RESEARCH

Remodeling of the tumor microenvironment via disrupting Blimp1+ effector Treg activity augments response to anti-PD-1 blockade

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Abstract

Background: Accumulation of Foxp3+ regulatory T (Treg) cells in the tumor often represents an important mechanism for cancer immune evasion and a critical barrier to anti-tumor immunity and immunotherapy. Many tumor-infiltrating Treg cells display an activated phenotype and express the transcription factor Blimp1. However, the specific impact of these Blimp1+ Treg cells and their follicular regulatory T (TFR) cell subset on tumor and the underlying mechanisms of action are not yet well-explored.

Methods: Various transplantable tumor models were established in immunocompetent wild-type mice and mice with a Foxp3-specific ablation of Blimp1. Tumor specimens from patients with metastatic melanoma and TCGA datasets were analyzed to support the potential role of Treg and TFR cells in tumor immunity. In vitro culture assays and in vivo adoptive transfer assays were used to understand how Treg, TFR cells and antibody responses influence tumor control. RNA sequencing and NanoString analysis were performed to reveal the transcriptome of tumor-infiltrating Treg cells and tumor cells, respectively. Finally, the therapeutic effects of anti-PD-1 treatment combined with the disruption of Blimp1+ Treg activity were evaluated.

Results: Blimp1+ Treg and TFR cells were enriched in the tumors, and higher tumoral TFR signatures indicated increased risk of melanoma metastasis. Deletion of Blimp1 in Treg cells resulted in impaired suppressive activity and a reprogramming into effector T-cells, which were largely restricted to the tumor-infiltrating Treg population. This destabilization combined with increased anti-tumor effector cellular responses, follicular helper T-cell expansion, enhanced tumoral IgE deposition and activation of macrophages secondary to dysregulated TFR cells, remodeled the tumor microenvironment and delayed tumor growth. The increased tumor immunogenicity with MHC upregulation improved response to anti-PD-1 blockade. Mechanistically, Blimp1 enforced intratumoral Treg cells with a unique transcriptional program dependent on Eomesodermin (Eomes) expression; deletion of Eomes in Blimp1-deficient Treg cells restored tumor growth and attenuated anti-tumor immunity.

Conclusions: These findings revealed Blimp1 as a new critical regulator of tumor-infiltrating Treg cells and a potential target for modulating Treg activity to treat cancer. Our study has also revealed two FCERIA-containing immune signatures as promising diagnostic or prognostic markers for melanoma patients.

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Background
The immune responses and self-tolerance are stringently controlled by Foxp3+ Treg cells, but accumulation of these Treg cells within the tumor represents a major obstacle to the development of effective anti-tumor immunity and immunotherapy [1–5]. The frequency of Foxp3+ Treg cells among tumor-infiltrating lymphocytes (TIL) is often associated with poor prognosis of patients with various cancers [1–5]. According to phenotypic and functional specialization, Foxp3+ Treg cells are categorized into central Treg and effector Treg (eTreg) subsets [6, 7]. The eTreg subset displays an activated phenotype and effector program, and expresses the transcription factor (TF) Blimp1 (encoded by Prdm1) [7, 8]. TIL Blimp1-expressing Treg cells have been recently proposed to be included for outcome prediction of some cancer patients [9]. We and others have also established that Blimp1 is required for the lineage stability and suppressive activity of Foxp3+ Treg cells during ongoing immune or inflammatory responses [10–13]. However, the contribution of Blimp1+ Treg cells to tumor progression remains largely unclear. Their tumor-specific regulatory activities and the impact of the tumor microenvironment (TME) on their function are entirely unknown.

Our recent study has also revealed that Blimp1 is required for the stability and suppressive activity of TFR cells that belong to a type of eTreg cells. Expression of Blimp1 in TFR cells ensures the proper regulation of follicular helper T (T FH) cells, B-cells and germinal center (GC) antibody (Ab) responses [10, 13]. While increased TIL T FH cells and B-cells as well as the formation of tertiary lymphoid structures (TLS) are associated with favorable outcomes in patients with certain types of cancer and better responses to checkpoint blockade therapies [14–18], the contribution of TFR cells and humoral Ab responses to the regulation of anti-tumor immunity remains poorly understood.

Here, using various transplantable tumor models, we evaluated how the TME imprints Blimp1+ Treg cells and how disruptions of their suppressive activity reshape local and systemic immune responses as well as responses to PD-1 checkpoint blockade.

Methods
All reagents or resources are listed in Additional File 1, if not specified in the text.

Mice and human samples
C57BL/6j (B6), Prdm1fl/fl, Foxp3YFP-Cre, Rosa26Cre-ERT2, Eomesfl/fl, Tcrα−/− and Prdm1YFP (Blimp1-YFP) (Jackson Labs) mice were housed in pathogen-free conditions. Prdm1fl/fl mice were bred onto Foxp3YFP-Cre or Rosa26Cre-ERT2 mice to generate Prdm1fl/flFoxp3YFP-Cre, Prdm1fl/flFoxp3YFP-Cre or Prdm1fl/flRosa26Cre-ERT2 mice, respectively. Prdm1fl/flFoxp3YFP-Cre mice were further crossed onto Eomesfl/fl mice to yield Eomesfl/flPrdm1fl/flFoxp3YFP-Cre (double knockout, DKO) mice. All mice were used at the age of 5 to 10 weeks unless otherwise specified. Both sexes (males or females) were randomly included for comparison groups in all experiments in an unblinded fashion. Generally, 3–7 mice were used per group unless otherwise indicated in each experiment. De-identified tissue samples from patients with stage IV metastatic melanoma and control tissues were provided from the University of Alabama at Birmingham (UAB) Tissue Collection and Banking Facility. The characteristic of these samples is listed in Additional file 2.

Cell lines
B16-F10 melanoma cells were purchased from American Type Culture Collection. B16-F10, B16 cells expressing the surrogate antigen ovalbumin (B16-OVA) or B16 expressing granulocyte-macrophage colony stimulating factor, GM-CSF (B16-GVAX) and MC38 colon cancer cells were cultured in complete Dulbecco’s Modified Eagle Medium (DMEM; Millipore Sigma) containing 10% FBS (Atlanta Biologicals) and 1 × Penicillin/Streptomycin (Millipore Sigma), as described previously [19, 20]. 250 μg/mL of G418 was added into the B16-OVA tumor cell line culture. All tumor cell lines were pathogen free, used within three to eight passages, and maintained at 37 °C with 5% CO2.

Tumor models
Mice were implanted subcutaneously (s.c.) with 2.5 × 105 B16-F10 or MC38 cells, or 2.5–4 × 106 B16-OVA cells on the flank on day 0. Mice implanted with B16-OVA cells were intraperitoneally (i.p.) immunized with NP-OVA in complete Freund’s adjuvant (CFA) on day 0 and NP-OVA in incomplete Freund’s adjuvant (IFA) on day 7. In some cases, mice implanted with B16-OVA cells were immunized s.c. with 1 × 106 irradiated (150Gy) GVAX on the opposite flank on day 1, and then 1 × 106 irradiated B16-OVA and GVAX on alternating flanks on days 3 and 7.
Tumor volume was measured 2–3 times per week using calipers and calculated as \((x \times y \times z)/2\) mm³. For mice treated with anti-PD-1, 200μg anti-PD-1 or rat IgG2a isotype control (BioXcell) was i.p. injected into mice at days 3,6,9 post-tumor inoculation, according to the protocol established by others [21–23]. Mice with the tumor reaching 2 cm on the longest axis or with >10% ulcerated tumor or with <2 body condition score, according to the UAB Animal Care and Use Committee (IACUC) guidelines, were euthanized before the end of the study. Mice were euthanized by CO₂ inhalation followed by cervical dislocation.

**Cell isolation**
The spleen was extracted, and a single cell suspension was obtained by mashing the spleen between frosted microscope slides. Red blood cells were then removed using the Ammonium-Chloride-Potassium (ACK) lysis buffer and the cell suspension was filtered through a 70μm filter membrane to eliminate debris. To isolate single cells from B16 melanoma or fresh human tissues, tumors or control tissues were mechanically dissociated into small pieces (<3 mm) followed by an agitated digestion for 1 h at 37°C in a dissociation solution (PBS supplemented with 2% FBS, 1 mg/ml collagenase/Dispase and 0.5 mg/ml DNase I for B16 or PBS supplemented with 2% FBS, 0.5 mg/ml collagenase/Dispase for human tissues). Digested samples were washed with DMEM/2% FBS and passed through a 70μm cell strainer, and then separated on a Ficoll-Paque 1.084 density gradient (40% mixed with cells/80% for B16, or 75%/100% with the cell solution layered on the top for human tissue) by centrifugation. Immune cells were collected for further analysis.

**Flow cytometry and sorting**
Single cell suspension was first stained with the fixable viability dye at 1:1000 in PBS for 10 min. After washing with flow activated cell sorting (FACS) buffer (PBS/2%FBS), cells were incubated with Fc block at 1:200 for 10 min, followed by staining with indicated antibody mixtures for 30 min before washing and flow cytometry analysis. For intracellular staining, including IgE, cells were fixed and permeabilized using the FoxP3 staining Buffer Set according to the manufacturer’s protocol. Cells were then incubated with Fc block and intracellular antibodies for 30 min before washing and flow cytometry analysis. All of the steps were performed at 4°C. For intracellular cytokine analysis, cells were stimulated with the BD Leukocyte Activating Cocktail, with BD GolgiPlug for 5 h at 37°C with 5% CO₂, prior to staining, as described above. Cells were acquired on a BD LSR II or FACSymphony using FACSDiva software (BD Biosciences) and analyzed using FlowJo software (Treestar).

For cell sorting, single cell suspensions isolated from spleens were enriched for CD4⁺ T-cells using the CD4 microbeads (Miltenyi Biotec). Enriched CD4⁺ T-cells or tumor cells were then stained with viability dye and surface antibodies as described above, followed by sorting on a FACSARia II using FACSDiva software.

**Adoptive transfer and tamoxifen treatment**
Donor mice were i.p. immunized with 100μg NP-OVA in CFA as described above. Splenocytes were isolated from donor mice and CD4⁺ T-cells were enriched using the CD4 microbeads before sorting CD4⁺PD-1⁺CXCR5⁺ follicular T-cells. 5 × 10⁵ sorted cells were intravenously injected into Tcrα⁻⁻⁻ mice followed by B16-OVA implantation and NP-OVA immunization, as described above. To deplete Blimp1 in TFR cells, mice were i.p. injected with 1 mg tamoxifen emulsified in sunflower oil once every 24 h for 5 consecutive days. Mice were monitored daily after injection.

**Enzyme-linked Immunosorbent assay (ELISA)**
In this study, peripheral blood (about 0.2 ml) was collected from each mouse at the experiment endpoint. Serum was separated via centrifugation and frozen at −20°C until testing. Total IgE titers were determined by the IgE OptEIA ELISA Set, according to the manufacturer’s protocol. Total IgG levels were measured using the purified goat anti-mouse IgG as the coating antibody, and goat anti-mouse IgG HRP as the detection antibody. The serum titers of anti-OVA IgE and anti-OVA IgG were measured using the OVA protein as the coating reagent, and biotinylated anti-mouse IgE followed by streptavidin HRP (contained in the IgE OptEIA ELISA Set) or goat anti-mouse IgG HRP as the detection antibody, respectively. The OD was read on the Ultra Micro EL 808 microplate reader (Biotek Instruments) at 450 nm.

