

**Inmunology
Oncology**

The Department of Immunology and Oncology (DIO) was created in 1996, with three main objectives:

- To contribute to the generation of scientific knowledge
- To improve society's wellbeing through biomedical research
- To collaborate with Spanish biotech companies to increase their competitiveness

Since its inception, the DIO has combined academic research and the establishment of strategic alliances with pharmaceutical and technology-based companies to seek synergies and to respond to the translational vocation of its scientists. The goal of DIO scientists is to develop new agents for the treatment of some of the major diseases that affect humankind in the 21st century, such as chronic inflammatory, infectious and autoimmune diseases, as well as cancer.

In this report, we summarize the 19 research projects under way in the department. In many of these projects, DIO researchers conjugate molecular analysis of the intracellular signal transduction pathways that control cell migration, differentiation, survival, proliferation and death, with the development of animal models that resemble human disease. The DIO has extensive experience with mouse models of systemic lupus erythematosus, rheumatoid arthritis, asthma, inflammatory bowel disease, multiple sclerosis, type I diabetes, and a number of models for cancer (breast, colon, leukaemia, etc.).

Last, but not less important, is our commitment to the preparation of new generations of scientists. Many DIO members participate in teaching activities, lectures and seminars in national and international institutions. Fourteen PhD dissertations directed by DIO scientists were defended in 2009-2010; most of these new young scientists are now postdoctoral researchers in international institutions, spreading the DIO values beyond our doors. We are indebted to all of them as well as to the technical, editorial and administrative staff, whose contribution makes possible the quality of work developed at the DIO.

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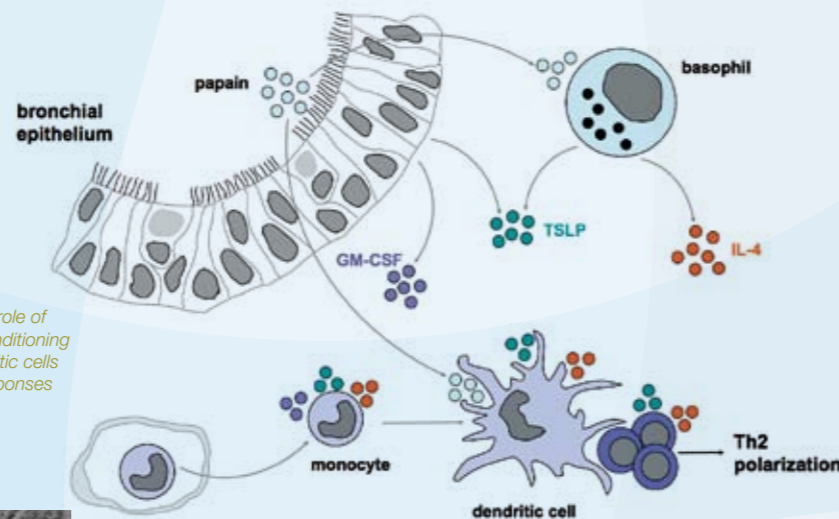
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Differentiation and Functional Specialization of Dendritic Cells during Inflammatory, Infectious and Allergic Processes

Our research aims at exploring the functional specialization of dendritic cells derived from monocytes during inflammatory responses caused by bacterial, yeast and parasitic infections, or by allergic reactions induced by allergens derived from plants, fungi or acaridae.



Working hypothesis on the role of the cytokine IL-4 on the conditioning of monocyte-derived dendritic cells for the induction of Th2 responses against allergens.

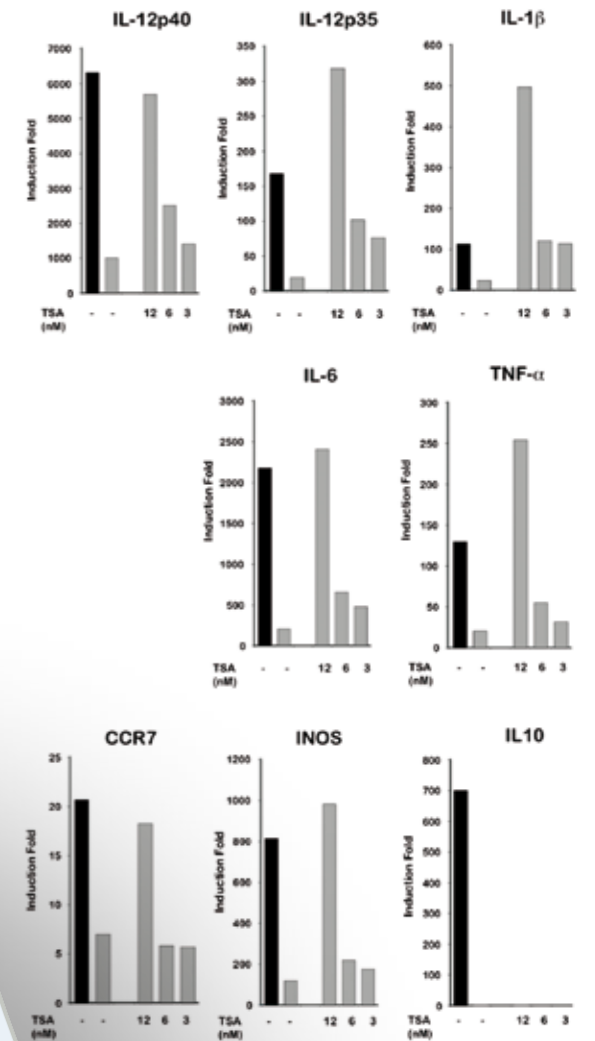
In particular our current research interests are focused on the following topics, that reflect the developmental and functional plasticity of monocytes and the relevance of monocyte-derived dendritic cells during infectious and allergic processes:

- Analysis of the effector functions of mouse monocytes in innate and adaptive immunity.
- Analysis of the differential migratory properties of mouse monocyte-derived dendritic cells and macrophages.
- Regulation of mouse monocyte differentiation into dendritic cells and macrophages during in vivo immune response to *Leishmania major*.

- Functional specialization of mouse dendritic cells for the induction of Th2 responses against pathogens and allergens.
- Gene expression profile of mouse monocytes and monocyte-derived dendritic cells exposed to allergens and Th2-polarizing mediators.
- Effect of statin treatment on proinflammatory cytokine production and nitric oxide metabolism by LPS-activated or *Listeria monocytogenes*-infected mouse monocyte-derived DCs.
- Analysis of the splenic innate immune response to in vivo *Listeria monocytogenes* infection on statin-treated mice.
- Role of type-I interferon in the induction of Th17 immune responses against *Candida albicans* in mice.

The methodology designed for addressing these objectives involves the following experimental approaches:

- Development of in vitro and in vivo infection models in different mouse strains, including mice deficient in cytokines (GM-CSF), cytokine/chemokine receptors (IFNAR, CCR2, CCR7), molecules involved in dendritic cell activation (MyD88, NOD 1/2, IRF-3, PPAR-γ) and mice transgenic for TCRs specific for ovalbumin-derived peptides expressed on MHC I (OT-I) or MHC II (OT-II, DO.11).
- Cell biology techniques designed for the purification or isolation of defined cell populations from mouse bone marrow, skin, lymph nodes and spleen, involving magnetic bead and FACS cell separation methods.
- Analysis of monocytes, dendritic cells and macrophages from *Leishmania*- or *Listeria*-infected mice on cell suspensions or tissue sections, by electron microscopy or confocal microscopy after immunofluorescent staining.
- In vitro differentiation of dendritic cells and macrophages on GM-CSF, IL-3 or Flt3L-driven cultures from monocytes or bone marrow precursors.
- Analysis of gene expression profiles at the protein level by flow cytometry, ELISA and electrophoresis.
- Analysis of gene expression at the mRNA level by real-time quantitative PCR, whole mouse genome microarray analyses and chromatin immunoprecipitation.



Epigenetic regulation of Th2 responses: reversal by the histone deacetylase inhibitor TSA of IL-4-dependent inhibition of pro-inflammatory cytokine production induced by TLR-4 engagement in monocyte-derived dendritic cells.

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**Interplay of Activation,
Apoptosis and Cell
Regulators Monitors
Autoimmune T Cell Memory
Responses and Inflammation**

**T cell memory responses and Homeostasis
in Immunity and Autoimmunity.**

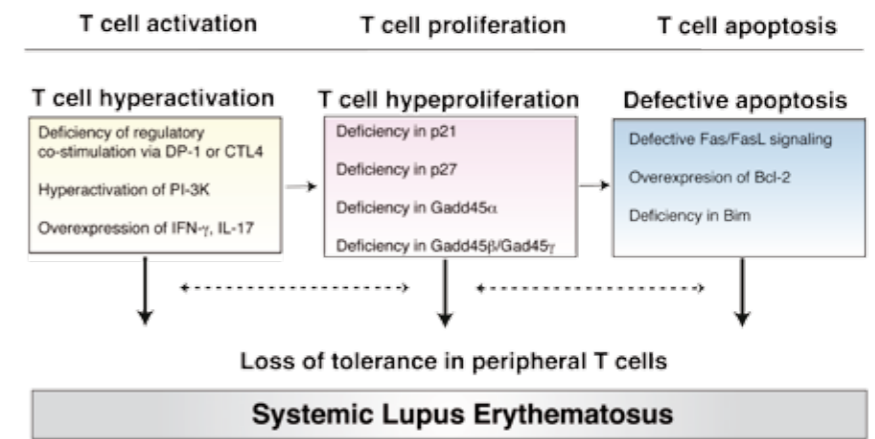
Apoptosis is considered a basic mechanism for limiting T cell memory expansion known as homeostasis. Nevertheless, we find that the control of activation of memory T cells and of proliferation are also fundamental for memory T cell homeostasis (Fig. 1). We suggest that memory responses and homeostasis require a combination of control in activation, apoptosis and proliferation (3).

The cell cycle inhibitor p21 suppresses autoimmunity and controls T cell memory responses.

We have shown that p21 is an autoimmunity suppressor, since p21 deficiency leads to autoimmunity (Balomenos D et al. Nat Med 2000, Arias CF et al. J Immunol. 2007. 178:2296-306). Indeed, p21 overexpression in T cells of autoimmune lupus-prone Fas-deficient (lpr/lpr) mice reduces autoimmunity development. (5) by limiting the expansion of autoreactive lpr/lpr memory T cells. p21 plays an attenuating role in T cell expansion upon persistent stimulation of T cells through TCR. Our recent work unveils a novel function of p21 regulating of TCR-dependent activation of memory T cells. Thus we propose that p21 exerts two functions in the response of memory T cells, by first regulating their level of activation and second by controlling their proliferative potential. We are now studying the mechanism by which p21 controls memory T cell activation.

An alternative function for the Fas/FasL apoptosis system. Our analysis of memory T cell activation, apoptosis and cell cycle regulation events in immunity and autoimmune disease, have revealed a previously unknown role for Fas. Similarly, to the function of p21 as a regulator of repeatedly

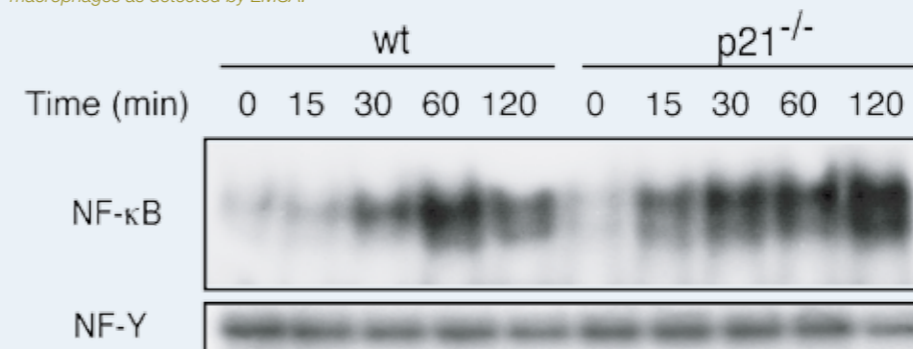
A schematic representation showing that defects in the control of T cell activation, apoptosis and proliferation may lead to Lupus autoimmunity.



activated T cells we have established that Fas also plays a crucial attenuating role in the response of previously activated T cells but not of primary T cells. We are currently investigating the mechanistic aspects of this new role of the Fas/FasL system.

p21 regulates the macrophage activation pathway. p21 has a general role in the immune response since independently of its cell cycle inhibitory capacity, regulates macrophage activation by controlling the (NF- κ B) pathway in the cytoplasm (1, Fig. 2). p21 regulation of NF- κ B activation is critical for progression of in vivo inflammation, since it decreases sensitivity to LPS-induced septic shock. We are studying the mechanism that defines the role of p21 in NF- κ B activation, and whether p21 suppresses glomerulonephritis development through its regulatory effect on innate immunity inflammation.

Lack of p21 leads to an increased activation of the NF- κ B pathway in LPS-stimulated macrophages as detected by EMSA.



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Lymphocytes in Physiological and Pathological Processes: Autoimmune Inflammatory Diseases, Cancer Immunotherapy, and Nanomedicine

Molecular and cellular mechanisms in autoimmune disease: identifying strategies for therapeutic intervention.

Approximately 5-10% of the population in the developed world is affected by at least one of >80 autoimmune disorders; these chronic, debilitating diseases have an enormous social and economic impact. To analyse the mechanisms that operate in autoimmune diseases and identify new drug targets and therapeutic strategies, we study intracellular signaling pathways that induce autoimmunity or inflammation when hyperactivated, as well as the mechanisms that maintain peripheral tolerance in the immune system.

Our lab studies several aspects of the initiation and progression of autoimmune disease in murine models: 1) the role of p110 γ PI3K in T cell activation and autoimmunity, 2) the role of p110 γ PI3K in secondary lymphoid organs and in the onset/ progression of immune responses and autoimmunity, 3) how p85 γ PI3K contributes to CD28 costimulation in the activation of effector and regulatory T cells, and how its absence affects rheumatoid arthritis onset and development, and 4) crosstalk between negative regulators of T cell activation that enforce T cell quiescence and PI3K isoforms, and how crosstalk affects autoimmunity.

NKG2D in autoimmunity and tumour immunotherapy

NKG2D is an activating receptor expressed by natural killer (NK) cells and T cells; it is implicated in immune responses to infections and tumours and in autoimmunity. NKG2D ligands are not expressed by most normal cells but are up-regulated on numerous tumor cell. In addition, their inappropriate expression in certain tissues can trigger or

exacerbate autoimmune disease. In fact, implicating NKG2D and its ligands in the pathogenesis of several autoimmune diseases.

