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Generation and identification of tumor-evoked regulatory B cells

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Abstract

The involvement of Bregs in cancer remains poorly understood despite their well-documented regulation of responses to the self and protection from harmful autoimmunity. We recently discovered a unique regulatory B cell subset evoked by breast cancer to mediate protection of metastasizing cancer cells. These results together with the wealth of findings of the last 40 years on B cells in tumorigenesis suggest the existence of additional cancer Bregs modulating anticancer responses. To facilitate the search for them, here we provide our detailed protocol for the characterization and generation of tumor-evoked regulatory B cells. Wherever applicable, we also discuss nuances and uniqueness of a Breg study in cancer to warn potential pitfalls.

Keywords

tBregs; Tregs; Metastasis; 4T1 breast cancer; TARC-PE38

1. Introduction

The outcome of two seemingly different diseases such as cancer and autoimmune disease often depends on a function of common immune regulatory machinery that controls responses to the self. Whereas the loss of regulation of immune responses facilitates autoimmune diseases, cancer is a result of over regulation and concurrent suppression of immune effectors. They involve a network of specialized regulatory immune cells representing almost every immune cell type, which includes myeloid (MSC) and myeloid-derived suppressive cells (MDSC), regulatory T cells (Tregs), NKT and regulatory B cells (Bregs). As name stands, regulatory immune cells regulate immune cells via both contact-dependent and independent mechanisms involving various immune modulatory cytokines and other soluble factors. As such, their accumulation (for example, MDSC and Tregs) is often a sign of poor disease outcome in humans and mice with cancer ¹⁻³, whereas their dysfunction is associated with autoimmune diseases ^{4,5}. Interestingly, Bregs are mostly known for the importance in regulation of responses to the self and protection from harmful autoimmunity ⁶⁻⁹, but their role in cancer remains poorly understood. This is despite the fact

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²⁴Both parental 4T1 cells and their subset 4T1.2 cells readily metastasize into the lungs upon subcutaneous (s.c.) challenge in to mammary gland of WT BALB/c mice.

²⁵Keep cells on ice to be injected as soon as possible, to avoid cell loss.

that B cells were often linked with cancer progression, including methylcholanthrene-induced carcinogenesis^{10,11} and transplanted tumors in mice¹². However, depending on the cancer type, the function of B cells may vary leading to opposing results. While B-cell deficiency retards tumor growth in mice unless replenished with B220⁺ or CD19⁺ B cells^{13,14}, the loss of CD27⁺CD19⁺ B cells appears to promote the advanced stage of melanoma and other solid tumors^{15,16}.

We recently identified a unique subset of Bregs that facilitates metastasis of orthotopic 4T1 breast adenocarcinoma (4T1 cancer) in BALB/c mice¹⁴. To date, these cells, designated tumor-evoked Bregs (tBregs), are also found in another model, C57BL/6 mice with spontaneous ovarian cancer (Bodogai & Biragyn, unpublished). The role of tBregs in cancer is to protect metastasizing cancer cells from immune effector cells by inducing immune suppression mediated by Tregs. tBregs convert non-regulatory CD4⁺ T cells into FoxP3⁺ Tregs and thereby neutralize cytolytic antitumor NK cells and GrB⁺CD8⁺ T cells. Phenotypically, tBregs are CD19⁺ B cells that express CD25⁺CD81^{High} B7H-1^{High} and CD20^{Low} 4-1BBL^{Low} and constitutively active transcription factor Stat3¹⁴. In 4T1 cancer model, tBregs are primarily induced by nonmetastatic 4T1 cancer cell subsets producing metabolites of 5-lipoxygenase¹⁷. As such, tBregs can be easily generated *in vitro* by treating murine splenic resting B cells with conditioned medium (CM) of non-metastatic 4T1 cancer cells.

In this chapter we provide the reader with our strategy to enrich for non-metastatic 4T1 cancer cells, that is based on preferential elimination of metastatic 4T1 cancer cells expressing chemokine receptor CCR4, using TARC-PE38. TARC-PE38 is a chimeric chemokine which only kills CCR4⁺ cells and which is made of CCL17 fused with a truncated form of Pseudomonas exotoxin (PE38). Of note, the selection of non-metastatic cells may not be needed for some tumors, as tBreg-like cells can be induced using CM of non-segregated human cancer cells¹⁴. Moreover, the detailed protocol for TARC-PE38 production provided in this chapter can also be adapted for immunotherapy and to elucidate CCR4-expressing immune cells in autoimmunity and cancer. We also describe our strategies for functional characterization of tBregs, such as *in vivo* evaluation of their metastasis-promoting activity and *in vitro* T cell suppression and FoxP3⁺ Tregs conversion assays. Finally we provide protocols for the generation and characterization of human cancer-associated tBreg-like cells.

2. Materials

2.1. Materials for the production of TARC-PE38 fusion protein

1. *E. coli* competent cells, BL21(DE3).
2. Ampicillin, carbenicillin.
3. Luria Bertani (LB) broth.
4. Amp-LB plates (LB plate with 100 µg/ml ampicillin).
5. Superbroth (SB medium, autoclave sterilized): 32 g tryptone, 20 g yeast extract, 5 g NaCl, 5 ml 1 N NaOH, 950 ml deionized water.

