Flow Cytometric Evaluation of Granzyme B May More Accurately Assess Effector Cell Function in CD8 Positive T Cells

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ABSTRACT

Cytotoxic effector lymphocytes (CTL) are the premier line of defense in viral infection or tumor infiltration. Granzyme B is the main weapon in the CTL arsenal. Granzyme B is a serine protease found primarily in natural killer (NK) cells and CTL and it acts by means of inducing apoptosis in target cells. Contact between CTL and target cells results in directed exocytosis of the CTL granules and penetrance into target by means perforin-polymerized target-cell pores. Studies suggest monitoring levels of granzyme B expression can indicate acute rejection episodes in patients that have received solid organ allografts. Our findings indicate a sharp increase in the percentage and amount of granzyme B in both CTL and NK following 48-hour in vitro activation with Staphylococcal enterotoxin B (SEB). Peripheral blood from healthy subjects was found to be activated in all subjects tested, as measured by characteristic 'blast' light scatter, expression of CD25 and production of interferon gamma. These results correlated with a demonstrable increase in granzyme B expression in these donors most notably in the CD8 T cell populations where mean fluorescence intensity of the granzyme B molecule increased from an average of 18 in control cultures to 166 in SEB-activated cultures. Simultaneous measurement of perforin in activated cells showed minimal changes in either the NK or CTL compartment and was overall significantly less than changes measured by granzyme B ($p < 0.004$ and $p < 0.04$ respectively). Overall data indicate that measurement of granzyme B may be a more accurate assessment of cytotoxic T cell and NK activation, and thus more indicative of an acute cytotoxic response.

INTRODUCTION

Cytotoxic T lymphocytes are in essence 'trained killers' whose premier function is to protect the host from invasion by pathogens and tumor cells. CTL pursue target cells following TCR recognition of MHC-peptide antigen complexes. Repeat encounters with target cells end in the emergence of an armed, cytotoxic effector cell population that activates distinct mechanisms of cell lysis ending in cell death by apoptosis. CTL initiate cell lysis by the release of the cytopathic proteins granzyme B and perforin contained within cytoplasmic granules.

Perforin is a 70 kD glycoprotein that is homologous to complement C9-related protein. In the presence of calcium, perforin polymerizes into transmembrane tubules. It is considered to facilitate the entry of serine proteases into the target cell and is also believed to be capable of lysing non-specifically a variety of target cells.

Granzyme B is a serine protease found in the lymphocyte granules of activated cytotoxic T cells (CTL) and natural killer (NK) cells. It is one of several homologous serine proteinases called granzymes. Expression of these proteins are reported to correlate with the cytolytic potential of cytotoxic lymphocytes. Granzyme B specifically, is critically involved in the rapid apoptotic signal delivered by CTL and NK cells. Following adhesion of the cytotoxic cell to the target, granzyme B is directly secreted and enters the target cell, assisted by perforin. Recently, the discovery of a receptor for granzyme B has indicated that granzymes might be taken up by receptor-mediated endocytosis and that perforin acts to release granzymes that are sequestered in endosomes into the cytosol of the target cell.

Granzyme B is necessary for target cell lysis in cell-mediated immune responses. The enzymatic activity of granzyme B is considered essential to its ability to induce cell death through the activation of caspases, but the exact mechanism remains unclear.

The primary application for monitoring granzyme B expression may be its ability to "predict" acute rejection following allogeneic transplant. Accurate diagnosis of rejection episodes in patients following stem cell or solid organ transplant is critical for clinical management. Data indicate that expansion of granzyme B-positive cells coincides with a temporary increase in cytotoxic T cell populations. Elevated expression of granzyme B may be useful as a diagnostic marker in transplant rejection and viral infection associated with immunosuppressive therapy concordant with allograft. Improvement in patient monitoring following transplantation will allow enhanced implementation of rescue therapies with improved patient outcome.

