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TESIS DOCTORAL

"Adoptive T cell therapy for the treatment of ovarian cancer"

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CERTIFICAN:

Que el trabajo de investigación titulado: **"ADOPTIVE T CELL THERAPY FOR THE TREATMENT OF OVARIAN CANCER"** ha sido realizado íntegramente por D. Alfredo Perales Puchalt bajo mi dirección. Dicha memoria está concluida y reúne todos los requisitos necesarios para su presentación y defensa como **TESIS DOCTORAL** ante un tribunal.

Y para que así conste a los efectos oportunos, firmo la presente certificación en Valencia, Octubre de 2015.
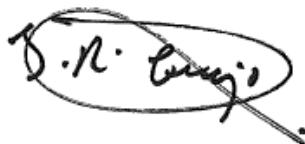
Fdo. Prof. Antonio Pellicer   Fdo. Prof. Jose R. Conejo-García

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I would first want to dedicate this work to God, who has been leading me around, from Valencia to Hershey, to Philadelphia; that has made me end up in the current lab where I'm writing this document and doing so many experiments; and has taken me through this time un unending work and failure and the few successes that I'm presenting here (nothing less than the invention of the FSHCER). Also I would like to dedicate it to Pedro Arrupe, for his company and his example, for giving me hope in all the difficult times.

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Abbreviations

Ab: Antibody

CAR: Chimeric Antigen Receptor

CER: Chimeric Endocrine Receptor

CGα: Chorionic Gonadotropin alpha

ELISA: Enzyme-Linked ImmunoSorbent Assay

ELISpot: Enzyme-Linked ImmunoSpot

FPKM: Fragments Per Kilobase of exon per Million fragments mapped

FSH: Follicle Stimulating Hormone

FSHβ: Follicle Stimulating Hormone subunit β

FSHR: Follicle Stimulating Hormone Receptor

GFP: Green Fluorescent Protein

Msln: Mesothelin

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

RPMI: Roswell Park Memorial Institute (culture media)

scFv: single chain variable fragment

TCGA: The Cancer Genome Atlas

Abstract

Despite de advances in surgery and chemotherapy, the outcome of ovarian cancer patients has improved very little over the last 40 years. Given that ovarian cancer is an immunogenic tumor, immunotherapies offer a great promise as treatment of this dismal disease. We show that follicle-stimulating hormone (FSH) receptor can be a very specific target that is expressed in >56% of human ovarian carcinomas and is a negative prognostic factor. Accordingly, we designed chimeric receptors expressing full-length FSH to redirect T-cell cytotoxic activity against these more aggressive tumors. *In vivo*, fully murine FSH-targeted T-cells and low affinity anti-mesothelin scFv-targeted Chimeric Antigen Receptor (CAR) T-cells were similarly safe and effective at increasing the survival of immunocompetent mice with established and aggressive ovarian carcinomas. Notably, both chimeric receptors enhanced the ability of endogenous, pre-existing tumor-reactive T-cells to abrogate malignant progression upon adoptive transfer into naïve recipients subsequently challenged with the same tumor. Interestingly, chimeric receptor-expressing CD4 T-cells were superior contributors to these consistent therapeutic benefits, compared to their CD8 counterparts. We also found that although chimeric receptor-targeted T-cells were able to persist as memory lymphocytes without noticeable PD-1-dependent exhaustion during end-stage disease, the shedding of FSH receptor from tumor cells diverted the effector activity of chimeric receptor-transduced T-cells away from targeted tumor cells. Accordingly, although tumor cells remained sensitive to chimeric receptor-driven activities, re-directed lymphocytes ultimately disappeared from tumor beds. Thus, although tumor microenvironmental factors could limit their effectiveness against established and aggressive epithelial tumors, lymphocytes redirected against tumor cells with full-length hormones or low-affinity scFvs induce

significant therapeutic benefits by boosting endogenous anti-tumor immunity and show negligible

toxicity even in the presence of cognate targets in tumor-free tissues.

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1 Introduction

1.1 Adoptive T cell transfer

Adoptive T cell transfer is a type of adoptive cellular immunotherapy in which T cells are infused into patients for their treatment (Tey et al., 2006). The ability of T cells to treat tumors was unveiled in the early 1990s. Two almost simultaneous discoveries proved the concept that the transfer of antigen specific T cells could eradicate tumors. On the one hand, clinical data showed that the transfer of mononuclear cells from haploidentical twins to patients bearing chronic lymphocytic leukemia (CLL) was able to produce complete remission (Kolb et al., 1990). On the other hand, the transfer of antigen specific T-cells (against Eppstein Bar Virus (EBV) or Citomegalovirus (CMV)) that had been isolated and expanded *ex vivo* we able to abrogate EBV or CMV associated tumors (Rooney et al., 1995, Heslop et al., 1996, Riddell et al., 1992).

The response of renal carcinoma and posteriorly melanoma with IL-2, a T-cell growth factor, fostered the idea that endogenous T-cells were important for tumor rejection (Rosenberg et al., 1984). The ability of interleukin-2 (IL-2) to allow growth and proliferation of tumor infiltrating lymphocytes (TILs) ex vivo from tumor patients led to the generation of T-cells specific for each patient's tumor that could be infused passively into the patient for its treatment (Rosenberg et al., 1986). This was the beginning of adoptive cell transfer (ACT).

An advantage of ACT is that the tumor microenvironment can be made more suitable for the treatment prior to the T-cell infusion. Tumor microenvironment accommodation has been done through nonmyeloablative chemotherapy, consisting in a combination of cyclophosphamide and fludarabine, which has a negligible effect on the tumor but facilitates the effect of the adoptively

transferred T-cells; and the coinfusion of IL-2 with the TILs (Dudley et al., 2005). The effect of ACT was further potentiated with a more aggressive lymphodepletion adding total body irradiation (TBI) of 1200 cGy to NMA (Rosenberg et al., 2011).

Another advantage of ACT is that large numbers of tumor specific T-cells could be generated from each patient. However, despite being able to obtain T-cells from multiple tumor types, only melanoma patients were able to respond to this therapy, because of the great immunogenicity of this this tumor type (Rosenberg et al., 1988). The poor responses in tumor other than melanoma led to the development of genetically modified T-cells engineered to express exogenous αβ T-cell receptors (TCRs) and chimeric antigen receptors (CAR) that allow the redirection of T-cells against specific tumor antigens.

1.2   Chimeric antigen receptors (CARs)

Redirecting T-cells against tumor antigens using engineered TCRs has the disadvantage that it is constrained by human leukocyte antigen (HLA) restriction. Antigen peptides need to bind major histocompatibility complex (MCH) molecules in order to go to the surface of the cells and be detected by the immune system. Humans express 6 MHC class I alleles (HLA-A, -B and –C from each progenitor) and 6 to 8 MHC class II alleles (one HLA-DP, -DQ and one or two –DR from each progenitor), each of which has multilple (generally more than 100) alleles. Therefore, the engineering of specific TCRs for each allele would suppose a practical problem. To solve this problem, T-cells have been engineered to express CARs. CARs consist on an antibody fragment (scFv) that binds a particular antigen on the cell surface, fused in frame with T-cell stimulatory and co-stimulatory molecules.

One of the first studies in which CAR T-cells were applied for solid tumors was renal cell carcinoma. The targeted antigen was carbonic anhydrase IX. However, the treated patients showed on-target off-tumor effects with liver toxicity that made the treatment not feasible (Lamers et al., 2006). Similarly, the scFv of Trastuzumab, an antibody raised against ERBB2 was used to generate a CAR for treating the wide array of tumors that overexpress this protein. Again, low expression of Her-2 in the lungs generated a fatal pulmonary failure that precluded the use of this target (Morgan et al., 2010). The difficulty of finding tumor antigens in the cell surface expressed by tumor cells and not healthy cells which would bear intolerable side effects has hampered the development of effective CAR therapies for solid tumors.

Mesothelin has also been targeted due to its overexpression in tumors such as mesothelioma, pancreatic carcinoma, ovarian cancer or triple negative breast cancer. To avoid the potential adverse effects on the healthy tissues that express mesothelin (mainly peritoneum, pleura and pericardium), a CAR expressed by RNA electroporation that only last in the T-cells for about a week has been tested (Beatty et al., 2014). However, this required multiple injections of the CAR T-cells, which elicited a lethal anaphylactic shock in a patient reacting against the murine scFv of the CAR.

The treatment of hematologic malignancies with CARs has shown impressive responses in multiple trials. The target that has been more widely used is CD19, which is expressed by virtually all B-cell malignancies (Uckun et al., 1988). Complete responses in patients with acute lymphocytic leukemia (ALL) and chronic lymphocytic leukemia (CLL) of 90% and 70% have been attained in clinical trials in the University of Pennsylvania and the Pediatric Oncology branch of the National Cancer Institute, respectively (Maude et al., 2014, Lee et al., 2015).

1.3   CARs and ovarian cancer

Epithelial ovarian cancer is one of the most deadly tumors, killing more than 14,000 women each year in the US (Jemal et al., 2009). Despite advances in surgical approach and chemotherapy, 5-year survival rates have barely changed in the last 40 years. Ovarian cancer is, however, an immunogenic tumor (Curiel et al., 2004, Zhang et al., 2003, Cubillos-Ruiz et al., 2009, Cubillos-Ruiz et al., 2010, Huarte et al., 2008, Nesbeth et al., 2009, Scarlett et al., 2009, Scarlett et al., 2012, Cubillos-Ruiz et al., 2012), and immunotherapies therefore offer great promise to reverse this dismal prognosis (Brayer and Pinilla-Ibarz, 2013, Nelson and Paulos, 2015).

In recent years, transferring autologous T-cells engineered to express chimeric antigen receptors (CARs) has shown impressive cures for patients with chemo-resistant hematologic malignancies (Porter et al., 2011, Maus et al., 2014, Kalos et al., 2011). However, several hurdles have so far prevented the success of this technology against solid tumors, including ovarian cancer. The most challenging is the paucity of specific antigens expressed on the surface of tumor cells that are not shared with healthy tissues, to avoid intolerable side effects.

The follicle-stimulating hormone receptor (FSHR) is selectively expressed in women in the ovarian granulosa cells (Simoni et al., 1997) and at low levels in the ovarian endothelium (Vannier et al., 1996). Most importantly, this surface antigen is expressed in 50-70% of ovarian carcinomas (Zhang et al., 2009, Al-Timimi et al., 1986, Zhang et al., 2013, Minegishi et al., 2000, Nakano et al., 1989, Parrott et al., 2001, Wang et al., 2003, Zheng et al., 2000). Given that oophorectomy is a standard procedure in the treatment of ovarian cancer, targeting the FSHR should not cause damage to healthy tissues. Therefore, FSHR could be an ideal therapeutic target to direct T-cells against ovarian cancer using chimeric receptors.

Preclinical testing of CARs has been done in immunodeficient xenograft-bearing mice, which do not share the targets of chimeric receptors and therefore cannot predict potential adverse effects that can happen in a clinical setting. To understand the interaction between CAR T-cells, the host immune system and the endogenous target potentially expressed in healthy tissues, we generated different fully murine chimeric receptors against mouse targets in immunocompetent mice. While CAR T-cells are typically re-directed against tumor antigens through the inclusion of an antibody (Ab) fragment (scFv), the FSHR receptor offers the advantage of having a highly specific, natural ligand (FSH). Receptor-ligand interactions have been tuned through evolution to maximize effectiveness, while Abs are not absolutely specific and Ab fragments often do not preserve the binding properties of the original Ig. In addition, FSH is an endogenous molecule, thus avoiding the potential immunogenicity of exogenous Ab fragments developed in different species. To exploit existing receptor-ligand interactions, we developed new Chimeric Endocrine Receptor (CER) constructs that include the two entire subunits of FSH, in frame with a transmembrane domain and the intracellular signaling domains successfully used in the treatment of leukemia (Porter et al., 2011). To detect potential side effects, we generated a fully murine CER that should target any unknown expression of FSHR in healthy tissues in tumor-bearing mice. In addition, we generated a fully murine CAR that targets the ovarian cancer antigen mesothelin through a classical scFv, and compare both effectiveness and toxicity.

2   Objectives

The PRIMARY OBJECTIVE of this project is to generate a chimeric antigen receptor that is useful for the treatment of ovarian cancer. In order to do that we first studied the potential surface proteins expressed in ovarian cancer cells. Mesothelin is a well-recognized tumor associated antigen overexpressed in ovarian cancer, however, its expression in healthy tissues makes its use in immunotherapy controversial. We found that follicle stimulating hormone receptor (FSHR) was a very specific antigen, not expressed outside of the ovary, which could be safely used to target ovarian cancer through CARs. After generating the CARs to redirect T-cells against FSHR we validated the CAR effectiveness in vitro through the ability of CAR T-cells to get activated and kill mouse and human tumor cells that expressed the FSHR. Following, we studied the ability of this CAR T-cells to treat ovarian cancer in an immunocompetent mouse model.

The SECONDARY OBJECTIVES were the following:

- Generate a CAR that should not elicit an anaphylactic response. The use of mouse-derived scFv for the generation of the CARs caused death in a patient after multiple infusions due to an anaphylactic shock. We had the objective to avoid this by being able to design a CAR that is fully human, with no murine counterparts that could elicit such a response.

- Study if the adoptively transferred T cells are able to boost the endogenous immune response. We hypothesize that part of the effect through which CAR T-cells elicit their anti-tumor effect is through the boost of the preexisting endogenous immune response, through the antigen spreading that would happen after the target cell killing by the CARs. We studied this mechanism by using congenic leukocyte markers (CD45.1 and CD45.2) that allowed us to differentiate between adoptively transferred T cells and the endogenous

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T-cells. After treatment, we isolated the endogenous T-cells and tested between the different treatment groups for their in vitro and in vivo anti-tumor responses.

- Study potential failure mechanisms of this therapy. The tumor microenvironment is tipically immunosuppressive and may account for the failure of immunotherapies in many occasions. In case we are not able to attain tumor rejections we plan to dissect the effects of tumor microenvironmental factors on the adoptive T cell transfer therapy. Examples of escape to the treatment are immunoediting, overexpression of PD-1 on the surface of the T-cells and microenvironment derived suppression through cellular or soluble factors.

- Study the distribution of the adoptively transferred T cells. In order to more deeply understand the therapy with CAR T-cells we will sacrifice tumor bearing mice treated with congenic CAR T-cells and study the distribution and phenotype of the adoptively transferred T-cells at different time points.

- Study the potential adverse effects of this therapy on the host. Past research with CARs has been done with immunodeficient mice bearing human tumor cell lines and targeting human tumor antigens that were not present in the mouse, so some of the adverse effects were not predictable. The advantage of using a murine CAR is that we can study, not only the interactions of the CAR therapy with the endogenous immune system, but also that the targeted molecules will also be present in the host and we will be able to see the adverse effects as we would in a clinical setting.

3 Materials and methods

Animals and cell lines

Wild-type C57BL/6 and Ly5.1 mice were purchased from National Cancer Institute or from Charles River. Animal experiments were approved by the Institutional Animal Care and Use Committee at The Wistar Institute.

Parental ID8 cells were provided by Katherine Roby (Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, KS) (Roby et al., 2000) and retrovirally transduced to express *Defb29* and *Vegf-a* (Conejo-Garcia et al., 2004).We generated ID8-*Defb29/Vegf-a* flank or intraperitoneal tumors as described previously (Cubillos-Ruiz et al., 2009). Tumors were measured with calipers and tumor volumes calculated as $1/2 \times$ (L x W x W), where L is length (longest dimension) and W is width (shortest dimension). To generate the ID8-*Defb29/Vegf-a*-mesothelin cell line, ID8-*Defb29/Vegf-a* cells were transduced 2 times with MIGR1-mesothelin retroviruses and sorted for GFP expression. To generate the ID8-*Defb29/Vegf-a*-FSHR cell line, ID8-*Defb29/Vegf-a* cells were transduced with pBMN-I-GFP-FSHR retroviruses and sorted for GFP expression.

Double transgenic LSL-K-ras$^{G12D/+}$p53$^{loxp/loxp}$ (p53/K-ras) mice were generated by obtaining LSL-K-ras^{tm4Tyj} (Jackson et al., 2001) and Trp53^{tm1Brn} (Jonkers et al., 2001) from the NCI mouse models of human cancer consortium and bred to a full C57BL/6 background as previously reported (Scarlett et al., 2012) . p53/K-ras mice were irradiated two consecutive days with 650 rads followed by reconstitution with bone marrow from XBP1$^{f/f}$ or XBP1$^{f/f}$CD11c-Cre mice. 8 weeks post bone-marrow reconstitution autochthonous ovarian tumors were initiated by delivery of adenovirus-

expressing Cre recombinase (ADV-Cre) into the ovarian bursa as previously reported (Flesken-Nikitin et al., 2003, Dinulescu et al., 2005).

Design of chimeric antigen receptors

We designed the chimeric antigen receptor constructs using the signal peptide of mouse CD8α, followed by either the scFv of the K1 anti-mesothelin antibody or a fusion of the full length FSHβ and CGα, peptides linked by a glycine/serine spacer, followed by mouse CD8α hinge and transmembrane domain, and an intracellular fragment of mouse 4-1BB and CD3. We ordered the construct from Genescript flanked by EcoRI and NotI and cloned into pBMN-I-GFP retroviral vector. The corresponding human sequences were ordered to generate the human FSH-targeted chimeric receptor.

