

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

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4 Running Title: Tumour protective low avidity CD8+ T cells

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29 **Abstract**

30 Regulatory T cells (Treg) play a major role in the suppression of protective anti-
31 tumour T cell responses. In the CT26 BALB/c murine model of colorectal carcinoma,
32 Tregs differentially suppress responses to two characterised CD8⁺ T epitopes, AH1
33 and GSW11, which results in an absence of detectable IFN- γ producing GSW11-
34 specific T cells in the spleen and lymph nodes of tumour challenged mice. Activation
35 of GSW11-specific T cells correlates with protection against tumour growth. Here we
36 show that GSW11-specific T cells are in fact induced in Treg-replete, CT26-bearing
37 mice, where they make up the majority of tumour infiltrating CD8⁺ lymphocytes, but
38 exhibit a dysfunctional 'exhausted' phenotype. This dysfunctional phenotype is
39 induced early in the anti-tumour response in draining lymph nodes, spleens and
40 tumours and is significantly more pronounced in GSW11-specific T cells compared to
41 other tumour-specific T cell responses. Depletion of Tregs prior to tumour challenge
42 significantly reduces the induction of exhaustion in GSW11-specific T cells and
43 correlates with altered T cell receptor (TcR) usage. Moreover, the avidity of GSW11-
44 specific TcRs that expanded in the absence of Tregs was significantly lower compared
45 to TcRs of cytotoxic T lymphocyte (CTL) populations that were diminished in
46 protective anti-tumour responses. This indicates that Tregs suppress the induction of
47 protective anti-tumour T cell responses and may signify that the induction of low
48 avidity T cells, while being more susceptible to exhaustion are the most efficacious in
49 tumour rejection.

50 **Introduction**

51 CD8+ T cell responses directed to tumours have been shown to occur in many human
52 cancers, where they are a positive prognostic indicator (1-5). Backed by studies in
53 preclinical mouse models, which clearly show that CD8+ T cells are important in the
54 clearance of tumours and may confer lifelong protection against malignancy (6, 7),
55 immunotherapies aimed at boosting anti-tumour cytotoxic T lymphocytes (CTL) are
56 showing promise in the clinic. Naturally occurring responses can be initiated during
57 tumour growth to establish immunosurveillance in which a dynamic process of
58 immunoediting can ensue; immunological pressure from anti-tumour CTL balances
59 tumour elimination against the emergence of tumour escape variants with no
60 accompanying net outgrowth of tumour (8). This process is responsible for shaping
61 the immunogenicity of the tumour (9). Breakdown of this equilibrium leading to
62 tumour outgrowth involves multiple factors, including the balance between T cell
63 activatory (TcR engagement and co-stimulation) and inhibitory signals (exhaustion
64 markers and immunosuppressive cytokines), and evasion of the T cell response
65 through downregulation of antigen processing machinery or antigen loss. Therapeutic
66 approaches designed to tip the balance back in favour of tumour elimination by
67 providing activation agonists or blockade of inhibition is an attractive strategy that is
68 currently investigated (reviewed in (10)). FoxP3+ CD4+ regulatory T cells (Treg) are
69 important in establishing an immunosuppressive tumour microenvironment, and their
70 infiltration into tumours is a negative prognostic biomarker (11) and a significant
71 obstacle to successful immunotherapy, correlating with a poorer outcome in clinical
72 trials (reviewed in (12)). Therefore, Treg depletion as a therapeutic option is being
73 pursued in the clinic, based on studies in mice that showed rejection of transplanted
74 tumours following ablation of Treg with anti-CD25 antibodies (13, 14).

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

91 To investigate the basis of differential suppression of the GSW11-specific T cell
92 response further, we utilised peptide-specific tetramers, to detect both functional
93 (IFN- γ ⁺) and inactivated (IFN- γ ⁻) antigen-specific T cells. We show that in Treg
94 replete tumour-bearing mice GSW11-specific T cells made up the majority of CD8+
95 tumour infiltrating lymphocytes (TIL), but exhibit an exhausted phenotype
96 characterised by PD-1 expression and absence of IFN- γ production upon stimulation.
97 We also found that Treg depletion permitted the proliferation of GSW11-specific T
98 cells with lower TcR/MHC avidity suggesting that this population may be more

- 99 efficacious at rejecting tumour than their higher avidity counterparts with the same
100 epitope specificity.

101 **Materials and Methods**

102 **Mice, Antibodies and *In vivo* depletion**

103 BALB/c mice were bred under specific pathogen-free conditions in Southampton.
104 Female or male mice (6-8 weeks old) were used in all experiments and during
105 experimental procedures mice were housed in conventional facilities. Hybridomas
106 secreting CD25 (PC61, rat IgG1) specific mAb have been described previously (13).
107 For depletion, mice received intraperitoneal (i.p.) injection of 1 mg of mAb PC61 in
108 100 μ l on days -3 and -1 prior to tumour challenge.

109

110 **Tumour cells and *In vivo* challenge**

111 CT26 tumour cells (American Type Culture Collection; ATCC) were maintained in
112 RPMI (Sigma) supplemented with 10% FCS (Globepharm), 2 mM L-glutamine,
113 penicillin/streptomycin (Sigma), 50 μ M 2-mercaptoethanol, 1 mM sodium pyruvate
114 (Gibco-BRL) and 1mM HEPES (PAA) and confirmed to be mycoplasma free. In all
115 experiments, mice were injected subcutaneously (s.c.) with 10^5 tumour cells in
116 endotoxin-low PBS. For analysis of PD-L1 expression, tumour cells were stained with
117 α -PD-L1 (10F.9G2; Biolegend) prior to s.c. injection and after 14-25 days of tumour
118 growth. All flow cytometry data acquisition was carried out on a FACS Canto II (BD
119 Biosciences) and all data analysed with FlowJo Software (Treestar). Tumour cells
120 were gated on live, single cells and the proportion of PD-L1⁺ cells assessed.

121

122 **DNA construct**

123 The H2-D^d single chain trimer (SCT) construct incorporating a *gp120* HIV peptide (a
124 kind gift from Dr. Keith Gould) was mutated into the GSW11 peptide via site directed
125 mutagenesis (SDM) PCR using KOD HotStart polymerase (Merck Biosciences)

126 according to manufacturer's instructions. The transmembrane domain of H2-D^d was
127 substituted for a biotinylation site using overlapping extension PCR. In addition, a
128 disulphide trap was incorporated into the construct (19) to tether the GSW11 peptide
129 onto the MHC I binding groove.

130

131 **Tetramer generation**

132 Tetramers were produced with the help and advice of the Cancer Research
133 UK/Experimental Cancer Medicine Centre Protein Core Facility (Cancer Sciences
134 Unit, University of Southampton, Southampton, U.K.) with few modifications. The
135 GSW11-SCT construct containing H2-D^d, β 2m and GSW11 peptide was cloned into
136 the pET-3a expression vector (Novagen) and expressed in BL-21 CodonPlus RIPL
137 cells (Stratagene). Concentrated refolded complexes were purified on a HiLoad 26/60
138 Superdex 200 column (GE Healthcare). Biotinylation was achieved with 50 μ M d-
139 biotin and 1 μ g/ml biotin protein ligase (Avidity) at 16°C overnight and then passed
140 through the column a second time. Biotinylated monomers were dialysed and
141 subsequently stored in 16% glycerol in PBS or tetramerised by incubation with 1:4
142 molar ratio of PE-labelled streptavidin (Thermofisher) at 4°C. Each batch of tetramers
143 were tested for binding against the GSW11-specific T cell hybridoma, CCD2Z. For
144 the analysis of AH1-specific T cells, AH1-specific dextramers were used
145 (Immunodex).

146

147 **Isolation and analysis of antigen-specific T cells and Tregs**

148 Splens, tumour draining lymph nodes and tumours from CT26 challenged mice
149 (Treg depleted or replete) were harvested between days 3-25 and disaggregated.
150 CD8⁺ T cell responses to CT26 antigens GSW11 and AH1 were assessed using

151 antigen-specific tetramers and the production of IFN- γ following peptide stimulation.
152 CD8⁺ T cells, APCs and peptides/tumours were cultured together for 4 hours in the
153 presence of brefeldin A (BD biosciences) before being stained for cell surface α -CD8
154 (63-6.7; BD biosciences), antigen-specific tetramer/dextramer, α -PD-1 (RMPI-30;
155 eBiosciences) and intracellular α -IFN- γ (XMG1.2; BD biosciences) using the
156 Cytotfix/Cytoperm kit (BD biosciences) according to manufacturer's instructions.
157 Cells were enumerated by flow cytometry. Numbers reported are those above the
158 background response of T cells alone, with no peptide stimulation. Single cell CD8⁺
159 lymphocytes were gated and assessed for tetramer binding and expression of IFN- γ .
160 For analysis of PD-1, CD8⁺ and tetramer⁺ were gated and assessed for PD-1 and
161 IFN- γ expression. Within these gates the T cell receptor clonality of GSW11-specific
162 T cells was assessed using a panel of 15 V β -specific antibodies (BD biosciences).
163 First, total CD8⁺ T cells were purified using a CD8 magnetic isolation negative
164 selection kit (Miltenyi) according to manufacturer's instructions. Purified CD8s were
165 stained with GSW11-specific tetramer, α -V β kit, α -CD8 and analysed by flow
166 cytometry. For the analysis of Tregs, cells were stained for cell surface α -CD4 (RM-
167 4-5; BD biosciences), α -CD25 (7D4; BD biosciences), α -PD-1 and α -PD-L1
168 (10F.9G2; Biolegend) and intracellular staining with α -FoxP3 (FJK-16S;
169 eBiosciences).

