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Premios Enrique Fuentes Quintana de Tesis Doctorales

STUDY OF THE INVOLVEMENT OF ANTIGEN CROSS-PRESENTATION IN THE ANTITUMOR ACTIVITY OF IMMUNOSTIMULATORY MONOCLONAL ANTIBODIES

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PREMIOS ENRIQUE FUENTES QUINTANA 2019

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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:

- 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).
- The infiltration of immune cells , especially CD8 T lymphocytes, into tumors (11, 12).
- A previously existing immune response in the tumor tissue, as indicated by transcription of IFN-γ response genes and PD-L1 expression (13).

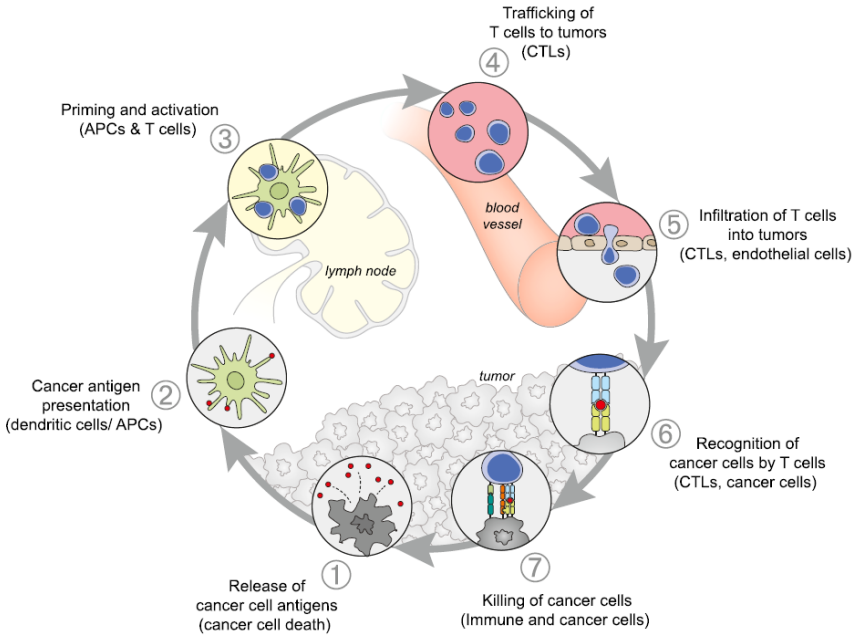
Most of the existing immunotherapeutic drugs operate based on potentiating T-cell activity. However, elimination of tumor cells by antigen-specific T lymphocytes is but the last step of a complex process that involves cellular components of both the innate and adaptive immunity.

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.

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

Figure 1.
The Cancer-Immunity Cycle



Source: Chen and Mellman (2013).

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

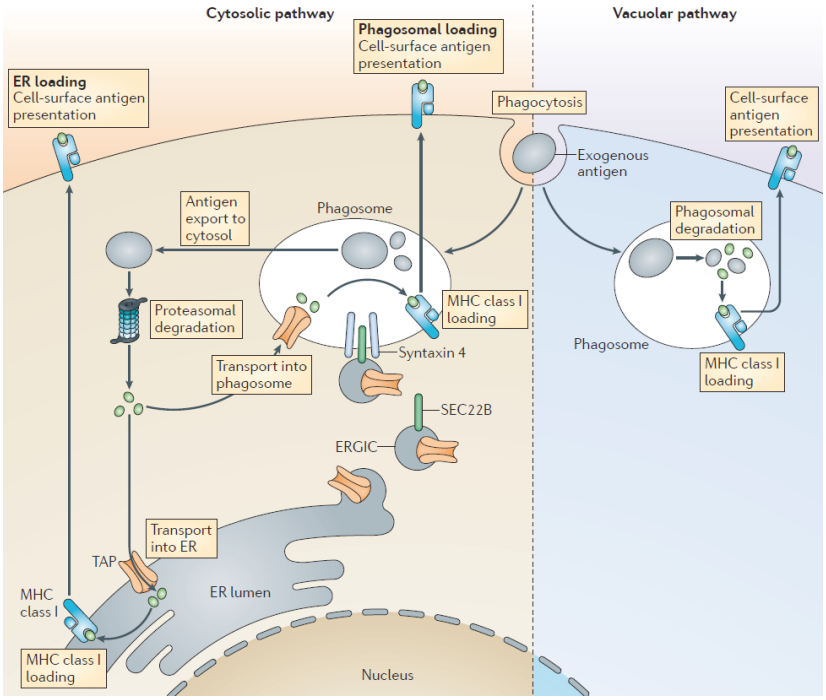
Conventional antigen presentation pathways on MHC molecules are divided in two categories: peptides derived from the proteins synthesized by the presenting cell, that we will call endogenous proteins, are presented on MHC-I molecules to CD8 T cells. This system allows a cell to present peptides from 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).

Figure 2.

Pathways for antigen cross-presentation



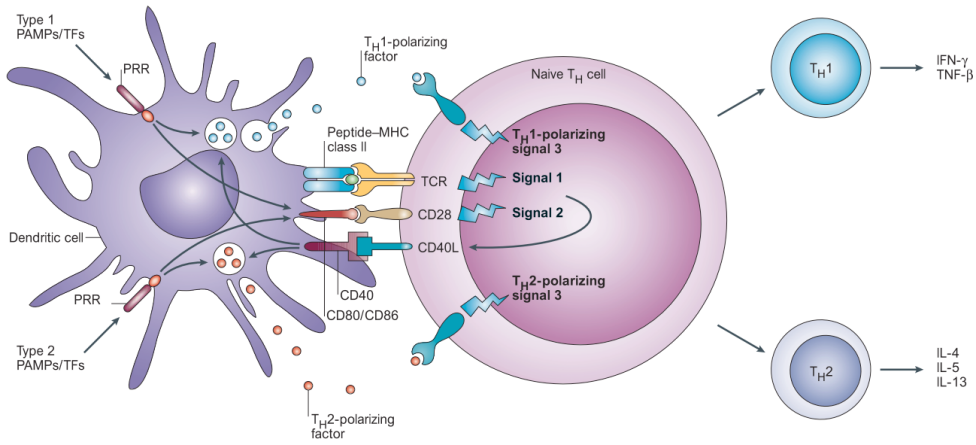
Source: Joffre *et al.* (2012).

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.

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



Source: Kapsenberg (2003).

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 95).

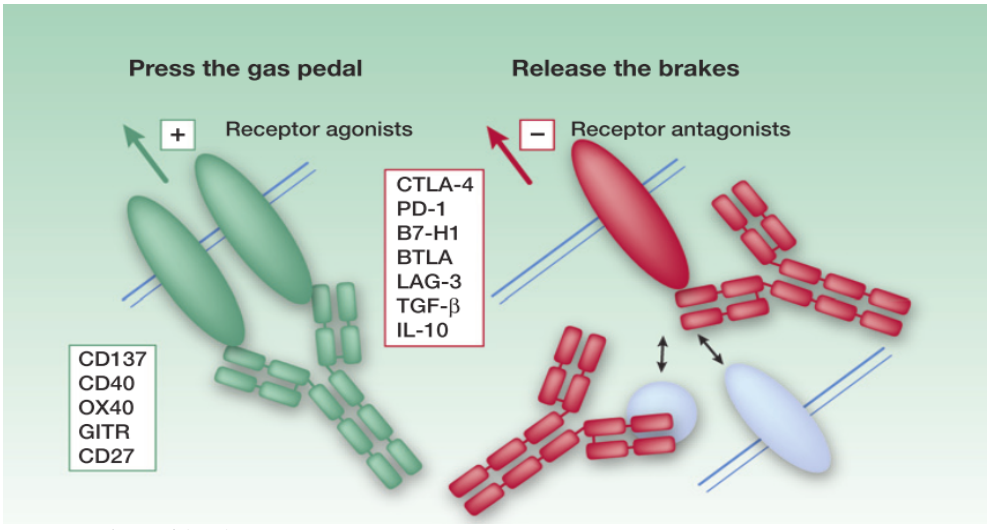
ACTING ON T-CELL COSTIMULATION/INHIBITION

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).

Figure 4.

T cell-targeted Immunostimulatory mAbs



Source: Melero *et al.* (2013).

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 109).

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.

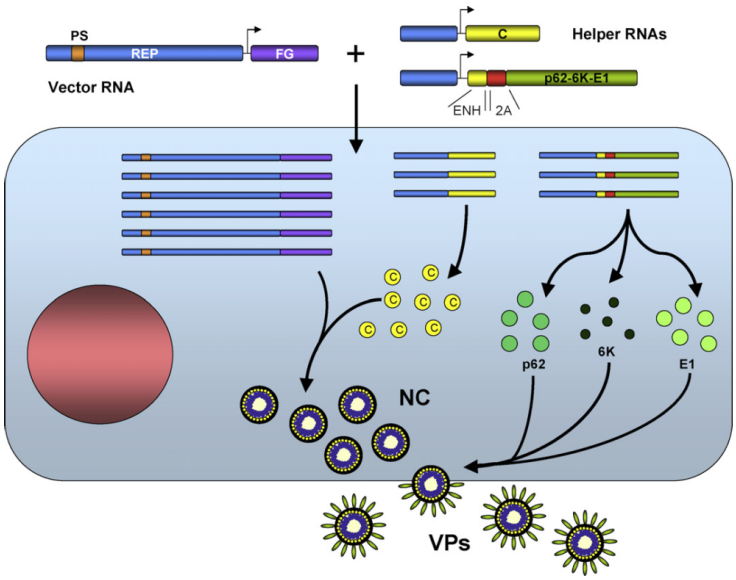
Cancer virotherapy strategies encompass two not mutually exclusive categories: oncolytic virotherapy and gene therapy with viral vectors.

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,

Figure 5.

Three-plasmid SFV vector production system



Source: Quetglas *et al.* (2010).

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).

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

RESEARCH BRIEF

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

Alfonso R. Sánchez-Paulete[1], Francisco J. Cueto[2,3], María Martínez-López[2], Sara Labiano[1], Aizea Morales-Kastresana[1], María E. Rodríguez-Ruiz[4], Maria Jure-Kunkel[5], Arantza Azpilikueta[1], M. Angela Aznar[1], José I. Quetglas[1], David Sancho[2], and Ignacio Melero[1,4]

ABSTRACT Weak and ineffective antitumor cytotoxic T lymphocyte (CTL) responses can be rescued by immunomodulatory mAbs targeting PD-1 or CD137. Using *Batf3*$^{-/-}$ mice, which are defective for cross-presentation of cell-associated antigens, we show that BATF3-dependent dendritic cells (DC) are essential for the response to therapy with anti-CD137 or anti-PD-1 mAbs. *Batf3*$^{-/-}$ mice failed to prime an endogenous CTL-mediated immune response toward tumor-associated antigens, including neoantigens. As a result, the immunomodulatory mAbs could not amplify any therapeutically functional immune response in these mice. Moreover, administration of systemic sFLT3L and local poly-ICLC enhanced DC-mediated cross-priming and synergized with anti–CD137- and anti–PD-1–mediated immunostimulation in tumor therapy against B16-ovalbumin–derived melanomas, whereas this function was lost in *Batf3*$^{-/-}$ mice. These experiments show that cross-priming of tumor antigens by FLT3L- and BATF3-dependent DCs is crucial to the efficacy of immunostimulatory mAbs and represents a very attractive point of intervention to enhance their clinical antitumor effects.

SIGNIFICANCE: Immunotherapy with immunostimulatory mAbs is currently achieving durable clinical responses in different types of cancer. We show that cross-priming of tumor antigens by BATF3-dependent DCs is a key limiting factor that can be exploited to enhance the antitumor efficacy of anti–PD-1 and anti-CD137 immunostimulatory mAbs. *Cancer Discov; 6(1); 71–9. ©2015 AACR.*

See related commentary by Robert-Tissot and Speiser, p. 17.

INTRODUCTION

Tumor cells are antigenic as a result of abundant mutated sequences in their exomes (1). However, they are poorly immunogenic to prime cytotoxic T lymphocyte (CTL) responses because antigen presentation takes place in the absence of appropriate co-stimulation and in a strongly immunosuppressive environment (2). The immune response to cell-associated antigens requires the interplay of specialized and professional antigen-presenting cells called dendritic cells (DC). Among the variety of DC subsets, certain DCs excel at redirecting cell-associated phagocytosed proteins to the

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Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

D. Sancho and I. Melero share senior authorship of this article.

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MHC class I antigen presentation pathway (3), a process termed cross-presentation, or cross-priming if it results in $CD8^+$ T-cell activation. There is evidence that tumor antigens are efficiently cross-presented *in vivo* (4).

Two DC subsets have been identified in mice as the most efficient at cross-priming *in vivo*: lymphoid-tissue resident $CD11c^+CD8\alpha^+$Clec9a/DNGR-1^+XCR1^+ DCs and migratory $CD11c^+CD103^+$Clec9a/DNGR-1^+XCR1^+ DCs (5). Differentiation of both DC subsets shows an absolute requirement for FLT3L and is largely affected by the absence of BATF3 (6). Notably, the absence of BATF3 impairs not only numbers but also functional responses in the remaining $CD11c^+$ Clec9a/$DNGR1^+$ $XCR1^+$ DCs, such as cell-associated cross-presentation or IL12 production (7, 8). Notably, *Batf3*$^{-/-}$ mice show impaired immunity against syngeneic immunogenic fibrosarcomas (6) and regulate T-cell infiltration in models of melanoma (9). However, other BATF3-independent DC subsets mediate the immune system–dependent antitumor activity of anthracyclines (10) and mediate tumor rejection under activating conditions in BATF3-deficient mice (11). Recent reports further support an important role for intratumoral BATF3-dependent $CD103^+$ DCs in priming a CTL response through IL12 production (12, 13). In humans, an equivalent BATF3-dependent DC subset characterized by expression of CD11c, CD141, Clec9a/DNGR-1, and XCR1 has been identified in peripheral blood and lymphoid organs (14).

Immunotherapy of cancer is currently being revolutionized by the use of immunomodulatory mAbs. Interaction of Programmed Cell Death 1 (PD-1; CD279), on activated and exhausted lymphocytes, with its ligands (PD-L1 or PD-L2, expressed on antigen-presenting DCs and tumor cells) downmodulates T-cell signaling (15, 16). Interference with these interactions using mAbs to PD-1 or PD-L1 has proved effective in patients with metastatic melanoma, renal cell carcinoma, non–small cell lung cancer, bladder cancer, head and neck cancer, and other malignancies (17). In addition, stimulation of the co-stimulatory receptor on activated T lymphocytes CD137 (4-1BB; ref. 18) results in complete tumor rejection in some transplantable tumor models (19). These promising findings have led to the clinical development of two anti-CD137 agents mainly for refractory lymphoma (BMS-663513/Urelumab and PF-05082566; NCT01775631, NCT02253992, NCT01307267).

The anti–PD-1 and anti-CD137 mAbs both induce tumor rejection by synergizing with vaccines (20), indicating that their function relies on a preexisting suboptimal CTL immune response that, if boosted, results in synergistic effects (1). Herein, we find an absolute need for BATF3-dependent DCs in cross-priming of tumor antigens to CTLs that subsequently upregulate PD-1 and CD137. This antitumor response can thus be manipulated with exogenous immunostimulatory mAbs. In consequence, expansion and activation of BATF3-dependent DCs concomitant with anti-CD137 mAb or anti–PD-1 treatment result in a suitable combined antitumor therapy.

RESULTS

Ineffective Antitumor Therapy with Immunomodulatory mAbs in *Batf3*$^{-/-}$ Mice

The absence of BATF3 affects the ontogeny and function of $CD8\alpha^+$ DCs in lymphoid organs and $CD103^+$ DCs in the periphery, impairing cell-associated cross-presentation and the ability to produce IL12 in response to infectious challenge. The antitumor effects of immunostimulatory anti–PD-1 and anti-CD137 mAbs are contingent on an already-present baseline immune response, which is rescued and amplified by treatment. Based on the proposed role for BATF3-dependent DCs in immune surveillance (6), we hypothesized that the preexisting immune response rescued by the immunostimulatory mAbs might be mediated by BATF3-dependent cross-priming. Grafted MC38-derived tumors were lethal in C57BL/6 wild-type (WT) and BATF3-deficient mice, with slightly faster progression in *Batf3*$^{-/-}$ mice (Fig. 1A). In WT mice, tumor growth was delayed or curtailed by a course of treatment with anti–PD-1 or anti-CD137 mAbs, starting on day 4 after tumor cell inoculation. Combination treatment with both mAbs had a synergistic effect on their antitumor action (Fig. 1A and B), as previously reported in other tumor models (21). The antitumor efficacy of anti-CD137 and anti–PD-1 mAbs, used alone or in combination, was abolished in *Batf3*$^{-/-}$ mice (Fig. 1A and B), suggesting that BATF3-dependent DCs are responsible for the baseline immune response that is potentiated by immunostimulatory mAbs, as *Batf3*$^{-/-}$ mice only present some functional defects in $CD8\alpha^+$ resident DC or $CD103^+$ migratory DC (6, 7, 12).

We explored whether the ability of BATF3-dependent DCs to specifically provide IL12 that boosts CTL function (8, 13) could underlie the advantage of BATF3-dependent DCs to mediate basal antitumor response. We analyzed the ability of intratumorally injected IL12 to rescue the antitumor effect of systemic anti-CD137 mAb in the absence of BATF3. Repeat injections of recombinant IL12 in tumor lesions clearly potentiated the antitumor effects of systemic anti-CD137 mAb in WT mice, leading to rejection of most of the tumors (Fig. 1C). In stark contrast, no therapeutic effect was seen in identically treated *Batf3*$^{-/-}$ mice (Fig. 1C). Administration of IL12 is thus unable to compensate for the loss of a key function of BATF3-dependent DCs in the synergy with immunostimulatory anti-CD137 mAb.

Impaired Ability of *Batf3*$^{-/-}$ DCs to Cross-Prime CTLs against Tumor Antigens

To investigate the possible involvement of deficient cross-presentation in the nonresponsiveness of *Batf3*$^{-/-}$ mice to anti–PD-1 and anti-CD137 mAbs, we analyzed the ability of $CD11c^+$ DCs to cross-present tumor-associated antigens to $CD8^+$ T cells *ex vivo*. For these experiments, we used MC38 cells transfected to express ovalbumin (OVA) as a surrogate tumor antigen (22). Two days after tumor-cell grafting, $CD11c^+$ DCs from tumor-draining lymph nodes (LN) were magnetically sorted and cocultured at different ratios with OT-I OVA-specific $CD8^+$ T cells. At all ratios tested, OT-I T cells cocultured with DCs from *Batf3*$^{-/-}$ mice produced markedly lower levels of intracellular and secreted IFNγ than cells cocultured with WT DCs (Fig. 2A and B), and also showed impaired proliferation (Fig. 2C), although there was some remaining cross-priming activity by *Batf3*$^{-/-}$ DCs.

