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Graphical abstract

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Autophagy orchestrates the regulatory program of tumor-associated myeloid-derived suppressor cells

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Abstract

Myeloid-derived suppressor cells (MDSCs) densely accumulate into tumors and potently suppress anti-tumor immune responses promoting tumor development. Targeting MDSCs in tumor immunotherapy has been hampered by lack of understanding on the molecular pathways that govern MDSC differentiation and function. Herein, we identify autophagy as a crucial pathway for MDSC-mediated suppression of anti-tumor immunity. Specifically, MDSCs in melanoma patients and mouse melanoma exhibited increased levels of functional autophagy. Ablation of autophagy in myeloid cells, significantly delayed tumor growth and endowed anti-tumor immune responses. Notably, tumor-infiltrating autophagy-deficient monocytic MDSCs (M-MDSCs) demonstrated impaired suppressive activity in vitro and in vivo, while transcriptome analysis revealed significant differences in genes related to lysosomal function. Accordingly, autophagy-deficient M-MDSCs exhibited impaired lysosomal degradation thereby enhancing surface expression of MHC class II molecules, resulting in efficient activation of tumor-specific CD4+ T cells. Finally, targeting of the membrane-associated RING-CH1 (MARCH1) E3 ubiquitin ligase, that mediates the lysosomal degradation of MHC II, in M-MDSCs, attenuated their suppressive function, and resulted in significantly decreased tumor volume followed by development of a robust anti-tumor immunity. Collectively, these findings depict autophagy as a molecular target of MDSC-mediated suppression of anti-tumor immunity.
Introduction

The effectiveness of current cancer immunotherapies is critically depended on the presence of activated effector T cells in the tumor microenvironment (TME) and pre-existing T cell infiltration in solid tumors holds a prognostic value since it is associated with clinical response (1). Despite the operation of immune surveillance mechanisms, tumors form endowed immunosuppressive networks that impede the elicitation of potent anti-tumor immune responses and impair the success of immunotherapy (2). Myeloid-derived suppressor cells (MDSCs), the progenitors of dendritic cells, macrophages and neutrophils, comprise a major component of the tumor-induced immunosuppressive circuit. In mice MDSCs are characterized as Gr-1+CD11b+ cells and can be further divided based on their morphology as monocytic (M-MDSCs/CD11b+Ly6G−Ly6Chi) or granulocytic (G-MDSCs/CD11b+Ly6G+Ly6Clow), while in humans MDSCs are frequently characterized as HLADRlo/−CD14−CD33+CD15+ cells (3-5). Multiple mechanisms have been attributed to MDSC-mediated inhibition of anti-tumor immune responses ranging from secretion of immunosuppressive mediators to direct cell-to-cell contact (6-7). To date, major therapeutic efforts in cancer aim to switch the differentiation and function of MDSCs towards an immunogenic phenotype.

Autophagy is a fundamental lysosomal catabolic pathway involving degradation of unwanted proteins and organelles to maintain nutrient and cell homeostasis (8). Autophagy pathway is induced under hypoxic conditions and hypoxia is a cardinal feature of most tumors that possesses a major role in tumor progression, metastasis as well as response to therapy (9). Although most studies have been focusing on the role of autophagy in tumor cells (10), how this pathway affects the immune components of TME and specifically MDSCs remains unknown. Several lines of evidence however have indirectly linked autophagy as a major regulator of MDSC function. To this end, hypoxia-inducible factor 1α (HIF-1α) that regulates
autophagy has been demonstrated to promote MDSC differentiation towards tumor-associated macrophages (11-13). Moreover, high mobility group box protein 1 (HMGB 1) has been shown to promote MDSC survival through induction of autophagy and pharmacologic inhibition of autophagy induced MDSC cell death (14). In a similar fashion, endoplasmic reticulum (ER) stress that activates autophagy as a compensatory mechanism for cell survival has been linked to TRAIL-R-mediated MDSC apoptosis (15-17). Finally, reactive oxygen species (ROS) are central in inducing autophagy and have been demonstrated to regulate the stress sensor CHOP in MDSCs that facilitates MDSC accumulation and function (18-19), suggesting that autophagy might be implicated in MDSC suppression. Although all aforementioned reports indicate a possible link of autophagy to the function of tumor-associated MDSCs, a direct role of this pathway as well as the precise autophagy-related molecular events in MDSC-mediated suppression of anti-tumor immunity remain elusive.

In this study we delineate an important role of autophagy in M-MDSC-mediated suppression of anti-tumor immune responses. We demonstrate that autophagy-deficient M-MDSCs are re-programmed, since they lose their suppressive activity and promote anti-tumor immune responses. Absence of autophagy de-regulates the lysosomal function of M-MDSCs that entails insufficient lysosomal degradation and subsequently elevated surface expression of MHC class II. Overall, these findings propose that manipulation of autophagy pathway in MDSCs could be considered as an immunotherapeutic protocol in cancer.
Results

Increased autophagy in MDSCs of melanoma patients and melanoma-bearing mice.

MDSC autophagy was first assessed in sorted highly pure HLA-DR\textsuperscript{low}/CD14\textsuperscript{-}CD33\textsuperscript{+}CD15\textsuperscript{+} MDSCs isolated from peripheral blood of melanoma patients (stage III-IV) and healthy controls (Figure 1A), following the updated guidelines for autophagy (20). Using immunofluorescence microscopy, we determined formation of functional autophagolysosomes based on the expression of LC3 that denotes formation of autophagosomes (20-21), the lysosomal-associated membrane protein 1 (LAMP-1) and the adaptor protein SQSTM1/p62 that targets ubiquitinated proteins for lysosomal degradation (22). Notably, increased formation of autophagosomes was demonstrated in MDSCs from melanoma patients compared to MDSCs from healthy individuals and co-localization analysis revealed increased puncta positive for both p62 and LC3, indicating operation of functional autophagy (Figure 1B). Next we examined MDSC autophagy levels in a clinically relevant melanoma mouse model that entails subcutaneous injection of B16-F10 melanoma cells in C57/BL6 mice. Upon melanoma establishment CD11c\textsuperscript{-}CD11b\textsuperscript{+}Gr1\textsuperscript{+} MDSCs were significantly enriched in the spleen of tumor bearing mice while the frequencies of the monocytic and granulocytic MDSC subsets were not altered (Figure 2A). Furthermore, assessment of autophagy in sorted MDSCs indicated increased levels of autophagosome formation and decreased levels of p62 compared to naïve animals (Figure 2B). Importantly MDSCs from tumors also demonstrated a prominent autophagolysosomal function based on LC3 and p62 expression (Figure 2B).