**Immunofluorescent microscopy**
Mouse tumor tissues were flash frozen in liquid nitrogen and embedded in optimum cutting temperature (OCT) compound. The frozen blocks were stored in −80°C until sectioning into 7μm sections. Sections were stored in −80°C before being thawed and fixed with acetone for 10 min at −20°C. Sections were then blocked with either PBS/5% BSA or PBS/5% animal serum matching the species of the secondary fluorescent antibody for 1 h. Sections were stained with FITC-conjugated anti-mouse CD3e and Alexa Fluor 594-conjugated rat anti-mouse CD45R/B220, or Alexa Fluor 647-conjugated rat anti-mouse CD31, or Alexa Fluor 488-conjugated rat anti-mouse CD68 and purified rat anti-mouse IgE that was visualized using Alexa Fluor 555-conjugated goat anti-rat IgG, or purified Armenian hamster anti-mouse FceR1α
that was visualized using Alexa Fluor 594-conjugated goat anti-hamster IgG. Nuclei were counterstained with DAPI. For analysis of TFR and TFI cells from formalin-fixed paraffin embedded (FFPE) melanoma tissues, tissue blocks were cut into 5–7 μm sections onto microscope slides followed by deparaffinizing and rehydrating. Sections then underwent antigen retrieval by boiling in IHC Antigen Retrieval Solution (high pH) before maintaining at a sub-boiling temperature for 20 min. After washing and sections were then blocked for any non-specific binding in PBS/Tween20/5% animal serum for 30 min at room temperature (RT) before staining with purified rabbit anti-human CD4, mouse anti-human CXCR5 and rat anti-human Foxp3 for 2 h at RT. After washing, Alexa Fluor 488-conjugated goat anti-rabbit IgG, Alexa Fluor 647-conjugated goat anti-mouse IgG and Alexa Fluor 555-conjugated goat anti-rat IgG were added respectively, and incubated for 1 h at RT prior to counterstaining nuclei with DAPI. Images were captured with a Leica DMRB microscope equipped with Hamatsu C4742-95 and 3CCD color cameras and appropriate filter cubes, and acquired using OpenLAB 3.1 software (Agilent Technologies). Image pseudo-coloring and quantification of stained areas or cells were performed using ImageJ software (NIH).

In vitro Treg suppression assay
The suppression assay by Treg cells was performed as previously described [24]. Briefly, CD8+ T-cells were enriched from spleens using the CD8 microbeads, and Treg cells from spleens and tumors were enriched using the CD4+CD25+ Regulatory T-Cell Isolation Kit. Enriched CD8+ T-cells were then labelled with the cell trace violet (CTV) and cultured alone, or with enriched Treg cells from indicated tissue and mice plus 5 μg/ml plate-bound anti-CD3ε and 2 μg/ml anti-CD28. 60 h later, CTV staining of CD8+ T-cells was analyzed by flow cytometry. The division index was retrieved using the FlowJo proliferation tool and the percent of suppression was calculated as 100−(division index in each experimental group / division index in CD8+ T-cell alone group)*100.

Isolation of mouse bone marrow-derived macrophages (BMDMs)
BMDMs were isolated and differentiated as previously described [25]. Bone marrow cells were flushed from the femurs and tibias of sacrificed mice using cold 1× PBS until the bones appeared clear. The cells were centrifuged and filtered through a 70 μm cell strainer and red blood cells lysed. Cells were cultured in complete DMEM containing 10 ng/mL M-CSF for 7 days with a media change every 2 days. Cultured cells were harvested and stained with the marker F4/80 to assess the purity before further analysis.

Macrophage-mediated phagocytosis and killing of tumor cells
2 × 10^5 CTV-labeled B16-OVA cells were co-cultured with 1 × 10^5 differentiated BMDMs in complete DMEM along with 2.5 μl serum (1.25% of the total culture) collected from tumor-bearing mice for 2 h or overnight. Sera pre-treated with anti-IgE (50 μg/ml) were included as controls. Following co-culture, the frequency of CTV+F4/80+ macrophages and CTV+B16-OVA cells positive for the viability dye, defined as dead cells, were measured by flow cytometry, respectively. The percent tumor killing is calculated using the following equation: [%dead cells (experimental group)−%dead cells (tumor alone)]/% dead cells (tumor alone).

TCGA dataset analysis
Correlation analyses of relevant genes with RSEM normalized TPM values from the TCGA-Skin Cutaneous Melanoma (SKCM) study were performed using Tier 3 standardized, normalized, batch corrected, and platform-corrected RNASEQ datasets downloaded from the Oncomlnc server (http://www.oncolnc.org). Linear regression model was used to calculate correlation coefficients and p value. To analyze multi-gene dependent fractions between primary and metastatic groups and geneset scoring based survival proportions, normalized gene expression data and clinical information of each patient from the TCGA-SKCM dataset were downloaded using UCSC Xena (https://genome-cancer.ucsc.edu/). Gene signature-based score was calculated by averaging log transformed transcript levels of indicated genes as described elsewhere [26]. Survival time was chosen based on the overall survival (OS) time and OS status. The OS status denotes survival time in days, and the status indicates whether the patient’s death was observed (status = 1) or that survival time was censored (status = 0). To investigate whether the differences in geneset-based computed scores can influence a patient’s survival, patient samples were grouped into high scoring or low scoring cohorts based on their top or bottom percentile scores. Survival curves were computed for the patients with each group using the Kaplan-Meier method. The log rank test was used to define whether patients with different geneset scores have significantly different survival time (p < 0.05).

RNA isolation and sequencing
Total RNA from sorted CD44+ Treg cells was extracted using a QIAshredder kit (QIAGEN) and an RNeasy Plus Micro Kit (QIAGEN) according to the manufacturer’s
RNA-seq data analysis
Sequence reads were trimmed to remove possible adapter sequences and nucleotides with poor quality using Trimomatic v.0.36. The trimmed reads were mapped to the *Mus musculus* GRCm38 reference genome available on ENSEMBL using the STAR aligner v.2.5.2b. The STAR aligner is a splice aligner that detects splice junctions and incorporates them to help align the entire read sequences. BAM files were generated as a result of this step. Unique gene hit counts were calculated by using featureCounts from the Subread package v.1.5.2. Only unique reads that fell within exon regions were counted. After extraction of gene hit counts, the gene hit counts table was used for downstream differential expression analysis. Using DESeq2, a comparison of gene expression of each group was performed. The Wald test was used to generate p-values and log2 fold changes. Genes with an adjusted p-value < 0.05 and absolute log2 fold change >1 were called as differentially expressed genes for each comparison. The Volcano plot shows the global transcriptional change across the groups compared. All the genes are plotted and each data point represents a gene. The log2 fold change of each gene is represented on the x-axis and the −log10 of its adjusted p-value is on the y-axis. The upregulated genes in WT eTreg cells with an adjusted p-value < 0.05 and a log2 fold change >1 are indicated by red dots. The downregulated genes in WT eTreg cells with an adjusted p-value < 0.05 and a log2 fold change < −1 are indicated by blue dots. Principal component analysis was performed to reveal the similarity within and between groups. Differentially expressed genes were also analyzed using Advaita’s "iPathwayGuide" (Advaita Bioinformatics) or g:Gost (g:Profiler) to reveal the biological pathways.

NanoString nCounter mRNA analysis
Total RNA from sorted CD45+ tumor cells was extracted using a QIAshredder kit (QIAGEN) and an RNeasy Plus Micro Kit (QIAGEN) according to the manufacturer’s protocol. RNA quantification was performed using the DeNovix DS-11 Spectrophotometer (DeNovix, Inc). 100ng of purified RNA was added to 3μL of Reporter CodeSet and 2μL Capture ProbeSet using an nCounter master kit as recommended (NanoString Technologies). Samples were processed on the NanoString nCounter Flex System per manufacturer instructions using the nCounter Mouse PanCancer Immune Profiling panel or the nCounter Mouse PanCancer Pathways panel (NanoString Technologies). Each gene set interrogates 750 cancer-related genes alongside 20 internal reference controls (full gene list and controls available on manufacturer’s website). Differentially expressed genes were identified in nSolver 4.0 Analysis Software (NanoString) as genes with a p value of less than 0.05 versus the respective baseline control. Reactome pathway analysis was performed using the NetworkAnalyst. The NanoString data have been deposited in the NCBI GEO under accession number GSE178135.

Statistics
Statistical analyses were performed using two-tailed, unpaired or paired Student’s t-test, or two-way ANOVA with GraphPad Prism V8 software. Error bars indicate mean ± SEM. A P value of <0.05 was considered to be statistically significant (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001). No exclusion of data points was used. Sample size was not specifically predetermined, but the number of mice used was consistent with previous experience with similar experiments.

Results
Blimp1+ Treg and TFR cells are accumulated in the tumor
To first understand the extent to which the TME could influence Blimp1+ Treg and TFR cells, we analyzed Treg cells from Blimp1-YFP reporter mice inoculated with B16-OVA. To facilitate the analysis of TFR cells and Ab response [10, 27], we immunized mice with NP-OVA, given that immunization or vaccination boosts the anti-tumor immune response [28]. When the tumor reached ~1 cm3, 80% TIL Treg cells but only 35% splenic Treg cells from an individual mouse displayed the eTreg phenotype (CD62LloCD44hiFoxp3+CD4+) (Fig. 1a and Additional file 3a,b and 4a). Unlike splenic eTreg cells that only had 5% positive for Blimp1 (YFP), about 50% TIL eTreg cells expressed Blimp1 (YFP) and almost all of the TIL Blimp1+ Treg cells expressed IL-10, consistent with the role of Blimp1 in the regulation of IL-10 expression [7]. TIL Blimp1+ (YFP+) Treg cells compared to their splenic counterparts also expressed higher levels of Blimp1, Foxp3, GITR, Helios and CTLA-4 (Fig. 1b and Additional file 4b), markers associated with an activated phenotype and robust suppressive activity [10, 29, 30]. Consistently, interrogating the transcriptome
of TIL versus splenic CD44+ Treg cells (i.e., eTreg cells) that were isolated and sorted from these mice prior to RNA sequencing (RNA-seq) showed that TIL eTreg cells displayed a distinct transcriptional signature (Additional file 3c and 4c-d), suggesting that eTreg cells could adapt to the TME to acquire unique features.

Further analysis of these tumor-bearing mice revealed that TFR cells (PD1+Bcl6+Foxp3+CD4+CD3+), TFH cells (PD1+Bcl6+Foxp3−CD4+CD3+) and GC B-cells (GL7+Fas+CD19+) were more enriched in the tumor than in Foxp3+ Treg, CD4+Foxp3− effector T-cells (Teff) and B-cell compartment, respectively, in the tumor than...
those in the spleen (Fig. 1c and Additional file 3a,b). A 2-3 fold increase in the ratios of TFR:Tfh or TFR:GC B in the tumor compared to those in the spleen suggested that the immunosuppression in the tumor might include suppression of Tfh-Ab response by TIL TFR cells.

