Title:

A novel antibody targeting ICOS increases intratumoural cytotoxic to regulatory T cell ratio and induces tumour regression.

Authors:

Richard C.A. Sainson^{1,*,&}, Anil K. Thotakura^{1, &}, Miha Kosmac¹, Gwenoline Borhis¹, Nahida Parveen¹, Rachael Kimber¹, Joana Carvalho^{1,4}, Simon Henderson¹, Kerstin Pryke¹, Tracey Okell¹, Siobhan O'Leary¹, Stuart Ball¹, Lauriane Gamand¹, Emma Taggart¹, Eleanor Pring¹, Hanif Ali¹, Hannah Craig¹, Vivian W. Y. Wong¹, Qi Liang¹, Robert J. Rowlands¹, Morgane Lecointre¹, Jamie Campbell^{1,2}, Ian Kirby^{1,3}, David Melvin¹, Volker Germaschewski¹, Elisabeth Oelmann¹, Sonia Quarantino¹ and Matthew McCourt¹.

Affiliations:

¹ Kymab Ltd., Babraham Research Campus, Cambridge CB22 3AT, U.K

² Current address: Abcam plc, Discovery Drive, Cambridge Biomedical Campus, Cambridge CB2 0AX

³ Current address: ADC Therapeutics, 42 New Road London, E1 2AX, UK

⁴ Current address: SNIPR BIOME, Lersø Parkallé 44, 2100 Copenhagen Denmark

[&] These authors contributed equally to this work.

*To whom correspondence should be addressed: (richard.sainson@kymab.com).

One Sentence Summary:

KY1044, an anti-ICOS antibody, depletes tumoural ICOS^{high} T_{Reg} cells, activates ICOS^{Low} T_{Eff} cells and has anti-tumour efficacy as monotherapy and in combination with anti-PD-L1.

Abstract:

The immunosuppressive tumour microenvironment constitutes a significant hurdle to the response to immune checkpoint inhibitors. Both soluble factors and specialised immune cells such as regulatory T cells (T_{Reg}) are key components of active intratumoural immunosuppression. Previous studies have shown that Inducible Co-Stimulatory receptor (ICOS) is highly expressed in the tumour microenvironment, especially on T_{Reg} , suggesting that it represents a relevant target for preferential depletion of these cells. Here, we used immune profiling of samples from tumour bearing mice and cancer patients to characterise the expression of ICOS in different tissues and solid tumours. By immunizing an *Icos* knockout transgenic mouse line expressing antibodies with human variable domains, we selected a fully human IgG1 antibody called KY1044 that binds ICOS from different species. Using KY1044, we demonstrated that we can exploit the differential expression of ICOS on T cell subtypes to modify the tumour microenvironment and thereby improve the anti-tumour immune response. We showed that KY1044 induces sustained depletion of $ICOS^{high} T_{Reg}$ cells in mouse tumours and depletion of $ICOS^{high}$ T cells in the blood of non-human primates, but was also associated with secretion of pro-inflammatory cytokines from $ICOS^{low} T_{EFF}$ cells. Altogether, KY1044 improved the intratumoural $T_{EFF}:T_{Reg}$ ratio and increased activation of T_{EFF} cells, resulting in monotherapy efficacy or in synergistic combinatorial efficacy when administered with the immune checkpoint blocker anti-PD-L1. In summary, our data demonstrate that targeting ICOS with KY1044 can favourably alter the intratumoural immune contexture, promoting an anti-tumour response.

Introduction

The last decade has seen a paradigm shift in cancer therapies, with the approval of antibodies targeting immune checkpoints (e.g. CTLA-4, PD-1 and PD-L1). These immune checkpoint inhibitors (ICIs) are associated with strong and durable responses in patients suffering from advanced malignancies, including but not limited to metastatic melanoma, non-small cell lung cancer (NSCLC), head and neck cancer, renal and bladder cancer (1). However, within all these indications, there are still a high proportion of patients who exhibit intrinsic or acquired resistance to ICIs. These patients represent a population with high unmet medical needs who may benefit from novel combinatory approaches with ICIs.

Multiple molecular and cellular mechanisms have been associated with the lack of response to immunotherapies (2). Accumulating evidence has shown that a low incidence of cytotoxic T cells and the presence of immunosuppressive cells represent major barriers to establishing a response to ICIs. One such class of immunosuppressive cells are regulatory T cells (T_{Reg}), which are characterised by the expression of the transcription factor FOXP3 and function by blocking the activation and cytotoxic potential of effector T-cells (T_{Eff}) through multiple mechanisms (3, 4). In fact, numbers of intratumoural T_{Reg} cells negatively correlate with patient survival in several cancer types including melanoma, NSCLC, breast cancer and hepatocellular carcinoma, where a high T_{Reg} content is associated with poor prognosis and minimal response to standard of care therapy (5, 6). Of relevance, T_{Reg} depletion modifies the immune contexture in the tumour microenvironment (TME) and favours an anti-tumour response in several different mouse tumour models (7–9). For these reasons, T_{Reg} cells have been investigated as a potential prognostic cell type and as therapeutic targets for depletion strategies.

The use of therapeutic antibodies for the preferential depletion of intratumoural T_{Reg} cells relies on the identification of a marker that is preferably highly expressed on the surface of these cells in the TME compared with other tissues. One such potential target is the Inducible Co-Stimulatory receptor (ICOS) which belongs to the CD28 and CTLA-4 family (10). Unlike CD28, ICOS is not expressed on naïve T_{Eff} cells but is rapidly induced upon T cell receptor (TCR) engagement (11, 12). Following activation, ICOS has been shown to be constitutively expressed on the surface of T cells where it can engage with its unique ligand (ICOS-LG, CD275) expressed primarily on antigen presenting cells. ICOS/ICOS-LG interaction initiates a co-stimulatory signal that results in production of either pro- or anti-inflammatory cytokines (IFN- γ and TNF- α by T_{Eff}

cells; IL-10 expression by T_{Reg}) (13). Overall the ICOS/ICOS-LG signalling pathway has been shown to regulate immune cell homeostasis and be implicated in the overall immune response (14, 15). Of relevance, the accumulation of ICOS⁺ T_{Reg} cells in the TME has been shown to be associated with disease progression in cancer patients (16, 17). In marked contrast, the upregulation of ICOS on CD4 T_{EFF} cells has been associated with an anti-tumour response and better prognosis in patients after treatment with anti-CTLA-4 (18–21). Interestingly, the relative expression levels of ICOS vary between different T cell subtypes, with T_{Reg} exhibiting the highest levels of ICOS followed by CD4⁺ and then CD8⁺ T_{EFF} cells (19). Similarly, some reports have suggested that ICOS expression is higher in the TME than in other tissues (12, 17, 22). This differential expression between T cell subtypes, and the high expression levels of ICOS in intratumoural T_{Reg} cells (22), suggests that ICOS represents a relevant target for a T_{Reg} depletion strategy. In fact, it has been demonstrated that anti-ICOS antibodies with depleting capability can specifically reduce numbers of ICOS⁺ T_{Reg} cells and induce a strong anti-tumour immune response when combined with a vaccine strategy (7). Given the co-stimulatory and pro-inflammatory effects of ICOS signalling in T_{EFF} cells, an ideal anti-cancer therapeutic strategy would be to promote the depletion of ICOS^{High} T_{Reg} cells while boosting the proinflammatory potential of ICOS^{Low} CD8 T_{EFF} cells in the TME.

In the present study, we used our antibody generating transgenic mouse platform (23, 24) to identify a fully human anti-ICOS IgG1 antibody called KY1044 that binds with similar affinity to mouse and human ICOS. *In vitro*, we established that KY1044 has a dual mechanism of action, namely a co-stimulatory effect on ICOS^{Low} T_{EFF} cells and a depleting effect on ICOS^{High} T_{Reg}. *In vivo*, pharmacodynamic analysis confirmed that KY1044 induces preferential T_{Reg} depletion and increased secretion of pro-inflammatory cytokines by activated T_{EFF} cells in the TME. Overall, KY1044 was shown to modulate the TME immune contexture by increasing the CD8⁺:T_{Reg} cell ratio. Importantly, this change in ratio was associated with anti-tumour efficacy when KY1044 was used as monotherapy and with synergistic activity when KY1044 was combined with anti-PD-L1. Altogether, our results highlight the potential of KY1044 as an anti-tumour therapeutic agent to diminish immune suppression in the TME and re-establish anti-tumour immunity in patients.

Results

ICOS is highly expressed on intratumoural T_{Reg} in several cancer indications

Many tumour types, such as ovarian, gastric and liver cancers, contain a high number of ICOS⁺ T_{Reg} cells (16, 17, 25). A high number of these cells in the TME is associated with poor prognosis, possibly due to the fact that they are highly immunosuppressive (17, 26). In order to assess ICOS expression on intratumoural T cells and to identify additional tumour types containing high ICOS⁺ T_{Reg} cells, we first analysed the content of T cells in tumours using different approaches.

We initially interrogated the TCGA (The Cancer Genome Atlas) mRNA datasets to identify tumours with high levels of *Icos* and *Foxp3* (a marker of T_{Reg}) mRNAs (Fig. 1A). Several cancers expressed high levels of these transcripts including tumours originating from the head and neck, stomach, cervix, thymus, testis, skin and lung. Since this approach does not establish which types of cells from the tumour expressed ICOS and/or FOXP3, we further assessed these markers at the mRNA and protein levels by single-cell transcriptomics and FACS analysis respectively. We obtained paired peripheral blood mononuclear cell (PBMC) and tumour scRNA-seq data for 79,544 cells from 5 NSCLC patients, which we further stratified into 27 immune cell subtypes based on their gene expression signatures (Supplementary Table 1). As expected, certain genes such as *foxp3*, *ccr8*, *il2ra*, *tnfrsf4* were shown to be highly expressed in intra-tumoural T_{Reg} thus validating the approach used (Fig. 1B). *Icos* mRNA expression in these NSCLC samples was higher in T_{Regs} than in other T cell subsets. Finally, *Icos* gene expression levels were also higher in the TME than in the periphery while the pattern of expression in both compartments followed the general trend of: T_{Reg} > CD4_{non-TReg} > CD8 > Other (Fig. 1B) suggesting that one could exploit the differential expression between the T cell subsets.

The T cells from these samples (tumours and PBMC) were further analysed by flow cytometry to confirm the levels of ICOS protein on the cell membrane. As shown in Fig. 1C, ICOS expression was higher on the surface of T_{Reg} (CD4⁺/FOXP3⁺) than on the other T cell subsets analysed (CD8⁺ and CD4⁺/FOXP3⁻). Similar to the mRNA levels, ICOS protein levels were higher in the TME than on circulating T cells. This difference between the two compartments was

strongly significant ($P < 0.001$) for the T_{Reg} subset. No differences in ICOS expression between PBMCs from healthy donors and NSCLC patients were observed on the T cell subsets analysed. As seen with the human samples, T cell immunophenotyping by flow cytometry analysis revealed that ICOS was also more highly expressed in the TME than in the spleen of CT26.WT syngeneic tumour bearing mice. This analysis also confirmed higher ICOS protein expression levels on T_{Reg} than on CD4/CD8 T_{Eff} cells (Supplementary Fig. 1).

Finally, we assessed tumour tissue microarrays containing cores from oesophageal, head and neck, gastric, lung, bladder and cervical cancer biopsies and carried out immunostaining to detect ICOS. We observed a significant up-regulation of ICOS in the TME as compared with matched healthy tissues (Fig. 1D). In order to confirm the ICOS expression on T_{Reg} , tumour cores were co-stained for both ICOS and FOXP3, and the number of cells co-expressing both markers was quantified by digital pathology (Fig. 1E). From this analysis, we further demonstrated the presence of a high number of ICOS⁺ T_{Reg} cells in the TME of the six selected indications, with only bladder cancer demonstrating a lower number of ICOS/FOXP3 double positive cells per mm² (Fig 1F).

Altogether, our data confirmed that ICOS is not homogeneously expressed on the different T cell subsets and is strongly induced in the TME, especially on the surface of T_{Reg} . In addition, we identified indications with high levels of intratumoural ICOS⁺ T_{Reg} cells and these include oesophageal, head and neck, gastric, lung, and cervical cancers.

KY1044: a fully-human anti-ICOS IgG1 antibody with both depleting and agonistic functions.

The preferentially high expression level of ICOS on intratumoural T_{Reg} cells suggests that the targeting of these cells with an effector enabled anti-ICOS antibody could lead to their preferential depletion (via antibody-dependent cellular cytotoxicity; ADCC) and hence remove their inhibitory effects on the anti-tumour efficacy of ICIs. The level of target expression and the amount of antibody bound to a target are key parameters influencing cell killing by ADCC (27). Therefore, it is expected that ICOS^{Low} cells (e.g. CD8⁺ T_{Eff} cells) will be less sensitive to depletion than ICOS^{High} T_{Reg} but yet could be sensitive to co-stimulatory potential via ICOS signalling if the antibody also harbours agonistic properties (via clustering through FcγR receptors) (28, 29). With this in mind, we identified an antibody called KY1044. KY1044 is a human monoclonal IgG1 (effector enabled) antibody that was generated in a transgenic mouse

line in which the endogenous *Icos* gene was knocked-out. Lack of endogenous ICOS expression was aimed to facilitate the generation of a mouse cross reactive antibodies. KY1044 binds ICOS from different species (human, Cynomolgus monkey, rat and mouse) with strong affinity (a KD below 3 nM for the Fab).

In vitro, KY1044 ADCC potential was tested in a series of cell-based assays using established cell lines as well as primary T cells and natural killer (NK) cells. The ability of KY1044 to induce ADCC was first assessed using a luciferase reporter assay. This assay quantifies the activation of FcγRIIIa signalling in response to KY1044 bound onto ICOS-expressing CHO cells. As shown in Fig. 2A, binding of KY1044 to human ICOS-expressing CHO cells significantly induced the luciferase signal with an average EC₅₀ of 0.15 nM (n=3). A similar EC₅₀ was obtained when using CHO cells expressing mouse ICOS (0.53 nM, n=3), rat ICOS (0.48 nM, n=3) or cynomolgus monkey ICOS (0.22 nM, n=3). No luciferase signal was induced with the human IgG1 isotype control. To validate further the ADCC potential of KY1044, we used purified human NK cells as effector cells. When incubated with KY1044 and CEM cells expressing human ICOS (5:1 Effector:Target cell ratio), these NK cells induced potent ADCC-mediated killing with an average EC₅₀ of 5.6 pM (n=8, Fig. 2B). Altogether, the data confirmed that KY1044 has the ability to trigger ADCC (and probably ADCP) of ICOS^{High} expressing cells through engagement with FcγRIIIa.

