Graduate Program in

THE MOLECULAR BASIS OF HUMAN DISEASE

Master Thesis

Investigate the role of autophagy in the Myeloid Derived Suppressor Cells-mediated tolerance of anti-tumor responses

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Abstract

Recruitment of suppressive immune cell types in tumor sites is a major manifestation of tumor tolerance. Myeloid-derived suppressor cells (MDSCs) play a very important role in the suppression of anti-tumor immunity by expressing several suppressive molecules and by expanding regulatory T cells. However, the exact molecular mechanism of MDSCs-Treg interaction is not yet fully understood. Autophagy, apart from its homeostatic role, has been implicated in antigen presentation through MHC class II molecules in antigen-presenting cells, but its implication in regulatory T cell expansion by MDSCs has not been studied. In this study, we observed an expansion of MDSCs in tumor-bearing mice that is accompanied by an increase in the expression of the autophagy-related genes *Atg5* and *Bcl1*. Mice with a conditional knock-out of Atg5 gene in myeloid cells ($LysM^{cre}Atg5^{fl/fl}$) showed decreased tumor growth compared to control tumor-bearing mice. Furthermore, $LysM^{cre}Atg5^{fl/fl}$ mice showed a markedly expansion of MDSCs in spleen compared to control mice and a differential expression of several effector molecules (TGFβ, arginase-1 and CEBP/β) in these cells. Collectively, these results support an important role for autophagy in the function of myeloid cells and additional experiments will elucidate the molecular mechanism underlying tumor tolerance by MDSCs.

Περίληψη

Η στρατολόγηση κατασταλτικών ανοσοκυττάρων στις περιοχές των όγκων είναι μία κύρια εκδήλωση της ανοσολογικής ανοχής κατά του όγκου. Τα κατασταλτικά κύτταρα της μυελικής σειράς (MDSCs) παίζουν έναν πολύ σημαντικό ρόλο στην καταστολή των ανοσολογικών αποκρίσεων εναντίον του όγκου, εκφράζοντας διάφορα κατασταλτικά μόρια και ενεργοποιώντας τα Τ ρυθμιστικά κύτταρα. Ο ακριβής μοριακός μηχανισμός, όμως, της αλληλεπίδρασης μεταξύ MDSCs και Τ ρυθμιστικών κυττάρων δεν είναι απόλυτα κατανοητός. Η αυτοφαγία, εκτός από τον ομοιοστατικό της ρόλο, έχει εμπλακεί και στην αντιγονοπαρουσίαση μέσω των μορίων MHC II στα αντιγονοπαρουσιαστικά κύτταρα, αλλά η συμβολή της αύξηση των Τ ρυθμιστικών κυττάρων από τα MDSCs δεν έχει μελετηθεί. Σε αυτή την μελέτη, παρατηρήσαμε μία αύξηση στον αριθμό των MDSCs σε ποντίκια με μελάνομα, που συνοδευόταν με αύξηση της έκφρασης των γονιδίων της αυτοφαγίας *Atg5* και *Bcl1*. Ποντίκια από τα οποία έχει αφαιρεθεί το γονιδίου Atg5 στα κύτταρα της μυελικής σειράς ($LysM^{cre}Atg5^{fl/fl}$) είχαν μειωμένη ανάπτυξη του όγκου συγκρινόμενα με τα ποντίκια ελέγχου. Επίσης, στα ποντίκια $LysM^{cre}Atg5^{fl/fl}$ παρατηρήθηκε μεγάλη αύξηση των MDSCs στον σπλήνα συγκρινόμενα με τα κοντρόλ ποντίκια και διαφορική έκφραση διάφορων δραστικών μορίων (TGFβ, arginase-1 and CEBP/β) σε αυτά τα κύτταρα. Συνολικά, τα δεδομένα μας δείχνουν έναν σημαντικό ρόλο για την αυτοφαγία στη δράση των κυττάρων της μυελικής σειράς και επόμενα πειράματα θα μας δείξουν τον μοριακό μηχανισμό που διέπει την ανοσολογική ανοχή κατά του όγκου από τα MDSCs.

introduction

Tumorigenesis

Tumors comprise of complex tissues with malignant cells that overproliferate and recruit normal cells that contribute to tumorigenesis by numerous interactions. The notion that tumors are just a mass of proliferating cells has been abandoned the last years because extensive research has shown that many steps and events are required for tumor cells to survive, proliferate and spread. Genomic instability and prolonged inflammation are the main contributors of the capabilities that tumor cells acquire during tumorigenesis. Tumor cells need to sustain a chronic proliferation state and break the homeostasis of normal cells. Growth-promoting signals through surface-binding growth factors that control the cell growth-and-division cycle are deregulated by tumor cells that eventually can control their own fate. The main mechanisms are: the establishment of an autocrine proliferative stimulation by expressing both the growth factors and their cognate receptors; stimulation of normal cells to supply growth factors; hyperresponse to growth factor ligands by overexpressing the receptor proteins levels, and constitutive activation of downstream signaling pathways in order to bypass the need for external stimuli. On the same time tumor cells need to inactivate the signaling pathways that negatively regulate cell proliferation. Tumor cells intervene with growth suppressor molecules, like the retinoblastoma-associated protein and TP53 protein that limit cell growth and proliferation.

After establishing infinite proliferation, tumor cells must protect themselves from senescence and cell death, the two mechanisms that normal cells employ to avoid abnormal growth. Normally, in each cell division, a small part at the end of each chromosome, the telomere, is not replicated and the chromosome shortens in length, giving the cell a finite number of divisions before entering into senescence state. Tumor cells on the other hand, acquire the ability to express the enzyme telomerase that adds telomere sequences at the ends of the chromosomes and thus protecting them from apoptosis and cell death. Tumor cells can also intervene directly to the cell death pathway of apoptosis that is deployed as a natural barrier to any form of abnormal growth. Each cell can receive and process extracellular death-inducing signals (Fas Ligand) through membrane receptors (Fas receptor), analyze them

intracellularly and activate the effector proteins of apoptosis, the caspases. Tumor cells can alter many components of the apoptotic circuit and resist cell death, like lowering the expression of damage sensor molecules or increasing the expression of antiapoptotic regulators.

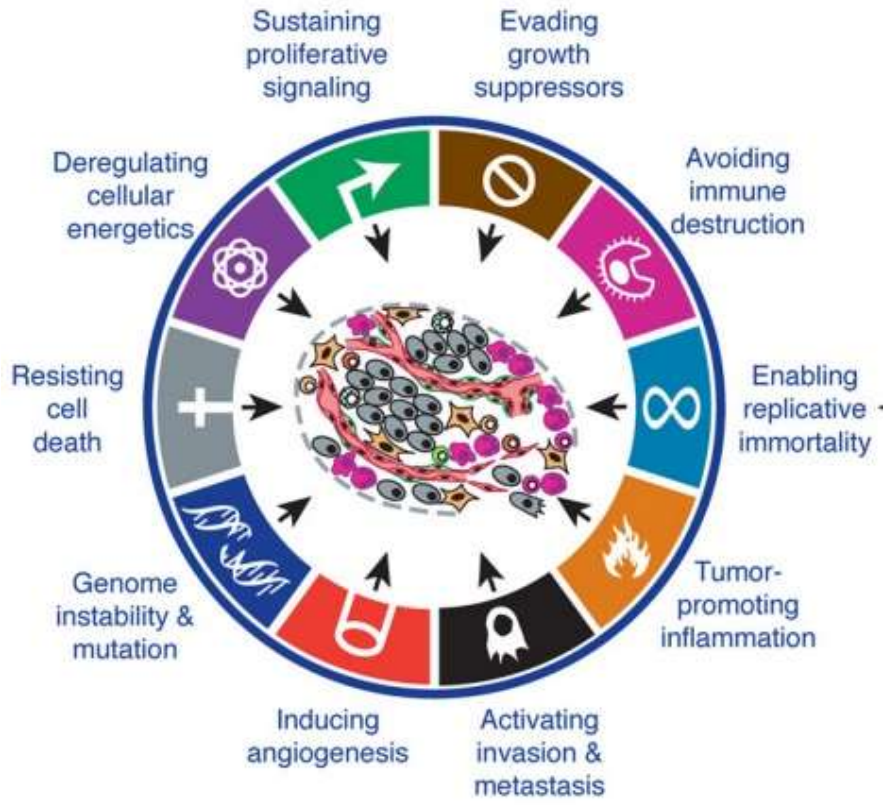


Figure 1. Hallmarks of cancer. Six biological capabilities described in 2000 by Hanahan and Weinberg and two additional described in 2011 (reprogramming energy metabolism and evading immune destruction) comprise the hallmarks of cancer; an organizing principle for rationalizing the complexities of neoplastic disease. Tumor-promoting inflammation and genomic instability are characterized as the main contributors of these hallmarks. (adopted from Hallmarks of Cancer: The Next Generation, Cell 144, March 4, 2011)

Tumors have also the ability to promote angiogenesis, a process that is normally activated during embryogenesis and in wound healing in adults. Hypoxic tumor microenvironment and oncogene signaling can upregulate the expression of the vascular endothelial growth factor (VEGF), the main orchestrator of new blood vessel formation. The vasculature that is formed, even in the very early stages of tumorigenesis, is characterized by excessive branching, leakiness and abnormal

proliferation and apoptotic rate of endothelial cells, but is capable of supplying tumor sites with nutrients and evacuate metabolic wastes and carbon dioxide. Invasion and metastasis of tumor cells to other sites of the body to form new tumors is controlled by cell-to-cell and cell-to-extracellular matrix adhesion proteins that normally help cells to form tissues or migrate to nearby areas.

A very important characteristic that tumor cells acquire during tumorigenesis is to evade immune destruction. Cancer and tumor immunology research over the past years, have started to appreciate the role that the immune system plays in the fight between eradicating and persisting of neoplasias and established tumors.[1]

Tumor tolerance

One major characteristic of the immune system is that it can distinguish between self and non-self and it eventually acts only against foreign antigens. Unresponsiveness to self antigens is achieved via a process called self-tolerance and is of great importance as it prevents the appearance of autoimmune diseases. Lymphocytes that have the ability to recognize self antigens are eliminated either in the thymus (central tolerance) or in the peripheral tissues (peripheral tolerance). Lymphocytes that recognize foreign antigens survive this selection process and circulate in the periphery waiting to encounter the antigen that they are designed to respond to. In a typical infection by a microbe, the immune system "senses" very effectively the foreign antigens and elicits anti-microbial immune responses to eradicate the threat. Cancer on the other hand, is a very complex disease as the abnormally proliferating cells in the body cannot be eradicated because they are recognized as self. Despite the absence of lymphocytes specific for self antigens due to self-tolerance, it is well established that tumors can elicit immune responses by expressing altered self-antigens which are recognized as foreign. The immunogenicity of tumors varies from low immunogenic tumors that express a few foreign antigens to high immunogenic tumors caused by oncogenic viruses that are recognized as foreign. Despite the existence of antigens within a tumor that can activate the immune system, in most cases tumor growth is not prevented because the rapid proliferation of abnormal cells overpasses the capacity of the immune system to eradicate tumor cells.[2, 3]

The immune system is designed in a way that contains all the necessary components and mechanisms to recognize and kill cancer cells without provoking autoimmune responses. Oncogenesis produces and releases neoantigens (antigens derived from host cells but are recognized as foreign) that are captured and processed by dendritic cells. DCs present these antigens on MHC class I and II molecules and prime effector T cell responses. Effector T cells traffic to tumour sites and infiltrate the tumour bed where they recognize cancer cells through the interaction between their T cell receptors and the cognate antigen bound to MHC molecules. Eventually tumour cells are eliminated, releasing more antigens that are captured by DCs and so on.[4]

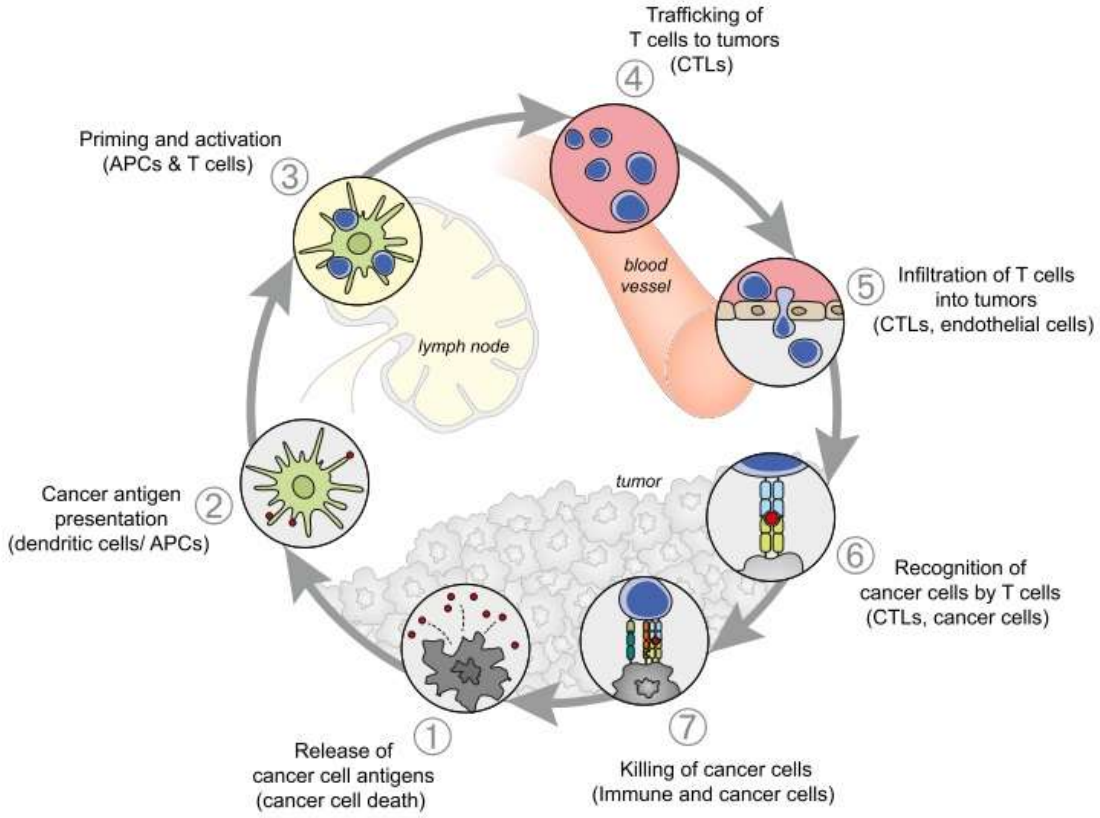


Figure 2. The cancer-immunity cycle. Anti-tumor response comprises of seven steps that lead to activation of T cell responses against malignant cells. (Oncology meets Immunology: The Cancer-Immunity Cycle, Immunity 39, July 25, 2013)

