Development of an *in vitro* T cell exhaustion model

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Introduction

The development of cancer or chronic infections is closely associated with the alteration of multiple immune cell functions that altogether account for an inefficient immune response. In particular, the induction of functionally exhausted T cells is a major factor for the lack of efficient immune activity against tumours[1].
As such, reversal of T cell exhaustion is viewed as a key aspect of numerous anti-cancer strategies through targeting of inhibitory checkpoint receptors[2].
Here, we describe the development of a model for the generation and study of functionally exhausted T cells *in vitro*. The ability to consistently generate large numbers of exhausted T cells is a key requirement for the development of robust *in vitro* screening assays to aid the identification of new anti-cancer therapies.

Setup

Exhausted T cells are characterised by high levels of inhibitory checkpoint receptor expression, loss of ability to produce inflammatory cytokines and reduced proliferation, as well as showing transcriptional and metabolic alterations.

Natural T cell exhaustion can be mimicked by repeatedly stimulating isolated T cells *in vitro*. To obtain robust exhaustion, we opted for a strong repeated stimulation using anti-CD3/CD28 Dynabeads™.
The progression of exhaustion phenotype was followed by flow cytometry.

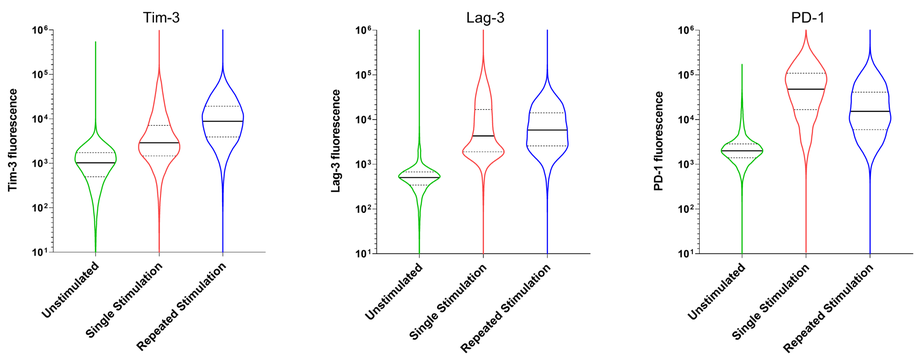

Figure 1: Repeated stimulation of T cells leads to an increased expression of checkpoint inhibitory receptors. 2x10^5 Human T cells isolated from healthy donors were stimulated with anti-CD3/CD28 Dynabeads™, once or 4 times, for "single stimulation" and "repeated stimulaton" respectively, over the course of 9 days. Fluorescence associated with Tim-3, Lag-3 and PD-1 is depicted in the violin plots.

Timecourse

To induce exhaustion, 2x10^5 T cells were subjected to 4 cycles of stimulation with anti-CD3/CD28 Dynabeads™ for a total of 9 days.

Three hallmarks of T cell exhaustion were assessed:

1) Reduced proliferative capacities following TCR engagement/co-stimulation [Figure 2A].
   - Maximal proliferation of naive cells with muted response following every subsequent cycle of stimulation.
2) Reduced inflammatory cytokine response [Figure 3A].
   - Peak of IL-2 production after the initial stimulation, quickly followed by a drastic decrease in the ability of the exhausted cells to produce pro-inflammatory cytokines.
3) Increase expression of checkpoint receptors on cell surface [Figure 3B].
   - PD-1 expression is reaching maximum level after a single cycle of stimulation, while both Lag-3 and Tim-3 levels progressively increase following repeated exposure to Dynabeads™.

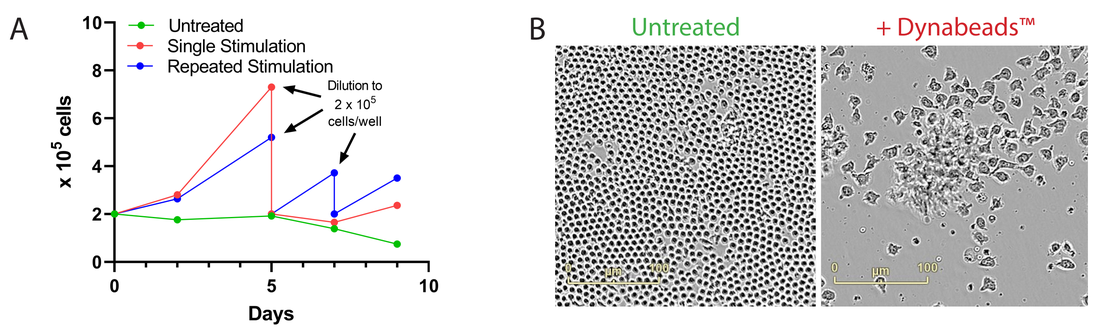

Figure 2: T cells repeatedly stimulated with anti-CD3/CD28 Dynabeads™ progressively lose their proliferative abilities. Growth curves (A) and representative morphological changes undergone by T cells following their activation (B).

