Universidad de Navarra

Facultad de Ciencias

Study of the involvement of antigen cross-presentation in the antitumor activity of immunostimulatory monoclonal antibodies

Estudio de la implicación de la presentación cruzada de antígenos en la actividad antitumoral de anticuerpos monoclonales inmunoestimulantes

Alfonso Rodríguez Sánchez-Paulete

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Memoria presentada por D./Dª Alfonso Rodríguez Sánchez-Paulete para aspirar al grado de Doctor por la Universidad de Navarra

El presente trabajo ha sido realizado bajo mi dirección en el Departamento de Inmunología e Inmunoterapia y autorizo su presentación ante el Tribunal que lo ha de juzgar.

Pamplona, 27 de marzo de 2018

Dr. Ignacio Melero Bermejo

Casi todos los que desconfían de sus propias fuerzas ignoran el maravilloso poder de la atención prolongada. Esta especie de polarización cerebral con relación a un cierto orden de percepciones afina el juicio, enriquece nuestra sensibilidad analítica, espolea la imaginación constructiva y, en fin, condensando toda la luz de la razón en las negruras del problema, permite descubrir en éste inesperadas y sutiles relaciones.

Santiago Ramón y Cajal

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GENERAL INTRODUCTION

Cancer immunotherapy, named breakthrough of the year by Science in 2013 (1), has drastically changed the landscape of clinical oncology and is immerse in a period of feverish activity. Immune checkpoint blocking monoclonal antibodies (mAbs) have revolutionized clinical oncology and pharmaceutical development, setting the pace of an era in which complete responses are obtained in patients suffering from highly aggressive disease (2,3). Still, not all patients derive benefit from treatment (4). The past decade has seen a great deal of effort invested in the identification of factors that can prospectively predict response to treatment. Among these can be found:

1. The incidence of non-synonymous mutations that give rise to immunogenic neoantigens (5–9), sometimes caused by mismatch repair deficiencies leading to accumulation of mutations (10).
2. The infiltration of immune cells , especially CD8 T lymphocytes, into tumors (11,12).
3. A previously existing immune response in the tumor tissue, as indicated by transcription of IFN-γ response genes and PD-L1 expression (13).

Most of the existing immunotherapeutic drugs operate based on potentiating T-cell activity. However, elimination of tumor cells by antigen-specific T lymphocytes is but the last step of a complex process that involves cellular components of both the innate and adaptive immunity.

The Cancer-Immunity Cycle

To bring together the understanding of the immune responses against cancer that immunotherapeutic drugs aim to potentiate, a model was proposed in 2013 that received the name “Cancer-Immunity Cycle” (14) (Figure 1). This model brought together the events required to achieve tumor eradication by the immune system, dividing them into discrete steps, from tumor antigen release and uptake to T-cell priming, and ending in tumor cell

destruction by T cells. Failure to successfully carry out the tasks involved in this cycle leads to tumor escape and progression (15). It comes as a matter of course that every active tumor exists as a consequence of this failure of the regulatory mechanisms set to stop it, the immune system being one among these.

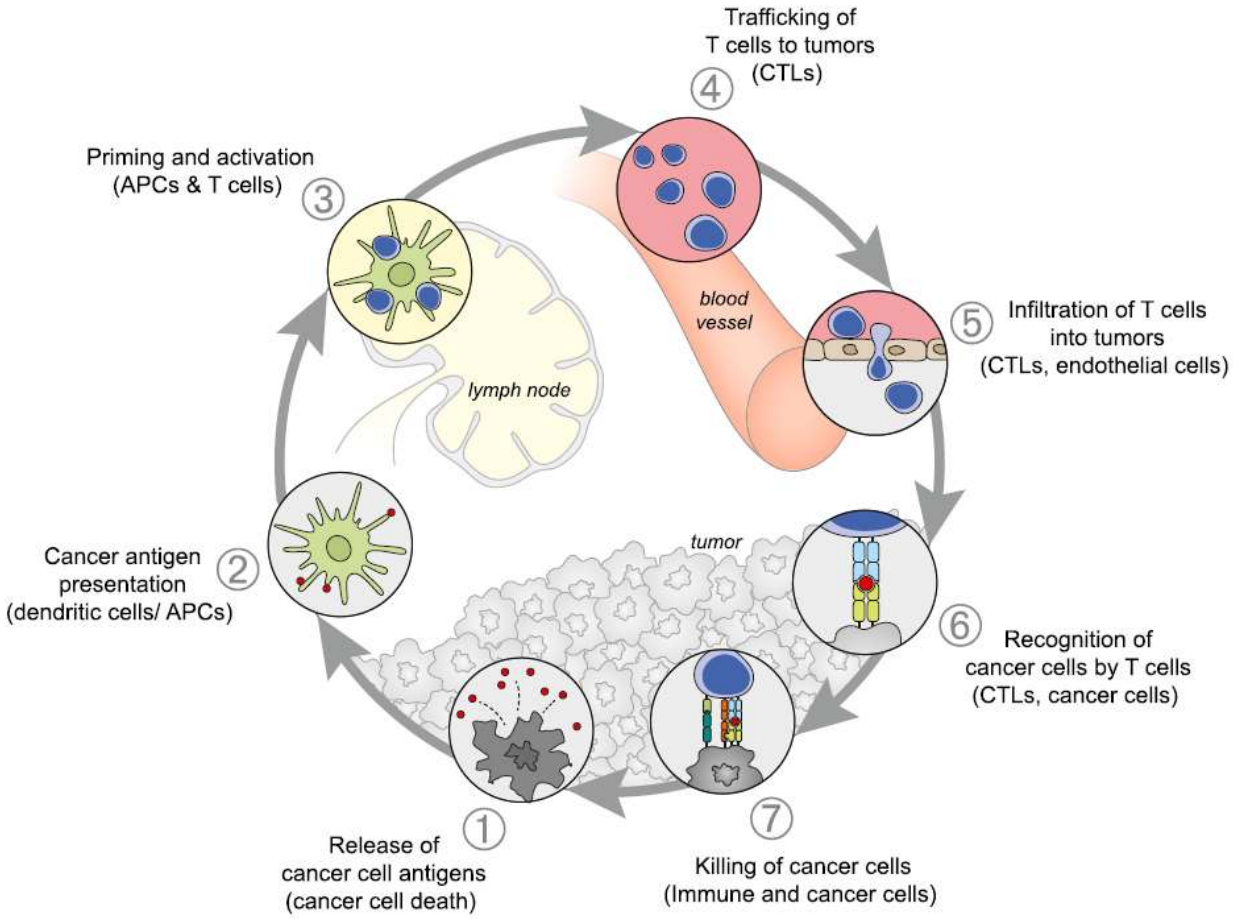

Figure 1. The Cancer-Immunity Cycle (14)

Tumor cell destruction by the adaptive immune system requires the presentation of antigenic peptides on MHC molecules on the surface of tumor cells. These presented antigens originate from unique mutations suffered by the tumor cell (neoantigens) or from aberrant expression of proteins that are normally expressed in immune privileged organs such as testes or embryonic stages of development. These can then be recognized by antigen-specific T lymphocytes. It is CD8 T cells that are best equipped to carry out tumor cell destruction

through recognition of antigen presented on MHC class I (MHC-I). T cells require to undergo a priming step when they first encounter their cognate antigen, which allows them to optimally expand and acquire effector and memory functions. Because tumor cells tend to lose MHC-I expression and because they lack the costimulatory signals required for this T-cell priming process, a different antigen-presenting cell is needed to kickstart CD8 T cell responses against cancer.

