# mTOR in the aging immune system

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## 1. Introduction

Aging represents a process of gradual functional decline affecting most physiological systems of the organism to varying degrees and leading to significantly increased vulnerability to disease and death. A complex network of cellular and molecular events appears to contribute to the progression of aging, including but not limited to genetic instability, proteostatic deregulation, epigenetic restructuring, cellular senescence and stem cell exhaustion. Metabolic and signaling perturbations are also common features of aging and in this regard extensive research has been lately focusing on the mTOR pathway. This direction of scientific investigation was fueled by the discovery that multiple models of mTOR inhibition have potent positive effects on mammalian longevity and age-related pathology. Unsurprisingly, mTOR appears to interact with numerous mechanisms involved in aging and therefore its effects on health and lifespan are likely non-specific, complex and cumulative (Stallone et al., 2019).

With the progression of age, the immune system undergoes a set of changes collectively referred to as immunosenescence. While some of the phenomena occurring in the aged immune system appear degenerative in nature and others are suspected to be adaptive, the general direction of these age-related changes tends to lead to impaired immune function, increased morbidity and mortality associated with infectious, autoimmune and inflammatory conditions. Data obtained from the use of rapamycin, the first studied mTOR inhibitory compound, have placed this pathway at an intriguing intersection between aging and immunity. Rapamycin recently emerged as a lifespan extending agent in several animal models, however long before that it accumulated a medical history of use as an immunosuppressive agent following transplant procedures. In the context of age-related immune decline, it appeared relatively surprising that a drug which exerts negative control on immune processes could have a beneficial influence on aging. The seeming contradiction is only superficial, as immunostimulatory effects of rapamycin began to emerge soon after its introduction, not only in animal models of bacterial and viral infection, but also in transplant recipients themselves, manifesting as increased protection against tumors and sometimes inflammatory complications (Bravo-San Pedro and Senovilla, 2013). The apparent paradox started to be unraveled following the discovery of unexpectedly diverse functions of mTOR within the immune

system. Indeed, the consequences of mTOR activation or suppression may rely immensely on cell type, duration of the intervention, timing of the response throughout distinct stages of cellular differentiation or phases of the immune reaction, as well as on the specific mTOR complex that is being targeted (Johnson et al., 2015). This creates the possibility for very careful modulation of mTOR activity during therapeutic and preventive interventions in aged patients, with the goal of obtaining optimal results while minimizing the side effects which are currently imposing restrictions on medical applications of rapamycin and other analogous compounds.

While a few successful trials are already exemplifying this approach, research on mTOR regulation in immune senescence is still in its infancy and an exploration of its physiological role and clinical significance is to a large extent speculative. For this reason, the structure of this essay will primarily consist of a sequential description of different functions and components of the immune system known to be affected both by aging and mTOR signaling. A side by side comparison is executed with the purpose of revealing the potential involvement of mTOR in aspects of immune aging, as well as suggesting interventions that may attenuate immune dysfunction in the elderly by interfering with the mTOR pathway. The theoretical approach will be consolidated by reported results in cases where the addressed immunological issues have already been subjected to clinical or pre-clinical investigation in the context of mTOR modulation in aged individuals.

## 2. **Overview of the immune system**

In the continuous arms race between highly specialized pathogens and their hosts, the mammalian immune system has evolved a high degree of complexity and adaptability. Distinct subsets of white blood cells are able to mount diverse responses to a wide variety of threats, while interacting with each other to coordinate their activity and regulate their function so as to achieve optimal protection against infection with minimal negative impact on the organism. The immune system is traditionally described as having two main branches, corresponding to innate and adaptive immunity. The innate immune system constitutes the first line of protection, as it recognizes conserved molecular signatures of infection and injury (pathogen- and damage-associated molecular patterns / PAMPs and DAMPs) and provides nonspecific defensive responses, while simultaneously initiating and controlling inflammatory processes and recruiting adaptive immune cells to the required site. Innate immunity is ensured by

granulocytes (most importantly neutrophils), monocytes and macrophages, natural killer (NK) cells and dendritic cells (DCs). Innate immune cells recognize their targets through classical pattern recognition receptors (PRR), of which the Toll-like receptors (TLR) are best described, and upon activation generate functional responses that can include phagocytosis, cytotoxicity or secretion of inflammatory mediators (Solana et al., 2012).

The adaptive immune system is characterized by a slower, but highly specific and effective response executed by lymphocytes. Adaptive immune cells comprise the B lymphocytes, which are responsible for humoral immunity, and the T lymphocytes, which are agents of cellular immunity and further subdivided into $CD8^+$ and $CD4^+$ lineages. CD8+ cells perform cytotoxic functions, while CD4+ (T helper) cells secrete cytokines which support the function of other immune cells. The CD4+ lineage also gives rise to regulatory T cells (Tregs) which limit unnecessary immune responses and maintain tolerance to self-antigens.

Despite their phenotypical and functional diversity, adult immune cells collectively originate from bone marrow hematopoietic stem cells (HSC), which need to retain their self-renewal and differentiation capacity in order to ensure the proper maintenance of a balanced immune system. While the exact stages of hematopoietic differentiation are still debated, one of the earliest occurring events in immune cell development is the commitment to either the lymphoid lineage (eventually giving rise to T, B and NK cells) or the myeloid lineage (which produces the remaining innate cells).

During their development, adaptive immune cells mature in different lymphoid organs and acquire their particular sets of surface receptors, which are essential not only for antigen recognition but also for efficient communication between the various components of the immune system. The specificity of adaptive immunity relies on the essential T- and B-cell receptors (TCR/BCR), which are generated with incredible diversity following genetic rearrangements to ensure affinity for a very large number of potential antigens. Immune responses are initiated when lymphocytes interact with their cognate antigen in secondary lymphoid organs. Mature lymphocytes are generally quiescent in the naïve state, however upon antigenic stimulation they become activated and resume proliferation and differentiation into effector cells. A small subset of activated lymphocytes further develops into memory cells of their respective kind, which persist for long periods of time in the body, conferring increased protection against recurrent infections and forming the basis of acquired immunity. Long thought to be a specific trait of adaptive immunity, immune memory is now acknowledged to

occur in the innate compartment as well. In this case it takes the form of trained immunity, representing an increased yet nonspecific responsiveness of previously stimulated innate immune cells to subsequent insults.

## 3. mTOR function and regulation

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase which constitutes the central element of a signaling hub capable of integrating multiple environmental and internal cues and of regulating cellular growth, proliferation and metabolic activity accordingly. Many of the signals that converge on the mTOR pathway are universal (energetic status of the cell, nutrient availability, growth factors) but some are also cell type specific. Accordingly, the activation of characteristic immune receptors (TCRs, BCRs, PRRs) is incorporated upstream in the mTOR signaling network specifically in cells of the immune system.

MTOR represents the catalytic component of two multi-protein complexes correspondingly named mTOR complex 1 and 2 (mTORC1 / mTORC2). MTORC1 is considered a master regulator of cellular growth as it stimulates anabolic transcriptional programs and biosynthetic processes such as protein translation, ribosome biogenesis and lipid synthesis, while suppressing catabolic functions such as autophagy. MTORC2 is involved in cell survival, proliferation and cytoskeletal actin organization (Araki et al., 2011; Bar-Peled and Sabatini, 2014). While they share several components, the two complexes contain distinct scaffolding proteins (Raptor for mTORC1 and Rictor for mTORC2), which can be conditionally deleted in experimental models in order to specifically inactivate each of the complexes and allow their independent study. Activation of mTORC1 occurs in the presence of high amino-acid concentrations and involves its recruitment by Rag GTPases to the lysosomal surface, where it can be stimulated by the small GTPase Rheb. The main substrates phosphorylated by mTORC1 are S6 kinase (S6K) and eukaryotic translation initiation factor 4E binding proteins (4E-BPs). The primary upstream negative regulator of mTORC1 is the tuberous sclerosis / TSC1-TSC2-TBC1D7 complex, which functions as a G-ATPase activating protein (GAP) and facilitates the conversion of Rheb to its non-functional GDP bound form, thereby limiting mTORC1 activation (Bar-Peled and Sabatini, 2014). Various extracellular signals including growth factors, insulin and cytokines can impinge on mTOR function through the phosphatidylinositol-3 kinase (PI3K) – protein kinase B (PKB/Akt) pathway. Detection of

these ligands by their corresponding receptors activates PI3K, leading to the production of the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3), which recruits Akt to the plasma membrane where it can be activated. Further downstream, phosphorylation of Tsc2 by Akt causes dissociation of TSC from the lysosomal surface and releases the barrier imposed on mTORC1 activation. In conditions of energy stress, mTORC1 is also negatively regulated at two different levels by AMP-activated protein kinase (AMPK), which can inhibit the complex both directly by phosphorylating Raptor and indirectly by activating Tsc2. The mechanisms controlling mTORC2 function are less complex, but closely tied to the regulation of mTORC1. The mSIN1 subunit of mTORC2 ensures autoinhibition of the complex, which can be directly relieved by PI3K-generated PIP3. Upon activation, mTORC2 can phosphorylate Akt at a different position than PI3K, thereby activating it fully and strengthening downstream signalling. The two complexes are therefore commonly activated at the same time and furthermore mTORC2 provides positive feedback to enhance Akt-dependent activation of mTORC1.

Regarding therapeutic intervention, a large proportion of existing data on pharmacological suppression of mTOR activity is obtained from the use of rapamycin, which is sometimes problematic due to the characteristics of its selectivity. Rapamycin acts preferentially on mTORC1 during short term treatment, however prolonged exposure to the drug eventually destabilizes newly formed mTORC2 complexes leading to a more comprehensive inhibition of mTOR function. Additionally, rapamycin is an allosteric regulator of mTOR which selectively impairs its ability to phosphorylate some targets (such as S6K) but not others (such as 4EBP1). More recent research has incorporated the use of rapalogs with slightly different properties such as Everolimus/RAD001, as well as catalytic inhibitors of mTOR such as Torin (Covarrubias et al., 2015), predictably leading to minor differences in results.

## 4. **Overview of immunosenescence**

Age related changes have been detected in most compartments of the immune system, however their fundamental causes and medical relevance may differ. Immunosenescence is a broad term encompassing both aspects of immune dysfunction resulting from degenerative processes associated with aging, as well as the persistent remodeling of the immune system reflecting its lifelong history of exposure to pathogens and its continued management of

accumulated threats. Indeed, the adaptability of the immune system is one of its essential traits and retaining the memory of past antigen encounters is central to its function. A healthy immune system is therefore expected to suffer changes throughout time by its own nature and many of the transformations it undergoes may be required to improve survival chances of the organism in the face of persistent antigenic challenges. It is of great clinical importance to separate controlled and accidental processes shaping the elderly immune system, so that by managing them differently, immune function can be improved in an age adjusted manner instead of simply being reversed to a youthful presentation. In order to distinguish between degenerative and harmless changes, the concept of an immune risk phenotype (IRP) was defined, representing a collection of immune parameters associated with increased frailty and greater overall mortality. Characteristics incorporated in the IRP include cytomegalovirus (CMV) seropositivity and clonal expansion of anti-CMV CD8+ cells, reduced B cell count, an impaired proliferative response of T cells and a ratio of CD4+ to CD8+ T cells below unity (Pawelec, 2012).

