IN VITRO BIOLOGICAL ACTIVITY CHARACTERIZATION AND CELL-BASED POTENCY ASSAY DEVELOPMENT FOR ALLOCETRA-OTS, A NOVEL MACROPHAGE REPROGRAMMING CELL THERAPY DEVELOPED FOR THE TREATMENT OF SEPSIS

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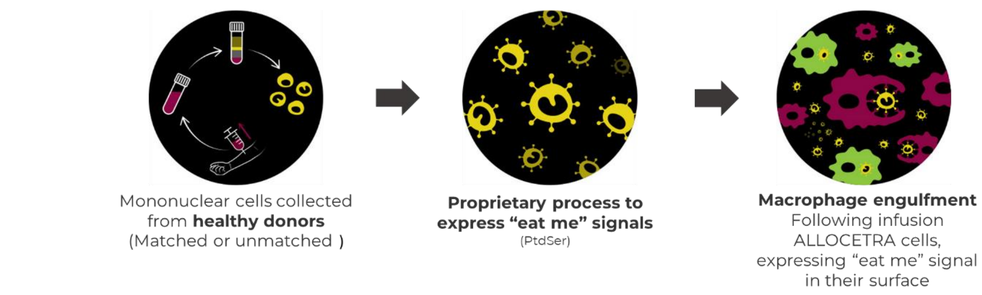
ENLIVEX
immune rebalancing

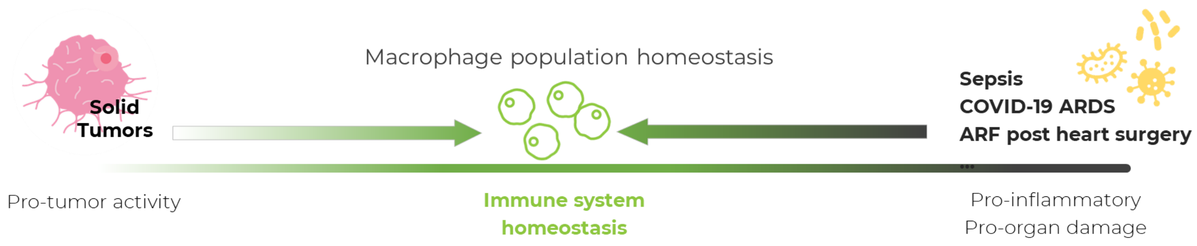
INTRODUCTION

The clearance of apoptotic cells by phagocytosis, termed efferocytosis, is an essential process in maintaining immune homeostasis. Enlivex Therapeutics is developing Allocetra-OTS, a clinical-stage allogeneic macrophage reprogramming cell therapy, consisting of mononuclear cells induced to an early, stable apoptotic state, for the treatment of life-threatening diseases such as sepsis and advanced solid tumors.

The manufacturing processes of Allocetra-OTS includes collection by leukapheresis of allogeneic mononuclear cells (MNCs) from unrelated, non-matched healthy donors. The MNCs are induced to an apoptotic state and developed as a frozen (cryopreserved) formulation drug product as a ready-to-use therapy.



Allocetra-OTS harnesses the naturally-occurring activity of apoptotic cells to induce an immunomodulated state. This effect is mediated by the engulfment of the Allocetra-OTS apoptotic cells by macrophages.



OBJECTIVES

- Development of *in-vitro* models, using primary human cells, to characterize the immunomodulatory effect of Allocetra-OTS cells.
- Development and optimization of an appropriate and controlled cell-based potency assay, based on biologic characterization models, in order to define the quality of Allocetra-OTS cells as acellular therapy products.

