1 **Title: Domain Binding and Isotype Dictate the Activity of Anti-human OX40**

2 **Antibodies**

3

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20 **Running Title:** Isotype and epitope dictate OX40 mAb activity

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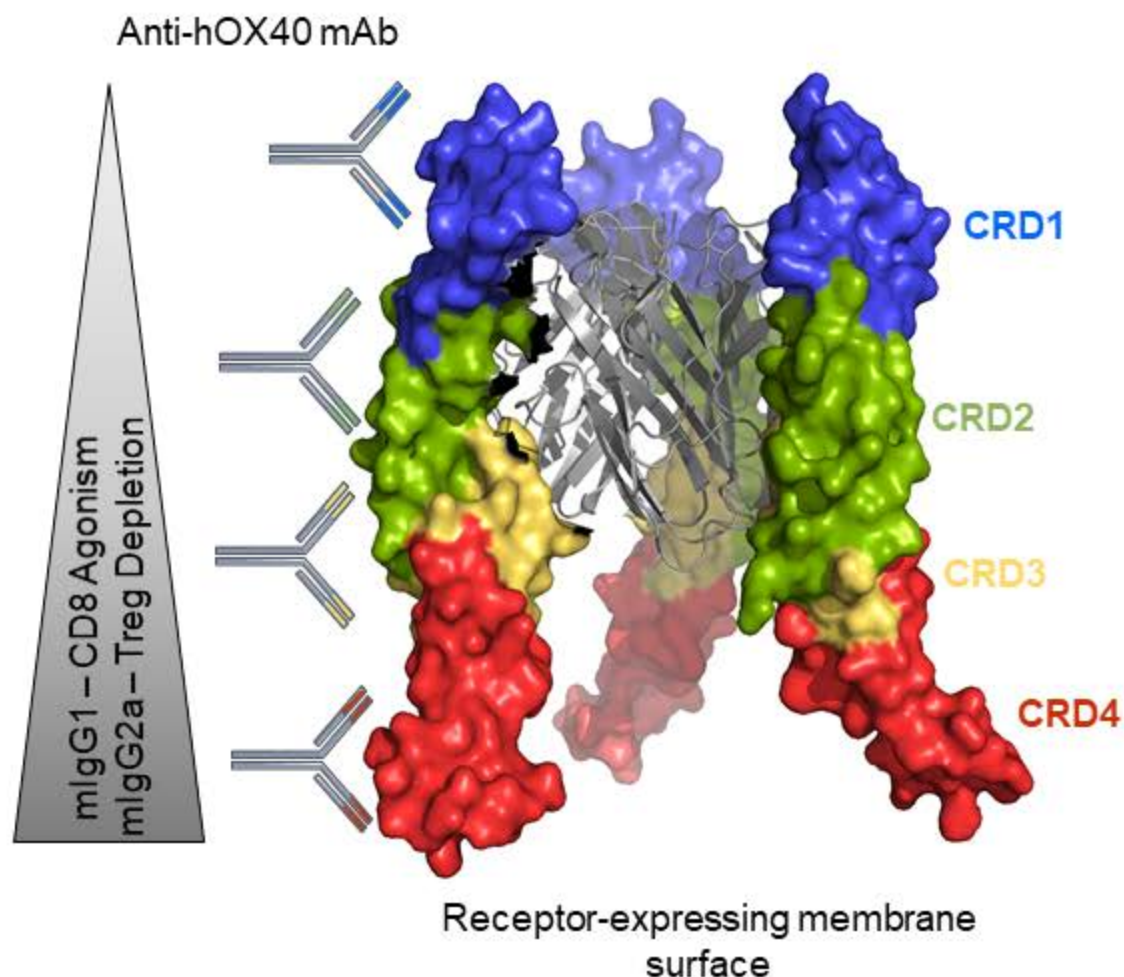22 **Keywords:** OX40, isotype, receptor agonism, therapeutic activity

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Abstract:

36	**Abbreviations:**	
37	ACK	Ammonium Chloride Potassium
38	ADCC	Antibody dependent cellular cytotoxicity
39	ADCP	Antibody dependent cellular phagocytosis
40	CDC	Complement dependent cytotoxicity
41	CRD	Cysteine rich domains (CRDs)
42	ECD	Extracellular domain
43	Eomes	Eomesodermin
44	I.P.	Intraperitoneal
45	I.V.	Intravenous
46	KI	Knock-in
47	KO	Knock-out
48	mAb	Monoclonal antibody
49	MPECs	Memory precursor cells
50	NOG	NOD/Shi-*scid*/IL-2Rγ^{null}
51	NSG	NOD scid gamma
52	PBMCs	Peripheral blood mononuclear cells
53	PCR	Polymerase chain reaction
54	SCID	Severe combined immune deficient
55	SLECs	Short lived effector cells
56	SPR	Surface Plasmon Resonance
57	tdLN	Tumour draining lymph node
58	TILS	Tumour inflitrating lymphocytes
59	TME	Tumour microenvironment
60	TNFR	Tumour necrosis factor receptors
61	WT	Wildtype
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Anti-hOX40 mAb

mIgG1 – CD8 Agonism
mIgG2a – Treg Depletion

CRD1
CRD2
CRD3
CRD4

Receptor-expressing membrane surface

Isotype and epitope dictate OX40 mAb activity;

- mIgG1 promotes direct agonism on CD8+ T cells

- mIgG2a depletes hOX40 expressing Tregs

- Strength of both agonism and depleting activity is greatest with membrane proximal binding mAb

- Provides insight into how anti-hOX40 mAb could be improved for clinical efficacy

70 **Abstract**

72 Background: Previous data suggests that anti-OX40 mAb can elicit anti-tumour
73 effects in mice through deletion of Tregs. However, OX40 also has powerful
74 costimulatory effects on T cells which could evoke therapeutic responses. Human
75 trials with anti-OX40 antibodies have shown that these entities are well tolerated but
76 to date have delivered disappointing clinical responses, indicating that the rules for
77 the optimal use of anti-human OX40 (hOX40) antibodies is not yet fully understood.
78 Changes to timing and dosages may lead to improved outcomes, however here we
79 focus on addressing the role of agonism vs depleting activity in determining
80 therapeutic outcomes. We investigated a novel panel of anti-hOX40 mAb to
81 understand how these reagents and mechanisms may be optimised for therapeutic
82 benefit.

84 Methods: This study examines the binding activity and in vitro activity of a panel of
85 anti-hOX40 antibodies. They were further evaluated in several in vivo models to
86 address how isotype and epitope determine mechanism of action and efficacy of
87 anti-hOX40 mAb.

89 Results: Binding analysis revealed the antibodies to be high affinity with epitopes
90 spanning all four cysteine rich domains of the OX40 extracellular domain. In vivo
91 analysis showed that their activities relate directly to two key properties: 1) isotype –
92 with mIgG1 mAb evoking receptor agonism and CD8+ T cell expansion and mIgG2a
93 mAb evoking deletion of Treg and; 2) epitope - with membrane-proximal mAb
94 delivering more powerful agonism. Intriguingly, both isotypes acted therapeutically in
95 tumour models by engaging these different mechanisms.

97 Conclusions: These findings highlight the significant impact of isotype and epitope on
98 the modulation of anti-hOX40 mAb therapy, and indicate that CD8+ T cell expansion
99 or Treg depletion might be preferred according to the composition of different
100 tumours. As many of the current clinical trials using OX40 antibodies are now using
101 combination therapies, this understanding of how to manipulate therapeutic activity
102 will be vital in directing new combinations that are more likely to improve efficacy and
103 clinical outcomes.

Introduction

The use of immunomodulating monoclonal antibodies (mAb) to generate anti-tumour immune responses offers an exciting approach to cancer immunotherapy. mAb against immune checkpoint inhibitors such as Ipilimumab and Nivolumab, which target the co-inhibitory receptors CTLA-4 and PD-1, respectively, pioneered this approach and have demonstrated success in treating a number of previously untreatable cancers [1, 2]. However, many patients do not respond to these reagents and additional therapeutic strategies are required. Agonistic mAb targeting co-stimulatory receptors have emerged as targets for clinical development, in particular tumour necrosis factor receptors (TNFR) superfamily members such as CD40 [3], 4-1BB [4], and OX40 [5-7]. However Freeman et al identified a intratumoural Treg signature which included TNFR family members with the hypothesis that they could be targeted instead by depleting antibodies in order to generate therapy [8]. TNFR family members are typically characterised by an extracellular domain (ECD) consisting of several cysteine rich domains (CRDs) which allow for binding of their respective trimeric ligands leading to receptor clustering and downstream signalling [9]. mAb targeting such receptors have been shown to depend on their interaction with the inhibitory FcγR (FcγRIIB) to generate sufficient cross-linking and resultant agonistic activity [10, 11]. More recently, the ability of a number of TNFR mAb to cause deletion of Tregs via engagement of activatory FcγR has been demonstrated [12, 13]. The anti-mouse OX40 mAb, OX86, has previously been shown to enhance effector T cell proliferation and survival leading to successful therapeutic outcomes in pre-clinical models [5, 14]. Recently it has also been demonstrated to be capable of deleting Tregs in an activatory FcγR-dependent manner [12]. This effect was directly influenced by isotype, with mIgG2a showing greater depleting capacity than the native rIgG1 isotype. Interestingly Tregs were preferentially deleted over effector T cells which correlated with mOX40 expression on these cells [12].

Work on several TNFRs has further highlighted the importance of the region targeted by the antibody in influencing the type and strength of effector function [15-17]. For anti-CD40 mAb, the membrane distal CRD1-binding mAb were shown to be strong agonists of CD40 with membrane proximal mAb less potent [16]. Furthermore, mAb binding CRD2-4 blocked CD40L and were potent antagonists. Additionally anti-4-

137 1BB mAb which bound membrane proximal domains engaged in more effective CDC
138 and ADCC killing mechanisms with ADCP less affected [15] . Moreover, Zhang et al.
139 reported that mAb binding to mouse (m)OX40 which blocked ligand binding and
140 bound CRD2, or bound at the membrane proximal domain (CRD4), provide stronger
141 agonistic and anti-tumour activity than mAb binding CRD1 and 3 [17]. These results
142 differed from those seen for hCD40, highlighting that the functional effects of mAb
143 domain binding are likely to require assessment for each of the TNFR family
144 members and validation for each species.

145 Given these discrepancies, we explored the optimal domain binding and isotype for a
146 novel panel of anti-human (h)OX40 mAb which collectively bound to all 4 CRDs of
147 the ECD of hOX40. We evaluated their function *in vitro* and *in vivo* as both mIgG1
148 and mIgG2a isotypes. Using a novel hOX40 knock-in (KI) mouse we found that
149 mIgG1 mAb were agonistic and engendered memory responses, whereas mIgG2a
150 mAb had depleting activity with poorer memory recall responses. The strength of
151 these effector functions appeared to correlate with domain binding; those mAb which
152 bound to the most membrane proximal domain (CRD4) which did not block ligand
153 binding, showed the strongest agonistic activity as mIgG1 as well as the most potent
154 depleting activity as mIgG2a. This data highlights how mAb to different TNFR (and
155 different species) exhibit different requirements in relation to optimal domain binding
156 and effector function and indicate how more active anti-hOX40 mAb might be
157 developed.

158
159
160 **Results**
161
162 **hOX40KI mice express hOX40 and develop normally**
163
164 To investigate the immunotherapeutic potential of a new panel of anti-hOX40 mAb
165 we generated a new knock-in (KI) mouse, designed to express hOX40 extracellular
166 domain and mOX40 transmembrane and intracellular domains (Supplementary Fig.
167 1A). PCR confirmed integration of the construct and identified WT, hOX40KI^{+/-} and
168 hOX40KI^{+/+} mice (Supplementary Fig. 1B). Analysis of OX40 surface expression
169 (mouse and human) on resting splenocyte T cells from WT, hOX40KI^{+/-} and
170 hOX40KI^{+/+} mice confirmed that the chimeric receptor was expressed at the cell

171 surface on relevant cell types (Fig. 1A and B) and in a gene dose-dependent manner

172 (Supplementary Fig. 1C). Expression of OX40 in all three genotypes was largely

173 restricted to T cell lineages (Fig. 1A, B and Supplementary Fig. 1D). In line with

174 previous reports [12, 18-20], a hierarchal expression pattern amongst the T cell

175 subsets was observed in all genotypes with expression highest on Tregs followed by

176 CD4+ effectors, with limited expression on resting CD8+ T cells (Fig. 1A and B). To

177 address whether the hOX40KI$^{+/+}$ mice represented a functional model to study the

178 OX40L:OX40 axis, we performed SPR analysis of mOX40L and hOX40L binding to

179 hOX40 (Supplementary Fig. 1E). Both mOX40L and hOX40L bound similarly to

180 hOX40, in agreement with earlier studies showing that mOX40L engages the same

181 domains on hOX40 as hOX40L [21]. Furthermore, OX40 KO mice are reported to

182 have a subtle defect in Treg numbers (reviewed in [22]). However expression of the

183 chimeric receptor did not affect normal immune cell development (Supplementary

184 Fig. 1F), further indicating that OX40L:OX40 signalling axis is intact in these

185 hOX40KI mice.

