

ORIGINAL RESEARCH



## Blocking CTLA-4 while priming with a whole cell vaccine reshapes the oligoclonal T cell infiltrate and eradicates tumors in an orthotopic glioma model

Cameron S. Field<sup>a,b</sup>, Martin K. Hunn<sup>a,c,d</sup>, Peter M. Ferguson<sup>d</sup>, Christiane Ruedl<sup>e</sup>, Lindsay R. Ancelet<sup>a,f,†</sup>, and Ian F. Hermans<sup>a,b,f,†</sup>

<sup>a</sup>Malaghan Institute of Medical Research, Wellington, New Zealand; <sup>b</sup>School of Biological Sciences, Victoria University of Wellington, Wellington, New Zealand; <sup>c</sup>Department of Neurosurgery, The Alfred Hospital, Melbourne, Australia; <sup>d</sup>Capital and Coast District Health Board, Wellington, New Zealand; <sup>e</sup>School of Biological Sciences, Nanyang Technological University, Singapore; <sup>f</sup>Maurice Wilkins Centre, Auckland, New Zealand

### ABSTRACT

Vaccine-mediated cancer treatment is unlikely to induce long-term survival unless suppressive mechanisms are overcome. Given the success of antibody-mediated immune checkpoint blockade in relieving regulation of endogenous anti-tumor T cell responses in tumor-burdened hosts, we investigated whether checkpoint blockade could improve the efficacy of responses induced with a whole tumor-cell vaccine. We show that administration of a single dose of blocking antibody was sufficient to significantly enhance antitumor activity of the vaccine, inducing complete radiological regression of established intracranial tumors. The antibody or vaccine alone were ineffective in this setting. The antibody had to be administered before, or close to, vaccine administration, suggesting CTLA-4 blockade had an impact on early priming events. The combined treatment resulted in enhanced trapping of leukocytes in the lymphoid tissues, including T cells that had undergone significant proliferation. There were no obvious changes in the stimulatory function of antigen-presenting cells or the number and function of regulatory T cells, suggesting T cells were the targets of the checkpoint blockade. While tumors regressing under combined treatment were highly infiltrated with a variety of leukocytes, tumor eradication was dependent on CD4<sup>+</sup> T cells. Analysis of the TCR repertoire showed that the addition of anti-CTLA-4 at priming reshaped the repertoire of tumor infiltrating T cells. In particular, the oligoclonal populations became greater in magnitude and more diverse in specificity. Using anti-CTLA-4 in a restricted way to promote the priming phase of an anti-cancer vaccine may offer a useful way of harnessing clinical benefit from this powerful agent.

### ARTICLE HISTORY

Received 25 May 2017  
Revised 16 August 2017  
Accepted 30 August 2017

### KEYWORDS



Vaccine; checkpoint blockade; anti-CTLA-4; glioma; TCR sequencing


### Introduction

The quality of T cell responses is regulated by a balance of co-stimulatory and inhibitory signals received by T cells at different times during their differentiation into effector cells. The inhibitory signals, which are mediated by a series of receptors on the T cell surface, are often referred to as immune checkpoints because they maintain self-tolerance and prevent autoimmunity by limiting the activity of self-reactive T cells. However, by influencing the expression of these receptors and their ligands, tumors exploit these same inhibitory pathways to limit the activity of endogenous anti-tumor T cell responses that are often induced in cancer patients.<sup>1</sup> The development of monoclonal antibodies to block immune checkpoints is a new treatment modality that is transforming cancer treatment, exemplified by the recent regulatory approval of blocking antibodies against cytotoxic T lymphocyte antigen-4 (CTLA-4)<sup>2</sup> and programmed death-1 (PD-1).<sup>3</sup> While these treatments have validated the concept of enhancing T cell-mediated immune responses for clinical impact, it is clear that not all patients respond. Given that checkpoint blockade relies on unleashing endogenous anti-tumor T cell responses, it is

possible that patients who fail to respond to treatment have not induced endogenous T cell responses of sufficient quality or quantity in the first place. There is therefore considerable interest in using vaccines to initiate anti-tumor responses (or boost weak responses), which could then potentially be enhanced through checkpoint blockade.

Despite the central nervous system (CNS) traditionally being considered an immune-privileged site, there is now accumulating evidence that malignant tissue in the CNS can be targeted by T cells,<sup>4</sup> giving impetus to develop vaccines for diseases like high-grade glioma for which current treatments are generally ineffective. However, the development of effective cancer vaccines faces numerous challenges, including a high level of tumor heterogeneity and a paucity of defined glioma-associated antigens. With surgery being a mainstay of glioma treatment, it is possible to use resected tumor material as a “personalized” source of tumor antigens. Indeed, intact irradiated tumor cells can provide a ready-made vehicle for delivery of these antigens to antigen-presenting cells (APCs) within the host. However, to elicit an effective T cell response it is critical that these APCs also receive strong licensing

**CONTACT** Ian F. Hermans  [ihermans@malaghan.org.nz](mailto:ihermans@malaghan.org.nz)  Malaghan Institute of Medical Research, PO Box 7060, Wellington, 6242, New Zealand.

 Supplemental data for this article can be accessed on the [publisher's website](#).

<sup>†</sup>L. R. Ancelet and I. F. Hermans contributed equally to the last authorship.

© 2018 Taylor & Francis Group, LLC

signals. We have recently explored the potential of a vaccine comprised of irradiated glioma cells pulsed with the glycolipid  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer), a compound that stimulates type I natural killer T cells (NKT cells). The glycolipid is acquired by host APCs and presented to NKT cells via the MHC-I like molecule CD1d;<sup>5</sup> the NKT cells then license the APCs through CD40 signaling to increase their stimulatory function. Since CD1d is a non-polymorphic molecule and the transgenic T cell receptor (TCR) of NKT cells is largely invariant, the licensing afforded by NKT cells is independent of tissue type, making  $\alpha$ -GalCer an attractive immune adjuvant with general utility. In an orthotopic murine model of glioma we have demonstrated that such  $\alpha$ -GalCer-adjuvanted vaccines prevent the development of tumors in a challenge setting,<sup>6</sup> but were insufficient to bestow long-term survival in a therapeutic setting unless regulatory T cells (Tregs) mediated suppression was overcome.

Here we investigated whether anti-tumor responses generated by an  $\alpha$ -GalCer-adjuvanted glioma vaccine can benefit from checkpoint blockade with anti-CTLA-4 ( $\alpha$ -CTLA-4). Because CTLA-4 is upregulated early after T cell activation, we reasoned that antibody administration close to vaccination may have most impact. We show that using  $\alpha$ -CTLA-4 in this limited fashion to reduce regulation around the priming phase reshapes the size and diversity of T cell populations that infiltrate intracranial tumors. This was associated with long-term survival in the majority of treated animals.

## Results

### ***Therapeutic vaccination can be enhanced by CTLA-4 blockade in an orthotopic murine model of glioma to induce complete radiological regression***

Cells from the glioma cell line GL261 engraft and form solid tumors when injected subcutaneously into C57BL/6 mice. This tumor model was used to assess the efficacy of a vaccine based on intravenous injection of  $\alpha$ -GalCer-pulsed, irradiated GL261 cells. When the vaccine was administered seven days before tumor challenge, significant protection was observed (Fig. 1A, Left). However, when administered seven days after challenge, vaccination failed to eradicate the established tumors (Fig. 1A, Right). To determine whether therapeutic outcomes could be improved by checkpoint blockade, vaccination was combined with blocking antibodies for CTLA-4. A single dose of  $\alpha$ -CTLA-4 administered by intraperitoneal injection one day prior to vaccination (day 6 after tumor challenge), or three days after vaccination (day 10) induced complete tumor regression after an initial period of tumor growth (Fig. 1B), although reduced tumor growth was observed when  $\alpha$ -CTLA-4 was delivered prior to vaccination.

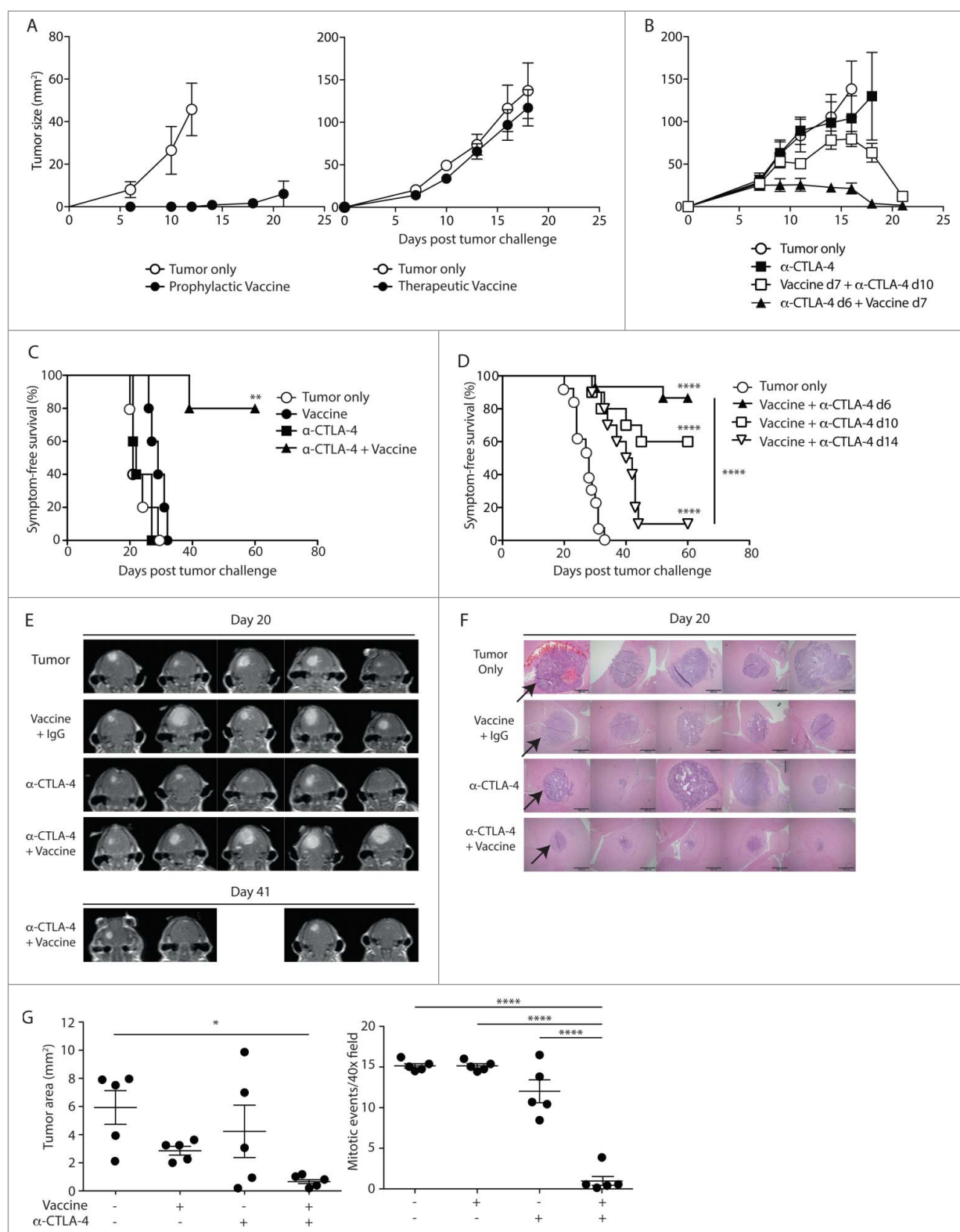
We next investigated whether anti-tumor vaccination could be improved by checkpoint blockade in an intracranial setting. Neither vaccination or  $\alpha$ -CTLA-4 alone had any impact on symptom-free survival in this setting. However, a single dose of  $\alpha$ -CTLA-4 prior to vaccination produced a significant anti-tumor response (Fig. 1C), preventing onset of tumor-associated symptoms in the majority of mice. As was observed in the subcutaneous setting, delaying administration

of  $\alpha$ -CTLA-4 until after vaccine delivery reduced tumor-free survival, suggesting that blockade of CTLA-4 signaling was most relevant when applied close to immune priming (Fig. 1D). No evidence of neurologic deficit was observed in any of the treated mice, and long-term survivors showed healthy weight gain suggesting no obvious morbidity (*data not shown*). Also, mice that responded completely to the combined treatment were able to reject subcutaneous tumor challenge 100 days after initial intracranial implantation, suggesting a response with immunological memory had been induced (Supplementary Fig. 1).

