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Germline Genetic Determination of Cancer Outcome

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GERMLINE GENETIC DETERMINATION OF CANCER OUTCOME

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Immunotherapy has transformed the treatment of melanoma patients. However, despite the enormous promise of immunotherapy, a major fraction of patients with advanced melanoma still succumb to this deadly disease. In addition, systemic immunotherapy can lead to relevant toxicities. Therefore, it is an unmet need to identify the factors that modulate the outcome and response to therapy of melanoma patients. One major factor accounting for individual differences in the host response to cancer is the genetic makeup of the germline. A thorough assessment of the role of germline genetics in melanoma outcome is lacking, partly because of the difficulty in assessing the vast number of genetic variants present in the human population.

In this thesis, I describe the discovery of the impact of three highly prevalent variants of the *Apolipoprotein E* (*APOE*) gene on melanoma progression and outcome. Using transgenic human *APOE* mice, we found that mice expressing the *APOE4* variant exhibit slower melanoma progression and metastasis than *APOE2* mice. The impact of *APOE* genotype on melanoma progression was mediated by modulation of anti-melanoma immunity. *APOE4* mice showed enhanced activation of anti-tumor immunity relative to *APOE2* mice, and T cell depletion abrogated the impact of *APOE* genotype on melanoma progression.

Importantly, analysis of large-scale human melanoma data validated the impact of *APOE* genotype on melanoma progression in humans. Amongst melanoma patients at high risk of melanoma-associated death, carriers of the *APOE2* variant showed worse survival outcomes relative to *APOE3* homozygotes and *APOE4* carriers in two independent studies.

APOE genotype also impacted melanoma outcome in the context of anti-PD1 immunotherapy in both mice and humans. Additionally, *APOE4* mice derived robust benefit of pharmacologic activation of liver-X-receptors, a class of transcription factors inducing *APOE* expression. In contrast, *APOE2* showed no treatment benefit, indicating that *APOE* genotype may serve as a biomarker for response to LXR-agonistic immunotherapy.

Overall, our data describe the first example of highly common germline variants that modulate the outcome of a common cancer type. These findings suggest *APOE* to be a potential biomarker for outcome and therapy response in melanoma. More generally, our findings suggest that common variants of the germline genetic makeup substantially modulate cancer outcome and will likely be a cornerstone of precision cancer management.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

APOE	Apolipoprotein E
ARIC	Atherosclerosis Risk in Communities Study
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
GWAS	Genome-wide association study
LDLR	Low-density lipoprotein receptor
LXR	Liver-X-receptor
MAPK	Mitogen-activated protein kinase
MC1R	Melanocortin 1 receptor
MDACC	MD Anderson Cancer Center
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
NK cells	Natural killer cells
PD-L1	Programmed death-ligand 1
PD1	Programmed cell death protein 1
SNP	Single nucleotide polymorphism
TAM	Tumor-associated macrophage
TCGA	The Cancer Genome Atlas
TLR	Toll-like receptor
Treg	Regulatory T cell

1 Introduction

1.1 Epidemiology and biology of melanoma

Malignant melanoma is a cancer arising from melanocytes, the pigment-producing cells found in the skin and other tissues of the body. Because of its propensity to spread to distant organs, melanoma is more dangerous than other types of skin cancer, such as squamous cell carcinoma and basal cell carcinoma. The incidence of melanoma has increased 17-fold for men and 9-fold for women between 1950 and 2007 (Geller *et al.*, 2013), making it the fifth most common cancer type in males and females in the United States in 2019 (National Cancer Institute). Globally, the incidence of melanoma is approximately 1-25 per 100,000 individuals, with substantial geographic and ethnic variation.

1.1.1 Risk factors for melanoma

The biggest risk factor for melanoma is environmental exposure to ultraviolet light (Elwood and Jopson, 1997), which leads to DNA damage with a characteristic accumulation of cytosine to thymine mutations (Lawrence *et al.*, 2013). The risk for sporadic melanoma is modulated by a number of prevalent germline variants with low penetrance, exemplified by the highly polymorphic melanocortin 1 receptor (*MC1R*) gene. Individuals with fair skin exihibit increased melanoma risk (Schadendorf *et al.*, 2015).

While environmental factors are the most important risk factor in sporadic cases of melanoma, germline genetic variations have been found in families with increased melanoma incidence (Hayward, 2003). In familial cases, which account for 10 % of melanoma cases, germline mutations in the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) locus are the most prevalent genomic alteration (Kamb *et al.*, 1994; FitzGerald *et al.*, 1996). The *CDKN2A* locus encompasses two open reading frames, which encode the distinct tumor suppressors $p16^{INK4A}$ and $p14^{ARF}$, both of which regulate cell cycle transition. In addition to *CDKN2A*, germline mutations have been identified in the *CDK4*, *BAP1*, *POT1* and other genomic loci. However, the genetic basis of a substantial fraction of familial melanomas remains unaccounted for (Schadendorf *et al.*, 2015).

1.1.2 Molecular landscape of melanoma

Hyperactivation of the mitogen-activated protein kinase (MAPK) pathway is a central molecular feature of melanoma. The MAPK-pathway consists of the small GTPase RAS (HRAS, KRAS, or NRAS), RAF kinase (ARAF, BRAF or CRAF), MAP/ERK kinase (MEK1 and MEK2) and MAPK (MAPK1 and MAPK3). Activating mutations in components of the MAPK-signaling pathway are present in approximately 75 % of melanomas: approximately 50 % of melanomas harbor the $BRAF^{V600E}$ mutation, which results in ~700-fold increased activation of the pathway relative to wildtype (Wan *et al.*, 2004). Activating mutations of *NRAS* are present in ~25 %

of all melanomas (The Cancer Genome Atlas Network, 2015). Of note, isolated activating mutations of MAPK pathway components result in cellular senescence because of cellular stress subsequent to MAPK-hyperactivation (Michaloglou *et al.*, 2005). Therefore, additional genetic aberrations are required for full malignant transformation. These codrivers comprise additional activating mutations in oncogenes, such as in *CDK4*, *CCND1* or *KIT*. The loss of tumor suppressors can act as alternative codrivers, such as deletions and transcriptional silencing of *PTEN*. Somatic deletions and transcriptional silencing of *CDKN2A* also occur in sporadic melanoma, consistent with its frequent mutant germline status in familial melanoma (The Cancer Genome Atlas Network, 2015).

1.1.3 Targeted therapy of melanoma

Given the addiction of melanoma to MAPK-signaling and the high frequency of *BRAF* and *NRAS* mutations, targeting the MAPK pathway has been a promising approach to molecular-guided therapy. Efforts to inhibit hyperactive mutant BRAF culminated in the approval of the small molecule inhibitor vemurafenib for the treatment of metastatic melanoma. Vemurafenib exhibits impressive response rates in over 80 % of melanoma patients (Chapman *et al.*, 2011). Unfortunately, however, responses to vemurafenib are short-lived, with most patients developing resistance and eventual progression of the disease (Sosman *et al.*, 2012). Resistance can develop due to amplification of the mutant $BRAF^{V600E}$ locus (Shi *et al.*, 2012), secondary mutations downstream from BRAF (Nazarian *et al.*, 2010), or activation of pathways other than MAPK (Villanueva *et al.*, 2010). In addition to vemurafenib, other inhibitors of BRAF as well as inhibitors targeting other components of the MAPK pathway have been developed, such as the MEK inhibitor trametinib. Combination therapy with both BRAF and MEK inhibition improves response rates, but the majority of melanoma patients similarly experiences relapse and/or progression under combination therapy (Robert *et al.*, 2019), highlighting the need for additional therapeutic modalities, as discussed below (see 1.2.3).

1.2 Anti-tumor immunity and its therapeutic modulation in melanoma

Cancer cells exhibit genomic alterations, which give rise to the expression of neoantigens and antigens physiologically only expressed during development or in immune-privileged sites. The altered antigen-profile of cancer cells enables immune cells to distinguish cancer cells from physiological cells (Schumacher and Schreiber, 2015). The hypothesis that the immune system has the capacity to fight cancer can be traced back to at least the 19^{th} century, when spontaneous regressions of cancer were noticed in patients that acquired erysipela infections. William Coley exploited these findings therapeutically by treating bone cancer patients with bacterial injections (Dobosz and Dzieciątkowski, 2019). However, it was not until 50 years later, that Thomas and Burnet proposed the cancer immunosurveillance theory, according to which lymphocytes identify and eliminate cancer cells. The work by Boon and colleagues provided proof that in cancer patients, CD8+ T cells spontaneously exist that recognize cancer-specific peptide-MHCI complexes (Boon *et al.*, 1994). Rapid advances in immunology, mouse models, and molecular biology firmly established the capacity of the immune system to ward off cancer

(Dunn *et al.*, 2004). A current model of how the immune system fights cancer is represented by the tumor immunity cycle, as described below (1.2.1).

1.2.1 The tumor immunity cycle

The tumor immunity cycle describes the cascade of events that need to take place for the elimination of cancer cells by the immune system (Figure 1.1) (Chen and Mellman, 2013). The cycle starts with tumor cells releasing antigens that get taken up by specialized antigen-presenting cells of the immune system, most importantly dendritic cells (Roberts *et al.*, 2016). Upon antigen ingestion, antigen-presenting cells migrate to secondary lymphoid organs, such as tumor-draining lymph nodes, where they present tumor-derived antigens on major histocompatibility complex (MHC) class I and class II molecules to naïve T cells. T cells whose T cell receptor recognizes a specific antigen/MHC complex get primed and activated, resulting in clonal expansion. Notably, for T cells to get activated and expand, antigen-presenting cells need to present co-stimulatory molecules in addition to the antigen/MHC-complex. Such co-stimulatory signaling comprises the interaction between B7 molecules (CD86 and CD80) on dendritic cells with CD28 on T cells. Without co-stimulation, antigen presentation can induce T regulatory cell responses rather than effector responses (Driessens *et al.*, 2009). Upon activation, T cells migrate to the tumor via the blood stream, where they recognize their cognate antigen presented on MHC molecules of the tumor, and engage in an effector response. The effector response leads to tumor cell killing via the secretion of cytokines and direct cell-cell interactions, and thereby perpetuates the tumor immunity cycle. It is still a matter of debate to which degree T cell priming can also take place within the tumor and alleviate the requirement of dendritic cell migration to secondary lymphoid organs (Roberts *et al.*, 2016; Thompson *et al.*, 2010).

1.2.2 The tumor immune microenvironment

Tumor cells, dendritic cells and T cells constitute the main components of the tumor immunity cycle. However, multiple other factors modulate and participate in the immune response to cancer. First, other cell types can assume certain functions of the tumor immunity cycle. For example, natural killer cells (NK cells) are potent cytotoxic lymphocytes of the innate immune system, which engage in tumor cell killing and cytokine secretion regulated by a suite of activating, co-stimulatory and inhibitory receptors (Shimasaki *et al.*, 2020). Second, other cell types modulate the function of the tumor immunity cycle at different steps of the cascade. CD4+ T cells, which recognize specific antigens presented by MHCII molecules, can directly participate in tumor cell killing (Kreiter *et al.*, 2015). In addition, CD4+ T cells modulate the activity of CD8+ effector T cells, even in tumors not expressing MHCII molecules (Alspach *et al.*, 2019). Multiple other cell types exist in the highly heterogeneous tumor immune microenvironment, including several myeloid cell types (Binnewies *et al.*, 2018). Intratumoral myeloid cells exist across a spectrum of differentiation and activation states (Broz and Krummel, 2015; Gabrilovich, 2017). Importantly, many intratumoral myeloid cells, including poorly differentiated myeloid cells termed myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs), exert pro-tumor functions. Myeloid cells can stimulate tumor progression by suppressing anti-tumor

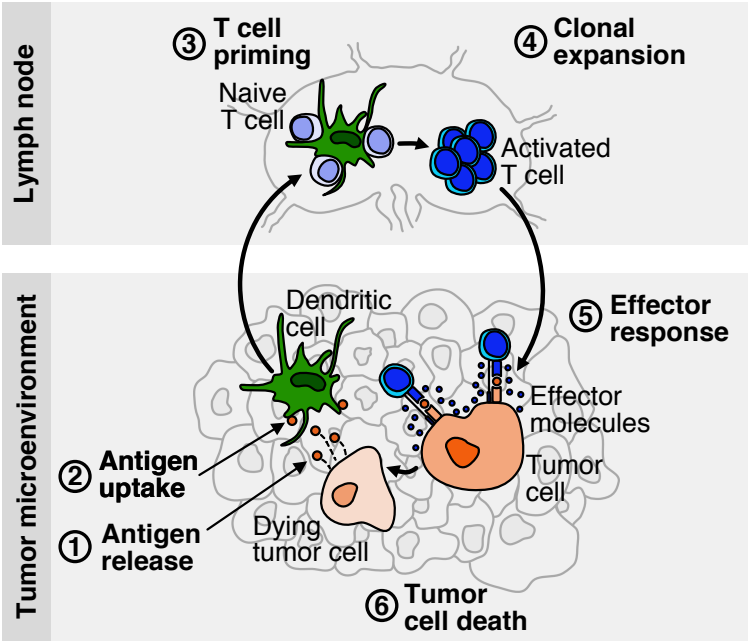

Figure 1.1. The tumor immunity cycle. The tumor immunity cycle is a model for an effective immune response against cancer. It consists of a cascade of events, during which antigen-presenting cells take up tumor-derived antigen and migrate to the tumor-draining lymph node. There, they prime naïve T cells, which clonally expand and migrate to the tumor. In the tumor bed, T cell killing of tumor cells perpetuates the cycle.

effector cells, as well as by stimulating angiogenesis and secreting protumoral survival factors (Coussens *et al.*, 1999; Ruffell *et al.*, 2014; Sippel *et al.*, 2011). Non-immune components also modulate anti-cancer immunity, such as cancer-associated fibroblasts (Kumar *et al.*, 2017; Lakins *et al.*, 2018) and vascular cells (Tian *et al.*, 2017). The tumor microenvironment is highly patient-specific; the factors that shape it and thereby modulate cancer and therapy outcome are described below (see 1.3).

1.2.3 Immunotherapy of melanoma

In patients with cancer, the immune system has failed to provide protection from cancer progression, indicating that the tumor immunity cycle does not work efficiently. Specific targeting of the rate-limiting step in the tumor immunity cycle offers the intriguing opportunity to reinvigorate anti-cancer immunity. Before the advent of checkpoint inhibition therapy, dacarbazine chemotherapy and high-dose interleukin-2 constituted the mainstay of melanoma therapy. Unfortunately, these showed poor efficacy in patients with advanced disease and usually did not provide curative outcomes (Pasquali *et al.*, 2018). The therapy of melanoma was transformed by the development of therapies targeting checkpoints of the immune response to cancer.

Cytotoxic T-lymphocyte-associated protein 4 (CTLA4) is a molecule expressed by T cells that competes with CD28 for binding to B7 molecules on antigen-presenting cells (see 1.2.2). In contrast to the stimulatory effects conferred by CD28, ligation of CTLA4 results in inhibition of T cell profileration and activation (Krummel and Allison, 1995), thereby acting as a negative checkpoint during T cell priming. Consistently, antibody-mediated inhibition of CTLA4 promotes anti-tumor imunity (Leach *et al.*, 1996). CTLA4 is also expressed on tumor-promoting regulatory CD4+ T cells (Treg), and the efficacy of CTLA4 blockade is partially mediated by modulating Treg function and abundance (Peggs *et al.*, 2009). The CTLA4-blocking antibody ipilimumab was approved for patients with advanced melanoma in 2011, leading to long-term survival in ~12 % of patients (Hodi *et al.*, 2010; Robert *et al.*, 2011).

Programmed cell death protein 1 (PD1) is another checkpoint molecule expressed by T cells that limits their activation. The main ligand for PD1 is programmed death-ligand 1 (PD-L1), which is expressed by a variety of cell types, including several immune subsets, cancer cells, and vascular cells. Importantly, the PD1/PD-L1 checkpoint acts at a later stage of T cell activation than CTLA4 by mainly regulating T cell effector functions after initial T cell priming (Freeman *et al.*, 2000). Notably, PD1 is also expressed by immune cell types other than T cells, including macrophages, and inhibition of PD1 has been shown to also promote anti-tumor activity in these non T cell populations (Gordon *et al.*, 2017; Hsu *et al.*, 2018). Two PD1-inhibiting antibodies have been approved for melanoma treatment, nivolumab and pembrolizumab. They exhibit more favorable efficacy and toxicity profiles than CTLA4 inhibitors, with impressive overall response rates of ~30 – 40 % (Topalian *et al.*, 2012; Hamid *et al.*, 2013). Their reduced toxicity relative to CTLA4 inhibition is likely due to the different mechanism of action, which targets the more downstream T cell effector phase. The complementarity of the mechanisms of action has prompted the assessment of combining CTLA4 and PD1 blockade, which further improves efficiacy of the individual agents (Larkin *et al.*, 2019). Of note, the inhibition of PD-L1 is also tested in cancer patients, but has not yet been approved for the treatment of melanoma (Mariathasan *et al.*, 2018). Inhibiting PD-L1 likely confers different effects from PD1 inhibition,

since T cells also express PD-L1 which binds to B7 proteins (Butte *et al.*, 2007). Additionally, PD-L1 inhibition does not affect the interaction of PD1 with another ligand, PD-L2, whose significance in cancer immunity warrants further assessment (Obeid *et al.*, 2016; Lakins *et al.*, 2018).

In addition to the widely applied inhibition of the CTLA4 and PD1 checkpoints in melanoma therapy, a range of alternative approaches have been explored to promote anti-tumor immunity. Cancer vaccination is one of the best studied of these. It comprises the injection of cancer derived antigenic peptides or proteins as well as the infusion of ex-vivo pulsed dendritic cells. Unfortunately, the efficacy of cancer vaccination attempts has been poor, with an objective response of $< 5\,\%$ across multiple trials (Melero *et al.*, 2014). However, it is possible that improving dendritic cell activation, enhanced antigen selection, and refined patient selection may increase the efficacy of cancer vaccination in the future (Carreno *et al.*, 2015; Palucka and Coussens, 2016). Adoptive T cell transfer constitutes another approach to enhance anti-melanoma immunity. Different strategies use naturally occurring tumor-specific T cells expanded ex-vivo as well as genetically engineered T cells transduced with either normal T cell receptors or chimeric antigen receptor (Hunder *et al.*, 2008; Rosenberg and Restifo, 2015). One major limitation to the widespread use of T cell adoptive transfer is the laborious and patient-specific process required for its application. Therefore, current efforts aim at generating T cell products that can be employed across patients (Crowther *et al.*, 2020). In addition, in contrast to B cell neoplasias, in which cell-based therapies have been paradigm-changing, the identification of cancer-specific neoantigens has been challenging (Rafiq *et al.*, 2020). Lastly, targeting of a multitude of other immune checkpoint molecules is currently undergoing clinical testing (Marin-Acevedo *et al.*, 2018).

Immunotherapy has transformed the prospect of patients with advanced melanoma. Moving forward, the identification of new targets for immunotherapy as well as the combination of different approaches, such as oncogene-targeted and immunotherapies hold promise to extend the benefit to an increasing share of patients (Havel *et al.*, 2019).

1.3 Determination of melanoma outcome

Despite the unprecedented success of immunotherapy in melanoma as outlined above (see 1.2.3), a large fraction of patients with advanced melanoma ultimately still succumb to their disease (Larkin *et al.*, 2019; Liu *et al.*, 2019). Additionally, systemic therapy is associated with relevant toxicities, such as immune-related adverse events in the case of checkpoint inhibition (Hodi *et al.*, 2010). For these reasons, it is critical to identify the factors that predict outcome of melanoma patients with and without therapeutic intervention. These factors are related to the tumor, the host, and the environment, as summarized below.

1.3.1 Modulators of melanoma immunotherapy

A major effect of immune checkpoint therapy consists of unleashing pre-existing immunity. It is therefore not surprising that the degree of pre-existing anti-tumor immunity correlates with the response to this therapeutic approach (Kim and Chen, 2016). Three basic immune profiles can be distinguished according to the degree of immune infiltration in the tumor before therapy

initiation: The immune-inflamed phenotype is characterized by infiltration of T cells into the tumor; these patients exhibit the best therapy outcome (Herbst *et al.*, 2014). In contrast, the immune-excluded phenotype is characterized by accumulation of immune cells in stromal tissue surrounding tumor cells without tumor penetration, and immune-desert tumors exhibit paucity of T cells in the tumor and the surrounding stroma. Notably, the predictive value of T cell infiltration for checkpoint therapy response is not universal: for example, there was no association in a study assessing anti-PD1 therapy in a patient cohort in which a large fraction had previously received anti-CTLA4 therapy (Riaz *et al.*, 2017). Similarly, the expression of PD-L1, which correlates with interferon signaling in the tumor microenvironment, has been shown to predict for response in immune checkpoint inhibition in certain cases, but patients with low PD-L1 expression can also benefit (Topalian *et al.*, 2012; Davis and Patel, 2019).