The agonistic potential of KY1044 was assessed using the MJ [G11] CD4⁺ cell line, which endogenously expresses ICOS and does not require addition of a primary stimulatory signal (e.g. TCR engagement) for cytokine production. KY1044 was either pre-coated on culture plates (mimicking cross-presentation/clustering) or used in solution with or without addition of a secondary cross-linking antibody to cluster KY1044. KY1044 did not increase proliferation of ICOS⁺ T cells. However, we demonstrated agonism by quantifying pro-inflammatory cytokines such as IFN-γ and TNF-α released in the media. While soluble KY1044 did not induce IFN-γ production, plate-bound and cross-linked KY1044 effectively induced IFN-γ secretion in a concentration-dependent manner with an EC₅₀ of 10.5 nM (±2.7 nM; n=2) and 0.50 nM (±0.18 nM, n=3), respectively (Fig. 2C and 2D). The agonism potential of KY1044 was also confirmed using purified primary T cells. Unlike MJ cells, T cells from PBMC were pre-activated with anti-CD3/CD28 beads (to induce ICOS expression; Supplementary Fig. 2) and cultured in the presence of KY1044 coated onto plates or presented in solution. As observed with the MJ

cell line, plate-bound (Fig. 2E) and cross-linked (Fig. 2F) KY1044 induced IFN- γ production in primary cells whilst soluble KY1044 did not. The levels of IFN- γ released in response to KY1044 agonism were variable between donors. Despite this variability, KY1044-dependent IFN- γ secretion was significantly higher than for the isotype treated cells in both plate-bound (2.4 $\pm$ 0.5-fold at 5 μ g/ml mAb, p <0.01) and cross-linked assays (2.5 $\pm$ 0.2-fold at 15 μ g/ml mAb, p <0.01). Similar data were obtained with induction of TNF- α (Fig. 2G). Importantly, a three-step stimulation-resting-costimulation experiment confirmed that TCR engagement was essential for KY1044 agonism. Indeed, the up-regulation of cytokines (IFN- γ and TNF- α) by ICOS⁺ T cells did not occur without concomitant TCR activation (Fig. 2G).

Finally, the direct agonistic potential of KY1044 to costimulate a pool of purified CD3⁺ T cells was confirmed using whole transcriptome RNA sequencing following 6-hours of combined anti-CD3 (to induce ICOS) and KY1044 stimulation. In line with previous reports (14) and our own cytokine measurements, we observed upregulation of key cytokine genes, most notably *Ifng*, *IL10* and *IL4* (Fig. 2H). Gene set enrichment analysis of the transcriptome confirmed that KY1044-dependent ICOS agonism induces genes involved in cytokine-cytokine receptor interactions as well as both leukocyte and lymphocyte activation, which did not seem to be significantly dependent on cell proliferation (Fig 2H). In fact, no significant change in genes associated with proliferation and cell cycle progression was observed. The analysis also showed an enrichment of genes involved in cell locomotion, adhesion and differentiation(30). This finding reflected our observations of MJ [G11] CD4⁺ morphology changes following long term anti-ICOS (C398.4A or KY1044) plate bound stimulation (Supp. Fig 2C and D). Even though ICOS signalling was previously reported to depend on the PI3K/AKT/mTOR axis (31), for KY1044 agonism, we mainly noticed an overrepresentation of genes downstream of the nuclear factor of activated T cells (NFAT) which is in agreement with recent findings (32). NFAT-dependent ICOS agonism was confirmed using another anti-ICOS (C398.4A) in a series of NFAT luciferase reporter cell lines also expressing ICOS with or without CD3 ζ (Supp Fig 2E).

Altogether the data presented here demonstrated that KY1044 has a dual mechanism of action with both depleting and agonistic co-stimulatory properties.

KY1044 (mIgG2a) monotherapy blocks tumour growth in lymphoma/myeloma syngeneic tumour models

KY1044 cross-reactivity to mouse ICOS facilitated the pre-clinical *in vivo* mouse pharmacology work for which the antibody was reformatted as a mouse IgG2a (the effector enabled format in mouse (33)). Outside of the solid tumour TCGA datasets (Fig. 1), we observed that ICOS expression was high in Diffuse Large B-Cell Lymphoma (DLBCL, Supplementary Fig. 3). Based on the known role of ICOS/ICOS-LG signalling in the generation and maintenance of germinal centres (10, 34) and on previously reported anti-tumour efficacy by anti-ICOS antibodies in lymphoma (35), therefore we assessed the anti-tumour efficacy of KY1044 mIgG2a in the ICOS-LG⁺ A20 tumour lymphoblast B cell model (36). Mice were dosed (2qw for 3 weeks at 10 mg/kg) starting from 6 days post tumour cell implantation with saline or with KY1044 mIgG2a. Both treatments were well tolerated, and no bodyweight loss was observed. Importantly, when compared to the control group, KY1044 mIgG2a resulted in an effective anti-tumour efficacy, with more than 90% of animals being free from measurable tumours at the end of the study (day 42, Fig. 3A). In addition, efficacy was confirmed (albeit to a lesser extent) in another B cell-derived syngeneic model, the J558 plasmacytoma model. As observed in the A20 model, KY1044 exhibited a clear anti-tumour efficacy, with around 70% (5 out of 7, Fig. 3B) of the KY1044 mIgG2a-treated mice being tumour free at the end of the study. Monotherapy response in non B-cell-derived tumour syngeneic models was also investigated. These models included models of haematological malignancies (T-cell lymphoma EL4) and models of solid tumours (the colorectal cell lines CT26.WT and MC-38 and the melanoma cell line B16.F10). Overall, the monotherapy response was absent or low in all these models. Mice harbouring CT26.WT or MC-38 tumours showed limited monotherapy anti-tumour response with only some tumour growth delay (supplementary Fig. 4). Altogether, our preclinical *in vivo* mouse tumour studies demonstrated that KY1044 mIgG2a displayed mild anti-tumour response in CT26.WT and MC-38 mouse colon cancer model but was highly effective as a monotherapy in B cell-derived mouse tumour models.

KY1044 (mIgG2a) synergises with anti-PD-L1 in models resistant to both monotherapies

As discussed above, only around 10% of the treated mice harbouring CT26.WT tumours responded to KY1044 mIgG2a monotherapy. Likewise, CT26.WT tumours are known to poorly

respond to anti-PD-L1 monotherapy (37). Since anti-ICOS and the anti-PD-L1 antibodies act on different but complementary immune pathways, we assessed whether the combination could improve the anti-tumour response. These studies (Fig. 4A) demonstrated a complete anti-tumour response in the majority (70 %) of mice treated with the KY1044 mIgG2a (equivalent of 3 mg/kg) and anti-PD-L1 (equivalent of 10 mg/kg) combination. In other in-house experiments, this response rate ranged from 50 to 90% of treated mice. Of relevance, when KY1044 was reformatted as a mouse IgG1 (low depleting potential in mouse) and combined at the same dose with anti-PD-L1, the resulting anti-tumour efficacy was weaker than the one observed with the KY1044 mIgG2/anti-PD-L1 combination, thus arguing for the contribution of ICOS-mediated depletion to the stronger anti-tumour efficacy seen in the CT26.WT model (Supplementary Fig 6A).

Notably, the mice that survived the first CT26.WT challenge in response to the KY1044 mIgG2a/anti-PD-L1 treatment were shown to be resistant to a CT26.WT re-challenge but sensitive to the growth of the EMT-6 tumour cell line (Fig. 4A). This response confirmed that the combination therapy not only induced an anti-tumour response but also generated a tumour antigen specific memory response in these “cured” mice. Finally, we demonstrated that the response to the KY1044 mIgG2a and anti-PD-L1 combination was primarily dependent on CD8⁺ T cells, with the improved survival fully abrogated when CD8⁺ T cells were depleted (Fig. 4B and supplementary Fig. 5). Conversely, addition of an anti-CD4 depleting antibody to the combination only reduced but did not fully prevent the anti-tumour response. Finally, pre-treatment with anti-CD4/CD8 prevented the response to the combination to a similar level to that observed with the anti-CD8 depleting antibody alone (Fig. 4B). Altogether, the data demonstrated that the co-administration of KY1044 mIgG2a and anti-PD-L1 mAbs triggers a strong and durable anti-tumour immune response in the CT26.WT syngeneic tumour model. Interestingly, the combination of anti-PD1 antibody (clone RMP1-14) with KY1044 was poorly effective in this model (supplementary Fig. 6B).

The KY1044 mIgG2a and anti-PD-L1 combination was also tested in additional BALB/c and C57Bl/6 syngeneic tumour models (Fig. 4C). Although the J558 model already responded well to either the anti-ICOS or the anti-PD-L1 monotherapy, a 100% response was achieved when combining both agents in this model. Similarly, the combination produced an effective response in the C57Bl/6 MC-38 model (with 62.5% complete response in comparison with 0%

complete response observed with the respective monotherapies; Supplementary Fig. 7). It should be noted that not all syngeneic tumours responded to the KY1044/anti-PD-L1 combination. For example, no response was observed in the B16F10, 4T1 and EL4 tumour models (Fig. 4C). Collectively, our preclinical tumour efficacy studies clearly demonstrate that the co-targeting of ICOS and PD-L1 results in a strong synergistic effect in selected tumour models, including those in which both monotherapies are poorly effective.

KY1044 depletes ICOS^{High} cells *in vivo* in mice and non-human primates

In order to assess the mechanism of action of KY1044 *in vivo*, we first conducted pharmacodynamic (PD) studies in the CT26.WT syngeneic tumour model. In this experiment, mice harbouring CT26.WT tumours were dosed twice with a dose response of KY1044 mIgG2a (ranging from 0.3 to 10 mg/kg) on day 13 and 15 post tumour cell implantation (Fig. 5A). The T cell content in the tumours and the spleens were then analysed by flow cytometry 24 hours after the second dosing. Although KY1044 monotherapy was not sufficient to induce strong anti-tumour efficacy in the CT26.WT model (Fig. 4A), the PD study shows a strong effect on the tumour immune contexture at all doses tested. KY1044 mIgG2a was strongly associated with intratumoural T_{Reg} cell depletion, even at doses as low as 0.3 mg/kg. Noteworthy, this decrease in T_{Reg} was significant for doses equal or superior to 1 mg/kg. T_{Reg} depletion was associated with an increase in the CD8⁺ T_{EFF} to T_{Reg} cell ratio (known to be associated with improved response to ICIs) in the TME at all tested doses (Fig. 5B). However, the highest dose of 10 mg/kg showed a lower (yet superior over saline control) CD8⁺ T_{EFF} to T_{Reg} cell ratio than the one resulting from the 1 and 3 mg/kg doses. This decrease in the ratio at the highest dose may have been caused by the depletory effect (albeit not significant) of CD8⁺ T cells (Supplementary Fig. 8A) in response to KY1044 mIgG2a. Noteworthy, when a similar analysis was performed on the spleens of treated mice, no depletion of T_{Reg} and no changes in the CD8⁺ T_{EFF} to T_{Reg} cell ratio were observed (Fig. 5C and Supplementary Fig. 8B) probably due to lower ICOS expression the spleen (Supplementary Fig. 1). We repeated similar PD experiment by dosing once CT26.WT tumour bearing mice with KY1044 mIgG2a. The tumours were then isolated for immunophenotyping up to seven days post treatment (Supplementary Fig. 8C). In this experiment, depletion of T_{Regs} was observed after a single dose of 0.3mg/kg, however at this dose, the incidence of T_{Regs} in the TME was back to the level observed in the control group. A significant and long-lasting T_{Regs} depletion was observed at higher dose (3 and 10mg/kg) and

as well as an improvement of the CD8 to T_{Regs} ratio which was significant at intermediate dose. Altogether this data suggests that KY1044 mIgG2a preferentially depletes ICOS^{high} T_{Reg} cells in the TME probably due to the higher ICOS expression described in the tumour (Supplementary Fig.1).

As for human immune cells (Fig.1), we confirmed that ICOS was expressed in cynomolgus monkeys on CD4⁺ memory T cells (T_M defined as CD3⁺/CD4⁺/CD95^{high}/CD28^{-/Dim/+}), CD4⁺ follicular T helper cells (T_{FH} defined as CD3⁺/CD4⁺/CD185⁺) and on T_{Reg} cells (defined as CD3⁺/CD4⁺/CD25⁺FoxP3⁺) (Fig. 5D). As previously shown for other species, circulating NHP CD8⁺ T cells showed the lowest levels of ICOS expression. We assessed the pharmacodynamic effects of KY1044 in cynomolgus monkeys after repeated weekly i.v. administration at dose levels of 0, 10, 30 and 100 mg/kg for 4 weeks (5 doses). Exposure and full occupancy of ICOS on circulating CD4⁺ T cells was maintained at all dose levels throughout the study (Supplementary Fig. 9A-B). Detailed immunophenotyping of different T cell subsets was performed by flow cytometry at various times up to 29 days after the first dose. This analysis indicated that there was a marked decrease in T_M and T_{FH} cells in peripheral blood (Fig. 5E-F) which was apparent soon after dosing. Absolute T_M counts at the end of the study (28 days post dose) were -5%, -60%, -75% and -77% of mean baseline at dose levels of 0, 10, 30 and 100 mg/kg, respectively (Fig. 5E). Similar reductions in T_{FH} cells were also observed and the mean number of absolute T_{FH} counts at 28 days post-dose were +7%, -66%, -81%, and -86% of mean baseline values at dose levels of 0, 10, 30 and 100 mg/kg, respectively (Fig. 5F). The magnitude of the reduction in T_M, and T_{FH} cells was therefore similar at dose levels of 30 and 100 mg/kg KY1044 did not appear to significantly affect the levels of circulating T_{Reg} (Supplementary Fig. 9C). However, this could be due to the fact that T_{Regs} are more difficult to quantify than T_M and T_{FH} cells since they only represent less than 3% of circulating CD4⁺ cells in monkeys (Fig. 5D). KY1044 did not notably affect CD8⁺ T cells in blood since the absolute counts were within the range observed during the pretreatment period (Supplementary Fig. 9D). As in the mouse study, we did not observe a significant decrease in ICOS^{high} cells such as T_M or T_{Reg} or in ICOS^{low} CD8⁺ T cells in the lymph node (or spleen) of treated monkeys at termination the day after the last dose (Supplementary Fig. 9E-G). In summary, these data confirmed that KY1044 elicited a rapid and long-term depletion of ICOS^{high} cells in peripheral blood but not in lymphoid tissues of NHP's.

