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GALECTIN-9 PROMOTES AN IMMUNE-SUPPRESIVE MICROENVIRONMENT IN GASTRIC CANCER

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Dedicated to my loving grandparents,

Mrs. Cecilia Garay (*R.I.P*), Mrs Meg Clark, Mr Angus Clark (*R.I.P*),

Rev. Leonora Hill and Prof. Peter Hill PhD.

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Index

Figure Index

Abbreviations

AP-1	Activator Protein 1
CD127	Cluster of Differentiation 127
CD137	Cluster of Differentiation 137
CD25	Cluster of Differentiation 25
CD28	Cluster of Differentiation 28
CD3	Cluster of Differentiation 3
CD4	Cluster of Differentiation 4
CD44	Cluster of Differentiation 44
CD8	Cluster of Differentiation 8
CEACAM1	Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1
CIN	Chromosomal Instability
CMV3	Cytomegalovirus Vector 3
CRDs	Carbohydrate Recognizing Domain
CTLA-4	Cytotoxic T-Lymphocyte Associated Protein 4
DC	Dendritic Cells
EBV	Epstein Barr Virus
ECM	Extra-Cellular Matrix
ELISA	Enzyme-Linked Immune-Sorbent Assay
EMT	Epithelial to Mesenchymal Transition
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration, USA
FGL	Forssman Glycosphingolipid
Gal-9	Galectin-9
GC	Gastric Cancer
GLUT-2	Glucose Transporter 2
GS	Genomic Stable
HCV	Hepatitis C Virus
HMGB1	High Mobility Group B1
HR	Hazard Ratio
HSV-1	Herpes Simplex Virus 1
HUVEC	Human Umbilical Cord Endothelial Cells
IAV	Influenza A Virus

ICB	Immune Checkpoint Blocker
IDO	Indoleamine 2,3-Dioxygenase 1
IFNγ	Interferon gamma
IgE	Immunoglobulin E
IgV	Immunoglobulin Variable domain
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-17	Interleukin 17
IL-1B	Interleukin 1 beta
IL-2	Interleukin 2
IL-35	Interleukin 35
IL-6	Interleukin 6
LPS	Lipopolysaccharides
MCP-1	Monocyte Chemo-attractant Protein 1, also known as C-C motif ligand 2 (CCL2)
MIP-2	Macrophage Inflammatory Protein-2
MSI	MicroSatellite Instability
NFkB	Nuclear Factor-kappa B
NFL6	Nuclear Factor for IL-6 expression
ORR	Objective Response Rate
OS	Overall Survival
PBMCs	Peripheral Blood Mononuclear Cells
PD-1	Programmed cell Death 1
PDI	Protein Disulfide Isomerase
PDL-1	Programmed cell Death Ligand 1
PDL-2	Programmed cell Death Ligand 2
PFA	Paraformaldehyde
PFS	Progression Free Survival
PI3K	Phosphatidylinositol 3-Kinase
PKC	Protein Kinase C
PMA	Phorbol 12-Myristate 13-Acetate
PstdSer	Phosphatidylserine
RA	Rheumatoid Arthritis
rhGal-9	recombinant human Gal-9

RPMI	Roswell Park Memorial Institute
SFM	Serum Free Media
SH2	Src Homology 2
TCF1	T cell- specific transcription factor 1 (also known as TCF7)
TCGA	The Cancer Genome Atlas
TCR	T Cell rReceptor
Th1	T helper 1
Th17	Tc helper 17
Th2	T helper 2
TILs	Tumour Infiltrating Lymphocytes
Tim-3	T-Cell Immunoglobulin And Mucin Domain-Containing Protein 3
TMB	Tumor Mutational Burden
TNFa	Tumour Necrosis Factor alpha
TOX	Thymocyte selection- associated high mobility group box protein
TRM	Tissue Resident Memory T cell
VEGF	Vascular Endothelial Growth Factor

Resumen

Las inmunoterapias con anticuerpos bloqueadores de proteínas inmuno-reguladoras o '*immune checkpoint*s' han revolucionado el tratamiento del cáncer gástrico (GC), particularmente los anticuerpos contra el receptor PD-1. Sin embargo, la presencia de un microambiente inmunosupresor y la redundancia entre proteínas inmuno-reguladoras podría ser responsable por el alto porcentaje de pacientes que no presenta un beneficio clínico. Una proteína imuno-reguladora emergente es el receptor Tim-3, una proteína de membrana capaz de promover inmunosupresión. A pesar de que este receptor ha adquirido recientemente relevancia en el campo de la inmuno-oncología, la expresión y rol de su ligando Galectina-9 (Gal-9) en GC aún no han sido determinados.

Se evaluó el patrón de expresión de Tim-3 y Gal-9 en el transcriptoma adenocarcinomas gástricos de la base de datos TCGA mediante análisis bioinformáticos (TIMER©, Cibersort y Xcell). Para evaluar el rol de Gal-9 en células de cáncer se transfectaron líneas celulares AGS y GES-1 y se evaluó el efecto de Gal-9 sobre su viabilidad, migración, invasión y expresión de proteínas inmuno-reguladoras. También se utilizó Gal-9 recombinante (rhGal-9) para tratar células endoteliales de cordón umbilical humanas (HUVEC) y evaluar su efecto sobre la capacidad angiogénica de estas células. Finalmente se evaluó el efecto de rhGal-9 sobre cultivos primarios de linfocitos T en presencia o ausencia de anticuerpos bloqueantes de los receptores inmuno-reguladoras PD-1 y Tim-3. Se evaluó la frecuencia de las poblaciones de células CD8+ disfuncionales y de células T reguladoras (Treg).

Los análisis de la base de datos TCGA demostraron que los niveles de mRNA de Gal-9 y Tim-3 están aumentados en STAD en relación a tejido gástrico sano. Los niveles de Tim-3 también presentaron un incremento significativo en STAD invasivos. También se observó que Gal-9 y Tim-3 presentan una correlación con otras proteínas inmuno-reguladoras como PDL-1 (el ligando de PD-1), resultado que fue posteriormente confirmado por nuestros experimentos in vitro donde la transfección de Gal-9 aumentó la expresión de su receptor Tim-3 y de PDL-1 en células de GC. Consistente con el aumento de los niveles de Tim-3 en tumores avanzados, Gal-9 incrementó la migración e invasión de las células AGS y GES-1, así como también la migración y angiogénesis in vitro en HUVEC. También a través de análisis bioinformáticos observamos una fuerte correlación positiva entre los niveles de Gal-9 y la firma molecular de células $CD8^+$ disfuncionales y Treg infiltrantes de tumores, sugiriendo que Gal-9 podría tener un rol en el aumento de la disfunción de células $CD8^+$ y en la expansión de Treg en el tumor. A través de experimentos in vitro, observamos que tanto rhGal9 como células de cáncer transfectadas con Gal-9 aumentan la frecuencia de las poblaciones disfuncionales $PD1^+Tim\text{-}3^+$ y $PD1^+Tim\text{-}3^+LAG3^+$, así como de la frecuencia de Treg de manera Tim-3 dependiente. Estos resultados sugieren una asociación entre la vía de Gal-9/Tim-3 y tumores más agresivos con un microambiente más inmunosupresor. Dado que la disfunción de células T ha sido asociada a la resistencia a inmunoterapia, evaluamos si es que Gal-9 pudiera ejercer efectos similares en presencia de un anticuerpo αPD1. Como era esperado, el αPD1 disminuyó las poblaciones disfuncionales, sin embargo no en presencia de rhGal-9. Esto indica que Gal-9 puede promover el fenotipo disfuncional a pesar del bloqueo de PD1, lo cual apoya el uso de estrategias combinatorias utilizando bloqueadores de PD1 y Tim-3 en la clínica. Debido a que Gal-9 es una proteína secretada y detectable en plasma, proponemos que sus niveles plasmáticos podrían servir como un biomarcador para la inmunoterapia combinada (αPD1 + αTim3). Más aún, un aumento en los niveles circulantes de Gal-9 en pacientes recibiendo la monoterapia con αPD1 podría indicar mayor probabilidad de resistencia adquirida y momento para iniciar la terapia combinada.

Abstract

The development of immune checkpoint inhibitors is revolutionizing gastric cancer (GC) therapy. However, the presence of an immune-suppressive tumor microenvironment and redundancy between inhibitory immune checkpoints may be responsible for why a high percentage of patients do not show clinical benefit. An emerging checkpoint target is Tim-3, a membrane protein that enhances immunosuppression. Despite this, the expression and the role of its ligand Galectin-9 (Gal-9) in GC is still undeciphered. We performed a bioinformatic analysis of stomach adenocarcinoma (STAD) from the TCGA database (TIMER©, Cibersort and Xcell were used). To access the potential role in GC in the tumor microenvironment, Gal-9 was transfected or recombinant Gal-9 (rhGal-9) was administrated to GC cell line AGS and Human Umbilical Vein Endothelial Cells (HUVECs), with biological effects assessed by migration, invasion and Matrigel tube formation assay. Western blotting assessed Tim-3 expression and PDL-1 expression. T cell primary cultures were treated with rhGal9 or co-cultured with Gal-9 transfected cancer cells and the frequency of dysfunctional $CD8^+$ populations and Tregs was evaluated in the presence or absence of Tim-3 and PD1 blocking antibodies.

Results from TCGA analysis demonstrated that both Gal-9 and Tim-3 are upregulated in gastric tumors. Tim-3 mRNA is significantly increased in invasive adenocarcinomas. A positive correlation was observed between Gal-9, PDL-1 and Tim-3, which were consistent with *in vitro* experiments, where increased expression of Gal-9 resulted in increased PDL-1 and Tim-3. In addition, Gal-9 presence *in vitro* increased cancer cell migration and invasion, and promoted HUVEC migration and angiogenesis. Further bioinformatic analysis revealed strong positive correlation of Gal-9 with $CD8^+$ T cell dysfunction and tumour infiltrating Treg molecular signatures, suggesting that in GC Gal-9 could promote T cell dysfunction and Treg expansion. Through in vitro experiments we demonstrated that rhGal-9 or Gal9 expressing cancer cells significantly increased the dysfunctional populations of $CD8^+$ T cells $PD1^+Tim3^+$ and $PD1^+Tim3^+LAG3^+$ in Tim-3 dependent manner. Furthermore, Gal-9 expanded Tregs *in vitro,* which was also prevented by the addition of a Tim-3 blocking antibody (αTim-3). These results suggest an association between the Gal-9/Tim-3 pathway with a more aggressive and increased immune suppressive microenvironment. Since T cell dysfunctionality has been associated to immunotherapy resistance, we evaluated if Gal-9 could exert the same effects in the presence of a PD1 blocking antibody (αPD1). As anticipated, αPD1 treatment reduced $CD8^+$ dysfunctional populations, however, this did not occur in the presence of Gal-9. This suggests that Gal-9 promotes T cell dysfunction regardless of PD-1 blockade and lends support for the clinical use of a combinatory strategy using αPD1 + αTim3 for cancer treatment. Moreover, as Gal-9 is a secreted protein and thus detectable in plasma, we propose that a blood test may serve as an effective companion diagnostic for the prescription of αPD1 + αTim-33 combinatory immunotherapy. Furthermore, an increase in circulating Gal-9 may signal when to initiate this combinatory therapy after initial αPD1 monotherapy.

Introduction

I. Overview of the cancer immune response

The cancer immune response or cancer-immunity cycle can be summarized in seven steps, beginning with the exposure of neoantigens by cancer cells to the elimination of these cells by T cells. First, neoantigens, which are mainly mutated proteins, are released and then captured by dendritic cells (DC) for processing. DC uptake of antigens accompanied by immunogenic signals such as Damage Associated Molecular Patterns (DAMPs) and pro-inflammatory cytokines will enable DC activation and migration to the draining lymph nodes. Here, DC present the neoantigens to T cells by exposing them on Major Histocompatibility Complexes (MHC) I and II, thereby activating effector T cell (Teff) responses against the cancer-specific antigens. At this stage the balance between Teff versus regulatory T cells (Treg) is critical for the nature of the immune response. Activated T cells then traffic and infiltrate the tumour guided by tropic chemokines and enabled by endothelial cell expression of adhesion molecules. Within the tumour bed, T cells specifically recognize and bind to cancer cells through the interaction between its T cell receptor (TCR) and its cognate antigen bound to MHCI. The TCR engagement enables the cytotoxic attack of $CD8^+$ T cells, cytokine release of $CD4^+$ helper T cells, and kills target cells. At this step, killing of cancer cells will release additional tumor-associated antigens, which can to increase the repertoire of recognized antigens and T cell recruitment in subsequent revolutions of the cycle.

Taken together, cancer immunity depends on cancer-specific T cells. Moreover, it has been demonstrated that mutant peptides that bind MHC I or II can generate protective immunity (1–4). Furthermore, there is direct evidence that mutant epitopes are recognized by T cells in cancer in humans, in particular it has been shown that $CD4^+$ and $CD8^+$ infiltrating T cells

recognize mutant epitopes (5–9). In accordance, cell immunotherapy based on the adoptive transfer of antigen-specific tumour-infiltrating lymphocytes has been shown to correlate with tumour regression in cancer patients (10–14). Of note, most studies are focused on $CD8^+$ T cells that eliminate cancer cells; however, $CD4^+$ T cells are also involved. $CD4^+$ T cells participate in both the antibody response and that of cytotoxic $CD8^+$ T cells, and they can produce Inteferon (IFN)-γ, contributing to an inflammatory environment.

The degree of inflammation can be assessed by the cellular content of the tumour, specifically the abundance of immune cells and their distribution within the tumour (15,16). Inflamed or '*hot*' tumours present higher levels of pro-inflammatory cytokines, thus providing a favourable environment for T cell activation and expansion, including type I and type II IFNs, Interleukin 2 (IL-2), IL-12, IL-23, IL-1β, and Tumour-necrosis factor (TNF)-α. Furthermore, the production of tropic chemokines by lymphocytes, myeloid cells and endothelial cells is likely to be a determinant factor for the increased infiltration of 'hot' tumours.

However, although a tumour may be infiltrated and present high levels of pro-inflammatory mediators, the presence of regulatory mechanisms present a major obstacle for an effective immune response. In this way, tumours take advantage of physiological tolerance mechanisms to avoid and escape the immune system. For example, tumour antigens may be recognized as 'self' and thereby promote Treg responses rather than effector responses. On the other hand, T cells may not be effectively recruited to the tumour and furthermore they can be subjected to active suppression mechanisms [reviewed in (17)]. Besides anti-tumoural cells such as T cells and Natural Killers (NK), there are other cell types regulating the local immune response. The tumour stroma is composed by different cell types, including fibroblasts, Treg, macrophage-lineage cells and vascular endothelial cells, and a variety of extracellular matrix proteins. In

addition to positively regulating tumor growth, the tumour microenvironment can impair host immune responses [Reviewed in (18)]. Noteworthy, endothelial cells also play a pivotal role as “gate-keepers” of tumour stroma by regulating immune cell recruitment and extravasation and playing an intricate cross-talk with immune cells [Reviewed in (19)].

Macrophages and other myeloid cells are universally found in the solid tumor microenvironment and can contribute to immune evasion. Tumour-Associated Macrophages (TAMs) display an M2-like phenotype and appear to contribute to immune suppression through the production of IL-10 and TGF-β (20) and increase Treg recruitment through CCL22 (21). Tregs are also a main source of local immunosuppression, directly regulating Teff cell function by IL-2 and consumption, secretion of anti-inflammatory cytokines such as IL-10 and TGF-β secretion and by locally depleting tryptophan through IDO activity, which also produces the toxic metabolite kyneureine [Reviewed in (22)]. Besides regulating the immune response, both TAMs and Treg are also known to promote tumour growth and angiogenesis by secreting growth factors (20,23).

Immune responses are tighly controlled by tolerance molecular mechanisms, including the activation of inhibitory receptors on T cells. These receptors are often referred as immune checkpoints and act as molecular breaks that limit T cell activation and effector functions (24–26). So far, the most studied checkpoints are the cytotoxic T-lymphocyte protein 4 (CTLA4) and programmed cell death protein 1 (PD1). CTLA4 and negatively regulates T-cell activation by binging to the co-stimulatory receptor CD28 binding partners, CD80 and CD86, triggering their trans-endocytosis and thus dampening TCR co-stimulation (27,28). PD1 is a cell surface receptor that is expressed on activated T cells and that binds to two ligands: Programmed cell Death Ligand (PDL)-1 and PDL-2 (29). Different cell types express PDL-1, including cancer

cells, endothelial cells and immune cells after exposure to cytokines such as IFNγ. In contast, PDL-2 is mainly expressed on DC. PD1 engagement by its ligands activates a signaling pathway that negatively regulates the TCR cascade, thereby interfering with T cell activation.

Interestingly, when a persistent antigen repetitively activates effector T cells, they acquire a dysfunctional or exhausted phenotype (30,31). This exhausted phenotype was first described on mice harbouring chronic viral infections in which the PDL-1/PD-1 axis was found to be an important negative feedback loop that ensures immune homeostasis. Furthermore it is also an important axis for restricting tumour immunity. T cell dysfunction is a differentiation process where T cells acquire the expression of different inhibitory receptors, including PD-1, T-cell immunoglobulin and mucin-domain containing protein 3 (Tim-3) and lymphocyte activation gene 3 (LAG-3). Importantly, Tumour-infiltrating lymphocytes (TILs) typically have the features of memory T cells and can possess an activated or dysfunctional phenotype, expressing markers including PD-1, Tim-3 and LAG-3.

Although the molecular mechanisms driving T cell dysfunction are not yet deciphered, it has been proposed that constant TCR activation accompanied by inhibitory signals such as checkpoint activation and metabolic signals lead to decreased proliferation and effector functions such as IFNγ, Granzyme B (GzmB), and perforin (Pfr) production (32). Dysfunctional T cells present high expression of the transcription factor Thymocyte selection-associated high mobility group box (TOX1) that associates with specific epigenetic changes (33,34). The increase of activity in TOX1 is often seen in a reduction of T cell specific transcription factor 1 (TCF1), which promotes T cell stemness, therefore it has been proposed that the balance between this transcription factors acts as a lineage commitment switch. Like other differentiation processes, there are degrees of dysfunction depending on effector

functions (30,35). While activated T cells express PD1, it has been described that 'early-dysfunctional' T cells are characterized by the co-expression of PD1 and Tim-3. On the other hand, 'late-dysfunctional' T cells co-express PD1, Tim-3 and Lymphocyte activation gene 3 (LAG-3) inhibitory receptors. It has also been shown that 'late-dysfunctional' T cells present the lowest degree of effector function and are less responsive to immunotherapy.

II. The Gal-9/Tim3 Pathway role in the immune system regulation and cancer

Gal-9, coded by the LGALS9 gene, has been reported to function in several biological processes, in a concentration-dependent manner and through different ligands. Among these functions are cell adhesion, cell surface recognitions, migration, chemo-attraction, growth, apoptosis, cytokine production, glycoprotein trafficking, protein folding, signal transduction, fertilization, development, and immune regulation [Reviewed in (36)].

Gal-9 is a tandem repeat galectin that possesses two Carbohydrate Recognizing Domains (CRDs), one at the N-terminal and one at the C-terminal, separated by a linker domain. Of note, there are different isoforms of Gal-9 that differ on the length of their linker domain and are able to bind to different glycan units with differential avidities and affinities. Gal-9 binding to glycan units has been reported to be highly pH-sensitive, implicating that acidification has a crucial regulatory role on the assembly and disassembly of galectin-glycan lattices for spatio-temporal control of signal transduction (37). Because Gal-9 possesses two-CRDs it can exhibit multivalency, even as a monomer, and therefore can act as a linking bridge between specific carbohydrates. Similar to antibody lattices, Gal-9 homo and hetero-oligomerization enables ordered arrays of complexes with multivalent glycol-conjugates. Due to the different oligosaccharide-binding affinities of Gal-9 CRDs, each of them can exert their own individual

biologically important activities, depending on the linker polypeptide length between them. Gal-9 can potentially cluster several multivalent glycol-conjugates mediating cell interactions and trigger cell-Extracellular cell matrix (ECM) interactions (38).

Gal-9 presents intracellular and extracellular expression. Within the cell, Gal-9 localized in the cytoplasm and nucleus; however, it can also be secreted most likely through exosomes (39). Extracellular Gal-9 selectively binds to galactose-containing oligosaccharides and glycoproteins (such as laminin, fibronectin, vitronectin)(40). Accordingly, the secreted form of Gal-9 can mediate cell adhesion, chemoattraction, receptor endocytosis, lipid recycling, raft clustering, etc. via binding to glycan ligand [Reviewed in (36)]. Different receptors or surface binging partners have been described for Gal-9, including GLUT-2 (41), protein disulfide isomerase (PDI) (42), Forssman glycosphingolipid (FGL) (37), IgE (43), CD44 (44), PD1 (45), CEACAM1(46), and Tim-3 (47). In contrast, cytoplasmic Gal-9 induces cancer cell aggregation leading to inhibition of invasion, detachment from tumour and attachment to vascular endothelium (48). It has also been proposed that Gal-9 may have a role in glycoprotein trafficking, protein folding, and signal transduction(37). Nuclear localization of Gal-9 has been observed in several cell types, including cancer and endothelial cells (49–53). Nuclear Gal-9 may play a role in gene expression regulation as it has been reported to bind to different transcription factors such as AP-1, NFkB, NFL6 (51), and βcatenin (54).