But despite all these mechanisms, in most cases cancer cells are not eradicated because tumors have developed mechanisms of immune evasion for almost every step of the anti-tumor immunity. The first line of defense is downregulation of the antigen-

presenting machinery by tumor cells themselves.[5] Genetic alterations in cancer cells like point mutations, large deletions or epigenetic silencing, can lead to reduced or complete loss of expression of MHC I class molecules. Defects in transport of antigen-presenting machinery has also been observed in some types of cancer due to mutated β2-microglobulin.[6, 7] Tumors also intervene in T cell homing to tumour sites and inhibit T cell infiltration to tumour bed. Post-translation modifications alter the functionality of chemokines, the main orchestrators of T cell homing and deprive T cells the signals necessary to reach tumour sites. CCL2, a key chemokine for this process, has been found to be nitrosylated and thus it loses its ability to attract cytotoxic T lymphocytes but this does not affect the homing of myeloid cells, like the suppressive population of MDSCs that will be discussed later.[8] Despite the blocking of the chemokine signaling, T cells can be found in tumour sites, but very often they fail to cross the tumour vasculature. Crossing of the endothelium requires adhesion of T cells to endothelial cells via adhesion molecules followed by extravasation. Endothelial cells are utilized by tumour to act as a physical barrier.[6] Vascular endothelial growth factor (VEGF) is overexpressed by tumour cells leading to reduced expression of adhesion molecules on the surface of endothelium cells.[9, 10] Endothelial cells themselves can turn into suppressive cells producing various suppressive and cytotoxic molecules like Fas Ligand, TNF-related apoptosis-inducing ligand, PD-L1, PD-L2, IL-10 and TGFβ.[11]

Tumour cells can also escape the cytotoxic effects of T cells that have managed to reach the intratumoral site by blocking the two major anti-tumour killing machineries. Perforin/granzyme pathway is a mechanism used by cytotoxic T cells to eliminate tumor and virus-infected cells. Tumour cells block the activity of granzymes by releasing PI-9/SPI-6, a serine protease inhibitor.[12] Binding of death receptors (CD95, TRAIL-R1/R2) in tumour cells with their ligands (CD95L, TRAIL) expressed by lymphocytes and natural-killer cells induces apoptotic events and activation of caspases.[13] Tumour cells evade this form of apoptosis by expressing antiapoptotic proteins, downregulating their death receptors or expressing truncated forms of these receptors that can bind with their ligands but cannot induce apoptotic signaling pathway.[7] Tumour cells have also the ability to counter-attack T cells and induce their death. Expression of enzyme indoleamine 2,3-dioxygenase (IDO) by tumour cells deprives tumour milieu from the essential amino acid tryptophan, activating apoptotic signals without the need of Fas/Fas Ligand interactions.[14]

One of the most important evasion mechanisms that has puzzled scientists over the years and has limited anti-tumour therapy is the peripheral tolerance against tumour antigens, involving cells of both the innate and the adaptive immunity. As discussed above, dendritic cells have the ability to capture and present tumor antigens to both B and T cells along with expression of co-stimulatory signals that will activate an effective immune response. Tumor microenvironment is enriched in molecules with suppressive function, like VEGF, TGFβ, IL-10, M-CSF and IL-6 which have all been found to disrupt DC maturation and function. Hypoxic conditions and low pH of tumor milieu enhances the immature state of DCs that have been found to express the immunosuppressive molecules IDO and prostaglandin E_2.[6] These partially mature or immature DCs have intermediate or low expression of MHC class I molecules respectively but can still present tumour antigens to lymphocytes. The absence of inflammatory signals along with the presence of immunosuppressive agents in tumor sites (mainly TGFβ) shifts DCs towards a tolerogenic state. T cells that recognize MHC/peptide complexes from immature DCs are not fully activated and they become anergic and eventually die of apoptosis, because DCs cannot provide with co-stimulatory molecules (CD40, B7.1/2).[7, 15] Functionally incompetent DCs are also incapable of secreting chemokines, like CCL19, to attract naïve T cells in tumour sites.[16]

Tumour, besides rendering DCs incapable of activating cytotoxic immune responses, recruits two more cell populations, best known for their suppressive functions. T regulatory (Treg) cells are very important for maintaining peripheral tolerance and preventing autoimmune diseases. Tregs are recruited in tumour sites (natural Tregs) by CCL22 chemokine secreted by tumour cells and tumour-infiltrating macrophages but can also be induced in situ (inducible Tregs) by TGFβ acting on naïve $CD4^+$ T cells.[17] Immunosuppressive molecules secreted by tumour cells (as discussed above) also contribute to abnormal myelopoiesis which leads to accumulation of immature myeloid cells. Myeloid-Derived Suppressor cells (MDSCs), which represent this premature state of myeloid cells, are released from the bone marrow under the influence of various chemokines secreted by tumour cells, expand in the spleen[18] and exert their immunosuppressive effects in tumour sites.[19]

Both cell populations suppress T cell responses with various mechanisms that will be discussed in the next section. The function and the interaction of Tregs and MDSCs are under intense research the last years because the understanding of the molecular mechanisms underlying the tumour-induced tolerance through these cells will give a very important target for cancer immunotherapy.

Regulatory T cells

introduction

Regulatory T (Treg) cells play a central role in maintaining immune homeostasis as a negative feedback mechanism for immune activation. Treg cells maintain peripheral tolerance, prevent autoimmune diseases (e.g. type I diabetes) and responses to allergens and limit chronic inflammatory diseases (e.g. inflammatory bowel disease, IBD). Apart from their beneficial role, Tregs also suppress sterilizing immunity and limit antitumor responses.[17] The transcription factor Foxp3 (forkhead box P3) is the main regulator of the Treg cell signature controlling the development, maintenance and function of these cells and is established as the main marker for the identification of this cell subset. The indispensable role of Foxp3 is clearly demonstrated in scurfy mice and IPEX syndrome in men; scurfy mice have a deletion in the forkhead domain of Foxp3, fail to generate Treg cells and develop a fatal lymphoproliferative syndrome with multi organ inflammation.[20] IPEX (immunodysregulation polyendocrinopathy and enteropathy, X-linked) syndrome is characterized by a variety of autoimmune phenomena caused by mutations in Foxp3 gene.[21]

mechanisms of suppression

Treg cells have a variety of mechanisms for the suppression of immune responses that target antigen-presenting cells and effector cells. The first mode of action is by secretion of inhibitory cytokines: TGFβ, IL-10 and IL-35 are up till now the main cytokines that have been found to be secreted by Tregs and play a suppressive role both *in vitro* and *in vivo*. TGFβ have been implicated in the prevention of colitis in the mouse model of IBD, in host response against *M. tuberculosis* and in suppression of allergic responses. In cancer, TGFβ has been implicated in limiting antitumor responses

in some forms of cancer by rendering T cells unresponsive and by limiting the activity of killer cells. Apart from secreted TGFβ, membrane-bound TGFβ has also been found to suppress immune responses by cell-to-cell contact mechanism.[17, 22] IL-10 secretion may only be partially necessary for the suppression of autoimmune responses, but is essential for controlling inflammatory responses induced by pathogens and environmental stress, as in a tumor microenvironment. IL-10 induced by tumor factors is responsible for a cell contact-independent mechanism of action.[17, 23] IL-35 is a newly characterized suppressive cytokine that is essential for maximal suppression *in vitro* and has been implicated in several inflammatory conditions, like IBD.[17] A second mechanism of action is cytolysis which is mediated by several cytotoxic molecules. The best characterized function is through the release of granzymes which enter target cells and activate the caspase pathway. Human Treg cells express the granzyme A and mouse Treg cells express the granzyme B, targeting effector T cells, B cells, Natural Killer cells and cytotoxic T lymphocytes. Activation of the TRAIL-DR5 and the galectin-1 pathways has also been shown to induce apoptosis.[17]

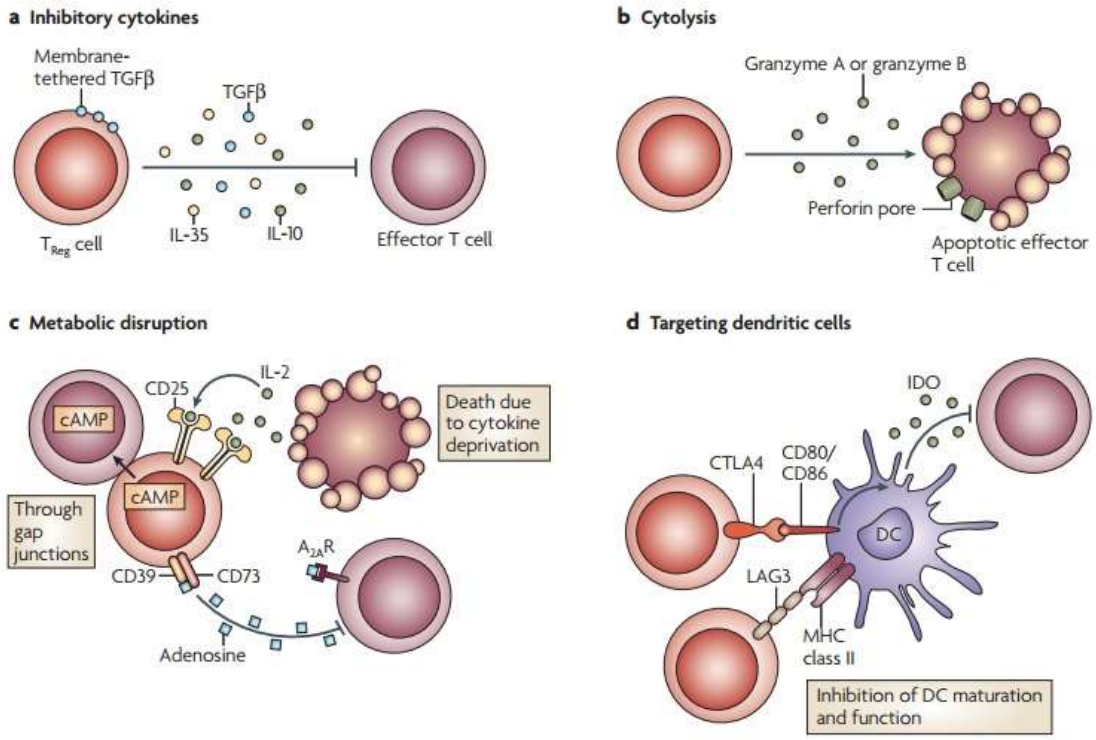


Figure 3. Mechanisms of suppression used by Treg cells. Suppression by Treg cells can be categorized as follows: secretion of inhibitory cytokines (TGFβ, IL-10, IL-35), cytolysis (Granzymes), metabolic disruption (mainly deprivation of IL-2) and targeting of dendritic cells (regulation of maturation and expression of co-stimulatory molecules). (How regulatory T cells work, Nature Reviews Immunology, Volume 8, July 2008)

Another mechanism of suppression is by metabolic disruption of the target cells. Treg cells deplete from their microenvironment the IL-2 cytokine which is very important for the proliferation and growth of T cells and is essential for the differentiation towards effector T cells. Expression of adenosine nucleosides not only suppresses effector T cells but enhances Treg cell generation by promoting TGFβ secretion. The last mode of action is the direct targeting of dendritic cells. Treg cells have the ability to intervene with the maturation and function of DCs. Downregulation of the co-stimulatory molecules CD80 and CD86 and direct interaction through cytotoxic T-lymphocyte antigen 4 (CTLA4) expressed on Tregs attenuates the ability of DCs to induce effective T cell responses. This interaction can be prolonged by constant expression of neuropilin-1 by Treg cells, a membrane receptor for VEGF and semaphorin. Treg cells also induce the expression IDO by DCs which depletes tryptophan resulting in suppression of T effector cells.[17]

origin

Regulatory T cells can be generated either in the thymus or in the periphery. Thymic differentiation occurs in parallel with positive selection of double positive $CD4^+$ $CD8^+$ T cells. Although the mechanism is not yet clear, $Foxp3^+$ cells in the thymus are T cells that have already been committed in $CD4^+$ or $CD8^+$ lineages. As in conventional T cells, positive selection of Treg cells requires interaction between T cell receptor (TCR) and major histocompatibility complex (MHC), but with stronger dependence on costimulatory signals through CD28. IL-2 is the main cytokine involved in the differentiation process of $Foxp3^-$ $CD25^{hi}$ cells into $Foxp3^+$ $CD25^+$, whereas a requirement for TGFβ has not been described. Downstream of TCR and IL-2 receptor, NF-κB pathway plays a positive role in Treg commitment, although the exact mechanism for specific activation of Foxp3 has not been found. Akt and mTOR pathway restricts thymic selection of Treg cells. Thymic-derived or naturally occurring Regulatory T cells (nTreg cells) are a functionally mature population.[24] Differentiation in peripheral lymphoid tissues occurs under inflammatory conditions where conventional $CD4^+$ $CD25^-$ T cells are "converted" into $Foxp3^+$ $CD25^+$ T cells in the presence of TGFβ. Constitutively activated type II TGFβ receptor along with high affinity TCR signaling and suboptimal costimulation induces Foxp3 expression. As stated before, tumor milieu in many mouse models and human tumor types is enriched

with TGFβ, which favors the survival of both nTreg and induced regulatory T cells (iTreg cells).[25, 26, 27]

regulatory T cells in tumor

Absolute numbers of Treg cells are elevated under tumor conditions by several mechanisms, apart from peripheral differentiation, leading to poor prognosis in cancer patients. Treg cells are recruited to tumor sites by various chemokines like CCL5 and CCL12, expressed by tumor or immune cells (like myeloid-derived suppressor cells and tumor-associated macrophages that will be discussed later). Treg cells that have reached the tumor sites can expand upon antigen stimulation by MDSCs, DCs and TAMs in the presence of inhibitory molecules TGFβ and IDO. Treg cells have also been found to be more resistant to oxidative stress-mediated cell death than conventional $CD4^+$ T cells induced by tumor and myeloid cells and downregulate proapoptotic genes.[25]

