency of PD-1^hi T-cells may therefore be useful as a surrogate marker for the functionality of TILs upon TCB activation as well as serve as a predictive marker for the therapeutic responses to TCB treatment. While this immune profile could guide the selection of patients who are likely to respond to immunotherapy such as TCBs, its correlation with in vivo T-cell exhaustion as a major therapy limiting component and clinical benefits remain to be determined in prospective clinical interventions.

To overcome insufficient T-cell activation and the thereby hampered therapeutic activity of TCBs, combinatorial

Figure 5. PD-1 blockade increases cytokine production but not their cytolytic function in T-cells from PD-1hi scarce tumors only. (A) FoR1^+ tumor digests or malignant effusions were cultured for 24h with FoR1-TCB in the presence or absence of a PD-1 blocking antibody. IFNγ, IL-2, TNF, and perforin in the cell culture supernatants were determined by Cytometric Bead Array or ELISA and normalized to the amount of 1 × 10^5 CD3^+ T-cells (IFNγ, IL-2, TNF) or CD3^+ CD8^+ T-cells (perforin). The increase in cytokine secretion upon combined FoR1-TCB and anti-PD-1 treatment compared with FoR1-TCB alone was determined in PD-1^hi scarce and abundant tumors. (B) Tumor digests or malignant effusions were co-cultured with exogenously added fluorescently labeled Skov3 cells at an E:T ratio of 1:1 for 24h in the presence or absence of a PD-1 blocking antibody and FoR1-TCB. The increase in specific killing by the α-PD-1 antibody was compared in PD-1^hi scarce and abundant tumors. P-values were calculated using the unpaired Mann–Whitney test.
approaches targeting immune checkpoints or additional activating receptors on T-cells may be promising strategies. Agonistic antibodies to costimulatory receptors such as CD28, 4-1BB, OX40, GITR, and LIGHT have been demonstrated to successfully induce antitumor immunity.\textsuperscript{43,44} The combination of bispecific antibodies with antibody-ligand fusion proteins targeting these receptors has been shown to significantly increase the efficacy of TCBs both \textit{in vitro} and in murine tumor models.\textsuperscript{45,46} However, the translation of such combinatorial approaches into the clinic requires an in-depth understanding of the effects elicited by potent T-cell stimulation as severe unexpected reactions such as massive cytokine release have been observed in a clinical phase I trial after superagonistic anti-CD28 treatment.\textsuperscript{47} Another promising avenue to improve the therapeutic efficacy of TCBs lies in the blockade of inhibitory signals on T-cells. Antibody-mediated interference with a single inhibitory receptor, namely of the PD-1/PD-L1 axis, has seen remarkable clinical success in multiple cancer types.\textsuperscript{4,5} and is currently being actively pursued around the globe. As PD-1 was the most prominently expressed inhibitory receptor in all tumors analyzed we assessed whether PD-1 blockade could enhance T-cell effector functions upon TCB activation. Of note, we observed increased secretion of effector cytokines upon combined FolR1-TCB and anti-PD-1 treatment, though only in PD-1\textsuperscript{hi} scarce tumors. These findings are in line with data from chronic viral infections where PD-1 blockade could only rescue T-cell subsets with intermediate but not high levels of PD-1 expression.\textsuperscript{47} Approaches combining blockade of the PD-1/PD-L1 axis with agonistic antibodies to either OX40 or CD27 have been shown to temporarily restore functionality to exhausted T-cells in a murine bone marrow transplantation model\textsuperscript{48} thereby leading to the loss of Tbet\textsuperscript{hi} PD-1\textsuperscript{int} T-cells and contraction of the effector pool capable to sustain the immune response. Similarly, administration of low-dose IL-2 combined with PD-L1 blockade has been demonstrated to decrease inhibitory receptor expression and viral load in a chronic murine LCMV model as IL-2 treatment may foster the generation of a pool of CD44\textsuperscript{hi} PD-1\textsuperscript{int} T-cells that are more responsive to PD-L1 blockade.\textsuperscript{49} Thus, novel therapeutic strategies, exploring the generation of more PD-1\textsuperscript{int} and PD-1\textsuperscript{hi} cells, instead of PD-1\textsuperscript{hi} cells to increase the susceptibility to PD-1/PD-L1 blockade, are clearly needed.