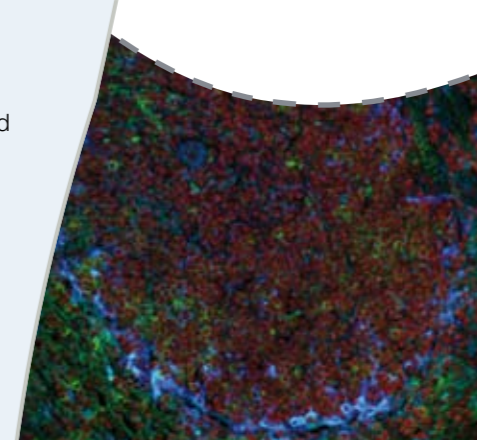
Our projects study the role of NKG2D in autoimmunity and tumour immunotherapy: 1) NKG2D associates with the adaptor protein DAP10, which binds and activates the PI3K p85 subunit. We are analyzing the specific contribution of each PI3K isoform to cell activation via NKG2D. 2) To study the contribution of NKG2D to the initiation/progression of autoimmune processes, we analyse NKG2D ligand expression in several mouse models of autoimmune disease, and study the correlation between ligand expression and disease onset/severity.

Nanomedicine as a therapeutic approach for treating cancer and autoimmune disease

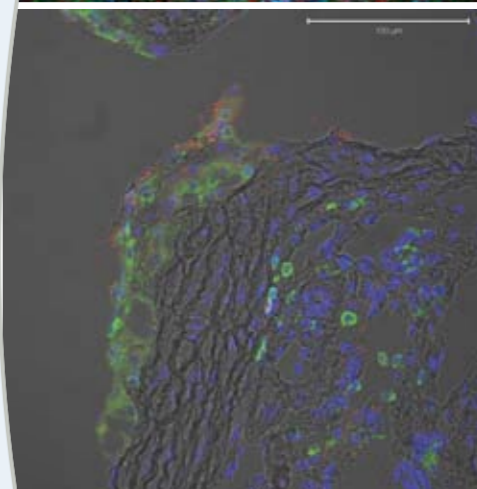
Radiotherapy- and chemotherapy-based cancer treatments affect both tumours and healthy tissue, leading to a search for more specific ways to fight tumours. Tumour immunotherapy is a promising treatment strategy, as it could enhance the immune system's natural capacity to control tumour development. Studies suggest IFN- γ as effective in tumour elimination, although it is difficult to deliver an appropriate cytokine dose to the tumour without causing toxicity to surrounding tissues. Directed targeting to the tumour could improve the efficiency of its delivery, increasing local dosage without augmenting the systemic concentration. Nanotechnology provides a means to target drugs using superparamagnetic iron oxide nanoparticles as drug delivery systems in conjunction with a magnetic field, applied externally or implanted internally.

In mouse models of cancer, we tested uniform dimercaptosuccinic acid (DMSA)-coated monodisperse magnetic nanoparticles (MNP) as a delivery system for IFN- γ . IFN- γ -adsorbed DMSA-coated MNP were targeted to the tumour site by applying an external magnetic field. We found nanoparticle accumulation and cytokine delivery at the tumour site, which led to increased T cell and macrophage infiltration and promoted an antiangiogenic effect, resulting in a notable reduction in tumour size. Our findings show that these nanoparticles can be an efficient in vivo drug delivery system for tumour immunotherapy.

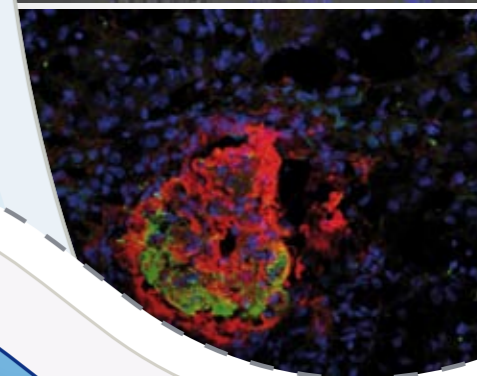
We are also developing and validating a nanoparticle-based system for controlled, localized release of small interfering RNA (siRNA), microRNA, antagomirs and aptamers for specific gene silencing and cell targeting, for treatment of cancer and autoimmunity.



This immunofluorescence staining was performed on spleen frozen sections from p110 $\delta^{WT/WT}$ mice. It shows a germinal center in which p110 δ PI3K is stained in green, marginal metallophilic macrophages in blue and nuclei in red.



This immunofluorescence staining was performed on aortic sections from LDLR^{-/-} p110 γ ^{-/-} mouse embedded in paraffin. A fragment of an atherosclerotic plaque is shown, as well as part of the tunica media and adventitia. T cells are stained in green, nuclei in blue (DAPI) and nuclear proliferation marker Ki67 as well as some erythrocytes in red.



Staining a glomerulus (in red) of a lupus prone MRL/lpr mouse showing expression and co-localization of ult-1 (in green), a Natural Killer cell ligand.

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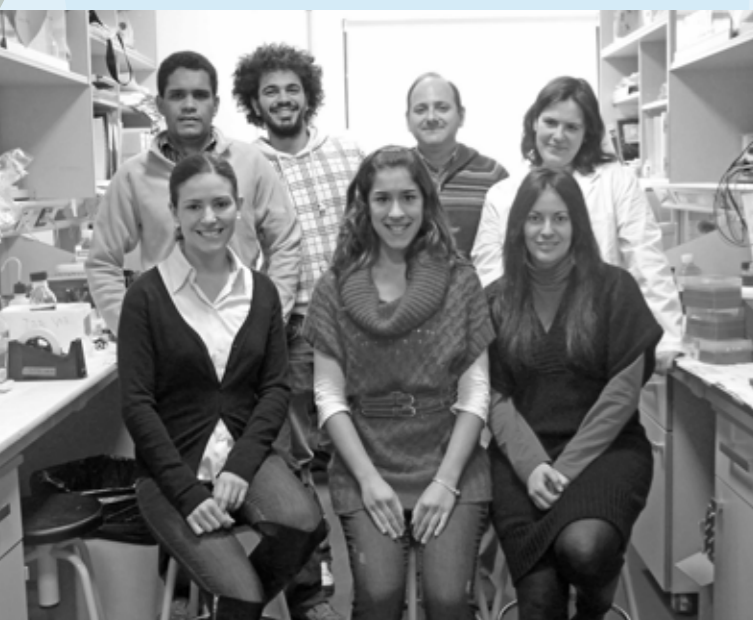
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Alcázar, I., Cortés, I., Zaballos, A., Hernández, C., Fruman, D., Barber, D.F., and Carrera, A.C. (2009) "p85beta phosphoinositide 3-kinase regulates CD28 coreceptor function". *Blood* 113:3198-3208. *Domingo F. Barber and Ana C. Carrera are senior authors on this paper.

PATENT

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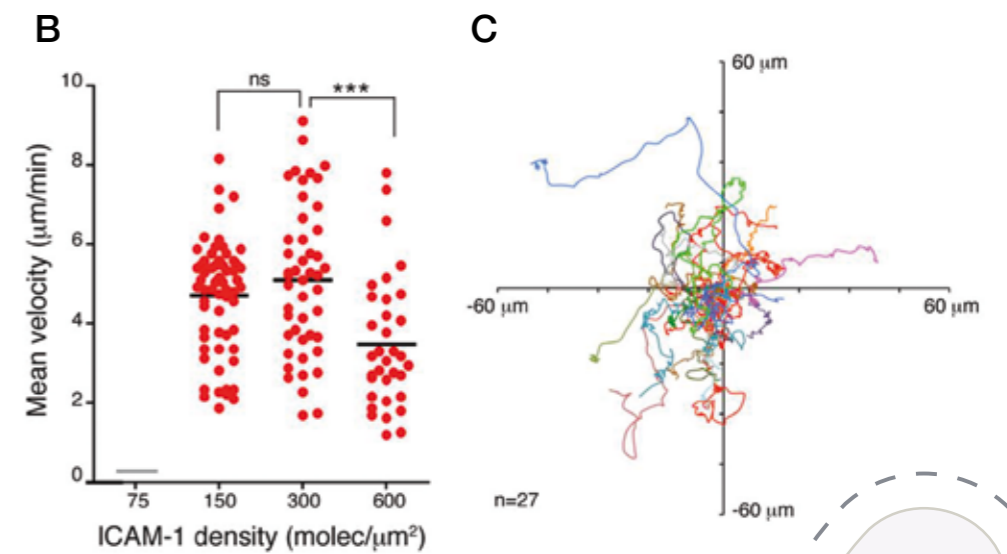
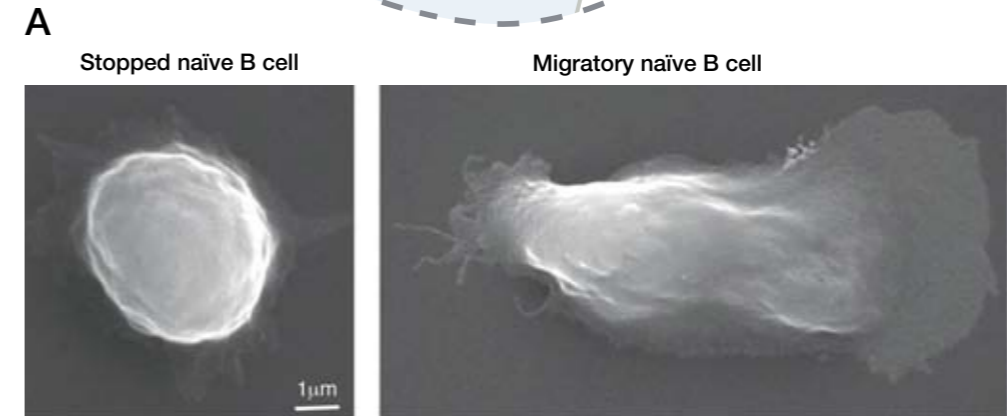
B Cell Dynamics

B cells are essential effectors of the adaptive immune response to pathogens.

They are responsible for pathogen neutralization and clearance through the production of antigen-specific antibodies. The prompt onset of the humoral immune response is thus crucial in the fight against invaders. This process depends critically on the ability of naïve B cells to search for antigen in secondary lymphoid organs (SLO).

Naïve B cells migrate incessantly seeking for specific antigen in SLO. Once they enter lymph nodes through the high endothelial venules, B cells move towards the follicles, guided by the chemokine CXCL13 and a network of stromal cells. B cells explore the entire follicular volume, moving by random walking at an average speed of 6 $\mu\text{m}/\text{min}$. CXCL13, produced mainly by follicular dendritic cells, underlies this B cell behavior by signaling through its receptor, CXCR5. Specific antigen recognition through the B cell receptor (BCR) alters steady-state B cell dynamics at the follicle. B cells stop to gather antigen into a central cluster at the site of contact with the antigen-presenting cell, establishing an immune synapse. Modulation of B cell dynamics thus becomes critical for shaping the process of antigen encounter and subsequent B cell activation.

To dissect the interplay between the BCR and CXCR5 in regulating B cell behavior, we established a two-dimensional model that allows study of CXCL13-mediated B cell migration and antigen encounter in real time (Figure 1). Our results identify a costimulatory function for CXCL13/CXCR5 signaling in BCR-triggered B cell activation by shaping cell dynamics. At limiting conditions of antigen density, naïve B cells establish an LFA-1-supported kinapse with the target membrane in response to CXCL13; through this migratory junction, they encounter antigen and integrate BCR signals. When antigen density is sufficient to trigger a stop signal through the BCR, naïve B cells establish a synapse with the target



*Two-dimensional model to study CXCL13-mediated migration and antigen encounter on naïve B cells. (A) Scanning Electron Microscopy images of two representative naïve B cells, in different dynamic stages (stopped vs. migratory), settled on ICAM-1-containing membranes coated with CXCL13. (B) Values for mean velocity and (C) tracks of migratory B cells on CXCL13-coated ICAM-1-containing membranes. Each dot in (B) corresponds to a single cell; ns, not significant; ***, $p < 0.0001$.*

membrane; CXCR5 signaling then promotes membrane ruffling and LFA-1/ICAM-1 contacts that increase antigen gathering near the synapse and thus, BCR signaling. Both mechanisms require a functional actin cytoskeleton and the activity of the motor protein non-muscle myosin-II. Based on our data, we also propose that B cells exploit both types of dynamic stages, kinapses and synapses, to integrate BCR signals; the use of one or the other will be determined mainly by antigen quality and abundance.

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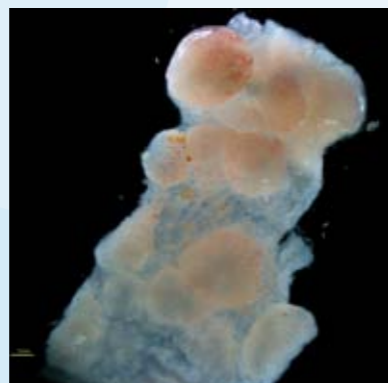
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PI3K in Cancer and Inflammation

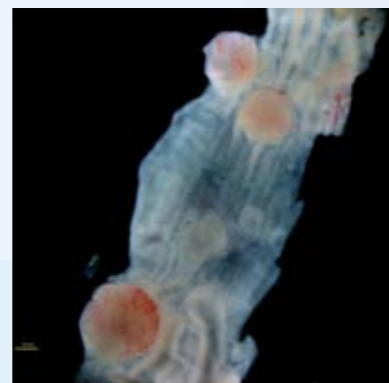
Two biological problems that have occupied the activity of our team (~10 –to-12 members): cancer and inflammation.

Our recent work is based in the assumption that the same biological activities that control physiological responses also control pathology when deregulated. The team is currently working in class I phosphoinositide 3-quinasa (PI3K) with special emphasis in the examining the specific function of each of the four class I PI3K isoforms in physiology and disease.

Reduced colon cancer upon interference with PI3K activation



Colon Carcinoma control mouse



Colon Carcinoma PI3K reduced

Starting with cancer we have recently showed that:

- (1) SADB kinase (that binds PI3K) controls centrosome duplication (*Nature Cell Biol.* 11, 1081-92, 2009).
- (2) PI3K ubiquitous isoforms PI3KCA and CB have different functions in cell division, PI3KCA regulates cell cycle entry and PI3KCB regulates DNA replication (*Mol Cell Biol.* 28: 2803-14,2008; *PNAS*106,7525-30,2009; *PNAS* 107: 7491-6, 2010).
- (3) Interference with PI3K reduces tumor formation *in vivo* (*Gastroenterology*, 138:1374-83, 2010).

These studies contributed to show that PI3K is a target for systemic lupus erithematosus and for cancer treatment and revealed an unexpected nuclear function for PI3KCB.

