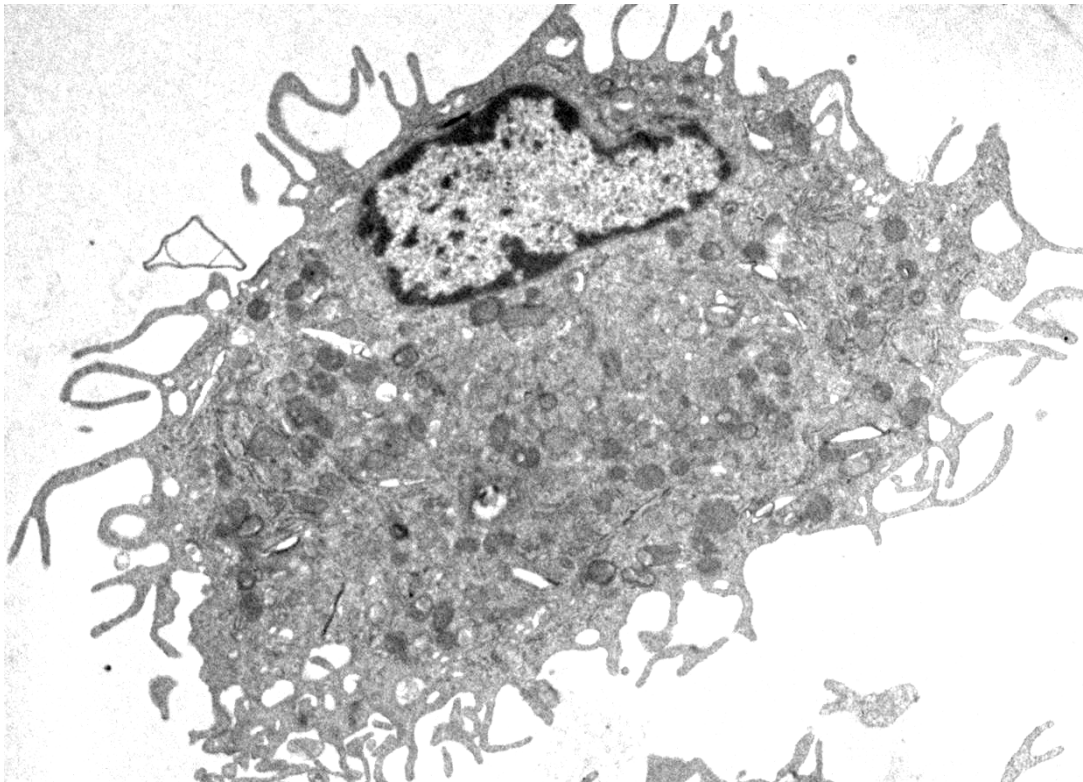# Early steps regulating proliferation

and activation in macrophages

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INTRODUCTION

INTRODUCTION

1. MACROPHAGES

Macrophages are extremely versatile phagocytic-monocytic cells involved in a number of complex functions in disease and health. They are critical to the establishment of proper defenses against invading pathogens and to the maintenance of homeostasis, by promoting angiogenesis, tissue remodeling and repair, and by scavenging debris (Mantovani, Sozzani et al. 2002).

Under the effect of growth factors, macrophages proliferate but the presence of microbial agents, cytokines or inflammatory molecules blocks this proliferation and induces functional activities such as secretion of proinflammatory cytokines (Plowden, Renshaw-Hoelscher et al. 2004). In inflammation, activated macrophages release toxic metabolites and consequently, microbes are eliminated by phagocytosis takes place (Schroder, Hertzog et al. 2004). In absence of stimulus, macrophages die through apoptosis, balancing the production and the elimination of effector cells (Celada and Nathan 1994; Xaus, Comalada et al. 2001). Macrophage heterogeneity reflects the plasticity and versatility of these cells in response to exposure to microenvironmental signals.

1.1 Origin of macrophages

The process of the formation and development of mature blood cells resulting from the survival, proliferation, lineage-commitment and differentiation of early progenitors is called haematopoiesis (Ogawa 1993). It provides a mechanism by which totipotent stem cells differentiate to all the cells present in blood giving a mechanism of normal cellular turnover. All these events are coordinated through interactions with cells (including adipocytes, fibroblastic reticular cells, endothelial cells and resident macrophages), extracellular matrix components of the surrounding microenvironment and soluble factors that are produced locally or have arrived from the circulation (Compston 2002).

Early in the development of a blood cell, pluripotent stem cells with self-renewing capacity reside inside specialized haematopoietic tissues such as bone marrow and spleen red pulp. These cells are in a quiescent state until they receive signals aimed at the up regulation of haematopoietic events. In response to specific growth factors, interleukins and hormones, these cells undergo two sequential differentiating processes. The first is commitment, by which stem cells lose their self-renewing capability

differentiating to other cells with a more limited differentiating potential. The second is maturation, which allows the terminal differentiation of those cells committed to a specific lineage. There are several soluble haematopoietic regulators acting on pluripotent stem cells known to arrive from the circulation or produced in a paracrine, juxtacrine or autocrine manner. Of these, the colony-stimulating factors have proven essential for the development of cells of different blood lineages (Watowich, Wu et al. 1996).

In bone marrow during adult life or in fetal liver, IL-1, IL-3 and/or IL-6 induce stem cell division that gives rise to a new stem cell and a pluripotent myeloid cell also referred to as granulocyte-erythrocyte-megakaryocyte-macrophage colony-forming unit (GEMM-CFU). In the presence of IL-1 and/or IL-3, this precursor is committed to becoming a progenitor of both macrophages and granulocytes known as the granulocyte-macrophage colony-forming unit (GM-CFU) which is also committed to the macrophage colony-forming unit (M-CFU) by the macrophage colony stimulating factor (M-CSF), the granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin IL-3. Subsequently, from the M-CFU (macrophage colony forming unit) there is diversion either to the osteoclast, myeloid dendritic cells or to the macrophage lineage. Differentiation along the macrophage lineage assumes that the macrophage colony forming unit (M-CFU) differentiates in the presence of the M-CSF to monoblast, promonocyte, monocyte and differentiated macrophages. Although monocytes are generally smaller than their immediate precursors, the differentiation of promonocytes to monocytes involves an enhanced phagocytic capability and the development of the lysosomal system (Valledor, Borras et al. 1998; Furukawa 2002).

Monocytes leave the bone marrow and travel through the peripheral blood vessels to all areas of the body. Circulating monocytes thus enter all tissue compartments recruited by chemotactic chemokines through selectin and integrin adhesion molecules to the endothelium (Penna, Vulcano et al. 2002). Once there, monocytes differentiate to macrophages increasing their lysosomal content, the amount of hydrolytic enzymes, the number and size of mitochondria and the extent of their energy metabolism. Although tissue macrophages have complete proliferative capability and do not depend on bone marrow monocytes to maintain their population in basal conditions, our bone marrow produces approximately 5×10^9 macrophages per day (Xaus, Comalada et al. 2001). In any case, as half-life of tissue macrophages could be very long (years), it is crucial that in the absence of cytokines or growth factors, apoptosis mechanisms become activated. In the presence of LPS and interferon gamma (IFN-γ), macrophages get activated; their proliferation program stops and they acquire effector functions (Xaus, Comalada et al. 2001).
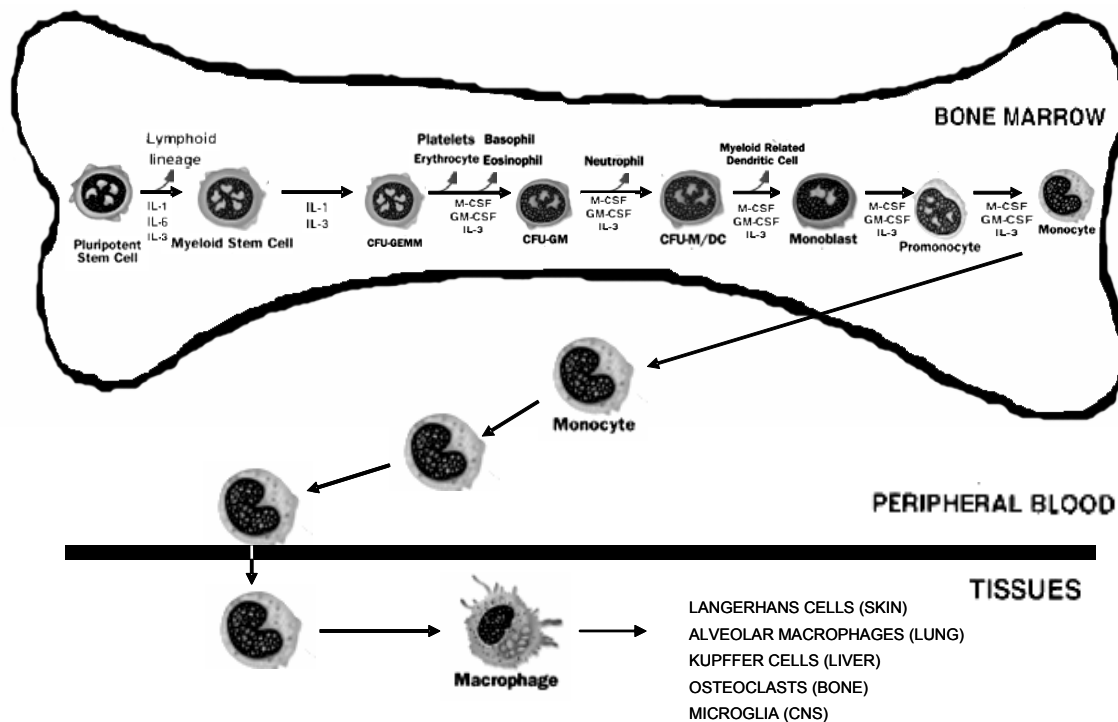
Figure 1. Differentiation of macrophages. The growth and differentiation of macrophages depends on lineage-determining growth factors and cytokines. After being distributed through blood stream, monocytes enter all tissue compartments of the body ceasing to proliferate and differentiating. M-CSF, macrophage colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; GEMM-CFU, granulocyte-erythrocyte-megakaryocyte-macrophage colony-forming unit; GM-CFU, granulocyte-macrophage colony-forming unit and M-CFU, macrophage colony-forming unit.

The function of macrophages depends on the tissue in which they reside. Dendritic cells, microglia (central nervous system), osteoclasts (bone), Kupffer cells (liver), alveolar macrophages (lung), class A cells (joints) and Langerhans cells (dermis) have their origin in the macrophage cell lineage. Macrophage development involves the regulation of several transcription factors regulating the lineage-specific expression of a number of genes and the subsequent establishment of the macrophage phenotype (Valledor, Borras et al. 1998). Known genes that determine a macrophage phenotype are those that code for the M-CSF receptor, essential for efficient response to M-CSF, lysozyme, adhesion molecules CD11b (Mac-1) and CD18, LPS receptor (CD14), IFN-γ receptor, Scavenger receptors and receptors for the constant region of IgG. Resident macrophages are also identified by specific F4/80 expression (McKnight, Macfarlane et al. 1996).

Conversely, macrophages may regulate erythropoiesis by secreting several cytokines, including IL-1, TNF-α, IL-12, M-CSF, GM-CSF and TGF-β, affecting cellular turnover (Miller, Carlino et al. 1992; Hamilton, Whitty et al. 1993).

1.2. Biochemistry of differentiation

The genetic program that leads to monocyte differentiation is determined by combinations of specific transcription factors (Valledor, Borras et al. 1998). The joint expression of PU.1, C/EBP proteins and AML1/CFBβ during myelopoiesis accounts for the myeloid-specific expression of the M-CSF and GM-CSF receptors required for differentiation, proliferation and survival of macrophages (Hohaus, Petrovick et al. 1995; Smith, Hohaus et al. 1996).

PU.1-coding gene Spi-1/Sfp-1 is a member of the ets family of transcription factors and is located on chromosome 11 in humans and chromosome 2 in mice (Klemsz, McKercher et al. 1990). Its tissue distribution is restricted specifically to haematopoietic lineage cells such as B cells, monocytes/macrophages, mast cells, neutrophils and early erythroblasts (Celada, Borras et al. 1996; Lloberas, Soler et al. 1999).

Regulation of PU.1 activity is mediated by post-transcriptional modifications such as serine phosphorylation by protein kinases such as casein kinase CKII which is involved in the phosphorylation of other transcription factors, including Myc and Myb (Celada, Borras et al. 1996). PU.1 null mice showed that this transcription factor is required for normal differentiation of myeloid cells to macrophages but not essential for commitment to the myeloid lineage (McKercher, Henkel et al. 1999). During the generation of macrophage colony forming units (M-CFUs) and their subsequent maturation, PU.1 also transactivates other genes involved in the acquisition of a functional macrophagic phenotype. Some of these genes code for adhesion molecules of terminal differentiation CD11b and CD18, the LPS receptor (CD14), IFN-γ inducible high-affinity FcγRI and low-affinity FcγRIIIA, required for recognition of the constant region of IgG, and scavenger receptors type I and II (Pahl, Scheibe et al. 1993; Moulton, Semple et al. 1994; Zhang and Dixon 1994; Rosmarin, Caprio et al. 1995). A number of additional promoters have been recognized to be regulated by PU.1, including those for the genes encoding IL-1β and TNF-α (Smith, Carl et al. 1998).

During monocyte maturation, the expression of other transcription factors is also induced. These include EGR-1, HOXB7, NF-Y and some members of the proto-oncogene Jun/Fos super family that participate in the proliferation and subsequent differentiation of macrophage colony forming unit (M-CFU) cells (Valledor, Borras et al. 1998).

The loss of proliferation associated with monocytic maturation seems to be caused by two parallel mechanisms. First, the expression of c-Myb and c-Myc is repressed early on during terminal monocyte differentiation, probably due to activation of certain Stat proteins, such as Stat3 and DIF. And second, the expression of the transcription factor IRF-1 is induced in promonocytes activating transcription of the IFN-α/β genes, which act as autocrine signals that inhibit monocyte proliferation (Valledor, Borras et al. 1998).

1.3. Biological functions of macrophages

Macrophages participate in innate and adaptative immune response recognizing, phagocytosing and clearing invading pathogens or apoptotic cells through the expression of pattern recognition receptors. Macrophages also participate in immune modulation through the production of cytokines and chemokines, antigen presentation and the regulation of T cell activation and differentiation. They also participate in resolution of inflammation and in the promotion of healing through induction of matrix synthesis, fibroblast proliferation, angiogenesis and the clearance of cellular debris (Stout and Suttles 2004).

Microbial antigens lead to the classical activated macrophages induced by proinflammatory microbial molecules such as LPS in a Th1 cytokine environment (IFN-γ produced by T lymphocytes), production of tumor necrosis factor alpha (TNF-α) and release of inflammatory and/or microbicidal products in tissues. These cells can be identified by their ability to produce nitric oxide (NO) combined with an increased expression of major histocompatibility complex (MHC) class II and CD86 enhancing their antigen-presenting capacity (APC, antigen presenting cell) to T lymphocytes. Activated macrophages play an essential role in protection against intracellular pathogens. They also exert anti-proliferative activities secreting NO and pro-inflammatory cytokines (TNF-α, IL-1, IL-6). However, the persistence of inflammatory processes often results in tissue damage and the immune system faces a permanent challenge for developing anti-inflammatory mechanisms (Gordon 2003).

The development of these activated macrophages is inhibited by Th2 cytokines; as a result, they switch to alternative activation program (Noel, Raes et al. 2004). M2 macrophages are activated in the presence of IL-4 and IL-13 and deactivated in the presence of IL-10. M2 Macrophages, in contrast to M1 macrophages, fail to generate NO from L-arginine and do not efficiently limit the growth of intracellular pathogens. Moreover, they exhibit enhanced endocytic and phagocytic ability and increased expression of MHC class II molecules for antigen presentation. As such, M2 Macrophages

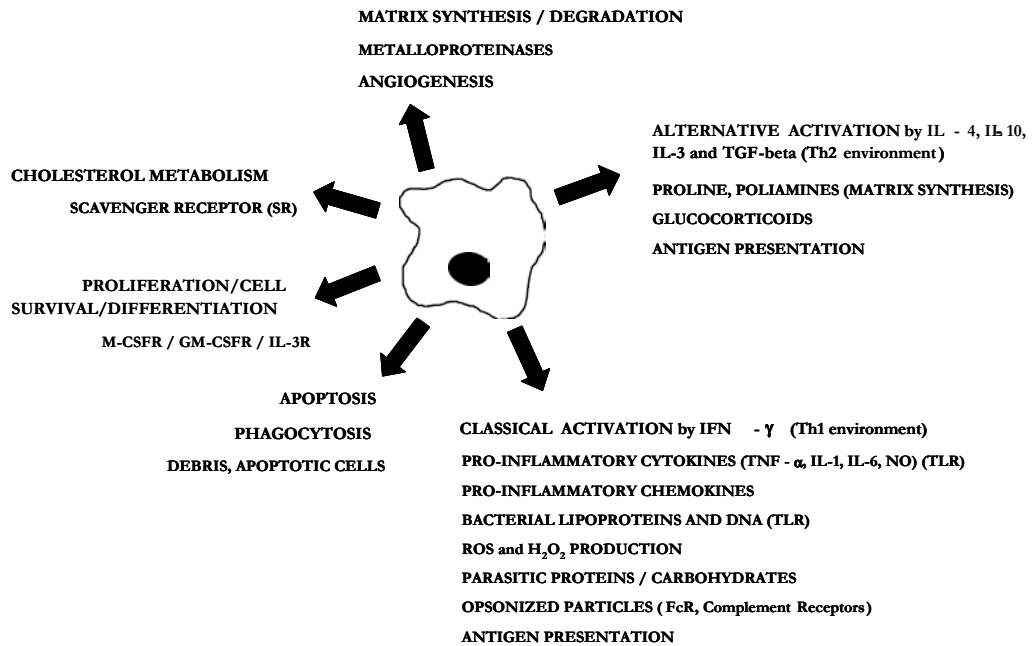protect organs and surrounding tissues against detrimental immune responses promoting tissue remodeling.

MATRIX SYNTHESIS / DEGRADATION
METALLOPROTEINASES
ANGIOGENESIS

ALTERNATIVE ACTIVATION by IL - 4, IL 10, IL-3 and TGF-beta (Th2 environment)
PROLINE, POLIAMINES (MATRIX SYNTHESIS)
GLUCOCORTICOIDS
ANTIGEN PRESENTATION

CHOLESTEROL METABOLISM
SCAVENGER RECEPTOR (SR)

PROLIFERATION/CELL SURVIVAL/DIFFERENTIATION
M-CSFR / GM-CSFR / IL-3R

APOPTOSIS
PHAGOCYTOSIS
DEBRIS, APOPTOTIC CELLS

CLASSICAL ACTIVATION by IFN - γ (Th1 environment)
PRO-INFLAMMATORY CYTOKINES (TNF - α, IL-1, IL-6, NO) (TLR)
PRO-INFLAMMATORY CHEMOKINES
BACTERIAL LIPOPROTEINS AND DNA (TLR)
ROS and H_2O_2 PRODUCTION
PARASITIC PROTEINS / CARBOHYDRATES
OPSONIZED PARTICLES (FcR, Complement Receptors)
ANTIGEN PRESENTATION

Figure 2. Biological functions of tissue macrophages. Depending on the stimulus, macrophages proliferate, activate or die through apoptosis. IL, interleukin; TGF-β, transforming growth factor-β; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; NO, nitric oxide; TLR, Toll-like receptor; M-CSFR, macrophage colony stimulating factor receptor; GM-CSFR, granulocyte-macrophage colony stimulating factor receptor and IL-3R, interleukin-3 receptor.

The terminal differentiation of macrophages involves the expression of the high- and low-affinity receptors for the constant region of IgG. Once expressed on the cell surface, these molecules allow macrophages to recognize and phagocytize IgG-opsonized bacteria. Macrophage/monocytes are important mediators of inflammation through the production of cytokines, free oxygen radicals, proteases and complement factors during antigen independent immune response. Complement factors together with antibodies opsonize surface antigens of pathogens recognized by receptors on the macrophage surface. CD18 is the β chain of integrins that, when associated with CD11b, constitute the membrane glycoprotein Mac-1 or complement receptor type 3 (CR3) which mediates the adhesion of monocytes to the endothelium and subsequent diapedesis and the phagocytosis of complement-opsonized particles (Plowden, Renshaw-Hoelscher et al. 2004) .

Moreover, macrophage stimulation induces the expression of scavenger receptor A involved in the capture and subsequent degradation of proteins that have been

chemically modified at inflammatory sites. This receptor recognizes the exogenous ligands lipid A and lipoteichoic acid as well as oxidized-low density lipoprotein and apoptotic cells, resulting in enhanced uptake of acetylated low density lipoproteins LDL. The uptake of modified lipoproteins by macrophages leads to the accumulation of cholesterol esters and formation of macrophage-derived foam cells. Macrophages also contribute to lesion remodeling and to plaque rupture by secreting matrix metalloproteinases.

2. MACROPHAGE PROLIFERATION

In monocytes, macrophages and their early precursors, M-CSF growth factor is required for the proliferation, differentiation and survival of cells. Cell proliferation is a balance between generation of new cells through cell cycle divisions and survival mechanisms. Therefore, cell cycle division is finely regulated by extrinsic factors (mitogens, growth factors and extracellular matrix proteins) or by intrinsic factors (cell cycle regulators).

2.1. Macrophage colony stimulating factor, M-CSF

M-CSF is the principal growth factor that regulates the survival, proliferation and differentiation of macrophages and their precursors, and also synergies with other cytokines such as GM-CSF or IL-3 to mediate the proliferation of early haematopoietic progenitors. M-CSF initiates a mitogenic response interacting with its specific receptor (M-CSFR) leading it to enter cell cycle after early and late gene expression through several signal transduction cascades. M-CSF has also been shown to be important in bone metabolism, atherogenesis, lipoprotein clearance, chemotaxis, degranulation, activation, adhesion, cytotoxicity and in female reproduction (Stanley, Berg et al. 1997; Barreda, Hanington et al. 2004).

2.1.1. Biochemical characteristics of M-CSF, GM-CSF and IL-3

M-CSF or CSF-1 was the first growth factor purified. It was found in murine serum, human urine and cell-conditioned medium from murine fibroblast L cell line. The normal range for serum CSF-1 concentration is between 150 and 500 U/ml or 3-8 ng/mL.

M-CSF is a homodimeric sialoglycoprotein synthesized by a variety of cell types including endothelial cells, fibroblasts, bone marrow stromal cells, osteoblasts, thymus epithelial cells, keratinocytes, astrocytes, myoblasts, mesothelial cells, endometrial gland cells and the placenta-trophoblast decidual stroma (Fixe and Praloran 1997). M-CSF

production can also be achieved following activation of cells such as monocytes, macrophages, microglial cells, T lymphocytes, B-lymphocytes, chondrocytes and mesangial cells. M-CSF synthesis is induced by GM-CSF, TNF-α, IL-1 and IFN-γ.

The human M-CSF gene is localized in chromosome 1 and includes ten exons and nine introns (Saltman, Dolganov et al. 1992). The full-length human M-CSF mRNA encodes a precursor protein of 522 amino acids. In the mouse, the M-CSF gene is in chromosome 3 (Gisselbrecht, Sola et al. 1989). The mouse gene encodes for a precursor transcript of 520 amino acids with 60% homology to the human transcript.

Although a single gene encodes M-CSF, several mRNA splice variants, ranging in size, have been identified. Alternative splicing has implications for the stability of the transcripts (AU-rich sequences) or for the structural characteristics; protein products are secreted to the medium either in glycoprotein or proteoglycan forms or as glycoprotein at the cell surface. This homodimer can be quickly released from the membrane as a result of proteolytic cleavage triggered by protein kinase C activation (Stein and Rettenmier 1991).

The alternative splicing mechanisms and different levels of glycosylation generate different species of M-CSF and differential modes of M-CSF mediated regulation: whereas the secreted glycoprotein may be involved in humoral regulation of various cellular targets, the membrane-bound surface glycoprotein and the proteoglycan form appear to be involved in local regulatory mechanisms (Stanley, Berg et al. 1997). As such, M-CSF can regulate cell function via direct cell-to-cell contact and/or autocrine, juxtacrine, paracrine, or endocrine mechanisms (Fixe and Praloran 1997).

Op/Op mice lacking M-CSF production due to a null mutation in coding region of M-CSF gene, which leads to the generation of a biologically inactive truncated form of the cytokine, have a severe osteoclast and macrophage deficiency, absence of teeth, abnormal bone remodeling and osteopetrosis, low body weight, abnormal breast development, decreased fertility and shortened life-span which can be reversed upon injection of recombinant M-CSF (Wiktor-Jedrzejczak and Gordon 1996). Antigen-presenting cells of the epidermis (Langerhans cells), thymus and lymph nodes do not depend on CSF-1 for their ontogenesis and survival (Barreda, Hanington et al. 2004).

GM-CSF is a growth factor that also promotes macrophage and granulocytic cell proliferation. Its gene is positioned in tandem to the IL-3 gene and in close proximity to the M-CSFR gene (Kluck, Wiegant et al. 1993). The full-length human GM-CSF mRNA encodes a 14.7-33 kDa protein. A signal peptide in its sequence has to be cleaved to give a mature protein. Human and murine GM-CSF proteins only share 52% amino acid identity and do not cross-react in their biological activities. Under normal conditions GM-CSF can be detected in serum at concentrations ranging from 20 to 100 pg/mml. This growth

factor is produced by a variety of cell types including macrophages, T lymphocytes, fibroblasts, endothelial cells, stromal cells, keratinocytes, placental cells, astrocytes, B-lymphocytes, mesothelial cells, osteoblasts, mast cells, eosinophils, neutrophils and blast cells (Martinez-Moczygemba and Huston 2003).

IL-3 is a soluble, highly glycosylated 26 kDa haematopoietic growth factor that plays a central role in the production of macrophages, neutrophils, eosinophils, basophils, mast cells, megakaryocytes and erythroid cells through stimulation of the pluripotent haematopoietic stem cells and their derivatives (Martinez-Moczygemba and Huston 2003). IL-3 is synthesized by T lymphocytes with a small contribution from mast cells.

2.1.2. Macrophage Growth Factor Receptor

The biological effects of M-CSF are mediated via the high affinity M-CSF receptor (M-CSFR, CD115) encoded by *fms* proto-oncogene (*c-fms*) (Stanley, Berg et al. 1997). The human M-CSFR is located on chromosome 5 and is composed of 22 exons and 21 introns. Two different tissue-specific promoters differentially dictate the transcription start site of the c-fms gene, one that is expressed in placental trophoblasts and mammary epithelial cells regulated by sex steroid hormones during pregnancy and lactation, and the other that is active in macrophages (Zhang, Fukuda et al. 2000). In addition, *c-fms* expression can be regulated through mechanisms including transcriptional attenuation, DNA methylation and regulation by lineage-specific transcription factors such as PU.1, Ets and C/EBP (Zhang, Hohaus et al. 1996; Xie, Chen et al. 2001). The Mouse M-CSF gene displays similar features (de Parseval, Bordereaux et al. 1993).

M-CSFR, a member of the class III receptor tyrosine kinase family (RTK), is a large integral plasma membrane glycoprotein (MW 140-150 kDa) with an N-terminal glycosylated extracellular portion containing five repeated immunoglobulin (Ig) domains that interacts with the corresponding growth factor, a short single transmembrane domain, a juxtamembrane domain, two intracellular kinase domains divided by a single kinase insert and a C-terminal domain (Wang, Myles et al. 1993; Reilly 2002). Other members of this family include stem cell growth factor receptor (c-kit), Flt3, platelet-derived growth factor receptor alpha (PDGFα) and PDGFβ (Rosnet, Marchetto et al. 1991).

The M-CSF receptor is expressed primarily on cells on the macrophage lineage, and, as such, is a useful marker to discriminate between monocytic and granulocytic progenitor cells and their differentiated progeny since the level of M-CSF receptor expression progressively increases from primitive haematopoietic precursors to

monocytes and macrophages. It can also be found in osteoclasts, placental trophoblasts and mammary epithelial cells during lactogenic differentiation (Hofstetter, Wetterwald et al. 1992; Sapi, Flick et al. 1998). Following injury or in pathological conditions, M-CSFR can be found in astrocytes and neurons, myeloid leukaemic blast cells and in some breast and ovarian cancers (Kacinski 1997).

Activation of the M-CSFR is achieved by M-CSF binding to the extracellular domain, which induces non covalent dimerization of the receptor. Activated receptors are thus able to autophosphorylate tyrosine residues outside the catalytic domain via cross-phosphorylation. The results of this auto-phosphorylation are the stabilization of the active receptor conformation and the creation of phosphotyrosine docking sites for proteins that transduce signals within the cell. Finally, the complex receptor ligand is internalised and degraded by lysosomes (Hamilton 1997).

There are several docking sites for phosphorylation on murine MCSFR. Y559 in the juxtamembrane region is associated with the Src family members with protein-protein Src–homology interaction domains (SH2 and SH3). Y697 in kinase insert (KI) interacts with Grb2 and Shc adaptor protein through its SH2 domain on p85 that also interacts with p110 phosphatidylinositol-3-kinase (PI3K) and Y721. Grb2, constitutively associated to mSOS, acts as an adaptor molecule of p21Ras activation. Other proteins associated to M-CSF signal transduction are phospholipase Cγ (Y706) and signal transducer and activator of transcription, proteins STAT1 and STAT3 (Bourgin, Bourette et al. 2000; Pixley and Stanley 2004).

The potent biological activities require mechanisms to effectively control their magnitude and duration. M-CSF has been shown to have a short half-life. The degradation of internalized M-CSF ligand-receptor complex is partially mediated through the action of c-Cbl, an ubiquitin-protein ligase that increases the rate of ubiquitination causing internalization via clathrin-coated pits and vesicles and degradation by lysosomes (Mancini, Koch et al. 2002; Suzu and Motoyoshi 2002).

There are also several phosphatases that negatively regulate M-CSF receptor signaling. SHP-1 (Src-Homology-2-domain-containing protein tyrosine phosphatase) functions to dephosphosphorylate the M-CSF receptor. Two other phosphatases, SHIP (SH2-containing inositol phosphatase) and PTPase (phosphotyrosine protein phosphatase) also appear to reduce the mitogenic response to M-CSF through similar mechanisms (Suzu and Motoyoshi 2002). An additional mechanism for preventing binding of soluble growth factor to its corresponding ligand is through the production and secretion of soluble receptors that will bind the ligand, thus preventing its association with the cellular-bound receptors (Iwasaki, Shimoda et al. 1999).
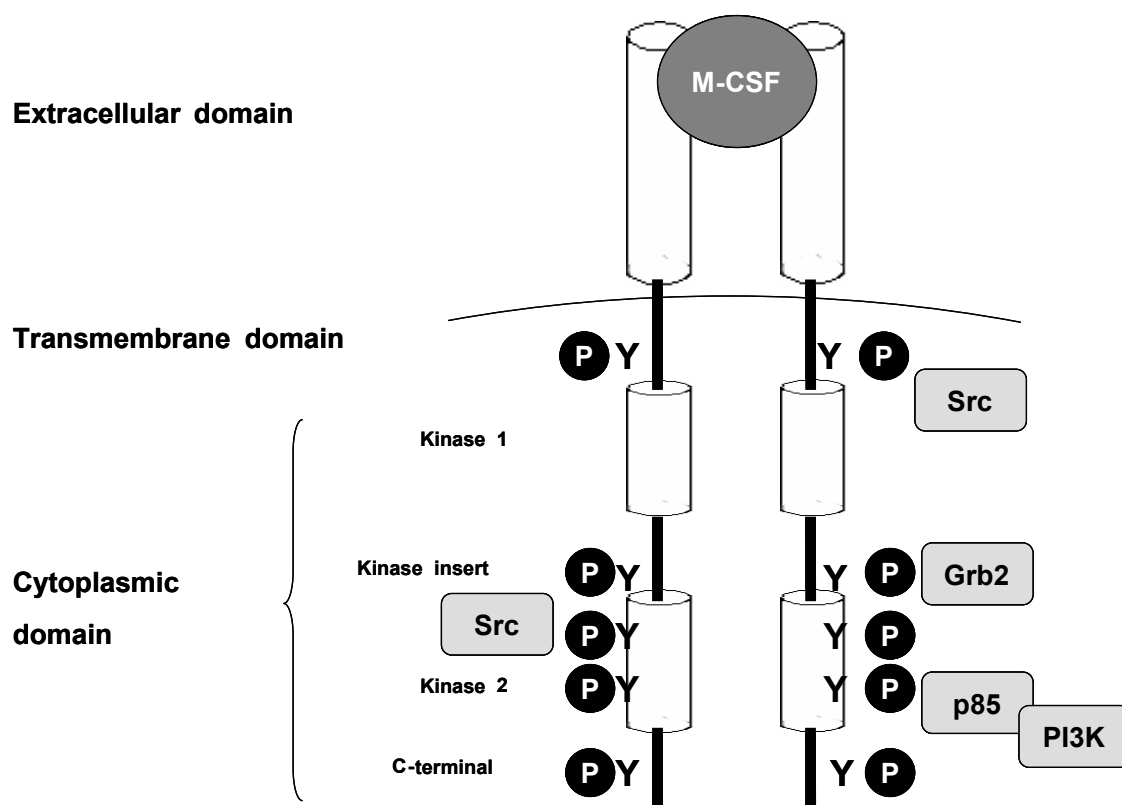
Figure 3. M-CSF receptor. M-CSF, macrophage colony stimulating; PI3K, phosphoinositide 3-kinase; Y, tyrosine residue and P, phosphate group.

On the other hand, GM-CSF Receptor is primarily expressed in low numbers in human and murine cells, as well as immature and mature granulocytes in the bone marrow. This receptor is composed of two distinct chains, α and β. The β-chain is shared with the receptor for IL-3 and has no intrinsic kinase activity. The IL-3 receptor (IL-3R) is a member of the cytokine receptor gene family and comprises α- and β-subunits. Both subunits contain extracellular domains common to the cytokine receptor family (Miyajima, Hara et al. 1992).

2.1.3. M-CSF transduction pathway

M-CSF interaction with the receptor results in activation of downstream signaling pathways from the cell membranes into the nucleus, eventually leading to a cellular response of growth and survival (Hamilton 1997; McCubrey, May et al. 2000). The tyrosine-phosphorylated receptor then serves as a docking site for a variety of intracellular signaling molecules that contain src-homology 2 (SH2) domains. Specific phosphorylated tyrosine residues are the binding site for Src family kinases (Courtneidge,

Dhand et al. 1993), Grb2 (van der Geer and Hunter 1993), Stat 1 (Novak, Harpur et al. 1995) and PI3K (Shurtleff, Downing et al. 1990).