The duality of damaging and useful modifications in the structure of the immune system is not entirely strict, as these categories can sometimes overlap, and the optimal balance maintained between them depends on the environmental conditions that the organism encounters. The pronounced accumulation of memory T cells associated with latent CMV infection perfectly exemplifies this ambiguity between adaptive responses and functional degradation. CMV is widespread, almost ubiquitous in some populations and likely constitutes a persistent selective pressure shaping adaptations of the human immune system. Long term management of CMV infection is necessary to avoid serious complications and the mechanisms involved in this task have become a normal part of immune aging. Thus, despite its characterization as a detrimental component of the IRP, the substantial expansion of anti-CMV memory T lymphocytes is one of the most common changes identified in the immune systems of the elderly and can be interpreted as an adaptation to the persistent presence of the virus.

Interestingly, immune traits correlated with increased mortality at extremely advanced ages deviate from the IRP defined for the general senior population. In very old individuals, an increased proportion of memory CD8+ cells is predictive of better survival, while high counts of naïve T cells confer no advantage, potentially because exposure to completely novel pathogens becomes negligible. Conversely, in non-industrialized societies subjected to higher antigenic stress, the amplification of memory cell pools is more extensive, occurs earlier and

ensures short term survival while average lifespan remains low (Pawelec, 2012). Extrapolating from these particular cases, strong immune responsiveness to certain pathogens may be advantageous when the risk of encountering them is high, while dampened immune reactions may instead be more supportive of healthy aging in a more sterile environment such as that artificially created in the modern world. This possibility may also impact the relevance of immunosenescence studies in animal models, which are normally kept in strictly controlled environments and exposed to a very limited range of pathogens.

While the inability to mount a fully effective immune response is a clearly dangerous development at advanced age, leading to low vaccination responsiveness rates, enhanced tumorigenesis and susceptibility to infections (Mannick et al., 2014), the idea that debilitating effects also occur in aged organisms as a result of amplified or abnormal immune reactions is additionally supported by the phenomenon of inflammaging and the increased frequency of autoimmune processes. Inflammaging refers to a persistent low-grade inflammatory state which commonly develops at old age and is often considered a key contributing factor in the progression of many age-related pathologies. Inflammaging likely reflects an excessive activation of the innate immune system, further compounded by the secretory activity of senescent cells which accrue paradoxically as a result of inappropriate immune clearance. While the assortment of immune disturbances associated with aging may seem contradictory in nature, simultaneously displaying characteristics of both over- and underactive immune function, it most likely indicates in fact an underlying perturbation of regulatory mechanisms. It is speculated that multiple imbalances in immune activity could be traced back to defective signaling processes, leading to an impaired ability of immune cells to both initiate and limit their responses, as well as to coordinate the function of different sub-compartments of the immune system. As a crucial signaling node controlling fundamental cellular functions, mTOR may likely have some degree of involvement in the age-related deregulation of immune pathways and may be targeted in attempt to re-establish balanced immune activity.

## 5. **mTOR in HSC aging**

As wide-ranging perturbations permeate the entire aged immune system, understanding its most fundamental changes warrants the inspection of the common origin of all immune cells. Indeed, old HSCs display a drop in function, manifesting as both reduced self-renewal and decreased hematopoiesis. Perhaps even more importantly, the aged HSC compartment is

characterized by a qualitatively abnormal output skewed towards the myeloid lineage, which may contribute to the imbalance between innate and adaptive immune responses in the elderly (Chen et al., 2009).

MTOR plays an essential role in HSCs, allowing them to integrate information about the conditions in the niche they inhabit. HSC quiescence requires low nutrient availability corresponding to reduced mTOR activation (Huang et al., 2012), in conjunction with low oxygen tension detected through the activity of HIF1α (Hoggatt and Pelus, 2011), which can also be regulated by mTOR. In mice, mTORC1 activity is increased in old HSCs independent of upstream signalling through the PI3K-Akt pathway. This constitutively enhanced mTOR phosphorylation may be at least in part directly responsible for the decline in HSC function. This hypothesis is supported by the fact that constitutive mTOR activation via Tsc1 deletion in young HSCs recapitulates traits of HSC aging such as reduced lymphopoietic capacity, progressive loss of self-renewal ability and upregulation of cyclin dependent kinase inhibitors (p16/p19/p21) which may be indicators of cellular senescence. Conversely, mTOR inhibition with rapamycin can restore youthful characteristics to aged HSCs including enhanced proliferative activity, diminished p16 and p19 expression, improved B cell production and reduced myelogenesis. It is of essence that manipulation of HSC function through the mTOR pathway translates into concrete clinical benefits, such as a substantially increased IgG antibody titre in response to influenza vaccination (Chen et al., 2009).

In addition to cell autonomous effects resulting from the modulation of intrinsic mTOR signalling, the contribution to HSC aging of extrinsic influences such as changes in the BM niche and the systemic cytokine milieu also needs to be considered. These factors may themselves be affected by age-related changes in mTOR signalling. Particularly the production of cytokines depends on mTOR activity, which supports their biosynthesis and participates in regulating the secretory function of immune and senescent cells. The mTOR pathway has been implicated in the function of some stem cell niches, thus a simple analogy should prompt investigation of its role in the BM niche, which is currently still unclear. It has been shown for instance that inhibition of mTORC1 in the Paneth cells of the intestinal niche improves the efficiency of stem cell renewal (Lamming et al., 2013).

Furthermore, the interactions of HSCs with their niche are not independent from intrinsic aspects of aging, as exemplified by the fact that old HSCs exhibit a decline in BM homing ability and modified selectivity for distinct niche subregions (Geiger et al., 2013). It

has been reported that Pten deletion, which causes hyperactivation of Akt-mTOR signalling, leads to reduced retention of HSCs in the bone marrow, which invites speculation about the involvement of mTOR in the age-related loss of niche specificity. Nevertheless, alternative downstream pathways and mTOR independent mechanisms are also employed in the absence of Pten and currently the role of mTOR in regulating HSC localisation within the niche remains an open question.

## 6. **mTOR in T cell aging**

The T cell compartment is most susceptible to age associated changes, partly as a natural consequence of its adaptive and dynamic nature, but also due to its thymic dependent development. Thymic involution contributes to the decline in T cell immunity with aging, albeit through different mechanisms in animal models and humans. In mice, the naïve T cell population is entirely of thymic origin and progressively shrinks with age, while this does not constitute a problem in humans, where homeostatic T cell proliferation continues peripherally and ensures a steady source of naïve T cells. While the size of the human naïve T cell compartment is relatively stable throughout life, its composition and functional characteristics are subjected to fluctuations with advancing age. In the absence of thymic activity and due to the reliance on continuous peripheral proliferation, the TCR repertoire and clonal distribution of naïve T cells remain vulnerable to the accumulation of alterations caused by stochastic events and unprogrammed selective pressures occurring throughout life. Overall, in the aging naïve T cell compartment CD8+ cells appear to be less apt than CD4+ cells at maintaining both their numbers and their clonal diversity (Goronzy and Weyand, 2017). In opposition to early thymic selection mechanisms, peripheral selective forces favour the proliferation of self-responsive T cells later in life. Additionally, age related disturbances in regulatory signalling pathways and recalibrations of the TCR activation threshold may allow these autoreactive T cells to overcome self-tolerance and mount effective autoimmune responses (Goronzy et al., 2013).

The memory T cell pool exhibits considerable plasticity and suffers the most significant changes with advancing age. Following a history of repeated exposure to diverse pathogens, the memory T cell compartment markedly expands at old age, a phenomenon termed memory inflation which involves preferentially CD8+ cells. Latent viral infections are primarily responsible for this process and especially the containment of CMV infection appears to be the

most important contributor to age-related immune memory inflation. While the size of the memory compartment increases, the epitope repertoire of antigen specific T cell subpopulations tends to narrow with age, exerting a negative influence on the ability to recognize multiple antigenically related viral strains. The reduced memory cell TCR variability has also been associated with improper CMV management, leading to repeated asymptomatic reactivations which may contribute to the general inflammatory state of the aged organism (Goronzy and Weyand, 2017). Despite being subjected to rapid turnover, the terminal effector T cell population is also frequently expanded in the elderly but also exhibits high oligoclonality, likely indicating continuous containment of latent infections and mirroring the same processes which shape the memory compartment.

Contrasting with their increase in number, effector and memory T cell pools display a progressive overall decline in functional responsiveness with advancing age, which may be partly traced to phenotypic alterations such as the gradual loss of the co-stimulatory CD28 receptor and deregulated cytokine secretion patterns (Bektas et al., 2017). Moreover, expression of the programmed death-1 (PD-1) inhibitory receptor is enhanced in old CD4+/CD8+ cells (Mannick et al., 2014), which may reflect the need for compensatory mechanisms of self-tolerance but may also negatively regulate TCR signalling in general and may interfere with the ability of T cells to attack cancer cells.

Effects of mTOR modulation have been most extensively studied in the T cell compartment, primarily in relationship to the therapeutic use of rapamycin for the prevention of allogenic transplant rejection. Studies uncovered predictable suppressive effects of rapamycin on T cells, however surprising stimulatory reactions have also emerged among the findings. Divergent cell type specific effects are among the reasons why this unexpected combination of results may occur, since interfering with mTOR signalling at the organismal level can have a profound impact both directly on intrinsic T cell function and indirectly on T cell activation and regulation through other immune cells which interact with them. The timing and context of interventions are likely additional contributors, as T cells appear to employ metabolic switches controlled by mTOR in order to progress through differentiation stages, requiring contrasting levels of mTOR activity at distinct points in their development and between separate lineages. In this context it is easily conceivable that dysregulated mTOR signalling during aging would contribute to changes in the composition of the T cell pool and its modified responsiveness to antigenic stimulation. A pragmatical interpretation of this situation is that knowing the ideal moment of intervention and time of exposure to mTOR

modulatory treatments could achieve superior results in immune function compared to the modest amelioration of age-related decline obtained through the continuous compensatory inhibition of the overall increased mTOR activity.

The functional T cell response involves a rapid expansion and differentiation of previously naïve T cells following stimulation with their specific antigen bound to major histocompatibility complex (MHC) molecules on antigen presenting cells, in conjunction with co-stimulatory and pro-inflammatory signalling. The clonal proliferation initially results in the generation of a large effector T cell pool (expansion phase), of which a small fraction will progress towards differentiation into memory T cells, while the rest undergo apoptosis once their function has been exerted (contraction phase). Serial changes in the expression of specific receptors characterize activated T cells, particularly the IL-7 receptor CD127 (high in naïve T cells, downregulated during expansion, upregulated again in memory cells) and KLGR-1 (high in short-lived terminal effector cells, low in memory precursor effector cells and the long-lived memory cells derived from them) (Araki et al., 2009). Surface proteins involved in T cell trafficking undergo similar fluctuations which are essential for optimizing functional responses by promoting relocation of T cells in accordance with their stage specific role. Accordingly, naïve and memory T lymphocytes express higher levels of the chemokine receptor CCR7 and the lymph node homing receptor CD62L, which facilitate their entry into peripheral lymph nodes where they can interact with antigen presenting cells. In contrast, effector T cells have an increased expression of tissue homing receptors which allow their migration to sites of infection. Upon stimulation T cells lose their lymph node homing factors and slowly re-acquire them during the memory phase (Sinclair et al., 2008). Additionally, newly generated memory T cells continue their differentiation and improve qualitatively by acquiring slow self-renewal ability, strengthening their antigen recall responsiveness and upregulating anti-apoptotic factors such as Bcl-2.

