Firmato digitalmente da MONACI SARA C=IT

UNIVERSITÀ DI SIENA 1240

DIPARTIMENTO DI MEDICINA MOLECOLARE E DELLO SVILUPPO

DOTTORATO DI RICERCA IN

MEDICINA MOLECOLARE

CICLO XXXIII

COORDINATORE: Prof. Vincenzo Sorrentino

TITOLO DELLA TESI

Hypoxia and physiological adaptations: autophagy as a cell survival pathway in dendritic cells

SETTORE SCIENTIFICO-DISCIPLINARE: BIO/09

DOTTORANDO:

Dott.ssa Sara Monaci

TUTOR:

Prof.ssa Antonella Naldini

ANNO ACCADEMICO: 2020/2021

INDEX

1. INTRODUCTION 5
1.1 Hypoxia in physiology and physio-pathology 5
1.1.1 Physiological features of hypoxia 6
1.1.2 Physio-pathological aspects of hypoxia 9
1.2 Hypoxia-inducible factors (HIFs) 11
1.2.1 HIF modulation of immune cell function 13
1.2.2 HIF and inflammation 15
1.3 Dendritic cells: from the discovery to *in vitro* generation 16
1.3.1 Dendritic cell classification 18
1.3.2 Dendritic cell maturation and antigen presentation 20
1.3.3 Toll-like receptors (TLRs) as pathogen sensors 22
1.4 Dendritic cells in the hypoxic microenvironment and the role of HIF 24
1.5 Autophagy 25
1.5.1 Induction of autophagy and initiation complex 26
1.5.2 Autophagosome formation 28
1.5.3 Degradation and reuse 29
1.6 Autophagy in dendritic cells 30
2. AIM OF THE THESIS 33
3. MATERIALS AND METHODS 34
3.1 Reagents 34
3.1.1 Autophagy inhibitors 34
3.2 Human Monocyte-Derived DC preparation and culture conditions 35
3.2.1 Monocytes differentiation into immature DCs (iDCs) and mature DCs (mDCs) 36
3.3 Cell viability and detection of mitochondrial membrane potential 37
3.4 Immunofluorescence staining and confocal microscope analysis 38
3.5 Lysotracker Staining 39
3.6 Immunoblotting and Antibodies 39
3.7 RNA Extraction and RT-qPCR 40
3.8 Statistical Analysis 40
4. RESULTS 41
4.1 Hypoxia upregulates the expression of HIF-1α and target genes in DCs 41
4.2 Hypoxia enhances autophagy-associated lysosomal activity mDCs 43
4.3 Impact of hypoxia on mitochondrial membrane potential in DCs 44
4.4 Hypoxia inhibits cell death and promote cell survival in mDCs 45
4.5 Hypoxia modulates LC3B, SQSTM1/p62 and Beclin-1 expression in DCs 46

4.6 Atg protein levels are modified in DCs 49
4.7 Hypoxia affects the expression of signaling molecules potentially involved in both DCs survival and autophagy 51
4.8 Hypoxia modulates cytokine expression in DCs 53
4.9 Autophagy inhibition impairs the acidic/lysosomal compartments in hypoxic mDCs 55
4.10 Autophagy inhibition modifies the expression of LC3B and SQSTM1/p62 in hypoxic mDCs 56
4.11 Autophagy inhibition impairs the expression levels of Atg in hypoxic mDCs 57
4.12 Inhibition of autophagy modifies pro-apoptotic and anti-apoptotic markers in hypoxic mDCs 58
5. Addendum 60
6. DISCUSSION AND CONCLUSIONS 61
7. REFERENCES 65
ACKNOWLEDGMENTS 74

ABSTRACT

Hypoxia consists of a reduction in oxygen availability that may occur because of an increased oxygen demand of an impaired oxygen supply. Hypoxia-inducible factors (HIFs) are transcriptional activators that are expressed in response to cellular hypoxia and mediates multiple cellular and systemic homeostatic responses to hypoxic conditions, as well as the regulation of immune system. HIF expression and stabilization in immune cells can be induced not only by hypoxia, but also by other factors such as inflammation and infectious microorganism. Dendritic cells (DCs) are the most potent antigen presenting cells and they have a broad range of functions including pathogens detection, phagocytosis, antigen processing and activation of adaptive immune system. During their lifespan DCs are exposed to different oxygen tensions, since they patrol several tissue microenvironments and interact with T cells in primary and secondary lymphoid organs, including thymus and lymph nodes, which are characterized by hypoxia (e.g., 2–10% oxygen tension against a 21% atmospheric oxygen tension). Oxygen deprivation can induce autophagy which is a catabolic process involving an intracellular degradation system that delivers cytoplasmic constituents to the lysosome, in order to maintain cellular homeostasis and facilitate adaptation to adverse conditions. Autophagy has a great variety of physiological and physio-pathological roles such as intracellular protein and organelle clearance, elimination of microorganisms, anti-aging activity, tumour suppression, antigen presentation, development and cell death. While the autophagy in DCs was shown to affect Tool-like receptors, its regulation by the lipopolysaccharides (LPS) is still unclear. For this reason, we investigated whether hypoxia can affect autophagy in unstimulated and LPS-stimulated DCs. Along HIF-1α expression, we observed a modulation in the expression of autophagic markers as well as enhanced lysosomal activity after exposure of DCs to hypoxia. To this purpose, using immunofluorescence confocal analysis, measure of mitochondrial membrane potential, Western blotting, and RT-qPCR, we showed that the ability of LPS to modulate autophagy was strictly dependent upon oxygen levels. Indeed, LPS inhibited autophagy in aerobic conditions whereas the autophagic process was induced in a hypoxic environment. Under hypoxic conditions, LPS induced a significant increase of functional lysosomes, LC3B-II accumulation, Atg protein upregulation, and reduction of SQSTM1/p62 protein levels. This was associated with the activation of signaling pathways and expression of cytokines typically associated with DC survival and autophagy. Finally, by using Bafilomycin A1 and chloroquine, which are well known autophagic inhibitors, we confirmed the induction of autophagy by LPS under hypoxia and its impact on DC survival. Our data underline the importance of hypoxia in the physiology of DCs and may contribute to further understand DC function. Moreover, understanding the autophagy role in DC adaptation to hypoxia may have a major impact on vaccine development since it may help to improve DC survival and antigen presentation. In conclusion, our results show that autophagy plays a pivotal role in the regulation of DC survival under hypoxia upon LPS stimulation.

1. INTRODUCTION

1.1 Hypoxia in physiology and physio-pathology

Oxygen is essential for the development and growth of multicellular organisms. Despite in the early atmosphere there were low oxygen levels, living microorganisms were able to survive because of its ability to adapt and flourish in changing atmospheres. This happened through metabolic evolution towards processes such as oxidative phosphorylation, which utilizes atmospheric oxygen as a final electron acceptor for highly efficient metabolism and because of the capability of living organisms to sense levels of atmospheric gasses and adapt consequently. For this reason, the mechanisms by which unicellular organisms sense changes in environmental gasses is an area of significant and growing interest in order to better understand the microbial physiology, virulence and antibiotic resistance with particular regards to immune system evolution [8].

For mammals, is crucial to maintain proper levels of oxygen in circulation and in tissues in order to have physiological conditions since oxygen partial pressure (pO_2) decrease (20–40 mmHg) from physiological levels is sufficient to consistently change basal ventilation [9]. This is mediated by pulmonary respiration and blood circulation through effective gas exchange mechanisms where O_2 is absorbed from the atmosphere through the lungs into the bloodstream and CO_2 is release to the atmosphere, thanks to a gradient of pressure that changes between different tissues and allows a correct oxygen distribution according to metabolic cellular need [10].

One of the critical aspects of this network is the ability to sense and respond to low-oxygen conditions. This initial response can be rapid and is mediated by transcriptional and posttranscriptional mechanisms. So far, the best characterized candidate for oxygen sensor is hypoxia inducible factor-1(HIF) (see below paragraph for details) together with the prolyl hydroxylase (PHD) family of enzymes, that require molecular oxygen for activity, the NAD(P)H oxidase family of enzymes that reduce reactive oxygen species (ROS), oxygen sensitive ion channels, and the electron transport chain [11]. Hypoxia is defined as the condition where the oxygen demand is higher than the oxygen supply [12]. The causes of this condition can be several such as low in arterial blood, reduced ability to carry oxygen (e.g., anemia), reduced tissue perfusion or due to the inability of cells to use oxygen (e.g., intoxication). In addition, hypoxia can be a result of insufficient oxygen diffusion (chronic hypoxia) or insufficient perfusion (acute, transient or ischemic hypoxia) [13].

1.1.1 Physiological features of hypoxia

There are many causes of hypoxia and some of them occur even when lung function is completely normal. The most frequent situation is represented by high altitude travel. As the height increases, the barometric pressure drops, even if the percentage of oxygen remains constant, around 21%; this reduction also reduces the pO_2 [14]. When a subject quickly reaches 3,000 m the alveolar pO_2 drops to approximately 60 mmHg and memory disturbances and other brain symptoms of hypoxia may occur. At higher altitudes arterial saturation drops rapidly and symptoms become more relevant: at 5000 m, unaccustomed subjects lose normal control of functions. It is therefore evident that at altitudes >3000 m the healthy individuals find themselves in conditions that in patients would be considered "respiratory insufficiency"[15].

Another example of physiological hypoxia is that due to the increased oxygen requirements of the tissues, such as during intense physical exercise [16]. If the increased consumption of oxygen in the tissues is not compensated by an increase in their perfusion, tissue hypoxia occurs, with a consequent decrease in the pO_2 in the blood. Numerous mechanisms can be activated to balance the increased tissue needs:

- Increase in cardiac output and ventilation and, therefore, in the supply of oxygen to the tissues;
- Dysfunction of blood supply in muscles at rest, from the skin and internal organs with preferential supply to the muscles in activity;
- The increase of O_2 extraction and therefore of the arteriovenous difference of oxygen;
- The reduction of pH at tissue and capillary level, which allows a greater release of oxygen by haemoglobin (Hb).

If these mechanisms turn out to be insufficient, tissue hypoxia appears in active muscles.
In addition to tumor tissues or tissues affected by inflammatory processes, low oxygen tensions have been found in various normal tissue microenvironments. The tissue compartments differ in the degree of vascularization and therefore in their level of oxygenation. In physiological situations, the oxygen tension is proportional to the distance from the nearest capillary. Detailed studies using different oxygen measurement methods have shown that the oxygen tensions in different tissues are low enough to be considered hypoxic [17]. It must be underlined that the oxygen pressure in the inspired air is ~ 159 mmHg and that in the thymus and spleen are ~ 10 mmHg and ~ 16 mmHg, respectively. In vivo measurements on the spleens of anesthetized mice, showed that the oxygen pressure is higher near the splenic artery and gradually decreases in proportion to the distance from the artery [18].

An important physiological process controlled by oxygen levels is erythropoiesis. The main function of erythropoietin (EPO) is to maintain the concentration of hemoglobin in the blood at normal levels during physiological conditions and to accelerate the recovery of red blood cells after hemorrhages. The concentration of circulating EPO increases exponentially with the decrease in hemoglobin levels because of diseases, such as anemia, but the variable that controls the production of EPO is not the concentration of erythrocytes or hemoglobin in the blood, but the pressure of oxygen in the tissues. This depends on the concentration of hemoglobin, blood pressure, oxygen-hemoglobin affinity and blood flow [19]. This feedback control system is very sensitive, and is regulated by a group of cells located in the kidney that act as a sensor able to perceive alterations in oxygen supply. In the presence of anemia or hypoxia, the synthesis of EPO grows rapidly, more than 100 times, and consequently increases the survival, proliferation and maturation of bone marrow progenitor cells through the inhibition of apoptosis. Normal levels of EPO in the blood are about 2-25 mU / ml, but can increase 100-1000 times in response to hypoxia. The oxygen sensor mechanism leads to the interruption of EPO production when the number of red blood cells and / or the supply of oxygen to the tissues returns to normal levels. The feedback mechanism ensures adequate production of red blood cells to prevent anemia and tissue hypoxia, but not too high to lead to polycythemia with excessive blood viscosity and consequent cardiovascular risks. The overproduction of EPO, which leads to polycythemia, can result from heart or respiratory diseases, from altitude, obstructions in blood flow to the EPO production site and from EPO-producing tumors [20].

Hypoxia is a condition that characterizes embryonic development; in fact, it has been shown that HIF-1α is necessary for the survival of mesenchymal cells during the development of the embryo. HIF-1α knockout embryos die in mid-gestation, showing cardiovascular malformations and neuronal defects. The importance of hypoxia is also related to the stimulation of the vascular endothelial growth factor, VEGF, whose levels are critical for establishing a vascular network during embryonic development [21]. Indeed, during the first trimester, extravillous trophoblast cells invade into the decidua, occluding uterine spiral arterioles. This restricts blood flow into the intervillous space resulted in a low oxygen environment that is essential for placental and embryonic development. Low oxygen tension is physiological for organogenesis and is the regulatory key of cellular events in the differentiation of trophoblasts during placental development. Cytotrophoblasts, the specialized cells of the placenta, proliferate rapidly during pregnancy, differentiate into cancer-like cells and create a blood flow to the placenta, invading the uterus and its vascularization. Grown in hypoxic conditions that mimic the microenvironment near the surface of the uterus, the cytotrophoblasts continue to proliferate, but differ little; while if they are grown in a condition that mimics the environment near the arterioles of the uterus, they stop proliferating and differentiate normally [22].

Therefore, oxygen tension is essential to determine whether cytotrophoblasts proliferate or invade, thereby regulating the growth of the placenta and cellular architecture. Other studies confirm the significant effect that hypoxia has on the development of the placenta, causing hypercapillarization of the vasculature of the villi. This is due to the hypoxic regulation of angiogenic mediators such as VEGF, placental growth factor and angiopoietin. Furthermore, hypoxia increases the number of trophoblasts in the villi, regulating their proliferation [23].

Physiological oxygen gradients exist in the intestinal mucosa where the epithelium covering all mucosal tissues is supported by a rich vasculature. Even at baseline, epithelial cells lining the mucosa exist at a relatively low pO_2, herein described as physiologic hypoxia. This has been measured using nitroimidazole dyes, a class of reactive compounds that are metabolized dependent on the level of tissue oxygenation revealing that epithelial cells at the luminal edge of the colon exist normally at a pO_2 <20 mmHg [24].

Finally, physiological hypoxia is an hallmark of immunological district such as thymus and spleen where the pO_2 correspond to ~ 10 mmHg and ~ 16 mmHg respectively [18]; in bone marrow where it ranges from ~ 0-30 mmHg [25] and in lymph nodes where the pO_2 ranges from ~ 16-40 mmHg [26] (**Figure 1**).

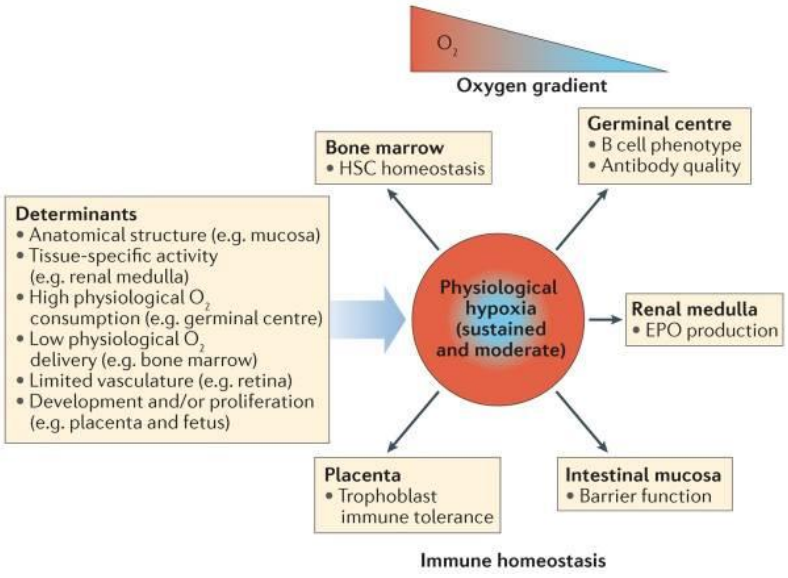


Figure 1: Schematic representation of physiological hypoxia [1]

1.1.2 Physio-pathological aspects of hypoxia

The correlation between growth factors, cytokines and hypoxia has been highlighted in various studies relating to neoplasms, immune functions and inflammatory processes. In fact, hypoxia is often associated with the inflammatory process [27]. Numerous growth factors and cytokines involved in the control of cell proliferation in hypoxic conditions have been highlighted. The production of growth factors and cytokines induces compensatory adaptation mechanisms that allow the hypoxic cell to survive and function adequately [28].

There are several factors that can contribute to tissue hypoxia during inflammation including an increase in the metabolic demands of cells and a reduction in metabolic substrates caused by thrombosis, trauma, compression (interstitial hypertension), or atelectasis (airway plugging). It must be underlined that in the case of inflamed tissue, hypoxia is not a bystander but instead can influence the environment of the tissue, particularly by regulating oxygen-dependent gene expression. Moreover, increase of intracellular pathogens can deprive infected cells of oxygen. Of interest, the increase in IL-6 levels reflects an increase in vascular permeability during prolonged hypoxia. This has been demonstrated in a variety of cell types: from vascular smooth muscle cells to mononuclear cells [29]. Changes in vascular permeability can be abrogated also by antioxidants, which are also involved in the response to hypoxia [30].

The other important cytokine involved in many physiological and physio-pathological situations, in which hypoxia is present, is IL-1[31]. Thanks to studies on HIF-1α conducted by Hellwig-Burgel and colleagues, an important correlation has been identified between IL-1 levels and hypoxia. In fact, liver cancer cells (HepG2), treated with IL-1, show a significant increase in the levels of the HIF-1α protein both in normal conditions and in hypoxia [31]. Finally, hypoxia is involved in the normal healing process where, following damage, hypoxic tissue is formed. During this process, angiogenesis is required as well as the budding of new capillaries from existing vessels and vasculogenesis, in which nonresident cells are recruited to participate in de novo blood vessel formation. Local and systemic responses to wounding contribute to the reparative vascularization process [32].

Pathological hypoxia occurs all those times that a pathological process alters the normal mechanisms of diffusion or use of oxygen. One of the most well studied pathological condition to be associated with hypoxia is cancer. Hypoxia can affect cancer cells both by acting as a stress factor, by inducing cell cycle arrest/ cell death (slowing of proliferation, apoptosis or necrosis) and as a factor stimulating

tumor progression and metastasis. Cancers contain hypoxic regions due to high rates of cell proliferation together with the formation of vasculature that is structurally and functionally abnormal [33]. Indeed, the vascular system triggered by the oxygen-deprived tumor is chaotic, leaky, and therefore inefficient in delivering oxygen [34]. The tumor mass is composed by different cell types that interact and determine tumor ability to survive and proliferate. In this context, the immune system is actively involved in the constitution of the tumor microenvironment. For example, macrophages and dendritic cells (DCs) release cytokines and chemokines to attract lymphocytes and at the same time, migrate to the lymph node to present the antigen and trig a massive adaptive response [35].
The relevance of hypoxia in cancers has been highlighted during the years thanks to studies showing that high HIFs levels are linked to a poor prognosis in cancer patients. Indeed, increased HIF-1α or HIF-2α levels in diagnostic tumor biopsies were associated with increased risk of mortality in cancers of the bladder, brain, breast, colon, cervix, endometrium, head/neck, lung, ovary, pancreas, prostate, rectum and stomach. Moreover, HIF-1α expression manipulation resulted in an increased tumor growth when HIF-1α was overexpressed, whereas loss of HIF activity results in decreased tumor growth. In breast cancer, the mean pO_2 is 10 mm Hg as compared to >60 mm Hg in normal breast tissue, and cancers with pO_2<10 mm Hg are associated with increased risk of metastasis and patient mortality [33]. Indeed, the low oxygen tension drives cellular adaptation through the alteration of several mechanisms, which control metabolism, pH regulation, migration, and angiogenesis [36]. Particularly, pH regulation through the modulation of carbonic anhydrases (CAs), leads to a better cell survival and enhances invasion and metastasis [37].

Another pathological condition that is characterized by hypoxia is ischemia. Ischemia occurs when oxygen and nutrient supply abruptly exceeds demand and can be due to arterial stenosis or acute blockage rendering the tissue oxygen and nutrient deprived. Of interest, ischemic tissue can produce chemotactic proteins that promote the migration of monocytes, which can contribute to angiogenesis through the production of pro-inflammatory cytokines with angiogenic properties [32]. Ischemia in organ grafts increases the risk of inflammation and graft failure or rejection [38]. Pro-inflammatory cytokines such as tumor necrosis factor (TNF) -α, and interleukin (IL) -1β, stimulate the progression of septic shock, while the anti-inflammatory cytokine IL-10 is a potent antagonist. In tissue ischemia, there are two important conditions: hypoxia and inflammation, considered fundamental stimuli for angiogenesis. This highly controlled process is associated with pathological conditions such as tumor growth, diabetic retinopathy, and ischemic diseases [39]. In the setting of obesity, an imbalance between the supply of and demand for oxygen in enlarged adipocytes causes tissue hypoxia and an

increase in inflammatory adipokines in fat. The resultant infiltration by macrophages and chronic low-grade systemic inflammation promotes insulin resistance [40].

Moreover, hypoxia has been documented in pathologies where blood supply to a tissue is compromised such as myocardial infarction, atherosclerosis, stroke, as well as preeclampsia due to placental hypoxia [41]. As far as the immune system is concerned, pathological immunological niches are characterized by chaotic and severe oxygen gradients that lead to areas of pathological hypoxia. This can be associated with immune cell dysfunction and tissue pathology since high levels of immune activity are paralleled with tumors, inflammation, infected or ischemic tissues. Indeed, chronic inflammation in immunological niches is associated with inflammation-driven carcinogenesis [24] (**Figure 2**).

.

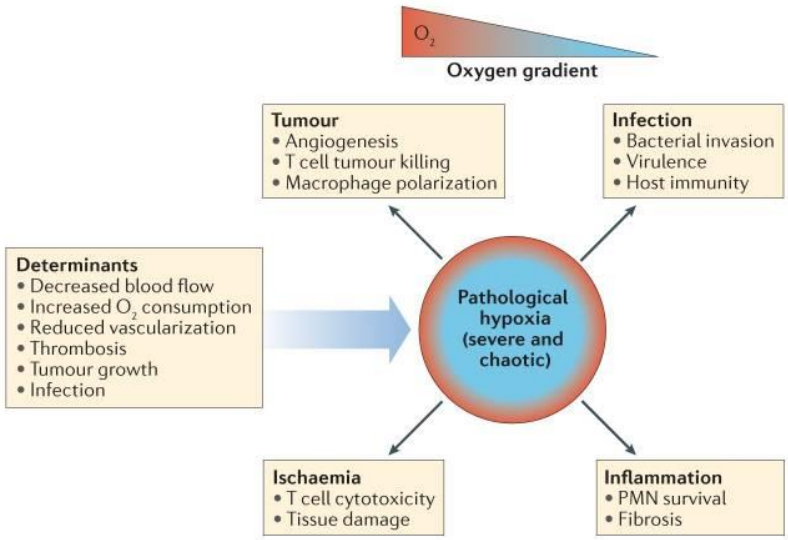


Figure 2: Schematic representation of pathological hypoxia [42]

1.2 Hypoxia-inducible factors (HIFs)

Because oxygen deprivation is a frequently occurring condition in health and disease, complex organisms have developed systemic and cellular responses in order to adapt to hypoxia. As we mentioned before, most cellular adaptive responses are mediated by (HIFs). In mammals, there are three different isoforms of HIF: HIF-1α, HIF-2α and HIF-3α [43] where HIF-1α and HIF-2α are the most structurally similar and best characterized. HIF-3α exists as multiple splice variants some of which inhibit the other two isoforms [44]. HIF-1α is expressed ubiquitously in all cells, whereas the other two isoforms are selectively expressed in certain tissues as vascular endothelium, type 2 pneumocytes, renal interstitial cells, liver parenchyma cells and cells belonging to the myeloid lineage [45]. However, HIF-1 firstly identified by Greg Semenza during his studies on EPO (erythropoietin)

[46], is the master regulator and mediator of oxygen homeostasis. HIF-1 is a heterodimer that acts as a transcription factor and regulates the expression of more than 100 genes ensuring functional, metabolic and vascular adaptation to oxygen shortages [47]. This heterodimer consists of the HIF-1β subunit (91-94 kDa), constitutively expressed by all cells, and the HIF-1α subunit (100-120 kDa) which is an oxygen labile protein [48]; indeed HIF-1α is usually unstable and is degraded by the ubiquitin-proteasome system under normoxic conditions. Under normoxic conditions HIF1-α is hydroxylated by three prolyl hydroxylases (PHDs), known as PHD1, PHD2 and PHD3, at the proline residues Pro-402 and Pro-564 in the ODDDs domain [49]. PHDs molecules are meticulously dependent on oxygen as a direct substrate, for this they are considered as an "oxygen sensors" coupling cellular oxygen concentration to HIF molecular response [44]. Hydroxylated HIF-1α is recognized by von Hippel-Lindau protein (pVHL) which is a component of E3 ubiquitin ligase complex, and thus mediates HIF-1α ubiquitination and degradation by proteasome [7]. HIF-1α subunit is also a substrate for another enzyme named FIH (factor-inhibiting HIF-1α) that is an asparagine hydroxylase enzyme that is depended on the oxygen concentration and for this, it acts as another important component on the oxygen-sensing machinery. Under normoxic condition, FIH prevents HIF-1α binding with its co-activators (p300/CBP) and therefore the HIF transcriptional activity is suppressed [44]. When O_2 availability is impaired, both PHDs and FIH are inhibited and HIF-1α can accumulate and translocate to the nucleus, where it interacts with HIF-1β and coactivators (p300/CBP) and induces the transcription of target genes containing Hypoxia Responsive Elements (HREs) contained in their promoters [50] (**Figure 3**).

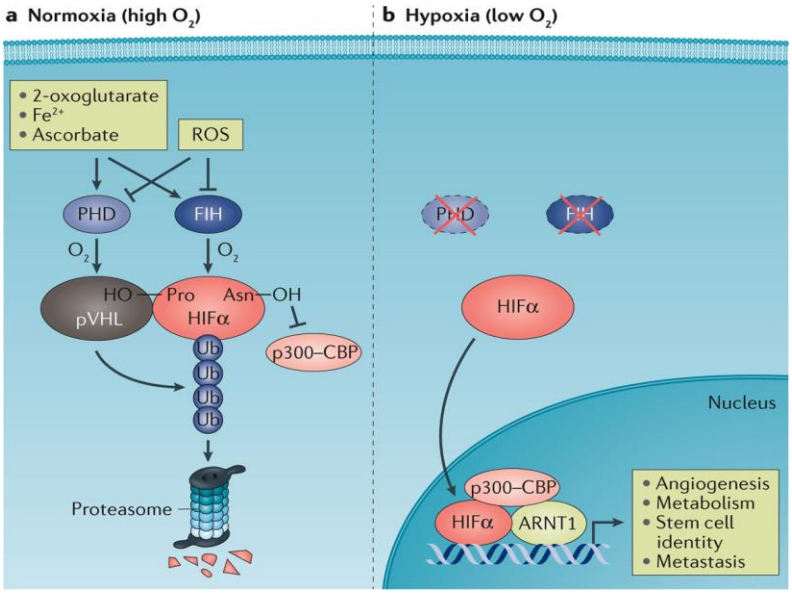


Figure 3: Regulation of HIF1- α (a) under normoxic and (b) hypoxic conditions [7]

HIF-1α is responsible for the regulation of several genes involved in angiogenesis, cell metabolism, development of metastasis, cell survival, proliferation and apoptosis [51] (**Figure 4**).

