Optimizing the Cryopreservation of Murine Splenocytes for Improved Antigen-Specific T Cell Function in ELISPOT

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Introduction

- ELISPOT assays are routinely used to measure immune responses of T cells in fresh and frozen splenocytes preparations. However, standardized methods for murine ELISPOT are not widely available. Freezing cells can significantly impact the function of T cells [1].
- The ability of cryopreserved splenocytes to retain antigen specific T cell function at levels similar to freshly isolated splenocytes greatly facilitates the studies of immune based therapies.
- We questioned whether an optimized cryopreservation protocol could be developed that would retain antigen specific T cell function to the same level of fresh ELISPOT after freezing splenocytes.
- To address the above question we studied the impact of freezing media, the temperature of media with thawing, and necessity of resting on cell viability

Methods

Mice: A TgMMTV-neu transgenic mouse colony was established and maintained as per protocol in our animal facilities. Breeding pairs obtained from The Jackson Laboratory (Bar Harbor, ME).

Vaccination: Mice were either immunized s.c. with 50 μg of three IGF-IR peptides, previously shown to be immunogenic by ELISPOT analysis (2) as a mixture in CFA for the first vaccine or IFA for all subsequent vaccinations, or adjuvant alone. Four immunizations were given 2 weeks apart. Two weeks after the last vaccination, mice were euthanized and spleens were collected in RPMI 1640.

Cryopreservation: Mouse splenocytes were cryopreserved using five different media: Medium 1 (50% X-Vivo media, 40% FBS, 10% DMSO), Medium 2 (25% RPMI 1640, 65% FBS, 10% DMSO), Medium 3 (90% FBS, 10% DMSO), Medium 4 (Amresco Media) and Medium 5 (EZ-CP2 Media).

ELISPOT Assay: Briefly, as previously described (2) 96-well FVDF plates were coated overnight at 4°C with 5 μg/ml anti-IFN-γ. The plates were washed 3 times with PBS and blocked with 2% bovine serum albumin for 2 h at 37°C. 3 × 10^5 splenocytes were plated in each well and were stimulated by different antigens: IGF-IR peptide mix (10 μg/ml each), HIV peptide (1μg/ml) as a negative control, either PMA, Con A, or PMA as positive controls, or media (no antigen). The plates were incubated at 37°C and 5% CO2 for 72 h or 96h depending on experimental conditions, and washed three times with PBS. Biotinylated anti-mouse IFNγ was used at 4°C overnight. After washing three times with PBS, streptavidin-horseradish peroxidase was added for 45 minute at room temperature. After another washing step with PBS, the plates were incubated with substrate for up to 20 minutes. Color development was stopped by rinsing the plate under running tap water. After drying overnight at room temperature, colored spots were counted using an AID ELISPOT High Resolution reader system and AID ELISPOT Software version 4.0. Corrected spots per well were calculated by subtracting the average of the no antigen wells from the average response in the antigen or mitogen stimulated wells. All plates were run with 4-6 replicates wells per stimulation.

Statistical Analysis: Statistical analysis was performed using GraphPad Prism version 5.04. All significance was considered at p<0.05. Comparison of a single variable between more than 2 groups was done by One-Way ANOVA with Tukey’s post-test. Repeated measures analysis was performed whenever appropriate. Two-Way ANOVA with Bonferroni’s post-test was performed in all ELISPOT grouped analysis. Comparison of two groups only was performed using a student's t-test.

Conclusions

- There was a significant reduction in the viability of the cells after thawing in 4°C media.
- Freezing times, time of splenocytes preparation, and cell resting did not impact the viability of cells.
- The method of cryopreservation can have a tremendous impact upon the viability and function of splenocytes.
- Freezing media with high percentage of FBS performed significantly better than commercial preparations of Amresco and EZ-CP2.
- By using an optimized cryopreservation protocol, it is possible to obtain antigen-specific T cell function at levels similar to freshly isolated cells.

References