

The Department of Immunology and Oncology (DIO) is mainly devoted to investigating the molecular and cellular basis of the immune response in health and disease from different but complementary perspectives. We are particularly interested in the study of inflammation-based diseases, infection and cancer with the aim of identifying new biomarkers for diagnosis and targets for the treatment of these pathologies. At the heart of our research is the investigation of inter- and intracellular signalling pathways in innate and adaptive immune cells, and in transformed cells. To unravel these molecular mechanisms, we use the most advanced methods of molecular biology, cell biology and immunology in cellular systems. For the study of cellular mechanisms and illness progression in vivo, we have generated numerous genetically modified mouse models.

Our common research objective provides an excellent environment for collaboration within the department as well as with other groups within and outside the CNB. Since its origins, the DIO has maintained productive collaborations with public and private partners that include prominent national and international research institutes, hospitals and pharmaceutical companies.

Immunology and Oncology

HEAD OF DEPARTMENT

Ana Cuenda

RESEARCH GROUPS

- 1. Dendritic cell and macrophage immunobiology**
Carlos Ardavín
- 2. Immune hyperactivity in autoimmunity and hyporesponsiveness in cancer depend on the level of mitochondrial activity**
Dimitrios Balomenos
- 3. Nanomedicine, cancer immunotherapy and autoimmune diseases**
Domingo F. Barber
- 4. Adult heart turnover**
Antonio Bernad
- 5. B cell dynamics**
Yolanda R. Carrasco
- 6. Molecular targets in health and disease: focus on PI3-kinase**
Ana Clara Carrera
- 7. Stress-activated protein kinase p38MAPK in inflammation and cancer**
Ana Cuenda
- 8. Physiopathology of chemokine receptor interactions**
Leonor Kremer
- 9. Signalling networks in inflammation and cancer**
Santos Mañes
- 10. Stem cells and immunity**
Carlos Martínez-A
- 11. Chemokine receptors: new targets for therapeutic intervention**
Mario Mellado
- 12. Diacylglycerol kinases in the control of immune response and cancer progression**
Isabel Mérida
- 13. Transcriptional control of B lymphocyte differentiation**
Ignacio Moreno de Alborán
- 14. Receptor-ligand interactions in immune responses to cancer and viruses**
Hugh T. Reyburn
- 15. T cell signalling in autoimmune diseases and cancer**
Jesús M. Salvador
- 16. Tumour immune activation and evasion**
Mar Valés-Gómez



Dendritic cell and macrophage immunobiology

66 IMMUNOLOGY & ONCOLOGY

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SELECTED PUBLICATIONS

Domínguez-Andrés J, Feo-Lucas L, Minguito de la Escalera M, González L, López-Bravo M, Ardavín C. Inflammatory Ly6C^{high} monocytes protect against candidiasis through IL-15-driven NK cell/neutrophil activation. *Immunity* 2017; 46: 1059-1072.

Domínguez-Andrés J, ..., Ardavín C, Netea MG. Rewiring monocyte glucose metabolism via C-type lectin signaling protects against disseminated candidiasis. *PLoS Pathog* 2017; 13: e1006632.

Nieto C, ..., Ardavín C, Castrillo A, Vega MA, Puig-Kröger A, Corbí AL. The Activin A-PPAR γ axis contributes to the transcriptome of GM-CSF-conditioned human macrophages. *Front Immunol* 2018; 9: 31.

Beceiro S, ..., Ardavín C, Partida-Sanchez S, Rodríguez-Fernández JL, Nagy L, Tontonoz P, Castrillo A. LXR nuclear receptors are transcriptional regulators of dendritic cell chemotaxis. *Mol Cell Biol* 2018; 38: 00534-17.

Alsina-Beauchamp D, ..., Ardavín C, Netea MG, Alemany S, JJ Sanz-Ezquerro, Cuenda A. Myeloid cell deficiency of p38 γ /p38 δ protects against candidiasis and regulates antifungal immunity. *EMBO Mol Med* 2018; 10: e8485.

Our research program aims at exploring the role of inflammatory monocytes and macrophages during infection, allergy and intraperitoneal tumour metastasis, and encompasses the following research lines:

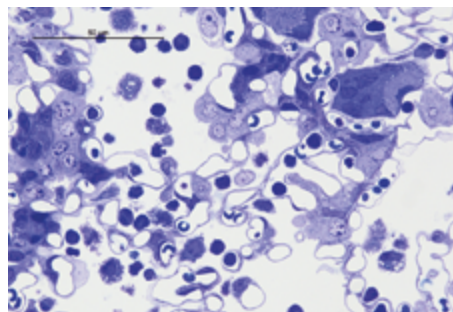
- Role of monocytes and type-I interferon in NK cell and neutrophil activation during the innate immune response against systemic *Candida albicans* infection.
- Alveolar macrophage dynamics and immunophysiology during airway allergic reactions caused by house dust mite-derived allergens.
- Role of the innate immune system of the peritoneal cavity in defence against intraperitoneal bacterial infections and colorectal tumour metastasis.

The experimental approach designed to address the role of monocytes during *Candida* infection involves the analysis of the early and cooperative spleen and kidney innate immune responses against intravenous infection with the fungus *Candida albicans* in a mouse model of systemic candidiasis, using wild type mice of the C57BL/6 strain as well as mice deficient in the type-I interferon receptor (IFNAR), the chemokine receptor CCR2 and the cytokine IL-15.

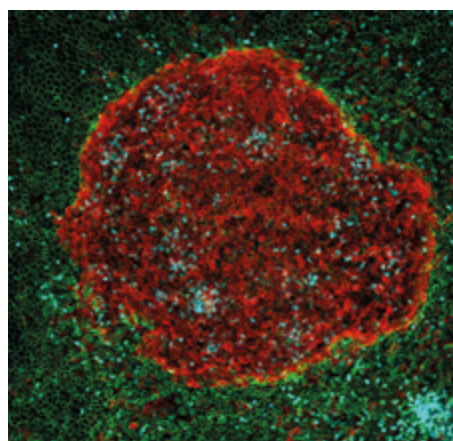
Our project on the dynamics of alveolar macrophages involves the study of the alveolar damage caused by strong airway allergic reactions against house dust mite-derived allergens, the process by which the alveolar macrophage subset is regenerated once the allergic process is resolved, and the mechanisms ensuring alveolar tissue repair and surfactant homeostasis. Wild type C57BL/6 and CCR2-deficient mice, parabiosis and progenitor transfer experiments, and immunofluorescent and electron microscopy are currently used in our laboratory to address these issues.

Experimental sepsis, peritoneal bacterial infection models using mouse intestinal strains of *E. coli*, and mouse models of intraperitoneal metastasis of colorectal tumours in wild type C57BL/6 and CCR2-deficient mice are employed to explore the role of the innate immune system of the peritoneal cavity in defence against intraperitoneal infection and tumour metastasis.

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1 Alveolar damage and pneumocyte hyperplasia caused by house-dust mite airway allergy. Light microscopy of a 1 μ m semithin section of the lung 4 days after intratracheal allergen challenge.

2 Macrophage cluster attached to the peritoneal wall mesothelium 4h after intraperitoneal bacterial infection. Confocal microscopy after whole mount immune-fluorescence using anti-F4/80 (macrophage marker; red), anti-Ly6G (neutrophil marker; blue) and anti-podoplanin (mesothelium marker; green) antibodies.



Immune hyperactivity in autoimmunity and hyporesponsiveness in cancer depend on the level of mitochondrial activity

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SELECTED PUBLICATIONS

Balomenos D, Shokri R, Daszkiewicz L, Vázquez-Mateo C, Martínez-A C. On how Fas apoptosis-independent pathways drive T cell hyperproliferation and lymphadenopathy in lpr mice. *Front Immunol* 2017; 8: 237.

Rodríguez-Frade JM, Guedán A, Lucas P, Martínez-Muñoz L, Villares R, Criado G, Balomenos D, Reyburn HT, Mellado M. Use of lentiviral particles as a cell membrane-based mFasL delivery system for *in vivo* treatment of inflammatory arthritis. *Front Immunol* 2017; 8: 460.

Rackov G, Shokri R, Álvarez De Mon M, Martínez-A. C, Balomenos D. The role of IFN-beta during the course of sepsis progression and its therapeutic potential. *Front Immunol* 2017; 8: 493.

In autoimmune diseases, hyperactive immunity provokes self-reactivity. In order to neutralise this damaging effect, the immune response needs to be deactivated. Alternatively, in cancer, immunosuppressed immunity requires reactivation.

Our studies suggest that p21 is a regulator of mitochondrial activity, controlling the balance between hyperactivation and immunosuppression. Therefore, high expression of p21 tempers T cell overactivity, while lack of p21 enhances T cell and macrophage responses (Figure 1).

