Sindbis Virus with Anti-OX40 Overcomes the Immunosuppressive Tumor Microenvironment of Low-Immunogenic Tumors

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Despite remarkable responses to cancer immunotherapy in a subset of patients, many patients remain resistant to therapies. It is now clear that elevated levels of tumor-infiltrating T cells as well as a systemic anti-tumor immune response are requirements for successful immunotherapies. However, the tumor microenvironment imposes an additional resistance mechanism to immunotherapy. We have developed a practical and improved strategy for cancer immunotherapy using an oncolytic virus and anti-OX40. This strategy takes advantage of a preexisting T cell immune repertoire in vivo, removing the need to know about present tumor antigens. We have shown in this study that the replication-deficient oncolytic Sindbis virus vector expressing interleukin-12 (IL-12) (SV.II12) activates immune-mediated tumor killing by inducing OX40 expression on CD4 T cells, allowing the full potential of the agonistic anti-OX40 antibody. The combination of SV.II12 with anti-OX40 markedly changes the transcriptome signature and metabolic program of T cells, driving the development of highly activated terminally differentiated effector T cells. These metabolically reprogrammed T cells demonstrate enhanced tumor infiltration capacity as well as anti-tumor activity capable of overcoming the repressive tumor microenvironment. Our findings identify SV.II12 in combination with anti-OX40 to be a novel and potent therapeutic strategy that can cure multiple types of low-immunogenic solid tumors.

INTRODUCTION

Immune checkpoint modulation has shown remarkable promise in treating advanced cancers.1–9 Although high response rates with immune checkpoint blockade have been documented in patients with highly immunogenic tumors, the proportion of patients who respond to treatment is often still low. Major challenges to overcome are the lack of T cell infiltration into the tumor microenvironment as well as the immunosuppressive nature of the tumor, which inhibits the intratumoral immune response.4,5 To improve response rates, combining immune checkpoint inhibitory antibodies with oncolytic virus (OV) therapy has become an attractive and promising novel strategy in patients.6–9 OVs can selectively infect and replicate in tumor cells, resulting in tumor cell lysis. Besides this direct virus-mediated anti-tumor activity, OVs also have the potential to induce an anti-tumor response indirectly by activating immune cells to help them target and kill cancer cells. However, major challenges are still ahead, as most OVs have to be administered intratumorally, which limits their application.10–13 Furthermore, the therapeutic efficacy of an OV is dependent on its ability to directly infect and kill tumor cells, thus narrowing the spectrum of OV therapy.

It has recently been appreciated that the metabolic landscape of the tumor microenvironment may represent an additional resistance mechanism to immunotherapy.14–18 T cell effector responses are energetically demanding, and T cells undergo substantial metabolic reprogramming during activation.19 However, due to the metabolic deregulation of tumor cells, the tumor microenvironment becomes restricted by oxygen and nutrients. Thus, even with a strong immunotherapy, such as with anti-PD-1 or anti-CTLA4 antibodies, once recruited to the tumor site, actively infiltrating anti-tumor T cells from the periphery experience metabolic stress and become dysfunctional. New immunomodulatory strategies are therefore needed to overcome these resistances. Besides the B7 co-inhibitory receptors (e.g., CTLA4 and PD-1), the tumor necrosis factor receptor (TNFR) superfamily contains many other immune checkpoints, which could become the next generation of immune modulators. One of these receptors is OX40, a co-stimulatory receptor that is expressed on activated T cells. Signaling through OX40 promotes survival signals in effector T cells through nuclear factor κB (NF-κB) and nuclear factor of activated T cells (NFAT) activation that enhance the expression of molecules such as survivin, cyclin A, Bcl-2, and cytokines.20 Furthermore, OX40 signaling impairs immunosuppressive regulatory T cell function through direct inhibition of Foxp3 expression.21 Thus, OX40 stimulation has the potential to improve anti-tumor therapy by prolonging survival of pre-existing tumor-specific effector T cells as well as enhancing T cell activation through depletion of immunosuppressive immune cells.

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In this study, we investigated the therapeutic efficacy of a replication-deficient oncolytic viral vector called Sindbis virus (SV). Because SV is a blood-borne pathogen, vectors from this virus can be administered in the bloodstream via the intravenous (i.v.) and intraperitoneal (i.p.) routes, which greatly facilitates their delivery. Furthermore, SV was genetically modified to be replication-defective by splitting its genome and deleting the packaging signal to block viral assembly after viral replication. We show that SV expressing the pro-inflammatory cytokine interleukin-12 (IL-12; SV.IL12) activates T cells as well as enhances the expression of OX40 on CD4 T effector cells and, therefore, potentiates efficacy of the agonistic anti-OX40 antibody therapy. Our data indicate that combination of SV.IL12 and anti-OX40 activates tumor immunity against low-immunogenic tumors through the metabolic rewiring of T cells into highly activated effector cells. This is in line with studies on other TNFRs, such as 41-BB, which reported a metabolic reprogramming of T cells after 41-BB stimulation. Furthermore, SV.IL12 in combination with anti-OX40 induces a marked immune cell infiltration into the tumor microenvironment. Considering that tumors tend to quickly escape the immune response by mutating or losing the expression of drug targets or tumor antigens targeted by the immune response, our treatment approach reduces the risk of developing tumor resistances and offers an attractive and safe strategy to change the immunogenic phenotype of various cancers without prior knowledge of tumor antigens.