In Inflammation we previously showed that:

- (1) Activation of T cells *in vivo* (in the mouse) induced by deregulated PI3K activation triggers a Lupus-like disease similar to human Systemic Lupus Erythematosus (SLE) (*FASEB J.* 14,895-903, 2000).
- (2) Deletion or pharmacological inhibition of PI3KCG in the mouse inhibit Lupus (*J. Immunol*176:589-593,2006, *Nature Medicine*11:933-935, 2005).
- (3) PI3K activation is frequent in human SLE (*preliminary results in Nature Medicine* 11:933-935, 2005).

We recently showed that:

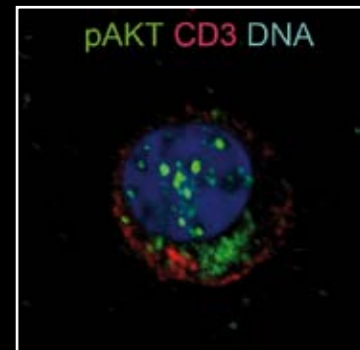
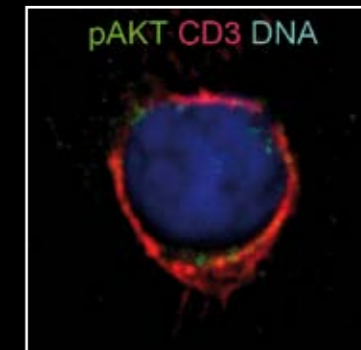
- (4) PI3K involvement in chronic inflammatory disease is at least partially due to its capacity to mediate memory T cell survival (*J. Exp Med* 204:2977-87, 2007; *Blood*,113:3198-208, 2009).

These studies contribute to show that hematopoietic PI3K isoforms are a target for chronic inflammatory disease treatment; we are currently studying human SLE.

Activation of the PI3K/AKT pathway in T cells from Systemic Lupus Erythematosus patients

T cell Control Individul

T cell Systemic Lupus



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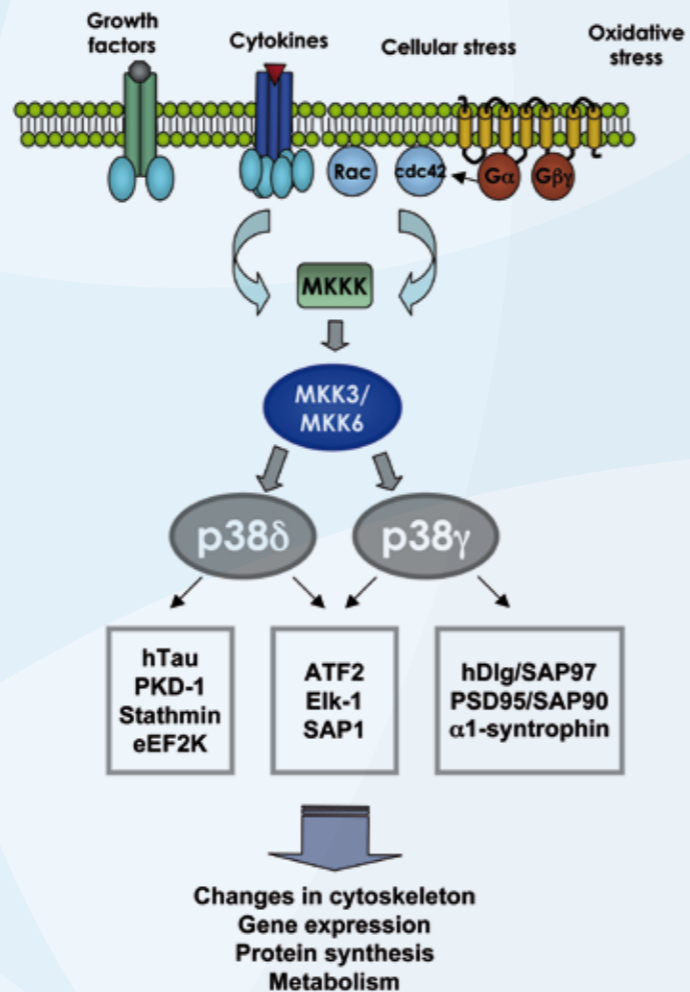
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Stress-Activated Protein Kinase: p38MAPK Signalling Pathways and their Role in Human Diseases

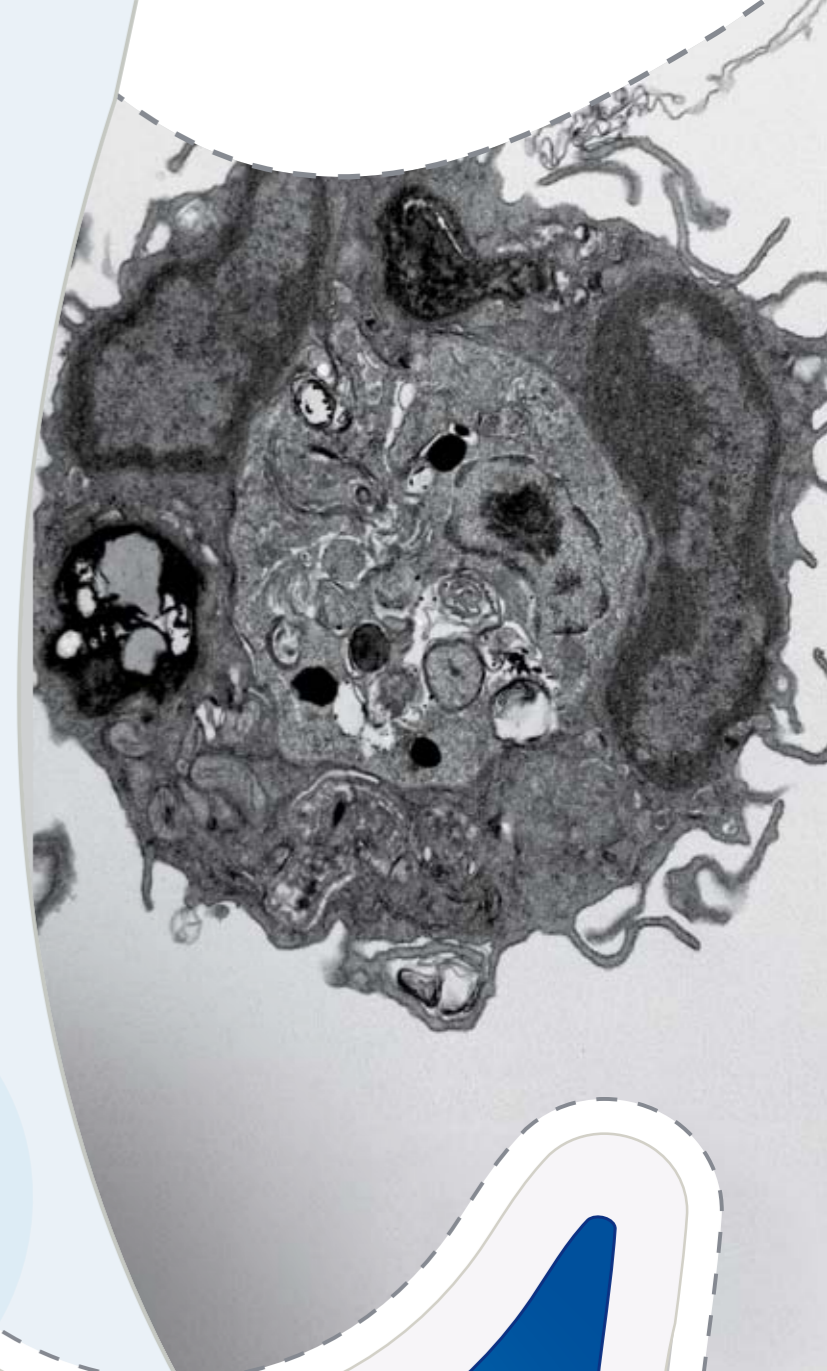
The aim of our group is both to discover how members of the p38MAPK family regulate cell function in physiological conditions and in response to environmental stresses, infection and proinflammatory cytokines, and to understand how they are deregulated in several human disease situations such as oncogenic transformation and inflammation.



Our research is focused on (1) the discovery of new substrates, interacting proteins and inhibitors for these kinases, and the study of their physiological roles using transgenic mice for the different p38 isoforms, and (2) the study of p38MAPK as a link between chronic inflammation and cancer, and as mediators of chronic inflammatory diseases. These studies utilize biochemical, cell biology as well as whole animal model approaches.

There are four members of the p38MAPK family (p38 α , p38 β , p38 γ and p38 δ), which are similar in amino acid sequence but differ in expression patterns, substrate specificities and sensitivities to inhibitors. In recent years, our group has centred on elucidating the regulation and roles of the p38MAPK family members p38 γ and p38 δ . We found that p38 γ interacts and is the physiological kinase of several PDZ domain-containing proteins. In particular, p38 γ interacts with and phosphorylates the tumour suppressor protein hDlg, regulating its association to the cytoskeleton and to nuclear protein-RNA complexes.

We are currently studying how p38 γ regulates the integrity of nuclear and intercellular-junctional complexes, cell adhesion, migration and polarity, as well as cell cycle and proliferation in response to many kinds of external stimuli. A large body of evidence indicated that p38 α MAPK activity is critical for production of proinflammatory cytokines, whose uncontrolled production is a major cause of chronic inflammatory diseases. Nonetheless, little is known about the role of p38 γ and p38 δ isoforms in these processes. We are currently undertaking further studies to study this, as well as their role in the development of cancer associated to inflammation, using the genetically modified mice we have generated.



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Inmunology Oncology

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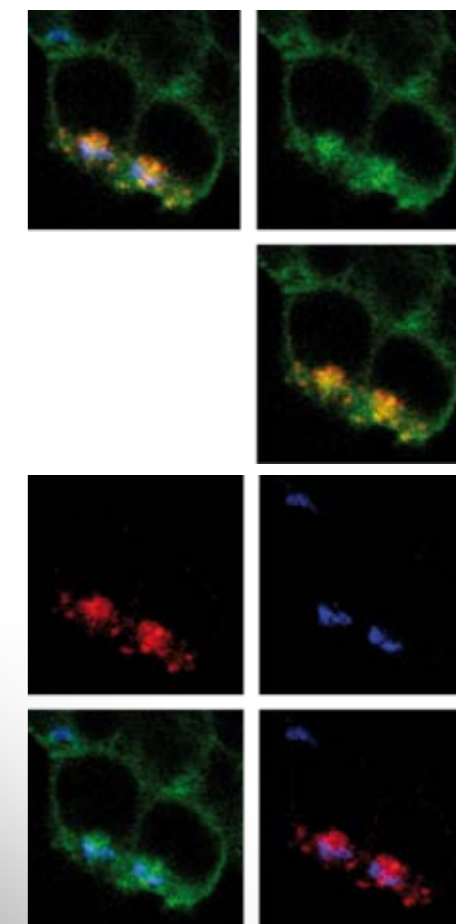
Chemokine-Mediated Cell Migration and Endocytosis

Chemokines are chemotactic cytokines that act through plasma membrane-tethered receptors.

They are involved in cell functions that include regulation of immune defense, as well as in tumor growth, atherosclerosis and asthma. Migration of immune cells from the blood into tissues is crucial in immune surveillance and host defense. In our group, we investigate the chemokine response of monocytic cells using cell biology and knock out animal models.

Cell migration is a complex biological function triggered by integrins, growth factors and chemokines. In order to evaluate the importance of membrane trafficking during cell migration towards a chemokine gradient, we monitored the number of the chemokine receptor CCR2 present at the cell surface. We found that cells that did not migrate needed higher concentrations of the chemokine CCL2 for the CCR2 internalization to occur. These data suggest that internalization occurs during chemotaxis and that non-migrating cells exhibit a reduced internalization. We manipulated clathrin/dynamin-mediated endocytosis to understand the molecular mechanisms implicated in CCL2-stimulated cell migration. Sucrose treatment of cells is described to interfere with endocytosis mediated by clathrin, a protein involved in transfer of material between cell organelles. We found that sucrose treatment impaired CCR2B internalization and migration of monocytic cells. When RNA interference was used to knock down clathrin, CCR2B internalization and transferrin uptake were prevented, as was cell migration. Dynamin is an endocytic pinchase implicated in the scission of vesicles. This function can be inhibited with the help of dynasore. When cells were treated with dynasore, we observed a reduction in cell migration. Our findings indicate that hindering membrane trafficking reflects in deficient cell migration. The relevance of membrane trafficking in the process of cell migration needs further evaluation using model of chemokinesis (wound assay) and live imaging techniques.

Cells expressing CCR2BmCherry (red) were CCL2-treated (150 nM; 5 min) and incubated on ice, then formaldehyde-fixed. Cells were labeled with antibodies to vesicular marker clathrin (green), the Golgi marker giantin (blue), followed by goat anti-rabbit AMCA and anti-mouse Alexa 488 labeled secondary antibodies. Images were obtained by confocal microscopy. Colocalization of receptor and clathrin is indicated (yellow), receptor and giantin (magenta) and clathrin and giantin (cyan). First panel represents the three labels. No overlap (white) was detected.



We have also found that the nuclear receptor RXR (Retinoid X Receptor) regulates the transcription of the chemokines CCL6 and CCL9 in vivo and in vitro. In the blood of mice lacking RXR in macrophages, we found lower levels of CCL6 and CCL9. When these mice suffer from peritonitis, they showed less inflammation and fewer leukocytes were attracted. In addition, these mice were less prone to sepsis and therefore our results put forward RXR as a target for treatment of inflammatory processes.

Our studies established CCR2 endocytic routes and their importance in monocytic cell migration. These insights into the molecular mechanisms of desensitization and signaling define potential targets for therapeutic intervention in diseases such as asthma and allergic inflammation.

In another collaborative work, we devised a method that assigns the topology of the membrane proteins using fluorescent proteins (FP). This so-called pH exchange assay (PEA) takes advantage of the pH sensitivity of GFP and YFP. Major advantages of the PEA are its technical simplicity, since it requires only imaging on a widefield or confocal microscope, its applicability to live, undisturbed cells, and the ability to quantify the proportion of inside/outside orientation for proteins with multiple topologies. We believe that this assay will be of interest to many groups working with membrane proteins.

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RXR α controls innate inflammatory responses through the upregulation of chemokine expression. V. Nuñez, D. Alameda, D. Rico, P. Gonzalo, R. Mota, T. Fischer, L. Boscá, C. Glass, A. García-Arroyo and M. Ricote (2010). *Proc. Natl. Acad. Sc. USA* 107:1026.

