Function of Peripheral Blood Eosinophils in Melanoma

Funktion der Eosinophilen Granulozyten aus dem peripheren Blut im Melanom

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Content

Summary

Despite accounting for only a small proportion of all skin cancers, malignant melanoma displays a serious health risk with increasing incidence and high mortality rate. Fortunately, advances in the treatment of malignant melanoma now prolong survival and enhance response and treatment efficacy. Established biomarkers help evaluate disease progression and facilitate choosing appropriate and individual treatment options. However, the need for easily accessible and reliable biomarkers is rising to predict patient-specific clinical outcome. Eosinophil infiltration into the tumor and high peripheral eosinophil counts prior and during treatment have been associated with better response in patients for various cancer entities, including melanoma. An analysis of a heterogeneous study cohort reported high serum ECP levels in non-responders. Hence, eosinophil frequency and serum ECP as a soluble eosinophil-secreted mediator were suggested as prognostic biomarkers in melanoma. We examined whether melanoma patients treated with first-line targeted therapy could also benefit from the effects of eosinophils. In total, 243 blood and serum samples from patients with advanced melanoma were prospectively and retrospectively collected before and after drug initiation. To link eosinophil function to improved clinical outcome, soluble serum markers and peripheral blood counts were used for correlative studies using a homogeneous study cohort. In addition, functional and phenotypical characterizations provided insights into the expression profile and activity of freshly isolated eosinophils, including comparisons between patients and healthy donors.

Our data showed a significant correlation between high pre-treatment blood eosinophil counts and improved response to targeted therapy and by trend to combinatorial immunotherapy in patients with metastatic melanoma. In accordance with previous studies our results links eosinophil blood counts to better response in melanoma patients. High pre-treatment ECP serum concentration correlated with response to immunotherapy but not to targeted therapy. Eosinophils from healthy donors and patients showed functional and phenotypical similarities. Functional assays revealed a strong cytotoxic potential of blood eosinophils towards melanoma cells in vitro, inducing apoptosis and necrosis. In addition, in vitro cytotoxicity was an active process of peripheral eosinophils and melanoma cells with bidirectional features and required close cell-cell interaction. The extent of cytotoxicity was dose-dependent and showed susceptibility to changes in physical factors like adherence. Importantly, we provide evidence of an additive tumoricidal function of eosinophils and combinatorial targeted therapy in vitro. In summary, we give valuable insights into the complex and treatment-dependent role of eosinophils in melanoma. As a result, our data support the suggestion of eosinophils and their secreted mediators as potential prognostic biomarkers. It will take additional studies to examine the molecular mechanisms that underlie our findings.

Zusammenfassung

Obwohl das Maligne Melanom nur einen geringen Anteil aller Hautkrebsarten ausmacht, stellt es ein ernstzunehmendes Gesundheitsrisiko mit steigender Inzidenz und hoher Sterblichkeitsrate dar. Durch Fortschritte in der Behandlung des malignen Melanoms konnten die Überlebenszeit verlängert und das Ansprechen und die Wirksamkeit der Behandlung verbessert werden. Etablierte Biomarker helfen bei der Bewertung des Krankheitsverlaufs und erleichtern die Wahl geeigneter und individueller Behandlungsoptionen. Der Bedarf an leicht zugänglichen und zuverlässigen Biomarkern zur Vorhersage patientenspezifischer klinischer Ergebnisse nimmt zu. Die Infiltration von Eosinophilen in den Tumor und hohe periphere Eosinophilenzahl vor und während der Behandlung wurden mit einem besseren Ansprechen bei Patienten mit verschiedenen Tumorarten, einschließlich des Melanoms, in Verbindung gebracht. Eine Analyse einer heterogenen Patientenkohorte berichtete über hohe ECP-Serumspiegel bei Patienten, die nicht auf eine Melanombehandlung ansprechen. Daher wurden periphere Eosinophile im Blut und ECP im Serum, als löslicher, von Eosinophilen sekretierter Mediator, als prognostische Biomarker für das Melanom vorgeschlagen. Wir untersuchten, ob sich die positive Wirkung der peripheren Eosinophilen beim Melanom auf Patienten übertragen lässt, die mit einer zielgerichteten Erstlinientherapie behandelt werden. Insgesamt wurden 243 Blut- und Serumproben von Patienten mit fortgeschrittenem Melanom prospektiv und retrospektiv vor und nach Einleitung einer medikamentösen Behandlung gesammelt. Um die Eosinophilenfunktion mit einem verbesserten klinischen Ergebnis in Verbindung zu bringen, wurden lösliche Serummarker und periphere Blutbilder für korrelative Studien in einer homogenen Studienkohorte analysiert. Darüber hinaus lieferten funktionelle und phänotypische Charakterisierungen Einblicke in das Expressionsprofil und die Aktivität von frisch isolierten Eosinophilen. Vergleiche von Patienten und gesunden Spendern wurden ebenfalls durchgeführt.

Unsere Daten zeigten, dass eine hohe prätherapeutische Eosinophilenzahl im Blut zu einer signifikanten Verbesserung des Ansprechens auf eine zielgerichtete Therapie und tendenziell zu einer Verbesserung des Ansprechens auf eine kombinatorische Immuntherapie bei Patienten mit metastasiertem Melanom beiträgt. In Übereinstimmung mit bereits publizierten Studien bringen unsere Ergebnisse eine erhöhte Eosinophilenzahl im Blut mit einem besseren Ansprechen bei Melanompatienten in Verbindung. Eine hohe prätherapeutische ECP-Serumkonzentration korrelierte mit dem Ansprechen auf eine Immuntherapie, nicht aber auf eine zielgerichtete Therapie. Eosinophile von gesunden Spendern und Patienten wiesen zudem funktionelle und phänotypische Ähnlichkeiten auf. Außerdem zeigten funktionelle Tests ein starkes zytotoxisches Potenzial von Eosinophilen gegenüber Melanomzellen in vitro. Periphere Eosinophile lösten Apoptose und Nekrose in den Melanomzellen aus. Darüber

hinaus war die Zytotoxizität in vitro ein aktiver Prozess zwischen peripheren Eosinophilen und Melanomzellen mit bidirektionalem Einfluss und erforderte eine enge Zell-Zell-Interaktion. Das Ausmaß der Zytotoxizität war dosisabhängig und zeigte eine Anfälligkeit für Veränderungen der physikalischen Faktoren wie der Adhärenz. Wir konnten Beweise für eine additive tumorizide Funktion von Eosinophilen und einer kombinatorischen zielgerichteten Therapie in vitro liefern. Zusammenfassend lässt sich sagen, dass diese Arbeit wertvolle Einblicke in die komplexe und behandlungsabhängige Rolle der Eosinophilen beim Melanom bietet. Unsere Daten unterstützen den Vorschlag, Eosinophile und die von ihnen sekretierten Mediatoren als potenzielle prognostische Biomarker zu verwenden. Weitere Studien sind erforderlich, um die molekularen Mechanismen unserer Beobachtungen zu entschlüsseln.

1   Introduction

The skin is the human's largest organ with its daily business to provide an intact barrier towards the outside world to function as the first line defense mechanism. Its importance to human life cannot be exaggerated. On a physical level, it is preventing infections and protects from external hazards, like allergens and toxic chemicals [1]. On the other hand, it maintains homeostasis and simply spoken, keeps the body from evaporating [2]. Despite its durability and strength towards a variety of biochemical and mechanical stress, the skins apparent impenetrability is illusional.

The human skin is susceptible to terrestrial ultraviolet (UV) radiation. Both UVA (400-315 nm) and UVB (315-280 nm) are linked to skin malignancies [3-5]. Despite the higher penetrance of UVA into skin, UVB rays are assigned to be more mutagenic and cause most skin cancers [5-7]. This is explained by the higher energy of UVB and consequently, its higher absorbance by DNA, ultimately resulting in genotoxic DNA damage [5, 8-9]. DNA damage manifests for instance in dimerization of adjacent nucleotides, more specific, in the generation of cyclobutene pyrimidine dimers (CPD) or in the formation of 6-4 photoproducts (6-4PP) [5, 10-12]. Modification and mutation of DNA and other cellular molecules by UVA can result in elevated generation of reactive oxygen species (ROS) and ultimately in inflamed skin [13-15]. In fact, besides environmental, ethnical and genetic variables as causative factors, the development of most cutaneous malignant melanoma is attributed to excessive UV exposure [16-19].

1.1   The origin of malignant melanoma – melanocytes and their mutations

The skins architecture is divided into three main layers, the adipocyte-containing subcutaneous layer, the collagen-rich dermis, mainly composed of fibroblasts, and the outmost epidermal layer [20]. The epidermis is primarily constituted by keratinocytes. Its main function is to provide a first-line protection to various external stressors. The basal layer, the deepest layer of the epidermis is connected to the dermis via the basal lamina. In this section, melanin pigment synthesizing melanocytes, Merkel cells and cells from the immune system can be encountered (Figure 1) [20].

Melanocytes originate from the highly migratory neural crest stem cells (NCSCs) [21]. While canonical WNT, NOTCH and BMP signaling are attributed to the initial lineage specification from multipotent NCSCs to bipotent glial-melanocyte stem cells, microphthalmia-associated transcription factor MITF, c-kit, tyrosinase related protein TRP1 and other factors are responsible for the later fate specification of melanocytes till their final reside in the epidermis [22-23].
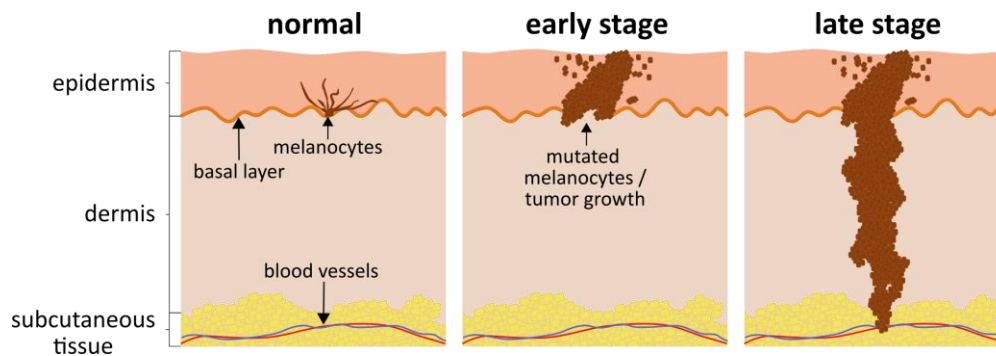
Figure 1. Schematic representation of the human skin and abnormal growth in the early and late stage of melanoma. Figure adapted from the Aim at Melanoma Foundation [24].