186

187 Consistent with previous findings [23], activated splenocytes isolated from WT,

188 hOX40KI$^{+/-}$ and hOX40KI$^{+/+}$ mice showed peak OX40 expression between 24-48

189 hours post activation (Fig. 1C). The kinetics of expression of hOX40 on hOX40KI$^{+/-}$

190 and hOX40KI$^{+/+}$ splenocytes correlated with that of hOX40 on activated human

191 PBMCs (Fig. 1D). A hierarchy of expression was also observed in both activated

192 splenocytes and hPBMCs with greatest expression detectable on Tregs (Fig. 1C and

193 1D). Samples taken from human cancer patients also showed the same pattern

194 (Tregs > CD4+ effectors > CD8+ T cells) with highest OX40 expression on T cells

195 isolated from tumour sites (Fig. 1E). Collectively, these results validated the use of

196 the hOX40KI model to assess anti-hOX40 mAb.

197

198 **Generation and characterisation of a panel of anti-hOX40 mAb**

199 A panel of seven anti-hOX40 mAb were subsequently generated by conventional

200 hybridoma technology and characterised. All mAb displayed a high affinity for hOX40

201 (KD values between 10^{-9}-10^{-10}M) as determined by SPR (Supplemental Fig. 2A) and

202 did not bind mOX40 (Supplementary Fig 2B and C). The crystal structure of hOX40

203 indicates four CRDs [21]. To determine which of these the anti-hOX40 mAb bound,

204 hOX40 domain mutants were generated, lacking CRD1, CRD1+2 or CRD1, 2+3 (Fig.

2A). A hCD20 epitope tag and mOX40 CRD3 domain were added to the final construct to stabilise its expression. Across the panel, at least one antibody bound to each of the different domains (Fig. 2B and Supplemental Fig. 2D). Cross-blocking experiments showed that anti-hOX40 mAb which bound to the same domain blocked the binding of one another, whereas mAb binding to different CRDs could bind simultaneously (Fig. 2C). The domain binding for each antibody is summarised in Fig. 2D. Finally, SPR analysis revealed that only mAb binding to CRD4 were able to bind in the presence of the ligand (Fig. 2E and Supplemental Fig 2E), indicating ligand binding blocks binding of mAb that recognise epitopes in CRD1-3.

Antibodies were then assessed for their ability to augment sub-optimal anti-CD3 mediated proliferation of hPBMC. All of the mIgG1 mAb increased proliferation of CD8+ T cells (Fig. 2F) whereas mIgG2a mAb reduced proliferation. Differences between mIgG1 and mIgG2a isotypes have previously been reported for other TNFR family members [24, 25] and so we class-switched the mAb so that both mIgG1 and mIgG2a isotypes were available. A similar trend was seen in hOX40KI splenocytes stimulated with anti-hOX40 mAbs with mIgG1 mAb showing a trend towards an increase in proliferation whilst mIgG2a mAb showed a clear reduction in proliferation (Supplementary Fig 3).

anti-hOX40 mIgG1 mAb are agonistic *in vivo*

To investigate the ability of the anti-hOX40 mAb to cause antigen-specific CD8$^+$ T cell expansion *in vivo* we used the OT-I model whereby antigen specific T cells are transferred into naive recipients. hOX40KI$^{+/-}$ OT-I T cells, which recognise the OVA$_{257-264}$/H-2K^b complex, were adoptively transferred into hOX40KI$^{+/+}$ mice before vaccination with OVA and administration of anti-hOX40 mAb as either mIgG1 or mIgG2a (Fig. 3A). Both mIgG1 and mIgG2a anti-hOX40 mAb expanded antigen-specific CD8+ OT-I T cells in blood compared to OVA alone and to a similar extent with the exception of SAP 28-2 which was notably weaker as a mIgG2a (Fig. 3B and Supplementary Fig. 4A). Despite reaching similar frequencies at the peak of the primary response, upon re-challenge with SIINFEKL peptide alone, a significantly smaller recall response was seen in mice that had received mIgG2a antibodies (Fig. 3B and Supplementary Fig. 4B). Given the time between antibody administration and rechallenge with SIINFEKL peptide, it is likely this lack of recall reflects responses

initiated during the priming stage as opposed to any effects of mAb persisting from the initial challenge. The frequency of OT-I cells pre-recall in mIgG1 treated mice strongly correlated with the strength of the recall peak (Supplementary Fig. 4C), however due to the lack of a recall response seen in the mIgG2a it is not possible to determine if this is also the case for the mIgG2a isotype. These data suggest that the number of OT-I cells present before re-challenge is a key determining factor for the strength of the recall response and that mIgG1 and mIgG2a mAb deliver signals during the primary response which results in different resting memory populations.

To understand this difference in memory response OT-I T cells in the blood were phenotyped during the primary and memory stages. Analsyis of CD127 and KLRG1 expression during the primary response can identify short-lived effector cells (SLECs - CD127-KLRG1+) and memory precurosor cells (MPECs - CD127+KLRG1-) [26]. The frequency of MPECs was higher in the mIgG1 groups at Day 18 (Fig 3C). Whilst frequencies of SLECs in the blood was similar between isotypes (Supplemental Fig 4D) granzyme B production was higher in SAP 9 and 25-29 mIgG2a treated mice compared to mIgG1 treated mice (Fig. 3C). Additionally these data indicated that there may be a domain related trend in granzyme B production in those mice receiving mIgG2a mAb, with the following hierarchy; CRD4 binding mAb (SAP 25-29) > CRD3 (SAP 9) > CRD2 (SAP 15-3) and CRD1 (SAP 28-2). In infection models, the relative frequencies of each subpopulation (SLECs vs MPECs) in the primary response does not always correlate with the accumulation of CD8+ cells during a recall response [26-28]. We therefore expanded our analysis to CXCR3 and CD43, shown to define three distinct populations of memory cells with a hierarchy of recall response ($CXCR3^{hi}CD43^{lo}$ > $CXCR3^{hi}CD43^{hi}$ > $CXCR3^{lo}CD43^{lo}$)[29, 30]. Mice that had been treated with mIgG1 anti-hOX40 mAb gave rise to a higher frequency of $CXCR3^{hi}CD43^{lo}$ and $CXCR3^{hi}CD43^{hi}$ cells in comparison to mice that had been treated with mIgG2a mAb (Fig. 3D and Supplemental Fig. 4E). This effect was most strongly seen with the SAP 25-29 CRD4-binding antibody (Fig. 3D and Supplemental Fig. 4E). This difference in frequencies between mIgG1 and mIgG2a treated mice, whilst slight during the contraction phase (D18), became more evident during the resting memory period prior to re-challenge (D53). Immediately following re-challenge with SIINFEKL peptide (D74), both mIgG1 and mIgG2a groups displayed expansion of cells with high proliferative capacity but further into the memory

273 response (D78) the higher prevelance of cells with greater proliferative capacity in
274 mIgG1 treated mice was re-established (Fig. 3D). Furthermore, mice treated with
275 mIgG2a anti-hOX40 mAb had a higher frequency of effector-like memory cells
276 (CXCR3^{lo}CD43^{lo}) in the resting memory phase (D53) (Fig 3D). Again this contrast
277 between mIgG1 and mIgG2a treated mice was re-established from D78 following re-
278 challenge. These data indicate, that in our OT-I transfer model, the choice of isotype
279 significantly impacts the development of a robust memory response.
280
281 These results also highlighted a discrepancy in the effect of the mIgG2a anti-hOX40
282 mAb between *in vitro* and *in vivo* experiments. mIgG2a anti-hOX40 mAb caused an
283 inhibition of proliferation *in vitro* (Fig. 2F), whereas T cell expansion was seen *in vivo*
284 (Fig. 3B). Therefore, hOX40KI$^{+/-}$ CD8+ OT-I T cells were purified and transferred into
285 WT C57BL/6 mice to see if both mIgG1 and mIgG2a isotypes were capable of acting
286 directly on CD8+ T cells (Fig 3E). Whilst mIgG1 mAb were able to drive a similar
287 expansion as before, mIgG2a mAb had no effect (Fig. 3F). These results indicate
288 that the mIgG2a mAb act indirectly, through non-CD8+ T cells, to facilitate OT-I
289 expansion in contrast to the ability of mIgG1 to act directly on the OT-I T cells.
290
291 **anti-hOX40 mIgG2a mAb deplete hOX40 expressing cells.**
292 We hypothesised that the indirect effect of mIgG2a mAb might involve deletion of
293 Treg [24]. Depletion of Tregs has previously been shown to allow greater expansion
294 of OT-I and Ova responses [31-33]. Therefore to address this hypothesis, spleens
295 were harvested from hOX40KI$^{+/+}$ mice four days after treatment with either anti-
296 hOX40 mIgG1 or mIgG2a mAb (Fig. 4A). Numeration of different T cell subsets in
297 the spleen showed that the mIgG2a mAb were able to significantly reduce the Treg
298 population and to a lesser extent CD4+ effectors whereas mIgG1 treated mice
299 evoked expansion of T cell populations (CD8+ and OT-I) (Fig. 4B and Supplemental
300 Fig. 5A). The relative deletion of T cell populations in the mIgG2a treated mice
301 correlated with the amount of hOX40 surface expression seen following activation *in*
302 *vitro* (Fig. 1C) as the most significant difference was seen in the Treg population
303 followed by CD4+ effectors. The CD8:Treg ratio was unchanged following treatment
304 with the mIgG1 mAb, however SAP 25-29 mIgG2a produced a significant increase in
305 the CD8:Treg ratio a with a trend towards an increase also seen for SAP 15-3
306 mIgG2a and SAP 9 mIgG2a (Fig. 4B). Interestingly SAP 9 mIgG2a also showed a

307   decrease in inter-sample variability when looking at OT-I cell numbers and a trend

308   towards a reduction in CD8+ T cell numbers, indicating superior deletion capacity

309   compared to other mAb. Therefore, we assessed the ability of SAP 9 to delete

310   human Tregs *in vivo*. Un-activated human Treg do not typically express appreciable

311   OX40 levels (Fig. 1D, [34]), so a NOG:PBMC transfer model was used whereby

312   hPBMCs are first activated through a xenoresponsive graft versus host response

313   before being transferred into a new NSG recipient mouse where deletion could then

314   be assessed. The transferred Treg upregulate hOX40 to levels similar to those

315   observed within tumours and significantly above the levels of CD8+ T cells

316   (Supplemental Fig. 5B). Using this approach, SAP 9 hIgG1 was shown to be capable

317   of deleting human Tregs at least as well as the clinically-approved Treg deleting anti-

318   CTLA-4 mAb Yervoy (Fig. 4D); thereby augmenting the CD8:Treg ratio (Fig. 4E).

319   Furthermore, when using a humanised version of SAP 9 hIgG1, significant depletion

320   of human Tregs was observed (Fig. 4F), alongside a significant improvement in the

321   CD8:Treg ratio (Fig. 4G), unlike CAMPATH-1 which deleted all T cells. These data

322   indicate that with the correct isotype and significant expression of hOX40, anti-

323   hOX40 antibodies are capable of specifically depleting Tregs and improving

324   CD8:Treg ratios *in vivo*.

325

326   Extent of isotype activity correlates with OX40 domain specificity

327   Throughout the data detailed above it became apparent that the strength of

328   deletion/agonism was associated with the domain of hOX40 bound by the various

329   mAb. To assess this further we grouped our results into membrane distal (CRD1+2)

330   and membrane proximal (CRD3+4) binding mAb (Fig. 5A and B). We also assessed

331   groups reflecting those able (CRD4) or unable to bind in the presence of ligand

332   (CRD1+2+3) to see if there was a correlation with ligand competition (Fig. 5C and

333   D). The strength of agonism seen with the mIgG1 mAb was highest for membrane-

334   proximal binding mAb for both expansion of OT-I cells and Tregs (Fig. 5A). In

335   contrast, with the mIgG2a isotype only depletion of Tregs correlated with domain,

336   again being greatest for membrane proximal mAb (Fig. 5B). Likewise, mAb binding

337   outside of the ligand-binding domain (i.e. CRD4) displayed the highest level of

338   mIgG1-mediated agonism (Fig. 5C). Interestingly the ability of mIgG2a mAb to to

339   deplete Tregs did not significantly correlate with binding to CRD4 (Fig 5D).

340

Ability of hOX40 mAb to control tumour growth

To determine the immunotherapeutic potential of the anti-hOX40 mAb *in vivo* in our hOX40$^{+/+}$ KI mice, we evaluated a mAb which bound to each CRD of hOX40 as both a mIgG1 and mIgG2a isotype. hOX40$^{+/+}$ KI mice were inoculated with E.G7-Ova lymphoma cells and subsequently treated with anti-hOX40 mAb once tumours had established. anti-hOX40 mAb, as both mIgG1 and mIgG2a, were able to elicit tumour control, with mice eradicating established tumours in most treatment groups (Fig. 6A). With the exception of SAP 25-29, the mIgG1 mAb caused a higher % survival than the mIgG2a mAb, although there was no obvious domain preference amongst the antibodies in terms of anti-tumour activity. Importantly, mice also appeared to form durable memory responses as upon re-challenge no mice developed a secondary tumour (Supplemental Fig 6A).

To determine the mechanism of tumour control, organs from tumour bearing mice were harvested and assessed for changes in immune inflitrate. Consistent with data from the OT-I model, the general trend seen in mIgG1 treated mice was expansion of T cells subsets whilst mIgG2a mAb caused T cell depletion (Fig. 6B and C and Supplemental Fig. 6B and C). This effect was most prominent in the spleen but also observed within the tdLN (CD4+ and Tregs). Cell numbers recovered from tumours were very small therefore it was difficult to ascertain clear trends within the TILs (Fig. 6B and C). A high CD8:Treg within human tumours is associated with prolonged survival [35] and depletion of Tregs allows for anti-tumour immunity and rejection [36, 37]. Our results show that mice treated with mIgG2a anti-hOX40 tended to increase the CD8:Treg ratio within the spleen, tdLN, and tumour (Fig. 6C). In mIgG1 treated mice whilst the spleen showed a significant reduction in the CD8:Treg ratio, in the tumour an increased trend was observed (Fig. 6B). It is unclear whether this discrepancy between locations occurs as a result of different response kinetics, with priming occurring in lymphoid organs and subsequent recruitment to the tumour or perhaps direct priming within the TME whereby the greater antigen density leads to a more rapid response and hence increase in CD8:Treg. A domain trend could also be seen within some of the T cell subsets; OT-I depletion in the spleen and CD4+/Treg expansion within the tdLN associated with membrane proximal domains (Fig. 6B and C and Supplemental Fig. 6B and C).

