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Novel molecular mediators in human dendritic cells adaptive responses to hypoxia.

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ABSTRACT

Tissue anatomical structure and cell specific energy requirements impair O_2 supply and increase O_2 demand, participating in the establishment of hypoxia (defined as a reduction in O_2 tensions) in several healthy tissues, including immunological niches. Moreover, O_2 shortage is associated with inflammatory areas and with the tumor microenvironment, where immune cells are recruited to exert their functions. Dendritic cells (DCs) are the most efficient antigen presenting cells and they provide a link between innate and adaptive immune responses. Indeed, they exist in as immature cells (iDCs), patrolling peripheral tissues for pathogens detection, and as mature DCs (maDCs), mediating naïve T lymphocyte priming in primary and secondary lymphoid organs. Thus, during their lifespan DCs are frequently exposed to different and lower O_2 tensions, such as in immunological niches, where pO_2 ranges between 10 and 40 mmHg against 100 mmHg of arterial blood. Hypoxia represent a stressful condition for several cell types, including DCs, and evolution has led to the development of cellular and systemic strategies to cope with it. Most of them are mediated by the Hypoxia-inducible factors (HIFs), but in last decades other cellular processes and molecular mediators of cell adaptation to hypoxia have been identified, including PI3Ks, Erk1/2 signaling and autophagy. We here investigated whether p62, a multifunctional protein that acts as a selective autophagy receptor, and Vps34, the only member of Class III PI3K classically associated with the autophagic process, could be involved in hypoxia-induced adaptative responses, in either iDCs or maDCs, especially in cell survival. To this end, we used several chemical compounds and siRNAs and a combination of Western blot, RT-qPCR, autophagy and viability assays. We observed that p62 inhibition, by RNA interference approach, resulted in an impaired hypoxic iDC viability by affecting the Erk1/2 pro-survival program. In addition, SAR405, a potent and selective Vps34 inhibitor, was able to dysregulate autophagy in maDCs, not only for their survival but also for the autophagic-dependent dampening of inflammation.

In addition, we evaluated whether RNASET2, a multifunctional ribonuclease, which also affects cell survival, could be involved in DCs responses to the hypoxic stress. We found out that prolonged hypoxic exposure significantly enhanced its expression along with the impairment of DC viability. Furthermore, RNASET2 upregulation was inhibited by LPS stimulation, which is known to exert not only DC final maturation but also a pro-survival function via activation of the PI3K/Akt pathway.

1. INTRODUCTION.

1.1 OXYGEN HOMEOSTASIS AND HYPOXIA IN HEALTH AND DISEASE.

Molecular oxygen (O_2) is the fuel of life: it is required for development, growth and survival of several prokaryotic and eukaryotic living organisms (Giaccia et al., 2004). Indeed, metabolic evolution has led to oxidative phosphorylation, which uses atmospheric O_2 as the final electron acceptor for ATP generation. Since its crucial role in maintaining an efficient metabolism, living organisms have developed several strategies to sense O_2 levels in the atmosphere and to adapt to changes in gas tension levels, maintaining tissue and cell homeostasis (Nakazawa et al., 2016).
Atmospheric air at sea level has a partial oxygen pressure (pO_2) of about 159 mmHg (21%).
In the human body, pulmonary respiration provides O_2 intake and gas exchange; indeed, after entering the lungs, where the pO_2 is about 150 mmHg, O_2 undergoes diffusion across the alveolar-capillary barrier, moving from the alveoli to the arterial circulation, where the pO_2 drops to 100 mmHg (Ortiz-Prado et al., 2019). Then, blood circulation provides O_2 delivery to peripheral tissues (Giaccia et al., 2004).
While O_2 is essential for cellular reactions common to different human cell types, the required levels of O_2 may vary among cells and tissues, depending on their energy requirements (Carreau et al., 2011; Nikinmaa, 1997). Indeed, the pO_2 in tissues is strictly dependent on the diffusion gradient between the vascular network, which provides O_2 delivery, and cells which represent the O_2 consumption site (Ortiz-Prado et al., 2010). Therefore, the reduction of O_2 availability, known as hypoxia, is a common feature in several human body districts and characterizes physiological processes (Semenza, 2020) (Figure 1). This condition occurs when O_2 demand exceeds supply (Cummins et al., 2016; MacIntyre, 2014) and it can result from various causes, including lower arterial blood oxygenation, reduction in oxygen-carrying capacity, tissue-specific structure and vascular network organization. Also external factors, such as altitude, latitude, temperature and barometric pressure, can influence the tissue pO_2 (Ortiz-Prado et al., 2019).
Although hypoxia is a physiological condition experienced by many cell types, it often characterizes pathological conditions, including inflammatory and autoimmune diseases (e.g. diabetes), ischemic disorders and tumors (Brahimi-Horn & Pouysségur, 2007).
The Hypoxia inducible factors (HIFs), and in particular HIF-1, are the master regulators of O_2 homeostasis and they are major mediators of responses to O_2 tension changes (Semenza, 2001) (HIF-1 regulation and functions are deeply discussed below).

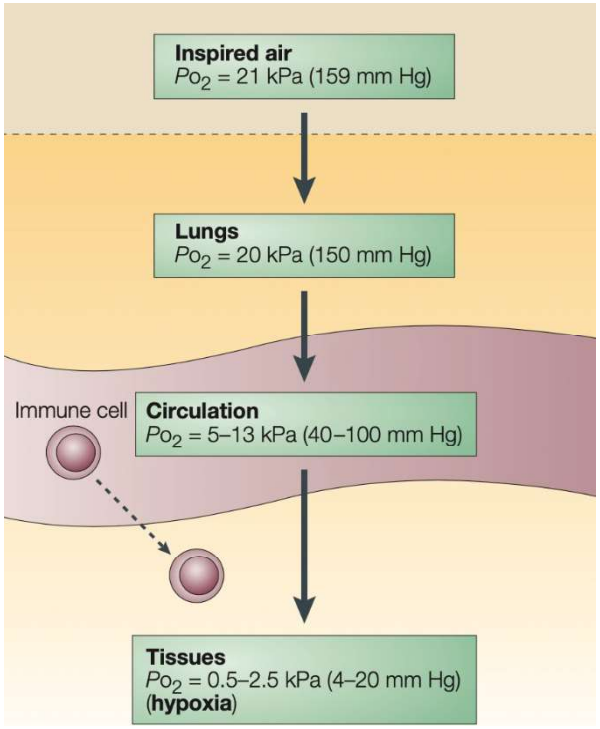


Figure 1. O_2 from inspired air to tissue hypoxia. pO_2 in lungs, circulation and peripheral tissues. From (Sitkovsky & Lukashev, 2005).

1.1.1 Physiological hypoxia.

Tissues vary in the organization of their vascular network, which has a major impact on their oxygenation levels. In healthy tissues, the pO_2 gradually decreases with the increasing distance to the nearest capillary (Simon & Keith, 2008; Sitkovsky & Lukashev, 2005).

Since their anatomical structure, immunological niches are characterized by tissue hypoxia. In particular, the pO_2 is ~10 mmHg in thymus, ~16 mmHg in spleen, and it ranges from 0 to 30 mmHg in bone marrow and from 16 to 40 mmHg in lymph nodes (Eltzschig & Carmeliet, 2011; Harrison et al., 2002; Huang et al., 2007). This condition influences innate and adaptive immunity by modulating immune cell proliferation, development and effector function, largely via transcriptional changes driven by HIFs (Taylor & Colgan, 2017).

In addition to its impact in immune regulation, hypoxia in bone marrow contributes to set up a special microenvironment for hematopoietic stem cells. Indeed, the hypoxic niche plays a crucial role in modulating self-renewal, quiescence, apoptosis, differentiation and immune privilege of hematopoietic stem cells (Zhang & Sadek, 2014). Moreover, HIF-1 and its target genes, Vascular endothelial growth factor-A (VEGFA) and erythropoietin (EPO), has been associated to hematopoietic stem cell maintenance in bone marrow and to inhibition of Reactive Oxygen Species (ROS)-induced apoptosis in the same cell population (Wang et al., 2022).

Among other physiological processes, which occur in hypoxic microenvironments, there is the embryonic development. Indeed, mammalian development takes place in a relatively low-oxygen (3% O_2) environment before the establishment of the circulatory system (Rodesch et al., 1992). In this context, hypoxia modulates Notch, WNT and other regulators of stem cell functions, has a major impact on mesenchymal stem cell survival and differentiation, and is involved in embryonic stem cell pluripotency maintenance (Imanirad & Dzierzak, 2013; Simon & Keith, 2008). In addition, hypoxia plays a crucial role in placental development: in early pregnancy, the uterine surface experiences low O_2 levels, since the occlusion of uterine spiral arterioles. This condition promotes proliferation of cytotrophoblasts from the embryo, which then invade into maternal spiral arterioles, leading to the establishment of the utero-placental circulation (Patel et al., 2010; Simon & Keith, 2008).

Although hypoxia physiologically characterized these tissues and processes, it may often be perceived as a stress by many cell types. For this reason, evolution has led to the development of several cellular adaptive strategies, ensuring cell survival maintenance even under O_2 deprivation, which are discussed below.

Other physiological situations, in which the human body experiences hypoxia, are represented by high-altitude travel and intense physical exercise. Indeed, with increasing altitude, barometric pressure decreases, even though the O_2 percentage remains constant (21%). This decrease in pressure results in pO_2 lowering. People traveling to high altitudes (>2,500 m elevation) commonly face the risk of high-altitude illnesses, exhibiting symptoms such as headache, nausea, fatigue, and gastrointestinal issues, which results from hypoxia-induced inflammation, vasogenic edema, and acidosis (Pham et al., 2021).

Concerning intense physical exercise, it requires a remarkable increase in O_2 consumption, at tissue level. To deal with this condition and to ensure the O_2 delivery to the active tissues, cardiopulmonary demand increases. In individuals with normal respiratory function, the O_2 supply to tissues is regulated by adjusting cardiac output and, simultaneously, by increasing pulmonary ventilation, to prevent decreases in arterial O_2 content (Garvey et al., 2012).

1.1.2 Hypoxia in physio-pathological conditions.

Hypoxia is strictly associated with inflammatory processes; indeed, tissue inflammation frequently leads to blood vessel damage or obstruction by phagocytes and, thus, to the impairment in blood supply and O_2 delivery (Sitkovsky & Lukashev, 2005). Other factors, which contribute to hypoxia establishment in inflamed areas, are thrombosis, interstitial hypertension and atelectasis (Eltzschig & Carmeliet, 2011). Moreover, infiltrating immune cells, such as neutrophils and macrophages, undergo metabolic shift and remarkably increase energy consumption in inflammatory sites (Taylor & Colgan,

2017): nutrient and O_2 exhaustion by these cells contribute not only to exacerbation of hypoxia but also to acidosis, lactate accumulation and hypoglycemia. An example of this condition is represented by gastrointestinal inflammation, which is typical of the Inflammatory bowel disease (IBD). Indeed, in IBD, exacerbation of O_2 shortage (gastrointestinal mucosa is physiologically hypoxic) occurs thanks to the interplay of several factors. In particular, in this condition, innate immune cells use O_2 in order to fuel cell migration toward the lesion site and to produce ROS, including superoxide anion and hydrogen peroxide, which play a pivotal role in microbicidal activity within phagocytic cells. In addition, lymphocytes contribute to O_2 depletion in IBD inflamed tissues, since they improve their proliferation rate and therefore are required to increase oxidative phosphorylation (Glover & Colgan, 2011).

Hypoxia characterizes also the tumor microenvironment (TME), where the high proliferation and growth rate of cancer cells is supported by enhanced energy consumption and therefore it is related to an increased O_2 demand (Chen et al., 2023; Zhao et al., 2017). In addition, the rapid growth of solid tumors is paralleled by an impairment in O_2 supply, due to the insufficient or functionally abnormal vascular network (Baluk et al., 2005; Husain et al., 2022; Jain, 2001). Taken together, these conditions play a crucial role in the establishment of hypoxia in TME.

The hypoxic condition in TME has a double effect on cancer cells: it can trigger apoptosis or necrosis, leading to the formation of necrotic areas within the tumor mass, especially in the central core which is characterized by severe O_2 and glucose deprivation (Brahimi-Horn & Pouysségur, 2007), or it can induce mutations or the activation of adaptive strategies, including metabolic reprogramming, which promote cell proliferation, actively contributing to tumor progression (Zhou et al., 2006), and angiogenesis. This dysregulated angiogenesis, which is mediated by the HIFs/VEGFA signaling pathway, leads to the formation of disordered, leaky and inefficiently perfused blood vessels (Brahimi-Horn & Pouysségur, 2007; Semenza, 2000).

Furthermore, hypoxia and HIFs are linked to the enhancement of tumor invasiveness and metastatic potential, by promoting depletion of E-cadherins, proteins located in adherent junctions (Sullivan & Graham, 2007), by inducing TWIST1, which is involved in epithelial mesenchymal transition (Petrella et al., 2005), and by upregulating metalloproteases, which enhance matrix remodeling (Brahimi-Horn & Pouysségur, 2007; Yang et al., 2006).

Therefore, tumor progression, invasiveness and metastatic potential are increased by hypoxia. All these processes are fueled by a metabolic shift, which is known as Warburg effect, and are tightly related to the impairment of the pH in TME. In this contest, Carbonic Anhydrases (CAs) play a pivotal role, since they catalyze the reversible hydration of carbon dioxide to bicarbonate, thus participating in cancer cell survival and tumor progression. Among CAs, CAIX and CAXII have been associated

to tumor hypoxia (Chiche et al., 2010) and, recently, Venturella et al. and Giuntini et al., have investigated their role in two different melanoma cell lines (SK-MEL-28 and A375). In particular, Venturella et al. have demonstrated that melanoma cells release CAIX-carrying sEVs (small extracellular vesicles) only under hypoxic conditions. In the same hypoxic context, Giuntini et al., reported that the Hedgehog pathway is tightly related to CAXII expression and function in melanoma cells, and that the crosstalk between them plays a crucial role in melanoma migration and invasion (Giuntini et al., 2022; Venturella et al., 2023).

1.1.3 The hypoxia inducible factor 1.

Although hypoxia is common feature in a great variety of physiological conditions, it represents a significant stress for cells and tissues. Indeed, it can induce programmed cell death and tissue damage (Martin et al., 2010). However, evolution has led to the development of several cellular (e.g. metabolic reprogramming) and systemic (e.g. erythropoiesis and neo angiogenesis) adaptive strategies to restore O_2 homeostasis and promote cell survival, which are mainly mediated by the HIFs (Maxwell, 2005; Semenza, 2012; Weidemann & Johnson, 2008).

Three HIF isoforms have been identified in mammals: HIF-1, HIF-2 and HIF-3. They are heterodimeric transcription factors, which consist of a β subunit, also known as aryl-hydrocarbon receptor nuclear translator (ARNT), constitutively active in all cells, even under normoxic conditions, and in an oxygen-labile α subunit. While HIF-1α is ubiquitously expressed in all cells, HIF-2α and HIF-3α have a tissue specific expression (e.g. vascular endothelium). Moreover, HIF-3α, the most recently identified member of the HIF family, exists in different splicing variants and often acts as an antagonist of the other two isoforms, which, instead, are structurally similar (Bao et al., 2021).

HIF-1 was discovered by Gregg Semenza in 1992, while he was studying EPO (Semenza & Wang, 1992) and it is the master regulator of O_2 homeostasis since it promotes the expression of more than 100 genes implementing metabolic, functional and vascular adaptation to O_2 deprivation (Palazon et al., 2014).

The activity of this transcription factor is inhibited under normoxia by the prolyl hydroxylases (PHDs), PHD1, PHD2 and PHD3, and by the factor inhibiting HIF (FIH). In particular, PHDs utilizes O_2 as a substrate to hydroxylate HIF-1α at Pro402 and Pro564 residues of the ODD domain (Giaccia et al., 2004). This step is required for the Von Hippel-Lindau protein (pVHL)-mediated polyubiquitination of HIF-1α which leads to its rapid proteasomal degradation under normoxic conditions (Haase, 2009; Koh et al., 2008). Furthermore, under normoxia FIH hydroxylates HIF-1α

asparaginyl residues, preventing its interaction with co-activators p300/CBP (Zhang et al., 2010) (Figure 2a).

Under hypoxia, PHDs and FIH activity, which depends on O_2 availability, is inhibited and HIF-1α can accumulate, translocate into the nucleus and interact with HIF-1β and p300/CBP. Then dimeric HIF-1 induces the transcription of target genes, by binding the Hypoxia Responsive Elements (HREs) in the promoter region, therefore mediating a great variety of intracellular and systemic responses to hypoxia (Sharma et al., 2022) (Figure 2b).

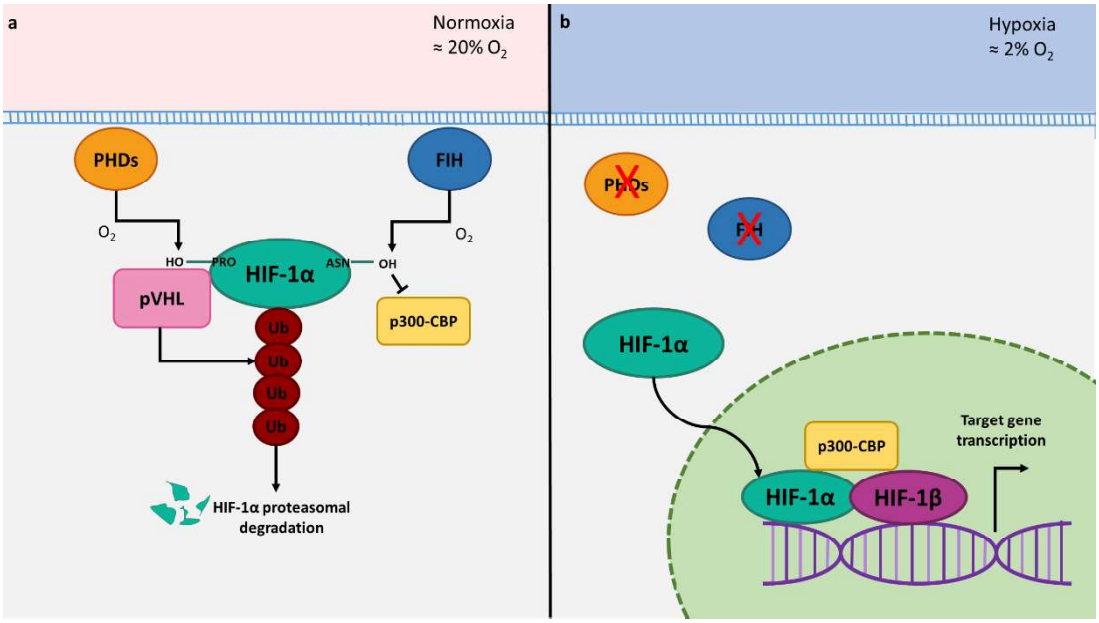


Figure 2. Regulation of HIF-1α: (a) under normoxic condition HIF-1α is hydroxylated and then degreaded by proteasome; (b) under hypoxia HIF-1α can migrate into the nucleus, where it interacts with its binding partners, regulating target gene expression.

The transcriptionally active HIF-1 can elicit proliferation and survival, apoptosis, and metabolic shift from oxidative to glycolytic pathways (Schofield & Ratcliffe, 2004; Semenza, 2003), or induce tissue specific adaptive processes (e.g. EPO production by interstitial renal fibroblasts and hepatocytes) (Haase, 2013) (Figure 3).

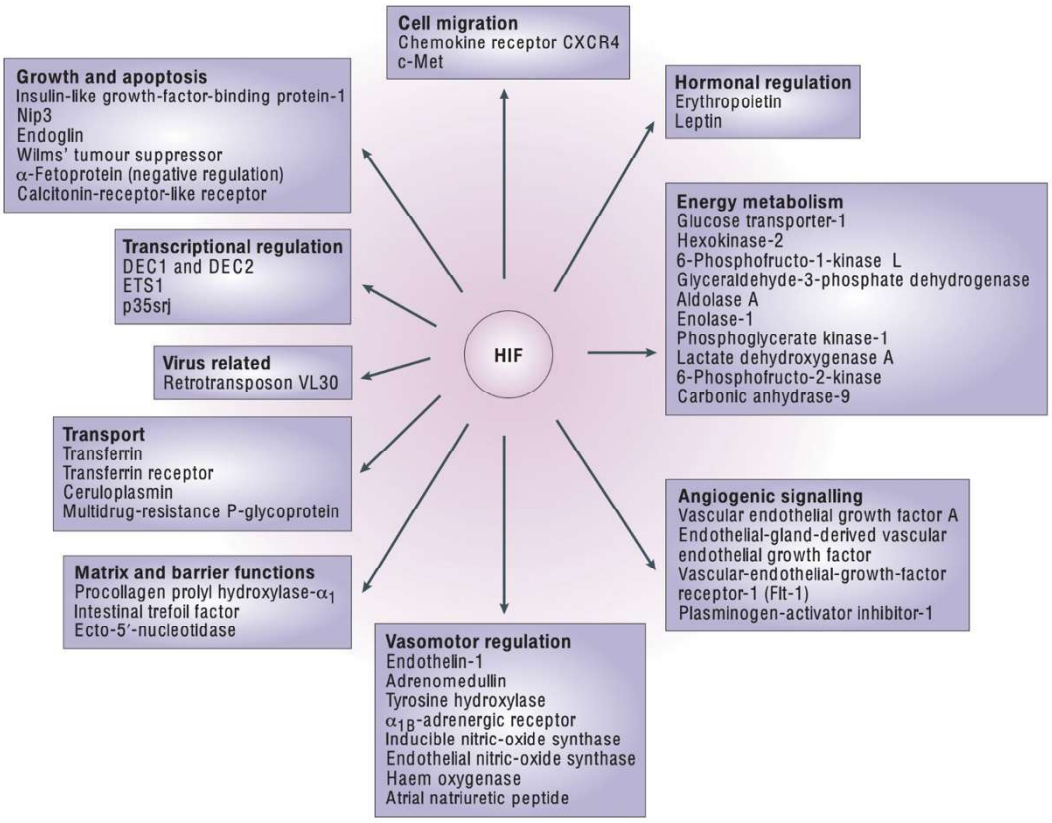


Figure 3. HIF targets and effector functions. HIFs modulates a great variety of cellular and systemic adaptive responses, by inducing the expression of its target genes. From (Schofield & Ratcliffe, 2004).