With a few exceptions, occurrence of melanoma is mainly attributed to somatic mutations in epidermal melanocytes but a small margin of around 5-12% of melanoma is linked to inherited germline mutations [25-27]. As discovered in 2002, approximately half of all malignant melanomas carry an activating oncogenic driver point mutation in the *BRAF* gene on chromosome 7 [28]. This gene encodes the serine-threonine BRAF kinase, participating in the mitogen-activated protein kinase (MAPK) signaling pathway, which is characterized by its pivotal function in modulating proliferation, cell growth, differentiation as well as senescence and apoptosis (Figure 2) [29-30]. The MAPK signaling pathway is frequently found hyperactivated in various tumors due to activating mutations in *RAS* or *BRAF* genes [31-34]. BRAF belongs to the rapidly accelerated fibrosarcoma (RAF) family, which share conserved structures, and is one out of three known mammalian isoforms (ARAF, BRAF, CRAF) [35-36]. Through cell external engagement of integrins or receptor tyrosine kinases (RTKs) by stimuli like growth factors or cytokines, a cell intrinsic signaling cascade is initiated. Signal transduction activates membrane anchored small GTP-binding protein RAS that in turn recruits inactive BRAF and enables phosphorylation of the activating sites within the BRAF kinase domain. Activation of the BRAF kinase is accompanied by the formation of homo- or heterodimers [37]. Further fractions of the MAPK signaling pathway core unit, MEK1, MEK2 and ERK, are subsequently phosphorylated and activated. Activated ERK dimer translocates to the nucleus where it regulates phosphorylation of various transcription factors, ultimately affecting the expression of a wide range of genes with numerous cell-affecting outcomes [38]. In melanoma, around 90% of the occurring somatic mutations in the *BRAF* gene are dominated by the mutation in codon 600. The most frequent aberration in this codon is *BRAFV600E*, caused by a substitution of the amino acid valine (V) to glutamate (E) in the activating segment of the kinase as a consequence of a nucleotide transversion from T to A (T1799A) [28, 39-40].
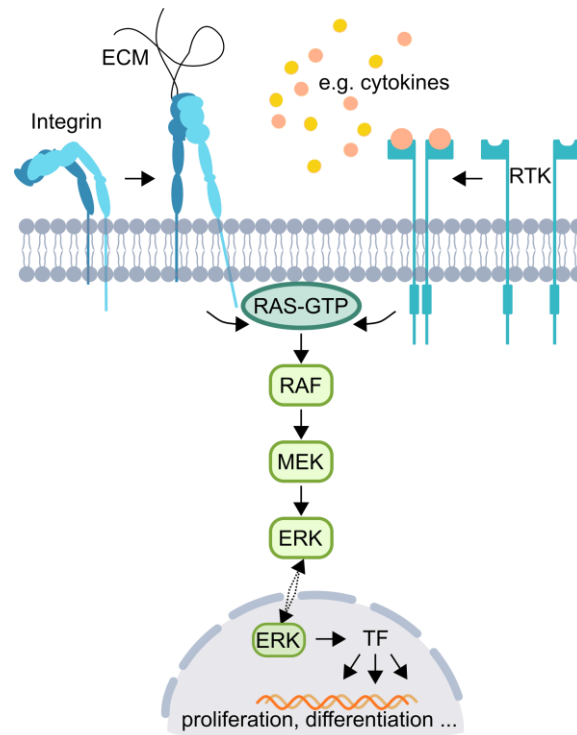
Figure 2. The MAPK signaling pathway and its core units. Binding of growth factors or cytokines to the receptor tyrosine kinase (RTK) or interaction of surface integrins with the extracellular matrix (ECM) triggers phosphorylation and activation of cell-internal downstream mediators like the membrane-bound RAS. RAS activation stimulates RAF phosphorylation and dimerization. Subsequently, MEK and ERK are activated. The latter regulates activation of transcriptions factors (TFs), which on the other hand decide for the cells fate. Depending on the initial signal, proliferation, differentiation but also other delicate processes like senescence and apoptosis are initiated. Mutations in the *RAS* gene (e.g., commonly in *NRAS*) or in *RAF* disrupt and dysregulate this highly intricate signaling cascade in favor of the outgrowth of a tumor. Figure adapted from Becchetti et al. [41].

Interestingly, mutated BRAFV600E is able to activate MEK and ERK RAS-independently as an active monomer [42-43]. Hence, mutation in this region causes elevated activity of the kinase and, due to its debated phospho-mimetic property, respectively in the constitutive sequential stimulation of the downstream MEK and ERK kinases [28, 44]. In case of melanoma, the ultimate result of the hyperactive MAPK pathway entails uncontrolled growth of the affected melanocyte and melanogenesis. Less common BRAF-mutation variants include the valine to lysine substitution V600K, attributed to around 5-12% of melanoma, the valine to aspartic acid V600D and the valine to arginine V600R-mutation representing only a small subgroup of ≤ 5% of melanomas [45-47]. High prevalence of the BRAF-mutation is not restricted to melanoma as it is frequently exhibited in other cancers like colorectal cancer (12%) and ovarian cancer with varying frequency, implicating its potential to promote a variety of oncogenic malignancies [48-52]. The amount of pre-existing and acquired dysplastic nevi raises the risk to develop melanoma [53-54]. Noteworthy, the mutation in the BRAF oncogene alone is insufficient to cause melanogenesis as found frequently in benign melanocytic lesions without apparent malignant transformation [55-58]. Cooperative acquisition of secondary genetic mutations for instance in tumor suppressors like p53, CDKN2A or cyclin D1, other protein kinases and mutations that impact the DNA damage repair mechanism like the

nucleotide excision repair (NER), are required favoring tumor development and correlate with progression and metastases formation [58-68]. This process is accompanied by increasing heterogeneity of the tumor cells, which underlines the need of surgical removal in the early stages of development as polyclonality might weaken the impact of therapeutic agents and intrinsic anti-tumor immunosurveillance. Invasiveness of melanoma is attributed to the inactivation of CDKN2A, with increasing copy-number alterations, while in the early progressive stages PTEN and TP53 mutations are frequently found [58, 69-71]. Predisposition to CDKN2A mutations is frequently found in inherited melanomas and increases the risk of pancreatic cancer development [26].

Melanoma harbors a diverse genetic landscape and its somatic mutation frequency is higher compared to some other solid cancers conducted in several studies [32, 72-75]. Interestingly, chromosomal aberration patterns significantly differ according to localization, for instance comparing neck and trunk, the pathohistological character and the sun-signature of the tumor and have been highly debated in defining the adequate therapeutic approach accordingly. Studies have put divergent oncogenic chromosomal aberration and mutation burden of melanoma types, their region of appearance and their UV signature into context with distinct developmental trajectories [76-80]. In fact, cutaneous melanoma in Caucasians can be categorized in chronically sun damaged (CSD), typically diagnosed in older patients >55 years due to higher level of cumulative UV exposure, and non-chronically sun damaged (non-CSD) melanoma [81]. Data propose that melanocytes may be displaying different susceptibility to transformation depending on the body's age, and therefore the number of accumulated UV-doses during life, and the location of melanocytes [81]. Intriguingly, *BRAFV600E* mutations in melanocytes do not necessarily coincide with exposure to light and commonly initiate non-CSD on skin sporadically exposed to UV and in naevi (≤ 50 years of age), while mutations in the oncogenic c-terminal cyclin D1 (*CCND1*) are recurrent in areas with frequent UV-exposure [28, 57, 77, 82-83]. With the absence of additional genetic aberrations (e.g., *TERT* or *CDKN2*) and as a result of functional immunosurveillance, these naevi typically remain benign, low-/non-proliferative and stable lesions. Although the majority of superficial spreading melanoma arise de novo, around 20% of melanoma are associated with pre-existing naevi [54, 84-89]. CSD melanomas commonly arise through mutations in the proto-oncogene *NRAS*, the second most common alteration found in cutaneous melanoma with a frequency of around 20%, or *BRAF-nonV600E* [90-91]. Other melanoma-associated mutations include e.g., a mutation in *KIT*. *KIT* mutations are rare and are more likely (in around 15 - 40% of the cases) encountered in non-UV-associated mucosal and acral melanomas [92-93]. Chromosomal aberrations are accounting for most of mucosal and acral melanomas [91]. The majority (< 80%) of uveal melanomas carry a *GNAQ* or *GNA11* mutation that rarely coincides with a mutation in *BRAF* or *NRAS* gene [94-96]. Other subtypes of melanoma harbor mutations in the *KRAS* gene,

accounting for 20%, *NF-1* mutation, accounting for around 15% and triple-wild-type melanoma, presented in around 10% of melanoma [97].

1.2 Clinical classification, diagnostic and treatment options

Malignant melanoma remains a serious threat to public health and its incidence worldwide has been rising continuously in the past decades [98-99]. The rising incidence might be explained by earlier diagnosis by general skin examinations in Germany and people's increasing awareness for the severity of this skin disease.

Wallace Clark and Dr. Richard Reed first classified malignant melanoma according to its histopathological features [59, 100]. Most clinical diagnosed form of melanoma is observed cutaneous. Although remaining controversial due to overlapping histological patterns, cutaneous melanoma is subdivided into different variants according to their spreading characteristics and their appearance. Around 70% of melanoma are attributed to superficial spreading melanoma, with initial radial growth and horizontal expansion in the basal membrane. In later stages, there is a high likelihood of vertical invasion accompanied by migration into the dermal layer with high metastatic potential [101-102]. A smaller portion of cutaneous melanoma is defined as nodular and lentigo maligna melanoma [100, 103]. Commonly found in darker skinned people, acral lentiginous melanoma make up only a small margin [104]. More rare forms of melanoma arise in UV-protected mucosal areas of the body and intraocularly [105].

Guidelines have remarkably improved precision in melanoma diagnosis and standardized clinical assessment. In 2017, the American Joint Committee on Cancer (AJCC) published the 8th edition of the melanoma staging panel, facilitating worldwide consistency in evaluating melanoma and providing recommendation of evidence-based patient-individual treatment approaches accordingly [106-107]. The AJCC guideline classifies melanoma by three categories [108]. The T(umor)-category records thickness (measured in mm) and ulceration status of the tumor. The N(ode)-category indicates spreading into adjacent lymph nodes while M(etastases)-category classifies tumors extending into distant lymph nodes and/or organs. The categorization helps defining the clinical and pathological staging group of the tumor providing information for establishing patient specific clinical care strategies and prognosis. In the early stage, primary local excision is conducted. The analysis of the lesion, in particular the vertical tumor thickness, dictates whether excision was sufficient or in case of extending a thickness limit and evidence of cancerous cells or ulceration, further examination e.g. the performance of a sentinel lymph node biopsy (SLNB) is required [108]. SLNB evaluates spreading of the primary tumor into adjacent lymph nodes and determines whether medication-based approaches are indispensable [108]. Due to increased tumor thickness, microscopic visualization of potential ulceration and increased metastatic risk in stage II, SLNB and a

potential treatment are discussed with the patients according to the biopsy analysis. Aside from the TNM classification and tissue markers (e.g., Melan-A, HMB-45) determined in histological samples, biomarkers like S100 and lactate dehydrogenase (LDH) are evaluated and the mutation burden of the tumor is detected [109]. The protein S100 in patients with malignant melanoma can be detected in immune histological samples and in the peripheral blood, where it is used as an independent biomarker to identify metastatic melanoma and evaluating its progression [108, 110-111]. Despite not being specific for melanoma, LDH is another useful prognostic marker in late-stage melanoma when found elevated in patients' serum [112-113]. Increased level of LDH is associated with poor prognosis and has been introduced into the current AJCC staging scheme [108, 114-116]. Identification of the underlying mutations attributing to distinct types of melanomas and the AJCC recommended staging panel have provided crucial knowledge in understanding the disease development and has led to the development of appropriate therapies [117-119].