375 The T-box transcription factors T-bet and eomesodermin (Eomes) cooperate to

376 promote cytotoxic lymphocyte formation, which correlates with the upregulation of

377 perforin and granzyme B in antigen specific cells [26, 38, 39], as well as sustaining

378 memory phenotypes [40]. Thus expression of T-bet and Eomes was examined in T

379 cells within the spleen, tdLN and tumour of anti-hOX40 treated mice to better

380 understand the mechanisms involved. Both isotypes increased the T-bet+Eomes+

381 double positive cells within the CD8+ populations (Fig. 6D) however in CD4+ T cells,

382 only mIgG1 increased this population and only in splenic populations

383 (Supplementary Fig 7A). A domain trend was also broadly observed in CD8+

384 populations from mIgG1 treated mice, most obviously within the spleen and tumour.

385 CD8+ T cells within the spleen and tumour also saw an increase in granzyme B

386 producing cells in both mIgG1 and mIgG2a treated mice (Supplementary Fig. 7B).

387 As with Tbet+Eomes+ populations, CD4+ GzmB+ cells were not significantly

388 increased with either isotype (Supplementary Fig. 7C), thus suggesting that in this

389 model, we do not see a significant impact on cytotoxic CD4+ T cells. However these

390 results do show that all anti-hOX40 mAb, irrespective of isotype or domain binding

391 region, are able to produce functional effector CD8+ T cells, as well as CD8+ T cells

392 expressing transcription factors important for effector and memory cell formation

393 within a tumour environment.

394

395 To determine if the ability of these mAb to evoke tumour control was consistent

396 across tumour models we utilised the subcutaneous MCA-205 sarcoma. Using SAP

397 25-29 as a paradigm, we again saw both isotypes providing tumour control, albeit

398 more limited in comparison to the E.G7 tumour model and with no isotype preference

399 (Figure 6E).

400

401 **Discussion**

402

403 In the current study, we generated and characterised a panel of mAb targeting

404 hOX40. Their ability to bind throughout the four different hOX40 CRDs and

405 expression as both mIgG1 and mIgG2a isotypes allowed investigation of the effects

406 of both isotype and domain binding on agonistic and therapeutic potential in a newly

407 developed hOX40 KI mouse model.

408 The hOX40 KI mouse model displayed an expression pattern of hOX40 largely

409 reflecting that seen on healthy hPBMCs and samples from ovarian cancer patients

410 with a hierarchy of expression of Treg>CD4+>CD8+. Nevertheless, differences were

411 observed such that higher levels of hOX40 were seen on peripheral CD8+ T cells in

412 the homozygous KI mouse than would be expected on hPBMC. Additionally,

413 constitutive expression of hOX40 was observed on peripheral Tregs in the mice, in

414 contrast to negligible levels on resting hPBMCs. However, this expression pattern

415 reflects that observed on Treg in TILS isolated from cancer patients and so appears

416 a reasonable model for studies in oncology.

417

418 In the hOX40 KI mouse, mOX40L not hOX40L is present which may have led to

419 immune defects due to the absence of OX40L:OX40 interaction. However, no overt

420 differences in immune development or homeostasis were observed in the

421 homozygous hOX40 KI mice. Furthermore mOX40L has been shown to make similar

422 contacts to hOX40 as hOX40L, [21], suggesting that the OX40L:OX40 signals would

423 be maintained in our model. It also indicates that the model is suitable to address

424 any influences of ligand binding on the activity of the mAb panel. Only mAb binding

425 to the most membrane proximal domain (CRD4), were able to bind in the presence

426 of the natural ligand OX40L (CD252), raising the possibility that certain effector

427 functions could be influenced by concurrent ligand binding for the CRD4 binding

428 mAb. Although not studied directly, no overt effects appeared to be driven by the

429 presence or absence of OX40L binding; i.e. all antibodies regardless of ligand

430 blocking were able to elicit function in an OT-I transfer model and in tumour models.

431

432 It is well known that isotype helps dictate mAb effector function due in part to

433 differences in FcγR interactions, and so isotype choice is important for delivering

434 therapeutic efficacy according to the mAb mechanism of action [24, 41, 42]. For

435 TNFR family members; mIgG1 antibodies have been agonistic with engagement of

436 FcγRII and mIgG2a being either inhibitory or with limited agonistic effects yet

437 capable of activatory FcγR-mediated target cell depletion [12, 18, 24, 25]. Our

438 hPBMC proliferation data added to this evidence, with hOX40 mIgG2a mAb resulting

439 in a decrease in the percentage of proliferating T cells whereas mIgG1 counterparts

440 evoked increases in T cell proliferation. However this was not reflected in our

441 hOX40$^{+/-}$ OT-I transfer studies in hOX40$^{+/+}$ KI mice, where both anti-hOX40 mAb

442 isotypes were able to cause strong expansion of OT-I cells. However, when hOX40$^{+/-}$

13

443   OT-I cells were transferred into WT mice the mIgG2a mAb were no longer able to

444   elicit expansion of OT-I cells, unlike the mIgG1 mAb, which retained this activity.

445   Together, these results indicate that the mIgG1 mAb causes direct agonism on the

446   hOX40$^{+/-}$ OT-I cells resulting in their expansion, whereas the mIgG2a mAb require

447   hOX40 expressing non-CD8+ cells to provide expansion. Previous experiments by

448   Ruby et al indicated a requirement for CD4+ T cells in the expansion of OT-I T cells

449   via anti-mouse OX40 mAb [43]. In those experiments in MHC Class II KO mice, a

450   reduction in the resting memory population was observed, with no significant change

451   at the peak of the primary response as we show here with the mIgG2a. There are

452   many differences in experimental set up, including the number of OT-I cells

453   transferred, and site of priming, as well as the isotype used. Experiments performed

454   by Ruby et al used the anti-mOX40 mAb OX86 which is a Rat IgG1 (rIgG1). rIgG1

455   interacts with only FcγRIIb and FcγRIII, giving it a low activatory FcγR: inhibitory

456   FcγR binding (A:I) ratio, in contrast to the mIgG2a which interacts strongly with all

457   activatory FcγR and has a higher A:I ratio [44]. Thus whilst the mIgG2a is likely to

458   mediate its effects through depletion, based upon strong activatory FcγR

459   interactions, the rIgG1 through its relatively greater interaction with FcγRIIb is likely

460   to have the additional capacity of direct agonism. Hence in the MHC Class II KO

461   mice there is the possibility for the OX86 rIgG1 to directly agonise the transferred

462   OT-I T cells via FcγRIIb mediated crosslinking, unlike the ahOX40 mIgG2a when

463   used in WT recipients, potentially explaining the disparity between the two data sets.

464

465   Enumerating the subpopulations of T cells within the spleen of hOX40$^{+/+}$ KI mice

466   revealed that the mIgG2a anti-hOX40 mAb uniquely caused depletion of Tregs with

467   lower levels of depletion seen in the CD4+ effector population. In contrast, the

468   mIgG1 anti-hOX40 mAb caused expansion of all T cell populations, most

469   significantly in the general CD8+ and OT-I populations. Assuming the presence of

470   relevant effector cells and normal distribution of FcγRs in our OT-I model, it is likely

471   that differential FcγR interactions of the different isotypes explains their disparate

472   effects. Furthermore the superior affinity for the activatory FcγR and resultant

473   deletion of suppressive Treg cells seen in mice treated with mIgG2a mAb likely

474   explain the mechanism behind the expansion of OT-I cells in the blood and spleen of

475   hOX40$^{+/+}$ KI but not WT mice.

476

Another disparity seen between the anti-hOX40 mIgG1 and mIgG2a treated mice was the recall response to SIINFEKL peptide in the OT-I model. Considering the similar levels of OT-I cells at the peak of the primary response it was surprising to observe such a difference in the frequency in the memory phase. A positive correlation between the frequency of OT-I cells pre-recall and the frequency of OT-I cells at the peak of the memory response highlighted the possibility that it was simply the result of the amount of cells present at the time of re-challenge, with mIgG1 mAb but not mIgG2a mAb providing signals for survival/persistence. Within the primary response the MPEC population (CD127$^+$KLRG1$^-$) was increased at day 18 in mice treated with mIgG1 mAb compared to those treated with mIgG2a. Those mice also generated a higher percentage of CXCR3^{hi}CD43^{lo} highly proliferative cells in comparison to mice treated with the mIgG2a mAb. These findings, alongside those relating to the frequency of antigen specific cells pre-rechallenge explain the disparity in the recall response comparing mIgG1 versus mIgG2a mAb. The exact mechanisms underpinning this dichotomy are not immediately clear but one possibility is that mIgG2a deplete Tregs alongside, to a lesser extent, CD4+ effectors, to influence priming. Importantly though, this higher proportion of effector cells in mIgG2a treated mice versus highly proliferative cells in mIgG1 treated mice may explain these differences in memory but moreover provide a rationale for how both isotypes are able to cause equivalent efficacy in mouse tumour models, despite exhibiting opposing mechanisms of action.

Analysis of affinity, on- and off-rates failed to reveal a correlation with activity. However, despite the limited numbers of antibodies against each individual domain, our data suggests a correlation between domain binding and strength of both mIgG2a depletion and mIgG1 agonism. anti-hOX40 mAb which bound to CRD4 were more potent agonists as mIgG1 when compared to mAb which bound to CRD1-3, although testing against a wider panel of antibodies would be required to strengthen this finding. This directly contrasts with our previous observations with anti-CD40 mAb [16] where CRD1 binding mAb were more agonistic. However, for both the anti-hCD40 and anti-hOX40 mAb tested, optimal agonistic function correlated with binding outside the natural ligand binding region. Potentially suggesting that the combined effects of both the ligand and the mAb in clustering the receptor are

510 required to elicit optimal agonism. This conjecture is partially supported by recent

511 evidence from Zhang et al. who also demonstrated strong agonistic function with a

512 CRD4 binding anti-mOX40 mAb. Those authors also documented equivalent

513 agonistic activity with a CRD2-binding, ligand blocking anti-mOX40 mAb [17],

514 indicating the fine epitope is also important, as we reported previously for anti-CD40

515 [16].

516

517 The ability of TNFR targeting mAb to cause depletion of intratumoral Tregs has been

518 clearly demonstrated in recent years [8, 12, 45, 46], both increasing their therapeutic

519 possibilities but confusing potential mechanisms of action. To assess, in a more

520 translational setting, the depleting ability of our anti-hOX40 mAb, we performed

521 depletion experiments in NSG mice engrafted with human target cells. In these

522 experiments, hOX40 and other TNFR family members become upregulated on the

523 activated Treg (Supplemental Fig. 5B and [13]), enabling them to serve as targets

524 akin to those seen in tumour samples. When compared with the clinically relevant

525 Treg depletor Yervoy [47], SAP 9 hIgG1 was not only as strong a Treg depleter but

526 also generated a higher CD8:Treg ratio indicating the therapeutic potential of this

527 anti-hOX40 mAb. Importantly, deletion of Treg was specific even though all T cell

528 subsets had expanded and were activated through the NSG passage.

529

530 Having established the agonistic and depletory capacity of our mAb, it was then

531 perhaps slightly surprising for both isotypes to act therapeutically to a similar extent

532 in tumour models but likely reflects their relative propensity to elicit different effector

533 functions in the tumour microenvironment (TME). The latter is known to have

534 profound effects on therapeutic efficacy [48]. A tumour with a high infiltrate of cells

535 expressing activatory FcγR such as NK cells and macrophages, is likely to be more

536 responsive to a mIgG2a mAb and depletion of detrimental target cells than a T cell-

537 agonising mIgG1 mAb. Conversely, if the inhibitory FcγRIIB is more prevalent,

538 mIgG1-mediated agonism may be more prominent. Lymphoid organs outside the

539 tumour may also be relevant. In the E.G7-Ova model, although the magnitude of

540 effect differed, the mIgG1 mAb largely caused T cell expansion and the mIgG2a

541 depletion in spleen and draining lymph nodes. It seems likely therefore that these

542 intrinsic differences underpin the relevant mechanism of action in each case.

543   Accordingly, mIgG2a reagents likely achieve therapeutic effects through depleting

544   Tregs, releasing T cell effector responses whereas mIgG1 expand T cell numbers

545   and hence increasing cytotoxic effectors within the tumour site. Despite the disparity

546   in recall responses in the OT-I model (mIgG1>>mIgG2a), in the E.G7-Ova model

547   both isotypes were able to elicit tumour control and generate memory sufficient to

548   prevent tumour growth upon rechallenge. This suggests that other factors must be

549   operational in the presence of tumour. One possibility is that the threshold for

550   memory is relatively low in the presence of an immunogeneic tumour and that the

551   limited level of memory recall seen following treatment with the mIgG2a is sufficient.

552   Alternatively, it may indicate that the mIgG2a mechanism of action, deleting Tregs,

553   allows the expansion of otherwise cryptic epitopes allowing T cell control as was

554   previously described in the CT26 model [49].

