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In vitro evaluation of human hybrid cell lines generated by fusion of B-lymphoblastoid cells and *ex vivo* tumour cells as candidate vaccines for haematological malignancies

Yehia S. Mohamed[a,b], Debbie Dunnion[a], Iryna Teobald[a], Renata Walewska[a,c], Michael J. Browning[a,d,*]

[a] *Department of Infection, Immunity & Inflammation, University of Leicester, Maurice Shock Building, University Road, Leicester LE1 9HN, United Kingdom*
[b] *Department of Microbiology and Immunology, Al-Azhar University, Cairo, Egypt*
[c] *Department of Haematology, Leicester Royal Infirmary, Leicester LE1 5WW, United Kingdom*
[d] *Department of Immunology, Leicester Royal Infirmary, Leicester LE1 5WW, United Kingdom*

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ABSTRACT

Fusions of dendritic cells (DCs) and tumour cells have been shown to induce protective immunity to tumour challenge in animal models, and to represent a promising approach to cancer immunotherapy. The broader clinical application of this approach, however, is potentially constrained by the lack of replicative capacity and limited standardisation of fusion cell preparations. We show here that fusion of *ex vivo* tumour cells isolated from patients with a range of haematological malignancies with the human B-lymphoblastoid cell line (LCL), HMy2, followed by chemical selection of the hybridomas, generated stable, self-replicating human hybrid cell lines that grew continuously in tissue culture, and survived freeze/thawing cycles. The hybrid cell lines expressed HLA class I and class II molecules, and the major T-cell costimulatory molecules, CD80 and CD86. All but two of 14 hybrid cell lines generated expressed tumour-associated antigens that were not expressed by HMy2 cells, and were therefore derived from the parent tumour cells. The hybrid cell lines stimulated allogeneic T-cell proliferative responses and interferon-gamma release *in vitro* to a considerably greater degree than their respective parent tumour cells. The enhanced T-cell stimulation was inhibited by CTLA4-Ig fusion protein, and by blocking antibodies to MHC class I and class II molecules. Finally, all of five LCL/tumour hybrid cell lines tested induced tumour antigen-specific cytotoxic T-cell responses *in vitro* in PBL from healthy, HLA-A2+ individuals, as detected by HLA-A2-peptide pentamer staining and cellular cytotoxicity. These data show that stable hybrid cell lines, with enhanced immunostimulatory properties and potential for therapeutic vaccination, can be generated by *in vitro* fusion and chemical selection of B-LCL and *ex vivo* haematological tumour cells.

1. Introduction

Dendritic cell (DC)/tumour fusion vaccines have shown promise as anti-tumour immunotherapies in animal models [1–7]. Based on these studies, a number of phase I clinical trials have been undertaken in humans [8–14]. However, fusion of DCs and tumour cells results in fusion cells that are short-lived and have restricted replicative capacity, with variable fusion efficiencies, and limited characterisation of the fusion products.

We have shown previously that the EBV-associated B-lymphoblastoid cell line (LCL), HMy2 [15], can be fused with human tumour cell lines *in vitro*, to produce stable hybridoma cells that grew spontaneously in culture, expressed tumour-associated antigens (TAA), and showed an enhanced ability to stimulate allogeneic T cell responses *in vitro*, when compared with the parent tumour cell lines [16]. In this paper, we extend these studies to demonstrate that HMy2 cells can be fused *in vitro* with tumour cells isolated directly from patients with a range of haematological malignancies to produce stable hybrid cell lines that grew continuously in tissue culture, survived freeze/thawing cycles, and induced strong allogeneic T-cell responses *in vitro*, including the induction of tumour antigen-specific cytotoxic T-cells in peripheral blood lymphocytes (PBL) from healthy, HLA-A2+ donors. The generation of stable APC/tumour hybrid cell lines, which replicate continuously in tissue culture and which induce tumour antigen-specific cytotoxic T cell responses, has implications for the potential use of fusion cells for therapeutic vaccination in haematological malignancies.

* Corresponding author at: Department of Infection, Immunity & Inflammation, University of Leicester, Maurice Shock Building, University Road, Leicester LE1 9HN, United Kingdom. Tel.: +44 1162586702.
E-mail address: mjb22@le.ac.uk (M.J. Browning).

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Table 1
Pentamer positive populations induced by the hybrid cell lines.

	Unstim PBMC	Unstim PBMC	HMy2	HMy2x ALL-52	HMy2x AML-24	HMy2x MCL-44	HMy2x MM-53	HMy2x MM-56
MAGE-A1	0.10	0.04	0.58	3.34	1.57	1.44	1.75	4.77
	(−)	(−)	(−)	(+)	(wk)	(+)	(+)	(+)
NY-ESO-1	0.09	0.07	0.93	1.66	1.63	1.57	1.87	0.47
	(−)	(−)	(wk)	(+)	(wk)	(+)	(+)	(−)
Survivin	0.04	0.09	1.81	3.16	4.49	3.0	2.07	3.06
	(−)	(−)	(+)	(+)	(+)	(+)	(+)	(+)
PRAME	0.08	0.12	0.33	2.39	3.95	1.17	0.40	0.94
	(−)	(−)	(−)	(+)	(+)	(+)	(−)	(+)
WT-1	0.26	0.17	0.54	0.48	1.87	0.29	0.33	0.49
	(−)	(−)	(−)	(−)	(wk)	(−)	(−)	(−)

The following PE-labelled Pro5 HLA-A2 pentamers were used: MAGE-A1, 278–286, sequence KVLEYVIKV; NY-ESO-1, 157–165, SLLMWITQV; survivin 96–104, LMLGEFLKL; PRAME, 300–309, ALYVDSLFFL; and WT-1, 235–243, CMTWNQMNL.
Table shows the proportion of pentamer-positive CD8+ T-cells as % of viable cells in the long-term T-cell cultures. TAA expression by the stimulating hybrid cell lines is given in parenthesis ('−' = negative; 'wk' = weakly positive; '+' = positive), as determined by RT-PCR (Fig. 3).

