A Comparison of Three Techniques to Induce Efficient *Ex Vivo* T Cell Expansion

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INTRODUCTION

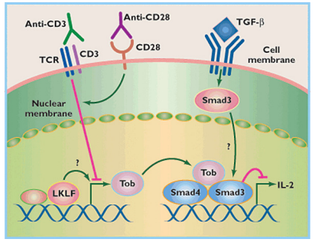
Much of our current knowledge of the immune system derives from the ability to initiate an *ex vivo* immune response in isolated T lymphocytes. This technology is particularly useful for functional testing of cytotoxic T cells relating to new peptide antigens for vaccine development or T cell immunotherapy. Expanded T cells can also be used in drug development to study the effect of candidate drugs on immune function.

The current study was undertaken to evaluate three common methods of inducing proliferation in a T lymphocyte population. In the first method cells are treated with purified monoclonal mouse anti-human CD3 antibody followed by addition of Interleukin-2. The second approach involves treatment with the mitogen Concanavalin A. The third technique makes use of monoclonal mouse antibodies against human CD3 and CD28 immobilized on the surface of superparamagnetic polystyrene-coated microspheres. Cell proliferation was monitored using CellTrace™ Violet, a novel cell proliferation tracing dye with a mode of action similar to carboxyfluorescein diacetate succinimidyl ester (CFSE).

MATERIALS AND METHODS

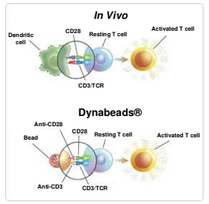
Human peripheral blood mononuclear cells were isolated from fresh whole blood using a centrifuge density gradient (Ficoll-Paque® Plus, GE Healthcare). Cells were resuspended in Dulbecco's phosphate-buffered saline (GIBCO), stained 20 minutes with 1 µM CellTrace™ Violet (Invitrogen), quenched five minutes with the addition of one volume of Fetal Bovine Serum, and resuspended in OpTmizer™ T-Cell Expansion SFM (GIBCO) supplemented with 2 mM L-glutamine (Invitrogen) and Penicillin-Streptomycin Solution (Sigma). Stained cells were aliquoted into a six well plate and treated with one of three stimulation techniques. One aliquot was treated with 200 ng mouse anti-human CD3 (Invitrogen, clone S4.1) and 100 ng IL-2 (Invitrogen, cat. no. PHC0026). A second aliquot was stimulated with 5 µg/ml Concanavalin A (Sigma). The third aliquot received 75 µl CD3/CD28 T cell expansion beads (3 beads per cell), while a fourth aliquot was reserved as an unstimulated control. Cells were incubated at 37°C and 5% CO_2 for 7 days. SYTOX® Red, a cell impermeant nucleic acid dye, was used to evaluate the effect of each treatment on cell viability. The specific subpopulations induced to proliferate with each treatment were characterized in multicolor flow cytometry using monoclonal antibody conjugates against human CD3, CD4, and CD8.

Figure 1. T Cell Activation by Anti-CD3 and IL-2



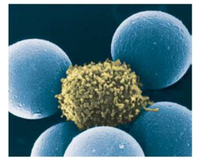
Antigen-presenting cells activate T lymphocytes by simultaneous binding and stimulation of the T cell receptor (CD3) and costimulatory complexes, such as CD28. This binding activates a pathway which triggers the production of cytokines such as IL-2 and eventually leads to T cell proliferation. IL-2 then stimulates the growth and differentiation of cytotoxic T cells.

Figure 2. Dynabeads® mimic *in vivo* T cell activation



In vivo, antigen presenting cells bind to CD3 (T cell receptor) and CD28 on the surface of resting T cells, activating them and initiating an immune response.

Figure 3. Micrograph of Dynabeads®

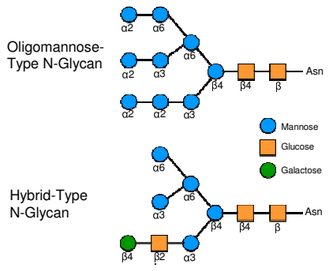


The cell-sized Dynabeads® (4.5 µm in diameter) are coated with mouse anti-human CD3 and CD28 monoclonal antibodies to mimic the effects of antigen presenting cells, stimulating cultured T cells to proliferate.

Figure 4. Structure of Concanavalin A

The mitogen Concanavalin A is perhaps the most widely used lectin and can be used as an alternative T cell stimulus. This lectin is frequently used as a surrogate for antigen-presenting cells in T cell stimulation experiments. Concanavalin A irreversibly binds to glycoproteins on the cell surface and commits cells to proliferation.