1.1.4 HIF-1 in immunity and inflammation.

Innate and adaptive immune cells are required to rapidly adapt to O_2 tension changes, since they move across different tissue microenvironments including blood circulation, where the pO_2 is significantly higher, immunological niches and inflammatory areas, which are instead hypoxic.

The correlation between immunity, inflammation and hypoxia is bidirectional: not only inflammation promotes local or systemic hypoxia and the recruitment of immune cells, but also hypoxia is able to exacerbate inflammation and modulate immune cell fate and functions (Watts & Walmsley, 2019).

This crosstalk is mainly mediated by HIF-1α, which stabilization, within immune cells, can be triggered by hypoxia, but it can also be elicited in an O_2 independent manner (e.g. by inflammation, bacterial infections and cancer). Furthermore, HIFs are involved in immune cell development and in the modulation of immune responses (Palazon et al., 2014).

Several studies have reported that bacterial infections are able to induce HIF-1α expression in macrophages independently from hypoxia. Indeed, Hartmann et al., have shown that Enterobacteriaceae trigger HIF-1 activation by repressing PHD-mediated HIF-1α degradation

(Hartmann et al., 2008). Furthermore, Lipopolysaccharide (LPS) from Gram-negative bacteria promotes HIF-1α accumulation in macrophages (Blouin et al., 2004) and dendritic cells (DCs) (Monaci et al., 2020) even under normoxia. Indeed, HIF-1α is required for DC metabolic shift, which occurs upon LPS stimulation, promoting cell survival, maturation, migration to draining lymph nodes and T cell activation (Guak et al., 2018; McGettrick & O'Neill, 2020).

Interestingly, HIF-1α deletion has been shown to induce metabolic defects in myeloid cells, leading to the impairment of innate immune responses. In particular, HIF-1α abolition in macrophages is associated with the impairment of aggregation, motility, bacterial killing and cytokine release.

Concerning neutrophils, HIF-1α accumulation promotes cell survival, delays apoptosis thanks to the crosstalk with the nuclear factor kB (NF-kB) (Palazon et al., 2014). In addition, it enhances neutrophil survival and extracellular traps (NET) release (McGettrick & O'Neill, 2020). However, excessive and inappropriate HIF-1 activity in neutrophils results in prolonged inflammation and tissue damage (Watts & Walmsley, 2019).

Moreover, HIF-1 is able to regulate T lymphocyte differentiation and function, under either normoxia or hypoxia. Different T cell subsets show different HIF-1α expression levels: lowest HIF-1α levels have been reported during the differentiation process into Treg (regulatory T cells), while higher levels have been detected in T lymphocytes undergoing Th17 differentiation, depending on STAT3 activity. The STAT3 involvement in HIF-1α expression has been described also in Th1 cells. Concerning cytotoxic T cells, following TCR stimulation, HIF-1 elicits metabolic reprogramming, implementing the effector functions (McGettrick & O'Neill, 2020).

HIF-dependent glycolytic switch is crucial also for B lymphocyte functions; indeed, it is required for B cell development, IgG class switching and IL-10 production (Taylor & Scholz, 2022).

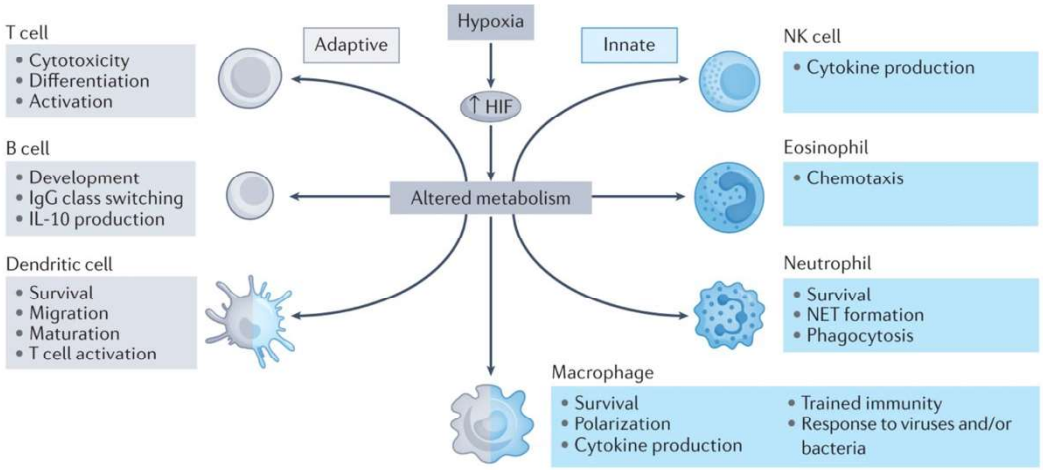


Figure 4. HIF in immune cells. HIF-dependent metabolic shift in innate and adaptive immune cells promotes cell survival and function maintenance. From (Taylor & Scholz, 2022)

1.1.5 The extracellular regulated kinase (Erk)-1/2 and cell survival under hypoxia.

Mitogen activated kinases (MAPKs) are a family of enzymes which mediate the transduction of extracellular signals to nuclear or cytoplasmic effectors, through sequential phosphorylation events. The first enzyme that triggers this activation cascade is the MAPK kinase kinase (MAPKKK), responsible for the phosphorylation of the MAPK kinase (MAPKK), which in turn leads to MAPK activation. Several MAPK signaling modules regulate mammalian cell fate and functions, including c-Jun N-terminal kinase (JNK), p38 and extracellular regulated kinase (Erk) pathways (Jin et al., 2002). The last one mediates a great variety of cellular processes, including cell survival, growth, differentiation and proliferation. Indeed, several Erk family members, with different roles, have been identified in mammals, including Erk 1/2 and Erks 3 to 8 (Bogoyevitch & Court, 2004; Kant et al., 2006).

Erk 1/2 activation relies on the phosphorylation, by MEK1/2, of Thr185 and Tyr187 residues which triggers a conformational change, promoting substrate binding and unmasking catalytic residues (Lavoie et al., 2020). Once activated, Erk 1/2 can exert several functions, by phosphorylating a great variety of substrates, including membrane proteins, such as CD120a, Syk, and calnexin, nuclear substrates, such as SRC-1, Pax6, NF-AT, Elk-1, MEF2, c-Fos, c-Myc, STAT3, and cytoskeletal proteins (Roux & Blenis, 2004). Beside its role in controlling cell growth, differentiation and proliferation, it positively regulates cell survival through the impairment of the intrinsic apoptotic pathway (Figure 5). Indeed, it is able to phosphorylate procaspase 9, preventing its processing and activation (Allan et al., 2003; Lavoie et al., 2020). Moreover, Erk 1/2 acts as an inhibitor of the BH3-only-protein BIM, downregulating its expression and inducing its dissociation form anti-apoptotic factors, such as BCL-xl and MCL1. In addition, it suppresses the pro-apoptotic activity of other factors, including BIK, which is degraded by proteasome immediately after Erk 1/2 phosphorylation, and BAD. Indeed, Erk-activated RSK (ribosomal protein S6 kinase) inhibits BAD interaction with BCL-xl and BCL-2, by inducing its translocation from the mitochondrial membrane to the cytosol, and thus enhances BCL-xl and BCL-2 anti-apoptotic activity (Lavoie et al., 2020).

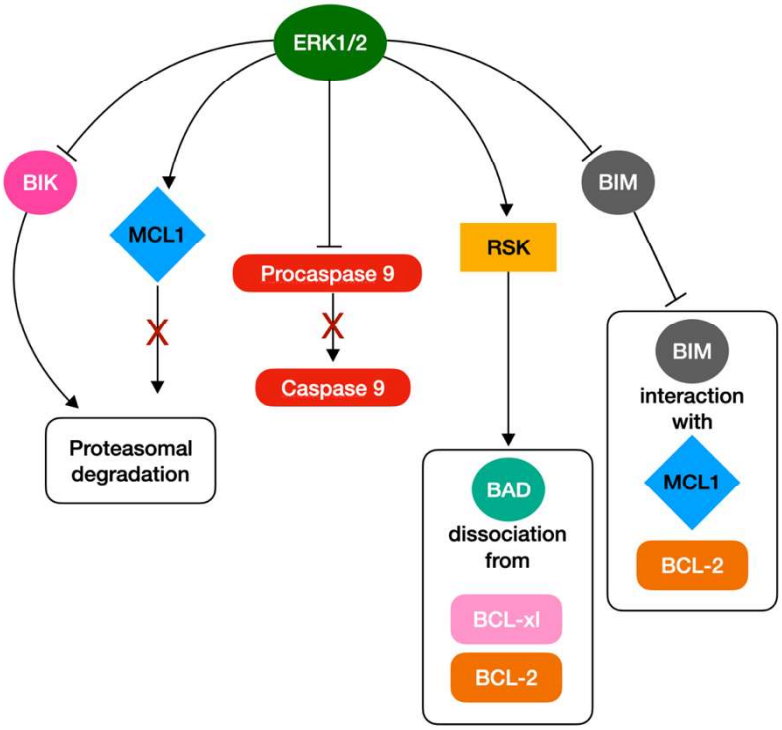


Figure 5. Erk1/2 regulation of cell survival. Erk1/2 promotes cell survival by phosphorylating several downstream molecules.

Interestingly, Erk 1/2 has been shown to exert a crucial role in protecting several cell types from hypoxia-induced cell death. Indeed, Jin et al., have demonstrated that hypoxia triggers the activation of MEK/Erk 1/2 pathways in cortical neuron cultures, which than leads to phospho-inactivation of BAD (Jin et al., 2002). The same Erk-dependent BAD inhibition was reported by Härtel et al., in response to transient hypoxia, in three different endothelial cell cultures. In addition, they showed that Erk 2 activation and BAD phosphorylation result in the maintenance of mitochondrial membrane integrity, reducing caspase 3 activation and promoting cell survival. Moreover, they found that PD98059 and U0126 (chemical compounds inhibiting MEK/Erk 1/2 pathway) are able to revert this effect, by downregulating Erk 1/2 phosphorylation and the consequent BAD inactivation (Härtel et al., 2010).

PD98059 has been shown to prevent also HIF-1 transcriptional activity in human microvascular endothelial cells-1 under hypoxia. Indeed, in their work, Minet et al. showed that Erk 1/2 is upregulated under hypoxia and that HIF-1α is phosphorylated in an Erk-dependent manner (Minet et al., 2000).

All these findings highlight the involvement of Erk 1/2 in HIF-1 transcriptional program induction and in cell survival maintenance in response to hypoxia.

1.2 PI3Ks INVOLVEMENT IN CELL SURVIVAL AND AUTOPHAGY.

Membrane lipid phosphoinositides are crucial mediators of a wide range of signaling pathways, since they act as second messengers, and their levels can change upon activation of cell surface receptors. Indeed, they are generated from phosphatidylinositol (PI) by the activity of phosphoinositide kinases, which leads to the formation of seven phosphoinositide species: three mono-phosphorylated phosphatidylinositol phosphates (PIPs), three bis-phosphorylated (PIP_2s) and a tris-phosphorylated (PIP_3) (Burke et al., 2023).

Phosphoinositide kinases are organized in three general families: Phosphoinositide 3 kinases (PI3Ks), phosphatidylinositol 4 kinases (PI4Ks) and PIP kinases (PIPKs) (Fruman et al., 1998).

PI3K family regulates a great variety of cellular processes, including membrane trafficking, cell survival, autophagy and metabolic programs. Three PI3K classes (I, II and III), with non-redundant functions, have been described. All three PI3K classes phosphorylate the 3′-position hydroxyl of the D-myo-inositol head group to generate specific phosphoinositide forms. PI3Ks are characterized by the 'PI3K signature motif', which includes a N-terminal C2 domain, involved in membrane binding, a central helical domain and a C-terminal catalytic domain (Jean & Kiger, 2014).

1.2.1 Class I PI3K/Akt signaling: the master regulator of cell survival.

Class I PI3K comprises several heterodimeric isoforms, formed upon the assembly of one of the four catalytic subunits (p110α, β, δ or γ) with a regulatory subunit (p85α, or its variants p55α and p50α, p85β, p55γ, p101 and p84). Class I PI3Ks are involved in RTKs (receptors with protein tyrosine kinase activity) and GPCRs (G protein-coupled receptors) signal transduction. Indeed, they catalyze the conversion of $PI(4,5)P_2$ to $PI(3,4,5)P_3$, which acts as a second messenger and is then recognized and bound by proteins, with FYVE or plekstrin homology (PH) domains, such as Akt (Osaki et al., 2004; Vanhaesebroeck et al., 2010).

Akt is a serine/threonine kinase, also known as PKB, involved in cell metabolism and survival and in cell cycle progression. Three Akt isoforms have been identified in mammals: Akt1 (or PKBα), Akt2 (or PKBβ) and Akt3 (or PKBγ), each characterized by a N-terminal PH domain, a central catalytic domain, responsible for the kinase activity, and a regulatory domain in the C-terminal region (Osaki et al., 2004).

PI3K/Akt signaling activation consists of a multistep process, which starts with PIP_3 generation, upon Class I PI3K activation. Then, PIP_3 accumulation induces Akt recruitment at plasma membrane level. The interaction of Akt amino-terminal PH domain with PIP_3, triggers a conformational change, which unmasks the T-loop of the Akt catalytic core; T-loop contains the T308 residue, that is phosphorylated by PDK1, leading to Akt activation. A second phosphorylation (of S473 in the C-terminal regulatory domain), which is required for Akt maximal activation, is then triggered by mechanistic target of rapamycin (mTOR) complex 2 (mTORC2) (Manning & Toker, 2017).

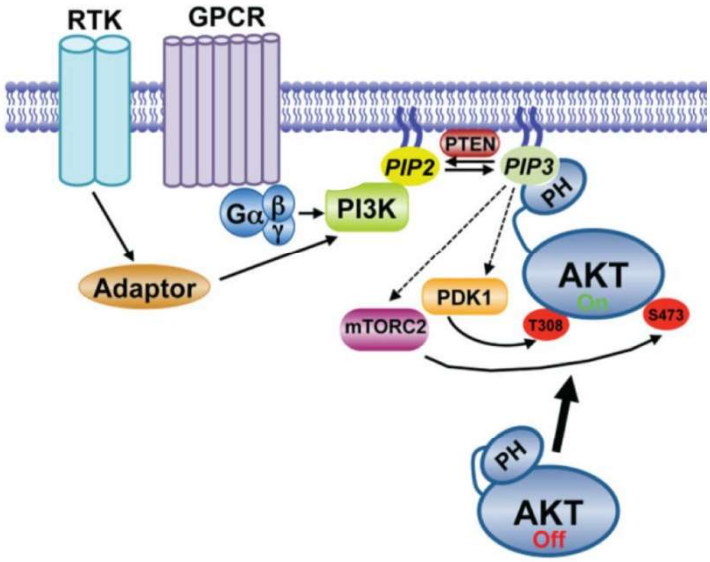


Figure 6. Akt activation. PIP_3-mediated Akt recruitment leads to its activation by PDK1- and mTORC2-mediated phosphorylation. Adapted from (Manning & Toker, 2017).

Upon activation, Akt is able to phosphorylate several substrates and to promote cell survival by acting at three different levels: transcriptional control, metabolic control and direct regulation of cell survival. The first one relies on Akt ability to control transcription factors involved in the expression of either pro- or anti-apoptotic mediators. Indeed, Akt has been shown to inhibit FoxO proteins, reducing the expression of their target genes (which include BIM and Fas ligand), and to enhance NF-kB and CREB transcriptional activity, leading to an increase in the expression of pro-survival factors such as BCL-xl, BCL-2 and MCL1 (Song et al., 2005).

Concerning metabolic regulation, Akt is able to inhibit both GSK3α and GSK3β, by direct phosphorylation. GSK3s negatively regulate (directly) metabolic enzymes, such as glycogen synthase, and (indirectly) transcription factors associated with metabolic programs (including HIF-1) as well as the stability of pro-survival BCL-2 family members, such as c-Myc and MCL1, inducing

their proteasomal degradation. So, Akt-dependent GSK3 inhibition, has been associated with restored glycogen synthesis and c-Myc / MCL1 activity (Manning & Toker, 2017).
Regarding direct regulation of cell survival, Akt phosphorylates BAD, at Ser136 level (Datta et al., 1997), triggering its dissociation form BCL-2 and BCL-xL, and therefore promoting the anti-apoptotic function of these two factors, and Caspase 9, at Ser196, reducing its pro-apoptotic activity (Song et al., 2005).

1.2.2 Class III PI3K and initiation of autophagy.

Autophagy is a catabolic process which provides clearance and turnover of cellular components. The name autophagy, which derives from the Greek words *auto*, meaning 'self', and *phagein*, meaning 'to eat', refers to the ability of cells to "self-eat" their components: cells select, degrade and recycle their own macromolecules and senescent organelles (which in this process are referred to as cargo) (Dikic & Elazar, 2018).
Autophagy is active at basal level in most eukaryotic cells, since it has a pivotal role in maintaining cellular homeostasis, which mainly relies on its ability to sustain an efficient metabolism and to prevent harmful components, misfolded proteins and damaged organelles accumulation. Furthermore, it can support the cell capacity to respond to environmental changes. Indeed, beside basal autophagy, the autophagic flux can be induced and/or enhanced by a great variety of stimuli, including stress conditions, such as nutrient starvation and pO_2 reduction, and signals which regulate cell survival, growth, differentiation and functions. Stress-induced autophagy is considered a double edge sword: it may either allow cells to survive during nutrient depletion, hypoxia and growth factor deficiency, or act as a mode of programmed cell death (Codogno & Meijer, 2005). However, as a matter of fact, autophagy is essential for functional maintenance of normal tissues and alterations in autophagic pathways and flux have been associated with cellular senescence and with the onset of cancer and neurodegenerative disorders (Aman et al., 2021).
Three major types of autophagy, which differ in their regulation, the nature of the cargo and the cargo trafficking, have been described: microautophagy, which relies on the ability of endosomes and lysosomes to modify their membranes (e.g. via invagination) in order to surround and capture the cargo; chaperone-mediated autophagy (CMA), in which Lamp-2A, Hsc70 and cochaperones directly mediate the translocation of substrates into lysosomal lumen; macroautophagy, which is instead mediated by specific organelles, named autophagosomes, and by ATGs (autophagy related genes) (Macian, 2019; Mizushima & Klionsky, 2007).
Macroautophagy (autophagy from now on) initiation is regulated by the vacuolar protein sorting 34 (Vps34), which is the only member of Class III PI3K and which catalyzes the conversion of PI in

PI(3)P, a phospholipid crucial for membrane trafficking and vesicle formation (Lindmo & Stenmark, 2006).

Vps34 has been shown to form two heterotetrameric complexes. Complex I is mainly involved in autophagy and forms upon interaction of the catalytic subunit Vps34 with the putative kinase Vps15, which acts as a regulatory subunit, Beclin1 and ATG14L, which is the sensor of membrane curvature. Instead, complex II, which is composed of Vps34, Vps15, Beclin1 and UVRAG (UV irradiation resistance-associated gene), is a master regulator of endocytic pathways and exerts a crucial role in endosome trafficking, lysosome recycling and LC3-associated phagocytosis (Ohashi et al., 2019).

Concerning complex I, it is involved in the first steps of the autophagic flux, since generation of PI(3)P at the phagophore formation site is a critical early event in autophagosome formation (Russell et al., 2013). The upstream events that regulate Vps34 complex I recruitment and activation, involve mTORC1 and ULK1. In particular, in the presence of nutrients, active mTORC1 acts as a potent autophagy repressor, inhibiting ULK1 kinase activity by direct phosphorylation of Ser757. Instead, the repression of mTORC1, induced by starvation or other inhibitory mechanisms, leads to ULK1 auto-phosphorylation and trans-phosphorylation of binding partners, ATG13L and FIP200, and therefore to activation of the kinase complex (Russell et al., 2014). Then, activated ULK1 kinase complex recruits, in the phagophore assembly site (PAS), Vps34 complex I by phosphorylating it subunits (Mercer et al., 2021; Ohashi, 2021), including Beclin1, which is essential also for proper complex I activation (Russell et al., 2013).