2. Materials and methods

2.1. Tumour cell collection and cell separation

Tumour cells were isolated from 14 patients with haematological malignancies, including B-cell chronic lymphocytic leukaemia (CLL; $n = 4$), follicular lymphoma (FCL; $n = 1$), mantle cell lymphoma (MCL; $n = 2$), multiple myeloma (MM; $n = 4$), precursor B-lymphoblastic leukaemia (ALL; $n = 1$) and acute myeloid leukaemia (AML; $n = 2$). All patients gave informed consent, and the study had the approval of the Local Research Ethics Committee. All samples were taken prior to commencement of therapy. Tumour cells were isolated from blood or bone marrow by Lymphoprep™ (Nycomed Pharma, Norway) separation, followed by positive selection using magnetic beads coated with monoclonal antibodies to lineage-specific surface molecules (CD19 for B-cell tumours, CD138 for myeloma, CD33 for myeloid leukaemias; Miltenyi Biotech, UK), according to the manufacturer's protocol. The method routinely yielded tumour cells with a purity of >90% by flow cytometric analysis.

2.2. Hybrid cell production

The tumour cells were fused *in vitro* with the EBV-associated B-LCL, HMy2 [15], as described previously [16]. Briefly, fresh or previously frozen tumour cells were mixed 1:1 with HMy2 cells, centrifuged, and polyethylene glycol/dimethyl sulphoxide (PEG/DMSO; Sigma–Aldrich UK; 50% v/v, pre-warmed to 37°C) was added dropwise to the cell pellet for one minute, with continuous mixing, after which the fusion reaction was diluted with pre-warmed Roswell Park Memorial Institute (RPMI) 1640 medium. The cells were centrifuged and transferred to RPMI 1640, 20% fetal calf serum, 5 mmol/l L-glutamine, 25 IU/ml penicillin, 25 μg/ml streptomycin (Gibco, UK). After 24 h, cells were transferred to selection medium containing hypoxanthine, aminopterin and thymidine (HAT) and ouabain (Sigma–Aldrich, UK), at concentrations that were lethal to HMy2 cells or parent tumour cells respectively. HMy2 cells are sensitive to HAT but resistant to ouabain, allowing for double chemical selection of hetero-hybridoma cells following cell fusion [15–17]. Following the outgrowth of hybrid cell lines, the cells were maintained in culture medium as above, except that FCS was reduced to 10% v/v.

2.3. Phenotypic analysis of hybrid cell lines and parent tumour cells

Hybrid cell lines and parent cells (tumour and HMy2) were analysed using direct immunofluorescence with FITC- or PE-labelled mouse anti-human monoclonal antibodies to CD11a (LFA-1), CD40, CD54 (ICAM-1), CD58 (LFA-3), CD80 (B7.1) and CD86 (B7.2) (Beckman Coulter, UK), HLA class I and class II (DAKO, UK), and HLA-A2 (BD Biosciences, UK). Fluorescently labelled isotype control antibodies were used as negative controls. Flow cytometry was performed on a FACSCalibur flow cytometer and analysed using CellQuestPro software (both Becton Dickinson, UK).

2.4. Tumour antigen-specific RT-PCR

Expression of known TAA by HMy2 and the hybrid cell lines was assessed by RT-PCR. Total RNA was extracted using RNeasy mini-kits, contaminating genomic DNA was removed using RNase-free DNase I (both Qiagen, UK), and RNA was reverse transcribed using SuperScript First-Strand system (Invitrogen, UK). TAA-specific PCR were performed in a T1 Thermo-cycler (Biometra, Germany) for 35 cycles, using previously described primer sequences and protocols, for MAGE-A1, NY-ESO-1, PRAME [18], WT-1 [19], survivin [20], and β-actin (Invitrogen, UK). Following PCR, the products were separated by agarose gel electrophoresis.

2.5. Short-term allogeneic T cell stimulation in vitro

Responder peripheral blood mononuclear cells (PBMC) were obtained by Lymphoprep separation of peripheral blood from healthy volunteer donors who were unrelated to the patients or HMy2 cells.

T-cell proliferation was assessed in 5 day mixed lymphocyte/tumour cell cultures (MLTC), by ^{3}H-Thymidine incorporation, using Mitomycin-C-treated hybrid cell lines or tumour cells as stimulators, as described previously [16]. Data are presented as counts per minute (cpm) of triplicate wells.

To identify the role of costimulatory and MHC molecules, CTLA4-Ig fusion protein (Ancell, UK), OX40-Ig fusion protein (Ancell, UK), anti-4-1BB ligand (Immatic, UK), (all at 0.5 μg/ml), anti-HLA class I (W6/32) or class II (L243) mAbs (at 0.75 μg/ml), or isotype control antibodies, were added to the stimulator cells for 45 min, prior to plating of the responder PBMC. The protocol was otherwise as described above.

Elispot assays were performed using human IFN-γ ELISpot kits (all reagents from Mabtech AB, Sweden), according to the manufacturer's instructions. Briefly, wells were coated with capture antibody (anti-IFN-γ clone 1-D1K), responder cells were added in triplicate wells, and Mitomycin-C-treated stimulator cells were added at a ratio of 3:1 responder to stimulator cells. Plates were incubated at 37°C in 5% CO_2 for 48 h. After incubation, biotinylated anti-IFNγ (clone7-B6-1) was added to the washed wells, followed by Streptavidin-conjugated alkaline phosphatase. Spots