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Research Article

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Research Article

Protective low-avidity anti-tumour CD8+ T cells are selectively attenuated by regulatory T cells

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Summary

Objectives Regulatory T cells (Treg) play a major role in the suppression of protective anti-tumour T cell responses. In the CT26 BALB/c murine model of colorectal carcinoma, Tregs differentially suppress responses to two characterised CD8+ T epitopes, AH1 and GSW11, which results in an absence of detectable IFN-γ-producing GSW11-specific T cells in the spleen and lymph nodes of tumour challenged mice. Activation of GSW11-specific T cells correlates with protection against tumour progression. We wanted to examine the presence of non-functional GSW11-specific T cells in Treg replete and depleted mice, assess their phenotype and their affinity compared to AH1-specific T cells.

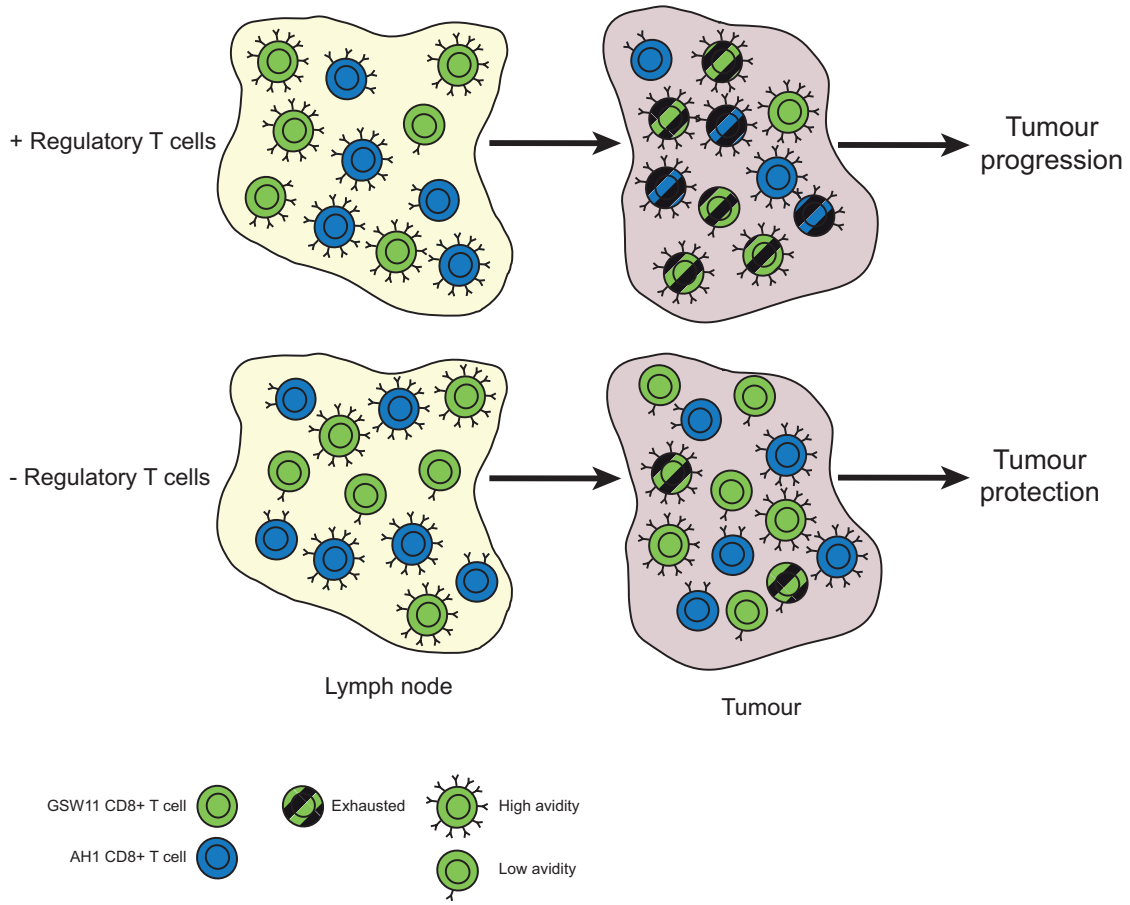
Methods We used peptide-specific tetramers to identify tumour-specific CD8+ T cells and assessed the cell surface expression of markers associated with exhaustion (PD-1, Tim3 and Lag-3) and their function by IFN-g production using flow cytometry. We also assessed the T cell receptor (TcR) clonality of tumour-specific T cells. Tetramer competition assays were performed to determine the relative affinity of identified TcR.

Results Here, we show that GSW11-specific T cells are in fact induced in Treg-replete, CT26-bearing mice, where they make up the majority of tumour-infiltrating CD8+ lymphocytes, but exhibit an 'exhausted' phenotype. This dysfunctional phenotype is induced early in the anti-tumour response in tumours. Depletion of Tregs prior to tumour challenge correlates with an altered T cell receptor (TcR) repertoire. Moreover, the avidity of GSW11-specific TcRs that expanded in the absence of Tregs was significantly lower compared with TcRs of CD8+ populations that were diminished in protective anti-tumour responses.

Conclusion Our results indicate that Tregs suppress the induction of protective anti-tumour T cell responses and may signify that low-avidity T cells play an important role in this protection.

Abbreviations: CTL: Cytotoxic T lymphocytes; IFN-γ: Interferon gamma; MHC I: MHC class I molecule; PCR: Polymerase chain reaction; SCT: Single-chain trimer; TAA: Tumour-associated antigens; Treg: Regulatory T cell; TcR: T cell receptor; TIL: tumour-infiltrating lymphocytes.

Graphical Abstract



Keywords: regulatory T cell, immunotherapy, tumour immunity, antigen presentation

Introduction

CD8+ T cell responses directed to tumours occur in many human cancers, where they are a positive prognostic indicator [1–5]. Backed by studies in preclinical mouse models, which clearly show that CD8+ T cells are important in the clearance of tumours and may confer lifelong protection against malignancy [6, 7], immunotherapies aimed at boosting anti-tumour cytotoxic T lymphocytes (CTL) are showing promise in the clinic. Naturally occurring responses can be initiated during tumour growth to establish immunosurveillance in which a dynamic process of immunoediting can ensue, where immunological pressure from anti-tumour CTL balances tumour elimination against the emergence of tumour escape variants with no accompanying net outgrowth of tumour [8]. This process is responsible for shaping the immunogenicity of the tumour [9]. Breakdown of this equilibrium leading to tumour outgrowth involves multiple factors, including the balance between T cell activatory (TcR engagement and co-stimulation) and inhibitory signals (exhaustion markers and immunosuppressive cytokines), and evasion of the T cell response through downregulation of antigen processing machinery or antigen loss. Therapeutic approaches designed to tip the balance back in favour of tumour elimination by providing activation agonists or blockade of inhibition are attractive strategies (reviewed in ref. [10]). FoxP3+ CD4+ regulatory T cells (Treg) are important in establishing an immunosuppressive tumour microenvironment, and their infiltration into tumours is a negative prognostic biomarker [11] and a significant obstacle to successful immunotherapy, correlating with a poorer outcome in clinical

trials (reviewed in ref. [12]). Therefore, Treg depletion as a therapeutic option is being pursued in the clinic, based on studies in mice that showed rejection of transplanted tumours following ablation of Treg with anti-CD25 antibodies [13, 14].

