

Video Article

Ex vivo Expansion of Tumor-reactive T Cells by Means of Bryostatin 1/Ionomycin and the Common Gamma Chain Cytokines Formulation

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Abstract

It was reported that breast cancer patients have pre-existing immune responses against their tumors^{1,2}. However, such immune responses fail to provide complete protection against the development or recurrence of breast cancer. To overcome this problem by increasing the frequency of tumor-reactive T cells, adoptive immunotherapy has been employed. A variety of protocols have been used for the expansion of tumor-specific T cells. These protocols, however, are restricted to the use of tumor antigens *ex vivo* for the activation of antigen-specific T cells. Very recently, common gamma chain cytokines such as IL-2, IL-7, IL-15, and IL-21 have been used alone or in combination for the enhancement of anti-tumor immune responses³. However, it is not clear what formulation would work best for the expansion of tumor-reactive T cells. Here we present a protocol for the selective activation and expansion of tumor-reactive T cells from the FVBN202 transgenic mouse model of HER-2/neu positive breast carcinoma for use in adoptive T cell therapy of breast cancer. The protocol includes activation of T cells with bryostatin-1/ionomycin (B/I) and IL-2 in the absence of tumor antigens for 16 hours. B/I activation mimics intracellular signals that result in T cell activation by increasing protein kinase C activity and intracellular calcium, respectively⁴. This protocol specifically activates tumor-specific T cells while killing irrelevant T cells. The B/I-activated T cells are cultured with IL-7 and IL-15 for 24 hours and then pulsed with IL-2. After 24 hours, T cells are washed, split, and cultured with IL-7 + IL-15 for additional 4 days. Tumor-specificity and anti-tumor efficacy of the *ex vivo* expanded T cells is determined.

Protocol

1. Isolation of Lymphocytes⁵

1. Isolate tumor-draining lymph nodes or spleens from tumor-bearing FVBN202 transgenic mice and prepare single cell suspension in ice-cold RPMI1640 supplemented with 10% FBS. B/I activation in 50-ml polypropylene conical tubes results in a greater T cell yield compared to polystyrene tubes. Ketamine and Xylazine are injected *i.p.* for anesthesia. Cervical dislocation is used as a method of euthanasia.
2. Culture the cells (10^6 cells/ mL) in complete medium containing 15% FBS with bryostatin-1 (5 nM) and ionomycin (1 μ M) along with 80 U/ mL of IL-2 (Peprotech) for 16 h.
3. Wash the cells three times with warm medium (37°C) and culture at 10^6 cells/ mL in complete medium with IL-7 (10 ng/ mL) and IL-15 (10 ng/ mL) (Peprotech) for 24 h.
4. Pulse the cells with IL-2 (40 U/ mL) for 24 h.
5. Split the cells and culture them with IL-7 and IL-15 (10 ng/ mL) for 4 more days. Change medium and split the cells if needed every 2 days.

2. Determine Fold Expansion of T Cells by Cell Counts and Flow Cytometry Analysis⁵

1. Cell counts by light microscopy
 1. Prepare appropriate cell dilution (1:100) in trypan blue and add few μ L onto hemocytometer
 2. Count 9 squares and determine total cell number by dividing cell counts to the number of chambers multiplied by the dilution factor. The results will present number $\times 10^4$ cells/ mL.
2. Determine proportion of CD8+ and CD4+ T cells in the expanded cells by flow cytometry
 1. Block non-specific binding of antibodies to Fc receptors by culturing the cells with anti-CD16/CD32 antibody (Biolegend) for 20 min on ice and then wash the cells two times with 2 mL of ice-cold PBS supplemented with 1% sodium azide.
 2. Stain the cells by culturing with FITC-CD4 and PE-CD8 antibodies for 20 min on ice and then wash the cells two times with 2 mL of ice-cold PBS supplemented with 1% FBS and 0.1% sodium azide.
 3. Fix the cells with 1% paraformaldehyde and run samples on a Beckman Coulter FC 500 and analyze using Summit version 4.3 software.