To further investigate the DC subsets responsible for tumor cross-priming in WT and *Batf3*$^{-/-}$ mice, we FACS-sorted DC subsets from MC38-OVA tumor-draining LNs into resident $CD11c^{hi}$MHC-$II^{int}CD11b^+$ and $CD11c^{hi}$MHC-$II^{int}CD8\alpha^+$ cells,

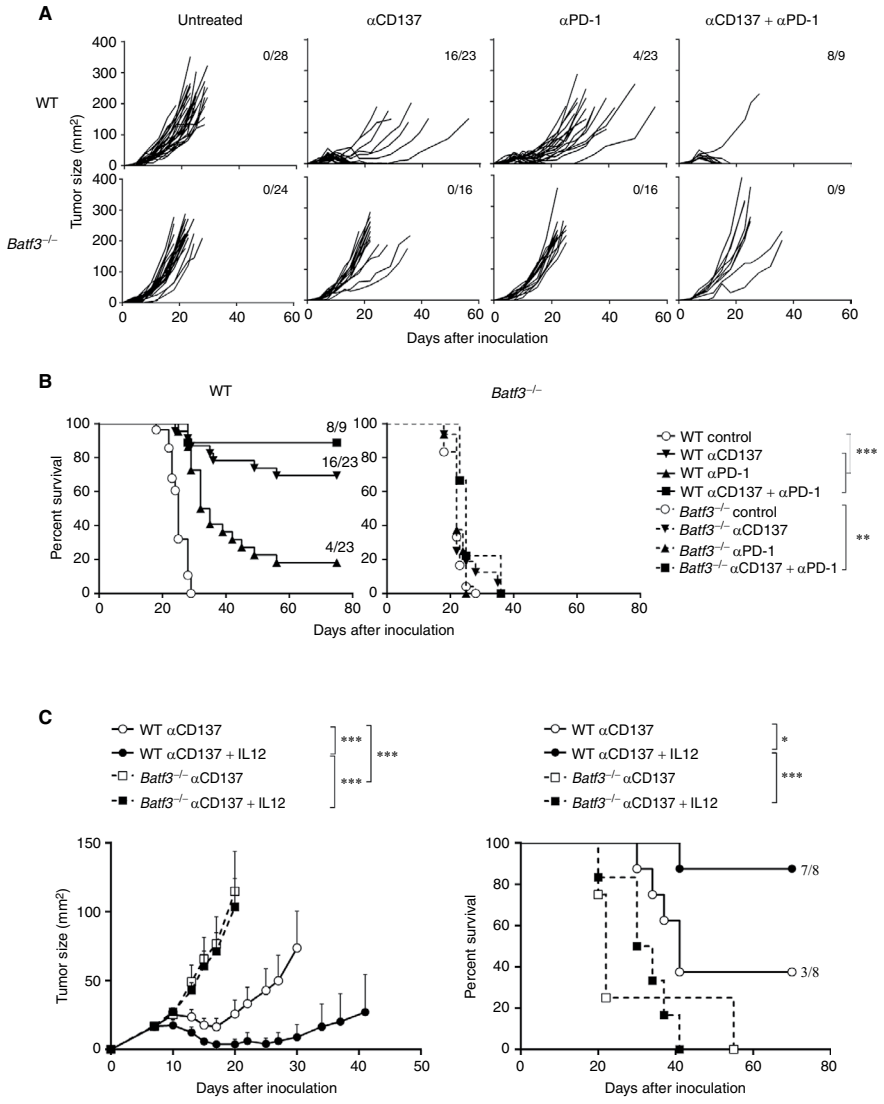

Figure 1. Antitumor therapy with immunomodulatory mAbs is abrogated in *Batf3*$^{-/-}$ mice and is not rescued by IL12 administration. WT or *Batf3*$^{-/-}$ mice were s.c. inoculated with 5×10^5 MC38 cells. **A** and **B,** mice were injected i.p. with 100 μg anti-PD-1 and anti-CD137 mAbs, alone or in combination (100 μg each), or with vehicle (untreated) on days 4, 7, and 10 after tumor cell inoculation. **A,** growth plots of individual tumors. **B,** overall survival charts show pooled results from 3 independent experiments with similar results. **C,** tumor-inoculated mice were injected i.p. with 100 μg anti-CD137 mAb on days 7, 10, and 13. The indicated groups of mice additionally received i.t. injections of recombinant mouse IL12 or saline on days 7, 9, and 11. IL12 was injected at 25 ng/dose into the tumor nodules. On the left, tumor area (mean ± SEM); on the right, overall survival. Fractions indicate the number of animals surviving at the end of the protocol. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

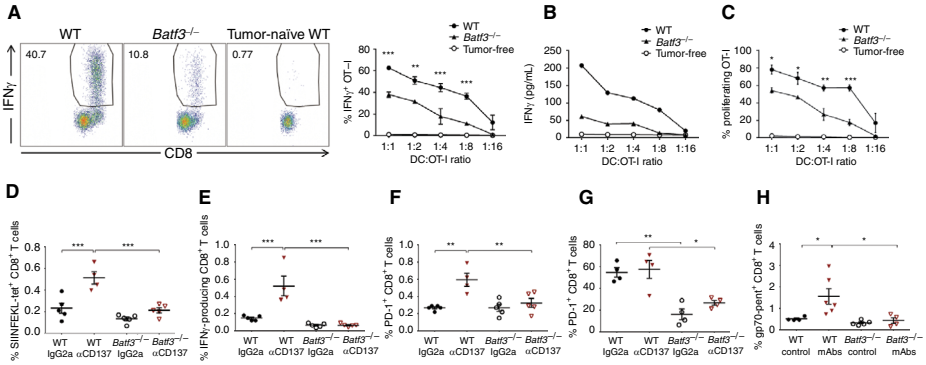

Figure 2. Reduced ability of *Batf3*$^{-/-}$ DC to cross-prime CTLs against tumor antigens both in steady state and after treatment with anti-CD137 and anti–PD-1 mAbs. **A–C,** CD11c^{+} DCs from WT and *Batf3*$^{-/-}$ mice bearing MC38-OVA tumors were magnetically sorted from tumor-draining LNs and cocultured (see Methods) with purified naïve CD8^{+} OT-I TCR transgenic T cells over a range of DC:T cell ratios. **A,** left: representative flow cytometry dot plots of intracellular IFNγ staining in OT-I T cells cultured at a 1:4 DC:T cell ratio. Right: percentages of IFNγ-positive OT-I T cells at all ratios tested. **B,** IFNγ concentrations in the culture supernatants. **C,** percentages of proliferating OT-I cells by dilution of Cell Violet dye. **D–F,** WT and *Batf3*$^{-/-}$ mice grafted with MC38-OVA cells were treated with anti-CD137 (days 5 and 7) and tumor-draining LN analyzed on day 9 (see Methods). **D,** frequency of H-2K^{b}-OVA-tetramer^{+} cells among CD8^{+} T cells. **E,** intracellular IFNγ production induced by restimulation with OVA$_{257\text{-}264}$ peptide in CD8^{+} T cells from tumor-draining LN. **F,** PD-1 surface staining on tumor-draining LN CD8^{+} T cells. **G,** frequency of PD-1^{+} lymphocytes among CD8^{+} TILs in mice treated as in **D**. **H,** WT and *Batf3*$^{-/-}$ mice grafted with MC38 cells were treated with anti-CD137 and anti–PD-1 mAbs on days 12 and 14, and tumor-infiltrating lymphocytes were analyzed on day 16 to detect CD8^{+} T lymphocytes specific for gp70 antigen (**A–C**) two-way and (**D–H**) one-way ANOVA with Bonferroni *post-hoc* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

and migratory CD11c^{int}MHC-II^{hi}CD103^{+} and CD11c^{int}MHC-II^{hi}CD103^{-} DCs and cocultured them with purified OT-I T cells as above. Notably, only migratory DCs were able to cross-present and, among these, migratory CD103^{+} DCs demonstrated better ability for cross-presentation of tumor-associated antigens in a BATF3-dependent fashion (Supplementary Fig. S1A–S1D).

We next tested whether deficiency in cross-presentation in the absence of BATF3 resulted in impaired cross-priming to tumor antigens *in vivo*. We analyzed priming of CD8^{+} T cells from the endogenous repertoire to grafted MC38-OVA tumors in WT and *Batf3*$^{-/-}$ mice treated or not treated with anti-CD137. In WT mice, treatment with anti-CD137 mAb increased the frequency and numbers of tumor antigen-specific CD8^{+} T cells from the endogenous repertoire in the tumor-draining LN (Fig. 2D), correlating with an increased effector response upon re-stimulation with tumor-antigen peptide (Fig. 2E). These effects were blocked in the absence of BATF3 (Fig. 2D and E). Notably, priming of CD8^{+} T cells resulted in upregulation of surface PD-1 in CD8^{+} T cells at the tumor-draining LNs in WT mice, and this was impaired in *Batf3*$^{-/-}$ mice (Fig. 2F). Tumor-infiltrating lymphocytes (TIL) were basally activated and expressed high PD-1 levels that were not further increased by anti-CD137 treatment (Fig. 2G). However, TILs expressed much lower levels of PD-1 in *Batf3*$^{-/-}$ mice (Fig. 2G), which correlates with their reduced potential to respond to immunomodulatory mAb therapy. These results show that BATF3-dependent DCs are crucial for the priming and concomitant induction of targets for immunostimulatory mAbs by tumor-specific CD8^{+} T cells.

We further analyzed the response against gp70, a well-described endogenous antigen in MC38 colon cancer cells (23). Notably, CD8^{+} TILs specific for gp70 were increased in a BATF3-dependent fashion upon anti-CD137 and anti–PD-1 mAb treatment, as detected by pentamer staining (Fig. 2H). A similar analysis of the response to the ADPGK-mutated neoantigen (24) showed some positive responses in WT but not BATF3-deficient mice (Supplementary Fig. S2A and S2B).

Priming of CD137^{+} PD-1^{+} Antigen-Specific TILs by Activated BATF3-Dependent DCs

We hypothesized that expansion and activation of BATF3-dependent DCs with sFLT3L and the TLR3 adjuvant poly-ICLC would synergize with immunostimulatory mAbs to enhance priming of tumor-specific CD8^{+} T cells. To extend our results to an alternative tumor model, we used B16-OVA melanoma cells grafted subcutaneously. Hydrodynamic injection of a plasmid expressing sFLT3L markedly promoted the expansion of cross-presenting DCs (Supplementary Fig. S3A). Intratumoral administration of poly-ICLC increased some activation markers including CD40 and PD-L1 in DCs from the spleen, tumor, and tumor-draining LNs, particularly in the TLR3-expressing CD103^{+} DCs (Supplementary Fig. S3B–S3D). Immunity to B16-OVA was estimated from the number of TILs detected by OVA-MHC–tetramer staining and was almost undetectable in control mice treated with empty vector and intratumoral saline buffer (Fig. 3A). Systemic hydrodynamic injection of sFLT3L combined with intratumoral injection of poly-ICLC raised a specific antitumor CTL response, and this induction was blocked in *Batf3*$^{-/-}$ mice (Fig. 3A). These events

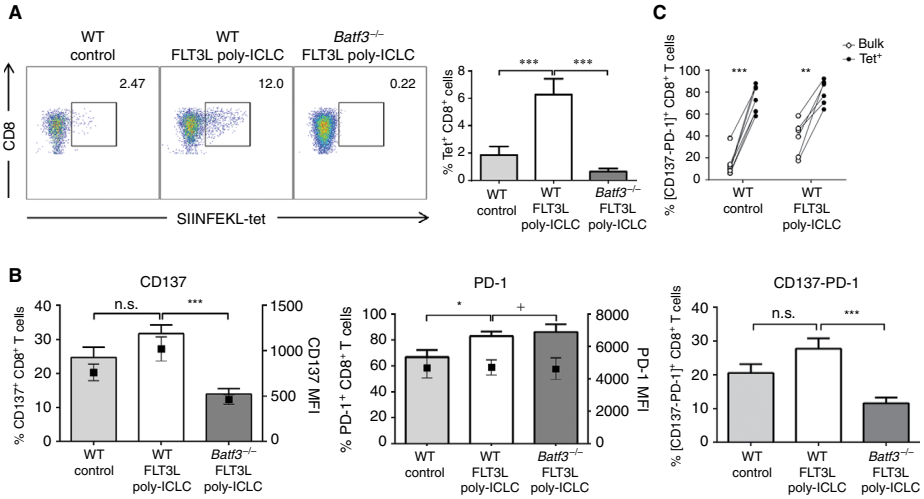

Figure 3. sFLT3L and poly-ICLC induce a BATF3-dependent increase in the numbers of tumor-antigen-specific TILs expressing CD137 and PD-1. WT or *Batf3*$^{-/-}$ mice were inoculated with B16-OVA melanoma cells on day 0, concomitant with hydrodynamic gene transfer of sFLT3L or control empty plasmid. On day 7, tumors were injected with poly-ICLC or control. Tumors were retrieved and TILs analyzed on day 10. **A,** H2Kb-OVA$_{257-264}$ tetramer staining in CD8$^+$ TILs. Left: representative plots. Right: graphs corresponding to a representative experiment ($n = 3$). **B,** surface CD137 and PD-1 immunostaining in CD8$^+$ TILs. **C,** PD-1 and CD137 surface immunostaining in SIINFEKL tetramer$^+$ gated T cells. One-way ANOVA with Bonferroni *post-hoc* test. $^+$, $P < 0.1$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

were paralleled by an increased frequency of CD137$^+$CD8$^+$ T cells in WT mice treated with sFLT3L and poly-ICLC and the impairment of this effect in *Batf3*$^{-/-}$ mice (Fig. 3B). Notably, antigen-specific TILs showed higher surface expression of PD-1 and CD137 compared with the bulk of CD8$^+$ infiltrating T cells (Fig. 3C). These results show that expansion and activation of BATF3-dependent DCs increase the frequency of primed CD8$^+$ T cells that upregulate markers of activation and exhaustion and are sensitive to immunostimulatory mAb treatment because of the expression of the targets for such agents.

BATF3-Dependent DC Activation Enhances Antitumor Ability of Immunomodulatory mAbs

We next sought to establish how FLT3L- and poly-ICLC–enhanced priming of CD8$^+$ T cells affects the antitumor efficacy of anti-CD137 and anti–PD-1 mAbs. For this analysis, we used the B16-OVA model, which in our hands responds weakly or not at all to anti–PD-1 or anti-CD137 mAb treatment (Fig. 4A and B). Hydrodynamic injection of sFLT3L was concomitant with tumor inoculation, and intratumoral injection of poly-ICLC at day 7 was administered with or without anti–PD-1 or anti-CD137 mAbs at days 4, 7, and 10 after tumor inoculation. The triple combinations retarded tumor progression and significantly extended overall survival in WT mice (Fig. 4A and B) but had no significant effect in *Batf3*$^{-/-}$ mice (Fig. 4C and D). Furthermore, we found that quadruple combination immunotherapy encompassing sFLT3L + poly-ICLC + anti-CD137 + anti–PD-1 mAbs exerted marked antitumor effects against parental B16F10-derived melanomas (Supplementary Fig. S4A), while completely eradicating B16-OVA–derived tumors (Supplementary Fig. S4B). Functional enhancement of BATF3-dependent DCs thus cooperates synergistically with anti-CD137 and anti–PD-1 mAbs, indicating that baseline BATF3-dependent cross-priming is a key limiting factor that can be targeted to enhance antitumor immunity.

DISCUSSION

This study shows the immunodynamic interactions between professional cross-priming DCs and immunostimulatory mAbs that target CD137 and PD-1. The observations are fully consistent with an essential presentation of tumor antigens to CD8$^+$ T cells by BATF3-dependent DCs. Both migratory CD103$^+$ DCs and LN-resident CD8α^+ DCs are functionally or ontogenically impaired in *Batf3*$^{-/-}$ mice (6, 7, 12), as they are also in *Irf8*$^{-/-}$ mice (12). Our results support a model in which at least one of these DC subsets is crucial for the basal antitumor response that is amplified by immunostimulatory mAbs.

BATF3-dependent DC subsets have been identified in the tumor environment, where they are functional and even have positive prognostic significance (12). These DCs are effective at taking up antigen from tumor cell debris for MHC class I cross-presentation. We find that these DCs mediate CTL priming at the malignant tissue or migrate via lymphatic afferent vessels to reach the draining LNs and meet naïve or

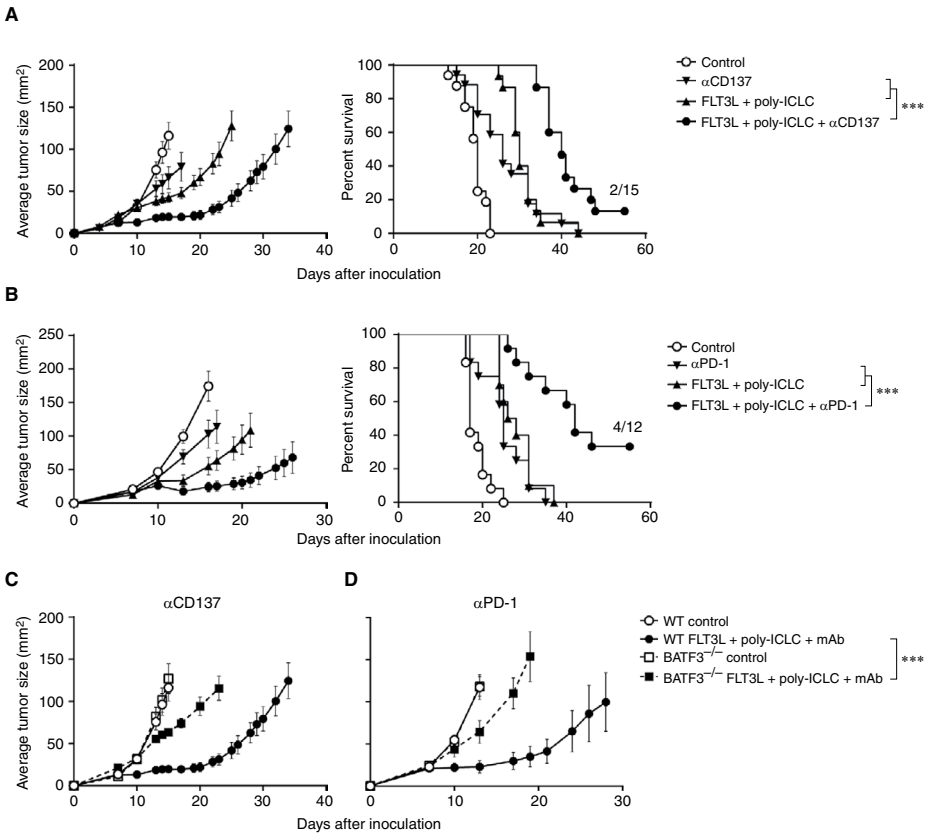

Figure 4. sFLT3L and poly-ICLC do not control the progression of B16-OVA–derived tumors in *Batf3*$^{-/-}$ mice. WT B16-OVA–bearing mice administered with hydrodynamic gene transfer with sFLT3L or control empty plasmid received i.p. injections of anti-CD137 mAb (**A**) or anti–PD-1 mAb (**B**), controlled by vehicle buffer, on days 4, 7, and 10. Poly-ICLC or control was administered i.t. on day 7. On the left, tumor areas (mean ± SEM). On the right, overall survival. **C** and **D,** comparison of the combined efficacy of sFLT3L + poly-ICLC with anti-CD137 mAb (**C**) or anti–PD-1 (**D**) in WT and *Batf3*$^{-/-}$ mice. Graphs represent pooled data from 4 (**A** and **C**) or 2 (**B** and **D**) independent experiments with similar results, for a total of 10 to 15 mice per group. ***, $P < 0.001$.

central memory CD8$^+$ T cells. These primed CTLs upregulate surface CD137 and PD-1, making them suitable targets for immunostimulatory mAbs. Our results show that expansion and activation of BATF3-dependent DCs result in increased antitumor priming and more effective tumor rejection in response to immunostimulatory mAbs. The dependency of anti-CD137 mAb treatment on DCs was suggested by the decreased efficacy of treatment upon depletion of CD11c cells (25). In the case of anti–PD-1 mAb, treatment synergizes with vaccines consisting of tumor cells transfected with GM-CSF or FLT3L, whose activity depends on attraction and differentiation of DC subsets (26).

Our data are consistent with the recent results from Gajewski and colleagues, elegantly showing that BATF3-dependent CD103$^+$ DCs play an important role in regulating the infiltration of T cells in the tumor. Notably, intratumoral injection of cultured FLT3L-derived DCs rescues the response to anti–CTLA-4 and anti–PD-L1 immunomodulatory mAbs in terms of inducing antitumor CTLs and exerting antitumor activity (9). Previous studies from the same group had indicated a role for CD8α$^+$ DCs in the baseline CTL response to a transplantable melanoma model (27).

CD103$^+$ DCs were recently shown to be responsible not only for priming in the draining LNs, but also for IL12-dependent

promotion of a productive CD8$^+$ T-cell response locally in the tumor (12, 13), suggesting that expansion and activation of BATF3-dependent DCs might favor the generation of antitumor responses at several levels. Although professional cross-priming DCs have been characterized as key IL12 producers in infections and also in the tumor environment (8, 12, 13), we find that treatment of tumor-bearing mice with exogenous IL12 is unable to rescue a key BATF3-dependent function needed for synergy with immunostimulatory mAbs. Therefore, although IL12 production might be involved in the action of BATF3-dependent DCs, other functions of cross-priming DCs are absolutely needed. It is becoming apparent that effective anti–CTLA-4 or anti–PD-1 mAb therapy requires the presence of a measurable preexistent CTL response to the tumor mutatome epitopes in both humans and mice (28). It is now crucial to identify whether such responses are caused by direct presentation of antigens by tumor cells or by cross-priming of tumor cell–associated antigens in the tumor or in the tumor-draining LNs. Our data suggest that basal antitumor responses that are amplified by immunostimulatory mAbs have a critical requirement for professional cross-priming by DCs.

The need for cross-priming in the antitumor immune response also indicates possible relationships with mechanisms of immunogenic tumor cell death (10). Recent results show a crucial role for BATF3-dependent CD103$^+$ DCs in priming a CTL response through IL12 production in the context of tumor cell death induced with paclitaxel (12, 13). However, doxorubicin-mediated immunogenicity against F244 sarcoma cells is BATF3-independent (10), and BATF3-deficient mice are able to reject tumors under conditions with exogenously provided IL12 (11). Therefore, the precise role of BATF3-dependent CD103$^+$ DCs may depend on the context of the ongoing baseline immune response in the tumor, which will be eventually modulated by the treatment with immunostimulatory mAbs.

Each addition to our knowledge in this area of tumor antigen cross-priming has the potential to provide predictive biomarkers for the efficacy of immunostimulatory mAbs, because cross-priming against tumor neoantigens seems to be a key determinant of the variable efficacy of these treatments in mice and humans (1, 12, 28). Moreover, more effective vaccines could be prepared by immune sorting or targeting these cross-priming DC populations or their differentiation in culture from precursors (29).

Overall, our results raise important pointers for improving therapy with immunostimulatory mAbs. The cross-priming function of DCs is essential for the therapeutic effect of immunostimulatory mAbs, but the baseline CTL-priming function is suboptimal. These observations suggest the potential to devise exogenous or *in situ* tumor vaccination therapies to enhance cross-priming of tumor antigens and thereby increase the efficacy of immunostimulatory mAbs.

METHODS

Mice

Mice were bred at the Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC) and the Center for Applied Medical Research (CIMA), University of Navarra, in specific pathogen-free conditions. *Batf3*$^{-/-}$ on C57BL/6 background (kindly provided by Dr. Kenneth M. Murphy, Washington University, St. Louis, MO) were further back-crossed with C57BL/6 mice at the CNIC to establish WT and *Batf3*$^{-/-}$ cousin colonies from the heterozygotes. Animal studies (protocol approval 150/12) were approved by the local ethics committee. All animal procedures conformed to EU Directive 2010/63EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enforced in Spanish law under Real Decreto 1201/2005.

Cell Lines, Culture Conditions, and Tissue Processing

MC38, MC38-OVA, B16F10, and B16-OVA cells were cultured in RPMI medium (Gibco) supplemented with 10% decomplemented and filtered FBS (Sigma Aldrich) containing 50 μmol/L β-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Gibco). MC38 cells were provided by Dr. Karl E. Hellström (University of Washington, Seattle, WA) in September 1998. B16F10 cells were purchased from the ATCC in June 2006. B16-OVA cells were a kind gift from Dr. Lieping Chen (Yale University, New Haven, CT) in November 2001. These cell lines were authenticated by Idexx Radil (Case 6592-2012) in February 2012. MC38-OVA–transfected cells were kindly provided by Dr. Cornelis Melief (Leiden University Medical Center, the Netherlands) in November 2013 and were not further verified. All cell lines were cultured at 37°C with 5% CO_2. Isolated LNs were incubated in collagenase/DNase for 15 minutes at 37°C, followed by mechanical disaggregation using frosted slides. Single-cell suspensions were then stained for flow cytometry.

