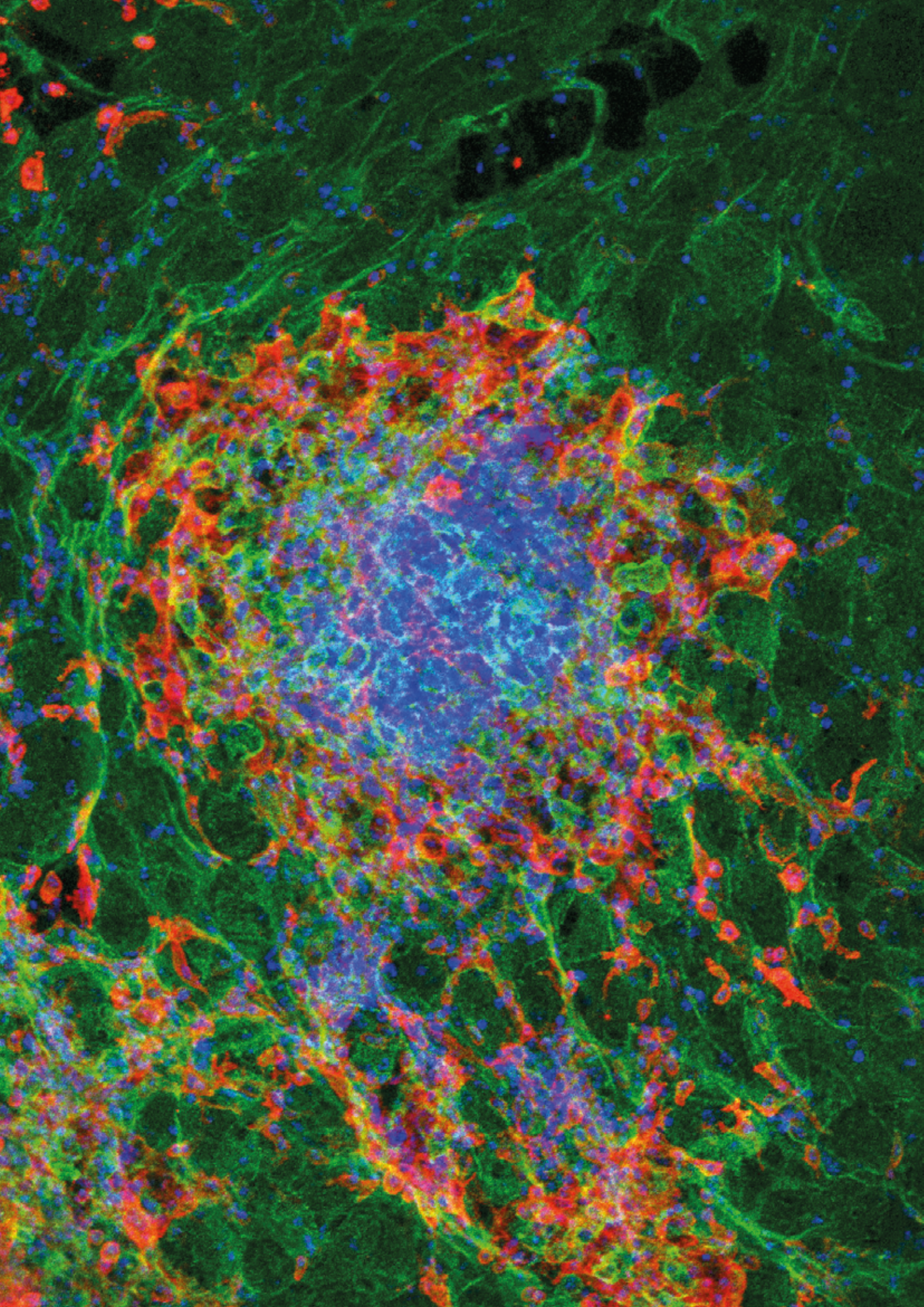

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IMMUNOLOGY AND ONCOLOGY

The Department of Immunology and Oncology (DIO) is devoted to the characterisation of the molecular and cellular bases of immune response in health and disease. We are interested in the study of the immune system function in tumour development, in inflammatory diseases as well as in infection by pathogens. Our aim is to identify new targets for the prevention, diagnosis and treatment of these pathologies, and to develop improved approaches for immune response modulation during cancer.

Various groups in the DIO address several aspects of cancer development and treatment, with special emphasis on the identification of new antitumour targets by characterising the cellular and molecular mechanisms that underlie (i) inflammation-driven carcinogenesis, as well as tumour immunology; (ii) the relationships among stem cells, metastasis, inflammation and cancer; and (iii) immunotherapy and diagnosis. The molecular and cellular mechanisms that underlie the immune response, inflammation and tumour development often overlap, providing many opportunities for collaboration among the groups in the Department as well as with other groups within and outside the CNB in the pursue of common research objectives.

During the COVID-19 pandemic, collaboration between groups in the DIO have led to the development of a serological test that includes several SARS-CoV-2 antigens and determines the presence of SARS-CoV2 antibodies with a 98% reliability. This antibodies test has been commercialised and approved for SARS-CoV2 diagnostics by the Spanish regulatory agency (Agencia Española del Medicamento y Productos Sanitarios, AEMPS).

HEAD OF DEPARTMENT

Ana Cuenda

Whole mount immunofluorescence and confocal microscopy image of the omentum, showing peritoneal resident macrophages infiltrating a milky spot, 4 hours after infection with the Escherichia coli *strain M6L4; anti-F4/80 (macrophages; red), anti-podoplanin (mesothelial cells; green) and DAPI (blue) staining. (Image from Carlos Ardavin's lab).*

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Immunobiology of monocytes, macrophages and dendritic cells

Our research program is currently focused on two major topics, alveolar dysfunction associated to airway allergy, and innate immunity against peritoneal infection and tumour metastasis. Using a mouse model of airway allergy induced by house dust mite extracts, our results demonstrate that airway allergic reactions caused a severe alveolar disorganization, involving the disappearance of alveolar macrophages, later replaced by monocyte-derived alveolar macrophages, and pneumocyte hypertrophy, associated with profound alterations in the composition and biophysical properties of pulmonary surfactant. These data support that the severe respiratory disorders caused by asthmatic reactions not only result from airway pathology due to bronchiolar inflammation, but also from profound alterations in the alveolar system. On the other hand, by using a mouse model of peritoneal bacterial sepsis, based on the intraperitoneal infection with a *E. coli* strain isolated from the mouse intestine, our group has defined the mechanisms by which resident peritoneal macrophages and inflammatory monocyte-derived macrophages control the defence against bacterial infection through the formation of complex, mesothelial bound macrophage aggregates, allowing the containment and elimination of bacteria.

Our results support that the formation of these aggregates require fibrin polymerisation, a process dependent on tissue factor release. The resolution of infection involves the disorganisation of macrophage aggregates, a process that involves fibrinolysis, controlled by monocyte-derived macrophages recruited to the peritoneal cavity. These results demonstrate that the ability of resident macrophages located in body cavities to fulfil their function depends on their attachment to the mesothelium and their clustering in cell aggregates, that in turn require a coagulation process for their formation. Similar cellular structures are formed in response to intraperitoneal injection of tumour-derived organoids, leading to peritoneal colorectal tumour metastasis. Overall these data support an important functional link between coagulation, inflammation and immunity for defence against peritoneal infection and tumour metastasis.