Once in the PAS, Vps34 induces PI(3)P increase in the isolation membrane, the first membranous structure that later gives rise to the autophagosome. Then, PI(3)P recruits proteins containing a FYVE, PX or WD40 domain, such as WIPI 1 and 2 and AMBRA1 (activating molecule in Beclin1-regulated autophagy 1), involved in autophagosome formation. So, Vps34 Complex I activation is required for autophagy induction. This was confirmed by specific inhibition of this kinase with a recently discovered molecule, SAR405. In fact, Ronan et al., showed that SAR405-dependent Vps34 inhibition was able to completely prevent starvation and rapamycin induced autophagy, along with the impairment of late endosome trafficking and lysosomal function (Ronan et al., 2014).

Moreover, the autophagy-specific Vps34 complex I is regulated by several interactors, including the Beclin1 activator AMBRA1, as well as the Beclin1 inhibitor BCL-2, which also interacts with ATG12 (Galluzzi et al., 2017).

1.2.3 Autophagosome formation and cargo degradation.

The isolation membrane, also known as phagophore, is a double membrane structure, which then elongates providing complete cargo sequestration and giving rise to the autophagosome. The expansion of the phagophore is characterized by two ubiquitination-like conjugation systems. The first one concerns the ATG12-ATG5-ATG16L1 complex, which prevents premature fusion between the rising autophagosome and the lysosome. It forms upon covalent conjugation of ATG12 to ATG5 in a process dependent on E1 activating enzyme, ATG7, and E2 conjugating enzyme, ATG10. Then, ATG16L1 noncovalently binds ATG12-ATG5, directly interacting with ATG5, and leads to the formation of a dimeric complex, which associates to the phagophore membrane. Complex formation is crucial for phagophore curvature and may be inhibited by either ATG5, ATG7 and ATG12 acetylation or interaction of RAB33A with ATG16L1 (Parzych & Klionsky, 2014).

The second ubiquitination-like system concerns LC3B (microtubule-associated protein light chain 3 β) processing. Firstly, LC3B is cleaved by ATG4 to expose a C-terminal glycine residue. Then, ATG7 and the E2-like enzyme, ATG3, catalyzes the conversion of LC3B-I to LC3B-II, by inducing the conjugation of the cytosolic LC3B-I to phosphatidylethanolamine (PE). LC3B-II is recruited, by the phagophore during elongation process, in both the exterior and the luminal side of the vesicle, and it is detectable not only in preautophagosomes and in autophagosomes, but also in autolysosomes. Indeed, it participates in the elongation of the isolation membrane and in cargo sequestration, via interaction with the selective autophagy receptor sequestosome-1 (SQSTM-1/p62). Moreover, it exerts a crucial role in autophagosome closure and trafficking, required for fusion with the lysosome, by interacting with Rab7-FYCO1 (FYVE and coiled-coil domain containing protein 1), which, in turn, interact with kinesin motor proteins. Interestingly, LC3B-II is considered an autophagic marker and may be used as an index of the ongoing autophagic flux, since it is possible to measure the conversion of LC3B-I to LC3B-II as well as the luminal LC3B-II decrease, which depends on lysosomal digestion and may be prevented by the V-ATPase proton pump inhibitor, bafilomycin A1 (BafA1) (Abounit et al., 2012; Lee & Lee, 2016).

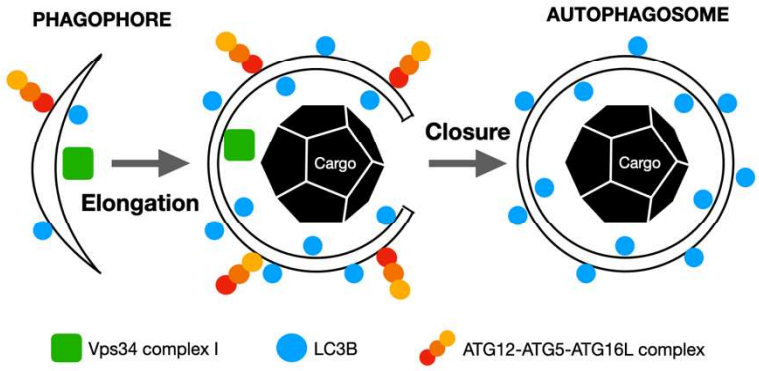


Figure 7. Autophagosome formation. Initiation of autophagy requires Vps34 complex I recruitment in PAS; there phagophore nucleation occurs. Later, phagophore undergoes elongation to form the autophagosome, in a process which is regulated by two systems: Atg5-Atg12 conjugation system and LC3B-II conjugation system.

Once autophagosomes are fully mature, they can fuse with lysosomes to form autolysosomes (Figure 8), which are positive for lysosomal enzymes and classical endo/lysosomal markers, including LAMP1, LAMP2 and V-type ATPase proton pump. This process is positively regulated by several factors, which include ATG14, LAMP2B, SNAREs, Rab7 and LC3B-II. Conversely, RUBICON (RUN and cysteine-rich domain containing Beclin-1 interacting protein) negatively affects the fusion of autophagosomes with lysosomes by interacting with Vps34. Upon autolysosome formation, acidic pH in vesicle lumen and proteinase B provides the degradation of both cargo and inner autophagosomal membrane. Then, monomeric units, generated upon macromolecule demolition, are exported to the cytosol and reused in anabolic processes (Galluzzi et al., 2017).

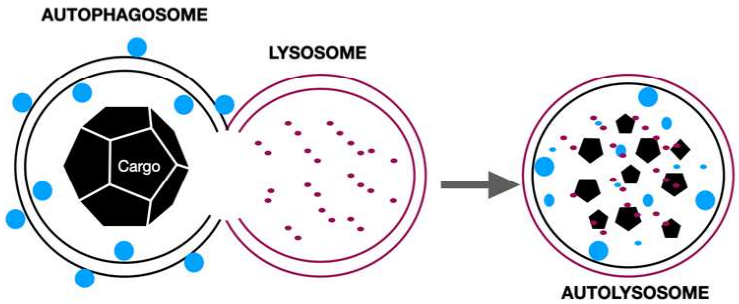


Figure 8. Autophagosome-lysosome fusion. Autolysosome formation occurs upon autophagosome-lysosome fusion, in a process which is regulated by several factors, including LC3B-II. This leads to cargo and autophagosome inner membrane degradation, which depends on acidic pH of the vesicle and lysosomal enzymes.

1.3 SQSTM-1/p62: A SIGNALING HUB PROTEIN.

SQSTM-1/p62 (hereafter p62), is a highly conserved, multidomain, multifunctional, adaptor protein involved in a great variety of cellular process, including ubiquitin-mediated autophagy, oxidative stress response, and metabolic reprogramming. Indeed, although p62 is mainly known for its role in cargo binding during selective autophagy, it has many interaction partners and acts as a hub, integrating signals of multiple pathways essential for cellular homeostasis maintenance (Berkamp et al., 2021).

Structurally, p62 is characterized by several domains, each with a specific function (Figure 9). The N-terminal Phox and Bpem1 (PB1) domain not only mediates p62 binding to atypical PKC (aPKC) and Erk1, but it is also required for self-oligomerization or heterodimerization (Puissant et al., 2012). In particular, the PB1 dependent oligomerization is essential for cargo collection and delivery to autophagosomes. Instead, the central zing finger ZZ domain, which is crucial for the interaction with receptor-interacting protein (RIP), cooperates with the TRAF6-binding segment (TBS) in order to mediate NF-kB signaling and inflammatory responses, mainly leading to IkB kinase (IKK) activation (Puissant et al., 2012). In addition, the ZZ domain is responsible for the interaction with RNAs and for p62-mediated recognition of N-degrons, which are specific single N-terminal amino acids acting as degradation signals for regulation of protein half-life (Berkamp et al., 2021; Zhang et al., 2018). In addition, downstream to the TBS, there is a segment which mediates p62 binding to the mTORC1 member Raptor; this interaction occurs only in response to amino acids and promotes mTORC1 activation (Berkamp et al., 2021). Another crucial p62 region is represented by the KIR (Keap-interacting region), engaged in the antioxidant response via binding to Keap1 which, in turn, induce the nuclear translocation of the NRF2 transcription factor (Puissant et al., 2012).

Concerning LC3-interacting region (LIR) and C-terminal ubiquitin-associated domain (UBA), they are responsible for p62 unique ability to act as a selective autophagy receptor. In particular, UBA domain mediates p62 binding to the polyubiquitinated cargo, while LIR domain interacts with autophagosome membrane LC3B, recruiting and keeping the p62-cargo complex within the autophagosome lumen. Therefore, autolysosomal digestion is accompanied by p62 intracellular level decrease, which is considered an index of the ongoing autophagic process (Berkamp et al., 2021; Puissant et al., 2012).

Concerning hypoxia and p62 interplay, Pursiheimo et al. have shown that p62 degradation is accelerated upon induction of autophagy in response to O_2 shortage (Pursiheimo et al., 2009). Moreover, Chen et al. have reported that p62 is involved in HIF-dependent glycolytic gene expression in renal cancer cells, since it promotes HIF-1α signaling by upregulating mTORC1 and NF-κB activity and downregulating of VHL E3 ubiquitin ligase activity (Chen et al., 2016). So p62, since its

structural properties, exerts a pivotal role in ensuring cell survival, even in response to stress conditions, by acting as a signaling hub for several intracellular pathways, including autophagy, nutrient/O_2 dependent signals and Erk1 activity.

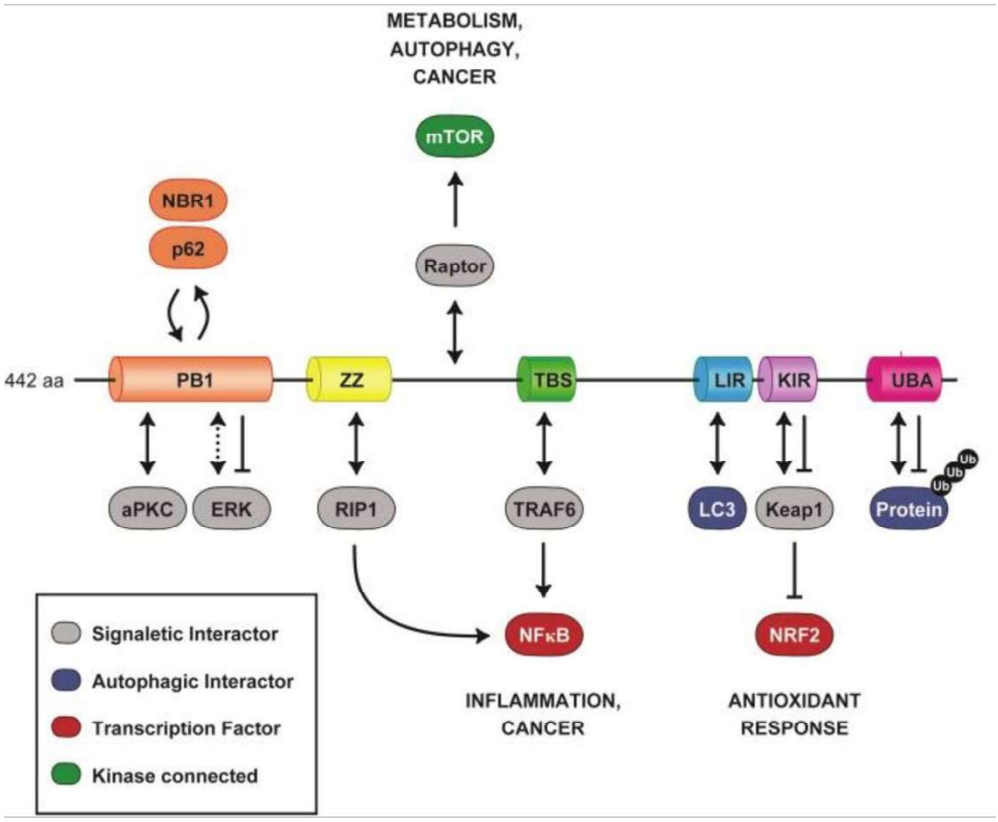


Figure 9. SQSTM-1/p62 domains. p62 acts as a signaling hub protein. Indeed, it is characterized by several domains providing its interaction with a great variety of binding partners. In particular, PB1 domain mediates p62 interaction with aPKC and Erk, while ZZ domain and TBS participate in NF-kB activation, interacting with RIP1 and TRAF6, respectively. Differently, LIR and UBA domains mediates p62 involvement in selective autophagy, while KIR domain binds Keap1, impacting NRF2 pathway. In addition, p62 can interact with Raptor, positively regulating mTORC1 activation. From (Puissant et al., 2012).

1.4 RNASET2: A KEY MOLECULE IN STRESS-INDUCED CELL DEATH?

T2 ribonucleases represent a subclass of endoribonucleases, characterized by two catalytic domains CAS (conserved active-site segments)-I and CAS-II, that cleave single-stranded RNAs (ssRNAs) producing mono- or oligonucleotides with a terminal 3′-phosphate. They are expressed in protozoans, plants, and animals and, interestingly, genes encoding these enzymes have been found also in virus and bacteria. RNASET2 is the only member of the T2 ribonuclease family identified in humans (Luhtala & Parker, 2010) and it exists in three different isoforms: the full-length protein, of 36 kDa, and the two products of its C-terminal proteolitic cleavage, of 31 and 26 kDa. Interestingly, RNASET2 has been detected not only in endoplasmic reticulum and Golgi apparatus, but also in lysosomes. Indeed, it has optimal catalytic activity at pH 5, as the majority of T2 ribonucleases. Moreover, RNASET2 can be secreted (Campomenosi et al., 2006) and may act as an immune modulator recruiting, activating and polarizing macrophages (Scaldaferri et al., 2018). Although this and other proposed biological functions remain to be fully elucidated, two recent works have shown that RNASET2 plays a crucial role in innate immune responses. Indeed, its degradation products are able to trigger the activation of the Toll like receptor 8 (TLR8), which is highly expressed in myeloid cells and mediates responses to pathogen ssRNAs (Greulich et al., 2019; Ostendorf et al., 2020).

In addition, other roles, beside its ribonuclease activity, have been hypothesized for this enzyme, including oncosuppressive functions. The latter have been explored, in cultured primary human melanocytes and keratinocytesby, by Wang et al., who have shown that RNASET2 overexpression, induced by stress conditions (UV radiation, oxygen peroxide and others), is paralleled by increased apoptosis, which is dependent on tumor necrosis factor receptor-associated factor 2 (TRAF2)–caspases pathway and triggered by physical interaction of RNASET2 with TRAF2 (Wang et al., 2014). A similar result was observed in an ovarian cancer model. Indeed, Lualdi et al. reported that RNASET2 knock down is associated with an increased proliferation rate of cancer cells exposed to stress conditions. Moreover, in the same work they showed that RNASET2 expression is upregulated upon hypoxic exposure and identified two putative HIF-1 binding sites in RNASET2 promoter (Lualdi et al., 2015).

All these data suggest that RNASET2, beside its role in innate immunity, plays a pivotal role in stress induced cell death.

1.5 DENDRITIC CELLS (DCs).

The immune system is the master regulator of tissue homeostasis and repair. Indeed, its cells and molecules orchestrate finely tuned responses, in order to protect the organism against pathogens and abnormal or damaged cells. Host immune machinery provides two main defense levels, which are represented by innate and adaptive responses. The innate response is immediately activated upon infection, since it is nonspecific. Indeed, it comprises chemical/physical barriers and the complement system, which are always ready to exert their functions, as well as cells, including monocytes and macrophages, mast cells, granulocytes, natural killer cells (NKs) and DCs, which are able to recognize molecular patterns common to different pathogens and directly kill them through several strategies, such as phagocytosis and natural cytotoxicity.

Moreover, innate immune cells are able to trigger adaptive responses, which are, instead, antigen specific and thus, slower but more precise. In addition, their rapidity and specificity can be improved upon multiple antigen encounters, thanks to the unique ability of adaptive immunity to develop an immunological memory. Adaptive responses are classified as cell-mediated or humoral-mediated responses and depends on T and B lymphocytes, respectively (Hoebe et al., 2004).

DCs draw a connection between innate and adaptive immunity. Indeed, DCs are the most potent antigen presenting cells (APCs) and are essential inducers of the primary immune responses, presenting pathogen-derived antigens to T lymphocytes via both Class-I and Class-II major histocompatibility complex molecules (MHC-I, MHC-II). In addition, DCs can modulate innate immunity, by producing and releasing cytokines (Steinman, 2012).

DCs, as their name suggests, have peculiar morphology, which has led to their identification by Ralph Steinman. Indeed, in 1973 Steinman discovered, in mice spleen, a subpopulation of immune cells with dendrite like protrusions and phagocytic properties (Steinman & Cohn, 1973).

Human DCs can be classified in two major subsets: conventional DCs (cDCs) and plasmacytoid DCs (pDCs). pDCs are similar to plasma cells and express CD123 and CD303 and higher CD4 levels than cDCs, but not CD11c. pDCs are specialized in anti-viral immunity: they promote cytolysis of infected cells by recruiting and activating NKs and cytotoxic CD8+ T cell, via secretion of type I interferon (INF-I). In addition, pDC INF-I drives CD4+ T lymphocytes polarization into T helper 1 cells (Th1) and prompt human B lymphocytes to differentiate into plasma cells and to produce IgG rather than IgM, implying that pDCs can specifically promote B cell immunological memory. Moreover, pDCs play pivotal role in Treg and Th17 responses, since they secrete indoleamine 2,3-dioxygenase (IDO) and inducible T cell costimulatory ligand (ICOS-L) (Bigley & Collin, 2020; Collin & Bigley, 2018).

Differently, cDCs, also known as myeloid DCs, represent a more heterogeneous population. Indeed, they can be further divided in different subgroups: type 1 conventional DCs (cDC1s) and type 2

conventional DCs (cDC2s). cDC1s, which are characterized by high levels of CD141, Clec9A, XCR1, and BTLA (while CD11c expression is relatively low), are specialized in defense against intracellular pathogens (e.g. virus, parasites and bacteria) and cancer cells, in triggering Th1 polarization and are the most potent subset for antigen cross-presentation, being major inducers of CD8+ T cell priming. Additionally, cDC1s are able to promote immune tolerance and drive Treg differentiation, via IDO and TGF-β secretion (Bigley & Collin, 2020; Guermonprez et al., 2019).
cDC2s, which express CD1c, CD2, SIRPA, CD11b, CD11c, CD13, and CD33, represent the predominant myeloid DC population. As cDC1s, they reside in peripheral tissues as well as in blood circulation and lymphoid organs, and, similarly to monocytes, they express a wide range of Pattern Recognition Receptors (PRRs), which include lectins, Toll-like receptors (TLRs), NOD-like receptors, and RIG-I-like receptors. However, cDC2s show lower efficiency in priming CD8+ T lymphocytes, through cross-presentation of exogenous antigens on MHC-I, when compared to cDC1s. This depends, at least in part, on their endocytic molecular machinery, which is more efficient in antigen processing in the MHC-II pathway. Indeed, they have a highly developed phago-lysosomal compartment (Collin & Bigley, 2018; Guermonprez et al., 2019).
A third, distinct, DCs subset is represented by monocyte-derived DCs (moDCs), which are also named inflammatory DCs, since they differentiate *in situ* from monocytes, during inflammation. They synergize with cDCs in inflammatory processes or upon infection, primarily contributing to innate defenses, processing and secreting IL-1, TNF-α, IL-12 and IL-23, and participating in initiation of T cell-mediated responses. Moreover, they reside in peripheral inflamed tissues as well as in draining lymph nodes; indeed, moDCs express CCR7, which is crucial for migration toward lymph node T-cell zone (Collin & Bigley, 2018; Marzaioli et al., 2020).
Interestingly, in 1994, Antonio Lanzavecchia devised a method for *in vitro* generation of human moDCs, which enable immature DC differentiation upon monocyte stimulation with granulocytes-macrophages colony stimulating factor (GM-CSF) and IL-4 (Sallusto & Lanzavecchia, 1994). Later, IL-13 was demonstrated to be as effective as IL-4 in *in vitro* moDC generation (Alters et al., 1999).

1.5.1 Functional differences between immature and mature DCs.

DCs exist in two functional states, immature DCs (iDCs) and mature DCs (maDCs), each with specific phenotype, morphology and function. iDCs are fully differentiated cells, distributed in various body districts, in both lymphoid and non-lymphoid tissues, where they act as professional sentinel cells, surveying the surrounding environment, monitoring for pathogens, and providing immune tolerance (Leblanc-Hotte et al., 2023; Mellman, 2013). Indeed, in healthy conditions, they continually capture self-antigens and present them to T cells, but in the absence of costimulatory

molecules, such as CD80 and CD86, this stimulation leads to autoreactive cell anergy, death or priming toward Treg phenotype, spreading tolerance toward self-antigens (Mahnke et al., 2002).

iDCs are characterized by high rates of macropinocytosis, phagocytosis, receptor-mediated and clathrin-mediated endocytosis, which provides antigen uptake. Upon internalization, antigens move from early endosomes or phagosomes to lysosomes, and, during this translocation, they undergo gradual proteolytic degradation. Indeed, DCs have highly developed lysosomal/proteasomal apparatus, which is essential for antigen processing and presentation and shows unique characteristics, such as incompletely assembled V-ATPase, crucial in preventing potential degradation of peptide epitopes required for T-cell activation (Alloatti et al., 2016).

