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Serum elevation of B lymphocyte stimulator does not increase regulatory B cells in glioblastoma patients undergoing immunotherapy

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Abstract

Regulatory B cells that secrete IL-10 (IL-10⁺ B_{reg}) represent a suppressive subset of the B cell compartment with prominent anti-inflammatory capacity, capable of suppressing cellular and humoral responses to cancer and vaccines. B lymphocyte stimulator (BLyS) is a key regulatory molecule in IL-10⁺ B_{reg} biology with tightly controlled serum levels. However, BLyS levels can be drastically altered upon chemotherapeutic intervention. We have previously shown that serum BLyS levels are elevated, and directly associated, with increased antigen-specific antibody titers in patients with glioblastoma (GBM) undergoing lymphodepletive temozolomide (TMZ) chemotherapy and vaccination. In this study, we examined corresponding IL-10⁺ B_{reg} responses within this patient population and demonstrate that the IL-10⁺ B_{reg} compartment remains constant

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Authorship Contributions

Contributions: Anirudh Saraswathula designed, performed and analyzed data and co-wrote manuscript; Elizabeth A. Reap designed and analyzed data and co-wrote manuscript; Bryan D. Choi designed initial experiments and co-wrote manuscript; Robert J. Schmittling performed experiments and analyzed data; Pamela K. Norberg provided critical samples for experimentation and analyzed data; Elias J. Sayour co-wrote the manuscript; James E. Herndon II, and Patrick Healy provided statistical expertise and analysis; Kendra L. Congdon analyzed data and co-wrote manuscript; Gerald E. Archer provided clinical samples and helped design experiments; Luis Sanchez-Perez designed, analyzed and co-wrote manuscript, and John H. Sampson provided funding.

Conflicts of Interest

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before and after administration of the vaccine, despite elevated BLyS levels in circulation. IL-10⁺ B_{reg} frequencies were not associated with serum BLyS levels and *ex vivo* stimulation with a physiologically relevant concentration of BLyS did not increase IL-10⁺ B_{reg} frequency. However, BLyS stimulation did increase the frequency of the overall B cell compartment and promoted B cell proliferation upon B cell receptor engagement. Therefore, using BLyS as an adjuvant with therapeutic peptide vaccination could promote humoral immunity with no increase in immunosuppressive IL-10⁺ B_{regs}. These results have implications for modulating humoral responses in human peptide vaccine trials in patients with GBM.

Keywords

BLyS; BAFF; B_{regs}; lymphopenia; GBM; immunotherapy

Introduction

Regulatory B cells (IL-10⁺ B_{regs}) play an immunosuppressive role in mouse models for autoimmunity and cancer (1–5). IL-10⁺ B_{regs} downregulate effector T cell responses through the secretion of immunosuppressive cytokines such as interleukin-10 (IL-10) and through cell-to-cell contact (1, 4, 6), and have been shown to promote cancer progression in murine models by upregulating immunosuppressive cell subsets such as regulatory T cells (T_{regs}) (7). However, the regulation of these cells, especially in human systems, is not well understood (8, 9). B lymphocyte stimulator (BLyS) is a B cell homeostatic cytokine that promotes differentiation and survival of B cells and has been linked to the promotion of IL-10⁺ B_{regs} in preclinical and clinical studies (10–13). Examination of BLyS and IL-10⁺ B_{regs} in the context of vaccination may provide important information on the induction of potentially immunosuppressive and detrimental IL-10⁺ B_{regs} responses.

Our Phase II trial of rindopepimut®, an Epidermal Growth Factor Receptor variant III (EGFRvIII) vaccine consisting of a 14-amino acid peptide containing the EGFRvIII mutation (PEPvIII, H-Leu-Glu-Glu-Lys-Lys-Gln-Asn-Tyr-Val-Val-Thr-Asp-His-Cys-OH) chemically conjugated to keyhole limpet hemocyanin (KLH) for the treatment of patients with glioblastoma (GBM) has shown a survival benefit in a multicenter, single-arm phase II clinical trial (ACT III) (14), likely through an antibody-dependent mechanism. In newly-diagnosed patients with GBM, this vaccine was administered with the standard-of-care (SOC) lymphodepleting chemotherapeutic alkylating agent temozolomide (TMZ) and these patients developed supraphysiological EGFRvIII-specific antibody titers (15–18). Further analysis revealed elevated serum BLyS levels (likely due to the lymphodepletive effects of TMZ) and these BLyS levels directly correlated with vaccine-induced immunoglobulin levels (19). This clinical observation is supported by preclinical studies demonstrating a role for BLyS in the promotion of humoral responses (20, 21).