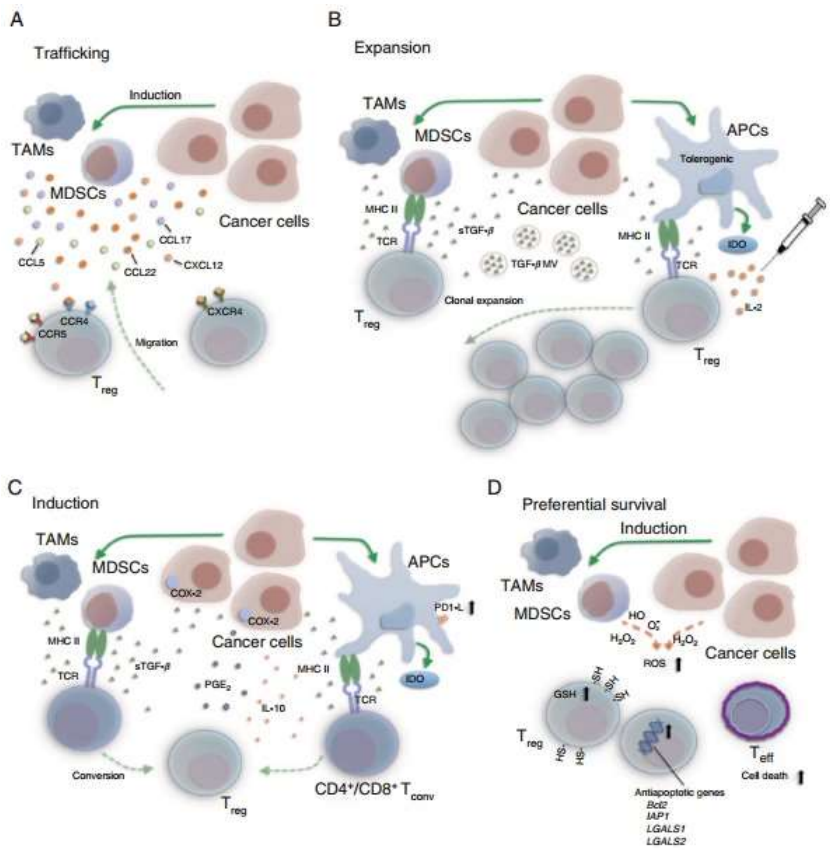


Figure 4. Regulatory T cells accumulation and expansion in tumor microenvironment. Natural Treg cells are chemoattracted to tumor sites by CCL5 and CCL15 where they expand by antigen presentation in the presence of TGFβ and IDO. Induced Treg cells are generated by suboptimal T cell activation in tumor sites. Both populations preferentially survive in tumor conditions. (Regulatory T cells in cancer, Advances in CANCER RESEARCH, 2010)

Myeloid-derived suppressor cells

introduction

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of cells of myeloid lineage that remain in an immature state and do not further differentiate into macrophages, dendritic cells and granulocytes. Their main function is to suppress immune responses through various mechanisms that will be discussed later. These immature cells consist of myeloid progenitor cells and immature myeloid cells (IMCs). In healthy steady state, haematopoietic stem cells differentiate into the common myeloid progenitors which in turn differentiate into immature myeloid cells. This process takes place in the bone marrow and is controlled by various soluble factors like GM-CSF, M-CSF and cell-surface molecules. IMCs migrate to peripheral tissues where they differentiate into macrophages, dendritic cells and granulocytes. During pathological conditions like cancer, traumatic stress, sepsis, autoimmune diseases and infections, IMC differentiation is partially blocked, resulting in the expansion of MDSCs and upregulation of their immune suppression mechanisms. Factors released by sites of inflammation and tumor sites chemoattract MDSCs in these tissues and in peripheral lymphoid organs where they expand and exert their immunosuppressive activities.[19, 28, 29]

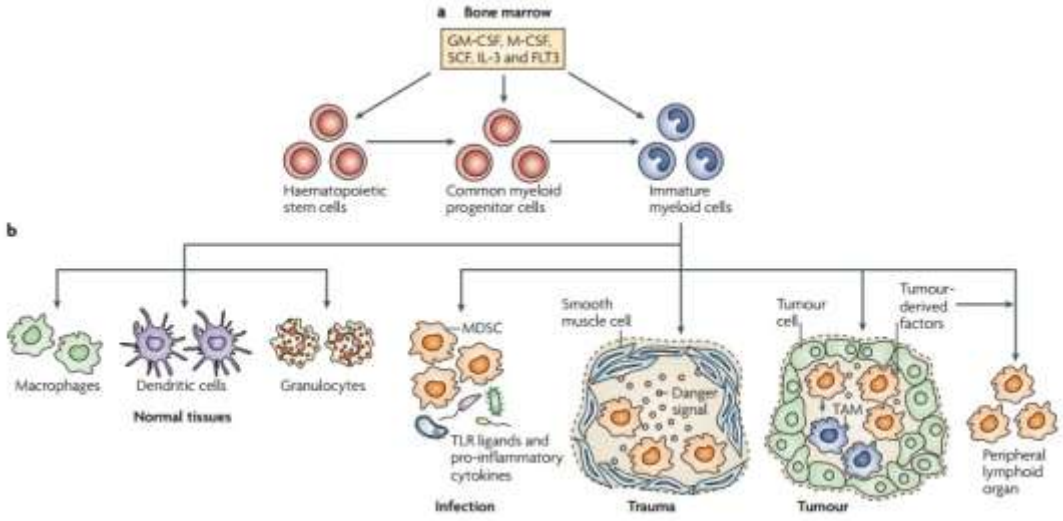


Figure 5. Myelopoiesis under normal and pathologic conditions. Immature myeloid cells (IMCs) in healthy state differentiate into macrophages, DCs and granulocytes in the periphery. In pathologic conditions, IMCs differentiation is blocked and immature cells expand and exert suppressive phenotype, now termed myeloid-derived suppressor cells. (Myeloid-derived suppressor cells as regulators of the immune system, Nature Reviews Immunology, Volume 9, March 2009)

subsets

Mouse MDSCs are characterized by the co-expression of CD11b and Gr-1 surface molecules and the absence of CD11c marker. The Gr-1 epitope is expressed on two surface molecules and characterizes the two distinct subsets of MDSCs that have been described. Granulocytic (G-MDSCs) and monocytic MDSCs (M-MDSCs) have different morphological characteristics, rate of expansion and mechanisms of suppression. G-MDSCs are characterized by high expression of Ly6G and low expression of Ly6C ($CD11b^{+}$ $Ly6G^{high}$ $Ly6C^{low}$) and are the predominant population in tumor-bearing mice with up to 5:1 ratio with M-MDSCs. Ly6G is the typical neutrophil marker which shows the commitment of G-MDSCs in the neutrophil lineage and explains the polymorphonuclear morphology. The main suppressive mechanism is through reactive oxygen species production (ROS). M-MDSCs are characterized by their mononuclear morphology and high expression of the Ly6C marker ($CD11b^{+}$ $Ly6G^{low}$ $Ly6C^{high}$). Expression of the typical monocyte/macrophage markers F4/80 and CD115 shows their commitment to the monocyte lineage. M-MDSCs use mainly the nitric oxide and peroxynitrite production as a suppressive mechanisms.[30, 31, 32]

induction and expansion in tumor milieu

MDSCs in tumor conditions expand by factors released by tumor cells that promote myelopoiesis and inhibition of IMC differentiation and by direct activation from T and stromal cells. GM-CSF is the key growth factor during myelopoiesis and is elevated in the tumor microenvironment promoting MDSCs generation, along with VEGF and IL-1b that blocks dendritic cell maturation and activation. IFN-γ, TGFβ, IL-13 and IL-4 are the main soluble factors that activate MDSCs through signaling via STAT1, STAT6 and NF-κB. IL-6, the main cytokine of chronic inflammation has been implicated in generation, migration and activation of MDSCs. Hypoxia-inducible factor 1α (HIF-1α) acting at tumor sites differentiates MDSCs into tumor-associated macrophages (TAMs).[33, 34, 35]

mechanisms of suppression

MDSCs show a variety of mechanisms that inhibit directly or indirectly T cell responses through direct cell-to-cell contact. MDSCs express the enzyme inducible nitric oxide synthase (iNOS) that uses the amino acid L-arginine as substrate to produce NO, which inhibits JAK3, STAT5 and MHCII and induces T cell apoptosis. L-arginine is used as a substrate from arginase-1 too and deprivation of L-arginine by these two enzymes inhibits T cell proliferation and downregulates TCRζ chain. MDSCs also produce reactive oxygen species (ROS). Superoxide anion, one of the main species of ROS, reacts with NO and produces peroxynitrite, a very powerful oxidant. Peroxynitrite nitrates and nitrosylates several amino acids (cysteines, methionines, tryptophans and tyrosines), TCR and CD8 molecules resulting in antigen-specific T cell unresponsiveness. These suppressive mechanisms are regulated by various cytokines. IFN-γ and its regulated genes play an important role in the suppressive function of tumor-induced MDSCs as it activates STAT1 through IFN-γ receptors and JAK kinases and eventually controls the expression of iNOS and arginase-1. IL-6 is another important cytokine as it activates STAT3 through gp130 and JAK kinases which controls ROS production.[28, 33, 36, 37]

Since the discovery that MDSCs can uptake, process and present tumor antigens[38], a great effort has been made to try to elucidate the antigen specificity of MDSCs suppression. Although there are some conflicting data, it is well established that suppression of $CD8^+$ T cells in peripheral lymphoid organs is antigen-specific and requires antigen presentation and direct cell-to-cell contact. At tumor sites, MDSCs function is enhanced by T cells and can suppress in an antigen-non-specific manner.[39] In a tumor microenvironment, MDSCs have been found not to upregulate the expression of CD80, which provides the necessary co-stimulatory signal for T cell priming[32], and their MHC II expression is detectable at high levels or remains unchanged after tumor induction, depending on the experimental setup.[40, 41]

The most important mechanism of immune suppression, but yet not fully understood, is the recruitment of regulatory T cells. Both the differentiation from naïve $CD4^+$ T cells and the expansion of pre-existing Treg cell populations have been described, through production of soluble factors (IFN-γ, IL-10 and TGFβ) in the presence of arginase-1 or

direct cell-to-cell contact through CD40-CD40L interaction, B7-H1 molecule and antigen presentation of tumor antigens. [19, 28, 39, 42, 43, 44]

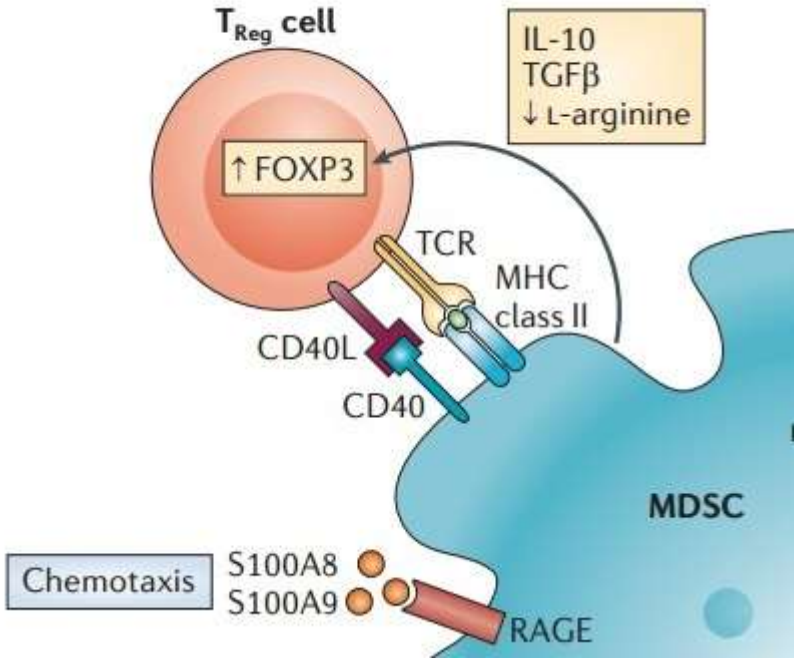


Figure 6. Regulatory T cell induction by myeloid-derived suppressor cells. MDSCs present tumor antigens under inflammatory conditions (IL-10 and TGFβ) and induce the expansion of regulatory T cells. (adopted from Coordinated regulation of myeloid cells by tumours, Nature Reviews Immunology, Volume 12, April 2012)

Dendritic cells

Dendritic cells are the most specialized antigen-presenting cells of the immune system and induce antigen-specific immune responses through antigen presentation to naïve T cells. In physiologic conditions, dendritic cells are generated in the bone marrow with the process of myelopoiesis discussed before. Soluble factors by the bone marrow stromal cells (GM-CSF and IL-3) and direct cell-to-cell contact drive the differentiation of common myeloid progenitors and immature myeloid cells towards immature DCs (iDCs). iDCs leave the bone marrow and are characterized by little or no expression of co-stimulatory molecules (CD40, CD80 and CD86) and produce low amounts of IL-12, the essential cytokine for T cell proliferation. Activation of iDCs by microorganisms or dying tumor cells upregulates the surface MHCII and co-stimulatory molecule levels and IL-12 secretion.

In a tumor tumor-bearing host, DCs are affected in many levels during differentiation and activation. These DC defects are systemic and are not localized to tumor tissues. The main observation is decreased production of mature functionally competent DCs in spleen and lymph nodes, as it is shown in many studies with tumor-bearing mice. Accumulation of immature DCs limits anti-tumor immunity, as these DCs have little or no expression of co-stimulatory and MHC molecules and induce T cell tolerance. Besides iDCs, MDSCs expansion is observed, as discussed before. These phenomena are induced by several tumor-derived factors (VEGF, GM-CSF, M-CSF, IL-6 and IL-10) that signal through STA3 which inhibits the differentiation and activation of iDCs and promotes their accumulation.[15, 45, 46, 47]

Tumor-associated macrophages

Macrophages are terminally differentiated myeloid cells, closely related to DCs and derive from monocytes circulating in blood. In a healthy individual they eliminate infectious agents, promote wound healing and regulate adaptive immunity. Two subsets of macrophages have been described; M1 macrophages, induced by IFNγ and bacteria, secrete high amounts of IL-12 and low amount of IL-10, thus promoting a Th1 response that can be tumoricidal. M2 macrophages, induced by IL-4, IL-10 and IL-13, secrete high amounts of IL-10 and promote regulatory T cell differentiation. The fate of macrophages is driven by the local tissue microenvironment where they reside.

In a tumor microenvironment, macrophages are M2-like, are characterized as tumor-associated macrophages (TAMs) and promote tumor evasion by several mechanisms. TAMs promote angiogenesis, tumor cell invasion and metastasis and protect tumor cells from chemotherapy-induced apoptosis. TAMs also use immune mechanisms, like elimination of M1 macrophage-mediated innate immune response and can impair T cell activation. IL-10 and TGFβ produced by TAMs can promote tumor progression by enhancing Treg cell activity.[39, 48]

Autophagy

introduction

Autophagy is a non-selective degradation system that delivers cytoplasmic constituents to the lysosome and it is distinct from the endocytotic route of extracellular materials. Autophagy is implicated in a variety of physiological and pathophysiological processes, like starvation adaptation, clearance of intracellular proteins and organelles, development, aging, elimination of microorganisms, cell death, tumor suppression and antigen presentation. The most typical trigger of autophagy is nutrient starvation and is a well-established method for autophagy induction in cultured cells.