Although they are considered relatively immobile cells, since the high expression of adhesion molecules (e.g. β1-integrins), iDCs can be recruited to inflammatory sites, thanks to their inflammatory chemokine receptors, which include CCR1, CCR2, CCR5, CCR6, CXCR1 and CXCR2 (Sallusto et al., 1998; Sozzani et al., 1997).

Upon antigen encounter, mediated by PRRs, CD40, TNF-R, or IL-1R activation, iDCs undergo maturation, which drives in the first few hours the reorganization of gene expression, and then a complete morphological and functional change, leading to a reduced potential for antigen uptake and a remarkable increase in DCs ability to prime T cell-mediated responses. DC maturation is almost complete 24 hours after activation (Granucci et al., 2005).

Indeed, maDCs show a re-distribution of the MHC molecules, from the intracellular compartment to the plasma membrane, concurrently with an increase in the expression levels of key costimulatory molecules, including CD40, CD58, CD80, and CD86.

In addition, the maturation process provides the downregulation of endocytic/phagocytic receptors and the loss of adhesive structures, which along with cytoskeleton reorganization and CCR7 upregulation, increases cellular motility (Rescigno et al., 1999; Watarai et al., 2005). In particular, CCR7 mediates maDC homing to lymph nodes, where they can interact with T lymphocytes and drive adaptive immune responses (López-Cotarelo et al., 2015).

Indeed, once in the lymph node, maDCs activates (a) CD8+ naïve T cells, presenting them antigenic peptides derived from intracellular pathogens, loaded onto MHC-I molecules, and (b) CD4+ helper T cells through MHC-II mediated presentation of exogenous antigens. In addition, maDCs have the unique ability to cross-present internalized antigens via MHC-I, leading to the development of a cytotoxic response against extracellular pathogens (Heath et al., 2004; Joffre et al., 2012).

Exogenous antigens may be cross-presented through either vacuolar or cytosolic pathways. The first one refers to antigen processed and loaded onto MHC-I molecules within the endocytic

compartments, while the second regards antigens processed, within the cytosol, by the proteasome system (Alloatti et al., 2016).

In addition, DC maturation is associated with an increase in cytokine production and release. In particular, maDCs increase IL-12 p40 expression and are major secretors of bioactive IL-12 p70, which plays a pivotal role in Th1 polarization (Moser & Murphy, 2000).

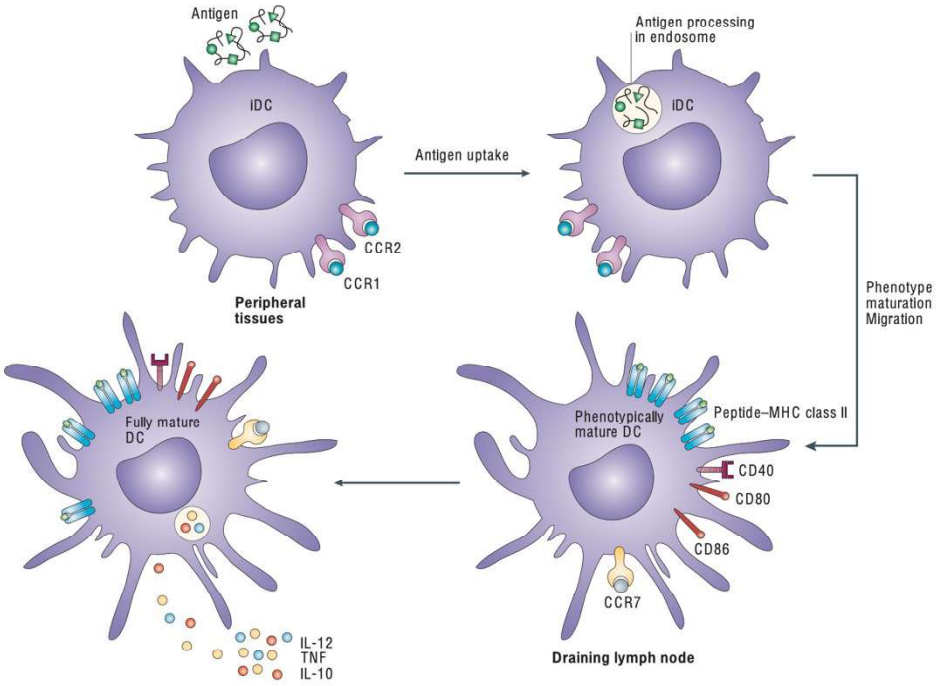


Figure 10. iDC and maDCs. iDCs are sentinel cells patrolling peripheral tissues. They are specialized in pathogen sensing, uptake and processing. Upon antigen encounter, they undergo maturation, which triggers their migration toward lymph nodes and promotes upregulation of MHC-II/peptide complex and costimulatory molecule expression. maDCs produce and secrete pro-inflammatory cytokines. Adapted from (Hackstein & Thomson, 2004).

1.5.2 The Toll-like receptor 4 and bacterial LPS.

Each innate immune cell, including DCs, differs from T and B lymphocytes, thanks to the ability to sense more than one infection or damage signal, through the expression of a broad repertoire of PRRs. Indeed, PRRs are able to recognize both pathogen and damage associated molecular patterns (PAMPs and DAMPs respectively) and to drive a great variety of intracellular events, including DC maturation. Among PRRs, is possible to mention RIG-I-like receptors (RLRs), NOD-like receptors (NLRs) and C-type lectin receptors (CTLs) as well as the Toll-like receptor (TLR) family which in

mammals comprises more than ten members, each with specific ligand and localization (Sameer & Nissar, 2021).

TLRs can be divided in two main categories: cell membrane TLRs, including TLR1,2,4,5,6 and TLR10 and intracellular TLRs, which can localize in endosome, endoplasmic reticulum and lysosome membranes, and include TLR3,7,8 and TLR9. Interestingly, in their active conformation, they exist as heterodimers (e.g. TLR1-TLR2 or TLR2-TLR6), homodimers, or associated with other proteins (such as MD-2 and CD14) (Moresco et al., 2011).

Concerning TLR4, it recognizes LPS from Gram negative bacteria, which is a large glycolipid, composed of a polysaccharidic core, linked to the lipid A, with several fatty-acid chains, and to the O antigen (Bertani & Ruiz, 2018). LPS-mediated activation of TLR4 requires the interaction of the receptor with several proteins: LPS binding protein (LBP), CD14 and MD-2. LBP shuttles LPS to CD14, which in turn promotes the binding of LPS to TLR4/MD-2 complex. Upon LPS recognition, TLR4/MD-2 complex recruits a second TLR4 molecule and this dimerization leads to the activation of the downstream signaling pathways, via recruitment of several adaptor proteins, which directly interact with TIR (Toll-interleukin-1 receptor) domains and include MyD88 (myeloid differentiation primary response gene 88), TIRAP (TIR domain-containing adaptor protein, also known as Mal, MyD88-adapter-like), TRIF (TIR domain-containing adaptor inducing IFN-β), TRAM (TRIF-related adaptor molecule). MyD88 dependent and independent pathways converge in NF-kB activation and leads to pro-inflammatory cytokines and INF-I expression, respectively (Figure 11) (Lu et al., 2008). Interestingly, TLR4 signaling has been associated also to DC survival, since it positively regulates Erk 1/2 (Rescigno et al., 1998) and PI3K/Akt activation (Naldini et al., 2012), even under hypoxia.

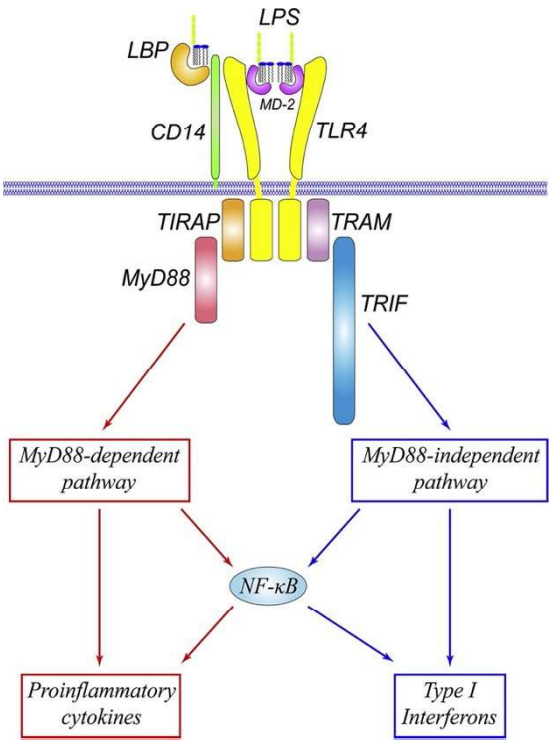


Figure 11. TLR4 signaling. Upon LPS recognition, TLR4 homodimerizes and recruits downstream molecules: MyD88 and TRIF. They lead to NF-kB activation and, thus, to pro-inflammatory cytokines and INF-I expression. From (Lu et al., 2008).

1.5.3 DCs and hypoxia crosstalk.

During their development, DCs often experience O_2 tension changes, since they move from peripheral tissues, which are characterized by variable O_2 levels, depending on their anatomical structure and physiological features, to secondary lymphoid organs, physiologically hypoxic, or to inflammatory sites and TME, where the pO_2 is even lower. As a matter of fact, hypoxia affects DC biology and physiology, with particular regards to the maturation stage, and the ability of DCs to adapt to this condition is crucial for them to survive and efficiently carry out their functions (Bosco & Varesio, 2012; Naldini et al., 2012).

Controversial results have been reported on how hypoxia modulates DC maturation and inflammatory properties. Jantsch et al. showed that hypoxia alone is not able to affect expression of pro-inflammatory cytokines, including IL-6, TNF, IL-10 and IL-12p40, in murine iDCs, but it can induce the enhancement of TNF and IL-6 levels in maDCs. Moreover, the combination of hypoxia and LPS stimulation, remarkable increases, in murine DCs, the expression of costimulatory molecules and the induction of allogeneic lymphocyte proliferation, compared with LPS alone (Jantsch et al., 2008;

McGettrick & O'Neill, 2020). On the contrary, Mancino et al. demonstrated that hypoxia impairs human moDC differentiation and full maturation. In particular, they reported that O_2 shortages reduces expression of the maturation markers CD40, CD80, CD83, CD86, and MHC-II along with their ability to prime T-cells, while it amplifies iDC capability to modulate innate immune responses, by increasing their secretion of inflammatory cytokines, thus enhancing their chemotaxis toward inflammatory sites (Mancino et al., 2008).

These data concerning the enhancement of DC function in the innate response are in accordance with a study of Ricciardi et al., which revealed that hypoxia is able to redefine completely iDC transcriptome. Indeed, elevated expression of genes associated with glycolysis, gluconeogenesis and the pentose phosphate pathways have been reported in their experimental model of iDCs generated from human monocytes under prolonged hypoxic conditions (H-iDCs). Moreover, H-iDCs showed increased migratory abilities along with the upregulation of chemokine receptors involved in the recruitment to inflammatory sites (Bosco & Varesio, 2012; Ricciardi et al., 2008). As demonstrated by Filippi et al., iDC migratory abilities, upon short term hypoxic exposure, mainly relies on HIF-1α. Differently, in maDCs, HIF-1α cooperates with the Akt pathway to enhance cell migration under hypoxia (Filippi et al., 2014).

Concerning DC adaptation to hypoxia, Naldini et al., demonstrated that prolonged hypoxic exposure promotes iDC cell death by HIF-1-induced upregulation of BINP3 and BAX expression, PARP cleavage, caspase 3 activation and BCL-2 downregulation. On the contrary, this effect is prevented by LPS-induced DC maturation, as they showed that maDCs survival under hypoxia is promoted by TLR4-mediated PI3K/Akt activation (Naldini et al., 2012). More recently, Monaci et al. demonstrated that, LPS-dependent promotion of DC survival relies also on autophagy induction under hypoxic conditions (Monaci et al., 2020). Indeed, cell survival under hypoxia can be promoted involving autophagy as a metabolic adaptive strategy (Mazure & Pouyssegur, 2010). Thus autophagy plays a pivotal role in DC biology since it not only modulates DC maturation, antigen presentation and cytokine production (Ghislat & Lawrence, 2018), but also promotes cell survival in response to environmental stresses.

2. AIM OF THE THESIS.

Tissue hypoxia is a characteristic in both physiological and pathophysiological conditions. Indeed, it is a hallmark of immunological niches as well as of TME and inflammatory areas. O_2 shortage is perceived as a stress condition by many cell types and evolution has led to the development of finely tuned adaptive responses to hypoxia. Although most of them are mediated by the hypoxia-inducible factor (HIF)-dependent transcriptional programs, more recently, other adaptive molecular signaling have been proposed. These include PI3Ks and Erk 1/2, as well the autophagic program, all associated with cell survival/death under hypoxia.

DCs, regardless to their maturation stage, are frequently exposed to O_2 tension changes. However, different hypoxia-induced responses have been described for iDCs and maDCs, being evidence of their distinct effector functions.

Autophagy participates in several DC functions and, more recently, its induction and the consequent p62 degradation have been associated to DC survival under hypoxia.

To establish novel molecular mechanisms involved in DC adaptive responses to hypoxia, in terms of autophagy, survival and cell death, we decided to evaluate the impact first of p62, a selective-autophagy receptor, then of Vps34, which plays a crucial role in the initial phase of the autophagic process, and, finally, of RNASET2 in hypoxic iDCs and maDCs.

3. MATERIALS AND METHODS.

3.1 PRIMARY CELL CULTURE.

3.1.1 Cell culture Reagents.

For human monocyte isolation, Fycoll/Lympholite and Percoll were purchased from Cederlane Labs, Burlington, ON, Canada and Amersham Bioscience, Pittsburgh, PA, USA, respectively.
For monocyte differentiation into DCs and DC culture RPMI 1640, fetal bovine serum (FBS), buffered saline solution (PBS) without Ca2+ and Mg2+, penicillin/streptomycin, and L-Glutamine (all purchased from Euroclone, Devon, UK), recombinant human granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-13 (IL-13), both by ProSpec TechnoGene, East Brunswick, NJ, USA, were used. Limulus Amebocyte Lysate assay was purchased from Cambrex, East Rutherford, NJ, USA, and used to assess that all reagents contained <0.125 endotoxin units/mL. For DC terminal maturation, LPS from Escherichia coli strain O26:B6, obtained from Sigma–Aldrich, Milano, Italy, was added to cell culture medium.

3.1.2 Human monocyte isolation and monocyte-derived DC preparation.

Human monocytes were isolated from anonymous healthy donor buffy coats, provided from South-East Tuscany Blood Establishment, AOUS, Siena, Italy.
In order to remove platelets, buffy coats were centrifuged for 15 minutes at 900 x g, then they were washed with sterile PBS (pH 7.4 and without Ca^{2+} and Mg^{2+} to avoid coagulation) and centrifuged at 900 x g for 10 minutes. Following these steps, blood was diluted with PBS in the ratio 1:1 and stratified on Ficoll (Lympholyte) in the ratio 2:1. Stratified blood was centrifuged at 800 x g for 30 minutes, allowing sedimentation of erythrocytes and granulocytes on the bottom of the tube, in parallel with the formation, at the interface between the sample and the separation medium, of a ring composed of PBMCs (peripheral blood mononuclear cells), which include lymphocytes and monocytes. PBMCs were recovered, washed for three times with PBS, stratified on Percoll 285 mmOsm, in the ratio 1:1, and centrifuged for 30 minutes at 771 x g. This step allowed the precipitation of lymphocytes in the tube bottom, while monocytes arranged to form a ring between the supernatant and the separation medium. The ring was collected, cells were washed and monocytes expressing CD14 (> 95%) were recovered. Then, monocytes were diluted in with RMPI with 5% FBS, at the concentration of $1x10^6$/mL, seeded in 6 well plates (Corning, New York, NY, USA) and incubated to allow monocytes adhesion.

After 2 hours, cells were washed twice with PBS and then RMPI with 10% FBS and supplemented with 50 ng/mL GM-CSF and 20 ng/mL IL-13, was added and cells were incubated for 6 days under normoxia (atmospheric pO_2 levels: 21% O_2, 5% CO_2, and 74% N_2 corresponding to a pO_2 ~ 140 mmHg).

3.1.3 DC culture conditions.

At the end of the 6 days, iDCs, expressing >90% CD1a and <5% CD14, were obtained. Upon differentiation, cells were collected, by using cold PBS and cell scraper, and seeded at a concentration of 500.000 cells/condition for Western blotting and RT-qPCR and 30.000 cells/condition for Lysotracker staining and Fluorescein diacetate assay. As indicated in results section, cells were cultured in the absence (iDCs) or in the presence of LPS (maDCs), at a concentration of 100 ng/mL, triggering their terminal maturation, and were exposed either to normoxia, in an incubator (New Brunswick Scientific, Edison, NJ) which was set at 5% CO2 and 20.9% O2 (pO2 ~ 140 mmHg; atmospheric pO2), or to hypoxia (5% CO2 and 2% O2, corresponding to a p O2 ~ 14 mmHg) in the workstation InVIVO O_2 400 (Ruskinn, Pencoed, UK). The hypoxic pO_2 was choose since it resembles the average O_2 tension experienced by immune cells in the lymphoid organs.
As indicated in results section, some cells were transfected either with a control sequence or with a p62-targeting siRNA, while others were treated with specific chemical compounds in the last 6 hours of the experiment.
In particular some cells were treated with 5μM U0126, purchased from Tocris Biosciences, Bristol, UK, which is a specific, potent and non-competitive MEK1/2 inhibitor. It prevents the phosphorylation and the consequent activation of Erk 1/2. Other cells were treated with SAR405, from CliniSciences, Nanterre, France, and Wortmannin, form Tocris Biosciences, Bristol, UK. In particular, SAR 405, which is a selective and ATP-competitive Vps34 inhibitor, was used at a concentration of 10μM, while Wortmannin, which is a cell-permeable, potent, selective, irreversible inhibitor of the PI3Ks, was used at a 5μM concentration, in order to prevent TLR4-induced Akt activation.

3.1.4 p62 inhibition by RNA interference approach.

SQSTM1/p62 inhibition was performed by an RNA interreference approach. Two specific p62-targerting siRNAs (NM_003900), siRNA p62-1 (SASI_Hs01_00118616) and siRNA p62-2 (SASI_Hs02_00336523), were purchased from Sigma-Aldrich, St. Louis, MO, USA. As a control (Ctr), the MISSION® siRNA Universal Negative Control #1 (SIC001) siRNA (Ctr), was selected

and purchased from Sigma-Aldrich, St. Louis, MO, USA. p62 siRNAs and Ctr were used at concentration of 46 nM. Transfection was performed by using Lipofectamine RNAi MAX (Invitrogen, Paisley, UK).

In particular, collected iDCs were seeded and incubated for 2 h to allow adhesion. Then, siRNA diluted with OPTI-MEM® (1X) (Gibco, Thermo Fisher Scientific, Cleveland, OH, USA) was added to tubes containing lipofectamine (diluted with OPTI-MEM® too), and the solution was gently mixed. Upon 20 minutes of incubation, the lipofectamine-siRNA complexes were added to the cells and DCs were incubated for 24 h under normoxia. After 24 h, the culture medium was replaced and DCs were exposed to normoxic or hypoxic conditions for indicated times.

3.2 GENE AND PROTEIN EXPRESSION ANALYSIS.

3.2.1 Western Blotting and antibodies.

Western Blot was performed in order to assess protein expression. To this end, 5 x 10^5 cells/mL were plated in 35x10 mm Petri's dishes (Corning, New York, NY, USA). At indicated times, supernatants were collected and centrifuged to recover suspended cells, while adherent cells were washed with cold PBS and lysed with RIPA buffer (40 µL) supplemented with a protease inhibitor cocktail (purchased from Sigma-Aldrich, St. Louis, MO, USA).

After centrifugation at 14000 x g for 20 minutes, Micro BCA Protein Assay Reagent kit (Rockford, USA) was used to assess the total protein concentration of each sample. Then, equal amounts of proteins were loaded onto SDS-PAGE gel and, at the end of the electrophoretic run, proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA).

Membranes were incubated overnight, at 4°C, with the following primary antibodies: HIF-1α (BD Biosciences, San Jose, CA, 1:500, Cat.n° 610958), SQSTM1/p62 (Cell Signaling Technologies, Danvers, MA, 1:1000, Cat.n° 5114), ph-Erk1/2 MAP Kinase (Cell Signaling Technologies, Danvers, MA, 1:1000 Cat.n° 9101), Erk1/2 (Cell Signaling Technologies, Danvers, MA, 1:1000, Cat.n° 9102), ph-AMPKα (Cell Signaling Technologies, Danvers, MA, 1:1000, Cat.n° 2535), AMPKα (Cell Signaling Technologies, Danvers, MA, 1:1000, Cat.n° 5831), ph-mTOR (Cell Signaling Technologies, Danvers, MA, 1:1000, Cat.n° 5536), ph-ULK1 (Ser757) Cell Signaling Technologies, Danvers, MA, 1:1000, Cat.n° 14202), Vps34 (Cell Signaling Technologies, Danvers, MA, 1:1000, Cat.n° 4263), Beclin1 (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n°3495), RNASET2 (rabbit polyclonal antibody raised against recombinant RNASET2 protein was kindly provided by Professor Francesco Acquati and diluted 1:500), ph-Akt (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n°4058), Akt (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n° 9272), and

β-actin (Sigma-Aldrich, 1:50000 Cat.n° A3854). Anti-mouse Cell Signaling Technologies, Danvers, MA, 1:5000 Cat.n°7076) and anti-rabbit (Cell Signaling Technologies, Danvers, MA, 1:2000 Cat.n°7074) IgG HRP-conjugated were used as secondary antibodies. Protein detection and image acquisition were performed using the ChemiDocXRS (Bio-Rad, Hercules, CA, USA) and the Image Lab software (Bio-Rad, Hercules, CA, USA) was used to quantify band intensity.