In this study, we examine the association of human IL-10⁺ B_{regs} with serum BLyS levels in the context of SOC TMZ treatment and PEPvIII-KLH vaccination in patients with GBM (22–24). Since BLyS levels are elevated in the context of TMZ-induced lymphopenia, we hypothesized that BLyS stimulation may enhance IL-10⁺ B_{regs} frequencies in these patients

and impede the ability to achieve maximal anti-tumor immune responses (19, 25, 26). Our studies demonstrated that after serial PEPvIII-KLH vaccination in the context lymphopenia, the frequency of IL-10⁺ B_{regs} in GBM patients did not significantly change. In addition, there was no association between the size of the IL-10⁺ B_{reg} compartment and BLYS serum levels. Further *in vitro* studies revealed that the frequency of IL-10⁺ B_{regs} did not increase upon stimulation with physiologically comparable BLYS levels. However, BLYS enhanced the proliferation and increased the frequency of the global B cell compartment. Taken together, these data suggest that using BLYS as an adjuvant with therapeutic peptide vaccination may not trigger IL-10⁺ B_{reg} expansion and instead promotes the proliferation of activated B cells and humoral immunity. These results implicate BLYS as a novel adjuvant for enhancing humoral responses in human peptide vaccine trials.

Materials and Methods

Patient samples

Cryopreserved patient samples were collected by blood draw or leukapheresis from the ACT-II trial, as described in (15). Briefly, adults with newly diagnosed GBM who had gross total resection of their EGFRvIII positive tumor and a Karnofsky performance status (KPS) score of ≥80 with no radiographic evidence of progression after radiation therapy were eligible for vaccination. The trial design and informed consent were approved by the FDA (under BB-IND-9944) and the local institutional review boards (15–17). After tumor resection and conformal external beam radiotherapy (XRT) with concurrent TMZ at a targeted dose of 75 mg/m², informed consent was obtained. The initial 3 vaccinations of a 14-mer peptide conjugated to KLH were given every two weeks starting within 6 weeks of completing radiation (15, 16). Subsequent vaccines were given until clinical or radiographic evidence of tumor progression or death. Patients were assigned to receive TMZ at a targeted dose of 100 mg/m² for the first 21 days of a 28-day cycle (*n* = 8).

Patient samples selected for analyses were taken at vaccine 1, prior to any vaccination or TMZ treatment at 100 mg/m² (**Pre**), and at a patient variable post-vaccination time point that was selected to occur after patient peak serum BLYS levels and near peak anti-EGFRvIII antibody titers (**Post**) (19). Table I includes the serum BLYS levels of GBM patients at the time of IL-10⁺ B_{reg} analysis. These time points were selected based on our hypothesis that serum BLYS elevation will directly translate into an enhancement of IL-10⁺ B_{reg} frequency, and based off of our available clinical samples. Normal donor samples were collected at Duke University Medical Center.

Ex vivo evaluation of IL-10 competency

In accordance with the protocols adopted from Iwata Y et al. (8), and with the studies from Khoder A et al. (9), peripheral blood mononuclear cells (PBMCs) were utilized for these studies because of the limited clinical sample availability. PBMCs were rested overnight (o/n) in RPMI Medium 1640 with GlutaMAX™-I (Gibco) plus 10% fetal bovine serum (FBS, GemCell™). Cells were then harvested and plated at 4.0×10⁶ cells/ml in 48-well plate for 43 hours in media supplemented with 1 µg/ml CD40L (R&D Systems) and 10 µg/ml lipopolysaccharide (LPS, Sigma-Aldrich catalog no. L4391). They were subsequently

stimulated for 5 hours with 50 ng/mL phorbol myristate acetate (PMA, Sigma-Aldrich), 1 µg/ml ionomycin (Sigma-Aldrich), 10 µg/ml LPS and 1 µl/mL of Brefeldin A (BFA, BD Biosciences GolgiPlug™ - Protein Transport Inhibitor).