Autophagosome is the major organelle involved during the process of autophagy; it is a double membrane structure that engulfs a portion of the cytoplasm. Three main events take place from the induction of autophagy to the degradation in lysosomes, with the involvement of several autophagy-related genes (Atg). Prior to autophagosome formation, an isolation membrane is formed that gradually encloses a portion of the cytoplasm; a process controlled by a complex of proteins formed by Atg6 (Beclin-1), PI3 kinase and the regulatory serine/threonine protein kinases ULK1 and ULK2. This isolation membrane can derive from the endoplasmatic reticulum (ER), the ER-mitochondria contact sites, the Golgi apparatus or from endosomal organelles. Formation of the autophagosome by elongation of isolation membrane and complete enclosure of the cytoplasm is controlled by two ubiquitin-like systems: the Atg8 (LC3) system that involves also Atg4, Atg3 and Atg7 proteins and the Atg12/Atg5 system that involves also Atg10 and Atg16. The targeting of proteins to autophagosomes is controlled by Alfy and p62 proteins.

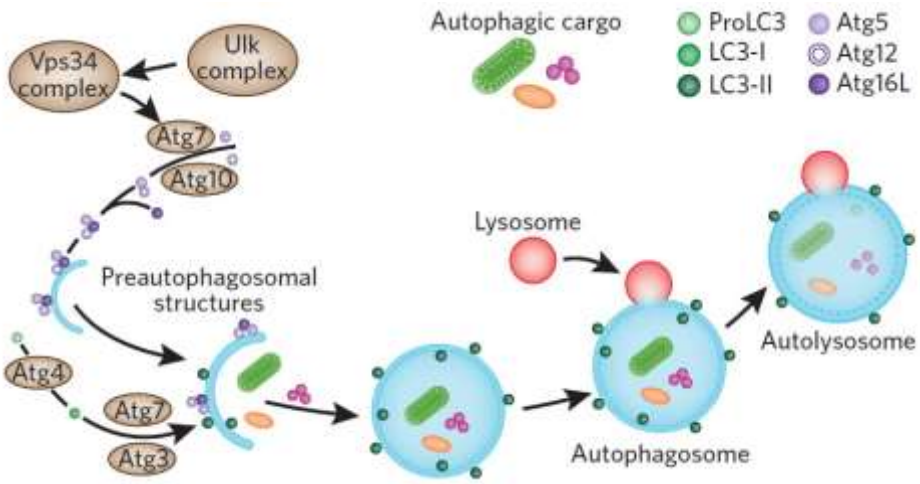


Figure 7. Autophagy pathway. Three main events characterize the pathway of autophagy; formation of isolation membrane, formation of autophagosome and fusion of autophagosome with lysosome. (Chemical modulators of autophagy as biological probes and potential therapeutics, Nature Chemical Biology, 7, 9-17, 2011)

Fusion of autophagosomes with lysosomes leads to the final maturation of autophagosomes into autolysosomes, a process controlled by Syntaxin 17 protein and Beclin-1/VPS34 complex. Fusion of the two organelles leads to acidification of the autophagosome lumen, acquisition of lysosomal hydrolases and degradation of enclosed proteins along with the inner membrane. [49, 50, 51, 52, 53, 54]

Autophagy is controlled at a signaling level by metabolic and immune signaling pathways. At resting state, TGFβ-activated kinases (TAK)-binding proteins 2 and 3 (TAB2, TAB3) bind Beclin-1 and suppress its function. During starvation, mTOR is inhibited and AMPK activity is increased, leading to activation of ULK1 and TBA proteins.[55] Immune signals also control autophagy through activation of Beclin-1 and engagement of several autophagic components. Pattern recognition receptor signaling and inflammatory cytokines like IL-1β and IFN-γ induce autophagy in effector cells, whereas Th2 cytokines (IL-4 and IL-13) and nitric oxide have been found to inhibit autophagy.[56]

immunity and inflammation

Autophagy has four major roles in immunity: elimination of intracellular microorganisms, control of inflammation, secretion of immune mediators and

regulation of adaptive immunity. Pattern recognition receptors (PRRs), the proteins that sense the invasion of microorganisms, are in close collaboration with the autophagic machinery to eliminate any incoming threat. Toll-like (TLRs) and Nod-like receptors (NLRs) are two classes of PRRs that sense microbial products (pathogen-associated molecular patterns; PAMPs) and have been shown to stimulate autophagy. Induction of autophagy by TLRs is one of the first steps against microbial invasion and there is a bidirectional influence that enhances both responses; whereas activation by NLRs promotes the accumulation of autophagy factors in the vicinity of incoming microorganisms. Nucleic acid sensors can either activate or inhibit autophagy (mainly in viral infections) and sequestosome 1-like receptors (SLRs) sense pathogen that have escaped control from conventional PRRs and activate autophagy to eliminate ubiquitin- or galectin-tagged targets.

Autophagy controls inflammation in several ways. Regulation of IFN-γ signaling generates feedback loops that can have either positive or negative effects. Generation of autoimmune plasma cells has been described in cases when autophagy delivers self DNA in TLR9 and activates B cells and promotes IFN-γ production by plasmacytoid DCs. Autophagy can also suppress pro-inflammatory protein complexes and inhibit IFN-γ production. The anti-inflammatory function of autophagy is enhanced by negatively regulating inflammasome, a cytoplasmic protein complex that is activated by PAMPs and danger-associated molecular patterns (DAMPs) and induces secretion of the highly inflammatory cytokines IL-1β and IL-18. The basal levels of autophagy under normal nutrient conditions help to clear cells from any defective protein and organelle that can act as inflammasome inducer and protects cells from sterile inflammation. Besides IL-1β, autophagy inhibits IL-1α secretion by controlling the calpain system.

Secretion of immune mediators like extracellular ATP, IL-6 and IL-18 has been shown to be influenced by autophagy. Excessive secretion of immunoglobulins by plasma cells is inhibited by autophagy, and serves as a protective mechanism against hyper-activation of the immune system. Autophagy is also involved in the unconventional secretion, under stress conditions, of IL-1β and IL-18 that lack the signal peptides for ER entry and conventional trafficking. Although autophagy inhibits inflammasome

under normal conditions, as discussed before, in response to PAMPs and DAMPs, autophagy increases inflammasome output during infection.

Autophagy regulates adaptive immunity in many aspects. Activation of autophagy has been linked with TCR signaling and CD28 co-stimulation in T cells during their activation. Activated T cells receive a pro-survival signal by autophagy that counteracts the FAS-FAS ligand apoptotic signal. Th17 polarization has been attributed to autophagy, as autophagy-deficient myeloid cells have been found to promote Th17 induction during bacterial infection. Autophagy is also important for B cell homeostasis, as it is important for preservation of the bone marrow plasma cell pool. Finally, autophagy has been described as a machinery for transporting proteins into the lumen of antigen-processing machinery of MHC II compartments and is hypothesized that it competes with proteasome for the degradation of cytoplasmic proteins and delivery for conventional MHC I presentation. Excessive discussion about the role of autophagy in antigen presentation will be made in the next section.[56]

Antigen presentation

overview

Antigens are loaded into major histocompatibility complex (MHC) molecules and prime T cell responses by the process of antigen presentation. Depending on their origin and subsequent intracellular trafficking, antigens can be loaded either on MHC I molecules and elicit $CD8^+$ T cell responses or on MHC II molecules and elicit $CD4^+$ T cell responses. The classical notion stated that exogenous proteins enter lysosomal/endosomal compartment and get loaded into MHC II molecules, whereas endogenous proteins enter proteasome and get loaded into MHC I molecules. Numerous studies have implicated autophagy in MHC II antigen presentation, whereas the involvement on MHC I pathway remains a controversial subject.[54]

MHC II pathway

MHC II molecules receive products of lysosomal degradation from either exogenous proteins that are endocytosed or by endogenous self-proteins and microorganisms. MHC II loading compartments (MIICs) then reach the surface and present their antigens in $CD4^+$ T cells; a process conducted exclusively by professional antigen presenting cells (APCs). Since autophagy is a pathway for protein delivery in lysosomal compartment, it was hypothesized that endogenous antigens gain access to the MIICs after being transported by autophagosomes. The viral antigen EBNA1 (nuclear antigen 1) of Epstein-Barr virus was the first endogenous antigen that have been found to be associated with MHC II presentation with the help of autophagosome trafficking.[57] Since then, more antigens have been identified, including the tumor antigen mucin 1, which can elicit $CD4^+$ T cell responses; manipulation of autophagy in antigen presenting cells affect T cell activation by these antigens.[58] Induction of autophagy by nutrient starvation increases the influx of resident intracellular antigens from mitochondria, cytoskeleton, and nucleus to lysosomes for display.[54, 59, 60, 61, 62]

MHC I pathway

The "classical" route for intracellular antigens (viral, tumor and self) that is conducted by all cell types is degradation by proteasome, traffic by transporters associated with antigen processing (TAPs) into the endoplasmic reticulum (ER) and binding with MHC I molecules for presentation to $CD8^+$ T cells. The role of autophagy in this pathway is very limited, since the autophagosome trafficking does not intersect with MHC I pathway; but it has been suggested that autophagy competes with the proteasome for the degradation of cytoplasmic proteins.[56, 63]

The role of autophagy in cross-presentation, the MHC I pathway that receives foreign and exogenous through phagocytosis, is controversial. Some studies support a role for autophagy as an effective vehicle for the delivery of exogenous tumor antigens and a requirement for efficient cross-presentation by DCs[64], whereas cross-presentation was not affected in autophagy deficient DCs in a viral infection setup.[56, 58]

i. Hypothesis

Myeloid-derived suppressor cells (MDSCs) have been established as a potent immune suppressor subset that have the ability to suppress T cell responses. A very important mechanism of suppression is the induction of Regulatory T cells, but the exact molecular mechanism remains unknown. Autophagy has been implicated in the delivery of antigens in MHC class II molecules, therefore we hypothesize that MDSCs present tumor antigens in an antigen-dependent manner to $CD4^+$ T cells under tolerogenic conditions and favor the differentiation of Regulatory T cells.

ii. Materials and Methods

Mice

Female C57BL/6 mice (6-12 weeks old), Foxp3-GFP mice (8-12 weeks old) and $Atg5^{fl/fl}$ mice (6-12 weeks old) were obtained from the Specific Pathogen Free unit of the Animal Facility in the Institute of Molecular Biology and Biotechnology (FORTH), Heraklion Crete, Greece. $LysM^{cre}$ mice on B6 background were housed at Medical School of the University of Crete (Greece) under the guidelines of the Animal Care committee of the University of Crete.

Tumor challenge

B16-F10 melanoma cells were a kind gift by Dr. A. Eliopoulos (Institute of Molecular Biology and Biotechnology, Heraklion Crete, Greece). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with high glucose concentration supplemented with 10% Fetal Bovine Serum and 1% penicillin/streptomycin (all from Life Technologies). Cell cultures were grown at 37°C in 5% CO_2 until 70-80% confluency and passaged with 0.05% Trypsin (Gibco, Life Technologies). Cells were washed with PBS prior to inoculation. 300,000 cells were injected subcutaneously at the dorsal area of the base of the tail. For tumor growth experiments, mice were shaved at the base of the tail one day before injection. Tumor volume was quantified by caliper measurement (length x $width^2/2$) after day 6 with the Cocraft Digital Vernier Caliper (Clas Ohlson, Sweden). Mice were anaesthetized with intra-peritoneal injection of xylazine/ketamine solution and photographed.

Flow Cytometry/sorting

Flow cytometric expression analyses were performed using the following anti-mouse fluorochrome-conjugated antibodies: CD11b (M1/70), CD11c (N418), F4/80 (BM8), Gr-1 (RB6-8C5), Ly-6G (1A8), Ly-6C (HK1.4), CD4 (GK1.5), CD8 (53-6.7), CD3 (145-2C11) (all from BioLegend). Intracellular staining for Foxp3 protein was performed using the Foxp3 Transcription Factor Staining Buffer Set (eBioscience) and Alexa Fluor® 488 Foxp3 (MF-14) antibody (BioLegend). Tumors were excised and treated with 1mg/ml Collagenase D (Roche) and 0.5 μg/ml DNase (Sigma-Aldrich) for 45 minutes at 37°C.

Spleens were erythrolysed with 2 minutes incubation with NH_4Cl. Treated tumor and spleen tissues along with bone marrow, thymus, inguinal and mesenteric lymph nodes were passed through 70μm cell strainers to obtain single cell suspensions. Staining of tissues with fluorochrome-conjugated antibodies was performed in dark, at 4°C for 20 minutes. Flow Cytometry and cell sorting were conducted in BD FACSCalibur and Dako Cytomation MoFlo cell sorter and analyzed with FlowJo software (version 7, Tree Star). The purity of sorted cells was 90-95%.

Real-Time PCR

RNA was extracted from sorted cells with PureLink® RNA Mini Kit and treated with DNase with TURBO DNA-free™ Kit (both from Ambion Life Technologies). RNA concentration and purity was measured in NanoDrop 2000 spectrophotometer (Thermo Scientific). cDNA was synthesized with SuperScript™ First-Strand Synthesis System for RT-PCR using oligo(DT) primers according to manufacturer's instructions (Invitrogen Life Technologies). Real-Time PCR was performed on CFX Connect™ Real-Time PCR Detection System (BIO-RAD) using SYBR green incorporation. cDNA was amplified with iTaq™ Universal SYBR® Green Supermix (BIO-RAD) in 20μl reaction and each sample was added in duplicate. Reactions were performed with the following thermal profile: 95°C for 2 min, 40 cycles of 95°C for 15 sec and 58°C or 60°C for 30 sec, followed by melting curve protocol from 65°C to 95°C with 0.5°C increment for 5 sec each. Data quantitation was performed using relative quantification with the 2-ΔΔCT method. The following primers were used (400nM final concentration each): *Atg5* (forward, 5'- AGCTCTGGATGGGACTG-3'; reverse, 5'- CTCCGTCGTGGTCTGAT-3'), *Bcl1* (forward, 5'-GGACAAGCTCAAGAAAACCAATG-3'; reverse 5'-TGTCCGCTGTGCCAGATGT-3'), *arginase-1* (forward, 5'-CAGAAGAATGGAAGAGTCAG-3'; reverse, 5'-CAGATATGCAGGGAGTCACC-3'), *CEBP/b* (forward, 5'-ACGGGACTGACGCAACACAC-3'; reverse, 5'-CCGCAGGAACATCTTTAAG-3'), *TGFβ* (forward, 5'-AGGTCACCCGCGTGCTAATG-3'; reverse, 5'- CCATTGCTGTCCCGTGCAGA-3'), *GAPDH* (forward, 5'- CCAGTATGACTCCACTCACG-3'; reverse, 5'-CTCCTGGAAGATGGTGATGG-3') and *HPRT* (forward, 5'-GTGAAACTGGAAAAGCCAAA-3'; reverse, 5'-GGACGCAGCAACTGACAT-3'). The expression of Atg5, Bcl1, arginase-1, iNOS, TGFβ and CEBP/b was normalized to GAPDH or HPRT.