170

171 **Tetramer competition assay**

172 Spleens and tumour draining lymph nodes were pooled from Treg depleted or replete
173 mice. CD8⁺ T cells were purified from disaggregated tissues using magnetic isolation
174 by negative selection (Miltenyi). Purified CD8⁺ T cells were incubated with 50 nM of
175 dasatinib (New England Biolabs) to prevent TcR internalisation before staining with

176 α -CD8, α -TCR β -chain (H57-597; Biolegend) and 5 μ g of PE-labelled GSW11-
177 specific tetramers. After two washes, cells were incubated with bleached tetramer at
178 varying ratios of the initial PE-labelled tetramers: 2.5 μ g, 5 μ g, 10 μ g or 20 μ g per test.
179 Bleached tetramers were tested for no/minimal PE-fluorescence before use. The β -
180 chain TCR staining was included to confirm the decreasing levels of PE-staining was
181 due to the fluorescently labelled tetramer being out-competed and not due to TcR
182 internalisation.

183

184 **Statistical analysis**

185 Analyses were performed using Prism software (GraphPad, San Diego, CA). The p
186 values were calculated using either two way ANOVA with Dunnett's post-test or two-
187 tailed unpaired t test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

188

189

190

191 **Results**

192 *Tumour infiltrating GSW11-specific CD8⁺ T cells are exhausted in CT26 tumour-*
193 *bearing mice*

194 Recent studies using mouse models have shown that many tumour infiltrating anti-
195 tumour CD8⁺ T cells have an exhausted phenotype, characterised by PD-1 expression
196 and a failure to express cytokines IL-2, TNF- α and IFN- γ (20, 21). Little is known
197 about whether, or to what extent, development of this phenotype relates to T cell
198 specificity. Clearly, a relationship exists inasmuch as T cell activation via TcR is a
199 prerequisite for the induction of exhaustion (reviewed in (22)). Given our previous
200 observations of differential suppression of anti-CT26 responses with different
201 specificity (17), we sought to investigate the induction of exhaustion in both AH1 and
202 GSW11-specific CD8⁺ T cells in CT26 tumour bearing mice using antigen-specific
203 tetramers as specificity probes for T cell populations, independent of their functional
204 phenotype. Due to the poor binding affinity of GSW11 for H2-D^d (17) we utilised
205 single chain trimer tetramers with GSW11 tethered to the binding groove using a
206 short linker polypeptide (19), allowing stable expression of GSW11-D^d monomers. In
207 CT26 challenged Treg replete mice, GSW11-specific T cells were the most abundant
208 CD8⁺ T cell population in tumours, making up >50% of all CD8⁺ T cell infiltration
209 after 14 days of tumour challenge (Figure 1A, B, C). However, similar to the situation
210 seen in many human cancers, these infiltrating CD8⁺ T cells do not confer protection,
211 and tumours continue to grow in these animals. The population of GSW11-specific T
212 cells was significantly larger than AH1-specific T cells, which constituted a maximum
213 of 20% of infiltrating T cells at later stages of tumour challenge (d17 and d22; Figure
214 1B, C). Notably, while the two T cell populations (anti-GSW11 and -AH1 CD8⁺ T
215 cells) made up the majority of tumour infiltrating CD8⁺ T cells (>60%) at later time

216 points (d14-22), they were in the minority in early anti-tumour responses (d7-10,
217 Figure 1C). Thus, an initial broad-specificity polyclonal TIL response becomes
218 focussed on two *gp90* derived epitopes during tumour growth (Figure 1C). We next
219 investigated the function, after *ex vivo* peptide stimulation, of GSW11- and -AH1
220 specific CD8⁺ T cells harvested from tumours during the course of the challenge. The
221 vast majority of GSW11-specific CD8⁺ T cells were unable to produce IFN- γ , with a
222 decrease in functional T cells as the tumour progressed (Figure 1D, E). Most of the
223 AH1-specific T cells were also non-functional, although, consistent with earlier
224 studies showing their presence in tdLN and spleen (17), there were significantly more
225 functional AH1-specific T cells at d22 compared to GSW11 (around 6% compared to
226 <1% functional; Figure 1D, E). This minor population of functional tumour-specific T
227 cells were, however, unable to control tumour growth.

228 To investigate whether the lack of effector function in anti-tumour T cells correlated
229 with the expression of checkpoint molecules commonly associated with T cell
230 dysfunction in tumours, we assessed the expression of PD-1 on GSW11- and AH1-
231 specific T cells following tumour challenge. The majority of non-functional (IFN- γ ⁻)
232 tumour infiltrating T cells of both specificities expressed PD-1 indicating the
233 induction of an exhausted phenotype (50-80%; Figure 2A, B). Anti-GSW11 CD8⁺T
234 cells acquired the non-functional phenotype more rapidly and to a greater extent
235 compared to AH1-specific T cells. Thus, a greater proportion of GSW11-T cells were
236 PD-1⁺ IFN- γ ⁻ at the first time-point, d7 ($p = <0.001$), which was sustained throughout
237 tumour growth (Figure 2B). Interestingly, a proportion of the small number of
238 functional (IFN- γ ⁺) GSW11- and AH1-specific T cells also expressed PD-1, ranging
239 from ~10% for AH1-specific T cells at day 7 to ~80% of GSW11-specific T cells at
240 day 22 (Figure 2C), indicating that these T cells are both activated and functional.

241 These results indicate that tumour infiltrating GSW11-specific T cells are highly
242 susceptible to the induction of an exhausted phenotype.

243

244 *The presence of regulatory T cells correlates with PD-1 expression on tumour*
245 *infiltrating lymphocytes.*

246 The tumour microenvironment has been shown to play a crucial role in modulating
247 anti-tumour T cell responses *in vivo* (23, 24) with the presence of tumour-infiltrating
248 Tregs impacting negatively on tumour rejection (11). We therefore investigated the
249 effect of Treg on the induction of exhaustion in infiltrating CD8⁺ T cells. In a
250 temporal analysis of TIL, Tregs comprised around 15% of total CD4⁺ T cells at the
251 earliest time point examined (d7). This rose to ~25% by d10 where it remained for the
252 duration of the tumour challenge (Figure 3A). Interestingly, the accumulation of
253 Tregs in the tumour followed similar kinetics to that observed for the loss of effector
254 function in GSW11-specific T cells (Figure 2B and 1D). Most Tregs expressed PD-L1
255 in both tumour and the tumour draining LN (tdLN) from the earliest time-point
256 onwards (Figure 3B), suggesting that Treg suppression of GSW11-specific T cells
257 may be mediated via PD-1/PD-L1 interaction. Since tumour-specific T cells also
258 engage with tumour cells for activation, we examined the expression of PD-L1 on
259 tumour cells over 25 days following inoculation. *In vitro* CT26 expressed low levels
260 of PD-L1, however this increased significantly both in terms of the proportion of
261 CT26 expressing, and the level of expression following their seeding and growth *in*
262 *vivo* (Figure 3C). These results suggest that during initial stages of growth, the tumour
263 provides a pro-inflammatory microenvironment, characterised by IFN- γ producing T
264 cells (and perhaps iNKT/NK cells). This rapidly changes to an immunosuppressive
265 microenvironment in both the tumour and tdLN, characterised by upregulation of

266 inhibitory ligands by the tumour, infiltration of Treg, and upregulation of PD1 on
267 infiltrating tumour-specific CD8+ T cells accompanied by their loss of effector
268 function.

