Role of Activin-A signaling in melanoma

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“Confusion, I was taught,
Is the beginning of understanding.”
Yongey Mingyur Rinpoche

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Summary

Cutaneous melanomas arise from transformed skin melanocytes. While accounting for only 1% of skin malignancies, melanoma is responsible for the majority of skin cancer-related deaths. Owing to lifestyle changes and increased exposure to sunlight, melanoma occurrence is on the rise in developed countries. Identification of the molecular mechanisms driving melanoma formation led to the development of new classes of therapeutics which revolutionized the standard of care. Among these, small molecule inhibitors targeting the MAPK pathway and immune checkpoint inhibitors resulted in unprecedented clinical success. Nevertheless, many patients still do not benefit from these therapies. Activin-A, a secreted ligand of the TGF-β superfamily, was recently found to be overexpressed in melanoma. However, whether Activin-A signaling promotes melanoma progression or therapy resistance is unknown. In this thesis, the contributions of autocrine and paracrine Activin-A signaling to melanoma growth are analyzed using lentiviral expression in melanoma cells of a constitutively active mutant type I Activin receptor (caALK4), of secreted Activin-A (gain-of-function), or of a ligand trap (loss-of-function). Paradoxically, while forced ALK4 signaling in melanoma grafts restored tumor suppression by inhibiting tumor cell survival and proliferation, transgenic Activin-A accelerated tumor growth. Conversely, expression of a ligand trap comprising the extracellular domain of Activin receptor IIB fused to human Fc (AIIB-Fc) slowed melanoma growth. Importantly, in immunodeficient $Rag1^{-/-}$ mice, effects of paracrine Activin-A gain and loss-of-function were abrogated, suggesting that Activin-A promotes melanoma progression by inhibiting the adaptive anti-tumor response. Flow cytometry analysis of tumor immune infiltrates combined with antibody-mediated cell depletion experiments confirmed that Activin-A promotes melanoma progression by inhibiting $CD8^{+}$ tumor-infiltrating lymphocytes (TILs). Furthermore, inhibition of endogenous Activin-A sensitized a treatment-resistant melanoma model to immune checkpoint inhibition by anti-PD1 and anti-CTLA4 antibodies. Activin-A therefore emerges as a promising target to improve immunotherapy outcome in melanoma.

Keywords: melanoma, Activin-A, immunosuppression, immunotherapy, checkpoint inhibitors.

Résumé

Le mélanome de la peau est le résultat de la transformation maligne des mélanocytes qui résident dans la couche inférieure de l'épiderme. Bien qu'il ne représente qu'un pourcent de tous les cancers de la peau, le mélanome est responsable de la majorité des décès liés à ces cancers. En raison de l'évolution des mœurs et de l'exposition croissante aux rayonnements solaires, les cas de mélanomes sont en nette croissance dans les pays développés. L'identification des mécanismes moléculaires conduisant au développement du mélanome a permis la mise-au-point de nouvelles classes d'agents thérapeutiques qui ont révolutionné la prise en charge de cette maladie. Parmi celles-ci, les inhibiteurs à petites molécules ciblant la voie de signalisation MAPK ainsi que les inhibiteurs de points de contrôle immunitaires ont abouti à des succès cliniques sans précédent. Malheureusement, une grande partie des patients ne bénéficient pas de ces nouvelles thérapies. Récemment, il a été découvert que l'Activin-A, un ligand sécrété membre de la superfamille TGF-β, est fréquemment surexprimé dans le mélanome. Cependant, l'implication de la signalisation par l'Activin-A dans le développement du mélanome et dans d'éventuelles résistances aux thérapies est à ce jour inconnue. Au cours de cette thèse, les contributions de la signalisation autocrine et paracrine par l'Activin-A dans la croissance du mélanome sont analysées à l'aide de l'expression induite par transduction lentivirale dans des lignées cellulaires de mélanome d'un récepteur à l'Activin de type I mutant constitutivement actif (caALK4), de l'Activin-A sécrétée (gain de fonction), ou d'un piège à ligands (perte de fonction). Paradoxalement, alors que la signalisation induite par caALK4 dans des greffes de mélanome a restauré la suppression tumorale en inhibant la survie des cellules tumorales ainsi que leur prolifération, l'expression transgénique de l'Activin-A a accéléré la croissance tumorale. À l'inverse, l'expression d'un piège à ligands constitué par le domaine extracellulaire du récepteur à l'Activin IIB fusionné au fragment Fc humain (AIIB-Fc) a ralenti la progression du mélanome. Remarquablement, les effets de l'Activin-A et d'AIIB-Fc ont été abrogés lorsque ces expériences ont été répétées dans des souris Rag1$^{-/-}$ immunodéficientes, indiquant que l'Activin-A stimule la croissance du mélanome en inhibant la réponse immune anti-tumorale adaptive. L'analyse de l'infiltrat immunitaire au sein de greffes de mélanome ainsi que l'ablation de certaines populations immunitaires induite à l'aide d'anticorps monoclonaux ont démontré que l'Activin-A contribue à la progression du mélanome en inhibant les lymphocytes T cytotoxiques infiltrant les tumeurs. En outre, l'inhibition de l'Activin-A a sensibilisé un modèle de mélanome à l'inhibition des points de contrôle immunitaires par des anticorps anti-PD1 et anti-CTLA4. L'Activin-A se profile ainsi comme une cible particulièrement prometteuse dans l'amélioration des immunothérapies.

Mots-clés : Mélanome, Activin-A, immunosuppression, immunothérapies, inhibiteurs des points de contrôle immunitaires.

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Abbreviations

ACT	Adoptive cell transfer
ACVR	Activin receptor
AE	Adverse effect
AIIB-Fc	ACVRIIB fused to human Fc
ALK	Activin receptor-like kinase
APC	Antigen-presenting cell
ASR	Age-standardized rate
ATP	Adenosine triphosphate
βA	*INHBA*
BMP	Bone morphogenetic protein
BRAFi	BRAF inhibitor
caALK4	Constitutively active mutant ALK4
CAF	Cancer-associated fibroblast
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
CRC	Colorectal cancer
CSD	Chronically sun-damaged
CTC	Circulating tumor cell
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cell
DETC	Dendritic epidermal T-cell
EC	Endothelial cell
ECM	Extracellular matrix
EdU	5-Ethynyl-2-deoxyuridine
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
FACS	Fluorescence-assisted cell sorting
FDA	Food and drugs administration
FGFR	Fibroblast growth factor receptor
FoxP3	Forkhead box P3
FST	Follistatin
GDF	Growth and differentiation factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
HIF	Hypoxia-inducible factor
ICB	Immune checkpoint blockade
ICD	Intracellular domain
I.d.	Intradermal

IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LAP	Latency-associated peptide
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony stimulating factor
MDSC	Myeloid-derived suppressive cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MITF	Microphtalmia-associated transcription factor
mMDSC	Monocytic myeloid-derived suppressive cell
MMP	Matrix metalloproteinase
MSS	Microsatellite stable
NO	Nitric oxide
NK	Natural killer
NR	Non-responder
NSCLC	Non-small cell lung cancer
ORR	Overall response rate
OS	Overall survival
OVA	Ovalbumin
PD1	Programmed cell-death protein 1
PDAC	Pancreatic ductal adenocarcinoma
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositide 3-kinase
PTEN	Phosphatase and tensin homolog
RCC	Renal cell carcinoma
ROS	Reactive oxygen species
rtTA	Reverse tetracycline transactivator
SCID	Severe combined immunodeficient
SIINFEKL	Class-I ovalbumin epitope
TAM	Tumor-associated macrophage
TCGA	The cancer genome atlas
TCR	T-cell receptor
TGF-β	Transforming growth factor β
TGFBR	TGF-β receptor
TIL	Tumor-infiltrating lymphocyte
TLR	Toll-like receptor
TME	Tumor microenvironment

TNF	Tumor necrosis factor
TRE	Tetracycline-responsive element
Treg	Regulatory T-cell
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
WT	Wild type
YUMM	Yale University melanoma cell lines

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1. Introduction

1.1 Cancer

The World Health Organization defines cancer as « the uncontrolled growth and spread of cells ». According to the Swiss Federal Statistical Office, cancer occurrence is on the raise in Switzerland, with 41'000 newly diagnosed patients in 2015. The age-standardized rate (ASR) has however remained stable between 1991 and 2010, indicating that this increase (Swiss Federal Statistical Office, 2015) may be attributed to population aging. Moreover, in the same timeframe, the ASR for cancer-related deaths has decreased by 33%. Despite these encouraging facts, cancer remains the leading cause of premature deaths in Switzerland, with 62'500 potential years of life lost (PYLL, defined as the difference between the patient's age at the time of death and a theoretical life expectancy of 70 years), far outweighing the 26'400 PYLL attributable to cardiovascular diseases. Considerable prevention and research efforts are therefore warranted to limit the burden of this life-threatening disease.

1.1.1 Cancer: a historical perspective

The earliest known description of cases evocative of cancer was discovered in an Egyptian papyrus dating from 3000 BC describing the untreatable cases of eight breast tumors ablated by cauterization, but it was only 2500 years later when Hippocrates first coined the term *carcinos*, referring to the venous projections observed within tumorous growth reminiscent of a crab's legs (American Cancer Society, 2018). In publications dating from the 1870s following careful examination of clinical cases of cancers, the British physician Campbell de Morgan offered the earliest known description of metastasis, where the disease was correctly thought to first arise locally and to later spread to distal sites through draining lymph nodes: "Today the glands may be free; tomorrow they may be affected. Today all disease may be within range of an operation; tomorrow disease may be distributed far beyond" (Grange et al., 2002). In 1902, the German biologist Theodor Boveri proposed following observation during mitosis in the sea urchin development the controversial idea that tumors may arise upon improper chromosome repartition during cell division, hypothesizing the genetic basis of cancer (Boveri, 1902). Nevertheless, it was only in the 1970s that the first oncogenes and tumor suppressor genes were identified. Scientists thus understood how carcinogens may lead to the uncontrolled growth of cells by inducing DNA damage known as mutations. Cancer research remained for long centered on the malignant cells themselves. In recent decades however, the influence on tumorigenesis of the tumor microenvironment (TME), constituted by a variety of host cells such as the endothelial cells and pericytes constituting blood vessels, as well as leukocytes and stromal fibroblasts, began to be fully appreciated, paving the way to a seminal review published in 2000 and updated in 2011 by Hanahan and Weinberg: The Hallmarks of cancer (Hanahan and Weinberg, 2000, 2011).

1.1.2 The Hallmarks of cancer

The hallmarks of cancer are defined as “distinctive and complementary capabilities that enable tumor growth and metastatic dissemination”, which all cancers acquire during the course of tumor progression, albeit potentially through different molecular mechanisms. Eight hallmark capabilities were described in the 2011 review:

1.1.2.1 Self-sufficiency in growth signals

Normal cells divide upon stimulation with mitogenic signals transmitted by transmembrane receptors, generally provided in the form of diffusible growth factors which can be secreted by surrounding cells in a paracrine fashion. Cancer cells use various strategies in order to gain independence from such extrinsic factors. Firstly, they may induce the secretion of a growth factor to which they are sensitive. Alternatively, proto-oncogenes which constitute the signaling pathways downstream of these growth factors, such as receptors or signal-transducing kinases, may be activated by mutations, leading to the synthesis of constitutively active onco-proteins.

1.1.2.2 Insensitivity to antigrowth signals

In order to sustain growth, cancer cells must evade the antiproliferative signals driven by tumor suppressors, such as TP53 and RB. TP53 may prevent cell cycle progression by integrating numerous stress signals from within a cell such as DNA damage, telomere shortening, metabolic imbalances, hypoxia or nutrient deprivation, whereas RB also processes signals from the extracellular milieu. Considering the central role of these tumor suppressors in regulating cell proliferation, it is not surprising to note that p53 inactivating mutations are found in over 50% of all human malignancies. Alternatively, escape from contact inhibition or alterations in receptor-mediated pathways involved in cell cycle regulation may allow sustained proliferation of transformed cells.

1.1.2.3 Evading cell death

Cell death constitutes an obstacle to cell proliferation that malignant cells must learn to overcome. Apoptosis, a form of programmed cell death which can be triggered by both cellular stress (intrinsic) and extrinsic stimulatory ligands such as FasL and TRAIL, has long been recognized as a negative regulator of cancer progression. Cancer cells may evade apoptosis by dysregulating the balance between anti- and pro-apoptotic proteins, mutations of the apoptosis regulator p53 or disruption of death receptor signaling.

1.1.2.4 Enabling replicative immortality

Normal cells dispose of a limited potential for division, after which they stop proliferating and enter a state of cellular senescence. The molecular basis for such a phenomenon is the shortening of telomeres, constituted by thousands of hexanucleotides repeats protecting the end of each chromosome, owing to the incomplete replication of chromosome 3’ ends by DNA polymerases during cell division. The

observation that most cultured cancer cell lines appear to be devoid of such a limitation suggests that replicative immortality may be required for the full acquisition of their malignant capabilities. Indeed, telomere length can be maintained through synthesis of telomeric repeats by the enzyme telomerase, which is upregulated in >90% of transformed cells.

1.1.2.5 Sustaining angiogenesis

Angiogenesis, the process by which new blood vessels emerge from existing ones by vascular sprouting, is generally quiescent in adulthood but can be temporarily induced in certain events such as wound healing. During the course of tumorigenesis, cancer cells gain the ability to disrupt the balance between pro- and anti-angiogenic factors, thereby activating the so-called angiogenic switch in order to sustain their substantial needs in oxygen and nutrients. During vascular sprouting, VEGF-A and Notch collaboratively determine endothelial tip cell and stalk cell identity (Potente et al., 2011). VEGF-A stimulates tip cell formation, induction of filopodia and responsiveness to guidance cues, thus governing vascular patterning. On the other hand, Notch-high stalk cells produce few filopodia but are highly proliferative and gain the ability to form tubes, constituting the vascular lumen. Vessel branching occurs by anastomosis upon tip cell interaction, a process facilitated by macrophages through interaction and stabilization of neighboring cell filopodias. Upon formation, new vessels must be stabilized to produce lasting networks. Blood flow onset participates to this process by downregulating VEGF-A in an oxygen dependent fashion, thus promoting vascular quiescence. Vessels are further matured upon PDGF-B-dependent recruitment of pericytes, which regulate blood flow and promote vessel stability. Owing to the co-option of angiogeneic pathways by cancer cells and the increased tissue hypoxia within malignant growths, tumor-associated blood vessels often feature anomalous function and structure, resulting in poor perfusion, leakiness and further stimulation of angiogenic pathways. Collectively, these defects promote metastasis by facilitating tumor cell extravasation, hinder immune cell infiltration and function and limit the efficacy of therapies by impairing drug delivery.

1.1.2.6 Tissue invasion and metastasis

Metastasis occurs through a multi-step process termed the invasion-metastasis cascade. Initially, epithelial cells at the invasive front gain the ability to dissociate and migrate away from the primary tumor. This process is thought to be facilitated by the induction of the epithelial to mesenchymal transition (EMT), a developmental program which plays crucial roles in the differentiation of tissues and the body plan formation. At the molecular level, the EMT is driven by a set of transcription factors which include Snail, Twist and Twist1/2, leading to downregulation of cell surface adhesion molecules, amongst which E-Cadherin, and adherens junction breakdown. Upregulation of matrix-degrading enzymes and cytoskeleton remodeling confer heightened migratory capabilities to carcinoma cells, whereas sensitivity to apoptosis is decreased, facilitating extravasation into blood and lymphatic vessels and survival of circulating tumor cells (CTCs). Ultimately, CTCs may extravasate from their host vessels and establish macroscopic lesions in distant organs. Fortunately, the invasion-metastasis cascade is particularly inefficient. This was experimentally illustrated by the observation that less than 0.02% of

intravenously injected B16F1 melanoma cells generated macroscopic metastases (Luzzi et al., 1998). Moreover, CTCs can be readily detected in the vast majority of carcinoma patients, including those who do not progress to metastatic disease (Nagrath et al., 2007). Notwithstanding, metastasis accounts for the death of about 90% of cancer patients and remains a major challenge for cancer therapy.

1.1.2.7 Reprogramming Energy metabolism

The metabolic adaptation of cells within a tumor constitutes the first of two new hallmarks introduced by the 2011 review. In the 1920s, Otto Warburg observed that unlike normal cells, cancer cells in normoxic conditions preferentially utilize glycolysis followed by lactic acid fermentation over oxidative phosphorylation to generate energy, a process termed aerobic glycolysis, or Warburg effect. While inefficient for the synthesis of the energy-carrying molecule adenosine triphosphate (ATP), this metabolic reprogramming allows for the generation of metabolic intermediates required to sustain the highly proliferative capabilities of cancer cells. The importance of this metabolic reprogramming is underlined by the relative success of glucose metabolism inhibitors in preclinical studies (Hamanaka and Chandel, 2012)

1.1.2.8 Evading immune surveillance

The last of the eight cancer hallmarks has received considerable attention in the past decades, leading to the approval of immunotherapeutic agents demonstrating unprecedented benefit for a subset of patients. Whether the immune system has a positive or negative impact on tumor development has long been debated. Owing to recent efforts in the field of immuno-oncology, it is now generally understood that immunity controls tumorigenesis in paradoxical ways. Whereas chronic inflammation may promote cell transformation, immunity can also control the growth of emerging tumors, thereby shaping tumor immunogenicity and eventually leading to the outgrowth of malignancies with reduced immune recognition and heightened immunosuppressive microenvironment. This concept of cancer immuno-editing, which occurs in three distinct phases termed “elimination, equilibrium and escape” was first proposed in a seminal study by Shankaran and colleagues in 2001 (Shankaran et al., 2001).

Elimination: The first phase occurs upon T-cell recruitment and activation by type I dendritic cells. Homing of CD8 T-cells to inflamed tumors was shown to be mediated by DC-derived CXCL9 and CXCL10, which engages CXCR3 signaling in lymphocytes [Spranger Cancer Cell 31 2017]. Cytotoxic T-cell activation requires two signals. In the first signal, $CD103^+$ dendritic cells (DCs) recruit activate CD8 T-cells by presenting exogenous antigens via major histocompatibility complex class I (MHC-I) molecules. This process, called cross-priming, can occur both within the tumor bed and in draining lymph nodes (Broz et al., 2014; Roberts et al., 2016). A second signal is delivered through the co-stimulatory TCR molecule CD28 by its ligands of the B7 family expressed on APCs, CD80 and CD86, promoting T-cell effector function and secretion of the T-cell growth factor Interleukin-2 (IL-2) (June et al., 1987; Linsley et al., 1990). Suboptimal co-stimulation induces T-cell anergy, a state a functional inactivation (Chen and Flies, 2013).Upon CCL5 and CXCL9-dependent migration to the tumor (Dangaj et al., 2019),

activated T-cells bearing a cognate TCR can trigger target cell death by releasing pore forming proteins, such as granzymes and perforins, or by expressing ligands of the extrinsic apoptosis pathway. Recruitment and effector function of CD8 T-cells can be facilitated by T helper 1 (Th1) CD4 T-cells, which secrete the pro-inflammatory molecule Interferon-γ (IFN-γ) and Th1-associated cytokines (Bos and Sherman, 2010; Haabeth et al., 2011). Components of the innate lymphoid immune system such as natural killer (NK) cells also participate in the elimination phase upon activation by inflammatory cytokines. In parallel, antigen-independent killing by NK cells leads to the release of tumor-associated antigens, facilitating DC activation, migration and cross-presentation capabilities (Kim et al., 2007).

Equilibrium: Eventually, rare tumor subclones may escape the elimination phase and participate in the maintenance of a stable tumor mass in the equilibrium phase, also termed tumor dormancy. At this stage, the continuous selective pressure applied by immunosurveillance leads to the selection of cancer cells with reduced immunogenic potential. The existence of this phase has been convincingly demonstrated in mice, where dormant tumors formed upon exposure to low dose carcinogen resurged following ablation of adaptive immunity by anti CD4, CD8 and IFN-γ antibodies (Koebel et al., 2007). Equilibrium is thought to be in many cases the longest phase. This can be exemplified by the clinical case of two patients, each grafted with kidneys from the same donor and who developed metastatic melanoma within 2 years post-transplantation. It was later found that the donor had been treated for melanoma 16 years before organ donation and considered disease-free (MacKie, 2003).

Escape: Many factors can contribute to the resurgence of dormant tumors and lead to the final phase of escape. At the tumor cell level, downregulation of MHC-I molecules occurs in 40-90% of human cancers and reduces cognate antigen stimulation of TCRs in CD8 T-cell (Dunn et al., 2004). Whilst IFN-γ signaling is essential for the activation of the adaptive anti-tumor immune response, it has also been shown to promote genetic instability in evading tumor cells, contributing to immune escape (Takeda et al., 2017). Another illustration of IFN-γ signaling's dark side came from the analysis of the susceptibility to T-cell-based therapies following genome-wide CRISPR-Cas9 deletions in melanoma cells. Using this strategy, an essential element in the IFN-γ pathway response was found to be inactivated in treatment-resistant tumors (Patel et al., 2017).

The tumor micro-environment plays an important role in promoting immune evasion. While many cellular components of the TME may hamper the anti-tumor immune response, regulatory T-cells (Tregs) myeloid-derived suppressive cells (MDSCs), tumor-associated macrophages (TAMs) have received particular attention.

- ***Tregs:*** In homeostasis, Tregs constitute a relatively rare population of immunosuppressive $CD4^+$ T-cells involved in the maintenance of immune tolerance. Driven by the forkhead box P3 (FoxP3) transcription factor, Treg differentiation occurs in the thymus in an IL-2-dependent manner upon high affinity interactions with MHC-II/self-antigen complexes (Plitas and Rudensky, 2016). Strong TCR signaling in conjunction with poor co-stimulation may also lead

to extra-thymic Treg differentiation of naïve T-cell precursors. Tregs affect CD8 T-cell function in multiple fashions. Secretion of inhibitory cytokines such as transforming growth factor β (TGF-β) or IL-10 directly limits T-cell effector function. Tregs can also impair the anti-tumor immune response by inducing effector cell cytolysis (Cao et al., 2007). Other mechanisms of Treg-mediated immunosuppression include inhibition of dendritic cell function or metabolic disruption of cytotoxic T-cells (Vignali et al., 2008). Nevertheless, while the proportion of Treg cells is significantly enriched in tumors compared to peripheral blood, the prognostic value of Treg infiltration in human cancer remains controversial (deLeeuw et al., 2012).

- ***MDSCs:*** Myeloid-derived suppressive cells form a highly diverse cell population. They can be grouped into neutrophil-like granulocytic MDSCs and mMDSCs, deriving from the monocytic lineage. Absent from the circulating blood in homeostasis, MDSCs are induced following exposure of immature precursor cells to tumor- and microenvironment-derived factors able to inhibit myeloid cell maturation and to promote suppressive differentiation (Kumar et al., 2016b). Several lines of evidence have identified the hypoxic tumor environment and its cellular mediator hypoxia-inducible factor 1α (HIF1α) as essential for this process (Corzo et al., 2010). Recruitment of mMDSCs to the tumor site is primarily mediated by the CCL2 and CCL5 chemokines, whereas granulocytic MDSC migration mainly depends on CXC molecules (Chun et al., 2015). As for Tregs, the immuno-suppressive functions of MDSCs are diverse: potent Arg1 expression induces depletion of arginine, an essential factor for T-cell proliferation and function, whereas the production of nitric oxide (NO) and reactive oxygen species (ROS) leads to the attenuation of T-cell activation and effector function (Raber et al., 2012; Raber et al., 2014). Moreover, akin to Tregs, MDSCs are an important source of the immune-suppressive cytokines TGF-β and IL-10 (Huang et al., 2006). Importantly, MDSC frequency is associated with poor overall and disease-free survival and resistance to immunotherapy in cancer patients (Ai et al., 2018; Weber et al., 2018).

- ***TAMs:*** Macrophages have pleiotropic functions in homeostasis. Critical in organogenesis during development, they also regulate metabolic functions and are essential for the immune response to pathogens (Wynn et al., 2013). Upon migration into the TME, $CD11b^{+}Ly6C^{hi}$ inflammatory monocytes quickly differentiate into tumor-associated macrophages (Italiani and Boraschi, 2014). Based on *in vitro* studies, TAMs have been categorized into the archetypical M1 (classically-activated) and M2 (alternatively-activated) polarizations according to their functions, but it is now fully appreciated that macrophage populations form a dynamic continuum in complex environments such as the TME (Shapouri-Moghaddam et al., 2018). M1 macrophages can differentiate in response to bacteria-derived lipopolysaccharides (LPS) and gain heightened anti-microbial activity through secretion of pro-inflammatory cytokines, ROS and NO. In the elimination phase, this phenotype can be evoked by granulocyte-macrophage colony-stimulation factor (GM-CSF) and Th1 immunomodulatory molecules to elicit anti-tumoral functions (Mantovani et al., 2017). In later stages of tumor progression, exposure to

macrophage colony-stimulation factor (M-CSF, or CSF1), Th2 cytokines such as IL-4 and IL-13 and hypoxia subverts TAM to exert a wide range of immunosuppressive functions which overlap, but are not limited to those of MDSCs (DeNardo et al., 2009). These mechanisms include secretion of immunosuppressive molecules such as TGF-β, IL-10, or prostaglandins (Park et al., 2015). Metabolic starving of T-cells through induction of the indoleamine 2,3-dioxygenase (IDO) pathway limits effector T-cell function (Grohmann and Bronte, 2010). Moreover, TAMs participate in the angiogenic switch and immuno-suppression by secreting important amounts of VEGF-A (Riabov et al., 2014). Finally, macrophages facilitate the metastatic process by helping extravasation in the neo-vasculature, promoting CTC survival and establishing the pre-metastatic niche (Aras and Zaidi, 2017).

In addition to these cellular mediators of immuno-suppression, the engagement of immune checkpoint pathways such as cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and programmed cell death protein 1 (PD1) is instrumental to the establishment of the escape phase. Evolved as negative feedback mechanisms to prevent exaggerated immune responses, these programs can be subverted by tumors to support their progression.

CTLA4 was the first co-inhibitory receptor to be identified (Walunas et al., 1994). Upregulated upon T-cell activation, CTLA4 prevents CD28-mediated T-cell co-stimulation by binding to CD80/CD86. Activation of the CTLA4 pathway leads to endocytosis and degradation of co-stimulatory receptors, blocks T-cell proliferation by preventing IL-2 secretion, and leads to T-cell anergy (Greenwald et al., 2001; Krummel and Allison, 1995; Qureshi et al., 2011). The CTLA4 pathway differentially regulates CD4 and CD8 T-cell activation and function. In CD4 T-cells, CTLA4 is already engaged during priming. Contrastingly, activation of stimulated CD8 T-cells isolated from $CTLA4^{-/-}$ mice was unaffected upon primary stimulation when compared to WT animals (Chambers et al., 1998). However, upon secondary stimulation, cytotoxic T-cells from CTLA4-deficient animals showed greater proliferative potential than wild-type, suggesting that CTLA4 preferentially dampens memory CD8 T-cell function. Another important difference in CTLA4 signaling between CD4 and CD8 T-cells lies in its constitutive expression by regulatory T-cells. Functionally, deletion of CTLA4 in Tregs has been shown to induce lymphoproliferation, loss of immuno-suppressive functions and to restore anti-tumor immunity in mice (Wing et al., 2008). The CTLA4 pathway therefore constitutes an important regulator of cytotoxic and memory T-cell-mediated anti-tumor immunity.

Originally identified by a genetic screen for pro-apoptotic genes in 1992, PD1 was only recognized as an immunomodulatory receptor in 2000 (Freeman et al., 2000; Ishida et al., 1992). Expression of the PD1 cell surface receptor is induced on T- and B-cells upon activation and acts in an overlapping yet non-redundant fashion compared to CTLA4 (Agata et al., 1996). Through the action of IFN-γ, the PD1 ligands PD-L1 and PD-L2 can be induced in cancer cells and other cells from the TME (Dong et al., 2002). Stimulation of PD1 by PD-L1 and PD-L2 leads to dephosphorylation and inhibition of the co-stimulatory receptor CD28 (Wei et al., 2018). Sustained PD1 signaling promotes T-cell exhaustion, a dysfunctional state characterized by reduced effector function and proliferation (Wherry and Kurachi,

2015). Moreover, PD1 signaling mediated by TAMs has been shown to evict cytotoxic T-cells from the tumor bed, a phenomenon that likely contributes to immune exclusion. In 2004, the PD1 pathway was shown to be engaged in already differentiated effector T-cells in tumors, providing a rationale for PD1 blockade to enhance effector functions of tumor-infiltrating lymphocytes and paving the way for the development of immune checkpoint inhibitors (Blank et al., 2004). In the last decade, immune checkpoint blockade strategies have led to the development of therapeutic agents demonstrating unprecedented clinical benefits (Postow et al., 2015). They will be discussed later in this manuscript.

Besides known cancer hallmarks, ongoing research efforts may reveal additional features that promote the progression of most, if not all cancers. In particular, the complex heterotypic cellular interactions occurring within the tumor micro-environment and how they might dictate response and resistance to diverse therapeutic interventions remain to be elucidated.

1.2 Cutaneous melanoma

According to statistics from the Swiss National Institute for Cancer Epidemiology and Registration (NICER), the overall age-standardized rate of cancer has decreased in Switzerland in the last 10 years. In sharp contrast, owing perhaps to lifestyle changes and increased exposure to sunlight, the occurrence of melanoma has been continuously rising since the 1990s. (Fig. 1) Accounting for merely 1% of all skin

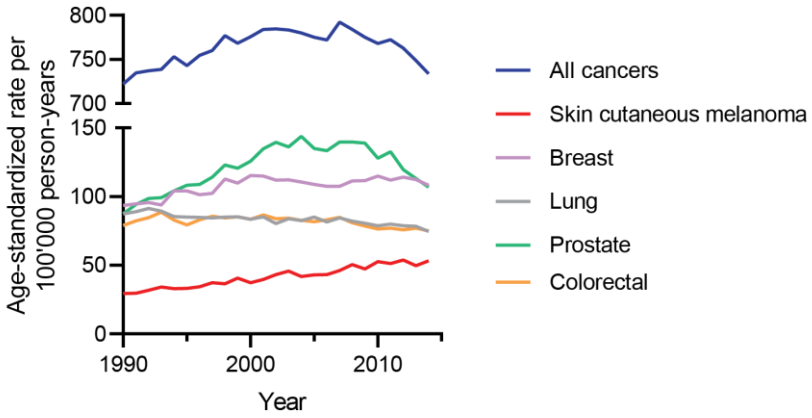


Figure 1: Age-standardized rate of cancer in Switzerland. Data from the Swiss National Institute for Cancer Epidemiology and Registration (NICER).

malignancies, melanoma is nonetheless responsible for the majority of skin cancer-related deaths. Although it can occur in multiple organs, cutaneous melanoma is the most frequent form to be diagnosed in Europe and the United States. Sustained prevention and improved therapeutic strategies are therefore needed to limit the burden of this disease.

1.2.1 Melanocytes in healthy skin

Melanoma arises upon malignant transformation of melanocytes, which migrate to the skin and other organs from the neural crest during development. In the skin, melanocytes populate the lower-most layer of the epidermis and hair follicles. The primary role of melanocytes is to secrete various melanin pigments, protecting the skin from ultra violet (UV)-induced radiations. Mechanistically, upon exposure to UV stimuli, keratinocytes secrete the α-melanocyte stimulating hormone (α-MSH), which promotes melanocyte proliferation and melanin production in neighboring melanocytes by binding to the melanocortin 1 receptor (MC1R). This process is regulated by the Microphtalia-associated transcription factor (MITF), a master regulator of the melanocytic lineage. Upon synthesis in multiple steps starting from the L-tyrosine precursor through hydroxylation by the enzyme tyrosinase, melanin is secreted and delivered to keratinocytes through dendritic contacts (Delevoye, 2014), where it efficiently absorbs UV radiation, preventing DNA damage and nutrient photolysis (Slominski et al., 2004).

1.2.2 Melanomagenesis

Melanoma occurs upon malignant transformation of melanocytes. UV-induced mutations due to sunlight exposure is the main environmental risk factor for the development of melanocytic malignancies (Gilchrest et al., 1999). Overall, activating mutations in the Mitogen-Activated Protein Kinase (MAPK) pathway are highly prevalent in melanoma as they can be found in >80% of patients. In physiological conditions, ligand-induced signaling through receptor tyrosine kinases leads to successive

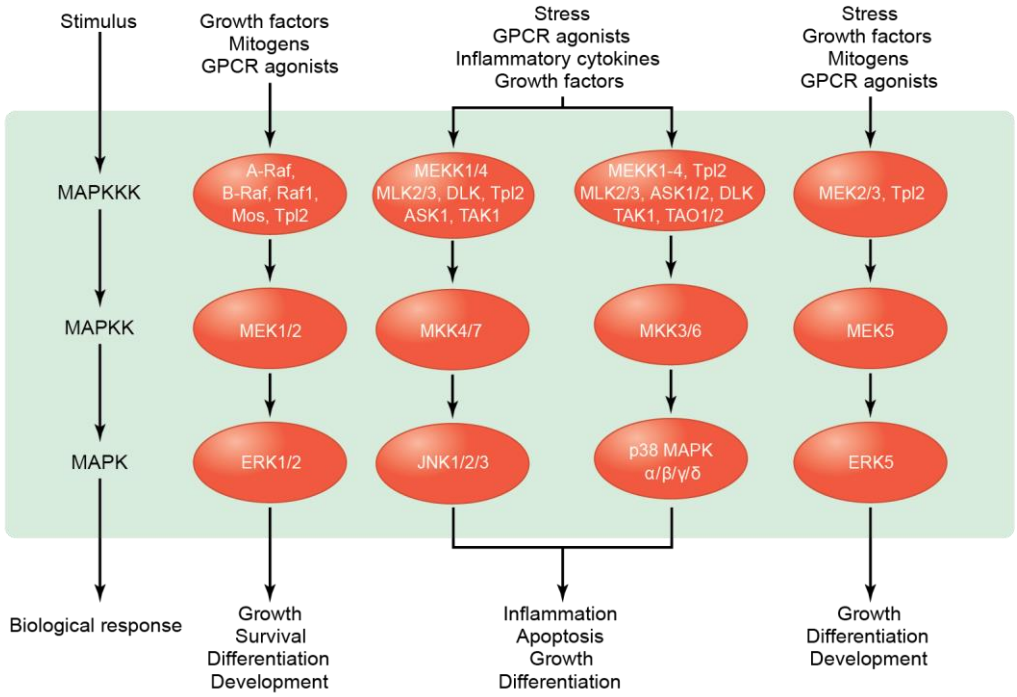


Figure 2: MAPK pathways (adapted from Morrison D.K., Cold Spring Harb Perspect Biol 4 2012)

phosphorylation events catalyzed by three successive kinases, starting from the generically named serine-threonine MAPK kinase kinases (MAPKKK) for the most upstream, followed by a serine/threonine-tyrosine MAPK kinase (MAPKK), ultimately leading to the activation of an effector serine/threonine MAPK. As depicted in Fig. 2, four different MAPK cascades co-exist and can be activated by various signals (Morrison, 2012). In melanoma, kinases of the ERK1/2 pathway are frequently affected by activating mutations. Extensive analysis of DNA, RNA and protein alterations of melanoma patients lead to the identification of four subtypes based on the most prevalent mutated genes, all of which affect the MAPK pathway: BRAF, RAS, NF1 and triple wild-type (WT) (Cancer Genome Atlas Network, 2015). In this landmark study, hotspot mutations in the BRAF gene was found in more than 50% of all patients, 75% of which were characterized by a valine to glutamic acid substitution on amino acid 600, a hotspot mutation termed $BRAF^{V600E}$. Mutations of Ras and NF1 were

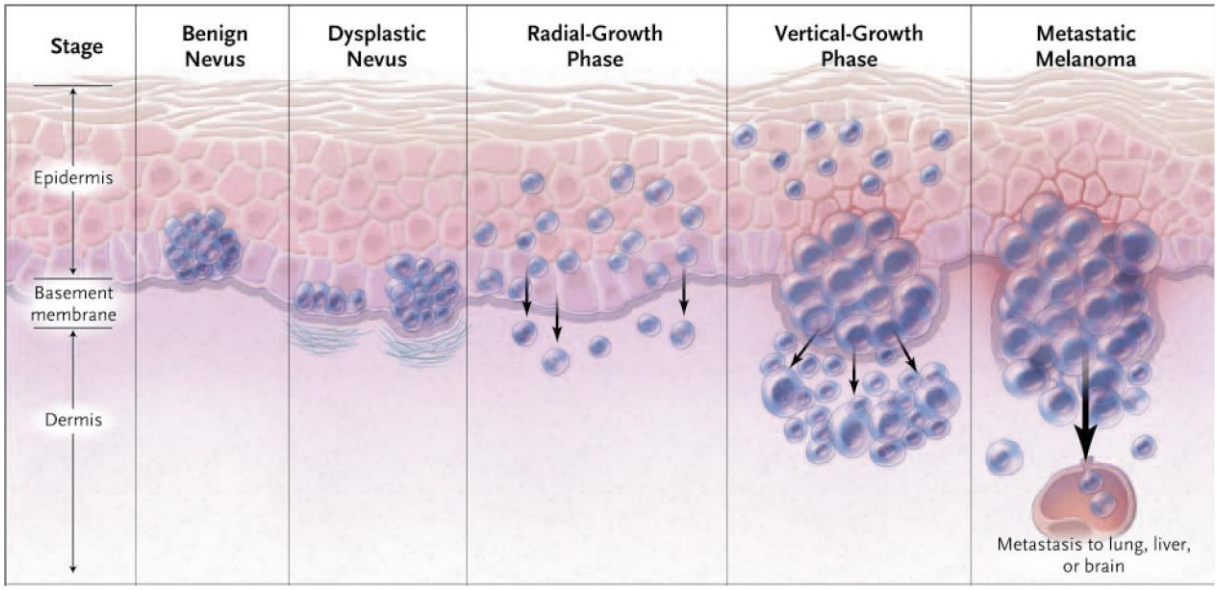


Figure 3: The Clark model of melanoma progression

Adapted from Miller AJ and Mihm MC, NEJM 355 2006

observed in 28% and 14% of the patients, respectively. The last subgroup, characterized by the absence of hotspot mutations in these three genes, was relatively rare and featured alternative driver mutations frequently observed in uveal melanomas such as KIT or CTNNB1. Interestingly, >90% of samples in the BRAF, RAS and NF1 subtypes compared to only 30% in the triple-WT subgroup bared UV mutational signatures characterized by C>T transitions.

Classically, melanoma progression is described by the Clark model, emphasizing a stepwise progression from a benign nevus, often developed early in life through $BRAF^{V600E}$-dependent transformation, following by the acquisition of dysplastic features and ultimately leading to metastatic disease (Fig. 3). The observation that $BRAF^{V600E}$ mutations are frequent in benign nevi consolidates this mutation as an early event in melanoma development (Pollock et al., 2003). These mutations are

thought to arise early in life, when melanocytes actively expand to maintain stable density in the skin, leading to a peak of melanoma onset two to three decades later (Shain and Bastian, 2016). Nevertheless, it is now appreciated that melanoma can arise *de novo* from a variety of precursor lesions, each bearing characteristic genetic alterations and leading to distinct pathogenesis. Whether the affected skin has been chronically sun-damaged (CSD) may partially dictate these factors. Whereas non-CSD melanomas often bear the $BRAF^{V600E}$ substitution and arise from nevi, CSD lesions are more frequently initiated by BRAF non-V600E, NRAS and NF1 mutations and *in situ* melanomas. In both CSD and non-CSD melanomas, subsequent progression is accompanied by the acquisition of additional mutations (Table 1). Unlike benign nevi, invasive melanomas frequently harbor inactivation of the Cyclin-dependent kinase inhibitor 2A gene (CDKN2A), which encodes two structurally unrelated tumor suppressors, $p16^{INK4a}$, a negative regulator of the cell cycle, and $p19^{ARF}$, mediator of p53 function. Inactivation of INK4A in an NRAS-mutant background leads to invasive capabilities in mice (Ackermann et al., 2005), demonstrating the tumor-suppressive function of CDKN2A in melanoma. TP53 mutations are found in 20% of metastatic melanomas and are more frequent in established metastatic lesions compared to primary tumors, suggesting that they might be involved in later stages of the invasive process. Similarly, Phosphatase and tensin homolog (PTEN) mutations are prominently detected in thicker primary tumors and metastases. These observations suggest that loss of CDKN2A might trigger early invasive capabilities in melanoma, whereas p53 and PTEN inactivation occurs in later stages. Consequently, the depth of a lesion correlates with the metastatic and lethal risks, and thus constitutes an important criteria for clinical melanoma staging.

1.2.3 Melanoma therapy

Owing to the apparent character of most skin lesions, most melanoma patients are diagnosed at early-stages of the disease. Surgical resection of localized lesions is curative in most cases. Treatment of metastatic melanoma has long been limited to palliative care. In the past, chemotherapeutic agents have been used as the only available treatment option for advanced metastatic melanoma. As they provide no benefit in overall survival (OS), they have been progressively abandoned as single agents (Domingues et al., 2018; Dummer et al., 2016). Fortunately, several new classes of therapeutic agents demonstrating remarkable response rates have been approved in the 2010s. These include small-molecule inhibitors targeting the MAPK signaling pathway and immune checkpoint inhibitors.

1.2.3.1 Inhibition of the MAPK pathway

Highly prevalent in melanoma, oncogenic MAPK signaling presents an interesting target for therapy. Two targeted BRAF inhibitors (BRAFi) with high selectivity towards $BRAF^{V600E}$ have been approved by the FDA: vemurafenib in 2011 and dabrafenib in 2013. Used as single agents, they have shown spectacular response rates. In a landmark study by Chapman and colleagues, treatment with vemurafenib led to initial tumor regression in over 50% of patients (Chapman et al., 2011). Progression-

free survival was also significantly improved from 1.6 to 5.3 months for patients treated with the chemotherapeutic agent dacarbazine or vemurafenib, respectively. Similar results were observed with dabrafenib (Hauschild et al., 2012). Unfortunately, these responses are often short-lived and patients invariably relapse. Resistance to BRAFi may occur through recovery of the MAPK pathway or activation of PI3K/Akt signaling (Luebker and Koepsell, 2019). Extensive analysis by Johnson and colleagues of 133 samples from 100 patients undergoing relapse following BRAFi treatment lead to the identification of a resistance mechanisms in 58.3% of cases (Johnson et al., 2015). BRAF amplifications or alternative splicing, and mutations in NRAS and MEK1/2 were frequently observed, providing a rationale for the combined inhibition of BRAF and MEK. Combination of dabrafenib and the MEK inhibitor trametinib lead to increased response rates and progression-free survival in clinical trials, but resistance ultimately occurs through similar mechanisms (Long et al., 2014). Upregulation of receptor tyrosine kinases such as epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR) or fibroblast growth factor receptor 1 (FGFR1) or AXL receptor tyrosine kinase (AXL) contribute to MAPK reactivation in BRAFi-resistant cell lines (Shaffer et al., 2017). Interestingly, a low ratio of MITF/AXL expression was identified in BRAFi-resistant melanoma cells and predicted resistance to BRAF inhibitors (Müller et al., 2014). It was further shown that an antibody-drug conjugate (ADC) targeting AXL-expressing cells collaboratively potentiated BRAF and MEK inhibition in melanoma (Boshuizen et al., 2018), highlighting the central role of AXL in mediating resistance to MAPK pathway inhibition.

Multiple treatment regimens featuring BRAF/MEK/ERK inhibitors are currently undergoing clinical trials. It is now apparent that further understanding of resistance mechanisms and identification of potential synergisms with alternative therapeutic strategies will be necessary for the development of treatment regimens offering durable response rates. Checkpoint inhibitors and other immunotherapies may reveal to be particularly relevant in this regard.

1.2.3.2 Immunotherapy

1.2.3.2.1 The “dark-age” of immunotherapies: pre-immune checkpoint era

The observation that T-cells can recognize tumor-associated neoantigens led to a surge of interest in immune approaches to cancer therapy (van der Bruggen et al., 1991). “Immunotherapy” is an umbrella term that encompasses various approaches.

Older strategies include unspecific stimulation of immunity using cytokines and interferons. In a pioneer study published in 1985 by Steven Rosenberg, high-dose injections of the T-cell growth factor interleukin-2 (IL-2) induced an overall-response rate of 16%, with complete response occurring in 4% of the patients (Domingues et al., 2018; Rosenberg et al., 1985). The relevance of this strategy remained limited by the modest response rates and the severe toxicity induced by systemic IL-2 administration. In 1996, Kirkwood and colleagues reported that injections with high doses of the type I interferon IFN-α-2b prolonged disease-free survival and overall survival of stage IIB and stage III melanoma patients as adjuvant therapy (Kirkwood et al., 1996). Despite limited long-term benefits, this treatment was approved

by the U.S. Food and Drugs Administration (FDA) and became the reference standard for subsequent evaluation of alternative treatment regimens.

