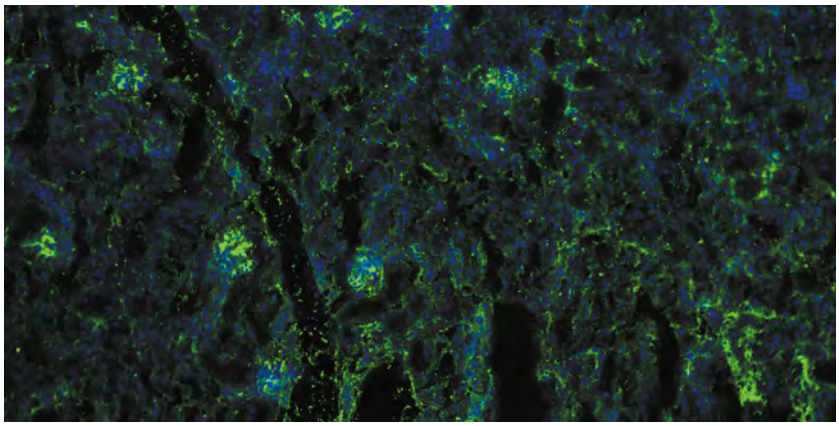

Immunology and Oncology

Research in the Department of Immunology and Oncology focusses on the molecular and cellular bases of immune system function and on tumour development. The Department comprises 16 Research Groups, including 1 Junior Group. Some of our groups are working in inflammatory and in autoimmune diseases; in the study of the innate and adaptive immunity in inflammation and in the tumour microenvironment; in the immune response against pathogens. Others are addressing several aspects of cancer development and treatment, with special emphasis on the identification of new anti-tumour targets by characterising the cellular and molecular mechanisms that underlie inflammation-driven carcinogenesis; in the relationships among stem cells, metastasis, inflammation and cancer; in tumour immunology; in tumour diagnosis and immunotherapy. Some research groups are focused on translational technologies such as nanoparticle-based nanomedicines, monoclonal antibodies, exosomes, or targeting peptides.

The department expertise combines multidisciplinary cutting-edge technologies such as advanced microscopy (total internal reflection fluorescence microscopy or real-time confocal microscopy), multi-parameter flow cytometry, nanoparticle production and characterization, exosome isolation and characterization, *in vivo* peptide phage display for the identification of targeting peptides, and next-generation "omics". In addition, we have extensive experience in the generation and use of genetically modified mouse models using the latest techniques.

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.

Since its origins, the DIO has maintained stable, productive collaborations with public and private partners that include prominent national and international research institutes, hospitals and pharmaceutical companies. As a recent example, during the COVID-19 pandemic, the 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 test has been approved for SARS-CoV2 diagnostics by AEMPS, and commercialised by the Spanish company Immunostep. An agreement with the public health organization Medicines Patent Pool (MPP), supported by the United Nations, and supervised by the World Health Organization (WHO) will allow this technology to reach developing countries.

Figure Legend: Immunofluorescence image from frozen kidney sections (Andrés París-Muñoz).

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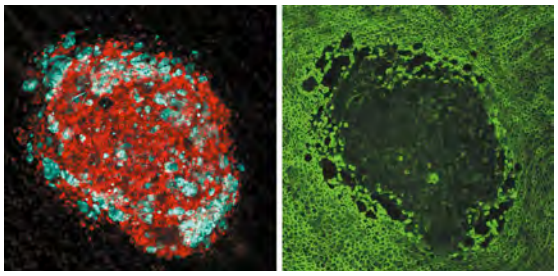
Immunobiology of macrophages and dendritic cells

Carlos Ardavín´s lab is focused on the immunobiology of monocytes, macrophages and dendritic cells during airway allergy and bacterial and fungal infections, and over the last years on the crosstalk between inflammation and coagulation in the control of innate immunity in the peritoneal cavity, using murine experimental models of bacterial sepsis and peritoneal metastasis.

Studies carried out in Ardavín's lab demonstrate that large peritoneal macrophages are crucial for defense against *E. coli* infection by coordinating the formation of mesothelium-bound, multicellular structures, composed by sequentially-recruited large peritoneal macrophages, B1-cells, neutrophils and monocyte-derived cells, that we termed resMØ-aggregates. ResMØ-aggregates are dynamic, transient structures, that provide a physical scaffold allowing the interaction and function of peritoneal immune cells, free in the peritoneal fluid in homeostasis, and enable efficient control of infection. The formation of resMØ-aggregates requires the development of a fibrin scaffold resulting from mesothelial tissue factor-dependent fibrin polymerisation.

Ongoing research in Ardavin's lab is focused on defining the molecular events driving fibrin polymerisation during peritoneal antimicrobial immunity, and in exploring whether resMØ-aggregate-like structures may also be formed during infection in other body cavities, such as the pleural cavity or the brain ventricular system, harboring MØs in a fluidic environment, that may need to attach to the epithelium lining these cavities to fulfill their immune defense functions.

An in-depth understanding of the response of the peritoneal immune system to peritoneal tumour metastasis is needed to develop immunotherapeutical treatments as an alternative to the current therapeutical strategies associated to severe adverse effects. By using an experimental model of colorectal cancer peritoneal metastasis based on intraperitoneal transfer of mouse tumor organoids derived from primary tumours, ongoing research in Ardavin's lab aims at exploring the interplay between peritoneal innate immunity and coagulation in the control of peritoneal metastatic growth.



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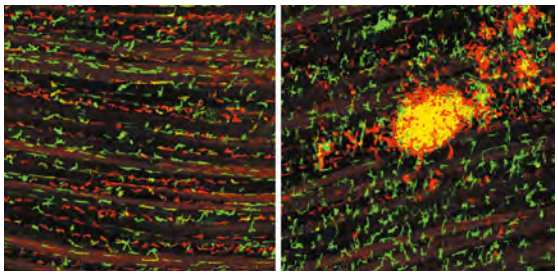

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1 *Whole mount immunofluorescence and confocal microscopy images of an early colorectal cancer (CRC) metastasis in the peritoneal wall, at 4 hours after intraperitoneal injection of mCherry-labeled mouse CRC-derived organoids. Turquoise blue: mCherry-labeled tumor cells. Red: resident peritoneal macrophages; anti-F4/80 staining. Green: mesothelial cells; anti-podoplanin staining.*

2 *Whole mount immunofluorescence and confocal microscopy images of the peritoneal wall in the steady state (left), and at 4 hours after infection with the Escherichia coli strain M6L4 (right), showing the response of MHC-II+ Tim4- and MHC-II- Tim4+ submesothelial macrophages to bacterial infection. Red: anti-Tim4 staining. Green: anti-MHC-II staining.*

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Regulation of inflammation by p21 and mitochondrial ROS in autoimmunity and cancer

Increased immune responses and hyper-inflammation govern the development and progression of diseases that extend from Autoimmunity, Cancer or COVID-19. In order to neutralise autoimmune inflammation, immune responses needs to be supressed. Alternatively, in cancer, immunosuppressed immunity requires reactivation. Therefore is it essential to understand how we could regulate the inflammatory responses. Notably, we have identified p21 as a regulator of the balance between hyper-activation 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*, 2022) and that inhibition of mROS results in reduction memory T cells (Figure 1). IFN-gamma orchestrates inflammatory responses in inflammation-induced diseases. Remarkably, Fas controls mROS and IFN-gamma induction independently of its apoptosis inducing potential. 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 p21expression lowers T cell overactivity, while lack of p21 enhances mROS production and cell responses.