Statistical analysis

The statistical significance between values was determined by t test. All data are expressed as the mean +/-SD. Statistical analyses were performed with the GraphPad Prism (Version 6.01) software.

iii. Results

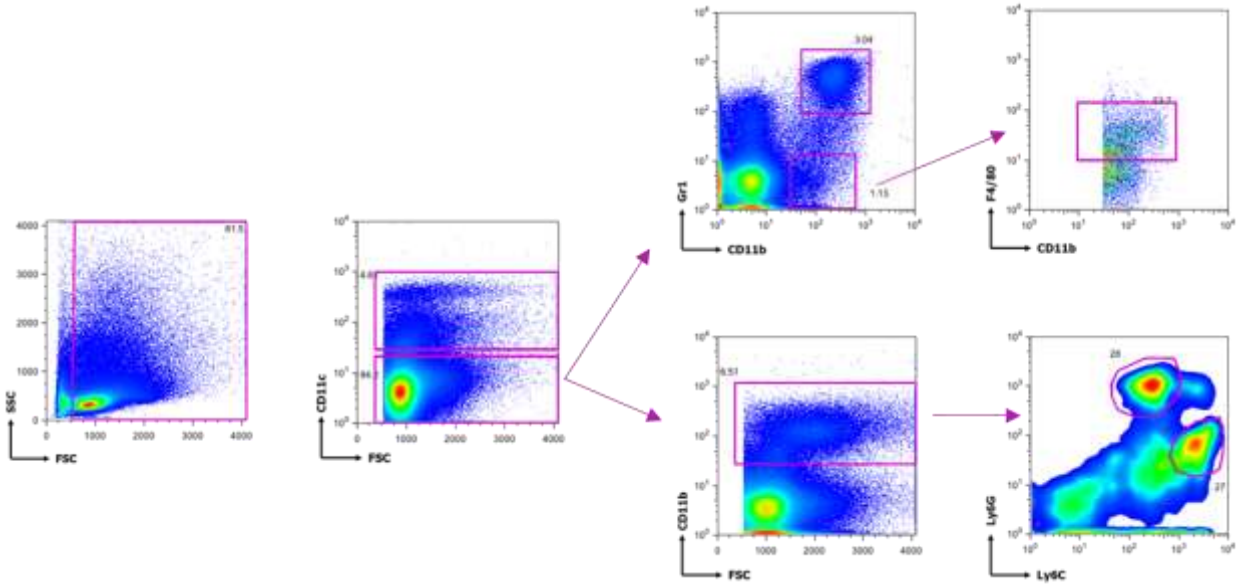
Upregulation of autophagy in myeloid cells during tumor growth

B16 melanoma mouse model is a low immunogenic tumor model that is widely used to study immune responses during tumor growth and evaluate the efficiency of possible therapies.[65] B16 melanoma cells can be administered in mice in two ways; a subcutaneous administration provides a solid "bleb" at the area of injection, whereas an intravenous injection is the common tool for study of metastasis. We utilized the subcutaneous model in C57BL/6 mice and evaluated the kinetics of myeloid cells in the spleen of tumor-bearing mice 8 and 10 days after injection. Specifically we examined dendritic cells (DCs) which are characterized as $CD11c^+$ cells, myeloid-derived suppressor cells (MDSCs) which are characterized as $CD11c^-$ $Gr\text{-}1^+$ $CD11b^+$ and tumor-associated macrophages (MΦs) as $CD11c^-$ $Gr\text{-}1^-$ $CD11b^+$ $F4/80^+$. The two MDSCs subsets are characterized as follows: granulocytic MDSCs are $Gr\text{-}1^{hi}$ $Ly6G^+$ $Ly6C^-$ and monocytic MDSCs are $Gr\text{-}1^{int}$ $Ly6G^-$ $Ly6C^+$ (Fig. 1A). Consisted with previous studies[32], MDSCs expanded both in absolute numbers and percentages; monocytic-like MDSCs showed the greater expansion among the subsets with a 2-fold increase (G-MDSCs: from 8590±546 to 14826±1844, *p=0.03, and M-MDSCs: from 4264±304 to 11291±473, *p=0.0002), but granulocytic-like MDSCs remained the predominant population with 1.5:1 ratio to M-MDSCs (Fig. 1B). Dendritic cells also showed significant expansion, whereas macrophage absolute numbers were decreased on day 8. Interestingly, the percentage of macrophages within the $CD11b^+Gr\text{-}1^-$ population was increased, suggesting an overall expansion of $CD11b^+$ cells. In all three myeloid cell populations, the greater difference, either increase or decrease, was observed 8 days after injection (Fig. 1B).

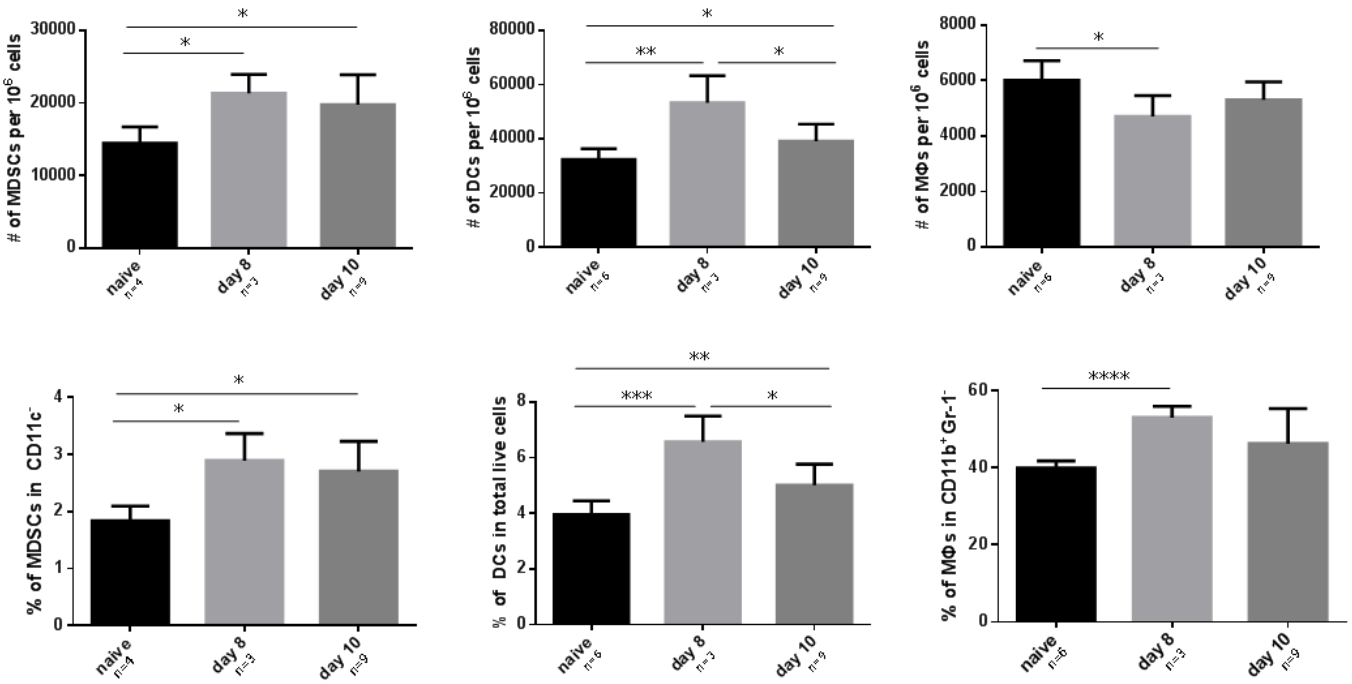
Next, we monitored the status of autophagy in myeloid cells of tumor-bearing mice by measuring the mRNA levels of two autophagy associated genes, Atg5 and Bcl1.[66] MDSCs, DCs and macrophages were sorted from spleens of naïve and tumor bearing-mice and the expression levels of Atg5 and Bcl1 were assessed with Real-Time PCR. Both genes increased in all myeloid subsets 10 days after injection (Fig. 1C). Specifically, the highest induction of autophagy was observed in MDSCs where it reached statistical significance in Bcl1 (Atg5: 1.014±0.125 versus 1.683±.02664 and

Bcl1: 1.034±0.185 versus 2.494±0.375, *p=0.03); DCs had moderate induction of autophagy that reached statistical significance in both genes (Atg5: 1.001±0.026 versus 1.317±0.090, *p=0.01 and Bcl1: 1.013±0.088 versus 1.378±0.075, *p=0.02), and in macrophages only Bcl1 reached statistical significance 10 days after injection (1.010±0.085 versus 1.676±0.179, *p=0.01). Overall, MDSCs expand in tumor-bearing mice and the inflammatory milieu of B16 melanoma upregulates autophagy.

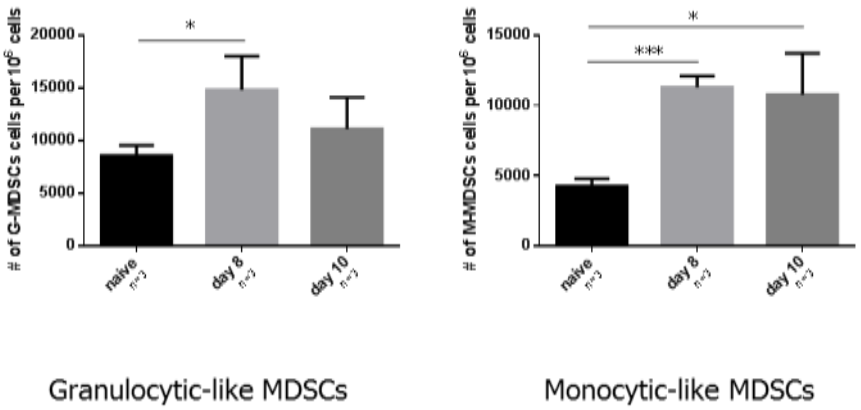
A. Gating strategy



B. Frequencies of myeloid cells in spleen during tumor growth

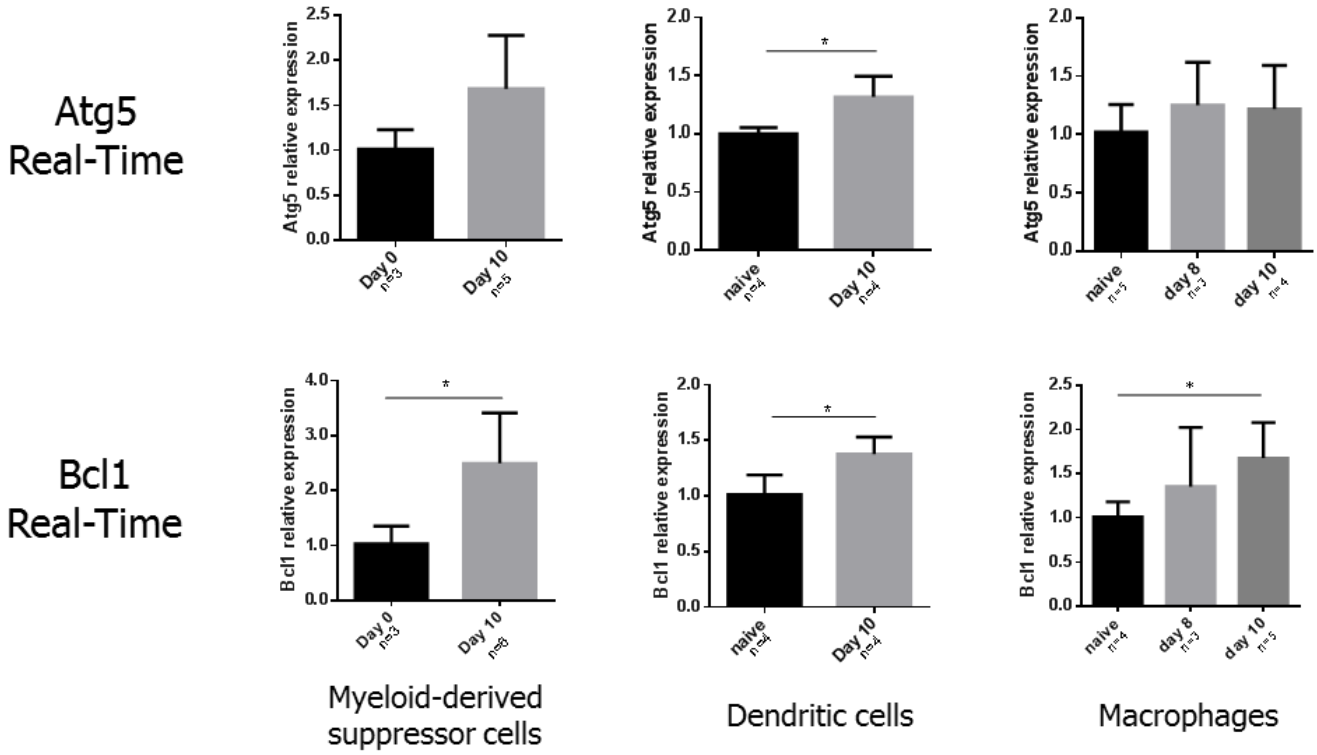


Myeloid-derived suppressor cells | Dendritic cells | Macrophages



Granulocytic-like MDSCs Monocytic-like MDSCs

C. Real-Time for autophagy markers in myeloid cells



Myeloid-derived suppressor cells Dendritic cells Macrophages

Figure 1. Upregulation of autophagy in myeloid cells during tumor growth
C57BL/6 mice were injected subcutaneously with $3*10^5$ B16/F10 melanoma cells and spleens were analyzed on day 8 and day 10. Naïve unimmunized mice were used as control.
A) Gating strategy for characterization of major myeloid cell populations. Dendritic cells (DCs) are characterized as $CD11c^+$ cells; myeloid-derived suppressor cells (MDSCs) as $CD11c^-$ $Gr\text{-}1^+$ $CD11b^+$ and tumor-associated macrophages (MΦs) as