Dendritic cells in cancer immunity

Ralph Steinman received a posthumous Nobel Prize in Physiology and Medicine in 2011 for his discovery of dendritic cells (DCs) in 1973 (16). DCs are potent, professional antigen-presenting cells and strong inducers of T-cell activation. Both in humans and in mice, DCs represent a heterogeneous group of cells with different origins, tissue distribution and functions (17,18), and can be grossly divided into three main categories: i) conventional DCs, specialized in antigen presentation; ii) plasmacytoid DCs, that have an important role in antiviral defense thanks to their capacity to rapidly produce high amounts of type-I interferon; and iii) monocyte-derived DCs, ontogenically less related to the previous two, that differentiate into DC-like cells from circulating monocytes under inflammatory conditions. Conventional DCs can be further subdivided into type 1 (cDC1) and type 2 (cDC2) cells, that differ in their ontogeny requirements and functional roles (17,19).

cDC1s are essential players in antitumor immunity. They are ontogenically dependent on the transcription factors BATF3 and IRF8 for their development (20), as shown in *Batf3*$^{-/-}$ and *Irf8*$^{-/-}$ mice, which are completely devoid of cDC1s (21). Elimination of cDC1s in these mice severely impairs CD8 T cell-mediated immunity against syngeneic tumors (22).

In addition to the ontogeny requirement for BATF3 and IRF8, cDC1s express receptors for several cytokines that favor their differentiation and maturation. One of the most important

is Flt3, also known as CD135, the receptor for Flt3L. Flt3 is expressed by mature DCs and DC precursors (23,24). Administration of soluble Flt3L (sFlt3L) to mice or humans leads to expansion of DC subsets (25–28) and can be used as an immune-modulating drug against tumors in mice (27,28). cDC1s also show expression of multiple chemokine receptors, among which CCR7 and XCR1 can be highlighted. CCR7 is required for peripheral tissue-resident DCs to migrate to tissue-draining lymph nodes in response to CCL19 or CCL21 and is expressed by cDC1s in a higher extent than it is by other DCs (29). XCR1 is receptor to XCL1, a chemokine produced by activated T and NK cells, and may serve as a means to bringing cDC1s close to activated T and NK cells for continued priming (30–32).

Homologous human cDC1s can be found in different tissues and are identified by expression of CD141, XCR1 and Clec9a (33–35).

The reasons behind the particularly central role cDC1s play in the Cancer-Immunity Cycle are their outstanding ability to:

i) Capture antigen from apoptotic and necrotic cells, thanks in part to expression of the C-type lectin receptor Clec9a that binds filamentous actin from necrotic cells (36,37).

ii) Process captured antigen to be presented to CD8 T cells on MHC-I molecules (cross-presentation) thanks to a series of molecular adaptations of the endosomal pathway for protein processing (38–41).

iii) Migrate to tumor-draining lymph nodes (TDLNs) in a CCR7-dependent fashion, transporting intact tumor antigen to be cross-presented (27,29,42).

Cross-presentation and cross-priming in cancer

Conventional antigen presentation pathways on MHC molecules are divided in two categories: peptides derived from the proteins synthesized by the presenting cell, that we will call endogenous proteins, are presented on MHC-I molecules to CD8 T cells. This system allows a cell to present peptides from intracellular pathogens such as viruses or intracellular bacteria and elicits a T cell response oriented toward cellular cytotoxicity mediated by CD8 T cells. All nucleated cells in mammals constantly present intracellular peptides on MHC-I. MHC-II antigen presentation to CD4 T cells, on the other hand, is carried out by specialized antigen-presenting cells (APCs): B cells, macrophages and dendritic cells. This pathway allows for presentation of antigens originated from outside of the cell (exogenous antigens). Back to the "self/non-self" logic, this would be useful for presentation of antigens acquired from extracellular pathogens such as bacteria or other parasites and would lead to a humoral response against the pathogen.

There is an additional pathway of antigen presentation that most APCs cannot carry out: antigen cross-presentation (43) (Figure 2). Cross-presentation defines the process through which a cell can present peptides derived from proteins of exogenous origin in MHC-I molecules, instead of routing them to the MHC-II machinery. Antigen cross-presentation is of vital importance for anticancer immunity because most of the cytotoxic activity unleashed by the immune system against tumor cells is performed by CD8 T cells. Thus, the need to have cells able to efficiently present tumor antigen in MHC-I molecules and activate CD8 T cells. The cells that carry out this task, almost exclusively at least in mice, are BATF3-dependent, type 1 conventional dendritic cells, cDC1s. Whether homologous $CD141^{+}$ DCs are as exclusively in charge of cross-presentation in humans remains controversial, since more human DCs seem well equipped for cross-presentation (44,45).

Antigen cross-presentation can be carried out through two different intracellular pathways: the proteasome-dependent cytosolic pathway, and the less frequent proteasome-independent vacuolar pathway (Figure 2). The specific contribution to either to cancer immunity remains to be fully understood.