Figure 5. Cell-surface binding specificity of Concanavalin A



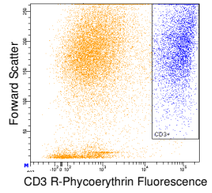
The lectin Concanavalin A, isolated from the Jack-bean plant Canavalia ensiformis, is an α-mannose/ α-glucose-binding lectin. On animal cell-surface glycoproteins, it is known to bind N-glycans, specifically oligomannose- and hybrid-type N-glycans with high affinity.

RESULTS

Figure 6. Live Cell Discrimination

The cell impermeant nucleic acid stain SYTOX® Red was applied to all samples to limit analysis to live cells.

Figure 7. T Cell Identification



A mouse anti-human CD3 monoclonal antibody conjugated to R-Phycoerythrin was used to further limit analysis to live, CD3+ T cells.

Figure 8. Flow Cytometric Analysis after Seven Day Stimulation

Cells were analyzed on a Becton Dickinson LSRII™ flow cytometer after seven days of incubation using a gating strategy to limit analysis to live CD3+ T Cells. CellTrace Violet Fluorescence was collected with 405 nm excitation and a 450/50 bandpass emission filter. The figures below illustrate two different ways of visualizing the same data set. The four figures on the left are histograms of fluorescence intensity – each peak represents one generation of proliferating cells. Figures on the right were generated using proliferation modeling software (ModFit LT™, Verity Software House). The location of each generation of cells is represented by a unique peak color.

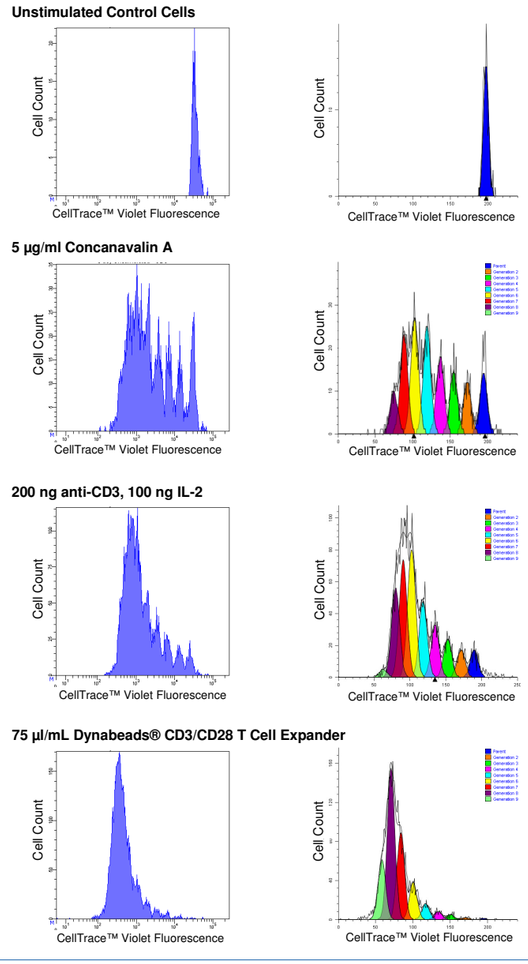


Figure 9. Cell Status Post-Stimulation

Stimulus	Viability Post-Expansion	Proliferation Index	Nonproliferative Fraction
Control	80.0%	1.00	100%
Concanavalin A	64.7%	8.27	40%
Anti-CD3 / IL-2	75.4%	4.95	49%
Dynabeads®	76.2%	41.96	8%

Cells treated with Concanavalin A or the combination of mouse anti-human CD3 and IL-2 showed a moderate, heterogeneous level of stimulation, with some cells undergoing nine cell divisions, others remaining senescent, and approximately equal amounts remaining in generations one through eight. The microsphere-bound antibodies on Dynabeads® provided a much stronger, more uniform stimulus as evidenced by the very high proliferation index of cells. Concanavalin A treatment was mildly cytotoxic, while viability of cells treated with free or bound antibodies was similar to unstimulated cells. CellTrace™ Violet permitted the visualization of distinct generations of cells in fluorescence histograms. Proliferation modeling software (ModFit LT™), was used to calculate the proliferation index (the average number of cells that each original cell became) and nonproliferative fraction (percent of cells that did not proliferate) for each treatment.

CONCLUSIONS

The strong, homogeneous stimulus provided by superparamagnetic polystyrene expansion beads is useful when massive numerical expansion of T lymphocytes is desired for subsequent research or adoptive transfer *in vivo*, but does not appear to be as useful when a population containing several discrete generations of cells is desired. The more moderate stimuli resulting from treatment with mouse anti-human CD3/IL-2 or Concanavalin A result in a less efficient numerical expansion, but provide an excellent tool to evaluate the phenotype and pedigree of cells as they proliferate through several generations. CellTrace™ Violet proved extremely useful for accurately tracking the proliferation of stimulated cells.

REFERENCES

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ACKNOWLEDGEMENTS

Thanks to Drew Bantly from The University of Pennsylvania for his advice and feedback.

TRADEMARKS