Cells were washed and stained with monoclonal fluorophore-conjugated antibodies for the following cell surface markers: extracellular peridinin chlorophyll protein complex (PerCP)-anti-CD19 and fluorescein isothiocyanate (FITC)-anti-CD14 as well as intracellular Allophycocyanin (APC)-anti-IL-10. Intracellular staining was performed using the Cytofix/Cytoperm™ Kit (BD Biosciences) as described by manufacturer.

Ex vivo BLyS-sensitivity assay

In order to examine cell sensitivity to BLyS, cells were stimulated in a similar protocol as used for the determination of IL-10 competency. BLyS (2,000 ng/ml; B cell activating factor (BAFF), R&D Systems) was added to the initial stimulation media as described above, and 4.0×10^6 cells/ml rested PBMCs were stimulated for 67 hours in a 48-well plate before being transferred for 5 hours to media containing 50 ng/mL PMA (Sigma-Aldrich), 1 µg/ml ionomycin (Sigma-Aldrich), 10 µg/ml LPS (Sigma-Aldrich) and 1 µl/mL of BFA (BD Biosciences). The intracellular staining was performed identically as described above.

B cell proliferation assay

To assess the B cell proliferative response to BLyS stimulation, we stained normal donor PBMCs with carboxyfluorescein succinimidyl ester (CFSE, Life Technologies), a fluorescent stain used to track cell proliferation, and stimulated with goat anti-Immunoglobulin (Ig)M (Southern Biotech) and BLyS (R&D; 2,000 ng/ml) in a 48-well plate (BD Falcon) for 72 hours. The cells were then stained for CD19 (PerCP) and analyzed for proliferation by flow cytometry.

Statistical analysis

Based on previous studies demonstrating the impact of BLyS on increasing the IL-10⁺ B_{reg} frequency amongst B cells (10–13), our a priori hypothesis was that due to the elevated serum BLyS levels experienced in our GBM patient population (19), this effect will directly translate into an enhancement of IL-10⁺ B_{reg} frequency within the B cells compartment. Since this hypothesis is unidirectional, a paired, one-tailed Student's t-test was used to compare IL-10⁺ B_{regs} frequency in pre- and post-vaccination samples. A Pearson linear regression was used to assess associations and statistical significance when examining associations with various other trial parameters. An unpaired, one-tailed Student's t-test was used when comparing BLyS-induced IL-10⁺ B_{regs} frequencies in GBM patients to normal donor samples. Statistical significance was defined at $p < 0.05$. All statistical analysis was completed in GraphPad Prism 5.00.

Results

The frequency of IL-10⁺ B_{regs} is unchanged following TMZ and peptide vaccine treatment

To understand the effect of tumor-specific peptide vaccination and TMZ-induced lymphopenia, on IL-10 secreting IL-10⁺ B_{regs} and based on the hypothesis that serum BLyS

elevation will directly translated into an enhancement of IL-10⁺ B_{reg} frequency, we examined PBMCs from cryopreserved samples from PEPvIII-KLH vaccinated GBM patients undergoing lymphodepletive TMZ treatment, utilizing a functional IL-10⁺ B_{reg} assay adopted from Iwata Y et al. (8) and Khoder A et al. (9) depicted in Figure 1a&b. IL-10⁺ B_{reg} frequency was analyzed at vaccine 1, prior to any vaccination or TMZ treatment at 100 mg/m² (**Pre**), and was also analyzed post-vaccination at a time-point chosen on a patient by patient basis that was selected to occur after peak serum BLyS levels and near peak anti-EGFRvIII antibody titers (**Post**) (19). Table I includes the serum BLyS levels of GBM patients at the time of IL-10⁺ B_{reg} analysis. In accordance with previous studies published from this patient population (15), analysis of the B cell population demonstrated an expected reduction in the frequency of B cells (Figure 1c; **p* = 0.0466). This reduction is largely attributed to the lymphodepletive effect of TMZ chemotherapy (15). Of interest, while the B cell frequency was reduced, there was no significant difference in IL-10⁺ B_{reg} frequency between these time points (Figure 1d).