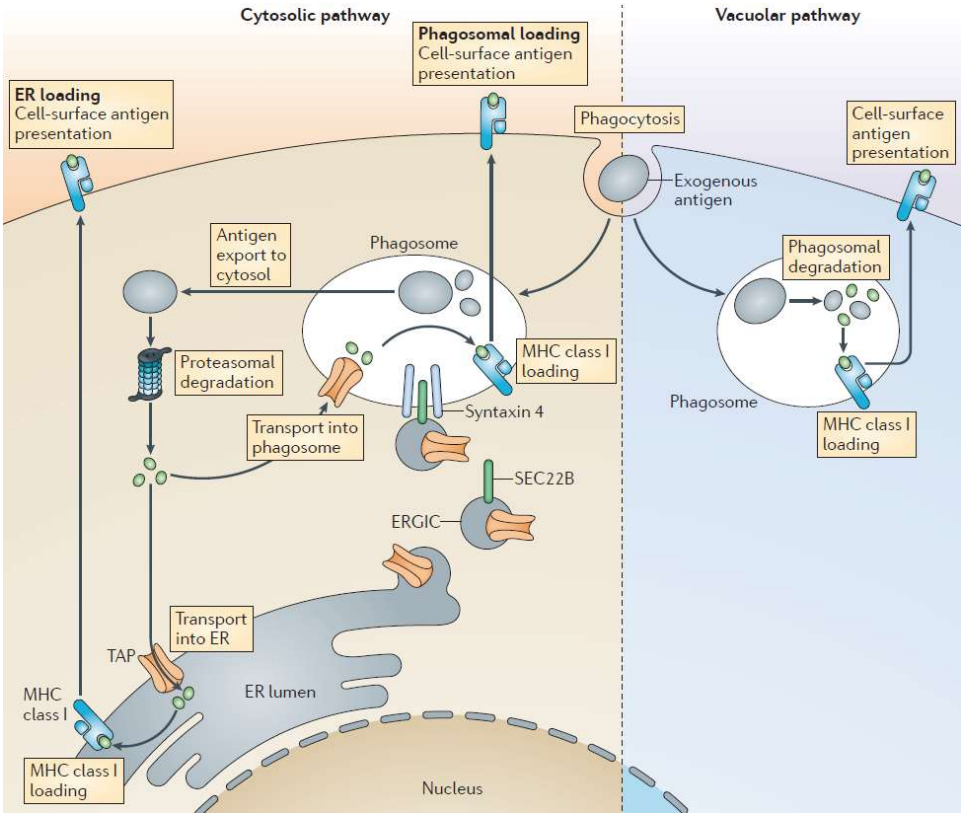

Figure 2. Pathways for antigen cross-presentation (43).

When antigen cross-presentation leads to CD8 T-cell expansion and activation, we speak of T-cell cross-priming (46). T-cell priming requires, besides antigen recognition, the presence of additional costimulatory signals and cytokines (Figure 3, the Three-Signal Model) (47). Dendritic cells are professional cells able to provide all three signals required for T-cell priming, but tumor cells are not (48–50). For this reason, cross-priming of tumor-specific T cells by DCs cross-presenting tumor antigen is key for the kickstarting of an antitumor CD8 T-cell response (51). DCs are, as Ralph Steinman said, “Nature’s adjuvants” (52).

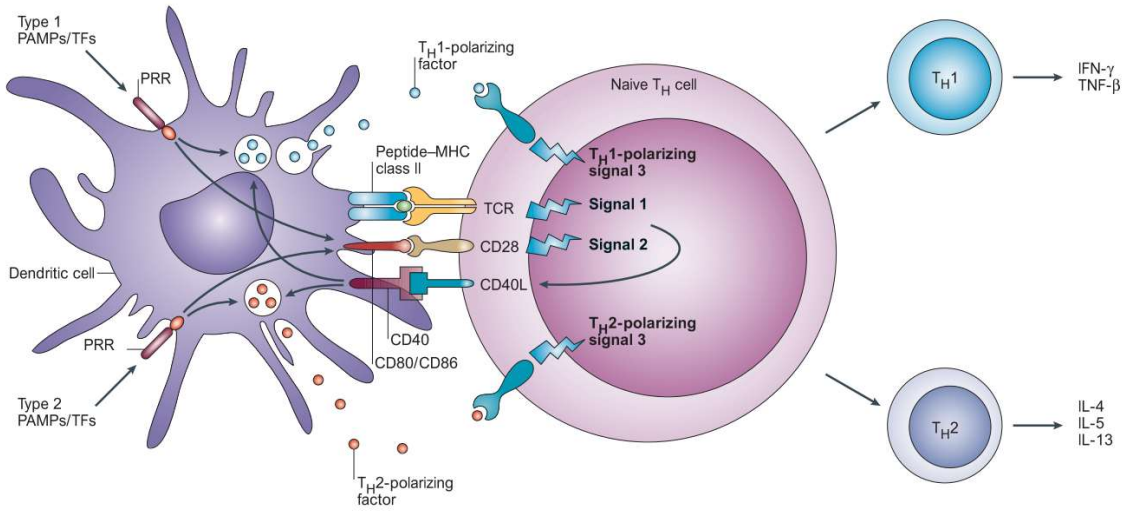

Figure 3. The three-signal model of T-cell activation (47)

For antigen cross-presentation to successfully drive T-cell cross-priming, a DC maturation process must take place that will drive DCs to upregulate antigen-presentation (signal 1) and T-cell costimulation machinery, including surface protein signals (signal 2) and soluble cytokines (signal 3) (53). The signals driving DC maturation include ligands for Toll-like receptors (TLRs) recognizing pathogen- or damage-associated molecular patters (PAMPs or DAMPs, respectively), such as viral RNA (54), bacterial lipopolysaccharide or the nuclear protein HMGB1 that is released upon necrotic or necroptotic cell death (55,56). In absence of maturation signals for DCs, T-cells recognizing their cognate cross-presented epitope will not acquire effector functions and will likely become anergic or apoptotic. This phenomenon is known as cross-tolerance (57,58).

It is important to note that during the maturation process DCs will also highly upregulate PD-L1 and other T-cell checkpoint ligands, as a means to regulate T-cell responses (27,28). The clinical relevance of the expression of these checkpoint ligands on DCs remains to be fully

understood, although expression of PD-L1 in immune cells infiltrating human tumors has predictive value for response to PD-1/PD-L1 blockade (59–61).

The involvement of cDC1s, cross-presentation and cross-priming in cancer immunity is described in depth in the review recently published by our group: "Antigen Cross-Presentation and T-Cell Cross-Priming In Cancer Immunology And Immunotherapy**"**, that can be found attached to this PhD thesis as **Annex 1** (page 97).

Acting on T-cell costimulation/inhibition

Immunotherapeutic modulation of T-cell activity with immunostimulatory mAbs to enhance antitumor activity comes in two complementary flavors (Figure 4) (62).

