Alternative p38 mitogen activated protein kinases p38γ and p38δ in 
Candida albicans infection and Colitis Associated Colon Cancer.

Dayanira Alsina Beauchamp

Madrid, 2015.
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PhD Thesis

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Tesis dirigida por la Dra. Ana I Cuenda
A mis abuelos,

A mis padres,

A mis hermanos y sobrinos,

A mi familia,

No hay océano que nos separe.
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**Resumen:**

La inflamación es un proceso por el cual el cuerpo se protege de la infección por microorganismos y estímulos nocivos como sustancias químicas. Cuando la respuesta inflamatoria se produce de una manera local y controlada resulta en la curación de la infección. Por otra parte si la respuesta inflamatoria está mal regulada y es prolongada, entonces puede resultar en una inflamación crónica que puede conllevar a la muerte. Por lo tanto, es importante estudiar la implicación de la inflamación en patologías y cuáles son los mecanismos moleculares que la controlan.

La vía de las p38MAPK está implicada en patologías asociadas a procesos inflamatorios como son el choqué séptico o la artritis reumatoide, controlando los mecanismos celulares importantes asociados a dichas enfermedades como son la apoptosis, proliferación, diferenciación y migración.

El objetivo principal de esta tesis doctoral es estudiar el papel de p38γ y p38δ, dos isoformas de la familia de p38 menos estudiadas, en procesos inflamatorios que se producen en la infección por Candida albicans y en la colitis y el cáncer de colon asociado a inflamación.

En esta tesis se demuestra por primera vez el papel de p38γ y p38δ en inflamación en respuesta a LPS y diferentes ligandos de TLRs y TNFα regulando la producción de citoquinas y regulando la vía de señalización de ERK1/2 a través de la regulación de la estabilidad de la proteína quinasa TPL-2.

También, se implica por primera vez a las isoformas p38γ y p38δ en la infección de Candida albicans en un modelo in vitro e in vivo regulando la expresión de citoquinas y modulando la infiltración de células del sistema inmune. Además se demuestra la implicación de TPL-2 en la vía de señalización de Dectin-1 en respuesta a Candida albicans en monocitos humanos.

Por último, se demuestra la importancia de p38γ y p38δ en el procesos de formación de tumores en un modelo de cáncer de colon asociado a colitis y modulando la inflamación en un modelo de colitis ulcerosa.

Estos resultados reafirman la importancia de p38γ y p38δ en procesos inflamatorios y cáncer, identificándolas como importantes dianas terapéuticas para el tratamiento de dichas enfermedades.
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Abbreviations:

ABIN-2- A20-binding inhibitor of Nuclear Factor κB
AOM- azoxymethane
APC- adenomatous polyposis coli
BMDM- bone marrow derived macrophage
CAC- colitis associated cancer
CARD9- caspase recruitment domain-containing protein 9
CCL- C-C motif chemokine ligand
CCR- C-C motif chemokine receptor
CD- crohn’s disease
CFU- colony forming unit
CLR-C-Type lectin receptor
COX-2- cyclooxygenase 2
CRC- colorectal cancer
DC- dendritic cells
DC-Sign- dendritic cell specific adhesion molecule-3-grabbing non-integrin
DSS- dextran sodium sulphate
ERK- extracellular signal regulated kinase
FAP- familial adenomatous polyposis
hDlg- human homologue of the Drosophila discs large tumor suppressor protein
HK Candida- heat killed candida
i.v.- intravenously
IBD- inflammatory bowel disease
IFN- interferon
IKKβ- inhibitor of nuclear factor kappa-B kinase subunit beta
IL- interleukin
IRF- interferon regulatory factor
IkB- nuclear factor of kappa light polypeptide gene enhancer in B-cells
JNK- c-Jun N-terminal kinase
KC- keratinocyte chemoattractant
LPS- lipopolysaccharide
MAPK- mitogen activated protein kinase
MBL- mannose-binding lectin
MCP- monocyte chemoattractant protein
MDSC- myeloid derived suppressor cells
MIP- macrophage inflammatory protein
MKK- mitogen activated protein kinase kinase
MKKK- mitogen activated protein kinase kinase kinase
MR- macrophage mannose receptor
MUC-2- mucin 2 protein
MyD88- myeloid differentiation factor 88
MyOD- myogenic differentiation-1 protein
NFκβ- nuclear factor kappa-light-chain enhancer of activated B cells
NK- natural killer
NOS- reactive nitrogen species
NOS-2- nitric oxide synthase
PAMP- pathogen associated molecular pattern
PBMC- peripheral blood mononuclear cell
PKD1- polycystin protein-1
PRR- pattern recognition receptor
ROS- reactive oxygen species
SYK- spleen tyrosine kinase
Thr- threonine
TJ- tight junctions
TLR- toll like receptor
TNF-R- tumor necrosis factor alpha-receptor
TNFα- tumor necrosis factor alpha
TPL-2- tumor progression locus 2
Tyr- tyrosine
UC- ulcerative colitis
INTRODUCTION
Introduction:

1. Immune Response:

The immune system is constantly challenged by microorganisms. There are different barriers to protect us from those microorganisms, such as the skin and the mucosa (Savage 1977). When these barriers fail, the microorganisms may become pathogenic and invade the body, if this happens the innate immune system gets activated. The mammalian immune system is divided into two branches of protection against pathogens: (i) the innate immunity, which works as a first line barrier and consists of a variety of cells such as granulocytes, macrophages, dendritic cells and mast cells; and (ii) the adaptive immunity, which is involved in the elimination of pathogens in the late phase of infection and consists of antibodies, B cells and T lymphocytes. Also, there are Natural Killer T cells and γδ T cells, which are lymphocytes that work between the innate and the adaptive responses (Akira, Uematsu et al. 2006) (Figure 1).

![Figure 1. Representation of the Innate and the adaptive response.](Image taken from (Dranoff 2004)).
1.1 Pattern recognition receptors

The important ability of the innate immune system to recognize and limit microbes early during infection is based primarily on different processes such as complement activation, phagocytosis, autophagy and immune activation by different families of pattern recognition receptors (PRRs) (Mogensen 2009).

Toll Like Receptors (TLRs) are a family of evolutionally conserved PRRs, which play an important role in microbe-host interactions. There are 13 members in mice and 10 members (TLR1 to TLR10) in human. All TLRs are transmembrane receptors expressed either at the cell surface, in the case of TLR1, 2, 4, 5, 6 and 10 or in intracellular membranes, in the case of TLR 3, 7, 9 (Plato, Hardison et al. 2015). There are two main pathways in TLR signaling, one is dependent and the other is independent of myeloid differentiation factor 88 (MYD88), which is an adaptor protein (Li, Ogino et al. 2014). TLR activation leads to subsequent activation of downstream signaling pathways and factors including the nuclear factor κβ (NFκβ), the mitogen activated protein kinases (MAPK) and the interferon (IFN) regulatory factors (IRF). The TLR family can be divided into subfamilies according to recognition of pathogen associated molecular patterns (PAMPs); thus TLR1, TLR2, TLR4 and TLR6 recognize lipids, whereas TLR3, TLR7, TLR8 and TLR9 recognize nucleic acids (Mogensen 2009).

Another important family of PRRs is the c-type lectin-like receptors (CLRs). CLRs comprise a large family of receptors including dectin-1, dectin-2, the macrophage mannose receptor (MR), the dendritic cell-specific ICAM3-grabbing nonintegrin (DC-SIGN) and the circulating mannose-binding lectin (MBL), that contribute to the recognition of a variety of species (van de Veerdonk, Kullberg et al. 2008). Several interactions between the CLR and TLR signaling pathways have been described, for example in fungal immunity, these pathways interact to induce an optimal immunity dependent on Syk/CARD9 and MyD88 signaling pathways (Hardison and Brown 2012).
1.2. MAPK

Signaling pathways activated by TLRs and CLRs response include the mitogen-activated protein kinase (MAPK) pathways. The multiple MAPK pathways present in all eukaryotic cells might be activated by diverse stimuli, including hormones, growth factors and cytokines (Kyriakis and Avruch 2012). MAPK can be divided into four well-characterized subfamilies; such as the extracellular signal regulated kinases (ERK) 1/2 and the stress activated protein kinases c-jun N-terminal kinase (JNK), p38MAPK and ERK5 (Banerjee, Gugasyan et al. 2006). These protein pathways are activated by a cascade of sequential phosphorylation mediated by three protein kinases: MAPK, MAPK kinase (MKK or MAP2K) and a MAPKKK kinase (MKKKK or MAP3K) (Roberts and Der 2007) (Figure 2). The activation of all MAPK relies on dual phosphorylation of threonine (Thr) and tyrosine (Tyr) residues in a conserved Thr-X-Tyr motif (where X amino acid depends on the MAPK) in the activation loop of the kinase subdomain VIII (Cuenda and Rousseau 2007).

![Figure 2: Activation of MAPK signaling pathways](image)

In this thesis we will focus mainly in the study of two important MAPK pathways: the ERK1/2 and p38MAPK signaling pathways.
1.2.1 ERK1/2 signaling pathway.

ERK1 and ERK2 are related protein-serine/threonine kinases that participate in the Ras-Raf-MEK (MKK1)-ERK1/2 signal transduction cascade (Figure 2). This cascade participates in the regulation of a large variety of processes including cell adhesion, cell cycle progression, cell migration, cell survival, differentiation, metabolism, proliferation and transcription (Roskoski 2012). The ERK1/2 pathway is a convergent signaling node that receives input from numerous stimuli, including internal metabolic stress, DNA damage pathways and altered protein concentrations, as well as through signaling from external growth factors, inflammatory cytokines, cell-matrix interactions, and communication from other cells. Several mutations involving the MEK/ERK1/2 pathway have been identified in human cancers and are important therapeutic targets (Burotto, Chiou et al. 2014). Mammalian cells activate ERK1/2 via two distinct types of MAP3K, members of the Raf family (Raf-1, B-Raf and A-Raf) or tumor progression locus-2 (TPL-2), which is also known as COT-1 and MAP3K8 (Gantke, Sriskantharajah et al. 2011).

Raf and TPL-2 activate ERK1/2 through direct phosphorylation of MEK (MKK1), the only ERK1/2 kinase. TPL-2 forms a ternary complex with NF-κβ1 (p105) and the ubiquitin binding protein ABIN-2 (A20-binding inhibitor of NF-κβ2) (Gantke, Sriskantharajah et al. 2011). In un-stimulated cells a substantial fraction of endogenous ABIN-2 is associated with both p105 and TPL-2, optimal TPL-2 stability in vivo requires interaction with both proteins (Lang, Symons et al. 2004). TPL-2 is activated by TLRs and TNF receptor (TNFR) family members, independently of Raf signaling. In macrophages and dendritic cells, TPL-2 can be activated by lipopolysaccharide (LPS), tumor necrosis factor α (TNFα) and IL-1β and by CD40 and LPS in B cells. Activation of the native TPL-2 complex by these agonists requires the phosphorylation of the p105 catalyzed by IKKβ (Inhibitor of NF-κβ (IκB) kinase β). p105 phosphorylation leads to its ubiquitylation and proteasomal degradation, which activates TPL-2 (Handoyo, Stafford et al. 2009).
1.2.2 p38 MAPK pathway.

The p38 MAPK subfamily consists of four isoforms: p38α, p38β, p38γ, p38δ encoded by different genes. All isoforms are widely expressed by most cell types, however p38γ is predominantly expressed in skeletal muscle and p38δ is enriched in kidney, testis, pancreas, and small intestine (Ono and Han 2000). The p38 MAPKs are strongly activated *in vivo* by environmental stresses and inflammatory cytokines. Canonical activation occurs by dual phosphorylation of their Thr-Gly-Tyr motif, in the activation loop, by two upstream kinases: MKK3 and MKK6 (Figure 3) (Wagner and Nebreda 2009, Risco and Cuenda 2012).

Among all p38 MAPKs the isoform p38α was the first identified by four independent groups (Freshney, Rawlinson et al. 1994, Han, Lee et al. 1994, Lee, Laydon et al. 1994, Rouse, Cohen et al. 1994) and is the most characterized. The p38 subfamily can be divided into two subgroups based on sequence homology on one hand p38α and p38β, which are 75% identical, and on the other, p38γ and p38δ, which share 70% homology. Also they can be classified by their susceptibility to inhibitors, it has been demonstrated that p38α and p38β are inhibited by the compounds SB203580 and SB202190, while p38γ and p38δ are unaffected by these inhibitors. Another difference between these two subgroups is found in their substrate selectivity, for example it has been shown that microtubule-associated protein Tau or the scaffold proteins a-syntrophin, PSD95 or hDlg are better *in vitro* substrates for p38γ and p38δ than for p38α and p38β (Figure 3) (Cuenda and Rousseau 2007, Risco and Cuenda 2012).
1.2.3 p38γ and p38δ.

The information about p38γ and p38δ biological roles is limited compared to the extensive knowledge about p38α and p38β functions. This could be due to the lack of specific inhibitors for these isoforms (Cuenda, Goedert et al. 1997). Although there are no specific inhibitors for these two isoforms, it has been demonstrated that the diaryl urea compound BIRB 796 is not only a potent inhibitor of p38α and p38β, but also inhibits p38γ and p38δ at higher concentrations (Kuma, Sabio et al. 2005). p38γ and p38δ knock-out mice have been generated (Sabio, Arthur et al. 2005), at the moment the use of specific p38γ, p38δ and p38γ/p38δ knock-out mice have been a great tool for the elucidation of the biological roles of these isoforms. Contrary to p38α, whose constitutive deletion causes death during embryonic development (Wagner and Nebreda 2009), p38γ and p38δ deficient mice are viable and have not apparent phenotype (Sabio, Arthur et al. 2005).

There are recent reports showing the implication of p38γ and p38δ in tissue regeneration, cancer and metabolic diseases. Thus, p38δ seems to be a regulator of processes related to the pathogenesis of diabetes, such as insulin secretion and β cells death, by controlling the activation of the protein kinase PKD1 (Sumara, Formentini et al. 2009, Goginashvili, Zhang et al. 2015). Studies in p38γ null mice reported that this kinase plays a cardinal role in blocking the premature
differentiation of skeletal muscle stem cells, the satellite cells that participate in adult muscle regeneration. p38γ phosphorylates the transcription factor MyoD, which promotes its association to the histone methyltransferase KMT1A and the repression of myogenin transcription (Gillespie, Le Grand et al. 2009).

Using ectopic over expression and knock-down model cell lines it has been shown that p38γ and p38δ pathway could be involved in the modulation of some processes implicated in cellular malignant transformation, such as proliferation, cell cycle progression or apoptosis and regulation of tumorigenesis (Nebreda and Porras 2000, Cerezo-Guisado, del Reino et al. 2011, Risco and Cuenda 2012). Using knock-out mice it has been shown that lack of p38δ reduced susceptibility to the development of TPA-induced skin carcinomas (Schindler, Hindes et al. 2009).

Growing evidence reveals important implications of p38γ and p38δ in pathological processes. Thus, p38γ and p38δ are key components in innate immune response by modulating cytokine production in a septic shock model (Risco, del Fresno et al. 2012) or in inflammation-induced acute lung injury (Ittner, Block et al. 2012). Additionally, p38γ and p38δ are implicated in linking tumor promotion and/or progression and inflammation in a colitis-associated colon cancer model (Del Reino, Alsina-Beauchamp et al. 2014). Also p38γ and p38δ have been implicated in the regulation of inflammatory joint destruction in the collagen-induced arthritis model (Criado, Risco et al. 2014).

2. *Candida albicans* infection.

There are approximately 1.5 million different species of fungi on Earth; however, only 300 cause infections in humans (Taylor, Latham et al. 2001). In the 20th century, fungi became important human pathogens primarily in host with impaired immunity as a consequence of medical interventions or of HIV infection (Garcia-Solache and Casadevall 2010). Among the most invasive fungi in humans is the *Candida* with over 150 species, from which more than 10 species appear in human (Ryan, K, Ray, G et al. 2014). In human, the infections caused by *Candida*, termed candidosis (plural) or candidiasis (singular), can be categorized as being systemic or superficial. Systemic infections generally develop in severely immunocompromised patients and are associated with high mortality. In contrast,
superficial infections on moist mucosal surfaces such as those of the mouth and vagina are more prevalent but have less damaging effects to the host (Williams, Jordan et al. 2013). *Candida albicans* has been the most prevalent microorganism isolated from candidemic patients (Wisplinghoff, Seifert et al. 2006).

*Candida albicans* is a commensal fungal microorganism, belongs to the *Saccharomycetaceae* family of ascomycete fungi, and is often part of the flora residing in the human oral cavity and genital tract. Under normal conditions it will not cause disease (Kim and Sudbery 2011); however, when mucosal or skin barriers are damaged and/or immune system is compromised, *Candida albicans* is able to cause disseminated infections that are associated with a high mortality (Wisplinghoff, Bischoff et al. 2004).

A distinctive characteristic of *Candida albicans* is its ability to grow with three distinct morphologies: yeast, pseudohyphae and hyphae (Figure 4). The ability to switch between yeast, pseudohyphal and hyphal morphologies is considered to be the main virulence factor for *Candida albicans*, both hyphae and pseudohyphae are invasive (Sudbery, Gow et al. 2004). Transcriptional factors responsible for this switch have been identified and they vary between *Candida* species (Whiteway and Bachewich 2007).