We performed radiological and histopathological analysis of the tumors subjected to vaccination with or without prior  $\alpha$ -CTLA-4 treatment to determine how combination therapy affected the rate of tumor progression. Analysis by MRI 20 days post tumor implantation revealed gadolinium enhanced regions in the brains of all challenged mice regardless of treatment. As expected, all untreated mice and those that received monotherapy developed glioma-associated symptoms and had to be culled within a few days of this analysis. In contrast, four of the five mice that received the combination treatment remained symptom-free, and could be scanned again at day 41. Of these, two now showed complete loss of the enhanced regions, another was substantially reduced in size, and the fourth increased in size only slightly. In a separate experiment, histopathological analysis of tumors on day 20 demonstrated that animals that received the combined treatment had significantly reduced tumor size compared to untreated animals (Fig. 1E, F). Mice treated with the vaccine or  $\alpha$ -CTLA-4 alone had hypercellular tumors with a high mitotic rate (Fig. 1G, Supplementary Table 1), whereas mice treated with the combination had relatively hypocellular tumors with low mitotic activity. Thus, tumors were clearly engrafted in all groups, but the combination treatment already showed some antitumor impact by day 20. It was notable that these tumors were more infiltrated with immune cells than tumors treated with either monotherapy (Supplementary Fig. 2, Supplementary Table 1). The apparent discrepancy between tumors that appeared larger on MRI than by histology may be analogous to the phenomenon of pseudoprogression observed in human patients undergoing treatment for glioma.<sup>7</sup> Lymphocytes were more commonly seen in the peritumoral brain of the combined treatment group but it is unlikely this increased cellularity alone could account for the discrepancy between MRI and histology. A more likely explanation is that the abundant cellular infiltrate in the tumor is associated with greater capillary permeability, contributing to accumulation of gadolinium and oedema fluid in perilesional tissues.

### ***Vaccine-induced activation of NKT cells is not affected by CTLA-4 blockade***

We next investigated how the combination of CTLA-4 blockade and vaccination affected early immune events in lymphoid tissues. The combined therapy failed to prevent tumor growth in CD1d-deficient animals (Fig. 2A), suggesting that even in the presence of  $\alpha$ -CTLA-4 the mode of activity of the vaccine



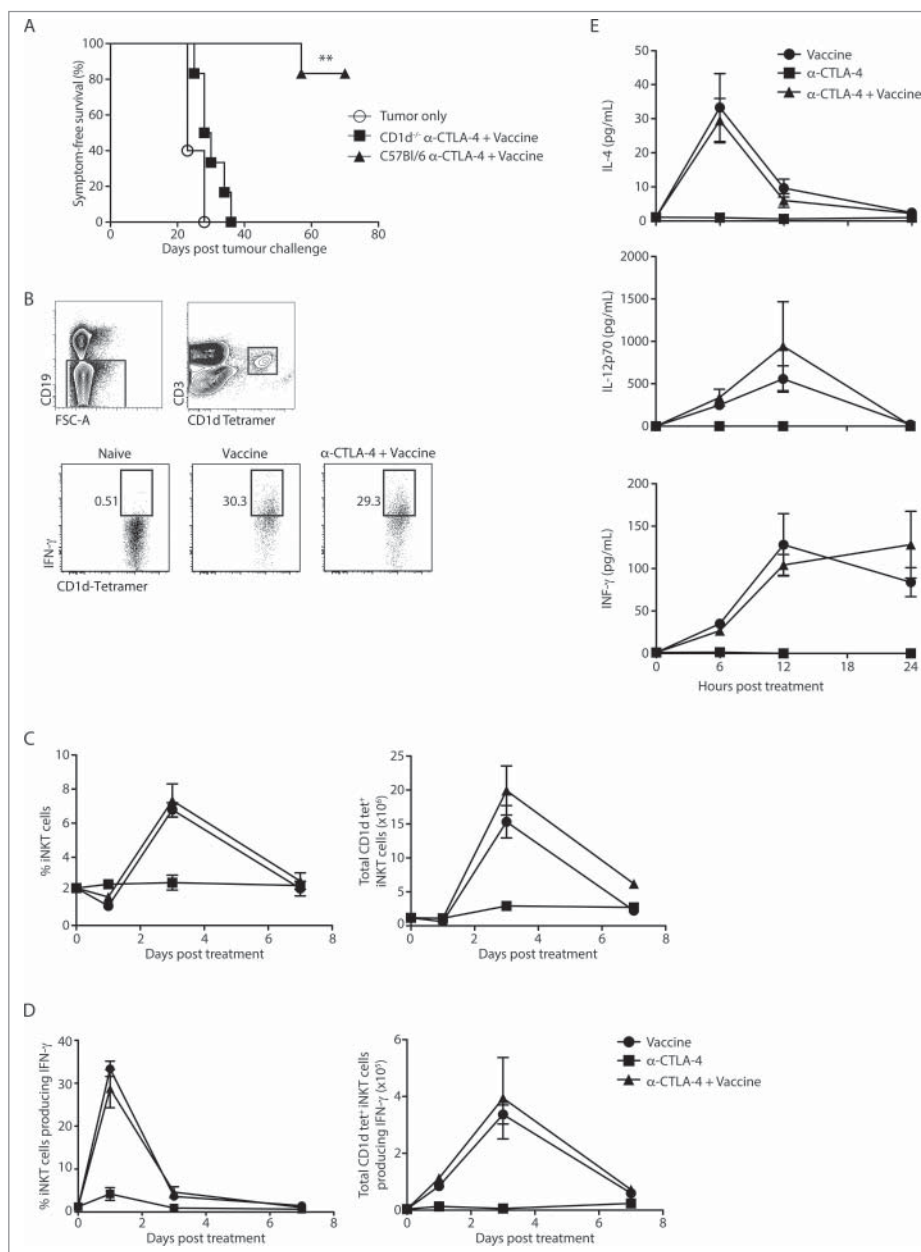
**Figure 1.** Treatment of subcutaneous or intracranial tumors with vaccination and checkpoint blockade. (A) Left, mean tumor size ( $\pm$  SEM) in groups of mice ( $n = 5$ ) subcutaneously challenged with GL261 cells 7 days following vaccination. Untreated mice served as tumor only controls. Right, mice were challenged with GL261 cells and treated with vaccine on day 7. (B) Mean tumor size ( $\pm$  SEM) in groups of mice ( $n = 5$ ) subcutaneously challenged with GL261 cells on day 0 and then treated with either  $\alpha$ -CTLA-4 alone on day 6, vaccine on day 7 combined with prior  $\alpha$ -CTLA-4 on day 6, or vaccine on day 7 combined with delayed  $\alpha$ -CTLA-4 on day 10. Untreated mice served as tumor only controls. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*\*  $P < 0.0001$ . Representative of three independent experiments. (C) Survival curves for mice with intracranial tumors treated with either vaccine alone on day 7,  $\alpha$ -CTLA-4 alone on day 6, or both \*\*  $P < 0.01$ . Results are representative of three independent experiments. (D) Survival curves for mice with intracranial tumors treated with vaccine on day 7 together with  $\alpha$ -CTLA-4 on either day 6, day 10 or day 14 \*\*\*\*  $P < 0.0001$ . Results represent combined data from two experiments. (E) MR images of brains of mice with intracranial tumors treated with either vaccine alone on day 7,  $\alpha$ -CTLA-4 alone on day 6, or both. (F) In a separate experiment, mice were challenged and treated as above and brains were removed on day 20 for histological analysis with hematoxylin and eosin staining. Tumor borders are indicated by arrows. (G) Mean tumor area  $\pm$  SEM was calculated per treatment group, together with mean number of mitotic events per high power field  $\pm$  SEM, as determined by a histopathologist blinded to sample groups. \*  $P < 0.05$  \*\*\*\*  $P < 0.0001$  ( $n = 5$  per group).

was still reliant on activated NKT cells, reflecting their ability to license APCs. It was possible that  $\alpha$ -CTLA-4 enhanced NKT cell activation, improving their licensing function. To address this, we assessed the proportion, number and function of CD1d-restricted NKT cells in the spleen following vaccine treatment with or without prior  $\alpha$ -CTLA-4 administration. Blocking CTLA-4 signaling had no impact on the percentage of NKT cells detected in the spleen seven days after vaccination, nor did it have any impact on their capacity to produce IFN- $\gamma$  (Fig. 2B-D). We have previously shown that the  $\alpha$ -GalCer-adjuvanted vaccine triggers NKT cell-dependent release of measurable quantities of IL-4 and IFN- $\gamma$  into the serum, as

well IL-12p70, which likely derives from the licensed APCs.<sup>6</sup> No differences in the kinetics or magnitude of cytokine accumulation in the serum were observed when  $\alpha$ -CTLA-4 was combined with the vaccine (Fig. 2E), again suggesting blocking CTLA-4 signaling did not enhance NKT cell activation.