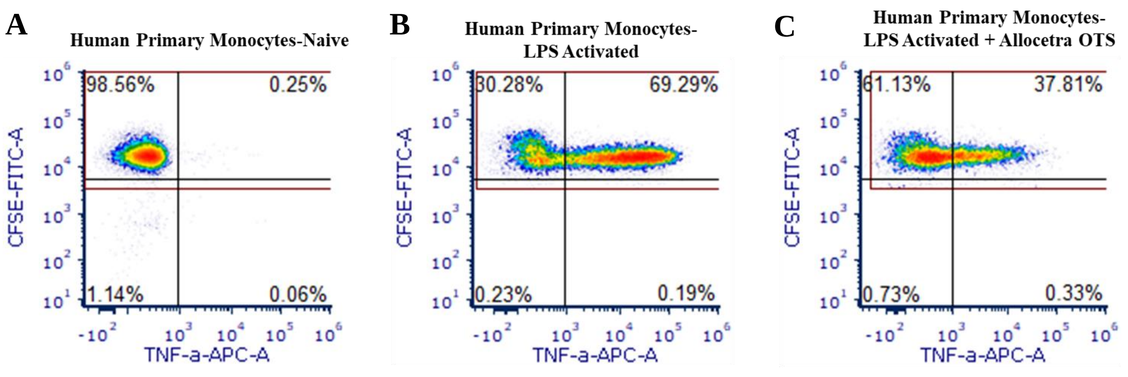
METHODS

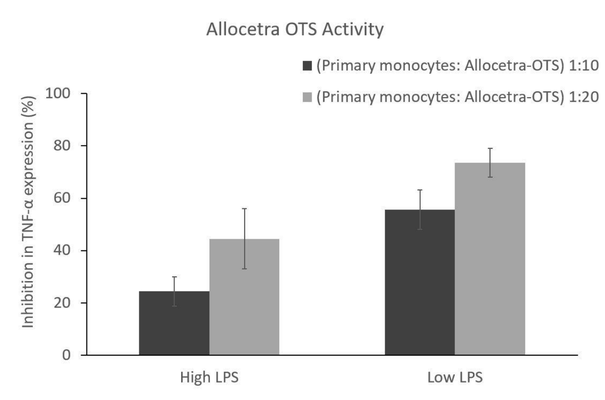
- **Biological characterization *in-vitro* models:** Human monocytes (CD14 positive cells) were stimulated with LPS and incubated with Allocetra-OTS cells in several ratios on several time points.
- **Monitoring of immunomodulating features:** The expression and secretion levels of the inflammatory cytokines TNF-α and IL-6 were analyzed by intracellular staining (ICS) using flow cytometry (Attune NxT), and ELISA for human cytokines. Allocetra-OTS activity is expressed as inhibition (%) of cytokines expression/secretion relative to activated monocytes
- **Cell-based potency model:** A robust model was developed using the murine macrophages cell line RAW 264.7. Following the initial activation of these cells with LPS, Allocetra-OTS cells are added in several ratios to additional incubation time. The immunomodulatory effect is expressed and quantitatively measured by the reduction of TNF-α secretion from LPS-activated macrophages by mouse TNF-α ELISA procedure. Allocetra-OTS activity is expressed as inhibition (%) of TNF-α secretion from activated murine macrophages cells at different RAW/Allocetra-OTS cell ratios in a dose-dependent pattern.

RESULTS

Inhibitory effect of Allocetra-OTS on TNF-α expression by LPS-induced human monocytes

Inhibition of TNF-α expression derived from LPS triggering of human primary monocytes in a dose-dependent pattern. Allocetra-OTS activity is expressed in a dose-dependent pattern as demonstrated by ICS and flow cytometry analysis.



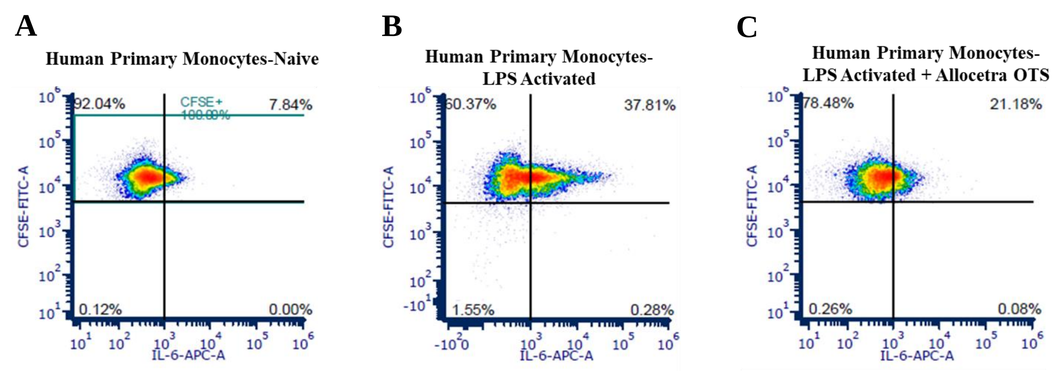


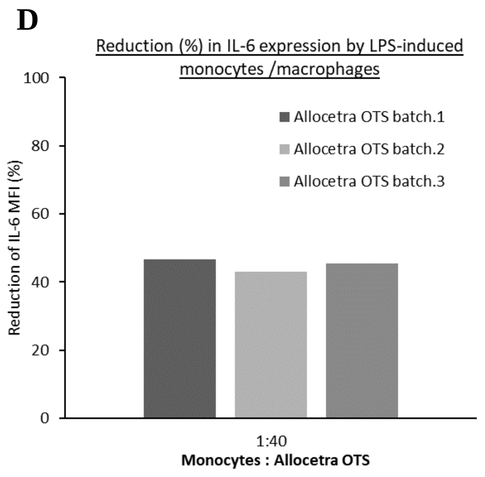
(A-C) Flow cytometry analysis: Expression of TNF-α from human monocytes following LPS exposure. 30 min following LPS exposure, monocytes (CFSE-labeled) were incubated with Allocetra-OTS for additional 2 hours of incubation. **(D)** Normalization of TNF-α inhibition according to LPS activation treatments (in two concentrations); N=3 representative Allocetra-OTS batches. Results are expressed as mean±SD from three independent experiments.