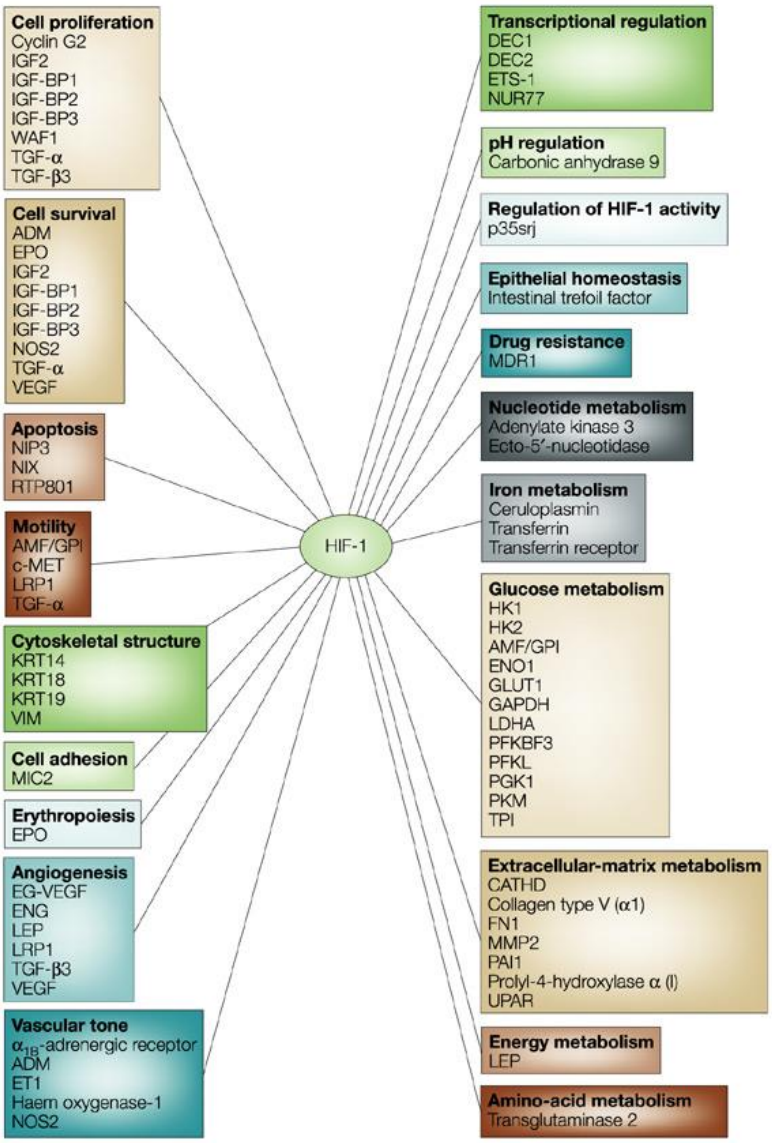


Figure 4: Genes that are transcriptionally activated by HIF-1 [48]

1.2.1 HIF modulation of immune cell function

Key aspects of the mammalian immune response take place within different immunological niches that are influenced by the microenvironment including lymphoid tissues and mucosal surfaces. Hypoxia is frequently a feature of the microenvironment in these immunological niches and may be physiological or pathophysiological in nature depending on the degree and duration of exposure. Indeed, it is now well known that HIF plays a key role in cell-type specific immune cell development and differentiation both in adaptive and innate immune responses. Circulating immune cells exist in

the oxygen-rich microenvironment of the blood and are recruited to hypoxic immunological niches when necessary [42]. During this process, immune cells must rapidly adapt to changes in pO_2 levels and to a hypoxic microenvironment.

Under hypoxic conditions, HIF has a great impact on immune cells functions since it increases the rate of glycolysis through the transcriptional upregulation of glycolytic enzyme expression. Therefore, number of immune cell types including macrophages, DCs, T lymphocytes, and B lymphocytes are activated [52]. Of interest, a reliable theme that has evolved is that HIFs play a crucial role in the regulation of immune cell development and function; therefore, HIFs should be considered key regulators of immunity and inflammation. These studies revealed cell type–specific roles for HIF in the regulation of several processes essential to immune cell activity [53]. Our knowledge of the role of HIFs in immune cell function is increasing, however it is remarkable that during inflammation, hypoxia occurs not in isolation but in the complex milieu of the inflammatory lesion. As such, several other factors such as the presence of cytokines and chemokines will combine with hypoxia to potently regulate immune cell development and function and the ultimate effects of hypoxia will depend upon which of these co-stimuli are present. For instance, macrophages can differentiate into M1 or M2 phenotypes, which are associated with first-line (proinflammatory) antibacterial defence and wound healing (anti-inflammatory) respectively [54].

Both HIF-1 and HIF-2 play important regulatory functional roles in the control of motility, bactericidal activity, and tumorigenic potential in macrophages [55]. Indeed, macrophages from mice HIF-1α deficient, show diminished capacity to kill Gram-negative and Gram-positive bacteria when compared to macrophages from wild-type littermates and HIF-1α deficient animals were more susceptible to invasive skin infection produced by *Streptococcus pyogenes*. [52]. HIF expression in tissue macrophages plays a critical role for the polarization of stromal inflammatory cells and the production of angiogenic factors, including, but not limited, to VEGF [56]. In agreement with the essential role of HIF-α in supporting macrophage effector functions, analysis of the transcriptional profile of hypoxic macrophages demonstrated that HIF-1α and HIF-2α are important transcriptional effectors regulating the responses of macrophages in hypoxia [57].

In neutrophils, HIF-1α and HIF-2α are critical in the control of cell survival and apoptosis since HIF-1α together with HIF-2α promotes neutrophil survival, bactericidal activity as well as neutrophilic inflammation [58]. In addition, hypoxia has been reported to play a crucial role in the neutrophil-

extracellular traps (NET) by Mclntruff et al., 2012 demonstrating an mTOR-dependent regulation of HIF-1α during NET formation after *lipopolysaccharides* (LPS) stimulation in primary neutrophils. HIF-1α has been shown to affect the differentiation and function of different T cell subsets in both hypoxic and normoxic conditions [59]. Data from literature demonstrated that HIF-1α is involved in the regulation of the glycolysis in Th1, Th2 and Th17 as well as in the induction of Th17 differentiation [60]. Makino et al., reported a key role for HIF-1α in regulating survival in human T cells (along with TCR activation) [61]. This was further investigated by Biju et al., using mice that conditionally lacked VHL and HIF. Thymocytes that lacked VHL (and consequently had elevated HIF-1α levels) had decreased survival levels because of HIF-1α dependent increase in caspase 8 enzymatic activity, at least in part [62]. In addition, T cell stably expressing the adaptor protein Rai, showed enhanced cell survival markers under hypoxia, suggesting that Rai plays an important role in hypoxic signaling and may be relevant in the protection of T cells against hypoxia [63] (**Figure 5**).

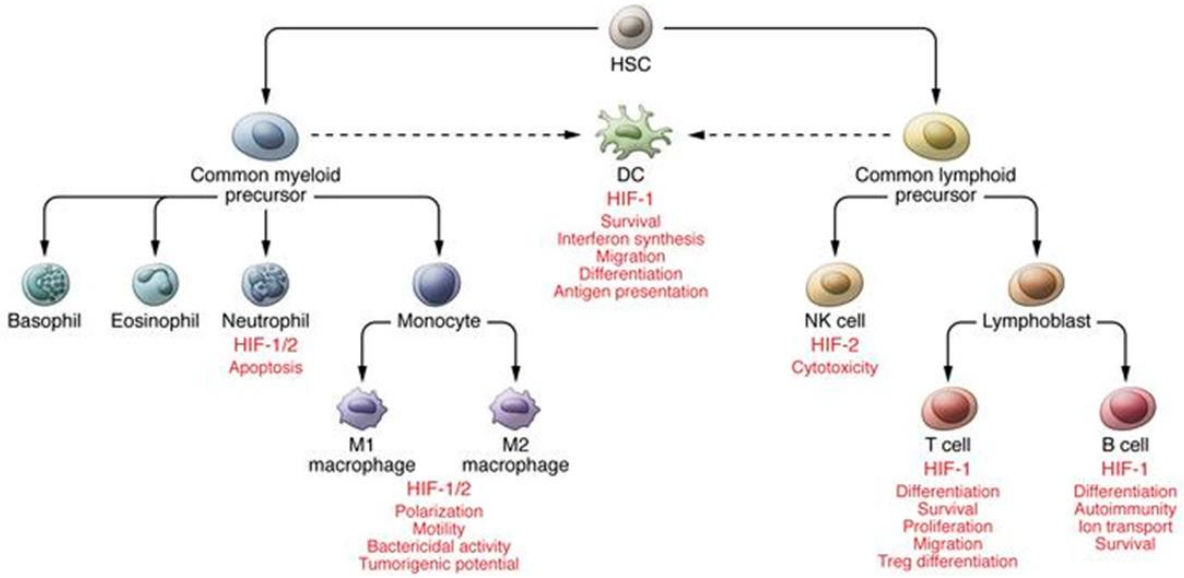


Figure 5: Regulation of immune cells by HIFs*. Functional immune cells differentiate from hematopoietic stem cells (HSC). Immune cells are frequently exposed to hypoxia when they enter the hypoxic niche of the inflammatory lesion where HIF can influence differentiation and function [4]*

1.2.2 HIF and inflammation

As stated in the "Physio-pathological aspects of hypoxia" section**,** the inflammatory process is characterized by hypoxic conditions, since in the sites of inflammation O_2 consumption is elevated in order to properly eliminate pathogens. In addition, blood supply is impaired because vessels are blocked by pathogens, damaged by primary insults or vasoconstricted by cytokines. However, the HIF system can be activated also independently upon hypoxia. Indeed, several inflammatory mediators are able to stabilize HIF-1α protein under aerobic conditions. These mediators include a

variety of small molecules like cytokines, ROS and nitric oxide (NO) that have been outlined for their ability to activate immune cells. NO, exogenously added or endogenously produced, Moreover, NO attenuates HIF-1α ubiquitination and decreases PHD activity, implying that hypoxia and NO use overlapping signaling pathways to stabilize HIF-1α [64]. It should be underlined that a number of pro-inflammatory cytokines including IL-1β and bacterial products, such as LPS, have been shown to increase also the basal transcriptional rate of HIF mRNA which contributes to further amplify HIF levels in chronically inflamed tissues. This is mediated, in part, by NF-kB that is upregulated in inflamed tissues and is a master regulator in inflammation and immunity. With regard to HIF regulation, NF-kB enhances HIF-1 transcription by its binding to the HIF-1a promoter. Therefore, HIF system could be further activated by both hypoxia and/or local mediators resulting in the amplification of HIF-regulated responses in inflamed tissues [65].

1.3 Dendritic cells: from the discovery to *in vitro* generation

The immune system is a well-organized machinery consisting of organs, cells and molecules with the unique ability of defending the organism against infections agents and abnormal cells. There are two defence levels against invading microbes: the innate immune responses (natural) that occur to the same extent however many times the infectious agent is encountered, and the acquired (adaptive) one that improve on repeated exposure to a given infection [66]. The innate responses use phagocytic cells including neutrophils, monocytes, and macrophages together with cells that release inflammatory mediators such as basophils, mast cells, eosinophil and natural killer cells [67]. An adaptive immune response is initiated when a pathogen overwhelms innate defence mechanisms. When pathogens replicates, sensor cells of the innate immunity become activated and activated the adaptive immune response. The adaptive immune response is antigen-specific and involves cell-mediated (T cell) and humoral-mediated (B cell) immunities which are both critical to drive tissue inflammation or repair [68]. The adaptive immune system mediates a slower but more finely tuned response against microorganisms, because of the immunological memory. Indeed, the proliferation of naive lymphocytes during the first encounter with an antigen, generates not only effector T and B but also memory T and B cells. The memory cells enable a quantitatively and qualitatively improved secondary immune response to be produced after a subsequent encounter with the same antigen [69]. While it was known by the 1960s that lymphocytes mediated adaptive immunity, it was unknown how antigens stimulated lymphocytes. Between 1967 and 1973, it has been reported that a rare cell type in murine spleen cells took up antigen and were essential for T cell dependent and independent antibody responses. We referred to them as A cells or the third cell type [70]. In 1973, Ralph Steinman and Zanvil Cohn described a rare cell type in murine spleen cells which was phagocytic but had

dendrite like protrusions; they named them DCs [71]. Thanks to this discovery, Ralph Steinman received half the 2011 Nobel Prize.

In the following 20 years, the research on this topic decreased due to the difficulties encountered in the processes of isolation from single tissues. Only in 1992, Jacques Banchereau was able to identify a method for cultivating DC in vitro, starting from stem cells of the bone marrow (CD34 +) [72]. In 1994, instead, Antonio Lanzavecchia, using a combination of granulocytes-macrophages colony stimulating factor (GM-CSF) and IL-4 succeeded in obtaining the ideal condition to differentiate monocytes in immature DC in vitro [73]. Later, in a study conducted by Alters and colleagues, the possibility of using IL-13 instead of IL-4 was evaluated. IL-13 is a cytokine that has numerous homologies with IL-4 since both are produced by activated T lymphocytes, promote the expression of class II MHC and inhibit the production of pro-inflammatory cytokines. Therefore, the DCs obtained in vitro from monocytes treated with GM-CSF and IL-13 have the same phenotypic and morphological characteristics of the DCs obtained using GM-CSF and IL-4 [74]. DC are irregularly shaped cells that occur in low frequency accounting for less than 1% of the cells in all organs. However, methods have been developed for their enrichment. DC in small numbers stimulate allogeneic and syngeneic mixed leukocyte reactions (MLR) and serve as accessory cells for the development of in vitro immune responses.

DC are the most potent antigen presenting (APC) cells and they are unique APCs because they are the only ones that can induce primary immune responses, thus permitting the establishment of immunological memory [75]. The DC population in the body is highly heterogeneous in phenotype and function, associated with a typical anatomical and tissue distribution. Although DCs constitute a heterogeneous group of cells, they all share the ability to modulate innate responses, via cytokine elaboration and release, and to initiate antigen specific responses. They induce T cell-mediated adaptive responses, presenting antigen-derived peptides to T lymphocytes [76]. DCs derived from CD34+ progenitor stem cells in the bone marrow, that in the presence of appropriate cytokines originates DCs and involves three functionally and phenotypically distinct stages for which the terms “precursors”, “immature” and “mature” are commonly used [77].

1.3.1 Dendritic cell classification

There have been relatively few studies on human DCs freshly isolated from tissues. Blood samples are the only readily available source, but human DCs are also isolated from lymphoid tissues derived from tonsil, thymus, and spleen in rare cases. Human DCs can be classified into multiple subsets in terms of the expression of a range of cell surface markers, but these might reflect differences in the maturation status rather than separate sublineages [5]. DCs can be derived from both myeloid and lymphoid precursor; CD8+ DEC205+ DCs originated from lymphoid progenitors, while CD8-33D1+ DCs were of myeloid origin. Most of DCs that have been studied are derived from a myeloid related progenitor[78]. Two main classes of DC can be distinguished from these precursors: plasmacytoid DCs (pDCs) and conventional or myeloid DCs (MDCs) [79]. pDCs have a similar morphology to plasma cells and are characterized by the CD11c-CD123+ phenotype; while MDCs are morphologically like monocytes and phenotypically characterized by CD11c+ CD123-[80]. pDCs are important in innate anti-viral immunity and autoimmunity and are localized in the blood and secondary lymphoid organs; besides expressing class II MHC they activate T lymphocytes and secrete high levels of IFN-α. The production of IFN plays a crucial role in the anti-viral immune response and in the activation of B lymphocytes and NK cells, whose cytolytic activity selectively affects infected cells. Additionally, pDCs, through the production of IFN-α and IL-6, induce human B cells to differentiate into plasma cells and to produce Ig. Thus, activated B cells secrete IgG rather than IgM, suggesting that pDCs they can specifically activate memory B cells [81].

Myeloid DCs (MDCs) in turn represent a group composed of three cell types:
(a) epidermal Langerhans cells (LCs)
(b) DCs derived from peripheral blood CD14 + monocytes and
(c) dermal or interstitial DCs.
LCs have long been considered as the exclusive DC of the skin, taking up pathogens or allergens that penetrate the epidermis. However, recent studies demonstrated the existence of a complex network of dermal DC suggesting that LCs might play an indirect role in T-cell priming, by the transfer of antigens to those DC that reside throughout their life cycle in the central lymph nodes[82]. These ‘‘resident’’ DC are generally denoted as lymphoid tissue-resident DC to distinguish them from nonlymphoid tissue-derived, migratory DC, such as LCs [83].

DCs derived from peripheral blood CD14+ monocytes are found in tissues and lymph and are more monocyte-like or macrophage-like and may arise from classical monocytes. These cells were originally identified as migrant CD14+ populations from human skin and were previously referred to

as ‘interstitial-type’ or ‘dermal-type’ DCs (to contrast with epidermal LCs) but this term is misleading because it ignores the major population of CD1c+ interstitial DCs. Many non-lymphoid tissues contain a modest population of these of DCs and express CD11c in common with monocytes, but lack typical activated DC markers such as CD1c or CD141, co-stimulatory molecules and CCR7. Since the low expression of CCR7, their migration ability to lymph nodes is questionable although discrete, possibly blood-derived CD14+ $CD209^{+}$ cells are found in the paracortex. Important functions have been ascribed to CD14+ DCs in the formation of follicular helper T cells or in providing direct B-cell help [84].

Interstitial dendritic cells (IDC) were first identified in the interstitium of non-lymphoid organs as leucocytes which displayed -MHC class II molecule. These cells are distinguished from tissue macrophages by their immunological phenotype and cytochemical and functional characteristics. IDC appear to be closely related to lymphoid dendritic cells, and have the ability to recognize antigen and stimulate T lymphocyte. It seems that they are represent in a stage of non-lymphoid dendritic cell differentiation necessary for antigen surveillance, similar to the LCs. The contact with antigen appears to induce migration of these cells into adjacent lymphatics and subsequent localization in the interfollicular areas of lymph node, where the DC present processed antigen to activate a primary T cell response. Of interest, IDC has been identified as the passenger leucocyte within organ allografts which contributes substantially to graft immunogenicity, so that eradication of donor organ IDC improves organ graft survival [85].

The term ‘inflammatory dendritic cell’ (inf-DC) has been attributed to monocyte-derived DC (mo-DC) that differentiate *in situ* during inflammation. These cells are a distinct subset of that can be found in several settings including eczema, psoriasis, skin sensitization, allergic rhinitis, coeliac disease, inflammatory bowel disease, synovitis and peritonitis. [84]. The main challenge is to discriminate inf-DCs from macrophages since resident tissue macrophages express a common gene signature that includes several molecules also expressed by inf-DCs, such as CD64. However, a cardinal feature of DCs, as opposed to macrophages, is their ability to migrate from tissues to lymph nodes. Indeed, inf-DCs have been identified in lymph nodes draining the sites of infection in several studies [86]. Of interest, inf-DCs after antigen recognition, can migrate to the lymph node and activate both CD4+ and CD8+ T lymphocytes in a process known as cross-presentation [87] via MHC molecules [88] (**Figure 6**).

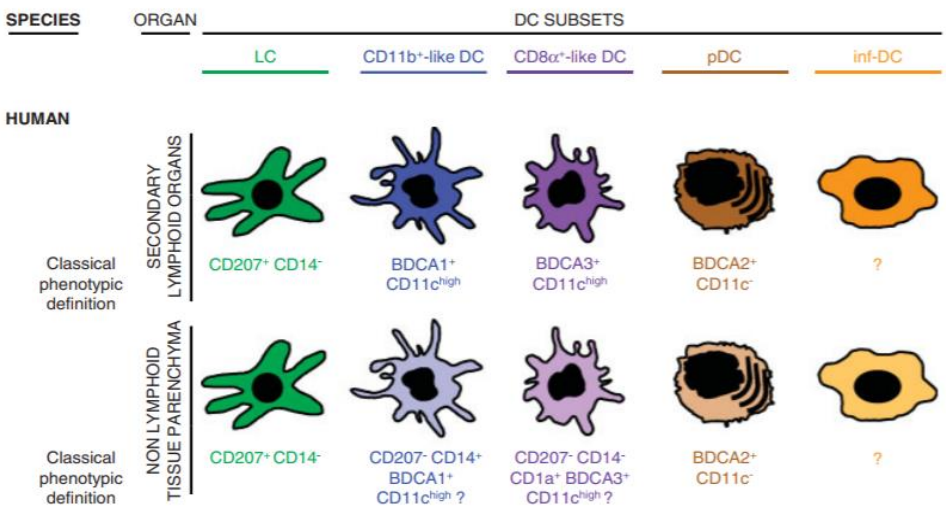


Figure 6: Human DC subsets. *Human DC subsets can be organized into five broad subsets irrespective of their primary location in secondary lymphoid organs or in the parenchyma of non-lymphoid organs. These five subsets correspond to: (i) langerhans cells (green), (ii) CD11b1 DC-like cells (blue), (iii) CD8a1 DC-like cells (violet), (iv) plasmacytoid DC (brown) and (v) monocyte-derived inflammatory-DC (orange) [83]*

1.3.2 Dendritic cell maturation and antigen presentation

In general, DCs are present in two functional states that reflect distinct abilities and roles: immature dendritic cells (iDCs) and mature dendritic cells (mDCs). iDCs have highly developed intracellular structures, such as endosomes, lysosomes, endoplasmic reticulum or Birbeck granules [88], suggesting that cytological features and expression profile of DCs fit their functions [76]. Indeed, microbial proteins are processed in the endosomal–lysosomal system as well as in endoplasmic reticulum and in cytoplasm by proteasome, and then presented to naive T lymphocytes.

Usually, antigenic peptides derived from intracellular sources are loaded on MHC class I molecules while antigens captured via phagocytosis or pinocytosis are loaded onto MHC class II molecules. However, DCs have the unique ability to cross-present exogenous antigens via MHC class I [89]. Under non-inflammatory conditions iDC circulate between tissue sites and lymphoid organs at a rapid rate with an estimated half-life of <2 day [90]. On their way DC constantly capture self-antigens from tissues and non-infectious environmental proteins as well as proteins from dying cells undergoing normal cell turnover [91]. Once in the regional lymph nodes, naive or resting T cells encounter self-antigen presented by those iDC but the interaction of the T cell receptor with MHC-bound peptides in the absence of an adequate costimulation from iDCs, that express no or low CD80 and CD86 levels, leads to anergy or apoptosis of auto-reactive T cells: thereby iDCs enhance peripheral tolerance [92]. Indeed, in the steady state iDC express low amounts of costimulatory molecules, but high intracellular MCHII levels, high endocytosis and phagocytosis activity since they are specialised in antigen

acquisition [75]. Although these cells have less migratory abilities than maDCs, as they show a greater expression of adhesion molecules like β1-integrins [93], iDCs can migrate to inflammatory areas and sites of injury; this ability is mainly conferred by expression of pro-inflammatory chemokines receptors including: CCR1, CCR2, CCR5, CCR6, CXCR1 and CXCR2 [94]. Numerous molecules including CD40, TNF-R, and IL-1R have been shown to activate DCs and to trigger their development from iDCs to mDcs. DC maturation is a continuous process that starts in the periphery upon Ag encounter and/or inflammatory cytokines and is completed during the DC–T cell interaction. Numerous factors induce and/or regulate DC maturation, including (a) pathogen-related molecules, such as LPS, bacterial DNA, and double-stranded RNA; (b) the balance between proinflammatory and anti-inflammatory signals in the local microenvironment, including TNF, IL-1, IL-6, IL-10, TGF-β, and prostaglandins; and (c) T cell–derived signals. The maturation process is also associated with several synchronized events such as (a) loss of endocytic/ phagocytic receptors; (b) upregulation of costimulatory molecules CD40, CD58, CD80, and CD86; (c) change in morphology, (d) modification in lysosomal compartments with downregulation of CD68 and upregulation of DC–lysosome-associated membrane protein and (e) change in class II MHC compartments to cell surface. Morphological changes accompanying DC maturation include a loss of adhesive structures, cytoskeleton reorganization, and acquisition of high cellular motility [95] **(Figure 7).**

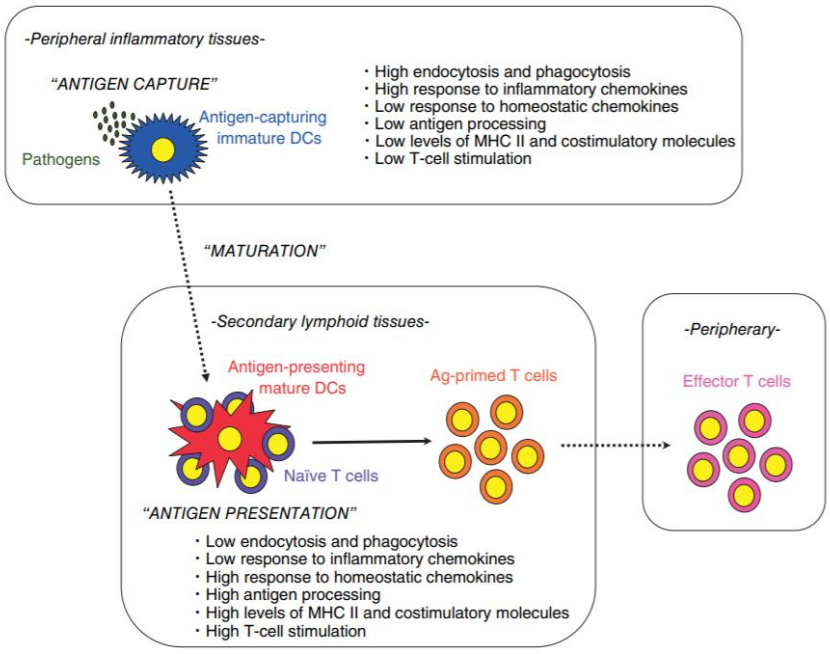


Figure 7: Schematic representation of iDC and mDc*. iDC express high levels of antigen uptake receptors and produce low levels of inflammatory mediators. mDC, in contrast, express low levels of antigen receptors but express molecules which are involved in the the presentation of antigen and stimulation of T cells mDC secrete high quantities of proinflammatory mediators and chemokines to attract and activate nearby T cells with utmost efficiency. mDC express particularly CCR7 for homing towards the T cell-rich areas in the lymph nodes[5].*

1.3.3 Toll-like receptors (TLRs) as pathogen sensors

In order to act a successful immune response, not only does the iDC have to be in the same nearby as the pathogen, but must also possess a means for its detection and acquisition. To suit this commitment, the iDC expresses proteins able to sense, recognize and uptake pathogens as well as receptors that can bind to material associated with cell damage. Direct recognition of pathogens is mediated by germline-encoded pattern-recognition receptors (PRRs), which bind to common features between pathogens termed pathogen-associated molecular patterns (PAMPs). One such family of PRRs is known as the Toll-like receptors (TLRs). TLRs function as homo- or heterodimers and with other PRRs are crucial for initiating key inflammatory responses, which can deeply impact the maturation state of the DC and the nature of immunogenicity [96]. TLRs can be found on the membrane or in the cytoplasm in the endosome membrane [97]. Among these, TLR1, TLR2, TLR4, TLR5 and TLR6 primarily recognize bacterial components, all are expressed at higher levels on iDCs compared to mDC[98]. The cellular location of TLRs is dependent on its maturation status. TLR2 is present both on the plasma membrane and in endosomal compartments, while TLR3, TLR7, TLR8 and TLR9 are expressed intracellularly and detect nucleic acids. In order to prevent signaling by host nucleic acids, these TLRs are sequestered to endosomal compartments. [99].

With regard to TLR4, it recognizes Gram- bacteria by binding to LPS. LPS differs in composition among distinct bacteria but basically consists of a polysaccharide core attached to an amphipathic lipid, lipid A, with a variable number of fatty-acid chains per molecule. To recognize LPS, the ectodomain of TLR4 needs an accessory protein, MD-2. MD-2 at first binds to TLR4 within the cell and is required both for the correct tracking of TLR4 to the cell surface and for the recognition of LPS. MD-2 associates with the central section of TLR4 and when the TLR4–MD-2 complex encounters LPS, part of lipid chains of LPS bind to a deep hydrophobic pocket of MD-2. This bound is mediated by a lipid chain that remains exposed on the surface of MD-2. Thanks to this last lipid chain and parts of the LPS polysaccharide backbone can then bind to the convex side of a second TLR4 ectodomain, inducing TLR4 dimerization that then activates intracellular signaling pathways [1] (**Figure 8**).