On the one hand, immunostimulatory mAbs antagonizing T-cell inhibitory molecules, known as immune checkpoints, work by neutralizing signals that refrain T-cell activity in the killing synapse with the tumor or during priming by a professional antigen-presenting cell (a DC, for example) (3). Immune checkpoint activation can lead to T-cell anergy, exhaustion, or apoptosis. The success of immunostimulatory mAbs blocking the interactions of the best-known members of this family, CTLA-4 (63) and PD-1 (64), with their respective ligands (CD80 and PD-L1/PD-L2), revolutionized clinical oncology and paved the way for the discovery of additional T-cell checkpoints (TIGIT, VISTA, TIM3, LAG3…) (65–68). The understanding of the roles each checkpoint molecule play in T-cell inhibition and the possible interactions between them are currently focus of strong R&D investment (69).

On the other hand, agonistic immunostimulatory mAbs directed towards T-cell activating receptors can be used to potentiate and optimize the activity of T cells against cancer. The receptors that can be targeted include members of the TNFR family such as CD137 (4-1BB), CD27 or OX40, as well as members of other families, such as CD28 or ICOS (70). CD137 is induced in activated T and NK cells (71,72), among other cell types, and its engagement has

long-lasting effects in their functional programming (73,74). The biology of CD137 is described in more detail in the review published recently by us "Deciphering CD137 (4-1BB) signaling in T-cell costimulation for translation into successful cancer immunotherapy**"** (75), that can be found attached as **Annex 2** (page 111).

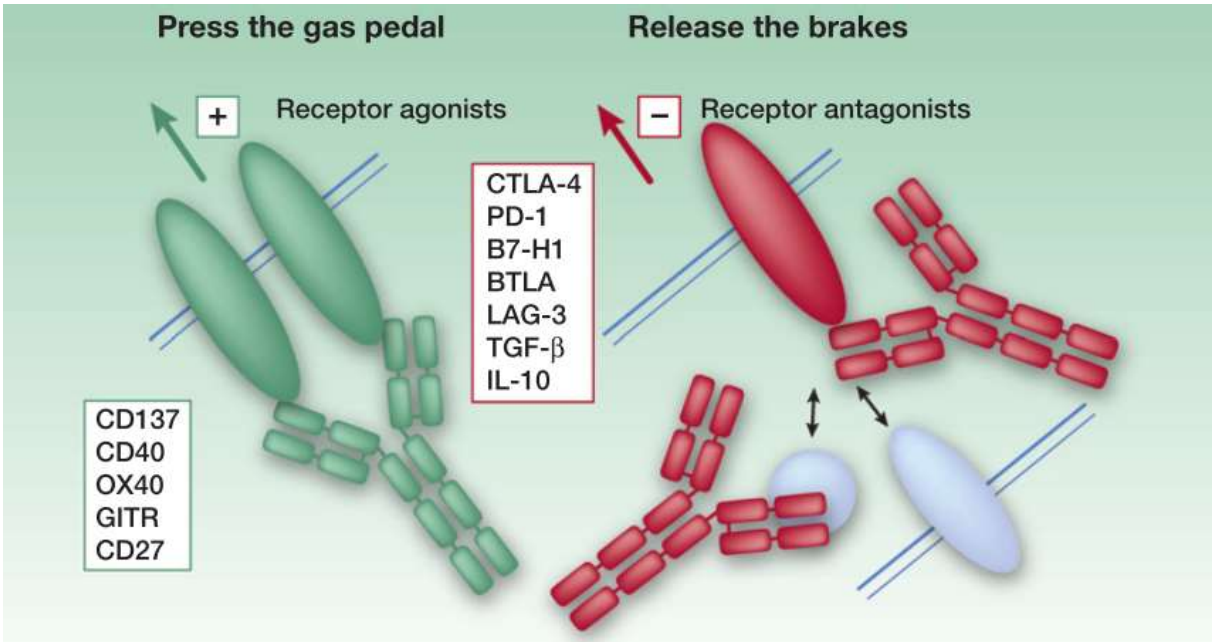

Figure 4. T cell-targeted Immunostimulatory mAbs (62).

Combined targeting of multiple activator or inhibitory receptors on T cells can improve the antitumor activity obtained by either agent separately (62). The most well-known combination treatment, which has been used against melanoma, lung cancer, and cancers from the digestive tract with unprecedented success, is the one making use of PD-1 plus CTLA-4 blockade (76,77). PD-1 blocking agents, especially, are today ubiquitous pipeline partners for other T-cell checkpoint inhibitors and costimulatory receptors, as well as non-immunotherapeutic drugs, in the search for improved combinations against cancer.

Cancer virotherapy

Infection by bacteria or viruses naturally elicits potent immune-activating effects. Cancer immunotherapy has, from its very beginnings, been closely related to the local administration of pathogens into tumors to obtain antitumor responses (78).

Cancer virotherapy defines the therapeutic use of attenuated viruses or viral vectors, usually administered directly into tumors, to achieve antitumor responses (79). Viral infection causes abundant tumor cell death and antigen release, and provides strong activating signals for innate immune cells, which makes it an attractive partner for checkpoint immunotherapy (80). Antigen acquired by activated tumor-infiltrating DCs can then be cross-presented and kickstart antitumor T-cell cross-priming to control tumor growth during and after viral clearance.

Cancer virotherapy strategies encompass two not mutually exclusive categories: oncolytic virotherapy and gene therapy with viral vectors.

Oncolytic viruses for cancer therapy are usually selectively able to replicate in tumor cells, that tend to have suffered modifications in the cell cycle and IFN-I signaling pathway that make them more susceptible for infection (81,82). Some oncolytic viruses are modified to allow for this specificity towards deregulated tumor cells (83), and may still induce transgene expression in infected cells (84).

Viral vectors for gene therapy take advantage of the gene transfer capabilities of viruses to introduce a gene of interest in the tumor microenvironment, added to the tumor cell death induction and adjuvant potency of the chosen vector (85,86). In 2015, FDA approval was granted to talimogene laherparepvec (T-vec), a Herpesvirus coding human GM-CSF, for treatment of metastatic melanoma (87,88), and that was recently shown to improve responsiveness to PD-1 blockade in this disease (89).

Semliki Forest Virus is an enveloped single-strand RNA alphavirus that has been used in the past by others and by us as a viral vector (90,91). The development of SFV vectors has been guided to ensure their safety and reduce the chances for the recombination of the wild-type virus. The current generation of SFV vectors is produced by co-electroporation of three different messenger RNA molecules coding the viral structural and non-structural proteins into BHK cells, which produces infective but non propagative viral particles (Figure 5) (91,92).