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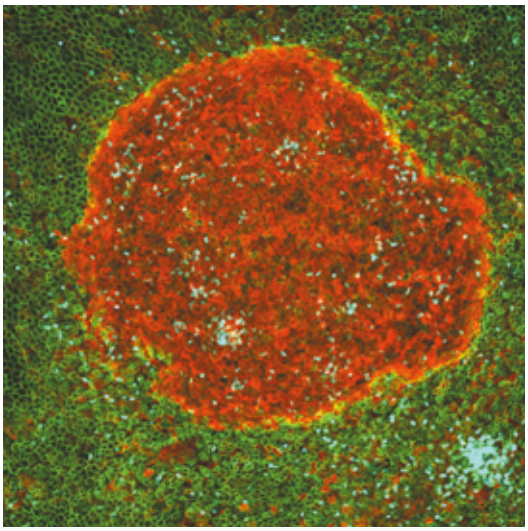

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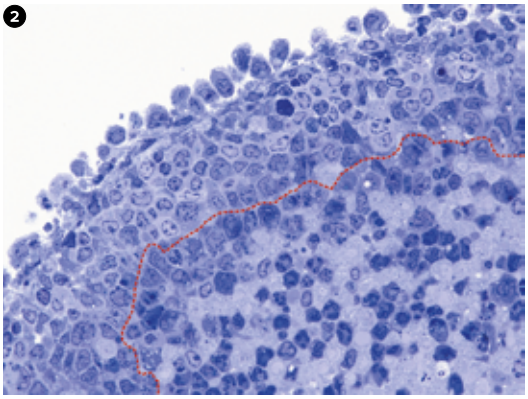

❶ *Whole mount immunofluorescence and confocal microscopy image of a resident macrophage aggregate in the peritoneal wall at 4 hours after infection with the* Escherichia coli *strain M6L4; anti-F4/80 (macrophages; red), anti-Ly6G (neutrophils; cyan) and anti-podoplanin (mesothelial cells; green) staining.*

❷ *Semi-thin section of a resident macrophage aggregate isolated 4 hours after infection with the* Escherichia coli *strain M6L4. The red dashed-line indicates the limits of central area harbouring necrotic macrophages and neutrophils. Toluidine blue staining.*

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Regulation of inflammation by p21 and mitochondrial ROS: from autoimmunity to COVID-19

Increased immune responses and hyperinflammation govern the development and progression of diseases that extend from Autoimmunity to COVID-19. In order to neutralise inflammatory responses, the immune response needs to be supressed. Alternatively, in cancer, immunosuppressed immunity requires reactivation. Therefore is it essential to understand systems that regulate these responses. Notably, our work points to p21 as a regulator of the balance between hyperactivation and immunosuppression by controlling mitochondrial Reactive Oxygen Species (mROS). Our recent work shows that mROS is essential for IFN-gamma production by memory T cells after IL-12 plus IL-18 challenge (Rackov *et al* 2020). IFN-gamma orchestrates inflammatory responses in inflammation-induced diseases. Remakably, Fas controls mROS and IFN-gamma induction independently of its apoptosis inducing potential (Figure 1). Our current work (in preparation) indicates that p21 modulates mROS and IFN-gamma production by memory T cells, corroborating our published data, showing that p21 overexpression tempers autoreactive T cells and IFN-gamma production (Daszkiewicz *et al*, 2015). Therefore, high expression of p21 lowers T cell overactivity, while lack of p21 enhances responses by regulating mROS production (Figure 1).

Similarly to memory T cells, p21 regulates the inflammatory potential in macrophages. We have shown a dual regulatory role for p21; first, in macrophage activation to M1 state (Trakala *et al*, 2009) and, second, in macrophage reprogramming from M1 to the M2 unresponsive state. Lack of p21 prevents macrophage reprogramming to M2 status (Rackov G *et al*, J Clin Invest 2016). Our present results firmly show that mROS, which is regulated by p21, is an early regulator of the inflammatory response of M1 macrophages as it enhances M1 responses as early as five minutes post-activation, and leads to NF-kB activation and ultimately to inflammatory cytokine production. The direct interaction of p21 and mitochondria in M1 macrophages is shown in Figure 2.

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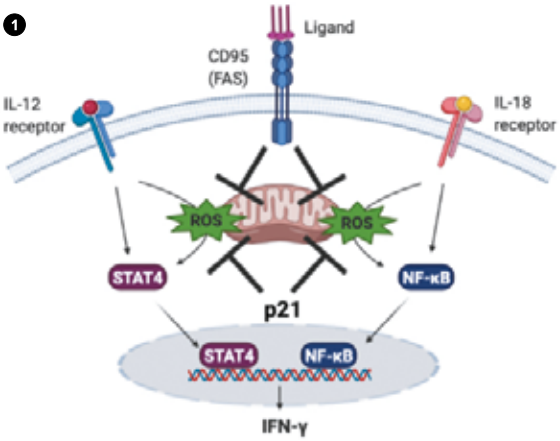

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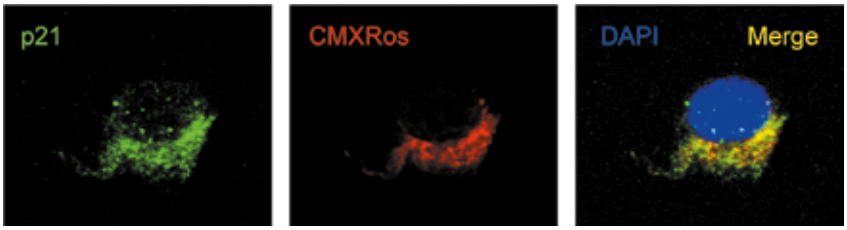

❶ *Schematic representation of how p21 and FAS regulate mitochondrial ROS production and consequently the pathway of memory T cells activation and IFN-gamma production in response to IL-12 plus IL-18.*

❷ *Confocal microscopy of stimulated macrophages shows direct interaction of p21 with mitochondria specifically stained by CMXRos. Polarisation of mitochondria is evident due to their activated status of macrophages.*

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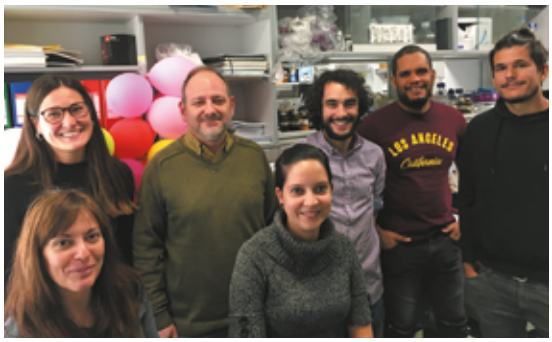

Nanomedicine, cancer immunotherapy and autoimmune diseases

Magnetic Iron oxide nanoparticles (MNPs) have considerable potential to be used as nanomedicines for targeted drug release or magnetic resonance imaging. Recently, we highlighted the promise of using MNPs in other therapeutic approaches to treat cancer, such as the induction of intracellular hyperthermia in tumour cells or the magnetic targeting/retention of lymphocytes in cell transfer therapies. We have also seen that the accumulation of MNPs by different cell types induces oxidative stress and its associated effects as a consequence of MNP degradation. Thus, here we aim to explore whether these responses could be used therapeutically to fight tumours at different levels.

The overall objective of our group is to fully understand the molecular and cellular mechanisms induced by MNPs at their different levels of action. This knowledge can be used to improve the functional design of MNPs for specific biomedical applications, such as therapies to combat tumours and autoimmune diseases, with the aim of bringing them closer to their clinical application. As such, we will pursue five specific objectives: 1) We will expand our studies on the magnetic retention/accumulation of MNP-functionalized anti-tumour lymphoid cells in ACT therapies in order to bring this therapy closer to the clinic; 2) We intend to explore whether the targeting to and/or retention of MNP loaded tolDCs in LNs could ameliorate the symptoms of lupus in the MRL/lpr mouse model of SLE; 3) We will evaluate the capacity of the oxidative stress induced in cells by MNPs to remodel the tumour microenvironment and to improve anti-cancer therapies; 4) We will assess how to improve the efficiency of intracellular heating of MNPs in AMF-induced hyperthermia strategies, studying the biological effects induced by MNPs of different physico-chemical characteristics (size, shape, anisotropy) after the application of an AMF of different intensity and frequency; 5) We will analyse whether oxidative and endoplasmic reticulum (ER) stress caused by MNPs inside tumour cells could affect the processing and presentation of antigens, and whether this might provoke the generation of neoantigens.

