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 responses[3]. 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, respectively[4]. 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.

Video Link

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Protocol

1. Isolation of Lymphocytes[5]

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 μM) 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 Analysis[5]

1. Cell counts by light microscopy
    1. Prepare appropriate cell dilution (1:100) in trypan blue and add few μL 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 x 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% CO_2.
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 age[7]. 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 naíve 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).

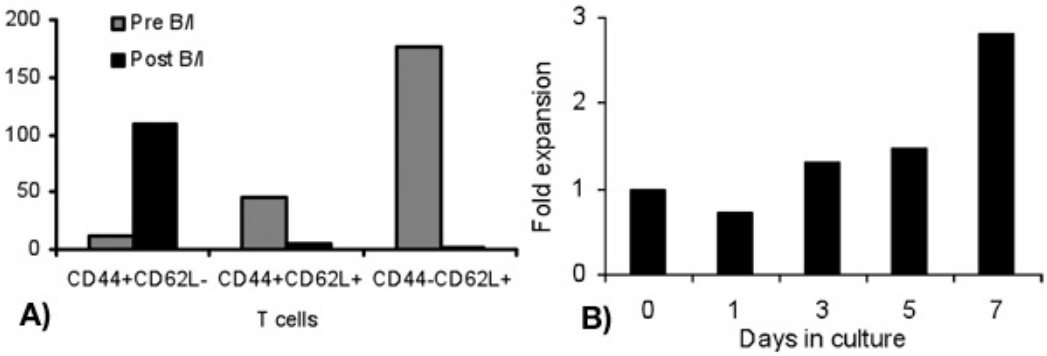

Figure 1. Fold expansion of lymphocytes at different time points following B/I activation (day 1) and *ex vivo* expansion with the gamma chain cytokines (days 3, 5, and 7)

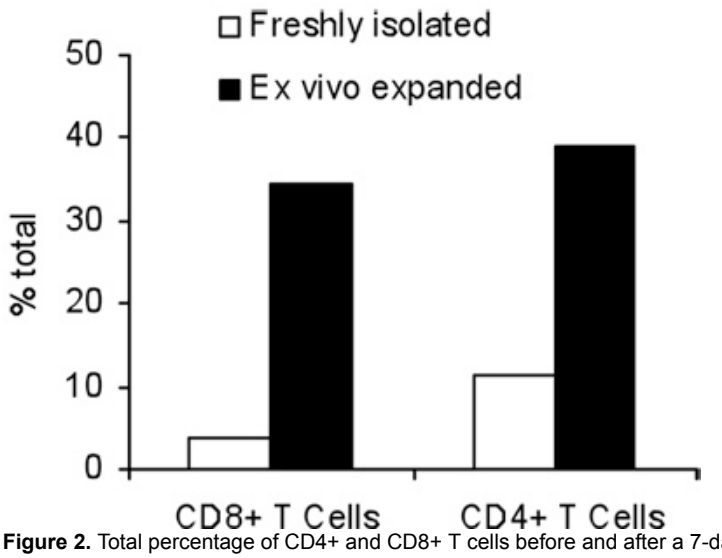

Figure 2. Total percentage of CD4+ and CD8+ T cells before and after a 7-day expansion with the gamma chain cytokines.

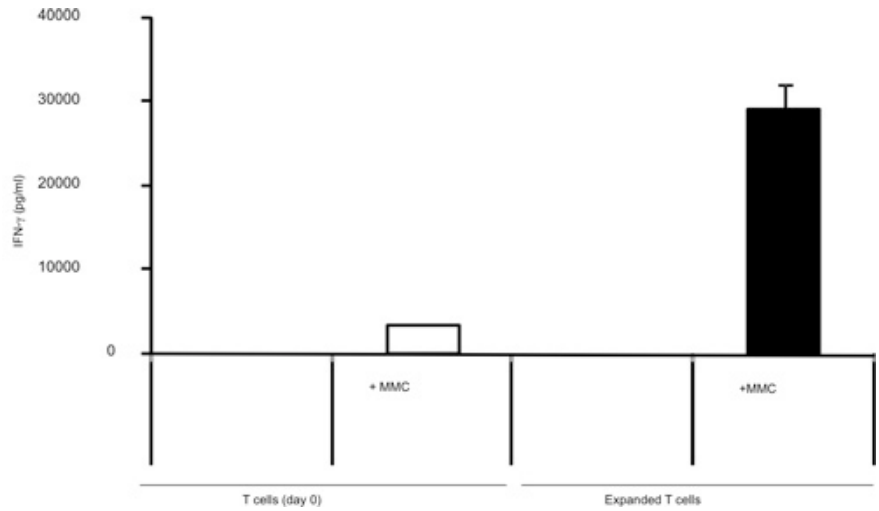

Figure 3. Tumor-stimulated IFN-γ production by T cells isolated from tumor-bearing mice prior to and after a 7-day expansion with the gamma chain cytokines, using IFN-γ ELISA