SCHEMATIC: TARGETED CELL LYSIS

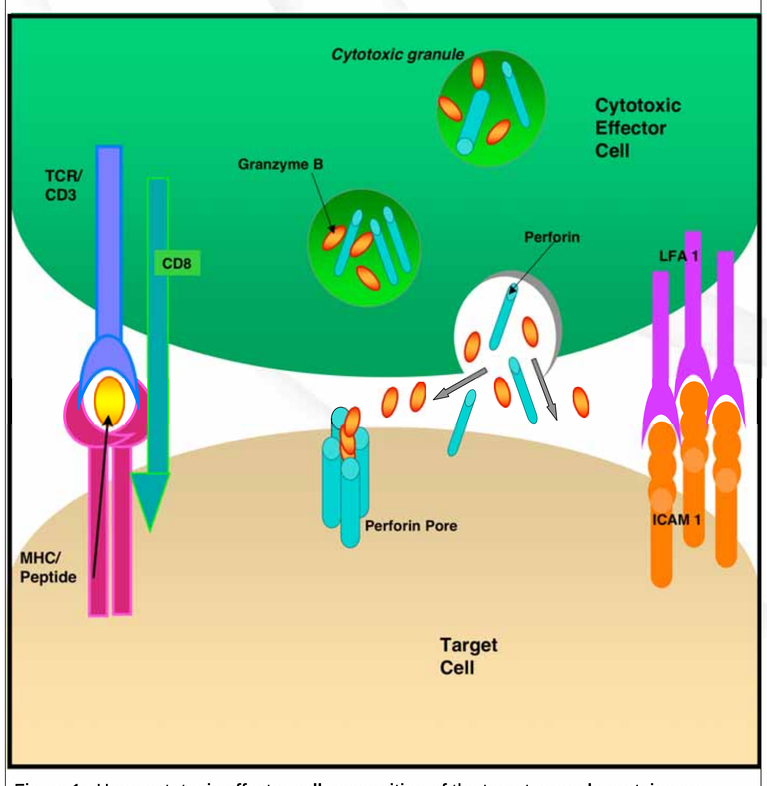


Figure 1: Upon cytotoxic effector cell recognition of the target, granule proteins are subsequently released. Granzyme B then initiates caspase-mediated apoptosis within the target cell.

METHOD

Peripheral blood mononuclear cells (PBMC) from 15 healthy subjects were isolated by Ficoll-Paque® according to manufacturer's recommendations. Cells were counted and cultured in 24-well plates with RPMI complete medium with 10% fetal calf serum according to the following conditions: Control, medium only; activated 1: medium with Staphylococcal Enterotoxin B (SEB) and antiCD28 antibody (CD28); activated 2: medium with antiCD3 conjugated to aminodextran and CD28. Cell cultures were then incubated at 37°C, 5% CO2, 95% humidity, for times stated.

After stimulation cells were harvested, washed and stained with monoclonal antibody combinations. Intracellular markers were stained following cell fixation/permeabilization with IntraPrep™ according to manufacturer's recommendations. Samples stained for cytokine expression were first incubated with Brefeldin A (10µg/mL) six hours prior to harvest. Isotype control antibodies were used to set instrument cursors.

The table below shows the panel of antibody reagents used. Cells were analyzed on a Beckman Coulter EPICS® XL-MCL™ equipped with a 488-argon laser. Data were analyzed using the EXPO32™ADC software.

DYE	REAGENT COMBINATION 1	REAGENT COMBINATION 2	REAGENT COMBINATION 3
FITC	PERFORIN	CD4	PERFORIN
PE	GRANZYME B	INTERFERON gamma	GRANZYME B
PC5	CD8	CD8	CD16
PC7	CD3	CD3	CD8

RESULTS

CONTROL		18 Hr. ACT. (SEB)	
NK Cells	CD8+ T Cells	NK Cells	CD8+ T Cells
26.4	6.2	30.2	41.3

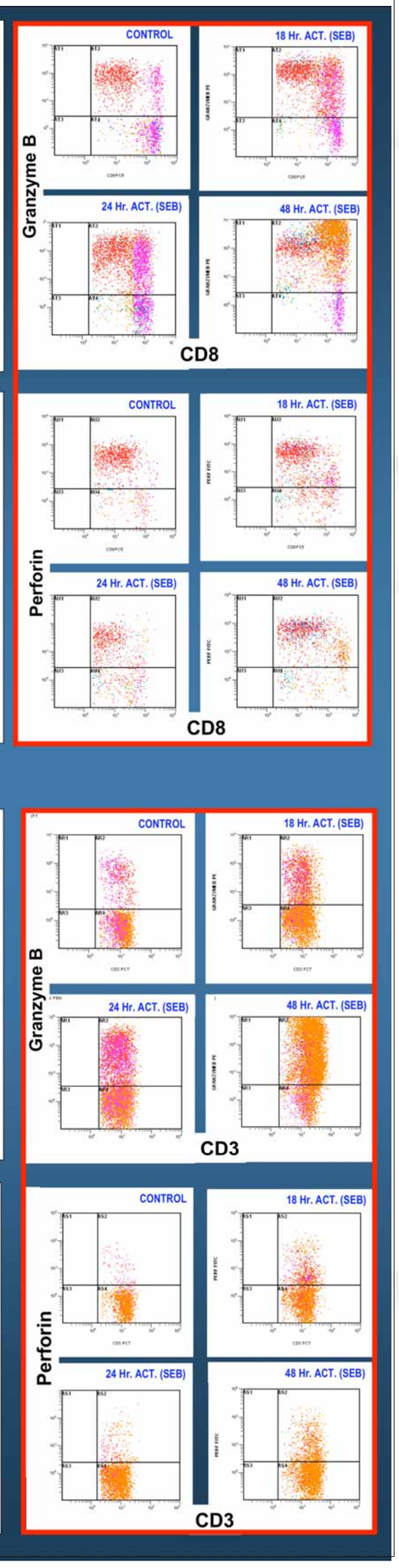
24 Hr. ACT. (SEB)		48 Hr. ACT. (SEB)	
NK Cells	CD8+ T Cells	NK Cells	CD8+ T Cells
25	41.4	24.78	60.72

Figure 1a. Flow cytometric histograms from a representative donor: Changes in co-expression with Granzyme B after in vitro activation.