The ability of *Candida albicans* to colonize and survive at different sites of the host is what makes it more harmful than other commensals of the human body (Khan, Ahmad et al. 2010). *Candida albicans* expresses several virulence factors that contribute to pathogenesis, these factors include host recognition biomolecules (adhesins), the ability to change morphogenesis, secreted aspartyl proteases and phospholipases (Calderone and Fonzi 2001). One of the major factors contributing to the virulence of *Candida albicans* in patients with medical devices such as pacemakers and artificial joints, is the formation surface-attached microbial communities known as biofilms (Seneviratne, Jin et al. 2008).
INTRODUCTION

The *Candida albicans* cell wall structure is composed of chitin, β-glucans, and mannoproteins. The polysaccharide structures of the cell wall of *Candida albicans* are recognized by two classes of PRRs, including the TLRs and CLRs (Cheng, Joosten et al. 2012). The innate immune system response to *Candida albicans* is determined by the recognition of the fungal cell wall components by different immune cells; neutrophils, monocytes and macrophages represent the first line of defense against fungal pathogens. Later on, recognition of fungal structures by dendritic cells leads to the activation of specific immunity, especially T-cell mediated. These various cell populations differ in their expression of TLRs and CLRs on their cell membrane, therefore they are capable of initiating different responses (Figure 5)(van de Veerdonk, Kullberg et al. 2008).

**Figure 4. Candida albicans morphology and cell cycle.** Image taken from (Sudbery, Gow et al. 2004).
The first PRRs discovered to recognize Candida albicans were the TLRs, such as TLR2, TLR4 and TLR9. Specifically TLR2 can recognize the phopholipomannans, while TLR4 recognizes the O-linked mannans and TLR9 is located in the cytosol and recognizes fungal DNA. They are involved in the Candida albicans recognition and in the induction of pro-inflammatory cytokine production (Gow, van de Veerdonk et al. 2012). It has been demonstrated that in vivo TLR2 and TLR4 are involved in the dissemination of candidiasis (Netea, Van Der Graaf et al. 2002). In contrast, TLR1 and TLR6 have redundant roles in host defense against Candida albicans (Gow, van de Veerdonk et al. 2012).

The second major PRR family that recognizes Candida PAMPs is the CLRs. Dectin-1 can bind β-glucans and dectin-2 together with the Fcγ receptor recognizes mannans (Gow, Netea et al. 2007). The N-linked mannans is recognized by the macrophage mannose receptor (MR) (Netea, Gow et al. 2006). DC-SIGN is another important receptor on the dendritic cells that recognizes Candida mannan. Also galectin-3 has been shown to play a role in recognizing the β-mannosides of Candida albicans (Figure 6) (Jouault, El Abed-El Behi et al. 2006).
Figure 6. *Candida albicans* main pattern recognition receptors and signaling pathway. Image taken from (Gow, van de Veerdonk et al. 2012).

Little is known about the role of MAPK on *Candida albicans* infection, studies *in vitro* have shown that *Candida albicans* activates NF-κβ and MAPK such as ERK1/2, p38 and JNK signaling (Moyes, Murciano et al. 2011). p38 MAPK family have been implicated *in vivo* with pathogenicity of *Candida albicans* in kidney (Choi, Choi et al. 2007). There is no data yet that implicates p38γ and p38δ in *Candida albicans* infection, our findings have implicated p38γ and p38δ in the regulation of TLR4, and therefore they might have a role on *Candida albicans* infection (Risco, del Fresno et al. 2012).

3. **Colitis-associated colon cancer.**

3.1 Colon

The colon is a highly specialized organ that is responsible for processing waste; it extracts water and salt from solid wastes before they are eliminated from the body. The colon is the last portion of the digestive system in most vertebrates; it makes the longest part of the large intestine and includes the cecum, appendix and ano-rectum (Grey. 2000).
The colon may be subdivided into four parts: ascending, transverse, descending and sigmoid colon. The microscopic anatomy is divided into the mucosa, submucosa, muscularis and serosa/adventitia. The mucosa is lined by simple columnar enterocytes with long microvilli; a layer of mucus that aids the transport of the feces covers it. Also, the mucosa contains many crypts of Lieberkuhn in which numerous goblet cells and enteroendocrine cells are found (Figure 7). The connective tissue is filled with macrophages, plasma cells and other immune cells. On the other hand, the submucosa comprises blood vessels, lymph nodes and fat tissues. The muscularis is constituted by muscle tissue divided into the inner circular musculature strongly pronounced and the outer longitudinal musculature. The longitudinal musculature is concentrated in three strong ribbon-like strips (taeniae coli). The serosa is the most external layer made of conjunctive tissue (Figure 7) (Kenhub. 2014).

Figure 7. Tissue layers of the colon with cut-away detailing layers. Taken from John Hopkins, School of medicine, Gastroenterology and hepatology (2015).

Many disorders affect colon’s ability to work properly, such as colon cancer, colonic polyps (extra tissue growing in the colon), ulcerative colitis (ulcers of the colon and rectum), diverticulitis (inflammation or infection of pouches in the colon) and irritable bowel syndrome (National Institute of Health, 2014).

The pathologies with more incidences in the colon are the inflammatory bowel diseases (IBDs), which primarily include Crohn’s disease and ulcerative colitis. Crohn’s disease is an IBD that causes inflammation anywhere in the digestive tract, while ulcerative colitis causes long-lasting inflammation in some part of the digestive tract (mainly the colon) (Fakhoury, Negrulj et al. 2014).
The frequency of IBDs has increased substantially over the last 50 years, Crohn's disease (CD) and ulcerative colitis (UC) are prevalent in highly industrialized regions and they are rare in less developed countries. This suggests that critical environmental factors affect the worldwide distribution of IBDs (Weinstock, Summers et al. 2004). The prevalence of both CD and UC are highest in Europe, with 322 and 505 per 100,000 per person per year respectively. Ongoing changes in environmental factors, including diet, lifestyle factors, infections and medication use have contributed to shifts in the global prevalence of these diseases. As IBDs are chronic disabling disorders without high mortality, prevalence rates may now be increasing due to earlier diagnoses and potentially to longer duration of diseases (Ponder and Long 2013).

IBDs are complex and multifactor disorders, besides the environmental factors, there is also a genetic influence associated to the diseases. Studies have demonstrated aggregation of cases of UC or CD in families, and of both diseases within the same families (Binder and Orholm 1996). Genome-wide association studies have identified approximately 100 loci that are significantly associated with IBDs. These loci implicate a diverse array of genes and pathophysiologic mechanisms, including microbe recognition, lymphocyte activation, cytokine signaling, and intestinal epithelial defense (Cho and Brant 2011). Also, the enteric microbiota are now accepted as a central etiologic factor in the pathogeneses of IBD. For example, Crohn’s disease patients exhibit defective microbial killing that results in increased exposure to commensal bacteria and activation of compensatory pathogenic T cells. It is likely that functional microbial alterations must interact with host genetic defects to cause disease (Sartor 2008).

3.2 Colon inflammation: Ulcerative colitis.

Multiple confluent ulcerations, pseudopolyps and histological crypt abscesses characterize ulcerative colitis. Symptoms include diarrhea and rectal bleeding with periods of acute exacerbation and remission of the symptoms (Kuhbacher, Schreiber et al. 2004). In the colon, the conjunction of severe inflammation and production of inflammatory mediators develops extensive superficial mucosal ulceration (Xavier and Podolsky 2007).
Intestinal permeability is the property that allows solute and fluid exchange between the lumen and tissues. Conversely, intestinal barrier function refers to the ability of the mucosa and extracellular barrier components, such as mucus, to prevent this exchange (Lee 2015). Intestinal barrier dysfunction is a main feature of intestinal inflammatory diseases like UC. The intestinal barrier is established by a polarized monolayer of epithelial columnar cells, which are connected by intercellular junctions (Hering, Fromm et al. 2012). Epithelial tight junctions (TJs) maintain the intestinal barrier while regulating permeability of ions, nutrients, and water. The TJ is a multi-protein complex that forms a selectively permeable seal between adjacent epithelial cells and demarcates the boundary between apical and basolateral membrane domains (Lee 2015). Including, the junction adhesion molecule-A that belongs to the family of cell adhesion molecules, localizes at intercellular junctions and mediates different physiological processes, such as junction assembly and leukocyte migration. This molecule has been described as fundamental for intestinal homeostasis in a colitis mouse model, suggesting a direct link between defects in epithelial barrier integrity and colitis (Vetrano, Rescigno et al. 2008).

The surface of the intestine is protected by a layer of mucus that is generated by goblet cells in the epithelium. Goblet cells exert a vital role secreting important molecules that have a protective role in the gut, such as mucins and trefoil factors (Wallace, Zheng et al. 2014). The secretory mucin MUC2 is mainly expressed in colonic epithelium and is the most important factor determining goblet cell morphology. It has been shown that MUC2 plays an important role in the suppression of intestinal cancer; mice lacking this protein spontaneously developed colitis (Van der Sluis, De Koning et al. 2006). Trefoil factor 3 is another important molecule expressed by goblet cells that has been shown to play an essential role for the maintenance and repair of the intestinal mucosa (Mashimo, Wu et al. 1996).

Furthermore, toll like receptors play an important role in the biological pathogenesis of UC. Aberrant TLR signaling can induce tissue damage and barrier destruction through the overproduction of cytokines and chemokines and the loss of commensal-mediated responses of colonic epithelial progenitors (Fan and Liu 2015). Specifically TLR2, TLR4 and TLR9 have been shown to have an important
role in ulcerative colitis; the mRNA expression levels of these TLRs were increased in UC patients (Tan, Zou et al. 2014).

Also, important cytokines have been identified to have an essential role in the regulation of UC, such as TNFα, IFNγ, IL-1β, IL-6 and IL-17. The over-production of these cytokines is known to disturb intestinal barrier function (Lee 2015). For example, TNFα has been shown in *in-vitro* studies to regulate the epithelial barrier by altering the structure and function of TJ (Schmitz, Fromm et al. 1999) and anti-TNFα therapy is being tested in patients with UC (Baki, Zwickel et al. 2015).

The intestinal immune system uses many different mechanisms to regulate the high concentrations of resident microbes and to protect the mucosal surface from pathogens. In adaptive immune response for example, B cell mediated responses are unique secreting high levels of IgA. This IgA assists in the defense against various intestinal pathogens. Also the exposure of the intestine to different antigens induces the T cell responses, such as Th1, Th2 and Th17 T regulatory cells responses, mainly mediated by dendritic cells (Abraham and Medzhitov 2011).

To understand completely the immunologic mechanisms implicated in gut inflammation it is necessary to study the interaction between various constituents of the innate and adaptive immune systems (Geremia, Biancheri et al. 2014).

**3.3 Colon Cancer.**

Colorectal cancer (CRC) develops as the result of the progressive accumulation of genetic and epigenetic alterations that lead to the transformation of normal colonic epithelium to colon adenocarcinoma. The cancer emerges via a multistep progression at both molecular and morphological levels. Also, the genetic and epigenetic alterations are key pathogenic events in cancer development that drive the initiation and progression of the polyp-cancer sequence (Grady 2005). CRC develops from dysplastic precursor lesion; in sporadic CRC the dysplastic precursor is the adenomatous polyp (adenoma), a discrete focus of neoplasia that is typically removed by endoscopic polypectomy. In contrast, dysplasia in patients with IBD can be polypoid or flat, localized, diffuse or multifocal and, once found, marks the entire colon as being at heightened risk of neoplasia, thereby needing surgery for removal of entire colon and rectum (Itzkowitz and Yio 2004).
CRC has high incidence and is associated with high case fatality, causing around 600,000 deaths annually (Hagland and Soreide 2015). About one third of patients diagnosed with CRC will develop a metachronous recurrence during the following years (Gilard-Pioc, Abrahamowicz et al. 2015). There are two types of causes for CRC, the sporadic and the hereditary, which develop by different molecular mechanisms (Hagland and Soreide 2015). The hereditary cancer includes the hereditary nonpolyposis colorectal cancer, also known as Lynch syndrome characterized by K-Ras mutations, and the familial adenomatous polyposis (FAP) characterized by benign growths (polyps) in the colon. The major pathways of this disease include the protein adenomatous polyposis coli (APC), p53, K-Ras, SMAD4, or the CMP/MSI pathway (Figure 8). The sporadic colorectal cancer may develop by two different pathways one is MS1 high (microsatellite instability high), which implies the instability of microsatellite regions of DNA, and the other is the canonical signaling pathway characterized by BRAF and K-Ras mutations (Wanebo, LeGolvan et al. 2012). Mutations in the APC and the DNA mismatch repair (MMR) genes are found in both sporadic and familial colorectal cancer syndromes (Figure 8) (Fearon and Vogelstein 1990).

The development of sporadic colorectal cancer is a multistep process, which can arise from a combination of mutations. A number of key oncogenes and tumor suppressor genes have been identified; whose progressive activation or loss of function by mutations drives the transition from adenoma to carcinoma. Inactivation of APC and p53 and activation of the oncogene K-Ras have been proposed to be particularly important determinants of tumor initiation and progression (Smith, Carey et al. 2002). Also important molecules mediate the development of colitis-associated cancer, such as IL-6 and STAT3 which are required for the survival of intestinal epithelial cells (Figure 8) (Foersch and Neurath 2014).
The major tumor suppressor function of the APC protein is being a negative regulator of Wnt signaling, where it forms part of the β-catenin stabilization and, consequently, to the deregulation of the Wnt pathway through the activation of TCF/LEF target genes, such as c-MYC. On the other hand, K-Ras signaling has been established in regulation of colorectal tumor cell proliferation, growth, survival, invasion and metastasis formation. It has been demonstrated that oncogenic activation of K-Ras/B-Raf/MEK signaling in intestinal epithelial cells activates the Wnt/β-catenin pathway which, in turn, promotes cell migration and invasion as well as tumor growth and metastasis (Lemieux, Cagnol et al. 2014).

3.4 Cancer associated to colitis.

Cancer and inflammation are highly associated (Hanahan and Weinberg 2011). Inflammation is an important risk factor for the development of colorectal cancer, and severity of inflammation has been directly linked to colorectal cancer risk (Triantafillidis, Nasioulas et al. 2009). Colorectal cancer represents a major cause
for excess morbidity and mortality by malignant disease in ulcerative colitis. The risk for ulcerative colitis associated colorectal cancer is increased at least 2-fold compared to the normal population and colorectal cancer is observed in 5.5-13.5% of all patients with ulcerative colitis (Pohl, Hombach et al. 2000).

IBD-related CRC is characterized by a dense infiltrate of both innate and immune cells, such as macrophages, neutrophils, myeloid derived suppressor cells (MDSC), dendritic cells (DC), and natural killer cells (NK), and adaptive immune cells, such as T and B lymphocytes (Monteleone, Pallone et al. 2012). The inflammatory cells that contribute to the colitis generate reactive oxygen and nitrogen species (ROS and NOS). Thus, neutrophils and macrophages generate free radicals and other pro-oxidant molecules, which generate oxidative stress (Figure 8). Cellular damage associated with oxidative stress, is thought to play a key role in the pathogenesis of the colitis itself as well as in colon carcinogenesis. It has been determined that inflamed tissues from patients with active UC showed increased expression of NOS and other ROS/NOS. Free radicals have the potential to affect a large array of metabolic processes, because their targets include DNA, RNA, proteins and lipids. If key genes, such as p53, or proteins responsible for colon cell homeostasis are targeted, dysplasia and subsequent carcinoma arise (Figure 9)(Itzkowitz and Yio 2004).
Proinflammatory factors of the innate and adaptive immune systems contribute to development and growth of colon neoplasia. In UC patients several inflammation-associated genes such as cyclooxygenase-2 (COX-2), nitric oxide synthase-2 (NOS-2) and the interferon-inducible gene 1-8U are increased in inflamed mucosa and remain elevated in colonic neoplasms (Ullman and Itzkowitz 2011). COX-2 is an intermediate response gene that encodes a cytoplasmic protein, which catalyzes the synthesis of prostaglandins and arachidonic acid. COX-2 expression is activated by numerous proinflammatory cytokines, including IL-1β, IL-1α and TNFα. COX-2 contributes to tumor development by determining sustained epithelial cell proliferation and inhibition of apoptosis, and triggering neoangiogenesis (McConnell and Yang 2009). Another important promoter of inflammation and colitis-associated cancer is TNFα. TNFα is released by activated macrophages and T cells, it binds to the receptor TNF-receptor (TNF-R) and can initiate carcinogenesis by promoting DNA damage, stimulating angiogenesis and inducing expression of COX-2 (Ullman and Itzkowitz 2011). Furthermore, activation of NF-κβ in epithelial cells contributes to tumor initiation and promotion primarily by suppressing apoptosis; when that suppression is removed precancerous cells are eliminated through cell death mechanisms. In tumor cells and epithelial cells at risk, as well as in inflammatory cells, NF-κβ activates the expression of genes encoding inflammatory cytokines, adhesion molecules, enzymes in the prostaglandin synthesis pathway, including COX-2, inducible nitric oxide synthase and angiogenic factors (Mantovani, Allavena et al. 2008).