555

556   In recent years, evidence for cytotoxic CD4+ T cells in tumour eradication has been

557   reported [50, 51]. Through the use of both adoptive transfer models and the B16 F10

558   melanoma model, Qui et al showed that OX40 mAb stimulation increased GzmB

559   production in CD4+ T cells and that dual co-stimulation with 4-1BB could expand

560   these cells [51]. In our E.G7 model however, we failed to see consistent increases in

561   either CD4+ GzmB+ T cells or CD4+ Eomes+Tbet+ T cells in response to anti-OX40

562   mAb (Supplementary Fig 7 A-C) regardless of isotype used. This potentially reflects

563   the differences in how GzmB was measured in the two tumour models as in the B16

564   F10 model, isolated cells were stimulated for 24 hours with anti-CD3 mAb prior to

565   GzmB detection. As we measured GzmB directly ex vivo we cannot rule out that

566   upon restimulation we would also have revealed CD4+ cytotoxic potential in

567   response to OX40 stimulation.

568

569   In summary, our findings show that immunomodulatory mAb directed against hOX40

570   can harness multiple mechanisms of action to elicit tumour control. They also show

571   that these mechanisms can be modulated dependent upon the choice of isotype and

572   domain binding region. Targeting the membrane proximal domains appears optimal

573   for both deletion and agonism; with the latter strongly driven by isotypes with low A:I

574   ratios such as mIgG1. Lowering the A:I ratio can be attained in many different ways,

575   for example by increasing the affinity for FcγRIIB, which has been shown to mediate

576 more effective agonism for anti-CD40 mAb [52] and a recent paper showed that this
577 may also be true for OX40 using a clinically relevant antibody [53] *in vitro* but as yet
578 it is unclear whether this will be true *in vivo* for anti-hOX40 mAb. These findings have
579 implications for the design of the next generation of anti-hOX40 mAb for the clinic.
580 Current reagents typically display an unmodified hIgG1 isotype and although safe
581 have not delivered strong anti-tumour effects [6]. These hIgG1 reagents would be
582 expected to deliver the Treg deleting function indicated here but to date this activity
583 has not been shown clearly in patients. Furthermore, their deletion may not be
584 sufficient to elicit tumour regression in most human cancers, unlike the mouse
585 models shown here. Therefore, mAb with appropriate isotypes and further
586 engineering (hIgG2B[16, 54], SELF[52, 55], V11[52, 56]) to elicit potent T cell
587 agonism may be warranted for further investigation for use both as a monotherapy
588 but more importantly in combination.

589
590 **Methods and Materials**
591
592 Human Samples
593 Peripheral blood mononuclear cells (PBMCs) were obtained from healthy adult
594 volunteers from either Southampton National Blood Service, UK or Hallands Hospital
595 Halmstad, Sweden. For NSG reconstitution experiments performed in Southampton,
596 hPBMCs were purchased from STEMCELL Technologies.

597

598 Mice
599 C57BL/6 mice and OT-I transgenic mice were obtained from Charles River
600 Laboratories. NSG mice were purchased from Jackson Laboratories. hOX40 knock-
601 in mice were generated by Ozgene. hOX40/OT-I mice were generated in house. For
602 all experiments young adult mice were sex- and age-matched and randomly
603 assigned to experimental groups. Experiments were not blinded.

604

605 Antibody Production and labelling
606 Anti-hOX40 mAb were produced and labelled using standard techniques as detailed
607 in supplementary methods. Cetuximab was a kind gift from Thomas Valerius and
608 Campath-1 was a kind gift from Geoff Hale.

609

610 Humanisation of SAP 9

611   The variable regions of SAP 9 heavy and light chains were sequenced from the

612   hybridoma by PCR. The sequence was humanised using Macromoltek's proprietary

613   humanisation algorithms. A generic antibody signal peptide sequence was then

614   added to the humanised variable region sequences and the amino acid sequences

615   converted into nucleotide sequences using

616   https://www.bioinformatics.org/sms2/rev_trans.html. Nucleotide sequences were

617   synthesized by GeneArt and subcloned into expression vector pEE6.4 (Lonza) for

618   expression.

619

620   Binding domain determination/ blocking experiments

621   hOX40 constructs were transiently transfected into 293F cells before addition of

622   10μg/ml anti-hOX40 mAb, binding was detected with a PE-labelled secondary anti-

623   mouse Fc antibody (Jackson Laboratories). For blocking experiments unlabelled

624   antibody was added for 30 minutes before addition of FITC-labelled anti-hOX40

625   mAb. Binding was assessed using flow cytometry (see below).

626

627   Surface Plasmon Resonance

628   A Biacore T100 upgraded to a T200 (GE Life Sciences) was used to measure

629   interactions with hOX40. 100nM of hOX40L-His was immobilised onto a CM5 chip

630   (GE Healthcare) coated with an anti-His mAb. 100nM hOX40-hFc was subsequently

631   captured followed by the injection of anti-hOX40 mAb (15μg/ml).

632

633   *In vitro* assays

634   Standard hPBMC proliferation assays were performed as detailed in supplementary

635   methods. For expression assays frozen hPBMCs were thawed and rested overnight,

636   then stimulated with plate bound anti-CD3 (OKT3, 15ng/ml) and soluble anti-CD28

637   (CD28.2, 0.5μg/ml). Cells were harvested, stained with appropriate antibodies and

638   assessed via flow cytometry.

639   Murine expression assays; standard activation was used as detailed in

640   supplementary methods. Cells were stained with appropriate antibodies and

641   analysed by flow cytometry.

642

643   Flow cytometry

644 Standard staining and analysis performed as detailed in supplementary methods.

645

646 **OT-I Adoptive Transfer**

647 1×10^5 hOX40 KI$^{+/-}$ OT-I cells were injected i.v. into hOX40 KI$^{+/+}$ or WT C57BL/6 mice.

648 24 hours later 5mg ovalbumin (Sigma) and 100μg anti-hOX40 or isotype control

649 were given i.p. Deletion was determined by harvesting spleens day 4 post i.p.

650 injection. OT-I kinetics were monitored in the blood through SIINFEKL tetramer

651 staining and mice were rechallenged between 6-10 weeks later with 30nM SIINFEKL

652 i.v. Based on preliminary experiments n=3 was determined as sufficient to see a

653 $p < 0.05$ for OT-I expansion. Mice with SIINFEKL tetramer responses less than 1% of

654 CD8+ lymphocytes at the peak of the response were excluded due to the likelihood

655 that the OT-I transfer had failed since isotype controls peak at an average 5.2% +/-

656 0.58 s.e.m. (mIgG1) and 4.65% +/- 0.65 s.e.m. (mIgG2a) in blood and 3.8% +/- 0.79

657 s.e.m (mIgG1) and 3.1% +/-0.89 on Day 4 in spleens. One mouse was excluded

658 from Figure 4 B groups SAP-28, 15-3 and 9 based on this criteria.

659

660 **Treg cell depletion in reconstituted NOG/SCID mice**

661 PBMC-NOG/SCID mice (primary human xenograft model) were generated by

662 injecting NOG or NSG mice with $1-1.5 \times 10^7$ PBMC isolated using Ficoll-Paque PLUS.

663 Approximately two weeks after reconstitution of NOG or NSG mice with hPBMCs,

664 spleens were harvested. 10×10^6 splenocytes were then injected into the peritoneal

665 cavity of naive SCID or NSG mice 1 hour prior to injection with 10 mg/kg of depleting

666 mAb or isotype control mAb. The peritoneal fluid was collected after 24 hours and

667 human T cell subsets were identified by flow cytometry.

668

669 **Tumour Models**

670 E.G7-Ova and MCA-205 tumour models: 5×10^5 tumour cells were injected

671 subcutaneously into the flank of mice. Based on preliminary experiments n=5 was

672 determined as sufficient to see a $p < 0.05$ for tumour therapy. Groups of 8 (E.G7-Ova)

673 or 6 (MCA-205) mice were set up to ensure that at treatment there would be a

674 minimum of 5 per group with established tumours with comparable size (between

675 5x5 and 8x8mm). Mice were then ranked according to tumour size and assigned to

676 treatments groups so that average tumour size per group was similar prior to

treatment. This ensured mixed treatment groups within cages to reduce influence of housing on treatment effect. Established tumours were treated with 3x100µg anti-hOX40 mAb or isotype i.p. every other day. For phenotyping experiments organs were harvested day 4 post final injection. Tumours were digested using 0.5 Units of liberase TL (Roche) and cells analysed via flow cytometry. For survival experiments tumour size mice culled once they reached a terminal size (E.G7: 20x20mm, MCA-205: 15x15mm). Mice which erradicated tumour after treatment were rechallenged with $5x10^5$ tumour cells s.c. into the flank.

Statistics

All results show mean ± SEM. One way Annova with multiple comparisons (Dunnett's, Tukey's or Sidak's as stated in legend) or Mann Whitney tests were used as stated in legends performed using GraphPad Prism. Survival curves evaluated using a Log-rank (Mantel-Cox) test. Significance shown relative to isotype control unless bar is shown. Where indicated ns = not significant, * P≤ 0.05, ** P≤ 0.01, *** P ≤ 0.001, **** P≤ 0.0001.

Declarations

Ethics Approval

All procedures were conducted in accordance with UK Home Office guidelines and were approved by the University of Southampton's ethical committee or at BioInvent under Dnr 14760/2016. For clinical samples, ethical approval was obtained from the Ethics Committee of Skåne University Hospital, Sweden.

Availability of Data and Material

All datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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865
866

867 **Figure Legends:**

868

869 Figure 1. hOX40KI mice express hOX40 in a hierachial manner. A. Expression of

870 OX40 (mouse (m) and human (h) – blue line) compared with isotype control (shaded

871 histogram) on Treg (top row), CD4+ Effectors (middle row) and CD8+ T cells (bottom

872 row). Representative plots shown. B Heat map summarising OX40 expression as a

873 percentage on resting mouse splenocytes (n=4). C. Expression of mOX40 (left

874 panel) and hOX40 (right panel) on splenocytes from WT or hOX40KI mice activated

875 with αCD3 and αCD28 (n=3). Isotype controls showed as dashed lines. D.

876 Expression of hOX40 on hPBMCs activated with αCD3 and αCD28. Histograms

877 show hOX40 expression on Tregs (blue line, isotype control black line), CD4+ (red

878 line, isotype control black dashed line) and CD8+ (green line, isotype control grey

879 filled histogram) from a representative donor on Day 0 (top panel) and Day 1

880 (bottom panel). Line graph (right panel) shows average expression, isotype controls

881 shown as dashed lines (n=3). E. hOX40 expression on CD4+ effector T cells (white

882 bars), CD8+ T cells (hatched bars) or Tregs (black bars) isolated from healthy

883   donors or blood, ascites and tumour from cancer patients (n =4-16). Mean +/-SEM

884   ****p<0.0001, ** p<0.01, * p<0.05 Tukey's multiple comparison test.

885

886   Figure 2. Characterisation of a panel of anti-hOX40 mAb. A. Schematic of the WT

887   and domain mutant hOX40 constructs generated. CRD3 from mOX40 was used to

888   stabilise the human CRD4 construct. B. Binding of anti-hOX40 mAb to domain

889   constructs detected by a PE labelled secondary Fab_2 fragments. Representative

890   histograms show hOX40 mAb binding (dark grey histogram) in comparison to an

891   isotype control (light grey histogram). C. Representative histograms show anti-

892   hOX40 FITC labelled mAb binding (dark grey histogram) in relation to an isotype

893   control (light grey histogram) after binding of unlabelled anti-hOX40 mAb. The

894   heatmap shows MFI of FITC labelled antibody binding in the presence of unlabelled

895   antibodies with the absence of colour indicating blocking. D. Diagram summarising

896   antibody binding domains in relation to the crystal structure of the OX40:OX40L

897   complex. E. SPR analysis of anti-hOX40 mAb binding to hOX40-hFc in the

898   presence of hOX40L-His fusion protein. F.Proliferation of hCD8+ T cells within

899   PBMC cultures in response to sub-optimal anti-CD3 and anti-hOX40 mAb stimulation

900   (representative of 4 individual donors). Mean +/- SEM ** p<0.01, * p<0.05 Dunnett's

901   multiple comparison test.