Cell-based approaches were first reported to induce objective responses in 1994 (Rosenberg et al., 1994). Adoptive-cell transfer (ACT) of autologous tumor-infiltrating lymphocytes (TILs) in conjunction with high-dose IL-2 induced an overall-response rate of 31%, but failed to produce durable responses or tumor shrinkage. In 2002, selection and in vitro expansion of tumor-specific T-cells combined with pre-treatment lymphodepletion was shown to ameliorate ACT outcome (Dudley et al., 2002) and lead to tumor regression in six of thirteen melanoma patients by allowing persistent T-cell clonal repopulation. However, in subsequent trials, complete remission was only obtained in 10-20% of patients, and most patients did not benefit from durable responses (Rosenberg et al., 2011).

Cancer vaccines of many sorts have undergone clinical trials. Shared melanosomal proteins, cancer testis antigens, autologous or allogeneic tumor antigens have all been used as immunizing agents. These approaches have so far failed to improve overall-survival (Weiss et al., 2019). Oncolytic viruses such as talimogene laherparepvec have been approved for treatment of metastatic melanoma, but their efficacy remains limited to the site of injection, and there is to date no evidence for prolonged survival (Andtbacka et al., 2016).

1.2.3.2.2 Immune checkpoint inhibitors: the new standard of care

Identification of the CTLA4 immune checkpoint pathway in 1994 (Walunas et al., 1994) paved the way for the development of a new class of drugs which revolutionized the management of metastatic melanoma and many other cancers. In 2010, a phase III clinical study of the anti-CTLA4 monoclonal antibody (mAb) ipilimumab achieved the formerly elusive goal of increased median overall survival from, 6.4 to 10.0 months compared to treatment with a gp100 peptide vaccine (Hodi et al., 2010). Moreover, long-term (24-months) survival was doubled from 13.7% to 23.5%, suggesting that durable responses could be achieved in a subset of patients. These encouraging results were quickly followed by trials of the fully human IgG4 antibody against PD1 nivolumab. FDA approval for this new agent was granted after the CheckMate 037 study, which showed improved objective response with anti-PD1 treatment compared to chemotherapy in melanoma patients previously treated with ipilimumab or ipilimumab + BRAFi whose disease had progressed (Weber et al., 2015). In previously untreated patients, nivolumab outperformed dacarbazine as first-line therapy with an objective response rate of 40% compared to 14%, and improving 1-year survival from 42% to 73% (Robert et al., 2014). Nivolumab was further shown to induce higher rates of recurrence-free survival in the adjuvant setting compared to ipilimumab (Weber et al., 2017). Importantly, PD1 blockade was well tolerated and only rarely led to severe treatment-related adverse events compared to CTLA4 inhibition. Other antibodies targeting PD1 and PD-L1 have since shown encouraging results in clinical trials and have been approved for the treatment of various solid tumors (Hargadon et al., 2018). The CTLA4 and PD1 pathways function non-redundantly, providing a rationale for combined inhibition in cancer therapy. In first-line treatments using nivolumab + ipilimumab, a 3-year survival rate of 58% was reached, with 39% of progression-free survival, thus

outperforming nivolumab or ipilimumab monotherapies (Wolchok et al., 2017). These impressive results have quickly established this new class of therapeutics as the treatment of choice for first-line and adjuvant therapies in patients with advanced melanoma.

1.2.3.2.3 Resistance to immune checkpoint blockade

These unprecedented results are mitigated by the fact that most patients do not benefit from immune checkpoint inhibitors. Whilst some tumors never respond to these treatments, others may initially shrink, but ultimately recur. Both tumor-intrisic and extrinsic factors can contribute to primary and acquired resistance to ICB.

1.2.3.2.3.1 Factors contributing to resistance to checkpoint inhibition

The most obvious factor that contributes to primary resistance to ICB is the lack of recognition of tumor cells by cytotoxic lymphocytes due to the absence of tumor-associated neoantigens (Gubin et al., 2014). In line with this idea, high tumor mutational burden strongly correlates with survival of patients treated with checkpoint inhibitors (Samstein et al., 2019). Accordingly, melanoma patients and lung cancer patients with a history of smoking most frequently benefit from such treatments. However, even when neoantigens are abundantly expressed, they may remain hidden from patrolling lymphocytes due to downregulation of MHC molecules by cancer cells (Sucker et al., 2014).

Alternatively, immune infiltration of the tumor bed can be inhibited by specific immuno-suppressive signaling pathways. Among these, alterations in the MAPK and PI3K pathways are particularly relevant for melanoma. Treatment of melanoma cells with selective BRAF inhibitors increases expression of melanoma-associated antigens such as gp100 and MART-1 without affecting T-cell effector function, suggesting that oncogenic BRAF signaling inhibits T-cell function by downregulating melanoma-specific antigens (Boni et al., 2010). Constitutive BRAF signaling inhibits anti-tumor immunity through upregulation of the immunomodulatory cytokines IL-6, IL-10 and VEGF (Sumimoto et al., 2006). Conversely, the same cytokines can be transcriptionally-repressed by rescuing PTEN expression in SKMEL-28 cells (Dong et al., 2014). Furthermore, low PTEN copy number correlates with reduced IFN-γ and granzyme B expression in the Cancer Genome Atlas (TCGA) dataset for melanoma, conferring clinical relevance to these findings (Peng et al., 2016). PTEN deletions may also lead to constitutive expression of PD1 ligands (Parsa et al., 2007). Primary resistance to ICB can also arise from alterations in the IFN-γ pathway. Inactivation of IFN-γ receptors and of downstream mediators were suggested to reduce the efficacy of both CTLA4 and PD1 blockade (Gao et al., 2016; Shin et al., 2017). Finally, the TGF-β pathway has long been recognized for its immunosuppressive abilities which will be discussed in details below.

Beyond tumor-intrinsic factors, the suppressive immune subsets which drive the escape phase of cancer progression can also induce primary resistance to checkpoint inhibitors. For example, in a GM-CSF-expressing B16 melanoma model, the efficacy of CTLA4 blockade depend on the balance between effector and regulatory T-cells (Quezada et al., 2006). However, it was recently reported that CTLA4

blockade by ipilimumab or tremelimumab promotes CD4 and CD8 T-cell infiltration within tumors but does not deplete FoxP3+ regulatory T-cells in patients with metastatic melanoma (Sharma et al., 2019). This observation suggests that the therapeutic activity of CTLA4 blockade might function independently of Tregs in human patients. In contrast, the number of circulating monocytic MDSCs in melanoma patients correlates with reduced response rates to anti-CTLA4 treatment, establishing MDSC blood count as a potential biomarker (Meyer et al., 2014). Resistance to ICB in various murine solid tumors can also be mediated by macrophages (Cassetta and Kitamura, 2018). For example, macrophage reprogramming using CSF1R blocking antibodies or PI3K-γ inhibitors sensitized both melanoma and breast tumor models to PD1 and CTLA4 blockade (De Henau et al., 2016; Neubert et al., 2018). Collectively, these results suggest that rational targeting of suppressive immune cells can ameliorate response to cancer immunotherapy.

While the list of factors that influence response to immune checkpoint inhibition is continually growing, reliable biomarkers are still needed to efficiently predict response in patients and to determine adequate course of treatment in the clinic. Complex genomic and transcriptomic signatures may help to achieve this goal (Hugo et al., 2016; Van Allen et al., 2015). Provided that these signatures are dependable and adaptable to the clinical setting, predicted ICB-sensitive patients could benefit from first line checkpoint inhibition, whilst others would receive prior sensitizing regimens. An important focus of current research on immunotherapies is the identification of combination strategies susceptible to overcome resistance mechanisms and increase both the amplitude and duration of response, as well as the fraction of patients that would respond to such treatments.

1.2.3.2.3.2 Overcoming resistance to immune checkpoint blockade

Chemotherapy: Pre-clinical and clinical data using conventional therapeutic approaches in combination to immune checkpoint inhibitors have already demonstrated encouraging results. The immunomodulatory effects of chemotherapies have long been hypothesized to contribute to the efficacy of these agents. By inducing DNA damage, and through direct cytotoxic functions, chemotherapy can increase tumor antigenicity and the release of tumor-associated antigens, thereby promoting T-cell priming by APCs. A recent meta-analysis of clinical trials for the combination of conventional chemotherapy plus PD1/PD-L1 blockade in non-small cell lung cancer (NSCLC) suggested increased overall survival compared to chemotherapy alone (Addeo et al., 2019). It is unfortunately unknown whether similar benefits could be obtained with checkpoint inhibitor monotherapy, as this group was not included in these studies. Promising results have been obtained with similar approaches in a small study cohort for metastatic melanoma. Treatment with chemotherapy following disease-progression after PD1 blockade increased median survival from 1.8 to 5.0 years (Aguilera et al., 2018).

Radiotherapy: In addition to chemotherapy, radiotherapy was also shown to improve tumor control by checkpoint inhibitors in various murine models of melanoma, breast, lung and colorectal cancers (Lamichhane et al., 2018). Synergistic mechanisms were suggested in breast tumors, where radiation therapy increased PD-L1 expression, ameliorating response to PD-L1 blockade and preventing MDSC accumulation within the tumor micro-environment (Deng et al., 2014). In melanoma, a phase 2 clinical

study of melanoma patients with brain metastases reported a 1-year overall survival of 31.8% amongst 51 patients, qualified as “higher than historical values” (Lopez-Martin et al., 2018). Further studies will however be necessary to confirm these results.

Antiangiogenic therapies: Targeting VEGF-A may synergize with immune checkpoint inhibitors by preventing VEGF-mediated immunosuppression, and promote drug delivery by normalizing the tumor-associated vasculature. Dual inhibition of the angiogenic factors VEGF-A and Angiopoietin 2 using the bispecific antibody A2V was shown to enhance immune control of B16.OVA melanoma when combined with PD1 blockade (Schmittnaegel et al., 2017). Mechanistically, A2V stimulated antigen-presentation by increasing tumor necrosis. In addition, vascular normalization facilitated extravasation of cytotoxic lymphocytes into the tumor bed. Early-phase clinical studies suggest safe toxicity profiles and encouraging response rates for the combination of checkpoint inhibitors together with the VEGF-A blocking antibody bevacizumab in NSCLC and renal cell carcinoma (RCC) (Schmidt, 2019). Importantly, combined inhibition of PD1/PD-L1 and VEGF-A results in ORR that exceed additive rates of corresponding monotherapies, suggesting synergistic mechanisms for these strategies in cancer patients.

Targeted therapies: Considering its immunosuppressive functions, inhibition of the MAPK pathway with BRAF and MEK inhibitors may potentiate immune checkpoint blockade. In line with this idea, depletion of CD8 T-cells abrogated the anti-tumor effects of the BRAF inhibitor PLX4720 in $BRAF^{V600E}$ mouse melanoma tumors (Knight et al., 2013). Using the same model, Hu-Lieskovan and colleagues demonstrated superior anti-tumor activity of PD1 blockade when combined with the BRAF and MEK inhibitors dabrafenib and trametinib (Hu-Lieskovan et al., 2015). Concerns were raised in clinical trials for the combination of anti-CTLA4 and vemurafenib, which have been interrupted due to severe liver toxicity events (Ribas et al., 2013). Targeting the PD1/PD-L1 pathway appears to be better tolerated when used in combination with inhibitors of the MAPK pathway in melanoma patients (Yan et al., 2018). Many clinical trials using these combination strategies are currently ongoing. Recently, a first-in-human trial of triple BRAF, MEK and PD1 inhibition in a small cohort improved the response rate to 73% (n=15) (Ribas et al., 2019). Future studies with larger patient cohorts and longer follow-up periods will be needed to verify the superiority of these treatment regimens over current standard of care.

Because of its well established immunosuppressive functions, the TGF-β pathway constitutes another interesting target for combination strategies. Two recent reports have underlined the pivotal role of TGF-β signaling in mediating resistance to immune checkpoint inhibition. Microsatellite stable (MSS) colorectal cancers (CRC) frequently harbor inactivating mutations in the TGF-β pathway, but retain a TGF-β signature which originates from cancer-associated fibroblasts. These cancers have to date shown poor response to ICB. In mouse genetic models which accurately reproduce the histological and micro-environment features observed in human patients, combination of the TGF-β inhibitor galunisertib with anti-PD1 antibodies eradicated established metastases and prolonged progression-free survival for over a year, despite treatment cessation at day 30 (Tauriello et al., 2018). In the same Nature issue, whole-exome and transcriptome sequencing by Mariathasan and colleagues identified a fibroblast-specific TGF-β signature, which correlated with immune desertification and with resistance to PD-L1

blockade by atezolizumab in metastatic urothelial cancer (Mariathasan et al., 2018). Clinical studies are eagerly awaited to validate these findings in human patients. Beyond its immunoregulatory functions, TGF-β signaling also plays many other fundamental roles in cancer development that will be the focus of the next chapter.

Over 3000 clinical trials using immune checkpoint inhibitors together with other therapeutic agents are currently in progress, illustrating the immense efforts currently undertaken to provide effective and rational treatments based on immunotherapies (Tang et al., 2018). To maximize response rates, the choice of a combination regimen should be patient-tailored: whereas immune-deserted tumors would likely benefit from immunogenic chemotherapy or radiation therapy, MAPK inhibitors could be more suited to patients bearing BRAF-mutant infiltrated tumors. Collectively, these personalized combination regimens are anticipated to significantly improve overall response and progression-free survival rates of patients treated with immuno-therapeutic agents, as well as expanding their applicability to cancers that have traditionally proved insensitive to single agent checkpoint inhibition.

1.3 TGF-β pathway

1.3.1 TGF-β signal transduction

TGFs were originally isolated from neoplastic cells as polypeptides that interacted with epidermal growth factor (EGF) to promote anchorage-independent growth in soft agar assay (Roberts et al., 1981; Todaro et al., 1980). Since then, a superfamily of 33 TGFβ-related ligands has been identified by sequence homology upon completion of human and mouse genome projects (Morikawa et al., 2016). These are subdivided in the TGF-β subfamily, which includes 3 TGF-β isoforms, Nodal and Activins, whereas the Bone Morphogenetic Protein (BMP) subfamily includes BMPs and Growth and Differentiation Factors (GDFs). TGF-β and other related ligands are secreted as inactive precursors bearing large N-terminal prodomains, which are required for their intracellular dimerization and activity (Gray and Mason, 1990), stability after secretion (Constam and Robertson, 1999; Johnson et al., 2016) and/or solubility (Pepinsky et al., 2017). Following cleavage by the prototypical proprotein convertase Furin, this prodomain remains bound as a latency-associated peptide (LAP) to latent TGF binding proteins (LTBP), thus forming latent TGF-β (L-TGF-β) (Constam, 2014). LAP dissociation, which can occur at acidic pH, under the action of matrix metalloproteases (MMPs), or through tensile strength induced by specific integrins, leads to the release of active mature TGF-β (Munger et al., 1999; Robertson and Rifkin, 2016; Yu and Stamenkovic, 2000).

Following the discovery in 1991 of the first Activin receptor, since then termed Activin receptor II (ACVRII), seven type I and five type II receptor serine/threonine kinases for TGF-β ligands were identified (Moustakas and Heldin, 2009). Upon binding of mature TGF-β, heterotetrameric complexes of type II (TGFBR2) and type I receptors (TGFBR1, or ALK5) assemble, allowing phosphorylation of

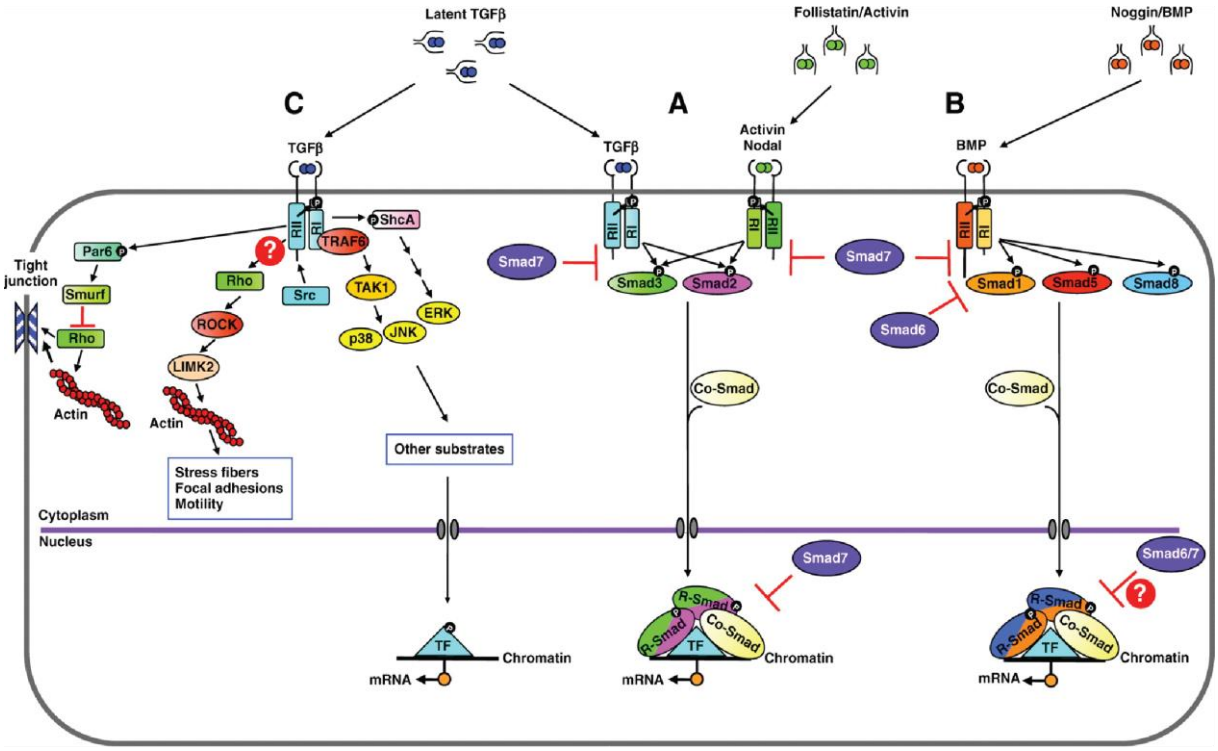


Figure 4: Signal transduction by the TGF-β superfamily
Adapted from Moustakas & Heldin (2009) Development.

TGFBR1 and replacement of the inhibitory partner FK506-binding protein (FKBP12) by receptor-regulated SMADs (R-SMADs) SMAD2 and SMAD3 (Huse et al., 2001) (Fig. 4). SMAD proteins comprise a DNA-binding Mad-Homology 1 (MH1) domain and an MH2 domain capable of homo- and hetero-dimeric interactions with SMADs and other co-regulators. Receptor-mediated phosphorylation of R-SMADs on two C-terminal Serine residues leads to association with the Co-SMAD SMAD4 and exposure of a nuclear import signal contained within the Smad2 MH2 domain (Xu et al., 2000). Activated trimers constituted by two R-SMADs and one SMAD4 molecules are then translocated in the nucleus, a process facilitated by nucleoporins (Chen and Xu, 2010). SMAD3 and some rare SMAD2 isoforms are endowed with DNA-binding capabilities (Dennler et al., 1998). Nonetheless, binding of the SMAD complex to SMAD-binding elements (SBE) most often requires association with other transcription factors, which dictate response to TGF-β signaling in a cell-type-specific manner by co-regulating specific groups of genes (Mullen et al., 2011).

Beyond regulating gene expression through SMAD proteins, TGF-β can also signal in a non-canonical manner, in particular by engaging the MAPK and PI3K pathways (Zhang, 2017). Treatment of epithelial cells by TGF-β was shown already in the early 1990s to induce ERK1/2 phosphorylation in a time frame similar to that of conventional MAPK activation by receptor tyrosine kinases, albeit less potently (Yan et al., 1994). TGF-β can also activate Akt signaling through PI3K, independently of SMAD proteins (Lamouille and Derynck, 2007). The regulatory PI3K subunit p85 can co-immunoprecipitate with TGFBR1, but only in presence of TGF-β (Yi et al., 2005). It was later shown that the formation of a

complex between p85α and TGFBR1 was induced by TGF-β through polyubiquitylation of p85α by the E3 ubiquitin ligase TRAF-6, leading to activation of PI3K and Akt (Hamidi et al., 2017). Indirect activation of PI3K signaling by TGF-β has also been reported (Vinals and Pouyssegur, 2001). In addition, TGFβ via TRAF6 stimulates TAK1, which in turn activates p38 MAPK in JNK by phosphorylating them (Fig. 4). TRAF6 also promotes proteolytic cleavage of TGFβR1, leading to nuclear translocation of a TGFβR1 intracellular domain (ICD) and promotion of migratory properties in cancer cells (Mu et al., 2011). However, the roles of SMAD-independent TGFβ signaling in human cancer remain poorly defined.

1.3.2 Regulation of TGF-β signaling

TGF-β signaling has pleiotropic functions in cell and tissue homeostasis that need tight regulation. Regulation can occur at all steps of signal transduction. Expression of the inhibitory SMADs (I-SMADs) SMAD6 and SMAD7 is directly induced by TGF-β and essential for the negative feedback control of TGF-β signaling (Afrakhte et al., 1998). At the receptor level, SMAD7 terminates R-SMAD recruitment and activation by competing for R-SMAD binding sites on TGFBR1 (Yan et al., 2009b). SMAD7 also prevents association of type I with type II receptors and recruitment of R-SMADs in cooperation with the pseudoreceptor BAMBI (Yan et al., 2009a). Upon recruitment of SMAD7, TGFBR1 is ubiquitinated by Smurf proteins and targeted to proteasomes for degradation (Ebisawa et al., 2001; Kuratomi et al., 2005). Phosphatases of the PPP family also regulate receptor activity in cooperation with I-SMADs (Liu and Feng, 2010). Recruitment by SMAD7 of GADD34, a regulatory subunit of the PP1 phosphatase, leads to TGFBR1 dephosphorylation, contributing to signal termination (Shi et al., 2004). Receptor endocytosis can promote or inhibit TGF-β signaling. Whereas clathrin-dependent internalization potentiates signaling by facilitating interactions of TGF-β receptors with R-SMADs through Smad anchor for receptor activation (SARA), receptor endocytosis through caveolae preferentially leads to interactions with SMAD7-Smurf2 complexes and induces receptor degradation (Di Guglielmo et al., 2003). I-SMADs may also regulate TGF-β signaling by interacting with R-SMADs. By binding to SMAD2/3 complexes, SMAD7 prevents SMAD4 recruitment and leads to R-SMAD degradation in cooperation with the NEDD4L ubiquitin ligase (Yan et al., 2016). Furthermore, SMAD7 prevents the formation of functional SMAD-DNA complexes by directly binding to SMAD-responsive elements (Zhang et al., 2007).

Regulation of TGF-β signaling also occurs independently of I-SMADs. When engaged in transcriptional regulation, SMAD proteins rapidly undergo polyubiquitylation followed by proteasome-mediated degradation, contributing to signal termination (Gao et al., 2009). Additionally, SMAD localization, which is determinant for signal transduction, is tightly regulated. R-SMADs nucleocytoplasmic shuttling occurs through the action of nucleoporins, and this equilibrium depends on various phosphorylation, ubiquitylation and sumoylation events (Moustakas and Heldin, 2009). Transcriptional co-repressors such as Ski and SnoN negatively regulate TGF-β signaling by forming inhibitory complexes with SMADs, preventing association with SMAD-binding elements in target genes (Tecalco-Cruz et al., 2018).

Interestingly, expression of Ski increases during melanoma progression, contributing to the gradual loss of TGF-β-mediated cytostasis (Chen et al., 2009).

1.3.3 TGF-β as a tumor suppressor

1.3.3.1 TGF-β inhibits cell proliferation

In normal epithelia and benign neoplasias, TGFβ signaling functions as a tumor suppressor by inhibiting cell proliferation. The cytostatic effects of TGF-β were reported in many cell lines as early as 1984 (Tucker et al., 1984). The mechanisms underlying the regulation of cell cycle progression by TGFβ involves induction of cyclin-dependent kinase (CDK) inhibitors, and suppression of proliferative signals, and/or induction of premature cellular senescence (Zhang et al., 2017). Direct induction of CDK inhibitors $p15^{INK4B}$ and p21CIP1 hinders cell cycle progression from G1 to the S phase by binding to CDK4 and CDK2, thereby preventing CDK-activation by cyclins (Matsuoka et al., 1995; Sandhu et al., 1997). In parallel, TGFβ signaling represses the expression of the oncogenic transcription factor c-MYC. This repression is frequently lost in malignant cells, contributing to their aberrant proliferation (Chen et al., 2001). Transcription factors of the Id family are similarly repressed by TGF-β, thus reducing cell proliferation by impeding Rb phosphorylation (Kang et al., 2003; Ouyang et al., 2002). Tumors frequently circumvent the cytostatic functions of TGF-β by altering components of this pathway. TGFBR2 was shown to be deleted, mutated or downregulated in various cancers (Izumoto et al., 1997; Markowitz et al., 1995). TGFBR1 mutations were also reported and contribute to disease aggressiveness in breast cancer (Chen et al., 1998). Interestingly, a 9 base pair deletion in the first exon of TGFBR1, which results in decreased signaling efficiency, was identified as a cancer susceptibility allele (Pasche et al., 1999). SMAD4 inactivation is found in >50% of invasive pancreatic cancers and correlates with poor prognosis (Xia et al., 2015). Nonetheless, inactivation of TGF-β receptors or SMAD4 alone is not sufficient to induce tumors, indicating that concomitant oncogenic stress is required (Meulmeester and Ten Dijke, 2011).

1.3.3.2 TGF-β regulates apoptosis

TGF-β can also promote or inhibit apoptosis depending on cell type and context (Siegel and Massague, 2003), and pro-apoptotic signaling specifically in cells undergoing EMT is now considered to be central for its tumor-suppressive activity. Mechanistically, TGF-β may participate in apoptosis initiation through induction of TGF-β-inducible early response gene 1 (TIEG1), which represses the anti-apoptotic factor Bcl2 (Chalaux et al., 1999). More recently, TGF-β was shown to induce apoptosis and mediate tumor suppression in $Smad4^{+}$ pancreatic ductal adenocarcinoma cells through repression of the lineage survival gene Klf5, which turned Sox4 into a pro-apoptotic transcription factor (David et al., 2016). Nevertheless, the precise mechanisms by which TGF-β induces apoptosis are still poorly understood

and further studies will be required to fully elucidate the intricate molecular basis underpinning this process and its contribution to tumorigenesis in human patients.

1.3.4 TGF-β as a tumor promoter

TGF-β signaling is frequently described as a double-edged sword in cancer progression. Indeed, beyond its role as a tumor suppressor, TGF-β can facilitate cancer progression by regulating several hallmarks of cancer.

1.3.4.1 TGF-β and the epithelial-to-mesenchymal transition (EMT)

Epithelial cells undergoing EMT lose cell-cell adhesion capabilities and apico-basal polarity, and gain mesenchymal traits such as increased migratory properties and secretion of ECM components. In development, several rounds of EMT and it opposite process, MET, are essential for the formation of the body plan and organogenesis (Thiery et al., 2009). In adults, EMT plays a crucial role in wound healing, were local keratinocytes migrate in order to restore epithelial function (Stone et al., 2016). As discussed above, the molecular events leading to EMT involve a set of transcription factors, including ZEBs, SNAILs and TWISTs, which are potently induced by TFG-β signaling to promote mesenchymal cell fates. Genes downregulated by these transcription factors include adhesion proteins such as E-cadherin as well as genes implicated in establishment of cell polarity, leading to adherens junction breakdown and loss of cell-cell adhesion (Batlle et al., 2000; Spaderna et al., 2008). In contrast, EMT-TFs induce expression of MMPs and cytoskeletal proteins associated with the mesenchymal state to promote migration (Lamouille et al., 2014). In cancer progression, the EMT program can be coopted by tumor cells to promote their invasive and survival capabilities, and it is considered by many to be essential for metastasis (Dongre and Weinberg, 2019). Several observations suggest TGF-β to play a pivotal role in this process. By immunohistochemistry, expression of TGF-β was observed to be confined to the primary tumor invasive front and to lymph node metastases in human mammary carcinoma (Dalal et al., 1993). Moreover, in a mouse model of breast cancer, TGF-β induced by radiation therapy promotes metastatic spread, an effect that can be abrogated by TGF-β-blocking antibodies or in tumors of mice that lack Tgfbr2 (Biswas et al., 2007). Intravital imaging of breast cancer cells undergoing metastatic spread revealed that transient activation of TGF-β signaling favors single cell motility and increased blood-borne metastasis (Giampieri et al., 2009). However, recent observations challenged the notion that EMT *per se* is necessary for tumors to metastasize. Using an EMT lineage-tracing allele in the PyMT breast cancer model, Fischer and colleagues observed that most lung metastases had never undergone EMT (Fischer et al., 2015). In good agreement, Zhen and colleagues deleted Snail or Twist in a model of PDAC and observed no reduction in the number of liver, lung or spleen metastasis (Zheng et al., 2015). These results question the necessity of EMT for dissemination of invasive cells. Nonetheless, in the same PDAC model, depletion of ZEB1 reduces grading and distant metastasis

without affecting primary tumor growth (Krebs et al., 2017) Therefore, discussions about the relative importance of EMT in metastasis continue (Aiello et al., 2017; Ye et al., 2017).

1.3.4.2 TGF-β and tumor angiogenesis

TGF-β can promote or inhibit angiogenesis according to context. This may be explained by the observation that endothelial cells respond to TGF-β in dose-dependent manner. Whereas low dose treatment promotes EC proliferation and migration, these effects are lost at high concentrations (Pardali and ten Dijke, 2009). Moreover, TGF-β can signal through distinct receptors. In human umbilical vascular endothelial cells (HUVEC), signaling through ALK1 induces expression of the inhibitory SMADs SMAD6/7 and of endoglin, leading to endothelial cell proliferation. In contrast, activation of TGFBR1 induces genes involved in ECM remodeling and perivascular cell differentiation, and restricts EC proliferation and migration (Goumans et al., 2002; Ota et al., 2002). This suggests that TGF-β signaling may regulate both the initiation and resolution phases of angiogenesis. TGF-β can also promote angiogenesis by upregulating VEGF in tumor cells and in the microenvironment (Teraoka et al., 2001). In mouse models of breast and colon cancers, as well as in several human cancer cell lines, inhibition of TGF-β reduces angiogenesis (Bandyopadhyay et al., 2002; Halder et al., 2005), although contrary effects have been reported in hepatoma and squamous cell carcinoma models (Kim et al., 2001; Yee et al., 2004).

1.3.4.3 TGF-β and immunosuppression

TGF-β1 knockout mice die after 3-4 weeks of age following onset of a dramatic inflammatory syndrome characterized by excessive immune infiltration of the heart, lungs, and other organs (Kulkarni et al., 1993). This phenotype strikingly illustrates the fundamental role of TGF-β signaling in maintaining immune homeostasis. During thymic T-cell development, adequate TCR signaling drives clonal survival and maturation. Although essential for the establishment of central tolerance, this selection process is imperfect and occasional autoreactive clones manage to reach the periphery where they must be constrained to prevent autoimmune disease. TGF-β signaling directly and indirectly regulates autoreactive lymphocytes to maintain peripheral tolerance, notably by suppressing T-cell proliferation and activation, and by inducing Treg differentiation (Ahmadzadeh and Rosenberg, 2005; Ishigame et al., 2013). The immunosuppressive functions of transforming growth factor β go well beyond regulation of T-cell function, as TGF-β signaling was shown to alter proliferation and function of most if not all immune subsets (Sanjabi et al., 2017). Notably, in inflammatory conditions, TGF-β promotes recruitment of immature monocytes but inhibits macrophage activation and function by preventing expression of inflammatory mediators and hampering phagocytosis (Tsunawaki et al., 1988; Wahl et al., 1987). Moreover, TGF-β induces macrophage expression of the receptor tyrosine kinase AXL, which upon induction by type-I interferons inhibits macrophage phagocytosis and innate inflammatory responses (Sharif et al., 2006). Dendritic cells respond to TGF-β by downregulating surface expression of MHC-II

and of the B7 family co-stimulatory ligands CD80 and CD86, impairing T-cell priming (Geissmann et al., 1999).

Considering these central immunomodulatory functions, it is unsurprising that cooption of the TGF-β pathway by various malignancies allows tumors to escape immune surveillance. In line with this idea, TGF-β inhibition reduces tumor infiltration by Tregs and inhibits tumor growth in various cancer models, including melanoma, providing synergistic opportunities when combined with immunotherapeutic approaches (Gil-Guerrero et al., 2008; Mariathasan et al., 2018; Tauriello et al., 2018; Xu et al., 2014).

1.3.5 TGF-β signaling in melanoma

TGF-β inhibits the proliferation of normal melanocytes. Upon malignant transformation, melanoma cell lines become insensitive to TGF-β-induced cytostasis and tend to increase the expression of all three TGFβ isoforms, albeit in their inactive latent form (Rodeck et al., 1994). Accordingly, TGF-β plasma levels correlate with metastatic disease and poor survival in melanoma patients (Krasagakis et al., 1998; Loffek, 2018). Several mechanisms underlying TGF-β signal attenuation in melanoma cells have been reported, including upregulation of Ski, SnoN and endoglin (Perrot et al., 2013). Once freed from its tumor-suppressive functions, melanoma tumors benefit from the aspects of TGF-β signaling that promote cancer progression by both autocrine and paracrine mechanisms. Autocrine TGF-β signaling induces a pseudo-EMT program marked by downregulation of E-cadherin and increased expression of integrin β1 and β3 as well as MMP-9 (Perrot et al., 2013). On the other hand, paracrine TGF-β signaling induces the recruitment and activation of stromal fibroblasts, which promote melanoma cell survival and migration upon secretion of ECM proteins (Berking et al., 2001). Interestingly, the presence of CAFs strongly correlates with resistance to PD-L1 blockade (Chakravarthy et al., 2018). TGF-β signaling in melanoma also regulates anti-tumor immunity. Recruitment of monocytes by CCL2, which is induced in melanoma cells upon exposure to TGF-β, is sufficient to potentiate malignant progression in a non-tumorigenic model (Nesbit et al., 2001). In mice intravenously injected with B16F10 melanoma cells, prior transplantation of bone marrow expressing a dominant negative TGFBRII strikingly prolongs survival and abrogates experimental metastasis (Shah et al., 2002). Furthermore, combination therapy of TGF-β and CTLA4 blockade reduced Treg infiltration and controlled tumor growth and metastasis in a $BRAF^{V600E}$ $PTEN^{-/-}$ mouse melanoma model, whereas both monotherapies were ineffective, suggesting synergistic mechanisms (Hanks et al., 2014).

Collectively, these reports support the notion that TGF-β signaling plays a central role in melanomagenesis and provide a rationale for its inhibition in melanoma therapy. Nevertheless, a word of caution was recently raised against careless use of TGF-β inhibitors. In $BRAF^{V600E}$ $PTEN^{-/-}$ mouse melanoma, inhibition of TGF-β signaling was paradoxically found to increase cancer-associated fibroblasts (CAFs). In turn, CAFs suppressed PD-L1 expression in an MMP9-dependent mechanism and mediated resistance to PD1 blockade (Zhao et al., 2018). Interestingly, this issue was circumvented by delaying delivery of the TGF-β inhibitor relative to anti-PD1 treatment initiation, suggesting that

temporal dynamics and other unexpected factors may dictate the outcome of such combination therapies.

1.4 Activin-A signaling pathway

1.4.1 Activin-A structure and receptors

Activins were originally purified as stimulators of follicle-stimulating hormone (FSH) release by the pituitary gland and identified as members of the TGF-β superfamily following molecular characterization (Vale et al., 1988; Vale et al., 1986). Over 30 years later, new physiological functions of Activins continue to be discovered in a wide variety of contexts. Activins are composed of two so-called inhibin β subunits linked by a disulfide bridge. To date, five Activins have been identified: dimers of inhibin βA, encoded by the *INHBA* gene, form Activin A. Activin B (βB βB), Activin AB (βA βB), Activin C (βC βC) and Activin E (βE βE) were also characterized (Namwanje and Brown, 2016). Like TGF-β, Activins are synthesized as large precursors, with N-terminal pro-domains enabling the dimerization and secretion of biologically active molecules (Gray and Mason, 1990). Upon proteolytic cleavage by Furin-like proprotein convertases, Activins are secreted in non-covalent complexes with their prodomains. This association has recently been shown to extend the serum-half-life of Activins *in vivo* (Johnson et al., 2016). Owing to the relatively low affinity of Activin's prodomain for the mature peptide, binding of Activin to its receptors does not require prodomain displacement by extrinsic factors (Walton et al., 2009). Signaling is initiated upon binding to Activin receptor type II A or B (ACVR2A or B), which only share moderate homology in their extracellular and transmembrane domains. Consequently, ACVRIIB binds Activin-A with a 4-fold stronger affinity compared to ACVRIIA (Attisano et al., 1992). Binding of Activins leads to type II receptor dimerization and cooperative recruitment and phosphorylation of Activin type I receptors, also termed Activin receptor-like kinases (ALKs) (Attisano et al., 1993). Whereas Activin-A signals through ALK4 (ACVR1B), Activin-B and Activin-AB predominantly act through ALK7 (ACVR1C) (Loomans and Andl, 2016; Tsuchida et al., 2004). Like TGF-β, activated Activin receptor complexes phosphorylate SMAD2 and -3. In contrast, TGF-β and Activin-A were suggested to differ in their non-canonical signaling. In CRC, TGF-β treatment used MAPK activation to induce tumor cell migration and EMT, whereas Activin-A did so through PI3K signaling (Bauer et al., 2015). In *in vitro* experiments, Activin-A was shown to be necessary for the induction of TGF-β-induced migration, but not of growth suppression in a CRC cell line (Staudacher et al., 2017). These results indicate that TGF-β and Activin-A signaling exert overlapping but non-redundant biological functions.

1.4.2 Regulation of Activin-A signaling

Due to the sharing of several pathway components, mechanisms that regulate Activin signaling are highly similar to TGF-β's. Nonetheless, several soluble molecules serve as specific Activin inhibitors.

For example, it is thought that Activin C does not bind Activin receptors but rather inhibits Activin signaling by forming inert βA-βC heterodimers (Mellor et al., 2003). Inhibins form by dimerization of inhibin β with inhibin α subunits to antagonize activin function (Lebrun and Vale, 1997). Follistatin is another important extracellular negative regulator of Activin signaling. Alternative splicing leads to the formation of two isoforms, termed FST-288 and FST-315 (Shimasaki et al., 1988). The less abundant isoform FST-288 is able to bind heparan sulfate proteoglycans, which thus locally inhibit Activin, in part by inducing its endocytosis and lysosomal degradation (Innis and Hyvonen, 2003). By contrast, the dominant form FST-315 preferentially binds and inhibits circulating Activin-A (Sugino et al., 1997). In addition to soluble inhibitors, various co-receptors regulate Activin signaling. Betaglycan and Cripto are important mediators of TGF-β and Nodal signal transduction, respectively (Namwanje and Brown, 2016). In contrast, betaglycan antagonizes Activin signaling as it enables binding of inhibin α subunits to type II Activin receptors (Chapman et al., 2002). On the other hand, Cripto has been reported to diminish recruitment of ALK4 by type II activin receptors when overexpressed (Gray et al., 2003; Wiater et al., 2009), although in our hands Activin-A potently signals even in cells transduced with Cripto lentivirus (Fuerer et al., 2014). These regulatory mechanisms are important to consider when studying the Activin pathway. Indeed, other factors beyond expression of Activins influence downstream signaling outputs.

1.4.3 Activin signaling in homeostasis

The biological functions of Activins are pleiotropic. Activin-A plays a central role in reproductive biology. In females, it is thought to be implicated in folliculogenesis and estrogen signaling, since it can stimulate FSH release and the expression of estrogen receptor β (Kipp et al., 2007). During pregnancy, levels of circulating Activin-A and follistatin progressively increase, and inadequate serum concentrations are associated with preterm labor, pre-eclampsia and other complications (Petraglia et al., 1995). In males, Activin-A is involved in testicular development and its expression correlates with testicular size (Mendis et al., 2011).

Activins and follistatin are potent regulators of muscle growth. In mice, injection of a soluble ligand trap consisting of human Fc fused to the extracellular domain of Acvr2B (AIIB-Fc), provokes dramatic increases in muscle mass which is attributed to blockade for the most part of Activin-A, and to a lesser degree Myostatin (Latres et al., 2017; Lee et al., 2005; Walton et al., 2019). Although the precise molecular mechanisms underlying Activin-A-induced muscle wasting are still unclear, reports have suggested induction of the muscle-specific ubiquitin ligases atrogin-1 and MuRF-1, reduced mTOR signaling and increased phagocytic degradation in muscle cells as possible mediators (Chen et al., 2016; Chen et al., 2014).

Mice overexpressing Activin-A display signs of excessive scarring. Moreover, the proliferation and production of extracellular matrix by skin fibroblasts is increased upon Activin exposure, suggesting that

like TGF-β, Activin-A may be an important regulator in wound healing (Antsiferova and Werner, 2012; Fumagalli et al., 2007).

Activin-A is also a critical mediator in innate inflammatory processes. Upon LPS challenge, serum concentrations of Activin-A rapidly increase and reach peak concentrations within 45 minutes, making it the first detectable cytokine released during acute inflammation (Jones et al., 2004). During septicemia, elevated serum concentrations of Activin-A correlate with that of the C-reactive protein and predict worse outcome (Lee et al., 2016). Activin-A release occurs upon activation of Toll-like receptor pathways, as it is lost in mice lacking functional Toll-like receptor 4 (TLR4) (Jones et al., 2007). Nevertheless, the functions of Activin-A in the immune system are highly context-dependent and incompletely understood. The monocyte-macrophage lineage appears to be an important effector of Activin signaling in the immune system. For example, macrophages and monocytes are a significant cellular source of Activin-A, in particular upon activation by inflammatory stimuli (Eramaa et al., 1992; Zhang et al., 2005). Interestingly, several lines of evidence suggest that Activin may regulate macrophage function in paradoxical ways. For example, Activin-A was shown to promote the release of nitric oxide and IL-1β at steady state in bone marrow macrophages, but had the opposite effect under inflammatory conditions (Nusing and Barsig, 1999; Wang et al., 2008). Another illustration of this ambivalent effect came from the observation that Activin-A stimulates MHC-II and CD80 expression in resting macrophages, but downregulates antigen-presentation pathways in macrophages stimulated by LPS (Ge et al., 2009; Zhou et al., 2009). In cultured RAW264.7 and peritoneal macrophages, Activin-A was shown to induce expression of MMP2, arginase-1 and to inhibit IFN-γ-induced production of NO^{2-}, suggesting a role for Activin signaling in alternative macrophage activation (Ogawa et al., 2006; Ogawa et al., 2000). Activin-A also regulates dendritic cell differentiation and function. Activin-A was shown to promote Langerhans cell (LC) differentiation from $CD14^{+}$ monocytes (Musso et al., 2008), but its role in DC function appears to be more complex. In human monocyte-derived and peripheral blood DCs, inhibition of autocrine Activin-A signaling was suggested to promote secretion of DC-derived cytokines, as well as expansion of viral antigen-specific T-cells (Robson et al., 2008). In contrast, Shurin and colleagues have reported increased proliferation and anti-tumor activity of CD8 and CD4 T-cells upon co-culture with Activin-treated DCs (Shurin et al., 2016). In relation to TGF-β, Activin-A exerts overlapping but non-redundant functions in the control of T-cell differentiation and effector function. This may be explained in part by differential expression patterns of TGF-β and Activin receptors in the T-cell lineage. Whereas ALK4 is predominantly expressed in immature $CD4^{-}CD8^{-}$ precursors, expression of ALK5 is highest in mature thymocytes (Rosendahl et al., 2003). Interestingly, while assisting in TGF-β-induced regulatory T-cell differentiation, Activin-A signaling alone fails to convert $CD4^{+}CD25^{-}$ T-cells into $FoxP3^{+}$ Tregs (Huber et al., 2009). Moreover, Activin-A was shown to be released by CD4 T-cells upon Th2 activation, thus skewing macrophage activation towards anti-inflammatory M2 polarization (Ogawa et al., 2006). Finally, Activin-A was reported to potently inhibit NK cell proliferation without affecting their cytotoxic functions (Robson et al., 2009). Collectively, it is becoming increasingly apparent that Activin signaling regulates maturation, differentiation and effector functions of multiple immune subsets in

complex manners. Further studies are warranted to elucidate its relevance in pathological conditions, notably in the context of anti-tumor immunity.

1.4.4 Activin-A in cancer development

INHBA mRNA is overexpressed by a subset of patients in most solid cancers, and high expression correlates with advanced disease and poor prognosis (Antsiferova et al., 2011; Bashir et al., 2015; Deli et al., 2008; Donovan et al., 2017; Kang et al., 2009; Lonardo et al., 2011; Seder et al., 2009; Wildi et al., 2001; Yoshinaga et al., 2008).