Historically, treatment with the alkylating chemotherapeutic agent dacarbazine (DTIC) approved 1975 by the Food and Drug Administration (FDA), high-dose immune-stimulator IL-2 (FDA-approved in 1998) and IFN α-2b represented the gold standard in treating melanoma [120]. Unfortunately, single-agent DTIC did not show beneficial impact in clinical response like overall survival (OS) [121-123]. Nowadays, chemotherapy is utilized as the last line palliative treatment option [123]. To evaluate the optimal choice of adjuvant therapy or for late-stage unresectable melanoma, the determination of the mutation status for BRAF, NRAS and c-Kit is recommended. Prior and during therapy, MRI, providing higher accuracy regarding cerebral metastasis, or PET/CT imaging techniques help visualizing distant metastasis and provide important tools for surveilling tumor progression and response to therapy [124-125]. NRAS mutation is detected in around 20% of melanoma. Determination of the c-Kit mutation is recommended for patients suffering from acral and mucosal melanoma [126]. This information can help determining whether immunotherapy or targeted therapy should be discussed with the patients.

2011 marked a turning point in the history of therapeutic strategies against metastatic melanoma. Two main treatment options were developed targeting melanoma on different levels, immunotherapy and targeted therapy. Immunotherapy utilizes immune intrinsic checkpoints that prevent indiscriminate cell attack and regulate T cell function [127]. The cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed death 1 (PD-1) present two major immune checkpoint regulators. T cell-mediated adaptive immune response requires the activation of T cells upon binding of their T cell receptor (TCR) of an antigen presented by the MHC molecule on the surface of an antigen-presenting cell (APC). Co-stimulants like the CD28:B7-interplay help orchestrate the functional outcome of the TCR-engagement [128]. CTLA-4 is a protein receptor expressed by T cells that binds B7 presented

on antigen presenting cells (APCs) and competes with CD28, as CTLA-4 it binds B7 with a higher affinity [128]. Engagement of CTLA-4 with B7 downregulates T cell activation and proliferation and inhibits autoimmunity to sustain self-tolerance correspondingly [127]. In 2011 ipilimumab (Yervoy®, Bristol-Myers Squibb) was approved by the FDA as the first human CTLA-4 monoclonal antibody (Figure 3) [129].
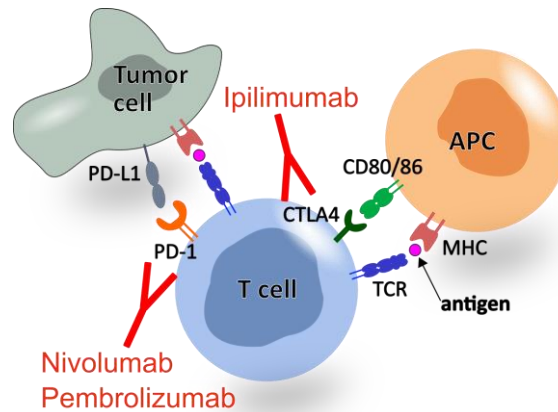
Figure 3. Immunotherapy and its targets. Activation of T cells is mediated by MHC-antigen recognition by TCR. T cells express CTLA4 and PD-1. Tumor cells regulate T cells via PD-L1. APCs, e.g., dendritic cells express B7. Binding of B7 (CD80/86) to CTLA4 or respectively PD-L1 to PD-1, regulates T cell activity and function. Ipilimumab inhibits CTLA4 on T cells, while nivolumab and pembrolizumab both inhibit PD-1 on T cells. Immunotherapy restores T cell activation. Modified from Ramos-Casals et al. [130].

Ipilimumab highjacks T cell regulation and initiates T cell activity and function especially in the lymph nodes, inducing enhanced tumor cell surveillance and optimizing anti-tumor activity [131]. Comparing patients receiving DTIC with patients additionally receiving Ipilimumab revealed significant increase in clinical response when treating with the CTLA-4 antibody [132-135]. Long-term survival analysis for Ipilimumab-monotherapy treated patients revealed a median overall survival (OS) of 9.5 months, while following the initiation of therapy, survival plateaued at around 3 years [136]. The 3-year survival rate when treating with ipilimumab-monotherapy has shown to be 25% [137]. Significant improvement in progression-free survival was achieved with the development of nivolumab and pembrolizumab, PD-1 inhibitors, optimally applied in combination with ipilimumab [127, 138]. The combination of nivolumab and ipilimumab led to a median progression-free survival (PFS) of 11.5 months and a median OS >5 years [139]. Like CTLA-4, PD-1 regulates T cell function by binding to its ligand programmed death ligand 1 (PD-L1). Unlike CTLA-4, PD-1/PD-L1 regulates the interaction axis between tumor cells and T cells. PD-L1 is expressed by tumor cells, promotes immune suppression and inhibits T cell cytotoxicity [140]. Although controversial, PD-L1 immunohistochemistry may assist to predict response to therapy as in some studies, PD-L1 expression was associated with PFS and OS in melanoma patients [141-146]. The inhibitor restores T cell immune function and merged with Ipilimumab prolongs overall survival in patients with metastatic melanoma. Nivolumab-monotherapy has shown to be superior to ipilimumab-monotherapy revealing a 5-

year OS rate of 44% and was outplayed by nivolumab-plus-ipilimumab treatment with a 5-year OS of 52% despite severe adverse effects (Figure 3) [138, 147].

Around 50% of melanomas harbor a *BRAF*-mutation [65]. *BRAF*-positive mutation status decides whether using selective BRAF-inhibitors is a reasonable treatment option [146, 148]. The *BRAF*-mutation is accompanied by a constitutive activation of the MAPK-signaling pathway, ultimately ensuring autonomous proliferation and survival of melanoma cells. Additionally, hyperactivation of the MAPK-signaling participates in the production of immunosuppressive mediators affecting tumor surveillance [149]. Thus, regulating MAPK-signaling in *BRAF*-mutated melanoma represents a powerful tool preventing tumor progression. So far three clinical BRAF-inhibitors have been approved by the FDA, vemurafenib (2011), dabrafenib (2013) and encorafenib (2018 in combination with the MEK-inhibitor binimetinib) for melanoma treatment, which selectively target BRAFV600-monomers (Figure 4) [150].
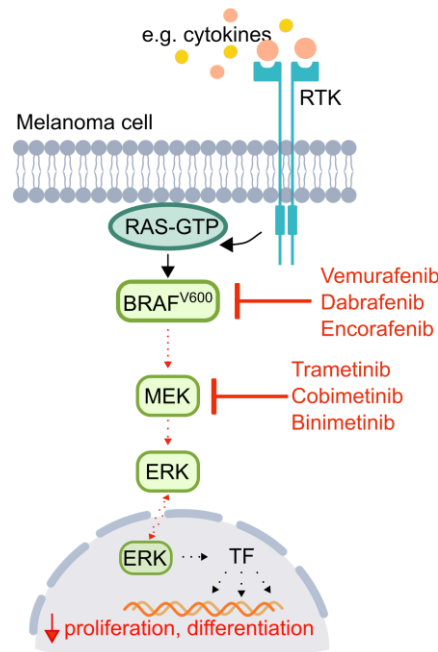
Figure 4. Targeted therapy and its targets. Mutation in the BRAF-kinase causes hyperactivation of the MAP-kinase pathway, inevitably affecting tumor cell survival and growth. By selective blockade of the mutated-BRAF-kinase using BRAF-inhibitors (e.g., vemurafenib, dabrafenib or encorafenib) or by unspecifically inhibiting downstream MEK utilizing MEK-inhibitors (e.g., trametinib, cobimetinib or binimetinib), proliferation and survival can be regulated and tumor growth prevented. Figure adapted from Becchetti et al. [41].

In 2011, vemurafenib (Zelboraf®, Genentech) received US FDA approval as the first selective BRAF-inhibitor for the treatment of unresectable or metastatic melanoma [151]. Vemurafenib is a small-molecule inhibitor that selectively blocks the mutated BRAFV600E-kinase and favorably influences the response rate by 48% and progression-free survival in patients when comparing to conventional chemotherapy with a response rate of only 5% [151-152]. Induction of tumor regression is not observed for wild-type BRAF cells [153-155]. Dabrafenib is indicated for patients harboring the BRAFV600E- or V600K-mutation and has been proven to prolong

PFS as well as showing superior effects on OS in a subset of patients when comparing to conventional chemotherapy [156-158]. Several studies have shown response rate reaching around 50% [146, 148, 159-160]. The PFS was significantly increased by 4.2 months (from 2.7 months to 6.9 months) compared to DTIC (HR = 0.37; 95%-KI: 0.24-0.58; p<0.0001) [158]. Encorafenib has shown superior pharmacological properties compared to vemurafenib and dabrafenib and improved clinical efficacy comparing with vemurafenib-monotherapy [161-163]. Schilling et al. reported a reduction of lymphocytes by around 24% when treating melanoma patients with vemurafenib but not with dabrafenib [164]. Interestingly, studies point at an enhancement of T cell function, while the release of immunosuppressive cytokines by melanoma cells was reduced when using BRAF-inhibitors [165-167]. However, several studies reported a paradoxical activation of the MAPK signaling using BRAF-inhibitors in wild-type BRAF cells through induction of RAF-dimerization [168]. Substantial clinical improvements have been achieved combining inhibition of BRAF and the inhibition for the downstream mediator, MEK (trametinib, binimetinib or cobimetinib) for patients with BRAFV600-mutated metastatic melanoma [148, 159-160]. The combination of vemurafenib and cobimetinib led to an increase of PFS by 3.7 months (from 6.2 months for vemurafenib plus placebo compared to 9.9 months for the combination treatment) [159]. Studies reported combinatorial therapy help combat acquisition of resistance by temporarily suppressing the tumor´s ability to escape using alternative signaling and survival pathways, thus extending treatment efficacy [169]. Comparing dabrafenib-trametinib combinatory treatment with dabrafenib-monotherapy a raise in response rate by 16%, the median PFS by around 2.2 months (11 months versus 8.8 months) and the median OS by 6.4 months (25.1 months versus 18.7 months) [148, 160]. Comparison of dabrafenib-trametinib with vemurafenib-monotherapy has been proven superior on both response rate (23% higher) as well as on median PFS (increase by 3.7%) and OS, raising the long-term perspective and effectiveness (p<0.0214) favoring the combination treatment [148, 159]. An indirect comparison of the three combination therapies disclosed highest median overall survival of 33.6 months for the combination encorafenib-binimetinib, followed by 25.6 months for dabrafenib-trametinib and 22.3 months for vemurafenib-cobimetinib [170].