3. Determine Tumor-specificity of the *ex Vivo* Expanded T Cells

1. Culture the *ex vivo* expanded lymphocytes in complete medium at a 10:1 ratio with irradiated neu positive MMC tumor cells (15,000 rad) for 24 h.⁵
2. Harvest supernatants and store at -80°C until used.^{5,6}
3. Detect IFN- γ using a Mouse IFN- γ ELISA Set (BD Pharmingen) according to the manufacturer's protocol.^{5,6}

4. Determine Anti-tumor Function of the *ex Vivo* Expanded T Cells^{5,6}

1. Incubate T cells with tumor cells in a 10:1 effector:target ratios for 48 hours in complete medium at 3 mL complete medium (RPMI-1640 supplemented with 100U/ mL of penicillin, 100µg/ mL streptomycin, 10% FBS, glutamine and β- mercaptoethanol) and 20U/ mL of IL-2 (Peprotech) in 6 well culture dishes 37°C /5% CO₂.
2. Perform three color antibody staining for neu (anti-c-Erb2/c-neu, clone-4, Calbiochem) followed by PE- anti mouse IgG, Annexin V-FITC and Propidium Iodide (PI) according to manufacturer's protocol (BD Pharmingen)
3. Gate on neu positive tumor cells and analyze viability (Annexin V-/PI-) of the tumor cells

5. Mouse Model of Breast Cancer

FVBN202 transgenic female mice (Charles River Laboratories) can be used for the source of tumor-reactive T cells. These mice overexpress an unactivated rat neu transgene under the regulation of MMTV promoter and as a result develop spontaneous mammary carcinoma between 4-10 months of age⁷. These mice develop premalignant mammary hyperplasia similar to ductal carcinoma *in situ* (DCIS) prior to the development of spontaneous carcinoma⁸. Spontaneous tumor-bearing mice are used as donors of T cells.

6. Representative Results:

Activation of T cells with B/I for 16 hours results in killing of naïve T cells that are not sensitized with the tumor *in vivo*. After the B/I selectivity of tumor-reactive T cells they expand up to 2.8-fold within a 6-day culture with the gamma chain cytokines (Figure 1). Both CD8+ and CD4+ T cells are equally expanded with the gamma chain cytokines (Figure 2). The *ex vivo*-expanded T cells show high responsiveness against the tumors that donor mice were sensitized to, as evaluated by the production of IFN-γ in the presence of neu positive mouse mammary carcinoma (MMC) tumor cells (Figure 3). The *ex vivo* expanded T cells can induce apoptosis in the neu positive MMC tumor cells such that viability of the tumor cells drops from 92% to 61% within 48 hours (Figure 4).

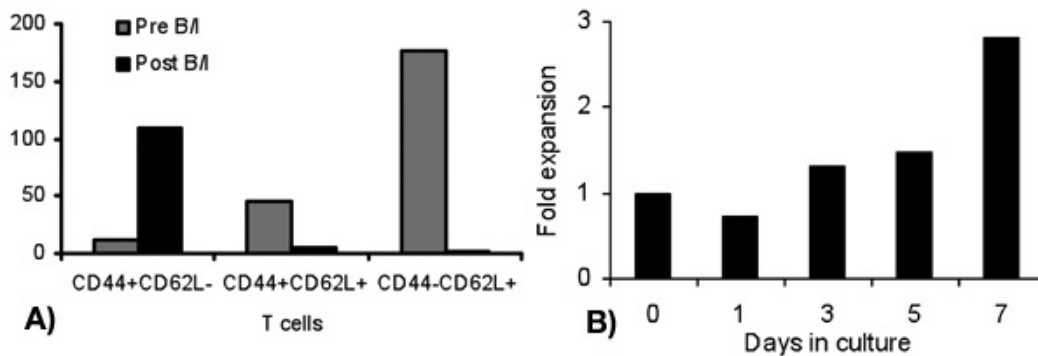


Figure 1. Fold expansion of lymphocytes at different time points following B/I activation (day 1) and *ex vivo* expansion with the gamma chain cytokines (days 3, 5, and 7)

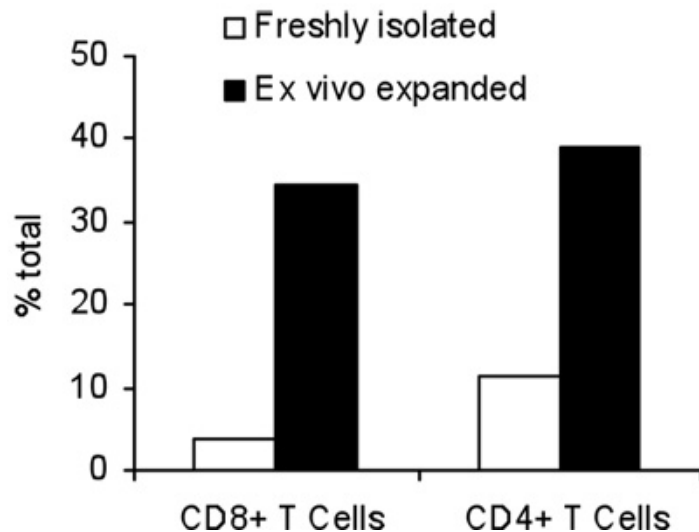


Figure 2. Total percentage of CD4+ and CD8+ T cells before and after a 7-day expansion with the gamma chain cytokines.