One of the most studied receptors of Gal-9 is Tim-3, a type 1 membrane protein with a structurally conserved IgV domain and a mucin stalk that anchors to an intracellular tail with SH2 phosphorylation domain (55). First identified on activated T helper 1 cells (Th1), Tim-3 has been described as an inhibitory immune checkpoint mediating T-cell apoptosis. However it may also mediate T cell migration, T cell exhaustion; Treg mediated immunosuppression,

DC differentiation and M2 macrophage polarization. As other immune checkpoints, Tim-3 localizes at lipid rafts at the immunological synapses of $CD8^+$ T cells, where its ligand Gal-9 also recruits other receptors such as CD45, CD148, CD44 and integrin, which enable TCR signaling (42,44,46). Furthermore, it has been proposed that Gal-9 acts by crosslinking receptors, binding to their glycosylated residues. In this way Gal-9 could stabilize Tim-3 interaction with its ligand CEACAM1 enhancing Tim-3 downstream signaling [Reviewed in (56)]. Interestingly, it was recently published that Gal-9 through its CRDs can also bind to PD-1, enabling a lattice between Tim-3 and PD-1 which stabilizes $PD1^+Tim3^+$ cells by preventing Tim-3 mediated cell death (45).

In Rheumatoid Arthritis models, Gal-9 possesses anti-inflammatory activity, offering a protective effect by promoting the differentiation of naïve T-cells to Treg and inducing apoptosis of $Tim\text{-}3^+$Th1 and pro-inflammatory Th17 cells (57). It has also been shown that recombinant Gal-9 (rGal-9) decreases pro-inflammatory cytokines such as IL-1β, IL- 6, TNF-α, MCP-1, MIP-2, IL-12 and IL-17. Similar results can be observed in viral infections models. In Herpes Simplex Virus 1 infection Gal-9 causes apoptosis in Tim-3 expressing effector cells and pro-inflammatory cells the reduction of pro-inflammatory molecules and simultaneous up-regulation of anti-inflammatory molecules (58). In Hepatitis C Virus, where infected cells express Gal-9, it has been shown that TGFβ and Gal-9 upregulate Tim-3 expression and regulatory cytokines TGFβ/IL-10 driving conventional $CD4^+$ T cells into $CD25^+FOXP3^+$ Treg-cells. In addition, rGal-9 also acts in synergy with TGFβ, transforming TCR-activated $CD4^+$ T cells into $Foxp3^+$ Treg in a dose-dependent manner (59). Furthermore, Gal-9 has been demonstrated to induce Th17 cell apoptosis, possibly through Tim-3 interaction, leading to a reduction in IL-17 expression and dampened antimicrobial response (60). However, Gal-9 role

in immune regulation is not limited to $CD4^+$ T cells. In an murine model of Influenza A virus infection, that lacked the regulatory effects of Gal-9/Tim-3 mounted a superior $CD8^+$ T cell response, were more refractory to viral infection and generated better virus-specific memory $CD8^+$ T cell responses (47).

Given the accumulated data in numerous biological responses, it is no surprise that Gal-9 has also been implicated in cancer biology. However, in the context of Gal-9 and malignancy, the literature presents not only inter-study variation, but also contradicting observations. Several studies have proposed an anti-metastatic role for Gal-9. In this context Gal-9 is shown to increase cancer cell aggregation and consequently decrease migration and invasion, while also promoting cancer cell apoptosis (Reviewed in (36)).

A recent study reported that 57% of gastric tumours are positive for Gal-9 and its expression it is associated to poor prognosis (61–63). Investigation in gastric cancer (GC) cell lines has shown that Gal-9 suppresses migration, invasion, and epithelial-mesenchymal transition (EMT) (64). Moreover, Tim-3 has also been proposed as a prognostic marker for solid tumours including GC, with high levels of Tim-3 expression associated with poor survival (65) .

Tumour angiogenesis is a limiting step for cancer growth. Interestingly, it has been shown on a chicken chorioallantoic model, that high concentrations of exogenous Gal-9 inhibited angiogenesis (39). However, at a lower concentration, Gal-9 displayed a trend towards an increased vessel length, suggesting regulation of endothelial cell migration. Noteworthy, angiogenesis and angiogenic factors such as VEGF-A are commonly associated to local immunosuppression (66–69). Accordingly, anti-angiogenic therapies have shown synergic effects with immunotherapy on mice models (70–72), and are currently on early phase clinical

trials (73,74). Of note, it has also been shown that Gal-9 expression is regulated during endothelial cell activation and stimulated upon inflammatory stimuli such as IFNγ and LPS (39,75). Of note Tim-3 is also expressed on tumour endothelial cells, and its blockade on LPS activated endothelial cells reduced cancer cell adhesion (76). Thus suggesting that the Gal-9 receptor Tim-3 could be involved in cell extravasation, a key step for immune infiltration and for cancer metastasis. Although the role of the Gal-9/Tim-3 axis in endothelial cell biology and angiogenesis remains elusive, Heusschen et al. speculated that Gal-9 levels on the endothelial cell layer might affect infiltration of anti-tumour immune effector cells, as well as immune-suppressive immune cells, and thereby interfering with a proper anti-tumour immune response (36). Given that endothelial cells also function as antigen presenting cells, and that Gal-9 is induced through inflammatory stimuli, it is possible that Gal-9 expressing endothelial cells may also regulate local immune responses. However, despite sporadic reports, the role and influence of Gal-9 on the modulation of the immune microenvironment and subsequent cancer cell behaviour, remains poorly understood.

As previously mentioned, Tim-3 is expressed on the most dysfunctional subset among tumour-infiltrating $CD8^+PD1^+$ T cells in cancer (77–79). Murine cancer models show that Tim-3 overexpression on T cells exacerbates tumour progression (80). Interestingly, Tim-3 expression is inversely correlated to TCF1 (81). Therefore, Tim-3 has been proposed as a marker that identifies both terminally differentiated effector cell and irreversibly exhausted or dysfunctional T cells (82–86). In line with these hypothesis, co-expression of Tim3 and PD1 is observed on both $CD4^+$ T cells and $CD8^+$ T cells in preclinical models, where $Tim\text{-}3^+PD1^+$ T cells have decreased IFNγ, TNF and IL-2 production when compared with their $Tim\text{-}3^-$ counterparts (78,83). Strikingly, Tim-3 up-regulation can be observed after anti-PD1 therapy,

an observation that has been attributed to activation of PI3K–AKT pathway (87). Accordingly, in cancer and viral chronic infection mouse models, the simultaneous blockade of Tim-3 and PD1 increases T cell responses compared with anti-PD1 mono-therapy (77,78,88–91).

An important connection has been established between Tim-3 and Treg function. It has been reported that Tim-3^+ Treg display increased production of IL-10 and enhanced immune-suppressive function that can be abrogated by anti-Tim-3 antibodies (92–95). In fact, Tim-3^+ Treg and correlate with tumour aggressiveness and progression (94,96,97). In a mouse model of colorectal cancer, tumour-infiltrating $Tim3^+$ Treg accounted for more than 50% of the Treg population, significantly exceeding their relative frequency reported in the blood, tumour-draining lymph nodes and the spleen. Importantly, in the colon cancer model anti-Tim-3 synergized with anti-PD1 therapy to reduce Treg infiltration and increase $CD8^+$ T cell infiltration (55). This suggests that Tim-3^+ Treg are required for tumour growth. In a lung cancer murine model, Li *et al*. demonstrated that anti-Tim-3 targeted Treg, where it not only reduced tumour burden but also TAM and co-inhibitory receptor expression, while re-establishing $CD8^+$ T cell effector functions (98). Importantly, in these murine models Tim-3^+ Treg accumulate in the tumour tissue prior to the $CD8^+$ T cell dysfunction being evident, and the depletion of Treg at this stage prevents $CD8^+$ T cells developing an exhausted phenotype (94).

III. Gastric Cancer and Immunotherapy resistance

Worldwide Gastric cancer (GC) takes the fifth place on most common cancers worldwide and it is currently the third cause of death by malignant tumours (99–101). Based on their histological characteristics, gastric tumours can be classified using the Lauren or the World Organization of Health systems. However, this classification has low clinical significance (102,103). Aiming to improve this, a new classification was proposed based on molecular characteristics of the tumour, such expression profiles and mutations present on the tumour (4). This classification is known as the TCGA classification ("The Cancer Genome Atlas"), and classify tumours into four kinds: Chromosomal Instable (CIN), Epstein Barr Virus positive (EBV), Genomically Stable (GS), and Microsatellite instability (MSI). Not only these tumours differ on genetic properties, but also show differential immune infiltration profiles (5), patient survival and therapy responses (103–106).

In Chile, GC is a principal cause of cancer death with an incidence and mortality of 15.6 and 13.8 per 100,000 inhabitants, respectively (100,101). According to 2012 national statistics, there were 3,354 deaths by GC with a mortality rate of 19.27 per 100,000 inhabitants for both sexes. This high mortality may be explained by the health system deficiencies and by the high percentage of patients been diagnosed with advanced or metastatic disease (107,108). Advanced disease is treated similarly between the public health system and private system, commonly prescribing palliative chemotherapy with schemes based on platinum salts combined with fluoropyrimidine (108–110). Median overall survival (OS) rates with these regimes are less than a year (110), raising the importance of new therapeutic approaches. While the five-year survival rate for GC patients is 30.4%, this rate falls to approximately 5% in metastatic gastric cancer patients (110). In the absence of a standard-of-care treatment for

patients with advanced or recurrent GC after chemotherapy failure, there is a clinical requirement for new treatment options.

Anti-PD1 immunotherapies such as Pembrolizumab or Nivolumab attempt to rescue the exhausted phenotype and restore T cell mediated anti-tumour response. The interaction between the checkpoint PD1 and its ligands PDL-1 or PDL-2 leads to inhibitory signal that reduces the TCR signalling and thus unable T cells to eliminate the target cell (111). Also it has been shown that PDL-1 signals backwards to target cells providing interferon resistance (112). This is a physiological mechanism commonly used to avoid autoimmunity that is exploited by tumours and rendering immune escape. Importantly, upon persistent antigen recognition under low cytokine stimulation, and high inhibitory signalling (such as PD1 pathway) T cells differentiate into a dysfunctional phenotype through a process known as T cell exhaustion. T cell exhaustion or dysfunction happens in a hierarchical manner and ultimately leads to senescence and apoptosis (Reviewed in (31,33,113,114)). Dysfunctional or exhausted phenotypes are characterized by high expression of inhibitory receptors such as PD1, Tim-3 and LAG-3 accompanied by weak TCR signalling. Studies in murine and human cancer suggest that intratumoral T cells display a broad spectrum of (dys-)functional states, that is most likely shaped by the multifaceted suppressive signals that occur within the tumor microenvironment. Different kinds of signals have been proposed to shape this differentiation process, including metabolic stress, hypoxia, anti-inflammatory cytokines, high antigenic load and/or persistent antigens and activation of immune checkpoint pathways. Importantly, there are PDL-1-independent mechanisms of immune escape; these include alternate immune checkpoints or co-inhibitory receptors such as Gal-9 and Tim-3 respectively, immune

suppressive cytokines, immune inhibitory metabolites, and immune suppressive cells (115–119).

Pembrolizumab, a PD1 blocking antibody, was granted accelerated approval for the treatment of patients with recurrent locally advanced or metastatic gastric or gastroesophageal junction adenocarcinoma. This therapy is prescribed for tumours that express PDL-1 [Combined Positive Score (CPS) > 1] which present disease progression on or after two or more prior lines of therapy including fluoropyrimidine and platinum containing chemotherapy and if appropriate, HER2/neu targeted therapy (120). Although this therapy showed a remarkable 56% ORR[1] on the KEYNOTE-052 trial, the most recent published study shows a 22.7% ORR on patients with PDL-1 positive tumours (121). Currently the only companion diagnostic available is an FDA approved test to determine tumour PDL-1 expression, but despite this selection 80% of patients show no clinical benefit (121), losing their opportunity to receive an effective treatment and suffering the elevated economic burden of this treatment. Similar results have been shown on CHECKMATE trials for another PD1 antibody, Nivolumab. In a phase 1/2 study in chemotherapy-refractory GC patients with or without PDL-1 expression, second-line Nivolumab treatment delivered a 26% ORR (44% ORR in PDL-1^{+} tumours) (120).

Over the past 5 years there has been an exhaustive search for reliable biomarkers or expression profiles that aid physicians to predict anti-PD1 responses and how to effectively combine checkpoint blockers. One of the main stumbling blocks in this goal is the scarcity of knowledge on how GC escapes systemic immune vigilance and the nature of the tumour

[1] ORR: Objective response rate, accounts for complete responses and partial responses determined by RECIST v1.1 criteria (EA Eisenhauser et al 2009)

properties, which permit a strong and robust immune suppressive microenvironment. It is documented that PDL-1 is over-expressed in the majority of GCs, and higher expression of PDL-1 correlates with worse overall survival (121,122); but PDL-1 negativity does not imply a lack of response. Some studies have suggested that *H. pylori* or EBV infection may be associated to favourable anti-tumour immune response, however their presence still has uncertain value as predictive biomarkers (123). An Asian cohort of 61 patients showed that patients with MSI or EBV-positive tumours had impressive ORR to anti-PD1, with 87.5% and 100% respectively (124). Although promising, further studies are needed to determine if EBV or MSI are good predictive biomarkers. In fact some of the published results in this article were different than those obtained in other cohorts (121). Other possible indicators of immunotherapy response such as tumour mutational burden (TMB) (125), IDO expression (126–128), and $CD8^+$ T cell infiltration have also been suggested in other cancers (129). Increased $CD3^+$, $CD45RO^+$ and $CD8^+$ tumour infiltrating lymphocytes (TILs) have also been suggested as possible predictive markers and as independent markers of better prognosis (130). A matter of controversy is the Treg marker, FOXP3, which has been suggested as both good (131,132) and poor (133,134) prognosis factor.

Of note, most proposed biomarkers are based on static measures and therefore cannot be evaluated through time and hence fail to rule out acquired resistance. Resistance to immunotherapy has been attributed to intrinsic tumour-cell properties, such a disruption on the antigen presenting machinery, production of anti-inflammatory cytokines and metabolic disruption of T cells [Reviewed in (118)]. However there are tumour-cell extrinsic mechanisms of resistance that involve cells from the tumour microenvironment including Treg, and the expression of co-inhibitory receptors such as Tim-3 (87,115,135–137).

Treg are a highly immune suppressive subset of $CD4^+$ T cells, characterized by expression of CD25 and FOXP3 (138–140). These cells play a central role in self-tolerance maintenance (141–145). In fact mutations in FOXP3 gene results in severe autoimmune disorders in both mice and humans (142–144). Besides their important role in immune homeostasis, Treg are also well known for their ability to suppress anti-tumour responses and thereby facilitating tumour growth and metastasis (145–149). Treg restrict anti-tumour responses by several mechanisms including the suppression of antigen-presenting cells via CTLA-4, secretion of inhibitory cytokines (IL-10, TGFβ and IL-35), expression of granzyme/perforin, consumption of IL-2 and tryptophan, and degradation of ATP (150). Tumour-infiltrating Treg have shown to promote immune tolerance by suppressing tumour-associated dendritic immunogenicity in a pancreatic duct cancer mouse model (148). A separate study showed that intra-tumour injection of αCD4 antibody in tumour-bearing mice caused rejection of late-stage tumours by depleting Treg and altering the cytokine milieu in the tumour microenvironment (149). Also when Treg are depleted by αCD25 antibody, either by in vivo antibody administration to mice or transfer of cell suspension depleted in vitro of $CD25^+$ Treg cells into hystocompatible T cell-deficient mice, effectively eradicates inoculated syngeneic tumours (147,151). Furthermore concomitant tumour immunity[2] was also suppressed by Treg in a melanoma murine model (152). In line with this finding it has been observed in patients that melanoma, non-small cell lung, gastric and ovarian tumours are heavily infiltrated by Treg (20–50% of $CD4^+$ TILs) (150,152). High Treg infiltration has also been associated to poor prognosis in different types of cancer, including GC (153). When it comes to how Treg affect GC

[2] *Concomitant tumour immunity is a phenomenon where tumour-bearing mice can reject the same tumour cells when inoculated at a distant site*

prognosis there is scattered information within the literature. In GC and peptic ulcer disease there is an increase in Treg both in the peripheral circulation and the gastric mucosa (154). A further study demonstrated that high levels of Treg among tumour-infiltrating $CD4^+$ T cells gave a favourable prognosis in gastric cancer (155).

While there is accumulating evidence that the removal of Treg cells enhances anti-tumour immune response, there are only few studies indicating their relevance on immunotherapy resistance. Wei et al. determined the infiltrating T cell population changes upon αPD1 and αCTLA4 immunotherapies on cancer murine models and showed that sensitive tumours had a decrease on Tregs and the expansion of exhausted $CD8^+$ T cells that correlated with tumour rejection (156). Within the same study, however using an immunotherapy resistant melanoma model, an inverse effect was observed which was similar to that found in human melanoma (61). Despite the absence of evidence that relates Treg infiltration to gastric cancer resistance to immunotherapy, it is established that Treg isolated from gastric tumours are proliferative and secrete IL-10, TGFβ (157) and that gastric cancer cell lines expand Treg population from peripheral blood mononuclear cells (PBMCs) (133). It was recently demonstrated that combined αPD1 therapy with Treg depletion using αCD25 antibodies promoted complete tumour rejection in murine models (158). Although the published literature suggests a major role of Treg in αPD1 resistance, there are yet no studies substantiating this theory in a clinical setting.

It has been described that $CD8^+$ dysfunctional states are associated to anti-PD1 therapy failure (159) and furthermore, they represent an obstacle for other immunotherapies such autologous T cell transfer. Among these dysfunctional phenotypes, those cells expressing PD1 and Tim-3

receptors are associated to a reversible phenotype, while the co-expression of PD1, Tim-3 and LAG3 have been associated to a terminally differentiated dysfunctional state.

From murine models, it has been proposed that the expression of two transcription factors rule the differentiation into an exhausted phenotype, TCF1 and TOX. TCF is mainly associated to T cell stemness and restrains effector differentiation, while TOX has been proposed as cell fate determinant factor that leads to the exhausted phenotype commitment. Interestingly, $PD1^-$ T cells and $PD1^+$ T cells that express TCF1, have been identified as essential for checkpoint blockade responsiveness (82,84).

Combinatory strategies using antibodies targeting Tim-3 and PD1 have shown a synergistic inhibition of tumour growth (78) and improving the tumour antigen- specific $CD8^+$ T cell responses of patient- derived T cells (79,160). Accordingly, Tim-3 expression on central memory CD8+ T cells showed and association to both primary and secondary immunotherapy resistance on lung cancer patients, where blockade of Tim-3 was able to restore PBMCs proliferation *ex vivo* (161).

Thus, while checkpoint therapy is offering enhanced overall survival where a significant number of patients, a better knowledge of gastric cancer immune escape mechanisms is required in order to identify possible predictive biomarkers and design new therapeutic approaches to overcome resistance. The literature to date has suggested that the Tim3/Gal9 axis is associated to both immunotherapy resistance and expansion and potentiation of Treg cells, and may play a key role in response to anti-PD1 therapy. Given that gastric cancer cells express Gal-9 and that Treg infiltration is frequently observed, we propose that Gal-9 expression on gastric cancer cells potentiates Treg immunosuppression and thus may renders resistance to anti-PD1 therapies.

Hypothesis

"Gal-9 promotes an immune-suppressive microenvironment and αPD1 resistance through its receptor Tim-3 on endothelial and T cells in gastric tumours"

General Objective

To better understand the influence of the axis Gal-9 and its receptor Tim-3 in the microenvironment of gastric cancer cells, endothelial cells and immune cells, utilizing the TCGA database, in vitro exogenous addition and gain of function models and determine the role of this axis in primary T cells fate, in the presence and absence of the αPD1.

Specific Aims

1. Evaluate the expression pattern of Galectin-9 and Tim-3 in gastric tumours.

i. Determine if Gal-9 and Tim-3 genes are overexpressed in gastric tumours

ii. Determine Gal-9 and Tim-3 levels according to cancer stage

iii. Determine Gal-9 and Tim-3 levels according to gastric cancer molecular subtype

iv. Determine if Gal-9 levels are associated to patient clinical outcome

v. Determine if Tim-3 levels are associated to patient clinical outcome

v. Determine if there is a correlation between Gal-9 and Tim-3 expression with other immune checkpoints in gastric tumours

2. Determine Gal-9 function in gastric cancer cells

i. Establish a Gal-9 gain of function in vitro model

ii. Evaluate the effect of Gal-9 in cancer cell viability/proliferation

iv. Determine the effect of Gal-9 on PDL-1 in gastric cancer cells

3. **Evaluate if Gal-9 promotes angiogenesis *in vitro***

i. Determine the effect of Gal-9 and Tim-3 expression in endothelial cells

ii. Determine the effect of Gal-9 on endothelial cell migration

ii. Determine the effect of Gal-9 on angiogenesis *in vitro*.

4. Determine if Gal-9 increases CD8+ T cell dysfunction and Treg frequency.

i. Determine if there is a correlation between Gal-9 and Tim-3 expression with Treg markers in gastric tumours

ii. Determine if there is a correlation between Gal-9 and Tim-3 expression with T cell exhaustion in gastric tumours

iii. Determine if secreted factors derived from gastric cancer cells expressing Gal-9 increase T cell exhaustion

iv. Determine if secreted factors derived from gastric cancer cells expressing Gal-9 promote Treg expansion

5. Determine if Gal-9 provides resistance to αPD1 therapy in vitro through Tim-3.

i. Evaluate the effect of αPD1 on the frequency dysfunctional populations of T cells in presence and absence of Gal-9.

i. Evaluate the effect of αPD1 + αTim-3 on the frequency dysfunctional populations of T cells in presence and absence of Gal-9.