The existence of distinct immune profiles in patients poses the important question which factors determine the composition and functional state of the tumor microenvironment. Oncogenic signaling is one major factor underlying the patient-specificity of the tumor microenvironment. The impact of oncogenic signaling pathways on the state of the tumor immune microenvironment is still incompletely understood, but associations have been shown for an increasing number of frequently altered genomic loci, including *p53* and *KRAS* (Pylayeva-Gupta *et al.*, 2012; Blagih *et al.*, 2020). In melanoma, the $BRAF^{V600E}$ mutation described above increases the expression of multiple cytokines that alter the phenotype of monocyte-derived dendritic cells (Sumimoto *et al.*, 2006). Tumors in the $BRAF^{V600E}Pten^{-/-}$ melanoma model exhibit constitutively active WNT/β-catenin signaling, which suppresses the recruitment of CD103+ dendritic cells via the inhibition of CCL4 production and thereby promotes cancer immune evasion (Spranger *et al.*, 2015). Moreover, loss of *PTEN* alone renders melanoma cells more resistant to cytotoxic T cells and checkpoint therapy (Peng *et al.*, 2016). Genomic alterations outside of oncogenes and tumor suppressors have also been shown to modulate antigen presentation and cell death as well as to exert immunosuppressive effects (Rooney *et al.*, 2015).

T cells distinguish tumor cells from healthy cells due to the expression of tumor-associated antigens. These comprise non-mutated self-antigens wich are aberrantly expressed or overexpressed and neoantigens that arise as a consequence of somatic mutations. Consistently, the overall mutational burden is one of the best predictors for response to checkpoint inhibition so far (Snyder *et al.*, 2014; Van Allen *et al.*, 2015). Melanomas exhibit the highest mutational burden of all human tumors, offering a potential explanation for the overall favorable outcome of immunotherapy in this cancer type (Hodis *et al.*, 2012). However, the association between mutational burden and immunotherapy outcome is not robust enough for individual patient stratification (Riaz *et al.*, 2017; Liu *et al.*, 2019). Heterogeneity is another characteristic of the tumor that impacts response to immunotherapy, since increased heterogeneity increases the likelihood of clonal tumor populations to evade the immune system. Consistently, the response to immune checkpoint blockade is more favorable in melanoma patients with low intratumor heterogeneity (McGranahan *et al.*, 2016; Riaz *et al.*, 2017).

Factors determined by the host also shape anti-tumor immunity. Recent research has revealed a major impact of the microbiome composition on anti-tumor immunity (Zitvogel *et al.*, 2018). In addition, age, sex, and obesity affect anti-tumor immunity (Pitt *et al.*, 2016; Galluzzi *et al.*, 2018). Importantly, germline variation also shapes patient-specific anti-tumor immunity, and its impact will be described in more detail below (see 1.3.2).

Lastly, several environmental factors participate in modulating the interplay between tumor and the immune system, including infectious agents, exposure to sunlight, and pharmacological agents (Chen and Mellman, 2017).

1.3.2 The role of germline variation in melanoma outcome

Variants of the germline genetic makeup are known to account for differences in the immune response between individuals. For example, genotype-phenotype association studies have identifed multiple single nucleotide polymorphisms (SNP) that modify activation of dendritic cells and T cells in the contexts of infectious disease and autoimmunity (Parkes *et al.*, 2013; Lee *et al.*, 2014; Raj *et al.*, 2014; Ye *et al.*, 2014; Cho and Feldman, 2015; Tian *et al.*, 2017). It is therefore plausible to assume that germline variations also impact anti-tumor immunity. Consistent with this hypothesis, polymorphisms in *FCGR3A*, a gene encoding a receptor with roles in antibody-dependent cell-mediated cytotoxicity, associate with outcome in melanoma patients treated with ipilimumab (Arce Vargas *et al.*, 2018). Polymorphism in genes encoding danger sensing Toll-like receptors associate with survival in melanoma (Tittarelli *et al.*, 2012; Gast *et al.*, 2011). In breast cancer, a loss-of-function polymorphism in the ATP-sensing $P2RX_7$ receptor that mediates dendritic cell activation predicts for worse metastasis-free survival (Ghiringhelli *et al.*, 2009). In the analysis of 1,535 patients with cancer treated with checkpoint blockade, a larger diversity of the MHCI encoding genes associated with enhanced outcome, potentially owing to the ability of the immune system to present a broader repertoire of tumor-associated antigen-derived peptides (Chowell *et al.*, 2018). A recent analysis of The Cancer Genome Atlas (TCGA) study identified the association of certain SNPs with the degree of immune infiltration in solid tumors (Shahamatdar *et al.*, 2019). Additionally, targeted assessment revealed SNPs known to modulate autoimmune disease to associate with outcome of checkpoint blockade in melanoma (Chat *et al.*, 2019). Therefore, multiple SNPs have been identified that shape anti-melanoma immunity. However, a global assessment of the association of germline variation and its causal role in modulating melanoma outcome is lacking.

1.4 The LXR/APOE-signaling axis

Apolipoprotein E (APOE) is a 34 kDa secreted glycoprotein that is produced mainly by the liver, the central nervous system, and macrophages (Utermann *et al.*, 1975; Holtzman *et al.*, 2012; Belloy *et al.*, 2019). A major function of APOE is tied to its presence on lipoprotein particles, whose uptake it facilitates by binding to members of the low-density lipoprotein receptor (LDLR) family, which includes LDLR, LRP1, VLDLR, LRP2 (megalin), LRP8, LRP4, and LRP1b (Lane-Donovan and Herz, 2017). Because of their expression in the brain, the interaction between APOE and the LDLR, VLDLR, LRP1, and LRP8 receptors is best characterized (Mahley *et al.*, 1979; Holtzman *et al.*, 2012).

In addition to its canonical role in lipid shuttling in organismal metabolism, several other functions of APOE have been described (Martinez-Martínez *et al.*, 2020). In immunity, APOE-deficient mice have been shown to be susceptible to infections with intracellular bacteria and virus (Ludewig *et al.*, 2001; Martens *et al.*, 2008; Toledo *et al.*, 2015). Paradoxically, *Apoe*-knockout mice exhibit hyperinflammation and chronic lymph node hypertrophy, but this was

shown to be due to impaired migration of dendritic cells to lymph nodes as well as impaired egress of lymphocytes from lymph nodes, explaining immune dysfunction in these mice (Angeli *et al.*, 2004; Tay *et al.*, 2019). The mechanism through which APOE modulates immunity is incompletely understood and seems context-specific (Zhang *et al.*, 2010). Consistent with a role in promoting immunity, APOE has been shown to mediate uptake of lipid antigens by antigen-presenting cells and subsequent priming of natural killer T cells (van den Elzen *et al.*, 2005). *Apoe*$^{-/-}$ mice also exhibit reduced delayed-type hypersensitivity (Laskowitz *et al.*, 2000). Conversely, consistent with APOE exerting immunosuppressive functions, a recent study indicated *Apoe*-deficiency to result in enhanced MHCII-mediated antigen presentation and subsequent CD4+ T cell activation (Bonacina *et al.*, 2018), and APOE has been shown to suppress T cell profileration (Laskowitz *et al.*, 2000).

In melanoma, APOE suppresses primary and metastatic progression. Melanoma cells with enhanced metastatic capacity upregulate a set of microRNAs that collectively repress the expression of the *Apoe* gene (Pencheva *et al.*, 2012). Mechanistically, APOE suppresses melanoma invasion by binding to the LRP1 receptor on cancer cells. In addition to this tumor-autonomous role, APOE suppresses endothelial recruitment by binding to LRP8 on endothelial cells (Pencheva *et al.*, 2012). Consistent with the suppressive role of extracellular APOE, genetic inactivation of *Apoe* in the stroma also promotes melanoma progression. Interestingly, both cancer- and stroma-derived APOE participate in the suppression of melanoma metastasis, while the contribution of tumoral APOE to suppression of primary tumor growth seems negligible (Pencheva *et al.*, 2014). In addition to its impact on melanoma invasion and angiogenesis, APOE also promotes anti-melanoma immunity by depleting MDSCs via its binding to the LRP8 receptor (Tavazoie *et al.*, 2018). Of note, APOE may also impact outcome of other cancer types, since *Apoe*-deficient mice exhibit increased progression of breast and ovarian cancer (Alikhani *et al.*, 2013; Lai *et al.*, 2018).

Importantly, the anti-tumor capacity of APOE can be exploited therapeutically. Liver-X-receptors (LXR) are transcription factors that promote the expression of *Apoe* (Evans and Mangelsdorf, 2014), and pharmacologic activation of LXRs suppresses melanoma progression through the transcriptional activation of *Apoe* (Pencheva *et al.*, 2014).

1.5 Germline variants of *APOE*

In humans, there are three prevalent variants of APOE that differ from one another in just one or two amino acids at positions 112 and 158 (Utermann *et al.*, 1977; Weisgraber *et al.*, 1981; Utermann *et al.*, 1984). The APOE2 variant has cysteines at both positions, the APOE3 variant has a cysteine at the 112 and an arginine at the 158 position, and APOE4 has arginines at both positions (Figure 1.2). These subtle sequence differences have major implications, since they impact the tertiary structure of APOE and thereby modulate the binding affinity to its receptors (Chen *et al.*, 2011; Weisgraber *et al.*, 1982; Kowal *et al.*, 1990; Xian *et al.*, 2018). Notably, the two *APOE*-defining SNPs seem in almost perfect linkage disequilibrium, since the *APOE1* genotype with the combination of an arginine at position 112 and a cysteine at position 158 has been described in only four individuals to date (Belloy *et al.*, 2019).

The worldwide distribution of *APOE* genotype varies substantially, with the *APOE3* variant being the most common in all human populations (Corbo and Scacchi, 1999). In Western

populations, the allelic frequencies of *APOE2* and *APOE4* are approximately 8 % and 14 %, respectively, rendering approximately 40 % of the Western population carriers of either the *APOE2* or *APOE4* allele (Farrer *et al.*, 1997). The allelic variation of *APOE* only exists in humans. Evolutionarily, the *APOE4* variant is considered the ancestral human allele (Hanlon and Rubinsztein, 1995). However, while the sequence of primate and rodent *APOE* is most similar to human *APOE4*, the tertiary struture and resulting function of APOE in these species resembles APOE3 more closely (Raffai *et al.*, 2001; McIntosh *et al.*, 2012).

Germline variation of *APOE* has major pathophysiological implications. The *APOE4* variant is the biggest monogenetic risk for Alzheimer's disease, while the *APOE2* variant is protective (Strittmatter *et al.*, 1993; Corder *et al.*, 1994). The risk for Alzheimer's disease increases by approximately 2- to 4-fold with one copy of *APOE4*, and by approximately 8- to 12-fold with two copies (Farrer *et al.*, 1997). In addition to their role in Alzheimer's disease, *APOE* variants modulate other inflammation-associated pathologies, including atherosclerosis, in which a similar association between *APOE*-genotype and risk is seen (*APOE4* > *APOE3* > *APOE2*) (Mahley, 2016; Bennet *et al.*, 2007; Xu *et al.*, 2016). *APOE* genotype is also one of the most robust predictors of longevity, with *APOE4* and *APOE2* carriers exhibiting the shortest and longest lifespans, respectively (Deelen *et al.*, 2019).

Despite these firmly established epidemiologic associations and more than 10,000 studies published on the association between *APOE* and Alzheimer's disease, the mechanisms through which *APOE* affects these outcomes remain incompletely understood. Indeed, in Alzheimer's disease, it is still debated whether the pathophysiological impact of *APOE4* represents a gain or loss of function (Belloy *et al.*, 2019). However, mice in which the endogenous murine *Apoe* locus is replaced with one of the three human *APOE* variants allow for the mechanistic dissection of the action of *APOE* genotype, and their use has confirmed the causal role of APOE in the aforementioned pathologies (Sullivan *et al.*, 1997, 1998; Knouff *et al.*, 1999).

Although an understanding of the molecular mechanism of APOE on these disease is lacking, APOE variants haven been shown to modulate specific organismal processes. Most importantly, given the role of APOE in modulating immune responses (see 1.4), it is not surprising that variants of APOE differentially impact immunity. Carriers of the *APOE4* genotype exhibit increased cytokine secretion relative to *APOE3* homozygotes (Gale *et al.*, 2014) and altered microglial phenotype (Krasemann *et al.*, 2017; Shi *et al.*, 2017). Additionally, *APOE4* carriers show expansion of CD4+ effector memory T cells and enhanced MHC-II mediated antigen presentation (Bonacina *et al.*, 2018). Consistently, human *APOE4* targeted-replacement mice show enhanced pro-inflammatory cytokine expression in microglia and macrophages upon LPS exposure (Vitek *et al.*, 2009; Ophir *et al.*, 2005).

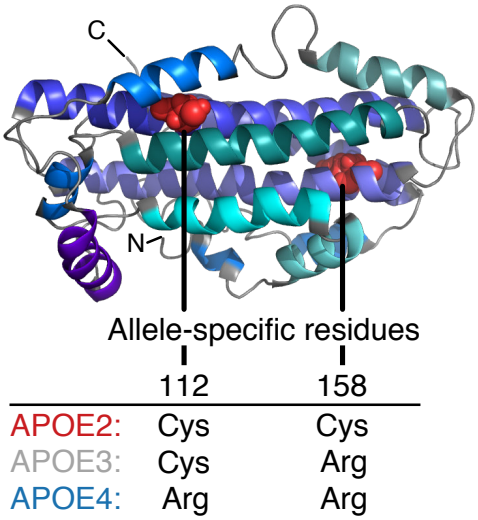

Figure 1.2. APOE variants. Representation of APOE3 according to structural analyses by Chen and colleagues (Chen *et al.*, 2011). There are three prevalent variants of APOE, which differ from one another in the presence of either cysteine or arginine residues in positions 112 and 158, as indicated above.

1.6 Overview and biological significance

As outlined above, APOE suppresses melanoma progression. Given the existence of three highly prevalent variants of *APOE*, this thesis investigates the hypothesis that *APOE* variants differentially modulate melanoma progression and outcome. Despite the established pathophysiological impact of *APOE* variants on several inflammation-related disease and their high prevalence, the association between *APOE* germline status and cancer outcome has remained inconclusive (Kulminski *et al.*, 2014; Anand *et al.*, 2014). To address this, we here analyze melanoma progression in genetic mouse models expressing human *APOE* germline variants and melanoma clinical trials to show that the *APOE4* variant confers enhanced melanoma outcome. In contrast, the *APOE2* variant confers detrimental outcome. These differences were due to differential anti-melanoma immune activation, and *APOE4* carriers showed enhanced immunotherapy outcome, indicating a potential role of *APOE* germline status in guiding melanoma therapy. Our findings show that the pathological impact of *APOE* variants is reversed in melanoma in comparison to Alzheimer's disease. More generally, *APOE* variants represent the first example of highly common germline variants that modulate the outcome of a common cancer type rather than its incidence.

2 *APOE* germline variants modulate progression of melanoma in-vivo

Previous studies from the Tavazoie laboratory showed that Apolipoprotein E (APOE) suppresses melanoma progression (Pencheva *et al.*, 2012, 2014). In humans there are three highly prevalent variants of APOE that differ from one another in one or two amino acids. This chapter explores the hypothesis that these variants differentially impact progression of melanoma. To this end, progression of several independent melanoma models was assessed in mice expressing distinct human *APOE* variants.

2.1 Human *APOE* germline variants modulate progression of murine YUMM1.7 melanoma in-vivo

To assess whether *APOE* genotype modulates melanoma progression, we injected syngeneic mouse YUMM1.7 melanoma cells into mice in which the endogenous mouse *Apoe* locus was replaced with one of the human *APOE* variants, resulting in human *APOE* targeted replacement (knock-in) mice (Knouff *et al.*, 1999; Sullivan *et al.*, 1997, 1998). The YUMM1.7 cell line was originally derived from a genetically induced $Braf^{V600E};Pten^{-/-};Cdkn2^{-/-}$ tumor (Dankort *et al.*, 2009). Consistent with a causal impact of *APOE* genotype on melanoma progression, tumors grew significantly faster in mice with the *APOE2* genotype relative to *APOE4* mice (Figure 2.1). Tumor growth in *APOE3* mice was intermediate between *APOE2* and *APOE4* mice, but the differences between tumors in *APOE3* mice and the other two genotypes were not significant. We therefore decided to focus on assessing tumor growth in *APOE2* versus *APOE4* mice for most subsequent experiments.

2.2 Human *APOE* germline variants modulate progression of murine YUMM3.3 and YUMMER1.7 melanomas in-vivo

To assess whether the impact of *APOE* germline status on melanoma progression was true across different models of melanoma, we next engrafted *APOE*-knock-in mice with cells from the independent YUMM3.3 melanoma model, which was originally derived from a genetically induced $Braf^{V600E};Cdkn2^{-/-}$ tumor (Meeth *et al.*, 2016). Consistent with our observations in the YUMM1.7 model, YUMM3.3 tumors progressed significantly faster in *APOE2* relative to *APOE4* mice (Figure 2.2a). Similarly, we observed faster tumor growth in *APOE2* relative to *APOE4* mice using the YUMMER1.7 melanoma model, a more immunogenic derivative of the YUMM1.7 model (Wang *et al.*, 2017) (Figure 2.2b). These data indicate that *APOE* genotype impacts melanoma progression across several syngeneic murine melanoma models.

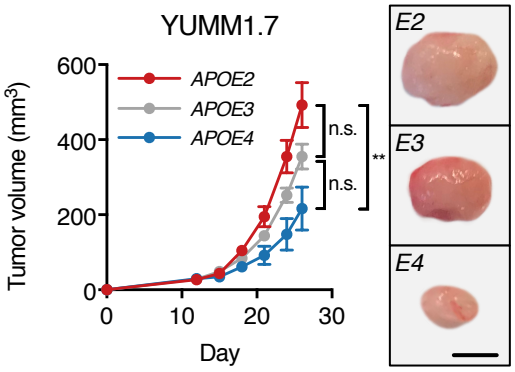

Figure 2.1. Stromal *APOE* genotype modulates progression of murine YUMM1.7 melanoma. Growth of syngeneic mouse YUMM1.7 tumors in human *APOE* targeted replacement mice (n = 11 per group, two-tailed t-test; representative of two independent experiments). Images correspond to representative tumors on day 26 after injection (scale bar, 3 mm).

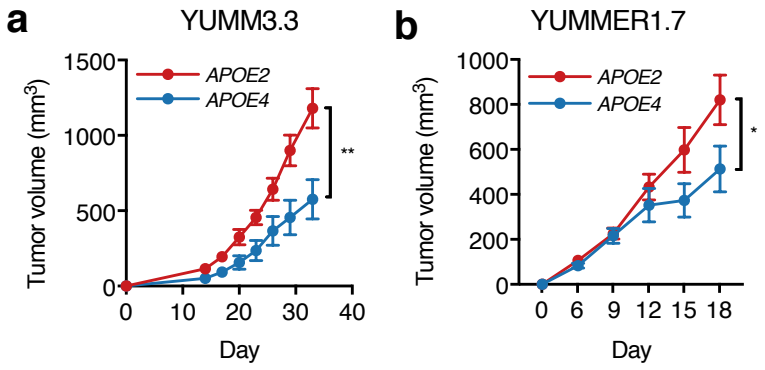

Figure 2.2. Stromal *APOE* genotype modulates progression of YUMM3.3 and YUMMER1.7 melanomas. (a, b) Growth of YUMM3.3 **(a)** and YUMMER1.7 **(b)** tumors engrafted in *APOE2* and *APOE4* targeted replacement mice (n = 13 per group for (a) and $\geq$ 11 for (b); two-tailed t-tests; each representative of two independent experiments).

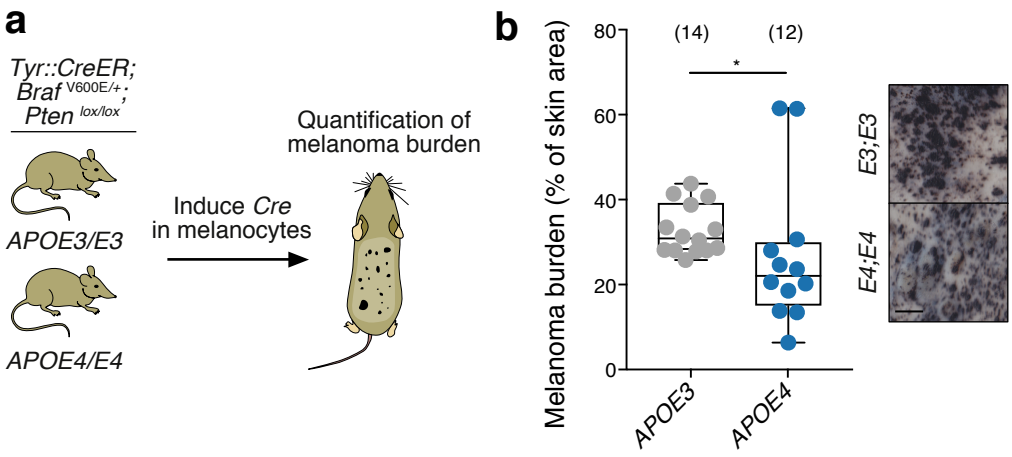

Figure 2.3. Human *APOE* germline variants modulate progression in a genetically induced melanoma model. (**a**) Experimental approach to assess the impact of human *APOE* alleles on genetically induced mouse melanoma progression. (**b**) Melanoma burden of mice described in (a) on day 35 after induction (P = 0.01, one-tailed Mann-Whitney test). Images of representative skins are shown on the right. Circles correspond to individual mice and numbers in parentheses indicate samples sizes.