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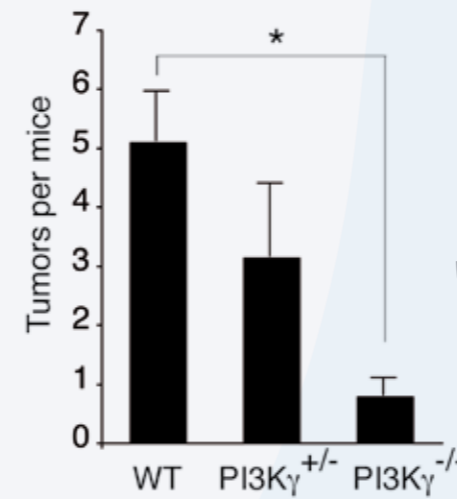
The Role of Ras Effectors in Inflammation and Cancer

The Ras GTPase is mutated in approximately 15% of human tumours, and these mutations are especially frequent in lung, colon and pancreatic carcinomas.

In normal cells, Ras participates in several biological responses, regulating cell cycle progression, migration, differentiation and cell survival as well as immune system development and function. Ras mediates these functions through the activation of three effector pathways, Raf/MAPK, PI3K and the Ral GTPases. The focus of our research is to analyse the contribution of these Ras effectors to several aspects of inflammation and tumour development.

We analyse the role of Ral GTPases in the immune system using genetically modified mice and biochemical tools to knock down the expression of these GTPases. In cytotoxic cells, both Ral isoforms, RalA and RalB, are activated rapidly after target cell recognition, and translocate to the cell-cell contact zone (Fig. 1). A critical step in cell-mediated cytotoxicity is the directed secretion of lytic granules at the immunological synapse, by which lytic molecules are delivered specifically to the target cell, and neighbouring cells are protected from damage. Ral GTPases have been shown to participate in polarized secretion in different cell types; we therefore study Ral function in the regulation of cell-mediated cytotoxicity.

A large body of evidence now supports a correlation between inflammation and cancer, although the molecular mechanisms that govern this process are poorly understood. This correlation is particularly clear in patients with inflammatory bowel disease, who have an increased risk of developing colorectal cancer. We use a murine model of inflammatory bowel disease to dissect the contribution of a PI3K isoform, PI3K γ , to inflammation-associated colorectal cancer. We found that PI3K γ -deficient mice had lower



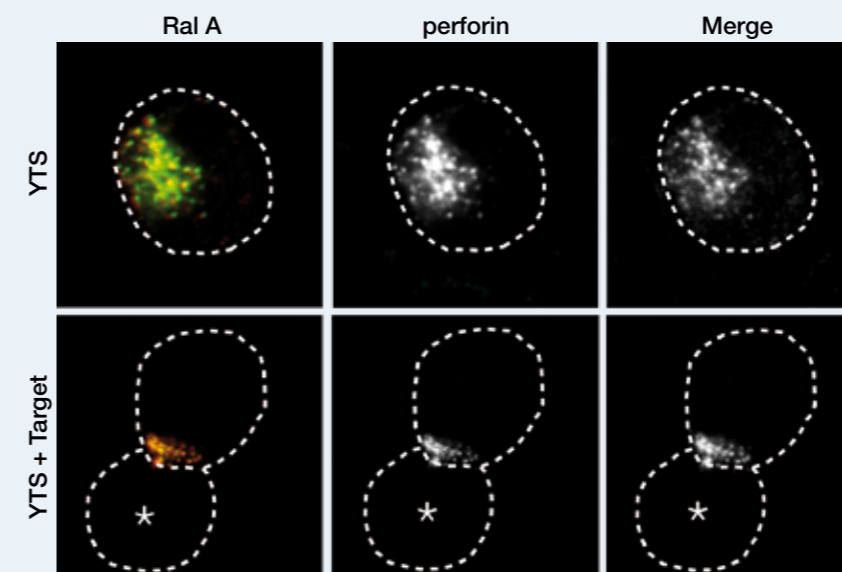
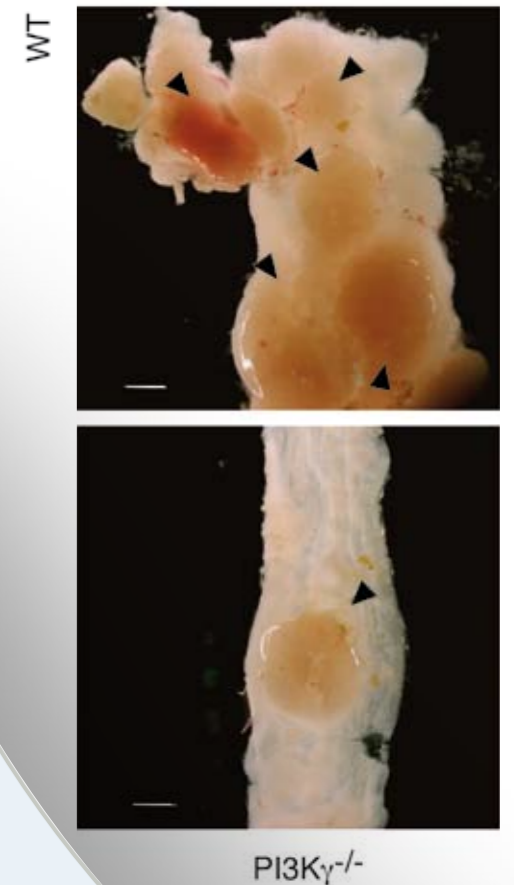
incidence and multiplicity of tumours than control mice (Fig. 2). PI3K γ ^{-/-} animals also showed a reduction in colon inflammation levels, with defective activation and infiltration of myeloid cells and, in consequence, defective recruitment of T cells to the colon. These data suggest that PI3K γ ameliorates inflammation-associated cancer by modulating the immune response.

By understanding the molecular mechanisms that regulate different steps during tumour formation, we can contribute to finding potential targets for the development of new cancer treatment therapies.

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Representative images of colons from WT and PI3K γ ^{-/-} mice showing tumours in the distal region. Quantification of tumour multiplicity is shown (right).



Ral localise to lytic granules in YTS cells. YTS cells were incubated alone or with 721.221 target cells at a 1:4 ratio (20 min), fixed, stained with anti-RalA (green) and anti-perforin (red) and analysed by confocal microscopy. *denotes 721.221 target cells.



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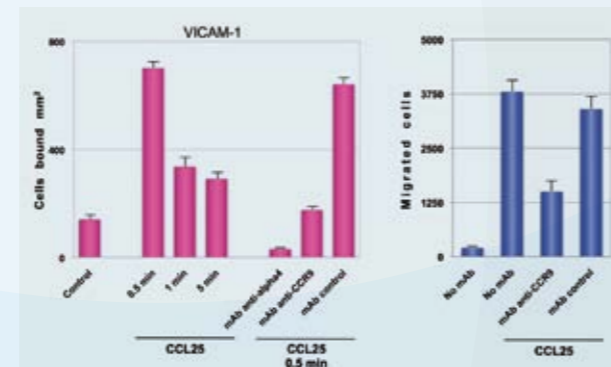
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Left, CCL25-stimulates the T cell adhesion mediated by $\alpha 4\beta 1$ integrin. BCECF-AM-labeled MOLT-4 T cells were treated with anti- $\alpha 4$, anti-human CCR9 or control mAb, incubated for the stated times alone or with soluble human CCL25, and tested in adhesion assays to VCAM-1. Right, Anti-human CCR9 mAb blocks migration of MOLT-4 T cells in response to CCL25. Chemotaxis assays were performed in transwell chambers, control or mAb-treated cells were added to the transwell inserts and placed in wells containing 200 nM CCL25. Following a 2 h incubation, cells migrating through the membranes into the lower wells were harvested and counted by flow cytometry.

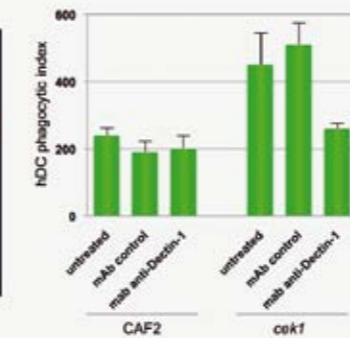
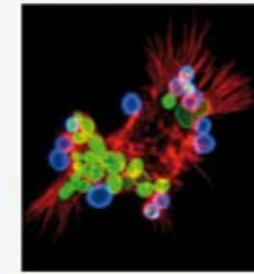
Chemokine Receptors in
Cancer Biology

Our main research interest is to understand how chemokines mediate the interaction between tumor cells and their microenvironment, and how they participate in the control of tumor growth and progression.

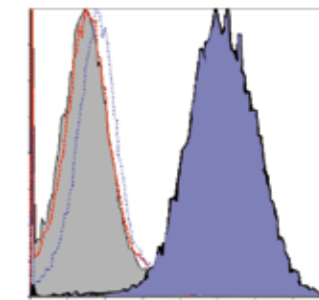
We study the contribution of chemokine receptors to cancer cell physiology, and evaluate their potential as antitumor targets.



Chemokines are small proteins that bind to G protein-coupled receptors and regulate leukocyte migration during homeostasis, inflammation and infection. Chemokine action extend to all cell types, however, and appears to be important in defining organ-specific metastatic destinations during cancer spread. In some solid tumors, levels of the chemokine receptors CCR7 and CXCR4 correlate with cell dissemination in lymph nodes or other sites; CCR6 and CX3CR1 expression is related to cancer cell homing to specific organs, suggesting chemokine receptors as promising therapeutic targets. The chemokine receptor CCR9 is expressed primarily on thymocytes and in a small subset of intraepithelial lymphocytes; its only natural ligand, CCL25, is expressed mainly in thymus and the small bowel. Recent reports showed that CCR9 overexpression increases the migratory and invasive capacity of



Left, phagocytosis and killing of *Candida albicans* by human monocyte-derived dendritic cells (hDC). Confocal microscopy images of dendritic cells showing extracellular yeast cells (blue), internalized yeast cells (green), and actin (red). Right, the fungal phagocytic indices of hDC exposed to wt CAF2 and the *cek1* deletion mutant were measured by differential immunofluorescence labeling. The phagocytic index was assessed by counting the number of internalized yeast cells per 100 phagocytes, after nontreatment or treatment of primary cells with isotype control mAb or the anti-dectin-1 MGD3 mAb.



Anti CCR9 mAb recognize specifically the CCR9 chemokine receptor in stably transfected HEK 293 cells. Human CCR4, CCR5, CCR9 or empty vector transfected cells were stained with anti-hCCR9 mAb, followed by PE-conjugated anti-mouse immunoglobulin antibodies and analyzed by flow cytometry.

prostate cancer cells, directs ovarian cancer and melanoma cell metastases to the small intestine, and increases proliferation of and resistance to apoptosis by acute lymphoblastic leukemia-derived cell lines. CCR9-mediated intracellular signaling activates the anti-apoptotic PI3K, Akt, PTEN, mTOR, ERK1/2 and GSK-3 β pathways, and downregulates activation of caspase-3, leading to survival and increased cell proliferation.

Our group is focused in determining the role of CCR9 in tumor physiopathology. We have generated anti-human CCR9 monoclonal antibodies (mAb) to characterize and follow CCR9 expression by flow cytometry, Western blot and immunomicroscopy, and for blocking receptor signaling. Our neutralizing anti-CCR9 mAb are being used to evaluate the CCR9 participation in tumor cell cycling, survival, migratory and invasiveness using human tumor lines, and in tumor progression and metastasis in xenogeneic mouse models. We use quantitative real-time PCR and immunohistochemistry to determine whether antibody action produces changes in mRNA and protein levels. CCR9-expressing human carcinoma cell lines and RNA interference approaches are also used to study the molecular mechanisms that underlie CCR9-mediated effects. In addition, we will test the usefulness of these mAb for diagnostic imaging and for antagonist screening of low molecular weight compounds for drug development.

We work in collaboration with Drs. Gabriel Márquez (Genetrix), Laura Carramolino (CNIC), Elena Fernández-Ruiz (Hospital La Princesa, Madrid), Joaquín Teixido (CIB/CSIC), Julio Gutiérrez, Ricardo Villares and Carlos Martínez-A (CNB/CSIC).

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Inmunology
Oncology

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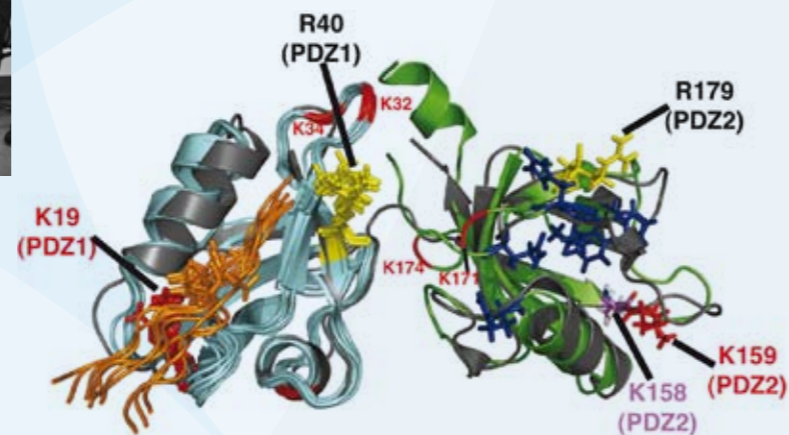


Signalling Networks in Inflammation and Cancer

Inflammation is a complex stereotypical response that is essential for effective defence of the organism against harmful stimuli such as pathogens, irritants or tissue damage.

Discovery of the detailed processes of inflammation has revealed a close relationship between the inflammatory reaction and the immune response. Indeed, a hallmark of inflammation is the directed migration (chemotaxis) of inflammatory cells (mostly leukocytes) through blood vessel walls to the site of injury. Once wound healing is complete, inflammation resolves and tissue homeostasis returns. Nonetheless, a deregulated response to tissue damage might lead to autoimmunity and chronic inflammatory diseases, and can also promote cancer.

Recent clinical and experimental evidence indicates that solid tumours exacerbate inflammation to promote their own progression. This leads to a tumour microenvironment largely orchestrated by inflammatory cells, altering the metabolic needs of the tissue and fostering neoangiogenesis, proliferation, survival, mutagenesis, migration and metastasis of malignant cells. Tumour-induced inflammation usually leads to immunosuppression, impeding the immune system surveillance function and clearance of the tumour; indeed, breaking immunosuppression has been demonstrated as a useful, efficient way to eradicate cancers. Immune cells might therefore provide both anti- and protumorigenic signals, which could be harnessed or attacked for therapeutic purposes.