6. SB medium with antibiotics: 100 µg/ml carbenicillin, 50 µg/ml ampicillin in SB.
7. Glycerol-SB medium: SB medium with antibiotics containing 1% glycerol.
8. Isopropyl β-D-1-thiogalactopyranoside (IPTG).
9. 20% sucrose solution in water, ice cold.
10. TE solution: 50 mM Tris-HCl, pH 7.5, 20 mM EDTA.
11. 6 M guanidine solution for proteins: 6 M guanidine-HCl, 0.1 M Tris-HCl, pH 8.0, 2 mM ethylenediaminetetraacetic acid (EDTA).
12. 1 M Tris-HCl solutions at pH 7.5 and pH 8.0.
13. 0.5 M EDTA, pH 8.0.
14. 50 mg/ml lysozyme solution in water, stored frozen.
15. Refolding solution: 0.1 M Tris-HCl, pH 8.0, 0.5 M L-arginine, 4 mM GSSG, 2 mM EDTA.
16. GSSG (Oxidized glutathione).
17. DTE (Dithioerythritol).
18. Heparin Sepharose CL-6B. (GE Healthcare).
19. Q Sepharose (GE Healthcare).
20. Buffer A: 50 mM sodium phosphate buffer (pH 8.0) containing 0.3 M NaCl.
21. Buffer B: buffer A with 1M imidazole.
22. Buffer A1: 20 mM sodium phosphate buffer (pH 7.4) containing 0.1 M urea.
23. Buffer B1: buffer A1 with 1M NaCl.
24. His-trap column (Talon metal affinity resin, Clontech).
25. 5% GL buffer: 5% glycerol, 50 mM NaCl, 20 mM Tris-HCl, pH 7.5.
26. 5 M NaCl in water.
27. 25% Triton X-100 in water.
28. Ultra pure Urea.
29. L-Arginine –HCl.
30. 100% Glycerol.
31. Imidazole.
32. 1 N NaOH.
33. BioLogic DuoFlow (BioRad) for fast protein liquid chromatography (FPLC).
34. Sample loading DynaLoop 25 (BioRad, or any loading system compatible with the BioLogic DuoFlow chromatography systems).
35. BCA Protein Assay Kit (Thermo Scientific).

36. Pierce LAL Chromogenic Endotoxin Quantitation kit.
37. Dialysis membrane (MWCO 6-8000, 50 mm width).
38. 60 L plastic container.
39. Tissue-Homogenizer.
40. Shaker incubator for use at 30°C and 37°C.

2.2. Materials for enrichment of 4T1-PE cells using TARC-PE38

1. Human acute T-lymphoblastic leukemia cell lines: CCRF-CEM (CCR4-positive cell line CCL-119) and MOLT-4 (CCR4-negative cell line CRL-1582, American Type Culture Collection, ATCC); 4T1 (ATCC) and 4T1.2 breast cancer cells (collectively designated 4T1 cells, the gift of Dr. Robin Anderson, Peter MacCallum Cancer Centre, Australia).
2. Complete RPMI (cRPMI): heat inactivated 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µM β-Mercaptoethanol in RPMI-1640.
3. WST-1 cell proliferation reagent (Roche Applied Science).
4. Elisa 96-well Plate Reader.
5. 75cm² cell culture flasks.
6. Trypsin-EDTA.
7. 0.2 µm filter.

2.3. Materials for *in vitro* generation of murine tBregs and for their phenotypical confirmation

1. Complete RPMI (see section 2.2, step 2).
2. Cell proliferation dye eFlour670 (eBiosciences).
3. Nylon mesh strainer 70µm.
4. Phosphate buffer saline (PBS).
5. Trypan blue.
6. Mouse spleen.
7. B-cell negative selection magnetic beads (ex: EasySep™ Mouse B cell Enrichment, StemCell Technologies).
8. Flow cytometry staining/washing buffer: 5% Bovine serum albumin (BSA), 0.05% Sodium azide in PBS.
9. Flow cytometry antibodies to murine CD19, CD81, CD25 and CD20.
10. Fixation buffer: 2% formaldehyde.

11. Permeabilization buffer: 0.1% Saponin, 0.009% sodium azide solution. Alternatively, any premade permeabilization buffer (available from R&D and eBioscience) can be used.
12. Cell staining/washing buffer (Cell Signaling Technology).
13. Western blot antibodies: PhosphoStat3 (Tyr705, clone 9138) and Stat3 (clone 9132, Cell Signaling Technology).
14. Recombinant mouse BAFF (R&D System).

2.4. Materials for functional confirmation of tBregs

1. Complete RPMI (see section 2.2, step 2).
2. Cell proliferation dye eFlour670 (eBiosciences).
3. Nylon mesh strainer 70 μ m (Fisher scientific).
4. PBS.
5. Trypan blue.
6. Mouse CD3-cell negative selection system (ex. Mouse CD3 enrichment columns, R&D Systems).
7. Mouse CD4⁺ T-cell negative selection system (ex. Mouse CD4⁺ T-cell isolation kit II, Miltenyi Biotec).
8. Mouse CD25⁺ cells selection system (ex. Dynabeads® CD25, Invitrogen).
9. Anti-mouse CD3 Ab (functional grade, BD Biosciences).
10. Anti-mouse CD3/CD28 Ab-coupled beads (Invitrogen).
11. Anti-mouse CD19 FITC/anti-FITC MicroBeads (Miltenyi Biotec).
12. Recombinant mouse IL-2 (Chiron).
13. Flow cytometry antibodies for CD4, CD8, Foxp3.
14. Female μ MT B-cell deficient mice in BALB/c background, 6-8 weeks old.
15. Female BALB/c mice, 6-8 weeks old.
16. Needles for injection (29G ½, 0.3mm $\times$ 13mm).
17. Fekete's solution: 70 ml absolute ethanol, 10 ml 37% formaldehyde, 5 ml glacial acetic acid and water to 100 ml.
18. 10% waterproof India ink in PBS.
19. Dissection tools: scissors, forceps, clamp.