1.4.4.1 Activin-A as a tumor suppressor

Like for TGF-β, the functions of Activin-A in cancer progression are numerous and pleiotropic. In prostate, breast, liver and pancreatic carcinoma cells, Activin-A was shown to exert growth-inhibitory effects (Antsiferova and Werner, 2012). In the T47D breast cancer cell line, Activin-A induced cell cycle arrest at the G0-G1 phase (Burdette et al., 2005). This was accompanied by increased expression of the cyclin-dependent kinase inhibitor p15 and concomitant decrease in Rb phosphorylation, suggesting that Activin-A regulates cell cycle progression in a mechanism reminiscent to that of TGF-β. Contrastingly, inhibin α was shown to act as a tumor suppressor in gonadal tumors in mice, consistent with the idea that Activin-A act as mitogens in these tissues (Matzuk et al., 1992; Woodruff et al., 1990).

1.4.4.2 Activin-A promotes tumor cell migration and metastasis

An increasing number of reports suggest that Activin-A signaling promotes tumor cell migration, invasion and metastasis in epithelial cancers. In prostate and breast cancers, elevated Activin-A serum concentrations correlate with bone metastasis occurrence (Leto et al., 2006). In colorectal cancer, combined Activin-A and TGF-β serum levels predict patient outcome (Staudacher et al., 2017) . Moreover, our lab found that overexpression of Activin-A in B16F1 melanoma promotes experimental lung metastasis (Donovan et al., 2017). Several cell intrinsic and extrinsic mechanisms were proposed for this increased metastatic propensity. In oral squamous cell carcinoma (OSCC), Bufalino and colleagues discovered that expression of Activin-A correlates with lymph node metastasis and increased risk of death (Bufalino et al., 2015). In the same study, treatment of OSCC cells with Activin-A induced expression of vimentin and N-cadherin and downregulated E-cadherin, suggestive of epithelial-mesenchymal transition. This was accompanied by increased invasive and anchorage-independent growth capabilities. Activin-A also participates in the maintenance of mesenchymal and invasive traits of non-small cell lung cancer cells (Wamsley et al., 2015). In metastatic breast cancer, Activin-A induces expression of IL13Rα2, and their combined expression correlates with metastasis and poor survival (Kalli et al., 2019). IL13Rα2 signaling was previously shown to mediate increased migratory properties of LM2 breast cancer in an ERK1/2-dependent manner (Zhao et al., 2015).

1.4.4.3 Activin-A regulates tumor angiogenesis

Activin-A exerts potent anti-proliferative functions and inhibits tube formation of human umbilical vascular endothelial cells (HUVECs) (Kaneda et al., 2011). Consequently, it is generally associated with anti-angiogenic activity, notably in gastric cancer and neuroblastoma (Kaneda et al., 2011; Panopoulou et al., 2005). Using the R30C breast cancer cell line, Krneta and colleagues report the curious observation that overexpression of Activin-A inhibits angiogenesis but paradoxically accelerates tumor growth upon injection into severe combined immunodeficient (SCID) mice, whereas follistatin leads to opposite outcomes (Krneta et al., 2006). The authors indicate that follistatin-expressing tumors feature increased susceptibility to apoptosis compared to those expressing Activin-A, but do not provide mechanistic insights to account for these uncoupled angiogenic and tumorigenic behaviors. In contrast, Activin-A stimulates corneal neo-vascularization in inflammatory conditions by upregulating VEGF-A in corneal epithelial cells (Poulaki et al., 2004). Moreover, recent studies from our lab and others have reported increased tumor angiogenesis in skin carcinomas and melanoma upon expression of Activin-A (Antsiferova et al., 2017; Donovan et al., 2017). The biological mechanisms underlying these discrepancies remain unknown.

1.4.4.4 Activin-A induces cachexia

Cachexia is characterized by involuntary loss of body weight and is estimated to be the cause of death in 20-30% of cancer patients (Murphy, 2016). Driven by a combination of factors which include elevated energy expenditure and anorexia, it can significantly affect the quality of life of cancer patients. Loss of skeletal muscle is a key characteristic of cancer-associated cachexia, and cachexia-induced cardiac atrophy is associated with cardiovascular complications and death in advanced malignancies. ACVRIIB signaling and its ligand myostatin have long been identified as critical mediators of muscle wasting in cancer. Nevertheless, amongst ACVRIIB ligands, Activin-A was shown to be the most potent negative regulators of muscle mass and Activin-A serum concentration correlates with loss of body weight in colorectal and lung cancer patients (Chen et al., 2014; Loumaye et al., 2015). Strikingly, pharmacological ACVRIIB inhibition using a soluble ligand-trap receptor fused to the human IgG2 Fc domain (AIIB-Fc) was shown to reverse prior loss of skeletal and heart muscle mass and to prolong survival of C26-tumor-bearing mice independently of tumor growth (Zhou et al., 2010). AIIB-Fc treatment of mice bearing Lewis lung carcinoma tumors similarly rescued body weight, but also decreased tumor size and the number of lung metastases (Busquets et al., 2012). Targeting Activin-A and other ACVR2B ligands therefore represents a promising strategy for the treatment of cancer-associated cachexia. Nevertheless, a clinical study in AIIB-Fc treated patients with Duchenne muscular dystrophy was recently discontinued due to adverse effects (AE), which included telangiectasia and epistaxis (Campbell et al., 2017). Concomitant inhibition of ACVR2B ligands which promote vascular quiescence, such as BMP9 and BMP10, could account for these AEs. Generation of novel therapeutic agents with

heightened specificity for Activin-A and myostatin are therefore warranted to ameliorate the safety profile of these anti-cachectic drugs. Recently, such purpose was achieved by the generation of an engineered Activin-A propeptide featuring increased affinity to its cognate mature ligand (Chen et al., 2017; Makanji et al., 2011). Upon systemic delivery, these propeptides efficiently rescued cachectic wasting induced by the injection of Activin-A-overexpressing CHO cells into the quadriceps of mice (Walton et al., 2019), supporting the therapeutic utility of this new class of agents in the treatment of cachexia.

1.4.4.5 Activin-A regulates anti-tumor immunity

Activin-A was first considered to promote cancer progression by inhibiting anti-tumor immunity in a model of skin carcinogenesis (Antsiferova et al., 2011). In this study, overexpression of Activin-A in keratinocytes accelerated tumor onset and metastasis upon treatment with a chemical skin carcinogenesis regimen. Co-expression of a dominant negative mutant of ALK4 (caALK4) in keratinocytes failed to rescue this phenotype, indicating that Activin-A promoted tumorigenesis via the stroma. Analysis of tumor-infiltrating lymphocytes revealed dramatic loss of dendritic epidermal T-cells (DETCs) in Activin-expressing tumor. DETCs form a subpopulation of resident epidermal γδ-T-cells involved in the maintenance of skin homeostasis and endowed with tumor-lytic abilities (Girardi et al., 2001). Loss of DETCs was accompanied by a concomitant increase in $FoxP3^+$ regulatory T-cells and of tumor-promoting Langerhans cells (Strid et al., 2008). However, evidence for the functional implication of these findings in the tumor-promoting effect of Activin-A was not provided. In a follow-up study, Antsiferova and colleagues demonstrate that Activin-A also promotes HPV-induced skin carcinogenesis and observe increased macrophage infiltration within Activin-expressing tumors (Antsiferova et al., 2017). RNAseq analysis of sorted tumor-associated macrophages revealed increased expression of gene sets implicated in angiogenesis, migration and immuno-suppressive functions. Finally, depletion of TAMs using anti-CSF1R antibodies delayed spontaneous tumorigenesis upon HPV exposure in the context of Activin signaling. Unfortunately, the lack of Activin-A-negative control groups in this experiment precludes formal implication of macrophages in the tumor-promoting function of Activin-A. Recently, Activin-A was proposed to inhibit anti-tumor immunity in melanoma by impairing NK cell proliferation and effector function, but evidence for the causal implication of these findings were, again, missing (Rautela et al., 2018). Clearly, further studies will be necessary to elucidate the role of Activin signaling in the regulation of anti-tumor immunity in skin malignancies and other solid tumors.

1.5 Goal of the study

Frequent upregulation of *INHBA* is part of a gene signature of human melanoma cell invasiveness (Hoek et al., 2006). Prior to the initiation of my thesis, our lab confirmed that *INHBA* mRNA is also frequently expressed in human melanoma sample (TCGA). Independently, Activin-A was also shown to be overexpressed in human melanoma by Heinz and colleagues in 2015 (Heinz et al., 2015). These authors reported that Activin-A negatively regulates lymphangiogenesis in human A375 melanoma xenografts

and promotes migratory properties of melanoma cells. In contrast, cell proliferation and tumor growth in immunodeficient mice remained unaffected. In good agreement, independent experiments in our lab using lentiviral AIIB-Fc in human melanoma xenografts expressing endogenous *INHBA* did not alter tumor progression (Kallioinen and Constam, 2012). Therefore, we hypothesized that the tumorigenic function of Activin-A signaling may depend on a functional immune system. The following work addresses three main questions related to the functions of Activin-A signaling in melanoma:

1. In a skin chemical carcinogenesis model, paracrine Activin-A signaling promotes tumor progression via the stroma, whereas tumor-cell autonomous signaling exerts tumor-suppressive functions (Antsiferova et al., 2011). The relative contributions of paracrine and autocrine Activin-A signaling in melanoma tumorigenesis and metastasis were unknown. To address this question, we generate melanoma cell lines expressing a constitutively active mutant type I receptor (caALK4) and analyze their proliferative potential *in vitro* as well as their tumor growth upon syngeneic grafting.

2. To verify whether Activin-A may promote melanoma tumorigenesis by inhibiting anti-tumor immunity, we generate Activin gain- and loss-of-function mouse melanoma cell lines and assess their tumorigenic potential in syngeneic grafts. The immune infiltrate of resulting tumors is analyzed by multiparameter flow cytometry. Moreover, the functional implication of selected immune subsets is verified by antibody-mediated depletion in tumor-bearing mice.

3. Checkpoint inhibitors are the new standard of care in melanoma therapy, but the majority of patients still do not benefit from these therapeutic agents in the long term. Anti-inflammatory cytokines such as TGF-β are associated with immune-suppressive tumor micro-environments and resistance to immune-checkpoint blockade (Mariathasan et al., 2018; Tauriello et al., 2018). To test for the first time whether resistance to checkpoint therapy in melanoma can be mediated by Activin-A, we compare efficacy of anti-PD1 and anti-CTLA4 antibodies in tumor-bearing mice in Activin gain- and loss-of-function models. Finally, the clinical relevance of these findings is validated by taking advantage of publicly available RNAseq datasets of human melanoma.

2. Results

2.1 Sustained cell-autonomous ALK4 signaling in melanoma cell lines inhibits tumorigenesis

To characterize the role of Activin-A signaling during melanoma progression, I first used the B16F1 mouse melanoma cell line, which can be grafted intradermally (i.d.) into syngeneic C57BL/6 hosts, and which metastasize to lungs at moderate frequency after injection into the tail vein (Fidler, 1973). No Activin-A was detected in the conditioned medium from cultured B16F1 cells by western blotting, and luciferase activity was not induced when the Smad3-dependent CAGA-luciferase reporter was transfected in B16F1 cells, indicating that *INHBA* is not expressed in functionally relevant amounts in these cells (Fig. 5A-B). As a surrogate to investigate the contribution of autocrine Activin signaling in melanomagenesis, B16F1 cells were transduced with lentiviruses expressing a phospho-mimicking T206D mutant form of HA-tagged ALK4 that is constitutively active (caALK4) even without ligand-binding extracellular domain (Attisano et al., 1996), or its loop 45 mutant variant that is unable to engage canonical Smad signaling (caALK4-L45m) owing to disruption of the Smad binding site (Persson et al., 1998). Surprisingly, anti-HA western blot analysis of infected cells after antibiotics-mediated selection of transduced cells readily detected the Smad signaling-deficient mutant caALK4-L45m, but no caALK4 protein (Fig. 5A). In good agreement, CAGA-luc failed to be induced in B16F1-caALK4 cells, even though robust luciferase activity was induced both upon transient transfection of a caALK4 plasmid, or after treatment with recombinant TGF-β (Fig. 5B).

We therefore asked if sustained cell-autonomous canonical ALK4 signaling might be detrimental to melanoma cell survival. To test this hypothesis, B16F1 cells were infected with doxycycline-inducible lentiviral constructs to generate B16F1 rtTA3G-caALK4 and B16F1 rtTA3G-L45m cell lines. Both caALK4 and caALK4-L45m were detected by anti-HA western blotting specifically after treatment with doxycycline for up to 36 hrs. As expected, only caALK4 and not caALK4-L45m induced Smad2,3 phosphorylation (Fig. 5C) and CAGA-Luciferase activity (Fig. 5D). Curiously, expression of caALK4, but not of caALK4-L45m, steadily declined until it became undetectable by western blotting within four cell passages (Fig. 5E). A similar trend was observed by qPCR analysis, although to an extent that could not fully account for the complete loss of protein expression (Fig. 5F). These results suggested that caALK4 expression might impair melanoma cell growth.

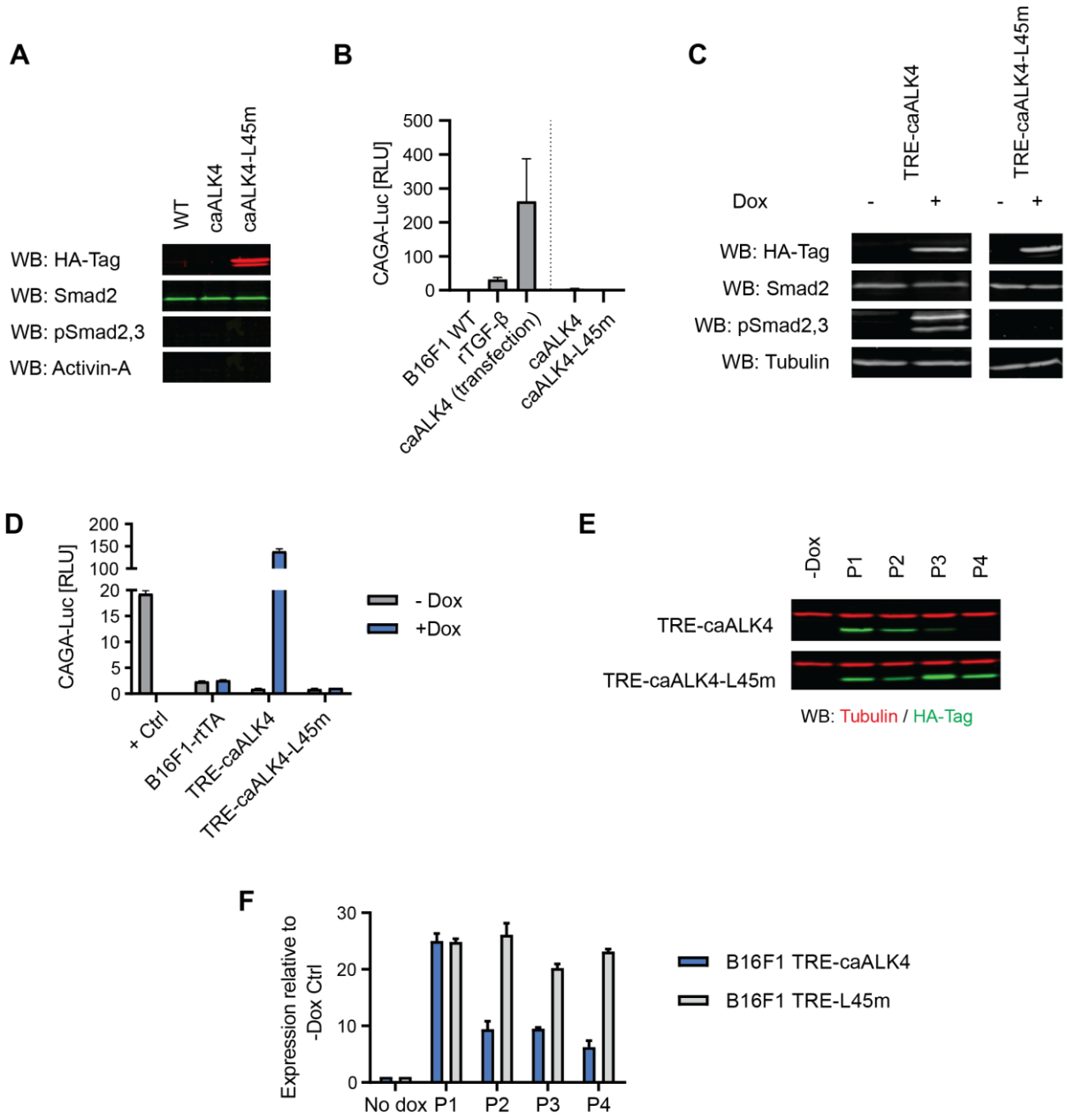


Figure 5: Loss of ALK4 expression in B16F1 melanoma cells

A. Western blot analysis of HA-tagged caALK4 or caALK4-L45m in B16F1 stable cell lines.

B. Induction of CAGA-luc reporter in B16F1 cells transduced with caALK4 or caALK4-L45m lentiviruses. Transient transfection of caALK4 plasmid or treatment with TGFβ1 [10 ng/mL] served as positive controls.

C. Dox-inducible caALK4 or caALK4-L45m expression in B16F1 cell lines treated with or without 1 µg/mL doxycycline for 36h and its effect on Smad2,3 phosphorylation analyzed by western blotting.

D. Induction of CAGA-luc expression in TRE-caALK4 or -caALK4-L45m B16F1 cells treated with or without 1µg/mL doxycycline for 36 hrs.

E. Anti-HA western blot of caALK4 expression during four subsequent cell passages in the continuous presence of 1µg/mL doxycycline.

F. RNA expression analyzed by qRT-PCR of caALK4 and caALK4-L45m in inducible cell lines cultured in the presence of doxycycline during four subsequent cell passages.

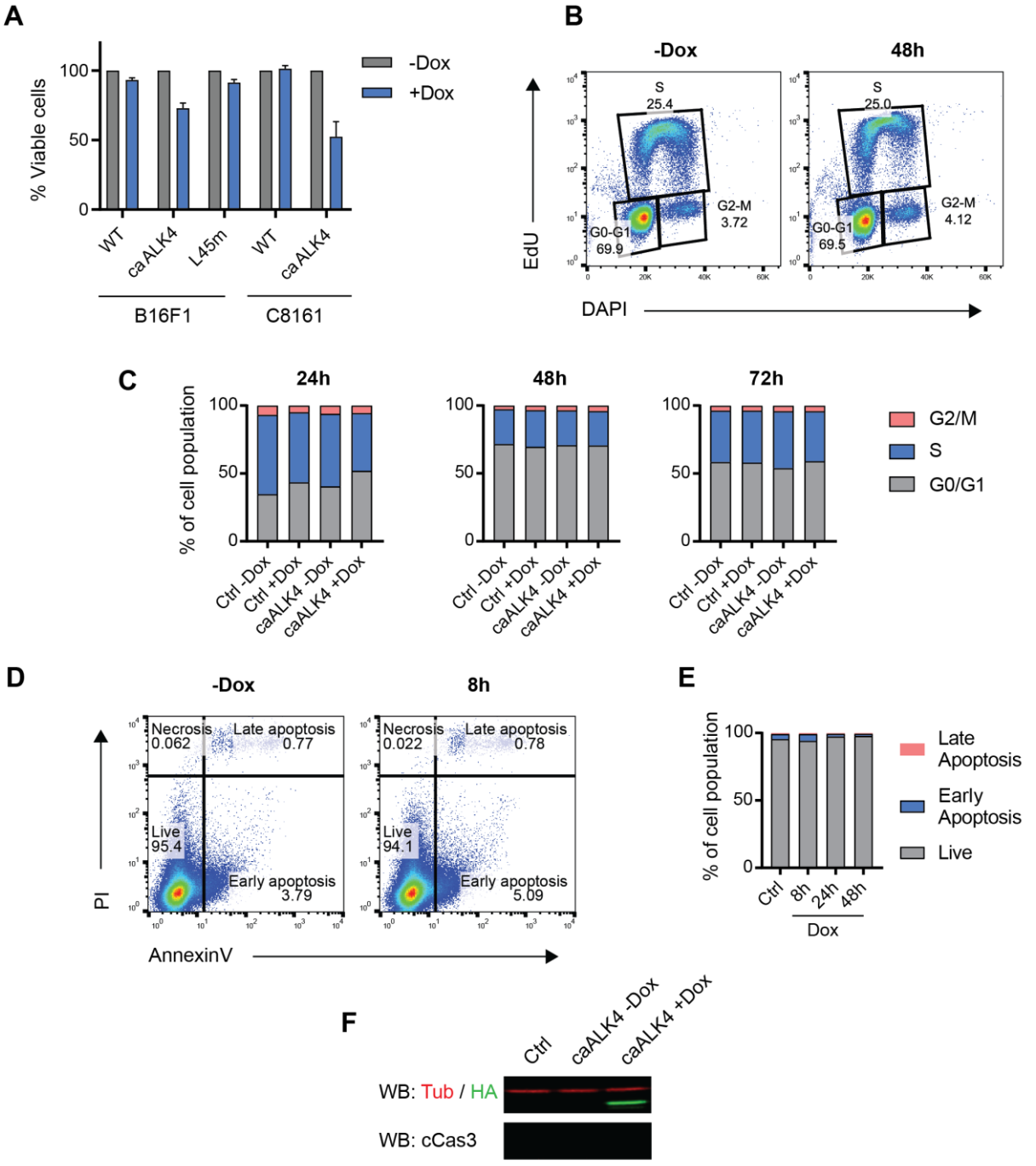


Figure 6: Sustained autocrine ALK4 signaling reduces melanoma cell proliferation *in vitro*

A. Alamar blue assays of B16F1 TRE-caALK4 and C8161 TRE-caALK4 cells cultured for 72h in absence or presence of 1 µg/mL doxycycline.

B. Representative flow cytometry plots of EdU incorporation by B16F1 TRE-caALK4 cells cultured in absence or presence of doxycycline for 48h.

C. Cell cycle analysis upon EdU incorporation by B16F1 Ctrl and B16F1 TRE-caALK4 cells cultured in presence or absence of doxycycline for 24h, 48h or 72h.

D. Representative flow cytometry plots of AnnexinV/PI staining of B16F1 TRE-caALK4 cells cultured in presence or absence of doxycycline for 8h.

E. Cell death quantification upon AnnexinV/PI staining of B16F1 TRE-caALK4 cells cultured in presence or absence of doxycycline for 8h, 24h or 48h.

To verify this hypothesis, B16F1 cell proliferation was assessed in Alamar blue assays. Induction of caALK4, unlike caALK4-L45m, significantly diminished cell viability within 72 hrs (Fig. 6A). Similar results were obtained in the C8161 human melanoma cell line, although the effect of caALK4-L45m was not tested in human cells. Importantly, treatment of wild type cells with doxycycline did not alter cell numbers. 5-Ethynyl-2-deoxyuridine (EdU) incorporation experiments did not reveal significant changes in cell cycle regulation upon treatment with doxycycline (Fig. 6B-C), and caALK4 did not induce apoptosis as assessed by AnnexinV/propidium iodide labeling (Fig. 6D-E) or cleaved caspase-3 staining (Fig. 6F). Collectively, these results indicate that sustained cell-autonomous canonical Activin-A signaling is detrimental to melanoma cell survival in a mechanism yet undetermined.

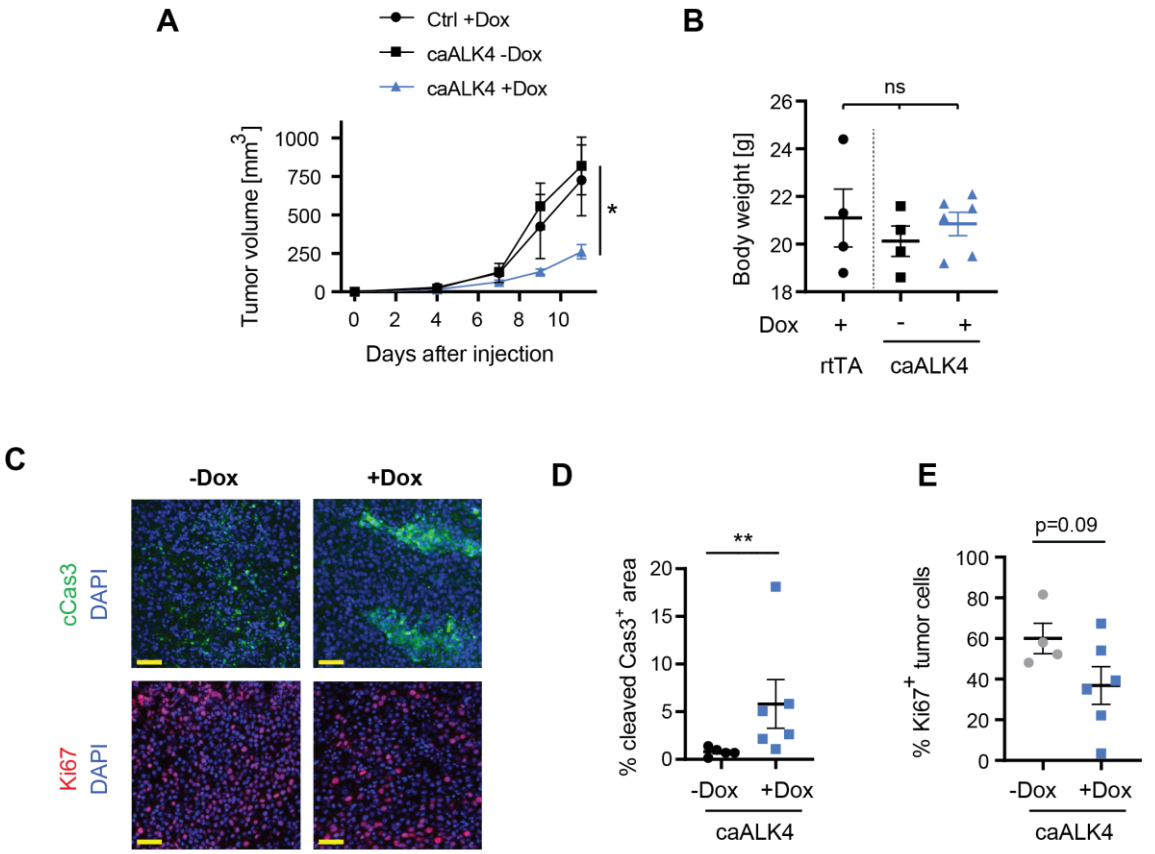


Figure 7: Autocrine ALK4 signaling inhibits B16F1 tumor growth *in vivo*

A. Tumor growth of B16F1 TRE-caALK4 cells grafted intradermally on the right flank of C57Bl/6 mice fed with (n=6) or without (n=5) doxycycline. B16F1 cells expressing the reverse tetracycline trans-activator (rtTA) served as control (n=5). (*p<0.05, Student's t-test)

B. Body weight of tumor-bearing C57Bl/6 mice at the time of sacrifice.

C. Immunofluorescent staining of cleaved Caspase 3 and Ki67 in B16F1 tumors expressing caALK4 (+Dox) or not (-Dox).

D. Quantification of cCas3⁺ areas in whole-sections of B16F1 tumors expressing caALK4 (+Dox, n=6) or not (-Dox, n=5). (**p<0.01, Student's t-test)

E. Flow cytometry analysis of Ki67⁺ proliferative cells in B16F1 tumors expressing caALK4 (+Dox, n=6) or not (-Dox, n=5). (p-value: Mann-Whitney t-test)

2.2 Cell-autonomous ALK4 signaling inhibits B16F1 melanoma progression *in vivo*

To verify whether the cell growth inhibition induced by caALK4 expression would translate into impaired tumor growth *in vivo*, B16F1-caALK4 cells were injected intradermally into C57BL/6 mice. Tumors resulting from animals fed with doxycycline grew significantly more slowly and without affecting body weight (Fig. 7A, B), indicating that caALK4 expression impairs tumor formation, and that Activin-A signaling induces cachexia independently of tumor cell-autonomous signaling. Cleaved caspase-3 immunofluorescent staining of tumor sections revealed increased apoptosis, often manifesting as clusters rather than dispersed cells (Fig. 7C-D). Moreover, Ki67 immunofluorescence and flow cytometry analysis suggested a trend for decreased tumor cell proliferation (Fig. 7C, E, p=0.09). These results suggest that tumor cell-autonomous Activin-A signaling is tumor-suppressive in B16F1 melanoma.

2.3 Activin-A gain-of-function does not affect the growth of cultured B16F1 melanoma cells

We next thought to assess whether paracrine Activin-A signaling may promote melanoma progression. We generated B16F1-βA and B16F1-Ctrl cells with lentiviruses bearing a myc-tagged *INHBA* transgene or an empty backbone, respectively. The expected 24 kDa band corresponding to mature Activin-A was

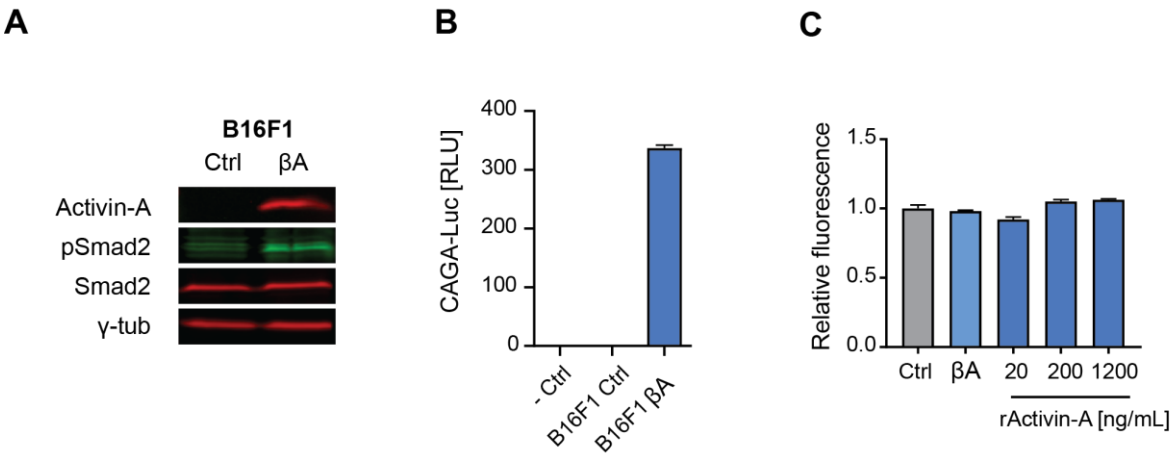


Figure 8: Activin-A gain-of-function does not alter B16F1 cell proliferation

A. Western blot analysis of Activin-A in conditioned medium from cultured B16F1-Ctrl and B16F1-βA cells, and of pSmad2, Smad2 and γ-tubulin in the corresponding lysates.

B. CAGA-luciferase activity in HepG2-CAGA reporter cells cultured in conditioned medium from B16F1-Ctrl and B16F1-βA cells.

C. Alamar blue cell viability assay of cultured B16F1-Ctrl and B16F1-βA cells, or B16F1 cells cultured in presence of recombinant Activin-A.

readily detected by western blot in conditioned medium from B16F1-βA cells, correlating with increased Smad2 phosphorylation in the corresponding cell lysates (Fig. 8A). The same conditioned medium potently induced luciferase activity in HepG2 reporter cells stably transduced with a CAGA-luciferase reporter transgene, suggestive of paracrine Activin signaling. Importantly, Alamar blue assays indicated that the growth and viability of cultured B16F1 were unaffected by *INHBA* overexpression or by treatment with recombinant Activin-A at physiological (20 ng/mL) or higher concentrations (Fig. 8C).

2.4 Secreted Activin-A only promotes melanoma growth in immunocompetent hosts

To determine whether Activin-A signaling influences tumor growth *in vivo*, 4 x 10^5 B16F1-βA or B16F1-Ctrl cells were intradermally injected into the right flank of 8- to 12-week-old female C57BL/6 mice. Tumors expressing Activin-A grew significantly faster and, unlike their control counterparts, induced a dramatic loss of body weight (Fig. 9A, B), reflecting at least in part systemic muscle wasting (Zhou et al., 2010). These data indicate that tumor-derived secreted Activin-A can drive tumor progression and cachexia.

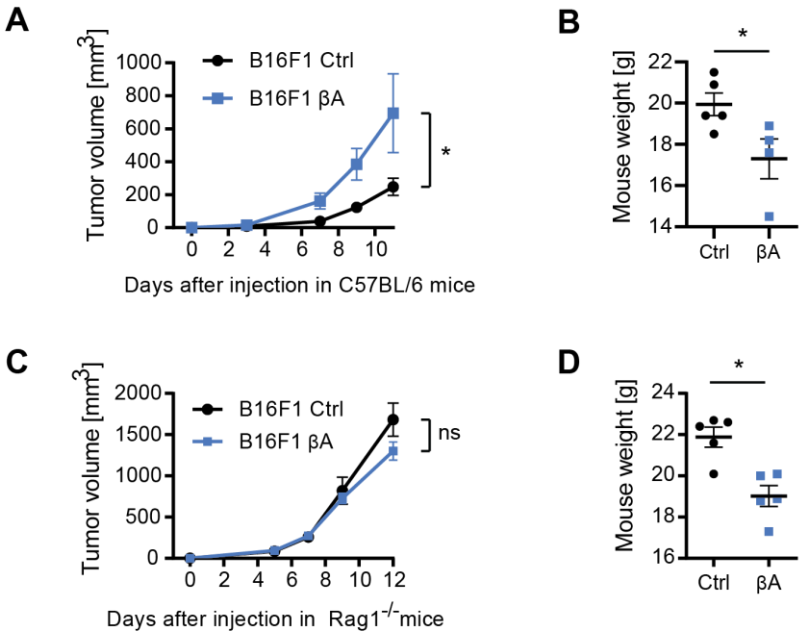


Figure 9: Secreted Activin-A accelerates B16F1 melanoma growth in immunocompetent mice

A. Tumor growth of B16F1-Ctrl or B16F1-βA cells grafted intradermally on the right flank of C57Bl/6 mice. (n=5 per group, *p<0.05, Student's t-test)

B. Body weight of tumor-bearing C57Bl/6 mice at the time of sacrifice. (n=5 per group, *p<0.05, Student's t-test)

C. Tumor growth of B16F1-Ctrl or B16F1-βA cells grafted intradermally on the right flank of C57Bl/6 Rag1$^{-/-}$ mice. (n=5 per group, Student's t-test)

D. Body weight of tumor-bearing C57Bl/6 Rag1$^{-/-}$ mice at the time of sacrifice. (n=5 per group, *p<0.05, Student's t-test)

To assess whether Activin-A stimulates melanoma progression by dampening adaptive anti-tumor immunity, I grafted B16F1 cells into Rag1$^{-/-}$ mice, which lack T- and B-cells due to deficient V(D)J gene rearrangements. Interestingly, in Rag1$^{-/-}$, B16F1-Ctrl tumors grew as fast as B16F1-βA grafts, suggesting that Activin-A signaling accelerates tumor growth by inhibiting the adaptive anti-tumor immune response in the B16F1 model (Fig. 9C). By contrast, cachexia mediated by B16F1-βA tumors was conserved in immunodeficient mice (Fig. 9D), in line with the observation that Activin-A leads to muscle wasting independently of pro-inflammatory cytokines (Zhou et al., 2010).

2.5 Activin-A stimulates angiogenesis independently of adaptive immunity

Solid tumors rely on neovascularization for oxygen and nutrient supply. Activin-A signaling has been shown to regulate angiogenesis in various cancer models (Kaneda et al., 2011; Krneta et al., 2006; Panopoulou et al., 2005), but whether it modulates melanoma vascularization is unknown. To investigate if increased tumor growth correlates with angiogenesis, I stained blood vessels from B16F1-Ctrl and B16F1-βA syngeneic tumors in thick cryosections (200 µm) using CD31 antibodies. Reconstruction of 3D z-stacks from confocal images of whole sections, followed by quantification of the CD31^{+} area revealed that Activin-A expression significantly increases tumor vascularization (Fig. 10A-B).

Conversely, hypoxia marked by pimonidazole staining was strongly decreased in βA compared to Ctrl tumors, indicating that the βA-induced neo-vasculature was perfused and functional (Fig. 10C-D). Interestingly, Activin-A similarly stimulated angiogenesis in B16F1 tumors grown in Rag1$^{-/-}$ hosts (Fig. 10E-F), where tumor growth was unaffected. These results show that secreted Activin-A stimulates neo-vascularization of B16F1 melanoma. Nevertheless, they also suggest that low vessel density and oxygenation are not rate-limiting for B16F1-Ctrl tumor growth in immunocompetent mice. Therefore, increased angiogenesis alone cannot account for the tumorigenicity of secreted Activin-A.

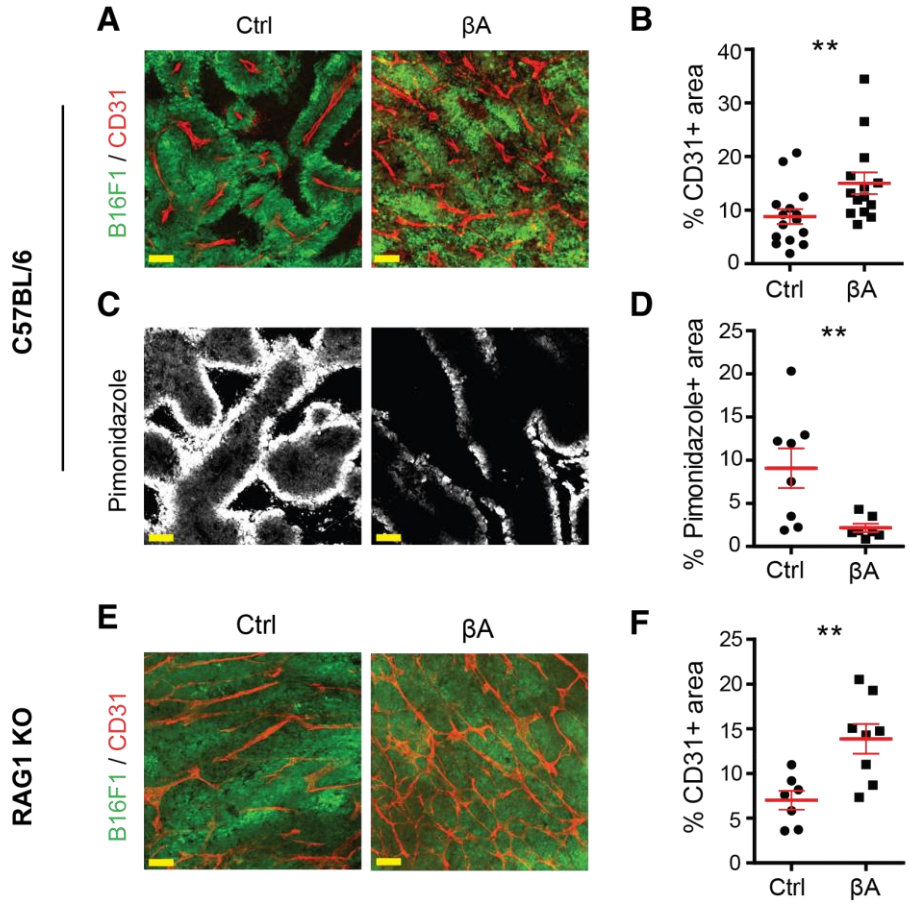


Figure 10: Activin-A stimulates angiogenesis independently of adaptive immunity in B16F1 tumors

A. CD31 immunofluorescent staining of 200µm-thick sections of syngeneic B16F1-Ctrl and B16F1-βA tumors expressing GFP. (Scale bars: 100µm)

B. Quantification of $CD31^+$ areas in whole-sections of B16F1-Ctrl and B16F1-βA tumors. (n=13 per group, **p<0.01, Student's t-test)

C. Pimonidazole immunofluorescent staining of hypoxic areas in 200µm-thick sections of syngeneic B16F1-Ctrl and B16F1-βA tumors. (Scale bars: 100µm)

D. Quantification of $pimonidazole^+$ areas in whole-sections of B16F1-Ctrl (n=8) and B16F1-βA (n=5) tumors. (**p<0.01, Student's t-test)

E. CD31 immunofluorescent staining of 200µm-thick sections B16F1-Ctrl and B16F1-βA tumors formed in $Rag1^{-/-}$ mice. (Scale bars: 100µm)

F. Quantification of $CD31^+$ areas in whole-sections of B16F1-Ctrl (n=7) and B16F1-βA (n=8) tumors formed in $Rag1^{-/-}$ mice. (**p<0.01, Student's t-test)

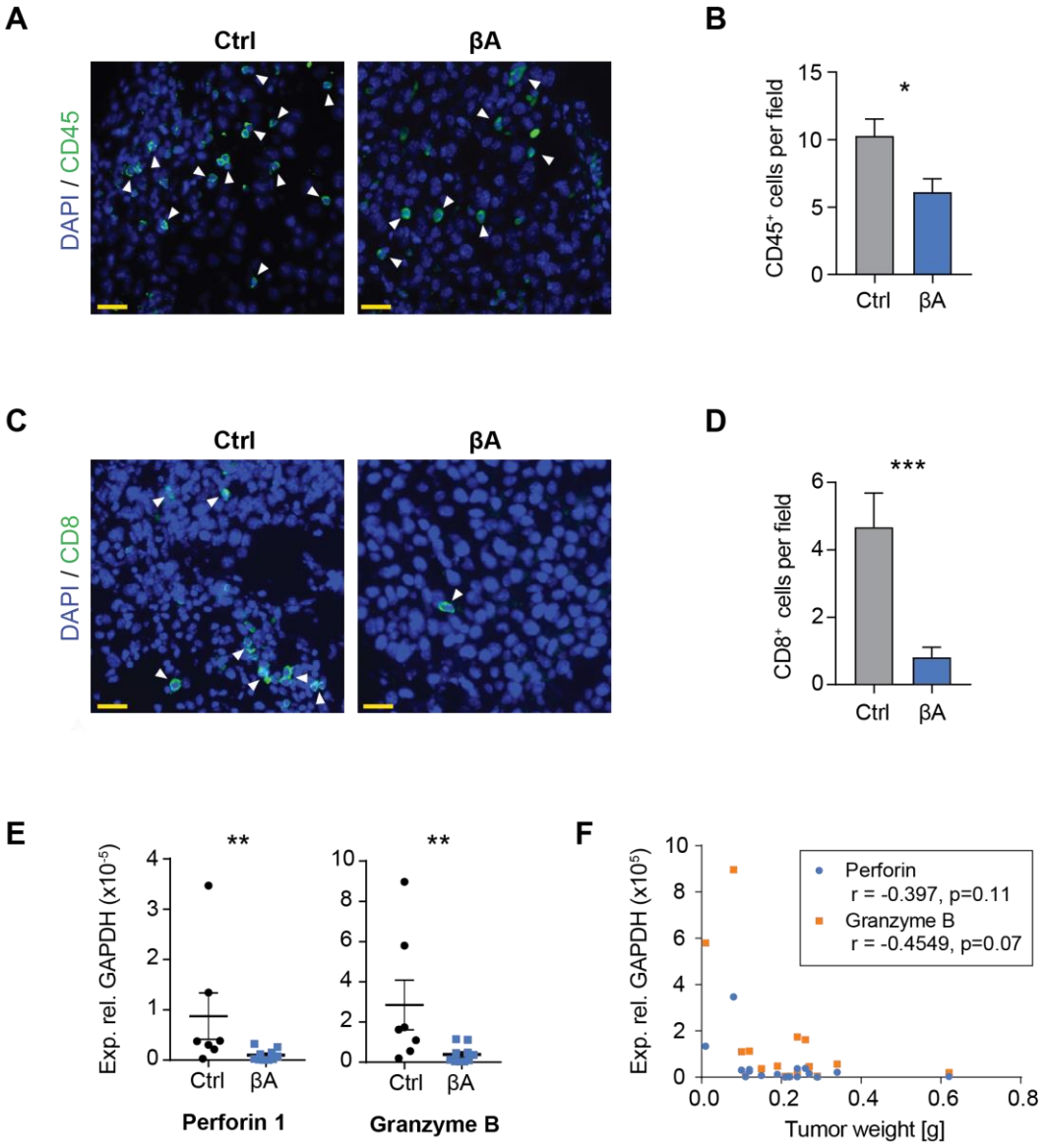


Figure 11: Activin-A reduces immune infiltration of B16F1 melanoma tumors

A. CD45 immunofluorescent staining of syngeneic B16F1-Ctrl and B16F1-βA tumors counterstained with DAPI.

B. Quantification of CD45⁺ cells per field in whole-sections of syngeneic B16F1-Ctrl (n=5) and B16F1-βA (n=4) tumors. (10 fields per section were counted, *p<0.05, Student's t-test)

C. CD8 immunofluorescent staining of syngeneic B16F1-Ctrl and B16F1-βA tumors counterstained with DAPI.

D. Quantification of CD8⁺ cells per field in whole-sections of syngeneic B16F1-Ctrl and B16F1-βA tumors. (n=5 per group ***p<0.001, Student's t-test)

E. Perforin-1 and Granzyme B RNA exression analysis by qRT-PCR in syngeneic B16F1-Ctrl (n=7) and B16F1-βA (n=10) tumors. (**p<0.01, Mann-Whitney t-test)

F. Perforin-1 and Granzyme B RNA expression negatively correlate with syngeneic B16F1 tumor weight at sacrifice. (n=17 per group, r = Pearson correlation coefficient, p = two-tailed p-value)

2.6 Activin-A negatively regulates immune cell infiltration in B16F1 melanoma grafts

Since the pro-tumorigenic effect of Activin-A depends on adaptive immunity (Fig. 6A, C), we decided to analyze tumor-infiltrating immune cells. As a first approach, hematopoietic cells and cytotoxic T-cells were stained using CD45 and CD8 antibodies, respectively. Quantification in whole-tumor cryosections revealed that infiltration of B16F1-βA tumors by both $CD45^+$ leukocytes (Fig. 11A-B) and $CD8^+$ T-lymphocytes (Fig. 11C-D) was significantly reduced when compared with B16F1-Ctrl tumors. Moreover, qPCR analysis of RNA extracted from whole tumors showed markedly reduced expression of Granzyme B and Perforin-1 (Fig. 11E), two key molecules mediating apoptosis in target cells upon release by cytotoxic lymphocytes. Despite not reaching statistical significance, expression of Perforin-1 and Granzyme B appeared to negatively correlate with tumor size, in line with the hypothesis that lymphocytic populations are functionally relevant in controlling tumor growth (Fig. 11F).