Increased IFN- γ and TNF- α expression in response to KY1044 *in vivo*

As presented above, KY1044 mIgG2a monotherapy modifies the intratumoural immune contexture with a significant decrease in T_{Reg} in the TME and an increase in the CD8 to T_{Reg} ratio. To demonstrate that KY1044 is also associated with activation of intratumoural T_{Eff} cells *in vivo*, we first assessed the expression of CD69 on the surface of CD8⁺ T cells. CD69 is a rapidly induced lymphoid marker used for the early detection of T cell activation (38, 39). We analysed T cells from CT26.WT tumours for CD69 expression, 24 hours after a second dose of KY1044 mIgG2a and compared this to the expression on T cells in the control group. As shown in Fig. 6A, KY1044 mIgG2a treatment was associated with an increase in CD69 expression, which was significant ($P < 0.05$) at doses of 1 and 3 mg/kg. Similarly, the analysis of CD69/CD44 double positive cells also demonstrated a significant increase ($p < 0.05$) of CD8 activation in response to KY1044 at a dose of 1mg/kg (Fig 6A).

In a separate experiment, we also examined KY1044-dependent production of IFN- γ and TNF- α by CD4 and CD8 T cells. Both are crucial cytokines that play important roles in for the surveillance and inhibition of tumour growth *in vivo* (40). Using an intracellular staining approach on intratumoral CD4⁺ and CD8⁺ T cells collected 7 days post treatment (3 mg/kg of KY1044 mIgG2a), we demonstrated a significant increase in intracellular IFN- γ and/or TNF- α production in response to KY1044 mIgG2a, arguing for an improved anti-tumour phenotype of the intratumoural T cells (Fig. 6B). Although, one cannot differentiate between direct activation (through direct ICOS engagement on T_{Eff} cells) or indirect activation (via T_{Regs} depletion), the present immunophenotyping of intratumoural T cells following KY1044 mIgG2a dosing *in vivo*, revealed that the anti-ICOS was effectively associated with activation of both ICOS^{Low} CD8⁺ and CD4⁺ T_{Eff} cells

Increased efficacy of KY1044 mIgG2a at an intermediate dose in combination with anti-PD-L1

The pharmacodynamic studies presented above confirmed that KY1044 mIgG2a effectively depletes ICOS^{high} cells such as T_{Reg} and result in activation of ICOS^{Low} T_{Eff} cells (albeit not sufficient to trigger anti-tumour efficacy as monotherapy in the CT26 model, Fig. 4A). However,

we noticed that when used at high doses, KY1044 mlgG2a also affected the numbers of intratumoural CD8⁺ T cells (Supplementary Fig 8), resulting in a lower CD8⁺ T_{Eff} to T_{Reg} cell ratio. Since a higher baseline CD8⁺ T_{Eff} to T_{Reg} cell ratio has been shown to correlate positively with a response to immune checkpoint blockers such as atezolizumab (41), we aimed to determine if the anti-tumour efficacy would be superior at an intermediate dose. For this, we repeated the CT26.WT efficacy study using a range of different doses of KY1044 mlgG2a (20, 60 and 200 µg/dose) combined with a fixed dose of anti-PD-L1 (200 µg/dose). As shown in Fig. 7, all the combination treatments were all associated with an improved response when compared with the saline or anti-PD-L1 monotherapy controls. Overall, this experiment demonstrated an improved response at the intermediate dose of 60 µg/dose of KY1044 mlgG2a, with 90% of the mice being tumour free and/or still on study by the study endpoint (day 54 post tumour cell implantation).

394 Discussion

395 The approval of ICIs as anti-cancer agents has been considered as a paradigm shift, providing
 396 a novel approach that adds to existing therapies used to fight cancer. However, responses to
 397 these checkpoint inhibitors are not universal, and many patients exhibit intrinsic or acquired
 398 resistance (2). Although the mechanisms of resistance are diverse, a strongly
 399 immunosuppressive TME can underlie the lack of response to ICIs in certain patients. Notably,
 400 intratumoural T_{Reg} are key cellular players involved in establishing and maintaining such an
 401 immunosuppressive TME. In addition, the high content of T_{Reg} is associated with poor prognosis
 402 for several tumour types and poor response to ICIs. For this reason, reducing T_{Reg} content and
 403 improving the ratio of effector T cells to T_{Reg} cells has emerged as an attractive strategy for
 404 improving the response to ICIs. This could be achieved via different strategies, including
 405 blocking the recruitment, function, expansion or survival of T_{Reg}, and a number of molecules
 406 that could be targeted to modulate T_{Reg} are currently under investigation (42). These include
 407 CTLA-4, CD25, GITR, OX40, CCR8, CD137 and CCR2. However, the differential expression of
 408 these targets (i.e. between different immune cell subsets) is crucial to preferentially target T_{Reg},
 409 since none of these targets are specific to T_{Reg} (8). Moreover, in order not to affect the
 410 homeostasis of non-tumoural tissues and trigger severe organ-specific autoimmune diseases,
 411 an ideal target should be expressed at different levels on T_{Reg} in the TME and in other organs.
 412 In the present study, we performed extensive immunophenotyping of human, NHP and mouse
 413 samples at the mRNA and protein levels and demonstrated that ICOS expression differs
 414 between T cell subsets, with the highest expression observed on the surface of T_{Reg}. In addition,
 415 using human and mouse tumour tissues, we confirmed that ICOS expression is higher in the
 416 TME than in the periphery (blood or spleen) or within the matching non-malignant tissue.
 417 Altogether our data suggests that due to its differential expression on different T cells subsets,
 418 ICOS represents a relevant and attractive target for an intratumoural T_{Reg} depletion strategy.

419

420 Another key aspect when developing novel anti-cancer therapies is to find the 'right' patients
 421 and the 'right' tumours. Our pre-clinical translational work has identified head and neck,
 422 gastric, oesophageal, lung, bladder, skin and cervical cancers as tumours with a high content
 423 of ICOS-positive cells. However, co-staining of ICOS with FOXP3 (used as a marker of T_{Reg})
 424 showed that these tumours differs by their incidence of ICOS⁺ T_{Reg}. For example, lower ICOS⁺

T_{Reg} content was observed in gastric and bladder cancers, while cervical, oesophageal and head and neck cancers were more infiltrated by ICOS⁺ T_{Reg}. Since the levels of ICOS expression on different cells subsets should affect the response to anti-ICOS therapies with a dual mode of action, our work suggests that the specific expression of ICOS on T_{Reg} may support a patient selection strategy. In addition, ICOS induction on T_{Reg} has been reported in chronic infectious disease (43). This is also relevant since several of the above preferred tumours that have high levels of ICOS⁺ T_{Reg} are also often associated with chronic viral or bacterial infections (44). At this stage, it is not yet clear if these T_{Reg} are pathogen-specific and are inhibiting the anti-tumour immune response as a “bystander effect”. However, one could hypothesise that the depletion of these cells should be beneficial for treating cancers associated with chronic viral and/or bacterial infections.

As discussed above, the expression of ICOS in tumours and the preferentially high levels of ICOS expression on intratumoral T_{Reg} highlight the potential of this protein for a depletion strategy through Fcγ receptor engagement on NK cells (ADCC) and macrophages (ADCP). Noteworthy, the fact that ICOS knockout is not embryonically lethal (unlike CTLA-4 knockout) also argues that the depletion of ICOS-positive cells would be better tolerated and not associated with severe autoimmune disease usually described with the targeting of CTLA-4 (11, 45–47). Using the Intelliselect™ platform (23, 24), we identified and characterised a fully human anti-ICOS antibody called KY1044 that is being developed as an effector enabled human IgG1 antibody. We demonstrated *in vitro* that KY1044 has a dual mechanism of action, namely the potential to deplete ICOS^{high} cells via ADCC (through the engagement of FcγRIIIa) but also as a co-stimulatory pro-inflammatory molecule on cells expressing lower levels of ICOS, such as CD8⁺ T_{Eff} cells (through FcγR-dependent clustering). By reformatting KY1044 into a mouse IgG2a antibody, we also showed monotherapy efficacy in lymphoma models and combination efficacy (with anti-PD-L1) in models of solid tumours that are known to be resistant to PD1/PD-L1 blockade. Interestingly, in the CT26.WT tumour model, we showed better efficacy when combining anti-PD-L1 with KY1044 mIgG2a (effector enable) than with a poorly depleting KY1044 mIgG1 antibody. Similarly, in this model, we showed better anti-tumour efficacy when combining KY1044 mIgG2a with anti-PD-L1 than with anti-PD1. Although it is not clear why such a difference was observed in this model, one could postulate that anti-PD1, which blocks both PD-L1 and PD-L2, may be associated with a different phenotype to anti-PD-L1, which

affects both PD1 and CD80 biology (48). Speculatively, there is also the possibility that both antibodies may interfere with each other's functions within the immunological synapse, as both ICOS and PD1 are often expressed on the same cells and can affect each other pathways (49–51).

There is ongoing debate regarding the value of mouse models when predicting the depletion potential of effector function enabled antibodies targeting T_{Reg} in human (8, 9, 22, 52, 53). Experiments in mouse models have shown almost complete depletion of intratumoural CTLA-4 positive T_{Reg} whereas depletion of these cells in patients treated with ipilimumab remains disputed. The differences in FcγR expression and Fc/FcγR interaction on effector cells (NK and macrophages) between rodents and primates may have contributed to these discrepancies(54). Here, we performed pharmacodynamic studies in both mice (using mlgG2a) and NHPs (using hlgG1) to confirm that KY1044 could decrease the frequency of ICOS^{high} cells *in vivo* in both species. With the mouse work, we clearly demonstrated that KY1044 modifies the tumour immune contexture through reduction of T_{Reg} and by improving the CD8⁺ T_{EFF} to T_{Reg} cell ratio. Although this effect on ICOS-expressing cells was not sufficient to induce monotherapy efficacy in the CT26.WT tumour model, the change in immune cell contexture strongly sensitized the tumours to respond to anti-PD-L1 in a synergistic manner. Importantly, we demonstrated a decrease in ICOS^{high} cells in the blood of NHPs treated with KY1044 hlgG1. Depletion was also observed in single dose study in cynomolgus monkeys at dose levels as low as 0.1 mg/kg (data not shown). Together, these data in NHP suggest that KY1044 should be able to deplete ICOS^{high} cells in humans.

While the depletion of T_{Reg} is an attractive therapeutic strategy to re-activate host antitumor immunity, it is important that such an approach does not affect all ICOS⁺ T_{Reg} cells in all tissues as this could be associated with autoimmune side effects(55, 56). Here, we showed that, while T_{reg} were depleted in the tumour and the blood of treated animals, no significant depletion was observed in the spleen of treated mice or in the lymph node or spleen of treated NHPs. Although the reasons behind this selective depletion is not fully understood, this response has been shown for other depleting antibodies and, in our case, could be explained by the lower expression of ICOS on the surface of T cells in lymphoid tissues (supplementary Fig. 1). Altogether, these data suggest that KY1044 has the potential to deplete ICOS^{high} cells without affecting all cells expressing ICOS in the circulation and lymphoid tissues. This was also

indirectly confirmed in the tumour rechallenge experiment, in which a long-term immune memory response was observed after treatment with KY1044 mlgG2a. Since memory T cells are known to express ICOS, this experiment suggests that that KY1044 mlgG2a does not kill all ICOS⁺ T cells. Finally, we also demonstrated that KY1044 has co-stimulatory properties, as shown by the activation and secretion of cytokines such as IFN- γ and TNF- α both *in vitro* and *in vivo*. Although no effect on T cell proliferation were observed, we noted a strong effect on cell morphology in response to KY1044-dependent ICOS stimulation. This co-stimulatory agonistic property was shown to be dependent on clustering of the antibody and on the concomitant engagement of the T cell receptor.

The depletion of ICOS-expressing cells other than T_{Reg} in the blood of cynomolgus monkeys was not associated with any adverse toxic effects and the depletion of up to about 80% of the T_M cells in peripheral blood is unlikely to present a significant risk of loss of immunologic memory to previous pathogenic antigen exposure or vaccinations. In humans, circulating T cells, of which memory cells represent about 40-60% in adults, represent <3% of total T cells in the body (57). T_M cells in peripheral blood only have a lifespan of about 5 months (58) and are replenished from a large pool of antigen-specific memory cells which reside in lymphoid tissues (mucosal sites, skin, spleen, lymph nodes and bone marrow; (57)) and KY1044 did not deplete T_M cells in lymph nodes or spleen of NHP.

Finally, while assessing the effect of KY1044 on the CD8⁺ T_{EFF} to T_{Reg} intratumoural ratio, we noticed that, although the treatment improved the ratio at all doses tested, a bell shape response pattern was observed, with a lower ratio obtained at the lowest and highest concentrations of KY1044 (0.3 and 10 mg/kg, respectively). At the highest dose, this could be explained by the reducing trend (albeit not significant) in the numbers of intratumoural CD8⁺ T cells. Although the cause of this decrease is not clear, there is a possibility that either cells expressing low levels of ICOS can be depleted at high doses of KY1044 or that the activation of T_{EFF} cells in response to KY1044 is associated with increased ICOS expression, which could make these cells more sensitive to depletion. It was also noted that, while all combinations of anti-PD-L1 with different doses of KY1044 mlgG2a were shown to trigger an anti-tumour immune response, the intermediate dose of 60 μ g of KY1044 resulted in the strongest response. Finding the most effective dose in patients will therefore require a thorough monitoring of immune cell contents in the blood and TME.

518 Altogether, our tumour efficacy and pharmacodynamic studies clearly demonstrate that
519 KY1044 is pharmacologically active, modifies the intratumoural immune contexture and
520 induces a strong and long-lasting anti-tumour immune response. These findings, therefore,
521 warrant the further assessment of KY1044 – as a monotherapy or in combination with anti-PD-
522 L1 as a potential treatment for solid tumours.

