Enhanced Generation of Cytotoxic T Lymphocytes Using Recombinant Vaccinia Virus Expressing Human Tumor-associated Antigens and B7 Costimulatory Molecules

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Abstract

In this work, we addressed the possibility to enhance the “in vitro” generation of CTLs recognizing tumor-associated antigens (TAA) by using an inactivated recombinant vaccinia virus encoding B7.1 and B7.2 costimulatory molecules (rVV-B7.1/2). Antigen presenting cells (APCs) infected by rVV-B7.1/2 and pulsed with MART-1/Melan-A27-35 HLA-A2.1-restricted peptide induced significantly higher specific cytotoxic activity than peptide-loaded APCs infected by wild-type VV, both in VV-sensitized and naïve donors. When APCs were infected with a rVV encoding both MART-1/Melan-A27-35 and B7-1/2 (rVV-B7.1/2-M), a significantly more effective CTL generation was observed as compared with cultures stimulated by APCs infected with a VV encoding the TAA epitope only (rVV-M). These enhancing effects were detectable irrespective of a previous VV-specific sensitization. Most importantly, fibroblasts, devoid of antigen-presenting capacity upon peptide pulsing or infection with rVV-M, could be turned into effective APCs after infection by rVV encoding TAA epitopes and costimulatory molecules. In these experiments, using separate recombinant viral constructs, we observed a predominant role of B7-1 as compared with B7-2 in the induction of TAA-specific CTLs. Taken together, our data indicate that replication-incompetent rVV encoding TAA epitopes and costimulatory molecules are able to induce highly effective generation of tumor-specific CTLs. Therefore, these vectors could represent valuable clinical tools for immunotherapy of melanoma patients.

Introduction

The molecular characterization of TAA and an improved understanding of the mechanisms underlying MHC class I- and class II-restricted antigen presentation have opened the way toward active specific immunotherapy trials (1). Optimal stimulation of T-cell responses requires at least two signals. The first is provided by the specific interaction between the MHC-antigen complex and the T-cell receptor. The second, nonspecific signal involves ligands able to trigger the CD28/CTLA4 pathway (2), playing a key role in lymphocyte activation; because T-cell response or anergy can result from the presence or absence of these costimulatory determinants on APCs (2, 3). The obvious potential significance of these findings in tumor immunology is underlined by a wealth of data, mostly obtained upon stimulation of T-cell reactivity or “in vitro” (4–6). rVV encode adjuvants to enhance the capacity of synthetic peptides to stimulate specific CTL “in vitro”; (b) we evaluated the CTL induction potential of rVV coexpressing both TAA epitope and costimulators, as compared with rVV only providing antigen expression; (c) we explored the role played by VV presentation in CTL induction; and (d) we investigated the ability of rVV expressing B7 genes to turn cells, physiologically devoid of antigen-presenting capacity, into effective APCs.

Materials and Methods

Cell Cultures. PBMCs and EBV-BLs from healthy donors were obtained and cultured as described previously (11, 14). These cells were maintained in RPMI 1640, supplemented with 1 mm sodium pyruvate, 2 mm nonessential amino acids, 2 mm L-glutamine, 10 mm HEPES buffer (all from Life Technologies, Inc., Paisley, UK), and 20 μg/ml Ciprofloxin (Bayer, Zurich, Switzerland), thereafter referred to as CM, to which 7.5% (v/v) of pooled heat-inactivated human AB serum (CM-AB) or 10% heat inactivated FCS (CM-FCS) was added.

Freshly purified PBMCs resuspended in CM-FCS were incubated in plastic culture flasks for 2 h to separate the adherent fraction, used as APCs, from the nonadherent cells that represented the responder-effector cells in the CTL priming experiments. Human fibroblasts derived from skin biopsies of healthy donors were cultured in CM supplemented with 20% FCS (12). CV-1 cells (ATCC CCL70) and BSC-40 cells (derived from BSC-1; ATCC CCL26) were used to amplify and titer the viruses.