One of the most widely used mouse models for preclinical testing of new immunotherapeutic drugs is the transplantable BALB/c-derived colorectal tumour CT26 [15, 16]. In this model, we have shown that depletion of Tregs induces robust protective anti-tumour immunity that effects tumour rejection in ~90% of mice, similar to responses observed in other mouse tumour models [13, 17]. The CT26-immune mice developed memory CTL responses and were able to reject a second challenge with CT26 as well as tumour lines of different histological origin following recovery of Tregs to normal levels. Anti-tumour responses in these mice are focussed on two epitopes derived from *gp90*; AH1 (SPSYVYHQF [18]) and GSW11 (GGPESFYCASW [17]). The anti-GSW11 response is more sensitive to Treg suppression *in vivo*, illustrated by the fact that functional (IFN-γ-producing) anti-GSW11 CTL can only be detected in tumour draining lymph nodes (tdLN) in the absence of Treg whereas anti-AH1 CTL are detected whether or not Treg are present [17]. Anti-GSW11 CD8+ T cells deliver the most potent anti-tumour response characterised by their ability to reject tumours expressing very low levels of antigen [13, 17].

To investigate the basis of differential suppression of the GSW11-specific T cell response further, we utilised peptide-specific tetramers, to detect both functional (IFN-γ^+) and inactivated (IFN-γ^-) antigen-specific T cells. We show that, in Treg replete tumour-bearing mice, GSW11-specific T cells made up the majority of CD8+ tumour-infiltrating lymphocytes (TIL), but a significant proportion exhibit an exhausted phenotype characterised by high expression of PD-1, Tim-3, and Lag3. We go on to show the preferential suppression of low-avidity anti-GSW11 T cells by Treg, which expand when Treg are depleted and correlate with protection.

Materials and Methods

Mice, antibodies, and *in vivo* depletion

BALB/c mice were bred under specific pathogen-free conditions in Southampton. Female or male mice (6–8 weeks old) were used in all experiments and during experimental procedures mice were housed in conventional facilities. Animal experiments were conducted according to the UK Home Office license guidelines and approved by the University of Southampton Ethical Committee. CD25-specific mAb (PC61.5.3, rat IgG1; Bio X Cell) previously described [13] and anti-horseradish peroxidase isotype control (HRPN, rat IgG1; Bio X Cell) were used in depletion experiments. Antibodies are very low endotoxin (<1EU/mg) determined by LAL gel clotting assay. For depletion, mice received intraperitoneal (i.p.) injection of 200 µg of mAb PC61 or isotype control in 100 µl sterile endotoxin-free PBS (Sigma-Aldrich) on days –3 and –1 prior to tumour challenge.

Tumour cells and *in vivo* challenge

CT26 tumour cells (American Type Culture Collection) were maintained in RPMI (Sigma) supplemented with 10% FCS (Globepharm), 2 mM L-glutamine, penicillin/streptomycin (Sigma), 50 µM 2-mercaptoethanol, 1 mM sodium pyruvate (Gibco-BRL), and 1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (PAA laboratories) and confirmed to be mycoplasma free. In all experiments, mice were injected subcutaneously (s.c.) with 10^5 tumour cells in endotoxin-low PBS. All flow cytometry data acquisition was carried out on a FACS Canto II (BD Biosciences) and all data analysed with FlowJo Software (Treestar).

DNA construct

The H2-D^d single-chain trimer (SCT) construct incorporating a *gp120* HIV peptide (a kind gift from Dr. Keith Gould) was mutated into the GSW11 peptide via site-directed mutagenesis polymerase chain reaction (PCR) using KOD HotStart polymerase (Merck Biosciences) according to the manufacturer's instructions. The transmembrane domain of H2-D^d was substituted for a biotinylation site using overlapping extension PCR. In addition, a disulphide trap was incorporated into the construct [19] to tether the GSW11 peptide onto the MHC I binding grove.

Tetramer generation

Tetramers were produced with the help and advice of the Cancer Research UK/Experimental Cancer Medicine Centre Protein Core Facility (Cancer Sciences Unit, University of Southampton, Southampton, UK) with few modifications. The GSW11-SCT construct containing H2-D^d, β2m, and GSW11 peptide was cloned into the pET-3a expression vector (Novagen) and expressed in BL-21 CodonPlus RIPL cells (Stratagene). Concentrated refolded complexes were purified on a HiLoad 26/60 Superdex 200 column (GE Healthcare). Biotinylation was achieved with 50 µM d-biotin and 1 µg/ml biotin protein ligase (Avidity) at 16°C overnight and then passed through the column a second time. Biotinylated monomers were dialysed and subsequently stored in 16%

glycerol in phosphate-buffered saline or tetramerised by incubation with 1:4 molar ratio of PE-labelled streptavidin (Thermofisher) at 4°C. Each batch of tetramers was tested for binding against the GSW11-specific T cell hybridoma, CCD2Z (Supplementary Fig. 1). For the analysis of AH1-specific T cells, AH1-specific dextramers were used (Immunodex).

Isolation and analysis of antigen-specific T cells and Tregs

Tumour draining lymph nodes and tumours from CT26 challenged mice (Treg depleted or replete) were harvested between days 7 and 22 and disaggregated. CD8+ T cell responses to CT26 antigens GSW11 and AH1 were assessed using antigen-specific tetramers and the production of IFN-γ following peptide stimulation. CD8+ T cells, APCs, and peptides were cultured together in the presence of brefeldin A (BD biosciences) for 4 h at 37°C. Cells were harvested and washed twice before being incubated with an FcγR-block (2.4G2; BD Biosciences) for 10 min at RT and stained for cell surface anti-CD8 (63–6.7; BD biosciences), antigen-specific tetramer/dextramer, anti-PD-1 (RMPI-30; eBioscience), anti-Tim-3 (8B.2C12; eBioscience), anti-Lag3 (C9B7W; Biolegend) for 30 min on ice. For intracellular staining, the cells were washed and fixed using the Cytofix/Cytoperm kit (BD biosciences) according to the manufacturer's instructions, before washing twice and incubating with anti-IFN-γ (XMG1.2; BD biosciences) for 30 min on ice. Cells were collected on the flow cytometer (FACSCanto; BD Biosciences) and data were analysed using FlowJo software (BD). Unstained and single antibody stained controls were used to measure autofluorescence and for compensation. Numbers reported for IFN-γ production are those above the background control response of T cells incubated with no peptide or irrelevant peptide. Single-cell CD8+ lymphocytes were gated and assessed for tetramer/dextramer binding and expression of IFN-γ. For the analysis of terminal exhaustion, cells were gated on CD8+ and tetramer/dextramer binding and the level of PD-1 expression assessed. Those expressing high levels of PD-1 were assessed for expression of Tim-3 and Lag-3 and cells expressing both Tim-3 and Lag-3 were further analysed for IFN-γ expression. To assess the effector CD8+ T cell population, CD8+, tetramer/dextramer binding, PD-1 intermediate expressing T cells were assessed for IFN-γ production. Gating strategies are shown in Supplementary Fig. 2. Enumeration of T cell populations was calculated from the indicated gated populations and normalised to numbers of cells/100,000 total cell counts for the sample. To examine the expression of PD-1 on naive antigen-specific T cells, splenocytes from naive BALB/c mice were harvested and stained with anti-CD8, tetramer/dextramer and anti-PD-1, collected on a flow cytometer (FACSCanto; BD biosciences) and analysed using FlowJo software (BD). Cells were gated for expression of CD8 and binding to tetramer/dextramer and the level of PD-1 expression assessed (Supplementary Fig. 2). To assess T cell receptor clonality of GSW11-specific T cells, we used a panel of 15 Vβ-specific antibodies (BD biosciences). First, total CD8+ T cells were purified using a CD8 magnetic isolation negative selection kit (Miltenyi) according to the manufacturer's instructions. Purified CD8s were stained with GSW11-specific tetramer, α-Vβ kit, α-CD8 and collected on a flow cytometer (FACSCanto; BD Biosciences). Data were analysed using FlowJo software (BD).