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 *et al*, 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 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.

1 *Confocal microscopy of naïve and memory-like T cells shows increased p65 nuclear translocation in the later and supports the increased responses of memory T cells, which is linked to mROS. DPI is an inhibitor of mROS generation and lowers the p65 translocation.*

2 *Confocal microscopy of stimulated macrophages showed direct interaction of p21 with mitochondria stained by CMXRos. Lack of p21 reduced staining by CMXRos, which reflects lower mitochondrial membrane potential in p21 macrophages at 15 and 30 minutes post LPS-stimulation.*

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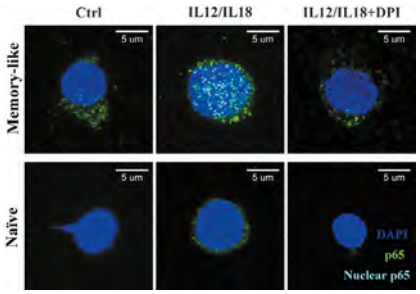

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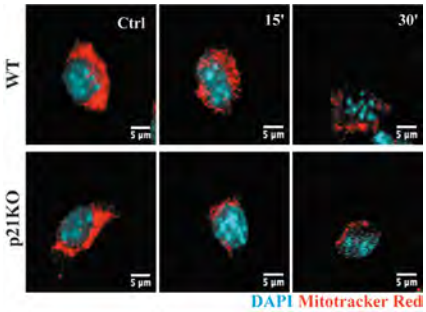

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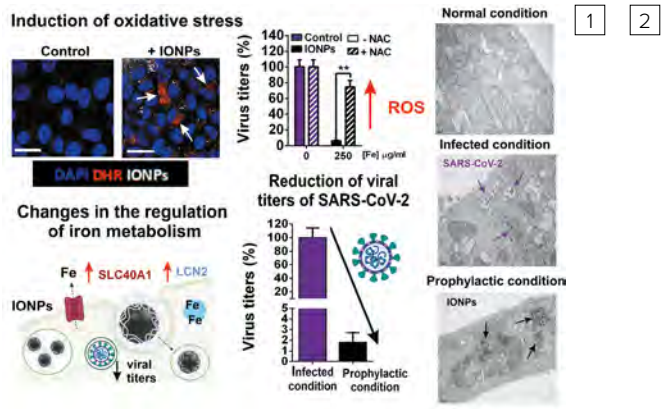
Nanomedicine, cancer immunotherapy and autoimmune diseases

During these years, our group has contributed to demonstrate the potential of iron oxide nanoparticles (IONPs) to be used as nanomedicines in different biomedical applications for cancer treatment: targeted drug release, reprogramming of macrophage responses and the tumour microenvironment, induction of intracellular hyperthermia in tumour cells, and magnetic targeting/retention of lymphocytes functionalised with IONPs in cell transfer therapies (ACT). We have also seen that the accumulation of MNPs in tumour cells induces oxidative stress as a consequence of IONP degradation, which affects mitochondrial metabolism. We are therefore investigating whether this effect could be used therapeutically to fight tumours at different levels.

Our group has also found a link between alterations in the tolerogenic dendritic cell (tolDC) compartment and the absence of CD8+ Tregs in two lupus-prone mouse models (MRL/MPJ and MRL/lpr), as well as the differential expression of Helios in several T-cell populations associated with lupus pathology. Based on these findings, we have extended the study of IONPs applications to other pathologies, and have investigated whether the functionalisation of TolDCs with IONPs and their subsequent magnetic retention could be used as an autoimmune therapy in the aforementioned mouse models of lupus.

We have also tested the antiviral capacity of some types of IONPs against some respiratory viruses such as influenza and SARS-CoV-2.

The overall goal of our group for the coming years is to fully understand the molecular and cellular mechanisms induced by IONPs at their different levels of action. This knowledge will be used to improve the functional design of IONPs for specific biomedical applications in the treatment of cancer and autoimmune diseases.



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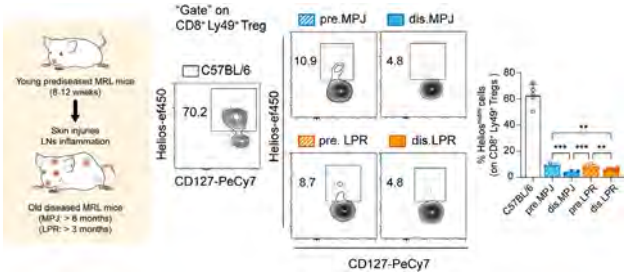

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1 *Iron oxide nanoparticles impair SARS-CoV-2 infection of cultured cells through two processes induced by the internalization of IONPs: the induction of oxidative stress and changes in the regulation of iron metabolism. (Yadileiny Portilla).*

2 *Helios expression on splenic CD8+ Ly49+ Tregs from C57BL/6, MRL/LPR and MRL/MPJ mice. (Andrés París).*

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Cardiac stem cells

Maintenance of organ functionality in mammals is determined by an efficient cell turnover. There are, however, large differences among adult organs both in steady-state, as well as in response to damages. It has been estimated that in adult mammalian heart only a 40% of total cardiomyocytes are replaced along lifespan but the mechanisms involved remains controversial.

We have identified and characterised a cardiac progenitor population, determined by the high levels of expression of the Bmi1 transcription factor (Bmi1+ Cardiac Progenitor Cells; B-CPC) which contributes to the turnover of the three main cardiac lineages. In response to a variety of cardiac damages B-CPC get proliferatively activated and their contribution to the mature lineages is enhanced. Thus, the B-CPC population contains cardiac progenitors contributing both to heart homeostasis and repair response to several modes of damage. In addition, *in vivo* genetic depletion of the B-CPC population provokes a pathological condition, when coupled to acute infarct, similar to human dilated cardiomyopathy (Figure 1). This is the first time that an association between a putative cardiac progenitor population and a human pathology (the most common cause of heart failure) has been established.