902

903   Figure 3. anti-hOX40 mIgG1 act agonistically *in vivo*. A. Schematic of the OT-I model

904   used in B-D. 1x10^5 hOX40KI$^{+/-}$ OT-I cells were transferred into hOX40KI$^{+/+}$

905   recipients. Mice were challenged with 5mg Ova and 100μg of antibody. B. Kinetic

906   analysis of OT-I expansion in response to anti-hOX40 mIgG1 mAb (left panel) or

907   mIgG2a mAb (right panel) (n=4 representative of 2 independent experiments). C

908   Analysis of memory and effector phenotyping of OT-I+ T cells in blood at Day 18.

909   MPECs - CD127+KLRG1- (left panel) and Granzyme B+ OT-I T cells (right panel)

910   (n=4 isotype controls and n=8 all treatment groups, pooled from 2 independent

911   experiments). D. CXCR3/CD43 profiling of OT-I T cells in blood at timepoints

912   indicated in response to SAP 25-29 stimulation (n=4). E. Schematic of the OT-I

913   model used in F. 1x10^5 hOX40KI$^{+/-}$ OT-I were transferred into WT C57BL/6

914   recipients. Mice were challenged with 5mg Ova and 100μg of antibody. F. Kinetic

915   analysis of OT-I expansion in response to anti-hOX40 mIgG1 mAb (left panel) or
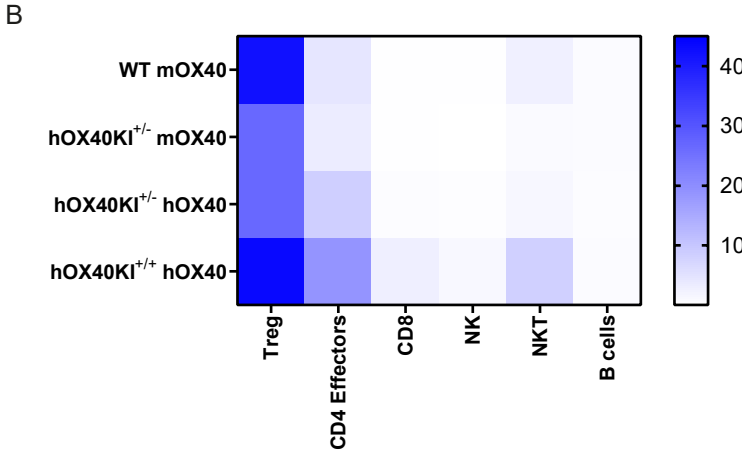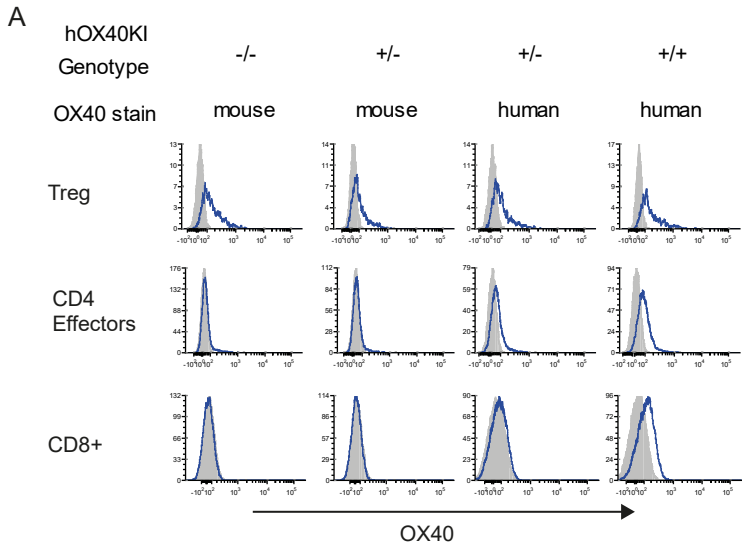
916   mIgG2a mAb (right panel) (n=4 representative of 2 independent experiments). Mean

917   +/- SEM ****p<0.0001, *** p< 0.001, ** p<0.01 * p<0.05 Sidak's multiple comparison

918   test.

919

920   Figure 4. anti-hOX40 mIgG2a mAb elicit Treg cell depletion *in vivo*. A. Schematic of

921   OT-I model used to assess cell depletion. B. Assesment of Tetramer+ve (left panel),

922   Treg cell numbers (middle panel) and CD8:Treg ratio (right panel) in response to

923   anti-hOX40mAb either as a mIgG1 (top row) or mIgG2a (bottom row) (n=4 SAP 28-

924   2, n=5 SAP 15-3 and SAP 9, n=6 all remaining groups, pooled from two independent

925   experiments). C. Schematic of the NSG/PBMC model to assess depletion of

926   hPBMCs. 1.5×10^7 (D, E) or 1×10^7 (F, G) hPBMC were transferred into NOG (D, E)

927   or NSG (F, G) mice. 2 weeks later splenocytes were harvested and transferred into

928   SCID (D,E; n=11 SAP-9, n=12 Yervoy, n=13 isotype control pooled from 2

929   independent experiments) or NSG (F, G; n=7 pooled from 2 independent

930   experiments) recipients which were then treated with depleting antibodies. Treg

931   numbers (D, F) and CD8:Treg ratio (E, G) were determined by flow cytometry. Mean

932   +/- SEM, ****p<0.0001, *** p< 0.001, ** p<0.01, * p<0.05 Sidak's (B) and Tukey's (F

933   and G) multiple comparison test.

934

935   Figure 5. Anti-hOX40 mAb agonism and depleting activity are related to domain

936   binding. Model used as in Fig 4A and B with spleens harvested on Day 4 post

937   treatment. A. Analysis of OT-I cell numbers (left panels) and Treg cell numbers (right

938   panels) in response to anti-hOX40 mIgG1 mAb grouped into proximal binders –

939   CRD3+4 or distal binders – CRD1+2. B. As in A except mIgG2a mAb was used. C.

940   Analysis of OT-I cell numbers (left panels) and Treg cell numbers (right panels) in

941   response to anti-hOX40 mIgG1 mAb grouped into those which can bind in the

942   presence of ligand - CRD4 vs those which cannot - CRD1-3. D. As in C except

943   mIgG2a mAb was used. Mean +/- SEM ****p<0.0001, *** p< 0.001, ** p<0.01, *

944   p<0.05 Tukey's multiple comparison test (A and B; n=6-16) (C and D; n=6-19).

945

946   Figure 6. anti-hOX40 mAb are therapeutic as both mIgG1 and mIgG2a. A. Survival

947   curves for mice challenged with E.G7 lymphoma cells (0.5×10^6) and treated with

948   3x100ug mAb once tumours are between 5x5 and 10x10mm. Data pooled from 2

949   independent experiments (n=5 SAP 28-2, n=10 SAP 15-3 and SAP 9, n=20 isotype
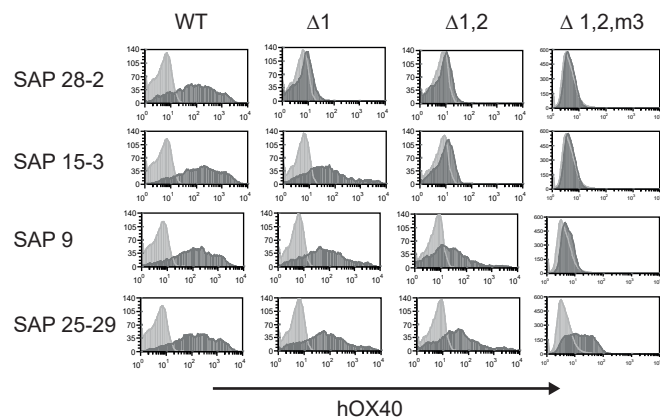
950   controls and SAP 25-29). Assessment of T cell populations in spleen (top panels),

951   Tumour draining lymph node (middle panels), and Tumour (bottom panels) isolated

952   24 hours post second mAb dose either as a mIgG1 (B) or mIgG2a (C). n=5 except

953   for SAP 28-2 mIgG2a n=4 for all organs and SAP15-3 and SAP 25-29 mIgG1 in

954   tumour n=3 due to tumour regression, representative of 2 independent experiments.

955   D. Analysis of Tbet and Eomes expression in CD8+T cells isolated from Spleen (left

956   panels), Tumour draining lymph node (middle panels) and Tumour (right panels)

957   from mice treated with either mIgG1 (top row) or mIgG2a (bottom row). Data pooled

958   from 2 independent experiments (n=8 SAP 28-2, all other groups n=9). E. Survival

959   graphs for mice challenged with MCA-205 cells (0.5×10^6) and treated with 3x100ug

960   mAb once tumours were 5x5mm. Data pooled from 3 independent experiments

961   (n=12 isotype mIgG1, n=15 isotype mIgG2a, n=18 SAP 25-29 mIgG2a and n=21

962   SAP 25-29 mIgG2a). Mean +/- SEM ****$p<0.0001$, *** $p< 0.001$, ** $p<0.01$, * $p<0.05$

963   Log-rank (Mantel-Cox) for survival graphs A and E and Dunnett's multiple

964   comparison test for B-D.

A

hOX40KI Genotype	-/-	+/-	+/-	+/+
OX40 stain	mouse	mouse	human	human

Treg

CD4 Effectors

CD8+

OX40

B

WT mOX40

hOX40KI$^{+/-}$ mOX40

hOX40KI$^{+/-}$ hOX40

hOX40KI$^{+/+}$ hOX40

Treg   CD4 Effectors   CD8   NK   NKT   B cells

C

WT C57/BL6   hOX40 KI$^{+/+}$

Treg
CD4
CD8

D

Day 0

Day 1

OX40

hPBMCs

Treg
CD4
CD8

E

CD4+ non-Treg
Treg
CD8+

Healthy hPBMCs   Patient Blood   Ascites   Tumour

A.

WT   Δ1   Δ1,2   Δ1,2,m3

CRD1
CRD2
CRD3
CRD4

Key
| 40a.a linker region
⌒ CD20 tag
◯ Mouse CRD3

Intracellular domain

B.

WT   Δ1   Δ1,2   Δ 1,2,m3

SAP 28-2
SAP 15-3
SAP 9
SAP 25-29

hOX40

C.

SAP 28-2 FITC   SAP 15-3 FITC   SAP 29-50 FITC   SAP 9 FITC

SAP 28-3

hOX40

FITC labelled mAb

SAP 28-2
SAP 15-3
SAP 9
SAP 29-50

Isotype Control   SAP 28-2   SAP 15-3   SAP 28-3   SAP 9   SAP 29-50   SAP 29-23   SAP 25-29

unlabelled mAb

D.

SAP 28-2
SAP 28-3 SAP 15-3
SAP 9
SAP 25-29 SAP 29-50 SAP 29-23

OX40L

CRD 1
CRD 2
CRD 3
CRD 4

OX40

E.

RU

Blank
SAP 28-2
SAP 28-3
SAP 15-3
SAP 29-50
SAP 25-29
SAP 29-23
SAP 9

seconds

F.

mIgG1   mIgG2a

% of cells 4+ divisions

Isotype   SAP 28-2   SAP 28-3   SAP 15-3   SAP 25-29   SAP 29-50   SAP 29-23   SAP 9

A.

hOX40KI^{+/-} OT-I splenocytes

hOX40
mOX40

Day -1

hOX40KI^{+/+}

Kinetic analysis via blood sampling

Day 0

Day 72

5mg Ova
100µg anti-hOX40

30nmol SIINFEKL

B.

mIgG1

mIgG2a

OT-I+ cells as % of CD8

Day

- Isotype
- SAP 28-2
- SAP 15-3
- SAP 9
- SAP 25-29

C.

CD127+KLRG-1-cells
(% of CD8+OT-I+T cells)

** ** ** * **** ****

Isotype SAP 28-2 SAP 15-3 SAP 9 SAP 25-29

GzmB+cells
(% of CD8+OT-I+T cells)

* * **** **

Isotype SAP 28-2 SAP 15-3 SAP 9 SAP 25-29

☐ mIgG1 ■ mIgG2a

D.

Day 18   Day 53   Day 74   Day 78

mIgG1

mIgG2a

- CXCR3^{hi}CD43^{lo}
- CXCR3^{hi}CD43^{hi}
- CXCR3^{lo}CD43^{lo}
- CXCR3^{lo}CD43^{hi}

E.

purified hOX40KI^{+/-} OT-I

hOX40
mOX40

Day -1

WT C57BL/6

Kinetic analysis via blood sampling

Day 0

Day 49

5mg Ova
100µg anti-hOX40

30nmol SIINFEKL

F.

mIgG1

mIgG2a

OT-I+ cells as % of CD8

Day

- Isotype
- SAP 28-2
- SAP 15-3
- SAP 9
- SAP 25-29

A

hOX40KI⁺ᐟ⁻ OT-I splenocytes

hOX40
mOX40

Day -1

hOX40KI⁺ᐟ⁺

Day 0

5mg Ova
100μg anti-hOX40

Day 4

Spleens harvested

B

OT-I+ T cells

OT-I+ cells (x10⁶)

Isotype | SAP 28-2 | SAP 15-3 | SAP 9 | SAP 25-29

* ** ****

Tregs

CD4+FoxP3+ T cells (x10⁶)

Isotype | SAP 28-2 | SAP 15-3 | SAP 9 | SAP 25-29

**

CD8:Tregs

CD8:Treg

Isotype | SAP 28-2 | SAP 15-3 | SAP 9 | SAP 25-29

mIgG1

OT-I+ cells (x10⁶)

Isotype | SAP 28-2 | SAP 15-3 | SAP 9 | SAP 25-29

** **

CD4+FoxP3+ T cells (x10⁶)

Isotype | SAP 28-2 | SAP 15-3 | SAP 9 | SAP 25-29

* ** *** ***

CD8:Treg

Isotype | SAP 28-2 | SAP 15-3 | SAP 9 | SAP 25-29

*

mIgG2a

C

NOG or NSG

1-1.5x10⁷ PBMCs I.V.

2 weeks

Splenocytes harvested

NSG or SCID

1 hour

24 hours

10x10⁶ Splenocytes I.P.

Facs analysis of hOX40 expression

Therapeutic mAb/ isotype 10mg/kg I.V.