The kinase mammalian target of rapamycin (mTOR)-dependent pathway is the best-characterized regulator of autophagy, and activation of PI3K/Akt axis is an upstream modulator of mTOR activity (23). To this end, we observed decreased phosphorylation of AKT (pAKT), mTOR (pmTOR) and the ribosomal protein S6 (pS6) in MDSCs from
melanoma-bearing mice compared to naïve controls (Figure 2C). In addition, phosphorylation of serine/threonine kinase UNC-51-like kinase-1 (ULK-1), that is required for activation of the pre-initiation complex in canonical pathway of autophagy (24-25), was significantly increased in MDSCs from spleen and tumors of melanoma mice (Figure 2D). Collectively, these findings demonstrate a significant up-regulation and completion of autophagy pathway in MDSCs from melanoma patients and melanoma-bearing mice.

Since, MDSCs comprise a heterogeneous population of monocytic and granulocytic progenitors (4), we sought to determine how autophagy pathway is regulated in the respective MDSC subsets. To this end, both subsets exhibited enhanced autophagy as demonstrated by the increase in LC3 and the decrease in p62 puncta formation (Supplemental Figure 1).

Attenuated tumor growth and induction of potent anti-tumor immune responses in mice deficient for autophagy in the myeloid compartment.

Next we assessed whether autophagy possesses a functional role in MDSC-mediated tumor immune evasion. To address this we generated LysMcreAtg5fl/fl mice (hereafter denoted as Atg5ΔLysM) that lack Atg5 expression, an essential autophagy component, in the myeloid compartment. qPCR and western blot analysis confirmed the significant reduction of Atg5 expression in MDSCs (Supplemental Figure 2A, B) but not in T cells, whereas its expression in CD11c+ DCs was 50% reduced in Atg5ΔLysM mice compared to control littermates (Supplemental Figure 2A). In addition, Atg5ΔLysM mice did not show any alterations in the frequencies of CD4+ T cells, CD8+ T cells and Foxp3+ Tregs either in the thymus or in the lymph nodes (LNs) (Supplemental Figure 2C, D). Interestingly, B16-F10 melanoma growth was significantly attenuated in Atg5ΔLysM mice as compared to control Atg5fl/fl mice (Figure 3A). This was not restricted to melanoma cells, since significant inhibition of Lewis Lung Carcinoma (LLC) cell growth was observed in Atg5ΔLysM mice.
compared to Atg5^fl/fl^ littermates (Figure 3B). Analysis of tumor-draining LNs (tdLNls) revealed no differences in the frequencies of CD4^+^ and CD8^+^ T cells whereas Foxp3^+^ T regulatory cells (Tregs) were significantly reduced in melanoma-bearing Atg5^{ΔLysM} mice (Figure 3C). However, analysis of tumor infiltrating cells demonstrated significantly increased frequencies of CD45^+^ cells and CD4^+^ lymphocytes in Atg5^{ΔLysM} mice, whereas the levels of tumor-infiltrating Foxp3^+^ Tregs were markedly decreased compared to control animals (Figure 3D). In addition, the expression of Foxp3 CTLA4 and CD73 were not altered on Tregs whereas expression of GITR was significantly decreased (Supplemental Figure 3). Immunohistological analysis of tumors confirmed the increased frequencies of CD4^+^ cells in Atg5^{ΔLysM} mice (Figure 3E). In addition, the frequencies of NK1.1^+^ cells significantly decreased in the tumor of Atg5^{ΔLysM} mice (Figure 3F). Importantly, CD8^+^ and NK1.1^+^ infiltrating cells in Atg5^{ΔLysM} mice expressed significantly increased levels of IFN-γ compared to control animals (Figure 3F, G). Overall these findings demonstrate that absence of autophagy in myeloid compartment elicits a robust anti-tumor immune response that attenuates tumor growth.

**Tumor-derived autophagy-deficient M-MDSCs highly accumulate into tumors and exhibit diminished suppressive activity.**

We next asked how the MDSC compartment was affected during tumor development in Atg5^{ΔLysM} mice. To this end, we observed increased frequencies of CD11b^+^Gr1^+^ MDSCs in the spleens of tumor-inoculated Atg5^{ΔLysM} mice compared to control animals whereas frequencies of CD11c^+^ DCs were not altered (Figure 4A). This was reflected to increased accumulation of G-MDSCs and decreased levels of M-MDSCs (Figure 4B). Surprisingly, we found markedly increased frequencies of total MDSCs in tumors of Atg5^{ΔLysM} mice (Figure 4C), with a prominent increase in M-MDSCs, while frequencies of G-MDSCs were not different (Figure 4D). Immunohistochemistry of tumor sections with CD206, a marker
expressed by tumor-associated monocytes (26), confirmed the increased infiltration of myeloid cells in Atg5ΔLysM tumors (Figure 4E). In addition, we found that all MDSCs expressed CD115 in the tumor milieu and CD40 expression, that has been linked to Treg induction by MDSCs (27) was significantly up-regulated in Atg5ΔLysM mice (Supplemental Figure 4A). Of interest, increased apoptosis was observed in tumor-infiltrating autophagy-deficient M-MDSCs (Supplemental Figure 4B) attributed likely to their increased turnover. The augmented frequencies of M-MDSCs in TME of Atg5ΔLysM mice along with the attenuated tumor growth prompted us to examine the functional properties of autophagy-deficient M-MDSCs. Thus we sorted highly pure M-MDSCs from tumors of Atg5ΔLysM and control mice and examined their suppressive properties in vitro. To this end, M-MDSCs from Atg5ΔLysM mice failed to suppress the proliferation, as well as activation (based on CD44 expression) of CD4+ T cells, in contrast to M-MDSCs isolated from control animals that were highly suppressive in vitro (Figure 4F). Importantly, co-injection of M-MDSCs from tumor-inoculated Atg5ΔLysM mice with B16-F10 cells resulted in significantly reduced tumor volume and weight (Figure 4G) and increased frequencies of CD4+ T cells in tdLN compared to control M-MDSCs (Figure 4H). Collectively these data demonstrate that tumor-derived autophagy deficient M-MDSCs exhibit diminished suppressive activity in vitro and in vivo.