Remarkably, we observed no improvement on tumor cell killing upon concomitant PD-1 blockade in all of the tumor samples. Thus, blockade of a single immune checkpoint may not be sufficient to restore the cytolytic capacity of TILs. In a mouse tumor model, however, blockade of the PD-1/PD-L1 axis has been shown to increase T-cell infiltration into tumors\textsuperscript{50} a characteristic of this treatment, which could not be addressed by our \textit{in vitro} approach. Thus, the therapeutic effect of PD-1 blockade \textit{in vivo} might not only result from improving T-cell cytotoxicity of residual intratumoral T-cells, but from the sustained functionality of newly infiltrating T-cells. TCB-induced T-cell activation has been shown to upregulate PD-1 expression, which may lead to secondary resistance in the presence of PD-L1 expressed on both tumor cells and infiltrating immune cells as recently demonstrated both with a Her2-specific TCB and with a carcinoembryonic antigen- (CEA) specific TCB.\textsuperscript{34,51} Importantly, blockade of the PD-1/PD-L1 axis could completely restore TCB-induced T-cell function both \textit{in vitro} and in a mouse tumor model. These observations indicate that co-administration of checkpoint inhibitors is capable of preventing secondary resistance, which may add to the dysfunctional state of TILs and limit the therapeutic efficacy of TCBs. Further work is clearly needed to determine optimal combination regimens of checkpoint inhibitors and TCBs. It will also be crucial to identify inhibitory and activating T-cell-receptors with non-redundant functions as potential therapeutic targets.

Our findings clearly indicate that bispecific antibodies such as FolR1-TCB are capable of causing T-cells to upregulate costimulatory molecules, produce inflammatory cytokines, and acquire cytolytic function. We have observed different states of T-cell dysfunction, which are orchestrated, at least in part, by the expression of inhibitory receptors and impair the therapeutic efficacy of the TCB. As FolR1-TCB-induced effector functions could only be partially restored by PD-1 blockade, our results suggest a rather complex immune regulation, which utilizes multiple and eventually non-redundant pathways to maintain T-cell dysfunction within the tumor environment. Future work will need to address the mechanistic role and significance of these receptors, their functional overlap, and importantly, their interplay with other inhibitory pathways. Additional combinatorial approaches of TCBs with other therapeutic strategies targeting T-cell dysfunction such as co-stimulation or the blockade of immune checkpoints need to be explored to unleash the full armamentarium of effector functions in tumor-infiltrating T-cells.

\section*{Material and methods}

\subsection*{Patients and tumor sample processing}

Fresh tumor tissues and malignant effusions were collected from 16 patients with NSCLC, 7 patients with EOC and two patients with RCC undergoing surgical resection and drainage, respectively, between January 2013 and August 2014 at the University Hospital Basel, Switzerland. Detailed patient characteristics are provided in Table S1. The analyzed samples comprised 15 solid tumor-derived single cell suspensions and 10 malignant effusions. The study was approved by the local Ethical Review Board (Ethikkommission Nordwestschweiz) and all patients consented in writing to the analysis of their tumor samples. An experienced pathologist carried out the histological analysis.

Solid tumor lesions were dissociated mechanically and digested using acctonate (PAA), collagenase IV (Worthington), hyaluronidase (Sigma), and DNase type IV (Sigma) directly after excision. Single-cell suspensions were prepared. The cellular fraction of malignant effusions was isolated by density gradient centrifugation using Histopaque-1119 (Sigma). All samples were stored in liquid nitrogen until further usage.

\subsection*{Reagents}

The T-cell bispecific antibodies engaging CD3 and folate receptor 1 (FolR1-TCB) or CD3 and an irrelevant antigen (DP47-TCB) were provided by Roche Innovation Center Zurich and the therapeutic PD-1 blocking antibody nivolumab (MDX5C4) antibody was...
commercially available. The following human mAbs were used: α-CD4-PE, α-CD8-PE-Cy7, α-CD45-PE-Cy7, α-CD45-PerCP-Cy5.5, α-CD137-FITC, α-CD4-PerCP-Cy5.5, α-BTLA-Biotin, α-CTLA-4-PE, α-ICOS-FITC, α-IFNgamma-FITC, α-Lag-3-APC (all eBioscience), α-CD3-PECF594, α-CD25-BV605, α-CD69-FITC, α-EpCAM-FITC, α-granzyme B-PE, α-active caspase 3-PE, α-PD-1-BV605, Streptavidin-BV711 (all BD Bioscience), α-FoxP3-AlexaFluor647, α-Tim-3-BV421 (all Biolegend), anti-FolR1-APC (aa25–233) (LifeSpanBiosciences). Dead cells were stained with LIVE/DEAD* Fixable Near-IR Dead Cell Stain Kit or LIVE/DEAD* Fixable Blue Dead Cell Stain Kit (Invitrogen). For intracellular staining, Fixation and Permeabilization Buffers from eBioscience were used. Samples were acquired for flow cytometric analysis on a BD LSR Fortessa. The human IL-2, IFNγ, and TNF ELISA sets and the human Th1/Th2/Th17 CBA Kit were all obtained from BD Bioscience; the human Perforin (PRF1) ELISA Kit was obtained from Abcam.

