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The *in vitro* generation of multi-tumor antigen-specific cytotoxic T cell clones: Candidates for leukemia adoptive immunotherapy following allogeneic stem cell transplantation

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ABSTRACT

Adoptive T-cell immunotherapy is a promising approach to manage and maintain relapse-free survival of leukemia patients, especially following allogeneic stem cell transplantation. Post-transplant adoptive immunotherapy using cytotoxic T lymphocytes (CTLs) of the donor origin provide graft-versus-tumor effects, with or without graft–versus-host disease. Myeloid leukemias express immunogenic leukemia associated antigens (LAAs); such as WT-1, PRAME, MAGE, h-TERT and others, most of them are able to induce specific T cell responses whenever associated with the proper co-stimulation. We investigated the ability of a LAA-expressing hybridoma cell line to induce CTL clones in PBMCs of HLA-matched healthy donors *in vitro*. The CTL clones were induced by repetitive co-culture with LAAs-expressing, HLA-A*0201$^+$ hybrid cell line, generated by fusion of leukemia blasts to human immortalized APC (EBV-sensitized B-lymphoblastoid cell line; HMy2). The induced cytotoxic T cell clones were phenotypically and functionally characterized by pentamer analysis, IFN-γ release ELISPOT and cellular cytotoxicity assays. All T cell lines showed robust peptide recognition and functional activity when sensitized with HLA-A*0201-restricted WT-$1_{235–243}$, hTERT$_{615–624}$ or PRAME$_{100–108}$ peptides-pulsed T2 cells, in addition to partially HLA-matched leukemia blasts. This study demonstrates the feasibility of developing multi-tumor antigen-specific T cell lines in allogeneic PBMCs *in vitro*, using LAA-expressing tumor/HMy2 hybrid cell line model, for potential use in leukemia adoptive immunotherapy in partially matched donor-recipient setting.

1. Introduction

Hematological tumors represent one tenth of malignancy cases worldwide and are characterized by significant mortality rates. However, recent advances in treatment have resulted in a significant improvement in the patient survival (Siegel et al., 2013). Allogeneic stem cell transplantation (ASCT) is a curative and accepted therapeutic choice for many chemotherapy incurable hematologic tumor patients (Thomas and Blume, 1999). In this approach, eradication of the emerging tumor cells is achieved by administration of high dose chemotherapy with or without total body irradiation. Subsequently, the patient's bone marrow aplasia is corrected by transfer of hematopoietic stem cells from compatible, HLA-matched volunteers. It is believed that ASCT

Abbreviations: AML, acute myeloid leukemia; ASCT, allogeneic stem cell transplantation; CFSE, carboxyfluorescein diacetate succinimidyl ester; FBS, fetal bovine serum; GVT, Graft-versus-tumor; GVHD, Graft-versus-host disease; GVL, graft-versus-leukemia; HAT, hypoxanthine-aminopterin-thymidine; h-TERT, human telomerase reverse transcriptase; IF, immunofluorescence; LAA, leukemia associated antigens; LCL, lymphoblastoid cell line; mHAgs, minor histocompatibility antigens; Mit-C, mitomycin-C; MLR, mixed lymphocyte reaction; PHA, phytohemagglutinin; PRAME, preferentially expressed antigen of melanoma; TAA, tumor associated antigens; TC, tissue culture; WT-1, Wilms' tumor-1.

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accomplishes its curative effect through destruction of the patient's whole hematopoietic system as well as the malignant clones. However, many studies showed that the less recurrence opportunities in leukemia patients were associated with the induction of acute or chronic Graft-versus-host disease (GVHD) (Weiden et al., 1979). In addition, many reports confirmed the inability of the hematopoietic conditioning, whatever its intensity, to cure the malignant tumor without other interventions. The essential role of the graft-versus-leukemia (GVL)effects of the transplanted immune cells of HLA-matched donors is greatly accepted due to the higher rates of post-transplantation relapse of chronic myelogenous leukemia (CML) patients receiving T-cell depleted allografts (Horowitz et al., 1990), as well as syngeneic transplant-patients as opposed to those receiving transplants from allogeneic, HLA-matched donors (Horowitz et al., 1990). Lastly, the ability of CML patients, who had relapsed following an allotransplant, to get back into a long-lasting remission following a single infusion of donor lymphocytes has convincingly proven both the reality and therapeutic potential of the GVL effect (Kolb et al., 1990). Similar GVL effects have been shown in acute myeloid leukemia, chronic lymphocytic leukemia, post-transplant Epstein-Barr virus-associated lymphoproliferative disease, multiple myeloma, and Hodgkin's and non-Hodgkin's lymphomas. (Oliansky et al., 2010; Mcclune et al., 2014; Copelan et al., 2015).