Human specimens

Human ovarian carcinoma tissues were procured under a protocol approved by the Committee for the Protection of Human Subjects at Dartmouth-Hitchcock Medical Center (#17702); and under a protocol approved by the Institutional Review Board at Christiana Care Health System (#32214) and the Institutional Review Board of The Wistar Institute (#21212263).

Analysis of TCGA data

Aligned Sequence files related to solid ovarian cancer samples were downloaded from TCGA data portal (2015). Downloaded files include whole exon sequencing and outcome data (404 patients).

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Scores (number of tags in each transcript) were obtained from each sample and normalized with respect to total tags in the sample as well as total tags in the chromosome. Tumors with FSHR expression values higher than the median FPKM (Fragments Per Kilobase of transcript per Million mapped reads) were identified as high expressors.

Retrovirus production

We generated retrovirus by transfecting Eco-Phoenix cells with pBMN-I-GFP or pBMN-I-GFP-CAR. Briefly, we plated $4.5e^6$ Phoenix Ecotropic packaging cells in a 10cm culture dish the evening before transfection. The next morning, when cells are at a 80-90% confluence we transfected them with a mix of DNA (20ug of FSHCER, K1CAR or pBMN), $CaCl_2$ 2M (in H_2O) and 2X HBS buffer (8.0g NaCl, 6.5g HEPES and 10ml of Na_2HPO_4 solution (5.5g in 500ml)) and Chloroquine 100 uM (dissolve 520mg in 10ml H_2O).

Mix DNA (20ug in xuL) with H2O (500-61-x uL) in a 15mL tube. Add 61uL of CaCl2 to mix drop by drop. Add 0.5mL of HBS2x to mix drop by drop. Bubble for 15 seconds. Add 2.5uL of Chloroquine 25mM to warm DMEM 10% FBS. Remove media from dish very gently (pipeting on the side-bottom). Add prewarmed DMEM 10% FBS (now with Chloroquine).Add the transfection mix (1mL total) drop by drop. Put dish in incubator. Refresh warm DMEM 10% FBS 9-11 hours after transfection. On the next morning we changed the media for RPMI 10% FBS containing b-mercapto ethanol (3.4ul in 1l) and collected the viral supernatant 24 and 48 hours later. Viral supernatants were stored at -80ºC until use.

Transduction of T-cells

We isolated naive T-cells from mouse spleens using a negative selection protocol. Briefly, non-T-cells were removed following red blood cell lysis using magnetic beads (BioMagR Goat anti-Rat IgG) to isolate splenocytes bound by the following antibodies: 2.4G2, M170.13, RA3 and M5114. Purified T-cells were resuspended in 10cm culture dishes in RPMI 1640 supplemented with 10% FBS, Penicillin, Streptomicin, L-Glutamine, 20 U/mL of IL-2 (Peprotech), 1µg/mL of IL-7 (Peprotech) and 2ug/mL of Concanavalin-A. After 36 hours we resuspended the T-cells at 10^6cells/mL in viral supernatant, with IL-2, IL-7 and 8µg/mL of Polybrene and spinfected for 90min at 32ºC and 1750rpm. Eight hours later, we resuspended the cells in RPMI containing 10%FBS IL-2 and IL-7 to remove the Polybrene. After 48 hours post-spinfection we sorted T-cells for GFP to be used for adoptive cell transfer.

Alternatively, after red blood cell lysis we resuspended splenocytes at $2x10^6$ cells/mL in a 24-well plate with 50U/mL of IL-2 (Peprotech), 1µg/mL of IL-7 (Peprotech) and 50µL/mL of anti-mouse CD3/CD28 beads (invitrogen). We performed 2 spinfections at 18 and 36 hours on Retronectin coated plates (Takara) and magnetically removed the CD3/CD28 beads at day 4 after isolation. We counted the T cell number every 2 days and added RPMI IL-2 IL-7 to keep a concentration of 10^6cells/mL. At day 7 T-cells were sorted for GFP and used for adoptive cell transfer.

Preparation of siRNA-PEI nanoparticles and therapeutic *in vivo* silencing

Endotoxin-free rhodamine-labeled and unconjugated polyethylenimine (PEI) for *in vivo* experiments "in vivo-jetPEI" was purchased from PolyPlus Transfection. To generate siRNA-PEI nanocomplexes, 50 µg of siRNA were complexed with "in vivo-jetPEI" at N/P ratio of 6, following the recommendations of the manufacturer and previously optimized conditions (Cubillos-Ruiz et

al., 2009). All siRNA oligonucleotides were from IDT and included 2′-OMe modified nucleotides and specific phosphorothioate linkages, as previously reported (Piret et al., 2002). Sequences for the sense and antisense strands are as follows: siLuc sense: 5′-CuUACgcUGAguaCUUcGAdTsdT-3′, siLuc antisense: 5′-UCgAAGUACUCAGCgUAAGdTdsT-3′, siXBP1 sense: 5′-cAcccuGAAuucAuuGucudTsdT-3′, siXBP1 antisense: 5′-AGAcAAUGAAUUcAGGGUGdTsdT-3′. siIRE1a sense: 5′-AuGccGAAGuucAGAuGGAdTsdT-3′, siIRE1a antisense: 5'-UCcAUCUGAACUUCGGcAUdTsdT-3'. 2′-OMe modified nucleotides are in lower case. Phosphorothioate linkages are represented by "s" and "d" indicates DNA bases.

For repeated siRNA treatments, wild-type C57BL/6 female mice were intraperitoneally injected with $1x10^6$ aggressive ID8-*Defb29/Vegf-A* ovarian carcinoma cells, and mice received nanocomplexes (50 μg of siRNA complexed with "in vivo-jetPEI" at N/P 6, per mouse) at days 8, 13, 18, 23 and 28 after tumor implantation.

Cytotoxicity assay

We plated 10000 ID8-*Defb29/Vegf-a-Fshr* in flat bottom 96 well plate. Before plating the T-cells we washed away the tumor conditioned media and added fresh media with no beta mercapto-ethanol and the appropriate number of T-cells per well (in 200uL). T-cells were FSHCER or mock transduced. Following 18 hours we collected T-cells, trypsinized tumor cells and stained them for Annexin V and Zombie Yellow or 7-AAD (Biolegend).

Interferon-γ determination

We plated 10000 ID8-*Defb29/Vegf-a-Fshr* in flat bottom 96 well plate and cocultured overnight with FSHCER or mock transduced T-cells. We measured interferon-γ using ELISA (Biolegend) and ELISPOT (eBioscience) following manufacturer instructions.

Flow cytometry

We used a BD LSRII flow cytometer or BD FACSAria cell sorter (BD Biosciences).

Anti-mouse antibodies used were directly fluorochrome conjugated. We used: anti-CD3e (17A2), CD4 (RM4-5), CD8b (YTS156.7.7), CD45 (30-F11), CD45.1 (A20), CD45.2 (104), CD44 (IM7), CD69 (H1.2F3), CD62L (MEL-14), PD-1 (29F.1A12) (all from Biolegend or Tonbo Biosciences), mesothelin (K1, from Santa Cruz Biotech). Live/dead exclusion was done with Zombie Yellow or 7-AAD (Biolegend).

Immunoblotting

Frozen human ovarian tumor specimens were mechanically disassociated. Protein extraction, denaturation and western blotting were performed as previously described (Rutkowski et al., 2015). Membranes were blotted with anti-FSHR (H-190, Santa Cruz Biotech) and anti-β-actin (a5441, Sigma-Aldrich).

Immunohistochemistry

Heart and lungs from mice treated with K1CAR or mock transduced T-cells were collected and embedded in OCT (Tissue-Tek) and frozen after which 8μm sections were made from frozen tissue

blocks. Slides were then fixed with acetone and washed with PBS. Sections were then blocked using α-CD32 followed by staining with biotinylated α-CD45 (30-F11) antibody and completion of immunohistochemical procedure according to manufacturer instructions (Vector Labs). Slides were viewed using Nikon ECLIPSE 80i microscope and the NIS-Element Imaging software.

Quantitative real-time PCR

Tissue RNA was isolated from snap-frozen samples by mechanical disruption and extracted using RNeasy kits (QIAGEN) according to manufacturer's instruction. RNA was reverse transcribed using High Capacity Reverse Transcription kits (Applied-Biosystems). Relative expression of murine CD3e (forward 5'-ACGTACTTGTACCTGAAAGCTC-3' and reverse 5'-CCTTCCTATTCTTGCTCCAGT-3'), CD45 (forward 5'-TGAAGAAGAGAGATCCACCCA-3' and reverse 5'-TTTCCAATGTGCTGTGTCCT-3') and GAPDH (forward 5'-CCTGCACCACCAACTGCTTA-3' and reverse 5'-AGTGATGGCATGGACTGTGCT-3') were quantified using SYBR Green (Applied Biosystems) on an ABI 7500 Fast Real-time PCR System (Applied Biosystems).

Statistical analysis

Unless noted otherwise, all experiments were repeated at least two times and results were similar between repeats. Animal experiments used between 3 and 6 mice per group. Differences between the means of experimental groups were calculated using a two-tailed unpaired Student's *t* test. Error bars represent standard error of the mean from independent samples assayed within the represented experiments. Survival rates were compared using the Log-Rank test. Tumor

growth experiments were compared using slope change through a linear regression analysis. All statistical analyses were done using Graph Pad Prism 5.0. A P value < 0.05 was considered to be statistically significant.

4   Results

4.1   FSH-targeted chimeric endocrine receptors effectively target human and murine ovarian carcinomas

To understand the relevance of FSHR as an immunotherapeutic target in human ovarian cancer, we first analyzed TCGA datasets from 404 high-grade serous ovarian carcinomas. We found that 56.5% of ovarian cancers express FSHR. Most importantly, FSHR overexpression is a negative prognostic factor in ovarian cancer (**Figure 4.1.1**), suggesting that FSHR is a good therapeutic target for aggressive tumors.
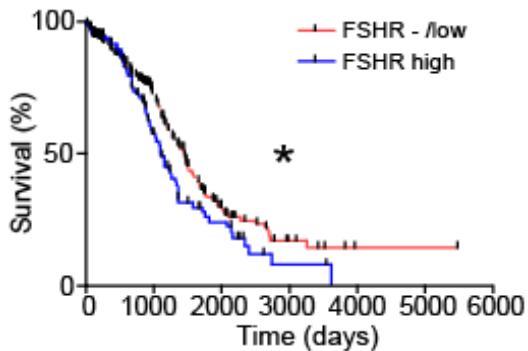
Figure 4.1.1 Overexpression of FSHR is a poor prognostic factor in ovarian cancer.

> Survival plot of 404 cases of ovarian cancer from TCGA dataset. We defined FSHR high as patients with tumors that had FSHR FKPM above the median, FSHR low as those with FKPM below the median and FSHR- as those with no expression measured by FKPM.

Widespread expression of FSHR was confirmed in western-blot analysis of randomly selected stage III/IV ovarian carcinomas from our tumor bank (Rutkowski et al., 2015, Stephen et al., 2014),

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which identified positive expression in ~70% of tumors (**Figure 4.1.2**), 25% of which exhibit

similar or higher levels than OVCAR-3 cells, a well-defined positive control (Nakano et al., 1989,

Choi et al., 2004).
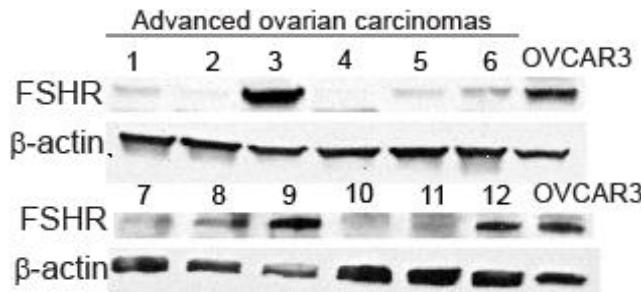
Figure 4.1.2 Approximately 66% of ovarian carcinomas in our tumor bank express FSHR

Western blot showing expression of FSHR in randomly selected ovarian tumors from
our tumor bank and OVCAR-3 ovarian cancer cell line.

To target the FSHR by redirecting T-cells, we generated a human chimeric receptor using the full-

length of the two subunits of the human FSH hormone, linked by a glycine/serine spacer, and in

frame with a transmembrane domain, the intracellular domain of co-stimulatory 4-1BB, and

CD3ζ (FSHCER; **Figure 4.1.3**).
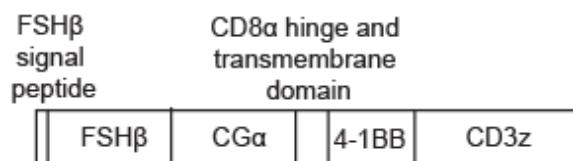
Figure 4.1.3 Schematic of FSHCER

FSHCER is composed by the full length FSHβ and CGα chains of FSH linked by a glycine/serine spacer between them and to a CD8α hinge and transmembrane domain, which are followed by the intracellular domains of 4-1BB and CD3ζ respectively

Unlike chimeric receptors employing small hormone fragments (Urbanska et al., 2015), FSHCER T-cells from HLA-A2[+] donors (to minimize allogenicity) killed target OVCAR-3 cells in dose-dependent, selective and highly effective manner, compared to mock transduced T-cells (**Figure 4.1.4**).
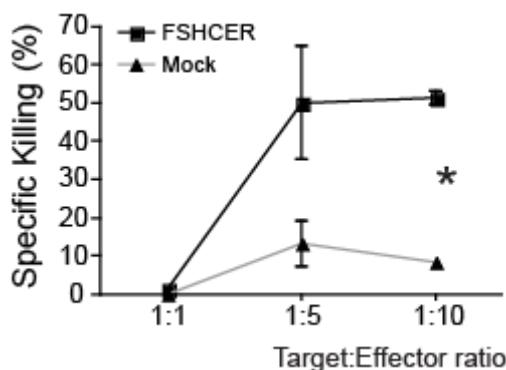
Figure 4.1.4 Human FSHCER cytotoxicity against OVCAR-3

Different numbers of human FSHCER or mock transduced T-cells were coincubated for 18 hours with 10000 OVCAR-3 cells and cytotoxicity measured by 7-AAD/Annexin V staining.

To define the potential of FSHCER T-cells to target established ovarian tumors in immunocompetent hosts in the unrestricted presence of targeted FSHR in healthy tissues, as in the

clinical setting, we generated a mouse FSHCER with the murine counterparts of all domains expressed in human T-cells. As expected, ID8-*Defb29/Vegf-a* tumor cells, an aggressive ovarian cancer model engineered to accelerate peritoneal carcinomatosis and ascites *in vivo* (Cubillos-Ruiz et al., 2012, Cubillos-Ruiz et al., 2009, Scarlett et al., 2009, Stephen et al., 2014), elicited robust secretion of IFN-γ by FSHCER mouse T-cells upon ectopic expression of FSHR, but not in the absence of FSHR expression (**Figure 4.1.5**).
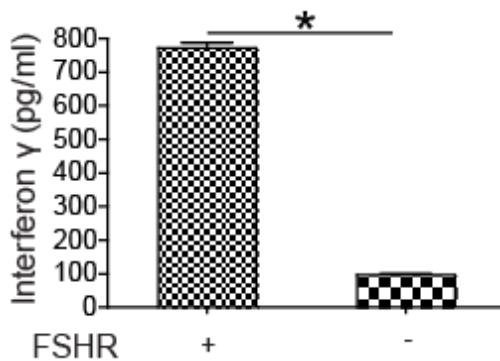
Figure 4.1.5 Mouse FSHCER T-cell interferon γ expression upon coculture of ID8-*Defb29/Vegf-a* with or without FSHR overexpression

Mouse T-cells transduced with the FSHCER were cocultured with ID8-*Defb29/Vegf-a* with or without FSHR ectopic expression. After 18 hours supernatants were collected and assayed for interferon γ by ELISA. * $p < 0.05$

FSHCER-specific T cell responses were consistent when CD4 and CD8 T-cells were independently stimulated (**Figure 4.1.6**).
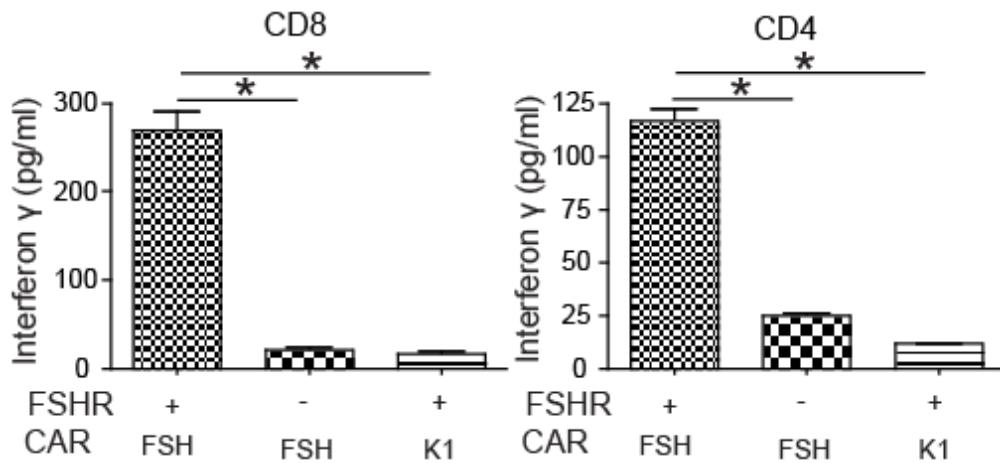
Figure 4.1.6 Mouse FSHCER CD4 and CD8 T-cell interferon γ expression