2.5. Materials for generation of human tumor-evoked regulatory B cells

1. Peripheral blood from healthy human donors.
2. ACK lysing buffer (Lonza)

3. Ficoll-Paque Plus (GE Healthcare Life Sciences).
4. Human cancer cells: OVCAR-3, B-2008, UCI101 and BG1 (ovarian cancer); MDA-MB-231 (HTB-26, breast cancer), FS11 (melanoma) and SW480 (colon cancer) (ATCC).
5. Human CD3⁺-cell negative selection system (ex. Human CD3⁺ T-cell enrichment columns, R&D Systems).
6. Human CD4⁺ negative selection system (ex. Human CD4⁺ T-cell isolation kit II, Miltenyi Biotec).
7. Human CD25⁺ cells selection system (ex. Anti-human CD25 PE / anti-PE MicroBeads, Miltenyi Biotec).

3. Methods

3.1. Production of TARC-PE38 fusion protein from bacteria

TARC-PE38 is a chimeric chemokine in which CCL17 is fused with a truncated form of *Pseudomonas* exotoxin (PE38). Since this protein only kills CCR4-expressing cells¹⁸, the use of TARC-PE38 represents a quite straightforward method for enrichment of nonmetastatic cancer cells (designated 4T1-PE cells). The use of TARC-PE38 is also a versatile and potent immunotherapeutic strategy that can be adapted to generate other chemokine-based toxins (designated chemotoxins). Here we provide a detailed protocol of its production, which represents a modified version of the protocol originally developed by Buchner and colleagues to purify single chain antibodies²¹. Since TARCPE38 cloning can be done using any simple cloning procedure, here we only provide methods for production and purification of TARC-PE38. Of note, since TARC-PE38 can be toxic for bacterial cells, it is advised to perform cloning procedures in *E. coli* strains that do not have T7 polymerase to reduce its background expression. Once the desired plasmid is created or obtained from the authors' laboratory, it should be transformed into *E. coli* strain designed for production of toxic proteins under the regulation of T7 polymerase, such as BL21(DE3). To produce TARC-PE38, the best yield is achieved by expressing TARC-PE38 as inclusion bodies.

3.1.1. Expression of TARC-PE38 in BL-21(DE3)—

1. Transform 20 µl BL21(DE3) competent cells with 1 µl TARC-PE38 plasmid (100 ng) for 10 min on ice.
2. Heat-shock the mixture for 1 min at 42°C.
3. Add 100 µl LB broth and plate bacteria on Amp-LB agar plate and incubate overnight at 37°C.
4. Next day, wash/resuspend all colonies off the plate using 6 ml SB medium.
5. Use 0.5 ml bacteria to inoculate 50 ml of glycerol-SB medium in a 2 L flask (see Note 1).

¹ Store the remaining bacterial suspension in 20% glycerol at -70°C.

6. Culture bacteria for 6-8 h at 30°C, shaking vigorously at 200 rpm.
7. Add 50 ml fresh glycerol-SB medium and continue shaking for 3-5 h.
8. Add 300 ml IPTG-containing SB without glycerol (0.8 mM IPTG in SB medium with antibiotics) and culture overnight in 2 L flasks.

3.1.2. Generation of bacterial spheroplasts and inclusion bodies with TARC-PE38—

1. Pellet bacteria by centrifuging at $5000 \times g$ for 15 min. Discard supernatant.
2. Resuspend bacteria in 20% sucrose solution, 60 ml.
3. Incubate on ice for 10 min.
4. Centrifuge as in step 1.
5. Resuspend bacteria in 100 ml ice-cold distilled water.
6. Incubate on ice for 10 min.
7. Pellet cells by centrifuging for 20 min at $19800 \times g$.
8. Resuspend the bacterial pellet in 30 ml ice-cold TE buffer. Homogenize pellet completely using tissue homogenizer.
9. Add 25 μ l of lysozyme solution.
10. Incubate at room temperature (RT) for 30-60 min.
11. Add 6 ml of 5 M NaCl and 5.2 ml of 25% Triton X-100 solution.
12. Incubate at RT for 30 min and then homogenize solution with tissue homogenizer (see Note 2).
13. Centrifuge for 45 min at $26000 \times g$. Discard supernatant.
14. Add 30-35 ml of ice-cold TE to the pellet and again thoroughly homogenize it.
15. Repeat steps 13 and 14 five more times (see Note 3).
16. To solubilize inclusion bodies, homogenize pellet in 5 ml of 6 M guanidine solution and collect supernatant after 30 min centrifugation as in step 13 (see Note 4).
17. Determine protein concentration using BCA Protein Assay Kit or Bradford assay.
18. Add 0.3 M DTE and incubate for 30 min at RT (see Note 5).