**Autophagy deficiency enhances the immunogenic properties of tumor-derived M-MDSCs through impaired lysosomal-degradation of MHC II molecules.**

To elucidate the molecular mechanism through which autophagy dictates the suppressive activity of M-MDSCs, we performed whole genome RNA sequencing of M-MDSCs isolated from tumor-inoculated Atg5ΔLysM and control animals and we found more than 1300 genes to be differentially regulated (Figure 5A). Clustering and gene ontology analysis pointed to an enrichment of genes encoding molecules that belong to lysosomal compartment (Figure 5B).
In support, flow cytometric analysis of LysoSensor Green (DND-189), a weak base that accumulates in acidic organelles and which fluorescence is increased upon protonation (28), revealed increased mean fluorescence intensity (MFI) in M-MDSCs from spleen and tumor of Atg5\(^{ΔlysM}\) mice (Figure 5C). Furthermore, we assessed the expression of Rab7 GTPase, that is known to regulate intracellular membrane trafficking of endosomal/lysosomal compartments (29), and expression of cathepsin D (cathD) in M-MDSCs isolated from Atg5\(^{ΔlysM}\) and Atg5\(^{fl/fl}\) mice. Although expression of Rab7 was significantly decreased, the number of LAMP1 puncta was not different in Atg5\(^{ΔlysM}\) tumor-in inoculated animals. Importantly the expression of cathD was also significantly decreased (Supplemental Figure 5). Finally we examined the lysosomal function by using a long-lived degradation assay, through pulsing M-MDSCs isolated from Atg5\(^{ΔlysM}\) and Atg5\(^{fl/fl}\) mice with \([\text{H}]\text{leucine},\) and further confirmed the impaired degradation capacity of lysosomes in autophagy-deficient M-MDSCs (Figure 5D). Since in tumor-bearing Atg5\(^{ΔlysM}\) we observe a prominent increase in CD4\(^+\) T cell responses and the lysosomal compartment has been linked to the regulation of MHC II turn over (30) we reasoned whether autophagy-deficient M-MDSCs have increased levels of MHC II surface expression due to impaired lysosomal degradation. To this end, flow cytometric analysis demonstrated a significant increase of surface MHC II levels in M-MDSCs from both spleen and tumors of Atg5\(^{ΔlysM}\) mice (Figure 5E) as well as blood M-MDSCs (Supplemental Figure 6) while the MHC II expression in DCs was not altered (Figure 5E). Of interest, significantly increased levels of IA\(^b\) co-localized with LAMP-1 in M-MDSCs from melanoma-bearing Atg5\(^{ΔlysM}\) mice (Figure 5F) suggesting that IA\(^b\) molecules do not efficiently degrade in lysosomal compartment. In support, blocking of lysosomal function with NH\(_4\)Cl or chloroquine in tumor explant supernatant (TES)-treated M-MDSCs from B16-F10-inoculated mice demonstrated significantly increased surface expression of IA\(^b\) molecules without affecting the transcription of IAb or Ciita (the master
regulator of MHC II expression) genes (Figure 5G).

To examine the functional importance of the increased MHC II expression in autophagy-deficient M-MDSCs, we co-cultured OVA peptide-pulsed M-MDSCs with sorted CellTrace-labeled CD4⁺CD25⁺Vα2⁺ OTII cells. Autophagy-deficient M-MDSCs demonstrated a superior ability to induce OTII T cell proliferation in vitro compared to WT M-MDSCs (Figure 6A). In a similar fashion, adoptive transfer of CD4⁺CD25⁺Vα2⁺ OTII T cells into B16-F10-OVA.GFP-inoculated Atg5ΔlysM mice resulted in enhanced activation (based on CD25 and CD44 expression) of the transferred OTII T cells compared to those transferred in Atg5fl/fl animals (Figure 6B), suggesting that autophagy-deficient M-MDSCs could efficiently present tumor-specific “neo-antigens” and this was greatly enhanced in autophagy-deficient M-MDSCs.

Finally, to provide direct evidence for the importance of the aberrant lysosomal degradation of MHC II molecules in the enhancement of anti-tumor immunity, we performed an siRNA-mediated knockdown of March1 (Figure 6C), membrane-associated RING-CH1 (MARCH1) E3 ubiquitin ligase, that mediates the MHC II internalization and trafficking to lysosomes (30), in M-MDSCs. Our results demonstrated a significant increase in the surface expression of IAb molecules in tumor-derived M-MDSCs transfected with March1-si RNA compared to control siRNA-transfected cells (Figure 6D). Importantly, adoptive transfer of March1-silenced M-MDSCs markedly reduced melanoma growth (Figure 6E) and induced a robust anti-tumor immune response (Figure 6F). Overall, these findings provide evidence for an important role of autophagy in dictating the suppressive program of M-MDSCs through regulation of lysosomal degradation of MHC class II molecules.
Discussion

Herein we provide evidence for a crucial role of autophagy pathway in the function of tumor-associated MDSCs. In the absence of autophagy, M-MDSCs are re-programmed to highly immunogenic cells eliciting an endowed anti-tumor immune response that promotes tumor eradication. Mechanistically, transcriptomic and functional analysis demonstrated an aberrant lysosomal degradation of MHC II in autophagy-deficient M-MDSCs that contributed to its increased surface expression and resulted in more efficient priming of anti-tumor CD4⁺ T cell responses.

