ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ

ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ

ΤΜΗΜΑ ΙΑΤΡΙΚΗΣ

ΜΕΤΑΠΤΥΧΙΑΚΟ ΠΡΟΓΡΑΜΜΑ

ΚΥΤΤΑΡΙΚΗ ΚΑΙ ΓΕΝΕΤΙΚΗ ΑΙΤΙΟΛΟΓΙΑ

ΔΙΑΓΝΩΣΤΙΚΗ ΚΑΙ ΘΕΡΑΠΕΥΤΙΚΗ ΤΩΝ ΑΣΘΕΝΕΙΩΝ ΤΟΥ ΑΝΘΡΩΠΟΥ.

Εργαστήριο Αυτοανοσίας και Φλεγμονής, Ίδρυμα Τεχνολογίας και Έρευνας – Ινστιτούτο Μοριακής Βιολογίας και Βιοτεχνολογίας

ΜΕΤΑΠΤΥΧΙΑΚΗ ΔΙΑΤΡΙΒΗ

Μελέτη του ρόλου της κινάσης Akt στην διαφοροποίηση και στη δράση των κατασταλτικών κυττάρων μυελικής σειράς (MDSCs).

ΑΝΤΩΝΙΟΣ Ι. ΠΙΚΟΥΛΑΣ

Ηράκλειο 2013

ΜΕΤΑΠΤΥΧΙΑΚΗ ΔΙΑΤΡΙΒΗ

Μελέτη του ρόλου της κινάσης Akt στην διαφοροποίηση και στη δράση των κατασταλτικών κυττάρων μυελικής σειράς (MDSCs).

Κύριος Επιβλέπων :

Πάνος Βεργίνης

3μελής συμβουλευτική επιτροπή

Πάνος Βεργίνης, Ερευνητής Δ τομέα Ανοσολογίας, ΙΙΒΕΑΑ

Χρήστος Τσατσάνης, Αναπληρωτής Καθηγητής Κλινικής χημείας, Ιατρική σχολή Πανεπιστημίου Κρήτης

Άρης Ηλιόπουλος, Καθηγητής κυτταρικής και μοριακής βιολογίας, Ιατρική σχολή Πανεπιστημίου Κρήτης

Η εργασία αυτή έγινε στο πλαίσιο της υλοποίησης του μεταπτυχιακού προγράμματος «Κυτταρική και Γενετική αιτιολογία, Διαγνωστική και θεραπευτική των ασθενειών του ανθρώπου» και αποτελεί προϊόν συνεργασίας των εργαστηρίων «Αυτοανοσίας και Φλεγμονής» και «Κλινικής Χημείας».

ΠΡΟΛΟΓΟΣ – ΕΥΧΑΡΙΣΤΙΕΣ

Η ολοκλήρωση της παρούσας εργασίας δεν θα μπορούσε να είναι δυνατή χωρίς την συνεισφορά αρκετών ατόμων οι οποίοι με στήριξαν και με βοήθησαν με τις συμβουλές τους κατά τη διάρκεια της εργασίας.

Αρχικά θα ήθελα να ευχαριστήσω όλα τα μέλη του Εργαστηρίου Αυτοανοσίας και Φλεγμονής για το καλό κλίμα και για τις καίριες και πολύτιμες συμβουλές τους : Θέμις, Μαριάννα, Κατερίνα Β., Λίτσα, Ειρήνη, Τόνια, Κατερίνα Γκ. καθώς και την κ.Χουλάκη γιατί οι συμβουλές της σε τεχνικά θέματα ήταν παραπάνω από κρίσιμες. Επίσης ευχαριστώ το Εργαστήριο Κλινικής Χημείας για τη βοήθεια που μου δώσανε και κυρίως την Ρίτσα η οποία βοήθησε πολύ σε ότι και αν ζήτησα βοήθεια.

Θα ήθελα να πω ένα μεγάλο ευχαριστώ στον κύριο Δημήτρη Μπούμπα που με δέχθηκε στο εργαστήριο του στο οποίο έγιναν και τα πειράματα τις εργασίας αυτής. Επίσης θα ήθελα να ευχαριστήσω τον κ. Πάνο Βεργίνη και τον κ. Χρήστο Τσατσάνη καθώς η αλληλεπίδραση και η συζήτηση μαζί τους σχετικά με την πορεία και τα πειράματα της εργασίας αυτής, ήταν παραπάνω από χρήσιμη και διδακτική για την αντιμετώπιση ερωτημάτων και προβληματισμών που μπορούν να προκύψουν σε ένα εργαστήριο καθώς και τον κ.Ηλιόπουλο , το 3^{ο} μέλος της τριμελούς επιτροπής που ήταν υπεύθυνη για αυτή την εργασία.

Τέλος θα ήθελα να πω ένα μεγάλο ευχαριστώ στην οικογένεια μου για την ψυχολογική κυρίως αλλά και οικονομική στήριξη που μου παρείχαν καθώς και στην Αγγέλα που ήταν πάντα δίπλα μου και έκανε υπομονή.

ΠΕΡΙΛΗΨΗ

Τα κατασταλτικά κύτταρα μυελικής σειράς (MDSCs) είναι ένας ετερογενής πληθυσμός μη-πλήρως διαφοροποιημένων κυττάρων που προέρχονται από τον μυελό των οστών και τα οποία έχουν την ικανότητα κάτω από ορισμένες συνθήκες να καταστείλουν την ανοσολογική απάντηση καταστέλλοντας τις διαδικασίες που γίνονται μέσω Τ-κυττάρων καθώς και αλληλεπιδρώντας με άλλα κύτταρα του ανοσοποιητικού συστήματος. Παρόλο που αρχικά τα κύτταρα αυτά μελετούνταν σε καρκινικά συστήματα όπου η σημασία τους είχε πλέον γίνει αποδεκτή, τα τελευταία χρόνια αποτελέσματα διαφόρων μελετών είτε με in vitro είτε με in vivo πειράματα, καταδεικνύουν την σημασία του ρόλου των κυττάρων αυτών καθώς και το ότι εμπλέκονται σε διάφορες παθολογικές καταστάσεις.

Η κινάση Akt είναι μια από τις σημαντικότερες και πιο μελετημένες πρωτεΐνες των ευκαρυωτικών κυττάρων. Αποτελεί βασικό κόμβο διαφόρων σηματοδοτικών μονοπατιών εντός του κυττάρου τα οποία δεν σχετίζονται υποχρεωτικά μεταξύ τους, αλλά ενεργοποιούνται από μια πληθώρα σηματοδοτικών μορίων που μπορεί να φθάσουν στην επιφάνεια του κυττάρου. Βιβλιογραφικά είναι γνωστές 3 διαφορετικές ισομορφές της Akt κινάσης οι οποίες έχουν τόσο διαφορετικές όσο και αλληλο-επικαλυπτόμενες λειτουργίες. Η σημασία του ρόλου των διαφόρων ισομορφών της κινάσης γίνεται κατανοητή αν παρατηρήσει κανείς τους φαινοτύπους των διαγονιδιακών ποντικιών τα οποία δεν τις εκφράζουν.

Η παρούσα εργασία στοχεύει στο να εξακριβωθεί ο ρόλος που έχουν δυο από τις ισομορφές της Akt στην διαφοροποίηση και λειτουργία των MDSCs είτε στον μυελό, όπου δημιουργούνται τα κύτταρα αυτά, είτε στην περιφέρεια όπου μεταναστεύουν και αναμένουν την ενεργοποίηση τους ή όπου μεταναστεύουν έπειτα από δευτερογενή σήματα ώστε να καταστείλλουν κάποια ανοσο-απάντηση.

Προκειμένου να γίνει κατανοητός ο ρόλος που έχουν οι κινάσες Akt στην διαφοροποίηση και στην λειτουργία των MDSCs χρησιμοποιήθηκαν διαγονιδιακά ποντίκια που δεν εκφράζουν τις επιθυμητές ισομορφές, είτε την Akt1 είτε την Akt2. Τα ποσοστά των κυττάρων που μελετήθηκαν, των MDSCs, μετρήθηκαν στα ποντίκια αυτά τόσο στον μυελό των οστών όσο και στην περιφέρεια, και συγκρίθηκαν με τα αγρίου τύπου ποντίκια σε δυο

διαφορετικές καταστάσεις : είτε σε στείρες συνθήκες (naïve conditions) είτε μετά από ενεργοποίηση ανοσολογικής απάντησης. Διαφορές μεταξύ των διαφορετικών στελεχών βρέθηκαν και στις δυο συνθήκες και αυτό θα μπορούσε να υποδηλώσει ένα πιθανώς ενεργό ρόλο των Akt πρωτεϊνών στην ωρίμανση ή/και στον πολλαπλασιασμό των MDSCs. Επίσης, μορφολογικές διαφορές βρέθηκαν μεταξύ κυττάρων MDSCs τα οποία απομονώθηκαν από $Akt1^{-/-}$ σε σχέση με αυτά που απομονώθηκαν από τα ποντίκια-αναφορείς των πειραμάτων. Ακόμα, η κατασταλτική λειτουργία των μυελικών αυτών κυττάρων που προέρχονται από τα διαγονιδιακά ζώα, φαίνεται να έχει τροποποιηθεί σε σχέση με τα ποντίκια αγρίου τύπου, γεγονός που καταδεικνύεται τόσο από πειράματα συν-καλλιεργειών των κυττάρων με ενεργοποιημένα Τ-κύτταρα όπου μετρήθηκαν οι παραγόμενες κυτοκίνες, όσο και από πειράματα που ελέγχθηκαν τα επίπεδα έκφρασης των μορίων arginase1 και CEBPβ, τα οποία αποτελούν βασικά κατασταλτικά μόρια για τα MDSCs.

Συμπερασματικά, από τα παραπάνω δεδομένα που προέκυψαν σχετικά με τις διαφορές στους αριθμούς των κυττάρων, στην μορφολογία τους και στην κατασταλτική λειτουργία τους , απουσία των πρωτεϊνών, καταδεικνύεται ένας σημαντικός ρόλος των Akt κινασών στην διαφοροποίηση και λειτουργία των κατασταλτικών κυττάρων μυελικής σειράς.

UNIVERSITY OF CRETE

SCHOOL OF HEALTH SCIENCES

FACULTY OF MEDICINE

GRADUATE PROGRAM IN

THE MOLECULAR BASIS OF HUMAN DISEASE

Laboratory Autoimmunity and Inflammation

Department of Rheumatology, Clinical Immunology and Allergy

Foundation of Research and Technology

Institute of Molecular Biology and Biotechnology

MASTER THESIS

Study of the role of Akt kinase in the differentiation and function of Myeloid derived suppressor cells (MDSCs).

ANTONIOS Y. PIKOULAS

HERAKLION 2013

ABSTRACT

Myeloid derived suppressor cells (MDSCs) are a heterogeneous population of immature cells generating from the bone marrow that have the ability under certain conditions to suppress the immune response. MDSCs contribute to the negative regulation of the immune response by suppressing the T-cell mediated responses and also by interacting with various other cells of the immune system. Although at the beginning these cells were only studied in various cancer models where their importance was proven, in the past few years, evidence is gathering from in vitro and in vivo studies concerning and underlining the important role of MDSCs' function or loss of function in various pathological conditions.

Akt kinase is one of the most important and known protein kinases of the eukaryotic cells. It is a central node for many signaling pathways in the cell and is activated after a variety of stimulatory molecules that reach the surface of cells. Three different isoforms are known and all of them have specific and also overlapping roles in many pathways. The importance of these kinases roles can be understood examining the phenotype and the pathology of Akt KO animals that depends on which Akt kinase isoform is absent.

This study aims to elucidate the role that two of the Akt isoforms have at the differentiation and function of MDSCs either in the bone marrow where these cells come from or in the periphery where they migrate in order to suppress an immune response or home until needed to suppress a response. In order to understand the role that Akt proteins have at the differentiation and function of MDSCs, Akt KO mice were used. The percentages of MDSCs were countered at both $Akt1^{-/-}$ and $Akt2^{-/-}$ mice in both bone marrow and spleen tissues and compared with B6 mice using two different conditions: naïve mice and mice that were immune triggered. Differences at different strains were revealed at both conditions that could indicate a role for Akt proteins at the maturation and proliferation of MDSCs. Also morphological differences were found between MDSCs isolated from $Akt1^{-/-}$ mice and B6 mice. Furthermore, the suppressive function of $Akt^{-/-}$ MDSCs seem to be altered compared to B6 mice, as results from co-culturing of these cells with T-cells show and also arginase1 and CEBPb expression levels differences reveal.

In conclusion, these data demonstrate differences at the numbers of MDSCs in the absence of Akt proteins and also morphological alterations and differences at the suppressive ability of these cells.

PURPOSE OF THIS STUDY

Myeloid derived suppressor cells are a population of cells that contributes to the negative regulation of the immune response in various conditions such as cancer, auto-immune diseases, sepsis, trauma and others. MDSCs were defined as a heterogeneous population of immature myeloid cells consisting of progenitors of macrophages, dendritic cells and immature myeloid cells. MDSCs have been associated with many diverse regulatory functions including tumor-linked immune defects, suppression of T-cell responses related to adaptive immune response and also regulation of the innate immune response. All of the above underline the importance of this population's functions and also indicate that MDSCs could be considered as a good possible target for therapeutic intervention.

Akt protein kinases signaling in response to cytokine receptor signaling promotes protein synthesis, cellular growth and proliferation. The importance of Akt signaling at the function of the immune response has been shown by many studies at the past, where it was shown that Akt has the ability to determine the polarization of macrophages into type 1 or type 2 macrophages.

As MDSCs and macrophages share a common myeloid lineage, the purpose of this study is to examine whether the absence of Akt proteins has any effect at the numbers of MDSCs both at the Bone marrow and the periphery. As Akt plays an important role in many pathways including differentiation of various cell types and migration, it would be interesting to see whether it affects also MDSCs during these procedures. Apart from this, the suppressive ability of MDSCs from Akt knock-out mice was examined in order to see whether Akt plays an important role at the pathways used from MDSCs in order to suppress the immune response.

INDEX

A) INTRODUCTION

A1. Akt protein

A1.i. General Information

The serine/threonine kinase Akt, also known as protein kinase B, is one of the central molecules involved in cell signaling downstream of various extracellular stimuli as growth factors, cytokines and other. Akt molecules participate in the regulation of metabolism, transcription, apoptosis and cell cycle. Deregulated or over-expressed Akt has been found in numerous cancer types, and sometimes the incidence rate exceeds 50% of the cases. On the other hand, the activation of Akt is reduced in human patients suffering from type II diabetes, furthermore indicating the importance of these molecules for the proper function of eukaryotic cells [1].

The critical role that Akt kinases can have in tumor models, regulating cell apoptosis, proliferation and protein synthesis has been recognized widely and supported by many studies during the past years. Last years, studies have shown that Akt kinases could also have an important role regulating the function and properties of cells of the immune system and thus affect the whole immune response. [22].

There are three different, highly homologous, isoforms of Akt kinase: Akt1, Akt2 and Akt3, also known as PKBα, PKBβ and PKBγ respectively. Analyses of Akt isoform knockout mice documented Akt isoform specific functions in the regulation of cellular growth, glucose homeostasis and neuronal development. PKBα is ubiquitously expressed, PKBβ is predominantly expressed in insulin target tissues (fat cells, liver and skeletal muscle) and PKBγ is less widely expressed, mainly in brain. Those results establish that the functions of the three isoforms are not completely overlapping and that each one through specific signaling pathways contributes to the diversity of Akt activities [2].