### **Inhibition of CTLA-4 signaling does not enhance the stimulatory capacity of APCs**

Trogoctosis of CD80 and CD86 by CTLA-4 expressing cells has been reported to maintain low activation status of APCs.<sup>8</sup> It was therefore possible that inhibition of CTLA-4

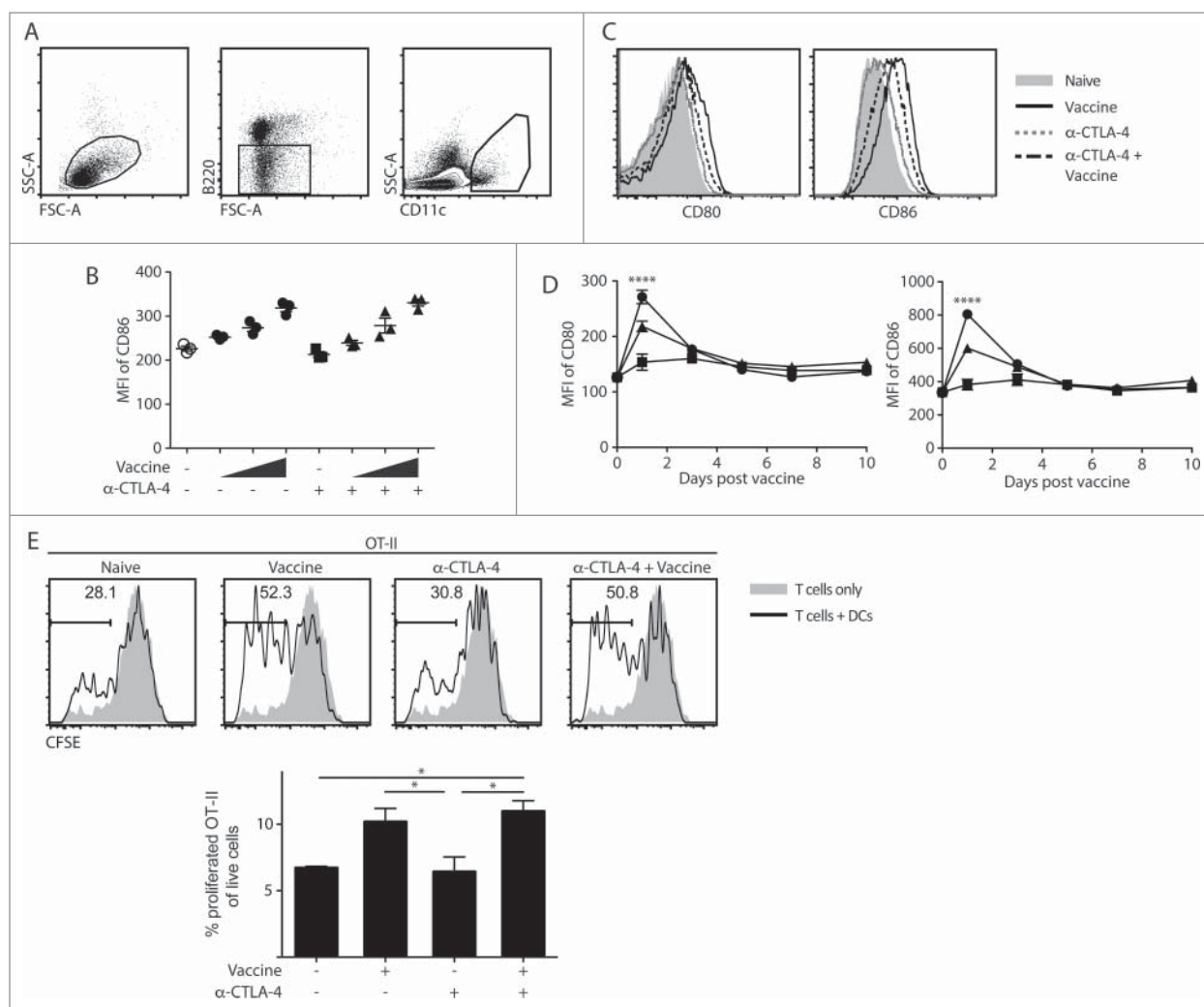


**Figure 2.** Improved therapeutic efficacy of vaccine by CTLA-4 blockade is dependent on NKT cells, but is not due to enhanced NKT cell activity. (A) Survival curves for wild-type C57BL/6 or CD1d<sup>-/-</sup> mice with intracranial tumors that were treated with vaccine and  $\alpha$ -CTLA-4. Untreated C57BL/6 mice served as tumor only controls \*\*  $P < 0.01$  ( $n = 5$  per group). Results are representative of three independent experiments. (B) Gating strategy used to enumerate NKT cells and examine their IFN- $\gamma$  expression in spleen after treatment with vaccine with or without  $\alpha$ -CTLA-4. (C) Mean percentage and number of NKT cells per treatment group ( $\pm$  SEM) at indicated times. (D) Mean percentage and number of IFN- $\gamma$ -producing NKTs on day 7. Results in B-D are representative of two independent experiments. (E) Mice subjected to the same treatment were bled at the indicated times to determine levels of cytokines IL-4, IL-12p70 and IFN- $\gamma$  in serum. Mean values per group ( $n = 5$ )  $\pm$  SEM are shown. Results are representative of two independent experiments.



during immune priming compromised this process, enhancing the co-stimulatory status of APCs involved in T cell activation. To explore this, the expression of CD86 was examined on splenocytes co-cultured with titrated doses of vaccine in the presence or absence of  $\alpha$ -CTLA-4 *in vitro*. While cultures containing the vaccine showed increased expression of CD86 on CD11c<sup>+</sup> dendritic cells (DCs) in a dose-dependent manner, reflecting the adjuvant activity of  $\alpha$ -GalCer, this was not affected by the presence of  $\alpha$ -CTLA-4 (Fig. 3A, B). No impact of combination with  $\alpha$ -CTLA-4 was seen on other APCs including plasmacytoid DCs and B cells, nor were other markers of APC activation affected, including CD40 (*data not shown*). In a similar *in vivo* experiment, activation of splenic APCs was assessed at various times following vaccination with or without prior administration of  $\alpha$ -CTLA-4, or following administration of  $\alpha$ -CTLA-4 alone. Peak expression of CD80 and CD86 on

CD11c<sup>+</sup> DCs was observed 24 hours following vaccination, with the highest expression observed in vaccinated animals that did not receive  $\alpha$ -CTLA-4 (Fig. 3C, D). To elucidate whether  $\alpha$ -CTLA-4 administration imparted functional differences in APCs, we assessed the ability of APCs isolated from treated mice to induce T cell proliferation *in vitro*. Splenocytes isolated from mice 18 hours after treatment were pulsed with peptide corresponding to the I-A<sup>b</sup>-binding epitope of chicken ovalbumin and cultured with CFSE-labelled CD4<sup>+</sup> T cells from OT-II mice. While splenocytes from vaccinated mice induced increased proliferation of the transgenic CD4<sup>+</sup> T cells relative to splenocytes from untreated controls, the proportion of proliferating cells was not affected by *in vivo* administration of  $\alpha$ -CTLA-4 (Fig. 3E). These data suggest that treatment with  $\alpha$ -CTLA-4 prior to vaccination does not enhance the stimulatory function of APCs in this model.



**Figure 3.** Blockade of CTLA-4 does not increase stimulatory function of APCs. Splenocytes from mice treated 24 hours earlier with  $\alpha$ -CTLA-4 were incubated with titrated doses of vaccine *in vitro*. Expression of the activation marker CD86 was assessed 16 hours later on DCs, defined as CD11c<sup>+</sup> B220<sup>-</sup> cells (A) Gating strategy used following exclusion of dead cells and doublets. (B) MFI of CD86  $\pm$  SEM ( $n = 3$ ). Triangles indicate increasing doses of vaccine cells from left to right ( $1 \times 10^5$ ,  $3.3 \times 10^5$ ,  $1 \times 10^6$ ). Results are representative of two independent experiments. (C) Mice were treated with  $\alpha$ -CTLA-4 alone,  $\alpha$ -CTLA-4 followed by vaccine 24 h later, or both. Expression of CD80 and CD86 was determined on DCs at the indicated times. Histograms are representative of analysis of CD80 and CD86 after 24 hours (D) Graphs show MFI of CD80 and CD86 ( $\pm$  SEM) for each treatment group ( $n = 4$ ); significance between vaccine alone, and combined treatment is indicated. \*\*\*\*  $P < 0.001$ . (E) Splenocytes from mice treated as in C were pulsed with OVA<sub>323-339</sub> peptide for 2 hours and used to stimulate CFSE-labelled OT-II cells *in vitro* for 6 days. The extent of proliferation induced was determined by examining CFSE of T cells cultured with splenocytes (black line) relative to T cells cultured alone (shaded grey). Mean percentage of proliferated cells ( $\pm$  SEM) per treatment group ( $n = 3$ ) are presented \*  $P < 0.05$ . Results representative of two independent experiments.

### Enhanced proliferation and accumulation of vaccine-induced T cells with CTLA-4 blockade

The expression of CTLA-4 is upregulated early after T cell activation, and signaling through this molecule is known to negatively impact on T cell proliferation.<sup>9,10</sup> To explore whether the early blocking of CTLA-4 affected the proliferation potential of the vaccine-stimulated T cells, CFSE-labelled splenocytes from CD45.1<sup>+</sup> congenic mice were transferred into naïve C57BL/6 mice (CD45.2<sup>+</sup>) one day prior to treatment, and splenocytes from host mice were analyzed one week later to assess the proliferation of the donor cells. While a modest trend towards increased proliferation of T cells was observed with vaccine treatment, it was interesting that significant proliferation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells was observed in mice treated with  $\alpha$ -CTLA-4 alone, suggesting inhibition of CTLA-4 signaling can unleash T cell proliferation in the absence of an obvious priming signal in this model. The greatest proliferative response, involving CD4<sup>+</sup> and CD8<sup>+</sup> T cells, was observed when mice were treated with vaccine and  $\alpha$ -CTLA-4 (Fig. 4A, B). To establish whether such enhanced proliferation corresponded with an augmented T cell response in the lymphoid tissues of mice that received the combined treatment in the absence of adoptive transfer, we next examined the number and proportion of T cells in the spleen on different days after vaccination. Significantly increased cellularity was observed after three days in mice administered the vaccine alone compared to untreated controls, which was reduced to normal levels by day 7. Increases were most pronounced in mice administered  $\alpha$ -CTLA-4 and vaccine, with splenocyte numbers remaining high at day 7 (Fig. 4C). This increase in cellularity involved all cell-types examined (*data not shown*). However, significant changes in the T cell compartment were observed, with numbers of CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells increased with the combined therapy in the spleen (Fig. 4E, F), while no changes in the number of regulatory T cells (CD3<sup>+</sup> CD4<sup>+</sup> FoxP3<sup>+</sup> cells; Tregs) were observed (Fig. 4D). The function of isolated Tregs was not altered by treatment (Supplementary Fig. 3). Interestingly, although significant proliferation of CFSE labelled cells had been observed in the earlier experiment in mice that received  $\alpha$ -CTLA-4 alone, this observation was not reflected in increased T cell accumulation in the spleen. Vaccine-induced stimulation was therefore required to drive the significant T cell accumulation seen with the combined treatment, and indeed was required for the phenomenon of increased cellularity.