**TIL Treg and TFR cells from melanoma patients express higher levels of Blimp1 with increased suppressive phenotype**

Next, we analyzed Treg and TFR cells from a group of patients with stage IV melanoma (Additional file 2). The abundance of Treg cells (CD25+CD127+CD4+CD3+ Tfh) in the metastatic tissues, including lung, liver and lymph node, were generally increased compared to Treg cells from adjacent uninvolved control tissues. These TIL Treg cells expressed higher levels of Foxp3, Helios and CTLA-4, except for slight decreases observed in the metastatic liver. Notably, these TIL Treg cells consistently expressed higher levels of Blimp1 than Treg cells from control tissues (Fig. 1d,e and Additional file 3d). Interestingly, there were substantially more TFR cells (PD-1+CXCR5+CD25+CD127+CD4+CD3+) in these metastatic tissues, including lung, liver and lymph node, compared to Treg cells from adjacent uninvolved control tissues. These TIL Treg cells being closely localized (Fig. 1h). This analysis suggested that TIL Treg and TFR cells might be involved in the regulation of tumor immunity in a set of melanoma patients. Notably, analysis of a SKCM patient cohort from TCGA datasets showed that PRDM1 mRNA expression was positively correlated with FOXP3 mRNA expression, and among the FOXP3hi population, PRDM1 expression was positively correlated with the levels of FUT4 and FUT7, genes encoding fucosyltransferase 4 and 7 that are enzymes responsible for the synthesis of CD15 and CD15s, respectively. The latter is a marker specific for highly suppressive human FOXP3hi eTreg cells [31] (Additional file 4e-g). Moreover, there were increased proportions of metastatic patients with higher expression of CXCR5 and PRDM1 in Treg cells (Fig. 1i), suggesting that high levels of CXCR5 and PRDM1 in Treg cells are correlated with increased risk of melanoma metastasis.

Delayed tumor growth and enhanced anti-tumor effector responses in mice with Foxp3-specific deletion of Blimp1

The above analysis of melanoma patients and mouse B16 models prompted us to investigate if Blimp1 expression in Treg and TFR cells regulates tumor immunity. We then implanted B16 or MC38 cells into Foxp3Cre mice (WT) and mice harboring a deletion of Prdm1 in Foxp3+ T-cells, which mainly affects eTreg subsets (Prdm1fl/flFoxp3Cre) [10]. While B16.F10 and MC38 tumors gradually developed in WT mice, Prdm1fl/flFoxp3Cre mice had delayed tumor growth with smaller volumes (Fig. 2a,b). As expected, immunization with NP-OVA or vaccination with irradiated GVAX [20] delayed tumor growth in both groups. However, Prdm1fl/flFoxp3Cre mice remained to develop much smaller tumors with a slower growth rate than WT mice, while a partial deletion of Blimp1 in heterozygous Prdm1fl/+Foxp3Cre mice also prevented the tumor growth (Fig. 2c,d).

Immune profiling revealed that tumor-infiltrating, but not splenic, CD4+Foxp3-Teff, CD8+ T-cells and NK cells expressed markedly higher levels of effector molecules, including IFNγ, TNFa and Granulysin B (GzmB), in Prdm1fl/flFoxp3Cre mice compared to WT mice, despite that the total frequency of these cells in the tumor was not significantly changed (Fig. 2e and Additional file 6a-c). In addition to these effector cells, dendritic cells (DC) (CD11c+MHCII+ and MHCII+ M1 type macrophages relative to CD206+ M2 type macrophages (CD11b+GR-1−F4/80−)) (but not the frequency of total macrophages) in the tumor of Prdm1fl/flFoxp3Cre mice were increased compared to WT mice (Fig. 2f,g and Additional file 6a). Taken together, these results suggested that the deletion of Blimp1 in Treg cells resulted in improved tumor control associated with enhanced activation of both adaptive and innate effector cells in the tumor.

**TIL Blimp1-deficient Treg cells convert into effector T-cells and display impaired suppressive activity**

We next analyzed the Treg compartment from these tumor-bearing mice. Despite an increased frequency, TIL Treg cells from Prdm1fl/flFoxp3Cre mice down-regulated Foxp3 but expressed increased effector molecules (IFNγ, TNFa and GzmB) compared to WT Treg cells (Fig. 3a-c), suggesting that TIL Blimp1-deficient Treg cells were unstable and reprogrammed into Teff. The conversion of TIL Treg cells was also observed in Prdm1fl/flFoxp3Cre mice vaccinated with GVAX (Fig. 3d). In contrast, there were no significant differences in the frequencies and effector molecule expression comparing splenic Treg cells from Prdm1fl/flFoxp3Cre mice to WT mice (Additional file 6d),
indicating that conversion of Blimp1-deficient Treg cells into Teff was largely restricted to the tumoral compartment.

The conversion of TIL Blimp1-deficient Treg cells may reflect a loss of suppressive activity by these cells. To test this proposition, we performed the in vitro suppression assays using Treg cells isolated from tumors compared to spleens from both groups of mice. Coculture of splenic Treg cells with CTV-labelled CD8+ T-cells showed that these Treg cells from both mice equally suppressed CD8+ T-cell proliferation. TIL Treg cells exhibited more suppressive activity than splenic Treg cells, and TIL Treg cells from WT mice were able to suppress CD8+ T-cell proliferation. However, TIL Treg cells from Prdm1fl/fl Foxp3YFP-Cre mice had greatly reduced suppression on CD8+ T-cell proliferation (Fig. 3e). These findings suggested that only Treg cells in the tumor were converted and no longer efficiently suppressed Teff.

**Foxp3 specific deletion of Blimp1 results in increased anti-tumor humoral immunity**

We next analyzed TFR, TFH and GC B-cells in Prdm1fl/fl Foxp3YFP-Cre and WT mice bearing B16-OVA tumors. Compared to WT mice, Prdm1fl/fl Foxp3YFP-Cre mice had increased frequency of TFH and GC B-cells in the tumor; this difference was not observed in the spleen and there was no significant difference of TFR cell frequency in the spleen and tumor from both groups of mice (Fig. 4a,b). Although there were high titers of serum IgG and anti-OVA (tumor) IgG Abs, only slight increases were observed in Prdm1fl/fl Foxp3YFP-Cre mice. In contrast, Prdm1fl/fo Foxp3YFP-Cre mice had significantly elevated titers of serum IgE and anti-OVA (tumor) IgE Abs compared
to WT mice, and anti-OVA IgE Abs were almost undetectable in WT mice (Fig. 4c). Consistent with the finding that serum specific IgE scores are inversely correlated with the risk of melanoma [32], serum IgE titers were inversely correlated with the tumor sizes of Prdm1<sup>fl/fl</sup> Foxp3<sup>YFP-Cre</sup> mice (Fig. 4d). The increased IgE and anti-OVA (tumor) IgE Ab titers were also observed in Prdm1<sup>fl/fl</sup> Foxp3<sup>YFP-Cre</sup> mice vaccinated with GVAX (Fig. 4e). IF analysis further confirmed that more CD3<sup>+</sup> and B220<sup>+</sup> cells were accumulated in the tumor of Prdm1<sup>fl/fl</sup> Foxp3<sup>YFP-Cre</sup> mice. Compared to the diffused distribution of these cells in WT mice, more of these cells were clustered in the tumor of Prdm1<sup>fl/fl</sup> Foxp3<sup>YFP-Cre</sup> mice (Fig. 4f and Additional file 7a). Interestingly, we also observed increased IgE deposition in the tumor along with increased IgE<sup>+</sup>B-cells in these mice compared to WT mice (Fig. 4f,g and Additional file 7b). Taken together, these results demonstrated how loss of suppressive function by Blimp1<sup>+</sup>Treg cells could lead to an increased cellular and humoral anti-tumor response, thus resulting in better tumor control. Despite the robust anti-tumor responses, no obvious autoimmune phenotype was observed throughout the experiments.

### Transfer of Blimp1-deficient T<sub>FR</sub> cells induces better anti-tumor response

Blimp1<sup>+</sup>Treg cells comprise both T<sub>FR</sub> cells and conventional non-T<sub>FR</sub> Treg cells. Our recent publication has indicated that Blimp1-deficient non-T<sub>FR</sub> Treg cells do not contribute significantly to the increased frequency of TFH and GC B-cells or dysregulated Ab responses observed in Prdm1<sup>fl/fl</sup> Foxp3<sup>YFP-Cre</sup> mice [10]. Instead, Blimp1-deficient T<sub>FR</sub> cells are capable of supporting GC-Ab response due to the acquisition of TFH-like properties post-immunization [10]. To further define the contribution of Blimp1<sup>+</sup>T<sub>FR</sub> cells independent of other Treg cells to the regulation of Ab responses and tumor growth, we used an inducible Blimp1 deletion system to circumvent potential developmental defects secondary to inflammation or other changes in the environment (Fig. 4h). We generated Prdm1<sup>fl/fl</sup>Rosa26<sup>Cre</sup>-ERT2 (del) mice to allow deletion of Blimp1 after administration of tamoxifen. Follicular T-cells (PD1<sup>+</sup>CXCR5<sup>+</sup>CD4<sup>+</sup>) (both Blimp1<sup>+</sup>T<sub>FR</sub> and Blimp1<sup>−</sup>T<sub>FH</sub>) were sorted from Prdm1<sup>fl/fl</sup>Rosa26<sup>Cre</sup>-ERT2 mice or Rosa26<sup>Cre</sup>-ERT2 (WT) mice 7 days after NP-OVA immunization and 1 day after tamoxifen administration. These cells were then transferred into Tcra<sup>−/−</sup> mice before B16-OVA implantation and injection of tamoxifen for 4 additional days (Fig. 4h,i). This method
can substantially reduce Blimp1 expression specifically by T FR cells from Prdm1^fl/fl^ Rosa26^Cre-ERT2^ mice along with increased T FR, TFH and GC B-cells [10]. We observed that Tcrα^−/−^ mice transferred with follicular T-cells bearing Blimp1-deleted T FR cells had smaller and delayed tumor growth associated with increased total and particularly OVA-specific IgG and IgE compared to mice transferred with follicular T-cells containing WT T FR cells (Fig. 4j). The results obtained from this adoptive transfer assay suggested that transfer Blimp1-deleted T FR cells compared to WT T FR cells can intrinsically contribute to increased Ab production and enhanced tumor control.

Sera from Prdm1^fl/fl^Foxp3^YFP-Cre^ tumor-bearing mice increase the anti-tumor activity of macrophages

Antibodies specific to tumor antigens bind directly to tumor cells while cross-linking with Fc receptors on effector cells, which triggers antibody dependent
cell-mediated cytotoxicity or phagocytosis of tumor cells. The Ab deposition in the tumor may contribute to effector cell-mediated tumor killing, and IgE has been reported to promote the macrophage polarization into M1 phenotype [33] and modulate macrophages against cancer [34, 35], while we noted increased intratumoral M1 macrophages in Prdm1fl/flFoxp3YFP-Cre mice (Fig. 2g).

To identify how increased IgE in the tumor could impact the cellular immunity against tumor cells, we first performed IF analysis of the IgE localization in relationship to macrophages in the tumor. Co-staining of the macrophage marker CD68 with IgE or its receptor FcεRIα revealed increased IgE+CD68+ and FcεRIα+CD68+ cells in the tumor of Prdm1fl/flFoxp3YFP-Cre mice compared to WT mice (Fig. 5a,b). Moreover, among all IgE+ cells in Prdm1fl/flFoxp3YFP-Cre tumors, over 85% cells were positive for CD68 while fewer CD68− cells were co-stained with IgE, suggesting that macrophages were the major IgE effector cells. We then explored if increased Ab production in these mice may promote macrophage-mediated phagocytosis and/or killing of tumor cells using an in vitro culture assay. BMDMs from adult WT mice were co-cultured with CTV-labelled B16-OVA cells treated with or without a same volume of sera collected from Prdm1fl/flFoxp3YFP-Cre or WT tumor-bearing mice. Using a published flow cytometry-based measurement of in vitro phagocytosis by macrophages, where macrophages containing CTV-labelled tumor cells were analyzed [36], we noted that the addition of sera increased F4/80+CTV+ cells and there were more of these cells after treatment with Prdm1fl/flFoxp3YFP-Cre sera than WT sera (Fig. 5c), suggestive of increased phagocytosis by macrophages. Correspondingly, the highest killing was observed for tumors incubated with both Prdm1fl/flFoxp3YFP-Cre sera and macrophages (Fig. 5d).