Mouse T-cells transduced with the FSHCER were first sorted for CD4 or CD8 expression. Each subset was independently cocultured with ID8-*Defb29/Vegf-a* with or without FSHR ectopic expression. As an additional control, we cocultured ID8-*Defb29/Vegf-a-Fshr* with T-cells expressing an irrelevant CAR (K1CAR). After 18 hours supernatants were collected and assayed for interferon γ by ELISA. * p<0.05

As with their human counterparts, mouse FSHCAR T-cells specifically killed FSHR-expressing tumor cells in a dose dependent manner (**Figure 4.1.7 and Figure 4.1.8**).
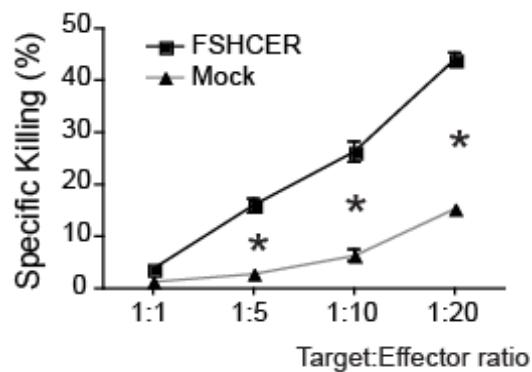
Figure 4.1.7 Mouse FSHCER cytotoxicity against ID8-*Defb29/Vegf-a-Fshr*

Different numbers of mouse FSHCER or mock transduced T-cells were coincubated for 18 hours with 10000 ID8-*Defb29/Vegf-a-Fshr* cells and cytotoxicity measured by 7-AAD/Annexin V staining.
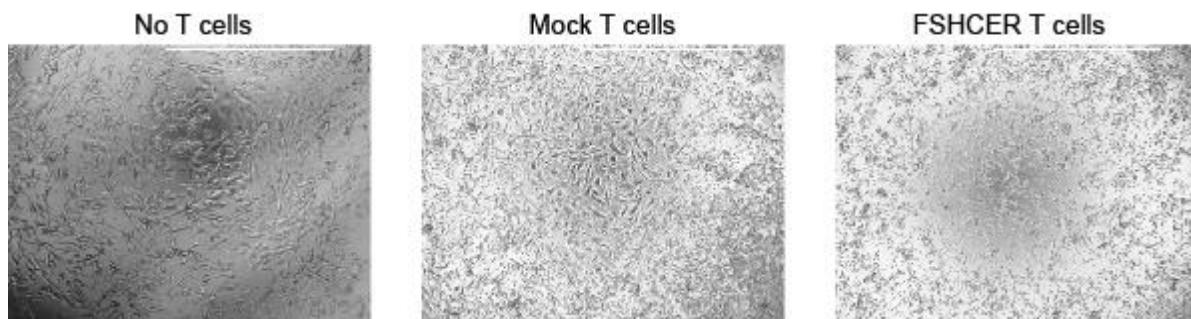
Figure 4.1.8 Mouse FSHCER cytotoxicity against ID8-*Defb29/Vegf-a-Fshr* - microscope

One hundred thousand mouse FSHCER or mock transduced T-cells were coincubated for 18 hours with 10000 ID8-*Defb29/Vegf-a-Fshr* cells and microscopic pictures of the corresponding wells were taken under a light microscope.

Taken together, these results indicate that chimeric receptors using entire subunits of the FSH hormone can effectively redirect the cytotoxic activity of T-cells against FSHR[+] ovarian carcinomas, which represent the majority of human tumors.

4.2 Chimeric receptor-expressing T-cells boost pre-existing (endogenous) T-cell-dependent anti-tumor immunity

Unlike with immunodeficient mice, the use of the immunocompetent mouse model allowed us to dissect the interaction of CAR/CER T-cells with an intact immune system. We hypothesized that the specific cytotoxic activity of FSHCER T-cells should result in antigen spreading and decreased immunosuppressive burden, which could have a significant effect on pre-existing anti-tumor immunity. To test this proposition, we treated different cohorts of CD45.2$^+$ mice growing established orthotopic FSHR$^+$ ID8-*Defb29/Vegf-a* tumors with either congenic CD45.1$^+$ FSHCER T-cells or vehicle (PBS). T-cells of host origin (CD45.2$^+$, to differentiate them from FSHCER T-cells) were sorted from the peritoneal cavity 1-2 weeks after treatment and subjected to IFN-γ ELISPOT analysis. As shown in **Figure 4.2.1**, the frequency of endogenous IFN-γ-producing T-cells responding to dendritic cells pulsed with double (UV + γ)-irradiated ID8-*Defb29/Vegf-a* tumor cells was dramatically higher in mice treated with FSHCER T-cells.
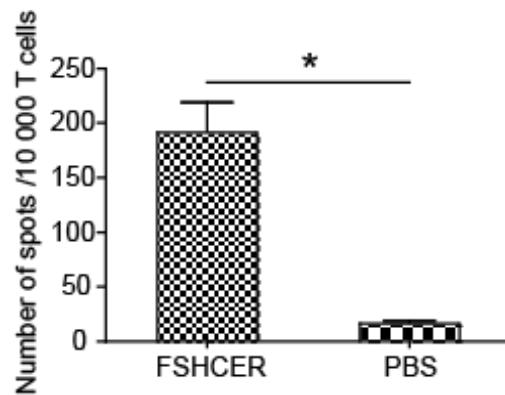
Figure 4.2.1 Endogenous T-cell ELISPOT against tumor antigen (FSHCER treated)

Representative interferon gamma ELISPOT of endogenous CD45.2 T-cells derived from ascites of FSHCER or PBS treated mice, primed with ID8-*Defb29/Vegf-a-Fshr* pulsed DCs (n=4 per group; 2 independent experiments). * $p<0.05$

Most importantly, this superior endogenous T-cell response was relevant to delay malignant progression, because adoptive transfer of host-derived (CD45.2[+]) splenic T-cell after treatment with FSHCER T-cells (CD45.1[+]) into naïve syngeneic mice resulted in significantly smaller tumors upon re-challenge with the same ID8-*Defb29/Vegf-a* cells, compared to the effect of T-cells from control tumor-bearing mice (**Figure 4.2.2**).
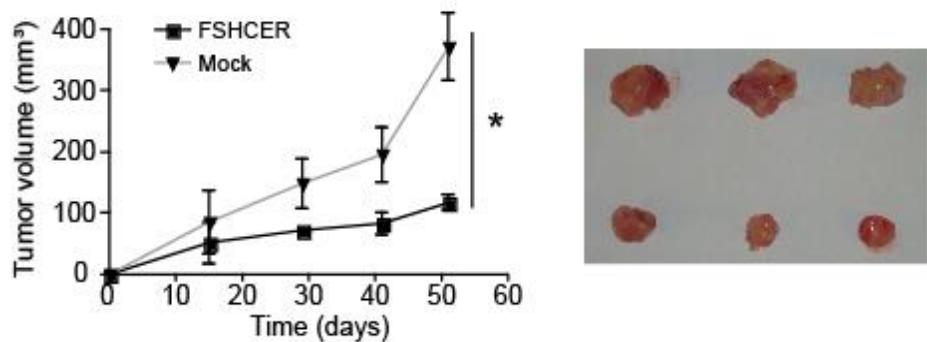
Figure 4.2.2 ID8-*Defb29/Vegf-a-Fshr* flank tumor growth after treatment with endogenous T-cells from FSHCER or Mock treated mice.

Growth kinetics and tumors after resection of ID8-*Defb29/Vegf-a-Fshr* flank tumors treated with endogenous (1e[6] CD45.2[+]GFP^{-} cells administered intraperitoneally) T-cells FACS sorted from the spleen of FSHCER or mock-transduced T-cell treated mice (n=4-5 per group; 2 independent experiments). * $p<0.05$

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To determine whether this boost in endogenous anti-tumor immunity is a general effect of T-cells re-directed against tumors by chimeric receptors, we generated an alternative, fully murine CAR. We focused on mesothelin, a glycophosphatidylinositol-anchored protein overexpressed in 66-93% non-mucinous ovarian carcinomas (Ordonez, 2003, Chang et al., 1992).

We found that the K1 antibody, an Ab that recognizes human mesothelin with low affinity (Chang and Pastan, 1996), also binds to mouse mesothelin in transduced ID8-*Defb29/Vegf-a* cells (**Figure 4.2.3**).
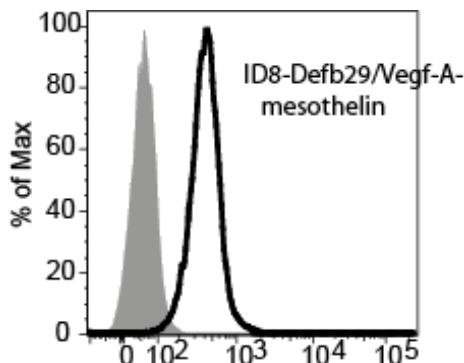
Figure 4.2.3 Mouse mesothelin staining of ID8-*Defb29/Vegf-a-Msln* with K1 antibody

Flow cytometry of ID8-*Defb29/Vegf-a-Msln* using anti-K1 antibody or an isotype control.

Further supporting specific targeting, K1 Abs recognized plate-bound mouse mesothelin but not control BSA in a custom ELISA (**Figure 4.2.4**).
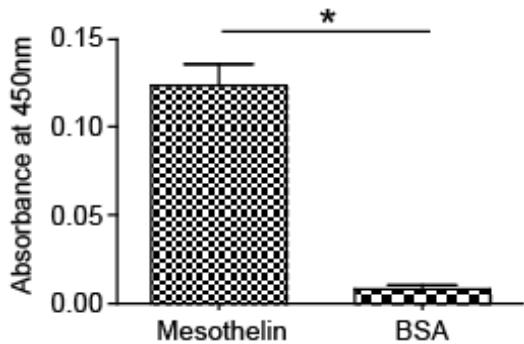
Figure 4.2.4 Mouse mesothelin ELISA with K1 antibody

Detection of plate bound mouse mesothelin with anti-K1 antibody compared to the levels bovine serum albumin by ELISA. * p<0.05

We therefore generated new mouse CAR T-cells using the K1 scFv as a targeting motif (**Figure 4.2.5**), and used transduced CD45.1[+] T-cells to treat mesothelin-expressing ID8-*Defb29/Vegf-a* tumor-bearing mice.
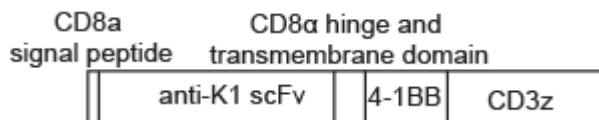
Figure 4.2.5 Schematic of K1CAR

K1CAR is composed by the heavy and light variable fragments (scFv) of the K1 antibody linked by a glycine/serine spacer between them and to a CD8α hinge and transmembrane domain, which are followed by the intracellular domains of 4-1BB and CD3ζ respectively

As with FSHCER T-cells, administration of K1CAR T-cells induced a significant boost in tumor-reactive endogenous (CD45.2$^+$) T-cells, as detected through ELISPOT analysis (**Figure 4.2.6**).
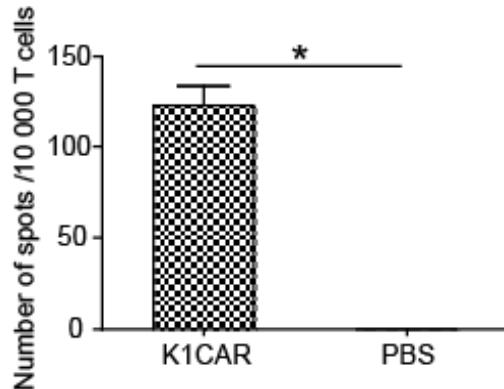

Figure 4.2.6 Endogenous T-cell ELISPOT against tumor antigen (K1CAR treated)

Representative interferon gamma ELISPOT of endogenous CD45.2 T-cells derived from ascites of FSHCER or PBS treated mice, primed with ID8-*Defb29/Vegf-a-Fshr* pulsed DCs (n=4 per group; 2 independent experiments). * p<0.05

Similarly, the adoptive transfer of host-derived T-cells stimulated *in vivo* by the administration of CAR T-cells was sufficient to delay malignant progression (

Figure 4.2.7).
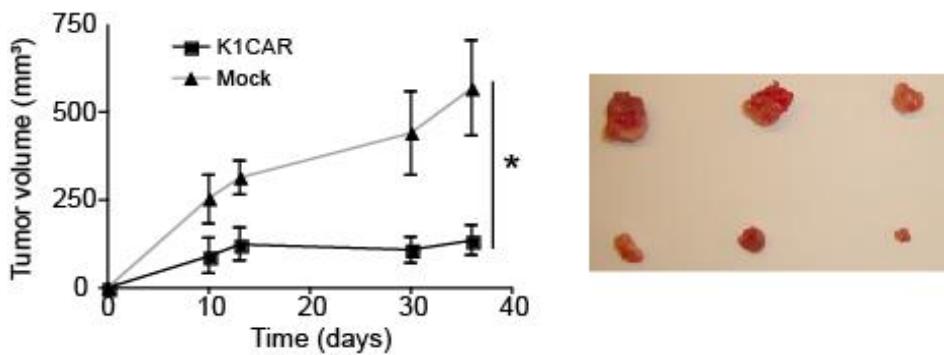
Figure 4.2.7 ID8-*Defb29/Vegf-a-Msln* flank tumor growth after treatment with endogenous T-cells from K1CAR or Mock treated mice.

Growth kinetics and tumors after resection of ID8-*Defb29/Vegf-a-Msln* flank tumors treated with endogenous (1e^6 CD45.2$^+$GFP$^-$ cells administered intraperitoneally) T-cells FACS sorted from the spleen of K1CAR or mock-transduced T-cell treated mice (n=4-5 per group; 2 independent experiments). * p<0.05

Taken together, these results indicate that the cytotoxic activity of T-cells re-directed against tumor cells with chimeric receptors boosts the activity of endogenous anti-tumor T-cells that should be able to delay malignant progression regardless of whether tumors lose targeted antigens through immunoediting.

4.3 Chimeric receptor-expressing T-cells delay malignant progression in immunocompetent ovarian cancer bearing mice

Given the striking ability of FSHCER T-cells to signal and kill FSHR tumor cells *in vitro*, and the significant boost in pre-existing anti-tumor immunity *in vivo*, we next determined the therapeutic potential of these cells in immunocompetent ID8-*Defb29/Vegf-a* tumor-bearing mice. Two

injections of 1-1.5x10^6 FSHCER T-cells at days 7 and 14 after tumor challenge were sufficient to significantly prolong the survival on this aggressive orthotopic model (Conejo-Garcia et al., 2004, Cubillos-Ruiz et al., 2012, Cubillos-Ruiz et al., 2009, Nesbeth et al., 2010, Rutkowski and Conejo-Garcia, 2015, Scarlett et al., 2009, Stephen et al., 2014), compared to mock-transduced T-cells, in multiple independent experiments (**Figure 4.3.1**). As importantly, no obvious adverse effects were observed in these experiments.
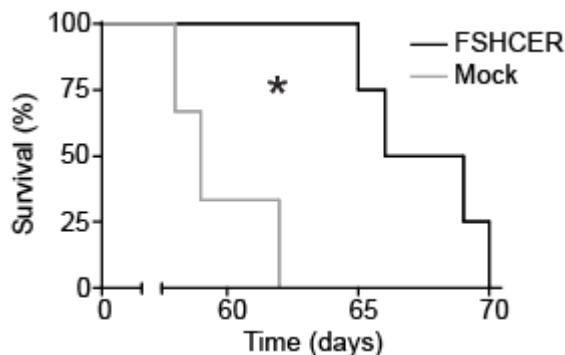
Figure 4.3.1 Survival plot of ID8-*Defb29/Vegf-a-Fshr* bearing mice

> Survival plot of mice bearing ID8-*Defb29/Vegf-a-Fshr* ovarian tumors treated with either T-cells bearing the FSHCER or mock-transduced (n=5 per group; 3 independent experiments). * p<0.05

Because the expression of FSHR has also been reported in certain metastatic lesions (Siraj et al., 2013), to demonstrate the general applicability of FSHCER T-cells, we also targeted FSHR-transduced tumors generated with highly aggressive p53/KRas-driven breast tumor cells (Rutkowski and Conejo-Garcia, 2015, Rutkowski et al., 2014) growing in the axillary flank.