One of the main cascades triggered after M-CSF exposure is the Raf→ MEK→ ERK-1/2 classical pathway, which has been shown to play an important role in cell proliferation. As a result of other phosphorylation processes there is also activation of other mitogen-activated protein kinase MAPK cascades, Rac/JNK and Rac/p38 pathways, culminating in proliferation or differentiation (Kyriakis, App et al. 1992).

On the other hand, interaction of GM-CSF or IL-3 with its receptor causes the subsequent transduction of intracellular signals that lead to proliferation, differentiation, survival and activation of haematopoietic cells, predominantly in the macrophage and neutrophil lineages. Phosphorylation of their receptors is responsible for induction of Shc-Grb2-SOS to the Ras-Raf-MEK-ERK and PI3K/Akt pathways, promoting c-fos and c-jun transcription and preventing apoptosis (de Groot, Coffer et al. 1998). But in contrast to M-CSFR, these receptors can also activate, through conformational changes, members of the JAK/STAT kinase family essential for the induction of STAT5, c-myc and pim-1 transcription factors (Liu, Itoh et al. 1999).
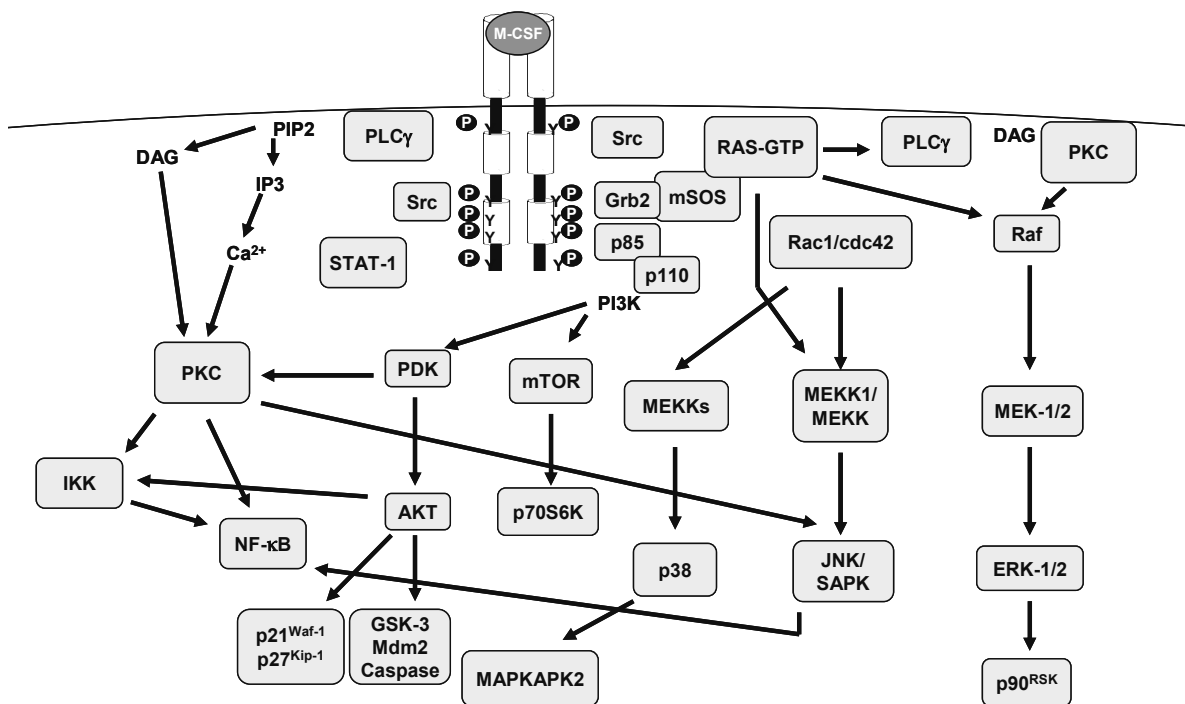

Figure 4. M-CSF receptor signaling. The main pathway activated by M-CSF is the classical Raf-1/MEK/ERK-1,2. However, MAPKs JNK and p38 are also activated through MEKK promoting MAPKAPK2 activation. Hydrolysis of phospholipids by PLC generates an increase in calcium and DAG levels activating PKC kinases responsible for increasing NF-κB levels through IKK phosphorylation. MEK, mitogen extracellular signal-

related kinase; ERK, extracellular regulated kinase; JNK, c-jun NH2-terminal kinase; MEKK, MEK kinase; MAPKAPK2, MAPK-activated protein kinase 2; PLC, phospholipase C; DAG, 1,2-diacylglycerol; PKC, protein kinase C; NF-κB, nuclear factor immunoglobulin kappa chain enhancer B cell transcription; IKK, I-kappa-b kinase; STAT-1, signal transducer and activator of transcription; PDK, phosphoinositide-dependent kinase; PI3K, phosphainositide-3 kinase; GSK, glycogen synthase kinase; PIP2, phosphatidylinositol-2-trisphosphate and IP3, inositol-3-phosphate.

2.1.3.1. PKC signaling

Protein kinase C (PKC) is a family of cyclic nucleotide-independent enzymes that phosphorylate serine and threonine residues in many target proteins. They have been implicated in development, memory, differentiation, proliferation and carcinogenesis (Saito, Hojo et al. 2002). At present, 13 isoforms have been identified, varying in terms of tissue expression and cellular compartmentalization. They have been divided into three groups differing in terms of the enzyme cofactor requirements; conventional cPKC isoforms (comprising α, βI/II and γ) that require calcium, phospholipids (phosphatidylserine) and 1,2-diacylglycerol (DAG) for activation; novel nPKC isoforms (comprising δ, ε, η, θ and μ) that require phospholipids and DAG, and atypical aPKC isoforms, namely ξ, ι and λ, that do not require calcium or DAG and, in contrast to c and n isoforms, do not respond to phorbol esters such as TPA (12-O-tetradecanoyl-phorbol-13-acetate) but are activated by PIP_3 (phosphatidylinositol (3,4,5)-triphosphate), ceramide and phosphatidic acid (Nishizuka, 1992; Nishizuka, 2003). Our group has demonstrated that bone marrow macrophages only express three PKC isoforms: PKCβI (conventional), PKCε (novel) and ξ (atypical). PKCε has been implicated in proliferation and activation (Valledor et al., 2000b).

All PKCs possesss a phospholipid-binding domain for membrane interaction. The general structure of a PKC molecule consists of a catalytic and a regulatory domain, composed of a number of conserved regions interspersed with variable domains. All PKCs, except the δ isoform, express PEST sequences (hydrophilic polypeptide segments enriched in proline (P), glutamic acid (E), serine (S) and threonine (T)) which target proteins for degradation by the proteasome.

Activation of c, n PKCs involves translocation from the cytosol to cell membranes. Following an increase in intracellular calcium levels, c, nPKCs interact with the cell membrane phosphatidylserine. DAG and phospholipid production facilitates penetration of these isoenzymes into the cell membrane through a conformational change that facilitates pseudosubstrate displacement.
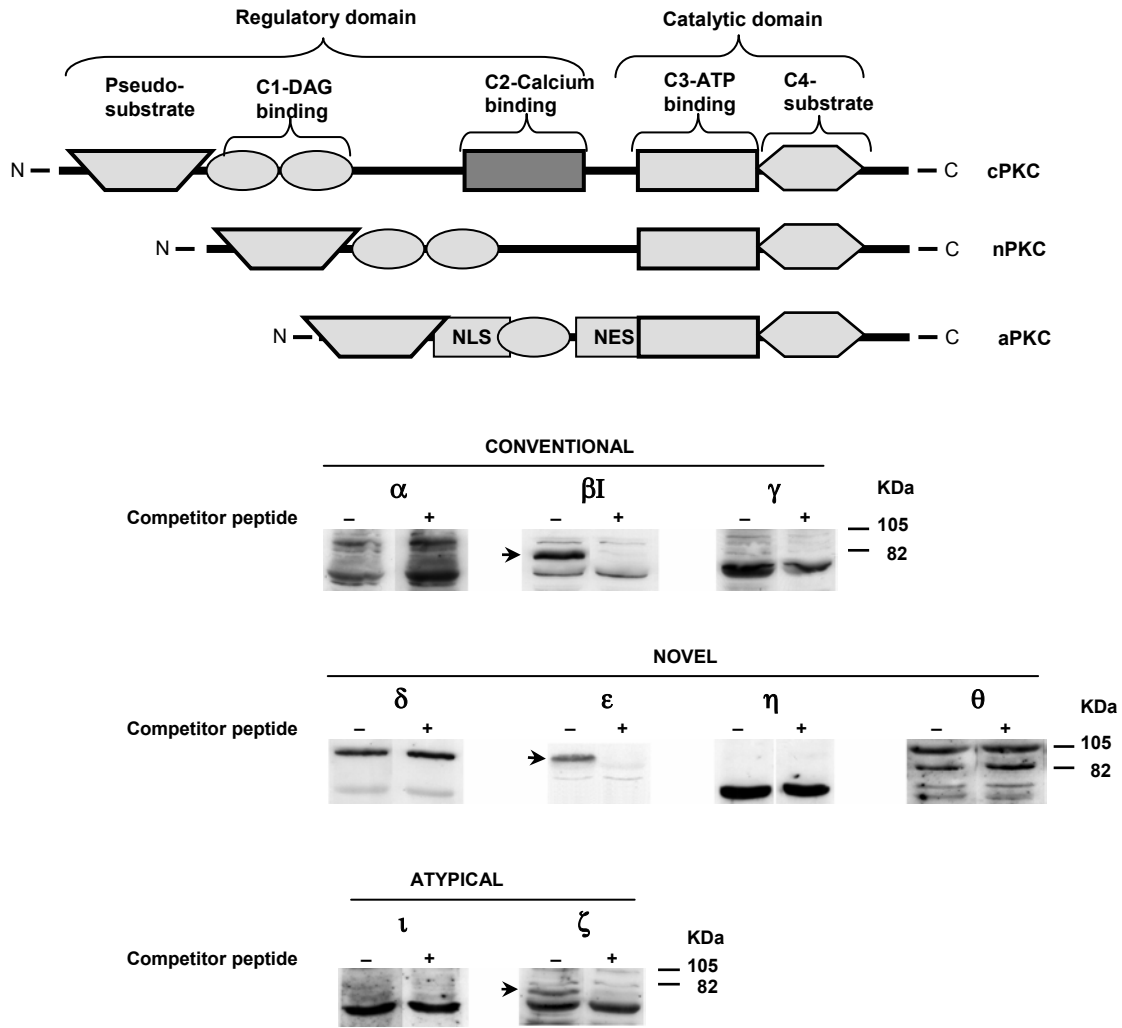
Figure 5. Structural characteristics of PKC isoforms expressed in bone marrow derived macrophages. Classical PKC isoforms (cPKC) possesss an autoinhibitory pseudosubstrate motif, two DAG-binding C1 domains and a calcium-binding C2 domain. Novel PKC isoforms (nPKC) lack a calcium-binding motif but are still regulated by DAG. Atypical PKC isoforms (aPKC) are independent of DAG and calcium but are regulated by their intracellular localization through nuclear localization signals (NLS) and nuclear export signals (NES). They all share the catalytic domain (adapted from Spitaler et al., 2004). Specific Western blot of different PKC isoforms in presence or absence of the competitive peptide used to generate specific antibodies (Valledor et al., 1999).

M-CSF receptor also regulates phospholipase C resulting in the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) and phosphatidylcholine and the production of inositol polyphosphates, which in turn regulate intracellular concentrations of calcium and DAG. There are other direct targets for DAG, apart from PKCs, such as the Ras guanyl-releasing protein (GRP) family of nucleotide-exchange factors (Ebinu, Stang et al. 2000). Another route for metabolization of inositol phospholipids is mediated by class

I phosphoinositide 3-kinases (PI3Ks), which phosphorylate the 3'-OH position of the inositol ring of PtdIns $(4,5)P_2$ to produce PtdIns $(3,4,5)P_3$ (Wymann, Bjorklof et al. 2003).

PKCs demonstrate relatively broad in vitro substrate specificity and several targets of PKC have been defined. Results indicate that PKC directly phosphorylates and activates Raf-1 both in vitro and in vivo with the PKC activator TPA (Sozeri, Vollmer et al. 1992). Some authors suggest that PKCε acts upstream of Raf-1 but downstream of protein-tyrosine kinase (e.g. Src) and Ras, consistent with a role of PKCε in growth factor-stimulated Raf-1 activation (Seibenhener, Roehm et al. 1999). However, mutant Raf-1 proteins lacking tyrosine phosphorylation sites are still activated in growth factor-stimulated cells, so an alternative mechanism of Raf-1 activation must also exist. The Rho family (Rho, Rac and Cdc42) of small GTPases mediate some of the Raf-independent effects of Ras (Matozaki, Nakanishi et al. 2000; Scita, Tenca et al. 2000) and is proposed to have a role in cytoskeleton regulation and activation of the JNK pathway (Wennerberg, Ellerbroek et al. 2002).

In addition, there is evidence for a role for PKCε in macrophage M-CSF and LPS signal transduction through the induction of mitogen-activated protein kinase phosphatase 1 (MKP-1) responsible for MAPK dephosphorylation, its mechanism is unknown (Valledor, Xaus et al. 1999; Valledor, Comalada et al. 2000). Previous studies have also suggested a role of PKCε as an important mediator of macrophage functions such as IL-4 induced NO production (Sands, Bulut et al. 1994). Some results demonstrate that atypical PKC can modulate MEK kinase to serve as a convergence point for the Ras/MAPK and Src/JNK pathways activated by M-CSFR. Inhibition of aPKC by pharmacological manipulation blocked growth factor induced activation of JNK, positioning PKC upstream of JNK (Diaz-Guerra, Bodelon et al. 1996; Schonwasser, Marais et al. 1998).

2.1.3.2. MAPK activation

The Raf protein family consists of A-Raf, B-Raf and Raf-1 (also c-Raf), which are involved in the regulation of the proliferation, differentiation and apoptosis induced after cytokine stimulation (Daum, Eisenmann-Tappe et al. 1994). They have three distinct functional domains: CR1, CR2 and CR3. The CR1 domain is necessary for Ras binding and subsequent activation and translocation by Ras GTPases to the cell membrane. The CR2 domain is a regulatory domain that has recently been shown in some cells to regulate negatively Raf-1 activity by Akt or PKA phosphorylation. CR3 is the kinase Ct domain (Morrison and Cutler 1997; Farrar, Tian et al. 2000). The highest level

of A-Raf expression in the adult is found in the urogenital tract, and testes and neuronal tissue for B-Raf expression (Wadewitz, Winer et al. 1993). The Raf-1 proto-oncogene produces a 74 kDa protein ubiquitously expressed in adult tissues with highest expression in muscle, cerebellum and fetal brain, and which has become the most intensely studied Raf family member. Drugs that block protein-protein interactions such as geldanamycin, currently used in clinical myelogenous leukaemia, have been used to study Raf activity (Tzivion, Shen et al. 2001).

It has been shown that stimulation of Raf protein serine/threonine kinase activation can be achieved through phosphorylation on S338 by Ras GTP-binding proteins (Morrison, Heidecker et al. 1993). Ras is a small guanine triphosphate (GTP)-binding protein that acts as a common upstream molecule of several downstream signaling pathways including Raf/MEK/ERK, PI3K/Akt-mTOR/S6K, NFκB pathways, several PKC isoforms depending on calcium, phospholipids and DAG levels produced by phospholipase C, PKA and RalEGF/Ral (Marais, Light et al. 1995; Chaudhary, King et al. 2000). Ras directly activates PI3K, which results in Akt membrane localization, phosphorylation and activation to induce cell survival.

Raf can also be activated by phosphorylation on S338-Y340-341 by Src protein-tyrosine kinases (Mason, Springer et al. 1999) or by oligomerization or by hydrolysis of phosphatidylcholine (PC) in NIH 3T3 cells (Dent and Sturgill 1994) or by phosphorylation of S497 and S499 by PKC inducing a cross talk between PKC and Raf/MEK/ERK signaling pathways (Cheng, Wung et al. 2001). Moreover, Raf activity can also be modulated by adaptor proteins including Bat1, Hsp70 (heat shock protein) and chaperonin proteins such as 14-3-3 and Hsp90 (heat shock protein)(Song, Takeda et al. 2001). It has been shown that PI3K/Akt and Raf/MEK/ERK pathways can cross talk in multiple ways. In some cells, Akt can contribute to Raf-1 inactivation through phosphorylation on S259 (Tzivion, Shen et al. 2001; Dhillon, Meikle et al. 2002) and it has been postulated that Raf signaling can activate the PI3K/Akt pathway to induce cell survival through mechanisms up-regulating p21$^{Waf-1/Cip1}$ activity and Cdk4-cyclinD activation, which lead to cell cycle progression (Chang, Steelman et al. 2003).

Activation of Raf serine/threonine kinases downstream of protein tyrosine kinase leads to phosphorylation and activation of MAPKs transmitting signals to transcription factors in the nucleus (Chang, Steelman et al. 2003). The MAPK family of serine/threonine kinases consists of both stress activated kinases (SAPK) and MAPK kinases. They form a network of signal transduction cascades that mediate cellular responses to a diverse range of stimuli including growth factors, chemical or osmotic stress, irradiation, bacterial infection and proinflammatory cytokines. Each MAPK is activated by dual phosphorylation of a Thr-Xaa-Tyr motif by upstream kinases, referred

to as MAPK kinases (MAPKK). MAPKKs are, in turn, activated by MAPKKK, over 30 of which have been described (Cano et al., 1995; Xu et al., 1995).

MAPK FAMILY MEMBERS

ERK	ERK-1, ERK-2, ERK-3, ERK-4, ERK-5, ERK-6, ERK-7
JNK / SAPK 1	JNK1 (MAPK8), JNK2, JNK3
SAPK 2	p38α, p38β, p38γ, p38δ

To date, several MAPK family members have been identified in mammalian cells: the classical MAPK extracellular signal regulated kinase (ERK-1/2), SAPK2 (p38α, β, γ, δ) and JNK/SAPK1 (c-jun NH2-terminal kinase or JNK1 (or MAPK8)/2/3 (Gupta, Campbell et al. 1995; Marshall 1995; Gupta, Barrett et al. 1996). The classical MAPK cascade is activated by mitogens and growth factors, and plays an important role in the control of cell growth and differentiation. All MAPK family members consist of a homodimer that contains the TXY sequence (X is glutamate, proline or glycine, respectively, in ERK, JNK or p38) (Payne, Rossomando et al. 1991; Robbins, Zhen et al. 1993). Phosphorylation of both the threonine and tyrosine is essential for MAP kinase activity and it is achieved through the action of MAP kinase kinases (MEKs). The MEK dual-specificity kinases are a family of genes that consists of five genes: MEK1 (MW 44 kDa), MEK2, MEK3, MEK4 and MEK5 (Crews, Alessandrini et al. 1992). This family of dual-specificity kinases has both Ser/Thr and Tyr kinase activity on MAPK target (Tanoue, Yamamoto et al. 2001). The specific role for Raf isozyme-specific function was determined by knockout studies in mice. A-Raf gene ablation resulted in neurological defects and the Raf-1/B-Raf double knockout resulted in almost complete loss of ERK activation and severely reduced expression of downstream targets (Kamata, Pritchard et al. 2004).

2.1.3.2.1. Extracellular regulated kinase, ERK

The main physiological substrates of MEK-1/2 are ERK-1/2 serine-threonine kinase family of genes. All three Raf family members and Mos proteins are able to phosphorylate and activate MEK-1/2 but different biochemical activities have been observed (B-Raf > Raf-1>> A-Raf) (Papin, Eychene et al. 1995; Wu, Noh et al. 1996). Small organic molecules, such as PD 98059, have been extensively used to inhibit MEK1 and ERK through non competitive mechanisms and investigate the functions of classical MAPK cascade (Bain, McLauchlan et al. 2003).

The ERK family consists of eight distinct groups of kinases: ERK1, ERK2, ERK3, ERK4, ERK5, ERK6, ERK7 and ERK8 kinases, which have different modes of activation. The ERK1 (42kDa) and ERK2 (44kDa) proteins are the most studied with regard to Raf signaling in haematopoietic cells. ERKs are activated through dual phosphorylation of threonine and tyrosine residues by MEK kinases. ERK dimerization occurs subsequent to phosphorylation and promotes nuclear localization of the protein (Khokhlatchev, Canagarajah et al. 1998). Activated ERK mainly phosphorylates Ser/Thr residues preceding a proline of cytoplasmic and nuclear proteins. However, ERK activity is down regulated by phosphatases such as MKP-1 (Pouyssegur, Volmat et al. 2002).

M-CSF treatment involves a 2-fold increase activity of ERK pathway, giving 2 peaks at around 5 and 15 min in bone marrow derived macrophages, measured by phosphorylation of myelin basic protein (MBP) (Valledor, Xaus et al. 1999). Upon activation, ERK kinases mediate key cellular events in the cytoplasm, including phosphorylation of membrane-associated and cytoplasmic proteins such as kinases, cytoskeletal elements, phospholipase A2 and stathmin. ERKs may translocate to the nucleus and directly phosphorylate a set of transcription factors including Ets-1, c-Jun, c-Fos and c-Myc leading to proliferation by expression of genes important for cell cycle progression, e.g., Cdks, cyclins, growth factors, and for mechanisms of apoptosis prevention, antiapoptotic Bcl-2 and cytokines (Karin and Hunter 1995; Chang, Steelman et al. 2003).

Ets is a family of transcription factors, which include Ets-1, Ets-2, Elk-1, SAP1, SAP2, E1AF, PEA3, PU.1 and others (Wasylyk, Hahn et al. 1993). They share an 85 amino-acid sequence called the ETS DNA-binding domain. The Ets transcription factors regulate many genes including transcription factor genes p53, c-Fos and NF-κB (Venanzoni, Robinson et al. 1996; Lambert, Ludford-Menting et al. 1997); cell cycle regulation genes, cyclin D1 (Albanese, Johnson et al. 1995), Rb (Tamir, Howard et al. 1999) and p21$^{\text{Waf-1/Cip1}}$ (Beier, Lee et al. 1999); apoptosis-related genes, Bcl-2, Bcl-X_L and Fas (Sevilla, Aperlo et al. 1999); cytokine genes GM-CSF and IL-3 (Nimer, Zhang et al. 1996), and growth factor genes PDGF and heparin binding epidermal growth factor HB-EGF (McCarthy, Chen et al. 1997). Ets proteins usually associate with other transcription factors to transactivate their target genes.

c-Myc is a transcription factor that regulates cell proliferation by promoting an increase in cell mass as well as a distinct set of target genes whose functions are associated with cell cycle progression or apoptosis (Prendergast 1999; Amati, Frank et al. 2001; Nasi, Ciarapica et al. 2001). ERK can also regulate the presence of c-Myc since phosphorylation of c-Myc on Ser62 increases its transactivation and half-life (Seth,

Alvarez et al. 1991) and phosphorylation on Thr58 facilitates rapid c-Myc proteolysis through the ubiquitin-proteasome pathway (Gregory and Hann 2000).

ERK can also phosphorylate and activate a downstream kinase RSK (pp90 ribosomal S6 kinase) that leads to the phosphorylation and activation of transcription factors such as CREB (cAMP-response element binding protein) (Seth, Gonzalez et al. 1992). CREB is a transcription factor of the CRE/ATF gene family that functions as a regulatory effector of the cAMP signaling pathway. The CRE/ATF gene family of basic leucine zipper DNA-binding proteins also includes CREM, ATF-1, ATF-2, ATF-3 and ATF-4, which can form homodimers, heterodimers or even in some cases cross-family heterodimers with other leucine zipper DNA-binding transcription factors of the Jun family (De Cesare, Jacquot et al. 1998; Boehlk, Fessele et al. 2000). Protein kinases including PKA, Ca^{2+}-calmodulin-dependent kinase IV (CaMKIV), mitogen and stress-activated kinase (MSK) and p70S6K can phosphorylate CREB (cAMP-response element binding protein) (de Groot, Ballou et al. 1994; Deak, Clifton et al. 1998). Genes regulated by the CREB/ATF transcription factors include cyclin A, cyclin D1, c-fos and Bcl-2 (Pugazhenthi, Miller et al. 1999; Wang and Murphy 2000).

Elk-1 contributes to the c-fos rapid and transient induction mediated by growth factors. The ternary complex factor (TCF) formed by two molecules of SRF (serum response factor) and one molecule of Elk-1 binds to the serum response element (SRE) at position –300 of c-fos promoter, while the CREB/ATF dimer binds to CRE (cAMP-response-element) at position –60 to induce its expression. Together with c-jun, c-fos is a component of the activating protein-1 (AP-1) transcription factor whose activity is important for cell proliferation (Chinenov and Kerppola 2001).

Moreover, indirectly ERK can lead to activation of Nuclear Factor immunoglobulin kappa chain enhancer B cell transcription factor (NF-κB) by translocation to the nucleus after phosphorylation and activation of its regulator inhibitor kinase (IKK), since, prior to growth factor stimulation, NF-κB is normally sequestered in the cell cytoplasm by binding to IκB inhibitors. Many target genes of NFκB play important roles in proliferation, prevention of apoptosis, angiogenesis, metastasis and immune responses (Schreck, Rieber et al. 1991; Baldwin 2001).
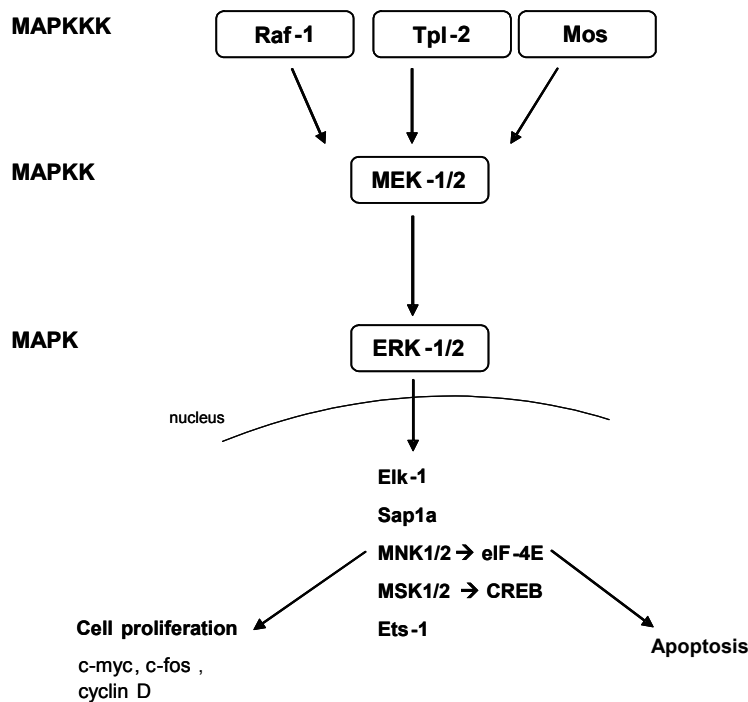
Figure 6. ERK activation signaling pathway. Specific transcription factors are activated by mitogen-activated protein kinase (MAPK), which are themselves activated by dual specificity MAPK kinase kinase (MAPKK), activated by MAP kinase kinase kinases (MAPKKK). ERK, extracellular-related kinase; MEK, mitogen extracellular signal-related kinase; Elk-1, ETS-domain protein; MNK, MAPK interacting kinases; Tpl2, tumor progression locus 2, and MSK, stress-activated protein kinase.

2.1.3.2.2. c-jun NH$_2$-terminal kinase, JNK

The SAPK1 (JNK) cascade is typically activated by cellular stress, bacterial infection and proinflammatory cytokines, although it has also been associated with cell proliferation, differentiation and apoptosis (Ip and Davis 1998; Shaulian and Karin 2001). JNK (p54MAPK) is activated through phosphorylation of both threonine and tyrosine residues by the dual specificity MAPK kinases, MEKK1 and SEK1, sequentially.

JNK activation results in the phosphorylation and activation of activating protein (AP-1) and other transcription factors (Chinenov and Kerppola 2001). AP-1 are dimers composed of Jun/Fos protein. All members of the proto-oncogene fos/jun superfamily code for transcription factors with a leucine zipper domain, which is necessary for dimerization and binding to a similar DNA site (Karin, Liu et al. 1997). Jun proteins (c-Jun, JunB, JunD) can form homodimers with Jun proteins or heterodimers with c-Fos, whereas Fos proteins (c-fos, Fos B, Fra1 and Fra2) only dimerize with Jun proteins. Both Jun and Fos proteins also dimerize with other transcription factors including members of the ATF/CREB or Maf/Nrl families of proteins to transactivate gene expression (Kerppola and Curran 1994; van Dam and Castellazzi 2001). Therefore, activity of AP-1 transcription

factors is finely regulated both by protein levels and post-translational modifications during the cell cycle and proliferation. Genes such as Cyclin D1, Cyclin A, Cdk4/6, p53, p16 INK4a, IL-2, IL-3 and GM-CSF are under AP-1 control in their promoter and enhancer regions (Wisdom, Johnson et al. 1999; Bakiri, Lallemand et al. 2000).

2.1.3.2.3. p38 MAPK

The SAPK2 (p38) is activated by growth factors through phosphorylation of both threonine and tyrosine residues by the dual specificity MAPK kinases, MKK6 or MKK3 (Clark, Dean et al. 2003). In the cytosol, MAPK p38 activates the kinase MAPKAPK-2 which, in turn, targets the adenosine/uridine rich elements (ARE)s of certain pro-inflammatory mRNAs 3'-untranslated regions (UTRs), typical of short mRNA half life for rapid deadenylation to bring about their stabilization (Shi and Gaestel 2002). AREs can be divided in three categories. Class I AREs (e.g. c-fos) contain one to three copies of AUUUA motifs with nearby U-rich regions. Class II AREs (e.g. GM-CSF, TNF-α, COX2 and other inflammatory mRNAs) contain multiple pentammers. Class III (e.g. c-jun) includes AREs that lack the pentammer sequence but contain U-rich regions. However, in the nucleus MAPK p38 regulates transcription via factors that include MEF2C, ATF-2 and NF-κB (Treisman 1996; Schmitz, Bacher et al. 2001).

The activation of ERK, p38 and JNK is terminated by removal of phosphate groups by serine/threonine-specific phosphatases, tyrosine-specific phosphatases or dual specificity phosphatases.

2.1.3.2.4. MAPK phosphatases

Activation of MAPKs by protein phosphorylation is opposed by phosphatases. Since MAPK pathways are regulators of gene expression in the immune response, phosphatases are important negative regulators of many aspects of cellular response (Hunter 1995; Keyse 2000). There are many distinct families of phosphatases acting on distinct protein targets: Serine/threonine-specific phosphatases (PSPs), tyrosine-specific phosphatases (PTPs) or dual specificity phosphatases (DSP).

Serine/threonine-specific phosphatases (PSPs) include PP1, PP2A, PP2B (also known as Calcineurin) and PP2C phosphatases differing in terms of their regulation and substrate specificity. They conserve catalytic subunit features between species and they can be positively regulated through phosphorylation by PKA and glycogen synthase kinase (GSK-3). The serine/threonine phosphatase calcineurin is directly activated by calcium and calmodulin. Calcineurin is the target of two immunosuppressant drugs, Cyclosporine

A and FK506, complexed with their intracellular receptors called immunophilins, cyclophilin and FK506-binding protein (FKBP) respectively (see section 5.1).

Tyrosine-specific phosphatases (PTPs) possess a conserved cysteine group in their catalytic domain and include several proteins acting as receptor or cytoplasmic phosphatases (Denu and Dixon 1998). Dual specificity phosphatases (DSPs) possess a conserved cysteine for catalysis and some conserved features of three-dimensional structure. There are two groups of dual specificity phosphatase (DSPs), one induced by the MAPK pathway (known as mitogen-activated protein kinase phosphatases, MKP), responsible for dephosphorylating MAPKs providing a negative feedback mechanism, and the other one constituted by Cdc25. MKPs inactivate MAPKs through dephosphorylation of both phospho-threonine and phospho-tyrosine residues in the activation loop. The other group consists of Cdc25 phosphatase, which plays a critical role in the control of cell cycle by removing the inhibitory phosphatases on amino-terminal threonine and tyrosine residues of Cdks (Busino, Chiesa et al. 2004).

To date, 13 DS-MKPs have been identified and are classified in four subgroups differing in terms of substrate specificity, tissue distribution, subcellular localization and target gene regulation (Ishibashi, Bottaro et al. 1994). Type I-DS-MKPs contain a Dual Specificity Phosphatase domain, including VHR (ERK), DSP2 (p38, JNK) (Aoyama, Nagata et al. 2001) and MKP6 (ERK/JNK) (Marti, Krause et al. 2001). Type II DS-MKPs contain an N-terminal MAP kinase–binding (MKB) domain in addition to the Dual Specificity Phosphatase (DSP) domain. That group includes MKP-1 (Chu, Solski et al. 1996), PAC1 (ERK=p38>JNK) (Ward, Gupta et al. 1994; Chu, Solski et al. 1996), MKP2 (ERK=JNK>p38) (Misra-Press, Rim et al. 1995; Chu, Solski et al. 1996), VH3/B23, MKP3 (ERK>>JNK=p38) (Muda, Theodosiou et al. 1998), PYST2 (Groom, Sneddon et al. 1996) and MKP4 (ERK>p38=JNK) (Muda, Boschert et al. 1997). The phosphatase MKP-1 mainly inactivates MAPK p38 and JNK, but under some circumstances may also dephosphorylate ERK (Franklin and Kraft 1997). Type III DS-MKP includes only MKP5 (JNK similar p38>>ERK) with Nt domain, MKB (N-terminal MAP kinase-binding) and DSP domains (Tanoue, Moriguchi et al. 1999). Type IV DS-MKPs contain a PEST sequence (enriched in proline, glutamic acid, serine and threonine) on DSP domain. They include VH5/M3/6 (Muda, Theodosiou et al. 1998) and MKP7 (Masuda, Shima et al. 2001), both of which are specific for JNK and p38 but not for ERK.