Adoptive T-cell transfer represents an alternative approach to active immunization, as a regimen of cancer immunotherapy. This immunotherapeutic approach has been used with significant clinical benefits in EBV-associated lymphoid disorders (Haque et al., 2007; Heslop et al., 2010), myeloma (Rapoport et al., 2005), and leukemia (Marijt et al., 2007; Warren et al., 2010), in addition to a group of solid tumors (Turin et al., 2007; Meehan et al., 2008) which demonstrated the feasibility of generating cytotoxic T lymphocyte lines to good manufacturing practice (GMP) standards, as well as the tolerability and therapeutic benefit of adoptive T cell immunotherapy.

In recent years, much of the immunotherapeutic approaches for cancer management have focused on the ability of antigen presenting cells (APCs) to activate tumor-specific T-cell responses (Browning, 2013). APC/tumor cell hybrids have been shown to induce defensive immunity to certain tumors induced in laboratory animals and, in some cases, to destroy established tumor masses (Gong et al., 1997; Wang et al., 1998; Siders et al., 2003; Yasuda et al., 2007). However, fusion of DCs (the most powerful APC) and tumor cells results in hybrid cells that are short-lived, with limited replicative ability, have variable fusion productivities, and have incomplete characterization profiles.

In previous studies, it has been shown that the EBV-associated B lymphoblastoid cell line (LCL), HMy2 (Edwards et al., 1982), could be fused with human tumor cells or cell lines *in vitro* to generate stable hybrids that grew spontaneously in tissue culture, expressed tumor-associated antigens (TAAs), and showed a higher ability to stimulate allogeneic T cell responses (Cywinski et al., 2006). Moreover, these hybrid cell lines induced tumor antigen-specific CTLs in peripheral blood lymphocytes (PBL) from healthy donors *in vitro*, as detected by HLA-A*0201-peptide pentamer staining and cellular cytotoxicity assays (Mohamed et al., 2012a, b). Such hybrid cell lines could potentially be used as *in vitro* inducers of antigen-specific CTLs for adoptive cellular transfer, if produced to GMP standards.

In this study, we extend our previous data by generating a hybrid cell line through fusion of *ex vivo* promyelocytic leukemia blasts with HMy2 cell line. The fusion product showed a phenotypic profile similar to the parent APC, regarding expression of co-stimulatory markers and HLAs, and simultaneously expressed tumor antigens of the parent leukemia cells. These hybrid cells survived immortally in tissue culture and, when co-cultured with allogeneic normal peripheral PBMCs, triggered primary T cell responses with tumor-specific cytotoxic potency, as revealed by pentamer analysis, IFN-ϒ release ELISPOT and Europium release cellular cytotoxicity assays. Our data indicate that this model of hybrid cell lines could be a candidate for use in tumor immunotherapy by *in vitro* generation of tumor-specific T cell lines or clones for adoptive cell transfer.

2. Materials and methods

2.1. Primary cells and cell lines

PBMCs were obtained from adult healthy HLA-A*0201^{+} volunteers, and primary leukemic tumor blasts were separated from marrow aspirate of a non-treated, acute promyelocytic leukemia case following informed consent, and after getting local Institutional Review Board approval (IRB-2014-01-11; University of Dammam). All human samples were used in accordance with the ethical standards of the appropriate institutional and national committees and with the Helsinki Declaration of 1975 (revised in 2008). PBMCs were separated from venous blood by density gradient centrifugation over Ficoll-Paque solution (GE Healthcare, UK). Tumor cells were separated from bone marrow lymphocytes using anti-CD33 magnetic micro-beads cell separation kit (Miltenyi Biotech, UK), and the purity of the promyelocytic cells was more than 95%. The separated cells were subsequently cryopreserved for future use. HMy2, T2 and K562 cell lines were used for hybrid generation or for other cellular assays in the study and have been described previously (Mohamed et al., 2012a).

2.2. Tissue culture

The primary cells and cell lines; HMy2, chronic myeloid leukemia cell line (K562), acute promyelocytic leukemia blasts and TAP-deficient lymphoblast cell line (T2) were maintained under tissue culture (TC) conditions using RPMI 1640 growth medium supplemented with 10% FBS, 100IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (all from Sigma Aldrich, UK), in 5% CO2-supplemented humidified incubator at 37 °C.

2.3. Generation of the hybrid cells

Acute promyelocytic leukemia blasts were fused to HMy2 (Edwards et al., 1982) using PEG/DMSO 1500 w/v as described previously (Cywinski et al., 2006). Following fusion, the cell mixture was transferred to chemical selection growth medium, RPMI with added hypoxanthine, aminopterin and thymidine (RPMI-HAT), for 7 days to select the growth of hetero-hybrid cells. Both homo-hybrids and non-fused HMy2 are unable to grow in the presence of the selective medium. In addition, the primary tumor cells did not survive in TC conditions for more than one week. After one week in the selection medium, the fusion mixture was transferred into supplemented RPMI growth medium.

2.4. Fusion protocol efficiency

Fusion efficiency was evaluated before and after the chemical selection through immunofluorescence (IF) staining and flow cytometry of the fusion mixture using monoclonal antibodies (mAbs) for lineage-specific markers, CD33 and CD19 for myeloid and B-lymphoblastoid lineages respectively. Percentage of the true hybrid fraction showing double positive for CD33 and CD19 was calculated.