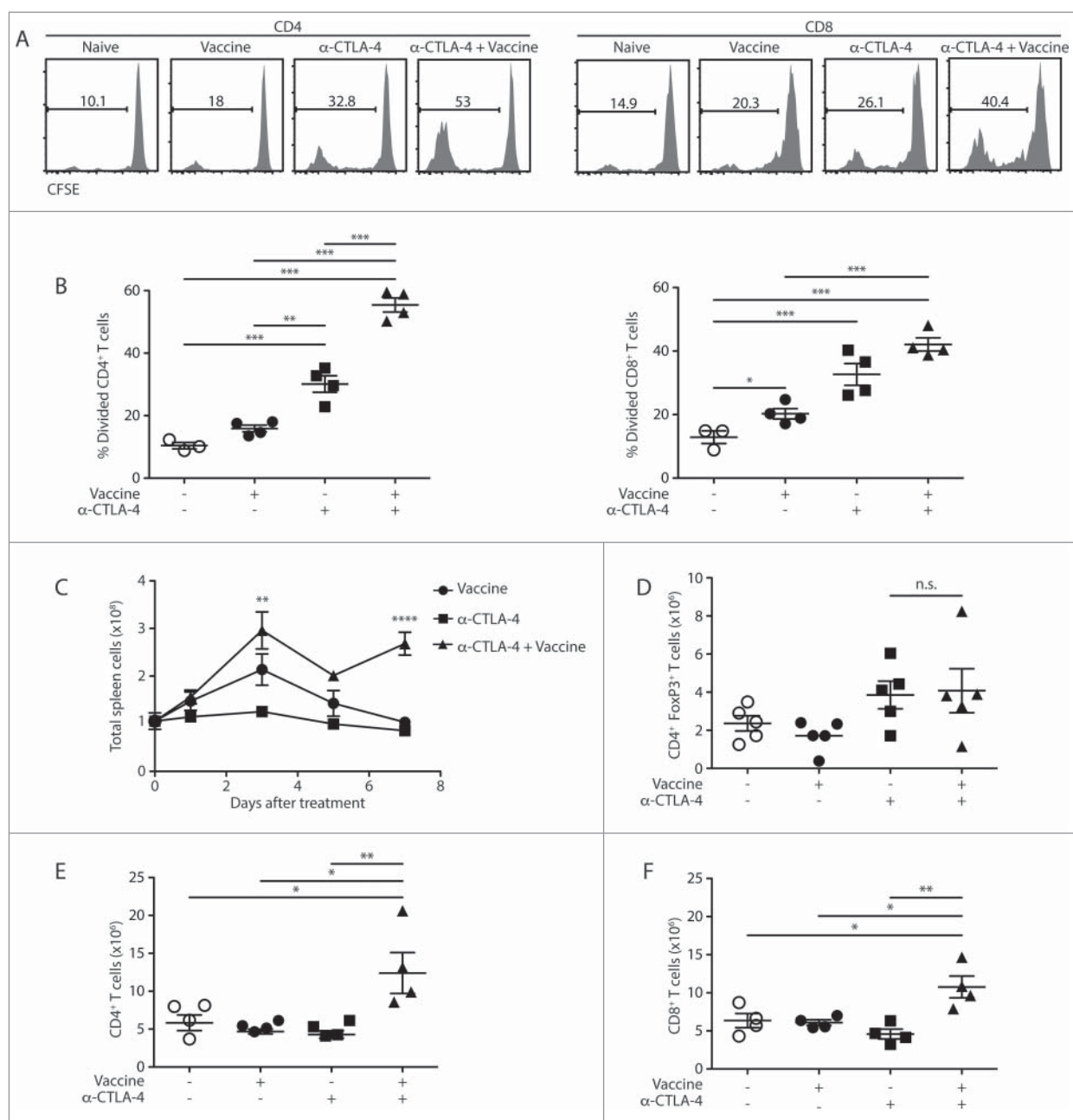
The phenotype of the CD4<sup>+</sup> and CD8<sup>+</sup> T cell population in the spleen was assessed at various times following the different treatment regimens based on expression of CD44 and CD62L (Fig. 5A). A greater than 5-fold increase in effector CD4<sup>+</sup> T cells (CD44<sup>hi</sup> CD62L<sup>lo</sup>) was observed in the spleens of mice treated with  $\alpha$ -CTLA-4 prior to vaccination, compared to vaccine or  $\alpha$ -CTLA-4 monotherapy, with peak expansion occurring at day 7 (Fig. 5B). Numbers of CD8<sup>+</sup> effector T cells were also increased, albeit to lower levels than CD4<sup>+</sup> T cells, and occurring earlier at day 3 (Fig. 5C, D). Similar results were observed in the mediastinal lymph nodes (Supplementary Fig. 4). Also, a greater proportion of splenic CD4<sup>+</sup> and CD8<sup>+</sup> T cells from mice subjected to combination therapy produced IFN- $\gamma$  and TNF- $\alpha$  following re-stimulation *in vitro* with GL261

tumor lysate (Fig. 5E, F), suggesting tumor-specific T cells were highly represented in the expanded population. Together these data suggest that the combined treatment creates an environment in which leukocytes accumulate in the lymphoid tissues, with T cells highly represented. Neither treatment alone had this outcome.

### Therapeutic efficacy of vaccine and $\alpha$ -CTLA-4 is CD4<sup>+</sup> T cell dependent

To explore the effector phase of the combined treatment strategy, immune infiltrates in the whole brain were analyzed seven days after therapy (Fig. 6). No obvious increases in infiltration were observed with  $\alpha$ -CTLA-4 treatment alone. A trend towards increased number of CD45<sup>+</sup> immune cells was observed in the brains of mice treated with vaccine alone compared to no treatment, but statistically significant increases were only seen when vaccination was combined with  $\alpha$ -CTLA-4, predominantly featuring macrophages (CD45<sup>hi</sup> CD11b<sup>+</sup> cells), microglia (CD45<sup>int</sup> CD11b<sup>+</sup>) and lymphocytes (CD45<sup>hi</sup> CD11b<sup>-</sup>) (Fig. 6A-C). Interestingly, within the T cell compartment, CD4<sup>+</sup> T cells were the most highly represented, remarkably accounting for 60–75% of CD3<sup>+</sup> cells in the brain. In contrast, no differences in numbers of CD8<sup>+</sup> or Tregs were seen with the different treatments (Fig. 6D, E). In MHC II-deficient mice, which are unable to mount CD4<sup>+</sup> T cell responses, the combined therapy failed to control tumor growth, whereas this treatment was still effective in TAP1-deficient mice, despite the significantly reduced CD8<sup>+</sup> T cell repertoire in these animals (Fig. 6F). These data suggest that CTLA-4 blockade can improve the outcome of vaccination by promoting potent CD4<sup>+</sup> T cell-mediated responses that effectively marshal the elimination of established glioma. Mechanistically, this may involve cytokine-mediated activation of myeloid cells, as most activity was lost when the combined treatment was used in animals deficient in IFN- $\gamma$ , or when CD11b<sup>+</sup> cells were depleted with diphtheria toxin (DT) in transgenic mice with human DTR expressed from the CD11b promoter (Supplementary Fig. 5).

To examine the impact of CTLA-4 blockade on vaccine-induced T cell responses, we sequenced TCR $\beta$  CDR3 regions from tumor-bearing mice, taking samples from pre- and post-treatment blood and from post-treatment brain tissue to determine whether antitumor efficacy was associated with general changes in TCR diversity. Examining responses induced by the vaccine alone, there were no changes in overall richness (number of unique productive rearrangements in test samples) or clonality of T cells in the blood after treatment, but lower richness and higher clonality of T cells in the brain. This indicates that the brain tissue (and likely the tumor) was infiltrated with a limited subset of T cells from the available repertoire (Fig. 7A). When the vaccine was combined with CTLA-4 blockade, increased clonality could be seen in the blood post-treatment. Furthermore, there was increased richness and clonality in the brain compared to animals in the vaccine only group, suggesting CTLA-4 blockade had provoked infiltration of a larger oligoclonal population. To emphasize this, heat maps were prepared based on numbers of templates for the top

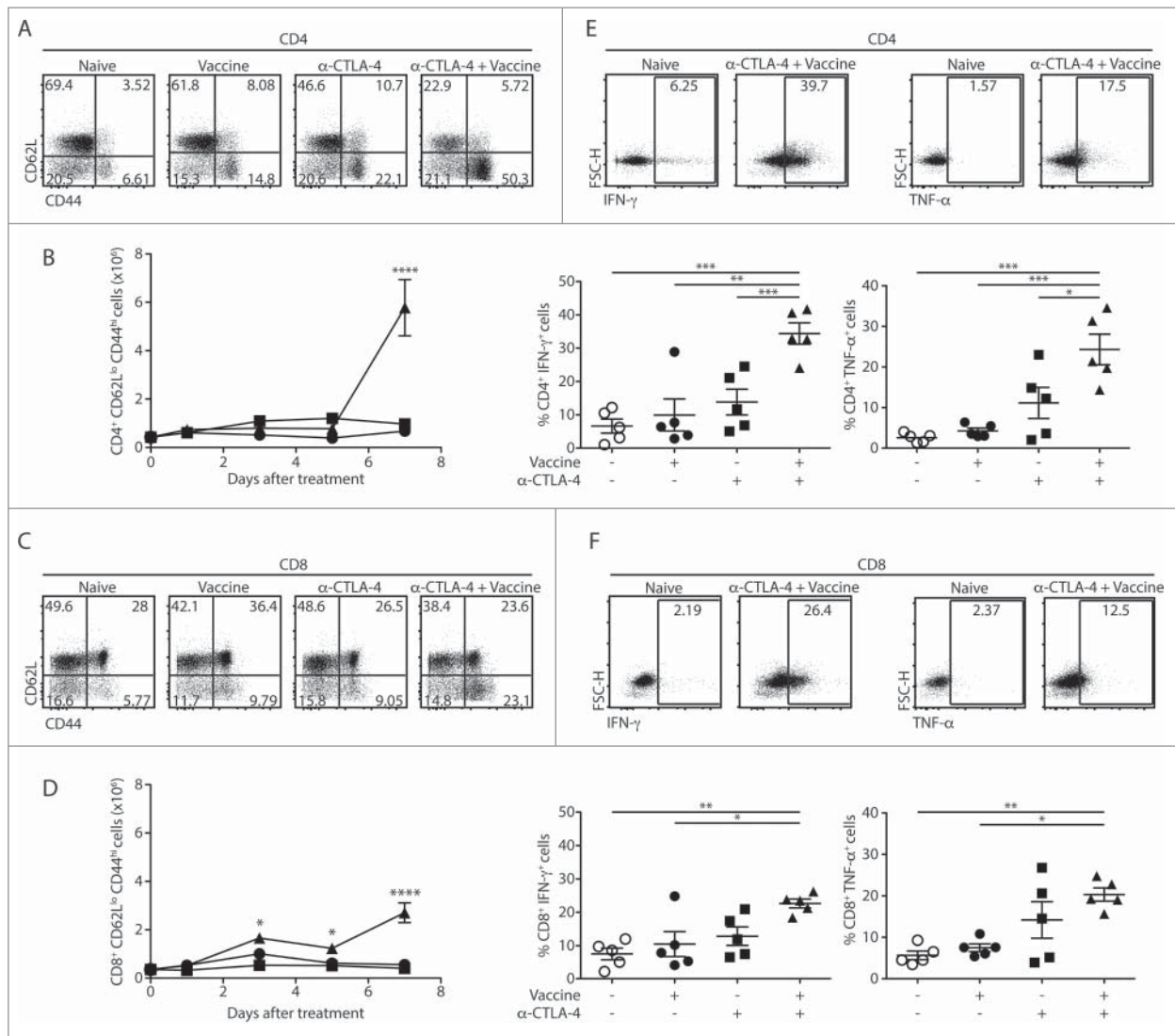


**Figure 4.** Combining vaccination with CTLA-4 blockade enhances T cell proliferation and accumulation in spleen. Proliferation of transferred CFSE-labelled CD45.1<sup>+</sup> splenocytes was examined in CD45.1<sup>-</sup> hosts treated with vaccine,  $\alpha$ -CTLA-4, or both. (A) Representative histograms of CFSE expression on transferred CD4<sup>+</sup> and CD8<sup>+</sup> cells seven days after vaccination. (B) Mean percentage ( $\pm$  SEM) of divided CD4<sup>+</sup> or CD8<sup>+</sup> cells on day 7 ( $n = 4$  per treatment group) \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.005$ , \*\*\*\*  $P < 0.001$ . Results are representative of two independent experiments. (C) Groups of mice ( $n = 5$ ) were treated with vaccine,  $\alpha$ -CTLA-4, or both. Mean cell count of spleens per treatment group  $\pm$  SEM are shown; significance between vaccinated mice, or mice treated with vaccine and  $\alpha$ -CTLA-4 is shown. \*\*  $P < 0.01$ , \*\*\*\*  $P < 0.001$ . Data are representative of two independent experiments. (D-F) Numbers of Foxp3<sup>+</sup>CD4<sup>+</sup> T cells, CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells were determined in groups treated as in C, together with untreated controls. Mean  $\pm$  SEM per group are shown; results are representative of two independent experiments.