In fact, expression of other phosphatases is highly restrictive. By this way, MKP-7 expression is very low in haematopoietic and lymphoid cells and contains predicted functional motifs such as nuclear export signal (NES) and nuclear localization signals (NLS) suggesting that it may be acting as a shuttle protein. MKP-5 expression has been related to exposure to LPS dephosphorylating JNK activity and limiting pro-inflammatory

cytokine levels of TNF-α and IL-6 in RAW cells (Theodosiou, Smith et al. 1999). On the other hand, the localization of MKP-3 and Pyst phosphatases is restricted to cytoplasmic compartment. Ubiquitous MKP-1 expression *in vivo*, is particularly physiologically relevant in cell types such as macrophages, mast cells, osteoblasts, gut and lung epithelia because it can efficiently dephosphorylate p42/44 MAPK ERK-1/2 (Engelbrecht, de Wet et al. 2003).

The *mkp-1* gene, originally named erp/3CH134 in mice or CL100 in humans, is mutated in certain tumors and is considered a tumor suppressor gene (Scimeca, Servant et al. 1997). MKP-1 was first identified because it is induced after growth factor and stress stimulation as a negative feedback mechanism for MAPK activity (Lewis, Groom et al. 1995). Furthermore, it was demonstrated that PKA, Ca^{2+} and protein kinase C (PKC) contribute to the activation of MKP-1 (Waskiewicz and Cooper 1995; Scimeca, Servant et al. 1997). In addition, activation of p42/44 MAPK is controversially important in stimulating the expression of MKP-1. Comparison of the 5' flanking sequences of the murine and human mkp-1 genes revealed a high degree of sequence homology in the proximal promoter region. There are several conserved consensus sequences that include two CREs and one E box motif. Further distal promoter regions show little homology (Ryser, Massiha et al. 2004).

Phosphatase family members (human)

Serine/Threonine-specific phosphatases		
PP1		
PP2A		
PP2B - Calcineurin		
PP2C		
Tyrosine-specific phosphatases		
Dual specificity phosphatases		
VHR	ERK	⎫
DSP2	p38, JNK	⎬ Type I
MKP-6 (MKP-L)-membrane	ERK, JNK	⎭
MKP-1 (CL100/DUSP1) nuclear	ERK, JNK, p38	⎫
PAC-1/DUSP2 nuclear	ERK = p38 > JNK	
MKP-2, TYP1 (DUSP4) nuclear	ERK = JNK > p38	
DSP5 (B23, hVH3) nuclear	ERK	⎬ Type II
MKP-3 (PYST1/DUSP6) cytoplasmic	ERK >> JNK = p38	
MKP-X (PYST2/DUSP7) cytoplasmic	ERK	
(MKP-4/DUSP9)- cytoplasmic	ERK > p38 = JNK	⎭
MKP-5 (MKP-5/DUSP10) nuclear/cytoplasmic	JNK = p38 >> ERK	⎬ Type III
MKP-7 (MKP-7) cytoplasmic	JNK, p38	⎫ Type IV
M3/6 (hVH5) cytoplasmic	JNK, p38	⎭
Cdc25	Cdk	

Table I. Phosphatase family members (in parenthesis, the human form).

2.1.3.3. Survival signaling by PI3K/AKT

After MCS-F activation there is a rapid increase in PI3K activity by tyrosine phosphorylation of the M-CSF receptor (Yusoff, Hamilton et al. 1994). Wortmannin and LY294002, inhibitors of the ATP binding site of PI3K, have been broadly used to study this pathway, although the individual role of PI3Ks is difficult to understand (Powis, Bonjouklian et al. 1994). However, in contrast to LY294002, Wortmanin can also inhibit FRAP/mTOR kinase activity (Brunn, Williams et al. 1996).

The PI3K-PKB/AKT axis governs many cellular events such as cell growth and survival rate, cytoskeletal remodeling and the trafficking of intracellular organelles (Koyasu 2003). The kinases associated with this pathway contain pleckstrin homology (PH) domains that bind to specific phosphoinositide lipids generated in the plasma membrane. The membranes of many types of cells and tissues contain substantial quantities of phosphatidylinositol (PtdIns) and small amounts of at least seven phosphorylated PtdIns derivatives (PPI). Phosphoinositide kinases synthesize phosphorylated PI derivatives (PPI) by adding phosphate groups to pre-existing inositol glycerophospholipids generating second messengers for intracellular signaling (Rameh and Cantley 1999).

PI3K is a lipid kinase family that consists of an 85 kDa regulatory subunit with SH2 and SH3 domains and a 110 kDa catalytic subunit. The phosphorylation of the p85 subunit leads to activation of the p110 catalytic subunit responsible for phosphorylating inositol ring 3'-OH group in inositol phospholipids (Fruman, Meyers et al. 1998). Mammals have three p110 isoforms (p110α, β and δ) encoded by three separate genes and at least seven adaptor proteins generated by alternative splicing of three different genes (p85α, β and p55γ). To date, nine different isozymes of PI3Ks have been identified in mammals and are classified into three major classes. Class I and II PI3Ks are expressed only in metazoans whereas class III can be found in unicellular eukaryotes as well (Wymann and Pirola 1998).

Class I PI3Ks generate PtdIns(3, 4, 5)P$_3$ (PIP$_3$) and contain two SH2 domains in their regulatory subunit to interact with phosphorylated residues of tyrosine receptors of growth factors and adaptor molecules (Arcaro, Volinia et al. 1998). Type I PI3Ks also use phosphatidylinositol (PI) and PI(4)P as substrates and produce PI-3-P and PI-3,4-P$_2$. Class II PI3Ks have a Ct C2 domain that can bind in vitro to phospholipids in a Ca^{2+} independent manner (Arcaro et al., 2000). Insulin, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), integrins and MCP-1 chemokine can activate class II PI3K activity involved in membrane trafficking (Zhang, Hogan et al. 1998). PtdIns(4,5)P2(PIP2)

biosynthesis also catalyses the formation of PtdIns(3,4,5)P$_3$ by PLC which triggers calcium and DAG flux activating PKC (Rhee and Bae 1997).

Various signaling molecules containing pleckstrin homology (PH) domains are regulated downstream of PI3Ks. Protein kinase B (PKB), as the main target, phosphoinositide-dependent kinase 1 (PDK1), Vav, PKC and PLC-γ are thought to function directly downstream of PI3K, because their PH domains bind to PI(3,4)P$_2$ or PI(3,4,5)P$_3$ (Alessi, Andjelkovic et al. 1996).
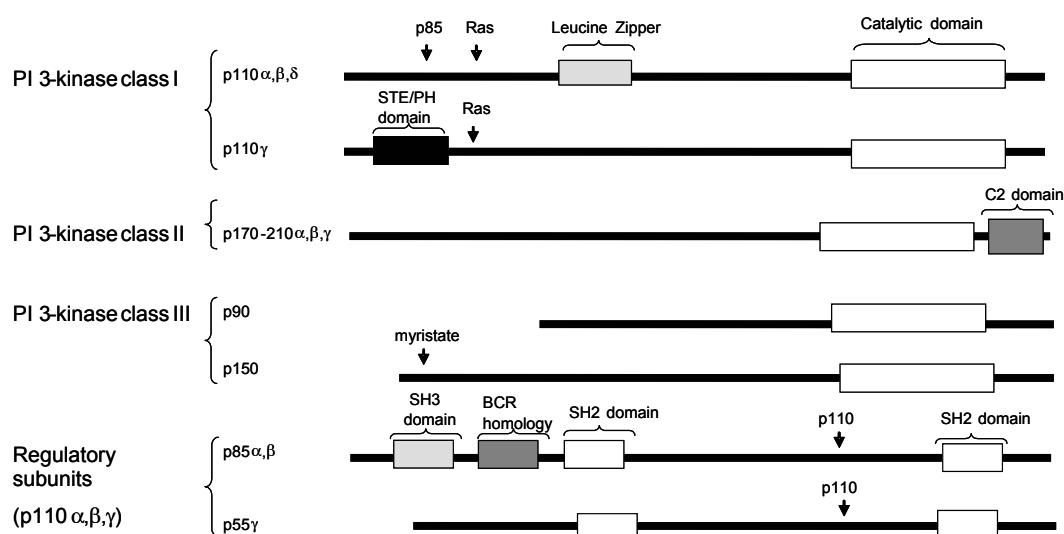

Figure 7. Classification of phosphoinositide 3-kinases. The enzymes are classified into three groups based on the molecular structure of the subunit that contains the kinase domain. The class I is subdivided into groups A and group B. Group A comprises α, β and δ which interact with the regulatory subunits p85 or p55. Group B has one member, p110γ. Class I comprises three isoforms, none of which requires a regulatory subunit for its activation. Class III has only one member.

PKB/Akt is a serine-threonine kinase similar to PKC and PKA activated by phosphorylation of threonine 308 in its catalytic domain and Serine 473 in the C terminal domain (Alessi, Andjelkovic et al. 1996). In mammals, three closely related isoforms of Akt are encoded by distinct genetic loci: Akt1, Akt2 and Akt3. They all have different tissue distribution, since Akt1 is expressed ubiquitously except the kidney, liver and spleen, Akt2 expression is restricted to muscle, intestinal organs and reproductive tissues, and Akt3 in brain and testis (Vanhaesebroeck, Leevers et al. 1997). Akt has been implicated in the regulation of cell survival, glucose transport/metabolism and inhibition of programmed cell death in several cell types in many different cell death situations, including withdrawal of extracellular signaling factors, oxidative and osmotic stress, irradiation, chemotherapeutic drugs and ischemic shock.

Once phosphorylated and activated, serine-threonine kinase PKB/Akt can phosphorylate many downstream targets in the cytoplasm. One of the first targets of Akt identified with direct involvement in cell survival was the proapoptotic Bcl-2 family member Bad activated by phosphorylation on Ser136 (Downward 1999). Akt also inhibits glycogen synthase (GSK) 3 avoiding c-Myc and cyclin D inactivation and degradation, forkhead transcription factor (Foxo-3) implicated in expression of the Fas ligand (Kops and Burgering 1999), IκB allowing NF-κB to up-regulate antiapoptotic proteins (Kane, Shapiro et al. 1999) and, in human cells, caspase 9 involved in the mitochondrial pathway of apoptosis (Blume-Jensen and Hunter 2001).

Another important class of Akt targets are proteins involved in the stress-activated protein kinase (p38/JNK SAPK) cascades and Raf-1 (Fulton, Gratton et al. 1999). In some cells, Akt can contribute to Raf-1 inactivation through phosphorylation of Ser259 (Zimmermann and Moelling 1999). Previous studies revealed that overexpression of PI3K resulted in activation of JNK but not MAPK, positioning PI3K upstream of JNK, which can modulate survival and activation of NF-κB (Kobayashi, Nagata et al. 1997; Liu, Ye et al. 1997).

An example of a downstream enzyme that is activated by Akt is 6-phosphofructo-2 kinase (PFK2) which regulates glucose flux to glycogen and glycolysis (Cross, Alessi et al. 1995). In addition, mixed lineage kinase-3 (MLK3) and apoptosis signal-regulating kinase 1 (ASK1) are functionally inhibited following Akt-dependent phosphorylation. On the other hand, after growth factor stimulation, Akt can phosphorylate Mdm2, which enters the nucleus inducing a decrease in p53 levels and transactivation. Consequently, the Mdm2-p53 complex leaves the nucleus, and in the cytoplasm p53 becomes degraded through a ubiquitine/proteasome-mediated process (Zhou, Liao et al. 2001). PKB/Akt also affects the phosphorylation state of the mammalian target of Rapamycin (mTOR, also known as FRAP and RAFT) either directly by phosphorylation or indirectly through the tuberous sclerosis complex (TSC1/TSC2), which acts as a modulator between PI3K and AKT (Gao, Gao et al. 2002; Inoki, Li et al. 2002).

PKB/Akt also modulates cell cycle progression, cell survival and cellular growth translocating to the nucleus and affecting the phosphorylation state of specific transcription factors (Testa and Bellacosa 2001). PKB promotes cell cycle entry by inducing E2F activity (Brennan, Babbage et al. 1997). Furthermore, PKB has been shown to negatively regulate p21[Waf-1/Cip1] and p27[Kip1] cdk inhibitors by phosphorylation, inducing their cytoplasmic localization, and thus inhibiting their interaction with proliferating cell nuclear antigen (PCNA), and promoting cicD1-Cdk4 activation and expression of target genes of FOXO transcription factors (Shin, Yakes et al. 2002).

The PI3K pathway is negatively regulated by cAMP (Kim, Park et al. 2001) and tumor suppressor PTEN (phosphatase and tensin homologue) (Leslie, Gray et al. 2000). PTEN is a dual-specificity lipid and protein phosphatase that acts directly on PI3K lipids and proteins. The gene encoding this protein is mutated and inactivated or overexpressed in a significant number of human cancers (Tsugawa, Jones et al. 2002).

2.2. Cell cycle

The cell cycle is a universal process by which cells divide in the presence of mitogenic signals maintaining genome integrity (Gutierrez, Ramirez-Parra et al. 2002). The mammalian cell cycle consists of several consecutive phases: G1, in which the cells prepare their machinery for DNA replication; S phase, in which duplication of genomic information occurs; G2, an interphase of preparation for mitosis, and M phase, in which nuclear and cellular division takes place. Stages of mitosis include prophase, metaphase, anaphase, telophase and cytokinesis. Cells thus generated can either start a new cycle or remain in a state of quiescence in the absence of growth factors, known as G0 phase. Cells in G0 phase stop growing and depress their protein synthesis accounting for the major part of non-proliferating cells.

Macrophages require CSF-1 for most of the G1 phase. Once the cells commit to the S phase, they are able to continue the rest of the cell cycle in the absence of growth factor stimulation. G1 lasts approximately 12 hours in macrophages and S, G2 and M phases last 6, 4, 2 hours, respectively (Rock, Cleveland et al. 1992). The transition from one phase to another occurs in an orderly fashion and is regulated by different cellular proteins. Diverse and partly overlapping checkpoint pathways operate in various cell cycle phases. Checkpoints were traditionally defined as molecular signaling cascades that promote cell cycle delay or arrest in response to abnormal damage, thereby providing more time for the repair of the damage and an orderly sequence of events in the cell cycle (Lukas and Bartek 2004).

The restriction point in G1/S checkpoint is activated transiently by DNA damage in order to provide time for DNA repair. DNA damage causes a p53 and Cdc 25 dependent arrest (Levine 1997). p53 stimulates the transcription of Cdk inhibitors such as p21[Waf-1/Cip-1] or negative autoregulatory molecules such as Mdm2 that inhibits p53 transcriptional activity and facilitates its ubiquitination (Agarwal, Mathur et al. 1999). Phosphorylation of Cdc25 phosphatase by Chk2 kinase enhances its ubiquitination and proteasome-mediated degradation (Sorensen, Syljuasen et al. 2003). The accumulation of p21[Waf1] and the disappearance of Cdc25 thus reduce the G1/S inhibiting Cdk2 activity required for recruitment of DNA polymerases into pre-replication complexes preventing initiation of

DNA synthesis and inhibiting Cdk4 dependent phosphorylation of pRb. Additional controls of checkpoints exist further in the cell cycle. In this route, there are also checkpoints during S phase and after DNA replication (G2/M checkpoint).

Mechanisms of S phase checkpoints are poorly understood, although some demonstrate inhibition of initiation and elongation of DNA replication by DNA polymerases (Tercero and Diffley 2001). There are three types of S-phase checkpoints. (i) The replication dependent intra S phase checkpoint initiated by defects in replication fork action such as depletion of deoxyribonucleotide pools (dNTPs) or presence of aberrant or damaged DNA structures. (ii) The intra-S-phase checkpoint induced by DNA double strand breaks (DSBs) is independent of the replication fork. (iii) The S-M checkpoint ensures that cells do not attempt to divide before their entire genomes become faithfully duplicated, through the evolutionary conserved cyclin B-Cdk1 complex.

The G2/M checkpoint prevents cells from initiating mitosis when progress in the cell cycle has experienced some damage that was not repair in previous phases. The key downstream target of the G2 checkpoint is the major mitosis promoting Cdk1 kinase operating through a similar mechanism to G1 and intra S phase checkpoints involving Cdc25 phosphatase down-regulation and up-regulation of Wee1, controlling Cdc2 activity (Donzelli et al., 2003).

Several methods for studying cell-cycle regulatory mechanisms have been developed. To synchronize cells, they are growth factor starved for 16-24-48 hours. Then, after growth factor addition, the cells re-enter the cell cycle in a synchronous fashion and can typically be analysed through most of the cycle. Microtubule-disrupting agents, such as nocodazole, arrest cells in M phase (Uzbekov, Chartrain et al. 1998). To determine the percentage of cells exiting quiescence and progressing through the cell cycle, cells entering S phase can be monitored by bromo-deoxy uridine (BrdU) incorporation (Wu, Hong et al. 2001), or by staining with propidium iodide (PI) for DNA content, followed by analysis using a flow cytometer (Schmid, Cole et al. 2000). The effects of growth factors on DNA synthesis can be measured by [³H]-thymidine incorporation as a pulse after growth factor addition.

2.2.1. Cdk-Cyclin complexes

Cell division has several checkpoint control mechanisms, coordinated by a number of proteins including cyclins (Cic) and cyclin-dependent kinases (Cdks) to ensure the progression through cell-cycle phases (Grana and Reddy 1995). Cdks exist as inactive serine/threonine kinase monomers that become activated when bound to specific cyclins. In contrast to yeast, where a single Cdk, cdc2 or cdc28, controls cell cycling, mammals

have evolved and have several Cdks. While levels of Cdks usually remain constant through the cell cycle, expression of cyclins varies by transcriptional or post-transcriptional regulators; thus each cyclin has a unique pattern of expression during the cell cycle.
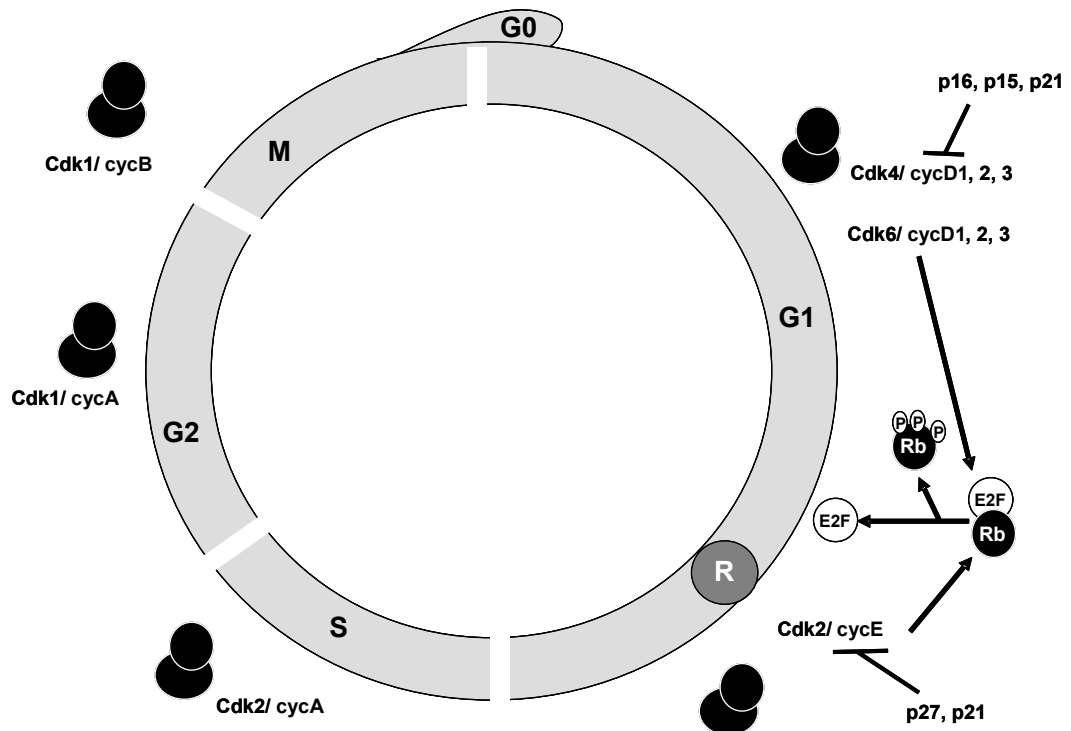

Figure 8. The cell cycle of eukaryotic cells. Cell cycle consists of G1, S, G2 and M phases regulated by CDK-Cyclin complexes. S, DNA replication; M, mitosis; G1 and G2, gaps separating periods of DNA replication; R, restriction point; G0, quiescent state; Cdk, cyclin dependent kinase; cic, cyclin; Rb, retinoblastoma tumor suppressor gene, and E2F transcription factor.

Control of the G0 quiescence-early G1 transition is mediated through mammalian D-type cyclins up-regulated in the presence of growth factors facilitating early G1 progression (Sherr 1994). D-type cyclins, in conjunction with their Cdk partners, Cdk4 and Cdk6, perform the initial inactivation of pRb through hyperphosphorylation at the late G1 restriction point. Two classes of cyclins are successively activated during the G1 phase of the cell cycle, D-type cyclins (cyclins D1, D2 and D3) (Matsushime, Roussel et al. 1991; Sherr 1994) and cyclin E (cyclins E1 and E2) (Payton and Coats 2002). These cyclins assemble with their catalytic partner, Cdk4 or Cdk6 for cyclin D at early G1 and Cdk2 for cyclin E at late G1, in order to be active. Cyclins A and B are then recruited by Cdk2 and Cdk1 (also known as Cdc2) to induce cell cycle progression respectively through S phase and towards mitosis (Arellano and Moreno 1997). Cyclins A and B contain a destruction box and cyclin D and E contain a PEST sequence (enriched in proline, glutamic acid,

serine and threonine) required for efficient ubiquitin-mediated cyclin proteolysis at the end of a cell cycle phase (Glotzer, Murray et al. 1991).

The activity of Cdks is regulated at three levels: cyclin association, phosphorylation and association with Cdk inhibitors (CDKIs) (Grana and Reddy 1995). The Cdk subunit is inactive as monomer or in an unphosphorylated state. To provide correct access to the binding sites within their catalytic cores a conformational change is necessary. Until cyclin binding, the substrate-binding site is blocked by a domain called the T loop that prevents binding of the target protein and access to ATP. Following cyclin binding, conformational modification induces correct positioning of the ATP phosphates. Additionally, a conserved threonine of the T loop becomes accessible for phosphorylation by the CAK (Cell cycle activated) protein kinase, and achieves complete activation.

In addition to cyclin binding, Cdk activity is also regulated by phosphorylation on conserved threonine and tyrosine residues by Wee1/Mik1/Myt1 protein kinases modifying the orientation of residues of the catalytic site inhibiting binding of cyclins (Donzelli and Draetta 2003). Furthermore, in humans, three phosphotyrosine phosphatases, Cdc25A, B and C dephosphorylate Cdks acting on proteins directly involved in cell-cycle transitions. Phosphorylation of Cdc25 proteins by checkpoint kinases inactivates them, excluding them from the nucleus and causing their proteolytic degradation. Cdc25 is tightly regulated at protein level being periodically synthesized and degraded via ubiquitin mediated proteolysis. In late G1, Cdc25 accumulates as a result of E2F and c-Myc mediated transcriptional activation dephosphorylating Cdk2 and activating Cdk2-cyclin E complexes (Ross, Tienhaara et al. 2002). In mitosis, Cdc25 is stabilized by phosphorylation by Cdk1-cyclin B. At mitotic exit and early G1, Cdc25 levels rapidly decrease by degradation.

2.2.2. Cdk inhibitors, CDKIs

Cdk activity can be counteracted by cell cycle inhibitory proteins called Cdk inhibitors (CDKI), which bind to Cdk alone or to the Cdk-cyclin complex and regulate its activity. Two distinct families of Cdk inhibitors have been discovered, the INK4 family and Cip/Kip family (Sherr and Roberts 1995).

2.2.2.1. INK4 family

The INK4 CDKI family is composed of specific inhibitors of G1 Cdks, Cdk4 and Cdk6, and includes $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$. These CDKIs form stable

complexes with the Cdk enzyme through ankirin-like domains before cyclin binding preventing its association (Carnero and Hannon 1998; Pavletich 1999).

p16 blocks cyclin D1/Cdk4-specific phosphorylation of pRb inducing cell cycle arrest in G1. A second member of the INK4 family, p15, is located adjacent to p16 gene and is codeleted in a high proportion of human cancer cell lines. Furthermore, p15 appears to act as an effector of TGF-β mediated cell cycle arrest. The third and fourth members of this family, p18 and p19, also block Cdk4 and Cdk6 activity and act as tumor suppressors (Roussel 1999).

2.2.2.2. CIP/KIP family

The Cip/Kip family of inhibitors includes p21$^{Waf-1/Cip-1}$, p27$^{Kip-1/Cip-2}$ and p57^{Kip-2} (Harper, Adami et al. 1993; Matsuoka, Edwards et al. 1995). These CDKIs contain a conserved region of sequence at the NH2 terminus that is required and sufficient for the inhibition of Cyclin-Cdk complexes, whereas the C terminal regions are variable in length and function (Chen, Jackson et al. 1995). Cip/Kip family inhibits a broader set of G1/S Cdks, Cdk2, Cdk4 and Cdk6 and to a lesser extent, Cdk1-cyclin B complexes (Harper, Adami et al. 1993). Unlike INK4 inhibitory proteins, Cip/Kip proteins do not dissociate cyclin-Cdk complexes. Because of their inhibitory activity on cell cycle progression, CDKIs are considered to be potential tumor suppressor genes (el-Deiry, Tokino et al. 1993).

2.2.2.2.1. p21$^{Waf-1/Cip-1}$

p21$^{Waf-1/Cip-1}$ is a CDKI with a wide spectrum of Cdk substrates that has been implicated in the mechanisms of cell cycle arrest that allow cell DNA repair (Li, Waga et al. 1994). p21$^{Waf-1/Cip-1}$ binds to the DNA polymerase δ processivity factor and proliferating-cell nuclear antigen (PCNA) through its C terminus, and inhibits PCNA-dependent DNA replication forming quaternary complexes with Cyclin-Cdk, but does not inhibit DNA repair.

p21$^{Waf-1/Cip-1}$ associates with cyclins-Cdks in two functionally distinct forms, one in which the kinase activity is inhibited and the other in which the kinase is still active, depending on stechiometry (Sherr and Roberts 1999). Under physiological conditions, p21$^{Waf-1/Cip-1}$ can bind to Cdk4/cyclin D and Cdk6/Cyclin D complexes and induce their kinase activity for early G1 until the middle of S phase. Also, p21$^{Waf-1/Cip-1}$ can bind to and inhibit kinase activity of Cdk2/cyclin A and Cdk2/cyclin E complexes from the late G1 until the S phase maintaining pRb inactivated since all genes of G1 are expressed correctly (Rank, Evans et al. 2000).

Based on structural studies, it is believed that an α-helix of a Cip/Kip protein initiates a first contact with the cyclin and that a second helix then inserts deep inside the catalytic cleft of the Cdk subunit, thereby blocking ATP loading. It is also believed that Cip/Kip proteins located in the cytoplasm act as a bridge between the two subunits to enhance the binding of cyclin D1 to the Cdk (Peter 1997).

p21$^{Waf-1/Cip-1}$ has also been related to cell survival promotion through an Akt dependent induction mechanism. It was shown that Akt could directly phosphorylate both threonine 145 and serine 146 near the C terminus of p21$^{Waf-1/Cip-1}$ decreasing its affinity for Cdk2 and releasing PCNA from the PCNA/p21$^{Waf-1/Cip-1}$/Cdk/cyclin complexes (Rossig, Jadidi et al. 2001). As a result of these phosphorylation events, DNA synthesis and Cdk activity was stimulated and cellular proliferation increases. Phosphorylated cytoplasmic p21$^{Waf-1/Cip-1}$ can then interact with the SAPK and ASK1 (apoptosis singal-regulating kinases) to inhibit their catalytic activities and prevent apoptosis induced by TNF-α and other stimuli (Suzuki, Tsutomi et al. 1999). In fact, previous studies by our group demonstrate that p21$^{Waf-1/Cip-1}$ is responsible for antiapoptotic effects induced by growth factor deprivation or LPS stimulation (Xaus, Valledor et al. 1999). Finally, p21$^{Waf-1/Cip-1}$ interacts with procaspase-3 in the mitochondria to inhibit its activation and to resist Fas-mediated cell death (Suzuki, Tsutomi et al. 1999).

Several studies suggest that Cip/Kip proteins might function as transcriptional cofactors. p21$^{Waf-1/Cip-1}$ regulates the activity of NF-κB, STAT-3 (Coqueret and Gascan 2000), Myc (Kitaura, Shinshi et al. 2000), C/EBP and E2F (Harris, Albrecht et al. 2001). The expression of genes involved in cell cycle progression such as DNA polymerase α, topoisomerase II, cyclin B1 and Cdk1 is regulated by p21$^{Waf-1/Cip-1}$. p21$^{Waf-1/Cip-1}$ expression has been shown to be regulated at transcriptional level by both p53-dependent and independent mechanisms (Peter 1997). After DNA damage, p21$^{Waf-1/Cip-1}$ is induced by two conserved p53-binding sites in p21$^{Waf-1/Cip-1}$ promoter. In addition, a variety of transcription factors induced by different signaling pathways activate p21$^{Waf-1/Cip-1}$ transcription by p53-independent mechanisms including Sp1, Sp3, Ap2, STATs, C/EBPα, C/EBPβ and the bHLH proteins BETA2 and MyoD. p21$^{Waf-1/Cip-1}$ expression may also be regulated posttranscriptionally by both ubiquitin-dependent and independent proteasome mediated degradation (Touitou, Richardson et al. 2001). In summary, p21$^{Waf-1/Cip-1}$ has both positive and negative regulatory effects on cell cycle progression and also as a regulator of cell survival.

2.2.2.2.2. p27^{Kip-1}

p27^{Kip-1} is a member of CDKI inhibitory proteins with an important role in inhibiting Cdk2 activity and DNA synthesis. Unlike p21$^{Waf-1/Cip-1}$, p27^{Kip-1} does not bind PCNA. During G1 progression, cyclin D1 associated with Cdk4 sequestrates p27^{Kip-1} releasing cyclin E/Cdk2 from its inhibitory effect, thereby inducing Cdk2 activity and progression toward S phase (Medema, Kops et al. 2000). The association of p27^{Kip-1} with Cdk2 involves an arrest of cell cycle. In fact, in human tumors, p27^{Kip-1} itself or its activity appear to be lost by increased degradation, cytoplasmic mislocalization or sequestration.

p27^{Kip-1} also regulates the exit of cells from the cell cycle in response to antimitogenic signals. The amount of p27^{Kip-1} increases in quiescent cells or cells undergoing differentiation inhibiting the cyclin E-Cdk2 complexes. Although it is unclear how differentiation signals trigger p27^{Kip-1} accumulation, it is known that mitogens promote p27^{Kip-1} degradation, allowing quiescent cells to re-enter the cell cycle. In early G1, p27^{Kip-1} inhibits the activity of AP-1 through down-regulation of its JAB1 cofactor blocking the activity of transcription factors in early G1 and preventing the activation of cell-cycle regulatory genes (Ghosh Choudhury, Kim et al. 1999).

p27^{Kip-1} activity can be altered through the PI3K/Akt and Ras/Raf/MEK pathways. The FOX family (AFX/FOXO4, FKHR/FOXO1 and FKHR-L1/FOXO3a) of transcription factors can be inactivated through direct phosphorylation of multiple S/T residues by Akt (Brunet, Roux et al. 1999). As a result of these phosphorylations, proapoptotic genes induced by FOX family are down regulated, including both p27^{Kip-1} and Fas (Liang, Zubovitz et al. 2002).

p27^{Kip-1} executes similar mechanisms to p21$^{Waf-1/Cip-1}$ in assembling and targeting Cdk and cyclin D to the nucleus. Cytoplasmic Cip/Kip proteins promote nuclear import of D-type complexes that do not possess signal motifs for nuclear localization, involving Cip/Kip proteins in the communication between cytoplasm and nucleus. p27^{Kip-1} can bind to the nuclear pore-associated protein mNPA60 and p21^{Waf-1} blocks the interaction between cyclin D1 and the exportin CRM1, leading to increased cyclin D levels in the nucleus (Gaubatz, Lees et al. 2001).

Cell cycle deregulation associated with cancer typically occurs through mutation of proteins that are important at different levels of the cell cycle. In cancer, mutations have been observed in genes encoding Cdk, cyclins, Cdk-activating enzymes, CDKIs, Cdk substrates and checkpoint proteins (Grana and Reddy 1995).

2.2.3. c-Myc

In mammalian cells, the highly regulated expression of nuclear Myc family transcription factors (c-, N- and L-Myc) is closely tied to cell growth and proliferation as well as inhibition of terminal differentiation and induction of apoptosis. C-Myb and c-Myc are highly expressed in proliferating myeloblastic cells and are strongly repressed after induction of their terminal differentiation. After a certain stage in monocytic maturation, the terminal differentiation program is no longer dependent on the downregulation of c-Myc. From that point on, the induction of c-myc expression has no effect on the phenotype of mature macrophages. By contrast, deregulation of Myc expression drives progression of many different types of cancer (Nesbit, Tersak et al. 1999).

c-Myc is a proto-oncogene discovered as the cellular homologue of the viral gene v-myc. The c-Myc protein consists of three domains, the N-terminal, the central and C-terminal domains. The N-terminal domain of c-Myc is involved in the activation of transcription and includes the highly conserved Myc box regions (MB1 and MB2) that are unique to the Myc family. The C-terminal DNA-binding domain of c-Myc contains both a basic region-helix-loop-helix and a leucine zipper motif (b-HLH-Leu zip) essential for DNA binding and interaction with other proteins. To recognize its binding site and activate gene transcription, c-Myc must form a heterodimer with another bHLH-Leu zip transcription factor, Max. Myc competes with bHLH-Leu zip Mad, Mxi1/Mad2, Mad3, Mad4 and Mnt cofactors for Max binding (Baudino and Cleveland 2001). These alternative heterodimers can compete with the Myc-Max complex repressing transcription through deacetylation of histones, thus antagonizing the transcriptional and transforming activities of c-Myc. After binding Max, the heterodimer binds to the cis-acting Myc E box element (5'-CACGTC) in the promoter of their target genes to transactivate their expression (Yang, Shen et al. 2001). The ability of c-Myc to modulate gene transcription is also dependent on the phosphorylation state of Myc: Myc can be phosphorylated on the central acidic and C terminal domain by Casein kinase-II (Hagiwara, Nakaya et al. 1992) and on the N terminal transactivation domain by glycogen synthase kinase-3β, EGF (epidermal growth factor) receptor and ERK kinase (Kubin, Parshley et al. 1999).

