CANCER IMMUNOLOGY MINIATURE

Virotherapy with a Semliki Forest Virus-based Vector encoding IL12 synergizes with PD-1/PD-L1 Blockade

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Disclosure of Potential Conflicts of Interest

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ABSTRACT

Virotherapy and checkpoint inhibitors can be combined for the treatment of cancer with complementarity and potential for synergistic effects. We have developed a cytolytic but non-replicative viral vector system based on Semliki Forest virus that encodes IL12 (SFV-IL-12). Following direct intratumoral injection, infected cells release transgenic IL12, die and elicit an inflammatory response triggered by both abundantly copied viral RNA and IL12. In difficult to treat mouse cancer models, such as those derived from MC38 and bilateral B16-OVA, SFV-IL-12 synergized with an anti-PD-1 monoclonal antibody (mAb) to induce tumor regression and prolong survival. Similar synergistic effects were attained upon PD-L1 blockade. Combined SFV-IL-12 + anti-PD-1 mAb treatment only marginally increased the elicited CTL response over SFV-IL-12 as a single agent, at least when measured by in vivo killing assays. In contrast, we observed that SFV-IL-12 treatment induced expression of PD-L1 on tumor cells in an IFNγ-dependent fashion. PD-L1-mediated adaptive resistance thereby provides a mechanistic explanation of the observed synergistic effects achieved by the SFV-IL-12 + anti-PD-1 mAb combination.
INTRODUCTION

Semliki Forest virus (SFV)-based RNA viral vectors have been developed for vaccination and immunotherapy of cancer (1, 2). These propagation-deficient vectors can accommodate expression cassettes and have been engineered to encode cytokines and other pro-immunogenic transgenes. A SFV vector encoding the heterodimeric murine interleukin-12 (IL12; SFV-IL-12) proteins (3-5) under the control of two independent subgenomic promoters is a powerful antitumor agent when directly injected into experimental tumors (6). The effect is the result of killing tumor cells in an immunogenic fashion, the local effects of the IL12 transgene and the innate immune activation caused by viral RNA, including a powerful type I IFN response (Melero and colleagues, manuscript submitted). In a previous study, we observed that intratumor injection of SFV-IL-12 and agonist costimulatory antibodies directed against the lymphocyte surface receptor CD137 were markedly synergistic (7).

Cancer immunotherapy is currently being revolutionized by the advent of monoclonal antibodies (mAb) blocking PD-1 and its PD-L1 ligand (8-10). Such antibodies exert antitumor effects in numerous mouse models and have shown frequent durable objective responses in melanoma, renal cell carcinoma, non-small cell lung cancer and other malignancies (11-13).

In this study we explored the combination of the local pro-immunogenic effects of SFV-IL-12 along with the relief of the co-inhibitory restraints mediated by PD-1 on the antitumor cytotoxic T-lymphocyte (CTL) response. Potent synergistic effects of the combination were observed with evidence for PD-L1 induction on tumor cells by the IL12-IFNγ axis. Such a train of events results in escape mechanisms that are tackled by anti-PD-1 mAb upon combination.
MATERIALS AND METHODS

Cell lines and animals

The BHK-21 cell line (ATCC CCL-10) was cultured as described previously (4). The murine melanoma cell line B16.F10 and the OVA-expressing variant B16-OVA (H-2b), the MC38 mouse adenocarcinoma cell line and the 4T1 murine breast cancer cell line, were tested to be mycoplasma-free and verified for identity. Five-week-old female C57BL/6 and Balb/c mice were purchased from Harlan Laboratories (Barcelona, Spain). Animal studies were approved by the institutional ethics committee for animal experimentation under Spanish regulations (protocol number 071-13).

Vector production

Plasmids pSFV-enhLacZ and pSFV-enhIL-12 have been described previously (4, 14). Plasmid pSFV-NS3 encodes the cytoplasmic Hepatitis C virus NS3 protein (Smerdou and colleagues, unpublished results). RNA synthesis from SFV plasmids, transfection into BHK-21 cells by electroporation, and packaging of recombinant RNA into SFV viral particles (vp) were performed as described previously (15, 16). Briefly, BHK-21 cells were co-electroporated with recombinant RNA and two helper RNAs (i.e., SFV-helper-C-S219A and SFV-helper-S2 RNAs), which provided in trans the SFV capsid and spike proteins, respectively (16). SFV vp were harvested and purified by ultracentrifugation as described previously (17). Indirect immunofluorescence was applied to SFV-infected BHK-21 cells to determine the titer of SFV-NS3, SFV-enhLacZ, and SFV-enhIL-12 (the last two designated in this manuscript as SFV-LacZ...
and SFV-IL-12, respectively) recombinant virus stocks as described previously (18). Viral vectors were kept frozen at -80°C until use.

