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Early exposure to interleukin-21 limits rapidly generated anti–Epstein-Barr virus T-cell line differentiation

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Abstract

Background aims. The adoptive transfer of *ex vivo*–expanded Epstein-Barr virus (EBV)-specific T-cell lines is an attractive strategy to treat EBV-related neoplasms. Current evidence suggests that for adoptive immunotherapy in general, clinical responses are superior if the transferred cells have not reached a late or terminal effector differentiation phenotype before infusion. The cytokine interleukin (IL)-21 has shown great promise at limiting late T-cell differentiation *in vitro*, but this remains to be demonstrated in anti-viral T-cell lines. *Methods.* We adapted a clinically validated protocol to rapidly generate EBV-specific T-cell lines in 12 to 14 days and tested whether the addition of IL-21 at the initiation of the culture would affect T-cell expansion and differentiation. *Results.* We generated clinical-scale EBV-restricted T-cell line expansion with balanced T-cell subset ratios. The addition of IL-21 at the beginning of the culture decreased both T-cell expansion and effector memory T-cell accumulation, with a relative increase in less-differentiated T cells. Within CD4 T-cell subsets, exogenous IL-21 was notably associated with the cell surface expression of CD27 and high *KLF2* transcript levels, further arguing for a role of IL-21 in the control of late T-cell differentiation. *Conclusions.* Our results show that IL-21 has profound effects on T-cell differentiation in a rapid T-cell line generation protocol and as such should be further explored as a novel approach to program anti-viral T cells with features associated with early differentiation and optimal therapeutic efficacy.

Key Words: *adoptive immunotherapy, CD27, Epstein-Barr virus, IL-21, T cell, T-cell memory*

Introduction

The adoptive transfer of *ex vivo*–expanded anti–Epstein-Barr virus (EBV) T-cell lines is an effective and safe approach to prevent or treat post-transplant lymphoproliferative disorders and other EBV-related cancers [1–6]. In recent years, the generation of T-cell lines targeting EBV along with several other viruses directly from peripheral blood mononuclear cells (PBMC) of healthy donors has been simplified and shortened by the use of synthetic overlapping antigenic peptide libraries and gas-permeable culture vessels [7,8]. This rapid method reliably expands virus-specific T-cell lines from seropositive but not from seronegative donors, which implies that a memory anti-viral T-cell repertoire is mobilized. The first trial demonstrating the safety and efficacy of infusing hematopoietic cell transplantation recipients with 5 to 20 $\times$ $10^6/m^2$ T cells generated with this approach was recently reported [9].

The *ex vivo* generation of antigen/pathogen-specific T cells requires exogenous cytokines to promote their differentiation and their expansion. However, the acquisition of effector functions *in vitro* may not correlate with optimal clinical efficacy. Terminally differentiated effector and effector memory (Tem) T cells, driven by strong activation signals *in vitro*, lose their capacity to further expand and persist after adoptive transfer. On the contrary, less differentiated antigen-specific T cells bearing a central memory phenotype (Tcm) can further expand, differentiate or self-renew *in vivo* [10–12]. Hence, a delicate balance must be sought to ensure antigen-specific recognition, adequate expansion and optimal differentiation.

It has been shown that a combination of interleukin (IL)-4 and IL-7 is superior to IL-2 or IL-15 for the rapid generation of interferon (IFN)-γ–producing anti-viral T cells *in vitro* [8]. Moreover, IL-4 and IL-7

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preserve the expression of Tcm markers on a large proportion of the *in vitro*−expanded T cells. Whether the addition of other cytokine signals could further enhance these characteristics is unclear.

The common γ-chain cytokine IL-21 has been shown to both stimulate T-cell responses and promote T-cell survival in humans and mice [13−18]. In the context of *ex vivo* T-cell cultures, previous studies have provided mixed results regarding the role of IL-21 on T-cell expansion and effector generation, perhaps in line with the heterogeneity of the culture protocols [19−27]. However, the bulk of available evidence suggests that overall, IL-21 inhibits CD8 T-cell terminal differentiation and programs long-term memory development, even if added only in the early stages of the culture [19], thereby improving the potential for *in vivo* cell persistence and clinical efficacy [10,13]. Now that Good Manufacturing Practice−grade IL-21 is available and can be incorporated in the preparation of cell therapy products [19], this cytokine is an attractive candidate to further improve T-cell line generation. However, the impact of IL-21 on the differentiation of a memory anti-viral repertoire in culture remains to be defined.