In general, for patients with BRAF-mutated metastatic melanoma, either immunotherapy or targeted therapy is recommended [171]. Mutation burden and pre-existing primary resistances may help evaluating the options. Despite immense improvements in the assessment and treatment of metastatic melanoma, numerous drawbacks need to be faced in order increase the number of long-term survivors.

1.3 Epidemiology

In disregard of age and sex, melanoma belongs to the top 20 (rank 19 out of 20) most common cancers [172]. Since the 1970s the global incidence of cutaneous melanoma has been rising in Caucasians, especially in those with high sun exposure [173-174]. The highest incidence rates are found in Australia, despite the record of stabilization in the incidence, and New Zealand (33.6 and 33.3 cases per 100,000 person-years respectively), followed by Europe with the highest incidence being recorded in Western Europe with 18.8 cases per 100,000 person-years and northern America with 14.7 cases per 100,000 person-years. Incidence rates in South America, Africa or Asia were below five cases per 100,000 person-years. The mortality rate varies depending on the country and its primary health care, with targeted early detection in patients with increased risk being beneficial for prognosis and outcome [173, 175]. Highest mortality rates worldwide were displayed in Australia and New Zealand (3.4 deaths per 100,000 person-years) as well as in Europe (≥ 1.5 deaths per 100,000 person-years) and northern America (1.4 deaths per 100,000 person-years) [172]. Increased risk factors for developing melanoma include multiple benign naevi (≥ 100), multiple atypical naevi and familial predisposition [176].

Compared to Caucasians, darker-skinned people can produce and store more melanin and make themselves less susceptible to the DNA damaging light. According to the American Cancer Society and according studies, the incidence of melanoma in non-whites is much lower and stable [177-179]. Nonetheless, melanoma development effects the whole population disregarding skin color variants but UV-shielding is superior in more pigmented skin [180]. Interestingly, most red-haired and light skin people carry a variant of the melanocortin-1-receptor (MC1R), a transmembrane receptor that regulates tyrosinase levels in melanocytes via cAMP, which in darker-skinned people induces eumelanin synthesis. Low levels of cAMP and tyrosinase due to the dysfunctional MC1R variant signaling result in pheomelanin pigment synthesis, which compared to eumelanin harbors inefficient photoprotective capacity and even induces ROS production, which all together increases the risk for developing melanoma [181-184].

1.4 Resistance mechanisms of melanoma against applied clinical therapeutics

Despite tremendous refinements in prolonging survival of patients with unresectable late-stage melanoma, the 5-year OS does not reach beyond 52% for immunotherapy (study for combined nivolumab-ipilimumab treatment) or 34% for dual targeted therapy (combination of dabrafenib and trametinib) with initial low level LDH and small number of metastases in distant organs associated with an advantageous clinical outcome [147, 185-187]. The response onset and

rate for immune checkpoint inhibitors is slow and low, however their long-term beneficial effect regarding clinical response is substantial [188-190]. Comparatively, targeted therapy tends to evoke a fast and robust response [171]. Unfortunately, due to acquisition of resistances within a few months, evading blockage of the MAPK signaling, the long-term response quickly reaches its limits [191-192].

Intrinsic and extrinsic resistances, which annul the benefit of therapy, pose a tremendous multifactorial challenge. The EORTC 18071 and KEYNOTE-001 trial have highlighted the limits of immune checkpoint inhibitors, as disease relapse or death was documented in around 55% and around 25% during follow-up (median 21 months) respectively [193-196]. Researchers have debated and investigated the different options that restrain therapies. Some patients show lack of response since onset of therapy caused by primary resistance, others with initial response relapse after a period due to acquisition of resistance [197]. For PD-1 inhibitors to work, tumor cell recognition by T cell must be fully functional. Intrinsic and extrinsic factors orchestrate adaption of resistance mechanisms in tumor cells. Intrinsic adaptions may directly alter signaling pathways essential for tumor cell survival like the MAPK, PI3K or WNT signaling or influence the antigen processing machinery. CD8$^+$ T cells secrete IFNγ cytokine in order to downregulate proliferation and induce apoptosis in tumor cells through activation of the JAK-STAT pathway [198]. In 2017, Sucker et al. reported the emergence of IFNγ resistance by acquiring an inactivating mutation in *JAK1* or *JAK2* in melanoma lesions accompanied by low HLA-DR and PD-L1 expression [199-200]. Furthermore, alterations in proteins like the transporter associated with antigen processing (TAP), which regulates peptide trapping in the endoplasmic reticulum (ER) for selective major histocompatibility complex (MHC) loading, or other modulators (e.g. beta-2-microglobulin (B2M)), ultimately suppressing recognition by the adaptive immune system have been reported [201-203]. Downregulation of MHC class I expression was described as well and was associated with de-differentiation in melanoma as a major driver resulting in a lack of tumor immunogenicity [204-205]. Aside from changes in the antigen presentation machinery, modification of the antigen repertoire generating a new neoantigen landscape represents a major advantage for tumor cells evading anti-tumor efficacy of immune checkpoint inhibitors [206-207]. Data also revealed the importance of various immune cells and their interaction in the tumor microenvironment [208-210]. Extrinsically, immune suppressive properties were attributed to regulatory T cells (Treg) by secreting cytokines like IL-10 [211-212]. Also, myeloid-derived suppressor cells (MDSCs) have been implicated in suppression of anti-tumor functionality of the adaptive immune system and correlate with reduced effectiveness of immunotherapy in melanoma patients [213-214]. Tumor cells secrete chemokines that attract Tregs and MDSCs and may enhance tumor escape [215].

Emergence of resistance during the course of therapy also pose an enormous problem for targeted therapy. A study from 2014 including 45 examined patients exhibited a frequency of 31% for early resistance in the first 12 weeks of treatment with a BRAF-inhibitor [216]. Detection of primary resistance in these patients may help choosing the proper therapeutic approach as alternative treatment with immune checkpoint inhibitors is recommended. Numerous multifactorial mechanisms and heterogeneity between patients have been identified eventually causing reactivation of the MAPK signaling pathway or activation of alternative pathways like the PI3K-AKT signaling pathway. A multi-center meta-analysis report defined the frequencies of *BRAF* splice variants (16%) and amplifications (13%) in melanoma, which have already been identified in previous studies, as potential resistance instrument [217-220]. Mutations in the BRAF downstream kinases MEK1 and MEK2 (around 7%) were described as well [221]. Bypassing MAPK-blockage by BRAF-inhibitors can be carried out in tumor cells by increased activity or adaption of receptor tyrosine kinases (RTKs) or through an activating mutation in the *NRAS* gene. Under healthy physiological conditions, cAMP activates BRAF and regulates PKA, which represses CRAF activity [222]. Mutation-induced increase in NRAS expression in combination with a dysregulation of cAMP signaling initiates switching from BRAF- to CRAF-mediated signaling resulting in a rewiring and reactivation of the MAPK signaling pathway accompanying the maintenance of proliferation in these tumor cells [222-226]. Further genes that were reported altered in resistant cells include PTEN accompanied by suppression of the pro-apoptotic marker BIM [216, 227-230], NF1 [231] and CDKN2A [227, 232] or MITF [216, 233-234]. It is highly debated, at which time point resistant cells evolve during treatment or whether resistant cells already preexist as a subset of the heterogeneous tumor lesion [235].

1.5 The need of biomarkers in management of patients with malignant melanoma

Considering the survival of patients with advanced melanoma, improving diagnostics and identifying prognostic biomarkers is key to assess and choose the optimal treatment option for each patient with malignant melanoma. Aside the aforementioned markers like LDH, Melan-A expression, S100, HMB-45, which recognizes the gp100 antigen, and the evaluation of the BRAF-status, there is rising evidence that hint towards prognostic importance of cell-based biomarkers [236]. This view reaches beyond the detection of circulating tumor cells (CTCs) that reflect tumor burden and which have been correlated with worse outcome in cancers [237-238]. Due to limiting proportions of cells in the peripheral blood, CTC detection remains challenging especially in the early stages [239]. In fact, several studies demonstrate the utility of peripheral leukocytes as predictive biomarkers in melanoma [240-242].

1.5.1 The two-faced role of the immune system in malignant melanoma

As a highly immunogenic cancer, malignant melanoma has a fragile relationship to its microenvironment harboring various classes of cells from the immune system. The immune system is capable of restraining tumor growth and spreading e.g., by targeting melanoma-associated antigens displayed on MHC class-I molecules [243]. Anti-tumor immunity was attributed to different T cell subsets, NK cells and granulocytes [244-247]. Importantly, the tumor microenvironment exceeds the restricted view of a simple relationship between adapted immune system and tumor but represents a multi-cellular mechanism with bidirectional outcome for both tumor and other involved cells. Numerous tumors have been reported to make use of and benefit from attracted granulocytes. In fact, pro- and anti-tumoral functions have been attributed to both eosinophilic and neutrophilic granulocytes [247-250].

Granulocytes develop in the bone marrow, where $CD34^+CD117^+$ pluripotent hematopoietic stem cells give rise to common myeloid progenitors (CMPs) [247]. These lineage-committed progenitors differentiate and mature into a variety of leukocytes during homeostasis like granulocytes, including neutrophils, eosinophils and basophils, and monocytes upon activation of distinct sets of transcription factors and exposure to a unique cytokine-cocktail [251-255]. The commitment to an either neutrophil or eosinophil lineage is not fully understood yet. However, classical priming factors of neutrophils are G-CSF and GM-CSF, while IL-3, IL-4, IL-5, CCL11, and GM-CSF favor the development of eosinophils [256-258]. IL-5 is a critical mediator of eosinophil development and has a wide impact on eosinophil biology, as it also promotes eosinophil activation and survival [248, 250]. Mature and terminally differentiated granulocytes are then released to and travel through the peripheral blood where they reach the sites of tissue infection or inflammation via a stimulation- and recruitment-cocktail to exert their effector function [257]. Eosinophils are tissue-dwelling cells with their primary homing into the gastrointestinal tract where they are implicated in immune regulation, but they can also be encountered in the mammary gland, and thymus [249, 258-259]. Under healthy conditions, the number of produced eosinophils is low, with eosinophils making up to 6% of circulating leukocytes [260]. This number is strongly altered during inflammation and may quickly rise during allergic responses [261]. Neutrophils are responsible for up to 70% of peripheral blood leukocytes, thus accounting for a majority of peripheral leukocytes in the blood [262].