Increased mitochondrial activity and lack of p21 increase memory T cell responses

Compared to normal memory T cells, autoreactive T cells become overactivated due to their repeated encounters with autoantigens. We have shown therapeutic potential for p21, as its overexpression deactivates hyperactivated autoreactive T cells (Daszkiewicz L *et al*, *Sci Rep* 2015; 5: 7691).

Our current work indicates that p21 does not act as a cell cycle inhibitor but modulates the activation of autoreactive T cells. Lack of p21 concurs with increased mitochondria activation, which drives T cell responses. We are currently examining a potential association between p21 and mitochondrial function.

The effect of p21 and mitochondrial activation in macrophage responses and its possible effects in cancer immunotherapy

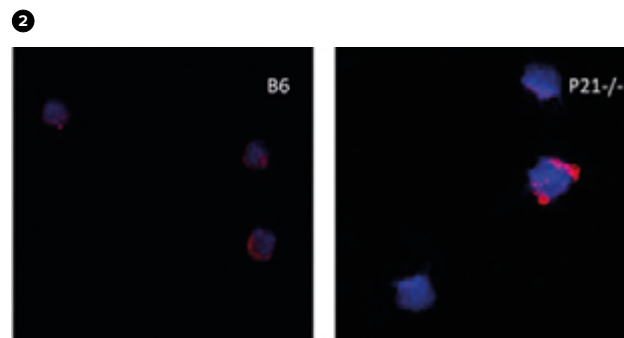
We have shown a dual regulatory role for p21; first, in macrophage activation to M1 state and, second, in macrophage reprogramming from M1 to the M2 unresponsive state. Lack of p21 prevents macrophage reprogramming to M2 status (Rackov G *et al*, *J Clin Invest* 2016; 126: 3089-3103).

Our data show that macrophage activation to M1 status is also associated to mitochondrial activation, which is linked to lack of p21 expression (Figure 2).

The role of p21 and mitochondria in macrophage activation may have an effect in immunotherapy of cancer, as tumour persistence neutralises M1 macrophages and attracts deactivated M2 cells.



1 Increased p21 expression is associated with controlled mitochondrial activity which contributes to T cell and macrophage hyporesponsiveness, while lack or decreased levels of p21 lead to increased mitochondrial activity that drive T cell and macrophage hyperactivation.



2 Lack of p21 increases mitochondrial activity in activated macrophages.



Nanomedicine, cancer immunotherapy and autoimmune diseases

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SELECTED PUBLICATIONS

Rojas JM, Gavilán H, Del Dedo V, Lorente-Sorolla E, Sanz-Ortega L, da Silva GB, Costo R, Pérez-Yagüe S, Talelli M, Marciello M, Morales MP, Barber DF, Gutiérrez L. Time-course assessment of the aggregation and metabolism of magnetic nanoparticles. *Acta Biomater* 2017; 58: 181-195.

Gonzalo-Gil E, García-Herrero C, Toldos O, Usategui A, Criado G, Pérez-Yagüe S, Barber DF, Pablos JL, Galindo M. Microthrombotic renal vascular lesions are associated to increased renal inflammatory infiltration in murine lupus nephritis. *Front Immunol* 2018; 9: 1948.

Fortes Brollo ME, Hernández Flores P, Gutiérrez L, Johansson C, Barber DF, Morales MDP. Magnetic properties of nanoparticles as a function of their spatial distribution on liposomes and cells. *Phys Chem Chem Phys* 2018; 20: 17829-17838.

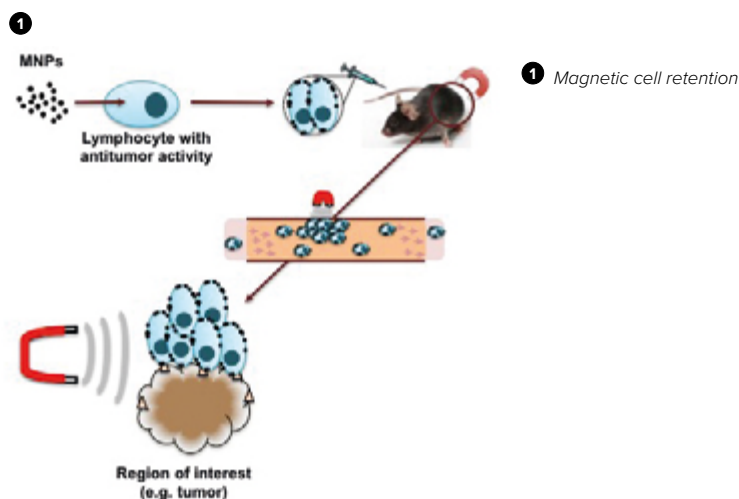
Risco A, Martin-Serrano MA, Barber DF, Cuenda A. p38γ and p38δ are involved in T lymphocyte development. *Front Immunol* 2018; 9: 65.

Mejías R, Hernández Flores P, Talelli M, Tajada-Herraiz JL, Brollo MEF, Portilla Y, Morales MP, Barber DF. Cell-promoted nanoparticle aggregation decreases nanoparticle-induced hyperthermia under an alternating magnetic field independently of nanoparticle coating, core size and subcellular localization. *ACS Appl Mater Interfaces* 2019; 11: 340-355.

Due to their small size and physicochemical properties, superparamagnetic iron oxide nanoparticles (SPION) have great potential as a nanomedicine in the fight against cancer, as they have proven effective for targeted drug release and in diagnosis by magnetic resonance imaging. SPIONs also show considerable promise for two additional cancer therapies, intracellular hyperthermia induction and targeting in cell transfer. Adoptive cell transfer is a type of immunotherapy that exploits the antitumor capacity of cytotoxic lymphocytes. The application of alternating magnetic fields (AMF) can magnetically induce intracellular hyperthermia in SPION-loaded cells, which can be used in cancer treatment. Results to date, using SPIONs *in vitro* studies and in animal models, suggest potential for rapid translation of these technologies to clinical practice. This development has nonetheless been delayed in part by a lack of basic knowledge of SPION-induced molecular and cellular mechanisms and the routes that regulate SPION degradation in the organism, both of which affect the therapeutic effectiveness of SPIONs and their accumulation and long-term toxicity.

The overall objective of our group is to understand SPION-mediated molecular and cellular mechanisms in distinct biomedical applications oriented to cancer and autoimmunity treatment, and to use this knowledge to improve SPION functional design for specific biomedical purposes in antitumour therapy. We pursue six specific objectives.

1. Investigate the ability of intracellularly loaded SPIONs to induce biological effects following the application of an AMF, to identify effects that depend on temperature increase or that are mediated by other mechanisms.
2. Comparative analysis of the effectiveness of various nanoparticle-targeting approaches in antitumour therapies.
3. Study SPION-induced immunogenic and epigenetic changes in cells and the possible contribution of AMF application in intracellular hyperthermia treatment on these changes.
4. Study of SPION degradation and transformation within lysosomes.
5. Potentiation of antitumour therapy by adoptive transfer of NK cells and CD8+ T cells using SPIONs.
6. Potentiation of autoimmunity treatment by adoptive transfer of immunosuppressive cells using SPIONs.





Adult heart turnover

IMMUNOLOGY & ONCOLOGY 69

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SELECTED PUBLICATIONS

Sanz-Ruiz R *et al.* Rationale and design of the first-in-man clinical trial to evaluate the safety and efficacy of intracoronary infusion of allogeneic human CARDiac stEm cells in patients with acute Myocardial Infarction and left ventricular dysfunction: the randomized multicenter double-blind controlled CAREMI trial. *Circulation Res* 2017; 121: 71-80.

Escudero B *et al.* Polu deficiency induces moderate shortening of P53^{-/-} mouse lifespan and modifies tumor spectrum. *DNA Repair (Amst)* 2017; 54: 40-45.

Herrero D *et al.* A. Redox-dependent BMI1 activity drives *in vivo* adult cardiac progenitor cell differentiation. *Cell Death Differ* 2018; 25: 807-820.

Herrero D *et al.* Bmi1-Progenitor Cell Ablation Impairs the Angiogenic Response to Myocardial Infarction. *Arterioscler Thromb Vasc Biol* 2018; 38: 2160-2173.

García-López S *et al.* Deregulation of the imprinted DLK1-DIO3 locus ncRNAs is associated with replicative senescence of human adipose-derived stem cells. *PLoS One* 2018; 13: e0206534.