3.5 p38 MAPK and colitis associated cancer.

p38 MAPKs have been implicated in the development of colorectal cancer via TLR recognition. It has been shown that commensal microorganisms normally activate TLRs in the intestinal epithelial cells, and that this symbiotic recognition is required for epithelial physiology (Formica, Cereda et al. 2014). Recent studies identified p38α as a mediator of resistance to various agents in CRC patients, its role as a negative regulator of proliferation has been reported in both normal and cancer cells. This function is mediated by the negative regulation of cell cycle progression and the transduction of some apoptotic stimuli (Grossi, Peserico et al.
p38α also mediates inflammation in patients with IBD, it is highly phosphorylated and active in the inflamed intestinal mucosa (Zhao, Kang et al. 2011). Moreover, it has been shown that p38α in intestinal epithelial cells is critical for chemokine expression, subsequent immune cell recruitment into the intestinal mucosa, and clearance of the infected pathogen (Kang, Otsuka et al. 2010). Additionally, it has been shown that p38α has a dual function in colon cancer development. p38α protects intestinal epithelial cells from colitis-associated cancer. Thus, p38α deletion enhances colitis induced epithelial damage and inflammation, therefore potentiating colon tumor formation. On the other hand, p38α inhibition in transformed epithelial cells reduces tumor number (Gupta et al. 2014).

The role of p38γ and p38δ in colorectal cancer associated to colitis has not been characterized yet. Although, their implication in inflammatory response has been demonstrated by us and others (Ittner, Block et al. 2012, Risco, del Fresno et al. 2012, Criado, Risco et al. 2014). In addition to their role in inflammation p38γ and p38δ have tumorigenic functions in certain contexts (Risco and Cuenda 2012) in vivo models have demonstrated p38δ has a protumorigenic role in skin carcinogenesis (Schindler, Hindes et al. 2009). Also p38γ regulates oncogenic proteins involved in colorectal cancer development, such as K-Ras (Qi, Pohl et al. 2007).

3.6 Cancer associated to colitis mouse model.

Experimental animal models are important to mimic the diseases in humans and to study the mechanisms underlying them. Azoxymethane (AOM) and its derivatives are the chemical agents that have been most successfully used in CRC mouse models (Neufert, Becker et al. 2007). AOM is a chemical agent that can initiate cancer by alkylation of DNA in cells, thereby facilitating base mispairings. A model has been proposed for AOM-induced colon carcinogenesis, AOM could induce mutations via two different signaling pathways K-Ras or β-catenin. Both pathways result in over expression of COX-2 producing excess of prostaglandins and causing an increase in cell proliferation and decrease of apoptosis (Takahashi and Wakabayashi 2004). It is important to take in account the genetic background
between mouse strains, which modifies the AOM-induced tumorigenesis (Neufert, Becker et al. 2007).

To focus the study in the tumor progression driven by chronic colitis as seen in ulcerative colitis, a pro-inflammatory reagent dextran sodium sulfate (DSS) is combined with the AOM. DSS dissolved in drinking water is very toxic to the epithelial lining of the murine colon, resulting in severe colitis characterized by bloody diarrhea (Neufert, Becker et al. 2007). Administration of a low dose of AOM (for initiation) and DSS in the drinking water for one week results in development of colon tumors within 20 weeks and mimics human CRC caused by chronic exposure to a small amounts of environmental mutagens and/or carcinogens and promoters (Tanaka, Kohno et al. 2003). Furthermore, to mimic a chronic inflammation, which is believed to be the driving force in tumor progression of colitis-associated CRC as seen in ulcerative colitis, a four-staged model has been proposed. The model is based on a single AOM injection followed by three cycles of DSS administration, which cause chronic colitis. With this protocol, tumor growth is accelerated resulting in multiple large tumors after 10 weeks (Neufert, Becker et al. 2007).
OBJECTIVES
Objectives:

The general objective of this doctoral thesis is to study the implication of p38\(\gamma\) and p38\(\delta\) in inflammation processes in *Candida albicans* infection and colon cancer associated to colitis.

To investigate this general objective we established the following partial objectives:

1. To study the implication of p38\(\gamma\) and p38\(\delta\) in Toll Like Receptors (TLR) and TNF Receptor (TNF-R) signaling and cytokine production.

2. To investigate the implication of p38\(\gamma\) and p38\(\delta\) in *Candida albicans* signaling in BMDM *in vitro* and in cytokine production in response to Curdlan and Heat Killed Candida.

3. To study the implication of p38\(\gamma\) and p38\(\delta\) in *Candida albicans* infection *in vivo* by injecting WT and p38\(\gamma/\delta/-/-\) mice with live *Candida albicans*.

4. To characterize the signaling pathways implicated in Heat Killed Candida response and cytokine production in human monocytes derived from peripheral blood mononuclear cells (PBMCs).

5. To analyze the implication of p38\(\gamma\) and p38\(\delta\) in colon tumor formation associated to colitis.

6. To study the implication of p38\(\gamma\) and p38\(\delta\) in colitis.

7. To determine the implication of p38\(\gamma\) and p38\(\delta\) in the hematopoietic in the tumor incidence in CAC model.
MATERIAL AND METHODS
Material and Methods:

1. General methods.

1.1 Mice.

All the mice used in the experiments were backcrossed onto C57BL/6 background for at least nine generations. Mice lacking p38γ (p38γ/-/-), p38δ (p38δ/-/-) and p38γ/δ (p38γ/δ/-/-) have been described previously (Sabio, Arthur et al. 2005). The colonies were maintained in the animal house of Centro Nacional de Biotecnología (CNB) on pathogen-free conditions. The experiments were performed in accordance with the European Union regulations and approved by CNB-CSIC ethical review.

1.2 Protein samples preparation.

For cell-derived protein extraction we used lysis buffer (see table A), added the cold lysis buffer to the cells and let them on ice for 10 minutes afterwards the cells were centrifuged at 14,000 rpm (Mikron 200R, Hettic zentrifugen) for 5 minutes at 4°C and protein in the supernatant was quantified with the Bradford method, using Coomassie Plus Protein reagent (Thermo Scientific).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
<th>Company and Reference number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris/HCl pH 7.5</td>
<td>50mM</td>
<td>Promega H5121</td>
</tr>
<tr>
<td>Triton-X-100</td>
<td>1% (v/v)</td>
<td>Sigma T8532</td>
</tr>
<tr>
<td>EDTA at pH 8</td>
<td>1mM</td>
<td>Sigma 60004</td>
</tr>
<tr>
<td>EGTA at pH 8</td>
<td>1mM</td>
<td>Sigma E3889</td>
</tr>
<tr>
<td>NaF</td>
<td>50mM</td>
<td>Sigma S7920</td>
</tr>
<tr>
<td>β-glycerol phosphate Na</td>
<td>10mM</td>
<td>Sigma 50020</td>
</tr>
<tr>
<td>Na₄P₂O₇</td>
<td>5mM</td>
<td>Sigma P8010</td>
</tr>
<tr>
<td>Sodium orthovanadate*</td>
<td>1mM</td>
<td>Sigma S6508</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.27M</td>
<td>Sigma S0389</td>
</tr>
<tr>
<td>Benzamidine (add at moment of use)</td>
<td>1mM</td>
<td>Sigma 434760</td>
</tr>
<tr>
<td>PMSF (add at moment of use)</td>
<td>0.1mM</td>
<td>Sigma P7626</td>
</tr>
<tr>
<td>β-mercaptoethanol (add at moment of use)</td>
<td>0.1% (v/v)</td>
<td>Sigma M6250</td>
</tr>
</tbody>
</table>

Table A. Lysis buffer preparation.

* In order to depolymerize the vanadate and enhance the ability of sodium orthovanadate to inhibit tyrosine phosphatases, the compound needs to be dissolved in water and
various cycles of heating to boil and cooled to room temperature, and adjusting to pH10 with sodium hydroxide, around 5 cycles until the solution is clear and colorless at a constant pH of 10.

1.3 Immunoblotting.

Reagents and Equipment:
- Poliacrylamide gel 12.5% agarose.
- Loading buffer (see table B)
- Running buffer (see table C)
- Nitrocellulose membrane
- Whatman filter paper
- Wet blotting system
- Voltage generator
- TBS pH 8 (10x)
  - 438g NaCl 1.5M
  - 60.5g Tris base 100mM
  - 5000ml H2O milliQ
- TBS-T wash buffer
  - 100ml TBS 10x
  - 900 ml distilled H2O
  - 500µl Tween-20
- Blocking buffer: 5% (w/v) milk in TBS-T
- Transfer buffer (see table D)

Proteins were separated using common protocol of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) based on their electrophoretic mobility. The protein samples were denatured in loading buffer for 5 minutes at 90°C. Equal amounts of protein from different samples were loaded on polyacrylamide gels using running buffer at 120V for 2 hours. In order to visualize specific proteins in our samples we used specific antibodies diluted in blocking buffer. First, we transferred the proteins from gel onto nitrocellulose blotting membrane by the wet blotting method using transfer buffer. Afterwards, the membrane was incubated with blocking solution shaking for 30 minutes at room temperature. Then we incubated with the antibody of interest (Table D) for 2 hours or overnight on a shaking platform. The membrane was washed with TBS-T and incubated with the corresponding fluorescent secondary antibody for 1 hour on a shaking platform at room temperature. Protein expression was visualized and quantified using the Odyssey ® infrared imaging system (LI-COR Bioscience).
### Table B. Loading buffer for samples used in Immunobloting.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>2M Tris-HCL pH 6.8</td>
<td>12.5ml</td>
<td>250mM</td>
</tr>
<tr>
<td>Glicerol (87%)</td>
<td>34.4ml</td>
<td>30% (v/v)</td>
</tr>
<tr>
<td>SDS</td>
<td>10g</td>
<td>10% (w/v)</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>5ml</td>
<td>5% (v/v)</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td></td>
<td>0.02%</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100ml</td>
<td>--</td>
</tr>
</tbody>
</table>

### Table C. Immunobloting running buffer preparation.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>14.4 g</td>
<td>192 mM</td>
</tr>
<tr>
<td>Tris base</td>
<td>3 g</td>
<td>25 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>1 g</td>
<td>0.1% (w/v)</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
<td>--</td>
</tr>
</tbody>
</table>

### Table D. Immunobloting transfer buffer preparation.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>2.9 g</td>
<td>39 mM</td>
</tr>
<tr>
<td>Tris base</td>
<td>5.8 g</td>
<td>48 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>0.037 g</td>
<td>0.04% (w/v)</td>
</tr>
<tr>
<td>Methanol</td>
<td>200 ml</td>
<td>20% (v/v)</td>
</tr>
<tr>
<td>Distilled water</td>
<td>800 ml</td>
<td>--</td>
</tr>
</tbody>
</table>
**Table E. Antibodies used for protein detection by Western Blot.**

*DSTT: provided by the Division of signal transduction therapy (Dundee, UK).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company and Reference number</th>
<th>Dilution</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-p38 (Thr180/Tyr182)</td>
<td>Cell Signaling 9211</td>
<td>1:1000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>p38α</td>
<td>Santa Cruz</td>
<td>1:1000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>p38γ</td>
<td>DSTT*</td>
<td>0.5µg/ml</td>
<td>Sheep</td>
</tr>
<tr>
<td>p38δ</td>
<td>DSTT*</td>
<td>0.2µg/ml</td>
<td>Sheep</td>
</tr>
<tr>
<td>p-ERK1/2(Thr202/Tyr204)</td>
<td>Cell Signaling 9101</td>
<td>1:1000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Cell Signaling 9102</td>
<td>1:1000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>p-JNK(Thr183/Tyr185)</td>
<td>Cell Signaling 44682</td>
<td>1:1000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>JNK</td>
<td>Cell Signaling 9252</td>
<td>1:1000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>p-MKK1 (S217/221)</td>
<td>Cell Signaling 9121</td>
<td>1:1000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>MKK1</td>
<td>Cell Signaling 9122</td>
<td>1:1000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>TPL2</td>
<td>Santa Cruz 204351</td>
<td>1:500</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Abin2</td>
<td>Was kindly provided by Dr. Steve Ley</td>
<td>1:500</td>
<td>Rabbit</td>
</tr>
<tr>
<td>IκBα</td>
<td>Cell Signaling 9242</td>
<td>1:1000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>p-p105(Ser 933)</td>
<td>Cell Signaling 4806</td>
<td>1:1000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>p105</td>
<td>Cell Signaling 4717</td>
<td>1:1000</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Alexa Fluour 680 Anti-Rabbit</td>
<td>Molecular Probes, A-21109</td>
<td>1:5000</td>
<td>Goat</td>
</tr>
<tr>
<td>Alexa Flour Anti-Sheep</td>
<td>Molecular Probes A-21102</td>
<td>1:5000</td>
<td>Donkey</td>
</tr>
</tbody>
</table>

1.4 RNA extraction.

Reagents and Equipment:
- TRI Reagent, Sigma Aldrich
- Chloroform
- 2-Propanol (isopropylalcohol)
- Glycoblue, Life Technologies
- 70% (v/v) Ethanol diluted in RNAsa free water
- Nanodrop spectrophotometer

To extract RNA from samples we used the method of TRI Reagent (Sigma Aldrich) mixing with isopropanol and glycoblue for RNA precipitation and following the manufactured instructions. The RNA was washed twice with 70% Ethanol, resuspending all samples in the same volume of RNAsa free water and quantified in Nanodrop spectrophotometer.
1.5 Real-time quantitative PCR (qPCR).

Reagents and Equipment:
- cDNA samples diluted in RNAsa free water
- 5x HOT FIREPol EvaGreen qPCR Mix Plus®, Solis BioDyne
- Primers 20µM
- 384 well plate, Applied Biosystems
- ABI PRISM 7900HT, Applied Biosystems

Quantitative PCR (qPCR) was performed after converting the RNA samples to cDNA by PCR method, using 500ng of RNA per sample with High Capacity cDNA Reverse Transcription Kit, Applied Biosystems. The qPCR reaction was performed in triplicate using 5µl of cDNA per sample and 3µl of Master Mix, the master mix includes Eva Green® buffer and the primers of interest all diluted in RNAsa free water. To analyze the qPCR we used the program SDS v2 and took in account the Threshold of the samples, making sure it was adjusted at straight part of lines and noise under the threshold line. Also, we check that Ct values were within the standard curve and below 35 cycles. The samples were quantified using the comparative Ct method and compared to unstimulated WT samples from the same experiment. As housekeeping gene we used between two or three making sure that the housekeeping gene used as reference did not change with treatment. The sequences of primers used in this thesis can be found on table F.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence (5→3)</th>
<th>Reverse Sequence (5→3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>TGGTGTTGAGGTCCATT</td>
<td>CAGCAGAGGCTTTTTGTTGG</td>
</tr>
<tr>
<td>TNFα</td>
<td>CTGTAAGCCACCTGAGTGAC</td>
<td>TTGAGATCCATGGCGTGG</td>
</tr>
<tr>
<td>IL-6</td>
<td>GAGGATACACTCCCAACAGCC</td>
<td>AACTGCTATGTTTTGCTATACA</td>
</tr>
<tr>
<td>IL-10</td>
<td>CAGGACCTTTAAGGTTACTTG</td>
<td>ATTTTCACAGGGGAGAAATC</td>
</tr>
<tr>
<td>IFNβ</td>
<td>TCAAGATGAGTGTTCGTTGC</td>
<td>AACCTTCAATGCAGTATAGTCA</td>
</tr>
<tr>
<td>MIP-2 (CXCL2)</td>
<td>CCTGTTGAGAAAATCATCCA</td>
<td>CTTCCGTTGAGGGACAGC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CCCATCACCATCTTTCCAGGA</td>
<td>CGACATACTCAGCAGGCC</td>
</tr>
<tr>
<td>β-actin</td>
<td>AAGGAGATTACTTGCTGGTCTCTCTCTCTT</td>
<td>ACTCATCGTACTCCTGCTTGGT</td>
</tr>
<tr>
<td>IL-17</td>
<td>CAGGAGAGCTTCATCTGTGT</td>
<td>GCTGAGGTTGAGGGATG</td>
</tr>
<tr>
<td>IFNγ</td>
<td>GCAACAGCAAGGGCAAAAA</td>
<td>TTCTTGCTGTGTAATGCAG</td>
</tr>
<tr>
<td>IL-12 p35</td>
<td>CACCCCTCTTTGCTTCTCTCTT</td>
<td>GCCACGCTTTGCTTGGT</td>
</tr>
<tr>
<td>IL-12 p40</td>
<td>GGAAGACGGGACAGCAGGAAA</td>
<td>AAAGGGCTGAGGATAGG</td>
</tr>
<tr>
<td>IL-23 p19</td>
<td>CACCGGGGACATATGGAATGCTACT</td>
<td>CTGGTGGTCAAGCCATCTTC</td>
</tr>
<tr>
<td>CCR1</td>
<td>CCTTGTGCTGAGAATGTCGA</td>
<td>GCCAGGACAAACAAAGTCT</td>
</tr>
<tr>
<td>CCR2</td>
<td>GCGGTGATGAACCATGGTAA</td>
<td>TGCGTCAAGGACGACAG</td>
</tr>
<tr>
<td>CCR5</td>
<td>TGGTCTCCTCCCTACAGGAG</td>
<td>TTGGGAGGCTGACGATA</td>
</tr>
<tr>
<td>KC</td>
<td>CCTTGACCCTCAGAGCTCCCT</td>
<td>CCGTGCACTGCAGCAGTC</td>
</tr>
</tbody>
</table>
Table F. Sequence of primers used to detect gene expression by qPCR.
*Cox-2 taqman gene expression numb. 4331182. Applied Biosystems(MM00478374_m1).