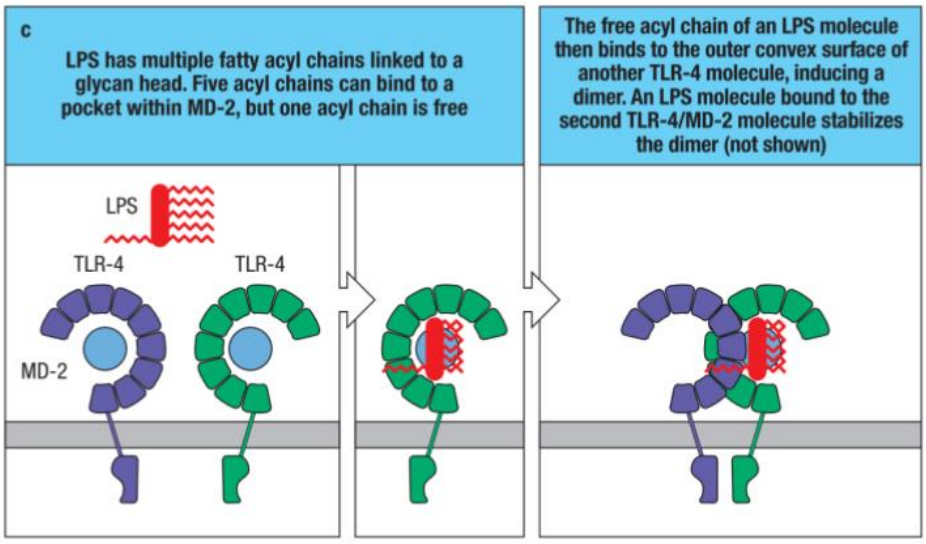


Figure 8: Schematic illustration of relative orientation of LPS binding to MD-2 and TLR4[1]

In addition, TLR4 activation by LPS in mediated by two other accessory proteins in addition to MD-2: LPS is normally a component of the outer membrane of Gram-negative bacteria, however during infections it can detached from the membrane and be picked up by the host LPS-binding protein present in the blood and in extracellular fluid in tissues. Therefore, LPS is transferred from LPS-binding protein to a second protein, CD14, which is present on the surface of several immune cells such as macrophages, neutrophils including DCs. On its own, CD14 can act as a phagocytic receptor, but on macrophages and DCs it also acts as an accessory protein for TLR4[1]. Once the antigen is recognized, DC set up a mechanism in order to preserve the antigen. However, the lack of proteolysis in iDCs contrasts with the endosomal and lysosomal compartments present in mDCs, which are exceptionally proteolytic. Several hypotheses have been assigned to the lack of proteolytic digestion in endosomes and phagosomes in iDCs. The first is that trafficking of some antigen leads to prolonged retention in early endosomal compartments, with a pH of 6.5, which is less digestive than other vesicles [100]. The second is that these late endosomal compartments are lacking proteolytic enzymes and MHC molecules. Third, there are not many activated hydrolases such as cathepsin B, D, L and S within iDCs. Fourth is the maintenance of a more alkaline pH within endosomes and phagosomes, which is achieved due to decreased activity in the vacuole proton pump. Together, these factors can result in the storage of antigen for up to 48h before stimulation with a maturation signal, making the iDC ideally suited to its role in antigen preservation [101].

1.4 Dendritic cells in the hypoxic microenvironment and the role of HIF

There is a general consensus that the type and the efficacy of an immune response depend on DC functional conditioning by the local tissue microenvironment, which regulate their orientation toward a tolerogenic or an immunogenic phenotype. DCs are exposed to changes in oxygen levels during development at and/or migration to inflammatory sites or secondary lymphoid organs, and adaptation to hypoxia is important for DCs to fulfill their functions in different tissues. DC response to hypoxia involved genes that control cell behaviour and function, indicating functional reprogramming by the hypoxic microenvironment [102]. Of interest, it has been shown that hypoxia-induced cell death in iDCs is mediated by HIF-1α and that LPS-induced maturation protects DCs against cell death [103]. The PI3K/Akt pathway is crucial for this protection. Increased level of HIF-1α is associated with an increase Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) expression as well as apoptotic markers. This was paralleled with the increase of genes involved in glycolysis, which are HIF-1 target genes, able to promotes DC maturation and T cell activation by DCs [104]. However, the role of hypoxia and/or HIF-1α on the production of costimulatory molecules is controversial. Several groups reported that hypoxia increases their expression [105] others showed a decrease [106] while other sowed no effect [107]. Hypoxia also affects cytokine production in DCs even if sometimes is controversial. Indeed, Jantsh et al., demonstrated the inability of hypoxia alone to induce the production of IL-6, TNF, IL-10 and IL-12p40. However, if DCs were stimulated with LPS, the level of IL-6 and TNF were increased especially if we compare it to the LPS stimulation under normoxic conditions. Similarly, Wobben et al., observed that DCs lacking HIF-1α have impaired interferon α and β production [108]. Though, Kohlet et al., by using mice with a conditional deletion of HIF-1α in DCs, reported that only IL-22 production was HIF-1 dependent [109].

Hypoxia is necessary for DC migration, indeed HIF-1α deficient mice showed impaired CCR7-mediated migration of mDCs both *in vivo* and *in vitro* [109]. Mancino et al., demonstrated that hypoxia strongly enhances the innate immune functions of DCs by inhibiting their maturation, but increasing both their production of inflammatory cytokines and their chemotactic response toward chemokines selectively expressed at peripheral sites of inflammation. This suggest that this modulation represents a safeguard against immune reactivity to damaged tissues [110]. In addition, recent studies have revealed a role for HIF-1α in defining the antigen-presenting function of DCs as well as promoting Treg expansion while limiting the expansion of CD8+ T cells [111]. Thus, taken together, there is clearly a role for hypoxia-dependent signaling and HIF-1α in shaping DC cell signaling supporting the concept of HIF-1α playing a key role in cell fate/survival in DCs exposed to hypoxia **(Figure 9**).

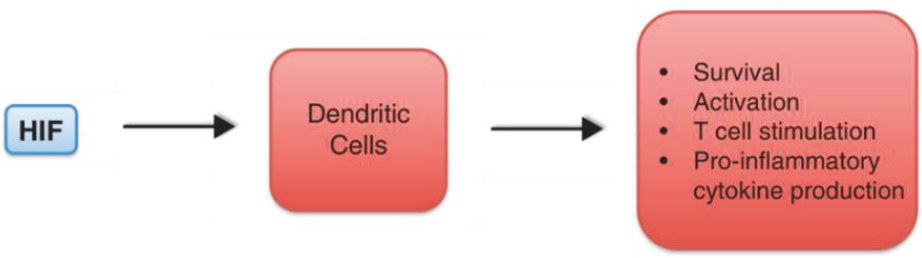


***Figure 9: HIF and DCs**. Adapted from [65]*

1.5 Autophagy

More than four decades ago, Clark and Novikoff observed mitochondria from mouse kidneys in membrane-bound compartments termed 'dense bodies', which were later shown to contain lysosomal enzymes [112]. Later, Ashford and Porter observed membrane-bound vesicles containing semi-digested mitochondria and endoplasmic reticulum in rats' hepatocytes after glucagon treatment [113], and Novikoff and Essner revealed that the same bodies contained lysosomal hydrolases [114]. One year later, in 1963, at the Ciba Foundation symposium on lysosomes, de Duve coined the term 'autophagy' to describe the presence of single- or double-membrane vesicles that contain parts of the cytoplasm and organelles in different states of disintegration. He indicated that these sequestering vesicles, or 'autophagosomes', were associated to lysosomes and occurred in normal cells [115]. The word autophagy derived from the Greek word for "self-eating" and refers to the catabolic processes through which the cell turns over its own constituents [116]. Autophagy is a general term for the degradation of cytoplasmic components within lysosomes. This process is different from endocytosis-mediated lysosomal degradation of extracellular and plasma membrane proteins. There are three types of autophagy: (a) microautophagy that involves the capture of cytosolic components by lysosome through various modification on their membranes, (b) chaperone-mediated autophagy by which protein containing KFERRQ-like motifs translocate into the lysosomal lumen and (c) macroautophagy that leads to bulk lysosomal degradation of the cytosol *via* double-membrane organelles called autophagosomes **(Figure 10).** The term "autophagy" usually indicates macroautophagy. Recent studies have clearly demonstrated that autophagy plays a pivotal role both in physiological and pathophysiological conditions, such as starvation adaptation, intracellular protein and organelle clearance, development, anti-aging, elimination of microorganisms, cell death, tumour suppression, and antigen presentation. It must be underlined that autophagy can be subclassified into "induced autophagy" and "basal autophagy". The former is used to produce amino acids following starvation, while the latter is important for constitutive turnover of cytosolic

components. Autophagy consists of several sequential steps: sequestration, degradation, and amino acid/peptide generation. Each step seems to exert different functions in a variety of cellular contexts. These step-dependent functions may allow autophagy to be multifunctional [117].

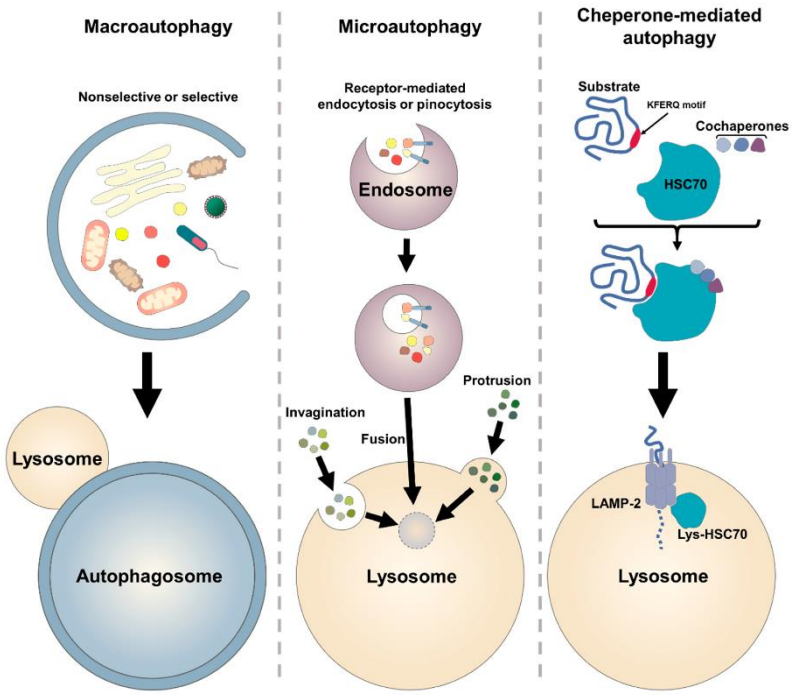


Figure 10: Overview of the types of autophagy*. Macro-autophagy encapsulates the cytoplasmic cargo by a delimiting membrane, which forms an autophagosome, which finally fuses with lysosome for degradation of the substrates. Micro-autophagy involves invagination or protrusion of the vacuole, which is formed by a lysosomal membrane. Chaperone-mediated autophagy is a selective degradation pathway, in which the protein substrates containing KFERQ-like motifs are recognised by chaperone HSC70 and cochaperones [118]*

1.5.1 Induction of autophagy and initiation complex

The most typical trigger of autophagy is nutrient starvation. Indeed, lack of any type of essential nutrient can induce autophagy. In yeast, nitrogen starvation is the most potent stimulus, but removal of other essential factors such as carbon, auxotrophic amino acids and nucleic acids, and even sulphate can induce autophagy, albeit less efficiently [119]. In mammals, regulation of autophagy seems to be more complicated. Depletion of total amino acids strongly induces autophagy in many types of cultured cells, but the effects of individual amino acids differ. However, such profiles depend on cell type because amino acid metabolism differs greatly among tissues. For example, only leucine has a leading effect on skeletal muscle and heart [120]. However, how cells sense amino acid concentration is not fully understood. Furthermore, recent Drosophila genetic studies have demonstrated the physiological importance of insulin signaling in vivo [121]. Other hormones and growth factors also

seem to contribute to autophagy regulation. In addition, it is well known that serum starvation can induce autophagy in many types of cultured cell. All these kinds of signals are thought to converge on mTOR (mammalian target of rapamycin), which is a master regulator of nutrient signaling. Indeed, treatment with inhibitors of TOR such as rapamycin and CCI-779 induces autophagy in yeast [122] and even in animals [123]. However, not all autophagy signals are transduced through mTOR; some amino acid signaling can suppress autophagy in an mTOR-independent manner [124]. Indeed, in addition to insulin and amino acid signaling, the involvement of many other factors in autophagy regulation has recently been reported [125]. Moreover, hypoxia (3–0.1% oxygen) can rapidly induce, via HIF-1α, a cell survival response engaging autophagy. This process is mediated by the atypical BH3-only proteins BNIP3 and the BNIP3 ligand (NIX) that are induced by HIF-1α. These mitochondrial associated BNIP proteins also mediate mitophagy, a metabolic adaptation for survival that can control ROS production and DNA damage [126]. Autophagy is initiated in yeast at a punctate structure called the Pre-Autophagosomal Structure (PAS, also sometimes called the Phagophore Assembly Site. In mammals, initiation is associated with an endoplasmic reticulum (ER) subdomain enriched for the lipid phosphatidylinositol 3-phosphate (PI(3)P), known as the omegasome. The conserved machinery for autophagosome formation contains two major initiation complexes (**Figure 11**): (a) the ULK1 complex and the (b) class III PI 3-kinase complex I (PI3KC3-C1). (a) ULK1/Atg1 is activated in at least three ways upon autophagy induction, and all three are essential. Protein kinase activity needs to be switched on; the active kinase needs to be recruited to the PAS, and essential non-catalytic scaffolding activities must be turned on. Following activation, ULK1 can be ubiquitinated by the Cul3-KLHL20 ligase complex and degraded, thereby switching off the autophagy-initiating signal. (b) Formation of PI(3)P is a critical early event in autophagy initiation, occurring just downstream of ULK1. PI3KC3 forms at least two different complexes, known as complexes–I and II (PI3KC3-C1 and –C2). Both complexes contain the catalytic subunit VPS34/Vps34, the putative protein kinase VPS15/Vps15, and Beclin-1-1/Atg6. PI3KC3-C1 contains Atg14L/Atg14, which directs the complex to phagophore initiation sites. PI3KC3-C1 facilitates elongation while complex II containing UVRAG directs endosome and autophagosome maturation [127]. The precise role of each protein remains to be understood, however, the most well studied is Beclin-1, which is a mammalian Atg6/Vps30 (vacuolar protein sorting 30) ortholog and a subunit of the class III PI3-kinase complex. Beclin-1 was initially identified as an interaction partner of Bcl-2, an anti-apoptotic protein [128]. This Bcl-2–Beclin-1 interaction is reduced upon starvation, freeing Beclin-1 to activate autophagy [129]. Another Beclin-1 partner is UVRAG (UV irradiation resistance-associated gene) [128], which interacts via the coiled-coil region of Beclin-1. UVRAG was shown to be a member of the class III PI3- kinase complex and a positive regulator of autophagy. Most recently, a WD-40

domain-containing protein named Ambra1, was shown to be a Beclin-1-interacting protein [130] and was shown to positively regulate Beclin-1 dependent autophagy. Ambra1 is mainly expressed in neural tissues and is indispensable for normal neural tube development. Consequently, mammalian Beclin-1 is likely to be regulated by its binding partners, which may not be present in yeast. Autophagosome membranes cannot recognize what they enclose; therefore, sequestration takes place primarily in a random manner. However, autophagosome membranes can recognize some proteins, and possibly organelles, at their surfaces. The best-studied example of such selective incorporation is the Cytoplasm-to-vacuole targeting pathway [131].

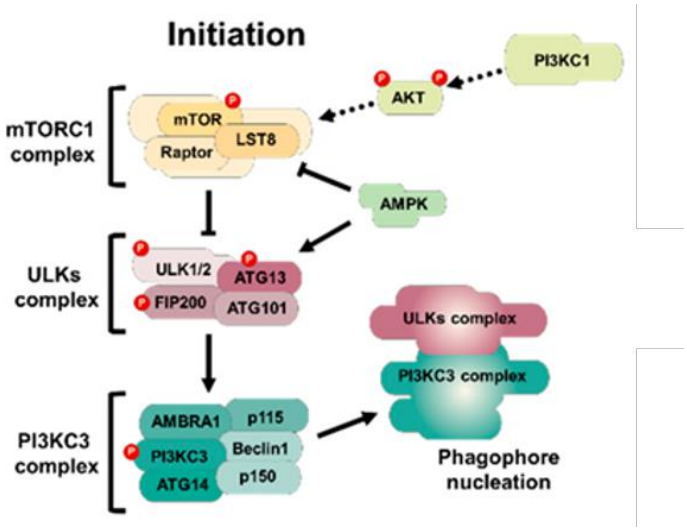


***Figure 11**: **Autophagy initiation complexes**. Under stress conditions, mTORC1 is inhibited and the ULKs complex, that encompasses ULK1/2, FIP200, Atg101 and Atg13, is activated. The ULKs complex provokes activation of the PI3KC3 complex via phosphorylation of Beclin-1 and AMBRA1. The active PI3KC3 produces PIP3 via phosphorylation of PI on the surface of the surface of the phagophore. Adapted from [118]*

1.5.2 Autophagosome formation

In the first stage of autophagosome formation, cytoplasmic constituents, including organelles, are sequestered by a unique membrane called the phagophore, which is a very flat organelle like a Golgi cisterna. Complete sequestration by the elongating phagophore results in formation of the autophagosome, which is typically a double-membraned organelle. In this step no degradation occurs. The phagophore is elongated to form the autophagosome, which is regulated by two ubiquitination-like conjugation systems: (a) Atg5-Atg12 conjugation and (b) microtubule-associated protein light chain 3 (LC3B) processing. The first mechanism is activated by Atg7 (E1-like activating enzyme), and is then conjugated to Atg5 by Atg10 (E2-like conjugating enzyme). The Atg5-Atg12 complex interacts non-covalently with Atg16L1 (E3-like ligase enzyme), which results in the formation of the Atg5-Atg12-Atg16L1 multimeric complex. The Atg5-Atg12-Atg16L1 complex is necessary to the

outer membrane of the phagophore, in order to avoid premature fusion with the lysosome. The C-terminal region of nascent LC3B (known as pro-LC3B) is converted to LC3B-I by Atg4. The exposed C-terminal glycine residue of LC3B-I is then activated by Atg7, and the LC3B-I is converted into LC3B-II by Atg3 through phosphatidylethanolamine (PE) conjugation. The main role of LC3B-II is the autophagosomes closure. Finally, the Atg5-Atg12-Atg16L1 complex is dissociated from completed autophagosomes. The LC3B-II is associated with the autophagosomal membrane until their fusion with lysosome and then is dissociated from the surface of the membrane by Atg4 and recycled [118] **(Figure 12)**. In addition, LC3 function as a receptor for a selective substrate, SQSTM1/p62[132]. SQSTM1/p62 significantly accumulates in autophagy-deficient cells [133], confirming that it is selectively recognized and degraded by autophagy. Additionally, since SQSTM1/p62 has a ubiquitin-binding domain, it has been proposed that ubiquitinated proteins and inclusion bodies can be recruited to the autophagosome membrane via SQSTM1/p62[132].

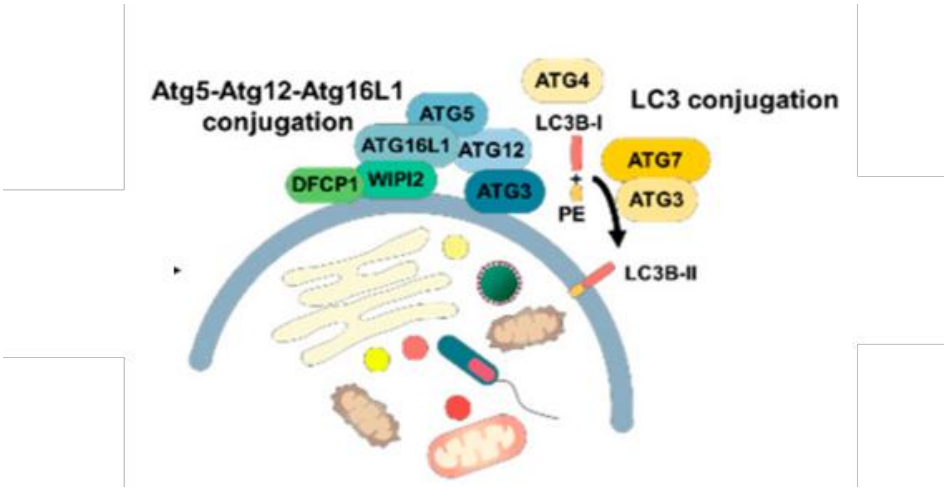


Figure 12: Phagophore elongation and phagosome formation*. The phagophore is elongated to form the autophagosome. This process is regulated by two systems: Atg5-Atg12 conjugation system and LC3B-II conjugation system. The Atg5-Atg12-Atg16L1 complex is involved in the curvature of the elongating phagophore through the insertion of processed LC3B. The Atg5-Atg12-Atg16L1 complex is recruited to the outer membrane of the phagophore to avoid premature fusion of the autophagosome and lysosome. proLC3B is converted to LC3B-I by Atg4, the exposed C-terminal glycine residue of LC3B-I is then activated by Atg7, and the LC3B-I is converted to LC3B-II via PE conjugation by Atg3. The LC3B-II is associated to the autophagosomal membrane until the forming of autophagolysosome. Adapted from [118]*

1.5.3 Degradation and reuse

When the autophagosome achieves fusion of the expanding ends of the phagophore membrane, the next step towards maturation in this self-degradative process is fusion of the autophagosome with the specialized endosomal compartment that is the lysosome to form the 'autolysosome'[117]. It has been

variously suggested that fusion of the autophagosome with early and late endosomes, prior to fusion with the lysosome, both delivers cargo and delivers components of the membrane fusion machinery and lowers the pH of the autophagic vesicle before delivery of lysosomal acid proteases. This process is relatively understudied but requires the small G protein Rab7 in its GTP-bound state. Within the lysosome, cathepsin proteases B and D are required for turnover of autophagosomes and, by inference, for the maturation of the autolysosome. Lamp-1 and Lamp-2 are also critical for functional autophagy, as evidenced by the inhibitory effect of targeted deletion of these proteins in mice on autolysosome maturation [134]. To successfully recycle cytoplasmic components, it is necessary to break down the single-membrane autophagic body that results from fusion of the double-membrane autophagosome with the vacuole. The vesicle lysis step is known to depend on an acidic pH of the vacuole lumen, and proteinase B [135]. When macromolecules have been degraded in the lysosome/vacuole, monomeric units (e.g., amino acids) are exported to the cytosol for reuse. However, little is known about this last process. Yeast Atg22, which was first identified as Aut4, a membrane protein required for the breakdown of autophagic bodies [136], was later identified as a putative amino acid effluxer [137] that collaborates with other vacuolar permeases, such as Avt3 and Avt4. Although a mammalian counterpart of Atg22 has not been found, equivalents of Avt3 and Avt4 have been identified as SLC36A1/LYAAT-1 (lysosomal amino acid transporter1) and SLC36A4/LYAAT-2, respectively. The contribution of autophagy to reuse of other macromolecules such as carbohydrates and lipids is unknown [137].

1.6 Autophagy in dendritic cells

It has been shown that autophagy is involved in DC functions at several levels including: DC maturation, triggers of DC maturation such as TLR stimulation, antigen presentation, cytokine production, DC migration and maturation and T-cell activation [2]. During DC maturation, the Hsc/Hsp70 co-chaperone network was shown to control the transient formation of DC aggresome-like-induced structures (DALIS) by modulating their autophagic degradation. In human DCs infected with Bacillus Calmette-Guerin, autophagy stimulation by starvation was reported to up-regulate the expression of CD86 and HLA-DR, as well as the secretion of IL-10 and IL-6[138, 139]. Moreover, DC maturation during viral infection has also been related with autophagy. For instance, inhibition of autophagy in murine bone marrow-derived DCs infected with respiratory syncytial virus (RSV) inhibited their maturation as assessed by the expression of MHC-II and co-stimulatory molecules CD40, CD80, and CD86[140]. This was supported by experiments conducted by Manuse et al., where they demonstrated the effects of autophagy inhibition in plasmacytoid pDCs infected with paramyxovirus Simian Virus 5 SV5, where a decrease in CD80 expression was observed [141]. Tregs

also reduce the immunogenic maturation of DCs through the inhibition of their autophagic machinery in DCs[142]. The migration of DCs was found to be regulated by autophagy using Atg16-deficient DCs that showed reduced migration to lymph nodes and increased cellular adhesion. This phenotype was due to a defect in DC cytoskeletal modulation through the loss of filopodia, altered podosome distribution, and increased membrane ruffling [143].

In addition, autophagy is involved in the regulation of TLRs in DCs. To this purpose, the first evidence of autophagy crosstalk with TLR signaling was shown in primary human and murine macrophages [144]. Next, the relevant role of autophagy in the TLR-mediated immune response in DCs was supported by a study showing the requirement of autophagy in the recognition of viral PAMPs by TLR7 and their transport to the lysosomes in pDCs that uniquely express this TLR [145]. This was paralleled by similar observations that reported a dramatic drop in the TLR4- and TLR8-mediated responses in autophagy-deficient DCs stimulated with LPS and ssRNA[146]. TLR4 stimulation in DCs was recently shown to inhibit autophagy because of mTORC1 activation suggesting that autophagy can regulate TLR stimulation [147]. As far as antigen presentation in concerned, autophagy was reported to be crucial in both self and non-self-antigens. Lee et al., reported that mice with DC conditional deletion in Atg5, showed impaired CD4+ T cell priming after herpes simplex virus infection and succumbed to rapid disease. The most pronounced defect of *Atg5*$^{-/-}$ DCs was the processing and presentation of phagocytosed antigens containing TLR stimuli for MHC class II. In contrast, cross-presentation of peptides on MHC I was intact in the absence of Atg5 revealing that DCs utilize autophagic machinery to optimally process and present extracellular microbial antigens for MHC II presentation[148].

Most of the peptides presented by MHC-I are derived from proteins processed by the ubiquitin-proteasome system (UPS) and then are transferred to the endoplasmic reticulum through the transporter associated with antigen processing (TAP). When autophagy is limited, some of its substrates, such as defective ribosomal products (DRiPs), accumulate in aggresome-like-induced structures (ALIS) and are subsequently presented by MHC-I after UPS-mediated processing [149]. Through the modulation of DALIS formation, the co-chaperones— CHIP, BAG-1 and HspBP1— were shown to alter MHC-I-mediated antigen presentation in DCs [138]. Autophagy was also shown to retain MHC-I internalization, leading to a lower stimulation of $CD8^+$ T-cell responses [150]. Recent reports have suggested a more direct role for autophagy in antigen presentation via MHC-I during viral infection through the non-classical TAP-independent presentation [151]. The involvement of autophagy in the cross-presentation of antigens by DCs met with debates: while no

defect was observed in Atg5$^{-/-}$ DCs to cross present antigens to MHC class I [148], α-alumina (Al_2O_3) nanoparticles induced efficient cross-presentation of conjugated antigens in bone marrow-derived DCs in an autophagy-dependent manner [152].