3.2.2 RNA extraction and qRT-PCR.

To analyze gene expression, DCs were seeded in 6-well plates (Corning, New York, NY, USA) at the density of 5 x 10^5 cells/well and lysed with EuroGOLD^{TM}Trifast reagent (500 µL), purchased from Euroclone, Devon, UK. Then, chloroform was used to separate the sample in three phases: the phase at the bottom of the tube and the intermediate one contained proteins and DNA respectively, while the upper phase contained total RNA. The latter was recovered and, following washes with isopropanol and cold ethanol (75%), was centrifuged at 12.000 x g. The obtained pellet was resuspended in nuclease-free water and boiled for 10 minutes at 56°C.
Total RNA was measured with Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Then, iScript^{TM}cDNA Synthesis kit (Biorad laboratories, Bio-Rad, Hercules, CA, USA) was used to convert 1 µg of RNA to complementary DNA (cDNA).
RT-qPCR was performed by using 10 ng of total RNA for each sample, the iTaq^{TM}SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and primers for SQSTM1/p62, BNIP3, IL-12, IL-6 and TNFα. β-actin and L-32 were used as housekeeping gene.
Gene expression was quantitatively analyzed with the iQ^{TM}5 Optical System Software (Bio-Rad, Hercules, CA, USA) by using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

3.3 CELL VIABILITY ASSAY.

Cell viability was assessed by using the fluorescein diacetate assay. Fluorescein diacetate (FDA) is a non-fluorescent and membrane permeable molecule. Upon its hydrolysis, which occurs only in viable cells, and which is mediated by esterase enzymes, it becomes fluorescent and stains live cells.
For viability assay, DCs were seeded in 96-well plates (Corning, New York, NY, USA) at the density of 3 x 10^4 cells/well. FDA was purchased from Sigma-Aldrich, St. Louis, USA, and solved in acetone (1 mg/mL). Then, it was diluted 1:200 in PBS and 100 µL of the working solution were added to each well, upon culture medium removal. Plates were incubated in the dark for 30 minutes, at 37°C, and fluorescence (λex: 494 nm and λem: 518 nm) was measured with a microplate reader, the Fluoroskan

Ascent (Thermolabsystem, Helsinki, Finland). The viability was indicated as the percentage of the ratio between the treated group and the control group.

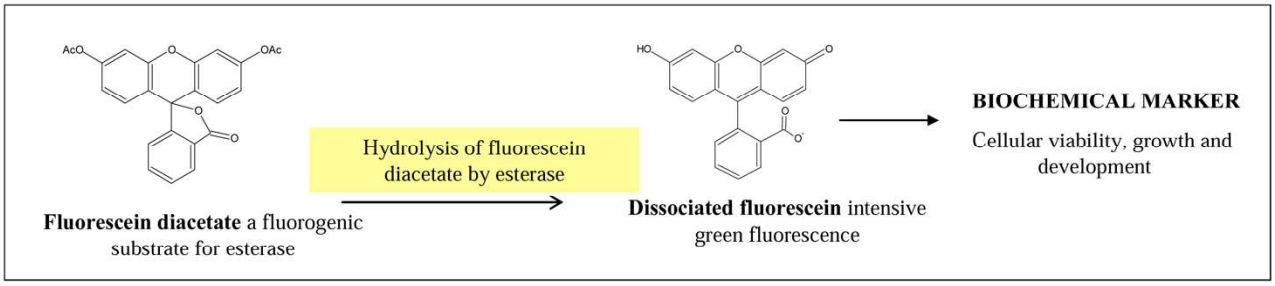


Figure 12. FDA structure and hydrolysis. Upon entering viable cells, FDA is hydrolyzed by esterase enzymes, and it becomes fluorescent. It is used to determine cell viability. Adapted from (Vitecek et al., 2007).

3.4 LYSOTRACKER STAINING.

LysoTracker staining was used to assess the ongoing autophagy flux, since it selectively marks acidic compartments, such as lysosomes and autolysosomes.

For LysoTracker staining, DCs, stimulated with LPS, were plated on 8-well coverglass slide (Sarstedt, Germany) and, upon culture medium removal, live cells were stained with the Lyso-ID Green Detection Kit, purchased from Enzo Life Sciences, Plymouth Meeting, PA, USA, and incubated in the dark for 30 minutes, at 37°C. DAPI was used for nuclear staining. Images were obtained with LSM-510 META confocal microscope (Carl Zeiss, Oberkochen, Germany) and ImageJ software was used to determine fluorescence intensity, as the mean pixel density of stained areas, in each cell. Fluorecence intensity values were shown as CTCF (corrected total cell fluorescence) = Integrated Density – (Area of selected cell X Mean fluorescence of background readings).

3.5 STATISTICAL ANALYSIS.

Data are shown as the mean ± SEM of at least 3 independent experiments. Analysis of variance (ordinary one-way ANOVA), with post hoc Tukey's multiple comparisons test, and unpaired two-tailed Student's t-test were performed with Graph-Pad Prism (San Diego, CA, USA). Difference of $p \leq 0.05$ was considered to be statistically significant. Statistically significant differences are indicated as * ($p \leq 0.05$), ** ($p \leq 0.01$) , *** ($p \leq 0.005$) and **** ($p \leq 0.001$).

4. RESULTS (PART I): INVOLVEMENT OF p62 IN IMMATURE DC ADAPTATION TO HYPOXIA.

4.1 Hypoxia induces HIF-1α accumulation along with p62 degradation.

Previous reports have shown that hypoxia affects DC survival, apoptosis, and autophagy with regards to DC maturation stage and hypoxic exposure times (Monaci et al., 2020; Naldini et al., 2012). Here we decided to investigate whether O_2 shortages may affect the autophagy-mediated degradation of p62 in a time-dependent manner and how it modulates other signaling pathways involved in cell survival and cell death.

To this end, we exposed human monocyte-derived iDCs to either normoxia (pO_2 of 140 mmHg) or hypoxia (pO_2 of 14 mmHg) and determined HIF-1α accumulation, at different time points (4, 8, 24 and 48 h), by Western blotting. As shown in Figure 13, already after 4 h, hypoxia was able to significantly increase HIF-1α protein levels in iDCs, and this increase was sustained up to 48 h. Interestingly, as shown in the same figure, HIF-1α accumulation was accompanied by reduction in p62 protein levels. This reduction was already detectable after 8 h of exposure to hypoxia, but it was more pronounced at 24 and 48 h. These results were in accordance with previous works showing that hypoxia-induced autophagy promotes p62 degradation in parallel with HIF-1α accumulation (Monaci et al., 2020).

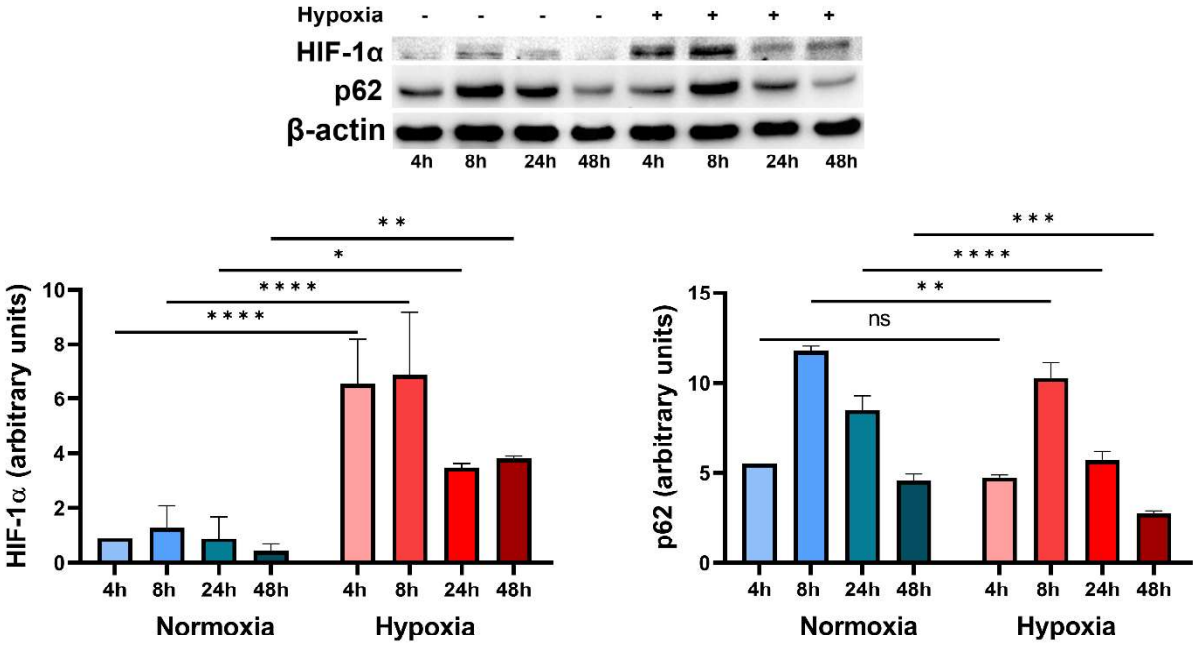
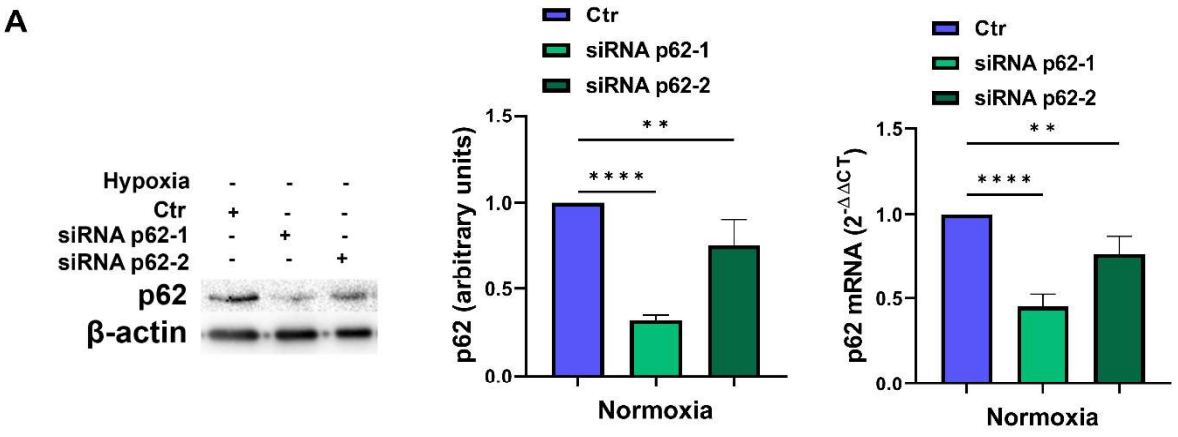


Figure 13. HIF-1α accumulation and p62 protein levels in normoxic and hypoxic iDCs. HIF-1α and p62 protein levels after 4, 8, 24 and 48 h exposure to normoxia and hypoxia as determined by Western Blot. Quantification of protein expression was performed by using β-actin as loading control. All blots shown are representative of at least three independent experiments and data are shown as the mean ± SEM of at least 3 independent experiments. * , **, *** and **** indicate statistically significant differences ($p \leq 0.05$, $p \leq 0.01$, $p \leq 0.005$ and $p \leq 0.001$ respectively).

4.2 Impairment of hypoxic iDC survival upon p62 inhibition.

To evaluate whether p62 is involved in iDC survival maintenance under hypoxia, we next inhibited p62 by siRNA, in iDCs exposed to either normoxia or hypoxia for 24 h, choosing this time point since it was the one associated with the most evident p62 degradation (Figure 13).

Firstly, we tested two different p62 targeting siRNAs under normoxia. As reported in Figure 14 A, both siRNA p62-1 and siRNA p62-2 were able to downregulate p62 at protein as well as at mRNA level, as assessed by Western blotting and RT-qPCR respectively, when compared to the negative control (Ctr). Then, we selected siRNA p62-1 (from now on siRNA p62) since it was the most efficient, and we used it to transfect also iDCs exposed to hypoxia for 24 h. As shown in Figure 14 B, hypoxic iDCs transfected with siRNA p62 were characterized by a significant reduction of p62 protein levels and mRNA expression. These results indicate that our RNA interference approach was effective either at mRNA and protein level, under both normoxia and hypoxia.



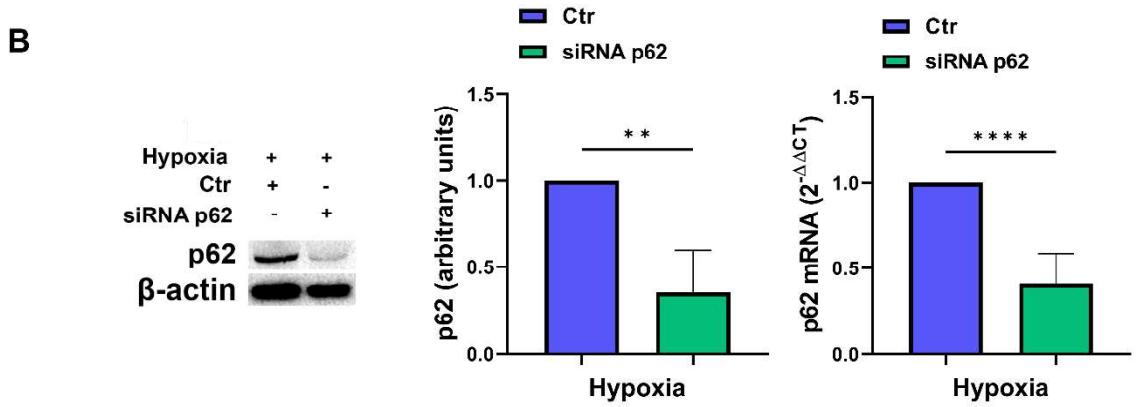


Figure 14. Efficiency of p62 inhibition by RNA interference, in normoxic and hypoxic iDCs. (A) p62 protein levels and mRNA expression, by Western blot and RT-qPCR respectively, in iDCs transfected with Ctr, siRNA p62-1 or siRNA p62-2 (46 nM) and exposed to normoxia for 24h. (B) p62 protein levels and mRNA expression, by Western blot and RT-qPCR respectively, in iDCs transfected with Ctr or siRNA p62-1 (46 nM) and exposed to hypoxia for 24h. Quantification of protein expression was performed by using β-actin as loading control. β-actin and L-32 were used as housekeeping genes for RT-qPCR analysis. All blots shown are representative of at least three independent experiments and data are shown as the mean ± SEM of at least 3 independent experiments. ** and **** indicate statistically significant differences ($p \leq 0.01$, and $p \leq 0.001$ respectively).

We next analyzed the impact of p62 inhibition on cell viability, in iDC exposed to either normoxia or hypoxia for 48 h, by using the fluorescein diacetate assay. Interestingly, although the p62 targeting siRNA was able to impair iDC viability under both normoxia and hypoxia, this impairment was more pronounced and statistically significant only in iDCs exposed to hypoxia (Figure 15).
This finding suggests that p62 exerts a pivotal role in protecting iDCs against the hypoxic insult that they physiologically experience in peripheral and inflamed tissues, where they act as sentinel cells.

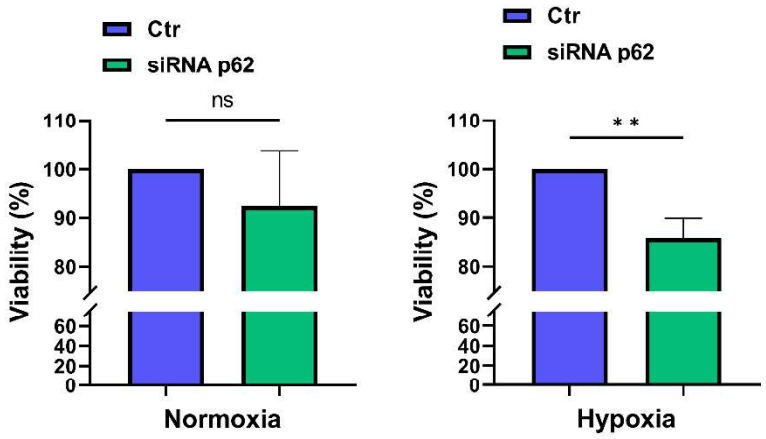


Figure 15. Effects of p62 inhibition on normoxic and hypoxic iDC viability. Cell viability, by fluorescein diacetate assay, in iDCs transfected with Ctr or siRNA p62 and exposed to normoxia or hypoxia for 48 h. Viability is expressed as the percentage of the ratio between the siRNA p62 transfected and Ctr transfected groups. Data are shown as the mean ± SEM of 3 independent experiments. ** indicates statistically significant differences ($p \leq 0.01$).

4.3 p62 inhibition does not affect the HIF-1 signaling pathway.

To elucidate the underlying molecular mechanisms, through which p62 can promote iDC survival under hypoxia, we took into account HIF-1 signaling and we considered whether p62 could affect it. Indeed, it has been previously shown that prolonged hypoxic exposure results in HIF-1-dependent upregulation of BNIP3 and other pro-apoptotic factors, and that such upregulation is implicated in iDC cell death (Naldini et al., 2012).

To this end, we determined HIF-1α accumulation, by Western blotting, as well as its transcriptional activity, by analyzing (through RT-qPCR) BNIP3 mRNA expression, in iDCs transfected either with the negative control sequence or with the p62 targeting siRNA and exposed to hypoxia for 24 h. Interestingly, has shown in Figure 16 A, HIF-1α protein levels were not affected by p62 inhibition. In addition, neither BNIP3 transcription was impaired by p62 knock down (Figure 16 B). These data suggest that p62-mediated iDC protection from hypoxic stress is independent of HIF-1 transcriptional programs.

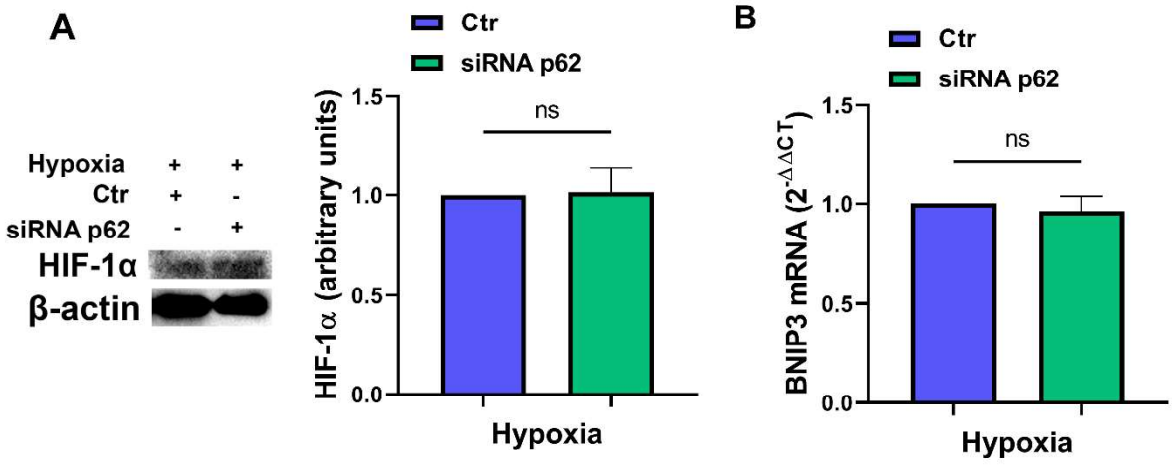


Figure 16. HIF-1α accumulation and BNIP3 expression upon p62 inhibition in hypoxic iDCs. (A) HIF-1α protein levels determined by Western Blot and (B) BNIP3 mRNA expression, by RT-qPCR, in iDCs transfected with Ctr or siRNA p62 and exposed to hypoxia for 24 h. Quantification of protein expression was performed by using β-actin as loading control. β-actin and L-32 were used as housekeeping genes for RT-qPCR analysis. Blot shown is representative of at least three independent experiments and data are shown as the mean ± SEM of at least 3 independent experiments.

4.4 Erk 1/2-AMPK crosstalk is involved in iDC adaptive responses to hypoxia.

Beyond HIF signaling pathway, evolution has led to the development of other adaptive strategies implicated in cell protection against hypoxic insult. Among them, we considered Erk 1/2, which has been shown to be activated by hypoxia, in response to which it mediates a pro-survival program (Minet et al., 2000). In addition, Erk 1/2 pathway has been demonstrated to be essential for DC survival and proper terminal maturation (Rescigno et al., 1998).