2. Candida albicans infection.

2.1 Bone Marrow derived Macrophages (BMDM) culture.

2.1.1. L929 culture and conditioned medium preparation.

Reagents and Equipment:
- L929 cells
- Dulbecco’s Modified Eagle’s Medium (DMEM)
- Penicillin (200 units/ml) / Streptomycin (0.2mg/ml)
- 4mM L-glutamine
- Fetal bovine serum (FBS)
- Trypsin-EDTA 0.025%
- Flasks with filter caps (T25, T75, T150)
- 0.2μM Filter
- 50 ml Falcon® Tubes

L929 is a murine fibroblast-like cell line that secretes the macrophage colony-stimulating factor (M-CSF), which is a lineage-specific growth factor that is responsible for the proliferation and differentiation of committed myeloid progenitors into cells of the macrophage/monocyte lineage. L929 cells are used as a conditioned medium for the culture of bone marrow derived macrophages.

We thawed the L929 cells in T25 flasks with 6-10ml of fresh culture medium, which is DMEM supplemented with penicillin/streptomycin, L-glutamine and 10% (v/v) FBS. When cells reached 90-100% confluence, they were sequentially passage as indicated in the table G and checked every 2-3 days under the microscope for confluence. To pass the cells, culture medium was removed and the cells washed twice with sterile PBS, then we added trypsin (see table G) and incubated the flasks for 3 minutes at 37°C 5% CO₂ to detach the cells. When cells were detached fresh culture medium was added to inhibit trypsin and cells placed in the new flasks (see Table G). When we had the last flasks of cells 100% confluent we add 50 ml of fresh medium and let them incubate at 37°C 5% CO₂ for 3 days, afterwards we collected the supernatant. The supernatant was filtered, divided into aliquots of 50ml per Falcon tube and frozen at -80°C.
Table G. Sequence of passes for L929 culture.

<table>
<thead>
<tr>
<th>Flask</th>
<th>Pass to</th>
<th>ml trypsin</th>
<th>Stop reaction fresh culture medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>T25</td>
<td>3x T75</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>T75</td>
<td>3x T150</td>
<td>1.5</td>
<td>20</td>
</tr>
<tr>
<td>T150</td>
<td>5x T150</td>
<td>3</td>
<td>30</td>
</tr>
</tbody>
</table>

2.1.2 Isolation of bone marrow derived macrophages (BMDM).

Reagents and Equipment:

- DMEM
- FBS
- Ethanol 70% (v/v) (ETOH)
- Scissors, tweezers, sterile surgical blades
- 50 ml syringe
- 25G needle
- 50 ml Falcon® Tubes
- Refrigerated Centrifuge
- L929 conditioned medium (Mikron 200R, Hettich zentrifugen)
- Penicillin (200 U/ml)/Streptomycin (0.2mg/ml)
- 4mM L-glutamine
- Cell culture petri dish 60x15 mm (P6)
- CASY® cell counter and buffer

BMDM were isolated from adult WT, p38γ-/-, p38δ-/- and p38γ/δ-/-. Mice were sacrifice in a CO₂ chamber. We extracted the femur, tibia and fibula with the help of scissors and blade and the bones were washed with 70% ETOH. All muscle tissue was removed, subsequently we cut the end of the bones and flush with 20ml of DMEM supplemented with 10% FBS using a syringe with a needle passing to a 50ml Falcon tube. Afterwards the cells were washed three times by centrifuging 1,200rpm (Mikron 200R Hettich zentrifugen) at 4°C and adding DMEM 10%FBS. The cells were resuspended in a known volume and counted. The cells were cultured at 2x10⁶ cells/ml in DMEM supplemented with 20%FBS, and 10% of L929 medium. Macrophages need 7 days to differentiate; the medium is changed to fresh medium at day 3 of culture and at day 6. At day 3 the medium is refreshed with DMEM supplemented with 20%FBS, and 10% of L929 and at day 6 changed to DMEM supplemented with 0.005% FBS. After 7 days, adherent cells were removed, counted and replated at a constant density (10⁶ cells/ml). At 4-8 h after replating in DMEM with 10% FBS, cells were stimulated as indicated below.
2.2 BMDM stimulus

The different stimuli were added to the BMDM culture medium at the final concentrations indicated in the table H for the times specified in each experiment (see Results).

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Company and Reference number</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS <em>E. coli</em></td>
<td>Sigma L3024</td>
<td>100ng/ml</td>
</tr>
<tr>
<td>Imiquimod –R837</td>
<td>InvivoGen 11D21MM</td>
<td>5µg/ml</td>
</tr>
<tr>
<td>ODN-1668</td>
<td>InvivoGen 11b17MM</td>
<td>250ng/µl</td>
</tr>
<tr>
<td>TNFα</td>
<td>Sigma Aldrich</td>
<td>100ng/ml</td>
</tr>
<tr>
<td>Curdlan</td>
<td>Sigma Aldrich 54724-00-4</td>
<td>10µg/ml</td>
</tr>
<tr>
<td>Heat Killed <em>Candida albicans</em></td>
<td>InvivoGen 08J30MM</td>
<td>10⁶ colonies or cells/ml</td>
</tr>
<tr>
<td>Pam3Cys</td>
<td>Was kindly provided by Dr. Carlos Ardavín.</td>
<td>200ng/ml</td>
</tr>
</tbody>
</table>

Table H. Stimulus used to activate BMDM.

2.3 *Candida albicans* in vivo experiments.

Mice used in these experiments were described in Section 1, general methods; all mice used were female from 6 to 12 weeks old.

2.3.1 *Candida albicans* culture.

Reagents and Equipment:

- Sterile inoculation loop
- Yeast extract- Peptone-Dextrose (YPD) Agar, Sigma Aldrich (Y1500)
- Autoclave
- Cell culture 10 cm dishes (P10)
- Sterile laminar flow hood
- *Candida albicans* strain SC5314 was kindly provided by Dr. Carlos del Fresno and Dr. Carlos Ardavín.

Preparation of *C. albicans* culture plates; 65 gm of YPD Agar were suspended in 1L of distilled water, heated to boiling while stirring to dissolve completely all ingredients and autoclaved for 15 minutes at 121°C. In a sterile laminar flow hood, the liquid YPD Agar was dividing equally on culture plates. Once the YPD Agar plates were solidified they were stored at 4°C. *C. albicans* was cultured in the YPD Agar plates at 30°C for 48 hours.
2.3.2 Mouse survival experiment.

**Reagents and Equipment:**
- *Candida albicans*
- PBS
- Sterile Inoculation loop
- 15ml Falcon® tube
- 1ml Eppendorf®
- Neubauer chamber
- 1ml Syringe with 25G needles

To prepare the inoculum one colony of *C. albicans* was isolated, diluted in 1ml of PBS and cells counted. The inoculum was prepared at a final concentration of $1 \times 10^5$ cfu/200µl per mice. In survival experiments groups of 10 mice per genotype were infected intravenously and monitored daily for weight, health and survival following the institutional guidance.

2.3.3. Measurement of Colony-forming units (cfu).

**Reagents and Equipment:**
- Homogenizer (IKA T10)
- 2 ml Eppendorf®
- PBS
- YPD agar plates
- Barrier pipette tips
- Dissection material (scissors, tweezers)

At day 3 of *C. albicans* infection (Section 2.3.2), mice were sacrificed in a CO$_2$ chamber and the right kidney was extracted to determine the cfu and kidney fungal burden. The kidneys were homogenized and serial dilutions of the homogenates were planted on YPD agar plates. After 48 hours colony-forming units were counted.

2.3.4. Flow cytometry analysis (FACS) of the immune cell infiltration in *C. albicans* infected kidneys.

**Reagents and Equipment:**
- Roswell Park Memorial Institute medium (RPMI) R0883 Sigma Aldrich
- Solution A: 25ml RPMI, 3% FBS, at 37°C. (per mouse)
- Solution B: 50ml PBS-EDTA 0.5M. Keep cold. (per mouse)
- Liberase Solution:
  - 2ml RPMI
  - 0.200ml liberase (0.178mg/ml). TM Roche #05401119001
• Cell strainer 40μm Falcon #352340 (one per mouse)
• 2%FBS in PBS
• Erythrocytes lysis buffer pH 7.4:
  o 4.15g ammonium chloride (NH₄Cl)
  o 0.5g sodium bicarbonate (NaHCO₃)
  o 20mg EDTA
  o 500ml distilled water
• Cell culture plate 24 wells
• Dissection material (scissors, tweezers)
• 50ml and 15 ml Falcon® tubes
• Refrigerated centrifuge (Mikron 200R Hettich zentrigugen)

After inoculation with C. albicans, mice were sacrificed, their kidneys extracted and cut into small pieces on a cell culture plate with RPMI. The kidney pieces were digested with 2 ml of liberase solution for 20 minutes at 37°C on a shaking platform. The reaction was stopped with 10 ml of solution A, filtered through cell strainers and centrifuged at 1640 rpm for 5 minutes at 4°C. The supernatants were aspirated and washed two times with solution B centrifuging at same conditions. The cells were lysed to remove erythrocytes for 2 minutes with 1.5 ml of lysis buffer, the reaction was stopped with PBS 2%FBS, centrifuged at same conditions and cells were counted. The flow cytometry analysis was performed with 1x10⁶ cells per condition; cells were stained with combinations of fluorescence-labeled antibodies to the cell surface markers CD45, CD4, CD8, Ly6G and F4/80 and analyzed in a FACScalibur cytometer (BD Biosciences).

2.3.5. Splenocytes re-stimulation with Candida albicans

Reagents and Equipment:
• RPMI 1640
• FBS
• Refrigerated centrifuge (Mikron 200R Hettich zentrigugen)
• Cell counter
• 1ml syringes
• Cell strainers 70μm
• 24 well culture plates

Mice were infected intravenously with Candida albicans 2x10⁵ cells/200μl for 3 or 7 days. The spleen was removed and placed in RPMI, afterwards we filter the spleen by crushing it on top of the cell strainer with the plunger of a 1 ml syringe collecting a single cell suspension on a 50 ml falcon. We flushed the filter with 5ml of RPMI supplemented with 20%FBS, centrifuged at 1700 rpm for 5 minutes at 4°C.
We discarded the supernatant, and resuspended the cells in 4ml of RPMI supplemented with 20% FBS and counted the cells. We adjust the volume to 1x10^7 cells/ml.

For the experiments we used 500µl cells (5x10^6 cells/well) in 24 wells plate and add 500µl of the stimulus. We incubated for 48 hours or 5 days at 37°C 5% CO₂ and measured cytokines in the supernatant by ELISA. The incubation time depends on the cytokine, at 48 hours we measured IFNγ, IL-10, TNFα and IL-6, at 5 days we measured IL-17 and IL-22.


3.1. Perypheral blood monocytes.

3.1.1. Isolation of Peripheral blood mononuclear cells (PBMC)

Reagents and Equipment:
- Blood drawn from healthy donors
- PBS
- RPMI 1640
- Ficoll-Paque PLUS G&E healthcare 17-1440-02
- Refrigerated centrifuge (Mikron 200R Hettich zentrifugen)
- Sterile Pasteur plastic Pipettes
- Cell counter
- 50ml Falcon® tube
- Syringe with long needle

The PBMCs were separated from the erythrocytes and granulocytes by gradient of density using the Ficoll method. First we divided the blood in aliquots of 10-20ml per tube, slowly we added 15 ml of Ficoll in the bottom of the tube, under the blood, using a syringe with a long needle. Afterwards, we centrifuged at 616 x g for 20 minutes at room temperature with no brake and slow acceleration. Then we remove 2/3 of the plasma with a Pasteur’s pipette and using another pipette we remove the PBMC layer (see figure). Cells were washed with cold PBS twice, centrifuging at 1700 rpm for 10 minutes at 4°C. Afterwards, the pellet was resuspended in RPMI (1ml/20ml of blood), cells counted and the concentration adjusted to the concentration specified in each experiment in Results Chapter.
3.1.2. Monocyte isolation with Percoll.

Reagents and Equipment:
- PBMC isolated by Ficoll
- PBS
- Sodium chloride (NaCl) 1.6M
- Sterile H₂O
- RPMI 1640
- Refrigerated centrifuge (Mikron 200R Hettich zentrifugen)
- Percoll, Sigma GE17-891-02
- Syringe with long needle
- Sterile Pasteur’s pipette

After isolating the PBMCs from the blood by Ficoll (section 3.1.1) we can separate the monocytes from the lymphocytes using Percoll, a hyper-osmotic density gradient medium. First we prepared the Percoll solution adding 48.5ml of Percoll, 41.5 ml of sterile water and 10 ml of NaCl 1.6M, shaking vigorously. We separated the PBMCs in 15ml tubes at 200x10⁶ of cells per tube and centrifuged 1700rpm for 10 minutes at 4°C. The medium was aspirated, and the cells were resuspend on 3ml RPMI. We added slowly 10 ml of the Percoll solution to the bottom of the tube under the cells using a syringe with a long needle. Afterwards we centrifuged at 580g for 15 minutes at room temperature with no brake and slow acceleration. The monocytes are in the interphase we removed them using a sterile pipette and combined all for washing. We washed with cold PBS two times by centrifuging at 350g for 7 minutes at 4°C. The cells were resuspended in 3ml of
RPMI, counted and adjusted to the concentration required for the each experiment specified in Results.

3.1.3 Monocyte stimulation with LPS, Heat Killed Candida and MAPK pathways inhibition.

Reagents and Equipment:
- 80x10^6 monocytes per donor (at least 2 donors)
- RPMI 1640 medium
- FBS
- Dimethyl Sulfoxide (DMSO)
- Eppendorf
- Kinase inhibitors were kindly provided by Dr. Phillip Cohen, MRC-PPU, University of Dundee (see table)
- Cytotox96, Promega, G1780

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB203580</td>
<td>5µM</td>
<td>p38α, p38β</td>
</tr>
<tr>
<td>BIRB 796</td>
<td>1µM for p38γ and 5µM for p38δ</td>
<td>p38α, p38β, p38γ, p38δ</td>
</tr>
<tr>
<td>PD184352</td>
<td>5µM</td>
<td>M KK1</td>
</tr>
<tr>
<td>C34</td>
<td>5µM</td>
<td>TPL2</td>
</tr>
</tbody>
</table>

Table I. MAPK inhibitors.

The cells were culture in eppendorf at a concentration of 1x10^6/eppendorf and pre-incubated with the kinase inhibitors at the concentrations indicated for 1 hour at 37°C 5% CO₂, before stimulation with either HK Candida or LPS, (See Table H) as a control. Cells were stimulated for 1 hour to examine MAPK pathways activation by western blot, or for 24 hour to measure cytokine production by ELISA.

Since the inhibitors are diluted in DMSO, Cytotox 96 assay from Promega was performed as described by the manufacturers to confirm that DMSO was not toxic for the cells.