Tumor sample characterization
All tumor samples were comprehensively characterized by multicolor flow cytometry. In detail, CD8+ and CD4+ T-cells (CD45+CD3+) were characterized for the expression of the surface markers PD-1, Tim-3, CTLA-4, Lag-3, and BTLA. Tumor cells (CD45+EpCAM+) were characterized for the expression of FolR1 by comparing the binding of a FolR1 specific antibody with its matched isotype control. Only samples that were positive for FolR1 expression were used for characterization of T-cell activation and cytokine secretion using FolR1-TCB.

Ex vivo treatment of tumor samples with FolR1-TCB
FolR1 positive tumor digests or malignant effusions were thawed, washed and plated in 96-well flat bottom cell culture plates (BD Falcon) at a density of 3 x 10^5 cells/200µL/well in complete medium (DMEM + sodium pyruvate (1mM) + MEM non-essential AA (1x) + L-glutamine (2mM) + penicillin/streptomycin (100ng/mL) + 2-mercaptoethanol (50nM) + ciproxin (1mg/mL) + 10% human serum). The samples were cultured in the presence or absence of FolR1-TCB or DP47-TCB at a concentration of 2 nM for 24 h. Where indicated, anti-PD-1 antibody was added at a concentration of 10µg/mL per well. Activation of CD8+ and CD4+ T-cells (CD45+CD3+) upon FolR1-TCB treatment was determined by multicolor flow cytometry by measuring the expression of the cell surface markers CD25, CD137, and ICOS. The concentration of IFNγ, IL-2, TNF, and perforin in the cell culture supernatants was measured by ELISA or Th1/Th2/Th17 CBA Kit (BD Biosciences) following the instructions of the manufacturers.

Killing assay
To determine the FolR1-TCB-induced tumor cell killing, 3 x 10^4 CFSE-labeled Skov3 cells were co-cultured with tumor digests or malignant effusions in the presence or absence of FolR1-TCB at a concentration of 2 nM for 24 h in 96-well flat bottom cell culture plates. Where indicated, anti-PD-1 antibody was added at a concentration of 10µg/mL per well. The E:T ratio (E: effector CD45+CD3+ cells; T: target FolR1+ cells from tumor and added Skov3 cells) was adjusted to 1:1 in each well and the cell number of the added tumor samples was calculated for each sample according to prior characterization by flow cytometry. Cell death of Skov3 cells was determined by flow cytometry by measuring activated caspase 3 and the live/dead marker Live/Dead-near-IR. The assay was performed in duplicate. The FolR1-TCB-mediated killing was calculated according to the following equation: % specific killing = 100 − [(% of Skov3 live cells in FolR1-TCB treated sample / % of Skov3 live cells in untreated sample) × 100].

Statistical analysis
Statistical analysis was performed using one-way ANOVA with Bonferroni post-hoc-test or the unpaired Mann–Whitney test, as indicated. The differences were considered as statistically significant if p < 0.05 (*), p < 0.005 (**), or p < 0.001 (**). For allocation of tumors to groups of low (i.e., PD-1hi scarcest tumors) and high frequencies of PD-1hi expressing T-cells (i.e., PD-1hi abundant tumors) a cut-off value of 30% PD-1hi expression on CD8+ T-cells was determined and validated in a second cohort consisting of 14 NSCLC and two ovarian cancer patients upon polyclonal stimulation by anti-CD3/anti-CD28 antibodies (Fig. S1)

Disclosure of potential conflicts of interest
J.S., D.S.T., P.M., P.H.U. and A.Z. received research funding from Roche Innovation Center Zurich, A.R. and A.B. are employed by Roche Innovation Center Penzberg, P.U., P.P., C.K., M.B., V.L. and V.K. are employed by Roche Innovation Center Zurich. The other authors have declared that no conflict of interests exists.

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Author contributions
J.S., D.S.T., P.M. and A.Z. designed and performed experiments and analyzed results; P.H.U. and F.U. performed experiments; A.R. and A.B. performed the statistical analysis; D.L., W.M., V.A.H.S and S.S. provided samples; P.U., P.P., C.K., M.B., V.L. and V.K. provided conceptual advice, generated and provided key reagents and wrote the paper; J.S., D.S.T., P.M. and A.Z. designed the research, made the figures, and wrote the paper. D.S.T., P.M. and A.Z. applied for funding.

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