We aim to identify and understand key molecules/pathways responsible for the aberrant inflammatory reaction involved in the development or outcome of inflammation-associated pathologies. Our research projects focus on distinct steps of this reaction:

- (i) Study at the cellular level of key signalling pathways that regulate acquisition of a motile phenotype in leukocytes
- (ii) Understand the role of specific chemokines/chemokine receptors in orchestrating the activation of the adaptive immune response
- (iii) Identify key regulators of terminal differentiation in innate immune cells within the inflammatory environment
- (iv) Examine the relevance of the vascular system in controlling inflammatory migration of specific leukocyte subtypes

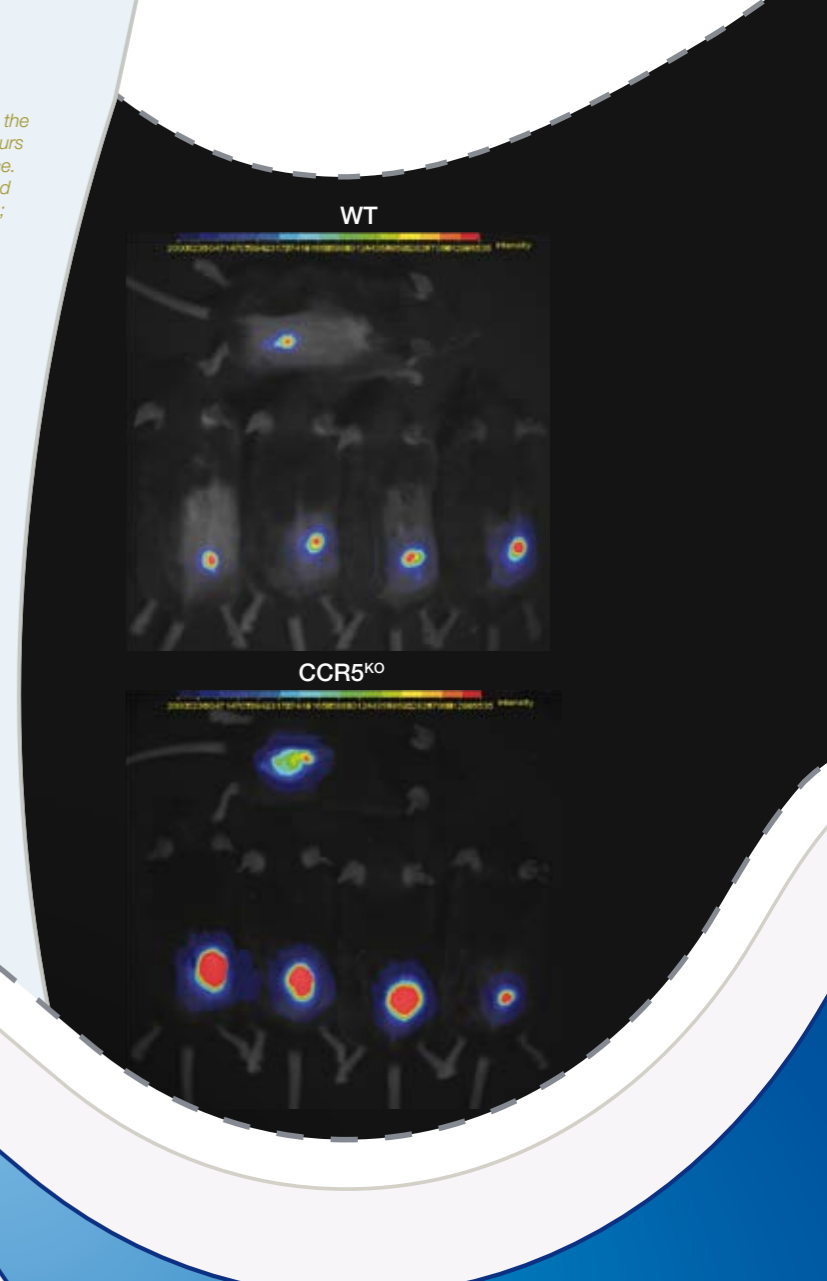
We hope to understand critical cellular, molecular, and chemical mediators through which tumours promote inflammation and subvert the immune system to favour their progression. Comprehension of the mechanisms that balance pro- and anti-tumour immunity could lead to the design of more effective anti-cancer therapeutics.

Although the major focus of our research is the inflammatory reaction to cancer, we are also interested in understanding the chronic inflammation associated to autoimmune diseases.

Legend:
 ■ syntein ■ EBP50-PDZ1 Sol. 1 ■ EBP50-PDZ2
 ■ PIP5Kβ C-terminal peptide

Model for the binding of the PDZ-binding motif identified in the Type1 PIP5K-beta isoform, with the tandem EBP50-PDZ domains, based on syntein structure. It is indicated the critical residues on EBP50 involved in binding to PIP5K.

Bioluminescence images showing the differential growth of pancreatic tumours in wild-type and CCR5-deficient mice. Luciferase activity was measured and transformed to a pseudocoloured image; red represents maximal light intensity.



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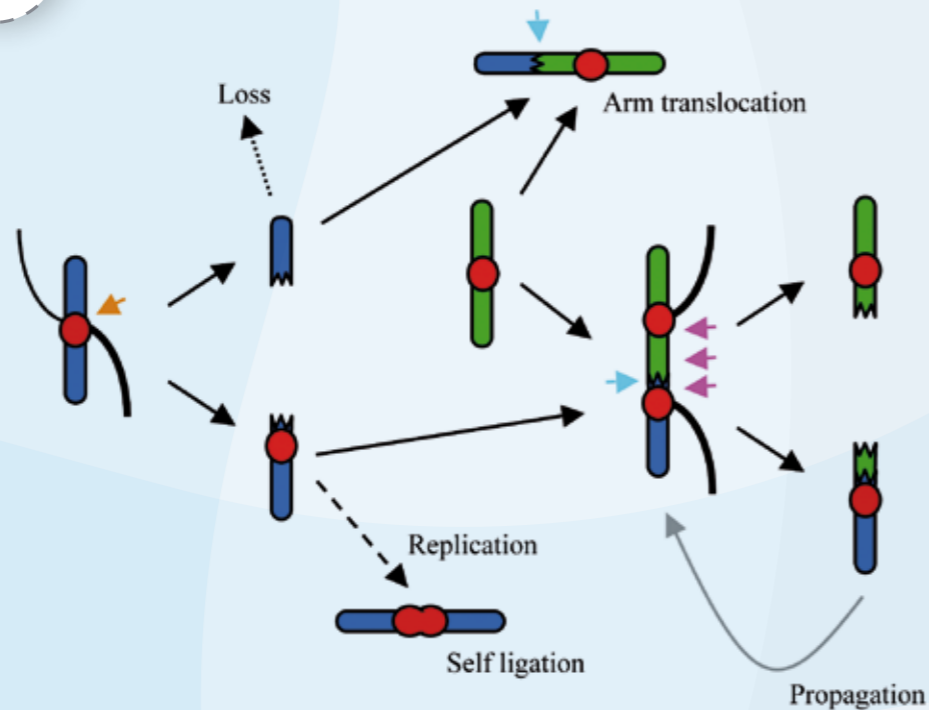
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Linking Centrosomal Aberrations to Chromosomal Instability

Our group studies the link between cell division and genomic instability, with a special interest in the death inducer obliterator (Dido) protein family.



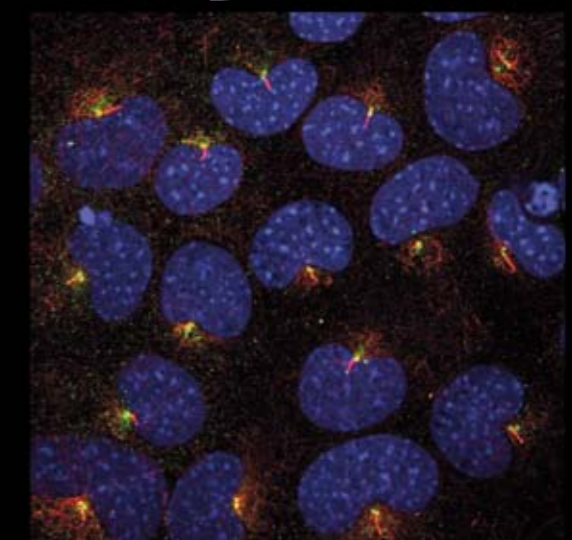
Model of centromere fission in chromosomal instability.

After Dido3 was found in the nucleus and centromeres from mitotic cells some years ago, we recently showed that Dido3 is associated with the synaptonemal complex in meiosis [1]. This observation points at a general role for Dido3 in cell division and chromosome maintenance. The localization of Dido3 in the synaptonemal complex is regulated by epigenetic modifications that are recognized by a histone-interacting domain in the protein's aminoter-

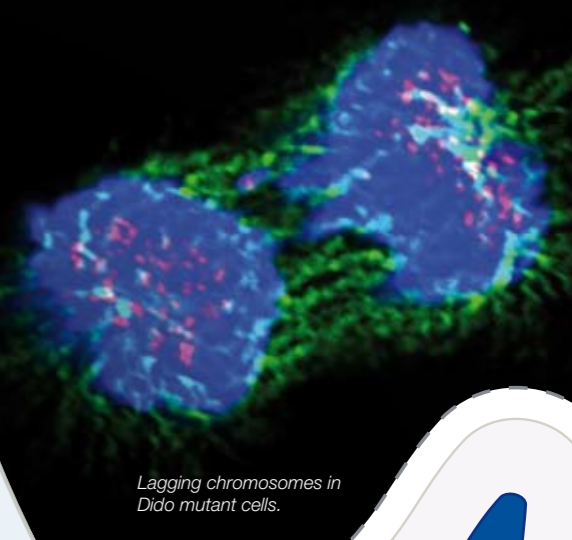
minus. A similar mechanism might govern the distribution of Dido3 between the nucleus and centromeres in mitotic cells. After having identified of the determinants for Dido3 localization, we plan to elucidate the mechanism by which Dido3 regulates target proteins in the centrosome and synaptonemal complex.

In addition to the role of Dido proteins in cell division, we are interested in the origins of genomic instability in sporadic carcinomas. The most common type of genomic instability in solid tumors is chromosomal instability (CIN), in which chromosome number changes occur together with segmental defects. This means that changes involving intact chromosomes accompany breakage-induced alterations in CIN tumors. Whereas numerical alterations are attributed to chromosome missegregation, the origins of breakage in CIN tumors remain disputed. Recently, we proposed a model of chromosome breakage based on spindle defects and kinetochore distortion [2]. Using *Dido* gene mutants, we obtained the first evidence of spindle-generated chromosome breakage [3]. Our data showed that reduced control over the mitotic spindle not only causes losses and gains of intact chromosomes, but also produces kinetochore distortion and shearing of kinetochore-associated chromatin.

Spindle-controlled shearing of kinetochore-associated chromatin, a phenomenon we termed centromere fission, occurs during mitotic chromosome segregation. Broken chromosomes generated by centromere fission are therefore separated from their counterparts, frequently end up in micronuclei, and are repaired when recombination is nearly inactive. As a consequence, chromosome arms containing centromeric breaks often fuse to healthy chromosomes, in a process that depends on non-homologous end joining [4]. A classification of genetic aberrations showed that centromere fission and capture of healthy chromosomes is the mechanism that best explains genomic instability in sporadic carcinomas. Our future work on chromosomal instability will be directed at elucidating the details of the mechanisms that underlie centromere fission and the identification of centromeric fusion products in cancer cells.



Ciliogenesis in Dido mutant cells.



Lagging chromosomes in Dido mutant cells.

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Chemokine Receptors: New Targets for Therapeutic Intervention

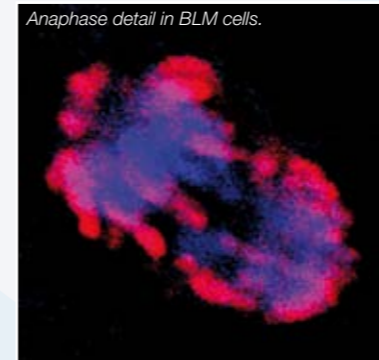
Since the first reports on chemokine function, much information has been generated on the implications of these molecules in numerous physiological and pathological processes, as well as on the signaling events activated through their binding to receptors.

Despite these extensive studies, no chemokine-related drugs have yet been approved for use in patients with inflammatory or autoimmune diseases. This discrepancy between efforts and results has forced a re-evaluation of the chemokine field.

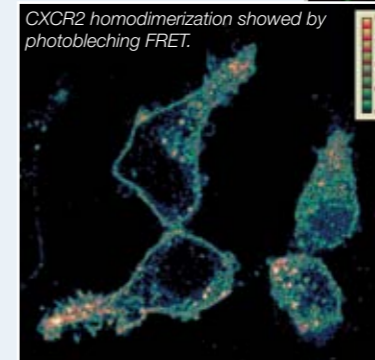
Using classic biochemical techniques and new methodologies based on energy transfer between fluorophores (FRET), we have explored chemokine receptor conformations at the cell surface and found that, as is the case for other G protein-coupled receptors, chemokine receptors are not isolated entities that 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 organized arrays that can be modified by receptor expression and ligand levels, indicating that they are dynamic structures.