Flow Cytometry

Acquisition was performed using a FACS Canto II flow cytometer (BD Biosciences). The antibodies used included FITC-conjugated αPD-1 (29F.1A12) and αCD40 (3/23); PE-conjugated αCD11b (M1/70), αCD137 (17B5), and αIFNγ (XMG1.2); PrCPCy5.5-conjugated αCD103 (2E7) and αCD11c (N418); APC-conjugated αCD11b (M1/70), αPDL1 (10F.9G2), αCD8 (53-6.7), and αXCR1 (ZET); BV570-conjugated αCD8 (53-6.7); and BV421-conjugated αCD4 (RM4-5). For identification of epitope-specific T cells, phycoerythrin-or Alexa Fluor 647–conjugated H-2K^b-OVA$_{257–264}$ tetramer (MBL and NIH Tetramer Facility), H-2K^b-KSPWFTTL pentamer (gp70, Proimmune), or H2-D^b-ASMTNMELM dextramer (ADPGK; Immudex) were used. For intracellular staining, cells were fixed and permeabilized using Cytofix/Cytoperm buffer and then incubated with fluorochrome-conjugated antibodies in PermWash buffer (BD Biosciences).

In Vivo *Tumor Experiments*

Cultured tumor cells were trypsinized before reaching confluence and suspended in PBS. Unless specified otherwise, 5×10^5 cells in 50 μL PBS were used for inoculation. Cells were injected s.c. using 29G syringes into the shaved right flank of 8-to-12-week-old C57BL/6 *Batf3*$^{-/-}$ and WT mice. Tumor size was measured twice weekly and calculated as the product of orthogonal diameters.

Anti-CD137 (1D8) antibody was produced as described (19). Anti–PD-1 (RMP1-14) antibody was purchased from BioXcell. Antibodies (100 μg) were administered i.p. in PBS on days 4, 7, and 10 after tumor inoculation. Recombinant mouse IL12 (25 ng/dose; Miltenyi) was administered intratumorally (i.t.) on days 7, 9, and 11. In experiments involving injection of IL12, anti-CD137 was administered on days 7, 10, and 13. For *in vivo* DC expansion, 10 μg of sFLT3L-coding plasmid (pUMVC3-mFLex, Aldevron) or a control empty plasmid were injected i.v. to achieve hydrodynamic liver gene transfer. For *in vivo* stimulation of DCs, 100 μg poly-ICLC (Hiltonol; Oncovir) were injected i.t. on day 7 or when tumors reached 25 to 50 mm^2. PBS was injected as control.

Ex Vivo *Cross-Presentation of Surrogate Tumor Antigen*

To test the *ex vivo* cross-presentation capacity of LN DCs, sFLT3L plasmid-injected mice were bilaterally inoculated s.c. with 2×10^6

MC38-OVA cells. LNs were extracted 48 hours later. $CD11c^+$ cells were magnetically sorted with CD11c microbeads in an AutoMACS Pro Separator (Miltenyi) and further FACS-sorted where indicated. OT-I CD8 T lymphocytes were magnetically sorted from the spleens of C57BL/6 mice using CD8 microbeads (Miltenyi). Cell Violet-labeled (Thermo Fisher) OT-I lymphocytes were cocultured with *Batf3*$^{-/-}$ and WT LN-derived $CD11c^+$ or FACS-sorted $CD11c^+$ subsets over a range of ratios. SIINFEKL peptide-pulsed DCs served as positive controls. After 72 hours, culture supernatants were collected, and OVA-reactive T cells were restimulated *ex vivo* with 1 μg/mL SIINFEKL peptide for 5 hours, with Brefeldin A (10 μg/mL; Sigma-Aldrich) added for the last 4 hours. Cells were then stained for membrane markers before being fixed and permeabilized for staining of intracellular IFNγ. Secreted IFNγ was measured in culture supernatants with the BD Biosciences OptEIA Mouse IFNγ ELISA Kit.

Analysis of T-cell Priming by Tumor Antigens

WT and *Batf3*$^{-/-}$ mice were inoculated s.c. with 2×10^6 MC38-OVA cells. Mice were injected i.p. with 100 μg anti-CD137 or an isotype control at days 5 and 7 after tumor inoculation. LNs and tumors were extracted at day 9. LNs were incubated at 37°C in Liberase TL (Roche; 20 minutes) and tumors in Liberase TL/DNase I (30 minutes). Then both LN and tumors were mechanically dissociated through a 70-μm cell strainer (Fisher Scientific). Single-cell suspensions were stained and analyzed by flow cytometry.

For OVA- or ADPGK-specific T-cell restimulation *ex vivo*, single-cell suspensions from LNs were cultured for 2 hours in 10% FBS RPMI medium containing 1 μg/mL SIINFEKL or ASMTNMELM peptide. Then Brefeldin A was added at a final concentration of 10 μg/mL, and cells were incubated for 10 hours. Cells were stained for surface markers, fixed, and permeabilized for intracellular IFNγ staining. Samples were analyzed by flow cytometry.

Statistical Analysis

Tumor growth data were analyzed with Prism software (GraphPad Software, Inc.). Mean diameters of tumors over time were fitted using the formula $y = A \times e^{(t\text{-}t0)}/(1 + e^{(t\text{-}t0)/B})$, where t represents time, A the maximum size reached by the tumor, and B its growth rate. Treatments were compared using the extra sum-of-squares F test. Tumor survival was compared with log-rank (Mantel–Cox) tests. All other analyses among groups were performed as described in figure legends.

Disclosure of Potential Conflicts of Interest

M. Jure-Kunkel has ownership interest (including patents) in Bristol-Myers Squibb. I. Melero reports receiving commercial research grants from Bristol-Myers Squibb and Pfizer and is a consultant/advisory board member for AstraZeneca, Boehringer Ingelheim, Bristol-Myers Squibb, and Roche-Genentech. No potential conflicts of interest were disclosed by the other authors.

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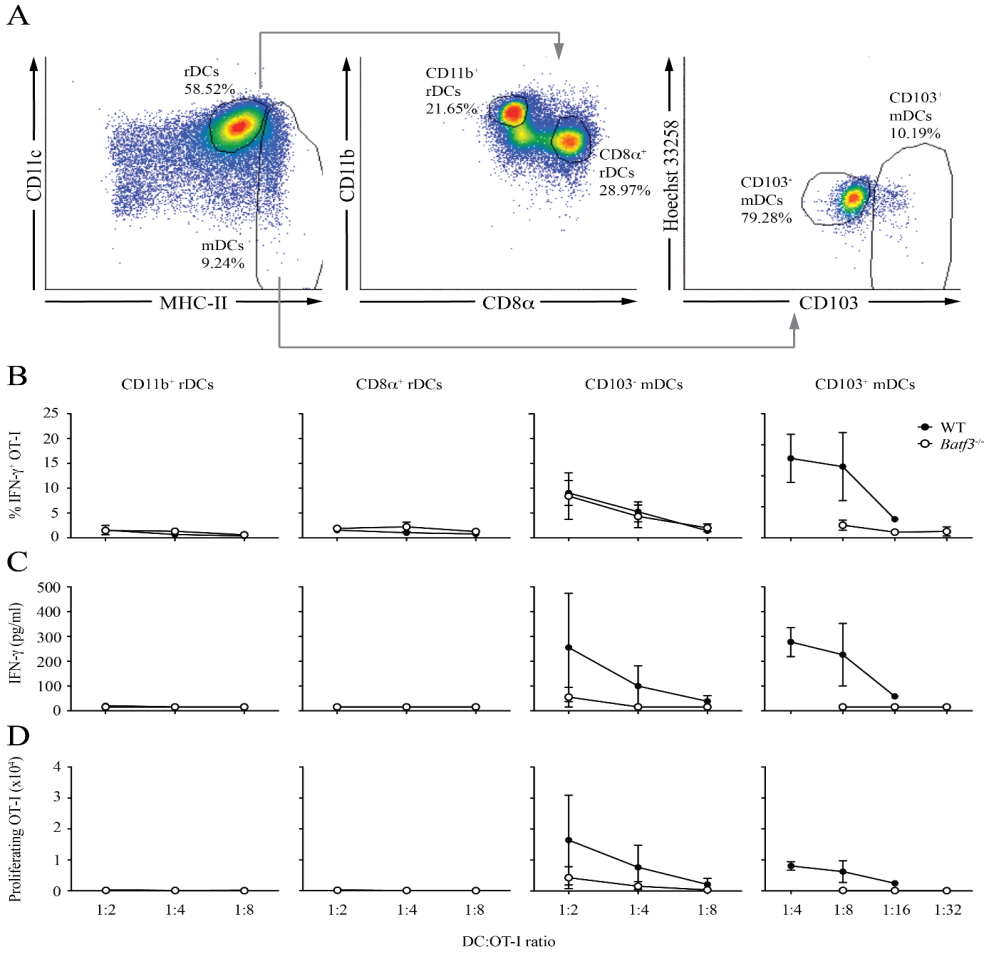
SUPPLEMENTARY FIGURES

Figure S1.

Migratory CD103⁺ DCs are the main mediators of cross-priming at the tumor-draining LNs

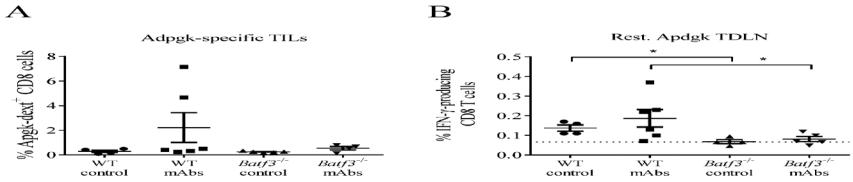


Magnetically presorted CD11c⁺ DCs from tumor-draining LNs of WT and *Batf3*$^{-/-}$ mice bearing MC38-OVA tumors were FACS-sorted into CD11c^{hi}MHC-II^{int}CD11b^{+}, CD11c^{hi}MHC-II^{int}CD8α^{+}, CD11c^{int}MHC-II^{hi}CD103^{+} and CD11c^{int}MHC-II^{hi}CD103^{-}, and cocultured with purified naive CD8⁺ OT-I OVA-specific T cells over a range of DC:T cell ratios. (A) Representative gating for FACS sorting of the indicated dendritic cell subpopulations. (B) Percentages of IFN-γ-positive OT-I T cells at all ratios tested upon coculture with the indicated DC subsets. (C) IFN-γ concentrations in the culture supernatants. (D) Numbers of proliferating OT-I cells by Cell Violet dye dilution.

Source: Sánchez-Paulete *et al.* (2016).

Figure S2.

CTLs against the Adpgk neoantigen of MC38 are induced by anti-CD137 and anti-PD-1 mAbs in a fraction of WT mice, but not in *Batf3*$^{-/-}$ mice

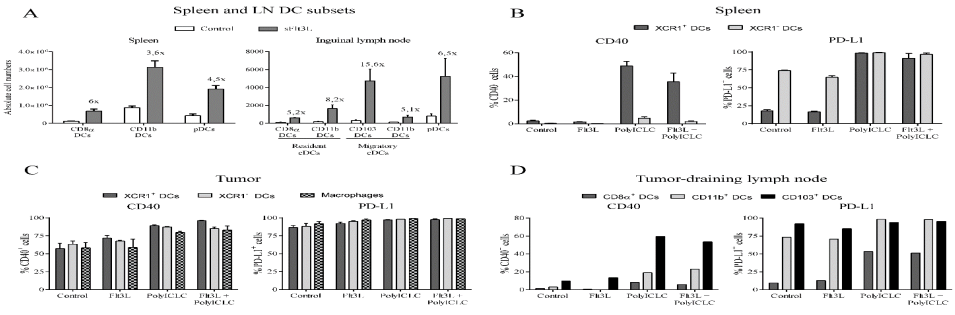


WT or *Batf3*$^{-/-}$ mice were s.c. inoculated with 5 x 10^5 MC38 cells. Mice were injected i.p. with 100 µg anti-PD-1 and 100 µg anti-CD137 mAbs, or with vehicle (control) on days 12 and 14 after tumor inoculation. On day 16, tumors and tumor-draining LNs were excised. (A) Tumors were stained with MHC-I dextramers for Adpgk (H-2D^b-ASMTNMELM). Percentage of Adpgk-specific CD8$^+$ T cells among tumor-infiltrating lymphocytes. (B) LN cell suspensions were restimulated overnight in the presence of Adpgk soluble peptide and BrefeldinA, and stained for intracellular IFN-γ. Percentage of IFN-γ cells among CD8$^+$ T cells. Mann-Whitney two-tailed test. * $p < 0.05$.

Source: Sánchez-Paulete *et al.* (2016).

Figure S3.

Systemic sFlt3L and local intratumoral poly-ICLC expand and mature DCs in B16-OVA bearing mice

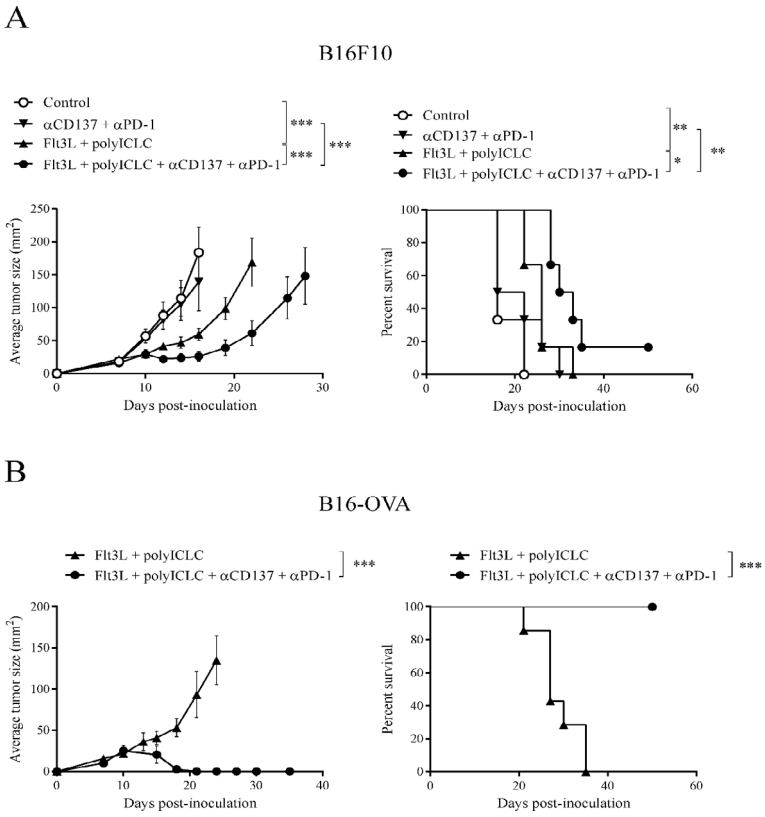


(A) WT mice were injected hydrodynamically in the tail vein with 10 µg sFlt3L-coding plasmid in 2 ml saline buffer. 10 days later, spleens and inguinal LNs were analyzed by flow cytometry to assess the absolute numbers of the indicated DC subsets. Numbers on each column indicate fold increase over baseline. (B-D) WT B16-OVA-bearing mice administered with hydrodynamic gene transfer with sFlt3L or control empty plasmid and received poly-ICLC or control buffer i.t. on day 11 post-tumor cell inoculation. (B-C) 24 or (D) 72 hours after poly-ICLC injection, mice were sacrificed and tumors, tumor-draining LNs and spleens were stained for flow cytometry to detect CD40 and PD-L1 expression on the gated DC subsets indicated in the figure.

Source: Sánchez-Paulete *et al.* (2016).

Figure S4.

Combinations of immunomodulatory anti-CD137 and anti-PD-1 mAbs synergize with sFlt3L and poly-ICLC against grafted B16F10 and B16-OVA melanomas



(A) WT B16F10-bearing mice (n = 6 per group) administered with hydrodynamic gene transfer with sFlt3L or control empty plasmid received i.p. injections of anti-CD137 mAb and anti-PD-1 mAb, controlled by vehicle buffer, on days 4, 7 and 10. Poly-ICLC or control buffer was administered i.t. on day 7. On the left, tumor areas (mean ± SEM). On the right, overall survival. (B) WT B16-OVA bearing mice (n = 7 per group) were treated as in (A). Mice treated with the quadruple combination remained alive and tumor-free 80 days after tumor cell inoculation. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Source: Sánchez-Paulete *et al.* (2016).

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

INTRATUMORAL IMMUNOTHERAPY WITH XCL1 AND SFLT3L ENCODED IN RECOMBINANT SEMLIKI FOREST VIRUS-DERIVED VECTORS TO FOSTER DENDRITIC CELL-MEDIATED T-CELL CROSS-PRIMING

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ABSTRACT

Multiple lines of evidence indicate a crucial role for antigen cross-presentation by conventional BATF3-dependent dendritic cells type 1 (cDC1s) in CD8-mediated antitumor immunity. Flt3L and XCL1 constitute, respectively, a key growth/differentiation factor and a potent chemoattractant for such antigen-presenting dendritic cells. To exploit their immunobiological functions in local immunotherapy, Semliki Forest Virus (SFV)-based vectors encoding soluble Flt3L (sFlt3L) and XCL1 were prepared. These vectors readily conferred transgene expression to tumor cells in culture and when engrafted as subcutaneous mouse tumor models. In syngeneic mice, intratumoral injection of SFV-XCL1-sFlt3L (SFV-XF) delayed progression of MC38- and B16-derived tumors. Therapeutic activity was observed but did not exert additive or synergistic effects in combination with anti-PD-1 or anti-CD137 immunostimulatory monoclonal antibodies. Therapeutic effects were abolished by CD8β T-cell depletion but were markedly enhanced by CD4 T-cell depletion. The role of CD4 cells was not explained by Tregs, since Treg pre-depletion with anti-CD25 mAb did not enhance efficacy. Antitumor effects were dependent on BATF3 and IFNAR, as observed in the corresponding gene-deficient mice. In B16-OVA tumors, SFV-XF increased the number of infiltrating CD8 T cells recognizing OVA. A clear increase of both resident and migratory BATF3-dependent DCs was found in tumor-draining lymph nodes following intratumoral treatment courses but not in the tumor microenvironment. In conclusion, viral gene transfer of sFlt3L and XCL1 is feasible, safe and biologically active in mice, exerting antitumor effects that are potentiated by CD4 T-cell depletion.

INTRODUCTION

Cancer immunotherapy is in the limelight of oncology therapeutics due to the efficacy of systemic administration of checkpoint inhibitors and chimeric antigen receptor-transduced T cells (1). Intratumoral approaches with immunotherapy agents are feasible (2), and include local administration of Toll-like receptor or STING agonists (3, 4) and recombinant oncolytic viruses (5) or viral vectors (6). Most immunotherapy approaches necessarily rely on the activation of CD8 T lymphocytes by mature dendritic cells (DCs) presenting cognate tumor antigens (7). A subset of DCs dependent on the transcription factors BATF3 and IRF8 for their ontogeny is critical for the activation of CD8 T lymphocytes (8, 9) and crucial for the antitumor efficacy of treatment with anti-PD1 and anti-CD137 mAbs in mouse models (10). BATF3-dependent DCs are also termed conventional DCs type 1 (cDC1s) and excel in uptaking antigens from dead cells and presenting their peptides on MHC-I molecules (cross-presentation), leading to the activation/expansion of specific cytotoxic T lymphocytes (cross-priming). Two subsets of mouse cDC1 have been identified. One of these resides in T-cell zones of lymphoid organs ($CD11c^+CD8\alpha^+CD103^-Clec9a^+$) (11) and the other ($CD11c^+CD8\alpha^-CD103^+Clec9a^+$) is deployed in peripheral tissues and migrates towards lymphoid tissue once activated (7, 12). Migratory $CD103^+$ cDC1s have been observed to carry tumor antigen to tumor-draining lymph nodes for cross-presentation (10, 13, 14). Flt3L is a critical growth/differentiation factor for this DC subpopulation (15) and XCL1 a chemokine that chemoattracts this DC lineage, which exclusively expresses the XCL1 receptor (XCR1) (16)

to allow for cDC1 *rendezvous* with NK and CD8 T cells (17, 18). cDC1s are endowed with abundant TLR3 expression that drives their activation/maturation once challenged with dsRNA denoting viral infection (19).

Local gene transfer into experimental tumors with Semliki Forest Virus (SFV)-derived vectors is feasible and has an attractive immunotherapeutic potential. Although SFV is not a replication-competent virus, it induces catastrophic death of infected cells (20), releases abundant viral dsRNA (21), induces local IFNα/β production (21), and is safe. Indeed, a vector encoding IL-12 (SFV-IL12) is highly efficacious in murine (22) and woodchuck (23) models of cancer and synergizes with other immunotherapies such as treatment with anti-PD-1 (24) and anti-CD137 (25) immunomodulatory mAbs.

Transfection of sFlt3L (26) or XCL1 (27) into tumor cells has been previously tested in culture and *in vivo* with immunotherapy purposes, achieving excellent vaccination effects in the case of sFlt3L (26).