We have demonstrated self-renewal capacity in B-CPC that seems to be linked to their loading in specialised structures, known as niches, in a perivascular 3D distribution (vascular niches). This 3D distribution seems to be functionally relevant because it is distorted by conditions that increase ROS. Both cell-cell interactions and locally secreted factors, within the niches, restrain proliferation, promoting concomitantly self-renewal programs (Figure 2). Altogether, results strongly suggest a plausible crosstalk between vessel structures and B-CPC implying bidirectional mechanisms to be studied. Furthermore, to gain a better knowledge of the B-CPC intrinsic activity, we are going deeper in the study of some identified putative players (Tbx3 and Mbd3) which seem involved in the regulation of the critical quiescence/differentiation equilibrium.

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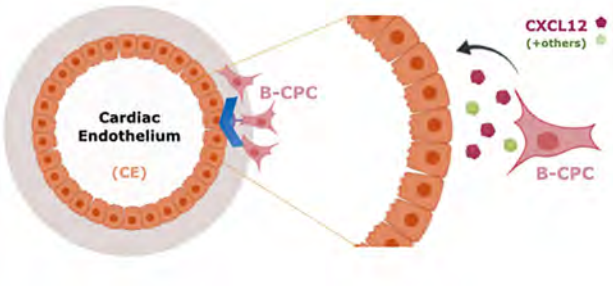

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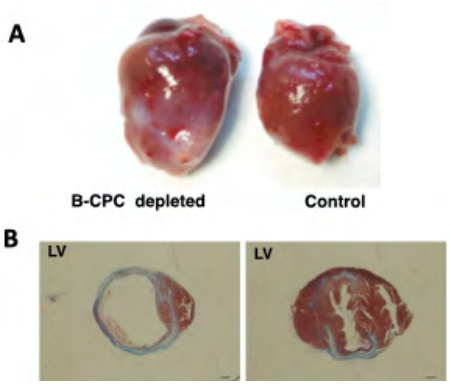

1 ***The vascular niche.*** *B-CPC secrete high levels of CXCL12 that promotes migration of cardiac endothelium to the close vicinity of B-CPC.*

2 ***Figure 1. B-CPC are essential for heart post-infarct repair.*** *Animals depleted of B-CPC (>95%), when a simultaneous infarct (AMI) was provoked, developed a lethal condition (2-3 months post-AMI), accompanied by heart hypertrophy (A) that derives from a large dilatation (B) of the left ventricle (LV).*

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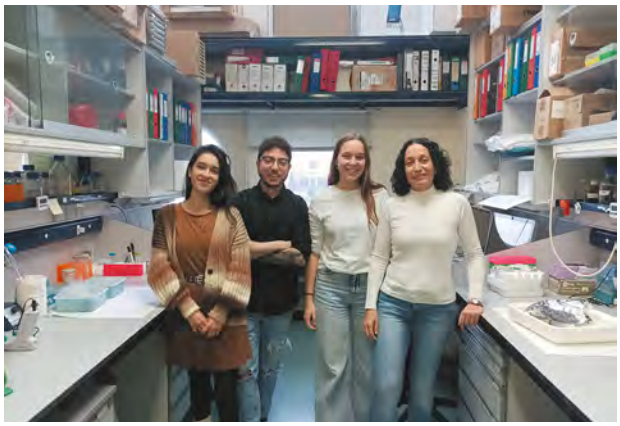

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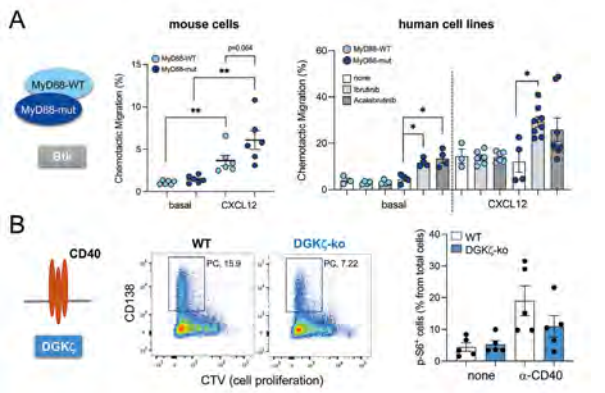
B lymphocyte dynamics

We investigate the molecular mechanisms that govern B lymphocyte dynamics and response, and how their dysfunction leads to or associates with B lymphocyte pathologies, in particular with B cell lymphomas. Our previous studies (Frontiers Immunol, 2018; Science Signaling, 2020) showed essential new functions for two proteins of clinical interest as therapeutic targets, the Bruton's tyrosine kinase (Btk) and the ζ isoform of Diacylglycerol-kinases (DGKζ). In the 2021-2022 period, we have studied the functional relation between Btk and the mutant active-form of MyD88 (MyD88-L265P), that features certain lymphomas, and the relevance of DGKζ for Plasma Cell differentiation downstream CD40 signalling, a main-actor at the germinal centre response.

While Btk mutations have not been linked to lymphoma development, increased protein levels and activation mediated by MyD88-L265P associates with Waldenström Macroglobulinemia and Activated B cell-Diffuse Large B cell Lymphoma (ABC-DLBCL). Using human ABC-DLBCL cell lines derived from patients and preclinical mouse models generated by our collaborator JA Martinez-Climent, CIMA-Navarra, we observed that the presence of mutant-MyD88 enhances cell invasion ability in mouse B cells. Its combination with the use of Btk pharmacological inhibitors (ibrutinib, acalabrutinib) augmented cell migration in human ABC-DLBCL cell lines (Figure 1A). Somehow MyD88 and Btk cooperate to regulate B lymphocyte motility/tissue residence, and this might be of clinical relevance. We are now investigating the molecular mechanisms behind. Regarding the CD40-DGKζ axis, we found that DGKζ-deficient B lymphocytes generate less Plasma Cells compared to wild type when stimulated *in vitro* via CD40. No differences in proliferation rate or CD98/CD71 markers upregulation were detected. Nonetheless, mTORC1 complex activity was reduced in deficient cells, estimated by measuring the levels of phosphorylated S6 ribosomal protein (Figure 1B). The data suggest that DGKζ has an important role downstream CD40 in regulating Plasma Cell fate via the mTORC1 pathway.



A, Migration frequency in basal and stimulated (CXCL12 chemokine) conditions of B lymphocytes carrying or not the mutant form of MyD88, in mouse models and human ABC-DLBCL cell lines. In the latter, they were also pre-treated with the specified Btk inhibitors. B, Representative dot-plots of Plasma Cell (PC) generation and cell proliferation for wild type and DGKζ-knockout B lymphocytes after 96 h stimulation with anti-CD40 antibodies plus IL-4/IL-5. Right, Quantification of S235/236-phosphorylated S6 protein levels by intracellular flow cytometry at 96h in the indicated culture conditions.