RESULTS

SV-Expressing IL-12 Enhances the Expression of OX40 on CD4 T Cells

Previous studies from our laboratory demonstrated that replication-deficient SV.IL12 elicits a therapeutic efficacy in immune-deficient severe combined immunodeficiency (SCID) mice bearing human ovarian carcinoma tumors. Furthermore, Granot et al. demonstrated that the therapeutic efficacy of SV.IL12 was natural killer (NK) cell mediated and enhanced by the presence of IL-12. Due to the fact that IL-12 also plays an essential role in regulating the adaptive immune response, we investigated the therapeutic effect of SV.IL12 in immune-competent tumor-bearing mice (colon cancer; CT26). To exploit SV.IL12 for cancer therapy, tumor cells were i.p. implanted, and after tumor establishment (4 days after tumor cell injection [day 0]), we i.p. injected SV, SV.IL12, or IL-12 on 4 consecutive days (days 1, 2, 3, and 4) for a total of 4 weeks (Figures 1A and 1B). While untreated (control), SV-treated, and IL-12-treated mice succumb to cancer after 3 weeks, treatment with SV.IL12 slightly prolonged survival time, with an overall long-term survival rate of 7.1%. These data suggest that SV-expressing IL-12 is needed to induce the observed therapeutic efficacy. Shortly after i.p. injection, SV infects macrophages in mediastinal lymph nodes where T cells get subsequently activated (Figure S1). Even though SV.IL12-infected cells secrete significant amounts of IL-12, as observed in in vitro experiments (Figure S2A), i.p. injection of SV.IL12 did not significantly change levels of plasma IL-12 in mice (Figure S2B), thus suggesting that IL-12 produced by SV acts locally and stimulates transduced macrophages (Figure S1) that present tumor antigens to correspond-
important for lowering risks of adverse events as well as being more convenient for patients in clinics. Interestingly, therapeutic efficacy in the CT26.Fluc tumor model was maintained with only one injection per week of SV.IL12 and anti-OX40 (Figure S5). This is in contrast with MyC-CaP.Fluc tumor-bearing mice for which the full treatment regimen was required (data not shown). Thus, in the following experiments mice bearing CT26 tumors were treated with one injection a week whereas MyC-CaP tumor-bearing mice received the full treatment regimen.

Combination Therapy Markedly Changes the Transcriptome Signature of T Cells

Due to the fact that OX40 is specifically expressed on T cells, which are crucial players during anti-tumor responses, we assessed the requirement of T cells during SV.IL12 and anti-OX40 (Figure S5). This is in contrast with MyC-CaP.Fluc tumor-bearing mice for which the full treatment regimen was required (data not shown). Thus, in the following experiments mice bearing CT26 tumors were treated with one injection a week whereas MyC-CaP tumor-bearing mice received the full treatment regimen.

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biological processes in T cells that are influenced by this treatment (Figure 3D). Although both upregulated and downregulated DEGs were included in the analysis, the vast majority of pathways were upregulated in T cells treated with combination therapy with the exception of four clusters (transforming growth factor β [TGF-β] signaling, ribosomal biogenesis, translation, and chromatin modification). The upregulated pathways were dominated by DNA replication, chromosomal organization, and cell cycle regulation, but they also included various metabolic and immunological processes, such as mitochondrial respiration, nucleotide metabolism, and adaptive immune responses.

Combination Therapy Enhances Systemic T Cell Responses, Favoring Th1-like ICOS CD4+ T Cells
As T cells from combination therapy express a marked change in their transcriptome signature compared with all other groups, markers for T cell differentiation and activation (e.g., PD-1, ICOS, OX40, TIM3, KLRG1, IL-7 receptor [IL-7R]) as well as T cell lineage transcription factors (e.g., EOMES, TBET, GATA3, BCL6, RORC, FOXP3) were analyzed (Figure 3E). Only T cells from combined therapy expressed the gene signature of terminally differentiated effector T cells, which are characterized by high expression of the killer cell lectin-like receptor G1 (KLRG1) and low expression of IL-7R. Furthermore, genes
encoding products associated with the differentiation and function of effector cells, such as **Batf, Id2, Tbet, Gzmb,** and **Ifng,** were also highly expressed in T cells isolated from mice treated with combined therapy compared with all other groups. The enhancement of effector T cells in combined therapy was confirmed by flow cytometry in both tumor models, as judged by the increased expression of the activation and proliferation markers **CD44** and **Ki-67,** respectively (Figures 3F and 3G; Figure S7). Interestingly, **CD4** T cells also expressed a marked anti-tumor effector phenotype (**ICOS**+/**T-bet**+T cells) that was on average 2- to 3-fold higher during combined therapy compared with **SV.IL12** or anti-**OX40** treatment (Figures 3H and 3I). Previous studies reported a correlation between expansion of **ICOS**+/**T-bet**+CD4 T cells and clinical benefit in cancer patients who received anti-**CTLA4** therapy.3,36,37 In summary, **SV.IL12** in combination with anti-**OX40** induces a marked systemic T cell response and favors the differentiation of terminal effector T cells. Furthermore, combined therapy induces a sustained increase in the frequency of **ICOS**+/**T-bet**+CD4 T cells, which has also been reported to be elevated during successful anti-**CTLA4** cancer therapy.

**CD4 and CD8 T Cells Are Metabolically Reprogrammed in Mice Treated with SV.IL12 and anti-**OX40****

The tumor microenvironment can be a very challenging milieu for an effector T cell, as it is characterized by hypoxia, acidosis, and low levels of nutrient sources such as glucose and glutamine.14,15,38 Even when T cell activation and initiation of effector function are allowed, T cells may be unable to generate the bioenergetic intermediates necessary to carry out effector function in the tumor microenvironment. Thus, providing a metabolic support for T cells is crucial for the success of cancer treatments, as previously reported.39-42 To test whether **SV.IL12** in combination with anti-**OX40** influences the metabolic state of T cells, gene set enrichment analysis (GSEA) of the RNA sequencing data was performed between T cells from combined therapy and control. GSEA analysis showed significantly higher expression of genes involved in oxidative phosphorylation and glycolysis pathways during combination therapy (Figure 4A). To confirm GSEA analysis, peripheral T cells from both tumor models were metabolically profiled using Seahorse analysis on day 7 (Figure 4B; Figures S8A and S8B). Oxidative phosphorylation and glycolytic profiles in T cells from naive, control, and mice treated with **SV.IL12** and/or anti-**OX40** were determined by measuring the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR), respectively. The basal OCR was enhanced in T cells from combined therapy and **SV.IL12** treatment, but only the former harbored a dramatic increase in spare respiratory capacity in the CT26 model (Figure 4B; Figure S8A). This was in contrast to T cells from combined therapy in the MyC-CaP.Fluc tumor model, which expressed 3.75-fold higher basal OCR with no spare respiratory capacity (Figure S8B). The reason for this discrepancy between the two models might be the differences in the number of treatments, as MyC-CaP.Fluc-bearing mice receive 3 and 4 times more injections of anti-**OX40** and **SV.IL12,** respectively.