2.7 Influence of Activin-A on the immune compartment of B16F1 tumors analyzed by multiparameter flow cytometry

Given the complex role of Activin-A in inflammation and immune regulation, we decided to analyze the tumor immune landscape using a 16-color flow cytometry panel, allowing unbiased identification and quantification of up to 60 immune cell populations across both lymphocytic and myeloid lineages (Table 1) (Faget et al., 2017). Activin-A appeared to reduce the total numbers of $CD45^+$ leukocytes in B16F1 tumors, although not significantly (Fig. 12A). The immune compartment of B16F1 tumors contained high myeloid cell infiltration, composed mainly of $CD11b^+$ cells, which were further enriched in INHBA expressing tumors (Fig. 12B). In this myeloid compartment, MDSCs ($CD11b^+Ly6C^{hi}$) were the most frequent population, and their prevalence significantly increased upon *INHBA* expression (Fig. 12C). A trend was observed with $CD11b^+F4/80^+$ macrophages (Fig. 12D), along with their tendency to increase CD206 expression, a marker of M2 polarization (Fig. 12E). Neutrophils were found in relatively low numbers in B16F1 tumors, and their presence seemed to be unaffected by *INHBA* expression (Fig. 12F). Interestingly, *INHBA* expression increased the number of dendritic cells (Fig. 12G). Whereas activation assessed by MHC-II mean fluorescence intensity was unchanged, (Fig. 12H), expression of the CD80 co-stimulatory molecule was strongly induced by Activin-A (Fig. 12I), indicating that Activin signaling stimulates antigen presentation rather than inhibiting it. *INHBA* expression also markedly affected tumor infiltration by lymphocytes: NK cells as well as cytotoxic CD8 T-cells significantly decreased (Fig. 12J, K) in line with our results obtained by immunofluorescent staining (Fig. 11C, D). Conversely, conventional CD4 T-cells were increased (Fig. 12L). Importantly, the number of $CD4^+FoxP3^+$ regulatory T-cells did not significantly change (Fig. 12M). B-cells were rare in B16F1 tumors, but moderately increased upon *INHBA* expression (Fig. 12N). Next, the proliferation of immune cells was assessed using a flow cytometry panel in which cells were stained for Ki67, a marker expressed exclusively by cycling cells (Table 2) (Sun and Kaufman, 2018).

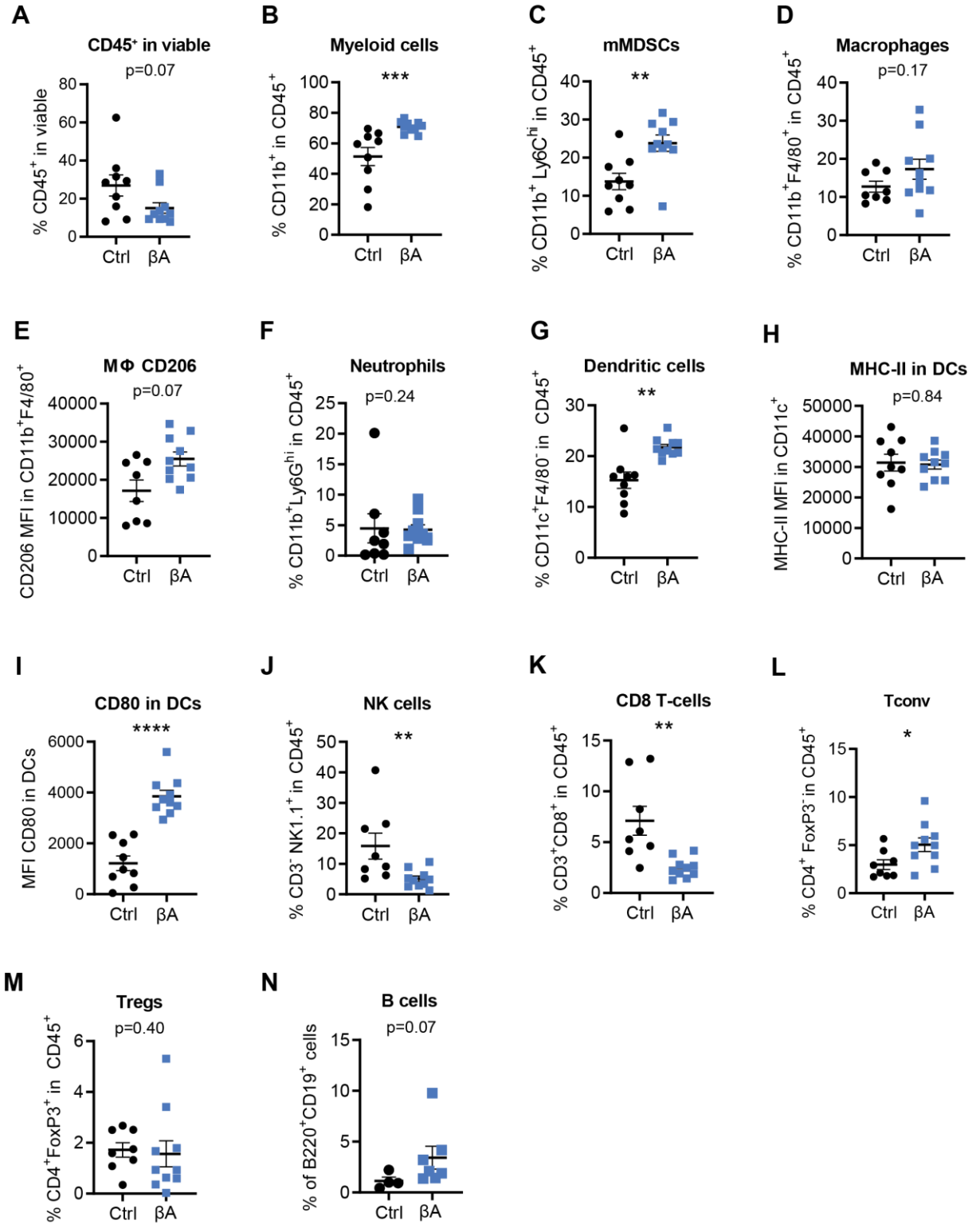


Figure 12: *INHBA* expression is immunosuppressive in B16F1 tumors

Flow cytometry analysis of the immune infiltration of syngeneic B16F1-Ctrl (n=8) and B16F1-βA (n=10) tumors at experimental endpoint. (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, Student's t-test)

Table 1: Immune populations in B16F1 tumors analyzed by flow cytometry

Fig. Panel	Population	Markers	Ctrl[1]	βA[1]	p-value
A	CD45+ leukocytes	CD45+	26.9% ± 7.6%	15.1% ± 8.7%	0.065
B	CD11b+ myeloid cells	CD11b+	51.4% ± 17.8%	70.8% ± 3.8%	0.0004
C	mMDSCs	CD11b+Ly6C^{hi}	13.8% ± 6.5%	23.8% ± 6.7%	0.004
D	Macrophages	CD11b+F4/80+	12.7% ± 4.1%	17.3% ± 8.4%	0.17
E	Macrophage CD206 MFI	CD206	17142 ± 7961	25501 ± 5789	0.068
F	Neutrophils	CD11b+Ly6G+	4.5% ± 6.7%	4.3% ± 2.5%	0.24
G	Dendritic cells	CD11c+F4/80-	13.0% ± 6.3%	21.4% ± 4.4%	0.003
H	Dendritic cells MHC-II MFI	IA/IE	31396 ± 8305	30757 ± 4751	0.84
I	Dendritic cells CD80 MFI	CD80	1216 ± 3853	3853 ± 758	<0.0001
J	NK cells	NK1.1+CD3-	15.8% ± 12.1%	5.1% ± 2.9%	0.006
K	CD8 T-cells	CD3+CD8+	7.1% ± 4.0%	2.4% ± 1.0%	0.001
L	Tconv	CD3+CD4+FoxP3-	2.9% ± 1.4%	5.0% ± 2.2%	0.04
M	Tregs	CD3+CD4+FoxP3+	1.7% ± 0.8%	1.6% ± 1.6%	0.41
N	B-cells	CD19+B220+	1.1% ± 0.8%	3.4% ± 3.0%	0.07

Flow cytometry analysis of immune populations in B16F1-Ctrl (n=9) and B16F1-βA (n=10) syngeneic grafts. [1] Data represent mean ± SEM in percentage of CD45^{+} population, or mean fluorescence intensity (MFI) when indicated. p-values calculated using Student's t-test.

INHBA expression in B16F1 melanoma diminished the fraction of infiltrating CD8 T-cells stained by Ki67 (Fig. 13A), while increasing it in conventional CD4 T-cells (Fig. 13B), in good agreement with the total size of each of these populations. Tregs remained unaffected (Fig. 13C). Interestingly, the proliferation of NK cells marked by Ki67 staining was not significantly impaired in tumors expressing *INHBA* (Fig. 13D), despite a three-fold decrease in population numbers. Thus, Activin-A inhibits the recruitment rather than the expansion of NK cells within the tumors. Myeloid cells were highly proliferative within B16F1 tumors, a phenotype that was further accentuated in monocytes, (Fig. 13E), macrophages (Fig. 13F) and dendritic cells (Fig. 13G) originating from *INHBA* tumors. Together, these results indicate that Activin-A signaling leads to decreased infiltration and/or proliferation of cytotoxic cells and increases immunosuppression in B16F1 tumors.

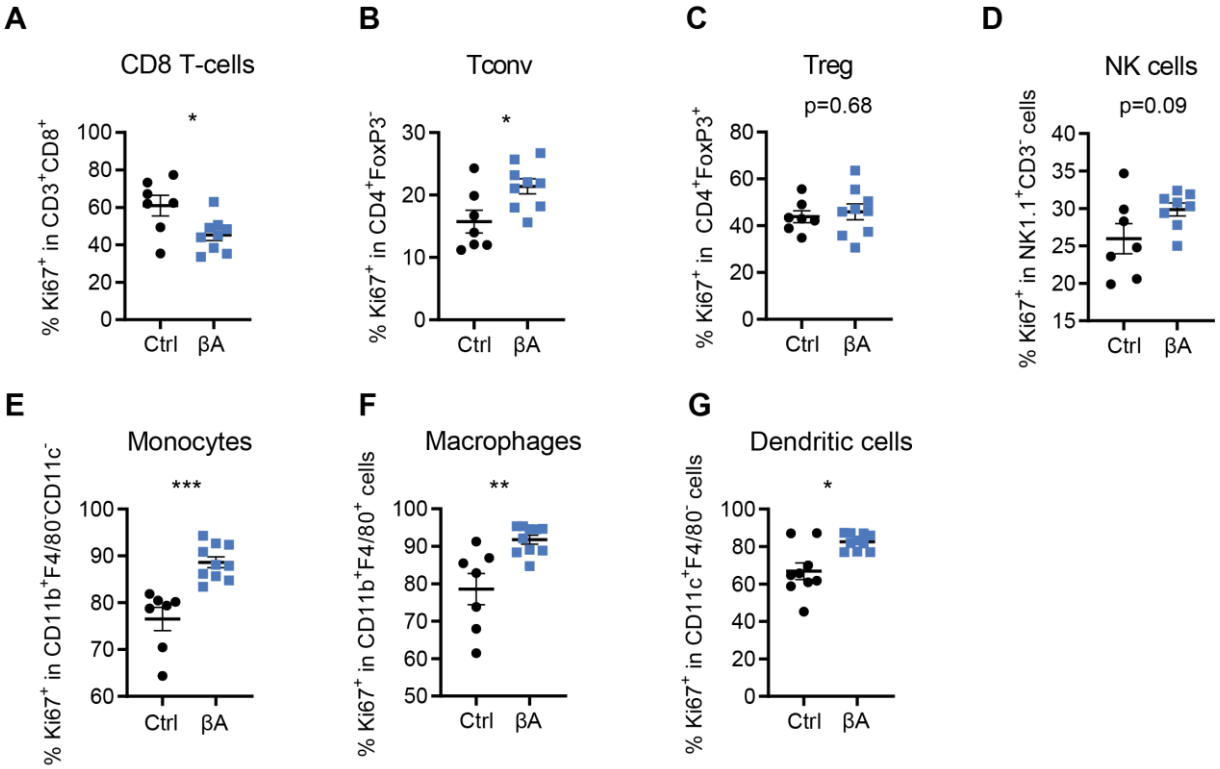


Figure 13: *INHBA* expression alters proliferation of immune subsets in B16F1 tumors

Flow cytometry analysis of immune subsets in syngeneic B16F1-Ctrl (n=7) and B16F1-βA (n=10) grafts stained intracellularly for the Ki67 proliferation marker. (*p<0.05, **p<0.01, ***p<0.001, Student's t-test)

Table 2: Proliferation of immune cells in syngeneic B16F1 grafts

Fig. Panel	Population	Markers	Ctrl[1]	βA[1]	p-value
A	CD8 T-cells	CD3+CD8+	60.9% ± 14.4%	45.3% ± 9.0%	0.03
B	Tconv	CD3+CD4+FoxP3-	15.7% ± 4.9%	21.4% ± 3.6%	0.03
C	CD4+ Tregs	CD3+CD4+FoxP3+	43.9% ± 6.8%	45.9% ± 10.2%	0.68
D	NK cells	NK1.1+CD3-	26.0% ± 5.3%	29.9% ± 2.4%	0.09
E	Monocytes	CD11b+F4/80-CD11c-	76.5% ± 6.5%	88.6% ± 3.7%	0.0001
F	Macrophages	CD11b+F4/80+	78.6% ± 11.0%	91.8% ± 3.8%	0.003
G	Dendritic cells	CD11c+F4/80-	66.8% ± 13.4%	82.6% ± 4.3%	0.02

Flow cytometry analysis of Ki67+ immune subsets in B16F1-Ctrl (n=7) and B16F1-βA (n=9) syngeneic grafts. [1] Data represent mean ± SEM. p-values calculated using Student's t-test.

2.8 Depletion of CD8$^+$, but not of CD4$^+$ cells, neutralizes the immunosuppressive function of Activin-A

We next asked whether changes in immune cell numbers were functionally relevant to the tumor-promoting function of Activin-A. Since NK cells still form in Rag1$^{-/-}$ hosts where Activin-A failed to accelerate tumor growth, we reasoned that Activin-A signaling likely inhibits CD4 or CD8 T-cell functions, and not simply NK cells. To test this hypothesis, we performed antibody-mediated cell depletion during the course of B16.OVA-Ctrl and B16.OVA-βA tumor growth using biweekly intraperitoneal injections of monoclonal antibodies for CD4, CD8 or both. Depletion was complete and specific, as virtually no CD4- and/or CD8 T-cell were detected in the circulation (Fig. 14A) and in tumors at endpoint (Fig. 14B, C). Depletion of CD8+ cells alone or together with CD4+ cells neutralized the tumor-promoting function of *INHBA* in B16.OVA tumors (Fig. 14E, G), indicating that secreted Activin-A likely inhibits anti-tumor immunity in a CD8 T-cell dependent manner. Curiously, *INHBA* expression in B16.OVA tumors failed to decrease the number of infiltrated cytotoxic T-cell (Fig. 14C). This result suggests that in the presence of ovalbumin, Activin-A still inhibits TIL effector function, but not T-cell infiltration, possibly due to increased immunogenicity of B16.OVA grafts compared to B16F1. Finally, ablation of CD4$^+$ cells led to regression of B16.OVA Ctrl tumors, whereas B16.OVA-βA tumors continued to grow aggressively (Fig. 14F). Since B16.OVA-βA tumor growth was not significantly altered in CD4 T-cell depleted mice when compared with IgG control injections, CD4+ Tregs unlikely mediate Activin-A-induced immuno-suppression in B16.OVA melanoma.

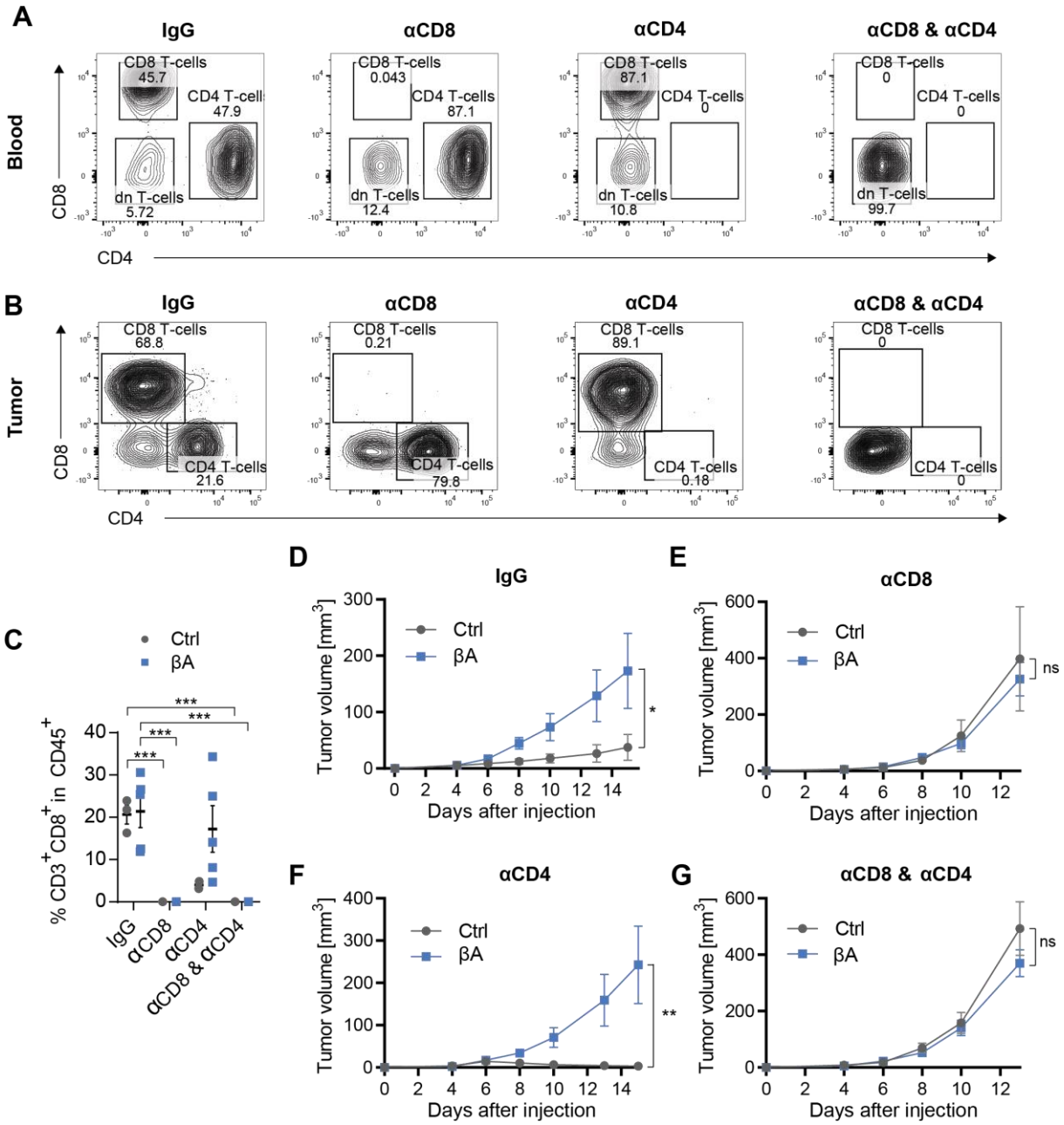


Figure 14: Depletion of CD8+, but not of CD4+ cells, neutralizes the immunosuppressive function of Activin-A

A. Representative flow cytometry plots of CD4 and CD8 T-cells in the circulating blood of B16.OVA-Ctrl and B16.OVA-βA tumor-bearing mice treated with IgG, αCD4, αCD8 or a combination of αCD4 & αCD8 antibodies.

B. Representative flow cytometry plots of CD4 and CD8 T-cells in syngeneic B16.OVA-Ctrl and B16.OVA-βA tumors of mice treated with IgG, αCD4, αCD8 or a combination of αCD4 & αCD8 antibodies.

C. Flow cytometry analysis of CD8 T-cells in B16.OVA-Ctrl and B16.OVA-βA tumors of mice treated with IgG, α CD4, αCD8 or a combination of αCD4 & αCD8 antibodies. (n=5 per group, ***p<0.001, Student's t-test)

D-G. Tumor growth of B16F1-Ctrl or B16F1-βA cells grafted intradermally on the right flank of C57BL/6 mice treated with IgG (D), αCD8 (E), αCD4 (F) or a combination of αCD4 & αCD8 antibodies (G). (n=5 per group, *p<0.05, **p<0.01, Student's t-test)

2.9 Anti-Gr-1 antibodies fail to deplete monocytic MDSCs in B16 melanoma

Activin-A may inhibit cytotoxic T-cell activity either directly or through the action of suppressive intermediates. Several subsets of the myeloid compartment are frequently implicated in cancer-associated immunosuppression (Kumar et al., 2016b; Mantovani et al., 2017). As monocytes and macrophages constitute ≥40-50% of total leukocytes in B16 melanoma (Fig. 12B, C), and given that the expression of suppressive markers is further increased in the monocytic lineage from *INHBA*-expressing tumors, myeloid-derived suppressive cells (MDSCs) constitute a likely candidate to mediate such an action. In particular, $CD11b^{+}Ly6C^{hi}$ mMDSCs have been shown to correlate with increased tumor burden in multiple cancer types (Ai et al., 2018), and with resistance to immunotherapy in melanoma (Meyer et al., 2014). By contrast, neutrophils are scarce in syngeneic tumors derived from B16 and other melanoma cell lines. Therefore, to assess the contribution of mMDSCs in the context of Activin-A signaling, we decided to use anti-Gr-1 antibodies, which bind both Ly6C and Ly6G antigens, to deplete $Ly6C^{hi}$ monocytes. Treatment of B16.OVA tumor-bearing mice with 100 µg anti-Gr-1 antibodies once per week completely depleted $Ly6G^{hi}$ neutrophils in peripheral blood, but unfortunately not $Ly6C^{hi}$ monocytes (Fig. 15A). Similar results were obtained in dissociated tumors at experiment endpoint (Fig. 15B). An alternative treatment regimen where 250 µg anti-Gr-1 antibodies were injected biweekly led to the same outcome. We conclude that in B16 melanoma, anti-Gr-1 antibodies deplete neutrophils, but not mMDSCs.

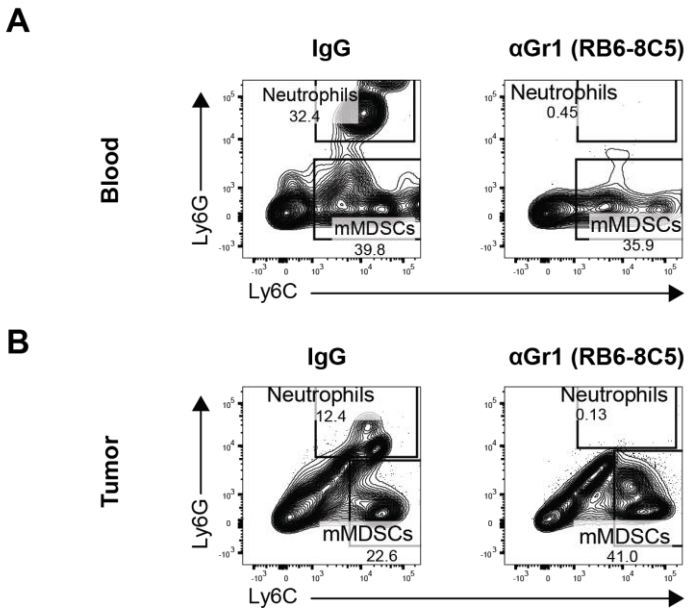


Figure 15: αGr1 antibodies fail to deplete myeloid-derived suppressive cells in B16F1 tumors

A. Representative flow cytometry plots of $CD11b^{+}$ cells in the circulating blood of B16.OVA-Ctrl and B16.OVA-βA tumor-bearing mice treated with IgG or αGr1 antibodies.

B. Representative flow cytometry plots of $CD11b^{+}$ cells in B16.OVA-Ctrl and B16.OVA-βA tumors from mice treated with IgG or αGr1 antibodies.

2.10 Activin-A may stimulate pro-inflammatory functions of macrophages in B16 melanoma

Tumor-associated macrophages (TAMs) express higher levels of CD206 in B16.OVA-βA than in B16.OVA CTRL tumors. CD206 marks alternatively activated (M2) TAMs, which frequently correlate with poor prognosis and treatment outcome (Aras and Zaidi, 2017). To test whether Activin-A inhibits anti-tumor immunity by polarizing TAMs, we tried to selectively deplete TAMs using monoclonal anti-CSF1R antibody (clone 2G2). Surprisingly, 2G2 failed to deplete macrophages from B16.OVA-Ctrl tumors, whereas TAMs from tumors expressing Activin-A were reduced to baseline levels observed in IgG-treated Ctrl tumors (Fig. 16A). Moreover, while 2G2 treatment did not affect the progression of Ctrl tumors, it tended to increase the tumorigenicity in B16.OVA-βA tumors (Fig. 16B). Neither Activin-A nor αCSF1R treatment altered CD8 T-cell infiltration of B16.OVA tumors (Fig. 16C). Nevertheless, TILs from

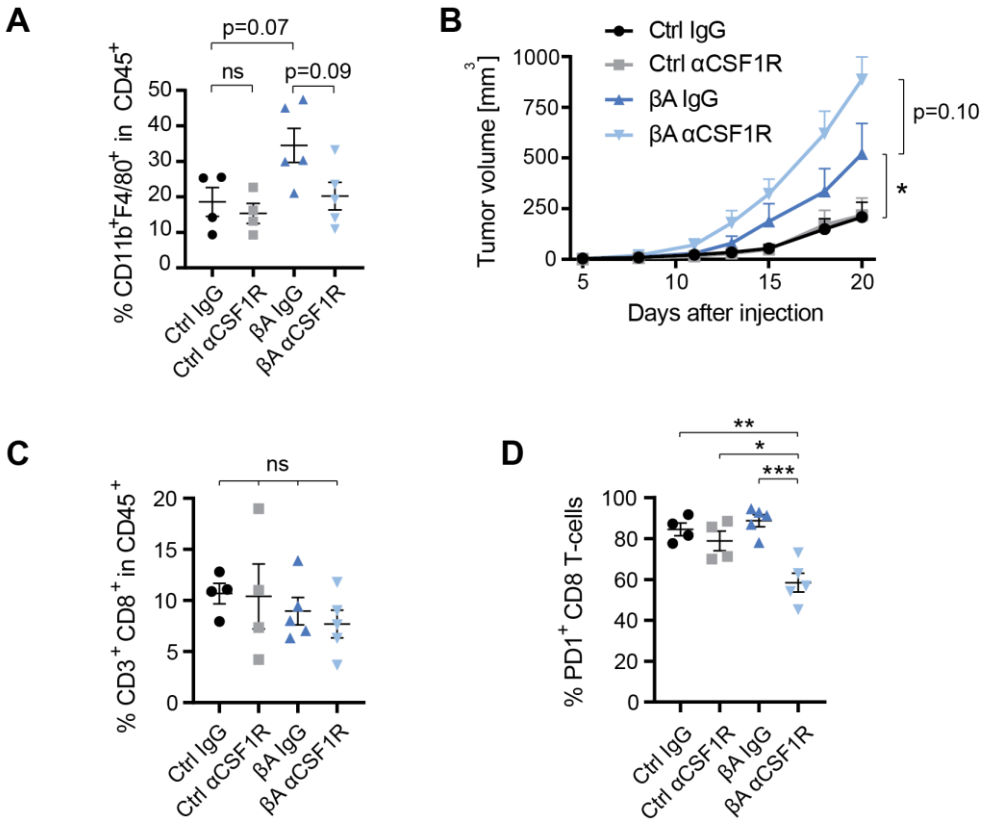


Figure 16: Macrophage depletion does not restore anti-tumor immunity in B16.OVA-βA tumors

A. Flow cytometry analysis of macrophage infiltration in B16.OVA-Ctrl (n=4 per group) and B16.OVA-βA (n=5 per group) tumors of mice treated with IgG or αCSF1R antibodies. (p-values: Student's t-test)

B. Tumor growth of B16.OVA-Ctrl and B16.OVA-βA cells grafted intradermally on the right flank of C57BL/6 mice treated with IgG or αCSF1R antibodies. (n=5 per group, *p<0.05, Student's t-test)

C. Flow cytometry analysis of CD8 T-cell infiltration in B16.OVA-Ctrl (n=4 per group) and B16.OVA-βA (n=5 per group) tumors of mice treated with IgG or αCSF1R antibodies. (p-values: Student's t-test)

D. Flow cytometry analysis of PD1⁺ CD8 T-cells in B16.OVA-Ctrl (n=4 per group) and B16.OVA-βA (n=5 per group) tumors of mice treated with IgG or αCSF1R antibodies. (*p<0.05, **p<0.01, ***p<0.001, Student's t-test)

INHBA tumors treated with 2G2 expressed less PD1, suggesting deficient activation (Fig. 16D). These results indicate that Activin-A overall promotes pro-inflammatory rather than suppressive capabilities of macrophages in B16 melanoma.

2.11 Activin-A does not inhibit MHC-I expression or antigen loading in melanoma cells

Cancer cells frequently evade tumor-specific CD8 T-cells by altering expression of key genes involved in antigen processing or presentation by MHC-I, and/or by reducing the repertoire of tumor antigens (Sucker et al., 2014). To address whether Activin-A regulates antigen processing, we analyzed ovalbumin expression and surface presentation of the class I OVA epitope SIINFEKL as a surrogate antigen. Western blot analysis indicated that expression of ovalbumin was comparable in B16F1.OVA and SM1.OVA cells, but barely above detectable levels in the B16.OVA cell line (Fig. 17A). Treatment with IFN-γ induced robust H2Kb expression in all three cell lines, as determined by flow cytometry (Fig. 17B, upper panels). Most importantly, mean fluorescence intensities for H2Kb (MHC-I) and H2Kb-SIINFEKL in B16F1.OVA and and SM1.OVA cells were unaffected by transgenic *INHBA* (Fig. 17B, lower panels). Presentation of the SIINFEKL peptide by MHC-I molecules was comparatively low in B16.OVA and SM1.OVA cells, suggesting that B16.OVA and SM1.OVA cells may inhibit SIINFEKL presentation by downregulating ovalbumin expression and by inhibiting antigen presentation pathways, respectively. To test whether alterations in antigen presentation my require prolonged exposure to Activin-A, the experiment was repeated using B16F1.OVA cells that were kept in culture for three weeks, corresponding to the time needed for tumors to reach endpoint *in vivo*. Although H2Kb-SIINFEKL staining was lost by <15% of the cells in both B16F1.OVA-Ctrl and B16F1.OVA-βA, continued exposure to Activin-A did not impair antigen presentation (Fig. 17C). FACS-sorting of H2Kb-SIINFEKL positive and negative populations followed by Western blot analysis of ovalbumin further indicated that H2Kb-SIINFEKL-negative cells had lost ovalbumin expression in both B16F1.OVA-Ctrl and B16F1.OVA-βA (Fig. 17D). Together, these results do not support the idea that Activin-A accelerates B16F1.OVA tumor growth by interfering with ovalbumin processing or MHC-I loading.

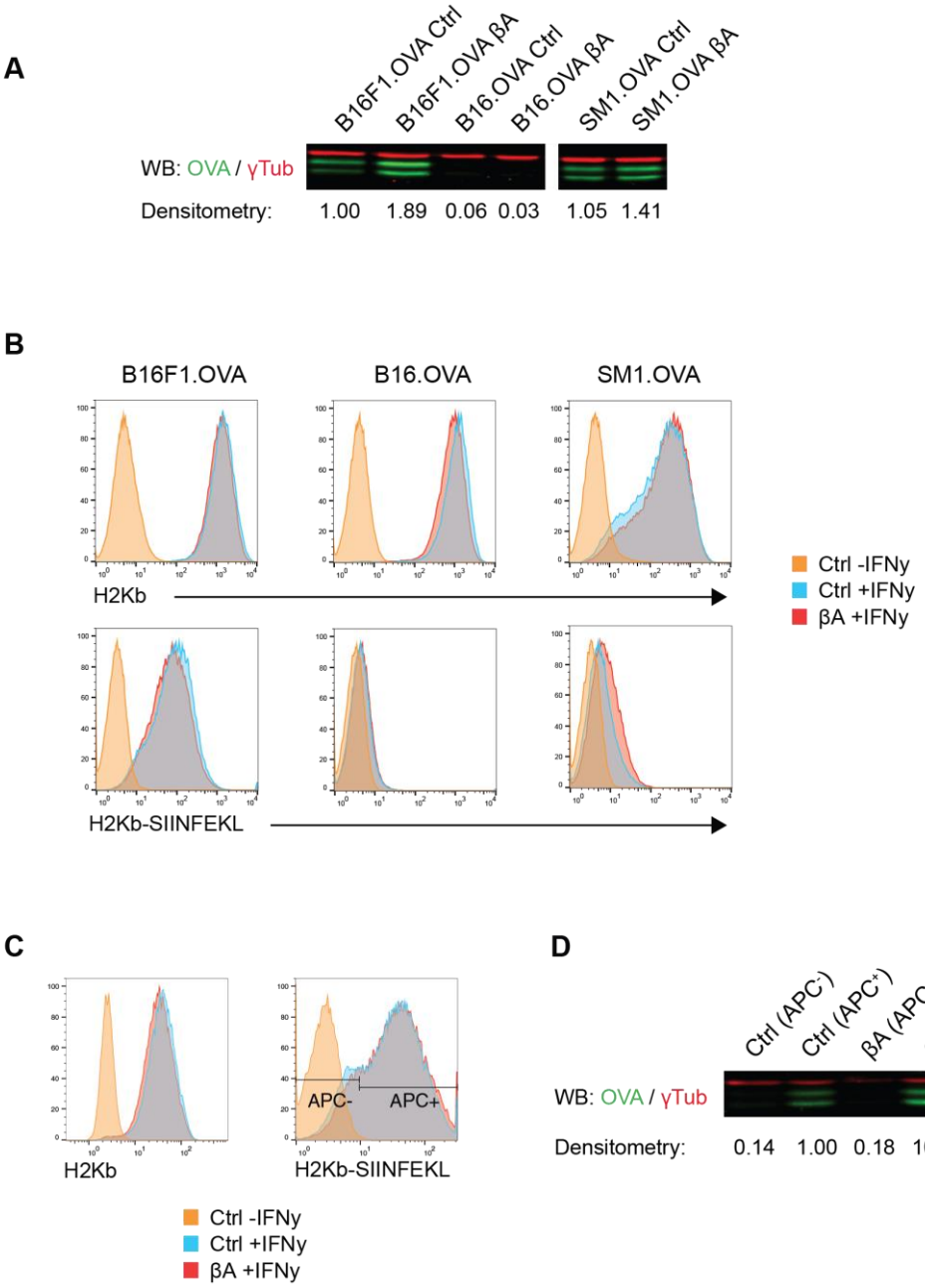


Figure 17: Activin-A does not inhibit antigen presentation in melanoma cells

A. Western blot analysis of OVA expression in B16F1.OVA, B16.OVA and SM1.OVA stable cell lines. γ-tubulin was used as loading control.

B. H2Kb expression (upper panels) and H2Kb-SIINFEKL presentation (lower panels) in B16F1.OVA, B16.OVA and SM1.OVA stable cell lines treated with or without 20µg/mL IFN-γ for 24h.

C. Flow cytometry plots of H2Kb expression (left) and H2Kb-SIINFEKL presentation (right) of B16F1.OVA stable cell lines cultured for three weeks and treated with or without 20µg/mL IFN-γ for 24h.

D. Western blot analysis of OVA expression in FACS-sorted H2Kb-SIINFEKL^{+} and H2Kb-SIINFEKL^{-} populations from (**C**).

2.12 Activin-A does not impair antigen cross-presentation by antigen-presenting cells *in vivo*

We next investigated *in vivo* whether Activin-A may regulate dendritic cells (DC) or their role in antigen cross-presentation by MHC-II. As described above, Activin-A signaling did not decrease DC infiltration or their MHC-II expression in B16F1 tumors (Fig. 12F, G). Similar syngeneic grafts were repeated using B16.OVA cells, allowing for analysis of antigen cross-presentation pathways and antigen-specific priming of $CD8^+$ T-cells. The numbers of antigen-presenting cells, $CD8^+$ APCs, and of OVA-specific (H2Kb-SIINFEKL+) APCs, as well as MHC-I MFI on APCs were comparable in draining lymph nodes from B16F1.OVA-Ctrl and B16F1.OVA-βA tumor-bearing mice (Fig. 18A-D). Interestingly, however, H2Kb-$SIINFEKL^+$ APCs were more frequent in *INHBA* compared to control tumors, despite similar total numbers of $H2Kb^+$ antigen-presenting cells (Fig. 18E, F). To test whether Activin-induced enrichment of OVA-specific APCs improves priming of cytotoxic T-cells, $CD8^+$ T-cells were stained with SIINFEKL-specific MHC-I dextramers to label OVA-specific TCR. Interestingly, OVA-specific $CD8^+$ T-cells were enriched 3-fold in *INHBA* compared to control tumors (Fig. 18G), even though their numbers in the circulation, in lymph nodes and in the spleen remained comparable (Fig. 18H, I). Taken together, these results suggest that Activin-A does not inhibit antigen-specific $CD8^+$ T-cell priming, but rather enhances it by stimulating cross-presentation of antigen-presenting cells within the tumor microenvironment.

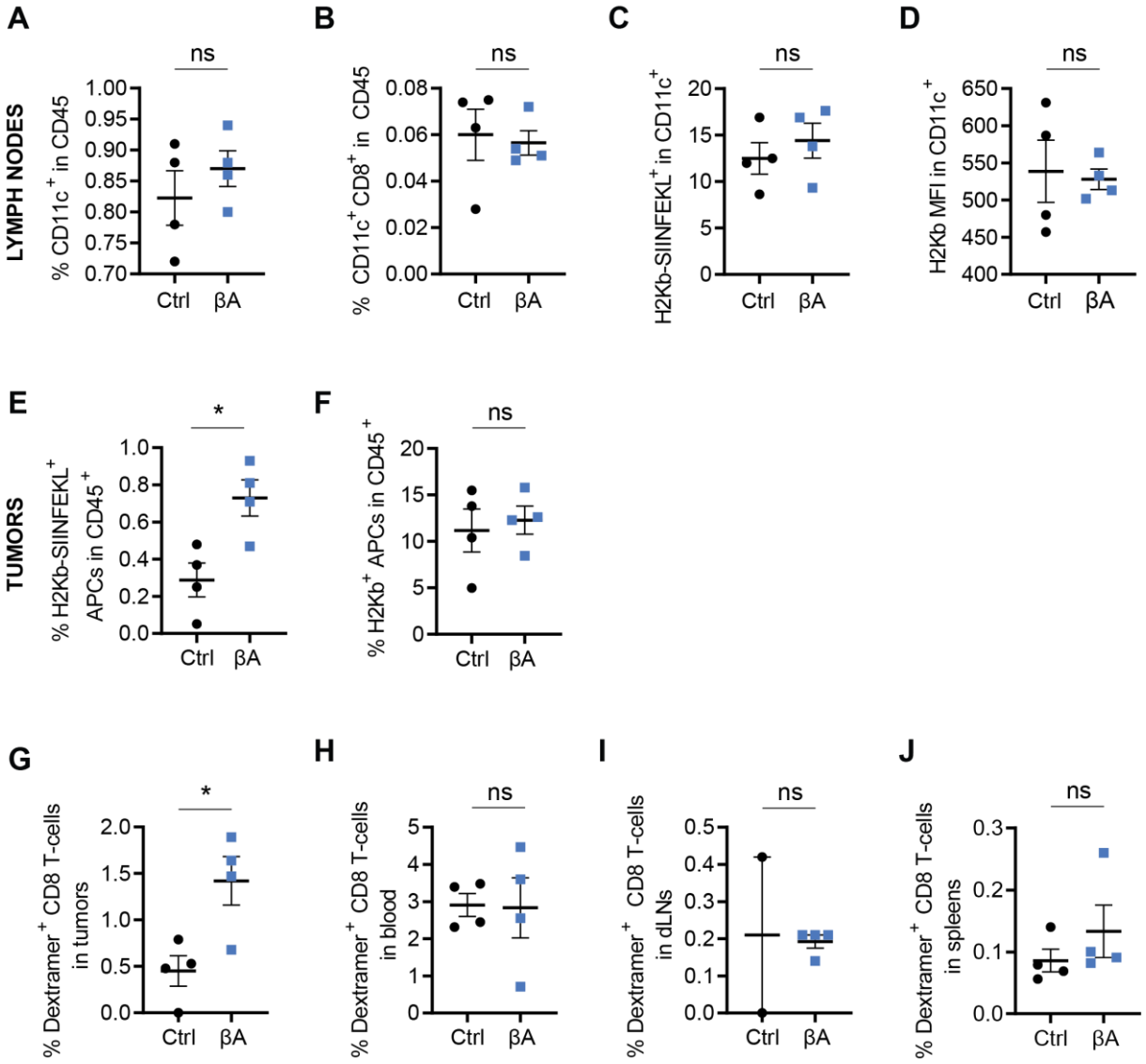


Figure 18: Activin-A stimulates cross-presentation in B16.OVA tumors but not in draining lymph nodes

A-D. Flow cytometry analysis of CD11c⁺ APCs (A), CD11c⁺CD8⁺ APCs (B), H2Kb-SIINFEKL presentation in CD11c⁺ APCs (C) and H2Kb expression in CD11c⁺ APCs (D) in draining lymph nodes from B16.OVA-Ctrl and B16.OVA-βA tumor-bearing mice. (n=4 per group, p-values: Student's t-test)

E-F. Flow cytometry analysis of H2Kb-SIINFEKL presentation in CD11c⁺ APCs (E) and H2Kb expression on CD11c⁺ APCs (F) in B16.OVA-Ctrl and B16.OVA-βA tumors. (n=4 per group, p-values: Student's t-test)

G-J. Flow cytometry analysis of H2Kb-SIINFEKL dextramer⁺ CD8 T-cells in B16.OVA-Ctrl and B16.OVA-βA tumors (G) and in the blood (H), draining lymph nodes (I) and spleens (J) of tumor-bearing mice. (n=4 per group, except for (I), where n Ctrl = 2. *p<0.05, Student's t-test)

2.13 A tentative approach to manipulate tumor hypoxia

Immune responses against solid tumors can be negatively regulated by hypoxia (Barsoum et al., 2014). However, recent reports suggest that low tissue oxygenation may instead improve effector functions of cytotoxic T-cells, both during viral infections and in tumors (Doedens et al., 2013; Palazon et al., 2017). Therefore, since forced *INHBA* expression in B16F1 melanoma leads to improved tumor vascularization and decreased hypoxia (Fig. 10), we asked whether Activin-A secretion blocks anti-tumor immunity indirectly by promoting normoxia. To inhibit angiogenesis and increase hypoxia within B16.OVA-βA tumors while avoiding the immunomodulatory functions of VEGF inhibitors, we chose to take advantage of the soluble chimeric fusion protein Alk1-Fc, which can inhibit tumor angiogenesis by sequestering proangiogenic factors BMP9 and BMP10 in plasma (Cunha and Pietras, 2011). B16.OVA- βA cells were transduced with an Alk1-Fc lentivirus and secretion of Alk1-Fc into the conditioned medium was verified by western blot (Fig. 19A). Expression of Alk1-Fc did not significantly alter the growth of B16.OVA-βA tumors (Fig. 19B). Hypoxia, monitored by pimonidazole immunofluorescent staining, appeared to be reduced by Alk1-Fc, albeit in only 2/4 tumors and without reaching statistical significance (Fig. 19C-D, $p=0.13$). Moreover, immunostaining with anti-CD8 antibodies indicated that Alk1-Fc expression significantly inhibited tumor infiltration by cytotoxic T-cell (Fig. 19E-F). Even though inhibition of angiogenesis by Alk1-Fc was incomplete, these results do not support a role for normoxia in mediating the pro-tumorigenic function of Activin-A in melanoma.