3.1.3. TARC-PE38 refolding—

²It is essential to break bacterial DNA to completely reduce the enhanced viscosity.

³Pellet mostly contains inclusion bodies with precipitated TARC-PE38. Its purity depends on a number of repeats in steps 13 and 14. Pellet can be stored frozen.

⁴Keep the total volume less than 10 ml.

⁵The sample can be stored at -20°C at this step.

1. Add sample drop-wise to pre-chilled (10°C) refolding solution while vigorously mixing with a magnetic stirrer to make final protein concentration 80 µg/ml. Let refold TARC-PE38 for 3-5 days at 10°C.
2. Fill dialysis tubes with the refolded protein solution and overnight dialyze in 20 mM Tris-HCl, pH 7.5 and 100 mM Urea at 4°C. Volume of dialysis buffer should be 10 times of refolding protein mixture, for example, use 40 L dialysis buffer for 4L of refolding protein mixture.
3. Clear the dialyzed sample by 15 min centrifugation at 4600 × g.
4. Gently remove supernatant into a clean container (see Note 6).

3.1.4. Heparin-sepharose (HP) purification of TARC-PE38—

1. Pack the HP column with 2-3 ml Heparin Sepharose CL-6B resin in 20 mM Tris-HCl, pH 7.5 (see Note 7).
2. Run TARC-PE38 through HP column at 4 ml/min flow at 4°C.
3. Wash HP column with 30 ml 5% GL buffer or until wash flowthrough is < OD₂₈₀ 0.05 (see Note 8).
4. Elute TARC-PE38 using 100-1000 mM NaCl gradient. It is usually eluted between 300-500 mM NaCl.
5. Collect 2 ml and determine protein content by UV reading at OD₂₈₀ or BCA assay.
6. Check fractions in reducing PAGE (MW of TARC-PE38 is about 50 kDa) and, if necessary, western blot hybridization to tags used such as c-Myc. Pool fractions containing TARC-PE38 to further purify using metal affinity column.

3.1.5. TARC-PE38 purification on His-trap column using FPLC (see Note 9)—

1. Set up FPLC. After washing with water fill the FPLC pump A with buffer A. For pump B, use buffer B (see Note 9). Connect 1 ml His-trap column.
2. Load 25 ml protein into a DynaLoop sample-loading loop.
3. Set the program for loading and elution at flow rate 2 ml/min as follows: buffer A: 10 ml; zero Baseline; buffer A: 10 ml; inject sample: 25 ml; buffer A: 10 ml; gradient increase in buffer B from 0% to 100%: 60 ml. At that time start collection of 1 ml fractions (total number of fractions will be 60). Stop collection and wash column with 10 ml buffer B.
4. Wash column with water (50 ml) and store it in 20% ethanol at 4°C.

⁶The pellet can be reused as in steps 18 (Section 3.1.2) through 4 (Section 3.1.3) or stored frozen.

⁷Keep resin volume low to improve purification purity. We usually use 2 ml resin, which is good for up to 80 mg total protein. Although purification can be done manually, the use of Fast Protein Liquid Chromatography system (FPLC), such as Biologic DuoFlow, BioRad, Hercules, CA, is highly recommended.

⁸For a higher purity of TARC-PE38 isolation, the column can be washed with GL buffer containing 100 mM NaCl.

⁹Heparin-sepharose purification only yields about 50% purity. For getting > 90% purity we add metal affinity purification, as TARC-PE38 contains His-tag. Although TARC-PE38 can be eluted from His-trap column by reducing pH of buffer A, we prefer to use imidazole. However, use freshly prepared imidazole.

5. Evaluate purity of TARC-PE38 in reducing PAGE. Pool fractions containing TARC-PE38.
6. Dialyze overnight in 20 mM sodium phosphate buffer (pH 7.4) containing 0.1 M urea for further purification and endotoxin removal (see Note 10).

3.1.6. Removal of endotoxin using ion exchange chromatography—

1. Clean FPLC system with 1 N NaOH followed with a thorough wash with endotoxin-free water (from now on endotoxin-free reagents and buffers should be used). Use buffer A1 for pump A and buffer B1 for pump B. Equilibrate 1 ml protein Q-sepharose column with A1 buffer and load with TARC-PE38.
2. Run TARC-PE38 at 2 ml/min speed at 4°C as in steps 1-5, Section 3.1.5.
3. Collect 1 ml fractions. Determine endotoxin content using an endotoxin measurement kit. Endotoxin is usually eluted after TARC-PE38 (see Fig. 1). Pool fractions with TARC-PE38 containing desired endotoxin purity (endotoxin 0.05 EU/μg protein is usually acceptable for most applications).
4. Dialyze TARC-PE38 in PBS containing 5% glycerol and 300 mM NaCl. Store TARC-PE38 at -70°C .