500 rearrangements detected in any of the brain samples (Fig. 7B); this analysis showed a clear increase in magnitude of infiltrates in animals that received the combined treatment. Furthermore, these animals had greater numbers of oligoclonal populations, defined as rearrangements that represented greater than 1 % of the sample (Fig. 7C). Also, these oligoclonal populations were generally of greater magnitude than those seen in the vaccine only group, as defined by mean number of templates detected per clone (Fig. 7D).

Tracking of the top 10 clones in the blood and brain samples from individual mice also revealed some significant differences

in response to the treatment strategies. In animals that received vaccine alone (Fig. 7E; left panels), the majority of high frequency clones in the brain had been at low frequency in the pre-treatment blood samples, and then increased in blood after vaccination - a profile that might intuitively be expected after a vaccine-induced T cell response. Nonetheless, this response was ineffective at limiting tumor growth. The brains of these mice also featured clones that had already been at a high frequency in the blood before treatment. These may have been expanded clonal populations triggered by initial tumor challenge that homed to the brain, or, because some expanded



**Figure 5.** Combining vaccination with CTLA-4 blockade increases accumulation of activated T cells in spleen. Mice were treated with vaccine,  $\alpha$ -CTLA-4, a combination of both, or were left untreated. After seven days, numbers of activated T cells were determined in spleen. (A, C) Representative flow cytometry plots for CD44 and CD62L on CD4<sup>+</sup> and CD8<sup>+</sup> cells (B, D) Mean number of activated T cells (CD44<sup>+</sup>CD62L<sup>+</sup>) per group ( $n = 5$ ); significance between vaccinated mice, or mice treated with vaccine and  $\alpha$ -CTLA-4 is shown. \*  $P < 0.05$ , \*\*\*\*  $P < 0.001$  (E-F) Splenocytes from groups of mice treated as above were restimulated *in vitro* with tumor lysate to assess tumor-specific effector cytokine production. Mean percentage of CD4<sup>+</sup> and CD8<sup>+</sup> T cells expressing IFN- $\gamma$  or TNF- $\alpha$  are shown ( $\pm$  SEM) \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.005$ . Results are representative of two independent experiments.

clones are present in blood of unmanipulated animals, may be unrelated clones that homed to the inflamed brain tissue in a non-specific manner.

In animals that underwent the combined treatment (Fig. 7E; right panels), high frequency clones in brain were also either undetectable or present at high frequency in pre-treatment blood. However, in striking contrast to mice treated with vaccine alone, these clones were not detected in the blood after treatment. It is possible that these “missing” clones had accumulated elsewhere at this time point as a consequence of trapping in lymphoid tissues, non-specific homing to inflamed brain tissue, and/or antigen-specific accumulation within brain tumors. The post-treatment blood samples were instead dominated by clones that, although expanded relative to pre-treatment blood, were not detected in the brain, and were therefore unlikely to have participated in the antitumor response. These clonal

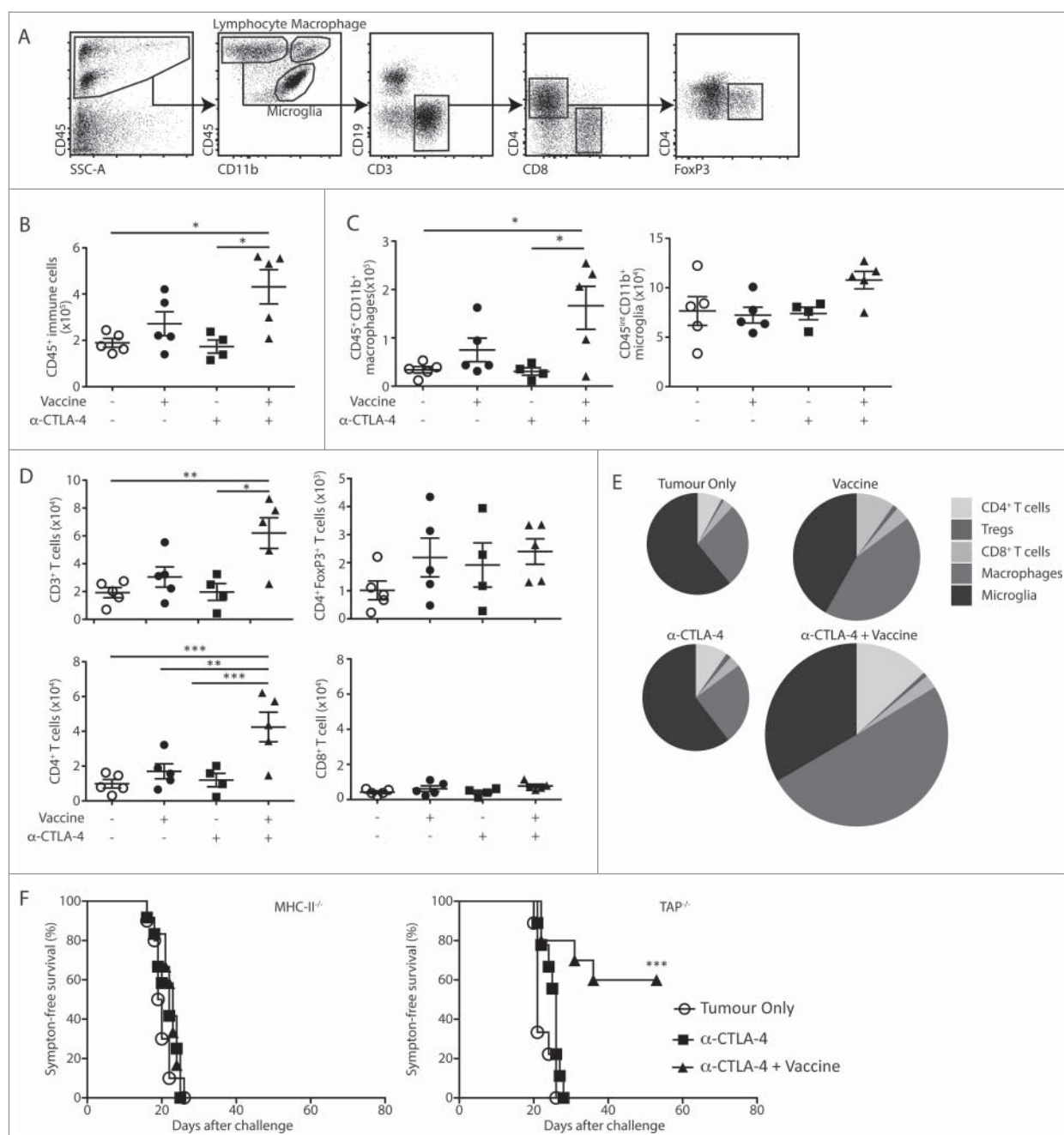
populations likely account for the increased clonality observed in post-treatment blood samples (Fig. 7A).

Overall, given the unique effects of combined treatment on proliferation and partitioning of T cells to blood or tissues, it is hard to draw conclusions on treatment-related TCR diversity based on analysis of post-treatment blood samples. However, in the brain, where the analysis is more clear-cut, the combination of treatments was associated with increased infiltration of a broader range of oligoclonal T cell populations.

## Discussion

Targeting of CTLA-4 and PD-1 with blocking monoclonal antibodies has resulted in unprecedented durable clinical responses in cancer patients, most notably in advanced melanoma.<sup>11</sup> In contrast, the induction of anti-tumor T cells through vaccination has been met with less clinical success, potentially because