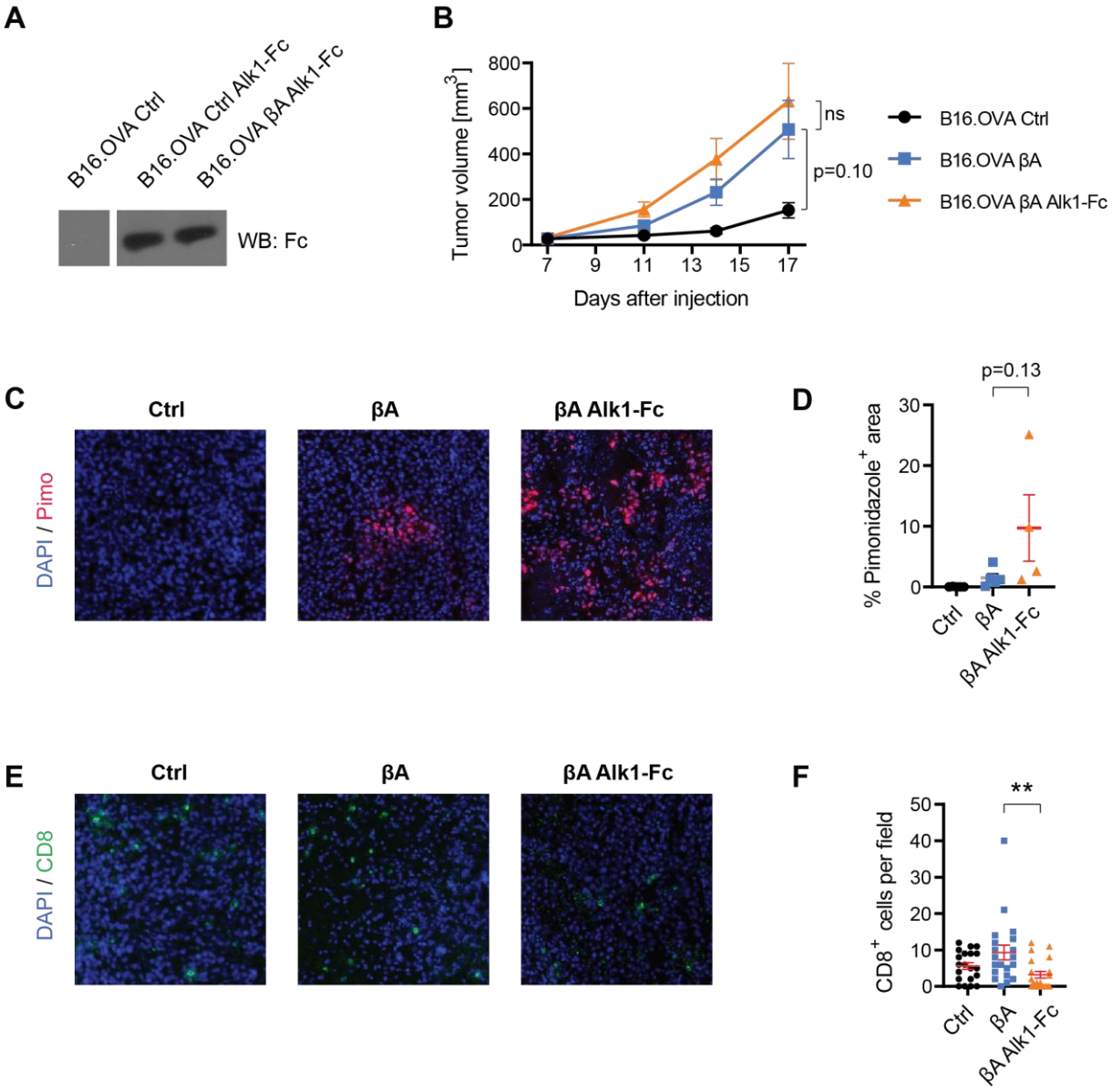


Figure 19: Expression of Alk1-Fc as an attempt to manipulate tumor hypoxia in B16.OVA-βA tumors

A. Western blot analysis of Fc in conditioned medium from B16.OVA-Ctrl and B16.OVA-βA cells stably transduced with lentiviral Alk1-Fc.

B. Tumor growth of B16.OVA-Ctrl, B16.OVA-βA and B16.OVA-βA/Alk1-Fc (n=5 per genoytpe) cells grafted intradermally on the right flank of C57BL/6 mice. (n=5 per group, p-values: One-way ANOVA)

C. Pimonidazole immunofluorescent staining of hypoxic areas in sections of syngeneic B16.OVA-Ctrl, B16.OVA-βA and B16.OVA-βA/Alk1-Fc grafts counterstained with DAPI.

D. Quantification of pimonidazole$^+$ areas in whole-sections of syngeneic B16.OVA-Ctrl (n=5), B16.OVA-βA (n=5) and B16.OVA-βA/Alk1-Fc (n=4) grafts. (p-value: One-way ANOVA)

E. CD8 immunofluorescent staining of syngeneic B16.OVA-Ctrl, B16.OVA-βA and B16.OVA-βA/Alk1-Fc grafts counterstained with DAPI.

F. Quantification of CD8$^+$ cells in syngeneic B16.OVA-Ctrl, B16.OVA-βA and B16.OVA-βA/Alk1-Fc grafts (n=5 per genotype, **p<0.01, One-way ANOVA).

2.14 Improved models for pre-clinical studies on Activin-A targeting: The YUMM cell lines

Melanoma research has long been hindered by the lack of genetically engineered mouse models and syngeneic cell lines. Despite their extremely wide use in melanoma research since the 1970s, the B16 cell line and its derivatives do not accurately depict genetic alterations that are most frequent in human patients. Additional limitations of B16 models include their low immunogenicity, poor stromal infiltration and severe necrosis, which can lead to the premature death of a subset of tumor-bearing animals. Therefore, to study the clinical implications of our findings, we decided to test Yale University melanoma lines (YUMM) derived from mice carrying specific genetic alterations in different combinations on the common C57BL/6 genetic background (Meeth et al., 2016). Analysis of the cancer genome atlas (TCGA) dataset for melanoma indicated that amongst the four genetic subtypes defined by the TCGA consortium (The cancer genome atlas network, 2015) and despite wide intrinsic variations, BRAF mutant melanoma patients express significantly higher *INHBA* mRNA levels than all other subtypes (Fig. 20), prompting us to select the YUMM3.3 ($Braf^{V600E/wt}$ $Cdkn2^{-/-}$) cell line for further investigation. No CAGA-Luc activity was detected in HepG2 reporter cells cultured in conditioned medium from this cell line, indicating that it does not secrete functional Activin-A. Cells were therefore transduced as described for B16F1 using lentiviruses for *INHBA* or a control empty backbone, followed by selection with puromycin. The conditioned medium from the resulting YUMM3.3-βA cell line induced robust CAGA-Luc activity in HepG2 reporter cells (Fig. 21A).

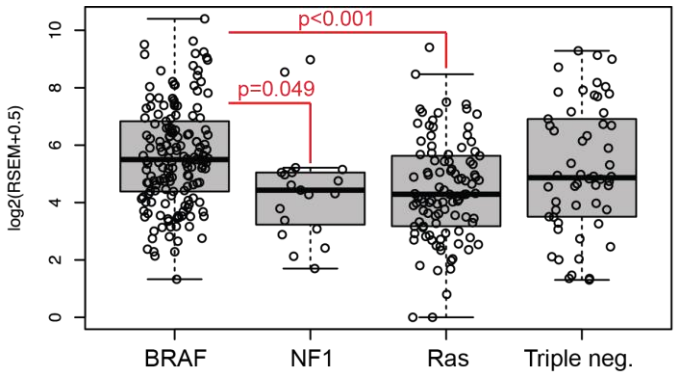


Figure 20: *INHBA* expression is elevated in BRAF-mutant melanoma patients

Expression analysis of *INHBA* in melanoma subtypes as defined by The Cancer Genome Atlas Network (Genomic Classification of Cutaneous Melanoma, Cell *161*, 2015). Expression data was obtained from the TCGA melanoma dataset. *Analysis by Sina Nassiri, Swiss Institute of Bioinformatics (SIB), University of Lausanne.*

2.15 Immunogenic rejection of YUMM tumors bearing the puromycin resistance gene (pac)

Surprisingly, while 2/3 syngeneic YUMM3.3 grafts expressing *INHBA* grew to endpoint, YUMM3.3 Ctrl tumors quickly regressed (n = 3/3), suggesting that the latter were more immunogenic than expected based on the literature (Fig. 21B) (Meeth et al., 2016). Alteration of several factors such as the mode of injection (subcutaneous vs. intradermal), increasing cell numbers ($100 \cdot 10^3$ – $2 \cdot 10^6$) or the age of the animals (8-20 weeks old) did not improve tumor take (data no shown). To verify whether lentiviral transduction could in itself lead to immunogenic rejection, the tumor growth of parental YUMM3.3 cells and derivatives transduced with Ctrl viral particles were compared. Strikingly, while 4/4 of non-transduced YUMM3.3 (Fig. 21C) tumors reached endpoint within three weeks, growth of all YUMM3.3-Ctrl tumors transduced with empty control lentivirus was significantly delayed. Despite being routinely used for the selection of transgenic cell lines in cancer research, immunogenicity of the puromycin *N*-acetyltransferase (pac) protein is poorly documented. We therefore cloned the *INHBA* transgene and an empty Ctrl into a lentiviral backbone bearing the blasticidin-S deaminase (bsd) selection cassette. Conditioned media from YUMM3.3 cell lines derived using these alternative lentiviruses induced CAGA-Luc activity in reporter cells as expected (Fig. 21D). Furthermore, YUMM3.3 cells infected with Ctrl lentiviruses bearing the bsd selection gene formed palpable tumors in all animals (Fig. 21E). Similar results were obtained with the YUMM2.1 cell line ($Braf^{V600E/wt}$ $Pten^{-/-}$ $Cdkn2^{+/-}$ $Bcat^{loxex3/wt}$, Fig. 21F-H). Together, these results strongly suggest that the puromycin *N*-acetyltransferase protein induces immunogenic rejection of YUMM tumors in syngeneic hosts.

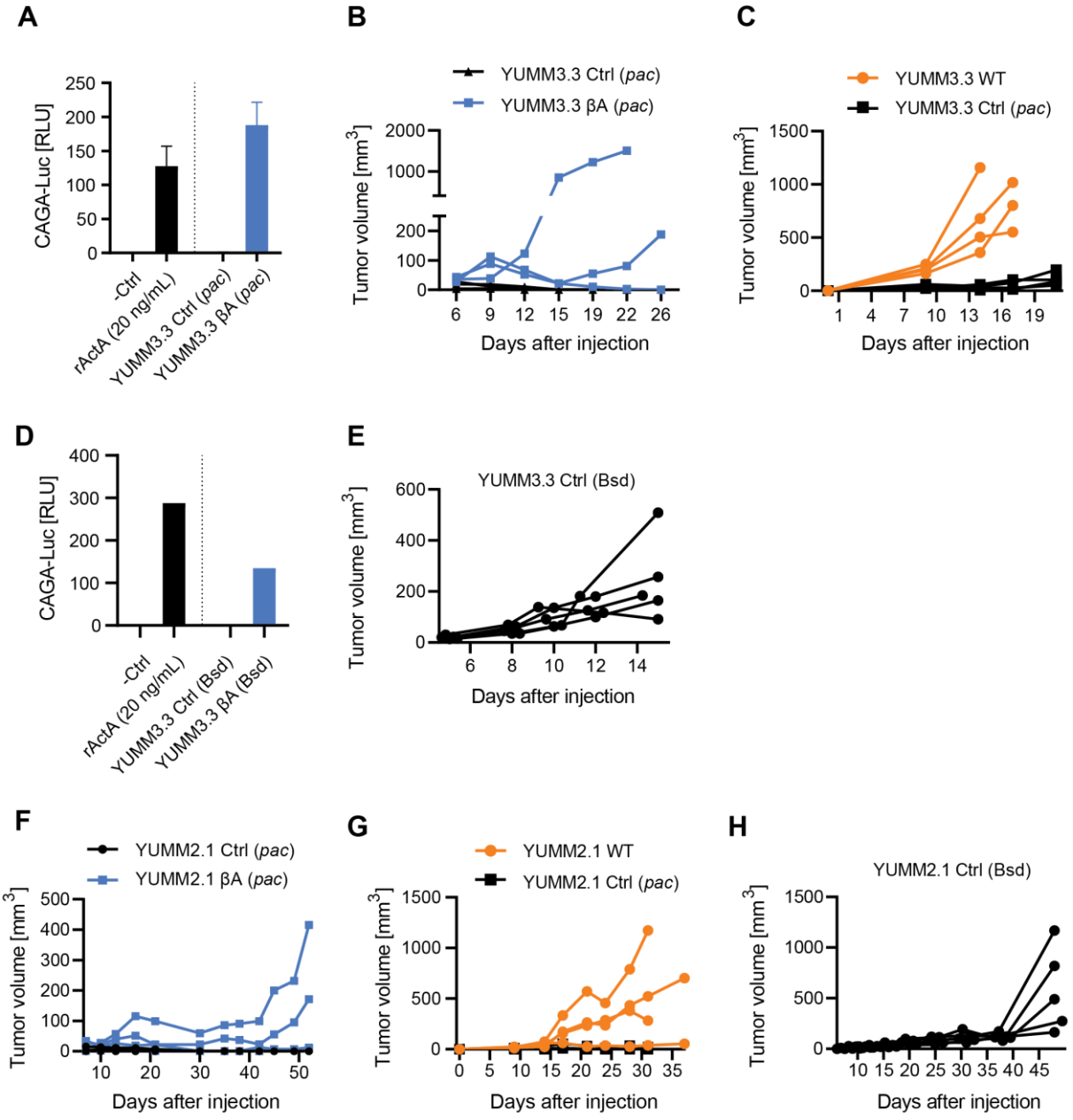


Figure 21: puromycin N-acetyltransferase (pac) mediates immunogenic rejection of YUMM syngeneic grafts

A. CAGA-luciferase activity in HepG2-CAGA reporter cells cultured in conditioned medium from YUMM3.3 cells stably transduced with lentiviral *INHBA* or ctrl vectors bearing puromycin N-acetyltransferase (*pac*).

B. Tumor growth of YUMM3.3-Ctrl and YUMM3.3-βA cells bearing *pac* grafted subcutaneously on the right flank of C57BL/6 mice.

C. Tumor growth of YUMM3.3-Ctrl (*pac*) and parental YUMM3.3 cells grafted subcutaneously on the right flank of C57BL/6 mice.

D. CAGA-luciferase activity in HepG2-CAGA reporter cells cultured in conditioned medium from YUMM3.3 cells stably transduced with lentiviral *INHBA* or ctrl vectors bearing blasticidin S deaminase (Bsd). (n=1)

E. Tumor growth of YUMM3.3-Ctrl (Bsd) cells grafted subcutaneously on the right flank of C57BL/6 mice.

F-H. As (**B**), (**C**) and (**E**) using YUMM2.1 cells.

2.16 Activin-A is immunosuppressive in YUMM3.3 tumors

Tumors formed by syngeneic injections of YUMM3.3 cells expressing *INHBA* grew faster than their control counterparts, confirming that the tumor-promoting function of Activin-A is not limited to the B16 model (Fig. 22A). This effect was not cell-intrinsic as *INHBA* expression or treatment of the cells with a physiological dose of recombinant Activin-A did not alter cell viability (Fig. 22B). Moreover, YUMM3.3-Ctrl tumors grew as fast as YUMM3.3-βA grafts when injected in Rag1$^{-/-}$ mice, suggesting that as in B16 melanoma, Activin-A signaling promotes YUMM3.3 tumor progression by blunting adaptive anti-tumor immunity (Fig. 22C).

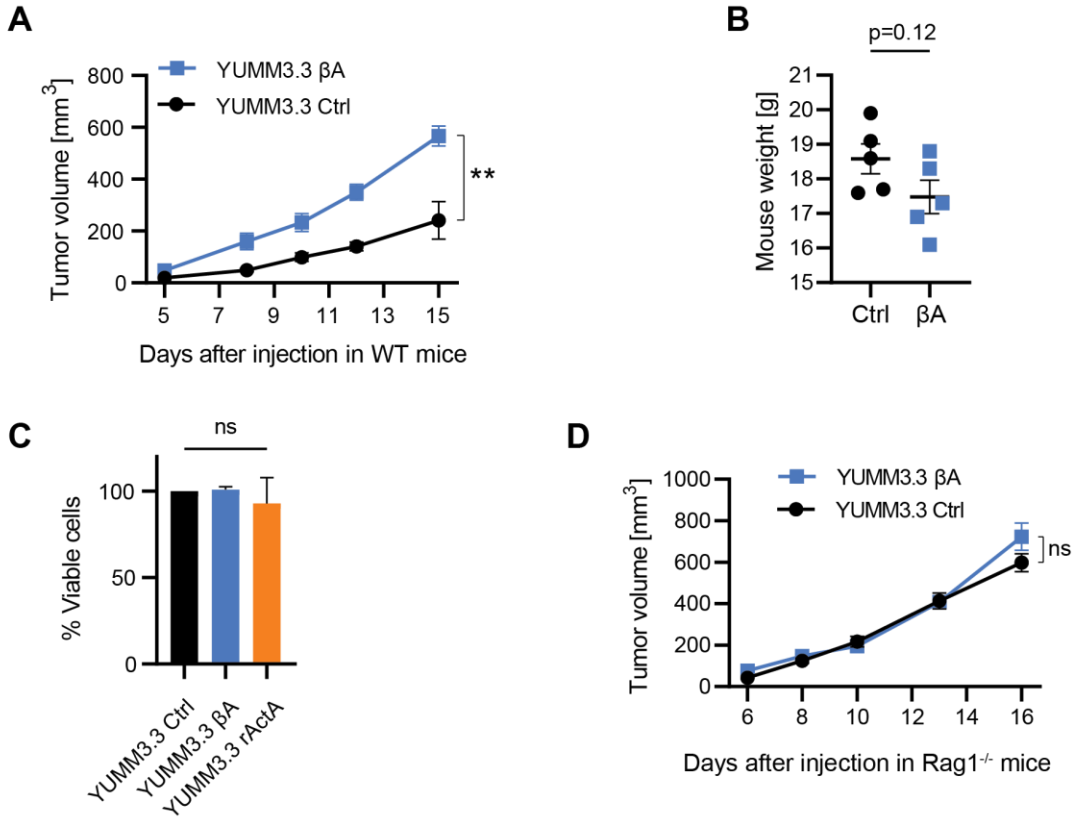


Figure 22: Secreted Activin-A accelerates YUMM3.3 melanoma growth in immunocompetent mice

A. Tumor growth of YUMM3.3-Ctrl or YUMM3.3-βA cells grafted intradermally on the right flank of C57Bl/6 mice. (n=5 per group, **p<0.01, Student's t-test)

B. Body weight of YUMM3.3-Ctrl or YUMM3.3-βA tumor-bearing mice at the time of sacrifice. (n=5 per group, p-value: Student's t-test)

C. Alamar blue cell viability assay of cultured YUMM3.3-Ctrl and YUMM3.3-βA cells, or YUMM3.3 cells cultured in presence of 20 ng/mL recombinant Activin-A. (p-values: Mann-Whitney test)

D. Tumor growth of YUMM3.3-Ctrl or YUMM3.3-βA cells grafted intradermally on the right flank of Rag1$^{-/-}$ C57Bl/6 mice. (n=5 per group, p-value: Student's t-test)

Analysis of the tumor immune infiltrate by flow cytometry revealed several features reminiscent of those observed in B16F1 tumors (Table 3), including high myeloid cell content and moderate lymphocytic populations in relation to total CD45$^+$ leukocytes, although *INHBA* expression did not significantly affect CD45$^+$ leukocyte infiltration in YUMM3.3 tumors (Fig. 23A). As in the B16 model, *INHBA* expression remodeled immune populations by increasing myeloid cell infiltration at the expense of the adaptive immune response. Specifically, CD11b$^+$ cells were increased in YUMM3.3-βA tumors (Fig. 23B). In contrast to observations made in B16F1 tumors, Activin-A did not significantly affect mMDSC counts (Fig. 23C) but macrophages were significantly enriched (Fig. 23D). Despite this increase in frequency, CD206 was not upregulated in TAMs exposed to Activin-A (Fig. 23E). Neutrophils were more abundant in YUMM3.3 compared to B16F1 tumors, but in the context of *INHBA* expression, their numbers appeared to decrease (Fig. 23F). Dendritic cells were also enriched in YUMM3.3 tumors when *INHBA* was overexpressed (Fig. 23G), and they expressed elevated MHC-II (Fig. 23H), possibly leading to improved antigen presentation. Importantly, CD8 T-cell infiltration was strongly decreased by Activin-A (Fig. 23I), whereas CD4 T-cell counts did not significantly change (Fig. 23J). Intracellular staining indicated that CD8 T-cells were less frequently IFNγ$^+$, expressed less TNFα, and downregulated granzyme B (Fig. 24A-C) when tumors expressed Activin-A. Altogether, these results suggest that Activin-A likely inhibits anti-tumor immunity in the YUMM3.3 model in a mechanism dependent on adaptive immunity, akin to that observed in B16 melanoma.

Table 3: Immune populations in YUMM3.3 tumors analyzed by flow cytometry

Fig. Panel	Population	Markers	Ctrl[1]	βA[1]	p-value
A	CD45+ leukocytes	CD45	25.7% ± 8.9%	33.2% ± 7.8%	0.23
B	CD11b+ myeloid cells	CD11b+	63.3% ± 7.6%	74.1% ± 4.4%	0.04
C	mMDSCs	CD11b+Ly6Chi	9.5% ± 3.3%	6.0% ± 3.2%	0.12
D	Macrophages	CD11b+F4/80+	19.6% ± 4.1%	31.3% ± 3.4%	0.003
E	Macrophage CD206 MFI	CD206	9740 ± 3316	11523 ± 3196	0.44
F	Neutrophils	CD11b+Ly6G+	12.0% ± 6.3%	4.8% ± 2.0%	0.06
G	Dendritic cells	CD11c+F4/80-	8.3% ± 2.3%	11.3% ± 1.2%	0.06
H	Dendritic cells MHC-II MFI	IA/IE	65612 ± 22162	97637 ± 4910	0.03
I	CD8 T-cells	CD3+CD8+	5.2% ± 1.6%	1.5% ± 0.5%	0.003
J	CD4 T-cells	CD3+CD4+	2.6% ± 1.1%	3.5% ± 0.8%	0.21

Flow cytometry analysis of immune populations in YUMM3.3-Ctrl (n=5) and YUMM3.3-βA (n=4) syngeneic grafts. [1] Data represent mean ± SEM in percentage of CD45$^+$ population, or mean fluorescence intensity (MFI) when indicated. p-values calculated using Student's t-test.

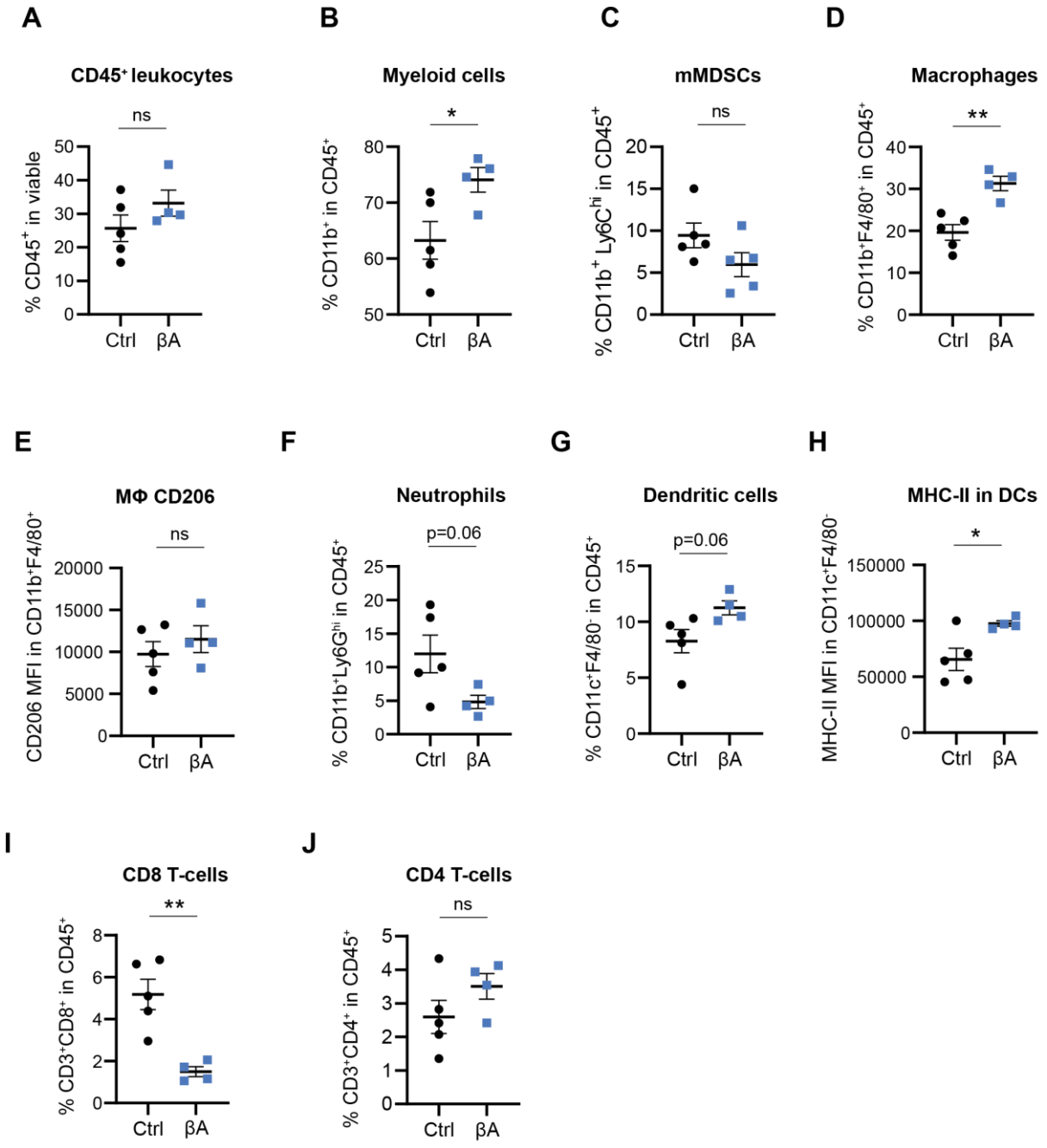


Figure 23: *INHBA* expression is immunosuppressive in YUMM3.3 tumors

Flow cytometry analysis of the immune infiltration of syngeneic YUMM3.3-Ctrl (n=5) and YUMM3.3-βA (n=4) tumors at experimental endpoint. (*p<0.05, **p<0.01, Student's t-test)

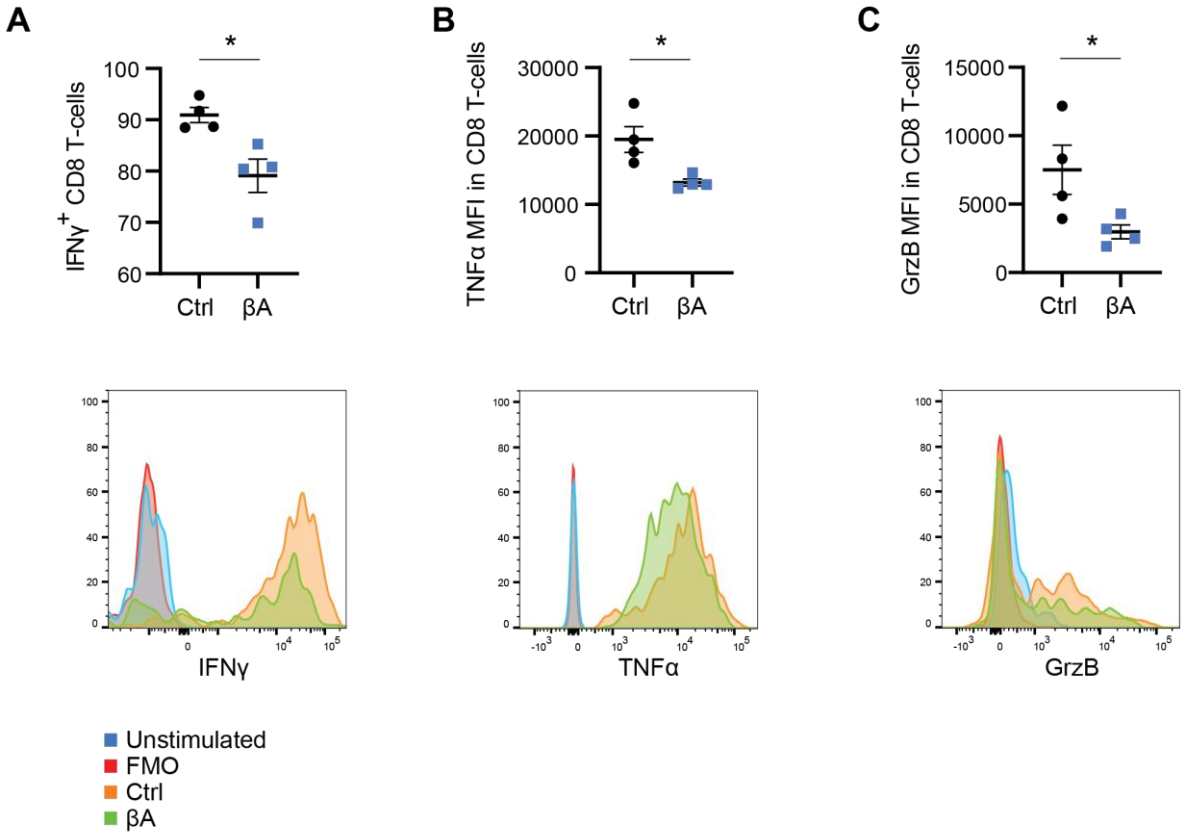


Figure 24: Secreted Activin-A impairs CD8 T-cell effector function in YUMM3.3 tumors

Flow cytometry analysis of CD8 T-cells from YUMM3.3-Ctrl and YUMM3.3-βA syngeneic grafts stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin and stained intracellularly for IFNγ (A), TNFα (B) and Granzyme B (C). Cells unstimulated with PMA/ionomycin and fluorescence minus one (FMO) staining were used as controls. (n=4 per group, *p<0.05, Student's t-test)

2.17 Activin-A may promote resistance to immune checkpoint blockade by αPD1 & αCTLA4 antibodies

Collectively, our results suggest that Activin-A mediates immuno-suppression in melanoma, prompting us to investigate whether Activin-A signaling could mediate resistance to immune checkpoint blockade. To verify this hypothesis, mice were challenged at experimental day 0 with YUMM3.3-Ctrl or YUMM3.3-βA tumors. Treatment with intraperitoneal injections of 10 mg/kg αPD1 and 5 mg/kg αCTLA4 antibodies was initiated five days later and repeated every 3 days for a total of five injections (Fig. 25A). 45 days after tumor challenge, 3/6 YUMM3.3-βA, but only 1/6 YUMM3.3-Ctrl tumor-bearing mouse had reached experimental endpoint (tumor volume >1cm^3, Fig. 25B, C). These results suggest that Activin-A signaling may promote resistance to combined anti-PD1 and anti-CTLA4 immune checkpoint inhibition in YUMM3.3 syngeneic grafts.

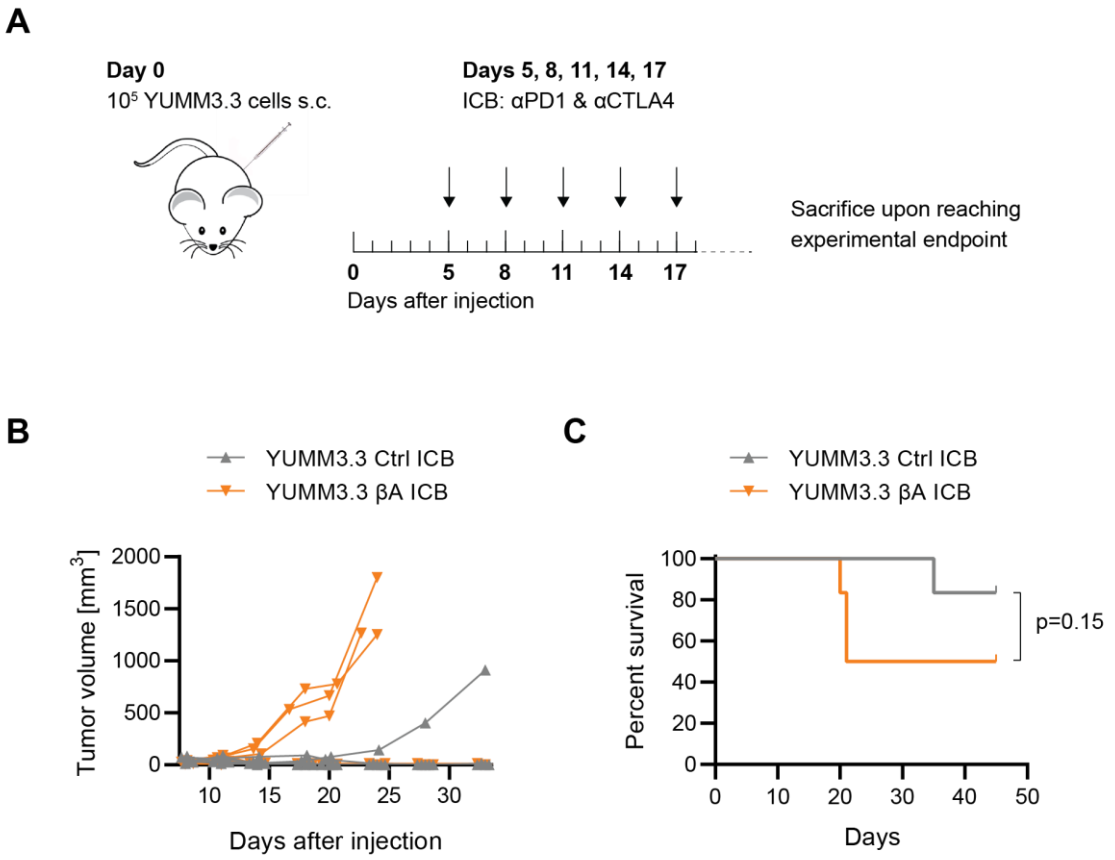


Figure 25: Activin-A signaling my promote resistance to immune checkpoint blockade in YUMM3.3 melanoma

A. Mice were injected at experimental day 0 with 10^5 YUMM3.3-Ctrl or YUMM3.3-βA cells. Treatment with 10 mg/kg αPD1 and 5 mg/kg αCTLA4 (ICB) or isotype Ctrl IgGs (IgG) was initiated at day 5 when tumors were palpable and repeated at days 8, 11, 14 and 17. Mice were sacrificed upon reaching experimental endpoint (tumor volume >1cm^3).

B. Individual tumor growth of YUMM3.3-Ctrl and YUMM3.3-βA cells grafted subcutaneously on the right flank of female C57BL/6 mice and treated with ICB or IgG control antibodies as indicated (n=6 per group).

C. Kaplan-Meier survival analysis of YUMM3.3-Ctrl and YUMM3.3-βA tumor-bearing mice. Mice were considered dead upon reaching experimental endpoint (tumor volume >1cm^3, p-value calculated using the Gehan-Breslow-Wilcoxon test).

2.18 Endogenous Activin-A signaling accelerates melanoma progression in a $BRAF^{V600E}$ model

Since B16 and YUMM3.3 models do not secrete endogenous Activin-A activity, we initially focused on gain-of-function studies using *INHBA* overexpression. To validate a tumorigenic role for Activin-A in loss of function experiments, we examined *INHBA* expression in the iBIP2 inducible mouse melanoma model ($Cdkn2a^{-/-}$ $Pten^{-/-}$ $BRAF^{V600E/WT}$) (Neubert et al., 2018). Single cell RNAseq indicated that a subset of tumor cells express *INHBA* (K. Homicsko & D. Hanahan, unpublished data), and several independent cell lines derived from this genetic model formed aggressive tumors when grafted in syngeneic FVB/N mice, including clones 2891L and 2104. Conditioned medium of 2891L, but not of 2104 cells, induced CAGA-Luc activity in reporter cells at levels comparable to stimulation with 20 ng/mL recombinant Activin-A (Fig. 26A). Treatment with 250µg/mL recombinant Follistatin 300 abrogated luciferase activity, indicating that Smad3 phosphorylation was likely induced by Activin-A. To study the role of endogenous Activin-A in melanoma progression, 2891L cells were transduced with AIIB-Fc or empty control lentiviruses. Expression of AIIB-Fc suppressed CAGA-Luc activity in conditioned medium (Fig. 26B) and, upon grafting into FVB/N hosts, significantly slowed tumor growth (Fig. 26C). Alamar blue assays indicated that cell proliferation was unaffected by AIIB-Fc expression (Fig. 26D). Moreover, as in the B16 and YUMM3.3 models, Activin-A did not accelerate tumor growth upon grafting in $Rag1^{-/-}$ mice (Fig. 26E). These results strongly suggest that endogenous Activin-A signaling promotes melanoma progression by inhibiting adaptive anti-tumor immunity, consistent with our previous observations in the B16 and YUMM3.3 gain-of-function models.

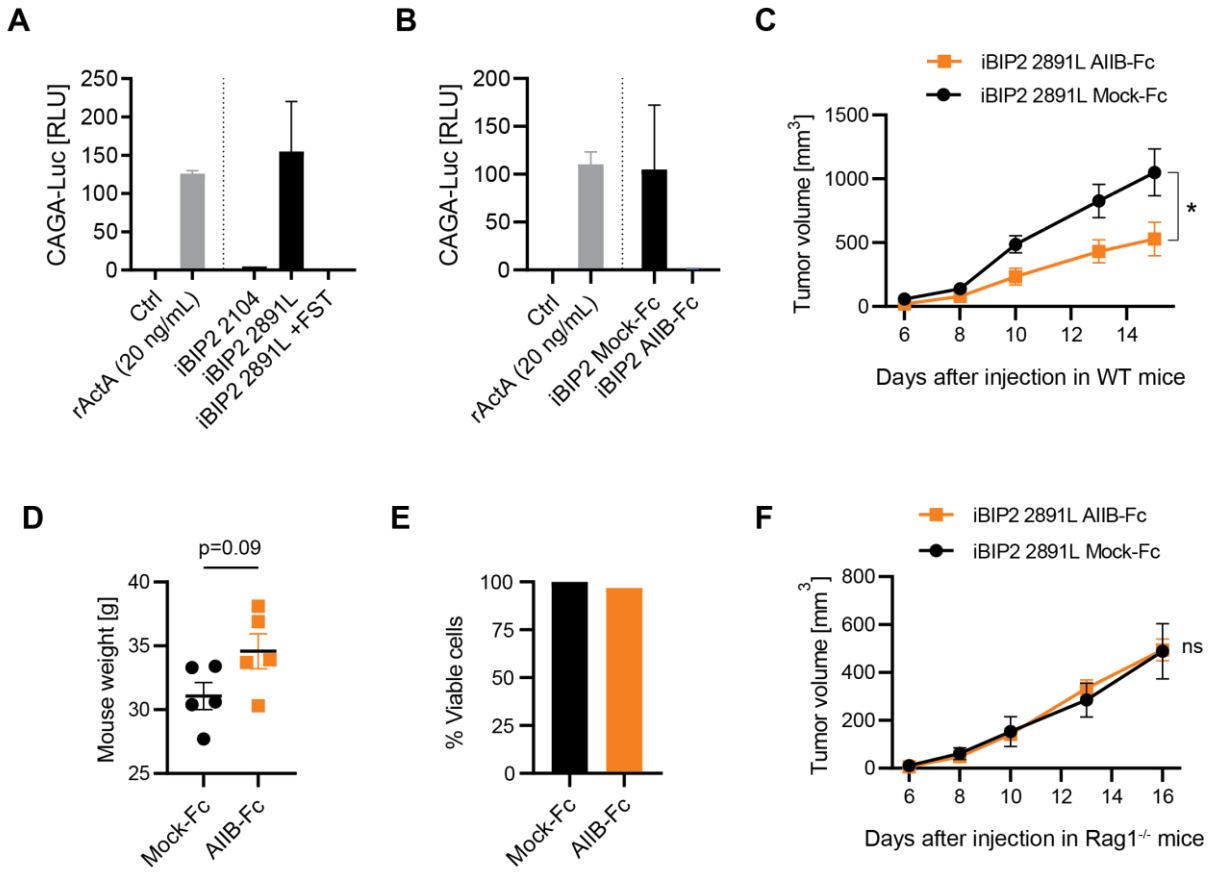


Figure 26: Inhibition of endogenous Activin-A inhibits iBIP2 melanoma growth in immunocompetent mice

A. CAGA-luciferase activity in HepG2-CAGA reporter cells cultured in conditioned medium from iBIP2 2104 and iBIP2 2891L in presence or absence of 100 ng/mL recombinant Follistatin. Treatment with 20 ng/mL recombinant Activin-A was used as positive control. (n=2)

B. CAGA-luciferase activity in HepG2-CAGA reporter cells cultured in conditioned medium from iBIP2 2891L cells stably transduced with lentiviral Mock-Fc or AIIB-Fc. Treatment with 20 ng/mL recombinant Activin-A was used as positive control. (n=2)

C. Tumor growth of iBIP2 2891L Mock-Fc and iBIP2 2891L AIIB-Fc cells grafted intradermally on the right flank of FVB/N mice. (n=5 per group, *p<0.05, Student's t-test)

D. Body weight of iBIP2 2891L Mock-Fc and iBIP2 2891L AIIB-FC tumor-bearing mice at the time of sacrifice. (n= 5 per group, p-value: Student's t-test)

E. Alamar blue cell viability assay of cultured iBIP2 2891L Mock-Fc and iBIP2 2891L AIIB-Fc cells. (n=1)

F. Tumor growth of iBIP2 2891L Mock-Fc and iBIP2 2891L AIIB-Fc cells grafted intradermally on the right flank of Rag1$^{-/-}$ C57BL/6 mice. (n=5 per group, p-value: Student's t-test)

2.19 Endogenous Activin-A is immunosuppressive in iBIP2 melanoma

To verify whether the impaired growth of iBIP2 tumors expressing AIIB-Fc correlated with improved immune surveillance, the infiltration of iBIP2 tumors by immune cells was analyzed by flow cytometry (Table 4). Total numbers of $CD45^+$ leukocytes were strikingly increased upon AIIB-Fc expression, suggesting that inhibition of Activin-A signaling improved infiltration of iBIP2 tumors by immune cells (Fig. 27A). This immune infiltrate was mostly constituted of myeloid cells (Fig. 27B), which included few MDSCs (Fig. 27C) and neutrophils (Fig. 27D), but macrophages were highly prevalent (Fig. 27E). Total numbers of myeloid cells decreased in AIIB-Fc-expressing tumors (Fig. 27B), whereas macrophage populations were not significantly affected (Fig. 27E). Dendritic cell infiltration was markedly reduced upon Activin-A inhibition (Fig. 27F), but DC activation assessed by MHC-II staining increased, albeit not significantly (Fig. 27G). Cytotoxic lymphocytes were particularly rare in iBIP2 tumors. Nevertheless, AIIB-Fc expression moderately increased NK cell and CD8 T-cell infiltration (Fig. 27H, I). CD4 T-cells were not significantly affected by AIIB-Fc (Fig. 27J). Collectively, these results suggest that AIIB-Fc ameliorates anti-tumor immunity by increasing immune infiltration and cytotoxic cell count within iBIP2 tumors.