The frequency of IL-10⁺ B_{regs} does not associate with anti-EGFRvIII antibody titers or patient survival

Published data indicates a potential role for IL-10⁺ B_{reg} in regulating humoral and cellular responses to vaccines as well as a regulatory role in immunosuppression and cancer progression. We therefore decided to analyze the relationship of IL-10⁺ B_{reg} frequency with peak anti-EGFRvIII antibody titers and patient survival outcomes. We found that post-vaccination, IL-10⁺ B_{reg} frequency is not related to peak anti-EGFRvIII antibody titers (Figure 2a; *r*² = 0.03185; *p* = 0.6724). Also, there does not appear to be any association with overall patient survival (Figure 2b; *r*² = 0.06793; *p* = 0.5330) progression-free survival, or time to radiographic progression of disease (Figure 2c; *r*² = 0.09114; *p* = 0.4674) measured in months (mo).

BLyS elevation does not affect the frequency of IL-10⁺ B_{regs} but increases the frequency and proliferation of B cells

Since previous preclinical studies have demonstrated a role for BLyS in promoting IL-10⁺ B_{reg} frequencies, we decided to evaluate whether BLyS serum levels were associated with IL-10⁺ B_{reg} frequencies in our GBM patients at time of IL-10⁺ B_{reg} analysis (Table I). Therefore, we compared IL-10⁺ B_{reg} frequencies to corresponding patient BLyS serum levels. There was no significant association (Figure 3a, *R*² = 0.3253; *p* = 0.1399) between IL-10⁺ B_{reg} and patient BLyS serum levels. Since our previous findings suggested a strong relationship between serum BLyS and B cell activity (19), we investigated these seemingly incongruous results by stimulating healthy, normal donor PBMC samples with BLyS in addition to *ex vivo* B cell receptor stimulation before intracellular IL-10 staining. We found no significant difference in IL-10⁺ B_{reg} frequency when cells were cultured with physiologically-relevant levels of BLyS (Figure 3b; *p* = 0.4882). However, both the frequency of B cells (Figure 3c; **p* = 0.0283) and the level of proliferation as determined by carboxyfluorescein succinimidyl ester (CFSE) did increase significantly (Figure 3d; **p* = 0.0284). Thus, BLyS does not appear to affect the IL-10⁺ B_{reg} compartment in *ex vivo* samples at the time of peak antibody titer or *in vitro* healthy donor samples, but does increase global B cell frequency and proliferation.

Discussion

In the context of tumor-specific vaccination during lymphodepletive TMZ chemotherapy in patients with GBM, we found that IL-10⁺ B_{reg} frequency was unchanged despite elevated BLyS levels at the time of analysis. While this therapy elicits robust production of anti-EGFRvIII antibodies and results in an increased overall survival when compared to historical controls (14, 15, 24), IL-10⁺ B_{reg} frequency was not associated with peak anti-EGFRvIII antibody titers, patient survival outcomes, or BLyS levels. These results were unexpected due the mouse and human literature demonstrating a link between BLyS levels and IL-10⁺ B_{reg} promotion (10–12).

However, recent studies suggest that IL-10⁺ B_{reg} subsets may not have the same activation-program response to BLyS observed in the greater CD19⁺ B cell compartment (27), which could account for the IL-10⁺ B_{reg} dynamics observed in our study. Another important point is the utilization of rested PBMCs for the IL-10⁺ B_{reg} assay. Many studies utilize purified B cells in stimulation assays in order to specifically stimulate the pure B cell population and to determine the IL-10⁺ B_{reg} fraction (8, 28). As we utilized PBMCs, there is the possibility of an indirect B cell stimulation by factors secreted by non-B cell subsets which might contribute to IL-10⁺ B_{reg} differentiation. Caveats of this study are the limited availability of the clinical samples as well as the decreased lymphocyte counts within these samples due to patient treatment with lymphodepletive TMZ chemotherapy. However, a recent study by Khoder A et al. (9) demonstrated that IL-10⁺ B_{regs} can be effectively examined from whole PBMCs. Therefore, we believe that our results represent the normal physiology of the IL-10⁺ B_{reg} compartment and that this compartment is not increased under elevated levels of serum BLyS.