A1.ii. Protein structure

Akt proteins are cytosolic, soluble proteins with a molecular weight of 57kDa and share many common features concerning their structure. Each one of Akt kinases has three conserved domains with specific roles, namely an N-terminal pleckstrin homology domain (PH domain), a central catalytic kinase domain and a C-terminal domain. Although various crystal structures are known for the protein kinase domain or PH domain, the complete structure of either of the three isoforms of the protein is not yet known [14, 15], an information that would be able to further elucidate the role of all and each isoform respectively.

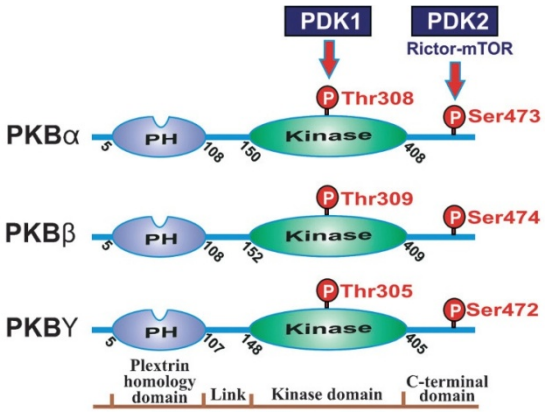

Figure 1: Structure of the different Akt isoforms [1]

The PH domain is a structure, with a size of around 100 amino-acidic residues, that binds phosphatidylinositol 3, 4, 5-trisphosphate [PI (3,4,5)P_3] and phosphatidylinositol 3,4, trisphosphate [PI(3,4)P_2] with very high affinity. The PH domain is unable to bind to phosphoinositides lacking a D3 or D4 phosphate, giving specificity concerning the binding of substrates and so giving specificity to the whole action of the kinase molecules. Phosphoinositides bind to a shallow pocket that is created in the PH domain and the phosphate side chains of the head group are forming salt bridges with specific basic residues in the protein [15-16].

The kinase domain of PKB, approximately 250 amino-acids in length, is highly similar to PKC, PKA, serum and glucocorticoids-regulated protein kinase. It contains a conserved amino-acidic residue that is a threonine (T309) and whose phosphorylation is necessary for the full activation of the molecule. This threonine residue is located between the DFG and Akt phosphorylation enhancer (APE).

The C-terminal domain, approximately 40 amino-acids in length, is the motif that is less conserved between the different Akt isoforms (70% instead of >80% for the rest of the protein) and is most closely related to the PKC family. This domain contains the characteristic hydrophobic motif of AGC kinases: F-X-X-F/Y-S/T-Y/F where X is any amino-acid. This region also contains a residue, which is a serine (S474), which must be phosphorylated in order for the protein molecule to be fully activated [14, 15-16].

The importance of the two conserved residues (T309-S474) was shown as point mutants of PKB with Ala substituting them, show significantly reduced activity and function. Other studies using mutant proteins with only one site changed to Ala or Asp furthermore showed the importance of these sites as these singly phosphorylated proteins were only partially active. All of the above indicate and underline the importance of the two sites for the activations of the protein molecule and that phosphorylation at both sites is required in order to fully activate the Akt protein molecule. [17].

A1.iii. Akt different isoforms

Akt kinase family consists of three highly homologous isoforms: Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ), with Akt1 sharing 81% and 83% of amino-acid identity with Akt2 and Akt3 respectively. The molecules seem to be conserved even across species (human-rodents) furthermore underlining the importance of the regulatory function of the proteins [1].

Phenotypic analyses of Akt knockout mice for the three different Akt molecules, underlined Akt isoform specific functions in the regulation of the various pathways they control, as well as common pathways that the isoforms participate into. Studies of Akt isoform-specific knockout mice suggest that Akt signaling diversity might be in part due to different functions of

the three isoforms and not only due to the different expression pattern. Akt1 knockout mice are smaller than their wild-type counterparts and Akt1-null cells display higher rates of apoptosis, indicating that Akt1 must have an important role in cell survival. On the other hand Akt2 knockout mice develop a type 2 diabetes-like phenotype suggesting that Akt2 has a central role in glucose homeostasis. Akt3 was proposed to have a role in brain development as mice lacking completely Akt3, displayed impaired brain development [7, 8, 9, 10 &11].

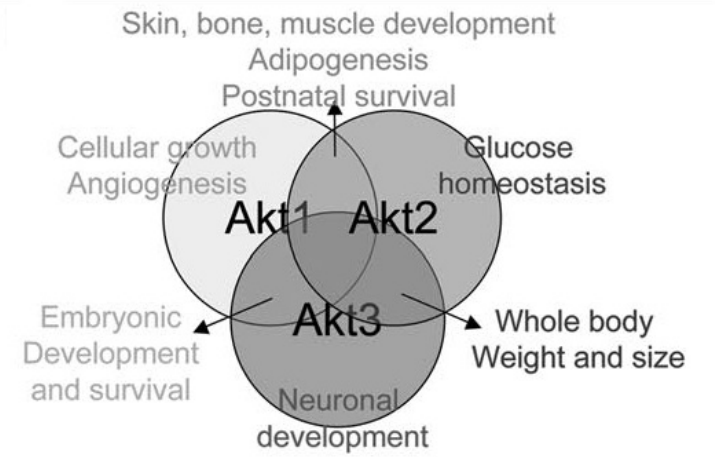

Figure 2: Overlapping and specific functions of the Akt family different members [3]

Different studies establish that the functions of the different Akt kinases are not completely overlapping and that isoform-specific signaling contributes to the diversity of Akt activities. All of the isoforms come from different genes, so common regulation of the expression of the three isoforms is not prerequisite. Although all of the above suggested that different isoforms have different role inside cells and organism, double mutants studies indicated that there may be some overlap of functions among the isoforms. The most important is to reveal the mechanism that contributes to Akt-isoform functions distinction. There have been many suggestions done concerning this mechanism but this topic will not be further studied at the present study [12, 13].

A1.iv. Akt protein activation and pathways involved

As mentioned before, in order for the Akt molecule to be activated, two phosphorylation events must occur at two different amino-acidic residues. These two residues are threonine 309 and a serine residue (different for each isoform but around 470) and both sites must be phosphorylated in order for the protein molecule to be fully activated.

External stimuli such as growth factors or cytokines, reaching the cell surface are "sensed" by receptors that cell membrane has and activate a downstream pathway that lead to the activation of PI3 Kinase (PI3K). This kinase after being phosphorylated, and thus activated, translocates to the plasma membrane, where it generates the key second messenger molecule PI (3, 4, and 5) P3. When PIP_3 is generated, it attracts Akt that binds to it using the PH domain that has a great affinity for PIP_3 molecules. That leads to the translocation of the Akt molecules at the cytosolic side of membrane of the cell. The interaction of Akt molecules with PIP_3 leads to conformational changes of Akt, making the phosphorylation sites available to kinases in order to activate the Akt protein molecules. Generation of PIP_3 molecules also leads to the translocation of other molecules at the plasma membrane. One of these proteins is PDK1 that also has a PH domain and binds to PIP_3 of the membrane. The co-localization of the two molecules leads to the phosphorylation of Akt molecules to the T309 site, an event that partially activates Akt but as mentioned before, Akt needs to be phosphorylated at the S474 also. This phosphorylation is done by another kinase, known as PDK2.

The phosphorylation of S474 is believed to be the most crucial step for the activation of the Akt molecule as it stabilizes the kinase motif of Akt in an active conformation state almost identical to that found in the constitutively phosphorylated and activated catalytic subunit of PKA. If not phosphorylated, the kinase domain of Akt protein interacts with its PH domain in a way that doesn't allow the kinase domain to be activated and thus phosphorylate the specific targets of the molecule. Because of the importance of phosphorylation of PKB on S473 in its activation, much effort has been expended in trying to identify which exactly is the cytosolic kinase that plays the role of PDK2 kinase [18].

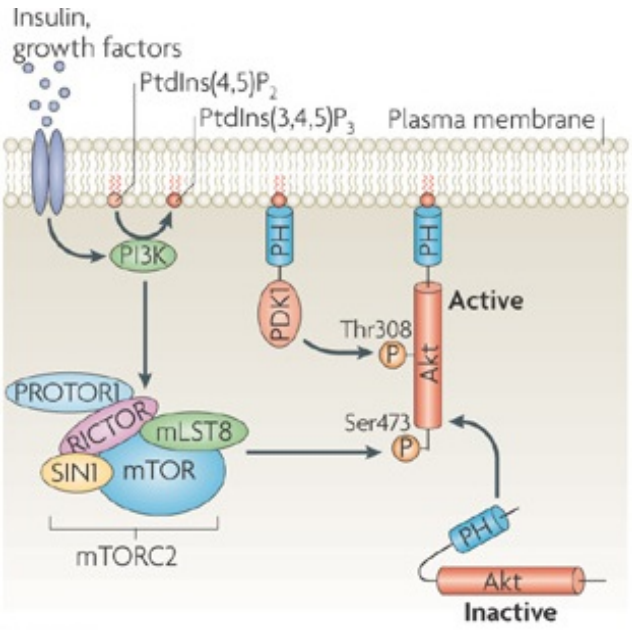

Figure 3: Activation pathway of Akt proteins via phosphorylation of specific residues []

The recent, most prominent candidates for playing the role of PDK2 kinase are mTORC2 and DNA-PK. Both of these proteins are supported to be the kinase that phosphorylates the S473 residue and there have been results that support each of them as the most probable protein that has the role of PDK2. Knock down of mTOR or rictor expression using the mechanism of RNAi leads to a decrease of S473 phosphorylation in cancer cells. Moreover, in vitro experiments have shown that mTORC2 can directly phosphorylate S473 and thus facilitate the phosphorylation of T308 by PDK1. The exact mechanism that leads to activation of the mTORC2 in the cell, because of external stimuli, still remains unknown [19]. Also mTORC2 is the main kinase of S473 during embryogenesis, as it was demonstrated in a whole body rictor knockout. On the other hand, results from experiments with skeletal muscle specific rictor knockouts have shown that activation of Akt doesn't require mTORC2. These results support the existence of another kinase that can possibly activate Akt under specific situations. For example, in stress situations following DNA damage or the presence of CpG DNA, there have been studies demonstrating that DNA-PK is the major Akt S474 kinase [20, 21]

After its activation, Akt dissociates from the plasma membrane and phosphorylates molecules-targets in the cytoplasm, transducting the signal downstream, affecting various

pathways, depending on the stimulus. Searching the available literature, more than 100 molecules have been reported as non-redundant Akt substrates. From all these, only 18 substrates meet some criteria (for example have the characteristic amino-acidic motif that Akt kinases recognize) and have been reported to be regulated by Akt in many, independent publications and thus can be examined as the most possible and common targets of Akt kinases.

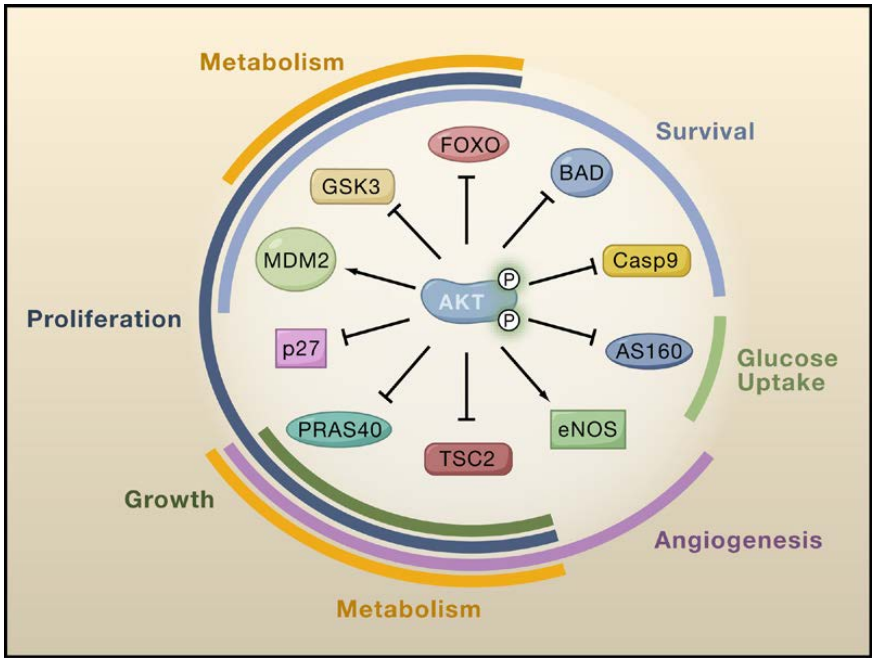

Figure 4: Akt kinase and the most known substrates. The pathway that each molecule participates in is indicated [14].

All of the above indicate that probably there are different proteins playing the role of the PDK2 kinase activating Akt protein, depending on the cell type and the situation or the stimuli that the cell has to face.

A1.v. Akt protein and the immune response/inflammation

Recently some studies have revealed a critical role of Akt protein in the immunity and autoimmunity. As it was previously known, Akt protein is involved in the regulation of the inflammatory response and several studies have underlined the important role of the PI3K-AKT signaling in inflammatory mediated diseases such as rheumatoid arthritis, multiple sclerosis, asthma, atherosclerosis and other [22, 23, 24, 25, and 26].

Many studies have shown that Akt-mTOR pathways are involved on adaptive immune cell expansion and function. [27-34] and apart from that, a large amount of evidence indicate that Akt predominantly expressed in the innate immune cells, including neutrophils, macrophages and dendritic cells, is critical to the inflammatory response and more specifically to the immune cells development and function.

Akt can activate different signaling molecules downstream and help to the activation of neutrophils in order for them to take effect in the immune response. It has been shown by studies that PI3K-dependent p38 kinase together with Akt and other molecules, form a stable complex in un-stimulated neutrophils which upon stimulation is dissociated and Akt is phosphorylated in order to transduct the signal downstream. Thus, by activating different signaling molecules, Akt participates to the activation of neutrophils in order to face possible threats such as viruses or microbes [35, 36, and 37].

Under normal conditions, in the circulation, neutrophils have a short lifespan and undergo apoptosis within less than 10hours. However, apoptosis is delayed after exposure to pro-inflammatory stimuli such as interleukin-8, TNFα, interleukin-12, lipopolysaccharide (LPS) and others. Anti-apoptotic stimuli lead to the activation of Akt which by de-activating phosphorylation of apoptotic molecules (among others caspase 9 one of the most known proteins involved in the apoptosis of eukaryotic cells) lead to the inhibition of the neutrophils apoptosis and contributes to the balance of the numbers of neutrophils. Supporting the above, recent studies report that de-activating Akt signaling leads to the spontaneous apoptosis of neutrophils, furthermore indicating the significant role of Akt signaling at the regulation of the apoptosis pathway [38].

Although many studies report a significant role for Akt as an anti-apoptotic molecule, other studies underline that Akt alone is not sufficient in order to prolong neutrophils survival. Neutrophils treated with G-CSF undergo apoptosis, even in the presence of high levels of p-Akt [39, 40] and in addition, inhibitors of Akt and downstream targets failed to alter neutrophils survival. In contrast, neutrophils precursors appear to be dependent on Akt signaling pathways for their survival whereas high levels of activated Akt inhibit proliferation [41].

All of the above suggest a controversial role for Akt at the survival and proliferation of early hematopoietic precursors and other cells as neutrophils. Although basal levels of Akt may be required for the brief life of neutrophils and their activation and function, further activations of the Akt expression is not able to extend neutrophils lifespan.