Supporting broad therapeutic potential, FSHCER T-cells were even more effective at abrogating the growth of established tumors at this additional location (**Figure 4.3.2**).
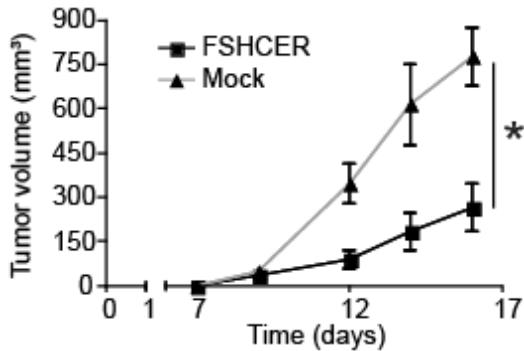
Figure 4.3.2 Tumor growth in A7C11-*Fshr* bearing mice

Growth kinetics of A7C11 axillary flank tumors expressing FSHR treated with FSHCER or mock transduced T-cells (n=5 per group; 3 independent experiments). * $p<0.05$

Despite the reproducible therapeutic effects, however, FSHCER T-cells did not induce complete rejection of established, aggressive tumors. To compare the effectiveness of hormone *vs.* scFv (antigenic) targeting, we treated established mesothelin-expressing ID8-*Defb29/Vegf-a* tumor-bearing mice with K1CAR *vs.* mock-transduced T-cells. As shown in **Figure 4.3.3**, expression of K1CARs empowered T-cells to delay malignant progression to a similar extent as FSHCER T-cells.
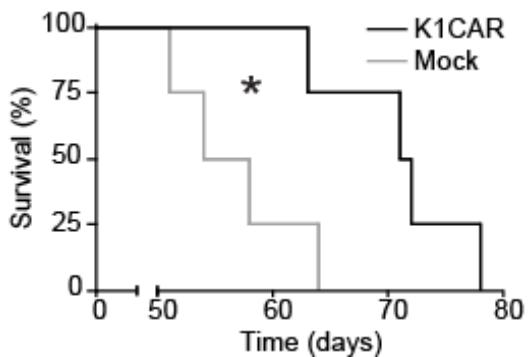
Figure 4.3.3 Survival plot of ID8-*Defb29/Vegf-a-Msln* bearing mice

Survival plot of mice bearing ID8-*Defb29/Vegf-a-Msln* ovarian tumors treated with either T-cells bearing the K1CAR or mock-transduced (n=5 per group; 3 independent experiments).

To understand the relative contribution of different T-cell subsets to the reproducible anti-tumor effectiveness of FSHCER lymphocytes, we next treated different cohorts of established ID8-*Defb29/Vegf-a* tumor-bearing mice with either FSHCER-transduced CD4 or CD8 T-cells, alone or in combination. Interestingly, CD4 T-cells expressing chimeric receptors were as effective as a mix of unsegregated lymphocytes, while CD8 T-cells alone induced reduced therapeutic benefits (**Figure 4.3.4**). A similar trend was observed with K1CAR-transduced T-cells (not shown).
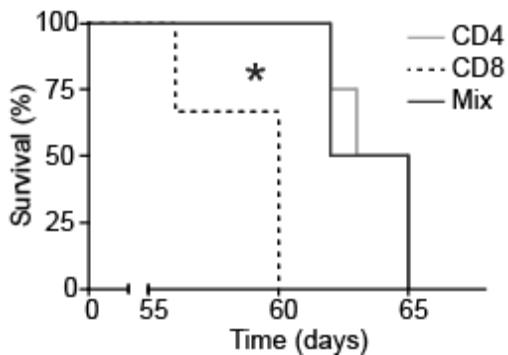
Figure 4.3.4 Survival plot of ID8-*Defb29/Vegf-a-Fshr* bearing mice (CD4 vs CD8)

Survival plot of mice bearing ID8-*Defb29/Vegf-a-Fshr* ovarian tumors treated with either CD4, CD8 or a mixed population of T-cells bearing the FSHCER (n=3-5 per group). * $p<0.05$

Together, our results indicate that T-cells redirected through chimeric receptors that take advantage of the natural affinity of a hormone for its receptor can be at least as effective as scFv-targeted CAR T-cells. Furthermore, CD4 T-cells are major contributors to consistent therapeutic benefits, although incapable of inducing complete rejection of established epithelial tumors.

4.4 Chimeric receptor-expressing T-cells persist in the absence of immunoediting

To understand tumor-induced mechanisms that could limit the effectiveness of chimeric receptor-expressing T-cells *in vivo*, we next tested whether tumor cells could lose the targeted surface molecules through immunoediting. Notably, fresh FSHCER T-cells reacted equally strongly by secreting IFN-γ in response to FSHR-transduced tumor cells sorted from the peritoneal cavity of mice previously treated with FSHCER- or mock-transduced T-cells, or PBS (**Figure 4.4.1**), indicating persistence of targeted FSHR.
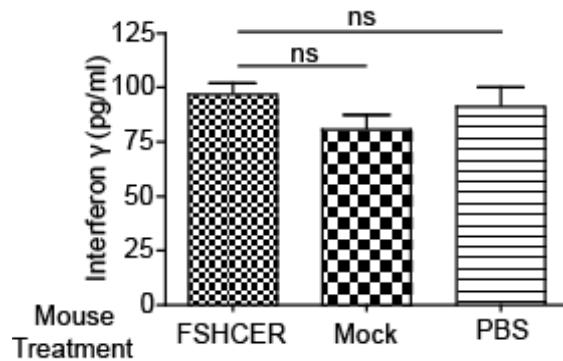
Figure 4.4.1 Interferon γ levels of FSHCER after coculture with ID8-*Defb29/Vegf-a-Fshr* from FSHCER, mock or PBS treated mice.

Levels of interferon-γ elicited by fresh FSHCER- or mock-transduced T-cells after incubation of ID8-*Defb29/Vegf-a-Fshr* cells (1:10 target:effector ratio), FACS-sorted from the peritoneal cavity of orthotopic ID8-*Defb29/Vegf-a-Fshr* bearing mice treated with either FSHCER, mock transduced T-cells or PBS. ns: not significant.

To determine whether chimeric receptor-targeted T-cells persist in ovarian cancer-bearing hosts shortly after adoptive transfer, we took advantage of the congenic marker CD45.1 and sacrificed FSHR-expressing ID8-*Defb29/Vegf-a* tumor-bearing mice at days 5, 10 and 15 after intraperitoneal administration of FSHCER T-cells. Five days after T-cell administration, we saw a peak of FSHCER T-cells in the peritoneal cavity, with similar proportions of CD8 *vs.* CD4 lymphocytes (**Figure 4.4.2 and Figure 4.4.3**).
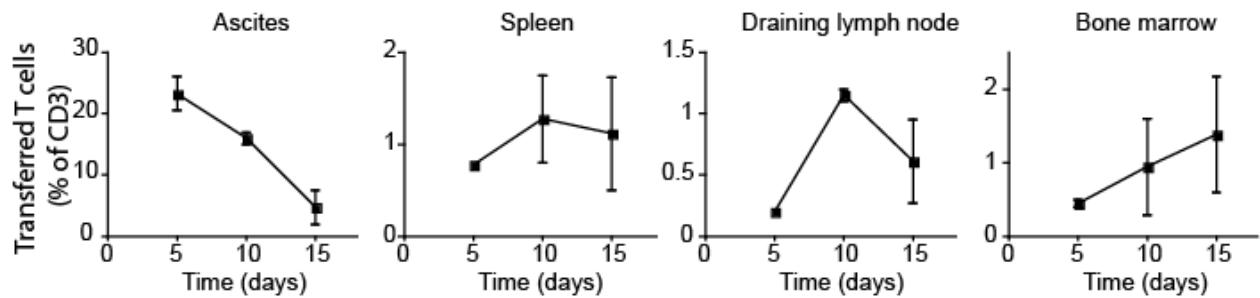
Figure 4.4.2 Persistance of transferred T-cells in treated mice

Five, ten and fifteen days after adoptive T-cell transfer we harvested the peritoneal wash, spleen, draining lymph node and bone marrow from ID8-*Defb29/Vegf-a-Fshr* treated mice and measured the percentage of transferred T-cells within the total T-cells in these samples.
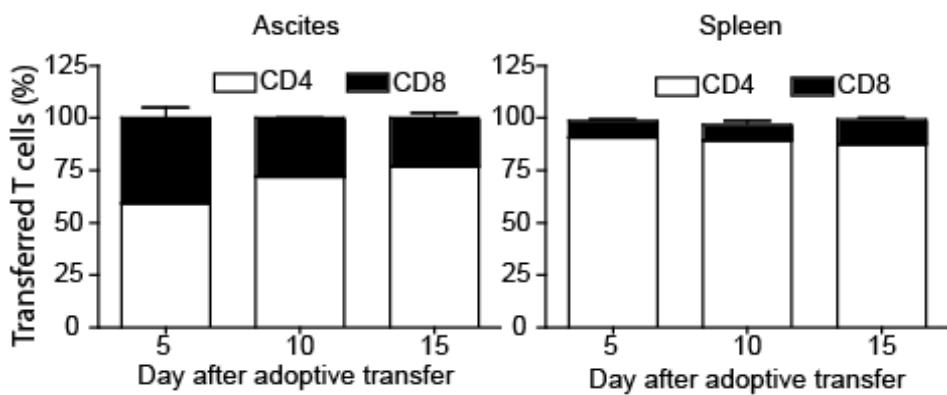
Figure 4.4.3 Proportion of CD4 and CD8 in the proportion of T cells transferred

CD4 T cells preferentially migrated to the spleen compared to CD8. In the peritoneal wash, at day 5 the proportion of CD4 and CD8 was similar, but in further timepoints, the CD4 T-cells outnumbered CD8.

Both subsets were activated (CD44$^+$CD69$^+$) at tumor (peritoneal) beds at this early stage (45.5% and 3.1%, respectively) (**Figure 4.4.4**).
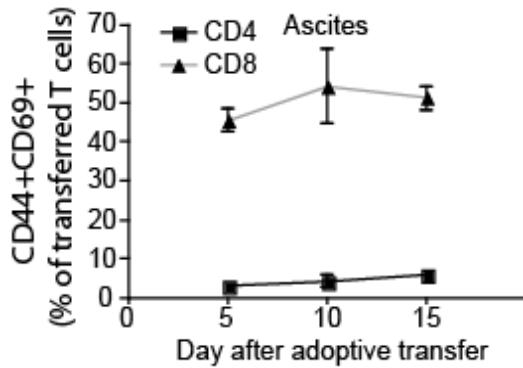
Figure 4.4.4 Percentage of activated CD4 and CD8 in the tumor microenvironment

We performed a peritoneal wash on days 5, 10 and 15 in ID8-*Defb29/Vegf-a-Fshr* and stained transferred T-cells for CD44+CD69+ to show the activated transferred T cells.

However, proportions and absolute numbers (not shown) of transferred T-cells decreased on days 10 and 15, accompanied by a higher percentage of CD4 T-cells relative to CD8, and retention of activation markers (**Figure 4.4.2, Figure 4.4.3 and Figure 4.4.4**, above). In both spleen and tumor draining lymph nodes, we observed a peak of accumulation at day 10, with trafficking as early as day 5. These T-cells are mostly CD4$^+$ (90.5%) with a central memory phenotype (CD44$^+$CD62L$^+$) (**Figure 4.4.5**) and represented 0.8% of total T-cells.
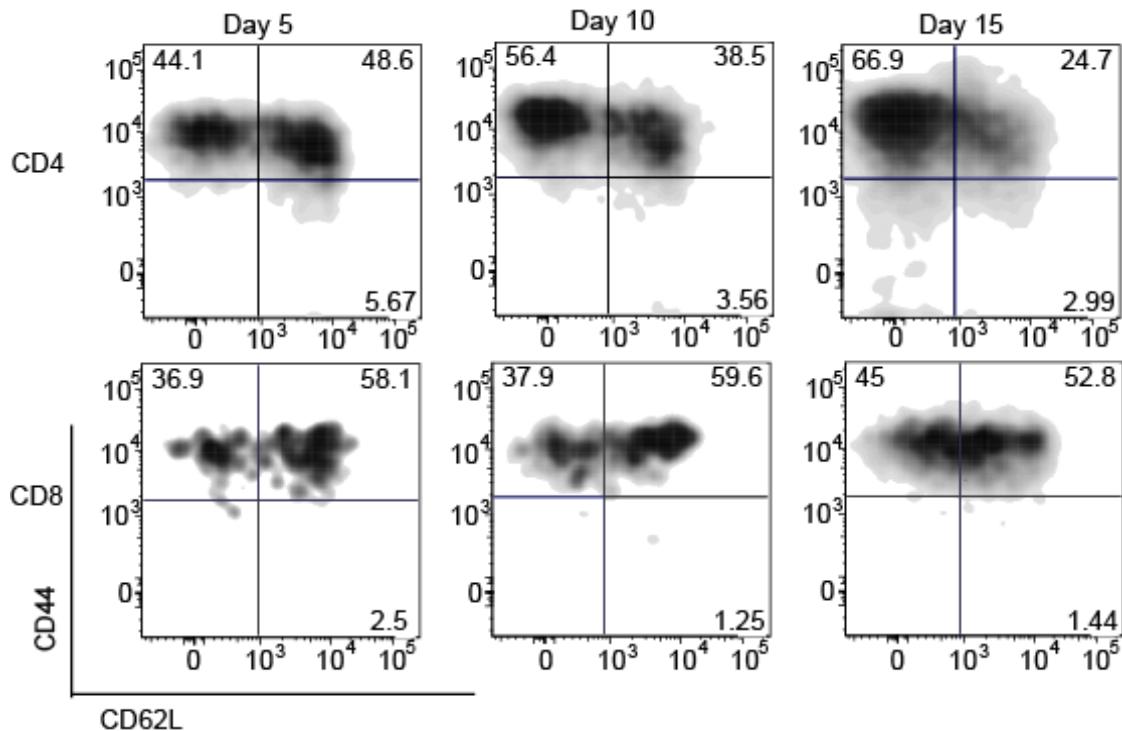
Figure 4.4.5 Memory phenotype of transferred T-cells

We stained transferred T-cells from the spleen of tumor bearing mice treated with FSHCER

or K1CER and stained with CD44 and CD62L to look for the distribution at each timepoint

of central memory (CD44-CD62L+), effector memory (CD44+CD62+) and late effector

(CD44+CD62-) T-cells.

At terminal stages of malignant progression, FSHCER T-cells were still clearly and reproducibly

detectable in the spleen (0.6% of total T-cells) (**Figure 4.4.6**). At this stage, however, no FSHCER

T-cells could be detected in tumor ascites or bone marrow.
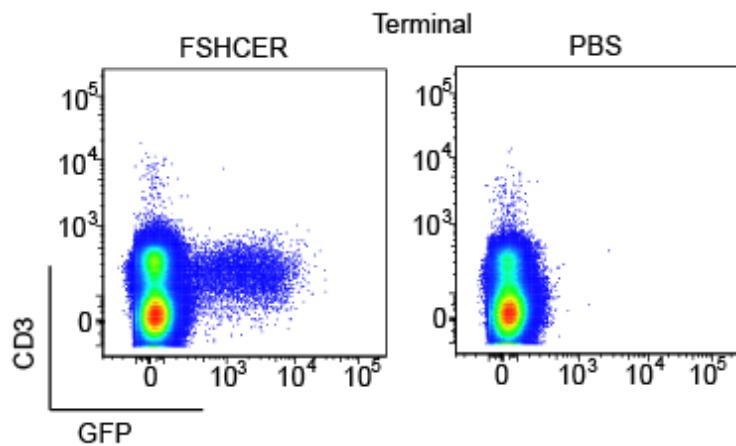
Figure 4.4.6 FSHCER T-cell are still present in the spleen at terminal tumor stage

We harvested spleens from ID8-*Defb29/Vegf-a-Fshr* bearing mice at terminal stage (with terminal ascites) and looked for GFP+ T-cells. We found a clear population in the spleen. We found no T-cells remaining in the tumor microenvironment (ascites) or bone marrow.

Of note, it is also unlikely that exhaustion is a major contributor to the eventual collapse of chimeric receptor-expressing T-cells because only a minority of T-cells (8% in CD8 and 31% in CD4) expressed measurable PD-1 on the cell surface (**Figure 4.4.7**).
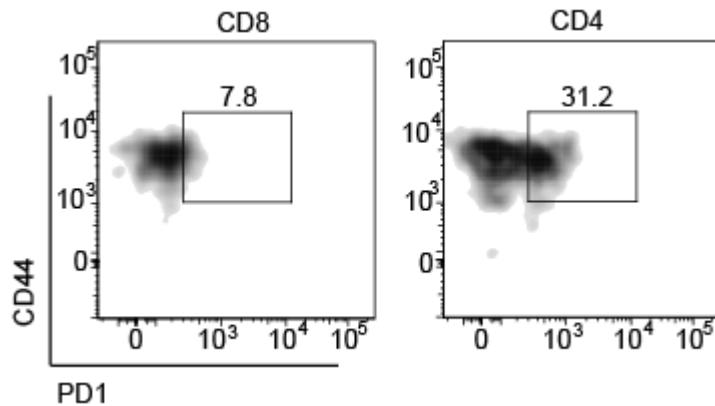
Figure 4.4.7 Transferred T-cells at terminal stage express low levels of PD1

CAR T-cells from the spleen at terminal stages showed low levels of PD1 expression.

To determine whether immunosuppressive factors in tumor ascites could induce T-cell death, we co-incubated FSHR-expressing ID8-*Defb29/Vegf-a* tumor cells and targeted FSHCER T-cells in the presence of increasing amounts of tumor-derived ascitic fluid. Unexpectedly, filtered (cell-free) tumor ascites increased the reactivity (IFN-γ secretion) of FSHCER T-cells against their cognate target in a dose-dependent manner (**Figure 4.4.8**).
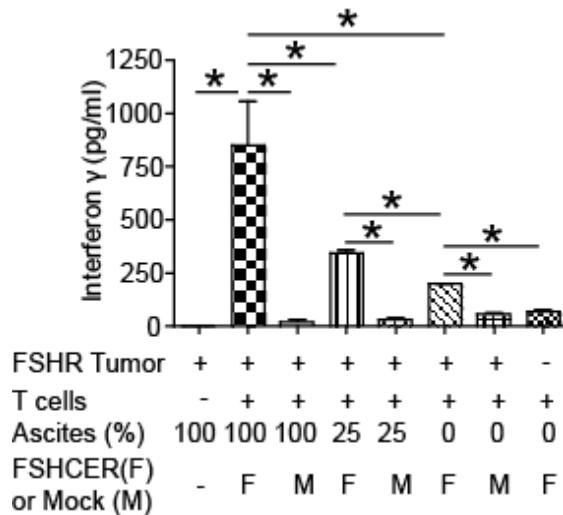
Figure 4.4.8 ID8-*Defb29/Vegf-a* ascites promotes interferonγ secretion of FSHCER T-cells in a dose dependent fashion

Levels of interferon gamma elicited after incubation of ID8-*Defb29/Vegf-a-Fshr* cells (1:10 target:effector ratio) with either FSHCER or mock transduced T-cells with the indicated percentages of cell-free ascites from ID8-*Defb29/Vegf-a-Fshr* bearing mice.