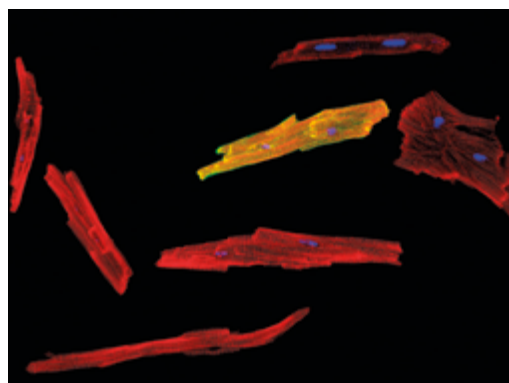
Adult mammalian heart can refresh damaged or aged cells during their lifetime but with low rate. However, mechanisms involved in the turnover remain highly controversial. We have defined a population of non-cardiomyocytic cells that expresses high levels of the polycomb Bmi1 transcription factor (Bmi1⁺), which contributes to the turnover of the three main cardiac lineages. In response to a variety of acute cardiac insults, the Bmi1⁺ cells progeny increases their contribution to the mature lineages.

In adult tissues, progenitors and stem cells are lodged in specialised structures (niches) that provide a protective microenvironment, essential for their correct regulation, and usually associated to low oxidative stress. In agreement with our working hypothesis, we found that Bmi1⁺ cells show low levels of reactive oxygen species (ROS). Interestingly, in homeostasis conditions, the analysis of cell distribution showed that Bmi1⁺ cardiac progenitor cells were located close to the vasculature, with an enrichment in quiescent Bmi1⁺ cells close to endothelial structures. These results strongly suggest the instructive role of cardiac vasculature that was confirmed by *in vitro* co-culture experiments with endothelial cells. In agreement with this role, *in vivo* genetic ROS amelioration altered the perivascular location of Bmi1⁺ cells, which resumed adolescent-like gene expression profiles. Altogether, we concluded that cardiac vasculature provides a protective and low-stress niche-like microenvironment that contributes to the maintenance of Bmi1⁺ cardiac progenitor cells in adult heart.

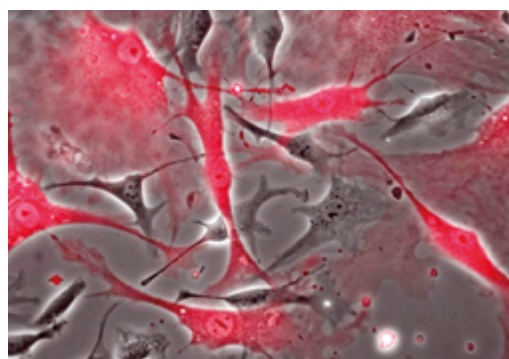
Finally, *in vivo* genetic depletion of the Bmi1⁺ population demonstrated that, although the population is not essential in homeostasis, its deletion in the context of acute

infarct recovery is highly deleterious; depleted animals demonstrated a significantly increased mortality, associated with major structural and functional heart alterations. Bmi1⁺-deficient infarcted hearts showed a severe decrease in neo-angiogenesis and ejection fraction function, that seemed to account for a severe ischemic-dilated cardiac phenotype.

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1 Plated murine cardiomyocytes isolated from adult heart. Cardiomyocytes are labelled with sarcomeric beta-actinin (red) and nuclei are labelled with DAPI (blue). *In vivo* lineage tracing study uncovers mature cardiomyocytes derived from Bmi1⁺ cardiac progenitor cells assessed by yellow fluorescent protein expression (yellow).

2 Cardiac progenitor cells (tomato+) in co-culture with 1g11 endothelial cells (not coloured).



B cell dynamics

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SELECTED PUBLICATIONS

Roman-García S, Merino-Cortés SV, Gardeta SR, de Bruijn MJW, Hendriks RW, Carrasco YR. Distinct roles for Bruton's tyrosine kinase in B cell immune synapse formation. *Front Immunol* 2018; 9: 2027.

Carrasco YR. *In vivo* tracking of particulate antigen localization and recognition by B lymphocytes at lymph nodes. *Methods Mol Biol* 2018; 1707: 163-169.

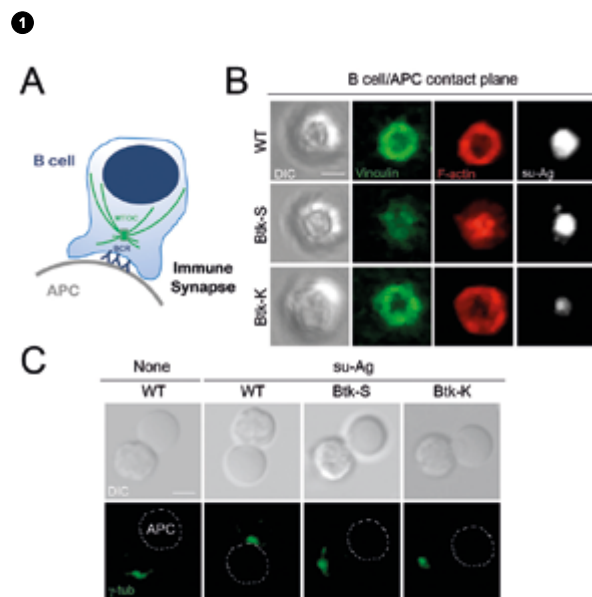
Perez-Olivares M, Trento A, Rodríguez-Acebes S, González-Acosta D, Fernández-Antoran D, Roman-García S, Martínez D, López-Briones T, Torroja C, Carrasco YR, Mendez J, Moreno de Alborán I. Functional interplay between c-Myc and Max in B lymphocyte differentiation. *EMBO Rep* 2018; 19: e45770.

Martínez-Muñoz L, Rodríguez-Frade JM, Barroso R, Sorzano COS, Torreño-Pina JA, Santiago CA, Manzo C, Lucas P, García-Cuesta EM, Gutiérrez E, Barrio L, Vargas J, Cascio G, Carrasco YR, Sánchez-Madrid F, García-Parajo MF, Mellado M. Separating actin-dependent chemokine receptor nanoclustering from dimerization indicates a role for clustering in CXCR4 signaling and function. *Mol Cell* 2018; 70: 106-119.

Bruton's tyrosine kinase (Btk) has a key role in the signalling pathways of receptors essential for the B cell response. Given its implication in B cell related immunodeficiency, leukaemias/lymphomas and autoimmunity, Btk is studied intensely and used as a target for therapy. Numerous clinical trials are ongoing, using Btk kinase activity inhibitors to treat patients suffering of B cell malignancies or B cell-related autoimmune disorders; while the clinical results are excellent, the underlying molecular mechanisms are hardly known. BCR recognition of antigen triggers the formation of the immune synapse (IS); this B cell/APC interaction platform provides a framework for signalling and polarised membrane trafficking to achieve B cell activation and antigen extraction. The actin cytoskeleton remodelling and adhesion-site dynamics have a crucial role in IS formation and stability. We reported that Btk controls the B cell ability to trigger IS formation and its appropriate intramolecular organisation mainly through shuttling/scaffold activities. Btk kinase function determines antigen accumulation at the IS by controlling the PLC γ 2/Ca $^{2+}$ axis. Impaired Btk shuttling/scaffold activity leads to defects in B cell activation and proliferation equivalent to those due to Btk kinase inhibition.

We also investigated Diacylglycerol kinases (DGK), a therapeutic target for fighting against immunosuppression in tumours. DGK limits antigen receptor signalling by DAG consumption, but the relevance of their product, phosphatidic acid (PA), in lymphocyte responses is quite unknown. Our findings suggest that PA generated by the DGK ζ isoform shapes B cell responses by controlling actin/adhesion-mediated force generation and cell polarity-related events at the synapse. An appropriate DAG/PA balance is key for B cell function.

The results derived from our studies reveal important aspects of Btk and DGK ζ /PA functions that enrich our knowledge and aid in the therapeutic targeting of these proteins.



1 Distinct roles for Btk scaffold and kinase activities in B cell synapse formation. A) Explicative scheme of the B cell IS established with an APC. B) DIC and fluorescence images of vinculin, F-actin and antigen (su-Ag) at the contact plane of the mature IS for representative B cells with normal Btk (WT), lacking kinase activity (Btk-K) or defective for the scaffold function (Btk-S); scale Bar, 2.5 μ m. C) DIC and fluorescence images of γ -tubulin (MTOC) for representative conjugates of B cells with APC unloaded (none) or antigen-loaded (su-Ag). Scale Bar, 5 μ m.