Analysis of mitochondrial mass (Figure 4C; Figure S8C) and activity (Figure 4D; Figure S8C), using flow cytometry with the mitochondrial stain MitoTracker Green and Deep Red, respectively, revealed that **SV.IL12** with or without anti-**OX40** induced higher mitochondrial mass and activity in CD8 T cells but not in CD4 T cells. These data suggest that the observed increase in basal OCR was mainly driven by CD8 T cells. Interestingly, a slight decrease of active mitochondria occurred in CD4 T cells from mice treated with combined therapy, which might explain the increase in spare respiratory capacity in this group. To test whether the reduction of active mitochondria in CD4 T cells is associated with a switch toward glycolysis, the master regulator for glycolysis c-MYC and basal ECAR were measured in T cells from all groups. Indeed, the addition of anti-**OX40** to **SV.IL12** induced elevated protein expression of c-MYC as well as basal ECAR (Figures 4E and 4F). T cells from naive and control as well as **SV.IL12**- or anti-**OX40**-treated mice showed no signs of elevations. Collectively, these findings reveal that **SV.IL12** induces enhanced oxidative phosphorylation in CD8 T cells, whereas the addition of anti-**OX40** to **SV.IL12** is needed to push CD4 T cells toward glycolysis by increasing the protein expression of c-MYC.

To determine the kinetics of peripheral T cell metabolism during the course of treatment with **SV.IL12** and anti-**OX40,** OCR and ECAR were measured on days 7, 14, and 40 in CT26.Fluc-bearing mice (Figure 4G). As shown above, T cells on day 7 shifted toward a glycolytic state, which is associated with the initial effector phase. Two weeks into the treatment, T cells switched to a highly energetic state utilizing both metabolic pathways, oxidative phosphorylation and glycolysis, as reported for highly activated T cells.40 Once tumors were fully rejected and mice were tumor-free for a month, T cells returned to a...
more quiescent state, such as naive cells. Interestingly, T cells from MyC-CaP.Fluc-bearing mice switched to a highly energetic state early on during treatment (day 7) and remained in this metabolic phenotype 2 weeks after treatment had stopped (Figure S8D). The reason for this discrepancy might be the differences in the number of treatments applied in both tumor models, as MyC-CaP.Fluc-bearing mice received 3 and 4 times more anti-OX40 and SV.IL12, respectively. T cells from control as well as anti-OX40- or SV.IL12-treated mice in both tumor models remained in a quiescent state during the course of treatment (Figure S8E). In summary, SV.IL12 in combination with anti-OX40 metabolically rewires T cells to an energetic state using both metabolic pathways, oxidative phosphorylation and glycolysis. This phenotype does not occur in SV.II.12- or anti-OX40-treated mice, which succumb to cancer. Thus, the changed metabolic state of T cells correlates with an efficient anti-tumor response and better survival rate.

**Metabolic Reprogrammed T Cells in SV.IL12 with Anti-OX40-Treated Mice Display Enhanced CD4-Mediated Cytokine Production and Anti-tumor Activity**

To test whether metabolic reprogrammed T cells in combined therapy possess enhanced effector functions, cytokine production and cytotoxicity were analyzed in T cells isolated from spleens on day 7. Genes encoding pro-inflammatory cytokines IFng and Il2 were upregulated in T cells from mice treated with SV in combination with anti-OX40 (Figure 5A). ELISpot (enzyme-linked immunospot) analysis of IFNγ by splenocytes confirmed RNA sequencing data, showing the strongest IFNγ secretion in mice treated with...
combined therapy in both tumor models (Figures 5B and 5C). Spleenocytes from SV.IL12-treated mice also produced IFN-γ but to a lesser extent. Interestingly, the main producers of IFN-γ were CD4+ T cells, as depletion of CD4 T cells but not CD8 T cells abolished IFN-γ secretion in splenocytes from mice treated with combined therapy.

In addition, RNA levels of the cytotoxic proteases granzyme A and B were upregulated in mice treated with combination therapy compared with all other groups (Figure 5A). Protein expression of granzyme B correlated with RNA levels as measured by flow cytometry in both tumor models (Figures 5D and 5E; Figures S9A–S9D). Furthermore, granzyme B-positive cells were detected in CD8 as...
Figure 6. Mice Treated with SV.IL12 in Combination with Anti-OX40 Display Enhanced T Cell Migration and Intratumoral T Cell Activity

(A and B) CT26.Fluc-bearing mice were left untreated or were treated with SV.IL12 and/or anti-OX40. On day 7, spleens were excised and a single-cell suspension was stained and analyzed by flow cytometry. (A) Percentages of CXCR3 expression by CD4 (left graph) and CD8 (right graph) T cells are shown. Representative flow cytometry plots are shown in (B). (C–F) Tumors were harvested after 2 weeks of treatment from control and treated mice. (C) Intratumoral gene expression of CXCL9 (top) and CXCL10 (bottom) analyzed by real-time PCR. Data are normalized to GAPDH. (D) Intratumoral T cell immune responses from indicated groups were assessed by flow cytometry. Percentages of CD4 expression by T cells (left graph), Ki-67 expression (middle graph), and granzyme B expression (right graph) by CD4 T cells are shown. (E and F) Multiplex