3.2. TARC-PE38-based strategy for the enrichment of 4T1-PE cells and generation of their conditioned media

Our procedure for the generation of murine tBregs requires treatment of B cells with cancer cell conditioned medium (CM)¹⁴ obtained from the non-metastatic subsets of murine 4T1 or 4T1.2 breast cancer cells (collectively designated 4T1 cells). We recommend enriching 4T1 cancer cells for non-metastatic cells, as they primarily induce tBregs¹⁴. Non-metastatic cells can be either selected utilizing the side population (SP) staining with Hoechst 33341 (Biosciences)^{14,20} or enriched using TARC-PE38 protein that specifically kills metastatic CCR4-expressing cells^{14,18}. The procedure for SP staining and selection of non-metastatic cancer cells is relatively simple and was described elsewhere¹⁴. In brief, we stain about 1×10^6 4T1 cells (harvested using trypsin-EDTA and resuspended in Iscove's DMEM (Invitrogen) supplemented with 2% fetal bovine serum) with 5 μg/ml Hoechst 33342 for 90 min at 37°C. Control cells (to verify specificity of staining) can be incubated with 100 mM verapamil hydrochloride, which abolishes the SP staining. Propidium iodide is added (1 μg/ml) to exclude dead cells during cell sorting with a Beckman-Coulter MoFlo high-speed cell sorter.

Here we describe the TARC-PE38-based method for the enrichment of nonmetastatic cancer cells. Before proceeding with the enrichment step, it is necessary to evaluate TARC-PE38 killing activity. Usually it is sufficient to use 1-5 μg/ml TARC-PE38 to kill 90% CCR4⁺ cells *in vitro*. We advise to standardize each batch of TARC-PE38 on CCR4⁺ human acute T-lymphoblastic leukemia cell line CCRF-CEM (CEM).

¹⁰At this stage, TARC-PE can be used for *in vitro* assays to kill CCR4⁺ cells (including enrichment of 4T1-PE cells). However, it is contaminated with endotoxin. If needed to generate endotoxin-low TARC-PE38 for *in vivo* and other *in vitro* functional assays, proceed to the following steps.

3.2.1. Evaluation of TARC-PE38 killing activity—

1. Plate CCRF-CEM and control MOLT-4 cells (5×10^4 per well) in 96-well flat-bottom plates in 100 μ l of cRPMI with titrated amounts of TARC-PE38 (for example: 100–10,000 ng/ml). Culture overnight at 37°C.
2. Add 100 μ l of 20% WST-1 reagent (see Note 11).
3. Incubate 1–3 h at 37°C until its color starts changing to dark red. Measure OD at 450 nm after each hour of incubation.
4. The measured absorbance correlates to the number of viable cells (see Note 11).

3.2.2. TARC-PE38 mediated enrichment of 4T1-PE cells—

1. Incubate 0.5×10^6 /ml 4T1 cells in 6-well plate with 10 μ g/ml TARC-PE38 in 1.5 ml cRPMI for three days at 37°C in humidified atmosphere with 5% CO₂.
2. Add 1.5 ml fresh cRPMI with 10 μ g/ml TARC-PE38 and continue the incubation.
3. Collect cells by centrifugation for 10 min at $500 \times g$. Resuspend cells in 5 ml cRPMI with 10 μ g/ml TARC-PE38 and culture them for two more days in T25 flask at 37°C in humidified atmosphere with 5% CO₂.
4. Collect cells by centrifugation for 10 min at $500 \times g$. Resuspend cells in fresh cRPMI. The majority (95%) of cells are 4T1-PE cells. Store them -100°C in 10% DMSO/40% FBS/cRPMI. They can now be grown in fresh cRPMI to generate tBreg-inducing conditioned medium (CM), as described in the following section.

3.2.3. Generation of CM from 4T1-PE cells—

1. Culture 5×10^6 4T1-PE cells in 8 ml cRPMI in 75 cm² flasks for two-three days at 37°C in humidified atmosphere with 5% CO₂.
2. Gently wash the cell monolayer with 10 ml PBS.
3. Detach 4T1-PE cells with 3 ml trypsin-EDTA solution for 3–5 min at 37°C.
4. Collect cells by pipetting several times and breaking cell clumps in 7 ml cRPMI (see Note 12).
5. Collect cells and determine cell number.
6. Plate 2×10^6 cells in 8 ml cRPMI in 75cm² flask and incubate for three days at 37°C in humidified atmosphere with 5% CO₂ (Passage # 1).
7. Collect CM after 5 min centrifugation at $500 \times g$.
8. Filter CM using 0.2 μ m filter (CM-4T1PE, see Note 13).

¹¹WST-1 reagent should be completely dissolved, if undissolved particles remain, filter the solution using 45 μ m syringe filter before use. Since formation of formazan dye (dark red) is a result of the glycolytic production of NAD(P)H in viable cells, the measured absorbance directly correlates to the number of viable cells.

¹²If cells are still attached to the plate, a cell scraper can be used.

¹³CM (CM-4T1PE) needs to be stored in aliquots at minimum -20°C . Repeated freeze thawing of CM is not recommended. The remaining cells can be further cultured as in steps 8–14 to prepare CM from additional cell passages (up to 3 consecutive passages).

3.3. *In vitro* generation of murine tumor-evoked regulatory B cells and their characterization

B cells of any mouse background can be used. Due to poor survival of murine splenic B cells, we use BAFF/Blys to keep control B cells viable. Since 4T1-PE cells express BAFF, no additional BAFF is needed for tBreg generation. To test the *in vitro* conversion of normal murine B cell into tBreg cells, a phenotypic and functional characterization of these cells should be performed. Functional criteria such as the abilities to suppress the proliferation of TCR-activated T cells (Section 3.3.3) and to convert naïve CD4⁺ T cells in regulatory T cells (Section 3.3.4) should be analyzed. In addition, tBregs should also be active *in vivo*, promoting lung metastasis in mice (Section 3.3.5).