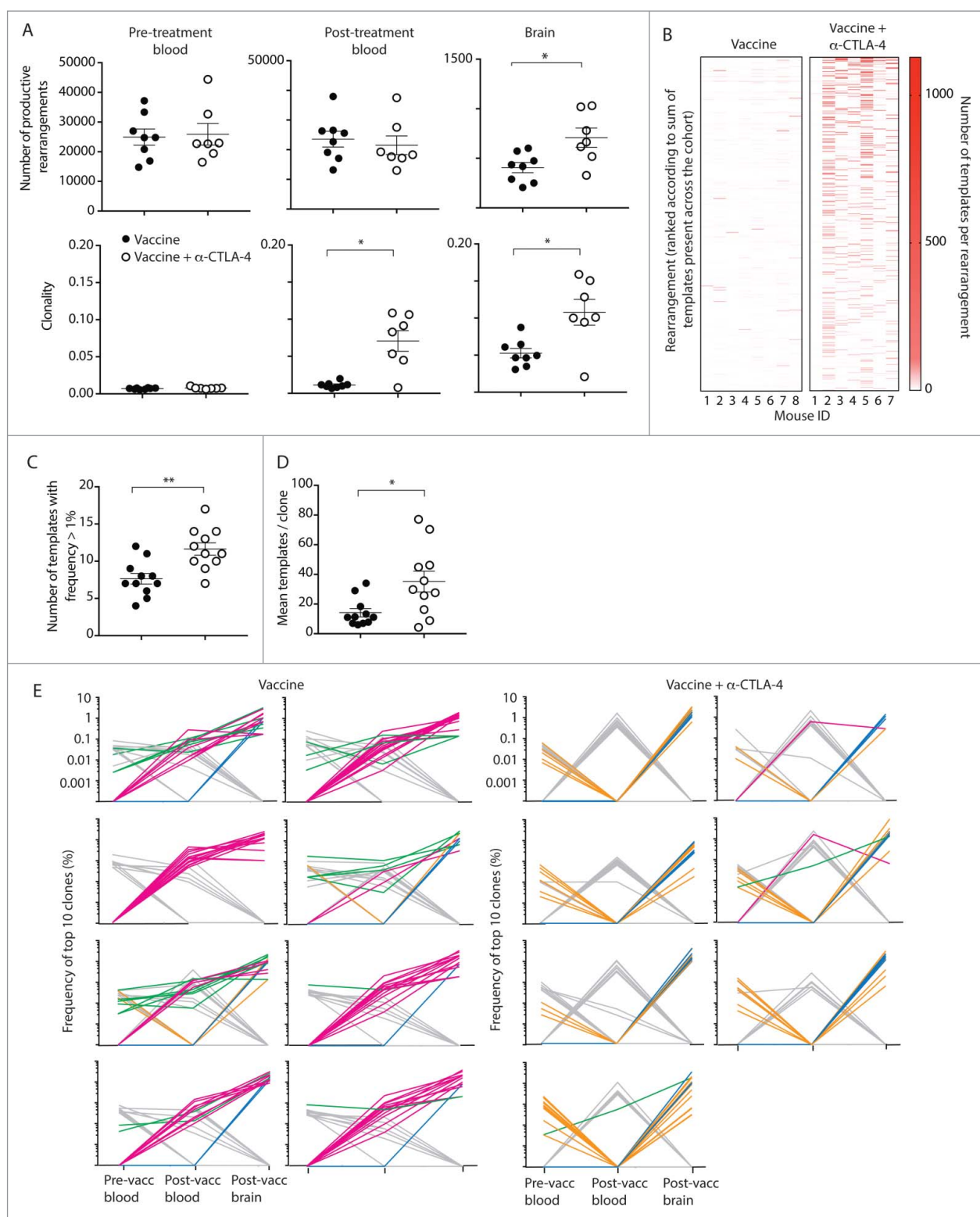


**Figure 6.** Therapeutic success of combining vaccination with CTLA-4 blockade is associated with accumulation of CD4<sup>+</sup> T cells in brain and is CD4-dependent. Wild-type mice with intracranial tumors were treated with vaccine,  $\alpha$ -CTLA-4, or combination of both. Controls were left untreated. The immune infiltrate within the brain was assessed by flow cytometry seven days after treatment, which was 14 days after tumor challenge. (A) Gating strategy for lymphocytes, macroglia and macrophages based on expression of CD45 and CD11b is shown, followed by gating of CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells and Foxp3<sup>+</sup>CD4<sup>+</sup> T cells. (B-D) Quantification of infiltrating CD45<sup>+</sup> immune cells, macrophages (CD45<sup>+</sup>CD11b<sup>+</sup>), microglia (CD45<sup>int</sup>CD11b<sup>+</sup>), T cells (CD3<sup>+</sup>), CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells and Foxp3<sup>+</sup>CD4<sup>+</sup> T cells \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.005$  ( $n = 5$  per group). Results are representative of two independent experiments. (E) Composition of T cell subsets, macrophages and microglia within the brains of intracranially challenged and early  $\alpha$ -CTLA-4, vaccine or combination treated mice. Size of pie chart reflects the size of the immune infiltrate and is relative to tumor only control (F) Survival curves for groups of MHC-II<sup>-/-</sup> and TAP<sup>-/-</sup> mice that were challenged with tumor intracranially and treated with vaccine, or vaccine combined with  $\alpha$ -CTLA-4. Controls were left untreated. \*\*  $P < 0.01$  Results represent combined survival of two independent experiments ( $n = 5$  per group in each experiment).

the induced responses are not potent or broad enough to generate a clinical response, or the T cells ultimately come under regulation, including through the same checkpoint pathways. To test the utility of combining vaccination with checkpoint blockade to overcome these hurdles, we examined combined therapy in an orthotopic model of glioma. We demonstrate significant therapeutic efficacy when  $\alpha$ -CTLA-4 was administered close to

immune priming with an  $\alpha$ -GalCer-adjuvanted whole cell vaccine, with long-term survival averaging 80 % in repeated experiments. This combined treatment was associated with extensive immune infiltration into the tumors, ultimately leading to regression that was apparent radiologically.

Through exploration of the early immune cascade involved in T cell priming, we found that therapeutic benefit of



**Figure 7.** Combining vaccination with CTLA-4 blockade reshapes the TCR repertoire in blood and brain. (A) Assessment of repertoire of TCR $\beta$  CDR3 sequences in mice bearing intracranial tumors that were treated with vaccine alone or in combination with  $\alpha$ -CTLA-4. ImmunoSeq analysis was conducted on blood samples taken on day 6 after challenge, which was before therapeutic treatment was initiated, and on blood and whole brain tissue collected on day 14. Richness and clonality scores were calculated for each animal (circular symbols), with mean values  $\pm$  SEM per treatment group shown. \*  $P < 0.05$  Mann-Whitney test. (B) Ranked rearrangements by frequency in brain for one example animal in each treatment group are shown. (C) The number of rearrangements represented by template frequency greater than 1% was calculated for each animal, with mean  $\pm$  SEM shown for each treatment group. \*\*  $P < 0.01$  Mann-Whitney test (D) The mean number of templates for the oligoclonal rearrangements (> 1% frequency) was calculated for each animal. \*  $P < 0.05$  Mann-Whitney test. (E) Graphs show the ten most frequent clones in each sample tracked across all samples taken from a given animal. The clones found at high frequency in the brain tissue are shown in color, with red, green, yellow or blue used to highlight clones that tracked similarly between the samples.

vaccination with  $\alpha$ -CTLA-4 could not be attributed to improved responses to the adjuvant used, or obvious improvements in APC function. A notable feature of the combined treatment was accumulation of leukocytes in the spleen, a phenomenon likely to be due, at least in part, to trapping of circulating immune cells. It is important to note that while the spleen was a convenient lymphoid organ to extensively examine the downstream consequences of treatment, we have previously shown that the mediastinal lymph nodes are also important sites of T cell priming with this vaccine,<sup>6</sup> and these lymph nodes were also enlarged with the combined treatment. This trapping may have been a consequence of the prolonged presentation and stimulatory activity of the vaccine, which we have previously shown can continue to provoke NKT cells in spleen to make cytokines a week after administration.<sup>6</sup> It is possible that enhanced leukocyte trapping in lymphoid tissues may improve responses to the vaccine by increasing the probability of APC:T cell contacts. A feature of the spleen (and mediastinal lymph nodes) after the combined treatment was the significant accumulation of T cells. By monitoring proliferative responses of transferred cells with treatment and assessing cytokine production after restimulation with tumor lysate in *ex vivo* assays, we attribute at least part of this accumulation to proliferation of tumor-specific T cells. Interestingly,  $\alpha$ -CTLA-4 treatment alone showed some activity in these assays, suggesting that the efficacy of the combined treatment may be the result of two activities; priming of a new cohort of antitumor T cells by the vaccine, or removal of CTLA-4-mediated checks on T cells within the endogenous repertoire that have antitumor activity.

Immune infiltrates featuring large numbers of macrophages and lymphocytes were observed in intracranial tumors seven days after treatment with vaccine and  $\alpha$ -CTLA-4. Within the T cell compartment, CD4<sup>+</sup> T cells were the most highly represented, despite both CD4<sup>+</sup> and CD8<sup>+</sup> T cell numbers being increased in the lymphoid tissues examined. This was not a result of accumulation of CD4<sup>+</sup> Tregs; indeed, Treg numbers and function were unaffected by the combined treatment. This is notable because depletion of Tregs in the tumor environment has received recent attention as a potential mode of activity of  $\alpha$ -CTLA-4 therapy.<sup>12</sup> The preferential accumulation of CD4<sup>+</sup> T cells in the tumor may reflect privileged access to the CNS due to expression of integrins important for transport across the blood-brain barrier.<sup>13</sup> Certainly, activation of CD4<sup>+</sup> T cells is critical to the success of the combined treatment, although the mechanisms by which they mediate anti-tumor activity have yet to be elucidated. We speculate that they may be acting on MHC II-expressing accessory cells, such as macrophages and microglia that infiltrate the tumor, as antitumor activity was much reduced in animals depleted of CD11b<sup>+</sup> cells, and also in animals deficient in IFN- $\gamma$ . However, in some models CD4<sup>+</sup> T cells have also been shown to have direct anti-tumor activity.<sup>14</sup> In this context, it is notable that GL261 tumors in culture are MHC-II negative, but can upregulate expression in response to IFN- $\gamma$ , and MHC-II can be observed on the tumor cells *ex vivo*.<sup>6</sup>

We were unable to observe any survival benefit when  $\alpha$ -CTLA-4 was used as a monotherapy against established tumors in the intracranial setting. In keeping with this, a previous report showed that  $\alpha$ -CTLA-4 alone could not provide

protection against intracranial GL261 tumors once established, but could induce limited protection when multiple treatments were started only three days after implantation. Established tumors could be effectively treated when  $\alpha$ -CTLA-4 was combined with a vaccine comprised of GM-CSF-transfected glioma cells.<sup>15</sup> However, another study showed that  $\alpha$ -CTLA-4 alone could be used to treat SMA-560 glioma in VM/Dk mice.<sup>16</sup> The authors attributed the activity of  $\alpha$ -CTLA-4 in this model to enhancement of T cell-proliferative capacity, which was otherwise severely reduced in the presence of this tumor.

Analysis of TCR repertoire by sequencing of TCR $\beta$  CDR3 regions showed that the effective combination was associated with infiltration of more oligoclonal T cell populations into the brain that were more diverse in specificity and greater in magnitude. While the specificity of the T cells is unknown, it can be speculated that this reshaping of the infiltrate reflects broadening of the response within the infiltrate to target a wider range of tumor antigens. It remains unclear whether this was a consequence of checkpoint blockade raising the amplitude of weak responses initiated by the vaccine, or whether checkpoint blockade 'unleashed' endogenous responses to an array of antigens unrelated to the vaccine that act cumulatively with the vaccine-induced response to eradicate the tumor.

The TCR repertoire analysis also revealed a strikingly different pattern in the blood of mice receiving vaccine plus  $\alpha$ -CTLA-4 compared with mice receiving vaccine alone. Following vaccination alone there was clear evidence of clonal expansion in the blood, and associated accumulation of these clones at the tumor site. In contrast, in mice that received the combination treatment, the post-treatment blood samples were dominated by clones that were not detectable either in pre-treatment blood or at the tumor site. The most prevalent clones at the tumor site in these mice, although often present in blood prior to treatment, were undetectable in post-treatment blood. A potential explanation for the apparent "disappearance" of these clones is that the addition of  $\alpha$ -CTLA-4 in some way led to enhanced trafficking or retention of tumor-specific clones at the tumor site. However, this explanation would require the majority of highest frequency clones present in blood prior to treatment to be tumor-specific, which seems unlikely. The enhanced trafficking would therefore have to include movement of non-tumor-specific T cells into the inflamed brain. Another possible explanation, not mutually exclusive, is that the missing clonal populations become trapped in the enlarged lymphoid tissues observed after combined treatment. However, neither of these explanations accounts for the corresponding influx of clones that appear at high frequency in blood after the combined treatment, but are not detectable in brain. Experiments in which transferred CFSE labeled cells were monitored after treatment (Fig. 4) showed that CTLA-4 blockade induces significant proliferation. It is possible that CTLA-4 blockade, when combined with DC activation induced by the vaccine, induces expansion of clones that are not vaccine- or tumor-reactive. Under these conditions, but in the absence of a strong TCR signal, they are perhaps not conditioned to home to the tumor, or as susceptible to trapping in lymphoid tissues. Regardless of the processes that led to the TCR repertoire profiles observed, overall these findings suggest that when a vaccine is combined with  $\alpha$ -CTLA-4, assessment of T cell

specificity in blood samples may be of limited value because it does not necessarily reflect the more relevant repertoire at tumor. Discordance between the post-treatment repertoire in blood and in tumor after combined treatments that include CTLA-4 blockade has been reported in a mouse model of melanoma<sup>17</sup> and in patients with breast cancer,<sup>18</sup> despite objective responses to treatment in both studies. This phenomenon does not appear to be particular to CTLA-4 blockade. In a recent study in renal cell cancer patients treated with a combination that included an anti-PD-L1 antibody, the dominant clones at the tumor site after treatment were found at much lower levels in blood while the dominant clones in post-treatment blood were not detected in tumors.<sup>19</sup>

Auto-immune side effects are a major limitation of CTLA-4 blockade in cancer patients, lessening enthusiasm for investigation in glioma patients in comparison with other checkpoint blockers. However, in the clinic,  $\alpha$ -CTLA-4 antibody is typically administered repeatedly over several months.<sup>2</sup> Similarly, in previous studies investigating CTLA-4 blockade in mouse models of glioma, multiple doses of  $\alpha$ -CTLA-4 have been administered either as monotherapy<sup>16</sup> or in combination with other treatments.<sup>15,20-23</sup> In contrast, when combined with a vaccine at time of priming, a single dose was sufficient for therapeutic benefit in our study. Using  $\alpha$ -CTLA-4 in a restricted way to promote the priming phase of an anti-cancer vaccine could present a way of harnessing clinical benefit from this powerful agent while potentially avoiding the auto-immune side-effects.