Of interest, recent study demonstrated the contribution of autophagy in cross-presentation in murine CD8α+ DCs through VPS34. [153]. However, in this study, the VPS34 deletion was not specific to the CD8α+ DC subset. Not all DCs but, only particular DC subsets, can cross-present antigens efficiently. Indeed, mouse CD8+ and CD103+ DC subsets are the dominant cross-presenters compared to CD8− and CD103− subsets. On the other hand, CD1a$^+$ DCs are dominant cross-presenting subtypes than CD14+ in human [154]. It is therefore expected that contribution of autophagy in cross-presentation is determined by subtypes of DCs that are capable of cross presenting or not. Clearly, inducing autophagy in DCs leading to enhanced immunogenicity is a strategy to increase the success of vaccination [155]. Moreover, better understanding the role of autophagic antigen processing and presentation has implications not only for infectious diseases, but also for cancer, autoimmunity, allergy, and transplantation medicine. It should be underlined that some pathogens have developed mechanisms that suppress autophagy and inhibit MHC class II-restricted antigen presentation [150] **(Figure 13).**

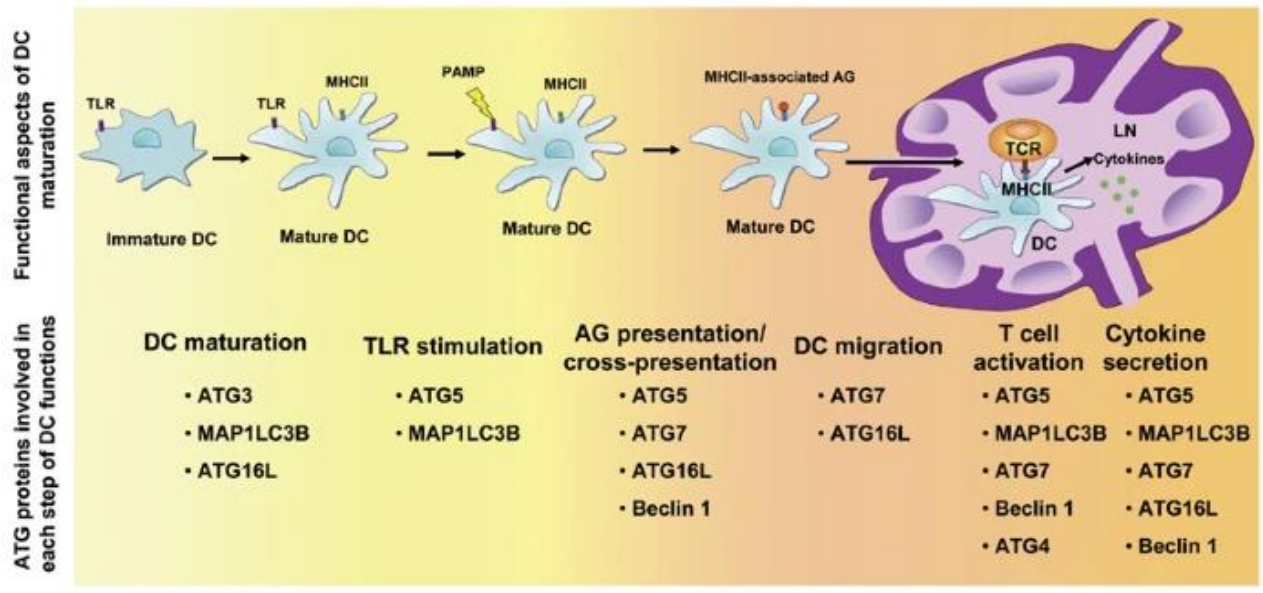


Figure 13: Scheme describing the involvement of ATG genes in the functional aspects of DC maturation [2]

2. AIM OF THE THESIS

Hypoxia is a common feature of the microenvironment of physiological immunological niches and lymphoid organs such as thymus and lymph nodes. Most of the adaptive responses to hypoxia are mediated by a family of transcription factors known as hypoxia inducible factors and include modulation of glycolytic metabolism, cell survival, pro-angiogenic cytokines, pro- and anti-apoptotic molecules and the regulation of the immune system. Immune cells are frequently exposed to hypoxia in both physiological and pathological states. Among this, dendritic cells (DCs) always encounter different physiological oxygen tension since they patrol different body district and are required to adapt to hypoxia; therefore, it is evident that low oxygen tensions can influence the physiology of DC. Indeed, the pO_2 has been found to be crucial for DC differentiation and especially during final maturation by *lipopolysaccharides*. Autophagy is one of the adaptive cellular responses that can be triggered in response to hypoxia and has shown to be involved in DC functions at several levels: induction of DC maturation such as Toll-like receptor stimulation, antigen presentation, cytokine production, DCs migration and maturation and T-cell activation. Autophagy is an evolutionarily conserved catabolic process involved in lysosomal degradation, recycling of macromolecules and organelles and it is essential to ensure maintenance of normal cell functions. Indeed, cells rely on autophagy to survive diverse cellular insults including, starvation, accumulation of protein aggregates, damaged mitochondria, or intracellular bacteria. By using human monocytes-derived DCs isolated from healthy donors, we investigated whether hypoxia can affect autophagy in unstimulated and LPS-stimulated DCs. We evaluated the expression of autophagic markers such as LC3B, SQSTM1/p62, the protein levels of Atg and cytokine expression associated with autophagy. In addition, we evaluated the molecular mechanisms and pathways activated by hypoxia and involved in cell survival and autophagy. To finally assess the impact of autophagy in DCs, we took advantages of two well-established autophagy inhibitors, with different mechanisms of action, such as Bafilomycin and Chloroquine.

3. MATERIALS AND METHODS

3.1 Reagents

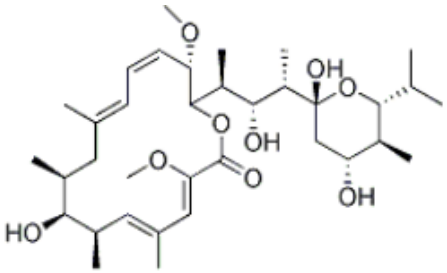
RPMI 1640, fetal bovine serum (FBS), buffered saline solution (PBS) penicillin/streptomycin, and L-Glutamine were purchased from Euroclone, Devon, UK. Fycoll was purchased from Cederlane Labs and Percoll from Amersham Bioscience, Pittsburgh, PA, USA. Recombinant human granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-13 (IL-13) were purchased from ProSpec TechnoGene, East Brunswick, NJ, USA. All reagents contained <0.125 endotoxin units/ml, as checked by the Limulus Amebocyte Lysate assay (Cambrex, East Rutherford, NJ, USA). LPS from Escherichia coli strain 055:B5 was obtained from Sigma–Aldrich, Milano, Italy.

3.1.1 Autophagy inhibitors

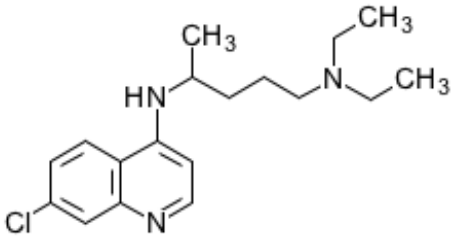
Baf 1A was purchased from VWR Chemicals BDH Milano, Italy, and CQ was obtained by Enzo Life Sciences, Plymouth Meeting, PA, USA.

Bafilomycin A1 (Baf A1) is a specific and potent inhibitor of the vacuolar type H+-ATPase (V-ATPase) in cells and belongs to a class of membrane ATPase inhibitors. It binds to the V0 sector subunit c of the V-ATPase complex and inhibits H^+ translocation, causing an accumulation of H^+ in the cytoplasm of treated cells Inhibits the acidification of organelles containing this enzyme, such as lysosomes and endosomes. Chloroquine (CQ) is a lysosomotropic agent and it accumulates preferentially in the lysosomes of cells; it was originally discovered and used to treat malaria, and subsequently inflammatory diseases. At physiological pH, it is roughly 10% deprotonated as calculated using the Henderson-Hasselbalch equation. This decrease to nearly 0.2% at a lysosomal pH of 4.6. Since the deprotonated form of the compound is more membrane permeable than the protonated form, CQ becomes quantitatively "trapped" in lysosomes. CQ mainly inhibits autophagy by impairing autophagosome fusion with lysosomes leading to the block of the delivery of sequestered cargo to the lysosomes (**Figure 14**). When used, 100nM Baf A1or 100 μM CQ were added directly in the final 6 h of treatments.

A



B



C

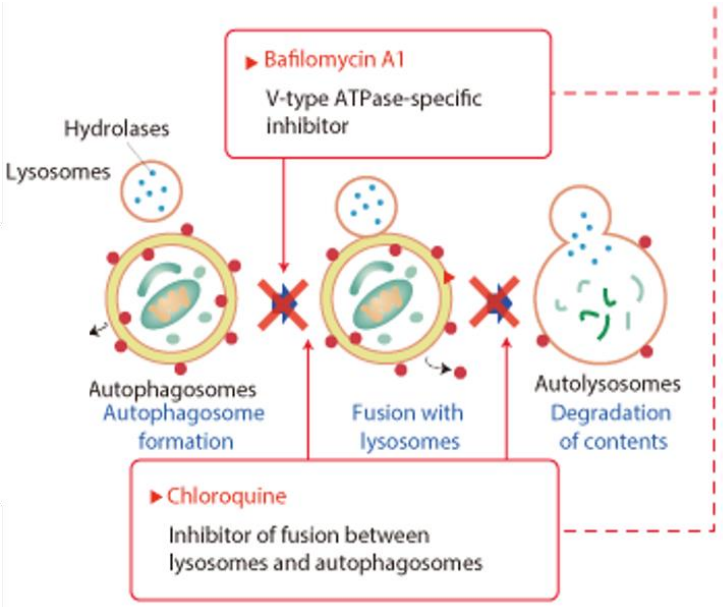


Figure 14: (A) Baf A1, (B) CQ structure and (c) schematic representation of their mechanisms of action. Structure adapted from https://www.selleckchem.com/products, schematic representation adapted from https://ruo.mbl.co.jp/bio/e/product/autophagy/pickup/autophagywatch.html.

3.2 Human Monocyte-Derived DC preparation and culture conditions

Human monocyte-derived DCs were generated from buffy coats supplied by the general hospital of Siena (Centro Emotrasfusionale, AOUS, Siena). Buffy coats were centrifuged for 15 minutes at 900 x g, a crucial step for platelets removal, and then they were diluted with a sterile, buffered saline solution at pH 7.4 (PBS without Ca^{2+} and Mg^{2+} in order to prevent triggering of the coagulation cascade) in the ratio 1:1. Blood diluted with PBS was stratified on Ficoll (Lympholyte, Cedarlane Labs), in the ratio 2:1, and centrifuged at 800 x g for 30 minutes. This step allowed the stratification of mononuclear cells at the interface between the sample and the separation medium, while erythrocytes and granulocytes settled on the bottom of the tube. Isolated mononuclear cells were collected and washed several times with PBS, resuspended in RPMI medium at 5% FBS and incubated overnight at 4° C. The following day, the cell suspension was recovered, stratified on PercollTM 285 mmOsm, a solution of colloidal silicates bound to polyvinylpyrrolidone, and

centrifuged for 30 minutes at 771 x g. After centrifugation, monocytes arranged to form a ring between the supernatant and the separation medium while lymphocytes sedimented on the bottom of the tube. Monocytes expressing CD14 (> 95%) were collected and plated 2 hours with RMPI with 5% FBS in a 6 well plates in order to allow monocytes to adhere.

3.2.1 Monocytes differentiation into immature DCs (iDCs) and mature DCs (mDCs)

At the end of the 2 hours, cells were washed twice with PBS and 50 ng/ml GM-CSF and 20 ng/ml IL-13 were added and cells were incubated for 6 days under normoxic conditions (atmospheric pO_2 levels: 21% O_2, 5% CO_2, and 74% N_2 corresponding to a pO_2 ~ 140 mmHg). At the end of the 6 days, we obtained immature DCs. Then, cells were collected by using cold PBS and cell scraper. In order to perform the experiments, cells were cultured in the absence (iDCs) or in the presence of LPS (100 ng/ml) to induce final maturation (mDCs) either under normoxia or hypoxia for 24 or 48 hours depending on the experiment. The experiments under hypoxic conditions were performed using a workstation INVIVO$_2$ 400 (Ruskinn, Pencoed Bridgend, UK) providing a customized and stable humidified environment through electronic control of CO_2 (5%), O_2 (hypoxia: 2%, ~14 mmHg) and temperature (37 °C) At the end of the treatment, iDCs and mDCs were assessed for cellular and molecular assays as follows (**Figure 15**).

Figure 15: Hypoxic workstation InVIVO O_2 400 (Ruskinn, Pencoed, UK)

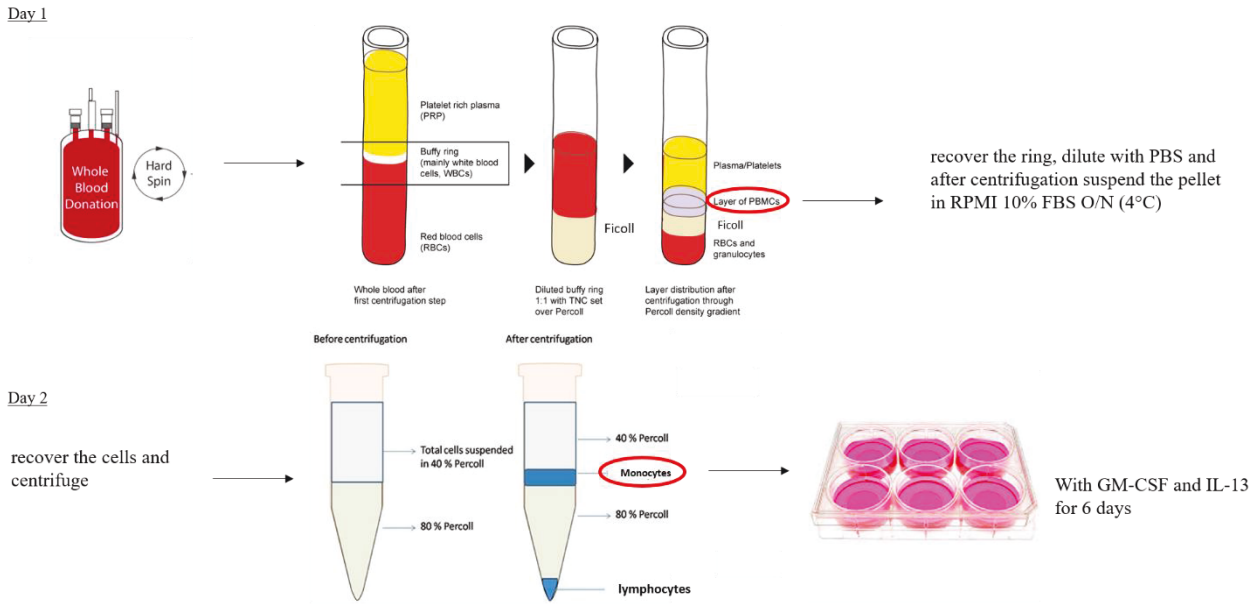


Figure 16: Schematic representation of Human Monocyte-Derived DCs preparation

3.3 Cell viability and detection of mitochondrial membrane potential

Cell viability was analysed by Trypan Blue exclusion assay by Bio-Rad TC20™ automated cell counter (Biorad laboratories, Bio-Rad, Hercules, CA, USA), which provides a total cell count, and it assesses cell viability using a digital image analysis algorithm [156]. Evaluation of mitochondrial membrane potential ($\Delta\Psi m$) was performed by a fluorogenic lipophilic cation (JC-1; Sigma-Aldrich). The JC-1 dye is a lipophilic, cationic dye (naturally exhibiting green fluorescence) which can enter the mitochondria where it accumulates and (in a concentration-dependent manner) starts forming reversible complexes called J aggregates. Differently, from JC-1 molecules, these J aggregates exhibit excitation and emission in the red spectrum (maximum at ~590 nm) instead of green. Thus, in healthy cells with a normal $\Delta\Psi_M$, the JC-1 dye enters and accumulates in the energized and negatively charged mitochondria and spontaneously forms red fluorescent J-aggregates. By contrast, in unhealthy or apoptotic cells the JC-1 dye also enters the mitochondria but to a lesser degree since the inside of the mitochondria is less negative because of increased membrane permeability and consequent loss of electrochemical potential. Under this condition, JC-1 does not reach a sufficient concentration to trigger the formation of J aggregates thus retaining its original green fluorescence [157] (**Figure 17**). Cells were seeded density of $2.00x10^4$ cells/well in a 96 well dish (Corning, New York, NY, USA). At indicated times, cells were washed with PBS and JC-1 was added for 12 minutes. Fluorescence was detected by using microplate reader Fluoroskan Ascent (Thermolabsystem, Helsinki, Finland)

protected from light. The ΔΨm was determined by the ratio between the red (~590 nm) and the green (~529 nm) fluorescent emission.

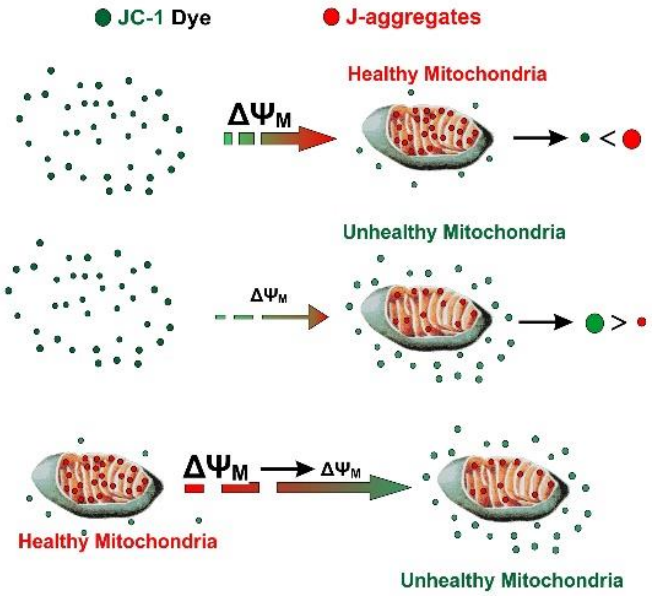


Figure 17: Schematic illustration of JC-1 entry into the mitochondria and the generation of J aggregate*. JC-1, a cationic carbocyanine dye (green) exhibits potential-dependent accumulation in mitochondria where it starts forming J aggregates (red); upon depolarization, it remains as monomer showing green fluorescence [157]*

3.4 Immunofluorescence staining and confocal microscope analysis

DCs were plated on sterile chamber slides (Nunc Lab-Tek) cultured and treated as indicated above. At the indicated time, cells were fixed in cold methanol at −20°C for 10 min and permeabilized with HEPES/Triton for 3 min. Then they were washed with PBS-BSA 0.2% and blocked with 10% goat serum. Cells were incubated with the primary antibody diluted in PBS-BSA 2% anti-HIF-1α (Thermo scientific, Rockford, USA, 1:200 Cat.n° MA-516), LC3B (Cell Signaling Technologies, Danvers, MA, 1:200 Cat.n° 2775S), SQSTM1/p62 (Cell Signaling Technologies, Danvers, MA.1:100 Cat.n°7695), or Atg12 (GeneTex, USA,1:1000 Cat.n° GTX629815) overnight at 4°C in a humidified chamber. The following day, cells were incubated with Cy2 (green) (Jekson Laboratories, 1:5000 Cat.n°711-225-152) or Cy3 (red) (Jekson Laboratories, 1:5000 Cat.n°111-166-045) conjugated secondary antibodies for 1 h at room temperature. Nuclei were visualized by DAPI (Calbiochem, San Diego, CA 1:10000 Cat.n° D9542-1MG). Coverslips were mounted on slides and imaged with LSM-510 META confocal microscope (Carl Zeiss, ObErkochen, Germany). The fluorescence intensity was determined by ImageJ software as the mean pixel density of staining area in each cell. After

subtraction of background, the intensity values were shown as arbitrary units relative to control: CTCF (corrected total cell fluorescence) = Integrated Density – (Area of selected cell X Mean fluorescence of background readings).

3.5 Lysotracker Staining

Cells were plated on 8-well coverglass slide (Sarstedt, Germany) and treated with LPS under normoxic or hypoxic conditions. For Baf A1 and CQ treatment, the compounds were added at a concentration of 100 nM and 100 μM, respectively, 6 h before the end of the experiment. After 24h, cells were labelled with Lyso-ID Green Detection Kit (Enzo Life Sciences, Plymouth Meeting, PA, USA) and nuclear staining was performed by using DAPI. Cells were analysed by confocal microscope and the fluorescence intensity was determined by ImageJ software, as described above.

3.6 Immunoblotting and Antibodies

DCs were seeded in 35x10 mm Petri's dishes (Corning, New York, NY, USA) containing 2 ml of medium at the density of $5.00x10^5$ cells/ml. Because DCs are both adherent and suspension cells, at indicated time, supernatant was collected and centrifugated in order to also recover suspension cells. The adherent cells were washed twice with cold PBS without Ca^{2+} and Mg^{2+}, lysed with 40 ul of RIPA buffer containing a cocktail of protease inhibitors (Sigma -Aldrich) and frozen at -20°C. Samples were then thawed on ice and centrifugated at 14000 x g for 20 minutes. Supernatant was recovered and protein concentration was determined using Micro BCA Protein Assay Reagent kit (Rockford, USA) and equal amounts of total proteins were loaded onto SDS-PAGE gel. After transferring, PVDF membranes were incubated with the specific primary antibodies over night at 4°C: HIF-1α (BD Biosciences, San Jose, CA, 1:200 Cat.n° 610958), Bax (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n° 2772), Bcl-xl (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n°2764), LC3B (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n°2775), Beclin-1 (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n°3495), Atg3 (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n°3415), Atg5 (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.8540), Atg7 (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n°8558), Atg12 (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n°4180), phNFkB (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n°3033), phAkt (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n°4058), php38 MAP Kinase (Thr180/tyr182) (Cell Signaling Technologies, Danvers, MA,1:1000 Cat.n°9211), php44/42 MAP Kinase (Thr202/Tyr204) (Cell Signaling Technologies, Danvers, MA1:1000 Cat.n°9101), PARP (Cell Signaling Technologies, Danvers, MA1:1000 Cat.n°9542), and β-actin (Sigma-Aldrich, 1:50000 Cat.n° A3854). Anti-mouse IgG HRP

(Cell Signaling Technologies, Danvers, MA, 1;2000 Cat.n°7076) and anti-rabbit IgG-HRP (Cell Signaling Technologies, Danvers, MA, 1;2000 Cat.n°7074) were used as secondary antibodies (Cell Signaling Technologies, Danvers, MA). Detection of images was performed by ChemiDoc™ MP System (Bio-Rad, Hercules, CA). The intensity of the band was quantified using Image Lab software (Bio-Rad).

3.7 RNA Extraction and RT-qPCR

DCs were seeded in 35x10 mm Petri's dishes (Corning, New York, NY, USA) containing 2 ml of medium at the density of $25.00x10^5$ cells/ml. Total RNA was extracted using EuroGOLD™Trifast reagent (Euroclone, Devon, UK) The addition of chloroform and the centrifugation of the samples triggered the separation of the sample into three phases: an organic phase on the bottom of the tube containing proteins and cellular debris, an intermediate opaque phase containing DNA and an aqueous upper phase containing the total RNA. The aqueous phase was transferred to a new tube and then washed in isopropanol and cold 75% ethanol, resuspended in nuclease free water, boiled 10 minutes at 56°C and stored at -80°C for short time. RNA quantification was evaluated with the Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Fisher, Thermo Fisher Scientific, Waltham, MA, USA). cDNA from total RNA extracted in Trifast reagent was synthesized using iScript™cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). RT-qPCR analysis was performed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA). mRNA levels of BNIP3, VEGF-A, IL-1β, IL-18, TNF-α, IL-6, IL-10, and TGF-β were determined by MiniOPTICON™ System (Bio-Rad Laboratories) and analysed on an iQ5™ Optical System Software (Bio-Rad Laboratories). Relative quantification was done by using the $2^{-\Delta\Delta CT}$ method [158] and β-actin as housekeeping gene. Primers were validated as previously described [159].

3.8 Statistical Analysis

The data are presented as the mean ± SEM of at least 3 independent experiments. Statistical analyses were performed with Graph-Pad Prism (San Diego, CA, USA). Analysis of variance (ANOVA) and unpaired two-tailed Student's t test were used to test for significant numerical differences among the group. Difference of $p \leq 0.05$ was considered to be statistically significant ($*p \leq 0.05$; $**p \leq 0.01$).

4. RESULTS

4.1 Hypoxia upregulates the expression of HIF-1α and target genes in DCs

To evaluate the impact of hypoxia on DC autophagy, we exposed DCs to normoxic (20% O_2) and hypoxic (2% O_2) condition for 24 hours in the presence or not of LPS. The TLR4 ligand, LPS, was used to get DC final maturation. Since adaptive responses to hypoxia are mainly mediated by HIF-1α, its expression was evaluated by using confocal microscopy and Western Blot analysis. As expected, a significant increase in HIF-1α protein levels was observed in DCs under hypoxic conditions, especially in the presence of LPS, compared to the normoxic control (**Figure 18A**). HIF-1α upregulation usually results in an enhanced transcription of genes which present HREs in the promoter region, including BNIP3 and VEGF-A. BNIP3 is directly regulated by HIF-1 because it contains HRE. HIF-mediated BNIP3 expression is required for the optimal induction of autophagy in hypoxia [6] and can either lead to cell death or promote cell survival driving mytophagy activation [126]. BNIP3 mechanism of action in autophagy induction seems to involve its atypical BH3 domain which mediates dissociation of the Bcl-2–Beclin-1complexes [6]. VEGF production is up-regulated by hypoxia, acidosis, and hypoglycemia. The cognate DNA recognition site of HIF-1α is HRE. HIF-1α binds to HRE of target genes such as VEGF, erythropoietin, and glycolytic enzymes. The binding of HIF-1α to HRE in the VEGF promoter is a predominant enhancer of VEGF production. In our model, HIF-1α accumulation was associated with an increased expression of BNIP3 and VEGF-A mRNA, particularly in LPS stimulated DCs, as determined by RT-qPCR analysis after DCs were exposed to normoxia or hypoxia for 24 hours (**Figure 18B)**.