Antigen recognition by the TCR engages the PI3K-Akt-mTOR pathway in a manner dependent on the duration of the interaction and dosage of the ligand. Several co-stimulatory signals including activation of the CD28 receptor also trigger this pathway and contribute to the magnitude of the response (Chi, 2012). While both complexes become activated upon naïve T cell stimulation, mTORC1 function in particular appears to be required for initiation of the

adaptive immune response, as it promotes increased protein translation, enhanced flux through the pentose phosphate pathway and a switch to glycolytic energy metabolism necessary for cell cycle re-entry and sustained proliferation (Stallone et al., 2019). Even more specifically, mTOR activity seems to be required for the implementation of the CD8+ effector program by supporting expression of the immune master regulatory transcription factor T-bet (Rao et al., 2010). Secondary to acute activation, cytokines such as IL-2, IL-7 and IL-12 in CD8 cells may prolong PI3K/Akt-mTOR signalling in order to support the maturation of terminal effector T cells. Sustained activation of mTOR during effector differentiation also controls T cell migration by limiting availability of the transcription factor KLF2 and consequently downregulating expression of CD62L and CCR7 (Sinclair et al., 2008).

Despite the essential role of mTOR in T cell activation, its pharmacological inhibition is currently investigated as a strategy to enhance vaccine responsiveness in the elderly, with several approaches already showing promising results. This is partly possible because optimal T cell effector function is not a critical aspect of artificial immunisation compared to active infection. Instead, improving vaccine efficiency relies primarily on increasing the number of newly formed memory $CD8^+$ T cells, with recent research also focusing on ensuring proper subsequent memory cell differentiation. The level of mTOR activity appears to be relevant in all stages of the T cell response to immunization, including expansion, contraction and memory cell differentiation, and its modulation during either of these processes has detectable effects on the clinical outcome of antigenic stimulation. Short term treatment with rapamycin during the progression of the T cell response exemplifies its surprising immunostimulatory potential. Inhibition of mTOR with rapamycin during the expansion phase of the response leads to the production of a heightened number of memory cell precursors, while a similar treatment during the contraction phase enhances survival and accelerates optimal differentiation of already formed memory $CD8^+$ T cells (Araki et al., 2010). Mice treated with rapamycin during LCMV infection develop a memory $CD8^+$ T cell pool characterized by enhanced CD127, CD62L and Bcl-2 expression and a higher proportion of KLGR-1 low cells, indicating improved functional quality, increased proliferative capacity and long-term survival potential. These results are effects of intrinsic mTORC1 downregulation, since they can be recapitulated by RNAi knock-down of Raptor in antigen-specific $CD8^+$ cells, while rendering CD8+ T cells insensitive to rapamycin through FKBP12 knock-down abolishes their beneficial responses to the treatment (Araki et al., 2009).

Other findings confirm that limiting mTORC1 activity promotes the implementation of a memory phenotype by facilitating the transition to a fatty acid oxidative metabolism characteristic of quiescent memory cells (Pearce et al., 2009), but also identify supportive roles of mTORC2 inhibition. Accordingly, lowered mTORC2 activity allows nuclear translocation of FoxO1 and consequent transcription of memory promoting factors such as CD127, CD62L and eomesodermin. Defective mTORC2 signalling due to Rictor deletion seems to confer slight improvements in memory CD8+ cell generation without affecting terminal effector cell production (Pollizzi et al., 2015). This could indicate a valid alternative therapeutic approach, as concerns still exists regarding the necessity of mTORC1 for the differentiation of functional effector cells. Additionally, mTORC1 facilitated reactivation of glycolysis appears to be required for effective recall responses of memory cells. Clearly, timing, context and pathway choice are essential aspects of mTOR regulation which may need to be adjusted for desired clinical outcome. Intermittent schedules of mTOR inhibition coordinated with predicted immune responses to vaccination or infection may prove to be more supportive of healthy aging than long term or randomly timed treatments.

While preclinical studies revealed promising results of mTOR modulation at different stages of the T cell response, successful trials in elderly human volunteers are investigating benefits of treatment well in advance of antigenic stimulation. Experiments with the allosteric mTOR inhibitory drug RAD001 were able to achieve enhanced responsiveness to influenza vaccination administered two weeks after discontinuation of treatment. The effect was attributed to downregulation of the PD-1 receptor in both CD4+ and CD8+ cells, leading to improved TCR signalling and stronger T cell functional responses (Mannick et al., 2014). Previous studies have shown that increased PD-1 signalling in old T cells is associated with reduced Akt phosphorylation and that inhibition of this receptor can restore normal Akt function (Henson et al., 2011). Collectively, these results suggest that suppression of mTOR in the steady state may in fact enhance mTOR activation upon stimulation, thereby indicating that an acute intervention can lead to long-term improvements in the regulation of this pathway in the elderly T cell compartment.

Calibrations of the RAD001 dosages required to improve vaccine efficiency revealed noticeable results even with low amounts of the pharmaceutical agent, corresponding only to partial mTOR inhibition assessed through the phosphorylation of its target effector S6K (Mannick et al., 2014). Because mTOR activity is still required to control other immune processes, the achievement of therapeutic benefit through limited interference with its function

offers the promise of minimal unpredictable side effects. Nevertheless, subsequent experiments produced improved immune responses through more extensive suppression of signalling downstream of mTOR. Synergistic inhibition of the mTORC1 pathway along multiple nodes, achieved through the concomitant use of RAD001 and the catalytic mTOR inhibitor BEZ235, lead to the development of an increased serologic titre in response to vaccination and additionally contributed to a decreased average risk of infections during the one year follow up period. As opposed to partial inhibition, the more comprehensive suppression of mTORC1 function also blocked the phosphorylation of 4EBP1 in addition to S6K and produced an improvement of downstream IFN signalling. In the elderly organism affected by perturbations of signalling pathways, this supplementary benefit may be particularly useful for ensuring effective anti-viral protection. Nevertheless, truly complete inhibition of mTOR reportedly produces the opposite effect by increasing vulnerability to infection. The effective dosages administered in this study were clearly still insufficient to repress mTOR activity to such a large extent (Mannick et al., 2018). Considering that mTOR is part of a relatively wide signalling network with multiple upstream and downstream components, it is not surprising that different degrees or methods of inhibition can produce varied results. Ideally this situation would be exploited to finely adjust treatment to the needs of the patient and the desired clinical outcome, and such calibrations may eventually even take aging biomarkers into account in order to accurately compensate for the advancement of immunosenescence.

Collectively, studies involving mTOR modulation in both mice and humans indicate that the T cell compartment is overall optimally responsive when mTORC1 activity is high at the moment of antigenic stimulation, but low during the anticipatory quiescence of naive T cells as well as during the formation of memory cells. Controversy exists regarding the ideal level of mTOR signalling during the proliferative phase and this may in fact vary depending on the intended outcome, with inhibition being conducive to improved memory development after vaccination, while sustained activation promotes superior effector responses during infection. In the elderly immune system, which struggles with both of these functions, inhibition of mTORC2 alone during the initial expansion period may be ideal for maintaining the balance between effector and memory responses, however development of more refined inhibitors is required before this can be clearly established. Most experiments showing the ability of mTOR modulation to alleviate detrimental aspects of immunosenescence have focused on individual stages of T cell differentiation and function. Future research will hopefully investigate whether the observed effects are cumulative and therefore whether

dynamic modulation of mTOR activity throughout the entire T cell development process could combat age-related functional decline to a more significant extent.

Due to the involvement of mTOR in T cell activation and the observed allograft tolerance in the presence of rapamycin, older models described mTOR inhibitory drugs as suppressors of T cell proliferation (Araki et al., 2011). Current reports of immunostimulatory benefits resulting from reduced mTOR activity are incompatible with this paradigm, however safety precautions still need to account for the context specific immunosuppressive effects of mTOR inhibition. Updated explanations of immune repression by mTOR inhibition reference increased generation of Tregs and impaired peripheral circulation of activated T cells, in addition to the destruction of antigen presenting cells by cytotoxic CD8+ effectors retained in lymph nodes (Sinclair et al., 2008). It appears that immunomodulatory effects achieved through the manipulation of mTOR activity have a more substantial impact on the functional properties of effector T cell populations than on their size. Optimal CD28 co-stimulatory signalling together with an increase in IL-2 levels may allow T cells to engage alternative pathways and maintain a proliferative response even in conditions of mTOR inhibition. Only in the absence of adequate amounts of IL-2, indicating a non-infectious context for T cell activation, rapamycin treatment can also impede further proliferation and promote the transition to an anergic phenotype (Mondino and Mueller, 2007). In this situation, sustained mTOR activation would be required for avoidance or reversal of immune tolerance but could be dispensable in the progression of an appropriate adaptive immune response to genuine antigenic challenges. In conjunction with Treg mediated suppression, the aforementioned intrinsic mechanisms illustrate how careful mTOR modulation could restore the balance between effective responses to foreign antigens and containment of self-reactivity. The same processes which justify the use of rapamycin in transplant cases may also be advantageous for elderly patients at risk of autoimmunity and, with an appropriate treatment plan, may even be able to coexist with immunostimulatory properties operating at different regulatory levels.

Particularities of the CD4+ compartment warrant special consideration in the analysis of the role of mTOR in immune aging. Despite its higher stability in size and diversity, the CD4+ T cell pool still manifests age-associated dysfunction at the cellular level, which interferes with the efficacy of the immune response. Even minor changes in aging CD4+ populations may have reverberating effects throughout the immune system due to the essential roles of this compartment in integrating immune signals and regulating the function of diverse immune effectors. While discoveries regarding CD4+ cell aging result predominantly from

experiments on the murine immune system, there are indications that human CD4+ T cells may undergo similar modifications and that some of these may not only be alleviated but fundamentally caused by changes in mTOR signalling.

While mTOR is involved to some extent in the differentiation of most immune cells and particularly lymphocytes, its role in CD4+ lineage development is of utmost importance and likely indispensable. After activation through the TCR (first signal), CD4+ cells have the possibility to differentiate into several subtypes depending on co-stimulatory cues and the composition of their microenvironment (second signal) including but not restricted to cytokine levels. Accurate integration of all contributing factors appears to involve selective mTOR activation. In the absence of mTOR signalling, the default differentiation pathway of CD4+ cells leads only to production of Treg effectors. MTOR is a negative regulator of the Treg master transcription factor FoxP3 and its activation promotes differentiation into alternate lineages. Development of Th1 and Th17 phenotypes strictly requires participation of mTORC1, while formation of Th2 cells is dependent on mTORC2 activity (Powell et al., 2013).