Tetramer competition assay

Spleens and tumour draining lymph nodes were pooled from Treg depleted or replete mice. CD8+ T cells were purified from disaggregated tissues using magnetic isolation by negative selection (Miltenyi). Purified CD8+ T cells were incubated with 50 nM of dasatinib (New England Biolabs) to prevent TcR internalisation before staining with α-CD8, α-TCR β-chain (H57-597; Biolegend), and 5 μg of PE-labelled GSW11-specific tetramers. After two washes, cells were incubated with bleached tetramer at varying ratios of the initial PE-labelled tetramers: 2.5, 5, 10 or 20 μg per test. Bleached tetramers were tested for no/minimal PE-fluorescence before use. The β-chain TCR staining was included to confirm the decreasing levels of PE staining was due to the fluorescently labelled tetramer being out-competed and not due to TcR internalisation.

Statistical analysis

Analyses were performed using Prism software (GraphPad, San Diego, CA). The *P* values were calculated using either two-way analysis of variance with Dunnett's post-test or two-tailed unpaired *t* test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$).

Results

GSW11-specific CD8+ T cells infiltrate CT26 tumour, but most are exhausted

Recent studies using mouse models have shown that many tumour-infiltrating anti-tumour CD8+ T cells have an exhausted phenotype, characterised by the expression of PD-1, Lag3, and Tim-3 and a failure to express cytokines IL-2, TNF-α and IFN-γ [20, 21]. Little is known about whether, or to what extent, development of this

phenotype relates to T cell specificity. Clearly, a relationship exists inasmuch as T cell activation via TcR is a prerequisite for the induction of exhaustion (reviewed in ref. [22]). Given our previous observations of differential suppression of anti-CT26 responses with different specificity [17], we sought to investigate the induction of exhaustion in both AH1 and GSW11-specific CD8+ T cells in CT26 tumour-bearing mice using antigen-specific tetramers as specificity probes for T cell populations, independent of their functional phenotype. Due to the poor binding affinity of GSW11 for H2-D^{d} [17], we utilised single-chain trimer tetramers with GSW11 tethered to the binding groove using a short linker polypeptide [19], allowing stable expression of GSW11-D^{d} monomers. In CT26 challenged Treg replete mice, GSW11-specific T cells were the most abundant CD8+ T cell population in tumours, making up >50% of all CD8+ T cell infiltration after 14 days of tumour challenge (Fig. 1A–C). However, similar to the situation seen in many human cancers, these infiltrating CD8+ T cells do not confer protection, and tumours continue to grow in these animals. The population of GSW11-specific T cells was significantly larger than AH1-specific T cells, which constituted a maximum of 20% of infiltrating T cells at later stages of tumour challenge (day 17 and day 22; Fig. 1B and C). Notably, while the two T cell populations (anti-GSW11 and -AH1 CD8+ T cells) made up the majority of tumour-infiltrating CD8+ T cells (>60%) at later time points (day 14–day 22), they were in the minority in early anti-tumour responses (day 7–day 10, Fig. 1C). Thus, an initial broad-specificity polyclonal TIL response becomes focussed on two *gp70* derived epitopes during tumour growth (Fig. 1C). We next investigated the function, after *ex vivo* peptide stimulation, of GSW11- and -AH1-specific CD8+ T cells harvested from tumours during the course of the challenge. The vast majority of GSW11-specific CD8+ T cells were unable to produce IFN-γ, with a decrease in functional T cells as the tumour progressed (Fig. 1D and E). Most of the AH1-specific T cells were also non-functional, although, consistent with earlier studies showing their presence in tdLN and spleen [17], there were significantly more functional AH1-specific T cells at day 22 compared with GSW11 (around 6% compared with <1% functional; Fig. 1D and E). This minor population of functional tumour-specific T cells was, however, unable to stop tumour progression.

The progressive expression of PD-1, Tim-3, and Lag3 is consistent with the development of terminal exhaustion in T cells resulting from persistent antigenic stimulation [23]. To investigate whether the lack of effector function in anti-CT26 tumour T cells correlated with the development of T cell exhaustion we assessed the expression of PD-1, Lag3, and Tim-3 on GSW11- and AH1-specific T cells and antigen-specific IFN-γ production following tumour challenge (Fig. 2A). The analysis of the number of antigen-specific T cells in tumours showed that both GSW11 and AH1 were similarly abundant at day 7, but these numbers decreased over time where a significantly greater number of GSW11-T cells where present compared with only a small number of AH1-T cells at day 22 (Fig. 2B). Examination of the development of terminal exhaustion in these antigen-specific T cells indicated a greater number of GSW11-T cells compared with AH1 had an exhausted phenotype at day 7 and day 22 (Fig. 2C). Further investigation of these exhausted T cells revealed that a small number of GSW11- and AH1-T cells retained effector function with GSW11-T cells more numerous than AH1 (Fig. 2D). Together, these data indicate that a proportion of tumour GSW11-T cells are exhausted and are affected to a greater extent than AH1-specific T cells. Examination of PD-1^{int}, Tim3^{-}, Lag3^{-} T cells, indicative of an effector phenotype, showed similar number of GSW11 and AH1-specific T cells produced IFN-γ (Fig. 2E). The number of effector T cells producing IFN-γ for both specificities decreased as the tumour progressed with only a few IFN-γ producing T cells detectable at day 22 (Fig. 2E). Although the numbers of samples were low, there was a clear trend indicating that the induction of an exhausted phenotype in tumour-specific CD8+ T cells is not equivalent between antigen specificities. This suggests that the dominant tumour-infiltrating GSW11-specific T cells are more susceptible to the induction of exhaustion, which occurs at an early stage following tumour challenge.

Presence of exhausted anti-tumourT cells in tumour draining lymph nodes

We next investigated whether this difference in susceptibility to exhaustion between GSW11- and AH1-specific T cells is a consequence of the tumour microenvironment or an intrinsic aspect of the T cells at the priming stage. Examination of naive T cell populations (GSW11 and AH1) showed that both expressed low levels of PD-1, consistent with previous studies [24], with expression on GSW11-specific T cells being slightly greater than AH1-specific T cells (Fig. 3A). Early after tumour challenge (day 7) GSW11-specific T cells accumulated in the tumour draining LN (tdLN), which decreased over time (Fig. 3B). By contrast, the number of AH1-specific T cells was similar throughout the course of tumour challenge (Fig. 3B). A similar proportion of AH1- and GSW11-specific T cells had an effector T cell phenotype (PD-1^{int}, Tim-3^{-}, Lag3^{-}, IFN-γ^{+}) early after tumour challenge