523

524

525 Material and Methods

526 Cell lines used in the study:

527 MC38 cells were obtained from National Institute of Cancer under a license agreement. J558
 528 (ATCC® TIB-6™), CT26.WT (ATCC® CRL-2638™), A20 [A-20] (ATCC® TIB-208™), EL4 (ATCC® TIB-
 529 39™), B16-F10 (ATCC® CRL-6475™) and MJ [G11] (ATCC® CRL-8294™) cell lines were obtained
 530 from the ATCC. The specific pathogen free status of these cells was confirmed by PCR screening
 531 for mouse/rat comprehensive panel (Charles River). MC38, J558 and B16-F10 cells were
 532 cultured in antibiotic free DMEM (Gibco, 41966-029) + 10% FBS (Gibco, 10270) complete cell
 533 culture media. CT26.WT cells were cultured in antibiotic free RMPI (Gibco, 2187) + 10% FBS
 534 complete cell culture media. A20 cells were cultured in antibiotic free RMPI (Gibco, 2187) +
 535 10% FBS + 0.05mM 2-mercaptoethanol (Gibco, 21985-023) complete cell culture media. MJ
 536 cells were culture in IMDM + 20% FBS (I20 media). The passage number of tumour cells were
 537 kept below 10 generations.

538 Gene expression analysis

539 *TCGA data analysis:* The standardized, normalised and batch corrected RNA sequencing data
 540 collected as part of the TCGA consortium was downloaded from the UCSC Xena platform (59).
 541 Samples classified as non-tumour tissue were excluded from the dataset. Single sample gene
 542 set enrichment analysis (ssGSEA (60)) was performed for a gene set consisting of Icos and
 543 Foxp3 using the R package GSVA (61). Samples were grouped by primary disease and the
 544 ssGSEA scores for each group were compared across the primary disease groups.

545 *Single-cell RNA sequencing:* PBMC and tumour cell suspensions from 5 NSCLC donors were
 546 processed using the BD Rhapsody single cell analysis system. Sequencing libraries were
 547 generated using the Immune Response Human targeted panel and sequenced using a 2 × 75
 548 bp paired-end run on the Illumina HiSeq 4000 System. Reads were processed by applying the
 549 BD Rhapsody processing pipeline to generate cell count matrices. The counts were then
 550 filtered, normalised and visualised using R and Bioconductor packages for single-cell RNA-seq
 551 data (62–65). Cell type specific gene sets were constructed by performing a literature search
 552 and cells were classified into one of 27 cell types (Supplementary Table 1) using the R
 553 package AUCell (60)

554 .

555 Flow Cytometry

556 *Mouse Studies:*

557 Tumours and spleens were harvested from CT26.WT tumour bearing mice at day 16 post
 558 tumour cell implantation. Tumours were dissociated into single cell suspensions using Miltenyi
 559 Tumour Dissociation Kit for mouse tissues (Catalogue no. 130-096-730) as per the
 560 manufacturer's instructions. Spleens were placed into C-tubes (Miltenyi Biotec, Catalogue no.
 561 130-096-334) dissociated into single cell suspensions using gentle MACS™ dissociator. Tumour
 562 samples were filtered through 70 µm nylon filters (Falcon, Catalogue no. 352350). Spleens
 563 samples were filtered through 40 µm nylon filters. Red blood cells in the spleens were lysed
 564 using RBC lysis buffer (Sigma, Catalogue no. R7757). For flow cytometry profiling, 2×10^6
 565 tumour samples or 1×10^6 spleen samples were plated in 96 well plate (Sigma, Catalogue no.
 566 CLS3957). Cell suspensions were pre-incubated with Live/Dead™ fixable Yellow Dead Cell Stain
 567 Kit (Invitrogen), according to the manufacturer's instructions. Prior to antibody labelling cell
 568 were incubated with Fc receptor blocking solution (Anti-CD16/CD32) at 4°C for 10 minutes.
 569 Cell surface staining was performed at 4°C for 30 minutes using fluorochrome-conjugated anti-
 570 mouse antibodies. Intracellular and intranuclear staining was performed using Foxp3 staining
 571 buffers (ThermoFisher Scientific, catalogue: 00-5523-00) according to manufacturer's
 572 instructions. All flow cytometry antibodies or isotype controls were purchased from
 573 ThermoFisher Scientific. Antibodies used include: anti-CD45 (30-F11), anti-CD3 (17A2), anti-
 574 CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD25 (PC61.5), anti-ICOS (7E.17G9), anti-Foxp3 (FJK-16s),
 575 anti-CD69 (H1.2F3).

576 For the tumour infiltrating T cell cytokine staining, single cell suspensions from CT26.WT
 577 tumours were plated at 1×10^6 cells per well in RPMI + 10% FBS cell culture media with $1 \times$
 578 Brefeldin A solution (eBioscience, catalogue no: 00-4506-51) for four hours in a cell culture
 579 incubator at 37°C 5% CO₂. Cells were surface stained as above and subsequently
 580 fixed/permeabilised for intracellular staining with anti-IFN-γ (XMG1.2) and anti-TNF-α (MP6-
 581 XT22). All flow cytometry data was acquired using Attune FxT flow cytometer and data was
 582 analysed using FlowJo™ software V10.

583 *Monkey study*

Blood samples (0.1 mL) were collected into tubes containing sodium EDTA or lithium heparin, mixed and red blood cells (RBCs) were lysed with lysis buffer containing ammonium chloride. The remaining leukocytes were washed with FACS buffer (PBS with 2% foetal bovine serum), stained with antibody cocktail by incubation in the dark for 45 minutes at room temperature and washed. Tissue samples (lymph node), taken from animals at termination were mechanically disrupted (Medimachine, Becton Dickinson GmbH, Heidelberg, Germany (BD)), single cell filtered and stained as for blood. Intracellular staining (for FoxP3) was carried out by permeabilization with 10x FACS lysing solution (1:5 with Aqua dest. +0.1% Tween-20) prior to incubation in the dark at room temperature for 45 minutes and wash steps. Antibody cocktails (including appropriate isotype antibodies) were prepared on the day of use and stored in the dark.

All flow cytometry antibodies or isotype controls were purchased from BD, eBioscience (via Fisher Scientific GmbH, Schwerte, Germany) or BioLegend (Koblenz, Germany). Antibodies used included: anti-CD3 (SP34), anti-CD20 (L27 or 2H7), anti-CD14 (M5E2), anti-CD4 (M-T477), anti-CD8 (SK1), anti-CD28 (CD28.2), anti-CD25 (M-A251), anti-Foxp3 (PCH101), anti-CD95 (DX2) and anti-CD185/CXCR5 (MU5UBEE). After staining, cells were washed and fixed in 1 × BD Stabilizing Fixative. Lymphocytes were gated by forward scatter (FSC), sideward scatter (SSC) and CD45. Multicolour flow cytometric analysis was performed using the following leukocyte phenotypic characteristics: CD4⁺ T helper cells: CD3⁺ /CD4⁺ /CD8⁻ /CD14⁻ /CD20⁻; CD8⁺ cytotoxic T cells: CD3⁺/CD4⁻/CD8⁺/CD14⁻/CD20⁻; Total memory CD4 T cells: CD28⁺/DIM⁻/CD95^{high}; Follicular T helper cells: (CD4⁺/CD185⁺) and Regulatory CD4 T cells: CD25⁺/ FoxP3⁺. Acquisition of flow data was performed on a FACSVerse™ flow cytometer (BD) and relative percentages of each of these subpopulations were determined using FlowJo™ software. Fifty thousand events were counted for all analyses. The absolute numbers of each of the subpopulations were determined for blood samples by calculations based on haemoanalyser analysis of whole blood.

Human studies

NSCLC tissue and whole blood from the same patients was obtained from consented subjects, with ethical approval for analysis of protein, RNA and DNA content. The NSCLC tumour samples were dissociated into single cell suspensions using enzymatic digestion, whilst whole blood was processed into PBMCs using density centrifugation, all specimens were cryopreserved in Gibco

Recovery™ cell culture freezing medium before used. Alongside NSCLC specimens, whole blood samples from 5 healthy individuals were collected and processed into PBMCs by density centrifugation. Flow cytometry was performed after cells were thawed and incubated in RPMI-1640 + 10% FBS supplemented with 100 units of RNase free DNase at 37°C 5% CO₂ for 30 minutes. Cell suspensions were pre-incubated with Live/Dead™ fixable Yellow Dead Cell Stain Kit (Invitrogen) and Fc receptor blocking solution (Biolegend), before staining with fluorochrome-conjugated anti-human antibodies specific to anti-CD3 (UCHT1)/anti-CD45 (2D1), anti-CD4 (2A3), anti-CD8 (RPA-T8), anti-CD25(MA251), anti-CD45RA (H100), anti-ICOS (C398.4A). Cells were incubated for 1 hour at 4°C, washed and fixed overnight at 4°C with eBioscience™ intracellular fixation and permeabilization buffer. Anti-FoxP3 (236A-E7) staining was then performed in permeabilization buffer for 1 hour at 4°C before washing and resuspending cells in DPBS (Gibco). All flow cytometry data was acquired using Attune FxT flow cytometer and data was analysed using FlowJo™ software V10.

Immunofluorescence and digital pathology:

An IHC protocol using a semi-automated method on the Ventana Discovery Ultra platform (Roche) was developed for co-staining of ICOS and FoxP3. Method was optimised in FFPE tonsil tissue, using clone D1K2T for the detection of ICOS (Cell Signaling), while clone 236A/E7 (Abcam) was chosen for FoxP3 detection. Methods were applied for the staining of tumour microarrays (TMA) from cervical (CR2088), oesophageal (ES2082), lung (LUC2281), head and neck (HN802a), gastric (STC2281) and bladder (BL2081a) cancer patients. The TMAs (tissue microarrays) were sourced from US Biomax, Inc. All stained slides were scanned at a magnification of 20x equivalent magnification, generating images which were quantified using the Indica Labs Halo platform to determine the number of ICOS/FoxP3 single and double positive cells per mm².

In vitro ADCC assays:

The ADCC activity of KY1044 human IgG1 (produced at Kymab) was first tested in vitro using an ADCC reporter bioassay (Promega) according to the manufacturer's instructions. In brief, CHO cells expressing either human, mouse, rat or cynomolgus ICOS as target cells were co-incubated with ADCC reporter cells expressing human FcγRIIIa (V158; Promega) at a 5:1 ratio. Serial dilutions of KY1044 or isotype control were added to the culture plates, incubated at

37°C overnight and the luciferase activity was measured using the Bio-Glo Luciferase Assay System (Promega) on the EnVision Multilabel Plate Reader (Perkin Elmer). Graph data were normalised to background and plotted versus Log10 antibody concentration.

For the primary cells ADCC assay, human NK cells were purified from PBMC isolated from whole blood by density gradient centrifugation. NK cells were subsequently purified from the by immunomagnetic negative selection using the NK Cell Isolation Kit (Stemcell) as per manufacturer instructions. ICOS-transfected CCRF-CEM (ICOS CEM, target cells) were preloaded with the fluorescence enhancing ligand (BATDA) for 30 minutes in the dark at 37 °C. At various occasions the cells were loaded and/or washed in the presence of an inhibitor of organic anion transporters (1mM Probenecid) to avoid spontaneous dye release from cells. KY1044 was diluted (1:4 dilutions, 10 points, starting from 33.3 nM) in assay buffer. The target ICOS CEM cells (50µl/well), effector cell (50µl/well) and reagent dilutions (50µl/well) were co-cultured with 50µl of the diluted antibody at 37 °C, 5% CO2 for 2-4hrs. The effector NK cells:target cell ratio was fixed at a 5 : 1 ratio. A digitonin-based lysis buffer (Perkin Elmer) was prepared in parallel and used to determine complete target cell lysis (100%).

In vitro agonism assays:

For the MJ cells assays, KY1044 human IgG1 was presented to the MJ cells in 3 different formats: plate-bound, soluble or soluble plus F(ab')₂ Fragments (Fc linker, 109-006-170, Jackson Immuno Research). For the plate bound assay, KY1044 and the hIgG1 isotype control were diluted 1:2 in PBS to give final antibody concentrations ranging from 10 µg/mL to 40ng/mL (10 points). 100 µL of diluted antibodies were coated in triplicate into a 96-well, high-binding, flat-bottom plate (Corning EIA/RIA plate) overnight at 4 °C and then washed. MJ cells (15,000 cells/well) were added to Ab-containing wells. For the soluble/cross-linked experiment: KY1044 and the isotype control were serially diluted 1:2 in I20 media (soluble Ab) or in I20 media containing 30 µg/mL of F(ab')₂ Fragments (cross-linked Ab) to give an 2X Ab stock concentrations ranging from 20 µg/mL to 80 ng/mL (10 points). 50 µL of diluted Abs were added to 96-well with 50 µl of MJ cells (3x10⁵/mL). For both assays the cells were cultured for 72 hrs at 37 °C and 5% CO2 and Cell free supernatants were then collected and used to perform IFN-γ ELISA using the Human IFN-γ DuoSet assay (R&D system, DY285).

For the primary T cell assays, leukocyte cones were obtained (HTA IRAS project number 100345). PBMCs were isolated from whole blood by density gradient centrifugation. T lymphocytes were then purified using the Stemcell EasySep Isolation kit (cat#17951) following manufacturer's instructions. In order to induce ICOS expression, isolated T-cells were cultured at 2×10^6 /mL in R10 media (RPMI 10% heat inactivated FBS) in the presence of 20 μ L/ml of Dynabeads Human T-Activator CD3/CD28 (from Life Technologies). These activated primary T cells were tested as for the MJ cells assays in 3 different formats: plate-bound (5 μ g/mL), soluble (15 μ g/mL) or soluble plus F(ab')₂ Fragments cross-linker. T-cell suspension were added to Ab-containing plates to give a final cell concentration of 1×10^6 cells/ml and cultured for 72 hrs at 37°C and 5% CO₂ until IFN- γ ELISA quantification. For the 3-step culture (stimulation-rest-costimulation assay), the T-cells were pre-stimulated by Dynabeads for 3-days to induce ICOS before being rested for 3-days to reduce their activation levels. These stimulated/rested T-cells were then cultured with KY1044 in the presence or absence of an anti-CD3 Ab (clone UCHT1, eBioscience) to assess the requirement of TCR engagement. The effect of ICOS co-stimulation was assessed after 72 hrs by measuring the levels of IFN- γ and TNF- α present in the culture (MSD multiplex assay).