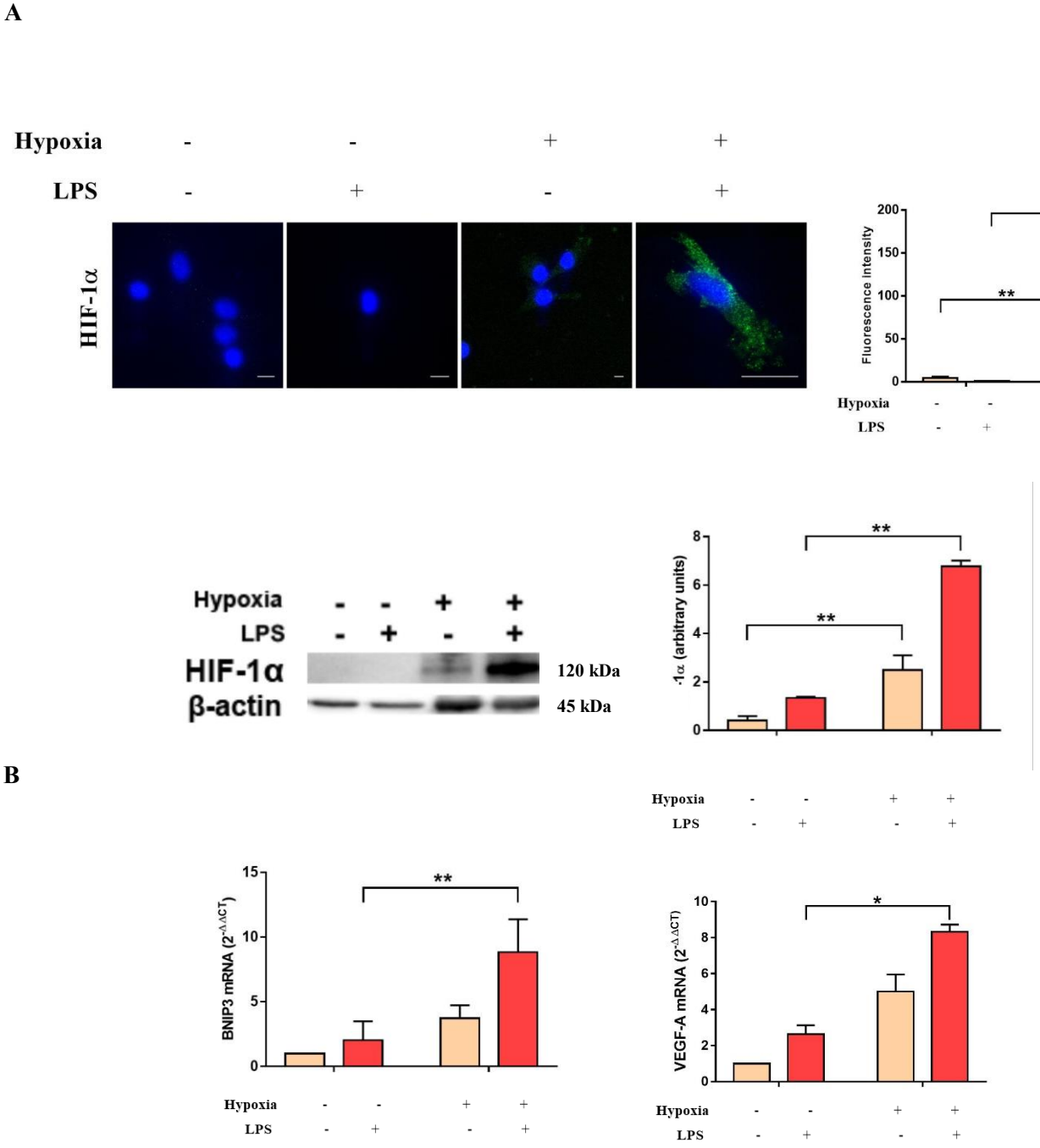


Figure 18: Expression of HIF-1α, BNIP3 and VEGF-A in iDCs and mDCs (100ng/ml LPS) at 24h of treatment either under normoxia and hypoxia (2% O_2) (A). *HIF-1α protein levels after 24 h exposure to normoxia and hypoxia as determined by confocal microscopy analysis (Scale bar: 8 µm; 15 µm only for LPS in hypoxia) and Western Blot (blot shown is representative of four independent experiments and β-actin was used as loading control)* ***(B)*** *RT-qPCR analysis of BNIP3 and VEGF-A mRNA expression (β-actin was used as a housekeeping gene). The values (mean ± S.E.M.) refer to three independent experiments. * and ** indicate*

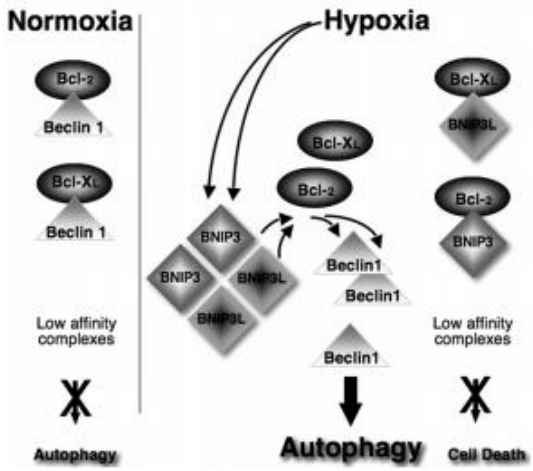


Figure 19: Hypothetical model of HIF-induced autophagy*. In normoxia (left), Beclin-1 forms low-affinity complexes with Bcl-xl and Bcl-2, thereby decreasing the rate of autophagy. In hypoxia (right), the rapid induction of the BH3-only proteins (BNIP3 and BNIP3L) displaces Beclin-1 from Bcl-xl and Bcl-2, leading to autophagy. The affinity of the BH3 domains of the BNIP proteins is too low to form tight complexes with Bcl-xl and Bcl-2. Therefore, BNIP3 and BNIP3L fail to induce cell death [6]*

4.2 Hypoxia enhances autophagy-associated lysosomal activity mDCs

Autolysosome formation and enhanced lysosomal activity are distinctive feature of cells undergoing autophagy; thus, these phenomena were studied in our cell model order to assess autophagy induction in response to reduced oxygen tensions. Autophagy-associated lysosomal activity was analysed in iDCs and mDCs by using Lyso-ID Green stain, which highlights lysosome structures within DCs exposed to normoxia and hypoxia for 24 hours. As shown in **Figure 20**, confocal analysis revealed a significant increase of acidic vesicles in mDCs under hypoxic conditions, as compared to the relative normoxic control suggesting that mDCs has a high lysosomal activity that is associated to autophagy.

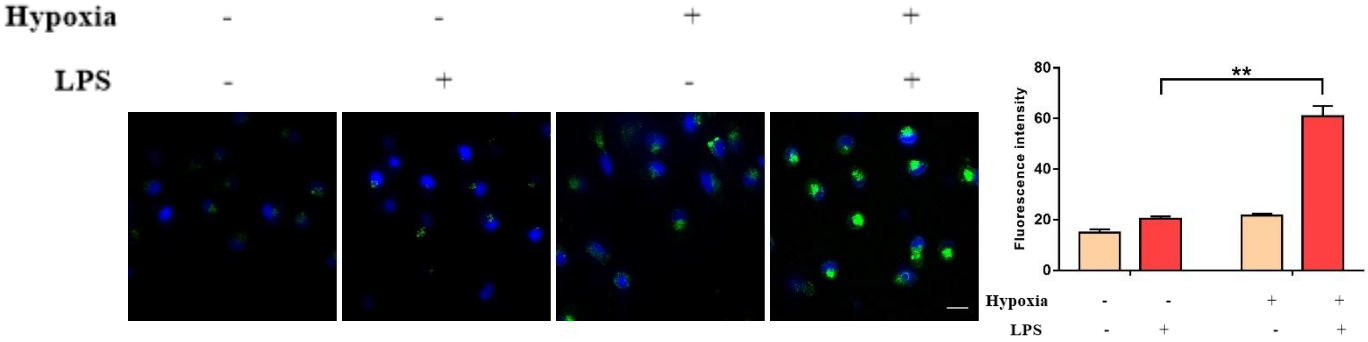


Figure 20: Detection of lysosomal structure in iDCs and mDCs (100ng/ml LPS) activity at 24h of treatment either under normoxia and hypoxia (2% O_2). *After 24h of incubation under normoxic and hypoxic conditions, cells were labelled with Lyso-ID Green Detection Kit, which accumulates in lysosomes. A nuclear staining was performed by using DAPI. DCs were then photographed with a confocal microscope (Scale bar: 15µm). Fluorescence intensity was assessed by using ImageJ software. The values (mean ± S.E.M.) refer to three independent experiments. * and ** indicate statistically significant differences ($p \leq 0.05$ and $p \leq 0.01$, respectively).*

4.3 Impact of hypoxia on mitochondrial membrane potential in DCs

In recent years, fluorescent dyes have been frequently used for monitoring mitochondrial membrane potential (ΔΨM) to evaluate mitochondrial viability and function. The key role of ΔΨM is to drive ATP synthesis using oxidative phosphorylation. The magnitude of ΔΨM, keeping the electron traveling across the electron transport chain to maintain an optimum electrochemical gradient, is determined by the balance between the production of ATP and its consumption. Increased ATP generation or mitochondrial dysfunction can lead to a decrease in ΔΨM. The study of ΔΨM is usefull to monitor the integrity of the cell in terms of cell survival. Indeed, in apoptotic or unhealthy cells the loss of ΔΨM, cause cell death. For this reason, we used JC-1 dye to evaluate mitochondrial integrity in DCs. It has to be kept in mind that in healthy mitochondria, JC-1 form the so called "J aggregates" that exhibit excitation and emission in the red spectrum, while in unhealthy mitochondria it exhibits its original green fluorescent spectrum. Under this condition, JC-1 cannot be retained into the cell to form J aggregates, since the inside of the mitochondria is less negative because of increased membrane permeability and consequent loss of electrochemical potential. Therefore, mitochondrial integrity is measured by the ration between red and green fluorescence. **Figure 21** shows that, LPS or hypoxia alone do not alter mitochondrial membrane potential, while synergistically they enhance it. The importance of this result is relevant since autophagy requires healthy mitochondria; indeed mitochondrial dysfunction is associated with the activation of mTOR and suppression of autophagy.

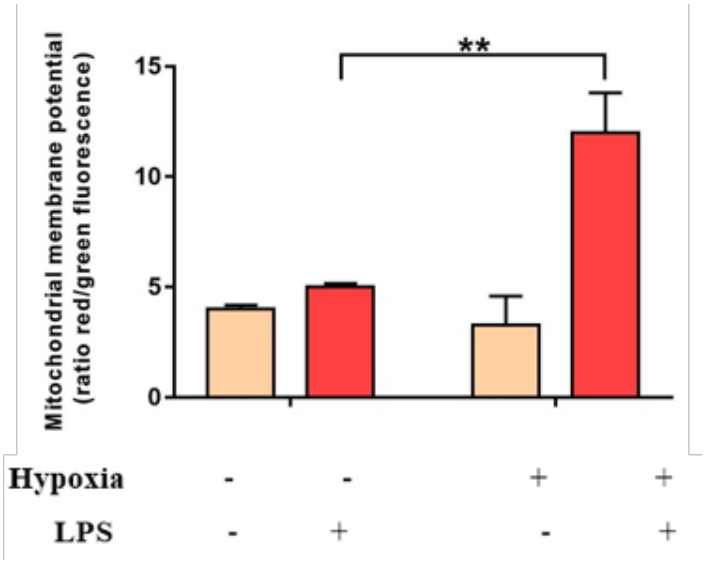


Figure 21: Mitochondrial membrane potential in iDCs and mDCs (100ng/ml LPS) at 48h of treatment either under normoxia and hypoxia (2% O_2). *Mitochondrial membrane potential analysis by JC-1 dye under normoxic or hypoxic conditions at 48h. The values (mean ± S.E.M.) refer to three independent experiments. * and ** indicate statistically significant differences ($p \leq 0.05$ and $p \leq 0.01$.*

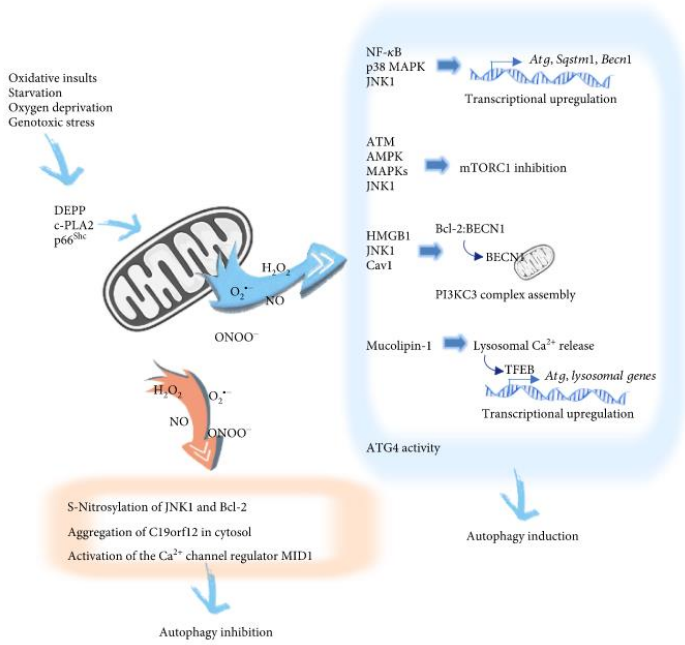


Figure 22: Cross talk between mitochondria and autophagy [3]

4.4 Hypoxia inhibits cell death and promote cell survival in mDCs

In previous reports, it has been shown that hypoxia affects DC death through the activation of a pro-apoptotic program that was antagonized by LPS [103]. To confirm the protective role of LPS against apoptosis in hypoxic DCs we analysed Bax and Bcl-xl, pro- apoptotic and anti-apoptotic markers, respectively. Indeed, Bax is a member of the Bcl-2 family that play key role in the regulation of apoptosis and plays a role in the mitochondrial apoptotic process. Under normal conditions, BAX is mostly cytosolic via constant retrotranslocation from mitochondria to the cytosol mediated by Bcl-2L1/Bcl-xL, which avoids accumulation of toxic Bax levels at the mitochondrial outer membrane. Under stress conditions, Bax undergoes a conformation change that causes translocation to the mitochondrion membrane, leading to the release of cytochrome c that then triggers apoptosis, promotes activation of caspase3, and thereby apoptosis. **Figure 23A** shows that Bax protein levels were reduced in hypoxic LPS-stimulated DCs. The antiapoptotic properties of Bcl-xl have been attributed to its ability to prevent translocation of cytochrome c to the cytosol, and thus interfere with the subsequent activation of cytosolic caspases and apoptosis. As reported in **Figure 23B** hypoxia induces the expression of Bcl-xl in LPS-stimulated cells. This was paralleled by an increased number of alive cells, as detected by cell viability assay (**Figure 23C).** All together these data support the theory that hypoxia promotes cell survival in mDCs.

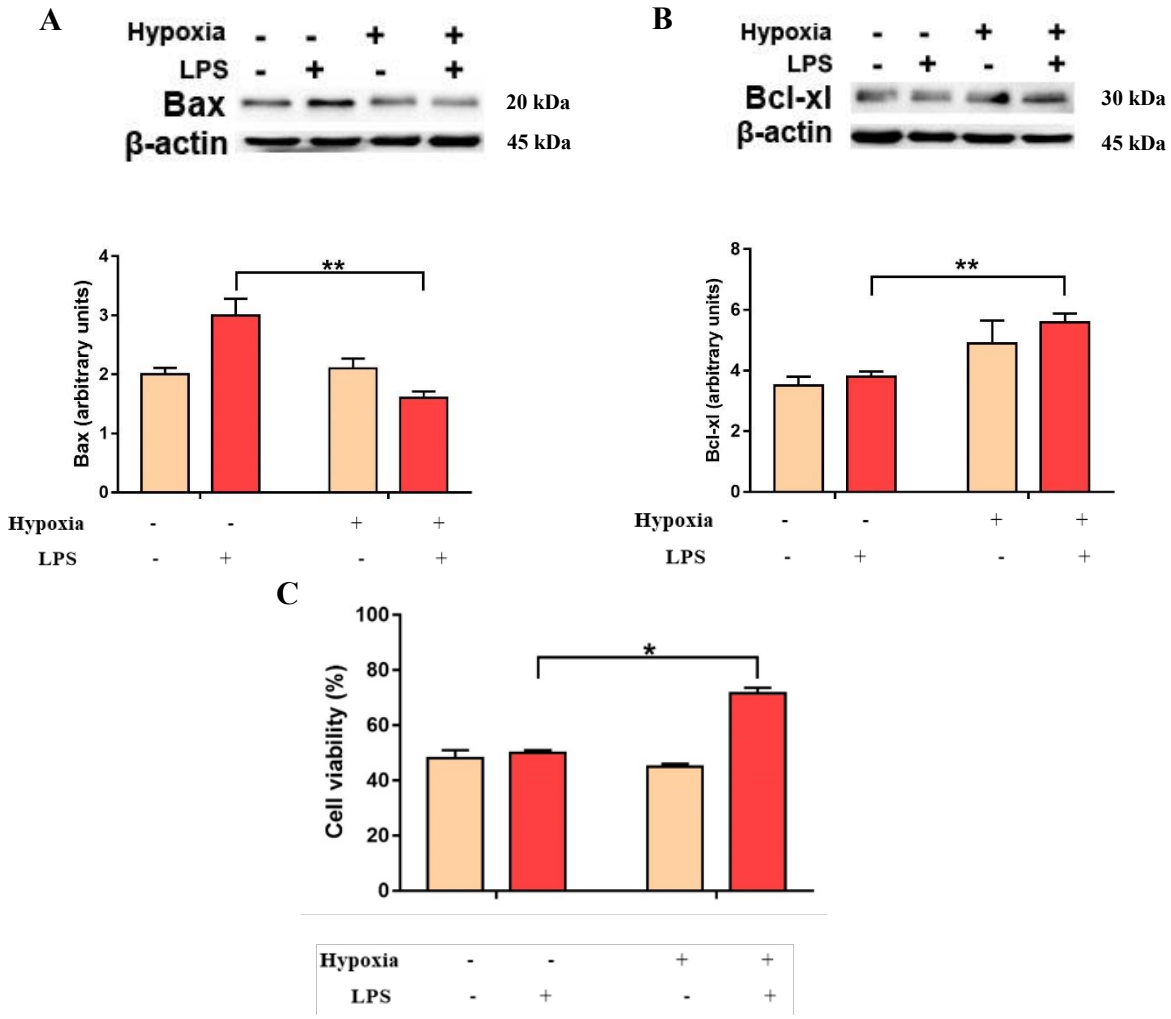


***Figure 23**. **Hypoxia decreases the expression of the pro-apoptotic protein Bax, enhances that of Bcl-xl and affects the survival of iDCs and mDCs (100ng/ml LPS) at 48h of treatment either under normoxia and hypoxia (2% O_2)**. **(A)** Bax, **(B)** Bcl-xl protein levels and **(C)** cell survival after 48h under normoxic or hypoxic conditions. The values (mean ± S.E.M.) refer to three independent experiments. * and ** indicate statistically significant differences ($p \leq 0.05$ and $p \leq 0.01$, respectively).*

4.5 Hypoxia modulates LC3B, SQSTM1/p62 and Beclin-1 expression in DCs

In order to investigate more deeply how hypoxia may affect DCs autophagy, we next analysed the protein levels of two key autophagic markers, LC3B and SQSTM1/p62. Two forms of LC3 are produced and found in different locations inside the cells. Indeed, LC3B-I is in the cytoplasm and is processed into another form, LC3B-II, associated with the autophagosome membrane. LC3 can bind to SQSTM1/p62 through a short 22-amino acid region located N-terminally to the UBA domain in SQSTM1/p62 that was found to be required for this interaction (**Figure 24**). SQSTM1/p62 is itself degraded by autophagy and may serve to link ubiquitinated proteins to the autophagic machinery to enable their degradation in the lysosome.

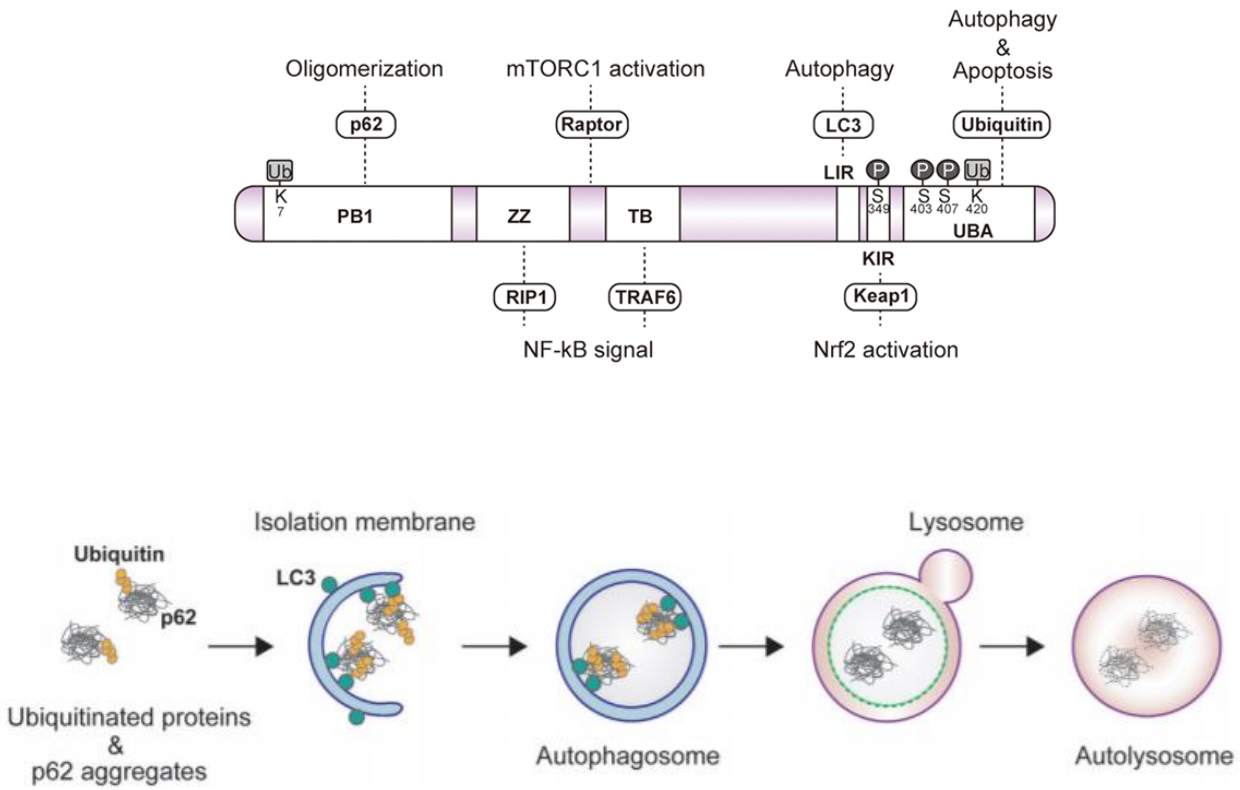


Figure 24: SQSTM1/p62-LC3B interaction*. SQSTM1/p62 interacts with ubiquitinated proteins through its C-terminal ubiquitin-associated (UBA) domain, and forms aggregates through its N-terminal Phox and Bem1 domain. The resulting protein aggregates are tethered to the autophagosome by direct interaction of p62 and LC3B. The isolation membrane elongates and sequesters SQSTM1/p62 and ubiquitin into the autophagosome. The autophagosome fuses with lysosome to form an autolysosome [160]*

Confocal immunofluorescent analysis shows that under normoxic conditions the effects of LPS on LC3B-II was not significant. However, LC3B-II was significantly enhanced in LPS-treated DCs under hypoxia. Accordingly, protein level of SQSTM1/p62 in DCs was reduced after hypoxic treatment in the presence of LPS when compared to the normoxic control, indicating a significant increase of autophagy. In contrast, LPS treatment under normoxia resulted in accumulation of SQSTM1/p62, suggesting that the induction of the autophagic process occurred only under hypoxic conditions. Of interest, SQSTM1/p62 was reduced under hypoxic condition in mDCs if compared to hypoxic iDC underlining the correlation between autophagy and the maturation state of DCs **(Figure 25).**

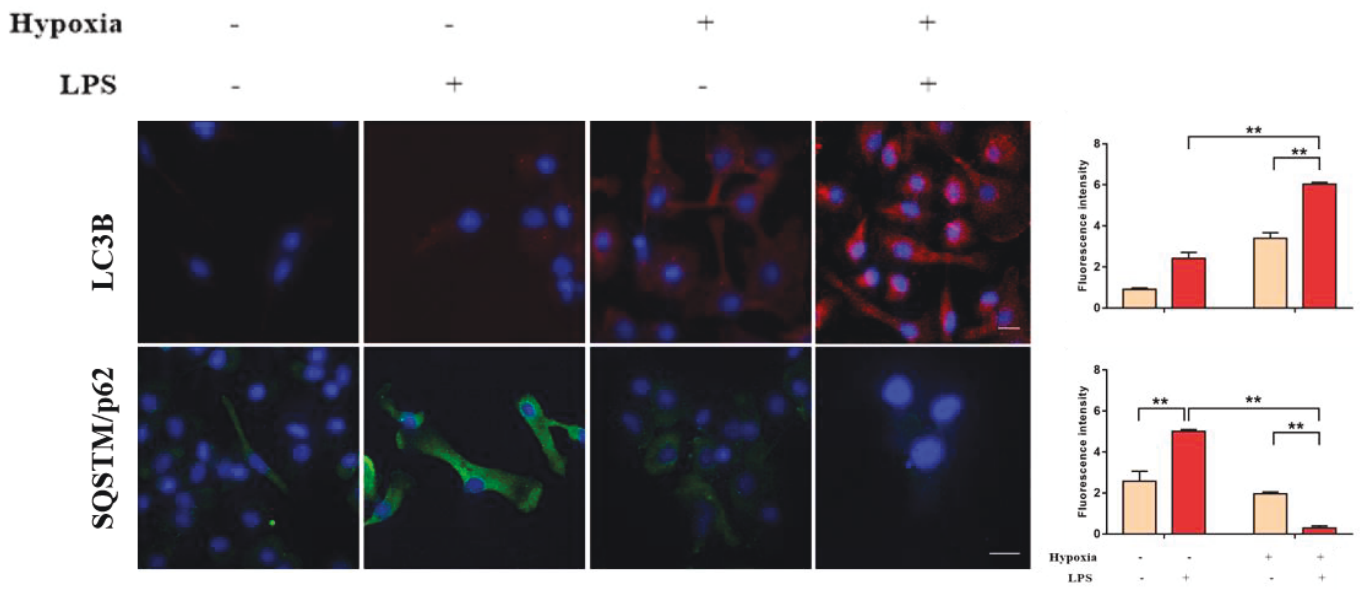


Figure 25: LC3B and SQSTM1/p62 protein levels in iDCs and mDCs (100ng/ml LPS) at 48h of treatment either under normoxia and hypoxia (2%O_2). *Confocal microscopy analysis of LC3B and SQSTM/p62 after 48h exposure to normoxia and hypoxia in DCs stimulated or not with LPS (Scale bar: 15)*

Kabeya and colleagues demonstrated that immunoblot analysis detected LC3B as two bands at 18 and 16 kDa in the lysates of various cells, implying that both the 18 and 16 kDa bands derived from the same mRNA. The 18 and 16 kDa forms of LC3B were called LC3-I and LC3-II, respectively. The amount of LC3-II is associated with the extent of autophagosome formation. LC3-II is the first mammalian protein identified that precisely associates with autophagosome membranes [161]. Under an analytical point of view the amount of LC3-II could be relatively overestimated by immunoblotting. Indeed the band intensity of LC3-II is sometimes stronger than that of LC3-I even in nutrient-rich conditions. For that reason, the amount of LC3-II or the LC3-II/LC3-I ratio is used for determining autophagic activity in mammalian cells [162]. To this purpose, we next analysed LC3B-II/LC3B-I ratio by Western blot. **Figure 26A** shows that this ratio was significantly increased in hypoxic LPS-treated DCs, as compared with normoxia. Accordingly, with the immunofluorescent confocal analysis, the protein level of SQSTM1/p62 was significantly enhanced upon LPS treatment under normoxia, while it was reduced under hypoxia, indicating a pro-autophagic process only in the latter condition **(Figure 26B)**. The fact that LPS-treated DCs were more autophagic under hypoxia was further confirmed by a significant increase of another marker of autophagy, Beclin-1, which is required for the autophagic flux induction thanks to the presence of BH3 domain with a conserved

molecular mechanism of binding to antiapoptotic Bcl-2 homologs **(Figure 26C).** All together results point out that autophagy is triggered by hypoxia in mDCs.