The effectiveness of checkpoint blockade immunotherapies is limited to patients with high proportion of tumor infiltrating lymphocytes (TILs) and thus major efforts have been placed to enhance the recruitment of TILs into TEM in order to induce potent anti-tumor immune responses (31). In addition, tumor-reactive effector CD4⁺ T cells have been shown to enhance and sustain the accumulation of CD8⁺ cytotoxic T cells within tumors (32-33) and tumor-specific Th17 cells potently inhibit melanoma growth (34) via recruitment of DCs intratumorally and development of potent anti-tumor cytotoxic T cell responses (35). Finally, adoptive transfer of tumor-specific CD4⁺ T cells into tumor-bearing lymphopenic hosts resulted in complete tumor eradication mediated by a potent cytotoxic activity of the CD4⁺ T cells (36). A prerequisite for efficient activation of tumor-specific T cells is presentation of tumor-associated antigens (TAA) by APCs in the context of MHC molecules and costimulation. MDSCs have been reported to express low levels of MHC in various tumor mouse models (37) and also in patients with tumors (38-40). One hypothesis is that low levels of MHC II expression by MDSCs would render them tolerogenic that would preferentially promote induction of Tregs. In support, in a B-cell lymphoma mouse model MDSCs expressing low levels of MHC II served as tolerogenic APCs, engulfed and presented tumor antigens and induced Treg-mediated tolerance (41). Furthermore, CD14⁺HLA-DR⁻/low
MDSCs isolated from patients with hepatocellular carcinoma, induced CD4⁺CD25⁺Foxp3⁺ Tregs in vitro upon culture with autologous T cells (42), however a direct link with HLA-DR expression was not shown. Our results demonstrate that autophagy sustains the low levels of MHC II surface expression in M-MDSCs, and thus contributes to their tolerogenic phenotype. Specifically, tumor-exposed autophagy-deficient M-MDSCs showed impaired lysosomal degradation accompanied by marked surface expression of MHC II molecules and enhanced proliferation of tumor-derived antigen-specific CD4⁺ T cells in vivo. In addition, we observed increased accumulation of IAᵇ molecules in the lysosomes of M-MDSCs from tumor inoculated Atg5ᵃᵇⁿʸˢᵐ mice suggesting an incomplete degradation of MHC II. Finally, in vitro blocking of lysosomal function significantly increased MHC class II surface expression without affecting IAᵇ or Ciita gene expression. All the above pointed towards an autophagy-dependent aberrant lysosomal degradation of MHC II molecules in tumor-associated M-MDSCs. In line with our results, earlier studies showed that DCs with limited lysosomal proteolysis are more potent APCs than macrophages with high proteolytic capacity (43). To date, lysosomal degradation of MHC II molecules is considered to play an instrumental role in determining the levels of surface MHC II expression (30, 44-45). Specifically, MHC II surface expression is regulated by the E3 ubiquitin ligase membrane-associated RING-CH 1 (March-I) that mediates, intracellular localization and lysosomal degradation of pMHC-II in DCs (46) and B cells (47). To this end, DCs isolated from March-I–KO or MHC-II ubiquitination-mutant mice, express very high levels of pMHC-II on the plasma membrane. In a similar fashion, our data provide additional evidence for the impaired lysosomal degradation of MHC II in determining the function of M-MDSCs since silencing of March1 in M-MDSCs, resulted in a marked expression of MHC II in the cell surface and importantly loss of M-MDSC suppressive activity by promoting tumor rejection and enhancing anti-tumor immunity.
NK cell responses play an important role in anti-tumor immunity by exerting strong cytotoxic activity and MDSCs have been shown to regulate their function not only in tumors but also in viral infections (48-50). Our findings show that the frequencies of NK1.1\(^+\) cells are decreased in tumor and spleen of Atg5\(^{ΔlysM}\) mice while the frequencies of intratumoral IFN\(γ\)-producing NK cells are increased. Whether NK cell responses are regulated by M-MDSCs that dominate the tumor microenvironment in Atg5\(^{ΔlysM}\) mice remain unknown. Indeed, only G-MDSCs have been closely linked to NK cell activity (48) suggesting that a thorough understanding of MDSC-mediated regulation of anti-tumor NK cell responses is required.

Our data demonstrate that mTOR pathway is down-regulated in MDSCs from tumor-inoculated mice, which is in agreement with the increased autophagy levels. Although mTOR signaling regulates various physiological as well as pathological processes and has been also shown to affect autophagosome-lysosome fusion (51), its role in MDSCs function just emerged. Specifically, it was demonstrated that lysosomal acid lipase (lal) in MDSCs regulated their metabolic reprogramming and expansion mostly in granulocytic lineage and also lal\(^−\) MDSCs exhibited increased activation of the mTOR signaling pathway (52). In a follow up study, Rab7 GTPase was identified to regulate the mTOR in lal\(^−\) MDSCs and inhibition of Rab7 function significantly reduced MDSC differentiation as well as trans-endothelial migration (53). Our data show that impaired lysosomal function in autophagy-deficient M-MDSCs is associated with decreased levels of Rab7 but no differences in total LAMP1 expression. Since MDSC constitute a heterogenous population of monocytic and granulocytic cells that potently suppress anti-tumor immune responses, the delineation of signals that regulate the metabolic activities and linked to lysosomal genesis and function in each subset are of great importance and warrants further investigation.

Given that MDSCs are the progenitors of granulocytes, macrophages, and DCs, delineation of the mechanisms involved in the MDSC differentiation are crucial for the development of
therapeutic regimens that will benefit cancer patients. Several methods have been developed to achieve this goal (4, 54-55), however the clinical implementation of these approaches and their specificity has not been examined. Our findings propose targeting of autophagy as a novel pathway to alter M-MDSC suppressive activity. Indeed, whole genome transcriptomic analysis of tumor-derived M-MDSCs provided evidence for a major impact of autophagy on their phenotype that was confirmed with in vitro and in vivo functional assays. Autophagy pathway was found to be significantly up-regulated in MDSCs from melanoma-bearing mice as well as melanoma patients. Several factors could contribute to increased MDSC autophagy in tumors. Reduced oxygen supply is a cardinal feature of TME and has been also linked to induction of autophagy (56) a process that is regulated through the HIF-1α (57). Expression of HIF-1α has been closely linked to MDSC differentiation since HIF-1α-deficient MDSCs failed to suppress and instead differentiated towards DCs and macrophages (12, 58). However, whether HIF-1α deficiency in tumor-exposed MDSCs affects operation of autophagy was not addressed. Alternatively, under hypoxic conditions, M-MDSCs up-regulated the expression of CD45 tyrosine phosphatase activity that subsequently decreased STAT3 function and halted M-MDSC differentiation (59). Our results unravel autophagy as a new pathway that its regulation could be exploited to drive MDSC differentiation and to impair their suppressive activity.

Collectively, autophagy has been shown to promote or suppress tumorigenic events in a highly context dependent manner (10, 60), but its role in shaping the function of tumor-associated immune regulatory cells remained obscure. Our findings delineate a pivotal role of autophagy in re-directing the differentiation of M-MDSCs to immunogenic cells that promote tumor rejection. Manipulating autophagy or the molecular events that lead to enhanced autophagy in MDSCs therapeutically could offer new opportunities to enhance activation and expansion of effector tumor-specific CD4+ T cells and to potentiate anti-tumor immune
responses. Currently a large number of clinical trials targeting autophagy in cancer have been launched (61) in combination with chemotherapy or other targeted agents. However, the generalized effect of autophagy inhibition in the development of an anti-tumor immunity remains a caveat of such approaches. Targeted delivery of autophagy inhibitors (i.e via nanoparticles) to MDSCs should be tested for the ability to generate immunity against tumors and thus could pave the way for the design of novel therapeutic protocols in cancer.
Materials and Methods

Mice. C57BL/6 mice were purchased from Jackson laboratory, $\text{Atg}5^\Delta\text{LysM}$ mice were generated by crossing $\text{Atg}5^{\text{flox/flox}}$ mice (62) (obtained from RIKEN BioResource Center) and $\text{LysM}^{\text{cre}}$ mice (obtained from Institute of Molecular Biology and Biotechnology institute (IMBB), OTII CD45.1 mice were kindly provided by Prof. Federica Sallusto (Institute of Research in Biomedicine, Bellinzona, Switzerland). Female mice 8-10 weeks old were used and maintained in the animal facility of Biomedical Research Foundation of Academy of Athens.