3.2. Cytokine production by ELISA.

Reagents and Equipment:
- Coating antibody (see table J)
- Coating buffer:
  - 0.1M NaHCO₃ pH 9.6
  - Solution A: 2.12 g Na₂CO₃ in 200ml distilled water
  - Solution B: 3.36 g NaHCO₃ in 400ml distilled water
  - Mix 70ml solution A + 175ml solution B until pH 9.6 is reached
- Dilution buffer: 5g BSA, Sigma A7030, in 500ml PBS
MATERIAL AND METHODS

- Blocking buffer: 2% milk powder in PBS
- Detection antibody (see table J)
- Streptavidin-HRP (see table J)
- Substrate buffer:
  - 15 g NaOAc. H₂O
  - Adjust pH to 5.5
  - Adjust volume to 1,000ml with distilled water
- TMB solution:
  - 60mg tetramethylbenzidine (TMB) Sigma T0440
  - 10ml Dimethyl Sulfoxide (DMSO) Sigma 472301
- H₂O₂ solution 3%: 100µl H₂O₂ + 900µl distilled water
- Substrate Solution: (per plate)
  - 12ml substrate buffer
  - 200µl TMB solution
  - 12µl 3% H₂O₂ solution
- Stop Solution: 1.8M H₂SO₄
  - 100ml 96% H₂SO₄
  - 900ml distilled water
- PBS wash buffer: 0.1% (v/v) Tween in PBS
- Standard curve: we reconstitute the standard for each cytokine in dilution buffer and made serial dilutions adding 100µl per well
- ELISA plate reader

First we coated the plates with the antibody of interest, the antibody was diluted in PBS 100µl were added per well and incubated overnight at room temperature. Next, the plate was washed (x5) with PBS buffer and 200 µl of blocking solution were added and incubated for 1 hour at room temperature. Afterwards, the plates were washed (x5) with PBS buffer and the samples of interest with the control standard curve were incubated for 2 hours shaking at room temperature. Next, 100µl per well of detection antibody were incubated for two hours at room temperature. Afterwards the plate was washed (x5) with PBS buffer and the streptavidin was added at the concentration indicated (see table J) and incubated for 30 minutes on a shaking platform at room temperature. Next, we washed the plate (x5) with PBS buffer and then 100µl per well of substrate solution was added and incubated at room temperature. When blue color appeared the reaction was stopped with the solution buffer. Finally, the plate was read on the reader at 450nm.
**MATERIAL AND METHODS**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Coating Ab</th>
<th>Detection Ab</th>
<th>Streptavidin</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα (R&amp;D)</td>
<td>Dilute 360x in PBS</td>
<td>Dilute 360x in Dilution buffer</td>
<td>Dilute 200x in Dilution buffer</td>
</tr>
<tr>
<td>IFNγ (Sanquin)</td>
<td>Dilute 200x in coating buffer</td>
<td>Dilute 200x in Dilution buffer</td>
<td>Dilute 20000x in dilution buffer</td>
</tr>
<tr>
<td>IL-10 (Sanquin)</td>
<td>Dilute 200x in coating buffer</td>
<td>Dilute 200x in Dilution buffer</td>
<td>Dilute 20000x in dilution buffer</td>
</tr>
<tr>
<td>IL-6 (Sanquin)</td>
<td>Dilute 200x in coating buffer</td>
<td>Dilute 200x in Dilution buffer</td>
<td>Dilute 20000x in dilution buffer</td>
</tr>
<tr>
<td>IL-17 (R&amp;D)</td>
<td>Dilute 180x in PBS</td>
<td>Dilute 180x in Dilution buffer</td>
<td>Dilute 200x in Dilution buffer</td>
</tr>
<tr>
<td>IL-22 (R&amp;D)</td>
<td>Dilute 180x in PBS</td>
<td>Dilute 180x in Dilution buffer</td>
<td>Dilute 200x in Dilution buffer</td>
</tr>
</tbody>
</table>

**Table J. Cytokines used for ELISA protocol.**

**4. Colitis-Associated Colon Cancer.**

**4.1 Colorectal cancer induction and Colitis induction.**

Reagents and Equipment:
- Azoxymethane (AOM), Sigma Aldrich,
- Dextran sodium sulfate (DSS), (MW 36,000-50,000 Da; MP Biomedicals)
- Syringe with 25G needle

Mice were described previously on Section 1. general methods, we used 10 to 14 week old female and male mice for these experiments. All mice were monitored for signs of distress and rectal bleeding.

Colorectal cancer associated to colitis was induced as described (Neufert, Becker et al. 2007). Briefly, 10- to 14-week-old mice were injected intraperitoneally with AOM (10 mg/kg). On day 5 post-injection, mice were treated with 2% DSS in drinking water for 5 consecutive days, followed by 16 days administration of normal drinking water. This DSS treatment was repeated for two additional cycles. During the course of the experiment, mice were monitored for body weight, diarrhea and macroscopic bleeding. On day 105 of the regime, mice were killed, the colon removed, washed with PBS and opened longitudinally for analysis.

In colitis experiments, mice were treated for 5 days with 3% DSS (w/v) in drinking water and then allowed to recover for additional days as indicated (see Results). Mice were monitored for signs of distress and rectal bleeding.
4.2 Colon disgregation for immune cells infiltration induced by DSS treatment.

Reagents and Equipment:
• Hank’s balanced salt solution medium (HBSS), Gibco, Invitrogen
• Penicillin (200U/ml)/Streptomycin (0.2mg/ml) (Pen/Strep)
• Blade
• HBSS 8 mM EDTA (160 µl of 0.5M EDTA in 10 ml HBSS)
• 37℃ chamber
• Falcon® tubes (50 ml, 15 ml)
• Refrigerated centrifuge (Mikron 200R Hettich zentrifugen)
• Disaggregation medium
  o HBSS
  o 0.4 mg/ml dispase, Life Technologies
  o 2x Penicillin/Streptomycin
• Fetal bovine serum (FBS)
• Cell strainers (100 µM, 70 µM, 40 µM)
• Cell counter

For crypt isolation, the colon was removed, flushed with ice-cold Hank’s balanced salt solution HBSS and incubated in HBSS with antibiotics for 15 min at room temperature (RT). The colon was cut into 0.5-cm pieces, which were incubated in HBSS with 8 mM EDTA for 15 min at 37℃ and then in HBSS; crypt epithelial cells were dissociated by repeated vigorous shaking. Tissue debris was removed with a cell strainer (100 µm) and crypts collected by centrifugation (200 x g, 15 min, 4℃).

Cell pellets were resuspended in 10 ml HBSS with 0.4 mg/ml dispase and 10,000 U/ml penicillin/streptomycin, and incubated for 30 min at 37℃. FBS was added at 5% (v/v) final concentration, and tissue debris was removed by sequential filtering through 100, 70 and 40 µm cell strainers. Cells were collected by centrifugation (150 x g, 10 min, 4℃). Colon cells were stained with combinations of fluorescence-labelled antibodies to the cell surface markers CD45, CD4, CD8, Ly6G and F4/80, and analysed in a FACS calibur cytometer (BD Biosciences). The profiles obtained were analysed with FlowJo software (BD Biosciences); leukocytes were gated as CD45+ cells.

Intracellular staining for CD27+ cells was performed using IntraPrep Permeabilization Reagent Kit. Beckman coulter (A07803).
4.3 Bone Marrow transplantation.

Reagents and Equipment:
- γ Irradiation chamber
- Donor male mice and recipient female mice
- Syringe with 25G needles
- PBS

WT and p38γ/δ-/- recipient female mice were lethally γ irradiated (10 Gy). Four hours after irradiation the mice received 4 x 10^6 cells/mouse intravenously. The bone marrow cells were obtained from femurs of WT or p38γ/δ-/- male mice. 60 days after transplantation, the chimerism was tested and mice were AOM/DSS-treated as above to induce acute colitis or CAC. The extent of bone marrow repopulation was determined by PCR analysis for the Y chromosome-linked Sry gene in genomic DNA extracted from blood samples as well as immunoblot to detect p38γ and p38δ in splenocytes.

4.4 Statistical analysis

Differences in tumour numbers and volume, in inflammation score, and in ulcers size were analyzed by the Mann-Whitney U test and the c² test. Other data were processed using the Student’s t or the one tail Mann-Whitney tests. Data were expressed as mean ± SD. p <0.05 was considered statistically significant. All data in this study were analysed statistically; for clarity of the figure, some non-significant differences are not indicated.
Results:

Chapter 1. p38γ and p38δ in inflammation and Candida albicans infection.

The recognition of pathogens is mediated by pattern recognition receptors (PRRs) such as Toll like receptors (TLRs) and C-type lectin receptors (CLRs) that interact with conserved structures of the microorganisms, the pathogen-associated molecular patterns (PAMPs) (van de Veerdonk, Kullberg et al. 2008). The activation of these receptors activates various signaling pathways including MAPKs signaling pathways, which play an important role in the response to pathogens, their activation leads to the transcription of cytokines and chemokines (Lee and Kim 2007). One of the families of MAPKs implicated in the recognition of pathogens is p38 family composed by p38α, p38β, p38γ and p38δ.

1.1 p38γ and p38δ key proteins in inflammation.

Inflammation is an important process by which the organism protects itself from pathogens. To elucidate the role of p38γ and p38δ in inflammation we started experiments with bone marrow derived macrophages (BMDM) from wild type (WT) mice, p38γ-/-, p38δ-/- and p38γ/δ-/- mice, stimulated with different ligands of toll like receptors (TLRs).

1.1.1 p38γ and p38δ modulate ERK1/2 pathway in bone marrow derived macrophages in response to the TLR4 ligand LPS.

TLR stimulation by pathogen-associated molecules such as the bacterial lipopolysaccharide (LPS), a TLR4 ligand, activates various signaling pathways crucial for the synthesis of proinflammatory molecules (Gaestel, Kotlyarov et al. 2009), which include the p38 MAPK pathway. To study the implication of p38γ and p38δ in this process we decided to perform experiments using a classical model of inflammation, the septic shock model induced by LPS.

As described in material and methods, we stimulated bone marrow derived macrophages (BMDM) from WT, p38γ-/-, p38δ-/- and p38γ/δ-/- mice with LPS from E.coli for different times and analyzed the activation of the different signaling pathways. TLR4 stimulation of myeloid cells by LPS activates all three major MAPK
RESULTS

pathways, cJUN N-terminal kinase (JNK), p38α MAPK, and ERK1/2 as well as the canonical NF-κβ pathway.

Our results showed that p38γ and/or p38δ deficiency did not affect the LPS-induced transient activation of p38α and JNK1/2 as determined by immunoblotting with phospho-specific antibodies. Although, the deactivation of JNK is less pronounced in p38γ/δ-/- compared to WT, p38γ-/- and p38δ-/- mice. Also, TLR4-induced proteolysis of the NF-κβ inhibitor IκBα was unaffected by the lack of p38γ, p38δ, or p38γ/δ (Figure 1.A).

In contrast, ERK1/2 and their activator MKK1 are phosphorylated in BMDM from WT, p38γ-/- and p38δ-/- mice stimulated with LPS but not in p38γ/δ double knock-out BMDM. Also, the expression of total protein levels of ERK1/2 and MKK1 were similar between genotypes (Figure 1.B), demonstrating that the deficiency of activation of ERK1/2 pathway in p38γ/δ-/- BMDM is not due to lower levels of these proteins in the cells. These results indicated that p38γ and p38δ together are necessary for LPS activation of ERK1/2 but not for other signaling pathways.

It has been shown that in response to LPS, MKK1 and ERK1/2 activation in macrophages is regulated by the MKK kinase TPL-2 (also known as tumor progression locus-2 and cancer Osaka thyroid Cot) (Gantke, Sriskantharajah et al. 2011). In unstimulated cells, TPL-2 is stoichiometrically complexed with the NF-κβ inhibitory protein p105 and the ubiquitin-binding protein ABIN-2, both of which are needed to maintain TPL-2 protein stability. We observed that in unstimulated and in LPS stimulated cells p38γ/δ-/- BMDM steady levels of TPL-2 and ABIN-2 were reduced compared to WT, p38γ-/- and p38δ-/- mice, whereas NF-κβ1 p105 activation and total protein levels were similar between genotypes (Figure 1.C).

These results indicate that p38γ and p38δ might modulate TPL2/ABIN2/p105 complex. Also, these results suggested that lack of p38γ/δ affects the ERK1/2 pathway regulating kinases upstream of MKK1.
Figure 1. Scheme of various signaling pathways activated in BMDM after LPS stimulation. BMDM from WT, p38γ-/-, p38δ-/- and p38γ/δ-/- mice were stimulated with 100ng/ml of LPS for the times indicated (A and B) and for 15 minutes (C). Cell lysates (50µg) were immunoblotted with the indicated specific antibodies; total protein levels of tubulin, p38αMAPK, JNK1/2, ERK 1/2 and MKK1 were measured in the same lysates as loading controls.

1.1.2 p38γ and p38δ implication in the activation of other TLRs and TNF receptor (TNFR).

After these observations, we decided to investigate if p38γ and p38δ are modulating ERK1/2 pathway activation in response to agonists that bind to other receptors. Since we did not observe any effect in ERK1/2 pathway activation in BMDM from p38γ-/- and p38δ-/- single knockout mice stimulated with LPS, we decided to use BMDM from WT and p38γ/δ-/- mice in the experiments from now on. We stimulated BMDM from WT and p38γ/δ-/- mice with different TLR agonist
and with TNFα (see the table below) and analyzed the signaling pathway activation and cytokine production.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imiquimod</td>
<td>TLR7</td>
</tr>
<tr>
<td>ODN1668</td>
<td>TLR9</td>
</tr>
<tr>
<td>TNFα</td>
<td>TNFR</td>
</tr>
<tr>
<td>Pam3Cys</td>
<td>TLR1/TLR2</td>
</tr>
</tbody>
</table>

Our data showed that there is activation of the three major signaling pathways such as JNK, p38 and NFκB (p105) with all the stimuli tested. JNK, p38α and p105 in WT and p38γ/δ-/- BMDM were activated in response to ODN, Imiquimod, Pam3Cys and TNFα at the time points indicated in Figure 2A. However, ERK1/2 and their activator MKK1 were phosphorylated only in BMDM from WT mice but not in p38γ/δ-/- cells with all these agonists (Figure 2A).

Furthermore, we also measured the cytokine production after stimulation with the different TLRs ligands, and after one-hour we observed that in response to: i) ODN, IL-1β and IL10 mRNA production was lower in p38γ/δ-/- BMDM compared to WT cells, whereas TNFα mRNA production was similar in both genotype; ii) imiquimod, the expression of all cytokines was lower in p38γ/δ-/- than in WT BMDM; and iii) TNFα, only the IL10 mRNA levels were slightly lower in p38γ/δ-/- macrophages (Figure 2B).

These results showed that, in general, in macrophages, the cytokine production is lower in p38γ/δ-/- than in WT BMDM in response to different TLR agonist and TNFR and suggest that p38γ and p38δ are modulating ERK1/2 pathway via TPL-2 probably controlling TPL-2 protein levels.
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Figure 2. Signaling pathways activated in WT and p38γδ−/− BMDM by TLRs and TNFR ligands. (A) BMDM were stimulated for the indicated times with ODN 250ng/ml ligand of TLR9, Imiquimod 5µg/ml ligand of TLR7, Pam3Cys 200ng/ml ligand of TLR2, TNFα 100ng/ml ligand of TNFR. The cell lysates (50µg) from WT and p38γδ−/− BMDM were immunoblotted with the indicated phospho-specific antibodies and the total protein antibodies as control. Representative blots are shown. (B) Relative mRNA expression was determined by qPCR for indicated genes in BMDM stimulated for 1h with the indicated
stimulus. Results were normalized to β-actin mRNA expression and the relative induction was calculated to WT expression at 0h. Data show mean ± SD (n = 3-6).

1.2 Role of p38γ and p38δ in Candida albicans infection on BMDM.

Since p38γ and p38δ are modulating ERK1/2 pathway activation in response to various TLRs ligands and TNFα, which are TPL-2 dependent, we decided to investigate their role in Curdlan signaling, in which is ERK1/2 activation is TPL-2 independent. Also, in Candida albicans infection, in which ERK1/2 activation is partially dependent on TPL-2 in response to TLRs signaling pathways. Candida albicans is a fungus that could cause death in immunocompromised patients (Wisplinghoff, Bischoff et al. 2004) and a ligand of Dectin 1, TLR -2, -4 and -9 (Brown 2006). First we examined the effect of p38γ and p38δ deletion in BMDM stimulated with Curdlan, a polymer recognized by the membrane bound Dectin-1 receptor leading to the CARD-9 dependent activation of NF-κβ and MAPK (Ferwerda, Meyer-Wentrup et al. 2008).

1.2.1 Effect of p38γ/δ deletion in BMDM stimulated with Curdlan.

BMDM from p38γ/δ−/− and WT mice were stimulated with Curdlan for the indicated times and cell lysates were immunoblotted with specific phospho-antibodies. We observed that there is activation of different major signaling pathways in BMDM stimulated with Curdlan, such as p38α, JNK and NFκB (p105) (Figure 3.A). As we have seen previously with other agonists, after stimulating cells with Curdlan there was no activation of ERK1/2 pathway in p38γ/δ−/− BMDM compared to WT. Subsequently, we measure IL-1β, TNFα and IL-10 cytokine production, which are important mediators of Dectin-1 signaling pathway, in BMDM from WT and p38γ/δ−/− mice stimulated with Curdlan for one hour. We observed a lower induction of IL-1β expression in p38γ/δ−/− BMDM compared to WT, whereas TNFα and IL-10 had similar expression in both genotypes in response to Curdlan (Figure 3.B).

These results indicate that p38γ and p38δ also be modulate Dectin-1 signaling pathway by regulating ERK1/2 pathway activation probably by controlling the TPL-2 protein level.
1.2.2 p38γ/δ deletion reduces cytokine production and ERK1/2 activation in BMDM activated by *Candida albicans*.

Induction of proinflammatory cytokines by PRRs is an important part of the immune response to *Candida albicans* to recruit and activate phagocytes to clear and control the infection (Heinsbroek, Taylor et al. 2008). To investigate if p38γ and p38δ are modulating the immune response to *Candida albicans* by modulating cytokine production we stimulated BMDM from WT and p38γ/δ-/- mice with heat...
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killed *Candida albicans* (HK Candida) for the indicated times. Afterwards, we measured by qPCR the mRNA expression levels of important cytokines involved in *Candida albicans* signaling pathway such as IL-1β, IL-6, TNFα, IL-10, IFNβ and the chemokine CCL2. The mRNA expression of all inflammatory mediators was transiently increased after stimulation with HK Candida in both WT and p38γ/δ-/- BMDM (Figure 4.A). IL-1β, IL-10 and CCL2 mRNA levels were significantly diminished in BMDM from p38γ/δ deficient mice at one hour of stimulus compare to WT. We did not observed significant differences between genotypes in the mRNA expression of other cytokines, such as IL-6, IFNβ and TNFα.