**Induction and treatment of tumor nodules**

Mice were subcutaneously (s.c.) injected with B16-OVA, B16.F10, MC38 and 4T1 cells (5 × 10^5 cells per animal) in the right flank. Mice injected with B16-OVA or B16.F10 cells were injected again in the contralateral flank 6 days following the first inoculation in the right flank. Treatments were administered 6-10 days following tumor cell inoculation as detailed for each experiment. SFV-LacZ and SFV-IL-12 were resuspended in 25 μl of PBS and injected intratumorally (i.t.) with 28 G needles. Three doses of 100 μg of anti-PD-1 mAb (clone RMP1-14; Bio X Cell, West Lebanon, NH, USA) and/or anti-CD137 mAb (clone 1D8, kindly provided by Maria Jure-Kunkel, BMS) or anti-PD-L1 mAb (10B5, hybridoma) were administered intraperitoneally (i.p.) as detailed for each experiment. The efficacy of treatment was evaluated by measuring the size of tumor nodules every 3-4 days.

**In vivo killing assay**

Splenocytes from naïve C57BL/6 mice were stained with 5 μM of Violet Proliferation Dye 450 (BD Biosciences, San Jose, CA, USA) and then divided into three different samples. Two of these samples were pulsed with 10 μg/ml of the OVA_{257-264} peptide (SIINFEKL, NeoMPS, Strasbourg, France) or the TRP-2_{180-188} peptide (SVYDFFVWL, NeoMPS, Strasbourg, France) for 30 min at 37°C in 5% CO_2 and extensively washed. The third sample received no peptide as an additional control. OVA-pulsed, TRP-2-pulsed and non-pulsed splenocytes were then labeled with 1μM (CFSE^{hi}), 100nM (CFSE^{med}) or 10nM (CFSE^{low}) of CFSE (Sigma-Aldrich, Dorset, UK) and with Violet
Proliferation Dye 450 (BD Horizon, San Jose, CA) at 10 μM in all cases. The three samples were mixed at the same ratio and injected intravenously (i.v.) (3 × 10^6 cells of each population) into B16-OVA-bearing or naïve mice. Twenty hours later, spleens were harvested and the transferred target cells were identified and gated using Violet Proliferation Dye 450. Specific cytotoxicity was analyzed by flow cytometry and calculated as follows: 100 − [100 × (% CFSE^{hi} or CFSE^{med} tumor-bearing mice / % CFSE^{low} tumor-bearing mice) / (%CFSE^{hi} or CFSE^{med} naïve mice/% CFSE^{low} naïve mice)].

**In vivo induction of PD-L1 expression on tumor cells**

B16-OVA tumors were generated as described above, and inoculated with 10^8 vp of SFV-LacZ, or SFV-IL-12, or an equivalent volume of saline. SFV-IL-12-injected mice also received 1 mg of a blocking anti-IFNγ mAb (clone HB170, hybridoma) or an equivalent volume of saline i.p.. After three days, tumors were harvested and treated with 400U/ml collagenase D and 50μg/ml DNase I (Roche Diagnostics, Indianapolis, IN, USA). After mechanical tissue dissociation, cells were passed through a 70μm nylon mesh filter (BD Falcon, BD Bioscience, San Jose, California, USA) and washed. Cells were also treated with ACK lysing buffer and washed before further use. Finally, cell suspensions were immunostained to assess H2-K^b and PD-L1 expression on gated CD45-negative malignant cells by flow cytometry.

**In vitro induction of PD-L1 expression on tumor cells**

B16-OVA cells were plated in triplicate in 12-well cell culture plates at a seeding density of 5 × 10^4 cells per well. When cells had attached to the wells, three hours later, fresh splenocytes from C57BL/6 mice were added or not at three different B16-
OVA/splenocytes ratios: 1:1, 1:5, and 1:10. Supernatants from SFV-enhIL-12- or SFV-NS3-infected BHK cells were added to the cell co-cultures, in the presence or absence of a blocking anti-IFNγ mAb (clone HB170, hybridoma) at 10 μg/ml. As an additional negative control, B16-OVA cells without splenocytes were similarly treated and analyzed. As a positive control, B16-OVA cells were cultured in the presence of 10³ U/ml of IFNγ (Miltenyi Biotec, Bergisch Gladbach, Germany). Three days later, B16-OVA cells were collected, washed with PBS and analyzed for H2-Kᵇ and PD-L1 surface expression on gated CD45-negative populations.