A2. Myeloid Derived Suppressor Cells

A2.i. General Information

The existence of a population of suppressive myeloid cells was firstly described more than two decades ago in patients suffering from cancer and mice bearing Lewis lung carcinoma but the importance of their role have only recently been elucidated. Various studies have shown that this population of cells with the suppressive activity contributes to the negative regulation of the immune system during various pathological conditions such as cancer or infectious diseases. [42]

MDSCs contribute to the negative regulation of the immune response in both innate and adaptive immunity. As it has been shown from previous studies, MDSCs not only have the ability to suppress various T-cell responses but also these cells can interact with macrophages and modulate the cytokine production from macrophages [50]. Initial studies of MDSCs were exclusively examining these cells in tumor-bearing mice or in patients suffering from various

types of cancer. However, in recent years, it is becoming clearer that MDSCs have an important role in different types of inflammation that are not directly associated with cancer.

The role of MDSCs as an important negative regulator of the immune response is extended beyond cancer and is observed in many pathological conditions. Although, as their name implies, these cells are mostly known for their immune-suppressive function, evidence are gathering pointing out that probably these cells contribute importantly in other aspects of the immune response. For example their role in the regulation of different $CD4^+$ T cell populations' numbers has recently been showed.

Recent publications elucidate the complex role and an interaction of MDSCs with the whole immune system as it seems that the interactions of MDSCs with $CD4^+$ Tcells is not one-directional and is also not limited to the immunosuppressive activity of MDSCs on Tcells. As mentioned in a review "it appears that T-cells can, in turn, regulate MDSC expansion and activity as well" [43] indicating that the interactions between these cell populations form a complex network that still has many unidentified interactions and pathways.

A2.ii. Characterization of cells and subpopulations of MDSCs

MDSCs are not terminally-differentiated cells and so they lack the expression of specific cell-surface molecules. These cells express markers also expressed by monocytes, dendritic cells or macrophages. In mice, MDSCs are characterized and isolated by the co-expression of GR-1, which is a characteristic antigen for all cells with myeloid origin, and also CD11b (also known as αM-integrin). The lack of specific surface molecules makes it difficult to isolate them separately from other cell populations that express common surface molecules such as neutrophils.

C57BL/6 mouse bone marrow under naïve conditions contains 20-30% of cells with this double positive phenotype ($GR1^+CD11b^+$) but in the periphery the percentage of these cells is significantly reduced to 2-4% in spleen under naïve condition and are also absent from the lymph nodes.

The population of MDSCs is a mixture of myeloid cells that have the morphology of granulocytes or monocytes. The morphological heterogeneity of the population implied the

possibility of more than one different subpopulations, a hypothesis which was proved using the appropriate molecules in mice models. Antibodies that are specific for GR1 have the ability to recognize two different epitopes, Ly6G and Ly6C that are produced from different genes. Using antibodies that bind specifically to one of these two epitopes, two different subsets of MDSCs have been characterized and isolated.

Granulocytic MDSCs that have an expression profile $CD11b^{+}Ly6G^{+}Ly6C^{low}$ (also known as polymorphonuclear MDSCs-PMN MDSCs) and also Monocytic MDSCs that have a profile $CD11b^{+}Ly6G^{-}Ly6C^{hi}$ [10, 14]. The phenotype of these subsets is well defined in mice and recently these cells were defined and characterized in cancer patients as well [63]. PMN-MDSCs consist of relatively immature and pathologically activated Neutrophils [64] whereas M-MDSCs are thought to be inflammatory monocytes activated under pathological conditions.

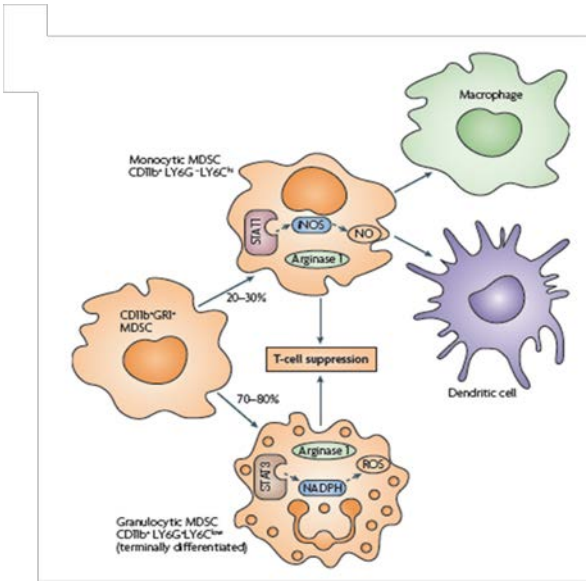

Figure 5: Subpopulations of MDSCs and differentiation possibilities [42]

These two subsets not only differ morphologically as evidence indicates that these two subsets may also have different functions and properties in autoimmune diseases and cancer. Data from ten different tumor models showed that although both subsets expanded, in most cases the expansion of granulocytic MDSCs (G-MDSCs) was much greater than that of the monocytic subset. Furthermore many studies show that the two subpopulations use different pathways to

suppress T-cell responses. In addition to the above, the finding that only monocytic MDSCs have the ability to differentiate in vitro into mature DCs and macrophages further supported the hypothesis that the two subsets have distinct roles at the immune response [51].

It was shown that, at least in cancer, M-MDSCs may have a central role in the development of immune suppressive myeloid cells. In the site of the tumor development, these cells differentiate to tumor-associated macrophages that are potent immunosuppressive cells and in the periphery these are able to differentiate into PMNs [65, 66].

A2.iii. Origin of myeloid derived suppressor cells

The normal process of myelopoiesis takes place in the bone marrow and is controlled by a complex network of soluble factors including various cytokines released (GM-CSF, M-CSF etc.) and intracellular expressed molecules (Notch). During this process, hematopoietic stem cells differentiate into common myeloid progenitor cells and then into IMCs. Normally, IMCs migrate to the periphery, into various tissues and organs, where they differentiate into macrophages, granulocytes and dendritic cells. However in various pathogenic conditions, such as trauma, sepsis, infection or tumor development, various factors are produced that lead to the accumulation of IMCs at these sites. These factors not only prevent IMCs from further differentiating but also induce their activation. These immature myeloid cells exhibit immunosuppressive properties and are therefore known as myeloid derived suppressor cells [42].

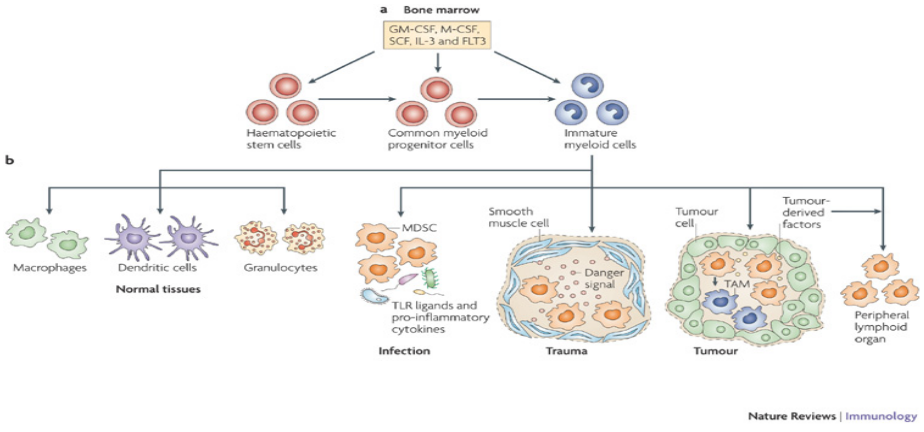

Figure 6: Process of myelopoiesis in Bone Marrow. Both the differentiation of IMCs under normal conditions and the generation of MDSCs because of various pathological conditions are presented [42].

A2.iv. Expansion and activation of MDSCs

Under normal conditions, a more or less stable population (concerning number of cells) of mature myeloid cells can be found at peripheral tissues and lymphoid organs. This situation is maintained via the release of progenitors and precursor cells from the bone marrow to the periphery, which is controlled by a complex network of factors produced [48]. In the setting of a pathogenic condition such as trauma, sepsis, tumor development, autoimmune disease, chronic infection etc. several different factors are produced to the site of inflammation that can influence both expansion and activation of myeloid cells and thus also affect the population of MDSCs.

These factors can be divided into:

1. Factors promoting myelopoiesis and inhibition of differentiation of immature myeloid cells (and thus leading to the expansion of MDSCs population).
2. Factors directly activating MDSCs at the site of inflammation.

A GM-CSF dependent pathway is used during an auto-immune de-myelinating disease in order to increase the release of $CD11b^{+}Gr1^{+}$ cells from the bone marrow to the periphery when needed. The importance of the GM-CSF factor was shown by studies using KO mice where after the immunization of the animals, no expansion of MDSCs population was observed [48]. Generally, many studies refer to GM-CSF as a factor un-dispensable to the expansion process of MDSCs & MDSC-dependent suppression of T cell responses [49].

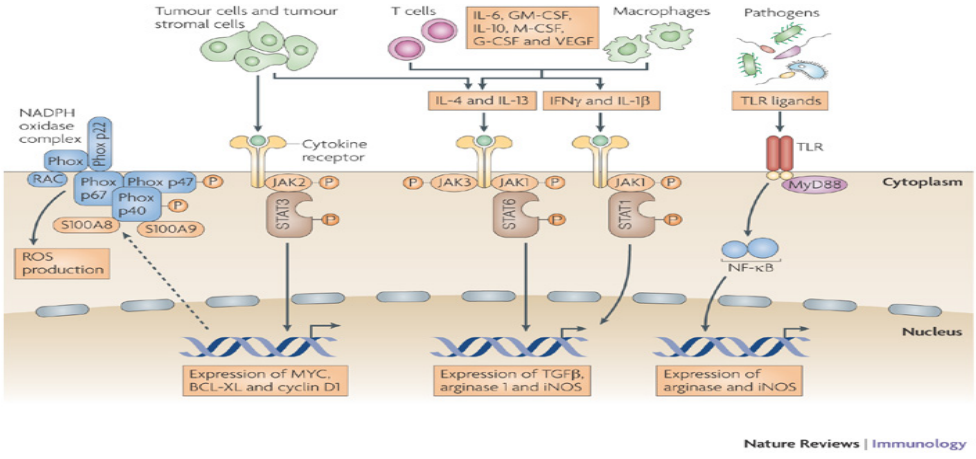

Figure 7: Pathways activated upon MDSCs stimulation by different types of stimuli []

A2.v. Cells of the immune system that interact with MDSCs

The role of MDSCs as an important regulator of the immune responses is extended beyond cancer and observed in many different pathological conditions such as autoimmune diseases, sepsis, trauma and others. Although the immunosuppressive ability is the most prominent feature of these cells, ample evidence points to their role in the regulation of different populations of T cells. The interaction of MDSCs with different populations of T cells is not one-directional and goes beyond the simple direct immunosuppressive activity of MDSCs on T cells. Importantly, it appears that T cells not only are regulated by MDSCs but also have the ability to regulate the MDSC expansion and activity as well [43].

- Interaction with $CD4^+$ T cells.
  - Many studies have shown a direct interaction between MDSCs and Th1 or Th2 cells. For example in a hepatitis model where immune mediated liver injury occurs, the accumulation of Th1 cells in the liver was associated with the accumulation of MDSCs and suppression of T cell proliferation. The rapid accumulation of MDSCs was abrogated when $CD4^+$ Tcells were depleted or unable to produce IFN-γ (TGF-b deficient mice) [68].

    In other mouse models, low dose of LPS promoted Th2 responses and allergic disease, while a high dose has been associated with suppression of allergic airway inflammation. The adoptive transfer of $CD11b^+GR1^+F4/80^+$ cells suppressed allergen-induced airway inflammation, suggesting that these cells may have regulatory functions in asthma [69]. Also in an adenoma carcinoma model, IL-4 expressing $CD4^+$ Th2 cells promoted the expansion of MDSCs and tumor associated macrophages.

  - It has been shown that MDSCs have the ability to induce Th17 polarization of naïve $CD4^+$ T cells. This interaction was cell contact independent and was due to the cytokines produced and secreted by MDSCs [70]. Experiments using gemcitabine to deplete

MDSCs showed reduction at the frequency of Th17cells in vivo furthermore showing the importance of the interaction between these two populations of cells [72].

This interaction is also two-directional as it was shown that IL-17 produced by Th17 cells could increase the suppressive ability of MDSCs through the up-regulation of Arg1, IDO and cyclo-oxygenase-2 while this phenomenon was abrogated at IL-17R deficient mice [71].

- The interaction of MDSCs and Tregs in various tumor models is well documented during the past years.

  MDSCs can regulate the numbers of the populations of Tregs in vivo. It has been shown that MDSCs have the ability to induce IL-10 and IFN-γ dependent $Foxp3^+$ $CD25^+$ Tregs in vivo that led to the suppression of the anti-tumor response in a mouse colon carcinoma model [78]. Also the in vivo depletion of MDSCs reduced the number of tumor infiltrating Tregs [80], furthermore showing that MDSCs can affect the numbers of Tregs populations. In order for the MDSCs to induce and activate Tregs, arginase1 and CD40 expression is very important [79, 81].

  Indications of interactions of MDSCs and Tregs in conditions other than cancer, now exist. In mice with allergic airway inflammation, M-MDSCs accumulated in lungs of these mice were able to downregulate T cell activation, recruit Tregs and dramatically decrease Ag-induced hyperresponsiveness [82]. Also the administration of MDSCs in mice with pancreatic islet transplants was associated with attenuation of $CD8^+$ T cells in grafts and a marked expansion of Tregs [83].

- <u>Interaction with $CD8^+$ T cells.</u>

  Apart from the above, MDSCs have been found to be able to interact with $CD8^+$ Tcells. MDSCs were found to be not only able to suppress CD8 T cell proliferation but also to induce their apoptosis in obese mice [84]. As studies conclude, the myeloid suppressor cells have the ability to nitrate CD8 and TCR molecules, leading to conformational changes of these membranic proteins via a peroxynitrate-mediated pathway that requires

cell to cell contact (PNT), thus leading to inhibition of Ag-specific $CD8^+$ or $CD4^+$ T cell responses [78, 85 and 86]. The interaction of MDSCs and $CD8^+$ T cells was also characterized in humans, were HIV and SIV infected patients were found to have a population of cells, similar to M-MDSCs, that expressed higher levels of STAT3 and iNOS2 and suppressed the expansion of $CD8^+$ T cells [2_33] proving the clinical significance of these interactions between MDSCs and other cell-populations of the immune system.

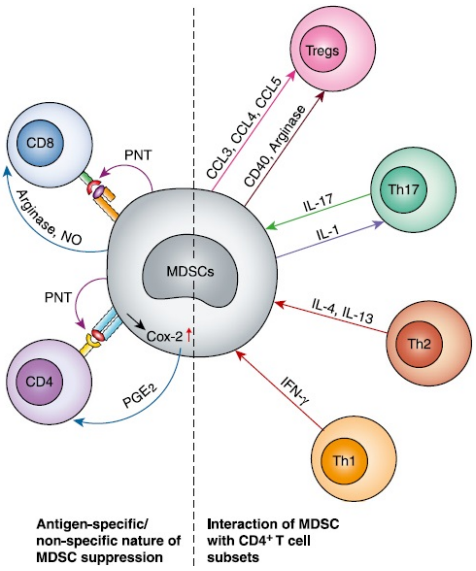

Figure 8: Interactions of MDSCs with other cell populations of the immune system [2]

A2.vi. Mechanisms used by MDSCs for suppression of the immune response

According to most studies, the immunosuppressive activities of MDSCs require cell to cell contact, which suggests that they function either through cell-surface receptors and/or through the release of short-lived soluble mediators in their microenvironment. Interestingly, the ligation of molecules (integrins) that are expressed on the surface of MDSCs was shown to contribute to an increased ROS production following the interaction of MDSCs with T cells [52].