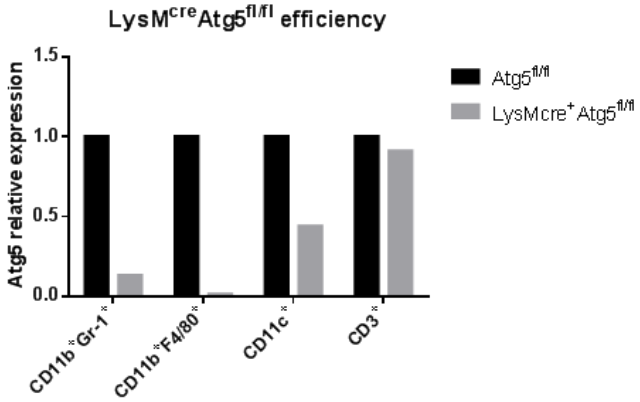
$CD11c^-$ $Gr\text{-}1^-$ $CD11b^+$ $F4/80^+$. MDSCs are divided in two distinct subsets; granulocytic like MDSCs ($Gr\text{-}1^{hi}$ $Ly6G^+$ $Ly6C^-$) and monocytic-like MDSCs ($Gr\text{-}1^{int}$ $Ly6G^-$ $Ly6C^+$).
B) Spleens were collected from naïve and B16-immunized C57BL/6 mice and analyzed by flow cytometry for the presence of myeloid cells. Absolute numbers and frequency of myeloid cells are presented. DCs frequency was calculated in total live cells; MDSCs frequency in gated $CD11c^-$ cells; MΦs frequency in gated $CD11b^+Gr\text{-}1^-$ cells; and G-MDSCs and M-MDSCs frequency in gated $CD11c^-CD11b^+$ cells. (*p<0.05, **p<0.0099, ***p<0.0009, ****p<0.00001) (n=3-9)
C) Dendritic cells, MDSCs and MΦs were sorted from spleens of naïve and B16 melanoma immunized mice using the previous gating strategy. RNA was extracted and Real-Time was performed for autophagy genes Atg5 and Bcl1. Relative expression is presented using as control group (expression=1) the unimmunized mice. (*p<0.05)

Immunophenotyping of LysM^{cre}Atg5$^{fl/fl}$ mice

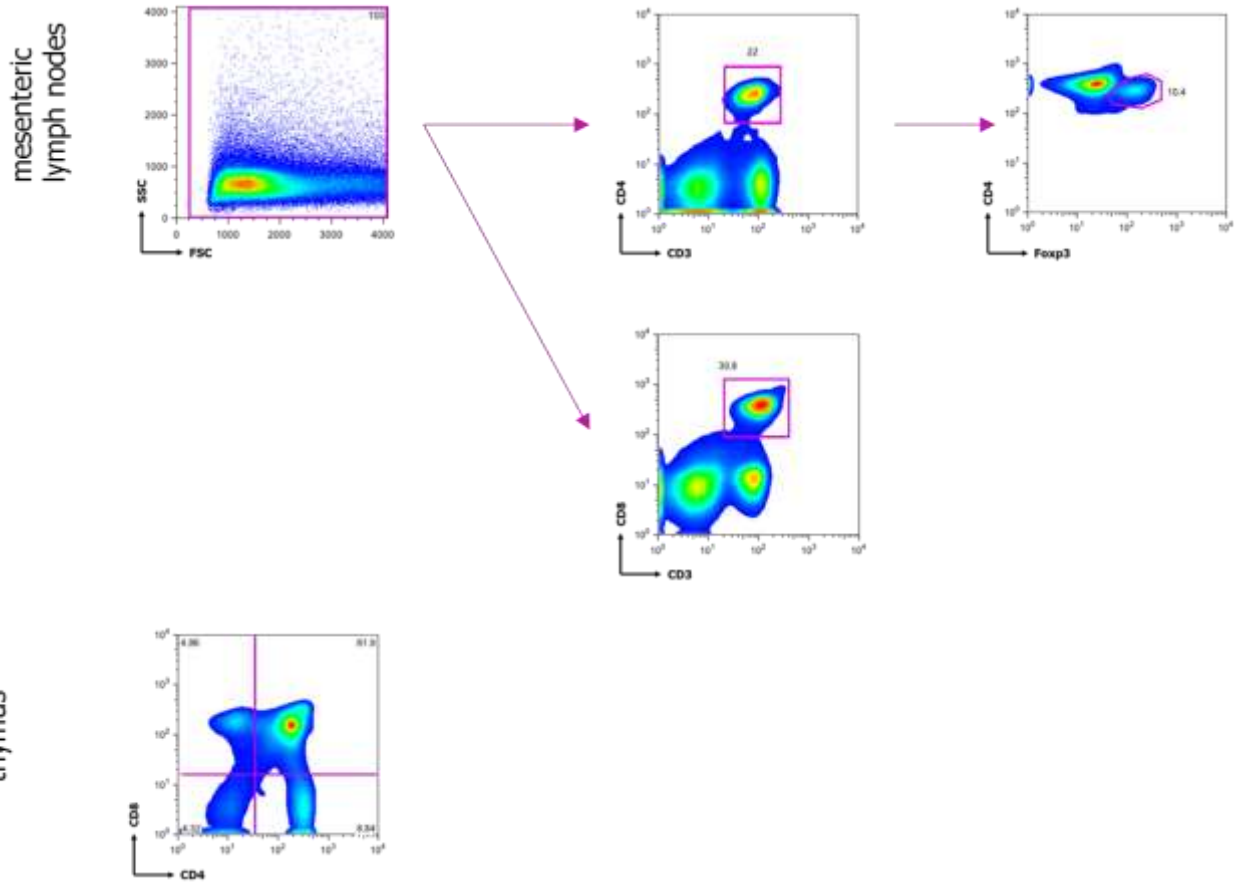
In order to study the impact of myeloid cells autophagy in tumor immune responses, we crossed LysM^{cre} mice, which have the Cre recombinase sequence under the lysozyme M promoter, with Atg5$^{fl/fl}$ mice that have the Atg5 gene flanked by loxP sites. The generated LysM^{cre}Atg5$^{fl/fl}$ mice have a conditional knock-out of the Atg5 gene in cells of myeloid origin that express the LysM gene. Deficit autophagy in myeloid cells is established in previous studies that showed an inefficient conversion of LC3-I to LC3-II.[67, 68] Before implicating these mice in any functional experiment, we assessed their immune phenotype under naïve conditions. First, we evaluated the efficiency of the Cre/loxP recombination system in our breedings by Real-Time PCR for the Atg5 mRNA using as control the Atg5$^{fl/fl}$ mice. As expected, Atg5 relative expression was very low in myeloid cells (MDSCs: 1.00 versus 0.13, DCs: 1.00 versus 0.44, and macrophages: 1.00 versus 0.011) and was not affected in CD3$^+$ T cells (1.00 versus 0.91) (Fig. 2A).

Next, we analyzed the frequencies of T and myeloid cells that are implicated in an anti-tumor response. The absence of autophagy from myeloid cells did not affect the generation of CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cells in the thymus and did not conflict with their presence in the periphery. Moreover, regulatory T cells are generated in the same rate in LysM^{cre}Atg5$^{fl/fl}$ and control mice and their frequencies in the periphery are at the same levels (Fig. 2B-C). Myeloid cells themselves were also not affected and appeared in similar frequencies in spleen (Fig. 2D). Collectively, the LysM^{cre}Atg5$^{fl/fl}$ mice are a suitable tool to study the effect of autophagy in myeloid cells, as their immune phenotype is the same as control mice.

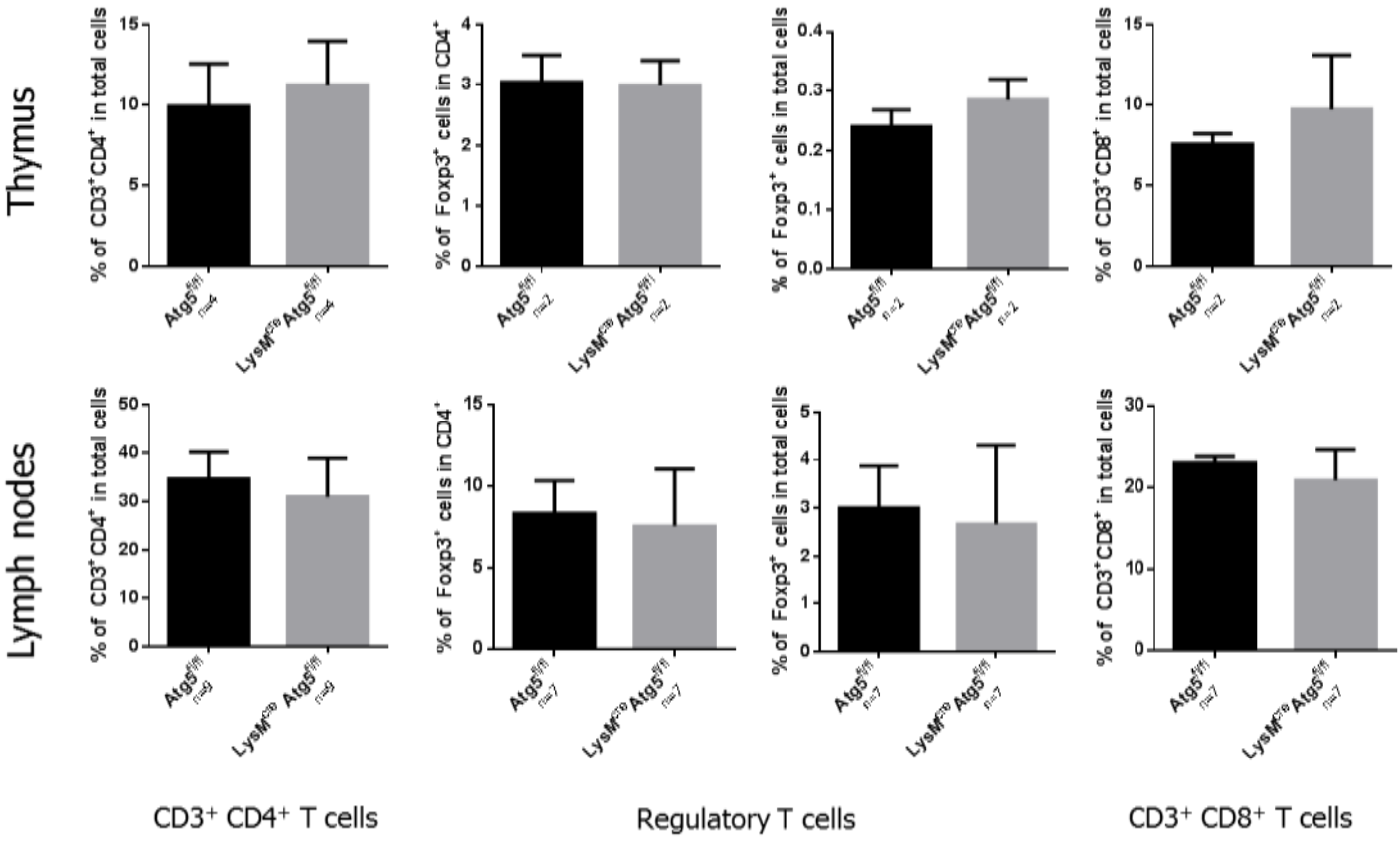
A. Real-Time for Atg5 in myeloid and T cells



B. Gating strategy of T cells



C. Frequencies of T cells



D. Frequencies of myeloid cells

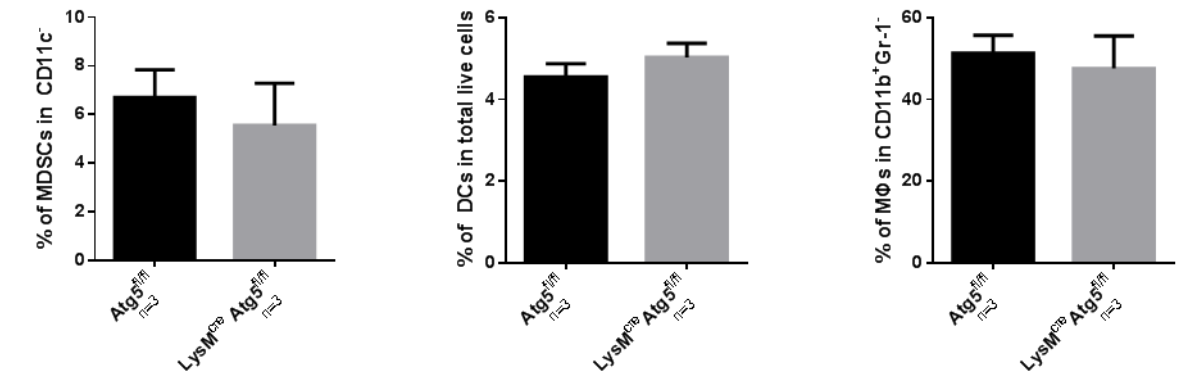


Figure 2. Immunophenotyping of LysM^{cre}Atg5$^{fl/fl}$ mice.
Evaluation of the cre/lox system in LysM^{cre}Atg5$^{fl/fl}$ and control Atg5$^{fl/fl}$ mice.
A) Myeloid-derived suppressor cells were sorted from bone marrow, dendritic cells and macrophages from spleen and CD3^{+} T cells from mesenteric lymph nodes of LysM^{cre}Atg5$^{fl/fl}$ and control Atg5$^{fl/fl}$ mice. RNA was extracted and Real-Time was performed for Atg5 mRNA. Relative expression is presented using as control group (expression=1) the Atg5$^{fl/fl}$ mice.
B) Gating strategy for the characterization of T cell populations. CD4 T cells are characterized as CD3^{+} CD4^{+}; CD8 T cells as CD3^{+} CD8^{+}; and T regulatory cells as CD3^{+} CD4^{+} Foxp3^{+}.
C) Thymus and mesenteric lymph nodes were collected from LysM^{cre}Atg5$^{fl/fl}$ and control Atg5$^{fl/fl}$ mice and analyzed by flow cytometry for the presence of T cell subsets CD3^{+} CD4^{+} and CD3^{+} CD8^{+}. Intracellular staining for Foxp3 was performed and regulatory T cells (CD3^{+} CD4^{+} Foxp3^{+}) levels were evaluated by flow cytometry. Absolute numbers and frequency are presented. CD3^{+} CD4^{+} and CD3^{+} CD8^{+} frequencies were calculated in total thymus and lymph nodes cells. Regulatory T cells frequency was calculated in gated CD3^{+} CD4^{+} and in total cells. (n=2-9)
D) Spleen and bone marrow were collected from LysM^{cre}Atg5$^{fl/fl}$ and control Atg5$^{fl/fl}$ mice and analyzed by flow cytometry for the presence of myeloid cells. Frequency of MDSCs, DCs and MΦs is presented was calculated as on Fig. 1B.

Impaired autophagy in myeloid cells decreased tumor growth

In order to address the role of myeloid cells autophagy in tumorigenesis, we subcutaneously injected LysM^{cre}Atg5$^{fl/fl}$ and control Atg5$^{fl/fl}$ mice with 3*10^5 B16-F10 melanoma cells and monitored tumor growth daily 6 to 12 days after injection. Interestingly, a decreased tumor growth in LysM^{cre}Atg5$^{fl/fl}$ mice was first observed 8 days after injection (LysM^{cre}Atg5$^{fl/fl}$: 193.1±22.80 versus control: 297.2±5.81, **p=0.0055), was established throughout the monitoring (day 12, LysM^{cre}Atg5$^{fl/fl}$: 443.5±19.25 versus control: 621.2±16.35, ***p=0.0003) (Fig. 3A) and could also be macroscopically observed (Fig. 3B).