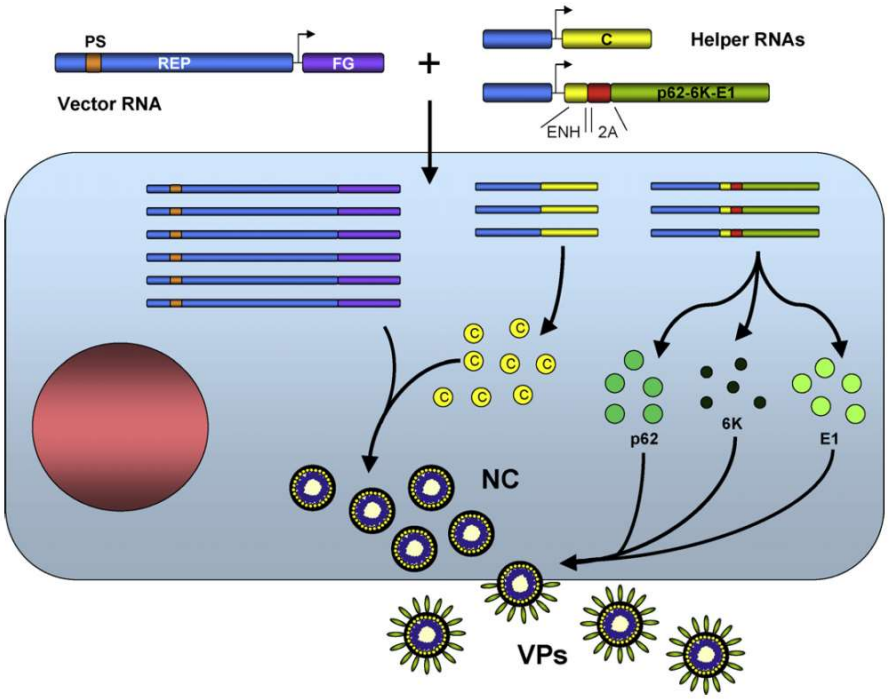

Figure 5. Three-plasmid SFV vector production system (91)

SFV-based vectors are potent tools for cancer immunotherapy: they induce caspase-dependent apoptosis of infected cells (93) and elicit strong type-I interferon (IFN-I) responses while forcing high, transient transgene expression in infected cells (94). Different components of the viral vector activate pattern recognition receptors in the host. However, the key element required for induction of IFN-I responses in hosts seems to be the intracellular RNA receptor RIG-I (95), that recognizes the vector's nucleic acids.

SFV vectors engineered to produce active chemokines and cytokines have been variably successful in cancer immunotherapy using rodent models. An SFV vector encoding mouse IL-12 was previously demonstrated to exert potent antitumor effects when injected intratumorally (96). Combined treatment of SFV-IL12 with anti-PD1 or anti-CD137 showed synergistic effects (97,98). Other transgenes cloned into SFV vectors for use in immunotherapy include IL-15, IL-18 or GM-CSF (91).

SFV has also been used as an oncolytic agent against a number of malignancies in rodent models (99).

OBJECTIVES

In the first part of this PhD project, we hypothesized that, in *Batf3*$^{-/-}$ mice devoid of cDC1s, immunostimulatory mAbs targeting PD-1 or CD137 would not be able to restore T-cell responses against subcutaneous tumors. Conversely, we designed gain-of-function experiments in which we systemically expanded and intratumorally activated DCs to increase T-cell cross-priming to obtain responsiveness to PD-1 and CD137 mAbs in previously unresponsive tumor models.

In a second project included in this thesis, we engineered a SFV vector coding XCL1 and sFlt3L (SFV-XF) for intratumoral administration into subcutaneous tumors in mice. Out hypothesis was that intratumoral injection of SFV-XF would increase tumor infiltration of cDC1s, augment tumor antigen uptake and cross-presentation by these cells and achieve antitumor efficacy through an increase in tumor-specific T-cell cross-priming.

The objectives of this PhD project will be three:

1. To identify the relations between cross-presentation of tumor antigens by dendritic cells and the antitumor activity of immunostimulatory monoclonal antibodies anti-PD-1 and anti-CD137, using subcutaneous tumor models engrafted in *Batf3*$^{-/-}$ mice.
2. To establish a combined immunotherapeutic treatment potentiating cDC1-mediated cross-presentation of tumor antigens for combination with anti-PD-1 and anti-CD137 mAbs.
3. To construct and characterize a Semliki Forest Virus coding XCL1 and sFlt3L for intratumoral immunotherapy of subcutaneous tumors in mice.

RESULTS

Chapter 1

Cancer Immunotherapy with Immunomodulatory Anti-CD137 and Anti-PD-1 Monoclonal Antibodies Requires BATF3-Dependent Dendritic Cells

Sánchez-Paulete AR, Cueto FJ, Martínez-López M, Labiano S, Morales-Kastresana A, Rodríguez-Ruiz ME, Jure-Kunkel M, Azpilikueta A, Aznar MA, Quetglas JI, Sancho D, Melero I. Cancer Immunotherapy with Immunomodulatory Anti-CD137 and Anti-PD-1 Monoclonal Antibodies Requires BATF3-Dependent Dendritic Cells. Cancer Discov. Jan 2016;6(1):71-9. doi: 10.1158/2159-8290.CD-15-0510.

Chapter 2

Intratumoral immunotherapy with XCL1 and sFlt3L encoded in recombinant Semliki Forest Virus-derived vectors to foster dendritic cell-mediated T-cell cross-priming

Sánchez-Paulete AR, Teijeira Á, Quetglas JI, Rodríguez-Ruiz ME, Sánchez-Arráez Á, Labiano S, Etxeberria I, Azpilikueta A, Bolaños E, Ballesteros-Briones MC, Casares N, Quezada SA, Berraondo P, Sancho D, Smerdou C, Melero I. Intratumoral Immunotherapy with XCL1 and sFlt3L Encoded in Recombinant Semliki Forest Virus-Derived Vectors Fosters Dendritic Cell-Mediated T-cell Cross-Priming. Cancer Res. Dec 2018;78(23):6643-6654. doi: 10.1158/0008-5472.CAN-18-0933.

GENERAL DISCUSSION

This PhD project has been oriented to the understanding and exploiting dendritic cell features, specially tumor antigen cross-presentation, in the consecution of therapeutic approaches against subcutaneous tumor models in mice.

This discussion will be divided in two chapters, each commenting on the findings presented in the first and second works that constitute this PhD thesis, followed by a few final commentaries before reaching the conclusions.

Chapter 1. Cancer Immunotherapy with Immunomodulatory Anti-CD137 and Anti-PD-1 Monoclonal Antibodies Requires BATF3-Dependent Dendritic Cells.