MDSCs have the ability to suppress T cell function through a variety of different mechanisms, including high level of Arginase activity, as well as the production of high levels of nitric oxide (NO) and reactive oxygen species (ROS) [1,3,4]. The pathways mentioned are the

main mechanisms used for suppression. Recently, several alternative pathways for suppression have been proposed by different studies such as: secretion of TGFβ [73, 74], induction of regulatory T cells [75, 76 and 77], depletion of cysteine from the microenvironment of the immune cells [87] and others.

-Arginase1 and iNOS

From the initial studies, the suppressive ability and activity of MDSCs has been associated with the metabolism of L-arginine. This amino-acid serves as a substrate for two different enzymes in MDSCs: iNOS and arginase1. Via the iNOS pathway, NO (nitric oxide) is generated while the arginase1 converts l-arginine into urea and l-ornithine. MDSCs have been found to express high levels of both and a direct role for both of these enzymes in the inhibition of T cell responses has been established [44,58]. In MDSCs there is an increased activation of arginase1 that leads to increased l-arginine metabolism. Subsequently that leads to the depletion of the amino-acid from the microenvironment of the cells. The shortage of l-arginine inhibits T cell proliferation through many different pathways and mechanisms including decreasing the expression of CD3 chains and affecting the production of cell cycle regulators such as cyclins and cyclin-dependent kinases [59, 60]. On the other hand, NO is used in different pathways in order to suppress T cell expansion as it is involved in the inhibition of JAK3 and STAT5 function in T cells, the reduction of MHC class II expression and the induction of cell-apoptosis pathway at the T cells. The importance of the Arginase1 protein at the suppressive mechanism

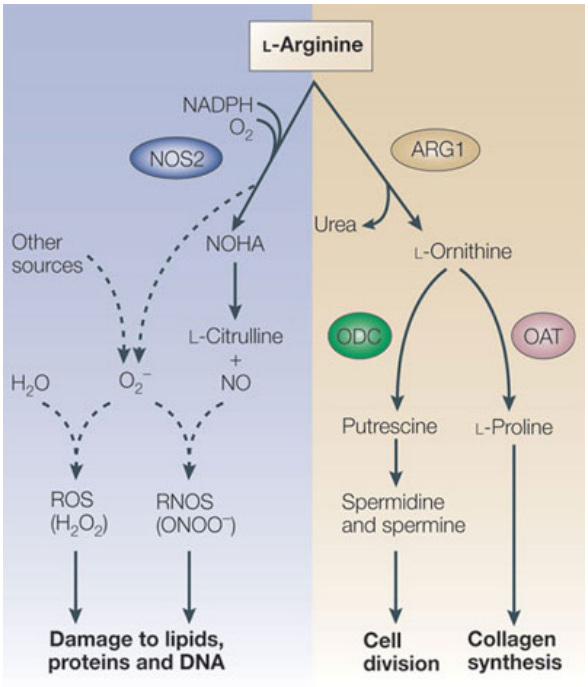

Figure 9: Suppressive pathways that involve Arginase1 and iNOS []

that MDSCs use was underlined as in Lupus-prone mice, MDSCs suppressive effect on T-cell proliferation was restored by an Arginase 1 inhibitor [67].

-ROS

Reactive oxygen species are another factor that has a critical role and contributes to the suppressive activity of MDSCs and many studies done on tumor-bearing mice and patients suffering from cancer have shown that ROS is one of the main characteristics of these cells [51, 52, 53 57, 61, 62]. The importance that ROS have at the suppressive activity of MDSCs was underlined by the results of studies done at patients suffering from cancer and tumor bearing mice, where the ablation of ROS production lead to the abrogation of suppressive ability that MDSCs had.

The involvement of the above molecules at the suppressive ability of MDSCs is not restricted to tumor models or neo-plasmatic pathological conditions. It has been shown that inflammation and various microbial products also have the ability to lead to the expansion and development of an MDSC population that produces ROS and NO after interacting with activated T cells [54]. The same phenomenon has been observed at auto-immune diseases such as EAE and also acute toxoplasmosis [55].

As mentioned before, various studies have shown few but basic differences between the two different subsets of MDSCs. Another difference can be noticed concerning their suppressive activity as studies have shown that the two different subpopulations of MDSCs might use different mechanisms in order to suppress T cell proliferation.

B) MATERIALS AND METHODS

B1. Mice Used

Female C57BL/6 mice (age 6-10 weeks) were obtained from the specific pathogen-free facility of the Institute of Molecular Biology and Biotechnology at Heraklion, Crete, Greece.

Female mice $Akt1^{-/-}$ and $Akt2^{-/-}$ (age 6-10 weeks old) that have a B6 genetic background, were kindly provided by Prof. C. Tsatsanis (Department of Clinical Chemistry, Medical School, University of Crete, Greece).

All procedures were in accordance to institutional guidelines and according to the Greek Federal Veterinary Office rules.

B2. Cell isolation from spleen and bone marrow

Reagents-Instruments

- Phosphate-buffered saline (PBS) (Gibco life technologies, Carlsbad, California, USA)
- Fetal Bovine Serum (FBS) (Gibco, Life Technologies, Carlsbad, California, USA)
- Cell strainer 40μm Nylon (BD Biosciences, NC, USA)
- Trypan Blue (Gibco, Life Technologies, Carlsbad, California, USA)
- Red Blood cell lysis buffer (R.B.C. lysis buffer) (0.155M NH_4Cl, 0.012M $KHCO_3$, 0.1M Na_2EDTA, final volume 1 Lt H_2O)
- Neubauer haemocytometer (Assistent, Germany)
- Light microscope (Nikon TMS, Tokyo, Japan)

Method

Mice were sacrificed in order to isolate the spleen and the bone marrow. Spleen tissues were homogenized and passed through cell strainers in order to remove any fat tissue remainings from the isolated cells, and resuspended into FBS/PBS 5%. Red blood cells lysis was done using 2 ml from R.B.C. lysis buffer for each sample. Samples were incubated for 2 minutes at room temperature and then washed using PBS. Bone marrow tissues were isolated using a syringe and were resuspended and washed using PBS. After the wash, all samples were centrifuged and the pellets were resuspended into 1ml volume of FBS/PBS 5%. After the isolation, the cells were countered using a Neubauer haemocytometer and after resuspending a small volume of the sample into Trypan blue (2μl of sample into 98μl of Trypan blue) and were counted by light microscopy.

B3. Mouse injections

Reagents-Instruments

- Myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}). MOG35–55 peptide [MEVGWYRSPFSRVVHLYRNGK] was synthesized by the Department of Chemistry, University of Patras, and was purified to 95% by HPLC
- Complete Freud's adjuvant (CFA) (Sigma Aldrich Inc, St.Louis, MO, USA)
- Phosphate-buffered saline (Gibco life technologies, Carlsbad, California, USA)

Method

To activate an auto-immune response, mice were injected with MOG-CFA. For the immunization, 100μg of MOG was given in each animal, diluted in a volume of CFA and an equal volume of PBS. MOG-CFA was emulsified with PBS in order to make a homogenized mix. All injections were done subcutaneously and to the lower left back of the animals. In order to have the immune response at its peak, any tissue isolation was performed 9 days post injection.

B4. Flow cytometry-Cell sorting

Reagents-Instruments

- Phosphate-buffered saline (PBS) (Gibco life technologies, Carlsbad, California, USA)
- Fetal Bovine Serum (FBS) (Gibco, Life Technologies, Carlsbad, California, USA)
- Anti-mouse antibodies
    - Fluorescein isothiocyanate (FITC)-conjugated CD11c, phycoerythrin (PE)-conjugated GR1, allophycocyanin (APC)-conjugated CD11b, fluorescein isothiocyanate (FITC)-conjugated Ly6C (Biolegend, Inc., San Diego, California, USA).
    - Phycoerythrin (PE)-conjugated Ly6G (BD biosciences, San Jose, California, USA).
- FACS Calibur (BD biosciences) flow cytometer
- MoFlo (Dako) high-speed cell sorter

Method

Cells isolated from Spleens and Bone marrows were stained using combinations of the antibodies mentioned before. Either CD11c-FITC, CD11b-APC & GR1-PE or CD11b-APC, Ly6G-PE & Ly6C-FITC. The staining was done using the appropriate volume of anti-mouse antibodies according to the manufacture's protocol and the combination of antibodies depended on the experimental settings. Samples were incubated for 20-30 minutes at 4^{o}C away from light, for the staining to be done. All antibodies were used with a concentration of 1/200. Afterwards, the cells were washed once using PBS and centrifuged for 10 minutes at 1400rpm at room temperature. The supernatants were removed and the cell pellets were resuspended into 1 ml of FBS/PBS 5%. Then the cells were subjected to flow cytometry or cell sorting depending on the experiment set up and using the appropriate facility located at the Medical School of Crete (Flow cytometer) or at the Institute of Molecular Biology and Biotechnology (IMBB) at Crete (cell sorter). The results were analyzed using the "FlowJo" software from Tree star Inc.

B5. Suppression assay and cytokine assessment

Reagents-Instruments

- Dulbecco's Modified Eagle Medium rich in glucose (DMEM-high glucose, Glutamax) (Gibco life technologies, Carlsbad, California, USA)
- Fetal Bovine Serum (FBS) (Gibco, Life Technologies, Carlsbad, California, USA)
- Penicillin G/Streptomycin Solution 100x (Biosera, Boussens, France)
- Myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}). MOG35–55 peptide [MEVGWYRSPFSRVVHLYRNGK] was synthesized by the Department of Chemistry, University of Patras, and was purified to 95% by HPLC
- Cell strainer 40μm Nylon (BD Biosciences, NC, USA)
- 96-well plate flat bottomed (Numc C8 White LockWell MaxiSorp) (Thermo Scientific, Waltham, Massachusetts, USA)

Method

FBS and penicillin/streptomycin solution was added in DMEM in order to make DMEM Complete medium (10%FBS). MDSCs were stained and isolated from the spleen of MOG-CFA injected B6, $Akt1^{-/-}$ and $Akt2^{-/-}$ mice, using the protocols described before (cell sorting-flow cytometry and cell isolation from Spleen). Total draining lymph node cells were taken, after smashing the lymph nodes of B6 mice, using a cell strainer to remove any tissue remainings. Co-cultures were done in DMEM-Complete medium with diluted MOG (working solution 20μg/ml). Co-culturing cells were incubated for 2 days at the tissue culture and two days later the supernatants were collected and stored at -80C until the time they were used.

B6. Enzyme Linked Immuno-Sorbent Assay (ELISA)

1) IFN-γ ELISA

Reagents and Instruments

- Mouse IFN-γ DuoSet ELISA development kit (containing Capture Antibody, Detection Antibody, Standard recombinant mouse IFN-γ, Detection enzyme (Streptavidin-HRP), 5X concentrated Assay Diluent (R and D Systems Inc., Minneapolis, USA).
- Phosphate-buffered saline (PBS) (Gibco life technologies, Carlsbad, California, USA).
- Wash buffer (0.05% Tween-20 in PBS).
- Block Buffer (1% BSA in PBS).
- Reagent Diluent (0.1% BSA, 0.05% Tween 20 in Tris-buffered Saline, pH = 7.2-7.4).
- Substrate solution (1:1 mixture of Color reagent A and color reagent B [tetramethylbenzine]) (R and D Systems Inc., Minneapolis, USA).
- Stop Solution (2N H_2SO_4) (R and D Systems Inc., Minneapolis, USA).
- 96-well plate (Numc C8 White LockWell MaxiSorp) (Thermo Scientific, Waltham, Massachusetts, USA)
- Model 680 Microplate Reader (Biorad, Hercules, California, USA)

Method

The assay was performed according to the kit's manufacturer guidelines and instructions. A 96-well plate was coated with 100µl/well of 1x Capture Antibody, diluted to the working concentration in PBS, and left overnight at room temperature. The plate was washed three times with Wash buffer and after that it was blocked by adding 200µl of Block buffer in every well and leaving the plate at room temperature for 2 hours. The plate was washed again three times using Wash Buffer and the samples as well as the standards (diluted according to manufacturer's guidelines) were added (100µl/well) and incubated for 2 hours at room temperature. The plate was washed again three times using Wash buffer and then Detection antibody was added

(100μl/well) and incubated for 2 hours at room temperature. Subsequently, three washes with Wash buffer were done followed by incubation with 100μl/well of Detection Enzyme (Streptavidin-HPRT) for 20 minutes at room temperature. The plate was once more washed with Wash buffer three times and 100μl/well of Substrate solution were added and incubated for 20 minutes at room temperature at a dark area to avoid direct light. The reaction was stopped by adding 50μl of Stop Solution to each well and the whole plate was read at 450nm and 540 nm at a microplate reader. Results were analyzed using Microsoft Excel and Graph Pad Prism 5 software.

The same protocol and method was used at the IL-17 ELISA as a kit from the same incorporation was used. Minimal differences that were needed, are fully described at the kit's guidelines.

2) IL-2 ELISA

Reagents and Instruments

- Mouse IL-2 Ready-SET-GO! Kit containing (Capture Antibody, Detection Antibody, Standard mouse IL-2 Recombinant Protein, Detection enzyme (Avidin-HRP), 5X concentrated Assay Diluent (eBioscience, Inc., San Diego, California, USA)
- Phosphate-buffered saline (PBS) (Gibco life technologies, Carlsbad, California, USA).
- Wash buffer (0.05% Tween-20 in PBS).
- Block Buffer (1% BSA in PBS).
- Reagent Diluent (0.1% BSA, 0.05% Tween 20 in Tris-buffered Saline, pH = 7.2-7.4).
- Substrate solution (1:1 mixture of Color reagent A and color reagent B [tetramethylbenzine])
- Stop Solution (2N H_2SO_4)
- 96-well plate (Numc C8 White LockWell MaxiSorp) (Thermo Scientific, Waltham, Massachusetts, USA)
- Model 680 Microplate Reader (Biorad, Hercules, California, USA)

Method

The assay was performed according to the kit's manufacturer guidelines and instructions. A 96-well plate was coated with 100μl/well of 1x Capture Antibody, diluted in coating buffer, and left overnight at 4C. The plate was washed five times with Wash buffer and after that it was blocked by adding 200μl of Assay diluent in every well and leaving the plate at room temperature for 1 hour. The plate was washed again five times using Wash Buffer and the samples as well as the standards (diluted according to manufacturer's guidelines) were added (100μl/well) and incubated for 2 hours at room temperature. The plate was washed again five times using Wash buffer and then Detection antibody, diluted in assay diluent, was added (100μl/well) and incubated for 1 hour at room temperature. Subsequently, five washes with Wash buffer were done followed by incubation with 100μl/well of Detection Enzyme (Avidin-HPRT) for 30 minutes at room temperature. The plate was once more washed with Wash buffer seven times and 100μl/well of Substrate solution were added and incubated for 15 minutes at room temperature at a dark area to avoid direct light. The reaction was stopped by adding 50μl of Stop Solution to each well and the whole plate was read at 450nm and 570 nm at a microplate reader. Results were analyzed using Microsoft Excel and Graph Pad Prism 5 software.