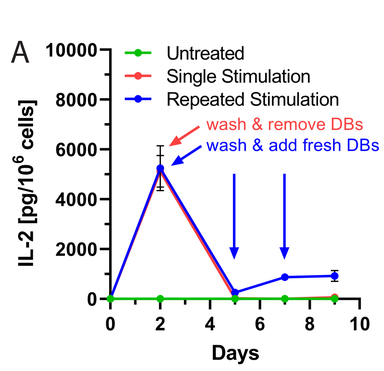

Figure 3: T cells repeatedly stimulated with anti-CD3/CD28 Dynabeads™ (DBs) show an increased expression of inhibitory checkpoint receptors and a reduced ability to produce IL-2. IL-2 release quantified by AlphaLisa (A) and expression of inhibitory checkpoint receptors assessed by flow cytometry (B).

Reproducibility

Next, the robustness of the induction of T cell exhaustion was assessed across multiple donors [Figure 4].

Despite the intrinsic variability associated with independent blood donors, we observed that the exhausted T cells displayed:

- A consistent increase in the expression of inhibitory checkpoint receptors [Figure 4A].
- A reduced ability to secrete IL-2 [Figure 4B].

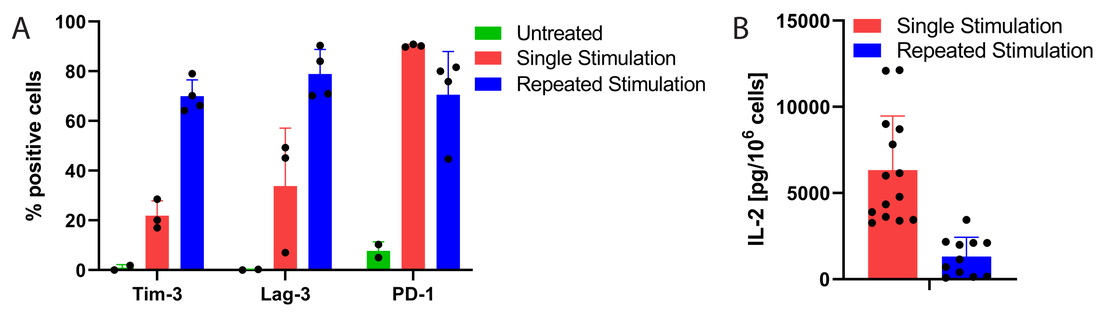

Figure 4: Repeated stimulation reproducibly induce T cell exhaution in independent donors. Expression of inhibitory checkpoint receptors assessed by flow cytometry (A) and IL-2 release quantified by AlphaLisa (B).

Reversal of T cell exhaustion using therapeutical agents *in vitro*

The effect of an inhhibitor of the HPK1 immuno-oncology target (HPK1-IN-3 from MedChemExpress) on the secretion of IL-2 by the exhausted T cells was then evaluated. HPK1 inhibition has been shown to lead to the reversal of the exhausted phenotype of T cells[3].

Inhibition of HPK1 for 48h in exhausted T cells increased the IL-2 secretion by up to 30-fold.
These results indicate that the cells:

- are not irreversibly exhausted.
- could be used as a tool to identify molecules able to reverse the T cell exhaustion phenotype induced by repeated stimulation.

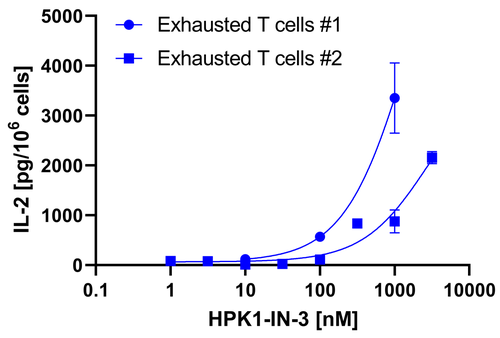

Figure 5: Treatment of exhausted T cells with an HPK1 inhibitor partially restores their function. Effect of a dose-response of HPK1 inhibitor on IL-2 release by exhausted T cells stimulated with Dynabeads™ in 2 independent donors.

Summary

We have shown that the repeated stimulation of isolated human T cells with anti-CD3/CD28 Dynabeads™ leads to *bona fide* exhausted T cells displaying 3 of the main hallmarks of exhaustion: reduced proliferative ability, decreased IL-2 secretion and increased expression of inhibitory checkpoint receptors on their surface.

While our preliminary results indicate that the exhaustion phenotype of these cells can be reversed by therapeutical agents, next steps will focus on the functional characterisation of those cells in a more complex cellular setting such as an MLR assay.

References

1. Reviewed in Zhang Z, Liu S, Zhang B, Qiao L, Zhang Y and Zhang Y (2020). T Cell Dysfunction and Exhaustion in Cancer. Front. Cell Dev. Biol. 8:17. doi: 10.3389/fcell.2020.00017.
2. Reviewed in Tabana Y, MoonT. C., Siraki A, Elahi S & Barakat K (2021). Reversing T-cell exhaustion in immunotherapy: a review on current approaches and limitations. Expert opinion on therapeutic targets, 25(5), 347–363.
3. Reviewed in Sawasdikosol S & Burakoff S (2020). A perspective on HPK1 as a novel immuno-oncology drug target eLife 9:e55122.

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