Batf3 deficiency leads to loss of CD8α and CD103-expressing cDC1s in mice (22). *Batf3*$^{-/-}$ mice have profound defects in control of tumor growth, because of the poor cross-priming of antitumor T cell responses in these mice. Because T-cell cross-priming is a requisite for the activation of tumor-specific CD8 T cells capable of expressing PD-1 and CD137, we hypothesized that Batf3-dependent DCs would be required for anti-PD-1 and anti-CD137 immunostimulatory mAbs to have antitumor activity in mice.

We demonstrated that the benefit of immunotherapy with anti-CD137 or anti-PD-1 was lost in *Batf3*$^{-/-}$ mice. Even when cross-presentation of tumor antigens is a most prominent capability of cDC1s, these cells are also strong producers of Th1-polarizing cytokines upon stimulation. IL-12 is a clear example of these (100–102) and a potent element of antitumor immunity that has been utilized in cancer immunotherapy in various forms (103). To rule out a deficiency in IL-12 as responsible for the lack of response of *Batf3*$^{-/-}$ mice to therapy, we performed intratumoral injection of IL-12 in

combination with systemic anti-CD137. IL-12, indeed, potentiated the response to anti-CD137 in wild-type mice. However, in absence of Batf3-dependent DCs, the same therapeutic dose of i.t. IL-12 was unable to overcome unresponsiveness to anti-CD137 therapy. These data showed that deficiency of Batf3-dependent DCs generates a more profound defect in antitumor immunity than exogenous administration of IL-12 can correct.

We suspected that CD8 T-cell cross-priming was the deficiency causing the loss of efficacy of the immunostimulatory mAbs. Therefore, we examined the capacity for tumor antigen cross-presentation by tumor-draining lymph node dendritic cells (TDLN DCs) and found a marked decrease in such function in *Batf3*$^{-/-}$ as compared to wild-type mice. Accordingly, the increase in number and activation status of antitumor CD8 T cells in response to therapy with anti-CD137 alone or in combination with anti-PD-1 did not take place in *Batf3*$^{-/-}$ mice *in vivo*. These data confirm the essential involvement of Batf3-dependent DCs in cancer immunity and show that the cross-priming of antitumor responses is a prerequisite for response to the T-cell oriented agents anti-CD137 and anti-PD-1.

In a complementary approach, we hypothesized that enhancing the same functions *Batf3*$^{-/-}$ mice lacked, and the loss of which compromised response to therapy, would synergize with treatment with the immunostimulatory mAbs anti-CD137 and anti-PD-1 in hard-to-treat tumor models such as B16-OVA and B16F10. To this end, we designed a treatment strategy encompassing systemic expansion of DCs via a gene therapy solution leading to an increased production of soluble Flt3L, and DC activation within tumor lesions through intratumoral injection of the TLR3 agonist Poly-ICLC (Hiltonol, Oncovir). Combinations of Hiltonol and Flt3L are currently being tested in clinical trials against

several malignancies and in combination with DC vaccines, immunostimulatory mAbs and radiotherapy. It is worth noting that the group of Miriam Merad from Mount Sinai Hospital, New York City, used the same treatment strategy against BRAF-driven mouse melanomas at the same time we did, and published it shortly afterwards (27). A set of experiments that can be found in their work includes the separate use of Flt3L and Poly-IC in experiments in vivo, demonstrating that the effect of either treatment element on its own was synergistically enhanced by their combination.

Treatment with sFlt3L and Poly-ICLC potentiated the CD8 response against B16-OVA, as measured by detection of CD8 tumor-infiltrating lymphocytes (TILs) recognizing the SIINFEKL OVA epitope. SIINFEKL-specific T-cells expressed CD137 and PD-1 to a higher extent than the bulk of CD8 TILs, consistent with a highly activated phenotype, and suggesting the possibility of targeting these molecules to further increase treatment efficacy. Accordingly, addition of anti-CD137 or anti-PD-1 to the DC-potentiation cocktail increased responsiveness of mice against B16-OVA tumors, with maximal efficacy obtained with the combination of all treatment elements. The question was raised that the high immunogenicity of this OVA-expressing tumor model might be artificially affecting response to treatment. To tackle this issue, we implanted mice with B16F10 tumors, which do not express OVA and are very poorly immunogenic and completely unresponsive to immunostimulatory mAbs. A very significant retardation of tumor growth could also be observed in B16F10-bearing mice when treated with the full combination of sFlt3L, poly-ICLC, anti-CD137 and anti-PD-1.

Both Flt3L and Poly-ICLC act on cells other than Batf3-dependent DCs: Flt3L mobilizes plasmacytoid and IRF4-dependent conventional DCs (104), and Poly-ICLC can trigger activation of innate immune cells expressing RIG-I or MDA-5 (105) and can have direct

antiproliferative effects on tumor cells (106). However, *Batf3*$^{-/-}$ mice bearing B16-OVA tumors and treated with the same sFlt3L-Poly-ICLC cocktail did not establish a CD8 T-cell response against SIINFEKL, and a recovery of response could not be achieved in these mice with the DC-potentiation combination treatment. This observation further highlights the unique and central role Batf3-dep DCs play in the cross-priming of antitumor responses and response to immunotherapy strategies also based on DC mobilization and activation.

The relevance of this work is derived from:

1. The identification of a key cellular component (Batf3-dependent cDC1s) driving response to immunotherapy with immunostimulatory agents anti-CD137 and anti-PD-1.
2. The design of a successful treatment strategy (systemic sFlt3L plus local Poly-ICLC) able to achieve antitumor response to immunotherapy with anti-CD137 and/or anti-PD1 in previously unresponsive or poorly responsive tumor models.

The involvement of cDC1s in T-cell antitumor responses had been previously shown (22,107). However, the necessary involvement of cDC1s in response to immunotherapy with anti-PD-1 and anti-CD137 in mice had not been explicitly demonstrated before the publication of this work.

Previous work had identified tumor infiltration by cDC1s as a factor predicting longer survival of cancer patients (42), and additional reports have shown correlation between cDC1 and NK or CD8 T-cell infiltration (32,108). Whether cDC1 presence in tumors, or cross-priming of antitumor T cells by cDC1 cells, predicts response to immunotherapy in cancer patients will be a very important piece of data for the understanding of the

variable outcomes of immunotherapy agents, especially those blocking PD-1/PD-L1 interaction, and the design of rational strategies to push forward the efficacy of these agents.