Importantly, neutralizing IgE activity by pre-incubating sera with anti-IgE markedly diminished macrophage-mediated phagocytosis and tumor killing (Fig. 5c,d). These findings suggested that sera from Prdm1fl/flFoxp3YFP-Cre mice, at least partly due to 4.5 fold more IgE included (Fig. 4c), had the potential to increase macrophage-mediated phagocytosis and killing of tumor cells, which may contribute to delayed tumor growth. Further analysis of TCGA datasets revealed that FCER1A was positively correlated with the M1 macrophage marker CD86 within the CD68hi population (Fig. 5e). A quantitative measure of the putative anti-tumor macrophage signature based on transcript levels of 3 factors extracted from the TCGA dataset, CD68, CD86 and FCER1A, further showed that the higher expression of this signature (named as Fcem1) was correlated with the better survival of SKCM patients (Fig. 5f).

TIL eTreg cells from Prdm1fl/flFoxp3YFP-Cre mice display distinct transcriptomic profiles

To understand why the Treg alteration in Prdm1fl/flFoxp3YFP-Cre mice was selectively induced in the tumor but not in the periphery, we performed RNA-seq analysis of splenic and TIL CD4+ Treg cells from WT compared to Prdm1fl/flFoxp3YFP-Cre mice in our B16-OVA/NP-OVA model (Fig. 6a and Additional file 3c). While only 78 genes (at a > 2 log2-fold change and P < 0.05) were differentially expressed in splenic Blimp1-deficient compared to WT eTreg cells, 734 genes were differentially expressed in TIL Blimp1-deficient compared to WT eTreg cells and only 13 of these differentially expressed genes (DEGs) were shared in splenic and TIL eTreg cells (Fig. 6a), suggesting that the extent of differential gene expression imposed by Blimp1 deficiency in eTreg cells depended greatly on the tissue microenvironment. Principal component analysis of the relationship of these splenic and tumoral eTreg cells also revealed that TIL eTreg cells formed groups that were mostly distant from splenic eTreg cells irrespective of Blimp1 genotype (Fig. 6b). Moreover, the segregation of TIL Blimp1-deficient from WT eTreg cells was much greater than that of splenic eTreg cells, despite that variations existed for the TIL eTreg samples (Fig. 6b). Interestingly, pathway analysis further revealed that genes

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**Fig. 5** Sera from Prdm1fl/flFoxp3YFP-Cre tumor-bearing mice increase the anti-tumor activity of macrophages. a-b) B16-OVA model was established as in Fig 2c. IF staining of IgE and CD68 in the tumor and quantitation of IgE+CD68+ and IgE−CD68− cells (a); or IF staining of FcεRIα and CD68 in the tumor (b). Each dot represents the counted numbers within a field of view (160 x). WT: n = 15 views from 6 mice; KO: n = 14 views from 6 mice. c-d) BMDMs were co-cultured with CTV-labelled B16-OVA cells treated with or without a same volume of sera (2.5 μl, 1.25% of the total culture) collected from WT or Prdm1fl/flFoxp3YFP-Cre (KO) tumor-bearing mice for 2 h (c) or overnight (d) in quadruplicates. In a group, sera were pre-treated with anti-IgE (a-IgE). c) BMDMs with phagocytosed B16-OVA cells were indicated as CTV+F4/80+ cells (upper left, representative F4/80+ plot; lower left, representative histogram of CTV expression gated on F4/80+ cells; right, quantitation). d) Percent tumor killing is shown after quantifying dead tumor cells (CTV+ cells positive for viability-dye). Tumor cells alone treated with sera were included as controls. Triangles, tumor cells and BMDMs with no sera added. ns, no significance; * P < 0.05; ** P < 0.01; *** P < 0.001 and **** P < 0.0001 (a, c, d, unpaired two-tailed Student’s t-test). Bars, mean ± SEM. e) The correlation of CD86 and FCER1A expression in top 25% CD68hi SKCM patients from the TCGA dataset (n = 110) was analyzed by Pearson correlation (two-tailed, no adjustment for multiple comparisons because of one correlation test for a gene pair). The values of the coefficients (r) and significance (p) are indicated. f) Kaplan-Meier analysis of OS of patient cohorts expressing differential Fcem1 signature (top 33% vs bottom 33%) based on combined log-averaging of CD68, CD86 and FCER1A transcript levels from the TCGA-SKCM dataset. P value is generated using two-tailed LogRank test. Median, median survival time.
related to the NK mediated cytotoxicity pathway were highly represented in TIL Blimp1-deficient eTreg cells (Fig. 6c). Zooming in genes that were significantly differentially expressed in TIL Blimp1-deficient compared to WT eTreg cells showed that TIL Blimp1-deficient eTreg cells not only downregulated genes reflecting Treg stability and suppressive activity (e.g., Foxp3, Ctla4, Il2ra, Ikrf2, Il10, Ebi3, Ikrf4) but also concomitantly upregulated genes characteristic of NK cell cytotoxic program (e.g., Eomes, Klrk1, Lamp1, Klrc2, Crtam, Ifng and genes encoding granzymes) (Fig. 6d). Consistent with the reduced expression of Il2ra and Foxp3 (Fig. 3a), there were reduced levels of phospho-Stat5 (pStat5), but not phospho-Smad2/3 (pSmad2/3), in TIL Blimp1-deficient...
Treg cells compared to WT Treg cells (Additional file 8). The tissue-specific genetic profile suggested that TIL eTreg stable phenotype could be influenced by both Blimp1 expression and the TME.

**Deletion of Eomes in Blimp1-deficient Treg cells promotes tumor growth**

We noted that the TF Eomes was highly upregulated in TIL Blimp1-deficient eTreg cells compared to WT eTreg cells (Figs. 6d and 7a). While Foxp3+ Treg cells express very low levels of Eomes and deletion of Eomes in Treg cells does not appear to affect their suppressive phenotype [37], Eomes is known to modulate the cytotoxic programs in Teff, including CD4+ cytotoxic T-cells [38]. Blimp1 can directly bind to the Eomes loci and regulate its expression [39]. We reasoned that the increased anti-tumor activity and cytotoxicity-like genetic program in TIL Blimp1-deficient eTreg cells may be at least in part mediated by the upregulation of Eomes. To test this proposition, we generated Eomesfl/fl mice.
Deletion of Eomes in Blimp1-deficient Treg cells promotes tumor growth. **a)** Comparison and quantitation of Eomes levels in TIL Treg cells from B16-OVA/NP-OVA mice (n = 8 per group), as in Fig. 2c. **b)** B16-OVA model was established in each mouse strain (WT: n = 7; KO: n = 6; DKO: n = 5), as in Fig. 2c. Tumor sizes are shown. **c)** Comparison ad quantitation of Eomes MFI in TIL Treg cells from b. The vertical dotted line represents the threshold for the gating of Eomes+ cells. **d)** Frequency of TIL Treg and TFR cells (PD-1+Bcl6+Foxp3+CD4+CD3+) and Foxp3 MFI of Treg cells as well as TIL Treg cells expressing IFNγ and GzmB or CD8+ T-cells expressing GzmB. **e)** Frequency of TIL TFH (PD-1+Bcl6+Foxp3−CD4+CD3+) and GC B-cells (GL-7+Fas+CD19+). **f)** Serum total IgG, IgE and anti-OVA IgG, IgE titers. The value 0 is used to indicate the undetectable anti-OVA IgE titers. In c-f, WT: n = 5-6; KO: n = 4-5; DKO: n = 5-8. Iso: Isotype control. WT: Foxp3YFP-Cre, KO: Prdm1fl/flFoxp3YFP-Cre, DKO: Eomesfl/flPrdm1fl/flFoxp3YFP-Cre. ∆MFI: MFI subtracted from the MFI of isotype controls. Data are pooled from two (a) or represent one of two independent experiments (b-f). ns, no significance, *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.001 (a,c,d-f, unpaired two-tailed Student’s t-test; b, two-way ANOVA with Tukey’s comparisons test, black: compared to WT, red: compared to KO). Bars, mean ± SEM.

Prdm1fl/flFoxp3YFP-Cre DKO mice with the dual deletion of Eomes and Blimp1 in Treg cells, and then established the B16-OVA/NP-OVA model in these mice and Prdm1fl/flFoxp3YFP-Cre as well as Foxp3YFP-Cre (WT) mice (Fig. 7b,c). The upregulation of Eomes in TIL Blimp1-deficient Treg cells was almost abolished in TIL Treg cells of DKO mice, and consistently, Prdm1fl/flFoxp3YFP-Cre mice had delayed and smaller tumor growth than WT mice (Fig. 7b,c). Notably, deletion of Eomes in Blimp1-deficient Treg cells expedited and enhanced tumor growth, even in a greater extent than WT mice. Analysis of TIL Treg cells revealed that DKO mice had Treg cells at a similar frequency as WT mice and expressed increased levels of Foxp3 compared to Treg cells from Prdm1fl/flFoxp3YFP-Cre mice, albeit at a lower level than WT Treg cells (Fig. 7d). Correspondingly, the increased expression of GzmB in TIL CD8+ T-cells and the increased expression of IFNγ and GzmB in TIL Treg cells of Prdm1fl/flFoxp3YFP-Cre mice were greatly reduced in TIL of DKO mice, and GzmB expression in DKO TIL Treg cells was even lower than WT TIL Treg cells (Fig. 7d). Although the TFH frequency did not change substantially, there were significantly reduced TFH and GC B-cells in DKO TIL compared to TIL of Prdm1fl/flFoxp3YFP-Cre mice (Fig. 7e). Accordingly, the serum titers of total IgG and anti-OVA IgG in DKO mice were decreased to levels as WT mice, despite that an increase of total IgG and unaltered anti-OVA IgG levels comparing DKO to Prdm1fl/flFoxp3YFP-Cre mice were also observed (Fig. 7f). Taken together, these results suggested that deletion of Eomes in Blimp1-deficient Treg cells prevented their reprogramming and restored their suppressive phenotype, which may at least partly contribute to the tampered anti-tumor response and greater tumor growth.
Deletion of Blimp1 in Treg cells remodels the TME and sensitizes the tumors to anti-PD-1 treatment

Finally, we determined what extent disruptions of Treg/TFR suppressive activity by a specific deletion of Blimp1 could impact on the tumor by analyzing gene expression of sorted CD45− cells using the NanoString PanCancer Immune Profiling Panel (Fig. 8a and Additional file 3e). Although only 38 genes were significantly differentially expressed (Additional file 9), pathway analysis revealed genes related to type 1 interferon (IFN-I) signature, including Mx2, Cxcl11, Oas2 and Ifi7, were enriched (FDR < 0.05) and downregulated, while the gene Vegfa encoding the angiogenic factor vascular endothelial growth factor A (VEGFA) was upregulated in CD45− cells from Prdm1fl/flFoxp3YFP-Cre mice compared to WT mice (Fig. 8a,b and Additional file 10). Analysis using the PanCancer Pathway Panel also revealed the gene Pgf encoding another angiogenic factor placental growth factor (PIGF), that was significantly upregulated in CD45− cells from Prdm1fl/flFoxp3YFP-Cre mice (Fig. 8a).
and Additional file 11). Accordingly, the tumor sections from Prdm1<sup>fl/fl</sup>Foxp3<sup>YFP-Cre</sup> mice had increased numbers of CD31<sup>+</sup> vessel-like structures, but with much smaller areas (Fig. 8c), suggesting a potential tumoral vasculature normalization. Further analysis showed that CD45<sup>−</sup> cells from Prdm1<sup>fl/fl</sup>Foxp3<sup>YFP-Cre</sup> mice also upregulated genes encoding MHCI and MHCII molecules as well as those related to MHCII-mediated antigen-presentation pathway, including CD74 and H2-DMβ1 (Additional file 12a). In addition to MHCII and CD74, CD45<sup>−</sup> cells from Prdm1<sup>fl/fl</sup>Foxp3<sup>YFP-Cre</sup> mice also upregulated PD-L1, albeit no significance achieved, and had fewer Ki-67<sup>+</sup> proliferating cells (Fig. 8d and Additional file 12b). All of these findings suggested that deletion of Blimp1 in Treg cells not only boosted TIL anti-tumor immune cells, but also improved tumor immunogenicity.