Table 4: Immune populations in iBIP2 2891L tumors analyzed by flow cytometry

Fig. Panel	Population	Markers	Mock-Fc[1]	AIIB-Fc[1]	p-value
A	CD45+ in viable cells	CD45	11.1% ± 1.2%	30.7% ± 12.1%	0.007
B	CD11b+ myeloid cells	CD11b+	81.8% ± 4.0%	66.7% ± 10.2%	0.15
C	MDSCs	CD11b+Ly6Chi	2.4% ± 0.7%	2.6% ± 0.8%	0.64
D	Neutrophils	CD11b+Ly6G+	2.2% ± 0.3%	2.8% ± 1.3%	0.41
E	Macrophages	CD11b+F4/80+	47.4% ± 3.2%	43.6% ± 10.0%	0.44
F	Dendritic cells	CD11c+F4/80-	26.5% ± 0.8%	16.2% ± 1.5%	<0.0001
G	Dendritic cells MHC-II MFI	IA/IE	12785 ± 5072	18406 ± 4371	0.10
H	NK cells	NK1.1+CD3-	1.0% ± 0.4%	3.6% ± 2.3%	0.04
I	CD8 T-cells	CD3+CD8+	0.8% ± 0.4%	2.6% ± 1.8%	0.06
J	CD4 T-cells	CD3+CD4+	7.9% ± 2.2%	10.5% ± 3.1%	0.16

Flow cytometry analysis of immune populations in iBIP2 2891L Mock-Fc and iBIP2 2891L AIIB-Fc (n=5 per group) syngeneic grafts. [1] Data represent mean ± SEM in percentage of $CD45^+$ population, or mean fluorescence intensity (MFI) when indicated. p-values calculated using Student's t-test.

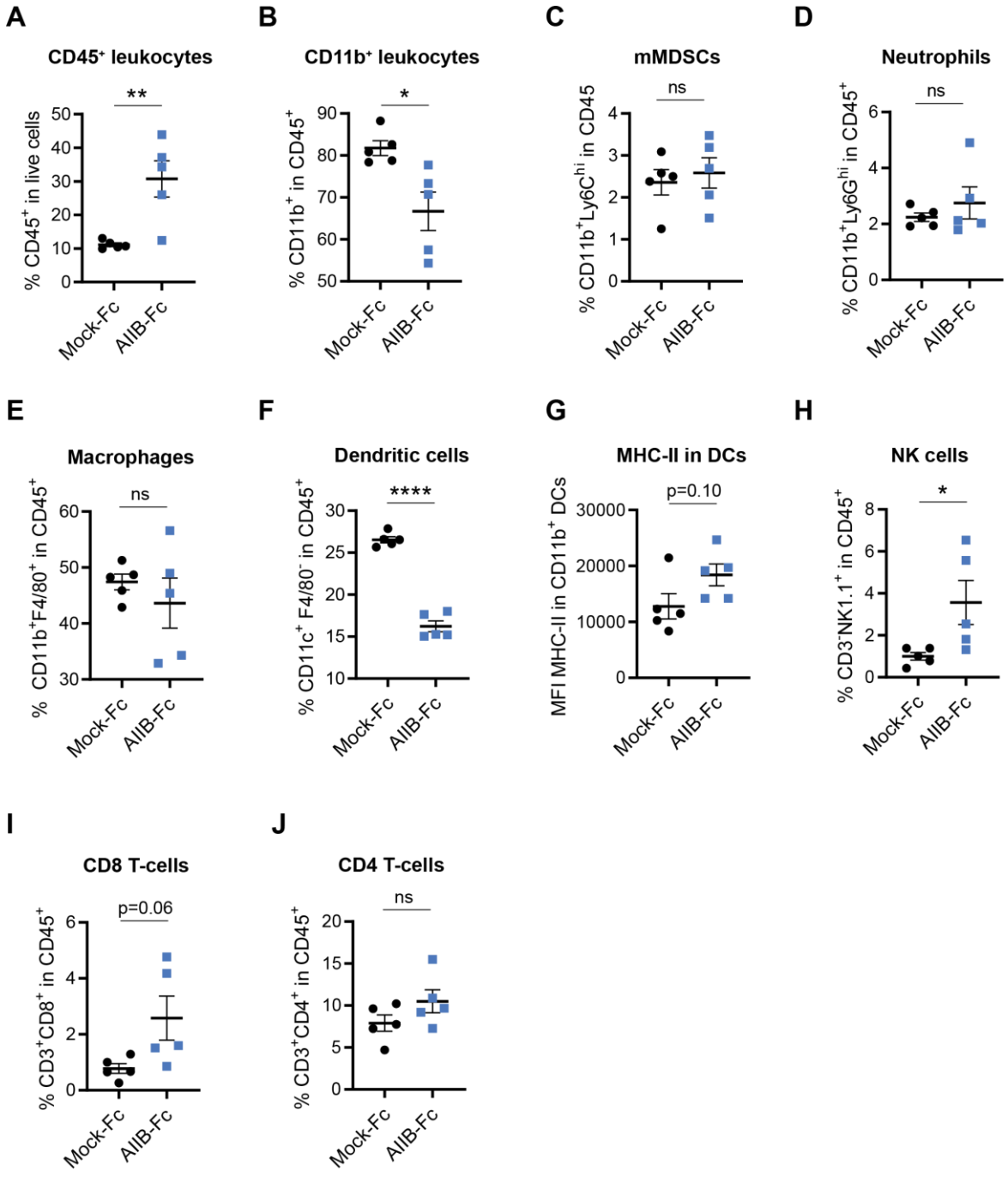


Figure 27: AIIB-Fc expression ameliorates anti-tumor immunity in iBIP2 2891L melanoma

Flow cytometry analysis of the immune infiltration of syngeneic iBIP2 2891L Mock-Fc and iBIP2 2891L AIIB-Fc tumors at experimental endpoint. (n=5 per group, *p<0.05, **p<0.01, ****p<0.0001, Student's t-test)

2.20 Inhibition of endogenous Activin-A signaling sensitizes iBIP2 tumors to immune checkpoint blockade

Since secreted Activin-A appeared to promote iBIP2 tumor progression by impairing the adaptive anti-tumor response, we next asked whether inhibition of endogenous Activin-A signaling in iBIP2 tumors could ameliorate immune checkpoint therapy outcome. Treatment with αPD1 and αCTLA4 or IgG control antibodies was initiated four days after tumor challenge (Fig. 28A). Whereas ICB treatment alone did not significantly alter tumor progression, the growth of iBIP2 grafts was strikingly inhibited when αPD1 and αCTLA4 were combined with AIIB-Fc (Fig. 28B). The infiltration of tumors by CD8 T-cells was

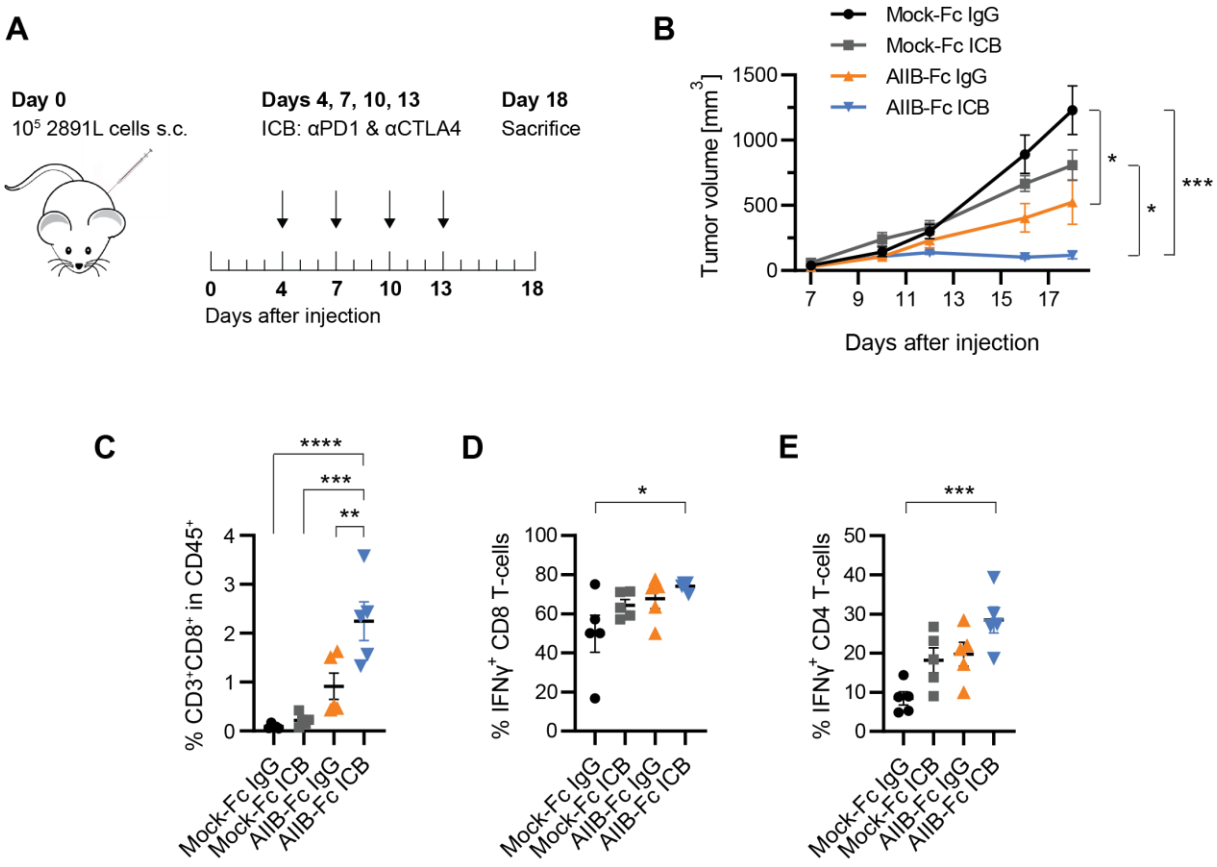


Figure 28: AIIB-Fc expression sensitizes iBIP2 2891L tumors to immune checkpoint blockade

A. Mice were injected at experimental day 0 with 10^5 iBIP2 2891L Mock-Fc or iBIP2 2891L Mock-Fc cells. Treatment with 10 mg/kg αPD1 and 5 mg/kg αCTLA4 (ICB) or rat IgG2a (IgG) antibodies was initiated at day 4 when tumors were palpable and repeated at days 7, 10 and 13. Mice were sacrificed at day 18 upon reaching experimental endpoint.

B. Tumor growth of iBIP2 2891L Mock-Fc and iBIP2 2891L AIIB-Fc cells grafted intradermally on the right flank of FVB/N mice and treated with ICB or IgG control antibodies as indicated. (n=5 per group, *p<0.05, ***p<0.001, One-way ANOVA)

C. Flow cytometry analysis of CD8 T-cells in iBIP2 2891L Mock-Fc and iBIP2 2891L AIIB-Fc tumors treated with ICB or IgG antibodies. (n=5 per genotype, **p<0.01, ***p<0.001, ****p<0.0001, One-way ANOVA)

D-E. Flow cytometry analysis of CD8 (D) and CD4 (E) T-cells from iBIP2 2891L Mock-Fc and iBIP2 2891L AIIB-Fc tumors treated with ICB or IgG antibodies (n=5 per genotype) stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin and stained intracellularly for IFNγ. (n=5 per genotype, *p<0.05, ***p<0.001, One-way ANOVA)

greatly ameliorated when ICB was combined with Activin-A inhibition (Fig. 28C). Moreover, this treatment regimen enhanced expression of IFNγ in CD8 and CD4 T-cells (Fig. 28D, E). Together, these results strongly suggest that inhibition of Activin-A signaling sensitizes iBIP2 tumors to immune checkpoint blockade.

2.21 *INHBA* expression correlates with resistance to immune checkpoint inhibitors in melanoma patients

Prompted by these results, we examined whether Activin-A mediates resistance to immune checkpoint blockade in melanoma patients. We took advantage of a human melanoma gene expression profiling dataset of treatment-naïve patients who would later receive anti-PD1 therapy (Hugo et al., 2016). Interestingly, expression of *INHBA* was significantly elevated in tumors from non-responder (NR) patients, suggesting that Activin-A signaling correlates with resistance to PD1 blockade in human melanoma patients (Fig. 29).

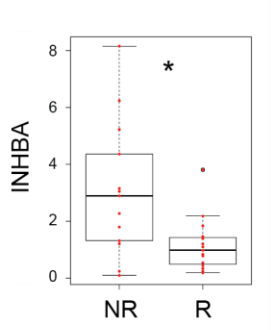


Figure 29: Elevated *INHBA* expression correlates with resistance to anti-PD1 therapy in melanoma patients

Expression analysis of *INHBA* in pre-treatment biopsies of treatment-naive melanoma patients undergoing anti-PD1 therapy and classified as responders (R) or non-responders (NR) based on irRECIST criteria (Wolchok et al. Clin. Cancer Res. *15* 2009). Datasets were from Hugo et al. Cell *165* 2016. *Analysis by Sina Nassiri, Swiss Institute of Bioinformatics (SIB), University of Lausanne.*

2.22 A role for Activin-A in the resistance to MAPK inhibition?

BRAF targeted therapies are highly effective in patients bearing the V600E hotspot mutation, but despite frequent initial tumor regression, relapse invariably occurs (Villanueva et al., 2011). Resistance to BRAF therapies may occur through various mechanisms. In particular, expression of the receptor tyrosine kinase AXL has been shown to circumvent BRAF inhibition by stimulating the AKT pathway (Müller et al., 2014; Zuo et al., 2018). Our analysis of TCGA datasets for melanoma patients revealed a strong correlation between *INHBA* and *AXL* expression (Donovan et al., 2017), prompting us to investigate a possible link between Activin-A signaling and the resistance to MAPK inhibitors. To address this, we chose the SM1 $BRAF^{V600E}$ melanoma cell line which is sensitive to the BRAF inhibitor vemurafenib (Koya

et al., 2012). Analysis of SM1 conditioned medium by CAGA-Luciferase assay suggested that SM1 cells secrete moderate amounts of Activin-A (Fig. 30A). Treatment with increasing concentrations of vemurafenib in the presence or absence of recombinant Follistatin or Activin-A revealed no overt Activin-induced drug resistance at any of the doses tested (Fig. 30B). Importantly, neither AIIB-Fc nor *INHBA* overexpression altered the expression level of *AXL* mRNA in SM1 melanoma cells when analyzed by qRT-PCR (Fig. 30C). Similar preliminary results were obtained in the *INHBA*-negative YUMMER1.7 cell line (n=1, Fig. 30D-F). These results do not support a role for autocrine Activin-A signaling in mediating

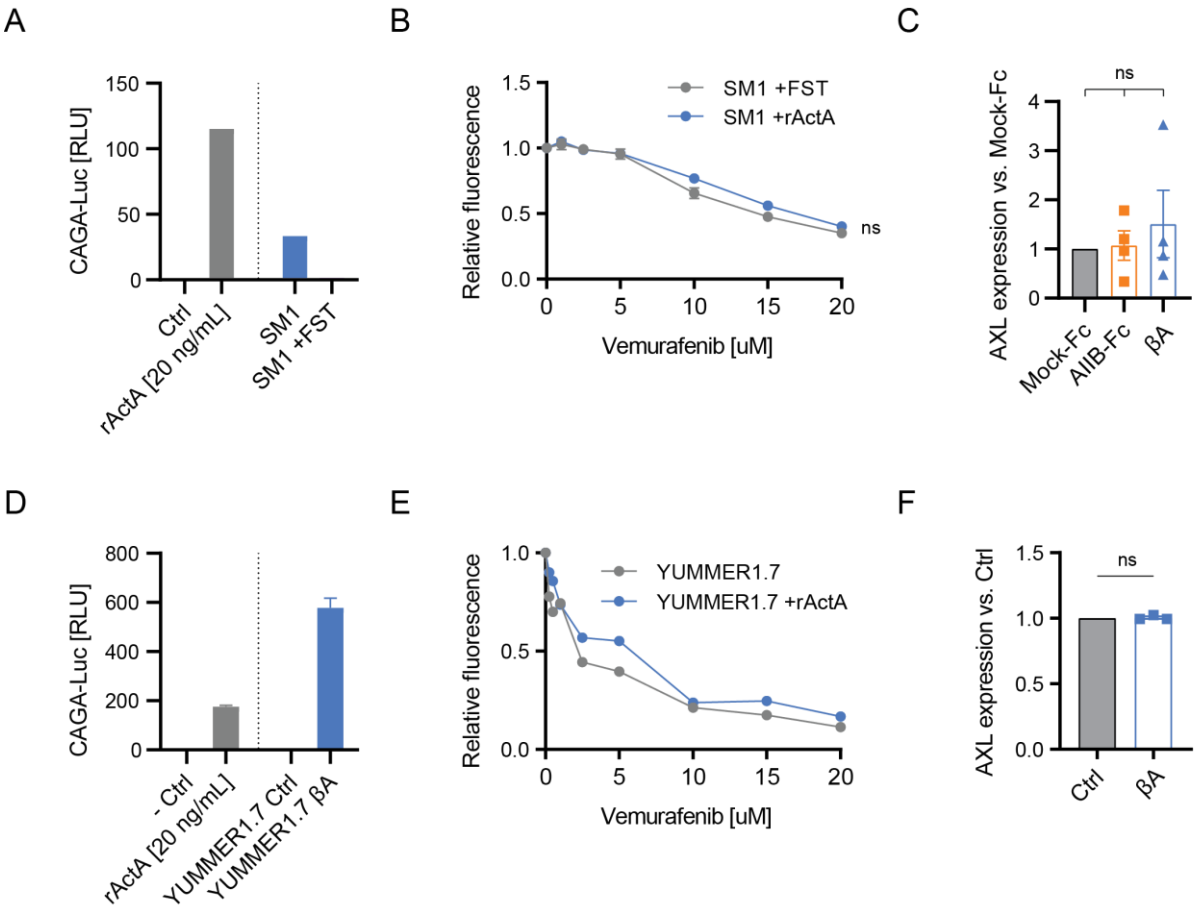


Figure 30: Tumor cell-autonomous Activin-A signaling does not induce resistance to BRAF inhibitors

A. CAGA-luciferase activity in HepG2-CAGA reporter cells cultured in conditioned medium from SM1 cells in presence or absence of 100 ng/mL recombinant Follistatin. Treatment with 20 ng/mL recombinant Activin-A was used as positive control.

B. Alamar blue cell viability assay of SM1 cells cultured in presence of 20 ng/mL recombinant Activin-A or 100 ng/mL recombinant Follistatin and with increasing concentrations of the BRAF inhibitor Vemurafenib.

C. qRT-PCR analysis of AXL RNA expression in SM1 cells stably transduced with Mock-Fc or AIIB-Fc, or SM1 parental cells cultured in presence of 20 ng/mL recombinant Activin-A for 24h. (p-values: One-way ANOVA)

D. CAGA-luciferase activity in HepG2-CAGA reporter cells cultured in conditioned medium from YUMMER1.7 cells stably transduced with *INHBA* or Ctrl lentiviruses. Treatment with 20 ng/mL recombinant Activin-A was used as positive control.

E. Alamar blue cell viability assay of YUMMER1.7 cultured in presence of 20 ng/mL recombinant Activin-A and with increasing concentrations of the BRAF inhibitor Vemurafenib. (n=1)

F. qRT-PCR analysis of AXL RNA expression in YUMMER1.7-Ctrl and YUMMER1.7-βA cells. (p-value: Mann-Whitney test)

resistance to BRAF inhibitors, but rather suggest that the observed correlation between *AXL* and *INHBA* expression in melanoma patients is linked to the microenvironment.

2.23 Interaction between Activin-A signaling and doxycycline

Doxycycline-inducible B16F1 TRE-βA cells were generated in parallel to B16F1 TRE-caALK4 cells (Fig. 5-6), initially with the aim to manipulate *INHBA* expression reversibly during metastasis. Induction of *INHBA* by doxycycline administration efficiently stimulated Smad-pathway activation (Fig. 31A). To test whether inducible Activin-A signaling accelerates tumor progression as observed upon constitutive

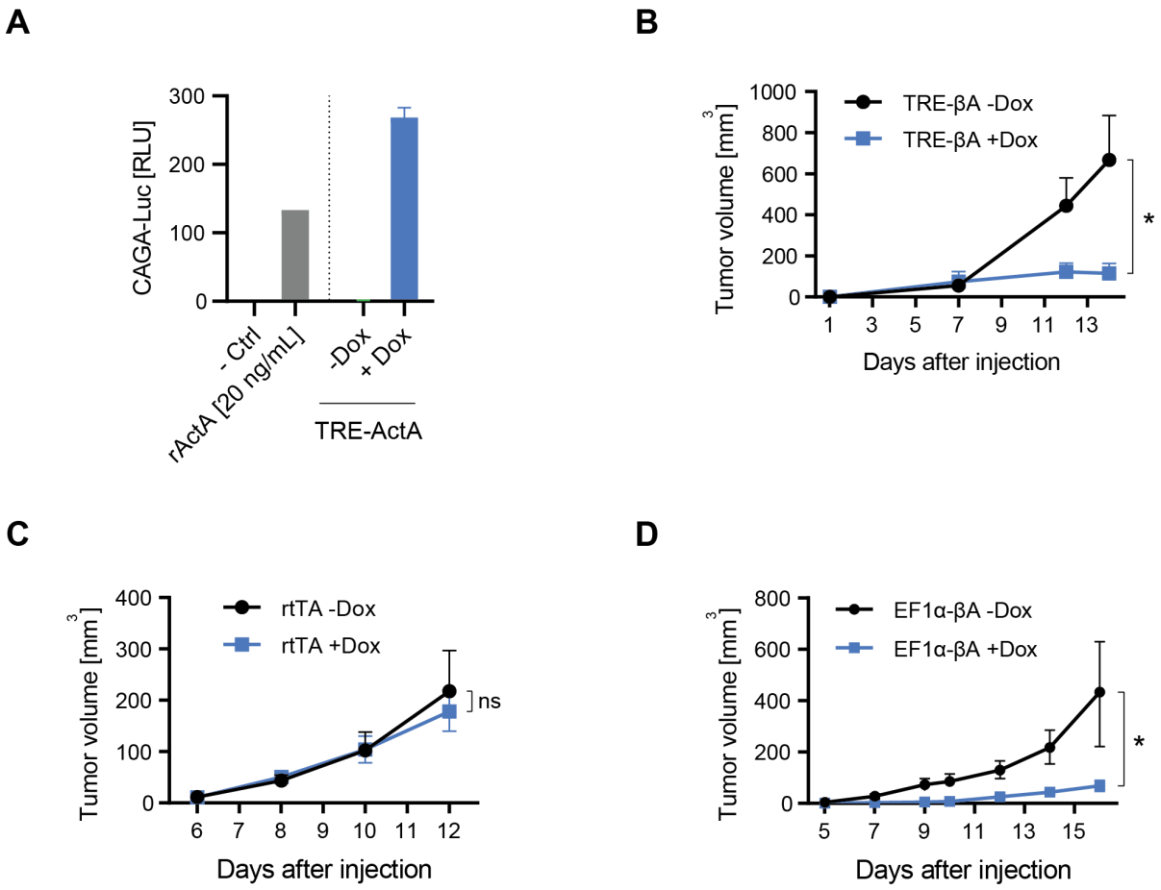


Figure 31: Doxycycline inhibits growth of B16F1-βA tumors

A. CAGA-luciferase activity in HepG2-CAGA reporter cells cultured in conditioned medium from B16F1 TRE-βA cells cultured in presence or absence of 1 µg/mL doxycycline. Treatment with 20 ng/mL recombinant Activin-A was used as positive control.

B. Tumor growth of B16F1 TRE-βA cells grafted intradermally on the right flank of C57BL/6 mice fed with or without doxycycline. (n=4 per group, *p<0.05, Student's t-test)

C. Tumor growth of B16F1 cells stably transduced with a reverse tetracycline transactivator (rtTA) lentivirus and grafted intradermally on the right flank of C57BL/6 mice fed with or without doxycycline. (n=5 per group, p-value: Student's t-test)

D. Tumor growth of B16F1-βA cells grafted intradermally on the right flank of C57BL/6 mice fed with or without doxycycline. (*p<0.05, Student's t-test)

overexpression (Fig. 9A), C57Bl/6 mice were intradermally grafted with B16F1-TRE-βA cells. Strikingly, the growth of tumors resulting from mice fed with doxycycline was significantly impaired, rather than accelerated (Fig. 31B). A similar experiment was repeated using B16F1 cells expressing the reverse tetracycline trans-activator (rtTA) alone. In these control conditions, treatment with doxycycline did not influence tumor progression (Fig. 31C), excluding experimental artifacts due to off-target effects. Furthermore, the growth of B16F1 tumors constitutively expressing *INHBA* was significantly impaired when mice were exposed to doxycycline (Fig. 31D). These results raise the intriguing possibility that doxycycline may circumvent Activin-A-induced tumor acceleration in a mechanism dependent on the tumor microenvironment.

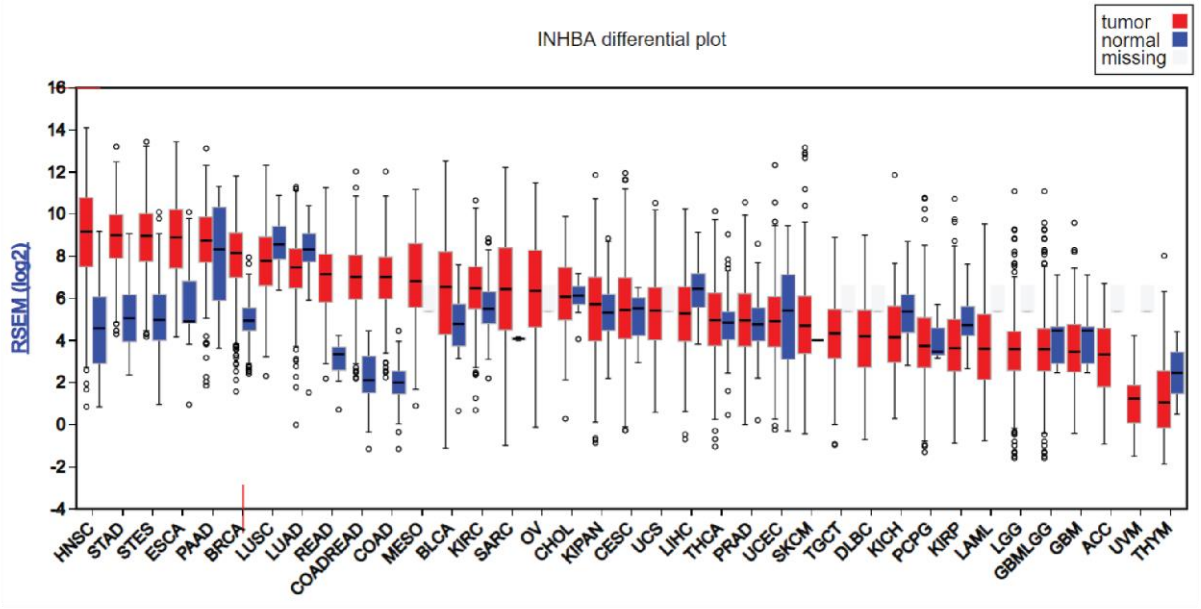
2.24 Beyond melanoma: Activin-A in cancer

Besides melanoma, Activin-A was previously shown to promote tumorigenesis in prostate, colorectal, lung, hepatocellular, pancreatic, esophageal and skin carcinomas (Antsiferova et al., 2011; Bashir et al., 2015; Deli et al., 2008; Kang et al., 2009; Lonardo et al., 2011; Seder et al., 2009; Wildi et al., 2001; Yoshinaga et al., 2008). Analysis of TCGA datasets indicate that *INHBA* mRNA is overexpressed in many cancers when compared to healthy controls (Fig. 34A & Table 5). Moreover, *INHBA* expression correlates with poor survival in renal, cervical, head and neck, as well as lung cancers, suggesting critical implication of Activin-A signaling in the pathogenesis of these diseases (Fig. 34B). However, the immuno-suppressive function of Activin-A has not been studied in the context of these malignancies. Our work thus paves the way for future studies of the role of this pathway in the development of other cancers, as therapeutic inhibition of Activin-A may provide extensive benefits beyond melanoma.

Table 5: *INHBA* mRNA fold-upregulation in cancer compared to healthy controls. Data from TCGA.

Cancer type	*INHBA* fold-upregulation
Colon adenocarcinoma	32.5
Colorectal adenocarcinoma	30
Head & Neck Squamous cell carcinoma	24
Stomach and esophageal carcinoma	15.9
Esophageal carcinoma	15.9
Stomach adenocarcinoma	15.5
Rectum adenocarcioma	13.9
Breast carcinoma	9.3
Bladder urothelial carcinoma	3.4

A



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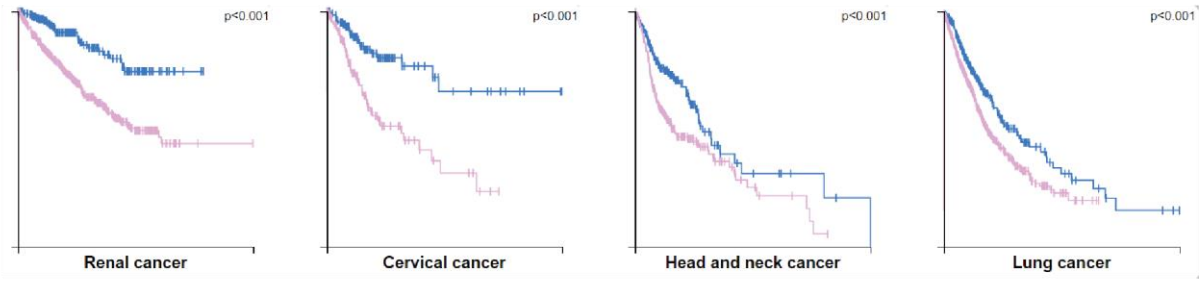

INHBA high
INHBA low

Figure 32: *INHBA* expression is upregulated in various cancers and correlates with poor survival

A. *INHBA* mRNA expression in cancers. Data from TCGA, plots from firebrowse.org.

B. Kaplan-Meyer survival analysis sorted by *INHBA* high and *INHBA* low mRNA expression in renal, cervical, head and neck and lung cancers.

3. Discussion

In this study, I investigated the role of Activin-A signaling in melanomagenesis and its relevance in the context of melanoma therapy. My results show that tumor autocrine and paracrine Activin-A signaling exert opposite effects on melanoma progression. Whereas tumor cell-autonomous signaling reduces cell proliferation in vitro and tumor growth in vivo, secreted Activin-A promotes tumorigenesis by inhibiting the adaptive anti-tumor immunity. Furthermore, my results show for the first time that Activin-A mediates resistance to immune checkpoint blockade in melanoma.

3.1 ALK4 signaling can inhibit melanoma cell growth and tumor progression

The relative contributions of autocrine and paracrine Activin-A signaling in melanoma development were previously unknown. To study the role of autocrine Activin-A signaling, B16F1 mouse melanoma cells were transduced with lentiviral vectors coding for a constitutively active mutant type I activin receptor 1b (caALK4) and bearing the puromycin selection cassette (pac). Surprisingly, western blot analysis of B16F1-caALK4 cells failed to detect transgene expression despite continuous exposure to puromycin. In contrast, expression of a Smad-signaling deficient caALK4 transgene (caALK4-L45m) was readily detected. Similar results were obtained with human melanoma cell lines. We therefore reasoned that sustained cell-autonomous Smad signaling might be detrimental to melanoma cell proliferation and/or survival. In line with this idea, expression of caALK4, but not of caALK5-L45m, was progressively lost in B16F1 cells upon induction by a doxycycline-responsive promoter despite continuous presence of doxycycline. Intriguingly, caALK4 expression did not alter cell cycle progression or apoptosis *in vitro*, suggesting that alternative cell death programs might be induced by cell-autonomous Activin-A signaling.

Downregulation of TGF-β receptors has been observed in many cancers and constitutes a critical step for malignant transformation (Ikushima and Miyazono, 2010). Similarly, ALK7, which mediates Activin-B signaling, was recently shown to suppress metastasis in pancreatic neuroendocrine tumors and its downregulation correlates with poor survival in human cancers (Michael et al., 2019). Downregulation of type II activin receptors was also observed in colorectal cancer (Zhuo et al., 2018). In contrast, ALK4 expression appears surprisingly unchanged in human cancers (TCGA), indicating that alternative mechanisms may be used by cancer cells to evade the tumor suppressive functions of tumor cell-autonomous ALK4 signaling. Mutations and alternatively spliced forms affecting the kinase domain of ALK4 have for example been reported in pancreatic cancer and pituitary adenomas (Alexander et al., 1996; Su et al., 2001).

In a chemically-induced mouse skin carcinogenesis model, inhibition of tumor cell-autonomous ALK4 signaling was shown to accelerate tumor growth, suggesting that Activin-A signaling may be detrimental to tumor progression *in vivo* (Antsiferova et al., 2011). In good agreement, our results indicate that expression of caALK4 greatly diminishes B16F1 melanoma progression by hampering cell proliferation and inducing apoptosis. These results nevertheless contrast with observations made in vitro, where proliferation and apoptosis of cultured B16F1 cells was not affected by caALK4 expression, suggesting that tumor-cell extrinsic factors might mediate these processes in the tumor context.

3.2 Paracrine Activin-A signaling promotes melanoma progression by inhibiting the adaptive anti-tumor immunity

Our data demonstrate that Activin-A promotes melanoma progression by inhibiting the adaptive anti-tumor immunity. In contrast to results obtained with caALK4 expression, exposure of B16F1 cells to Activin-A did not alter cell proliferation *in vitro*. This differential response of melanoma cells to ligand-induced signaling may be explained by signal attenuation. Previous studies have indicated that TGF-β receptors are endocytosed following ligand exposure, leading to a refractory period where sustained exposure to TGF-β does not lead to SMAD2 phosphorylation (Vizan et al., 2013). Recently however, data from the same group suggested that such a refractory state does not exist in the context of Activin-A signaling, and neither ACVR1B or ACVR2B are depleted from the cell surface upon ligand binding (Miller et al., 2019). Despite being frequently induced by Activin-A signaling [Zhang, BBA 1997], Follistatin RNA expression was unchanged in B16F1 cells exposed to recombinant Activin-A. Alternatively, caALK4 overexpression may lead to artificially high signaling strength and cellular toxicity.

Activin-A was previously reported to inhibit lymphangiogenesis in melanoma without affecting tumor growth or metastasis (Heinz et al., 2015). The function of Activin-A in the regulation of anti-tumor immunity was masked in this study due to the use of A375 human cell lines xenografted into severe combined immunodeficient mice. Using several syngeneic cell lines in gain- and loss-of-function experiments, our data show that Activin-A accelerates melanoma growth in immunocompetent, but not $Rag1^{-/-}$ mouse models, suggesting that Activin-A inhibits the adaptive anti-tumor immunity across multiple mouse melanoma models. Coherent with this idea, CD45 and CD8 immunofluorescent staining suggested decreased infiltration of leukocytes and $CD8^+$ cells in tumors expressing Activin-A. The tumor-promoting function of Activin-A appeared even more apparent in B16.OVA compared to B16F1 grafts, owing perhaps to the increased immunogenicity elicited by expression of the ovalbumin surrogate antigen. This cell line was therefore chosen for antibody-mediated $CD8^+$ and $CD4^+$ cell depletion. Depletion of $CD8^+$, but not of $CD4^+$ cells led to similar growth rates of B16.OVA-Ctrl and B16.OVA-βA grafts, indicating that Activin-A likely inhibits CD8 T-cell function.

3.3 Activin-A stimulates angiogenesis in B16F1 melanoma

Our data indicated that Activin-A stimulates angiogenesis in B16F1 melanoma, both in WT and Rag1$^{-/-}$ mice. However, Ctrl tumors grew as fast as *INHBA*-expressing grafts in Rag1$^{-/-}$ animals, indicating that basal tumor angiogenic activity in the absence of Activin-A is sufficient to sustain accelerated tumor growth. These observations suggest that the pro-angiogenic activity of Activin-A alone is not sufficient to promote tumor progression. Antsiferova and colleagues reported a similar pro-angiogenic activity of Activin-A in an HPV-induced skin carcinogenesis model, where *INHBA* expression promoted vascularization in the upper dermis (Antsiferova et al., 2017). However, since Activin-A was shown to inhibit proliferation and tubule formation of endothelial cells (Kaneda et al., 2011), the pro-angiogenic function of Activin-A is likely mediated indirectly through the tumor micro-environment. In line with this idea, *VEGFA* mRNA expression was increased in *INHBA*-expressing tumors (data not shown). The increased vascularization induced by Activin-A correlated with decreased hypoxia in WT animals, suggesting that the neo-vasculature was perfused. Pimonidazole staining of hypoxia was also performed in Rag1$^{-/-}$ hosts; however extreme necrosis in control tumors impaired proper cryosectioning and the sample number was too low.

Whether hypoxia stimulates or inhibits anti-tumor immunity may depend on the context (Petrova et al., 2018). Low oxygen pressure within the tumor microenvironment has long been recognized to promote differentiation and recruitment of suppressive immune subsets, including Tregs, MDSCs and TAMs (Noman et al., 2015). Interestingly, hypoxic culture conditions delay CD8 T-cell development but stimulate cytotoxic functions (Caldwell et al., 2001; Doedens et al., 2013). Moreover, HIF-1α was recently shown to be essential for CD8 T-cell effector functions (Palazon et al., 2017). Considering this emerging tumor-suppressive role, we attempted to manipulate intra-tumoral hypoxia in B16.OVA tumors by expressing anti-angiogenic Alk1-Fc (Cunha and Pietras, 2011). As a result, hypoxia was only increased in 2/4 tumors, and Alk1-Fc expression did not significantly alter B16.OVA-βA tumor growth. In contrast to results obtained with B16F1 grafts, no hypoxic areas were detected in the control group, owing perhaps to small tumor sizes at the time of sacrifice. Moreover, CD8 T-cell infiltration was found to be reduced 3-fold in tumors expressing Alk1-Fc, suggesting that factors beyond hypoxia are affected by the use of this strategy, thus confounding interpretation of experimental results. Consequently, alternative experimental approaches will be necessary to elucidate whether Activin-A may inhibit anti-tumor immunity by increasing intratumoral oxygenation. In particular, syngeneic melanoma grafts may be performed in mice after conditional deletion of HIF pathway components in cytotoxic T-cells.

3.4 Mechanistic study of Activin-A-induced immunosuppression

Our data indicate that Activin-A signaling generally decreases CD45^{+} leukocyte infiltration in melanoma tumors. Extravasation of leukocytes into the tumor bed depends on endothelium maturation and pericyte

coverage (Rudziak et al., 2019; Strell and Entschladen, 2008). Activin-A promotes angiogenesis, but vessel maturation was not assessed in our study. Nevertheless, upregulation of VEGF-A that was observed in tumors expressing Activin-A may correlate with poor vessel maturation in such tumors.

Several studies have recently confirmed TGF-β as a key mediator of immune exclusion and as a druggable target in cancer (Mariathasan et al., 2018; Newsted et al., 2019; Tauriello et al., 2018). A similar function for Activin-A has not been reported. Here, we provided evidence that Activin-A induces immune exclusion of cytotoxic T lymphocytes and NK cells in melanoma. Whereas a functional role for the implication of CD8 T-cells was confirmed by antibody-mediated CD8 depletion, the role of NK cells in the control of tumor progression is unclear. Studies have reported an inhibitory effect for Activin-A in NK cell proliferation in vitro (Robson et al., 2009). However, NK cell proliferation was not inhibited by Activin-A in B16.OVA grafts, suggesting that *INHBA* expression might impair recruitment rather than proliferation of NK cells. Importantly, depletion of CD8+ cells led to similar growth rates of B16.OVA-Ctrl and B16.OVA-βA tumors, indicating that NK cells are not sufficient to mediate the immuno-suppressive function of Activin-A.

Our results show that Activin-A impairs both infiltration and effector functions of CD8 T-cells in melanoma. Flow cytometry analysis indicated that CD8 T-cell numbers were reduced in the context of Activin-A signaling in B16F1, YUMM3.3 and iBIP2 tumors. By contrast, CD8 T-cell infiltration was unchanged by Activin-A in B16.OVA tumors, owing perhaps to increased immunogenicity. Interestingly, Activin-A still accelerated tumor growth in this model. Two factors may explain this apparent paradoxical observation. First, as the data was quantified as a percentage of $CD45^+$ populations, total numbers of cytotoxic lymphocytes may still be lower within B16.OVA-βA tumors compared to B16.OVA-Ctrl grafts. Additionally, intracellular cytokine staining performed in the YUMM3.3 model indicate that Activin-A impairs not only infiltration, but also activation and effector functions of CD8 T-cells. Indeed, IFNγ, TNFα and Granzyme B staining were all reduced by *INHBA* expression. A direct regulatory function of Activin-A in CD8 T-cell proliferation and effector function has not been reported. Further experiments will therefore be needed to assess whether Activin-A directly impairs CD8 T-cell function, or whether its immuno-suppressive function may be mediated through the tumor microenvironement. Importantly, Activin-A did not alter MHC-I expression nor presentation of the ovalbumin class I epitope SIINFEKL in several melanoma cell lines.

We next attempted to identify potential cellular mediators of this immunosuppressive function. mRNA expression data from the Immunological Genome Project (ImmGen) indicate that all major immune subsets express Activin receptors, albeit to various levels (Fig. 32), suggesting that the immune compartment as a whole may be responsive to Activin-A signaling. Here, we began to analyze dendritic cells, monocytes and macrophages, as their functions were previously shown to be affected by Activin-A (Antsiferova et al., 2017; Ogawa et al., 2006; Robson et al., 2008; Salogni et al., 2009; Seeger et al., 2014; Shurin et al., 2016; Sierra-Filardi et al., 2011). Collectively, our results suggest that Activin-A stimulates dendritic cell function in melanoma. Several lines of evidence support this idea. First, tumor

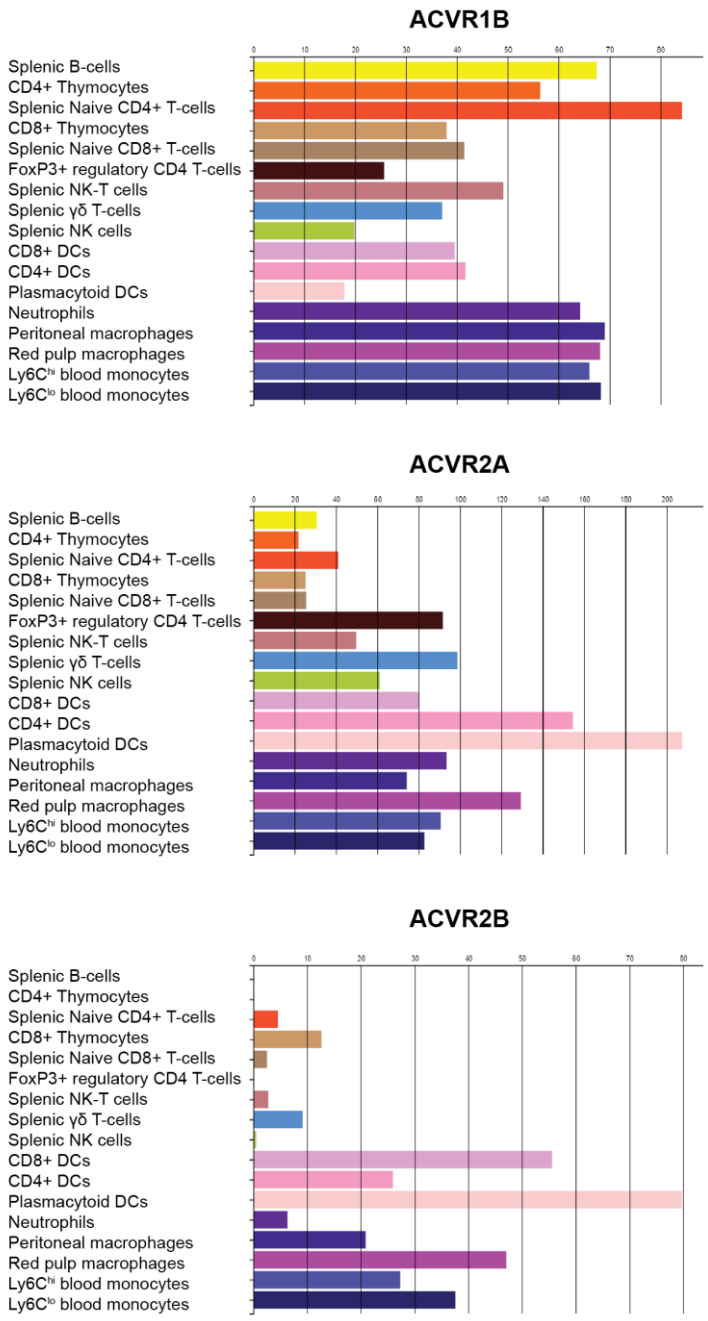

Figure 33: Expression of Activin receptors in immune subsets

RNAseq data of Activin receptor expression compiled from the Immunological Genome Project (ImmGen).

infiltration by DCs was consistently higher in the context of Activin-A signaling. The vast majority of these DCs were CD11b$^+$, indicative of myeloid origin. This is in good agreement with a report by Salogni and colleagues which demonstrated a pro-migratory role for Activin-A on immature myeloid dendritic cells (Salogni et al., 2009). Moreover, whereas DC activation assessed by MHC-II was not consistently altered by Activin-A, CD80 staining was dramatically increased in B16F1.βA compared to B16F1.Ctrl tumors, indicating that Activin-A may stimulate critical co-stimulatory signals necessary for T-cell activation, in sharp contrast to TGF-β-induced CD80 downregulation (Geissmann et al., 1999).