This was the result of recognition of FHSR of tumor cell origin in ascites through recent surface shedding, because, firstly, identically treated mock-transduced T-cells did not react against FSHR⁺ tumor cells in the presence of ascites (**Figure 4.4.8**).

Also, when we cultured T-cells with ascites in the absence of tumor cells, there was only a relevant response when FSHCER T-cells were exposed to ascites that had been previoiusly coincubated with FSHR expressing cells, not in the case of ascites without tumor coincubation or with media previously incubated with tumor cells (**Figure 4.4.9**). These results indicate that the acute release of FSHR from the tumor cells into the ascitic fluid is necessary for the specific activation of the FSHCER.
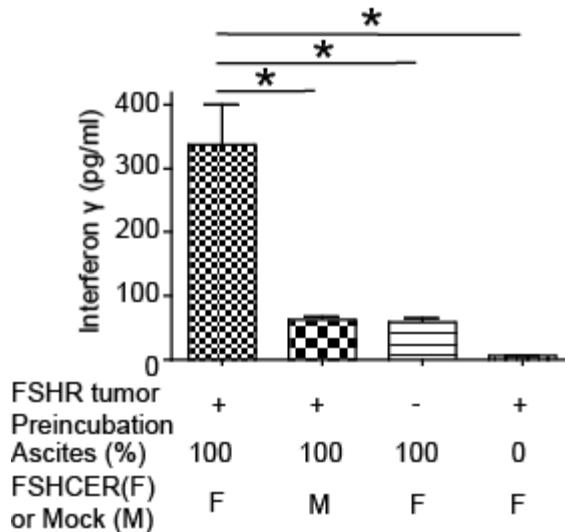
Figure 4.4.9 Ascites from ID8-*Defb29/Vegf-a* can activate FSHCER to a lower extent

Levels of interferon γ elicited after incubating FSHCER or mock transduced T-cells with ascites from ID8-*Defb29/Vegf-a* or media previously coincubated or not with ID8-*Defb29/Vegf-a-Fshr*. * $p<0.05$.

Together, these results indicate that chimeric receptor-targeted T-cells persist as memory cells in treated ovarian cancer-bearing hosts but they eventually disappear from tumor beds. Shedding of continuously expressed molecular target into ascites elicits T-cell activity distally from targeted tumor cells, likely contributing to the eventual absence of transferred T-cells from ascites, despite apparent lack of exhaustion and persistence in lymphoid organs.

4.5 Treatment with FSHCAR and K1CAR is safe

An important concern regarding the use of chimeric receptor-targeted immunotherapies is the occurrence of off-tumor on-target effects. Supporting the restricted pattern of expression of FSHR, we did not observe obvious toxicity upon transfer of FSHCER T-cells, in terms of weight loss or any signs of discomfort.

Because mesothelin is expressed in the pleura, pericardium and peritoneum (Chang et al., 1992), we focused our attention on potential adverse effects upon adoptive transfer of K1CAR T-cells, which have the capacity to recognize mouse mesothelin in tumor-free tissues. Again, treated mice showed no obvious adverse effects during follow up. Most importantly, we found no differences in the infiltration of total (CD45$^+$) leukocytes in the pleura and pericardium of mice treated with K1CAR T-cells, compared to control mice identically treated with mock-transduced T-cells, as determined by immunohistochemistry (**Figure 4.5.1**).
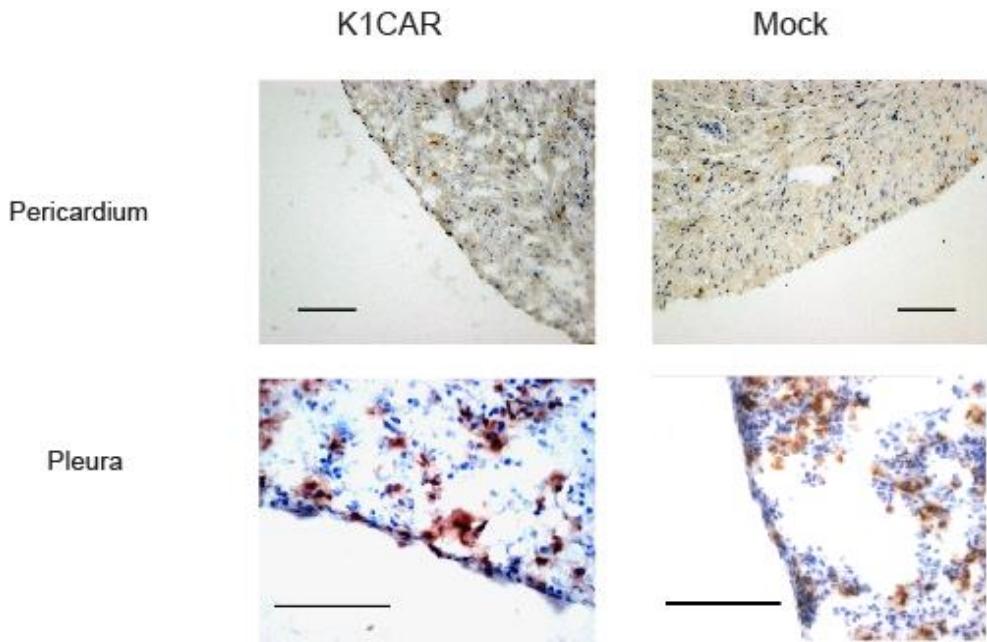
Figure 4.5.1 Leukocyte infiltration does not differ in pericardium and pleura of K1CAR vs mock transduced T-cell treated mice

We harvested the heart and lungs of mice treated with K1CAR or mock transduced T-cells, embebbed the tissues in OCT blocks, cut in slides and stained for CD45.

Further supporting the absence of increased inflammation in mesothelin-expressing healthy tissues, q-PCR did not detect any differences in mRNA expression levels of both CD45 and CD3 between K1CAR treated vs. mice receiving either mock-transduced T-cells or, most importantly, untreated mice (**Figure 4.5.2**).
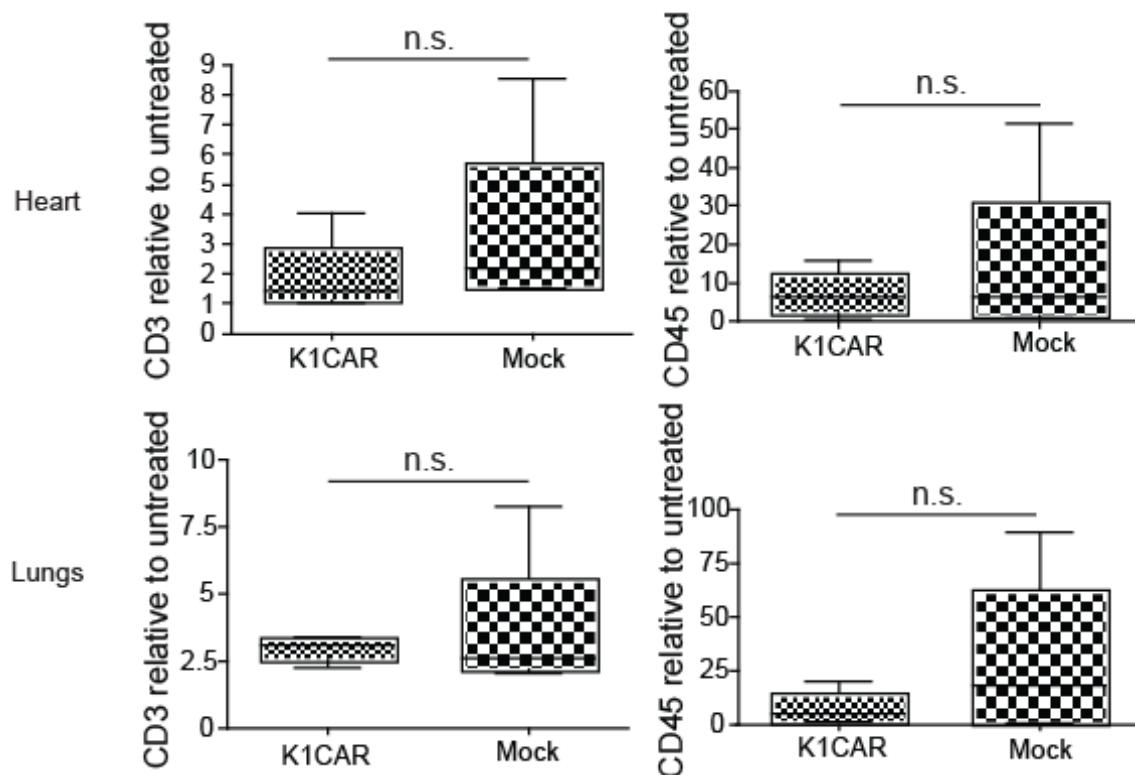
Figure 4.5.2 qRT-PCR shows non-different levels of CD3 and CD45 transcripts in heart and lungs of K1CAR or mock transduced T-cells.

We harvested the heart and lungs of mice treated with K1CAR or mock transduced T-cells, isolated RNA, made cDNA and analyzed through qRT-PCR for the expression of CD3 and CD45.

Overall, our results demonstrate that, although with limited effectiveness due to tumor microenvironmental factors, T-cells re-directed against ovarian cancer cells with hormone- or scFv-targeted chimeric receptors induce measurable therapeutic benefits with negligible toxicity, despite the expression of targeted antigens in tumor-free tissues.

4.6 XBP-1 expression in dendritic cells promotes immunosuppression in the ovarian cancer microenvironment

Aberrant accumulation of unfolded proteins in the Endoplasmic Reticulum (ER) triggers "ER stress", a hallmark feature of many diseases including cancer (Hetz and Glimcher, 2009). XBP1, a transcription factor that govenrs ER stress response (Fang and Declerck, 2013), has been suggested to operate in cancer cells to influence their survival and growth (Koong et al., 2006, Romero-Ramirez et al., 2004). However, its role in modulating the immune cells to allow a tumor-permissive microenvironment has not been explored.

To define how constitutive activation of XBP1 in tDCs might influence ovarian cancer progression, we developed orthotopic p53/K-ras-driven primary ovarian tumors (Scarlett et al., 2012) in conditional knockout female mice (Lee et al., 2008) that selectively delete exon 2 of *Xbp1* in CD11c$^+$ DCs. Notably, ovarian cancer-bearing female mice lacking functional XBP1 in CD11c$^+$ DCs (XBP1$^{f/f}$ CD11c-Cre) demonstrated reduced tumor growth (**Figure 4.6.1 and Figure 4.6.2**) compared with control gene-sufficient (XBP1$^{f/f}$) littermates.

Figure 4.6.1 Orthotopic p53/Kras ovarian tumors

p53/K-ras-driven ovarian tumors were generated in hosts reconstituted with bone marrow from either XBP1$^{f/f}$ (top) or XBP1$^{f/f}$ CD11c-Cre (bottom) donor mice as described in the methods and primary tumors were resected 48 days after intrabursal injection of Cre-expressing adenovirus (ADV-Cre).
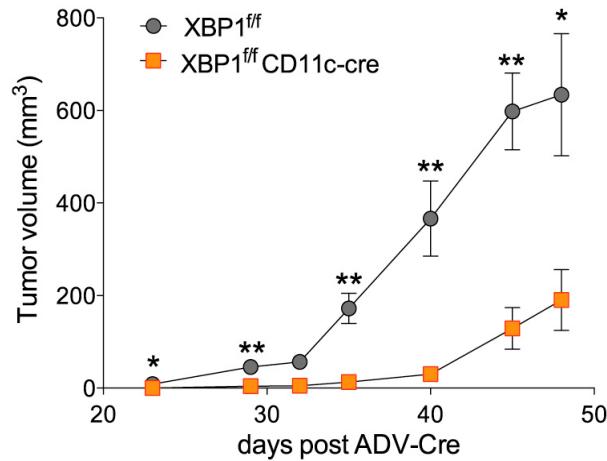
Figure 4.6.2 Growth kinetics of p53/K-ras ovarian tumors in hosts reconstituted with bone marrow from the indicated genotypes.

P53/K-ras ovarian tumors grew significantly faster in hosts reconstituted with XBP1 deficient dendritic cells compared to their sufficient counterparts.

These results indicate that XBP1 expression in CD11c$^+$ DCs is necessary for the accelerated progression of ovarian cancer.

To determine whether abrogating XBP1 function selectively in tDCs had significant therapeutic effects, mice bearing aggressive ID8-*Defb29/Vegf-a* ovarian tumors were intraperitoneally treated with saline, non-targeting siRNA or XBP1-specific siRNA-PEI nanocomplexes. Treatments were administered on days 8, 13, 18, 23 and 28 after tumor implantation, following conditions previously optimized by our group (Cubillos-Ruiz et al., 2009). Ascites accumulation and overall host survival were monitored over time. Consistent with prior observations (Cubillos-Ruiz et al., 2009), treatment with immunostimulatory PEI-based nanoparticles carrying non-targeting siRNA prolonged survival in cancer-bearing mice compared with untreated controls. Nevertheless, superior therapeutic effects were evident in hosts treated with nanoparticles silencing XBP1 in tDC (**Figure 4.6.3**).
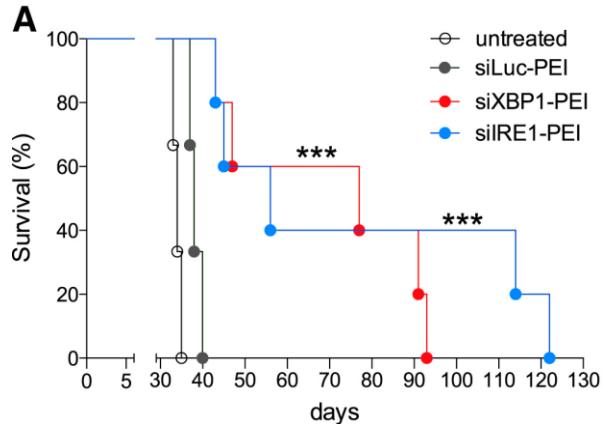
Figure 4.6.3 PEI-nanocomplexes carrying XBP1 siRNA increased survival in mice bearing ID8-*Defb29/Vegf-a* ovarian tumors

Mice bearing ID8-*Defb29/Vegf-a* ovarian tumors were treated on days 8, 13, 18, 23 and 28 after tumor implantation with PEI-nanocomplexes associated with siRNA against XBP1, luciferase or with vehicle (PBS) and survival analyzed.

Therefore, while immunogenic PEI-based nanocomplexes inherently induce beneficial outcomes, concomitant abrogation of aberrant XBP1 function in ovarian cancer-associated DCs results in substantially enhanced therapeutic benefits (Cubillos-Ruiz et al., 2015).

5 Discussion

Here we showed that T-cells expressing different chimeric receptors mediate their therapeutic activity in part by boosting endogenous, pre-existing anti-tumor immunity, which is relevant to delay malignant progression. In addition, the use of the two subunits of the FSH hormone effectively redirects the cytotoxic activity of T-cells against ovarian cancer cells, resulting in reproducible therapeutic benefits in different preclinical models, eventually limited by tumor-induced diversion of effector activities distally from tumor cells.

Our study demonstrates for the first time that host-derived T-cells influenced by chimeric receptor-transduced lymphocytes contribute to overall effectiveness. Accordingly, recent studies showed that short-lived mesothelin-targeting CAR T-cells can induce epitope-spreading and raise Ab titers in patients (Beatty et al., 2014). Our work in immunocompetent preclinical models expand these mechanisms by demonstrating that CAR T-cells increase endogenous T-cell activity, which is sufficient to delay tumor progression. These effects may be particularly important in tumors undergoing loss of targeted antigens through immunoediting or deletion of CAR T-cells, because polyclonal responses provide additional immune pressure against malignant progression even after transferred lymphocytes become ineffective. Interestingly, we did not observe any loss of antigenicity in our systems at end-stage disease, despite the fact that FSH-targeted chimeric receptor-expression T-cells remained in the host until tumor-bearing mice succumbed to terminal disease. Although CAR T-cells have been shown to be reversibly inactivated in solid tumors in a PD-1-dependent manner (Moon et al., 2014), in our ovarian tumors we observed PD-1 expression in only a minority of FSHCER T-cells persisting in terminal mice. Rather, we found that shedding of targeted molecules from the surface of tumor cells into the ascitic fluid is sufficient to divert the

effector activity of chimeric receptor-expressing T-cells away from tumor cells. Our results therefore unveil a new mechanism of tumor-induced immune evasion that should be considered for, at least, the treatment of ovarian cancer patients.