Peritoneal Lavage

D

T_reg cells (% of hCD45⁺ cells)

Isotype | Yervoy | SAP 9

**** ****

E

CD8/Treg ratio

Isotype | Yervoy | SAP 9

** ****

F

T_reg cells (% of hCD45⁺ cells)

Cetuximab | Campath-1H | SAP 9

**** ****

G

CD8/Treg ratio

Cetuximab | Campath-1H | SAP 9

* ****

Target	Clone	Company
mOX40	OX-86	ThermoFisher-eBioscience
mCD8a	53-6.7	ThermoFisher-eBioscience
mCD4	GK1.5	ThermoFisher-eBioscience
mCD3	145-2C11	ThermoFisher-eBioscience
mNK1.1	PK136	ThermoFisher-eBioscience
mFoxp3	FJK-16s	ThermoFisher-eBioscience
mB220	RA3-6B2	ThermoFisher-eBioscience
mCD11c	N418	ThermoFisher-eBioscience
mF4/80	BM8	ThermoFisher-eBioscience
mCD11b	M1/70.15	ThermoFisher-eBioscience
mCD62L	MEL-14	ThermoFisher-eBioscience
mCD44	IM7	ThermoFisher-eBioscience
mKLRG1	2F1	ThermoFisher-eBioscience
mCD127	A7R34	ThermoFisher-eBioscience
mCXCR3	CXCR3-173	ThermoFisher-eBioscience
mCD45.2	104	ThermoFisher-eBioscience
mT-bet	eBio4B10	ThermoFisher-eBioscience
mEOMES	DAN11MAG	ThermoFisher-eBioscience
mCD43 (activation glycoform)	1B11	BioLegend
mLy6C	HK1.4	BioLegend
mLy6G	1A8	BioLegend
h/m Granzyme B	GB11	ThermoFisher-Invitrogen
H-2K^b/SIINFEKL tetramer		In house
hCD4 (healthy samples)	RPA-T4	ThermoFisher-eBioscience
hCD8 (healthy samples)	SK1	ThermoFisher-eBioscience
hOX40 (healthy samples)	ACT35	ThermoFisher-eBioscience
hCD127 (healthy samples)	eBioRDR5	ThermoFisher-eBioscience
hFoxp3 (healthy samples)	236A/E7	ThermoFisher-eBioscience
hCD56 (healthy samples)	CMSSB	ThermoFisher-eBioscience
hCD14 (healthy samples)	61D3	ThermoFisher-eBioscience
hCD3 (healthy samples)	SK7	BioLegend
hCD25 (healthy samples)	M-A251	BioLegend
hCD19 (healthy samples)	HIB19	BioLegend
hCD4 (ovarian cancer and human cells in NSG samples)	RPA-T4	BD Biosciences
hCD25 (ovarian cancer and human cells in NSG samples)	M-A251	BD Biosciences
hCD127 (ovarian cancer and human cells in NSG samples)	HIL-7R-M21	BD Biosciences
hCD8 (ovarian cancer and human cells in NSG samples)	RPA-T8	BD Biosciences
hOX40 (ovarian cancer and human cells in NSG samples)	ACT35	BD Biosciences
isotypes		Corresponding companies
fixable eFluor780 Live/Dead stain		ThermoFisher-eBioscience
aqua live/dead viability stain		ThermoFisher-Invitrogen

1   Supplementary Methods

2

3   PCR genotyping of mice

4   hOX40 KI was detected using the following primers F –

5   AGTGCCCACGCTTCCTGAGGA and R - CTTGAGGATGCCAGAGGAGGC

6   which give a 290bp product. mOX40 was detected using the following primers

7   F -TCTCCACCCACCTTGGTGACT and R – GCCAGCAGGACAGTCAAGGA

8   which give a 174bp product.

9

10   Antibody Production and labelling

11   Anti-hOX40 mAb were created using conventional hybridoma technology [49]**.**

12   Isotype switching was carried out as previously described [50] and mAb

13   purified either using a protein-G or a protein-A column. All preparations were

14   deemed endotoxin low (<1ng/mg protein). Anti-hOX40 mAb were FITC

15   (Sigma) labelled at a ratio of 1:10 FITC:mAb and purified using a desalting

16   column (GE Healthcare).

17

18   Surface Plasmon Resonance

19   A Biacore T100 upgraded to a T200 (GE Life Sciences) was used to measure

20   interactions with hOX40. For affinity determination 1μg/ml of hOX40-hFc was

21   immobilised onto a CM5 chip (GE Healthcare) and a range of concentrations

22   (0-500nM) of anti-hOX40 mAb was injected over the chip. For ligand binding

23   to hOX40; 1μg/ml of hOX40-hFc was immobilised onto a CM5 chip coated

24   with an anti-Fc antibody and hOX40L, mOX40L, h4-1BBL or m4-1BBL

25   (15μg/ml) was injected over the chip.

26

27   *In vitro* assays

28   hPBMC proliferation assays: hPBMCs were isolated using lymphoprep

29   separation. For proliferation assays cells were labelled with 1μM CFSE

30   immediately following isolation, cultured at high density (1.5x10^7 cells/ml) for 2

31   days at 37°C, then plated at 1x10^5 cells/well. Cells were stimulated with

32   soluble anti-CD3 (OKT3, 5ng/ml) and anti-hOX40 mAb (5μg/ml). Cells were

33 stained with appropriate antibodies and CFSE dilution assessed via flow

34 cytometry.

35 Murine expression assays; splenocytes were isolated using a 100μm strainer

36 and red cell lysed using ACK lysis buffer. Cells were plated at 2x10^5 cells/well

37 and stimulated with soluble anti-CD3 (145-2C11, 0.1μg/ml) and anti-CD28

38 (37.51, 1μg/ml). Cells were stained with appropriate antibodies and analysed

39 by flow cytometry.

40 Murine proliferation assays; splenocytes were isolated using a 100μm strainer

41 and red cell lysed using ACK lysis buffer. Cells were labelled with 5μM CFSE,

42 plated at 1x10^5 cells/well and stimulated with platebound anti-CD3 (145-2C11,

43 1μg/ml) and anti-OX40 mAb (10μg/ml). Cells were incubated for 72 hours,

44 stained with appropriate antibodies and analysed for CFSE dilution by flow

45 cytometry.

46

47

48 Flow cytometry

49 Flow cytometry antibodies are listed in Supplementary Table 1. Intracellular

50 staining was performed using Foxp3 staining buffer kit (ThermoFisher-

51 eBioscience) according to the manufacturer's protocol. Cells from ovarian

52 cancer patients were incubated with 10mg/ml KIOVIG (Baxalta) prior to

53 staining with relevant antibodies. All flow cytometry experiments were

54 performed using either a FACSCalibur, FACSCanto, FACSAria or FACSVerse

55 machine (all BD Bioscience). Data analysed using Cellquest Pro, FACSDiva

56 (V6.1.2), FlowJo (all BD Bioscience) or FCS Express (V.3) software (De Novo

57 Software).

58

59
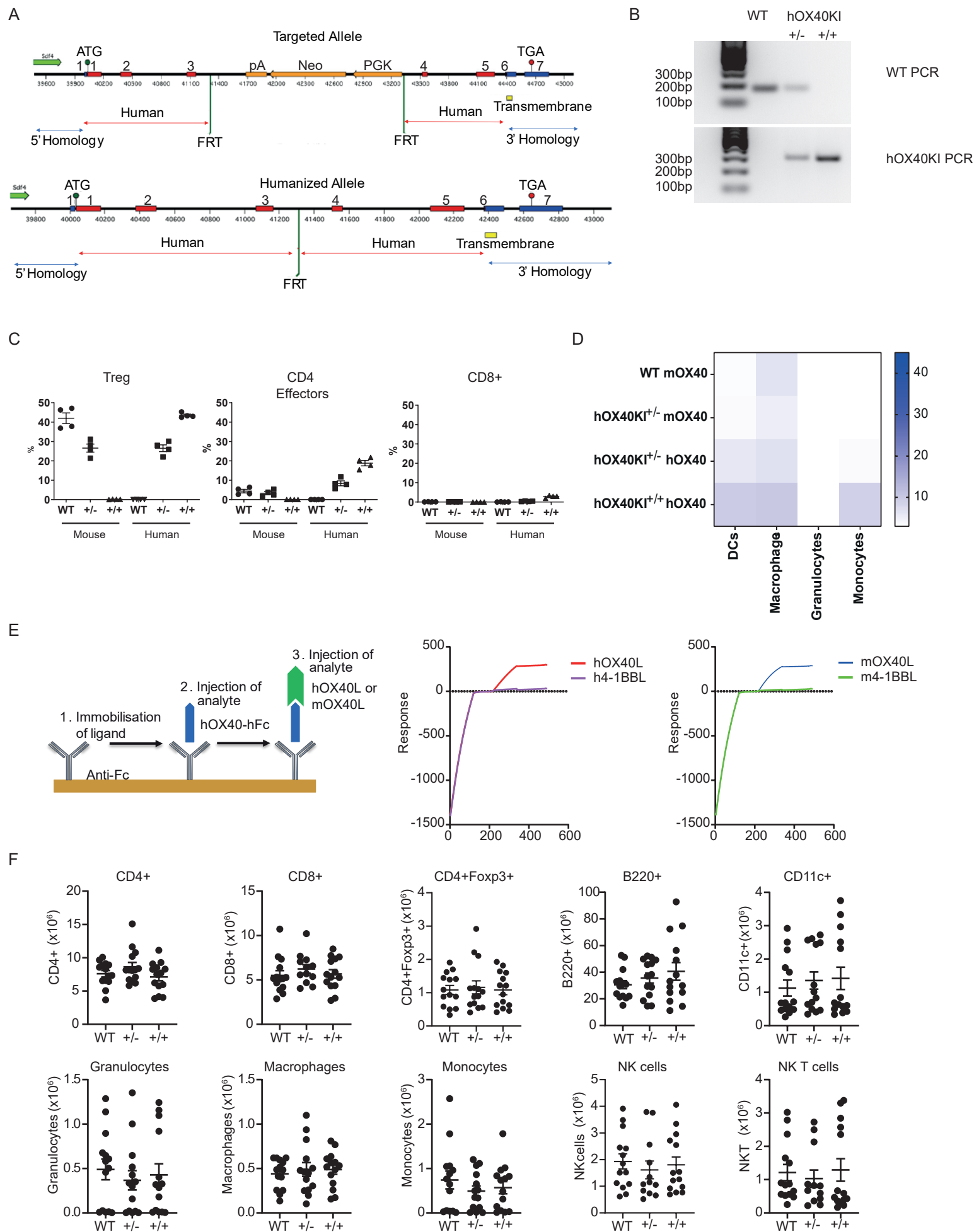
Supplementary Figure 1. hOX40 expression in hOX40KI mice is dose dependent. A. Schematic of hOX40 chimeric receptor construct used to generate the hOX40KI mice. B. PCR showing the genotyping of WT, hOX40KI$^{+/-}$ and hOX40KI$^{+/+}$ mice. C. Expression of mouse and human OX40 on Treg (left panel), CD4 effectors (middle panel) and CD8 T cells (right panel) isolated from WT, hOX40KI$^{+/-}$ and hOX40KI$^{+/+}$ n=4. D. Heat map showing expression levels on myeloid populations. E. SPR analysis of OX40 L binding to hOX40. Left panel shows schematic, h4-1BBL and m4-1BBL were used as negative controls. Middle panel shows hOX40L (red) and h4-1BBL (purple) binding and right panel shows mOX40L (blue) and m4-1BBL (green) F. Lymphocyte and Myeloid populations in WT, hOX-40KI$^{+/-}$ and hOX40KI$^{+/+}$ mice (6-10 weeks age, n=14).

A

Antibody	Parental Isotype	Domain binding	ka (M-1·s-1)	kd (s-1)	KD (M)
SAP 28-2	mIgG1	1	4.2×10^4	2.57×10^{-4}	6.1×10^{-9}
SAP 28-3	mIgG1	2	2.5×10^5	0.46×10^{-4}	1.84×10^{-10}
SAP 15-3	mIgG1	2	14.3×10^4	4.68×10^{-4}	3.3×10^{-9}
SAP 9	mIgG2a	3	50.9×10^4	3.98×10^{-4}	7.82×10^{-10}
SAP 25-29	mIgG1	4	2.56×10^4	1.31×10^{-4}	5.1×10^{-9}
SAP 29-50	mIgG1	4	13.8×10^4	0.62×10^{-4}	4.49×10^{-10}
SAP 29-23	mIgG2a	4	29×10^4	1.81×10^{-4}	6.24×10^{-10}
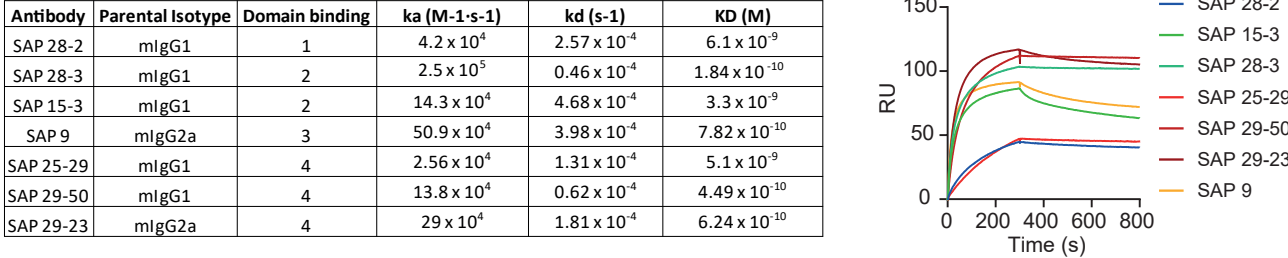
B
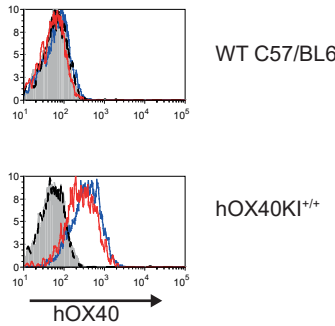
C
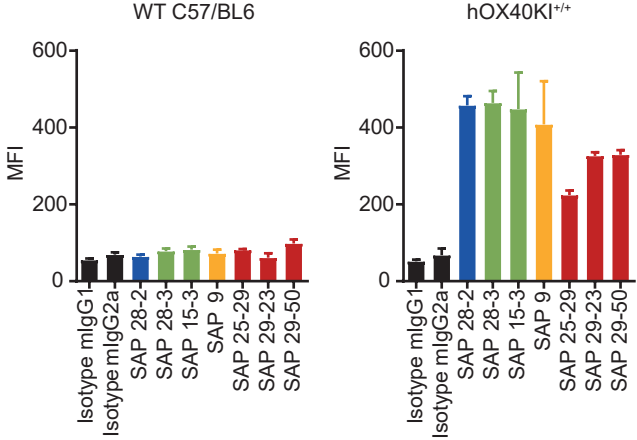
D