Proinflammatory Th17 cells are normally maintained in homeostatic balance with suppressive Tregs, however during aging their basal ratio shifts in favour of Th17 effectors, potentially contributing to the systemic inflammatory state. In contrast, old Tregs can expand substantially upon stimulation, reflecting the general state of the aged immune system, characterized by increased basal activation in conjunction with blunting of acute responses. Aged Tregs can sometimes lose their immunosuppressive potential due to decreased expression of FoxP3, which in conjunction with their retained ability to recognize self-antigens may lead to the initiation of autoimmune reactions (Bektas et al., 2017). In this context it can be speculated that some of the benefits of artificially downregulating mTOR signalling during aging might be exerted through effects on CD4+ subpopulations, by promoting optimal production and functioning of Tregs and therefore achieving better containment of dysregulated immune processes such as sterile inflammation and autoimmunity.

Notably, the general cytokine milieu of the aged organism develops towards a composition reminiscent of the secretory profile of Th2 cells, with an increase in IL-4 and IL-10 production, to the detriment of Th1 typical factors such as IL-2 and IFN-ɣ (Bektas et al., 2017). This potentially indicates that a focus on mTORC2 rather than mTORC1 inhibition in the CD4+ compartment may be more effective at restoring a youthful systemic environment.

Whether this is indeed a desirable goal is still debatable, since some of the shifts in cytokine production may only reflect secondary adaptive responses to underlying perturbations in immune function and their correction would not resolve the original disruption. In support of this view, the secretion of both pro- and anti-inflammatory cytokines increases with age, indicating that instead of a clearly directional degenerative process there is rather an increased stress placed on homeostatic mechanisms attempting to balance each other and maintain a functional state. On the other hand, concrete changes have been detected in the transcriptional and secretory responses of old CD4+ cells to TCR stimulation, demonstrating a non-negligible involvement of intrinsic age-related functional decline. As is suspected for many physiological and biochemical developments observed in aging, these processes are in fact not only compatible, but likely locked in a feedback loop in which functional disruption and compensatory mechanisms reinforce each other until a part of the system collapses leading to concrete pathology. Effective therapeutic intervention through modulation of signalling pathways like mTOR will require a refined understanding of the structure of such feedback networks, in order to ensure that the balance will not be wrongly tipped towards the acceleration of detrimental processes.

Despite concerns regarding potential regulatory imbalance, the CD4+ compartment appears harmonious in its regulatory requirements for internal stability versus responsiveness to the dynamic system at large. Specifically, downregulation of mTORC2 constitutes both a hypothetical measure to optimize the control of disrupted immune reactions through Tregs and T helper cells, as well as a potential key to attenuating features of the aged CD4+ phenotype at a cellular level. In fact, at least in murine models, upregulated mTORC2 signalling may even be one of the primary drivers of CD4+ cellular aging. Old murine CD4+ cells exhibit increased size, modified cytoskeletal organization, diminished Rac-/RhoA-GTPase activity, dampened TCR and CD28 signalling, limited proliferative capacity, decreased expression of the proapoptotic factor BIM and reduced levels of the phosphorylated cytoskeletal proteins Ezrin and Moesin (pERM) which may lead to improper formation of the immunological synapse. These phenotypical features were replicated in young CD4+ cells following overexpression of the mTORC2 components Rictor and Sin1 which stabilize the complex and increase its activation, while no similar effects could be obtained by enhancing mTORC1 signalling. Long-term rapamycin treatment was able to partially prevent the development of aging traits in CD4 cells, but also had potentially detrimental effects on parameters not normally affected by aging such as the substantial reduction in CD4+ pool size (Perkey et al., 2013).

Such findings underscore the necessity of developing treatments which specifically target the mTORC2 complex, which may hold promise for therapeutically addressing precise facets of adaptive immunosenescence. This strategy is currently approached with caution because mTORC2 inhibition is not considered a contributor to longevity in animal models and in fact may even produce adverse health outcomes and a reduction of lifespan in male mice (Mannick et al., 2018). Nevertheless, most data on mTORC2 downregulation is obtained from models of irreversible conditional deletion or long-term rapamycin treatment, which do not exclude potential advantages of transient, precisely timed intervention. Additionally, differences in the healthspan and longevity outcomes of mTORC2 inhibition between sexes indicate the existence of compensatory pathways which mitigate its negative influence, and which may provide future solutions to minimize side effects while still deriving therapeutic benefit. Present observations suggest however that mTORC2 is less likely than mTORC1 to be targeted as a preventive measure in support of healthy immune aging but may still be useful for adjusting regulatory processes in the old immune system in specific medical situations. Understanding the precise mechanisms behind mTORC2 mediated detrimental effects will be necessary to determine which clinical presentations and immune phenotypes are compatible with the modulation of this pathway.

## 7. **mTOR in B cell aging**

Unlike T cells, B lymphocytes are continuously generated in the bone marrow throughout life, which leaves each of their essential developmental stages vulnerable to age associated damage. The early steps of B cell development, from B cell progenitors to immature B cells, are contained in the bone marrow. The pro-B stage is characterised by the initiation of immunoglobulin heavy chain gene rearrangements. As cells progress to the pre-B stage, surrogate light chains are produced and pre-BCRs are expressed on the cell surface in order to facilitate selection of functional heavy chains. In this phase, cells that receive satisfactory pre-BCR signalling proliferate and undergo rearrangement of the immunoglobulin light chain. The immature phase is defined by the expression of the full BCR and selective elimination of autoreactive clones. The few B cells that pass this essential checkpoint are released into circulation and migrate to the spleen, where they undergo a series of transitional developmental stages. In the red pulp B cells encounter additional self-antigens and the ones displaying autoreactivity become anergic. Remaining immunocompetent cells migrate to the white pulp,

where they become naïve mature B lymphocytes of the follicular or marginal zone and may subsequently transit to other secondary lymphoid tissues. Completion of the transitional phase and subsequent persistence in the mature B cell pools require tonic BCR stimulation and competition for survival factors, primarily B lymphocyte stimulator (BLyS) (Scholz et al., 2013).

Humoral adaptive immune responses are classically initiated upon specific antigen recognition by the BCR, however alternative innate-like B cell activation can also be achieved through TLR signalling. The two activating mechanisms can either function independently or they can synergize and additionally cooperate with CD40 co-stimulatory signalling from follicular T helper cells. BCR ligation prompts a proliferative response, followed either by rapid differentiation of activated B cells into low-affinity IgM secreting plasmablasts or by initiation of a germinal center (GC) reaction conditioned by interaction with activated T cells (Limon and Fruman, 2012). In the latter case, B lymphocytes recruited to the GC undergo antibody class switching and affinity maturation through somatic hypermutation and selection by follicular T helper cells (Jones et al., 2016). TCR stimulation of B cells sustains proliferation, enhances GC processes, induces cytokine secretion and antigen presenting behaviour and ensures escape from Treg inhibition. The GC response eventually generates either memory B cells or mature high-affinity antibody producing plasma cells, which either function as short lived effectors or migrate to the bone marrow where they can endure for extensive periods (Jones et al., 2016).

During aging, B cell production suffers a decline from the onset of lymphoid specification, as a result of skewed HSC potency. All bone marrow cellular populations of the B lineage consequently drop in number and this trend is additionally supported by intrinsic differentiation defects, changes in the composition of the microenvironment and homeostatic feedback mechanisms induced by the peripheral B cell compartment. Progression from the pro-B to the pre-B stage is particularly problematic due to dysfunctional IL-7 signalling (Ma et al., 2019). Conversely, murine transitional, follicular and marginal zone B cell pools remain at a constant size despite having a very dynamic composition. In the elderly organism this stability translates to an equilibrium between reduced turnover and increased longevity of established mature cells. Human studies produce more conflicting observations, with some reports indicating that similar homeostatic adjustments are unable to prevent the decline in peripheral B cell numbers. In a manner comparable to changes in the T cell compartment, old B lymphocyte populations exhibit an accrual of antigen-experienced cells, albeit accompanied by

a decrease in the proportion of memory cells that display signs of class switching (Scholz et al., 2013).

Decreased interclonal competition resulting from the shrinking output of immature B cells, together with the occurrence of oligoclonal expansions in mature B lymphocyte subsets, can lead to lower BCR repertoire diversity and selective accumulation of self-responsive cells during aging. GC formation is defective in the elderly and the associated processes of affinity maturation and class switching become less effective. As a result of this, antibodies produced at old age tend to be less specific and more frequently auto- or cross-reactive (Cancro et al., 2009). Primary responses are delayed in the elderly, potentially because the decreased variability of the naïve B cell pool may need to be compensated by longer periods of affinity maturation (Kogut et al., 2012). Recall responses are also poor, irrespective of the availability of large numbers of memory B cells and regardless of the fact that individual plasmablasts maintain a high antibody production capacity. This indicates functional impairment of old memory B cells, which may manifest as reduced responsiveness to stimulation, limited proliferative capacity and difficulty differentiating into antigen secreting cells (Kogut et al., 2012). Additionally, the persistence of long-lived plasma cells appears to be affected by aging (Cancro et al., 2009).

The main hallmark of immune senescence in the B cell compartment is the accumulation in the spleen and bone marrow of an unconventional subset of B lymphocytes termed age-associated B cells (ABCs), which progressively replace an increasing fraction of the standard B cell pools. ABCs likely originate from the exhaustive expansion of follicular B cells but they are quiescent, more prone to respond to TLR than BCR stimulation and have altered cytokine secretion profiles, indicating their likely contribution to inflammatory processes in the elderly. ABCs function independently of BLyS, however they are still capable of binding and depleting it, thereby restricting the access of other B cell subsets to essential pro-survival signalling. Additionally, the secretory output of ABCs and especially their increased production of TNFα may further limit the generation of new conventional B cells by promoting apoptosis of their precursors. ABCs develop several properties which may implicate them in the higher occurrence of autoimmune conditions at old age. Firstly, they are commonly implicated in autoantibody production, a process which is sustained by TLR signalling and critically dependent on the heightened expression of T-bet. Secondly, ABCs upregulate CCR7, which facilitates their unnatural migration to T cell zones where they engage in vigorous antigen presentation, a behaviour which is believed to enhance reactivity to limitedly available

self-antigens. Moreover, ABCs skew CD4 T cell differentiation towards the Th17 phenotype, thereby supporting secretion of cytokines which favour autoimmune processes. (Ma et al., 2019).