In this study, repeated injections of an SFV vector simultaneously expressing sFlt3L and XCL1 were tested in an attempt to attract and expand cDC1 cells, while killing a fraction of tumor cells and providing viral RNA-mediated activation of innate immunity (28). Partial antitumor activity was substantiated against transplantable established tumors. This antitumor effect was dependent on CD8 T cells and on the integrity of the BATF3 and IFNAR genes in tumor-bearing mice.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

MC38 cells were a kind gift from Dr. Karl E. Hellström (University of Washington, Seattle, WA) in September 1998. B16-OVA cells were provided by Dr. Lieping Chen (Yale University, New Haven, CT) in November 2001. These cell lines were authenticated by Idexx Radil (Case 6592-2012) in February 2012. MC38 and B16-OVA cells were cultured in RPMI medium (Gibco) supplemented with 10% decomplemented and filtered FBS (Sigma Aldrich), containing 50 μmol/L β-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Gibco). Baby Hamster Kidney (BHK) cells were cultured in GMEM-BHK21 medium (Gibco) supplemented with 5% decomplemented and filtered FBS (Sigma Aldrich), containing 20 mM Hepes (Invitrogen), 10% Tryptose Phosphate Broth, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (all from Gibco). When indicated, BHK cells were cultured in CHO medium (Sigma) supplemented with the same components as indicated for BHK, save for the FBS. For infection, cells were incubated in MEM medium (Gibco) containing 0.2% bovine serum albumin (Sigma).

Construction of SFV-derived vectors

To generate the XCL1-sFlt3L construct, the coding sequence for soluble Flt3L was amplified by PCR from its expression plasmid (mFlex, Aldevron, Fargo, ND) and coding

sequences for the autocatalytic peptide 2A from foot and mouth disease virus and a furin binding site were added upstream of the protein-coding region, together with Mlu I restriction sites for cloning onto a mouse XCL1 expression plasmid (MR200473, Origene, Rockville, MD). The amplified product was isolated, digested with Mlu I and cloned into the MR200473 vector, downstream of the XCL1-coding region and without altering the translation reading frame. The accuracy of the cloning process was verified by DNA band analysis following enzymatic digestion with Sac I and by sequencing of the region surrounding the insertion site. XCL1, sFlt3L and XCL1-Flt3L were amplified by PCR and had Xma I target sites added at both 5' and 3' regions. All three PCR products were digested with Xma I for insertion into the pSFV-b12a vector backbone (22), which includes genes for the viral replicase. Clones that were demonstrated to be correctly inserted as assessed by digestion [sFlt3L: Nhe I; XCL1: EcoR V.HF-Msc I; XF: Nhe I] and sequencing were selected and amplified. The plasmid vector for SFV-LacZ (pSFV-enhLacZ)has been previously reported (29). mRNAs were produced in vitro from the transgene-coding and two helper plasmids coding the viral structural proteins, as previously described (30) Viral particles were produced by co-electroporation of transgene-coding and helper mRNAs into BHK cells. Electroporated cells were incubated for 48 h at 33ºC in GMEM BHK-21 medium. Debris was cleared from the supernatant by centrifugation at 2,000 g. The cleared supernatants were ultracentrifuged at 160,000 g using a SW40Ti rotor (Beckman Coulter) and resuspended in Tris-NaCl buffer, aliquoted and immediately frozen in liquid N_2. Aliquots were kept at -80ºC until used. The generated vectors were titrated by immunofluorescent detection of viral replicase on BHK cell monolayers infected by serially diluted SFV particles in MEM-0.2% BSA (infection medium), followed by an overnight culture in GMEM BHK-21 medium for protein expression. An in-house anti-replicase rabbit polyclonal antibody was used for staining to demonstrate viral gene transfer.

mRNA quantitative analysis

BHK, MC38 or B16-OVA cells were cultured on 6-well culture plates to confluence. Infection was carried out using 3 x 10^7 SFV particles, and cells were allowed an overnight incubation to ensure transgene expression. RNA was extracted from cell suspensions using the RNAeasy kit (Qiagen, Hilden, Germany) and according to the manufacturer's instructions and cDNA was generated. We designed primers to amplify the coding sequences for mouse sFlt3L (FW TGTGGCAGGGTCTAAGATGC; RV CTTCTAGGGCTATGGGACTCC), XCL1 (FW TAGCTGTGTGAACTTACAAACCC; RV ACAGTCTTGATCGCTGCTTTC), β-actin (FW AGCCTCGCCTTTGCCGA; RV CTGGTGCCTGGGGCG), and the viral replicase (FW GACGCGTCGTCAGCCAGGG; RV CCACGACCCCTGCACCTGC). The generated cDNAs were amplified by real-time PCR (BioRad, Hercules, CA) and results were analyzed using CFX manager software.

For *in vivo* RNA extraction, MC38 tumors were established and 10^8 SFV particles were administered intratumorally when tumors reached an approximate size of 25 mm^2. 24h later, tumor single cell suspensions were generated by 15 minute collagenase/DNAse digestion and mechanical disruption. mRNA was extracted from cell suspensions using the RNAeasy kit, cDNA was generated and Flt3L, XCL1, β-actin and the viral replicase were amplified and analyzed by real-time PCR (BioRad iQ5).

Western Blotting

Infection and incubation of BHK cells were performed as described above. After trypsinization, cells were lysed in RIPA buffer in the presence of a protease inhibitor (Complete, Roche, Basel, Switzerland) and the lysate protein concentration was quantified by BCA (Thermo Fisher Scientific, Waltham, MA). The lysate was boiled for 5 minutes in β-mercaptoethanol-containing loading buffer. Electrophoresis on polyacrylamide gel was carried out and proteins were transferred to PVDF membranes. Membranes were blocked with TBS-5% skimmed milk and stained with primary antibodies against mouse Flt3L (R&D AF427) or XCL1 (R&D AF486), followed by secondary staining with HRP-conjugated Goat Anti-Rat IgG (Pierce, Appleton, WI). SuperSignal™ Femto Substrate (Thermo Scientific) was used for detection. After detection, membranes were washed with azide-containing TBS buffer and re-stained with anti-mouse β-actin (Sigma, St. Louis, MO). Secondary staining was carried out with HRP-conjugated Goat Anti-Rabbit IgG (BioRad) and Pierce™ ECL Western Blotting Substrate (Thermo Scientific) was used for detection.

Functional assays for transgene products

BHK cells were infected with SFV vectors at an MOI of 10 as described above and incubated overnight in serum-free CHO medium (Sigma) for XCL1 bioactivity testing or GMEM BHK-21 (Gibco) for Flt3L bioactivity testing. Supernatants were collected and kept frozen until use. For Flt3L testing, bone marrow cell suspensions were flushed out of hind limb bones and cultured in RPMI medium conditioned with 20% infected BHK-derived supernatants. After 9 days, classical BM-DC ($CD11c^+CD11b^+$) and plasmacytoid BM-DC ($CD11c^+CD11b^-B220^+$) cells were assessed by flow cytometry to demonstrate sFlt3L-dependent differentiation. For XCL1 testing, standard transwell chemotaxis assays were performed on iCD103 BM-DCs (31). 10^5 iCD103 cells were suspended in serum-free CHO medium and plated onto 5 μm transwell inserts (Costar). Cells were allowed to migrate for four hours toward infected BHK-derived supernatants and the total number of cells in the lower well was quantitated by flow cytometry.

Mice and *in vivo* tumor experiments

Experiments involving mice were carried out in the animal facility of the Center for Applied Medical Research (CIMA, Pamplona, Spain) under study approvals 150/12 and 082/16 from the University of Navarra Ethics Committee. C57Bl/6 *Batf3*$^{tm1Kmm/J}$ (Batf3 KO) (8), *Tmem173*$^{gt/J}$ (STING KO) (32) and *IFN-a/bR*$^{o/o}$ (IFNAR KO) (33) mice were bred at CIMA in specific pathogen-free conditions. C57Bl/6 mice were obtained from Envigo (Barcelona, Spain). Batf3 KO, STING KO and IFNAR KO mice were kindly provided, respectively, by Dr. Kenneth M. Murphy, Washington University, St. Louis, MO, by Dr. Gloria González Aseguinolaza (CIMA, Pamplona) and by Dr. Matthew Albert (Institut Pasteur, Paris). Cultured tumor cells were cultured and trypsinized for injection before reaching confluence.

5×10^5 MC38 or B16-OVA cells were injected subcutaneously in 50 µl PBS into the right flank of 6- to 12-week old mice. SFV viral particles (VPs) were diluted in PBS and kept ice-cold until administration. Intratumoral injection of 50 µl suspension containing 10^8 VPs or vehicle control was performed using 29G syringes and under inhalatory anesthesia. When indicated, 100 µg anti-CD137 (1D8) or anti-PD-1 (RMP1-14) were administered intraperitoneally (i.p.) in PBS. Depletion of lymphocyte subsets was performed by i.p. injection of anti-CD4 (GK1.5, Bioxcell, West Lebanon, NH), anti-CD8 (H35-17.2, in-house) or anti-NK1.1 (PK136, in-house) mAbs. 200 µg of each mAb were injected two days before SFV administration; 100 µg on SFV treatments days and three days after the last SFV administration. A single 300 µg dose of anti-CD25 (PC61, in-house) was administered two days before SFV administration. Depletion was verified by peripheral blood flow cytometry staining. 100 µg p60 peptide (34) were administered i.p. daily for 10 days, starting two days before SFV administration. Tumor area was measured twice weekly and calculated as the product of orthogonal diameters.

Tissue Processing and Flow cytometry

Excised tumors and tumor-draining lymph nodes were incubated in collagenase/DNAse for 30 minutes at 37ºC, followed by mechanical disaggregation and filtering through a 70-µm cell strainer (Thermo Fisher Scientific). Single-cell suspensions were then stained for flow cytometry. The fluorochrome-tagged mAbs used are listed in Supplementary Table 1. For identification of epitope-specific T cells, phycoerythrin-conjugated H-$2K^b$-$OVA_{257-264}$ tetramer (MBL, Woburn, MA) was used. For intranuclear staining, cells were fixed and permeabilized using the TrueNuclear transcription factor staining kit (Biolegend, San Diego, CA) and then stained according to manufacturer's instructions. Acquisition was performed using a FACS Canto II flow cytometer (BD Biosciences, Franklin Lakes, NJ).

Software and statistical analyses

Flow cytometry data were analyzed using FlowJo software (BD Biosciences). Statistics on tumor growth data were analyzed with Prism software (GraphPad Software, La Jolla, CA). Mean diameters of tumors over time were fitted using the formula $y = A \times e^{(t-t0)}/(1 + e^{(t-t0)/B})$, where t represents time, A the maximum size reached by the tumor, and B its growth rate. Treatments were compared using the extra sum-of-squares F test (10). Tumor survival was compared with log-rank (Mantel-Cox) tests. All other analyses between groups were performed using unpaired One-way ANOVA with Turkey's post-hoc test. Unless specified otherwise, graphs depict mean ± SEM.

RESULTS

Characterization of SFV-derived vectors encoding sFLt3L and XCL1

Non-replicative SFV vectors were constructed by replacing the viral structural proteins with the mouse sequences of XCL1 or sFlt3L, generating vectors SFV-XCL1 and SFV-sFlt3L, respectively (Fig. 1A). An SFV vector expressing β-galactosidase encoded by LacZ gene (SFV-LacZ) was used for control purposes. An SFV vector encoding both XCL1 and sFlt3L as a single ORF was made by placing a 2A cis-protease sequence to permit post-translational efficient proteolytic separation of both transgene products. A furin cleavage site was also inserted to eliminate the remaining 2A target sequence from XCL1. Three cell lines were infected in culture with the different SFV vectors and quantitative RT-PCR detected strong transcription of the transgenes (Fig. 1B). Moreover, gene expression was readily detected in subcutaneous MC38-derived tumors excised 24h post-intratumoral injection of the corresponding SFV vectors (Fig. 1C). Of note, both *in vitro* and *in vivo,* the vector expressing the two transgenes showed comparatively lower quantities of each transgene mRNA as compared to single-gene SFV vectors, indicating less efficient expression in the double-transgene vector. Translation was confirmed by analyzing tissue culture cell-lysates of 24h-infected BHK cells by Western Blot (Fig. 1D). The differences in the sizes of the detected proteins encoded by the single-transgene and double-transgene vectors are due to the presence of a C-terminal myc tag from the XCL1 parental expression plasmid. Due to the cloning strategy used, the tag is present in the C-terminus of the XCL1 protein from SFV-XCL1 and from the sFlt3L protein from SFV-XF, thus slightly modifying their detected molecular weights in the Western Blot analysis.

Next, we examined the functionality of the expressed transgenes (Fig. 1E). For this purpose, we analyzed the chemotactic activity of XCL1 from tissue culture supernatants of SFV-infected BHK cells on iCD103 DCs derived in culture from bone marrow precursors as previously described (31) (Fig. 1F). sFlt3L bioactivity was assessed by studying the effect of infected BHK culture supernatants to promote the differentiation of bone marrow cell suspensions into conventional and plasmacytoid DCs (cDCs and pDCs) (Fig. 1G). In both instances, transgene products appeared to be fully functional.

Antitumor activity of SFV vectors encoding sFlt3L and/or XCL1

To study the antitumor effects of the constructed SFV vectors, a single injection of 10^8 viral particles (VPs) was given into day 8 established MC38 subcutaneous tumors (Fig. 2A). A certain degree of tumor growth retardation was observed with all sFlt3L-containing SFV vectors, but it was more prominent with the vector encoding both XCL1 and sFLt3L (SFV-XF). To enhance antitumor effects, three doses of vectors were given every two days starting at day 8 after tumor cell inoculation. Again, MC38 tumors were more efficiently delayed in their growth by the SFV-XF vector (Supp. Fig. 1A). Treatment of B16F10-derived melanomas with three doses of SFV-XF also indicated the therapeutic effects of SFV-XF (Supp. Fig. 1B). In a series of experiments represented in Figures 2 B and C, evident tumor growth delays were achieved by repeated intratumoral administration of SFV-XF into established MC38 (Fig.

2B) and B16-OVA (Fig. 2C) tumors. This treatment resulted in survival prolongation in both models but seldom in tumor eradication.

Given the clinical success of immunomodulatory monoclonal antibodies (mAbs), we explored whether local SFV therapeutic activity could be potentiated by its combination with systemic antagonist anti-PD-1 or agonist anti-CD137 mAbs. As shown in Fig. 3, while the anti-CD137 mAb was able to delay tumor growth in both models, anti-PD-1 was only partially effective against B16-OVA-derived tumors (Fig. 3A and B). Contrary to our expectations, no increase in the efficacy of SFV-XF was found upon combination with repeated doses of either immunomodulatory mAb.

Antitumor activity of SFV-XF was dependent on CD8 T cells but enhanced by CD4 T-cell depletion

To study the cellular requirements for the activity of SFV-XF, selective depletion of T-cell subsets and NK1.1$^+$ NK and NKT cells were performed prior to treatment in MC38 tumor-bearing mice. As shown in Fig. 4A, depletion of CD8β cells abolished therapeutic activity whilst CD4 and NK1.1 depletion enhanced the therapeutic effects, leading to extended survival. This result indicates that the antitumor effect mediated by SFV-XF is mainly mediated by CD8$^+$ T cells.

One interpretation of the enhanced antitumor activity following CD4 depletion is the ensuing elimination of CD4$^+$ Tregs. However, pre-depletion of Tregs with an anti-CD25 mAb (35) or inhibition of Foxp3 with an antagonist peptide (34) did not enhance therapeutic effects (Supp. Fig. 2). In contrast, CD4 T-cell depletion gave rise to 4 out of 5 mice eradicating their tumor upon intratumoral treatment with SFV-XF. In mice bilaterally engrafted with MC38 tumors, SFV-XF treatment in the context of CD4 T-cell, but not NK1.1 depletion, undoubtedly delayed the growth of distant non-injected tumors (Fig 4B and C). SFV-XF as a single agent did not have therapeutic effects on distant tumors, even though a trend for delay of tumor growth was observed in some of the experiments (Fig. 4C).

In B16-OVA-derived tumors, there was an increase of CD4 and CD8 T-cell content in the tumor microenvironment (Supp. Fig. 3A). In these B16-OVA tumors, we observed a rapid increase in the number of H-2K^b-tetramer-positive CD8 T cells recognizing the OVA-specific SIINFEKL epitope (Supp. Fig. 3B). These results indicate increases in tumor-reactive CTLs consistent with the CD8 depletion experiments.

SFV-XF therapeutic activity is contingent on BATF3-dependent DC integrity and causes cDC1 accumulation in tumor-draining lymph nodes (TDLNs)

Experiments were performed in mice deficient in BATF3, which are virtually devoid of cDC1s (8). In these animals, the antitumor effects of SFV-XF seen in wild type (WT) control mice were completely lost (Fig. 5A, B). The integrity of the type-I interferon (IFN-I) system is required for the function of BATF3-dependent DCs (36) and for CD8 immunity (37). As

seen in spaghetti plots in Figure 5A, when treatment was given to *Ifnar*$^{-/-}$ mice, efficacy was also lost. However, tumor growth delay was preserved to some degree in STING KO mice, indicating an at least partial independence of our therapy of the cGAS-STING pathway.

Given the activity of the SFV-encoded transgenes, we expected tumors to become infiltrated by cDC1s, a feature reported to correlate with better prognosis in human cancer (38,39). However, as seen in Fig. 6, the tumor myeloid infiltrate did not significantly change following three intratumoral doses of SFV-XF over control or SFV-LacZ (Fig. 6A,B). In contrast, harvested TDLNs showed marked increases in absolute numbers of both migratory ($CD11c^{+}IAb^{hi}CD103^{+}CD11b^{-}$) and resident ($CD11c^{hi}IAb^{+}CD8\alpha^{+}CD11b^{-}$) cDC1 cells (Fig. 6C). In addition, there was a detectable increase in $CD11b^{+}$ cDC2 cells (Fig. 6B). FACS gating strategies for analysis are shown in supplementary Fig. 4.

In conclusion, dependency on BATF3 and the increase of cross-presenting DCs in TDLNs are consistent with the immunotherapeutic activity of XCL1 and sFlt3L as SFV-encoded transgenes.

DISCUSSION

In this study, SFV vectors engineered to increase cross-priming of tumor antigens were tested following intratumoral injection. Although all SFV constructions encoding sFlt3L delayed tumor growth, the combination of the chemokine XCL1 and sFlt3L showed more marked antitumor effects.

Intratumoral injection of viral vectors including HSV (40), measles virus (41), Vaccinia virus (42), VSV (43) and reovirus (44) is gaining momentum in tumor immunotherapy (6). Their intratumoral administration frequently leads to meaningful therapeutic effects, particularly when combined with anti-CTLA-4 or anti-PD-1 checkpoint inhibitors (5, 45). In the case of alphavirus vectors, an SFV virus encoding IL-12 exerts potent antitumor effects dependent on CD8 T-cell antitumor immunity (22). SFV-XF was therapeutically less potent than an SFV vector encoding IL-12 (data not shown), although it has the advantage that IL-12 uncontrolled production might have safety problems, as reported in human patients systemically given the recombinant protein (46). In this regard, Flt3L recombinant protein is reportedly safe in humans following subcutaneous administration (47).

The original objective of the SFV-XF vector was to enhance tumor antigen cross-presentation by means of attracting and differentiating cDC1s and thereby enhancing CD8 T-cell cross-priming. Indeed, the SFV-XF encoded transgenes exert these effects on cells in culture. We had previously shown two important features of SFV-based local immunotherapy: (i) it provides abundant viral RNA that enhances TLR3 and helicase-dependent innate signals, and (ii) it enhances local IFNα/β through these mechanisms (48). These two effects, in conjunction with a more prominent cDC1 function should prime and sustain cellular antitumor immunity. In this context, it was surprising that SFV-XF showed a rather modest curative immune activity, although most tumors were delayed in their growth after treatment. In this line, treatment failed to synergize with anti-PD-1 and anti-CD137 mAbs as we

were wrongly anticipating, despite the fact that each agent exerted its reported individual therapeutic effects. Of note, intratumoral SFV-IL12 is reportedly highly synergistic with these immunomodulatory antibodies (24,25).

Experiments upon depletion of CD8 T cells were consistent with a necessary involvement of CTLs in the antitumor effects. Surprisingly, CD4 T-cell depletion and NK/NKT depletion gave rise to enhanced therapeutic activity. Having ruled out a simple explanation based on the elimination of Tregs by CD25 depletion, our next hypothesis was that lymphopenia secondary to CD4 depletion augmented the availability of homeostatic cytokines such as IL-7 or IL-15 for CD8 T cells. However, we were unable to detect circulating levels of these cytokines following depletion (data not shown). The mechanistic interplay of NK and NKT cells to dampen the efficacy of SFV-XF remains to be elucidated, although some reports suggest an inhibitory activity of NK cells on recently activated CD8 T-cell blasts (49, 50).

In keeping with the function of the XCL1 and sFlt3L transgenes, antitumor effects were contingent on BATF3-dependent DCs. However, we did not observe any increase in such DCs in the tumor microenvironment at various time points following SFV-XF intratumoral administration. This is in contrast with the increases found in TDLNs that were minimally seen in non-draining lymphoid organs (data not shown). Such increased cDC1 cells belonged to both resident and migratory phenotypes, suggesting that perhaps part of these cDC1 cells seen in TDLNs might have been in the tumor tissue at some earlier time points. Yet, the absence of increases of cDC1 in the tumor microenvironment warrants further research.

The striking effect of SFV-XF combination with CD4 depletion which led to a certain degree of efficacy against distant tumors is difficult to translate into the clinic, since CD4 depletion is highly immunosuppressive and in practice could only be induced transiently. CD4 T-cell immunity is complex and encompasses both antitumor and protumor activities. Transplanted tumors in mice, as opposed to human malignancies, grow fast in the two weeks following tumor cell inoculation and the mechanism of action of SFV-XF, relying on cross-priming, might take longer to properly begin. In fact, DC numbers kept increasing in TDLNs from treated mice over time. Little is known about the interplay of CD4 T cells and cDC1s, and our results call for an in-depth study.

All in all, our results indicate interesting immunobiological effects of SFV-mediated XCL1 and sFlt3L local gene transfer into tumors that might find suitable combination partners for effective cancer immunotherapy. The strategy is of much interest due to its effects on antigen-presenting cells specialized in CD8 T-cell cross-priming.

