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Comparative analysis of MVA-CD40L and MVA-TRICOM vectors for enhancing the immunogenicity of chronic lymphocytic leukemia (CLL) cells

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ABSTRACT

Adenoviral transduction with CD40L and poxviral transduction with B7-1, ICAM-1, and LFA-3 (TRICOM) have been used to enhance the antigen-presenting capacity of chronic lymphocytic leukemia (CLL) cells. This study compares the same vector (modified vaccinia virus strain Ankara (MVA)) encoding CD40L or TRICOM for its ability to enhance the immunogenicity of CLL cells. CLL cells from some patients showed differential responses to each vector in terms of induction of autologous T-cell responses. This study supports the rationale for the use of CLL cells modified *ex vivo* with pre-specified recombinant MVA vectors as a whole tumor-cell vaccine for immunotherapy in CLL patients.

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1. Introduction

Chronic lymphocytic leukemia (CLL) is a malignancy characterized by the accumulation of clonal $CD5^+$ B lymphocytes [1]. With current treatments consisting of combinations of various cytotoxic drugs, CLL remains an incurable disease; resistance to chemotherapy develops in the majority of patients, and significant toxicities are seen [2]. A better understanding of the molecular and cellular mechanisms of CLL is leading to the development of alternative forms of therapy that might efficiently work against the disease. One such therapy involves *ex vivo* modified CLL cells which can be used as a whole tumor-cell vaccine to stimulate an anti-tumor immune response in CLL patients [3]. CLL cells express tumor-associated antigens and major histocompatibility complex (MHC) molecules, but have been shown to be inefficient at antigen presentation due to a lack of expression of T-cell costimulatory molecules on the cell surface [4,5]. Therefore, strategies are being investigated in an effort to increase the immunogenicity of CLL cells for use as a whole tumor-cell vaccine. CD40 activation has been used as one means to increase expression of various costimulatory molecules on the surface of CLL cells and thus enhance their antigen-presenting ability. CD40 ligand (CD40L)-induced signaling has been achieved via coculture of CLL cells with $CD40L^+$ feeder cells [4,6,7], as well as direct gene transfer of CD40L into CLL cells by adenoviral vectors [8,9]. A phase I clinical trial in which adenovirally-CD40L-transduced autologous CLL cells were intravenously infused to patients with CLL showed increases in the number of leukemia-specific T cells following treatment, as well as reductions in leukemia cell counts [9]. Further, combined expression of CD40L and IL-2 or OX40L by CLL cells, via direct or indirect adenoviral transduction, was shown to augment the T-cell activation induced by CD40L alone [10,11].

Replication-defective poxviral vectors encoding for the costimulatory molecules B7-1, ICAM-1, and LFA-3 (designated TRICOM) constitute another strategy to increase the immunogenicity of CLL cells. We have previously shown that both murine and human normal B cells could be infected with a nonreplicative fowlpox vector encoding TRICOM to enhance their APC potency [12,13]. In animal studies, B lymphoma cells transduced with a recombinant fowlpox vector encoding TRICOM induced anti-tumor responses more effectively than non-transduced lymphoma cells [14]. In human cells *in vitro*, we previously compared the ability of replication-defective poxviral vectors encoding TRICOM to infect CLL cells and showed that a recombinant modified vaccinia virus strain Ankara (MVA)-TRICOM vector, but not recombinant fowlpox-TRICOM, was most efficient at enhancing the antigen-presenting capacity of CLL cells

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[15]. MVA-TRICOM-infected CLL cells induced proliferation of autologous T cells and generated cytotoxic T lymphocytes with reactivity against unmodified CLL cells [15].