Clusters of chemokine receptors are expressed at the cell surface. It is thus plausible that receptor dimers organize in such clusters, like bundles of cigars. Ligands then modulate and stabilize specific receptor conformation to trigger functional responses without disrupting cell surface receptor arrays. Ligand-mediated internalization of a given receptor pair does not necessarily alter the levels of other receptors in the 'bundle'. It is nonetheless possible that the conformation of resting receptors in an array might be affected by ligand binding to a responding receptor. Such 'allosteric' conformational changes might not be restricted

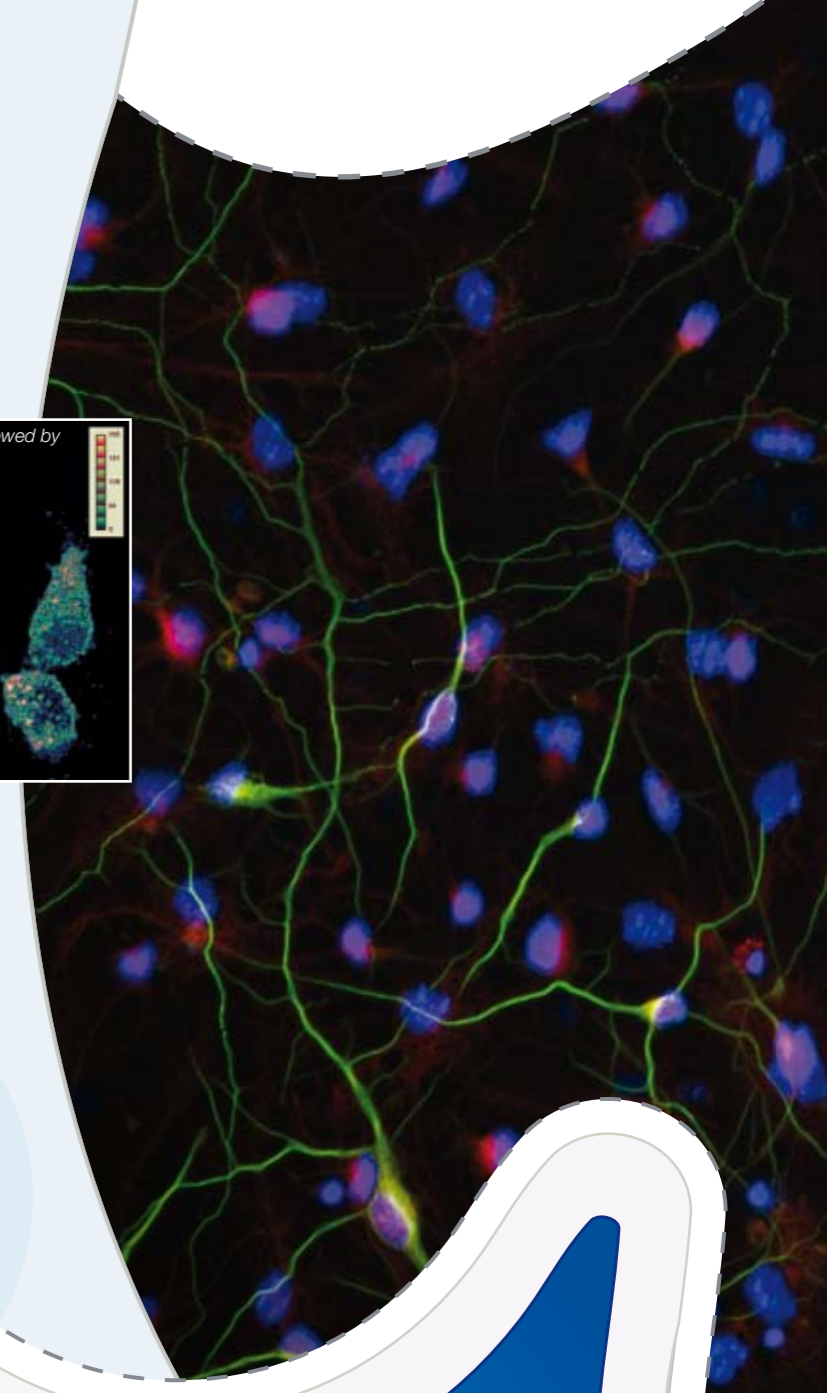
Anaphase detail in BLM cells.



CXCR2 homodimerization showed by photobleaching FRET.



Neural stem movement.



to neighboring dimers, but might extend through the array in a domino effect.

The chemokines also activate a tyrosine kinase pathway that shares many components with the biochemical pathway activated by the cytokine receptors. We have reported that chemokines activate the JANUS kinases (JAK), which associate to the chemokine receptor and promote its rapid tyrosine phosphorylation. Through the JAK/STAT pathway, the chemokines trigger suppressor of cytokine signaling (SOCS) expression. The SOCS intracellular proteins are thus key physiological regulators of cytokine and chemokine responses, SOCS proteins regulate signal transduction by binding directly to JAK or by competing with STAT for the phosphorylated receptor; in addition, they target ubiquitinated signaling intermediates for degradation by the proteasome pathway through ECS (elongin-Cullin-SOCS) E3 ligase formation. As consequence of this last effect, SOCS1 function as a tumor suppressor blocking cell cycling in human melanoma by affecting G1/S and mitosis.

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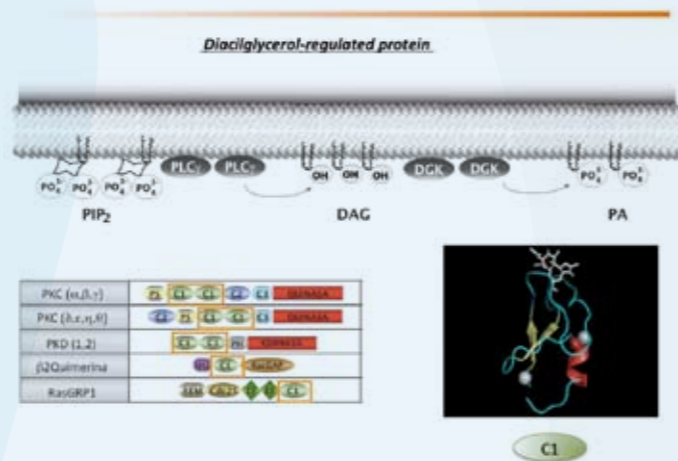
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Lipid Signalling

Diaclycerol (DAG) is a lipid with unique functions as a basic membrane component, as a lipid metabolism intermediate and as a signaling molecule.



DAG regulates C1 containing proteins. Top. DAG metabolism at the membrane. Bottom left. C1 domain containing proteins. Bottom right. Ribbon model of PLCdelta C1 domain

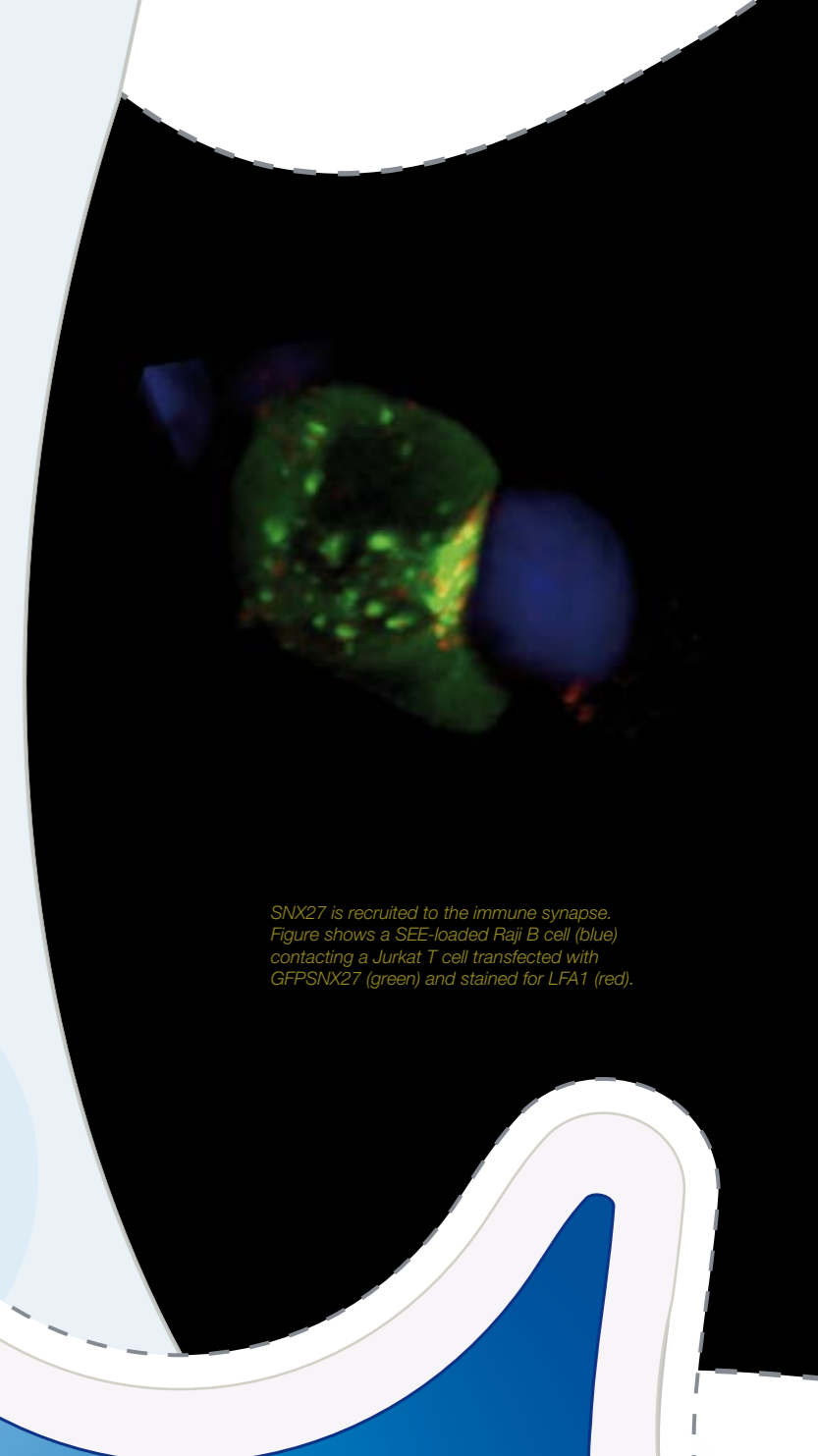
In eukaryotes, a host of proteins have evolved the ability to bind to DAG and are thus modulated by this lipid, creating additional levels of control to meet the complex needs of multicellular organisms. DAG-regulated proteins participate in neuronal and vascular patterning, synapse transmission, glucose transport and are critical for the correct immune response. Altered DAG functions are related to transplant rejection, inflammation, diabetes, allergy and autoimmune disease. Sustained DAG generation, on the other hand, is associated with malignant transformation. The complexity of DAG-regulated processes emphasizes the need for studies aimed to evaluate the potential therapeutic manipulation of DAG generation and clearance.

Our group studies the contribution of DAG-regulated mechanisms to T cell activation and oncogenic transforma-

tion, so that steps of these processes can be manipulated for possible therapeutic benefit. Our final goal is to demonstrate that modification of DAG metabolism represents a novel and understudied strategy directed to the management of a more effective immune response and/or treatment of cancer.

We investigate the mechanisms by which DAG binds and regulate C1 domain-containing proteins. In addition to the well characterized PKC family, vertebrates express six additional families of DAG regulated proteins: chimaerins, DGK (beta and gamma), PKD, Munc13, RasGRP and MRCK. The specific expression in T cells of C1-containing GEFS and GAPS for small GTPases of the Ras and Rho family has uncovered new and strategic functions for DAG in the regulation of Ras and Rac. We study the spatial generation of DAG using fluorescent DAG sensors. Studies from our laboratory have also provided new insight into the mechanisms that govern DAG-mediated regulation of RasGRP1 and β2 chimaerin during T cell activation.

Another important area is the study of Diacylglycerol kinases (DGK), which transform DAG into phosphatidic acid, and represent important modulators of DAG-dependent functions. We use biochemical and genetic approaches to better understand how antigen mediated stimulation determines membrane localization/activation of DGKα and ζα, their site of activation and the nature of the interacting partners. We also explore the role of these isoforms in the maintenance of transformed state through regulation of the PI3K/MTOR pathway. We expect that our findings will contribute to assess the therapeutic potential of the DGK enzyme family as tools for a better and more effective management of the immune response and treatment of cancer.



SNX27 is recruited to the immune synapse. Figure shows a SEE-loaded Raji B cell (blue) contacting a Jurkat T cell transfected with GFP-SNX27 (green) and stained for LFA1 (red).

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**Function of the
c-Myc Proto-Oncogene
*in vivo***

At present, with 1.7 million deaths each year, cancer represents the second most important cause of death in Europe.

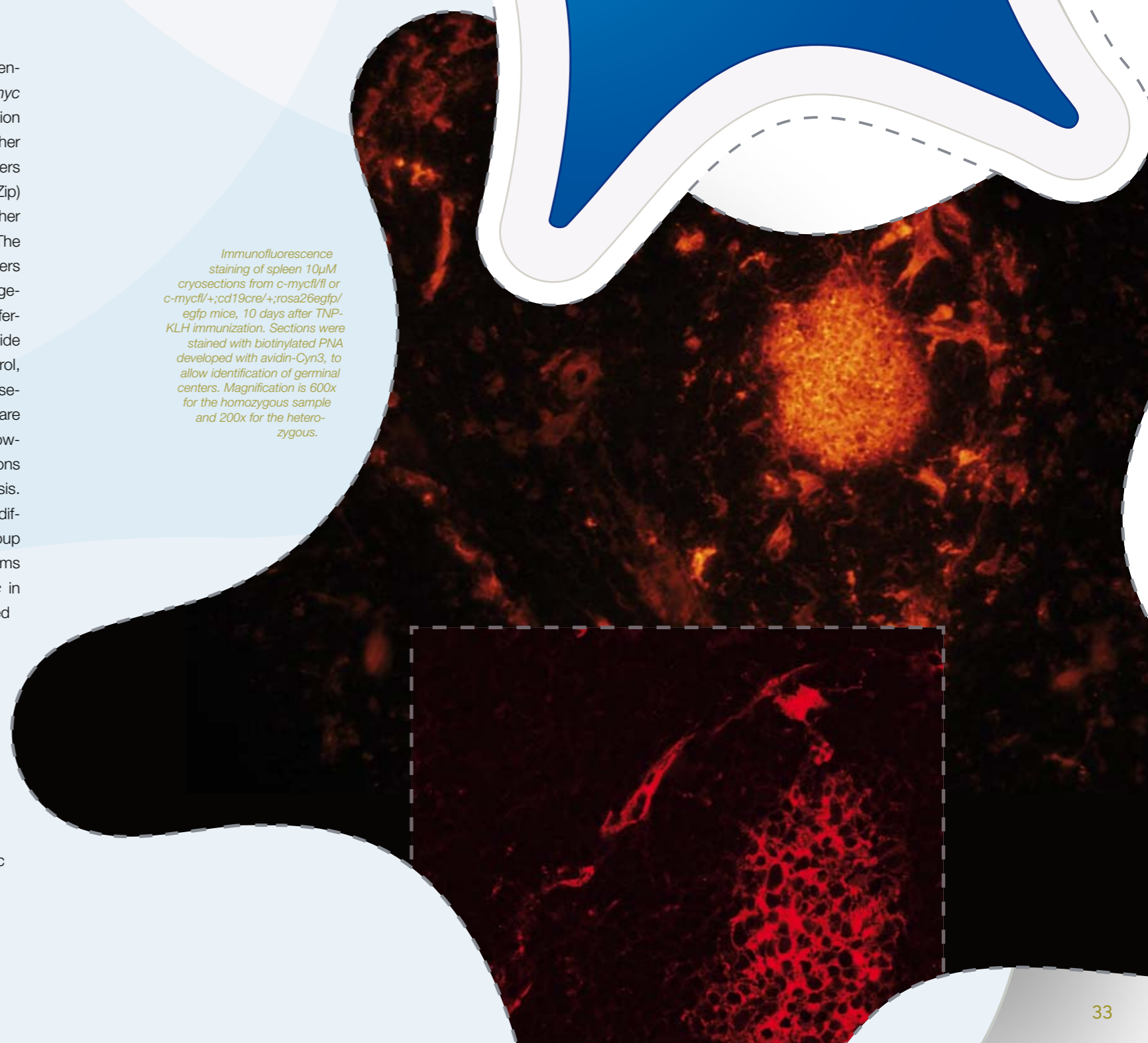
In up to 50% of all human cancers, constitutively enhanced expression of proto-oncogenes of the *myc* family is a characteristic signature. *Myc* deregulation is due to rearrangements or other mutations in either one of the three *myc* genes. Myc proteins are members of a basic region/helix-loop-helix/leucine zipper (bHLHZip) transcription factor family (N-, L- and c-Myc) that can either activate or repress expression of their target genes. The study of Myc function is a complex task. Myc members have been shown to bind to several thousand loci in the genome of humans and mice and regulate a variety of different genes. This large number of Myc targets affects a wide range of biological processes such as cell cycle control, apoptosis, protein synthesis, energetic metabolism, senescence, cell polarity and cell differentiation, which all are known to play a role in human cancer development. However, it remains to be determined which of these functions are the most relevant in Myc-dependent tumorigenesis. Among all these Myc functions, the role of c-Myc in cell differentiation is poorly understood. In this context, our group is interested in understanding the molecular mechanisms that mediate the action of the proto-oncogene *c-myc* in cell differentiation. With this aim in mind, we have focused our efforts to address this question in a well-defined setting *in vivo* such as B lymphocyte differentiation. In recent years, we have developed several conditional mouse models to inactivate c-Myc at different developmental stages in B lymphocytes. These models have proved to be very useful to place c-Myc in the context of the transcription factors necessary for B lymphocyte differentiation. Finally, we expect to translate all this knowledge to pathological situations caused by deregulation of c-Myc expression in B lymphocytes.