Materials and Methods

1. TCGA Database Bioinformatics Analysis

The bioinformatics analysis of gastric cancer data of patients with gastric adenocarcinoma from TCGA Database was performed with TIMER (Tumor Immune Estimation Resource; © X Shirley Liu Lab & Jun Liu Lab 2018). The parameters subjected to this analysis include sample purity (tumour cell/stromal cells relationship of the sample), and mRNA levels of inhibitory checkpoints, T cell exhaustion markers, Treg markers, adhesion molecules and Endothelial cell activation genes. Spearman and Pearson's regression analysis were performed considering a p-value of 0.05 or less significant. TIMER analysis considered data of 387 tumour samples from 1 data set (Stomach Adenocarcinoma 415 dataset) of untreated patients from the TCGA database. Tumour infiltrating Treg and CD8+ dysfunction signatures were calculated as previously described (159,162). Briefly, each signature was calculated using the mean z-scores of gene expression values corresponding to each gene least. Then, specific correlation of LGALS9 levels with molecular signatures were performed by Spearman's regression analysis.

Overall Survival (OS) and Progression-Free Survival (PFS) from patients was obtained from the TCGA STAD 2018 data set along with HAVCR2 and LGALS9 mRNA levels. High and low levels were defined as the upper and low quartile respectively.

2. Gastric cancer and epithelial cell lines transfection

The human gastric adenocarcinoma cell line AGS and human gastric epithelial cell line GES-1 were kindly provided by Dr. Alejandro Corvalán at the Pontificia Universidad Católica de Chile. Gastric cell lines were used for transfection with Gal-9 overexpression, Gal-9 knockdown or empty vectors. AGS and GES-1 were cultured in RPMI 10% FBS and

transfected using a CMV3-LGALS9 expression vector o empty vector as control. Transfections were performed during 4-6 hours on OPTIMEM using a transfection solution with 1:1 DNA:Fugene according to manufacturer. After transfection cells were harvested and seeded in 6 well plates and 96 well plates for further experiments. Gal-9 expression was assessed by western blot (human anti-Gal9, CST) and ELISA (Gal-9 DuoSet R&D). Besides transfections, 0.5μg/mL recombinant human Galectin-9 (rhGal-9, R&D) was used to treat cells. After transfection or rhGal-9 treatment, viability was evaluated by WST-1 assay (Sigma), migration and invasion assay were performed as previously described (163) in presence or absence of 30mM α-Lactose (Sigma).

4. Human Umbilical Vein Endothelial Cells:

Primary Human Umbilical Vein Endothelial Cells (HUVECs) were kindly provided by Dr Alejanadro Godoy at the Pontificia Universidad Católica de Chile at passages 1-2 and maintained in 1% gelatin coated flasks (Nunc) with 10% fetal bovine serum (FBS) in DMEM/F12 culture media (Gibco) supplemented with Endothelial Cell Supplement (Gibco) in standard cell culture conditions of 37^{o}C and 5% CO_2. To determine the effect of galectin-9 on endothelial cell activation and *in vitro* tube formation (hereafter referred to as angiogenesis), we performed in vitro experiments using HUVEC cells treated with human recombinant Galectin-9 in presence or absence of 30mM α-Lactose in culture media. Tube formation assay (in vitro angiogenesis) was performed with $4x10^4$ cells seeded on Matrigel coated plates (angiogenesis slides®IBIDI). Culture medium containing or not 40ng/mL VEGF was used as a positive and negative control, respectively. To evaluate migration, scratch assays were performed on 24 well culture plates; images were taken at time 0h, 4h, and 8h to access and quantify wound closure (Olympus CKX41, 10x). Proteins were extracted from

treated cells after 8h and 24h of treatment, and conditioned media (media that had been in presence of culture cells) was obtained after 8h of treatment and stored at -80ºC for further use.

4. Primary T cell cultures & Flow Cytometry.

To determine if secreted factors derived from gastric cancer cells expressing Gal-9 expands Treg population and increases T cell exhaustion, T cells were isolated from peripheral blood mononuclear cells (PBMC) from healthy donors with written consent and ethical approval from the Ethical Committee of the Pontificia Universidad Católica de Chile (Annexes 2 and 3). First, PBMCs were isolated by Ficoll gradient (Ficoll-Plus®), and then T cells were isolated by magnetic separation using the Untouched Human Pan T Cells isolation kit (Life Technologies) according to manufacturer's indications. Upon isolation, T cells were counted and stimulated with Dynabeads® CD3/CD28 T Cell Expander and rhGal-9 (0.25ug/mL, 0.5ug/mL and 1ug/mL) in presence or absence of blocking antibodies: 20ug/mL α-Tim3 (Biolegend) and 20ug/mL α-PD1 (InVivoMab®) and cultured for 96 hours in T cell media (10% FBS, 1% Penicillin-Streptomycin, 1% Glutamine, B-Mercaptoethanol, HEPES, RPMI Glutamax). After culture cells were stained with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit (LifeTechnologies) and labelled antibodies against CD4, CD8, CD127, CD25, PD-1, Tim-3, and LAG-3. Subsequently cells were fixed and permeabilized for intracellular staining with antibodies against FOXP3 (BD Bioscience). For IFNγ staining cells were first re-stimulated with the Protein Kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA, Sigma), Ionomycin (Sigma) and Brefeldin A (Biolegend) for 4 hours and subsequently stained as previously described, fixed and permeabilized for IFNγ staining (BD Biosciences). Results were obtained by flow cytometry using FACS BD –Canto II and FACS DIVA. Compensation

ArC® and AbC® compensation beads and unstained cells were used to determine voltages and compensation. Upon acquisition, the results were analysed using FlowJo 10.5

5. Immunofluorescence

AGS cells were grown to 40% confluence state on glass coverslips. Then treated with rhGal-9 (0.5ug/mL) in Supplemented RPMI and incubated for 24h. After treatment, cells were washed with warm PBS and fixed with 4% PFA at room temperature. Cells were then washed with 1X PBS and incubated with PBS-Triton 0.1% for 5 min and blocked with 2% BSA-PBS for 30 min at room temperature. PDL-1 primary antibody (E1LN3®, CST) was incubated overnight in a humid chamber at 4 °C and the secondary antibody (Anti-rabbit Alexa Fluor 488) were incubated for 1h at room temperature. Cell nuclei were stained by DAPI and coverslips were mounted using Fluoromount (Fisher Scientific). Images were obtained from independent fields for each coverslip in a Nikon C2s1 microscope and processed/analysed in ImageJ (NIH).

6. WST-1 viability/proliferation assay

Cell viability was assessed by seeding $5x10^3$ cells on 96well culture plates, and transfected or treated with 0.5µg/mL rhGal-9 for 24h. After treatments, cells were incubated with the colorimetric reagent WST-1 (1:10) at 37ºC for 15 min approximately. The absorbance (at 490nm vs 620nm) was analysed and graphed as the percentage of cell viability.

7. Western Blot

Cell lysates were obtained using Misao lysis buffer supplemented with protease inhibitors (Roche). Proteins were quantified by the Bradford method and separated by SDS-PAGE (in 12% gels) for 1.5h at 100 volts. Samples were transferred to nitrocellulose membranes for 2h at 300mA (Wet/Tank Blotting Systems, Bio-Rad). The membranes were blocked with TBS-Tween 5% Milk and the primary antibodies against Gal-9 (D9R4A, CST, 1:1000), PDL-1

(D8TX5, CST, 1:500), Tim-3 (MAB2, R&D, 1:1000), β-Actin (15G5A11/E2, Invitrogen, 1:1000) were incubated overnight at 4°C. The secondary antibody (anti rabbit/mouse-HRP, Biorad, 1:3000 dilution) was incubated for 1h at room temperature and labelled proteins were identified using the myECL system (Pierce Biotechnology). Membrane stripping was performed using Restore™ Plus (Thermofisher Scientific) for 15min at RT under gentle shacking, then membranes were washed three times with TBS and incubated with primary antibodies.

7. ELISA Assay

Galectin-9 ELISA assay was performed using Gal-9 DuoSet kit according to manufacturers instructions. Briefly, MaxiSorp plates (Falcon) were coated over night with anti-Gal-9 in PBS-BSA solution at RT. Then washed with PBS-Tween and blocked for 1h at room temperature. After blocking, plates were washed and incubated with cell conditioned media for 2h at RT, after incubation plates were washed and incubated with capture antibodies and streptavidin. Colorimetric assay using TMB was performed and after 15min stop solution was added. Absorbance was measured at 450nm and 690nm and the concentration of samples was calculated by applying a 4-PL regression to the standard curve.

8. Statistic Analysis.

Statistical differences were evaluated using Kruskal Wallis and Mann Whitney statistic tests. Correlations were assessed either by Pearson's or Spearman regressions. Hazard ratio (HR) was calculated using LogRank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests. All statistic analysis were performed using Graphpad®Prism 8.0.

Results

1. Evaluation of the expression pattern of Galectin-9 and Tim-3 in gastric tumours.

i. Galectin-9 and TIM-3 expression patterns in human stomach adenocarcinoma (STAD).

Galectin-9 (LGALS9) and Tim-3 (HAVCR2) mRNA levels were evaluated in registered normal gastric tissue samples (n=35) and tumours (n=376) from the TCGA database (STAD 2018). All clinical characteristics of the studied cohort have been previously reported and are summarized on (Table 1, Annex 1, p.92). LGALS9 was significantly increased in tumour samples (2.773±0.18) compared to normal tissue (1.708±0.61) (Fig.1A), while it did not show significant differences between stages; stage I and II had a mean level of 6.412±0.17 and stages III and IV a mean of 7.723±0.08 (Fig.1B). HAVCR2 levels were also significantly higher on tumour samples (2.517±0.19) than in normal tissue (1.188± 0.69) (Fig. 1D). Unlike its ligand, HAVCR2 levels were increased in advanced-stage (7.723±0.08) compared to early-stage disease (6.419±0.08) (Fig. 1E).

As molecular subtypes of gastric cancer have been associated to patient outcome, including immunotherapy response, we evaluated if there was a differential expression of LGALS9 and HAVCR2 among each reported subtype (Fig. 1C, F). LGALS9 mean levels were 3.483±0.50, 3.241±1.06, and 4.944±0.89 on CIN, EBV and MSI subtypes respectively. On the other hand, HAVCR2 mean levels were 5.692±0.84, 3.236±1.00, and 5.16±0.98 on CIN, EBV and MSI subtypes respectively. No significant differences between the CIN, MSI or EBV subtypes were observed with either of the Gal-9 or TIM-3 mRNA levels (Fig. 1C, F). Interestingly, LGALS9 (13.6±2.53) and HAVCR2 (8.07±1.49) displayed higher levels within the Genomically Stable (GS) molecular subtype of gastric tumours. However, no significant differences were observed on LGALS9 levels between GS and MSI (p=0.09).

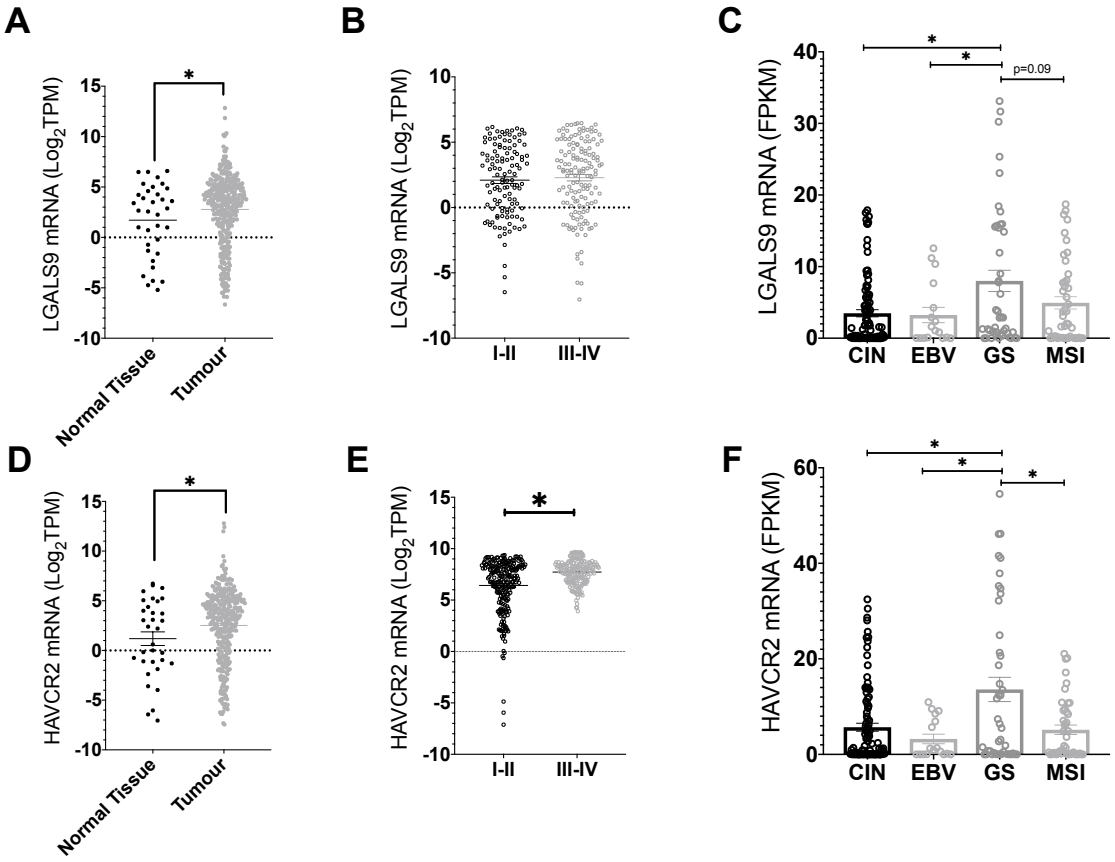

Figure 1. Galectin-9 and Tim-3 are overexpressed in stomach adenocarcinoma and associate with advanced stages and genomical stable subtypes. Galectin-9 (LGALS9) and Tim-3 (HAVCR2) mRNA levels were evaluated in normal gastric tissue samples and tumors from the TCGA database (STAD 2018). LGALS9 was significantly increased in tumour samples (A), while it did not show significant differences between stages (B). HAVCR2 levels were also significantly higher on tumour samples (D) and in advanced stages (E). Both LGALS9 and HAVCR2 showed higher levels on the genomically stable (GS) molecular subtype of gastric tumours (C, F) *p<0.05, *unpaired t-Student. CIN: chromosomal instability; EBV: Epstein Barr Virus-positive; GS: Genomically Stable; MSI: Microsatellite Instable*

ii. Expression of Galectin-9 in gastric cancer, tumour endothelium and in areas of immune cell infiltration.

There is compelling evidence of high Gal-9 expression in gastric tumours. However, expression patterns are not well studied. To determine which cells express Gal-9, we evaluated the contribution of cancer cells and tumour microenvironment cells to the reported mRNA levels through the relationship between mRNA levels with sample purity (Fig. 2G and 2H).

Spearman's regression analysis indicated slopes of -0.126 and -0.273 for Gal-9 and Tim-3 mRNAs respectively, thus indicating that the mRNA expression in both cancer cells and microenvironment cells. To determine which cell types expressed Gal-9 and Tim-3 at protein level, immunohistochemistry (IHC) analysis is required. In a preliminary approximation, we evaluated deposited images of gastric cancer tumours analysed by IHC for Gal-9 and Tim-3 from available images of samples from TCGA database (Fig 2A-F). Positive staining for Gal-9 was observed in cancer cells, tumour endothelium and in areas of immune cell infiltration (Fig.2A-D). These samples were classified by pathologists as moderate and weak based in cancer cell staining, but as it can be seen in Figure 2, there is evident staining in all samples, including the samples considered as negative (Fig. 2C). Within the available samples deposited in the TCGA database, Tim-3 expression was not observed in cancer cells, although weak staining could be observed on highly infiltrated areas.

It has been reported that endothelial cells express both Gal-9 and Tim-3 upon contact with cancer cells in different tumour types (Heusschen et al 2014). However, this has not been assessed in gastric cancer. Our observations demonstrate that Gal-9 has a ubiquitous expression within gastric tumours (Figure 2). Although Tim-3 expression within gastric tumours still requires further detailed evaluation (preferable multiplex staining with specific markers), these analyses suggest that Tim-3 expression is concentrated principally on immune cells. Further analysis in patient samples will be required to determine the specific expression patterns of these proteins in gastric cancer and a possible impact of their expression in patient prognosis.

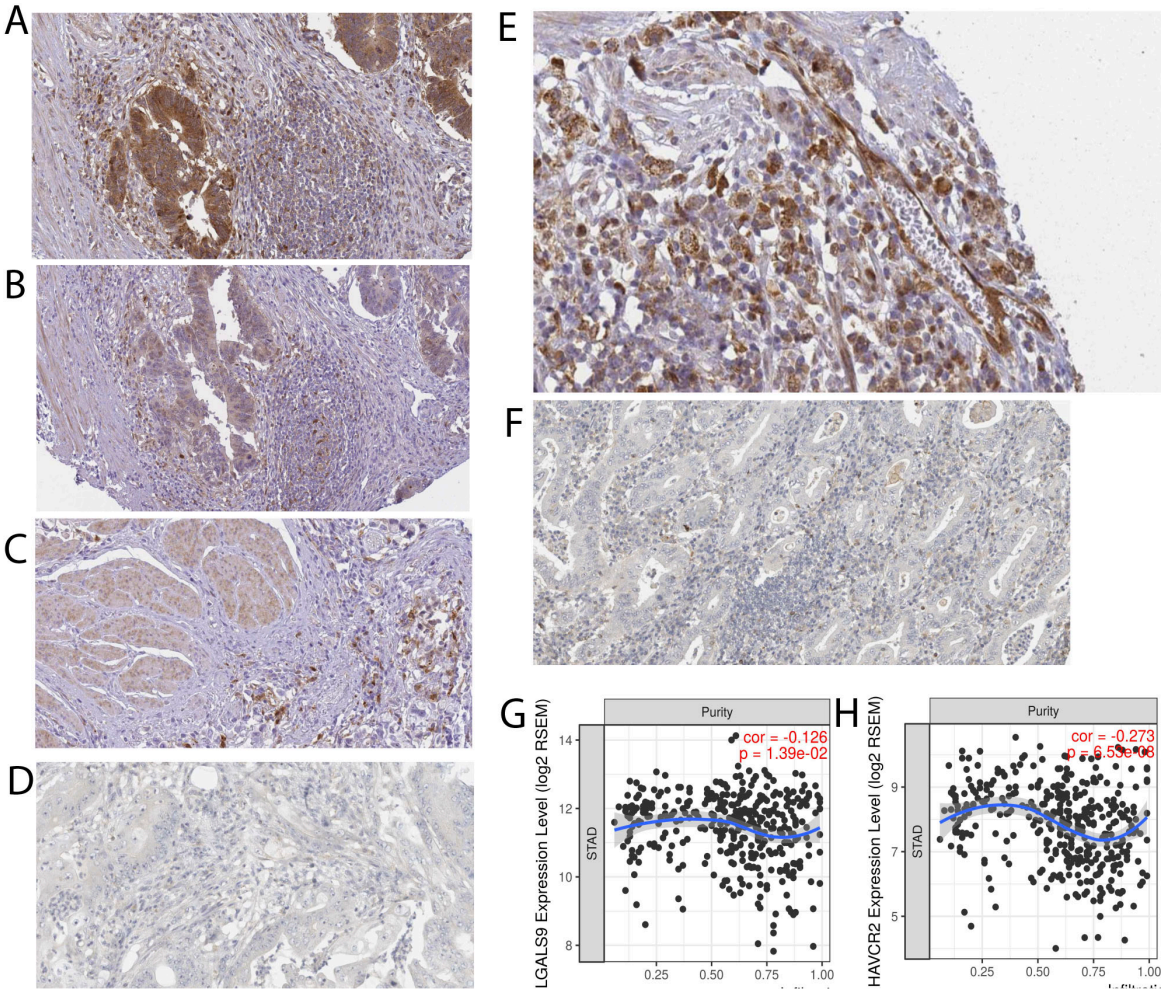

Figure 2: Galectin-9 and Tim-3 expression in Stomach adenocarcinoma
Gal-9 IHC on stomach adenocarcinoma tissue samples from TCGA database revealed different protein expression patterns classified as strong expression (A), Low expression (B) and negative expression (C) according to cancer cell staining (*Human Protein Atlas*). Gal-9 positive endothelium can also be found on moderate expression stomach adenocarcinomas (E). There is also Gal-9 positive staining surrounding the tumour infiltrated immune cells, which can be observed at al expression levels. Tim-3 IHC were classified as negative in all available samples from the TCGA according to cancer cell staining, some samples did not have any staining (D) while others had weak staining on infiltrated areas (F). Pearson's correlation was performed using *TIMER*® to evaluate mRNA expression levels of Gal-9 (*LGALS9*) and Tim-3 (*HAVCR2*) with sample purity, obtaining significative correlation with slopes that tend to 0 (F,G), suggestive of expression in both cancer cells and tumour microenvironment cells.

iii. Elevated Gal-9 and Tim-3 mRNA levels associated with higher levels of other inhibitory immune checkpoints in stomach adenocarcinoma

To evaluate if there was an association between Gal-9 and Tim-3 with other immune checkpoint expressions in gastric tumours. We performed Spearman's regressions using the stomach adenocarcinoma database from TCGA and TIMER© software. A strong association

between Gal-9 and herpes virus entry mediator (HVEM) mRNAs was observed and also moderate positive associations between Gal-9 mRNA and PDL-1, LIGHT, V-domain Ig suppressor of T cell activation (VISTA) and negative associations with CD40L, B7 Homolog 3 (B7-H3), Immunoglobulin Superfamily Member 11 (IGSF11) (Fig.3 A, C, F, H, I) and moderate negative associations with B7-H3 and IGSF11 (Fig.3 B, C). No significant associations were observed between Gal-9 and B7 Homolog 4 (B7-H4), and a weak positive association was observed with PDL-2 mRNA levels (Fig 3. D).