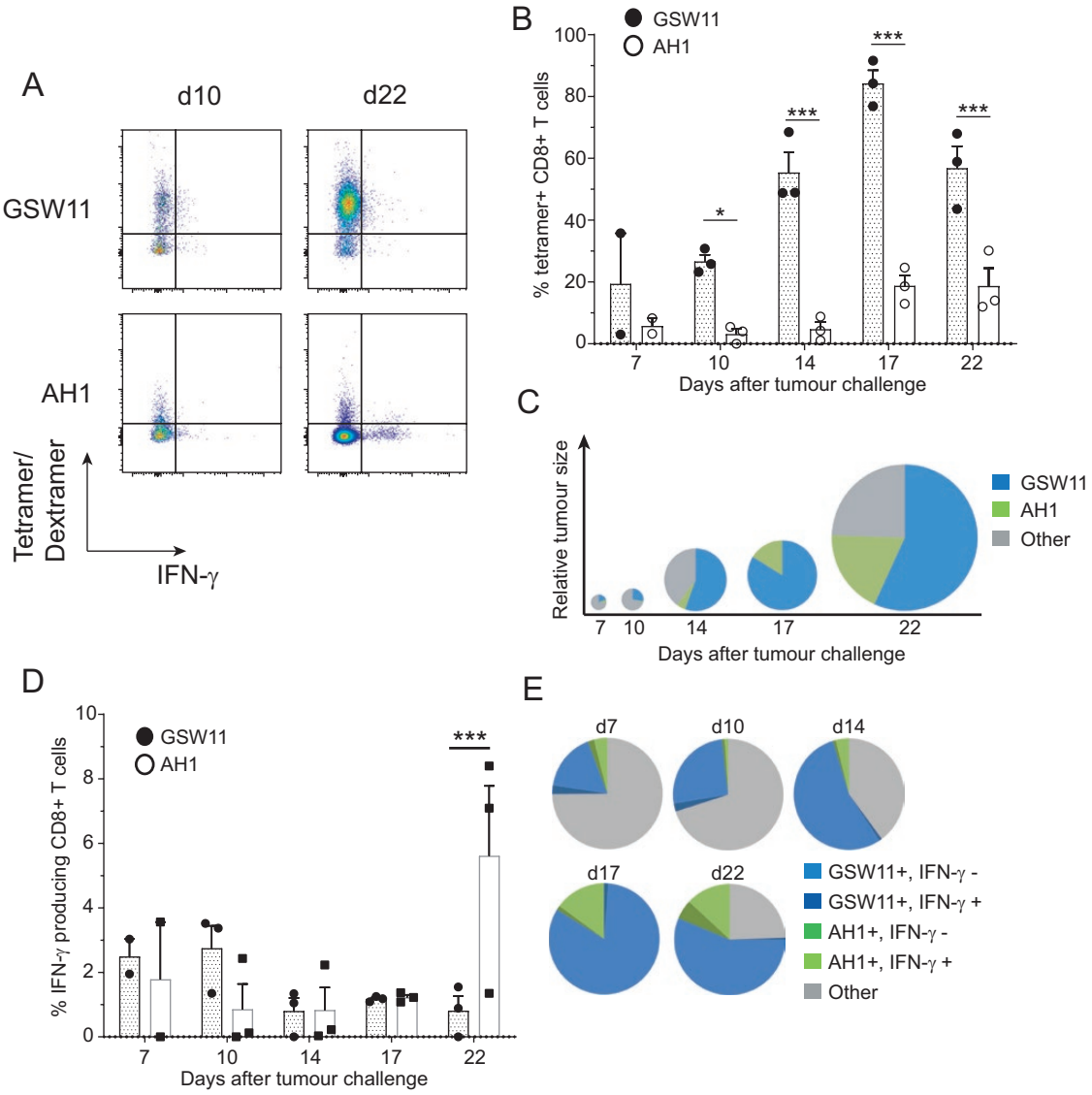


Figure 1. The majority of tumour-infiltrating GSW11-specific T cells are non-functional. BALB/c mice were challenged with CT26 tumour cells and the presence of tumour-infiltrating AH1- and GSW11-specific T cells was assessed over the indicated time course. (A) Assessment of AH1- and GSW11-specific T cells using tetramer/dextramer and IFN-γ production at day 10 and day 22. (B) Percentage of antigen-specific T cells detected by tetramer/dextramer. (C) Diagram representing the tumour size (diameter) and relative proportions of antigen-specific tumour-infiltrating T cells over time. (D) Percentage of functional AH1- and GSW11-specific CD8+ T cells. (E) Relative proportion of functional and non-functional antigen-specific tumour-infiltrating T cells. (B, D; mean and SEM of two/three mice at each time point; $*P < 0.05$, $***P < 0.001$).

(day 7), which remained comparable throughout tumour challenge (Fig. 3C). We next examined whether tumour challenge led to the induction of exhausted T cells in tdLN. The proportion of exhausted antigen-specific T cells (PD-1^{hi}, Tim-3^{+}, Lag3^{+}) was low for both T cell specificities at day 7 after tumour challenge. These levels increased as the tumour progressed with ~20% of GSW11 and AH1-specific T cells having an exhausted phenotype at day 22 (Fig. 3D). Interestingly, the proportion of IFN-γ producing exhausted CD8+ T cells diminished earlier for GSW11-specific T cells (day 14) compared with AH1 (day 22) (Fig. 3E). These data indicate that a greater number of GSW11- compared with AH1-specific T cells are activated following tumour