Neutrophils are easily distinguishable from eosinophils, as they exhibit multilobed nuclei while eosinophils contain characteristic bilobed nuclei [250]. Characteristic for granulocytes are their cytoplasmic granules. Neutrophils harbor three main granules, primary (azurophilic granules: including e.g., myeloperoxidase (MPO) and neutrophil elastase (ELANE)), secondary (specific granules: including e.g., lactotransferrin (LTF)) and tertiary (gelatinase granules: including e.g., arginase 1 (ARG1) and matrix metallopeptidase 9 (MMP-9)) granules [263]. Eosinophils on the

other hand are equipped with primary and secondary granules. The latter serve as a repository of the potent basic proteins like major basic protein 1 (MBP1), mainly found in the crystalloid core, matrix-located eosinophil peroxidase (EPX), a ribonucleases from the RNase A superfamily eosinophil cationic protein (ECP) and for eosinophil derived neurotoxin (EDN) [250, 263-264]. Among them, high amounts of pro-inflammatory proteins, various enzymes, growth factors and enzymes are stored in granules. Upon activation, eosinophil content is released during piecemeal or cytolytic degranulation, where they carry out their toxic potential against both healthy but also cancerous tissues [250, 261, 265-266].

Eosinophils express a range of receptors, which enables reactivity to growth factors, adhesion markers, induction of granulation and importantly, to cell-cell interactions (demonstrated in Figure 5) [250].
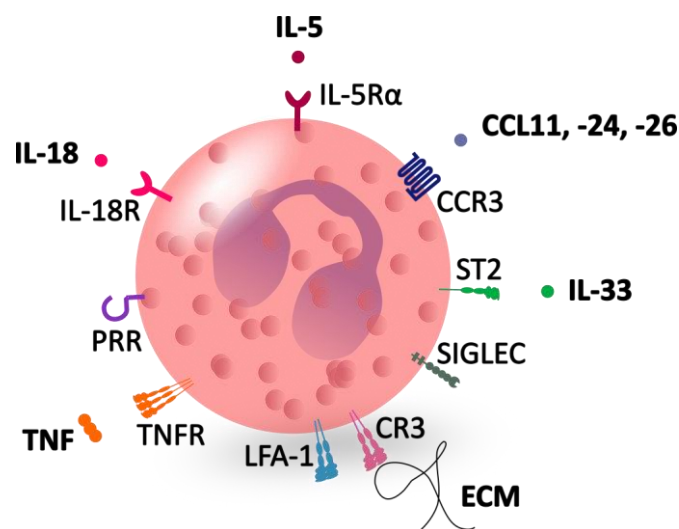
Figure 5. **The receptor repertoire of peripheral eosinophils.** Eosinophils possess characteristics that facilitate interaction with their environment. Activation and survival are majorly regulated by recognition of IL-5 via IL-5R and engagement of the CCR3 by the ligands CCL11 (eotaxin 1), CCL24 (eotaxin 2) and CCL26 (eotaxin 3). Adhesion is mediated by the expression of adhesion receptors like LFA-1 and CR3. The latter is recognized by the extracellular matrix (ECM). Additionally, adhesion is partially regulated by engagement of the ST2 receptor and the IL-18R by their respective ligands. Pattern-recognition receptors (PRRs), including various Toll-like receptors, receptor for advanced glycation end-products (RAGE) and TNFR enable capture of pathogens and reactivity to inflammation. Scheme adapted from Grisaru-Tal et al. [267].

The unique receptors of eosinophils are the interleukin-5 receptor (IL-5R), CC-chemokine receptor 3 (CCR3) and sialic acid-binding immunoglobulin-like lektin 8 (Siglec-8). As IL-5, the engagement of the CCR3 receptor by CC-chemokine ligand 11 (CCL11; eotaxin), CCL24 (eotaxin 2) or CCL26 (eotaxin 3) promotes chemotaxis, activation and survival of eosinophils, both in an IL-5-dependent and -independent manner. Additional eosinophilia regulating factors include the alarmin IL-33, an IL-1 cytokine family member produced by epithelial and endothelial cells, recognized by the ST2 receptor, or the IL-25 cytokine produced by T helper 2 (Th2) cells, which contributes to the regulation of IL-5 production by e.g., Th2 cells [248, 250, 261]. Ubiquitously expressed high-mobility group protein B (HMGB1), an eosinophil migration- and degranulation-promoting factor involved in inflammatory responses is recognized by

eosinophils via the receptor for advanced glycation end-products (RAGE) or through engagement with Toll-like receptors (TLR2 and TLR4) [268-269]. Extravasation of eosinophils from the peripheral blood into tissue is mainly attributed to attraction mediated by chemoattractants and involves integrins heterodimers, e.g. the lymphocyte function-associated antigen 1 (LFA-1; CD11a-CD18) and the complement receptor 3 (CR3; CD11b-CD18) enabling diapedesis [270-271]. Adhesion of eosinophils to cancer cells was stimulated by upregulation of LFA-1 expression through IL-18 and facilitated anti-tumoral function by eosinophils. IL-18 is recognized by the IL-18 receptor (IL-18R) [272-273].

1.5.2 Eosinophils – a novel biomarker in melanoma?

Eosinophil degranulation is a major function by which these cells cause intruders to undergo apoptosis, subsequently removing pathogens or even tumor cells [274]. Canonically, detrimental roles have been attributed to eosinophils and their potent granular mediatory. With rising interest for the pleiotropic multi-functional effector cells, the role of eosinophils in immune regulation and inflammation has become increasingly recognized over the years. In fact, eosinophils are involved in diseases like asthma, allergy, cancer and even obesity [275-280]. Already in 1893, tumor-associated tissue eosinophils (TATE) have been identified and investigated later on in gastric carcinoma utilizing electron microscopy [281-284]. Numerous studies have highlighted the beneficial prognostic value of eosinophils in most cancers. However, it is evident that high eosinophil counts and the degree of TATE can also be non-beneficial in other cases [261, 285-286]. Eosinophilia's prognostic value is therefore subject controversy. This might be reflected by the plasticity and heterogeneity of different cancers. The latter influencing eosinophil function in the tumor-microenvironment and their interaction with the adjacent immune cells. Eosinophils have been linked to tumor invasion in Hodgkin´s lymphoma and cervical carcinoma, showing a negative association with outcome and prognosis [261, 285-286]. Eosinophil infiltration into solid tumors like colon carcinoma [287-288], bladder carcinoma, oral squamous cell carcinoma and prostate cancer [248, 289-290] have been linked to a good prognosis. As for melanoma response to treatment was correlated with the presence of tumor-infiltrating immune cells and by trend survival was prolonged in patients with eosinophilia to any given time of the disease [267, 291-294]. Additionally, baseline relative eosinophil counts not only positively correlated with the overall survival of patients receiving ipilimumab (anti-CTLA-4) but high blood eosinophil counts also showed enhanced response of melanoma patients exposed to nivolumab (anti-PD-1) or combinatory treatment (anti-CTLA-4 and anti-PD-1/anti-PD-L1), thus significantly prolonging survival during treatment [294-297]. Interestingly, in rare cases exposure to anti-PD-1/anti-PD-L1 caused a massive increase of peripheral eosinophil counts in patients, which were proven not to be driven by

allergy or a parasitic infection [295]. In spite of the fact that eosinophils appear to be beneficial in melanoma, studies also suggest an association with immune-related adverse events (irAEs) during immune checkpoint inhibitor treatment [298]. Eosinophilia was also observed upon administration of IL-4, GM-CSF and CTLA-4 inhibitor [242, 299-302]. Moreover, eosinophil activation was reported upon IL-2 therapy, thus rejecting the hypothesis of tissue eosinophilia being an epiphenomenon arising only from impetuous secretion of IL-5 by T cells or cancer cells [261, 301-305]. Clinical observations regarding TATE have been supported by in vitro studies showing direct eosinophil-mediated cytotoxicity towards a variety of cancers, including melanoma and colon cancer but not again Hodgkin lymphoma [267, 272-273, 306-307]. For instance, an in vitro study on prostate cancer revealed a tumor growth-inhibiting function of eosinophils by secretion of IL-10 and changes in E-cadherin expression, potentially influencing metastatic seeding [308]. Tumoricidal effects are assigned to immunoregulatory cytokines, like IFN-γ and TNF. These factors are produced, stored and rapidly secreted by eosinophils upon activation [306, 309-312]. Eosinophil-derived TNF-α and granzyme A production and release of EDN and ECP, which were responsible for tumor cell apoptosis and necrosis, were shown inducible both in a colon carcinoma cell line (Colo-205) and in a melanoma mouse model (Figure 6) [270, 306].
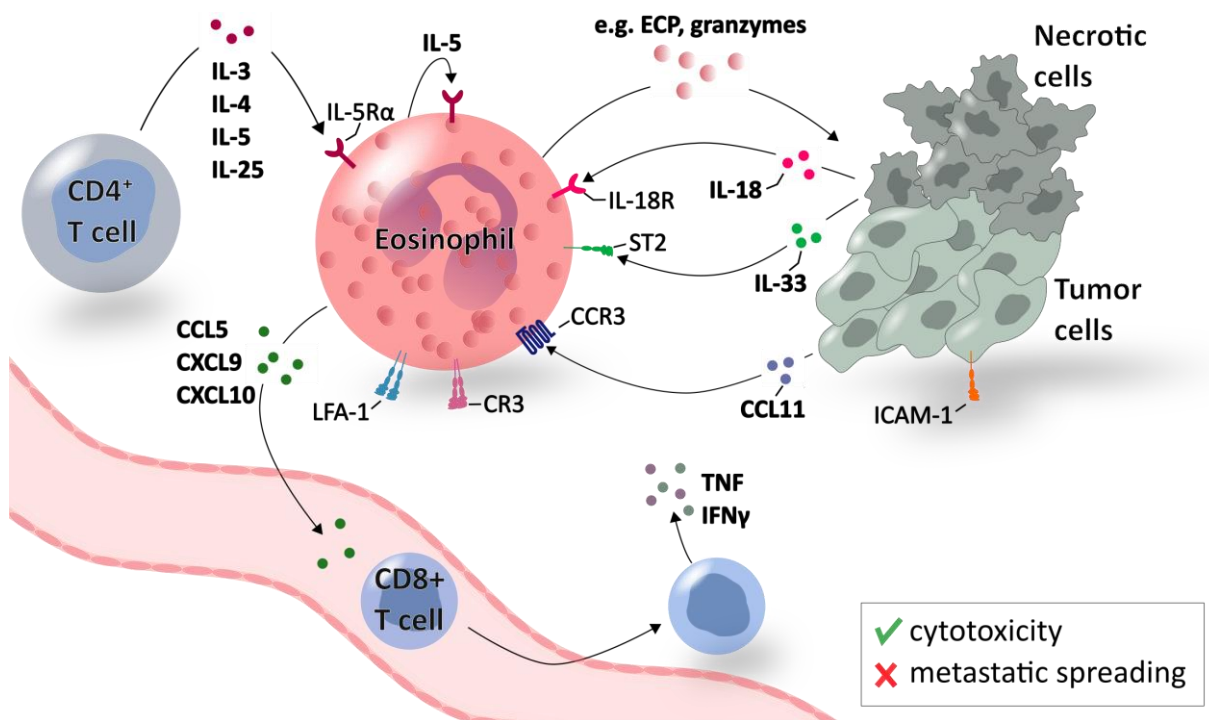
Figure 6. Indirect and direct eosinophil-mediated anti-tumoral functions. Production and release of cytokines like IL-5 and IL-25 by CD4+ T cells and the autocrine-secretion of IL-5 by eosinophils mediates eosinophil activation and survival. Cell-cell contact between eosinophils and tumor cells is regulated by IL-33 and IL-18, causing an increase in expression of the adhesion receptor LFA-1 on eosinophils and an increase in ICAM-1 expression on tumor cells. The adhesion process triggers the release of cytotoxic soluble mediators such as ECP and granzymes, causing tumor cells to undergo apoptosis. Aside from the direct eosinophil-tumor cell cytotoxicity, eosinophils induce infiltration of CD8+ T cells into the tumor-microenvironment, which themselves release tumoricidal factors facilitating the removal of tumor cells. Overview adapted from Grisaru-Tal et al. [267].