Another interesting aspect of our study is that, despite superb cytotoxic activity *in vitro* even at low effector:target ratios, and although FSHCER T-cells elicited reproducibly significant survival increases in mice with established aggressive ovarian malignancies, all treated mice eventually succumb to the disease. Because ovarian cancer is a very challenging disease with a dismal prognosis, for these proof-of-concept experiments we limited treatments to two injections and used particularly aggressive (and established) tumors. The anti-tumor effects of other CAR T-cells could be augmented by additional injections of the CAR T-cells, or by combination with other immunotherapeutic interventions (Wang et al., 2014). It is therefore likely that combinatorial interventions and applying immunotherapies soon after surgical resection plus chemotherapy will further increase the survival of ovarian cancer patients receiving similar treatments. Our results provide a rationale for these interventions because we did not observe any noticeable toxicity during the course of our treatments. This was expected for FSHCER T-cells because the only healthy tissue where FSHR has been found to be expressed is the ovary (Simoni et al., 1997), where ID8 tumor cells preferentially home (not shown). However, we also found any absence of toxic effects, or even inflammation, upon the use of mesothelin-targeted CAR T-cells.

The requirement of CD4 T cells to attain a more effective delay in tumor progression supports the relevance of the boost in the endogenous response in the CAR T cell antitumor activity. Both CD4 and CD8 CAR T cells can elicit a similar cytotoxic activity, however, CD4 T cells may be able to orchestrate an effective endogenous antitumor response.

Mesothelin is broadly expressed, including in pericardium and pleura. As mesothelin is being currently targeted through CAR T-cell interventions in the clinic (Adusumilli et al., 2014, Moon et al., 2014, Beatty et al., 2014), concerns about potential off-tumor effects arise. So far, no fatal off-tumor specific responses have been reported, but multifactorial T-cell hypofunction in the microenvironment of very advanced tumors has also limited the effectiveness of mesothelin CAR T-cells (Moon et al., 2014). If CAR T-cells are eventually optimized to induce tumor rejections and they persist in treated patients, it is theoretically possible that toxicity will become an issue. This could be diminished with the addition of a suicide gene (Hoyos et al., 2010), but long-term protection against recurrences would be eliminated. It is therefore possible that low-affinity scFvs (such as the K1 clone used in this study) (Chang and Pastan, 1996)) offer a better compromise between effectiveness and low toxicity, by targeting (tumor) cells overexpressing the target while sparing healthy cells expressing lower levels.

Overall, our study demonstrates the effectiveness of targeting chimeric receptors for T-cell activation using full-length hormones and unveils mechanisms of activity and eventual suppression of these interventions that should help understanding previously unknown effects in ongoing and future clinical trials.

6 Current work/Future directions

6.1 In vivo testing of human FSHCER

Our development and study of the murine FSHCER has been a tool for the better understanding and optimization of the targeting of human FSHR expressing tumors in the clinical setting with human FSHCER.

We have already shown that human T-cells transduced FSHCER show a potent cytotoxicity against OVCAR-3, a FSHR expressing ovarian cancer cell line in vitro. However, human tumors are heterogeneous, and that heterogeneity cannot be recapitulated with the use of cancer cell lines. To accurately recapitulate the heterogeneity of human ovarian cancer in different patients we are engrafting fresh advanced ovarian carcinomas that we receive from the long-term collaboration between Wistar and the Helen Graham Cancer in NOD-SCID (NSG) mice. We dissect and engraft ~1mm^3 chunks into the ovarian bursa of NSG (severely immunodeficient) mice. Importantly, we are also receiving peripheral blood from the same patients, and we are cryopreserving peripheral mononuclear cells from each patient. Tumors that engraft (approximately 10-20%) become palpable and visible in ~2 months (**Figure 6.1.1**).

Figure 6.1.1 Ovarian cancer patient derived xenografts

Three cases of human ovarian carcinomas that we have engrafted in the ovaries of NSG mice.

Although their initial progress is slow, we have found that tumors grow faster when they are serially engrafted for a second time. Our goal is to generate xenografts for ≥5 primary tumors from different patient. These mice will be used to define the effectiveness of FSHCER T cells against human ovarian tumors. For that purpose, we will CD3/CD28-expand and transduce the FHSR-targeting CAR on T cells from the same patient, thus mimicking its potential clinical application.

As a complement, thanks to a collaboration with Dr Julia Tchou, MD, from the Hospital of the University of Pennsylvania, we are also collecting and engrafting triple negative breast cancer tissue into the mammary fat pad of NSG mice (**Figure 6.1.2**). Unlike with ovarian cancer, the engraftment rate of breast cancer into NSG mice has been 100% so far (5 out of 5 tumors).

Figure 6.1.2 Orthotopic breast cancer patient derived xenograft

NSG mouse bearing a human triple negative breast cancer (arrow) that was engrafted as a single cell suspension of tumor cells in the mammary fat pad.

We have previously found mesothelin to be a negative prognostic factor for breast cancer (Li et al., 2014). We have analyzed Dr Tchou's tumor bank for mesothelin expressing tumors and used those to generate patient derived xenografts in NSG mice. Once we are able to grow the mesothelin positive tumors we will pass them into a higher number of NSG hosts and use an established mesothelin targeting CAR (Beatty et al., 2014) to study the efficacy of CAR immunotherapy in breast cancer as a step for its clinical application.

6.2 Generation of exhaustion resistant T cells by ablation of FoxP1

TGFβ is lymphocyte inhibitor secreted by multiple cells and frequently overexpressed in aggressive cancers (Wrzesinski et al., 2007, Flavell et al., 2010). TGF-β can suppress anti-tumor CD8 lymphocyte response (Ahmadzadeh and Rosenberg, 2005). We recently reported Forkhead box P1 (FoxP1) as a transcription factor that mediates TGFβ immune suppression. This

transcription factor is upregulated in the T-cells present in the tumor microenvironment and prevent tumor reactive T-cells from proliferating and eliciting tumor rejection (Stephen et al., 2014). Ablation of Foxp1 in tumor antigen-primed or chimeric receptor anti-tumor T-cells could therefore empower lymphocytes to resist immunosuppressive networks in solid tumors.

In order to generate Foxp1 deficient T-cells we engineered two CRISPR guides for targeting Foxp1 in the most constant sequence of the gene (towards the 3'). We used the following sequences: 5'-GCCCGGGCTGAATTGTCAGA-3' and 5'-GGCCTTGGCGCTGCAAAGAC-3'. We electroporated the T-cells with the guide and the plasmid Cas9-GFP using the Amaxa electroporation kit P3 and following instructions for naïve T-cells.

As initial readout of transfection we performed flow cytometry staining for IL-7 receptor α (upregulated in Foxp1 deficient cells) and GFP (which the Cas9-GFP plasmid has a reporter) (**Figure 6.2.1**).

So far, we haven't been successful in ablating Foxp1 in human T-cells and we are still optimizing the protocol. Once we make it work, we plan to ablate Foxp1 in CAR T cells and study their potential superior effectiveness in the tumor microenvironment of solid tumors.
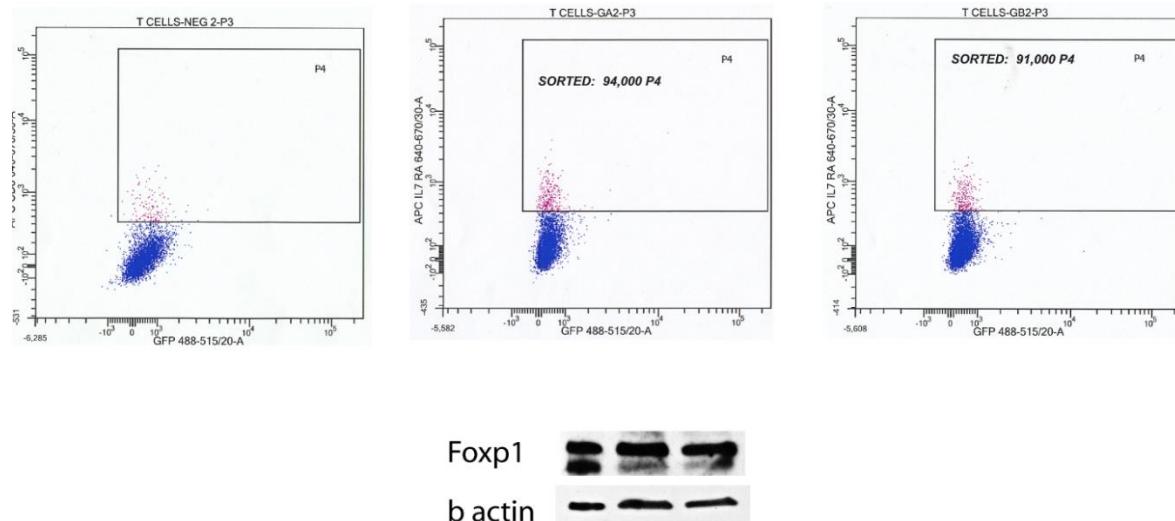
Figure 6.2.1 IL-7 receptor α expression by transfected cells and western blot for Foxp1 expression

After T-cell electroporation we incubated the T-cells with IL-2 (20U/ml) and IL-7 (1ug/ml) for 48 hours and stained for IL-7 receptor α and sorted for IL-7 receptor α and GFP + (for the detection of the plasmid transfection) T-cells. Above we see the result of FACSorting for the mentioned markers (from left to right: no guide, guide 1, guide 2). After sorting we extracted proteins from the T-cells and blotted for Foxp1 to ensure the absence of the protein due to the gene ablation with the CRISPR. Below is an example of an experiment where we did not attain ablation.

6.3   Adoptive T cell therapy optimization by modulation of the microbiome

As we recently published (Rutkowski et al., 2015), commensal microbiota is of paramount importance in distal tumor progression. We found that antibiotic depletion of the microbiota in mice delayed tumor progression in a sarcoma model. On the other hand, chemo and

immunotherapy effectiveness have been shown to be modulated by the gut microbiota (Viaud et al., 2015). Hence, we were interested in the effect that selective populations of microbiota depletion could have in the presence of immunotherapy. To validate the differential tumor progression in different tumor types, we treated with FSHCER ovarian cancer ID8-*Defb29/Vegf-a-Fshr* and A7C11-FSHR with or without microbiota depletion from an antibody cocktail containing ampicillin (0.5 mg/ml), neomycin (0.5 mg/ml) and vancomycin (0.25 mg/ml), that we diluted in water. In this preliminary experiment we saw that in the A7C11-FSHR breast tumors, antibiotic treatment promoted tumor growth and we could see no effect of the FSHCER (**Figure 6.3.1**).
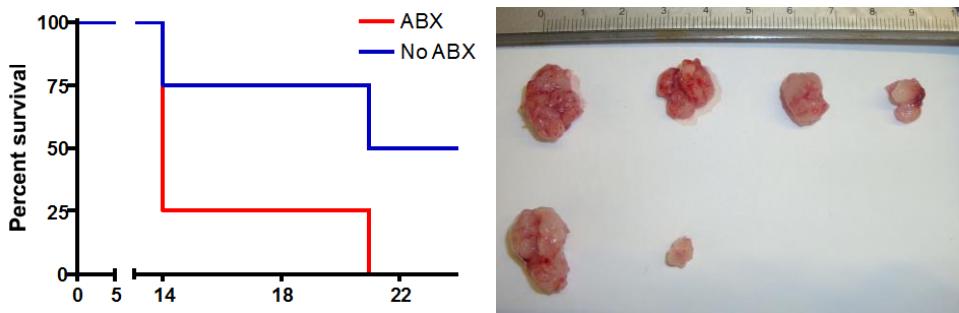
Figure 6.3.1 A7C11-Fshr tumor bearing mice treated with FSHCER and antibiotic cocktail

(left) Time at which tumors become palpable after tumor cell injection in the flank. Mice that received the antibiotic cocktail (Abx) presented a faster breast tumor growth compared to those that did not receive antibiotic treatment (No Abx). Two of the tumors in the non-antibiotic treated group had not developed at the time of sacrificing the mice for analysis. (right) top: tumors of antibiotic treated mice; lower: tumors of non-antibiotic treated mice.

In the ovarian ID8-*Defb29/Vegf-a-Fshr*, on the contrary, there seemed to be a slower tumor progression associated with antibiotic treatment. At the time of sacrificing the mice for analysis, the antibiotic treated mice had smaller spleens (splenomegaly is correlated with tumor progression due to the accumulation of myeloid derived suppressor cells) and less ascites than the non-antibiotic treated mice (**Figure 6.3.2**).
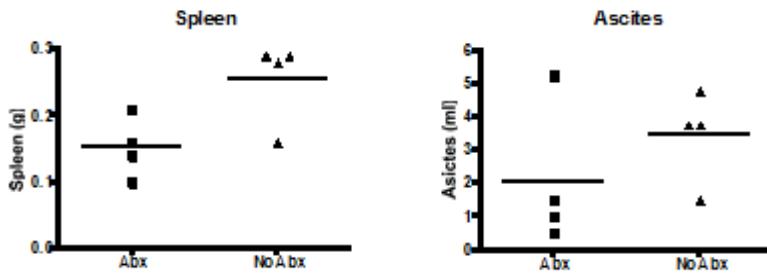
Figure 6.3.2 ID8-*Defb29/Vegf-a-Fshr* bearing mice treated with FSHCER and antibiotic cocktail

Spleen weight (p=0.05) and ascites volume (p=0.3) suggested a lower tumor burden in mice treated with antibiotic cocktail (Abx) vs non-antibiotic treated (No Abx).

After obtaining this preliminary data, we are planning to start tumors with or without antibiotic treatments with and without treatment with FSHCER. Antibiotic treatments will have either all three antibiotics or each single antibiotic. After detecting if the effect of antibiotic treatment is direct on tumor progression, in the immunotherapy or both, we will sequence the gut microbiota in the different groups to study if the depletion of a selective bacterial population is responsible of the seen effects.

7   Conclusions

Treatment of ovarian cancer using immunotherapies offers great promise. For being able to develop an immunotherapeutic tool for the treatment of ovarian cancer we focused on FSHR, a specific molecule that we found to be expressed in 56% of epithelial ovarian carcinomas, and the expression of which is associated with a poor prognosis. Given that the target is a receptor, we were able to use its natural ligand (FSH) to redirect the T-cells against it generating a CAR that will be constituted by protein fragments all from the same species, avoiding the chance of an anaphylactic shock.

Once generated, we were able to test and confirm its activation and cytotoxic activity in vitro which was specific towards cells that expressed the FSHR. Accordingly, it was able to delay tumor progression both in an ovarian tumor model and a breast tumor model, which we tested due to the high expression of this receptor in metastasis and vascularization of other tumors.

We then tried to get a deeper understanding of the mechanisms by which CAR therapy elicited its antitumor response. We found that CAR T-cells were able to boost the preexisting endogenous immune system to respond against the tumor. Accordingly, in survival experiments we saw that the adoptive transfer of CD4 T-cells was as effective as transferring a mix of CD4 and CD8 T-cells, whereas the transfer of CD8 T-cells elicited a less pronounced antitumor activity.

The transferred T-cells migrated from the tumor microenvironment to the different lymphoid organs, especially CD4 T-cells and were able to obtain a central memory phenotype. However, in advanced stages of tumor progression, transferred T-cells only remained in the spleen and could no longer be found in the tumor microenvironment.

The tumor microenvironment of ovarian cancer is very immunosuppressive. Therefore, we hypothesized that transferred T-cells were not able to be effectively activated in this microenvironment and unable to kill the tumor cells. When we cocultured the T-cells and FSHR-expressing tumor cells in the presence of cell-free ascites we found a higher activation of the CAR T cells compared to the incubation in media. However, this activation also happened in the presence of cell-free ascites that had been previously incubated with FSHR-expressing tumor cells. Therefore, we hypothesized that proteases in the ascites are causing shedding of the FSHR ectodomain, which is activating the T-cells away from the target tumor cells. This is a novel immunoevasion mechanism that may be relevant at least in T-cell therapies used in the treatment of ascitic tumors.

Finally, the fact that we could use CARs against mouse targets allowed us to study the potential adverse effects that the CAR therapy could have in the clinical setting. We found no clinical evidence of distress in the FSHCAR treated mice, however, we did not expect adverse effects due to the specificity of the distribution of FSHR. We tested a low affinity CAR targeting mesothelin, and analyzed the lymphocytic infiltration of the CAR versus the transfer of non-specific T-cells showing no difference and proving that low affinity CARs against targets which are present in healthy tissues can be a viable option in the immunotherapeutic approach to cancer.

8 Resumen en lengua oficial de la Universidad de Valencia

8.1 Introducción

El cáncer epitelial de ovario es uno de los tumores más letales, matando a más de 14.000 mujeres cada año en los EE.UU. A pesar de los avances en cirugía y quimioterapia, las tasas de supervivencia a 5 años apenas han cambiado en los últimos 40 años. El cáncer de ovario es, sin embargo, un tumor inmunogénico (Curiel et al., 2004, Zhang et al., 2003, Cubillos-Ruiz et al., 2009, Cubillos-Ruiz et al., 2010, Huarte et al., 2008, Nesbeth et al., 2009, Scarlett et al., 2009, Scarlett et al., 2012, Cubillos-Ruiz et al., 2012), y las inmunoterapias suponen una gran esperanza para la reversión de este mal pronóstico (Brayer y Pinilla-Ibarz, 2013, Nelson y Paulos, 2015).