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Molecular targets in health and disease: focus on PI3-kinase

1) Boosting PTEN phosphatase as an anti-tumoral strategy

PIP3 (3-poly-phosphoinositides) control cell survival, division, and migration. Whereas PI-3-kinase (PI3K) upregulates PIP3, PTEN phosphatase reduces PIP3 levels. The mechanisms connecting PI3K and PTEN activities were unknown.

We have examined the activation kinetics of PTEN and those of the PIP3-effector AKT after growth factor addition for different times. AKT and PTEN activities were complementary (Figure 1). Maximal pAKT levels coincided with low PTEN activity and vice versa. The inactivation of PTEN was induced by UBIquitination and required cCBL and CBLb expression. At later time points, when pAKT levels decreased and PTEN phosphatase activity is recovered, PTEN exhibits high SUMOylation and translocates to the plasma membrane. These results reveal a mechanism for PI3K/PTEN crosstalk, and suggest that cCBL and SUMO machinery could be actionable strategies to rescue PTEN activity in PTEN +/-tumours.

2) The action of PI3-kinase beta on hESC stemness/ differentiation decision

The mechanism governing the transition of human embryonic stem cells (hESCs) toward differentiated cells is only partially understood. To gain insight of the role of PI3K pathway in this process, the activity and expression of the ubiquitous PI3Kα and PI3Kβ have been modulated in primed hESCs using different approaches.

The study reveals a pathway that dismantles the restraint imposed by the EZH2 (Enhancer Of Zeste 2 Polycomb Repressive Complex 2) repressor on an essential stemness gene, NODAL. PI3Kβ requirement for stemness is illustrated by OCT4 faded signal in cells with low PI3Kβ content (Figure 2). Control cells were greener (high OCT4 content), similar to the single cell in the image centre (not PI3Kβ depleted). At later time points, when ESC are prone to differentiate through the formation of the primitive streak (the site where gastrulation begins), PI3Kβ will contribute to tissue differentiation by modulating EZH2 release from promoters of transcription factors essential for primitive streak formation.

The pathway involves a noncatalytic action of PI3Kβ that controls nuclear- RAC1 levels, activation of Jun N-terminal kinase and nuclear β -catenin accumulation. These findings highlight how EZH2 is erased from promoters and points at new targets for directing tissue regeneration.

Additionally, our results support that PI3Kβ expression is also required for cancer stem cells phenotype maintenance in Lung Squamous Cell Cancer.

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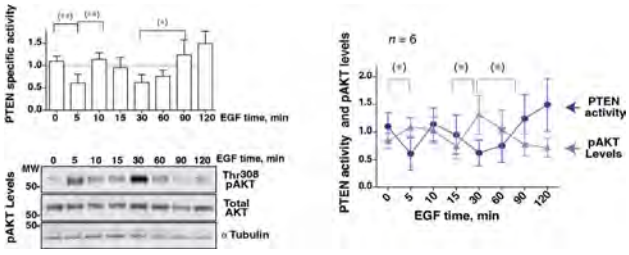

1 *Kinetics of PTEN activity and pAKT levels at different time points after EGF addition suggest PTEN inactivation contribution for maximal AKT activation.*

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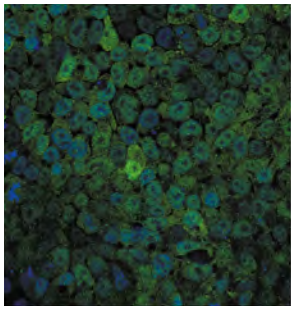

2 *Human stem cells with low PI3Kbeta levels (most of them) show low levels of the stemness marker OCT4 (green).*

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Stress-activated protein kinases in inflammation and cancer

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 colorectal cancer (CRC) associated to colitis. CRC is the second leading cause of cancer death. Patients with inflammatory bowel disease (IBD), ulcerative colitis or Crohn's disease are at increased risk of developing CAC; however, our understanding of the interplay inflammation-cancer at the molecular level is limited. We have reported that p38γ/p38δ have a pro-oncogenic function in CRC by regulating the production of inflammatory molecules and miRNAs, in humans. We found that p38γ/p38δ protein can be detected in human plasma, and that p38γ is significantly upregulated in IBD and CRC patients indicating that can mediate inflammation signaling to promote tumorigenesis. Our work suggests that p38γ/p38δ can be useful biomarkers for CRC/IBD diagnostic, as well as potential treatment targets, for colitis and early-stage CRC. Additionally, our data support the role of p38γ/p38δ in promoting tumour growth and give further evidence that one of the mechanisms by which p38γ promotes tumorigenesis is linked to its elevated protein expression and activation, thus increasing inflammation and providing an inflamed tumour environment that would promote tumour growth.

We have further investigated the molecular mechanism by which p38γ/p38δ regulate cytokine production and found that these kinases regulate inflammation, in part by controlling the expression of the ERK1/2 upstream kinase, tumour progression locus 2 (TPL2), in myeloid cells. We have demonstrated that TPL2 protein level is regulated by p38γ/p38δ at two different levels: 1) interacting with the TPL2/A20 Binding Inhibitor of NF-κB2 (ABIN2)/Nuclear Factor κB1p105 (NF-κB1p105) complex, increasing TPL2 protein stability; and 2) controlling TPL2 mRNA translation by modulating the repressor function of TPL2 3' Untranslated region (UTR) mediated by its association with aconitase-1 (ACO1).

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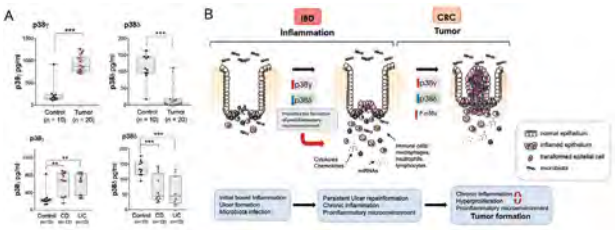

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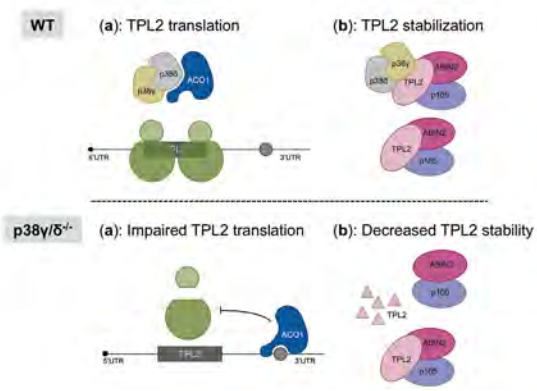