3.3.1. Use of CM-4T1PE for *in vitro* generation of tBregs—

1. Generate a single cell suspension from murine spleen using a 70 µm cell strainer.
2. Pellet cells by centrifuging 5 min at 500 × g at RT.
3. Purify B cells using the negative selection system of the EasySep™ Mouse B cell Enrichment Kit. Follow manufacturer's instructions (see Note 14).
4. Count viable cells using trypan blue. Resuspend cells in cRPMI at the concentration of 4 × 10⁶ cells/ml.
5. Incubate 2 × 10⁶ cells/ml in cRPMI with 50% CM-4T1PE (from step 8, Section 3.2.3) at 37°C in humidified atmosphere with 5% CO₂.
6. Control B cells: incubate with 100 ng/ml recombinant mouse BAFF in cRPMI (B +BAFF) (see Note 15).

3.3.2. Phenotypic characterization of *in vitro* generated tBregs—

1. After 2 days of incubation check for the expression of CD19, CD81, CD25, CD20 and phospho Stat3 (pStat3) by flow cytometry (see Note 16).
2. Phenotypically, tBregs are pStat3⁺ CD25⁺ CD19⁺ cells that express high levels of CD81 but low levels of CD20 (see Fig. 2A,B).
3. B cells treated with BAFF are used as controls, as they do not have the phenotype of tBregs.

3.3.3. Functional confirmation of *in vitro* generated tBregs by T-cell suppression assay—

1. Count tBregs and control B cells (B+BAFF) in trypan blue to determine the quantity of viable B cells.

¹⁴Any other negative selection method is suitable, including Milteny Biotec system.

¹⁵Due to poor survival of murine splenic B cells, we use BAFF/Blys to keep control B cells viable.

¹⁶For pStat3 staining, follow the protocol provided by the manufacturer (Cell Signaling Technology). Alternatively, western blot can be performed to check pStat3 expression. Use β-actin as internal control (see Fig. 2B).

2. Wash cells twice and resuspend cells in cRPMI at the final concentration of 10^6 cells/ml (see Note 17).
3. Isolate CD3⁺ T cells from a congenic mouse spleen using the mouse CD3-cell enrichment columns.
4. Label T cells with 4 μ M eFluor670 dye by incubating them in PBS for 10 min at 37°C.
5. Centrifuge cells for 5 min at $500 \times g$ at RT and resuspend the pellet in cRPMI at the final concentration of 10^6 cells/ml.
6. Incubate T cells with tBregs (or B+BAFF) at 1:1 ratio for 5 days in the presence of 1.5 μ g/ml anti-mouse CD3 Ab or of anti-CD3/28 activator beads (bead:cell ratio 1:3).
7. After 5 days of co-culture, stain cells with anti-mouse CD4 and CD8 Ab and determine T cell proliferation using flow cytometry analysis. Decrease in eFluor670 within CD4⁺ and CD8⁺ T cells represents the proportion of proliferated T cells (see Fig. 2C).
8. B cells treated with BAFF do not show the regulatory function of tBregs. (see Note 18)

3.3.4. Functional confirmation of *in vitro* generated tBregs by Treg conversion assay (see Note 19)—

1. Generate clumps-free splenic single cell suspension.
2. Negatively isolate CD4⁺ T cells using the mouse CD4⁺ T-cell isolation kit II following the manufacturer's instructions.
3. Deplete CD25⁺ cells using any method, including commercially available CD25 selection kits.
4. Count viable CD4⁺CD25⁻ cells using trypan blue. Resuspend them at 10^6 cells/ml in cRPMI.
5. Incubate equal numbers of non-Tregs and tBregs (or control B+BAFF) for 5 days in the presence of bead-conjugated anti-CD3/CD28 Abs and 500 U/ml mouse recombinant IL-2.
6. After 5 days of co-culture, stain cells for intracellular Foxp3 to evaluate the percentage of Foxp3⁺ within CD4⁺ T cells (see Fig. 2D).

3.3.5. Functional confirmation of *in vitro* generated tBregs by *in vivo* characterization of tBreg activity—The induction of lung metastasis in B-cell deficient μ MT mice is used as a readout for the *in vivo* characterization of *in vitro* generated tBregs.

¹⁷Since B cells can be sensitive to thermic changes, we advice to prepare B cells just before mixing with T cells.

¹⁸B cells activated with lipopolysaccharides (LPS) or CpG can be also used as negative controls for tBregs, unlike Bregs in autoimmunity.

¹⁹Outcome may depend on the quality of purification of non-Treg cells, such as CD25⁻CD4⁺ T cells.

Unlike in WT mice, in μ MT mice 4T1 cancer can progress at the primary site, i.e. in the mammary gland, but poorly metastasize into the lungs²². However, lung metastasis is restored in these mice upon adoptive transfer of *in vitro*-generated tBregs or tBregs from tumor-bearing mice (Section 3.4).