While IFN-I typically promotes anti-tumor immunity, persistent tumoral IFN-I signaling renders tumor resistance to checkpoint blockade therapy [40]. The increased IFNγ production in the TME of Prdm1<sup>fl/fl</sup>Foxp3<sup>YFP-Cre</sup> mice could potentially drive prolonged IFN-I and induce adaptive resistance [40]. However, the improved tumor immunogenicity and reduced IFN-I response led us to reason that tumors from Prdm1<sup>fl/fl</sup>Foxp3<sup>YFP-Cre</sup> mice may display an increased responsiveness to anti-PD-1 treatment. Indeed, anti-PD-1 greatly reduced tumor growth of Prdm1<sup>fl/fl</sup>Foxp3<sup>YFP-Cre</sup> mice, while it did not significantly improve tumor control in WT mice (Fig. 8e). We also noted that PD-1 expression did not change significantly in splenic and TIL Treg cells and TFH cells, as well as splenic CD8<sup>+</sup> T-cells, but was increased in TIL CD8<sup>+</sup> T-cells in Prdm1<sup>fl/fl</sup>Foxp3<sup>YFP-Cre</sup> mice compared to WT mice (Additional file 12c). Further analysis of these CD8<sup>+</sup> T-cells revealed that there were reduced terminally-differentiated PD-1<sup>+</sup>CD44<sup>+</sup>Tim3<sup>+</sup>TCF1<sup>−</sup>CD8<sup>+</sup> T-cells but significantly increased stem-like PD-1<sup>−</sup>CD44<sup>−</sup>Tim3<sup>−</sup>TCF1<sup>−</sup>CD8<sup>+</sup> T-cells [41] in the tumor of Prdm1<sup>fl/fl</sup>Foxp3<sup>YFP-Cre</sup> mice compared to WT mice (Additional file 12d). Moreover, TIL PD1<sup>−</sup>CD8<sup>+</sup> T-cells from Prdm1<sup>fl/fl</sup>Foxp3<sup>YFP-Cre</sup> mice expressed increased levels of Ki-67 compared to WT counterparts (Additional file 12e). Given the recent finding that PD-1 expression balance between Teff and Treg cells can predict the clinical efficacy of PD-1 blockade therapy [42], the shifted balance of PD-1 towards TIL CD8<sup>+</sup> T-cells in Prdm1<sup>fl/fl</sup>Foxp3<sup>YFP-Cre</sup> mice may explain the favorable response of PD-1 blockade in these mice.

Taken together, our results suggest that deletion of Blimp1 in Treg cells specifically converted TIL Treg and T<sub>Fr</sub> cells into Teff, which cooperated with both cellular and humoral anti-tumor components to reprogram the immunosuppressive TME into an immunostimulatory milieu and to enhance tumor immunogenicity, resulting in better tumor control and augmented response to anti-PD-1 blockade (Fig. 9a). This finding was further evidenced by using a quantitative measure of a putative immunostimulatory TME signature based on transcript levels of 3 factors extracted from the TCGA dataset, CD74, FCRER1A and PDCD1 (CFP), or levels of 20 factors comprising all HLA genes in addition to CFP (CFPHLA). These factors highly represented enhanced anti-tumor cellular and humoral immunity, particularly IgE response, in our Prdm1<sup>fl/fl</sup>Foxp3<sup>YFP-Cre</sup> mice. The higher expression of both CFP and CFPHLA signatures was correlated with the better survival of SKCM patients (Fig. 9b and Additional file 12 f).

Discussion

Understanding the symbiotic relationship between the tumor and TIL Treg cells is crucial for the manipulation of Treg activity for cancer therapy. The present study has revealed that Treg cells in the tumor were imprinted by the TME and regulated by Blimp1 which imposed TIL Treg cells with a unique signature responsible for their stable suppression and cytotoxicity. Deletion of Blimp1 in Treg cells reprogrammed these cells into Teff, which was specific to the TIL Treg cells but not Treg cells in the periphery, leading to increased anti-tumor cellular and humoral immunity, and decreased tumor growth. Moreover, this study has also demonstrated that remodeling the TME by disrupting Treg activity improved response to anti-PD-1 treatment.

The functional stability of Treg cells has been extensively investigated and relies on many factors under various conditions, including growing tumors [1, 3, 21, 43–46]. For example, disruption of the CARMA1–BCL10–MALT1 signalosome complex or targeting Nrp1 or Helios or ligation of GITR in Treg cells has been shown to destabilize TIL Treg cells and effectively control tumor without peripheral autoimmune effects reported [19, 21, 47–49]. Based on the finding that Blimp1 marks a subset of TIL Treg cells with the highly suppressive activity and the specific effect of Blimp1 depletion on the stable suppression of TIL Treg cells, our study has revealed Blimp1 as another central regulator of TIL Treg cells. The converted TIL Blimp1-deficient Treg cells constitute a new source of anti-tumor effector activity and targeting Blimp1<sup>+</sup> Treg cells can generate robust anti-tumor effects while limiting systemic toxicity.

The mechanisms for the Blimp1-dependent regulation of stable TIL Treg cells are likely multifactorial. Under inflammation, Blimp1 can stabilize the conserved non-coding sequence 2 (CNS2) of Foxp3 by either preventing its methylation or ensuring the
activation of CD25-STAT5 pathway [10, 11, 13], which also operated in TIL Treg cells, as reflected by the reduced expression of Il2ra and pStat5 in TIL Blimp1-deficient Treg cells compared to WT Treg cells. However, other genetic or epigenetic regulation of Foxp3 expression cannot be excluded. Despite that Bcl6 antagonizes Blimp1 in many cell types [10, 27], Bcl6 was not altered in TIL Blimp1-deficient Treg cells, suggesting a Bcl6-independent role of Blimp1 in the regulation of tumor immunity. We also noted that deletion of Blimp1

Fig. 9 Disrupting Blimp1+ Treg activity reshapes the TME for improved tumor control and response to checkpoint blockade. a) Left, Blimp1-sufficient Treg. Treg and Tfh cells mainly suppress the cellular and humoral anti-tumor immune responses, respectively. Conversely, tumor cells impose suppression on both cellular and humoral immune responses, but foster the immune suppression by Treg and Tfh cells (not depicted). Right, Blimp1-deficient Treg. Deletion of Blimp1 in Treg cells specifically destabilizes and reprograms TIL Treg and Tfh cells into Teff, which upregulate Eomes, display impaired suppressive activity and cooperate with both cellular and humoral anti-tumor components to control tumor growth. Disrupting Blimp1+ Treg activity also increases tumor immunogenicity by upregulating MHC-related molecules, reduces IFN-I signature and augments response to checkpoint blockade therapy. MΦ: macrophage. The unclear events are indicated by dashed lines. Not depicted: peripheral Tfh and B-cells and their migration into the tumor; expansion of Treg/Tfh cells and anti-tumor effector cells; other cells regulating anti-tumor responses (e.g., myeloid-derived suppressor cells, etc.). b) Kaplan-Meier analysis of OS of patient cohorts expressing differential CFP signature (top 33% vs bottom 33%) based on combined log-averaging of CD74, FCER1A and PDCD1 transcript levels from the TCGA-SKCM dataset. P value is generated using two-tailed LogRank test. Median, median survival time.
reduced the TIL Treg expression of IL10 (encoding IL-10) and Ebi3 (encoding a subunit of IL-35). Both IL-10 and IL-35 are critical cytokines for Treg suppressive activity and important for inducing TIL CD8+ T-cell exhaustion [50]. Their decreased expression may partly account for the activated status of TIL CD8+ T-cells in Prdm1fl/fl/Foxp3YFP-Cre mice. Additionally, the gene Tcf7 that encodes the TF TCF1 was upregulated in Blimp1-deficient Treg cells, consistent with the antagonistic regulation between TCF1 and Blimp1 [51]. Ablation of TCF1 in Treg cells blocks the development of TFR cells [52], however, the frequency of TIL TFR cells was not altered in mice with Blimp1-deficient Treg cells. Moreover, TCF1 partners with Foxp3 to repress the proinflammatory program in Treg cells, but not the core Treg cell transcriptional signature [53, 54]. Future investigation is required to understand the mechanisms for the overall reprogramming of Blimp1-deficient Treg cells in our system and other tumor models.

Blimp1 instructs a universal transcriptional program of tissue residency in lymphocytes [55], and Treg cells display progressive and transcriptional dynamics of adaption to the non-lymphoid tissues, including tumor [56]. Interestingly, our RNA-seq analysis clearly showed that TIL Blimp1+ Treg cells developed adaption to the TME. However, the modulation of a cytokotoxic program in TIL Treg cells by Blimp1 is unexpected, although it is known that Treg cells can mediate suppression via killing. This genetic reprogramming appears to be dependent of the expression of Eomes. Although Treg cells in the periphery were not significantly altered, ablation of Eomes in TIL Blimp1-deficient Treg cells not only reduced the cytokotoxic signature (e.g., IFNγ and GzmB), but also restored the stable phenotype to some degrees. Reduced IFNγ in DKO TIL Treg cells may also facilitate their stabilization, as increased IFNγ induces the Treg cell “fragility” [21]. The overall outcome may result in enhanced suppression by DKO TIL Treg cells, as reflected by reduced GzmB+CD8+ Teff, partially explaining the increased tumor growth in DKO mice compared to WT mice. It is possible that the Eomes-mediated cytokotoxic program of TIL Treg cells is decoupled from the Foxp3-dependent gene signature for their stability, but both are controlled by Blimp1. Future studies are required to understand if the Eomes-dependent regulation is required for TIL Treg cells to kill tumor cells or suppress anti-tumor effector cells, and how Blimp1 regulates the TIL Treg heterogeneity.

Our study has also supported a potential role of TFR cells in tumor immunity. Despite a few reports showing that TFR cells are significantly increased in cancer patients compared to healthy controls [57, 58], and a recent study showing that TIL TFR cells curtail anti-PD-1 therapeutic efficacy [59], their mechanisms of action in the tumor remain unclear. Consistent with the increased proportions of metastatic melanoma patients expressing high levels of FOXP3, PRDM1 and CXCR5, we have detected TFR cells in melanoma across the different metastatic tissues. Although the tissue environment may affect the Treg suppressive phenotype, the TFR cell frequency was consistently and inversely correlated with the abundance of activated B-cells. Importantly, the TFR adoptive transfer assay has established that dysregulated TFR cells due to the deletion of Blimp1 boosted anti-tumor Ab responses, although co-transfer of other Teff, e.g., CD8+ T-cells, may enhance the overall tumor control. We have also shown for the first time that disruption of TFR suppressive activity modulated anti-tumor immune responses, albeit no changes in TFR cell numbers. It is interesting to observe that Eomes ablation in Blimp1-deficient Treg cells did not alter the TFR frequency, but regulated the Tfh-GC Ab response. Although there was no significant difference in the cellular and humoral response in mice with a Treg-specific deletion of Eomes compared to WT mice, future studies are required to understand if the changes in the Tfh-GC Ab response in DKO mice are attributed to a direct or indirect effect of the Eomes deletion in Blimp1-deficient Treg cells. To facilitate analysis of TFR cells and Ab response, we used the B16-OVA/NP-OVA model that generated strong humoral responses. However, the cellular and humoral changes in mice with the Treg-specific deletion of Blimp1 compared to WT mice were consistent across all of the models we have used. The analysis of various models has informed us that in addition to inducing Treg destabilization, targeting Blimp1+ Treg cells also induces potent humoral responses, thus achieving multifaceted anti-tumor effects.