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❶ *Lysosomal degradation of superparamagnetic iron oxide nanoparticles inside of macrophages. (Yadileiny Portilla).*

❷ *Retention of tolerogenic dendritic cells (tolDCs) associated to Magnetic nanoparticles (MNPs) using a neodymium magnet of 1.45 T in a flow chamber assay. (Andrés París).*

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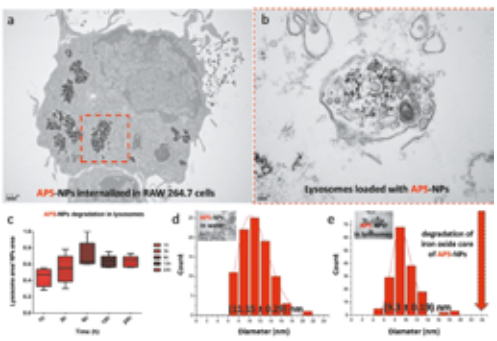

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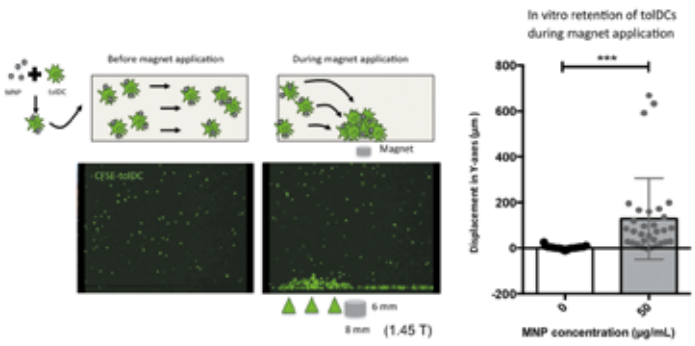

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Cardiac stem cells

Adult mammalian heart refresh damaged or aged cells during their lifetime but with low rate, particularly regarding cardiomyocytes. However, the mechanisms involved in heart turnover remains controversial. We have characterised cardiac progenitor cells that express high levels of the polycomb Bmi1 transcription factor, which contributes to the turnover of the three main cardiac lineages. In response to a variety of cardiac insults these Bmi1^{+} Cardiac Progenitor Cells (B-CPC), get proliferatively activated and their progeny contribution to the mature lineages is enhanced with special proness towards the endothelial lineage. In addition, *in vivo* genetic depletion of the B-CPC population provokes a deleterious condition during acute infarct recovery. Thus, the B-CPC population contains cardiac progenitors contributing both to heart homeostasis and in response to several modes of damage.

In adult tissues, progenitors and stem cells are lodged in specialised structures (niches) that provide a protective microenvironment, essential for their correct regulation. These niches are usually associated to a low oxidative stress environment, where adult progenitors show a restrained proliferative status essential for maintenance of their self-renewal capacity. In good agreement with our working hypothesis, we found that B-CPC show low levels of ROS and, interestingly, in homeostasis conditions, they are located close to the cardiac vasculature, showing a proliferative gradient coincident with Bmi1 expression levels; low-proliferative B-CPC are closer to endothelial structures. These results, together with *in vitro* co-culture experiments, strongly suggested a plausible crosstalk between vessel structures and B-CPC. In addition, we confirmed by transgenic manipulation of ROS levels *in vivo*, that B-CPC cardiac location and their activity are susceptible to oxidative stress modifications. Altogether, we concluded that cardiac vasculature provides a protective and low-stress microenvironment that contributes to the maintenance of B-CPC promoting their self-renewal in adult heart. Currently, we are trying to dissect the specific bidirectional mechanisms involved and defining the B-CPC vascular niche.

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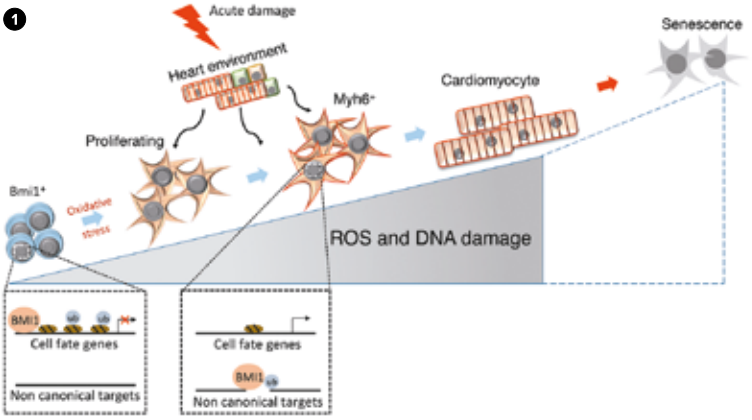
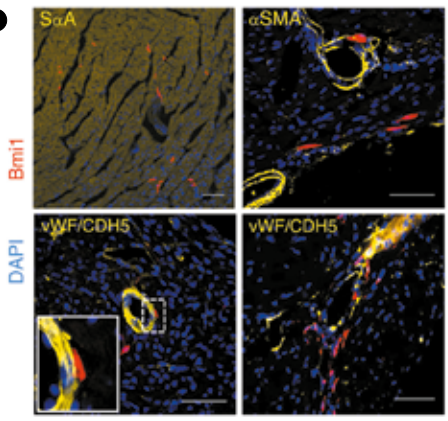
❶ *Scheme of regulation of B-CPC activity in homeostasis and in response to oxidative stress and acute damage.*

❷ *Heart cryosections of reporter mice (B-Tmt) 5-days post Tx induction. B-CPC (Tomato+) are located close to endothelial (yellow markers) cells. Inset (2x).*





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B Lymphocyte dynamics

B lymphocytes patrol our body seeking for pathogen-derived antigens. Recognition of antigen activates the B cell immune response, which leads to the production of highly specific antibodies that will neutralise and eliminate the pathogen, and of memory B cells that confer long-term immunity. The complexity of the B cell response involves changes in lymphocyte behaviour, switching from highly motile states to stable cell-to-cell interactions (immune synapse), adjustments of the cell mechanical properties (flexibility, stiffness) and cell polarity (MTOC and organelle distribution). Gene mutations or functional alterations in proteins related with these events are frequent in B cell pathologies (immunodeficiency, lymphomas), stressing their relevance for B cell function.

Our research focuses on the mechanisms that govern B lymphocyte dynamics, and how their dysfunction leads to B cell pathology. We recently revealed essential new functions of two proteins, Bruton's tyrosine kinase (Btk) and the ζ isoform of Diacylglycerol kinases (DGKζ), both of interest for the clinic as therapeutic targets. Btk has a key role in the signalling of the B cell receptor for antigen and clinical trials with kinase inhibitors are on-going for B cell-lymphoma treatment. We found that Btk promotes the cell-cytoskeleton and adhesion-site remodelling needed for immune synapse formation mainly through its shuttling/scaffold activity. Impairment of that leads to B cell activation defects equivalent to those due to Btk kinase inhibition. Related with DGKζ, known for diminishing antigen receptor signalling through DAG consumption, our findings showed that it also stimulates the B cell immune response. DGKζ facilitates antigen extraction at the immune synapse by promoting actin-cytoskeleton remodelling and mechanical forces. B cell ability of antigen extraction is essential for antigen presentation to CD4 T cells and the germinal centre response. Both events are reduced for DGKζ-deficient B cells compared to wild type (Figure 1).

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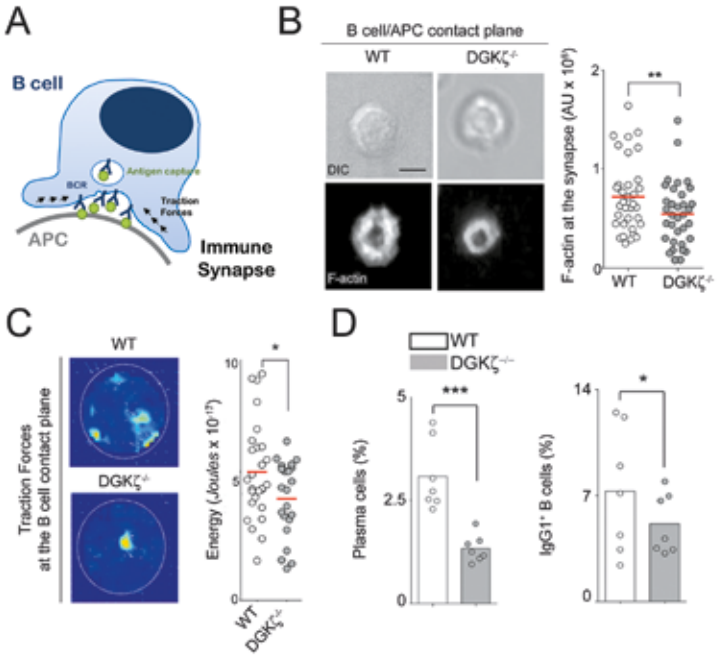