## Materials and methods

### Mice

Inbred C57BL/6 mice and the CD45.1 congenic strain B6.SJL-*Ptprca*<sup>a</sup>*Pep3*<sup>b</sup>/BoyJArc were purchased from Jackson Laboratories, Bar Harbor, Maine. Also used were: CD1d-deficient mice;<sup>24</sup> an F1 cross of OT-II mice that express a TCR specific for the I-A<sup>b</sup>-restricted epitope of chicken ovalbumin,<sup>25</sup> with the CD45.1 congenic strain; I-A $\alpha$ -deficient (MHC-II deficient) mice;<sup>26</sup> TAP1-deficient mice;<sup>27</sup> IFN $\gamma$ -deficient mice;<sup>28</sup> and CD11b-DTR transgenic mice. The latter line was generated via BALB/c ES cells transfected with recombiner bacterial artificial chromosome (BAC) clones (CD11b: RP23-373D19, BAC-PAC Resources Children's Hospital Oakland, USA) carrying insertions of human DTR sequence with its polyadenylation site in the initiation codons replacing the first coding exons of the CD11b gene. Recombineering was performed using RED/ET recombination kits following the instructions of the manufacturer (Gene Bridges GmbH, Heidelberg, Germany). SceI linearized BACs were electroporated in ES cells and those ES cell clones containing intact BAC sequences (i.e. both vector ends and middle modification verified by PCR) were selected for further transgenesis. Experiments were conducted in F1 crosses between CD11b-DTR and C57BL/6 mice. All animals were maintained by the Biomedical Research Unit at the Malaghan Institute of Medical Research. The Victoria University Animal Ethics Committee approved all experimental protocols (reference 2012R15M). Mice were 6–12 weeks of age and matched for age and gender.

### Cell lines and reagents

The murine glioma cell line GL261 was obtained from the DCRD Tumor Repository (National Cancer Institute, Frederick, USA), authenticated genotypically by IDEXX BioResearch (Columbia, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% FBS (Sigma-Aldrich, St. Louis, USA), 2 mmol/L GlutaMax, 100 U/mL penicillin and 100 mM streptomycin (Life Technologies, Carlsbad, USA). The NKT cell ligand  $\alpha$ -GalCer was manufactured as previously described.<sup>29</sup>

### Tumor implantation

For intracranial implantation, mice were anaesthetized by intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine. Lacrilube (Allergan, Parsippany, USA) was applied to the cornea to prevent desiccation. Buprenorphine (Renckitt Benckiser Pharmaceuticals, North Chesterfield, USA) and Carprofen (Norbrook Laboratories, Corby, UK) were used as peri-operative analgesic. A total of  $2.5 \times 10^4$  GL261 cells in 2  $\mu$ L of phosphate-buffered saline (PBS) were injected via a 32-gauge needle into the right striatum 2 mm lateral from the bregma and at a depth of 3 mm using a stereotactic frame (Harvard Apparatus, Holliston, USA). Time to symptom appearance was defined as time to 10% weight loss or overt behavioral symptoms (hunching, reduced activity). The presence of tumors was confirmed at necropsy. For subcutaneous implantation, mice were injected with  $1 \times 10^6$  GL261 cells in 100  $\mu$ L DMEM in the flank. Mice were euthanized when tumor area (the product of two perpendicular diameters) was  $>150 \text{ mm}^2$ .

### Vaccine generation and administration

GL261 cells were cultured in complete DMEM supplemented with 200 ng/mL  $\alpha$ -GalCer for 24 hours at 37°C before being harvested and irradiated on ice to 150 cGy. Cells were frozen in 10% DMSO, 90% complete DMEM until use. Prior to use, the vaccine was thawed by gentle agitation in incomplete DMEM, centrifuged and resuspended in PBS. Mice received  $1 \times 10^5$  cells in 200  $\mu$ L PBS, injected intravenously.

### Checkpoint blockade

Blocking  $\alpha$ -CTLA-4 (4F10, Armenian hamster IgG) was purified from hybridoma supernatant using protein G affinity columns, or purchased from BioXCell (West Lebanon, NH, USA). Control Armenian Hamster IgG was purchased from BioXCell. Mice were injected intraperitoneally with 1 mg of antibody.

### Magnetic resonance imaging

Magnetic resonance imaging (MRI) was conducted on anaesthetized animals using a clinical 1.5-T MR scanner (Philips Medical Systems) equipped with a wrist solenoid coil. T1 weighted images were acquired with the following parameters: TE = 20 milliseconds; TR = 800 milliseconds; thickness = 1 mm. Contrast was enhanced by intravenous administration of 100  $\mu$ L gadolinium diethylenetriaminepentaacetic acid (Gd-



DPTA; Magnevist, Bayer Schering Pharma). Image analysis was performed using Philips DICOM Viewer R2.5 Version 1.

### ***In vitro* T cell proliferation assay**

Spleens were isolated from mice 18 hours following treatment and single-cell suspensions were prepared by filtering through a 70  $\mu\text{m}$  filter, followed by red blood cell lysis (RBC Lysis Solution, Qiagen). Cells were incubated with 10  $\mu\text{M}$  OVA<sub>323–339</sub> (ISQAVHAAHAEINEAGR) for 2 hours at 37°C and then fixed for 10 minutes in 2% paraformaldehyde. Cells were quenched with 0.1 mM glycine (Sigma-Aldrich), resuspended in Iscove's Modified Dulbecco's Media (IMDM) supplemented with 5% FBS, 2 mmol/L GlutaMax, 100 U/mL penicillin and 100 mM streptomycin and 50  $\mu\text{M}$  2-ME (Sigma-Aldrich), and serially diluted across a 96-well plate. Cell suspensions were prepared from the spleens of OT-II mice, stained with 1  $\mu\text{M}$  carboxy-fluorescein succinimidyl ester (CFSE), and  $2 \times 10^5$  cells were added to wells containing fixed splenocytes. Preparations were cultured for 6 days and CFSE dilution of OT-II T cells, indicative of cell proliferation, was determined by flow cytometry.

### ***Assessment of T cell proliferation in vivo***

Cell suspensions were prepared from spleens of OT-II mice, stained with 1  $\mu\text{M}$  CFSE and  $1 \times 10^7$  cells were adoptively transferred into C57BL/6 recipients intravenously. Mice were treated the following day with  $\alpha$ -CTLA-4 and/or vaccine and the CFSE dilution of OT-II T cells in the spleen was analyzed 1 week later by flow cytometry.

### ***Analysis of lymphoid and brain tissues***

Single-cell suspensions from spleens were prepared as above. For analysis of brain tumor infiltrates, brains were collected and dissociated with a scalpel before digestion in incomplete IMDM, supplemented with 4  $\mu\text{g}/\text{mL}$  DNase I and 2.4 mg/mL collagenase II (Gibco, Life Technologies, NY, USA). Digested tissue was homogenized by passage through an 18G needle. Filtered tissue was centrifuged on a 37% percoll gradient (GE Healthcare, Little Chalfont, UK), with cells recovered from the bottom of the gradient.

### ***Flow Cytometry***

Antibody staining was conducted for 15 minutes at 4°C in PBS supplemented with 1% FBS and 2 mmol/L EDTA. Non-specific FcR binding was blocked with anti-CD16/32 (clone 2.4G2, prepared in-house). LIVE/DEAD<sup>®</sup> Fixable Dye (Life Technologies) was used for dead cell exclusion. Antibodies used were specific for: CD3 (145-2C11; eBioscience, BD Pharmingen or Biolegend); CD4 (RM4-5), CD45 (30-F11), FoxP3 (FJK-16S), IFN- $\gamma$  (XMG1.2), CD86 (GL1), B220 (RA3-6B2), CD44 (1M7), all eBioscience; CD11b (M1/70) and NK1.1 (PK136) from BD Biosciences; CD8 (53-6.7), CD11c (N418), CD19 (6D5) from Biolegend; TNF- $\alpha$  (MP6-XT22; BD Biosciences or eBioscience). Invariant NKT cells were detected using  $\alpha$ -GalCer-loaded CD1d tetramers (National Institutes of Health Tetramer Core Facility, Atlanta, GA). Flow cytometry was conducted on a BD

LSRII Flow Cytometer with data analyzed using FlowJo 9.7.5 software (TreeStar Inc). Doublets and dead cells were excluded from analysis.

### ***Histology***

Mice were perfused PBS through the left ventricle before brains were harvested. Tissue was fixed in 4% paraformaldehyde for 48 hours before dehydration in 70% ethanol. Brains were sagittally sectioned at 2 mm intervals and embedded in paraffin. Sections for microscopy were cut 10  $\mu\text{m}$  thick and stained with hematoxylin and eosin. Slides were analyzed by a histopathologist in de-identified treatment groups. Tumor area on sections at the tumor midpoint by was calculated by indicating the tumor perimeter for analysis by Cell<sup>^</sup>F Software (Olympus, Hamburg, Germany).

### ***TCR sequencing and repertoire analysis***

DNA was isolated from pre- and post-treatment blood and brain samples using the DNeasy blood and tissue kit (Qiagen). Amplification and sequencing of TCR $\beta$  CDR3 regions was performed using ImmunoSeq assay (Adaptive Biotechnologies, Seattle, WA) at survey resolution, performed by Adaptive Biotechnologies as previously described.<sup>30</sup> This multiplex PCR system amplifies rearranged TCR $\beta$  CDR3 regions in a quantitative manner, with an amplification bias feature based on assessment of a synthetic repertoire of TCRs.<sup>31</sup> Data analysis was performed using the ImmunoSEQ Analyzer (Adaptive Biotechnologies). Shannon's entropy was used a measure of the richness and uniformity of TCR frequency distribution for each sample, calculated by summing the frequency of each clone times the  $\log_2$  of the same frequency over all productive reads in a sample. Because sample size influences the number of unique sequences in a sample, this value was normalized based on the total number of productive unique sequences and subtracted from 1, to give 'clonality' scores. Values ranged from 0 to 1, with values approaching 1 nearing monoclonality. ImmunoSEQ Analyzer also permitted identification and tracking of the top 10 clones in each sample from individual mice.

### ***Statistical Analysis***

Unless otherwise stated, statistical significance was determined by one-way analysis of variance with a Bonferroni post-test. The log-rank test was used to determine significance between Kaplan-Meier survival curves. Analysis was performed using Prism 5.0 software (GraphPad Software, Inc.); *P* values of <0.05 were considered significant.

### ***Potential conflicts of interest***

None.

### ***Disclosure of potential conflict of interest***

No potential conflicts of interest were disclosed.