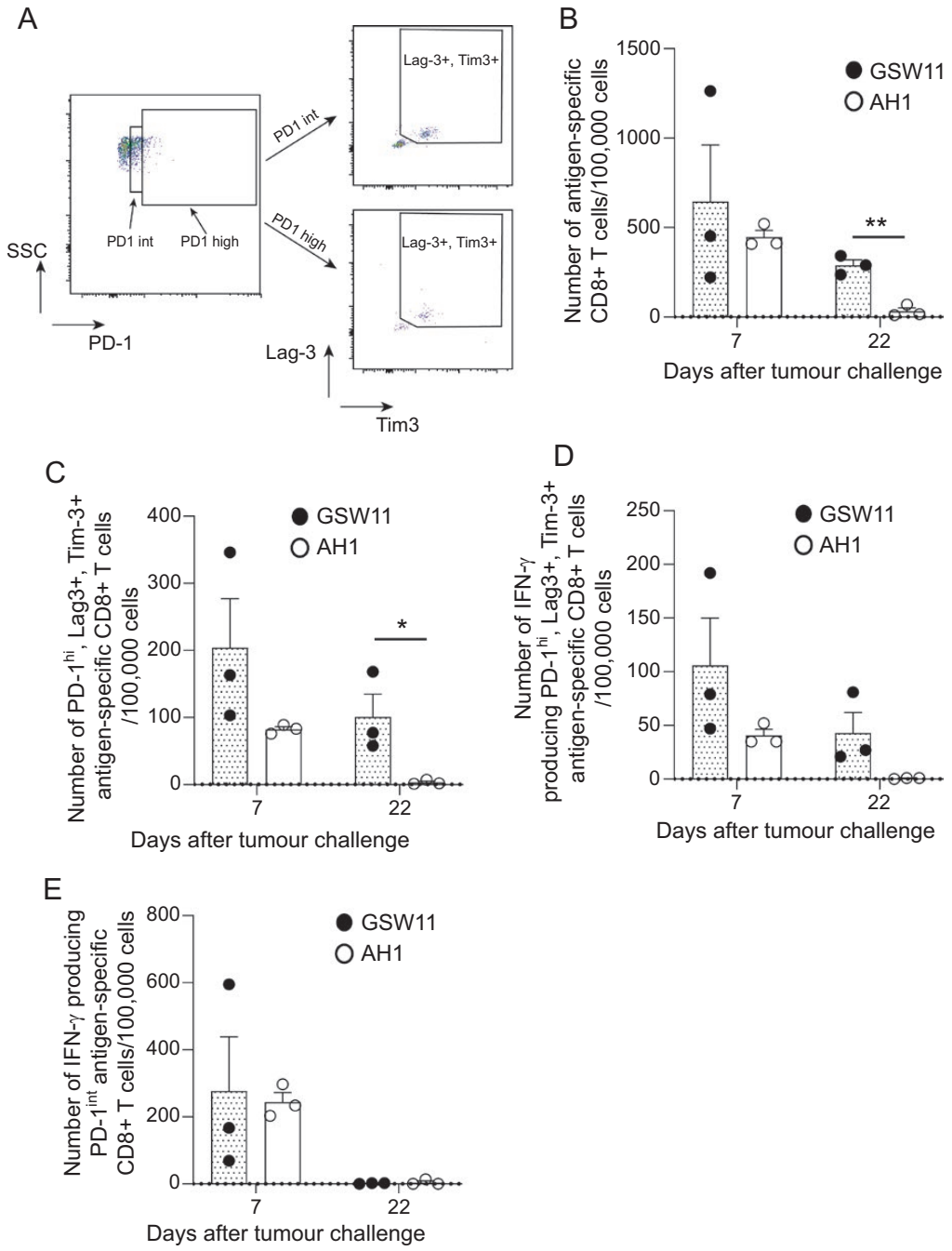


Figure 2. Tumour-infiltrating GSW11-specific T cells are more exhaustible than AH1-specific T cells. (A) A representative gating strategy to assess Lag-3 and Tim3 expression on PD-1^{int} and PD-1^{hi} CD8+ T cells. BALB/c mice were challenged with CT26 tumour and the number of antigen-specific CD8+ T cells/100,000 cells was assessed at early (day 7) and late (day 22) stages of tumour progression was assessed (B). The number of tumour-infiltrating PD-1^{hi}, Lag3+, Tim-3+ GSW11-, and AH1-specific CD8+ T cells (C) and the number of those cells that produced IFN-γ following peptide antigen stimulation was evaluated (D). Assessment of the number of functional tumour-infiltrating PD-1^{int} GSW11- and AH1-specific CD8+ T cells (E). B–E; mean and SEM of three mice at each time point; $*P < 0.05$, $**P < 0.01$).

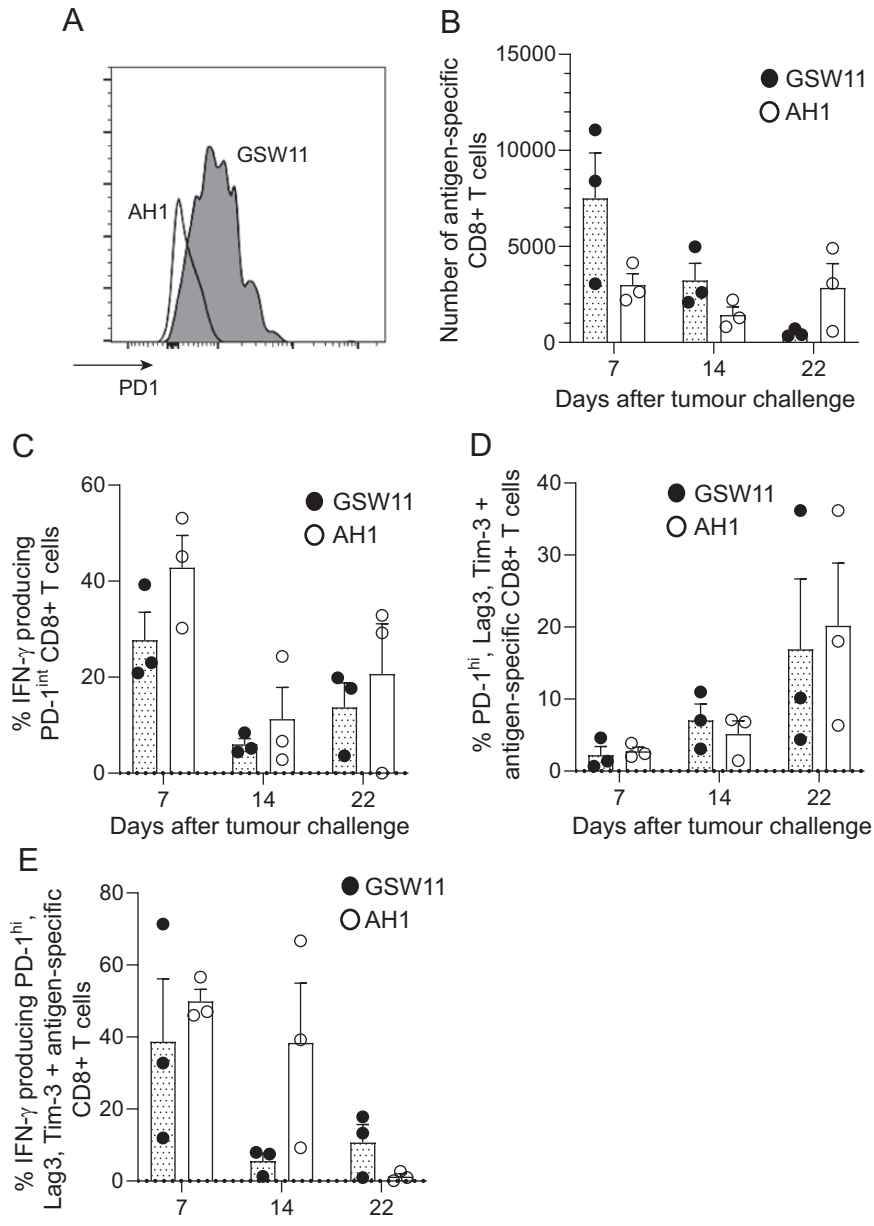


Figure 3. Draining lymph node tumour-specific CD8+ T cells become exhausted as tumour progresses. BALB/c mice were challenged with CT26 tumour and antigen-specific CD8+T cells in tumour draining lymph nodes were characterised. (A) A representative histogram showing PD-1 expression on naive GSW11- and AH1-specific CD8+T cells. (B) The number of antigen-specific CD8+ T cells present in tumour draining lymph nodes during tumour progression. (C) The percentage of functional PD-1^{int} GSW11- and AH1-specific CD8+ T cells in draining lymph nodes. Assessment of the percentage of PD-1^{hi}, Lag3+, Tim-3+ GSW11- and AH1-specific CD8+T cells (D) and the percentage that produced IFN-γ following antigen stimulation (E) in tumour draining lymph nodes. (B–E; mean and SEM of three mice at the indicated time points).

challenge. In addition, despite similar proportions of both AH1- and GSW11-T cells becoming exhausted as the tumour progresses, GSW11-T cells are more susceptible to terminal exhaustion (loss of effector function; day 14 Fig. 3E). The observation of an increase in exhaustion of tumour-specific T cells in tdLN as the tumour progresses suggests an influence of the tumour and/or immune regulatory cells on proximal LN. Indeed,

tumour material is detected in tdLNs following challenge (Supplementary Fig. 3).