We here determined, in iDCs, the impact of hypoxia on Erk 1/2 activation, which depends on its phosphorylation at Thr185 and Tyr187 residues (Lavoie et al., 2020). As presented in Figure 17 A by Western blotting, hypoxia enhanced Erk 1/2 phosphorylation in iDCs, after both 24 and 48 h of hypoxic exposure. This finding suggests that hypoxia exerts a pivotal role in Erk 1/2 activation even in iDCs.

Interestingly, Erk 1/2 pro-survival program also includes the crosstalk with AMPK, which has been demonstrated to be essential in regulating maDC lifespan and immune function. Indeed Lopez-Cotarelo et al. have shown that AMPK activation results in the induction of maDC apoptosis and that it may be prevented by Erk 1/2, which is responsible for the inhibitory phosphorylation of AMPK on Ser485 (López-Cotarelo et al., 2015). Moreover, AMPK activation has been associated to immunosuppressive phenotype of tumor myeloid-derived suppressor cells (Trillo-Tinoco et al., 2019), highlighting the opposite effect of this kinase when compared to Erk 1/2. However, little is known about AMPK activity in iDCs.

We here reported that hypoxia, in parallel with Erk 1/2 activation enhancement, almost completely abolishes AMPK activation. Indeed, as shown in Figure 17 B, we assessed, by Western blotting, a significant decrease in the phosphorylation of the AMPK α-subunit (AMPKα), which is responsible for its activation, in hypoxic iDCs, when compared with the normoxic controls, at both time points.

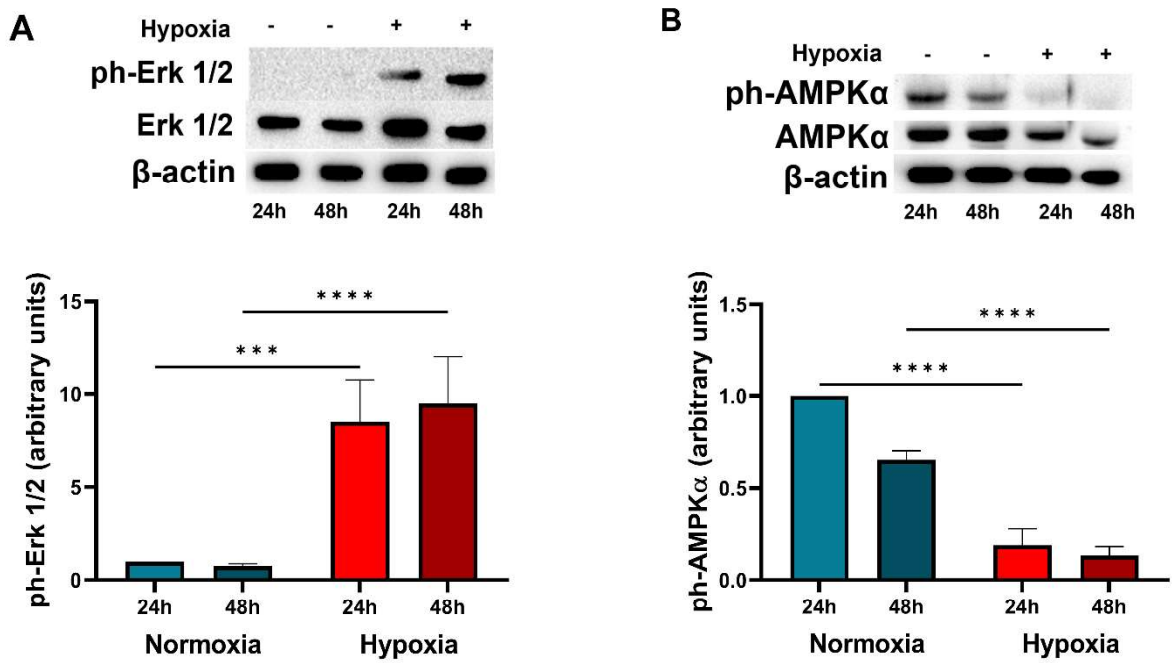


Figure 17. Erk 1/2 – AMPK crosstalk in normoxic and hypoxic iDCs. (A) ph-Erk 1/2 protein levels after 24 and 48 h exposure to normoxia and hypoxia as determined by Western Blot. (B) ph-AMPKα protein levels after 24 and 48 h exposure to normoxia and hypoxia as determined by Western Blot. Quantification of protein expression was performed by using β-actin as loading control. All blots shown are representative of at least three independent experiments and data are shown as the mean ± SEM of at least 3 independent experiments. *** and **** indicate statistically significant differences ($p \leq 0.005$ and $p \leq 0.001$ respectively).

To further determine the involvement of the Erk 1/2-AMPK crosstalk in iDC adaptation to O_2 shortages, we took advantage of U0126, a selective MEK1/2 inhibitor that prevents Erk 1/2 phosphorylation. In particular, we investigated whether this compound, along with Erk inhibition, could impact AMPK activation, affecting iDC survival. To this end, we exposed iDCs, treated or untreated with U0126, to hypoxia for 24 h. Firstly, we assessed, by Western blotting, the efficiency of this inhibitor which completely abolished Erk 1/2 phosphorylation in hypoxic iDCs (Figure 18 A). In parallel, we reported a significant increase in the phosphorylation of AMPKα (Figure 18 B). As shown in Figure 18 C, the inhibition of Erk 1/2 and the increase in AMPK activation, induced by U0126, were accompanied by a significant decrease in the percentage of viable iDCs exposed to hypoxia, which was assessed by fluorescein diacetate assay (Figure 18 C).

Therefore, we concluded that Erk 1/2-AMPK crosstalk is essential to ensure iDC survival upon hypoxic exposure.

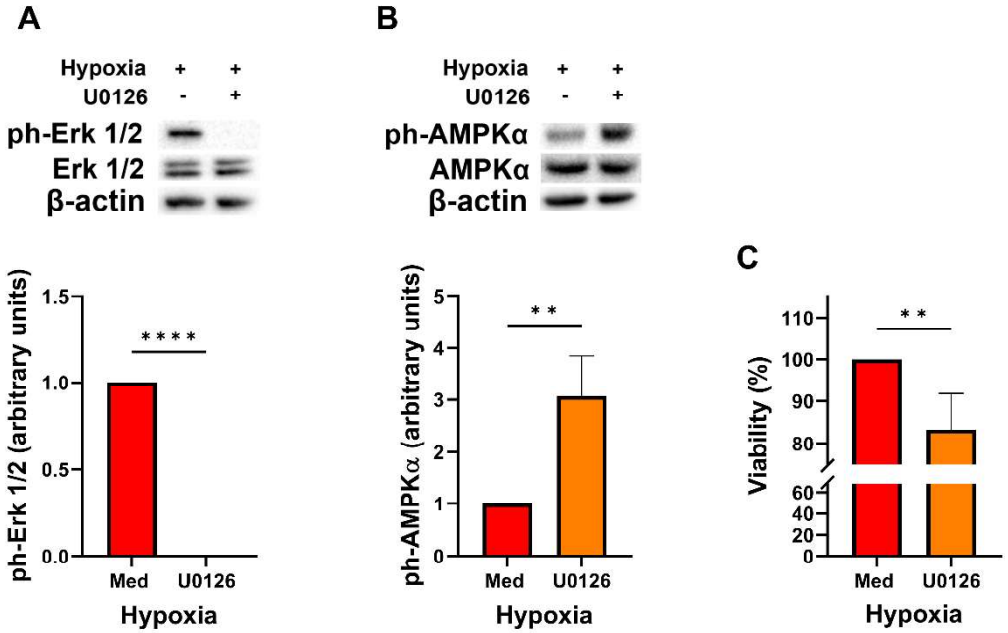


Figure 18. Erk 1/2 inhibition by U0126 affects AMPK activation and cell viability, in hypoxic iDCs. (A) ph-Erk 1/2 and (B) ph-AMPKα protein levels, in iDCs exposed to hypoxia for 24h and treated or untreated with U0126 (5 μM), as determined by Western Blot. (C) Cell viability, by fluorescein diacetate assay, in iDCs exposed to hypoxia for 48h and treated or untreated with U0126 (5 μM). Quantification of protein expression was performed by using β-actin as loading control. Viability is expressed as the percentage of the ratio between the treated and untreated groups. All blots shown are representative of at least three independent experiments and data are shown as the mean ± SEM of at least 3 independent experiments. ** and **** indicate statistically significant differences ($p \leq 0.01$ and $p \leq 0.001$ respectively).

4.5 p62 inhibition impairs hypoxic iDC viability by affecting the Erk 1/2-AMPK crosstalk.

Since the impairment of iDC viability under hypoxia, upon p62 knock down, did not correlate with alteration in HIF-1 transcriptional activity, and keeping with the finding that Erk 1/2-AMPK crosstalk participates in iDC survival maintenance upon O_2 shortages, we investigated whether p62 could modulate the balance between these two factors. To this end, we firstly assessed, by Western blotting, their phosphorylation levels in iDCs transfected with either Ctr or p62 targeting siRNA and exposed for 24 h to normoxia. As reported in Figure 19 A, p62 inhibition did not significantly affect Erk 1/2 and AMPK activation in normoxic iDCs. Differently, hypoxic iDCs transfected with the p62 targeting siRNA showed a significant downregulation of Erk 1/2 phosphorylation, which was paralleled by a strong increase in AMPKα activation (Figure 19 B). These data indicate that p62 inhibition, by RNA interference, impairs iDC adaptation to hypoxia, by affecting cell viability in a manner which, at least in part, depends on Erk 1/2-AMPK axis impairment.

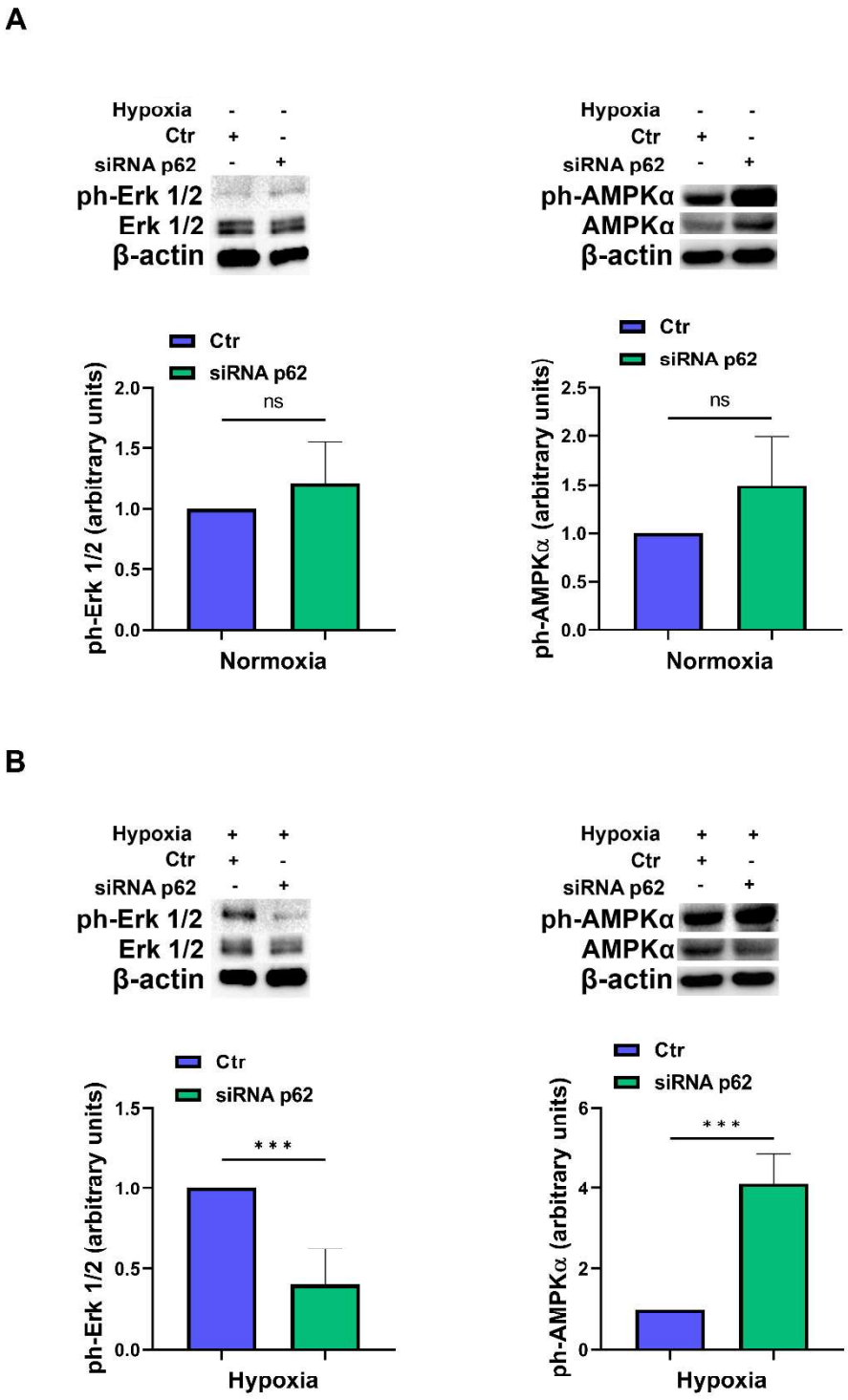


Figure 19. Impairment of Erk 1/2 and AMPK upon p62 inhibition, in normoxic and hypoxic iDCs. (A) ph-Erk 1/2 and ph-AMPKα protein levels, in iDCs transfected with Ctr or siRNA p62 and exposed to normoxia for 24 h, as determined by Western Blot. (B) ph-Erk 1/2 and ph-AMPKα protein levels, in iDCs transfected with Ctr or siRNA p62 and exposed to hypoxia for 24 h, as determined by Western Blot Quantification of protein expression was performed by using β-actin as loading control. All blots shown are representative of at least three independent experiments and data are shown as the mean ± SEM of at least 3 independent experiments. *** indicate statistically significant differences ($p \leq 0.005$).

5. RESULTS (PART II): CLASS III PI3K/ Vps34 INVOLVEMENT IN HYPOXIA-INDUCED AUTOPHAGY IN MATURE DCs.

5.1 Hypoxia induces autophagy in maDCs.

It has been previously demonstrated that, differently from iDCs, maDC adaptation to hypoxia is mainly mediated by PI3Ks. Indeed, TLR-4 activation, upon LPS stimulation, results in the induction of several pro-survival programs, in which the Class I PI3K/Akt pathway plays a pivotal role. In addition, in the last years, evidence has emerged concerning autophagy enhancement upon hypoxic exposure and its involvement in maDC survival (Monaci et al., 2020; Naldini et al., 2012).

We here evaluated whether PI3Ks and autophagy cooperate to protect maDCs from the hypoxic insult and we investigated the underlying molecular mechanism. To this end, we took into account Class III PI3K, also known as Vps34, and we tried to address whether it was involved in hypoxia-induced autophagy in maDCs.

Firstly, we determined the ability of hypoxia to promote the autophagic process in maDC.

We analyzed, by Western blotting, HIF-1α accumulation in LPS stimulated DCs, upon 24 h of hypoxic treatment. As expected, hypoxia induced a significant increase in HIF-1α protein levels (Figure 20 A). As shown in Figure 20 B, this finding was paralleled by a remarkable downregulation of p62 protein levels and by a significant increase in Erk 1/2 activating phosphorylation, in hypoxic maDCs when compared to the normoxic control. p62 decrease, which is known to be dependent on its degradation within autolysosomes, and ph-Erk upregulation suggested that hypoxia triggered the induction of autophagy along with the Erk 1/2 mediated pro-survival program.

To further assess the ongoing autophagic flux, we analyzed, by Western blotting, the activation of mTOR, which is responsible for autophagy inhibition. Such activation was evaluated by its phosphorylation at Ser2448 and by determining its ability to inhibit ULK1, by directly phosphorylating it at Ser757.

As shown in Figure 20 C, maDCs exposed to hypoxia for 24 h were characterized by a strong reduction in mTOR activating phosphorylation, which was paralleled by a significantly reduced ULK1 inhibitory phosphorylation.

Together these data indicated that hypoxia is able to promote autophagy by reducing mTOR activation and the consequent ULK1 inhibition.

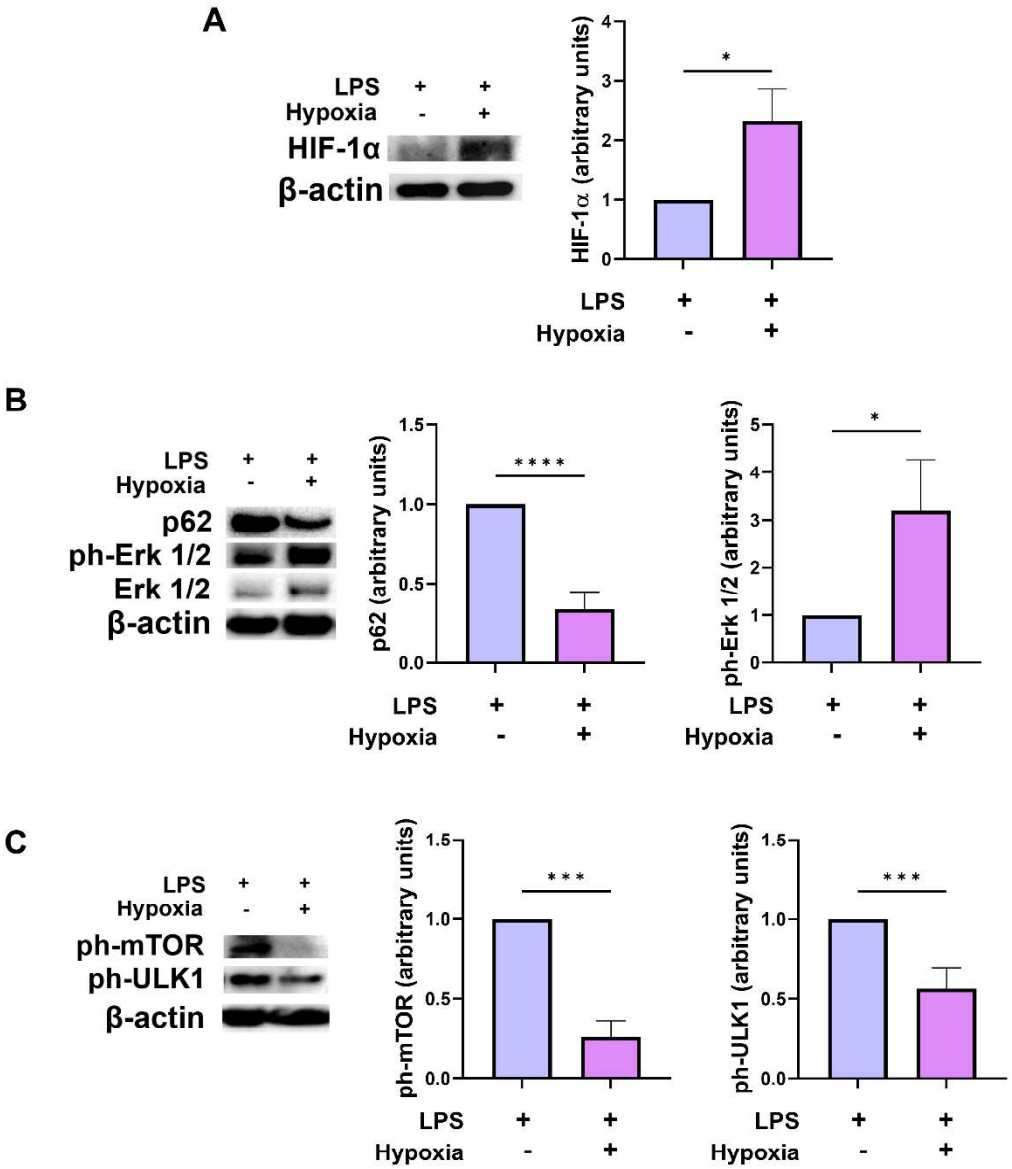


Figure 20. Hypoxia induces HIF-1α accumulation along with p62 degradation a mTOR inhibition in maDCs. (A) HIF-1α, (B) p62 and ph-Erk 1/2, (C) ph-mTOR and ph-ULK1 (Ser757) protein levels in DCs stimulated with LPS and exposed to either normoxia or hypoxia for 24 h, as determined by Western Blot. Quantification of protein expression was performed by using β-actin as loading control. All blots shown are representative of at least three independent experiments and data are shown as the mean ± SEM of at least 3 independent experiments. *, *** and **** indicate statistically significant differences ($p \leq 0.05$, $p \leq 0.005$ and $p \leq 0.001$ respectively).

5.2 Vps34 inhibition by SAR405 impairs hypoxia-induced autophagy in maDCs.

Keeping with the finding that hypoxia induced autophagy in our maDCs, we next tried to address the potentially involved molecular mechanism. In particular, we determined whether Vps34 was implicated in this process, by inhibiting it with SAR405. Indeed, this compound was demonstrated to prevent autophagy induction in other cellular models (Ronan et al., 2014).