Furthermore, we analyze major signaling pathways activated by *Candida albicans* in BMDM from WT and p38γ/δ-/- activated with HK Candida. Cell lysates were immunoblotted with specific phospho-antibodies and we observed there was activation of different mayor signaling pathways such as p38α and NFκβ(p105) in BMDM from both genotypes. We did not detect JNK activation in our experimental conditions; however, we found that JNK basal activation was higher in WT than in p38γ/δ-/- BMDM (Figure 4.B). In p38γ/δ-/- BMDM there is no activation of ERK1/2 in response to HK Candida (Figure 4.B), as we have seen with other agonists. Since TPL2 expression is very low in p38γ/δ deficient cells compare to WT. p38γ/δ might be modulating *Candida albicans* pathway on a TPL2 dependent way.

These results indicated that p38γ and p38δ regulate important cytokines expression in response to HK Candida by controlling ERK1/2 pathway activation, and suggest that these two kinases might modulate *Candida albicans* infection *in vivo*. 
Figure 4. p38γ and p38δ deletion modifies the response of BMDM to Candida albicans. (A) Relative mRNA expression was determined by qPCR for the indicated genes in WT and p38γ/δ-/- BMDM stimulated with HK Candida 10^6 cfu/ml for the indicated times. Results were normalized to β-actin mRNA. Data show mean ± SD (n = 3-6). *p ≤0.05 and ***p ≤0.001, relative to WT mice in the same conditions. (B) BMDM from WT and p38γ/δ-/- mice were stimulated with HK Candida 10^6 cfu/ml for the indicated times. Cell
lysates (50µg) were immunoblotted with the indicated antibodies. Representative blots are shown.

1.3 p38γ and p38δ modulate *Candida albicans* infection *In vivo*.

Next, we decided to investigate *Candida albicans* infection in p38γ/δ-/- mice compared to WT. The infection was induced injecting intravenously (i.v.) groups of WT and p38γ/δ-/- mice with *Candida albicans* as described in material and methods. We measured different parameters in infected mice, such as the survival, the fungal burden, the infiltration of immune cells and the cytokine production in splenocytes from *Candida albicans* infected mice and then re-stimulated *in vitro* with HK Candida.

1.3.1 p38γ/δ-/- mice are protected from *Candida albicans* infection.

To determine if p38γ and p38δ are modulating *Candida albicans* infection on a mouse infection model, we started monitoring the survival of p38γ/δ-/- mice compared to WT after inoculation with a lethal dose of 1x10⁵ cfu of *Candida albicans*. We found that WT mice started dying earlier than p38γ/δ-/- mice. Ten days after infection 90% of WT mice had died versus 30% of p38γ/δ-/- mice, also p38γ/δ-/- mice survive significantly longer to the infection (Figure 5.A).

To determine the role of p38γ/δ-/- on fungal dissemination we measure the fungal burden in the major organs of infected mice. We collected kidney, liver and spleen from WT and p38γ/δ-/- mice infected with *Candida albicans* and analyzed the presence of fungal cells by counting the colony forming units (cfu). We observed more infection in kidney compare to other organs, which is in agreement with previous reports (MacCallum 2009). Therefore, to investigate the inflammatory response to *Candida albicans* we focused on the immune response and pathology of the kidney. Groups of p38γ/δ-/- and WT mice were infected with indicated doses of *Candida albicans* and kidney samples were collected at day 3 of infection. Since our data showed that WT mice started dying at day 5 of infection to avoid premature death of the animals before sample collection we chose day 3 as a referring point. We found that at lower doses of *Candida albicans* the fungal burden was smaller in p38γ/δ-/- than in WT kidney; nonetheless, this difference between genotypes is lost at high dose and there were no statistically significant
differences in the fungal burden between genotypes at any doses of *Candida albicans* (Figure 5.B).

To investigate if p38γ and p38δ are modulating cytokine production *in-vivo*, we injected p38γ/δ-/- and WT mice with 2x10^5 cfu of *Candida albicans* for 0, 1 or 3 days and measured in mouse serum important cytokines implicated in *Candida albicans* immune response, such as IL-1β, TNFα, IL-17 and IFNγ. We observed a general decreased in cytokine production in p38γ/δ-/- mice compared to WT. IL-1β and TNFα were slightly decreased in p38γ/δ-/- at day 1 and 3 of infection compared to WT mice. Whereas, IL-17 and IFNγ production was notably decreased in p38γ/δ-/- mice at day 1 compared to WT.

These results suggest that p38γ and p38δ are modulating the immune response to *Candida albicans* infection but do not alter significativelly fungal clearance, indicating that other mechanism confer resistance in p38γ/δ-/- mice to *Candida albicans* infection.
Figure 5. p38γ and p38δ deletion provokes protection to Candida albicans infection. (A) p38γ/δ−/− and WT mice were i.v. injected with a lethal dose of 1x10⁵ cfu of Candida albicans and survival was monitored for 30 days. The data presented as a Kaplan-Meier survival curve is a summary of three independent experiments (n=10 mice per genotype) **p<0.001. (B) WT and p38γ/δ−/− mice were infected with the indicated doses of Candida albicans and kidney fungal burden was analyzed at day 3 of infection. Data show mean ± SD. Data presented are from three independent experiments per dose (n= 3-5 mice per genotype and per experiment). (C) p38γ/δ−/− and WT mice were i.v. injected with 2x10⁵ cfu of Candida albicans for 1 or 3 days and cytokine production was measured in mouse serum by Luminex. Representative data is shown (n=3 mice per genotype).

1.3.2 p38γ and p38δ deletion reduces innate immune response to Candida albicans infection.

Since we did not found a significant difference on the fungal burden between genotypes, we decided to study the infiltration of immune cells in these mice during Candida albicans infection. WT and p38γ/δ−/− mice were infected i.v. with
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*Candida albicans* 2x10⁵ cfu and mouse kidneys were collected at day 0 and at an early stage of infection days 1 and 3. We measured the infiltration of various leukocyte (CD45+ cells) such as macrophages F4/80+ cells, neutrophils Ly6G+ cells and T cells CD4+ and CD8+ by flow cytometry. The number of total renal-infiltrating leukocytes (CD45+ cells) increased after *Candida albicans* infection in both genotypes. At day 1 the leukocyte number was similar between genotypes, whereas at day 3 was significantly lower in p38γδ-/− compared to WT (Figure 6.A). The characterization of leukocytes from control and *Candida albicans* infected animals showed that at day 1 the number of macrophages (F4/80+ cells), neutrophils (Ly6G+ cells) and CD8+ cells was higher in p38γδ-/− infected kidneys than in WT. However, at day 3 the recruitment of macrophages and neutrophils was significantly smaller in p38γδ-/− mice than in WT (Figure 6.A). CD4+ T lymphocyte numbers were similar between genotypes (Figure 6.A). When we compared the percentage of cells, we observed a significant reduction of Ly6G+ cells neutrophils in p38γδ-/− treated mice at day 1 and 3 of infection compare to WT. Also, we observed a slight reduction of macrophages F4/80+ cells at day 1 of infection in p38γδ-/− mice compare to WT, although at day 3 of infection F4/80+ were slightly higher in p38γδ-/− mice compared to WT. There were no significant differences on CD4+ and CD8+ T cells between genotypes. (Figure 6B-C).

These results suggest that p38γ and p38δ modulate an earlier immune response to *Candida albicans* infection.
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Figure 6. Reduced neutrophil recruitment in p38γδ−/− mouse kidney. WT and p38γδ−/− mice were i.v. injected with Candida albicans 2x10⁵ cfu for 0, 1, 3 days and kidney cells were stained with anti-CD45, anti CD4, anti CD8, anti-Ly6G and anti F4/80 antibodies. (A) The total cell number and (B and C) percentage of positive cells were analyzed by flow cytometry. Representative day 3 of infection profiles are shown (B).
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(B) CD45^+ cells were gated and percentages of CD4^+, CD8^+, Ly6G^+ and F4/80^+ cells analyzed by flow cytometry. In (A) and (C) Data show mean ± SD. Data presented are from three independent experiments (n= 3-5 mice per genotype and per experiment).ns, not significant; * p ≤0.05; ** p ≤0.01, *** p ≤0.001, relative to either control mice or to WT mice in the same conditions (as indicated with lines).

1.3.3 p38γ/δ modulate cytokine production upon in-vitro splenocytes re-stimulation.

After finding differences in the infiltration of neutrophils, we decided to measure the amount of IL-17 production in the Candida albicans infection model. The IL-17A expressed by T cells controls the neutrophil-mediated response in inflammation(van de Veerdonk, Gresnigt et al. 2009). Since the spleen is the major source of T cells, we performed experiments using splenocytes from WT and p38γ/δ^-/- mice infected with Candida albicans and then re-stimulated in-vitro with HK Candida. Re-stimulation of the cells is necessary to be able to detect IL-17 production.

Groups of WT and p38γ/δ^-/- mice were infected i.v. with Candida albicans 2x10^5 cfu for 3 or 7 days. The spleens were collected at 0, 3 or 7 days post-infection and splenocytes were cultured and re-stimulated with HK Candida, or LPS as a control, for 48 hours or 5 days. Cytokine production was measured by ELISA, after 48 hours of re-stimulation with HK Candida we analyzed IFNγ and IL-10 production. We found a higher production of IFNγ in splenocytes from WT mice infected for 3 days compared to p38γ/δ^-/- mice, whereas at day 3 of infection IL-10 expression was higher in p38γ/δ^-/- mice compared to WT and at day 7 of infection IL-10 expression was lower in p38γ/δ^-/- mice compared to WT. After 5 days of in vitro splenocytes re-stimulation with HK Candida we analyzed IL-17 and IL-22 expression. The production of these two cytokines by splenocytes from mice infected for 3 and 7 days was lower in p38γ/δ^-/- compared to WT mice. However, when cells were re-stimulating with LPS, we observed a very small cytokine expression and we did not find differences between genotypes (Figure7).

These results suggest that p38γ and p38δ might be modulating Candida albicans infection in a non T cell dependent way.
Figure 7. Re-stimulation of splenocytes with HK Candida modulates cytokine expression in p38γδ-/- mice. WT and p38γδ-/- mice were infected with Candida albicans 2x10⁵ cfu for 0, 3 and 7 days, afterwards splenocytes were re-stimulated in vitro with HK Candida 1x10⁶ cfu/ml or LPS 100ng/ml for 48 hours or 5 days. Cytokine production was measured in the supernatants by ELISA. Data show mean ± SD. Data presented are from three independent experiments (n= 3-5 mice per genotype and per experiment).

Chapter 2. Human monocytes infection by Candida albicans.

The implication of p38γ/p38δ and TPL-2 in the response of BMDM to Candida albicans in vitro and also in vivo in the infection model, prompted us to continue researching their implication in a human cell model using peripheral blood mononuclear cells (PBMCs) derived monocytes using a combination of various kinase inhibitors.
2.1 TPL-2 modulates ERK1/2 activation in HK Candida activated PBMCs monocytes.

To investigate the implication of TPL-2 in a human cell activation model, we started studying the expression of p38 MAPK in PBMCs derived monocytes of healthy donors. Cell lysates were immunoblotted with specific antibodies. We observed there is expression of p38 MAPK; p38α, p38γ and p38δ (Figure 8.A).

Furthermore, to elucidate the mechanism by which ERK1/2 activation is regulated in the Candida albicans pathway we performed experiments with various MAPK inhibitors, such as PD184352 inhibitor of MKK1, C34 inhibitor of TPL-2, and the p38 inhibitors: SB203580 that inhibits p38α and p38β. PBMCs derived monocytes from healthy donors were treated with the different MAPK inhibitors for one hour and afterwards stimulated for one hour with HK Candida or LPS as a control. Cell lysates were immunoblotted with specific antibodies. We observed that in cells treated with PD184352, specific inhibitor of MKK1 (Risco, del Fresno et al. 2012), or with C34, specific inhibitor of TPL2 (Wu, Green et al. 2009), there was impaired activation of ERK1/2 pathway after stimulation with Heat Killed Candida, compared to control PMBCs or PMBCs pre-treated with SB203580, specific inhibitor of p38α/p38β (Risco, del Fresno et al. 2012). Also, we observed treating the cells with PD184352 and stimulating afterwards with LPS there was no phosphorilation of ERK1/2.

These results indicate that TPL-2 might be regulating Candida albicans signaling pathway by modulating ERK1/2 activation.
Figure 8. MAPKs activation in Candida albicans stimulated PBMC. (A) PBMCs derived monocytes from healthy donors cell lysates (30µg) were immunoblotted with the indicated antibodies. Representative blots are shown from three donors (D1,D2 and D3) (B) PBMCs derived monocytes were treated with indicated inhibitors for one hour and then stimulated with HK Candida or LPS for one hour. Cell lysates (30µg) were immunoblotted with indicated antibodies. Representative blots are shown from two donors (D1 and D2).
2.2 TPL2 is essential for *Candida albicans* induced cytokine production in PBMCs derived monocytes.

PBMCs derived monocytes from healthy donors were treated for one hour with the inhibitors previously mentioned and then stimulated for 24 hours with HK Candida or LPS as a control. Supernatants were collected and expression of important immune modulators cytokines, such as TNFα, IL-6 and IL-10 were measured by ELISA. We did not observe a significant effect on TNFα and IL-6 cytokine expression induced by HK Candida on PBMCs derived monocytes treated with p38 inhibitor SB203580, although there is a slight inhibition of IL-10 production in the same conditions. However, we observed inhibition of TNFα, IL-6 and IL-10 production induced by LPS in these cells treated with SB203580 (Figure 9) as it has been previously reported in BMDM and monocytes (Kuma, Sabio et al. 2005). Treatment with the compound PD184352 caused a decrease in all cytokine production in response to HK Candida at high concentrations of the inhibitor, although this was only statistically significant in IL-10 production at high concentrations. In response to LPS the MKK1 inhibitor significantly impaired the production of TNFα, but not of the other cytokines (Figure 9). When cells were treated with the TPL-2 inhibitor, the compound C34, we found that in response to HK Candida, and also to LPS, the production of all cytokines was severely impaired (Figure 9).

These data indicates that TPL-2 is an important modulator of the immune response to *Candida albicans* infection and confirm the implication of ERK1/2 pathway in *Candida albicans* infection in humans.
Figure 9. TPL2 modulate cytokine production in the immune response to *Candida albicans* infection. PBMCs derived monocytes were treated with different MAPK inhibitors, C34 inhibitor of TPL2, PD184352 inhibitor of MKK1 and SB203580 inhibitor of p38 MAPK family at 0.5µM, 1.5µM and 5µM, for 1 h and then stimulated with HK Candida 10⁶ cfu/ml or LPS 100ng/ml. Cytokine production was measured on the supernatants by ELISA. Representative graphs of three independent experiments are shown. Data show mean ± SD (n= 3 donors). ns, not significant; * p ≤0.05; ** p ≤0.01, *** p ≤0.001, relative to cells stimulated in the absence of inhibitor.
Chapter 3. Role of p38γ and p38δ in linking inflammation and cancer.

Cancer and chronic inflammation are intimately associated, yet the precise mechanism of this association remains largely unknown (Hanahan and Weinberg 2011). p38MAPK signaling has been implicated in the regulation of processes leading to cancer development and progression. To study more deeply these implications we decided to investigate the role of p38γ and p38δ in colitis-associated colon cancer model. Previous results from our group demonstrated that p38γ/δ−/− affects the process of tumor formation (del Reino, P .2012). For a better understanding of the implication of p38γ/δ in this process we analyze the effect of p38γ and p38δ deletion in colon tumor formation and determine the inflammation and cytokine production in the colon of p38γ/δ−/− mice compare to WT.

3.1 p38γ/δ deletion decreases colitis-associated tumor incidence (CAC)

To study the role of p38γ and p38δ in CAC cancer, we used a well-described protocol that combines the use of the carcinogen AOM with DSS-induced colitis (Figure 10.A). AOM/DSS protocol induces tumors in the distal part of the colon of rodents and is commonly used to elicit colorectal cancer in experimental animals. This protocol was applied to WT, p38γ−/−, p38δ−/− and p38γ/δ−/− and at week 15 the mice were sacrificed and observed for tumor development. All mice developed tumors mainly in the distal to middle colon (Figure 10.B), which is the predominant site of human colorectal tumors. We observed no differences in the histopathology of the tumors between all genotypes (Figure 10.C). In contrast, p38γ−/−, p38δ−/− and p38γ/δ−/− mice showed a significant decrease in tumor numbers and in total tumor volume per mouse compared with WT mice; the decrease in p38γ/δ−/− mice was more pronounced than in the other genotypes (Figure 10.D and 10.E). Individual tumor size distribution and average tumor size were similar in all genotypes, although p38γ−/− and p38γ/δ−/− mice had slightly fewer large tumors than p38δ−/− and WT mice (Figure 10.F).