Diversity of GSW11-specific T cell responses in Treg replete and depleted mice

We observed that GSW11- and AH1-specific T cells were present in the tumour at the height of a primary response; however, GSW11-T cells were more prone to the induction of an exhausted phenotype as the tumour progressed. One important aspect of T cell priming, activation and exhaustion is TcR engagement with peptide/MHC. In priming and activation, TcR signals are essential in initiating T cell activation and for antigen-specific effector functions. The strength of TcR/pMHC interaction is a critical determinant of T cell proliferation and effector function in acute responses [25], and when antigen persists, is likely to be a critical determinant of exhaustion via upregulation of checkpoint inhibitor receptors. The induction of exhaustion is overcome in mice depleted of Treg, which preferentially target GSW11-T cells, prior to CT26 tumour challenge and induces protective anti-tumour responses, consisting of both AH1- and GSW11-specific effector T cells [17]. Therefore, we determined the diversity of the anti-GSW11 T cell response with a view to identifying oligoclonal populations that are preferentially suppressed by Treg and therefore potentially more prone to exhaustion. We determined TcR Vβ usage of GSW11-specific T cells from CT26 challenged Treg depleted or replete mice using a panel of V-region specific antibodies. This revealed that the anti-GSW11 response was very diverse with at least 15 different clonotypes observed (Fig. 4A). Despite the broad response, only three Vβ represented >10% of GSW11-specific T cells (Vβ8.1/8.2, Vβ8.3, and Vβ14; Fig. 4A), indicating a predominantly oligoclonal response despite the broad Vβ usage. In Treg depleted mice, the anti-GSW11 response was similarly broad, although some populations were significantly increased, such as those expressing Vβ3 and Vβ13 and others diminished, such as Vβ10b and Vβ14 compared with Treg replete responses (Fig. 4A). Some responses such as Vβ8.1/8.2 and Vβ8.3 (which make up ~30% and ~12% of the response, respectively) were largely unchanged following Treg depletion. We next characterised the phenotype of clones selectively expanded in Treg replete and depleted mice both individually and grouped together. Interestingly, a significantly greater proportion of the clones which correlated with tumour progression in Treg replete mice (Vβ10b, Vβ14, and Vβ17), had an exhausted phenotype (PD-1^{hi}, Tim-3+, Lag3+ IFN-γ^{-}) compared with those clones enhanced in Treg depleted mice (Vβ3 and Vβ13), correlating with protection (Fig. 4B and C). These findings suggest that Tregs modulate the anti-tumour GSW11-specific response by selectively suppressing certain clonotypes, while allowing the expansion of others that subsequently become exhausted.

Tregs target lower avidity anti-GSW11 T cells

We next investigated the mechanistic basis for this selectivity. Previous studies have shown that the presence of Treg during priming to a transplantation antigen preferentially inhibits the priming of T cells with low-avidity TcR [26]. We therefore sought to determine whether clonotypes that were more susceptible to suppression by Treg had a lower avidity for GSW11 using tetramer competition assays as described previously (Fig. 5A and ref. [27]). The two dominant T cell oligoclones that expanded following Treg depletion and correlated with tumour clearance (Vβ3 and Vβ13) had a lower avidity compared with Vβ8.1/8.2 and Vβ8.3 T cells, which were similarly abundant whether Tregs were present or not (Fig. 5B and C). In addition, two oligoclones, Vβ10b and Vβ14, which correlated with tumour progression in Treg replete mice, displayed a high avidity similar to that observed for Vβ8.1/8.2 and Vβ8.3 T cells (Fig. 5B and C). The levels of cell surface TcR β chain were similar regardless of the amount of competing tetramer added (data not shown), confirming that the reduction in tetramer staining was due to competition and not decreased TcR. In pooled samples from groups of three out of four mice, these low-avidity T cells account for ~16% of the total anti-GSW11 response in Treg depleted animals, but only ~2.5% in Treg replete animals (red shades; Fig. 5D). In addition, high-avidity T cells make up >70% and ~55% of the total anti-GSW11 T cell response in Treg replete and depleted mice, respectively (blue shades; Fig. 5D). Therefore, in the CT26 model, it seems likely that Treg depletion results in the preferential expansion of some low-avidity GSW11-specific sub-clones which are tumoricidal and escape the induction of terminal exhaustion.

Discussion

Immune surveillance of cancers starts with the priming of T cells to tumour-associated antigens (TAA) and their infiltration into the diseased tissue. Here, their anti-tumour function is modulated by the evolving microenvironment, often leading to tumour escape via multiple mechanisms including the induction of T cell tolerance/anergy (through lack of co-stimulation during

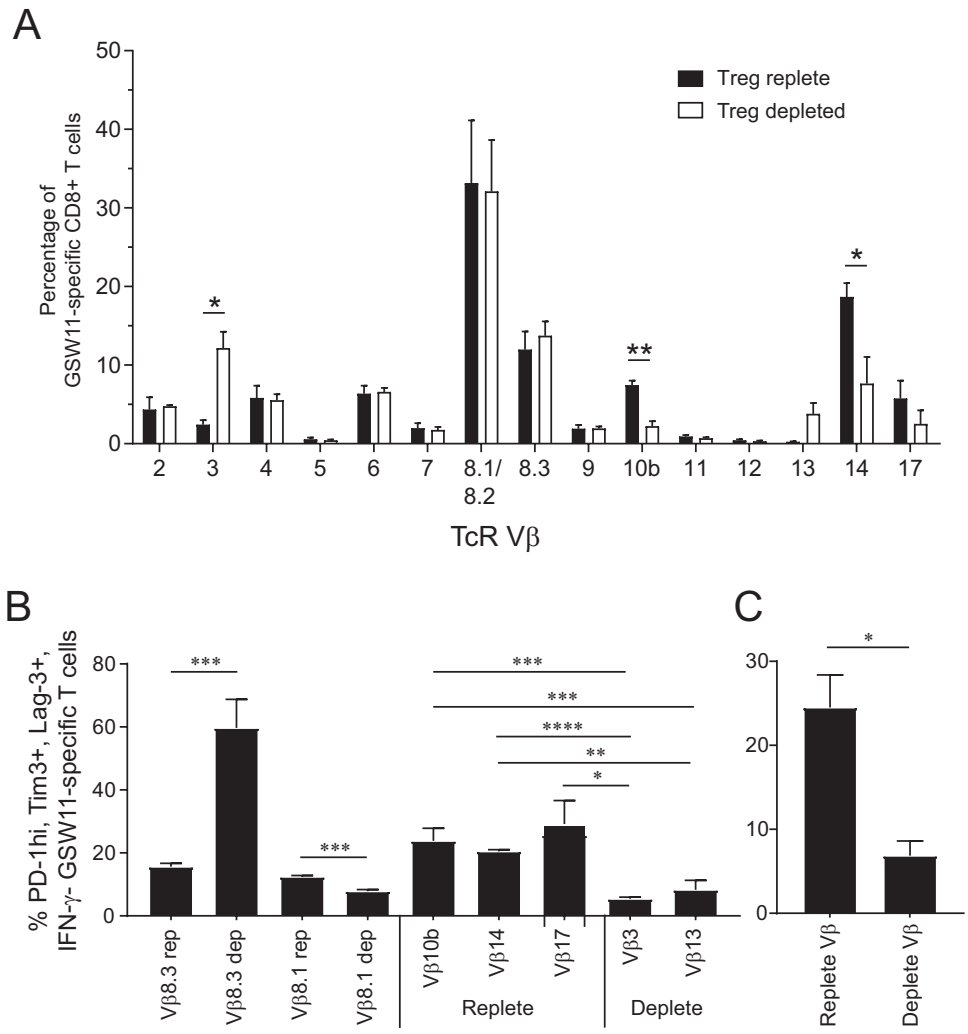


Figure 4. GSW11-specific T cell clonalities are modulated by regulatory T cells. Treg replete or depleted BALB/c mice were challenged with CT26 and the TcR expression of GSW11-specific T cells assessed at day 7. (A) Percentage of GSW11-specific CD8+ T cells expressing the indicating TcR Vβ in Treg replete or depleted CT26 challenged mice. The percentage of exhausted PD-1^{hi}, Tim3+, Lag-3+ GSW11-specific CD8+ T cells expressing TcR unchanged, increased or decreased in Treg depleted mice as individual TcR (B) or grouped (C). (A; mean and SEM of 10 mice in three pools, B; mean of ten mice from three pools, C and D; mean of at least three mice from two independent experiments; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