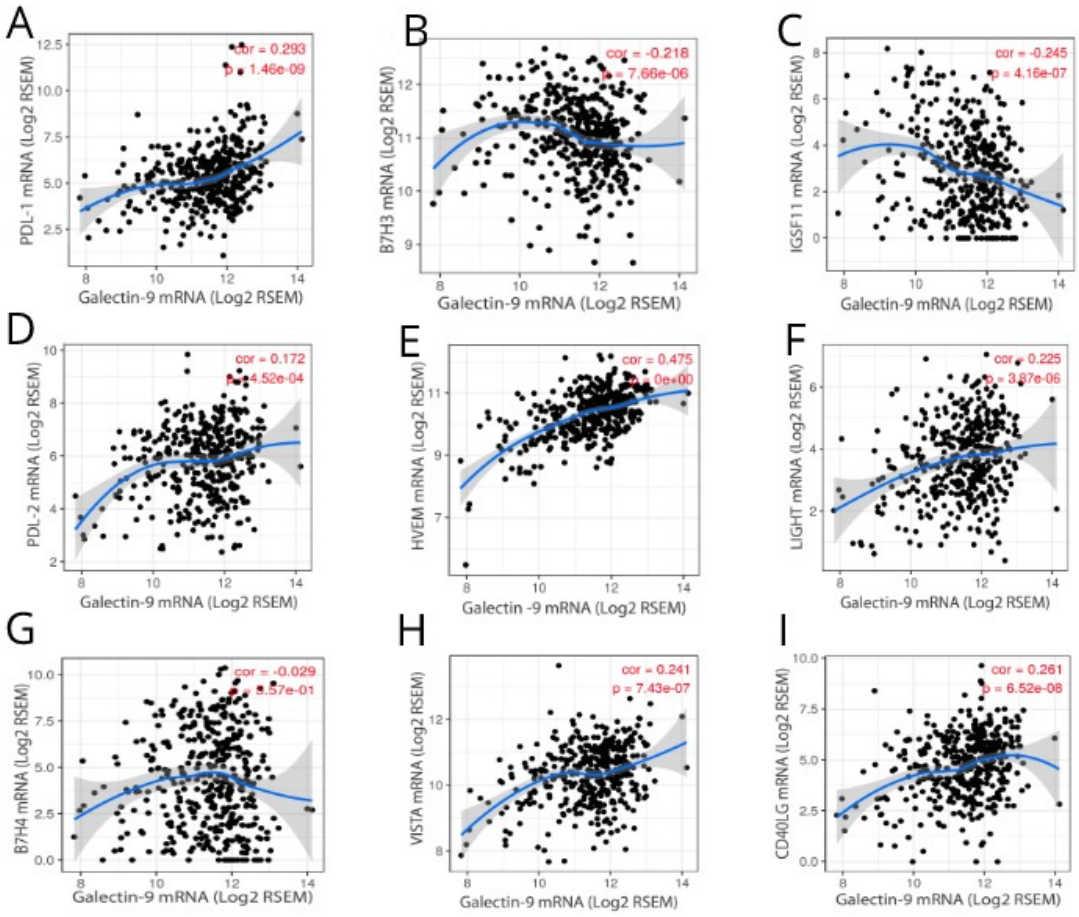

Figure 3: Immune Checkpoints expression correlates with Gal-9 mRNA in stomach adenocarcinoma tumors. Correlation between Galectin-9 mRNA levels and Immune checkpoints mRNA levels within stomach adenocarcinoma tumors was assessed by Spearmans regressions using TIMER© software and the from the STAD 415 data set of TCGA database (n=387). The immune checkpoints assessed were: PDL-1 (A), B7H3 (B), IGSF11 (C), PDL-2 (D), HVEM (E), LIGHT (F), B7H4 (G), VISTA (H), CD40L (I).

In contrast, Tim-3 mRNA levels presented a strong positive association with both PDL-1, PDL-2, LIGHT and CD40L mRNA levels (Fig 4. A,D,F,I). Weak positive associations with B7-H3, HVEM and VISTA. A negative association was observed between Tim-3 mRNA levels and B7-H4, while no association was observed with IGSF11.

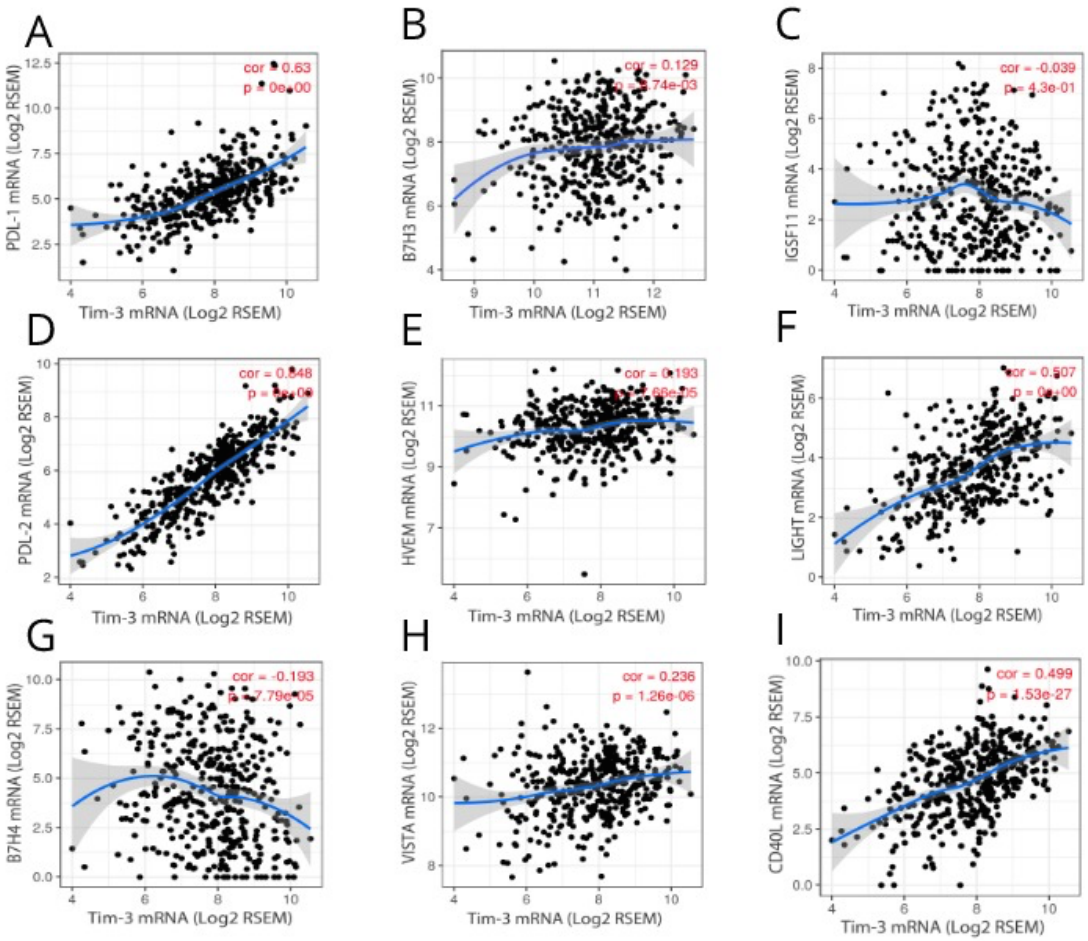

Figure 4: Immune Checkpoints expression correlates with Tim-3 mRNA in stomach adenocarcinoma tumors. Correlation between Tim-3 mRNA levels and Immune checkpoints mRNA levels within stomach adenocarcinoma tumors was assessed by spearman's regressions using TIMER© software and the from the STAD 415 data set of TCGA database (n=387). The immune checkpoints assessed were: PDL-1 (A), B7H3 (B), IGSF11 (C), PDL-2 (D), HVEM (E), LIGHT (F), B7H4 (G), VISTA (H), CD40L (I).

Interestingly, we observed a significant increase in PDL-1 and a strong tendency in Tim-3 expression when Gal-9 was transfected into AGS cells, which are in line with the results observed through bioinformatic analysis from patient samples. Moreover, here we show that

other relevant proteins in immune regulation such as HVEM, VISTA and CD-40L are also associated with Gal-9 and Tim-3 mRNA expression. Taken together, our results suggest the presence of a regulatory network between immune checkpoints, where the lack of one is most likely compensated by another.

iv. Elevated levels of Gal-9 and Tim-3 may be associated with cancer progression and reduced patient survival.

Given that LGALS9 and HAVCR2 levels were associated to increased immune checkpoint expression, and that HAVCR2 was associated to late stages, we evaluated if higher reported levels of mRNA expression were associated to poor clinical outcomes on stage IV patients from the TCGA database.

As shown in figure 5, PFS of highest quartile LGALS9 and lowest LGALS9 quartile presented a median survival of 8.48 and 20.52 respectively, with a Hazard Ratio (HR) of 1.014 and p=0.37 (Fig 5A). In a similar pattern the OS of highest quartile LGALS9 and lowest LGALS9 quartile presented a median survival of 9.99 and 17.48 months respectively, with a HR=1.39 and p=0.07. PFS of highest quartile HAVCR2 and lowest LGALS9 quartile presented a median survival of 4.86 and 14.39 months respectively, with a HR=1.04 and p=0.35 (Fig. 5C). OS of highest quartile HAVCR2 and lowest LGALS9 quartile had a median survival of 6.57 and 13.01 months respectively, with a HR=1.101 and p=0.25 (Fig. 5D). Although no statistical significance was observed, these results show a tendency to reduced PFS and OS in patients with high LGALS9 and HAVCR2 levels.

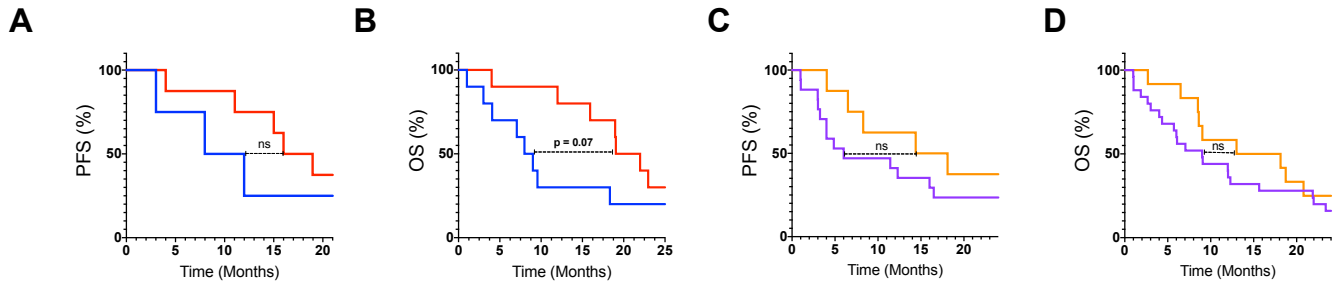

Figure 5. Higher LGALS9 and HAVCR2 levels demonstrate a tendency towards poorer patient survival in stage IV gastric cancer. Kaplan Mayer plots showing progression free survival (PFS) and Overall Survival (OS) in stage IV patients qith Gal-9 high (blue) and Gal-9-low (red) tumors (A,B), and Tim-3 high (purple) and Tim-3 low (orange) tumors (C,D). TCGA STAD 2018, Gehan-Breslow-Wilxocoxn.

2. Determine Gal-9 function in gastric cancer cells.

i. Galectin-9 gain of function upregulates PDL-1 and Tim-3 expression in the AGS cell line

To determine the consequences of Galectin-9 overexpression in gastric cancer cells we transfected AGS cells with a Gal-9 expression vector (CMV3-Gal9) or empty vector as control (CMV3-Empty). To confirm that our transfection model was stable during the time that experiments lasted (24h), we performed WB against and ELISA assay to determine the cellular expression and secretion of Gal-9 on transfected cells (Fig. 6). Gal-9 transfection led to a 10-fold increase in protein expression and secretion without altering cell viability or proliferation as shown in Figure 6 (Fig.6 A-D).

Given our observations from the TCGA database analysis were Gal-9 and Tim-3 levels correlate with the expression of other immune checkpoints, we evaluated the expression of PDL-1 in AGS cell lines transfected with Gal-9 or treated with 0.5µg/mL rhGal-9 after 24 hours. We observed through WB a significant increase in PDL-1 expression in transfected cells (Fig 6.C, E), which was also evidenced by immunofluorescence on rhGal-9 treated cells using a different antibody (Fig 6.C).

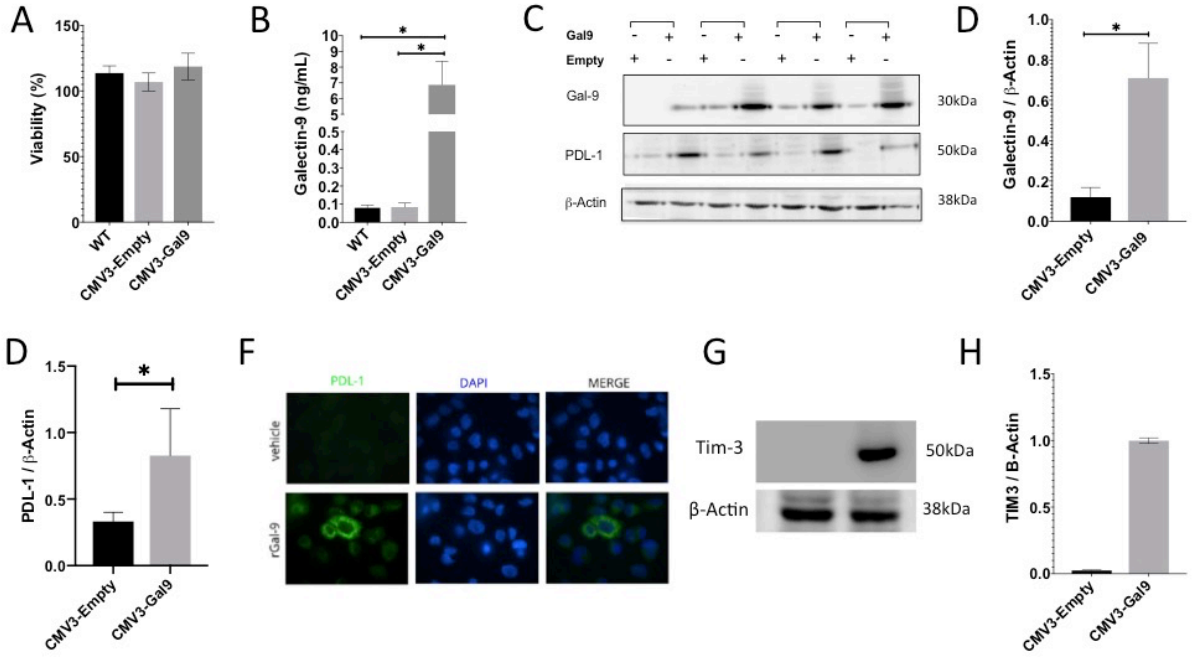

Figure 6. Gal-9 transfected cells display elevated protein levels of cellular Gal-9, secreted Gal-9 and PDL-1. The gastric cancer cell line AGS was transfected with CMV-3 empty vector or containing the Gal-9 gene. Viability was evaluated through WST-1 assay at 24 hours after transfection (A), with no significant differences being observed among transfected cells (Kruskal Wallis). A 700-fold increase in Gal-9 secretion (B) and a 6-fold increase in cellular protein levels (C,D) was observed in Gal-9 transfected cells by ELISA and WB respectively (n=7). PDL-1 protein levels were significantly upregulated in CMV3-Gal-9 transfected cells, which was evidenced by both WB (C,E) and immunofluorescence microscopy (n=3) (F). Gal-9 transfection also resulted in increased protein levels of its receptor Tim-3 (n=2) (G,H). *p<0.05, Kruskal Wallis-One Tail Mann Whitney.

iii. Galectin-9 gain of function in AGS gastric cancer cell line enhances cell migration and invasion

Because Gal-9 has been previously associated to cell migration and invasion and given our results from TCGA database analysis were Gal-9 showed a tendency to poorer patient outcome, we evaluated if Gal-9 could increase cancer cell migration and invasion. Addition of rhGal-9 to the AGS cell line caused a significant increase in cell migration as assessed by the transwell assay (Fig.7 A,B). To evaluate if this phenomenon was restricted only to cancer cells and this particular cell line, we repeated this assay in the GES-1 cell line, which is derived from the immortalization of normal epithelial cells. As observed in figure 7C and 7D, Gal 9

also promotes the migration in gastric epithelial cells. Interestingly when cells were treated with α-Lactose, to block extracellular Gal-9 CRDs, the increase in cell migration was prevented in both cell lines.

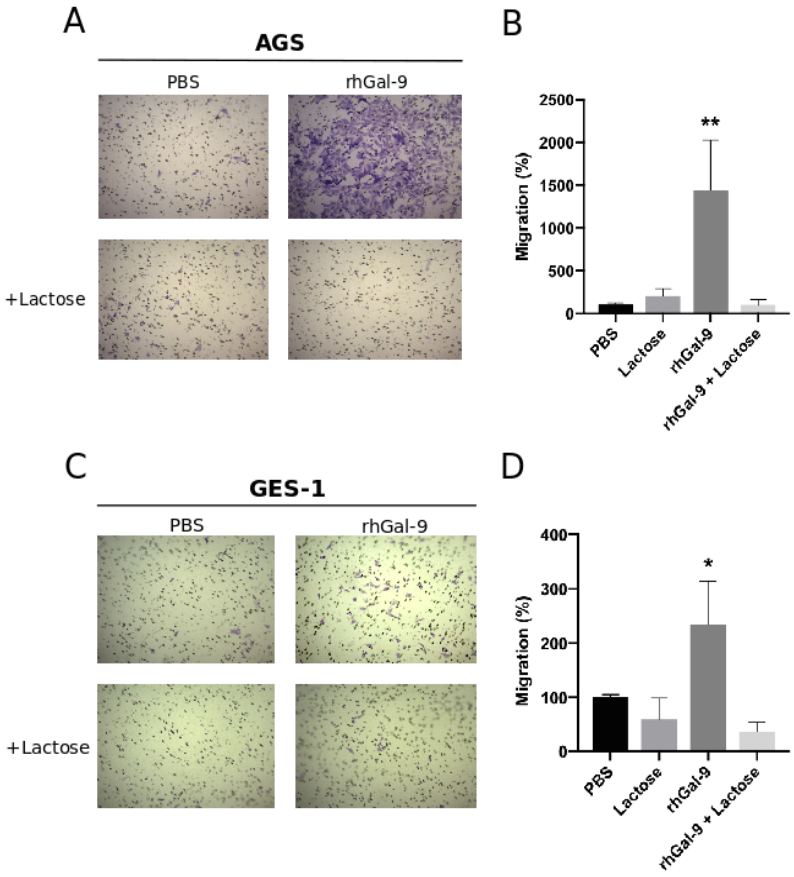

Figure 7. Galectin-9 promotes cancer cell and gastric epithelial cell migration in vitro, an effect blocked by the addition of Lactose. Migration assays were performed using the gastric cancer cell line AGS and the normal gastric epithelial cell line (GES-1). Cells were treated with vehicle (PBS), Lactose 30mM, rhGal-9 0.5ug/mL or rhGal9 0.5ug/mL + Lactose 30mM for 16h. After incubation cells were fixed and stained with Crystal Violet, representative photographs of stained membranes show that the increase in cell invasion can be prevented by addition of Lactose (A, C). Cells were counted and migration percentage was calculated (B,D). A significant increase in migration was observed in both cell lines, which was effectively prevented by the addition of Lactose. *$p<0.05$ Kruskal-Wallis, U-Man Whitney, n=3.

The invasive potential of the cancer is directly related to poor patient prognosis. To investigate if Gal-9 enhances cancer cell invasion we repeated our migration assays in the presence of

Matrigel, which acted as a surrogate extracellular matrix. AGS cells that had increased Gal-9 due to their transfection with an expression vector showed a significant increase in invasion (Fig. 8). To determine if this was an effect of extracellular Gal-9 we also treated AGS cells with rhGal-9 and used α-Lactose to block Gal-9. Similar to our results from the migration assays, α-Lactose treatment significantly reduced invasion on Gal-9 transfected and rhGal-9 treated cells (Fig.8A-C). Taking together, hese results suggest that extracellular secreted Gal-9, but not intracellular Gal-9, has an effect on migration/invasión.