1. Wash tBregs or B-BAFF cells twice with 50 ml PBS.
2. Resuspend cells in PBS at 50×10^6 /ml ice-cold PBS to inject 5×10^6 cells per mouse (see Note 20).
3. Intravenously inject (adoptively transfer via tail vein) μ MT mice with 5×10^6 tBregs or control B-BAFF cells in 100 μ l PBS one-day before and 5 days after tumor challenge (see Note 20).
4. Subcutaneously inject 1×10^6 4T1.2 cells (in 100 μ l) into the fourth-mammary pad of donor WT BALB/c mice (see Section 3.4 and Note 21).
5. Repeat step 3 after 5 days post tumor challenge.
6. Starting from 16 days post tumor challenge, measure tumor size every other day. Euthanize mice between days 28 and 36 to assess tumor weight and lung metastasis.
7. To analyze lung metastasis, inject India ink through the trachea of euthanized mice. Remove lungs and overnight fix in Fekete's solution.
8. Wash lungs several times with water and count tumor nodules that appear as white spots (see Note 22).

3.4. *In vivo* generation of murine tumor-evoked regulatory B cells (see Note 23)

Here we provide the method for the *in vivo* generation of tBregs from mice with 4T1 cancer (see Fig. 3).

1. Prepare 4T1.2 cells for tumor challenge by plating 5×10^6 cells in 10 ml cRPMI in 75 cm² flask for 2 days.
2. Discard the supernatant and wash cells with 10 ml PBS.
3. Split cells 1:3 in 75 cm² flasks and incubate them for one more day.
4. Wash cells twice with 20 ml PBS and add 3 ml trypsin-EDTA for 5 min at 37°C.
5. Collect and wash cells twice with 50 ml ice-cold PBS. Count viable cells in trypan blue.
6. Resuspend cells at 5×10^5 /ml ice-cold PBS (see Notes 24, 25).

²⁰B cells must be kept on ice and injected as soon as possible.

²¹The injection site of mice need to be shaved prior to tumor challenge. Although WT BALB/c mice succumb of metastasis by 28 days, longer time (up to 40 days) is required to get comparable metastasis in μ MT mice adoptively transferred of tBregs.

²²Fixation time longer than 24 h will diminish contrast of metastatic nodules.

²³4T1 cancer-bearing WT mice readily generate tBregs, which can be detected at many sites such as the secondary lymphoid organs (spleen and lymph nodes), blood, at the primary tumor site, lungs or liver^{17,22}. Since spleen and blood also contain cancer-expanded Gr1⁺ myeloid cells, they can affect the purity of tBreg isolation. To avoid potential contamination with MSC cells, we suggest to isolate tBregs from lymph nodes to perform functional studies.

7. Subcutaneously inject 1×10^6 4T1.2 cells (in 100 μ l) into the fourth-mammary pad of donor WT BALB/c mice.
8. Euthanize mice at days 13 and 15 post-tumor challenge and remove draining lymph nodes (axillar and inguinal).
9. Prepare a single lymph node cell suspension using a cell strainer.
10. Positively isolate CD19⁺ B cells using magnetic beads system (see Note 26).
11. Evaluate suppressive function of tBregs ex vivo by testing the ability to suppress T cell activity (see Section 3.3.3) and induce conversion of Foxp3⁺ Tregs (see Section 3.3.4).
12. To test the ability of tBregs to promote lung metastasis, adoptively transfer ($> 4 \times 10^6$ cells/mouse) into μ MT mice with 1–13 days old 4T1.2 cancer (challenged as in step 7).

3.5. Generation of human tumor-evoked regulatory B cells

Human tBreg-like cells can be generated from peripheral blood B cells of healthy donors using CM of various human cancer cells¹⁴. However, human cancer cells do not need to be segregated into non-metastatic cell subsets. In addition, some phenotypic markers of human tBreg-like cells differ from mouse tBregs.

3.5.1. Generation of human cancer cell CM—

1. After an initial step of expansion of a human cancer cell line (for example, MDAMB-231 cells), plate the cells at 2×10^6 cells in 8 ml cRPMI in a 75cm² flask.
2. CM is collected when tumor cells reach 90% confluence (see Note 27).
3. As for mouse 4T1.2 tumor cells, collect CM by centrifugating for 10 min at 500 $\times$ g.
4. Filter CM with 0.2 μ m filters and store at -80°C in small aliquots (see Note 28).

3.5.2. Use of human cancer cell-CM to generate human tBregs—

1. Human peripheral blood mononuclear cells (PBMC) are obtained after Ficoll gradient separation following the manufacturer's instructions.
2. Lyse red blood cells with ACK lysing buffer. Wash twice with PBS and count viable cells using trypan blue.
3. Isolate B cells using a negative magnetic selection system (see Note 29).

²⁶B cells from naïve mouse draining LN (without tumor) can be used as controls.

²⁷We advice not exceed 5-6 days of cell expansion.

²⁸CM should not be repeatedly frozen/thawed, and do not use CM of more than 3 consecutive passages.

²⁹B cells only represent 2-5% of normal donor PBMCs.

4. Pellet cells (5 min, $500 \times g$ at RT), count viable cells using trypan blue and resuspend cell at 4×10^6 B cells/ml in cRPMI.
5. Add CM to reach a final B cell density of 2×10^6 /ml in 50% tumor CM (see Note 30).
6. tBregs are usually generated after 48 h incubation at 37°C .
7. Verify the generation of human tBregs using surface marker expression (see Section 3.3.2).
8. Verify tBregs by performing functional assays, such as the ability to suppress the proliferation of TCR-activated T-cells (see Section 3.3.3) and to convert naive $\text{CD4}^+\text{CD25}^-$ into Foxp3^+ Treg cells (see Section 3.3.4 and note 31)^{22,24}.