B7. RNA isolation from MDSCs

Reagents and Instruments

1. PureLink RNA Mini kit (containing Spin Cartridges with collection tubes, Lysis buffer, Wash buffer I , Wash buffer II, RNase-free water) (Ambion, Life Tehnoles Carlsbad, California, USA)
2. 70% Ethanol, RNase –free
3. Nanodrop 2000 Spectrophotometer (Thermo Scientific Waltham, Massachussetts, USA)

Method

Isolated MDSCs from the animals, were centrifuged at 1400rpm for 10 minutes at room temperature and the pellet was resuspended and homogenized in the appropriate volume of Lysis buffer + 1% b-mercaptoethanol as a reducing agent, according to the protocol provided by the manufacturer. After being transferred into the spin cartridges and added equal volume of 70% ethanol (RNase-free) in order to provide suitable binding conditions, samples were centrifuged at 12.000g for 15 seconds at room temperature. After that, the spin cartridges were washed three times (once with Wash buffer I and two times with Wash buffer II that contains ethanol) followed by a centrifugation for 15 seconds at 12.000g at room temperature after every wash. After the last wash, the spin cartridges were centrifuged for 2 minutes at room temperature at 12.000g in order to make sure the membrane with the bound RNA is as dry as possible. Water was added to the center the membrane of the cartridges and left incubate for 2 minutes at room temperature and followed by a centrifugation at 12.000g for 2 minutes at room temperature so as for the RNA to be eluted. The concentration of the RNA samples was quantified using Nanodrop 2000 Spectrophotometer according to the manufacturer's protocol and the samples were stored at -80C.

B8. Reverse Transcription- Real Time quantitative Polymerase Chain Reaction

Reagents and Instruments

1. PrimeScript 1st strand cDNA Synthesis Kit [containing PrimeScript RTase (200U/μl), 5x PrimeScript Buffer, RNase Inhibitor (40U/ μl), dNTP Mixture (10mM), Oligo dT Primer, RNase-free water] (Takara, Otsu, Shiga, Japan)
2. KAPA SYBR FAST Universal 2x qPCR Master Mix (Kapa Biosystems Inc. Woburn, MA, USA)
3. PCR machine (Veriti 96 well thermal cycler)(Applied Biosystems, Life Technologies, Carlsbad, California, USA)

4. RT-PCR machine (CFX Connect, Real-Time system)(Biorad, Hercules, California, USA)

Method

CDNA was prepared from isolated RNA using PrimeScript 1st strand cDNA Synthesis Kit according to manufacturer's protocol. The isolated RNA was used as a template for every reaction and was mixed initially with appropriate volume of Oligo-dT primers and mixture of dNTPs. After incubation for 5 minutes at 65o C at a Veriti 96 well thermal cycler, the samples were cooled immediately on ice. Next, appropriate volumes of 5x PrimeScript buffer, RNase inhibitor, PrimeScript Reverse Transcriptase and RNase-free water were added and the samples were incubated at 50o C for 45 minutes and at 95o C for 5 minutes at a Veriti 96 well thermal cycler, followed by cooling on ice. cDNA was stored at -20o C.

PCR amplification of the resulting cDNA samples was performed using appropriate volumes of KAPA SYBR® FAST Universal 2x qPCR Master Mix and specific for each gene primers at a CFX Connect™, Real-Time System.

Primers used (mouse)

All primers used, were designed to anneal at a intron-exon junction in order to exclude the possibility of polymerizing RNA molecules and lead to a biased result.

B-actin forward: 5'- GGCTGTATTCCCCTCCATCG-3'
B-actin reverse: 5'- CCAGTTGGTAACAATGCCATGT-3'

Arginase 1 forward: 5'-CAGAAGAATGGAAGAGTCAG-3'
Arginase 1 reverse: 5'- CAGATATGCAGGGAGTCACC-3'

CEBPβ forward: 5'- ACGGGACTGACGCAACACAC-3'
CEBPβ reverse: 5'-CCGCAGGAACATCTTTAAG-3'

iNOS forward: 5’-CAAGAGTCGGGTTGTTATGTTCT-3’

iNOS reverse: 5’-GTGGACGGGTCGATGTCAC-3’

B9. May Grunwald-Giemsa staining

Reagents and Instruments

1. Giemsa stain solution
2. May-Grunwald stain solution
3. Slides
4. Coverslips
5. Xylene
6. Cytospin machine
7. Microscope

Method

Cells were centrifuged using the appropriate centrifugation machine and equipment in order to be katakrhminontai on the slide. Slides were left for 5-10 minutes at room temperature in order to dry. The cells were stained using May-Grunwald working solution for 3 minutes away from direct contact with light. After being washed with tap water, slides were also stained using Giemsa staining (working solution 1/10 from stock solution diluted in tap water) for 10 minutes and then washed again with tap water. Slides were dried in upright position at room temperature and then mounted with coverslips with xylene for storing. After all of the above, slides are ready to be examined using the appropriate microscope.

C) RESULTS

C1. Differences at the numbers of MDSCs in the bone marrow and periphery of Akt KO compared to control mice

To examine possible differences in the numbers of myeloid derived suppressor cells (MDSCSs) between Akt KO mice and control mice (C57BL/6), animals were used from each genotype: Akt1 KO, Akt2 KO and C57BL/6. All animals were under naïve conditions, in order to examine possible differences at the population of MDSCs at a steady state without being triggered.

Spleen and Bone marrow tissues were isolated from each animal. This selection of tissues was done to examine differences at the bone marrow, where MDSCs are generated, and at the periphery (spleen) where they migrate when needed upon pathological conditions (42, 92). All samples were stained using anti-GR1, anti-CD11b and anti-CD11c antibodies and double positive cells ($CD11c^{-}GR1^{+}CD11b^{+}$) were gated. CD11c antibody was used in order to exclude dendritic cells and other myeloid-derived cells.

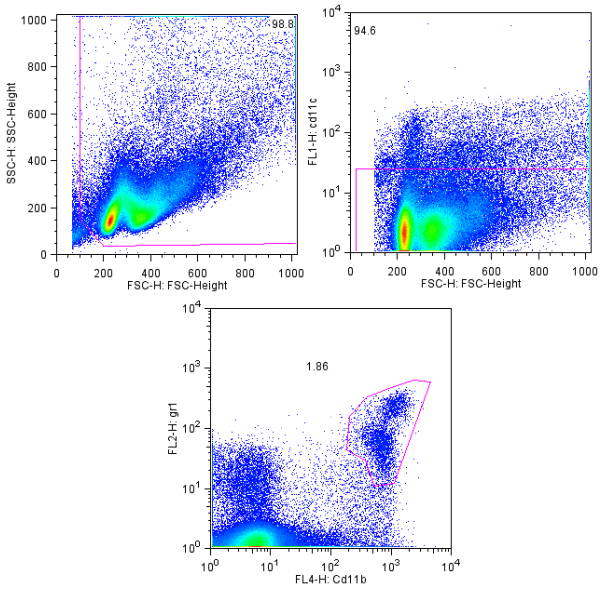

Figure 10: Spleen from C57BL/6 mouse. The gates used during this study are presented. First gating is to exclude background and dead cells. The second gate excludes only CD11c+ cells and the last gate it to select double positive $GR1^{+}CD11b^{+}$ cells. The desired populations is well separated as shown in figure.

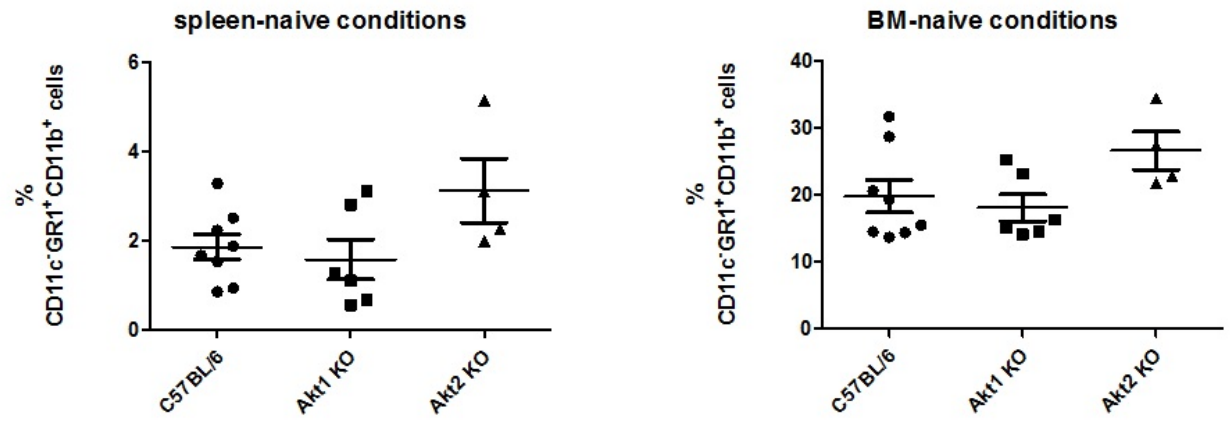

Figure 11: Percentages of $CD11c^{-}GR1^{+}CD11b^{+}$ cells at the bone marrow and the periphery of naive animals

As it can be noticed on figure 11, no significant differences were detected at both bone marrow and spleen samples between different strains of mice. In mice lacking Akt1 protein, MDSCs are slightly reduced while in Akt2 KO mice, the same cell population was found to be slightly increased both in the bone marrow and in the periphery, compared to the control B6 mice.

The above differences were not found statistically significant ($p>>0.05$) using a t-test analysis at both types of samples, spleen and bone marrow. The analysis was done comparing B6 to Akt1 KO and B6 to Akt2 KO. Although found to be non-significant, more animals should be used in order to have a more stable and solid result and to be able to say that there are or not any changes in numbers.

C2. Differences at the numbers of MDSCs of Akt KO mice after triggering an immune response

MDSCs are a population of suppressive cells that upon inflammation and initiation of the immune response are activated, migrate to the site of inflammation and proliferate in order to suppress that response (42, 49 and 92). In order to examine for possible differences in the process of activation and proliferation of MDSCs in the absence of Akt proteins, mice were injected subcutaneously with CFA (Complete Freund's Adjuvant) in order to trigger an immune

response. Nine days after injection, bone marrow and spleen tissues were isolated from the animals used. All samples were stained using anti-GR1, anti-CD11b and anti-CD11c antibodies and double positive cells ($CD11c^{-}GR1^{+}CD11b^{+}$) were gated as before. The cells that were isolated had a phenotype $GR1^{+}CD11b^{+}CD11c^{-}$

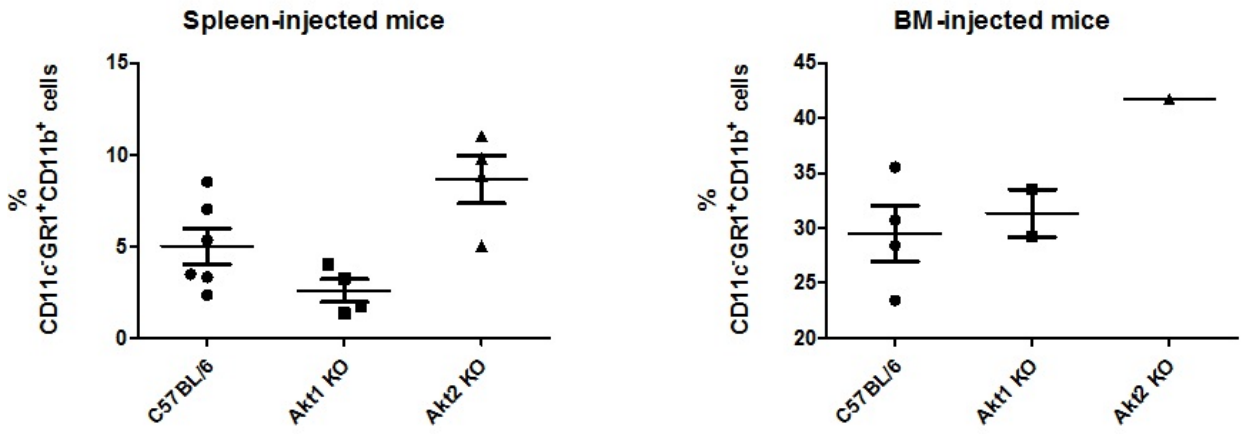

Figure 12: Percentages of $CD11c^{-}GR1^{+}CD11b^{+}$ cells at the bone marrow and the periphery of injected animals.

Comparing Akt1KO mice to C57BL/6 animals, no significant differences were noticed at the bone marrow samples as in the injected animals, like the naïve ones, only show slight differences at the population of MDSCs in these samples. On the other hand spleen samples seem to have more obvious differences at the numbers of MDSCs. In the Akt1 KO animals, the population of MDSCs seems to be less induced and less increased compared to the same population in C57BL/6 mice as a result of the injection. In each sample, bone marrow or spleen, t-test analysis was performed for each KO strain with B6 control type. As before with the naïve animals, every difference observed at the percentages of cells was found to be not-statistically significant ($p>0.05$) but again, this could be explained by the fact that only few animals were used during this study and an increase at the numbers of animals used could alter this result.

As the percentages of the population are similar in the Bone marrow samples but differ at the periphery of the mice, it could be assumed that Akt1 plays an important role concerning the migration of MDSCs from the bone marrow to the periphery or that the differentiation of MDSCs into other myeloid cells is increased or accelerated in the absence of Akt1.

Also, the comparison between Akt2KO and C57BL/6 mice, revealed a trend at the differentiation of the percentages of the cells at both the bone marrow and the spleen samples. The percentages of MDSCs are increased in Akt2 KO mice, indicating that Akt2 may have an even more significant role compared to Akt1 role, at the whole process of maturation and migration of MDSCs from the bone marrow to the periphery.

C3. Morphology of MDSCs

In order to better understand differences between MDSCs population at the different strains of mice that were used, an assay that would allow us to examine the morphology of these cells was crucial. In order to examine the probable differences between the Akt KO derived MDSCs and the control MDSCs, Giemsa staining was used.