Myc expression is also rate limiting for G1 progression in response to growth factors, and deregulated expression of c-Myc is sufficient to drive continuous cell proliferation or apoptosis in response to growth-promoting or inhibitory signals respectively. Moderate c-Myc expression modulates important proliferation-associated gene expression and leads to cell cycle progression. However, strong c-Myc expression induces apoptotic gene expression and leads to apoptosis.

Figure 9. Myc family members, Myc, Max, Mad and Mxi1. Domains involved in heterodimerization. bHLH, basic helix-loop-helix; LZ, leucine zipper.

c-Myc directly targets and activates the expression of genes that regulate cell cycle progression which include cyclin D1, cyclin D2, cyclin A, cyclin E, Cdk4 and Cdk1. c-Myc also directly represses transcription of Cdk kinase inhibitors including $p27^{Kip-1}$, $p21^{Waf-1/Cip-1}$, $p15^{Ink4b}$ and $p16^{Ink4a}$ (Amati, Alevizopoulos et al. 1998). By direct transcriptional effects on cyclin E and Cdc25A expression or indirect mechanisms such as sequestration of the Cdk inhibitor $p27^{Kip-1}$ into cyclin D-Cdk4/6 complexes away from cyclin E-Cdk2, and phosphorylation and subsequent ubiquitination and proteasome-mediated degradation of the $p27^{Kip-1}$ protein at G1/S, c-Myc can also activate Cdk2-cicE complexes (Mateyak, Obaya et al. 1999).

In addition to inducing cell proliferation, c-Myc can also induce apoptosis (Soucie, Annis et al. 2001). The apoptosis induced by c-Myc is a protective mechanism to control tumor development with therapeutic applications. C-Myc-mediated cell apoptosis is afforded through its ability to stimulate the proapoptotic Bax activity in the mitochondria leading to cytochrome–c (Cit c) release and eventually apoptosis in response to apoptosis-inducing agents. Other apoptosis associated genes including tumor necrosis factor receptor-associated protein-1 (TRAP1) are also c-Myc targets (Dong, Naito et al. 1997).

2.2.4. Retinoblastoma family

One of the major substrates of the Cdks in G1 progression is the retinoblastoma tumor suppressor gene (pRb) and pocket proteins, p107 and p130 (Harbour and Dean 2000). When cells are quiescent in the G0 state, pRb is underphosphorylated. As the cells progress through G1, pRb becomes more and more phosphorylated by Cdk4/6 and Cdk2 to the point where cells are committed to S phase.

Phosphorylation of Rb is absolutely required for the exit from G1 and the commitment to S phase, because in underphosphorylated form it acts as a transcriptional

repressor complex that sequesters E2F factors inhibiting the expression of genes involved in G1/S transition, S phase and DNA synthesis. When pRb is hyperphosphorylated, it releases E2F and DP-1 factors to activate transcriptional expression of S-phase genes, including cyclin A, E and Cdc25 (Flemington, Speck et al. 1993). pRb remains hyperphosphorylated for the remainder of the cell cycle and Cdk2-cyclin E complex participates in maintaining this hyperphosphorylated state, acting as a repressor. Pocket proteins inhibit expression of E2F-regulated genes in two ways: by directly binding and blocking of the activation domain of E2F proteins; or by active repression through the recruitment of histone deacetylases (HDACs) and SWI/SNF factors (Rayman, Takahashi et al. 2002). Disruption of pRb pathway by either overexpression of cyclin D or loss of p16^{INK4} inhibitor, or even loss or mutation of pRb itself, are common events associated with increased proliferation of cancer cells.

2.2.5. E2F proteins

In mammalian cells, the E2F family of transcription factors plays a pivotal role in regulating the expression of genes involved in the G1/S transition and DNA synthesis during cell cycle progression. There are at least six E2F proteins that can be subdivided into three categories based on their transcriptional properties and their interaction potential with pocket proteins. E2Fs heterodimerize with DNA binding protein partners called DP1 and DP2/3. They have been identified in mouse (mE2F1 and mE2F2) and Drosophilae melanogaster (dE2F) (Stevaux and Dyson 2002). Murine E2F1 is 86% identical to human E2F1.

E2F1, -2, and –3 are potent transcriptional activators that interact mainly with pRb. They share an N-terminal cyclin A-Cdk binding domain and a canonical basic nuclear localization signal (NLS). E2F1, E2F2 and E2F3 are required at late G1 to induce S-phase entry and activation of E2F target gene expression as can be seen in E2F1/2/3 TKO. The human E2F1, 2 and 3 mRNAs are present in a broad range of mammalian tissues (Trimarchi and Lees 2002).

E2F4 and –5 act as repressors of activation and appear to be able to interact with all three pocket proteins, predominantly with p107 and p130 . They possess a nuclear export signal (NES), thus, E2F4 and –5 are mainly cytoplasmic and need to assemble with a pocket protein to be transported into nuclei. They can occupy E2F activating DNA-binding sites, but cannot activate gene expression. Second, the pRb-E2F complex, while still bound to DNA, recruits various factors, e.g. HDACs (Histone deacetylase), SWI/SNF, Polycomb group proteins and histone methyl transferase (SUV39H10) that are able to switch off transcription causing active repression (Robertson, Ait-Si-Ali et al. 2000). E2Fs

can promote transcriptional activation by increasing histone acetylation (Polanowska, Fabbrizio et al. 2001). HDACs (histone deacetylase) regulate the expression of the cyclin E gene, and in turn, cyclin E/cdk2 complexes play a role in regulation of histone gene expression induced during G1/S phase transition for chromatin remodeling. It has been shown that the acetylation of histones is important for increasing the accessibility of DNA to many transcription factors (Polanowska, Fabbrizio et al. 2001).

E2F6 does not associate with pocket proteins and it is believed to repress transcription through its interaction with Polycomb group proteins (Morkel, Wenkel et al. 1997). In fact, E2F6 diverges considerably from the other E2F family members, sharing almost no homology outside the core DNA binding and dimerization domains, and possessing truncated C and N terminal regions relative to those of the other E2F subgroups.

The regulation of the different E2F family members is carried out by transcriptional control, post-translational modifications and interactions with other proteins. Different E2F complexes accumulate in different phases of cell cycle. For example, E2F4-p130-HDAC (histone deacetylase) complexes are detected in quiescent cells, whereas E2F4-p107 or E2F4-pRB complexes accumulate in G1. Free E2F1, -2, and -3 and E2F4-p107 complexes and increase in acetylation of histones H3 and H4 are most prevalent in S phase fractions, as E2F target genes are highly expressed and pocket proteins hyperphosphorylated by Cdk4/Cdk6-cicD and Cdk2-cicE complexes. Cyclin A-Cdk2 turn off E2F target genes because of phosphorylation of DP-1 and subsequent loss of E2F DNA binding activity (Krek, Xu et al. 1995). This event, in addition to the rapid turnover of cyclin E mediated by ubiquitin dependent proteolysis, is required for cell cycle exit from S phase.

Target genes of E2F family have been studied by array technology. Typical targets include those encoding S-phase entry regulators such as cyclin E, cyclin A, cyclin D1, cdc2, Cdc25A, c-Myc and Cdk2, products involved in the assembly of the pre-replication complex at origins of replication such as ORC proteins, MCMs (mini chromosome maintenance proteins), Cdc6 and enzymes needed for the direct synthesis of DNA such as ribonucleotide reductase, dihydrofolate reductase (DHFR), thymidine kinase (TK) and DNA polymerase α. Also genes involved in chromosome segregation by centrosomes, mitotic spindle checkpoints, chromatin assembly/condensation apoptosis, differentiation and development are regulated by E2Fs (Ren, Cam et al. 2002). Several genes involved in DNA repair and recombination repair, closely linked to DNA synthesis, are also regulated by E2F, such as msh2 and mlh1 (mismatch repair genes), Fanconi anaemia, rpa3 (replication protein of excision repair), rad 51, recQ1 and rad 54 (double-strand break recombination repair).
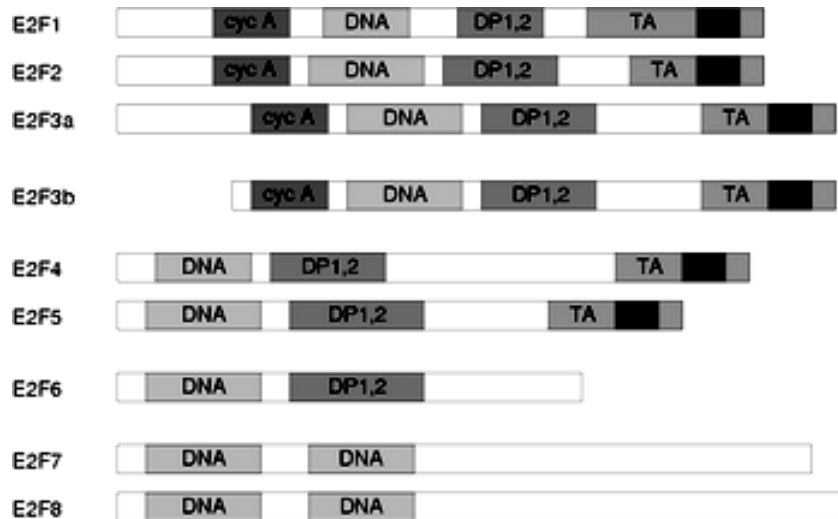
Figure 10. E2F family proteins. cyc A - Cyclin A binding domain; DNA - DNA-binding domain ; DP1,2 - domain for dimerization with DP1,2;TA - transcriptional activation domain; PB - pocket protein binding domain

E2F-1 can trigger apoptosis through both p53-dependent and independent pathways (Phillips, Bates et al. 1997). Thus, E2F1 augments p53 dependent apoptosis through the transcriptional activation of the ARF gene, which in turn modulates the activity of Mdm2 (Tolbert, Lu et al. 2002). Also, E2F-1 signals apoptosis independent of p53 via direct transcriptional activation of the p53 family member, p73, induces cell cycle arrest and may trigger apoptosis (Irwin, Marin et al. 2000). In DNA-damage responses, E2F express DNA-repair and pro-apoptotic genes including Apaf-1 (apoptosis protease-activating factor 1) which regulates cytochrome c release from mitochondria and activates caspase 9 leading to the activation of downstream effector caspases, caspase 3 and caspase 7 (Moroni, Hickman et al. 2001).

Another level of control of E2F activity includes targeted proteolysis and acetylation. The rapid degradation of E2F-1 at the S to G2 transition requires an interaction between the E2F-1 and the F-box-containing protein p45SKP2, the cell cycle–regulated component of the ubiquitin-protein ligase SCF (skip/cullin/F-box) SKP2, targeting E2F-1 for degradation. Therefore, cyclin A regulates the duration of S phase since skip/cullin/F-box (SCF) complex targets E2F-1 for destruction once S phase is complete (Marti and Ballmer-Hofer 1999).

E2F protein degradation is also regulated by p14ARF (p19ARF, the mouse homologue) that binds to the C terminus of E2F-1 and flags it for ubiquitination and degradation via the proteasome pathway (Martelli, Hamilton et al. 2001). The subcellular localization of various E2F members also seems to play a crucial role in their activity. Only the activating E2Fs have a nuclear localization signal (NLS) in their protein, whereas

the repressive E2Fs lack NLS and depend on either DP-1 or pocket protein for their nuclear localization.

E2F gene knockout in mice has revealed distinct roles for E2F family members in mouse development and physiology. Disruption of E2F1 results in formation of tumors in older adults (Yamasaki, Jacks et al. 1996). They also exhibit decreased T cell apoptosis. Mouse embryonic fibroblasts (MEFs) derived from E2F1-/- mice show a delayed exit from G0 phase, indicating that E2F-1 has an important role in timing S phase entry from G0 (Wang, Yang et al. 1998). E2F2 mutant mice exhibit increased proliferation of haematopoietic cells and develop autoimmunity and tumors. E2F3 mutant mice exhibit embryonic lethality, and reduced cell cycle entry. E2F4 -/- mice have defective erythropoiesis and craniofacial defects. E2F5 -/- mice develop hydrocephalus. The combined loss of E2F1 and E2F2 results in defects on spermatogenesis and on B cell differentiation but induces T cell proliferation and increases percentages in S phase. They develop diabetes as a result of destruction of the pancreatic β islet cells (Li, Zhu et al. 2003).

2.3. Inhibitors of M-CSF dependent proliferation

Several mechanisms inhibit M-CSF dependent proliferation of macrophages. Protein kinases such as cAMP-dependent protein kinase (PKA) are known to regulate Raf-1 activity, consequently inhibiting proliferation dependent on Raf/MEK/ERK pathway. PKA also diminishes c-Myc, cic D1 and Cdk4 expression in macrophages and increases $p27^{Kip-1}$ levels (Xaus, Valledor et al. 1999). Consequently, agents that increase cAMP levels such as adenosine and E-prostaglandin (PGE_2) act as potent inhibitors of DNA synthesis induced by M-CSF (Xaus, Valledor et al. 1999). There are two PKA isozymes, I and II, that conserve their catalytic subunit but differ in the regulatory subunit. PKA I is typically associated to cell membrane while PKA II is localized in the cytoplasm (Laxminarayana and Kammer 1996). Binding of cAMP to its regulatory subunit releases it, thus phosphorylating several targets in the cytoplasm or entering the nuclei to transactivate CRE (cAMP-response elements) sequences of target promoters (Sassone-Corsi 1998).

Transforming growth factor-β (TGF-β) is a potent growth inhibitor of various cell types, causing growth arrest in G1 by down regulating c-Myc expression in order to prevent cell proliferation (Polyak 1996). A Smad3/ E2F-4/E2F-5/ DP-1/p107 complex pre-exists in the cytoplasm and it is translocated into the nucleus in response to TGF-β, where it associates with Smad 4. Smad 4 then allows the complex to recognize a

composite Smad-E2F binding site in the c-myc promoter leading to repression (Massague 2003).

Although lymphocytes undergo a clonal expansion when they are activated either after interaction with a peptide presented by the major histocompatibility complex (T cells) or after the direct recognition of an antigen (B cells), macrophage activation is linked to growth arrest and an enhancement of their ability to perform specialized functions in the immune system. Therefore, activation of macrophages by type I (IFN-α, IFN-β) and type II (IFN-γ) interferons, LPS or TNF-α, stops the proliferating program at G1 phase of cell cycle through different mechanisms (Vadiveloo 1999). However, whereas LPS induces apoptosis program, no such effect has been observed with IFN-γ. Moreover, pre-treatment of macrophages with IFN-γ protects them against apoptosis induced by LPS, glucocorticoids or M-CSF withdrawal through expression of p21$^{\text{Waf-1/Cip-1}}$ and blockage of the cell cycle at the G1/S boundary (Xaus, Cardo et al. 1999).

3. MACROPHAGE ACTIVATION

The classical activation of macrophages is induced by proinflammatory and/or microbial molecules such as lipopolysaccharide (LPS) in a Th1 cytokine environment (IFN-γ produced by T lymphocytes) or tumor necrosis factor alpha (TNF-α). Activated macrophages thus produce nitric oxide (NO) combined with an increased expression of major class II histocompatibility complex (MHC) and CD86, enhancing their antigen-presenting capacity. They also exert anti-proliferative and cytotoxic activities, resulting partly from their ability to secrete NO and pro-inflammatory cytokines (TNF-α, IL-1, IL-6) activating mechanisms of host survival (Klimp, de Vries et al. 2002).

These classical activated macrophages are inhibited by Th2 cytokines triggering an alternative activation program, and inversely. M2 macrophages are thus activated in the presence of IL-4 and IL-13, and deactivated in the presence of IL-10 (Stein, Keshav et al. 1992). They fail to generate NO from L-arginine and do not efficiently limit the growth of intracellular pathogens. M2 macrophages enhance expression of anti-inflammatory IL-1 receptor antagonist (IL-1Ra), scavenger receptors, IL-1 decoy receptor, alternative macrophage activation-associated CC chemokine (AMAC-1) and thymus activation regulated chemokine (TARC) (Imai, Nagira et al. 1999). M2 macrophages express similar levels to Th1 macrophages of CD11a, CD40, CD54, CD58, CD80 and CD86 costimulatory molecules. Moreover, they exhibit enhanced endocytic and phagocytic ability, increased expression of class II MHC molecules and can perform antigen presentation (Gordon 2003).

3.1. Type II Interferons

IFNs were classified into type I and type II according to receptor specificity and sequence homology. The type I IFNs comprise IFN-α, IFN-β and other subsets such as IFNι (Baccala, Kono et al. 2005). IFN-γ is the sole type II IFN. It is structurally unrelated to type I IFNs, binds to a different receptor and is encoded by a separated chromosomal locus. Type I IFNs are secreted at low levels by almost all cell types although haematopoietic cells are the major producers of IFN-α, whereas fibroblasts of IFN-β (Pestka, Krause et al. 2004). IFN-β is also produced by macrophages under appropriate stimulus (Puddu, Fantuzzi et al. 1997).

3.1.1. Biochemical characteristics of IFN-γ

Interferon-γ is involved in antiproliferative, antiviral, immuno modulatory, immune surveillance and tumor suppression responses (Boehm, Klamp et al. 1997). In fact, it has been reported that IFNs regulate the induction of more than 300 different genes through different mechanisms . IFN-γ is a homodimeric glycoprotein made from the non-covalent binding of two subunits encoded by the same gene.

Production of IFN-γ is principally carried out by CD4+ and CD8+ T lymphocytes and natural killer (NK) cells (Yoshimoto, Wang et al. 1998; Pestka, Krause et al. 2004), but some data suggest that antigen-presenting cells (APCs) and B cells also produce IFN-γ (Puddu, Fantuzzi et al. 1997; Frucht, Fukao et al. 2001). IFN-γ production by professional APCs (monocytes/macrophages and dendritic cells) acting locally may be important in cell self-activation and activation of nearby cells (Munder, Mallo et al. 1998). IFN-γ secretion by natural killer cells and possibly professional APCs is likely to be important in early host defense against infection, whereas T lymphocytes become the major source of IFN-γ in the adaptative immune response.

APCs regulate this process by producing IL-12 and IL-18, which induce the production of IFN-γ by natural killer cells and make direct naïve CD4+ T cells differentiate into IFN-γ producing T helper 1 (Th1) cells and CD8+ T cells. Several cytokines down-regulate the production of IFN-γ by APCs, including IFN-α, TGF-β, glucocorticoids, IL-4 and IL-10.

3.1.2. Signaling of type II interferon receptors

IFN-γ exerts its effects on cells by interacting with a different specific type II IFN receptor composed of two subunits, IFNγR1 and IFNγR2 (Bernabei, Coccia et al. 2001). Type II IFN receptor transduces signals via activation of receptor-associated tyrosine kinases of the Janus family (JAK kinases). Binding of IFN-γ to its receptor induces receptor oligomerization and activation of the receptor-associated JAK1 and 2 kinases by trans-phosphorylation (Sakatsume, Igarashi et al. 1995). The activated JAKs phosphorylate the intracellular domain of the receptor, which serves as a docking site for STAT binding. Additional tyrosine residues of the receptor and the JAKs themselves are also phosphorylated. Defects in IFN-γ or IFN-γ receptor result in profound deficiencies in their responses to certain intracellular pathogens, including mycobacterium and Salmonella (Ottenhoff, De Boer et al. 2003).

The JAK/STAT pathway was first identified as being involved in IFN-stimulation; later, new receptors appear to activate Stat-like activities such as IL-3, IL-7, SCF, G-CSF

and TGF-β receptors. The JAK/STAT pathway consists of three families of genes: the JAKs, the STAT (Signal transducers and activators of transcription) family and the CIS/SOCS family, which serves to down-regulate the activity of the JAK/STAT pathway (Kisseleva, Bhattacharya et al. 2002). Stimulation of the JAK/STAT pathway by activation of cytokine receptor results in STAT transcription factor activity. JAKS are a family of large tyrosine kinases of 120-140 kDa. Four JAKS (JAK1, JAK2, JAK3 and Tyk2) of seven different conserved domains (JH1-JH7) have been identified in mammals (Darnell 1998).
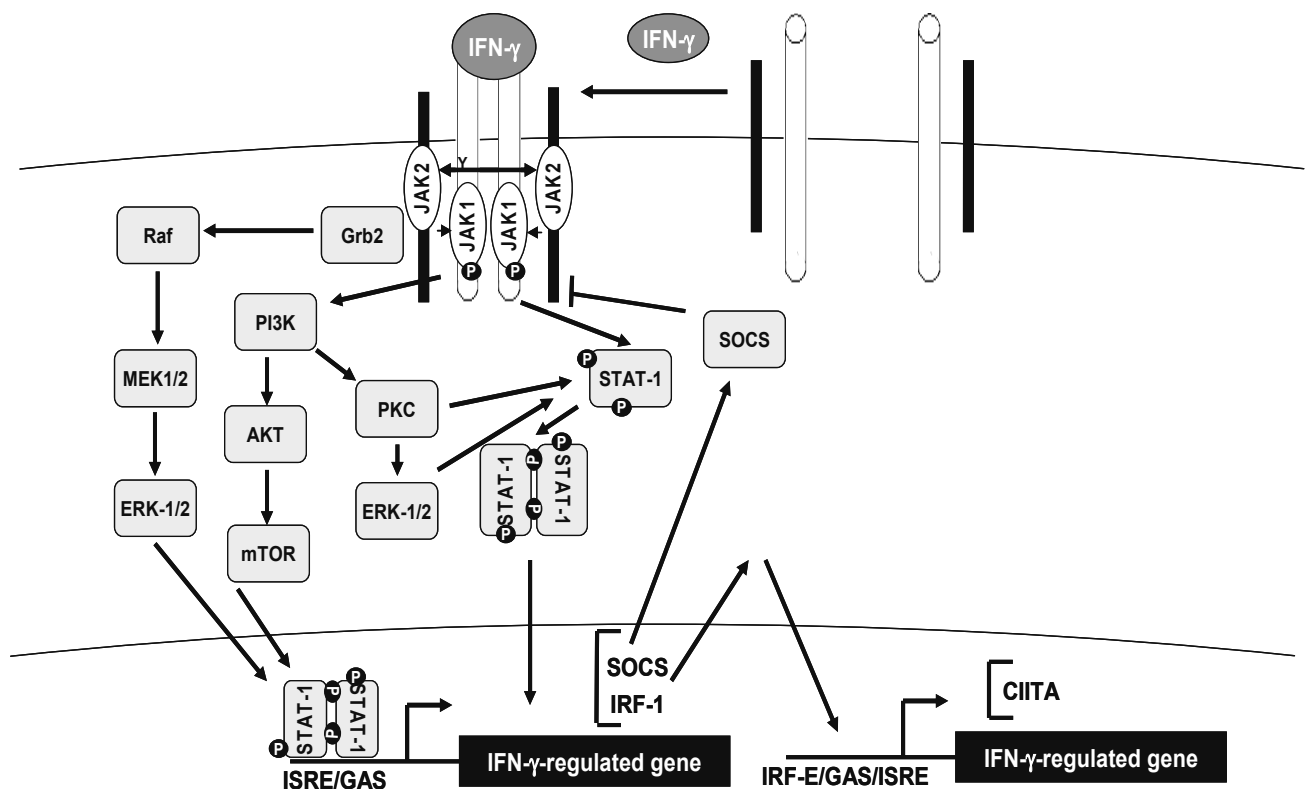

Figure 11. IFN-γ signal transduction. IFN-γ binding causes a conformational change in the receptor that autophosphorylates and activates Jak2 kinase, which, in turn, transphosphorylates Jak1 kinase. The activated Jak1 phosphorylates STAT-1 (signal transducer and activator of transcription) inducing its homodimerization. STAT-1 homodimers travel to the nucleus and bind to promoter IFN-γ activation site (GAS) elements to initiate/suppress transcription of IFN-γ-regulated genes such as IRF-1 (immediate-early inducible factor) and SOCS which are able to drive regulation of transcription or to inhibit the pathway. Simultaneously, the MAPK and PI3K pathways are activated.

The STAT gene family consists of seven proteins (Stat1, Stat2, Stat3, Stat4, Stat5α, Stat5β and Stat6) of 75-95 kDa. Structure of STAT family proteins consists of an N terminal oligomerization domain, a DNA-binding domain in the central part of the protein, a SH2 domain and a transactivation domain near the C terminal (Darnell 1998).

Activation of STATs genes by IFN-γ involves a rapid tyrosine 701 phosphorylation of the oligomerization domain by JAKs, allowing the phosphotyrosine product to interact with the SH2 domains of other STAT proteins forming homo/heterodimers. Subsequently, STAT dimers move to the nucleus where they bind to specific DNA sequences called IFN-sensitive response elements (ISREs) in the promoter of IFN-regulated genes to activate gene transcription, as well as increase protein stability. The DNA-binding domain recognizes a consensus site, TTCC(G/C)GGAA, present in IFN-γ activation site (GAS) elements (Kisseleva, Bhattacharya et al. 2002).

At some point, early in activation, STAT-1 is also phosphorylated on serine 727 by a process involving mTOR kinase activity which is required for maximal transcriptional activity of STATs (Goh, Haque et al. 1999). The mTOR kinase activity is independent of p70 S6Kinase in a Rapamycin-insensitive fashion. This activation forms a STAT1-mTOR macromolecular complex that can interact with atypical PKC isoforms supporting a dual role for PI3K and mTOR. STAT1-null mice develop normally but lack many classical responses to IFN-α/β and IFN-γ and are thus extremely susceptible to microbial and viral infections. In addition to the classical JAK-STAT pathway, there are other cascades such as creatinine kinase and insulin receptor substrate (IRS) pathways important in type II IFN signaling resulting in downstream activation of PI3K (Platanias and Fish 1999).

STAT activation is inhibited within 1 hour of IFN-γ treatment despite the continued presence of extracellular IFN-γ, through mechanisms controlling the extent of ligand stimulation. In fact, one of the most inducible targets of IFN-γ is a specific feedback inhibitor, SOCS-1, which associates with JAK-1/2 interfering with tyrosine kinase activity and inhibiting downstream IFN-γ signaling (Kile and Alexander 2001). Following signal transduction, the IFN-γ/IFN-γR1 complex internalizes and enters the endosomal pathway, where the complex dissociates (Celada and Schreiber 1987). In many cell types, the IFNγR1 chain is eventually recycled to the cell surface in its uncoupled dephosphorylated form and the ligand is degraded. But, in others, it may induce degradation of the internalised receptor, thereby down-regulating IFN-γR1 surface expression.

3.1.3. Physiological function of IFN-γ

Anti-microbicidal activities of IFNs are afforded through increased pinocytosis and receptor mediated phagocytosis in macrophages. IFN-γ activated microbicidal ability includes induction of the NADPH-dependent phagocyte oxidase system (the "respiratory burst"), tryptophan depletion and up-regulation of lysosomal enzymes promoting microbe destruction (Gupta, Kubin et al. 1992). IFN-γ up-regulates argininosuccinate synthetase

(producer of L-arginine substrate), GTP-cyclohydroxylase I (supplier of tetra hydrobiopterin cofactor required for NO production) and the NOS2 enzyme (MacMicking, Xie et al. 1997). Activation of this enzyme by IFN-γ requires STAT-1 activation through JAK, MAPK and atypical PKC isoform phosphorylation. NO and reactive nitrogen intermediates produced can then easily penetrate the microbial cell wall/coat to inflict injury (MacMicking, Xie et al. 1997). Furthermore, the toxic oxidants produced by the respiratory burst are also able to react with those produced by NOS2, thereby forming a large number of different toxic species to mediate cytotoxicity by a wide variety of mechanisms (Radi, Beckman et al. 1991; Radi, Beckman et al. 1991).

IFN-γ and NO produced at the site of inflammation cause local dilatation of the blood vessels, thereby decreasing the local blood flow rate and causing extravasation of specific leukocyte subsets in tissues via interactions between adhesion molecules. In this sense, IFN-γ up-regulates expression of chemokines (e.g. IP-10, MCP-1, MIG, MIP-1a/b, RANTES) and adhesion molecules (ICAM-1, VCAM-1) (Vaday, Franitza et al. 2001). IFN-γ also promotes microbe destruction by augmenting the surface expression of the high-affinity FcγRI on mononuclear phagocytes, thereby promoting antibody-dependent cell-mediated cytotoxicity and directly promoting B cell isotype switching to IgG2a (Capsoni, Minonzio et al. 1994). Complement-mediated phagocytosis is also up-regulated by IFN-γ through increased complement secretion and complement receptor surface expression on mononuclear phagocytes.

IFN-γ promotes the Th1 phenotype promoting innate cell-mediated immunity via activation of natural killer effector functions, specific cytotoxic immunity via growth inhibition of Th2 populations and up-regulating the antigen processing presentation and antigen presenting cell (APC) costimulatory molecules on dendritic cells and macrophages, promoting the killing of intracellular pathogens such as bacteria, viruses, protozoa, helminths, fungi and tumor cells. On the other hand, IFN-γ enhances IL-2 production by phagocytes in a positive feedback mechanism to produce IFN-γ (Yoshida, Akiba et al. 1994).

IFN-γ primes macrophages for more rapid and stronger responses to LPS and bacterial DNA (CpG DNA) (Lorsbach, Murphy et al. 1993). In fact, IFN-γ receptor KO mice are highly resistant to LPS-induced toxicity (Car, Eng et al. 1994). IFN-γ influences LPS-dependent signaling by promoting transcription of TLR4 and myeloid differentiation factor 88 (MyD88) adaptor, subsequent TLR4 surface expression and LPS-binding ability in macrophages (Mita, Dobashi et al. 2001; Bosisio, Polentarutti et al. 2002).

Suppression of CSF-1 dependent cell growth of bone marrow derived macrophages after IFN-γ treatment is caused by G1/S arrest (Xaus, Cardo et al. 1999). This effect is

primarily caused by up-regulation of protein levels of CDKI inhibitors such as p27^{Kip-1} which inhibit the activity of cyclin E-Cdk2 and cyclin D-Cdk4 complexes, thereby causing hypophosphorylation of pRb which sequesters E2F factors from activation of genes required for cell cycle progression such as c-myc (Matsubara, Katayama et al. 1999; Matsuoka, Kitamura et al. 1999). In addition to decreasing the abundance of myc, IFN-γ increases Mad levels, thereby further antagonizing myc activity (Dey, Kim et al. 1999). Apart from that, through STAT-1 dependent mechanisms, IFN-γ also inhibits MKP-1 expression increasing the time during which ERK is phosphorylated (Xaus, Comalada et al. 2001). Furthermore, IFN-γ provides a survival signal against pathogen-induced apoptosis allowing macrophages to survive at the inflammatory loci when IFN-γ is present. This prevention of apoptosis induction is mediated through the expression of the cdk inhibitor p21^{Waf1}.

3.1.4. Major histocompatibility complex II, MHC-II

Of all the IFNs, only IFN-γ can efficiently up-regulate the class II antigen presenting pathway essential for the generation of a cell mediated immune response promoting antigen presentation and peptide-specific activation of CD4+ T cells and cytotoxic lymphocytes, as well as the phenomenon known as major histocompatibility restriction through interaction with T-cell receptors (TCR) (Guermonprez, Valladeau et al. 2002). This is important for host response to intracellular pathogens as it increases the potential for recognition of foreign peptides. In fact, the lack of class II MHC expression in humans can lead to one type of severe combined immunodeficiency disease, whereas the abnormal expression may cause autoimmune diseases (Kisseleva, Bhattacharya et al. 2002). Engagement of MHC II – peptide complexes by the TCR is essential for selection of the mature CD4+ T cell repertoire during T cell development in the thymus and for the initiation, propagation and regulation of adaptive immune responses by mature T cells in the periphery (Darnell 1998; Kisseleva, Bhattacharya et al. 2002).

Class II MHC molecules are membrane proteins expressed constitutively on B lymphocytes and in an inducible way after exposure to IFN-γ in cells of the monocyte-macrophage lineage and dendritic cells (professional antigen presenting cells –APCs-) and non professional APCs such as endothelial cells and fibroblasts that mediate a wide variety of immunologic phenomena. MHC class II antigens are readily found on activated human T cells but are absent on murine T lymphocytes even in the presence of IFN-γ. In any case, recent studies suggest class II MHC induction on Th1 cells. Although the exact

function of MHC class II molecules on T cells is not completely resolved, it is thought that although initiation of the T cell response is dependent on professional APCs, it is likely that with time and under conditions of crowding, T cells can capture, process and present antigens that costimulate each other and become autonomous (Pichler and Wyss-Coray 1994). Recently, T cell APC activity has also been suggested to be a mechanism for self-tolerance (Mannie and Norris 2001).

MHC class II genes HLA-DR, -DP and –DQ (I-A and I-E in mice) encode cell-surface polymorph heterodimeric complexes consisting of two transmembrane glycoprotein chains, α (33-35kDa) and β (27-29kDa), whose expression is a critical point in the control and maintenance of the immune response. The membrane distal domains of both subunits form a peptide binding site that consists of a β-pleated sheet topped by two α-helical regions, thus forming an open groove where peptides can be accommodated in an extended conformation with their ends protruding at both sides of the groove. The polymorphism of the β chain concentrates in this area and is responsible for the peptide binding specificity of each MHC II allotype.

The MHC II $\alpha\beta$ dimers assemble in the endoplasmic reticulum with the chaperone invariant chain (Ii), whose CLIP region occupies the peptide binding site of the dimer (Ghosh, Amaya et al. 1995). The invariant chain Ii also contains in its cytoplasmic tail a double leucine motif that directs the delivery and retention of the complexes into the endocytic route to encounter the antigenic peptides through Golgi traversion (Bakke and Dobberstein 1990). Once there, the invariant chain Ii must be removed to allow the formation of $\alpha\beta$-peptide complex, which is then delivered to the cell surface in transport vesicles. The invariant chain Ii is degraded in stages in the CIIV and MIIC compartments of the endocytic route. The last stage is the cleavage of invariant chain Iip10 by IFN-γ induced protease cathepsin S, in B cells and dendritic cells; Cathepsin L, in thymus epithelial cells; or Cathepsin F, L and S, in macrophages, and the antigenic peptides are loaded into the MHC II peptide binding groove with the assistance of the MHCII-like chaperones HLA-DM and HLA-DO (Shi, Feinberg et al. 1999; Shi, Villadangos et al. 1999). MHC class II molecules present peptide antigens derived mostly from endogenous and exogenous proteins that access the endocytic route by a variety of mechanisms such as pinocytosis, phagocytosis, receptor-mediated endocytosis or autophagocytosis, where they are degraded by lysosomal proteases and other hydrolases. The MHC II-peptide complexes are turned over by endocytosis followed by destruction in lysosomal compartments. The internalised MHC II-peptides complexes can thus acquire new peptides and be recycled back to the cell surface (Bakke and Nordeng 1999). Thus, IFN-γ also up-regulates the quantity of peptide-MHC II complexes on the cell surface by

promoting expression of key molecules such as the Ii invariant chain, cathepsins, lysosomal proteases implicated in production of antigenic peptides for class II MHC loadings, and DM, a regulator of peptide accessibility to the peptide-binding cleft of class II MHC.