CD4+ and CD8+ T-Cell Isolation. CD4+ and CD8+ T-cell populations were purified by using a magnetic bead-coupled antibody system (Mylteny Biotec, Bergisch-Gladbach, Germany) according to the manufacturer’s instructions. Purity of at least 95% in the resulting populations was verified by flow cytometry.

rVV Encoding Melan-A/Mart-127-35, B7.1, and B7.2 (rVV-B7.1/2-M). Human B7.1 and B7.2 genes were inserted into the VV genome (rVV-B7.1/2) as described recently (12). Endoplasmic reticulum-targeted Melan-A/Mart-127-35 minigenes were inserted into rVV-B7.1/2 in a different locus (11). Inactivation of viral replication was achieved by PLUV treatment as described previously (15).
**Proliferation Assays.** Nonadherent PBMCs from VV immune or naive donors (10^5 cells) were cultured in CM-AB in the presence of VV-infected autologous adherent cells (2 × 10^6 cells) in 0.2 ml volumes in 96 flat-bottomed wells, in triplicate samples. Cultures were pulsed with ^3H]thymidine (0.5 μCi/well) on day six and harvested on filter paper according to standard procedures 18 h later. Incorporated radioactivity was then measured with a liquid scintillation counter.

**Induction of CTLs.** Adherent cells from healthy donors' PBMCs or primary cultures of skin fibroblasts were infected at a m.o.i. of 10–20 with recombinant or wild-type viruses and cultured overnight in CM-AB (or in CM-FCS for fibroblasts). These infected APCs were then mixed with autologous nonadherent effector cells in CM-AB. After 7 days, one-half of the medium was replaced by fresh CM-AB containing IL-2 (10 units/ml) and IL-4 (0.4 unit/ml). On day 10, cells were restimulated with synthetic peptide (20 μM), pulsed, irradiated (10,000 rad) autologous EBV-BL or fibroblasts, in the presence of the cytokine cocktail described above (11). After an additional cytokine supplementation on day 13, cytotoxic activity was tested on day 17.

**Cytotoxicity Assays.** The cytotoxic activity of the CTL cultures was tested in standard ^51Cr release assays as reported previously (14). As targets, peptide-pulsed or rVV-infected HLA-A2.1* EBV-BL, or selected melanoma cell lines, were used. Specific lysis was measured in triplicate cultures at different E:T ratios.

**Immunophenotyping.** Direct immunostaining was performed by using the following commercially available monoclonal antibodies: FITC-labeled anti-human B7.1 (Immunokontact, Frankfurt, Germany); FITC-labeled anti-human B7.2 (PharMingen, San Diego, CA); and FITC-labeled anti-CD4 and phycoerythrin-labeled anti-CD8 (Becton Dickinson, San Jose, CA).

**TNF-α Release Assay.** PBMC-derived nonadherent cells were stimulated according to the standard priming protocol described above. Seven days after initial stimulation, 10^5 effector cells and 2 × 10^5 stimulator cells (autologous PBMCs, VV-infected, cultured for 15 h and irradiated prior to mixing) were cocultured in triplicates in 200 μl of CM-AB final volumes. After overnight incubation at 37°C, TNF-α release was measured in 100 μl of each culture supernatant using a specific ELISA assay (CLB, Pelikine Compact; human TNF-α ELISA kit; Amsterdam, the Netherlands).

**Results**

**Characterization of VV-responsive and VV-naive Donors.** To evaluate the role of VV presensitization in the generation of immune responses directed against recombinant gene products, initial experiments addressed specific responsiveness in representative healthy donors.

Only serum from immunized donors inhibited the plaque-forming capacity of VV on BSC-40 cell monolayers (data not shown). PBMCs from VV immunized but not naive donors proliferated "in vitro" in response to a viral challenge. Data from representative experiments are depicted in Fig. 1A. Furthermore, when mixed with autologous VV-infected EBV-BL, unstimulated PBMCs from at least one VV-immune donor showed evidence of a modest, but reproducible, cytotoxic activity (Fig. 1B).