Cell lines. The melanoma cell line B16-F10 and the Lewis Lung Carcinoma cell line (LLC1) were kindly provided by Dr. Aris Eliopoulos (Medical School, University of Crete, Heraklion, Greece). The B16-F10 cell line stably expressing ovalbumin with gfp (B16-F10-OVA.GFP) was kindly provided by Dr. Caetano Reis e Sousa (The Francis Crick Institute, London, United Kingdom).

Solid tumor induction. Induction of solid tumors was performed as previously described (63). Briefly, C57BL/6, $\text{Atg}5^\Delta\text{LysM}$ and $\text{Atg}5^{\text{flox/flox}}$ mice were injected subcutaneously (s.c.) with $3 \times 10^5$ B16-F10 melanoma or LLC1 cells. Tumor volume was monitored from day 7 to day 15 and was calculated using the equation ($\text{length} \times \text{width}^2)/2$. Analysis was performed 12-15 days following tumor inoculation.

Flow cytometry and cell sorting. For analysis of TILs, tumors were dissected and incubated for 45 min at $37^\circ$C in RPMI medium containing 0.1 mg/ml DNaseI (Sigma-Aldrich), 0.2 mg/ml collagenase D (Roche). Single-cell suspensions from TILs, peripheral blood mononuclear cells (PBMCs), spleen or dLNs were stained with conjugated antibodies to mouse: CD11c (Catalog N. 117310 / Clone N418), Gr-1 (108408 / RB6/8C5), CD11b (101216 / M1/70), I-A$^b$ (116406 / AF6-120.1), Ly6G (127608 / 1A8), Ly6C (128032 / HK1.4), CD4 (100406 / GK1.5), CD8 (100722 / 53-6.7), CD45 (103132 / 30-F11), CD3
17
(100222 / 17A2), CD25 (101918 / 3C7), Va2 (127808 / B20.1), NK1.1 (108710 / PK136),
CD115 (135511 / AFS98), CD73 (127224 / TY/11.8), CTLA4 (106313 / UC10-4B9), GTR
(126312 / DTA-1) CD40 (124625 / 3/23) (Biolegend), pmTOR (12-9718-41 / MRRBY),
pAKT (17-9715-41 / S473), pS6 (12-9007-41 / S235/S236) (eBioscience) and Lysosensor
(L7535 / DND189) (Molecular Probes). For Foxp3 intracellular staining, cells were fixed and
stained using the Foxp3 Staining Set (anti-Foxp3 12-4774-42 / 150D, eBioscience Inc.)
according to manufacturer instructions. For intracellular cytokine staining, CD45+
cells sorted
from tumors were incubated with 50 ng/ml PMA (Sigma-Aldrich), 2 μg/ml Ionomycin
(Sigma-Aldrich) and Golgi plug (1/1000) (Becton Dickinson Biosciences) for six hours at
37°C, and stained for IFN-γ (505808 / XMG1.2, Biolegend) using the BD Cytofix/Cytoperm
Plus Fixation/Permeabilization kit (Becton Dickinson Biosciences). For intracellular phospho
protein staining, cells were permeabilized with the intracellular Fixation & Permeabilization
buffer set (eBioscience Inc.) according to manufacturer instructions and stained with antibody
against phosphor proteins. CD11c−CD11b+Gr-1+ MDSCs, CD11c−CD11b+Ly6C+ M-MDSCs,
CD4+CD25+Va2+ OTII T cells, CD11c+ DCs, CD3+ T cells were sorted on a FACS ARIA III
(Becton Dickinson Biosciences). Cell purity was above 95%.

Immunofluorescence. Cells were seeded in coverslips pretreated with poly-L-lysine (Sigma
Aldrich), fixed with 4% PFA (Sigma-Aldrich) for 15 min in room temperature followed by
10 min of fixation with ice cold methanol. Cells were permeabilized by using 0.1% saponin
(Sigma-Aldrich), 2% BSA and stained with mouse anti-LC3 antibody (1:20, 0231 / 5F10
nanoTools), rat anti-LAMP-1 (1:400, sc-19992 / 1D4B Santa Cruz Biotechnology), rabbit
anti-p62 (1:500, PM045 / SQSTM1, MBL), rabbit anti-phospho-ULK1 (Ser556, 5869S /
D1H4, 1:70, Cell Signaling Technology), mouse anti-LAMP-1 (sc-17768 / E-5, 1:400 Santa
Cruz Biotechnology), rat anti-IA/IE (14-5321-81 / M5/114.15.2, 1:50, Biolegend), mouse
anti-Rab7 (ab50533 / Rab7-117, 1:2000, Abcam), goat anti-cathepsin D (sc-6486 / C-20,
1:100, Santa Cruz) and followed by incubation with Alexa fluor® 555 anti-mouse IgG (1:500, A28180 / Invitrogen), Alexa fluor® 647 anti-rabbit IgG (1:200, A21245 Invitrogen), Alexa fluor® 488 anti-rat IgG (1:250, A-11006, Invitrogen). For visualization of the nuclei DAPI (Sigma-Aldrich) was used. Samples were coverslipped with moviol and visualized using a 63x oil lens in inverted confocal live cell imaging system Leica SP5.

Puncta of LC3/cell, p62/cell, Rab7/cell, LAMP-1/cell and cathepsin D/cell were calculated using a macro developed in Fiji software as described (64).

Co-localization of IAβ with LAMP-1 and LC3 with p62 was calculated using cross-correlation analysis with volocity software (65).

For immunofluorescence staining of frozen tissues, tumors were embedded in OCT Tissue-Tekk specimen matrix and were cut in 7μm-thick sections using Cryostar NX50 cryotome (Thermo Fisher) at -20°C. The sections were fixed in cold acetone for 10 minutes, and left for at least 30 minutes at room temperature. Sections were blocked with 5% goat serum in TNT buffer (20mM Tris pH 7.6, 0.9% NaCl, 0.05% Tween) for 30 minutes in room temperature and stained with rat anti-mouse CD206 (1:200, Santa Cruz, MR5D3) and rat anti-mouse CD4 (1:200, eBioscience, 14-0041-82 / GK1.5) either at 37°C for 1 hour in humidified chamber or at 4°C overnight, followed by staining with goat anti-rat IgG Alexa 555 (1:500, A-21434, Cell Signaling) for 30 minutes at room temperature. For visualization of the nuclei DAPI (1mg/ml, 1:5000 in PBS) was used. Slides were mounted with fluorescent mounting medium (Dako) and visualized using a digital slide scanner (Zeiss Axio Scan).