269

270 *Dysfunction of anti-tumour T cells is induced in the periphery*

271 We next investigated whether GSW11- and AH1-specific T cells are primed
272 effectively to induce T cell effectors. Examination of naïve T cell populations
273 (GSW11 and AH1) showed that both expressed low levels of PD-1 (consistent with
274 previous studies; (25)) with expression on GSW11-specific T cells being slightly
275 greater than AH1-specific T cells (Figure 4A). Following CT26 seeding in Treg
276 replete mice we observed functional (IFN- γ producing) AH1- and GSW11-specific T
277 cells in spleen and tdLN, appearing at d3 and detectable through to the humane end
278 point at d22, with AH1-specific T cells dominating over GSW11-specific T cells at
279 later time points (Figure 4B and C). AH1-specific responses were similar throughout
280 the experiment in lymphoid organs, with the greatest response in spleen (Figure 4C).
281 By contrast, GSW11-specific IFN- γ responses were greatest at early time points (d3-
282 10) and declined over time (Figure 4B). This confirmed that a co-dominant, functional
283 (IFN- γ) response to AH1 and GSW11 was established in tdLN and spleen
284 immediately following tumour seeding in Treg replete mice and that over time,
285 progressive loss of functional anti-GSW11, but not anti-AH1-specific T cells, was
286 seen. In Treg depleted mice, in which tumours are rejected, GSW11-specific T cells
287 were dominant at most time points, in particular at time points d3-17, in both spleen
288 and tdLN with the greatest response observed in spleen (in line with our previous
289 observations (17) and Figure 4B and C). In addition, the magnitude of anti-GSW11
290 responses were much greater in Treg depleted compared to Treg-replete mice. AH1-

291 specific responses became co-dominant after d17. Indeed, the magnitude of the anti-
292 AH1 response was not sensitive to the presence or absence of Treg (Figure 4C).
293 Analysis of PD-1 expression on non-functional (IFN- γ ⁻) T cells revealed that a
294 significant proportion (~20%) of GSW11-specific T cells in the spleen and tdLN
295 expressed PD-1 throughout the time-course, even at the earliest time-point of 3 days;
296 rising to ~40% at d22 in tdLN (Figure 4D). By contrast, only a very small proportion
297 of IFN- γ ⁻ AH1-specific T cells expressed PD-1, with a maximum of ~10% of cells in
298 tdLN reached by d22 (Figure 4E). A similar pattern of dysfunctional GSW11- and
299 AH1-specific T cells was observed in the spleens of tumour challenged Treg depleted
300 mice, although, there was a much greater reduction in GSW11-specific T cells
301 compared to AH1, illustrating the differential suppression of anti-CT26 T cells by
302 Tregs ((17) and Figure 4D and E). Importantly, the proportion of dysfunctional
303 GSW11-specific T cells was significantly lower in tdLN of Treg depleted mice (d7
304 and d22; Figure 4D), suggesting that Treg can exert their effect on T cells in tdLN and
305 spleen and their removal reduces the induction of dysfunction in tumour-specific T
306 cells following priming.

307 It is likely that the precursors of IFN- γ ⁻, PD-1⁺ T cells are IFN- γ ⁺, PD-1⁺ (22),
308 therefore, to gain a better understanding of the dynamics of transition from functional
309 to dysfunctional phenotypes we assessed PD-1 expression on functional (IFN- γ ⁺)
310 CD8⁺ T cells. In Treg replete mice, levels of PD-1⁺ IFN- γ ⁺ GSW11- and AH1-
311 specific T cells were similar, although more AH1-specific T cells were observed at
312 later time points in spleens (d14-22; Figure 4F and G). Similar levels of PD-1⁺ IFN- γ ⁺
313 GSW11- and AH1-specific T cells were observed in Treg depleted mice, however
314 more PD-1⁺ IFN- γ ⁺ GSW11-specific T cells were seen at d17 and d22 in spleens
315 (Figure 4F and G). These results indicate that, although the level of primed functional

316 AH1- and GSW11-specific responses is similar, GSW11-specific T cells are more
317 susceptible to the induction of a dysfunctional phenotype.

318

319 *Diversity of GSW11-specific T cell responses in Treg depleted mice*

320 Previous studies have shown that the presence of Treg during priming to a
321 transplantation antigen inhibits the priming of T cells with low-avidity TcR (26). To
322 investigate this possibility in the context of Treg-dependent tumour rejection, we first
323 investigated the oligoclonality of the anti-GSW11 T cell response with a view to
324 identifying oligoclonal populations that are preferentially suppressed by Treg. To this
325 end, we determined TcR V β usage of GSW11-specific T cells from CT26 challenged
326 Treg replete mice using a panel of V-region specific antibodies. This revealed that the
327 anti-GSW11 response was very diverse with at least 15 different clonotypes observed
328 (Figure 5A and B). Despite the broad response only three V β represented >10% of
329 GSW11-specific T cells (V β 8.1/8.2, V β 8.3 and V β 14; Figure 5A) indicating a
330 predominantly oligoclonal response despite the broad V β usage. In Treg depleted
331 mice the anti-GSW11 response was similarly broad, although some populations were
332 significantly increased, such as those expressing V β 3 and V β 13 and others
333 diminished, such as V β 10b and V β 14 compared to Treg replete responses (Figure 5A,
334 B). Intriguingly, some responses such as V β 8.1/8.2 and V β 8.3 (which make up ~30%
335 and ~12% of the response respectively) were largely unchanged following Treg
336 depletion. These findings suggest that Tregs modulate the anti-tumour GSW11-
337 specific response by preferentially suppressing some T cell clones leading to the
338 expansion of others.

339

340 *Tregs target lower avidity anti-GSW11 T cells*

341 We next estimated the TcR avidity of anti-GSW11 T cell oligoclonal populations that were
342 preferentially suppressed by Treg (V β 3 and V β 13) compared to oligoclonal populations that were
343 largely unaffected by the presence of Treg (V β 8.1/8.2 and V β 8.3), using tetramer
344 competition assays as described previously (Figure 6A and (27)). The two dominant T
345 cell oligoclonal populations that expanded following Treg depletion, V β 3 and V β 13, had a lower
346 avidity compared to V β 8.1/8.2 and V β 8.3 T cells, which had similar populations
347 whether Tregs were present or not (Figure 6B and C). In addition, two oligoclonal populations,
348 V β 10b and V β 14, which were proportionally better represented in Treg replete mice,
349 displayed a high avidity similar to that observed for V β 8.1/8.2 and V β 8.3 T cells
350 (Figure 6B and C). Levels of cell surface TcR β chain were similar regardless of the
351 amount of competing tetramer added (Supplementary figure 1). This confirmed that
352 the reduction in tetramer staining was due to competition and not decreased TcR. In
353 pooled samples from groups of 3/4 mice, these low avidity T cells account for ~16%
354 of the total anti-GSW11 response in Treg depleted animals compared to ~2.5% in
355 Treg replete animals (red shades; Figure 5B). In addition, high avidity T cells make
356 up >70% and ~55% of the total anti-GSW11 T cell response in Treg replete and
357 depleted mice respectively (blue shades; Figure 5B). This indicates that sensitivity of
358 a T cell to Treg suppression might be linked to TcR avidity, with lower avidity T cells
359 preferentially targeted. Therefore, in the CT26 model, Treg depletion results in the
360 elaboration of a tumoricidal GSW11-specific T cell response, which appears to
361 correlate with preferential expansion of some low avidity GSW11-specific sub-clones.
362 The response in Treg depleted mice represents a 6-fold increase in low avidity TcR,
363 which make up ~1/6 of the GSW11-specific response compared to only 1/40 in Treg
364 replete mice.

365

366 **Discussion**

367 Immune surveillance of cancers starts with the priming of T cells to tumour associated
368 antigens (TAA) and their infiltration into the diseased tissue. Here, their anti-tumour
369 function is modulated by the evolving microenvironment, often leading to tumour
370 escape via multiple mechanisms including the induction of T cell tolerance/anergy
371 (through lack of co-stimulation during priming) or exhaustion (through the
372 progressive loss of effector function following activation) (22, 28). Using antigen-
373 specific multimers, we show that, in a commercially important preclinical mouse
374 model, growing CT26 tumours are highly infiltrated with tumour-specific CTL
375 recognising one of two non-mutated epitopes (GSW11 and AH1) from a single highly
376 abundant TAA, *gp90*. Most TIL, however, exhibit an exhausted phenotype, which is
377 at least in part mediated by Tregs.

378 In human cancer, high levels of T cell infiltration generally correlate with good
379 prognosis. This infiltration is marked by a signature that includes increased
380 transcription of genes associated with antigen processing and presentation (MHC I
381 and MHC II), T cell markers (CD8, CD4, CD3) and genes associated with T cell
382 homing (*CCL2*, *CCL3*, *CCL4*, *CXCL9*, *CXCL10* – the latter two being CD8+T-cell
383 specific), signalling (ICOS, IRF1) and CTL function (granzymes, IFN- γ) (29, 30).
384 These are all consistent with the tumour milieu supporting ongoing peptide:MHC I
385 (pMHC I)-driven T cell proliferation via TcR engagement. However, because of their
386 secretion of the inflammatory cytokine IFN- γ , CD8+ TIL also drive evolution of the
387 immunosuppressive microenvironment including expression of PD-L1, IDO and the
388 infiltration of Tregs (31). In addition, T cells derived from highly infiltrated tumours
389 express the highest levels of inhibitory receptors (such as PD-1) (31). Immunotherapy
390 targets all three key stages of anti-tumour immunity: priming, infiltration and

391 regulation, and the gene signature for T cell inflamed (so-called ‘hot’) tumours
392 encompasses patterns of differentially expressed transcripts associated with positive
393 responses to checkpoint blockade immunotherapy (PD-1, PD-L1, CTLA4) (32).