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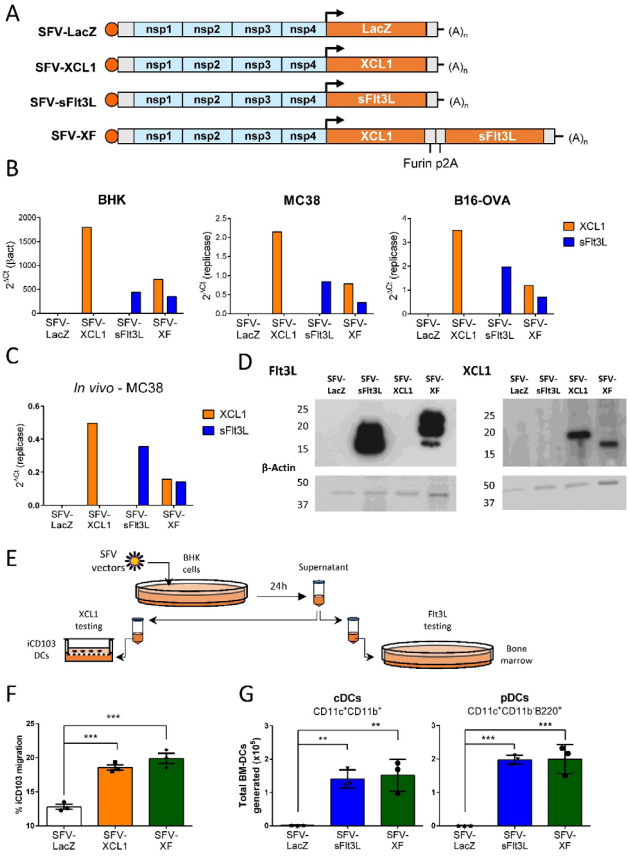
FIGURES

Figure 1.

SFV-based vectors confer functional expression of XCL1 and/or Flt3L in infected cells

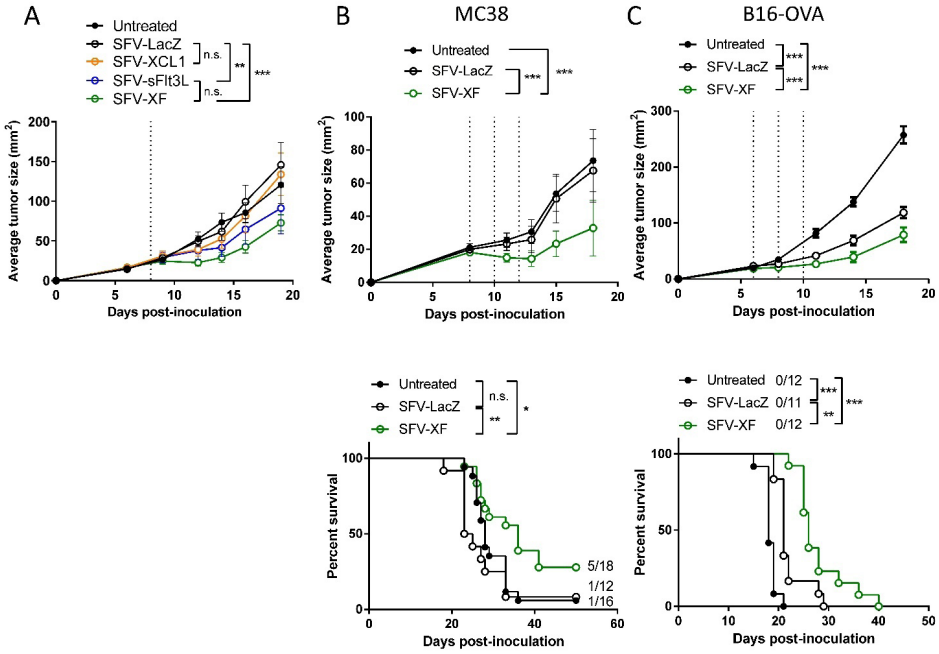


(A) WT mice were injected hydrodynamically in the tail vein with 10 µg sFlt3L-coding plasmid in 2 ml saline buffer. 10 days later, (A) XCL1 and/or soluble Flt3L (sFlt3L) cDNAs were cloned into the SFV vector backbone encoding SFV non-structural proteins (nsp 1-4). (B and D) BHK, MC38 and B16-OVA cell lines were infected in culture with SFV-derived vectors and transgene expression was assessed 24h later by quantitative RT-PCR (B) or Western Blot analysis with antibodies specific for the indicated proteins (D). Ct values were normalized for β-actin (βact) or SFV replicase (replicase). (C) MC38 subcutaneous tumors were established and intratumorally injected with 108 SFV viral particles when they reached an approximate size of 25 mm2. Transgene expression was assessed 24h later by quantitative RT-PCR. (E) BHK cells were infected with SFV-derived vectors at a multiplicity of infection (MOI) of 10 and cell-free supernatants were collected 24h later and used for the indicated assays. (F) iCD103 cells were derived from bone marrow in 14-day cultures in the presence of sFlt3L and GM-CSF as described (31). For chemotaxis assays, 105 iCD103 cells were placed onto a 5-µm transwell membrane and allowed to migrate towards infected BHK-supernatants for 4h. Total migrated cells in the lower chamber were quantified by flow cytometry. One representative experiment is shown out of three. (G) Bone marrow cell suspensions flushed out of mouse bones were differentiated ex vivo for nine days using infected BHK supernatant-conditioned media. On day 9, cultures were analyzed by flow cytometry. Conventional DCs (cDCs) were identified as CD11c+CD11b+ and plasmacytoid DCs (pDCs) as CD11c+B220+CD11b-. One representative experiment is shown out of three. **p<0.01; ***p<0.001. (A)n, polyA; furin, target sequence for furin protease; p2A, 2A autoprotease from foot and mouth disease virus.

Source: Own elaboration.

Figure 2.

Intratumoral injection of SFV-XF exerts antitumor effects against MC38 and B16-OVA subcutaneous tumors

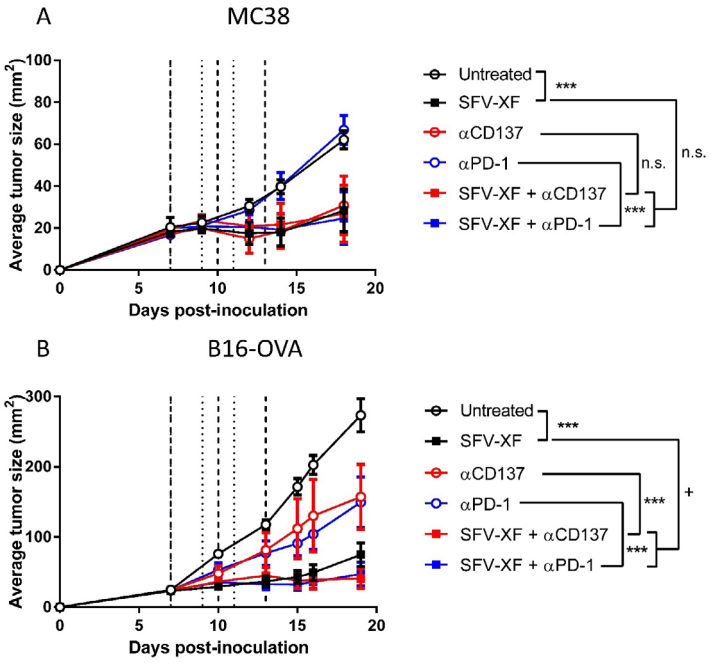


(A and B) 5 x 10^5 MC38 cells were inoculated subcutaneously into the right flank of C57Bl/6 mice. (A) Mice received one intratumoral dose of 10^8 VPs of SFV-derived vectors on day 8 (indicated by the dotted line). Results represent mean tumor sizes from one representative experiment with 6 mice per group of four experiments performed. (B) Mice received three intratumoral doses of 10^8 VPs of SFV-derived vectors on days 8, 10, and 12 (dotted lines). Data represent mean tumor sizes over time (upper panel) from one representative experiment with six mice per group of three experiments performed and survival of the mice (Kaplan-Meier curves in lower panel) summarizing three pooled experiments. Fractions indicate surviving mice at the end of the experiment. (C) 5 x 10^5 B16-OVA cells were inoculated subcutaneously into the flank of C57Bl/6 mice. Mice received three intratumoral doses of 10^8 VPs of SFV-derived vectors on days 6, 8, and 10 (indicated by dotted lines). Mean tumor sizes over time (upper panel) from one representative experiment with seven mice per group of two experiments performed and survival of the mice (lower panel) from the two pooled experiments are represented. *p<0.05; **p<0.01; ***p<0.001.

Source: Own elaboration.

Figure 3.

Intratumoral treatment with SFV-XF shows no synergy with anti-CD137 or anti-PD-1 mAbs

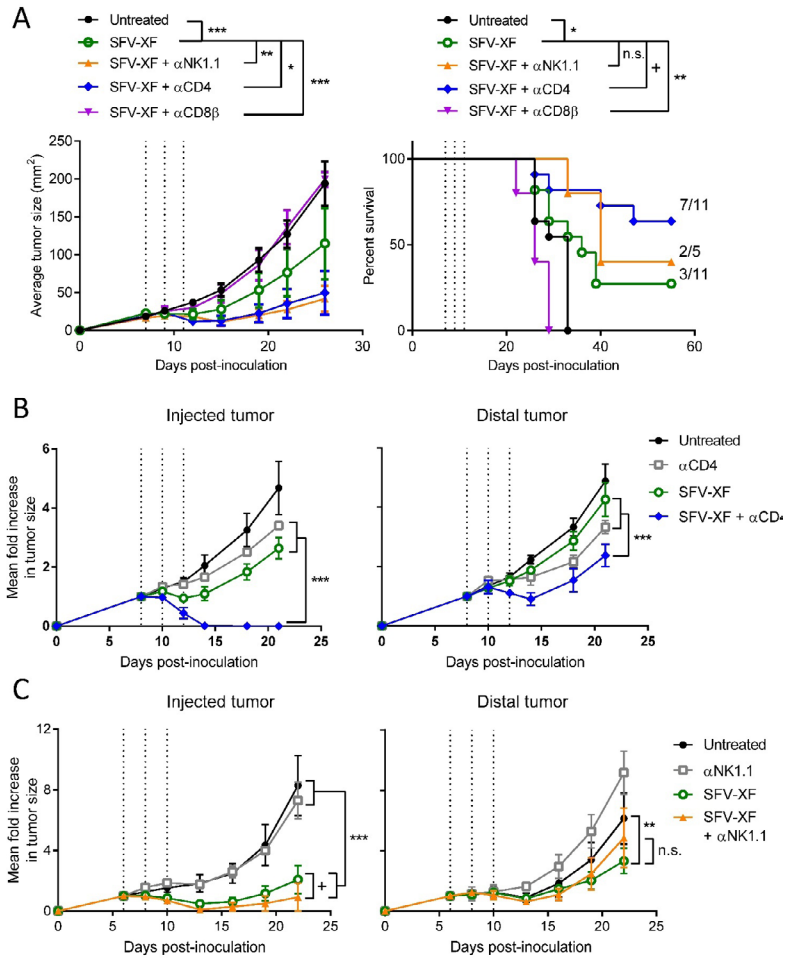


(A) 5 x 10^5 MC38 or (B) 5 x 10^5 B16-OVA cells were inoculated subcutaneously into the flank of C57Bl/6 mice. Mice received three intratumoral doses of 10^8 VPs of the indicated SFV vectors on days 7, 9, and 11 (dotted lines) and three intraperitoneal doses of anti-CD137 or anti-PD-1 mAbs on days 7, 10, and 13 (dashed lines). Mean tumor sizes over time are represented (n = 5-6 mice per group).

Source: Own elaboration.

Figure 4.

CD8 T-cell depletion abrogates SFV-XF therapeutic effects, whereas CD4-T cell depletion markedly improves efficacy

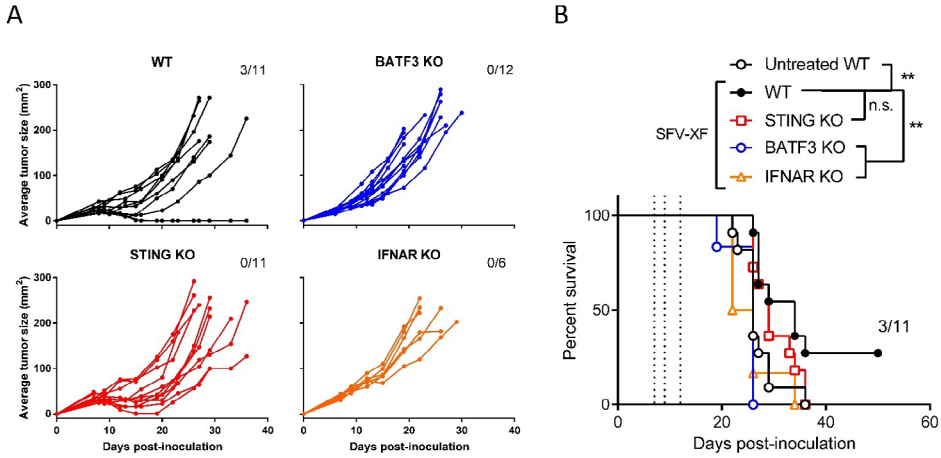


(A) 5 x 10^5 MC38 cells were inoculated subcutaneously into the flank of C57Bl/6 mice. Three intratumoral doses of 10^8 VPs of SFV-XF were given on days 7, 9, and 11 (dotted lines). Results show mean tumor progression from one representative experiment of two performed (left panel) and survival summarizes two pooled experiments (right panel). Fractions in the caption indicate surviving tumor-free mice at the end of the experiment. (B, C) 5 x 10^5 and 3 x 10^5 MC38 cells, respectively, were inoculated into the right and left flanks of C57Bl/6 mice and the right flank tumor was treated as described in (A). Results represent mean fold increase in tumor growth over time. All mice received intraperitoneal injections of depleting antibodies and depletions were confirmed as described in Materials and Methods. Fractions indicate surviving mice. $^{+}p<0.1$; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Source: Own elaboration.

Figure 5.

SFV-XF requires Batf3-dependent DCs and IFNAR for therapeutic activity

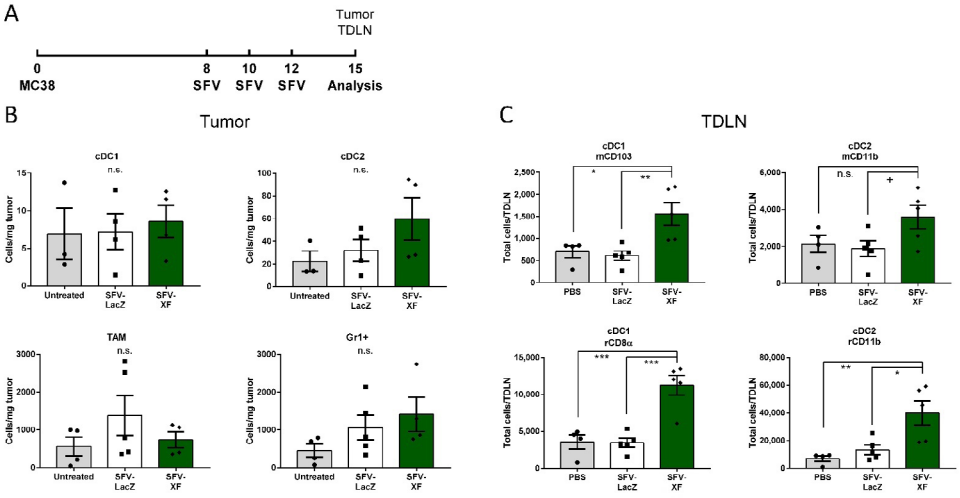


5 x 10^5 MC38 cells were inoculated subcutaneously into the flank of WT, *Batf3*$^{-/-}$, *Tmem173*$^{-/-}$, or *Ifnar*$^{-/-}$ mice with C57Bl/6 background. Three intratumoral doses of 10^8 VPs of SFV-derived vectors were given on days 7, 9, and 12 (dotted lines). Tumor sizes over time (A) and survival (B) from two pooled experiments are shown. Fractions in each graph indicate surviving mice. **p<0.01.

Source: Own elaboration.

Figure 6.

Conventional DCs become enriched in treated tumor-draining LNs but do not augment their numbers in the tumor microenvironment



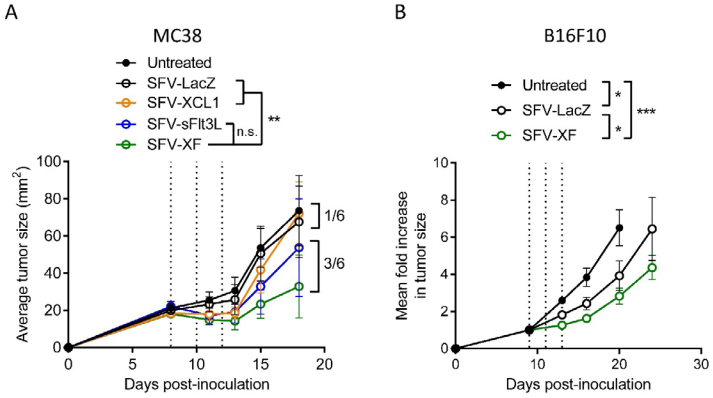
(A) Schematic design of the experiment. 5 x 10^5 MC38 cells were inoculated subcutaneously into the flank of C57Bl/6 mice, which received three intratumoral doses of 10^8 VPs of SFV-derived vectors on days 8, 10, and 12. Three days after the last administration of SFV, tumors and TDLNs were excised, digested, and single cell suspensions analyzed by flow cytometry. (B) Numbers of infiltrating cells per mg of tumor from one representative experiment of three are presented. (C) Absolute number of dendritic cells per LN is presented. Gating strategies are shown in Supplementary Figure 3. $^+$p<0.1; *p<0.05; **p<0.01; ***p<0.001.

Source: Own elaboration.

SUPPLEMENTARY FIGURES

Supplementary Figure 1.

The SFV-XF vector exerts maximal antitumor efficacy as compared to SFV vectors encoding each single transgene and is effective against B16F10-derived melanomas

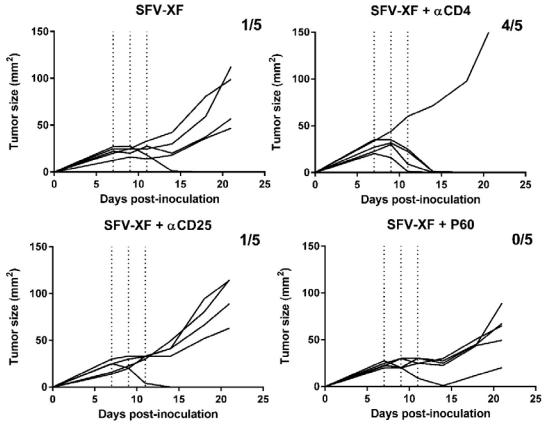


(A) 5 x 10^5 MC38 cells or (B) 5 x 10^5 B16F10 cells were inoculated subcutaneously into the flank of C57Bl/6 mice as in Figure 1. Mice received three intratumoral doses of 10^8 VPs of SFV-derived vectors on days 8, 10, and 12 (A) or days 9, 11 and 13 (B) (dotted lines). Mean tumor sizes over time (A) or mean fold increase in tumor size (B) are represented. *p<0.05; **p<0.01; ***p<0.001.

Source: Own elaboration.

Supplementary Figure 2.

CD4 Treg depletion or inhibition does not recapitulate the enhancement of efficacy of SFV-XF treatment found with CD4 T-cell depletion

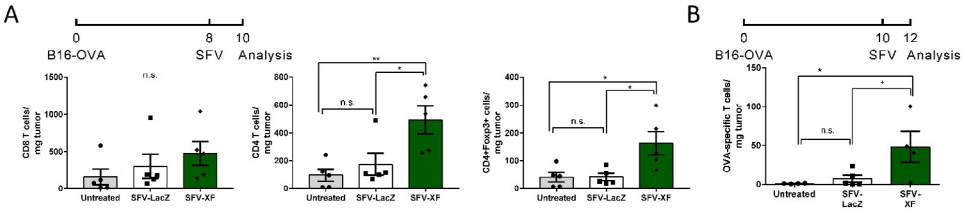


5 x 10^5 MC38 cells were inoculated subcutaneously into the flank of C57Bl/6 mice. Three intratumoral doses of 10^8 VPs of SFV-XF were given on days 7, 9, and 11 (dotted lines). Mice received intraperitoneal injections of depleting antibodies or Foxp3-inhibitor peptide (p60) as indicated and described in Materials and Methods and depletions were confirmed by immunostainings in peripheral blood. Results represent individual tumor growth over time. Fractions indicate surviving mice.

Source: Own elaboration.

Supplementary Figure 3.