As there was a problem with Akt2 KO strain and no animals were available, Akt1KO and C57BL/6 mice were used at this assay. G-MDSCs and M-MDSCs were isolated from the spleen tissue of these animals using the appropriate staining for each of these populations:

- $CD11b^{+}Ly6G^{+}Ly6C^{int}$ phenotype for the Granulocytic MDSCs
- $CD11b^{+}Ly6G^{-}Ly6C^{+}$ phenotype for the Monocytic MDSCs

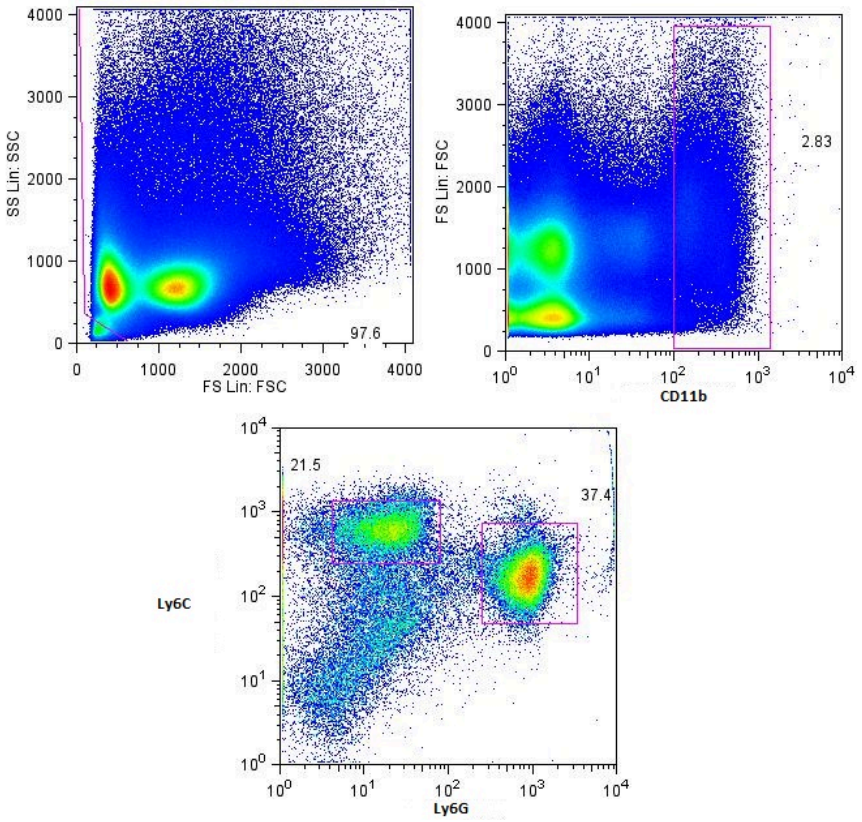

Figure 13: Spleen from C57BL/6 mouse. The gates used during this experiment are presented. First gating is to exclude background and dead cells. The second gate includes only CD11b^{high} cells and the last gate it to sort the two different subsets of MDSCs.

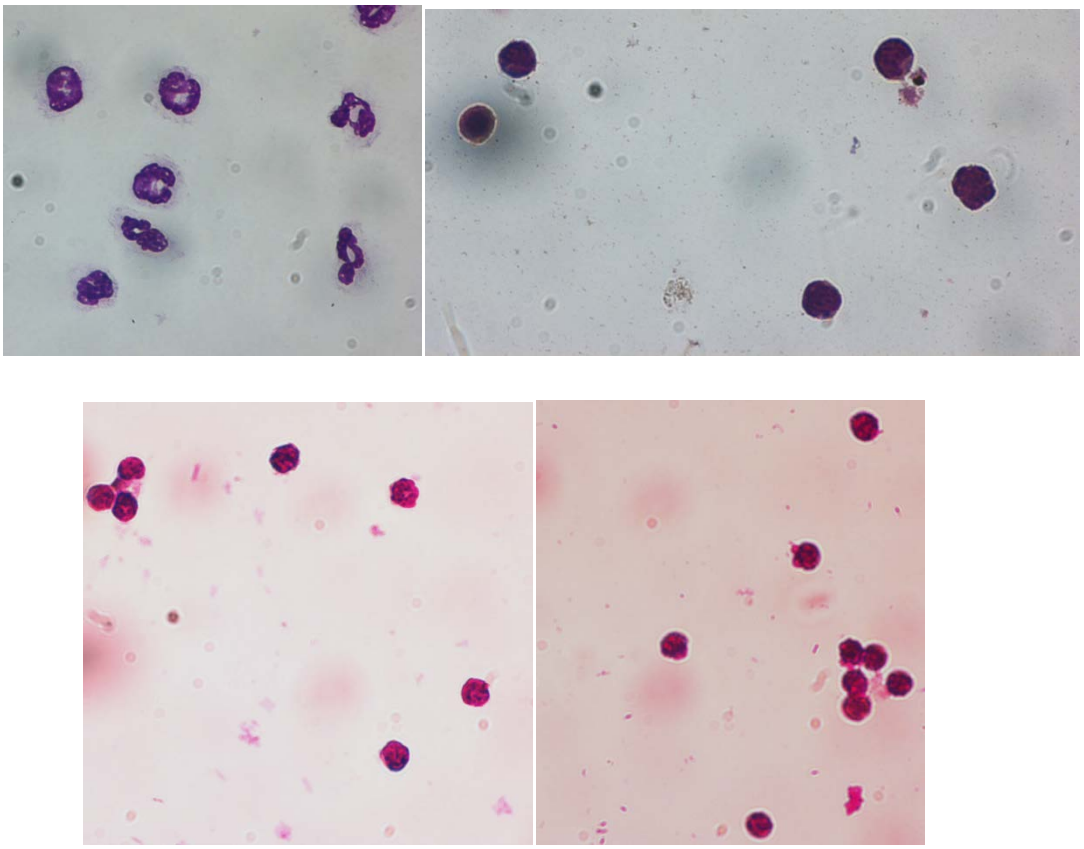

Figure 14: Top row: MDSCs isolated from the spleen of C57BL/6 mice. On the left part G-MDSCs and on the right M-MDSCs. Bottom row: MDSCs isolated from the spleen of Akt1 KO mice. On the left part G-MDSCs and on the right M-MDSCs.

Comparing the pictures taken with confocal microscopy (presented at Figure 14) of the MDSCs subpopulations from different strains interesting differences were found. The granulocytic MDSCs clearly differ from the monocytic subset of MDSCs when looking at the cells that came from the C57BL/6 mice. On the other hand comparing the different subsets of MDSCs isolated from Akt1KO mice, was more difficult. That is because the G-MDSCs and the M-MDSCs from Akt1 KO mice seem to have very few, if any, morphological differences according to the Giemsa staining result.

This observations generated lots of questions on whether these cells, in the absence of Akt proteins, apart from their numbers and morphology face an alteration concerning their function and their suppressive ability.

C4. Suppression assays revealed pro-inflammatory cytokine production from MDSCs

As Akt proteins are very important signaling molecules that are involved in many different pathways, a suppression assay was done, in order to check the suppressive ability of the MDSC population in the absence of the Akt proteins. Animals were used from each strain that we use (Akt1KO, Akt2KO and C57BL/6) and all mice were injected with MOG/CFA subcutaneously in order to initiate an antigen-specific immune response. Nine days post injection; spleens were isolated from all animals and also draining inguinal lymph nodes from the injected control C57BL/6 mice. Spleen samples were stained using anti-GR1, anti-CD11b and anti-CD11c antibodies and isolated cells with $CD11b^{+}GR1^{+}CD11c^{-}$ phenotype.

The lymph nodes were smashed and total lymph node cells were isolated and co-cultured with isolated MDSCs in the presence of MOG peptide in the medium, in order to induce and activate T cells and examine the suppressive ability of the myeloid cells from all strains. Two different ratios of LN cells/ MDSCs were used at the specific set up: 10 to 1 and 5 to 1. As controls we used total lymph node cells in medium (DMEM complete) with or without MOG in order to see the production of the cytokines in triggered or un-triggered conditions respectively without the presence of suppressive cells. Two days after co-culturing the cells, supernatants were collected and used in an ELISA assay to detect the presence of IL2, IL-17 and IFN-γ or not, which are molecules implicated in the active immune response (42, 43, 47, and 49).

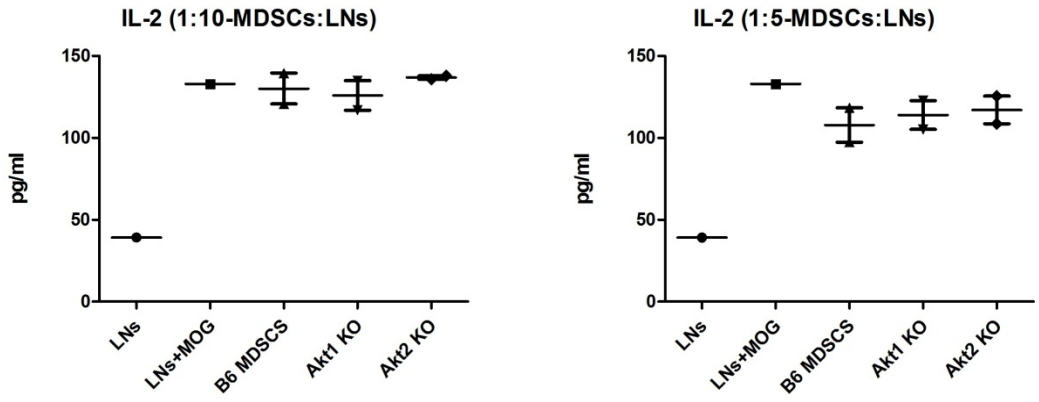

Figure 15: Co-cultured total lymph node cells with MDSCs from B6, Akt1 KO or Akt2 KO mice. ELISA result in order to detect IL-2 in the supernatant of the co-culturing. On the left graph, the result from the wells with a ratio of 1:10 MDSCs:LNs and right graph with a ratio of 1:5

MDSCs:LN cells. The presence of MDSCs (B6, akt1KO or akt2KO does not seem to have a significant result upon the production of IL-2 by triggered total lymph node cells.

According to the results that are presented to the graph above, concerning IL-2, the presence of MDSCs doesn't seem to clearly suppress the cytokine production from the activated LN cells. The same result can be seen either in presence or absence of Akt proteins as the graphs shows and independently of the MDSCs to LN cells ratio (graph3).

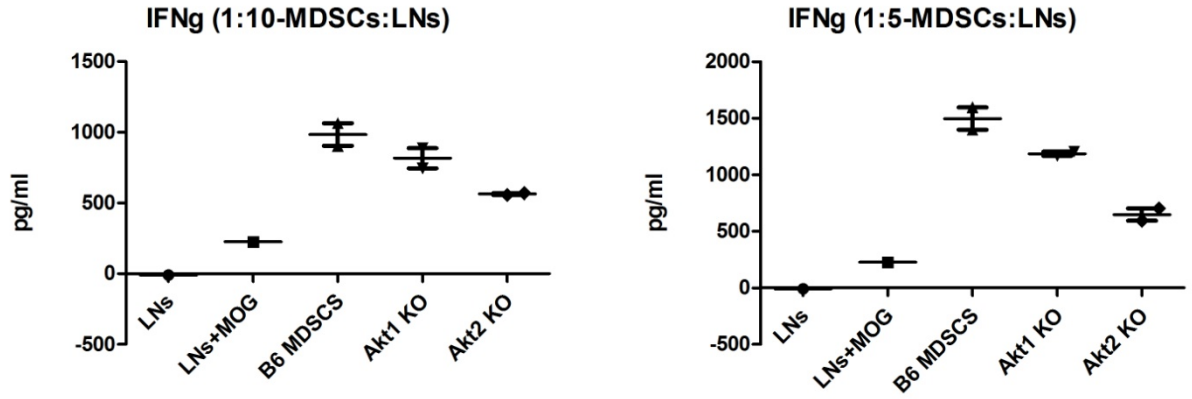

Figure 16: Co-cultured total lymph node cells with MDSCs from B6, Akt1 KO or Akt2 KO mice. ELISA result in order to detect IFN-γ in the supernatant of the co-culturing. On the left graph, the result from the wells with a ratio of 1:10 MDSCs:LN and right graph with a ratio of 1:5 MDSCs:LN cells.

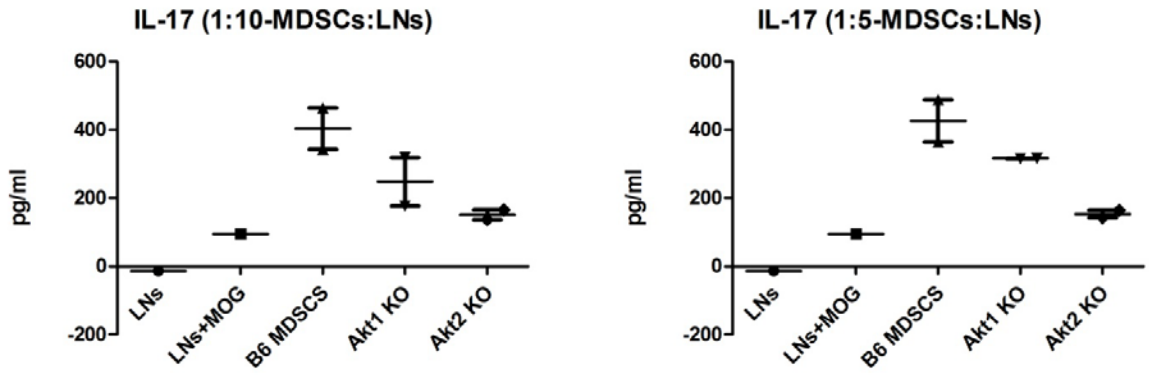

Figure 17: Co-cultured total lymph node cells with MDSCs from B6, Akt1 KO or Akt2 KO mice. ELISA result in order to detect IL-17 in the supernatant of the co-culturing. On the left graph, the result from the wells with a ratio of 1:10 MDSCs:LNs and right graph with a ratio of 1:5 MDSCs:LN cells.

On the other hand, concerning the production of the IL-17 and IFN-γ cytokines, the assay used gave an interesting result. According to our experiment the co-culturing of total MDSCs with total LNs in the wells not only suppresses the expression of the pro-inflammatory cytokines from the LN cells, but seems to enhance it (figures 16 and 17). Also, the absence of Akt proteins in the knock-out mice seem to alter the suppressive ability and function of MDSCs as the MDSCs sorted from KO animals seem to be more suppressive than total B6 MDSCs or less activatory than B6 MDSCs.

C5. Differences in the expression of suppressive molecules

Based on the results of the suppression assay that it was done, it was shown that there are differences at the suppressive ability of MDSCs in the absence or not of Akt proteins. In order to better understand and describe the molecular pathways that may be affected by the absent Akt proteins and elucidate the way that these pathways affect the whole suppressive ability of the myeloid-derived suppressor cells, real time polymerase chain reaction was used.

As it was mentioned in the introduction, molecules characteristic of the suppressive function of the myeloid derived suppressor cells are many but the most critical ones are Arginase1and iNOS (59, 60 and 67). Another molecule that its expression levels were checked

was CEBPβ which is a basic transcription factor found by previous studies to play an important role in the maturation and polarization of macrophages as well as in the activation of MDSCs (94, 95) . As macrophages are myeloid derived cells and CEBPβ was found to be critical in order to have suppressive or pro-inflammatory macrophages, it seemed more than appropriate to also check for alterations of its expression.

Control and Akt1 KO mice were used under naïve conditions. Total MDSCs were isolated from both spleen tissue and bone marrow from these mice. Also, the two distinct subsets of MDSCs (Granulocytic and Monocytic) were isolated from the bone marrow of the mice used, giving the ability to check each subset separately for the expression of the molecules mentioned above.