All measurements were done only in the tumor area, not considering any positive signal in stroma surrounding areas. Positive area for CD206 was measured with Fiji and represented as percentage of tumor area positive for CD206. Positive cells for CD4 were counted within the tumor area and represented as number of CD4 per tumor.
Quantitative PCR analysis. Cells were lysed in RLT buffer (Qiagen) and RNA was extracted using Qiagen RNeasy mini kit according to manufacturer instructions. First-strand cDNA synthesis was performed using Superscript II reverse transcriptase (Invitrogen). qPCR was carried out using the iTaq Universal SYBR Green Supermix (BioRad). Relative expression of target genes was calculated by comparing them to the expression of the housekeeping gene Hprt. The following primers were used: mouse Atg5 forward, 5’-AGCTCTGGATGGGACTG-3’, Atg5 reverse 5’-CTCCGTCGTGGTCTGAT-3’, mouse Hprt forward, 5’-GTGAAACTGGAAAAGCCAAA-3’, Hprt reverse, 5’-GGACGCAGCAACTGACAT-3’, mouse Ciita forward, 5’-TGCGTGTGATGGATGTCCAG-3’, Ciita reverse, 5’–CCAAAGGGGATAGTGGGTGTC–3’, mouse IA^b (Qiagen), mouse March1 forward, 5’-AAGAGAGCCCACTCATCACACC-3’, March1 reverse, 5’-ATCTGGAGCTTTTCCACTTCC-3’.

Suppression assays. Splenocytes from tumor bearing mice were incubated with biotin anti-mouse Ly6C (HK1.4, 1:200 Biolegend) followed by streptavidin microbeads (Miltenyi Biotec) and then Ly6C^+ cells were positively selected on a magnetic field according to the manufacturer’s instructions (MACS separation columns MS, Miltenyi Biotec). 10^6 Ly6C^+ cells from Atg5^{5ΔLysM} or Atg5^{5fl/fl} mice were co-injected s.c. with 3x10^5 B16-F10 cells in C57BL/6 mice. The tumor volume was monitored from day 7 to day 15. For in vitro suppression assays, highly purified M-MDSCs were sorted from tumors of Atg5^{5ΔLysM} and Atg5^{5fl/fl} mice and cultured in 96-well round-bottomed plates with 1.5x10^5 whole CellTrace-labeled (10μm, Invitrogen) LN cells (LNCs) of naïve C57BL/6 mice in a 1:2 ratio, in the presence of Dynabeads mouse T-activator CD3/CD8 (Life Technologies). Cells were analyzed 4 days later.
Antigen presentation assay. 10^5 magnetically isolated Ly6C^+ cells were cultured in 1:1 ratio with CellTrace-labeled CD4^+CD25^−Va2^+ T cells isolated from OTII naïve mice in the presence of OVA_{323–339} peptide (20 μg/ml, Caslo ApS). Cells were analyzed 4 days later.

Adoptive transfer experiments. Atg5^{ΔLysM} and Atg5^{fl/fl} mice were implanted s.c. on the back with 3x10^5 B16-F10-OVA.GFP melanoma cells. 7 days post injection, sorted 10^6 CD4^+CD25^−Va2^+ T cells from OTII naïve mice labeled with CellTrace (50μm, Invitrogen) were transferred intravenously (i.v.) and 4 days later tdLN were isolated and analyzed.

Preparation of tumor explant supernatants (TES). Tumors from C57/BL6 mice were dissected at day 15 and single cell suspensions were plated in 6-well plates (10^6 cells/ml). Supernatants were collected 16 hours later.

Lysosomal inhibition experiments. 2.5x10^5 Ly6C^+ magnetically isolated cells were plated in 96-well round-bottom plates and treated with tumor explant supernatants (TES 20% v/v) in the presence or absence of the inhibitors: ammonium chloride (NH_4Cl 20mM, Sigma-Aldrich) and chloroquine diphosphate (CQ 50mM, Sigma-Aldrich) for 16 hours.

Measurement of lysosomal function (long-lived protein degradation assay). M-MDSCs were magnetically isolated from the spleens of B16-F10 melanoma cell-inoculated Atg5^{ΔLysM} and Atg5^{fl/fl} mice. Lysosomal function was assessed with the long-lived protein degradation assay using [^3]H leucine. In brief, 7x10^4 cells were plated in 48/well plates. 24 hours later, [^3]H leucine (Perkin Elmer) was added in the culture media. The next day the medium was replaced with starvation-inducing medium and excess of unlabeled leucine. After 6 hours cells were treated with lysosomal inhibitors (NH_4Cl 20mM and leupeptin 20μm (Sigma-Aldrich) or bafilomycin 100 nM (Sigma-Aldrich)) or left untreated for 16 hours. For precipitation of the degraded proteins, aliquots of culture supernatants were treated with 20% trichloroacetic acid and BSA (20 mg/ml). For isolation of non-degraded proteins (proteins in media and cell lysates), cells were lysed with a mild lysis buffer containing 0.1N NaOH and
0.1 % w/v sodium deoxycholate. Counts per minute (cpm) were obtained using a beta counter. The protein degradation is calculated as degraded proteins/(non-degraded proteins + lysed cells).

**RNA sequencing analysis.** M-MDSCs were isolated from the spleens of B16-F10 melanoma cell-inoculated Atg5<sup>ΔLysM</sup> and Atg5<sup>fl/fl</sup> mice, using magnetic beads. RNA was extracted with Macherey-Nagel Nucleo-spin RNA kit. RNA sequencing was employed and single end 75 bp length reads were generated. Data were aligned to mouse genome (mm9) version with top hat 2 algorithm. HT-seq and DESeq algorithm were used in order to measure gene expression and identify differential expression between the two groups of patients. Genes with p-value ≤0.05 and fold change ≥ 1.5 or ≤-1.5 were considered to be up- and down-regulated respectively. Gene ontology analysis, pathway annotation, transcription factor enrichment and comparison with various immunological and oncogenic gene signatures were performed with the use of DAVID knowledge base, Ingenuity Pathway Analysis software and Molecular Signature Database (MSigDB) from Broad Institute. SRA accession number: PRJNA395259.