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

IMMUNOLOGY & ONCOLOGY 71

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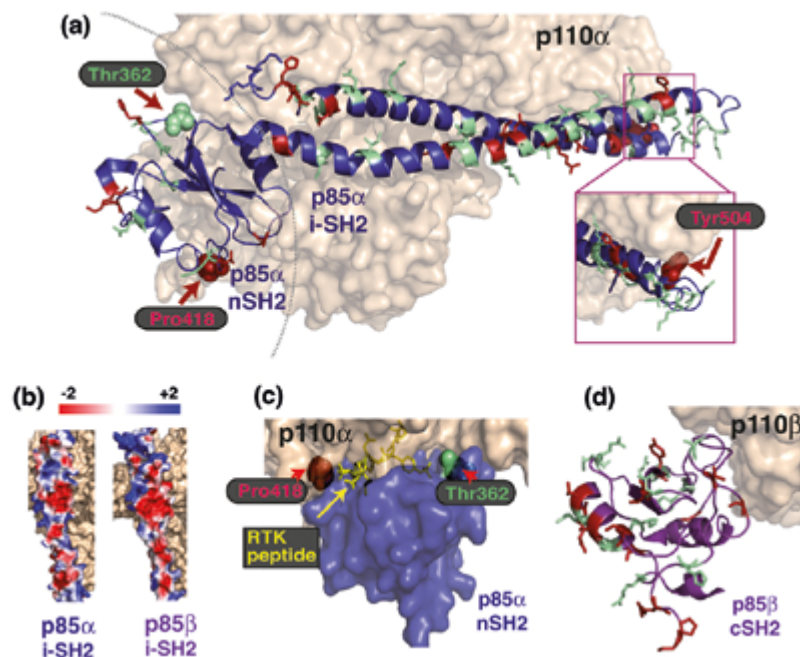
Varona R, Ramos T, Escribese MM, Jimeno L, Galán A, Würtzen PA, Vega F, Marín A, Martín S, Carrera AC, Blanco C, Barber D. Persistent regulatory T-cell response 2 years after 3 years of grass tablet SLIT: Links to reduced eosinophil counts, sIgE levels, and clinical benefit. *Allergy* 2019; 74: 349-360.

Olazabal-Morán M, González-García A, Carrera AC. Functions of Nuclear polyphosphoinositides. *Handbook of Experimental Pharmacology* 2018; Chapter 8 (Springer, Heidelberg).

Two biological problems have occupied the activity of our team of 12 to 15 members: cancer and inflammation. Our work is based on the assumption that the same biological activities that control physiological responses also control pathology when deregulated. The team is currently working on class I phosphoinositide 3-kinase (PI3K), with emphasis on examining the specific function of each of different catalytic and regulatory isoforms in physiology and disease.

- Function of PI3K in cancer using animal models and biochemistry approach.
- Alternative cancer treatments based on interfering molecules.
- New therapeutic targets on cancer based on the tumour hypoxic and oxidative environment.
- Mechanism for PI3-kinase beta action on DNA/chromatin remodelling.

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1 Structural differences between p85α and p85β. Residue differences of p85α and p85β in complex with PI3K/p110. Distinct residues could explain why the tumour suppressor p85α restrains p110 activity, while p85β is less inhibitory and drives tumour progression.

(a) p110α (ochre)/p85α (blue) interacting surface. Non-conserved (red) and semi-conserved (turquoise) residues. Only three residues: Thr362, Pro418 and Tyr 504 are in contact with PI3K (b) Different electrostatic surface of p85α and p85β (red-blue), p110α surface behind. (c) p110α (ochre) and nSH2 region of p85α (blue) showing that Thr362 and Pro418 are close to receptor Tyr Kinases (yellow). (d) p110β (ochre) and p85β iSH2 domain (purple) showing that distinct residues are not in contact with p110β and most likely mediate association with different partners.



Stress-activated protein kinase p38MAPK in inflammation and cancer

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SELECTED PUBLICATIONS

Cuenda A, Sanz-Ezquerro JJ. p38 γ and p38 δ : from spectators to key physiological players. *Trends Biochem Sci* 2017; 42: 431-442.

Cuenda A., Lizcano, Lozano J. Editorial: Mitogen activated protein kinases. *Front Cell Dev Biol* 2017; 5: 80.

Risco A, Martín-Serrano MA, Barber DF, Cuenda A. p38 γ and p38 δ are involved in T lymphocyte development. *Front Immunol* 2018; 9: 65.

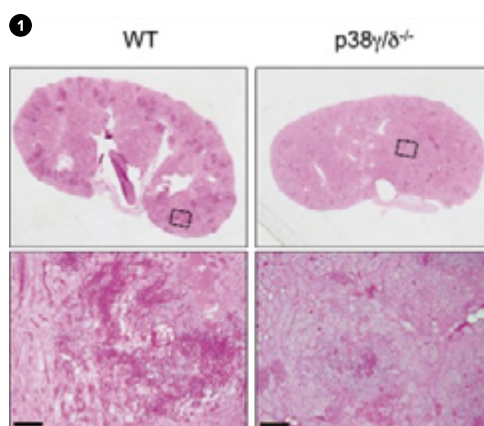
Alsina-Beauchamp D, Escós A, Fajardo P, González-Romero D, Díaz-Mora E, Risco A, Martín-Serrano MA, Del Fresno C, Domínguez-Andrés J, Aparicio N, Zur R, Shpiro N, Brown GD, Ardavin C, Netea MG, Alemany S, Sanz-Ezquerro JJ, Cuenda A. Myeloid cell deficiency of p38 γ /p38 δ protects against candidiasis and regulates antifungal immunity. *EMBO Mol Med* 2018; 10: 8485.

Cuenda A. MAP Kinases. Editorial: *Encyclopedia of Cancer*, Third Edition (editors in chief: Paolo Boffetta and Pierre Hainaut), Elsevier.

Our group is studying the physiological and pathological functions of the p38MAPK family in the context of inflammation and cancer. Inflammation, in the right place and at the right time, controls a healthy host response; however, uncontrolled inflammation causes many diseases, including some types of cancer. In these two years, we have expanded our knowledge on the molecular mechanisms involved in the inflammatory response in the setting of infection.

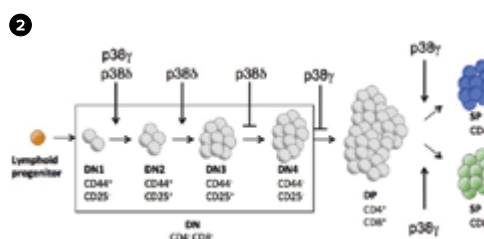
Previously, we have demonstrated that alternative p38MAPK (p38 γ and p38 δ) are key elements in the control of the inflammatory response in several models of inflammatory disease. p38 γ and p38 δ regulate many immune cell functions such as cytokine production, migration, or T cell activation. More recently, we have shown that p38 γ /p38 δ deletion protects against *Candida albicans* infection and increases mice survival. *C. albicans* is normally a benign member of the microbiota that colonise the gastrointestinal tract. p38 γ /p38 δ -/- mice exhibit increased fungicidal activity and decreased systemic inflammation. We also have defined a novel Dectin-1 signalling pathway by which p38 γ and p38 δ are essential for ERK pathway activation and contribute to production of inflammatory cytokines in macrophages infected by *C. albicans*. We demonstrated that genetic and chemical inhibition of p38 γ /p38 δ reduce fungal burden in mice, establishing p38 γ /p38 δ as potential therapeutic targets in humans.

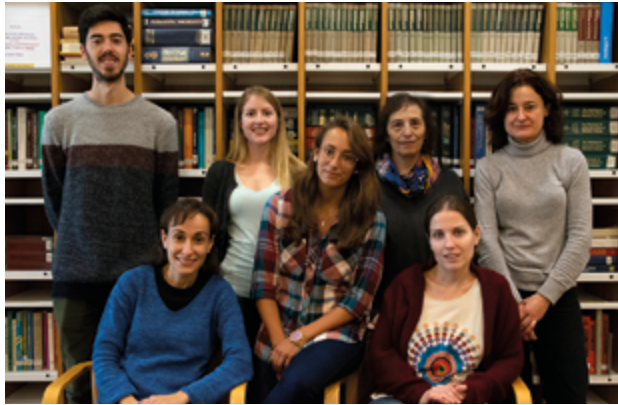
We have also investigated the role of alternative p38MAPK and in the development of immune cells such as T lymphocytes. We found that the T cell differentiation program in thymus was affected at different stages in p38 γ -, p38 δ -, and p38 γ / δ -deficient mice; peripheral T cell homeostasis was also compromised. Particularly, p38 δ deletion affects different stages of early CD4-CD8- double-negative thymocyte development, whereas lack of p38 γ favours thymocyte positive selection from CD4+CD8- double-positive to CD4+ or CD8+ single-positive cells. Our results have identified unreported functions for p38 γ and p38 δ in T cells.



1 Haematoxylin and eosin staining of kidney sections infected with *Candida albicans*, where fungus hyphae are seen as filaments.

2 Schematic representation indicating the different stages of T cell development partially controlled by p38 γ and/or p38 δ .