Recently, serum ECP was proposed as a potential biomarker for melanoma patients. Krückel et al. reported that even though ECP is cytotoxic and tumoricidal, high serum levels were independent of LDH values and correlated with a worse outcome in patients with metastatic melanoma [313]. Idiopathic hypereosinophilic syndrome (IHES) is one of the more common eosinophilic diseases. Patients with eosinophilia exhibited a higher plasma ECP level than the control group, suggesting that plasma ECP could assist in the clinical diagnosis of the disease [314-316]. As additional evidence, several studies suggest the combined measurement of ECP and eosinophil count ratio is an excellent marker for asthmatics [314, 317-318]. Diverse studies shed light on the interaction of eosinophils and T cells. Infiltration of CD8^{+} T cells into the mouse tumor model was improved through production of CCL5, CXCL9 and CXCL10 by eosinophils, demonstrating the collaboration between the adapted and innate immune system in tumor rejection (Figure 6) [275]. This view was supported by a study conducted by Lucarini et al. describing a significant tumor growth delay mediated by IL-33 injection in a subcutaneous melanoma of a mouse model. IL-33 injection was accompanied by recruitment and accumulation of eosinophils and CD8^{+} T cells into the tumor subsequently preventing pulmonary metastasis formation [307]. Eosinophil-mediated cytotoxicity against colon carcinoma also involved IL-18 and the upregulation of adhesion markers like LFA-1 and ICAM-1, implying the need of close contact between eosinophils and their target [272-273]. Angiogenesis is crucial for metastasis and tumor growth as it provides an extension route of tumor cells into the circulation [319]. Attenuated secretion of angiogenic factors like vascular endothelial growth factor (VEGF) by eosinophils have been linked to normalization of tumor vessels and induced eosinophil infiltration by CCL11-mediated stimulation impeded angiogenesis and caused necrosis in a fibrosarcoma mouse model (Figure 6) [275, 320]. Despite extensive research on eosinophils in the tumor microenvironment, it was just recently that researchers discovered eosinophil-mediated cytotoxicity against a human colon carcinoma cell line via CD11a/CD18-dependent mechanisms [272, 306].

1.5.3 Aim of this study

All these aforementioned studies highlight that eosinophils are underestimated innate immune cells that play potent anti-tumoral roles in the vast majority of cancers. A rapid increase in personalized therapies necessitates the development of prediction models that facilitate balancing clinical and patient well-being, especially for high-risk patients [321]. Currently, the question remains to how to optimize the use of knowledge about how eosinophils function and their ability to fight tumors in standard clinical practice.

In this thesis, we pursued two specific aims.

I.   To evaluate the prognostic importance of peripheral blood eosinophil counts and their soluble mediators, like ECP, in patients with advanced melanoma.
II. To unravel the relationship between blood-derived eosinophils and melanoma cells utilizing phenotypical and functional analysis and to describe eosinophil-mediated cytotoxicity in vitro.

2 Materials and methods

2.1 Materials

2.1.1 Patient cohort and healthy donors

All consecutive patients with newly diagnosed metastatic or unresectable cutaneous melanoma presenting at the Department of Dermatology, University Hospital Würzburg, were enrolled in this study (Table 1) [274]. Enrollment was not restricted to certain lines of therapy. Patients undergoing adjuvant therapy were not included. Patients with metastatic melanoma and no evidence of disease at the time of blood being drawn were identified in the database and enrolled during follow-up visits. In addition, all consecutive patients with newly diagnosed stage I or II melanoma were enrolled after surgical treatment. None of the early-stage patients received therapy. Patients were enrolled between 07/2015 and 12/2021. The study had been approved by the ethics committee of the University of Würzburg (50/17-mk). Additionally, we received 21 retrospective serum samples from advanced melanoma patients receiving targeted therapy as a first-line therapy from the Department of Dermatology, University Hospital Erlangen, and nine serum samples from the multi-centric blood bank of the Department of Dermatology, University Hospital Tübingen that were derived from patients recruited at the Department of Dermatology, University Hospital Dresden. All patients enrolled provided written informed consent. Serum samples were collected prior to and during treatment. For samples from patients receiving dual targeted therapy, the median of days between the first sample (pre-treatment) and second (on-treatment) sample was 98 days (range 58–178 days). On-treatment serum samples were obtained close to the first response assessment. For samples from patients receiving immunotherapy, the median time span was 175 days (range 52–269 days). To obtain ECP reference values, three healthy volunteers were included. White blood cell count and serum lactate dehydrogenase (LDH) were assessed 0–63 days prior to collection of the pre-treatment ECP samples and 0–63 days prior to collection of the on-treatment serum ECP samples. The closest peripheral blood draw was considered when multiple values were available. Responders were defined by RECIST 1.1. as CR (complete remission) or PR (partial remission) and non-responders as PD (progressive disease) or SD (stable disease) to the respective treatment. Demographic and clinical data were collected from all patients listed in Table 1. For phenotypical analysis of eosinophils and assessment of cytotoxicity, a second cohort of 49 samples from patients diagnosed with stage IV melanoma before the implementation of therapy, six patients with no evidence of disease (NED) in stage I and II and a total of 12 healthy donors (HD) serving as controls were included. Clinical parameters were not collected for the second cohort (Table 2) [274].

Table 1. Patients included for evaluation of serum ECP, peripheral blood counts and association of REC with response to MAPKi in patients with advanced malignant melanoma receiving dual targeted therapy (total n = 94). Patients receiving immunotherapy served as control cohort (total n = 149).

Variables		Patients	
Age	median (range)	70 years (27–93)	
			%
Individual Patients		243	100
Sex	male	115	47.3
	female	98	40.3
	unknown [1]	30	12.4
Stage	III	17	7
	IV	226	93
M-Category	M1a	24	9.9
	M1b	59	24.3
	M1c	85	35
	M1d	58	23.9
First-Line Therapy	yes	205	84.4
	no	38	15.6
Therapy after Study Inclusion	anti-PD-1	86	35.4
	anit-PD-1 + anti-CTLA-4	63	25.9
	BRAFi + MEKi [2]	94	38.7
LDH	>1x ULN [3]	86	35.4
	<1x ULN [3]	153	63
	missing	4	1.6

[1] For patient serum samples from Tübingen/Erlangen, this information was not provided.
[2] All patients receiving dual MAPKi (BRAFi + MEKi) showed a BRAFV600-mutation.
[3] Upper limit of normal (ULN).

Table 2. Patients and healthy donors included in phenotyping eosinophils and for cytotoxicity evaluation [1].

Variables			Donor	
				%
Individual Donors			67	100
Patient	Stage	I/II	6	9.0
		IV [2]	49	73.1
Healthy Donor			12	17.9

[1] For functional and phenotypic analyses, additional blood samples were collected from donors. Clinical parameters were not collected for this cohort.
[2] Treatment-naïve stage IV patient samples.

2.1.2 Cell lines and cell culture

All melanoma cell lines, aside from MaMel114, carried the BRAFV600E mutation and grew adherently in culture flasks. The primary human cutaneous melanoma cell line MaMel63a was mainly used to assess the in vitro function of eosinophils, including the cytotoxicity evaluation of eosinophils from melanoma patients compared to healthy donors, phenotypic analysis and analysis of the relationship between melanoma cells and eosinophils. Further BRAF-mutated cell lines (MaMel06, MaMel80a and MaMel51) served as controls to evaluate the extent of cytotoxicity in different cell lines. All MaMel cell lines were derived from patient biopsies as described previously [322]. Evaluation of specificity and sensitivity of the effects observed by eosinophils was conducted using non-melanoma-derived and non-BRAF-mutated cell lines including the non-small-cell lung cancer cell line H460 and the suspension Merkel cell

carcinoma cell line WaGa, kindly provided by D. Schrama and R. Houben (University Hospital Würzburg). All cell lines and solutions for cell culture are listed in Table 3 and 4.

Table 3. List of melanoma and non-melanoma cell lines.

Name	Disease	Site of Derivation	BRAF	NRAS	TERT	Gender
MaMel63a	melanoma	cutaneous/ subcutaneous	V600E	wt	mut	f
MaMel51	melanoma	lymph node	V600E	-	mut	m
MaMel06	melanoma	lymph node	V600E	-	mut	m
MaMel80a	melanoma	lymph node	V600E	-	mut	f
MaMel114	melanoma	brain	wt	-	mut	m
WaGa	merkel cell carcinoma	ascites	-	-	-	m
H460	non-small-cell lung cancer	pleural effusion, lung	-	-	-	m

Abbreviations: mut = mutated, wt = wild type, f = female, m = male.

Table 4. List of solutions for cell culture.

Product	Supplier
DPBS	Sigma-Aldrich # D8537-500ML
FBS superior	Biochrom # S 0615
Penicillin-Streptomycin	Sigma-Aldrich # P4333-100ML
RPMI-1640 Medium with L-glutamine with sodiumbicarbonate	Sigma-Aldrich # R8758-500ML
Trypan Blue Solution	Sigma-Aldrich # 93595-250ML
10x Trypsin/EDTA	Sigma-Aldrich # E7889-100ML

2.1.3 Consumables

Table 5. List of consumables.