Recent studies have shown that individuals with higher levels of B-cell class switches in the tumor, not only the total B-cell infiltration levels, have significantly better clinical outcomes in melanoma and other tumors [60, 61]. However, it is of interest to observe that IgE and anti-tumor specific IgE but not IgG were mainly increased in mice with a deletion of Blimp1 in Treg cells. The increased IL-4 and IL-21 produced by both Tfh and TFR cells in Prdm1fl/fl/Foxp3YFP-Cre mice may account for the elevated IgE as we reported in other settings [10, 13], but other factors are likely involved, as revealed by recent reports [62, 63]. Consistent with these studies, our findings point to Blimp1+ TFR cells as key suppressors of IgE production in the context of tumor, although at other settings, TFR cells have been shown to induce IgE [64, 65]. Considering the IgE's emerging anti-tumor activity [66–68], the first ongoing clinical trial using an IgE anti-tumor Ab in cancer patients (NCT02546921),
the inverse correlation of serum IgE scores and risk of melanoma [32], the use of ultra-low IgE as a biomarker for cancer risk [69], and the associated usage of omalizumab, a monoclonal Ab that blocks IgE, with more cancer incidences [70], further definition of biological consequences and mechanisms of action for IgE in tumor immunity is of key importance. Although IgE may exhibit anti-tumor responses via activation of various effector cells, the preferential colocalization of IgE and its receptor FcεRIα with CD68+ macrophages in the tumor and increased macrophage function after treatment with sera from Prdm1fl/fl/Foxp3YFP-Cre tumor-bearing mice suggest that macrophages are likely major effector cells participating in the IgE-mediated anti-tumor response, in line with other reports [34, 35]. Notably, the finding that SKCM patients with the higher FcεRI or CFP signatures have better survival suggests that FcεRI or CFP could be used as diagnostic and prognostic markers for these patients. It is also important that a portion of IgE is specific to tumor antigen. However, we cannot exclude other portions of IgE that may exhibit autoreactive anti-tumor activity, as reported for its role in carcinogen-induced skin cancer [66]. Future delineation of the specificity and effector activity of IgE may facilitate us to understand its anti-tumor potential and any systemic reactivity.

The alterations of genes related to angiogenesis and IFN-I response in the tumor of Prdm1fl/fl/Foxp3YFP-Cre mice appear contradictory to their conventional roles in tumor control. However, the increased tumor immunogenicity along with the reduced IFN-I signature in the tumor of Prdm1fl/fl/Foxp3YFP-Cre mice, which can potentially sensitize tumors to checkpoint blockade and to potentially destabilize Treg cells [40, 71], justifies a better therapeutic outcome by combining Treg-specific deletion of Blimp1 with anti-PD-1 treatment, as proved in this study. The enhanced responsiveness to anti-PD-1 treatment may also result from the increased TIL Tfh/GC B-cell responses, as reported by recent clinical studies [15, 16], despite no success in detecting the TLS formation in our mouse models. It should be noted that only TIL CD8+ T-cells but no other cells, including Treg cells, expressed increased PD-1 in Prdm1fl/fl/Foxp3YFP-Cre mice with all of the tumor models that we have evaluated. These TIL CD8+ T-cells displayed more of a stem-like phenotype with increased proliferation potential [41], which may contribute to the overall strong anti-tumor immunity and improved responses to anti-PD-1 blockade. Moreover, the unaltered PD-1 expression in TIL Blimp1-deficient Treg cells may suggest that PD-1 does not negatively impact these cells, as higher PD-1 levels in Treg cells impair their suppression [72, 73]. Blimp1 could act as a repressor or activator of PD-1 expression in CD8+ T-cells depending on the stages of immune responses [74, 75], but no definitive reports show that Blimp1 also regulates PD-1 in Treg cells, particularly TIL Treg cells, which requires further investigation.

Conclusions

Our study has revealed that the Blimp1-dependent regulation of Treg suppression in tumor immunity extends beyond its conventional role in other settings. Although depletion or inhibition of systemic Treg cells can enhance anti-tumor responses, autoimmune sequelae have diminished the enthusiasm for such approaches. By virtue of the unique transcriptional signature of TIL Blimp1-deficient Treg cells, specific reprogramming of TIL Blimp1+ Treg cells and reshaping the TME are highly desirable and important for treating cancer patients, including those treated with immunotherapy, as it will direct the development of effective, targeted immunotherapies with reduced adverse events. This represents a new direction for how to manipulate Treg activity for cancer treatment and how to design combination checkpoint blockade therapies. The immune signatures that are revealed in this study as an outcome of targeted disrupting Blimp1+ Treg activity positively correlate with better survival of SKCM patients, suggesting the applicability of this approach for cancer therapy.

Abbreviations

Ab: Antibody; ACK: Ammonium-Chlorine-Potassium; BMDMs: Bone marrow-derived macrophages; CFA: Complete Freund’s adjuvant; CNS2: Conserved non-coding sequence 2; CTV: Cell trace violet; DC: Dendritic cell; DEGs: Differentially expressed genes; DKO: Double knockout; DMM: Dulbecco’s Modified Eagle Medium; ELISA: Enzyme-Linked Immunosorbent Assay; Eomes: Eomesodernin; eTreg: Effector regulatory T-cell; FACS: Flow activated cell sorting; FBS: Fetal bovine serum; FFPE: Formalin-fixed paraffin embedded; GC: Germinal center; GM-CSF: Granulocyte-macrophage colony stimulating factor; GzmB: Granzyme B; GVAX: B16 expressing GM-CSF; IFN-I: Type I interferon; IF: Immunofluorescence; IFA: Incomplete Freund’s adjuvant; IL: Interleukin; i.p.: Intraperitoneally; MFI: Mean fluorescence intensity; MHC: Major histocompatibility complex class I or class II; NP-OVA: 4-Hydroxy-3-nitrophenylacetyl-Ovalbumin; OCT: Optimum cutting temperature; OVA: Ovalbumin; OS: Overall survival; pAcc: Probability of accumulation; PBS: Phosphate-buffered saline; PIGF: Placental growth factor; pORA: Probability of over-representation; RSEM: RNA-Seq by Expectation-Maximization; RT: Room temperature; s.c.: Subcutaneously; SEM: Standard error of the mean; SKCM: Skin Cutaneous Melanoma; TCGA: The Cancer Genome Atlas; TCR: T cell receptor; Teff: Effector T-cells; Tfh: Follicular helper T cells; TF: Transcription factor; TFH: Follicular helper T cells; TFR: Follicular regulatory T cells; TLS: Tertiary lymphoid structure; TME: Tumor microenvironment; TPM: Transcripts Per Million; Treg: Regulatory T-cell; VEGFA: Vascular endothelial growth factor A.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12943-021-01450-3.

Additional file 1: Table 1. Reagents and Resources.
Additional file 2: Table 2. Characteristics of de-identified metastatic melanoma tissues. Only patients 1-3 have matched control tissues.

Additional file 3: Gating strategy used for flow cytometry analysis and sorting. a-b) Gating strategy used for analysis of immune cells from spleens (a) or tumors (b) isolated from tumor-bearing mice presented on Fig. 1a-c, Fig. 2e-g, Fig. 3a-d, Fig. 4a-b, Fig. 7a, c-e, Fig. 8d. Additional file 4 a-b, 6, 12b-e: c) Gating strategy used for sorting of Foxp3+/YFP+ CD44+ Treg from spleen (1→2, followed by steps 1-4 in a) and tumor (3→4, followed by steps 1-5 in b) for RNA-seq analysis presented in Fig. 6 and Additional file 4 c-d. d) Gating Strategy used for analysis of immune cells from metastatic tissues of patients with melanoma presented on Fig. 1d-e. e) Gating strategy used for sorting of CD45+ cells for NanoString analysis presented in Fig. 8a-b and Additional file 9-11, 12. The number at the right lower corner in each plot indicates the order of sub-gating for each condition.

Additional file 4: Blimp1+ Treg cells are accumulated in the tumor. a-b) Blimp1-YFP reporter mice (n = 5) were inoculated with B16-OVA and immunized as in Fig. 1a. Flow plots of CD62L+CD44+Foxp3+ Treg, Blimp1+YFP+ eTreg and IL-10+ Blimp1+ Treg subset (a) as quantitated in Fig. 1a, and MFI of each marker of Blimp1+ Foxp3+ Treg cells (b) as presented in Fig. 1b-c.d) Foxp3TCF3+ mice were established with B16-OVA/NP-OVA model as in Fig. 1a. eTreg cells (CD45+CD44+YFP+CD4+CD3+CD4+) from spleens or tumors were sorted for RNA-seq (duplicates). Principle component analysis of splenic and TIL eTreg cells (c), top KEGG pathways that are differentially expressed in splenic versus TIL eTreg cells (analyzed by gGost) (d). P < 0.05, **P < 0.01 and ***P < 0.001 (b, c), unpaired two-tailed Student’s t-test. Bars, mean ± SEM. e-g) The correlation of PRDM1 and FOXP3 expression in all SKCM patients (n = 458) (e) or the correlation of PRDM1 and FUT4 expression (f) or PRDM1 and FUT7 expression (g) in top 50% FOXP3+ SKCM patients (n = 229) (extracted from the TCGA dataset) was analyzed by Pearson correlation (two-tailed, no adjustment for multiple comparisons because of one correlation test for a gene pair). The values of the coefficients (r) and significance (p) are indicated.

Additional file 5: Table 3. Quantitation of each marker or subset in metastatic melanoma tissues compared to control tissues. *P value: unpaired two-tailed Student’s t-test. Numbers indicate mean ± SEM. ns, no significance.

Additional file 6: TIL effector cells and expression of effector molecules in TIL or splenic effector cells and Treg cells. B16-OVA/NP-OVA model was established in Foxp3TCF3 (WT) and Prdm1+/Foxp3TCF3 (KO) mice, as in Fig. 2c. a) Frequency of TIL immune cells (n = 7 per group, except n = 5 (WT) and n = 4 (KO) for F4/80+ cells). b-c) Frequency of each effector subset in spleens (WT: n = 8; KO: n = 6) (b) or tumors (n = 7 per group) (c) expressing IFNγ, TNFα and GM-CSF. d) Analysis and frequency of splenic Treg cells expressing IFNα, TNFα and GM-CSF (WT: n = 8; KO: n = 6), no significance. ***P < 0.001 and ****P < 0.0001 (a-d, unpaired two-tailed Student’s t-test). Bars, mean ± SEM.

Additional file 7: Immunofluorescence (IF) staining of CD3, B220 and IgE in the spleens of Prdm1+/Foxp3TCF3+ mice, as positive controls for Fig. 4F. Spleens were taken from mice bearing B16-OVA (as in Fig. 2c). Representative IF staining of T (CD3) and B (B220) (a), or B220 and IgE (b, left) or CD3, IgE and IgD (b, right) (100×). IgE+ cells are localized outside germinal centers. Arrowheads, T/B clusters, *, IgE.