E
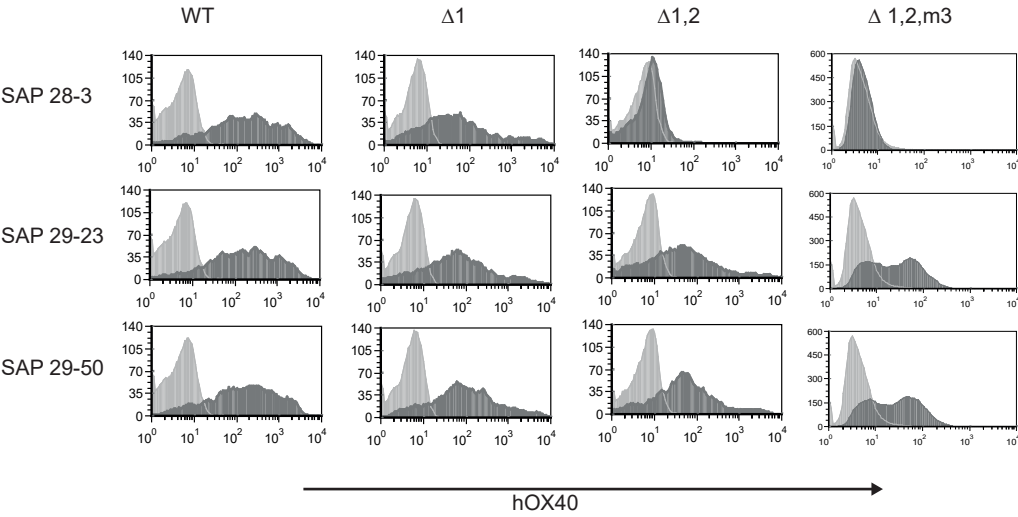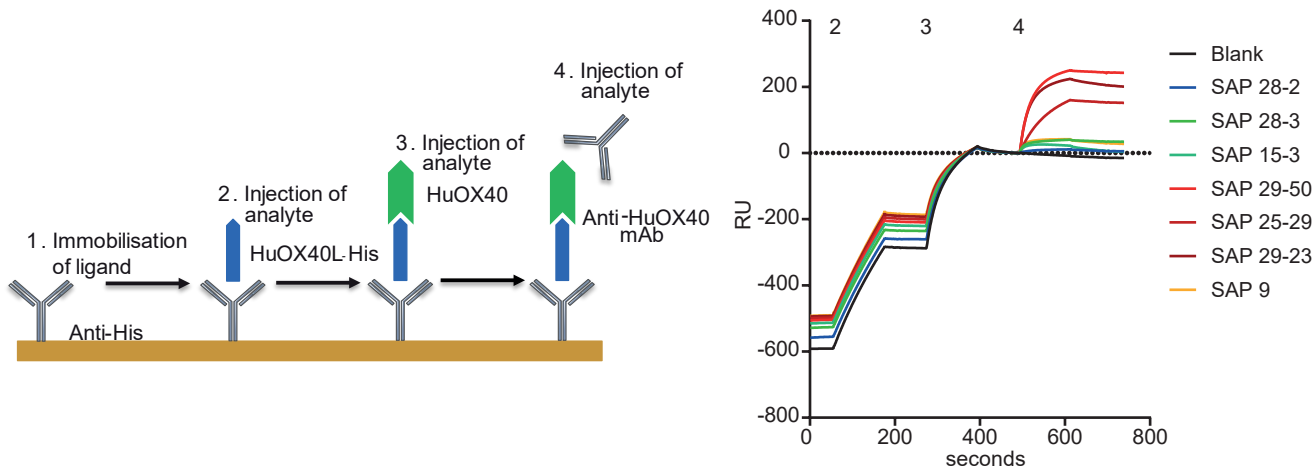
Supplementary Figure 2. Characterisation of anti-hOX40 mAb. A. SPR analysis of anti-hOX40 mAb binding and affinity. B. Binding of anti-hOX40 mAb SAP28-2 mIgG1(blue line) and SAP 29-23 mIgG2a (red line) to activated Tregs (cells activated with αCD3 (0.1μg/ml) and αCD28 (5μg/ml) for 24 hours) from WT (top panel) or hOX40KI$^{+/+}$ mice (bottom panel). Isotype controls shown as filled grey histogram (mIgG1) and black dotted line (mIgG2a). C. Binding of all parental anti-hOX40mAb to activated Tregs (cells activated as in B) isolated from WT (left bar chart) or hOX40KI$^{+/+}$ mice (right bar chart) n=3. D. Binding of FITC-labelled anti-hOX40 mAb to hOX40 domain mutants. E. Schematic and full SPR analysis of anti-hOX40mAb binding in the presence of hOX40L.
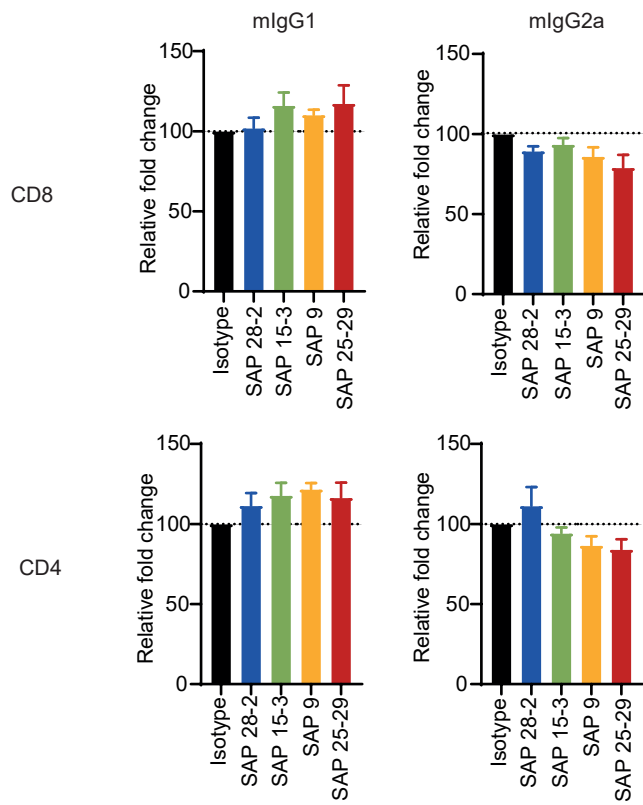
Supplementary Figure 3. anti-hOX40mAb mIgG1 promotes proliferation whilst mIgG2a reduces proliferation. hOX40KI$^{+/+}$ splenocytes were activated with αCD3 (1μg/ml) and αhOX40 mAb (10μg/ml) for 72 hours and then CFSE dilution analysed. Data presented as fold change over isotype contol, n=5.
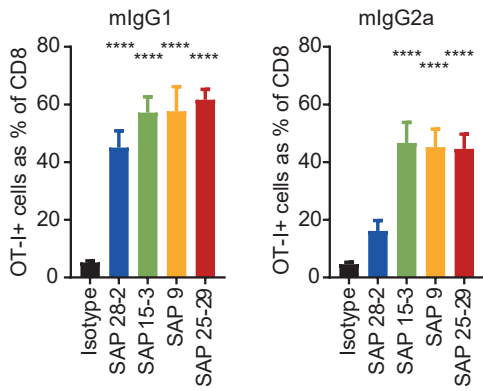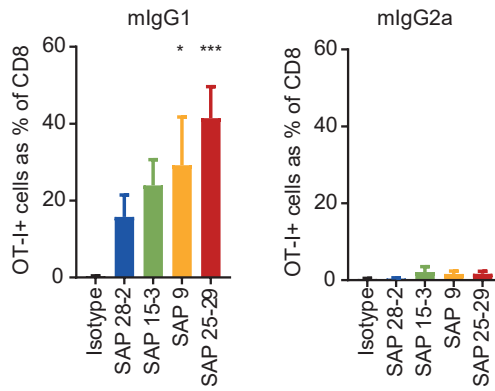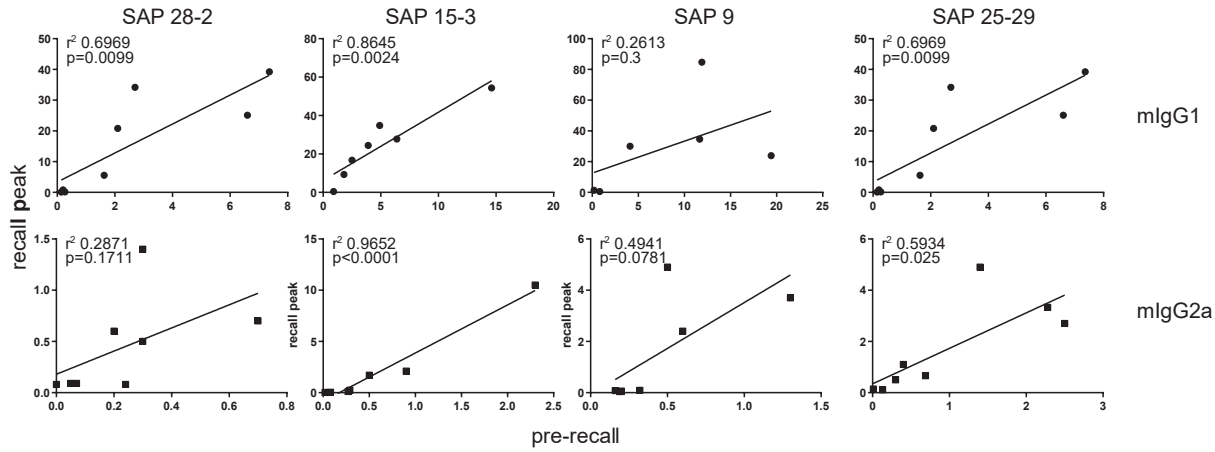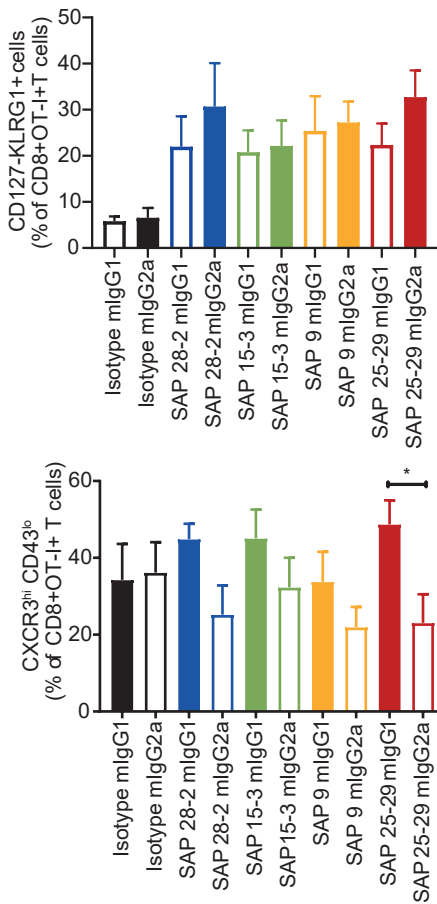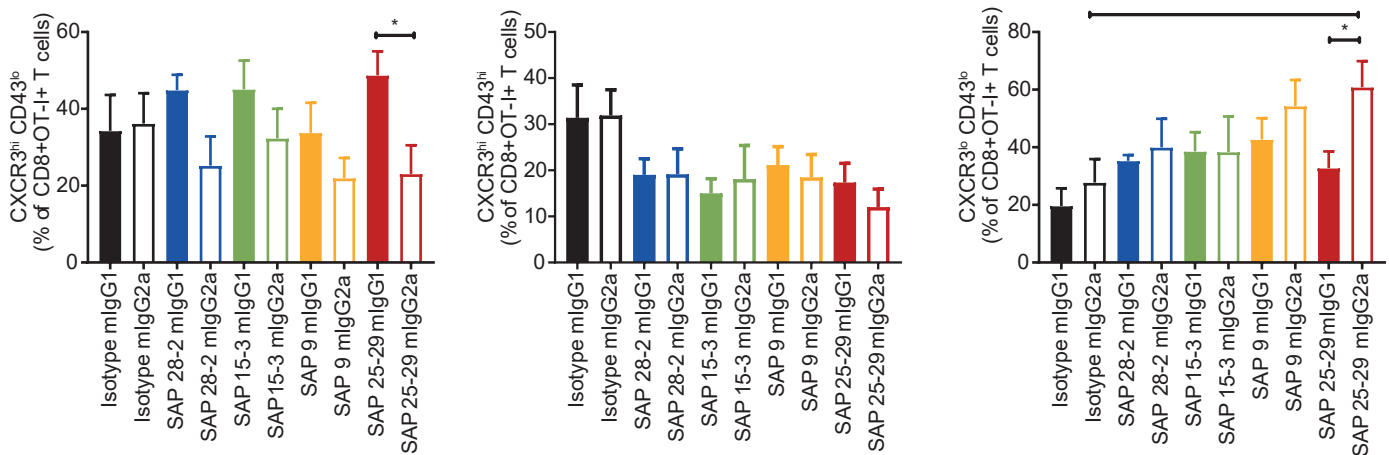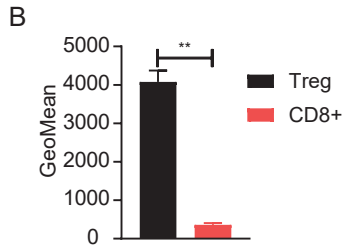
Supplemental Figure 4. anti-hOX40 mIgG1 act agonistically *in vivo*. A. Primary peak response of OT-I expansion in blood to anti-hOX40 mIgG1 (left panel) or mIgG2a (right panel) (n=6-8, pooled from 2 independent experiments). B. Recall peak following SIINFEKL rechallenge of mice previously treated with anti-hOX40 mIgG1 (left panel) or mIgG2a (right panel) (n=6-8, pooled from two independent experiments). C. Correlation graphs between recall response and pre-recall OT-I levels in anti-hOX40 mIgG1 (top row) and mIgG2a (bottom row) treated mice. n=6-8, pooled from two independent experiments. D. Analysis of OT-I SLECs CD127-KLRG1+ in the blood at D18 (n=8). E. CXCR3 and CD43 analysis of OT-I in the blood pre-rechallenge with SIINFEKL peptide (n=7-8 pooled from 2 independent experiments). CXCR3hiCD43lo (left panel), CXCR3hiCD43hi (middle panel) and CXCR3loCD43lo (right panel). *p<0.05 Sidak's test.