For the gene expression analysis, T cells were harvested from the 3-step culture (stimulation-rest-costimulation assay) after 6- hrs of plate-bound antibody stimulation. Total RNA was extracted from the cell pellets with the RNeasy Micro Kit (QIAGEN), quality controlled on the Agilent 2100 Bioanalyzer (Agilent Technologies) and subjected to SE50 sequencing following mRNA enrichment (BGI). The sequence reads were aligned using kallisto (67) and further processed using limma (68) and metasplice (69) and GSVA (61).

Mice

All mice for *in vivo* work was cleared through local ethical committee and was performed under Home Office license. 8 to 10 week-old wild-type female Balb/C or C57BL/6J mice were sourced from Charles River UK Ltd (Margate, UK) and housed in transparent plastic cages with wire covers (391 W x 199 L x 160 H mm, floor area: 500 cm²) containing Grade 6 Wood Chip which can be replaced with Lignocell (IPS Product Supplies Ltd, BCM IPS Ltd. London. WC1N 3XX) and bale shredded nesting material (IPS Product Supplies Ltd, BCM IPS Ltd. London. WC1N 3XX). Four to five Mice were housed per cage in a room with a constant temperature (19-23°C) and humidity (40-70 %) and a 12-hour light-dark cycle (lighting from 7 A.M. to 7 P.M.). Mice were

provided with pellet food (CRM(P), Specialist Diet Services, Witham, UK) and RO water *ad libitum* using an automatic watering system.

Tumour cell implantation and tumour measurement

Early passage (below P10) MC38 (3×10^6 cells), J558 (1×10^6 cells) CT26.WT (1×10^5 cells), A20 (5×10^6 cells), EL4 (1×10^4 cells) and B16-F10 (1×10^5 cells) tumour cells were prepared in either PBS or Matrigel (Corning, 354230) and the resulting cell suspension were injected subcutaneously into the flank of the mice (Study day 0). Prior to tumour cell implantation, mice were anaesthetised with isoflurane and the right flank of the mice was shaved. For the implantation, 100 μ l of the cell suspension were injected using 25 G needles (BD Microlance TM 3. VWR 613-4952). Cell numbers and viability (required to be above 90%) were determined pre-implantation by the trypan blue assay. Tumour growth was measured using digital callipers three times a week until end of the study. The tumour volumes (mm^3) was estimated using a standard formula: $(L \times W^2) / 2$ (with L being the larger diameter, and W the smaller diameter of the tumour. All data were plotted using GraphPad Prism V10.

Antibody dosing

For the *in vivo* tumour efficacy studies, both KY1044 mIgG2 and anti-PD-L1 (AbW) mIgG2a were produced by Kymab Ltd. The anti-PD1 (clone RMP1-14) monoclonal antibody was purchased from BioXcell. Antibodies were dosed between 0.1 and 10 mg/kg or by flat dose of 20 to 200 μ g/dose via the intraperitoneally route. For the efficacy and pharmacodynamic studies the treatment groups were not blinded. The *in vivo* depletion of CD8⁺ and CD4⁺ T cells were conducted using a flat dose 200 μ g of anti-CD8a (53-6.7) and/or anti-CD4 (GK1.5). Dosing was performed twice a week for 3 weeks starting from day 3 post tumour cell implantation. The efficiency of the antibody mediated CD8 and CD4 T cell depletion was determined by flow cytometry of tumour, spleen, tumour draining lymph node (inguinal lymph node) and blood cells using an anti-CD3 antibody (17A2).

Cynomolgus monkey Study

The pharmacodynamic effects of KY1044 in non-human primates were studied as part of a repeat dose toxicity study. Naïve male cynomolgus monkeys (*Macaca fascicularis*) were obtained from a certified supplier, group housed, allowed access to water *ad libitum* and fed

on a pelleted diet for monkeys supplemented with fresh fruit and biscuits. Animals ranged from 4 to 7 years old and weighed 3-6 kg at time of dosing. Four groups of three cynomolgus monkeys received weekly intravenous (i.v.) doses of KY1044 (slow bolus over approximately 1 minute) for a month (5 doses in total) at dose levels of 0 (vehicle control; phosphate-buffered saline pH 7.4), 10, 30 or 100 mg/kg at a dose volume of 2 mL/kg. Blood samples were taken at 1 or 2 timepoints prior to dosing and at multiple timepoints up to 29 days after the first dose for measurement of serum KY1044 using a qualified ELISA assay, ICOS occupancy on CD4+ cells in blood determined using a validated flow cytometry method and/or immunophenotyping of whole-blood (described above). Scheduled necropsies were conducted 1 day after the final dose (Day 30) and spleen and mesenteric lymph nodes were taken for immunophenotyping.

Statistical analyses:

Unless otherwise stated in the Figures legend, the efficacy observed for the different treatment groups were compared using either T-test or one-way analysis of variance (ANOVA) and Tukey's multiple-comparison post-hoc test. Differences between groups were significant at a *P* value of <0.05. Statistical analyses were performed with GraphPad Prism 10.0 (GraphPad Software, Inc., San Diego, CA).

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752 References

- 753 1. M. A. Postow, M. K. Callahan, J. D. Wolchok, Immune Checkpoint Blockade in Cancer
754 Therapy, *J. Clin. Oncol.* 33, 1974–1982 (2015).
- 755 2. P. Sharma, S. Hu-Lieskovan, J. A. Wargo, A. Ribas, Primary, Adaptive, and Acquired
756 Resistance to Cancer Immunotherapy, *Cell* 168, 707–723 (2017).
- 757 3. Y. Takeuchi, H. Nishikawa, Roles of regulatory T cells in cancer immunity, *Int. Immunol.* 28,
758 401–409 (2016).
- 759 4. H. Wang, F. Franco, P.-C. Ho, Metabolic Regulation of Tregs in Cancer: Opportunities for
760 Immunotherapy, *Trends Cancer* 3, 583–592 (2017).
- 761 5. B. Shang, Y. Liu, S. Jiang, Y. Liu, Prognostic value of tumor-infiltrating FoxP3+ regulatory T
762 cells in cancers: a systematic review and meta-analysis., *Sci. Rep.* 5, 15179 (2015).
- 763 6. W. H. Fridman, L. Zitvogel, C. Sautès-Fridman, G. Kroemer, The immune contexture in
764 cancer prognosis and treatment, *Nat. Rev. Clin. Oncol.* 14, 717–734 (2017).
- 765 7. L. Mo, Q. Chen, X. Zhang, X. Shi, L. Wei, D. Zheng, H. Li, J. Gao, J. Li, Z. Hu, Depletion of
766 regulatory T cells by anti-ICOS antibody enhances anti-tumor immunity of tumor cell
767 vaccine in prostate cancer, *Vaccine* 35, 5932–5938 (2017).
- 768 8. F. Arce Vargas, A. J. S. Furness, I. Solomon, K. Joshi, L. Mekkaoui, M. H. Lesko, E. Miranda
769 Rota, R. Dahan, A. Georgiou, A. Sledzinska, A. Ben Aissa, D. Franz, M. Werner Sunderland,
770 Y. N. S. Wong, J. Y. Henry, T. O'Brien, D. Nicol, B. Challacombe, S. A. Beers, S. Turajlic, M.
771 Gore, J. Larkin, C. Swanton, K. A. Chester, M. Pule, J. V. Ravetch, T. Marafioti, K. S. Peggs,
772 S. A. Quezada, L. Spain, A. Wotherspoon, N. Francis, M. Smith, D. Strauss, A. Hayes, A.
773 Soultati, M. Stares, L. Spain, J. Lynch, N. Fotiadis, A. Fernando, S. Hazell, A. Chandra, L.
774 Pickering, S. Rudman, S. Chowdhury, C. Swanton, M. Jamal-Hanjani, S. Veeriah, S. Shafi, J.
775 Czyzewska-Khan, D. Johnson, J. Laycock, L. Bosshard-Carter, G. Goh, R. Rosenthal, P.
776 Gorman, N. Murugaesu, R. E. Hynds, G. Wilson, N. J. Birkbak, T. B. K. Watkins, N.
777 McGranahan, S. Horswell, R. Mitter, M. Escudero, A. Stewart, P. Van Loo, A. Rowan, H.
778 Xu, S. Turajlic, C. Hiley, C. Abbosh, J. Goldman, R. K. Stone, T. Denner, N. Matthews, G.
779 Elgar, S. Ward, J. Biggs, M. Costa, S. Begum, B. Phillimore, T. Chambers, E. Nye, S. Graca,
780 M. Al Bakir, J. A. Hartley, H. L. Lowe, J. Herrero, D. Lawrence, M. Hayward, N.
781 Panagiotopoulos, S. Kolvekar, M. Falzon, E. Borg, C. Simeon, G. Hector, A. Smith, M.
782 Aranda, M. Novelli, D. Oukrif, S. M. Janes, R. Thakrar, M. Forster, T. Ahmad, S. M. Lee, D.
783 Papadatos-Pastos, D. Carnell, R. Mendes, J. George, N. Navani, A. Ahmed, M. Taylor, J.
784 Choudhary, Y. Summers, R. Califano, P. Taylor, R. Shah, P. Krysiak, K. Rammohan, E.
785 Fontaine, R. Booton, M. Evison, P. Crosbie, S. Moss, F. Idries, L. Joseph, P. Bishop, A.
786 Chaturved, A. M. Quinn, H. Doran, A. Leek, P. Harrison, K. Moore, R. Waddington, J.
787 Novasio, F. Blackhall, J. Rogan, E. Smith, C. Dive, J. Tugwood, G. Brady, D. G. Rothwell, F.
788 Chemi, J. Pierce, S. Gulati, B. Naidu, G. Langman, S. Trotter, M. Bellamy, H. Bancroft, A.
789 Kerr, S. Kadiri, J. Webb, G. Middleton, M. Djearaman, D. Fennell, J. A. Shaw, J. Le Quesne,

D. Moore, A. Nakas, S. Rathinam, W. Monteiro, H. Marshall, L. Nelson, J. Bennett, J. Riley, L. Primrose, L. Martinson, G. Anand, S. Khan, A. Amadi, M. Nicolson, K. Kerr, S. Palmer, H. Remmen, J. Miller, K. Buchan, M. Chetty, L. Gomersall, J. Lester, A. Edwards, F. Morgan, H. Adams, H. Davies, M. Kornaszewska, R. Attanoos, S. Lock, A. Verjee, M. MacKenzie, M. Wilcox, H. Bell, N. Iles, A. Hackshaw, Y. Ngai, S. Smith, N. Gower, C. Ottensmeier, S. Chee, B. Johnson, A. Alzetani, E. Shaw, E. Lim, P. De Sousa, M. T. Barbosa, A. Bowman, S. Jorda, A. Rice, H. Raubenheimer, C. Proli, M. E. Cufari, J. C. Ronquillo, A. Kwayie, H. Bhayani, M. Hamilton, Y. Bakar, N. Mensah, L. Ambrose, A. Devaraj, S. Buder, J. Finch, L. Azcarate, H. Chavan, S. Green, H. Mashinga, A. G. Nicholson, K. Lau, M. Sheaff, P. Schmid, J. Conibear, V. Ezhil, B. Ismail, M. Irvin-sellers, V. Prakash, P. Russell, T. Light, T. Horey, S. Danson, J. Bury, J. Edwards, J. Hill, S. Matthews, Y. Kitsanta, K. Suvarna, P. Fisher, A. D. Keerio, M. Shackcloth, J. Gosney, P. Postmus, S. Feeney, J. Asante-Siaw, Fc-Optimized Anti-CD25 Depletes Tumor-Infiltrating Regulatory T Cells and Synergizes with PD-1 Blockade to Eradicate Established Tumors, *Immunity* 46, 577–586 (2017).

9. M. J. Selby, J. J. Engelhardt, M. Quigley, K. A. Henning, T. Chen, M. Srinivasan, A. J. Korman, Anti-CTLA-4 antibodies of IgG2a isotype enhance antitumor activity through reduction of intratumoral regulatory T cells., *Cancer Immunol. Res.* 1, 32–42 (2013).

10. F. Amatore, L. Gorvel, D. Olive, Inducible Co-Stimulator (ICOS) as a potential therapeutic target for anti-cancer therapy, *Expert Opin. Ther. Targets* 22, 343–51 (2018).

11. A. J. McAdam, R. J. Greenwald, M. A. Levin, T. Chernova, N. Malenkovich, V. Ling, G. J. Freeman, a H. Sharpe, ICOS is critical for CD40-mediated antibody class switching., *Nature* 409, 102–5 (2001).

12. A. Hutloff, A. M. Dittrich, K. C. Beier, B. Eljaschewitsch, R. Kraft, I. Anagnostopoulos, R. A. Krocze, ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28, *Nature* 397, 263–266 (1999).

13. Y. Burmeister, T. Lischke, A. C. Dahler, H. W. Mages, K.-P. Lam, A. J. Coyle, R. A. Krocze, A. Hutloff, ICOS controls the pool size of effector-memory and regulatory T cells, *J. Immunol.* 180, 774–82 (2008).

14. D. J. Wikenheiser, J. S. Stumhofer, ICOS Co-Stimulation: Friend or Foe?, *Front. Immunol.* 7, 304 (2016).

15. J. Leconte, S. Bagherzadeh Yazdchi, V. Panneton, W.-K. Suh, Inducible costimulator (ICOS) potentiates TCR-induced calcium flux by augmenting PLC γ 1 activation and actin remodeling., *Mol. Immunol.* 79, 38–46 (2016).

16. H. Nagase, T. Takeoka, S. Urakawa, A. Morimoto-Okazawa, A. Kawashima, K. Iwahori, S. Takiguchi, H. Nishikawa, E. Sato, S. Sakaguchi, M. Mori, Y. Doki, H. Wada, ICOS(+) Foxp3(+) TILs in gastric cancer are prognostic markers and effector regulatory T cells associated with *Helicobacter pylori*., *Int. J. Cancer* 138, 1328–36 (2016).