In light of the positive results from the phase I clinical trial using CD40L-transduced CLL cells and our results with MVA-TRICOM-modified CLL cells *in vitro*, here we evaluated for the first time an MVA vector platform for delivery of CD40L to CLL cells (MVA-CD40L) and compared it to MVA-TRICOM for their ability to enhance the immunogenicity of CLL cells *in vitro*. Our results demonstrated that the novel MVA-CD40L vector is very efficient, even at low multiplicity of infection (MOI), at inducing CD40L-expression on the surface of CLL cells. Moreover, MVA-TRICOM-infected and MVA-CD40L-infected CLL cells appeared equally potent at inducing autologous T-cell proliferation among the patients assayed, although a level of disparity was observed among patient T-cell responses to either vector-modified CLL cells. Therefore, the results from this study further support the rationale for the use of CLL cells modified *ex vivo* with recombinant MVA as a whole tumor-cell vaccine for the immunotherapy of CLL, either by modification with the MVA-CD40L or MVA-TRICOM vector. The choice of vector could potentially be predetermined by *in vitro* analyses prior to therapy.

2. Materials and methods

2.1. PBMCs from CLL patients and healthy donors

Peripheral blood was collected at the University of Pittsburgh Cancer Institute from patients diagnosed with CLL, after informed consent was obtained and following approval by the University of Pittsburgh Institutional Review Board. Demographics of patients included in this study are presented in Supplemental Table 1. Peripheral blood was collected at NIH from healthy donors, after informed consent was obtained and following approval by the NIH Institutional Review Board. Peripheral blood mononuclear cells (PBMCs) were isolated as previously described [15]. Unless otherwise noted, cells were cultured in RPMI 1640 medium (Mediatech, Inc., Herndon, VA) supplemented with 2 mM glutamine, 1× antibiotic/antimycotic solution (Mediatech, Inc.), and 10% human AB serum (Gemini Bio-Products, West Sacramento, CA).

2.2. Recombinant MVA

Recombinant MVA virus expressing genes encoding for the human B7-1, ICAM-1, and LFA-3 costimulatory molecules (designated MVA-TRICOM) has previously been described [15]. MVA-CD40L contains an 870 bp DNA fragment with the open reading frame of human CD40L under the control of the vaccinia virus 40K promoter [16]. Wild-type MVA virus (designated MVA-WT) was used as a control vector. All viruses were obtained as part of a CRADA with Therion Biologics (Cambridge, MA).

2.3. Infection of CLL cells with recombinant MVA

CLL cells were resuspended at 4×10^6 cells/mL in Opti-MEM (Invitrogen, Carlsbad, CA), plated at 2×10^6 cells/well (in 0.5 mL) on a 24-well plate, and infected with MVA virus for 1 h at 37 °C. Following infection, 1.5 mL of prewarmed medium containing 10% human AB serum was added to the cells, and cells were cultured for an additional 24 h. At 24 h post-infection, the time at which cells were harvested for analysis of expression of costimulatory molecules or for setup of proliferation assay, no significant decrease in cell viability was observed for MVA-infected CLL cells versus uninfected control cells. However, MVA is cytopathic and apoptosis of infected cells has been shown at 48–96 h post-infection in other systems [17,18]. For antibody blocking experiments, CLL cells were pre-treated with purified antibody to human CD40L (clone 24–31; eBioscience, San Diego, CA) or control mouse IgG1 (Serotec, Inc., Raleigh, NC) at a concentration of 10 μg/mL for 1 h at 37 °C, then infected with MVA as above in the presence of the same concentration of antibody.

2.4. Flow cytometry

Twenty-four hours after infection, CLL cells were analyzed by flow cytometry for expression of costimulatory molecules. CD19-FITC, CD19-PE-Cy5, CD40L-FITC, CD40L-PE, B7-1-PE, ICAM-1-PE, and LFA-3-PE were purchased from BD Biosciences Pharmingen (San Diego, CA). To determine whether upregulation of costimulatory molecules occurred following infection with recombinant MVA vectors, values were compared to uninfected CLL cells from the same patient. For expression of CD40L and B7-1, which exhibit negative/low expression on uninfected CLL cells, samples which showed a total expression of 10% or less following infection were considered to be negative for upregulation; for samples with a percentage expression of more than 10%, upregulation was defined as an increase of greater than 50% in percentage expression and/or an increase of 2-fold or greater in mean fluorescence intensity (MFI) of expression compared to the uninfected control cells. For expression of ICAM-1 and LFA-3, which exhibit significant expression on most uninfected CLL cells, upregulation was defined as an increase of greater than 20% in percentage expression and/or an increase of 2-fold or greater in MFI of expression compared to the uninfected control cells.