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Immunofluorescence staining of spleen 10µM cryosections from c-mycfl/fl or c-mycfl/+;cd19cre/+;rosa26egfp/egfp mice, 10 days after TNP-KLH immunization. Sections were stained with biotinylated PNA developed with avidin-Cy3, to allow identification of germinal centers. Magnification is 600x for the homozygous sample and 200x for the heterozygous.



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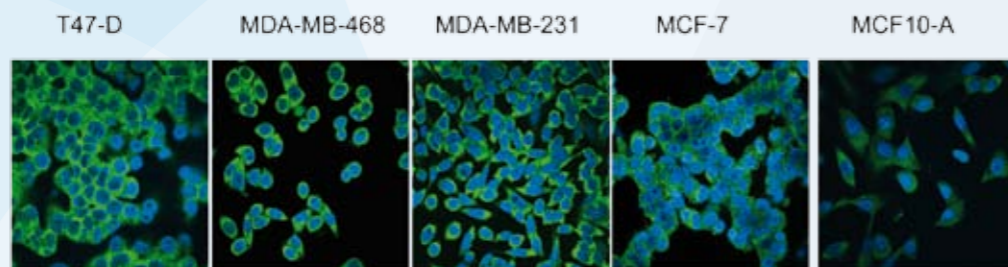
Function and Regulation of APRIL, a TNF Protein: Implications in Pathology

We focus on the study of APRIL (a proliferation-inducing ligand), a member of the TNF family proteins named for its ability to stimulate the proliferation of tumour cells *in vitro*.

APRIL binds to two known TNF receptors, TACI and BCMA. In addition, APRIL binds to heparan sulphate proteoglycans (HSPG), although the biological significance is not yet clear. APRIL is expressed by several cell types (dendritic cells, macrophages, epithelial cells, osteoclasts) and secreted as a soluble factor.

APRIL is known to enhance B cell proliferation and cell survival; it also enhances T-independent humoral responses and promotes immunoglobulin class-switch recombination to IgG and IgA. Altered APRIL expression has been detected in pathological situations such as autoimmunity and cancer. We described that APRIL transgenic mice develop lymphoid tumours that originate from the expansion of peritoneal B cells. Tumours in these mice resemble human chronic lymphocytic leukaemia (CLL), and our analysis of CLL patient sera shows an increase in circulating APRIL levels that correlates with reduced overall survival. In these

Human breast cancer cells express APRIL protein. APRIL expression (green) and DAPI (blue) by confocal microscopy in a panel of breast cancer cell lines (MDA-MB213, MDA-MB468, T47D and MCF7) and control cells (MCF10A).

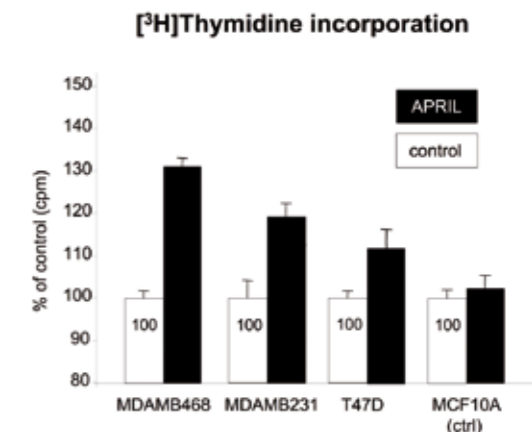


B cell malignancies, APRIL activates NFκB transcription factor, promotes tumour cell survival and protects cells from apoptosis.

Our main goal is to dissect APRIL function in the immune system and in pathological conditions. One research line studies the relevance of APRIL in epithelial breast cancer, using cell lines and mouse models. We detected high APRIL mRNA levels in 30% of the human primary breast tumours analysed and found that APRIL protein is expressed in various human breast cancer cell lines and promotes their proliferation. We identified molecules that stimulate APRIL secretion in these cancer cells and characterised the signalling transduction pathways activated by the cytokine. We also generated the MMTV-neu/APRIL double transgenic mouse and used syngeneic tumour transplant models in APRIL-Tg and APRIL-KO mice to study the influence of APRIL in epithelial tumour development *in vivo*. We are also exploring the potential clinical use of APRIL and analysing its expression in human solid primary tumours.

Another line of research uses mouse models and human samples to focus on autoimmune diseases (RA, SLE) and B cell deficiencies (XLA). We are examining the cell types responsible for APRIL/BAFF secretion and the factors that regulate it, the effects mediated by APRIL/BAFF, the target cells involved and the signalling pathways activated.

Finally, we are working on the generation of APRIL antagonist molecules that effectively block this cytokine as a strategy for blocking tumour development.



APRIL promotes proliferation of breast cancer cells. Control and cancer cells were cultured with APRIL (black) or alone (white) and a thymidine incorporation assay was performed. Graphic shows the percentages of proliferation normalized to the cpm of unstimulated cells (100%).

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PATENT

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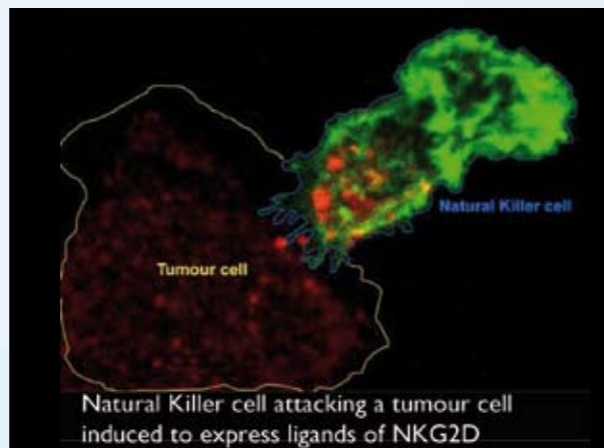
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**Receptor-ligand interactions
in immune responses to
cancer and viruses**

Current research in the laboratory addresses various issues related to the biology of NK cells and in particular the receptor NKG2D.

Some of topics of investigation represent the continuation of projects ongoing in the lab, before the move from Cambridge in October 2008, while others are new projects begun in the CNB:



Deposition of NKG2D containing lytic granules from an NK cell onto a tumour cell induced to express ligands of NKG2D.

Traffic and function of the NKG2D receptor

We have identified amino acids in the cytoplasmic tail of NKG2D and DAP10 that regulate internalisation of the receptor complex, and are now analysing how receptor recycling alters the threshold for signalling.

We have described how NKG2D/DAP10 receptor complexes polarise to the cytotoxic immune synapse in secretory lysosomes/lytic granules. We are now studying how the presence of this receptor complex in the lytic granules affects the fusion of the granules with the target cell membrane for delivery of the lethal hit.

We, and others, have described that chronic interactions with NKG2D-ligand-expressing target cells produces NK cell exhaustion/anergy. We are now analysing the molecular basis of this defect.

The use of human cytomegalovirus as a tool to study the regulation of expression of NKG2D ligands

We have a longstanding interest in studying the interactions between viruses and cells of the immune system as a strategy to gain insight into functionally important features of the immune system. We have noted that infection with human cytomegalovirus (HCMV) induces high levels of shedding of NKG2D ligands, and are characterising the biochemical basis of this effect *in vitro*. We have also gone on to show that the NK cells and CD8⁺ T cells of patients with active CMV-related disease have markedly reduced levels of NKG2D receptor expression *in vivo*, and are now working to understand the mechanisms underlying this phenomenon. These experiments are being done in collaboration with groups in the university teaching hospitals Gregorio Marañón and 12 de Octubre in Madrid, and we are beginning to collaborate with investigators in other regions of Spain.

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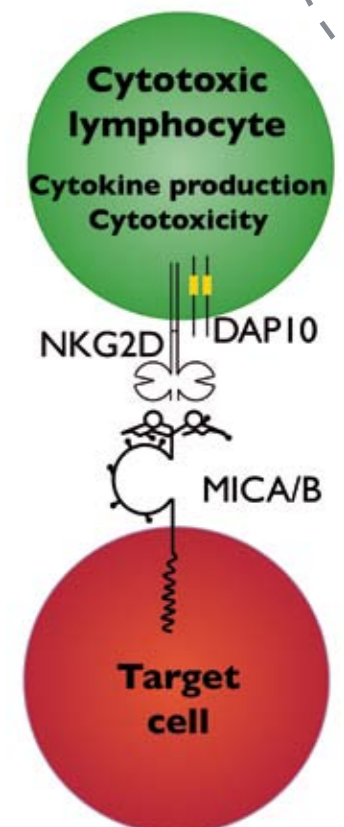
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The roles of NKG2D in regulation of the immune response

- 1) Immune surveillance of tumours
- 2) Autoimmune disease
e.g. Coeliac disease, rheumatoid arthritis, diabetes
- 3) Transplant rejection
- 4) Immune recognition of pathogens (e.g. HCMV, HIV)



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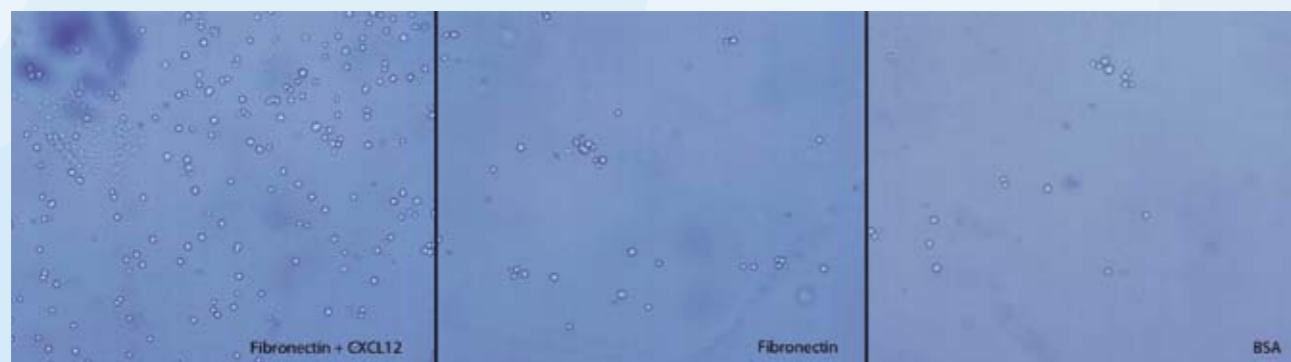
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Chemokine Signaling

Chemokines are a family of proinflammatory cytokines that, through interaction with seven transmembrane G protein-coupled receptors, play a key role in numerous biological processes, from organogenesis and leukocyte trafficking to host immune responses.

Signalling through chemokine receptors involves ligand-induced conformational changes in the receptor, allowing binding of JAK kinases and interaction with G proteins, initiating signalling cascades that lead to cytoskeletal rearrangement, gene expression, and receptor desensitization via internalization.

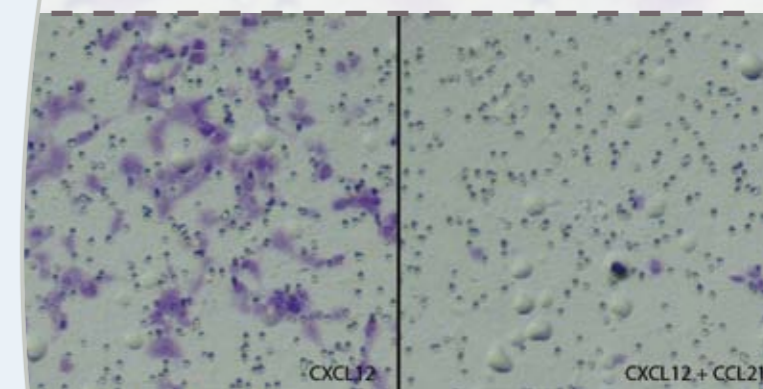


Melanoma cell adhesion to Fibronectin under flow conditions.

Chemokine receptors were thought to exist as isolated entities, allowing a 1:1 interaction with the corresponding ligand as the basic unit that triggers signalling and functional consequences. Chemokine biology is nonetheless more complex than initially predicted, as several studies suggest that chemokines can dimerise and that their receptors are found as dimers and/or higher order oligomers at the cell surface. Receptor oligomerisation might alter G protein specificity, coupling to signalling pathways, attenuate signalling, facilitating synergism between chemokine pairs and allowing GPCR crosstalk. The potential of chemokine receptor oligomerisation greatly increases the number of potential phenotypes, with implications for physiology and pharmacological intervention.

In recent years, we have studied chemokine-induced signalling pathways, including changes in receptor conformation, activation of tyrosine kinases and the various possibilities of chemokine signalling dependent on the cell microenvironment. Using the chemokine receptors CXCR4 and CCR7, we observed differences in coupling to distinct signalling pathways depending on the cell type analyzed. JAK kinases, G proteins and PI3 kinases are essential for some functions but dispensable for others. These effects are not only cell-type specific, but also chemokine receptor-specific; in some cases, there are also differences depending on the chemokine that activates a given receptor.