Product	Supplier
Centrifuge Tubes	Greiner CELLSTAR®
MS Columns	Miltenyi Biotec
Neubauer counting chamber 0,100 mm depth	Assistent
Reagent Tubes	Sarstedt
Serological Pipettes	Greiner CELLSTAR®
SuperFrost® Plus microscope slides 25 x 75 x 1.0mm	R. Langenbrinck GmbH
Spin-X® Centrifuge Tube Filter	Corning Costar®
Test Tubes (flow cytometry tubes, polypropylene)	Beckman Coulter
Test Tubes (flow cytometry tubes, polystyrene)	Hartenstein
Tissue Culture Flasks (t-flask)	Greiner CELLSTAR®
24-Well Plates	Greiner CELLSTAR®

2.1.4 Chemicals

Table 6. List of chemicals and their final concentration (f.c.).

Product	Solved in	Concentration	CAS	Supplier
Brefeldin A	DMSO	f.c.: 5 µg/mL		Biolegend # 420601
BSA		f.c.: 0.1-0.5%	6381-92-6	Sigma Life Science # A7906-100G

Cisplatin	DMSO	stock: 3.33 MM f.c.: 20 µM	15663-27-1	Ratiopharm Supplied by the UKW pharmacy
Cobimetinib (GDC-0973)	DMSO	stock: 1 mM f.c.: 100 nM	934660-93-2	Cayman Chemical # 19563
DMSO		stock: ≥99.7%	67-68-5	Sigma-Aldrich # D2650-100ML
Ethanol absolute		stock: 99.9%	64-17-5	Chemsolute® # 2246.1000
Phorbol-12- myristate-13- acetate (PMA)	DMSO	stock: 5 mg/mL f.c.: 50 nM	16561-29-8	Sigma-Aldrich # 79346
Propidium iodide (PI)	1% FCS in DPBS	0.1 mg/mL		Sigma-Aldrich # P-4170
RNAseA		0.1 mg/mL		ThermoFisher Scientific # EN0531
Vemurafenib (PLX4032)	DMSO	stock: 10 mM f.c.: 100 nM - 10 µM	918504-65-1	Cayman Chemical # 10618

2.1.5   Buffers and solutions

Table 7. Materials for crystal violet staining.

Product	Composition	Amount	F.c.	Supplier
Crystal violet staining solution	0.5% crystal violet solution in ddH$_2$O	25 mL	0.25%	Roth # T123.1
	Methanol	10 mL	20%	VWR Chemicals # 20847.295P
	ddH$_2$O	15 mL	30%	

Table 8. List of buffers and solutions for granulocyte separation and purity staining.

Product	Application	Supplier
10x Annexin V Binding Buffer	diluted 1:10 in ddH$_2$O	BD Pharmingen™ # 51-66121E
autoMACS™ Running Buffer MACS Separation Buffer		Miltenyi Biotec # 130-091-221
autoMACS™ Pro Washing Solution		Miltenyi Biotec # 130-092-987
Biocoll Separating Solution	density: 1.077 g/mL, isotonic	Biochrom # L6115
Flow Cytometry Staining Buffer	0.1% BSA in DPBS	
10x Red Blood Cell Lysis Buffer	diluted 1:10 in ddH$_2$O	Biolegend # 420302

2.1.6   Instruments

Table 9. List of instruments.

Product	Supplier
Analytical lab scale BP121S	Sartorius
AutoMACS® Pro Separator	Miltenyi Biotec
Block Heater SBH130DC	Stuart
Centrifuge Heraeus™ Pico™ 17	ThermoFisher Scientific
Centrifuge Megafuge™ 16R	ThermoFisher Scientific
CytoFLEX LX	Beckman Coulter
Cytospin 2	Shandon
FACS Canto™	BD
Incubator Heracell™ VIOS 160i	ThermoFisher Scientific

25

IKA-Combimag REO magnetic stirrer	IKA
Microscope DM IL	Leica
Microscope DM750	Leica
Micro scale scout	OHAUS
M1 Minishaker	IKA
Nikon TI-E microscope	Nikon
Rotating platform RM5-30 V	Engineering buro CAT
Tecan Reader (Infinite M Nano)	Tecan
Tissue Culture Hood Safe 2020	ThermoFisher Scientific

2.1.7 Kits

Table 10. List of kits.

Product	F.c. or volume	Supplier
CellTrace CFSE Cell Proliferation Kit	2 µM	ThermoFisher Scientific # C34554
ECP ELISA	-	Cusabio # CSB-E11729h
Eosinophil Isolation Kit human	-	Miltenyi Biotec # 130-092-010
Multicolor CompBeads	1 drop = 60 µl	BD Biosciences # 644204

2.1.8 Antibodies

Table 11. List of human antibodies and dyes including their final concentration (f.c.).

Product	Clone	F.c. or volume	Supplier
7-AAD		1:1000 in 1x Annexin V binding buffer	Life technologies # A1310
Annexin V-APC		1:33 in 1x Annexin V binding buffer	BD Bioscience # 550475
CD3-PE		4 µl	Beckman Coulter # A07747
CD3-PE	HIT3a	4 µl	Biolegend # 300307
CD14-PerCP-Cy5.5	HCD14	2 µl	Biolegend # 325622
CD16-FITC	3G8	1 µl (phenotyping) 2 µl (purity control)	Biolegend # 302006
CD16-Pacific Blue™	3G8	1 µl	Biolegend # 302032
CD19-PE		4 µl	Beckman Coulter # A07769
CD19-PE	HIB19	4 µl	Biolegend # 302208
CD29-APC-Cy7	TS2/16	2 µl	Biolegend # 303014
CD31-PerCp-Cy5.5	WM59	2 µl	Biolegend # 303131
CD45-PE-Cy7	HI30	4 µl	Biolegend # 304015
CD49d-PE-Cy7	9F10	2 µl	Biolegend # 304313
CD56-PE	CMSSB	4 µl	eBioscience # 12-0567-42
CD66b-APC	G10F5	2 µl	eBioscience # 17-0666-42
CD193-APC-Cy7 (CCR3)	5E8	1 µl (phenotyping) 4 µl (purity control)	Biolegend # 310712
CD193-PE (CCR3)	5E8	1 µl	Biolegend # 310706
CD274-PE-Cy7 (B7-H1, PD-L1)	29E.2A3	2 µl	Biolegend # 329718
Ki-67 APC	Ki-67	4 µl	Biolegend # 350514
HLA-A, -B, -C-PE	W6/32	2 µl	Biolegend # 311405
HLA-DR-APC-Cy7	L243	2 µl	Biolegend # 307618
Mouse IgG1 κ-FITC Isotype control	MOPC-21	2 µl	Biolegend # 400108
Mouse IgG1 κ-PE Isotype control	MOPC-21	4 µl	Biolegend # 400112
Mouse IgG1 κ-PE-Cy7 Isotype control	P3.6.2.8.1	4 µl	eBioscience # 25-4714-42
Mouse IgG1 κ-PerCP-Cy5.5 Isotype control	MOPC-21	2 µl	Biolegend # 400150

Mouse IgG1 κ-APC Isotype control	P3.6.2.8.1	2 µl	eBioscience # 17-4714-41
Mouse IgG1 κ-APC Isotype control	MOPC-21	2 µl	Biolegend # 400122
Mouse IgG1 κ-APC-Cy7 Isotype control		4 µl	BD Pharmingen # 557873
Siglec-8-PerCP-Cy5.5	7C9	2 µl	Biolegend # 347107

Table 12. Blocking antibodies and DNAseI for dissolving EETs.

Product	Clone	F.c. or volume	Supplier
CD11a (LEAF) Host: mouse	HI111	2 µg/mL	Biolegend # 301213
CD11b (LEAF) Host: mouse	ICRF44	2 µg/mL	Biolegend # 301311
CD18 (LEAF) Host: mouse	TS1/18	2 µg/mL	Biolegend # 302111
CD49d (LEAF)	9F10	2 µg/mL	Biolegend # 304309
CD54 (LEAF) = ICAM-1 Host: mouse	HCD54	2 µg/mL	Biolegend # 322704
QIAamp DNA Mini including DNaseI		1:50 in digestion buffer	Qiagen # 51306
Mouse IgG1, κ Isotype	P3.6.2.8.1	2 µg/mL	eBioscience™ # 16-4714-82

2.2 Methods

2.2.1 ELISA

To determine ECP levels in serum of late-stage melanoma patients and healthy volunteers, freshly drawn blood was centrifuged (800 g, 10 minutes, room temperature (RT); serum was aliquoted and stored at -80 °C until use. After thawing, samples were centrifuged for 15 minutes at 1,000 g and ECP concentration was measured by ELISA. According to the manufacturer, the detection range lays between 1.56 ng/mL and 100 ng/mL. Normal ECP values in serum from healthy donors range (95%) from 2.3 ng/mL to 15.9 ng/ml [313, 323]. Duplicates of each sample were assessed. ECP serum levels were correlated with patient´s response to targeted therapy or to immunotherapy and with laboratory values like relative eosinophil counts (REC) and absolute eosinophil counts (AEC). Responders were defined as CR and PR at time of assessment and non-responders as PD and SD according to RECIST 1.1. Absorbance was measured at 450 nm and a reference absorbance was measured at 540 nm using the Tecan Reader (Infinite M Nano).

2.2.2 Multiplex-Analysis

Customized human panels were used for LegendPlex™ multi-analyte analysis including eosinophil-related soluble mediators such as RANTES, sRAGE, Eotaxin, GM-CSF and APRIL. Serum from melanoma patients before and during administration of targeted therapy as first-line therapy was centrifuged before processing. Serum analysis was carried out as described in the protocol provided by the manufacturer. Pre- and on-treatment concentrations of appropriate analytes were correlated with clinical response. Responders were defined as CR and PR at time of assessment and non-responders as PD and SD according to RECIST 1.1. Analyte intensity was measured using the CytoFLEX LX from Beckman Coulter. Raw data was analyzed using the QOGNIT software platform (version 2022-02-10) provided by Biolegend.

2.2.3 Cell culture

Cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FCS) and 1% penicillin/streptomycin, referred to as complete medium (CM). For harvesting adherent melanoma cells, 1x Trypsin/EDTA in DPBS was used to detach cells from culture flasks. Trypan blue staining helped to determine cell amount. Cells were routinely tested for mycoplasma infection and found to be negative. All cells were grown at 37 °C with 5% CO_2.

2.2.4 CFSE-staining of tumor cells

CFSE-stainings of tumor cells was conducted to distinguish cell population in co-cultures. CFSE was warmed up to RT, covered and protected from light at all times. 2×10^6 cells were used for following staining procedure. After detaching cells from culture flask, cells were washed twice with DPBS with alternating centrifugation steps at 300 g for five minutes. Cells were resuspended in 800 µL DPBS and 200 µL of 10 µM CFSE, with a final CFSE concentration of 2 µM. After thorough mixing, cells were incubated for 10 minutes at 37 °C. The reaction was stopped by adding 10 mL CM for 10 minutes at RT in the dark. Cells were washed twice with CM with alternating washing steps and centrifugation at 300 g for five minutes. In a final step, cells were resuspended in 10 mL CM and counted.