Physiopathology of chemokine receptor interactions

IMMUNOLOGY & ONCOLOGY 73

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SELECTED PUBLICATIONS

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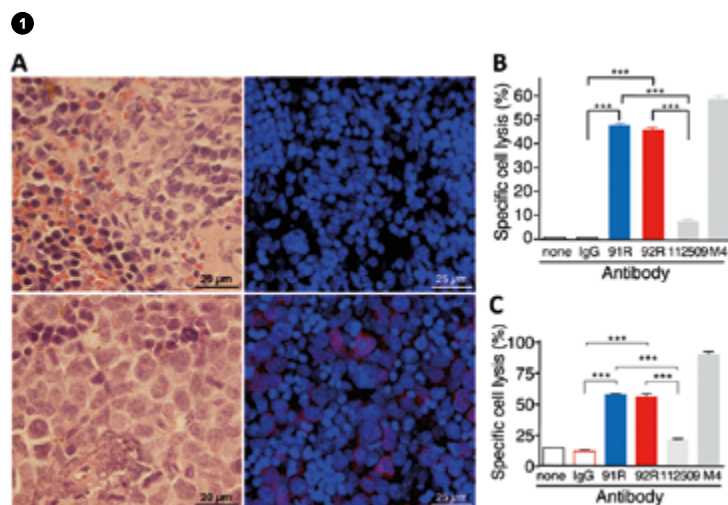
There is an increasing interest in the development of new immunotherapies for cancer treatment. This interest correlates with the therapeutic success obtained using these strategies, in particular with Chimeric Antigen Receptor (CAR) T-cell therapies and antibody-based medicines.

Chemokines and their receptors are key players in cancer biology, where they have relevant roles in tumour progression and metastasis. These proteins modulate tumour-associated angiogenesis and host anti-tumour immunological responses, and stimulate tumour cell survival and proliferation.

Our group studies the physiopathology of chemokine receptors involved in inflammatory diseases and cancer, and is currently focused on the human CCR9 receptor, a seven-transmembrane domain receptor that is highly expressed in a number of different haematological malignancies.

We have generated a panel of CCR9-specific monoclonal antibodies. Two of them were selected based on their effectiveness in reducing the growth of human CCR9+ tumours in immunodeficient mouse models. The results of *in vitro* experiments suggest that these antibodies might eliminate tumour cells through complement- and antibody-dependent cellular cytotoxicity. These antibodies have been licensed to a biopharmaceutical company.

Recent results of our work, in collaboration with Dr. J. A. García-Sanz (CIB-CSIC) and SunRock Biopharma, demonstrated that both the chimeric and humanised variants of these antibodies have the same specificity, affinity and *in vivo* anti-tumour activity as the original antibodies. We also observed that these antibodies strongly inhibit the growth of human CCR9+ leukaemia cell tumours in an immunodeficient NSG mouse model; these mice lack T cells, B cells and have compromised NK and complement activities. These findings support the notion that other mechanisms, including antibody-dependent cellular phagocytosis or direct apoptosis, might also play a role in tumour elimination mediated by these anti-CCR9 antibodies.



1 (A) Haematoxylin and eosin staining (left) and fluorescent immunohistochemical analysis (right) of spleens of NSG mice. Images of structural changes in the spleen an untreated mouse (top) and of a mouse inoculated with CCR9+ human tumour cells stained with red fluorescence (bottom). (B) Specific complement-dependent cytotoxicity and (C) specific NK cell-mediated cytotoxicity of CCR9+ human tumour cells opsonised with anti-CCR9 mAb (91R, 92R or 112509), isotype-matched mAb or positive control M4 serum, evaluated by flow cytometry.



Signalling networks in inflammation and cancer

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SELECTED PUBLICATIONS

Lacalle RA, Blanco R, Carmona-Rodríguez L, Martín-Leal A, Mira E, Mañes S. Chemokine receptor signaling and the hallmarks of cancer. *Int Rev Cell Mol Biol* 2017; 331:181-244.

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Inflammation is a defence response of the organism against internal and external harmful stimuli. Nonetheless, a deregulated inflammatory response can promote cancer and other diseases such as Alzheimer's.

In the 2017-2018 period we worked in the following four areas.

1. Extracellular superoxide dismutase (SOD3) in normalisation of tumour-associated vasculature.

The endothelium is a semipermeable barrier that regulates the transfer of oxygen, endo- and xenobiotics. Progressing tumours are characterised by an exacerbated angiogenesis but, unexpectedly, they are highly hypoxic. We have shown that elevation of extracellular superoxide dismutase (SOD3) in the tumour microenvironment or in perivascular regions normalise the tumour vasculature through a nitric oxide-dependent mechanism. We are now studying how SOD3 regulates tumour infiltration by effector immune cells.

2. Identification signalling pathways associated to PD-1-induced immunosuppression.

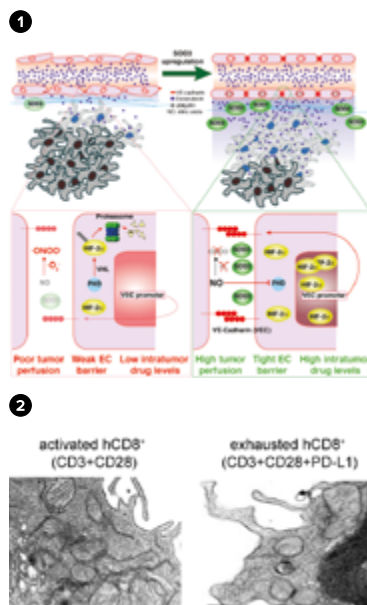
The immune system is able to identify and delete neoplastic cells, and blockade of immune checkpoints (such as PD-1) has transformed the clinical practice. Nevertheless, little is known about how PD-1 blocks the effector function in T cells. We have used RNA-seq and bioinformatics to identify the metabolism and mitochondrial structure as new targets of the inhibitory program elicited by PD-1 in CD8+ T cells.

3. CCR5 effects on T-cell receptor (TCR) organisation and the response of memory CD4+ T cells.

The chemokine receptor CCR5 not only work as a chemoattractant receptor for immune cells, but also provides costimulatory signals required for optimal CD4+ T cell activation. We have found that in addition to its role in primary activation of these cells, it regulates the function of CD4+ memory T cells *in vivo*. This activity is associated to changes in the nanoscale organisation of the TCR due to alterations on sphingolipid metabolism.

4. Innate immune cell differentiation in neurological diseases.

Innate immune cells, particularly macrophages, are major conductors of the inflammatory reaction. Depending on their polarisation, macrophages may activate a "healing program", which in the case of cancer, or a "tissue destruction program" as this occurs in inflammatory diseases. As part of a multidisciplinary European consortium, we are investigating the metabolic changes associated to apolipoprotein E (APOE) epsilon-4 genotype, a variant associated to late-onset Alzheimer's disease, and its influence on monocyte/macrophage/microglia functionality.



1 SOD3 increases the delivery of chemotherapeutics into tumours. tumours express low levels of SOD3, and prolyl hydroxylases (PHD) trigger HIF-2α degradation. SOD3 re-expression prevents nitric oxide (NO) oxidation, which stabilizes HIF-2α by NO-mediated PHD inhibition. HIF-2α then enhances VE-cadherin (VEC) transcription.

2 PD-1 induces structural defects in the mitochondria of human CD8+ T lymphocytes. Magnified mitochondria from human CD8+ T cells activated ex-vivo with anti-CD3 and anti-CD28 antibodies, or with the same anti-CD3 and anti-CD28 antibodies plus the ligand for PD-1 (Fc-PD-L1).



Stem cells and immunity

IMMUNOLOGY & ONCOLOGY 75

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Fütterer A, de Celis J, Navajas R, Almonacid L, Gutiérrez J, Talavera-Gutiérrez A, Pacios-Bras C, Bernascone I, Martín-Belmonte F, Martínez-A C. DIDO as a switchboard that regulates self-renewal and differentiation in embryonic stem cells. *Stem Cell Rep* 2017; 8: 1062-1075.

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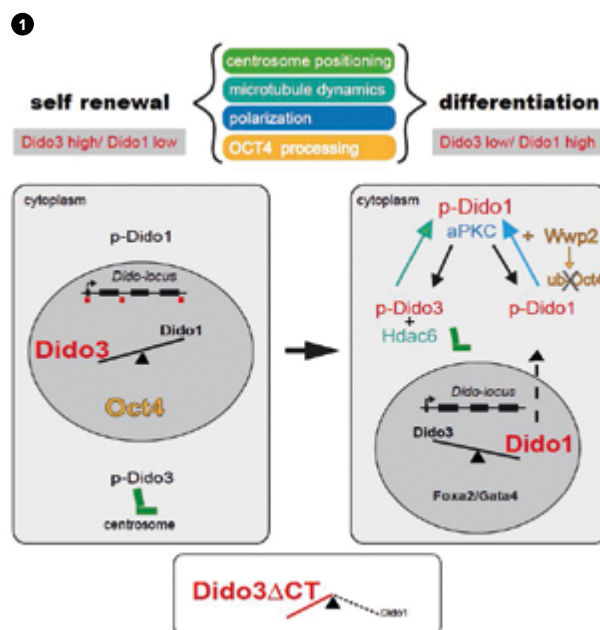
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We work to identify the molecular mechanisms of stem cell renewal and differentiation, and their role in regulating transcription and the cell cycle. Genome-wide screens have identified potential regulators including the *Dido* (death-inducer obliterator) gene, a locus that encodes three proteins generated by alternative splicing. From smallest to largest, *Dido1*, *Dido2*, and *Dido3* have a common N-terminal region with a PHD domain, and isoform-specific C-terminal parts.