(legend continued on next page)
well as CD4 T cells, suggesting the presence of cytotoxic CD4 T cells in mice treated with combined therapy.44–46 Upregulation of granzyme B was associated with downregulation of the transcription factor Foxo1, which is known to control granzyme transcription through repression of the transcription factor T-bet (Figure 5A).47 Last, the enhanced cytotoxic potential of T cells from combined therapy was also supported by elevated expression of the NKG2D receptor, which has been shown to trigger T cell receptor (TCR)-independent cytotoxicity in activated T cells (Figure 5A; Figures S9E and S9F).48

Having observed upregulation of granzymes and cytotoxic receptors in combination therapy, the function of T cells was investigated using an ex vivo tumor growth assay. Splenocytes obtained from all groups were co-cultured at an effector-to-target cell ratio of 10:1 with either CT26.Fluc (Figure 5F) or MyC-CaP.Fluc (Figure 5G) tumor cell lines. The anti-tumor activity of splenocytes was determined by measuring the luciferase activity of cell lines, which correlates with cell growth. Tumor growth was markedly reduced when co-cultured with splenocytes from mice receiving combined therapy compared with splenocytes from naive, control, and mice treated with anti-OX40 in both tumor models. The anti-tumor activity of splenocytes from mice treated with SV.II12 alone was weaker than that from combined therapy. Surprisingly, tumor growth inhibition was mediated by CD4 T cells, as depletion of CD4 T cells but not CD8 T cells abolished the inhibitory effect on tumor cells. Taken together, these results clearly show that T cells from combined therapy elicit enhanced anti-tumor and functional activity, such as granzyme B and IFNγ production driven by CD4 T cells.

**Mice Treated with SV.II12 in Combination with Anti–OX40 Display Enhanced T Cell Migration and Intratumoral T Cell Immunity**

Only a minority of the total of treated patients respond to current immunotherapy, and the presence of tumor-infiltrating lymphocytes (TILs) has been shown to be one of the main factors that influence the responsiveness toward various therapies in multiple cancers.49,50 Due to the fact that SV-elicited anti-tumor responses do not necessarily require direct infection of the tumor or intratumoral injection, we wondered whether SV.II12 therapy in combination with anti-OX40 could nevertheless alter the local tumor microenvironment and favor intratumoral immunity. To assess whether SV.II12 in combination with anti-OX40 induces T cell infiltration into the tumor, the chemokine receptor CXCR3 on peripheral T cells was analyzed after 1 week of treatment. We observed that in the CT26.Fluc model CXCR3 levels were significantly upregulated on CD4 T cells during combination therapy compared with all other groups, and CXCR3 levels remained elevated during the course of treatment (Figures 6A and 6B; Figure S10A). In contrast, CXCR3 expression on CD8 T cells only appeared later on in treatment, suggesting that CD4 T cells are first recruited to the inflamed site followed by CD8 T cells (Figure S10A). MyC-CaP.Fluc tumor-bearing mice showed elevated levels of CXCR3 on CD4 and CD8 T cells after 1 week of combination treatment (Figures S10B and S10C). Furthermore, SV.II12 treatment also increased CXCR3 expression on T cells but to a lesser extent. The reason for this discrepancy between the two models might be the differences in the number of treatments, as MyC-CaP.Fluc-bearing mice receive 3 and 4 times more injections of anti-OX40 and SV.II12, respectively. Cells expressing CXCR3 follow the gradient of their ligands CXCL9, CXCL10, and CXCL11.51 Indeed, combination therapy also enhanced the production of CXCL9 and CXCL10 in the tumor microenvironment, as judged by real-time PCR, suggesting that CXCR3-positive T cells migrate to the tumor site (Figure 6C). Treatment of SV.II12 or anti-OX40 alone did not alter the expression of these ligands. In line with these observations, an overall increase in T cells was observed in CT26.Fluc and MyC-CaP.Fluc peritoneally disseminated tumors from mice treated with combined therapy compared with control and anti-OX40-treated mice (Figures 6E and 6F).

SV.II12-treated mice also showed enhanced T cell infiltration but to a lesser extent. Furthermore, dissecting CD4 and CD8 T cells by flow cytometry revealed that combination therapy increases the proportion of CD4 T cells in CT26.Fluc tumors, which is consistent with the elevated CXCR3 expression on peripheral CD4 T cells (Figure 6D). These results clearly show that SV.II12 in combination with anti-OX40 alters the tumor microenvironment by facilitating T cell infiltration via modulation of the CXCR3/CXCL9–11 axis. Not only did combination therapy increase T cell infiltration in both tumor models, but CD4 as well as CD8 T cells also demonstrated enhanced functional activity in the tumor, as judged by the Ki-67 and granzyme B expression (Figure 6D; Figures S10D and S11). In line with these results, a decrease in proliferation, as judged by the expression of Ki-67 in tumor cells, was observed in CT26.Fluc and MyC-CaP.Fluc tumor cells when treated with combination therapy compared with all other treatments (Figures 6E and 6F). These results suggest that the presence of activated T cells in the tumor microenvironment exerts anti-tumor activity, which inhibits tumor growth. Besides from T cell activation, we also observed enhanced inducible nitric oxide synthase (iNOS) production in MyC-CaP.Fluc tumors treated with combination therapy compared with control or anti-OX40 treatment (Figure 7). SV.II12 treatment alone also induced iNOS production but to a lesser extent. Interestingly, the amount of iNOS inversely correlated with arginase1 production, suggesting a repolarization of tumor-associated macrophages from the M2-like (pro-tumor) into the M1-like (anti-tumor) phenotype during combination therapy. These trends were only observed in MyC-CaP.Fluc and not in CT26.Fluc tumors, which might be a consequence of SV directly infecting MyC-CaP cells.
In summary, our findings clearly show that even in absence of direct SV tumor targeting, SV.IL12 in combination with anti-OX40 alters the tumor microenvironment in two distinct solid tumors through an indirect and immunity driven mechanism that enhances T cell infiltration and intratumoral T cell immunity.