**Effects of PLUV Inactivation on VV Antigenic Determinant Expression.** Safety concerns recommend inactivation of viral replication prior to a possible clinical use. Because this process might alter viral immunogenicity, we investigated the viral antigenic profile in cells infected with either a PLUV-inactivated or with the replicating VV form. Upon incubation with VV-immune human serum and staining with FITC-labeled anti-human immunoglobulin antibodies, cells infected with the replicating but not with the PLUV-inactivated virus displayed a strong positivity as observed by flow cytometry (data not shown). On the other hand, lytic activity of VV-specific CTLs was enhanced when target cells were infected with nonreplicating virus (Fig. 1C). These observations confirm the general characteristics of VV-driven immune responses against viral antigens; cellular responses are mostly directed against early expressed proteins, whereas humoral responses predominantly target antigens produced after virus replication (16–18).

**Direct CD8+ Cell Stimulation by rVV-encoded B7.1/2.** B7-mediated costimulation acts predominantly on T-helper cells (19, 20). Indeed, we have reported previously that APCs infected with rVV-B7.1/2 can efficiently stimulate VV-specific CD4+ T-cells in vitro (12, 21).

The direct effect of virally encoded B7.1/2 on CD8+ T-cell activation was evaluated. Using CD4+ and CD8+ purified cells, we compared the capacity of rVV-B7.1/2 or wild-type VV-infected adherent cells to stimulate the antiviral responses of autologous purified
T cells from VV-immune donors. As shown in Table 1, TNF-α release by CD8+ cells was 3-fold higher upon priming by rVV-B7.1/2 as compared with wild-type VV stimulation. Thus, vaccinia-encoded B7 molecules display a potent direct effect on CD8+ cell activation, in the absence of CD4+ T cells. Remarkably, stimulation of a CD4+CD8+ mixed population in vitro does not appear to result in the production of higher amounts of TNF-α compared with CD8+ alone.

Enhanced CTL Priming with Synthetic Peptides Using rVV-B7-infected APCs. The capacity of B7.1/2-transduced APCs to improve the induction of CTLs upon stimulation with synthetic TAA epitopes was then tested. HLA-A2.1-positive adherent cells from VV-immune healthy donors were infected with rVV-B7.1/2 or control VV and incubated in the presence of Melan-A/MART-1-27-35 peptide. Subsequently, they were cocultured with autologous nonadherent cells. Cultures were restimulated and tested as described above. A significantly higher specific CTL generation could be observed (Fig. 2A) in cultures stimulated with APCs infected with rVV-B7.1/2, compared with wild-type VV.

Generation of TAA-specific CTLs by Recombinant Virus Encoding Both Signals: B7 Costimulatory Molecules and Antigenic Epitopes. Next, we engineered the Melan-A/MART-1-27-35 TAA epitope together with B7.1/2 costimulatory molecules in a single recombinant vaccinia virus (rVV-B7.1/2-M). The in vitro immunogenic activity of this construct was compared with that of VV-M, expressing only the epitope (11). PBMC-derived adherent cells were infected with either virus and used as APCs in CTL priming.

B7 ligand expression during the primary stimulation significantly increased the efficiency of TAA-specific CTL generation in both VV-naive and -immune PBMCs (Fig. 2B). Although a lower TAA-specific cytotoxicity, eventually related to anti-VV response, was observed in the immunized donor, this did not prevent the generation of CTLs against the recombinant antigen.

CTL Priming with VV-B7-infected Human Fibroblasts as APCs. Recently, we have demonstrated the possibility to use primary cultures of human fibroblasts infected with rVV-B7.1/2 as APCs to stimulate class II-restricted, virus-specific CD4+ T lymphocytes (12). Based on this background, using these cells as APCs, we attempted the generation of TAA-specific, MHC class-I restricted, cytotoxic responses.

Although adherent PBMC constitutively express low levels of costimulatory molecules, the absence of B7 determinants on short-term cultured fibroblasts ensures higher reliability and relevance to the comparison of the respective effects of B7.1 and B7.2 in our CTL stimulation protocol. Thus, we performed these experiments by using rVV encoding MART-1-27-35 epitope (rVV-M) together with rVV encoding either costimulatory molecule (rVV-B7.1 and rVV-B7.2).