En los últimos años, la transferencia de células T autólogas que expresan receptores de antígenos quiméricos (CAR) han producido una gran proporción de remisiones completas en pacientes con neoplasias hematológicas quimio-resistentes (Porter et al., 2011, Maus et al., 2014, Kalos et al., 2011). Sin embargo, varios obstáculos han impedido hasta ahora el éxito de esta tecnología frente a tumores sólidos, incluyendo el cáncer de ovario. El mayor desafío es la escasez de antígenos específicos expresados en la superficie de las células tumorales que no se comparten con los tejidos sanos, para evitar efectos secundarios intolerables.

El receptor de la hormona folículo estimulante (FSHR) se expresa selectivamente en las mujeres en las células granulosas de ovario (Simoni et al., 1997) y en niveles bajos en el endotelio ovárico (Vannier et al., 1996). Además, este antígeno de superficie se expresa en el 50-70% de los carcinomas de ovario (Zhang et al., 2009, Al-Timimi et al., 1986, Zhang et al., 2013, Minegishi et al., 2000, Nakano et al ., 1989, Parrott et al., 2001, Wang et al., 2003, Zheng et al., 2000). Dado que la ooforectomía es un procedimiento estándar en el tratamiento de cáncer de ovario, redirigir

el sistema inmune contra el FSHR no debería dañar los tejidos sanos. Por lo tanto, FSHR podría ser una diana terapéutica ideal para dirigir las células T contra el cáncer de ovario usando CAR.

Los estudios preclínicos con CAR se han realizado en ratones inmunodeprimidos portadores de injertos de tumores humanos, por tanto no presentan las dianas de los CAR en sus tejidos sanos y no pueden predecir los efectos adversos. Para comprender la interacción entre el CAR, el sistema inmune del huésped y la presencia de la diana del CAR potencialmente expresada en tejidos sanos, generamos CAR totalmente murinos contra dianas presentes en el ratón y los utilizamos en ratones inmunocompetentes. Mientras los CAR se han dirigido típicamente contra antígenos tumorales a través de la inclusión de un fragmento de anticuerpo (scFv), FSHR ofrece la ventaja de tener un ligando natural altamente específico (FSH). Además, la FSH es una molécula endógena, evitando así la inmunogenicidad potencial de los fragmentos de anticuerpo exógenos desarrollados en diferentes especies. Para explotar las interacciones receptor-ligando existentes, hemos desarrollado nuevo receptor quimérico endocrino (CER) que incluyen las dos subunidades enteras de FSH, en marco con un dominio transmembrana y los dominios de señalización intracelular utilizados con éxito en el tratamiento de la leucemia. Para detectar posibles efectos secundarios, generamos un CER totalmente murino que debería atacar cualquier expresión desconocida de FSHR en los tejidos sanos de los ratones portadores de tumores. Además, hemos generado un CAR totalmente murino cuya diana es la mesotelina a través de un scFv clásico para comparar eficacia y toxicidad.

8.2 Objetivos

El objetivo principal de este proyecto es generar un receptor de antígeno quimérico que pueda utilizarse para el tratamiento del cáncer de ovario. Con este fin, estudiamos primero las proteínas

de superficie expresadas en las células de cáncer de ovario. La mesotelina es un antígeno cuya sobreexpresión en cáncer de ovario es bien conocida, sin embargo, su expresión en los tejidos sanos hace que su uso en la inmunoterapia controvertido. Hemos visto que el receptor de la hormona folículo estimulante (FSHR) es un antígeno muy específico, al no expresarse fuera del ovario, y podría utilizarse de forma segura para tratar el cáncer de ovario utilizando CARs. Tras generar los CAR para redirigir las células T contra FSHR, validamos la eficacia del CAR in vitro a través de la capacidad de células T con CAR para activarse y matar células tumorales humanas y murinas que expresen la FSHR. Después, se estudió la capacidad de este CAR para tratar el cáncer de ovario en un modelo de ratón inmunocompetente.

Los objetivos secundarios fueron los siguientes:

- Generar un CAR que no produzca una respuesta anafiláctica. El uso de scFv derivado de ratón para la generación de los CAR causó la muerte en un paciente después de múltiples infusiones debido a un shock anafiláctico. Uno de nuestros objetivos es evitar esto por mediante el diseño de un CAR plenamente humano, sin fragmentos murinos que pudieran provocar una respuesta anafiláctica.

- Estudiar si las células T transferidas son capaces de estimular la respuesta del sistema inmune endógeno. Nuestra hipótesis es que parte del efecto a través del cual los CAR provocan su efecto antitumoral es a través de la potenciación de la respuesta inmune endógena preexistente, a través de la difusión de antígenos que ocurre después de la destrucción de células diana por el CAR. Estudiamos este mecanismo mediante el uso de marcadores leucocitarios congénicos (CD45.1 y CD45.2) que nos permiten diferenciar entre células T adoptivamente transferidas y las células T endógenas. Después del tratamiento, aislamos las células T endógenas y estudiamos su respuesta antitumoral in vivo e in vitro.

- Estudiar posibles mecanismos de fracaso de la inmunoterapia mediante CAR. El microambiente tumoral es típicamente inmunosupresor y puede explicar el fracaso de inmunoterapias en muchas ocasiones. En caso de que no seamos capaces de conseguir rechazar el tumor tenemos la intención de disecar los efectos de los factores del microambiente tumoral en la terapia adoptiva de transferencia de células T. Ejemplos de escape al tratamiento pueden ser la inmunoedición, la sobreexpresión de PD-1 en la superficie de las células T y la supresión por parte del microambiente tumoral debida a factores celulares o solubles.

- Estudio de la distribución de las células T transferidas adoptivamente. Para comprender más profundamente el tratamiento con CAR vamos a sacrificar los ratones con tumor tratados con CAR y estudiar la distribución y el fenotipo de las células T transferidas a diferentes puntos temporales.

- Estudiar los posibles efectos adversos de esta terapia en el paciente. Las investigaciones anteriores con CAR se han hecho con ratones inmunodeficientes con líneas de células tumorales humanas y dirigidos contra antígenos tumorales humanos que no estaban presentes en el ratón, por lo que algunos de los efectos adversos que se han visto a posteriori no eran previsibles. La ventaja de usar un CAR murino es que podemos estudiar, no sólo las interacciones de la terapia CAR con el sistema inmune endógeno, sino también que las moléculas contra las que dirigimos los CAR estarán presente en los tejidos habituales y podremos ser capaces de ver los efectos adversos como lo haríamos en la clínica.

8.3 Material y métodos

Animales y líneas celulares

Los ratones C57BL/6 y Ly5.1 se adquirieron del National Cancer Institute o de Charles River. Los experimentos con animales fueron aprobados por el Comité de Cuidado y Uso de Animales Institucional del Wistar Institute.

Las células ID8 parentales fueron proporcionadas por Katherine Roby (Departamento de Anatomía y Biología Celular de la Universidad de Kansas Medical Center, Kansas City, KS) (Roby et al., 2000) y transducidas con retrovirus para expresar Defb29 y VEGF-A (Conejo-García et al., 2004). Generamos tumores en el flanco axilar o intraperitoneales con ID8-*Defb29/Vegf-a* como describimos previamente (Cubillos-Ruiz et al, 2009). Los tumores se midieron con calipers y calculamos los volúmenes tumorales como $1/2 \times$ (L x W x W), donde L es la longitud (dimensión más larga) y W es la anchura (dimensión más corta). Para generar las células ID8-*Defb29/Vegf-a-Msln*, las células ID8-*Defb29/Vegf-a* fueron transducidas 2 veces con retrovirus MIGR1-mesotelina y seleccionadas mediante la expresión de proteína verde fluorescente (GFP). Para generar las ID8-*Defb29/Vegf-a-Fshr*, las ID8-*Defb29/Vegf-a* fueron transducidas con un retrovirus que contenía pBMN-I-GFP-FSHR y seleccionadas mediante la expresión de GFP.

Diseño de los receptores de antígeno quimérico

Diseñamos los constructos del receptor de antígeno quimérico usando el péptido señal de CD8α murino, seguido de la scFv del anticuerpo anti-mesotelina K1 o una fusión de las subunidades FSHβ y CGα, unidos por un espaciador glicina/serina, seguidos del dominio bisagra y transmembrana de CD8α, y el fragmento intracelular de 4-1BB y CD3z murinos. Pedimos la construcción de Genescript, flanqueado por EcoRI y NotI, y la clonamos en el vector pBMN-I-GFP retroviral. Pedimos las correspondientes secuencias humanas para generar el receptor quimérico contra FSHR humano.

Muestras humanas

Las muestras de carcinoma de ovario humano fueron adquiridos bajo un protocolo aprobado por los comités de ética de la Universidad de Darmouth y The Wistar Institute.

Análisis de los datos del TCGA

Descargamos las secuencias procedientes de muestras de cáncer de ovario del portal de datos TCGA (2015). Los archivos descargados incluyen datos de secuenciación de exones y de resultados integrales de 404 pacientes. Los tumores con valores de expresión de FSHR superiores a la mediana fueron identificados como de expresión alta.

Producción Retrovirus

Generamos retrovirus mediante la transfección de células Eco-Phoenix con pBMN-I-GFP o PBMN-I-GFP-CAR.

Transducción de las células T

Se aislaron las células T de bazos de ratones utilizando un protocolo de selección negativa. Tras lisis de glóbulos rojos usamos partículas magnéticas asociadas a anti-IgG de rata junto con los siguientes anticuerpos: 2.4G2, M170.13, RA3 y M5114 que se unen a todos los esplenocitos excepto a las células T y nos permiten aislarlas. Las células T purificadas se resuspendieron en placas de cultivo en RPMI 1640 suplementado con 10% FBS, penicilina, estreptomicina, L-glutamina, 20 U/ml de IL-2 (Peprotech), 1μg/ml de IL-7 (Peprotech) y 2 μg/ml de Concanavalina-A. Después de 36 horas se resuspenden las células T en un millón de células/ml en el sobrenadante viral, con IL-2, IL-7 y 8μg/ml de Polybrene y se centrifugan durante 90 min a 32ºC y 1750rpm. Ocho horas más tarde, se resuspenden las células en RPMI que contiene 10% de FBS IL-2 e IL-7

para eliminar el Polybrene. Después de 48 horas post-centrifugación separamos las células T infectadas por citometría de flujo para realizar los ensayos.

Alternativamente, después de la lisis de glóbulos rojos se resuspenden los esplenocitos a 2 millones de células/ml en una placa de 24 pocillos con 50 U/ml de IL-2 (Peprotech), 1µg/ml de IL-7 (Peprotech) y 50µl/ml de anti- ratón de partículas asociadas a anti-CD3/anti-CD28 (Invitrogen). Se realizan dos centrifugaciones a las 18 y 36 horas en placas recubiertas de RetroNectin (Takara) y quitamos magnéticamente las particulas anti-CD3/anti-CD28 en el día 4 después de establecer el cultivo. Contamos el número de células T cada 2 días y añadimos RPMI IL-2 IL-7 para mantener una concentración de 1 millón de células/ml. En el día 7 las células T se seleccionan por la expresión de GFP y se utilizan para los diferentes ensayos.

Ensayo de citotoxicidad

Incubamos 10000 ID8-*Defb29/Vegf-a-Fshr* en una placa de fondo plano de 96 pocillos. Antes de añadir las células T, lavamos el medios acondicionado por el tumor y añadimos medio fresco sin beta-mercapto etanol y el número apropiado de células T por pocillo (en 200uL). Después de 18 horas se recogieron las células T y las células tumorales y se tiñeron para Anexina V y Zombie Yellow o 7-AAD (Biolegend).

Determinación de interferón-γ

Medimos el interferón-γ en el sobrenadante del cocultivo entre células tumorales y linfocitos T mediante ELISA (Biolegend) y ELISPOT (eBioscience) siguiendo las instrucciones del fabricante.

Citometría de flujo

Utilizamos un citómetro de flujo BD LSRII o "cell sorter" BD FACSAria (BD Biosciences). Los anticuerpos anti-ratón utilizados estaban directamente fluorocromo-conjugados. Utilizamos: anti-CD3e (17A2), CD4 (RM4-5), CD8b (YTS156.7.7), CD45 (30-F11), CD45.1 (A20), CD45.2 (104), CD44 (IM7), CD69 (H1.2F3), CD62L (MEL-14), PD-1 (29F.1A12) (todos de Biolegend o Tonbo Biosciences), mesotelina (K1, desde Santa Cruz Biotech).

Immunoblot

Disociamos mecánicamente muestras de tumor de ovario humano congeladas. La extracción de proteínas, la desnaturalización y transferencia se realizaron como se ha descrito previamente (Rutkowski et al., 2015). Las membranas se incubaron con anti-FSHR (H-190, Santa Cruz Biotech) y anti-β-actina (a5441, Sigma-Aldrich).

Inmunohistoquímica

El corazón y los pulmones de ratones tratados se recogieron y conservaron en OCT (Tissue-Tek) y se congelaron. Después se hicieron secciones a partir de bloques de tejido congelado. Los portaobjetos se fijaron a continuación con acetona y se lavaron con PBS. Las secciones se bloquearon a continuación utilizando α-CD32 seguido por tinción con α-CD45 biotinilado (30-F11) y la finalizamos el procedimiento de acuerdo con las instrucciones del fabricante (Vector Labs).

PCR cuantitativa en tiempo real

Aislamos ARN de tejidos de muestras congeladas mediante destrucción mecánica y realizmos transcripción reversa a ADN copia. Medimos la expresión relativa de CD3e murino (cebadores 5'-ACGTACTTGTACCTGAAAGCTC-3 'y 5'-CCTTCCTATTCTTGCTCCAGT-3'), CD45 (cebadores 5'-TGAAGAAGAGAGATCCACCCA-3 'y 5'-TTTCCAATGTGCTGTGTCCT-3') y GAPDH (cebadores 5 '-CCTGCACCACCAACTGCTTA-3' y 5'-AGTGATGGCATGGACTGTGCT-3 ') utilizando SYBR Green (Applied Biosystems) en un Sistema de PCR ABI 7500 Fast en tiempo real (Applied Biosystems).

Análisis estadístico

A menos que se indique lo contrario, todos los experimentos se repitieron al menos dos veces y los resultados fueron similares entre las repeticiones. En los experimentos con animales utilizaron entre 3 y 6 ratones por grupo. Las diferencias entre las medias de los grupos experimentales se calcularon utilizando la prueba t de Student no pareada de dos colas. Las barras de error representan el error estándar de la media a partir de muestras independientes ensayadas dentro de los experimentos representados. Las tasas de supervivencia se compararon mediante la prueba de log-rank. Los experimentos de crecimiento tumoral se compararon mediante el cambio de pendiente a través de un análisis de regresión lineal. Todos los análisis estadísticos se realizaron utilizando Graph Pad Prism 5.0. Un valor de $p < 0,05$ fue considerado estadísticamente significativo.

8.4   Resultados

Los receptores endocrinos quiméricos redirigen las células T eficazmente frente al carcinoma de ovario humano y murino

Para entender la relevancia de FSHR como un diana en el tratamiento del cáncer de ovario, primero analiza datos de la base de datos del TCGA, que tiene 404 carcinomas de ovario serosos de alto grado. Se encontró que el 56,5% de los cánceres de ovario expresan FSHR. Además, la expresión elevada de FSHR es un factor pronóstico negativo en cáncer de ovario (Figure 4.1.1 Overexpression of FSHR is a poor prognostic factor in ovarian cancer.), lo que sugiere que FSHR es una buena diana terapéutica para tumores agresivos. Confirmamos la expresión generalizada de FSHR con el análisis de Western-blot de tumores de ovario estadio III / IV seleccionados al azar de nuestro banco de tumores (Rutkowski et al., 2015, Stephen et al., 2014), que identificó la presencia de FSHR en ~ 70% de los tumores (Figure 4.1.2 Approximately 66% of ovarian carcinomas in our tumor bank express FSHR), el 25% de las cuales exhiben niveles similares o mayores que las células OVCAR-3, un control positivo bien definido (Nakano et al., 1989, Choi et al., 2004).

Para utilizar el FSHR como diana mediante lar redirección de las células T, generamos un receptor quimérico humano usando la longitud completa de las dos subunidades de la hormona FSH humana, unidas por un espaciador glicina/serina, en marco con un dominio transmembrana y el dominio intracelular de 4-1BB, y CD3ζ (FSHCER; Figure 4.1.3). A diferencia de los receptores quiméricos que emplean fragmentos pequeños de hormonas (Urbanska et al., 2015), el FSHCER transducido en células T de donantes HLA-A2 + (para minimizar la reacción alogeneica) causó en células OVCAR-3 una citotoxicidad dosis-dependiente, selectiva y eficaz, en comparación con células T transducidas con un vector control (Figure 4.1.4).

Para definir el potencial del FSHCER para tratar tumores ováricos establecidos en modelos inmunocompetentes en presencia no restringida de FSHR en los tejidos sanos, como sucedería en

el ámbito clínico, generamos un FSHCER murino con los homólogos murinos de todos los dominios expresadas en células T humanas. Como era de esperar, las células tumorales ID8-*Defb29/Vegf-a*, un modelo de cáncer de ovario agresivo diseñado con una acelerada producción de carcinomatosis peritoneal y ascitis in vivo (Cubillos-Ruiz et al., 2012, Cubillos-Ruiz et al., 2009, Scarlett et al., 2009, Stephen et al., 2014), provocó la secreción robusta de IFN-γ por células T con FSHCER murino tras la expresión ectópica de FSHR, pero no en la ausencia de expresión FSHR (Figure 4.1.5). La respuesta específica del FSHCER se mantuvo cuando las células T CD4 y CD8 se estimularon de forma independiente (Figure 4.1.6). Al igual que con sus homólogos humanos, las células T de ratón con FSHCAR mataron específicamente células tumorales que expresan FSHR de una manera dosis-dependiente (Figure 4.1.7 y Figure 4.1.8). Tomados en conjunto, estos resultados indican que los receptores quiméricos utilizando subunidades enteras de la hormona FSH pueden redirigir eficazmente la actividad citotóxica de las células T contra carcinomas de ovario que expresan FSHR, que representan a la mayoría de los tumores humanos.