17. J.-F. Tu, Y.-H. Ding, X.-H. Ying, F.-Z. Wu, X.-M. Zhou, D.-K. Zhang, H. Zou, J.-S. Ji, Regulatory T cells, especially ICOS+ FOXP3+ regulatory T cells, are increased in the hepatocellular carcinoma microenvironment and predict reduced survival, *Sci. Rep.* 6, 35056 (2016).

18. D. Ng Tang, Y. Shen, J. Sun, S. Wen, J. D. Wolchok, J. Yuan, J. P. Allison, P. Sharma, Increased Frequency of ICOS⁺ CD4 T Cells as a Pharmacodynamic Biomarker for Anti-CTLA-4 Therapy, *Cancer Immunol. Res.* 1, 229–234 (2013).
19. X. Fan, S. a Quezada, M. a Sepulveda, P. Sharma, J. P. Allison, Engagement of the ICOS pathway markedly enhances efficacy of CTLA-4 blockade in cancer immunotherapy., *J. Exp. Med.* 211, 715–25 (2014).
20. S. C. Wei, J. H. Levine, A. P. Cogdill, Y. Zhao, N.-A. A. S. Anang, M. C. Andrews, P. Sharma, J. Wang, J. A. Wargo, D. Pe'er, J. P. Allison, Distinct Cellular Mechanisms Underlie Anti-CTLA-4 and Anti-PD-1 Checkpoint Blockade, *Cell* , 1–14 (2017).
21. T. Fu, Q. He, P. Sharma, The ICOS/ICOSL pathway is required for optimal antitumor responses mediated by anti-CTLA-4 therapy, *Cancer Res.* 71, 5445–54 (2011).
22. F. Arce Vargas, A. J. S. Furness, K. Litchfield, K. Joshi, R. Rosenthal, I. Solomon, M. H. Lesko, N. Ruef, C. Roddie, J. Y. Henry, L. Spain, A. Ben Aissa, A. Georgiou, Y. N. S. Wong, M. Smith, D. Strauss, A. Hayes, D. Nicol, T. O'Brien, L. Mårtensson, A. Ljungars, I. Teige, B. Frendéus, M. Pule, T. Marafioti, M. Gore, J. Larkin, S. Turajlic, C. Swanton, K. S. Peggs, S. A. Quezada, A. Melcher, A. Wotherspoon, N. Francis, B. Challacombe, L. Pickering, J. Lynch, S. Rudman, S. Chowdhury, K. Harrison-Phipps, M. Varia, C. Horsfield, A. Polson, G. Stamp, M. O'Donnell, W. Drake, P. Hill, D. Hrouda, E. Mayer, J. Olsburgh, G. Kooiman, K. O'Connor, G. Stewart, M. Aitchison, M. Tran, N. Fotiadis, H. Verma, J. Lopez, J. Lester, F. Morgan, M. Kornaszewska, R. Attanoos, H. Adams, H. Davies, D. Fennell, J. Shaw, J. Le Quesne, A. Nakas, S. Rathinam, W. Monteiro, H. Marshall, L. Nelson, J. Bennett, J. Riley, L. Primrose, L. Martinson, G. Anand, S. Khan, M. Nicolson, K. Kerr, S. Palmer, H. Remmen, J. Miller, K. Buchan, M. Chetty, L. Gomersall, S. Lock, B. Naidu, G. Langman, S. Trotter, M. Bellamy, H. Bancroft, A. Kerr, S. Kadiri, J. Webb, G. Middleton, M. Djearaman, Y. Summers, R. Califano, P. Taylor, R. Shah, P. Krysiak, K. Rammohan, E. Fontaine, R. Booton, M. Evison, P. Crosbie, S. Moss, F. Idries, J. Novasio, L. Joseph, P. Bishop, A. Chaturvedi, A. Marie Quinn, H. Doran, A. leek, P. Harrison, K. Moore, R. Waddington, F. Blackhall, J. Rogan, E. Smith, C. Dive, G. Brady, D. Rothwell, S. Gulati, F. Chemie, J. Tugwood, J. Pierce, D. Lawrence, M. Hayward, N. Panagiotopoulos, R. George, D. Patrini, M. Falzon, E. Borg, R. Khirya, M. Jamal-Hanjani, G. Wilson, N. Juul Birkbak, T. Watkins, N. McGranahan, C. Abbosh, S. Horswell, R. Mitter, M. Escudero, A. Stewart, A. Rowan, C. Hiley, J. Goldman, A. Ahmed, M. Taylor, J. Choudhary, P. Shaw, R. Veeriah, J. Czyzewska-Khan, D. Johnson, J. Laycock, R. Hynds, M. Werner Sunderland, J. Reading, M. Novelli, D. Oukrif, S. Janes, M. Forster, T. Ahmad, S. Ming Lee, P. van Loo, J. Herrero, J. Hartley, R. Kevin Stone, T. Denner, M. Costa, S. Begum, B. Phillimore, T. Chambers, E. Nye, S. Ward, G. Elgar, M. Al-Bakir, D. Carnell, R. Mendes, J. George, N. Navani, D. Papadatos-Pastos, M. Scarci, P. Gorman, H. Lowe, L. Ensell, D. Moore, M. MacKenzie, M. Wilcox, H. Bell, A. Hackshaw, Y. Ngai, S. Smith, N. Gower, C. Ottensmeier, S. Chee, B. Johnson, A. Alzetani, E. Shaw, E. Lim, P. De Sousa, M. Tavares Barbosa, A. Nicholson, A. Bowman, S. Jordan, A. Rice, H. Raubenheimer, C. Proli, M. Elena Cufari, J. Carlo Ronquillo, A. Kwayie, H. Bhayani, M. Hamilton, Y. Bakar, N. Mensah, L. Ambrose, A. Devaraj, S. Buder, J. Finch, L. Azcarate, H. Chavan, S. Green, H. Mashinga, K. Lau, M. Sheaff, P. Schmid, J. Conibear, V. Ezhil, V. Prakash, S. Danson, J. Bury, J. Edwards, J. Hill, S. Matthews, Y. Kitsanta, K. Suvarna, M. Shackcloth, J. Gosney, P. Postmus, S. Feeney, J. Asante-Siaw, P. Russell, T. Light, T. Horey,

- 874 K. Blyth, C. Dick, Fc Effector Function Contributes to the Activity of Human Anti-CTLA-4
875 Antibodies, *Cancer Cell* 33, 649-663.e4 (2018).
- 876 23. E.-C. Lee, Q. Liang, H. Ali, L. Bayliss, A. Beasley, T. Bloomfield-Gerdes, L. Bonoli, R. Brown,
877 J. Campbell, A. Carpenter, S. Chalk, A. Davis, N. England, A. Fane-Dremucheva, B. Franz, V.
878 Germaschewski, H. Holmes, S. Holmes, I. Kirby, M. Kosmac, A. Legent, H. Lui, A. Manin, S.
879 O'Leary, J. Paterson, R. Sciarrillo, A. Speak, D. Spensberger, L. Tuffery, N. Waddell, W.
880 Wang, S. Wells, V. Wong, A. Wood, M. J. Owen, G. a Friedrich, A. Bradley, Complete
881 humanization of the mouse immunoglobulin loci enables efficient therapeutic antibody
882 discovery, *Nat. Biotechnol.* 32, 356–363 (2014).
- 883 24. V. Tkachev, S. N. Furlan, B. Watkins, D. J. Hunt, H. B. Zheng, A. Panoskaltsis-Mortari, K.
884 Betz, M. Brown, J. B. Schell, K. Zeleski, A. Yu, I. Kirby, S. Cooley, J. S. Miller, B. R. Blazar, D.
885 Casson, P. Bland-Ward, L. S. Kean, Combined OX40L and mTOR blockade controls effector
886 T cell activation while preserving T reg reconstitution after transplant, *Sci. Transl. Med.* 9,
887 eaan3085 (2017).
- 888 25. C. Conrad, J. Gregorio, Y. H. Wang, T. Ito, S. Meller, S. Hanabuchi, S. Anderson, N.
889 Atkinson, P. T. Ramirez, Y. J. Liu, R. Freedman, M. Gilliet, Plasmacytoid dendritic cells
890 promote immunosuppression in ovarian cancer via ICOS costimulation of Foxp3+ T-
891 regulatory cells, *Cancer Res.* 72, 5240–5249 (2012).
- 892 26. L. Strauss, C. Bergmann, M. J. Szczepanski, S. Lang, J. M. Kirkwood, T. L. Whiteside,
893 Expression of ICOS on human melanoma-infiltrating CD4+CD25highFoxp3+ T regulatory
894 cells: implications and impact on tumor-mediated immune suppression., *J. Immunol.* 180,
895 2967–80 (2008).
- 896 27. F. Hoffman, D. Gavaghan, J. Osborne, I. P. Barrett, T. You, H. Ghadially, R. Sainson, R. W.
897 Wilkinson, H. M. Byrne, A mathematical model of antibody-dependent cellular
898 cytotoxicity (ADCC), *J. Theor. Biol.* 436, 39–50 (2018).
- 899 28. J. D. Waight, D. Chand, S. Dietrich, R. Gombos, T. Horn, A. M. Gonzalez, M. Manrique, L.
900 Swiech, B. Morin, C. Brittsan, A. Tanne, B. Akpeng, B. A. Croker, J. S. Buell, R. Stein, D. A.
901 Savitsky, N. S. Wilson, Selective FcγR Co-engagement on APCs Modulates the Activity of
902 Therapeutic Antibodies Targeting T Cell Antigens, *Cancer Cell* 33, 1033-1047.e5 (2018).
- 903 29. B. C. Barnhart, M. Quigley, Role of Fc-FcγR interactions in the antitumor activity of
904 therapeutic antibodies, *Immunol. Cell Biol.* 95, 340–346 (2017).
- 905 30. J. L. Franko, A. D. Levine, Antigen-independent adhesion and cell spreading by inducible
906 costimulator engagement inhibits T cell migration in a PI-3K-dependent manner, *J Leukoc*
907 *Biol* 85, 526–38 (2009).
- 908 31. H. Chen, T. Fu, W.-K. Suh, D. Tsavachidou, S. Wen, J. Gao, D. Ng Tang, Q. He, J. Sun, P.
909 Sharma, CD4 T cells require ICOS-mediated PI3K signaling to increase T-Bet expression in
910 the setting of anti-CTLA-4 therapy., *Cancer Immunol. Res.* 2, 167–76 (2014).

- 911 32. Q. Chen, L. Mo, X. Cai, L. Wei, Z. Xie, H. Li, J. Li, Z. Hu, ICOS signal facilitates Foxp3
912 transcription to favor suppressive function of regulatory T cells, *Int. J. Med. Sci.* 15, 666–
913 673 (2018).
- 914 33. P. Bruhns, B. Iannascoli, P. England, D. A. Mancardi, N. Fernandez, S. Jorieux, M. Daëron,
915 Specificity and affinity of human Fcγ receptors and their polymorphic variants for human
916 IgG subclasses, *Blood* 113, 3716–3725 (2009).
- 917 34. A. Pratama, C. G. Vinuesa, Control of TFH cell numbers: why and how?; *Immunol.*
918 *Cell Biol.* 9269, 40–48 (2014).
- 919 35. K.-S. Le, M.-L. Thibult, S. Just-Landi, S. Pastor, F. Gondois-Rey, S. Granjeaud, F. Broussais,
920 R. Bouabdallah, R. Colisson, C. Caux, C. Ménétrier-Caux, D. Leroux, L. Xerri, D. Olive,
921 Follicular B Lymphomas Generate Regulatory T Cells via the ICOS/ICOSL Pathway and Are
922 Susceptible to Treatment by Anti-ICOS/ICOSL Therapy, *Cancer Res.* 76, 4648–4660
923 (2016).
- 924 36. B. Wang, N. Ma, H. Cheng, H. Zhou, H. Qiu, J. Yang, J. Wang, Effects of ICOSLG expressed
925 in mouse hematological neoplasm cell lines in the GVL reaction., *Bone Marrow*
926 *Transplant.* 48, 124–8 (2013).
- 927 37. S. J. Dovedi, A. L. Adlard, G. Lipowska-Bhalla, C. McKenna, S. Jones, E. J. Cheadle, I. J.
928 Stratford, E. Poon, M. Morrow, R. Stewart, H. Jones, R. W. Wilkinson, J. Honeychurch, T.
929 M. Illidge, Acquired Resistance to Fractionated Radiotherapy Can Be Overcome by
930 Concurrent PD-L1 Blockade, *Cancer Res.* 74, 5458–5468 (2014).
- 931 38. A. Moretta, A. Poggi, D. Pende, G. Tripodi, A. M. Orengo, N. Pella, R. Augugliaro, C.
932 Bottino, E. Ciccone, L. Moretta, CD69-mediated pathway of lymphocyte activation: anti-
933 CD69 monoclonal antibodies trigger the cytolytic activity of different lymphoid effector
934 cells with the exception of cytolytic T lymphocytes expressing T cell receptor alpha/beta.,
935 *J. Exp. Med.* 174, 1393–1398 (1991).
- 936 39. S. F. Ziegler, F. Ramsdell, M. R. Alderson, The activation antigen CD69, *Stem Cells* 12, 456–
937 465 (1994).
- 938 40. T. Calzascia, M. Pellegrini, H. Hall, L. Sabbagh, N. Ono, A. R. Elford, T. W. Mak, P. S. Ohashi,
939 TNF-α is critical for antitumor but not antiviral T cell immunity in mice, *J. Clin. Invest.*
940 (2007), doi:10.1172/JCI32567.
- 941 41. D. F. McDermott, J. A. Sosman, M. Sznol, C. Massard, M. S. Gordon, O. Hamid, J. D.
942 Powderly, J. R. Infante, M. Fassò, Y. V. Wang, W. Zou, P. S. Hegde, G. D. Fine, T. Powles,
943 Atezolizumab, an Anti–Programmed Death-Ligand 1 Antibody, in Metastatic Renal Cell
944 Carcinoma: Long-Term Safety, Clinical Activity, and Immune Correlates From a Phase Ia
945 Study, *J. Clin. Oncol.* 34, 833–842 (2016).
- 946 42. K. Shitara, H. Nishikawa, Regulatory T cells: a potential target in cancer immunotherapy,
947 *Ann. N. Y. Acad. Sci.* 1417, 104–115 (2018).

