## Human and monkey Perforin ELISpot

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Perforin is a hallmark effector molecule of cell-mediated destruction of target cells. Perforin can permeabilize target cells directly as well as facilitate the delivery of granzymes which in turn induce apoptosis (Fig.1). A number of granzymes are involved in the induction of apoptosis whereof granzyme B is thought to be one of the most important. There is an increasing interest in methods enabling the measurement of the release of these molecules in studies of e.g. infectious diseases and for the development of vaccines targeting the cellular immune system.

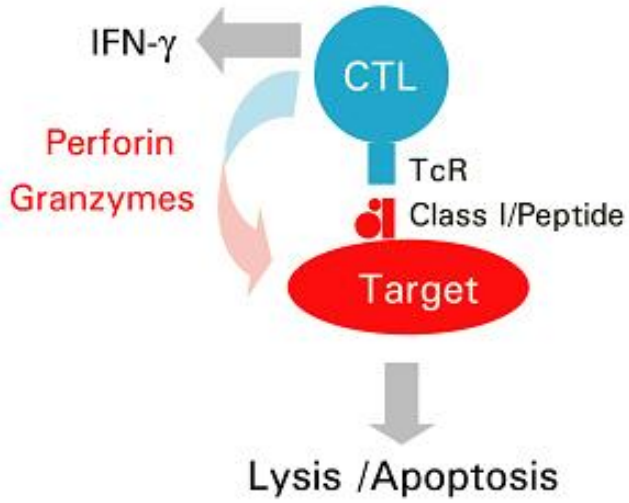


Fig.1. Cytotoxic T cells that recognize MHC-I + peptide release Perforin, granzymes and cytokines, such as IFN-γ.

Antigen-induced IFN-g secretion by cytotoxic T cells (CTLs) is often determined by ELISpot and used as an immunological correlate of cytotoxic activity. Since perforin and granzyme B are more directly involved in cytotoxicity, the analysis of these molecules can serve as a complement to IFN-γ ELISpot. Antigen-activated CD8+ T cells can display a dissociated release of granzyme B, perforin and IFN- γ i.e. a cell can express all three molecules but may also express one or two of these molecules (Kelso et al., 2002, Kleen et al., 2004). Perforin and granzyme B ELISpot assays can thus provide the tools necessary for establishing the relation between the release of these different molecules and the CTL activity.

A human granzyme B ELISpot assay was described a few years ago (Rininsland et al., 2000, Shafer-Weaver et al., 2003) whereas the perforin ELISpot was developed recently (Zuber et al., 2005). Initial results using the perforin ELISpot have shown that antigen-specific CD8+ T-cell responses can be detected after *ex vivo* stimulation of peripheral blood mononuclear cells (PBMC). However, limited amounts of perforin appear to be released form each cell which results in spots of limited size and thus requires optimized ELISpot protocols in order

to achieve good results. Potential applications for the perforin ELISpot and recommendations for optimization of the assay protocol will be discussed in this letter.

**The perforin ELISpot assay**

The perforin ELISpot assay was developed in order to meet the needs for a capture immunoassay that in a simple and direct way can be used to quantify the release of perforin by e.g. cytotoxic T cells in response to antigenic stimuli. The performance of the perforin ELISpot was initially evaluated using the YT cell line (human T/NK leukaemia cell) which is known to secrete both perforin and granzyme B. The perforin spots obtained using YT cells resembled the spots obtained in other highly sensitive ELISpot assays with regard to intensity and the number of spots obtained closely matched the number of input cells per well i.e. most cells put in to a well generated a spot.

**Applications for perforin ELISpot**

***Ex vivo* ELISpot analysis of human CD8+ T-cell responses to viral peptides**

To analyze perforin release induced in response to antigen stimulation, we used a mix of class I-restricted peptides, known to be frequently recognized by human CD8+ T cells, derived from Cytomegalovirus, Epstein-Barr virus and Influenza virus (CEF peptide pool) (Currier et al., 2002). Human PBMCs from healthy blood donors were stimulated with the CEF peptide pool and analyzed by perforin, granzyme B and IFN- γ ELISpot. Out of fifteen donors, five responded to CEF with increased PFN release, these donors also responded with IFN- γ and granzyme B to CEF.

**Vaccine-induced T-cell responses in monkeys**

The human perforin ELISpot (but not the granzyme B ELISpot) cross-reacts with perforin from cynomolgus and rhesus macaques making it possible to use the assay for animal studies of e.g. SIV vaccination and infection. Pilot data from a study comparing perforin and IFN- γ ELISpot in cynomolgus macaques immunized with DNA-MVA constructs containing SIV/HIV genes showed an antigen-specific induction of both perforin and IFN- γ. Interestingly, preliminary data obtained by cell depletion experiments suggest that both CD4+ and CD8+ responded by IFN- γ production whereas only CD8+ cells released perforin. In this system, it thus seems like perforin release could be a better marker for cytotoxic activity.

**Studies of Cytotoxic T-cell clones**

The $^{51}$chromium release assay is well established for measurement of cytotoxicity but the assay has a number of disadvantages such as being time consuming and labour intensive and requiring generation and labeling of target cells. Although the perforin and granzyme B ELISpots do not measure killing of cells directly, the results obtained correlate well with the results from $^{51}$Cr release experiments (Zuber et al., 2005, Shafer-Weaver et al., 2004, Shafer-Weaver et al., 2003). The Grz-B ELISpot has also been shown to correlate to the number of effector cells expressing the degranulation marker CD107a after interaction with target cells (Shafer-Weaver et al., 2004).

