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Long-lived fusions of human haematological tumour cells and B-lymphoblastoid cells induce tumour antigen-specific cytotoxic T-cell responses *in vitro*

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ABSTRACT

Tumour-specific cytotoxic T-cells (CTL) are important anti-cancer immune effectors. Most tumour cells, however, do not stimulate effective anti-tumour immune responses, *in vivo* or *in vitro*. To enhance tumour cell immunogenicity, we fused human tumour cells from haematological malignancies with the B-lymphoblastoid cell line (LCL), HMy2, to generate a panel of long-lived, self replicating LCL/tumour hybrid cell lines. The LCL/tumour hybrid cell lines expressed HLA class I and class II molecules, CD80 and CD86, and a range of known tumour associated antigens (TAAs). *In vitro* stimulation of PBLs from healthy, HLA-A2+ individuals by hybrid cell lines induced tumour antigen-specific CTLs to TAAs, including survivin, MAGE-A1, NY-ESO-1 and WT-1. Individual hybrid cell lines simultaneously induced CTL to multiple TAAs. *In vitro* stimulation of PBL from 2 patients with acute myeloid leukaemia by autologous LCL/tumour hybrid cell lines induced CTL capable of killing the patient's own tumour cells. Our data show, for the first time, that hybrid cell lines formed by fusion of HMy2 cells and haematological tumour cells induce tumour- and tumour antigen-specific cytotoxic T-cell responses *in vitro*. Hybrid cell lines such as those described may represent novel reagents for use in the immunotherapy of haematological malignancies.
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Introduction

The development of immunotherapeutic strategies for treating cancers has received considerable interest in recent years. Much of this work has focussed on the potential of dendritic cells (DCs) for inducing tumour-specific T-cell responses. One strategy for DC-based cancer immunotherapy has been the use of fusions of DCs and tumour cells as therapeutic cancer vaccines. DC/tumour cell fusions have been shown to induce protective immunity to tumour cell challenge in animal models and, in some cases, to eradicate established tumours (Gong et al. 1997; Wang et al. 1998; Siders et al. 2003; Yasuda et al. 2007). In human studies, several phase I clinical trials have reported clinical and/or immunological responses in individual patients (Marten et al. 2003; Avigan et al. 2004; Trefzer et al. 2004; Avigan et al. 2007; Rosenblatt et al. 2011). The clinical use of DC/tumour cell fusions, however, is hampered by the labour-intensive processes required to generate fusion cells for patient-specific treatment. Furthermore, current techniques have relatively low fusion efficiencies, and generate short-lived hybridomas with limited replicative capacity and non-standardised fusion products. These features potentially limit the broader application of DC/tumour cell fusions for cancer immunotherapy in humans.

The generation of stable antigen presenting cell (APC)/tumour hybrid cell lines, which replicate continuously in tissue culture and retain the capacity to induce antigen-specific T-cell responses *in vitro* or *in vivo*, would circumvent most of these problems. We have previously reported that the chemically selectable B-lymphoblastoid cell line, HMy2 (Edwards et al. 1982), can be fused *in vitro* with a range of tumour cells to generate long-lived hybrid cell lines that replicate continuously in tissue culture, and survive repeated freeze/thaw cycles in liquid nitrogen. The hybrid cell lines were phenotypically and functionally stable, showed enhanced immunogenicity in allogeneic mixed lymphocyte/tumour cell cultures (MLTC) compared with the parent tumour cells, and these responses were inhibited by addition of CTLA4-Ig to the cultures, indicating a role of CD80/CD86 costimulation in the enhanced

Abbreviations: ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; APC, antigen presenting cell; CTL, cytotoxic T cell; DC, dendritic cell; EBV, Epstein–Barr virus; HAT, hypoxanthine, aminopterin, thymidine; HLA, human leukocyte antigen; LCL, lymphoblastoid cell line; MHC, major histocompatibility complex; MLTC, mixed lymphocyte/tumour cell culture; MM, multiple myeloma; PBL, peripheral blood lymphocytes; PBMC, peripheral blood mononuclear cells; PCR, polymerase chain reaction; TAA, tumour associated antigen.