Flow cytometry

Single-cell suspensions obtained from in vivo growing tumors or in vitro experiments were pretreated with anti-CD16/32 mAbs (clone 2.4G2; BD Biosciences Pharmingen) to reduce non-specific binding to Fc receptors. Then, cells were stained as needed with the following fluorochrome-conjugated antibodies: CD45.2 (100), PD-L1 (10F.9G2) and H2-Kᵇ (AF6-88.5) purchased from Biolegend, San Diego, CA. Dead cells were discarded during the analysis by staining with Sytox Green or 7-AAD (Molecular Probes, Eugene, OR). A FACS-Canto II (BD-Biosciences) was used for cell acquisition and data analysis was carried out using FlowJo software (Tree Star Inc.).

Statistical analyses

All error terms are expressed as standard deviation (SD). Prism software (GraphPad Software Inc., San Diego, CA) was used for statistical analysis. Survival of tumor-bearing animals was represented by Kaplan-Meier curves and analyzed by log-rank tests. Data were analyzed first by the Kolmogorov-Smirnov Normality test. Kruskal–Wallis tests followed by Dunn’s Multiple comparison tests were used to permit
comparisons in experiments with four experimental groups. P values <0.05 were considered to be statistically significant. Synergy index (S) was studied using the formula described by Mazat and co-workers (19).
RESULTS

SFV-IL-12 and anti-PD-1 mAb synergistic effects.

To test the combined effects of intratumoral SFV-IL-12 and anti-PD-1 mAb blockade, we chose the B16-OVA bilateral melanoma model. In this setting, mice were s.c. inoculated twice with tumor cells in each flank with a six day interval between injections. On day 10, SFV-LacZ or SFV-IL-12 were given i.t. in the nodule induced first, along with systemic anti-PD-1 mAb or saline given on days 10, 13, and 17. Figure 1A shows the progression of individual tumors and demonstrates that combined treatment was superior to achieve control of the bilateral experimental disease, resulting in an evident survival advantage (Fig. 1B) with over 80% of mice remaining completely tumor-free at the end of the experiment. According to the formula of synergy index (S) described by Mazat and co-workers (19), we calculated an S value of +9.11, clearly superior to zero. Therefore we can conclude that SFV-IL-12 + anti-PD-1 synergized to reject treated tumors completely.

Such synergistic effects were also observed upon treatment of MC38-derived tumors that were injected with a suboptimal dose of SFV-IL-12 that by itself only cured one of six mice (Fig. 1C). However, upon treatment with the combined regimen, most mice were completely cured in an experimental setting in which anti-PD-1 mAb as a single agent only resulted in a marginal retardation of tumor growth with no survival benefit (Fig. 1D). In this experiment a positive S (+3.88) value was computed thus demonstrating a synergistic rather than a merely additive effect of the combined treatment. Again, a majority of mice treated with the combination were tumor-free at the end of the experiment.
The PD-1/PD-L1 pathway is targeted in the clinic with agents directed against either one of these counter receptors. In our combination regimen, we tested in parallel both options (anti-PD-1 or anti-PD-L1 mAb) and obtained similar results on B16-OVA-derived tumors (Supplementary Fig. S1).

These experiments were extended to the untransfected bilateral B16.F10 tumor model and the 4T1 breast cancer model. In both instances, a less evident but reproducible therapeutic effect was observed with the combined treatment (Supplementary Fig. 2A and B). In the case of 4T1-derived breast carcinomas, a group of mice was treated with a triple combination of SFV-IL-12 i.t. + anti-PD-1 + anti-CD137 mAbs with a trend suggestive of better survival (Supplementary Fig. 2B).

Our data strongly support synergistic therapeutic effects of intratumoral injection of SFV-IL-12 with PD-1 blockade that were exerted both against the SFV-IL-12-injected tumor and distantly implanted lesions that were not injected with the viral vector.