- 948 43. S. A. Redpath, N. van der Werf, A. M. Cervera, A. S. MacDonald, D. Gray, R. M. Maizels, M.
949 D. Taylor, ICOS controls Foxp3 + regulatory T-cell expansion, maintenance and IL-10
950 production during helminth infection: Immunity to infection, *Eur. J. Immunol.* 43, 705–
951 715 (2013).
- 952 44. D. van Elsland, J. Neefjes, Bacterial infections and cancer, *EMBO Rep.* 19, e46632 (2018).
- 953 45. E. A. Tivol, F. Borriello, A. N. Schweitzer, W. P. Lynch, J. A. Bluestone, A. H. Sharpe, Loss of
954 CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction,
955 revealing a critical negative regulatory role of CTLA-4, *Immunity* 3, 541–547 (1995).
- 956 46. C. Dong, A. E. Juedes, U. A. Temann, S. Shresta, J. P. Allison, N. H. Ruddle, R. A. Flavell,
957 ICOS co-stimulatory receptor is essential for T-cell activation and function., *Nature* 409,
958 97–101 (2001).
- 959 47. A. Tafuri, A. Shahinian, F. Bladt, S. K. Yoshinaga, M. Jordana, A. Wakeham, L. M. Boucher,
960 D. Bouchard, V. S. Chan, G. Duncan, B. Odermatt, A. Ho, A. Itie, T. Horan, J. S. Whoriskey,
961 T. Pawson, J. M. Penninger, P. S. Ohashi, T. W. Mak, ICOS is essential for effective T-
962 helper-cell responses., *Nature* 409, 105–9 (2001).
- 963 48. K. M. Zak, P. Grudnik, K. Magiera, A. Dömling, G. Dubin, T. A. Holak, Structural Biology of
964 the Immune Checkpoint Receptor PD-1 and Its Ligands PD-L1/PD-L2, *Structure* 25, 1163–
965 1174 (2017).
- 966 49. S. Suvas, R. Channappanavar, B. Twardy, Inducible costimulator (ICOS) negatively regulate
967 programmed death-1 (PD-1) induced apoptosis of effector memory CD4 T cells under
968 homeostatic condition (47.11), *J. Immunol.* 188, 47.11-47.11 (2012).
- 969 50. Y. Zhang, Y. Luo, S.-L. Qin, Y.-F. Mu, Y. Qi, M.-H. Yu, M. Zhong, The clinical impact of ICOS
970 signal in colorectal cancer patients., *Oncoimmunology* 5, e1141857 (2016).
- 971 51. G. Beyrend, E. van der Gracht, A. Yilmaz, S. van Duikeren, M. Camps, T. Höllt, A. Vilanova,
972 V. van Unen, F. Koning, N. F. C. C. de Miranda, R. Arens, F. Ossendorp, PD-L1 blockade
973 engages tumor-infiltrating lymphocytes to co-express targetable activating and inhibitory
974 receptors, *J. Immunother. Cancer* 7, 217 (2019).
- 975 52. A. Marabelle, H. Kohrt, I. Sagiv-Barfi, B. Ajami, R. C. Axtell, G. Zhou, R. Rajapaksa, M. R.
976 Green, J. Torchia, J. Brody, R. Luong, M. D. Rosenblum, L. Steinman, H. I. Levitsky, V. Tse,
977 R. Levy, Depleting tumor-specific Tregs at a single site eradicates disseminated tumors, *J.*
978 *Clin. Invest.* 123, 2447–2463 (2013).
- 979 53. A. Sharma, S. K. Subudhi, J. Blando, J. Scutti, L. Vence, J. A. Wargo, J. P. Allison, A. Ribas, P.
980 Sharma, Anti-CTLA-4 immunotherapy does not deplete FOXP3+ regulatory T cells (Tregs)
981 in human cancers, *Clin. Cancer Res.* , clincanres.0762.2018 (2018).
- 982 54. M. B. Overdijk, S. Verploegen, A. Ortiz Buijsse, T. Vink, J. H. W. Leusen, W. K. Bleeker, P.
983 W. H. I. Parren, Crosstalk between Human IgG Isotypes and Murine Effector Cells., *J.*
984 *Immunol. Baltim. Md* 1950 189, 3430–8 (2012).

985 55. J. S. Ellis, X. Wan, H. Braley-Mullen, Transient depletion of CD4 + CD25 + regulatory T cells
986 results in multiple autoimmune diseases in wild-type and B-cell-deficient NOD mice,
987 *Immunology* 139, 179–186 (2013).

988 56. L. A. Stephens, D. Gray, S. M. Anderton, CD4+ CD25+ regulatory T cells limit the risk of
989 autoimmune disease arising from T cell receptor crossreactivity, *Proc. Natl. Acad. Sci.*
990 102, 17418–17423 (2005).

991 57. D. L. Farber, N. A. Yudanin, N. P. Restifo, Human memory T cells: generation,
992 compartmentalization and homeostasis, *Nat. Rev. Immunol.* 14, 24–35 (2014).

993 58. L. Westera, J. Drylewicz, I. den Braber, T. Mugwagwa, I. van der Maas, L. Kwast, T.
994 Volman, E. H. R. van de Weg-Schrijver, I. Bartha, G. Spierenburg, K. Gaiser, M. T.
995 Ackermans, B. Asquith, R. J. de Boer, K. Tesselaar, J. A. M. Borghans, Closing the gap
996 between T-cell life span estimates from stable isotope-labeling studies in mice and
997 humans, *Blood* 122, 2205–2212 (2013).

998 59. M. Goldman, B. Craft, J. Zhu, D. Haussler, Abstract 2584: The UCSC Xena system for
999 cancer genomics data visualization and interpretation, *Cancer Res.* 77, 2584–2584
1000 (2017).

1001 60. D. A. Barbie, P. Tamayo, J. S. Boehm, S. Y. Kim, S. E. Moody, I. F. Dunn, A. C. Schinzel, P.
1002 Sandy, E. Meylan, C. Scholl, S. Fröhling, E. M. Chan, M. L. Sos, K. Michel, C. Mermel, S. J.
1003 Silver, B. A. Weir, J. H. Reiling, Q. Sheng, P. B. Gupta, R. C. Wadlow, H. Le, S. Hoersch, B. S.
1004 Wittner, S. Ramaswamy, D. M. Livingston, D. M. Sabatini, M. Meyerson, R. K. Thomas, E.
1005 S. Lander, J. P. Mesirov, D. E. Root, D. G. Gilliland, T. Jacks, W. C. Hahn, Systematic RNA
1006 interference reveals that oncogenic KRAS-driven cancers require TBK1., *Nature* 462, 108–
1007 12 (2009).

1008 61. S. Hänzelmann, R. Castelo, J. Guinney, GSEA: gene set variation analysis for microarray
1009 and RNA-Seq data, *BMC Bioinformatics* 14, 7 (2013).

1010 62. A. T. L. Lun, D. J. McCarthy, J. C. Marioni, A step-by-step workflow for low-level analysis of
1011 single-cell RNA-seq data with Bioconductor, *F1000Research* 5, 2122 (2016).

1012 63. D. J. McCarthy, K. R. Campbell, A. T. L. Lun, Q. F. Wills, Scater: pre-processing, quality
1013 control, normalization and visualization of single-cell RNA-seq data in R, *Bioinformatics*
1014 33, 1179–1186 (2017).

1015 64. M. D. Young, S. Behjati, SoupX removes ambient RNA contamination from droplet based
1016 single cell RNA sequencing data, *bioRxiv*, 303727 (2018).

1017 65. L. McInnes, J. Healy, J. Melville, UMAP: Uniform Manifold Approximation and Projection
1018 for Dimension Reduction, (2018) (available at <https://arxiv.org/abs/1802.03426>).

1019 66. S. Aibar, C. B. González-Blas, T. Moerman, V. A. Huynh-Thu, H. Imrichova, G. Hulselmans,
1020 F. Rambow, J.-C. Marine, P. Geurts, J. Aerts, J. van den Oord, Z. K. Atak, J. Wouters, S.
1021 Aerts, SCENIC: single-cell regulatory network inference and clustering, *Nat. Methods* 14,
1022 1083–1086 (2017).

67. N. L. Bray, H. Pimentel, P. Melsted, L. Pachter, Near-optimal probabilistic RNA-seq quantification, *Nat. Biotechnol.* 34, 525–527 (2016).
68. M. E. Ritchie, B. Phipson, D. Wu, Y. Hu, C. W. Law, W. Shi, G. K. Smyth, limma powers differential expression analyses for RNA-sequencing and microarray studies, *Nucleic Acids Res.* 43, e47–e47 (2015).
69. Y. Zhou, B. Zhou, L. Pache, M. Chang, A. H. Khodabakhshi, O. Tanaseichuk, C. Benner, S. K. Chanda, Metascape provides a biologist-oriented resource for the analysis of systems-level datasets, *Nat. Commun.* 10, 1–10 (2019).

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Competing interests: With the exception of EM, EP, JaC and IK, all authors were employees of Kymab Ltd at time of writing of this manuscript. EM, EP, JaC and IK are former employees of Kymab Ltd. Several of the authors are inventor on patents relating to anti-ICOS antibodies, including US9957323 in the name of Kymab Limited

Figure legends

Figure 1: ICOS is highly expressed in cancer T_{Reg}s. (A) Density plots showing the combined *Icos* and *Foxp3* expression in different solid tumour types from the TCGA datasets. Combined expression was scored using single-sample gene set enrichment analysis (ssGSEA) and density distributions plotted by indications. Stacked histograms are arranged by order of descending median ssGSEA score (marked by vertical ticks). The TCGA standard cancer type abbreviations were used (<https://gdc.cancer.gov/resources-tcga-users/tcga-code-tables/tcga-study-abbreviations>). (B) Immune response genes were sequenced in each cell. Sequenced cells were then classified into one of 27 immunological subtypes based on their gene signature (cell types and the corresponding gene signatures are described in Supplementary table 1). Heatmap (top) of scaled gene expression in T cells from PBMC (left panel) and tumours (right panel) from NSCLC patient samples (n=5). Genes relevant to T cell lineage and function are presented. Each gene was scaled individually across all cells of the dataset and the mean scaled expression for each of 17 T cell subtypes is presented. Scatter plots (bottom) showing ICOS mRNA expression in T_{Reg}, CD4_{non-TReg}, CD8 and all other T cells. Each dot corresponds to a single cell. Full lines indicate the mean ICOS gene expression of the total cell compartment and the dashed lines indicate the mode (density peak) of the ICOS+ve cell population. (C) Relative ICOS expression (as determined by flow cytometry analysis) on CD4+, CD8+ and T_{Reg} (CD4+/FOXP3+) in healthy donor PBMCs (n=5), NSCLC tumour samples (n=5) and matched NSCLC patient PBMCs (n=4). *** = P value ≤0.001 and **** = P value ≤0.0001 (2-way ANOVA with Tukey's multiple comparison). (D) Graph showing the number of ICOS+ve cells per mm² in tumour cores (n=995) from 6 indications and cores from matching healthy tissues (n=48). ****p≤0.0001 (Mann-Whitney unpaired T test). (E) Example of ICOS/FOXP3 staining of gastric tumour core showing (a); original whole core image with ICOS staining in purple and FOXP3 in brown (b); classifier analysis overlays showing tissue ROI (lilac) and white space (white); (c) cellular analysis overlay showing single FOXP3 positive (green overlay), single ICOS positive (red overlay) and ICOS/FOXP3 dual positive (cyan overlay); (d - f) x20 magnified detailed area (F) Quantification of ICOS/FOXP3 double positive cells per mm² in TMAs from six indications (cervical, oesophageal, head and neck, lung, bladder and gastric) by image digital pathology.

Figure 2: KY1044 triggers ADCC and has co-stimulatory agonistic properties in ICOS positive T cells (A) KY1044 induces FcγRIIIA signalling in “effector” cells when cocultured with ICOS positive cells. Serial dilutions of KY1044 hlgG1 and the isotype control were incubated O/N with engineered Jurkat effector cells containing the reporter NFAT-luciferase and FcγRIIIa and CHO cells expressing ICOS from different species. (B) KY1044 induces ADCC in a human primary NK cells assay. Serial dilutions of KY1044 or isotype control were co-incubated with ICOS CEM target cells and NK for 4 hours. ADCC activity was quantified by measuring Specific Dye Release (mean of triplicates±SEM). (C) and (D) Plate bound and cross-linked soluble KY1044 induces the release of IFN-γ from MJ cells. The concentration of IFN-γ was assessed in the supernatant of MJ cultured for 72hrs in the presence of serial dilutions of plate bound and soluble antibodies with or without the addition of an Fc cross-linking anti-human F(ab')₂ Fragments (D). (E) and (F) Levels of IFN-γ induced in human primary T-cell activated with anti-CD3/CD28 (to induce ICOS) and cultured with either 5 µg/mL of plate-bound KY1044 or the isotype control (IC) IgG1 (E, n=10) or with either 15µg/mL of soluble KY1044 or soluble IC, with or without the addition of an Fc cross-linking anti-human F(ab')₂ Fragments (F, n=10). (G) Cytokine production by KY1044 requires anti-CD3. Levels of TNF-α in T-cell culture with 5 µg/mL of KY1044 or IC IgG1 (plate-bound, n=8) in the presence or not of anti-CD3. * p<0.05, ** p<0.01 (Wilcoxon statistic test). (H) RNA sequencing analysis comparing T cell stimulation with either KY1044 + anti-CD3 or control (anti-CD3 only) antibodies. Genes reported to be downstream of ICOS signalling show a pattern of upregulation following KY1044 co-stimulation, with significant p-values obtained for IFNG, IL10 and IL4 following 6 hrs of stimulation (left). Metascape pathway enrichment analysis of the differentially expressed genes from the 6-hr time point. Enriched gene sets are ranked based on the significance of their p-values (top right). Heatmap showing unsupervised clustering of samples based on the expression levels of the top GO terms from the Metascape enrichment analysis. KY1044 stimulated samples show specific gene ontology modules that segregate them from control stimulated samples (bottom right).