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Table 1
Tumour cells and cell lines used in the study.

Tumour designation	Tumour type	Source of tumour cell	Hybrid cell designation
HMy2	EBV B-LCL	Cell line	N/A
ALL-052	B-ALL	*Ex vivo*/marrow	HMy2xALL052
AML-024	AML	*Ex vivo*/blood	HMy2xAML024
AML-050	AML	*Ex vivo*/marrow	HMy2xAML050
KG-1	AML	Cell line	HMy2xKG-1
MM-053	Myeloma	*Ex vivo*/marrow	HMy2xMM053
MM-056	Myeloma	*Ex vivo*/marrow	HMy2xMM056
U266	Myeloma	Cell line	HMy2xU266

EBV B-LCL, Epstein–Barr virus-associated lymphoblastoid cell line; B-ALL, precursor B cell acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; myeloma, multiple myeloma.

allo-immunogenicity of the hybrid cell lines (Dunnion et al. 1999; Walewska et al. 2007). In this study, we demonstrate for the first time that HMy2-derived LCL/tumour hybrid cell lines induce tumour antigen-specific cytotoxic T lymphocyte (CTL) responses to a range of tumour associated antigens (TAAs), including MAGE-A1, NY-ESO-1, WT-1 and survivin, following *in vitro* stimulation of peripheral blood lymphocytes (PBL) from healthy individuals. Furthermore, stimulation of PBL from two tumour-bearing patients with their autologous LCL/tumour hybrid cell lines induced CTLs that killed the patients' own tumour cells *in vitro*. Our data indicate that long-lived LCL/tumour hybrid cell lines present an alternative to DC-based strategies for generating tumour-specific T-cell responses, and may therefore have potential for use in tumour immunotherapy.

Materials and methods

Generation and culture of hybrid cell lines. The EBV-associated B-LCL, HMy2 (Edwards et al. 1982; gift from Prof. Alan Rickinson, Birmingham, UK), was used to generate LCL/tumour hybrid cell lines (Table 1) by fusion *in vitro* with a range of human haematological tumour cells, as described previously (Dunnion et al. 1999; Walewska et al. 2007). Following fusion, HAT and ouabain (both from Sigma, UK) were added to the culture medium at concentrations that were lethal to non-fused parent HMy2 cells or tumour cells respectively. Control experiments confirmed that HMy2 cells and tumour fusion partner cells were unable to grow under the selection conditions used. *Ex vivo* tumour cells used in the generation of hybrid cells lines were purified from blood or bone marrow samples from patients with haematological malignancies (with appropriate ethical approval and informed consent) by positive selection using magnetic beads (Miltenyi Biotech, UK) coated with monoclonal antibodies specific for CD19 (B-ALL), CD33 (AML) or CD138 (MM), according to the manufacturer's instructions, prior to cell fusion.

Flow cytometric analysis. Hybrid cell lines, parent tumour cells and HMy2 were analysed using direct immunofluorescence with FITC- or PE-labelled mouse anti-human monoclonal antibodies to CD40, CD80 (B7.1) and CD86 (B7.2) (Beckman Coulter, UK), HLA class I and class II (DAKO, UK), and HLA-A2 (BB7.2; BD Biosciences, UK). Fluorescently labelled isotype control antibodies were used as negative controls. Flow cytometry was performed on a FACSCalibur flow cytometer and data were analysed using CellQuestPro software (both Becton Dickinson, UK).

Expression of tumour-associated antigens. Expression of TAA (MAGE-A1, MAGE-A3, PRAME, WT-1, NY-ESO-1, MUC-1 and survivin) was assessed by antigen-specific reverse transcriptase polymerase chain reaction (RT-PCR), using primer sequences and cycling conditions as previously described, for survivin (Schmidt et al. 2003), WT-1 and PRAME (Greiner et al. 2004), NY-ESO-1 (Sugita et al. 2004), MAGE-1 and MAGE-A3 (Van Baren et al. 1999), and MUC-1 (Brossart et al. 2001). For each antigen-specific PCR, cDNA from normal peripheral blood mononuclear cells (PBMC) was included as a negative control, and a known positive cell line was included as a positive control. PCR of β-actin was used as a control of cDNA template integrity. Following RT-PCR, the products were separated by agarose gel electrophoresis in the presence of 0.5 μg/ml ethidium bromide (Sigma, UK), and visualised by UV transillumination (UVP, USA).