Nevertheless, these results are coherent with the observation that *in vivo* injection of DCs treated with Activin-A enhances tumor-specific cytotoxic T-cell function and diminishes B16 and Lewis Lung Carcinoma tumor growth (Shurin et al., 2016). Whether this also occurs in YUMM3.3 and iBIP2 tumors remains to be established. Finally, the functional relevance of these findings was confirmed by the observation that tumor-antigen-specific T-cells were increased in B16.OVA tumors when they expressed *INHBA*. Interestingly, both H2Kb-SIINFEKL$^+$ DCs and SIINFEKL-dextramer$^+$ CD8 T-cells were specifically enriched in tumors, but not in lymphoid organs, indicating that antigen presentation occurs directly within the tumor microenvironment in this model. Using CCR7 knock-out mice, Roberts and colleagues recently reported the critical requirement of CD103$^+$ DC trafficking to the draining lymph nodes for proper CD8 T-cell activation in melanoma (Roberts et al., 2016). Activin-A did not promote lymph node trafficking of antigen-specific DCs. This is unlikely to be caused by temporal factors or the use of different tumor models: Roberts and colleagues further demonstrated that antigen-specific DCs progressively migrate to draining lymph nodes in the course of tumor progression, and this DC migration was observed in multiple tumor models, including a B16F10 variant. Nevertheless, our results suggest that Activin-A promotes antigen-specific cytotoxic T-cell activation by stimulating myeloid-derived dendritic cell recruitment to the tumor bed and by increasing surface expression of the co-stimulatory molecule CD80. Therefore, we conclude that dendritic cells unlikely mediate the immuno-suppressive function of Activin-A. Of note, we did not specifically investigate the role of the skin-specific DC subset Langerhans Cells in this study. The relevance of LCs for the activation of cytotoxic T-cells in melanoma was questioned by the observation that LCs are weak inducers of T-cell alloreactivity (van de Ven et al., 2011). Moreover, Activin-A promoted tumor growth upon intradermal, but also subcutaneous grafting, where Langerhans Cells are likely absent.

Myeloid-derived suppressive cells (MDSCs) are highly relevant in cancer as they correlate with poor survival and limited efficacy of immunotherapies (Weber et al., 2018). The role of granulocytic MDSCs (CD11b$^+$Ly6G^{hi}) was not analyzed in detail here for two main reasons. First, neutrophils were particularly rare in the B16F1 and iBIP2 tumor models, and their numbers were decreased by Activin-A in the YUMM3.3 model. In contrast, monocytic MDSCs (CD11b$^+$Ly6C^{hi}) were highly prevalent in B16F1 tumors and enriched by *INHBA* expression, constituting an interesting T-cell-suppressive candidate. We therefore attempted to deplete mMDSCs in tumor-bearing mice by injecting anti-Gr1 antibodies. Unfortunately, in two independent pilot experiments, Ly6G$^+$ neutrophils were efficiently suppressed in tumors and blood from tumor-bearing mice, but Ly6C$^+$ mMDSCs were resistant to αGr1 treatment, even when the antibody dosage was increased. Interestingly, similar results were obtained by Kumar and colleagues in a mouse model of mammary carcinoma (Kumar et al., 2016a). The efficacy of anti-Gr1 antibodies for the depletion of MDSCs is debated and studies using this approach often do not disclose depletion plots (Xing et al., 2016). Moreover, even when such plots are provided, authors frequently use identical antibody clones for cell depletion and for flow cytometry staining, raising the issue of antigen masking (Srivastava et al., 2012). Another limitation of anti-Gr1 antibodies lies in their lack of specificity. In addition to binding Ly6G on neutrophils, αGr1 may also target Ly6C$^+$ subsets of CD8 T-cells, including memory CD8 T-cells, complicating interpretation of experiments. Considering these drawbacks,

alternative approaches will be required to inhibit mMDSC recruitment in B16F1 tumors. mMDSCs have been characterized to express the CCL2 chemokine receptor CCR2 (Lesokhin et al., 2012). Moreover, mRNA expression of CCR2 in the myeloid lineage was shown to be potently induced *in vitro* in the presence of recombinant Activin-A (Sierra-Filardi et al., 2014). In a preliminary experiment, I observed a two-fold enrichment of $CD11b^+CCR2^+$ in B16.OVA tumors expressing *INHBA* (Fig. 33). Grafting of B16F1 or B16.OVA cells in CCR2-deficient mice could potentially be informative for the implication of mMDSCs in the immuno-suppressive phenotype induced by Activin-A. A limitation to such an experiment is that it could confound the relative contributions of MDSCs and macrophages, as both these subsets depend on CCL2/CCR2 signaling for their recruitment (Argyle and Kitamura, 2018; Lesokhin et al., 2012). Importantly, mMDSC infiltration was much less prominent in the YUMM3.3 and iBIP2 models and their relative frequency did not increase in the context of Activin-A signaling. Opposingly, macrophages were more prevalent in these models, particularly in iBIP2 tumors. As TAMs differentiate from monocyte progenitors under the control of various cytokines, it is likely that melanoma cell lines intrinsically differ in their cytokine secretome, leading to different polarization within the monocytic lineage. Whether these factors could determine the populations which mediates the immuno-modulatory function of Activin-A remains to be determined.

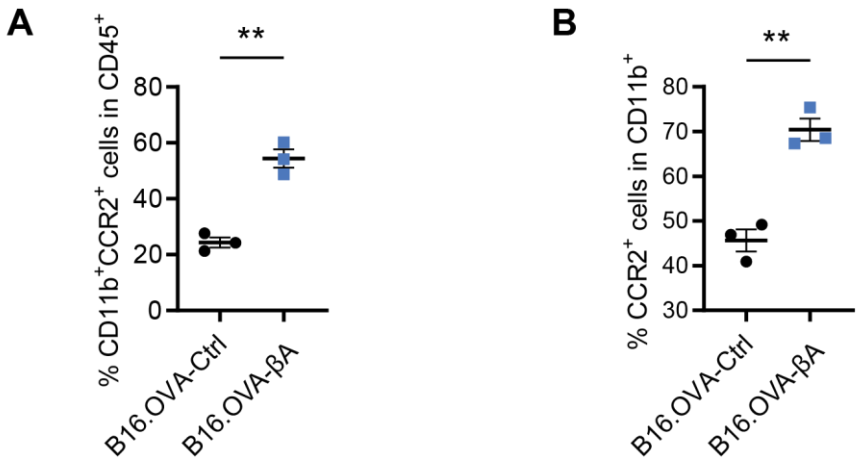

Figure 34: Activin-A stimulates infiltration of $CCR2^+$ myeloid cells in B16.OVA tumors

A. Flow cytometry analysis of $CD11b^+CCR2^+$ cell infiltration in the $CD45^+$ leukocyte compartment.
B. Flow cytometry analysis of $CCR2^+$ cells in $CD11b^+$ myeloid cells.

Like MDSCs, tumor-associated macrophages are associated with tumor progression, metastasis and therapy resistance in cancer (Mantovani et al., 2017). Activin-A has long been implicated in macrophage biology. In vitro, Activin-A acts as a Th2 cytokine and promotes M2 alternative-activation of macrophages (Ogawa et al., 2006; Sierra-Filardi et al., 2011). However, our results do not consistently support a role for macrophages in the tumor-promoting function of Activin-A in melanoma. First, macrophage numbers were only significantly increased by Activin-A in YUMM3.3 tumors. In this model,

expression of CD206 by macrophages was unchanged upon *INHBA* expression, suggesting that Activin-A did not promote M2-polarization. Moreover, while anti-CSF1R-mediated macrophage depletion in did not alter B16.OVA-Ctrl tumor growth, it tended to accelerate rather than slow B16.OVA-βA tumorigenesis. This surprising result suggests that Activin-A signaling promotes anti-tumorigenic functions of macrophages in this model, thus contrasting a mouse model of HPV-induced skin carcinogenesis, where expression of Activin-A induced CCR2-dependent macrophage recruitment and pro-tumorigenic reprogramming (Antsiferova et al., 2017). These observations illustrate the highly context-dependent functions of Activin-A in the regulation of macrophage function. Further experiments will be needed to fully decipher the influence of Activin-A on macrophage function in the context of melanoma progression. Such experiments could be performed in YUMM3.3 and iBIP2 tumor models, where macrophages are highly prevalent. Since anti-CSF1R antibodies inefficiently depleted macrophages in B16.OVA grafts, alternative approaches are needed. PI3Kγ was recently identified as a key inducer of macrophage immuno-suppressive function (Kaneda et al., 2016). Macrophage-specific PI3Kγ signaling resulted in suppression of CD8 T-cell activation in part by stimulating arginase 1 expression. Consequently, PI3Kγ inhibition reduced tumor growth in a CD8 T-cell-dependent manner and synergized with anti-PD1 therapy. Treatment PI3Kγ inhibitors could therefore be an interesting strategy to analyze the functional consequences of Activin-A signaling in the regulation of macrophages in melanoma tumors. Nevertheless, selective manipulation of the myeloid compartment is notoriously difficult *in vivo* due to the lack of specific markers, high cellular plasticity and compensatory hematopoiesis in the bone marrow upon myeloid cell depletion. To circumvent these issues, *in vitro* experiments may be indicated. Notably, T-cell proliferation and killing assays could be performed in presence of selected myeloid populations sorted from $INHBA^+$ or $INHBA^-$ tumors.

In our experiments, the influence of Activin-A on Treg induction was only explored in B16F1 tumors, where *INHBA* expression did not consistently induce Treg differentiation. This could be explained by a report which suggested that TGF-β is required for the Activin-A-induced conversion of conventional $CD4^+$ T-cells into FoxP3+ Tregs *in vitro* (Huber et al., 2009). Moreover, depletion of $CD4^+$ cells did not alter B16.OVA-βA tumor growth, strongly suggesting that Tregs do not mediate the immuno-suppressive function of Activin-A in B16F1 melanoma.

Beyond immune cells, components of the stroma, and in particular cancer-associated fibroblasts (CAFs) have potent immunoregulatory functions. In homeostasis, fibroblasts are generally quiescent and exert minor metabolic and transcriptomic activity (Kalluri, 2016). Upon activation by inflammatory cytokines, fibroblasts acquire contractile, migratory and proliferative properties and become transcriptionally active to secrete immuno-modulatory cytokines and components of the extracellular matrix (Ziani et al., 2018). The biological function of activated fibroblasts was first observed in wound healing, but it soon became apparent that these cells are also implicated in the pathogenesis of various inflammatory conditions such as fibrosis, but also cancer, which has been elegantly termed as a “wound that does not heal” (Dvorak, 1986). During carcinogenesis, tumor-derived TGF-β plays a central role in the recruitment and activation of CAFs, which in turn regulate innate and adaptive immune responses by secreting

immunosuppressive cytokines and by upregulating programmed cell death ligands (Khalili et al., 2012; Ziani et al., 2018). Activin-A has also been implicated in fibrotic processes of multiple organs, and it stimulates fibroblast proliferation in keloid scars (Mukhopadhyay et al., 2007; Werner and Alzheimer, 2006). Interestingly, Activin-A expression is upregulated in fibroblasts upon activation by TGF-β (Staudacher et al., 2017). In preliminary experiments, immunostaining of B16F1 grafts with the CAF marker αSMA did not reveal overt desmoplasia in tumors expressing *INHBA*. Nevertheless, future investigations are warranted to further investigate a potential role for CAFs in the pro-tumorigenic function of Activin-A.

In summary, our results clearly demonstrate that Activin-A promotes melanoma progression by inhibiting adaptive anti-tumor immunity. Nevertheless, further experiments are needed to elucidate whether Activin-A directly regulates cytotoxic T-cell proliferation and function, or whether this occurs through yet unidentified mediators in the tumor microenvironment.

3.5 Role of Activin-A in the resistance to melanoma therapies

Targeted therapies and immune checkpoint inhibitors have revolutionized the management of advanced-stage melanoma. Unfortunately, the efficacy of these approaches is mitigated by moderate response rates and frequent relapses owing to the manifestation of intrinsic and acquired resistance mechanisms. Our results strongly support a role for Activin-A signaling in the resistance to checkpoint inhibitors in melanoma. Inhibition of endogenous Activin-A by a soluble form of ACVRIIB bound to human Fc (AIIB-Fc) sensitized iBIP2 melanoma tumors to combined PD1 and CTLA4 blockade. This was accompanied by a significant influx of CD8 T-cells in the tumor microenvironment, as well as improved T-cell activation as assessed by intracellular IFNγ staining. Furthermore, analysis of a publicly available dataset indicated that expression of *INHBA* was higher in tumors from melanoma patients whose disease would progress upon anti-PD1 therapy, supporting the clinical relevance of these findings. In parallel, experiments performed using the YUMM3.3 cell line suggest that *INHBA* overexpression may also mediate resistance to ICB in this model. Analysis of Kaplan-Meier survival curves of YUMM3.3-Ctrl and YUMM3.3-βA tumor-bearing mice treated with anti-PD1 and anti-CTLA4 antibodies suggest increased hazard ratios in the context of Activin-A signaling. Nevertheless, as statistical analysis did not reach significant p-values, a verification experiment should be performed to strengthen these observations.

In a previous study, we observed a striking correlation between *INHBA* and *AXL* mRNA expression in melanoma (Donovan et al., 2017). Since the tyrosine kinase receptor AXL contributes to MAPK reactivation and therapy resistance upon treatment with BRAF inhibitors (Müller et al., 2014; Shaffer et al., 2017), we asked if Activin-A signaling contributes to this resistance mechanism. Presence of recombinant Activin-A or its inhibitor Follistatin did not alter the response of two $BRAF^{V600E}$ melanoma cell lines to treatment with the BRAF inhibitor vemurafenib. Moreover, *AXL* mRNA expression was unchanged in these cells upon treatment with Activin-A or Follistatin. These results do not support a role

for autocrine Activin-A signaling in promoting resistance to BRAF inhibitors. Interestingly, AXL expression is strongly induced in macrophages in inflammatory conditions (Myers et al., 2019). It is therefore conceivable that the correlation observed between *INHBA* and *AXL* mRNA expression in melanoma stems from the tumor microenvironment.

3.6 Melanoma-derived Activin-A induces cachexia

Cachexia is a significant co-morbidity in cancer and is thought to be the cause of death in 20-30% of cancer patients (Murphy, 2016). Preventing cancer-associated cachexia has proven difficult, but the recent identification of molecular pathways leading to muscle wasting and cardiac atrophy may allow development of efficient therapeutic agents. Our results are in agreement with an increasing body of evidence suggesting that Activin-A is a potent inducer of cachexia (Chen et al., 2014; Loumaye et al., 2015). *INHBA* expression significantly decreased body weight of B16F1 tumor-bearing mice, and a similar trend was observed with the YUMM3.3 model. Conversely, AIIB-Fc expression partially relieved cachexia in iBIP2 tumor bearing mice. Therapeutic inhibition of Activin-A may therefore provide several benefits, by reverting cancer-associated cachexia, as well as relieving immunosuppressive signals .

3.7 Doxycycline reverts Activin-A-mediated B16F1 tumor progression

One of the most intriguing results of this work was the observation that doxycycline abrogated the tumor-promoting function of Activin-A in the B16F1 model. B16F1-TRE-βA tumors from mice treated with doxycycline grew significantly slower than their control counterparts. This was specifically due to *INHBA* expression, as doxycycline did not affect the growth of cells transduced only with the reverse tetracycline transactivator (rtTA), but potently diminished B16F1-βA tumor growth. Following their development in the early 1990s, tetracycline-inducible expression systems have been widely used in biological research as they provide efficient and reversible transcriptional control of a gene of interest upon exposure to tetracycline or its derivatives. Doxycycline is most commonly used for this purpose since it is functional at low dosage, relatively stable in solution and well tolerated by cultured cells and animals (Krueger et al., 2004). Despite these undeniable advantages, doxycycline can diminish mitochondrial function in cell metabolism, proliferation, and survival, at least at high concentrations (Ahler et al., 2013). Of note, doxycycline may also prevent TGF-β-induced secretion of matrix metalloproteases by inhibiting Smad-pathway activation in a dose-dependent fashion (Kim, 2005; Shin et al., 2016) Further experiments are warranted to identify the precise mechanism by which doxycycline impairs B16F1-βA tumor growth. In the iBIP2 model, which requires continuous doxycycline presence due to its inducible $BRAF^{V600E}$ allele, inhibition of Activin-A reduced tumor growth, suggesting that the effects of doxycycline are likely model-dependent.

3.8 Conclusions and outlook

In this work, we analyzed the role of Activin-A signaling in melanoma progression and its implications for melanoma therapy. Three main question were addressed:

1. By taking advantage of cell lines expressing a constitutively active type I Activin receptor (ALK4), we show that cell-autonomous Activin-A signaling is tumor suppressive, both *in vitro* and *in vivo*. Induction of caALK4 in melanoma cells inhibited cell proliferation and resulted in diminished tumor growth upon injection in syngeneic hosts. Opposingly, we show in gain- and loss-of-function experiment that paracrine Activin-A signaling promotes melanoma growth, but only in immunocompetent mice, suggesting that Activin-A inhibits adaptive anti-tumor immunity.

2. To study how Activin-A promotes melanoma progression, the immune infiltrate of syngeneic grafts was analyzed by multiparameter flow cytometry. These experiments revealed that Activin-A decreases immune infiltration by $CD45^+$ leukocytes and CD8 T-cells within the tumor microenvironment but stimulates dendritic cell function in melanoma. This was accompanied by monocytic myeloid-derived suppressive cells influx upon *INHBA* expression in B16F1 tumors, but the implication of the myeloid compartment in mediating Activin-A-induced immunosuppression was less apparent in YUMM3.3 and iBIP2 tumor models.

3. For the first time, we show that Activin-A mediates resistance to immune checkpoint blockade in melanoma. Whereas combined CTLA4 and PD1 blockade did not control iBIP2 tumor progression, inhibition of Activin-A sensitized iBIP2 grafts to checkpoint inhibitors. Moreover, the clinical relevance of this result was suggested by the observation that tumors of treatment-naïve melanoma patients whose disease would later progress under anti-PD1 therapy expressed elevated *INHBA* mRNA levels.

Activin-A thus emerges as a particularly promising therapeutic target for melanoma therapy, as well as in other cancers.

4. Materials and methods

4.1 Cell lines, recombinant protein treatments & transfection

B16F1, HepG2 and HEK293T cells (ATCC) were maintained in DMEM (Sigma). C8161 cells were provided by Dr. Mary Hendrix and maintained in RPMI (Gibco). YUMMER1.7 were provided by Dr. Marcus Bosenberg and maintained in RPMI (Gibco). YUMM2.1 & YUMM3.3 cells were provided by Dr. Anna Obenauf (Vienna) and maintained in DMEM/F12 (Gibco). iBIP2 cell lines were provided by Mélanie Tichet (Hanahan lab, EPFL) and maintained in RPMI in presence of 1µg/mL doxycycline (Sigma-Aldrich). All media were supplemented with 10% fetal bovine serum, 50 µg/mL gentamicin (Gibco), and 1% GlutaMAX (Gibco). HepG2 reporter cells were established by stable lentiviral transduction of a CAGA-Luc reporter and Renilla luciferase for normalization and have been described previously (Fuerer et al., 2014). When necessary, stable cell lines were maintained under selective pressure with antibiotics at the following doses:

Cell line	Puromycin (µg/mL)	Blasticidin (µg/mL)	G418 (µg/mL)
B16F1	1	5	800
YUMMER1.7	2	2	300
YUMM2.1	2	8	N/A
YUMM3.3	1	3	N/A
iBIP2 8193L (BL6)	4	N/A	N/A
iBIP2 2891L (FVB)	2	4	N/A

When indicated, cells were treated with 20 ng/mL recombinant human/mouse/rat Activin-A, 10 ng/mL recombinant human TGF-β or 250 ng/mL recombinant human Follistatin 300 (all from R&D).

For transfection, $5 \cdot 10^5$ B16F1 cells were seeded in 6-well plates. The next day, cells were incubated with 2µg plasmid DNA in Lipofectamine 3000 (Invitrogen) for 6h in OptiMEM medium (invitrogen). Medium was then replaced with complete DMEM. Cells were routinely tested for mycoplasma infections using Mycoplasma detection kit (Southern biotech).

4.2 Melanoma grafts and *in vivo* antibody injections

B16F1, B16.OVA, YUMM3.3: 10^5 cells were injected intradermally (B16F1 & B16.OVA) or subcutaneously (YUMM3.3) into the right flank of 8-12 weeks old female WT (ENVIGO & Charles River laboratories) or $Rag1^{-/-}$ (gift from Michele De Palma, EPFL) C57BL/6 mice. iBIP2 2891L: 10^5 cells were injected subcutaneously into the right flank of 8-12 weeks old male FVB/N mice (Charles River). Tumors were measured 3 times/week and volume was calculated using the formula $V=\pi/6 \cdot 1.58 \cdot (length \cdot width)^{3/2}$

(Feldman et al., 2009). For αCD4 & αCD8-mediated depletion, treatment was initiated one day before tumor challenge. Mice were intraperitoneally injected with 10mg/kg anti-CD4 (clone YTS191), anti-CD8 (clone YTS169.4) or rat IgG2b (clone LTF-2, all from BioXcell) in 200µL PBS twice weekly. For αGr1-mediated depletion, treatment was initiated 2 days after tumor challenge. Mice were intraperitoneally injected three times a week with 5-12.5 mg/kg anti-Gr1 antibodies (clone RB6-8C5, BioXcell). For αCSF1R-mediated depletion, treatment was initiated 3 days after tumor challenge. Mice were treated once weekly with 30 mg/kg anti-CSF1R antibodies (clone 2G2, gift from Michele De Palma) or mouse IgG1 (clone MOPC-21, BioXcell). For anti-PD1 and anti-CTLA4 pre-clinical studies, mice were intraperitoneally injected every 3 days with 10 mg/kg anti-PD1 (clone RMP1-14), 5 mg/kg anti-CTLA4 (clone 9H10) or 10mg/kg rat IgG2a and 5mg/kg Syrian hamster IgG (all from BioXcell) in 200µL PBS. All procedures were approved by the cantonal veterinary office.

4.3 Expression vectors and cloning

Spe1 fragment of INHBA (NM_002192.2, nucleotides 218-1609, plus myc tag) was subcloned into XbaI-digested pLenti-EF1alpha-MCS-SV40-puro vector. pLenti-EF1alpha-OVA-bsd was generated by replacing GFP by the OVA sequence from pcDNA3-OVA (Addgene #64599) into pLENTI-EF1alpha-GFP-bsd. In brief, the BamHI and MluI restriction sites were flanked on OVA by PCR using the following primers: 5'- CCA GGA TCC TCC TCG AAA GAC AAC TCA GA -3' and 5'- ACA ACG CGT TGC TCT GGG TCT TGT TGG A -3'. BamHI/MluI digested OVA fragment was then inserted in BamHI/MluI digested pLENTI-EF1alpha-GFP-bsd. pLV-EF1alpha-ALK1-Fc-PGKneo was generated by subcloning the extracellular domain of human Alk1 fused to Fc-gamma portion of human IgG (gift from Peter ten Dijke) into pLV-EF1alpha-mcs-PGKneo using EcoRI digestions. pLenti-EF1alpha-ActA-SV40-bsd was generated by subcloning INHBA from pLenti EF1a-MycActA SV40puro into pLenti EF1a-OVA bsd. In brief, a BglII site was flanked on the 5'- end of INHBA using the following primers: 5'- GGG GAG ATC TAA GGC AAT CAC AAC AAC TTT TGC -3' and 5'- CAC AGG GTC GAC CAC TGG TC -3'. The resulting fragment was digested with BglII/MluI and inserted into BglII/MluI-digested pLenti EF1a-OVA bsd. pLenti-EF1alpha-AIIB-Fc-SV40-bsd and pLenti-EF1alpha-Mock-Fc-SV40-bsd were cloned using the same strategy, but using the following primers: 5'- GGG GAG ATC TGC CTC GAG AAT TGC TTC CAC -3' and 5'- GGG TCG ACC ACT GGT CGA C -3'.

4.4 Lentiviral transduction

To generate lentiviral particles, $8 \cdot 10^6$ HEK293T cells were seeded 24h before transfection in 15-cm plates in 22 mL complete DMEM. 2h before transfection, medium was replaced with fresh DMEM. Cells were transfected using 9µg pMD2.VSVG, 16µg pCMVΔR8.74 and 30µg transfer plasmid using calcium phosphate precipitation in HEPES buffered saline (HBS) buffer. Medium was replaced 16h later by fresh complete DMEM buffer. Supernatant was collected after 24h and 40h, filtered using a 22µm membrane (Milipore). Lentiviral particles were concentrated by ultracentrifugation at 22'000 rpm for 2h20 using an S32 Ti rotor (Beckman). Supernatant was discarded and pellet was resuspended in 50µL PBS. $1 \cdot 10^5$

target cells were transduced in 16-well plates using 10µL concentrated viral supernatant. Antibiotics selection was started 48h after transduction.

4.5 Western blot analysis

Cells were lysed in homemade RIPA buffer supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitors when necessary (Sigma-Aldrich). Conditioned medium was precipitated using ice-cold acetone and resuspended in RIPA buffer. Protein concentration was measured by BCA assay (Thermo Fisher Scientific). Proteins were separated on 10% SDS-PAGE gels under reducing conditions and transferred on nitrocellulose membranes. After membrane blocking with 5% skim milk (Sigma) in Tris-buffered saline + 0.1% Tween-20, proteins were stained overnight at 4°C with primary antibodies against HA-tag (Sigma H-6908), SMAD2 (Cell Signaling 3122), pSMAD2/3 (Cell Signaling 8828), cleaved Caspase3 (Cell signaling 9664) and γ-tubulin (Sigma GTU88) at 1:1000 dilutions. Proteins from cell lysates were revealed using secondary antibodies fluorescently coupled with IRDye 800CW and IRDye 680RD on an Odyssey CLx instrument (all from Licor). Proteins from conditioned media were revealed on X-ray film (Kodak) using HRP-coupled secondary antibodies and ECL reagents (Thermo Fisher).

4.6 qPCR analysis

Cells cultured in 6-well plates were lysed in 700µL QIAzol (Qiagen) followed by chloroform extraction. Total RNA was isolated using RNeasy mini kit according to manufacturer's protocol. cDNA was synthesized from 2.5µg RNA using SuperScript III transcriptase (Invitrogen) and 200ng random hexamers as primers. Analysis was performed using SYBR Green GoTaq Master Mix (Promega) on a QuantStudio 6 instrument (Applied Biosystems). qPCR primers are listed in Table 6.

4.7 CAGA-Luciferase assay

$1 \cdot 10^5$ HepG2-CAGA reporter cells were seeded into 24-well plates in conditioned medium and cultured for 24 hours. Cells were lysed in 100µL 10mM potassium buffer containing 0.2% Triton-X100. 5µL of lysate were transferred into white 96-well plates (nunc). Firefly and renilla luciferase luminescence were measured using a Centro LB960 luminometer by addition of 50µL P/R A (Firefly) and P/R B (Renilla) reagents (Hampf and Gossen, 2006). Firefly luciferase measurements were normalized using renilla values.

4.8 Whole mount immunofluorescent staining

Tumors were fixed with 4% PFA in PBS overnight, cryoprotected with 20-30% sucrose and embedded in OCT. When needed, mice were intraperitoneally injected with 60mg/kg pimonidazole (Hypoxyprobe) 45 minutes before sacrifice. For CD31 and pimonidazole double staining, free-floating 200µm sections were washed for 1h in PBS, digested using 20µg/mL proteinase K in TE buffer and permeabilized in 100% MetOH at room temperature for 30min. After washing, sections were blocked overnight in 3% milk + 0.3% Triton X-100 in PBS overnight at 4°C. After washing, sections were stained with α-mouse CD31 (1:300, Clone MEC13.3, BD Pharmingen) and α-pimonidazole (Hypoxyprobe) antibodies for 2h at 4°C. After washing, sections were incubated with α-rat biotinylated (1:200, EPFL HCF #30), followed by Streptavidin-Alexa647 (1:800, EPFL HCF #68). Images were acquired by Z-stack reconstitutions of whole sections using a Zeiss LSM700 confocal microscope and quantified using ImageJ. For CD45, CD8, cleaved Caspase 3 and Ki67 immunostainings, 8µm cryosections were washed in PBS for 10 min, fixed and permeabilized with MetOH at -20°C for 20 minutes and blocked with 5% BSA + FcR block (1:500, Miltenyi) in PBS for 1h at RT. Sections were stained overnight with αCD8 (1:100, BD Horizon), αCD45 (1:100, eBioscience), αcCas3 (1:800, Cell Signaling) or Ki67 (1:400, Abcam) antibodies in 5% BSA in PBS. After washing, sections were stained with conjugated secondary antibodies (EPFL HCF) and counterstained with DAPI (1:10'000). Whole section images were acquired using a Leica DM5500 microscope and quantified using ImageJ.

4.9 Cell viability assay

$5 \cdot 10^3$ cells were seeded into 96-well plates and cultured for 3 days in 100µL complete medium. 10µL AlamarBlue reagent (Invitrogen) were added to wells 3-4h before fluorescent measurement at 560nm (excitation) and 590nm (emission). Viability was calculated by normalizing vs ctrl values.

4.10 Cell cycle analysis

$3 \cdot 10^5$ cells were seeded into 6-well plates and cultured in presence of 1µg/mL doxycycline for 24-72h. 5-ethynyl-2'-deoxyuridine (EdU) was added to cells for 30 minute at 10µM concentration. Cells were harvested, washed with 2% FBS in PBS and fixed with 4% PFA for 20 minutes. After washing, cells were permeabilized with 0.5% Triton X-100 in PBS. After washing, cells were incubated for 30 minutes in a solution containing $CuSO_4$, fluorescent azide and sodium ascorbate (FCCF, EPFL). After washing, cells were stained with DAPI and acquired using a Cyan-ADP flow cytometer.

4.11 AnnexinV/PI analysis

$3 \cdot 10^5$ cells were seeded into 6-well plates and cultured in presence of 1µg/mL doxycycline for 0-48h. Cells were stained using Annexin V apoptosis detection kit (BioLegend) according to manufacturer's protocol. In brief, cells were washed and resuspended in Annexin V binding buffer, stained with Annexin V FITC and propidium iodide for 15 minutes at room temperature. Cells were then analyzed using a Cyan-ADP flow cytometer.

4.12 Staining of dissociated tumors and analysis by flow cytometry

Tumors were dissected, minced using rounded scissors and digested in DNase I (0.02 mg/mL, Sigma) and collagenase (1mg/mL, Sigma) in RPMI using a gentleMACS Octo Dissociator (Miltenyi). Red blood cells were lysed using PharmLyse buffer (BD Biosciences). After washing in PBS, cells were counted and $1 \cdot 10^6$-$5 \cdot 10^6$ cells were used for staining. Cells were incubated with mouse FcR blocking solution (1:200, Miltenyi) and Live/Dead fixable blue dead cell stain (1:1000, Life Technologies) for 30 minutes. After washing, cells were stained for surface marker for 45 minutes in FACS buffer (2% FBS, 2mM EDTA in PBS). When needed, cells were fixed and permeabilized using the FoxP3 staining buffer set (eBioscience) before intracellular staining for 45 minutes. Cells were washed in FACS buffer and data was acquired using an LSRII flow cytometer (Becton Dickinson). When necessary, 2µL MHC-I SIINFEKL dextramers (Immudex) were added before antibody staining. MHC-I SIYRYYGL dextramer was used as staining control. Antibodies used for staining are listed below in Table 7.

4.13 Intracellular cytokine staining

After tumor dissociation, cells were counted and $1 \cdot 10^6$ cells were plated in complete RPMI medium and incubated for 6h in presence of PMA (20ng/mL), ionomycin (1µg/mL) and monensin. Cells were then washed and stained for surface markers, followed by fixation and permeabilization using FoxP3 staining buffer set (eBioscience) before intracellular staining. Cells were washed in FACS buffer and data was acquired using an LSRII flow cytometer (Becton Dickinson). Antibodies used for staining are listed below in Table 7.

4.14 Statistical analysis

Statistical tests were performed using the Prism software (GraphPad). Unless indicated, data represent mean±SEM of at least 2 independent experiments. When comparing two groups, normal distributions were analyzed by Shapiro-Wilk normality test, and p-values calculated by Student's t-test (normal

distribution) or Mann-Whitney's test (non-parametric test). One-way ANOVA was used to compare several groups of unpaired values. Kaplan-Meier survival curves were analyzed using the Gehan-Breslow-Wilcoxon test.

Table 6: Primers used for qRT-PCR analysis

Primer	Species	Sequence
AXL fw	Mouse	GGT GTT TGA GCC AAC CGT GGA A
AXL rev	Mouse	GCC ACC TTA TGC CGA TCT ACC A
VEGF fw	Mouse	GCA CAT AGG AGA GAT GAG CTT CC
VEGF rev	Mouse	CTC CGC TCT GAA CAA GGC T
Perforin fw	Mouse	ACA CAG TAG AGT GTC GCA TGT AC
Perforin rev	Mouse	GTG GAG CTG TTA AAG TTG CGG G
Granzyme B fw	Mouse	CAG GAG AAG ACC CAG CAA GTC A
Granzyme B rev	Mouse	CTC ACA GCT CTA GTC CTC TTG G
GAPDH fw	Mouse	ACT GAG GAC CAG GTT GTC TCC
GAPDH rev	Mouse	GTT GGG ATA GGG CCT CTC TTG C

Table 7: List of flow cytometry antibodies

Reagent	Source	Clone	Reference	Dilution
CD3 PE	BioLegend	145-2C11	100308	1:200
CD3e PE-Cy5.5	eBioscience	145-2C11	35-0031-82	1:200
CD3 APC-eFluor780	eBioscience	500A2	47-0033-82	1:200
CD4 BV785	BioLegend	RM4-5	100552	1:200
CD4 PE-Cy7	eBioscience	GK1.5	25-0041-82	1:200
CD8 BV510	BD Horizon	53-6.7	563068	1:200
CD11b BV711	BioLegend	M1/70	101241	1:200
CD11c BV421	BioLegend	N418	117330	1:200
CD19 BV510	BioLegend	6D5	115545	1:200
CD45 APC	eBioscience	30-F11	17-0451-82	1:200
CD45 PerCP	BioLegend	30-F11	103129	1:200
CD45.2 BUV737	BD Horizon	104	564880	1:200
CD45R (B220) APC	Miltenyi	RA3-6B2	130-102-259	1:200
CD80	Miltenyi	16-10A1	130-102-372	1:200
CD103 PE	eBioscience	2.00E+07	12-1031-82	1:200
CD206 FITC	BioLegend	C068C2	141703	1:200
F4/80 BV605	BioLegend	BM8	123133	1:200
FoxP3 PE-eFluor610	eBioscience	FJK-16s	61-5773-82	1:100
GranzymeB AlexaFluor647	BioLegend	GB11	515406	1:50
H2Kb FITC	BioLegend	AF6-88.5	116505	1:200
H2Kb-SIINFEKL APC	BioLegend	25-D1.16	141605	1:200
IA/IE (MHC-II) AlexaFluor700	BioLegend	M5/114.15.2	107622	1:600
IA/IE (MHC-II) APC-Cy7	BioLegend	M5/114.15.2	107627	1:600
IFNγ PE	BioLegend	XMG1.2	505808	1:100
Ki67 eFluor450	Invitrogen	SolA15	48-5698-80	1:200
Ly6C PerCP/Cy5.5	BioLegend	HK1.4	128012	1:400
Ly6C AlexaFluor700	BioLegend	HK1.4	128023	1:400
Ly6G FITC	BioLegend	1A8	127605	1:200
Ly6G PE	BioLegend	1A8	127607	1:200
NK1.1 BV650	BioLegend	PK136	108735	1:200
TNFα PE-Cy7	BioLegend	MP6-XT22	506323	1:200

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Annexes

Paracrine Activin-A Signaling Promotes Melanoma Growth and Metastasis through Immune Evasion

ORIGINAL ARTICLE

Paracrine Activin-A Signaling Promotes Melanoma Growth and Metastasis through Immune Evasion

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The secreted growth factor Activin-A of the transforming growth factor β family and its receptors can promote or inhibit several cancer hallmarks including tumor cell proliferation and differentiation, vascularization, lymphangiogenesis and inflammation. However, a role in immune evasion and its relationship with tumor-induced muscle wasting and tumor vascularization, and the relative contributions of autocrine versus paracrine Activin signaling remain to be evaluated. To address this, we compared the effects of truncated soluble Activin receptor IIB as a ligand trap, or constitutively active mutant type IB receptor versus secreted Activin-A or the related ligand Nodal in mouse and human melanoma cell lines and tumor grafts. We found that although cell-autonomous receptor activation arrested tumor cell proliferation, Activin-A secretion stimulated melanoma cell dedifferentiation and tumor vascularization by functional blood vessels, and it increased primary and metastatic tumor burden and muscle wasting. Importantly, in mice with impaired adaptive immunity, the tumor-promoting effect of Activin-A was lost despite sustained vascularization and cachexia, suggesting that Activin-A promotes melanoma progression by inhibiting antitumor immunity. Paracrine Activin-A signaling emerges as a potential target for personalized therapies, both to reduce cachexia and to enhance the efficacy of immunotherapies.

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INTRODUCTION

In melanoma research, targeted inhibitors and immune checkpoint therapies are available, but their efficacies remain limited by acquired and intrinsic drug resistance mechanisms (Lau et al., 2016). Patients with resistant melanoma have a poor prognosis due to high probability of metastasis, correlating with progression from superficial spreading to invasive vertical growth. Tumor-initiating potential and metastasis also correlate with low immunogenic profiles of subpopulations of cells and phenotypic plasticity marked by pseudoepithelial-to-mesenchymal transitions and the ability to reversibly switch between proliferative and invasive gene signatures (Caramel et al., 2013; Hoek et al., 2008; Schatton and Frank, 2009; Widmer et al., 2012). Thus, elucidating mechanisms of tumor immune evasion and their coupling to cancer cell plasticity is critical to develop effective immunotherapies.

Known local cues in the tumor microenvironment that regulate melanoma cell plasticity and antitumor immunity include transforming growth factor β (TGFβ). In many tumors including melanoma, TGFβ facilitates or inhibits tumor progression, depending on the context (Bellomo et al., 2016; Perrot et al., 2013). In normal melanocytes, TGFβ induces cell cycle arrest and apoptosis (Alanko and Saksela, 2000; Rodeck et al., 1991), but this response is attenuated in cells from benign nevi despite sustained activation of downstream SMAD2,3 transcription factors (Rodeck et al., 1999). SMAD-binding sites in the *PAX3* gene mediating repression of pigment synthesis likely contribute to melanoma cell plasticity and phenotype switching (Pinner et al., 2009; Yang et al., 2008). TGFβ immunostaining was found to correlate with the invasive vertical growth phase and metastasis (Van Belle et al., 1996), and blockade of downstream SMAD2,3 transcription factors by overexpression of antagonistic SMAD7 in 1205Lu melanoma cells inhibited tumorigenicity and bone metastasis (Javelaud et al., 2005, 2007). In metastatic B16F10 mouse melanoma, TGFβ also reduces natural killer cell-mediated tumor rejection while promoting the differentiation of immunosuppressive Foxp3+ regulatory T cells in tumor beds and draining lymph nodes (Chen et al., 2003; Gorelik and Flavell, 2001; Turk et al., 2004). However, the functionally relevant active TGFβ in this model is provided by immature myeloid dendritic cells in draining lymph nodes, likely because B16F10 and other melanoma cells themselves secrete TGFβ only in latent form (Ghiringhelli et al., 2005; Yin et al., 2012).

Melanoma cell lines and tumors also frequently express Activin-A (Heinz et al., 2015; Hoek et al., 2006). Activin-A

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Abbreviations: caALK4, constitutively active mutant Activin receptor-like kinase 4; FST, Follistatin; INHβA, Inhibin βA; TGFβ, transforming growth factor β

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stimulates the same SMAD transcription factors as TGFβ, even though it derives from distinct precursor dimers encoded by the *Inhibin βA* (*INHβA*) gene and binds distinct complexes of the Activin/Nodal type I and II receptors Acvr1b/ALK4 or Acvr1c/ALK7 and Acvr2 (reviewed in Hedger et al., 2011; Sozzani and Musso, 2011; Walton et al., 2011). Activin-A can mediate protective or tumorigenic effects (reviewed in Loomans and Andl, 2014). For example in mouse models of pancreatic cancer, Activin-A promotes tumor progression by inhibiting cell differentiation (Lonardo et al., 2011; Togashi et al., 2015). Paradoxically, its receptor ALK4 (ACVR1B) primarily mediates tumor suppressive functions and is frequently deleted in clinical samples (Qiu et al., 2016; Togashi et al., 2014). Cytostatic and proapoptotic signaling by Activin-A has also been reported in human melanoma cell lines, although this activity is counteracted by the secreted antagonist Follistatin (FST-1) (Stove et al., 2004). Immunohistochemical analysis detected Activin-A staining in superficially spreading melanoma, whereas benign nevi and metastatic lesions showed elevated expression of FST (Heinz et al., 2015). Interestingly, however, gain of transgenic Activin-A expression in the A375 human melanoma xenograft model did not alter tumor growth or metastasis, even though it reduced tumor lymphangiogenesis, a risk factor of poor prognosis (Heinz et al., 2015). Also in xenograft models of other cancers, overexpression of a dominant negative mutant receptor revealed no adverse functions for Activin-A or related ligands, with the exception of tumor-induced systemic weight loss (cachexia) through remote Smad2,3 activation in skeletal muscles (Li et al., 2007; Zhou et al., 2010).

Here, we asked whether a potential tumorigenic role for Activin signaling in melanoma may be curtailed in xenografts by the absence of a functional immune system. To address this, we monitored the expression of *INHβA* in public datasets and in a panel of melanoma tumors and cell lines, and we performed loss- and gain-of-function studies, respectively, in human xenografts and in the moderately metastatic syngeneic B16F1 mouse melanoma model using dominant negative or ligand-independent mutant Activin/Nodal receptors or Activin-A lentiviral transgenes. Our comparison of melanoma grafts in tumor hosts of different genetic backgrounds reveals a protumorigenic and prometastatic function specifically for paracrine Activin-A signaling acting on adaptive immunity, and that this function can be uncoupled from immune-independent anorexic and proangiogenic effects and from autocrine effects on melanoma cell differentiation. Our findings suggest that gain of Activin-A expression in human melanoma should be considered as a potential target for improved immunotherapies.

RESULTS

Human melanoma and other skin cancers frequently express Activin-A rather than NODAL

To assess potential roles of *INHβA* in melanoma, we queried a gene expression profiling dataset of 42 primary cutaneous tumors (Riker et al., 2008). We found that *INHβA* expression was significantly elevated both in primary cutaneous cancers and in metastatic melanoma, whereas the antagonist FST tended to decrease in melanoma in situ ($n = 2$, not significant) compared with normal skin. In contrast to *INHβA*, other Activin receptor ligands encoded by *NODAL* or *GDF3* were neither upregulated in this dataset nor in a TCGA collection of 474 skin cutaneous melanomas (Figure 1a, Supplementary Figure S1 online). Elevated *INHβA* expression significantly clustered with a standard gene expression signature of invasive rather than proliferative melanoma cell lines as determined by Heuristic Online Phenotype Prediction analysis (Figure 1b) (Widmer et al., 2012). Furthermore, although RT-PCR analysis confirmed *INHβA* expression in more than half of a panel of 20 human melanoma cell lines, the related ligands encoded by *INHβB* or *NODAL* were rarely transcribed or undetectable, respectively (Figure 1c, Supplementary Figure S2 online). Although survival did not correlate with either *INHβA* or *NODAL*, low expression of the Activin antagonist *Follistatin* in the TCGA dataset was associated with worse outcome (Supplementary Figure S3 online). These results are consistent with a potential role for Activin-A signaling in melanoma progression.

Inhibition of endogenous ActRIIB ligands in human C8161 melanoma cells inhibits cachexia but not tumor progression in immunocompromised nude mice

Previous analysis of Activin-A functions in tumor xenograft models uncovered potent anorexic activity but no important roles in tumor progression (Heinz et al., 2015; Zhou et al., 2010). Instead, increased aggressiveness in human melanoma has been attributed to a secreted Nodal-like activity that was initially detected by injecting C8161 melanoma cells into zebrafish embryos (Topczewska et al., 2006). Because we observed no *NODAL* expression in human melanoma, we decided to reassess how C8161 cells stimulate Nodal/Activin receptors in zebrafish. To address this, we grafted C8161 cells into wild-type or mutant zebrafish embryos lacking maternal and zygotic expression of the Nodal coreceptor *one-eyed pinhead* (MZ*oep*) (Gritsman et al., 1999). To validate that human NODAL signaling requires Oep in zebrafish, we injected 50 pg of mRNA encoding human Nodal at the one-cell stage and monitored induction of the Smad2,3 target gene *gsc* during early gastrulation. We found that both human Nodal and the zebrafish homolog Squint were active in wild-type but not in MZ*oep*, whereas human Activin-A induced *gsc* independently of Oep (Supplementary Figure S4a online). Moreover, both wild-type and MZ*oep* embryos ectopically upregulated *gsc* as well as *ntl* around grafted C8161 melanoma cells (Supplementary Figure S4b). These results strongly argue for secreted Activin-A and against Nodal as the mediator of Smad2,3 signaling secreted by C8161 melanoma grafts in zebrafish.