CONTROL		18 Hr. ACT. (SEB)	
NK Cells	CD8+ T Cells	NK Cells	CD8+ T Cells
24.4	4.2	28.8	6.7

24 Hr. ACT. (SEB)		48 Hr. ACT. (SEB)	
NK Cells	CD8+ T Cells	NK Cells	CD8+ T Cells
11.9	2	20.3	4.8

Figure 1b. Flow cytometric histograms from a representative donor: Changes in co-expression with Perforin after in vitro activation.



CONTROL	18 Hr. ACT. (SEB)
CD3+ T Cells	CD3+ T Cells
8.8	28.7

24 Hr. ACT. (SEB)	43 Hr. ACT. (SEB)
CD3+ T Cells	CD3+ T Cells
34.58	76.6

Figure 2a. Flow cytometric histograms from a representative donor: Changes in co-expression with Granzyme B after in vitro activation.

CONTROL	18 Hr. ACT. (SEB)
CD3+ T Cells	CD3+ T Cells
1.43	10.4

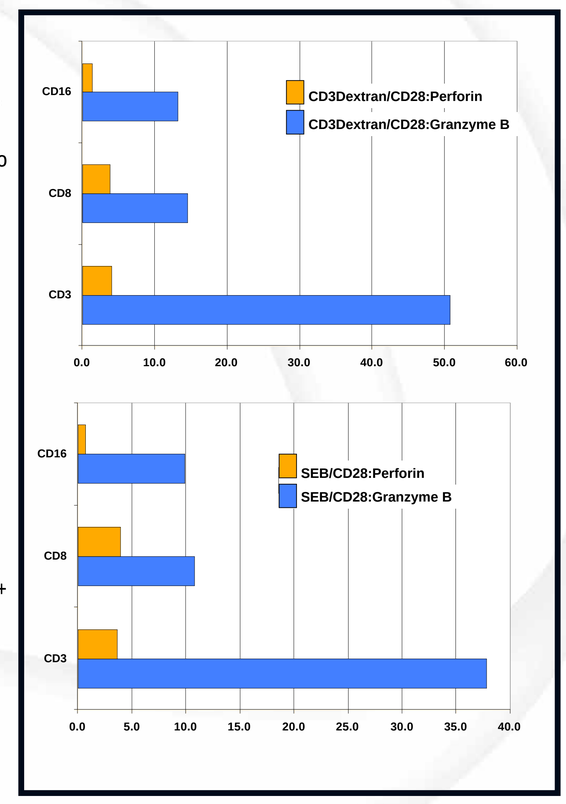
24 Hr. ACT. (SEB)	43 Hr. ACT. (SEB)
CD3+ T Cells	CD3+ T Cells
2.93	8.75

Figure 2b. Flow cytometric histograms from a representative donor: Changes in co-expression with Perforin after in vitro activation.

RESULTS

Figure 3. Expression (%+) of Granzyme B or Perforin after in vitro activation (with either CD3Dextran/CD28, or SEB/CD28); summarized results from 15 donors.

Fluorescent intensity (MFI) increased accordingly, reaching maximum levels after 48 hr. in vitro activation: with avg. MFI of Granzyme B in CD3+ cells = 142 and avg. MFI of Perforin in CD3+ cells = 39.



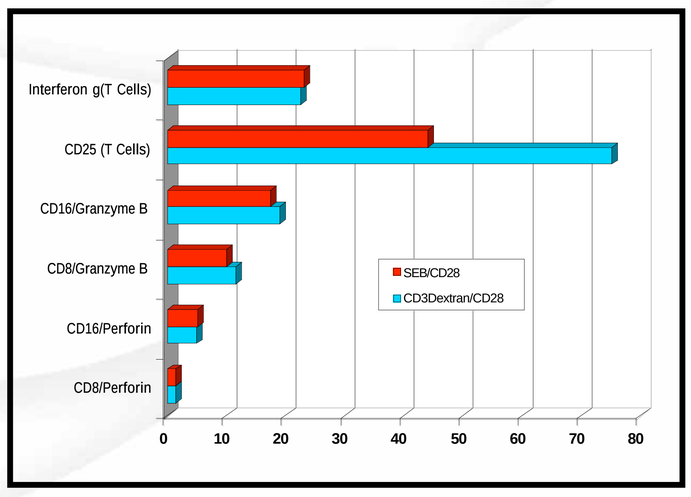


Figure 4.

Expression (%+) of Granzyme B or Perforin after in vitro activation. Comparison to CD25, Interferon gamma expression in T cells.

CONCLUSIONS

- *Recognition of changes in host blood cell populations in reaction to allograft rejection or viral infection is critical to clinical immune monitoring post transplantation.*
- *Effector cell populations in cases of solid organ transplant have been proven to be directly associated with rejection. Accurate measurement of these populations could have direct impact on treatment decisions related to immunosuppressive medication.*
- *Monitoring differences in perforin and granzyme B expression may provide improved understanding of the fundamental biology behind the immune system's targeted elimination of pathogenic cells.*

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