**DISCUSSION**

We have developed a practical strategy for cancer immunotherapy using an OV and anti-OX40. This strategy takes advantage of the preexisting T cell immune repertoire *in vivo*, removing the need to know about present tumor antigens. We have shown in this study that the combination of replication-deficient SV.IL12 and anti-OX40 amplifies these antitumor T cells and induces their action throughout the body against two distinct solid tumors, reversing effectively local tumor-mediated immune suppression. This effect was specific for combination therapy and was not observed during SV.IL12 or anti-OX40 treatment alone.

Interestingly, both tumor models required different treatment frequencies to achieve the observed therapeutic efficacy of combination therapy. The reason for this discrepancy is not fully understood, but we suggest that it is multifactorial. First, both tumor models are being tested in two different mouse strains, which have different immune responses against infection/inflammation and cancer formation. For example, FVB mice have a Th2 bias in cellular immunity as compared to a strain such as BALB/c. The phenotype bias may, therefore, greatly impact the immunologic surveillance as well as the ability to respond to immune-based cancer therapies, as previously reported.53–55 Furthermore, several studies comparing the sensitivity of FVB and BALB/c mice to a chemical or viral induction of cancer reported that FVB mice are more susceptible to tumor formation than are BALB/c mice.56,57 Taken together, these results suggest that BALB/c mice are naturally more resistant to cancer compared to FVB mice and, therefore, might require less frequent injections of SV.IL12 and anti-OX40. Second, the tumor microenvironment is very different in both tumor models, with differences in the cell type and composition. It has been published that the tumor mutational load correlates with better response rates to immunotherapies.61,62 Prostate cancer models have been described to have low tumor mutational burden and, therefore, are less sensitive to immunotherapies.63–65 In contrast, CT26 colon cancer cells are relatively more immunogenic with a higher tumor mutational burden and show better response rates to immunotherapies.66,67

The synergistic therapeutic effect between peritoneally delivered SV.IL12 and anti-OX40 antibody can be explained by the fact that SV.IL12 induced the expression of the OX40 target on CD4 T cells *in vivo*. The mechanism by which SV.IL12 enhances OX40 expression on CD4 T cells is unclear, but viruses are known to stimulate OX40 expression on anti-viral CD 4 T cells in the presence of their cognate antigen. Cytokines IL-12 and IFNγ are likely to be important, as previously reported by Sagiv-Barfi et al.69 They prolong survival and allow maintenance of these cells. The study of Sagiv-Barfi et al. demonstrated that stimulation with either the Toll-like receptor 9 ligand CpG or the Toll-like receptor 7/8 ligand single-stranded RNA (ssRNA) enhances OX40 expression on CD4 T cells through myeloid-derived cytokine production of IL-12, IFNγ, and TNFα.

Due to the fact that SV.IL12 carries a ssRNA, infects myeloid cells,
and induces IL-12 expression in infected cells as well as induces IFN-γ production in T cells, the same mechanism is likely to apply.

Using SV.IL12 in combination therapy has many advantages compared to other immunostimulatory agents, such as CpG. OVs can promote tumor killing via various mechanisms, including direct tumor lysis, stimulation of enhanced local and systemic immune responses, modulation of the tumor microenvironment, and providing virally encoded immunomodulatory molecules. First, we show that Sindbis replication-deficient OV vector expressing IL-12 overcomes the need to rely on adequate tumor immune infiltration prior to treatment start. Pre-clinical and clinical data have shown that replication-competent OV vectors can markedly increase immune cell infiltration, including macrophages, CD4 T cells, and cytotoxic CD8 T cells into the local tumor microenvironment.8,9,26,90 This “priming” by viral infection can change a “cold” tumor microenvironment into a “hot” one with the influx of a multitude of immune cells and cytokines. Interestingly, immunohistochemistry of CT26.Fluc and MyC-CaP.Fluc tumor sections clearly showed that non-spreading SV.IL12 enhances T cell infiltration in both tumor models. However, the intensity of T cell infiltration was correlated with the ability of SV to transduce tumor cells directly, with much more infiltration in the SV-susceptible tumor model MyC-CaP.Fluc. Second, SV therapy does not require intratumoral injection, which is a potential drawback for many immunotherapies. SV overcomes this issue by being a blood-borne viral vector. It can travel to the tumor microenvironment and bind to overexpressed laminin receptors on cancer cells.73 In addition to its oncolytic potential, SV enters peripheral lymphoid organs, such as mediastinal lymph nodes, where it infects macrophages and activates T cells.32,93 Thus, even in absence of oncolytic activity, SV induces a systemic immune response, which has been shown to be required for effective immunotherapy.74 The addition of IL-12 into SV vector adds an additional support to the immune system, as IL-12 is a potent pro-inflammatory cytokine that promotes CD4 T cell differentiation into Th1 cells as well as directly activates CD8 T cells and enhances their cytotoxic potential.26,75

Our findings reveal that SV.II12 in combination with anti-OX40 induces a dramatic expansion of ICOS+ T-bet+ CD4 T cells, which was not observed in other treatment groups. The expansion of ICOS+ CD4 T cells is of interest, as previous studies also reported expansion of these cells during successful anti-CTLA4 therapy.3,36,74,77 In murine models, ICOS expression during anti-CTLA4 or anti-OX40 therapy was required for inducing optimal anti-tumor responses and tumor rejection, suggesting a crucial role of ICOS+ CD4 T cells on disease outcome.78,79 The increase in the frequency of ICOS+ CD4 T cells was also correlated with clinical benefit in a small cohort of patients with melanoma who received anti-CTLA4 therapy.87 Although many reports indicate a crucial role of ICOS+ CD4 T cells during anti-CTLA4 therapy, the precise function of these cells remains an open question. It is tempting to speculate that expansion of ICOS+ CD4 T cells improves the anti-tumor responses by enhancing T cell infiltration, cytolytic CD8 activity, and T cell memory formation. Addressing these possibilities is of great importance given the fundamental understanding that CD4 help is critical for the development of robust T cell responses, as well as recent findings that peripheral CD4 T cells are critical for effective immunotherapy.74