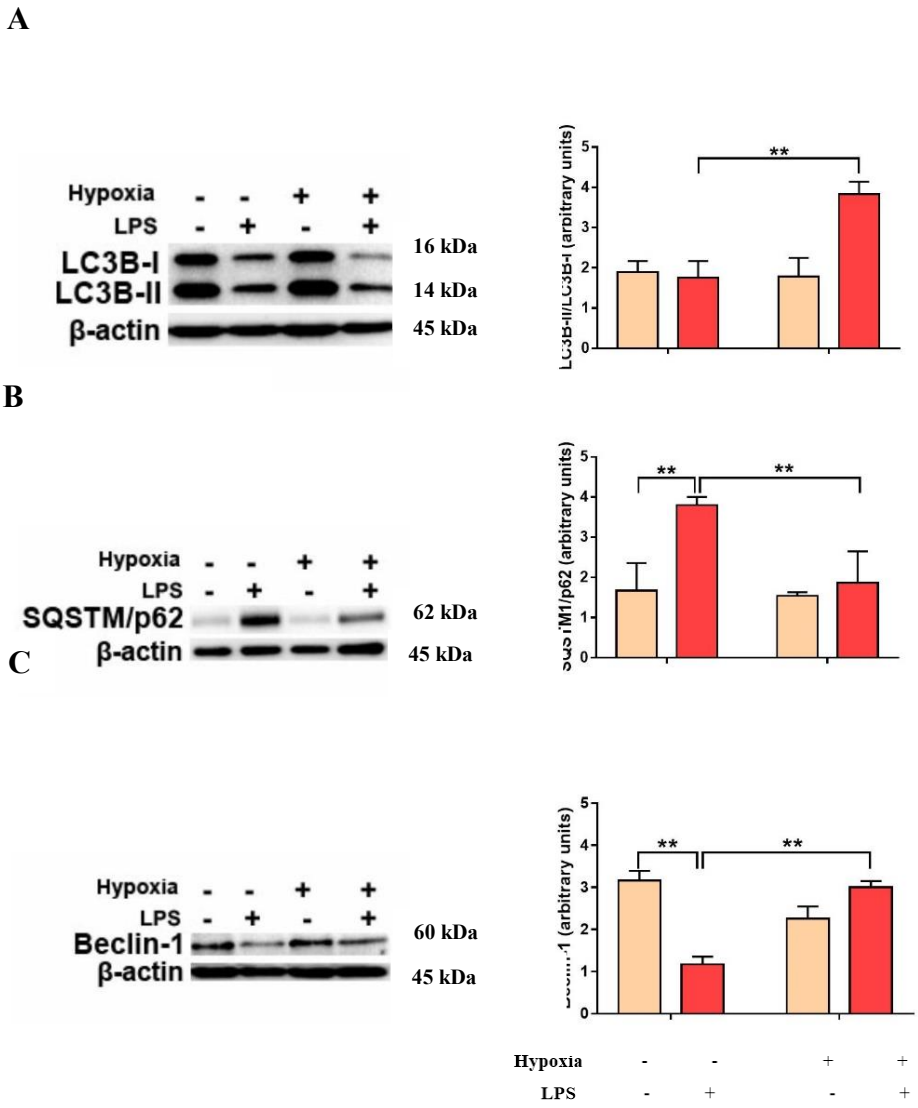


Figure 26: Autophagic markers iDCs and mDCs (100ng/ml LPS) at 48h of treatment either under normoxia and hypoxia (2%O_2). (A) *LC3B-II/LC3B-I,* ***(B)*** *SQSTM1/p62, and* ***(C)*** *Beclin-1 protein levels as determined by western blotting (blot shown is representative of three independent experiments and β-actin was used as loading control). ** indicate statistically significant differences ($p \leq 0.01$; $n = 4$).*

4.6 Atg protein levels are modified in DCs

Autophagosome formation is a dynamic event, which is executed by the sequential function of Atg proteins. Recently, there is growing evidence showing that some ATG proteins can directly interact with membranes, transfer lipids between membranes and regulate lipid metabolism. For these reasons we analysed the effect of hypoxia on the level of several Atg proteins in DCs treated with LPS. We first analysed the protein level of Atg12 by confocal immunofluorescent analysis. Atg12 is activated by Atg7, transferred to Atg10 and finally conjugated to Atg5. The Atg12-Atg5 conjugate is an irreversible complex essential for autophagosome elongation. As shown in **Figure 27A**, confocal

microscopy analysis revealed that Atg12 was reduced upon LPS treatment under normoxic conditions. However, when DCs were exposed to hypoxia, LPS treatment resulted in significant increase of Atg12 protein level, when compared with normoxic LPS-treated DCs. These results were confirmed by Western blot analysis (**Figure 27B**). Indeed, antibodies against Atg12-Atg5 complex and to Atg5 alone revealed a significant reduction in LPS-treated DCs under normoxia, while under hypoxia LPS treatment resulted in significant increase in the protein levels of both Atgs. This data was supported by the results obtained also for other Atg proteins, including Atg3 and Atg7, which are crucial for autophagosome formation [163]. Even in these cases, when DCs were treated with LPS under normoxia, we observed a significant decrease in the Atgs that we had analysed. Of interest, in all cases, Atg levels in LPS-treated cells were lower than in control cells. Indeed, this could be due to the turnover that is associated with autophagy, since it is a degradative process. These results are in line with the above data, and in particular with those of SQSTM1/p62, supporting the fact that autophagy is inhibited in normoxic mDCs, while it is induced in mDCs.

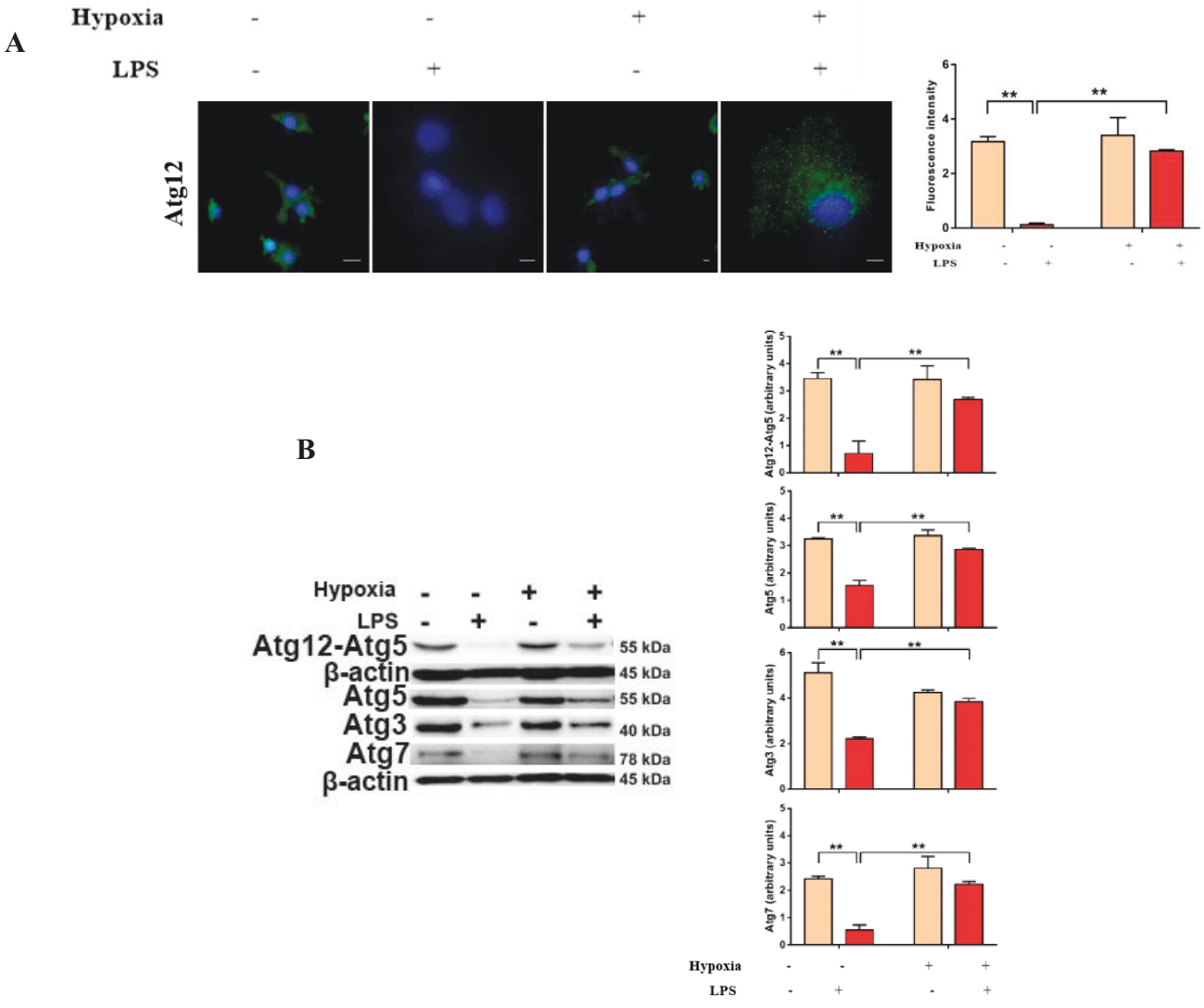


Figure 27: Levels of Atg proteins in iDCs and mDCs (100ng/ml LPS) at 24h of treatment either under normoxia and hypoxia (2%O_2) (A) *Atg12 protein levels after 24h exposure to normoxia and hypoxia in the presence of LPS, as determined by confocal microscopy analysis (Scale bar: 15 µm for medium, normoxia, and hypoxia, and 4 µm for LPS, in normoxia and hypoxia)* ***(B).*** *Atg12-Atg5, Atg5, Atg3, and Atg7 protein levels as determined by western blot analysis (β-actin was used as loading control). The blots are representative of three independent experiments. ** indicate statistically significant differences ($p \leq 0.01$; $n = 3$).*

4.7 Hypoxia affects the expression of signaling molecules potentially involved in both DCs survival and autophagy

To further establish the effect of hypoxia on DCs, we next analysed the activation of several signaling pathways associated with DC survival and, so far, with autophagy [164]. Mitogen-activated protein kinases (MAPK) cascades have been shown to play a pivotal role in transduction extracellular signal to cellular responses. MAPK pathways transfer, amplify and integrate signals from diverse range of stimuli, including hypoxia, and provoke appropriate physiological response including cellular, differentiation, development and inflammatory response [165]. The Erk (extracellular regulated kinases) pathway has been the best characterized MAPK pathway and represents one of the most studied one belonging to this family [166]. More recently, Erk activity has been associated with autophagy in many cellular models, including monocytes and macrophages. Moreover, direct Erk activation by overexpression of constitutively active MEK can promote autophagy without any other stimulus [167]. As shown in **Figure 28A**, LPS treatment resulted in an increased phosphorylation of Erk in normoxic conditions. However, Erk phosphorylation was significantly enhanced in hypoxic mDCs when compared to hypoxic iDCs. More interestingly, Erk phosphorylation was significantly enhanced in hypoxic mDCs compared to normoxic mDCs. We observed a similar pattern also for Akt that, along with Erk, is essential to inhibit DC apoptosis and to promote DC survival [168]. These results confirm that LPS promotes the activation of a pro-survival program in DCs

We also investigated NFkB and p38 pathways which are both involved in DC maturation and activation, including the expression of several cytokines, which are released by DCs [169]. In this case, LPS treatment resulted in an increased phosphorylation of NFkB and p38 in mDCs under normoxia and hypoxia if compared to iDCs. More interesting, and in line with the results obtained for Erk and Akt, NFkB and p38 were significantly enhanced in hypoxic mDCs when compared to normoxic mDCs (**Figure 28B)** underlining, once again, that hypoxia may enhanced cell survival in mDCs.

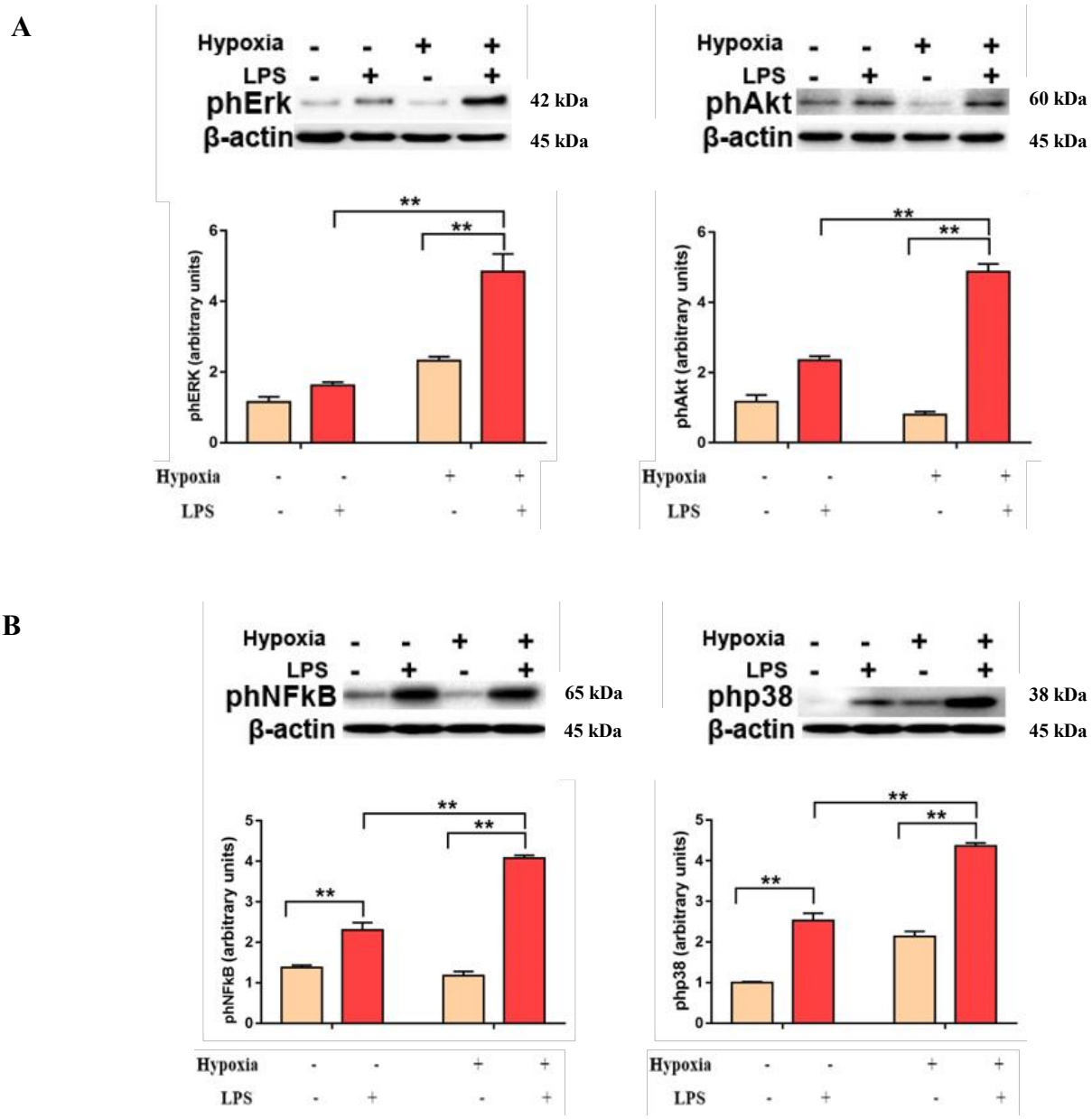
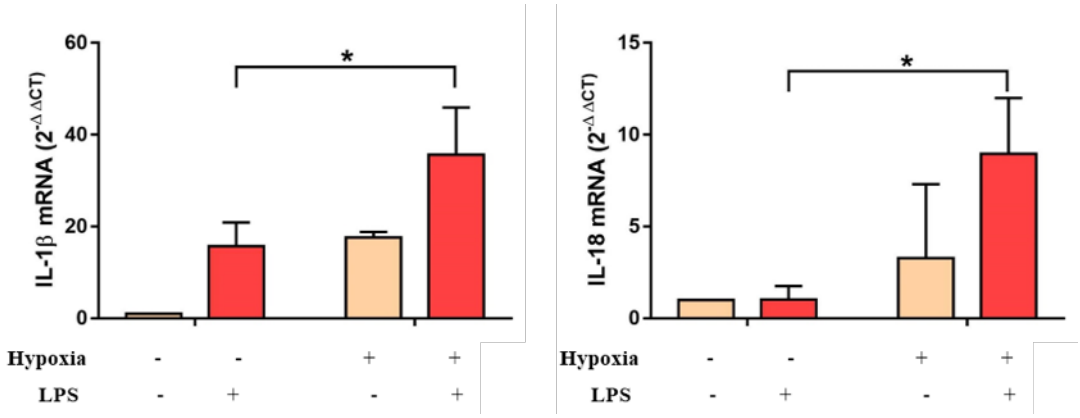


Figure 28: Signaling pathways expression in iDCs and mDCs (100ng/ml LPS) at 24h of treatment either under normoxia and hypoxia (2% O_2). ***(A)****Erk, Akt and* ***(B)*** *NFκB, and p38 phosphorylation in DCs after a 24h exposure to normoxia and hypoxia with or without LPS, as determined by western blotting (the blots are representative of three independent experiments and β-actin was used as loading control) ** indicate statistically significant differences ($p \leq 0.01$; $n = 3$).*

4.8 Hypoxia modulates cytokine expression in DCs

Interleukin 1 family cytokines include IL-1β, IL-18, as well as other cytokines, and orchestrate a wide range of immune and physiological roles [170]. In particular, IL-1β, have strong pro-inflammatory effects and are responsible for the recruitment of myeloid cells to sites of inflammation. IL-18 is similarly pro-inflammatory and both IL-1β and IL-18 are tightly regulated; they are produced as inactive pro-forms that are cleaved by caspase-1 to form the mature, bioactive, cytokines. Recently, studies have suggested that IL-1β can drive the release of other cytokines involved in inflammation, such as, IL-1α and IL-23, to further highlighting the importance of this cytokine in regulating inflammatory responses. In addition, autophagy can itself directly regulate the transcription process and secretion of IL-1β and IL-18[171] in response to several environmental stimuli, such as amino acid deprivation, but also by both host- and pathogen-derived molecules, including TLRs ligands. Therefore, we decided to investigate how hypoxia could affect the expression of some of the mediators that could have a major impact on autophagy. **Figure 29A** clearly shows that when DCs were stimulated with LPS under hypoxic conditions, the expression of IL-1β and IL-18 mRNA was significantly higher as compared with the normoxic control. This suggest that mDCs under hypoxic conditions may be autophagic since, in particular IL-1β, is able to activate autophagosome formation [171].

A



B

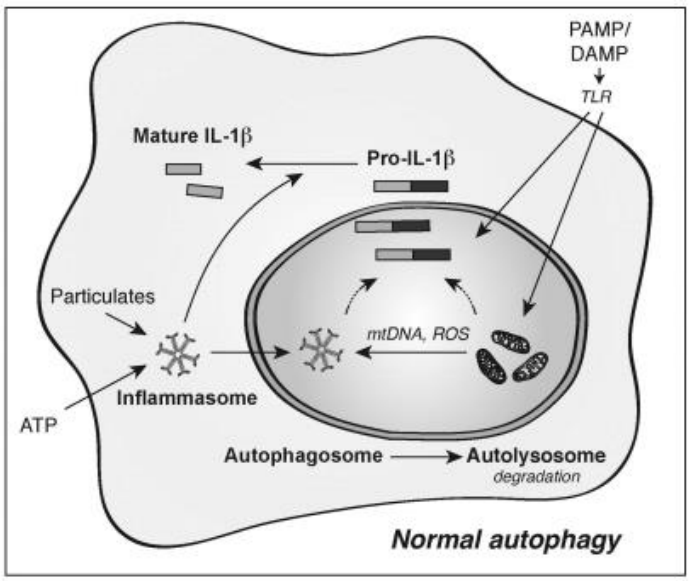
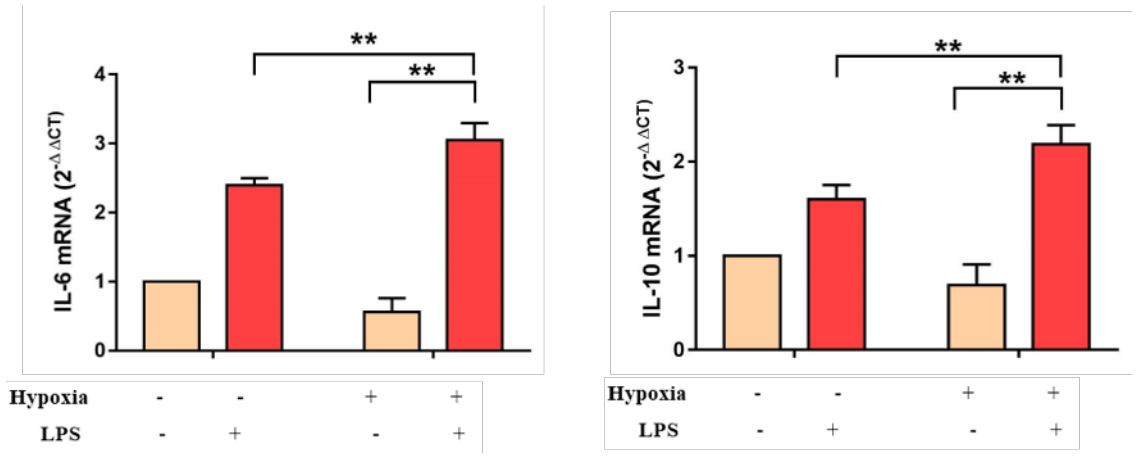


Figure 29: (A) expression of IL-1β in iDCs and mDCs (100ng/ml LPS) at 24h of treatment either under normoxia and hypoxia (2%O_2)(B) Schematic illustration of IL-1β secretion regulated by autophagy*. Secretion of IL-1β by macrophages and DCs cells requires the production of an inactive precursor (pro-IL-1β) and the assembly and activation of an inflammasome, which, in turn, activates caspase-1 to process pro-IL-1β into the mature, active cytokine. Stimulation of TLRs by endogenous danger signals (DAMPs) and pathogen-associated molecules (PAMPs) stimulates the production of pro-ILβ [172].*

Since enhanced levels of IL-6 and IL-10 are associated with neutrophil autophagy [173], we decided to investigate whether the expression of these cytokines was modified in our model. **Figure 30A** shows that IL-10 and IL-6 expression pattern were similar to those of IL-1β and IL-18 since when mDCs were stimulated with LPS, under hypoxic conditions, the expression of IL-10 and IL-6 mRNA was significantly higher as compared with normoxic mDCs. However, LPS was able to significantly enhanced the expression of these two cytokines also in mDCs if compared to hypoxic controls.

A



It has been demonstrated that TNF- α is able to stimulate autophagy in several cell models by inducing the expression of Beclin-1/Atg7, and that autophagy can itself regulates the production of several cytokines in order to induce autophagosome formation depending on TNF-α production [171]. **Figure 30B** shows that hypoxia enhances the expression of TNF-α production in mDCs, if compared with normoxic mDCs, confirming that autophagy may be involved in the regulation of TNF- α expression in mDCs. So far, we focused out attention on cytokines that are associated with inflammation, thus we newt wanted to evaluate the expression of cytokine with opposite effect such as TGF-β. This protein has unique and potent immunoregulatory properties since is produced by every leukocyte lineage, including DCs. Its expression serves in both autocrine and paracrine modes to control the differentiation, proliferation, and state of activation of immune cells. TGF-β inhibit macrophages activation and DCs differentiation, inflammation and T cell proliferation [1]. In agreement with previous reports [174], **Figure 30C** shows that in mDCs, TGF-β expression was reduced both under normoxia and hypoxia, while we do not appreciated differences between normoxic and hypoxic iDCs, suggesting that in mDCs a proinflammatory program may be triggered.

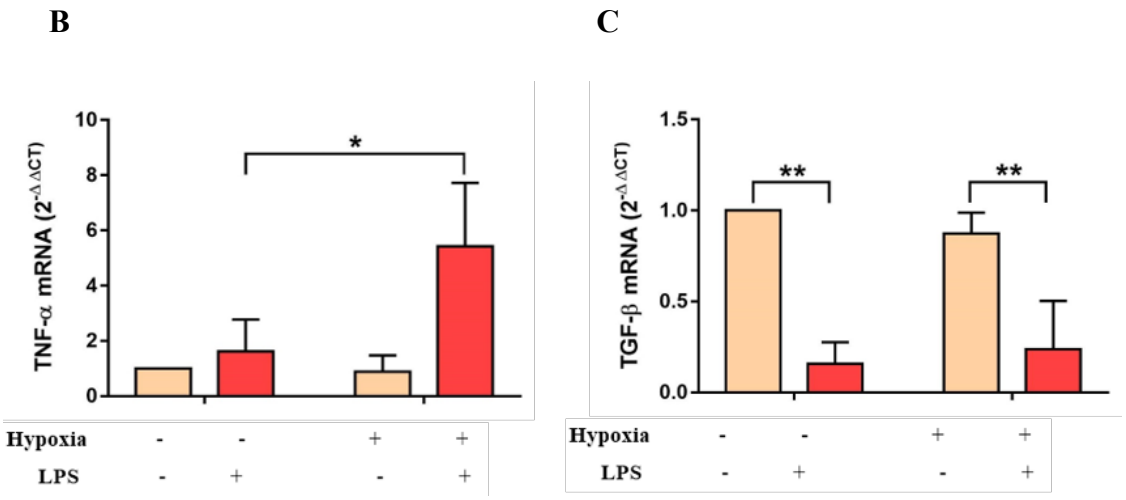


Figure 30: Modulation of IL-6, IL-10, TNF-α and TGF-β expression in iDCs and mDCs (100ng/ml LPS) after 24h of treatment either under normoxia and hypoxia (2% O_2) *RT-qPCR analysis of* ***(A)*** *IL-6, IL-10 and* ***(B)*** *TNF-α and* ***(C)*** *TGF-β mRNA expression (β-actin was used as housekeeping gene) at the end of 24h treatment. * and ** indicate statistically significant differences ($p \leq 0.05$ and $p \leq 0.01$, respectively; $n = 4$)*

4.9 Autophagy inhibition impairs the acidic/lysosomal compartments in hypoxic mDCs

In order to better understand the mechanism of LPS-induced autophagy under hypoxia, we used two well-known autophagy inhibitors, such as Baf A1 and CQ. The experiments were performed under hypoxia, since our data suggested that the effects of LPS on DCs were not significant under aerobic conditions. To this end we used the pH-sensitive lysosomal dye LysoTracker in hypoxic LPS-treated

DCs. Confocal microscopy analysis revealed that Baf A1 treatment led to a rapid decrease of fluorescence due to the decreased the acidity of lysosomes, as it is expected from an inhibitor of the vacuolar proton pump [175]. For many years it has been reported that CQ blocks the autophagic flux through the same mechanism as BafA1, which increases lysosomal pH and thus inhibits the activity of resident hydrolases. However, recent reports demonstrated that CQ severely affects the endo-lysosomal system and the Golgi complex *in vitro* and *in vivo*, thereby probably impairing the basal autophagic flux by decreasing autophagosome-lysosome fusion, and not by inhibiting lysosomal degradation capacity as Baf A1 does [176]. This effect is clearly demonstrated in our study were CQ did not decrease LysoTracker-positive structures, which tended to be more and much larger after CQ treatment compared to control or Baf A1 treatment because of the inhibition between autophagosome and lysosome **(Figure 31).**

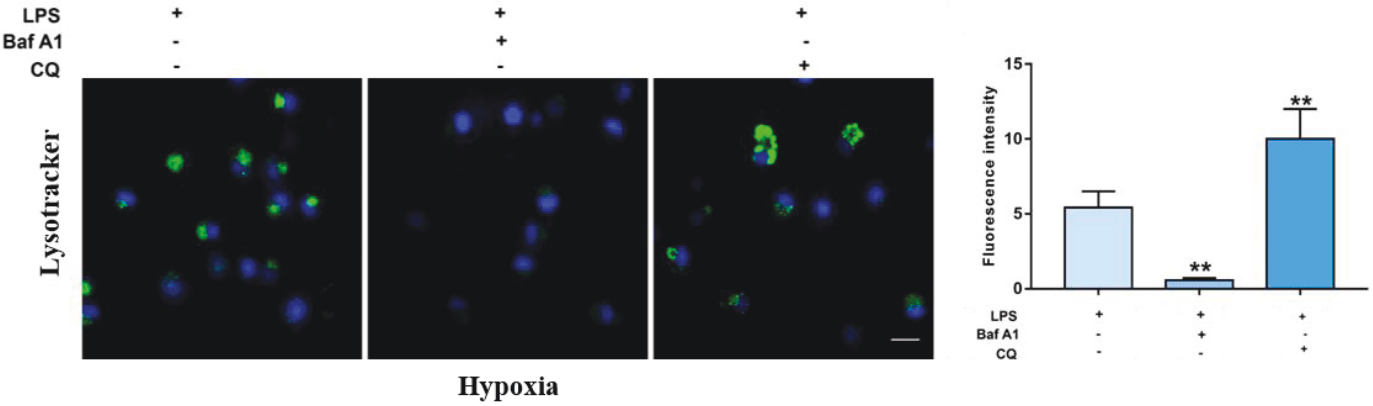


Figure 31: Detection of acidic/lysosomal compartments by Lysotracker and confocal analysis in hypoxic (2%O_2) LPS-stimulated (100ng/ml LPS) DCs cells after 24h of treatment with Baf A1 and CQ *(Scale bar: 15 μm)* ***at the end of 24h treatment****. * and ** indicate statistically significant differences ($p \leq 0.05$ and $p \leq 0.01$, respectively; n = 4).*

4.10 Autophagy inhibition modifies the expression of LC3B and SQSTM1/p62 in hypoxic mDCs

Since LC3B and SQSTM1/p62 were modulated in the previous results, we decided to evaluate the effects of Baf A1 and CQ on these two well-known autophagic. Figure 32 shows that autophagy inhibition by Baf A1, resulted in SQSTM1/p62 accumulation and in a higher LC3B-II/LC3B-I ration. SQSTM1/p62 accumulation is established to be associated with autophagy inhibition, while increased amount of LC3B-II is usually associated with an induction of autophagy. However, LC3B-II after Baf A1 treatment, can correlate with block of the autophagic process at the late steps of this pathway, due to autophagosome fusion with lysosomes and/or lysosomal degradation impairment. Similarly, also CQ treatment resulted in a significant enhancement of LC3B-II/LC3B-I ratio. However, CQ

reduces the protein level of SQSTM1/p62, probably because CQ inhibits autophagy by decreasing autophagosome-lysosome fusion, but does not substantially decreases lysosomal activity of the cells, preserving their capacity to degrade delivered material (**Figure 32**).