IL-10⁺ B_{regs} represent a suppressive compartment of the B cell lineage. While several markers exist to identify different B_{reg} subsets in humans, the reported phenotype of these cells varies between studies (8, 28, 29). A B cell population with the phenotype CD19⁺CD24^{high}CD38^{high} has been shown to secrete large amounts of IL-10 upon stimulation through CD40 (28). Another human B_{reg} cell subset equivalent to murine B10 cells, which expresses CD24 and the memory-related marker CD27 (8, 30), has also been identified. While different subsets exist for IL-10⁺ B_{regs} in both humans and mice, the IL-10⁺ B_{reg} subset evaluated in this study universally displays the phenotypic markers of CD19⁺CD24^{high}CD38^{high}. Therefore, our study focuses on the evaluation of CD19⁺CD24^{high}CD38^{high} IL-10⁺ B_{regs}, which are largely considered to be the main contributors of IL-10 secretion and regulation. Our study demonstrates that this population might not be as sensitive to the homeostatic cytokine BLyS as previously expected from studies that demonstrate the role of BLyS on IL-10⁺ B_{reg} homeostasis. It remains to be elucidated whether another distinct regulatory B cell subset characterized by CD1d^{high}CD5⁺ expression, which was previously determined to be sensitive to BLyS induced expansion (10–13), will be elevated in these patients with elevated serum BLyS levels.

These data indicate that in the context of TMZ-induced lymphopenia and humoral immunity-targeting vaccines, that IL-10⁺ B_{regs} may not be as responsive to BLyS as non-regulatory B cell subsets. These discrepancies may pertain to differences in BLyS receptor

expression or the expression of intracellular adaptor molecules known to regulate receptor mediated IL-10 induction (13). Alternatively, in response to BLyS, serial vaccinations with PEPvIII-KLH inducing anti-EGFRvIII immunoglobulins may differentially affect activation and maturation between regulatory and non-regulatory B cell subsets. Unlike the T_{reg} compartment (31–34), our current and previous findings (19) indicate that there may be diminished IL-10⁺ B_{reg} responses to tumor-specific vaccines.

These results have implications for interpreting and more effectively modulating humoral responses in human peptide vaccine trials. By delineating the interplay between BLyS, IL-10⁺ B_{regs}, and antigen-specific humoral responses, we may ultimately be able to deliver tangible benefits for patients with refractory tumors (i.e. GBM) expressing tumor-specific surface antigens. Importantly, our findings indicate that IL-10⁺ B_{regs} may not be a regulatory barrier in the use of BLyS as a humoral adjuvant for vaccine-based cancer immunotherapy.

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List of Abbreviations

APC	Allophycocyanin
BAFF	B cell activating factor
BLyS	B lymphocyte stimulator
BFA	brefeldin A
CFSE	carboxyfluorescein succinimidyl ester
EGFRvIII	epidermal growth factor receptor variant III
XRT	external beam radiotherapy
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FDA	Food and Drug Administration
GBM	glioblastoma
Ig	immunoglobulin
IL-10	interleukin-10
KPS	Karnofsky performance status
KLH	keyhole limpet hemocyanin

LPS	lipopolysaccharide
mo	months
o/n	overnight
PEPvIII	peptide containing the EGFRvIII mutation
PerCP	peridinin chlorophyll protein complex
PBMCs	peripheral blood mononuclear cells
PMA	phorbol myristate acetate
IL-10 IL-10⁺ B_{regs}	regulatory B cells that secrete
B_{regs}	regulatory B cells
T_{regs}	regulatory T cells
SOC	standard-of-care
TMZ	temozolomide