Figure 3: KY1044 mIgG2 monotherapy triggers anti-tumour efficacy in the A20 and J558 syngeneic tumour models (A) Spider plots showing individual mouse tumour volumes from BALB/c mice (n=10 per group) harbouring subcutaneous A20 tumours. Tumour bearing mice were dosed i.p with 200 µg of KY1044 mIgG2a or 200 µl saline starting from day 8 post tumour cell implantation. (B) Spider plots showing individual mouse tumour volumes from BALB/c mice

(n=7 per group) harbouring subcutaneous J558 tumours. Tumour bearing mice were dosed i.p with 60 µg of KY1044 mIgG2a or 200 µL saline starting from day 9 post tumour cell implantation. For both experiments the dosing days are indicated by vertical dotted lines. The Survival curves depicting the control and KY1044 mIgG2a groups are shown on the right end side. ** P<0.01, **** P<0.0001 (Statistics calculated using Log-rank (Mantel-Cox) test. Data are representative of at least 2 experiments.

Figure 4: KY1044 mIgG2a synergises with anti-PD-L1 to promote durable anti-tumour immune response in model of solid tumours. (A) Survival curves of mice harbouring CT26.WT tumours and treated with control, KY1044 mIgG2a monotherapy at 60 µg/dose, anti-PD-L1 monotherapy 200 µg/dose and the combination of KY1044 mIgG2a and anti-PD-L1 at 60 and 200 µg/dose, respectively (Left panel). The dosing days are indicated by vertical dotted lines. ** P<0.01, *** P<0.001 and **** P<0.0001 (Statistics calculated using Log-rank Mantel-Cox test). The data is representative of 6 independent experiments. Tumour-bearing mice cured of CT26.WT tumours were randomly allocated to two groups and rechallenged with either CT26.WT or EMT6 tumour cells (Right panel). Tumour growth was only observed for EMT6 tumours but not CT26.WT tumours. (B) The anti-tumour response to the combination is primarily dependent on the presence CD8 (and to a lesser extent on CD4) T Cells. Mice implanted with CT26.WT cell (n=10 per group) were depleted of CD8 and/or CD4 T cells and treated with saline or with KY1044 mIgG2a and anti-PD-L1 combination as described in panel (A). Data is representative of two independent experiments. (C) Table summarising the antitumour efficacy of KY1044 mIgG2a monotherapy, anti-PD-L1 monotherapy and the combination in different syngeneic models.

Figure 5: KY1044 mlgG2a preferentially depletes ICOS^{high} T_{Reg} cell *in vivo*. (A) In vivo experimental protocol of the pharmacodynamic study in the CT26.WT tumour bearing BALB/c mice. Mice were dosed twice with saline or different doses of KY1044 mlgG2a (ranging from 0.3 to 10 mg/kg) on days 13 and 15 and the tumour and spleen were harvested on day 16 and the immune cells analysed by flow cytometry. KY1044 depletes T_{Reg} and increase the CD8+ T_{EFF} to T_{Reg} cell ratio in the tumour microenvironment (B) but not in the spleen (C) of treated mice (n=7/8 mice per groups). * p<0.05, ** p<0.01 and ***p<0.001. (D) Relative expression of ICOS and frequency of T cells subsets in the blood of NHP (the number of NHP used for each analysis are indicated in brackets). ICOS is highly expressed on T_{Reg} cell followed by CD4 memory (T_M) and T follicular helper cells (T_{FH}). NHP CD8 T cells express low levels of ICOS. (E) (F) and (G) Graphs showing the changes (vs baseline pre-treatment) of total memory CD4 T cells and follicular T Helper cells in the blood of NHP at different timepoints following a single dose KY1044 hlgG1 at 0, 10, 20 and 100 mg/kg (n=3 NHP per groups)

Figure 6: KY1044 mlgG2a activates intratumoural CD8 T cells as shown by the increase in CD69 and CD44 expression and increases the expression of Th1 cytokines IFN- γ and TNF- α in vivo. (A) KY1044 mlgG2a i.p injection results in an increase percentage of intratumoural CD8 T cells expressing the activation marker CD69 on their surface (n=7/8 mice per groups). Measurement of CD69 and CD69/CD44 double positive cells were performed 24 hours after a second dose of KY1044 mlgG2a (see figure 5A). (B) Graphs showing the flow cytometry analysis of Intracellular IFN- γ and/or TNF- α expression in intratumoural CD8+ and CD4+ T cells from CT26.WT tumour collected 7 days after i.p injection of saline or KY1044 mlgG2a at 3 mg/kg. Groups of mice (n = 4 mice/group). * p<0.05 Tukey's multiple comparisons test

Figure 7: Better combination efficacy was obtained at intermediate dose of KY1044 mlgG2a. Spider plots showing individual mouse tumour volumes from BALB/c mice (n=9 per group) harbouring subcutaneous CT26.WT tumours and treated with different doses of KY1044 mlgG2a (20, 60 and 200 µg/dose) with a fixed dose of anti-PD-L1 (200 µg/dose). Kaplan-Meier plot depicting the survival of mice injected with CT26.WT-tumour and treated with different doses of KY1044 mlgG2a/anti-PD-L1 combination. The dosing days are indicated by vertical dotted lines.

Supplementary Materials

Materials and Methods

Luciferase assays:

Luciferase reporter assays: Jurkat-Lucia™ NFAT cells (Invivogen) stably expressing luciferase under the control of NFAT response elements were further transfected with either the human *Icos* gene sequence or a chimeric construct of *Icos* fused with *CD247* (CD3ζ). Following selection of stable transgene integration, a luciferase activity bioassay was performed. High binding 96-well assay plates were coated overnight with either anti-CD3 (UCHT-1, BD Biosciences; 10 µg/ml), anti-ICOS (C398.4A, BioLegend; 10 µg/ml), isotype control (HTK888, BioLegend; 10 µg/ml), anti-CD3 + anti-ICOS (5 µg/ml each) or anti-CD3 + isotype control (5 µg/ml each). Transgene expressing cells or control cells (untransfected Jurkat-Lucia™ NFAT cells) were seeded at 50,000 cells / well. Following overnight incubation luciferase activity was measured by adding BioGlo reagent (Promega) and reading luminescence on a plate reader. Luciferase activity was normalised and scaled to 100% by comparing to cells stimulated using Cell Stimulation Cocktail (eBioscience).

NHP ethics statement

The cynomolgus monkey study was conducted at Covance preclinical Services GmbH, Muenster, Germany in strict accordance with a study plan reviewed and approved by the local Institutional Animal Care and Use Committee (IACUC) of the testing facility and the German Animal Welfare Act. The study was performed according to DIRECTIVE 2010/63/EU OF THE

EUROPEAN PARLIAMENT AND OF THE COUNCIL of 22 September 2010 on the protection of animals used for scientific purposes and the Commission Recommendation 2007/526/EC on guidelines for the accommodation and care of animals used for experimental and other scientific purposes (Appendix A of Convention ETS 123). The study was in compliance with the Kymab Working Practice on Experiments involving Animals and was approved by the Kymab Ethics Committee.

Supplementary figure legends:

Supplementary figure 1: FACS analysis showing the (A) percentage of ICOS positive cells and (B) the relative ICOS expression in T cells derived for the spleen and tumours of CT26.WT tumour bearing mice. (C)) visNE (visualization of t-distributed stochastic neighbor embedding) map of the T cell subpopulations (CD3+ cells) from untreated CT26.WT tumour samples (Cytobank). The colour scales depict the expression levels of selected markers (CD4, CD8, FOXP3, CD25 and ICOS) ranging from blue (low expression) to red (high expression) from the total T cell population that was organised in 2D based on similarity of expression.

Supplementary figure 2: Percentage of ICOS positive cells and relative ICOS expression on CD4 and CD8 T cells at baseline (Day 0) and at day 3 and day 6 post anti-CD3/CD28 stimulation (A and B). (C) Time lapse microscopy over 24 hours of MJ cells cultured on plate precoated with an isotype control or with anti-ICOS C398.4A (20µg/ml). (D) images capture after 48 hours of MJ cells grown on plates pre-coated with different concentrations of KY1044. (E) Graph showing the NFAT luciferase reporter assays using untransfected or ICOS and ICOS-CD3ζ transfected Jurkat cells treated with different plate bound stimuli including anti-CD3 and anti-ICOS (C398.4A)

Supplementary figure 3: Density distributions showing the levels of *Icos* expression in haematological and solid tumour types from the TCGA datasets.

Supplementary figure 4: Examples of syngeneic tumour models resistant or poorly responsive to KY1044 mIgG2a monotherapy. Kaplan-Meier plot depicting the survival of mice injected sub-

cut with different syngeneic tumour types (EL4, B16-F10, CT26 and MC38) and treated with KY1044 mIgG2a (3 or 10 mg/kg, vertical dotted lines).

Supplementary figure 5: Graph summarising the analysis of T cells content in different mouse tissues (tumour, spleen, tumour draining lymph node [TDLN] and blood) treated with an isotype control antibody or anti-CD4 and anti-CD8 depleting antibodies. *** = P value ≤ 0.001 and ** = P value ≤ 0.01 (2-way ANOVA with Tukey's multiple comparison).

Supplementary figure 6: (A) Kaplan-Meier plot depicting the survival of mice injected sub-cut with CT26.WT syngeneic tumour cells line and treated with isotype control, KY1044 mIgG2a and anti-PD-L1 (3 and 10 mg/kg, respectively vertical dotted lines) or KY1044 mIgG1 and anti-PD-L1 (3 and 10 mg/kg, respectively vertical dotted lines). (B) spider plots showing the growth of CT26 tumours implanted in mice and treated with a combination of KY1044 mIgG2a with anti-PD-L1 (equivalent of 3 and 10 mg/kg, respectively vertical dotted lines, left) or a combination of KY1044 mIgG2a with anti-PD1(clone RMP1.14, equivalent of 3 and 10 mg/kg, respectively vertical dotted lines).

Supplementary figure 7: The combination of KY1044 mIgG2a with anti-PD-L1 is highly effective in the MC38 syngeneic tumour model. Spider plots showing individual mouse tumour volumes from C57Bl/6 mice (n=8 per group) harbouring subcutaneous MC38 tumours. Tumour bearing mice were dosed i.p (vertical dotted lines) with 60 μ g of KY1044 mIgG2a and/or 200 μ g of anti-PD-L1 starting from day 8 post tumour cell implantation

Supplementary figure 8: Flow cytometry analysis showing the percentage of CD8 T cells in the tumour and spleen of CT26 tumour bearing mice treated with different doses of KY1044. High dose KY1044 only marginally affects the percentage of CD8⁺ T in CT26 tumours but not in the spleen of tumour bearing mice (n=7/8 mice per groups) (A and B). (C) Longitudinal in vivo pharmacodynamic study looking at the T_{regs} content over time and the CD8 to T_{reg} ratio (Day 20) in CT26 tumours treated with different doses of KY1044. Note that the intermediate dose of 3mg/kg was associated with long term Treg depletion and the highest increase in the CD8 to T_{reg} ratio. (2-way ANOVA with Tukey's multiple comparison).

Supplementary figure 9: KY1044 hIgG1 exhibits dose-proportional linear pharmacokinetics in cynomolgus monkeys after weekly repeated i.v. dosing (serum profiles after the first and penultimate dose (Days 1 and 22; A) and full ICOS occupancy was maintained on blood CD4+

1248 cells for all treated animals for the duration of the study (B). KY1044 hIgG1 does not have a
 1249 notable effect on circulating T_{Reg} or CD8+ T cells in blood (C and D) or the proportion of T_M, T_{Reg}
 1250 or CD8+ T cells in the lymph nodes of NHP (E to G).

1251 **Supplementary table 1:** Table summarizing the genes used to define the 27 immune cell
 1252 subtypes analysed from the single cell RNA sequencing data generated from paired PBMC
 1253 and tumour samples from 5 NSCLC cancer patients.

Figure 1

A.

B.

Figure 1

C.

Figure 1

D.

Figure 1

E.

Figure 1

Figure 2

A. ADCC on ICOS⁺ cell lines*

*Promega assay

B. ADCC using primary NK cells on human ICOS+CEM cells

Figure 2

C.

D.

Cell lines assays (MJ ICOS + cells)

Figure 2

E.

F.

Figure 2

Figure 2
H.

Figure 3

A.

B.

Figure 4

Figure 4

C.

	Models	KY1044 Monotherapy	Anti-PD-L1 Monotherapy	Combo KY1044 with anti-PD-L1
Sensitive models	CT26 (Balb/c)	+/-	+/-	++
	A20 (Balb/c)	+++	+/-	+++
	J558 (Balb/c)	++	++	+++
	MC38 (C57Bl/6)	+/-	+	++
Resistant models	B16F10 (C57Bl/6)	-	+/-	+/- (*)
	EL4 (C57Bl/6)	-	-	-
	4T1 (Balb/c)	-	-	-

(-) no efficacy; (+/-) tumour growth delay or CR <20%; (+) CR (20-39%); (++) CR (39-99%); (+++) CR (100%)

(*) efficacy similar to anti-PD-L1 monotherapy

Figure 5

Figure 5

D.

Cynomolgus monkey blood data	TCM	TRegs	TFH
Cell frequency (% of CD3+CD4+ cells) in the blood	20-40%	2-3%	10-30%

Figure 5

E.

CD3⁺CD4⁺CD95^{high} (total memory) CD4⁺ T cells in blood

F.

CD3⁺CD4⁺CD185⁺ (T follicular helper) cells in blood

Figure 6

A.

Figure 6

B.

Figure 7

Supplementary figures

Supplementary Figure 1:

Supplementary Figure 1:

C.

Supplementary figure 2

C

ISOTYPE CONTROL (20ug/mL)

ANTI-ICOS (20ug/mL)

Supplementary figure 2

D

ICOS agonism changes the cell morphology in a dose dependent manner (timepoint 48hours)

Supplementary figure 2

E

Supplementary figure 3

Supplementary figure 4

Supplementary figure 5

Supplementary figure 6

B.

70 % cure rate
with KY1044 + anti-PD-L1 combination

10% cure rate
with KY1044 + anti-PD-1 combination

Supplementary figure 6

MC38 model

Supplementary figure 7

Dunn's multiple comparisons test: all non-significant

Dunn's multiple comparisons test: all non-significant

Supplementary figure 8

C.

No depletion observed in spleen or lymph nodes

Dunnett's multiple comparisons test

Supplementary figure 8

Supplementary Figure 9

A.

B.

Supplementary Figure 9

C.

T_{REG} cells in blood

D.

CD3⁺CD8⁺ (cytotoxic) T cells in blood

Supplementary Figure 9

E.

F.

G.