**Immune mechanisms driving the SFV-IL-12 + anti-PD-1 synergy.**

Both SFV-IL-12 and anti-PD-1 mAb rely on amplified CTL antitumor responses to exert therapeutic effects (4, 20). Hence, an alternative explanation of the combined effects could be the induction or amplification of a more robust antitumor CTL response. In the same therapeutic setting of Figure 1A and B, we performed *in vivo* killing experiments testing CTLs against the OVA-derived peptide SIINFEKL and an endogenous melanosomal peptide antigen (TRP-2) also presented by H2-K<sup>b</sup>. Results indicated only a marginal improvement in cytotoxicity with the combination regimen on the CTL response in the case of the TRP-2 antigen but none in the case of the OVA-derived antigen (Fig. 2). In the case of the OVA epitope, SFV-IL-12 as a single agent induced a nearly optimal response, which is difficult to be improved upon by the PD-1
blockade. Of note, PD-1 as a single agent induced a very modest increase in CTL responses, at least in this difficult-to-treat tumor model. Thus, we reasoned that the cooperative mechanism for synergy ought to be different and hypothesized that the virally encoded IL12 could act as an inducer of PD-L1 on tumor cells via IFNγ (20), thereby resulting in a tumor-protective factor against CTLs as elicited by SFV-IL-12. Indeed, as shown in Figure 3A and B, SFV-IL-12 intratumor injection induced bright expression of PD-L1 on CD45-negative tumor cells analyzed by flow cytometry. This was paralleled by a concomitant increase in surface expression of MHC class I molecules. Induction of both MHC-I and PD-L1 only took place with the vector encoding IL12 but not with a similar SFV vector encoding β-galactosidase as a control transgene. Moreover, IFNγ blockade in vivo with a neutralizing mAb abolished the induction of both PD-L1 and MHC-I. Similar results were observed in MC38-derived tumors (data not shown). Therefore, IL12 induction of IFNγ in the tissue microenvironment is responsible for the induction of PD-L1 and MHC-I. While PD-L1 would be detrimental for CTL tumor destruction (21), MHC-I would probably enhance tumor-antigen presentation.

This train of phenomena could be mimicked in cell cultures, for examples, the co-culturing B16-OVA melanoma cells with syngeneic splenocytes as a putative source of IFNγ in response to IL12. Indeed, when such co-cultures were supplemented with virus-free and IL12-containing supernatants from BHK cells that had been infected with SFV-IL-12, tumor cells increased their surface expression of MHC-I (data not shown) and PD-L1, an effect that was also abolished with the addition of an anti-IFNγ mAb to neutralize this cytokine as produced by the co-cultured lymphocytes (Fig. 3C and D).
DISCUSSION

This study provides strong preclinical evidence to combine virotherapy and checkpoint blockade for cancer treatment. Previous results have pointed to the synergistic effects of tumoral infection with Newcastle Disease Virus (NDV) and blockade with anti-CTLA-4 mAbs (22). Immunostimulatory mAbs directed against CD137 to enforce T-cell costimulation are also strongly synergistic with intratumoral vaccinia virus-derived vectors (23) and SFV-IL-12 (7).

Viral vectors, even if not replicative, mimic an acute pathogenic viral infection sending a compendium of alarm signals that call for a CTL-mediated immune response (24). We hypothesize that mimicking viral infection of the tumor should trigger or sustain the proper type of cytotoxic inflammation in the tumor microenvironment. With this rationale in mind, checkpoint inhibitors operating in the malignant tissue can unleash the efficacy of CTL response that had resulted from tumor infection or from the expression of pro-immunogenic transgenes such as IL12.

In our present study, an undesirable loop is elicited by SFV-IL-12, which in an IFNγ-mediated fashion induces PD-L1 on tumor cells. Hypoxia via HIF-1α may also contribute to PD-L1 overexpression (20) since IL12 is known for its antiangiogenic effects (3). We have observed that blockade of such adaptive resistance mechanisms results in powerful synergistic effects that can control distant tumor lesions not treated with the viral SFV vector.

Combinations of local virotherapy and systemic checkpoint blockade are suitable for testing first in metastatic melanoma with accessible cutaneous metastasis in which treatment is provided to one or some of the lesions in an attempt to turn transduced lesions into an in situ cancer vaccine (25). Combined strategies of this kind, based on
knowledge of the immunodynamic interaction of the viral vector and check-point blocking antibody, hold much promise for clinical translation since both types of agents are currently undergoing clinical trials.

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FIGURE LEGENDS

Figure 1. Treatment efficacy of SFV-IL12 + anti-PD-1 combination.