3.6 Concluding remarks

Here we present a basic protocol sufficient to run a competitive Breg study in cancer. However, the outcome of the study may depend on a number of nuances primarily associated with Bregs in cancer. First, unlike Bregs/B cells involved in autoimmunity, signaling via toll-like receptors does not augment regulatory activity of tBregs, instead it can disable tBregs converting them to pathogenic B cells²². As a result, tBregs can be successfully inactivated in tumor-bearing mice by delivering immune-stimulatory CpGODN. Thus, if CM contains endotoxin or other TLR-activating ligands, the in vitro generation of tBregs would fail. Second, tBreg numbers are usually low and can be disguised within overwhelmingly expanded non-Breg (potentially-beneficial) B cells. Since tBregs only express low levels of CD20, they can be enriched in mice with 4T1 cancer by differentially depleting non-Breg B cells using CD20-targeting antibody. As a result, anti-CD20 Ab treatment increases metastasis and worsens survival of BALB/c mice with 4T1 cancer²². Third, large-sized tumors produce sufficient levels of cytotoxic factors to directly kill immune cells. As such, their progression may be less dependent on regulatory immune cells. Regulatory immune cells appear to be required to protect cancers at their vulnerable to immune attack state. For example, as we reported, tBregs induce a chain of suppressive responses protecting metastasizing cancer cells^{14,22,23}. Thus, the in vivo assessment of tBregs may be best to conduct at early stages of tumor progression. Lastly, the tBregs induction is dependent on a cancer type. To date, tBregs are found in two murine tumor models, such as orthotopic 4T1 breast adenocarcinoma (4T1 cancer) in BALB/c mice¹⁴ and spontaneous ovarian cancer in C57BL/6 mice (Bodogai & Biragyn, personal communication). At present, we can only speculate that other cancer Bregs may exist, and the protocol described here can be used to answer this question. On the other hand, in vitro-generated tBregs can also be utilized to understand immune regulation even if tumors do not naturally induce tBregs. For example, to evaluate tBreg-induced Tregs in mice bearing B16 melanoma by adoptively transferring congenic tBregs generated using CM of 4T1-PE cells,

³⁰Use B+BAFF as control B cells, although for human B cells BAFF can be omitted.

³¹If CD3^+ T cells (for both assays) are obtained from the same donor as for B cells, it is necessary to use an anti-human CD3 antibody to stimulate TCR. CD3 cells can be isolated at the same time as B cells and kept at 4°C for 48 h in cRPMI until use without significant loss of viability. However, CD3 cells from HLA-mismatched donors can also be used (a mixed-lymphocyte reaction). Then, we usually omit anti-CD3 Ab activation.

which enhance B16 melanoma progression via *in vivo* conversion of FoxP3⁺ Tregs and suppression of cytolytic effector cells^{22,23}. Although B-cell or/and T-cell deficient mice cannot support 4T1 cancer metastasis, the mice should have NK cells to see the metastasis-promoting role of tBregs and Tregs. Thus, instead of B-cell deficient μ MT mice, NOD/SCID mice deficient in B and T cells (but sufficient in NK cells) can also be used. Moreover, in NOD/SCID mice, to restore metastasis, tBregs need to be adoptively transferred together with non-Treg CD4⁺ T cells to convert them into FoxP3⁺ Tregs and thereby kill NK cells¹⁴. Thus, if NK cells impaired in B and T cell-deficient mice, 4T1 cancer would metastasize as good as in WT mice.

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Figure 1. Elution patterns of TARC-PE38 and endotoxin from an ion-exchange Q column
Using Q column, TARC-PE38 can be efficiently separated from endotoxin, as it is eluted earlier. Protein and endotoxin concentrations are determined with BCA and Endotoxin assay kits, respectively.

Figure 2. Characterization of tBregs

(A) Flow cytometry staining (dot plots) to phenotype tBregs within CD19⁺ B cells, as determined according to the forward scatter (FSC), side scatter (SSC), and expression of CD19, CD81, CD25 CD20 and phosphoStat3 (pStat3). (B) Western blot that shows pStat3 expression levels in lysates of tBregs or B+BAFF cells. (C) Functionally, tBregs are defined by their ability to suppress CD8⁺ and CD4⁺ T cell proliferation. tBregs (black line) or B +BAFF (grey dotted line) cells are mixed with CD3⁺ T cells at 1:1 ratio in the presence of mouse anti-CD3/CD28 activators beads. Activated T cells without B cells (grey line) and non-activated T cells (full grey) are used as positive and negative control for proliferation, respectively. (D) tBregs also convert Foxp3⁺ Tregs from naïve non Tregs CD4⁺CD25⁻ T cells. T cells are mixed with tBregs or B+BAFF at 1:2 ratio and stimulated with a bead-conjugated mouse anti-CD3/CD28 Abs and 500U/ml recombinant mouse IL-2.

Figure 3. Schema of adoptive transfer experiment to assess tBregs *in vivo*

Readout of the experiment is the ability to promote lung metastasis in B-cell deficient μ MT mice.