1 ***A***. *p38γ and p38δ expression in the plasma of healthy donors (control), colon cancer patients (tumor) or of IBD (CD and UC) patients. Each dot is a single patient or donor. (Upper panel) p38γ expression in CRC patients with (grey square) or without (red square) IBD; p38δ expression in CRC patients with (grey triangle) or without (blue triangle) IBD. Student's t-test. **P ≤ 0.01; ***P ≤ 0.001.* ***B.*** *Schematic illustration of the function of alternative p38MAPKs, p38γ and p38δ, in IBD and CRC formation.*

2 *Model for the regulation of TPL2 protein levels by p38γ and p38δ. In WT cells, p38γ and p38δ associate with ACO1 and also with the TPL2/ABIN2/p105 complex. (a) In WT cells, the p38γ/p38δ/ACO1 complex prevents ACO1 from binding to TPL2 3´UTR, and TPL2 mRNA is translated. In p38γ/δ-/- cells, free ACO1 binds to TPL2 3´UTR, and impairs TPL2 mRNA translation. (b) In WT cells, the p38γ/p38δ/TPL2/ABIN2/p105 complex stabilises TPL2 protein, whereas p38γ/p38δ absence decreases TPL2 stability and increases its degradation.*

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Physiopathology of chemokine receptor interactions

Chemokines and their receptors play an important role in homeostasis and inflammation, but they also regulate the tumour microenvironment, the generation of metastases and the anti-tumour immune response. Our group has generated monoclonal antibodies (mAbs) against human CCR9, which is overexpressed in different types of haematological malignancies. Two of these mAbs strongly inhibit the growth of human CCR9+ tumours in immunodeficient mouse models. These mAbs have been protected by an international CSIC patent, licensed to SunRock Biopharma. We have also generated human leukaemia cell lines expressing a modified CCR9 epitope which is not bound by these antibodies. Using these gene-edited cells in animal models of tumour progression, we have confirmed that the presence of the native epitope is a necessary and sufficient condition for the antitumor activity of our anti-CCR9 mAbs.

With the aim of developing naked antibody and antibody-drug conjugate (ADC) cocktails that allow simultaneous targeting of multiple tumour antigens, we generated a wide panel of new hybridomas producing antibodies from immunizations with whole human leukemic cells, from both primary cultures and stable cell lines. Twelve hybridomas were selected and cloned, and the corresponding mAbs were purified for their characterisation. Using flow cytometry, immunoprecipitation, protein arrays, and proteomics analyses, we have identified three different proteins, highly expressed on the surface of malignant cells, which are recognised with high affinity and specificity by their respective antibodies. These novel mAbs strongly reduce leukaemia tumour growth in xenograft models.

In collaboration with other research groups, we have generated and characterised mAbs against the macrophage protein CD5L, which modulate the immune response and are being evaluated in mouse models of liver fibrosis and liver cancer. We have also generated a broad panel of mAbs against the spike protein of the SARS-CoV-2 virus. Four of these mAbs protect mice from a lethal SARS-CoV-2 infection.

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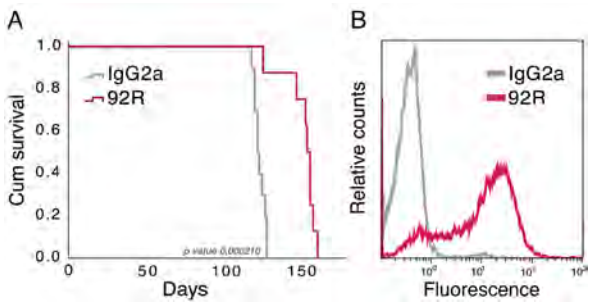

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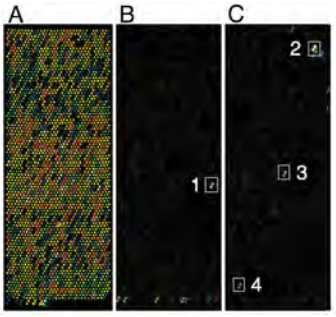

1 *92R increases survival of mice inoculated with CCR9+ early T-cell precursor lymphoblastic leukaemia cells. (A) Kaplan-Meier cumulative survival curves of mice carrying human xenotransplants injected in the tail vein on day 1. Mice were treated on days 2, 9 and 70 with either an anti-CCR9 mAb (92R) or an isotype control mAb (IgG2a). (B) Flow cytometry analysis of the leukaemia cells labelled with 92R or IgG2a.*

2 *Discovery of antibody specificity using HuProtTM v4.0 array analysis, representing more than 16,000 different human genes. Control subarray block with glutathione S-transferase staining (A). Detection of individual hits for mAbs 21 (B) and 35 (C). Staining intensity is shown in a false colour scale: blue-green-yellow-orange-red, increasing.*

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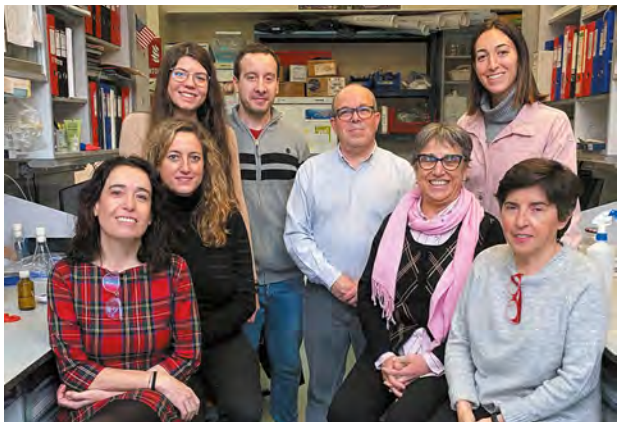

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Signaling networks in inflammation and cancer

Immune evasion is a fundamental hallmark of cancer. The immune system recognises tumour cell-derived neoantigens and generates tumour-specific T cell responses, distinguishing neoplastic from healthy cells. But cancers escape this control. We aim to identify, understand and manipulate tumour-induced resistance mechanisms and to develop new immunotherapies against cancer.

Our main research topics are:

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 are working on basic aspects of a new normaliser of the vasculature (SOD3), and collaborate in clinical trials to demonstrate the synergistic activity of anti-angiogenic and immunotherapy in breast cancer treatment.

2. Signals involved in T cell dysfunction

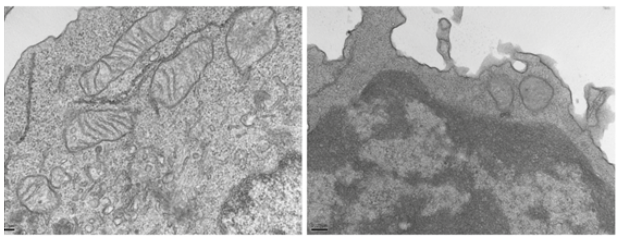
Tumour-specific T cells are usually dysfunctional in the tumour microenvironment (TME). Using RNA-seq and bioinformatics, we identified a genetic program associated to T cell exhaustion. In collaboration with Industry, we are deciphering the mechanisms of new intermediates causing T cell dysfunction and their potential clinical application.