The high metabolic activity of cancer cells together with the poor vasculature blood supply in the tumor microenvironment can induce nutrient deprivation.14,25,28 These conditions can impair TCR signaling, glycolytic and mitochondrial metabolism, as well as decrease mitochondrial mass, all hallmarks of T effector cells, resulting in impaired anti-tumor effector functions of tumor-specific T cells.39,42 Previous studies have reported the importance of metabolic reprogramming of T cells to rescue mitochondrial function, which resulted in increased anti-tumor responses in the tumor.39,80 Our data in two distinct models of cancer immunotherapy support the notion that SV.II12 in combination with OX40 signaling provides the necessary metabolic support to T cells to generate an efficient anti-tumor response. This metabolic support is characterized most prominently by elevated mitochondrial function and mass in CD8 T cells as well as a switch to aerobic glycolysis in CD4 T cells. Previous reports demonstrated that aerobic glycolysis is crucial for T cell expansion and proliferation during the effector phase as well as promotes Th1 differentiation.19,81,82 These studies are in line with our results, showing enhanced Th1 differentiation and increased proliferation of T cells in mice treated with combined therapy. Furthermore, Myc has been shown to act as a key regulator of aerobic glycolysis in T cells.83 In accordance with that study, T cells from mice treated with SV.II12 in combination with anti-OX40 demonstrated enhanced protein expression of c-Myc compared with all other groups. Thus, our study clearly shows that T cells are metabolically reprogrammed in the periphery during combination therapy. However, further experiments have to be performed to investigate whether the observed metabolic changes in peripheral T cells are also maintained in tumor-infiltrating T cells during SV treatment.

In conclusion, our data strongly show that the therapeutic efficacy of SV.II12 with anti-OX40 is driven by T cell modulation and reprogramming of its metabolic state, in order to enhance the anti-tumor response in the periphery and in the tumor microenvironment. Furthermore, the use of SV allows these metabolically reprogrammed T cells to better infiltrate the tumor microenvironment, which is crucial for an adequate immunotherapy. Anti-OX40 antibody is currently being studied in phase 1 and 2 clinical trials. SV will be tested as a single agent in its first clinical trial in the third quarter of 2020. The results from our current preclinical studies provide a strong rationale for combining SV.II12 with agonistic anti-OX40 antibodies in a therapeutic format in patients with solid tumors.

MATERIALS AND METHODS

Cell Lines

Baby Hamster kidney (BHK), BALB/c colon carcinoma (CT26.WT [ATCC CRL-2638]), and FVB prostate carcinoma (MyC-CaP [ATCC CRL-3255]) cell lines were obtained from the American Type Culture Collection. Fluc-expressing CT26 and MyC-CaP cells
BHK cells were maintained in α-modified minimum essential medium (α-MEM) (Corning Cellgro) with 5% fetal calf serum (FCS, Gibco) and 100 mg/mL penicillin-streptomycin (Corning Cellgro). CT26.Fluc and MyC-CaP.Fluc cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/L glucose (Corning Cellgro) supplemented with 10% FCS, 100 mg/mL penicillin-streptomycin, 7.5 μg/mL puromycin, or 400 μg/mL gentamycin, respectively. All cell lines were cultured at 37°C and 5% CO₂.

**SV Production**

All SV viral vectors used in these studies are replication defective. Vectors were produced as previously described. SV.II.12 plasmid used in this study was published as published in 2002. SV empty is the same plasmid without an additional gene of interest (e.g., IL-12). SV.Luc was generated as described. SV.GFP was generated as published in 2002. Briefly, plasmids carrying the replicon (e.g., Sin Rep-IL12) or DHBB helper RNA were linearized with XhoI. In vitro transcription was performed using the mMESSAGE mMACHINE RNA transcription kit (Ambion). Helper and replicon RNAs were then electroporated into BHK cells and incubated at 37°C in α-MEM supplemented with 10% FCS. After 12 h, the media were replaced with Opti-MEM (Gibco-BRL) supplemented with CaCl₂ (100 mg/L) and cells were incubated at 37°C. After 24 h, the supernatant was collected, centrifuged to remove cellular debris, and frozen at −80°C. Vectors were titrated as previously described.

**In Vivo Experiments and Tumor Models**

All experiments were performed in accordance with the Institutional Animal Care and Use Committee of New York University Health. Six- to 12-week-old female BALB/c mice were purchased from Taconic (Germantown, NY, USA) and age-matched male FVB/NJ mice were purchased from Jackson Laboratory.

**Tumor Inoculation and Animal Studies**

Treatment started on day 4 after i.p. inoculation of 7 × 10⁴ CT26.Fluc cells or 10⁵ cells of MyC-CaP.Fluc in 500 μL of Opti-MEM. For treatments, mice were randomized and SV (10⁷ transduction units [TU]/mL), in a total volume of 500 μL, was injected i.p. into the left side of the animal once for CT26.Fluc and 4 days a week (days 1, 2, 3, and 4) for a total of 4 weeks for MyC-CaP.Fluc-inoculated mice. The immune checkpoint inhibitor anti-OX40 (clone OX-86, Bio X Cell) was injected i.p. into the left side of the animal at a dose of 250 μg per injection (once per week for the CT26.Fluc and three times per week for the MyC-CaP.Fluc tumor-bearing mice). Therapeutic efficacy of the treatment was monitored in two ways: tumor luminescence and survival. Noninvasive bioluminescent imaging was performed using the IVIS (in vivo imaging system) Spectrum imaging system (Caliper Life Sciences) at the indicated time points, and tumor growth was quantified using the Living Image 3.0 software (Caliper Life Sciences) as previously described. Relative tumor growth for each mouse was calculated by dividing total body counts of the first IVIS image. Survival was monitored and recorded daily.