2.3 Human *APOE* germline variants modulate progression of genetically induced syngeneic melanomas in-vivo

The *APOE3* and *APOE4* variants are the most prevalent germline variants of *APOE*, prompting us to assess more thoroughly whether these genotypes differentially impact melanoma progression. We reasoned that a genetically induced mouse model of melanoma might provide more resolution than a transplanble model to detect a potential difference in tumor progression. To this end, we crossed *APOE3* and *APOE4* knock-in mice with the *Braf*^{V600E}*;Pten*$^{-/-}$ mouse model, in which melanocyte-specific expression of *Cre* can be induced through the administration of Tamoxifen, resulting in the deletion of the tumor suppressor *Pten* and subsequent formation of melanomas (Figure 2.3a) (Dankort *et al.*, 2009). Mice with the *APOE3* genotype showed significantly increased melanoma burden on day 35 after induction in comparison with *APOE4* mice (Figure 2.3b), consistent with *APOE* genotype significantly impacting progression in this genetically induced model.

2.4 Human *APOE* germline variants modulate metastatic progression of murine melanoma

To assess whether *APOE* germline variants also modulate progression of melanoma metastasis, we used the syngeneic murine B16F10 melanoma model, which readily metastasizes to th lung upon tail vein injection (Fidler, 1975). We observed that expression of *Apoe* was significantly higher in B16F10 cells relative to YUMM1.7 cells (Figure 2.4a). To limit the contribution of

murine APOE to total APOE levels in-vivo, we decided to use previously described B16F10-shApoe cells, in which expression of *Apoe* is silenced using RNA-interference (Pencheva *et al.*, 2012). Consistent with our findings in the primary tumor models, metastatic progression of B16F10 melanomas was faster in *APOE2* mice relative to *APOE4* mice (Figure 2.4b), indicating that *APOE* genotype also modulates metastatic progression of melanoma.

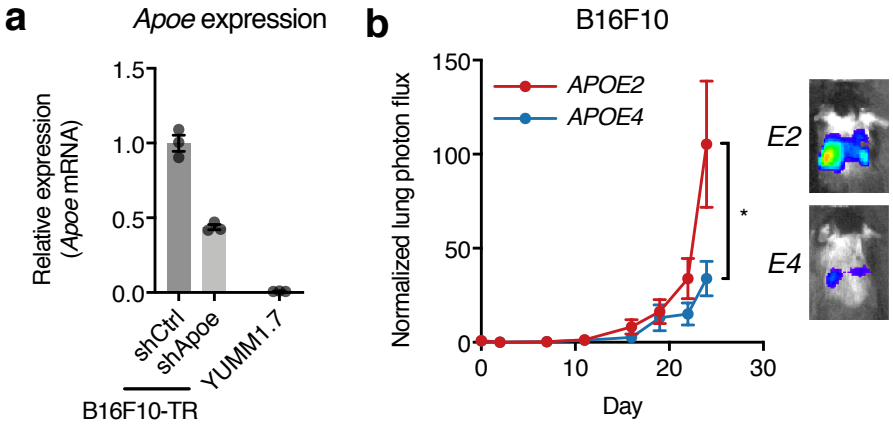

Figure 2.4. Human *APOE* variants modulate metastatic progression of murine melanoma. (a) Relative expression of murine Apoe as determined by qRT-PCR in B16F10 cells expressing shCtrl and shApoe hairpins and in YUMM1.7 cells. **(b)** Bioluminescence imaging of metastatic progression of murine melanoma B16F10-TR-shApoe cells intravenously injected into *APOE* knock-in mice (n = 10 per group; one-tailed Mann-Whitney test; representative of two independent experiments). Images correspond to representative mice on day 24 after injection.

3 *APOE* germline variants modulate the immune microenvironment in melanoma

APOE exerts pleiotropic functions in metabolism and immunity (Mahley, 2016; Shi and Holtzman, 2018). In cancer, APOE suppressess progression by inhibiting cancer cell invasion and angiogenesis (Pencheva *et al.*, 2012). It also promotes anti-tumor immunity by depleting immunosuppressive myeloid-derived suppressor cells (Tavazoie *et al.*, 2018). This chapter assesses the mechanism through which different alleles of *APOE* impact melanoma progression. Using flow cytometry, single cell RNA-sequencing and selective depletion experiments, we show that *APOE* genotype modulates melanoma progression through the modulation of anti-tumor immunity.

3.1 The melanoma microenvironment in *APOE4* mice exhibits an anti-tumor immune phenotype

To assess whether *APOE* genotype affects the melanoma immune microenvironment, we performed flow cytometry on dissociated YUMM1.7 melanomas hosted by *APOE2* and *APOE4* mice (gating strategy depicted in Figure 3.1). Remarkably, *APOE4* mice exhibited increased recruitment of leukocytes into the tumor (Figure 3.2a).

Consistent with a shift towards an anti-tumor immune phenotype, the relative abundance of immune cells known to exert pro-tumor functions such as Ly6G+ granulocytic MDCSs and tumor-associated macrophages was diminished in the *APOE4* microenvironment (Figure 3.2b), while anti-tumor effector cells, such as natural killer cells (NK cells) and T cells, were expanded (Figure 3.2c). Consistent with increased activation in *APOE4* mice, these effector cell populations also expressed higher levels of effector molecules, such as Granzyme B and Interferon-γ (Figure 3.2d-f).

To validate these flow cytometry results, we stained sections of YUMM1.7 tumors hosted by *APOE2* and *APOE4* mice for CD8+ T cells using immunofluorescence. Consistent with the flow cytometry results, tumors hosted by *APOE4* mice exhibited increased infiltration of CD8+ T cells (Figure 3.3).

3.2 Single-cell RNA-sequencing reveals profound modulation of the melanoma immune microenvironment by *APOE* genotype

The tumor immune microenvironment is highly complex (Binnewies *et al.*, 2018). Flow cytometry is limited by the number of parameters that can be simultaneously assessed, and particularly the assessment of the functional status of myeloid cells in the tumor microenvironment is challenging by flow cytometry (Broz *et al.*, 2014; Binnewies *et al.*, 2019). To more comprehensively

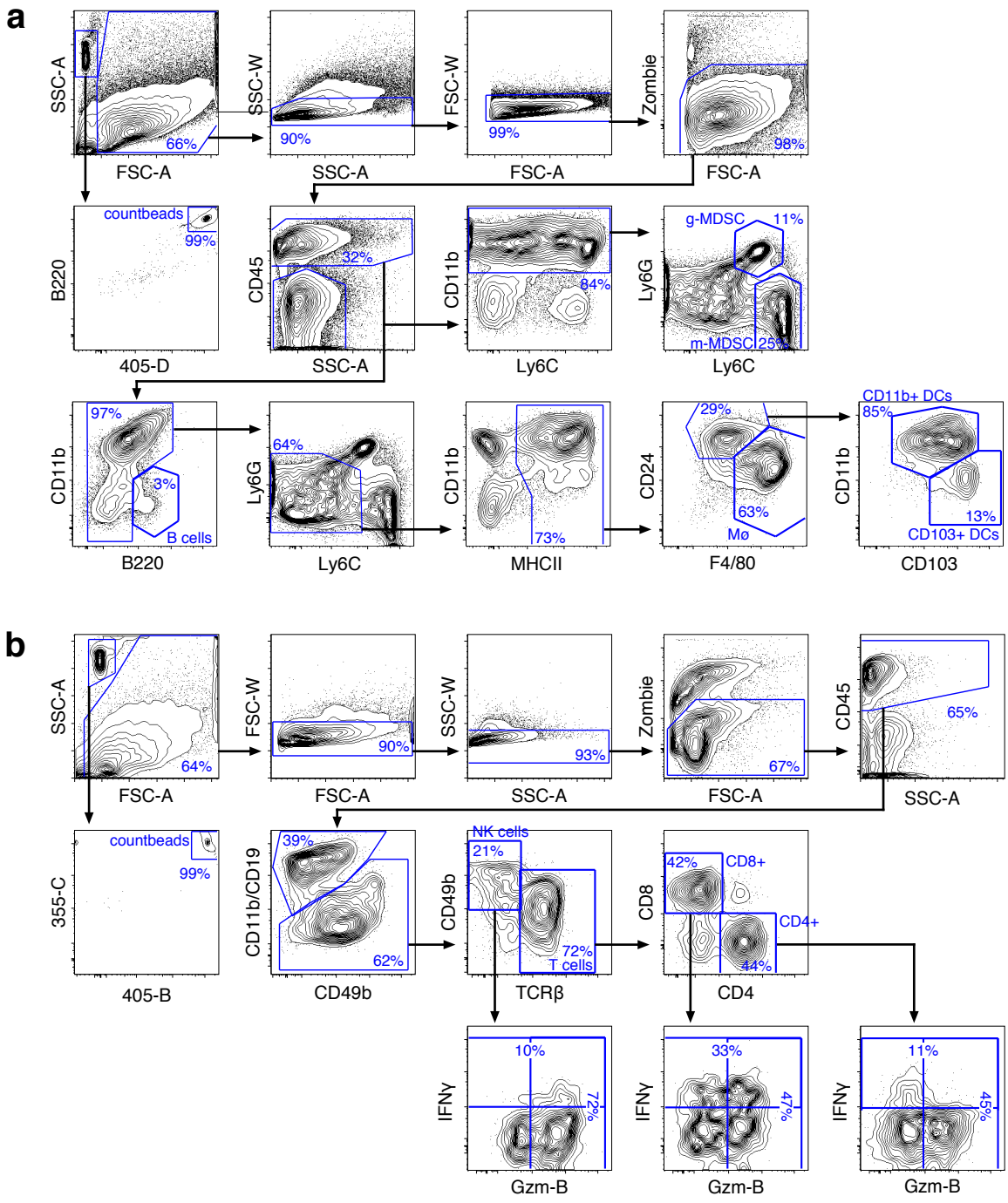

Figure 3.1. Gating strategy for immunoprofiling of the melanoma microenvironment in *APOE2* versus *APOE4* mice. (a-b) Representative flow cytometry plots demonstrating the gating strategy to identify major myeloid **(a)** and lymphoid **(b)** cell subsets in the tumor microenvironment.

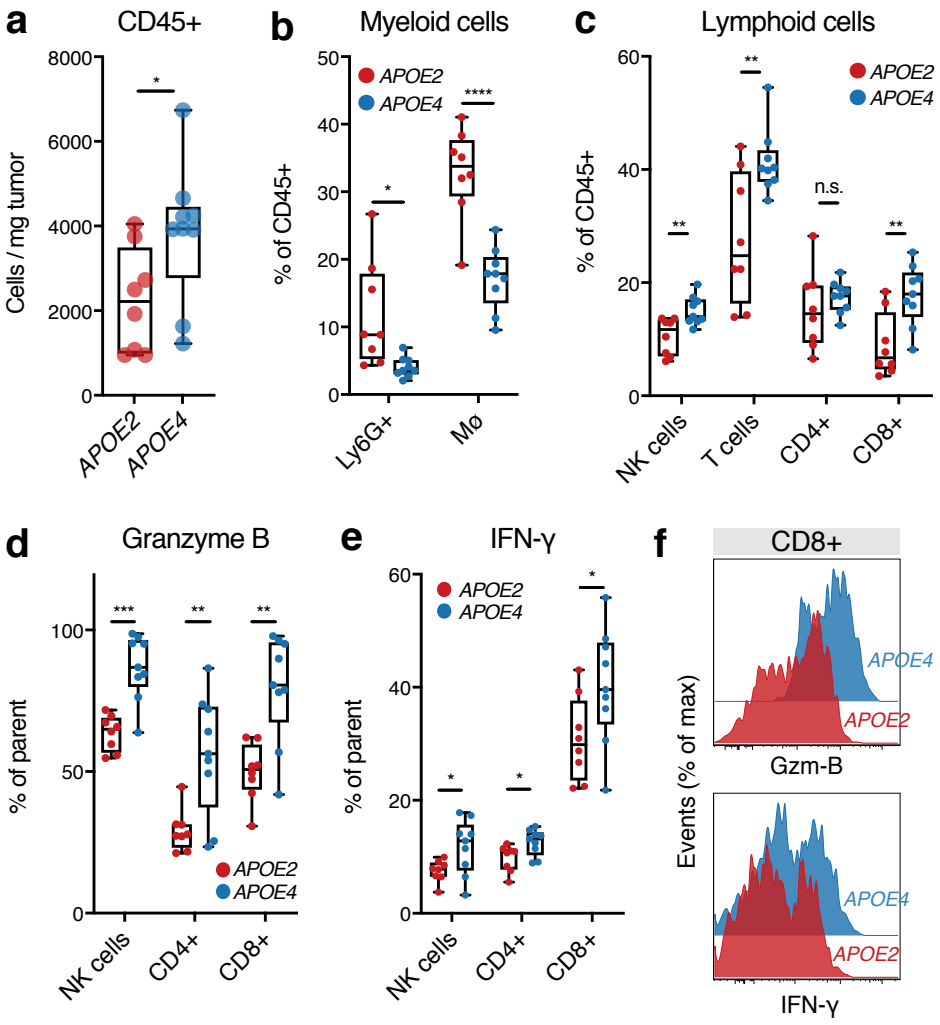

Figure 3.2. Human *APOE* variants modulate the tumor immune microenvironment. **(a)** Abundance of CD45+ leukocytes in YUMM1.7 tumors in *APOE2* and *APOE4* knock-in mice (P = 0.04, two-tailed t-test; representative of two independent experiments). **(b-c)** Proportion of myeloid **(b)** and lymphoid **(c)** immune subsets in YUMM1.7 melanoma-bearing *APOE2* and *APOE4* mice (two-tailed t-tests; representative of two independent experiments). **(d-e)** Expression of Granzyme B (Gzm-B) **(d)** and Interferon-γ (IFN-γ) **(e)** in immune effector cells infiltrating YUMM1.7 melanomas in *APOE2* versus *APOE4* mice (one-tailed t-test; representative of two independent experiments). **(f)** Representative flow cytometry plots illustrating the expression of activation markers in YUMM1.7-infiltrating CD8+ T cells in *APOE2* and *APOE4* mice.

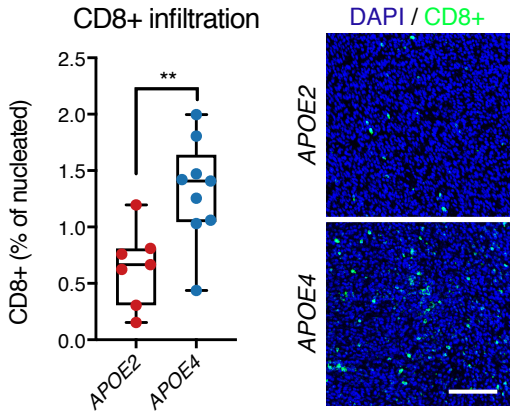

Figure 3.3. Melanomas in *APOE4* mice exhibit enhanced infiltration of CD8+ T cells. Intratumoral CD8+ T cell infiltration in YUMM1.7 tumors from *APOE2* and *APOE4* mice ($n \geq 7$ per group; two-tailed t-test). Images show representative sections (scale bar, 100 µm).

profile the immune microenvironment, we performed single cell RNA-sequencing on CD45+ leukocytes isolated from YUMM1.7 tumors in *APOE2* and *APOE4* mice. We profiled 10,050 CD45+ cells and clustered them into 26 groups using differential gene expression analysis and cross-referencing top differentially expressed transcripts with the immunological genome project (Yoshida *et al.*, 2019) (Figure 3.4). Single cell RNA-sequencing revealed profound heterogeneity in the tumor immune microenvironment, allowing us to capture small cell populations, such as skin-resident macrophages and basophils.

Consistent with our flow cytometry results, the immune microenvironment in *APOE4* mice showed a pronounced shift towards an anti-tumor phenotype, with expansion of anti-tumor effector cells, such as NK and T cells, and depletion of tumor-promoting cells, such as macrophages (Figure 3.5a). Single-cell RNA-sequencing also validated enhanced activation of anti-tumor effector cells in *APOE4* mice (Figure 3.5b-d).

To assess the functional status of different clusters in the tumor immune microenvironment more globally, we performed pathway analysis on the gene expression profiles of clusters in *APOE4* versus *APOE2* mice. Remarkably, many clusters in *APOE4* mice showed enrichment of pathways implicated in anti-tumor immunity, such as Interferon-signaling and allograft rejection (Figure 3.6). Conversely, genes upregulated in clusters of *APOE2* mice showed enrichment for pathways implicated in tumor progression, such as angiogenesis. Overall, single cell RNA-sequencing of CD45+ tumor-infiltrating leukocytes showed a shift in *APOE4* relative to *APOE2* mice in terms of both abundance and activation status towards an anti-tumor phenotype.

3.3 APOE receptors are differentially expressed in tumor-infiltrating leukocytes between *APOE2* and *APOE4* mice

Several receptors are known to bind APOE, many of which bind APOE variants with different affinites (Holtzman *et al.*, 2012). To assess whether *APOE* genotype associated with differential

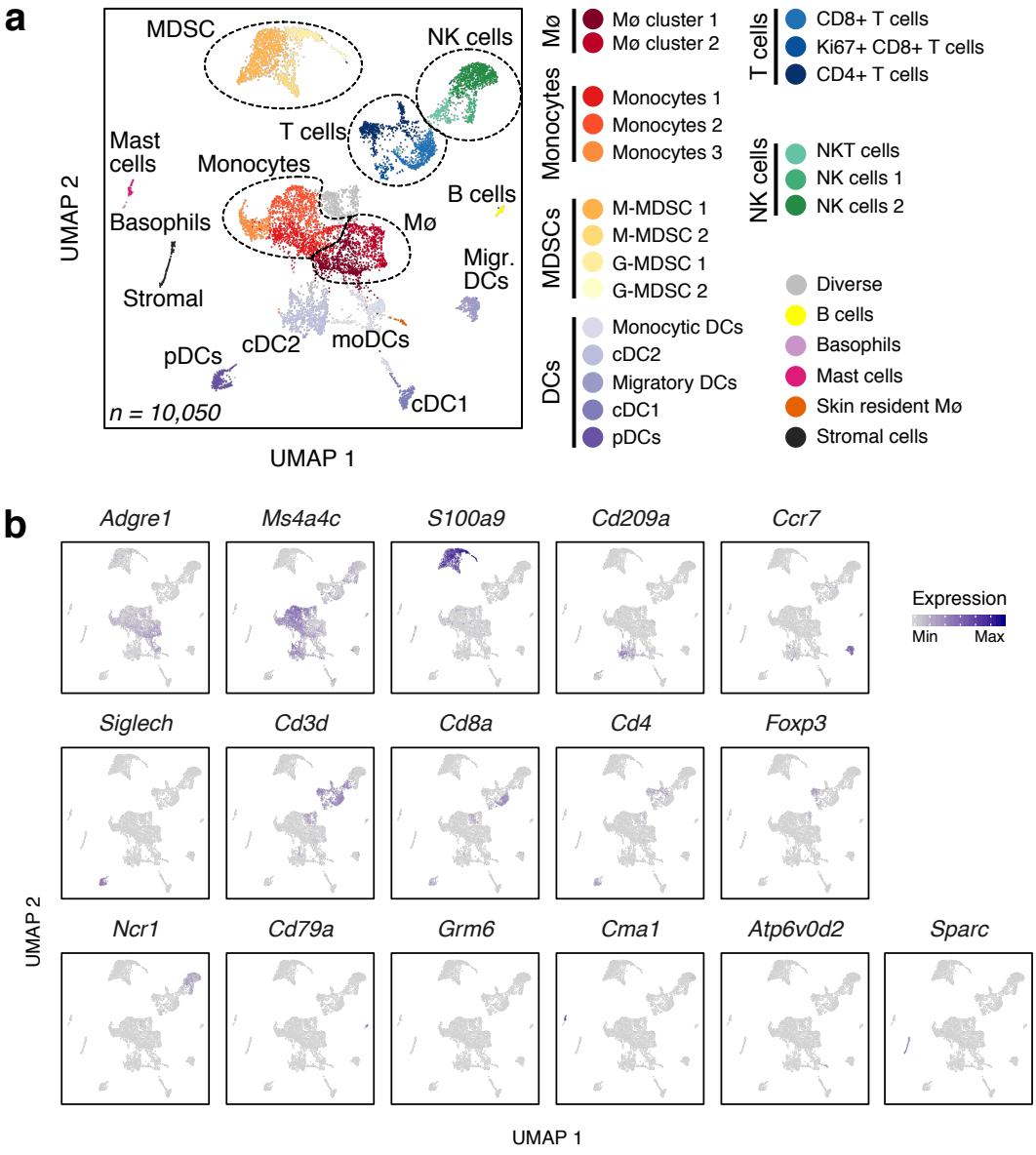

Figure 3.4. Single-cell RNA-sequencing of the melanoma immune microenvironment. (a) Uniform manifold approximation and projection (UMAP) plot of 10,050 CD45+ RNA-sequenced tumor-infiltrating cells from *APOE2* and *APOE4* hosts. **(b)** Uniform manifold approximation and projection (UMAP) plots illustrating the distribution of the expression of manually curated, lineage-defining genes.