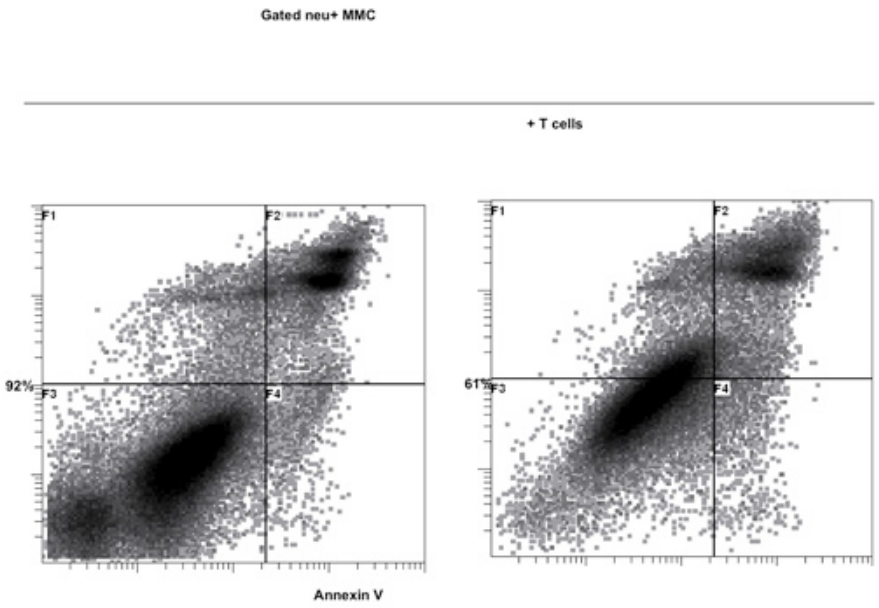

Figure 4. Cytotoxic function of the *ex vivo* expanded T cells with the gamma chain cytokines against neu positive mouse mammary carcinoma (MMC) tumor cells

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 therapy[9-11]. 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 efficacy[12]. 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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References

1. Goodell, V., Waisman, J., Salazar, L.G., de la Rosa, C., Link, J., Coveler, A.L., Childs, J.S., Fintak, P.A., Higgins, D.M. & Disis, M.L. Level of HER-2/neu protein expression in breast cancer may affect the development of endogenous HER-2/neu-specific immunity. Mol Cancer Ther. 7, 449-454 (2008).
2. Disis, M.L., Knutson, K.L., Schiffman, K., Rinn, K. & McNeel, D.G. Pre-existent immunity to the HER-2/neu oncogenic protein in patients with HER-2/neu overexpressing breast and ovarian cancer. Breast Cancer Res Treat. 62, 245-252 (2000).
3. Liu, S., Riley, J., Rosenberg, S. & Parkhurst, M. Comparison of common gamma-chain cytokines, interleukin-2, interleukin-7, and interleukin-15 for the *in vitro* generation of human tumor-reactive T lymphocytes for adoptive cell transfer therapy. J. Immunother. 29, 284-293 (2006).
4. Bear, H.D., Roberts, J., Cornell, D., Tombes, M.B., Kyle, B. Adoptive immunotherapy of cancer with pharmacologically activated lymph node lymphocytes: a pilot clinical trial. Cancer Immunol Immunother. 5, 269-274 (2001).
5. Morales, J.K., Kmieciak, M., Graham, L., Feldmesser, M., Bear, H.D. & Manjili, M.H. Adoptive transfer of HER2/neu-specific T cells expanded with alternating gamma chain cytokines mediate tumor regression when combined with the depletion of myeloid-derived suppressor cells. Cancer Immunol Immunother. 58, 941-953 (2009).
6. Cha, E., Graham, L., Manjili, M.H. & Bear H.D. IL-7 + IL-15 are superior to IL-2 for the *ex vivo* expansion of 4T1 mammary carcinoma-specific T cells with greater efficacy against tumors *in vivo*. Breast Cancer Res Treat. 2009 Oct 14. [Epub ahead of print]
7. Guy, C.T., Webster, M.A., Schaller, M., Parsons, T.J., Cardiff, R.D. & Muller W.J. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc. Natl. Acad. Sci. U. S. A. 89, 10578-10582 (1992).
8. Kmieciak, M., Morales, J.K., Morales, J., Bolesta, E., Grimes, M. & Manjili M.H. Danger signals and nonself entity of tumor antigen are both required for eliciting effective immune responses against HER-2/neu positive mammary carcinoma: implications for vaccine design. Cancer Immunol Immunother. 57, 1391-1398 (2008).
9. Stern, J.B. & Smith, K.A. Interleukin-2 induction of T-cell G1 progression and c-myb expression. Science. 233, 203-206 (1986).
10. Kittipatarin, C. & Khaled, A.R. *ex vivo* expansion of memory CD8 T cells from lymph nodes or spleen through *in vitro* culture with interleukin-7. J Immunol Methods. 344, 45-57 (2009).
11. Kokaji, A.I., Hockley, D.L. & Kane, K.P. IL-15 transpresentation augments CD8+ T cell activation and is required for optimal recall responses by central memory CD8+ T cells. J Immunol. 180, 4391-401 (2008).
12. Le, H.K., Graham, L., Miller, C.H., Kmieciak, M., Manjili M.H. & Bear, H.D. Incubation of antigen-sensitized T lymphocytes activated with bryostatin 1 + ionomycin in IL-7 + IL-15 increases yield of cells capable of inducing regression of melanoma metastases compared to culture in IL-2. Cancer Immunol Immunother. 58, 1565-1576 (2009).