Other genes located within the class II MHC locus in chromosome 6 (in mice, chromosome 17) are the antigen processing (TAP) subunits, which are coordinately expressed with MHC class I and are vital in peptide transport from the cytosol to the endoplasmic reticulum lumen to associate with class I MHC to aid in efficient peptide loading for cytotoxic T lymphocyte (CTL) responses (Epperson, Arnold et al. 1992). IFN-γ stimulation also induces a replacement of the constitutive proteasome subunits (β1, β2 and β5, encoded outside the MHC locus) with "immunoproteasome" subunits (LMP2, MECL-1 and LMP7) (Nandi, Jiang et al. 1996; Groettrup, Khan et al. 2001; Groettrup, van den Broek et al. 2001). In this way the immmunoproteasome can increase the quantity, quality and repertoire of peptides for class I MHC loading (Groettrup, Khan et al. 2001; Groettrup, van den Broek et al. 2001).
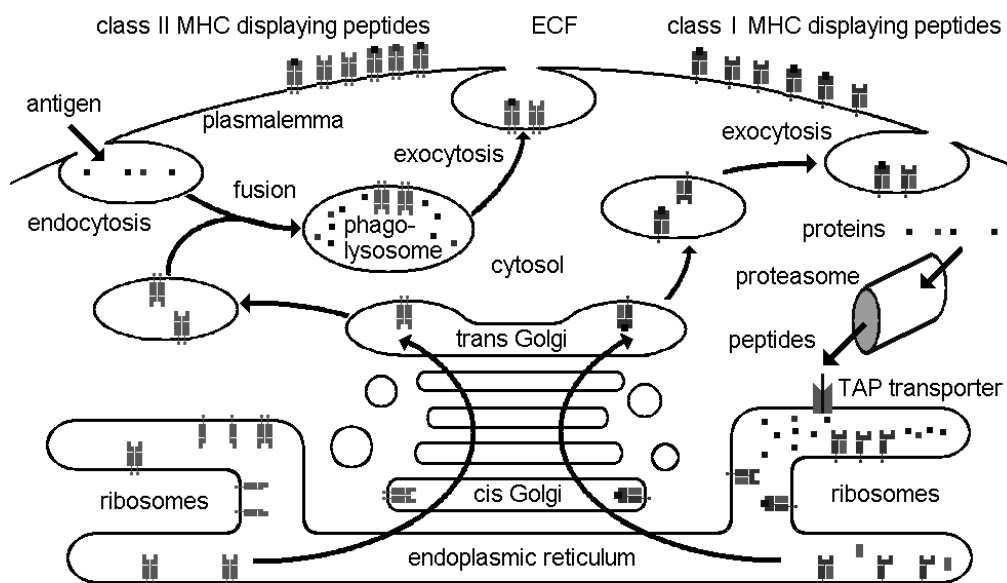

Figure 12. Antigen presentation. Class I MHC display peptides from cytosolic proteins. Class II MHC display fragments from phagocytosed matherial.

However, although by convention presentation of MHC class I-restricted epitopes involves processing by cytosolic proteasomes, whereas MHC class II-restricted epitopes are generated by endosomal proteases, recent studies suggest that MHC class II-restricted epitopes can also be generated by a proteasome- and TAP-dependent pathway in dendritic cells and that this pathway may have an important role in responses to viral

infections and transformed cells (Tewari et al., 2005). Furthermore, some studies have implicated the proteasome in the MHC class II-restricted presentation of certain endogenously expressed cytosolic antigens (Mukherjee, Ginardi et al. 2001).

Regulation of MHC class II expression occurs essentially at the transcriptional level by control elements located upstream of the gene. There are four conserved upstream cis-acting sequences referred to as the X1, X2, Y and W (or S) box, located within 50 to 300 bp 5' of transcription initiation site (Herrero, Sebastian et al. 2002). These four boxes are present in a tightly conserved arrangement with respect to orientation and spacing, and they function together as a single composite regulatory unit.
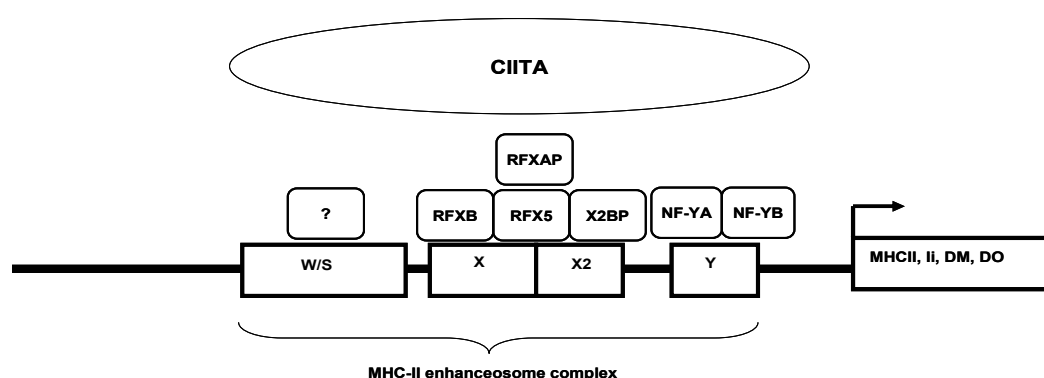

Figure 13. Transcriptional complex for the regulation of MHC class II expression. RFX, regulatory factor X; NF, nuclear factor; CIITA, class II transactivator, MHC, major histocompatibility complex; Ii, invariant chain.

The trimer complex RFX (regulatory factor X), composed of RFX5 (a member of the regulatory factor X (RFX) family of DNA-binding proteins), RFXANK (also called RFX-B) and RFXAP, binds to the X1 region (Masternak, Barras et al. 1998). X2BP (a complex that includes CREB) binds to the X2 box and a trimer complex, NF-Y, composed of NF-YA, NF-YB and NF-YC, recognizes the Y box (Celada, McKercher et al. 1996). A number of proteins can bind to the W box in vitro; including RFX (regulatory factor X), but none of them has been formally shown to be the functionally relevant W-box-binding protein in vivo (Celada, McKercher et al. 1996). However, all these transcription factors form the MHCII enhanceosome but are not sufficient for HLA expression since the components are expressed more or less ubiquitously (Kretsovali, Agalioti et al. 1998). An additional non-DNA binding protein, the class II transactivator (CIITA), is required for the formation of the transcriptional complex (Kretsovali, Agalioti et al. 1998). In fact, CIITA functions as a transcriptional scaffold to coordinate NF-Y, CREB and RFX (regulatory factor X) transcription factors in forming enhanceosome structures at MHC class II promoters

inducing histone acetylation and/or recruiting transcription initiation and elongation factors (Masternak, Peyraud et al. 2003). Therefore, in contrast to enhanceosome components, CIITA exhibits a cell-type-specific, cytokine inducible and differentiation-stage-specific pattern of expression that parallels that of MHC II genes. Ectopic expression of CIITA can induced MHC class II in all cells tested so far. Regulation of MHC-II mRNA stability does not seem to be an important mechanism of regulation since study of their sequences has not revealed any adenosine/uridine rich (ARE) elements. In any case, although MHC II mRNA stabilization mechanism is unknown, endonuclease cleavage could be involved (Cullell-Young, Barrachina et al. 2001).

3.1.4.1. Class II Transactivator, CIITA

The class II transactivator (CIITA) is the major physiological controller of the coordinated expression of MHC class II genes. Targeted disruption of CIITA in mice causes complete loss of constitutive or inducible display of class II MCH molecules (Chang, Guerder et al. 1996). Loss of constitutive/inducible class II MHC display is found in humans with CIITA mutation in Bare Lymphocyte Syndrome-BLS, a rare severe combined immunodeficiency accompanied by greatly reduced CD4+ T lymphocytes (Waldburger, Masternak et al. 2000). As CIITA is the limiting factor in a complex that directs transcriptional regulation, it acts as a switch for rapid up-regulation of class II MHC-relatnes by IFN-γ (Boss 1997). In addition to its essential role in classical MHC class II expression, CIITA controls the expression of several genes encoding accessory proteins required for MHCII-restricted antigen presentation, invariant chain, HLA-DO and HLA-DM molecules (Harton and Ting 2000) and represses the transcription of different genes such as IL-4 (Sisk, Gourley et al. 2000), Fas ligand (Gourley and Chang 2001), collagen alpha2, cyclin D1 and thymidine kinase (Zhu and Ting 2001).

CIITA is a non-DNA binding protein that has a complex structure with an N terminal acidic region identified as a transactivation domain, which binds components of the basal transcription machinery, RFX (regulatory factor X), and NF-Y and CREB-binding protein (CBP). In addition, different motifs have been implicated in the nuclear translocation of CIITA, a GTP-binding motif necessary for nuclear translocation of CIITA, nuclear localization sequences and C terminal leucine-rich repeats (Cressman, O'Connor et al. 2001). More recently, both the central GTP-binding domain and the leucine-rich region have been shown to be essential for CIITA dimerization (Raval, Howcroft et al. 2001).
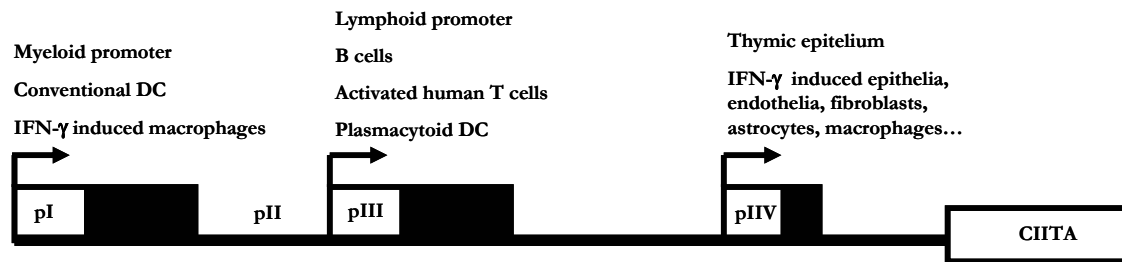
Figure 14. Schematic representation of the four independent promoters that control expression of the CIITA gene. Cell-specific expression from each promoter produces a different mRNA isoform with unique first exons differing only at their N-terminal ends. First exons are represented by black boxes following each promoter. pI is active in cells of myeloid origin, pIII is used in cells of lymphoid origin and pIV is essential for responsiveness to IFN-γ (adapted from Reith, 2004).

In contrast to RFX (regulatory factor X) and other class II MHC enhanceosome components, the expression of CIITA is highly regulated and imposes a tight qualitative and quantitative control over MHC expression. Cell-specific CIITA expression is under the control of four differential upstream promoters on the MHC2TA gene (Pai, Askew et al. 2002). These promoters are numbered pI, pII, pIII and pIV according to the location of their transcriptional start site from 5' to 3'. Myeloid pI is expressed in dendritic cells and macrophages induced by IFN-γ (Pai, Askew et al. 2002).

pII is expressed at very low levels only in humans and it is not well characterized. In fact, pII is absent from mouse sequence (Pai, Askew et al. 2002). Lymphoid pIII is constitutively active in B and is induced by IFN-γ in other cell types such as dendritic cells (Landmann, Muhlethaler-Mottet et al. 2001). The promoter region that is required for activity in B cells is contained inside the first 319 bp while the region required for the IFN-γ response requires an additional 5 kb of distal sequences (Ghosh, Piskurich et al. 1999). Finally, pIV is expressed in response to IFN-γ in various cell types such as macrophages and dendritic cells but also controls MHC class II in murine non haematopoietic cells such as endothelial, epithelial, astrocytes and fibroblast cells (Barbieri, Deffrennes et al. 2002). The different promoters do not share any sequence homology and are not co-regulated. Each promoter has its own unique transcriptional start site and precedes a distinct first exon that is spliced alternatively to the shared downstream exons resulting in the production of three CIITA isoforms (of apparent molecular weights of 132, 124 and 121 kDa) distinguished by their 5' N-terminal sequences (Barbieri, Deffrennes et al. 2002).

Although the initial analysis of CIITA promoters in macrophages showed that pIV was involved in IFN-γ induction of class II MHC, it is now clear that in the induction of bone marrow derived macrophages, pI could also be implicated, suggesting that induction

of pIV is only transient while that of pI is sustained. It remains unknown how IFN-γ activates pI but it has been demonstrated that the concomitant binding of three nuclear factors to 300 bp proximal region of pIV is essential for activation of CIITA transcription by IFN-γ. This region contains a GAS element, E box and IRF-1 binding site where phosphorylated STAT-1 homodimers, upstream regulatory factor-1 (USF-1), a constitutive ubiquitous nuclear factor of the basic helix-loop-helix/leucine zipper family, and the immediate-early inducible factor (IRF-1) bind respectively (Dong, Rohn et al. 1999). In fact, IRF-1 expression is itself under the control of phosphorylated STAT-1 active homodimers. Thus, STAT-1 acts directly on CIITA transcription by binding to the pIV promoter and indirectly by allowing IRF-1 synthesis.

In addition, the CIITA expression is altered by posttranslational modifications since CIITA phosphorylation or GTP binding can regulate CIITA nuclear localization (Sisk, Nickerson et al. 2003). Nonetheless, the currently accepted model is that MHC-II transcription is controlled primarily by regulation of CIITA transcription.

3.2. Lipopolysaccharide, LPS

Bacterial lipopolysaccharide (LPS) or endotoxin is a glycolipid composed of a hydrophilic O-antigen-like polysaccharide moiety and a hydrophobic domain known as lipid A. LPS is a major component of the outer membrane of Gram-negative bacteria and an initiator of macrophage effector functions (Alexander and Rietschel 2001). The lipid A portion of LPS represents the invariant pattern and it is responsible for the proinflammatory effects of LPS, while the O-antigen portion is variable in LPS from different species of bacteria. Not all lipid A have the same stimulatory activity. Lipid A preparations from *Salmonella* strains possesss very little stimulatory activity in human macrophages, but are very potent in murine macrophages. By contrast, lipid A preparations from *E. coli* are equally active.

3.2.1. LPS receptor

Macrophage recognition of lipid A requires TLR4, a Toll-like receptor (TLR) family member. The LPS receptor complex is thus composed of 4 proteins, CD14, LBP, TLR4 and the myeloid differentiation protein-2 (MD-2).

CD14 is a 55-kDa glycoprotein present in soluble form in blood or as glycosylphosphatidylinositol (GPI)-linked form on the surface of myeloid lineage cells such as macrophages, monocytes, neutrophils, lymphocytes and mast cells. Enterobacterial LPS first binds to a serum LPS binding protein (LBP) which in turn

transfers a LPS monomer from the bacterial cell wall to membrane-bound CD14 on monocytes and other myeloid cells, subsequently causing LPS responses. CD14 then presents the LPS/LBP complex to the myeloid differentiation protein-2 MD-2/TLR4 complex triggering the dimerization of TLR4 (da Silva Correia, Soldau et al. 2001). CD14-negative cells such as endothelial cells and epithelial cells also respond to LPS because soluble CD14 can substitute the membrane-bound CD14. Besides LPS, CD14 is also required for the recognition of other bacterial products including peptidoglycans, lipoteichoic acids and lipoarabinomannans. In mouse CD14, the C terminal half of the molecule is required for the signal transduction.

To date, 10 mammalian TLRs have been identified (TLR-1/10), which represent type I transmembrane proteins characterized by an extracellular domain containing multiple Leucine-rich repeats (LRR), a transmembrane domain and an intracellular Toll/IL-1 receptor/plant R gene product homology domain ("TIR domain") shared with IL-1 receptor (Imler and Hoffmann 2003; Imler and Zheng 2004).
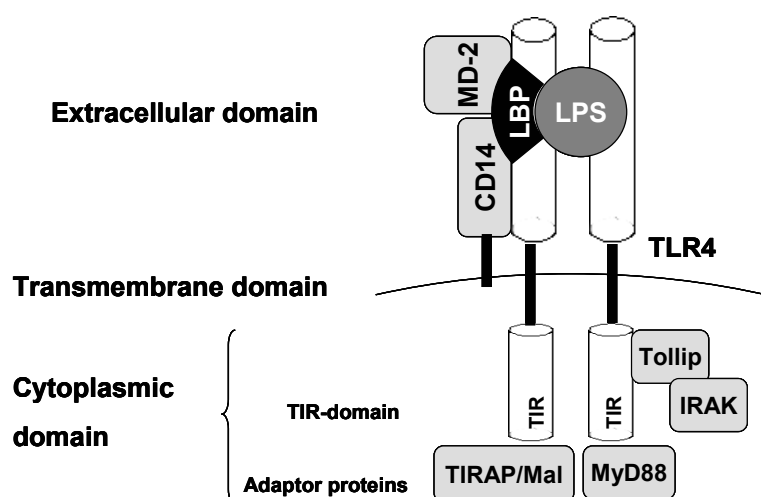

Figure 15. LPS receptor. LPS bound to LBP interacts with CD14 activating the MD-2/TLR4 complex and causing its dimerization and recruitment of adaptor proteins. LPS, lipopolysaccharide; LBP, LPS binding protein; MD-2, myeloid differentiation protein-2; TLR, toll-like receptor; TIR, Toll/IL-1 receptor/plant R gene product homology domain; Tollip, Toll-interacting protein; IRAK, IL-1R-associated kinase; MyD88, myeloid differentiation factor 88, and TIRAP/Mal, Toll receptor IL-1R domain with adaptor protein/aka MyD88-adaptor.

Human TLR4 was the first TLR characterized and it is expressed predominantly in immune cells, including macrophages and dendritic cells. TLR4 mutations are also associated with hyporesponsiveness to endotoxin in humans. TLR4 is also implicated in the recognition of several other ligands apart from LPS, including heat-sensitive cell-

associated factor derived from *Mycobacterium tuberculosis,* hsp60 and the fusion protein of respiratory syncytial virus (Imler and Hoffmann 2003; Imler and Zheng 2004).
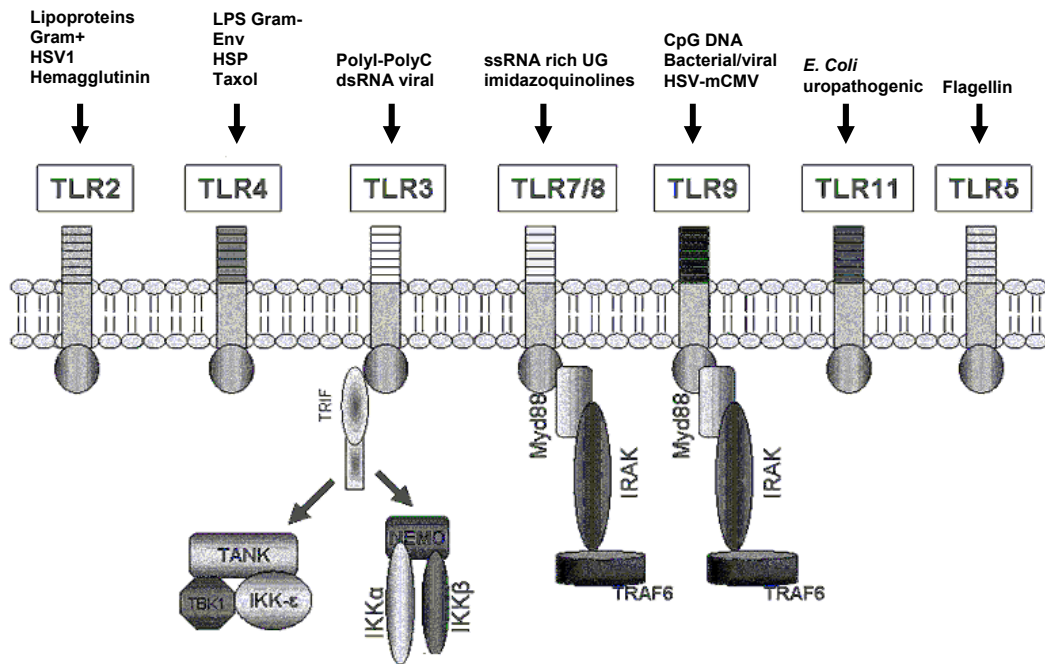

Figure 16. Toll Like receptors and their ligands.

Originally mammalian TLR2 was though to confer a LPS-responsive phenotype. However, TLR2, in conjunction with TLR6, and possibly other TLRs, is essential for the recognition of lipoproteins and peptidoglycan molecules found on pathogens such as Gram-negative and positive bacteria, mycobacterium, spirochetes and zymozan from fungi (Kirschning and Schumann 2002). TLR3 is associated with the binding and activation of double stranded RNA signaling. TLR5 is responsible for the recognition of flagellin, a monomeric constituent of bacterial flagella and TLR9 is essential for the recognition of bacterial CpG DNA, a motif found in non-methylated bacterial DNA (Imler and Hoffmann 2003; Imler and Zheng 2004).

Myeloid differentiation protein-2 (MD-2) is a small protein (18kDa) that lacks a transmembrane domain and it is associated with the extracellular domain of TLR4. Therefore, the functional integrity of the LPS receptor depends on the cell surface expression of three molecules but also on N-linked glycosilations of both myeloid differentiation protein -2 (MD-2) and TLR4 (Palsson-McDermott and O'Neill 2004).

3.2.2. LPS Transduction pathway

LPS-TLR4 interaction triggers a signaling cascade which ultimately results in activation of NF-κB, AP-1 and transcriptional control over genes involved in the immune function of macrophages (Aderem and Ulevitch 2000). The adaptor protein myeloid differentiation factor 88 (MyD88) functions as an adaptor to connect the intracellular portion of TLR4 to downstream signaling components and it is required for many, but not all, LPS-induced effects. In fact, in myeloid differentiation factor 88 (MyD88)-deficient macrophages, the nuclear translocation of NF-κB and phosphorylation of MAPK remain intact following stimulation with LPS (Kawai, Adachi et al. 1999). Other downstream events of TLR4 signaling are mediated by TIRAP/Mal (Toll receptor IL-1R domain containing adaptor protein/aka MyD88-adaptor like), TICAM-1 (TIR-containing adaptor molecule), TRIF (Aka TIR domain-containing adaptor inducing IFN-β), TRAM (TRIF-related adaptor molecule) and other adaptor proteins (Yamamoto, Nakane et al. 2003).

Myeloid differentiation factor 88 (MyD88) associates with the TIR domain of TLR and IL-1 receptor, whereas the death domain interacts with the N-terminal death domain of IRAK (IL-1R-associated kinase) recruiting it to the receptor complex. IRAKs are subsequently autophosphorylated and dissociated from the receptor complex to interact with other adaptor molecule, TRAF6 (tumor necrosis factor receptor-activated factor 6). TRAF6 is responsible for activating MAPK kinases, which can lead to AP-1 activation. TRAF6 also activates the inhibitors of IκB kinase, allowing NFκB to translocate into the nucleus and activate the gene transcription, including TNF-α gene (Muzio, Ni et al. 1997).

In addition to this myeloid differentiation factor 88 (MyD88)-dependent pathway, TLR4 recruits TICAM-1 (TIR-containing adaptor molecule) in a MyD88-independent manner (Hoebe, Du et al. 2003). TICAM-1 (TIR-containing adaptor molecule) then, via two protein kinases (TBK-1) and IKKε (I-kappa-b-kinase) activates NFκB and AP-1 up-regulating IFN-β via the transcription factor IRF-3 (Fitzgerald, Rowe et al. 2003). Signaling transduction pathways that participate in molecular mechanisms of LPS-induced macrophage activation involve kinases such as G proteins, tyrosine kinases, PLC, PKA, PKC, Src-related kinases, and the three MAPKs: ERK-1/2, p38MAPK and JNK (Reimann, Buscher et al. 1994).
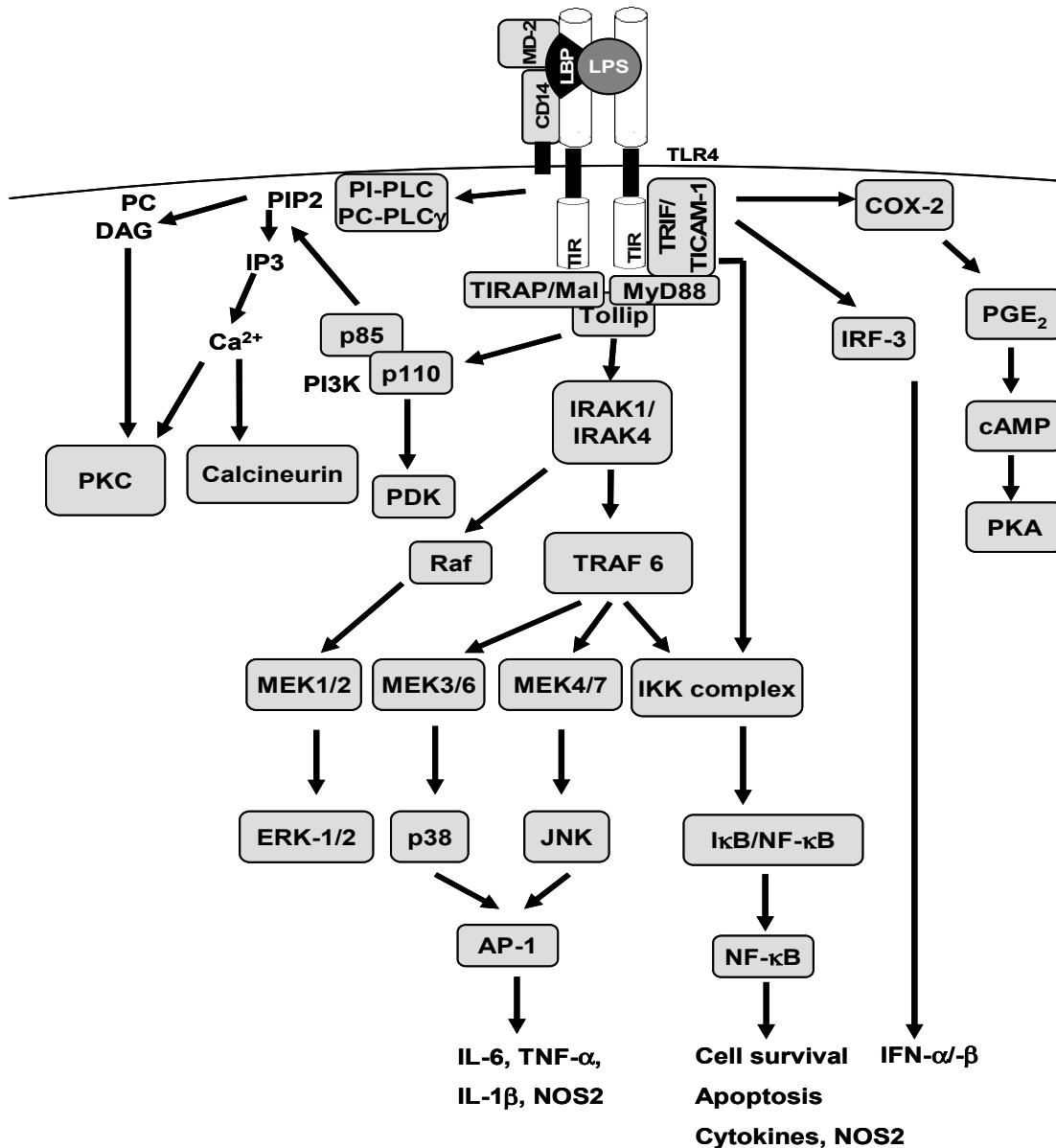
Figure 17. LPS signaling pathway. LPS induces the activation of several signal transduction pathways such as MAPKs, NF-κB, PKC, PKA or PI3K that lead to induction of gene transcription in the nucleus. LPS, lipopolysaccharide; LBP, LPS-binding protein; TLR, Toll-like receptor; ERK, extracellular signal regulated kinase; IKK, I-kappa-b kinase; NF-κB, nuclear factor; IRAK, interleukin-1 receptor associated kinase; JNK, c-Jun N-terminal kinase; MEK, mitogen-extracellular signal related kinase; PDK, phosphoinositide-dependent kinase; PI3K, phosphoinositide 3-kinase; PIP2, phosphatidylinositol (4,5)-bisphosphate; IP3, inositol triphosphate; TLR, Toll-like receptor; TOLLIP, Toll-interacting protein; TRAF6, tumor necrosis factor receptor-associated factor 6; PI-PLC, phosphatidylinositol-phospholipase C; PC-PLC, phosphatidylcholine-PLC; PKC, protein kinase C; PKA, protein kinase A; NOS2, inducible nitric oxide synthase; DAG, diacylglycerol; AP-1, activating protein-1; IL, interleukin; TNF-α, tumor necrosis factor-alpha; IFN, interferon; PGE2, prostaglandin; and IRF, immediate-early inducible factor.

3.2.2.1. PKC and PKA signaling

LPS activates heterotrimeric G proteins consisting of a α catalytic subunit and two β and γ regulatory subunits. Its activation is afforded through GTP binding that triggers α subunit dissociation, thus activating other effector molecules such as PLC, phosphatidylinositol PLC (PI-PLC) and adenilate cyclase enzyme increasing DAG and cAMP production.

PKCs are important mediators of macrophage activation after DAG and cAMP induction. Macrophage-specific inhibition of PKCε has been linked to an inhibition of LPS induced NO production in the J774 macrophage cell line (Fujihara, Connolly et al. 1994). In addition, there is evidence of a role for PKCε in macrophage LPS signal transduction (Weinstein, Sanghera et al. 1992). MKP-1 phosphatase is also induced after LPS treatment of macrophages. The use of PKC inhibitors revealed that LPS induces MKP-1 expression through a PKC-dependent pathway similar to M-CSF, although the signal transduction mechanism seems to be different (Valledor, Xaus et al. 2000). Lost of PKCε also reduce dramatically the levels of NO, PGE$_2$ (E-prostaglandin), TNF-α and IL-1β in response to LPS and IFN-γ co-stimulation. Experiments using PKC-isoenzyme-specific pseudosubstrate-based peptide inhibitors also demonstrate that PKCε is critical for LPS-induced NF-κB activation.

3.2.2.2. ERK MAPK

Although triggering opposed responses, both M-CSF and LPS induce the activation of ERK-1/2 through Raf/MEK pathway in macrophages (Reimann, Buscher et al. 1994). However, the time-course of ERK activation is different for each type of stimulus. Proliferating agents induce a rapid ERK activation and deactivation (i.e. between 5 and 30 minutes), whereas ERK activation by LPS occurs after 15 minutes of stimulation and lasts until 45-60 minutes. The use of PD98059 inhibitor, which specifically blocks MEK activation, demonstrates that ERK activity is necessary for macrophage proliferation (Valledor, Comalada et al. 2000). Moreover, PD98059 inhibits the expression of cytokines induced by LPS, suggesting that ERK activity is also required for macrophage activation (Sebolt-Leopold 2004).

Active ERKs phosphorylate and regulate several cellular proteins, including additional protein kinases, cytoskeletal components, phospholipase A2 (responsible for araquidonic acid synthesis) and nuclear transcription factors, such as Elk1/TCF and c-Jun, which regulate the expression of immediate early genes (Boulton, Nye et al. 1991). (See section 2.1.3.2.1)

3.2.2.3. JNK MAPK

The JNK and p38 kinases serine/threonine kinases or stress-activated protein kinases (SAPKs) are also activated following exposure of a variety of cells to cytokines and growth factors. Therefore, JNK is phosphorylated through MKK4 (also known as SEK1) and MKK7 MAPKKs in a cooperative way (Derijard, Raingeaud et al. 1995). In turn, MAPKKs are activated by MAPKKK MEKK (1-4), MLK (mixed-lineage protein kinase) (MLK1-3, DLK, LZK), ASK1-2 (Apoptosis signal-regulating kinase), TAK (TGF-β-activated kinase-1) and Tpl-2 (tumor progression locus 2). Furthermore, recent studies have supported a role for scaffold proteins in the activation of JNK. Four groups of potential scaffold proteins have been reported, Creatin kinase II, filamin, β-arrestin and JIP (JNK interacting protein). Although their mechanism of action has not been well established, they may act by increasing the local concentration of the components of the kinase module increasing signal transmission (Whitmarsh, Cavanagh et al. 1998). No differences in the kinetics of JNK in proliferation/activation state have been observed in bone marrow derived macrophages (Valledor, Comalada et al. 2000).

In mammals, there are three JNK genes, JNK1, JNK2 and JNK3, each on a different chromosome, 10, 5 and 4 in humans or 14, 11 and 5 in mice (Kyriakis and Avruch 1996). Each mammalian JNK gene can be subjected to alternative splicing giving rise to at least ten different JNK proteins. JNK3 expression is restricted to brain, heart and testis tissues. In contrast, JNK1 and JNK2 proteins are widely distributed.

JNKs have been characterized by their ability to associate with and phosphorylate regulatory sites in the N-terminus of the transcription factor c-Jun (Sanchez, Hughes et al. 1994). However, JNKs can also phosphorylate a variety of additional transcription factors such as JunB, JunD, ATF-2, Elk-1, NFAT and p53 (Gupta, Campbell et al. 1995; Yang, Mark et al. 1998; Buschmann, Potapova et al. 2001). Furthermore, JNK can play a wider intracellular role through phosphorylation of non-nuclear proteins related to regulation of apoptosis. In fact, JNK directly phosphorylates substrates localized in the mitochondria such as the Bim-related members of the Bcl-2 family leading to Bax-dependent apoptosis (Lei and Davis 2003).