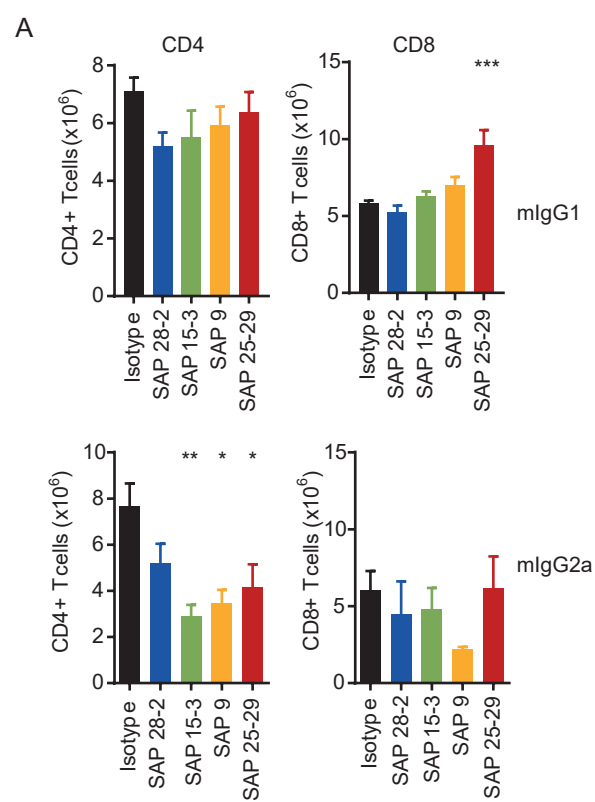
Supplemental Figure 5. Isotype of anti-hOX40 mAb influences effector function. A. CD4 and CD8 T cell numbers assessed on Day 4 in splenocytes isolated from hOX40KI$^{+/+}$ mice receiving 1×10^5 hOX40KI$^{+/-}$OT-I followed by 100μg anti-hOX40 mAb as either a mIgG1 or mIgG2a (n=7-8, pooled from 2 independent experiments). ** p<0.01, * p<0.05 Dunnett's test. B. hOX40 expression on Treg and CD8+ T cells isolated from NOG mice 2 weeks post injection of hPBMCs (n=5, representative of 2 independent experiments). ** p<0.01 Mann Whitney U test.
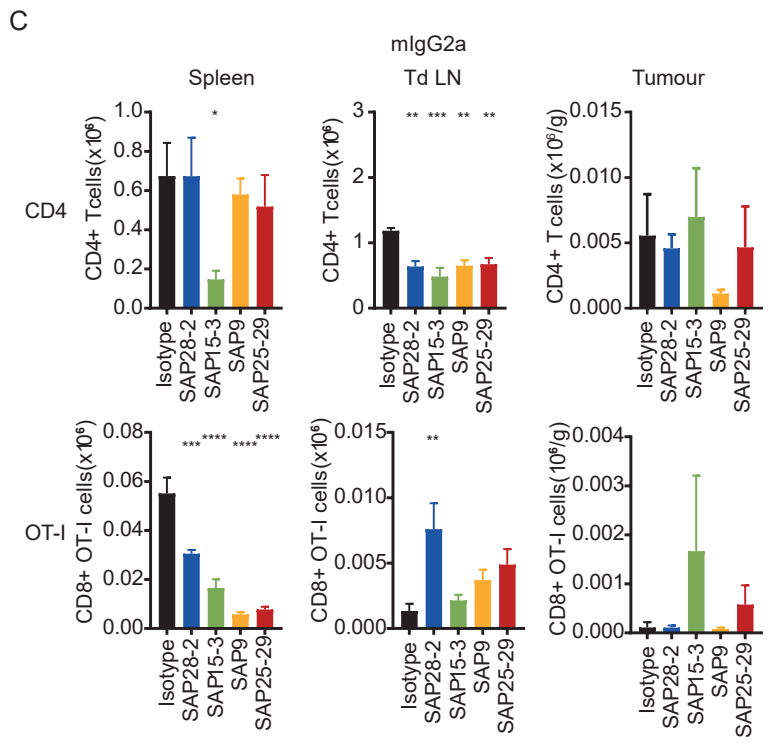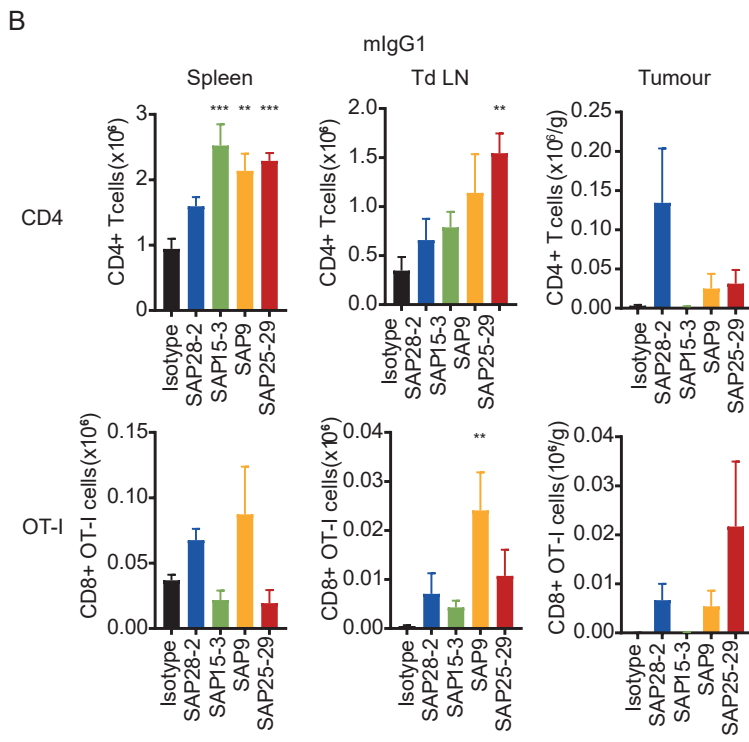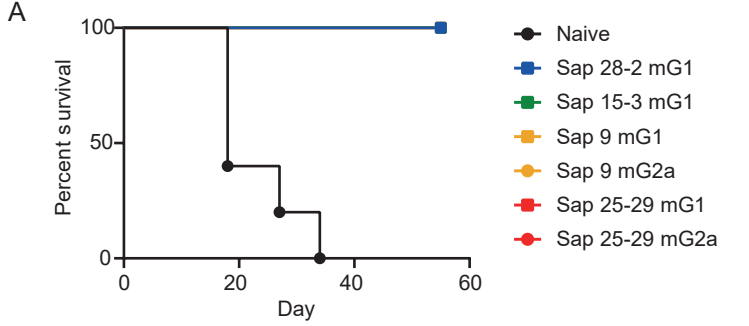
Supplementary Figure 6. hOX40mAb are therapeutic as both mIgG1 and mIgG2a. A. Survival graphs of mice rechallenged with E.G7 lymphoma cells shown alongside naive recipients. n=1-5 representative of 2 independent experiments. B and C. Assessment of CD4+ (top rows) and CD8+OT-I (bottom rows) T cell populations in Spleen (left panels), Tumour draining lymph node (middle panels), and Tumour (right panels) isolated 24 hours post second mAb dose either as a mIgG1 (B) or mIgG2a (C). n=3-5, representative of 2 independent experiments. ****p<0.0001, *** p< 0.001 ** p<0.01 * p<0.05 Log-rank (Mantel-Cox) for survival graphs A and Dunnett's multiple comparison test for B-C.
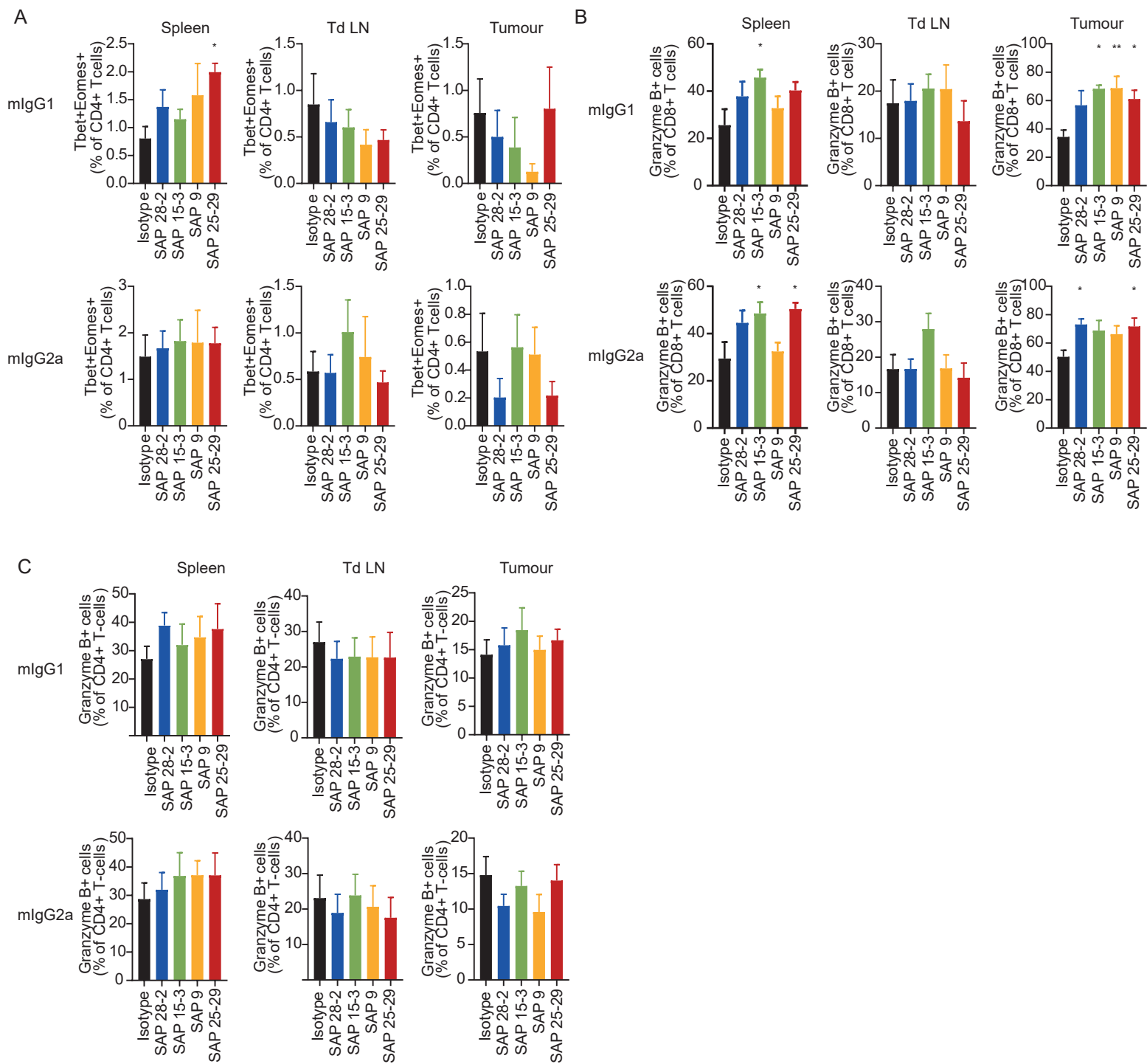
Supplementary Figure 7. Both mIgG1 and mIgG2a promote the induction of Granzyme B+ CD8+ T cells but not CD4+ T cells. A-C. Assessment of CD4+Tbet+Eomes+ (A), CD8+ GranzymeB+ (B) and CD4+GranzymeB+ (C) populations isolated from Spleen (left panels), Tumour draining lymph node (middle panels) and Tumour (right panels) from mice treated with either mIgG1 (top row) or mIgG2a (bottom row). Data pooled from 2 independent experiments (n=8-9). ****p<0.0001, *** p< 0.001 ** p<0.01 * p<0.05 Dunnett's multiple comparison test.