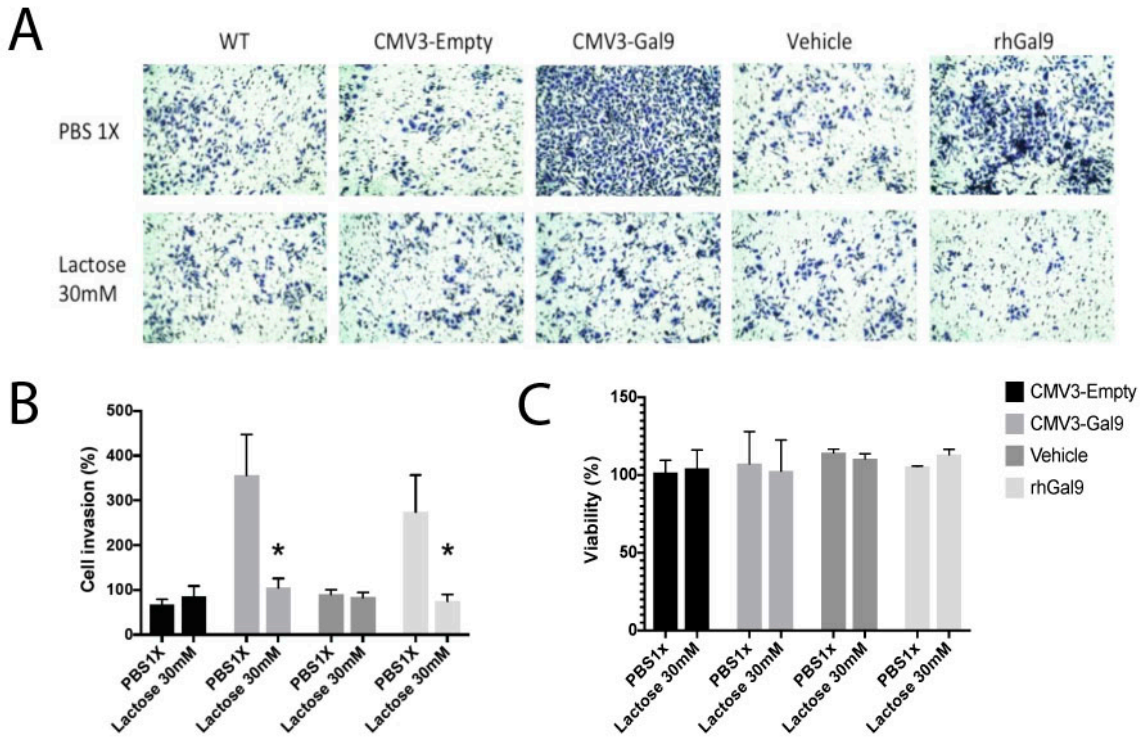

Figure 8. Blockade of extracellular Galectin-9 with Lactose prevents cell invasion of gastric cancer cells.
Matrigel invasion assay was performed with transfected AGS cells and human recombinant Galectin-9 treated AGS cells or vehicle with or without lactose. Representative photographs of stained membranes show that the increase in cell invasion can be prevented by addition of Lactose 30mM (A). Lactose induces a significant reduction in cell invasion in Gal-9 transfected cells and recombinant Gal-9 treated cells (B). Lactose treatment did not affect cancer cell viability (C). *$p>0.05$ *in paired t-Student Test.*

When performed in transfected GES-1 cell line, this same experimental procedure also demonstrated an increase in invasion in the presence of extracellular Gal-9 (Fig 9). The lower levels of invasion observed in the GES-1 cell line are in accordance with normal epithelial cells have less invasive potential than the cancer cell. These results suggest that Gal-9 promotion of cell migration and invasion may be both the physiological and pathophysiological.

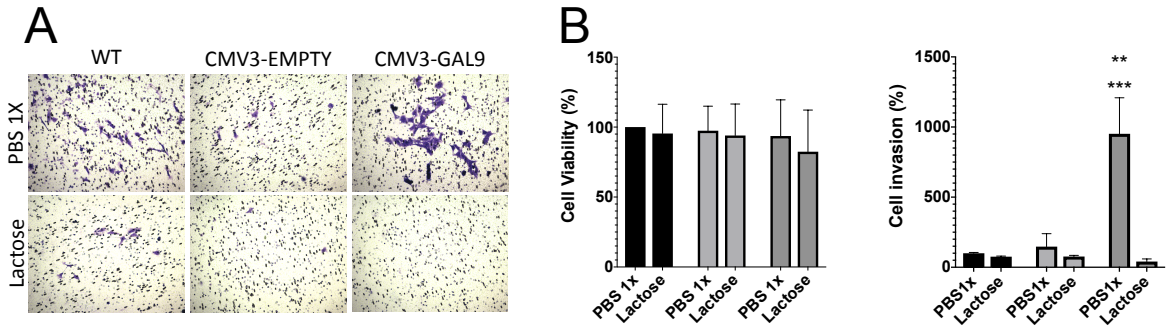

Figure 9. Blockade of extracellular Galectin-9 with Lactose prevents cell invasion of gastric epithelial cells. Matrigel invasion assay was performed with transfected GES-1 cells with or without Lactose. Representative photographs of stained membranes show that the increase in cell invasion can be prevented by addition of Lactose 30mM (A). Quantifications of cell invasion are represented in (B) were lactose induces a significant reduction in cell invasion in Gal-9 transfected cells and recombinant Gal-9 treated cells (B). Lactose treatment did not affect cancer cell viability (C). **p>0.05 paired t-Student Test*.

3. Evaluate if Gal-9 promotes angiogenesis *in vitro*

It has been previously described that tumour endothelial cells express Gal-9 in other cancer types such as breast and lung cancer (39). Analyzing Gal-9 immunohistochemestries in stomach adenocarcinoma samples from the TCGA database (Fig 2.E) we observed that 60% of patients with high or moderated Gal-9 expression within cancer cells had positive Gal-9 staining on vessel walls.

To determine if Gal-9 may play a role in angiogenesis, we assessed if rhGal-9 could promote tubule formation (in vitro angiogenesis assay) in HUVEC cells. As shown in figure 10, when endothelial cells grown on Matrigel are treated with rGal-9 there is an increase in tubular

structure formation, an effect that is inhibited by the addition of lactose. No changes in cell viability were observed at 24h of treatment (Fig 10. A). As migration is a principal component of angiogenesis, using scratch assays we assessed if GAL9 was enhancing cancer cell migration. Once again, rhGAL9 presence resulted in a significant increase in migration, which was abrogated by the presence of lactose. The specificity of lactose for rhGAL-9 is demonstrated by the failure of this disaccharide to inhibit extracellular VEGF form increasing tube formation (Fig 10.B, C).

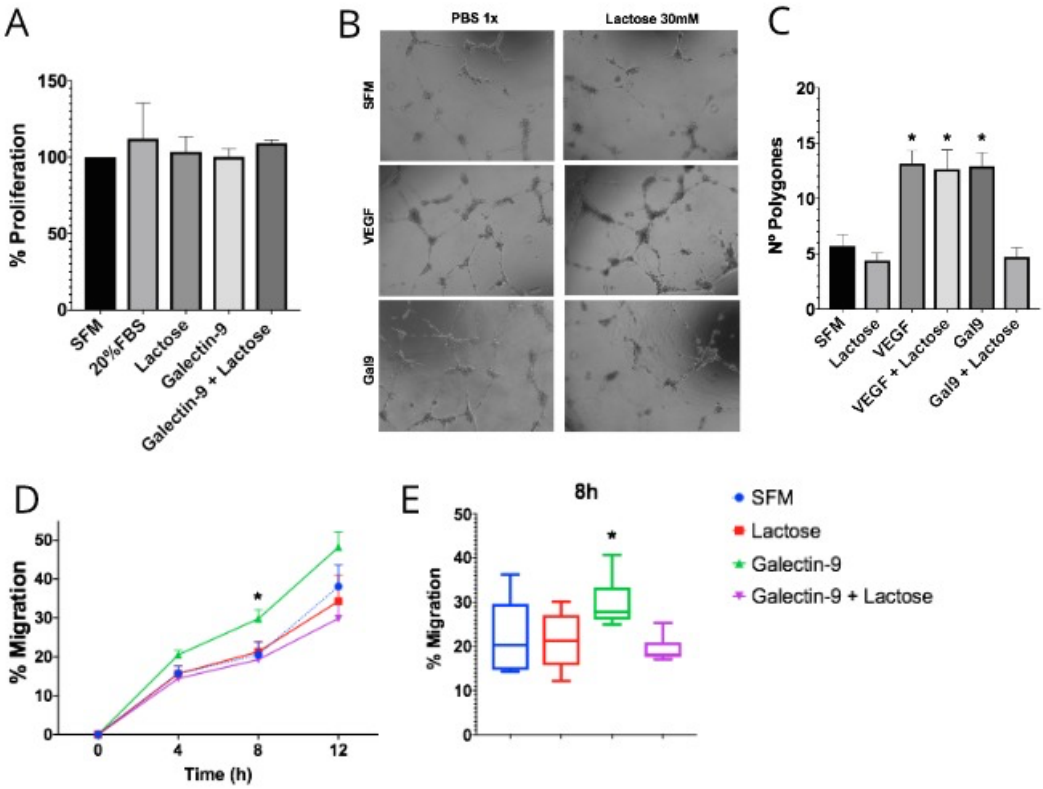

Figure 10. Galectin-9 increases endothelial cell tube formation and migration
HUVEC cells treated with rGal-9 in presence or absence of Lactose 30mM. No significant changes in cell viability/proliferation were detected after 24 hours; n=4 (A). Tube formation was significantly increased in Gal-9 treated cells, an effect that was prevented by lactose addition; n=5 (B,C). Migration of HUVEC cells was assessed by scratch assays, were a significant increase in %migration was observed with rGal-9 treatment, but not in rGal-9 and lactose treated cells (n=5). *p<0.05, Kruskal-Wallis, Mann-Whitney.

4. Determine if Gal-9 increases CD8+ T cell dysfunction and Treg frequency

An immunosupressive microenviroment is clinically associated to reduce patient survival. To determine if there is an association between Gal-9 and Tim-3 mRNA levels with T cell exhaustion marker expression in gastric tumours, we performed Spearman's regressions using the stomach adenocarcinoma database from TCGA and TIMER© software. Figure 11 demonstrates a significant correlation between Galectin-9 mRNA levels with PD1 levels and LAG-3 mRNA levels was observed in stomach adenocarcinomas (Fig 11. A, D), while there was no relationship with TOX and Eomesodermin (EOMES) mRNA expression (Fig 11. B, C). Strong positive correlations were observed between Tim-3 mRNA levels and PD1, EOMES and LAG-3 mRNA levels (Fig 11 E, G, H). No association between Tim-3 and TOX mRNA levels was observed (Fig 11 F).

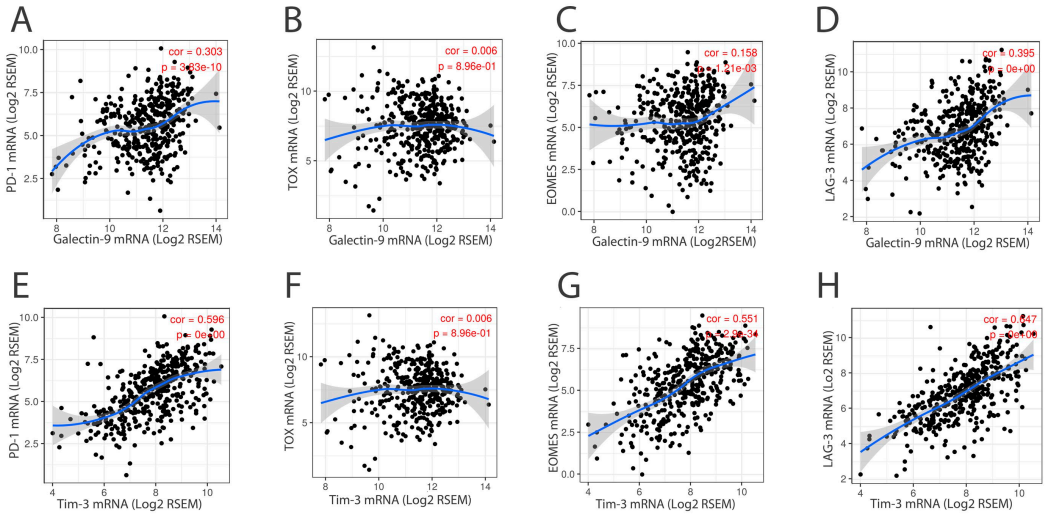

Figure 11. Galectin-9 and Tim-3 mRNA levels correlation with expression of T cell exhaustion genes in Stomach Adenocarcinoma samples.
Weak correlation between Galectin-9 mRNA levels with PD-1 levels and LAG-3 mRNA levels (A,D) was observed in stomach adenocarcinomas, while there was no relationship with TOX and EOMES mRNA expression (B,C). Strong positive correlations were observed between Tim-3 mRNA levels and PD-1, EOMES and LAG-3 mRNA levels (E,G,H). No associations between Tim-3 and TOX mRNA levels were observed (F). Spearman's regressions were performed using TIMER© software and the from the STAD 415 data set of TCGA database (N=387).

Moreover, using the Cibersort algorithm on TIMER® we observed that Gal-9 correlated with $CD8^+$ T cell infiltration with a Rho value of 0.355 and $p<0.001$ using Spearman's regression (Fig. 12). Due to the correlation with exhaustion associated genes, we further performed a gene enrichment analysis according to $CD8^+$ T cell dysfunctional signature that was previously published in the literature, and had demonstrated association with immunotherapy resistance (Jiang et al 2018). Spearman's regression analysis showed that Gal-9 mRNA levels within the tumour were significantly associated with a $CD8^+$ T cell exhaustion molecular signature (Fig. 12 B).

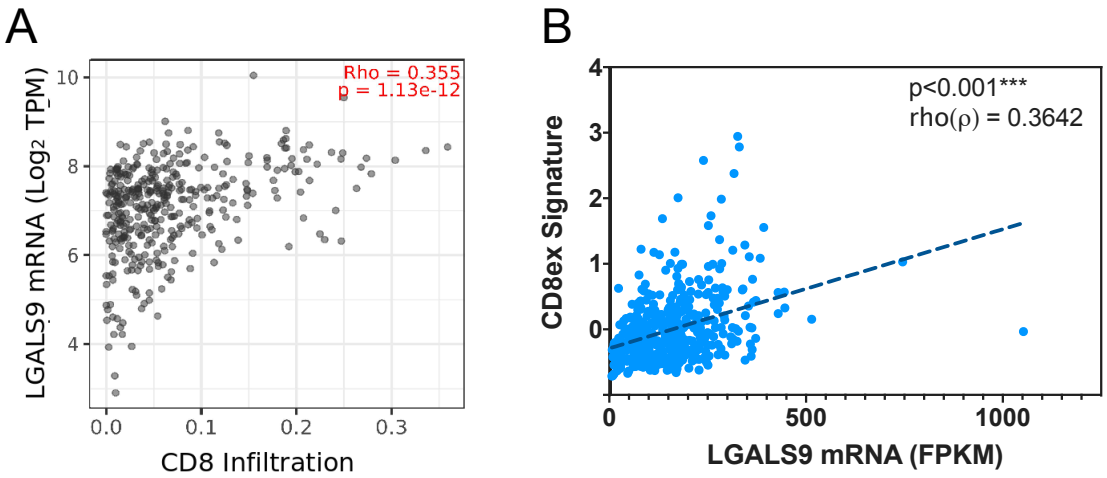

Figure 12: Galectin-9 mRNA correlates with CD8 infiltration and an exhausted CD8 T cell transcriptional signature. Positive correlation between LGALS9 mRNA and Treg signature was observed using Cibersort ABS data analysis of the STAD 415 data set from TCGA database (N=387), Spearman's regression was performed using TIMER© software with a rho value of 0.386 and $p<0.001$ (A). An independent gene enrichment analysis using an exhausted $CD8^+$ (CD8ex) on TCGA dataset was also performed, observing with a positive correlation through Spearman's regression, Rho value=0.3642 and $p<0.001$ (B).

Association between Gal-9 and Tim-3 with Treg markers was also evaluated. Weak and moderate correlations between Galectin-9 mRNA levels with CD25 and FOXP3 mRNA levels were observed (Fig 15. A, B), although there was no relationship with TGFβ or IL-10 mRNA expression (Fig 15. C,D). Strong positive correlations were observed between Tim-3 mRNA

levels and CD25, TGFβ and IL-10 mRNA levels (Fig. 15 E, G, H), while a strong association was found with FOXP3 mRNA levels (Fig 15.F).

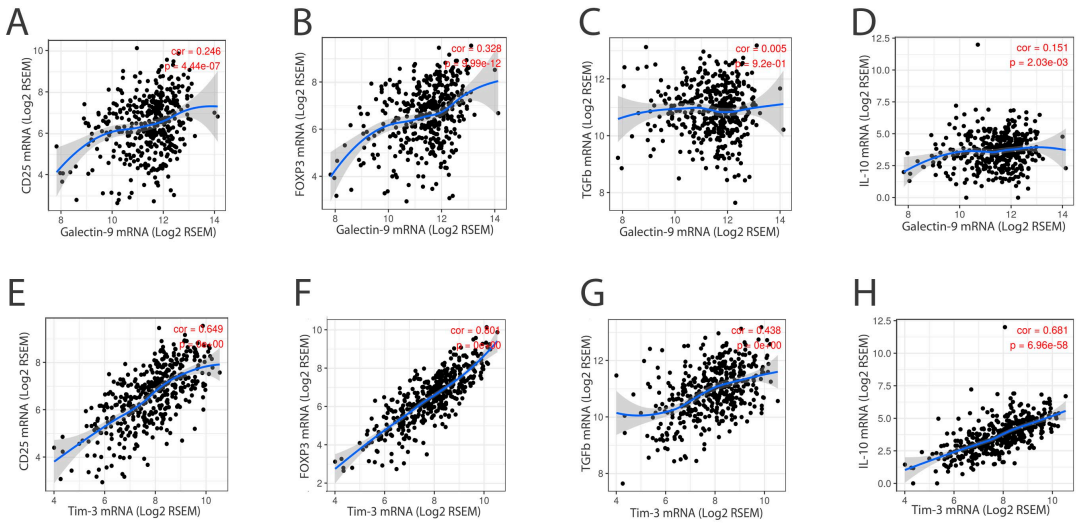

Figure 13. Galectin-9 and Tim-3 mRNA levels correlation with expression of Treg associated genes in Stomach Adenocarcinoma samples.
Weak and moderate correlations between Galectin-9 mRNA levels with CD25 and FOXP3 mRNA levels were observed (A,B), although there was no relationship with TGFβ or IL-10 mRNA expression (C,D). Strong positive correlations were observed between Tim-3 mRNA levels and CD25, TGFβ and IL-10 mRNA levels (E,G,H), while a very strong association was found with FOXP3 mRNA levels (F). Spearman's regressions were performed using TIMER© software and the from the STAD 415 data set of TCGA database (N=387).

We further evaluated if there was a correlation between Gal-9 mRNA and Treg infiltration using CIBERSORT ABS on TIMER® and observed that there was a positive correlation with a Rho value of 0.234 and $p<0.001$ (Fig 14A). This analysis may suggest that tumours with higher Gal-9 levels also present increased Treg infiltration. However, as Cibersort utilizes molecular signatures based on natural Treg and mice models, we further evaluated if similar results could be obtained with a specific signature for tumour infiltrating Tregs. To this end, we performed a gene enrichment analysis based on Magnunson *et al*. "Tumour infiltrating Treg" signature which has been validated in both mice and human tumours (162). Our analysis of gastric adenocarcinomas noted a strong association between Gal-9 mRNA levels and this

Treg signature, with a Rho value of 0.965 and p<0.001 after Spearman's regression (Fig 12.B). These results support the hypothesis that Gal-9 may be driving Treg expansion and T cell immune suppression, most likely through Tim-3 signalling.

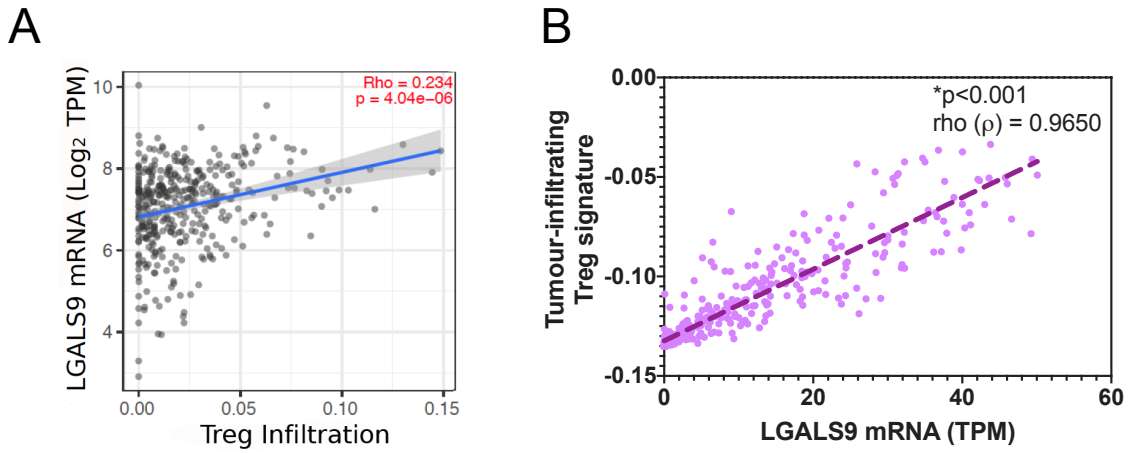

Figure 14. Galectin-9 mRNA levels correlate with Treg transcriptional signatures in Stomach Adenocarcinoma. Positive correlation between LGALS9 mRNA and Treg signature was observed using Cibersort ABS data analysis of the STAD 415 data set from TCGA database (N=387), Spearman's regression was performed using TIMER© software with a rho value of 0.386 and p<0.001 (A). An independent analysis using a specific Tumor infiltrating Treg transcriptional signature was performed on TCGA dataset, obtaining a positive correlation through Spearman's regression, Rho value=0.3642 and p<0.001 (B).