**The Perforin ELISpot – notes for optimization**

Perforin in cytotoxic cells is normally expressed and released in lower amounts compared to Granzyme-B and IFN-γ. As a consequence, in the ELISpot assay, Perforin spots tend to be smaller and less intense. Therefore, for best results, it is crucial to use this test under optimal conditions.

To define the best conditions for the assay, a number of parameters which we know are critical also to other ELISpot assays were investigated:

**Antibodies:**

Several different Perforin specific monoclonal antibodies, developed by Mabtech or obtained from other sources were evaluated to find optimal combinations and concentrations.

**Plates:**
Filter plates from Millipore Corp. USA with either PVDF (MSIP, ELIIP) or nitrocellulose (MAHA) membranes were tested. For the PVDF plates, which require preactivation with ethanol for optimal binding, different preactivation protocols were compared.

**Substrates:**
Substrates from different suppliers were compared. BCIP/NBT-plus (Moss Inc. USA) was used for alkaline phosphatase and TMB, AEC (both from Moss Inc. USA) and NovaRed (Vector Laboratories Ltd. UK) for horseradish peroxidase.

**Results**

**Antibodies:**
-Optimal results were obtained by coating over night (+4-8˚ C) with Pf-80/164 which represents a mixture of two monoclonal antibodies. The antibodies were used at a concentration of 30 µg/ml (i.e. 15 µg/ml of each antibody) in sterile PBS and 100µl/well.

**Plates:**
- Good and consistent results were obtained with ELIIP plates that had been preactivated with 50 µl/well of 70% ethanol for 2 min before coating with antibody (Fig.2).
- We also got good results using MSIP plates but the preactivation step with ethanol is here more critical and it is essential to use a lower percentage of ethanol (35%), a smaller volume (15 µl/well) and not incubate for more than one (1) minute,
- The omission of ethanol treatment of ELIIP or MSIP plates resulted in poor spot quality with greyish and blurry spots.
- The use of nitrocellulose plates (MAHA) is not recommended, since few or no Perforin spots were detected using these plates.

**Substrates:**

BCIP/NBT-plus was the overall best substrate considering spot size, intensity and morphology. For HRP-conjugate, TMB worked better than AEC and NovaRed which both resulted in fewer and weaker spots.
The Perforin spots also demand longer incubation with the substrate than e.g. human IFN-γ spots. Development time is best established by monitoring the substrate reaction in a dissection microscope (spots may be difficult to see with the naked eye) but was typically 40-50 minutes with both BCIP/NBT-plus and TMB.

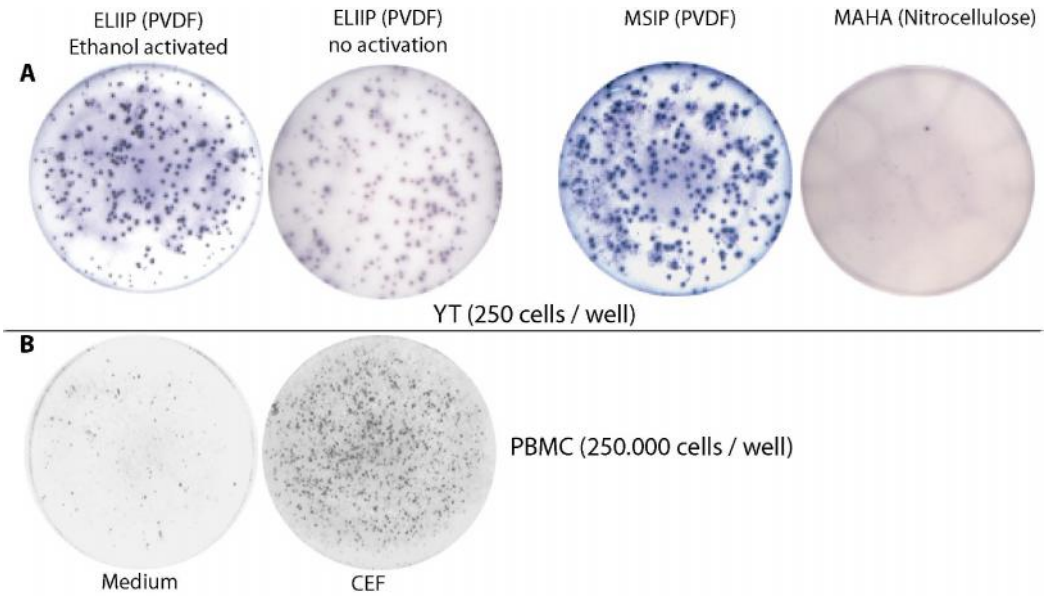


Fig.2. A: Comparison of different plates in the Perforin ELISpot assay using the YT lymphoma cell line B: Perforin release from PBMC stimulated with the CEF peptide pool or medium alone on ethanol activated ELIIP plates. The substrate used was BCIP/NBT-plus.

**Conclusions**

We defined a number of parameters as critical in order to obtain optimal results with the Perforin-ELISpot. Several of these, such as the choice of plates and pre-activation conditions, are essential for ELISpot in general and we strongly advice optimization of all ELISpot assays. However, due to its low secretion from cells, this may be particularly critical when assessing Perforin.

*Please feel free to discuss all the above issues with us.*
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*2005-05-30*References

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