394 The CT26 tumour microenvironment resembles cancers with a strong T cell
395 inflamed phenotype, and a signature that includes elevated transcripts for T cell
396 infiltration and activation, CD8, CD4, CD3, CD45, CD62L, CD80, CD86, CD40,
397 OX40L, CD25 and immunosuppression including FoxP3, CTLA-4 and IDO (32).

398 Indeed, we show here that although the tumour mass is infiltrated with tumour-
399 specific CD8+ T cells, they are functionally inert. Despite the obvious differences
400 between spontaneously arising cancer and a transplantable mouse model, studies that
401 have directly compared spontaneous and transplantable tumour models in mice have
402 found only small differences in the tumour-host interaction including immune gene
403 profiles (32, 33). Thus, our time-course of CT26 growth might reasonably
404 approximate to a model for the evolution of the tumour-immune interaction in
405 spontaneously arising cancers in terms of immune-editing and the development of
406 host immune modulation mechanisms within the microenvironment. This is
407 encouraging because transplantable models are more experimentally tractable and
408 permit a more rapid turnaround of preclinical immunotherapy studies.

409 With this in mind, we observed two key features of the CT26:BALB/c
410 interaction that are relevant to understanding human disease and its response to
411 immunotherapy. Firstly, Treg can differentially suppress different CTL clones
412 recognising TAA and this even applies to CTL recognising the same pMHC I
413 complex; and secondly that differential suppression of GSW11 and AH1-specific T
414 cells, characterised by upregulation of PD-1 expression and loss of effector function
415 (IFN- γ production) was observed very early after tumour challenge (d3) in tdLN. This

416 indicated that a large proportion of GSW11-specific CD8+ T cells were dysfunctional
417 at the site of priming.

418 The early preferential suppression of anti-GSW11 T cells persists in peripheral
419 tissues where a disparity between dysfunctional GSW11- and AH1-specific T cells is
420 evident in tdLN and spleen. By contrast, in tumours, the difference between GSW11
421 and AH1 T cells diminishes after d10. This difference between tdLN and tumour
422 indicates that there may be separate mechanisms that operate to establish T cell
423 dysfunction at the early priming and late effector phase. The differential induction of
424 dysfunction in GSW11 at early time points in tdLN, which is significantly reduced in
425 the absence of Tregs, suggests that Tregs play a key role in this process. The rapid
426 expression of PD-L1 on Tregs and PD-1 expression on GSW11-specific T cells
427 indicates this may be an important interaction. Indeed, stimulation of PD-1 (rapidly
428 upregulated following activation) by PD-L1 expressed on antigen presentation cells
429 can cause T cell anergy, defined by reduced proliferative capacity, in a peptide
430 induced model (34). The difference observed in PD-1 expression between AH1- and
431 GSW11-T cells may therefore be a reflection of the relative susceptibility to Treg
432 suppression at the priming stage mediated by PD-1/PD-L1 interaction. Once at the
433 tumour, there is much less difference between AH1 and GSW11 with respect to a
434 dysfunctional 'exhausted' phenotype. This change may be due to the increased
435 expression of PD-1 on AH1-T cells, similar to that observed on GSW11-T cells at the
436 tumour, due to continued antigenic stimulation (22). In addition, the more suppressive
437 microenvironment at the tumour site with PD-L1 expression on tumour cells as well
438 as Tregs, which accumulate over time, and the presence of immunosuppressive
439 cytokines such as TGF- β , may overcome any ability of AH1-specific T cells (and to a
440 lesser degree GSW11-T cells) to resist the induction of exhaustion.

441 The presence of Tregs in several cancer types is a negative prognostic
442 indicator. In addition, tumour-infiltrating Tregs have been shown to express gene
443 signatures, including markers of activation and function, which distinguish them from
444 not only blood Tregs, but also tissue resident Tregs from healthy tissue of the same
445 origin (35, 36). Clinical trials are underway to determine the outcome of agents
446 designed to reduce Treg numbers by targeting the IL-2 receptor or GITR. To date
447 these results have been varied, with daclizumab (anti-CD25) reducing Treg numbers
448 in patients while allowing the induction of T cell responses to targeted tumour
449 antigens (37, 38) and a phase 1 trial of an anti-GITR antibody (TRX518) decreasing
450 Treg numbers in the tumour and circulating blood (39). However, depletion of Tregs
451 in stage IV melanoma with an IL-2/diphtheria toxin conjugate (DAB/IL-2) showed
452 partial responses in only 16.7% of patients, although there was a greater one year
453 survival in partial responders compared to those with progressive disease (80% vs.
454 23.7%; (40)). This study provides a fuller mechanistic framework for the further
455 rational development of Treg-targeted immunotherapy. Treg suppression of GSW11-
456 specific T cells primarily affected those with lower avidity TcR. These clones
457 expanded in the absence of Tregs, and correlated with survival, suggesting they are
458 important in tumour rejection. It will be interesting to know whether these expanded
459 CTL have a similar profile to the recently described CD103⁺ CD8⁺T_{RM} that
460 characterise T cell infiltrates of NSCLC associated with prolonged survival (41). The
461 expansion of protective low Ka T cell clones in Treg depleted mice is consistent with
462 previous studies. For example, Pace et al showed that the presence of Treg during
463 priming to a transplantation antigen increased the affinity of the CD8⁺ T cell response
464 by inhibiting the priming of T cells bearing low affinity TcR via a mechanism

465 involving CCL3/4 dependent destabilisation of T cell interactions with dendritic cells
466 (26).

467 The therapeutic efficacy of the low-avidity T cell clones was somewhat
468 unexpected since it is generally assumed that high avidity T cells have a competitive
469 advantage in an immune response due to stronger and prolonged activation signals
470 (42, 43). However, in a situation of persistent antigenic stimulation, as is encountered
471 in the tumour, it is likely that these T cells progress to exhaustion as observed in
472 chronic viral infection. Low avidity CTL may escape the same fate through lower
473 expression of PD-1 or by receiving a TcR signal below the threshold required for
474 exhaustion while maintaining some effector function (44, 45).

475 The identification of low K_a GSW11-specific TcR, which correlate with
476 protection, may have implications for epitope selection in immunotherapy. Current
477 strategies concentrate on the identification and use of tumour epitopes with a high
478 K_a /slow off-rate in an attempt to induce CD8⁺ T cell responses with a strong K_a TcR
479 (46-48). This approach has had some success with antigen-specific T cell responses
480 directed to TAA such as NY-ESO-1 and MART-1 and neoantigens. However, only a
481 small proportion of these patients show a partial or complete clinical response (49,
482 50). These studies show that, while the induction of high-avidity T cells to dominant
483 TAA occur, in a therapeutic setting, it may be more efficacious to induce a broad
484 repertoire of TcR affinities using peptide epitopes presented at sufficient levels
485 regardless of their affinity for MHC. With this in mind, it is notable that the dominant
486 target peptide recognised by TIL in CT26, GSW11, binds weakly to its presenting
487 MHC I, H2-D^d with a half-life of ~20min at the cell surface, though it is presented in
488 high abundance (17). It will therefore be important to understand the relationship
489 between antigen processing/presentation and the induction of low avidity T cells. For

490 example, algorithms predicting the affinity of candidate peptides could be better
491 deployed for selecting candidate epitopes for targeted immunotherapy if used in
492 combination with computational models that take into account antigen abundance and
493 mechanistic details of the antigen processing pathway.