References

1. Candando KM, Lykken JM, Tedder TF. B10 cell regulation of health and disease. Immunological reviews. 2014; 259:259–72.10.1111/imr.12176 [PubMed: 24712471]
2. Kim KM, Ishigami T, Hata D, Yamaoka K, Mayumi M, Mikawa H. Regulation of cell division of mature B cells by ionomycin and phorbol ester. Journal of immunology. 1992; 148:1797–803.
3. Matsushita T, Tedder TF. Identifying regulatory B cells (B10 cells) that produce IL-10 in mice. Methods in molecular biology. 2011; 677:99–111.10.1007/978-1-60761-869-0_7 [PubMed: 20941605]
4. Yanaba K, Bouaziz JD, Haas KM, Poe JC, Fujimoto M, Tedder TF. A regulatory B cell subset with a unique CD1dhiCD5+ phenotype controls T cell-dependent inflammatory responses. Immunity. 2008; 28:639–50.10.1016/j.immuni.2008.03.017 [PubMed: 18482568]
5. Katz SI, Parker D, Turk JL. B-cell suppression of delayed hypersensitivity reactions. Nature. 1974; 251:550–1. [PubMed: 4547522]
6. Kessel A, Haj T, Peri R, Snir A, Melamed D, Sabo E, Toubi E. Human CD19(+)CD25(high) B regulatory cells suppress proliferation of CD4(+) T cells and enhance Foxp3 and CTLA-4 expression in T-regulatory cells. Autoimmunity reviews. 2012; 11:670–7.10.1016/j.autrev.2011.11.018 [PubMed: 22155204]
7. Olkhanud PB, Damdinsuren B, Bodogai M, Gress RE, Sen R, Wejksza K, Malchinkhuu E, Wersto RP, Biragyn A. Tumor-evoked regulatory B cells promote breast cancer metastasis by converting resting CD4(+) T cells to T-regulatory cells. Cancer research. 2011; 71:3505–15.10.1158/0008-5472.CAN-10-4316 [PubMed: 21444674]
8. Iwata Y, Matsushita T, Horikawa M, et al. Characterization of a rare IL-10-competent B-cell subset in humans that parallels mouse regulatory B10 cells. Blood. 2011; 117:530–41.10.1182/blood-2010-07-294249 [PubMed: 20962324]
9. Khoder A, Sarvaria A, Alsuliman A, et al. Regulatory B cells are enriched within the IgM memory and transitional subsets in healthy donors but are deficient in chronic GVHD. Blood. 2014; 124:2034–45.10.1182/blood-2014-04-571125 [PubMed: 25051962]
10. Yang M, Sun L, Wang S, Ko KH, Xu H, Zheng BJ, Cao X, Lu L. Novel function of B cell-activating factor in the induction of IL-10-producing regulatory B cells. Journal of immunology. 2010; 184:3321–5.10.4049/jimmunol.0902551