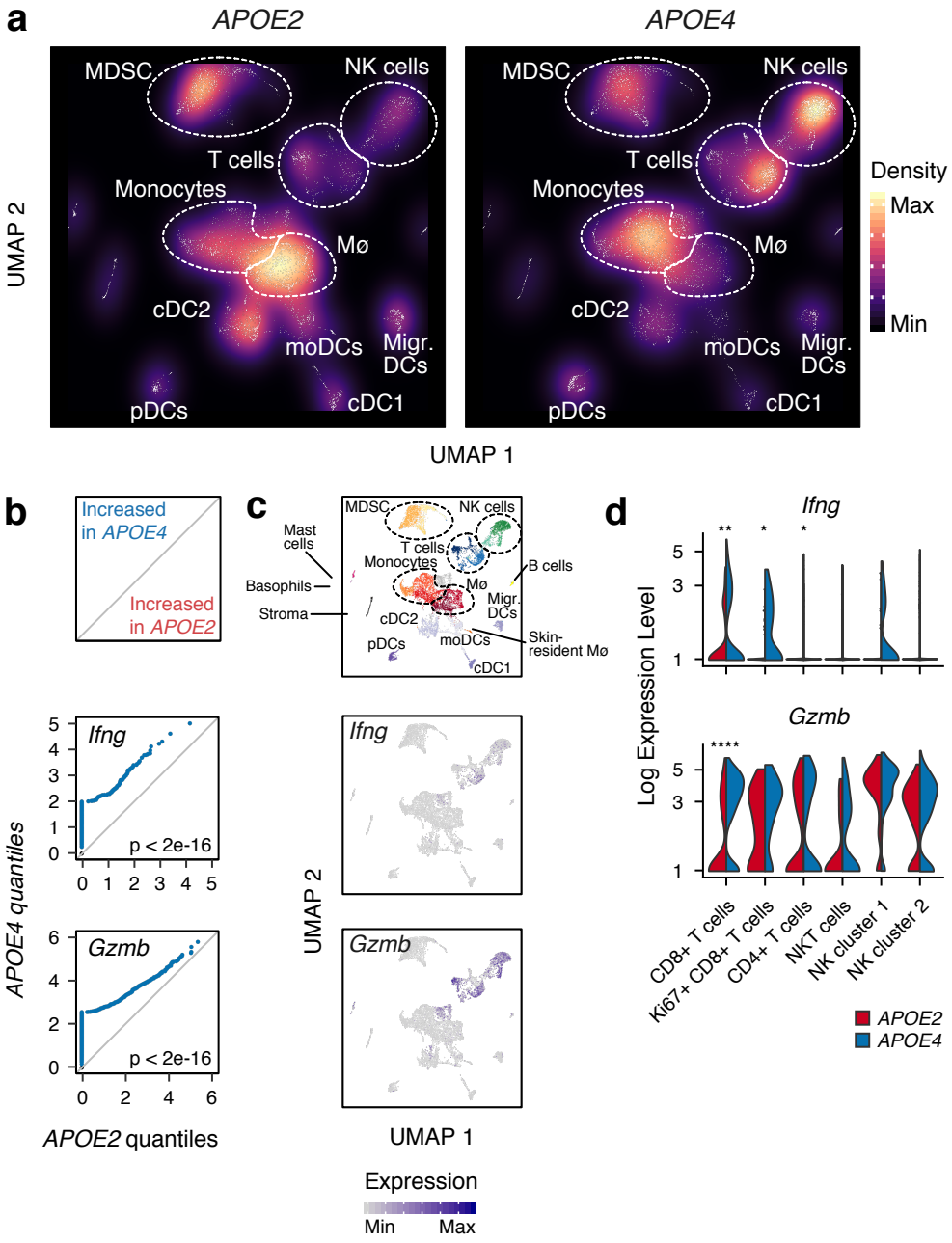

Figure 3.5. Single-cell RNA-sequencing of melanoma-infiltrating leukocytes in *APOE2* and *APOE4* mice. **(a)** Density plot of single cell sequenced immune clusters in tumors of *APOE2* and *APOE4* mice. **(b)** Paired quantile-quantile plots for the expression of *Ifng* and *Gzmb* in CD45+ cells infiltrating tumors in *APOE2* and *APOE4* mice (*P* values according to Wilcoxon rank-sum test). **(c)** Uniform manifold approximation and projection (UMAP) plots illustrating the distribution of *Ifng* and *Gzmb* expression across immune cell clusters. **(d)** Expression of *Ifng* and *Gzmb* across T and NK cell subsets from (b-c) (significance levels according to Wilcoxon rank-sum test adjusted for total number of clusters by FDR).

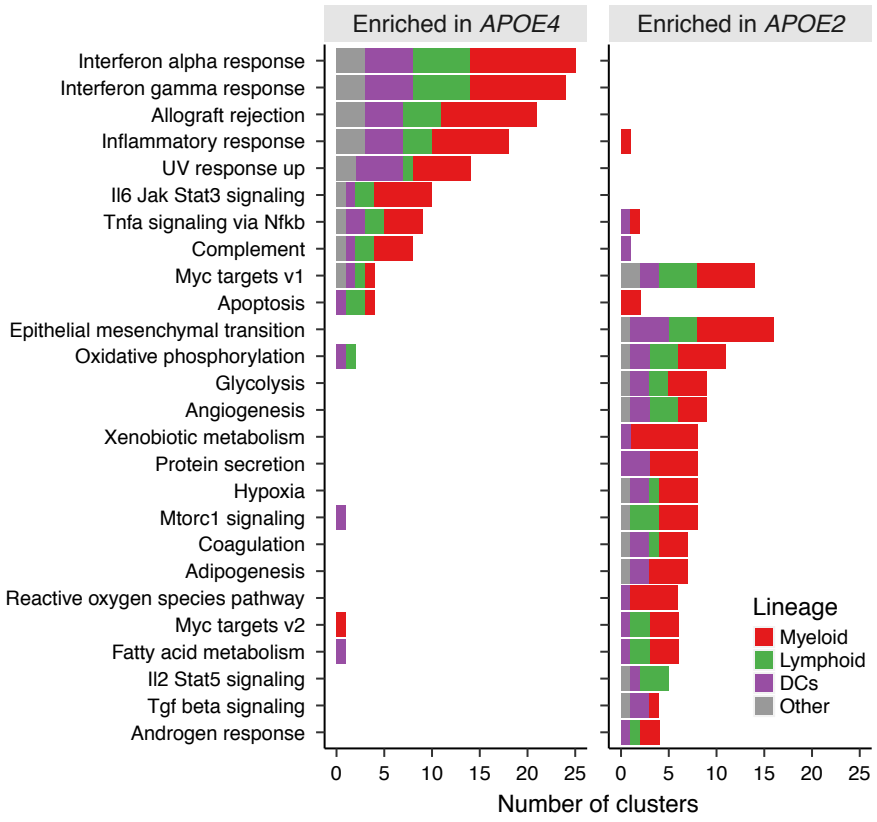

Figure 3.6. Analysis of pathways enriched in differentially expressed genes between immune cell clusters in tumors of *APOE2* and *APOE4* mice. Number of immune cell clusters as identified by scRNA-seq with significant pathway enrichment in genes differentially expressed between *APOE4* and *APOE2* (pathways listed with significance in > 3 clusters).

expression of such APOE receptors, we assessed their expression in the immune microenvironment. Analzying all cells together, we observed upregulation of *Ldlr* and downregulation of *Lrp1* and *Trem2* in leukocytes of *APOE4* mice (Figure 3.7a). Expression of these receptors was mostly limited to myeloid cells (Figure 3.7b), and several individual clusters showed significantly differential expression between *APOE4* and *APOE2* mice (Figure 3.7c). These data suggest that particularly *Lrp1* and *Trem2* may be promising candidates for further study of the mechanistic basis of modulation of anti-tumor immunity by *APOE* genotype.

3.4 The impact of *APOE* genotype on melanoma progression depends on adaptive T cell immunity

We next asked whether the impact of *APOE* genotype on melanoma progression was mediated by modulating anti-tumor immunity. To this end, we assessed YUMM1.7 melanoma progression in *APOE2* versus *APOE4* mice and depleted CD4+ and CD8+ T cells in two cohorts of mice. Weekly administration of anti-CD4+ and anti-CD8+ antibodies resulted in efficient depletion of T cells, as evidenced by flow cytometry of samples from tumors, lymph nodes, and spleens (Figure 3.8a). Remarkably, while melanomas expectedly progressed faster in *APOE2* than in *APOE4* mice receiving PBS injections, this effect was completely abrogated in mice depleted for T cells (Figure 3.8b). These data suggest that the adaptive immune system is a critical mediator of the impact of *APOE* genotype on melanoma progression.

3.5 Distinct *APOE* genotypes in the hematopoietic cell compartment are sufficient to differentially impact melanoma progression

Next to the liver, hematopoietic cells are a major source of APOE in the body (Holtzman *et al.*, 2012). We therefore sought to assess whether distinct *APOE* genotypes in the hematopoietic compartment would be sufficient to differentially impact melanoma progression. To this end, we transplanted hematopoietic stem cells from *APOE2* or *APOE4* donors into lethally irradiated C57Bl6j wildtype mice. Upon reconstitution, we injected these mice with YUMM1.7 melanomas. Consistent with our findings in *APOE2* versus *APOE4* mice, YUMM1.7 melanomas progressed faster in mice transplanted with *APOE2* hematopoietic stem cells than in *APOE4* transplanted mice (Figure 3.9). These data implicate hematopoietic *APOE* genotype as a modulator of melanoma progression.

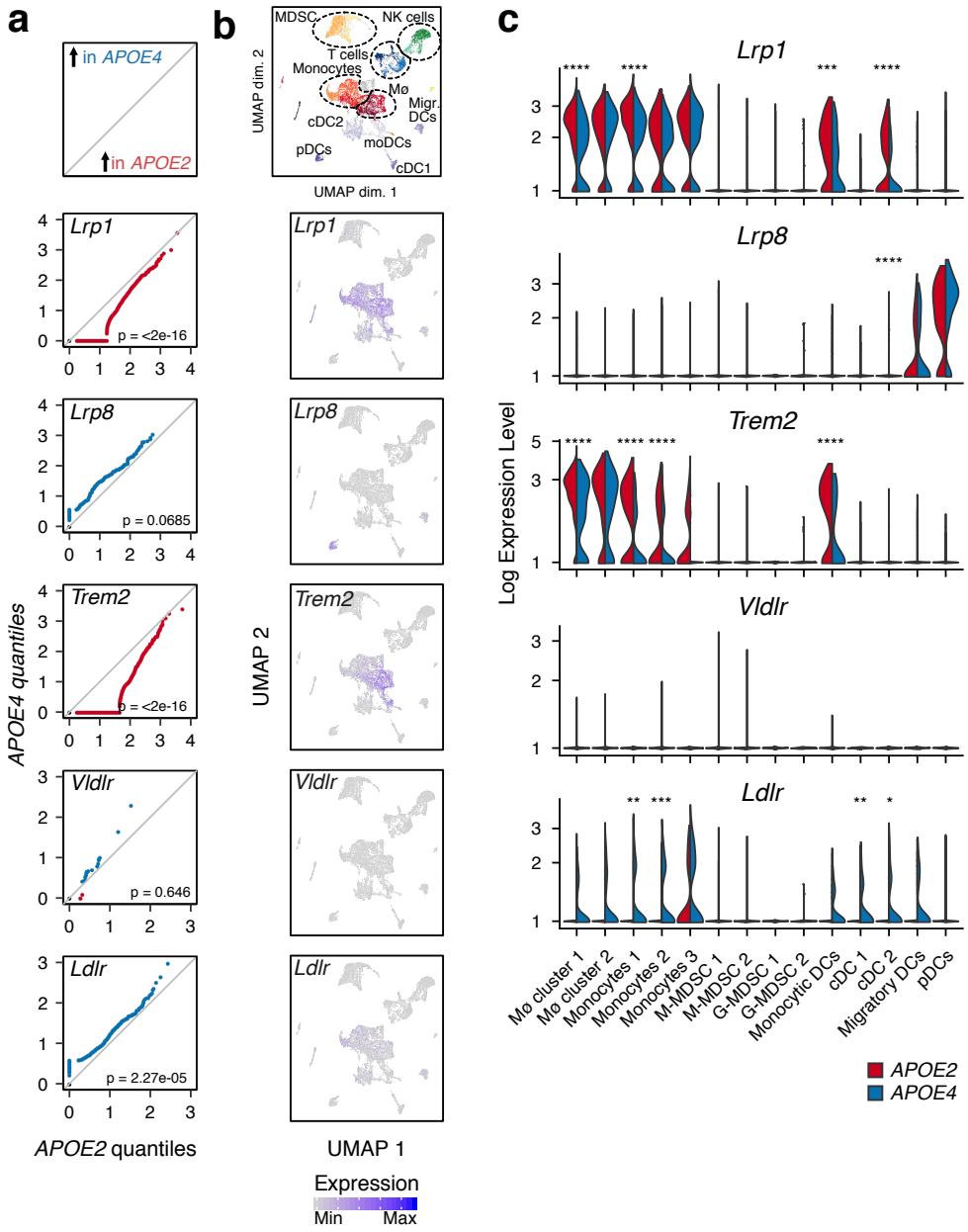

Figure 3.7. Expression of APOE receptors in the immune microenvironment. **(a)** Paired quantile-quantile plots for the expression of APOE receptors in CD45+ cells infiltrating tumors in *APOE2* and *APOE4* mice (*P* values according to Wilcoxon rank-sum test). **(b)** Uniform approximation mapping (UMAP) plots illustrating the distribution of APOE receptor expression across immune cell clusters. **(c)** Expression of APOE receptors cross myeloid and dendritic cell clusters from (a-b) (significance levels according to Wilcoxon rank-sum test adjusted for total number of clusters by FDR).

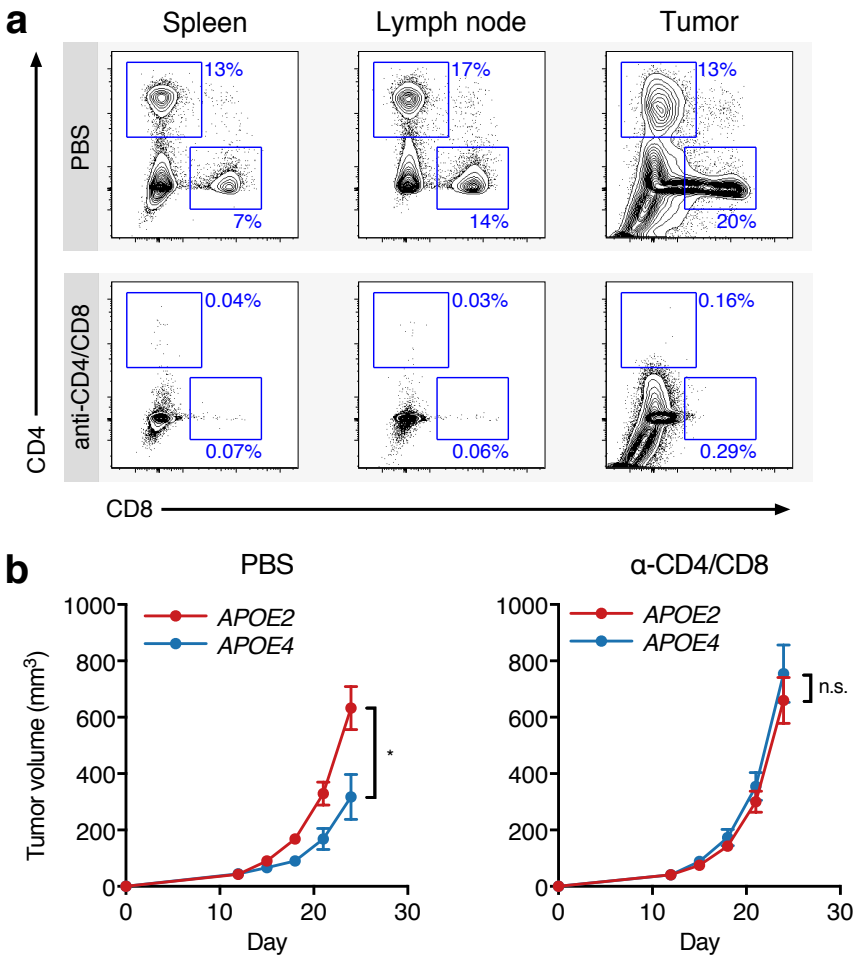

Figure 3.8. T cell depletion abrogates the impact of *APOE* genotype on melanoma progression. (a) Representative flow cytometry results illustrating the efficacy of CD4+ and CD8+ T cell depletion in YUMM1.7 tumor-bearing mice. **(b)** Impact of T cell depletion on YUMM1.7 tumor growth in human *APOE* knock-in mice ($n \geq 10$ per group, two-tailed t-tests; representative of two independent experiments).

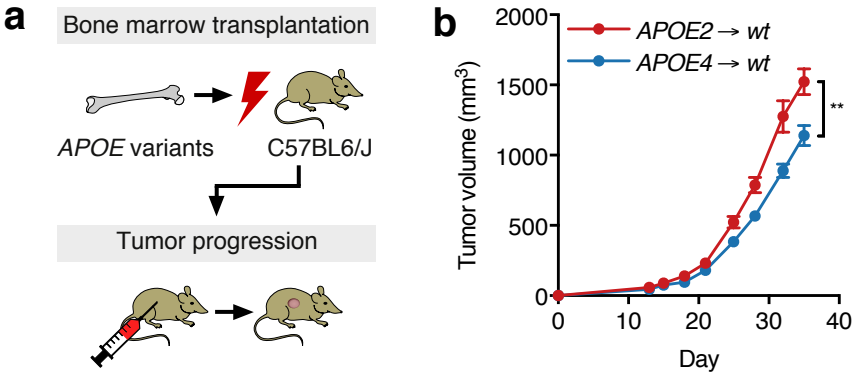

Figure 3.9. Hematopoietic cell *APOE* genotype modulates melanoma progression. (a) Experimental approach to determine the impact of hematopoietic cell-derived *APOE* variants on melanoma progression. **(b)** Growth of YUMM1.7 tumors in C57Bl6j wild-type mice transplanted with bone marrow from human *APOE* knock-in mice (n = 15 per group, two-tailed t-test).

4 *APOE* germline variants associate with outcome in human melanoma

The previous chapters have established that *APOE* germline variants causally modulate melanoma progression by modulating anti-melanoma immunity. This chapter explores whether these results translate to human patients with melanoma. To this end, we analyzed large studies of melanoma patients and correlated *APOE* genotype with melanoma outcome.

4.1 *APOE* germline variants associate with survival in melanoma patients of the TCGA study

To assess the association of *APOE* germline genotype and and melanoma outcome in humans, we analyzed the melanoma study of The Cancer Genome Atlas (TCGA) (The Cancer Genome Atlas Network, 2015) (Figure 4.1a). Using normal tissue whole exome sequencing data, sufficient read depth was available to genotype 460 out of 470 patients of the study (Figure 4.1b). We focused on patients at increased risk of relapse and melanoma-associated death (stage II/III at diagnosis).

We first compared the distribution of *APOE* genotypes in melanoma patients to the general population. In comparison to the study with healthy participants of the Atherosclerosis Risk in

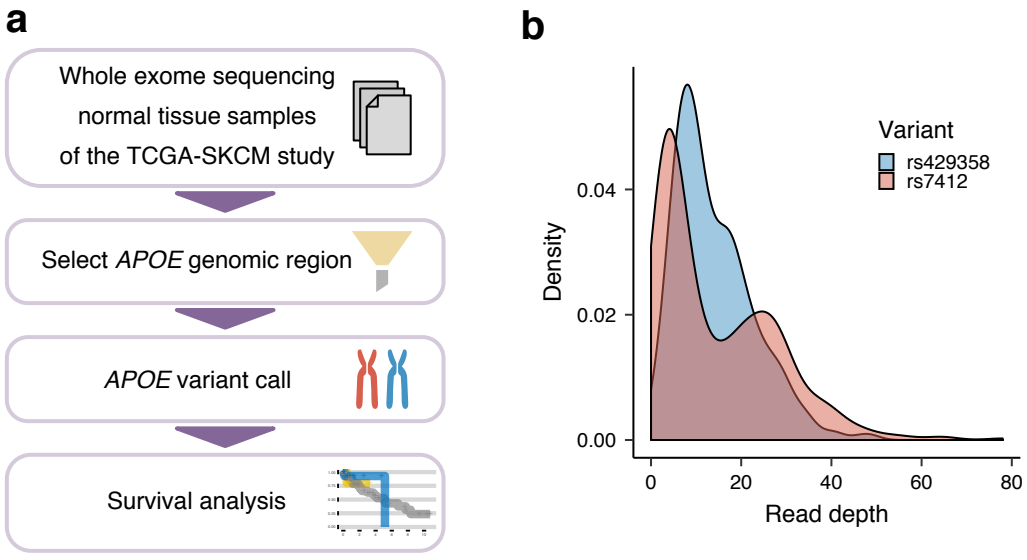

Figure 4.1. Overview of the analysis of melanoma patients in the TCGA study. **(a)** Schematic of approach to analyze melanoma patients in the TCGA study. **(b)** Read depth for the two *APOE* genotype-determining genomic loci in normal tissue whole exome sequencing data of melanoma patients.