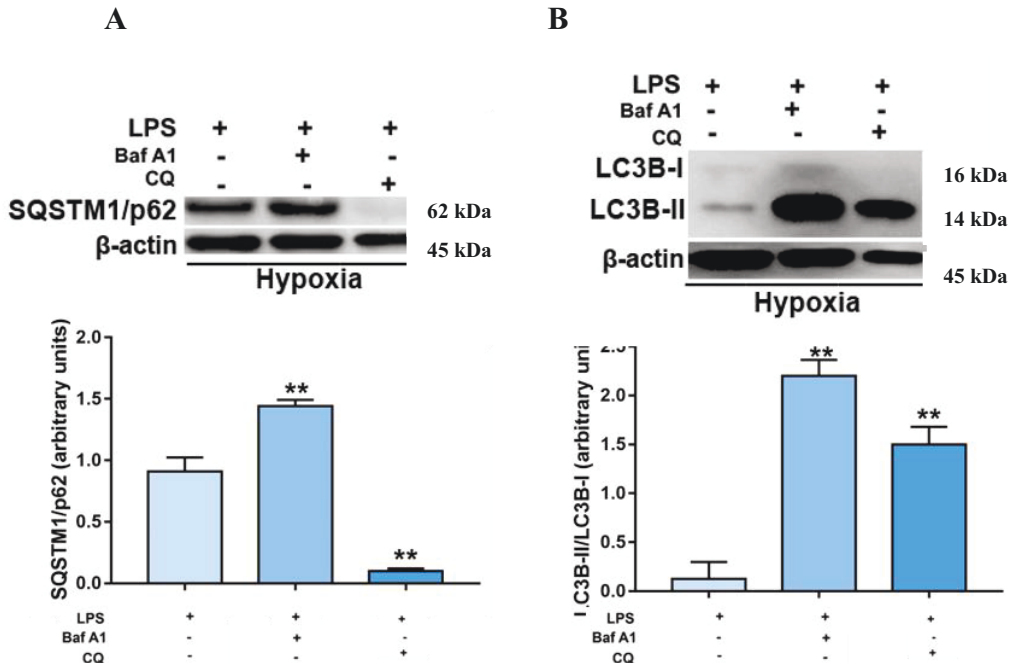


***Figure 32**: (A) SQSTM1/p62 and (B) LC3B-II/LC3B-I protein levels in 24h hypoxic (2%O_2) LPS-stimulated (100ng/ml LPS) cells after treatment with Baf A1 and CQ, as determined by western blotting (blot is representative of three independent experiments and β-actin was used as loading control) and cell viability. * and ** indicate statistically significant differences ($p \leq 0.05$ and $p \leq 0.01$, respectively; $n = 4$).*

4.11 Autophagy inhibition impairs the expression levels of Atg in hypoxic mDCs

Because of the importance of Atgs proteins in the autophagic process, we evaluated the protein levels of several Atgs, which are involved in different steps of the autophagic process to further test the effects of Baf A1 and CQ. Of interest, Atg12 and Atg5 are involved in the elongation process of the autophagosome and are present in the outer membrane of the phagophore, in order to avoid premature fusion with the lysosome. Atg3 is an autophagy E1-like enzyme involved in the two ubiquitin-like conjugation systems that are essential for the autophagosome biogenesis, Atg7 is essential for autophagy and vacuole transport and together with Atg5, is considered to be crucial for the induction of autophagy. **Figure 33** demonstrated that both Baf A1 and CQ treatments reduced the protein levels of all the Atg mentioned, clearly indicating that autophagy is inhibited in hypoxic mDCs.

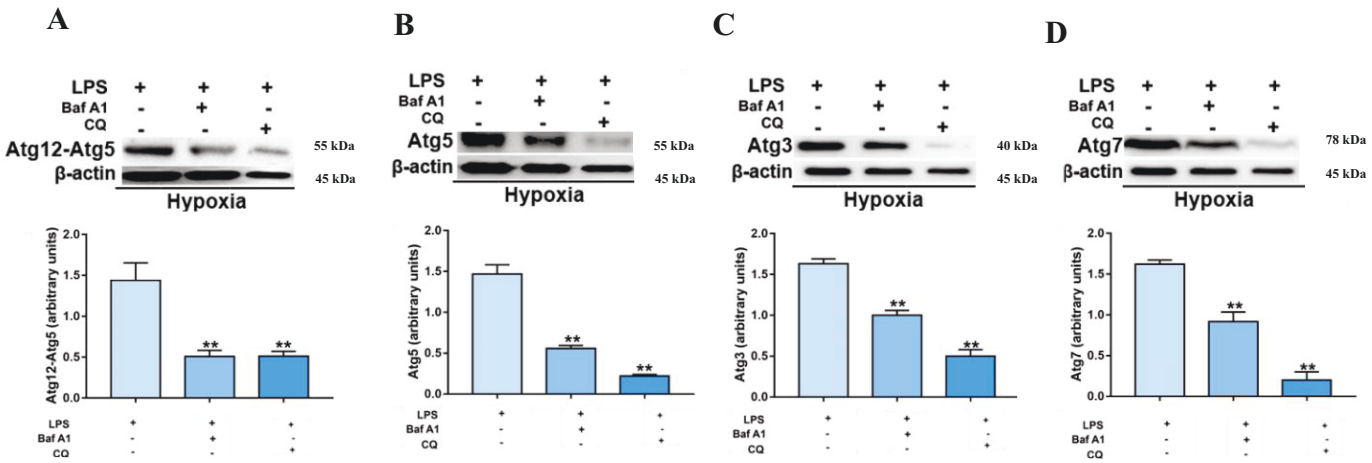


***Figure 33**: (A) Atg12, (B) Atg5, (C) Atg7, and (D) Atg3 protein levels in 24h hypoxic (2%O_2) LPS-stimulated (100ng/ml LPS) cells after treatment with Baf A1 and CQ as determined by western blotting (blot is representative of three independent experiments and β-actin was used as loading control) and cell viability. * and ** indicate statistically significant differences ($p \leq 0.05$ and $p \leq 0.01$, respectively; $n = 4$).*

4.12 Inhibition of autophagy modifies pro-apoptotic and anti-apoptotic markers in hypoxic mDCs

Undoubtedly, there are several connections between the apoptotic and autophagic processes [177]. Therefore, we finally, evaluated apoptotic and survival marker in order to better understand the relationship between autophagy inhibition and apoptosis in hypoxic mDCs. **Figure 34** shows that Baf A1 and CQ increased the protein level of the pro-apoptotic protein Bax and reduced that of Bcl-xl, which are pro- apoptotic and pro-survival proteins, respectively [178]. This was associated with a significant increase of PARP cleavage, which is another marker of apoptosis [179]. Indeed, PARP cleavage is followed by degradation of DNA and therefore apoptosis. Finally, the inhibition of autophagy by Baf A1 and CQ resulted in a significant decrease of alive cells. These results demonstrated that inhibition of autophagy may induce DC death, suggesting that autophagy is essential for DC survival.

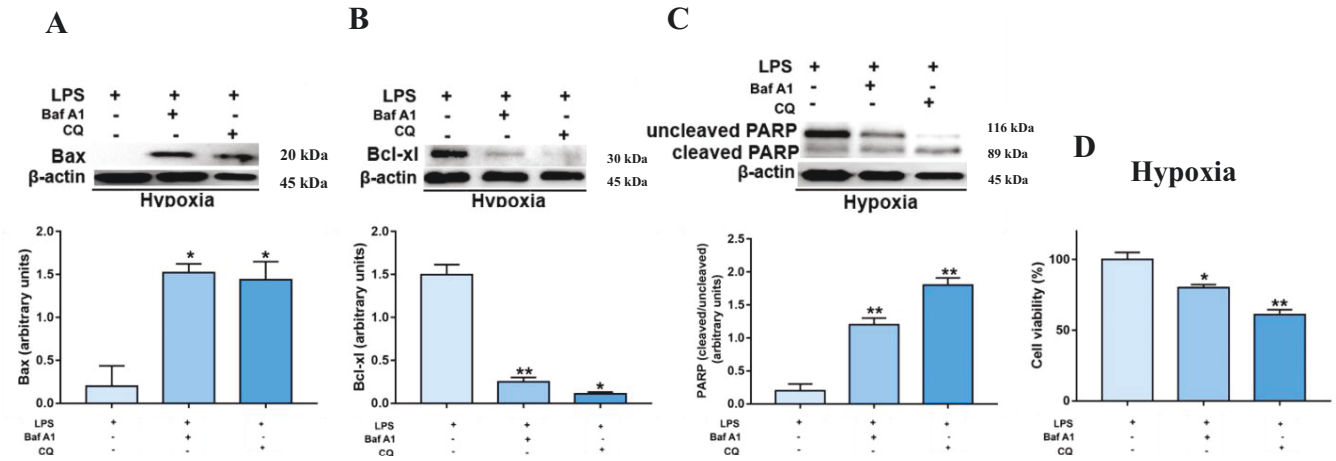


***Figure 34**: (A) Bax, (B) Bcl-xl, and (C) PARP protein levels as determined by western blotting (blot is representative of three independent experiments and β-actin was used as loading control) and **(D) cell viability in 24h hypoxic (2% O_2) LPS-stimulated (100ng/ml LPS) cells treated with Baf A1 and CQ**. * and ** indicate statistically significant differences ($p \leq 0.05$ and $p \leq 0.01$, respectively; $n = 3$).*

5. Addendum

Part of these data have been published in: Monaci, S., Aldinucci, C., Rossi, D., Giuntini, G., Filippi, I., Ulivieri, C., Marotta, G., Sozzani, S., Carraro, F., & Naldini, A. (2020). Hypoxia Shapes Autophagy in LPS-Activated Dendritic Cells. Frontiers in Immunology, 11, 3071.

6. DISCUSSION AND CONCLUSIONS

Hypoxia is a condition of low oxygen tension that characterizes lymphoid organs as well as almost every site of inflammation, tissue damage and neoplasia. Of interest, DCs are professional antigen-presenting cells patrolling tissues to sense danger signals, to activate specific immune responses and to promote inflammation and tissue repair [180]. During their lifespan, DCs are frequently exposed to hypoxic microenvironments that affects their differentiation and functions. Immune response may occur under different oxygen tensions depending on the tissue raising the question of the relationship between the quality of immune responses and oxygen levels in the tissue. Additional data suggests that low oxygen tensions modulate the immune response by affecting T-cell response, monocyte migration and neutrophil functions [181]. It is therefore evident that low oxygen tensions, like those present in the lymphoid organs, can influence the physiology of DCs. The molecular pathways activated by hypoxia are mainly mediated by HIF-1α accumulation and can be associated with angiogenesis, cell survival, migration and autophagy. However, divergent effects of hypoxia on DC maturation were reported in previous studies based on the expression of maturation markers, costimulatory molecules, chemokine receptors, and T cell priming ability, suggesting the great interest in understanding the impact of hypoxia in DCs [110].

Although extensive studies have been conducted to define the role of HIF-1α in the induction of the expression of cytokines / chemokines and chemokine receptors in cells of the immune system, little is known about the impact of hypoxia on DCs autophagy under hypoxic microenvironment. In our lab, the first approach to this field was when Naldini et al., demonstrated that hypoxia affects DC survival and induced the expression of one of the most studied autophagic markers such as BNIP3 [103]. For this reason, we decided to deeply investigate the role of hypoxia on DCs autophagy. We used a pO_2 that was equal to 14 mmHg ($2\%O_2$) to mimic, at least in part, the hypoxic microenvironment physiologically present in lymphoid organs. Indeed, when DCs were exposed to hypoxia, the expression levels of the master regulator of hypoxia, HIF-1α, were significantly enhanced when compared to the normoxic control. This effect was even more evident when hypoxic cells were treated with LPS. This data was in line with other reports [104], suggesting that those synergistic effects of LPS and hypoxia potentially determine a sustained inflammatory activation of DC, resulting in an enhanced immunogenic response. Previous reports indicate that hypoxia promotes autophagy resulting in prolonged cell survival [182]. Indeed, the expression of BNIP3, which is transcriptionally regulated by HIF1-α, is tightly related to autophagy because is capable of binding to Bcl-2, thereby disrupting the interaction between Bcl-2 and Beclin-1, and inducing autophagy. Accordingly, we here report that hypoxia enhances BNIP3 mRNA expression together with the

mRNA level of VEGF-A. Both have the HRE, therefore the binding of HIF-1α to HRE in the VEGF-A promoter is a predominant enhancer of VEGF-A and BNIP3 production.

The impact of hypoxia on DCs autophagy was evident regarding the number of acidic/functional lysosomes, since autophagy is a lysosome-dependent homeostatic process. Indeed, according to literature [183, 184], we show here that lysosomal activity was significantly enhanced in hypoxic DCs stimulated with LPS. The theory that the induction of autophagy leads to cell survival and to a modulation of apoptosis, was supported by the results that we obtained analysing pro-apoptotic and anti-apoptotic markers. Indeed, hypoxic DCs stimulated with LPS showed a significantly higher mitochondrial membrane potential, suggesting that the combination of hypoxia and LPS enhanced cell survival in mDCs. This was associated with an increased number of alive cells, as detected by cell viability assays, a decrease of the pro-apoptotic protein Bax and enhancement of the antiapoptotic molecule Bcl-xl. These results confirm and support the theory that LPS protect hypoxic DCs from apoptosis, by promoting autophagy.

Autophagy is a complex process that can be triggered after several external stimuli, such as pathogens, thanks to the danger signals perceived by TLRs. While it is widely acknowledged that autophagy activates TLR4 downstream signaling, the effect of LPS activation on autophagy is still a material of debate. Indeed, Delgado et al., reported that TLRs stimulation promoted the autophagy flux [185]. In contrast, and in agreement with our observation, Terawaki at al reported that TLR stimulation under normoxic conditions activates mTOR and suppresses autophagy in DCs [147]. Indeed, in our model we observed that under normoxic conditions the protein level of SQSTM1/p62 was significantly enhanced by LPS treatment. Furthermore, we did not observe a significant increase of LC3B-II, a phenomenon usually predictable during the autophagic process. However, when LPS-treated DCs were cultured under hypoxic conditions, we observed a significant reduction of SQSTM1/p62 protein level, paralleled by an increase of LC3B-II protein level. Thus, this suggest that the induction of the autophagic process in DCs is related to their maturation state and to the microenvironment in which they localize.

Several proteins are involved in the elongation and formation of autophagosomes and they are differently regulated during DC maturation steps [2]. Here we analysed one of the two ubiquitin-like systems involved in the autophagosome formation and maturation: the Atg12 system. This process is controlled by a series of ubiquitin-like conjugation reactions catalysed by the E1-like enzyme Atg7 and E2-like enzyme Atg3. The ATG conjugation systems in autophagy. Atg7 mediates the binding

of Atg12 and Atg5. Of interest, Atg7, Atg5, and Atg3 are key proteins in DC autophagy and functional activities [186]. We here demonstrate a dual role of LPS in DCs: Atgs protein levels were reduced upon LPS stimulation under normoxic conditions, but were significantly increased under hypoxia, confirming that the trigger of autophagic process in DCs is related to the microenvironment and to maturation stage.

The promotion of autophagy under hypoxia correlates with the induction of several signaling pathways and molecules known to be involved in cell survival [187]. In agreement with the hypothesis that autophagy promotes cell survival in LPS-treated DCs, we here show that the phosphorylation of Akt and Erk were significantly enhances in hypoxic mDCs. We should underline that both molecules are part of signaling pathways associated with DC autophagy and survival. Indeed, mammalian cells regulate autophagy via both class I and class III PI3K. Class I PI3K plays an inhibitory role, whereas class III PI3K kinase complex, which includes Beclin-1, plays a stimulatory role in autophagy by promoting the nucleation of autophagic vesicles. It has been demonstrated that Erk modulates autophagy via regulating the Beclin-1 level and by the inhibition on mTORC1, causing protective autophagy [188].

Besides Erk and Akt, there are others signaling pathways involved in autophagy, including p38 MAPK and NFkB. p38 has a dual role in autophagy, both as a positive and negative regulator. It is possible that the autophagic response may depend upon the nature of the stimulus and the strength and duration of the activated MAPK pathways. Our results, show an enhanced phosphorylation of p38 that could be associated with DCs activation in an autophagic context [169]. With regard to NFkB, there are a lot of evidence indicating its crosstalk with autophagy, both in physiological and pathological processes. In fact, it has been demonstrated that NFkB could trigger autophagy by directly inducing the expression of genes or proteins involved in the autophagosome machinery [189]. This observation was evident also in our study where the phosphorylation of NFkB was associated with DC autophagy.

To further support the hypothesis that hypoxia promotes survival and activation of DCs, we showed the upregulation of several cytokines, which are associated with autophagy. Indeed, IL-1β and IL-18 have been demonstrated to be triggered and regulated by autophagy [185]. Accordingly, we reported that both IL-1β and IL-18 are significantly enhanced under hypoxia in mDCs. In contrast, by in agreement with previous reports[174], we here demonstrated that the expression of the anti-inflammatory cytokine TGF-β was inhibited in mDCs, underlining, once again, the tight relationship

between inflammation and autophagy. However, its role as a regulator of immune and inflammatory functions depends on balancing acts between positive and negative effects on gene expression

Finally, the impact of autophagy on hypoxic mDCs was further confirmed by Baf A1 and CQ, that are two of the mostly used autophagy inhibitors [176]. Data from literature have widely shown that Baf A1 treatment affected autophagy in several cell types, including bone marrow–derived DCs [190]. Accordingly, in our study Baf A1 inhibited the autophagic process in hypoxic mDC by reducing the number of acidic compartments and upregulating SQSTM1/p62. However, we observed Baf A1 enhanced the protein level of LC3B-II. Accordingly with previous reports, this enhancement may be related to (a) LC3-I sequestration into p62 aggregates, (b) reduced autophagosome turnover, probably due to delayed trafficking to the lysosomes, (c) reduced fusion between compartments, (d) impaired lysosomal proteolytic activity [191]. In addition, accumulation of LC3B-II was also observed after CQ treatment. However, this is validated by the results obtained for functional lysosomes, where Lysotracker positive structures tended to be much larger after CQ treatment compared to control or Baf A1 treatment. Of interest, CQ treatment resulted also in SQSTM1/p62 reduction. This is in line with previous reports showing that the degradative capacity of the cells is intact after CQ treatment and the lysosomes preserve their capacity to degrade delivered material [192]. However, both Baf A1 and CQ reduced the protein levels of all the Atgs that we have analysed, confirming autophagy inhibition. The fact that autophagy may correlates with a prosurvival program, [193] is further supported by Baf A1 and CQ treatment, which resulted in a reduction of alive mDCs, in the upregulation of pro apoptotic markers, such as Bax and PARP cleavage, as well as the inhibition of prosurvival protein such as Bcl-2.

In conclusion, our data indicate that hypoxia shapes DCs autophagy and survival. Since hypoxia is a hallmark of lymphoid tissues, where DCs are recruited to exert their main effects, our results may give a contribution on how DCs adapt to changes of pO_2 to preserve their functions. Thus, this study underlines the relevance of DCs autophagy, not only within a physiological lymphoid microenvironment, but also in physio-pathological conditions, with important implication in the orchestration on immune response.

7. REFERENCES

1. Murphy, K. and C. Weaver, *Janeway's immunobiology*. 2016: Garland science.
2. Ghislat, G. and T. Lawrence, *Autophagy in dendritic cells.* Cellular & molecular immunology, 2018. **15**(11): p. 944-952.
3. Roca-Agujetas, V., et al., *Recent insights into the mitochondrial role in autophagy and its regulation by oxidative stress.* Oxidative Medicine and Cellular Longevity, 2019. **2019**.
4. Taylor, C.T., et al., *Hypoxia-dependent regulation of inflammatory pathways in immune cells.* The Journal of clinical investigation, 2016. **126**(10): p. 3716-3724.
5. Sato, K. and S. Fujita, *Dendritic cells-nature and classification.* Allergology International, 2007. **56**(3): p. 183-191.
6. Bellot, G., et al., *Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains.* Molecular and cellular biology, 2009. **29**(10): p. 2570-2581.
7. Nakazawa, M.S., B. Keith, and M.C. Simon, *Oxygen availability and metabolic adaptations.* Nat Rev Cancer, 2016. **16**(10): p. 663-73.
8. Cummins, E.P., M.J. Strowitzki, and C.T. Taylor, *Mechanisms and Consequences of Oxygen and Carbon Dioxide Sensing in Mammals.* Physiol Rev, 2020. **100**(1): p. 463-488.
9. De Vito, E.L., et al., *Effects of spontaneous and hypercapnic hyperventilation on inspiratory effort sensation in normal subjects.* Am J Respir Crit Care Med, 1998. **158**(1): p. 107-10.
10. Veras, M.M., et al., *Before the first breath: prenatal exposures to air pollution and lung development.* Cell Tissue Res, 2017. **367**(3): p. 445-455.
11. Giaccia, A.J., M.C. Simon, and R. Johnson, *The biology of hypoxia: the role of oxygen sensing in development, normal function, and disease.* Genes Dev, 2004. **18**(18): p. 2183-94.
12. Semenza, G.L., *The genomics and genetics of oxygen homeostasis.* Annual Review of Genomics and Human Genetics, 2020. **21**.
13. Buja, L.M., *Myocardial ischemia and reperfusion injury.* Cardiovasc Pathol, 2005. **14**(4): p. 170-5.
14. Netzer, N., et al., *Hypoxia-related altitude illnesses.* J Travel Med, 2013. **20**(4): p. 247-55.
15. Taylor, A.T., *High-altitude illnesses: physiology, risk factors, prevention, and treatment.* Rambam Maimonides Med J, 2011. **2**(1): p. e0022.
16. Pedersen, B.K. and A. Steensberg, *Exercise and hypoxia: effects on leukocytes and interleukin-6-shared mechanisms?* Med Sci Sports Exerc, 2002. **34**(12): p. 2004-13.
17. Sitkovsky, M. and D. Lukashev, *Regulation of immune cells by local-tissue oxygen tension: HIF1α and adenosine receptors.* Nature Reviews Immunology, 2005. **5**(9): p. 712-721.
18. Braun, R.D., et al., *Comparison of tumor and normal tissue oxygen tension measurements using OxyLite or microelectrodes in rodents.* Am J Physiol Heart Circ Physiol, 2001. **280**(6): p. H2533-44.
19. Bunn, H.F., *Erythropoietin.* Cold Spring Harb Perspect Med, 2013. **3**(3): p. a011619.
20. Beleslin-Cokic, B.B., et al., *Erythropoietin and hypoxia stimulate erythropoietin receptor and nitric oxide production by endothelial cells.* Blood, 2004. **104**(7): p. 2073-80.
21. Dunwoodie, S.L., *The role of hypoxia in development of the Mammalian embryo.* Developmental cell, 2009. **17**(6): p. 755-773.
22. Patel, J., et al., *Regulation of hypoxia inducible factors (HIF) in hypoxia and normoxia during placental development.* Placenta, 2010. **31**(11): p. 951-7.
23. Fryer, B.H. and M.C. Simon, *Hypoxia, HIF and the placenta.* Cell Cycle, 2006. **5**(5): p. 495-8.
24. Taylor, C.T. and S.P. Colgan, *Regulation of immunity and inflammation by hypoxia in immunological niches.* Nature Reviews Immunology, 2017. **17**(12): p. 774-785.

25. Harrison, J.S., et al., *Oxygen saturation in the bone marrow of healthy volunteers.* Blood, 2002. **99**(1): p. 394.
26. Huang, J.H., et al., *Requirements for T lymphocyte migration in explanted lymph nodes.* J Immunol, 2007. **178**(12): p. 7747-55.
27. Eltzschig, H.K. and P. Carmeliet, *Hypoxia and inflammation.* N Engl J Med, 2011. **364**(7): p. 656-65.
28. Haddad, J.J. and H.L. Harb, *Cytokines and the regulation of hypoxia-inducible factor (HIF)-1alpha.* Int Immunopharmacol, 2005. **5**(3): p. 461-83.
29. Chao, J., J.G. Wood, and N.C. Gonzalez, *Alveolar hypoxia, alveolar macrophages, and systemic inflammation.* Respir Res, 2009. **10**: p. 54.
30. Wood, J.G., et al., *Systemic hypoxia increases leukocyte emigration and vascular permeability in conscious rats.* J Appl Physiol (1985), 2000. **89**(4): p. 1561-8.
31. Hellwig-Burgel, T., et al., *Review: hypoxia-inducible factor-1 (HIF-1): a novel transcription factor in immune reactions.* J Interferon Cytokine Res, 2005. **25**(6): p. 297-310.
32. Semenza, G.L., *Hypoxia-inducible factors in physiology and medicine.* Cell, 2012. **148**(3): p. 399-408.
33. Semenza, G.L., *HIF-1: upstream and downstream of cancer metabolism.* Curr Opin Genet Dev, 2010. **20**(1): p. 51-6.
34. Schoch, H.J., S. Fischer, and H.H. Marti, *Hypoxia-induced vascular endothelial growth factor expression causes vascular leakage in the brain.* Brain, 2002. **125**(Pt 11): p. 2549-57.
35. Allinen, M., et al., *Molecular characterization of the tumor microenvironment in breast cancer.* Cancer Cell, 2004. **6**(1): p. 17-32.
36. McDonald, P.C., S.C. Chafe, and S. Dedhar, *Overcoming Hypoxia-Mediated Tumor Progression: Combinatorial Approaches Targeting pH Regulation, Angiogenesis and Immune Dysfunction.* Front Cell Dev Biol, 2016. **4**: p. 27.
37. Meehan, J., et al., *Inhibition of pH regulation as a therapeutic strategy in hypoxic human breast cancer cells.* Oncotarget, 2017. **8**(26): p. 42857-42875.
38. Kruger, B., et al., *Donor Toll-like receptor 4 contributes to ischemia and reperfusion injury following human kidney transplantation.* Proc Natl Acad Sci U S A, 2009. **106**(9): p. 3390-5.
39. Oechmichen, M. and C. Meissner, *Cerebral hypoxia and ischemia: the forensic point of view: a review.* J Forensic Sci, 2006. **51**(4): p. 880-7.
40. Ye, J., *Emerging role of adipose tissue hypoxia in obesity and insulin resistance.* Int J Obes (Lond), 2009. **33**(1): p. 54-66.
41. Bernards, R. and R.A. Weinberg, *A progression puzzle.* Nature, 2002. **418**(6900): p. 823.
42. Taylor, C.T. and S.P. Colgan, *Regulation of immunity and inflammation by hypoxia in immunological niches.* Nat Rev Immunol, 2017. **17**(12): p. 774-785.
43. Ke, Q. and M. Costa, *Hypoxia-inducible factor-1 (HIF-1).* Molecular pharmacology, 2006. **70**(5): p. 1469-1480.
44. Kaelin Jr, W.G. and P.J. Ratcliffe, *Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway.* Molecular cell, 2008. **30**(4): p. 393-402.
45. Bertout, J.A., S.A. Patel, and M.C. Simon, *The impact of O 2 availability on human cancer.* Nature Reviews Cancer, 2008. **8**(12): p. 967-975.
46. Semenza, G.L. and G.L. Wang, *A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation.* Molecular and cellular biology, 1992. **12**(12): p. 5447-5454.
47. Palazon, A., et al., *HIF transcription factors, inflammation, and immunity.* Immunity, 2014. **41**(4): p. 518-528.
48. Semenza, G.L., *Targeting HIF-1 for cancer therapy.* Nature reviews cancer, 2003. **3**(10): p. 721-732.
49. Jaakkola, P., et al., *Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation.* Science, 2001. **292**(5516): p. 468-472.