Our data clearly indicate that, upon infection with rVVs encoding a TAA epitope and B7 molecules, skin fibroblasts became capable to efficiently present the relevant class I-restricted antigen and to stimulate the generation of specific CTLs (Fig. 3). This capacity was critically dependent on the simultaneous expression of costimulatory and antigenic molecules because the rVV encoding the antigenic epitope alone was ineffective. Most remarkably, rVV-B7.1 induced a significantly higher specific CTL response as compared with the rVV-B7.2 construct. These results confirm, in MHC class-I restricted responses, the observations made for MHC class II-restricted responses with the same recombinant viruses (12).

All of the Melan-A/Mart-1-27-35 CTLs generated during these experiments were also able to lyse HLA-A2.1-positive (but not HLA-A2-negative) melanoma cell lines expressing this TAA (data not shown).

Discussion

In recent studies, we demonstrated the high capacity of a replication-incompetent rVV encoding a defined human TAA epitope (rVV-M) to induce specific CTLs (11). The ability of a rVV encoding human B7.1 and B7.2 ligands (rVV-B7.1/2) to provide effective costimulation of CD4+ T cells was also shown (12, 21).

Table 1  TNF-α release from VV-stimulated T cells

<table>
<thead>
<tr>
<th>Primary stimulus</th>
<th>CD4+</th>
<th>CD8+</th>
<th>CD4+/CD8+</th>
</tr>
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<tbody>
<tr>
<td>VV-wt</td>
<td>61</td>
<td>47</td>
<td>1.32</td>
</tr>
<tr>
<td>VV-B7.1</td>
<td>170</td>
<td>157</td>
<td>1.09</td>
</tr>
</tbody>
</table>

*Peripheral blood T cells from a VV-immune donor were subdivided into CD4+ and CD8+ populations and stimulated with wild-type (wt) or rVV-B7.1-infected autologous adherent cells. Seven days later, they were cocultured for 16 h together with wild-type infected APCs. Background values related to TNF-α production by cells cultured in the absence of virus (<50 pg/ml) were subtracted.
Capitilizing on this background, we investigated the costimulatory effects of rVV-B7.1/2 on the generation of TAA-specific CTLs. We used it as an “adjuvant” in the presentation of synthetic peptides, and we demonstrated its capacity to enhance the induction of specific CTLs. Remarkably, in a parallel study with computer-defined HLA-A2.1 binding epitopes, rVV-B7.1/2 infection of peptide-pulsed APCs appeared to be decisive for the generation of specific CTLs (22). These data suggest that rVV encoding human costimulatory molecules could be considered as a powerful adjuvant for effective stimulation of cellular immune responses.

We then constructed and tested the functional activity of a rVV simultaneously encoding both antigen and costimulatory molecules. Data reported here demonstrate that coexpression by rVV of the human B7 and MHC class I molecules was shown to be a more powerful costimulator than B7.2.

Part of the strong immunogenic capacity of the rVV-B7.1/2 could be related to the expression of viral antigens, triggering specific antibody and cytotoxic T lymphocytes from healthy donors (25). In different experiments using PBMCs from VV-immune donors, we observed no impairment of the immune response against the recombinant antigen when compared with nonimmunized donors. However, the immune response against the recombinant antigen was not notably affected.

In previous work, the capacity of cells constitutively devoid of APC capacity was demonstrated to stimulate a class II-restricted response after infection with a rVV encoding costimulatory molecules (12, 21). The present results indicate that human skin fibroblasts can be used to generate APCs able to effectively initiate MHC class I-restricted responses upon infection with these rVV. This feature is of relevance for the clinical use of these reagents because it suggests that rVV-B7.1/2-derived vectors might bypass the stringent requirement for highly professional APCs. In this context, experiments comparing the rVV encoding individual B7 gene products indicate that B7.1 is a more powerful costimulator than B7.2.

Taken together, the data reported here indicate that rVV encoding both human B7 molecules and defined TAA epitopes comply with several critical requirements of vaccines for potential clinical relevance: (a) replication incompetent rVV are safe while still displaying a strong immunogenic capacity, enabling generation of TAA-specific CTLs from healthy donors’ PBMCs; (b) their immunogenicity in vitro does not appear to be affected by the immune response against the vaccinia vector itself; and (c) these vectors are able to take advantage of nonprofessional APCs to prime and stimulate specific effector cells.

Acknowledgments

The friendly contribution of Dr. M. Tsichard Eberti is gratefully acknowledged.

References


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