Additional file 8: Comparison and quantitation of pSTAT5 (a) and pSmad2/3 (b) levels in TIL Treg cells from Foxp3+YFP+ (WT) and Prdm1+/Foxp3TCF3+ (KO) mice (n = 3 per group) established with B16-OVA, as in Fig. 2c. A) MFI: MFI subtracted from the MFI of isotype controls (IgG), ns, no significance and * P < 0.05 (unpaired two-tailed Student’s t-test). Bars, mean ± SEM.

Additional file 9: Table 4. NanoString mouse PanCancer Immune Profiling of sorted CD45+ cells (KO (n = 2) vs WT (n = 3)).

Additional file 10: Table 5. Reactome pathway analysis of DEGs revealed in Table 4 (KO (n = 2) vs WT (n = 3)) via NetworkAnalyst.

Additional file 11: Table 6. NanoString mouse PanCancer Pathway analysis of sorted CD45+ cells (KO vs WT, n = 2 per group).

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Authors’ contributions

J.W.L., M.L.D. and L.L. designed and performed experiments, analyzed data and interpreted the results. S.G. performed the TCGA dataset analysis. S.G., J.M.G. and J.D.L. assisted with experiments and participated in discussion and manuscript writing. J.W.L., M.L.D. and J.D.L. wrote the paper. J.W.L. conceived and supervised the study. The author(s) read and approved the final manuscript.

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Availability of data and materials

The RNA sequencing and NanoString data have been deposited in the NCBI GEO under accession number GSE178135. All data generated or analyzed during this study are included in this article [and its supplementary information (Additional files)].

Declarations

Ethics approval and consent to participate

All animal experiments were performed in compliance with federal laws and institutional guidelines as approved by the UAB IACUC. Approval from the UAB Institutional Review Board for Not Human Subjects Research (IRB-300003295) was used for study of melanoma patient samples.

Consent for publication

Not applicable.

Competing interests

The authors have declared that no conflict of interests exists.

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Additional file 12: MHC and PD-L1 expression in CD45+ cells and PD-L1 expression in each immune subset. B16-OVA/NP-OVA model was established in Foxp3YFP+ (WT) and Prdm1+/Foxp3TCF3+ (KO) mice, as in Fig. 2c. a) DEGs related to MHCI and MHCII in WT (n = 3) and KO mice (n = 2), as revealed by NanoString analysis in Fig. 8a. b) PD-L1 MFI in CD45+ cells (WT: n = 3; KO: n = 4). c) PD-L1 MFI in each subset (WT: n = 9; KO: n = 8). d) Flow plots of Tim3 and TCF-1 expression in PD-L1+CD44+CD8+ T-cells in the tumors from B16-OVA mice (WT: n = 3; KO: n = 4), as in Fig. 2c. Right, frequency of indicated CD8+ T-cell subsets. e) Comparison and quantitation of Ki-67 in TIL PD-L1+ CD8+ T-cells from B16-OVA mice (WT: n = 3; KO: n = 4), as in Fig. 2c. Data represent one of two (b,d,e) or are pooled from two (c) independent experiments. ns, no significance and * P < 0.05 (unpaired two-tailed Student’s t-test). Bars, mean ± SEM. f) Kaplan-Meier analysis of OS of patient cohorts expressing differential CFFHLA signature (top 33% vs bottom 33%) based on combined log-averaging of transcript levels of 20 genes (right) from the TCGA-SKCM dataset. P value is generated using two-tailed LogRank test. Median, median survival time.
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References

1. Dixon ML, Leavenworth JD, Leavenworth JW. Lineage Reprogramming of Effector Regulatory T Cells in Cancer. Front Immunol. 2021;12:717421. Epub 2021/08/17. doi: https://doi.org/10.3389/fimmu.2021.717421. PubMed PMID: 34394124; PMCID: PMC8355732.

2. Scott EN, Gocher AM, Workman CJ, Vignali DAA. Regulatory T Cells: Barriers of Immune Infiltration Into the Tumor Microenvironment. Front Immunol. 2021;12:702726. Epub 2021/06/29. doi: https://doi.org/10.3389/fimmu.2021.702726. PubMed PMID: 34177968; PMCID: PMC8222776.

3. Li C, Jiang P, Wei S, Xu X, Wang J. Regulatory T cells in tumor microenvironment: new mechanisms, potential therapeutic strategies and future prospects. Mol Cancer. 2020;19(1):116. Epub 2020/07/19. doi: 10.1016/j.molonc.2020.07.014. PubMed PMID: 32821275; PMCID: PMC7637382.

4. Zhao J, Savage PA. Unlocking the Complexities of Tumor-Associated Regulatory T Cells. J Immunol. 2018;200(2):415-21. doi: https://doi.org/10.4049/jimmunol.1701188. PubMed PMID: 29311383; PMCID: PMC5763514.

5. Tanaka A, Sakaguchi S. Regulatory T cells in cancer immunotherapy. Cell Res. 2017;27(1):109-18. doi: https://doi.org/10.1038/cr.2016.151. PubMed PMID: 28077791; PMCID: PMC5223231.

6. Smigiel K, Richards E, Srivastava S, Thomas KR, Dudda JC, Klanoyski KD, Campbell DJ. CCR7 provides localized access to IL-2 and defines homeostatically distinct regulatory T cell subsets. J Exp Med. 2014;211(1):121-36. doi: https://doi.org/10.1084/jem.20131142. PubMed PMID: 24378538; PMCID: PMC3892972.

7. Ctenery E, Kallies A, Nutt SL. Differentiation and function of Foxp3(+) effector regulatory T cells. Trends Immunol. 2013;34(2):74-80. 23219401.

8. Liston A, Gray DH. Homeostatic control of regulatory T cell diversity. Nat Rev Immunol. 2014;14(3):154-165. Epub 2014/02/01. doi: https://doi.org/10.1038/nri3605. PubMed PMID: 24841837.

9. Ward-Hartstone MC, McCullough TR, Kamps AK, Girardin A, Luebeck VS, Ng J, Zhao H, Marini AM, Flavell RA. Antigen-specific Treg activity results in expansion of T follicular regulatory T cells by the Bmp1 transcription factor. Cell Rep. 2019;29(7):1848-61 e6. Epub 2019/11/14. doi: https://doi.org/10.1016/j.celrep.2019.10.012. PubMed PMID: 31722202; PMCID: PMC6897316.

10. Ward-Hartstone MC, McCullough TR, Kamps AK, Girardin A, Luebeck VS, Ng J, Zhao H, Marini AM, Flavell RA. Antigen-specific Treg activity results in expansion of T follicular regulatory T cells by the Bmp1 transcription factor. Cell Rep. 2019;29(7):1848-61 e6. Epub 2019/11/14. doi: https://doi.org/10.1016/j.celrep.2019.10.012. PubMed PMID: 31722202; PMCID: PMC6897316.

11. Garg G, Muschaweck A, Moreno H, Vasanthakumar A, Floess S, Obenauf AC, Angell G, Fredriksen T, Lafontaine L, Berger A, Bruneval P, Fridman WH, Becker C, Pages F, Speicher M, Trajanoski Z, Galon J. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. Immunity. 2013;39(4):577-95. doi: 10.1016/j.immuni.2013.10.003. PubMed PMID: 24138663; PMCID: PMC3895716.

12. Ogawa C, Banko T, Nguyen T, Hanashadze-Klubi N, Nadeau S, Porritt RA, Couse M, Fan X, Dhali D, Eberl G, Ohmacht M, Martins GA. Blimp-1 Functions as a Molecular Switch to Prevent Inflammatory Activity in Foxp3(+)-Regulatory T Cells. Cell Rep. 2015;21(19):247-58 e18. doi: https://doi.org/10.1016/j.celrep.2018.09.016. PubMed PMID: 30282028; PMCID: PMC6237548.

13. Luo L, Hu X, Dixon ML, Pope BJ, Leavenworth JD, Ramen C, Meador WR, Leavenworth JW. Dysregulated follicular regulatory T cells and antibody responses exacerbate experimental autoimmune encephalomyelitis. J Neuroinflammation. 2018;15(1):27. Epub 2018/01/21. doi: https://doi.org/10.1186/s12974-021-02070-6. PubMed PMID: 33468194; PMCID: PMC7814531.

14. Bindean G, Mlecnik B, Tosolini M, Kirovskiy L, Waldner M, Obenauf AC, Angell G, Fredriksen T, Lafontaine L, Berger A, Bruneval P, Fridman WH, Becker C, Pages F, Speicher M, Trajekoski Z, Galon J. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. Immunity. 2013;39(4):577-95. doi: 10.1016/j.immuni.2013.10.003. PubMed PMID: 24138685.

15. Cabrita R, Lauss M, Sanna A, Donia M, Skarups Larsen M, Mitra S, Johanson I, Phung B, Harbst K, Volland-Chersterson J, van Schoiack A, Lovgren K, Warren S, Jirstrom K, Olsson P, Kietats C, Isaaksen K, Schadendorf D, Schmidt H, Bastholt L, Carneiro A, Wargo JA, Svane IM, Jonsson G. Tertiary lymphoid structures improve immunotherapy and survival in melanoma. Nature. 2020;577(7791):561-565. doi: 10.1038/s41586-020-1919-8. PubMed PMID: 31942071.

16. Helmsik BA, Reddy SM, Gao J, Zhang S, Basar R, Thakur R, Yizhak K, Sade- Feldman M, Blando J, Han G, Gopalakrishnan V, Yi X, Zhao H, Marini AM, Taohi HA, Gogidi AP, Liu W, LeBlou VS, Kugsrati RS, Patel D, Davies MW, Hsu P, Lee JG, Gershentwald J, Laccia A, Arora R, Woodman S, Keung EZ, Gaudreau PA, Reuben A, Spencer CN, Burnen EM, Haydu LE, Lazar AJ, Zapassodi R, Hughes CW, Ledesma DA, Ong S, Bailey M, Warren S, Rao D, Krigsman O, Rozemen EA, Peepker D, Blank CU, Schumacher TN, Butterfield LH, Zelazowska MA, McBride KM, Kalluri R, Allison J, Petropet F, Fridman WH, Sauers-Fridman C, Hacohen N, Rezvani K, Sharma P, Tetzlaff MT, Wang L, Wargo JA. B cells and tertiary lymphoid structures promote immunotherapy response. Nature. 2020;577(7791):540-55. doi: 10.1038/s41586-020-1919-8. PubMed PMID: 31942071.

17. Cello AP, Kurnen CH, Tabiti T, Qi Z, Onkar S, Wang T, Liu A, Duvvuri U, Kim S, Sooie RJ, Oestereich S, Chen W, Lafayris R, Bruno TC, Ferris RL, Vignali DAA. Immune landscape of viral- and carcinogen-driven head and neck cancer. Immunity. 2021;55(2):183-199 e9. doi: https://doi.org/10.1016/j.immuni.2021.01.014. PubMed PMID: 32709447; PMCID: PMC8355732.

18. Collison LW, Vignali DA. In vitro Treg suppression assays. Methods Mol Biol. 2011;724:19-37. doi: https://doi.org/10.1007/978-1-61737-979-4_2. PubMed PMID: 21287326; PMCID: PMC3043080.

19. Trupolin V, Boucher N, Gorrel L, Conti F, Motolla G, Giglio E. Bone marrow-derived macrophage production. J Vis Exp. 2013(81):50966.
33. Zhang X, Li J, Luo S, Wang M, Huang Q, Deng Z, de Febbo C, Daoui A, Lupar E, Brack M, Garnier L, Laffont S, Rauch KS, Schachtrup K, et al. Molecular Cancer (2021) 20:150.

31. Miyara M, Chader D, Sage E, Sugiyama D, Nishikawa H, Bouvry D, Claer L, Wing JB, Ise W, Kurosaki T, Sakaguchi S. Regulatory T cells control antigen-specific T cell responses. Nature. 2019;572(7769):392-6. doi: https://doi.org/10.1038/s41586-019-1456-0. PubMed PMID: 32367045; PMCID: PMC6669206.