RESULTS (cont.)

Inhibitory effect of Allocetra-OTS on IL-6 expression by LPS-induced human monocytes

Allocetra-OTS mediated inhibition of IL-6 expression in human monocytes, following incubation with LPS-activated monocytes as assessed by ICS and flow cytometry analysis.

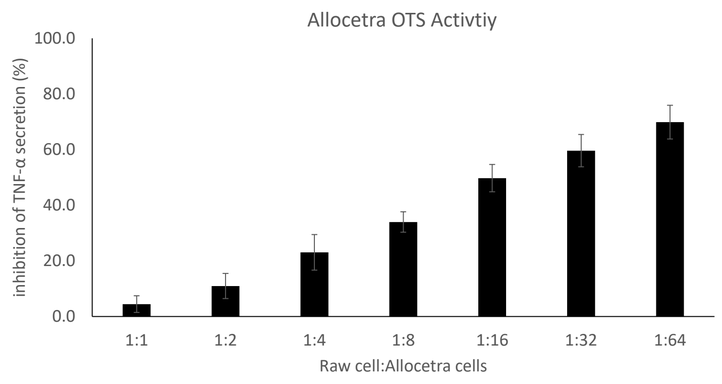




(A-D) Flow cytometry analysis: Expression of IL-6 in human primary monocytes following LPS exposure. Following LPS exposure, monocytes (CFSE-labeled) were incubated with Allocetra-OTS for additional 4 hours of incubation. **(D)** Inhibition of IL-6 expression derived from LPS triggering of human monocytes, expressed as reduction in IL-6 MFI from activated cells and demonstrated by ICS and flow cytometry analysis. The reduction in IL-6 MFI was normalized to the LPS activation treatments (without Allocetra-OTS) and calculated for three independent batches (representative batches).

Cells-based potency model: Inhibitory effect of Allocetra-OTS on TNF-α secretion by LPS-induced murine macrophages

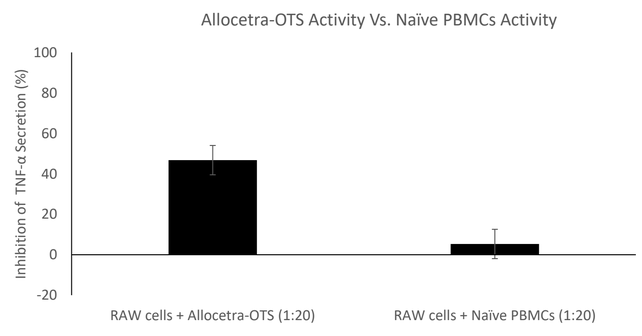
Incubation of murine macrophage RAW 264.7 cell line with Allocetra-OTS cells leads to a reduction in the LPS-induced TNF-α secretion in a dose-dependent pattern.



1×10^5 RAW 264.7 cells were stimulated with LPS at concentrations of 0.1 ng/ml and Allocetra-OTS cells were added in different ratios. TNF-α was measured by ELISA. Results are expressed as mean ± SD from 4 independent experiments

Allocetra-OTS, rather than naïve PBMCs, inhibit TNF-α secretion from activated RAW cells

To demonstrate the inhibition effect derived specifically by Allocetra-OTS, human naïve live PBMCs were added to the activated RAW cell culture in parallel to the Allocetra-OTS cells, and their ability to mediate inhibition in TNF-α secretion was measured.



Inhibition of mouse TNF-α secretion from LPS activated RAW 264.7 cells, mediated by Allocetra-OTS or naïve PBMCs and demonstrated by ELISA. The inhibition in mouse TNF-α was normalized according to the LPS activation treatments without Allocetra cells or PBMCs. Results are expressed as mean ±SD from three independent experiments.

CONCLUSIONS

- Our data provide evidence that Allocetra-OTS induces immune modulation and rebalancing of immune responses as demonstrated through the downregulation of various pro-inflammatory cytokines such as TNF-α and IL-6.
- This evidence provided the basis for the development of a robust potency assay, which plays a key role in the quantitative measurement of Allocetra-OTS's biological activity related to the drug mechanism of action.
- The development of the potency methodology includes the development of reference material to confirm the consistency of potency determination, and the evaluation of additional assay parameters, to determine that this assay is suitable as a potency bioassay.
- Taken together, these findings indicate an important mechanism mediated by Allocetra-OTS cells, which enables their successful use as therapeutic modalities in various immune disorder diseases such as sepsis.

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