In the presence of reversible ATP-competitive inhibitors or equal potency toward the three gene products such as SP600125 anthrapyraxole (Bennett, Sasaki et al. 2001), the selective disruption of JNK genes or genes encoding JNK pathway components has provided an effective approach for evaluating their contribution. JNK3 disruption is not associated with any obvious abnormal phenotype (Dong, Yang et al. 1998). The loss of JNK1 results in hyperproliferative T cells, less activation-induced apoptosis and preference for differentiation to helper cell Th2 antigen-presenting cells (Dong, Yang et

al. 1998). Furthermore, JNK1 KO mice is significantly protected from obesity-induced insulin resistance (Hirosumi, Tuncman et al. 2002). JNK2 expression is required for IFN-γ production by Th1 cells and efficient activation and apoptosis of T cells (Sabapathy, Hu et al. 1999). Some results emphasize that in many tissues in which JNK1 and JNK2 are co-expressed, they may have redundant or at least overlapping roles. In contrast, the loss of JNK1 and JNK2 in combination has been shown to be embryonically lethal (Kuan, Yang et al. 1999). At the level of MKK4 and MKK7, knockouts are also lethal; neither alone prevents JNK activation though both attenuate it. c-jun has been specifically targeted by gene disruption by knocking out the gene or by conditional gene targeting using Cre/LoxP mediated recombination (Yang, Kuan et al. 1997).
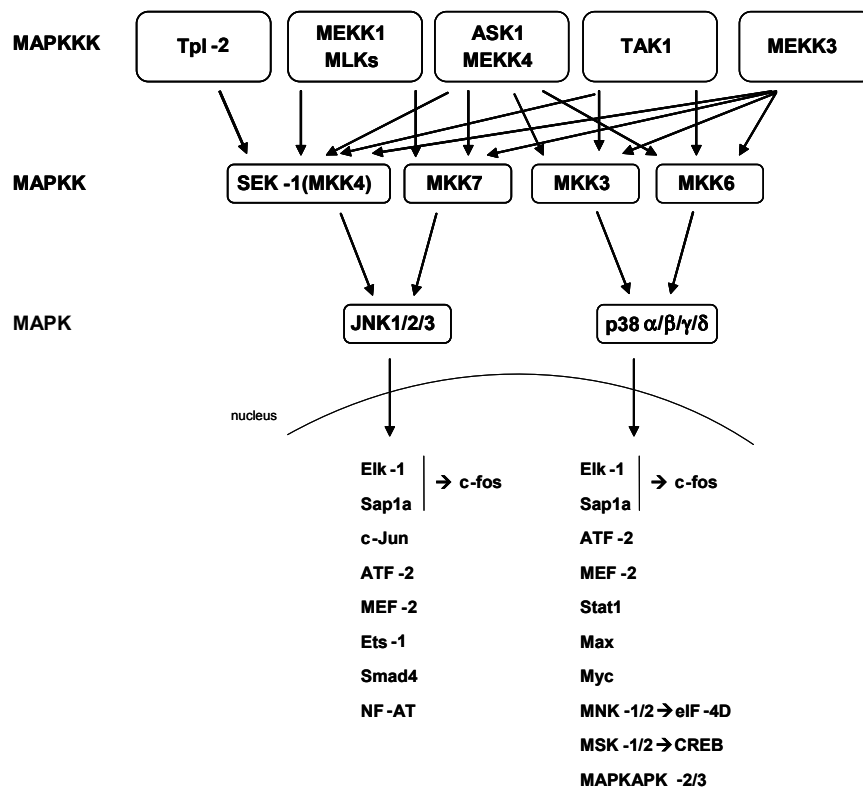

Figure 18. Stress-activated MAP kinase signaling pathway, the JNK and p38 MAPK pathway. JNK and p38 are activated by different MAPKK which are also activated by MAPKKK. Tpl-2, tumor progression locus 2, JNK, c-jun N-terminal kinase; ASK, apoptosis signal-regulating kinase; MEK, MAPK/ERK kinase; MLK, mixed-lineage protein kinase; TAK, TGF-beta activated kinase; SEK, SAPK/ERK kinase; p38, MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; Elk, Ets-domain protein; ATF-2, activating transcription factor-2; MEF-2, mouse embryonic fibroblast-2; and MAPKAPK, MAPK-activated protein kinase, Mnk-1/2, MAPK interacting kinases, Msk-1/2, stress-activated protein kinase; NF-AT, nuclear factor activator of T lymphocytes.

3.2.2.4. p38 MAPK

The p38 pathway is activated through phosphorylation by dual specificity MAPKKs, MKK-3 and 6, and in certain cases by MKK4 implicated in JNK activation (Han, Enslen et al. 1998). The activation of these MAPKK is regulated by other upstream serine/threonine kinases, the MAPKKK. Proteins that have been shown to act as MAPKKK in the p38 pathway include Tak1, Ask1 and Mlk3 (Tibbles, Ing et al. 1996). The activation of these kinases is regulated by upstream regulatory signals that involve activation of G-proteins such as Rac1 and Cdc42 (Frost, Xu et al. 1996).

There are four known isoforms of p38 which share significant amino acid homology with each other: p38α (also known as CSAIDS binding protein, CSFBP or SAPK2a), p38β (SAPK2b or p38-2), p38γ (SAPK3 or ERK6) and p38δ (SAPK4) (Jiang, Li et al. 1997). p38 α and β are ubiquitously expressed, whereas the expression of the γ and δ isoforms is limited to tissues such as brain. Our knowledge of the function of the different isoforms is largely limited to that of p38 α and β because they are inhibited by pyridimylimidazole compounds such as SB203580, whereas p38 γ and δ are not (Lee, Kwack et al. 2000).

p38 MAPKs can activate Elk-1, CHOP (a member of the C/EBP family of transcription factors), ATF-2/6, the ternary complex factor (TCF) Sap1α and MEF2C factors by phosphorylation (Kyriakis and Avruch 2001). Other immediate targets include Mnk1, Msk2, MAPKAPK-2, MAPKAPK-3, Msk1 and hnRNP. A major physiological substrate of MAPKAPK-2 is the small heat shock protein, hsp27, involved in cytoskeleton reorganization (Lathey, Kanangat et al. 1994). Early work using inhibitors of p38 in LPS-treated monocytes suggested that p38 regulates transcription of IL-1β and TNF-α causing their mRNA stabilization (Lee, Laydon et al. 1994).

Therefore, the activation of AP-1 in response to cytokines and genotoxic stress is mediated largely by JNK and p38 MAPKs. The physiological outcomes of AP-1 activation are apoptosis, inflammation, responses to cellular stress and cytokine activation (Yang, Tournier et al. 1997). However, several additional proteins crucial for survival of cancer cells are also substrates for JNK, including p53, c-myc and several Bcl-2 family members such as Bcl-2, Bcl-X$_L$, Bad, Bax and Bak (Noguchi et al., 1999; Kharbanda et al., 2000). It has also been shown that p38 activity mediates platelet aggregation, regulation of hepatocyte growth and organization of the actin cytoskeleton (Polnowska-Grabowska et al., 2002). Furthermore, p38 exhibits regulatory effects in the induction of apoptosis and cell differentiation (Nagata, Takahashi et al. 1998).

Inactivation of these MAPK occurs relatively rapidly and is mediated by phosphatases such as protein phosphatase 1, protein phosphatase 2A and MKP-1 (Chu, Solski et al. 1996).

3.2.2.5. PI3K/Akt

The PI3K/Akt pathway is an additional inducible negative regulator of TLR signaling induced by TLR-4. This system differs from the described tolerance systems that produce PI3Ks to negatively regulate TLR signaling at an early phase at the first encounter with pathogens. Notably, IL-1 receptors and members of the TNF receptor family also activate class I PI3Ks in macrophages and dendritic cells (Bellacosa, Testa et al. 1991).

Signal transduction pathways responsible for activating PI3K downstream of TLRs are not completely characterized; PI3K is activated by TAK (transforming growth factor β-activated kinase 1) downstream of TLR4 and interactions of PI3K with myeloid differentiation factor 88 (MyD88) in response to LPS have also been reported.

In monocytes, PI3Ks suppress both MAPK and NF-κB cascades in response to LPS, resulting in decreased production of TNF-α. In dendritic cells, PI3Ks block the p38 activation pathway essential for transcriptional activation of IL-12 genes. The hallmark of dendritic cell function is the unique ability of these cells to present antigens to naïve T cells, whereas macrophages and B cells can only activate primed T cells. PI3K have been involved in certain diseases (Th2-dominant chronic allergic diseases such as asthma and atopic dermatitis) associated with the disruption of the Th1-Th2 balance. Thus PI3K-mediated negative feedback in IL-12 production seems to be important in preventing excessive innate immune responses (Martin, Schifferle et al. 2003).

3.2.2.6 Calcineurin

Calcineurin serine/threonine phosphatase (PP2B) is a heterodimer composed of a catalytic subunit, calcineurin A (60kDa), and a myristoylated regulatory subunit, calcineurin B (19kDa) (Rusnak and Mertz 2000). The calcineurin A subunit binds one molecule of the Ca^{2+}/calmodulin complex. The regulatory Calcineurin B subunit contains four Ca^{2+}-binding hand motifs, a calmodulin-binding domain and an "autoinhibitory" domain (Yuan, Gomes et al. 2004).

All eukaryotic organisms possess one or more genes for each subunit. In mammals, there are two calcineurin B genes, one ubiquitously expressed and the other only found in testes (Nakatomi and Yazawa 2003). Therefore, calcineurin is widely distributed in mammalian tissues with the highest levels found in brain (Bahl, Bradley et al. 2001).

In absence of Ca^{2+}/calmodulin, B subunit remains tightly associated with the A-subunit and the autoinhibitory domain binds in the active site cleft and inhibits the phosphatase activity of the enzyme. In a rise in intracellular Ca^{2+}, the binding of Ca^{2+} to calmodulin results in a conformational change that displaces the autoinhibitory domain from the catalytic domain of calcineurin, thereby allowing it to bind to calcineurin activating its phosphatase activity (Groenendyk, Lynch et al. 2004). Blc-2 also regulates calcineurin forming a complex that targets calcineurin to the cytoplasmic membrane and although the complex still maintains phosphatase activity it is unable to promote nuclear translocation of NF-AT (Srivastava, Sasaki et al. 1999). Calcineurin has also been shown to be associated with the cytoskeleton including tau, microtubule-associate protein 2, tubulin, dystrophin and dynamin (Li and Handschumacher 2002).

3.2.3. Macrophage response to LPS

LPS activates monocytes and macrophages to produce various proinflammatory cytokines such as TNF-α, IL-1, IL-6, IL-8 (which induces IFN-γ production), IL-12 (which up-regulates IFN-γ production), TGF-β and macrophage inhibitory factor (MIF), which play important roles in the immune response. Macrophages also secrete, in response to LPS, a wide variety of other biological response mediators including arachidonic acid metabolites (e.g. platelet-activating factor, prostaglandin and leukotriens), proteases, eicosinoids and free radicals, such as reactive oxygen and nitrogen intermediates (NO) (Miller, Ernst et al. 2005; Miller, Viriyakosol et al. 2005).

The production of these inflammatory cytokines and mediators by monocytes/macrophages contributes to the efficient control of growth and dissemination of invading pathogens. Conversely, TNF-α can act in an autocrine manner to mediate many LPS-induced effects via NF-κB (Cheshire and Baldwin 1997). However, excessive and uncontrolled production of these inflammatory cytokines and mediators may lead to serious systemic disorders such as microcirculatory dysfunction, tissue damage and septic shock characterized by fever, myocardial dysfunction, acute respiratory failure, hypotension, multiple organ failure, and in some cases, high mortality.

LPS can also induce interferon IFN-α/β expression in murine macrophages through myeloid differentiation factor 88 (MyD88) independent pathways. In turn, these type I IFNs act in an autocrine/paracrine fashion through their cell surface receptors to activate the JAK/STAT1 pathway and trigger the transcription of NOS2 and NO production.

Nitric oxide (NO) has been identified as an important signaling molecule involved in regulation of a wide range of biological activities in the neural, vascular and immune systems. NO and its metabolites mediate a number of host defense functions mediated by

activated macrophages, including antimicrobial and tumoricidal activity, involved in the pathogenesis of tissue damage associated with acute and chronic inflammation. Macrophages generate NO gas from L-arginine via a reaction catalysed by the inducible form of NOS2 although NF-κB as well as AP-1 activation by LPS is required. NO can then act as an intracellular or extracellular messenger. There are several isoforms of NOS2 (NO1, 2 and 3) encoded on three different chromosomes with a tissue specific distribution. However, NO production is regulated at two levels, at the level of NOS2 transcription over a period of several hours and by substrate availability (Mitrovic, Ignarro et al. 1995).

3.3. Alternative activation

L-arginine is a substrate for NOS2 and for arginase enzymes. Unlike NOS2, arginase drives the production of ornithine, a precursor for polyamine and proline synthesis. Although the effects of NO are primarily cytotoxic, production of ornithine by macrophages promotes cell proliferation and matrix synthesis.

IL-4, a potent inhibitor of NO production, accomplishes this task by up-regulating arginase expression through the activation of signal transducer and activator of transcription Stat6 and limiting the amount of L-arginine available to NOS2 (Nelms, Keegan et al. 1999). In fact, Modolell and colleagues have established that expression of arginase is induced in macrophages by Th2-type cytokines and that the balance between NOS2 and arginase correlates with the balance between Th1-and Th2-type activities (Munder, Eichmann et al. 1998).

Alternative activation of macrophages by IL-4 and IL-13 produces M2-type responses, particularly in allergic, cellular and humoral responses to parasitic and extracellular pathogens. This alternative activation results in up-regulation of the mannose receptor and MHC class II molecules expression by macrophages, which stimulates endocytosis and antigen presentation respectively. They also induce the expression of selective chemokines such as macrophage-derived chemokine (MDC; also known as CCL22) and thymus and activation regulated chemokine (TARDC, CCL17), and intracellular enzymes, such as arginase, which are involved in cell recruitment and repair of granuloma formation, thereby counteracting the effects of NOS activation and nitric oxide release. Inhibition by IL-4 of the expression of LPS-induced pro-inflammatory cytokines (such as TNF) of the respiratory burst is moderate (Nelms, Keegan et al. 1999).

IL-10 acts on a distinct plasma-membrane receptor to those for IL-4 and IL-13, and its effects on macrophage gene expression are different, involving a more profound

inhibition of a range of antigen-presenting and effector functions, together with the activation of selected genes or functions.
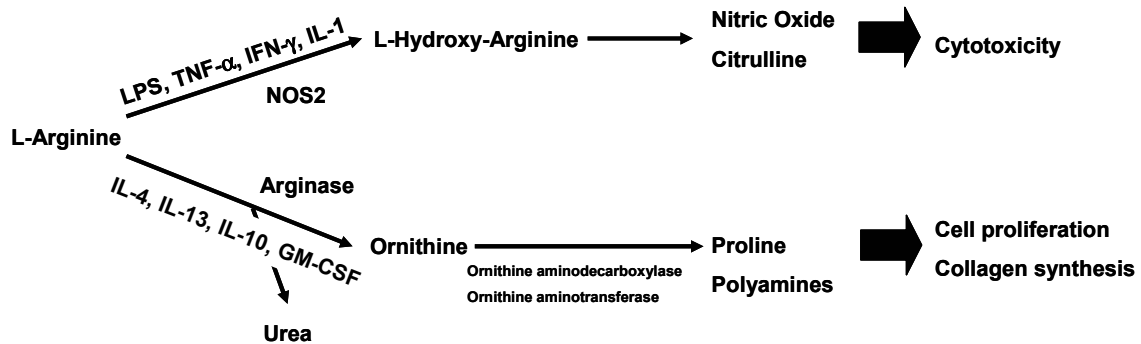

Figure 19. Differential catabolism of L-arginine by classical or alternative activated macrophages. L-Arginine is a substrate for NOS2 and Arginase enzymes. Consequently, cytotoxicity or reparation occurs respectively. LPS, lipopolysaccharide; IL, interleukin; TNF, tumor-necrosis factor; IFN, interferon, NOS2, inducible nitric oxide synthase; GM-CSF, granulocyte-macrophage colony-stimulating factor.

3.4. Inhibitors of macrophage activation

TGF-β antagonizes IFN-γ-driven processes of macrophage activation, such as the production of H_2O_2, NO, up-regulation of NOS2, release of TNF-α and the IFN-γ-induced death of intracellular microorganisms (Ulloa, Doody et al. 1999). The inhibitory mechanism of TGF-β seems to involve inhibition of CIITA transcription; and surprisingly, it does not affect IFN-γ induced phosphorylation of JAK-1, JAK-2 or STAT-1 or interfere with DNA-binding of STAT-1, USF-1 (upstream regulatory factor-1) or IRF-1 to pIV. It has been reported that Smad-3 is essential for TGF-β inhibitory effect. Vice versa, the TGF-β/SMAD signaling cascades are inhibited by IFN-γ/STAT pathways (Werner, Jain et al. 2000).

In addition, TGF-β inhibits LPS-induced activation of macrophages. This cytokine inhibits LPS-induced NOS2 expression and reduces the expression of proinflammatory cytokines during septic shock (Imai, Kurokawa et al. 2001). Also extracellular matrix such as decorin modulates proliferation and activation of macrophages reversing TGF-β repressive effects (Comalada, Cardo et al. 2003).

Other mechanisms involved in blocking macrophage activation have also been described. For example, at the inflammatory loci, adenosine is produced and secreted into the extracellular medium. Adenosine interacts with macrophages through the A2B receptor and leads to an increase in cAMP levels causing PKA activation and inhibition of class II MHC molecules, the expression of NOS2 and proinflammatory cytokines (Xaus,

Valledor et al. 1999). IFN-γ activated expression of CIITA can also be suppressed by a number of different stimuli including IL-1, IL-4 and IL-10 (O'Keefe, Nguyen et al. 1999).

Furthermore, the NO produced by macrophages upon IFN-γ stimulation may act as a feedback inhibitor of MHC II synthesis by inhibiting IFN-γ-induced CIITA expression (Kielar, Sicher et al. 2000). In addition, statins (HMG-CoA reductase inhibitors), known for their cholesterol-lowering effect, exhibit a number of anti-inflammatory properties among them the inhibition of CIITA gene activation (Kwak, Mulhaupt et al. 2000).

4. CELL DEATH

Apoptosis or programmed cell death is a regulated form of cell death evolutionarily conserved from pnematodes to mammals that does not activate the immune response. Apoptotic cells die in a manner morphologically distinct from necrotic cell death, characterized by membrane ruptures, release of cellular content and recruitment of inflammatory cells to the scene (Sauter, Albert et al. 2000).

Morphologically, necrotic cells are characterized by an overall increase in cellular size since the cytoplasm becomes disorganized and mitochondria begin to accumulate lipid-rich particles and swell. At later stages, there is release of proteases, nucleases and lysosomal content due to rupture of the cell membrane. The chromatin becomes flocculent and then disperses, leading to recruitment of monocytes and macrophages (Proskuryakov, Konoplyannikov et al. 2003).

4.1. Programmed cell death: apoptosis

In contrast to necrotic cells, as described by Kerr, Wyllie and Curie in 1972, apoptotic cells shrink in size and exhibit marked alterations in their chromatin structure at early stages (Mondello and Scovassi 2004). The chromatin becomes highly condensed within the nucleus. The organelles within the cytoplasm remain intact and show little change apart from some swelling of the endoplasmic reticulum. One of the earliest morphological manifestations of imminent death is that membranes become convoluted, often referred as "blebbing", causing the loss of phospholipid asymmetry of the cell membrane, leading to exposure of phospholipids such as phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) on the outside of the cell membrane.

Exposure of phospholipids is a major factor in the recognition of apoptotic cells by phagocytes, involving membrane-bound receptors, such as the phosphatidyl serine(PS)-receptor, vitronectin receptor and several scavenger receptors (SR), as well as

soluble opsonins (Fadok, Bratton et al. 2001). Specific binding of Annexin V to phosphatidyl serine has been used as a marker for identifying cells dying by apoptosis (Fadok, Bratton et al. 2001). Apoptotic cells in vitro shed apoptotic bodies, membrane-bound vesicles containing intracellular organelles and condensed chromatin.
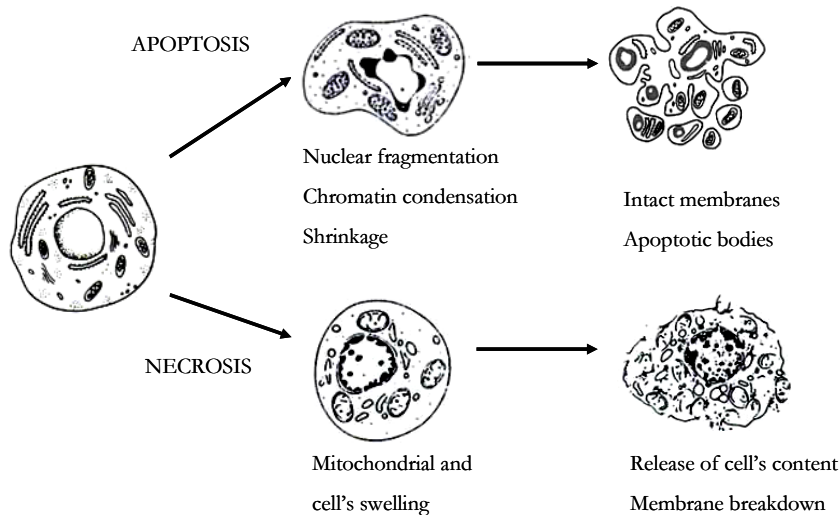

APOPTOSIS

Nuclear fragmentation

Chromatin condensation

Shrinkage

Intact membranes

Apoptotic bodies

NECROSIS

Mitochondrial and

cell's swelling

Release of cell's content

Membrane breakdown

Figure 20. Apoptosis versus necrosis.

Apoptotic cells are removed as intact cells rapidly by their nearest viable neighbor or passing phagocytic cells such as macrophages and immature dendritic cells ensuring the protection of neighboring cells from being damaged by the cellular content (Gregory and Devitt 2004) . This recognition of apoptotic cells is believed to be mediated through lectin-like molecules on the macrophage surface and cell-surface carbohydrates on dying cells.

Two main pathways for apoptosis induction in the cell have been defined: 1) the extrinsic death receptor pathway and 2) the intrinsic mitochondrial pathway.

4.1.1. Death signaling by death receptors

Death receptors are members of the TNF Receptor superfamily that are activated in response to external ligands, (Idriss and Naismith 2000). These receptors have conserved protein-protein binding domains termed death domains (DD) at C terminal through which they recruit and activate a caspase cascade (Sartorius, Schmitz et al. 2001). The death domains are required for the binding of adaptor protein molecules in Fas receptor/CD95 receptor, TNFR1, DR3, DR4, DR5 and DR6. Although other members of

the TNF receptor family such as CD30, CD40, CD27, TNF-R2 do not contain a death domain, they also trigger cell death (Sartorius, Schmitz et al. 2001).

TNF binds to two receptors TNF-R1 (55kDa) and TNFR2 (kDa75) of which TNFR1 is the main receptor mediating TNFα-induced cytotoxicity. TNF-R1 and R2 are type 1 transmembrane proteins that have conserved cysteine-rich domains (CRD) within their extracellular domains. The FasL/Apo1/CD95L (apoptosis-1) ligand is able to induce apoptosis when crosslinked with Fas/CD95 receptor (Idriss and Naismith 2000).

The cytoplasmic proteins involved in generating downstream signaling events are FADD (Fas-associated death domain), which contains a death effector domain (DED) that binds to caspase-8, and TRADD (TNF receptor-associated protein with death domain) which binds to two other proteins, FADD (Fas-associated death domain) and RIP. FADD recruits caspase 8 and RIP that can produce two different outcomes, activation of NF-κB or programmed cell death through TRAF (tumor necrosis factor receptor-activator factor) binding and caspase 9 binding (Wallach, Varfolomeev et al. 1999). There are 6 TRAFs (tumor necrosis factor receptor-activated factor) identified in mammalian cells that mediate the activation of NF-κB and JNK.

Cytokine	Cells	Receptor	Cells
LTα	NK, T and B cells	TNFR1	Most normal and transformed cells
		TNFR2	Endothelial cells and immune cells
TNF	Macrophages, NK, T and B cells	TNFR1, TNFR2	See above
LTβ	DCs, macrophages, T, B and NK cells	LTβR	NK cells, CD4$^+$ and CD8$^+$ T cells
CD95L	Activated splenocytes, thymocytes and non-lymphoid tissues, such as the eye and testis	CD95	Most normal and transformed cells
		DCR3	Lung and colon cells
TRAIL	NK cells, T cells and DCs	DR4, DR5	Most normal and transformed cells
		DCR1, DCR2, OPG	Most normal and transformed cells
TWEAK	Monocytes	FN14	Endothelial cells and fibroblasts
CD27L	NK, T and B cells	CD27	CD4$^+$ and CD8$^+$ T cells
CD30L	T cells and monocytes	CD30	Reed–Sternberg cells
CD40L	T and B cells	CD40	Reed–Sternberg cells
4-IBBL	B cells, DCs and macrophages	4-IBB	Activated T cells, monocytes and NK cells
OX40L	T and B cells	OX40	T cells
APRIL	Macrophages, lymphoid cells and tumour cells	BCMA	B cells, PBLs, spleen, thymus, lymph nodes, liver and adrenals
		TACI	B cells, PBLs, activated T cells, spleen, thymus and small intestine
BAFF	T cells, DCs, monocytes and macrophages	TACI	See above
		BCMA	See above
		BAFFR	B cells, PBLs, resting T cells, spleen and lymph nodes
LIGHT*	T cells, granulocytes, monocytes and DCs	HVEM	T cells
		LTβR	Non-lymphoid haematopoietic cells and stromal cells
VEGI*	Endothelial cells	DR3, DCR3	Activated T cells
GITRL	N. D.	GITR	CD4$^+$CD25$^+$ T cells
RANKL	Activated T cells and osteoblasts	RANK	Osteoclasts, osteoblasts and activated T cells
		OPG	Osteoclast precursors, endothelial cells and others
EDA1	Skin	EDAR	Ectodermal derivative
EDA2	Skin	XEDAR	Ectodermal derivative
N. D.		DR6	Resting T cells
N. D.		RELT	Lymphoid tissues
N. D.		TROY	Embryo skin, epithelium, hair follicles and brain

Table II. Cellular expression of ligands and receptors of the tumor-necrosis factor superfamily.

TNF receptor superfamily-induced cell death can also be modulated by adaptor proteins. Thus FLIP (FLICE inhibitory protein) binds to FADD (Fas-associated death domain) preventing release of caspase 8 and induction of apoptosis with a mutated caspase site. TRAFs (tumor necrosis factor receptor-activated factor) in turn bind the inhibitors of apoptosis (IAPs) (Nachmias, Ashhab et al. 2004).

4.1.2. The Caspases

Caspases are a family of cysteine proteases that cleave after an aspartic acid residue. Caspases are expressed in the cell as inactive enzymes (proenzymes) and are proteolytically activated cleaving their N terminal prodomain (2-25kDa) between the large (20kDa) and small subunits (10kDa).
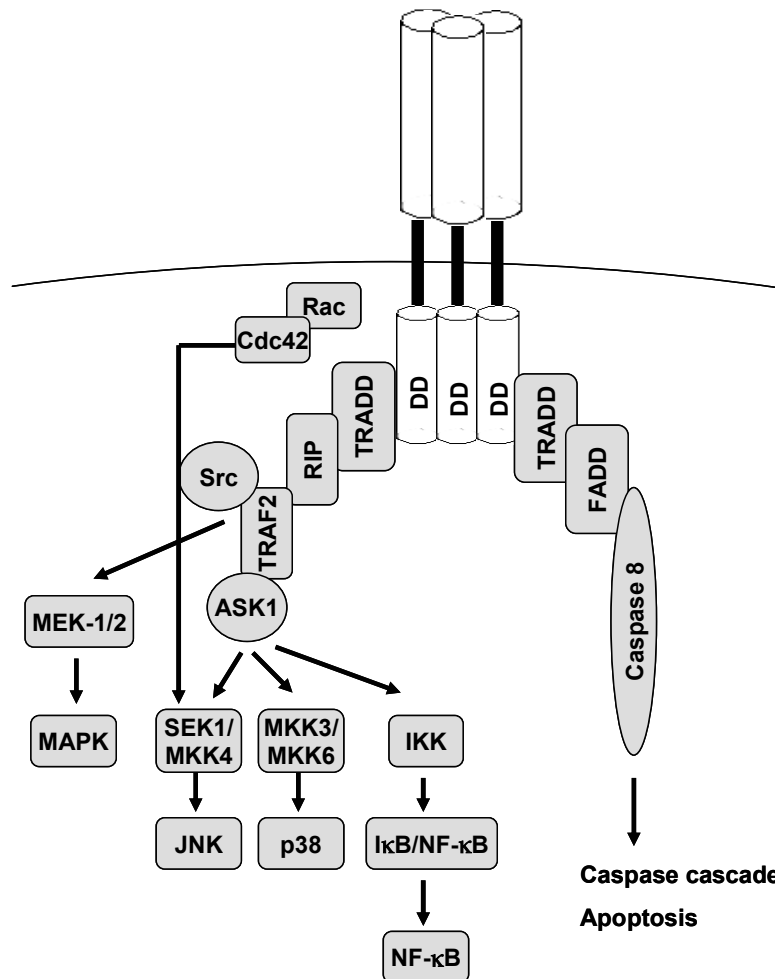

Figure 21. TNF-R1 receptor signaling. TRADD binds to death domains and promotes binding of FADD or RIP through a DD interaction. FADD facilitates, through its DED, the binding of procaspase-8 and its activation. This in turn leads to activation of the caspase cascade and death. RIP mediates binding of TRAF2 which in turn binds ASK1 and activates both NF-κB (survival signals) and JNK and p38 (possible death signals). DD, death domain; TRADD, TNF receptor-associated protein with death domain; FADD, Fas-associated death

domain; RIP, receptor-interacting protein; ASK, apoptosis signal-regulating kinase; SEK, SAPK/ERK kinase; IKK, IκB kinase complex; JNK, c-Jun kinase; IκB, inhibitor of κB; NF-κB, nuclear factor κB.

An active enzyme is formed by a heterotetramer of two small and two large subunits. The active site of the enzyme contains a conserved active site QACXG (where A is Alanine, C is Cysteine and X is Arginine R, glutamine Q or glycine G) in the larger subunit, while the smaller subunit determines substrate specificity. The two subunits bind to form a central 6 strand β sheet core, flanked on either side by α helices (Philchenkov 2004).

Caspases can be activated by transactivation, proteolysis by other proteases or by autoactivation. In addition to the caspases themselves other proteases may cleave and activate the procaspases. For example, Granzyme B, a serine protease directly cleaves and activates caspase-3 leading to the destruction of the cell (Riedl and Shi 2004).

At present time, there are 14 members of the caspase family, referred to as caspase 1-14, which can be subdivided in various ways (Fig. 22). Most commonly they are grouped according to their substrate specificity or their functional role. Mammalian caspases can be broadly grouped into upstream or downstream caspases. The upstream initiator caspases such as procaspase 8 or 10 are recruited by death adaptor molecules to activate other caspases while downstream ones are executioner caspases cleaving specific substrates that aid apoptosis (Shiozaki and Shi 2004).

However, they can also be grouped according to their N terminal prodomain structure. Long prodomains (Caspase 8, 10, 1, 2, 4, 5, 9, 12, 13) cleave other procaspases (Caspase 3 and 7) at the cell membrane and interact with other proteins with similar motifs (Shi 2004; Shi 2004). Short or no prodomains present in caspase 3, 6, 7, 11 and 14 mediate assembly of caspase activating complex. Thus, the prodomain of caspases 8 and 10 contain two death effector domain regions and interact with death adaptor molecules containing DEDs (death effector domains) such as FADD (Fas-associated death domain) adaptor molecule.

Several other caspases such as caspase 1, 2 and 9 contain CARD (Caspase Recruitment Domain) regions in their prodomain that result in aggregation and activation of other adaptor molecules such as CARDIAK, RAIDD, Apaf-1 and CED-4 recruiting a death-inducing signaling complex (DISC). This complex contains death domain adaptor proteins such as FADD (Fas-associated death domain) and caspases 8 and 10, which can then initiate the process of apoptosis (Boatright and Salvesen 2003).
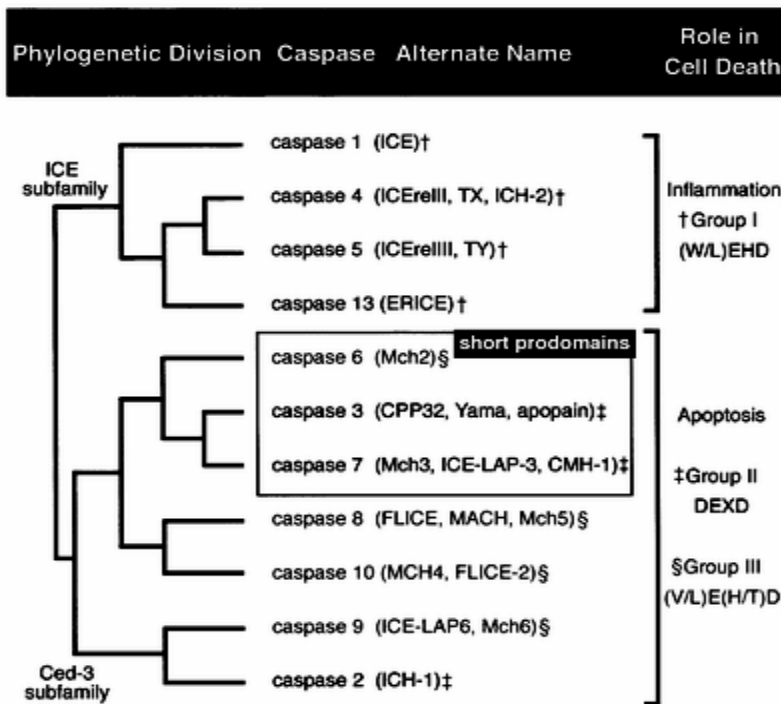
Phylogenetic Division	Caspase	Alternate Name	Role in Cell Death

Figure 22. Caspase classification. Caspases are synthesized in their zymogen form containing prodomains encoding CARD (Caspase recruitment domain) or DED (death effector domain). L, large subunit, S, small subunit.

4.1.3. Mitochondria in apoptosis

Permeabilization of mitochondrial membranes is a rate-limiting event of the apoptotic process induced by death stimuli. At least 5 different classes of effector molecules are released from the intermembrane space: cytochrome c (Cyt c), which participates in the activation of caspases; procaspases (2, 3, and 9), heat shock proteins hsp10 and hsp60 which facilitate the activation of procaspase-3, apoptosis-inducing factor (AIF) which induces large scale chromatin fragmentation and DNase (Lorenzo and Susin 2004).