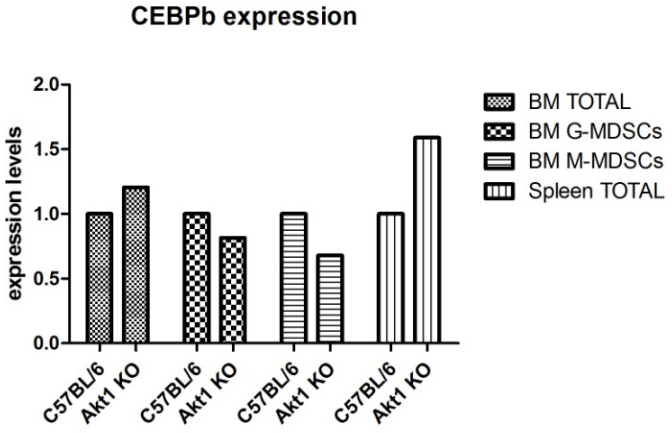

Figure 18: Q-pcr showing differences at the CEBPβ expression between B6 MDSCs and the Akt1KO MDSCs. Total MDSCs from bone marrow and spleen as well as separately G and M MDSCs from bone marrow

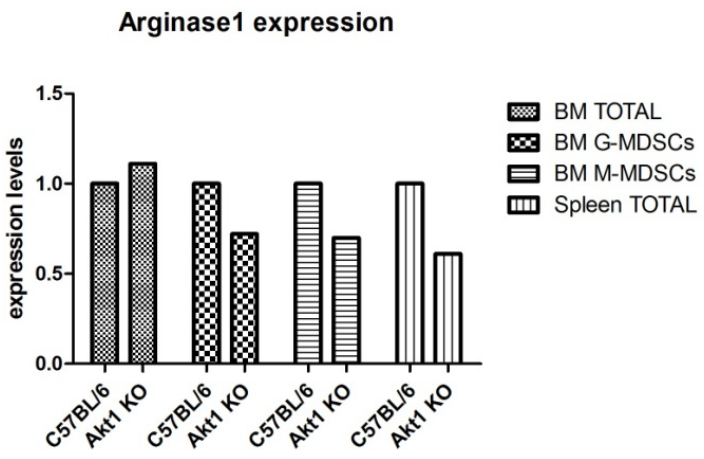

Figure 19: Q-pcr showing expression levels of arginase1 at B6 and Akt1KO MDSCs. Total MDSCs from bone marrow and spleen of the animals as well as distinct subsets G and M MDSCs from bone marrow of mice.

Based on the results of the quantitative pcr, shown on figures 18 and 19 above, the expression of the transcription factor CEBPβ at total MDSCs from the bone marrow of Akt1 KO mice is slightly up-regulated compared to the B6 population's expression. The interesting result is that when the two subsets of MDSCs are separated and checked for the expression of the same molecule, both of the subsets seems to have lower expression than the B6 populations. The same expression "pattern" can be seen when looking at the expression levels of the Arginase1 molecule at the same populations.

On the other hand, the absence of the Akt1 protein from mice seem to up-regulate the expression of CEBPβ at the population of MDSCs in the periphery (spleen) but also down-regulates the expression of the suppressive enzyme Arginase1 based on our results.

In order to better elucidate whether the suppressive function of the MDSCs isolated is altered, qpcr to check for iNOS expression was performed. Based on the result of this pcr (data not shown) all samples had very low expression of iNOS and so no comparison or hypothesis can be done.

D) DISCUSSION

During the past years many studies have shown that Akt protein kinases may play a significant role during the maturation and polarization of various myeloid cell populations such as macrophages and neutrophils (14, 22, 26, 37, 88-91). Interestingly, in the literature, there are no studies examining the relationship between Akt proteins activation or inhibition and the myeloid derived suppressor cells population. These cells are a heterogeneous population of myeloid cells that contribute to the negative regulation of the immune response by directly inhibiting the response or by activating other suppressive populations of cells (42, 43, 49 and 50). Because of their important role in the immune response and as Akt have been shown to greatly affect myeloid cells, examining the function and suppressive ability of MDSCs in the absence or inhibition of Akt proteins could help clarify and further elucidate the importance of function of these cells.

As there was no study available about the MDSCs population in the absence of Akt proteins, the first thing required was to examine the population numbers in the different mice strains used for this study: C57BL/6 (control), Akt1 knock out and Akt2 knock out. The population percentages were countered at both naïve conditions and after triggering an immune response, in the bone marrow where they generate and at the periphery (spleen). According to the results, under naïve conditions in both bone marrow and spleen, the absence of Akt1 protein seems to lead to a minimal decrease of the population of MDSCs while the absence of Akt2 kinase leads to an increased population of MDSCs which is more obvious at the periphery. When animals were triggered for an immune response, checking the numbers of MDSCs lead to similar observations with minimal differences at the bone marrow but with greater decrease for Akt1 KO MDSCs in the periphery compared to B6 and with a great increase in the numbers of Akt2 KO MDSCs in both bone marrow and periphery. When the differences were analyzed using a t-test no statistical significance was found for both Akt1 and Akt2 compared to B6 mice, a result that should be checked again by using a greater number of animals in order to be more certain as the number of mice used is not sufficient to make solid hypotheses.

Because of the differences between the percentages of the cell population between the different strains of mice, an assay that would allow examining morphological differences between MDSCs isolated from these mice was done. Cells from both subsets of MDSCs, G and

M, were isolated from B6 control mice and Akt1 KO mice and were stained using a May-Grunwald Giemsa staining. After examining the photographs taken, significant differences between B6 and Akt1 KO mice MDSCs phenotype were found. The granulocytic MDSCs clearly differ from the monocytic subset of MDSCs when looking at the cells isolated from C57BL/6 mice. On the other hand comparing the different subsets of MDSCs isolated from Akt1KO mice, was more difficult. That is because the G-MDSCs and the M-MDSCs from Akt1 KO mice seem to have very few, if any, morphological differences according to the Giemsa staining result. Based on the morphological differences and similarities that this assay revealed, it could be assumed that Akt1 has an important role in the whole maturation process of MDSCs and so could affect the suppressive ability of these cells. Thus in the absence of it, these cells either cannot be differentiated as well as in B6 control mice or they lack their suppressive ability and no alterations occur at their phenotype.

In order to check the above hypothesis, the suppressive ability of the MDSCs from control and knock-out strains of mice, a suppressive assay was performed by taking the supernatants from co-cultures of activated whole lymph node cells(from B6 mice) and MDSCs from all different strains used at this study. In order to measure the suppressive ability of these cells, IL2, IL17 and IFN-γ produced by co-culturing were measured using ELISA assay. All three cytokines mentioned before are characteristic pro-inflammatory cytokines that it was expected to be produced by activated T cells that can be found in lymph nodes. Adding MDSCs was expected to reduce the levels of these cytokines as a result of T-cell suppression by MDSCs and thus a measure of suppressive ability of these cells.

The unexpected result was that when MDSCs were added into the activated total lymph nodes cells not only did they not suppressed T cells leading to the reduction of cytokines' levels, but activated them furthermore, leading to increased levels of the cytokines in the co-culture supernatants. So it was shown that the adding of MDSCs lead to an increase in the levels of cytokines, a phenomenon that the absence of Akt proteins (Akt1 or Akt2) partially reduced. A solid hypothesis cannot be done by only this assay, as it cannot be defined whether the Akt KO MDSCs are **more suppressive or less activatory** than the B6 control MDSCs. Although this result was surprising as it showed an absence of a suppressive role for MDSCs it was supported by the findings of another group that were able to show that one of the subpopulations of

MDSCs has the ability to efficiently enhance the differentiation of naïve $CD4^+$ T cell precursors into T_{h17} cells. That led to a significantly increased total number of T_{h17} cells in the culture and thus increased indirectly the levels of IL-17 produced (72).

To understand the previous result, and better examine the suppressive ability of MDSCs in KO mice compared to control animals, used naïve animals and RNA was isolated from Total MDSCs (coming from spleen or bone marrow) and subsets (coming from bone marrow) and used in order to generate cDNA which was used with real time qpcr. Using the quantitative pcr the expression levels of Arginase1, iNOS and CEBPβ were compared between different mice. The two first molecules examined are the characteristic molecules that give MDSCs their suppressive ability and the CEBPβ is a basic transcription factor found by previous studies to play an important role in the maturation and polarization of macrophages. As macrophages are myeloid derived cells and CEBPβ was found to be critical in order to have suppressive or pro-inflammatory macrophages examining the expression levels of this molecule allows making hypotheses on whether Akt proteins truly affect maturation or polarization of MDSCs.

According to the results of this experiment, CEBPβ expression is higher in Akt1 KO mice total MDSCs either spleen-derived or BM-derived, but seems to be less expressed when each subpopulations was examined alone and compared to the control one. On the other hand, Akt1 KO MDSCs express lower levels of Arginase1 when examined as subpopulations or total population from spleen, but seem to have same expression levels when examined as total MDSCs in the bone marrow of animals. Also, when iNOS levels were checked using qpcr, it was not detectable in any of these populations examined before, showing that maybe the levels of iNOS used is beneath the minimum detection limit of the method or it is a pathway not used by these non-activated MDSCs.

Taken all of the above under consideration, it seems that Akt1 KO mice have reduced numbers of MDSCs in either naïve condition or after triggering an immune response and these MDSCs seem to be less suppressive than the control ones. Also these MDSCs when examined in total population and under naïve conditions seem to have increased expression of the transcription factor CEBPβ which has been shown to play a role in the maturation of the myeloid-derived macrophages. Also the different subsets of MDSCs coming from Akt1KO mice have very similar phenotype and cannot be easily distinguished as the control subpopulations of

MDSCs can. From these result it can be assumed that in the absence of Akt1 protein, MDSCs maybe differentiate more than in the control mice, losing their MDSCs “phenotype”, are less suppressive and so express lower levels of Arginase 1. Also this could explain why these cells don’t have the same phenotype as the control MDSCs and also why it was observed that in Akt1KO mice the numbers of MDSCs are reduced in both bone marrow and the periphery. The reduced numbers of Akt1 could also imply that the apoptosis pathways is altered and affected as it has been shown from previous studies that akt1 null cells show increased apoptosis in vitro compared to control cells []. Concerning Akt2 KO MDSCs no hypothesis can be done as the data available are very few.

E) FUTURE PERSPECTIVES-EXPERIMENTS TO BE DONE

As mentioned before, this study is a first approach to examine whether the absence of Akt proteins alters the numbers or the suppressive ability and function of MDSCs in both naïve and after triggering an immune response conditions. Based on data, many questions are generated and many future experiments can be planned in order to have most of these answered and also better elucidate the importance of Akt proteins in the normal function and physiology of MDSCs.

Firstly, in order to better understand the similarities that were found between G and M MDSCs in Akt1, it would be interesting not only to see the morphology of Akt2 subsets of MDSCs but also to examine the ratios of G and M MDSCs in Akt-KO mice compared to B6 control mice in both naïve and immunized mice. This experiment will give the opportunity to make more safe assumptions concerning maturation and differentiation process of MDSCs before and after stimulation and how it is affected by the absence of Akt proteins.

As mentioned at the result section, the suppression assay using MDSCs from B6 and Akt KO mice revealed that these cells don't really suppress the production of pro-inflammatory cytokines but seem to activate it. In order to understand if one of the two subsets of MDSCs is responsible for this result or the interaction of both is needed, a suppression assay using separated the two subsets of MDSCs from B6 and Akt-KO mice is needed to understand the suppressive ability or not of these cells. Results from another study indicate the suppressive ability of G-MDSCs upon T-cell activation and proliferation in mice with EAE, when these populations of cells are co-cultured [93] furthermore indicating the need to do this experiment in order to understand the interaction between the two subpopulations of MDSCs.

Also, it would be really useful to examine the expression of Arginase1, iNOS and CEBPβ in MDSCs that would be isolated from immunized mice, in order to examine whether the activation of the immune response would reveal a bigger difference or a different expression profile of MDSCs isolated from Akt-KO mice compared to B6 control mice.

Finally as mentioned already several times during this thesis, the small number of animals being available for experiments, was a restraining factor and if available, it would be very useful to make more experiments, have more data probably and thus make more stable and solid hypothesis or conclusions.

F) REFERENCES

1. E. M. Sale and G. J. Sale. Protein Kinase B: signaling roles and therapeutic targeting. *Cell. Mol. Life Sci*. 65,113-127.
2. Lana Bozulic and Brian A Hemmings. PIKKing on PKB: regulation of PKB activity by phosphorylation. *Current Opinion in Cell Biology.* 2009; 21:256-261.
3. Eva Gonzalez and Timothy E. McGraw. The Akt kinases. Cell *Cycle* 8:16, 2502-2508, 2009.
4. Chen WS, Xu PZ et al. Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. *Genes Dev*. 2001; 15:2203-8.
5. Cho H, Thorvaldsen JL et al. Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. *J. Biol Chem.* 2001; 276:38349-52.
6. Garofalo RS, Orena SJ et al. Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKBbeta. *J. Clin. Invest*. 2003; 112:197-208.
7. Cho H, Mu J et al. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2. *Science* 2001; 292:1728-31.
8. Tschopp O, Yang ZZ et al. Essential role of protein kinase Bgamma (PKBgamma/Akt3) in postnatal brain development but not in glucose homeostasis, *Development* 2005; 132:2943-54.
9. Peng XD, Xu PZ et al. Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. *Genes Dev*. 2003; 17:1352-65.
10. Yang ZZ, Tschopp O et al. Dosage dependent effects of Akt1/PKBalpha and Akt3/PKBgamma on thymus, skin, and cardiovascular and nervous system development in mice. *Mol. Cell Biol*. 2005; 25: 10407-18.
11. Fayard E, Tintiggnac L et al. Protein kinase B/Akt at a glance. *J. Cell Sci*. 118, 5675-5678.

12. Kumar C.C. and Madison V. Akt crystal structure and Akt specific inhibitors. *Oncogene* 24, 7493-501 (2005).
13. Thomas C.C., Deak M. et al. AKT crystal structure and AKT-specific inhibitors. *Oncogene* 24, 7493-501.
14. Manning B.D. and Cantley L.C. AKT/PKB signaling, navigating downstream. *Cell* 129,1261-1274.
15. Hanada M, Feng J et al. Structure, regulation and function of PKB/Akt- a major therapeutic target. *Biochim.Biophys.Acta* 11, 3-16.
16. Sarbassov D. D., Guertin D.A. et al. Phosphorylation and regulation of Akt/PKB by the rector-mTOR complex. *Science* 307, 1098-1101.
17. Shiota C, Woo JT et al. Multiallelic disruption of the rector gene in mice reveals that mTOR complex2 is essential for fetal growth and viability. *Dev.Cell* 2006; 11:583-589.
18. Bentzinger CF, Romanino K, Cloetta D, et al. Skeletal muscle-specific ablation of raptor, but not of rector, causes metabolic changes and results in muscle dystrophy. *Cell Metabol*.2008; 8:411-424.
19. Bozulic L, Surucu B et al. PKBalpha/Akt1 acts downstream of DNA-PK in the DNA double strand breaks response and promotes survival. *Mol.Cell* 2008; 30:203-213.
20. Boehme KA, Kulikov et al. p53 stabilization in response to DNA damage requires Akt/PKB and DNA-PK. *Proc.Natl.Acad.Sci* USA 2008; 105:7785-7790.
21. Dragoi AM, Fu X et al. DNA-PKcs, but not TLR9, is required for activation of Akt by CpG-DNA. *EMBO J*. 2005; 24:779-789.
22. Yang Zhang, Xiao Wang et al. Kinase AKT controls innate immune cell development and function. *Immunology* 2013; Oct; 140(2):143-52.
23. Chi YC, Lin CY, Chen CP, et al. Peptidoglycan enhances IL-6 production in human synovial fibroblasts via TLR2 receptor, focal adhesion kinase, Akt, and AP-1-dependent pathway. *J Immunol* 2009; 183:2785-92.
24. Chang JD, Sukhova GK, Libby P et al. Deletion of the phosphoinositide 3-kinase p110gamma gene attenuates murine atherosclerosis. *PNAS* 2007; 104:8077-82.
25. Ma B, Dela Cruz CS, Hartl D et al. RIG-like helicase innate immunity inhibits vascular endothelial growth factor tissue responses via a type I IFN-dependent mechanism. *Am.J.Respir.Crit.Care Med*. 2012; 183:1322-35.