priming) or exhaustion (through the progressive loss of effector function following activation) [22, 28]. T cell exhaustion describes a state of T cell dysfunction in response to chronic antigen stimulation resulting in hyporesponsiveness. This change in state can be categorised by, but not limited to, altered, reduced effector functions such as a reduced capacity to secrete cytokines and increased persistent expression of multiple inhibitory receptors, e.g. CTLA-4, PD-1, TIM3, and LAG3. Recent studies have indicated that these exhausted T cells derive from progenitors that express the transcription factor TCF1 and have low expression of PD-1 and TIM3. These stem cell-like populations produce terminally differentiated dysfunctional PD-1^{hi}, TIM3^{hi} TCF1-cells [29–32]. For this study, we used high expression of inhibitory molecules PD-1, TIM3, and LAG-3 to define a dysfunctional/exhausted T cell population. Using antigen-specific multimers, we show that, in a commercially important pre-clinical mouse model, growing CT26 tumours are highly infiltrated with tumour-specific CTL recognising one of two non-mutated epitopes (GSW11 and AH1) from a single highly abundant TAA, *gp*90. Most TIL,

however, exhibit an exhausted phenotype, which correlates with the presence of Tregs.

In human cancer, high levels of T cell infiltration generally correlate with good prognosis. This infiltration is marked by a signature that includes increased transcription of genes associated with antigen processing and presentation (MHC I and MHC II), T cell markers (CD8, CD4, and CD3) and genes associated with T cell homing (*CCL2*, *CCL3*, *CCL4*, *CXCL9*, *CXCL10* – the latter two being CD8+T-cell specific), signalling (ICOS, IRF1), and CTL function (granzymes, IFN-γ) [33, 34]. These are all consistent with the tumour milieu supporting ongoing peptide:MHC I (pMHC I)-driven T cell proliferation via TcR engagement. However, because of their secretion of the inflammatory cytokine IFN-γ, CD8+ TIL also drive evolution of the immunosuppressive microenvironment including expression of PD-L1, IDO, and the infiltration of Tregs [35]. In addition, T cells derived from highly infiltrated tumours express the highest levels of inhibitory receptors (such as PD-1) [35].

The CT26 tumour microenvironment resembles cancers with a strong T cell inflamed phenotype, and a signature that includes elevated transcripts for T cell infiltration and activation, CD8, CD4, CD3, CD45, CD62L, CD80, CD86, CD40, OX40L, CD25 and immunosuppression including FoxP3, CTLA-4, and IDO [36]. Transcriptomic profiling of transplantable tumours reveals only small differences in the tumour–host interaction when compared with spontaneous tumour models [36, 37]. Thus, our time course of CT26 growth might reasonably approximate to a model for the evolution of the tumour–immune interaction in terms of immune-editing and the development of host immune modulation mechanisms within the microenvironment. This is encouraging because transplantable models are more experimentally tractable and permit a more rapid turnaround of pre-clinical immunotherapy studies.

With this in mind, we observed two key features of the CT26:BALB/c interaction that are relevant to understanding human disease and its response to immunotherapy. Firstly, Treg can differentially suppress different CTL clones recognising TAA and this even applies to CTL recognising the same pMHC I complex; and secondly that differential suppression of GSW11 and AH1-specific T cells, characterised by upregulation of PD-1 expression and loss of effector function (IFN-γ production) was observed early after tumour challenge in tdLN. This indicated that a large proportion of GSW11-specific CD8+ T cells were dysfunctional at the site of priming.

As tumour progresses, the proportions of effector tumour-specific T cells decrease and inversely correlate with the induction of an exhausted phenotype in tdLN, indicating an influence of tumour cells and/or immune regulatory cells, which may migrate from the tumour to the tdLN as it progresses [38]. At the tumour, GSW11-T cells are more prone to exhaustion, likely induced as a consequence of persistent antigenic stimulation in tumours [22] and/or the more suppressive microenvironment at the tumour site. Interestingly, this susceptibility may be imprinted at priming as observed in a spontaneous arising tumour model where exhausted T cells in late-stage tumours are imprinted at an earlier stage of tumour development and can arise from naive T cells subjected to continuing TcR stimulation without adequate co-stimulation [39].

The presence of Tregs in several cancer types is a negative prognostic indicator with tumour-infiltrating Tregs expressing gene signatures, including markers of activation and function, which distinguish them from both blood Tregs, and tissue resident Tregs from healthy tissue of the same origin [40, 41]. Treg suppression of GSW11-specific T cells primarily affected those with lower avidity TcR and correlated with survival. This observation is consistent with previous studies, for example, Pace et al showed that the presence of Treg during priming to a transplantation antigen increased the affinity of the CD8+ T cell response by inhibiting the priming of T cells bearing low-affinity TcR via a mechanism involving CCL3/4 dependent destabilisation of T cell interactions with dendritic cells [26].

The therapeutic efficacy of low-avidity T cell clones was somewhat unexpected since it is generally assumed that high-avidity T cells have a competitive advantage in an immune response due to stronger and prolonged activation signals [42, 43]. However, in a situation of persistent antigenic stimulation and suppressive microenvironment, as is encountered in the tumour, it is likely that these T cells progress to exhaustion [44–46]. Low-avidity CTL may escape the same fate through lower expression of PD-1 or by receiving a TcR signal below the threshold required for exhaustion while maintaining some effector function [47, 48].

The identification of low Ka GSW11-specific TcR, which correlate with protection, may have implications for epitope selection in immunotherapy. Current strategies concentrate on the identification and use of tumour epitopes with a high Ka/slow off-rate in an attempt to induce CD8+ T cell responses with a strong Ka TcR [49–51]. This approach has had some success with antigen-specific T cell responses directed to TAA such as NY-ESO-1 and MART-1 and neoantigens. However, only a small proportion of these patients