Las células T que expresan receptores quiméricos potencian la inmunidad antitumoral endógena pre-existente

A diferencia de los ratones inmunodeficientes, el uso del modelo de ratón inmunocompetente nos permite disecar la interacción de las células T con receptores quiméricos con un sistema inmunológico intacto. Tenemos la hipótesis de que la actividad citotóxica específica de las células T con FSHCER da lugar a difusión de antígenos lo que permite una disminución de la carga inmunosupresora, lo que podría tener un efecto significativo sobre la inmunidad preexistente antitumoral. Para probar esta hipótesis, se trataron diferentes cohortes de ratones CD45.2[+] con tumores ID8-*Defb29/Vegf-a-Fshr* con células T congénicos CD45.1[+] transducidas con FSHCER

o vehículo (PBS). Entre una y dos semanas después del tratamiento extrajimos las células T endógenas y las realizamos con ellas un ELISPOT de interferón γ. Como se muestra en la Figure 4.2.1, la frecuencia de células T endógenas productoras de IFN-γ que responden a células dendríticas pulsadas con doblemente (UV + γ) irradiadas ID8-*Defb29/Vegf-a-Fshr* era dramáticamente mayor en los ratones tratados con FSHCER. Además, esta respuesta de las células T endógenas superior era suficiente para retrasar la progresión tumoral, ya que la transferencia adoptiva de células T endógenas del bazo después del tratamiento con FSHCER en ratones singénicos resultó en el desarrollo de tumores más pequeños al volver inyectar los mismos con ID8-*Defb29/Vegf-a-Fshr*, en comparación con el efecto de las células T de ratones tratados con el tratamiento control (Figure 4.2.2).

Para determinar si este aumento en la inmunidad antitumoral endógena es un efecto general de las células T redirigidos contra los tumores por los receptores quiméricos, generamos otro CAR totalmente murino. Nos centramos en la mesotelina, una glicoproteína que está sobreexpresada en 66-93% carcinomas de ovario no mucinosos (Ordonez, 2003, Chang et al., 1992).

Vimos que el anticuerpo K1, un anticuerpo que reconoce la mesotelina humana con baja afinidad (Chang and Pastan, 1996), también se une a la mesotelina ratón en células ID8-*Defb29/Vegf-a* transducidas con mesotelina (Figure 4.2.3). Además, el anticuerpo K1 reconoce la mesotelina ratón, pero no el control (albúmina) mediante ELISA (Figure 4.2.4). Por lo tanto, generamos un nuevo CAR murino utilizando el scFv de K1 (Figure 4.2.5), y utilizamos células T transducidas para tratar ratones portadores de tumores ID8-*Defb29/Vegf-a* que expresan mesotelina. Al igual que con el FSHCER, la administración de células T con K1CAR indujo un aumento significativo en la actividad endógena antitumoral de las células T, detectada a través ELISPOT (Figure 4.2.6). Del mismo modo, la transferencia adoptiva de células T derivadas de ratones tratados con K1CAR

fue suficiente para retrasar la progresión tumoral (Figure 4.2.7). Tomados en conjunto, estos resultados indican que la actividad citotóxica de las células T redirigidas contra las células tumorales con receptores quiméricos aumenta la actividad antitumoral de las células T endógenas que deberían ser capaces de retrasar la progresión maligna, independientemente de si los tumores pierden los antígenos contra los que están dirigidos los receptores quiméricos a través inmunoedición.

Las células T que expresan el receptor quimérico retrasan la progresión maligna en ratones inmunocompetentes con cáncer de ovario

Dada la capacidad de ataque de las células T con FSHCER para matar a las células tumorales que expresan FSHR in vitro, y la potenciación de la inmunidad endógena antitumoral in vivo, decidimos determinar el potencial terapéutico de estas células en ratones inmunocompetentes con tumores ID8-*Defb29/Vegf-a*. Dos inyecciones de 1-1.5x106 células T que expersaban el FSHCER en los días 7 y 14 después de la inyección del tumor fueron suficientes para prolongar significativamente la supervivencia en este agresivo modelo ortotópico (Conejo-Garcia et al., 2004, Cubillos-Ruiz et al., 2012, Cubillos-Ruiz et al., 2009, Nesbeth et al., 2010, Rutkowski and Conejo-Garcia, 2015, Scarlett et al., 2009, Stephen et al., 2014), en comparación el tratamiento con células T control en múltiples experimentos independientes (Figure 4.3.1). No se observaron efectos adversos obvios en estos experimentos.

Debido a que la expresión de FSHR también se ha reportado en ciertas lesiones metastásicas (37), para demostrar la aplicabilidad general del FSHCER, también tratamos tumores de mama murinos altamente agresivos transducidas-FSHR (Rutkowski et al., 2014) que crecen en el flanco axilar.

Apoyando amplio potencial terapéutico, las células T con FSHCER eran aún más eficaces en freanr el crecimiento tumoral en esta ubicación adicional (Figure 4.3.2).

A pesar de los efectos terapéuticos reproducibles el FSHCER no consiguió inducir el rechazo completo de estos tumores. Para comparar la eficacia de la hormona contra el scFv, tratamos ratones portadores de tumores ID8-*Defb29/Vegf-a* que expresan mesotelina con células T transducidas con K1CAR o células T control. Como se muestra en la Figure 4.3.3, la expresión de K1CAR retrasa la progresión maligna en un grado similar al FSHCER.

Para entender la contribución relativa de los diferentes subtipos de células T a la eficacia antitumoral del FSHCER, tratamos diferentes cohortes de ratones con ID8-*Defb29/Vegf-a*, ya sea con FSHCER en CD4 o CD8 solos o en combinación. Curiosamente, las células T CD4 que expresan receptores quiméricos eran tan eficaces como una mezcla de linfocitos no segregados, mientras que CD8 células T inducidas por sí solo presentaron un menor beneficio terapéutico (Figure 4.3.4). Una tendencia similar se observó con las células T K1CAR transducidas. En conjunto, nuestros resultados indican que las células T redirigidas a través de receptores quiméricos que se aprovechan de la afinidad natural de una hormona por su receptor pueden ser al menos tan eficaz como los CAR redirigidos mediante scFv. Además, las células T CD4 son los principales contribuyentes a los beneficios terapéuticos, aunque son incapaces de inducir el rechazo total de los tumores epiteliales establecidos.

Las células T que expresan receptores quiméricos persisten en ausencia de inmunoedición
Para entender los mecanismos que podrían limitar la eficacia de las células T que expresan el receptor quimérico in vivo, estudiamos si las células tumorales podrían perder las moléculas de superficie mediante un mecanismo de inmunoedición. Las células T con FSHCER reaccionaron

de manera semejante en cuanto a secreción de IFN-γ en respuesta a las células tumorales transducidas-FSHR aisladas desde la cavidad peritoneal de los ratones tratados previamente con células T transducidas con vector control, FSHCER o PBS (Figure 4.4.1), lo que indica la persistencia del FSHR en las células tumorales.

Para determinar si las células T con receptores quiméricos persisten en los ratones tras el tratamiento, utilizamos el marcador congénico CD45.1 y sacrificamos los ratones tratados a días 5, 10 y 15 después de la administración intraperitoneal de las células T FSHCER. Cinco días después de la administración de las células T, vimos un pico de células T FSHCER en la cavidad peritoneal, con proporciones similares de CD8 frente a los CD4 (Figure 4.4.2 y Figure 4.4.3). Ambas poblaciones estaban activadas (CD44 + CD69 +) en el microambiente tumoral (45,5% y 3,1%, respectivamente, a día 5) (Figure 4.4.4). Sin embargo, proporciones y números absolutos de las células T transferidas disminuyeron en los días 10 y 15, acompañados de un incremento de células T CD4 en relación a CD8, y retención de los marcadores de activación (Figure 4.4.2-Figure 4.4.4). En bazo y ganglios linfáticos de drenaje tumoral, observamos un pico de acumulación en día 10, aunque ya había células presentes a día 5. Estas células T son en su mayoría CD4 + (90,5%) con un fenotipo de memoria central (CD44 + CD62L +) (Figure 4.4.5) y representaban 0,8% de las células T totales. En las etapas terminales de progresión tumoral, las células T con FSHCER todavía eran detectables en el bazo (0,6% de las células T totales) (Figure 4.4.6). En esta etapa, sin embargo, no hay células T con FSHCER en la ascitis tumoral o la médula ósea.

Tampoco el agotamiento parecce un importante factor que explique el colapso de las células T que expresan el receptor quimérico porque sólo una minoría de las células T (un 8% en CD8 y el 31% en las células CD4) expresa PD-1 en la superficie celular (Figure 4.4.7).

Para determinar si los factores inmunosupresores en ascitis tumoral podría inducir la inhibición de las células T, coincubamos ID8-*Defb29/Vegf-a-Fshr* y las células T con FSHCER en presencia de cantidades crecientes de líquido ascítico derivado del tumor. Inesperadamente, la ascitis acelular aumentó la reactividad (secreción de IFN-γ) de las células T con FSHCER contra su objetivo de una manera dosis-dependiente (Figure 4.4.8). Pensamos que esto es el resultado de reconocimiento de FHSR procedente de las células tumorales en la ascitis través de la liberación reciente desde la superficie de las células tumorales por diversas razones. En primer lugar, las células T transducidas con el vector control no reaccionaron contra las células tumorales FSHR + en presencia de ascitis. Además, si cultivamos la ascitis con células T en ausencia de células tumorales, la ascitis sólo potencia el incremento de la reactividad de las células T con FSHCER si ha sido incubada previamente con células tumorales, pero no de manera directa o cuando el medio de cultivo no ascítico ha sido preincubado con células tumorales. Esto último que indica que es necesaria la liberación aguda de FSHR a partir de células tumorales activadas por líquido ascítico para la activación específica del FSHCER (Figure 4.4.9).

En conjunto, estos resultados indican que las células T con FSHCER persisten como células de memoria en los ratones portadores de cáncer de ovario tratados pero finalmente desaparecen del microambiente tumoral. La liberación a la ascitis de la diana terapéutica provoca la activación de células T distalmente a las células tumorales, probablemente contribuyendo a la ausencia de células T transferidas en la ascitis a largo plazo, a pesar de la aparente falta de agotamiento y la persistencia de las células T en los órganos linfoides.

El tratamiento con FSHCAR y K1CAR es seguro

Una preocupación importante con respecto al uso de inmunoterapias con CAR es la aparición de efectos en las moléculas diana que se encuentran en tejidos sanos. Apoyando el patrón restringido de expresión de FSHR, no observamos toxicidad evidente tras la transferencia de células T FSHCER, en términos de pérdida de peso o cualquier signo de malestar.

Debido a la mesotelina se expresa en la pleura, el pericardio y el peritoneo (33), estudiamos los posibles efectos adversos sobre la transferencia adoptiva de células T con K1CAR, que tienen la capacidad de reconocer la mesotelina de ratón. Una vez más, los ratones tratados no mostraron efectos adversos evidentes durante el seguimiento. No encontramos diferencias en la infiltración de leucocitos totales (CD45 +) en la pleura y el pericardio de ratones tratados con células T K1CAR, en comparación con los ratones control idénticamente tratados con las células T control por inmunohistoquímica (Figure 4.5.1). Además, no detectamos diferencias en los niveles de expresión de ARNm de ambos CD45 y CD3 entre K1CAR o control por q-PCR (Figure 4.5.2).

En general, nuestros resultados demuestran que, aunque con una eficacia limitada debido a factores procedentes del microambiente tumoral, las células T re-dirigidas contra las células de cáncer de ovario con hormonas o scFv inducen beneficios terapéuticos medibles con una toxicidad insignificante, a pesar de la expresión de antígenos específicos en los tejidos libres de tumor.

8.5   Discusión

Aquí demostramos que las células T que expresan diferentes receptores quiméricos median su actividad terapéutica, en parte, al aumentar la inmunidad antitumoral endógena, que es capaz de retrasar la progresión maligna. Además, el uso de las dos subunidades de la hormona FSH redirige de manera eficaz la actividad citotóxica de las células T contra las células de cáncer de ovario,

resultando en beneficios terapéuticos reproducibles en diferentes modelos preclínicos, aunque con un aumento de supervivencia limitado en caso de la ascitis por la activación distal debida a la probable secreción de molélulas diana en el microambiente tumoral.

Nuestro estudio demuestra por primera vez que las células T endógenas potenciadas por los CAR contribuyen a la eficacia antitumoral. Estudios recientes mostraron que los CAR pueden inducir difusión de antígenos y elevar los títulos de anticuerpos en pacientes (Beatty et al., 2014). Nuestro trabajo en modelos preclínicos inmunocompetentes amplia estos mecanismos al demostrar que los CAR son capaces de aumentar la actividad de las células T endógenas, que es suficiente para retrasar la progresión tumoral. Estos efectos pueden ser importantes en los tumores sometidos a pérdida de antígenos específicos a través inmunoedición o supresión de las células T transferidas, porque las respuestas policlonales del sistema inmune endógeno proporcionan presión inmune adicional contra la progresión maligna. Curiosamente, no observamos pérdida de antigenicidad en nuestros tumores en fase terminal, a pesar de que las células T con FSHCER se persistieron hasta que los ratones portadores de tumores sucumbieron a la enfermedad terminal. Aunque otros autores han mostrado que los CAR pueden ser inactivados de forma reversible en los tumores sólidos de una manera PD-1-dependiente (Moon et al., 2014), en nuestros tumores de ovario se observó que la expresión de PD-1 sólo ocurría en una minoría de células transferidas. Sin embargo, encontramos que la liberación de moléculas específicas de la superficie de las células tumorales en el líquido ascítico es suficiente para desviar la actividad efectora de las células T que expresan el receptor quimérico lejos de las células tumorales. Por lo tanto, nuestros resultados revelan un nuevo mecanismo de evasión inmune inducida por el tumor que debe ser considerado para, al menos, el tratamiento de pacientes con cáncer de ovario.

Otro aspecto interesante de nuestro estudio es que, a pesar de excelente actividad citotóxica in vitro, y aunque las células T con FSHCER provocaron aumentos de supervivencia reproducible de forma significativa en los ratones con cáncer de ovario agresivo, todos los ratones tratados finalmente sucumben a la enfermedad. Dado que el cáncer de ovario es una enfermedad con muy mal pronóstico, para estos experimentos de prueba de concepto limitamos tratamientos a dos inyecciones y a tumores particularmente agresivos. Los efectos podrían ser aumentadas mediante inyecciones adicionales de células T, o por la combinación con otras intervenciones inmunoterapéuticas (Wang et al., 2014). Por tanto, es probable que las intervenciones combinatorias e inmunoterapias aplicadas después de la resección quirúrgica aumentará aún más la supervivencia de pacientes con cáncer de ovario que reciben tratamientos similares. Nuestros resultados proporcionan un fundamento para estas intervenciones porque no observamos ninguna toxicidad apreciable en el curso de nuestros tratamientos. Esto se esperaba para el FSHCER porque el único tejido sano donde se ha encontrado FSHR es el ovario (Simoni et al., 1997). Sin embargo, también encontramos una ausencia de efectos tóxicos e inflamación con el uso de un CAR de baja afinidad para la mesotelina.

El requisito de la presencia de células T CD4 para lograr un retraso más efectivo en la progresión tumoral apoya la relevancia de la potenciación en la respuesta endógena en la actividad antitumoral de los CAR. Tanto las células T CD4 y CD8 pueden provocar una actividad citotóxica similar, sin embargo, las células T CD4 pueden ser además capaces de orquestar una respuesta antitumoral endógena eficaz.

La mesotelina se expresa en pericardio, pleura y peritoneo sanos. Hoy en día, se está utilizando como diana terapéutica para el uso de CAR en diferentes tumores (Adusumilli et al., 2014, Luna et al., 2014, Beatty et al., 2014) y existe la preocupación por la aparición potencial de efectos

adversos en este tejido sano. Hasta el momento, no se han publicado efectos adversos específicos letales, pero la hipofunción multifactorial de las células T en el microambiente de tumores muy avanzados también ha limitado la eficacia del CAR (Luna et al., 2014). Si se optimiza el tratamiento con CAR para inducir el rechazo de tumores y que persistan las células T en los pacientes tratados, es posible que la toxicidad se convertirá en un problema. Esta podría disminuir con la adición de un gen suicida (Hoyos et al., 2010), pero la protección a largo plazo contra las recurrencias se eliminaría. Por tanto, es posible que los scFv de baja afinidad (tales como el clon K1 utilizado en este estudio) (Chang y Pastan, 1996)) ofrezcan una mejor relación entre la eficacia y baja toxicidad, por dirigirse a las células (tumor) que sobreexpresan la diana terapéutica sin afectar a las células sanas que expresan niveles más bajos.

En general, nuestro estudio demuestra la eficacia de los receptores quiméricos para la activación de las células T utilizando hormonas y da a conocer mecanismos de la actividad y supresión de estas intervenciones. Estos datos deben ayudar a la comprensión de efectos previamente desconocidos en la realización de ensayos clínicos en curso y la optimización de los futuros.

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