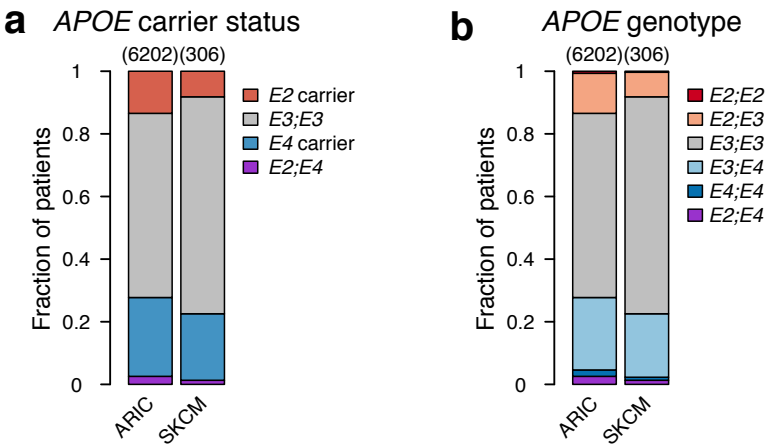

Figure 4.2. Distribution of *APOE* carrier status and bi-allelic genotype in stage II/III melanoma patients of the TCGA study. (a-b) Proportion of *APOE2* and *APOE4* carrier status **(a)** and bi-allelic genotype **(b)** in the Atherosclerosis Risk in Communities study (ARIC) and in patients with stage II/III melanoma in the TCGA-SKCM study (P = 0.0017 and 0.0066, respectively; χ^2 test).

Communities study (ARIC), which is similar in age and ethnic composition (Blair *et al.*, 2005), neither the *APOE2* nor the *APOE4* was enriched in melanoma patients (Figure 4.2), indicating that neither of these variants predisposed for increased melanoma incidence.

We next assessed *APOE* genotype in matched tumor samples of the same patients. The distribution of *APOE* genotypes was comparable between normal and tumor tissue samples (Figure 4.3a), and we identified the same carrier status in sample pairs of 95.6 % of patients with available results (94.2 % of all samples; Figure 4.3b). These congruent findings between normal tissue and tumor samples suggest that our genotyping approach was robust and that loss of heterozygosity events in the tumor were rare.

We then assessed the association between *APOE* genotype and survival outcome. Remarkably, *APOE* genotype was significantly associated with survival in stage II/III melanoma patients, whereby patients carrying at least one *APOE2* or *APOE4* allele showed the shortest and longest survival, respectively (median survival of 2.4, 5.2, and 10.1 years in *APOE2* carriers, *APOE3* homozygotes, and *APOE4* carriers, respectively; P = 0.0038, log-rank test; Figure 4.4a). *APOE2* carriers exhibited an increased hazard ratio versus *APOE3* homozygotes (HR = 2.08, P = 0.01) and versus *APOE4* carriers (HR = 3.69, $P < 0.001$) in this data, as revealed by Cox proportional hazard regression analysis (Figure 4.4b).

To assess variables that may act as potential confounders we investigated the distribution of major clinical and tumoral characteristics in patients stratified by *APOE* genotype. We did not detect significant differences between any of these variables (Figure 4.5). Consistently, multivariate analysis revealed *APOE* genotype to remain significantly associated with survival (Figure 4.6).

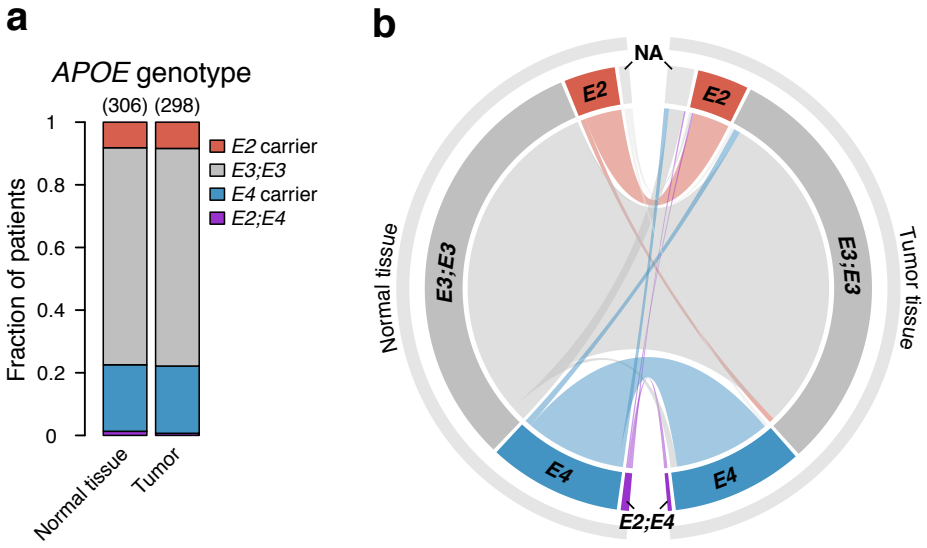

Figure 4.3. *APOE* genotype in matched normal tissue and tumor samples of stage II/III patients in the TCGA-SKCM study. (a) Proportion of *APOE2* and *APOE4* carrier status in normal tissue and tumor samples of patients with stage II/III melanoma in the TCGA-SKCM study (P = 0.8899; χ^2 test). **(b)** Chord diagram of *APOE* carrier status as identified in paired normal and tumor tissue samples of stage II/III melanoma patients in the TCGA-SKCM study.

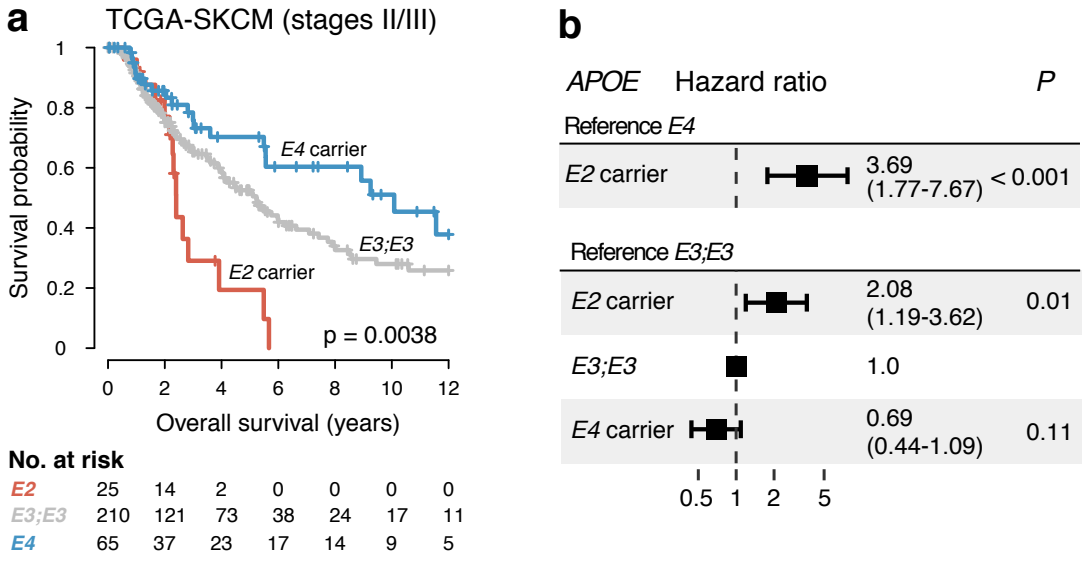

Figure 4.4. *APOE* germline variants predict survival in human melanoma. (a-b) Survival **(a)** and hazard ratios **(b)** of stage II/III melanoma patients in the TCGA-SKCM study stratified by *APOE* carrier status (P values according to log-rank test (a) and Cox proportional hazards model (b); numbers in parentheses indicate 95 % confidence interval).

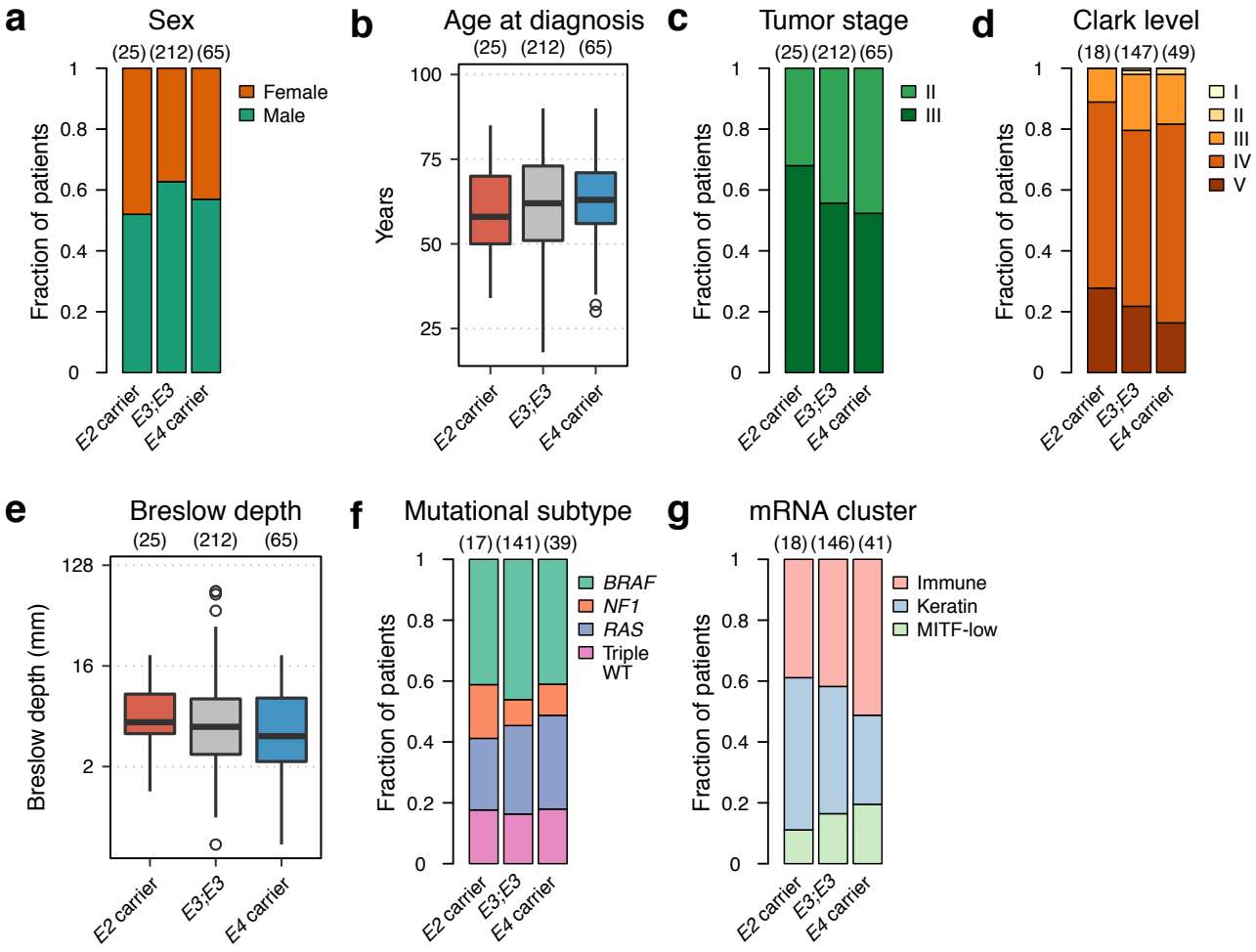

Figure 4.5. Clinical characteristics of stage II/III patients in the TCGA-SKCM study. **(a)** Sex proportions were not significantly different between *APOE* carrier groups (P = 0.46, χ^2 test). **(b)** Age at diagnosis was not significantly different between *APOE* carrier groups (P = 0.45, Kruskal-Wallis rank sum test). **(b)** Tumor stage at diagnosis was not significantly different between *APOE* carrier groups (P = 0.4, χ^2 test). **(d)** Melanoma Clark level at diagnosis was not significantly different between *APOE* carrier groups (P = 0.95, χ^2 test). **(e)** Breslow depth was not significantly different between *APOE* carrier groups at diagnosis (P = 0.24, Kruskal-Wallis rank sum test). **(f)** Proportion of molecular subtypes stratified by *APOE* carrier status in the TCGA-SKCM study (P = 0.93, χ^2 test). **(g)** Proportion of *APOE* carrier status across transcriptional clusters of the TCGA-SKCM study (P = 0.55, χ^2 test). Hinges of boxplots represent the first and third quartiles, whiskers extend to the smallest and largest value within 1.5 × interquartile ranges of the hinges and points represent outliers.

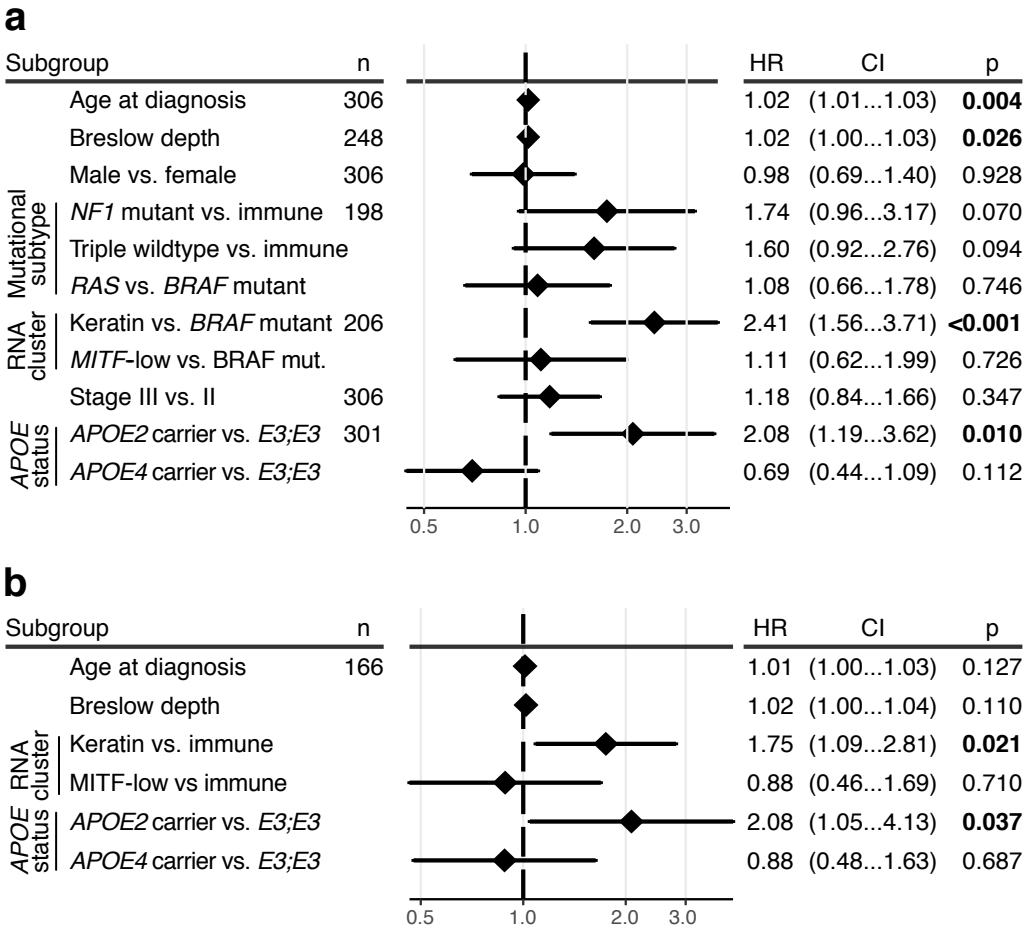

Figure 4.6. Univariate and multivariate survival analysis of stage II/III melanoma patients of the TCGA study. (a) Univariate analysis of the impact of clinical and molecular characteristics on survival of stage II/III melanoma patients (*P* values according to univariate Cox proportional hazards model). **(b)** Multivariable analysis of the impact of clinical and molecular characteristics with significant impact in univariate analysis on survival of stage II/III melanoma patients (*P* values according to multivariable Cox proportional hazards model).

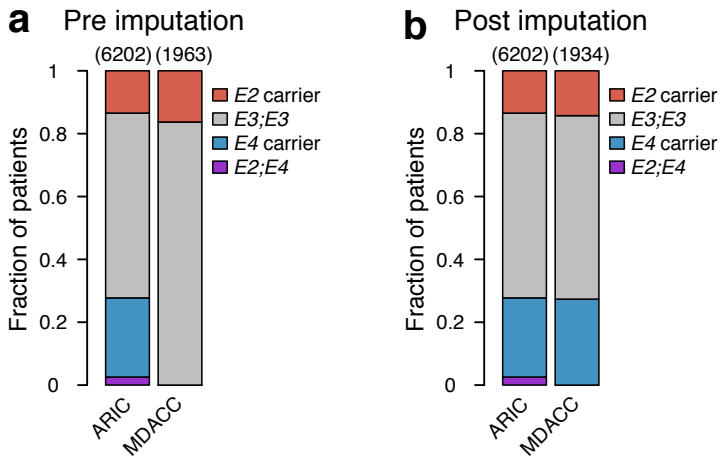

Figure 4.7. *APOE* genotype imputation in the MDACC melanoma GWAS. (a-b) Distribution of *APOE* carrier status in the Atherosclerosis Risk in Communities study (ARIC) and the MDACC melanoma study before **(a)** and after **(b)** imputation of *APOE* genotype ($P < 2.2\times10^{-16}$ and $< 1.821\times10^{-11}$, respectively; χ^2 test).

4.2 *APOE* germline variants are associated with survival in high-risk melanoma patients of an independent study

To validate these findings in an independent study, we analyzed a genome-wide association study (GWAS) reporting on the outcome of melanoma patients (Amos *et al.*, 2011). Array chips often used in GWAS studies have been reported to not adequately genotype the *APOE* defining loci (Radmanesh *et al.*, 2014). Consistent with inadequate genotyping, we found no individual of the large MDACC study to exhibit the minor allele at the rs429358 locus, prompting us to impute genotype at this locus based on a previously validated approach (Radmanesh *et al.*, 2014) (Figure 4.7).

Consistent with the association of *APOE* genotype with survival in the TCGA study, *APOE* genotype was associated with survival in patients at high risk of melanoma-associated death in the MDACC study (Clark level 5), with *APOE2* carriers surviving the shortest. In contrast, *APOE2* patients with early stage melanomas – and therefore low risk of melanoma-associated death – trended towards better survival (Figure 4.8). These findings are consistent with the established association of *APOE2* with enhanced lifespan in the general population (Deelen *et al.*, 2019).

Notably, patients in the TCGA study exihibited features conferring higher risk for melanoma-associated death, such as older age and more advanced Clark level (Figure 4.9a-c). These factors are likely reasons underlying the overall better outcome in patients in the MDACC study relative to the TCGA-SKCM study (Figure 4.9). When restricting the analysis of MDACC patients to those at hightest risk for relapse and melanoma-associated death as defined by advanced age and high Clark level, *APOE* genotype exhibited a similarly strong association with survival outcome as in the TCGA study (Figure 4.9e). Therefore, the known impact of germline genetic variants of *APOE* on survival was reversed in patients with advanced melanoma, who were at increased risk for melanoma-associated death.

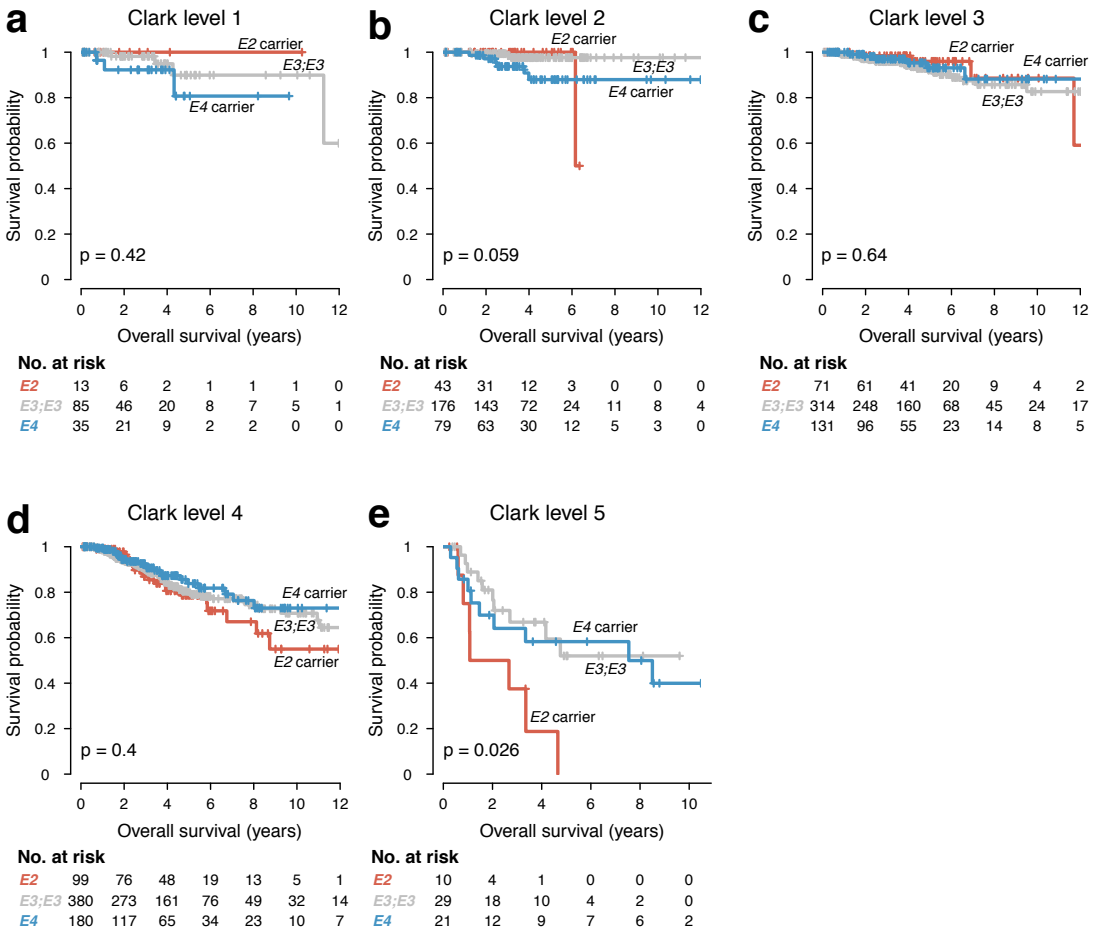

Figure 4.8. Impact of *APOE* genotype on survival of melanoma patients in the MDACC study. Survival of melanoma patients in the MDACC study stratified by local melanoma stage and *APOE* genotype (log-rank tests).