11. Yehudai D, Snir A, Peri R, Halasz K, Haj T, Odeh M, Kessel A. B cell-activating factor enhances interleukin-6 and interleukin-10 production by ODN-activated human B cells. *Scandinavian journal of immunology*. 2012; 76:371–7.10.1111/j.1365-3083.2012.02752.x [PubMed: 22725135]
12. Xu LG, Wu M, Hu J, Zhai Z, Shu HB. Identification of downstream genes up-regulated by the tumor necrosis factor family member TALL-1. *Journal of leukocyte biology*. 2002; 72:410–6. [PubMed: 12149433]
13. Xu LG, Shu HB. TNFR-associated factor-3 is associated with BAFF-R and negatively regulates BAFF-R-mediated NF-kappa B activation and IL-10 production. *Journal of immunology*. 2002; 169:6883–9.
14. Schuster J, Lai RK, Recht LD, et al. A phase II, multicenter trial of rindopepimut (CDX-110) in newly diagnosed glioblastoma: the ACT III study. *Neuro-oncology*. 2015; 17:854–61.10.1093/neuonc/nou348 [PubMed: 25586468]
15. Sampson JH, Aldape KD, Archer GE, et al. Greater chemotherapy-induced lymphopenia enhances tumor-specific immune responses that eliminate EGFRvIII-expressing tumor cells in patients with glioblastoma. *Neuro-oncology*. 2011; 13:324–33.10.1093/neuonc/noq157 [PubMed: 21149254]
16. Sampson JH, Heimberger AB, Archer GE, et al. Immunologic escape after prolonged progression-free survival with epidermal growth factor receptor variant III peptide vaccination in patients with newly diagnosed glioblastoma. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*. 2010; 28:4722–9.10.1200/JCO.2010.28.6963 [PubMed: 20921459]
17. Sampson JH, Archer GE, Mitchell DA, et al. An epidermal growth factor receptor variant III-targeted vaccine is safe and immunogenic in patients with glioblastoma multiforme. *Molecular cancer therapeutics*. 2009; 8:2773–9.10.1158/1535-7163.MCT-09-0124 [PubMed: 19825799]
18. Heimberger AB, Sampson JH. The PEPvIII-KLH (CDX-110) vaccine in glioblastoma multiforme patients. *Expert opinion on biological therapy*. 2009; 9:1087–98.10.1517/14712590903124346 [PubMed: 19591631]
19. Sanchez-Perez L, Choi BD, Reap EA, et al. BLYS levels correlate with vaccine-induced antibody titers in patients with glioblastoma lymphodepleted by therapeutic temozolomide. *Cancer immunology, immunotherapy: CII*. 2013; 62:983–7.10.1007/s00262-013-1405-y [PubMed: 23591978]
20. Dosenovic P, Soldemo M, Scholz JL, et al. BLYS-mediated modulation of naive B cell subsets impacts HIV Env-induced antibody responses. *Journal of immunology*. 2012; 188:6018–26.10.4049/jimmunol.1200466
21. Gor DO, Ding X, Li Q, Sultana D, Mambula SS, Bram RJ, Greenspan NS. Enhanced immunogenicity of pneumococcal surface adhesin A (PsaA) in mice via fusion to recombinant human B lymphocyte stimulator (BLYS). *Biology direct*. 2011; 6:9.10.1186/1745-6150-6-9 [PubMed: 21306646]
22. Mackay F, Schneider P. Cracking the BAFF code. *Nature reviews. Immunology*. 2009; 9:491–502.10.1038/nri2572 [PubMed: 19521398]
23. Woodland RT, Schmidt MR, Thompson CB. BLYS and B cell homeostasis. *Seminars in immunology*. 2006; 18:318–26.10.1016/j.smim.2006.06.001 [PubMed: 16931037]
24. Swartz AM, Li QJ, Sampson JH. Rindopepimut: a promising immunotherapeutic for the treatment of glioblastoma multiforme. *Immunotherapy*. 2014; 6:679–90.10.2217/imt.14.21 [PubMed: 25186601]
25. Neyns B, Tosoni A, Hwu WJ, Reardon DA. Dose-dense temozolomide regimens: antitumor activity, toxicity, and immunomodulatory effects. *Cancer*. 2010; 116:2868–77.10.1002/cncr.25035 [PubMed: 20564393]
26. Su YB, Sohn S, Krown SE, et al. Selective CD4+ lymphopenia in melanoma patients treated with temozolomide: a toxicity with therapeutic implications. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*. 2004; 22:610–6.10.1200/JCO.2004.07.060 [PubMed: 14726505]
27. Garraud O, Borhis G, Badr G, Degrelle S, Pozzetto B, Cognasse F, Richard Y. Revisiting the B-cell compartment in mouse and humans: more than one B-cell subset exists in the marginal zone and beyond. *BMC immunology*. 2012; 13:63.10.1186/1471-2172-13-63 [PubMed: 23194300]

28. Blair PA, Norena LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, Mauri C. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. *Immunity*. 2010; 32:129–40.10.1016/j.immuni.2009.11.009 [PubMed: 20079667]
29. Yang M, Rui K, Wang S, Lu L. Regulatory B cells in autoimmune diseases. *Cellular & molecular immunology*. 2013; 10:122–32.10.1038/cmi.2012.60 [PubMed: 23292280]
30. Bouaziz JD, Calbo S, Maho-Vaillant M, Saussine A, Bagot M, Bensussan A, Musette P. IL-10 produced by activated human B cells regulates CD4(+) T-cell activation in vitro. *European journal of immunology*. 2010; 40:2686–91.10.1002/eji.201040673 [PubMed: 20809522]
31. Sampson JH, Schmittling RJ, Archer GE, et al. A pilot study of IL-2Ralpha blockade during lymphopenia depletes regulatory T-cells and correlates with enhanced immunity in patients with glioblastoma. *PloS one*. 2012; 7:e31046.10.1371/journal.pone.0031046 [PubMed: 22383993]
32. Mitchell DA, Cui X, Schmittling RJ, et al. Monoclonal antibody blockade of IL-2 receptor alpha during lymphopenia selectively depletes regulatory T cells in mice and humans. *Blood*. 2011; 118:3003–12.10.1182/blood-2011-02-334565 [PubMed: 21768296]
33. Fecci PE, Sweeney AE, Grossi PM, et al. Systemic anti-CD25 monoclonal antibody administration safely enhances immunity in murine glioma without eliminating regulatory T cells. *Clinical cancer research: an official journal of the American Association for Cancer Research*. 2006; 12:4294–305.10.1158/1078-0432.CCR-06-0053 [PubMed: 16857805]
34. Fecci PE, Mitchell DA, Whitesides JF, et al. Increased regulatory T-cell fraction amidst a diminished CD4 compartment explains cellular immune defects in patients with malignant glioma. *Cancer research*. 2006; 66:3294–302.10.1158/0008-5472.CAN-05-3773 [PubMed: 16540683]