In previous work, we showed that the ligands for CXCR4 and CCR7 can couple to distinct signalling pathways in tumours and lymphoid cells. Our current research program is related to analysis of chemokine receptor conformations in different cell microenvironments. Using classical biochemical techniques and resonance energy transfer, we are analyzing interactions between chemokine receptors, which can indicate novel, specific activities triggered by chemokines. The main objective is to envisage new ways of modulating chemokine responses that could be therapeutically relevant by promoting or disrupting specific chemokine receptor complexes that indicate specific signalling events and therefore, cell-specific chemokine functions.



Matrigel invasion of BLM cells in response to CXCL12 in the presence or not of CCL21.

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Laura Martínez Muñoz, Pilar Lucas, Gemma Navarro, Ana I. Checa, Rafael Franco, Carlos Martínez-A., José Miguel Rodríguez-Frade and Mario Mellado. Dynamic regulation of CXCR1 and CXCR2 homo- and heterodimers. *J Immunol* 183:7337-7346 (2009).

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T cell Signalling in Autoimmune Diseases and Cancer

The main goal of our group is to study the molecular mechanisms that regulate T cell signalling in the development of autoimmune diseases and cancer.

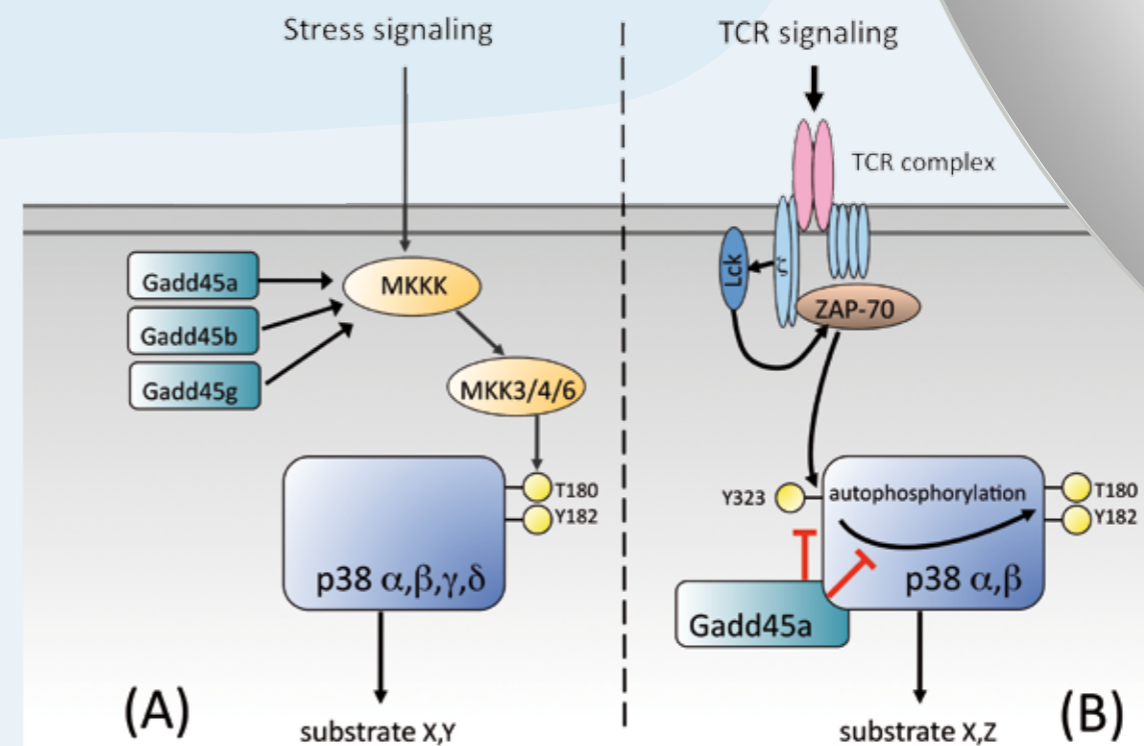
We focus on the role of the Gadd45 (growth arrest and DNA damage-inducible genes) and p38 MAPK (mitogen-activated protein kinase) families in suppression of autoimmunity and cancer. In mammalian cells, the best-characterised mechanism for p38 activation is via a phosphorylation cascade termed the classical MAPK pathway, in which a MAPK kinase kinase (MAPKKK) phosphorylates MKK3, MKK4 and MKK6. These kinases then phosphorylate p38 on the Thr180-Tyr182 motif, enhancing substrate access to the catalytic site and increasing its activity. We found that in T cells, p38 is activated by an alternative mechanism in response to antigen T cell receptor (TCR) signalling. TCR ligation increases activity of the Src kinase Lck, which phosphorylates Zap70. This tyrosine kinase phosphorylates p38 on Tyr323, which in turn induces autophosphorylation at Thr180, resulting in p38 activation. Gadd45a is a negative regulator of the alternative pathway. Gadd45a binds to p38, preventing Zap70-mediated p38 phosphorylation at Tyr323.

p38 MAPK is important in the pathogenic immune response in rheumatoid arthritis (RA). We addressed the function of the Tyr323-dependent and the classical MAPKK pathways in T cells from patients with distinct types of inflammatory arthropathies. We quantified phosphorylation of Tyr323p38 and (Thr180-Tyr182)p38 on T cells from healthy controls and patients with RA or ankylosing spondylitis (AS) to identify variables associated with p38 phosphorylation and disease activity.

In a cross-sectional study, we measured p38 phosphorylation on Tyr323 and Thr180-Tyr182 on T cells from 30 control individuals, 33 patients with AS, 30 with RA in remission and 79 with active RA. We collected clinical characteristics and

analysed the correlation between clinical variables, disease activity score (DAS) and p38 phosphorylation levels. Multivariate regression analysis was used to identify variables associated with p38 phosphorylation on Tyr323 and Thr180-Tyr182. We found that p38 phosphorylation on Tyr323 was higher in T cells from patients with active RA than in patients with RA in remission or with AS. Tyr323p38 phosphorylation was associated with disease activity as determined by the DAS28. Enhanced p38 phosphorylation was linked to Lck-mediated activation of the Tyr323-dependent pathway in the absence of upstream MAPKK activation (López-Santalla et al Arthritis Rheum, in press). Our results indicate that the Tyr323-dependent pathway has a central function in T cell-mediated p38 activation in RA patients and correlates with disease activity, suggesting selective inhibition of this pathway as an attractive target for specific downregulation of p38 activity in RA patients.

Gadd45a regulation of p38 activity in response to stress and TCR signalling. (A) In T cells, stress signals and cytokines induce dual phosphorylation of the Thr-X-Tyr motif (Thr180, Tyr182) and activation of all p38 MAPK isoforms, by triggering an upstream MAPK kinase kinase (MKKK) that activates MAPK kinases MKK3, 4 and 6. (B) Stimulation through the T cell receptor (TCR) activates Lck and ZAP70. ZAP70 phosphorylates p38 α and p38 β on the non-canonical Tyr323 residue; this induces autophosphorylation of Thr180 and p38 activation. Gadd45a binds p38 and blocks Tyr323 phosphorylation, Thr180 autophosphorylation, p38 activation, and alters p38 substrate specificity.



SELECTED PUBLICATION

López-Santalla, M., Salvador-Bernáldez, M., González-Álvarez, I., Castañeda, S., Ortiz, A.M., García, M.I., Kremer, L., Roncal, F., Mulero, J., Martínez-A, C. and Salvador JM. Tyr323-dependent p38 activation is associated with rheumatoid arthritis and correlates with disease activity. *Arthritis Rheum*, in press.

Johnen, H. and Salvador JM. The role of Gadd45a in suppression of autoimmunity. *Mol Cell Pharmacol* 1(5): 290-298, 2009.

PATENT

Jesús M Salvador, M López-Santalla, Carlos Martínez-A. Method for the diagnosis and treatment of diseases which develop with alteration of kinase p38. Elements necessary therefor and applications thereof. **WO 2009/053514, PCT/ES2008/070193.** Consejo Superior de Investigaciones Científicas.

**Inmunology
Oncology**

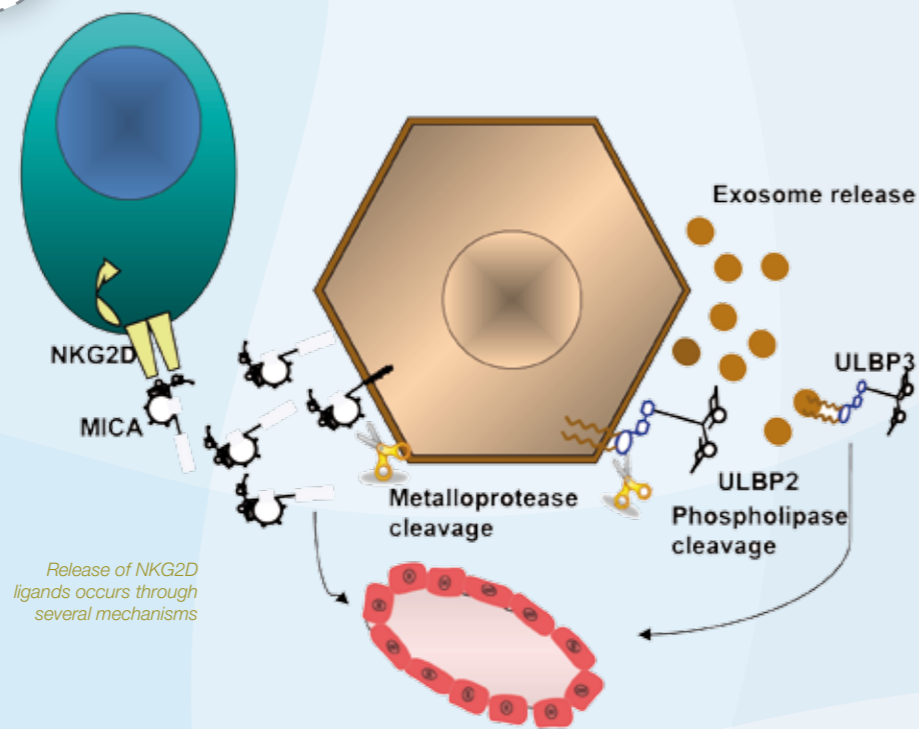
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PREDOCTORAL SCIENTISTS

Lola Fernández Messina
Sheila López Cobo

**Biochemical
Characterization of the
Ligands for the Immune
Receptor NKG2D:
Implications of their
Heterogeneity for Pathology
and Therapy**



Release of NKG2D ligands occurs through several mechanisms

NKG2D is an activating immune receptor constitutively expressed in humans in most cytotoxic lymphocytes including NK and CD8+ T cells; in mice it is expressed on NK cells, but on T cells only after activation.

After binding of its ligands, NKG2D activates the mechanisms that lead to lysis and cytokine secretion by immune effector cells. It thus seems reasonable that NKG2D ligands are not expressed constitutively on all cell types. Instead, their pattern of expression is affected by cell stress. In

humans, NKG2D ligands (NKG2D-L) belong to two families of "stress-inducible" proteins: the polymorphic family of MHC-I related chain A/B (MICA/B) and the multi-gene family of UL16-binding proteins (ULBP, now termed RAET1A-E). The existence of such a large number of ligands for a single receptor is not fully understood, but might reflect a differential role for distinct ligands in immune surveillance or an evolutionary response to selective pressures exerted by pathogens or cancer. Our hypothesis is that the different biochemical properties of NKG2D ligands could lead them to follow distinct cellular pathways, and that these biological features would allow the cell to adapt to a variety of stress stimuli (pathogen, tumour transformation).

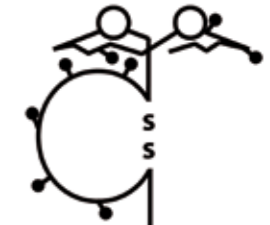
Interestingly, NKG2D ligands can also be shed as soluble molecules and induce a state of unresponsiveness in T and NK cells. This phenomenon is of particular importance in immune recognition of cancer, since the presence of soluble ligands for NKG2D in serum of cancer patients has been linked to poor disease prognosis.

Recent work from the laboratory, initiated at the University of Cambridge, focuses on studying the cellular and molecular differences that would explain the heterogeneity among NKG2D ligands. Our data demonstrate that release of NKG2D ligands occurs through several cellular mechanisms, including metalloprotease cleavage and release in exosomes.

Tumour release of proteins is a vehicle of communication with the immune system that can lead either to activation of the response and elimination of the tumour, or to suppression and immune escape. In the case of NKG2D ligands, the presence of soluble protein leads to inhibition of NK cell cytotoxicity, and exosomes containing NKG2D ligands are potent downmodulators of the NKG2D receptor.

MHC Class I-related protein (MIC)

MICA, MICB
Polymorphic



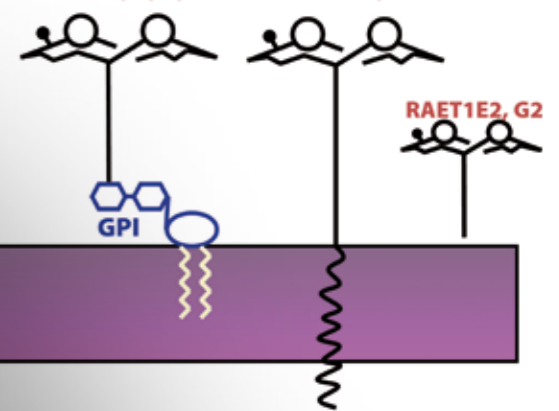
NKG2D ligands belong to two families of stress-related proteins

**UL16-Binding Proteins (ULBPs)
Retinoic acid early transcript (RAET1)**

ULBP-1, 2, 3 and 6
RAET1 I, H, N, L

ULBP-4, 5
RAET1E, G

RAET1E2, G2



SELECTED PUBLICATIONS

Fernández-Messina L, Ashiru O, Boutet P, Agüera-González S, Skepper JN, Reyburn HT, Valés-Gómez M (2010) Differential mechanisms of shedding of the glycosylphosphatidylinositol (GPI)-anchored NKG2D ligands. **J Biol Chem** 285:8543-51.

Agüera-González S, Boutet P, Reyburn HT, Valés-Gómez M (2009) Brief residence at the plasma membrane of the MHC class I-related Chain B (MICB) is due to clathrin-mediated cholesterol-dependent endocytosis and shedding. **J Immunol** 182:4800-8.

Boutet P, Agüera-González S, Atkinson S, Pennington C, Edwards D, Murphy G, Reyburn HT, Valés-Gómez M (2009) ADAM17/TACE regulates proteolytic shedding of MICB and this occurs in lipid rafts. **J Immunol 'Cutting Edge'** 182:49-53.