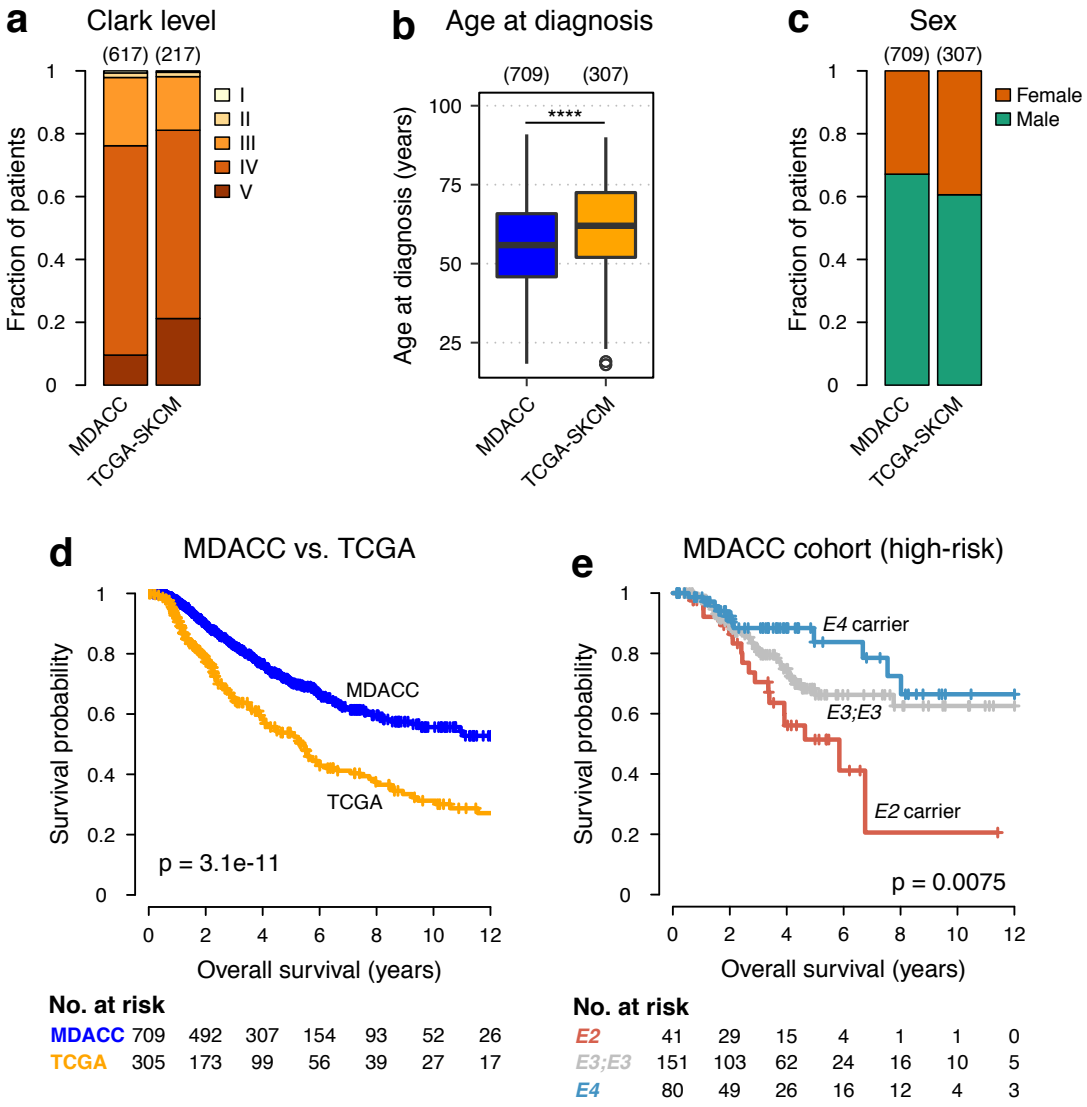

Figure 4.9. Characteristics of the MDACC GWAS study and comparison to the TCGA-SKCM study. **(a-c)** Distribution of age **(a)**, melanoma Clark level **(b)**, and sex **(c)** in stage II/III patients of the MDACC and TCGA-SKCM melanoma studies (respective significance tests: $P < 6.416 \times 10^{-9}$, Kruskal-Wallis rank sum test; $P < 0.00046$, χ^2 test; $P = 0.05231$, χ^2 test). **(d)** Survival of stage II/III melanoma patients in the MDACC and TCGA-SKCM studies (log-rank test). **(e)** Survival of high-risk melanoma patients in the MDACC GWAS study as defined by advanced local melanoma and older age (*P* value according to log-rank test).

5 *APOE* germline variants modulate melanoma outcome in the context of immunotherapy

As outlined in the introduction, immunotherapy has transformed the therapy of melanoma patients (see 1.2.3). Our observations that *APOE* genotype impacts melanoma progression and outcome described above were mainly focused on mice and humans that did not receive immunotherapy. This chapter explores whether the association between *APOE* genotype and melanoma outcome is also true in the context of checkpoint-inhibition immunotherapy. Using mice and human studies, we found that *APOE* genotype also associates with outcome in mice and humans receiving anti-PD1 checkpoint inhibition.

5.1 *APOE* germline variants modulate melanoma progression in the context of PD1-checkpoint inhibition in-vivo

To assess whether *APOE* genotype also modulates progression and outcome of melanoma in the context of anti-PD1 immunotherapy, we injected highly immunogenic YUMMER1.7 melanoma cells into *APOE2* and *APOE4* mice and treated these mice with anti-PD1 inhibition. We used the YUMMER1.7 melanoma model because of its susceptibility to checkpoint inhibition (Wang *et al.*, 2017). A larger fraction of mice with the *APOE4* genotype achieved a complete remission with anti-PD1 inihibition relative to *APOE2* mice, translating into significantly longer survival in *APOE4* versus *APOE2* mice (Figure 5.1)

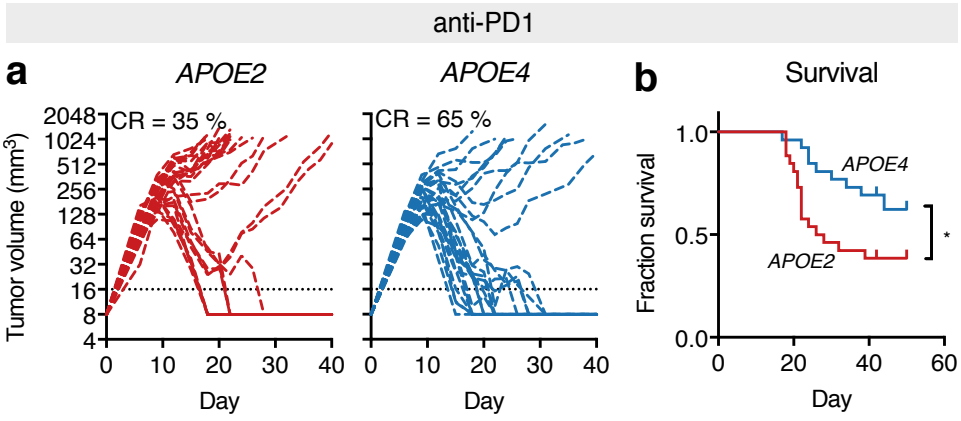

Figure 5.1. *APOE* genotypes modulates melanoma progression in the context of anti-PD1 immunotherapy. (a-b) Tumor growth **(a)** and survival **(b)** of human *APOE* knock-in mice injected with YUMMER1.7 tumors and treated with anti-PD1 antibody (n = 26 per group; P = 0.022, two-tailed log-rank test; data pooled from two independent experiments; CR = complete remission).

5.2 *APOE* germline variants associate with survival in melanoma patients receiving second-line immunotherapy

5.2.1 Analysis of the Roh *et al.* study

To assess whether the association of *APOE* genotype with outcome in melanoma in the context of immunotherapy also applies to humans, we assessed a study of advanced-stage melanoma patients that received anti-PD1 immunotherapy after failing or progressing on first-line immunotherapy with anti-CTLA4 inhibition (Roh *et al.*, 2017). The distribution of *APOE* carrier status was not significantly different between this study and the healthy population in the ARIC study (Blair *et al.*, 2005) (Figure 5.2a). In analogy to our approach analyzing the TCGA study, we also compared genotyping results from matched normal and tumor tissue samples. Genotyping results were identical for 32 out of 34 patients (94 %), confirming our genotyping approach to be robust and tumor loss of heterozygosity events to be rare (Figure 5.2b).

We next assessed clinical response and survival in patients that sequentially received both anti-CTLA4 and anti-PD1 targeting therapy. While there was a trend towards a better response in patients with the *APOE4* genotype according to the RECIST criteria, this was not significant (Figure 5.2c). Remarkably, however, *APOE4* carriers showed the best survival outcomes, while patients with at least one copy of the *APOE2* variant survived the shortest (Figure 5.2d). These data are consistent with our observations in the TCGA and MDACC studies, in which most patients did not receive immunotherapy.

5.2.2 Validation in the Riaz *et al.* study

To validate these results, we analyzed the study by Riaz *et al.*, in which patients received anti-PD1 therapy either as first-line immunotherapy or after failing or progressing on anti-CTLA4 therapy (Riaz *et al.*, 2017). Consistent with our findings in the Roh *et al.* study, in patients receiving PD1-blockade after failing or progressing under anti-CTLA4 therapy, there was no significant difference in the distribution of *APOE* carrier status (Figure 5.3a), but *APOE4* and *APOE2* carriers surved the longest and shortest, respectively (Figure 5.3b). In patients receiving upfront immunotherapy with PD1-inhibition, genotype distribution was also not significantly different between melanoma patients and healthy individuals (Figure 5.4a). However, in contrast to patients receiving second-line immunotherapy with anti-PD1, we did not observe a significant association of *APOE* genotype and survival in these patients (Figure 5.4b). This may be due to either the small sample size, or it may point to a context-specific impact of *APOE* genotype on melanoma progression. Overall, our findings from both mouse models and human clinical data show that *APOE* genotype impacts melanoma progression in the context of anti-PD1 immunotherapy.

5.3 *APOE* germline variants predict response to LXR-agonistic immunotherapy

Activation of Liver-X-receptor (LXR) transcription factors has been shown to promote anti-tumor immunity via transcriptional activation of *APOE* (Tavazoie *et al.*, 2018). We reasoned that

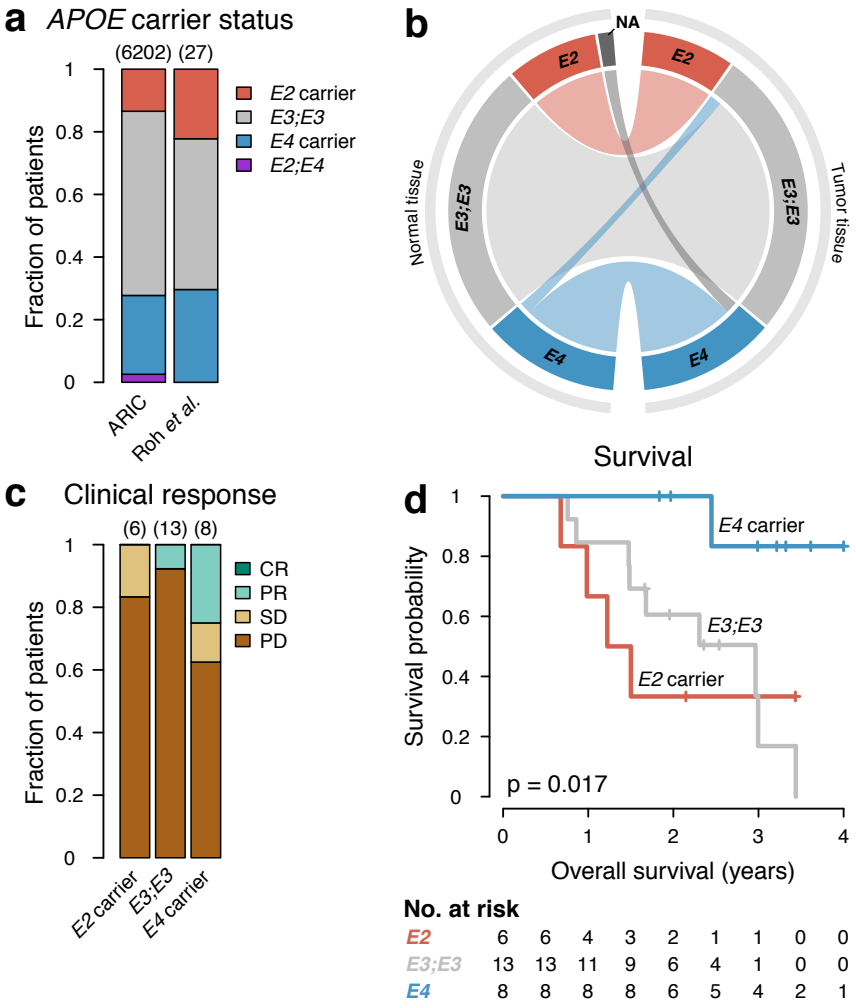

Figure 5.2. Analysis of the impact of *APOE* genotype on outcome of melanoma patients receiving second-line immunotherapy with anti-PD1 in the Roh *et al.* study. **(a)** Comparison of the distribution of *APOE* carrier status in patients of the Roh *et al.* study with healthy individuals of the ARIC study (P = 0.3978, χ^2 test). **(b)** Chord diagram of *APOE* carrier status as identified in paired normal and tumor tissue samples of melanoma patients in the Roh *et al.* study. **(c)** Clinical response to anti-PD1 therapy according to RECIST criteria in melanoma patients stratified by *APOE* genotype (P = 0.171, asymptotic linear-by-linear association test). **(d)** Survival of patients from (a-c) stratified by *APOE* genotype (log-rank test).

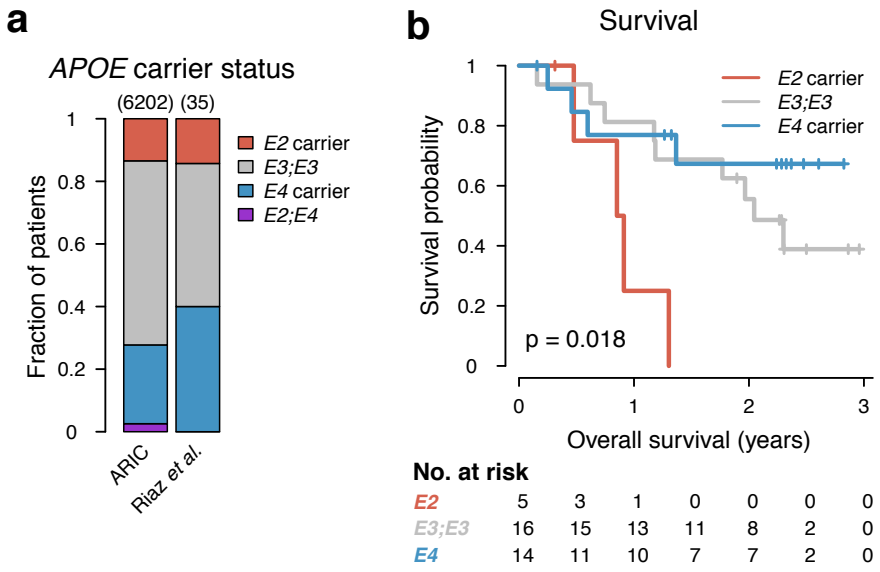

Figure 5.3. Analysis of the imapct of *APOE* genotype on second-line immunotherapy-treated melanoma patients from the Riaz *et al.* study. (a) Comparison of the distribution of *APOE* carrier status in melanoma patients receiving second-line immunotherapy from the Riaz *et al.* study with healthy participants from the ARIC study (P = 0.1744, χ^2 test). **(b)** Survival of patients from (a) stratified by *APOE* carrier status (log-rank test).

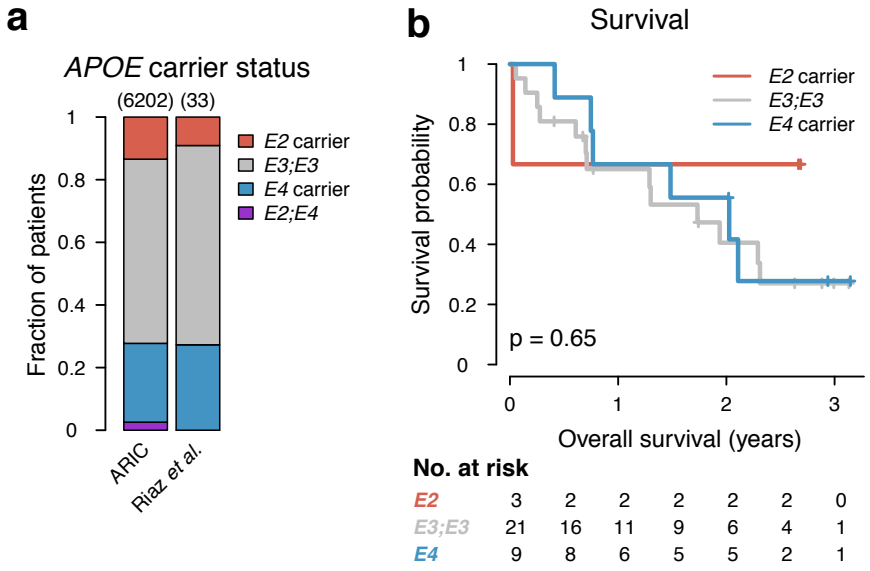

Figure 5.4. Association of *APOE* genotype with outcome in upfront anti-PD1 immunotherapy-treated melanoma patients. (a) Comparison of the distribution of *APOE* carrier status in melanoma patients receiving first-line immunotherapy from the Riaz *et al.* study with healthy participants from the ARIC study (P = 0.6829, χ^2 test). **(b)** Survival of melanoma patients treated with anti-PD1 therapy with no prior checkpoint therapy from the Riaz *et al.* study stratified by *APOE* carrier status (log-rank test).

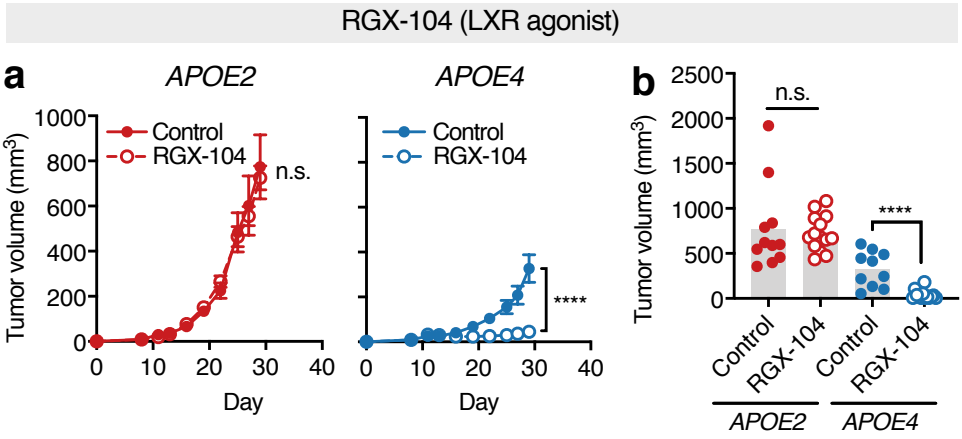

Figure 5.5. *APOE* genotype predicts response to LXR-agonistic immunotherapy. (a) Effect of LXR-agonistic treatment on growth of YUMM1.7 tumors in human *APOE* knock-in mice ($n \geq 10$ per group, two-tailed t-test; representative of two independent experiments). **(b)** Individual tumor volume on day 29 post injection from (a).