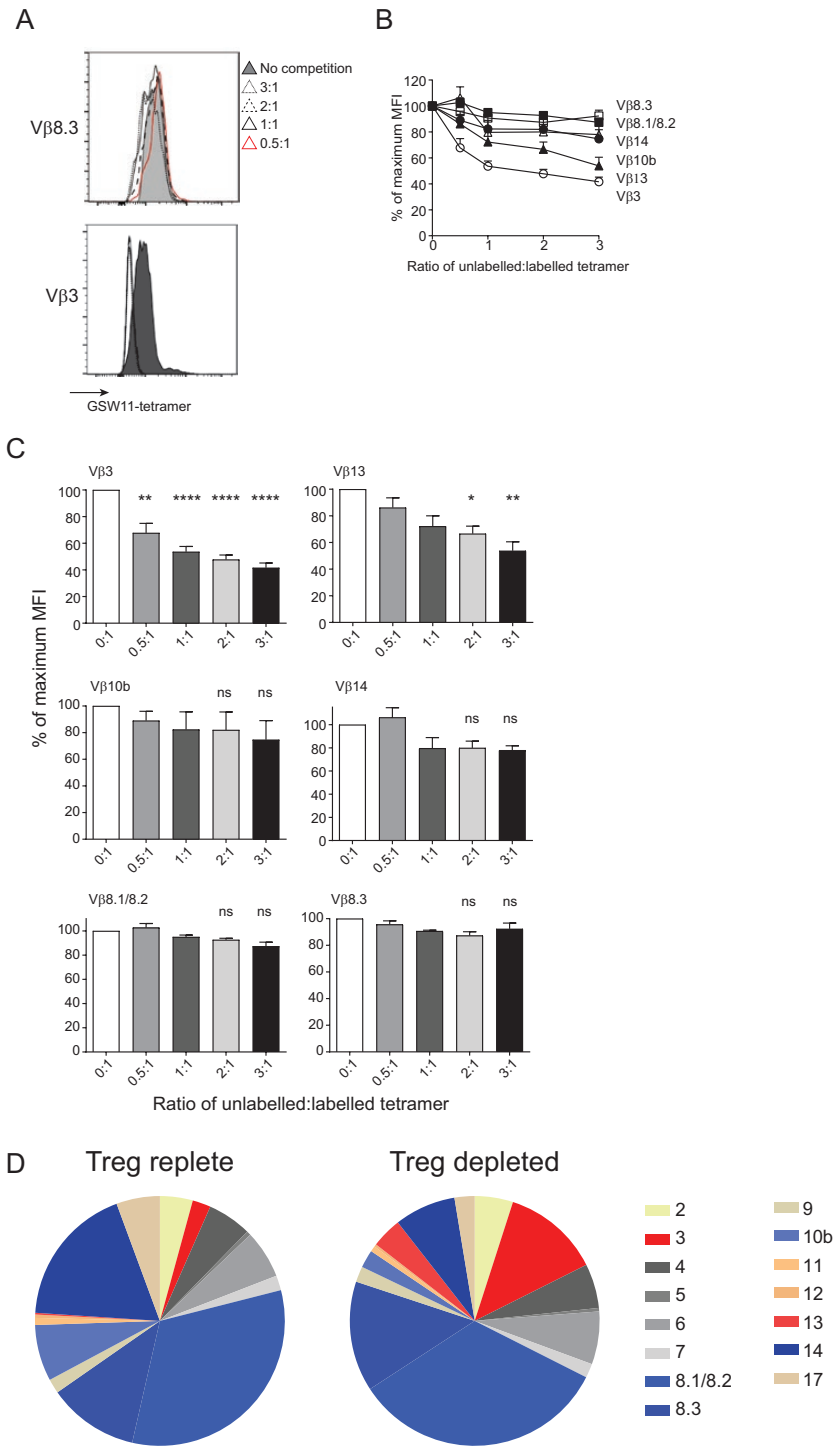


Figure 5. Tumour protective GSW11-specific CD8+ T cells have low TcR avidity. The TcR avidity of anti-GSW11 T cell oligoclones that showed either increased, decreased, or similar levels in tumour challenged Treg depleted mice was assessed using tetramer competition. (A, B) A representative histogram (A) and analysis of relative change in tetramer MFI (B) of the TcR tetramer competition assay in indicated TcR clones. (C) Relative avidity of indicated GSW11-specific T cell oligoclones following tetramer competition. (D) The relative proportion of TcR Vβ usage in GSW11-specific T cells following tumour challenge (blue segments indicate TcR with high avidity and red indicate those with low avidity). (C; mean and SEM of at least three independent experiments and D; mean of at least three mice from two independent experiments; $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$).

show a partial or complete clinical response [52, 53]. These studies together with the data presented here show that, while the induction of high-avidity T cells to dominant TAA occur, in a therapeutic setting, it may be more efficacious to induce a broad repertoire of TcR affinities using peptide epitopes presented at sufficient levels regardless of their affinity for MHC. With this in mind, it is notable that the dominant target peptide recognised by TIL in CT26, GSW11, binds weakly to its presenting MHC I, H2-D^{d} with a half-life of ~20 min at the cell surface, though it is presented in high abundance at steady state [17]. In future, it will therefore be important to understand the relationship between antigen processing/presentation and the induction of low-avidity T cells. For example, algorithms predicting the affinity of candidate peptides could be better deployed for selecting candidate epitopes for targeted immunotherapy if used in combination with computational models that take into account antigen abundance and mechanistic details of the antigen processing pathway.

Supplementary material

Supplementary data are available at *Immunology Advances* online.

Supplementary Figure 1. GSW11 tetramer specificity. (A) GSW11-specific T cell hybridoma cells, CCD2Z, were incubated with 10mg GSW11 tetramer for 30 min on ice. Assessment of cell surface staining by flow cytometry, as shown in the gating strategy (right panel is an overlay of unstained and tetramer stained CCD2Z cells), demonstrated tetramer binding to CCD2Z cells. (B) To confirm specificity, GSW11 tetramer was incubated with CCD2Z (red histogram), B3Z (SIINFEKL-specific; orange histogram) hybridomas or unstained (blue histogram) and assessed for binding by flow cytometry. The staining indicates specific binding of CCD2Z and not B3Z hybridoma cells. (C and D) To further confirm specificity of GSW11 tetramer and AH1 dextramer, naive spleen cells (C) and inguinal lymph node cells (D) were incubated with GSW11 tetramer and AH1 dextramer and assessed for binding by flow cytometry. CD8+ T cells were gated as indicated in the far left and middle left panels (C, D). Examination of CD8+ T cells showed minimal tetramer (middle right panel) and dextramer (far right panel) binding.

Supplementary Figure 2. Gating strategy for identifying GSW11/AH1-specific CD8+ T cells. Tumour or lymph nodes cells from tumour challenged BALB/c mice at d7-22 were analysed. The gating strategy for experiments performed in Figs 1–3 is given as a representative. Gates for tetramer or dextramer and IFN-g positivity were set on CD8+ lymphocytes (Panels 1 and 2) and used to calculate the proportion of tetramer/dextramer positive and IFN-g producing CD8 T cells (Panel 3; Fig. 1 data). PD-1 expression was assessed on gates of tetramer/dextramer positive cells (Panel 4). The level of PD-1 expression was sub-divided into intermediate and high expression and these gates were used to assess expression of Lag-3 and Tim-3 (Panel 5; Figs 2 and 3 data).

Supplementary Figure 3. Detection of CT26 transcripts in tumour draining lymph nodes. BALB/c mice were challenged with CT26 and after 3 days tumour draining lymph nodes were harvested and assessed for the presence of CT26-specific gp70 transcripts. (A) Primers which spanned across the join formed from a 100bp deletion (highlighted in black) in transcript #2 only expressed in CT26 tumour cells were used. (B) The two sets of primers generate PCR products of 800bp and 350bp no observed in Balb/c tissue.

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Author contributions

G.S. designed and performed experiments, analysed the data, and critically interpreted the data; D.S. performed experiments, analysed the data, and critically interpreted the data; O.D. performed experiments and analysed the data; E.D.A-A. performed experiments and analysed the data; TE developed the project, designed the experiments, critically interpreted the data, wrote and revised the manuscript, and provided funding for the project; E.J. developed the project, designed the experiments, analysed the data, critically interpreted the data, wrote and revised the manuscript, and provided funding for the project.

Conflict of interest

E.J. is a consultant for GreyWolf Therapeutics.

Ethics approval

All institutional and national guidelines for the care and use of laboratory animals were followed.

Data availability

All data generated or analysed during this study are included in this published article.

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