1 *DGKζ stimulates the B cell immune response. A, Events at the B cell immune synapse. B, DIC and fluorescence images of F-actin at the synapse of wild type (WT) and DGKζ-deficient (DGKζ$^{-/-}$) B cells; scale bar, 2.5 µm. Values of the total amount of F-actin at the synapse are shown; each dot is a cell. C, Colour maps of stress forces at the immune synapse of WT and DGKζ-/- B cells, measured by Traction Force Microscopy. Average values of synaptic traction forces per cell over time are shown; each dot is a cell. D, Plasma cells (CD138^{+}) and IgG1+ B cells generated in mice adoptively transferred with WT or DGKζ$^{-/-}$ B cells at day 7 after immunization; each dot is a mouse. *, p<0.05, **, p<0.01, ***, p<0.001.*

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Molecular targets in health and cancer: special focus on PIP3

Our group studies the molecular mechanisms by which signalling proteins control cell behaviour, and how these proteins, when mutated, influence the course of human cancer. In recent years we have focused on the enzymes that control PIP3 (phosphatidylinositol 3-phosphate), a little-abundant molecule in "resting tissues" but which is required when cells need to divide or migrate - including in cancer. We have been involved in the following studies:

1) The action of PI3-kinase beta action on hESC stemness/differentiation decisions
PI3-kinase beta, one of the enzymes generating PIP3, localises to the nucleus and regulates DNA replication, segregation and repair. We are studying its function in human stem cell (hESC) stemness/differentiation decisions.

2) Regulation of PTEN phosphatase activity under near-physiological conditions
PTEN phosphatase, which reduces PIP3 levels, is altered in many human tumours, most commonly during the metastatic phase. The main therapeutic approach for limiting PIP3 action has been to inhibit PI3-kinase enzymes, but boosting tumour PTEN phosphatase activity could be an alternative. We are involved in the study of how PTEN phosphatase activity is modulated after growth factor receptor activation.

3) PIP3 actions in TUMOR microenvironment: hypoxia and oxidative stress
Solid tumours commonly grow under low oxygen conditions (hypoxia). The adaptation of cells to hypoxia is regulated by HIF transcription factors. We are in the process of examining how PI3-kinases modulate HIF-mediated transcription.

Solid tumours also show high levels of reactive oxygen species (ROS), a stress to which their cells have to adapt if they are to survive. Many lung tumours show activation of the **NFE2L2** pathway under high ROS conditions. We are therefore investigating the mechanism of action of NFE2L2 and how to interfere with it in lung cancer.

SELECTED PUBLICATIONS

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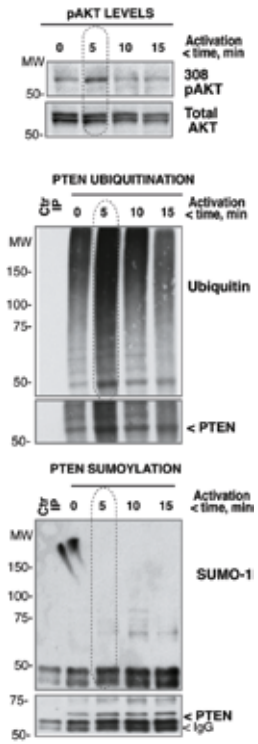

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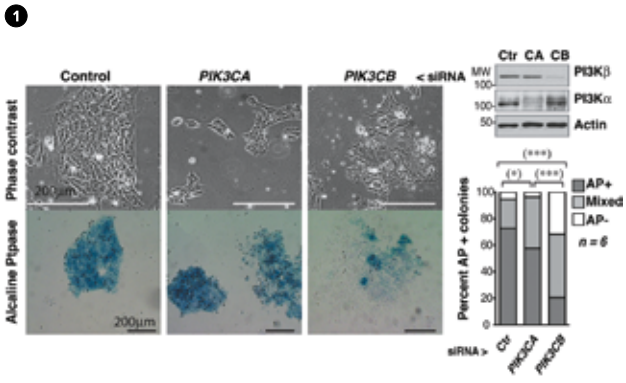

❶ *PI3Kβ is required for human embryonic stem cells stemness. Representative phase-contrast images or alkaline phosphatase (AP)-stained hESC transfected with siRNA control, or for PIK3CB (encoding PI3-kinase beta), or PIK3CA (encoding PI3-kinase alpha) (96 h). Right: Western blotting illustrates silencing efficiency. The graph shows the percentages of AP+, AP- and mixed colonies. (*) P<0.05; (***) P<0.001 (Chi squared test).*

❷ *Maximum pAKT levels correlate with PTEN ubiquitination, while pAKT decrease concurs with PTEN SUMOylation. HEK-293T cells were stimulated with serum (15%) for different times. PTEN was immunoprecipitated from whole cell extracts (for SUMOylation) or from extracts that were enriched in ubiquitinated proteins in appropriate columns (for ubiquitination). WB tested 308-pAKT levels, PTEN ubiquitination, and PTEN SUMOylation.*

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Stress-activated protein kinases in inflammation and cancer

Inflammation is a defensive response against pathogens and a natural process of the immune system to repair tissue damage. However, uncontrolled inflammation is pathological and is the cause of many chronic diseases, which have been steadily increasing in recent decades, especially in western developed countries. This represents a major challenge for modern medicine. Thus, understanding how the inflammatory process is regulated is essential to find new ways to control it, either endogenously or with external therapeutic intervention.

In these two years we have expanded our knowledge on the molecular and cellular mechanisms involved in the inflammatory response in the settings of chronic inflammation leading to tumour development, as occurring in colon cancer associated to colitis; and also, the development of new tools (e.g. kinase inhibitors) for the treatment of inflammation-driven tumours and other inflammatory diseases.

We have also investigated the role of p38MAPK in the development of immune cells such as B lymphocytes in bone marrow and spleen, using mice lacking p38γ and p38δ, or conditional knockout mice that lack both p38γ and p38δ specifically in the B cell compartment. We found that p38γ/δ-deficient mice had reduced numbers of peripheral B cells as well as altered marginal zone B cell differentiation in the spleen. Expression of co-stimulatory proteins and activation markers in p38γ/δ-deficient B cells are diminished in response to BCR and CD40 stimulation; p38γ and p38δ are necessary for B cell proliferation induced by BCR and CD40 but not by TLR4 signalling. Furthermore, p38γ/δ-null mice produced significantly lower antibody responses to T-dependent antigens. Our results identify novel functions for p38γ and p38δ in B cells and in the T-dependent humoral response; and show that the combined activity of these kinases is needed for peripheral B cell differentiation and function.

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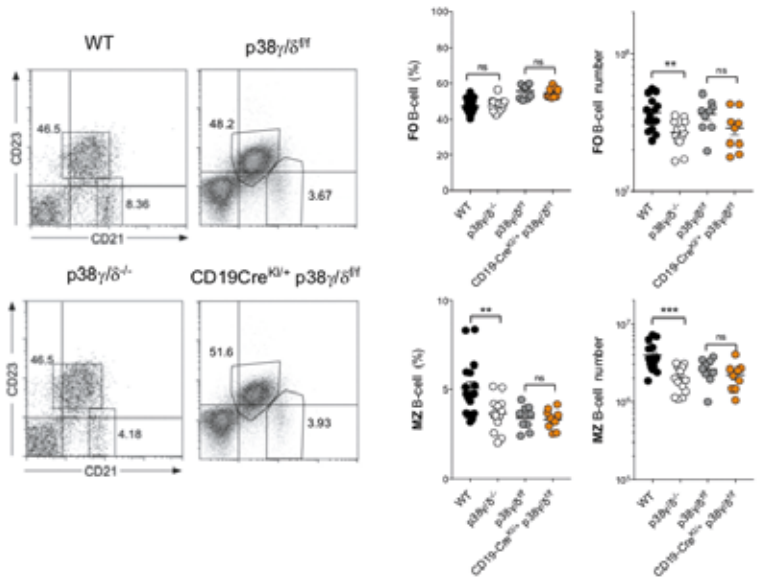

1 *Analysis of B cell populations in the spleen. Representative dot plots for CD21 and CD23 expression in splenocytes from mice of the specified genotypes. Frequencies of gated follicular (FO) B cells (CD21+CD23+) and marginal zone (MZ) B cells (CD21hiCD23-) (gates) are indicated. Frequency and total cell number of FO and MZ B cells in adult mouse spleens. Each dot represents a single mouse.*

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SELECTED PUBLICATIONS

Perez-Zsolt D, Erkizia I, Pino M, García-Gallo M, Martín MT, *et al.* Anti-Siglec-1 antibodies block Ebola viral uptake and decrease cytoplasmic viral entry. Nat Microbiol 2019; 4: 1558-1570.