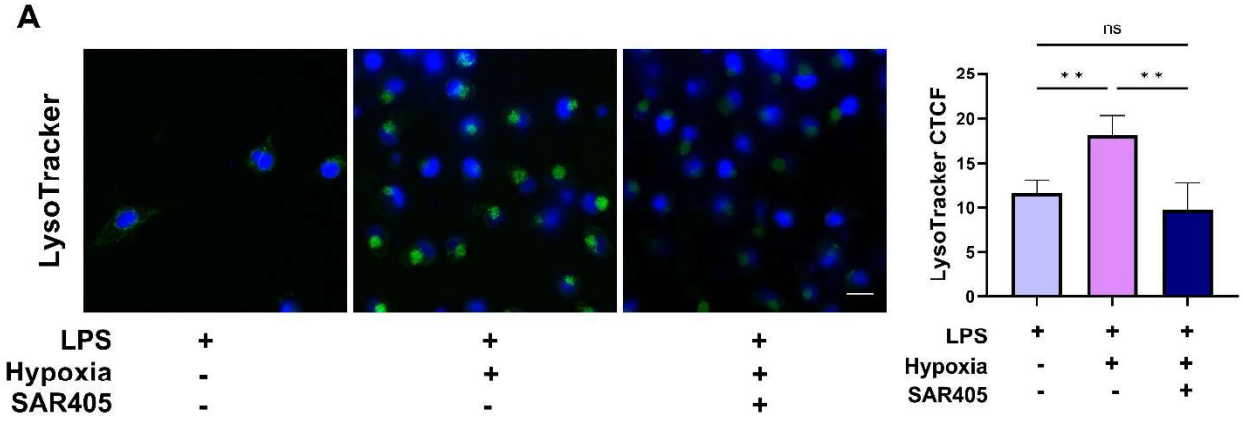
As a first step, we measured, by LysoTracker staining and confocal microscopy, the acidification of the auto-lysosomal compartment, which represent an index of the ongoing autophagic flux, in maDCs exposed to nromoxia or hypoxia for 24 h and treated or untreated with SAR405, to determine the impact of SAR405 in autophagy inhibition.

Interestingly, we reported, in Figure 21 A, that while hypoxia promoted an enhancement in lysosomal acidification, SAR405 completely reverted this effect in hypoxic maDCs.

Then, we determined the effect of this inhibitor on Vps34 protein levels as well as its impact on Beclin1, since it is a component of Vps34 complex I and it is required for proper complex activation and autophagy induction.

We observed (Figure 21 B) that hypoxic maDCs treated with SAR405 were characterized by a significant downregulation of both Vps34 and Beclin1 protein levels, when compared to the untreated control.

These data indicated that SAR405 was able to impair hypoxia-induce autophagy in maDCs, by affecting protein stability of Vps34 complex I members (Vps34 and Beclin1) and reducing the flowing autolysosomal acidification. Thus, we concluded that class III PI3K/Vps34 plays a pivotal role in hypoxia-induced autophagy in LPS-stimulated DCs.



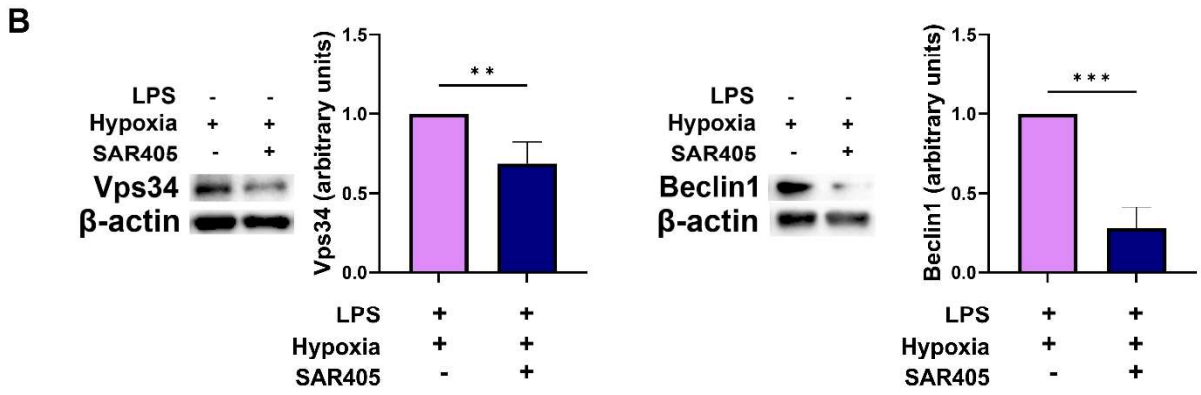


Figure 21. Vps34 inhibition by SAR405 prevents activation of hypoxia-induced autophagy in maDCs. (A) Detection of acidic/lysosomal compartments, by LysoTracker staining and confocal analysis (Scale bar: 15 μm), in DCs stimulated with LPS, treated or untreated with SAR405 (10 μM) and exposed to either normoxia or hypoxia for 24 h. (B) Vps34 and Beclin1, as determined by Western blot, in DCs stimulated with LPS, treated or untreated with SAR405 and exposed to hypoxia for 24 h. and Quantification of protein expression was performed by using β-actin as loading control. All blots shown are representative of at least three independent experiments and data are shown as the mean ± SEM of at least 3 independent experiments. ** and *** indicate statistically significant differences ($p \leq 0.01$ and $p \leq 0.005$ respectively).

5.3 Vps34 inhibition affects hypoxic maDC viability and function.

Upon assessment of Vps34 involvement in hypoxia-induced autophagy, we investigated whether it was required for maDC viability and function maintenance in response to O_2 shortages. In particular, we evaluated whether Vps34 inhibition could affect pro-survival and pro-inflammatory pathways in hypoxic maDCs.

To this end, maDCs, treated or untreated with SAR405, were exposed to hypoxia for 24 h; then, Erk 1/2 and p38 activating phosphorylations were assessed by Western blotting. Indeed, while Erk 1/2 positively regulates DC viability, p38 was demonstrated to exert an opposite effect (Xie et al., 2005). Interestingly, as shown in Figure 22 A, along with autophagy impairment, Vps34 inhibition resulted in a significant decrease in Erk 1/2 activation in hypoxic maDCs. On the contrary, p38 activating phosphorylation was remarkably upregulated in the same condition (Figure 22 B). These findings suggest that Vps34 inhibition was able to impair maDC viability under hypoxia. This hypothesis was further confirmed by the fluorescein diacetate assay, through which we observed a significant decrease in percentage of viable maDC upon SAR405 treatment (Figure 22 C).

These data indicated that Vps34 exerts a crucial role in maDC adaptation to hypoxia, promoting cell survival via induction of autophagy and by modulating Erk 1/2 and p38 signaling pathways.

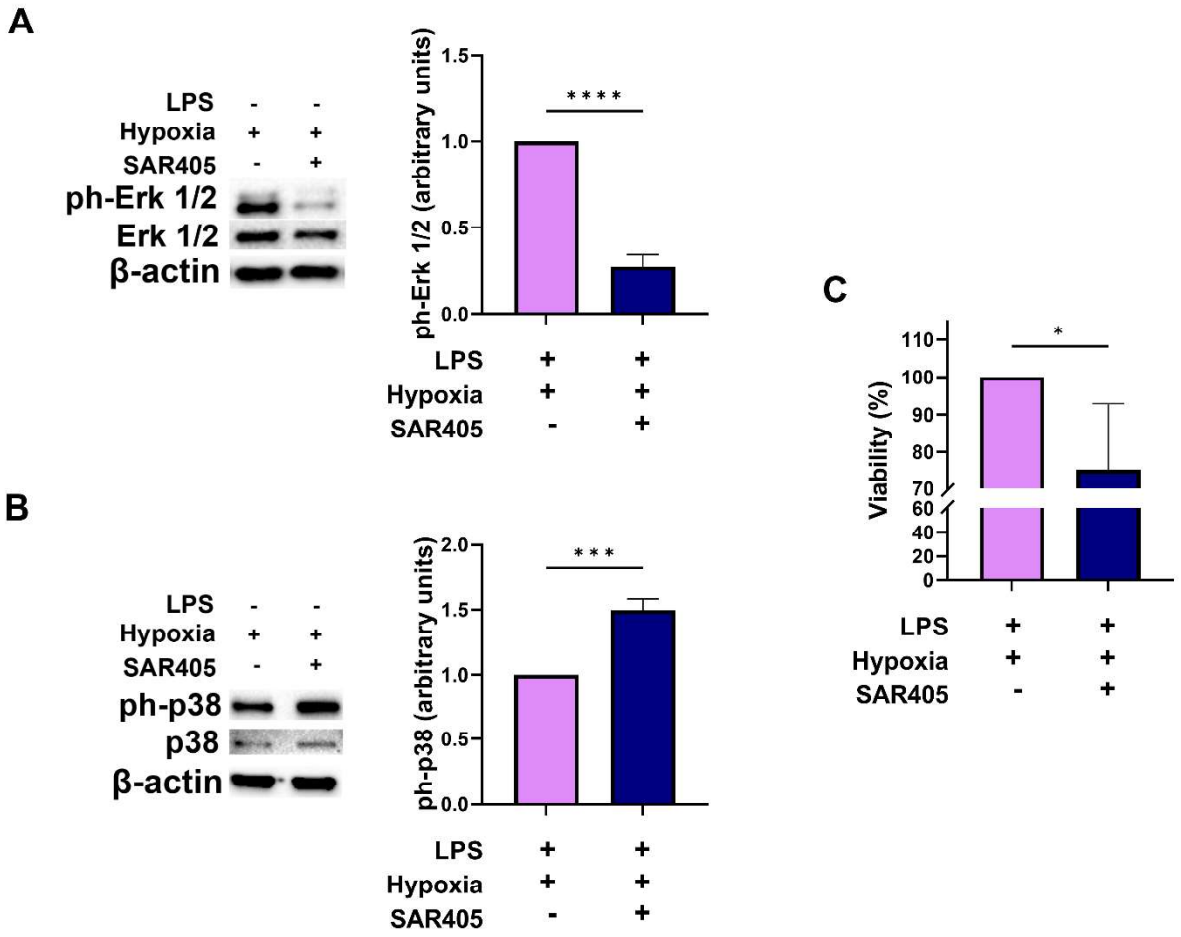


Figure 22. Vps34 inhibition by SAR405 impairs maDC viability. (A) ph-Erk 1/2 and (B) ph-p38 protein levels as determined by Western blot, in DCs stimulated with LPS, treated or untreated with SAR405 and exposed to hypoxia for 24 h. (C) Cell viability, by fluorescein diacetate assay, DCs stimulated with LPS, treated or untreated with SAR405 and exposed to hypoxia for 24 h. Quantification of protein expression was performed by using β-actin as loading control. Viability is expressed as the percentage of the ratio between the treated and untreated groups. All blots shown are representative of at least three independent experiments and data are shown as the mean ± SEM of at least 3 independent experiments. *, *** and **** indicate statistically significant differences ($p \leq 0.05$, $p \leq 0.005$ and $p \leq 0.001$ respectively).

Since p38 not only modulates maDC survival but it is also involved in their immune function, we next investigated whether its enhancement upon Vps34 inhibition was associated to the impairment of pro-inflammatory signals. To this end, we took into account IL-12 (p40), IL-6 and TNFα and we measured, by RT-qPCR, their mRNA expression levels in maDCs exposed to hypoxia for 24 h and treated or untreated with SAR405. As presented in Figure 23, Vps34 inhibition resulted in a significant upregulated mRNA expression of these three pro-inflammatory cytokines in hypoxic maDCs, highlighting the importance of Vps34-mediated autophagy in maDC adaptation to hypoxia, since it not only prevents cell death but also promotes cell function maintenance.

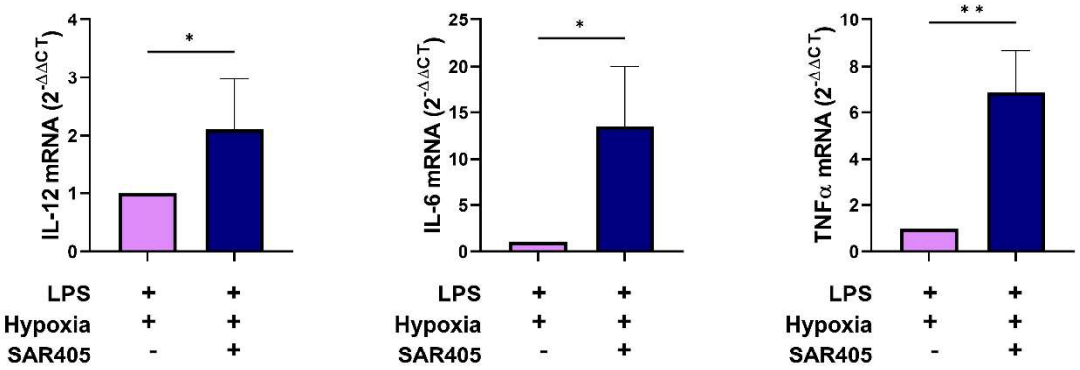


Figure 23. Vps34 inhibition by SAR405 enhances pro-inflammatory cytokine production in hypoxic maDCs. IL-12 (p40), IL-6 and TNFα mRNA expression levels, as determined by RT-qPCR, in DCs stimulated with LPS, treated or untreated with SAR405 and exposed to hypoxia for 24 h. β-actin and L-32 were used as housekeeping genes for RT-qPCR analysis. Data are shown as the mean ± SEM of at least 3 independent experiments. * and ** indicate statistically significant differences ($p \leq 0.05$ and $p \leq 0.01$ respectively).

5.4 *Addendum.*

Part of these data have been published in:

- Coppola, F., Monaci, S., Falsini, A., Aldinucci, C., Filippi, I., Rossi, D., Carraro, F. & Naldini, A. (2024). SQSTM1/p62 inhibition impairs pro-survival signaling in hypoxic human dendritic cells. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1871*(2), 119625.
- Monaci, S., Coppola, F., Rossi, D., Giuntini, G., Filippi, I., Marotta, G., Sozzani, S., Carraro, F. & Naldini, A. (2022). Hypoxia induces autophagy in human dendritic cells: involvement of class III PI3K/Vps34. *Cells*, *11*(10), 1695.

6. DISCUSSION (PART I and II).

Hypoxia characterizes healthy tissues, including lymphoid organs and immunological niches, in which the pO_2 varies among 10 and 40 mmHg (against 100 mmHg of arterial blood) (Sitkovsky & Lukashev, 2005) as well as inflammatory process and the TME, that, together, represent the sites where immune cells are required to exert their functions. Thus, DCs frequently experience O_2 tension changes, regardless to their maturation stage. Indeed, during their lifespan, DCs continuously move across normoxic and hypoxic microenvironments, which may affect their viability and function, modulating DC-mediated immune responses and influencing their quality (McGettrick & O'Neill, 2020).

DCs have developed a great variety of cellular strategies to cope with hypoxia and most of them are mediated by the HIFs, and in particular by HIF-1. Indeed, hypoxia-induced HIF-1 transcriptional activity has been demonstrated to modulate DC physiology in a manner, which depends on their maturation stage. Indeed, while in iDC, which are more susceptible to the hypoxic stress, HIF-1α accumulation is accompanied by a pro-apoptotic program, in maDCs it is involved in migration enhancement and in functional activity modulation (Filippi et al., 2014; Winning & Fandrey, 2016).

Beyond HIF-1, recent studies have highlighted the involvement of other molecules and processes in cellular adaptation to O_2 shortages and deprivation (Mazure & Pouyssegur, 2010), which may influence DC biology and positively regulate their viability and function maintenance upon hypoxic exposure. Among them, autophagy and Class I PI3K have emerged as major mediators of DC responses to hypoxia, even in this case acting differently in iDCs and maDCs (Ghislat & Lawrence, 2018; Monaci et al., 2020; Naldini et al., 2012).

Thus, we here tried to identify, among autophagic proteins and PI3Ks, novel molecular mediators of DC adaptation to hypoxia, being careful to distinguish between iDC and maDC specific responses.

To this end, we evaluated p62 and Class III PI3K (Vps34), which are involved in cargo selection and in phagophore nucleation, respectively, in our experimental model by using human monocyte-derived DC, stimulated or not with LPS. The hypoxic experiments were conducted at a pO_2 of 14 mmHg (2% O_2) to mimic the physiological hypoxic microenvironment, characteristic of immunological niches.

Firstly, we investigated the impact of p62 on hypoxic iDC survival, since previous reports have demonstrated that autophagy-dependent p62 degradation under hypoxia is promoted in DCs (Monaci et al., 2020) and that its inhibition in several cancer cell lines is paralleled by enhanced autophagy-mediated programmed cell death (Nihira et al., 2014). Accordingly, we here reported that HIF-1α accumulation was paralleled by decreased p62 protein levels at different time points in hypoxic iDCs, and that p62 inhibition, by RNA interference, was associated with the impairment of DC viability upon exposure to hypoxia. Since p62 acts not only as a selective autophagy receptor, but also as a

signaling hub for several intracellular pathways, we evaluated whether the underlying molecular mechanism, by which it regulates iDC survival, involved HIF-1. However, in contrast with a precious report showing that p62 knockdown affects HIF-1 activity (Chen et al., 2016), in our study, p62 inhibition did not affect HIF-1α protein levels neither the expression of BNIP3 mRNA, which is transcriptionally regulated by HIF-1. Thus, we next considered Erk 1/2, which directly interacts with the PB1 domain of p62 (Puissant et al., 2012). In accordance with a previous work showing that Erk 1/2 is enhanced by hypoxia, and so promoting a pro-survival program (Minet et al., 2000), we here observed that hypoxia strongly upregulated its activation in iDCs. Of note, it has been reported, in a previous study, that a crosstalk between Erk 1/2 and AMPK controls the lifespan of maDC, since the ability of Erk to prevent AMPK activation and thus to inhibit its pro-apoptotic function (López-Cotarelo et al., 2015). Accordingly, we here found out that Erk 1/2 induction in hypoxic iDCs was accompanied by a significant decrease of AMPKα phosphorylation. Moreover, we reported that p62 knock down completely reverted the balance between these two kinases, downregulating Erk 1/2 and upregulating AMPK activation in iDC exposed to hypoxia. Interestingly, Erk 1/2-AMPK crosstalk was not affected by p62 inhibition in normoxic iDCs, explaining why viability impairment was more pronounced and statistically significant only in iDCs exposed to hypoxia. Together these data suggest that p62 plays a pivotal role in iDC adaptation to hypoxia positively regulating Erk 1/2 mediated pro-survival program.

Next, we investigated the impact of Vps34 in maDC adaptive responses to O_2 shortages, in terms of survival and autophagy as well as of function maintenance. Indeed, previous studies have shown that the autophagic flux in DCs is enhanced by hypoxic exposure, especially upon LPS/TLR4 induced terminal maturation, protecting maDCs from hypoxic cell death (Monaci et al., 2020). Moreover, evidences of class I and class III PI3Ks involvement in autophagy induction have been collected in several cell types (Mercer et al., 2021), including DCs (Monaci et al., 2020).

Accordingly, we here showed that hypoxia, in parallel with HIF-1α accumulation, induced autophagy in DCs stimulated with LPS, consequently promoting p62 protein level decrease, which depends on its degradation within autolysosomes. Moreover, in agreement with the hypothesis that autophagy protects cells from the hypoxic insult, as previously described for other cell types (Hu et al., 2012; Zhang et al., 2015), we reported that p62 degradation was paralleled by a strong increase of Erk 1/2 activating phosphorylation, which is known to promote cell survival.

Then, we further confirmed the autophagic induction by analyzing mTOR activation and its capability to phosphorylate ULK1 at Ser757. Indeed, mTOR negatively regulates autophagy through direct phosphorylation of ULK1 Ser757 (Kim et al., 2011). We found out that hypoxia almost completely abolished mTOR activation and ULK1 inhibitory phosphorylation in LPS stimulated DCs.

Once assessed that hypoxia enhanced autophagy, we inhibited Vps34 in hypoxic maDCs to establish its involvement in this process and we observed, in accordance with literature (Ronan et al., 2014), that such inhibition by SAR405 impaired the autophagic flux. Indeed, it reverted the hypoxia-induced enhancement of lysosome acidification, which is an index of lysosomal activity upregulation. Moreover, SAR405 not only induced a remarkable decrease of Vps34 protein levels, but it also downregulated Beclin1 which is essential for the activation of Vps34 complex I and thus for phagophore nucleation and initiation of autophagy.

Interestingly, we determined that SAR405-mediated autophagy inhibition in hypoxic maDCs not only strongly impaired their viability, decreasing Erk 1/2 phosphorylation and upregulating p38 activation, but also increased IL-12, IL-6, and TNF-α expression, in agreement with a previous work showing that SAR405 treatment enhances expression of pro- and anti-inflammatory cytokine in bone marrow-derived DCs (Pittini et al., 2016). Therefore, we concluded that Vps34 not only is essential to ensure maDC survival, but is also required for proper function maintenance in response to O_2 shortages, since Vps34-mediated autophagy provides inflammation dampening and immune modulation.

These data clearly demonstrated that Vps34 is essential in hypoxia induced autophagy and highlighted its involvement in LPS-stimulated DC adaptation to hypoxia.

7. RESULTS (PART III).

7.1 Hypoxia induces RNASET2 expression along with HIF-1α accumulation in iDCs.

RNASET2, which plays a pivotal role in innate immunity, has been proposed to have oncosuppressive properties, modulating normal and cancer cell viability and proliferation rates, in response to a variety of stress conditions, which may include hypoxia.