Administration of SFV-XF into B16-OVA, but not MC38 tumors, increases T-cell tumor infiltration

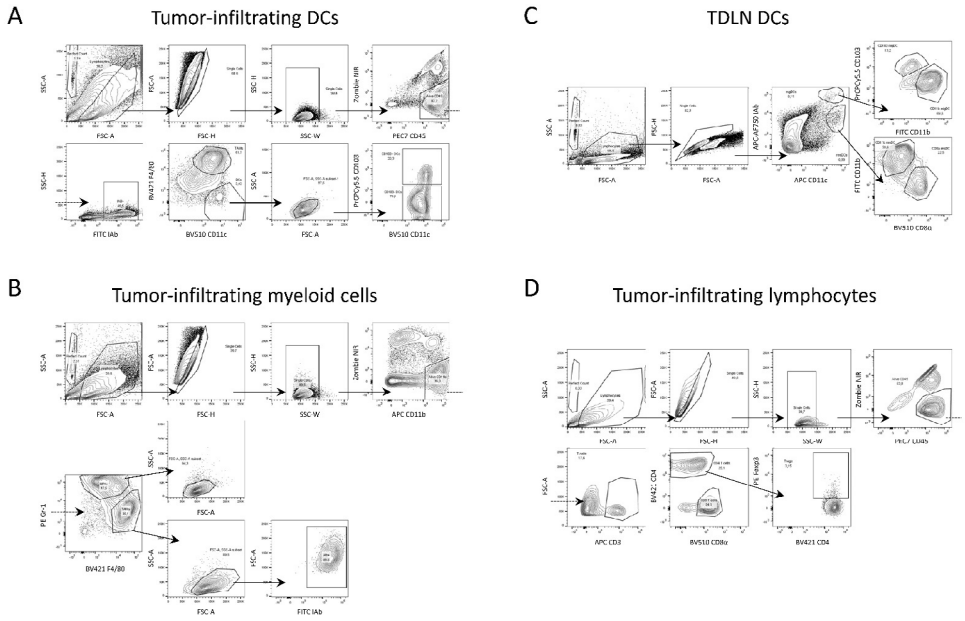


5 x 10^5 B16-OVA cells were subcutaneously injected into C57Bl/6 mice. Mice received one intratumoral dose of 10^8 VPs of SFV-derived vectors on the days indicated in the figure. Two days later, tumors were excised, digested, and cell suspensions analyzed by flow cytometry. The numbers of infiltrating cells per mg of tumor are shown in panels A and B. In panel B, H-2Kb-SIINFEKL tetramers were used to evaluate tumor-specific CD8 T cells. Data represent one representative experiment out of two performed.

Source: Own elaboration.

Supplementary Figure 4.

FACS Gating strategies to analyze tumor-infiltrating and lymph node cell suspensions



FACS strategies used to identify tumor-infiltrating DC subsets (A), tumor-infiltrating myeloid cells (B), DC subsets in TDLN (C) and tumor-infiltrating T lymphocytes (D) are shown.

Source: Own elaboration.

Supplementary Table 1.

Antibodies and reagents used in flow cytometry experiments

Reagent	Source (mAb clone)
Zombie NIR	Biolegend
SAV-APCAF750	Invitrogen
FITC B220	Biolegend (RA3-6B2)
APC CD11b	Biolegend (M1/70)
FITC CD11b	Biolegend (M1/70)
PE CD11c	Biolegend (N418)
APC CD11c	Biolegend (N418)
BV510 CD11c	Pharmingen (HL3)
FITC CD25	Pharmingen (7D4)
PE Foxp3	eBioscience (FJK-16S)
APC CD3	Biolegend (145-2C11)
PEC7 CD45	Biolegend (30-F11)
BV421 CD4	Biolegend (RM4-5)
BV510 CD8	Biolegend (53-6.7)
FITC IAb	Pharmingen (AF6-120.1)
Biotin IAb	Pharmingen (KH74)
PE Gr1	Biolegend (RB6-8C5)
BV421 F4/80	Biolegend (BM8)
PrCPCy5.5 CD103	Biolegend (2E7)
APC XCR1	Biolegend (ZET)

Source: Own elaboration.

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:

- The identification of a key cellular component (Batf3-dependent cDC1s) driving response to immunotherapy with immunostimulatory agents anti-CD137 and anti-PD-1.
- 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

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REVIEW

Antigen cross-presentation and T-cell cross-priming in cancer immunology and immunotherapy

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Dendritic cells (DCs) are the main professional antigen-presenting cells for induction of T-cell adaptive responses. Cancer cells express tumor antigens, including neoantigens generated by nonsynonymous mutations, but are poor for antigen presentation and for providing costimulatory signals for T-cell priming. Mounting evidence suggests that antigen transfer to DCs and their surrogate presentation on major histocompatibility complex class I and II molecules together with costimulatory signals is paramount for induction of viral and cancer immunity. Of the great diversity of DCs, BATF3/IRF8-dependent conventional DCs type 1 (cDC1) excel at cross-presentation of tumor cell-associated antigens. Location of cDC1s in the tumor correlates with improved infiltration by $CD8^+$ T cells and tumor-specific T-cell immunity. Indeed, cDC1s are crucial for antitumor efficacy using checkpoint inhibitors and anti-CD137 agonist monoclonal antibodies in mouse models. Enhancement and exploitation of T-cell cross-priming by cDC1s offer opportunities for improved cancer immunotherapy, including *in vivo* targeting of tumor antigens to internalizing receptors on cDC1s and strategies to increase their numbers, activation and priming capacity within tumors and tumor-draining lymph nodes.

Key words: cross-presentation, cross-priming, cancer immunotherapy, dendritic cells, T cells

Introduction

In a series of experiments involving immunization with major histocompatibility complex (MHC)-incompatible mouse splenocytes osmotically loaded with chicken ovalbumin (OVA), Michael Bevan discovered that the antigen-presenting cells for MHC class I restricted OVA epitopes were necessarily recipient antigen-presenting cells [1]. This phenomenon was termed cross-priming, since the read-out was the ensuing activation of antigen-specific T cells. The set of mechanisms involving uptake, processing and presentation of cell-associated or soluble extracellular antigens receives the name of cross-presentation. Interestingly, MHC class I cross-presentation can lead to antigen-specific tolerance that can be referred to as "cross-tolerance" [2]. Dendritic cells (DCs) were identified as the subset of myeloid cells most efficient at cross-presentation [3]. Discoveries over recent years suggest that a very specific subset of DCs excels at cross-presentation [4, 5], and equivalent subsets have been characterized in humans [6–9]. While the demonstration of the relative cross-priming ability in different human DCs subsets requires further study [10, 11], understanding and exploiting cross-priming is becoming very important in cancer immunotherapy, as it affects a variety of key issues ranging from the development of more efficacious vaccines [12] to understanding the effect of immunostimulatory monoclonal antibodies [13]. Figures 1 and 2 summarize antigen capture and cross-presentation by DCs in the tumor microenvironment (TME) and tumor-draining lymph nodes (LNs), and how targeting such DCs offers translational opportunities for the development of cancer therapies.

DC subsets specialized in cross-priming

Steinman and Cohn [14] first described DCs as a phagocytic cell type in mouse spleen with dendrite-shaped protrusions, which could

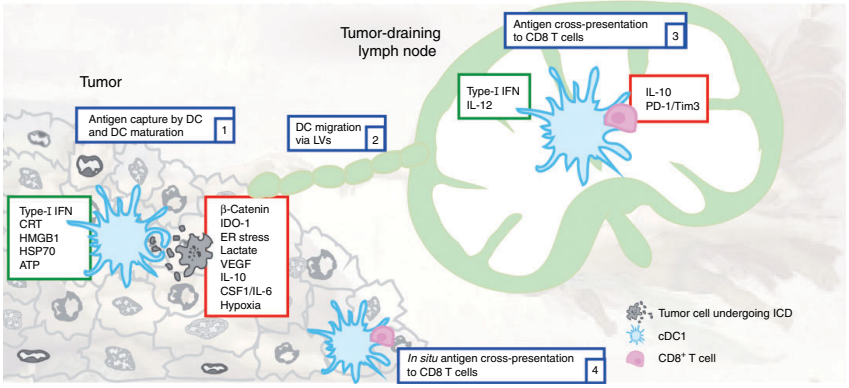

Figure 1. Depiction of the processes and factors involved in tumor antigen cross-presentation to T cells. Numbered boxes represent the stages of T-cell cross-priming in draining lymph nodes and at the tumor site. Intrinsic and environmental factors promoting antitumor T-cell responses are depicted in green, while those linked to inhibition of antitumor immune responses are depicted in red. DC, dendritic cell; ER, endoplasmic reticulum; ICD, immunogenic cell death; LVs, lymphatic vessels.

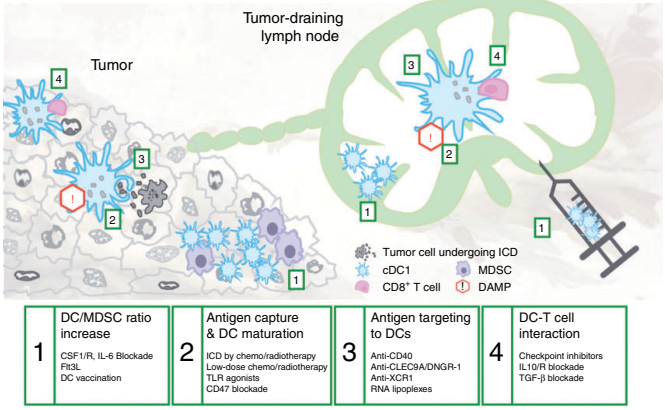

Figure 2. Summary of current therapeutic strategies that improve cross-priming of antitumor T cells. The intervention strategies for cancer treatment relying on tumor-antigen cross-priming are schematically represented in relation with the anatomical site of action. Of note, the doses of chemo or radiotherapy eliciting immunogenic cell death (ICD) are likely to be greater than those causing immunomodulation in the tumor microenvironment. DAMP, damage-associated molecular pattern; DC, dendritic cell; MDSC, myeloid-derived suppressor cell; TLR, toll-like receptor.

prime and activate naive T cells upon antigen presentation [3]. Michael Lotze in mice [15, 16] and Ron Levy in humans [17] pioneered work to use DCs in tumor immunotherapy by incubation of DCs with tumor antigens in different forms to elicit tumor-specific T-cell immunity upon reinfusion of the antigen-loaded DCs into the tumor-bearing hosts. In most of these instances, the DCs used for immunotherapy were differentiated from monocytes in culture. Following exciting results against transplantable mouse tumors [18–20], a large series of therapeutic vaccine clinical trials have been carried out but with as yet limited clinical efficacy [21].

Over the years since their discovery, it has been revealed that DC lineage is very complex and encompasses a variety of subsets both in mice and in humans. DC heterogeneity adds an extra layer of complexity to instructing and manipulating immunity. Several DC subsets are functionally defined by their capacity to activate naive T cells, including conventional DCs (cDCs), plasmacytoid DCs (pDCs), Langerhans cells and monocyte-derived DCs [22–25]. These DCs are subdivided based on their dependence on specific transcription factors in their ontogeny and show diverse functional responses, phenotypic markers and tissue

distribution [22–31]. In addition, DCs can be differentiated in culture from monocytes or bone marrow precursors under the influence of granulocyte–macrophage colony-stimulating factor (GM-CSF), Flt3L or other cytokines [32–34]. These GM-CSF-derived DCs generated *ex vivo* have been extensively used in experimentation with the caveat that they imperfectly reflect their naturally existing counterparts.

pDCs comprise a subgroup of DCs dependent on the E2-2 transcription factor and co-express CD11c and PDCA1 (CD317) in mice, and BDCA2 (CD303) and BDCA4 (CD304) in humans. The main role of pDCs seems to be the abundant production of type I interferon (IFN-α/β) associated with viral sensing. IFN-α/β is a factor known to enhance cross-priming [35] and reportedly, pDCs themselves can cross-present melanoma shared antigens *in vitro* [36]. The involvement of pDCs in cross-priming *in vivo* could be mostly indirect via type I IFN production although pDC direct involvement cannot be excluded.

Langerhans cells that are found in the epidermis are endowed with some antigen cross-presentation capability in humans [37, 38] and can migrate to draining LNs [37]. Probably, their main physiological role is antiviral defense of the skin [39].

cDCs are best known for their high efficiency in initiating and directing T-cell responses [22, 24, 26, 27, 29]. In mice, cDCs express CD11c and MHC class II and can be subdivided into $CD11b^+$ (cDC2) and $CD11b^-$ (cDC1) subsets [25]. cDC2 can be identified by surface coexpression of CD11b and SIRPα (CD172a) in mice, and BDCA1 (CD1c) in humans. cDC2 are dependent on the transcription factor IRF4 for ontogeny and include subsets defined by ontogenic dependence on Notch 2 or KLF4, associated with Th17 and Th2 immunity, respectively [40–42]. Indeed, cDC2s direct Th2 immunity in allergic asthma [43].

$CD11b^-$ "CD8α-like" cDC1s comprise $CD8\alpha^+$ DCs in lymphoid organs and their $CD103^+$ $CD11b^-$ counterparts in nonlymphoid tissues that share gene expression patterns and depend on specific transcription factors, including IRF8 and BATF3 [44]. They have been recently reported to derive from a unique myeloid precursor [45, 46]. cDC1 express XCR1, CLEC9A/DNGR-1, CD8α and/or CD103 in mice, while in humans they can be best identified by XCR1, CLEC9A/DNGR-1 and BDCA3 (CD141) staining [47]. This subset very efficiently cross-presents extracellular antigens, particularly cell-associated antigens, to $CD8^+$ T cells [4, 44, 48–50]. When activated, cDC1s also produce high amounts of Th1-differentiating cytokines including IL-12, as observed both in human and in mice [8, 51–53] and provide essential signals for generation of resident memory $CD8^+$ T cells [54]. Although probably sculpted by evolution to initiate and sustain anti-viral immune responses [55], the superior capacity of cDC1s for the induction of cytotoxic T lymphocyte (CTL) and Th1 responses makes them uniquely suitable for combatting cancer [4, 13, 56]. Recent evidence in transplanted mouse tumors shows the key role of cDC1s in the baseline CD8-mediated immune response against tumor antigens [4, 57], while their presence in the TME of human tumors correlates with the intensity of CD8 T-cell infiltrates [58–60]. cDC1s come in two forms similarly fit for cross-priming. In the mouse, $CD8\alpha^+$ DCs are naturally resident in lymphoid tissues, whereas $CD103^+$ DCs lacking CD8α expression are deployed in peripheral tissues and upon activation migrate to LNs to meet T cells for antigen presentation. Given that these subsets are mainly involved in antiviral immune responses, it is likely that LN-resident cDC1s mainly deal with infections causing widespread viremia, while non-lymphoid tissue migratory cDC1s would handle viral infection at the point of entry.

More recently, cancer vaccination attempts have been made using reinfusion of defined populations of DCs obtained *ex vivo* upon immunomagnetic sorting from peripheral blood, including the use of $BDCA1^+$ and pDCs [61–63]. The paucity of $BDCA3^+$ cDC1s in peripheral blood has so far precluded similar approaches with these cells although efforts to separate such a professional cross-priming subset in clinical-grade conditions are ongoing [www.procrop.eu (28 March 2017, date last accessed)].

Intracellular molecular players in cross-presentation

MHC-I cross-presentation requires the processing and trimming of the endocytosed protein material. This processing takes place through two main intracellular routes: the cytosolic and the vacuolar pathways [49]. The cytosolic pathway requires antigen export of polypeptides from endosomal compartments into the cytosol [64], proteasomal digestion [65, 66] and transporter associated with antigen processing (TAP)-dependent transport of polypeptides to the endoplasmic reticulum (ER) or endosomes, where final peptide trimming and MHC-I peptide loading take place. Inhibition of TAP in endosomes or inhibition of endosomal trafficking to the cell membrane leads to abrogation of soluble OVA protein cross-presentation in a cathepsin-independent fashion [67]. Trimming is carried out by ER-located aminopeptidase 1 [68] and the early endosome-associated protein insulin-regulated aminopeptidase (IRAP) [69]. Both peptidases are required for optimal cell-associated antigen cross-presentation. An interesting experimental approach to deplete cross-presenting DCs is to inject cytochrome C *in vivo*, such that only those DCs with ability to cross-present that leak this pinocytosed protein to the cytosol undergo apoptosis [70].

The vacuolar MHC-I pathway is proteasome- and TAP-independent and does not require antigen to exit the endosomal compartment. In this case, endosomal protein cargo is degraded by lysosomal enzymes (cathepsins) and peptides are locally generated and trimmed to directly bind onto MHC-I molecules [71]. The exact relative contribution of the cytosolic and vacuolar pathways to tumor antigen cross-presentation *in vivo* remains unknown.

A distinctive feature of DCs specialized in cross-priming is their ability to maintain a higher pH in endosomal compartments, as compared with non-specialized DCs or macrophages. A higher endosomal pH delays antigen protein degradation, since lysosomal enzymes optimally perform in acidic conditions. Delayed acidification of prelysosomal or lysosomal compartments allows for protein export to the cytosol or its loading onto recycled MHC-I molecules in the endosome. This slow acidification mechanism is mediated by the phagosomal NADPH oxidase NOX2, which catalyzes reactive oxygen species production and proton consumption in phagosomes [72, 73]. In this context, the G-protein Rac2 is required for the effective action of NOX2 in lysosomes [74]. Sec22b is reportedly another key molecular player, bringing together ER-derived vesicles (ER-Golgi Intermediate Compartments, ERGIC) and phagosomes for fusion, while delaying antigen proteolytic

degradation in endosomes [75]. It must be acknowledged that the molecular machinery defining uptake and MHC-I cross-presentation of tumor cell-associated antigens still defies complete understanding.

Evidence for cross-presentation and cross-priming in cancer immunology

Tumor antigen cross-presentation is postulated to be naturally and constantly taking place. Batf3-deficient mice, in which cross-presentation is severely reduced, are more susceptible to tumor engraftment than their wild-type counterparts [4]. Cross-presentation of tumor antigens is frequently demonstrated with the help of known surrogate antigens expressed by tumor cells, the most common being chicken OVA, although other viral or neoantigens known to be present in tumor cell lines could be used in this same way. These surrogate antigens stimulate T-cell receptor transgenic lymphocytes, e.g. OT-I $CD8^+$ T cells recognizing an $H2\text{-}K^b$-restricted peptide of OVA. Most tumor antigens are probably cross-presented as cell-associated material by Batf3-dependent cDC1s [44], rather than soluble individual proteins. cDC1s show high efficiency at endocytosis of material from dying or dead cells, and from subcellular vesicles such as exosomes [76–80]. However, the superior capacity of cDC1s for cross-presentation is attributable to their specialized antigen-processing capacity [81, 82]. The cross-presentation ability of cDC1s is also favored by the selective expression of receptors such DNGR-1 (CLEC9A) on their surface [83–85]. DNGR-1 facilitates cross-presentation of necrotic material upon interaction with filamentous actin onto which other proteins can be adsorbed and complexed [86, 87]. *In situ* tumor antigen capture is similar among different tumor-infiltrating DCs (TIDCs), monocytes and tumor-associated macrophages (TAMs) [58, 88], but cDC1s uniquely mediate the transport of antigens for cross-presentation from the tumor to the draining LN for cross-priming of $CD8^+$ T cells [60, 88].

Some controversy exists surrounding the superiority of $BDCA3^+$ cDC1s in cross-presentation of cell-associated antigens in humans [6–9, 11]. Whether or not $BDCA3^+$ cDC1s outperform other DC subsets in cross-presentation activity in cancer patients still remains unclear. However, mounting evidence suggests that the presence of $BDCA3^+$ cDC1s in the TME is associated with more abundant T-cell infiltration and better prognosis in cancer patients and the success of immunotherapy approaches [57–59]. Of note, there is no published formal experimental evidence that neoantigens can be cross-presented yet.

Does T-cell cross-priming take place in the TME and/or in tumor-draining LNs?

As stated above, although macrophages and other DC subsets phagocytose tumor antigens, $CD103^+$ cDC1s mediate tumor antigen transport and cross-presentation from established tumors and early metastases to LNs [13, 58, 60, 79, 88, 89]. The role of LN-resident DCs in tumor antigen cross-presentation is unclear. A potential tumor antigen transfer mechanism from $CD103^+$ to other LN DC populations has been proposed [60].

Whether $CD103^+$ cDC1s or other tumor-infiltrating myeloid cells mediate cross-priming *in situ* remains uncertain although such a phenomenon is probably important. Transcriptomic analysis of tumor-infiltrating $CD103^+$ DCs revealed superior expression of genes involved in cross-presentation, costimulation ability and IL-12 production over non-BATF3-dependent DCs, suggesting that their role could be carried out in the TME [58]. Depletion of cDCs hampered an adoptive T-cell therapy experiment in which LN priming would be dispensable, suggesting that the intratumoral presence of $ZBTB46^+$ cDCs is a requirement for the continuous priming of the transferred T cells [58]. In line with this, *in situ* activation of naive T cells in tumors was possible in experiments in which T-cell recirculation was blocked with FTY-720 and even in mice lacking LNs and spleen, thus pointing to T-cell activation by TIDCs and/or tumor cells themselves [90, 91]. However, other studies have reported no tumor-associated antigen (TAA) cross-presentation from CD11c-sorted cells from the TME [92]. A potential limitation of this and other reports is the use of CD11c expression as the exclusive marker to identify TIDCs, which may include a majority of TAMs in the subsequent functional analyses [93]. Recent reports refining the isolation of cDC1 from the tumor site confirmed that these cells are able to cross-present tumor antigen with a higher efficiency than other DC subsets [58, 89]. In addition to DCs, it is possible that other cells such as lymphatic endothelial cells cross-present TAA in the TME and in TDLNs, but their function seems to be more closely related to cross-tolerance than to eliciting antitumor immunity [94].

Immunosuppressive factors for DCs in the TME

Tumor-derived factors influencing DC function have been recently reviewed in detail by the group of Michael Shurin [95]. TIDCs are exposed to tumor-associated and extracellular immunoregulatory factors that may render DCs non-functional or even actively immunosuppressive [96]. These deleterious mechanisms comprise metabolic, immune-mediated, biochemical or mechanical factors (Figure 1).

A very important signaling route that is involved in cross-priming inhibition in tumors is controlled by the β-catenin pathway. Previous work suggested that the activation of β-catenin signaling favors a tolerogenic state in DCs [97, 98]. Wnt ligands and other molecules promoting β-catenin signaling, both in tumor cells and inside DCs, mediate DC exclusion from the TME and the inhibition of their antitumor immune functions, respectively. The group of Thomas Gajewski identified melanoma cell-intrinsic β-catenin signaling as the main cause for a downregulation of CCL4 production and hence of DC chemoattraction. As a result, there is T-cell exclusion from the TME [57] (While this review was in editorial production, the findings in [99] were confirmed and cDC1 cells were found, in an experimental melanoma model, to be key to chemoattract CD8+ T cells to the TME by means of CXCL9 and CXCL10 production. Also, CXCL9 and CXCL10 mRNA in human melanomas were found to correlate with a gene signature denoting cDC1 infiltrate.). DC-intrinsic β-catenin signaling is also active in TIDCs, and it both disrupts cross-presentation and reprograms DC to induce tolerance, generating T regulatory cells (Tregs) as a

result of their TGFβ production [100]. In some cases, Wnt ligands are tumor derived [101]. Ensuing IDO-1 expression has been proposed as one of the mechanisms underlying tolerization by DCs [102]. This enzyme causes tryptophan depletion and production of immunosuppressive kynurenine and other metabolites in the TME [103–105].