Figure 1. IL-10⁺ B_{regs} frequency remains constant in glioblastoma patients undergoing TMZ and peptide vaccine

(a) PBMCs were rested overnight (o/n) in media (RPMI Medium 1640 with GlutaMAX™-I plus 10% FBS). Cells were harvested and stimulated in a 48-well plate at 4.0×10^6 cells/ml for 43 hours in media supplemented with 1 $\mu\text{g/ml}$ CD40L and 10 $\mu\text{g/ml}$ LPS. They were subsequently stimulated for 5 hours in media with 50 ng/mL PMA, 1 $\mu\text{g/ml}$ ionomycin, 10 $\mu\text{g/ml}$ LPS, and 1 $\times$ Brefeldin A (BFA) before being stained for CD19, CD14, IL-10, and, in some experiments, the EGFRvIII tetramer. (b) A representative flow cytometry sample from a patient sample is shown, compared to the appropriate negative control. (c) In the same experiments, the frequency of total B cells was found to decrease over the course of vaccination ($*p = 0.0466$). (d) No significant difference was observed in the frequency of IL-10⁺ B_{regs} in patient PBMC samples ($N = 8$) from the ACT II trial ($p = 0.20165$). Paired one tailed t-test analyses were used to determined statistical significance ($*p < 0.05$).

Figure 2. The frequency of IL-10⁺ B_{regs} is not associated with anti-EGFRvIII Ig titer or patient survival

Associations between IL-10⁺ B_{regs} frequencies and (a) patient peak serum anti-EGFRvIII titer ($r^2 = 0.03185$; $p = 0.6724$), (b) patient overall survival in months (mo) ($r^2 = 0.06793$; $p = 0.5330$) and (c) patient progression-free survival in months ($r^2 = 0.09114$; $p = 0.4674$) were evaluated. Pearson correlation statistics was used to determined statistical significance.

Figure 3. BLyS elevation does not impact frequency of IL-10⁺ B_{regs}, but significantly impacts B cell levels and proliferation

(a) There was no significant relationship observed between the frequency of IL-10⁺ B_{regs} and patient serum BLyS levels at the time of IL-10⁺ B_{regs} analysis ($R^2 = 0.3253$; $p = 0.1399$). Dotted line represents the average serum levels of BLyS in normal donors (b) *Ex vivo* experiments with healthy donor samples found no significant change in the frequency of IL-10⁺ B_{regs} in healthy donor PBMC samples in the context of soluble BLyS (2,000 ng/ml) (c). However, *ex vivo* experiments demonstrated a significant change in the frequency of B cells in healthy donor PBMC samples upon soluble BLyS addition ($p = 0.0283$). (d) B cells stimulated with anti-IgM and BLyS proliferated significantly more, as measured by a CFSE proliferation assay ($p = 0.0284$). Paired one tailed t-test analysis was used to determine statistical significance (* $p < 0.05$).

Table IBLyS serum levels of GBM patients at the time of IL-10⁺ B_{reg} analysis

All Patients	BLyS serum levels (pg/ml)	BLyS (fold increase)
ACTII(B6)	1357	1.346
ACTII(B7)	1786	1.772
ACTII(B10)	2397	2.378
ACTII(B11)	1372	1.361
ACTII(B12)	1688	1.675
ACTII(B14)	1439	1.428
ACTII(B15)	1386	1.375
ACTII(B17)	1337	1.326