3. Rewiring tumour cell metabolism

Metabolic reprogramming is another new hallmark of cancer. Neoplastic cells develop a number of metabolic adaptations to survive and proliferate without restrictions, but also induce metabolic stress affecting the activity of endothelial and T cells in the TME. We aim to manipulate the metabolism of tumour, cells to turn the immunosuppressive TME into an immunodominant one.

4. CD4 T cell differentiation in the TME

The number and nature of the immune populations that infiltrate tumours determine the immune-mediated control of cancer and the response to immunotherapy. The cells may come from the bloodstream or lymph, or can be differentiated in the TME. We are investigating the function of the extracellular matrix in the generation of immunosuppressive immune cell phenotypes and its relevance in tumour progression.



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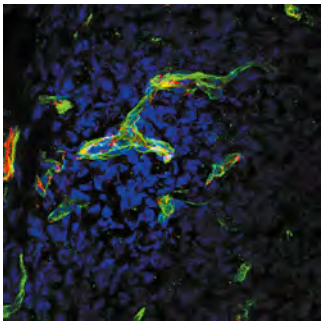

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1 *PD-1 signalling reduces mitochondrial cristae length. Note the different structure of mitochondria in activated (left) and PD-1-exhausted CD8+ T cells (right).*

2 *Restoration of SOD3 expression in the tumour microenvironment normalises tumour vasculature. Tumour section stained with SOD3 (red), CD31 (green) and DAPI (blue).*

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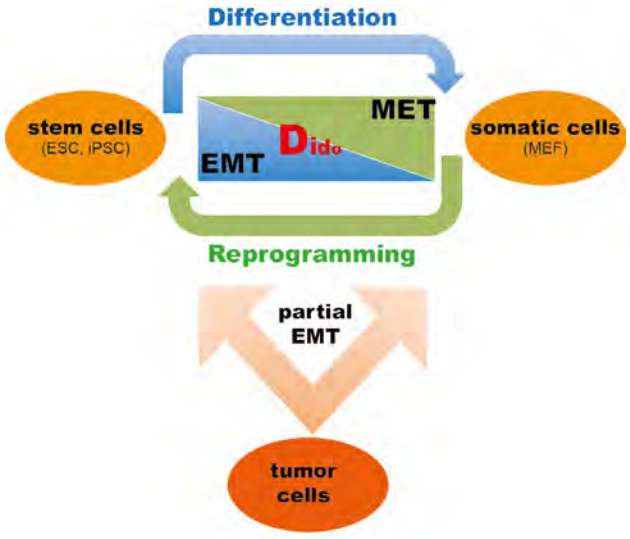
Stem cells and immunity

We previously identified the *DIDO* locus, which regulates chromatin remodeling and RNA metabolism such as RNA splicing and alternative termination. We now used mouse embryo fibroblast (MEF) mutants in the Death Inducer Obliterator (*DIDO*) gene to study the effect on 3′ end processing and on regulation of alternative polyadenylation. In *in vivo* experiments in mutant mice lacking *Dido* exon 16, we also observed the development of mild hepatitis, testicular degeneration, and progressive ataxia, in association with systemic alterations in mRNA splicing and transcriptional readthrough.

As we determined previously, the *DIDO* locus also participates in early embryonic development and ESC differentiation through processes involving chromatin remodeling and RNA metabolism; these include RNA splicing and alternative termination. Moreover, as embryonic stem cell (ESC) differentiation and somatic cell reprogramming are highly regulated biological processes that display a largely common set of genes, we characterised the role of *Dido* in somatic cell reprogramming.

Using a conditional deletion mutant for *Dido* in combination with transcriptomic, protein interaction, and cellular studies, we have identified the underlying molecular mechanism through which *Dido* orchestrates the effects on both differentiation and reprogramming. We show that truncation of the exons 16 of the *DIDO* gene alters RNA splicing and transcription termination in ESC and MEF. In addition, we found that DIDO3, the largest *Dido* isoform, interacts with the helicase DHX9, which is involved in R-loop processing and transcription termination, and that DIDO3-exon16 deletion increases nuclear R-loop content and causes DNA replication stress. These effects result in failure of ESC to differentiate and of MEF to be reprogrammed. MEF immortalization restored impaired reprogramming capacity. DIDO3 therefore has essential functions in ESC differentiation and somatic cell reprogramming by supporting accurate RNA metabolism, with its exon16-encoded domain playing the main role. These data allow us to postulate an underlying molecular mechanism of an additional *DIDO* role in somatic cell reprogramming.

In an exhaustive computational study that integrated multi-omics data and *Dido* domain composition, we analyzed the significance of the *Dido* gene in EMT and MET processes, in differentiation, cell reprogramming, and tumour formation (see figure). Experiments in progress will allow us to understand Dido gene function in tumour formation and metastasis, and will permit exploration and clarification of its role in oncogenic transformation and cancer progression, in which EMT and MET are fundamental.



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Chemokine receptors: biology and clinical relevance in inflammation, cancer and AIDS

Cell migration involves myriad signaling proteins and receptors that act coordinately to activate intracellular pathways and promote polarised cell states and directional migration. To migrate directionally in response to external stimuli, the internal machinery of cells needs to be spatially organised, which involves the integration of biochemical and mechanical factors to generate force in a specific direction to move the cell forward.

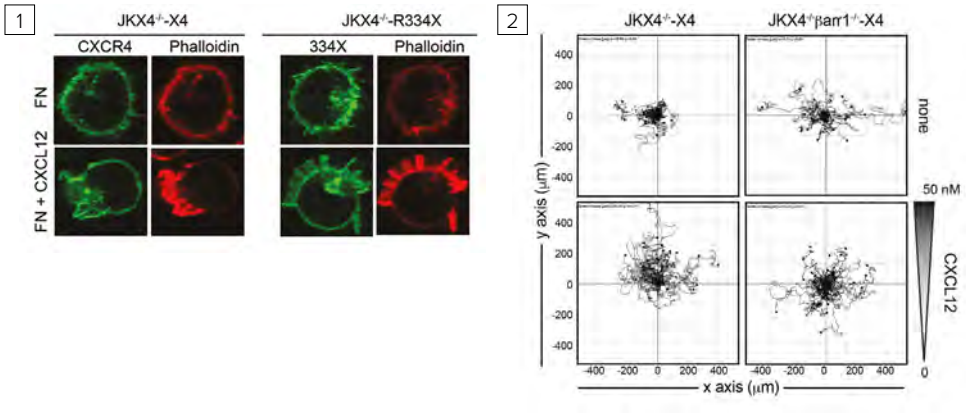
Chemokine receptors are membrane-expressed seven-transmembrane receptors linked to G proteins. Through interaction with the corresponding ligands, they induce a wide variety of cellular responses including cell polarisation, movement, immune and inflammatory responses, as well as prevention of HIV-1 infection. For achieving their function, chemokine receptors undergo ligand-dependent conformational changes that are influenced by the local cellular microenvironment.