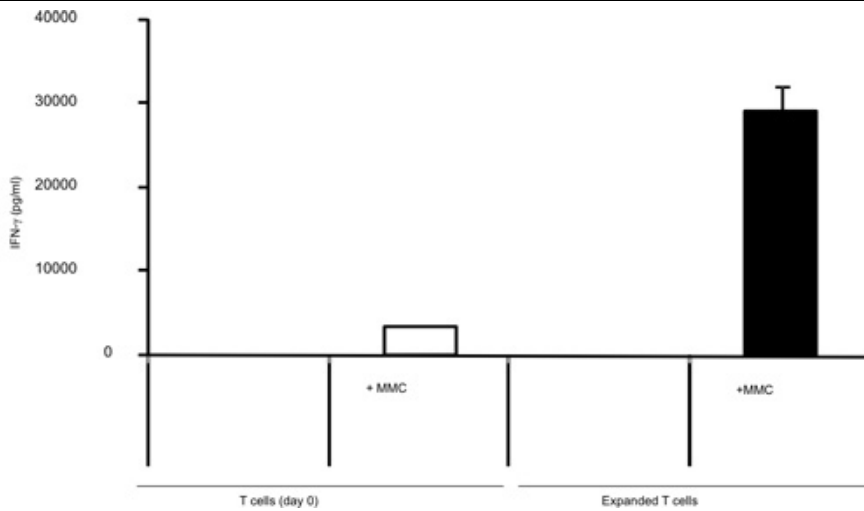


Figure 3. Tumor-stimulated IFN-γ production by T cells isolated from tumor-bearing mice prior to and after a 7-day expansion with the gamma chain cytokines, using IFN-γ ELISA

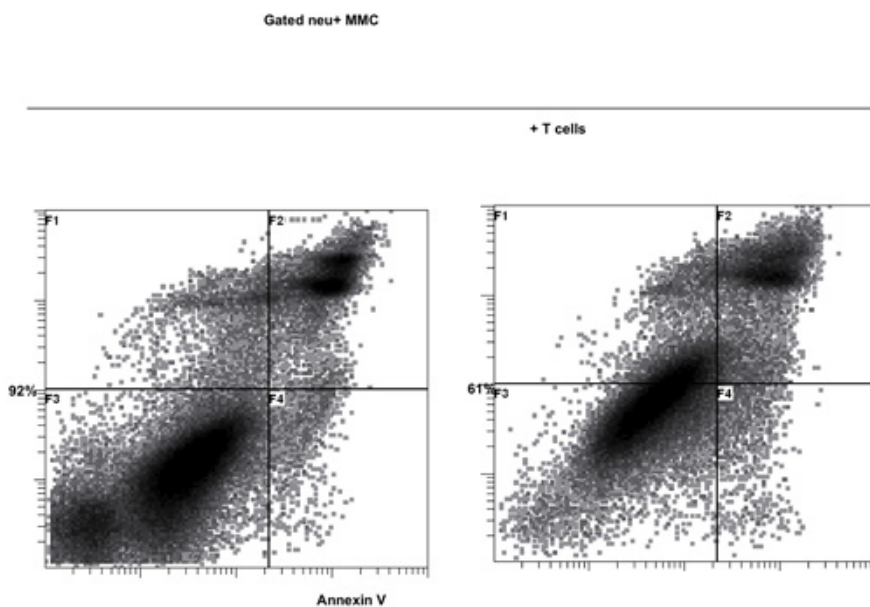


Figure 4. Cytotoxic function of the *ex vivo* expanded T cells with the gamma chain cytokines against neu positive mouse mammary carcinoma (MMC) tumor cells

Discussion

Selective expansion of tumor-reactive T cells with effector anti-tumor function can be achieved by the proposed protocol using B/I activation and *ex vivo* expansion with the gamma chain cytokines IL-2, IL-7 and IL-15. While IL-2 is a T cell growth factor that can support the differentiation and expansion of antigen-specific T cells, IL-7 can inhibit apoptosis of T cells and support their viability during expansion. IL-15 can support memory T cells that are important for generating long-term anti-tumor responses upon adoptive T cell therapy⁹⁻¹¹. Changing the order and combination of these cytokines could affect differentiation of the expanded T cells which in turn may improve or reduce their anti-tumor efficacy¹². The proposed protocol will not require the identification of tumor antigens. Selective expansion of tumor-reactive T cells results in the production of high numbers of anti-tumor T cells that can be used for adoptive T cell therapy of cancer patients. We have previously shown that the *ex vivo* expanded T cells protected animals against breast cancer following adoptive T cell therapy.

Disclosures

No conflicts of interest declared.

Acknowledgements

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