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499 **References:**

- 500 1. Hiraoka, K., M. Miyamoto, Y. Cho, M. Suzuoki, T. Oshikiri, Y. Nakakubo, T.
501 Itoh, T. Ohbuchi, S. Kondo, and H. Katoh. 2006. Concurrent infiltration by
502 CD8+ T cells and CD4+ T cells is a favourable prognostic factor in non-small-
503 cell lung carcinoma. *Br J Cancer* 94: 275-280.
- 504 2. Naito, Y., K. Saito, K. Shiiba, A. Ohuchi, K. Saigenji, H. Nagura, and H.
505 Ohtani. 1998. CD8+ T cells infiltrated within cancer cell nests as a prognostic
506 factor in human colorectal cancer. *Cancer Res* 58: 3491-3494.
- 507 3. Nakano, O., M. Sato, Y. Naito, K. Suzuki, S. Orikasa, M. Aizawa, Y. Suzuki,
508 I. Shintaku, H. Nagura, and H. Ohtani. 2001. Proliferative activity of
509 intratumoral CD8(+) T-lymphocytes as a prognostic factor in human renal cell
510 carcinoma: clinicopathologic demonstration of antitumor immunity. *Cancer*
511 *Res* 61: 5132-5136.
- 512 4. Pages, F., A. Berger, M. Camus, F. Sanchez-Cabo, A. Costes, R. Molitor, B.
513 Mlecnik, A. Kirilovsky, M. Nilsson, D. Damotte, T. Meatchi, P. Bruneval, P.
514 H. Cugnenc, Z. Trajanoski, W. H. Fridman, and J. Galon. 2005. Effector
515 memory T cells, early metastasis, and survival in colorectal cancer. *N Engl J*
516 *Med* 353: 2654-2666.
- 517 5. Sato, E., S. H. Olson, J. Ahn, B. Bundy, H. Nishikawa, F. Qian, A. A.
518 Jungbluth, D. Frosina, S. Gnjjatic, C. Ambrosone, J. Kepner, T. Odunsi, G.
519 Ritter, S. Lele, Y. T. Chen, H. Ohtani, L. J. Old, and K. Odunsi. 2005.
520 Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high
521 CD8+/regulatory T cell ratio are associated with favorable prognosis in
522 ovarian cancer. *Proc Natl Acad Sci U S A* 102: 18538-18543.
- 523 6. Shankaran, V., H. Ikeda, A. T. Bruce, J. M. White, P. E. Swanson, L. J. Old,
524 and R. D. Schreiber. 2001. IFN γ and lymphocytes prevent primary
525 tumour development and shape tumour immunogenicity. *Nature* 410: 1107-
526 1111.
- 527 7. Smyth, M. J., K. Y. Thia, S. E. Street, D. MacGregor, D. I. Godfrey, and J. A.
528 Trapani. 2000. Perforin-mediated cytotoxicity is critical for surveillance of
529 spontaneous lymphoma. *J Exp Med* 192: 755-760.
- 530 8. Dunn, G. P., A. T. Bruce, H. Ikeda, L. J. Old, and R. D. Schreiber. 2002.
531 Cancer immunoediting: from immunosurveillance to tumor escape. *Nat*
532 *Immunol* 3: 991-998.
- 533 9. Schreiber, R. D., L. J. Old, and M. J. Smyth. 2011. Cancer immunoediting:
534 integrating immunity's roles in cancer suppression and promotion. *Science*
535 331: 1565-1570.
- 536 10. Dempke, W. C. M., K. Fenchel, P. Uciechowski, and S. P. Dale. 2017.
537 Second- and third-generation drugs for immuno-oncology treatment—The
538 more the better? *European Journal of Cancer* 74: 55-72.
- 539 11. Shang, B., Y. Liu, S. J. Jiang, and Y. Liu. 2015. Prognostic value of tumor-
540 infiltrating FoxP3+ regulatory T cells in cancers: a systematic review and
541 meta-analysis. *Sci Rep* 5: 15179.
- 542 12. Olson, B. M., and D. G. McNeel. 2013. Monitoring regulatory immune
543 responses in tumor immunotherapy clinical trials. *Front Oncol* 3: 109.
- 544 13. Golgher, D., E. Jones, F. Powrie, T. Elliott, and A. Gallimore. 2002. Depletion
545 of CD25+ regulatory cells uncovers immune responses to shared murine
546 tumor rejection antigens. *Eur J Immunol* 32: 3267-3275.

- 547 14. Onizuka, S., I. Tawara, J. Shimizu, S. Sakaguchi, T. Fujita, and E. Nakayama.
548 1999. Tumor Rejection by *in Vivo* Administration of Anti-CD25
549 (Interleukin-2 Receptor α) Monoclonal Antibody. *Cancer Research* 59: 3128-
550 3133.
- 551 15. Grosso, J. F., and M. N. Jure-Kunkel. 2013. CTLA-4 blockade in tumor
552 models: an overview of preclinical and translational research. *Cancer Immun*
553 13: 5.
- 554 16. Stewart, R., M. Morrow, S. A. Hammond, K. Mulgrew, D. Marcus, E. Poon,
555 A. Watkins, S. Mullins, M. Chodorge, J. Andrews, D. Bannister, E. Dick, N.
556 Crawford, J. Parmentier, M. Alimzhanov, J. S. Babcook, I. N. Foltz, A.
557 Buchanan, V. Bedian, R. W. Wilkinson, and M. McCourt. 2015. Identification
558 and Characterization of MEDI4736, an Antagonistic Anti-PD-L1 Monoclonal
559 Antibody. *Cancer Immunol Res* 3: 1052-1062.
- 560 17. James, E., A. Yeh, C. King, F. Korangy, I. Bailey, D. S. Boulanger, B. J. Van
561 den Eynde, N. Murray, and T. J. Elliott. 2010. Differential suppression of
562 tumor-specific CD8⁺ T cells by regulatory T cells. *J Immunol* 185: 5048-
563 5055.
- 564 18. Huang, A. Y., P. H. Gulden, A. S. Woods, M. C. Thomas, C. D. Tong, W.
565 Wang, V. H. Engelhard, G. Pasternack, R. Cotter, D. Hunt, D. M. Pardoll, and
566 E. M. Jaffee. 1996. The immunodominant major histocompatibility complex
567 class I-restricted antigen of a murine colon tumor derives from an endogenous
568 retroviral gene product. *Proc Natl Acad Sci U S A* 93: 9730-9735.
- 569 19. Truscott, S. M., L. Lybarger, J. M. Martinko, V. E. Mitaksov, D. M. Kranz, J.
570 M. Connolly, D. H. Fremont, and T. H. Hansen. 2007. Disulfide bond
571 engineering to trap peptides in the MHC class I binding groove. *J Immunol*
572 178: 6280-6289.
- 573 20. Duraiswamy, J., G. J. Freeman, and G. Coukos. 2013. Therapeutic PD-1
574 pathway blockade augments with other modalities of immunotherapy T-cell
575 function to prevent immune decline in ovarian cancer. *Cancer Res* 73: 6900-
576 6912.
- 577 21. Sakuishi, K., L. Apetoh, J. M. Sullivan, B. R. Blazar, V. K. Kuchroo, and A.
578 C. Anderson. 2010. Targeting Tim-3 and PD-1 pathways to reverse T cell
579 exhaustion and restore anti-tumor immunity. *J Exp Med* 207: 2187-2194.
- 580 22. Wherry, E. J., and M. Kurachi. 2015. Molecular and cellular insights into T
581 cell exhaustion. *Nat Rev Immunol* 15: 486-499.
- 582 23. Gajewski, T. F., H. Schreiber, and Y. X. Fu. 2013. Innate and adaptive
583 immune cells in the tumor microenvironment. *Nat Immunol* 14: 1014-1022.
- 584 24. Marvel, D., and D. I. Gabrilovich. 2015. Myeloid-derived suppressor cells in
585 the tumor microenvironment: expect the unexpected. *J Clin Invest* 125: 3356-
586 3364.
- 587 25. Agata, Y., A. Kawasaki, H. Nishimura, Y. Ishida, T. Tsubata, H. Yagita, and
588 T. Honjo. 1996. Expression of the PD-1 antigen on the surface of stimulated
589 mouse T and B lymphocytes. *Int Immunol* 8: 765-772.
- 590 26. Pace, L., A. Tempez, C. Arnold-Schrauf, F. Lemaitre, P. Bousso, L. Fetler, T.
591 Sparwasser, and S. Amigorena. 2012. Regulatory T cells increase the avidity
592 of primary CD8⁺ T cell responses and promote memory. *Science* 338: 532-
593 536.
- 594 27. Holmberg, K., S. Mariathasan, T. Ohteki, P. S. Ohashi, and N. R. Gascoigne.
595 2003. TCR binding kinetics measured with MHC class I tetramers reveal a