Chapter 2. Intratumoral immunotherapy with XCL1 and sFlt3L encoded in recombinant Semliki Forest Virus-derived vectors to foster dendritic cell-mediated T-cell cross-priming

Virotherapy strategies for cancer treatment can be grossly divided into two categories, not always mutually exclusive: oncolytic virotherapy and gene therapy with viral vectors. Oncolytic virotherapy typically makes use of modified viruses in which a specificity towards cancer cell infection and destruction is achieved by the removal of viral elements in charge of dysregulating cell cycle, so that viral replication will only take place in cells in which cell cycle regulation is already damaged; in this case, tumor cells. To the reduction in the number of live tumor cells following viral infection is added the adjuvant effect the presence of the virus has on the immune system, activating the type I IFN system. Activation of DCs in the context of abundant tumor cell death and antigen release should result in increased priming of tumor-specific T cells. This is as analogous approach to the one used in the first chapter, in which tumor-infiltrating immune cells were activated using poly-ICLC, that in fact mimics a viral infection.

Among the molecules introduced in viral vectors for use in immunotherapy can be found cytokines aimed to polarize myeloid and T-cell populations towards a phenotype that can resist tolerization and anergy in the tumor microenvironment to obtain potent

cytotoxic activities (85,86). T-vec (Sipuleucel-T) is a Herpesvirus vector coding human GMCSF that was recently shown to induce responsiveness to PD-1 blockade in melanoma patients. A Semliki Forest Virus coding mouse IL-12 (SFV-IL12) has antitumor activity against B16-OVA subcutaneous tumors in mice and can be used in combination with anti-CD137 and anti-PD-1, synergistically enhancing the effects of either treatment alone (97,98).

We chose sFlt3L and XCL1 as genes of interest for our SFV vector (SFV-XCL1-sFlt3L or SFV-XF). cDC1s are dependent on Flt3 engagement for differentiation and survival *in vivo* (109). Systemic treatment with sFlt3L is a very interesting cancer immunotherapy approach, as we have shown in the first chapter of this PhD project and others have shown before. Induction of expression of sFlt3L by tumor cells has also been used for cancer vaccination purposes (110). XCL1 is a chemokine whose receptor, XCR1, was recently discovered to be expressed exclusively on Batf3-dependent DCs (30). XCL1 is produced by activated CD8 T cells and NK cells (111,112). The XCL1-XCR1 axis is probably involved in sustaining contacts between DCs and activated T and NK cells for continued priming (32,112).

Both Flt3L and XCL1 transgenes had been used in cancer virotherapy before. An adenovirus expressing Flt3L is active against different mouse tumor models in vivo (113,114). However, transgenic expression of XCL1 in a similar approach failed to elicit antitumor responses in an earlier work (115), a result that in fact we replicated in this project. Our original hypothesis was that antitumor responses would be obtained via an augmentation of DC infiltration into subcutaneous tumors injected with SFV-XF, and the subsequent increase in the cross-priming of antitumor T-cell responses. Although we did see expansion of DC populations in tumor-draining lymph nodes after repeated doses of

SFV-XF and robust antitumor responses were obtained, we did not detect the sought increase in DC tumor infiltration.

The differences in antitumor efficacy between SFV-sFlt3L and SFV-XF were small, but significant and robust across several experiments. We chose to remain with SFV-XF during this study after comparing both virus side-by-side against MC38 tumors and achieve slightly better tumor growth delay with SFV-XF.

The SFV-XF vector successfully elicits functional transgene expression in mouse tumor cell lines in vitro and in subcutaneous tumors in vivo. We observed a delay in the growth of MC38, B16F10- and B16-OVA-derived subcutaneous tumors when they were injected intratumorally with three doses of 10^8 SFV-XF viral particles, as compared to a control SFV vector.

Strikingly for us, we did not observe synergistic activity between the antitumor effects of SFV-XF and those of anti-CD137 or anti-PD-1 against MC38. This is, however, in consonance with the failure of SFV-XF treatment to increase T-cell infiltration into MC38 tumors and with the failure of existing infiltrating T cells to increase their expression of the activation markers and therapy targets CD137 and PD-1. Still, some mutual enhancement between treatment regimens (SFV-XF and anti-CD137 or anti-PD-1) was observed in B16-OVA tumor models, but it was observed in similar degree in combination with SFV-LacZ control vectors (data not shown), pointing at the IFN-I triggering capacity of the SFV vector as the reason for synergy. Also, the SFV-LacZ control vector caused B16-OVA and B16F10 tumor delay, but was innocuous against MC38, indicating differences in the biology of both tumor models, maybe regarding sensitivity to IFN-I. These differences in model behavior upon SFV vector administration in fact highlight the relevance of the efficacy of treatment with SFV-XF in these tumors.

It is puzzling to observe the different outcomes that both DC-enhancing approaches taken during this PhD have had in combination with anti-CD137 and anti-PD-1 mAbs (sFlt3L + Poly-ICLC on the one hand, and SFV-XF on the other). The reasons behind this divergence are not know to us at the time. However, it must be noted that, in B16-OVA melanomas, both Flt3L + poly-ICLC and the intratumoral administration of SFV-derived vectors enhanced the efficacy of either mAb. In the case of MC38, we have observed a different pattern of responses against the agents tested, specially SFV-LacZ, but we did not test responses against the Flt3L + Poly-ICLC combination. It should be of great interest to explore whether the success of intratumoral therapy with TLR agonist agents and their ability to potentiate T-cell responses depend on tumor-intrinsic parameters such as antigenicity, and to determine if this divergence is such a case or not.

We found that treatment with SFV-XF was ineffective when CD8 T cells were depleted before treatment. In contrast, CD4 or NK cell depletion not only did not abrogate the antitumor effects of SFV-XF, but in fact increased the found responses and, in the case of CD4 depletion, significantly prolonged the survival of treated mice and caused delay of uninjected tumors. A number of hypotheses can be listed to account for this observation, the most obvious of which, in the case of CD4 T-cell depletion, is the T regulatory cell (Treg) elimination. However, depletion of Tregs with anti-CD25 mAb (118) or inhibition of Foxp3 with the Foxp3-inhibitor p60 peptide (119) did not increase responses to SFV-XF administration. One critic to be made to these results is the suitability of the agents used for Treg depletion: the anti-CD25 clone PC61 has been shown to inefficiently deplete Tregs in tumor tissue (120). Also, it could be argued that a more prolonged administration of the p60 Foxp3 inhibitor could have altered the result of the experiment (inhibitor was given until day 14 after MC38 inoculation). More

sophisticated systems in which to explore the role of Tregs in the context of SFV-XF would be the use of Foxp3-DTR mice (121) or monoclonal antibodies against CD25 or CTLA4 optimized for Treg depletion (120). We are currently exploring if CD4 T-cell depletion can cause an increase in the levels of homeostatic T-cell cytokines such as IL-7or IL-15 that could potentiate a CD8 T-cell response against MC38 tumors upon treatment with SFV-XF (122).