5. Determine if Gal-9 provides resistance to αPD1 therapy in vitro through Tim-3.

i. Galectin-9 decreases CD8$^+$ T cells while increasing the frequency of dysfunctional like CD8$^+$ T cells through Tim-3

Given that the Gal-9 mRNA levels are associated with a CD8$^+$ T cell dysfunction signature in gastric tumours, we evaluated if Gal-9 promoted the frequency of dysfunctional CD8$^+$ T cells *in vitro*. T cells were isolated with informed consent from healthy donors (n=8) and stimulated with CD3/CD28 activating beads in presence of rhGal-9 at increasing concentrations (0.25µg/mL, 0.5µg/mL and 1µg/mL). T cells were then cultured for 96 hours, stained with anti-CD8, anti-PD1, anti-Tim3, anti-LAG3, anti-IFNγ and NeaR IR Dead Cell stain

(Thermofisher). $CD8^+$, $IFN\gamma^+$, $PD1^+Tim3^+$ and $PD1^+Tim3^+LAG3^+$ populations were evaluated through flow cytometry.

We first observed that rhGal-9 significantly reduced the frequency of $CD8^+$ T cells, without a significant effect on total viability (Fig 15A-C). Although Gal-9 effectively decreased $CD8^+$ T cell frequency, IFNγ production within this population was not changed (Fig. 15D). This result indicates that Gal-9 not only affects $CD4^+$ T cells as previously reported in the literature (44,57,80), but also alters $CD8^+$ T cell frequency. To determine if Gal-9 could promote $CD8^+$ T cell dysfunction we assessed the frequency of $PD1^+Tim3^+$ and $PD1^+Tim3^+LAG3^+$ T cells within the $CD8^+$ population. Interestingly, we observed that Gal-9 significantly increased not only the frequency of $PD1^+Tim3^+$ (Fig. 15E,F) cells, but also the $PD1^+Tim3^+LAG3^+$ T cell population (Fig. 15H,G), which have been described as 'late-dysfunctional' or terminally dysfunctional T cells.

Given that Gal-9 has been proposed as a Tim-3 ligand, we then treated cells with rhGal9 in presence of a Tim-3 blocking antibody. Blockade of Tim-3 significantly abrogated (albeit in part) the decrease in $CD8^+$ population (Fig. 16A) and the increase in dysfunctional $PD1^+Tim3^+$ (Fig 16B). The increase in $PD1^+Tim\text{-}3^+LAG3^+$ T cell population by Gal-9 was completely abolished by the inhibition of Tim-3 (Fig 16C). This evidence suggests that the increase in $CD8^+$ T cell dysfunction produced by Gal-9 is mediated by the Tim-3 receptor.

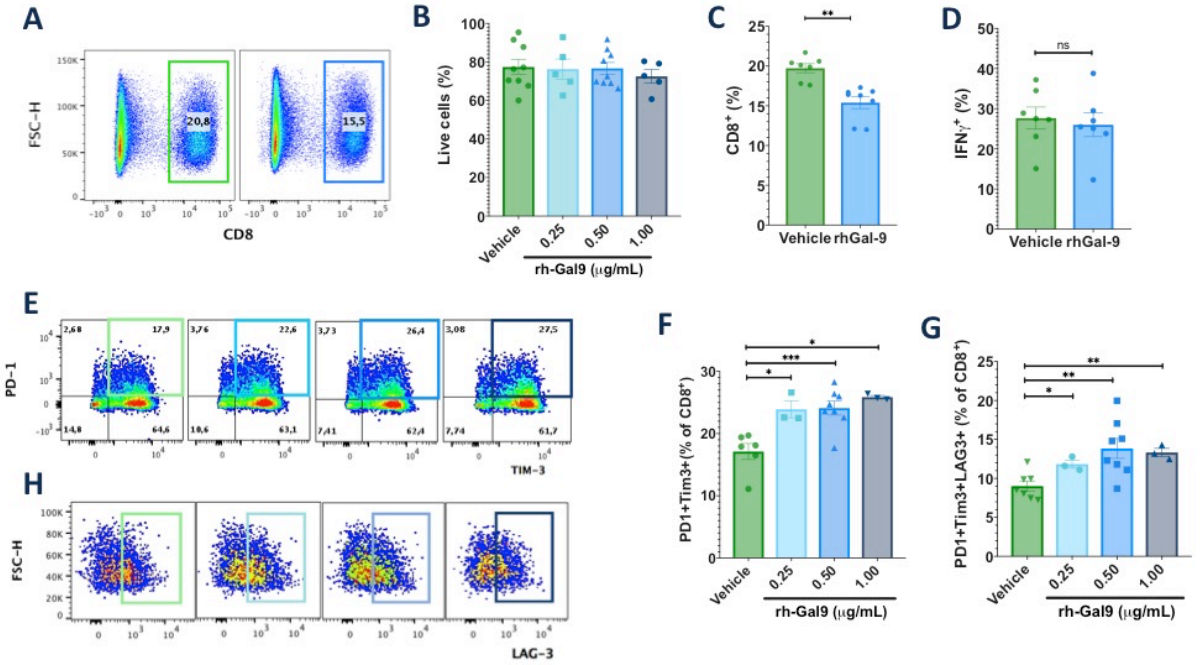

Figure 15. Galectin-9 decreases CD8$^+$ T cell population while increasing the frequency of CD8$^+$ dysfunctional populations PD1$^+$Tim3$^+$ and PD1$^+$Tim3$^+$LAG3$^+$. Treatment of isolated T cells from healthy donors (n=8) were activated with aCD3/aCD28 beads and treated with rhGal-9 or vehicle (PBS). After 96h cells were harvested, stained and fixed for flow cytometry. rhGal9 decreased the frequency of CD8+ T cells (A,C) however it did not have an effect on total cell viability or IFNγ production (B,D). Within the CD8+ T cell population, both PD1+Tim3+ (E,F) and PD1+Tim3+LAG3+ frequencies were increased with rhGal9 (G,H). **p<0.05; **p<0.01; ***p<0.001 Kruskal Wallis – Mann Whitney.*

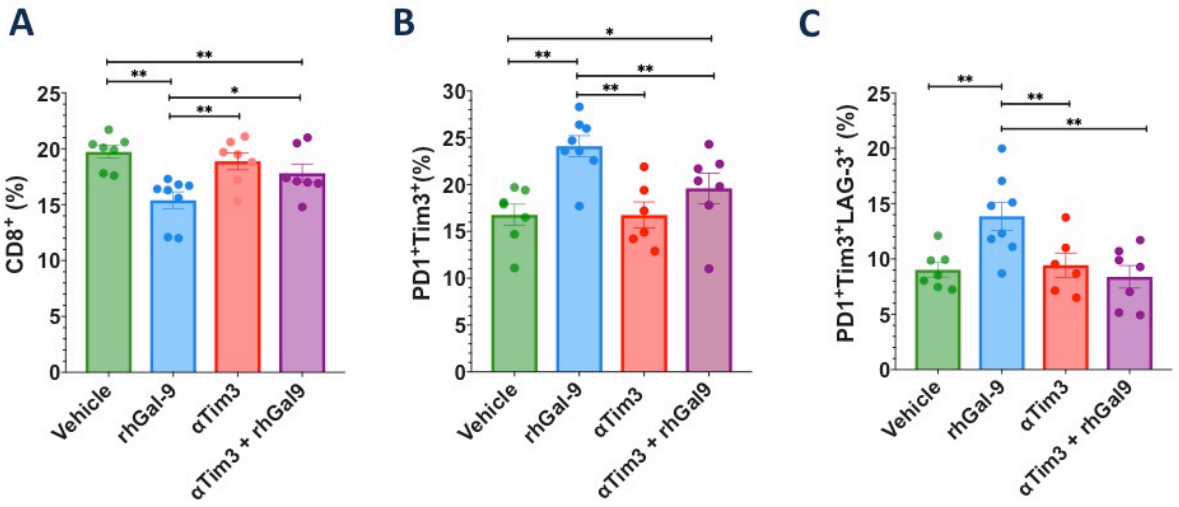

Figure 16. Tim-3 blockade prevents the decrease in CD8$^+$ T cell population and the increase in the frequency of CD8+ dysfunctional populations PD1$^+$Tim-3$^+$ and PD1$^+$Tim-3$^+$LAG3$^+$ mediated by rhGal-9. Treatment of isolated T cells from healthy donors (n=8) were activated with aCD3/aCD28 beads and treated with rhGal-9 or vehicle (PBS) in presence or absence of a Tim-3 blocking antibody. After 96h cells were harvested, stained and fixed for flow cytometry, addition of aTim-3 significantly reduced the effect of rhGal-9 on the frequency of CD8+ (A), PD1+Tim3+ (B) and PD1+Tim3+LAG+ (C) T cells. **p<0.05; **p<0.01; ***p<0.001 Kruskal Wallis – Mann Whitney.*

ii. Galectin-9 increases the frequency of Treg through Tim-3

Considering that Treg presence in the TME clinically correlates with poor survival and that LGALS9 levels strongly correlated with Tumour Treg transcriptional signature, we evaluated if rhGal-9 could be expanding Treg *in vitro*. First, we stimulated peripheral T cells with αCD3/αCD28 activating beads and then incubated them in the presence of rhGal-9 at different concentrations (0.25, 0.5 and 1.0µg/mL). After 96h, cells were stained with antibodies against the surface markers CD25 and CD127, fixed and permeabilized for intracellular staining of CD4 and FOXP3. The frequency of Treg, defined by the percentage of CD25+FOXP3+ cells within the CD4+CD127+ population, was evaluated by flow cytometry. In line with our results from bioinformatic analysis, we observed that rhGal-9 significantly increased Treg frequency *in vitro* (Fig. 17A,B) at each of the three concentrations used.

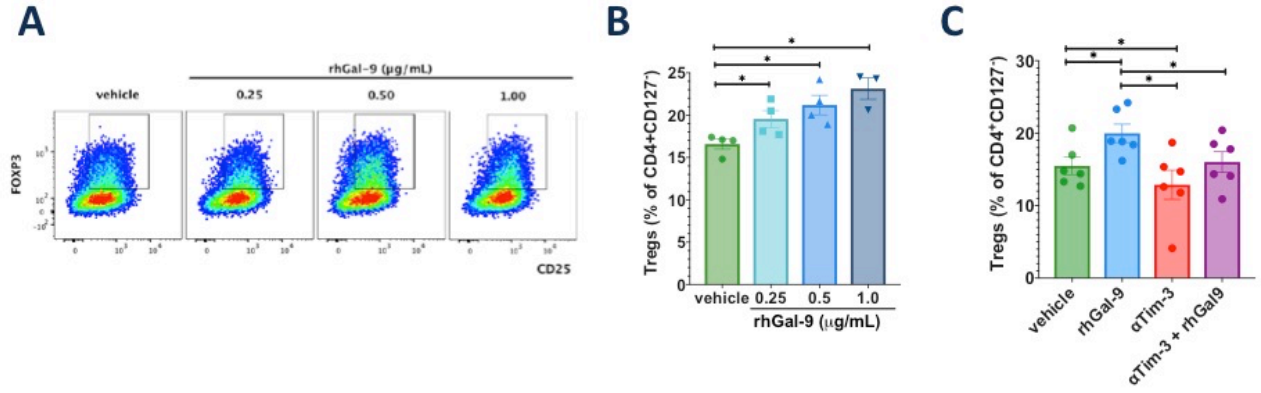

Figure 17. Gal-9 increases Treg frequency in a Tim-3 dependent manner. Isolated T cells from HD were activated with aCD3/aCD28 beads and treated with rhGal-9 or vehicle (PBS) in presence or absence of a Tim-3 blocking antibody. After 96h, Treg frequency (CD4+CD127-CD25+FOXP3+ %) was assessed by flow cytometry. rhGal-9 significantly increased Treg frequency (n=4)(A,B), while the addition of an aTim-3 blocking antibody effectively reduced the increase in Tregs mediated by rhGal-9 (n=6) (C) **p<0.05; **p<0.01; ***p<0.001 Kruskal Wallis – Mann Whitney.*

Since Treg express the Gal-9 receptor, Tim-3, we then evaluated if the increase in Treg frequency required Tim-3 by adding the αTim-3 blocking antibody. Similarly to our results with $CD8^+$ T cells, aTim-3 prevented the increase in Treg frequency. Interestingly, αTim-3 treatment also significantly reduced Treg frequency in absence of rhGal-9, suggesting that Tim-3 may be necessary to maintain the Treg population.

iii. Galectin-9 expressing cancer cells promote $CD8^+$ T cell dysfunction and increase Treg frequency.

We evaluated if Gal-9 expressing cancer cells, were capable of increasing $CD8^+$ T cell dysfunction and Treg expansion, as was observed upon the addition of the recombinant Gal-9 protein. For this we transfected the AGS cell with CMV3-empty or CMV3-Gal9 and co-cultured with isolated T cells from healthy donors in a 1:50 ratio of cancer cells to T cells. Upon 96 hours of incubation, T cells were harvested, and stained with antibodies against CD4, CD8, CD127, CD25, LAG3, FOXP3, PD-1 and Tim-3 for flow cytometry analysis (Fig. 18). The co-culture of T cells with transfected cancer cells significantly decreased T cell viability; however, no significant differences were observed between T cells co-cultured with CMV3-Empty or CMV3-Gal9 transfected cells (Fig 18A). No significant changes were observed in $CD8^+$ (Fig. 18B) or $CD4^+$ populations (Fig. 18C), although a tendency to decrease in $CD4^+$ frequency can be observed on T cells co-cultured with cancer cells. In accordance with the results obtained with rhGal-9, cancer cells transfected with Gal-9 significantly increased the percentage of $CD8^+PD1^+Tim3^+$ (Fig. 18D), $CD8^+PD1^+Tim3^+LAG3^+$ (Fig. 18E) T cell populations and the Treg frequency (Fig. 18F). These results indicate that Gal-9 expressing cancer cells can promote $CD8^+$ T cell dysfunction and Treg expansion.

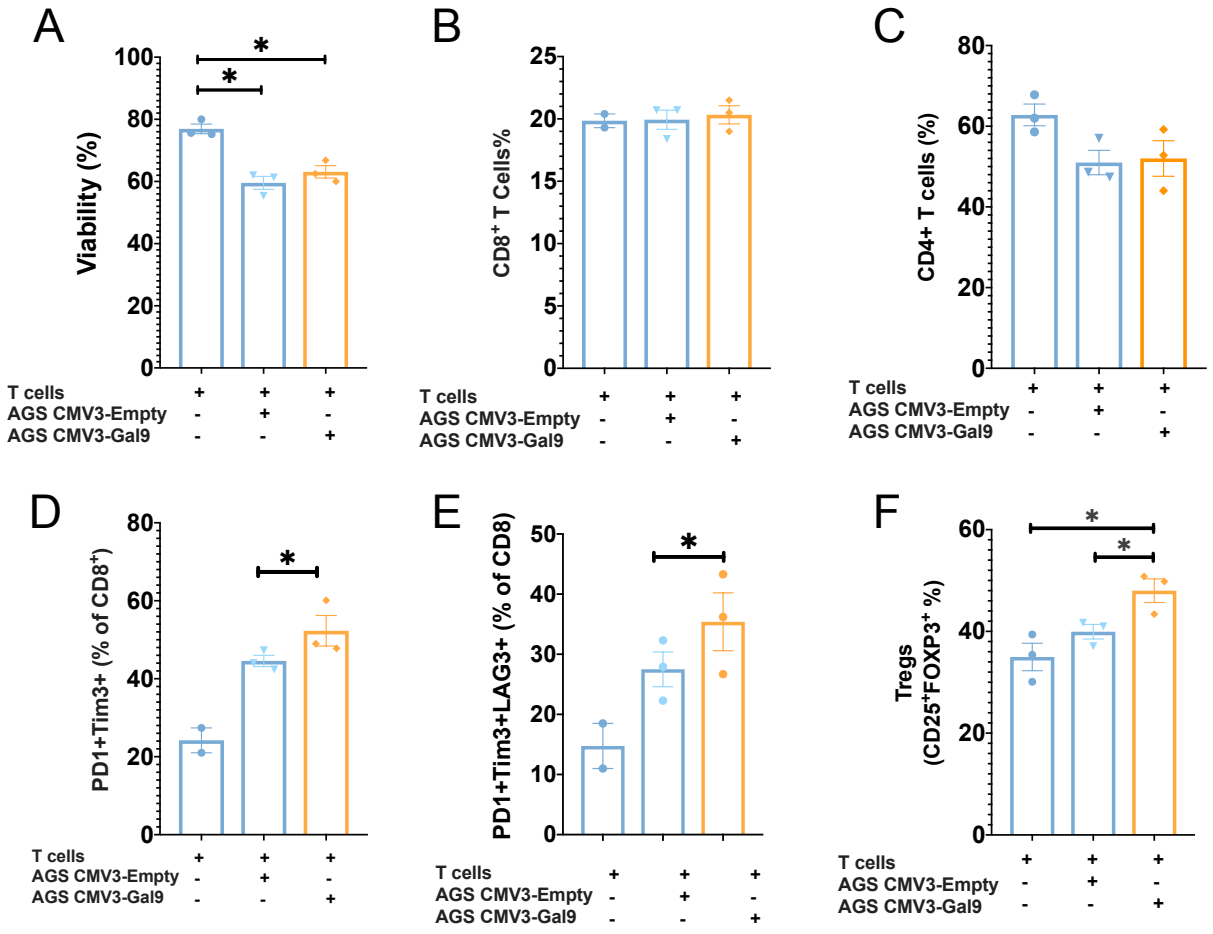

Figure 18. Co-culture of Gal-9 expressing cancer cells and isolated T cells increases $CD8^+$ dysfunctional populations and Treg frequencies *in vitro*. AGS cells were transfected with CMV3-empty or CMV3-Gal9 vectors and co-cultured with isolated T cells from healthy donors in a 1:50 ratio of cancer cells:T cells. After 96h, T cells were harvested, and stained with antibodies against CD4, CD8, CD127, CD25, LAG3, FOXP3, PD-1 and Tim-3 for flow cytometry analysis. Co-culture of T cells with cancer cells significantly decreased T cell viability (A), while no significant changes were observed in $CD8^+$ or $CD4^+$ populations (B,C). Gal-9 expressing cancer cells significantly increased the percentage of $PD1^+Tim3^+$ (D), $PD1^+Tim3^+LAG3^+$ (E) and $CD25^+FOXP3^+$ (Treg) (F) populations. *$p<0.05$, $n=3$, One-tail Mann Whitney test.*

6. The Galectin-9 mediated increase in dysfunctional like $CD8^+$ T cells is maintained in the presence of an anti-PD1 blocking antibody.

Dysfunctional or exhausted T cells have been associated to αPD1 resistance in different cancer types, including gastric cancer. Given that both rhGal-9 and Gal-9 expressing cancer cells

increased $PD1^{+}Tim3^{+}$ and $PD1^{+}Tim3^{+}LAG^{+}$ frequencies, we evaluated if this could be maintained in the presence of a PD1 blocking antibody. Briefly, isolated T cells were activated with αCD3/CD28 beads and treated with rhGal-9 in presence or absence of αPD1 and αTim3 antibodies. The percentage of $CD8^{+}$ T cells, and the dysfunctional populations $PD1^{+}Tim3^{+}$ and $PD1^{+}Tim3^{+}LAG3^{+}$ were assessed by flow cytometry (Fig. 19).

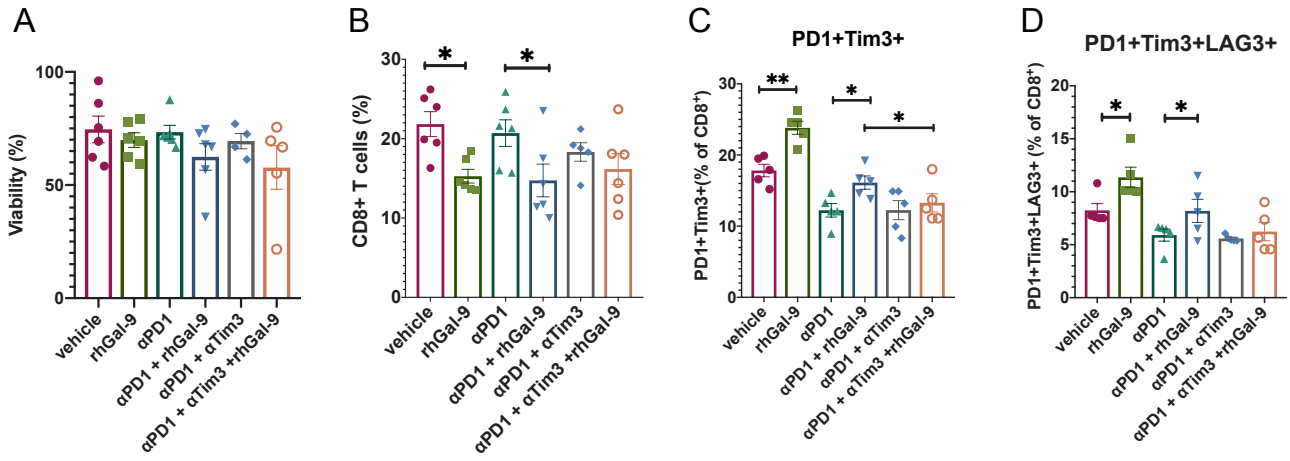

Figure 19. Gal-9 promotes T cell dysfunction in the presence of aPD1 blocking antibody.
T cells isolated from healthy donors were activated with αCD3/CD28 beads and treated with rhGal9 in presence or absence of αPD1 blocking antibodies or a combination of αPD1 and αTim3 antibodies. After 96h, cells were harvested and stained for flow cytometry. No significant changes were observed in total T cell viability (A), however a significant decrease was observed in the percentage of $CD8^{+}$ cells treated with rhGal in presence or absence of the αPD1 antibody (B). $PD1^{+}Tim3^{+}$ and $PD1^{+}Tim3^{+}LAG3^{+}$ frequencies were increased when cells were treated with rhGal9 in presence or absence of the aPD1 antibody (C,D), however only $PD1^{+}Tim3^{+}$ increase was prevented by the combination of aPD1+aTim3 antibodies. **p<0.05, **p<0.01, n=5, Kruskal Wallis, Mann Whitney.*

As shown in figure 19, despite inter-experimental fluctuation, no significant changes in cell viability were observed (Fig. 19A). However, $CD8^{+}$ frequency was decreased by rhGal9 addition as reported previously, however this persisted in the presence of the αPD1 blocking antibody (Fig.19B), an effect that was not prevented by adding αTim3. Interestingly, rhGal-9 increased the frequency of $PD1^{+}Tim3^{+}$ and $PD1^{+}Tim3^{+}LAG3^{+}$ cells, even in presence of an αPD1 antibody (Fig. 19C-D). Furthermore, addition of an αTim-3 antibody effectively

decreased $PD1^{+}Tim3^{+}$ cells (Fig.19C), while a similar tendency was observed on the frequency of the $PD1^{+}Tim3^{+}LAG3^{+}$ cells (Fig. 19D). These results indicate that the presence of Gal-9/Tim-3 may promote T cell dysfunction and Treg frequency even in the presence of an αPD1 antibody.