**Flow Cytometry**

For flow cytometry analysis, spleens and tumors were harvested from mice and processed as previously described. The extracted tumors were chopped into small pieces and incubated with a digestive mix containing RPMI 1640 with collagenase IV (50 μg/mL) and DNase I (20 U/mL) for 1 h at 37°C. Tumor samples had additional hyaluronidase V (50 μg/mL) in the digestive mix.

Spleens and digested tumors were mashed through a 70-μm strainer before red blood cells were lysed using ammonium-chloride-potassium (ACK) lysis buffer (Gibco). Cells were washed with PBS containing 1% FCS, and surface receptors were stained using various antibodies. Fluorochrome-conjugated antibodies against mouse CD3, CD4, CD44, ICOS, OX40, CD69, Foxp3, granzyme B, and T-bet were purchased from BioLegend. Fluorochrome-conjugated antibodies against mouse CD8α were purchased from BD Biosciences. MitoTracker Deep Red FM, MitoTracker Green, and MitoTracker Deep Red FM were purchased from Thermo Fisher Scientific. Stained cells were fixed with PBS containing 4% formaldehyde. For intracellular staining, the forkhead box P3 (FOXP3) staining buffer set was used (eBioscience). Flow cytometry analysis was performed on an LSR II machine (BD Biosciences) and data were analyzed using FlowJo (Tree Star).

**T Cell Isolation**

Total T cells were freshly isolated with the EasySep mouse T cell isolation kit. Freshly isolated lymphocytes were depleted of either CD4- or CD8-specific T cells using EasySep mouse CD4⁺ and CD8⁺ selection kits II. Isolation of T cells and depletions were performed according to the manufacturer’s protocols (STEMCELL Technologies).

**ELISPOT**

ELISPOT was performed as previously described. Splenocytes and T cells were prepared as described for flow cytometry. Mouse IFNγ ELISPOT was performed according to the manufacturer’s protocol (BD Biosciences). Lymphocytes (4 × 10⁵ cells) and isolated T cells (8 × 10⁵ cells) were plated per well overnight in RPMI 1640 supplemented with 10% FCS. No additional stimulus was used in the ELISPOT. As positive control, cells were stimulated with 5 ng/mL phorbol myristate acetate (PMA) + 1 μg/mL ionomycin.

**Ex Vivo Cytotoxic Assay**

T cells were isolated on day 7 and day 14 during treatment. T cells (8 × 10⁵/mL) were co-cultured with CT26.Fluc cells (2 × 10⁴/mL) or MyC-CaP.Fluc cells (2 × 10⁴/mL) in a 24-well plate for 2 days in 1 mL of RPMI 1640 supplemented with 10% FCS. Cells were washed with PBS and lysed with 100 μL of M-Per mammalian protein extraction reagent (Promega) per well. Cytotoxicity was assessed based on the viability of CT26 cells, which was determined by measuring the luciferase activity in each well. Luciferase activity was measured by adding 100 μL of steady-Glo reagent (Promega).
to each cell lysate and measuring the luminescence using a GloMax portable luminometer (Promega).

**CD8+ and CD4+ T Cell Depletion In Vivo**

CD8+ T cells were depleted using anti-CD8 antibody (clone 2.43) (Bio X Cell, Lebanon, NH, USA). 0.1 mg of antibody in 0.2 mL of PBS was injected into each mouse, starting 1 day before the first SV treatment, and then every 4 days for 2 weeks. CD4+ T cells were depleted using anti-CD4 antibody (clone GK 1.5) (Bio X Cell, Lebanon, NH, USA). 0.4 mg was injected into each mouse, starting day 1 before the first treatment. Control mice were injected with PBS and isotype controls.

**Quantitative Real-Time PCR**

RNA was extracted from tumor samples using a RNeasy kit (QIAGEN), followed by cDNA synthesis with the iScript II kit (Bio-Rad). qRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad) and a StepOne real-time PCR detection system (Applied Biosystems). PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles (94°C for 30 s, 58°C for 30 s) of amplification. For quantitation, CT values were normalized to GAPDH and expression was analyzed using the 2^-ΔΔCT method. Primers for CXCL9, CXCL10, and GAPDH were used, which included the following: CXCL9 (forward, 5'-GAAGTCCGTGTTCTTCTCC-3', reverse, 5'-TGTACCTCCGTCTCTCAGT-3') and CXCL10 (forward, 5'-GCCTGAACTGCATCCATATC-3', reverse, 5'-AGGAGCCCTTTAGACCTTT-3').

**Transcriptome Analysis of T Cells**

Total RNA was extracted from freshly isolated T cells on day 7 of treatment from spleens using an RNeasy kit (QIAGEN). For each group, three BALB/C mice or three FVB/J mice were used for biological repeats. RNA sequencing was done by the NYU Langone Genomic Technology Center. RNA quality and quantity were analyzed. RNA sequencing libraries were prepared and loaded on the automated HiSeq 4000 sequencing system (Illumina) and run as single 50-nt reads.

**Alignment and Differential Expression Analysis**

Sequences were aligned to the mm10 mouse genome using Bowtie software (version 1.0.0) with two mismatches allowed. Uniquely mapped reads were further processed removing PCR duplicates with Picard ("Picard Tools," Broad Institute, GitHub repository: http://broadinstitute.github.io/picard/). MarkDuplicates and transcripts were counted using HTSeq and differential gene expression was performed between all groups using DESeq. Differences in gene expression were considered significant with an adjusted p value <0.05.