29. Sage PT, Paterson AM, Lovitch SB, Sharpe AH. The coinhibitory receptor PD-1 on CD8+ T cells limits the magnitude of the effector response and the generation of memory. Proc Natl Acad Sci U S A. 2015;112(23):7225-30. Epub 2014/12/20. doi: https://doi.org/10.1073/pnas.1508224112. PubMed. PMC7047522.

28. Slagel JD, Varga J, Tozzi A, Beyer M, Chavakis T, Boumpas D, Tsirigos A, Verginis P, Jandus C. Tumor-specific cytolytic CD4+ T cells in humans. Proc Natl Acad Sci U S A. 2015;112(23):7225-30. Epub 2014/12/20. doi: https://doi.org/10.1073/pnas.1508224112. PubMed. PMC7047522.

27. Okabe M, Kodama T, Ishikawa M, Hara K, Nakamura K, Yokota T, Nakamura N, Kato H, Okada S, Fujita M, et al. Tumor Interferon Signaling Regulates a Multigenic Resistance Program to Immune Checkpoint Blockade. Cell. 2016;167(6):1540-54 e12. Epub 2016/05/28. doi: https://doi.org/10.1016/j.cell.2016.05.028. PubMed. PMC5385895.

26. Rooney MS, Shukla SA, Wu CJ, Getz G, Hacohen N. Molecular and genetic complexity causes T (reg) cells to prime tumours for immune checkpoint blockade immunity. Immunity. 2019;50(1):195-211 e10. Epub 2019/01/13. doi: https://doi.org/10.1016/j.immuni.2019.12.016. PubMed. PMC6612021.

25. Crescioli S, Canevari S, Figini M, Montes A, Downes N, Dombrowicz D, Corrigan CJ, Czirok A, Reisfeld RA, Jordan R, et al. Tumor-specific cytolytic CD4+ T cells limit the magnitude of the effector response and the generation of memory. Proc Natl Acad Sci U S A. 2015;112(23):7225-30. Epub 2014/12/20. doi: https://doi.org/10.1073/pnas.1508224112. PubMed. PMC7047522.
Miragaia RJ, Gomes EA, Ji Y, Huang B, Harly C, Sen JM, Berg LJ, Gattinoni L, McGavern DB, Schwartzberg PL. TCF1 Is Required for the T Follicular Helper Cell Response to Viral Infection. Cell Rep. 2015;12(12):2099-110. Epub 2015/09/15. doi: 10.1016/j.celrep.2015.09.061.

Yang BH, Wang K, Liang Y, Yuan X, Dong Y, Cho S, Xu W, Jepsen N, Clarke J, Ramirez-Suastegui C, Panwar B, Madrigal A, Eschweiler S, Krausgruber T, Shields M, Tucci A, Chen X, Lindeman I, Emerton G. Hobit and Bley N instruct a universal transcriptional program of tissue residency for the T Follicular Helper Cell Response to Viral Infection. Immunity. 2019;50(2):493-504 e7. Epub 2019/02/10. doi: 10.1016/j.immuni.2019.02.003.

Yang BH, Wang K, Liang Y, Yuan X, Dong Y, Cho S, Xu W, Jepsen N, Clarke J, Ramirez-Suastegui C, Panwar B, Madrigal A, Eschweiler S, Krausgruber T, Shields M, Tucci A, Chen X, Lindeman I, Emerton G. Hobit and Bley N instruct a universal transcriptional program of tissue residency for the T Follicular Helper Cell Response to Viral Infection. Immunity. 2019;50(2):493-504 e7. Epub 2019/02/10. doi: 10.1016/j.immuni.2019.02.003.

Blimp1 instruct a universal transcriptional program of tissue residency. Obici D, Fiebiger E, Gould HJ, Hartmann K, Jappe U, Jordakieva G, Josephs K, Kazemian M, Gounari F, Khazaie K, Gounari F. Wnt-beta-catenin activation epigenetically reprograms Treg cells in inflammatory bowel disease and dysplastic progression. Nat Immunol. 2021;22(4):471-484. Epub 2021/03/06. doi: 10.1038/s41590-021-00889-2. PubMed PMID: 33664518.

Osman A, Yan B, Li Y, Favelko KD, Quandt J, Saadalla A, Singh MP, Kazemian M, Gounari F, Khazaie K. TCF-1 controls Treg cell functions that regulate inflammation, CD8+ T cell cytotoxicity and severity of colon cancer. Nature Immunology. 2021;22(9):1152-62. Epub 2021/08/14. doi: 10.1038/s41590-021-00987-1. PubMed PMID: 34385712; PMCID: PMC8428683.

Mackay LK, Minnich M, Kragten NA, Liao Y, Noto B, Seillet C, Zaid A, Man K, Preston S, Freeston D, Braun A, Wynne-Jones E, Behr FM, Stark R, Pellicci DG, Godfrey DJ, Belz GT, Pellegrini GM, Gebhardt T, Busselinger M, Shi W, Carbone FR, van Lier RA, Kallies A, van Gisbergen KP. Hobit and Blimp1 instruct a universal transcriptional program of tissue residency in lymphocytes. Science. 2016;352(6284):459-463. doi: 10.1126/science.aad2035.

Miraigaia RJ, Gomes EA, Ji Y, Huang B, Harly C, Sen JM, Berg LJ, Gattinoni L, McGavern DB, Schwartzberg PL. TCF1 Is Required for the T Follicular Helper Cell Response to Viral Infection. Cell Rep. 2015;12(12):2099-110. Epub 2015/09/15. doi: 10.1016/j.celrep.2015.09.061.

Chiaurutti G, Mele S, Opzoomer J, Crescioli S, Ilieva KM, Lacy KE, Karagiannis SN. B cells and the humoral response in melanoma: The overlapped players of x-hu the tumor microenvironment. Oncoimmunology. 2017;6(4):e1294296. doi: 10.1080/2162402X.2017.1294296. PubMed PMID: 28507802; PMCID: PMC5414880.

Clement RL, Daccache J, Mohammed MT, Diaglo A, Blazir BR, Kuchroo VK, Lovitch SB, Sharpe AH, Sage PT. Follicular regulatory T cells control humoral and allergic immunity by restraining early B cell responses. Nat Immunol. 2019;20(10):1360-71. doi: 10.1038/s41590-019-0472-4. PubMed PMID: 3177921.

Gonzalez-Figueroa P, Rocca JA, Papa I, Nunez Villacis L, Stanley M, Linter MA, et al. Follicular regulatory T cells produce neuritin to regulate B cells. Cell. 2021;184(7):1775-89 e19 e3371126. https://doi.org/10.1016/j.cell.2021.02.027. Epub 2021/03/13.

Koh B, Ulrich BJ, Nelson AS, Panangipalli G, Khawraddar R, Wu W, Xie MM, Fu Y, Turner MJ, Pacessey S, Janga SC, Dent AL, Kaplan MH. Bcl6 and Blimp1 reciprocally regulate ST2(+) T-cell development in the context of allergic airway inflammation. J Allergy Clin Immunol. 2020;146(5):1121-36 e9. Epub 2020/03/18. doi: 10.1016/j.jaci.2020.03.003.

Xie MM, Chen Q, Liu H, Yang Y, Koh B, Wu W, Maleki SJ, Hurlbut BK, Cook-Mills J, Kaplan MH, Dent AL. T follicular regulatory cells and IL-10 promote food antigen-specific IgE response. J Clin Invest. 2020;130(7):3820-32. Epub 2020/04/08. doi: 10.1172/JCI132249. PubMed PMID: 32555767; PMCID: PMC7234176.

Crawford G, Hayes MD, Seoane RC, Ward S, Dalessandri T, Lai C, Healy E, Kipling D, Proby C, Moyes C, Green K, Best K, Haniffa M, Botto M, Dunn-Walters D, Strid J. Epithelial damage and tissue gammatdelta T cells promote a unique tumor-protective IgE response. Nature immunology. 2019;20(8):859-70. doi: 10.1038/s41590-2019-0161-8. PubMed PMID: 30013146; PMCID: PMC6071860.

Nigro EA, Brini AT, Yenagi VA, Ferreira LM, Achatz-Straussberger G, Ambrosi A, Sarvito F, Sopranza E, van Anken E, Achaz G, Siggia AC, Vangelista L. Cutting edge: IgE plays an active role in tumor immune-surveillance in mice. J Immunol. 2019;167(7):2583-2588. doi: 10.4049/jimmunol.1601026. PubMed PMID: 27566822.

Jensen-Jarolim E, Turner MC, Karagiannis SN. AllergyOncoology: IgE- and IgG4-mediated immune mechanisms linking allergy with cancer and their translational implications. J Allergy Clin Immunol 2017;140(4):982-984. doi: 10.1016/j.jaci.2017.04.014. PubMed PMID: 28526623.

Ferastarau D, Bax HJ, Bergmann C, Capron M, Castells M, Dombrowicz D, Fiebiger A, Müller V, Poli A, Rosenstrech D, Roth-Walter F, Shamy M, Steveling-Klein EH, Turner MC, Untersmayr E, Karagiannis SN, Jensen-Jarolim E. AllergyOncoology: ultra-low IgE, a potential novel biomarker in cancer-a Position Paper of the European Academy of Allergy and Clinical Immunology (EAACI). Clin Transl Allergy. 2020;10:32. Epub 2020/07/23. doi: 10.1186/s13601-020-00335-w. PubMed PMID: 32695309; PMCID: PMC7366896.

Strunk RC, Bloomberg GR. Omalizumab for asthma. N Engl J Med. 2006;354(25):2689-2695. doi: 10.1056/NEJMct055184. PubMed PMID: 17697070.

Lee SE, Li X, Kim JC, Lee J, Gonzalez-Navajas JM, Hong SH, Park IK, Rhee JH, Raz E. Type I interferon maintains Foxp3 expression and T regulatory cell functions under inflammatory conditions in mice. Gastroenterology. 2012;143(1):145-54. Epub 2012/04/06. doi: 10.1053/j.gastro.2012.03.042. PubMed PMID: 22475334; PMCID: PMC3729390.

Tan CL, Kuchroo JR, Sage PT, Liang D, Francisco LM, Buck J, Thaker YR, Zhang Q, McArule SL, Junea VR, Lee SJ, Lovitch SB, Lian C, Murphy GF, Blazir BR, Vignali DAA, Freeman GJ, Sharpe AH. PD-1 restraint of regulatory T cell suppressive activity is critical for immune tolerance. J Exp Med. 2021;218(1): doi: 10.1084/jem.20182232. PubMed PMID: 33045061; PMCID: PMC7543091.

Sage PT, Francisco LM, Carman GV. T cell beta2-Microglobulin. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. Nature Immunology. 2013;14(2):152-61. doi: 10.1038/ni.2496. PubMed PMID: 23242415; PMCID: 3788614.
74. Bally AP, Austin JW, Boss JM. Genetic and Epigenetic Regulation of PD-1 Expression. J Immunol. 2016;196(6):2431-7. doi: https://doi.org/10.4049/jimmunol.1502643. PubMed PMID: 26945088; PMCID: PMC4780223.

75. Bally APR, Neeld DK, Lu P, Majumder P, Tang Y, Barwick BG, Wang Q, Boss JM. PD-1 Expression during Acute Infection Is Repressed through an LSD1-Blimp-1 Axis. J Immunol. 2020;204(2):449-58. doi: https://doi.org/10.4049/jimmunol.1900601. PubMed PMID: 31811020; PMCID: PMC6946872.

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