It should not be forgotten that the physical and chemical conditions of the TME affect the functions of the leukocytes that dare to infiltrate the malignant tissue. Solid tumors contain large hypoxic areas, due to poor vascularization and the leaky nature of tumor-irrigating blood vessels. Hypoxia has been shown to cause a shift toward glycolytic metabolism and increased responsiveness to LPS stimulation in DCs [106]. It has also been observed that hypoxia exposure reduces IL-12 production by DCs [107], which is partially rescued by HIF-1α silencing [108]. The specific contribution of the hypoxic tumor environment to the maturation status and function of TIDCs has still to be determined. The overall picture is that while hypoxia dampens the antitumor functions of myeloid cells, it improves the performance of T cells [109].

A glycolytic switch is characteristic of both DC and T-cell activation to an effector phenotype [110]. Glucose availability in the TME is a critical limiting factor for T-cell activation and function [104, 111]. The local concentration of certain aminoacids and waste metabolites also dramatically influences T-cell and DC function in the tumor, often dampening antitumor immune responses [112, 113]. TIDCs are prone to accumulation and oxidization of lipid bodies [114], which can hamper efficiency of cross-priming and produce other dysfunctions through chronic induction of the ER stress response [115–117]. Hence, targeting metabolic pathways in TIDCs might represent an interesting opportunity for cancer immunotherapy [118, 119].

There is ample evidence that functional immune cell receptors acting as checkpoints [120] repress anti-cancer immunity [121]. DCs express high levels of PD-L1 and PD-L2 upon stimulation [88]. PD-1 expression has also been demonstrated on TIDCs in human cancerous tissue and blood [122], as is also the case with the coinhibitory receptor Tim-3 [123]. The expression of these checkpoints and their counter-receptors on DCs interferes with the DC maturation processes inhibiting NF-κB activation [122], HMGB1 function as TLR4 agonist [123, 124] and cytosolic nucleic acid recognition in the TME. Therefore, checkpoint surface molecules on DCs ultimately exert a negative effect on the cross-priming of T cells. Whether or not the expression of these checkpoint molecules on DCs is directly involved in the clinical antitumor efficacy of PD-1/PD-L1 blockade is an issue that remains to be elucidated.

TIDC differentiation from circulating monocytes is also affected by tumor-derived factors such as M-CSF (CSF1) and IL-6, which favor macrophage differentiation [125]. TAMs are great producers of IL-10 in the TME [126], which is known to act as an immunosuppressive factor for cross-priming DCs [51].

Activation of TIDCs by administration of TLR agonists such as poly:IC (TLR3) or imiquimod (TLR7/8), among other strategies, aims to reverse their tolerogenic status [13, 127–129] (Figure 2). A strategy currently being tested in clinical trials against melanoma involves local transfection of TIDCs using mRNA encoding for T-cell costimulatory molecules [130–132]. Transfection of IL-12 into ex vivo-generated DCs for intratumoral injection has also been reported to improve antitumor responses in mice and humans [133, 134].

Immunogenic cell death and cross-priming

The concept of immunogenic cell death (ICD) proposed by Guido Kroemer and collaborators [135–137] is intimately bound to the concept of tumor immunogenicity, cross-priming and DC function. ICD can induce an adaptive effector immune response against antigens present in the dying cell [138]. It is important to remember that ICD is an active process within the dying cell, which releases alarmins and chemotactic factors leading to DC attraction and activation (Figure 3).

DCs are key mediators in the building of an immune response against cells undergoing ICD. ICD activates antigen cross-presentation in several ways: (i) attracting cross-presenting DCs to dying cells (i.e. ATP, mitochondrial formyl peptides) [91, 139], (ii) increasing the uptake and processing/presentation of dead cell-associated antigens by DCs (i.e. exposure of calreticulin, heat shock protein 70, exposure of phosphatidylserine) [140, 141, 142] and (iii) licensing DCs for CTL activation (i.e. HMGB1 acting on TLR4 or ATP acting in $P2X_7$) [141, 143, 144]. An interesting mechanism has been reported in this regard: CD24 on cDC1s can adsorb HGMB1 to be trans-presented to RAGE on T cells [124]. Accordingly, in the absence of DCs, responses against vaccines or conventional anti-cancer treatments inducing ICD are impaired [91, 143, 145].

Hypericin-based photodynamic therapy [145], radiotherapy [146, 147], certain chemotherapeutics [91, 138] and other interventions [148] have been demonstrated to elicit ICD *in vitro* and are candidate strategies for cancer vaccine preparation. Cell freezing and thawing is widely regarded as generator of a non-immunogenic necrotic death and, as a result, does not lead to efficient antigen cross-priming [145]. However, a simple heating step following cell lysis might halt protein degradation by peptidase inactivation and allow for T-cell cross-priming [149].

A recent paper by the group of Matthew Albert identified a cancer cell-intrinsic RIPK1-NF-κB signaling pathway that was required for a form of programmed necrosis called necroptosis [150]. Mice immunized with necroptotic cells established stronger responses than those immunized with apoptotic or frozen/thawed cells. Immunization again was dependent on cross-priming by Batf3-dependent DCs. Similar results were obtained in an additional publication using CT26 necroptotic cells [151]. No mechanism has been reported so far linking necroptosis to facilitated cross-priming.

This concept of ICD is reminiscent of the postulates of the danger model originally proposed by Polly Matzinger, according to which the immune system is set up to respond to agents causing tissue and cell damage [152]. The overall concept is that alarmins released or exposed [153] during ICD change the functional profile of DCs, even in sterile conditions, in a process known as maturation or activation. As a consequence, costimulatory molecules for T cells become expressed on the plasma membrane along with abundant MHC-antigen complexes and IL-15Rα coupled to IL-15 on the DC surface that is thereby trans-presented to signaling receptors on T cells [154]. The induction of IL-12 and ligands for T-cell costimulatory receptors of the tumor necrosis factor receptor (TNFR) family such as CD27L (CD70), CD137L, OX40L [155–157] are considered paramount in this process (Figure 3).

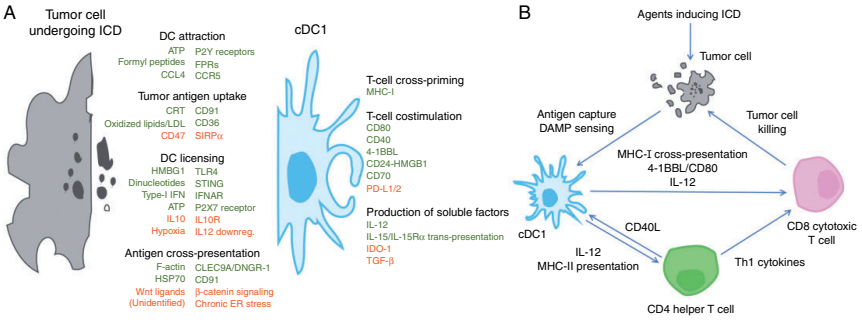

Figure 3. Schematic representation of the mechanisms reportedly coupling immunogenic tumor cell death with T-cell cross-priming by dendritic cells (DCs). (A) Molecular players involved in cell-associated antigen uptake and processing for cross-presentation by DCs and DC activation/maturation. Mechanisms linked to antitumor effects are depicted in green, and those linked to protumor effects are depicted in red. (B) Postulated key cell-to-cell interactions mediating antitumor T-cell cross-priming against tumor antigens.

Targeting tumor antigen to DCs to favor its cross-presentation

An attractive way that has been explored for immunization against tumors is the targeting of tumor antigens to DCs using monoclonal antibodies (mAbs) directed to DC surface receptors that internalize upon ligation.

The group of Ralph Steinman efficiently targeted antigen to the DC surface receptor DEC205 [158]. Using this strategy, CD8- and CD4-mediated responses were generated, the former being TAP-dependent. Without coadministration of an agonist anti-CD40 monoclonal antibody (mAb) as a DC-activating adjuvant, vaccination was actually tolerogenic. This effect was mainly mediated by CD8α$^+$ cDC1s in the mouse. DEC205 targeting directs the antigens to late endosomes and lysosomes [159]. Targeting antigens to CD40, unlike DEC205, delivers antigen to early EEA1$^+$ endosomes and is a more efficient strategy for cross-presentation. This is consistent with the notion that intracellular trafficking to early endosomes is required for efficient cross-presentation. Targeting to CD40 potentiates cross-priming by both Batf3-dependent and Batf3-independent DCs, reportedly achieving better responses than those obtained by anti-DEC205 antigen complexes [159]. This strategy is being pursued in clinical trials with anti-DEC205 mAb linked to NYESO-1 antigen (NCT01834248, NCT02166905).

DNGR-1 (CLEC9A) is an internalizing receptor with high expression narrowly restricted to cDC1s in mouse and humans, although it shows low expression on other cell types [84, 85, 160, 161]. Its main function may be the routing of necrotic cell-derived material into nonlysosomal compartments for cross-presentation [55, 162, 163]. Targeting cDC1s with protein antigens coupled to anti-DNGR-1 mAbs was much superior to control IgG-bound antigen in generating antitumor immune responses, when combined with adjuvants such as anti-CD40 or poly:IC [84]. In a similar manner, coupling TAA to a short peptide that targets DNGR-1 has been shown to induce antitumor immunity [164].

Since cDC1s selectively express the chemokine receptor XCR1, targeting of this receptor with a construct of its ligand XCL1 coupled with antigen was also effective in inducing CD4 and CD8 T-cell-mediated responses against viral infection [165].

A caveat for the formulation of antigens targeted to DC receptors is that the nature of the most immunogenic tumor antigens is usually ignored. Indeed, the most powerful tumor antigens are the result of unique non-synonymous mutations in their translated genes whose peptide sequences fit the autologous MHC-I and MHC-II alleles acting as antigen-presenting molecules. Such antigens specific to each tumor are named neoantigens. The use of cancer neoantigens for vaccination holds much promise for the delivery of efficacious immunotherapy strategies [166], particularly when combined with checkpoint inhibitors [167]. Targeting neoantigens to cross-priming DCs seems to be a reasonable strategy, but preparing individual DC-targeting moieties for each patient is a daunting biotechnical challenge. mRNA coding for neoantigens and/or shared antigens has been complexed with liposomal carriers and administered systemically, generating potent vaccine-specific antitumor immunity in a DC-dependent way, provided that the charge and size of the lipoplexes is optimized [168]. This approach, using neoantigens and shared tumor antigens, is currently being tested in clinical trials against melanoma and breast cancer (NCT02410733, NCT02316457). Alternatively, naked synthetic mRNA encoding cancer neoantigens can be injected inside LNs with ultrasound guidance achieving powerful vaccine effects [169].

Cross-priming involvement in various cancer therapies

We will briefly discuss the involvement of cross-priming in currently used therapeutic strategies and the potential for improvement of both cytotoxic therapy and immunotherapy upon combination with cross-priming enhancers.

Chemotherapy

Chemotherapy can improve immunotherapeutic approaches in two main ways: first, by inducing ICD of tumor cells, allowing for

antitumor T-cell cross-priming by native DCs; second, by modulating the phenotype of tumor-associated regulatory populations such as regulatory T cells (Tregs), TAMs or myeloid-derived suppressor cells. It is now well known that not all chemotherapeutic agents induce ICD [136]: anthracyclines such as doxorubicin or mitoxantrone [138, 140] and cyclophosphamide [170] are strong inducers of ICD and tumor antigen cross-presentation, while cisplatin is not [171]. Additionally, systemic gemcitabine was shown to recover dysfunctional cross-presentation by TAMs and TIDCs [92] whereas it was ineffective in cDC1-deficient Batf3$^{-/-}$ mice [172]. One report pointed to a Batf3-independent subset of tumor-infiltrating CD11c^{+} CD11b^{+} Ly6C^{hi} cells as responsible for the ensuing immune response to ICD induced by anthracyclines [91]. This suggests a more complex interplay of immune cells involved in the response to chemotherapy. The proimmune effects of chemotherapy may need lower doses than the maximally tolerable dose levels used as a standard [173]. All in all, the line of work pioneered by Guido Kroemer and Laurence Zitvogel puzzled the world of clinical oncology, since in mouse models some forms of chemotherapy act against tumors with an absolute need for cellular immune responses dependent on ICD [135].

Radiotherapy

Ionizing radiation is an ICD inducer, and therefore a good candidate for successful combination with immunotherapy [138, 174, 175]. Radiotherapy (RT) has been shown to potentiate tumor antigen cross-presentation in mouse models [176]. Several groups explored the intratumoral injection of DCs into irradiated mouse tumor models with positive results [177, 178]. The functions of cDC1s sensitive to IFNα have been found to be very important for the immune-mediated therapeutic effects of local irradiation [179]. These findings are consistent with the requirement for DC-mediated cross-priming in mouse models in which RT induces abscopal effects to concomitant non-irradiated tumors, that can be greatly potentiated with immunomodulatory anti-PD-1, anti-CTLA-4 and anti-CD137 mAbs [180–184]. It should be kept in mind that TIDCs under the irradiation beam also undergo functional changes [185]. Curiously, a conversion from pro- to anti-tumor myeloid populations occurs in the TME of tumors irradiated at low doses [186]. Active combinations of RT and local TLR agonists have been preclinically reported [187] and clinically tested against follicular lymphoma [188] and breast cancer [189].

Immunotherapy

Type I IFN (IFNα/β) potentiates cross-presentation by DCs [35] and it has been found to be clinically active against a number of malignancies [190]. The antitumor activity of type I IFN requires type-I IFN receptor (IFNAR) function on cDC1s in mouse models [191, 192]. IFNAR absence in CD11c cells leads to reduced intratumoral accumulation of DCs and decreased cross-presentation capability on a per-cell basis. The antitumor effect of anti-CD47 is also dependent on IFNAR and this agent is known to potentiate antigen cross-presentation by DCs and macrophages both at the tumor site and in TDLNs [193]. CD47 functions as a ligand for SIRPα, acting as a don't-eat-me signal. Accordingly, if anti-CD47 mAb disrupts this inhibitory interaction, more phagocytosis takes place. Conceivably IFNα/β enhances cross-presentation and cross-priming against the cell-associated endocytosed material.

Stimulator of IFN genes (STING) agonists are potent type I IFN inducers [194]. Not surprisingly, local immunotherapy based on STING agonist cyclic dinucleotides given intratumorally absolutely requires STING expression in Batf3-dependent DCs [195] and this function is required to enhance the therapeutic results of immune checkpoint blockade in the B16 melanoma mouse model [196].

Immune checkpoint blockade with anti-PD-1/PD-L1 and anti-CTLA4 has been demonstrated to be ineffective in Batf3-deficient mice [13, 88, 167]. Moreover, Batf3-dependent DCs are critical for the antitumor activity of anti-CD137 agonist immunostimulatory mAbs [13]. In fact, systemic DC expansion and local stimulation with Flt3L and poly-ICLC synergized with PD-1/PD-L1 blockade and CD137 stimulation [13] or mutant BRAF inhibition [88]. These results suggest that the numbers of such DCs mediating cross-priming and their activation status can be modulated to enhance other immunotherapy interventions.

Conclusion

Direct presentation by malignant cells of tumor antigen to T cells is crucial at the effector killing phase, but inefficient to prime and sustain the cytotoxic immune response [197]. Cytotoxic T lymphocytes need therefore to recognize their cognate antigen on professional antigen-presenting cells. Only a few years ago, cross-priming was a black box in terms of our mechanistic knowledge [198]. The molecular and cellular details on how, where and under which circumstances cross-presentation of tumor antigens efficiently takes place are crucial for understanding immune responses against tumors and will certainly provide multiple opportunities for progress in cancer immunotherapy.

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Disclosure

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ANNEX 2

REVIEW ARTICLE

DECIPHERING CD137 (4-1BB) SIGNALING IN T-CELL COSTIMULATION FOR TRANSLATION INTO SUCCESSFUL CANCER IMMUNOTHERAPY

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Review

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

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CD137 (4-1BB, TNF-receptor superfamily 9) is a surface glycoprotein of the TNFR family which can be induced on a variety of leukocyte subsets. On T and NK cells, CD137 is expressed following activation and, if ligated by its natural ligand (CD137L), conveys polyubiquitination-mediated signals via TNF receptor associated factor 2 that inhibit apoptosis, while enhancing proliferation and effector functions. CD137 thus behaves as a bona fide inducible costimulatory molecule. These functional properties of CD137 can be exploited in cancer immunotherapy by systemic administration of agonist monoclonal antibodies, which increase anticancer CTLs and enhance NK-cell-mediated antibody-dependent cell-mediated cytotoxicity. Reportedly, anti-CD137 mAb and adoptive T-cell therapy strongly synergize, since (i) CD137 expression can be used to select the T cells endowed with the best activities against the tumor, (ii) costimulation of the lymphocyte cultures to be used in adoptive T-cell therapy can be done with CD137 agonist antibodies or CD137L, and (iii) synergistic effects upon coadministration of T cells and antibodies are readily observed in mouse models. Furthermore, the signaling cytoplasmic tail of CD137 is a key component of anti-CD19 chimeric antigen receptors that are used to redirect T cells against leukemia and lymphoma in the clinic. Ongoing phase II clinical trials with agonist antibodies and the presence of CD137 sequence in these successful chimeric antigen receptors highlight the importance of CD137 in oncoimmunology.

Keywords: Cancer immunotherapy · CD137 (4-1BB) · Costimulation · K63-polyubiquitin · TRAF-2

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Introduction

CD137 (4-1BB, *tnfsfr9*) was originally reported by the group of B. Kwon in 1992 as a cDNA clone whose sequence showed homology to TNF receptors and as being selectively expressed in activated versus resting T cells [1, 2]. With the first monoclonal antibodies specific for this surface glycoprotein, these same investigators demonstrated that ligation of CD137 could result in costimulatory signals for T lymphocytes, which cooperate with those elicited via the TCR–CD3 complex [3]. Their studies in mouse [2] and human [4] T lymphocytes showed consistent results between species in terms of inducing T-cell proliferation, enhancing IL-2 production and inhibiting apoptosis [5]. The next landmark discovery in the study of CD137 was the identification of CD137-Ligand (4-1BBL or *tnfsf9*), a molecule of the TNF family, by Alderson et al. [6, 7]. To date, CD137L remains the only intercellular ligand known for CD137, although the extracellular domain of CD137 reportedly binds to fibronectin [8] and to galectin-9 [9]. Coimmunoprecipitation of CD137 with the signaling adaptors TRAF-2 (TNF receptor associated factor 2) and TRAF-1 (TNF receptor associated factor 1) has been reported [10–12], as well as the sequences required for the interaction between CD137 and TRAF-2 [13]. The crystal structure of the CD137L trimer has been resolved, and a model for interaction with CD137 has been proposed that is analogous with that of other members of the TNFR family [14].

A proposed model for CD137 signaling and its regulation

Signaling via CD137 proceeds from ligated molecules at the cell surface, which become cross-linked either by trimerized ligand [14] or multivalent antibodies [3] (Fig 1). CD137 has been immunoprecipitated both as a monomer and as a dimer [2]. Extracellular binding of galectin-9 to CD137 has shown to be a factor keeping preassembled CD137 complexes together [9], which are then further cross-linked by antibody or by CD137 ligand (Fig 1). Across the TNFR family, it seems that trimers are the optimal signaling complexes [15], although a role for the formation of multimers of higher order is likely. The orientation of the monomers in the assembled complexes does not appear to be relevant for signaling, since mAbs binding different distant epitopes over the molecule have been shown to induce the same functional effects [16]. Although a conformational change of CD137 in these complexes cannot be definitively ruled out, this molecular event has not been observed with other members of the TNF-TNFR family and its requirement would not be absolute. CD137 associates with the adaptors TRAF-2 and TRAF-1 in its cytoplasmic tail, resulting in coimmunoprecipitation, which is enhanced upon CD137 activation in T cells [12, 17]. TRAF-2 is expressed in resting T lymphocytes, while TRAF-1 increases its levels of expression following activation [18]. In this way, the composition of the membrane CD137–TRAF complexes changes during lymphocyte activation.

The intrinsic biochemical activity of TRAF-1 is still unknown [19], although it has been reported to link CD137 receptor signaling to alternative NF-κB activation via NIK (NF-κB-inducing kinase) in TCR-stimulated T cells [20, 21]. TRAF-2 encompasses an E3 ubiquitin ligase domain (Really Interesting New Gene (RING) domain) predicted to polyubiquitinate substrate proteins in conjunction with the Ubc13 (ubiquitin-conjugating protein 13) as its only E2 enzyme companion [22, 23] (Fig 1). However, the RING domain of TRAF-2 might not be able to accommodate ubiquitin moieties [24] and it is possible that the polyubiquitination reactions are mediated instead by cIAP-1 and cIAP-2 (cellular inhibitor of apoptosis protein), which physically associate with TRAF-2 [25]. In fact, an inactive mutant c-IAP protein in transgenic mice impairs NF-κB and ERK activation via CD137 [26].

Polyubiquitin chains linking the carboxyl terminus of ubiquitin molecules to the Lys63 of the next ubiquitin are well known to offer docking sites for downstream signaling components, giving rise to activation complexes that recruit other signaling molecules that dock to the scaffold [27, 28].