A, C57BL/6 female mice were inoculated subcutaneously with $5 \times 10^5$ B16-OVA cells in the right flank on day 0 and in the left flank on day 6 as indicated in the drawing. They then received an intratumoral injection of saline (top panels), $10^8$ vp of SFV-LacZ (middle panels), or $10^8$ vp of SFV-IL-12 (bottom panels) on day 10 as indicated. On days 10, 13 and 17 mice received i.p. 100 µg of anti-PD-1 mAb (right panels), or an equivalent volume of vehicle (left panels). Black curves represent the follow-up of the directly SFV-IL-12-treated tumor diameters (tumors in the right flank) and red curves represent the follow-up of the non-treated tumor diameter (left tumors). Each curve represents an individual mouse. The fractions in black in the right upper corner of each graph indicate the number of rejected treated tumors and fractions in red the number of rejected contralateral tumors relative to the total number of animals in each group, with the percentage of complete tumor regressions. C, C57BL/6 female mice were inoculated subcutaneously in the right flank with $5 \times 10^5$ MC38 cells on day 0 and then received an intratumoral injection of saline (top panels), or $10^8$ vp of SFV-IL-12 (bottom panels) on day 9. On days 9, 12 and 16 mice received i.p. 100 µg of anti-PD-1 mAb (right panels), or an equivalent volume of saline vehicle (left panels). Each curve represents the evolution of the mean tumor diameter for each individual mouse. The numbers in the right lower corner of each graph indicate the fraction of tumor-free mice on day 80 relative to the total number of animals in each group, and the percentage of complete tumor regressions, respectively. B and D, Kaplan–Meier plots of mouse survival. The SFV-IL-12 + anti-PD-1-treated group was compared with the rest of the groups with the
log-rank test. *p<0.05; ***p<0.001. A and B, the graphs correspond to pooled data of two independent experiments with similar results. α, anti-.

**Figure 2. Minor enhancements in *in vivo* killing assays induced by SFV-IL-12 + antiPD-1 mAb combined treatment.**

C57BL/6 female mice were subcutaneously inoculated with 5 x 10⁵ B16-OVA cells on day 0 and then received i.t. saline or 10⁸ vp of SFV-IL-12 on day 10. On days 10 and 13 mice received i.p. 100 µg of anti-PD-1 mAb or an equivalent volume of saline. On day 17, tumor-antigen peptide-pulsed splenocytes from naïve mice were injected as target cells into treated mice. Twenty hours later mice were sacrificed and spleens collected. The percentage of specific cell lysis was quantified by flow cytometry. Specific *in vivo* cytotoxicity against SIINFEKL-pulsed targets (left) and against SVYDFFVWL (TRP-2)-pulsed targets is indicated for each individual mouse with the mean of the group represented by a horizontal line. *p<0.05; ***p<0.001. α, anti-.

**Figure 3. Induction of PD-L1 expression on tumor cells is mediated by IFNγ.**

A and B, study of PD-L1 and H2-Kᵇ expression on B16-OVA tumor cells. C57BL/6 mice were subcutaneously inoculated with 5 x 10⁵ B16-OVA cells on day 0. On day 10, mice were injected i.t. with saline, or 10⁸ vp of SFV-LacZ, or 10⁸ vp of SFV-IL-12. Additionally, a group of SFV-IL-12-inoculated mice received i.p. 1 mg of anti-IFNγ mAb or an equivalent volume of vehicle. At 72h post-vector inoculation, mice were sacrificed and tumors were excised for evaluation of H2-Kᵇ and PD-L1 surface expression by flow cytometry. A, histograms showing the expression of PD-L1 (left) and H2-Kᵇ (right) on CD45.2-negative tumor cells of one representative mouse per group. B, graphs showing mean fluorescence intensity (MFI) of PD-L1 (left) or H2-Kᵇ
(right) immunostainings gated on CD45.2-negative tumor cells for each individual mouse with the mean of the group represented by a horizontal line. The graphs represent pooled data from two independent experiments with similar results. C and D, study of PD-L1 expression on B16-OVA cells in vitro. B16-OVA cells were cultured alone or co-cultured in triplicate with different ratios of splenocytes from a naïve C57BL/6 mouse. Supernatants of control SFV-NS3- or SFV-IL-12-infected cells were added to the co-cultures. Recombinant IFNγ (10^3 U/ml) as a positive control or anti-IFNγ mAb (10 μg/ml) were added when indicated. C, graph showing the MFI of PD-L1-positive cells gated on CD45.2-negative cells. D, histograms showing the expression of PD-L1 on CD45.2-negative cells as observed in one representative well per condition. n.s.: not significant; *p<0.05; **p<0.01; ***p<0.001. α, anti-. Isot. ctrl., isotype control. Splen., splenocytes. Suprnt., supernatant.
Figure 2
Figure 3

A

B

C

D

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