To test whether melanoma may grow faster when forced to express Nodal, C8161 cells transduced with Nodal lentivirus (Supplementary Figure S2d) were grafted subcutaneously into FoxN1$^{nu/nu}$ mice. C8161 xenograft tumor growth was not increased by Nodal compared with cells transduced with empty vector, indicating that it was not limited by the lack of Nodal expression (Supplementary Figure S4e and f). To assess the function of endogenous ligands in melanoma xenografts, we transduced human C8161 cells with lentivirus expressing an Fc fusion of Activin type IIB receptor extracellular domain (AIIB-Fc) or Fc alone. Lentivirally transduced AIIB-Fc inhibited

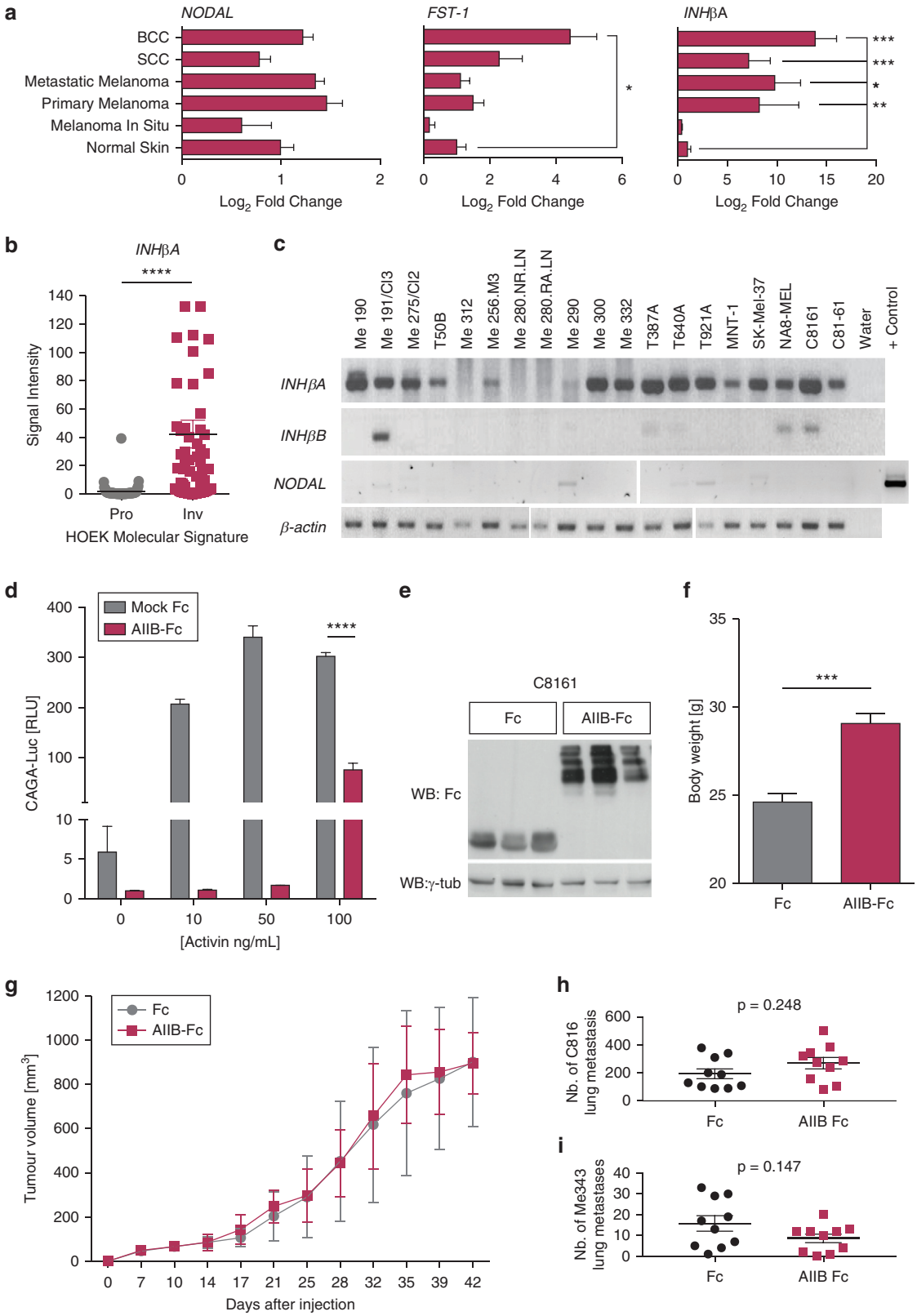

Figure 1. Cachexia induced by endogenous Activin-A in human melanoma xenografts does not promote tumor progression. (**a**) Expression of *NODAL*, Follistatin (*FST-1*), and *INHβA* (encoding Activin-A) mRNAs in normal skin compared with 42 primary cutaneous tumors comprising 14 melanoma, 11 squamous cell, 15 basal cell skin cancers, and 40 melanoma metastases (Riker et al., 2008). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (**b**) Relative *INHβA* mRNA levels in 536 human melanoma cell lines distinguished by proliferative (Pro) or invasive (Inv) gene expression signatures (Widmer et al., 2012). $^{****}P < 0.0001$. (**c**) RT-PCR analysis of the indicated mRNAs in total RNA from human melanoma cell lines or fetal brain (+Control). (**d**) Normalized expression of the Smad3 luciferase reporter CAGA-Luc in HEK293T cells treated with the indicated concentration of Activin-A or with conditioned media of C8161 melanoma cells expressing lentiviral AIIB-Fc or Fc alone (mock). Data show the average fold change of three experiments ± SD, $^{****}P < 0.0001$ at all concentrations.

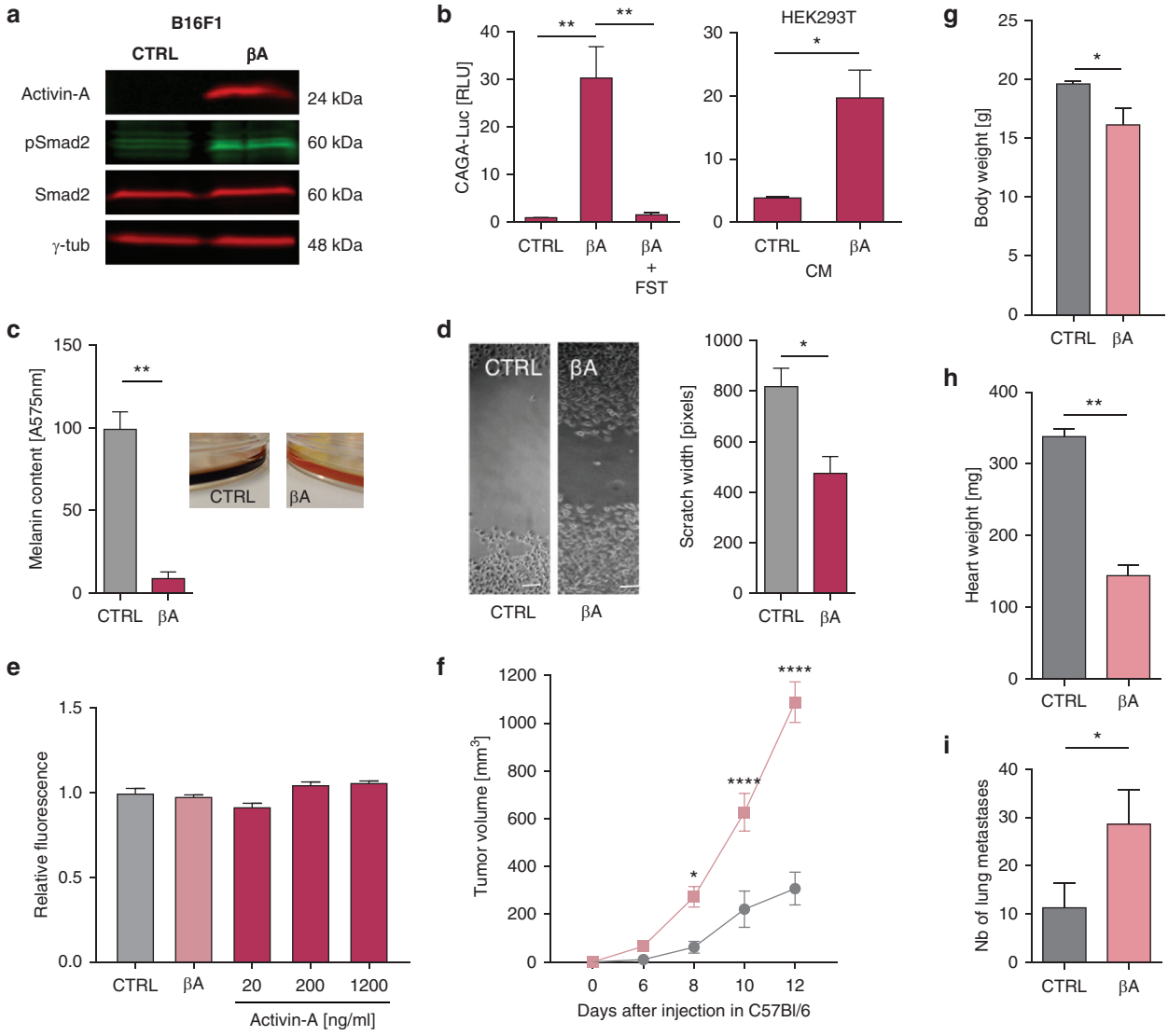

Figure 2. Gain of Activin-A expression stimulates phenotype switching and syngeneic tumor growth in B16F1 mouse melanoma. (**a**) Western blot of secreted Activin-A and (**b**) CAGA-Luc expression in B16F1:GFP cells (left panel) transduced with *INHβA* (βA) or control lentivirus (CTRL). Where indicated, cells were treated with Follistatin (FST, 300 ng/ml). CAGA-Luc expression in HEK293T cells treated with conditioned media of B16:GFP.βA or B16:GFP.CTRL cells is also shown (right panel in **b**). Data represent mean ± SEM of three experiments; $*P < 0.05$, $**P < 0.01$. (**c**) Light absorbance by pigment in medium of transduced B16F1:GFP cells (mean ± SEM, n = 3; $**P < 0.01$). (**d**) Representative micrographs and widths of scratch wounds 20 hours after wounding of cell monolayers (mean ± SEM from three experiments, $*P < 0.05$). (**e**) Alamar blue quantification of live B16F1 cells transduced with control or *INHβA* lentivirus, or treated with the indicated concentrations of Activin-A for 48 hours (mean ± SEM, n = 3, no significant differences). (**f**) Growth curves of B16F1:GFP syngeneic intradermal grafts with Activin-A (βA) or without (CTRL) in C57BL/6 mice, and (**g**) body and (**h**) heart weights at the time of tumor resection. Data represent mean ± SEM of 20 animals/group; $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$. (**i**) Lung metastases of tail-vein injected B16F1:GFP βA or CTRL cells (n = 15 each). Data represent mean ± SEM of three experiments; $*P < 0.05$. GFP, green fluorescent protein; INHβA, Inhibin βA; SEM, standard error on the mean.

endogenous Activin activity in the conditioned medium of C8161 cells in vitro and was readily detected by immunoblotting in C8161 tumor xenografts (Figure 1d and e). As expected, expression of AIIB-Fc in C8161 xenografts significantly protected FoxN1$^{nu/nu}$ hosts against loss of body weight and muscle mass (Figure 1f). However, despite potent systemic inhibition of cachexia, soluble AIIB-Fc receptor neither diminished intradermal tumor growth nor experimental lung metastases after tail vein injection (Figure 1g and h). Additionally, secondary tumor outgrowths after resection of the primary C8161 graft and spontaneous lung or lymph node metastases were not significantly inhibited (Supplementary Figure S5a–c online), nor did AIIB-Fc transduction inhibit experimental lung metastases of

(**e**) Western blot analysis of lentiviral AIIB-Fc or Fc alone in intradermal C8161 melanoma xenograft tumors, and (**f**) body weights of Foxn1nu/nu hosts at the time of tumor resection ($***P < 0.01$). (**g**) Growth curves of C8161 melanoma were unaffected by AIIB-Fc expression. (**h**) Number of superficial lung metastases of C8161-Fc (MockFc) or C8161-AIIBFc melanoma cells 4 weeks after injection into the tail vein of Foxn1$^{nu/nu}$ mice (n = 10 per group, $P = 0.25$, two-tailed Mann-Whitney). (**i**) As in (**h**), but using human Me343 melanoma cells (n = 10 per group, $P = 0.14$). INHβA, Inhibin βA; SD, standard deviation.

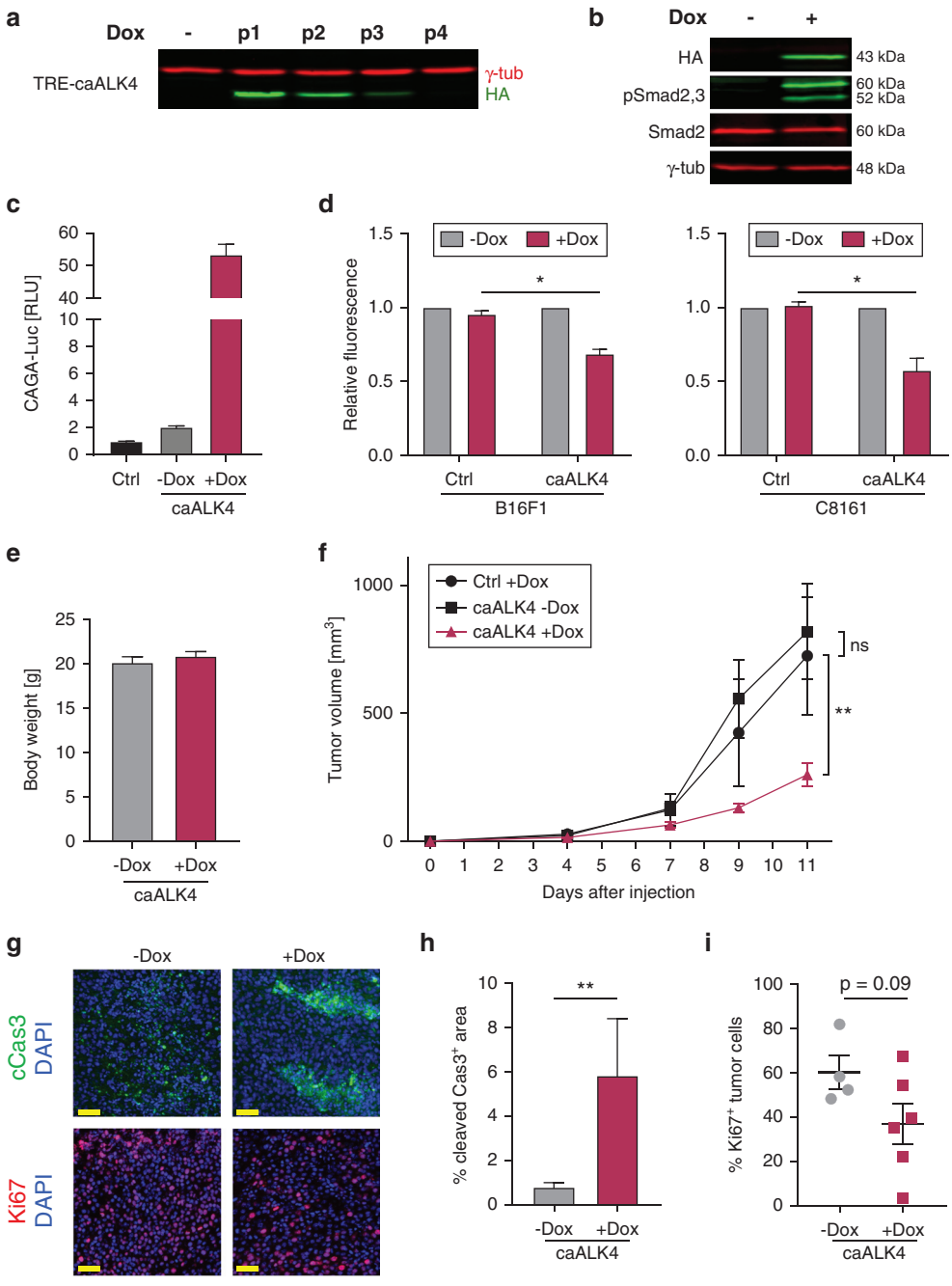

Figure 3. Sustained autocrine Activin receptor signaling inhibits B16F1 cell survival and tumorigenesis rather than stimulating it. (**a, b**) Anti-HA and phospho-Smad2,3 Western blot of B16F1 cells treated with doxycycline during (**a**) 4 cell passages or (**b**) 24 hours after lentiviral infection of inducible HA-tagged caALK4 (rtTA-caALK4). (**c**) CAGA-Luc expression in B16F1 (Ctrl) or B16F1 rtTA-caALK4 cells ± doxycycline for 24 hours. Data represent mean ± SEM of three experiments. (**d**) Alamar blue assay of B16F1 or C8161 (Ctrl) and B16F1 rtTA-caALK4 or C8161 rtTA-caALK4 cells cultured for 3 days in medium with or without doxycycline. Data represent mean ± SEM of two experiments, $*P < 0.05$. (**e**) Body weights of C57BL/6 hosts at the time of tumor resection after feeding with or without doxycycline, and (**f**) growth curves of intradermal syngeneic grafts of B16F1 rtTA (Ctrl, n = 15) and B16F1 rtTA-caALK4 cells (n = 15). Data represent mean ± SEM of three experiments, $**P < 0.01$. (**g**) Cleaved Caspase-3 (cCas3) and Ki67 immunofluorescence staining of syngeneic B16F1 tumors expressing caALK4 (+Dox) or not (−Dox). Scale bar: 50 μm. Corresponding whole-section images are shown in Supplementary Figure S7f. (**h**) Quantification of cCas3+ area from entire sections revealed increased apoptotic areas in +Dox (n = 6) compared with −Dox tumors (n = 5), $**P < 0.01$. (**i**) Flow cytometry of proliferative (Ki67+) cells in syngeneic B16F1 tumors expressing caALK4 (+Dox) or not (−Dox). A trend for decreased proliferation in caALK4 tumors (n = 6) compared with controls (n = 4) was not significant ($P = 0.09$). caALK4, constitutively active mutant Activin receptor-like kinase 4; Dox: doxycycline; HA, human influenza hemagglutinin; SEM, standard error on the mean.

Me343 cells that also express *INHβA* (Figure 1i, Supplementary Figure S2g). These results suggest that C8161 xenografts grow and metastasize independently of Activin signaling and of associated systemic cachexia, possibly because of the lack of functional adaptive immunity.

Activin-A gain of function promotes phenotype switching of mouse melanoma cells and tumor progression

To evaluate potential tumorigenic or prometastatic effects of Activin-A in immunocompetent hosts, we transduced *INHβA* in the moderately metastatic syngeneic B16F1 mouse melanoma model (Fidler, 1973) using lentiviruses for green fluorescent protein alone (CTRL) or green fluorescent protein together with Activin-A (INHβA). Western blot analysis of B16F1-conditioned medium and cell lysates confirmed Activin-A secretion that increased the accumulation of pSmad2 compared with CTRL cells that do not secrete Activin-A (Figure 2a). Transfection of the SMAD3 luciferase reporter CAGA-Luc showed elevated autocrine Activin-A signaling in *INHβA*- compared with CTRL-transduced B16F1 cells, and this increase was blocked on treatment with FST (Figure 2b, left panel). Conditioned medium from *INHβA*-transduced B16F1 cells also stimulated the expression of transfected CAGA-Luc in HEK293T reporter cells (Figure 2b, right panel). Furthermore, gain of Activin-A expression in B16F1 cells stimulated their migration in a scratch wound assay and markedly reduced their melanin content without altering their cell proliferation or viability (Figure 2c–e),

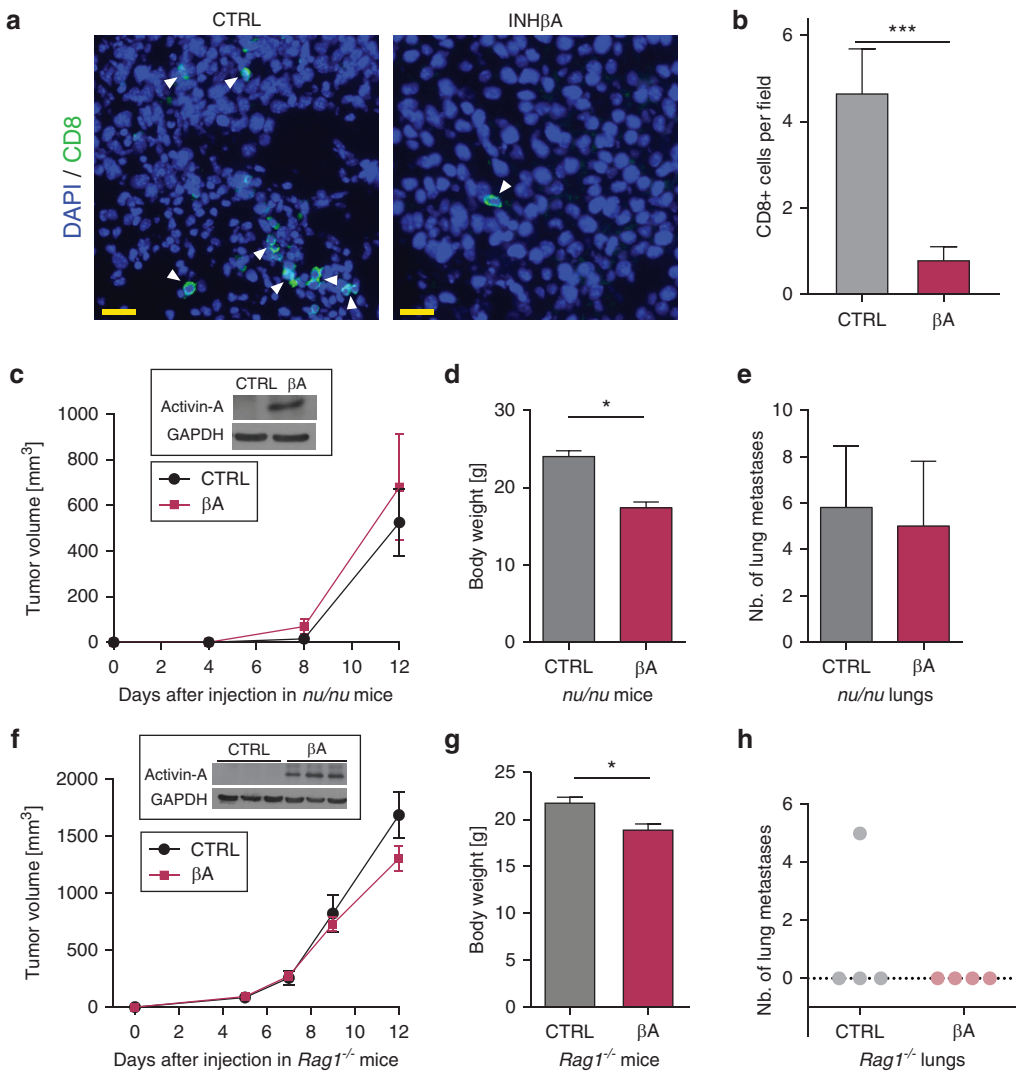

Figure 4. Tumorigenic activity of paracrine Activin-A signaling is mediated by the adaptive immune system. (**a**) CD8 immunostaining (green) of B16F1:GFP CTRL (n = 4) or βA (n = 3) syngeneic intradermal grafts counterstained with DAPI (blue). Arrowheads point to cells positive for CD8 staining. Scale bars: 25 μm. (**b**) Quantification of tumor-infiltrating lymphocytes (mean ± SEM, ***$P <$ 0.001). Cells positive for CD8 staining were counted in 1,000 pixel-wide fields. (**c**) Growth curves of CTRL or βA-expressing B16F1:GFP tumors (n = 5 each) after intradermal injection in the right flank of Foxn1$^{nu/nu}$ mice. Inset: Western blot of Activin-A in randomly chosen tumor extracts at the experimental endpoint. GAPDH served as loading control. (**d**) Body weights of the hosts of the tumors shown in (a) (mean ± SEM, *$P < 0.05$). (**e**) Experimental lung metastases observed after tail vein injection of CTRL or βA-expressing B16F1:GFP (n = 10 each) in Foxn1$^{nu/nu}$ mice. (**f**) Volumes of intradermal CTRL- (n = 4) or βA-B16F1:GFP tumor grafts (n = 3) in the right flank of *Rag1*$^{-/-}$ mice. Western blots were as in panel (**a**). (**g**) Body weight at the time of tumor resection. *Rag1*$^{-/-}$ mice injected with βA expressing B16F1:GFP develop cachexia (n = 4) compared with CTRL injected animals (n = 4). Data represent mean ± SEM; *$P < 0.05$. (**h**) Numbers of experimental lung metastases after tail vein injection of B16F1:GFP ± CTRL (n = 4) or βA (n = 4) in *Rag1*$^{-/-}$ mice. GAPDH, glyceraldehyde-3-phosphate dehydrogenase, GFP, green fluorescent protein; INHβA, Inhibin βA; SEM, standard error on the mean.

consistent with a potential autocrine function in promoting a phenotypic switch.

To determine if the oncogenic effects of Activin-A gain of function may promote tumor growth in vivo, B16F1 grafts were intradermally inoculated on the right flank of 8- to 10-week-old female C57BL/6 mice. Mice injected with *INHβA*-transduced cells grew significantly larger tumors, combined with loss of body weight and cardiac muscle wasting (Figure 2f–h). Compared with vector control, *INHβA* also increased the number of experimental lung metastases formed by tumor cells that were injected into the tail vein (Figure 2i). Taken together, these results show that Activin-A signaling can promote melanoma growth and metastasis in immunocompetent hosts.

Sustained cell autonomous ALK4 signaling inhibits B16F1 tumorigenesis rather than stimulating it

To validate whether the tumorigenic function of Activin-A involves cell nonautonomous paracrine signaling, we transduced B16F1 cells with lentivirus expressing a constitutively active ligand-independent truncated form of ALK4 (caALK4). Induction of caALK4 by doxycycline led to increased Smad2,3 phosphorylation and expression of the SMAD3-dependent luciferase reporter CAGA-Luc (Figure 3a–c) and reduced pigment secretion (Supplementary Figure S6a online). However, caALK4 expression decreased below detection within four subsequent cell passages despite the continuous presence of doxycycline (Figure 3a). 5-ethynyl-2′-deoxyuridine incorporation, AnnexinV and propidium iodide flow cytometry, and cleaved Caspase3 immunofluorescence staining after up to 3 days of caALK4 induction in vitro revealed no overt cell cycle inhibition or apoptosis (Supplementary Figure S7a–d online and data not shown), even though the number of cells detected by Alamar blue assays after 3 days of caALK4 induction *in vitro* was reduced (Figure 3d), indicating impaired cell viability. A similar reduction in the number of viable cells was induced by ligand-independent caALK4 in human C8161 melanoma cells. To investigate the impact on tumor growth, B16F1 cells transduced with caALK4 were grafted intradermally into

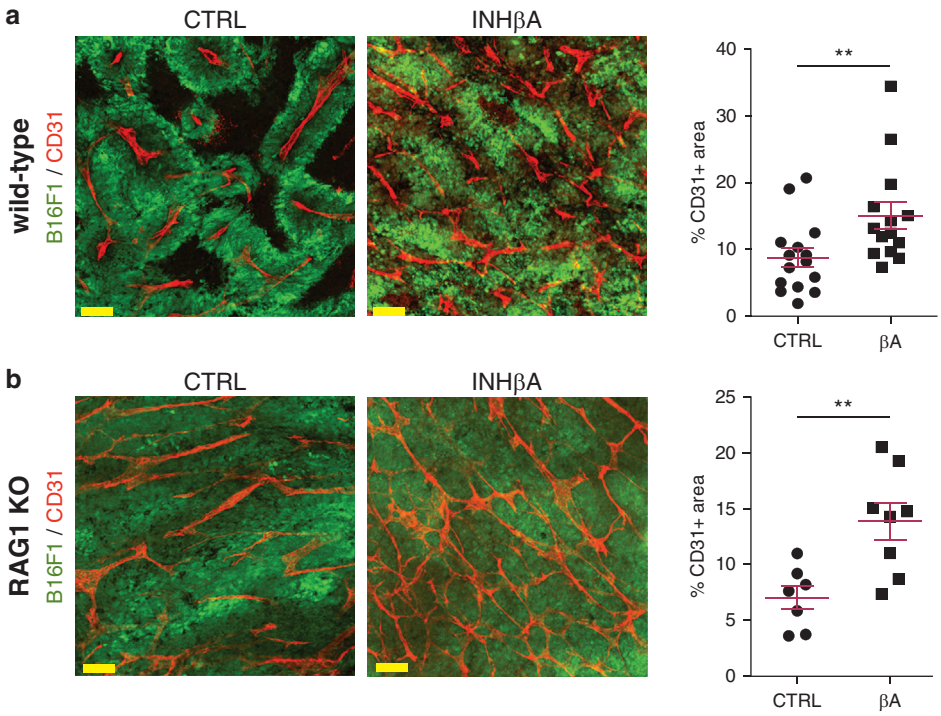

Figure 5. Paracrine Activin-A can stimulate tumor angiogenesis and oxygenation independently of adaptive immunity. CD31 immunofluorescence staining (red) of thick sections of B16F1:GFP CTRL or βA syngeneic intradermal grafts (green) in wild-type and *Rag1*$^{-/-}$ C57BL/6 mice. Quantifications (right panels) show that βA-expressing tumors had significantly increased vascular coverage compared with CTRL tumors both in wild-type (βA: n = 14; CTRL: n = 15) and in *Rag1*$^{-/-}$ hosts (βA: n = 8; CTRL: n = 7). $^{**}P < 0.01$. GFP, green fluorescent protein; INHβA, Inhibin βA.

syngeneic C57BL/6 hosts, followed by treatment with doxycycline or empty vehicle until the endpoint of the experiment. Tumors induced no overt loss of body weight (Figure 3e), and they grew significantly less in doxycycline-fed animals than in vehicle-treated controls (Figure 3f), indicating that sustained autocrine Activin receptor signaling does not stimulate but rather suppresses tumor growth in vivo. Immunostaining at the endpoint of the experiment revealed increased apoptosis marked by cleaved Caspase-3 (Figure 3g, Supplementary Figure S7e), whereas Ki67-stained proliferating cells appeared to decrease, albeit not significantly (Figure 3h and i, Supplementary Figure S7f). We conclude that whereas paracrine functions of Activin-A promote cachexia and tumor growth, sustained autocrine receptor signaling in vivo reduces B16F1 cell survival and tumorigenicity.

Activin-A-dependent B16F1 melanoma growth is mediated by impaired tumor immune surveillance

One possible mechanism how paracrine Activin-A signaling accelerates tumor growth is by inhibiting tumor immune surveillance. In keeping with this idea, immunostaining of tumor sections with anti-CD8 antibody revealed that Activin-A expression significantly inhibited tumor infiltration by cytotoxic T-lymphocytes (Figure 4a and b). To directly test whether paracrine Activin-A signaling promotes tumor progression by inhibiting antitumor immunity, B16F1 cells were inoculated subcutaneously or intravenously into FoxN1$^{nu/nu}$ that lack functional T cells, or into *Rag1*$^{-/-}$ mice devoid of V-D-J recombination in antigen receptors. In sharp contrast to immune-competent wild-type hosts, loss of either *FoxN1* or *Rag1* enabled CTRL tumors to grow as fast as those with sustained *INHβA* expression, even though Activin-A provoked cachexia irrespective of the host genotype (Figure 4a–f). In good agreement, the same lentiviral *INHβA* transgene also failed to stimulate tumor growth or experimental lung metastases in xenografts of human melanoma cell lines in immunodeficient FoxN1$^{nu/nu}$ mice (Supplementary Figure S5d–g). These results strongly suggest that Activin-A accelerates B16F1 melanoma growth by blunting T-cell-mediated antitumor immunity.

Angiogenesis is enhanced by Activin-A but is not sufficient to promote B16F1 melanoma growth

Depending on the context, Activin-A signaling may also promote or inhibit angiogenesis that can be rate-limiting for tumor oxygenation and nutrient supply (Lewis et al., 2016). To assess whether increased tumor growth correlates with increased tumor vascularization, we labeled blood vessels in thick cryosections of syngeneic B16F1 grafts using CD31 antibodies. Quantification in z-stack reconstructions of entire sections of 15 CTRL and 14 INHβA tumors showed that Activin-A significantly increased the vascular density (Figure 5a). Conversely, pimonidazole staining of hypoxic areas in the same sections was 4-fold reduced, indicating that Activin-induced blood vessels were functional (Supplementary Figure S8a and b online). Because Activin-A has been shown to inhibit endothelial cell growth and tubule formation in vitro (Kaneda et al., 2011), we asked whether its proangiogenic effect in B16F1 melanoma could be mediated by macrophages. However, FACS sorting of dissociated B16F1 melanoma at the experimental endpoint revealed no changes in total myeloid populations, and M2-like macrophages marked by CD206 staining were decreased in *INHβA* compared with CTRL tumors (Supplementary Figure S8c). Also in human melanoma with the highest levels of Activin-A, markers of lymphocytic or myeloid infiltrates appeared to be reduced rather than increased (Supplementary Figure S9 online), although recruitment of such infiltrates or their functions may vary depending on Activin-A

signaling strength or duration. Interestingly, angiogenesis was similarly increased in *Rag1*$^{-/-}$ hosts where tumor growth remained unchanged on *INHβA* overexpression, suggesting that increased vascularization alone cannot account for the tumorigenic effects of Activin-A (Figure 5b).

DISCUSSION

Previous studies reported both tumor-suppressive and oncogenic effects of Activin-A, but a role in antitumor immunity and the relative contributions of autocrine versus paracrine signaling in an in vivo model of melanoma remained to be evaluated. Here, we propose that the net outcome of a gain in paracrine Activin-A signaling in mouse and human melanoma grafts is determined by whether or not the tumor host has functional adaptive immunity. Tumorigenic paracrine effects on adaptive immunity trumped proapoptotic autocrine signals within cancer cells to overall facilitate primary and metastatic growth. Ectopic Activin-A signaling also stimulated tumor vascularization and, concurring with previous reports, systemic cachexia, and these effects were preserved in *Rag1*$^{-/-}$ mice albeit without accelerating tumor growth. Thus, a potential boost in nutrient supply by recycled tissue breakdown products was either insufficient to fuel tumorigenesis or neutralized by growth-inhibitory autocrine Activin receptor signaling within tumor cells. To our knowledge, these results furnish direct evidence that adaptive immunity is required for a tumorigenic Activin function, and that autocrine and paracrine signaling mediates opposite effects on melanoma growth in vivo. Based on these findings, future strategies to boost the efficacy of immunotherapies should consider targeting Activin-A.

Activin-A only stimulates melanoma growth in mice that have functional T cells

Our main finding is that paracrine Activin-A signaling was tumorigenic specifically in immunocompetent hosts, despite a proapoptotic function of autocrine Activin/Nodal receptor signaling within melanoma cells. Thus, a lentiviral *INHβA* transgene encoding secreted Activin-A in syngeneic B16F1 mouse melanoma grafts increased intradermal tumor growth and the frequency of experimental lung metastases specifically in wild-type C57BL/6 mice. By contrast, in syngeneic *Rag1*$^{-/-}$ hosts that lack T- and B-lymphocytes, or in athymic *FoxN1*$^{nu/nu}$ mice devoid of only T cells, neither blockade of endogenous Activin/Nodal receptor ligands in the human C8161 melanoma cell line nor overexpression of Activin-A revealed a tumorigenic function. Technical artifacts arising from clonal variation or specific cell lines are unlikely because we used nonclonal pools of lentivirally transduced cells, and reproducible results were obtained with multiple independent batches of cells and with additional human melanoma cell lines. In keeping with our observations, also other tumor types in immunocompromised mice revealed minimal or no oncogenic effects of Activin-A (Zhou et al., 2010). Notable exceptions are pancreatic and esophageal cancer and R30C mammary carcinoma cells where gain of autocrine Activin signaling directly stimulates tumor cell stemness or survival (Krneta et al., 2006; Lonardo et al., 2011).

Induction of metastatic disease in chemically induced squamous cell carcinoma by a keratinocyte-specific *INHβA* transgene facilitates infiltration by immunosuppressive Tregs while reducing the number of resident γδ-TCR-positive dendritic epidermal T-lymphocytes, consistent with a potential role in promoting immune evasion (Antsiferova et al., 2011). Depletion of CD4-positive T cells, including Tregs, did not suppress the tumorigenicity of transgenic Activin-A in this skin carcinoma model (Antsiferova et al., 2017). However, it will be interesting to compare in future studies the potential of Tregs or tumor-infiltrating cytotoxic T-lymphocytes or other T-cell subsets to mediate effects of Activin-A on antitumor immunity in melanoma. A role in suppressing antitumor immunity may explain why *INHβA* expression is more frequently upregulated in human melanoma and other solid human tumors than expected for a neutral bystander (Hoda et al., 2016; Wu et al., 2015).

Inhibition of melanoma growth by autocrine signaling may curtail oncogenic effects of secreted Activin-A on the tumor microenvironment

We found that in contrast to secreted Activin-A, autocrine signaling by a doxycycline-inducible ligand-independent mutant ALK4 transgene inhibited B16F1 tumor growth rather than stimulating it. This confirms that oncogenic Activin-A activity in immunocompetent syngeneic hosts was mediated by paracrine signaling. Induction of caALK4 also inhibited the expansion of cultured B16F1 cells ex vivo. Although we cannot rule out that forced ALK4 signaling may induce non-physiological levels of Smad2/3 phosphorylation, cell proliferation and survival are also inhibited in normal melanocytes treated with Activin-A. By contrast, Activin-A-treated B16F1 cells proliferated normally. Because melanoma cell survival was impaired by caALK4 but not by secreted Activin-A, autocrine signaling activity of the ligand is likely attenuated in transformed cells compared with normal melanocytes. In the hepatocyte lineage, autocrine antiproliferative Activin-A signaling is frequently attenuated by secreted antagonists in hepatocarcinoma (reviewed in Deli et al., 2008). Also in human melanoma, dynamic changes in the levels of FST expression during progression of melanoma in situ to metastatic growth may modulate Activin-A responses (Heinz et al., 2015; Stove et al., 2004). However, whether FST influences the balance between autocrine and paracrine Activin signaling remains to be determined.

Interplay of tumor growth and cachexia

Commonly associated with advanced cancer in human patients, cachexia reduces life quality and drug responses while increasing morbidity and mortality (Fearon et al., 2013). In pancreatic MIA PaCa-2 xenografts, a comparison of tumor growth with the time course of Activin-induced cachexia suggests that associated metabolic changes or general weakening curtails a growth-promoting effect of autocrine Activin signaling (Togashi et al., 2015). If cachexia similarly curbs the growth of B16F1 melanoma growth, tumor growth should slow down concurring with the onset of Activin-induced cachexia at least in immunodeficient hosts. Such a trend in *Rag1*$^{-/-}$ hosts was not statistically significant and not seen in *nu/nu* hosts. The observed tumor growth curves also do not support a model that cachexia is rate-limiting for the supply of essential amino acids and other metabolites to cancer cells in a process of "autocannibalism" (Theologides, 1979).

Activin-induced tumor angiogenesis and its uncoupling from tumor growth

We found that paracrine Activin-A signaling in the syngeneic B16F1 melanoma model also stimulated tumor vascularization. However, a similar increase of blood vessels in *Rag1*$^{-/-}$ hosts was not sufficient to facilitate tumor growth. Although we could not stain enough tumors in *Rag1*$^{-/-}$ mice with pimonidazole to formally exclude a stimulatory effect of adaptive immunity on vessel functionality and hypoxia, it is interesting to note that tumor growth was also uncoupled from angiogenesis in immunodeficient SCID mice bearing mammary carcinoma xenografts, where gain of Activin-A signaling diminished tumor angiogenesis without affecting vascular perfusion (Krneta et al., 2006). Interestingly, however, Activin signaling within endothelial cells in this breast cancer model and other tumors was cytostatic and reduced sprouting and blood vessel density (Breit et al., 2000; Kaneda et al., 2011; Krneta et al., 2006), indicating that proangiogenic activity is likely indirect. T-cell-derived cytokines are unlikely involved because Activin-A stimulated the vascularization of B16F1 tumors even in *Rag1*$^{-/-}$ syngeneic hosts. Possibly, class I inflammatory macrophages that stimulate vascularization in Activin-induced skin squamous cell carcinoma mediate proangiogenic activity (Antsiferova et al., 2017). Although the total number of infiltrating macrophages was unchanged by Activin-A in B16F1 melanoma under the conditions examined, and because increased angiogenesis was not sufficient to promote tumor growth, we did not further investigate whether Activin-A directly polarized a proangiogenic macrophage subtype in this model.

Overall, our findings suggest that paracrine Activin-A should be considered as a target for personalized therapies not only to reduce cachexia and melanoma vascularization, but also to enhance the efficacy of immunotherapies.

MATERIALS AND METHODS

Melanoma grafts

A total of 1×10^6 B16F1 cells were injected intradermally into the right flank of 8- to 12-week-old female wild-type (Harlan) or *Rag1*$^{-/-}$ C57BL/6 (EPFL animal core facility) syngeneic hosts, or of Hsd-athymic *nu/nu* mice (Harlan). Animal body weights and tumor sizes were measured every 2 days. Tumor volumes were calculated using the formula *length* × *width* × *depth*. Experimental lung metastases were obtained by injecting 3×10^5 B16F1 cells into the tail vein. Pigmented metastases visible at the surface of each lung lobe were counted 3 weeks after injection. Where indicated, animals were fed with chow containing 0.625 g/kg doxycycline (Provimi Kliba AG, Gossau, Switzerland). All procedures were according to Swiss legislation and approved by the cantonal veterinary administration. Generation of cell lines and in vitro assays are further documented in Supplementary Materials and Methods online.

Gene expression analysis

Total RNA from melanoma cell lines and tumors was isolated using Trizol reagent (Thermo Fisher Scientific, Waltham, MA) and guanidinium/CsCl gradient, respectively, or by using the RNeasyMini kit (Qiagen, Venlo, Netherlands). One microgram of total RNA was used for cDNA synthesis using the Superscript III Reverse Transcription Kit (Invitrogen, Carlsbad, CA). Quantitative polymerase chain reaction assays were performed using SYBR green chemistry according to manufacturers' instructions (Applied Biosystems, Waltham, MA), or commercial Taqman probe for INHβA (Eurogentec, Liège, Belgium). PCR primer sequences are listed in Supplementary Table S1 online.

Cell cycle and Ki67 analysis

After treatment with 250 μg/ml doxycycline for 24, 48 or 72 hours in 6-well plates, cells were incubated with 10 μM 5-ethynyl-2′-deoxyuridine for 30 minutes and then trypsinized, washed with phosphate buffered saline, and fixed for 20 minutes with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 in phosphate buffered saline, and stained for 30 minutes with Alexa-647-coupled azide in the presence of copper sulfate and sodium ascorbate. Cells were then washed and stained with DAPI and analyzed using a CyAn ADP analyzer (Beckman Coulter, Brea, CA). For Ki67 immunostaining, tumors were dissociated into single cells in 0.02 mg/ml DNase I and 1 mg/ml collagenase mix (Sigma-Aldrich, St. Louis, MO) using a GentleMACS tissue octo dissociator (Miltenyi, Bergisch Gladbach, Germany). Dissociated cells were stained with live and dead blue dye (Life Technologies, Carlsbad, CA), and washed and labeled with antibodies against CD45-APC-Vio770 (Miltenyi, 130-105-463), CD31-BV605 (BD Biosciences, Franklin Lakes, NJ, 740356), and CD140a-PE (eBioscience, Waltham, MA, 12-1401-81) to exclude leukocytes, endothelial cells, and fibroblasts, respectively. After washing, cells were fixed and permeabilized using FoxP3 fix and perm buffer set (eBiosciences) and stained with eFluor 450-conjugated Ki67 antibodies (eBiosciences, 48-5698-80) using permeabilization buffer (eBiosciences). Samples were acquired using an LSRII cytometer (Becton Dickinson, Franklin Lakes, NJ).

Statistical analysis

All statistical analyses were performed using GraphPad Prism v6 software. Data were analyzed using Mann-Whitney (for nonparametric data), *t*-tests, and 1-way or 2-way analysis of variance with Bonferroni correction for parametric data. A *P*-value <0.05 was considered significant.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2017.07.845.

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CONFERENCE ATTENDANCE

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