B cell development appears to unfold optimally within a very specific range of mTOR activity, as the differentiation process has been reportedly disrupted both by mTOR hypomorphism and by mTOR hyperactivation secondary to Tsc1 knockout (Araki et al., 2011). The function of mTOR in early stages of the B lineage is likely closely linked to IL-7 signalling. MTORC1 activation occurs predominantly in pro-B and pre-B cells downstream of IL-7 stimulation, whereas immature and mature B lymphocytes exhibit lower engagement of this pathway in the resting state. Raptor deletion blocks B cell development in the early pre-B stage (Iwata et al., 2017) in a way that is reminiscent of differentiation difficulties observed in aging due to IL-7 signalling impairment. Conversely, reduced mTORC2-Akt signalling enhances expression of IL-7 receptors in B cell progenitors (Limon and Fruman, 2012) and Rictor deletion was shown to increase the proportion of pro-, pre- and immature B lymphocytes in the bone marrow (Scholz et al., 2013). Such observations suggest that the selective inhibition of mTORC2 represents a potential therapeutic opportunity for restoring a substantial output of immature B cells from the aged bone marrow. Nevertheless, irreversible impairment of mTORC2 activity also leads to the loss of mature B cell populations (Iwata et al., 2017), indicating that mTORC2 focused approaches can be problematic if they are not appropriately regulated.

In peripheral B lymphocytes the level of mTOR activity appears to play a role in regulating cell fate decisions at various stages. Signal transduction from the BCR involves activation of the PI3K-Akt-mTORC1 pathway, therefore in order to complete their transitional stage development B cells rely on a basal level of mTORC1 activity in accordance with their requirement for tonic BCR signalling. MTORC2 function also appears to be required in this phase for the expression of pro-survival genes regulated by NF-kB downstream of BLyS and BCR. Increased mTORC1 activation during transitional differentiation stages impedes B cell maturation, as it constitutes a potential indicator of self-reactivity which necessitates induction of anergy (Iwata et al., 2017). A question that can arise from this observation is whether mTOR suppression could modify the selection criteria of transitional stage B lymphocytes to the advantage of cells that respond to self-antigens. In aged lymphoid tissues where selection is already less stringent, reducing mTOR activity could hypothetically bestow an additional anergy escape route on autoreactive cells, while preventing less responsive B cells from

achieving minimal signalling conditions for survival. While mTOR inhibition tends to confer increased acute protection against autoimmunity, its influence on the dynamics of the peripheral B cell repertoire may compromise beneficial long-term results. Such concerns are currently purely speculative, however as mTOR modulating interventions are increasingly considered for therapeutic use in elderly patients, potential risks will need to be carefully evaluated.

MTOR signalling in mature B cells influences multiple aspects of GC formation, including expansion after stimulation, chemokine directed migration and expression of anti-apoptotic factors necessary to ensure B cell survival in the GC (Keating et al., 2013). Engagement of the PI3K-Akt-mTORC1 pathway is necessary for proliferation after BCR stimulation. Additional signalling through TLR and CD40 can bypass this condition but nonetheless maintain a PI3K/Akt-independent basal level of mTORC1 activity. Indeed, despite the numerous publications addressing the effects of rapamycin on T cell proliferation, it appears that mTOR inhibition has significantly more profound consequences for B cell responses. Rapamycin treatment markedly reduces B cell proliferation upon TLR-dependent activation and completely blocks it in the context of BCR signalling alone (Limon and Fruman, 2012).

Moderate fluctuations in mTOR activity are known to affect composition of the serologic spectrum but the clinical significance of this effect is still incompletely understood. Class switching and somatic hypermutation depend on Foxo1 directed expression of the mutator enzyme AID (activation induced cytidine deaminase) and can therefore be suppressed by Akt signalling and indirectly by mTORC2. Indeed, high Akt activity promotes early differentiation of stimulated B cells into plasmablasts instead of participation in GC processes (Limon and Fruman, 2012). The opposite effect is reported for mTORC1, as Raptor deletion prevents induction of AID expression (Keating et al., 2013) and treatment with rapamycin interrupts ongoing GC responses and inhibits class switch recombination even at dosages much lower than those that interfere with proliferation (Jones et al., 2016). This could indicate the potential of mTORC1 suppression to exacerbate age-associated traits of newly generated plasma and memory B cells and this outcome would be further aggravated if mTORC2 remains active. The block in affinity maturation leads to the production of immunoglobulins with decreased specificity which confer reduced protection upon secondary exposure to the same antigen. On the other hand, low affinity antibodies generated in conditions of limited mTORC1 activity could potentially mediate heterosubtypic immunity in response to vaccination (Keating

et al., 2013). The utility of such interventions during aging is debatable, since GC reactions are already impaired in the elderly and resulting antibodies display increased cross-reactivity without any associated improvement in humoral immunity. Nevertheless, increased protective properties of influenza immunization correlated with a broader serologic affinity spectrum have already been reported in elderly patients subjected to pharmacological mTOR inhibition (Mannick et al., 2014). Arguably, the modified epitope repertoire produced in conditions of restricted mTORC1 activity might compensate to an extent the decreased antibody diversity seen at old age, despite compromising production of high affinity immunoglobulins.

Interestingly, engagement of the Akt-Foxo1 axis in T cells has an inverse effect on GC responses compared to its role in B cells. Because reduced Foxo1 function facilitates effective Tfh differentiation and limits Treg activity, intense Akt signalling in T cells ensures optimal support of GCs (Limon and Fruman, 2012). The global effects of mTORC2 modulation on humoral immune responses are therefore difficult to predict and will need to be carefully investigated if this approach is ever considered for therapeutic application.

While the involvement of mTOR in ABC formation and function has not been thoroughly investigated, analogies with processes observed in T cells might suggest a potential role of mTOR in sustaining the ABC phenotype. Specifically, it can be speculated that mTOR could facilitate autoimmune reactions mediated by ABCs by inducing upregulation of CCR7 and T-bet and consequently increasing antigen presentation to T cells and autoantibody production. A useful direction for future research would be to investigate whether reducing mTOR activity in the B lineage could limit conversion of regular B cells to ABCs or whether it could promote reversal of self-reactivity in existing ABCs.

## 8. **mTOR in aging of the innate immune system**

Contrary to the decrease in function observed in the adaptive branch, a heightened activity of the innate immune system tends to be conserved in aging. The factors that contribute to this outcome may include the early myeloid skewing of haematopoiesis and the development of innate immune memory following repeated encounters with pathogens. Despite the net constancy or even increase in innate immune engagement, deeper investigations usually reveal a more complex collection of subtle gains and losses of function within different cellular subsets of this compartment. In this context, overactivity is not equivalent to improved

functionality but rather indicates persistent disturbances in signalling pathways and feedback mechanisms which constitute major factors in the maintenance of inflammaging.

In the mature innate immune system mTOR does not hold a central regulatory role but still participates in the execution of immune cell functions. Activation of innate immune cells through TLR4 stimulation triggers multiple signalling pathways, either mediated by the adaptor protein MyD88 or independently of it, with the former also engaging the PI3K-Akt-mTOR axis. Activation of mTOR is thus a secondary mechanism which supports the functional reorganization of innate immune cells upon stimulation and enhances main immune responses by regulating the metabolic and biosynthetic processes which sustain them. Additionally, it has been suggested that the Akt-mTOR pathway serves to prolong TLR4 signalling, as both kinases remain in an activated state for a substantial duration after the receptor is internalized and degraded (Saric et al., 2016). Attempts to modulate mTOR function in the aged immune system should therefore not discount potential effects mediated by the innate compartment.

Aside from its intrinsic effects on cellular processes and phenotypes, the mTOR pathway has an important role in the innate immune compartment in regulating cytokine production and therefore has the ability to influence the functional cohesion of the entire immune system and the general inflammatory state of the organism. Considering the application of mTOR inhibitors to combat transplant rejection and age-related pathology, it is perhaps paradoxical that mTORC1 activation reportedly supports a predominantly anti-inflammatory cytokine secretion pattern. In innate immune cells, engagement of mTORC1 through TLR stimulation promotes expression of the anti-inflammatory cytokine IL-10 while downregulating the pro-inflammatory cytokine IL-12. Additionally, mTORC2 activation can also negatively regulate inflammatory responses by limiting expression of pro-inflammatory cytokines through Akt mediated phosphorylation and subsequent cytoplasmic retention of the transcription factor FoxO1 (Katholnig et al., 2013). Some reported side effects of protracted rapamycin treatment courses could be traced to this facet of mTOR modulation and its clinical implications may require serious consideration in the context of age-related destabilization of inflammatory regulation. In kidney transplant patients treated with rapamycin, complications of an inflammatory nature such as pneumonitis or inflammatory anaemia are surprisingly common, and it is a reasonable concern that such risks may be elevated in the background of inflammaging. In mouse models of inflammatory lung disease, the inflammatory signalling facilitated by mTOR inhibition was shown to increase deleterious recruitment of activated macrophages to the affected tissue (Katholnig et al., 2013). Since similar processes of

inflammatory macrophage infiltration are also involved in the aetiology of some age-related pathologies, increased caution should be exercised regarding therapeutic mTOR modulation in elderly patients depending on their specific clinical presentation.

### 8.1. mTOR in neutrophil aging

Neutrophils are the first immune cells recruited to the site of infection or tissue damage, where they engulf pathogens through phagocytosis and neutralize them intracellularly using reactive oxygen or nitrogen species and proteolytic enzymes released from their granules. Neutrophils can also target extracellular pathogens through the extrusion of chromatin fibers coated with antimicrobial proteins, known as neutrophil extracellular traps (NETs). Throughout these processes, neutrophils release a series of pro-inflammatory mediators intended to recruit and activate other innate immune cells. Neutrophils have a short cellular lifespan and, while activation enhances their longevity, they normally still undergo programmed cell death after completing their antimicrobial function (Solana et al., 2012) Neutrophils retain their numbers during aging but suffer a drop in phagocytic and ROS generation capacity, potential alterations of chemotaxis, accompanied by a heightened inflammatory basal state mediated through TGFβ upregulation (Tomay et al., 2018). Additionally, apoptosis resistant neutrophil subpopulations with prolonged functionality have been identified in elderly patients and it can be speculated that their presence might exacerbate the persistent inflammatory state characteristic of aging. Old neutrophils also exhibit alterations in TLR signalling pathways despite retaining a normal number of receptors. (Stallone et al., 2019)

Dual roles have been proposed for mTOR in regulating neutrophil function. MTORC2 signalling directs the cytoskeletal reorganization steps required to support polarization and chemotaxis. On the other hand, effective NET release involves autophagic activity, which places it under the negative control of mTOR. Indeed, interventions which inhibit mTOR signalling can lead both to impaired chemotaxis and enhanced NET extrusion upon stimulation (Malik et al., 2018). The global effects of mTOR modulating treatments on neutrophil functionality during aging may therefore be complex and difficult to predict, potentially depending on pre-existing disturbances in mTOR signalling in aged neutrophils.

Experimental observations in elderly mice revealed a reduction in NETosis coupled with defects in autophagy (Xu et al., 2017), as well as abnormal infiltration of neutrophils in

the splenic white pulp which could interfere with B cell development (Tomay et al., 2018). Such findings indicate potential benefits of mTOR inhibition in the aged neutrophil compartment, by improving bactericidal capacity and protecting against opportunistic infections, while reducing the disruptive migration of activated neutrophils into sterile tissues. Nevertheless, concrete evidence of modified mTOR activity in elderly neutrophils is still required and currently only indirect signs of disruption are reported, such as reduced initiation of macroautophagy upon TLR engagement, perturbed PI3K-Akt signalling downstream of TLRs (Solana et al., 2012) and hypersegmented nuclear morphology, a phenotype assumed to depend on mTOR (Tomay et al., 2018).