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Physiopathology of chemokine receptor interactions

Our group studies the role of chemokines and their receptors in tumour progression and metastasis, since they are involved in tumour cell survival and proliferation, tumour-associated angiogenesis and the antitumour immune response. There is growing interest in the development of new antibody-based immunotherapies for cancer treatment. We have generated a panel of mouse monoclonal antibodies (mAbs) specific for the human CCR9 receptor, which is overexpressed in different haematological malignancies. Two antibodies that were selected for their efficacy in reducing the growth of human CCR9$^+$ tumours in different immunodeficient mouse models have been protected by an international patent and have been licensed to SunRock Biopharma. Chimeric and humanised variants of these antibodies also effectively inhibit tumour growth. Recently, using the CRISPR/Cas9 system, tumour cell lines with modified variants of CCR9, were generated and are being used in ongoing experiments on animal models to evaluate whether the candidate antibodies for clinical use exhibit any off-target side effects.

With the aim to generate antibody cocktails that can simultaneously attack different molecular targets on leukaemia cells, we generated mAbs against surface antigens present on human T-cell acute lymphoblastic leukaemia cells. Several of them strongly reduce tumour size in animal models. Using proteomic techniques to identify the antigens recognized by these mAbs, we are selecting those directed against cell surface molecules that are potential therapeutic targets.

In collaboration with different research groups, we are also generating and evaluating mAbs that can be used to modulate the immune response in other pathologies. We have contributed to analyse the role of the CCL1-CCR8 axis in atherosclerosis, to study CD5L in liver fibrosis and to generate new tools for inhibiting Ebola and HIV-1 viral uptake. We have also generated mAbs against SARS-CoV-2 that are being evaluated as potential therapeutic agents for the treatment of COVID-19.

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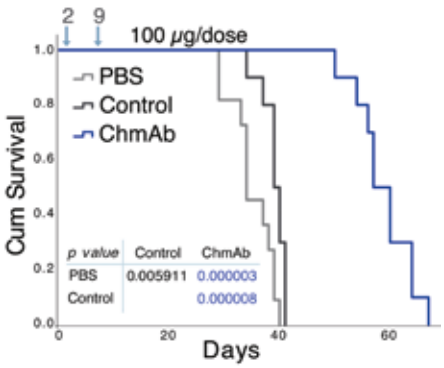

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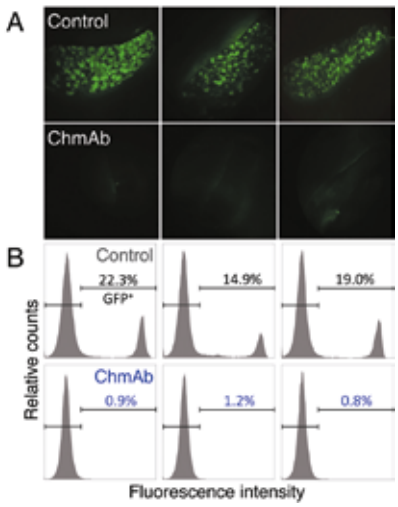

❶ *Kaplan-Meier survival curves of NSG mice carrying xenotransplants. MOLT4-GFP cells were injected into the tail vein of mice on day 1. Mice were subsequently treated on days 2 and 9 with either chimeric anti-CCR9 mAb (ChmAb), isotype control mAb or PBS.*

❷ *A. Stereomicroscopic images of a representative spleen from each treatment group, where the accumulation of tumour cells (MOLT-4-GFP) in the isotype-control treated animals but not in the ChmAb-treated group, could be observed. Data from 3 mice of each 10 mice group are shown. B. Flow cytometry analyses of mouse bone marrow showing the fraction of MOLT-4-GFP cells from isotype control and ChmAb-treated animals. Data from 3 mice of each 10 mice group are shown.*

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Signalling networks in inflammation and cancer

We aim to understand the molecular cues that regulate Inflammation in pathological conditions, such as cancer or Alzheimer's disease. Using multidisciplinary approaches, we study key immune and non-immune cell elements that participate in the inflammatory reaction. In the 2019-2020 period we worked in four areas:

1. Normalisation of tumour-associated vasculature to improve immunotherapy.
Angiogenesis is a common feature of cancer. Tumour vessels are, however, dysfunctional, leading to hypoxia and tumour aggressiveness. We discovered that restoring the levels of Extracellular superoxide dismutase (SOD3) in the tumour microenvironment normalises the tumour vasculature and increases the specific tumour infiltration by effector immune cells (details in Carmona *et al*). Moreover, anti-angiogenic agents synergise with tumour immunotherapy to improve the survival of patients with metastatic breast cancer (details in Quintela *et al*).

2. Identification of signalling pathways downstream of PD-1.
PD-1 blockade is common immunotherapeutic treatment in cancer. Yet little is known about how PD-1 blocks the effector function in T cells. Using RNA-seq and bioinformatics we have identified a PD-1-induced genetic program that elicits immunosuppression by targeting the metabolism and mitochondrial ultrastructure of $CD8^+$ T cells (details in Ogando *et al*).

3. CCR5 effects on T-cell receptor (TCR) organisation and the memory $CD4^+$ T cell response.
The chemokine receptor CCR5 not only causes chemoattraction of immune cells, but also provides costimulatory signals required for optimal $CD4^+$ T cell activation. We have now found that CCR5 regulates the functionality of $CD4^+$ memory T cells. This activity is associated to changes in the nanoscale organisation of the TCR due to alterations on sphingolipid metabolism (details in Martín-Leal *et al*).

4. Innate immune cell differentiation in neurological diseases.
The high co-morbidity of Alzheimer's disease with cardiovascular and metabolic disorders suggest that systemic alterations might be determinant for Alzheimer's evolution. Using isogenic iPSC we have studied the influence of APOEε4 polymorphism in macrophage polarisation, metabolism and cholesterol efflux activity, and the association of these parameters to the development of late-onset Alzheimer's disease (in preparation).

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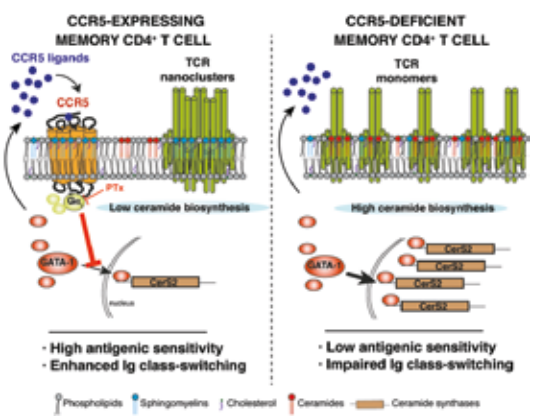

1 *CCR5 primes memory CD4+ T-cell function. In memory cells, CCR5 signals inhibit the nuclear translocation of GATA-1. This reduces ceramide biosynthesis, enables T cell receptor (TCR) nanoclustering and increase the response after antigenic re-encounter. Lack of CCR5 (ccr5Δ32 persons) enhances ceramide levels, which rigidify the cell membrane and impedes TCR nanoclustering.*

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Mora-Gallardo C, Sánchez de Diego A, Gutiérrez-Hernández J, Talavera-Gutiérrez A, Thierry Fischer T *et al.* Dido3-dependent SFPQ recruitment maintains efficiency in mammalian alternative splicing. Nucleic Acids Res 2019; 47: 5381–5394.