**Silencing of March1 by siRNA** Splenocytes from tumor bearing C57BL/6 mice were incubated with biotin anti-mouse Ly6C (HK1.4, 1:200 Biolegend) followed by streptavidin microbeads (Miltenyi Biotec) and then Ly6C<sup>+</sup> cells were positively selected on a magnetic field according to the manufacturer’s instructions (MACS separation columns MS, Miltenyi Biotec). Ly6C<sup>+</sup> cells were seeded in 12-well plates (1.5x10<sup>6</sup>), cultured with 250ng/ml LPS and transfected with 20 µl (10µM) of either March1 siRNA cocktail (Santa Cruz Biotechnology, Inc. #sc-106199) or scramble si-RNA sequence (Santa Cruz Biotechnology, Inc. #sc-37007), using Lipofectamine® 2000 (Thermo Scientific, # 11668019) according to the manufacturer protocol. 48 hours post wash of transfection, cells were harvested and tested with qPCR for knockdown efficiency. The expression of MHCII in transfected cells was assessed with FACS. 4x10<sup>5</sup> transfected Ly6C<sup>+</sup> cells, with either scramble or si-March1, were
co-injected s.c. with $3 \times 10^5$ B16-F10 cells in C57BL/6 mice. The tumor volume was monitored from day 7 to day 12.

**Human subjects and isolation of MDSCs from peripheral blood.** Peripheral blood from melanoma patients (stage IV) and healthy individuals was collected in EDTA-coated tubes prior to systemic treatment. Peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque® 1077 (Sigma Life Science) (1800rpm, 30 min, room temperature) washed and stained with antibodies against human CD14 (325604/HCD14), CD33 (303404/WM53), CD15 (323018/W6D3) and HLA-DR (307616/L243) (Biolegend) prior to MDSC sorting. Sorted MDSCs were processed for autophagy pathway analysis with confocal microscopy.

**Statistics.** Statistical analyses were performed using unpaired two-tailed Student's $t$ test. Two-way ANOVA statistical tests were applied in experiments with multiple comparisons. Data are presented as means ± S.E.M. Differences were considered statistically significant at $P < 0.05$. All data were analyzed using GraphPad Prism v5 software.

**Study approval.** Melanoma patients (stage IV) and healthy individuals were recruited through the Oncology Department, University Hospital “Laiko” (Athens, Greece). The Clinical Research Ethics Board of National and Kapodistrian University of Athens, Medical school, University Hospital “Laiko” (Athens, Greece) approved this study and informed consent form was obtained from all patients and healthy individuals prior to sample collection. All procedures in mice were in accordance to institutional guidelines and were reviewed and approved by the Greek Federal Veterinary Office (Protocol N. 1474, Athens, Greece).
Author contributions

T.A. and A.H. designed and performed experiments, analyzed data, generated figures and wrote the manuscript. K.M., R.M.B. and A.B. performed experiments, analyzed data and generated figures. S.S. and B.W. performed immunohistochemical analysis and participated in interpretation of data. A.P. and M.X. assisted with experiments and analyzed data, H.G. performed clinical evaluation of the patients and provided human specimens, P.V. designed and supervised the study, performed data analysis and writing of the manuscript.