This event promotes the formation of a caspase-activating complex. This complex is constituted after mitochondrial Cyt C release in presence of ATP, and Cyt C binding to Apaf-1 in the cytosol. Finally conformational changes occur to bind procaspase-9 via caspase recruitment domains (CARD) present in both molecules leading to autoproteolysis and activation of downstream caspases. Most recently the mitochondrial protein SMAC/DIABLO has been shown to be released during apoptosis at the same time as Cyt c, promoting caspase activation (Khosravi-Far and Esposti 2004). Smac/DIABLO is able to bind IAPs (inhibitors of apoptosis) bound to active caspases 9 and 3, blocking their action.

Thus, without the release of Smac/DIABLO from the mitochondria, caspases may well remain inactive due to the presence of bound inhibitors of apoptosis (IAPS). As TNF Receptor family involves caspase 8, they are not affected by inhibitors of apoptosis (IAPs).

4.1.4. Regulation of apoptosis

Death can be prevented by the presence of specific anti-apoptotic proteins that stop the downstream caspases binding to the receptor. The Bcl-2 family of proteins are structurally related molecules that play an instrumental role in the regulation of apoptosis (Tsujimoto 2003). This family includes both proapoptotic proteins such as Bad, Bax and Bik as well as anti-apoptotic proteins such as Bcl-2 and Bcl-X_L. The balance of antiapoptotic to proapoptotic proteins dictate whether a cell will survive or undergo apoptosis (Tsujimoto 2003). In addition to the independent functions of Bcl-2 proteins, they also determine the life or death of cells by homo and dimerization between anti-apoptotic and pro-apoptotic members (Festjens, van Gurp et al. 2004). Bcl-2 was shown to prevent apoptosis induced by several different factors (except induced by death receptors), including serum deprivation, heat shock, chemotherapy agents, ethanol and other stimuli.
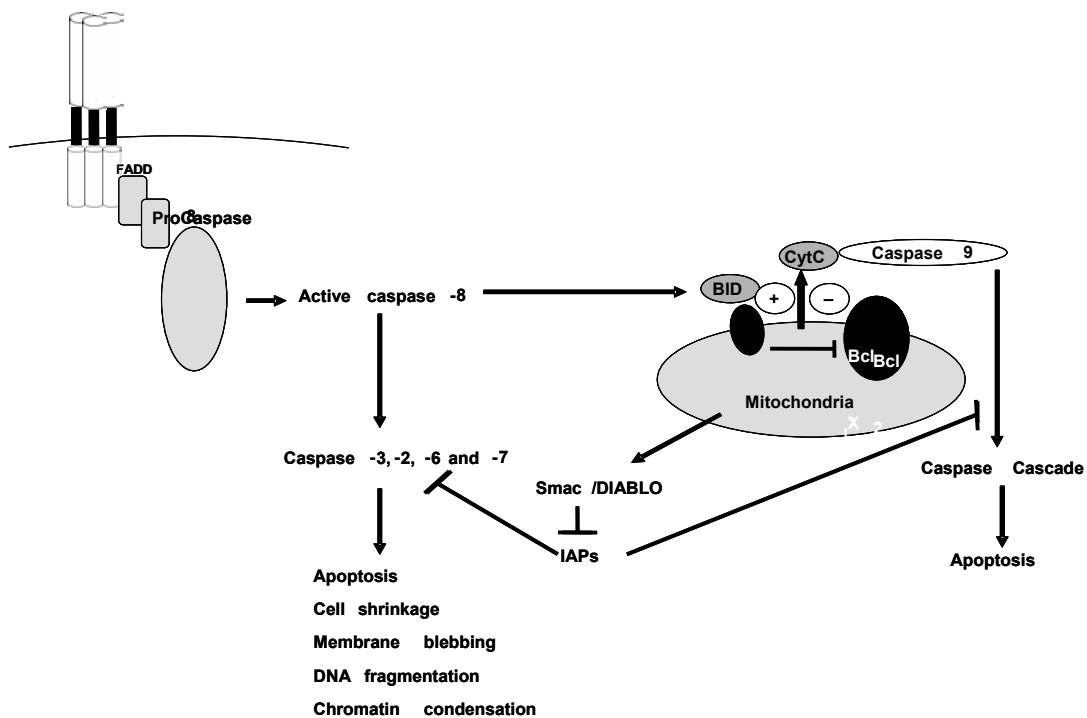

Figure 23. Death receptor and mitochondrial signaling pathway, two ways of apoptosis. The death receptor pathway triggers the binding of FADD (Fas-associated death domain) to the receptor, which in turn

recruits caspase-8, cleaving downstream caspases and triggering the death of the cell. Alternatively, caspase-9 can be activated by release of cytochrome c from mitochondria. Active caspase-9 cleaves other downstream caspases triggering apoptosis. Inhibition of caspases by the IAPs (inhibitor of apoptosis) is overcome by release of Smac/DIABLO from the mitochondria.

Anti-apoptotic Bcl-2 family members localized to mitochondria appear to regulate the release of Cytochrome c from mitochondria, which acts in concert with specific downstream caspases to induce apoptosis (Donovan and Cotter 2004). Bcl-X_L can bind Apaf-1 inhibiting its association with caspase-9 (Kuwana and Newmeyer 2003). Bcl-2 also regulates the mitochondrial membrane permeability through the permeability transition pore (PT) forming channels with a cation preference to maintain the mitochondrial membrane potential. Bcl-2 can also bind to calcineurin to inhibit the dephosphorylation of NF-AT, keeping NF-AT in the cytoplasm and arresting T cells in G1 (Burlacu 2003).

Protein	Function
Bcl-2 subfamily	
Bcl-2	antiapoptotic
Bcl-X_L	antiapoptotic
Bcl-X_S	proapoptotic
Mcl-1	antiapoptotic
A1 (Bñ-1)	antiapoptotic
Boo	antiapoptotic
Bax subfamily	
Bax	proapoptotic
Bak	proapoptotic
Mtd (Bok)	proapoptotic
Diva	propapoptotic
BH3-only subfamily	
Bik	proapoptotic
Bid	propapoptotic
Bad	proapoptotic
Bim	proapoptotic

Table III. Bcl-2 family proteins. Bcl-2 proteins can have an anti or pro apoptotic role.

Proapoptotic Bcl-2 proteins, Bad and Bax bind the antiapoptotic proteins Bcl-2 and Bcl-X_L preventing from exerting their antiapoptotic function. When it is not phosphorylated, Bad inhibits antiapoptotic Bcl-2 family members by direct binding (Sheikh and Huang 2004). Furthermore, Bax can form larger ion channels with a Cl$^-$ preference to destroy ion homeostasis of the outer mitochondrial membrane, which leads to a loss of membrane potential (Sharpe, Arnoult et al. 2004). In the presence of survival

factors, PKB/Akt and PKA phosphorylate Bad which binds to the cytoplasmic protein 14-3-3, and is unable to antagonize anti-apoptotic effects. In Ca^{2+}-induced apoptosis, Bad is dephosphorylated by calcineurin.

There is also evidence that caspase activation could be negatively regulated by inhibitors of apoptosis (IAPs) repressing different apoptotic pathways. The inhibitors of apoptosis (IAPs) can directly inhibit the active form of caspase 3, 7 and 9 blocking their interaction with Apaf-1. The caspase activator Smac DIABLO however binds the inhibitors of apoptosis (IAPs) and blocks their inhibitory function presumably by disabling their interaction with caspase-9. A homologous mammalian protein was identified with a similar function termed FLIP (FLICE inhibitory protein)/CasperI-FLIC/FLAME-1/CASH/CLARP/MRIT (Daniel, Anderson et al. 2003). This protein resembles the structure of caspase 8 and 10 but lacks enzymatic activity, thus competing with other caspases for binding to FADD (Fas-associated death domain) in case of receptor-mediated apoptosis.

4.2. Biological relevance of apoptosis

Apoptosis can be found in many physiological and pathological processes, such as development, differentiation, tumorigenesis and infections (Rodenburg, Raats et al. 2000). During immune responses, apoptosis is also important in cell-mediated killing of target cells, in the eradication of pathogens and in the pathogenesis of autoimmune disorders. Furthermore, disruption of pathways that regulate apoptosis can give rise to various diseases, including cancer and neurodegenerative disorders (Samowitz, Curtin et al. 2002).

There are several roles for apoptotic cells in the regulation of immune responses. Macrophages and dendritic cells seem to be the main phagocytes involved in the immune regulation of apoptotic cells. Immunosuppressive cytokines secreted by phagocytes upon recognition of apoptotic cells, or produced by apoptotic cells per se, are critical in the establishment of immune tolerance. After apoptotic cells are phagocytosed, breakdown of apoptotic cells by endosomal and lysosomal enzymes generates peptides that are loaded into the grooves of major histocompatibility complex molecules (Levine and Koh 1999). Thus, apoptotic cells are a source of autoantigens, and insufficient clearance of apoptotic cells may result in the accumulation of autoantigens in the circulation, which may activate the immune system and lead to systemic autoimmunity such as systemic lupus erythematosus (Lorenz, Grunke et al. 1997).

Macrophages ingesting apoptotic neutrophils decreased pro-inflammatory cytokine production and increased immunosuppressive cytokines, such as TGF-β,

prostaglandin E2, and platelet-activating factor critical in the establishment of immune tolerance (Savill, Dransfield et al. 2002). In contrast to apoptotic cells, a non-inflammatory process, phagocytosis of necrotic cells leads to dendritic cell maturation and macrophage activation linked to release of heat shock proteins (Basu, Binder et al. 2000).

In cytotoxic T lymphocytes (CTL)-mediated cytotoxicity against tumorigenic and virus-infected cells, the elimination of cells is mediated in part by the perforin-granzyme B pathway that facilitates the entry of granzymes into the target cell to activate caspase-3 leading to cell apoptosis (Hayashida, Shimaoka et al. 2000).

Another mechanism of non invasive cell death is opsonization. Opsonization increases the efficiency of the interaction between phagocyte and apoptotic cell and induces receptor-mediated phagocytosis. Deficiency of opsonins such as complement factors delays the in vivo uptake of apoptotic material by professional phagocytes, increasing the risk of induction of autoimmunity.

5. IMMUNOSUPPRESSION

5.1. Immunophilins

Immunophilins, also referred to as peptidyl prolyl cis-trans isomerases (PPIases), were initially identified in the search for binding partners for immunosuppressive agents. Their functions are poorly understood but have been linked to both prokaryotic and eukaryotic cellular processes, such as protein folding, trafficking, chaperone activity, oxidative stress response, functional arrangement of components within receptor complexes and apoptosis (Schiene-Fischer and Yu 2001).

Isomerase activity of immunophilins catalyses cis-trans isomerization of peptide bonds. Though this activity affects the conformation of a few known proteins, the immunophilins may primarily serve as scaffold or chaperone proteins assisting protein folding during maturation and protein-protein binding. Ligands of the immunophilins like Cyclosporine A (CsA), Rapamycin and FK506 immunosuppressors inhibit their isomerase activity, a mechanism for their immunosuppressant effects (Ruhlmann and Nordheim 1997).

Eukaryotes express three families of PPIases acting as intracellular receptors that are grouped according to the structure of their catalytic domains. These families are the cyclophilins (receptors for CsA), FK506 binding protein –FKBP- (receptor for FK506) and parvulins (Taylor, Husi et al. 1997).

5.1.1. Cyclophilins

The cyclophilin family consists of 15 members characterized by the presence of domains that confer subcellular localization mediating interactions with other proteins or nucleic acids. Found in all subcellular compartments, cyclophilins have been implicated in such diverse processes as protein translocation across membranes or through the secretory pathway, mitochondrial function, pre-RNA processing and control of transcription. In humans, four main cyclophilin-like proteins have been described: the cytosolic Cyclophilin A, the Cyclophilin B and Cyclophilin C in the endoplasmic reticulum and secretory pathway; and the mitochondrial Cyclophilin F.

Protein	Localization	Characteristics
CypA	Cytoplasm	Receptor for CsA
CypB, SCYLP	Endoplasmatic reticulum, secretory pathway	Heat shock
CypD	Mitochondria	CypB, SCYLP
CypC	Secretory pathway	Heat shock
Cyp 40	Cytoplasm, nucleus	Associated to steroid receptor
CypF, Cyp3	Mitochondrial inner membrane	
PPIL1, CypM	cytoplasm	
USA-CyP, Cyp20	nucleus	Human U4/U6 snRNP-associated protein
CypE, Cyp33A	nucleus	RNA-binding protein
Cyp33B	nuclear	RNA-binding protein
Cyp60	nucleus	
KIAA0073, Hal539-CyP	unknown	
CARS-CyP, Clk-associating RS cyclophilin	nucleus	Associated to nuclear matrix and splicing factors
NK-TRCyP	nucleus	Associated to nuclear matrix and splicing factors
RAN-BP2, NUP-358	Cytoplasm-nucleus	Associated to nuclear pore, cytoplasmic face

Table IV. The known human cyclophilins. Localization and characteristics of human cyclophilins.

The most abundant cyclophilin, Cyclophilin A, is a 18 kDa cytoplasmatic protein found in all cells, which consists of an eight stranded β barrel with an hydrophobic pocket that serves as the active site of the enzyme as well as the binding site for CsA (Dornan, Taylor et al. 2003). In presence of CsA, Cyclophilin A inhibits the Ca^{2+} and calmodulin-regulated protein phosphatase, calcineurin (CaN), a key intermediate of the T cell activation cascade causing a blockage of their proliferation program by blocking IL-2

production (Clipstone and Crabtree 1992). Independently of its action on CsA, Cyclophilin A exerts chaperone activity in protein refolding (Kern, Kern et al. 1994) and inflammatory effects since it may be secreted by mouse resident peritoneal macrophages and RAW 264.7 cell line in response to LPS, and cause inflammatory cell infiltration through chemotactic activity (Sherry, Yarlett et al. 1992). Biochemical studies have shown that a small group of proteins interact with Cyclophilin A, including heat shock protein (Hsp90) (Duina, Marsh et al. 1996), the transcription factor YY1 (Yang et al., 1995) and the antioxidant protein Aop1 (Jaschke, Mi et al. 1998).

The second discovered cyclophilin, Cyclophilin B (20kDa), is a β-barrel protein containing endoplasmatic reticulum leader sequence (Mariller, Haendler et al. 1996). Cyclophilin B has been associated with compartments of the secretion pathway, including the endoplasmatic reticulum, Golgi and biologic fluids such as breast milk and blood (150 ng/mL). It has also been found in the nucleus through nuclear localization signals at N terminal and the proteolysis of the C terminal endoplasmatic reticulum retention motif, resulting in the secretion of Cyclophilin B.

It has been suggested that Cyclophilin B can act as a chaperone with nascent peptides during their transport across the endoplasmatic reticulum. Furthermore, some surface binding sites for Cyclophilin B are found in specific cell populations including platelets, endothelial cells and T lymphocytes (Denys, Allain et al. 1997). Two binding sites have been identified, one composed of basic amino acid clusters in N terminal responsible for binding of Cyclophilin B to glycosaminoglycans, and another in the central core of Cyclophilin B, responsible for binding to type I receptor such as CD147 (Yurchenko, O'Connor et al. 2001). Cyclophilin B is thus responsible for an increased platelet adhesion to collagen regulating the activity of receptor membrane Ca^{2+} channel (Allain, Durieux et al. 1999). In vascular smooth muscle cells, oxidative stress has been associated with increased secretion of Cyclophilin B, suggesting that it may be an important mediator of the effects of reactive oxygen species on vascular function. In inflammatory processes, Cyclophilin B stimulates the influx of Ca^{2+} and initiates chemotaxis in peripheral blood T lymphocytes promoting their adhesion to the extracellular matrix (Allain, Durieux et al. 1999).

Cyclophilin C (18-23kDa) is located at the endoplasmatic reticulum. It is involved in several cellular processes. Similar to Cyclophilin A, Cyclophilin C can inhibit calcineurin activity in the presence of CsA (Schneider, Charara et al. 1994). CsA also interacts with murine cyclophilin C-associated protein (CyCAP hMac-2-BP), a member of the scavenger receptor cysteine-rich domain superfamily identified in the class A types I scavenger receptor implicated in mediating specific protein-protein interaction in cell adhesion (Jalkanen, Leu et al. 2001). In macrophages, inflammatory cytokines such as

TNF-α and IFN-γ up-regulate cyclophilin C-associated protein (CyCAP)/hMac-2-BP expression exerting an immune regulation effect through down-modulation of LPS signaling by altering the association of CD14 with its receptor. Cyclophilin C may also have a role in apoptosis since it is able to fragment DNA (Montague, Hughes et al. 1997).

Other cyclophilins have also been described with a specific cellular localization. For example, human Cyclophilin 23 and Cyclophilin 22 possess secretory and mitochondrial targeting signal peptides respectively. The cyclophilin NK-TR is part of the tumor recognition complex on the surface of natural killer cells. Another subfamily is Cyclophilin 40-like proteins which contain tetratricopeptide repeats that can bind calmodulin and steroid receptor–Hsp 90 heat shock protein chaperone complex (Ratajczak, Carrello et al. 1993). It has also been reported that the DNA binding activity of c-Myb transcription factor could specifically be regulated by Cyclophilin40 PPIase activity (Leverson and Ness 1998). Nup358 cyclophilin seems to take part in the trafficking of macromolecules through the nuclear pore complex (Wu, Matunis et al. 1995).

Cyclophilin 33 has been implicated in RNA metabolism, gene transcription and mRNA splicing, since it has been shown to bind poly(A) or poly(U) polyribonucleotides and bind to RNA through a RNA-binding motif. Another nuclear cyclophilin, Cyclophilin-20, is involved in nuclear splicing of pre-mRNA since it is a component of the human U4/U6 small nuclear-ribonucleoprotein particle (snRNP) (Horowitz, Kobayashi et al. 1997). Recently a Moca family of nuclear cyclophilins has been implicated in the regulation of gene expression. These proteins are characterized by a Moca domain and serine/arginine repeats and belong to the family of serine/arginine rich phosphoproteins involved in splicing and processing of mRNA (Cavarec, Kamphausen et al. 2002).

5.1.2. FKBPs

The family of cytoplasmatic FKBP immunophilins are the intracellular targets of Rapamycin and FK506. Members of the FKBP family (12-52 kDa) are found in all organisms. Using different mechanisms, FK506-FKBP complexes inhibit calcineurin activity, and the Rapamycin complex, binds to mTOR and inhibits its kinase activity and, subsequently, the down-stream targets (Gingras, Raught et al. 2001). FKBP (12kDa) is abundant and ubiquitously expressed and is reported to be a component of the intracellular calcium release channel complex. Another FKBP, FKBP52 has been associated with heat shock Hsp90 proteins in mammalian steroid receptor complexes (Peattie, Harding et al. 1992). Furthermore, FKBP can associate with other proteins; in

this way, FKBP12 and FKBP12.6 active sites can interact with steroid and arylhydrocarbon receptors and calcium release channels.

Protein	Localization	Characteristics
FKBP12	Cytoplasm	Receptor for FK506 and rapamycin
FKBP13	Secretory pathway	Heat shock
FKBP25	Nucleus	
FKBP12.6	Cytoplasm	Associated with cardiac calcium channel
FKBP33	Cell membrane	
FKBP51	Cytoplasm	
FKBP52	Cytoplasm	Steroid hormone receptor binding protein

Table V. Human FKBPs, their localization and characteristics.

mTOR (also known as FRAP or RAFT) is a 289 kDa serine/threonine kinase involved in regulating many aspects of cell growth as well as cell cycle progression, initiation and elongation phases of translation, amino-acid import, membrane trafficking, transcription of enzymes, protein degradation and both protein kinase signaling and transcription (Gingras, Raught et al. 2001). In presence of mitogens, mTOR regulates translation via phosphorylation of eukaryotic initiation factor 4E-binding protein (4E-BP1) and p70 ribosomal protein S6 kinase (S6K). mTOR also regulates antiapoptotic signaling by phosphorylation of Bad.

5.1.3. Parvulins

In contrast to the cyclophilins and the FKBPs, only a limited number of parvulins and homologues are known. Two essential parvulin homologues are known in eukaryotic cells: the human Pin1 and hPar14 both acting as regulators in mitotic cell cycle (Fujimori, Gunji et al. 2001). Pin 1 has a role in cell cycle regulation at the G1 phase targeting multiple proteins such as Sin3-Rpd3 histone deacetylase, RNA polymerase II and Cdc25 (Lu, Hanes et al. 1996). To date, one inhibitor, juglone, has been described for parvulins.

5.2. Immunosuppressants

The immunosuppressants permit organ transplantation without the toxicity associated to general antimitogenic agents (MacDonald 2003). Of all the

immunosuppressants, CsA has been the most widely used. CsA is a fungal cyclic polypeptide with low myelotoxicity. Patients receiving CsA treatment often develop autoimmunity upon cyclosporine withdrawal (Damoiseaux and van Breda Vriesman 1998).

CsA interacts with cyclophilins with high affinity, inhibiting their PPIase activity and the action of the phosphatase calcineurin, necessary for NF-AT-transactivated expression of IL-2 in T lymphocytes (Resch and Szamel 1997). CsA also protects cells against oxidative injury caused by anoxia/reoxygenation, peroxides and treatment of various cytotoxic agents. Through calcineurin-independent process, CsA can also block the mitochondrial permeability transition pore involved in cell necrosis during oxidative stress and Ca^{2+} homeostasis (O'Neill, Edwards et al. 2004).

Rapamycin (sirolimus) is a macrocyclin lactone antibiotic that was isolated from soil *Streptomyces* organisms and was identified as a potent immunosuppressor and antifungic, effective in a variety of organ transplantations preventing allograft rejection. It is a structurally analogue of tacrolimus (FK506) but using different mechanism of action.
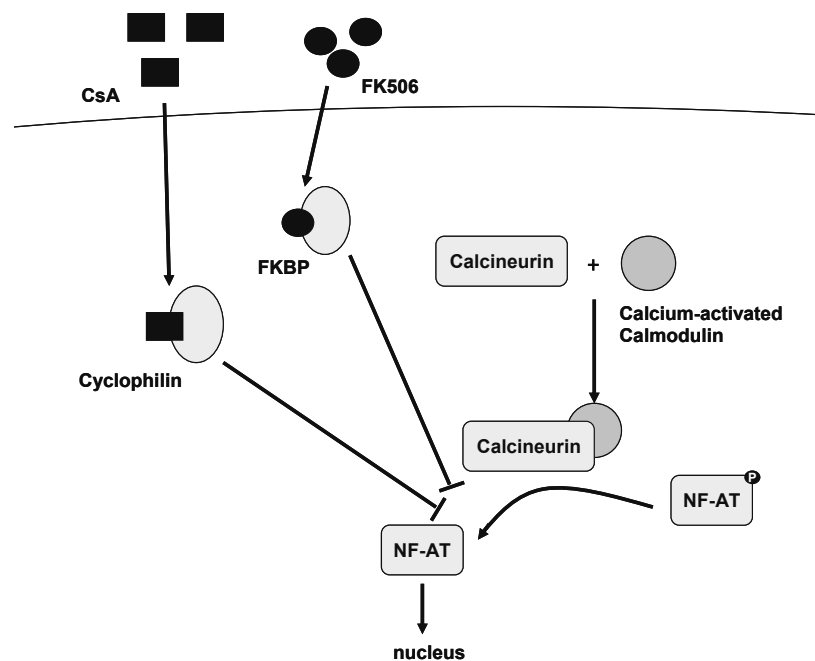

Figure 24. Mechanism of action of macrolide immunosuppressants. Calcium release activates calmodulin which in turn activates calcineurin. Activated calcineurin dephosphorylates cellular NF-AT (nuclear factor of activated T-cell) enabling it to enter the nucleus. CsA (cyclosporin A) binds to its immunophilin, cyclophilin; FK506 has a different immunophilin, FKBP. Both of them inhibit calcineurin-induced dephosphorylation of NF-AT.

Rapamycin and FK506 bind to a member of the cytoplasmatic immunophilin family, the FK506-binding protein, FKBP (Dumont and Su 1996). FK506 thus inhibits calcineurin phosphatase activity and rapamycin inhibits mTOR without inhibiting calcineurin. It has been observed that rapamycin blocks T and B cell proliferation at G1 by inhibiting mTOR. Thus, rapamycin inhibits S6K required for cell growth and promotes apoptosis enhancing tolerance induction (Li, Li et al. 1999).

Sanglifehrin A (SfA) is a recently developed immunosuppressive macrocyclic compound produced by the actinomycetes strain *Streptomyces* (Fehr, Kallen et al. 1999; Sanglier, Quesniaux et al. 1999). Like CsA, SfA binds to Cyclophilin A but with an affinity that is 20-fold higher than that of CsA (Fehr, Kallen et al. 1999; Zenke, Strittmatter et al. 2001; Zhang, Youn et al. 2001). The Cyclophilin-SfA complex does not interact with calcineurin and does not affect Ca^{2+}-dependent IL-2 production by T lymphocytes. Rather, it has been demonstrated to inhibit IL-2 dependent T cell proliferation, through a G1 blockage which resembles the action of rapamycin (Zhang, Youn et al. 2001; Allen, Zheng et al. 2004). The exact biochemical and molecular mechanism accounting for SfA's ability to suppress T cell function have yet to be elucidated since SfA does not inhibit mTOR activity, measured by 4E-BP1 (eukaryotic initiation factor 4E-binding protein) phosphorylation (Zenke, Strittmatter et al. 2001). Furthermore, SfA shows some inhibitory activities on IgG production by B cells, on TNF-α production by monocytes and on IL-12 production by dendritic cells (Comalada, Valledor et al. 2003; Steinschulte, Taner et al. 2003; Woltman, Schlagwein et al. 2004).

INTRODUCCIÓ

ELS MACRÒFAGS

Els macròfags participen tant en la resposta immunitària en front a patògens com en l'homeostasis reparant teixits (Mantovani, Sozzani et al. 2002). Durant la resposta innata els macròfags fagociten els patògens i modulen la resposta immune a través de la producció de citocines i quimiocines. També es caracteritzen per poder presentar antigens a limfòcits T activant una resposta específica.

Els macròfags s'originen a partir de cèl·lules mieloides precursores presents a la medul·la òssia dels adults. En presència de citocines com M-CSF, IL-3 o GM-CSF, els precursors mieloides maduren i es diferencien a monòcits. Els monòcits formats són alliberats a la sang i són atrets als diferents teixits on es diferencien a macròfags. Depenent del tipus de teixit, els macròfags reben diferents denominacions, microglia en el sistema nerviós central, cèl·lules de Langerhans a la pell, cèl·lules de Kuppfer al fetge, etc... (Xaus, Comalada et al. 2001).

En els teixits, els macròfags poden proliferar en presència de factors de creixement i en absència d'aquests factors són induïts a l'apoptosi. Quan la intervenció dels macròfags és requerida, degut a la presència d'agents microbians o de citocines, els macròfags aturen la seva proliferació i indueixen funcions antimicrobianes com la fagocitosi, la secreció de citocines proinflamatòries (TNF-α, IL-1, IL-6) i òxid nítric (NO) que ajuden a eliminar els patògens (Xaus, Comalada et al. 2001; Schroder, Hertzog et al. 2004). D'igual forma, la resposta imflamatòria ha de ser regulada quan es resol la inflamació per evitar danys tissulars (Gordon 2003).

LA PROLIFERACIÓ DELS MACRÒFAGS

L'M-CSF és un factor de creixement que regula la supervivència, proliferació i diferenciació dels macròfags. L'M-CSF interacciona amb el seu receptor específic *c-fms (CD115)* conduint a l'activació de diferents cascades de transducció de la senyal. L'M-CSF és sintetitzat per molts tipus cel·lulars, principalment per fibroblasts i cèl·lules endotelials en presència de GM-CSF, TNF-α, IL-1 i IFN-γ. La seva expressió és crucial ja que la soca de ratolins Op/Op, que no produeixen M-CSF, presenten deficiències en la població de macròfags (Wiktor-Jedrzejczak and Gordon 1996).

El receptor del M-CSF és de la família de receptors del tipus III tirosina quinasa. L'expressió del receptor s'observa al llarg de la diferenciació dels macròfags, tot i que també es troba en osteoclasts i trofoblasts (Hofstetter, Wetterwald et al. 1992; Sapi, Flick et al. 1998).

La unió del M-CSF al seu receptor provoca la seva dimerització no covalent autofosforilant residus de tirosina en l'extrem citoplasmàtic estabilitzant la seva conformació. Les diferents fosforilacions del receptor del M-CSF impliquen l'activació de diferents senyals intracel·lulars que activen la funcionalitat i l'expressió de factors de transcripció nuclears (Hamilton 1997).

La principal via activada pel M-CSFR a través de molècules adaptadores (Src, SOS, Grb2, Ras) és la clàssica via de Raf→ MEK→ ERK-1/2 necessària per la proliferació cel·lular. Tot i això, l'M-CSF també indueix l'activació de les altres dues MAPKs, Rac/JNK i Rac/p38 (Kyriakis, App et al. 1992). A través de l'activació de la fosfolipasa C, M-CSF també regula els nivells d'inositols, Ca^{2+} i de diacilglicerol, activant la família de les proteïnes quinases C. Els fosfolípids generats també poden ser metabolitzats per la PI3K (Wymann, Bjorklof et al. 2003).

Proteïna quinasa C

Actualment es coneixen 13 isoformes diferents de proteïnes quinases C implicades en múltiples funcions biològiques (Saito, Hojo et al. 2002). Segons els requeriments de cofactors, es classifiquen en isoformes convencionals (α, βI/II i γ) que necessiten Ca^{2+}, fosfolípids i DAG per activar-se, formes noves (δ, ϵ, η, θ i μ) que necessiten de fosfolípids i DAG; i en isoformes atípiques com ξ, ι i λ, que s'activen amb ceramida, àcid fosfatídic i PIP$_3$ (Nishizuka, 2003). Estudis portats a terme prèviament en el nostre grup demostren que els macròfags de medul·la òssia només expressen 3 isoformes de PKC: βI , ϵ i ξ.

S'ha descrit que les PKC regulen l'activitat de Ras o Raf-1 tant *in vitro* com *in vivo* regulant el creixement cel·lular (Sozeri, Vollmer et al. 1992; Seibenhener, Roehm et al. 1999). Estudis previs realitzats en el nostre grup també demostren que la PKCϵ està implicada en la inducció de la fosfatasa MKP-1, tant per M-CSF com per LPS, a través d'un mecanisme encara desconegut (Valledor, Xaus et al. 1999; Valledor, Comalada et al. 2000). També s'ha descrit que les PKC atípiques poden modular la kinasa MEK influenciant tant en la via d'activació de Ras/MAPK com en la de Src/JNK. La inhibició de PKCs atípiques bloqueja l'activació de JNK, posicionant les JNK com a substrats de les PKC (Diaz-Guerra, Bodelon et al. 1996; Schonwasser, Marais et al. 1998).

MAPKs

La principal via necessària per la proliferació dels macròfags és la de Raf → MEK → ERK. De les 3 isoformes de Raf, A-Raf, B-Raf i Raf-1 (també anomenada c-Raf), l'isoforma Raf-1 s'expressa de forma ubiqua en els teixits d'adult i ha estat l'isoforma més estudiada. Raf és activada per la proteïna Ras d'unió a GTP (Morrison, Heidecker et

al. 1993) que també està implicada en la senyalització de PI3K/Akt-mTOR/S6K, NFκB, PKCs i PKA (Marais, Light et al. 1995; Chaudhary, King et al. 2000). La seva supressió en ratolins deficients en Raf-1/B-Raf implica una pèrdua pràcticament complerta de l'activació d'ERK i la disminució de l'expressió depenents de la seva activació (Kamata, Pritchard et al. 2004).

L'activació de les MAPKKKs condueix a l'activació de MAPKK-MEK i finalment de les MAPKs (Cano et al., 1995; Xu et al., 1995). Apart de la via clàssica ERK, hi ha 2 grups més de serina-treonina MAPKs activades per M-CSF, JNK (JNK1/2/3) i p38 (p38α, β, γ, δ), que típicament s'activen amb estímuls d'estrés (Gupta, Campbell et al. 1995; Marshall 1995; Gupta, Barrett et al. 1996). D'aquesta manera, les MAPKs s'activen per fosforilació dual en motius Thr-Xaa-Tyr (Payne, Rossomando et al. 1991; Robbins, Zhen et al. 1993) transmeten les senyals extracel·lulars fins al nucli.

L'ús de PD 98059 com a inhibidor de MEK-1/2, ha permès estudiar la implicació d'ERK en molts processos fisiològics (Bain, McLauchlan et al. 2003). La família de les ERK està constituïda per ERK1, ERK2, ERK3, ERK4, ERK5, ERK6, ERK7 i ERK8. D'elles, ERK1 (42kDa) i ERK2 (44kDa) han estat les més estudiades. Després de ser fosforilades per MEK-1/2, les ERK dimeritzen i es transloquen al nucli on fosforilen factors de transcripció regulant l'expressió gènica (Khokhlatchev, Canagarajah et al. 1998). Entre les diferents dianes de ERK-1/2, cal destacar Ets-1, c-Jun, c-Fos, NF-κB i c-Myc, responsables de regular l'expressió de gens necessaris pel cicle cel·lular i per la supervivència i apoptosi (Schreck, Rieber et al. 1991; Karin and Hunter 1995; Prendergast 1999; Amati, Frank et al. 2001; Baldwin 2001; Nasi, Ciarapica et al. 2001; Chang, Steelman et al. 2003). En macròfags l'M-CSF provoca un augment de l'activitat d'ERK, als 5 i 15 min (Valledor, Xaus et al. 1999).