- 596 positive selecting peptide with relatively high affinity for TCR. *J Immunol*
597 171: 2427-2434.
- 598 28. Schietinger, A., and P. D. Greenberg. 2014. Tolerance and exhaustion:
599 defining mechanisms of T cell dysfunction. *Trends Immunol* 35: 51-60.
- 600 29. Gajewski, T. F., J. Louahed, and V. G. Brichard. 2010. Gene signature in
601 melanoma associated with clinical activity: a potential clue to unlock cancer
602 immunotherapy. *Cancer J* 16: 399-403.
- 603 30. Spranger, S., J. J. Luke, R. Bao, Y. Zha, K. M. Hernandez, Y. Li, A. P.
604 Gajewski, J. Andrade, and T. F. Gajewski. 2016. Density of immunogenic
605 antigens does not explain the presence or absence of the T-cell-inflamed tumor
606 microenvironment in melanoma. *Proc Natl Acad Sci U S A* 113: E7759-
607 E7768.
- 608 31. Spranger, S., R. M. Spaapen, Y. Zha, J. Williams, Y. Meng, T. T. Ha, and T.
609 F. Gajewski. 2013. Up-regulation of PD-L1, IDO, and T(regs) in the
610 melanoma tumor microenvironment is driven by CD8(+) T cells. *Sci Transl*
611 *Med* 5: 200ra116.
- 612 32. Lechner, M. G., S. S. Karimi, K. Barry-Holson, T. E. Angell, K. A. Murphy,
613 C. H. Church, J. R. Ohlfest, P. Hu, and A. L. Epstein. 2013. Immunogenicity
614 of murine solid tumor models as a defining feature of in vivo behavior and
615 response to immunotherapy. *J Immunother* 36: 477-489.
- 616 33. Murphy, K. A., M. G. Lechner, F. E. Popescu, J. Bedi, S. A. Decker, P. Hu, J.
617 R. Erickson, M. G. O'Sullivan, L. Swier, A. M. Salazar, M. R. Olin, A. L.
618 Epstein, and J. R. Ohlfest. 2012. An in vivo immunotherapy screen of
619 costimulatory molecules identifies Fc-OX40L as a potent reagent for the
620 treatment of established murine gliomas. *Clin Cancer Res* 18: 4657-4668.
- 621 34. Chikuma, S., S. Terawaki, T. Hayashi, R. Nabeshima, T. Yoshida, S.
622 Shibayama, T. Okazaki, and T. Honjo. 2009. PD-1-mediated suppression of
623 IL-2 production induces CD8+ T cell anergy in vivo. *J Immunol* 182: 6682-
624 6689.
- 625 35. De Simone, M., A. Arrigoni, G. Rossetti, P. Gruarin, V. Ranzani, C. Politano,
626 R. J. Bonnal, E. Provasi, M. L. Sarnicola, I. Panzeri, M. Moro, M. Crosti, S.
627 Mazzara, V. Vaira, S. Bosari, A. Palleschi, L. Santambrogio, G. Bovo, N.
628 Zucchini, M. Totis, L. Gianotti, G. Cesana, R. A. Perego, N. Maroni, A. Pisani
629 Ceretti, E. Opocher, R. De Francesco, J. Geginat, H. G. Stunnenberg, S.
630 Abrignani, and M. Pagani. 2016. Transcriptional Landscape of Human Tissue
631 Lymphocytes Unveils Uniqueness of Tumor-Infiltrating T Regulatory Cells.
632 *Immunity* 45: 1135-1147.
- 633 36. Plitas, G., C. Konopacki, K. Wu, P. D. Bos, M. Morrow, E. V. Putintseva, D.
634 M. Chudakov, and A. Y. Rudensky. 2016. Regulatory T Cells Exhibit Distinct
635 Features in Human Breast Cancer. *Immunity* 45: 1122-1134.
- 636 37. Rech, A. J., and R. H. Vonderheide. 2009. Clinical use of anti-CD25 antibody
637 daclizumab to enhance immune responses to tumor antigen vaccination by
638 targeting regulatory T cells. *Ann N Y Acad Sci* 1174: 99-106.
- 639 38. Jacobs, J. F., C. J. Punt, W. J. Lesterhuis, R. P. Suttmuller, H. M. Brouwer, N.
640 M. Scharenborg, I. S. Klasen, L. B. Hilbrands, C. G. Figdor, I. J. de Vries, and
641 G. J. Adema. 2010. Dendritic cell vaccination in combination with anti-CD25
642 monoclonal antibody treatment: a phase I/II study in metastatic melanoma
643 patients. *Clin Cancer Res* 16: 5067-5078.
- 644 39. Zappasodi, R., Y. Li, M. Abu-Akeel, J. Qi, P. Wong, C. Sirard, M. Postow, D.
645 A. Schaer, W. Newman, H. Koon, V. Velcheti, M. K. Callahan, J. D.

- 646 Wolchok, and T. Merghoub. 2017. Intratumor and peripheral Treg modulation
647 as a pharmacodynamic biomarker of the GITR agonist antibody TRX-518 in
648 the first in-human trial [abstract]. In: *Proceedings of the Annual Meeting of*
649 *the American Association for Cancer Research; 2017 Apr 1-5; Washington,*
650 *DC: AACR; 2017: Abstract nr CT018.*
- 651 40. Telang, S., M. A. Rasku, A. L. Clem, K. Carter, A. C. Klarer, W. R. Badger,
652 R. A. Milam, S. N. Rai, J. Pan, H. Gragg, B. F. Clem, K. M. McMasters, D.
653 M. Miller, and J. Chesney. 2011. Phase II trial of the regulatory T cell-
654 depleting agent, denileukin diftitox, in patients with unresectable stage IV
655 melanoma. *BMC Cancer* 11: 515.
- 656 41. Ganesan, A. P., J. Clarke, O. Wood, E. M. Garrido-Martin, S. J. Chee, T.
657 Mellows, D. Samaniego-Castruita, D. Singh, G. Seumois, A. Alzetani, E.
658 Woo, P. S. Friedmann, E. V. King, G. J. Thomas, T. Sanchez-Elsner, P.
659 Vijayanand, and C. H. Ottensmeier. 2017. Tissue-resident memory features
660 are linked to the magnitude of cytotoxic T cell responses in human lung
661 cancer. *Nat Immunol* 18: 940-950.
- 662 42. Busch, D. H., and E. G. Pamer. 1999. T cell affinity maturation by selective
663 expansion during infection. *J Exp Med* 189: 701-710.
- 664 43. Zehn, D., S. Y. Lee, and M. J. Bevan. 2009. Complete but curtailed T-cell
665 response to very low-affinity antigen. *Nature* 458: 211-214.
- 666 44. Conrad, J. A., R. K. Ramalingam, R. M. Smith, L. Barnett, S. L. Lorey, J.
667 Wei, B. C. Simons, S. Sadagopal, D. Meyer-Olson, and S. A. Kalams. 2011.
668 Dominant clonotypes within HIV-specific T cell responses are programmed
669 death-1high and CD127low and display reduced variant cross-reactivity. *J*
670 *Immunol* 186: 6871-6885.
- 671 45. Harari, A., C. Cellera, F. Bellutti Enders, J. Kostler, L. Codarri, G. Tapia, O.
672 Boyman, E. Castro, S. Gaudieri, I. James, M. John, R. Wagner, S. Mallal, and
673 G. Pantaleo. 2007. Skewed association of polyfunctional antigen-specific CD8
674 T cell populations with HLA-B genotype. *Proc Natl Acad Sci U S A* 104:
675 16233-16238.
- 676 46. Castle, J. C., S. Kreiter, J. Diekmann, M. Löwer, N. van de Roemer, J. de
677 Graaf, A. Selmi, M. Diken, S. Boegel, C. Paret, M. Koslowski, A. N. Kuhn, C.
678 M. Britten, C. Huber, Ö. Türeci, and U. Sahin. 2012. Exploiting the
679 Mutanome for Tumor Vaccination. *Cancer Research* 72: 1081-1091.
- 680 47. Robbins, P. F., Y.-C. Lu, M. El-Gamil, Y. F. Li, C. Gross, J. Gartner, J. C.
681 Lin, J. K. Teer, P. Cliften, E. Tycksen, Y. Samuels, and S. A. Rosenberg.
682 2013. Mining Exomic Sequencing Data to Identify Mutated Antigens
683 Recognized by Adoptively Transferred Tumor-reactive T cells. *Nature*
684 *medicine* 19: 747-752.
- 685 48. Rooij, N. v., M. M. v. Buuren, D. Philips, A. Velds, M. Toebes, B.
686 Heemskerk, L. J. A. v. Dijk, S. Behjati, H. Hilkmann, D. e. Atmioui, M.
687 Nieuwland, M. R. Stratton, R. M. Kerkhoven, C. Keşmir, J. B. Haanen, P.
688 Kvistborg, and T. N. Schumacher. 2013. Tumor Exome Analysis Reveals
689 Neoantigen-Specific T-Cell Reactivity in an Ipilimumab-Responsive
690 Melanoma. *Journal of Clinical Oncology* 31: e439-e442.
- 691 49. Carreno, B. M., V. Magrini, M. Becker-Hapak, S. Kaabinejadian, J. Hundal,
692 A. A. Petti, A. Ly, W. R. Lie, W. H. Hildebrand, E. R. Mardis, and G. P.
693 Linette. 2015. Cancer immunotherapy. A dendritic cell vaccine increases the
694 breadth and diversity of melanoma neoantigen-specific T cells. *Science* 348:
695 803-808.