### 8.2. mTOR in monocyte/macrophage aging

The monocyte macrophage system comprises innate immune cells with intense phagocytic activity, namely circulating monocytes and mature tissue resident macrophages which result from them. Macrophages scavenge cellular debris, apoptotic or transformed cells and foreign substances, but also internalize pathogens and employ oxygen dependent and independent mechanisms to destroy them. In practice macrophages have a wide variety of tissue-specific roles and can be polarized towards different activation states to fulfil particular context-dependent tasks. Based on their functional polarization, activated macrophages have been traditionally classified into two major categories, M1/classical and M2/alternative, which have been recently reinterpreted as ends of a phenotypical spectrum resulting from the implementation of different transcriptional programs and the recruitment of distinct metabolic pathways that support versatile effector functions. The M1 type is catabolic and serves pro-inflammatory and antimicrobial purposes, while the M2 type is anabolic, secretes anti-inflammatory cytokines and supports tissue repair (47, 48).

MTOR is not a critical determinant of macrophage polarization, however it is still engaged during macrophage activation and contributes to the required metabolic reprogramming and implementation of different effector programs. The Akt-mTOR axis is activated both by IL-4R signalling during M2 polarization and by TLR4 signalling during M1 polarization (Covarrubias et al., 2015). MTORC1 supports M1 macrophage function by promoting lipogenesis via activation of Srebp transcription factors, which provides necessary constituents for the formation of cellular membranes and prostaglandin synthesis. Rapid membrane growth facilitated by this transcriptional program is required by phagocytic

compartments and also supports the expansion of the ER and Golgi, which are involved in cytokine production. Additionally, mTORC1 mediated translation of HIF1α reinforces M1 typical glycolytic metabolism. While less important for M2 macrophage activation, mTORC1 may still potentially contribute to their function by supporting glutamine metabolism and lectin N-glycosylation.

Outside of normal physiological conditions, aberrant mTOR activation selectively enhances M1 function, as it promotes hyperinflammatory M1 responses but limits induction of M2 transcriptional programs. Such polarization skewing has been observed in models of TSC deletion but is also suspected to occur in metabolic pathology. This process is likely specifically dependent on mTORC1 and appears to rely on low Akt function, implying that it can be paradoxically aggravated by suppression of mTORC2 (Covarrubias et al., 2015). In addition to its intrinsic roles, mTOR can also influence macrophage polarization indirectly through its control of CD4+ T cell differentiation, as Th1 cells facilitate M1 activation, while Th2 cells drive M2 polarization (Solana et al., 2012).

Macrophages are likely major contributors to age related inflammatory pathology. Being extremely responsive and adaptive to their surroundings, macrophages are strongly affected by the aged environment. Additionally, due to their longevity, tissue resident macrophages have the tendency to accumulate intrinsic damage similarly to parenchymal cells (Van Beek et al., 2019). Old macrophages exhibit a decline in phagocytosis, generation of free radicals, chemotaxis, expression of MHC-II and associated antigen presenting ability, as well as altered homing behaviour (Stallone et al., 2019). Both monocytes and macrophages exhibit a steady state increase in pro-inflammatory cytokine production, while the magnitude of their secretory response upon stimulation becomes reduced in aging (Solana et al., 2012).

Normal polarization is typically dysregulated in aging, as increasing numbers of macrophages display an overall M2-like phenotype in conjunction with pro-inflammatory properties. This unusual immunophenotype may be associated with the development of senescent features favouring an inflammatory secretory activity. Other explanations implicate progressive ER stress coupled with mitochondrial dysfunction, as the former stimulates expression of M2 markers while the latter activates pro-inflammatory pathways. Such intracellular disturbances are difficult to resolve due to the decreased efficiency of the unfolded protein response and autophagy associated with aging (Van Beek et al., 2019). MTOR activity can further aggravate the dysfunction of both processes and can support the secretory output of

senescent macrophages. Nevertheless, it is unclear whether mTOR is truly overactive in aged macrophages or whether normal levels of mTOR activity simply become incompatible with optimal maintenance of cellular functions. In fact, metabolic responses which are normally supported by mTOR function during stimulation exhibit a decline in old murine macrophages. Accordingly, aged bone marrow derived macrophages become impaired in their ability to activate glycolysis and arginine metabolism (Van Beek et al., 2019), indicating a reduced functionality of the M1 phenotype which could be potentially further accentuated by mTOR inhibition.

Ideal modulation of macrophage activity in the aged organism would likely not consist of a full reversal of the elderly phenotype, but rather of limiting inflammatory responses while retaining M2 traits. Indeed, several interventions known to increase longevity, such as activation of SIRT or AMPK, appear to promote the implementation of M2 characteristics while combating pro-inflammatory mitochondrial disruption (Van Beek et al., 2019). If the goal for healthy aging is restoring potent M2 polarization with anti-inflammatory properties, mild but highly selective mTORC1 inhibition is likely to facilitate this conversion. Suppression of mTORC1 activity could be expected to limit initial M1 activation and subsequent excessive inflammatory responses, while also supporting the repair of damage in aged M2 macrophages by releasing restraints placed on proteostatic mechanisms. Nevertheless, treatments which secondarily inhibit TORC2 function may need special consideration as they risk maintaining pro-inflammatory signalling by preventing activation of Akt. Clinical applications of mTOR inhibition in macrophages are already being investigated in age-related disease. In pre-clinical studies, topically delivered Everolimus has been shown to induce autophagic death in macrophages infiltrated in atherosclerotic plaques, thereby limiting plaque rupture, which is a common complication in elderly patients (Zhai et al., 2014).

Interestingly, mTORC1 signalling is employed by CMV in macrophages to sustain viral protein synthesis during active infection and its inhibition has been reported to effectively prevent virus reactivation in transplant patients treated with rapamycin (Katholnig et al., 2013). This phenomenon might also be relevant in the context of immunosenescence, as natural control of the CMV infection takes a substantial toll on the aging immune system and alternative pharmacological methods of maintenance might halt the progression of age associated changes.

### 8.3. mTOR in dendritic cell aging

DCs are the primary antigen presenting cells and constitute a tight link between innate and adaptive immunity. Their main function is to scan the intra- and extracellular environment for antigens, which they subsequently process and expose on their surface via the MHC-I and MHC-II complexes for the purpose of activating naïve T cells. Concomitant with their antigen sampling, DCs integrate signals from multiple PRRs and generate distinct T cell reactions depending on their detection of PAMPs / DAMPs (Sukhbaatar et al., 2016). Antigen presentation by immature DCs in the absence of these signals induces peripheral tolerance in responding T cells. In contrast, PRR activated DCs undergo a maturation process and gain the ability to stimulate effector T cell responses (Agrawal and Gupta, 2011). During this process tissue resident endocytosing DCs suffer a morphological and metabolic transformation, begin secreting cytokines and later migrate to secondary lymphoid organs where their effective interaction with T cells takes place (Sukhbaatar et al., 2016). DCs have been classified into several subpopulations, of which the myeloid DCs (mDCs) in humans and equivalent conventional DCs (cDCs) in mice develop directly from myeloid precursors, while the common plasmacytoid DCs (pDCs) originate from lymphoid progenitors. Additionally, differentiation of monocytes can give rise to a separate subset of DCs, termed monocyte-derived DCs (moDCs) (Katholnig et al., 2013).

While observations are still inconclusive, it appears that all DC subsets maintain relatively steady circulating numbers during healthy aging, while mDCs tend to be decreased in frail subjects. Circulating aged DCs also display normal levels of MHC molecules, co-stimulatory receptors and possibly TLR receptors. In contrast, phagocytic capacity and migratory ability are impaired in elderly mDCs and moDCs (20, 46). PDCs, which are functionally specialized for the secretion of high quantities of type I interferons, exhibit a substantially decreased output of IFN-α with aging. Suboptimal priming of T cells can lead to anergy and preferential generation of Tregs, which could in theory be an expected outcome of DC functional decline during aging, however experimental results are conflicting and point towards a predominant role of T cell intrinsic factors. Despite their reduced capacity to phagocytose self-antigens derived from apoptotic cells, elderly DCs paradoxically promote an increased reactivity to autoantigens, in part due to a heightened level of basal activation sustained by NF-kB overactivity. This situation contributes not only to the loss of peripheral self-tolerance and subsequent increase in autoimmune responses, but also to continuous

cytokine production and maintenance of an inflammatory environment (Agrawal and Gupta, 2011).

Induction of bone marrow derived DC development relies on the engagement of the PI3K-Akt-TOR axis downstream of Flt3L signalling (Araki et al., 2011). In both mice and in vitro human cells, normal development of all DC subsets appears to involve a meticulous control of mTOR function, as both inhibition and constitutive activation can have severely damaging effects on the proliferative capacity, differentiation and survival of DC progenitors. Despite these observations, treatment with rapamycin does not affect the size of the mDC and pDC populations in human patients. Similarly, monocyte conversion to functional moDCs depends on mTORC1 signalling in response to GM-CSF and IL-4 stimulation. Exposure to rapamycin during this process amplifies apoptosis and promotes the development of a tolerant DC phenotype, which loses the ability to stimulate effector T cell responses but retains Treg induction properties, however these results remain to be verified in vivo (Araki et al., 2011; Katholnig et al., 2013).

In mature DCs, mTOR is engaged by TLR signalling via PI3K in order to support the anabolic processes required for the phenotypical conversion towards the activated state (Sukhbaatar et al., 2016). Accordingly, there are reports of immunosuppressive effects exerted on DCs by rapamycin, such as decreased IFN production by pDCs, downregulation of co-stimulatory surface molecules and reduced total cytokine output of moDCs (Araki et al., 2011). Nevertheless, partial inhibition of mTOR signalling during TLR stimulation appears to have a surprisingly positive influence on the functional abilities of mDCs. Suppression of mTOR during DC activation decreases expression of PD-L1, the ligand of the T cell inhibitory PD-1 receptor which negatively regulates effector T cell proliferation and Treg apoptosis. At the same time, expression of the costimulatory protein CD86 on the surface of DCs is enhanced during mTOR inhibition. Antigen presentation on MHC I/II molecules depends on efficient autophagic processing and is predictably enhanced upon administration of rapamycin. Additionally, reduced mTOR activity appears to increase the lifespan of DCs, thereby allocating more time for their interaction with T cells. Collectively these results indicate an improved ability of DCs to activate T cells in conditions of reduced mTOR signalling and this effect has been experimentally confirmed especially in the case of Th1 cells (Katholnig et al., 2013). Similar to cells of the adaptive immune system, a model of spatiotemporal mTOR signalling fluctuations has been proposed to explain the contradictory effects observed in activated DCs. According to this hypothesis, DCs employ high mTOR activity to detect the

nutrient rich tissue milieu and support early activation steps, but decrease their level of mTOR signalling as they migrate to the metabolically distinct environment of lymph nodes, while switching functionally from an inflammatory role to an antigen presenting task. In this context, mTOR inhibition might limit DC activation to some extent, but this effect would be sufficiently compensated by the increased ability of responsive DCs to stimulate T cell responses. The observed behaviour of DCs thus corroborates the potential of short term pharmacological mTOR inhibition to enhance vaccine responsiveness in the elderly.