Reduced tumor incidence with no apparent change in size suggests a role for p38γ and p38δ early in colon tumor initiation or development.
**RESULTS**

Figure 10. Reduced incidence of CAC cancer in p38γ/δ−/− mice. WT, p38γ−/−, p38δ−/− and p38γ/δ−/− mice were treated with AOM/DSS, and colon tumors were analyzed at week 15. (A) Scheme of AOM/DSS protocol. (B) Representative colons showing tumors in the distal region (arrow). (C) Representative H&E-stained colon sections showing colon tumors; bars 200µm. (D) Tumors were counted at week 15, dot represents a single mouse.
3.2 p38γ/δ regulates cytokine production in DSS-induced colitis mouse model.

Transcription of inflammatory cytokines is largely mediated by NF-κβ and MAPK signaling pathways (Kim and Pasparakis 2014), and we have shown that p38γ/δ regulates cytokine production by modulating ERK1/2 pathway in macrophages and in dendritic cells (Risco, del Fresno et al. 2012). To measure the effect of p38γ/δ in the immune response to colitis we treated p38γ/δ-/- WT and compared to WT mice with 3% DSS for the indicated time points (in collaboration with Paloma del Reino) and measure the cytokine production. DSS treatment induced lower expression of the inflammatory mediators TNFα, IL1β, IL6 and COX-2 in p38γ/δ-/- than in WT mice. IL-12p35, IFNβ, IL23p19 and IL17A expressions were higher in p38γ/δ-/- mice, whereas IL-10 and IL12p40 expressions were similar to WT (Figure 11).

These results suggest that p38γ/δ, modulate the inflammatory response in colon in response to DSS.

![Figure 11. p38γ/δ reduces DSS-induced cytokine production.](image) Relative mRNA expression was determined by qPCR for indicated genes in DSS-treated WT and p38γ/δ-/- mouse distal colon and normalized to GAPDH mRNA. Data mean ± SD (n=5-6), *p < 0.05, relative to WT mice in the same conditions.
3.3 Reduced immune cells infiltration in p38γ/δ-/- DSS-treated colon.

In addition to impaired activation of signaling pathways involved in cytokine production (Risco, del Fresno et al. 2012, Del Reino, Alsina-Beauchamp et al. 2014), reduced leukocyte infiltration might cause the reduced inflammation and cytokine production in DSS-treated p38γ/δ-/- mouse colon. To test this possibility we studied the hematopoietic cell recruitment after DSS treatment in WT and p38γ/δ-/- mice.

We measured by FACs the infiltration of immune cells in colon DSS-treated for 0 and 5 days and observed significantly less macrophage F4/80+ cells and neutrophil Ly6G+ cells in p38γ/δ-/- compare to WT mice. However, we observed no variation on the percentage of CD4+ and CD8+ T lymphocytes between genotypes (Figure 12).

![Figure 12. Immune cells infiltration in DSS-treated WT and p38γ/δ-/- mice.](image)

Colon crypt cells from 0 to 5 day DSS-treated WT and p38γ/δ-/- mice were stained with anti CD45, anti-CD4, anti-CD8, anti-Ly6G and anti-F4/80 antibodies and the percentage of CD45 gated positive cells were analyzed by flow cytometry. Representative profiles are shown. Data mean ± SD (n>20 mice/condition); *P ≤ 0.05; ** P ≤ 0.01; ***P ≤ 0.001.
RESULTS

Furthermore, we analyzed the expression of chemokines receptors implicated in macrophage and neutrophil migration CCR1, CCR2, CCR5 by qPCR and observed similar expression in both genotypes (Figure 13.A). Since we did not observe differences in cytokine production between genotypes at the last time points of the DSS treatment (15 and 20 days; Figure 10) we decided to analyze only at short times, 0, 5 and 10 days the expression on chemokines for macrophage (MCP1) and neutrophil (KC, MIP-2) migration. We observed in response to DSS a higher production of MCP1, KC and MIP-2 in the colon from WT mice compared to p38γ/δ mice (Figure 13.B).

These results indicate that a decrease in chemokine production in p38γ/δ-/- mice cause a reduction in macrophages and neutrophils infiltration.

We also examined the percentage of T regulatory cells, which are involved in intestinal inflammation (Serebrennikova, Tsatsanis et al. 2012). After DSS treatment, p38γ/δ-/- colon had a slightly lower percentage of Treg cells than WT

Figure 13. Reduced chemokine production in the colon from p38γ/δ-/- mice.
(A-B) Relative mRNA expression for indicated genes in DSS-treated WT and p38γ/δ-/- mouse colon for the indicated time points was determined by qPCR and normalized to GAPDH mRNA. Data mean ± SD (n=5-6), *p ≤ 0.05.
mice, whereas Treg cells in the thymus (where these cells are generated) are similar in p38γ/δ−/− and WT mice (Figure 14).

All these findings suggest that p38γ and p38δ modulate the procarcinogenic local environment and inflammation in CAC.

**Figure 14.** T regulatory cells infiltration in p38γ/δ−/− and WT DSS-treated mice. Colon crypt and thymus cells from 0 to 5 day DSS-treated WT and p38γ/δ−/− mice were stained with anti-CD4 and anti-CD127 the percentage of positive cells were gated on CD127 and analyzed by flow cytometry. Representative profiles are shown, data mean ± SD (n>20 mice/condition).

3.4 p38γ/δ in hematopoietic cells modulate colon tumor formation.

To identify the cell compartment responsible for decreased tumorigenesis in p38γ/δ−/− mice, we generated reciprocal bone marrow chimeric mice by transplanting WT bone marrow into p38γ/δ−/− mice (WT→p38γ/δ−/−) or WT (WT→WT) hosts, and p38γ/δ−/− into p38γ/δ−/− (p38γ/δ−/−→p38γ/δ−/−) or WT (p38γ/δ−/−→WT) mice. After the bone marrow transplantation we treated the mice with 3% DSS to cause inflammation as described before and measured cytokine and chemokine production by qPCR. We observed less production of the cytokines IL-1β, TNFα and less expression of neutrophil migration chemokine KC in mice reconstituted with p38γ/δ−/− bone marrow than WT bone marrow reconstituted mice (Figure 15).
These findings suggest that p38γ/δ depletion in hematopoietic cells is important for cytokine and chemokine production.

![Graph showing cytokine production in DSS-treated Wt and p38γ/δ chimeric mice.](image)

**Figure 15. Cytokine production in DSS-treated Wt and p38γ/δ chimeric mice.** Relative mRNA expression was determined by qPCR for indicated genes in 5-day DSS-treated chimeric mouse colon and normalized to GAPDH mRNA. Data mean ± SD (n=6-9); ns, not significant; *, P < 0.05.

Also, we analyzed immune cells infiltration by flow cytometry in these mice. Mice reconstituted with p38γ/δ−/− bone marrow had less amount of macrophage F4/80+ cells and neutrophils Ly6G+ cells in DSS-induced cell recruitment compared to WT bone marrow reconstituted mice (Figure 16). These results suggest that p38γ and p38δ play an important role in hematopoietic cells for the DSS-induced immune cells recruitment.

![Graph showing immune cell infiltration](image)
RESULTS

Figure 16. Immune cells infiltration on DSS-treated WT and p38γ/δ chimeric mice. Colon crypt cells from 5-day DSS-treated chimeric mice were stained with anti-CD45, anti-Ly6G, and anti-F4/80 antibodies and the percentage of positive cells analyzed by flow cytometry. Data mean ± SD (n>10 mice/condition); *, P < 0.05.

Furthermore, after the bone marrow transplantation we treated the mice with AOM/DSS colitis associated to cancer protocol described before and analyzed in the colon of these mice the tumor incidence and tumors histopathology. When mice were treated with the AOM/DSS protocol, WT→p38γ/δ-/- and WT→WT had more tumors than p38γ/δ-/-→WT and p38γ/δ-/-→p38γ/δ-/- mice (Figure 17.A). Tumors from all chimeric mice were histologically similar (Figure 17.B).

These results confirm that the depletion of p38γ/δ in hematopoietic cells is essential for the immune response resulting in decreased tumorigenesis.

Figure 17. WT chimeric mice with p38γ/δ-/- bone marrow show reduced CAC incidence.(A) Chimeric mice were AOM/DSS- treated to induce CAC and colon tumors analyzed at week 15. Tumors were counted and total volume determined by summing all volumes for a given mouse. Dot, a single mouse; ns, not significant, *, P < 0.05; ** P ≤ 0.01. (B) Representative H&E- stained colon sections showing colon tumors; bars, 200μM.

All of these data showed that p38γ/δ expression in hematopoietic cells is critical for production of inflammatory mediators and cell recruitment that lead to colon tumor development, and indicate that absence of p38γ and p38δ in the hematopoietic compartment is responsible for decreased tumorigenesis in p38γ/δ -/- mice.
DISCUSSION
Discussion:

Chapter 1. Candida albicans infection

Despite the research on fungal immunity and the discoveries on fungal treatment, the cause of death from Candida albicans infection remains very high in immunocompromised patients (Wisplinghoff, Seifert et al. 2006). Therefore, the complete understanding of Candida albicans infection mechanism is necessary for the development of new therapeutic approaches. In this thesis we described for the first time the role of two alternative p38MAPK, p38γ and p38δ, in Candida albicans infection.

1.1 p38γ and p38δ are key proteins in inflammation.

Inflammation is the body’s attempt to protect itself from harmful stimuli it is an important part of the immune response. The innate immune response constitutes the first line of host defense during infection (Mogensen 2009). p38 MAPK have been implicated in innate immunity in processes such as apoptosis, survival, cell differentiation and proliferation (Cuenda and Rousseau 2007). Also, studies have implicated the p38 family in inflammatory diseases and cancer (Hommes, van den Blink et al. 2002, Kim, Sano et al. 2008, Otsuka, Kang et al. 2010), the majority of research is based in p38α and little is known about the alternative isoforms p38γ and p38δ biological roles.

We showed in Section 1.1.1 the implication of p38γ and p38δ in inflammation using a septic shock model induced by LPS. First, we demonstrated that these two kinases modulate ERK1/2 pathway in response to the TLR-4 ligand LPS. In bone marrow derived macrophages (BMDM) the deletion of p38γ/δ blocks the ERK1/2 pathway activation, showing lower levels of phospho-MKK1, a kinase upstream of ERK1/2 and also of phospho-ERK1/2 itself. p38γ/δ did not affect the activation of other MAPK pathways such as JNK and p38α, as well as the NFκB pathway in response to LPS. Also we observed that BMDM from p38γ/- and p38δ/- mice had no impaired the activation of ERK1/2 pathway in response to LPS, this indicates that both kinases are necessary for LPS induced modulation of ERK1/2 pathway and that p38γ and p38δ have redundant functions. Furthermore, we showed that p38γ and p38δ are modulating ERK1/2 pathway through the MKK kinase TPL-2,
which forms a ternary complex with the proteins p105 and ABIN-2 (Gantke, Sriskantharajah et al. 2011). It has been described that in vivo p105 and ABIN-2 positively regulate the ERK1/2 signaling pathway by stabilizing TPL-2 (Lang, Symons et al. 2004). Our data demonstrate that the steady protein levels of TPL-2 and ABIN-2, but not p105, were reduced in p38γ/δ-/- BMDM and indicate that p38γ and p38δ control TPL-2 and ABIN-2 protein levels. The reduction in TPL-2 and ABIN-2 proteins resulted from post-transcriptional effects since Tpl2 and Abin2 mRNA levels were unaffected by p38γ/δ deficiency (Risco, del Fresno et al. 2012). In rescue experiments in p38γ/δ-/- mouse embryonic fibroblasts (MEF), over-expression of p38δ and p38γ, restored TPL-2 and ABIN-2 protein to levels similar to those in WT MEF. Also, in p38γ/δ-/- BMDM the expression of TPL-2 increased ERK1/2 activation and TNFα production in response to LPS (Risco, del Fresno et al. 2012). Therefore, we conclude that p38γ and p38δ together are modulating TLR-4 signaling pathway by regulating TPL-2 and ABIN-2 protein levels, which subsequently modulates MKK1-ERK1/2 pathway activation.

TLRs are pattern recognition receptors (PRRs) implicated in immune response to microbes. Their implication in microbe recognition has been showed to be essential for innate immunity (Mogensen 2009), and agonists of several TLRs have been used for therapeutic interventions, such as cancer immunotherapy (Damm, Wiegand et al. 2014). As observed with the TLR-4 ligand LPS, p38γ and p38δ might be modulating other TLR pathways via ERK1/2. To further demonstrate the implication of p38γ and p38δ in the immune response we used cells lacking these two isoforms and stimulated them with different TLR ligands.

In Section 1.1.2 we demonstrated that p38γ and p38δ modulate ERK1/2 pathway via TLR1/2, TLR7, TLR9 and TNFR. First we showed in BMDM from WT and p38γ/δ-/- mice that ODN1668 induced MAPKs activation such as p38 and JNK, and the phosphorylation of p105. ODN1668 are synthetic CPGs oligonucleotids, which are recognized by TLR-9 and have been used to study DNA-associated autoimmune diseases (Yoshida, Nishikawa et al. 2011). The stimulatory effect of bacterial DNA on TLR9 depends on unmethylated cytosine-phosphate-guanosine (CpG) dinucleotides (Damm, Wiegand et al. 2014). We showed that ODN1668 induction diminished activation of MKK1-ERK1/2 pathway in p38γ/δ-/- BMDM
and did not affect p38MAPK, JNK or NFκβ. Same results were observed with the TLR7 ligand Imiquimod, an imidazoquinoline that has been approved for viral treatments (Kang, Park et al. 2009); also, with the TLR1 and TLR2 ligand Pam3Cys, a synthetic tryacilated lipopeptide potent activator of NFκβ (Peng and Zhang 2014), and with the TNFR ligand TNFα. Therefore, p38γ and p38δ are modulating TNFα and various TLR pathways by regulating ERK1/2 pathway activation, probably by controlling TPL2 protein levels, which indicates an important role of these p38 isoforms in inflammation.

Overall, we showed that after stimulation with ODN1668, Imiquimod and TNFα, the production of important inflammatory cytokine was reduced in BMDM lacking p38γ/δ compared to WT macrophages. These results indicate that p38γ and p38δ have a role in inflammation by modulating cytokine production induced by TLR9, TLR7 and TNFα signaling. Also, we observed that the modulation of TNFα, IL1β and IL-10 production by p38γ and p38δ was not similar with the different ligands; this indicates that the molecular mechanism(s) by which these p38s regulate cytokine production depend on the stimuli and shows the complexity of the innate immune responses.

1.2 p38γ and p38δ regulate Candida albicans infection and BMDM stimulation.

Candida albicans is recognized by different PRRs, it has been demonstrated that the implication of MAPKs is important for the immune response to the infection (van de Veerdonk, Kullberg et al. 2008). To determine the unknown role of p38γ and p38δ in the inflammatory response to Candida albicans infection we first studied their implication in Dectin-1 pathway. Dectin-1 is a CLR, which is important for the recognition of Candida albicans by recognizing the β-glucans of the fungi (Marakalala, Vautier et al. 2013).

In Section 1.2.1 we demonstrated for the first time the implication of p38γ and p38δ in Dectin-1 pathway. For this, we stimulated BMDM from WT and p38γ/δ/-/- mice with Curdlan, which is a β-glucan high-molecular-weight polymer of glucose and has been shown to be a specific ligand of Dectin-1 (Ferwerda, Meyer-Wentrup et al. 2008). BMDM lacking p38γ/δ stimulated with Curdlan showed diminished
activation of MKK1-ERK1/2 pathway although the activation of other pathways such as JNK, p38α and NFκβ were unaffected compared to WT BMDM. These results demonstrated that p38γ and p38δ are modulating ERK1/2 pathway via Dectin-1 and suggest that TPL-2 might be implicated in Dectin-1 signaling pathway, as happen in TLR-4 signaling (Figure 1). Further studies need to be done to elucidate the exact mechanism by which p38γ and p38δ are regulating TPL-2 and ABIN-2 protein levels in macrophages and their implication in Dectin-1 signaling pathway.

![Figure 1. p38γ and p38δ implication in Dectin-1 signaling pathway.](image)

Furthermore, p38γ and p38δ might be implicated in the inflammatory response in Dectin-1 signaling pathway by modulating ERK1/2 pathway and cytokine expression, since we observed that in response to Curdlan the IL-1β cytokine production is diminished in BMDM lacking p38γ/δ compared to WT.

After observing an implication of p38γ and p38δ in Dectin-1 pathway we attempted to study the role of these two isoforms in Candida albicans infection, using Heat Killed Candida as a stimulus. Heat Killed Candida has been used in many studies to investigate the effect of Candida albicans in inducing host cytokine
production; this form of the fungus exposes the β-glucans of the wall producing a Dectin-1 dependent response in both human mononuclear cells and murine macrophages (Gow, Netea et al. 2007). In Section 1.2.2, we demonstrated for the first time an implication of p38γ/δ in Candida albicans infection. BMDM lacking p38γ/δ showed impaired activation of ERK1/2 pathway after Heat Killed C.albicans induction, although other important pathways such as, JNK, p38α and NFκβ were not affected. Therefore, p38γ/δ might be also modulating ERK1/2 pathway by regulation of TPL2.