Using quantitative single-molecule spatio-dynamic imaging, our group studies how the formation of large receptor nano-clusters in response to the ligand is critical for the maintenance of correct actin dynamics in migrating cells that allows directed cell migration. We have determined that altered cluster formation, using natural mutant chemokine receptors or altering the lipid composition of the cell membrane, results in the inability of these cells to correctly sense chemokine gradients. This inability to form clusters is responsible of the complex phenotype manifested by individuals carrying this mutated receptor and suggest that receptor clustering can be exploited as therapeutic target in inflammatory diseases and cancer.

In addition, our group is also involved in studying the effect of Growth hormone (GH) pretreatment of immune cells and its functional relevance. For instance, we observe that GH treatment prevents inflammatory joint destruction in a CIA model or that GH improved remission of inflammation and mucosal repair during recovery in the acute dextran sodium sulfate-induced colitis. The effects occur in several immune cells, i.e. GH reprograms inflammatory macrophages to an anti-inflammatory phenotype and improves resolution during pathologic inflammatory responses.

1 ***CXCL12 induces multiple lamellipodia through interaction with mutant CXCR4R334X.*** *F-actin (phalloidin-TRITC, red) and CXCR4 (AcGFP, green) visualised by confocal microscopy in JKX4-/--X4 and JKX4-/--R334X cells adehered to fibronectin and treated or not with CXCL12.*

2 ***β-arrestin regulates directional cell migration in response to CXCL12.*** *Spider plots showing the trajectories of tracked JKX4-/--X4 and JKX4-/-βarr-/--X4 cells migrating on fibronectin-coated μ-slide chemotaxis chambers along a CXCL12 gradient.*



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Diacylglycerol kinases in the control of immune response and cancer progression

Over the past years the emergence of immunotherapies, namely adoptive T cell transfer (ATC) and immune checkpoint blockade (ICB) has shifted the paradigm of cancer therapies, directing them to rescue the ability of T lymphocytes to eliminate tumours. The efficacy of these treatments is still quite limited due to the development of either innate or acquired resistance. Diacylglycerol Kinase (DGK) alpha and zeta are two cytosolic checkpoint inhibitors that limit T cell function by transforming diacylglycerol (DAG) into phosphatidic acid. The abnormal elevation of these two DGK isoforms in tumour-infiltrating natural and engineered T lymphocytes contributes to driving these cells into non-functional states.

Our group works to better understand the consequences of limiting the activity and/or expression of these two DGK isoforms in the regulation of antitumor T cell responses. To this we use cell-based and mouse preclinical models to investigate the consequences of 1) genetic deletion 2) pharmacological inhibition and 3) altered subcellular distribution of DGK alpha and zeta in the immune response against tumours. Some of our recent results have demonstrated that DGK alpha targeting potentiates the effect of targeting PD-1.

Unleashing the potential of targeting these kinases offers additional possibilities to enhance the effectiveness of actual therapies and improve its success rates in the treatment of advanced tumours. 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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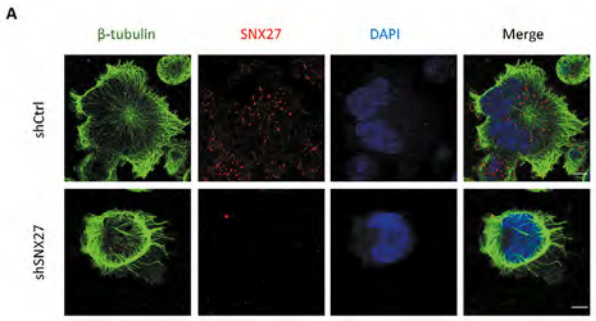

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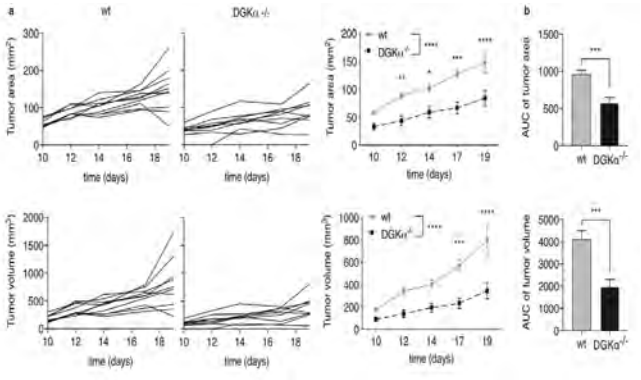

1 *SNX27 silenced Jurkat T cells display abnormal microtubule reorganization and cell shape*

2 *DGKa deficiency limits subcutaneous implanted MC38 tumor growth*

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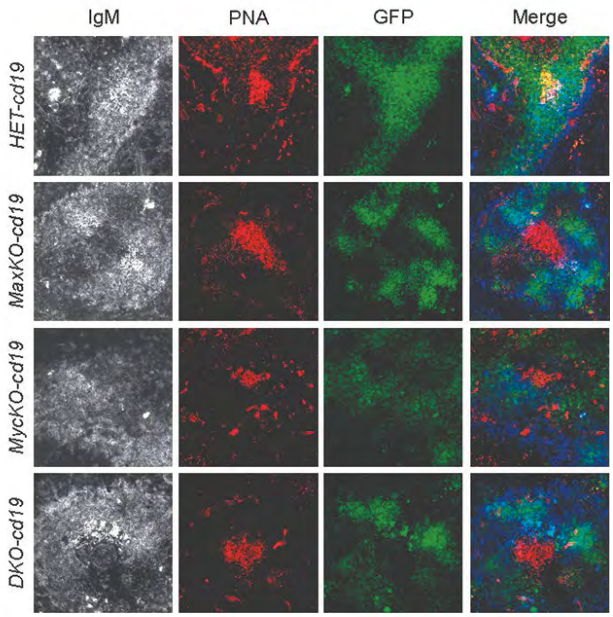
Transcriptional regulation of B lymphocyte differentiation

With more than 3 million new cases and 1.7 million deaths each year, cancer is currently the second most important cause of death in Europe. In up to 50% of all human cancers, constitutively enhanced expression of MYC family proto-oncogenes is one of the hallmarks (Burkitt lymphoma, breast and lung cancers). Myc proto-oncogenes are deregulated by various mechanisms such as rearrangements or other mutations in one of the three myc genes or by alteration of the signaling pathways that control their expression. The Myc proteins are members of a basic region/helix-loop-helix/leucine zipper (bHLHZip) transcription factor family (N-, L- and c-Myc) and are implicated in many biological functions such as regulation of cell cycling, differentiation, and apoptosis. Experiments in mouse tumour models strongly suggest that interference with the function of deregulated myc will have clear therapeutic impact on a wide range of aggressive and hitherto incurable tumors.