APOE genotype may associate with response to LXR-agonistic therapy. To test this, we treated YUMM1.7 melanoma-bearing *APOE2* and *APOE4* mice with the synthetic LXR agonist RGX-104 (Tavazoie *et al.*, 2018). Remarkably, LXR agonistic treatment conferred robust anti-tumor activity in mice with the *APOE4* genotype, while efficacy was completely blunted in *APOE2* mice (Figure 5.5). These data indicate that *APOE* genotype may serve as a biomarker to predict response to LXR-agonistic therapy.

6 Discussion

Germline variants of the *APOE* gene have major pathophysiological implications. The *APOE4* variant, present in approximately 25 % of Western populations, is the biggest monogenetic risk factor for Alzheimer's disease, while *APOE2* is protective. *APOE* variants also impact cardiovascular disease. Despite their high prevalence and their impact on inflammation-associated diseases, the role of *APOE* variants in modulating cancer outcome has been unclear.

APOE was previously shown to suppress melanoma progression and metastasis. Therefore, this thesis investigated the hypothesis that common *APOE* variants differentially modulate melanoma outcome. Using genetic mouse models and large human melanoma trial data, we show that, in a reversal of their impact on neurodegeneration and cardiovascular disease, the *APOE4* and *APOE2* variants confer favorable and poor outcomes in melanoma, respectively, by impacting anti-tumor immunity.

6.1 Overview of major findings

To elucidate whether *APOE* germline variants modulate melanoma progression, we assessed the growth of three transplantable melanoma models in mice expressing human *APOE* variants. Across these models, *APOE4* mice showed slower melanoma progression than *APOE2* mice. Consistent with these primary tumor data, progression of melanoma metastasis was slower in *APOE4* mice relative to *APOE2* mice. Additionally, melanoma burden in a genetically inducible model was lower in *APOE4* mice relative to *APOE3* mice. Importantly, these models allowed us to conclude that the impact of *APOE* genotype on melanoma progression is causal. Previous work showed that stroma-derived APOE is the main modulator of melanoma primary tumor progression (Pencheva *et al.*, 2014). It is consistent with these previous results that we detected significant effects of stromal *APOE* genotype on melanoma progression when using transplantable melanoma models, in which *APOE* genotype is different only in the stroma and not in the tumor compartment.

To assess how *APOE* variants impact melanoma progression, we characterized the tumor immune microenvironment in *APOE* variant mice. Flow cytometry and single cell RNA-sequencing approaches showed enhanced anti-tumor immune activation in *APOE4* relative to *APOE2* mice. Consistent with an essential role of the immune system in mediating the impact of *APOE* variant status on melanoma progression, depletion of T cells completely abrogated this impact. Additionally, by transplanting hematopoietic stem cells from *APOE2* and *APOE4* hosts, we showed that hematopoietic cell *APOE* genotype is sufficient to differentially modulate melanoma progression. Enhanced immune activation in *APOE4* mice in the context of anti-tumor immunity described in this thesis is consistent with the pro-inflammatory cytokine profile of *APOE4* versus *APOE3* microglia and macrophages (Vitek *et al.*, 2009; Ophir *et al.*, 2005; Shi *et al.*, 2017) and enhanced cytokine secretion in human *APOE4* carriers (Gale *et al.*, 2014).

One important question that arose from these in-vivo data was whether this effect could also be observed in humans. To investigate this, we assessed *APOE* genotype in normal tissue whole-exome sequencing data from stage II/III melanoma patients of the TCGA-SKCM study (The Cancer Genome Atlas Network, 2015). Remarkably, *APOE* genotype significantly associated with survival. Consistent with our observations in human *APOE* knock-in mice, patients that carried the *APOE2* allele showed significantly worse survival than *APOE3* homozygotes and *APOE4* carriers. As alluded to above (see 1.4), it was previously established that *APOE4* and *APOE2* correlate with reduced and enhanced longevity, respectively (Belloy *et al*., 2019). It is therefore surprising that we detected enhanced survival in *APOE4* carriers, since the impact of *APOE* genotype on longevity would be expected to counter the reversed impact on melanoma outcome. It is likely that our ability to detect this reversal on survival in melanoma patients is due to the pronounced risk of melanoma-associated death in the high-risk group of TCGA-SKCM patients (The Cancer Genome Atlas Network, 2015). To validate these results, we analyzed data from a genome-wide association study in melanoma patients (Amos *et al*., 2011). Consistent with our observations in the TCGA-SKCM study, amongst patients at high risk of melanoma-associated death (Clark level V), *APOE2* carriership associated with detrimental survival outcome. In contrast, survival outcomes trended worse for *APOE4* carriers in patients with lower-risk melanomas, consistent with the previously established impact of *APOE* genotype on longevity.

We also sought to assess the impact of *APOE* genotype on melanoma outcome in the context of immunotherapy. *APOE4* melanoma-bearing mice survived longer than *APOE2* mice upon PD1-checkpoint blockade, and the analysis of two independent studies confirmed favorable survival in *APOE4* relative to *APOE2* carriers in patients receiving PD1-blockade after failing or progressing under CTLA4-blockade. These results are consistent with our observations in the absence of immunotherapy and indicate that *APOE* genotype also modulates melanoma outcome in the context of immunotherapy. As will be discussed in more detail below, these results have major potential clinical implications, since immunotherapy has become the mainstay of advanced melanoma therapy. Finally, previous work from our laboratory has shown that activation of LXRs confers anti-melanoma efficacy through the transcriptional activation of *APOE* (Tavazoie *et al*., 2018). We therefore assessed whether *APOE* genotype predicts for response to LXR agonism. While *APOE4* mice derived robust treatment benefit from LXR agonism, there was no effect in *APOE2* mice. These data indicate that *APOE* genotype may be a useful biomarker for LXR agonism in melanoma patients.

6.2 Biological and clinical relevance

Our findings of an impact of highly prevalent *APOE* germline variants on melanoma progression are novel in multiple regards. They constitute the first example of highly common germline variants that causally modulate cancer outcome. Of note, our observations are different from the established role of germline mutations in tumor suppressor genes that modulate cancer incidence, rather than cancer outcome, such as *BRCA1/2* (Miki *et al*., 1994; Wooster *et al*., 1995). Additionally, in contrast to *APOE* variants, such previously described mutations play a role in the tumor compartment rather than in the stroma. One previous study revealed that polymorphisms in the *FCGR3A* gene are associated with the outcome of ipilimumab treatment

in melanoma patients (Arce Vargas *et al.*, 2018). However, in contrast to our findings presented here, this association was merely correlative.

While immunotherapy has transformed the treatment of melanoma, it is important to identify biomarkers that predict for (i) whether patients are at high risk of relapse and melanoma-associated death and for (ii) treatment outcome (Larkin *et al.*, 2019). Several biomarkers have previously been identified, such as tumor mutational burden and PD-L1 expression, but these are not robust enough for individual patient stratification (see 1.3.1). Therefore, it is a major unmet need to characterize biomarkers that identify patients that are most likely to benefit from therapeutic intervention. Our findings constitute the first example of common germline variants having a major causal impact on anti-tumor immunity, and they show that variants within the same gene can either promote or blunt immune responses. Importantly, *APOE* genotype correlated with outcome of melanoma patients also in the context of immunotherapy, indicating that *APOE* genotype is a promising candidate for a biomarker to both predict risk for adverse outcome in melanoma patients as well as to predict for response to therapy. Prospective clinical trials will need to validate the potential role of *APOE* as a biomarker.

Another clinical implication concerns Alzheimer's disease, for which recent efforts have assessed the feasibility of converting the *APOE4* variant into *APOE2* to mitigate *APOE4*'s detrimental impact (Abudayyeh *et al.*, 2019). Our results call for careful investigation into the potential harmful effects of such efforts, such as deleterious effects on cancer outcomes.

6.3 Perspectives

Our findings raise several important questions that will need to be addressed in the future. The most important points will be discussed below.

6.3.1 The molecular link between *APOE* genotype and anti-tumor immunity

In this work, we show that *APOE* variants modulate melanoma outcome by impacting anti-tumor immunity. Moving forward, it will be important to determine the molecular mechanisms that mediate this impact. Previous work from the Tavazoie laboratory showed that APOE promotes anti-tumor immunity by depleting immunosuppressive MDSCs via binding to the APOE receptor LRP8 (Tavazoie *et al.*, 2018). It is conceivable that different APOE variants exhibit differential capacities to deplete MDSCs via differential activation of LRP8 signaling. For some APOE receptors, such as LDLR and LRP1, different binding affinites to distinct APOE variants have been shown, whereby APOE4 and APOE2 exhibit the highest and lowest binding affinities, respectively (Weisgraber *et al.*, 1982; Kowal *et al.*, 1990). This binding affinity pattern also seems to apply to LRP8, the receptor involved in MDSC depletion, for which APOE2 exhibits the lowest binding affinity (Xian *et al.*, 2018). To complicate matters, the differential impact of APOE variants on receptor signaling is not limited to different binding affinities: in the case of LRP8, pH-dependent conformational changes of APOE4 lead to an endosomal trafficking defect, which modulates receptor surface expression and is likely to exert secondary effects (Xian *et al.*, 2018). To assess whether the APOE/LRP8 axis in MDSCs accounts for the differential impact of APOE variants on anti-melanoma immunity, it will be necessary to perform in-vitro experiments exposing MDSCs to recombinant APOE variants and assess cell viability. In

addition, to conclude whether myeloid LRP8 mediates the in-vivo impact of APOE variants, tumor growth experiments will need to be performed with conditional deletion of LRP8 on myeloid cells in the *APOE2* and *APOE4* contexts.

Of note, APOE is known to bind to several other receptors of the LDLR family as well as to TREM2 in addition to LRP8 (see 1.4). Our single cell RNA-sequencing data allowed us to assess the expression of APOE receptors in immune subsets with the hypothesis that differential activation of APOE receptors may lead to transcriptional feedback. Expression differences of the LRP1 and TREM2 receptors were most pronounced between *APOE2* and *APOE4* immune cells, indicating the potential participation of these receptors in mediating the effect of APOE variants. Of note, it has been previously shown that profiling of granulocytic MDSCs by single cell RNA-sequencing is not optimal (Martin *et al.*, 2019). Therefore, the expression of receptors in granulocytic MDSCs should be assessed using orthogonal approaches. Interestingly, myeloid LRP1 has previously been implicated in anti-tumor immunity (Staudt *et al.*, 2013; Sedlacek *et al.*, 2019). In addition, TREM2 variants are known to associate with Alzheimer's risk and modulate microglial immune phenotypes (Krasemann *et al.*, 2017; Shi and Holtzman, 2018). These previous observations suggest a potential role of APOE receptors other than LRP8 in mediating the impact of APOE variants on melanoma progression and render it necessary to perform the experiments proposed above for LRP8 for other APOE receptors as well.

It is conceivable that systemic effects of distinct *APOE* genotypes also participate in modulating anti-tumor immunity in addition to direct APOE/APOE-receptor signaling on immune cells. One major systemic factor that is impacted by *APOE* gentoype is cholesterol homeostasis (Bennet *et al.*, 2007). Hypercholesterolemia is known to modulate immunity, as exemplified by its suppression of antiviral T cell cytotoxicity in the context of viral liver infection (Ludewig *et al.*, 2001). However, immune modulation of APOE was maintained in the context of altering APOE status only in the hematopoietic system, which allows to decouple the role of hematopoietic APOE from hypercholesterolemia (Bonacina *et al.*, 2018). Additionally, microglia from *APOE3* and *APOE4* mice showed differential cytokine secretion when cultured in-vitro (Vitek *et al.*, 2009). These findings indicate that APOE may impact immunity independent from its effect on systemic cholesterol homeostasis.

An alternative hypothesis potentially explaining the impact of APOE on immunity is based on its impact on the composition and turnover kinetics of cell membranes. Specifically, APOE has been shown to modulate lipid rafts, which can act as scaffolds for the assembly of signaling molecules. The APOE4 variant was shown to be less effective than APOE3 in inducing cholesterol efflux from cells, thereby leading to cholesterol retention in the cell membrane and prolonged signaling and immune activation (Gale *et al.*, 2014; Bonacina *et al.*, 2018).

It is important to note that, due to APOE's pleiotropic actions and its binding to several receptors, the determination of its precise mechanism of action in cancer is challenging. Indeed, despite a quarter century of research, there is still no definitive conclusion for how APOE variants modulate the risk of Alzheimer's disease. This is best exemplified by the notion that it remains unresolved whether the *APOE4* variant constitutes a gain- or loss-of-function variant (Belloy *et al.*, 2019). Part of this complexity arises from the fact that the function of APOE is context- and process-specific. For example, the APOE4 variant exhibits higher binding affinity to the LDLR and LRP1 receptors, thereby acting in a gain-of-function manner compared to the other variants (Weisgraber *et al.*, 1982; Kowal *et al.*, 1990). Additionally, *APOE4* mice showed

increased susceptibility to Tauopathies relative to *Apoe*$^{-/-}$ mice (Shi *et al.*, 2017). In contrast, however, its impact on cholesterol efflux is more akin to APOE deletion (Bonacina *et al.*, 2018), consistent with a loss-of-function. Regarding the impact of *APOE* genotype on anti-melanoma immunity described in this work, previous work showed blunting of anti-tumor immunity in *Apoe*-knockout mice, suggesting that the *APOE4* variant exerts a gain-of-function impact. It is likely that pleiotropic mechanisms account for the profound impact of *APOE* genotype on anti-cancer immunity, and future work will need to systematically dissect these.

6.3.2 *APOE* variants in cancer beyond melanoma

In this work, given previous data showing that APOE suppresses melanoma progression (Pencheva *et al.*, 2012), we focused on assessing the impact of *APOE* variants on melanoma outcome. It is an important question whether *APOE* genotype also impacts other cancer types. The only published data approaching this question are epidemiologic studies. A meta-analysis published in 2014 did not find an assocation between *APOE* genotype and cancer incidence, but did not report on cancer outcome (Anand *et al.*, 2014). Other epidemiologic studies suggest a later onset of cancer in male *APOE4* carriers (Kulminski *et al.*, 2011, 2014). In addition, *APOE4* carriership correlated with better survival in female patients with chronic lymphocytic leukemia (Weinberg *et al.*, 2008).

Several reasons explain why the link between *APOE* genotype and melanoma progression described here has not been readily established by previous studies. Most studies assessing the impact of genetic variability on melanoma have focused on incidence, rather than survival (Brown *et al.*, 2008; Bishop *et al.*, 2009; Barrett *et al.*, 2011; Macgregor *et al.*, 2011). Additionally, most chip arrays used for cancer genome-wide association studies do not adequately capture one of the *APOE* genotype-defining SNPs (Radmanesh *et al.*, 2014). Lastly, the statistical power of unbiased genome-wide studies is hampered by testing a vast number of hypotheses in parallel. In contrast, our approach was guided by previous molecular biology findings from our laboratory showing an impact of APOE on melanoma, thereby circumventing this problem.

In the absence of experimental data on the role of *APOE* variants in other cancer types, it is useful to examine whether *Apoe*-deletion impacts progression of a given cancer type. Interestingly, *Apoe*$^{-/-}$ mice exhibit faster progression of breast and ovarian cancers (Alikhani *et al.*, 2013; Lai *et al.*, 2018). In contrast, repression of tumoral APOE blunted ovarian cancer progression, hinting at potential differences between the roles of stromal and tumoral APOE (Chen *et al.*, 2005).

Overall, the sparse existing data support a potential role of APOE in modulating cancer types beyond melanoma. A combination of assessing tumor progression in human *APOE*-transgenic mice with the analysis of large-scale patient data will be required to address which cancer types are impacted by *APOE* genotype.

6.3.3 Interaction with the impact of *APOE* genotype on melanoma progression

Previous studies have shown the effect of *APOE* genotype on certain phenotypes to be modulated by other variables. In statistical terms, such variables are said to interact with the impact of *APOE* genotype. Two of the best characterized interacting factors are ethnicity

and sex. In Africans, the association of *APOE4* with Alzheimer's disease is only observable in *APOE4* homozygotes, while East-Asians seem more susceptible to the impact of *APOE4* (Farrer *et al.*, 1997). Ethnicity also seems to interact with the impact of *APOE* on cardiovascular disease, longevity, and lobar hemmorhage (Bennet *et al.*, 2007; Tzourio *et al.*, 2008; Garatachea *et al.*, 2014; Sawyer *et al.*, 2018). It is equally well established that sex interacts with the impact of *APOE* genotype, since the association of *APOE4* with Alzheimer's disease is stronger in women and in female mice (Payami *et al.*, 1994; Farrer *et al.*, 1997; Raber *et al.*, 1998). Interestingly, the sex-by-*APOE* interaction has also been found in macrophages cultured in-vitro (Colton *et al.*, 2005; Brown *et al.*, 2007). Epidemiologic data suggest that a sex-by-*APOE* interaction may also exist in cancer. The *APOE4* variant associates with a delay in cancer onset, but this association was only observed in males (Kulminski *et al.*, 2011, 2014).

Further analysis of variables interacting with the impact of *APOE* genotype on cancer has the potential to reveal its context-specificity. It may also offer cues to the mechanisms underlying *APOE*'s impact on cancer. To identify interacting factors, large-scale patient datasets should be analyzed for interaction terms with *APOE*'s impact on known associated traits, such as melanoma outcome (reported in this thesis), and Alzheimer's disease. Additionally, factors that are known to interact with *APOE* in certain contexts should be assessed in targeted experiments. For example, the sex-by-*APOE* interaction can be readily addressed in mouse experiments. Of note, for experiments in this thesis, an impact of *APOE* genotype on melanoma progression was observed in both female (YUMM3.3 and YUMMER1.7 experiments) and male mice (YUMM1.7). However, a more systematic assessment of such potential interactions is desirable.

6.3.4 The impact of *APOE* genotype on other immune-related contexts

As outlined above, APOE plays a major role in several pathophysiological contexts, such as Alzheimer's disease, cardiovascular disease, and cancer progression. A common element in most of these processes is the impact of immunity. Indeed, as outlined in section 1.4, *Apoe*-knockout mice exhibit immune dysfunction, and *APOE*-transgenic mice show altered immune responses (Ophir *et al.*, 2005; Vitek *et al.*, 2009). In humans, *APOE* variants associate with altered immune profiles (Gale *et al.*, 2014; Bonacina *et al.*, 2018). It has been discussed before whether the differential impact of *APOE* variants on different immune processes may have shaped its heterogeneous global distribution due to selective pressure (van Exel *et al.*, 2017). One hypothetical example would be pleiotropic antagonism, whereby *APOE4* protects from processes in which immune activation is beneficial, such as infection, while conferring detrimental consequences in diseases promoted by inflammation, such as neurodegeneration. However, it remains an unsolved question, to which degree *APOE* variants modulate the outcome of common infectious diseases. This question should be approached using *APOE*-transgenic mice. Additionally, *APOE* genotyping of infectious disease patients may provide epidemiologic evidence supporting a potential association, as partially performed in the past (van Exel *et al.*, 2017).

6.3.5 The germline genetic makeup in cancer progression

Our findings uncover an impact of highly prevalent germline variants on anti-cancer immunity. Importantly, these findings suggest the possibility that other common polymorphisms at other immune-related loci also participate in modulating disease outcome. A global assessment of their role in outcome and therapy response is outstanding. This is in part due to the sheer number of genetic variants, complicating their unbiased assessment because of power limitations. Additionally, as outlined above, most studies analyzing germline variation in cancer patients have so far focused on incidence rather than outcome (Brown *et al.*, 2008; Bishop *et al.*, 2009; Barrett *et al.*, 2011; Macgregor *et al.*, 2011). The predicted use of routine germline and tumor sequencing of cancer patients will provide opportunities to more globally define the role of germline variation in cancer outcome. Determining how germline genetics shape an individual patient's outcome and response to therapy will be a key element of precision cancer therapy and will help to optimize treatment efficacy while limiting unnecessary toxicity.

6.4 Conclusions

APOE was previously shown to suppress melanoma progression and metastasis. Because of the presence of three highly prevalent *APOE* variants in humans, this thesis investigated the hypothesis that *APOE* variants differentially modulate melanoma progression and outcome. In a reversal of their impact on Alzheimer's disease, we found that the *APOE4* and *APOE2* variants imparted favorable and poor outcomes in melanoma, respectively. These effects were mediated by modulating anti-melanoma immunity, and the impact of *APOE* genotype on melanoma outcome was also true in the context of immunotherapy. Our results show that highly prevalent germline variants modulate the outcome and immune response in a common cancer type. Additionally, they suggest that *APOE* genotype may be a valuable biomarker to predict for adverse outcome and therapy response in melanoma patients.

7 Material and methods

7.1 Animal studies

All animal experiments were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee at The Rockefeller University. Human *APOE2* (strain #1547), *APOE3* (#1548), and *APOE4* (#1549) targeted replacement (knock-in) mice were obtained from Taconic Biosciences. C57BL6/J (#000664) and *Tyr::CreER;Braf*$^{V600E/+}$*;Pten*$^{lox/lox}$ (#013590) mice were obtained from Jackson laboratories.