Mice carrying the *Dido3* C-terminal truncation (*Dido3 Δ CT*) die at day 8 *post coitum*, and embryonic stem cells (ESC) derived from these mutants retain self-renewal capacity but fail to undergo differentiation, a process rescued by ectopic expression of wild type *DIDO3*. The mechanism (see Figure 1) shows that *Dido3* binds the *Dido* locus through the PHD domain via H3K4me3 and RNA PolII, and induces *DIDO1* expression, necessary for lineage commitment and differentiation into the primitive endoderm [PE]. In addition, *Dido3* must be phosphorylated and translocate to centrosomes, which ensures their correct positioning for PE cell polarisation and maintenance of daughter cell self-renewal capacity. The interesting ability of the *Dido* gene itself to regulate production of the distinct *Dido* isoforms is the subject of further study.

The PHD binding of *Dido* to H3K4me3, an epigenetic marker involved in histone recognition, indicates a possible role of *Dido* in transcription regulation. Using cells from the (*Dido3 Δ NT*) mice lacking the PHD domain, we showed that *Dido* is expelled from the histones during chromatin condensation. To preserve long-term histone trimethylation, adjacent residues are rapidly phosphorylated by mitotic kinases. This process ejects the transcription machinery through steric hindrance, promoting access for cohesins and condensins, and subsequent chromatin compaction. At the end of cell division, dephosphorylation unmasks the prior epigenetic state, allowing for resumption of an unchanged transcription program.



1 *DIDO* act as a switchboard that regulates the genetic programs for Embryonic Stem Cells (ESC) transition from symmetric to asymmetric cell division. *DIDO3*, binds the *Dido* locus via H3K4me3 and RNA POL II and induces *DIDO1* expression. *DIDO1*, which is exported to cytoplasm, associates with, and is N-terminally phosphorylated by PKC α . It binds the E3 ubiquitin ligase WWP2, which contributes to cell fate by OCT4 degradation, to allow expression of primitive endoderm (PE) markers. PE formation also depends on phosphorylated *DIDO3* localisation to centrosomes, which ensures their correct positioning for PE cell polarisation.



Chemokine receptors: new targets for therapeutic intervention

76 IMMUNOLOGY & ONCOLOGY

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SELECTED PUBLICATIONS

Collins PJ, McCully ML, Martínez-Muñoz L, Santiago C, Wheeldon J, *et al.* Epithelial chemokine CXCL12 synergizes with CXCL12 via allosteric modulation of CXCR4. *FASEB J* 2017; 31: 3084-3097.

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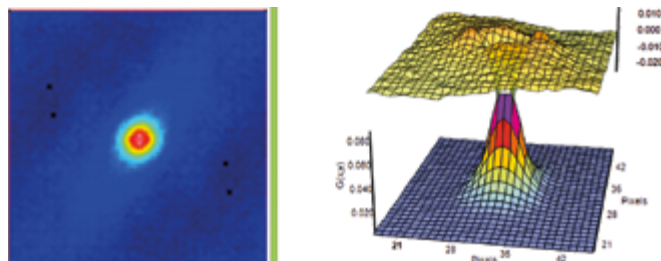
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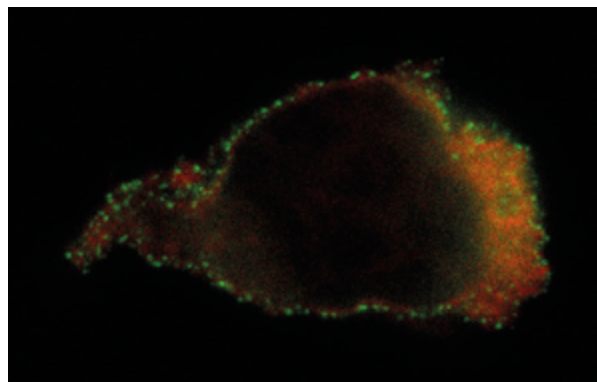
A broad array of biological responses including cell polarisation, movement, immune and inflammatory responses, cancer metastasis and prevention of HIV-1 infection are triggered by the chemokines, a family of secreted chemoattractant proteins that bind to class A-specific G protein-linked seven-transmembrane receptors.

In the last quarter century, the field has accumulated much information regarding the implications of these molecules in different immune processes, as well as mechanistic insight into the signalling events activated through their binding to their receptors. Today, we know that chemokine receptors must not only be considered isolated entities that are activated following ligand binding; rather, they are found as dimers and/or higher order oligomers at the cell surface, even in the absence of ligands. These complexes form organised arrays that can be modified by receptor expression and ligand levels, indicating that they are dynamic structures. The way in which these receptor complexes are stabilised modulates ligand binding as well as their pharmacological properties and the signalling events activated. These conformations thus represent a mechanism that increases the broad variety of chemokine functions. However, in the last five years, the use of new biophysical approaches, i.e. super-resolution microscopy and total internal reflection microscopy that allow precise analysis of protein–protein interactions in living cells, are revealing an unanticipated level of complexity among chemokine receptors at the cell surface. The dynamic interactions between these receptors, as well as their interplay with other proteins co-expressed by the cells, lipids that form the cell membrane, the cytoskeleton, and downstream signalling machinery will be crucial for defining the context-specific functions triggered. This new information is transforming our working model of chemokine-associated functions and allows us to identify new targets and to devise innovative pharmacological therapies to modulate certain cell activities without affecting others.

1



2



1 Analysis of lipid diffusion in Jurkat cells using the RICS (Raster Image Correlation Spectroscopy) approach.

2 Analysis by STED (Stimulated Emission Depletion) microscopy of a polarized primary CD4⁺ T cell. (Green: human CXCR4; Red: Phalloidin)



Diacylglycerol kinases in the control of immune response and cancer progression

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SELECTED PUBLICATIONS

Tello-Lafoz M, Rodríguez-Rodríguez C, *et al.* SNX27 links DGK ζ to the control of transcriptional and metabolic programs in T lymphocytes. *Sci Rep* 2017; 7: 16361.

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The Diacylglycerol Kinase (DGK) family of lipid kinases regulate the conversion of Diacylglycerol (DAG) into phosphatidic acid (PA). Altered DAG/PA homeostasis resulting from DGK malfunction causes several human diseases (Figure 1). In T lymphocytes, DGKs limit DAG-dependent activation of effector functions. We work to get a better understanding of the mechanisms underlying DGK regulation in T cells so that steps of the process can be manipulated for therapeutic benefit.

1. DGK and cancer

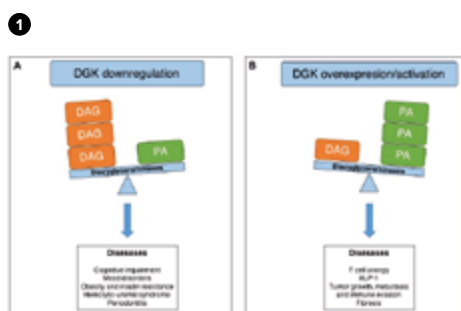
Overcoming the hypofunctional state imposed by solid tumours to T cells has become a critical strategy in the fight against cancer. Clinical progress is challenging due to the complex strategies that tumours employ to evade the immune system. Our group works to demonstrate that targeting specific DGK isoforms represents a novel and understudied strategy to manipulate antitumoural immune responses (Figure 2).

2. DGK and aplastic anaemia

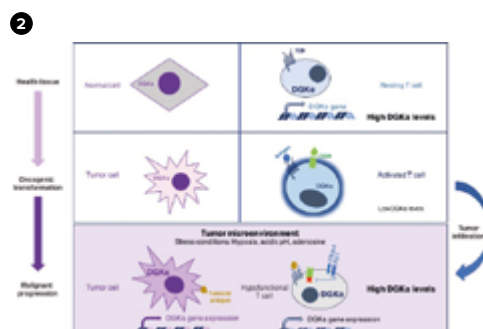
Aplastic anaemia (AA) is a disease in which the bone marrow gradually stops producing cells. In most cases, AA results from spontaneous T cell attack to bone marrow cells. We recently discovered that deficiency of specific DGK isoforms facilitates T cell activation in the bone marrow. We are working to better understand whether DGK malfunction may contribute to AA triggering.