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Stem cells and immunity

We have identified *Death Inducer-Obliterator (Dido)*, which produces three protein isoforms termed DIDO1, 2, and 3, as an important gene in stem cell (SC) differentiation. Several lines of investigation link the *Dido* gene to SC biology through its function in transcription and its relation to chromatin biology. Our studies have characterised the effects of the 5' and 3' regions of the Dido gene individually *in vivo* and in *in vitro* cell lines. Our analysis of the *Dido* gene 5' region identified nuclear localisation of all three isoforms and their interaction with chromatin. The recent characterisation of the 3' regions of the gene focused on DIDO3, the only isoform to comprise a complete domain architecture. DIDO3-specific sequences are encoded by a separate exon located in the 3' region, found in all vertebrates but not in organisms without SC such as yeast.

Mammalian cells that lack Dido3 but can produce the other isoforms show widespread defects in the processing of RNA derived from spliced genes. This finding indicates that DIDO3 has a role in splicing (see Figure 1), and possibly in transcription termination (in review process). This hypothesis is supported by preliminary data, since part of the aberrant RNAs found by RNA sequencing involve readthrough beyond the constitutive 3' UTR. Dido3 thus appears to have evolved to compensate for the increased dependency on RNA processing.

In addition, Dido3 mutations cause blockade of stem cell differentiation, defects in chromosome segregation, and genomic instability. Analysis of cells derived from the Dido3 mutant shows centrosome amplification, cytokinesis defects, binucleated cells, and genomic instability. Previous work in our laboratory showed that mice lacking the N-terminal domain of Dido develop myelodysplasia/myeloproliferative disorders (MDS). Based on these results, we propose that Dido3 defects contribute notably to the pathologies associated with the aberrant production of Dido isoforms.

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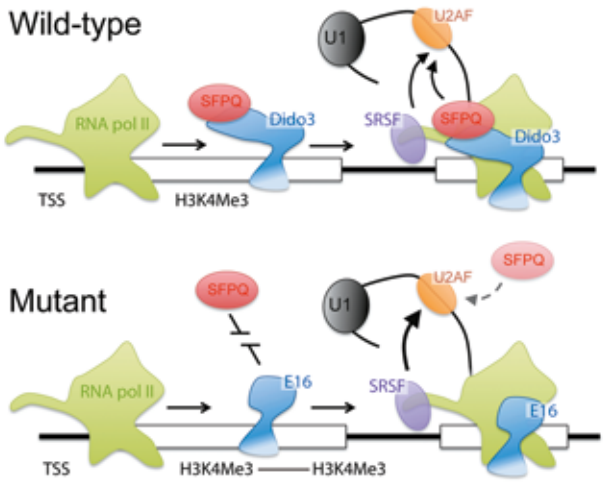

1 *Model for the role of Dido3 in transcription and splicing. Protein interaction studies and deletion mutants attribute a bridging role to Dido3, in which Histones H3 acts as a reservoir from which the protein is recruited by RNA Polymerase II. In turn, Dido3 facilitates the binding of SFPQ to nascent RNA for subsequent spliceosome assembly.*

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Chemokine receptors: New targets for therapeutic intervention

The chemokine receptors are members of the GPCR family that, through interaction with their ligands, induce a wide variety of cellular responses including cell polarisation, movement, immune and inflammatory responses, as well as prevention of HIV-1 infection. Like a Russian matryoshka doll, the chemokine receptor system is more complex than initially envisaged. The chemokines and their receptors exist as monomers, dimers and oligomers, their expression pattern is highly regulated, and the ligands can bind distinct receptors with similar affinities. The use of novel imaging-based technologies, particularly real-time imaging modalities, has shed new light on the very dynamic conformations that chemokine receptors adopt, and that affect chemokine responses. To date, all of the chemokine receptors tested form homo- and heterodimers during their synthesis and maturation, and in such conformations reach the cell membrane.

Knowledge of the dynamic interactions between ligands and receptors, as well as their interplay with other proteins co-expressed by the cell, lipids at the cell membrane, the cellular cytoskeleton, and downstream signalling machinery will be crucial to determine how they modulate cell responses. Using STimulated Emission Depletion (STED) microscopy and single particle tracking and Total Internal Reflection Fluorescence Microscopy (TIRFM) we have evaluated the receptor organisation and signalling in living cells on the spatial and temporal scales and determined the presence of basal nanoclusters of CXCR4 in resting T cells, whose extent, dynamics, and signalling strength are modulated by the orchestrated action of the actin cytoskeleton, other molecules expressed at the cell membrane, and the ligands. This new information will transform our vision of the chemokine-mediated functions, and will hopefully identify exciting opportunities for drug discovery.

In parallel, our group has also a research line to investigate inflammatory and autoimmune disease models to test the targets and hypothesis identified on the chemokine projects.

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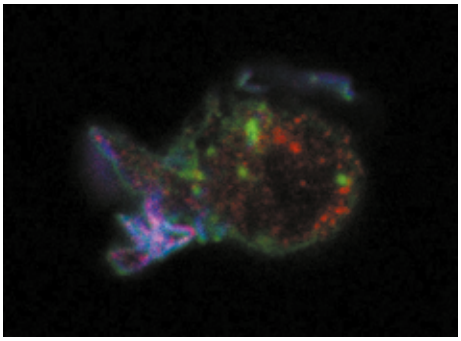

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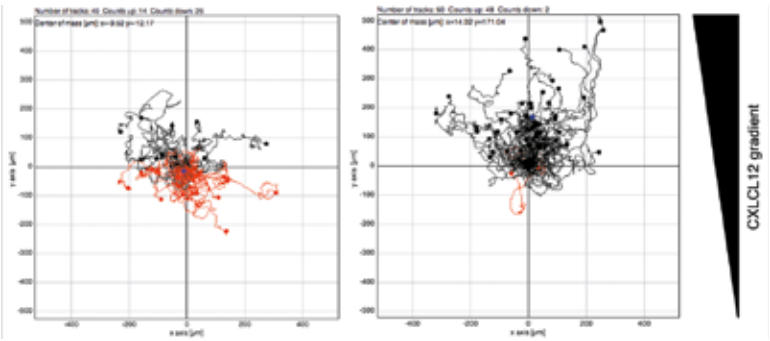

1 *CXCL12 triggers rapid cell polarisation and lamellipodia formation. Jurkat cells expressing CXCR4-AcGFP (green) were added on coverslips coated with fibronectin plus CXCL12, fixed and stained with anti-Rac1-GTP mAb (blue) and phalloidin (red).*

2 *CXCL12 triggers directed cell migration. Spider graphs showing directed JK cells migration towards CXCL12 (right) vs control in the absence of gradient (left). The black triangle indicates the direction of the chemokine gradient.*

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Diacylglycerol kinases in the control of immune response and cancer progression

T cell tolerance is the mechanism that protects healthy tissue from damage during immune attack. Solid tumours employ similar mechanisms, expressing ligands for co-inhibitory receptors to avoid immune destruction (Fig). The Diacylglycerol Kinase (DGK) family of enzymes transform diacylglycerol (DAG) in to phosphatidic acid. Several DGK isoforms have been related to cancer but only the alpha and zeta have been extensively characterised as negative regulators of T cell responses. The abnormal elevation of these two DGK isoforms in tumour infiltrating lymphocytes drives T cells into anergic non-functional states. Manipulation of DGK activity/expression enhances antitumour T cell functions, suggesting potential for pharmacological intervention.

Our group works to better understand the redundant and specific actions of DGK alpha/zeta in cancer. We have identified high DGKα expression, that in healthy cells is mostly restricted to T cells, in mesenchymal cancer types. Targeting DGKα thus not only re-instates immunological tumour recognition and destruction, but may also help to destroy tumours by interfering with oncogenic signals. DGKζ on the other hand, is broadly expressed and operates in others systems different from T cells. In the laboratory we use combinations of genetical and biochemical approaches to explore the consequences of isoform-specific DGK targeting in distinct preclinical models of cancer. We also investigate the immunomodulatory potential of small molecules with potent inhibitory action against purified enzymes.