La cascada JNK i p38 s'activa típicament per estrès cel·lular, infecció bacteriana o per citocines proinflamatòries, tot i que també s'han vist associades a la proliferació cel·lular, diferenciació i apoptosi (Ip and Davis 1998; Shaulian and Karin 2001). Les MEKK responsables de fosforilar a JNK són MEKK1 i SEKK1 i MKK6 o MKK3 en el cas de p38 (Clark, Dean et al. 2003). L'activació de JNK implica l'activació de la proteïna activador (AP-1), homo i heterodímers dels factors de transcripció Jun i Fos i d'altres factors de transcripció que regulen l'expressió de gens com ciclina D1, ciclina A, Cdk4/6, p53, p16 INK4a, IL-2, IL-3 i GM-CSF (Wisdom, Johnson et al. 1999; Bakiri, Lallemand et al. 2000). Per altra banda, l'activació de p38 fosforila la quinasa MAPKAPK-2 citosòlica que regula seqüències riques en adenosina i uridina (ARE)s de regions 3'-no traduïdes, modificant l'estabilitat de la vida mitja del ARN missatger (Shi and Gaestel 2002). En el nucli, p38 també regula l'activació de factors de transcripció com ATF-2, MEF2C i NF-κB (Treisman

1996; Schmitz, Bacher et al. 2001). L'extensió de l'activació de les tres MAPKs està regulada per proteïnes fosfatases específiquesl (Pouyssegur, Volmat et al. 2002).

Proteïnes fosfatases

Les fosfatases de les MAPKs, MKPS, són crucials per regular negativament la resposta cel·lular (Hunter 1995; Keyse 2000). Hi ha diferents tipus de fosfatases depenent de la seva diana: fosfatases específiques de serina/treonina (PSPs), específiques de tirosina (PTPs) o d'especificitat dual (DSP).

A destacar les PSPs inclouen la PP2B també coneguda com a Calcineurina. L'activació de la calcineurina és depenent de calci i és la diana d'immunosupresors com la Ciclosporina A i el FK506.

Les 13 DSPs conegudes en la actualitat es classifiquen en diferents grups depenent de l'especificitat pel substrat, distribució en teixits, localització subcel·lular i la regulació de gens (Ishibashi, Bottaro et al. 1994). Les DSPs tipus I inclouen la VHR (ERK), la DSP2 (p38, JNK) (Aoyama, Nagata et al. 2001) i la MKP6 (ERK/JNK) (Marti, Krause et al. 2001). Les tipus II inclouen MKP-1 (Chu, Solski et al. 1996), PAC1 (ERK=p38>JNK) (Ward, Gupta et al. 1994; Chu, Solski et al. 1996), MKP2 (ERK=JNK>p38) (Misra-Press, Rim et al. 1995; Chu, Solski et al. 1996), VH3/B23, MKP3 (ERK>>JNK=p38) (Muda, Theodosiou et al. 1998), PYST2 (Groom, Sneddon et al. 1996) i MKP4 (ERK>p38=JNK) (Muda, Boschert et al. 1997). Les tipus III inclouen només la MKP5 (JNK similar a p38>>ERK). Les tipus IV inclouen la VH5/M3/6 (Muda, Theodosiou et al. 1998) i la MKP7 (Masuda, Shima et al. 2001), les quals són específiques de JNK i p38, però no d'ERK.

Centrant-nos en MKP-1 (o CL100 o DUSP1), aquesta fosfatasa principalment inactiva a p38 i JNK, però en certes circumstàncies també defosforila a ERK (Franklin and Kraft 1997). La seva expressió ubiqua és particularment rellevant en tipus cel·lulars com macròfags, mastocits, osteoblasts i epitelis pulmonars i intestinals (Engelbrecht, de Wet et al. 2003). De fet, l'expressió de MKP-1 es troba mutada en certs tipus de tumors (Scimeca, Servant et al. 1997). Diferents estudis han demostrat que tant PKA, el calci i les proteïnes quinases C participen en l'expressió de MKP-1 (Waskiewicz and Cooper 1995; Scimeca, Servant et al. 1997).

Via de supervivència PI3K/Akt

L'estimulació per M-CSF provoca un augment de l'activitat PI3K que es pot bloquejar utilitzant inhibidors com Wortmanina i LY294002 (Yusoff, Hamilton et al. 1994). L'eix PI3K-PKB/Akt regula diferents fenòmens cel·lulars tals com el creixement cel·lular, la supervivència i el metabolisme/transport de glucosa en diferents tipus

cel·lulars. Les PI3Ks estan implicades en la regulació d'altres molècules com PKCs i PLC-γ (Alessi, Andjelkovic et al. 1996). També regulen l'expressió de molècules proapoptòtiques de la família Bcl-2 (Downward 1999), les caspases, la glicogen sintasa 3 i els factors de transcripció Foxo-3 necessaris per l'expressió del lligand de Fas (Kops and Burgering 1999). També s'ha observat que Akt pot contribuir a l'inactivació de Raf-1 (Fulton, Gratton et al. 1999) i a l'activació de JNK (Kobayashi, Nagata et al. 1997; Liu, Ye et al. 1997). La via PI3K/Akt també modula la progressió del cicle cel·lular.

El cicle cel·lular

El cicle cel·lular és el procés pel qual les cèl·lules es divideixen mantenint l'integritat genòmica (Gutierrez, Ramirez-Parra et al. 2002). El cicle cel·lular dels mamífers es pot dividir en una fase G1 de preparació de la maquinària cel·lular, en una fase S on es duplica l'informació gènica, en una fase G2 de preparació per la mitosi, la fase M. En absència de factors de creixements, les cèl·lules es troben en fase G0.

Els macròfags només requereixen de la presència del M-CSF durant la major part de la fase G1 que en macròfags dura aproximadament unes 12 hores (Rock, Cleveland et al. 1992). La transició d'una fase a una altra està altament regulada per diferents proteïnes cel·lulars (Lukas and Bartek 2004).

Els estudis de cicle cel·lular requereixen una sincronització de les cèl·lules per l'absència del factor de creixement respectiu, de manera que reentrin en el cicle de forma sincrònica. Per determinar el nombre de cèl·lules progressant en el cicle cel·lular s'utilitza la incorporació de bromo-desoxiuridina (BrdU) (Wu, Hong et al. 2001) o la tinció amb iodur de propidi que tenyeix el ADN (Schmid, Cole et al. 2000). Els efectes dels estímuls sobre la síntesi de ADN també es poden avaluar mesurant la incorporació de timidina tritiada [^{3}H].

Les diferents fases del cicle cel·lular estan coordinades per diferents proteïnes com les ciclines (Cic) i les quinases depenents de ciclina (Cdks) (Grana and Reddy 1995). Les Cdks són serina/treonina quinases inactives en forma monomèrica que s'activen quan s'uneixen a ciclines específiques. En mamífers, normalment els nivells de les Cdks són constants i només l'expressió de les ciclines és regulada a nivell transcripcional i post-transcripcional al llarg del cicle.

La fase inicial de G1 està regulada per ciclines tipus D induïdes pels factors de transcripció (Sherr 1994). D'aquesta manera les ciclines D1, D2 i D3 s'activen al unir-se a Cdk4 i a Cdk6(Matsushime, Roussel et al. 1991; Sherr 1994). Durant la fase G1 tardana, és la ciclina E (E1 i E2) conjuntament amb la Cdk2, les que regulen la transició a la fase S (Payton and Coats 2002). L'activació dels complexes ciclina-Cdk és necessària per passar a la fase S a través de la hiperfosforilació i la inactivació de la proteïna del

retinoblastoma (pRb) i l'alliberament dels factors E2F necessaris per l'expressió de gens de la fase S, com ciclina A, E i Cdc25 (Flemington, Speck et al. 1993; Harbour and Dean 2000).

Per altra banda, l'activitat de les Cdks també està regulada per associació a inhibidors de Cdks (CDKIs) (Grana and Reddy 1995). Actualment es coneixen dos famílies de CDKIs, la família INK4 i la família Cip/Kip (Sherr and Roberts 1995). La família INK4 està formada per p16^{INK4a}, p15^{INK4b}, p18^{INK4c} i p19^{INK4d}, i només regula l'activitat de les Cdks de la fase G1, Cdk4 i Cdk6, provocant l'aturada del cicle cel·lular a G1 (Carnero and Hannon 1998; Pavletich 1999). Per contra, la família Cip/Kip inclou a p21$^{Waf-1/Cip-1}$, p27$^{Kip-1/Cip2}$ i p57^{Kip-2} (Harper, Adami et al. 1993; Matsuoka, Edwards et al. 1995). Els inhibidors d'aquesta família inhibeixen pràcticament l'activitat de totes les Cdks independentment de la fase del cicle (Harper, Adami et al. 1993).

Específicament, s'ha implicat a p21$^{Waf-1/Cip-1}$ en la inhibició de la ADN polimerasa δ i de PCNA. També s'ha relacionat p21$^{Waf-1/Cip-1}$ amb la supervivència cel·lular a través de mecanismes depenents d'Akt (Rossig, Jadidi et al. 2001). De fet estudis previs en el nostre grup, han demostrat que p21$^{Waf-1/Cip-1}$ és responsable dels efectes antiapoptòtics observats en absència de factors de creixement o en presència de LPS (Xaus, Valledor et al. 1999). També s'ha descrit que els CDKIs poden actuar com a cofactors transcripcionals regulant l'activitat de NF-κB, STAT-3 (Coqueret and Gascan 2000), Myc (Kitaura, Shinshi et al. 2000), C/EBP i E2F (Harris, Albrecht et al. 2001).

Per altra banda, el paper de p27^{Kip-1} s'ha relacionat més amb la inhibició de l'activitat Cdk2 i el bloqueig del pas a fase S (Medema, Kops et al. 2000). La p27^{Kip-1} també regula la sortida del cicle cel·lular en quiescència. L'entrada a cicle induïda per factors de creixement promou la degradació de p27^{Kip-1} a través d'un augment de l'activitat del proteosoma (Liang, Zubovitz et al. 2002). Tots aquests reguladors del cicle cel·lular, Cdks, ciclines i CDKIs o bé els seus substrats s'han vist desregulats en molts càncers (Grana and Reddy 1995).

L'expressió de la família de factors de transcripció Myc (c-, N- and L-Myc) és essencial per la progressió de la fase G1 i la seva desregulació condueix a diferents tipus de càncers (Nesbit, Tersak et al. 1999). C-Myc és un protooncogene que pot interaccionar amb el ADN a través de dominis de cremallera de leucina. Per reconéixer els llocs d'unió cal que c-Myc formi heterodímers amb el factor de transcripció Max (Baudino and Cleveland 2001) i que estigui fosforilat (Hagiwara, Nakaya et al. 1992; Kubin, Parshley et al. 1999). D'aquesta manera c-Myc dirigeix l'expressió de gens com ciclina D1, ciclina D2, ciclina E, Cdk4 i Cdk1. La c-Myc també reprimeix la transcripció de p27^{Kip-1}, p21$^{Waf-1/Cip-1}$, p15^{Ink4b} i p16^{Ink4a} (Amati, Alevizopoulos et al. 1998)

Inhibidors de la proliferació depenent de M-CSF

La proliferació depenent d'M-CSF es pot inhibir a través de diferents mecanismes. Proteïnes quinases com PKA, depenents d'un augment d'AMPc, poden regular l'activitat Raf-1 inhibint en conseqüència la proliferació depenent de Raf/MEK/ERK. La PKA també provoca una disminució de c-Myc, ciclina D1 i Cdk4 en macròfags augmentant l'expressió de p27^{Kip-1}(Xaus, Valledor et al. 1999). Altres agents que també augmenten els nivells de cAMP com l'adenosina i les prostaglandines E (PGE$_2$), també actuen com a inhibidors de la síntesi d'ADN induïda per M-CSF (Xaus, Valledor et al. 1999). El factor de creixement transformador β (TGF-β) també és un potent inhibidor del creixement en diferents tipus cel·lulars a través de la inhibició de l'expressió de c-Myc a través de complexes Smad (Polyak 1996; Massague 2003).

L'activació dels macròfags implica necessàriament un bloqueig de la seva proliferació i un augment de funcions especialitzades. Per tant, l'activació dels macròfags amb interferons tipus I (IFN-α, IFN-β) i tipus II (IFN-γ), LPS o TNF-α, atura la proliferació dels macròfags a G1 a través de diferents mecanismes (Vadiveloo 1999). D'aquesta manera mentre que el LPS indueix un programa apoptòtic, el tractament amb IFN-γ protegeix els macròfags de l'apoptosi induïda per LPS, glucocorticoids o absència d'M-CSF, a través de l'expressió de of p21$^{Waf-1/Cip-1}$ i un bloqueig del cicle cel·lular a G1/S (Xaus, Cardo et al. 1999).

ACTIVACIÓ

L'activació clàssica dels macròfags s'indueix en presència de molècules microbianes o inflamatòries com el LPS en un ambient tipus Th1 (en presència d'IFN-γ produït pels limfòcis T) o per TNF-α. D'aquesta manera, els macròfags produeixen òxid nítric (NO) i augmenten l'expressió del complexe major d'histocompatibilitat de classe II (MHC-II) i de CD86, augmentant la seva capacitat de presentació d'antigens. La producció de citocines proinflamatòries i de NO provoca una activitat antiproliferativa i citotòxica (Klimp, de Vries et al. 2002).

L'activació clàssica dels macròfags és inhibida per citocines tipus Th2 que indueixen un programa d'activació alternatiu, i a l'invers. La presència de IL-4 i IL-3, doncs, activa a macròfags tipus M2 que no poden generar NO utilitzant L-arginina. Els macròfags M2 augmenten l'expressió de l'antagonista del receptor de la IL-1 (IL-1Ra), de receptors scavenger i de certes quimiocines (Imai, Nagira et al. 1999). De forma similar als macròfags M1, els macròfags M2 presenten un augment de MHC-II, capacitat fagocítica i endocítica junt amb molècules coestimuladores CD86 (Gordon 2003).

Interferó gamma IFN-γ

L'IFN-γ és un interferó tipus II produït principalment pels limfòcits CD4$^+$ o CD8$^+$ positius i per cèl·lules NK (Yoshimoto, Wang et al. 1998; Pestka, Krause et al. 2004), tot i que també s'ha descrit la seva producció per cèl·lules presentadores d'antigen professionals i per limfòcits B (Puddu, Fantuzzi et al. 1997; Frucht, Fukao et al. 2001). L'IFN-γ genera una resposta antiproliferativa, antiviral, immunomoduladora i supresora de tumors a través del seu receptor específic (Boehm, Klamp et al. 1997; Bernabei, Coccia et al. 2001). L'augment de quimiocines (IP-10, MCP-1, MIG, MIP-1a/b, RANTES) i de molècules d'adhesió (ICAM-1, VCAM-1) induïda per IFN-γ afavoreix el reclutament de limfòcits específics (Vaday, Franitza et al. 2001). També promou l'augment d'expressió en superfície de FcγRI d'alta afinitat, augmentant la citotoxicitat depenent d'anticossos (Capsoni, Minonzio et al. 1994).

Així doncs, l'IFN-γ provoca un augment de la fagocitosi, indueux l'esclat respiratori i provoca un augment d'enzims lisosomals que promouen la destrucció del patogen (Gupta, Kubin et al. 1992).

La unió de l'IFN-γ al seu receptor provoca la seva oligomerització i trans-fosforilació de tirosina quinases Janus associades al receptor, JAK-1/2 (Sakatsume, Igarashi et al. 1995). Alhora, les JAKs fosforilen els factors de transcripció STAT que dimeritzen. Els dímers d'STAT-1 fosforilats es trasloquen al nucli on s'uneixen a seqüències del ADN (ISREs en elements GAS), regulant l'expressió de gens depenents de IFN-γ (Darnell 1998) (Kisseleva, Bhattacharya et al. 2002). També s'ha descrit que una efectiva activitat transcripcional, STAT-1 requereix ser fosforilada en serina. En aquesta fosforilació s'hi han vist relacionades certes quinases com la mTOR (Goh, Haque et al. 1999), la família PKC i les diferents MAPKs.

Simultàniament a l'activació de la via JAK/STAT, l'IFN-γ també indueix l'expressió de reguladors negatius de la seva pròpia activació, la família CIS/SOCS (Kisseleva, Bhattacharya et al. 2002). Les SOCS s'associen a JAK-1/2 bloquejant la seva activitat tirosina quinasa i les consegüents vies associades (Kile and Alexander 2001).

La presència d'IFN-γ millora la resposta dels macròfags a LPS i ADN bacterià (Cpg ADN) promovent un fenotip M1 (Lorsbach, Murphy et al. 1993). De fet, els ratolins deficients pel receptor del IFN-γ són resistents a la toxicitat per LPS (Car, Eng et al. 1994). Contràriament al cas amb l'LPS, l'IFN-γ provoca un arrest a G1/S dels macròfags en presència de M-CSF (Xaus, Cardo et al. 1999). S'ha hipotetitzat que aquest efecte ve mediat per augment de inhibidors de Cdks com p27^{Kip-1} i la inhibició de l'activitat Cdk2 i Cdk4. En conseqüència, no s'expressen gens com c-myc depenents dels factors E2F (Matsubara, Katayama et al. 1999; Matsuoka, Kitamura et al. 1999). A més a més, també

s'ha descrit un augment dels nivell de Mad que antagonitzen l'activitat myc (Dey, Kim et al. 1999). IFN-γ també provoca una inhibició de l'expressió de MKP-1 per M-CSF a través d'un mecanisme STAT-1 depenent (Xaus, Comalada et al. 2001).

Una de les principals característiques de l'IFN-γ és l'augment de l'expressió de MHC-II, que és un element essencial per la selecció i l'activació adequada de limfòcits T CD4 (Darnell 1998; Guermonprez, Valladeau et al. 2002; Kisseleva, Bhattacharya et al. 2002). Les molècules MHC-II s'expressen de forma constitutiva en limfòcits B i només en resposta a IFN-γ en macròfags i cèl·lules dendrítiques (APCs) o en cèl·lules endotelials i fibroblasts.

Les molècules de MHC-II presenten principalment pèptids derivats de proteïnes exògenes o endògenes endocitades per diferents mecanismes (Bakke and Nordeng 1999), mentre que les molècules de classe I expressades en pràcticament tots els tipus cel·lulars processen les d'origen endogen (Epperson, Arnold et al. 1992). Els gens de classe II HLA-DR, -DP i –DQ (I-A i I-E en ratolins) presenten una regulació principalment transcripcional. Els seus promotors presenten unes seqüències conservades dites X, Y i W on s'hi uneixen diferent factors de transcripció com RFX, CBP i NF-Y (Celada, McKercher et al. 1996; Herrero, Sebastian et al. 2002). L'expressió d'aquests factors, però, sol ser constitutiva i no és suficient per l'expressió del MHC-II (Kretsovali, Agalioti et al. 1998). De fet, la seva expressió depèn de la inducció del CIITA, el transactivador de classe II, el qual interacciona amb els factors de transcripció i amb la maquinària transcripcional general (Masternak, Peyraud et al. 2003).

A diferència dels factors de transcripció que s'uneixen a les caixes X, Y i W, l'expressió del CIITA està molt regulada, és induïble per citocines i depèn del tipus cel·lular. De fet, l'expressió ectòpica del CIITA és suficient per induir l'expressió del MHC-II i la seva deficiència provoca la pèrdua completa d'expressió del MHC de classe II tant en la induïda com en la constitutiva (Chang, Guerder et al. 1996).

L'expressió del CIITA està regulada per 4 promotors, el pI, pII, pIII i pIV. D'ells, el pI mieloid, actua en cèl·lules dendrítiques i macròfags induït per IFN-γ (Pai, Askew et al. 2002). El pII actua molt poc en humans i no s'expressa en ratolins (Pai, Askew et al. 2002). El pIII limfoid participa de forma constitutiva en limfòcits B i de forma induïda per IFN-γ en cèl·lules dendrítiques (Ghosh, Piskurich et al. 1999; Landmann, Muhlethaler-Mottet et al. 2001). Per acabar, el pIV participa en resposta a IFN-γ en diferents tipus cel·lulars com macròfags i cèl·lules dendrítiques però també en cèl·lules no hematopoiètiques (Barbieri, Deffrennes et al. 2002).

Els estudis fets en macròfags indiquen que el pIV està implicat en la inducció de molècules del MHC de classe II per IFN-γ, però que també ho pot estar el pI, amb una

activació transitòria del pIV i sostinguda del pI. Aquests promotors contenen elements GAS, caixes E i llocs d'unió a IRF-1 on s'uneixen els dímers d'STAT-1 fosforilats, USF-1 i IRF-1 respectivament (Dong, Rohn et al. 1999). A més a més, l'expressió del CIITA també pot patir modificacions postranscripcionals regulant la seva localització cel·lular (Sisk, Nickerson et al. 2003).

El lipopolisacàrid, LPS

El LPS és un glicolípid de la membrana dels bacteris Gram negatius. La regió hidrofòbica coneguda com a lípid A és l'iniciadora dels efectes proinflamatoris del LPS en macròfags (Alexander and Rietschel 2001).

Els macròfags reconeixen l'LPS a través del Toll like receptor 4 (TLR4). Per ser reconegut, l'LPS s'uneix a la LBP sèrica (la proteïna d'unió al LPS). Aquesta transfereix l'LPS al CD14 soluble o unit a la superfície dels macròfags. Aquest correceptor presenta el complex al TLR4 unit a la proteïna 2 de diferenciació mieloid (MD-2) provocant la dimerització del TLR4 (da Silva Correia, Soldau et al. 2001).

Actualment s'han identificat 10 TLRs (TLR-1/10) en mamífers i permeten el reconeixement de diferents antigens com lipoproteïnes, peptidoglicans (TLR2 i TLR6), RNA de doble cadena (TLR3), flagelina (TLR5), ADN bacterià CpG (TLR9) (Kirschning and Schumann 2002; Imler and Hoffmann 2003; Imler and Zheng 2004). Els diferents TLRs es caracteritzen per un "domini TIR" compartit amb el receptor de la IL-1 (Imler and Hoffmann 2003; Imler and Zheng 2004).

El TLR4 s'expressa predominantment en macròfags i cèl·lules dendrítiques i activa diferents vies de senyalització intracel·lular a través de molècules adaptadores com el factor de diferenciació mieloid (MyD88), TIRAP/Mall, TRIF, TRAM i d'altres (Yamamoto, Nakane et al. 2003). Mitjançant l'activació d'IRAK (quinasa associada al receptor de la IL-1) per fosforilació, s'activa TRAF6 responsable d'activar les MAPKs. Els principals factors de transcripció induïts pel LPS són NFκB i AP-1 activant l'expressió de gens com el TNF-α (Muzio, Ni et al. 1997).

L'LPS, doncs, activa diferents quinases com PKC, PI3K, PKA i les 3 MAPKs, ERK-1/2, p38MAPK and JNK (Reimann, Buscher et al. 1994). L'activació de les PKCs per un augment de DAG i de cAMP en presència de LPS s'ha relacionat amb l'expressió d'òxid nítric, PGE$_2$ (prostaglandina E), TNF-α i IL-1β (Weinstein, Sanghera et al. 1992; Fujihara, Connolly et al. 1994). L'expressió de la fosfatasa MKP-1 pel LPS també depèn de l'activació de PKC de forma similar al M-CSF (Valledor, Xaus et al. 2000).

Per altra banda, l'LPS també indueix l'activació de les ERK-1/2 a través de la via Raf/MEK (Reimann, Buscher et al. 1994). Però els estudis fets indiquen que el patró

d'activació ERK varia depenent de l'estímul. Els agents proliferadors indueixen una activació i desactivació d'ERK ràpida (entre 5 i 30 minuts), mentre que l'activació d'ERK per LPS és als 15 min i s'atenua als 45-60 minuts. La seva activació és doncs, requerida tant per la proliferació dels macròfags (Valledor, Comalada et al. 2000) com en la inducció per LPS de citocines (Sebolt-Leopold 2004).

JNK i p38

Les principals MAPK activades pels estímuls d'estrès i activació són les serina/treonina quinases JNK i p38 (Derijard, Raingeaud et al. 1995). Els mamífers expressen 3 gens de JNK: JNK1, JNK2 i JNK3, però només JNK1 i 2 estan expressats de forma ubiqua. Les JNKs es caracteritzen per fosforilar principalment el factor de transcripció c-Jun (Sanchez, Hughes et al. 1994), però també d'altres com JunB, JunD, ATF-2, Elk-1, NFAT and p53 (Gupta, Campbell et al. 1995; Yang, Mark et al. 1998; Buschmann, Potapova et al. 2001).

La manca de JNK3 no implica un fenotip anormal, però la pèrdua de JNK1 implica l'hiperproliferació de limfòcits T, una menor apoptosi i una diferenciació decantada cap a una resposta Th2 (Dong, Yang et al. 1998). Els KO de JNK1 també presenten protecció a la resistència a la insulina associada a l'obesitat (Hirosumi, Tuncman et al. 2002). L'expressió de JNK2 s'ha vist implicada en la producció d'IFN-γ pels limfòcits Th1 i la seva apoptosi (Sabapathy, Hu et al. 1999). Alguns resultats semblen indicar que JNK1 i JNK2 poden suplir les seves funcions, per contra, la pèrdua tant de JNK1 com de JNK2 resulta mortal a nivell embrionari (Kuan, Yang et al. 1999).

L'LPS també indueix atenuadors de la seva pròpia resposta com l'activació de la via PI3K/Akt (Bellacosa, Testa et al. 1991). Les PI3Ks bloquegen les cascades de les MAPKs i de NF-κB en resposta al LPS, provocant una disminució de la producció del TNF-α. La desregulació de la PI3K s'ha relacionat en la regulació del balanç Th1-Th2 associat en malalties com l'asma, la dermatitis atòpica i al·lèrgies) (Martin, Schifferle et al. 2003).

La producció d'aquests mediadors contribueix al control dels patògens. Alhora, els productes generats poden actuar de forma autocrina (Cheshire and Baldwin 1997). La producció descontrolada d'aquests mediadors pot provocar patologies importants com el shock sèptic.

Inhibidors de l'activació dels macròfags

El TGF-β antagonitza l'activació dels macròfags per IFN-γ, disminuint la producció d'H_2O_2, NO i TNF-α (Ulloa, Doody et al. 1999). El mecanisme pel qual el TGF-β bloqueja

aquesta resposta és la inhibició de l'expressió del CIITA, a través d'Smad3 sense afectar a l'unió d'STAT-1 al ADN, l'expressió de USF-1 o IRF-1 (Werner, Jain et al. 2000). A més a més, el TGF-β inhibeix l'expressió de NOS2 i de citocines inflamatòries induïdes per l'LPS (Imai, Kurokawa et al. 2001). Diferents elements de la matriu extracel·lular, com la decorina, també modula la proliferació i activació dels macròfags revertint els efectes del TGF-β (Comalada, Cardo et al. 2003).

L'adenosina a través de la interacció amb el receptor A2B també inhibeix l'activació dels macròfags augmentant els nivells de cAMP i activant la PKA que inhibeix l'expressió de molècules MHC de classe II, l'expressió de NOS2 i de citocines (Xaus, Valledor et al. 1999). A més a més, el NO produït pels macròfags també pot actuar com inhibidor de la síntesi de classe II inhibint l'expressió del CIITA (Kielar, Sicher et al. 2000).

MORT CEL·LULAR

L'apoptosi o mort cel·lular programada es diferencia de la necrosis, per no produïr el trencament de la membrana ni alliberar el contingut cel·lular al medi, evitant l'activació de processos pro-inflamatoris no desitjats (Sauter, Albert et al. 2000).

Les cèl·lules necròtiques es caracteritzen per un augment del tamany cel·lular, la desorganització citoplasmàtica i finalment l'alliberament de proteases, nucleases i el contingut lisosomal, pel trencament de la membrana cel·lular provocant el reclutament dels macròfags (Proskuryakov, Konoplyannikov et al. 2003).

Per contra, l'apoptosi implica la condensació de la cromatina en el nucli. Es caracteritza per mantenir intactes els orgànuls del citoplasma i presentar fosfolípids a la cara externa de la membrana plasmàtica. Aquests fosfolípids seran reconeguts pels fagòcits a través de receptors de fosfatidil serina, vitronectina i per receptors scavenger (SR) (Fadok, Bratton et al. 2001). Finalment, les cèl·lules apoptòtiques formen cossos apoptòtics, que contenen els orgànuls intracel·lulars i fragments de la cromatina condensada, que són fagocitats pels macròfags (Gregory and Devitt 2004). Les cèl·lules presenten dos vies principals d'inducció de l'apoptosi: 1) la via de receptors de mort extrínseca i 2) la via mitocondrial intrínseca.

Els receptors de mort són membres de la família del receptor de TNF que s'activen per lligands externs (Idriss and Naismith 2000). Alguns d'aquests receptors (Fas receptor/CD95 receptor, TNFR1, DR3, DR4, DR5 i DR6) es caracteritzen per tenir dominis de mort (DD) a l'extrem C terminal intracel·lular a través del qual recluten i activen la cascada de caspases (Sartorius, Schmitz et al. 2001).

El TNF és reconegut per 2 receptors, TNF-R1 (55 kDa) i TNFR2 (75 kDa), tot i que el TNFR1 és el responsable de la major part de la citotoxicitat induïda pel TNFα. Una sèrie de proteïnes citoplasmàtiques generen la resposta cel·lular incloent-hi FADD que activa a la caspasa 8, TRADD que s'uneix a FADD i a RIP que pot activar a NF-κB, a JNK i a la caspasa 9 (Wallach, Varfolomeev et al. 1999). Les caspases són cisteina proteases que s'expressen com a proenzims inactius i s'activen al ser proteolitzats per altres caspases o proteases. La forma activa de l'enzim està constituïda per un heterotetràmer format per 2 subunitats grans i 2 subunitats petites (Riedl and Shi 2004).

Actualment es coneixen fins a 14 caspases que es classifiquen segons la seva especificitat de substrat, extrem N terminal i funció (Fig. 22). Les caspases iniciadores engloben la procaspasa 8 o 10 i són caspases que interaccionen amb molècules adaptadores i poden activar les caspases efectores (caspasa 3, 7, 11, 14) (Shi 2004; Shi 2004).

D'altra banda, la via mitocondrial d'inducció d'apoptosi, implica l'alliberament de citocrom c (Cyt c) i s'SMAC/DIABLO de la membrana mitocondrial en presència d'ATP, els quals participen en l'activació de procaspases 2, 3 i 9 a través de la unió amb Apaf-1 i en l'alliberament del factor inductor d'apoptosi (AIF) que provoca la fragmentació de la cromatina (Khosravi-Far and Esposti 2004; Lorenzo and Susin 2004).

La mort induïda pel receptor del TNF també està modulada a través de FLIP (proteïna inhibidora de FLICE) que s'uneix a FADD evitant l'alliberament de la caspasa 8. Per altra banda, a través de TRAF s'activen els inhibidors de l'apoptosi (IAPs) que bloquegen l'activació de la caspasa 9, 7 i 3 (Nachmias, Ashhab et al. 2004). Per altra banda, l'apoptosi està regulada per la presència de proteïnes anti-apoptòtiques de la família Bcl-2 (Tsujimoto 2003). Aquesta família inclou tant proteïnes proapoptòtiques com Bad, Bax i Bik, així com proteïnes anti-apoptòtiques com Bcl-2 i Bcl-X$_L$ que regulen l'alliberament del citocrom c i la unió d'Apaf a la caspasa 9. El balanç entre les unes i les altres decidirà si una cèl·lula sobreviurà o patirà el procés d'apoptosi (Tsujimoto 2003; Donovan and Cotter 2004; Sheikh and Huang 2004).

L'apoptosi és crucial el molts processos fisiològics com el desenvolupament, la diferenciació, infeccions i tumorogènesis (Rodenburg, Raats et al. 2000). Durant la resposta immunitària també ajuda a erradicar els patògens i eliminar cèl·lules danyades (Samowitz, Curtin et al. 2002). L'apoptosi també és important com a font d'autoantigens per establir tolerància immunològica (Levine and Koh 1999).

IMMUNOSUPRESIÓ

Les immunofilines són peptidyl prolyl cis-trans isomerases (PPIases) implicades en el plegament, unió i tràfec de proteïnes i en el control transcripcional i de processament del pre-ARN en processos de resposta a estrès i d'apoptosi (Schiene-Fischer and Yu 2001).

Els principals lligands de les immunofilines són immunosupresors com la Ciclosporina A (CsA), la Rapamicina i l'FK506 (Ruhlmann and Nordheim 1997). Hi ha 3 famílies de PPIases segons el domini catalític, les ciclofilines (receptors per CsA), FKBP (receptor de FK506 i rapamicina) i parvulines (Taylor, Husi et al. 1997).

La CsA s'uneix principalment a la ciclofilina A, però s'han descrit fins a 15 membres diferents de ciclofilines (Ciclofilina B, C, F, etc..) (Dornan, Taylor et al. 2003). El complexe CsA-Ciclofilina A inhibeix la fosfatasa calcineurina (CaN) regulada per Ca^{2+} i calmodulina, que està implicat en la producció de IL-2 a través de NF-AT en limfòcits T (Clipstone and Crabtree 1992; Resch and Szamel 1997). De fet, també s'ha descrit que independentment de la CsA, la Ciclofilina A pot actuar com a xaperona i agent quimiotàctic al ser secretada per macròfags peritoneals en resposta al LPS (Sherry, Yarlett et al. 1992; Kern, Kern et al. 1994).

Els complexes FK506-FKBP també són capaços d'inhibir l'activitat calcineurina mitjançant diferents mecanismes. Les FKBPs quan estan acomplexades amb la FK506 també poden inhibir l'activitat calcineurina, però els complexes FKBP-Rapamicina inhibeixen la serina/treonina quinasa mTOR sense afectar a la calcineurina (Dumont and Su 1996; Gingras, Raught et al. 2001). D'aquesta manera, la Rapamicina regula el cicle cel·lular de cèl·lules T i B, provocant una aturada en G1 (Li, Li et al. 1999).

La Sanglifehrin A (SfA) és un nou immunosuppressor produït pels *Streptomyces* que s'uneix a la ciclofilina amb una afinitat vint cops superior a la que ho fa la CsA (Fehr, Kallen et al. 1999; Sanglier, Quesniaux et al. 1999; Zenke, Strittmatter et al. 2001; Zhang, Youn et al. 2001). Tot i això, sembla que el complex de ciclofilina-SfA no interacciona amb la calcineurina ni amb la mTOR. D'aquesta manera, s'ha descrit que no interfereixen en la producció de IL-2 pels limfòcits T, tot i que provoca una aturada del cicle cel·lular a G1 (Zenke, Strittmatter et al. 2001; Zhang, Youn et al. 2001; Allen, Zheng et al. 2004). Pel moment també se sap que la SfA pot inhibir la producció de IgG i la producció de TNF-α i IL-12 per monòcits i dendrítiques (Comalada, Valledor et al. 2003; Steinschulte, Taner et al. 2003; Woltman, Schlagwein et al. 2004).