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References


**Figure 1. Enhanced autophagy in MDSCs from melanoma patients.** (A) Gating strategy and frequencies of MDSCs (HLA-DR CD14+CD33+CD15+) in PBMCs of healthy individuals (n=18) and melanoma patients (n=17) (**p<0.0001). (B) Representative confocal microscopy images for LC3 (red)/LAMP-1 (green)/p62 (silver white)/DAPI (blue) and Pearson's correlation of LC3 vs p62 (**p<0.0001) in sorted MDSCs from peripheral blood of healthy individuals (n=4) and melanoma patients (n=4). Scale bar 10 μM. One representative experiment of 3 is shown. Results are expressed as mean ± SEM. Statistical significance was obtained by unpaired student’s t test.
Figure 2. Up-regulation of autophagy pathway in MDSCs from melanoma-bearing mice. (A) Representative flow cytometric analysis and frequencies of total MDSCs (CD11c<CD11b−Gr-1+) (n=5 mice per group, ***p<0.0001) and subsets from spleens of naive or B16-F10 inoculated mice, n=4. (B) Representative immunofluorescence confocal images for LC3 (red), LAMP-1 (green), p62 (silver white), and DAPI (blue) and LC3 puncta/cell and p62 puncta/cell in sorted MDSCs from spleens and tumors of naive and B16-F10-inoculated mice n=4 mice per group (LC3: ***p<0.0001, p62: *p=0.0459, ***p=0.0003, **p<0.0001). Scale bars: 10 μm. (C) MFI of pAkt (*p=0.0483), pmTOR (**p=0.0515) and pS6 (***p=0.0027) in MDSCs from spleens of naive or B16-F10 inoculated mice, n=5 mice per group. (D) Representative immunofluorescence confocal images for pULK-1 (silver white), and DAPI (blue) and pULK-1 puncta/cell in sorted MDSCs from spleens and tumors of naive and B16-F10-inoculated mice (pULK-1: ***p<0.0001). Scale bars: 10 μm., n=5 mice per group. One representative experiment of 3 is shown. Results are expressed as mean ± SEM. Statistical significance was obtained by unpaired student's t test (A,C) or Two-way ANOVA (B,E).
Figure 3. Deficiency of autophagy pathway in the myeloid compartment attenuates tumor growth and enhances anti-tumor immune responses. (A) Tumor volume (**p=0.0005) and representative image of excised tumors of B16-F10 inoculated Atg5ΔLysM and Atg5Δ/Δ control mice. Representative results from three independent experiments are shown, n=5 mice per group. (B) Tumor volume (**p=0.0028) of LLC inoculated Atg5ΔLysM (n=4) and Atg5Δ/Δ control (n=5) mice. Representative results from three independent experiments are shown. (C) Frequencies of CD4+, CD8+ T cells and CD4+Foxp3+ Tregs (**p=0.0032) in tDLNs of B16-F10-inoculated Atg5ΔLysM and Atg5Δ/Δ control mice, n=8 mice per group. (D) Gating strategy and frequencies of CD4+ (*p=0.0150, n=4), CD4+ (*p=0.0088, n=6), CD8+ (n=10), and CD4+Foxp3+ Tregs (**p=0.0030, n=11), in tumor sites of Atg5ΔLysM and Atg5Δ/Δ control mice. For (C,D) Representative results from four independent experiments are shown. (E) Representative digital slide scanner images of CD4+ T cells (red) and DAPI (blue) in tumor section from B16-F10 inoculated Atg5ΔLysM and Atg5Δ/Δ control mice are shown. n=5 mice per group, scale bar: 50 μm (F) Representative flow cytometric analysis and frequencies of NK cells (*p=0.0364) gated on CD45+ and CD3 NK1.1+IFNγ+ NK cells (**p=0.0008) in tumor site, n=4 mice per group. (G) Gating strategy and frequencies of CD8+IFNγ+ T cells (*p=0.0456) in tumor site. Representative results from four independent experiments are shown, n=5 mice per group. Results are expressed as mean ± SEM. Statistical significance was obtained by unpaired student’s t test.
Figure 4. Impaired suppressive function of tumor-infiltrating autophagy-deficient M-MDSCs from melanoma bearing mice. (A) Frequencies of MDSCs (CD11c CD11b^Gr-1^) (***p=0.0036, n=7) and DCs (CD11c^+^, n=11) in spleens of B16-F10 inoculated Atg5^-/-^LysM and Atg5^-/-^ control mice. (B) Representative flow cytometric analysis and frequencies of G-MDSCs: CD11c CD11b^Ly6G^Ly6C^- (***p<0.0001) and M-MDSCs: CD11c CD11b^Ly6G^Ly6C^- (***p=0.0067) in spleens of B16-F10 inoculated Atg5^-/-^LysM and Atg5^-/-^ control mice (n=10 mice per group). (C) Representative flow cytometric analysis and frequencies of tumor infiltrating MDSCs (**p=0.006) in B16-F10 inoculated Atg5^-/-^LysM and Atg5^-/-^ control mice (n=7 mice per group). (D) Frequencies of G-MDSCs and M-MDSCs (**p=0.0050) in tumors of B16-F10 inoculated Atg5^-/-^LysM and Atg5^-/-^ control mice (n=8 mice per group). (E) Representative digital slide scanner images and percentages of CD206^+^ cells (*p=0.0310) (red) and DAPI (blue) per tumor section isolated from B16-F10 inoculated Atg5^-/-^LysM and Atg5^-/-^ control mice are shown. (n=5 mice per group, scale bar: 40 μM). (F) Representative histograms of CD4^+^ T cell proliferation and flow cytometric analysis of CD44 in CellTrace labeled LNCs cultured with sorted M-MDSCs from tumors of Atg5^-/-^LysM and control Atg5^-/-^ B16-F10 inoculated mice, n=4 mice per group. For (G, H) Ly6C^-+^ cells from spleens of Atg5^-/-^LysM and Atg5^-/-^ control B16-F10 inoculated mice, were mixed with B16-F10 melanoma cells (3:1 ratio) and were s.c. injected into C57BL/6 mice (n=8 mice per group). (G) Tumor volume (*p=0.0082) and tumor weight (**p=0.007) are shown. (H) Frequencies of CD4^+^ (***p=0.0499) and CD8^-+^ T cells from tdLNs. Results are expressed as mean ± SEM. Statistical significance was obtained by unpaired student’s t test. Representative results from three independent experiments are shown.
Figure 5. Impaired lysosomal degradation and increased surface expression of MHCII molecules in autophagy deficient M-MDSCs. (A) Heat map of differentially expressed genes in M-MDSCs isolated from spleens of B16-F10 inoculated Atg5\textsuperscript{ΔLysM} and control mice (n=3 mice/group). (B) Heat map of differentially expressed genes related to the lysosomal function in M-MDSCs isolated from the spleens of B16-F10 inoculated Atg5\textsuperscript{ΔLysM} and control mice (n=3 mice/group). (C) MFI of lysosensor in M-MDSCs from spleen (p=0.0470) and tumor (p=0.0335) of B16-F10 inoculated Atg5\textsuperscript{ΔLysM} and control mice (n=5 mice/group). (D) Percentage of protein degradation, using [\textsuperscript{3}H] leucine, in M-MDSCs isolated from the spleens of B16-F10 inoculated Atg5\textsuperscript{ΔLysM} and control mice treated with lysosomal inhibitors (NH\textsubscript{4}Cl and leupeptin or bafilomycin) or left untreated (n=3 mice/group), (p=0.0134, **p=0.0084, *p=0.0195, *p=0.0128, *p=0.0179, **p=0.0088, ***p=0.0264). (E) Representative histograms for the expression of IA\textsuperscript{b} by M-MDSCs of spleen or tumor of Atg5\textsuperscript{ΔLysM} and control mice, n=5 mice per group. (F) Representative confocal microscopy images for LAMP-1 (red)/IA\textsuperscript{b} (green)/DAPI (blue) and Pearson’s correlation of IA\textsuperscript{b} vs LAMP-1 (**p<0.0001) in sorted M-MDSCs from from splenocytes of B16-F10 inoculated Atg5\textsuperscript{ΔLysM} and control mice (n=4 mice/group). Scale bar 10 \textmu M. (G) Representative histograms for the expression of IA\textsuperscript{b} by M-MDSCs isolated from spleens of B16-F10 Atg5\textsuperscript{ΔLysM} inoculated mice after in vitro stimulation with TES in the presence of NH\textsubscript{4}Cl or chloroquine. Geometric mean of IA\textsuperscript{b} (**p<0.0001, *p=0.048), relative expression of Ciita and IA\textsuperscript{b} (**p<0.0001) are shown, (n=8 mice per group). Results are expressed as mean ± SEM. Statistical significance was obtained by unpaired student’s t test, or Two-way ANOVA (D, G). Representative results from three independent experiments are shown.
Figure 6. Sustained IAα expression in autophagy deficient tumor-derived M-MDSCs endows their immunogenic properties. (A) Representative flow cytometric analysis of CellTrace labeled OTII CD4+ T cells cultured with M-MDSCs of Atg5ΔlysM and control B16/F10 inoculated mice in the presence of OVA peptide, n=5 mice per group. (B) Gating strategy and frequencies of CD25+ (*p=0.0236) and CD44+ (**p=0.0116) OTII CD4+ T cells adoptively transferred in Atg5ΔlysM and control tumor bearing mice, n=3 mice per group. (C) Relative March1 expression in M-MDSCs following transfection with si RNA for March1 or scramble si (**p=0.0006, n=3 mice per group). (D) Representative histograms and MFI for IAα expression (*p=0.0129) in M-MDSCs following transfection with si RNA for March1 or scramble si, (n=4 mice per group). (E) For (E, F) 4x10⁵ M-MDSCs, transfected with scramble si or si-March1, were co-injected s.c. with 3x10⁵ B16-F10 cells in C57BL/6 mice (n=7 mice per group). (E) Tumor volume (**p=0.0044, **p=0.017, ***p<0.0001) and tumor weight (**p=0.0036, ***p=0.0001) are shown. (F) Numbers of CD45+ (***p=0.0035), CD4+ (***p<0.0001) and CD8+ (*p=0.0307) T cells/ 6x10⁵ tumor cells are depicted. One representative experiment of three is shown. Results are expressed as mean ± SEM. Statistical significance was obtained by unpaired student’s t test.