## Acknowledgments

We thank Gavin Painter (The Ferrier Research Institute) for providing  $\alpha$ -GalCer, the personnel of the Biomedical Research Unit of the Malaghan Institute of Medical Research for animal husbandry, the Hugh Green Cytometry Core for flow cytometry support, Angela Slocombe (Department of Radiology, Wellington Hospital) for MR imaging, and Jane Anderson (Wellington School of Medicine, University of Otago) for histology. The authors acknowledge the National Institutes of Health Tetramer Core Facility (contract HHSN272201300006C) for provision of CD1d tetramers.

## Funding

This work was supported by the Health Research Council of New Zealand IROF fund under grant number 14/1003, the Genesis Oncology Trust, and the Maurice Wilkins Centre. CSF was supported by a post-graduate scholarship from the Genesis Oncology Trust.

## Abbreviations

$\alpha$ -GalCer	$\alpha$ -galactosylceramide
APCs	antigen-presenting cells
CDR	complementarity-determining region
CFSE	carboxyfluorescein succinimidyl ester
CNS	central nervous system
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
Gd-DPTA	gadolinium diethylenetriaminepentaacetic acid
MRI	magnetic resonance imaging
NKT cells	type I natural killer T cells
PD-1	programmed death-1
TCR	T cell receptor
Tregs	regulatory T cells

## ORCID

Ian F. Hermans  <http://orcid.org/0000-0002-7584-887X>

## References

- Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: A common denominator approach to cancer therapy. *Cancer Cell*. 2015;27:450-61. <https://doi.org/10.1016/j.ccell.2015.03.001>. PMID:25858804
- Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010;363:711-23. <https://doi.org/10.1056/NEJMoa1003466>. PMID:20525992
- Hamid O, Robert C, Daud A, Hodi FS, Hwu WJ, Kefford R, Wolchok JD, Hersey P, Joseph RW, Weber JS, et al. Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. *N Engl J Med*. 2013;369:134-44. <https://doi.org/10.1056/NEJMoa1305133>. PMID:23724846
- Fecci PE, Heimberger AB, Sampson JH. Immunotherapy for primary brain tumors: no longer a matter of privilege. *Clin Cancer Res*. 2014;20:5620-9. <https://doi.org/10.1158/1078-0432.CCR-14-0832>. PMID:25398845
- Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo E, et al. CD1d-restricted and TCR-mediated activation of  $V\alpha 14$  NKT cells by glycosylceramides. *Science*. 1997;278:1626-9. <https://doi.org/10.1126/science.278.5343.1626>. PMID:9374463
- Hunn MK, Farrand KJ, Broadley KW, Weinkove R, Ferguson P, Miller RJ, Field CS, Petersen T, McConnell MJ, Hermans IF. Vaccination with irradiated tumor cells pulsed with an adjuvant that

stimulates NKT cells is an effective treatment for glioma. *Clin Cancer Res*. 2012;18:6446-59. <https://doi.org/10.1158/1078-0432.CCR-12-0704>. PMID:23147997

- Kruser TJ, Mehta MP, Robins HI. Pseudoprogression after glioma therapy: a comprehensive review. *Expert Rev Neurotherapeutics*. 2013;13:389-403. <https://doi.org/10.1586/ern.13.7>
- Qureshi OS, Zheng Y, Nakamura K, Attridge K, Manzotti C, Schmidt EM, Baker J, Jeffery LE, Kaur S, Briggs Z, et al. Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4. *Science*. 2011;332:600-3. <https://doi.org/10.1126/science.1202947>. PMID:21474713
- Krummel MF, Allison JP. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med*. 1995;182:459-65. <https://doi.org/10.1084/jem.182.2.459>. PMID:7543139
- Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity*. 1995;3:541-7. [https://doi.org/10.1016/1074-7613\(95\)90125-6](https://doi.org/10.1016/1074-7613(95)90125-6). PMID:7584144
- Postow MA, Callahan MK, Wolchok JD. Immune checkpoint blockade in cancer therapy. *J Clin Oncology*. 2015;33:1974-82. <https://doi.org/10.1200/JCO.2014.59.4358>
- Simpson TR, Li F, Montalvo-Ortiz W, Sepulveda MA, Bergerhoff K, Arce F, Roddie C, Henry JY, Yagita H, Wolchok JD, et al. Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *J Exp Med*. 2013;210:1695-710. <https://doi.org/10.1084/jem.20130579>. PMID:23897981
- Engelhardt B. Molecular mechanisms involved in T cell migration across the blood-brain barrier. *J Neural Transmission*. 2006;113:477-85. <https://doi.org/10.1007/s00702-005-0409-y>
- Quezada SA, Simpson TR, Peggs KS, Merghoub T, Vider J, Fan X, Blasberg R, Yagita H, Muranski P, Antony PA, et al. Tumor-reactive CD4<sup>+</sup> T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. *J Exp Med*. 2010;207:637-50. <https://doi.org/10.1084/jem.20091918>. PMID:20156971
- Agarwalla P, Barnard Z, Fecci P, Dranoff G, Curry WT, Jr. Sequential immunotherapy by vaccination with GM-CSF-expressing glioma cells and CTLA-4 blockade effectively treats established murine intracranial tumors. *J Immunother*. 2012;35:385-9. <https://doi.org/10.1097/CJI.0b013e3182562d59>. PMID:22576343
- Fecci PE, Ochiai H, Mitchell DA, Grossi PM, Sweeney AE, Archer GE, Cummings T, Allison JP, Bigner DD, Sampson JH. Systemic CTLA-4 blockade ameliorates glioma-induced changes to the CD4<sup>+</sup> T cell compartment without affecting regulatory T-cell function. *Clin Cancer Res*. 2007;13:2158-67. <https://doi.org/10.1158/1078-0432.CCR-06-2070>. PMID:17404100
- Twyman-Saint Victor C, Rech AJ, Maity A, Rengan R, Pauken KE, Stelekati E, Benci JL, Xu B, Dada H, Odorizzi PM, et al. Radiation and dual checkpoint blockade activate non-redundant immune mechanisms in cancer. *Nature*. 2015;520:373-7. <https://doi.org/10.1038/nature14292>. PMID:25754329
- Page DB, Yuan J, Redmond D, Wen YH, Durack JC, Emerson R, Solomon S, Dong Z, Wong P, Comstock C, et al. Deep sequencing of T-cell receptor DNA as a biomarker of clonally expanded TILs in breast cancer after immunotherapy. *Cancer Immunol Res*. 2016;4:835-44. <https://doi.org/10.1158/2326-6066.CIR-16-0013>. PMID:27587469
- Wallin JJ, Bendell JC, Funke R, Sznol M, Korski K, Jones S, Hernandez G, Mier J, He X, Hodi FS, et al. Atezolizumab in combination with bevacizumab enhances antigen-specific T-cell migration in metastatic renal cell carcinoma. *Nat Commun*. 2016;7:12624. <https://doi.org/10.1038/ncomms12624>. PMID:27571927
- Belcaid Z, Phallen JA, Zeng J, See AP, Mathios D, Gottschalk C, Nicholas S, Kellett M, Ruzevick J, Jackson C, et al. Focal radiation therapy combined with 4-1BB activation and CTLA-4 blockade yields long-term survival and a protective antigen-specific memory response in a murine glioma model. *PLoS one*. 2014;9:e101764. <https://doi.org/10.1371/journal.pone.0101764>. PMID:25013914
- Reardon DA, Gokhale PC, Klein SR, Ligon KL, Rodig SJ, Ramkissoon SH, Jones KL, Conway AS, Liao X, Zhou J, et al. Glioblastoma

- eradication following immune checkpoint blockade in an orthotopic, immunocompetent model. *Cancer Immunol Res.* 2016;4:124-35. <https://doi.org/10.1158/2326-6066.CIR-15-0151>.
22. Vom Berg J, Vrohling M, Haller S, Haimovici A, Kulig P, Sledzinska A, Weller M, Becher B. Intratumoral IL-12 combined with CTLA-4 blockade elicits T cell-mediated glioma rejection. *J Exp Med.* 2013;210:2803-11. <https://doi.org/10.1084/jem.20130678>. PMID:24277150
  23. Wainwright DA, Chang AL, Dey M, Balyasnikova IV, Kim CK, Tobias A, Cheng Y, Kim JW, Qiao J, Zhang L, et al. Durable therapeutic efficacy utilizing combinatorial blockade against IDO, CTLA-4, and PD-L1 in mice with brain tumors. *Clin Cancer Res.* 2014;20:5290-301. <https://doi.org/10.1158/1078-0432.CCR-14-0514>. PMID:24691018
  24. Chen YH, Chiu NM, Mandal M, Wang N, Wang CR. Impaired NK1<sup>+</sup> T cell development and early IL-4 production in CD1-deficient mice. *Immunity.* 1997;6:459-67. [https://doi.org/10.1016/S1074-7613\(00\)80289-7](https://doi.org/10.1016/S1074-7613(00)80289-7). PMID:9133425
  25. Barnden MJ, Allison J, Heath WR, Carbone FR. Defective TCR expression in transgenic mice constructed using cDNA-based alpha and beta-chain genes under the control of heterologous regulatory elements. *Immunol Cell Biol.* 1998;76:34-40. <https://doi.org/10.1046/j.1440-1711.1998.00709.x>. PMID:9553774
  26. Kontgen F, Suss G, Stewart C, Steinmetz M, Bluethmann H. Targeted disruption of the MHC class II Aa gene in C57BL/6 mice. *Int Immunol.* 1993;5:957-64. <https://doi.org/10.1093/intimm/5.8.957>. PMID:8398989
  27. Van Kaer L, Ashton-Rickardt PG, Ploegh HL, Tonegawa S. TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and CD4<sup>-</sup>8<sup>+</sup> T cells. *Cell.* 1992;71:1205-14. [https://doi.org/10.1016/S0092-8674\(05\)80068-6](https://doi.org/10.1016/S0092-8674(05)80068-6). PMID:1473153
  28. Dalton DK, Pitts-Meek S, Keshav S, Figari IS, Bradley A, Stewart TA. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science.* 1993;259:1739-42. <https://doi.org/10.1126/science.8456300>. PMID:8456300
  29. Lee A, Farrand KJ, Dickgreber N, Hayman CM, Jurs S, Hermans IF, Painter GF. Novel synthesis of alpha-galactosyl-ceramides and confirmation of their powerful NKT cell agonist activity. *Carbohydr Res.* 2006;341:2785-98. <https://doi.org/10.1016/j.carres.2006.09.006>. PMID:17014832
  30. Robins HS, Campregher PV, Srivastava SK, Wachter A, Turtle CJ, Kahsai O, Riddell SR, Warren EH, Carlson CS. Comprehensive assessment of T-cell receptor beta-chain diversity in alphabeta T cells. *Blood.* 2009;114:4099-107. <https://doi.org/10.1182/blood-2009-04-217604>. PMID:19706884
  31. Carlson CS, Emerson RO, Sherwood AM, Desmarais C, Chung MW, Parsons JM, Steen MS, LaMadrid-Herrmannsfeldt MA, Williamson DW, Livingston RJ, et al. Using synthetic templates to design an unbiased multiplex PCR assay. *Nat Commun.* 2013;4:2680. <https://doi.org/10.1038/ncomms3680>. PMID:24157944