3. DGK and Alzheimer's disease in Down Syndrome

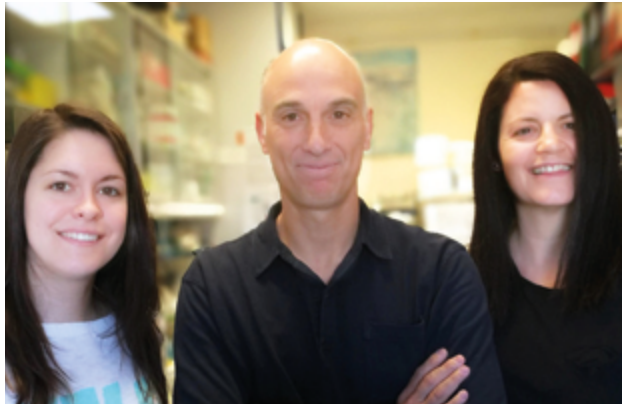
Down syndrome (DS), the most prevalent chromosomal disorder, results in mild cognitive impairment, high frequency of infections and elevated risk of leukaemia and autoimmune diseases. Virtually all DS people develop Alzheimer's disease (AD) by their 40s and at least 70% develop dementia. Notably, people with DS are protected from solid tumours. We recently identified SNX27, a protein that is diminished in DS, as a DGK partner and have demonstrated that SNX27/DGK ζ interaction contributes to the control of T cell responses. We work to investigate whether reduced SNX27 expression, due to trisomy 21, favours immune disorders that may contribute to DS associated pathologies.



1 General model of DGK contribution to human disease. (A) Deficiency of selected DGK isoforms results in an excess of DAG that impairs synaptic transmission, favours insulin resistance and triggers abnormal platelet or neutrophil activation. (B) Upregulation of DGK isoforms limits T and NK cytotoxic function and contributes to oncogenic transformation.



2 High DGK ζ expression in tumours and tumour infiltrating T cells. Expression of DGK ζ decrease in activated T lymphocytes and increases during oncogenic transformation. Stress conditions lead to enhanced expression of DGK ζ in tumour and tumour infiltrating T cells. These cells become no functional since TCR and costimulatory signals are blocked by the high expression of immune checkpoints such as CTLA-4 and PD-1. This limits the T cell response to cancer cells and leads to tumour persistence.



Transcriptional control of B lymphocyte differentiation

78 IMMUNOLOGY & ONCOLOGY

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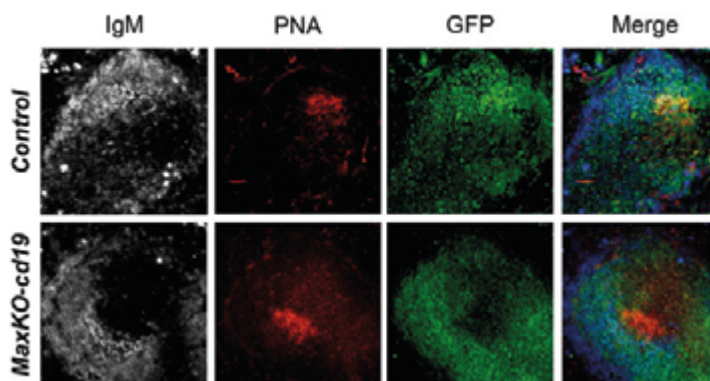
SELECTED PUBLICATIONS

Pérez-Olivares M, Trento A, Rodríguez-Acebes S, González-Acosta D, Fernández-Antorán D, Román-García S, Martínez D, López-Briones T, Torroja C, Carrasco YR, Méndez J, Moreno de Alborán I. Functional interplay between c-Myc and Max in B lymphocyte differentiation. *EMBO Rep* 2018; 19: e45770.

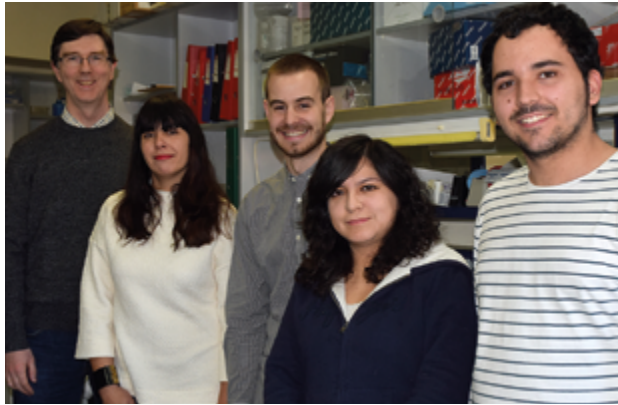
B lymphocytes are essential cellular components of the immune response. They undergo a differentiation process in the bone marrow and in secondary lymphoid organs in which a number of transcription factors play a prominent role. Our general biological question is to understand the transcriptional program that governs this process. Among the spectrum of transcription factors involved, we focused our attention on 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 various 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.

The Myc proteins contain a basic region/helix-loop-helix/leucine zipper domain that mediates DNA binding and heterodimerisation with its mandatory partner Max. It is generally assumed by the majority of scientific reports that, in order to activate or repress target genes, Myc proteins must heterodimerise with Max and bind to specific regulatory regions. However, no definitive data have addressed the role of this Myc/Max interplay *in vivo*. Previous data from our group showed that Myc/Max functional collaboration in B lymphocytes is more complex than initially anticipated. In our lab, we are currently interested in the study of the functional relationship between Myc and Max in physiological and pathological scenarios *in vivo*. For this purpose, we have generated new and complex genetically modified mouse models that specifically allow to address these questions. Due to the central role of Max, we expect that our results will have a relevant impact on the current knowledge of Myc biology.

1



1 Analysis of Germinal Centre (GC) formation in the spleens of *MaxKO-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*-deficient B cells; green).



Receptor ligand interactions in immune responses to cancer and viruses

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SELECTED PUBLICATIONS

Esteso G Guerra S, Valés-Gómez M, Reyburn HT. Innate immune recognition of double-stranded RNA triggers increased expression of NKG2D ligands after virus infection. *J Biol Chem* 2017; 292: 20472-20480.

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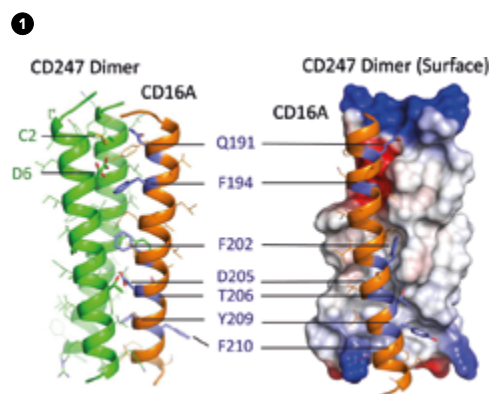
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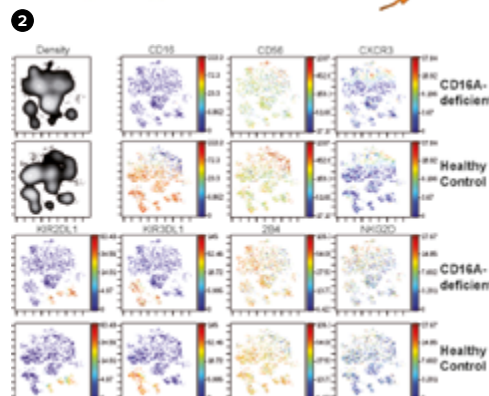
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Natural killer (NK) cells kill infected cells and secrete cytokines, to play an important role in defence against viral infection. Although NK cells are often perceived as rather primitive lymphocytes; always ready to kill unless checked by inhibitory 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 the knowledge of the cell biology of various NK cell receptors and their ligands. 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 investigate in detail the molecular bases of the changes observed *in vivo*.



1 Model of CD16A assembled with CD247 dimer. The key residues for this interaction, identified in experiments of site-directed mutagenesis, are highlighted.



2 ViSNE plots of NK cells from a healthy control and a CD16A-deficient patient. Cryopreserved PBMC were thawed, stained with a panel of 45 mAbs and analysed by mass cytometry. ViSNE plots for the indicated NK receptors are shown. Density plots are shown in grayscale, and visualise cell quantities in each area of the plot. Expression plots are represented in a colour scale, from blue (low) to red (high).