The production of inflammatory molecules such as IL-1β, IL-10, CCL-2 and IFNβ, that play an important role in Candida albicans immune response (van de Veerdonk, Joosten et al. 2009) is decreased in BMDM from p38γ/δ-/- mice compared to WT, whereas IFN-β mRNA induction is mildly increased in p38γ/δ-/- BMDM in response to Candida albicans. These results agree with what found in p38γ/δ-/- BMDM stimulated with LPS (Risco, del Fresno et al. 2012) and p38γ/δ-/- colon treated with DSS (Del Reino, Alsina-Beauchamp et al. 2014), and suggest that p38γ and p38δ play an important role in the antifungal host defense. Since, it has been demonstrated that IL-1β is crucial for this process (van de Veerdonk, Joosten et al. 2009), IL-1β cytokine production has been reported to induce the development of Th17 cells. Th17 responses are associated with chronic inflammation and autoimmune diseases and are critical for mucosal and epithelial host defense against extracellular bacteria and fungi (van de Veerdonk, Gresnigt et al. 2009).

1.3 p38γ and p38δ regulate Candida albicans infection in an in vivo mouse infection model.

The immune response to a pathogen is a key factor to the host resistance to it; in many cases the response is more important than the pathogen itself. Many studies have shown that cytokine impairment is a key factor to candidiasis and that fungus induced-inflammation contributes to tissue damage and mortality (Majer, Bourgeois et al. 2012). Examining the fungal Candida albicans burden in kidney we found that both WT and p38γ/δ-/- mice were infected, however p38γ/δ-/- mice were more resistant to Candida albicans infection and showed a significant longer
survival compared to WT mice. Also, cytokine production in p38γ/δ-/- mice serum was lower compared to WT coinciding with our observations in BMDM from p38γ/δ-/- mice that showed, in general, less cytokine production compare to WT, these results indicate that the decrease observed in cytokine production in p38γ/δ-/- mice could be essential for the survival to *Candida albicans* infection in this mice. For example, it has been shown that IL-10 plays an early regulatory innate response to *Candida albicans* infection (Romani, Puccetti et al. 1994), and that low IL-10 expression increases resistance to *Candida albicans* in mouse (Moore, de Waal Malefyt et al. 2001).

It has been reported that in a mouse model of infection to *Candida albicans*, the organ most infected is the kidney which leads to the overall pathology of the disease (MacCallum 2009). *Candida albicans* fungal burden analyses indicate that p38γ and p38δ modulate an early immune response to the infection. Immune cell infiltration is an important process of the initial immune response. Studies have shown that neutrophils are key factors of immune response to *Candida albicans* infection (Trautwein-Weidner, Gladiator et al. 2015). Interestingly, we show a significantly reduced amount of CD45+ immune cells, including F4/80+ cells (macrophages) and Ly6G+ cells (neutrophils) at day 3 in response to *C. albicans* infection in the kidney of p38γ/δ-/- mice compare to WT. As previously reported by others significantly more neutrophils accumulate in the kidney during the first 24 hours after the infection, when neutrophil presence is critical for *Candida* control (Lionakis, Lim et al. 2011). On the other hand, we showed that total numbers of immune cells such as, CD45+ cells, F480+ cells and Ly6G+ cells were significantly higher at day 1 of infection in p38γ/δ-/- mice compared to WT.

These results suggest that p38γ/δ-/- mice survive longer to the infection because p38γ and p38δ might be modulating an early immune response. Also, we demonstrated that at 24 hours of infection the accumulation of neutrophils is slightly higher in p38γ/δ-/- mice compare to WT. However, at day 3 post infection the neutrophil infiltration in WT mice increases while in p38γ/δ-/- mice decreases. These results support our hypothesis indicating that p38γ/δ modulate an earlier response to *Candida albicans* infection, producing less inflammatory cytokines, less immune cells infiltration and longer survival.
DISCUSSION

Important cytokines implicated in *Candida albicans* infection such as, IL-17, IL-22 and IFNγ are detected at a longer stage of the infection. They are dependent on T-cell immune response, to be able to measure these cytokines we stimulated WT and p38γ/δ knockout mice with a lethal dose of *Candida albicans*, afterwards we re-stimulated the splenocytes of infected mice with Heat Killed *C. albicans in vitro*. In Section 1.3.3, we showed that p38γ/δ knockout mice had lower expression of IL-17, IL-22, IFNγ at day 3 and day 7 post infection compare to WT, coinciding with our results observed in the serum of *Candida albicans* infected mice. It has been shown that IL-17 and IL-22 are both involved in T helper Th17 response; they mediate a protective antifungal host defense (van de Veerdonk, Gresnigt et al. 2009). Also, IL-17/-/ mice are more susceptible to *Candida albicans* infection showing a reduced survival and an increase in fungal burden compare to WT mice (Huang, Na et al. 2004). Our data demonstrates that p38γ/δ regulate T cell function and that p38γ/δ-/- mice have a reduction of cytokines involved in a T helper response compared to WT. Also we observed no differences on T cell CD4+ and CD8+ between genotypes. These results suggest that p38γ and p38δ might be mediating the immune response to *Candida albicans* infection in non T cell dependent regulation pathway.

Chapter 2. Human monocytes infection by *Candida albicans*

The use of a mouse model has been of great help for the understanding of *Candida albicans* infection; however, the use of primary human cells could be a closer approach to elucidate the infection mechanism in humans. MAPK pathways play an important role in the immune response and they have been implicated in the response to *Candida albicans* infection (Netea, Brown et al. 2008). JNK, ERK1/2 and p38αMAPK are expressed and activated in response to *Candida albicans* in human monocytes. To study the implication of these MAPKs signaling pathways in the regulation of *Candida albicans* infection, we perform experiments using specific inhibitors of the different MAPK pathways. In Section, 2.2 we showed for the first time the implication of TPL-2 in *Candida albicans* infection in human monocytes. Our data demonstrates that PMBCs derived monocytes treated with PD184352 (specific inhibitor of M KK1) (Risco, del Fresno et al. 2012) or with C34 (specific inhibitor of TPL2) (Wu, Green et al. 2009) showed impaired activation of ERK1/2
pathway after stimulation with Heat Killed Candida, compared to control PMBCs or PMBCs pre-treated with SB203580 (specific inhibitor of p38α/p38β).

Also, we showed that PBMCs derived monocytes treated with C34, specific inhibitor of TPL2, showed a highly significant inhibition of TNFα, IL-6 and IL-10 cytokine production in HK Candida stimulated cells. As it has been described we also observed inhibition of cytokine production by C34 pre-incubation when stimulating the cells with LPS (Green, Hu et al. 2007). Also we observed there is a slight lower cytokine expression in cells treated with PD184352 inhibitor of MKK1. However, the use of SB203580 inhibitor of p38α/β MAPK did not affect the cytokine production after HK *C. albicans* stimulation. Although, PBMCs cytokine production after LPS stimulation was inhibited by pre-treatment with SB203580 as it has been described (Kuma, Sabio et al. 2005). Therefore, these results confirm ERK1/2 pathway is important for *Candida albicans* infection and implicates for the first time TPL2 in *Candida albicans* pathway. TPL2 has been described as a key factor in immune response (Risco, del Fresno et al. 2012) also, plays an important role in cytokine release of inflammatory cells, which is critical for the immune response (Lee, Choi et al. 2015).

Taken together, our work shows for the first time the implication of TPL2 in *Candida albicans* infection. Our data in a mouse infection model suggest that p38γ and p38δ are regulating ERK1/2 pathway by modulating TPL2 expression. Also, our data indicate that p38γδ-/- mice are protected from *Candida albicans* infection (having a longer survival to the infection) by modulating the infiltration of immune cells and by controlling cytokine production through ERK1/2 pathway activation. In addition, we showed that cytokine production in HK Candida stimulated PBMCs was inhibited by C34, TPL2 specific inhibitor. Further, studies need to be performed to understand the mechanism behind the p38γδ modulation of TPL2 pathway.

**Chapter 3. p38γ and p38δ decreases colitis-associated cancer.**

Growing evidence shows that cancer and chronic inflammation are intimately associated (Hanahan and Weinberg 2011); however, the exact mechanism connecting these two processes remains unknown. It is important to understand this connection since the incidence of cancer is growing on a daily basis (Hagland
and Soreide 2015). In this thesis, we addressed the implication of p38γ and p38δ in colorectal carcinogenesis and intestinal inflammation, using the well established cancer-associated to colitis model AOM/DSS in mice lacking p38γ/−, p38δ/− or both isoforms.

In Section 3.1, we showed that after AOM/DSS treatment mice lacking both p38γ/δ develop a significant reduced amount of tumors in the distal part of the colon, whereas p38γ and p38δ single knock-out mice had partial effect on tumor incidence compared to WT mice. These results indicate that is important to block both p38γ and p38δ to observe significantly less number of tumors after this protocol, indicating that these isoforms might be modulating each other. Also, since we observed no differences in the histopathology of the tumors and there was no apparent change in tumor size between genotypes, p38γ and p38δ might be modulating early tumor initiation or development.

Since the immune response is important for the tumor initiation, in order to study the implication of p38γ and p38δ in this process we analyzed the cytokine production in DSS induced colitis model. Cytokine production is crucial for the immune response, studies have shown the importance of TLRs signaling pathways activation and subsequent cytokine production in human colorectal cancer (Lu, Kuo et al. 2015). In Section 3.2, we demonstrated that the DSS-induced expression of important inflammatory mediators such as, TNFα, IL-1β, IL-6 and COX-2 was diminished in p38γ/δ/− mouse colon compare to WT. Also, we observed that the expression of IL-12p35, IFNβ, IL-23p19 and IL-17A cytokines were higher in p38γ/δ/− mice, whereas IL-10 and IL-12p40 expressions were similar to WT. These results demonstrate that p38γ and p38δ are modulating the immune response in a colitis model. Previous data from our group showed that p38γ and p38δ are modulating the immune response by controlling the expression of TPL-2 (Risco, del Fresno et al. 2012). Consistent with our results, it has been shown that TPL-2/− mice are more resistant to a DSS colitis induced model. Also, DSS-treated TPL2/− mice showed lower expression of IL-1β, TNFα and IL-6 (Lawrenz, Visekruna et al. 2012). Furthermore, similar results in IL-12p35 and IFNβ mRNA expression in p38γ/δ/− DSS-treated mouse colon were observed in LPS treated p38γ/δ/− and TPL-2/− macrophages (Gantke, Sriskantharajah et al. 2011, Bandow,
Kusuyama et al. 2012, Risco, del Fresno et al. 2012). Therefore, our findings suggest that p38γ and p38δ are modulating inflammatory response by regulating, at least in part, the expression of TPL-2.

Common pathological changes associated with inflammatory bowel disease (IBD), which one of the major conditions for developing colorectal cancer, include a massive increased of immune cells infiltration (Wang, Dubois et al. 2009). Thus in Section 3.3, we measured the infiltration of immune cells in colons from p38γ/δ−/− DSS-treated mice and compared with WT DSS-treated mice. Our data shows significantly less macrophage and neutrophil infiltration in p38γ/δ−/− mice compare to WT. However, we did not observe significant differences in T lymphocytes, CD4+ and CD8+ cells, between genotypes. It has been described that macrophages and neutrophils are essential for tumor microenviorment and progression (Grivennikov 2013). Therefore, the lower infiltration of macrophages and neutrophils observed in DSS-treated p38γ/δ−/− mice could explain the reduced incidence of CAC observed in these mice.

Chronic inflammation associated with development of cancer is partly driven by the chemokine system. Chemokines are chemoattractant cytokines that recruit leukocytes from the circulatory system to local inflammatory sites (Wang, Dubois et al. 2009). To explain the reduced immune cells infiltration observed in p38γ/δ−/− mice compare to WT, we analyzed the expression of chemokines receptors implicated in macrophage and neutrophil migration such as, CCR1, CCR2 and CCR5. Our data showed similar expressions of these chemokines between genotypes. Therefore, we decided to analyze the expression on chemokines for macrophage (MCP1) and neutrophil (KC, MIP-2) migration. Our results showed a significantly higher production of MCP1, KC and MIP-2 chemokines in WT mice compare to p38γ/δ−/− mice. Indicating that the observed reduction in macrophage and neutrophil infiltration in p38γ/δ−/− mice might be caused by the decrease in their chemokine production.

T regulatory cells have been described as important mediators of intestinal inflammation. They have a protective role in a variety of human tumors, including colon cancer (Serebrennikova, Tsatsanis et al. 2012). Therefore, we decided to investigate if they are modulating the colitis-associated tumor incidence in p38γ/δ−/− mice. Our data demonstrated that after DSS treatment p38γ/δ−/− colon
had a slightly lower percentage of Treg cells compare to WT mice, whereas Treg cells in the thymus, where these cells are generated, were similar between genotypes. All these results together indicate that p38γ and p38δ modulate the procarcinogenic local environment in CAC.

To identify the cell compartment responsible for decreased tumorigenesis in p38γδ−/− mice we did bone marrow transfer experiments. In Section 3.4, we demonstrated for the first time that p38γ and p38δ expression in hematopoietic cells is the main contributor to colon tumor formation. Our data showed that when WT mice where reconstituted with bone marrow from p38γδ−/− mice they express lower levels of DSS-induced cytokines and chemokines compare to WT bone marrow reconstituted mice. Also, these mice showed less macrophage and neutrophil recruitment. Furthermore, we analyze tumor development with AOM/DSS protocol after bone marrow transplantation. We demonstrated that WT mice reconstituted with p38γδ−/− bone marrow developed less tumors compare to WT bone marrow reconstituted mice. Indicating that the deletion of p38γ and p38δ in the hematopoietic compartment is critical for production of inflammatory mediators and for the infiltration of immune cells leading to the decreased tumorigenesis in p38γδ−/− mice.

Taken together our results demonstrated that p38γ and p38δ are critical in the immune response, modulating the cytokine production and immune cells infiltration probably by modulating ERK1/2 signaling pathway and TPL-2 stability. The different models studied in this thesis indicate that these isoforms are important therapeutic targets in inflammatory diseases and cancer. Further studies need to be done to elucidate the mechanism by which p38γ and p38δ are modulating ERK1/2 signaling pathway and cytokine production.
CONCLUSIONS
Conclusions:

1. p38\(\gamma\) and p38\(\delta\) might regulate ERK 1/2 pathway activation and cytokine production in response to different TLR agonist, TNFR and *Candida albicans* in BMDM by modulating TPL2 levels.

2. p38\(\gamma\) and p38\(\delta\) modulate in vivo the immune response to *Candida albicans* infection by regulating neutrophil infiltration and cytokine production.

3. TPL-2 is an important modulator of the immune response to *Candida albicans* infection in humans monocytes, suggesting an implication of ERK1/2 in Dectin-1 signaling pathway.

4. p38\(\gamma\) and p38\(\delta\) play an important role in colon tumor initiation and development, and modulate the procarcinogenic local environment and inflammation in CAC.

5. p38\(\gamma/\delta\) depletion in hematopoietic cells is crucial for the immune response in a CAC model.
Conclusiones:

1. p38γ y p38δ regulan en macrófagos derivados de medula (BMDM), la activación de la vía de ERK1/2 y la producción de citoquinas en respuesta a diferentes ligandos de TLR, TNFR y Candida albicans modulando los niveles TPL-2.

2. p38γ y p38δ modulan la respuesta inmune a Candida albicans regulando la infiltración de neutrófilos y la producción de citoquinas.

3. TPL-2 es un factor importante en la respuesta inmune a la infección por Candida albicans en monocitos humanos, lo que sugiere una implicación de ERK1/2 en la vía de señalización de Dectin-1.

4. p38γ y p38δ tienen un papel importante en la iniciación y desarrollo de tumores en el colon y modulan el ambiente procarcinógeno e la inflamación en el cáncer de colon asociado a colitis.

5. La depleción de p38γ y p38δ en células hematopoyéticas es crucial para la respuesta inmune en el modelo de cáncer de colon asociado a colitis.


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CURRICULUM VITAE

Personal Details
First Name, surname: Dayanira Alsina Beauchamp
Date of birth: 7-03-1985
Nationality: American

Current professional status
FPI grant, PhD student
Department of Immunology and Oncology
National Research Center (CNB-CSIC), Madrid, Spain
Phone number: 915854558
E-mail: dalisna@cnb.csic.es

Internships:
(1) Radboud University Medical Center, Nijmegen. Internal Medicine Department. Project: Role of 
$p38_{\gamma}$ and $p38_{\delta}$ in 

Research lines: Study of the physio-pathological role of signal transduction pathways involved in 
the response to cellular stress, environmental insults and inflammatory cytokines.

Publications


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Professional organizations
2013 Member of Spanish Biochemistry and Molecular Biology Society (SEBBM)

Co-tutor students
Sundary Sormendy: “Role of p38γ and p38δ in the immune response” AECC student (2012)
Carmen Vela: laboratory practice (February-May 2013)
Noelia Aparicio: laboratory practice (October 2013- June 2014)

Others
2014/present Toastmaster International Organization member
2012/present Organization Committee “Poster Session” Immunology and Oncology Department CNB-CSIC
2013 Organization Committee “Retreat” Immunology and Oncology Department CNB-CSIC