Finally, we explore the potential adverse effects of DGK manipulation using Down syndrome-associated comorbidities as a model. Our final purpose is dual: on one hand we seek to demonstrate the full potential of DGK targeting so inhibitors of these kinases can be considered in the arsenal of cancer immunotherapies. On the other we want to identify possible adverse consequences derived from targeting DGK-regulated pathways.

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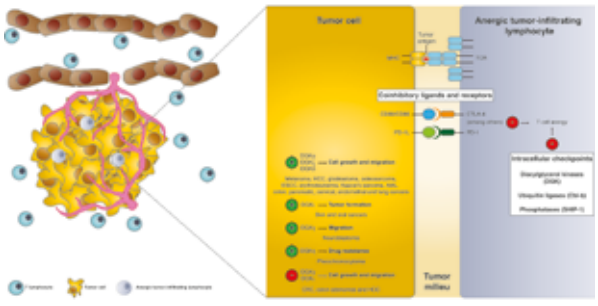

1 *DGK as intracellular checkpoints. Left: cartoon shows how tumour-infiltrating lymphocytes become anergic and unable to destroy tumours. Right: Membrane-bound immune checkpoints in T lymphocytes act as coinhibitory receptors upon recognition of tumour-expressed ligands. In tumours DGKs promote malignant traits whereas in T lymphocytes specific isoforms act as intracellular checkpoints that limit T cell cytotoxic potential. DGK blockade could reinstate T cell attack on tumours, limiting at the same time tumour growth and metastasis. MHC, mayor histocompatibility complex; TCR, T cell receptor; CD80/86, cluster of differentiation 80/86; CTLA-4, cytotoxic T-lymphocyte antigen 4; PD-1/L, Programmed cell death protein 1/ligand; HCC, hepatocellular carcinoma; ESCC, esophageal squamous cell carcinoma; AML, acute myeloid leukemia; CRC, colorectal carcinoma.*

SELECTED PUBLICATIONS

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González-Mancha N, Mérida I. Interplay between SNX27 and DAG metabolism in the control of trafficking and signaling at the IS. Int J Mol Sci 2020; 21 (12): 4254.

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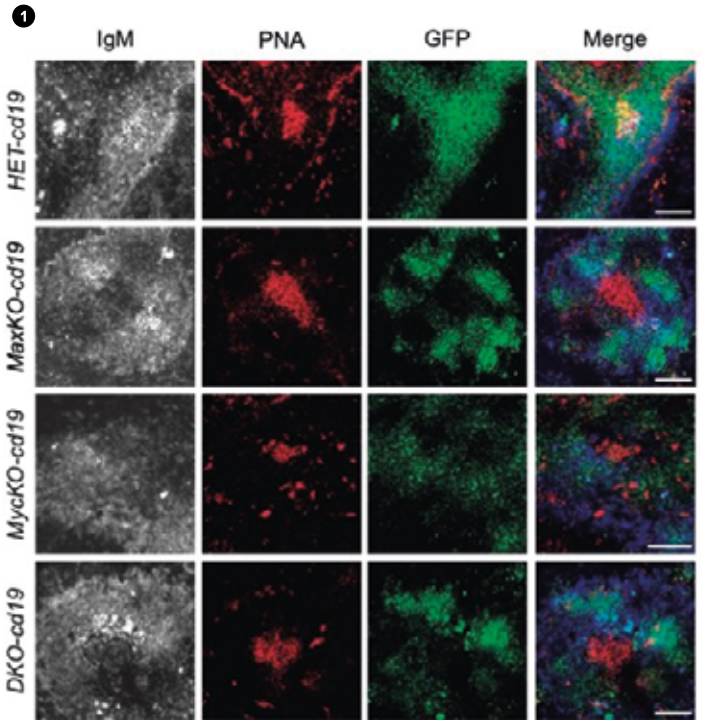
Transcriptional control of lymphocyte differentiation

Our major biological question is to understand how cell differentiation is regulated by transcription factors and how this process is altered in pathological scenarios such as cancer. We approach this question by analysing the transcriptional program in a well-defined setting *in vivo* such as B lymphocyte differentiation. Among the wide spectrum of transcription factors involved in this process we focused our efforts in the function of the proto-oncogene *c-myc* for two reasons. First, the c-Myc protein is a member of the Myc family (N-, L- and c-Myc) of transcription factors involved in numerous biological functions including the regulation of cell proliferation, differentiation and apoptosis in multiple cell types. This pleiotropic function confers this protein an essential and distinct role at different differentiation stages in numerous cell types. Second, in animal models and humans, deregulated c-Myc expression leads to the development of tumours, including B and T lymphomas. This oncogenic potential provides an interesting dimension in terms of possible therapeutic applications of our research.



1 *Analysis of germinal centre (GC) formation in the spleen of Max KO (MaxKO-cd19), Myc KO (Myc-KO-cd19), Double KO (DKO-cd19) and heterozygous control mice immunised with TNP-KLH. Representative images of frozen spleen sections stained with IgM (grey/blue), PNA (GC marker; red), and GFP (Max-, c-Myc- or c-Myc/Max-deficient B cells; green). Scale bar, 80µm.*

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Bravo García-Morato M, Calvo Apalategi A, Bravo-Gallego LY, Blázquez Moreno A, Simón-Fuentes M, *et al,* Impaired control of multiple viral infections in a family with complete IRF9 deficiency. J Allergy Clin Immunol 2019; 144: 309-312. e10.

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Receptor ligand interactions in immune responses to cancer and viruses

Natural killer (NK) cells kill infected cells and secrete cytokines, to play an important role in defence against viral infection. Although NK cells are often perceived as rather primitive lymphocytes; always ready to kill unless checked by inhibitory receptors binding to MHC Class I molecules. It is now clear that the behaviour of an NK cell when confronted by a potential target cell depends on the integration of multiple signals coming from a range of activating and inhibitory receptors. Inhibitory receptor expression is largely under genetic control, whereas activation receptor expression is heavily environmentally influenced and NK cells adapt their expression of activating receptors in response to pathogens and tumours so giving rise to the multiple discrete NK cell subpopulations that can be found in human peripheral blood. Thus, to understand NK cells in disease requires detailed knowledge of the biochemistry of individual activating and inhibitory receptors and the subpopulations of NK cells expressing different receptor repertoires.

We have contributed extensively to knowledge of the cell biology of various NK cell receptors and their ligands and recently, to address the wider roles of NK cells in immunity, we have initiated collaborations with clinical colleagues to study patients suffering from primary immunodeficiencies that affect NK cell function. Inherited human immunodeficiencies are experiments of nature in which gene defects compromise immune function and our hypothesis is that the study of congenital defects affecting NK cells will help to increase our understanding of NK cell biology and function *in vivo*. We use innovative flow cytometry and molecular genetic technologies to characterise these primary immunodeficiency diseases at high resolution. These studies are complemented and enhanced by *in vitro* experiments involving the study of NK cells and the use of genome-editing technologies to study in detail the molecular bases of the changes observed *in vivo*.

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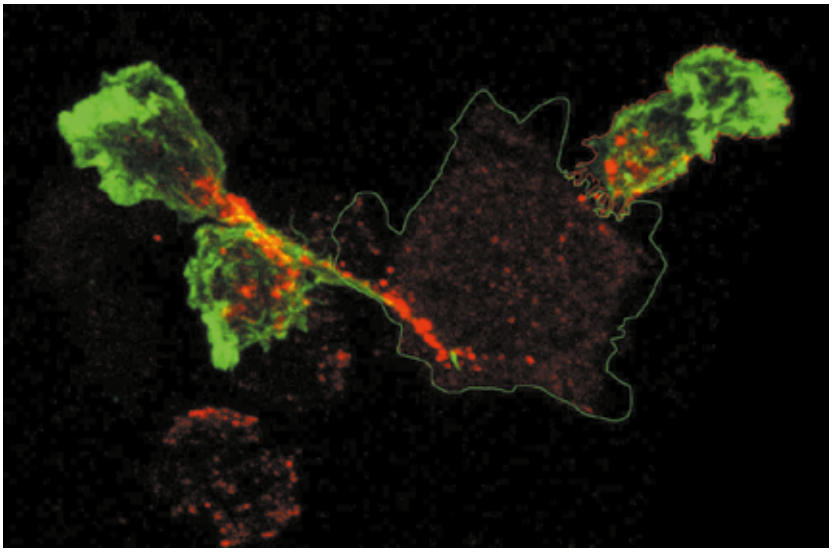