- 696 50. Rosenberg, S. A., J. C. Yang, and N. P. Restifo. 2004. Cancer immunotherapy:
697 moving beyond current vaccines. *Nat Med* 10: 909-915.
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- 699

700 **Figure Legends**

701 **Figure 1. The majority of tumour infiltrating GSW11-specific T cells are non-**
702 **functional.** Balb/c mice were challenged with CT26 tumour cells and the presence of
703 tumour infiltrating AH1- and GSW11-specific T cells was assessed over the indicated
704 time course. (A) Assessment of AH1- and GSW11-specific T cells using
705 tetramer/dextramer and IFN- γ production at d10 and d22. (B) Percentage of antigen-
706 specific T cells detected by tetramer/dextramer. (C) Diagram representing the tumour
707 size (diameter) and relative proportions of antigen-specific tumour infiltrating T cells
708 over time. (D) Percentage of functional AH1- and GSW11-specific CD8⁺ T cells. (E)
709 Relative proportion of functional and non-functional antigen-specific tumour
710 infiltrating T cells. (B, D; mean and s.e.m. of three mice at each time point; * $p < 0.05$,
711 *** $p < 0.001$).

712

713 **Figure 2. GSW11-specific T cells are exhausted in tumour challenged mice.** (A) A
714 representative histogram showing PD-1 expression of functional and non-functional
715 AH1- and GSW11-specific T cells. (B, C) Percentage of PD-1 expressing tumour
716 infiltrating non-functional (B) and functional (C) AH1- and GSW11-specific T cells
717 (B, C; mean and s.e.m. of at least three mice from two independent experiments; ***
718 $p < 0.001$).

719

720 **Figure 3. Expression of PD-1 and PD-L1 on tumour infiltrating and LN resident**
721 **regulatory T cells and tumour cells.** (A) Percentage of tumour infiltrating Tregs
722 over time. (B, C) Expression of PD-L1 (B) and PD-1 (C) on Tregs in tumours and
723 tumour draining LN. (D) Percentage and level of PD-L1 expression on CT26 tumour

724 cells prior to and during tumour challenge. (A-C; mean and s.e.m. of three mice at the
725 indicated time points).

726

727 **Figure 4. Exhaustion of anti-tumour T cells is induced in the periphery and**
728 **regulated by regulatory T cells.** Treg replete or depleted Balb/c mice were
729 challenged with CT26 and anti-tumour T cell responses in spleens and tumour
730 draining LN assessed. (A) A representative histogram showing the relative level of
731 PD-1 expression on AH1- and GSW11-specific T cells as indicated. (B, C) Percentage
732 of functional GSW11- (B) or AH1- (C) specific T cells in spleen and tdLN in Treg
733 replete and depleted mice following tumour challenge over the indicated time course.
734 (D-G) Proportion of PD-1 expressing non-functional (D, E) and functional (F, G) anti-
735 GSW11 (D, F) or -AH1 (E, G) T cells in spleen and tumour draining LN in Treg
736 replete and depleted mice following tumour challenge. (B-G; mean and s.e.m. of three
737 mice at the indicated time points; d25 data only available for Treg depleted samples).

738

739 **Figure 5. GSW11-specific T cell clonalities are modulated by regulatory T cells.**
740 Treg replete or depleted Balb/c mice were challenged with CT26 and the TcR
741 expression of GSW11-specific T cells assessed. (A) Percentage of GSW11-specific
742 CD8+ T cells expressing the indicating TcR V β in Treg replete or depleted CT26
743 challenged mice. (B) The relative proportion of TcR V β usage in GSW11-specific T
744 cells following tumour challenge (blue segments indicate TcR with high avidity and
745 red indicate those with low avidity). (A; mean and s.e.m. of ten mice in three pools,
746 B; mean of ten mice from three pools; * $p < 0.05$, ** $p < 0.01$).

747

748 **Figure 6. Tumour protective GSW11-specific CD8+ T cells have low TcR avidity.**

749 The TcR avidity of anti-GSW11 T cell oligoclones that showed either increased,
750 decreased, or similar levels in tumour challenged Treg depleted mice was assessed
751 using tetramer competition. (A, B) A representative histogram (A) and analysis of
752 relative change in tetramer MFI (B) of the TcR tetramer competition assay in
753 indicated TcR clones. (C) Relative avidity of indicated GSW11-specific T cell
754 oligoclones following tetramer competition (C; mean and s.e.m. of at least three
755 independent experiments; * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$).