26. Bozinovski S, Jones JE, Vlahos R et al. Granulocyte/macrophage-colony-stimulating factor (GM-CSF) regulates lung innate immunity to lipopolysaccharide through Akt/Erk activation of NFkappa B and AP-1 in vivo. *The journal of biological chemistry* 2002; 277: 42808-14.
27. Liu G, Yang K, Burns S, Shrestha S, Chi H. The S1P(1)-mTOR axis directs the reciprocal differentiation of T(H)1 and T(reg) cells. *Nature immunology* 2010; 11:1047-56.
28. Liu G, Burns S, Huang G, Boyd K, Proia RL, Flavell RA, Chi H. The receptor S1P1 overrides regulatory T cell-mediated immune suppression through Akt-mTOR. *Nature immunology* 2009; 10:769-77.
29. Haxhinasto S, Mathis D, Benoist C. The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells. *The Journal of experimental medicine* 2008; 205:565-74.
30. Ohkura N, Sakaguchi S. A novel modifier of regulatory T cells. *Nature immunology* 2009; 10:685-6.
31. 16 Delgoffe GM, Kole TP, Zheng Y, et al. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity* 2009; 30:832-44.
32. 17 Araki K, Youngblood B, Ahmed R. The role of mTOR in memory CD8 T-cell differentiation. *Immunological reviews* 2007; 235:234-43.
33. 18 Bi Y, Liu G, Yang R. mTOR regulates T-cell differentiation and activation in immunity and autoimmunity. *Critical reviews in eukaryotic gene expression* 2012; 21:313-22.
34. 19 Zhang L, Zhang H, Li L, et al. TSC1/2 signaling complex is essential for peripheral naive CD8+ T cell survival and homeostasis in mice. *PloS one* 2011; 7:e30592.
35. Essin K, Gollasch M, Rolle S, et al. BK channels in innate immune functions of neutrophils and macrophages. *Blood* 2009; 113:1326-31.
36. Wong CK, Cheung PF, Ip WK, Lam CW. Intracellular signaling mechanisms regulating toll-like receptor-mediated activation of eosinophils. *American journal of respiratory cell and molecular biology* 2007; 37:85-96.
37. Rane MJ, Coxon PY, Powell DW, Webster R, Klein JB, Pierce W, Ping P, McLeish KR. p38 Kinase-dependent MAPKAPK-2 activation functions as 3-phosphoinositide-

dependent kinase-2 for Akt in human neutrophils. *The Journal of biological chemistry* 2001; 276:3517-23.

38. Xu Y, Loison F, Luo HR. Neutrophil spontaneous death is mediated by down-regulation of autocrine signaling through GPCR, PI3Kgamma, ROS, and actin. *PNAS* 2010; 107:2950-5.
39. Souza LR, Silva E, Calloway E, Cabrera C, McLemore ML. G-CSF activation of AKT is not sufficient to prolong neutrophil survival. *Journal of leukocyte biology* 2013; 53: 1235-9.
40. Jun HS, Lee YM, Song KD, Mansfield BC, Chou JY. G-CSF improves murine G6PC3-deficient neutrophil function by modulating apoptosis and energy homeostasis. *Blood* 2011; 117:3881-92.
41. Herrera BS, Ohira T, Gao L, *et al.* An endogenous regulator of inflammation, resolvin E1, modulates osteoclast differentiation and bone resorption. *British journal of pharmacology* 2008; 155:1214-23.
42. D.Gabrilovich and Srinivas Nagaraj. Myeloid derived suppressor cells as regulators of the immune system. *Nature Reviews Imm.* 2009; 162-174.
43. Srinivas Nagaraj, Je-In Youn and D. Gabrilovich: Reciprocal relationship between Myeloid-Derived Suppressor cells and T cells. *The Journal of Immunology*. 2013; 191: 17-23.
44. Vincenzo Bronte & Paola Zanovello: Regulation of immune responses by L-arginine metabolism. *Nat.Reviews.Imm*. 2005
45. Thomas Condamine and Dmtry I. Gabrilovich. Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function. *Trends in immunology* 2010.
46. Elisa Peranzoni, Serena Zilio, Ilaria Marigo et al. Myeloid-derived suppressor cell heterogeneity and subset definition. *Current opinion in immunology*, 2010.
47. Bing Zhu, Yoshio Bando, Sheng Xiao et al. $CD11b^{+}Ly6C^{hi}$ Suppressive Monocytes in Experimental Autoimmune Encephalomyelitis. *The Journal of Immunology*, 2007.
48. Irah L. King, Travis L. Dickendesher et al. Circulating $Ly6C^{+}$ myeloid precursors migrate to the CNS and play a pathogenic role during autoimmune demyelinating disease. *Blood*, 2009.

49. James E. Talmadge. Pathways mediating the expansion and immunosuppressive activity of Myeloid-Derived Suppressor Cells and their relevance to cancer therapy. *Clin.Cancer Res*. 2007.
50. Sinha P, Clements VK, Bunt SK, Albelda SM, Ostrand-Rosenberg S. Crosstalk between myeloidderived suppressor cells and macrophages subverts tumor immunity toward a type 2 response. *The Journal of Immunology*. 2007; 179:977–983.
51. Youn JI, Nagaraj S, Collazo M, Gabrilovich DI. Subsets of myeloid-derived suppressor cells in tumorbearing mice. *The Journal of Immunology*. 2008;181:5791–5802.
52. Kusmartsev S, Nefedova Y, Yoder D, Gabrilovich DI. Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. *The Journal of Immunology*. 2004;172:989–999.
53. Schmielau J, Finn OJ. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of T-cell function in advanced cancer patients. *Cancer Res* 2001;61:4756–4760.
54. Dietlin TA, et al. Mycobacteria-induced Gr-1+ subsets from distinct myeloid lineages have opposite effects on T cell expansion. *J Leukoc Biol* 2007; 81:1205–1212.
55. Zhu B, et al. CD11b+Ly-6Chi Suppressive Monocytes in Experimental Autoimmune Encephalomyelitis. *The Journal of Immunology*. 2007;179:5228–5237.
56. Goni O, Alcaide P, Fresno M. Immunosuppression during acute Trypanosoma cruzi infection: involvement of Ly6G (Gr1(+))CD11b(+)immature myeloid suppressor cells. *Int Immunol.* 2002;14:1125–1134.
57. Kusmartsev S, Nagaraj S, Gabrilovich DI. Tumor-associated CD8+ T cell tolerance induced by bone marrow-derived immature myeloid cells. *The Journal of Immunology*. 2005;175:4583–4592.
58. Rodriguez PC, Ochoa AC. Arginine regulation by myeloid derived suppressor cells and tolerance in cancer: mechanisms and therapeutic perspectives. *Immunol Rev* 2008;222:180–191.
59. Rodriguez PC, et al. Regulation of T cell receptor CD3zeta chain expression by L-arginine. *J Biol Chem* 2002;277:21123–21129.
60. Rodriguez PC, Quiceno DG, Ochoa AC. L-arginine availability regulates T-lymphocyte cell-cycle progression. *Blood* 2007;109:1568–1573.

61. Szuster-Ciesielska A, Hryciuk-Umer E, Stepulak A, Kupisz K, Kandefer-Szerszen M. Reactive oxygen species production by blood neutrophils of patients with laryngeal carcinoma and antioxidative enzyme activity in their blood. *Acta Oncol* 2004;43:252–258.
62. Agostinelli E, Seiler N. Non-irradiation-derived reactive oxygen species (ROS) and cancer: therapeutic implications. *Amino Acids* 2006;31:341–355.
63. Gabrilovich, D. I., S. Ostrand-Rosenberg, and V. Bronte. 2012. Coordinated regulation of myeloid cells by tumours. *Nat. Rev. Immunol*. 12: 253–268.
64. Youn, J.-I., M. Collazo, I. N. Shalova, S. K. Biswas, and D. I. Gabrilovich. 2012. Characterization of the nature of granulocytic myeloid-derived suppressor cells in tumor-bearing mice. *J. Leukoc. Biol*. 91: 167–181.
65. Youn, J. I., V. Kumar, M. Collazo, Y. et al. 2013. Epigenetic silencing of retinoblastoma gene regulates pathologic differentiation of myeloid cells in cancer. *Nat. Immunol*. 14: 211–220.
66. Corzo, C. A., T. Condamine, L. Lu, et al. 2010. HIF-1a regulates function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment. *J. Exp. Med*. 207: 2439–2453.
67. Iwata, Y., K. Furuichi, K. Kitagawa, et al. 2010. Involvement of CD11b+ GR-1low cells in autoimmune disorder in MRL-Faslpr mouse. *Clin. Exp. Nephrol*. 14: 411–417.
68. Cripps, J. G., J. Wang, A. Maria, I. Blumenthal, and J. D. Gorham. 2010. Type 1 T helper cells induce the accumulation of myeloid-derived suppressor cells in the inflamed Tgfb1 knockout mouse liver. *Hepatology* 52: 1350–1359.
69. Arora, M., S. L. Poe, A. Ray, and P. Ray. 2011. LPS-induced CD11b+Gr1intF4/80+ regulatory myeloid cells suppress allergen-induced airway inflammation. *Int. Immunopharmacol*. 11: 827–832.
70. Chatterjee, S., S. Das, P. Chakraborty, A. Manna, M. Chatterjee, and S. K. Choudhuri. 2013. Myeloid derived suppressor cells (MDSCs) can induce the generation of Th17 response from naive CD4+ T cells. *Immunobiology* 218: 718–724.
71. He, D., H. Li, N. Yusuf, C. A. Elmets, J. Li, J. D. Mountz, and H. Xu. 2010. IL-17 promotes tumor development through the induction of tumor promoting

microenvironments at tumor sites and myeloid-derived suppressor cells. *The Journal of Immunology*. 184:2281–2288.

72. Yi, H., C. Guo, X. Yu, D. Zuo, and X. Y. Wang. 2012. Mouse CD11b+Gr-1+myeloid cells can promote Th17 cell differentiation and experimental autoimmune encephalomyelitis. *The Journal of Immunology*. 189: 4295–4304.
73. Li, H. et al. (2009) Cancer-expanded myeloid-derived suppressor cells induce anergy of NK cells through membrane-bound TGF-beta 1. *The Journal of Immunology*. 182, 240–249
74. Yang, L. et al. (2008) Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promotemetastasis. *Cancer Cell* 13, 23–35
75. Huang, B. et al. (2006) Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. *Cancer Res*. 66, 1123–1131
76. Pan, P.Y. et al. (2010) Immune stimulatory receptor CD40 is required for T-cell suppression and T regulatory cell activation mediated by myeloid-derived suppressor cells in cancer. *Cancer Res.* 70, 99–108
77. Serafini, P. et al. (2008) Myeloid-derived suppressor cells promote cross-tolerance in B-cell lymphoma by expanding regulatory T cells. *Cancer Res*. 68, 5439–5449.
78. Huang, B., P. Y. Pan, Q. Li, A. I. Sato, D. E. Levy, J. Bromberg, C. M. Divino, and S. H. Chen. 2006. Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumorbearing host. *Cancer Res*. 66: 1123–1131.
79. Pan, P. Y., G. Ma, K. J. Weber, J. Ozao-Choy, G. Wang, B. Yin, C. M. Divino, and S. H. Chen. 2010. Immune stimulatory receptor CD40 is required for T-cell suppression and T regulatory cell activation mediated by myeloid-derived suppressor cells in cancer. *Cancer Res*. 70: 99–108.
80. Zhang, Y., Q. Liu, M. Zhang, Y. Yu, X. Liu, and X. Cao. 2009. Fas signal promotes lung cancer growth by recruiting myeloid-derived suppressor cells via cancer cellderived PGE2. *The Journal of Immunology*. 182: 3801–3808.

81. Serafini, P., S. Mgebroff, K. Noonan, and I. Borrello. 2008. Myeloid-derived suppressor cells promote cross-tolerance in B-cell lymphoma by expanding regulatory T cells. *Cancer Res*. 68: 5439–5449.
82. Deshane, J., J. W. Zmijewski, R. Luther, A. Gaggar, R. Deshane, J. F. Lai, X. Xu, M. Spell, K. Estell, C. T. Weaver, et al. 2011. Free radical producing myeloidderived regulatory cells: potent activators and suppressors of lung inflammation and airway hyperresponsiveness. *Mucosal Immunol*. 4: 503–518.
83. Chou, H. S., C. C. Hsieh, R. Charles, L. Wang, T. Wagner, J. J. Fung, S. Qian, and L. L. Lu. 2012. Myeloid-derived suppressor cells protect islet transplants by B7-H1 mediated enhancement of T regulatory cells. *Transplantation* 93: 272–282.
84. Xia, S., H. Sha, L. Yang, Y. Ji, S. Ostrand-Rosenberg, and L. Qi. 2011. Gr-1+CD11b+ myeloid-derived suppressor cells suppress inflammation and promote insulin sensitivity in obesity. *J. Biol. Chem*. 286: 23591–23599.
85. Gabrilovich, D. I., M. P. Velders, E. M. Sotomayor, and W. M. Kast. 2001. Mechanism of immune dysfunction in cancer mediated by immature Gr-1+ myeloid cells. *The Journal of Immunology*. 166: 5398–5406.
86. Sinha, P., V. K. Clements, and S. Ostrand-Rosenberg. 2005. Interleukin-13- regulated M2 macrophages in combination with myeloid suppressor cells block immune surveillance against metastasis. *Cancer Res*. 65: 11743–11751.
87. Srivastava, M.K. et al. (2010) Myeloid-derived suppressor cells inhibit T-cell activation by depleting cystine and cysteine. *Cancer Res*. 70, 68–77.
88. Alicia Arranz, Christina Doxaki, Eleni Vergadi et al. Akt1 and Akt2 protein kinases differentially contribute to macrophage polarization. *PNAS* 2011 vol.109,no.24 9517-9522.
89. Ariadne Androulidaki, Dimitrios Iliopoulos et al. The kinase Akt1 controls macrophage response to LPS by regulating microRNAs. *Immunity* 2009 31, 220-231.
90. Jim Hee Kim, Tae Heung Kang, Kyung Hee Noh et al. Enhancement of DC vaccine potency by activating the PI3K/AKT pathway with a small interfering RNA targeting PTEN. *Immunology Letters 2010* 134, 47-54.
91. Thomas J.Cremer, Prexy Shah, Estelle Cormet-Boyaka et al. Akt-Mediated Proinflammatory response of mononuclear phagocytes infected with Burkholderia

cenocepacia occurs by a novel GSK-3β-Dependent, IkB kinase-independent mechanism. *The Journal of Immunology* 2011, 187: 635-643.

92. Peter Boros, Jordi C. Ochando et al. Myeloid-derived suppressor cells: Natural regulators for transplant tolerance. *Human Immunology 2010.*
93. Marianna Ioannou, Themis Alissafi et al. Crucial role of granulocytic myeloid-derived suppressor cells in the regulation of central nervous system autoimmune disease. *The journal of Immunology*, 2012, 188: 1136-1146.
94. Nada Sonda, Maria-cristina Chioda et al. Transcription factors in myeloid-derived suppressor cell generation. *Current opinion in Immunology 2011, 23:1-7.*
95. Cain DW, O'Koren EG et al. Identification of a tissue specific, CEBP/β-Dependent Pathway of differentiation for murine peritoneal macrophages. *The Journal of Immunology*, 2013, 191.