We propose that the main action of CD137 is to place two or more TRAF-2 molecules in close molecular proximity to each other. Under these circumstances, a constitutive process of transubiquitinating sister TRAF-2 molecules would be set in action. Transubiquitination would proceed as long as the short molecular distance between sister TRAF-2 molecules is maintained. Accordingly, sister TRAF-2 molecules would be the first substrates of the ensuing reaction. Growing K63 polyubiquitin chains would then act to recruit TAK-1–TAB1/2 (transforming growth factor beta-activated kinase 1—TAK-1 binding proteins 1 and 2) into these complexes and this kinase complex would in turn phosphorylate other downstream substrates, leading to activation of the canonical route of NF-κB via IKKβ and NEMO (NF-κB essential modulator) [27] as well as MAP kinases via MEKK1 [29, 30] (Fig 1).

Hence, the major factor driving CD137 signaling is postulated to be the relative density of TRAF-2-assembled CD137 moieties in micropatches of plasma membrane, as predicted to occur in immune synapses, formed by $CD137^+$ lymphocytes and $CD137L^+$ antigen-presenting cells [31].

Spontaneous signaling from unligated CD137 should however be avoided to prevent uncontrolled or overstimulation of lymphocytes. In our recent research, we have observed that the K63 deubiquitinases (DUBs) A20 [32] and CYLD [33] downregulate CD137-elicited ubiquitination and signaling toward NF-κB activation in transfected cell lines as well as on primary T cells (Azpilikueta, A. et al., manuscript in preparation). Therefore, it can be envisioned that these proteases are constantly removing polyubiquitin chains. Degradation by DUBs is proposed to take place either when polyubiquitin chains are not protected by the trimerized CD137 complex, or when K63 polyubiquitin chains are not made faster than the protease enzymatic speed of the DUBs. In other words, constant deubiquitination may keep the pathway under control and terminate signaling in the absence of ligand binding.

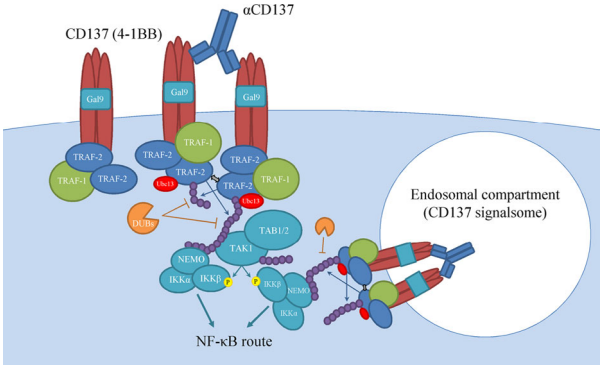

Figure 1. TRAF-2 transubiquitination model of CD137 signaling. Schematic representation of the mode of action of TRAF-2 attached to the cytoplasmic tail of CD137. According to this model, TRAF-2 has constitutive K63 polyubiquitin ligase E3 activity. When CD137 becomes multimerized by ligand or antibody, it brings TRAF-2 molecules into proximity so they can start transubiquitinating one another with the help of the Ubc13 E2 enzyme donating activated ubiquitins. These structures generate docking sites for the TAK-1-TAB1/2 complex and potentially other signaling proteins. This pathway is postulated to be quenched by rapid deubiquitination by K63 DUBs that are constantly removing polyubiquitin chains. This complex keeps signaling from endosomes once internalized by agonistic anti-CD137 mAbs [16, 123].

It has also been reported that CD137 becomes internalized upon ligation with anti-CD137 antibodies, and is trafficked to an endosomal compartment in a K63-polyubiquitin-dependent fashion [16]. Whether the natural ligand, CD137L, causes internalization as well remains to be seen; this process could serve to be another level of regulation of the pathway. CD137 internalization on dendritic cells as also been observed upon binding to CD137L fusion proteins used to target antigens for vaccination [34]. Intriguingly, recent research has shown that CD137L$^{-/-}$ T cells express higher levels of CD137. This was attributed to undetectable CD137L protein expression, albeit detectable at the mRNA level, leading to the interpretation that without CD137L, CD137 could not be internalized and therefore higher levels on the cell surface are observed [35].

TRAF-1 is chiefly induced via NF-κB signaling [36], and hence is predicted to more avidly assemble into the complexes once T cells are costimulated. Its molecular function is incompletely understood, but TRAF-1 may also operate by molecular proximity to other functional partners when recruited to multimolecular complexes. Although TRAF-2 has been coimmunoprecipitated with CD137 from cells at baseline, the CD137-TRAF-2 interaction has been shown to be enhanced upon ligand binding as a result of as yet unknown mechanisms [10–12]. It would be important to investigate how TRAF-2 and TRAF-1 functionally interact in these complexes.

Overall, CD137 signaling is fostered by multimerization, and we propose that cross-linking CD137 molecules and their adaptors within short molecular reach is the key factor. The enzymatic activity of TRAF-2, which self-ubiquitinates, or more likely K63-transubiquitinates close sister TRAF-2 molecules, is postulated to be the key triggering event. Regulation of this pathway by K63 DUBs modulates the intensity of the signal and prevents undesired ligand-independent activation. Figure 1 summarizes the proposed molecular events to turn on and regulate downstream CD137 signaling.

Agonist anti-CD137 monoclonal antibodies in the treatment of malignant diseases

The acceptance of CD137 as a costimulatory molecule has engendered fruitful research into using it in cancer immunotherapy. A collection of anti-mouse CD137 mAbs [37] were able to induce rejection of transplanted tumors in syngeneic mice, or at least to delay tumor progression [38]. Among the mAbs able to cause this effect were rat IgG antibodies that blocked or did not block ligand binding [36, 37], suggesting an agonist activity of the antibodies, which was also observed in in vitro T-cell cultures [37]. The therapeutic activity of anti-CD137 antibodies was critically dependent on CD8$^+$ T cells and also dependent on NK cells in certain models [38, 39]. Furthermore, the costimulatory molecule CD28 was not essential for the antitumor effect of anti-CD137 monoclonal antibody therapy, even though CD28 strongly contributes to eliciting CD137 surface expression on CD8$^+$ T cells following antigen stimulation [40].

The contribution of dendritic cells to the therapeutic effect was studied in CD11c-DTR (diphtheria toxin receptor) transgenic mice, which self-ablate CD11c$^+$ cells upon repeated diphtheria toxin treatment [41]. This study suggested a role for dendritic cell-mediated antigen presentation in anti-CD137 antibody therapy, leading to the interpretation that dendritic cell-mediated presentation of tumor antigens was critical to prime the baseline antitumor immune response that anti-CD137 mAbs potently costimulate. More recently, we have found a key role for Batf3 (basic leucine zipper transcription factor ATF-like 3)-dependent dendritic cells, which are the main mediators of tumor antigen cross-priming [42].

With regard to the role that CD4$^+$ T cells play in anti-CD137 therapy, there are paradoxical effects. On the one hand, depletion of CD4$^+$ T cells negatively affects therapy in some models [36], while in others, CD4$^+$ T-cell elimination potentiates the therapeutic effects [43]. The potentiating effects of the CD4$^+$ T-cell

depletion [44] are likely due to the destruction of the Treg-cell compartment in the tumor microenvironment.

To complicate the therapeutic picture even more, several groups explored the effects of the same anti-CD137 antibodies that had been previously shown to elicit curative anti-tumor immunity, in mouse models of autoimmunity. It was found that anti-CD137 mAbs improved murine autoimmune conditions mediated by autoreactive $CD4^+$ T cells, such as experimental autoimmune encephalomyelitis (EAE) [45], lupus-like syndromes [46], and collagen-induced arthritis [47]. However, anti-CD137 treatment worsened CD8-mediated autoimmune diabetes in NOD (nonobese diabetic) mice [48, 49] and exacerbated graft versus host disease [37, 50]. In fact, in healthy mice, anti-CD137 mAbs have been shown to cause polyclonal CD8-dominated infiltrates in the liver, which in turn raise transaminase serum levels [51].

The effects of anti-CD137 mAb on the functionality of regulatory T cells remain an active area of discovery. It is clear that CD137 is expressed on the plasma membrane of natural and induced Treg cells [52], including those infiltrating experimental tumors [53]. Anti-CD137 mAb can regulate function [54, 55] and differentiation [52] of Treg cells. However, the extent of the contribution of Treg-cell modulation by the anti-CD137 mAb on the overall antitumor therapeutic activity is still under investigation.

When agonist anti-CD40 mAbs were described to rely on the CD32 FcR (Fc receptor) to crosslink the antibody in order to mediate the antitumor effects of anti-CD40 therapy [56–59], we performed experiments in $FcRIIB^{-/-}$ mice, and showed that the activity of anti-CD137 therapy against solid tumors was preserved in the absence of such FcR crosslinking (Morales-Kastresana, A., unpublished observations). Similarly, subsequent experiments indicated that the anti-CD137 antibodies were able to induce internalization in vivo without CD32 involvement for its agonistic activity (Morales-Kastresana, A., unpublished observations). The involvement of other FcRs in the activity of anti-CD137 antibodies has not yet been explored.

In mouse models of cancer, successful combinations between anti-CD137 antibodies and peptide vaccines [60–62], dendritic cell vaccines [62–64], chemotherapy [65, 66], radiotherapy [67–69], virotherapy strategies [70–72], cytokine gene therapy [73, 74], adoptive T-cell therapy [75–77] and other strategies have been shown to lead synergistic, often curative, anti-tumor activity, as summarized in Figure 2. Soluble forms of trimerized CD137L have been also shown to be synergistic with TLR agonists [78]. Importantly, anti-CD137 mAbs have been shown to exert synergistic effects in conjunction with checkpoint inhibitors [79], such as anti-CTLA-4 [80] and anti-PD-1 (programmed cell death 1) mAbs [53, 81, 82], against difficult-to-treat mouse tumor models such as B16 melanomas or 4T1 breast carcinomas.

Combination therapies involving CD137 mAbs were shown to be effective in inducing complete tumor rejections on larger and less immunogenic tumors if given in higher order combinations (triplets or quadruplets) with other immunostimulatory monoclonal antibodies, such as those directed against CD40 [83], CTLA-4 [84], OX40 [64, 85], and PD-1/PD-L1 (programmed death-ligand 1) [86]. These combinations have shown beneficial effects even against primary carcinogen-induced sarcomas [83] and hepatocellular carcinomas arising in oncogene transgenic mice [87].

Recombinant forms of multimeric CD137L, either in the form of a soluble agent or as a gene construct transfected to tumor cells [88, 89], have also been used with less potency in gene therapy strategies. Of note, in a gene therapy approach involving mouse models of transplantable colon cancer, the CD137L construct showed strong synergy with IL-12 cotransfer [88, 89]. Gene transfer of membrane-bound, single chain anti-CD137 mAb was shown to be therapeutically more potent than the CD137 L constructs [90, 91], giving rise to strong systemic antitumor immunity in these mice that was mediated by $CD8^+$ T cells, with a prominent role for NK cells [90, 91].

Two fully human IgG4 anti-CD137 mAbs (Urelumab and PF-05082566) are currently being developed in phase I/II trials in the clinic, either as monotherapies or in combination with mAbs blocking PD-1 (NCT02253992, NCT02534506, NCT02179918, NCT01307267). Both antibodies, when tested as monotherapy agents, show evidence of partial antitumor activity at least against melanoma and lymphoma [92, 93]. In the case of Urelumab, but not PF-05082566, a dose-dependent liver inflammation was shown to occur in a fraction of patients. The mechanisms behind liver inflammation as an on-target side effect remain obscure, but probably resemble the observations made in mice [51, 94]. A possible explanation arises from the fact that any recombinant antibody administered to animals or human beings tends to accumulate passively in the liver, as evidenced by PET imaging [95, 96]. Hence, it is possible that the selectively high bioavailability in the liver may explain hepatitis because of the proinflammatory actions of the antibodies on yet to-be-determined liver-resident $CD137^+$ cells. Variable antigen-independent absorption into the liver, perhaps mediated by FcRs, may explain differences in liver toxicity observed among the anti-CD137 mAbs under clinical development and also differences in terms of susceptibility to these adverse reactions among individual patients.

As clinical trials on immunotherapy combinations progress [97], we have recently reported evidence for antitumor effects of Urelumab when used in conjunction with anti-PD-1 (Nivolumab) to treat immunodeficient Rag-/-IL-2Rγ-/-mice, which had been coengrafted with human tumors and human lymphocytes [98]. In a setting of these mice coengrafted with a gastric carcinoma and lymphocytes from the same patient, it was possible to study tumor infiltrates of human lymphocytes using multiplex immunofluorescence on tumor sections. Interestingly, $CD137^+$ human T lymphocytes were prominent in the infiltrates of mice treated with the immunostimulatory mAbs that were able to curtail tumor growth.

Another exciting discovery was the finding that anti-CD137 mAbs strongly enhance, in both mice and humans, the ADCC (antibody-dependent cell-mediated cytotoxicity) activity mediated by NK cells [99]. In this study, it was shown that when FcRγIII (CD16A) on NK cells recognize IgG antibodies coating target tumor cells, this induces CD137 expression on the NK cells, which greatly enhances ADCC if the NK cells are stimulated via CD137 [99]. Synergy of anti-CD137 mAbs with ADCC-eliciting anti-tumor mAbs in the clinic, such as Rituximab [100], Trastuzumab [101],

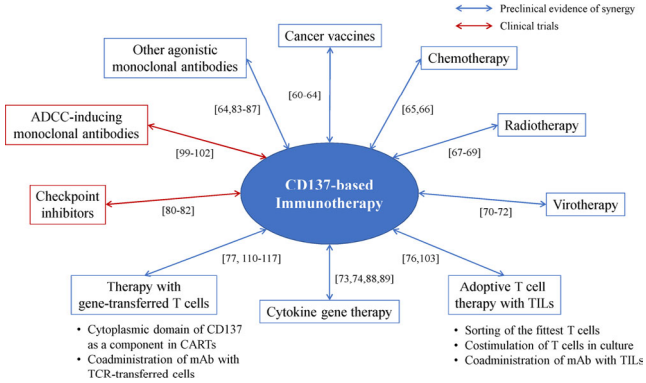

Figure 2. Landscape of synergistic interactions of immunotherapies based on the combination of CD137-based and other anticancer therapeutics. Arrows represent described combinations with main references to the literature provided.

or Cetuximab [102], is currently being addressed in clinical trials (NCT02420938, NCT02110082, NCT02252263, NCT01307267).

CD137 in synergy with adoptive T-cell therapy

Infusion of cultured T cells is becoming a prominent strategy in cancer therapy. For example, the infusion of expanded autologous tumor-infiltrating T lymphocytes has been shown to yield excellent results in a fraction of melanoma patients [103]. More recently, ex vivo gene engineering of the lymphocytes to be infused, via transfecting either TCRs recognizing tumor antigens, or single-chain, antibody-based chimeric antigen receptors (CARs), is taking center stage [103].

Recently, adoptive cell therapy and CD137-mediated costimulation have been shown to cooperate (Fig 2) in a four-pronged manner. These effects are as follows:

(i) CD137 and PD-1 are expressed precisely by those tumor-infiltrating lymphocytes (TILs) showing a stronger response to tumor antigens [104–106]. Hence, immunomagnetic and FACS techniques have been implemented to select CD137$^+$ TILs as the fittest population to generate therapeutic lymphocyte cultures for adoptive transfer.

(ii) CD137 agonist antibodies can be used to effectively deliver costimulation during ex vivo culture achieving a better yield in terms of the numbers of lymphocytes and their anti-tumor activity [76]. Costimulation of these cultures could also be achieved with the cognate CD137 ligand [107].

(iii) In mouse tumor models, combined treatment with adoptive T-cell therapy and anti-CD137 mAb synergize at various levels. CTLs under the influence of the infused anti-CD137 antibody perform better effector functions against the tumor [77], and show greater penetration of the malignant tissue, as observed by in vivo microscopy. This is due, in part, to stimulation of CD137 ectopically expressed on endothelial cells in tumor vessels [108, 109]. These CD137-stimulated tumor vessels go on to express adhesion molecules and chemokines in a proinflammatory response that facilitates T-cell homing to the tumor site [109].

(iv) The signaling domain of CD137 is a key constituent of the cytoplasmic tail of successful CARs [110, 111]. Its function is critical for T-cell persistence and expansion following infusion [110, 111]. In this respect, CD137 surpasses CD28 as a T-cell stimulatory molecule and provides a tonic signal that avoids exhaustion [112]. However, CD137 can be replaced by other members of the TNFR family, such as CD27 [113], to construct CARs. Nevertheless, CARs combining the cytoplasmic tail of CD137 are achieving astonishing clinical efficacy against B-cell leukemias, lymphomas, and myelomas [114–117].

Future directions and conclusions

The tumor microenvironment is rich in CD137, as it is expressed by effector and regulatory T lymphocytes at this location [53]. This rich CD137 expression is likely to be maintained by TCR-mediated antigen recognition, and potentiated by hypoxia, acting in a HIF1α (hypoxia-induced factor 1α) dependent fashion [53, 118]. Ascertaining the direct and indirect effects of CD137 ligation on the migration and function of TILs will be of much interest. More importantly, biotechnology strategies must be deployed to target or locally deliver CD137 agonists to tumors to maximize exposure and limit systemic toxicity (e.g., in the liver and bone

marrow). In fact, most CD137 expressed at a given time point is present only in the tumor microenvironment [53].

A better understanding of the CD137 signaling pathways may permit pharmacological or genetic manipulation, although these signaling mechanisms are shared by other members of the TNFR family and other surface receptor systems [119, 120], and as such could encompass off-target side effects.

Combination is the key word to make the most of CD137-based immunotherapy (Fig 2). As mentioned, clinical trials are in progress to exploit its synergy with PD-1/PD-L1 blockade and cytotoxic monoclonal antibodies such as Rituximab and Cetuximab. Vaccines, including neoantigen-based vaccines, and adoptive T-cell transfer, should follow in this strategy of immunotherapy combinations [97, 121, 122]. Overall, there can be no doubt that CD137-based immunotherapy clearly offers many interesting opportunities for clinical and translational development.

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Abbreviations: **ADCC**: antibody-dependent cell-mediated cytotoxicity · **CAR**: chimeric antigen receptor · **DUB**: deubiquitinases · **FcR**: Fc receptor · **PD-1**: programmed cell death 1 · **TRAF**: TNF receptor associated factor

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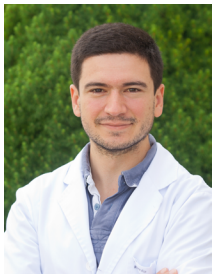

ACADEMIC FORMATION AND RESEARCH EXPERIENCE

As of July 2019, I am working as a postdoctoral fellow in the Icahn Institute of Medicine at Mount Sinai Hospital, in New York (USA), under the direction of Dr. Brian Brown and Dr. Miriam Merad.

I studied a Bachelor's degree in Biotechnology in University Francisco de Vitoria (Madrid, Spain) between 2007 and 2012. During this time, I carried out laboratory training in Carlos III Health Institute in Majadahonda, Madrid, under the direction of Dr. Sara Ballester, and in the Spanish National Research Council, in the laboratory of Dr. Clara Uriel.

I worked in Dr. Ignacio Melero's laboratory at the Center for Applied Medical Research (University of Navarra) between 2012 and 2018: as a Biomedical Research Master's degree student at first (2012-2013), and as a PhD student between 2013 and April 2018. Between 2016 and 2017 I mentored the Master's degree project of a student in this laboratory.

I obtained my PhD degree in Biomedical Research from the University of Navarra in April 26, 2018. My PhD project orbited around the role of dendritic cells in cancer immunotherapy with immunomodulatory antibodies. This PhD work produced two research papers (Cancer Discovery, 2016; and Cancer Research, 2018) and two review articles (European Journal of Immunology, 2015; and Annals of Oncology, 2017) as first author, among my participation in other publications by the team (please see *Scientific Publications ahead*).

I am able to design, carry out, analyze and interpret experiments, work as part of collaborative research projects, present and discuss data, stay up to date with scientific literature on a subject, and respond to unexpected results. I am also fluent in written, read and spoken English (C1 by Cambridge ESOL, 2005).

SCIENTIFIC PUBLICATIONS

Sanchez-Paulete, A. R., Cueto, F. J., Martinez-Lopez, M., Labiano, S., Morales-Kastresana, A., Rodriguez-Ruiz, M. E., Jure-Kunkel, M., Azpilikueta, A., Aznar, M. A., Quetglas, J.

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ATTENDANCE TO SCIENTIFIC MEETINGS

- "Frontiers in Immunodulation and Cancer Therapy", CNIO, Madrid, July 2018.
- AACR Annual Meeting 2016, New Orleans, April 2016, poster 4098 – attendance funded by the Thematic Network for Cooperative Cancer Research (RTICC).
- European Congress of Immunology, Vienna, September 2015, poster 2015-A-999-ECI.
- "Cancer, Inflammation and Immunity" by Cell Symposia, Sitges, June 2015, poster P1.079 – attendance funded by the Thematic Network for Cooperative Cancer Research (RTICC).
- International symposium "Cellular Immunotherapies for Cancer", Pamplona, December 2016.
- International symposium "Immunostimulatory monoclonal antibodies and immunomodulation: Harvesting the crop", Pamplona, October 2015.

- National Symposium "3rd Madrid Meeting on DCs and Macrophages", Madrid, April 2014.
- International symposium "Routing Cancer Immunology and Immunotherapy from the Lab to the Clinic", Pamplona, March 2014.
- Speaker in the seminar course "Principles and applications of flow cytometry", Pamplona, June 2018.

SCHOLARSHIPS AND FELLOWSHIPS

- Postdoc Fellowship: Fundación Alfonso Martín Escudero, between January 2019 and December 2020.
- PhD scholarship: Foundation for Applied Medical Research (University of Navarra), between September 2013-2017.
- Master's degree scholarship: UN-Grupo Santander between, September 2012-2013.
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