Figure 1

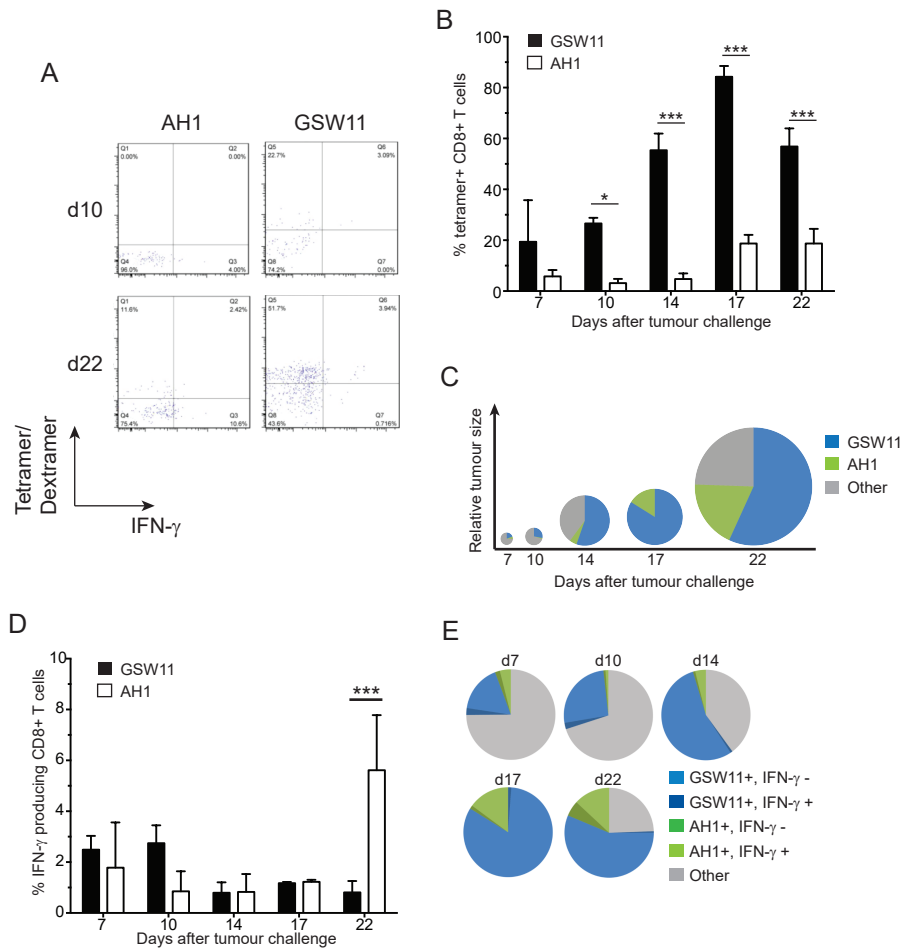


Figure 2

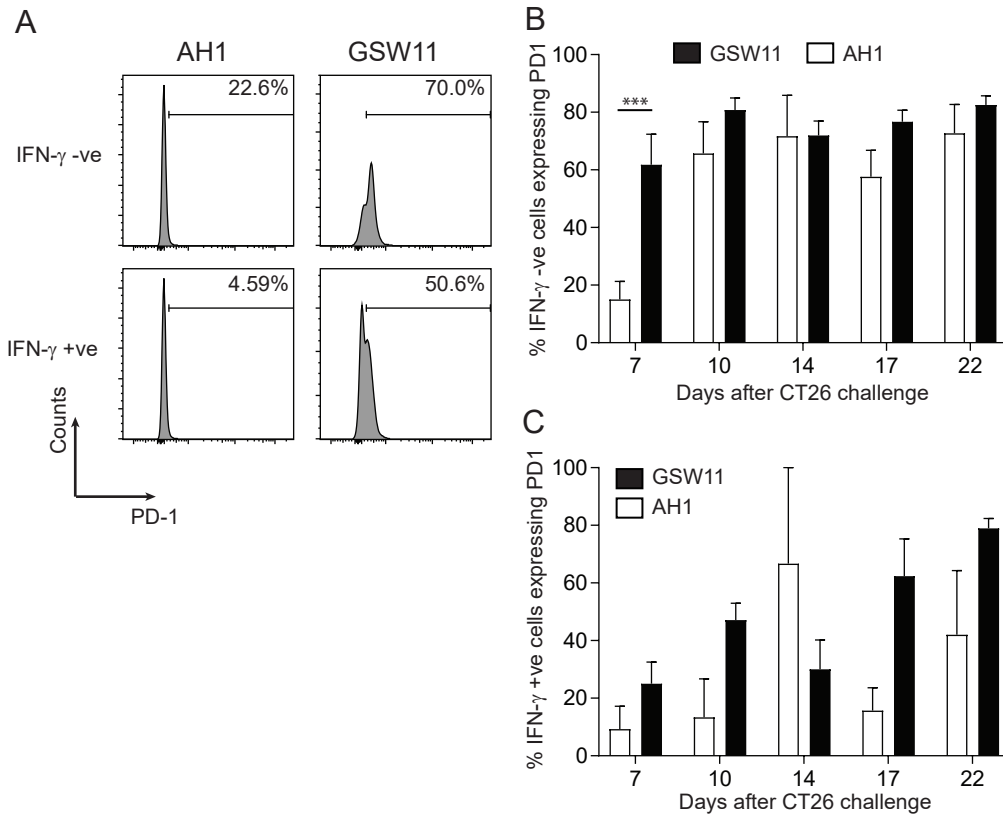


Figure 3

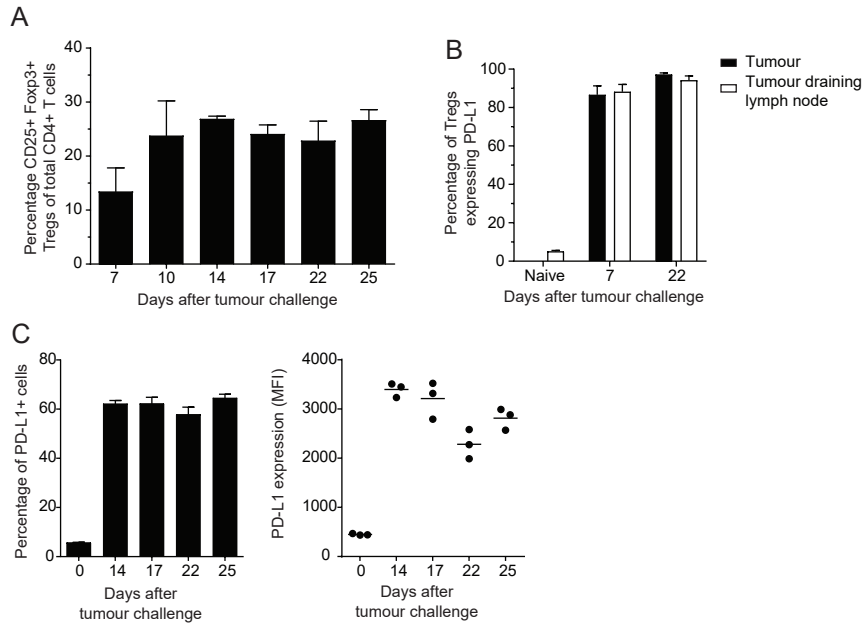


Figure 4

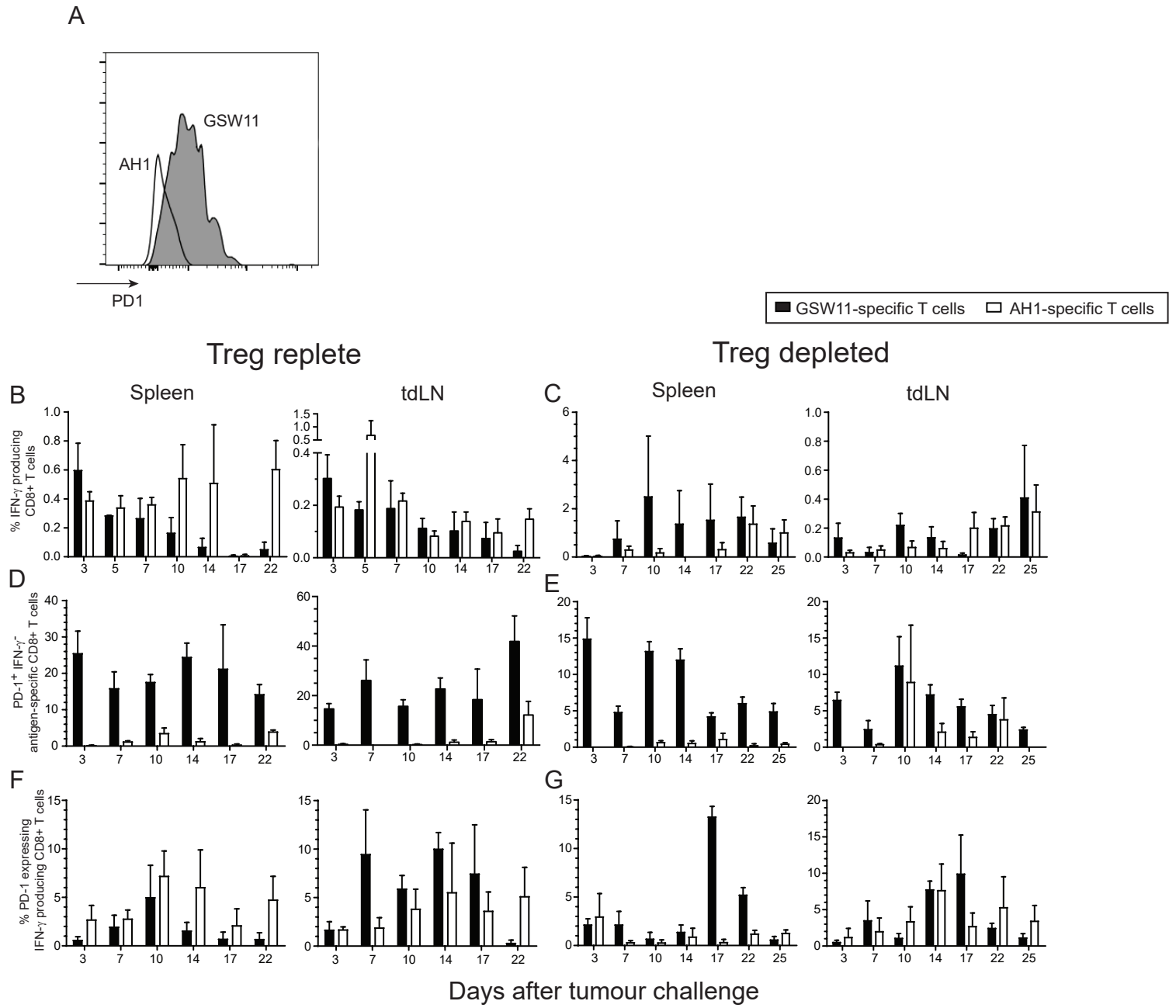


Figure 5

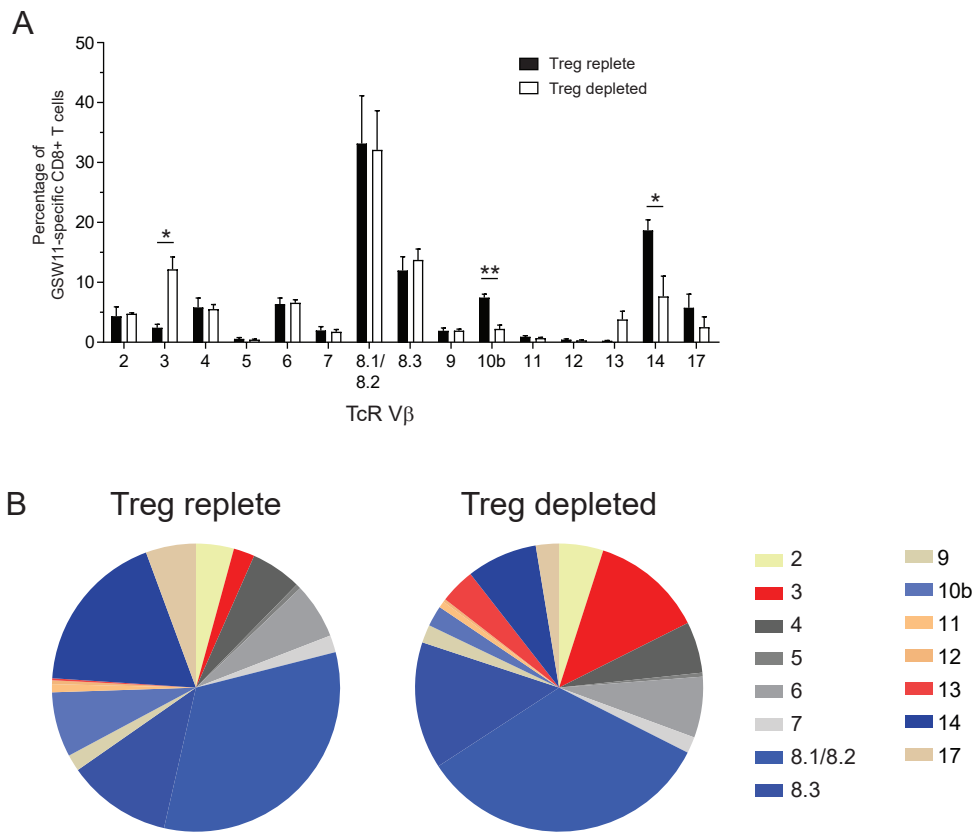


Figure 6