Discussion

The development of Immune Checkpoint Blockers (ICB) is revolutionizing GC therapy. However, the presence of an immune-suppressive tumor microenvironment and redundancy between inhibitory immune checkpoints may be responsible for why a high percentage of patients do not show clinical benefit. An emerging target is the inhibitory receptor Tim-3. Despite this, the expression and the role of its ligand Gal-9 in GC is still undeciphered.

I. Galectin-9 and Tim-3 levels increase in advanced stage stomach adenocarcinoma and may impact clinical outcome

Using the stomach adenocarcinoma RNAseq database (STAD 2018) from chemotherapy naïve patients from the TCGA database (n=379), we evaluated the mRNA levels of Galectin-9 and Tim-3 mRNA. First we observed that both were upregulated in tumour samples when compared to adjacent tissue, and that Tim-3 levels were elevated on advanced stage patients. Furthermore, GS tumours presented increased levels of Gal-9 and Tim-3. Then we evaluated the expression pattern on IHC from The Human Protein Atlas, and observed that Gal-9 was mainly expressed on tumour endothelial cells and cancer cells, especially on areas with dense immune cell infiltration. Interestingly, we observed a significant increase in PDL-1 and a strong tendency in Tim-3 expression when Gal-9 was in extracellular environment, either added as a recombinant protein or secreted by transfected AGS cells, which are in line with the results observed through bioinformatic analysis from a patient sample. Relevant proteins in immune regulation such as HVEM, VISTA and CD-40L are also associated with Gal-9 and Tim-3 mRNA expression in gastric tumours. Suggesting that there is a robust regulatory

network promoting immune escape and that is possible that by blocking one pathway to restore the immune response, others can compensate and provide a bypass to the treatment.

In line with our findings, a 'mesenchymal-like' subtype of cancer has been recently described in peritoneal carcinomatosis (PC) from gastric cancer patients, associated to increase T-cell exhaustion phenotype and high expression of Tim-3, Gal-9, VISTA and TGF-β1 (164). Furthermore, Wang et al. showed that proliferative cells from PC samples stained positive for Tim-3, an observation that was interpreted as cancer cells expressing Tim-3. We here showed a similar observation, were advanced or metastatic tumours presented significantly higher levels of Tim-3. Given the results of our purity analysis, the Tim-3 is most likely expressed not only on stromal cells but also on cancer cells. Interestingly, Wang et al. also showed that patients with the mesenchymal-like subtype were less responsive to standard chemotherapy. Additionally, it was recently described that Gal-9 expression on gastric cancer cells was associated to poor survival on an Asian cohort of patients (63). Although our analysis failed to reach statistical significance, there existed a strong tendency for both Gal-9 and Tim-3 levels to associate with decreased patient PFS and OS. Taken together the presence of the Gal-9/Tim-3 axis on gastric tumours may contribute to metastasis and cancer progression by a direct action upon both the cancer cell and the promotion of a immunosuppressive microenvironment (Fig. 20).

II. Gal-9 promotes cancer cell invasion and migration in cancer cells and gastric epithelial cells

Gal-9 role in gastric cancer biology has been understudied, and published reports are contradictory. Some studies support the hypothesis that Gal-9 expression is protective in gastric cancer, improving survival only when late stages were ruled out of the analysis (64). Although it has been shown that Gal-9 expression on gastric cancer cell lines suppresses migration, invasion, and epithelial-mesenchymal transition, these functions can be attributed to a predominant expression of cytoplasmic Gal-9 (46). In fact, it has been shown that cytoplasmic Gal-9 induces cancer cell aggregation and inhibits metastasis (48). Importantly, in this study the expression of Gal-9 was neither seen in the nucleus nor on the surface of the cells, and that cell aggregation was surprisingly not induced by exogenous Gal-9 (81). Interestingly, we show that extracellular Gal-9 induces cell migration and invasion, and that this requires its CRDs. There was also a tendency showing that rhGal-9 had a lower impact on invasion when compared to Gal-9 transfection, however no significant differences were detected. An increase in invasion was not only observed on cancer cells but also on gastric epithelial cells, suggesting that this could be in fact a physiological function of Gal-9. Taken together, it is likely that Gal-9 has different functions depending on its subcellular expression, where nuclear, cytoplasmic and extracellular Gal-9 display different biological functions.

III. Gal-9 upregulates PDL-1 on cancer cells

A recent study reported that 57% of gastric tumours are positive for Gal-9 and this protein was associated to poor prognosis (63). By sequencing cells present in Peritoneal Carcinomatosis (PC) in a cohort of 43 patients it was observed that the less responsive 'mesenchymal-like' subtype exhibited T-cell exhaustion phenotype, with high expression of Gal-9, Tim-3, VISTA and TGF-β1(164). Our *in vitro* analyses are in line with these clinical results, showing that Gal-9 promotes a more aggressive phenotype in AGS gastric cancer cell line, an observation that is also substantiated by observations by Wang *et al*. Furthermore, we observed that Gal-9 transfection and rhGal-9 increased PDL-1 on cancer cells. The evaluation of whether this action is mediated by cellular of extracellular Gal-9 is still to be determined, however there is evidence in the literature to speculate on a biological role for this regulation. PDL-1 regulation is reported to be mediated by inflammatory signalling that leads to the activation of the transcription factors STAT, NFkB, AP-1, Myc and Jun. Interestingly, the promoter activity of IL-1A, IL-1β and IFN-γ was regulated by the small isoform of Gal-9 (51). The expression of these genes is modulated by different transcription factors, including AP-1, NF-IL6, and NF-kB. Interestingly, Gal- 9 is implicated in increasing the activity of AP-1 and NF- IL6, in fact a physical interaction of Gal-9 and NF-IL6 has been observed (40). Moreover, LPS-stimulated monocytes have been reported to show nuclear translocation of Gal-9-NF-IL6 and activate IL-8, IL-1 and IL-β production (40). Another pathway that is implicated in the regulation of PDL-1 expression is the Wnt/β -catenin pathway. Treatment of triple negative breast cancer cells with selective Wnt inhibitors or activators can either down-regulate or upregulated PD-L1 expression, respectively, implying a functional cross-talk between Wnt activity and PD-L1 expression (165), Furthermore, β-catenin signalling by Wnt or EGF treatment increases the

expression of PD-L1 in an AKT and β-catenin–dependent manner, and blocking the AKT pathway synergizes with anti–PD-1 in a glioblastoma model (166). Immunoprecipitation experiments demonstrate that there is a physical interaction between Gal-9 and β-catenin (54). On one hand it is possible that a physical interaction between Gal-9 and the abovementioned transcription factors mediate transcription from the PDL-1 promoter, while on the other hand, Gal-9 could be activating a signalling pathway that in turn activates the PDL-1 promoter and induces PDL-1 expression. This latter mechanism could involve the activation of specific receptors such as Tim-3 (we demonstrate that Gal-9 transfected cells increases expression of this receptor).

IV. Galectin-9 promotes endothelial cell migration and angiogenesis *in vitro*.

Gal-9 promoted endothelial cell migration and increased tube formation on a Matrigel assay, an effect that was prevented by the addition of α-Lactose. Thus, a physiological of pathophysiological role of secreted (extracellular) Gal-9 could be to promote angiogenesis by binding of protein glycosylations. As Tim-3 is also expressed on endothelial cells, it is possible that Tim-3 activation by Gal-9 on these cells is promoting angiogenesis. Previous reports in other models, such as a rheumatoid arthritis model, also suggested that Gal-9 increased angiogenesis (167). O'Brien et al. proposed that Gal-9 induced angiogenesis by MAPK activation, but this study did not evaluate if this was dependent on Tim-3 binding. However a recent study demonstrated that Tim-3 promotes tube formation and focal adhesion disassembly in HUVECs (168). Previous studies showed that Tim-3 expression on endothelial cells promotes cancer cell survival and metastasis, and was necessary for stable cell adhesion during endothelial transmigration of murine melanoma cells (76). It has been reported that

Tim-3 overexpression on endothelial cells enhances IFNγ, NOS and TNFα expression (Yang et al 2015), which is also associated with endothelial cell activation, a key step for immune cell recruiting and metastasis. Future experiments using *in vivo* cancer models will help determine the exact role of Gal-9 in the regulation of angiogenesis. However, it is worth pointing out that angiogenesis is in general associated with an immunosuppressive microenvironment. Firstly, angiogenic factors such as VEGF and hypoxia are strongly associated to macrophage M2 polarization and Treg recruitment (66–69). Secondly, tumour infiltration is known to be compromised, and the current line of thought hypothesizes that by stabilizing the tumour capillary network, the immune cell access will be enhanced and an anti-tumour immune response improved. In support of this reasoning, it has been observed that anti-angiogenic therapies have limited potential as monotherapies, yet are reported to synergize with immunotherapies in both murine cancer models(70–72) and are currently on early phase trial evaluation (73,74).

V. Gal-9 promotes CD8+ T cell dysfunction through the Tim-3 receptor

$CD8^+$ T cells have been for long described as key elements of the cancer immune response and their dysfunctional states widely associated to poor clinical outcomes and immunotherapy resistance. We demonstrate herein that the percentage of $CD8^+$ T cells is significantly decreased by rhGal-9. No significant changes were observed in total T cell viability, thus this result could be explained by either a reduction on $CD8^+$ T cell proliferation or a direct effect on their viability. Gal-9 was initially described as a pro-apoptotic factor on $CD4^+$Th1 T cell apoptosis (169), and it has also been associated to a decrease in PBMC proliferation (87). Furthermore, it has been reported that both Gal-9 or Tim-3 blockade restores $CD8^+$ T cell

proliferation (170). Interestingly, this effect on $CD8^+$ frequency was not observed on T cells when co-cultured with Gal-9 expressing cancer cells. The reduction of $CD8^+$ T cells may depend on Gal-9 concentration, as when treated with rhGal-9 0.25ug/mL, no significant differences on $CD8^+$ T cell percentage was observed. It is also possible that other signals maintain $CD8^+$ frequencies and are masking out the effect of Gal-9, such as antigen drive proliferation. Further experiments will be required to evaluate these hypotheses. Of note, although it has been shown that Gal-9 induces a wave of $CD4^+$ T cell apoptosis on PBMCs after 1 day of stimulation after which a $CD4^+IFN\gamma^+$ population was expanded (171). We did not observe significant differences on the frequency of $CD4^+$ T cells when cells were co-cultivated with Gal-9 transfected cancer cells. Non the less, similar to our results with rhGal-9 it was reported that Gal-9 could promote a CD4-dominated response, shifting the CD4/CD8 balance.

Our database analysis brought to light that a molecular signature of dysfunctional $CD8^+$ had a positive correlation with tumour LGALS9 levels, suggesting that Gal-9 is associated with $CD8^+$ dysfunction in gastric tumours. To further evaluate this hypothesis, we performed *in vitro* experiments with T cell primary cultures. In agreement with our bioinformatics analysis, we show that rhGal-9 and the co-culture of cancer cells expressing Gal-9 increased $PD1^+Tim\text{-}3^+$ and $PD1^+Tim\text{-}3^+LAG3^+$ frequencies. Moreover, co-treatment with a Tim3-blocking antibody prevented this effect. Therefore, it is likely that Gal-9, acting through Tim-3 engagement, is sufficient to increase the frequency of these populations.

A reduced effector function, including the production of TNF, interleukin-2 (IL-2) and IFNγ under (semi)physiological conditions has been previously reported in dysfunctional $CD8^+$ T cells (172–175). Interestingly, when we evaluated IFNγ production, no significant changes

were observed on the frequency of IFNγ$^+$ cells within the CD8$^+$ subset, although there was a significant reduction on total CD8$^+$ cell number. These results indicate that Tim-3 stimulation through Gal-9 could be sufficient to increase inhibitory receptor expression on CD8$^+$ T cells, but not to reduce their functionality in terms of cytokine production under PMA/Ionomycin stimulation. As has been previously described, dysfunctional or exhausted T cells have unpaired cytokine production, however most of these studies evaluate this directly ex vivo or after stimulation with cognate antigen or low- dose anti- CD3 (172–175). Our experiments were performed with healthy donor peripheral T cells and thus to determine IFNγ production we had to re-stimulate cells with PMA/Ionomycin, which provide TCR independent stimuli via Ca2$^+$ and MAPK pathways. Thus, it is possible that the Gal-9/Tim-3 inhibitory pathway acts upstream of these events. Further experiments, preferably on isolated CD8$^+$ T cells will be required to determine the molecular mechanism underlying our observations.

It must be noted that dysfunctional T cells are a highly heterogeneous population. Taking this into consideration, it has been suggested that this T cell subset can be classified into 'pre-dysfunctional', 'early dysfunctional' and 'late dysfunctional' cells (176). However, it has to be kept in mind that T cell dysfunction is not a binary state, but rather a continuum of different states that culminate into a terminally exhausted T cell phenotype leading to senescence.

In our experiments, Gal-9 increased both PD1$^+$Tim3$^+$ and PD1$^+$Tim-3$^+$LAG3$^+$ populations, which have been respectively suggested as early dysfunctional and late dysfunctional T cells. Interestingly, the appearance of these sub-populations was accompanied by a decrease on the total CD8$^+$ cell frequency. This may indicate that Gal-9 is pushing the differentiation towards a late dysfunctional phenotype and therefore leading to senescence and cell death. In line with these hypothesis, it has been proposed that CD8$^+$ T cells retain proliferative capacity during

their transition from the pre-dysfunctional state to an early dysfunctional state, but lose this capacity at the stage of more profound, 'late', dysfunction, either because of an intrinsic block or because their high inhibitory receptor expression suppresses T cell activation (173,176–180).

Many ligands have been proposed for Tim-3, including PstdSer, HMGB1, CEACAM1 and Gal-9, the latest reports suggest that Tim-3 binds to CEACAM1 though Gal-9, where Gal-9 CRDs crosslink these two membrane proteins stabilizing downstream signalling [Reviewed in (56)]. Importantly, in our hands Tim-3 blockade prevented the increase in dysfunctional subsets of $CD8^+$ T cells, which indicates that this effect is at least partly mediated through Tim-3. Interestingly, some studies have suggested that Tim-3 blocking antibodies do not prevent Gal-9 binding (181), however our results suggest that at least the clone used in this study (F38-2E2) effectively impairs the Gal-9 mediated effects.

A variety of transcription factors regulate CD8+ T cell dysfunctionality, including TOX and TCF1 [Reviewed in (176)]. TCF1 and TOX appear to be determinant for T cell fate regarding a commitment into a stem-like phenotype or a dysfunctional phenotype in murine models (34). Since our results indicate that Gal-9 is driving the differentiation process into late-dysfunctional cells, further studies should evaluate if this protein can regulate these transcription factors and furthermore and increase the expression of the recently described late-dysfunction marker CXCL13 (173,176).

VI. Gal-9 promotes Treg expansion through the Tim-3 receptor

Tumour-resident regulatory T (Treg) cells have been shown to counteract tumour- specific immune responses by suppressing the infiltration and anti-tumour activity of, among other cells, CD8+ T cells and macrophages (182). These cells have also been shown to directly promote tumour growth by secreting VEGF-A and other growth factors such as TGFβ (67). Moreover, Treg accumulation has been associated to immunotherapy resistance in mice and patients (183,184). Therefore, given that we reported LGALS9 correlated with several checkpoints commonly expressed on Treg, we evaluated if LGALS9 levels were also associated with increased Treg infiltration. Through bioinformatics, we analysed the TCGA database testing if there was a correlation between a tumour infiltrating Treg signature and LGALS9 levels. Strikingly, we observed that there was a strong positive correlation, thus suggesting that Gal-9 could increase Treg infiltration, possibly by expanding this population or increasing their trafficking into the tumour.

Seki et al. showed that Lgals9 knockout (KO) mice displayed a reduced frequency of Foxp3+ Treg (57). A separate study showed that this KO mouse had impaired Foxp3 expression, and that exogenous Gal-9 could restore Foxp3 function, and furthermore, that TGFβ1 induces Gal-9 expression in a feedforward loop (44). In addition, Gal-9 promotes TGFβ1-dependant induction of Treg via the TGFβ/Smad signalling pathway. Given the evidence from mice models and our bioinformatics results, as a proof of concept we evaluated if Gal-9 could expand human Treg *in vitro*, using T cell primary cultures from healthy donors. In accordance with our hypothesis, we observed that Gal-9 at the three concentrations used effectively increased Treg frequency.

It has been shown that Tim-3 blockade relieves Treg mediated immunosuppression in head and neck cancer. Using the Tgfbr1/Pten double KO mouse model, the application of an aTim-3 antibody reduced Treg numbers, restored IFNγ production and impaired tumour growth (93). In a similar manner, Tim-3 expression is associated with tumour growth as demonstrated by Tim-3 blockade reverting Treg-mediated immunosuppression (95). Tim-3 appears not only to play a key role on Treg function in cancer, but also in autoimmune diseases such as osteoarthritis, where a reduction of Tim-3 expression on Treg associates with a decreased production of IL-10 (92).

Besides Tim-3, Gal-9 has been reported to act through other receptors such as CD44 and CD137 (185). In murine models where Gal-9 could expand Treg, this action was principally attributed to an interaction with the CD44 receptor that enhanced the stability and function of these cells (44). However, in our observations in human peripheral T cells, Gal-9 action appears to be predominantly mediated through the Tim-3 receptor, although we cannot rule out that Gal-9 is also, albeit partially, mediating changes through alternative receptors. While this remains to be evaluated, we can conclude that that Tim-3 is required for Gal-9 mediated Treg expansion.

VII. Gal-9 may contribute to aPD1 immunotherapy resistance by increasing CD8+ dysfunctionality in a Tim3 dependent manner

In the field of immunotherapy, they still remains an open question of whether ICB acts by reinvigorating a tumour-resident T cell (TRM) population or mobilization of T cells from outside the tumour also occurs. Studies in mice models revealed that $TCF1^{+}$ cells, thereby stem-like T cells, were critical for obtaining a positive tumour response upon application of

single agent αPD1 therapy or αPD1 and αCTLA4 combination therapy. However, by depleting TCF1-expressing T cells and thus leaving late-dysfunctional T cells, treatment with ICB still reduced tumour growth (84), thus indicating that ICB not only prevent dysfunctionality, but can also act directly over dysfunctional T cells. In human lung cancer, TRM cell clones with a dysfunctional profile (expressing HAVCR2 and other inhibitory receptor genes) displayed increased expression of cytotoxic genes after anti- PD1 treatment (179), thus indicating that their effector functions were restored. On the same line, a $PD1^{hi}$ T cell subset, that expressed activation markers as well, was enriched for tumour reactivity and associated with clinical response to αPD1 therapy (173). Hence, it appears that early-dysfunctional T cells, but not late-dysfunctional T cells, may be responsible for the favourable responses observed with ICB therapies [Extensively reviewed in (176)].

Herein we report that a $CD8^+$ dysfunction molecular signature (TIDE) derived from bioinformatics analysis of untreated patient samples (186), is positively correlated with LGALS9 levels. Importantly, when Jiang *et al.* applied this signature to pre-treatment transcriptomic data from patients with melanoma who subsequently received ICIs, TIDE consistently out-performed all other candidate predictive biomarkers tested, including PDL-1 levels, tumour mutational burden, and an IFNγ signature (186).

Given the results of our bioinformatic analysis, we evaluated if Gal-9 could provide a bypass to the presence of a PD1 blocking antibody. After treatment of isolated T cells with αCD3/CD28beads and simultaneous exposure rhGal9, we observed a significant decrease in $CD8^+$ frequency, even in presence of the αPD1. Interestingly, in presence of the αPD1, Gal-9 increased the frequency of both the early ($PD1^+Tim\text{-}3^+$) and late ($PD1^+Tim\text{-}3^+LAG3^+$) dysfunctional phenotypes. The participation of the Tim-3 receptor is highlighted by the

effectively decreased $CD8^+$ frequency and $PD1^+Tim\text{-}3^+$ cells, while showing a similar tendency on the frequency of $PD1^+Tim\text{-}3^+LAG3^+$ cells. It should be noted that our experiments were performed using peripheral T cells from healthy donnors, and further studies should evaluate the effect of Gal-9 on TILs response to immunotherapies. Given that TILs are reported to present higher abundance of dysfunctional T cells, testing αPD1 and αTim-3 on these cells in presence of Gal-9 could reveal if these therapies can revert or partially revert dysfunctional phenotypes and if Gal-9 could reduce their efficacy.