Signalling perturbations occur in aged DCs, but it is unclear how they affect mTOR activity. PI3K-Akt signalling is impaired in old DCs (Agrawal and Gupta, 2011), which should lead to reduced mTOR activation, however excessive NF-kB activity is commonly associated with increased mTOR function. It is possible that mTOR activity is enhanced in the steady state while also being less receptive to TLR stimulation, which would be consistent with the reported basal activation of elderly DCs paired with suboptimal functional responses. In this case the greatest improvement in old DC responses may be achieved through preliminary pharmacological suppression of mTOR which is then temporarily relaxed during contact with the antigen. The fact that mTOR modulation can influence the function of DCs without affecting their number may be a positive aspect in the context of aging, as the elderly DC compartment is stable in size and would optimally benefit from functional rescue alone.

It is important to note that both phagocytic function of macrophages and effective antigen display by DCs rely on processes in the lysosomal compartment which require a certain level of mTOR activity. In DCs, exogenous antigens are loaded on MHC-II class molecules while transiting through endolysosomal equivalent intracellular compartments and are then delivered to the plasma membrane through the formation of tubular structures. Accordingly, mTORC1 promotes exogenous antigen presentation by stimulating lysosomal biogenesis, assembly of the V-ATPase and subsequent lysosomal acidification, as well as lysosomal tubulation. In this context, the previously described enhanced ability of DCs to stimulate T cells could clearly only be obtained in conditions of partial mTOR inhibition. Indeed, potent repression of mTOR with Torin1 reportedly impedes the delivery of MHC-II molecules to the cell surface in murine bone marrow DCs (Saric et al., 2016). Conversely, mTOR activity may have a more pronounced suppressive influence on endogenous antigen presentation, which relies primarily on autophagic flux. This differential regulation of endogenous and exogenous

antigen processing is functionally relevant, as the spike in mTOR function upon DC stimulation facilitates the transition from presentation of self-antigens to preferential exposure of foreign ones. As a result, while mild inhibition of mTOR signalling improves presentation of antigens from both sources, the display of endogenous antigens is expected to be more potently enhanced (Sukhbaatar et al., 2016). The implications of this phenomenon in the elderly immune system will need to be carefully assessed. In conditions of healthy immune function, suppression of mTOR in the absence of TLR stimulation promotes a tolerogenic DC phenotype. It remains to be determined whether old DCs which support autoreactivity will benefit from the application of the same principle or whether an enhanced ability to present self-antigens will aggravate their dysfunctional phenotype.

### 8.4. mTOR in NK cell aging

NK cells are innate immune cells of lymphoid origin which play essential roles in the elimination of cancerous and virally infected cells. NK cells react quickly and without prior sensitisation to cells lacking MHC-I class molecules and mount either a cytolytic or a cytokine secretory response. During their developmental progression NK cells transiently express the CD27 marker, which is considered a characteristic of immature NK cells, broadly corresponding to the classically described $CD56^{bright}$ NK subset and associated with peak regulatory cytokine production capacity. At later stages NK cells gain the integrin CD11b, regarded as a trait of mature NK cells, likely equivalent to the classical $CD56^{dim}$ NK subset and correlated with maximal cytotoxic ability (Fu et al., 2011). Maintenance of high NK cytotoxic activity may be particularly important in the aged organism, which requires reliable defences against the increased risk of tumour formation and latent viral infection. While cytolytic ability declines during aging on a single cell level, the $CD56^{dim}$ NK population concomitantly expands, compensating the loss of function and ensuring a stable overall cytotoxicity of the NK compartment. This dynamic has been explained primarily as a consequence of CMV infection, however the prolonged peripheral lifespan of NK cells due to increased IL-15 signalling may be an additional contributor. Conversely, the $CD56^{bright}$ subset decreases in number in the elderly independently of viral infection, potentially due to reduced generation of progenitors in the bone marrow (Solana et al., 2014). While old $CD56^{bright}$ NK cells do not appear functionally impaired, the composition of their cytokine output may suffer changes and may have reverberating influences on both innate and adaptive immunity, since the secretory activity of this cellular subpopulation is a critical supporting factor of DC and monocyte activation (Solana et al., 2012).

The two mTOR complexes engage in intricate interactions to control NK differentiation and function. MTORC2 activity is required for early specification of the NK lineage and Rictor deletion reportedly causes a block in NK development shortly after acquisition of the CD27 molecule. Nevertheless, mature mTORC2 deficient NK cells display increased cytotoxic potential, involving significant upregulation of perforin and granzyme B as well as enhanced degranulation measured per cell. MTORC1 signalling is needed throughout the lifespan of NK cells, as it supports their maturation, activation and effector function, but also restricts their proliferation and survival, preventing their excessive increase in number in the bone marrow. Raptor deletion causes the accumulation of proliferating NK progenitors in the BM, but blocks NK development after expression of the CD11b protein. In complete opposition to the effects of Rictor deletion, Raptor deficiency leads to the downregulation of perforin / granzyme B coupled with significantly reduced cytotoxic responses against cancer cells. It appears that both mTOR complexes cooperate to regulate NK cell maturation by sustaining the expression of the transcription factors Tbx21 and eomesodermin, which are required for effective NK differentiation. While NK activation promotes signalling through both mTORC1 and mTORC2, the two pathways have surprisingly contradictory effects on NK cytolytic function. In fact, it seems that specifically in NK cells the two complexes engage in an Akt-independent regulatory feedback loop, characterized by the ability of mTORC2 to exert negative control on mTORC1 by repressing the STAT5 pathway. IL-15 signalling, a critical determinant of NK responsiveness, is also involved in a delicate regulatory relationship with mTOR. While IL-15 stimulation activates both complexes, mTORC1 exerts a positive feedback response by supporting expression of IL-15 receptors, while mTORC2 dampens downstream signalling through this pathway (Wang et al., 2018).

It remains to be determined whether mTOR plays an active role in the natural age-associated changes of the NK compartment, however its modulation could clearly be expected to affect multiple aspects of NK aging. The most pronounced effects can be predicted to occur in response to the selective targeting of a single mTOR complex, which would disturb the fine regulatory balance maintained through the coordinated activity of mTORC1 and mTORC2. Partial inhibition of mTORC1 could hypothetically achieve a moderate reversal of the elderly NK subset distribution, as it would promote improved survival of progenitors, reduced progression to late developmental stages and limited peripheral persistence in response to IL-15 stimulation, thereby restoring a higher ratio of immature to mature cells. It is questionable whether this would actually have a positive effect, since NK subset restructuring at old age

could be an adaptive or compensatory mechanism. Additionally, mTORC1 inhibition may even reinforce detrimental aspects of the aged NK phenotype, such as the decreased cytotoxic responses of individual cells. Suppression of mTORC2 may have therapeutic potential as a means of enhancing NK cytotoxicity for the containment of tumoral processes and viral infections, however it may also severely limit the production of regulatory immature NK cells and further aggravate the age-associated disturbances in cytokine production. Fluctuations in NK subsets and their functional responses might need to be considered even when other immune compartments constitute the primary targets of mTOR modulatory treatment. Nevertheless, aging NK populations display exceptional homeostatic ability as they can finely balance functional perturbations with changes in number, indicating that short term interventions will likely have an acceptable safety profile.

### 8.5. The role of mTOR in MDSCs as agents of age-related immune dysregulation

Still only recently understood, myeloid derived suppressive cells (MDSCs) provide an excellently integrated example of the delicate balance that the innate immune system struggles to maintain between under- and overactivity, of its significance in aging and of the role mTOR plays in immune homeostasis. MDSCs are a heterogeneous collection of myelocytic precursors at different stages of development which possess strong immunosuppressive abilities and might play an important role in the termination of acute immune responses, protecting tissue integrity and promoting repair mechanisms after threats have been effectively eliminated. MDSCs were first studied in the context of emergency myelopoiesis, a process of rapid but abnormal myeloid expansion induced in conditions of prolonged inflammatory stress such as chronic infections and cancer. The mild but persistent inflammatory state associated with aging, together with the affiliated myeloid skewing of haematopoiesis, are in many ways reminiscent of this phenomenon and could potentially implicate MDSCs in similar ways. Indeed, the increased myeloid output affects all concerned cellular subsets of the elderly, not only the mature myeloid types but also different progenitor stages including those with a MDSC phenotype.

Heightened numbers of MDSCs accumulate in the bone marrow and secondary lymphoid organs during aging, but also increase in circulation and infiltrate in chronically inflamed tissues. As MDSCs attempt to suspend local immune responses but are unable to resolve the systemic cause of inflammation, a positive feedback loop can be established whereby suppression of homeostatic immune processes leads to increased vulnerability of the

affected tissues to infection and accumulation of damage so that underlying sources of inflammation are maintained and continued recruitment of MDSCs becomes a self-sustaining process. Aspects of tissue maintenance that become impaired in the protracted presence of MDSCs may include clearance of dead or senescent cells, proteostasis and antitumoral immune surveillance. Senescent cells in particular may engage in an especially damaging relationship of reciprocal reinforcement with MDSCs, as colony stimulating factors and chemokines included in the SASP are potent inducers of MDSC differentiation and chemotaxis, while MDSCs themselves not only prevent the elimination of senescent cells but also promote paracrine senescence through secretion of TGF-β. In the bone marrow, accumulation of activated MDSCs may disturb the stem cell niche and reinforce the reduction in B lymphopoiesis. From this perspective, increased MDSC activity is more than just a symptom of aging; paradoxically in respect to its original function, it may instead adopt a causal role in the perpetuation of age associated tissue deterioration and inflammation. In chronic conditions such as inflammaging MDSCs constitute a compromise, as they thwart rampant immune reactions which pose an immediate danger, despite promoting slower acting degenerative processes in exchange for their protective task (Salminen et al., 2018).

While human MDSCs are still incompletely described, murine equivalents are classified into two major subpopulations of granulocytic and monocytic origin respectively (G-MDSCs / M-MDSCs). Together they can suppress the functions of almost all immune cells, with G-MDSCs preferentially restricting humoral immune responses and M-MDSCs inhibiting the activation of T cells, NK cells, DCs and macrophages. Conversely, MDSCs cooperate with other immunosuppressive cells such as Tregs and Bregs and support their differentiation (Salminen et al., 2018). MDSCs block T cell activation through enzymatic and oxidative mechanisms that alter the structure of TCRs and deplete the microenvironment of vital nutrients required for T cell proliferation (Wu et al., 2016). These processes are primarily mediated by arginase and inducible nitric oxide synthase (iNOS) secreted by MDSCs. Functional properties are enhanced in elderly MDSCs and translate into a strong inhibitory effect on T-cell cytotoxicity and T-cell mediated humoral responses, thereby reinforcing problematic aspects of immunosenescence (Salminen et al., 2018).