50. Wenger, R.H., D.P. Stiehl, and G. Camenisch, *Integration of oxygen signaling at the consensus HRE.* Sci STKE, 2005. **2005**(306): p. re12.
51. Harris, A.L., *Hypoxia--a key regulatory factor in tumour growth.* Nat Rev Cancer, 2002. **2**(1): p. 38-47.
52. Cramer, T., et al., *HIF-1alpha is essential for myeloid cell-mediated inflammation.* Cell, 2003. **112**(5): p. 645-57.
53. Cummins, E.P., et al., *The role of HIF in immunity and inflammation.* Mol Aspects Med, 2016. **47-48**: p. 24-34.
54. Mills, E.L. and L.A. O'Neill, *Reprogramming mitochondrial metabolism in macrophages as an anti-inflammatory signal.* Eur J Immunol, 2016. **46**(1): p. 13-21.
55. Gondin, J., et al., *Myeloid HIFs are dispensable for resolution of inflammation during skeletal muscle regeneration.* J Immunol, 2015. **194**(7): p. 3389-99.
56. Murdoch, C., et al., *The role of myeloid cells in the promotion of tumour angiogenesis.* Nat Rev Cancer, 2008. **8**(8): p. 618-31.
57. Fang, H.Y., et al., *Hypoxia-inducible factors 1 and 2 are important transcriptional effectors in primary macrophages experiencing hypoxia.* Blood, 2009. **114**(4): p. 844-59.
58. Thompson, A.A., et al., *Hypoxia-inducible factor 2alpha regulates key neutrophil functions in humans, mice, and zebrafish.* Blood, 2014. **123**(3): p. 366-76.
59. McInturff, A.M., et al., *Mammalian target of rapamycin regulates neutrophil extracellular trap formation via induction of hypoxia-inducible factor 1 alpha.* Blood, 2012. **120**(15): p. 3118-25.
60. Shi, L.Z., et al., *HIF1alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells.* J Exp Med, 2011. **208**(7): p. 1367-76.
61. Makino, Y., et al., *Hypoxia-inducible factor regulates survival of antigen receptor-driven T cells.* J Immunol, 2003. **171**(12): p. 6534-40.
62. Biju, M.P., et al., *Vhlh gene deletion induces Hif-1-mediated cell death in thymocytes.* Mol Cell Biol, 2004. **24**(20): p. 9038-47.
63. Criscuoli, M., et al., *The Shc protein RAI promotes an adaptive cell survival program in hypoxic neuroblastoma cells.* J Cell Physiol, 2018. **233**(5): p. 4282-4293.
64. Dehne, N. and B. Brüne, *HIF-1 in the inflammatory microenvironment.* Experimental cell research, 2009. **315**(11): p. 1791-1797.
65. Scholz, C.C. and C.T. Taylor, *Targeting the HIF pathway in inflammation and immunity.* Current opinion in pharmacology, 2013. **13**(4): p. 646-653.
66. Delves, P.J. and I.M. Roitt, *The immune system.* New England journal of medicine, 2000. **343**(1): p. 37-49.
67. Varol, C., A. Mildner, and S. Jung, *Macrophages: development and tissue specialization.* Annual review of immunology, 2015. **33**: p. 643-675.
68. Sun, L., et al., *Innate-adaptive immunity interplay and redox regulation in immune response.* Redox Biology, 2020: p. 101759.
69. Bonilla, F.A. and H.C. Oettgen, *Adaptive immunity.* Journal of Allergy and Clinical Immunology, 2010. **125**(2): p. S33-S40.
70. Rowley, D.A. and F.W. Fitch, *The road to the discovery of dendritic cells, a tribute to Ralph Steinman.* Cellular immunology, 2012. **273**(2): p. 95-98.
71. Steinman, R.M. and Z.A. Cohn, *Identification of a novel cell type in peripheral lymphoid organs of mice: I. Morphology, quantitation, tissue distribution.* The Journal of experimental medicine, 1973. **137**(5): p. 1142-1162.
72. Caux, C., et al., *GM-CSF and TNF-α cooperate in the generation of dendritic Langerhans cells.* Nature, 1992. **360**(6401): p. 258-261.
73. Sallusto, F. and A. Lanzavecchia, *Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus*

interleukin 4 and downregulated by tumor necrosis factor alpha. The Journal of experimental medicine, 1994. **179**(4): p. 1109-1118.
74. Alters, S.E., et al., *IL—13 Can Substitute for IL—4 in the Generation of Dendritic Cells for the Induction of Cytotoxic T Lymphocytes and.* Journal of1mmmini/wrap, 1999. **22**(3): p. 229-236.
75. Banchereau, J., et al., *Immunobiology of dendritic cells.* Annual review of immunology, 2000. **18**(1): p. 767-811.
76. Banchereau, J. and R.M. Steinman, *Dendritic cells and the control of immunity.* Nature, 1998. **392**(6673): p. 245-252.
77. Thomas, R. and P.E. Lipsky, *Dendritic cells: origin and differentiation.* Stem cells, 1996. **14**(2): p. 196-206.
78. Liu, K. and M.C. Nussenzweig, *Origin and development of dendritic cells.* Immunological reviews, 2010. **234**(1): p. 45-54.
79. Bonasio, R. and U.H. von Andrian, *Generation, migration and function of circulating dendritic cells.* Current opinion in immunology, 2006. **18**(4): p. 503-511.
80. Adams, S., D.W. O'Neill, and N. Bhardwaj, *Recent advances in dendritic cell biology.* J Clin Immunol, 2005. **25**(2): p. 87-98.
81. Colonna, M., G. Trinchieri, and Y.-J. Liu, *Plasmacytoid dendritic cells in immunity.* Nature immunology, 2004. **5**(12): p. 1219-1226.
82. Carbone, F.R., G.T. Belz, and W.R. Heath, *Transfer of antigen between migrating and lymph node-resident DCs in peripheral T-cell tolerance and immunity.* Trends in immunology, 2004. **25**(12): p. 655-658.
83. Guilliams, M., et al., *From skin dendritic cells to a simplified classification of human and mouse dendritic cell subsets.* European journal of immunology, 2010. **40**(8): p. 2089-2094.
84. Collin, M., N. McGovern, and M. Haniffa, *Human dendritic cell subsets.* Immunology, 2013. **140**(1): p. 22-30.
85. Hart, D.N. and J.L. McKenzie, *Interstitial dendritic cells.* International reviews of immunology, 1990. **6**(2-3): p. 127-138.
86. León, B., M. López-Bravo, and C. Ardavín, *Monocyte-derived dendritic cells formed at the infection site control the induction of protective T helper 1 responses against Leishmania.* Immunity, 2007. **26**(4): p. 519-531.
87. Segura, E., et al., *Human inflammatory dendritic cells induce Th17 cell differentiation.* Immunity, 2013. **38**(2): p. 336-348.
88. Lipscomb, M.F. and B.J. Masten, *Dendritic cells: immune regulators in health and disease.* Physiological reviews, 2002.
89. Dudek, A.M., et al., *Immature, Semi-Mature, and Fully Mature Dendritic Cells: Toward a DC-Cancer Cells Interface That Augments Anticancer Immunity.* Front Immunol, 2013. **4**: p. 438.
90. Kamath, A.T., et al., *The development, maturation, and turnover rate of mouse spleen dendritic cell populations.* J Immunol, 2000. **165**(12): p. 6762-70.
91. Huang, F.-P., et al., *A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes.* Journal of Experimental Medicine, 2000. **191**(3): p. 435-444.
92. Granucci, F., M. Foti, and P. Ricciardi-Castagnoli, *Dendritic cell biology.* Adv Immunol, 2005. **88**: p. 193-233.
93. Watarai, H., et al., *Plasma membrane-focused proteomics: dramatic changes in surface expression during the maturation of human dendritic cells.* Proteomics, 2005. **5**(15): p. 4001-11.
94. Sozzani, S., et al., *Human monocyte-derived and CD34+ cell-derived dendritic cells express functional receptors for platelet activating factor.* FEBS Lett, 1997. **418**(1-2): p. 98-100.

95. Rescigno, M., et al., *Coordinated events during bacteria-induced DC maturation.* Immunology today, 1999. **20**(5): p. 200-203.
96. Hopkins, R.A. and J.E. Connolly, *The specialized roles of immature and mature dendritic cells in antigen cross-presentation.* Immunologic research, 2012. **53**(1-3): p. 91-107.
97. Medzhitov, R. and C. Janeway, Jr., *The Toll receptor family and microbial recognition.* Trends Microbiol, 2000. **8**(10): p. 452-6.
98. Visintin, A., et al., *Regulation of Toll-like receptors in human monocytes and dendritic cells.* The Journal of Immunology, 2001. **166**(1): p. 249-255.
99. Nilsen, N.J., et al., *Cellular trafficking of lipoteichoic acid and Toll-like receptor 2 in relation to signaling; role of CD14 and CD36.* Journal of leukocyte biology, 2008. **84**(1): p. 280-291.
100. Zehner, M., et al., *Mannose receptor polyubiquitination regulates endosomal recruitment of p97 and cytosolic antigen translocation for cross-presentation.* Proceedings of the National Academy of Sciences, 2011. **108**(24): p. 9933-9938.
101. Duclos, S., et al., *The endosomal proteome of macrophage and dendritic cells.* Proteomics, 2011. **11**(5): p. 854-864.
102. Bosco, M.C. and L. Varesio, *Dendritic cell reprogramming by the hypoxic environment.* Immunobiology, 2012. **217**(12): p. 1241-1249.
103. Naldini, A., et al., *Hypoxia affects dendritic cell survival: role of the hypoxia-inducible factor-1alpha and lipopolysaccharide.* J Cell Physiol, 2012. **227**(2): p. 587-95.
104. Jantsch, J., et al., *Hypoxia and hypoxia-inducible factor-1α modulate lipopolysaccharide-induced dendritic cell activation and function.* The Journal of Immunology, 2008. **180**(7): p. 4697-4705.
105. Doedens, A.L., et al., *Hypoxia-inducible factors enhance the effector responses of CD8+ T cells to persistent antigen.* Nature immunology, 2013. **14**(11): p. 1173.
106. Ricciardi, A., et al., *Transcriptome of hypoxic immature dendritic cells: modulation of chemokine/receptor expression.* Molecular Cancer Research, 2008. **6**(2): p. 175-185.
107. Paardekooper, L.M., et al., *Hypoxia potentiates monocyte-derived dendritic cells for release of tumor necrosis factor alpha via MAP3K8.* Biosci Rep, 2018. **38**(6).
108. Wobben, R., et al., *Role of hypoxia inducible factor-1alpha for interferon synthesis in mouse dendritic cells.* Biol Chem, 2013. **394**(4): p. 495-505.
109. Kohler, T., et al., *Influence of hypoxia-inducible factor 1alpha on dendritic cell differentiation and migration.* Eur J Immunol, 2012. **42**(5): p. 1226-36.
110. Mancino, A., et al., *Divergent effects of hypoxia on dendritic cell functions.* Blood, 2008. **112**(9): p. 3723-34.
111. Hammami, A., et al., *IRF-5-Mediated Inflammation Limits CD8+ T Cell Expansion by Inducing HIF-1alpha and Impairing Dendritic Cell Functions during Leishmania Infection.* PLoS Pathog, 2015. **11**(6): p. e1004938.
112. Novikoff, A.B., *The proximal tubule cell in experimental hydronephrosis.* J Biophys Biochem Cytol, 1959. **6**(1): p. 136-8.
113. Ashford, T.P. and K.R. Porter, *Cytoplasmic components in hepatic cell lysosomes.* The Journal of cell biology, 1962. **12**(1): p. 198.
114. Novikoff, A.B. and E. Essner, *Cytolysomes and mitochondrial degeneration.* The Journal of cell biology, 1962. **15**(1): p. 140.
115. De Duve, C. and R. Wattiaux, *Functions of lysosomes.* Annual review of physiology, 1966. **28**(1): p. 435-492.
116. Boya, P., F. Reggiori, and P. Codogno, *Emerging regulation and functions of autophagy.* Nature cell biology, 2013. **15**(7): p. 713-720.
117. Mizushima, N., *Autophagy: process and function.* Genes & development, 2007. **21**(22): p. 2861-2873.
118. Yun, H.R., et al., *Roles of Autophagy in Oxidative Stress.* International Journal of Molecular Sciences, 2020. **21**(9): p. 3289.

119. Takeshige, K., et al., *Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction.* The Journal of cell biology, 1992. **119**(2): p. 301-311.
120. Mortimore, G.E. and A.R. Pösö, *Intracellular protein catabolism and its control during nutrient deprivation and supply.* Annual review of nutrition, 1987. **7**(1): p. 539-568.
121. Rusten, T.E., et al., *Programmed autophagy in the Drosophila fat body is induced by ecdysone through regulation of the PI3K pathway.* Developmental cell, 2004. **7**(2): p. 179-192.
122. Noda, T. and Y. Ohsumi, *Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast.* Journal of Biological Chemistry, 1998. **273**(7): p. 3963-3966.
123. Ravikumar, B., et al., *Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease.* Nature genetics, 2004. **36**(6): p. 585-595.
124. Mordier, S., et al., *Leucine limitation induces autophagy and activation of lysosome-dependent proteolysis in C2C12 myotubes through a mammalian target of rapamycin-independent signaling pathway.* Journal of Biological Chemistry, 2000. **275**(38): p. 29900-29906.
125. Codogno, P. and A.J. Meijer, *Autophagy and signaling: their role in cell survival and cell death.* Cell Death & Differentiation, 2005. **12**(2): p. 1509-1518.
126. Mazure, N.M. and J. Pouysségur, *Hypoxia-induced autophagy: cell death or cell survival?* Current opinion in cell biology, 2010. **22**(2): p. 177-180.
127. Hurley, J.H. and L.N. Young, *Mechanisms of autophagy initiation.* Annual review of biochemistry, 2017. **86**: p. 225-244.
128. Liang, C., et al., *Autophagic and tumour suppressor activity of a novel Beclin1-binding protein UVRAG.* Nature cell biology, 2006. **8**(7): p. 688-698.
129. Pattingre, S., et al., *Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy.* Cell, 2005. **122**(6): p. 927-939.
130. Fimia, G.M., et al., *Ambra1 regulates autophagy and development of the nervous system.* Nature, 2007. **447**(7148): p. 1121-1125.
131. Klionsky, D.J., et al., *A unified nomenclature for yeast autophagy-related genes.* Developmental cell, 2003. **5**(4): p. 539-545.
132. Bjørkøy, G., et al., *p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death.* The Journal of cell biology, 2005. **171**(4): p. 603-614.
133. Wang, Q.J., et al., *Induction of autophagy in axonal dystrophy and degeneration.* Journal of Neuroscience, 2006. **26**(31): p. 8057-8068.
134. Tanaka, Y., et al., *Accumulation of autophagic vacuoles and cardiomyopathy in LAMP-2-deficient mice.* Nature, 2000. **406**(6798): p. 902-906.
135. Teter, S.A., et al., *Degradation of lipid vesicles in the yeast vacuole requires function of Cvt17, a putative lipase.* Journal of Biological Chemistry, 2001. **276**(3): p. 2083-2087.
136. Suriapranata, I., et al., *The breakdown of autophagic vesicles inside the vacuole depends on Aut4p.* Journal of cell science, 2000. **113**(22): p. 4025-4033.
137. Yang, Z., et al., *Atg22 recycles amino acids to link the degradative and recycling functions of autophagy.* Molecular biology of the cell, 2006. **17**(12): p. 5094-5104.
138. Kettern, N., et al., *The Hsc/Hsp70 co-chaperone network controls antigen aggregation and presentation during maturation of professional antigen presenting cells.* PloS one, 2011. **6**(1): p. e16398.
139. Min, Y., et al., *Autophagy promotes BCG-induced maturation of human dendritic cells.* Acta Biochim Biophys Sin, 2010. **42**(3): p. 177-182.
140. Morris, S., et al., *Autophagy-mediated dendritic cell activation is essential for innate cytokine production and APC function with respiratory syncytial virus responses.* The Journal of Immunology, 2011. **187**(8): p. 3953-3961.

141. Manuse, M.J., C.M. Briggs, and G.D. Parks, *Replication-independent activation of human plasmacytoid dendritic cells by the paramyxovirus SV5 Requires TLR7 and autophagy pathways.* Virology, 2010. **405**(2): p. 383-389.
142. Xiong, A., et al., *Flt3L combined with rapamycin promotes cardiac allograft tolerance by inducing regulatory dendritic cells and allograft autophagy in mice.* PloS one, 2012. **7**(10): p. e46230.
143. Wildenberg, M., et al., *The ATG16L1 risk allele associated with Crohn's disease results in a Rac1-dependent defect in dendritic cell migration that is corrected by thiopurines.* Mucosal Immunology, 2017. **10**(2): p. 352-360.
144. Xu, Y., et al., *Toll-like receptor 4 is a sensor for autophagy associated with innate immunity.* Immunity, 2007. **27**(1): p. 135-144.
145. Lee, H.K., et al., *Autophagy-dependent viral recognition by plasmacytoid dendritic cells.* Science, 2007. **315**(5817): p. 1398-1401.
146. Blanchet, F.P., et al., *Human immunodeficiency virus-1 inhibition of immunoamphisomes in dendritic cells impairs early innate and adaptive immune responses.* Immunity, 2010. **32**(5): p. 654-669.
147. Terawaki, S., et al., *RUN and FYVE domain–containing protein 4 enhances autophagy and lysosome tethering in response to Interleukin-4.* Journal of Cell Biology, 2015. **210**(7): p. 1133-1152.
148. Lee, H.K., et al., *In vivo requirement for Atg5 in antigen presentation by dendritic cells.* Immunity, 2010. **32**(2): p. 227-239.
149. Wenger, T., et al., *Autophagy inhibition promotes defective neosynthesized proteins storage in ALIS, and induces redirection toward proteasome processing and MHCI-restricted presentation.* Autophagy, 2012. **8**(3): p. 350-363.
150. Loi, M., et al., *Macroautophagy proteins control MHC class I levels on dendritic cells and shape anti-viral CD8+ T cell responses.* Cell reports, 2016. **15**(5): p. 1076-1087.
151. Tey, S.-K. and R. Khanna, *Autophagy mediates transporter associated with antigen processing-independent presentation of viral epitopes through MHC class I pathway.* Blood, The Journal of the American Society of Hematology, 2012. **120**(5): p. 994-1004.
152. Li, H., et al., *Alpha-alumina nanoparticles induce efficient autophagy-dependent cross-presentation and potent antitumour response.* Nature nanotechnology, 2011. **6**(10): p. 645-650.
153. Parekh, V.V., et al., *Autophagy-related protein Vps34 controls the homeostasis and function of antigen cross-presenting CD8α+ dendritic cells.* Proceedings of the National Academy of Sciences, 2017. **114**(31): p. E6371-E6380.
154. Nierkens, S., et al., *Antigen cross-presentation by dendritic cell subsets: one general or all sergeants?* Trends in immunology, 2013. **34**(8): p. 361-370.
155. Ravindran, R., et al., *Vaccine activation of the nutrient sensor GCN2 in dendritic cells enhances antigen presentation.* Science, 2014. **343**(6168): p. 313-317.
156. Criscuoli, M., et al., *The Shc protein Rai enhances T-cell survival under hypoxia.* Journal of Cellular Physiology, 2020.
157. Sivandzade, F., A. Bhalerao, and L. Cucullo, *Analysis of the mitochondrial membrane potential using the cationic JC-1 dye as a sensitive fluorescent probe.* Bio-protocol, 2019. **9**(1).
158. Livak, K.J., et al., *Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization.* Genome Research, 1995. **4**(6): p. 357-362.
159. Guerrini, G., et al., *Inhibition of smoothened in breast cancer cells reduces CAXII expression and cell migration.* Journal of cellular physiology, 2018. **233**(12): p. 9799-9811.
160. Ichimura, Y. and M. Komatsu, *Activation of p62/SQSTM1–Keap1–Nuclear Factor Erythroid 2-Related Factor 2 Pathway in Cancer.* Frontiers in oncology, 2018. **8**: p. 210.

161. Kabeya, Y., et al., *LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing.* The EMBO journal, 2000. **19**(21): p. 5720-5728.
162. Kabeya, Y., et al., *LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation.* Journal of cell science, 2004. **117**(13): p. 2805-2812.
163. Etna, M.P., et al., *Mycobacterium tuberculosis-induced miR-155 subverts autophagy by targeting ATG3 in human dendritic cells.* PLoS pathogens, 2018. **14**(1): p. e1006790.
164. Cadwell, K., *Crosstalk between autophagy and inflammatory signalling pathways: balancing defence and homeostasis.* Nature Reviews Immunology, 2016. **16**(11): p. 661-675.
165. Minet, E., et al., *ERK activation upon hypoxia: involvement in HIF-1 activation.* FEBS letters, 2000. **468**(1): p. 53-58.
166. Zhang, W. and H.T. Liu, *MAPK signal pathways in the regulation of cell proliferation in mammalian cells.* Cell research, 2002. **12**(1): p. 9-18.
167. Corcelle, E., et al., *Disruption of autophagy at the maturation step by the carcinogen Lindane is associated with the sustained mitogen-activated protein kinase/extracellular signal–regulated kinase activity.* Cancer research, 2006. **66**(13): p. 6861-6870.
168. Rescigno, M., et al., *Dendritic cell survival and maturation are regulated by different signaling pathways.* The Journal of experimental medicine, 1998. **188**(11): p. 2175-2180.
169. Arrighi, J.-F., et al., *A critical role for p38 mitogen-activated protein kinase in the maturation of human blood-derived dendritic cells induced by lipopolysaccharide, TNF-α, and contact sensitizers.* The Journal of Immunology, 2001. **166**(6): p. 3837-3845.
170. Mantovani, A., et al., *Interleukin-1 and related cytokines in the regulation of inflammation and immunity.* Immunity, 2019. **50**(4): p. 778-795.
171. Harris, J., *Autophagy and cytokines.* Cytokine, 2011. **56**(2): p. 140-144.
172. Jones, S.A., K.H. Mills, and J. Harris, *Autophagy and inflammatory diseases.* Immunology and cell biology, 2013. **91**(3): p. 250-258.
173. An, Q., et al., *Enhanced neutrophil autophagy and increased concentrations of IL-6, IL-8, IL-10 and MCP-1 in rheumatoid arthritis.* International immunopharmacology, 2018. **65**: p. 119-128.
174. Morelli, A.E., et al., *Cytokine production by mouse myeloid dendritic cells in relation to differentiation and terminal maturation induced by lipopolysaccharide or CD40 ligation.* Blood, The Journal of the American Society of Hematology, 2001. **98**(5): p. 1512-1523.
175. Peña-Llopis, S., et al., *Regulation of TFEB and V-ATPases by mTORC1.* The EMBO journal, 2011. **30**(16): p. 3242-3258.
176. Mauthe, M., et al., *Chloroquine inhibits autophagic flux by decreasing autophagosome-lysosome fusion.* Autophagy, 2018. **14**(8): p. 1435-1455.
177. Mukhopadhyay, S., et al., *Autophagy and apoptosis: where do they meet?* Apoptosis, 2014. **19**(4): p. 555-566.
178. Iyer, S., et al., *Robust autoactivation for apoptosis by BAK but not BAX highlights BAK as an important therapeutic target.* Cell Death & Disease, 2020. **11**(4): p. 1-13.
179. Zandarashvili, L., et al., *Structural basis for allosteric PARP-1 retention on DNA breaks.* Science, 2020. **368**(6486).
180. Sica, A., G. Melillo, and L. Varesio, *Hypoxia: a double-edged sword of immunity.* Journal of molecular medicine, 2011. **89**(7): p. 657-665.
181. Sitkovsky, M. and D. Lukashev, *Regulation of immune cells by local-tissue oxygen tension: HIF1 alpha and adenosine receptors.* Nat Rev Immunol, 2005. **5**(9): p. 712-21.
182. Hubbi, M.E. and G.L. Semenza, *Regulation of cell proliferation by hypoxia-inducible factors.* American Journal of Physiology-Cell Physiology, 2015. **309**(12): p. C775-C782.
183. Ryter, S.W., D. Bhatia, and M.E. Choi, *Autophagy: a lysosome-dependent process with implications in cellular redox homeostasis and human disease.* Antioxidants & Redox Signaling, 2019. **30**(1): p. 138-159.

184. Shacka, J.J., K.A. Roth, and J. Zhang, *The autophagy-lysosomal degradation pathway: role in neurodegenerative disease and therapy.* Front Biosci, 2008. **13**: p. 718-736.
185. Delgado, M.A., et al., *Toll-like receptors control autophagy.* The EMBO journal, 2008. **27**(7): p. 1110-1121.
186. Cooney, R., et al., *NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation.* Nature medicine, 2010. **16**(1): p. 90-97.
187. Wang, J., et al., *A non-canonical MEK/ERK signaling pathway regulates autophagy via regulating Beclin 1.* Journal of Biological Chemistry, 2009. **284**(32): p. 21412-21424.
188. Kihara, A., et al., *Two Distinct Vps34 Phosphatidylinositol 3–Kinase complexes function in autophagy and carboxypeptidase Y Sorting inSaccharomyces cerevisiae.* Journal of Cell Biology, 2001. **152**(3): p. 519-530.
189. Verzella, D., et al., *Life, death, and autophagy in cancer: NF-κB turns up everywhere.* Cell Death & Disease, 2020. **11**(3): p. 1-14.
190. Zang, F., et al., *Autophagy is involved in regulating the immune response of dendritic cells to influenza A (H1N1) pdm09 infection.* Immunology, 2016. **148**(1): p. 56-69.
191. Runwal, G., et al., *LC3-positive structures are prominent in autophagy-deficient cells.* Scientific reports, 2019. **9**(1): p. 1-14.
192. Yucel-Lindberg, T., H. Jansson, and H. Glaumann, *Proteolysis in isolated autophagic vacuoles from the rat pancreas.* Virchows Archiv B, 1992. **61**(1): p. 141.
193. Talla, U., et al., *Prolonged exposure to hypoxia induces an autophagy-like cell survival program in human neutrophils.* Journal of Leukocyte Biology, 2019. **106**(6): p. 1367-1379.

ACKNOWLEDGMENTS

First, my sincerest and profound thanks go to my supervisor Professor Antonella Naldini for her extraordinary mentorship. Her immense knowledge, motivation and patience have guided me for three years to succeed in my research. I am immensely grateful for the support she gave me and for the possibility that she offered to me. Words will never be enough to express my gratitude.

I would like to express my gratitude also for Professor Fabio Carraro for the encouragement and for all his precious suggestions.

I will not forget to express my gratefulness to rest of the previous and present lab team: Dr. Carlo Aldinucci, Dr Irene Filippi, Dr Mattia Criscuoli, Dr Giuditta Guerrini and Dr Gaia Giuntini. They all have played a major role in polishing my research skills. Their endless guidance is hard to forget throughout my life.

From the depth of my heart, I would like to thank Marco, you are my biggest fan and I can't put into words my gratitude for your endless support.

Thanks to my parents for being silently always by my side.