2.5. T-cell proliferation assays

Allogeneic mixed lymphocyte reaction (MLR) and autologous lymphocyte proliferation assays were conducted as previously described [15].

3. Results

3.1. Phenotypic and functional modification of CLL cells following infection with MVA-TRICOM or MVA-CD40L

We previously compared MVA and other replication-defective poxviral vectors encoding for TRICOM molecules and showed that MVA-TRICOM was the most efficient at augmenting the antigen-presenting ability of CLL cells [15]. Here MVA-TRICOM and MVA-CD40L were compared for their ability (a) to increase expression of the costimulatory molecules CD40L, B7-1, ICAM-1, and LFA-3 on the surface of CLL cells and (b) to enhance antigen-presenting capacity of the CLL cells. As shown in Tables 1 and 2, at a multiplicity of infection (MOI) of 5, the optimal dose determined by titration, MVA-CD40L increased expression of CD40L on the surface of the CLL cells, by percentage and/or MFI, in 12 of 12 patients analyzed, while MVA-TRICOM was able to enhance the expression of CD40L on the surface of CLL cells in only 5 of 12 patients analyzed. The expression of B7-1, normally very low among uninfected CLL cells, was enhanced by both vectors in 10 out of 12 samples (Table 2), with 2 additional samples showing upregulation of B7-1 expression only in response to infection with MVA-TRICOM. The pattern of upregulation of ICAM-1 was similar to that of B7-1 in that both vectors upregulated expression of ICAM-1 in 8 of 12 samples, with 4 additional samples responding only to MVA-TRICOM (Table 2). LFA-3 upregulation was more efficient after infection with MVA-TRICOM than with MVA-CD40L, with 11 out of 12 samples showing upregulation in response to MVA-TRICOM and only 4 out of 12 samples showing upregulation in response to MVA-CD40L (Table 2). In general, the MFI of B7-1, ICAM-1, and LFA-3 expression was markedly increased by MVA-TRICOM compared to MVA-CD40L (Table 1).

Despite differences in upregulation of costimulatory molecules on CLL cells, both MVA-TRICOM and MVA-CD40L increased the immunogenicity of CLL cells, as measured by their ability to induce proliferation of allogeneic $CD3^+$ T cells from healthy donors in an MLR (Fig. 1). In 4 patients assayed, infection of CLL cells with either MVA-TRICOM or MVA-CD40L enhanced the proliferation of healthy donor T cells above the levels induced by uninfected CLL cells or CLL cells infected with control vector. MVA-TRICOM infection was greater in enhancing immunogenicity of the CLL cells in 1 of 4 patients analyzed (patient 7, Fig. 1) and slightly inferior to MVA-CD40L in 1 of 4 patients (patient 8, Fig. 1). MVA-TRICOM-infected and MVA-CD40L-infected CLL cells induced similar levels of allogeneic T-cell proliferation in the remaining 2 patients (patients 3 and 5, Fig. 1). CLL cells were subsequently infected with MVA-CD40L at increasing MOI (10 or 20) to determine if the immunogenicity of CLL cells could be further increased. While increased MOI resulted in greater expression of CD40L, it did not correlate with enhanced expression of B7-1, ICAM-1, and LFA-3 on the CLL cells (data not shown). Further, infection with MVA-CD40L at increasing MOI resulted in a decreased antigen-presenting capacity of the CLL cells in an allogeneic MLR (data not shown). We concluded that, at an optimal MOI of 5, both MVA-TRICOM and MVA-CD40L could upregulate costimulatory molecules on CLL cells