SFV-XF administration did not significantly alter the T-cell composition of MC38 tumor immune infiltrates. Treated B16-OVA tumors, however, saw an increase in CD4 effector and regulatory cells, as well as CD8 cells recognizing the SIINFEKL epitope. These differences in the response of the TIL compartment between MC38 and B16-OVA tumors, both responsive in similar grade to SFV-XF treatment, is striking and maybe suggests SFV-XF can exert antitumoral activity through additional mechanisms not identified by us in this work.

As was expected, the antitumor effect of SFV-XF was dependent on BATF3 and IFNAR. The lack of effect of SFV-XF in *Batf3*$^{-/-}$ mice is consistent with the dependency on CD8 T cells in this chapter and with the non-responsiveness of these mice to immunotherapy with sFlt3L+Poly-ICLC from chapter 1. These results indicate that absence of Batf3-dependent DCs is a defect that is not overcome by sFlt3L administration in vivo, nor by intratumoral activation of remaining DCs by molecular danger signals such as a TLR3 ligand or a SFV vector. On the other hand, type I IFN signaling is essential for the activation of innate immunity and for CD8 T-cell cross-priming and antitumor immunity (107). Our findings are concordant with previous reports by our lab showing that antitumor responses elicited by SFV-IL12 require an intact IFNAR system (94).

Contrary to our expectations and our hypothesis, SFV-XF administration into MC38 or B16-OVA tumors caused no changes in tumor-infiltrating dendritic cell density. The original aim of both SFV-coded transgenes was to i) attract mature cDC1s expressing the XCL1 receptor, XCR1, towards locally infected tumor cells, and ii) favor the differentiation of infiltrating DC precursors into DCs, specially into Batf3-dependent cDC1s, using sFlt3L. Despite these goals not having been met, we did observe an expansion of cDC1 and cDC2 subsets in SFV-XF-treated TDLNs, and to a lesser extent, in distant non-tumor draining lymph nodes. This observation accounts for the activity of SFV-XF transgenes, likely sFlt3L, and serves to establish the hypothesis that it may be at least partially responsible for the antitumor efficacy observed with the SFV-XF vector. Further work will aim to ascertain whether tumor antigen capture *in situ* and transport to TDLNs by $CD103^+$ cDC1s is potentiated by SFV-XF administration.

After completing the programmed experimentation, we have not obtained a clear indicator of the contribution of XCL1 to the effects of the vector *in vivo*. To understand the role XCL1 is playing in this setting and to explore whether it could be replaced by a different molecule would help optimize the antitumor effect of a vector of this kind. At the top of the list of attractive chemokines to test in this regard would be the T-cell chemoattractors CXCL9/10 (116) and the DC-chemoattractors CCL4/5 (32,117).

Final remarks of the discussion

This PhD project has served to uncover the essential role cDC1s and cross-presentation play in the success of the immunotherapeutic agents anti-PD-1 and anti-CD137, analogous to those available in the clinic and that have revolutionized treatment of cancer. We have done so in loss-of-function settings using mouse genetically deficient for *Batf3* and devoid of cDC1s, which displayed complete unresponsiveness to immunotherapy. Next, we have devised gain-of-function experiments aimed to systemically and locally expand cDC1 populations, while at the same time providing local activation signals to mature them. In the first chapter, we chose to expand cDC1s by systemically administering sFlt3L through hydrodynamic injection of sFlt3L-coding plasmid, and to locally activate them by intratumoral injection of Hiltonol®, Poly-ICLC, a TLR3 agonist available in the clinic. In the second chapter, we cloned XCL1 and sFlt3L into a Semliki Forest Virus vector (SFV-XF) for intratumoral administration. In this setting, both transgenes were intended to cause chemoattraction and differentiation of cDC1s, while viral RNA would provide the activation signals to drive DC maturation and potentiate CD8 T-cell cross-priming. Although we did not manage to detect increased cDC1 infiltration into injected tumors, SFV-XF showed robust antitumor efficacy against different tumor models in mice and promoted accumulation of conventional DCs in tumor-draining and distant lymph nodes.

CONCLUSIONS

1. Antitumor therapy with immunomodulatory mAbs is abrogated in *Batf3*$^{-/-}$ mice and is not rescued by IL12 administration.
2. *Batf3*$^{-/-}$ DCs have reduced ability to cross-prime CTLs against tumor antigens both in steady state and after treatment with anti-CD137 and anti–PD-1 mAbs.
3. sFLT3L and poly-ICLC induce a BATF3-dependent increase in the numbers of tumor-antigen-specific TILs expressing CD137 and PD-1.
4. sFLT3L and poly-ICLC do not control the progression of B16-OVA–derived tumors in *Batf3*$^{-/-}$ mice.
5. Semliki Forest Virus(SFV)-based SFV-XF vectors confer functional expression of XCL1 and sFlt3L in infected cells.
6. Intratumoral injection of SFV-XF exerts antitumor effects against MC38 and B16-OVA subcutaneous tumors.
7. Intratumoral treatment with SFV-XF shows no synergy with anti-CD137 or anti-PD-1 mAbs.
8. CD8 T-cell depletion abrogates SFV-XF therapeutic effects, whereas NK1.1 or CD4-T cell depletion improves efficacy.
9. SFV-XF requires Batf3-dependent DCs and the type-I IFN receptor IFNAR for therapeutic activity.
10. Conventional DCs become enriched in SFV-XF-treated tumor-draining LNs but do not augment their numbers in the tumor microenvironment.

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ANNEX 1

Review article

Antigen Cross-Presentation and T-Cell Cross-Priming In Cancer Immunology And Immunotherapy

Sánchez-Paulete AR, Teijeira A, Cueto FJ, Garasa S, Pérez-Gracia JL, Sánchez-Arráez A, Sancho D, Melero I. Antigen cross-presentation and T-cell cross-priming in cancer immunology and immunotherapy. Ann Oncol. Dec 2017;28(suppl_12):xii44-xii55. doi: 10.1093/annonc/mdx237.

ANNEX 2

Review article

Deciphering CD137 (4-1BB) signaling in T-cell costimulation for translation into successful cancer immunotherapy

Sanchez-Paulete AR, Labiano S, Rodriguez-Ruiz ME, Azpilikueta A, Etxeberria I, Bolaños E, Lang V, Rodriguez M, Aznar MA, Jure-Kunkel M, Melero I. Deciphering CD137 (4-1BB) signaling in T-cell costimulation for translation into successful cancer immunotherapy. Eur J Immunol. Mar 2016;46(3):513-22. doi: 10.1002/eji.201445388.