We here investigated whether RNASET2 was involved in DC responses to the hypoxic stress. In particular, we evaluated whether hypoxia could upregulate RNASET2 expression in DCs and whether such upregulation was associated to DC viability impairment.

To this end, we considered that prolonged hypoxia, as previously demonstrated, impairs iDC survival through a mechanism involving HIF-1 transcriptional reprogramming, which promotes the expression of pro-apoptotic factors. Indeed, it has been demonstrated that, up to 48 h hypoxic exposure, iDC cell death is strongly enhanced and that this effect can be prevented by HIF-1α inhibition (Naldini et al., 2012).

Thus, we exposed human monocyte-derived iDCs to normoxia (pO_2 of 140 mmHg) or hypoxia (pO_2 of 14 mmHg) for 48 h, and we analyzed HIF-1α accumulation, by Western blotting, and iDC viability, by fluorescein diacetate assay. As expected, the remarkable increase of HIF-1α protein levels in hypoxic iDCs was accompanied by the finding that the percentage of viable iDCs was significantly reduced under hypoxia, when compared to the normoxic control.

Then, we measured RNASET2 expression, at both protein and mRNA level, by Western blotting and RT-qPCR, in normoxic and hypoxic iDCs.

Interestingly, we found out that prolonged hypoxia (48 h) was able to significantly upregulate RNASET2 protein as well as mRNA expression (Figure 24 C).

These data suggest that not only RNASET2 can be induced in response to the hypoxic insult, but also that such enhancement is associated with a pro-cell death phenotype of iDCs.

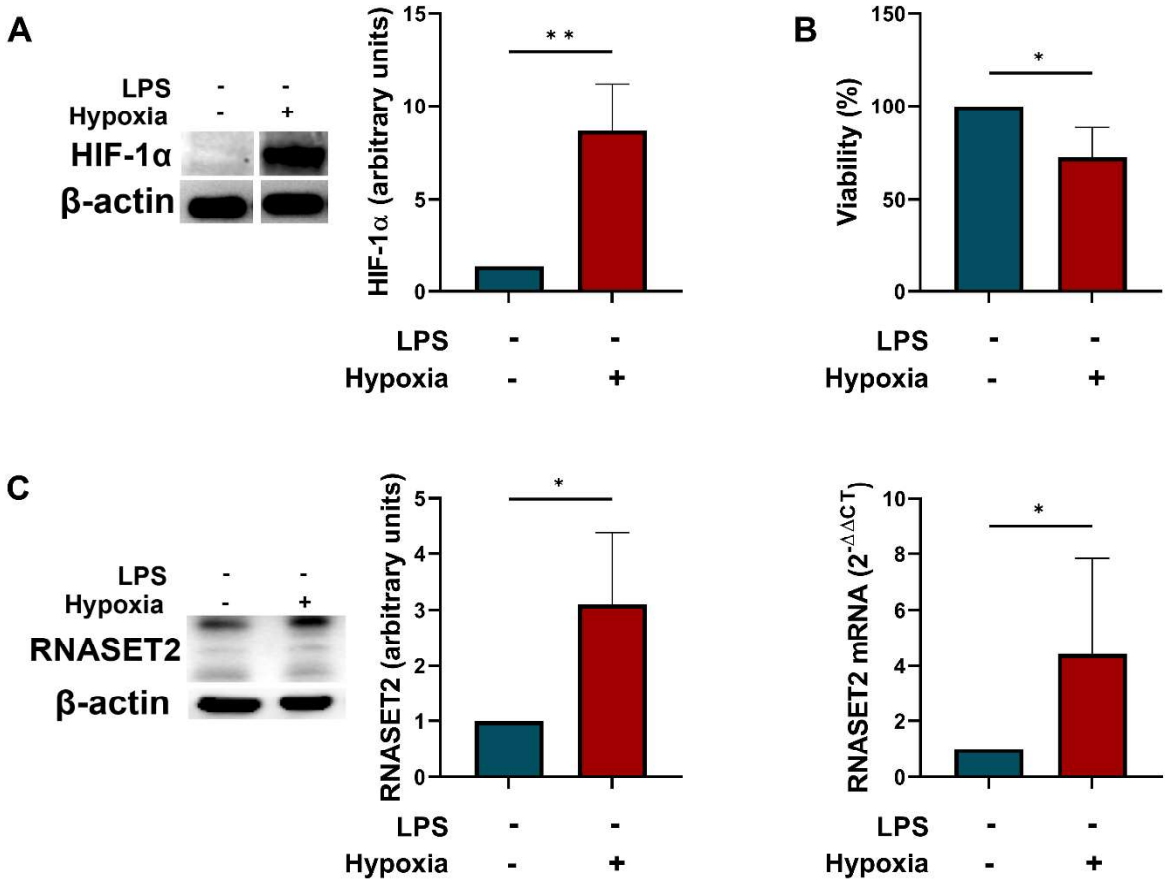


Figure 24. HIF-1α accumulation, iDC viability and RNASET2 expression in nromoxic and hypoxic iDCs. (A) HIF-1α protein levels determined by Western Blot and (B) Cell viability, by fluorescein diacetate assay, in iDCs exposed to normoxia or hypoxia for 48 h. (C) RNASET2 protein levels (37, 31 and 27 kDa) and mRNA expression, as determined by Western Blot and. RT-qPCR respectively. Quantification of protein expression was performed by using β-actin as loading control. β-actin and L-32 were used as housekeeping genes for RT-qPCR analysis. Viability is expressed as the percentage of the ratio between the hypoxic and normoxic groups All blots shown are representative of at least three independent experiments and data are shown as the mean ± SEM of at least 3 independent experiments. * and ** indicate statistically significant differences ($p \leq 0.05$, and $p \leq 0.01$ respectively).

7.2 LPS-induced DC terminal maturation prevents RNASET2 expression.

Keeping with the finding that hypoxia-induced RNASET2 upregulation was associated with the impairment of iDC viability, we next evaluated whether pro-survival stimuli can affect the expression of this novel ribonuclease.

To this end, we induced DC terminal maturation, by stimulating monocyte-derived DCs with LPS, and we investigated whether LPS-induced TLR4 activation could prevent RNASET2 expression. Indeed, it has been previously demonstrated that, upon LPS binding, TLR4 promotes Class I PI3K/Akt signaling pathway activation, which in turn enhances maDC viability and function, both under normoxia and hypoxia (Naldini et al., 2012; Rescigno et al., 1998).

After exposing, both iDCs and maDCs, to normoxia for 48 h, we analyzed Akt activating phosphorylation to assess the pro-survival effect of LPS on DCs.

As expected, Akt phosphorylation was enhanced by LPS stimulation in normoxic DCs (Figure 25 A). Interestingly, we observed that Akt activation was paralleled by a significant decrease of RNASET2 protein and mRNA expression in maDCs, when compared to LPS-unstimulated cells, as shown in Figure 25 B.

We next exposed iDCs and maDCs to prolonged hypoxia (48 h) and analyzed, even in this condition, Akt activating phosphorylation as well as RNASET2 expression.

We found out that, under hypoxia, maDCs were characterized by a significantly higher Akt phosphorylation then iDCs (Figure 25 C), in accordance with the previous finding, by Naldini et al., that LPS protects DC from hypoxia-induced cell death by triggering PI3K/Akt pro-survival pathway. Surprisingly, in parallel, LPS stimulation completely prevented hypoxia-induced RNASET2 expression (at both protein and mRNA level), as shown in Figure 25 D.

These data strongly highlight the correlation between RNASET2 and DC viability impairment, since in our experimental model LPS, which represent a pro-survival stimulus, was able to almost completely abolish RNASET2 expression.

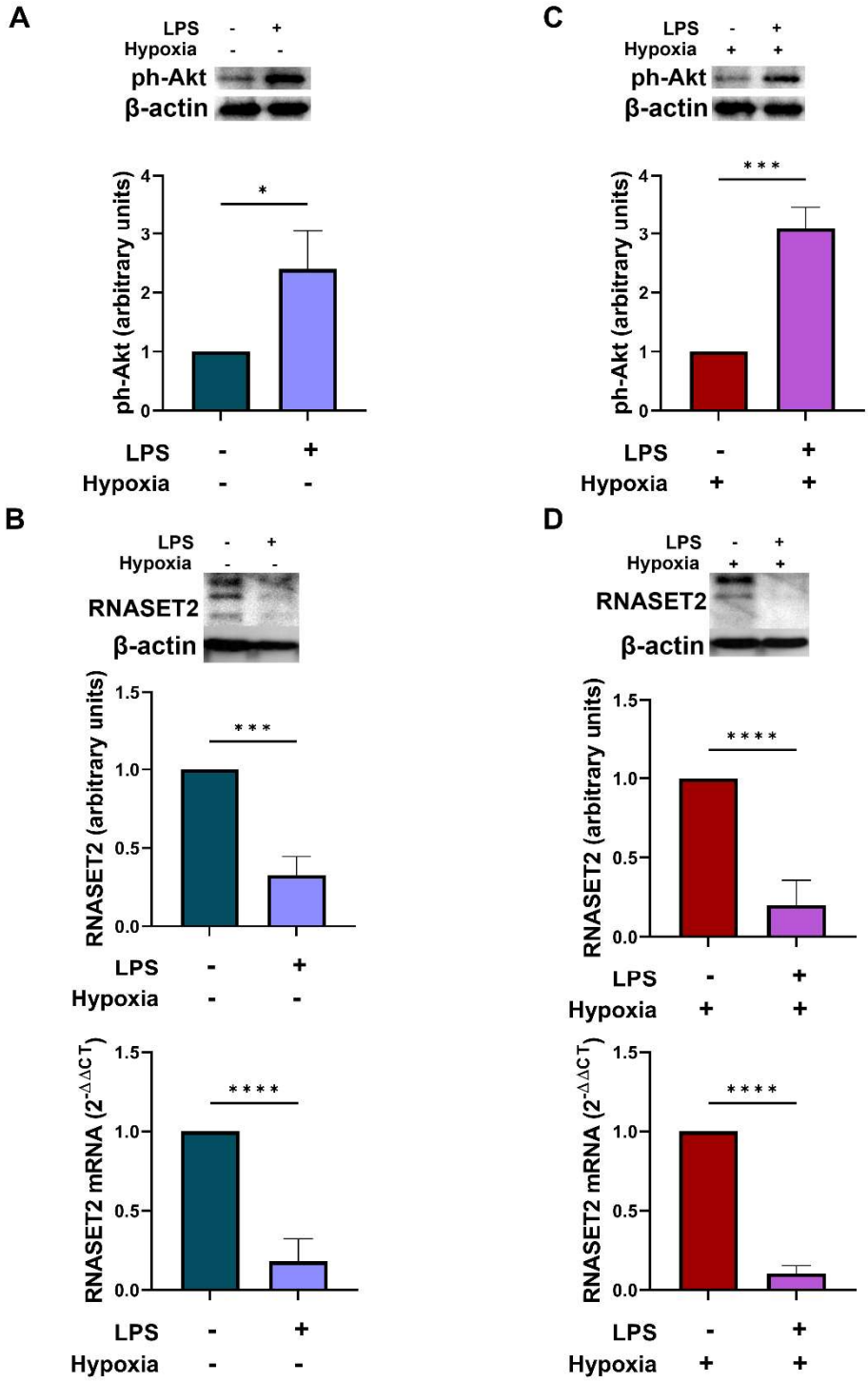


Figure 25. LPS promotes Akt phosphorylation and reduces RNASET2 expression in normoxic and hypoxic DCs. (A) ph-Akt protein levels determined by Western Blot and (B) RNASET2 protein levels (37, 31 and 27 kDa) and mRNA expression, as determined by Western Blot and RT-qPCR respectively, in DCs stimulated or unstimulated with LPS exposed to normoxia for 48 h. (C) ph-Akt protein levels determined by Western Blot and (B) RNASET2 protein levels and mRNA expression, as determined by Western Blot and RT-qPCR respectively, in DCs stimulated or unstimulated with LPS exposed to hypoxia for 48 h. Quantification of protein expression was performed by using β-actin as loading control. β-actin and L-32 were used as housekeeping genes for RT-qPCR analysis. All blots shown are representative of at least three independent experiments and data are shown as the mean ± SEM of at least 3 independent experiments. *, *** and **** indicate statistically significant differences ($p \leq 0.05$, $p \leq 0.005$ and $p \leq 0.001$ respectively).

7.3 PI3K/Akt inhibition enhances hypoxia-induced RNASET2 expression in maDCs.

Since we hypothesized that RNASET2 expression is associated with cell death promotion in response to the hypoxic insult, having an opposite behavior when compared to the pro-survival PI3K/Akt pathway, we here investigated whether Class I PI3K inhibition, by Wortmannin, could enhance hypoxia-induced RNASET2 expression, even in DCs stimulated with LPS, and how it affected maDC survival.

To this end, we exposed maDCs, treated or untreated with Wortmannin, to prolonged hypoxia (48 h) and then we analyzed, by Western blotting, Akt activating phosphorylation and RNASET2 expression.

Interestingly, as shown in Figure 26 A and B, we found out that, along with PI3K/Akt inhibition, Wortmannin was able to restore hypoxia-induced RNASET2 expression in maDCs, which was prevented by LPS-mediated pro-survival effects. This finding was accompanied by the observation that hypoxic maDC viability, assessed by fluoresceine diacetate assay, significantly decreased upon Wortmannin treatment.

These data further confirm that RNASET2, which expression is strongly upregulated in response to hypoxic stress, is associated with DC viability impairment and it is negatively affected by LPS, which, in turns, promotes DC survival and adaptation to hypoxia, mainly through the PI3K/Akt pathway.

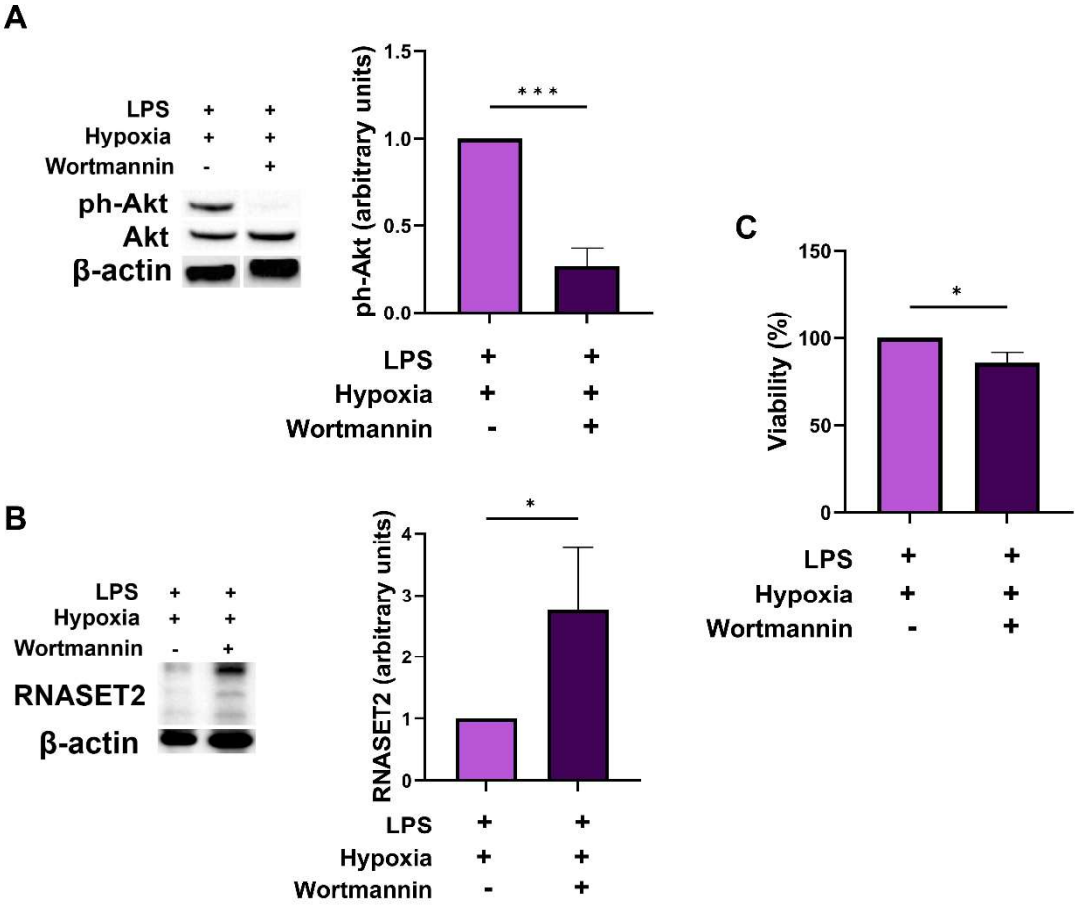


Figure 26. Wortmannin, along with PI3K/Akt inhibition, enhances RNASET2 expression and decreases maDC viability. (A) ph-Akt and (B) RNASET2 protein levels (37, 31 and 27 kDa) as determined by Western Blot, in DC stimulated with LPS, treated or untreated with Wortmannin (5μM) and exposed to hypoxia for 48 h (C) Cell viability, by fluorescein diacetate assay, i in DC stimulated with LPS, treated or untreated with Wortmannin (5μM) and exposed to hypoxia for 48 h. Quantification of protein expression was performed by using β-actin as loading control. Viability is expressed as the percentage of the ratio between the treated and untreated groups. All blots shown are representative of at least three independent experiments and data are shown as the mean ± SEM of at least 3 independent experiments. * and *** indicate statistically significant differences ($p \leq 0.05$, and $p \leq 0.005$ respectively).

7.4 *Addendum.*

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8. DISCUSSION (PART III).

Among T2 endoribonucleases, RNASET2 is the only one discovered in humans, and, in the last years, several studies have tried to address its function and regulation. We here investigated whether RNASET2 expression was modulated by hypoxia and how it affected DC viability, keeping with the finding that RNASET2 not only plays a crucial role in modulating cancer cell survival and proliferation, but also participates in innate immune responses. Indeed, it is able to trigger the activation of TLR8 through its degradation products (Greulich et al., 2019; Ostendorf et al., 2020).

To this end, we firstly exposed LPS-unstimulated human monocyte-derived DCs to prolonged hypoxia (48 h of exposure to a pO_2 of 14 mmHg), which has been reported to promote an apoptotic program in this cellular model (Naldini et al., 2012). We observed that RNASET2 overexpression, at both protein and mRNA level, was dependent upon hypoxia, in agreement with Lualdi et al., who have identified two putative HIF-1 binding sites in RNASET2 promoter, suggesting a role for this newly discovered ribonuclease in cellular responses to the hypoxic stress (Lualdi et al., 2015). Of interest, RNASET2 overexpression was accompanied by the decrease of iDC viability, in accordance with a previous work of Wang et al., showing that the enhancement of RNASET2 expression is strictly associated with melanocyte apoptosis (Wang et al., 2014).

Then, to further establish the involvement of RNASET2 in DC death, we evaluated whether LPS-stimulation, which is known to mediate, along with DC terminal maturation, a pro-survival program by inducing the PI3K/Akt pathway (Li et al., 2017), was able to affect RNASET2 expression. Although a previous study has shown that LPS positively regulates RNASET2 in *Hirudo verbana* granulocytes (Baranzini et al., 2020), we here reported that TLR4 activation upon LPS stimulation, almost completely abolished its expression. This effect, in parallel with the increase of Akt phosphorylation, in both normoxic and hypoxic human DCs, confirmed the evidence of the crosstalk between this ribonuclease and TLRs. However, the LPS-induced impairment of RNASET2 expression was more evident and significant in DCs exposed to hypoxia. Our results support the hypothesis that this enzyme plays a pivotal role in DC death in response to the hypoxic insult and, in general, in cell responses to stressful conditions, accordingly to a previous report concerning UV irradiation and hydrogen peroxide-mediated RNASET2 increase (Wang et al., 2015).

Moreover, with our data we highlighted the inverse correlation between RNASET2 and the PI3K/Akt pathway, already reported in a previous work, in other cellular models (Xu et al., 2018). Indeed, we further characterized the PI3K/Akt signaling as a negative regulator of RNASET2 expression, by using a potent and selective inhibitor of this pathway, which, along with the impairment of Akt activating phosphorylation, restored RNASET2 expression in hypoxic LPS-stimulated DCs and

decreased their viability. This was in agreement with a work of Caputa et al. showing the association of RNASET2 expression with cell death and survival impairment in different cell types (Caputa et al., 2016).

Taken together these data indicate that a prolonged exposure to hypoxia strongly enhances RNASET2 at both protein and mRNA level, and that it may be involved in hypoxia-induced DC death. However, further studies are required to deeply assess these findings and the molecular mechanism which inversely correlates PI3K/Akt pathway and RNASET2.

9. CONCLUSIONS.

We here proposed a novel role for p62 and Vps34 in the adaptation to hypoxia of iDC and maDC respectively. We also indicated RNASET2 as a marker of DC viability impairment upon prolonged hypoxic exposure. Our study opens new perspectives on how DC respond to the hypoxic microenvironment. Indeed, the identification of novel the molecular mediators, with the underlying mechanisms, involved in hypoxia-induced modulation of DC survival and function, could be relevant not only for the treatment of autoimmune disorders but also in the improvement of cancer immunotherapy.

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