**GSEA and Enrichment Map Analysis**

The network-based method enrichment map was used for gene-set enrichment visualization and interpretation of data. As a follow-up analysis of GSEA2, it reduces redundancy and helps in the interpretation of large gene sets and helps to quickly identify major enriched functional themes in the gene expression data. To perform this analysis, we first assigned a unique row identifier for each transcript and obtained DEGs through DESeq. These genes were then ranked and GSEA was performed in the GenePattern server using the GSEA pre-ranked module. We then obtained the gene identifiers corresponding to the gene names using the Bioconductor package org.Mm.eg.db, and the resulting positively and negatively regulated gene identifiers were used to generate enrichment maps in Cytoscape. The expression heatmap as drawn by Morpheus (https://software.broadinstitute.org/morpheus/). Highest and lowest expressions for each gene (row minimum and row maximum) were displayed as red or blue, respectively.

**Measurement of OCRs and ECARs of T Cells**

T cell metabolic output was measured by Seahorse technology as previously described. Purified T cells were plated at 6 x 10^5 cells/well in an Agilent Seahorse XF24 cell culture microplate. OCR and ECAR were measured using an Agilent Seahorse XFe24 metabolic analyzer following the procedure recommended by the manufacturer (Agilent Technologies). For the mitochondrial stress test, (1) oligomycin (1 μM), (2) carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (1.5 μM), and (3) rotenone (100 nM) and antimycin A (1 μM) were injected sequentially through ports A, B, and C.

**Immunoblot Analysis**

Cells were lysed in M-PER mammalian protein extraction reagent according to the manufacturer’s protocol. Lysates were separated by SDS-PAGE on 4%–12% Bio-Rad gels, transferred to polyvinylidene fluoride (PVDF) membranes, blocked in 5% milk in Tris-buffered saline (TBS) buffer with 0.1% Tween 20 (TBST). Primary antibodies to c-Myc (Santa Cruz Biotechnology) and GAPDH (Thermo Fisher Scientific) were added at room temperature or overnight at 4°C. Secondary fluorescent antibodies (IRDye, LI-COR Biosciences) were added in 5% milk in TBST for 1 h at room temperature. An Odyssey Classic infrared imaging system was used for visualization.

**Histochemistry and Multiplex Immunofluorescence (MIF)**

Tumors of mice were collected, fixed in 4% paraformaldehyde (PFA) for 2 days and embedded in paraffin, sectioned, and H&E stained. For MIF staining and imaging, 5-μm paraffin sections were stained with an Akoya Biosciences Opal multiplex automation kit on a Leica BondRX autostainer, according to the manufacturers’ instructions. Prior to incubation with the first primary antibody, sections underwent heat retrieval with Bond Epitope Retrieval Buffer 2 (Leica ER2, AR9640) and blocking. Primary antibodies in panel 1 were against CD3 (1:200, Bio-Rad, MCA1477T), CD8 (1:2,000, Cell Signaling Technology, 98941S), and Ki-67 (1:200, Abcam, AB16667). Primary antibodies in panel 2 recognized iNOS (1:1,000, GeneTex, GTX130246), Arg1 (1:750, Abcam, AB133357), and F4/80 (1:250, Cell Signaling Technology, 70076S). Each primary antibody was followed by a cocktail of horseradish peroxidase-conjugated secondary antibodies against mouse and rabbit immunoglobulin G (IgG) (RTU, Akoya/PerkinElmer, catalog no. ARH1001) and then tyramide-mediated signal amplification (TSA).
with covalent linkage of the individual Opal fluorophore (each 1:250, Opal 520 [FP1496001KT], 540 [FP1487001KT], 570 [FP1494001KT], 620 [FP1488001KT], 650 [FP1495001KT], or 690 [FP1497001KT], Akoya/PerkinElmer [catalog nos.]) to the tissue antigen. Antibodies were subsequently stripped using either ER1 (Leica, AR9961) or ER2 (Leica, AR9640) heat retrieval buffer, and the next round of immunostaining was initiated. After completion of the sequential incubations and stripping, slides were counterstained with spectral DAPI (Akoya/PerkinElmer, FP1490). Monoplex controls were used to confirm appropriate staining for antibodies integrated into the multiplex panels. Multispectral imaging was performed on a Vectra3 imaging system (Akoya/PerkinElmer) at \(\times 20\). The fluorophore emission signatures were captured by a multispectral camera and then unmixed with INFORM software (Akoya/PerkinElmer). Autofluorescence, obtained from an unstained slide, was removed from the composites and pseudo-colored images were exported as tif files.

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism 7.0 as described in the figure legends. All data are shown as mean ± SEM. Figures were prepared using GraphPad Prism 7, Adobe Photoshop, and ImageJ software. Treated groups were compared with a one-way analysis using GraphPad Prism 7, to naive mice. Differences with a \(p\) value of \(<0.05\) were considered significant: \(*p < 0.05\), **\(p < 0.005\), ***\(p < 0.001\).

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at [https://doi.org/10.1016/j.omto.2020.04.012](https://doi.org/10.1016/j.omto.2020.04.012).

**AUTHOR CONTRIBUTIONS**

I.S., S.O., and D.M. conceived the study. I.S., S.O., C.L., and K.K. designed experiments. I.S., S.O., C.L., K.K., A.M.H., C.P., and M.Y. performed mouse experiments and/or data analysis. I.S., O.S., and D.M. wrote the manuscript. All authors reviewed and edited the manuscript.

**CONFLICTS OF INTEREST**

All authors are employed by NYU Langone School of Medicine and have no employment relationship or consultancy agreement with Cynvec. I.S., A.M.H., C.P., and D.M. are inventors on one or several issued patents and/or patent applications held by NYU that cover Sindbis technology to Cynvec. This work was also supported, in part, by the Experimental Pathology Research Laboratory at NYU Langone, which is partially supported by the Cancer Center Support Grant P30CA016087. The Vectra3 multispectral imaging system was purchased through Shared Instrumentation Grant S10 OD021747. We would like to thank the NYU High Throughput Biology Laboratory for Seahorse usage, training, and assistance. We are grateful to Shanshan Feng for support in viral vector preparation and titration.

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