Differentiation of M-MDSCs requires intense glycolytic metabolism supported by mTORC1 through upregulated expression of glycolytic pathway components. M-MDSCs also rely functionally on mTOR signalling to promote transcription of iNOS and Arg-1 (Wu et al., 2016) and overexpression of both genes has been detected in old MDSCs upon stimulation

(Salminen et al., 2018). Inhibition of mTOR with rapamycin limits M-MDSC development, reduces their number in circulation and at sites of injury, and abrogates their immunosuppressive abilities. Myeloid specific knockout experiments indicate that mTORC2 is dispensable for M-MDSC differentiation and function, while G-MDSCs seem able to develop independently of mTOR altogether (Wu et al., 2016).

A challenging aspect of therapeutic mTOR modulation will be predicting the result of the interplay between distinct immune cell subsets in which mTOR signalling may perform very different functions that could have either synergistic or counterbalancing effects. A good example of this balancing act is the interaction between MDSCs and T cells, during which mTOR inhibition can release the external suppressive constraints on T cell activation while simultaneously exerting a complex intrinsic influence on T cell function. In allograft or tumour environments these may be conflicting processes, as the direct tolerance promoting effects of mTOR inhibition in CD4+/CD8+ cells compete with the increased TCR stimulation allowed by removal of the M-MDSC immunosuppressive checkpoint (Wu et al., 2016). A similar dynamic could possibly occur in aged tissues affected by autoimmune processes and sterile inflammation. Conversely, in conditions of intense antigenic stimulation, the removal of immunosuppressive barriers may collaborate with the enhanced adaptive memory responses promoted by mTOR inhibition to confer improved vaccine responsiveness and protection against infection which is critically needed by elderly patients. This issue is further complicated by the coexistence of multiple regulatory cell subsets of different origins, each impacted independently by mTOR modulation while responding to the cues of the others.

### 8.6.mTOR in trained immunity

Trained immunity has been proposed among the mechanisms that support detrimental increases in the activation of the innate immune system during aging. Indeed, myeloid cells involved in the inflammaging process often express traits indicative of immune memory (Salminen et al., 2018). In innate immune cells, memory is established fundamentally through epigenetic reprogramming, which ensures enhanced gene expression upon reactivation and stronger non-specific defensive responses against secondary infections. Trained cells may be primed to react more effectively to proliferation signals, they exhibit improved metabolic responses, enhanced phagocytic activity and, importantly for the aging organism, increased production of proinflammatory cytokines. Epigenetic signatures of innate immune memory

reveal active promoters for genes involved in immune signalling, components of the glycolytic pathway, as well as mTOR itself and its downstream effector EIF4EBP1. Acute activation of monocytes following recognition of β-glucan by the dectin-1 receptor occurs through Akt-mTOR signalling and consequent upregulation of glycolytic genes mediated by the transcription factor HIF1α. These pathways are employed upon initial pathogen contact and are required for the induction of training; however they also form the basis of vigorous subsequent immune responses upon re-stimulation. Based on their epigenetic landscape, trained cells are highly responsive to mTOR activation and can rapidly implement new metabolic programs to sustain aggressive functional reactions. Early pharmacological inhibition of mTOR can prevent implementation of innate immune memory in a dose dependent manner (Cheng et al., 2014). Such a gradual response may prove advantageous for elderly patients, who would often derive the greatest benefit from the restoration of balance between different immune functions rather than the complete abrogation of any processes which remain necessary despite their dysregulation. On the other hand, inhibition of mTOR during reactivation of already trained immune cells might, in principle, be able to counteract unrestrained acute responses without disturbing the underlying reprogramming which allows retention of memory. In this case intermittent treatments would be the most useful clinical approach, as they would alternate between controlling the unfettered reactivity of the old innate immune system and allowing it to accumulate further experience or address genuine danger with effective responses. Therefore, through careful dosing and timing of treatments targeting mTOR signalling, the benefits of trained immunity in fighting infection and compensating the decline of adaptive immune responses may be reaped without the undesired consequence of fuelling inflammaging. Since not only mature myeloid cells but also MDSCs could develop trained phenotypes (Wu et al., 2016), modulating innate memory through the mTOR pathway may temper both inflammatory and anti-inflammatory reactions simultaneously and prevent unnecessary reinforcement of regulatory feedback loops.

## 9. Discussion and perspectives

MTOR is critically needed during the development and activation of most immune cells, as it supports the metabolic and biosynthetic requirements which fuel fast proliferation and phenotypic conversion. At the same time, mTOR activity needs to be strictly limited during specific differentiation phases and periods of quiescence, and regulatory failure in this regard

can lead to immune dysfunction. Dynamic control of mTOR function is normally employed as immune cells progress through their life cycle. At important checkpoints the level of mTOR activity can determine cell fate decisions and functional adaptions. Deviations from optimal ranges of mTOR signalling have been associated with improper maturation of immune cells, survival failure, exhaustion of the haematopoietic stem cell and lymphoid compartments, as well as abnormal secretory activity of myeloid cells. Due to the intricate and sometimes divergent regulation of mTOR activity in different processes which support the function of the immune system, therapeutic inhibition of this pathway can produce either immunostimulatory or immunosuppressive effects in a context dependent manner.

The aged immune system suffers signalling perturbations which characteristically lead to aberrant activation in steady state conditions, as well as to the inability of mounting effective responses in the face of genuine threats. Consequently, aging of the immune system generates an intriguing combination of issues resulting both from reduced functionality (increased vulnerability to infections and cancer, lower vaccine efficacy, improper maintenance of tissue homeostasis), as well as from hyperactive function (persistent inflammation and autoimmune reactions). Attempting to contain some of these problems, regulatory cells and adaptive responses often aggravate others. As additional hallmarks of aging develop and join the interplay of dysfunctional processes and compensatory mechanisms, cellular homeostasis decays under increasing stress, runaway physiological and biochemical phenomena ensue, and balance becomes progressively more difficult to restore to the aged biological system.

As mTOR participates downstream in transduction pathways from a variety of essential immune cell receptors, it becomes inevitably involved in the age-related degradation of signalling network integrity and function. Changes in the absolute and relative activity of the two mTOR complexes may directly contribute to aspects of the age-related functional decline of different immune compartments. Conversely, absent or limited fluctuations in mTOR function might also be problematic as they could be insufficient for cells to compensate the myriad perturbations unfolding in the aged system. At the same time, changes in the responsiveness of the mTOR pathway to microenvironmental stimuli and feedback mechanisms may lead to the inability of immune cells to accurately adjust their specific responses to external challenges and to internal resources and capacities. Overall, the basal activation level of mTOR tends to be increased in the elderly organism, while individual cells may encounter difficulties upregulating it further in order to meet specific functional requirements upon stimulation.

The interplay between the range of mTOR activity and various facets of immunosenescence is elaborate and only minimally understood. Despite the intimidating complexity of the aging process and its dynamic impact on signalling networks, very simple external interventions that modulate mTOR activity are able to improve the function of the elderly immune system in a significant and reliable manner. Pharmacological inhibition of mTOR supports healthy aging in animal models and many of its benefits are traceable to effects exerted through components of the immune system. Optimally calibrated suppression of mTOR activity can be predicted to improve most complications of immune aging, from stunted primary and recall responses to reduced self-tolerance and aberrant regulation of inflammatory processes. An elegant symmetry emerges at the intersection between age-related dysfunction and mTOR signalling regulation in immunity. The paradoxical coexistence of immune hyperactivity and impaired immune responsiveness in aging mirrors the regulatory intricacies of mTOR signalling which allow both immunosuppressive and immunostimulatory effects to be achieved through its inhibition. Thus, the flexibility of mTOR modulation in exerting contrasting influences on different immune processes depending on adjustable factors is what allows it to address distinct types of immune dysregulation stemming from aging.

The versatility of mTOR within the immune system, together with its well-known involvement in longevity and healthy aging, promise numerous potential applications of mTOR modulation in preventing and alleviating age-associated morbidity related to immune dysfunction. Before progressing to clinical interventions, it will be necessary to determine the parameters within which interfering with mTOR function is likely to produce a desirable and reliable result. The overlap between enhancement and suppression of immune functions underlies not only the advantages, but also the potential side effects of mTOR targeted therapies. Both payoff and risks might be higher in elderly patients whose immune regulatory mechanisms are already strained. Potential outcomes would need to be delineated and assessed in accordance with dosages, durations and specificities of treatments in order to determine the safest and most effective approaches. Treatment courses may eventually be customized to individual immune phenotypes, by predicting which immune effectors are likely to respond positively to specific interventions and which ones pose risks of complications.

Theoretical interpretations of the role of mTOR in the immune system together with several experimental models indicate that very intense suppression of mTOR function is counterproductive, as it blocks development and responses of most immune cell types and aggravates immune dysfunction instead of correcting it. Authors currently working on

developing mTOR inhibitory treatment courses for the elderly have suggested that the degree of suppression that would achieve maximal therapeutic benefit could be the one that restores basal mTOR activity to the levels measured in youthful cells (Mannick et al., 2014). This hypothesis appears simple and intuitive in cases where metabolic and signalling dysregulation is the primary cause of immune failure. Nevertheless, until this approach is verified it can also be argued that optimal levels of mTOR function might be different in old cells which accumulate various age-related changes at multiple levels and may therefore face different challenges and requirements for maintaining homeostasis.

Despite the many unknowns, tentative clinical and pre-clinical studies of mTOR inhibition in the elderly have obtained impressive successes and proved some of the hypothesised benefits of modulating mTOR activity for improving immune function. Reported results indicate that limiting mTOR activity in the aged immune system can restore HSC quiescence, normal lymphopoiesis, lymphocyte homing ability, antigen presentation and T cell responsiveness to stimulation, broader humoral responses, adaptive memory development and consequent protective abilities of vaccination. Limitations include the fact that the most comprehensive experiments primarily focused on adaptive immunity and specific inhibition of mTORC1. In order to construct a complete perspective on the potential of mTOR as a therapeutic target for regulating the immune function of senior patients, responses of the innate compartment and methods of mTORC2 suppression will also require serious investigation.

## 10. Conclusion

Strict regulation of mTOR activity is required for optimal maintenance of the immune system and aging interferes with this process through signalling dysfunction affecting the pathways which converge on mTOR. Medical interventions which modulate mTOR function can restore youthful traits to the elderly immune system and compensate for impaired or aberrant immune responses associated with aging. Nevertheless, the consequences of mTOR activation or inhibition are highly variable, depending on context, cell type, developmental stage, duration and intensity of the response. The interplay of these diverse factors needs to be elucidated in detail before clinical applications can be developed safely and optimized to achieve desired therapeutic goals with precision. This area of research has great potential of leading to concrete methods of preventing age-related frailty and minimising morbidity resulting from immune dysregulation in elderly patients.

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