T cell signalling in autoimmune diseases and cancer

80 IMMUNOLOGY & ONCOLOGY

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SELECTED PUBLICATIONS

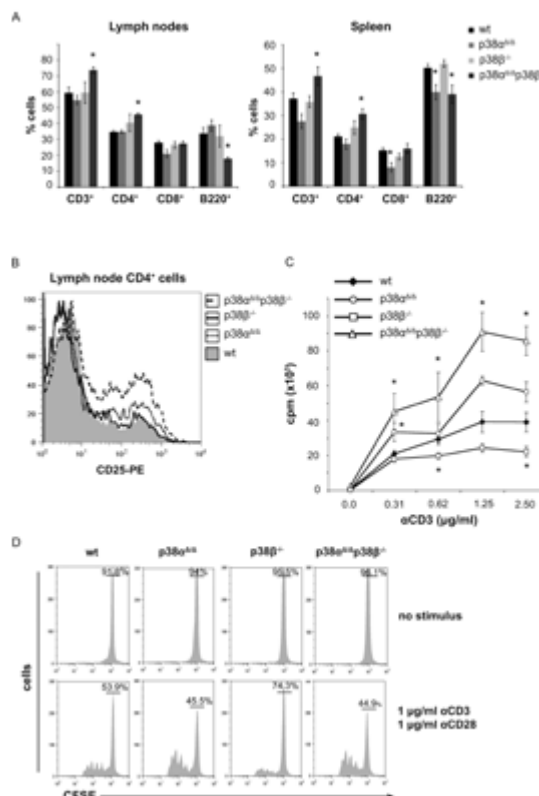
Salvador-Bernáldez M, Mateus SB, Del Barco Barrantes I, Arthur SC, Martínez-A C, Nebreda AR, Salvador JM. p38 α regulates cytokine-induced IFN γ secretion via the Mnk1/eIF4E pathway in Th1 cells. *Immunol Cell Biol* 2017, 95: 814-823.

Herrero D, Cañon S, Pelacho B, Salvador-Bernáldez M, Aguilar S, Pogontike C, Carmona RM, Salvador JM, Pérez-Pomares JM, Klein OD, Prósper F, Jiménez-Borreguero LJ and Bernad A. Bmi1+ progenitor cell ablation impairs the angiogenic response to myocardial infarction. *Arterioscler Thromb Vasc Biol* 2018, 38: 2160-2173.

Our group is focused on the identification and characterisation of the molecular mechanisms that regulate T cell functions involved in the development of autoimmune diseases and cancer. p38 MAPKs pathways have a critical role in the regulation of the immune response and inflammatory processes. The precise function of p38 α and p38 β in T cell proliferation and cytokine production nonetheless remains controversial, because it has been addressed mostly using chemical inhibitors. To dissect p38 α and p38 β functions in T cells, we have characterised mice deficient for each isoform. Since p38 α -deficient mice are not viable, we used a conditional knockout mouse model to analyse p38 α function in CD4 $^{+}$ T cells; in addition, we characterised mice lacking p38 β , and generated double-knockout mice. Notably, our results indicate that p38 α and p38 β have distinct regulatory roles in CD4 $^{+}$ T cell proliferation: p38 α is a negative regulator, whereas p38 β plays the opposite function.

We have analysed the role of p38 α and p38 β in Th1 and Th2 effector function as well as cytokine production. Our results demonstrate that p38 α and p38 β are essential for normal Th1, but not for Th2 effector function. p38 α and p38 β control T cell receptor-induced IFN γ and TNF α production, but only p38 α modulates cytokine-induced IFN γ production. Our findings demonstrate that p38 α , but not p38 β , controls IFN γ production through the activation of the Mnk1/eIF4E pathway of translation initiation in T cells.

These findings could be useful in generating new anti-inflammatory treatments. Our data indicate that selective inhibition of p38 α activity is not sufficient to block production of proinflammatory cytokines, and that combined inhibition of p38 α and p38 β should be considered for targeting the inflammatory response in autoimmune diseases.



1 Opposite roles of p38 α and p38 β in CD4 $^{+}$ T cell proliferation. (A) Percentage of lymph node and spleen cells showing increased CD4 $^{+}$ T cell population in p38 α $^{-/-}$ p38 β $^{-/-}$ mice. (B) Representative histogram overlay of CD25 expression in CD4 $^{+}$ T cells from wt, p38 α $^{-/-}$, p38 β $^{-/-}$ and p38 α $^{-/-}$ p38 β $^{-/-}$ mice. (C) Compared to wt cells, p38 α $^{-/-}$ and p38 α $^{-/-}$ p38 β $^{-/-}$ CD4 $^{+}$ T cells showed more rapid proliferation in response to TCR activation. (D) CD4 $^{+}$ T cells from wt, p38 α $^{-/-}$, p38 β $^{-/-}$ and p38 α $^{-/-}$ p38 β $^{-/-}$ mice were activated with anti-CD3 and anti-CD28 antibody and the proliferative response was monitored by CFSE staining.



Tumour immune activation and evasion

IMMUNOLOGY & ONCOLOGY 81

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SELECTED PUBLICATIONS

López-Cobo S, Pieper N, Campos-Silva C, García-Cuesta EM, Reyburn HT, Paschen A and Valés-Gómez M. Impaired NK cell recognition of vemurafenib-treated melanoma cells is overcome by simultaneous application of histone deacetylase inhibitors. *Oncoimmunology* 2017; 7: e1392426.

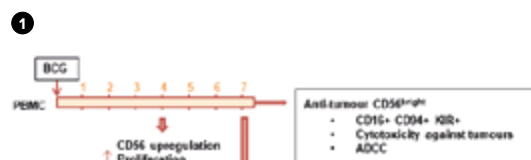
García-Cuesta EM, Esteso G, Ashiru O, López-Cobo S, Álvarez-Maestro M, Linares A, Ho M, Martínez-Piñeiro L, Reyburn HT and Valés-Gómez M. Characterisation of a human anti-tumoral NK cell population expanded after BCG treatment of leukocytes. *Oncoimmunology* 2017; 6: e1293212.

S López-Cobo, C Campos-Silva, A Moyano, M Oliveira-Rodríguez, A Paschen, M Yáñez-Mó, MC Blanco-López, M. Valés-Gómez. Immunoassays for scarce tumour-antigens in exosomes: detection of the human NKG2D-Ligand, MICA, in tetraspanin-containing nanovesicles from melanoma. *J Nanobiotechnology* 2018; 16: 47.

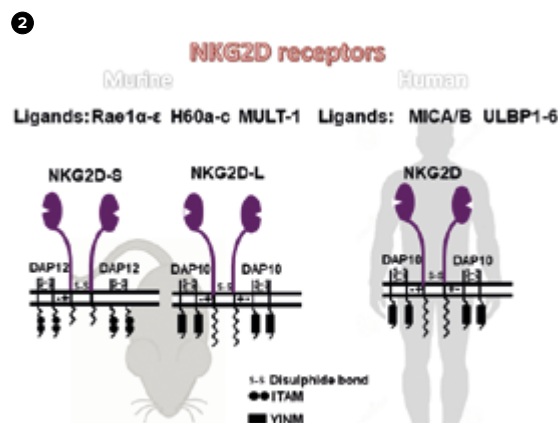
Campos-Silva C., Kramer MK, Vales-Gomez M. NKG2D-ligands: Putting everything under the same umbrella can be misleading. *HLA* 2018; 91: 489-500.

The group is interested in cancer immunity mediated by Natural Killer (NK) cells. These studies pose many difficulties, because of the complexity of the response, arising from the large number of cell subtypes and soluble factors that can be recruited to the tumour environment. NK cells can be affected by tumour recognition or evasion events and, thus, directly contribute to the outcome of the immune interaction. We use the treatment of bladder cancer patients with intra-vesical instillations of BCG as a model for the study of the stimulation of immune cells to eliminate tumours. In this context, *in vitro* experiments involving the culture of PBMCs with non-pathogenic Mycobacteria have shown that, after stimulation with BCG, a subpopulation of CD56^{bright} NK cells expands and acquires the ability to recognise tumour cells, including bladder cancer. CD56^{bright} cells were defined in other systems as a subpopulation of immature NK cells, usually with a high ability to secrete cytokines. However, the subpopulation that we have discovered derives from CD56^{dim} cells and mediates cytotoxic activity due to the presence of other NK receptors.

We have also described that, in melanoma, NKG2D-mediated immune modulation can occur in the context of therapies directed to proliferation pathways, such as the activation of BRAF. This could represent a mechanism of immune evasion for therapies directed against the MAPK route. Since NKG2D-ligands can be released as soluble molecules or in extracellular vesicles, the consequence of their modulation in the context of BRAF inhibitors could be followed analysing patient plasma. To investigate this idea, we have developed techniques for the study of NKG2D-ligands in extracellular vesicles.



1 Immune activation during one week of co-incubation of PBMCs with BCG. PBMCs from healthy donors incubated with BCG provoke the expansion of an anti-tumour NK cell population characterised by the upregulation of CD56 and CD16. This CD56^{bright} NK population has an increased capacity to eliminate different bladder cancer cell lines by cytotoxic degranulation. The activation depends on cytokine release, presumably by other immune cell subtypes.



2 Murine and human NKG2D receptors and their ligands: different things under the same umbrella. Mouse NKG2D (left) exists as two different splicing forms and can trigger different signalling routes. Human NKG2D only associates with DAP10. Many different proteins bind to human NKG2D: the genes for MICA and MICB are highly polymorphic and ULBPs also have different variants (modified from Campos-Silva, Kramer and Valés-Gómez, *HLA* 2018).