We adapted the protocol from Gerdemann *et al.* [8] to rapidly expand anti-EBV T-cell lines and addressed whether IL-21 exposure could program their differentiation. The addition of IL-21 at the beginning of the culture consistently resulted in lower expansion and decreased proportion of Tem cells in favor of less-differentiated subsets. In all cases, antigen specificity was preserved, although globally, IFN-γ release was decreased in IL-21−exposed T-cell lines. Moreover, IL-21 prevented the downregulation of CD27 on CD4 cells and favored the expression of gene transcripts associated with early-stage differentiation in CD4 Tem. We conclude that IL-21 limits the differentiation of EBV-specific T cells in rapidly generated T-cell lines. These cells would be expected to have a greater capacity to persist, further differentiate or self-renew after adoptive transfer.

Methods

PBMC procurement

Healthy donors were recruited through the Hematopoietic Cell Bank at Hôpital Maisonneuve-Rosemont after approval of the protocol by the local ethics committee. Blood was collected by means of venipuncture in heparinized tubes and centrifuged for plasma collection. The PBMC were isolated with the use of Ficoll-Paque Plus density gradient separation as recommended by the manufacturer (GE Healthcare).

T-cell line generation

We used the approach from Gerdemann *et al.* [8], with slight modifications. Briefly, 15×10^6 PBMCs (either fresh or recovered after thawing and overnight incubation in T-cell media) were pelleted and pulsed with 100 ng of both latent membrane protein 2 (LMP2) and Epstein-Barr virus nuclear antigen 1 (EBNA1) overlapping peptide libraries (JPT Peptides). After 30 min at 37°C, the cells were counted and mixed with 30 mL of T-cell media (45% advanced Roswell Park Memorial Institute [RPMI] 1640 medium, 45% Click's medium, L-glutamine 2 mmol/L and 10% autologous plasma) and supplemented with IL-4 (1666 U/mL; Feldan), IL-7 (10 ng/mL; Miltenyi) and IL-21 (30 ng/mL or as indicated) (Feldan). Cell suspensions were incubated at 37°C and 5% CO_2 in G-Rex10 culture vessels (Wilson Wolf Manufacturing). At days 5, 8−9 and 12, half of the media was removed and cultures were replenished with fresh T-cell media and cytokines (IL-4 and IL-7). Cells were counted by use of trypan blue exclusion, with the use of an automated cell counter (Countness, Invitrogen). If cell concentration exceeded 1.5×10^6/mL, cultures were split (1:2). Cultures were terminated at day 12 if cell count at day 8−9 was superior to 45×10^6 cells. Otherwise, the culture was extended to day 14. Paired cultures (with or without IL-21 from the same donor) were always terminated on the same day.

Flow cytometry

Cells were harvested and washed once in phosphate-buffered saline supplemented with 2% fetal bovine serum and counted. Intracellular cytokine detection was performed after cell stimulation for 4 h, with dimethyl sulfoxide, peptide library or phorbol 12-myristate 13-acetate (PMA; 50 ng/mL) and ionomycin (500 ng/mL), followed by intracellular transport blockade with Brefeldin A (7.5 μg/mL) (Sigma-Aldrich) for 12 h. Cells were then permeabilized and fixed (buffer set from eBioscience). Antibodies were purchased from eBioscience (anti-CD8), BioLegend (anti-CD45RA, CD62L, PD-1, CD57 and 7-amino-actinomycin D) or BD biosciences (anti-CD3, CD4, CD8, CD45RO, CD45RA, CCR7, CD28 and CD27). The IOTest Beta Mark TCR Repertoire kit (Beckman Coulter) was used to estimate T-cell receptor repertoire. The data were acquired on an LSRII flow cytometer (BD Biosciences) and analyzed with Flow Logic software (Inivai Technologies).

Enzyme-linked immunospot and cytotoxicity assays

The enzyme-linked immunospot (ELISpot) assay was performed with 5×10^4 cells per well, according

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