7.2 Tumor growth studies and treatments

To assess the impact of *APOE* genotype on the growth of syngeneic melanoma, 1×10^5 YUMM1.7 cells were subcutaneously injected into the flank of 6-10-weeks-old human *APOE* targeted replacement mice. The sex of the mice for experiments with YUMM1.7, YUMM3.3 and B16F10 cells was matched to the sex of the tumor cell line (males for YUMM1.7 and B16F10, females for YUMM3.3). YUMMER1.7 cells were injected into female mice. Cells were injected in a total volume of 100 µL, and YUMM1.7 cells were mixed 1:1 with growth factor reduced Matrigel (356231, Corning) before injection. Tumor size was measured on the indicated days using digital calipers and tumor volume was calculated as (small diameter)^2 $\times$ (large diameter) / 6. In experiments employing YUMMER1.7 cells, 5×10^5 cells resuspended in PBS were injected subcutaneously into the flank.

To deplete T cells in-vivo, 400 µg of each anti-CD4 (BioXCell, clone GK1.5) and anti-CD8 (BioXCell, clone 53-6.7) antibodies were injected intraperitoneally on days 7, 14, and 21 post tumor cell injection. Control mice received PBS injections on the same days. Efficient depletion was verified by flow cytometry on day 27 post tumor injection. For LXR-agonistic treatment, mice were administered chow supplemented with the synthetic LXR-agonist RGX-104 (Rgenix (Tavazoie *et al.*, 2018)) at 628.5 mg/kg chow (Research Diets, approximate target dose of 100 mg/kg body weight) starting on day 3 post injection. For anti-PD1 treatment, mice were injected intraperitoneally with 250 µg and 125 µg of anti PD-1 antibody (BioXCell, clone RMP1-14) on days 6 and 9 post tumor cell injection, respectively. Control mice received PBS injections on the same days. For survival analysis in the YUMMER1.7 model, mice were euthanized when the tumor volume exceeded 1000 mm^3. Therapy responses were considered complete (CR, complete response) when tumor volumes fell below 16 mm^3 (lowest limit of detection).

7.3 Genetically initiated model of melanoma progression

Human *APOE* targeted replacement mice were crossed with *Tyr::CreER;Braf*$^{V600E/+}$*;Pten*$^{lox/lox}$ mice. To induce melanoma, 6-7-weeks-old female mice were injected intraperitoneally with

1 mg of Tamoxifen (T5648, Sigma-Aldrich) on three consecutive days. Tamoxifen solution was prepared by dissolving Tamoxifen powder in 100 % ethanol at 50 ℃ for five minutes and subsequently diluted tenfold in peanut oil to yield a 10 mg/mL working solution. To assess melanoma burden, dorsal skin samples stretching from ears to hips were harvested on day 35 after induction, depilated with commercial depilation cream (Nair), washed with water and fixed in 4 % PFA. Skins were then scanned and the percentage of pigmented melanoma lesion area was quantified using Cellprofiler v3.

7.4 Tail-vein metastasis assays

For tail-vein assays, B16F10-shApoe cells stably expressing a retroviral construct encoding luciferase were used in order to assess cancer progression by bioluminescence imaging as described previously (Pencheva *et al.*, 2014). To assess whether *APOE* genotype impacts metastatic progression, 1×10^5 cells were resuspended in 100 µL of PBS and injected into the tail vein of 6-8-weeks-old male human *APOE* knock-in mice. Bioluminescence imaging was performed approximately twice a week and the signal was normalized to the signal obtained on day 0.

7.5 Mouse genotyping

Genotyping to distinguish between mouse and human *APOE* was performed using standard PCR with independent reactions for mouse and human *APOE* (PCR product lengths of 200 bp and approximately 600 bp, respectively). In order to distinguish between human *APOE* alleles, PCR-based restriction fragment length polymorphism genotyping was used (Hixson and Vernier, 1990). In brief, a 244 bp fragment of *APOE* was amplified using standard PCR and digested with HhaI (R0139S, New England Biolabs), and allele-specific products were resolved on a 15% polyacrylamide gel. Primer sequences are listed in Table 7.1.

Table 7.1. Oligonucleotides for genotyping.

Target	Sequence (5' to 3')
Tyr::CreER;Braf$^{V600E/+}$*;Pten*$^{lox/lox}$ mice	
Cre transgene forward	GCG GTC TGG CAG TAA AAA CTA TC
Cre transgene reverse	GTG AAA CAG CAT TGC TGT CAC TT
Cre internal control forward	CAC GTG GGC TCC AGC ATT
Cre internal control reverse	TCA CCA GTC ATT TCT GCC TTT G
Braf forward	TGA GTA TTT TTG TGG CAA CTG C
Braf reverse	CTC TGC TGG GAA AGC GGC
Pten forward	CAA GCA CTC TGC GAA CTG AG
Pten reverse	AAG TTT TTG AAG GCA AGA TGC
Mouse versus human knock-in *APOE*	
Common forward	TAC CGG CTC AAC TAG GAA CCA T
Mouse *Apoe* reverse	TTT AAT CGT CCT CCA TCC CTG C
Human *APOE* reverse	GTT CCA TCT CAG TCC CAG TCTC

Continued . . .

... continued

Target	Sequence (5' to 3')
Human *APOE* allele restriction length polymorphism	
Human *APOE* forward	ACA GAA TTC GCC CCG GCC TGG TAC AC
Human *APOE* reverse	TAA GCT TGG CAC GGC TGT CCA AGG A

7.6 Hematopoietic stem cell transplantation

Six to 7-weeks-old C57BL6/J mice were whole-body irradiated with 10.5 Gray (two doses of 525 rad each 3.5 hours apart). Six hours after the last dose of irradiation, 2-3 × 10^6 nucleated bone marrow cells isolated from 6-8-weeks old *APOE*-knock-in mice (n = 5 per group) were infused into recipient mice by retroorbital injection. Bone marrow chimeras were reconstituted for 8 weeks before experimental use.

7.7 Cell lines

YUMM1.7 cells, originally derived from the genetic *Braf*^{V600E}*;Pten*$^{-/-}$*;Cdkn2*$^{-/-}$ mouse melanoma model, as well as their more immunogenic derivative YUMMER1.7 were a kind gift from Marcus Bosenberg (Meeth *et al.*, 2016; Wang *et al.*, 2017). YUMM3.3 *Braf*^{V600E}*;Cdkn2*$^{-/-}$ mouse melanoma, B16F10 mouse melanoma, and human umbilical vein endothelial cells (HUVEC) were obtained from American Tissue Type Collection and cultured according to the supplier's conditions. B16F10 cells expressing luciferase (Ponomarev *et al.*, 2004) and shRNA targeting murine Apoe (shRNA clone TRCN0000011799; B16F10-TR-shApoe) were described previously (Pencheva *et al.*, 2014). MeWo melanoma cells were obtained from American Tissue Type Collection, and their highly metastatic MeWo-LM2 subclone was described previously (Pencheva *et al.*, 2012). B16F10 and MeWo-LM2 cells were cultured in DMEM medium with Pyruvate and Glutamine (11995, Gibco) supplemented with 10 % FBS (F4135, Sigma), Penicillin-Streptomycin (15140, Gibco), and Amphotericin B (17-936E, Lonza). For culture of YUMM1.7, YUMM3.3 and YUMMER1.7 cells, DMEM/F-12 medium supplemented with L-Glutamine, 15mM HEPES (11330, Gibco), 10 % FBS, Penicillin-Streptomycin, Amphotericin B, and 1 % non-essential amino acids (111400, Gibco) was used. Intermittent PCR testing according to standard protocols was performed to rule out contamination with mycoplasma (Young *et al.*, 2010).

7.8 Quantitative real-time PCR

Total RNA from cells cultured in triplicates was isolated with the Total RNA Purification Kit (17200, Norgen Biotek). The SuperScript III First-Strand Synthesis System (18080051, ThermoFisher) was used to reverse-transcribe 1 µg of total RNA into cDNA according to the manufacturer's instructions using oligo(dT) primers. Subsequently, quantitative real-time PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems) and an Applied Biosystems 7900HT

system. Expression of *Apoe* was normalized to *Gapdh* expression for each sample. Primer sequences are listed in Table 7.2.

Table 7.2. Oligonucleotides for qRT-PCR.

Target	Forward primer (5' to 3')	Reverse primer (5' to 3')
Apoe	CTG ACA GGA TGC CTA GCC G	CGC AGG TAA TCC CAG AAG C
Gapdh	GCA CAG TCA AGG CCG AGA AT	GCC TTC TCC ATG GTG GTG AA

7.9 Isolation of tumor-infiltrating leukocytes

To isolate tumor-infiltrating leukocytes, YUMM1.7 tumors were resected on day 21 after injection and thoroughly minced on ice using scalpels. Tumor pieces were incubated in HBSS2+ (HBSS with Calcium and Magnesium (24020, Gibco) supplemented with 2 % FBS, 1 mM sodium pyruvate (11360, Gibco), 25 mM HEPES (15630, Gibco), 500 U/mL Collagenase IV (LS004188, Worthington), 100 U/mL Collagenase I (LS004196, Worthington), and 0.2 mg/mL DNAse I (10104159001, Roche)) for 30 minutes at 37 ℃ on an orbital shaker (80 rpm). After thorough trituration, the mixture was passed through a 70 µm strainer and diluted with HBSS2- (HBSS without Calcium and Magnesium (14170, Gibco), 2 % FBS, 1 mM sodium pyruvate, and 25 mM HEPES). After centrifugation, the cell pellet was resuspended in a 35 % Percoll solution (170891, GE Healthcare) and a phase of 70 % Percoll was underlaid using a glass Pasteur pipette. The resulting gradient was centrifuged at 800 × g for 20 minutes at room temperature without brakes. After removal of the red blood cell-containing pellet on the bottom and excess buffer containing cellular debris on the top, the cell population at the Percoll interphase enriched for tumor-infiltrating leukocytes was washed twice with HBSS2-.

7.10 Flow cytometry

Unless otherwise mentioned, all steps were performed on ice and under protection from light. Fc receptors were blocked by incubation with 2.5 µg/mL anti-CD16/32 antibody (clone 93; 101320, BioLegend) in staining buffer (25 mM HEPES, 2 % FBS, 10 mM EDTA (351-027, Quality Biological), and 0.1 % sodium azide (7144.8-16, Ricca) in PBS) for 10 minutes. Cells were incubated with antibodies diluted in staining buffer for 20 minutes, washed with PBS, incubated with Zombie NIR Fixable Live/Dead Stain (423105, BioLegend) for 20 minutes at room temperature, and washed twice with staining buffer. Cells were analyzed on an LSR Fortessa (BD Biosciences). For cell quantification, CountBright counting beads (C36950, Thermo Fisher) were added to the samples before analysis.

For intracellular staining of cytokines, cells were incubated with 500 ng/mL ionomycin (I0634, Sigma), 100 ng/mL Phorbol 12-myristate 13-acetate (P8139, Sigma), and 10 µg/mL Brefeldin A (B7651, Sigma) for 3-4 hours at 37 ℃ prior to surface labelling and live/dead staining as described above. Cells were then incubated in fixation/permeabilization buffer (00-5523, eBioscience) for 30 minutes, washed with permeabilization buffer (00-5523, eBioscience), and

incubated with antibodies diluted in permeabilization buffer for 20 minutes. Finally, cells were washed with permeabilization buffer and subsequently with staining buffer.

7.11 Single cell RNA-sequencing of tumor-infiltrating leukocytes

Human *APOE* knock-in mice (n = 6 per group) were subcutaneously injected with 1×10^5 YUMM1.7 cells on the flank (mixed 1:1 with growth factor reduced Matrigel (356231, Corning)). Tumors were resected on day 19 after injection and tumor-infiltrating leukocytes were isolated as outlined above. Fc receptors were blocked with anti-CD16/32 antibody (clone 93; 101320 BioLegend) and cells were stained with an anti-CD45 antibody and DAPI in flow cytometry buffer without sodium azide. Subsequently, 10,000 CD45+/DAPI- leukocytes from each mouse were independently sorted on a BD FACSAria II cell sorter and samples from the same genotype were pooled (i.e., total of 60,000 cells per genotype). Five-thousand cells per genotype were targeted for single-cell RNA-sequencing (scRNAseq) on a Chromium Single Cell System (10x Genomics). Samples were processed as per the manufacturer's instructions (Chromium single cell 3' reagents, v3 chemistry) and libraries were sequenced on an Illumina NextSeq sequencer.

Pre-processing of sequencing results to generate transcript matrices was performed using the 10x genomics Cell Ranger pipeline with default settings (v3.0.1). Further downstream analysis was performed in R using the Seurat package v3.0.2. Cells were excluded if fewer than 200 or more than 6000 genes were detected, or if mitochondrial transcripts accounted for more than 10 % of reads; genes were excluded if they were detected in fewer than five cells. The two datasets were integrated using Seurat's default settings, resulting in an expression matrix of 10,050 cells by 15,495 genes. Data were scaled and principal component analysis performed using Seurat's default settings. Cells were clustered using the FindNeighbors (20 dimensions of reduction) and FindClusters functions at default settings; uniform manifold approximation and projection (UMAP) was calculated for visualizing clusters. Differential gene expression analysis between each cluster was performed using a Wilcoxon rank sum test. The identity of cell clusters was determined by cross-referencing top differentially expressed transcripts with the immunological genome project (Yoshida *et al.*, 2019)(Figure 3.4). The identity of one cell cluster remained ambiguous; since further sub-clustering revealed the presence of a mixed population (data not shown), this cluster was labeled "diverse".

For gene set enrichment analysis (GSEA), differential expression of genes between *APOE4* and *APOE2* for each cluster was calculated using a Wilcoxon rank sum test and genes were ranked using the metric [-log10(p-value)]/[sign of log-fold change]. The ranked gene list was used for calculating GSEA using the clusterProfiler package with the Hallmark gene sets in the MSigDB database (Yu *et al.*, 2012; Subramanian *et al.*, 2005). For visualization, the number of clusters with significant enrichment were plotted for pathways that were significant in more than three clusters. For lineage summarization, all macrophage, monocyte, MDSC, basophil, and mast cell clusters were grouped as "myeloid", T cell, B cell, and NK clusters as "lymphoid", all DC clusters as "DCs", and the remaining clusters as "other".

7.12 Antibodies

The following anti-mouse fluorophore-conjugated antibodies were used for flow cytometry: CD45-BV785 (clone: 30-F11, cat#: 103149, supplier: BioLegend, dilution: 1:3,000), B220-BUV395 (RA3-6B2, 563793, BD Biosciences, 1:400), CD11b-BV605 (M1/70, 101257, BioLegend, 1:6,000), CD11b-FITC (M1/70, 101206, BioLegend, 1:4,000), Ly6G-PerCP/Cy5.5 (1A8, 127616, Biolegend, 1:500), Ly6C-BV711 (HK1.4, 128037, BioLegend, 1:12,000), I-A/I-E-BV421 (M5/114.15.2, 107632, BioLegend, 1:9,000), F4/80-FITC (BM8, 123108, BioLegend, 1:500), CD24-PE (M1/69, 101808, BioLegend, 1:5,000), CD103-APC (2E7, 121414, BioLegend, 1:500), CD19-FITC (1D3/CD19, 152404, BioLegend, 1:1,500), TCRβ-PerCP/Cy5.5 (H57-597, 109228, BioLegend, 1:200), CD49b-APC (HMa2, 103516, BioLegend, 1:300), CD4-BV605 (GK1.5, 100451, BioLegend, 1:200), CD8α-AF700 (53-6.7, 100730, BioLegend, 1:1,000), Granzyme B-PE (QA16A02, 372208, BioLegend, 1:200), IFNγ-PE/Cy7 (XMG1.2, 25-7311-82, eBioscience, 1:500).

7.13 Immunofluorescence microscopy

YUMM1.7 tumors were excised and fixed in 4 % paraformaldehyde at 4 ℃ for 24 hours. Fixed tumors were embedded in paraffin and sectioned in 5 µm thick slices. Sections were dewaxed and rehydrated by incubation with xylene and descending ethanol concentrations. Antigen retrieval was performed by microwaving samples in citrate buffer (C9999, Sigma) for 30 minutes. Samples were blocked by incubation with 5 % goat serum in PBST (PBS with 0.1 % Tween-20) for one hour. Subsequently, the sections were stained with anti-Endomucin (clone V.7C7, Santa Cruz; 1:200 in PBST with 5 % goat serum) or anti-CD8 antibody (rabbit polyclonal, Synaptic Systems, 1:200 in PBST with 5 % goat serum) at 4 ℃ overnight. Slides were washed three times with PBS and stained with AF555-conjugated anti-rat or AF488-conjugated anti-rabbit antibody (1:200 in PBST, ThermoFisher Scientific) for 45 minutes. Slides were washed with PBS and nuclei were counterstained with DAPI (2.5 µg/ml, Roche) before mounting with Prolong Gold (ThermoFisher Scientific). Images of tumor sections were acquired using an RS-G4 confocal microscope (Caliber I.D.). Images were quantified using CellProfiler (v3.1.8). Four sections per tumor were analyzed and averaged. Samples without addition of primary antibody served as negative controls.

7.14 Analysis of *APOE* genotype in the TCGA-SKCM study

To assess *APOE* genotypes in patients with melanoma, aligned whole exome sequencing BAM files sliced for the genomic coordinates chr19:44904748-44910394 (GRCh38) were downloaded from the TCGA-SKCM project using the Genomic Data Commons API (The Cancer Genome Atlas Network, 2015). *APOE* variants were called using the samtools/bcftools package, providing allele frequencies for chr19:44908684 (rs429358) and chr19:44908822 (rs7412) as determined in the Atherosclerosis Risk in Communities (ARIC) study (Blair *et al.*, 2005) as a prior distribution. Normal tissue samples (either blood, solid tissue, or buccal cells) were available for 470 patients. No genotype could be determined in 10 patients. Additionally,

patients that exhibited the *APOE2;APOE4* genotype (n = 5) were excluded from analyses except for genotype frequency assessment. *APOE* genotype abundance in the normal population was based on the assessment of Caucasian patients in the ARIC study.

Clinical data including survival times and clinical response were used as recently curated (Liu *et al.*, 2018). The R package 'TCGAbiolinks' was used to add clinical data for Breslow depth and Clark level. To assess the impact of *APOE* genotypes on survival, Kaplan-Meier survival analyses were performed, and statistical significance was assessed with the log-rank test using the 'survival' and 'survminer' packages. Hazard ratios were calculated according to a Cox proportional hazards regression model using the 'survival' R package. For multivariate analysis, variables found to be significantly associated with survival in univariate analysis were tested for significance in a multivariate Cox proportional hazard model. For visualization purposes, survival data were truncated at 12 years. All analyses were performed using R v3.5 (The R Foundation for Statistical Computing) and RStudio v1.1.3.

7.15 Analysis of *APOE* genotype in the MDACC melanoma study

GWAS genotyping results of the MDACC melanoma study (Amos *et al.*, 2011) were downloaded from dbGap, and the *APOE* variant-defining SNPs rs429358 and rs7412 were selected using Plink. Genotyping data were filtered to exclude variants with minor allele frequency < 1 %, genotyping rate < 95 %, and departure from the Hardy-Weinberg equilibrium at P < 1E-06. Samples were excluded if the missing genotype call rate exceeded 5 %. Genomic coordinates were lifted from genome assembly hg18 to hg19 using the UCSC liftOverPlink utility, and strands were aligned using GenotypeHarmonizer and the 1000 genomes reference genome. Since no individual was found to exhibit the minor allele at rs429358, inadequate genotyping at this locus was assumed as described by others (Radmanesh *et al.*, 2014), prompting us to impute the genotype at this locus based on a previously validated approach (Radmanesh *et al.*, 2014) prior to performing survival analysis. Pre-phasing was performed using ShapeIt v2, and variants in the genomic region 19:45411941-45422946 were imputed using Impute2 with parameters as suggested specifically for *APOE* imputation (-NE 20000 -iter 100 -call_thresh 0.8 -align_by_maf_g) (Radmanesh *et al.*, 2014). Subsequent analysis of the association between clinical variables and *APOE* genotype was performed as described for the TCGA-SKCM study above.

7.16 Analysis of *APOE* genotype in the anti-PD1 melanoma studies by Riaz *et al.* and Roh *et al.*

Analyses of the Roh *et al.* (Roh *et al.*, 2017) and Riaz *et al.* (Riaz *et al.*, 2017) studies were performed as described for the TCGA-SKCM study. In brief, normal tissue whole-exome sequencing data were downloaded from dbGaP (BioProject IDs PRJNA369259 and PRJNA359359) and *APOE* genotype was called as detailed above. No genotype could be determined from the normal tissue sample of one patient in the Roh *et al.* study. For the Roh *et al.* study, only patients that received both anti-CTLA4 and anti-PD1 treatment were considered. In the Riaz

et al. study, patients were stratified by prior CTLA4 treatment status. Kaplan-Meier survival analyses were performed using the 'survival' and 'survminer' packages, as detailed above.

7.17 Statistical analysis

Unless otherwise noted, all data are expressed as mean $\pm$ standard error of the mean. Groups were compared using tests for significance as indicated in the figure legends and the text. A significant difference was concluded at $P < 0.05$. Throughout all figures: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$. Unless indicated otherwise, all box plots show median, first and third quartiles, and whiskers represent minimum and maximum.

8 Publications

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