Of note, it was recently published that Gal-9 could also bind to PD1, in a way that promoted a lattice formation between PD1 and Tim-3, which was proposed to increase the stability of dysfunctional T cells, possibly by reducing Tim-3 mediated cell death (45). These results have clinical applicability as they suggest that the axis Gal-9/Tim-3 T cells can push T cells into a dysfunctional state even in the presence of an ICB antibody. Interestingly these results indicate not only that Gal-9 presence could provide a bypass to αPD1 therapy and thus contribute to ICB resistance, but also shines a light on how αPD1 ICB works. In our experiments, αPD1 treatment effectively reduced the amount of dysfunctional T cells and thus a principal *modus operandi* of αPD1 therapies may be to obstruct the differentiation of T cells into a dysfunctional state. Although the main discussion within the immunotherapy field has been that a durable response to αPD1 therapy requires the presence of tumour-specific T cells with low levels of dysfunction, our results point to another possibility. Whether it is merely the levels of dysfunction T cells, or also the presence of inhibitory ligands that are the determinants is yet to be ascertained. As T cell dysfunctionality is led by a robust system of inhibitory pathways, under the activation of other co-inhibitory pathways such as Gal-9/Tim-3, T cells may undergo dysfunctionality regardless of the αPD1 treatment. In line with this

idea, Auslander *et al.* developed an immuno-predictive score for neuroblastoma, IMPRES, calculated on the basis of 15 rational pairwise relationships between the expression levels of inhibitory and activating immune-checkpoint genes. As well as predicting neuroblastoma regression, high IMPRES scores were found to be associated with immunologically hot tumours and longer overall survival in patients with untreated metastatic melanoma (187). In predicting responses to ICB in this setting, IMPRES strikingly achieved an overall accuracy of AUC = 0.83, outperforming existing predictors and capturing almost all true responders while misclassifying less than half of the non-responders. This work strengths the hypothesis that a robust network of regulatory checkpoints operates to maintain the tumour immunosupresive microenvironment.

Several published articles on mice models have shown that combinatory ICB is more effective for control of tumour growth and survival than using monotherapies (87,188). Noteworthy, our results showed that a combinatory strategy using αPD1 + αTim3 effectively prevented the effects of rhGal9 on T cell destiny, further supporting the idea of combining ICB for cancer treatment. Encouragingly, there are 63 clinical trials of antibodies targeting Tim-3 either alone or in combination with PD1 blockers. Moreover, Gal-9 is a secreted protein that can be detected in plasma, and thus future studies should examine the clinical benefit of evaluating Gal-9 and intra-tumour Tim-3 levels as a companion diagnostic for αPD1 + αTim-3 combinatory immunotherapy. Furthermore plasmatic Gal-9 may prove value as a resistance risk indicator for patients receiving ICB monotherapy and could signal when to begin combinatory therapy.

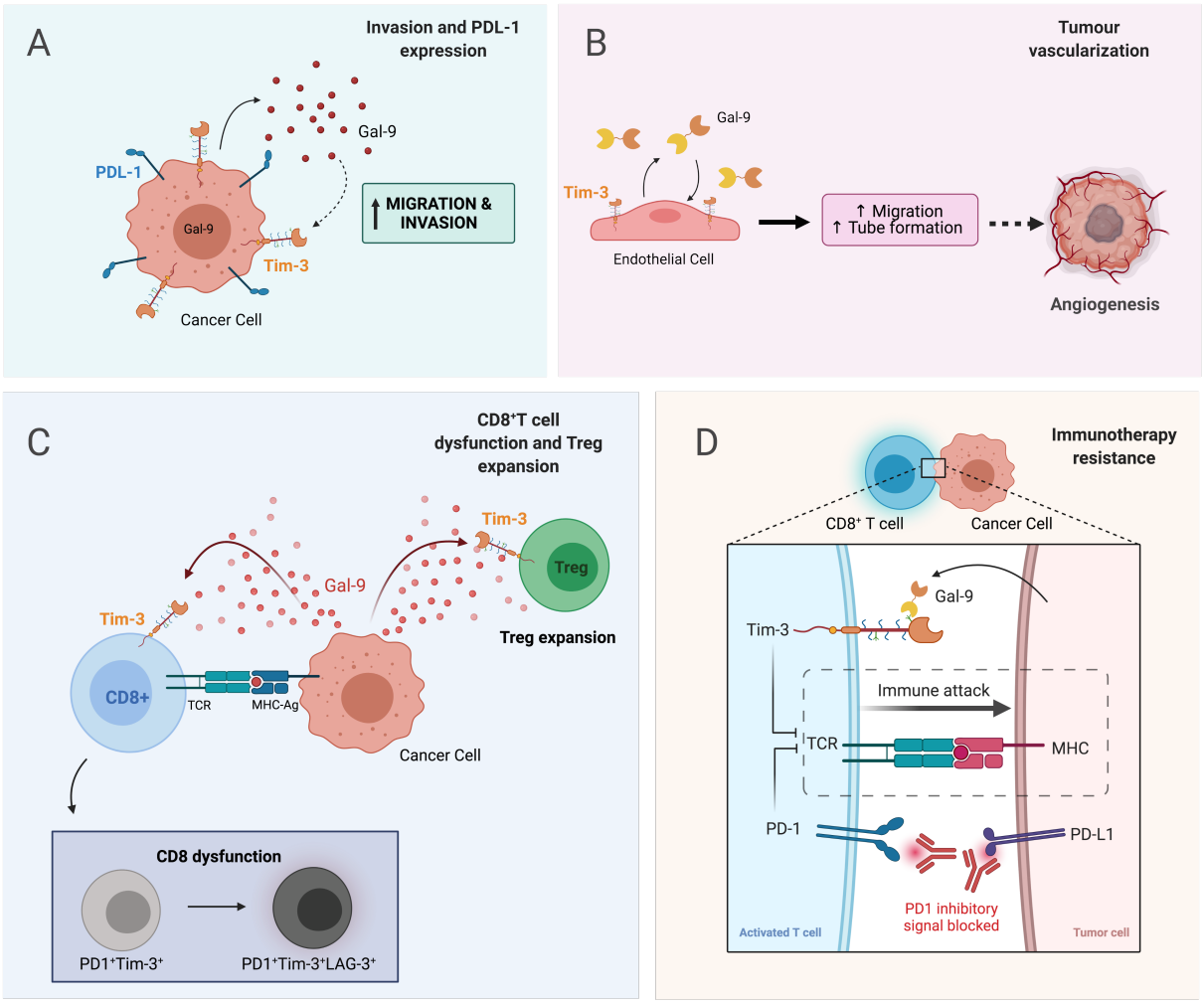

Figure 20. The Gal-9/Tim-3 pathway in the regulation of the tumour microenvironment and cancer progression. Gal-9 expression in cancer cells increases PDL-1 expression and cancer cell migration and invasion, possibly through Tim-3 activation (A). Endothelial cells express Gal-9, extracellular Gal-9 increases cell migration and tube formation in a CRD dependant manner, which could contribute to tumour angiogenesis (B). Gal-9 expression on cancer cells increases CD8+ T cell dysfunctionality and expands Tregs in a Tim-3 dependant manner (C), which occurs independently of PD1 blockade (D).

Conclusions

Immunotherapy based on checkpoint inhibition is designed to restore immune response in cancer patients. We speculated in this thesis that single agent immunotherapy may fail in gastric cancer as other checkpoint inhibition signals are present and provide a bypass to this treatment. Using bioinformatic analysis we observed that Gal-9 and Tim-3 mRNA levels were associated to immune-regulatory genes, suggesting the presence of a robust regulatory network promoting immune escape. A tendency toward poor clinical outcome was observed in stage IV GC patients with higher levels of Gal-9 and Tim-3, and increased levels of Tim-3 were observed on advanced stage tumours. In accordance with these clinical observations, our *in vitro* experiments showed that Gal-9 promotes cell migration, invasion and angiogenesis. Furthermore, Gal-9 increased the populations of $CD8^+$ T dysfunctional cells and Tregs *in vitro,* an effect that was dependent on Tim-3 receptor. Thus, our results suggest that the Gal-9/Tim-3 axis may contribute to metastasis and cancer progression by a direct action upon both the cancer cell and the promotion of an immunosuppressive microenvironment. In line with our hypothesis, we proposed that under the activation of the co-inhibitory pathway Gal-9/Tim-3, T cells may undergo dysfunctionality regardless of the presence of αPD1. Accordingly, we demonstrated that Gal-9 prevented the reduction in dysfunctional T cell populations observed by the presence of PD1 blockade. Based on our results we propose that future studies should examine the clinical benefit of αPD1 + αTim-3 combinatory immunotherapy. As elevated levels of Gal-9 and intra-tumour Tim-3 levels may indicate the enhanced possibility of single agent PD1 blockade bypass, we further propose that these levels are evaluated as a companion diagnostic for combinatory immunotherapy. Furthermore, plasmatic Gal-9 may prove value as a resistance-risk indicator for patients receiving ICB

monotherapy and could signal when to begin combinatory therapy. While ICB has offered the potential of complete response to a minority of patients, the future understanding of immunoregulatory pathways and the clinical exploitations of these, may convert advanced stage gastric cancer from a prognosis of little hope to a manageable chronic disease.

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Annex 1: Clinicopathological characteristics of the TCGA-STAD 2018 cohort

Table I. Clinicopathological characteristics of the TCGA-STAD 2018 cohort

Characteristics	N (%)
Sex Female Male	 156 (35.6%) 284 (64.6%)
Age (mean ± SD)	65.63 ± 10.74
Stage* I II III IV	 58 (18.8%) 131 (31.1%) 188 (44.7%) 44 (10.5%)
Molecular Classification# CIN EBV GS MSI	 223 (59.31%) 30 (7.98%) 50 (13.30%) 73 (19.42%)
Overall Survival (OS) (mean ± SD)	17.88 ± 17.86
Progression Free Survival (PFS) (mean ± SD)	17.00 ± 17.71

*Of a total of 436 patients, *15 cases did not have available information regarding their stage and # 64 cases had unreported molecular classification. CIN: Chromosomal Instability, EBV: Epstein Barr Virus, GS: Genomic Stable, MSI: Micro-Satellite Instability.*

Annex 2: Ethical approval certificate

PONTIFICIA UNIVERSIDAD CATÓLICA DE CHILE

ACTA DE RESOLUCIÓN
COMITÉ ÉTICO CIENTÍFICO CIENCIAS DE LA SALUD UC
Re-acreditado por SEREMI de Salud
Resolución Exenta N°012321 del 07 de junio de 2017

NUEVO ESTUDIO
Fecha y N° de Sesión: 17 de septiembre de 2020, Sesión N°15
Investigador(a) Responsable: Charlotte Nicole Hill Machado

ID Protocolo: 180629007

Título del Proyecto: Galectin-9 expression within gastric tumours and Tim3 expression on the tumour microenvironment is associated to anti-PD1 resistance and increased Treg and MDSCs infiltration and immunosuppressive effects.

Facultad/Unidad Académica: Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile
Académico Responsable: Gareth Ivor Owen
Sitio de realización: Facultad de Cencias Biológicas, Pontificia Universidad Católica de Chile
Financiamiento: Milenio (indicar) IMII P09016F / Fondecyt Regular 1180173

Miembros del Comité que participaron en la aprobación del estudio:
Dra. Colomba Cofré Dougnac, Vice-Presidente
Mg. Andrea Villagrán Torres, Secretaria Ejecutiva
Sr. Jorge Muñoz Castillo, Abogado miembro externo
Dr. Gustavo Kaltwasser González, Miembro externo
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Dr. César Sánchez Rojel, Departamento de Hemato-Oncología
Dra. Carolina Méndez Orellana, Carrera de Fonoaudiología
Dr. Luis Villarroel Del Pino, Departamento de Salud Pública
Dr. Pablo Brockmann Veloso, Departamento de Enfermedades Respiratorias del niño

Documentos recibidos por el Comité:
- Carta Presentación Investigador Responsable
- Carta Apoyo Jefe de Departamento

Documentos revisados y aprobados por el Comité:
- Formulario presentacion CEC Salud .pdf
- Consentimiento Informado voluntarios

PONTIFICIA
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DE CHILE

- Proyecto de Tesis
- Dispsensa de consentimiento informado para datos clínicos y muestras anonimas provenientes de proyecto ID 170905007

Considerando que:

1- Los investigadores referidos cuentan con la experiencia necesaria para la conducción y el desarrollo de este tipo de estudio;
2- La metodología descrita es apropiada para el cumplimiento de los objetivos del estudio, de acuerdo con los estándares internacionales de rigor científico;
3- Durante la conducción del estudio se garantiza un balance riesgo/beneficio favorable para los participantes, por cuanto sólo se realizan procedimientos de bajo riesgo;
4- La población por estudiar no es considerada vulnerable . El protocolo contempla todos los resguardos necesarios para la seguridad y bienestar de los participantes;
5- Se ha contemplado el resguardo de la confidencialidad de la información sensible e identificable en la difusión de los resultados, por lo que no introduce un riesgo de menoscabo para la intimidad de los participantes; y
6- Los participantes ingresarán voluntariamente luego de ser adecuadamente informados sobre los aspectos esenciales del estudio, sus deberes y derechos, y los plazos estipulados para el cumplimiento de los objetivos de la investigación.

Constatado que, el texto del documento de Consentimiento Informado (on-line):

1- La descripción general de los objetivos de la investigación;
2- El detalle de los procedimientos que involucra la participación en este estudio;
3- Los antecedentes sobre el uso que se dará a la información obtenida a partir de cada procedimiento de la investigación;
4- El compromiso respecto a la utilización actual y futura de la información, la que sólo se realizará dentro de los marcos del presente estudio y para el logro de dichos objetivos;
5- El resguardo de la confidencialidad y el anonimato de la información recogida, según corresponde a cada procedimiento del estudio;
6- El detalle respecto del costo en tiempo que significa la participación en el estudio;
7- La información sobre los beneficios, derechos frente a riesgos y cobertura de daños por la participación en la investigación; y
8- La voluntariedad de la participación y la garantía para cada participante de hacer abandono del estudio, sin repercusión alguna.

Resolución CEC-Salud UC:

Este proyecto cuenta con la opinión favorable del Comité con fecha **17 de septiembre de 2020**, en la **sesión ordinaria N°15**, la que tiene vigencia de un año.

Para iniciar el proceso de consentimiento y de reclutamiento se debe disponer previamente de la última versión aprobada y timbrada del documento de Consentimiento Informado . De este modo, el Investigador Responsable velará por la realización de estos procedimientos, utilizando las copias de cada versión original (timbradas, fechadas y firmadas por el CEC-Salud UC).

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DE CHILE

El Investigador Responsable deberá solicitar la renovación anual de la presente aprobación ética con al menos 45 días de anticipación si desea continuar con el estudio. Si no ha recibido la respuesta oficial a su solicitud, el investigador deberá detener las actividades del proyecto, no podrá enrolar a ningún nuevo participante y no podrá proceder con el análisis de los datos.

En la eventualidad de requerir cualquier modificación al estudio o a los documentos aprobados originalmente, el investigador deberá notificarlo al Comité por medio de una enmienda (a través de plataforma) para la evaluación y emisión de una nueva acta de resolución ética.

El Investigador Responsable debe descargar, revisar, firmar y subir a plataforma, documento de Responsabilidad del Investigador, la que se encuentra disponible en sección "Plantillas" del estudio de la referencia (ID: 180629007).

Comité Ético Científico UC de Ciencias de la Salud

SRA. ANDREA VILLAGRÁN TORRES
Secretaria Ejecutiva CEC-Salud UC

DRA. COLOMBA COFRÉ DOUGNAC
Vice- Presidenta CEC-Salud UC

EN CASO DE CUALQUIER DUDA SE LE SOLICITA CONTACTARSE CON EL CEC-Salud UC

Se certifica que la información contenida en el presente documento es correcta y que refleja el Acta del Comité Ético Científico de Ciencias de la Salud UC (CEC-Salud UC). Este Comité adhiere a los principios éticos de la Pontificia Universidad Católica de Chile, que considera como eje fundamental el respeto a la dignidad de la persona humana en cualquier condición. Este Comité cumple además con las Guías de buena práctica clínica definidas por la Conferencia Internacional de Armonización (GCP-ICH); y con las leyes chilenas 19.628; 20.120; 20.584 y 20.850 que modifica el Código Sanitario

Annex 3: Informed Consent

DOCUMENTO DE CONSENTIMIENTO INFORMADO

Nombre del Estudio: **Expresión de galectina-9 desde tumores gástricos y expresión de Tim3 en el microambiente tumoral asociado con la resistencia anti-PD1, aumento de la infiltración y efectos inmunosupresores de Treg y MDSCs.**

Patrocinador del Estudio / Fuente Financiamiento: Instituto Milenio de Inmunología e Inmunoterapia (**IMIIP09/16F**)

Investigador Responsable: **Charlotte Hill Machado**

Teléfono de contacto**: +56982976001**

Depto/UDA: **Departamento de Fisiología**, Facultad de Ciencias Biológicas UC

El propósito de esta información es ayudarle a tomar la decisión de participar en una investigación médica. Tome el tiempo que necesite para decidirse, lea cuidadosamente este documento y hágale las preguntas que desee al médico o al personal del estudio.

OBJETIVOS DE LA INVESTIGACIÓN

Usted ha sido invitado/invitada a participar en este estudio como donante voluntario
El propósito de este estudio es determinar el efecto de la proteína galectina-9 sobre las células del sistema inmune. Específicamente queremos determinar el efecto de esta proteína cuando es liberada de células de cáncer.

PROCEDIMIENTOS DE LA INVESTIGACIÓN

Si usted acepta participar en el estudio, se le hará/pedirá lo siguiente:

1.- Se tomara una muestra de sangre de 15 ml, equivalente a tres cucharas de sopa para estudiar la respuesta de las células del sistema inmune al cáncer in vitro.
2.- Su participación en este estudio durará aproximadamente 15 minutos (lo que demorará la toma de muestra de sangre).
3.- Las muestras obtenidas serán usadas únicamente para el propósito de esta investigación. Si en el futuro son usadas para propósitos diferentes a los de esta investigación médica, se le solicitará un nuevo consentimiento.
4.- En este estudio no se harán estudios genéticos.
5.- Las muestras serán almacenadas por 2 meses en el Laboratorio del Dr. Gareth Owen, bajo la responsabilidad de Charlotte Hill.

BENEFICIOS

Usted no se beneficiará directamente por participar en esta investigación médica. Sin embargo, la información que se obtendrá será de utilidad para conocer más acerca de la biología del cáncer y eventualmente podría beneficiar a otras personas con su misma condición.

RIESGOS

Este estudio posee riesgos asociados a la toma de muestra de sangre como dolor, hinchazón, moretón en la zona de punción.

COSTOS

Su participación en este estudio no le significara ningún costo para usted.

COBERTURA DE DAÑOS

Cualquier daño que pudiese sufrir durante su participación en la investigación serán cubiertos por el Dr. Gareth Owen.
No habrán compensaciones por complicaciones inherentesa su condición clínica.
El pago es a todo evento y no contingente al uso o existencia de seguro de salud del sujeto.

COMPENSACIONES

Este estudio no contempla ningún tipo de compensación por la participación en él.

CONFIDENCIALIDAD DE LA INFORMACIÓN

No se almacenará información personal para este estudio. Las muestras obtenidas serán anonimizadas de forma inmediata y no serán vinculadas a su información personal.
Es posible que los resultados obtenidos sean presentados en revistas y conferencias científicas, sin embargo, su nombre no será conocido.

VOLUNTARIEDAD

Su participación en esta investigación es completamente voluntaria. Usted tiene el derecho a no aceptar participar o a retirar su consentimiento y retirarse de esta investigación en el momento que lo estime conveniente. Al hacerlo, usted no pierde ningún derecho que le asiste como paciente de esta institución y no se verá afectada la calidad de la atención médica que merece.
Si usted retira su consentimiento, sus muestras serán eliminadas y la información obtenida no será utilizada.

PREGUNTAS

Si tiene preguntas acerca de esta investigación médica puede contactar o llamar al Charlotte Hill Machado, Investigador Responsable del estudio, al teléfono: 223541935 y email: cnhill@uc.cl.

Si tiene preguntas acerca de sus derechos como participante en una investigación médica, usted puede llamar a la Dra. Claudia Uribe Torres., Presidente del Comité Etico Cientifico de Ciencias de a Salud de la Pontificia Universidad Católica de Chile, a los teléfonos 223542309 / 223548173, o enviar un correo electrónico a: eticadeinvestigacion@uc.cl

DECLARACIÓN DE CONSENTIMIENTO *(texto obligatorio)*

- Se me ha explicado el propósito de esta investigación médica, los procedimientos, los riesgos, los beneficios y los derechos que me asisten y que me puedo retirar de ella en el momento que lo desee.
- Firmo este documento voluntariamente, sin ser forzado/forzada a hacerlo.
- No estoy renunciando a ningún derecho que me asista Se me comunicará de toda nueva información relacionada con el estudio/ fármaco en estudio/ aparato médico que surja durante el estudio y que pueda tener importancia directa para mi condición de salud
- Se me ha informado que tengo el derecho a reevaluar mi participación en esta investigación médica según mi parecer y en cualquier momento que lo desee.
- Al momento de la firma, se me entrega una copia firmada de este documento.

_____
Participante (nombre, firma y fecha)

_____
Investigador Responsable (nombre, firma y fecha)

_____
Director Institucional o delegado (nombre, firma y fecha)