Induction of tumour antigen-specific CTL in vitro. PBMC were obtained by Lymphoprep separation of heparinised peripheral blood, either from patients whose tumours were used in the generation of hybrid cell lines, or from healthy, HLA-A2+ volunteers. The study had the approval of the Local Research Ethics Committee, and informed consent was obtained. T-cell cultures were established by *in vitro* stimulation of PBMCs, using selected hybrid cell lines as stimulator cells. Responder PBMCs were mixed with Mitomycin-C-treated hybrid stimulator cells, at a ratio of 2:1 in 6 well plates at 2×10^6 responder cells per ml, and in a total volume of 2 ml/well of complete medium. Fresh medium was added on day 3. After 7 days, cells were harvested, counted and re-stimulated with a new batch of Mitomycin-C-treated hybrid stimulator cells in ratio of 3:1 in a total volume of 2 ml/well of complete medium in the presence of 50 U/ml IL-2 (R&D system, UK), with fresh medium added on days 9 and 11. On day 14, and weekly thereafter, responder cells were harvested, counted and re-stimulated as above, at a ratio of 3:1 responder: stimulator cells, in a total volume of 2 ml/well of complete medium in the presence of 300 U/ml IL-2. Fresh medium was added on days 2 and 4 after restimulation.

HLA-A2-peptide pentamer staining. Tumour antigen-specific CTLs were detected by staining T-cell cultures with PE-labelled Pro5 MHC class I pentamers and FITC-labelled anti-CD8, clone LT8 (both from Proimmune Limited, UK), according to the manufacturer's instructions. Flow cytometric analysis was carried out using a FACSCalibur flow cytometer and analysed using CellQuest Pro software (Becton Dickinson, UK). At least 500,000 total events were acquired per sample, with gating on lymphocytes. The HLA-A2-peptide pentamers used were: Survivin, 96–104, sequence LMLGEFLKL; WT-1, 235–243, CMTWNQMNL; MAGE-A1, 278–286, KVLEYVIKV; NY-ESO-1, 157–165, SLLMWITQV; PRAME, 300–309, ALYVDSLFFL.

^{51}Cr release assays. To investigate the antigen specificity of the T-cell lines, T2 cells (Hosken and Bevan 1990) were pulsed with known HLA-A2-restricted, tumour antigen-derived synthetic peptides (Survivin, 96–104, LMLGEFLKL; WT-1, 235–243, CMTWNQMNL; MAGE-A1, 278–286, KVLEYVIKV; NY-ESO-1, 157–165, SLLMWITQV; PRAME, 300–309, ALYVDSLFFL; 50 μg/ml peptide) plus 3 μg/ml β2-microglobulin for 4 h at 37 °C, or with an irrelevant peptide (*P. falciparum* CSP, 334–342, YLNKIQNSL, under identical conditions; all peptides from ProImmune, UK; β2m from Sigma, UK). After peptide loading, target cells were labelled with 100 μCi ^{51}Cr (Perkin Elmer, UK), and used as target cells in ^{51}Cr release assays as described previously (Dunnion et al. 1999).

Statistical analysis. Data were analysed by paired *t*-tests or ANOVA as appropriate, using GraphPad PRISM 5.5 software (GraphPad Software, San Diego, CA, USA). Fisher's exact test was used to study the relationships between TAA expression by the hybrid cell lines, and induction of pentamer-positive T-cell populations and CTL activity in allogeneic PBMCs.

Results

The hybrid cell lines used in the study were generated by fusion of the EBV-associated B-lymphoblastoid cell line, HMy2 (Edwards et al. 1982), with tumour cells or cell lines isolated from

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