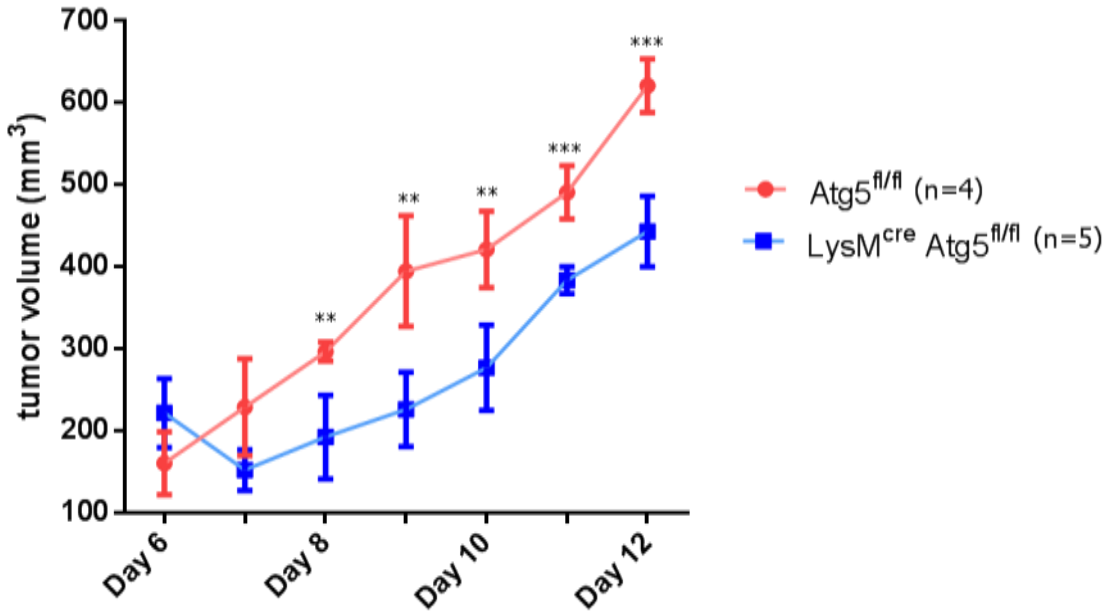
To elucidate this phenomenon, we first evaluated the levels of T cells in the draining lymph nodes of tumor-bearing mice. Interestingly, cytotoxic CD8$^+$ T cells were decreased in LysM^{cre}Atg5$^{fl/fl}$ mice (17.40±1.24 versus control: 25.57±0.12, **p=0.0027) which was attributed to the overall decrease of CD3$^+$ T cells in LysM^{cre}Atg5$^{fl/fl}$ mice (41.32±2.11 versus control: 62.70±2.15, ***p=0.0006) that also resulted in decreased CD4$^+$ T cells. Consisted with these differences were also the reduced frequencies of Foxp3$^+$ regulatory T cells in total lymph node cells, although the frequencies of regulatory T cells within the CD4$^+$ T cells were at the same levels between LysM^{cre}Atg5$^{fl/fl}$ and control mice (Fig. 3C).

Further analysis in spleens of tumor bearing-mice revealed a markedly expansion of MDSCs in LysM^{cre}Atg5$^{fl/fl}$ mice, reaching a two-fold increase compared to control mice (LysM^{cre}Atg5$^{fl/fl}$: 34460±4498 versus control: 17955±2715, *p=0.04). Dendritic cells reached the same levels in the two groups of mice, whereas macrophage absolute numbers were decreased in LysM^{cre}Atg5$^{fl/fl}$, but on the same time their frequencies within the CD11b$^+$Gr-1$^-$ population were increased (Fig. 3D).

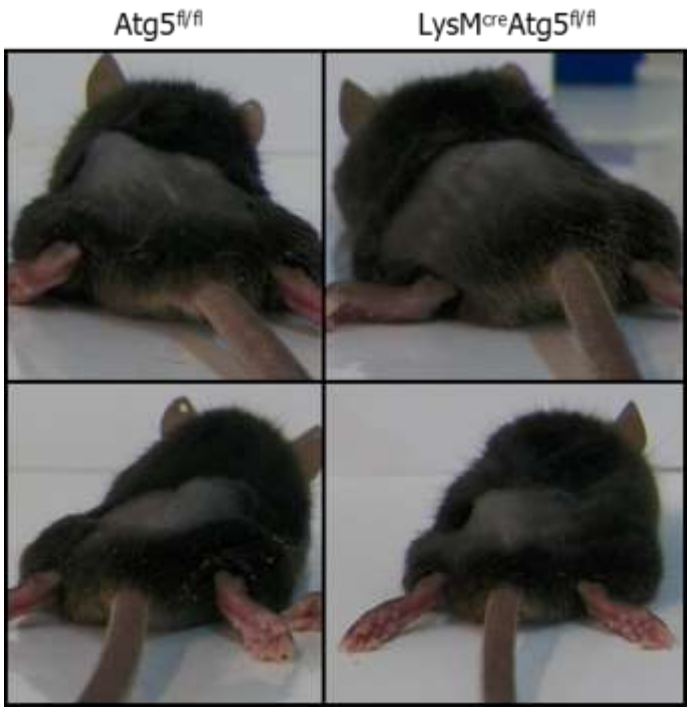
Besides the affected presence of myeloid cells between LysM^{cre}Atg5$^{fl/fl}$ and control mice, a more detailed analysis of their function would give an insight on how the absence of autophagy affects their function and subsequent the activation of T cells. We evaluated the levels of the effector molecules arginase-1, TGFb and CEBP/b with Real-Time PCR in sorted MDSCs, dendritic cells and macrophages from the spleens of LysM^{cre}Atg5$^{fl/fl}$ and control tumor-bearing mice. Arginase-1, a major suppressive molecule of MDSCs, was undetectable in our experimental setup in control mice, but present in LysM^{cre}Atg5$^{fl/fl}$ mice. Arginase-1, which has also been implicated in the

suppressive function of dendritic cells in tumor bearing-mice, reached a four-fold upregulation in autophagy-deficient dendritic cells (4.157±0.12 versus control: 1.003±0.07, ***p=0.0003). TGFβ had lower expression in autophagy-deficient myeloid cells, but did not reach statistical significance in any case. CEBP/b, that regulates the immunosuppressive function of MDSCs and controls the differentiation of macrophages, showed the same expression levels between the two groups (Fig. 3E). Overall, $LysM^{cre}Atg5^{fl/fl}$ mice have a distinct phenotype that is characterized by reduced tumor growth, reduced T cell numbers in draining lymph nodes and great expansion of MDSCs in the periphery.

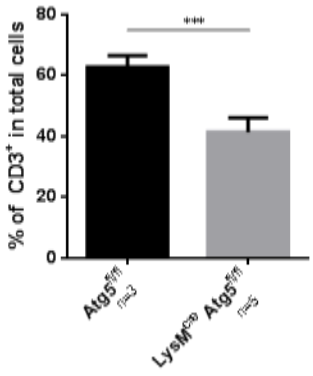
A. Tumor growth



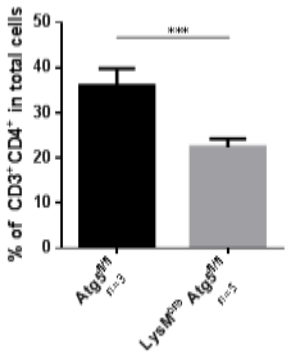
B. Photographs



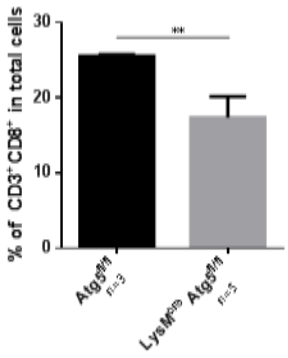
C. Frequencies of T cells



CD3+ T cells

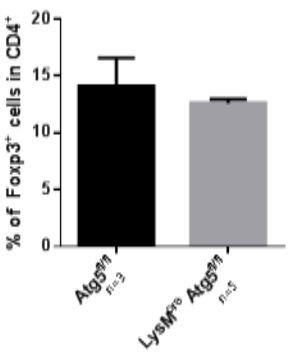


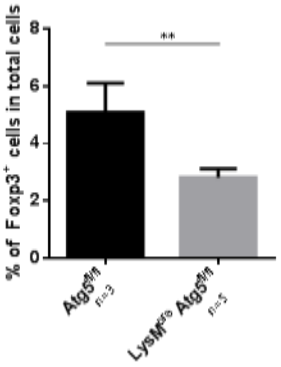
CD3+ CD4+ T cells



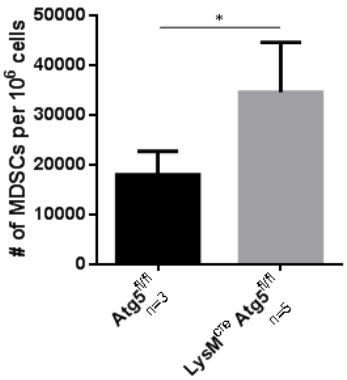
CD3+ CD8+ T cells

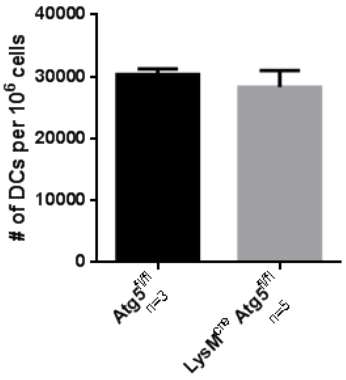
Regulatory T cells

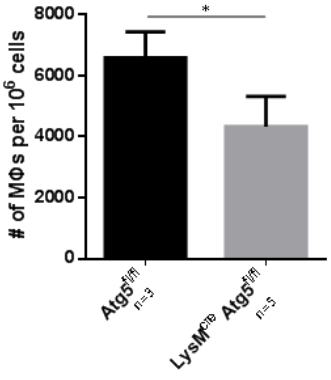


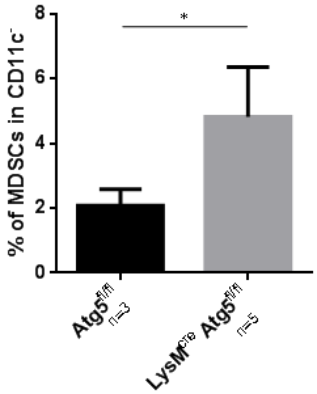


D. Frequencies of myeloid cells

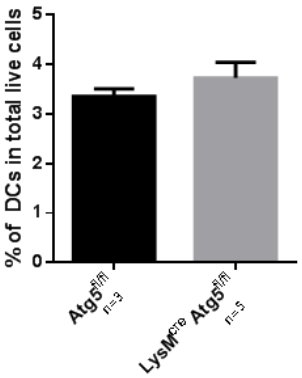




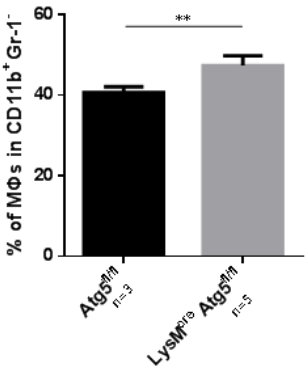




Myeloid-derived suppressor cells



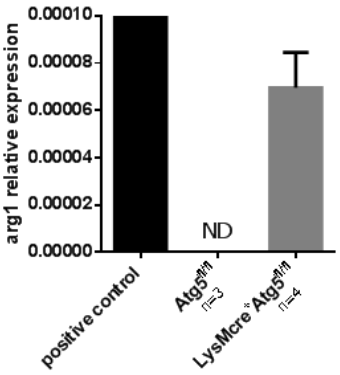
Dendritic cells

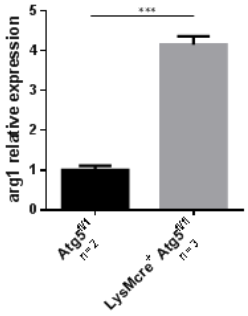


Macrophages

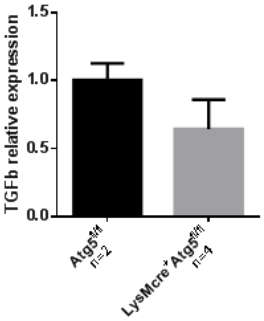
E. Effector molecules in myeloid cells

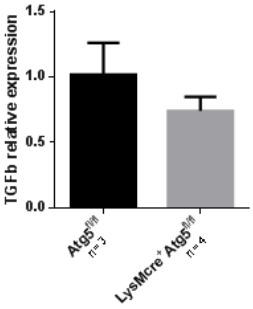
Arginase-1
Real-Time

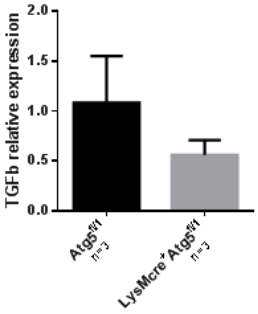




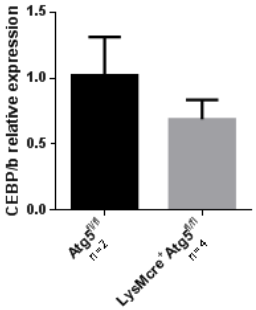
TGFb
Real-Time

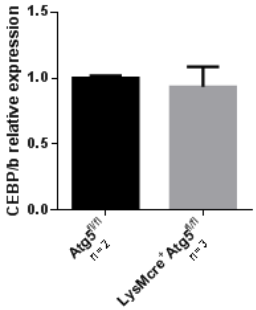






CEBP/b
Real-Time





Myeloid-derived suppressor cells Dendritic cells Macrophages

Figure 3. Impaired autophagy in myeloid cells decreases tumor growth
$LysM^{cre}Atg5^{fl/fl}$ mice and control $Atg5^{fl/fl}$ mice were subcutaneously injected with $3*10^5$ B16-F10 melanoma cells and monitored for tumor growth; spleens, inguinal lymph nodes and tumors were analyzed.
A) Tumor growth in $LysM^{cre}Atg5^{fl/fl}$ and control $Atg5^{fl/fl}$ mice. Tumor volume was measured daily after day 6.
B) Macroscopic observation of tumor growth 10 days after B16-F10 injection.
C) Draining inguinal lymph nodes were collected from $LysM^{cre}Atg5^{fl/fl}$ and control $Atg5^{fl/fl}$ mice 13 to 14 days after injection and analyzed by flow cytometry for the presence of T cell subsets as on Fig 2C. Absolute numbers and frequency are presented. CD3+, CD3+ CD4+ and CD3+ CD8+ frequencies were calculated in total lymph nodes cells. Regulatory T cells frequency was calculated in gated CD3+ CD4+ and in total cells.
D) Spleens and tumors were excised from $LysM^{cre}Atg5^{fl/fl}$ and control $Atg5^{fl/fl}$ mice and analyzed by flow cytometry for the presence of myeloid cells. Frequency of MDSCs, DCs and MΦs is presented and was calculated as on Fig. 1B.
E) Dendritic cells, MDSCs and TAMs were sorted from spleens of $LysM^{cre}Atg5^{fl/fl}$ and control $Atg5^{fl/fl}$ mice using the previously described gating strategy. RNA was extracted and Real-Time was performed for arginase 1, TGFβ and CEBP/b mRNA. Relative expression is presented using as control group (expression=1) the $Atg5^{fl/fl}$ mice.