1 *Human natural killer cells attacking a tumour cell induced to express ligands of the activated receptor NKG2D (granzyme stained in red) and polymerised actin in green)*

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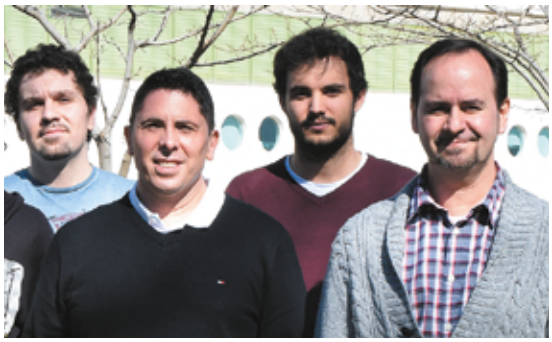

T cell signalling in autoimmune diseases and cancer

The main goal of our group is the characterisation of the molecular mechanisms involved in cell activation, proliferation and apoptosis in the context of inflammation and tumour development. In particular, we are focused on the study of the biological function of the Gadd45 and p38 MAPK family in these processes.

Gadd45 family proteins have an important role in cell cycle control, proliferation, cell survival, and maintenance a genomic stability in response to environmental and physiological stress. In general, there is a large body of evidence that Gadd45 proteins play a key role in tumor suppression. Reduced expression of Gadd45a and Gadd45b has been observed in many tumours and cell lines. Often, this is correlated with promoter methylation in several types of human cancer. In order to identify novel regulators involved in tumorigenesis and/or inflammation, we have developed specific knockout mouse lines of potential autoimmune disease and tumor suppressor genes.

Notably, our recent findings on the role of Gadd45 on carcinogenesis challenge current dogmas on cell regulation and demonstrate a novel role for Gadd45 in tumor promotion. Currently, we are studying the molecular mechanisms that regulate this process. We hypothesise the lack of Gadd45 could affect the molecular mechanisms that control cell death, proliferation, cytokine production or immune cell infiltration in acute and chronic inflammation as well as tumorigenesis. To dissect the molecular pathways involved in carcinogenesis, we are analysing the expression of pro-inflammatory and pro-tumorigenic genes, apoptotic proteins and MAPKs activation by different techniques. The characterisation of novel regulators involved in inflammation-mediated carcinogenesis will help identify new molecular targets for tumour treatment.

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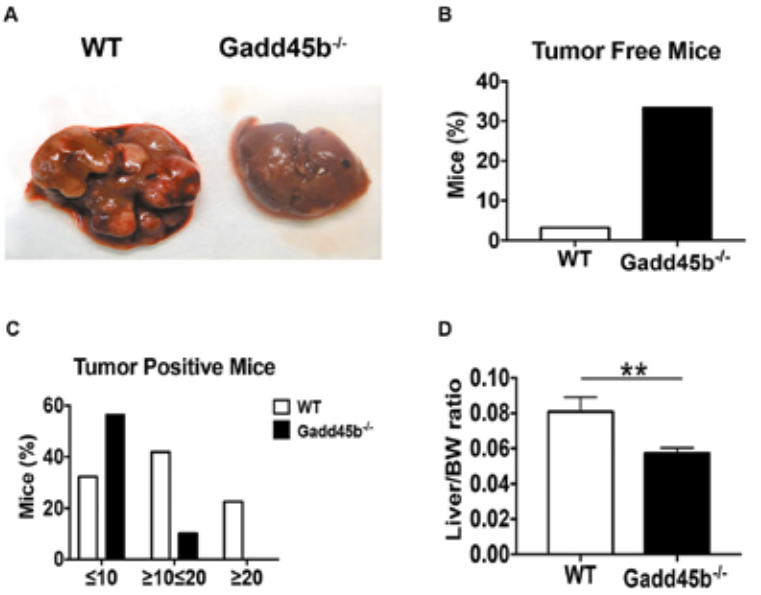

1 *Liver tumour formation in WT and Gadd45b$^{-/-}$ mice. (A) Representative picture of induced liver tumors in wild-type and Gadd45b$^{-/-}$ mice. (B) The percentage of tumour-free mice at 9 months. Statistically significant differences between WT and Gadd45b$^{-/-}$ mice are indicated (P<0,01). (C) The percentage of tumour-positive mice after nine months. (D) The liver/body weight ratio was calculated and expressed as the mean percentage ± S.E.M.*

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Tumour immune activation and evasion

The group is interested in the immune response against cancer, in particular mediated by Natural Killer (NK) cells. These cells respond against tumours after integration of activating and inhibitory signals coming from a large number of receptors. One of the main cytotoxic receptors, NKG2D, recognises ligands that can also be released as soluble molecules either truncated by metalloproteases or in extracellular vesicles (EVs), resulting in immune evasion. In the last years, we have developed several methodologies to examine NKG2D-ligands and other tumour markers carried in EVs. In addition, we have used models of bladder cancer and melanoma to understand successful treatments for cancer that involve activation of the immune system. Since the treatment of bladder cancer patients with intra-vesical instillations of BCG (Bacille Calmette-Guérin) has been used successfully for decades, *in vitro* models that include PBMCs and mycobacteria are used. In parallel, *ex vivo* samples from patients treated with BCG have revealed that urine, collected one week after instillations, provides information on long lasting immune responses that continuously release soluble factors. We have described the presence of CXCL10, a chemokine that could be used to follow the effect of the treatment in patients.

During the COVID-19 pandemic, we have developed a serology test including several SARS-CoV-2 antigens and have described that the main protease of the virus (3CLpro, Mpro) is antigenic in COVID-19 patients. A patent has been filed and the know-how licensed in a non-exclusive manner. The kit commercialised by Immunostep, S.L. was approved for diagnostics by the Spanish regulatory agency (AEMPS).

SELECTED PUBLICATIONS

Valés-Gómez M. Bacillus Calmette Guérin in bladder cancer: is more immune stimulation better? Transl Androl Urol 2019; 8: S517-S520.

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Portilla AP, Moraru M, Moreno AB, Kolb P, García-Morato MB, *et al.* Identification of the first cases of complete CD16A deficiency: association with persistent EBV infection. J Allergy Clin Immunol 2020; 145 (4): 1288-1292.

❶ *Detection of immune soluble factors in urine from bladder cancer patients treated with BCG. Treatment of non-muscle invasive bladder cancer consists on weekly instillations with Bacillus Calmette-Guérin (BCG), the tuberculosis vaccine. After instillations, patients activate the immune response with recruitment of cells and soluble factors, such as cytokines and chemokines, to the bladder. The identity of these chemokines can be detected in urine even 7 days after the contact with the mycobacteria. Data in Ashiru et al. Cancers, 2019.*

❷ *Detection of SARS-CoV-2 Mpro-specific antibodies by ELISA. Plates were coated with SARS-CoV-2 Mpro and sera dilutions (1/50 to 1/1600, as indicated) were tested. Black symbols correspond to COVID-19 patients; grey symbols correspond to sera collected pre-COVID.*

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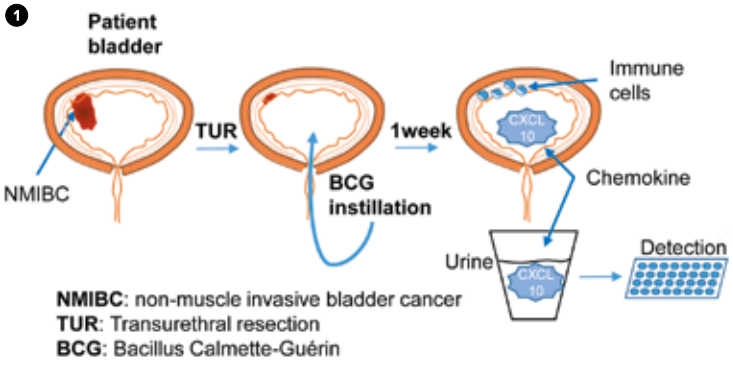

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