To activate or repress target genes, Myc proteins bind to conserved DNA sequences (E-boxes) on gene regulatory regions, for which Myc must form heterodimers with its partner, Max. The vast majority of related scientific reports to date assume that Myc/Max partnership is needed for all Myc functions. Max is highly conserved in evolution and is constitutively expressed in many cell types. Max can also heterodimerize with other proteins and antagonise Myc functions. Max thus has a central role in modulating the complex Myc protein network.

Our group is interested in characterising whether Myc action on developing and mature B cells relies exclusively on its association with Max. Thus, we will challenge the current idea and explore the possibility of Myc function without Max *in vivo* using unique genetically modified mouse models.

The prevailing strategies for combating cancer in patients rely mainly on the use of non-specific cytotoxic drugs, whose severe side effects are a major drawback. New therapies that target specific key molecules involved in cell transformation are therefore pursued. c-Myc is a therapeutic target for human cancer, due to the broad range of malignancies in which this gene is activated. As the contribution of Max to Myc-induced B lymphoma *in vivo* is also unknown, we will also study whether Max is needed for generation and/or maintenance of these lymphomas. Hopefully, our work will open new strategies to tackle Myc-induced tumorigenesis and thus, improve human health.



*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 immunized 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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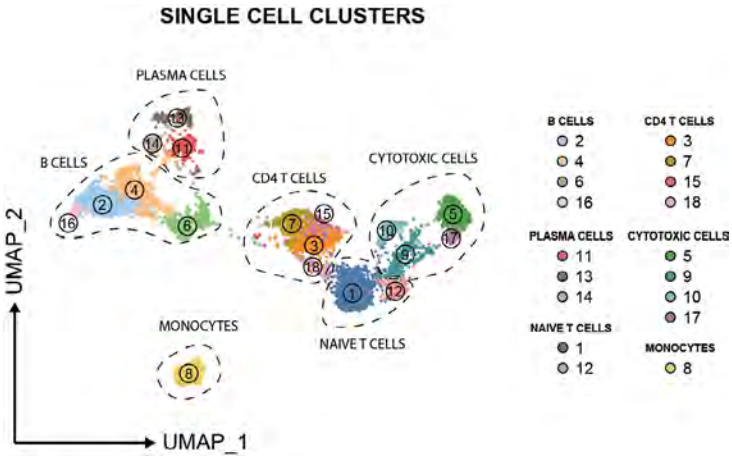
Receptor ligand interactions in immune responses to cancer and viruses

Immunity to virus infection, particularly the innate immune response to viruses, has been the central focus of my research since my PhD working on immune responses occurring in lymph nodes. Current research in the lab is still focussed on innate immune recognition of cells infected by viruses. This research is driven by the study of patients with primary immunodeficiencies as a source of *in vivo* insights into the effects of defective innate immunity on disease susceptibility in these individuals. Once the genetic defect has been identified, we aim to characterise the phenotypic and functional defects in detail with the goal of achieving a mechanistic explanation for the genotype-phenotype observations. These studies typically involve the use of genome-editing technologies to establish relevant in vitro models to study in detail the molecular bases of the changes observed in vivo. For example, we have studied a number of individuals with novel genetic deficiencies including patients unable to make an interferon αβ response after virus infection and patients that lack expression of the cellular Fcγ receptor CD16.

Since infection with Epstein-Barr virus is a major cause of mortality for these patients we have also embarked on a systematic characterisation of the cellular immune response to this virus in healthy, yet seropositive individuals where the host manages to achieve sufficient control of viral replication that an equilibrium consistent with health is established. These experiments are enhanced by an extensive network of collaborations, both national and international, that we have established with basic scientists and clinical colleagues.

Single cell RNASeq analysis of peripheral blood mononuclear cells responding to Epstein-Barr Virus identifies clusters of B and plasma cells, CD4 T cells, cytotoxic lymphocytes and monocytes.



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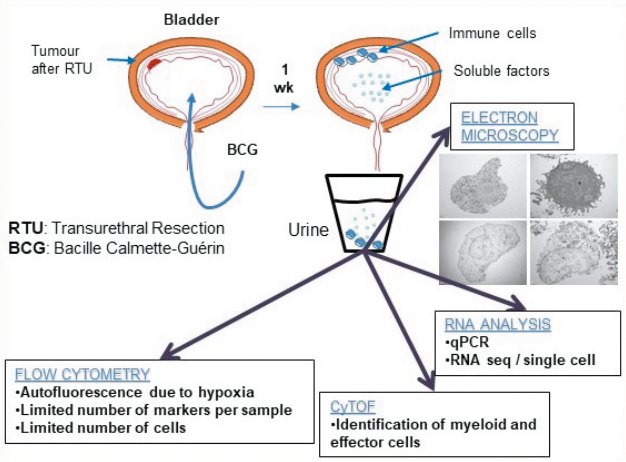
Tumour immune activation and evasion

To understand the immune response against cancer, we study a successful therapy for bladder cancer that involves activation of the immune system. Intra-vesical instillations of BCG (Bacille Calmette-Guérin) have been used for decades achieving great response rates, with 70% of patients free of tumour five years after therapy. In the last years, we have developed *in vitro* models that allow us to explore in detail the mechanisms underlying these therapeutic effects, and have described the activation phenotype of anti-tumour Natural Killer (NK) cells, both in the presence of live and dead mycobacteria. In parallel, analysis of ex vivo urine samples from patients treated with BCG, collected one week after instillations, has provided information on long lasting immune responses, such as the presence of cells with a phenotype consistent with tumour associated neutrophils (TAN).

Moreover, we study the biology of NK receptors, that can also be expressed by other effector lymphocytes, including αβ and γδ T cells. One key activating receptor, NKG2D, recognises tumour-associated ligands that mediate immune evasion when released as soluble molecules, either by metalloprotease cleavage or in extracellular vesicles (EVs). We have identified an unexpected role of this system in targeted therapies, using BRAFV600E and other MAPK inhibitors, for melanoma. After studying the physico-chemical properties of EVs, we optimised immunocapture assays using flocculation methods, that allow us to examine NKG2D-ligands, and other EV-associated tumour markers directly in biological fluids.

Finally, as part of the CNB response to the COVID-19 pandemic, we developed a multi-antigenic serology test (4 viral proteins + 3 antibody isotypes in one test tube) using flow cytometry, that has been commercialised by Immunostep, S.L., as well as conventional ELISAs that have since been transferred to the WHO.



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. We use several approaches to analyse cells one week after instillations. Data in Castellano et al. Front Immunol, 2022.*

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