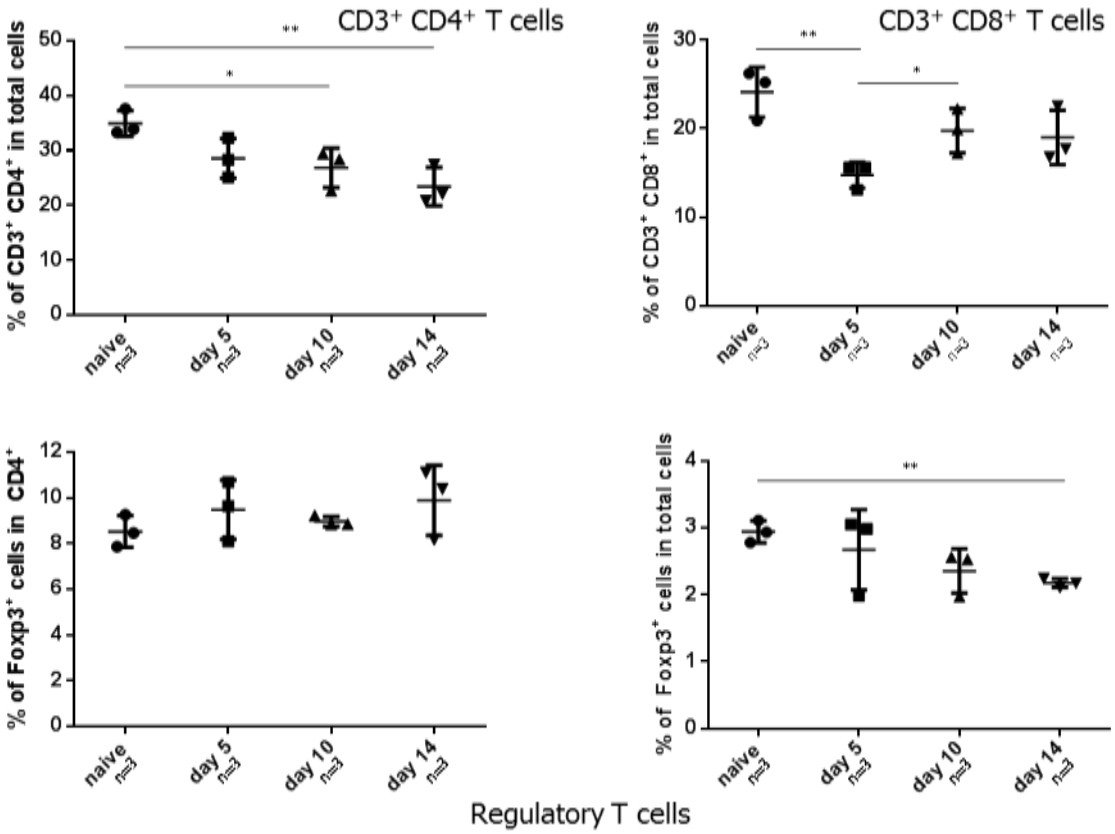
Kinetics of immune response during tumor growth

The importance of regulatory T cells during an anti-tumor immune response led us to analyze their kinetics in parallel with myeloid cells to try and find if any a quantitative relation between these two immune cell subsets. We subcutaneously injected the Foxp3-GFP reporter mice with $3*10^5$ B16-F10 melanoma cells and evaluated the frequencies of T and myeloid cells in draining lymph nodes and spleens 5, 10 and 14 days after injection. Lymph node $CD4^+$ and $CD8^+$ T cells decreased as tumor started to establish; the largest decrease of $CD4^+$ T cells was observed on day 14 (naïve: 34.93±1.34 versus day 14: 23.43±2.01, **p=0.009) and of $CD8^+$ T cells on day 5 (naïve: 24.10±1.62 versus day 5: 14.77±0.83, **p=0.0069). The frequencies of regulatory T cells in total lymph node cells followed the decrease of $CD4^+$ T cells, but their percentage within the $CD4^+$ T cells remained level. Circulating $CD4^+$ T cells found in the spleens slightly decreased on day 14 (naïve: 18.00±1.07 versus day 14: 13.97±0.86, *p=0.04), whereas $CD8^+$ T cells numbers were equal throughout tumor growth. Regulatory T cells frequencies within $CD4^+$ T cells remain unchanged and on the same time their frequencies in total splenocytes increased on day 5 and eventually dropped below naïve levels (naïve: 2.043±0.07 versus day 14: 1.763±0.05, *p=0.034) (Fig. 4A).

MDSCs and dendritic cells in spleens increased during tumor growth, but these differences did not reach statistical significance. The levels of macrophages remained level throughout the experiment (Fig. 4B).

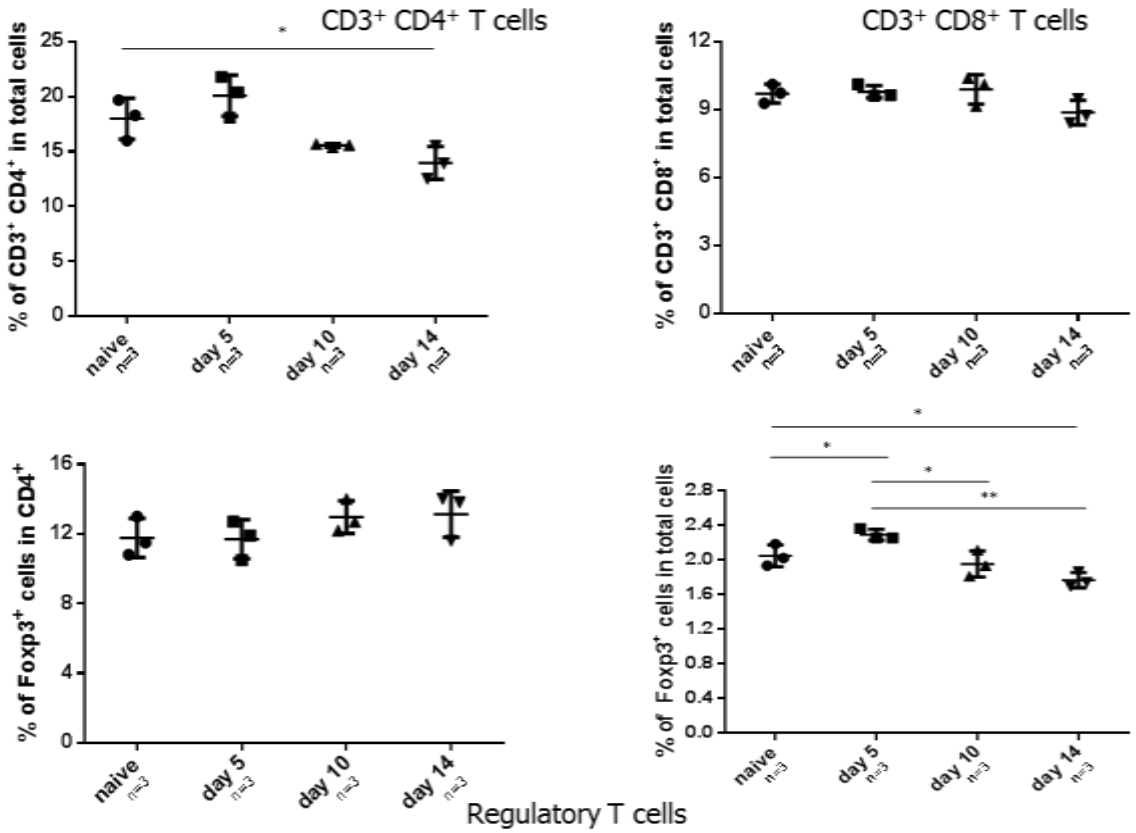
A. Frequencies of T cells during tumor growth

inguinal lymph nodes



Regulatory T cells

spleen



Regulatory T cells

B. Frequencies of myeloid cells during tumor growth

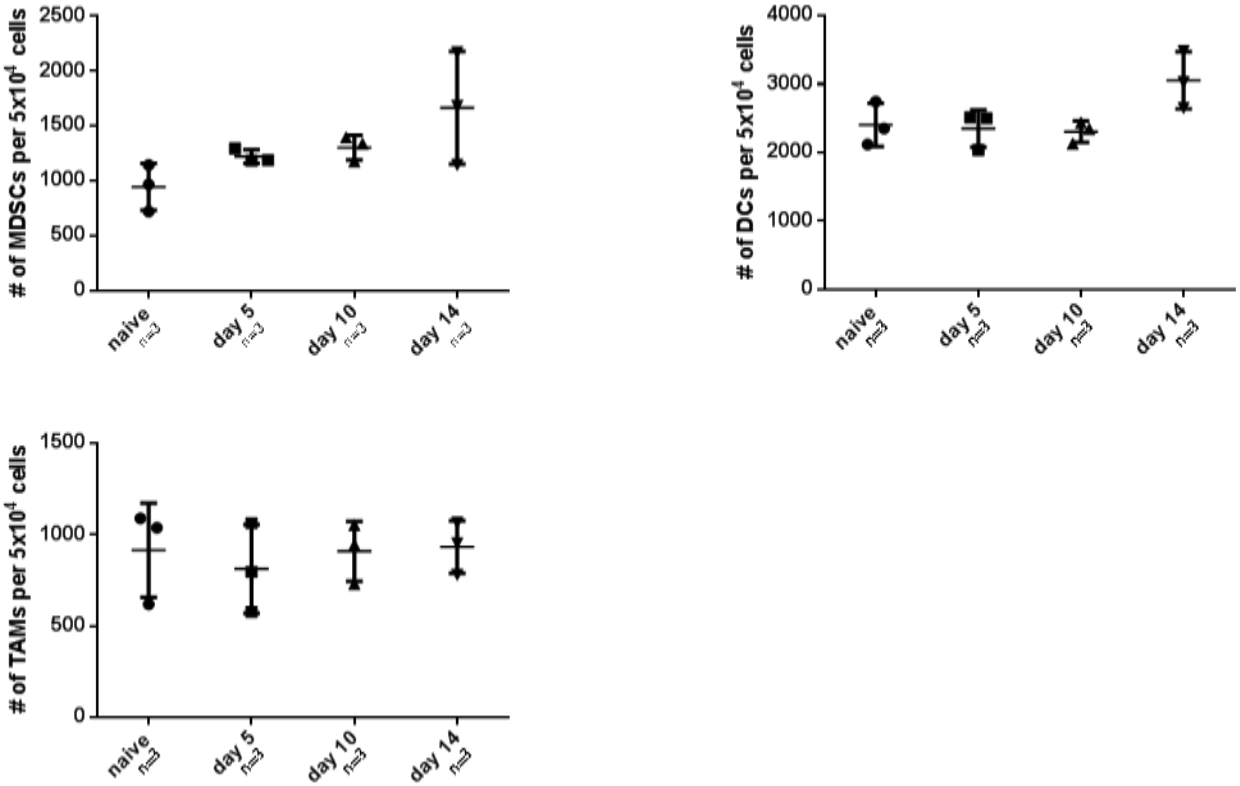


Figure 4. Kinetics of T cells in Foxp3-GFP mice
Foxp3-GFP mice were injected with B16-F10 melanoma cells and spleens, inguinal lymph nodes and tumors (when applicable) were analyzed on day 5, day 10 and day 14. Naïve unimmunized Foxp3-GFP mice were used as control.
A) Draining inguinal lymph nodes were collected and analyzed by flow cytometry for the presence of T cell subsets, as on Fig 2C. Absolute numbers and frequency are presented. CD3+ CD4+ and CD3+ CD8+ frequencies were calculated in total lymph nodes cells. Regulatory T cells frequency was calculated in gated CD3+ CD4+ and in total cells. (n=3)
B) Spleens were excised and analyzed by flow cytometry for the presence of myeloid cells. Frequency of MDSCs, DCs and MΦs is presented and was calculated as on Fig. 1B. (n=3)

iv. Discussion and future directions

Our results supported a very important role for autophagy in the myeloid cell compartment (MDSCs, dendritic cells and macrophages) since LysM^{cre}Atg5$^{fl/fl}$ mice had reduced tumor growth. Tumor-bearing mice with deficit autophagy in myeloid cells showed reduced T cell numbers in draining lymph nodes, great expansion of MDSCs in spleen and differential expression of several functional molecules and enzymes. Collectively these results provide evidence to directly address our hypothesis and to delineate the molecular mechanism underlying this phenomenon.

Interpretation of the observed phenotype in LysM^{cre}Atg5$^{fl/fl}$ mice requires additional experiments to decipher the pathways that are affected by the absence of autophagy and the implications of autophagy-deficient MDSCs in regulatory T cell expansion and/or induction. First, in order to evaluate the suppressive status of autophagy-deficient MDSCs, we will perform a co-culture proliferation assay. MDSCs will be sorted from control and LysM^{cre}Atg5$^{fl/fl}$ tumor-bearing mice and will be co-cultured with naïve CD4^{+}CD25^{-} T cells in the presence of anti-CD3/anti-CD28. Using the same experimental setup, we can evaluate the induction of regulatory T cells from CD4^{+}CD25^{-} T cells and the proliferation of regulatory T cells under the influence of MDSCs. Finally, using an antigen-specific tumor model, we will test the involvement of autophagy in antigen presentation of myeloid cells. B16-OVA-GFP is a mouse melanoma cell line that expresses the antigen ovalbumin on the surface of B16 cells. Because of the foreign origin of ovalbumin, it is recognized, processed and presented by antigen presenting cells. MDSCs will be sorted from B16-OVA-bearing mice (control and LysM^{cre}Atg5$^{fl/fl}$) and co-cultured with OT-II T cells that have a specific TCR for OVA. Proliferation, activation status and regulatory T cell induction we will monitored at the end of the experiment. The same interaction will be tested in vivo with adoptive transfer of CFSE-labeled OT-II T cells in B16-OVA-bearing mice.

v. References

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