2.2.5 Flow cytometry

Flow cytometric analysis was carried out measuring 5,000 events in the gate of interest for co-cultures and 10,000 events for phenotypical analysis and purity control of eosinophils and neutrophils. Multicolor CompBeads were stained with appropriate control anti-mouse IgG1 κ antibodies listed in Table 11 for appropriate compensation of multicolor assays. For accurate analysis, fluorochrome signal was measured at the same setting used for the corresponding experiment. Samples were measured using the Canto™ FACS device from BD or the CytoFLEX LX from Beckman Coulter. Samples from the same experiment were measured on the same device. Flow cytometry data were analyzed and visualized using FlowJo V10 (BD Biosciences).

2.2.6 Isolation and purification of granulocytes from whole blood

Granulocytes obtained from healthy volunteers and patients were isolated from peripheral blood samples collected in sterile vacutainers heparin tubes. Polymorphonuclear leukocytes (PMNs) were separated from heparinized blood by density-gradient centrifugation. Blood samples diluted 1:1 with DPBS was layered on Biocoll and centrifuged at 360 g for 20 minutes without the centrifuge brake at room temperature. The PBMC and Biocoll layer was carefully removed without disturbing the erythrocyte-PMN layer. To remove erythrocytes, the PMN/erythrocyte suspension was incubated with a hypotonic 1x RBC lysis buffer for 10 minutes by constant rocking. Cells subsequently were washed at 300 g for 10 minutes at RT. PMNs were resuspended once more in 1x RBC lysis buffer and centrifuged at 300 g for five minutes at RT. Eosinophils and neutrophils were purified using an automatic magnetic labelling-based system with a multi-antibody eosinophil isolation kit from Miltenyi Biotec. PMN

yield was determined via trypan blue staining; cells were centrifuged at 300 g for 10 minutes and resuspended in 40 µl DPBS per 10^7 cells. Purification was carried out by negative selection of eosinophils. The positive selected fraction contained neutrophils and a small fraction of basophils. The purity of isolated eosinophils and neutrophils and their phenotypic characterization was evaluated flow cytometrically using the Canto™ FACS device from BD.

2.2.7 Extracellular staining for flow cytometry to determine purity and phenotype

Dry eosinophil pellets were stained using the following antibodies to ascertain purity after isolation and purification: anti-CD16-FITC, anti-CD66b-APC, anti-CD14-PerCP-Cy5.5, anti-CD193-APC-Cy7, anti-CD45-PE-Cy7, anti-CD56-PE, anti-CD3-PE and anti-CD19-PE. Eosinophils were defined as CD45$^+$/CD16$^-$/CD66b$^+$/CD193$^+$. A high purity of >90% was routinely obtained. For phenotypic surface characterization, 5 x 10^5 eosinophil dry pellets were stained with following lineage antibodies to define the population: anti-CD16-FITC or -PB, anti-CD66b-APC and anti-CD193-PE or -APC-Cy7. These antibodies were combined with two or three antibodies for the following target epitopes: HLA-DR, HLA-A/B/C, PD-L1, Siglec-8, TNFR2, CD49d, CD69, CD66b, CD31 and CD29. The surface expression of granulocytes derived from melanoma patients was compared to those obtained from patients without tumor burden and healthy donors.

To evaluate the effect of the metastatic cell line MaMel63a on the phenotypic pattern of eosinophils prior and after 24 and 48 hours in in vitro co-culture with a target to effector cell ratio 1:7.5, co-cultured cells were centrifuged at 1036 g for 5 minutes, washed once with DPBS and stained as described. MaMel63a cells were labelled with CFSE prior co-culture to distinguish between the two cell subtypes in co-cultures. Antibodies were incubated for 30 minutes at RT in the dark. Subsequently, cells were washed with 1 mL staining buffer and centrifuged at 1036 g for 5 minutes. The supernatant was discarded, and cells were resuspended in staining buffer. Measurements were performed using the Canto™ FACS device from BD. Antibodies are listed in Table 11.

2.2.8 Adherent and non-adherent culture assays

In order to identify a suitable concentration of vemurafenib and cobimetinib to treat BRAF-mutated melanoma cells, a gradient of different concentrations was tested. MaMel63a cells were treated with 10 µM, 5 µM, 1 µM, 500 nM or 100 nM vemurafenib or with 1 µM, 500 nM, 100 nM, 50 nM or 10 nM cobimetinib. As control, DMSO was used. Cells were cultured non-adherently for 24 and 48 hours with or without treatment. As 1 µM vemurafenib and 100 nM cobimetinib was determined to be optimal, MaMe51 and MaMel63a cells were treated with

combination of both vemurafenib and cobimetinib and viability was determined serially (3-50 hours).

For cytotoxic assays, freshly isolated eosinophils were co-cultured with 2×10^4 CFSE-labelled tumor cells in a target to eosinophil (T:E) ratio of 1:1, 1:5, 1:7.5 and 1:10. Cells were cultured in 1 mL CM with or without addition of 1 µM vemurafenib and/or 100 nM cobimetinib or 20 µM cisplatin. Co-cultures were carried out under non-adherent culture conditions in polypropylene tubes or under adherent conditions in 24-well plates for 24 or 48 hours as indicated. After the incubation, 7-AAD and Annexin V-APC staining determined time the viability of tumor cells and granulocytes.

2.2.9   7-AAD/Annexin-V viability staining

Cell viability was determined by 7-AAD and Annexin-V staining. To do so, 300 µL 1x Annexin-V binding buffer was prepared per condition. 100 µL of the 1x Annexin-V binding buffer was used to dilute 7-AAD 1:1000 and Annexin-V 1:33. Cells were centrifuged at 1036 g for 5 minutes after indicated culture duration, washed with 1 mL DPBS and centrifuged at 1036 g for 5 minutes. The supernatant was discarded. Cell pellet was resuspended with the prepared 100 µL buffer containing 7-AAD and Annexin-V. After an incubation time of 15 minutes at RT in the dark, 200 µL 1x Annexin-V binding buffer was added to each condition. Viable cells were defined as being 7-AAD- / Annexin V-double negative. Early apoptotic cells were defined as Annexin-V-positive, necrotic cells as 7-AAD-positive and late apoptotic cells as 7-AAD- and Annexin-V-double positive. Measurements were performed using the Canto^{TM} flow cytometry system from BD.

2.2.10   Proliferation and cell cycle assays

Ki-67 is a protein that is highly expressed in proliferating cells but downregulated in cells in the G_0 resting phase of the cell cycle [324-325]. Although Ki-67 has been used as a biomarker in various type of cancers [326-329], its relevance as a prognostic marker in cutaneous melanoma remains controversial as it does not capture the exact number of cells proceeding into mitosis [325, 330-331]. Propidium iodide (PI) on the other hand intercalates into the DNA, enabling the study on cell cycle status.

To reveal the effect of granulocytes on melanoma cell proliferation and cell cycle under non-adherent and adherent conditions, melanoma cells were stained with a Ki-67-specific antibody or PI after 24 hour or 48-hour co-culture under stated treatment conditions. Cells were washed with DPBS and centrifuged at 350 g for five minutes. For Ki-67 staining cell pellet was slowly resuspended in 2 mL cold 70% ethanol while vortexing to prevent clogging during fixation.

Cells were fixed for 1 hour at -20°C, followed by two washing steps with 1 mL 1% FCS in DPBS. The cell pellet was resuspended in 100 µL 1% FCS in DPBS and 4 µL conjugated Ki-67-APC antibody and stained for 30 minutes at room temperature in the dark. To remove unbound antibody, cells were washed twice with 1% FCS in DPBS and resuspended in 200 µL DPBS.

For PI staining, pelleted cells were washed and resuspended in 250 µL 1% FCS in DPBS and 2 mL cold 100% ethanol was added to the cells while vortexing. Fixation was carried out for at least 1 hour at 4 °C. Fixed cells were centrifuged at 350 g for five minutes and resuspended in 1 mL DPBS containing 1% FCS, 0.1 mg/mL PI and 0.1 mg/mL RNAseA. Staining was carried out for 1 hour at 37 °C in the dark. After Ki-67 or PI staining, cells were subsequently measured at the flow cytometer to determine proliferation or cell cycle using the Canto™ FACS device.

2.2.11 Colony formation assay

To visualize the impact of granulocytes on the ability of melanoma cells forming colonies and spreading on a given surface, and to determine melanoma cell viability, co-cultures were stained for crystal violet. Co-cultures of melanoma cells and eosinophils were carried out as described in 2.2.8. After co-culture incubation time, melanoma cells were counted and seeded on a 6-well plate for additional 48 hours. Cells were then stained with crystal violet to visualize cell density. Cells were washed once with DPBS and 0.25% crystal violet solution (containing 20% methanol) was added for one hour at room temperature under the fume hood. Wells were washed three times with deionized H_2O and plates dried overnight. Representative images were taken. For quantification, equal amount of methanol was added to each well (maximum of 1 mL). After 20 minutes of incubation, triplicates were transferred to a 96-well plate. Absorbance of crystal violet dye was measured at 570 nm using a Tecan Reader (Infinite M Nano).

2.2.12 Experiments with conditioned medium

Non-adherent co-cultures were performed as described for 48 hours using a ratio of 1:7.5 tumor to eosinophils. After the incubation time, the supernatant, referred to as conditioned medium, was collected by centrifuging the prepared co-cultures at 300 g for 5 minutes. The conditioned medium was transferred into a fresh 1.5 mL Eppendorf tube. To avoid any contamination with residual cells, another centrifugation step at 300 g for 5 minutes was added and the supernatant transferred into a final 1.5 mL Eppendorf tube. The samples were stored at -20°C or used immediately for further analysis. For the latter, freshly prepared melanoma

cells or eosinophils were cultured with the prepared conditioned medium for 48 hours. The cell viability was measured as described.

2.2.13 Transwell-experiments

For Transwell-experiments, CFSE-stained tumor cells were cultured separately or together with freshly isolated eosinophils in Spin-X columns equipped with a 0.22 µm porous membrane. Tumor cells were kept in the bottom of the column. Eosinophils were seeded on top of the membrane or together with tumor cells. The column was filled with 1 mL and the Transwell inlay with 700 µL CM with or without 1 µM vemurafenib and 0.1 µM cobimetinib. To remove residual air bubbles the column with the insert was centrifuged for 1 minute at 200 g. Culture was incubated for 24 hours and viability of cells was determined as described.

2.2.14 Interaction blockade and dissolving EETs in co-cultures

For blocking experiments, CFSE-stained melanoma cells or freshly isolated eosinophils were pre-incubated with blocking antibodies for target structures like ICAM-1 (CD54), CD11a, CD11b, CD18, CD49 or with the respective control antibody, anit-IgG1. Dry cell pellets were incubated with blocking antibodies at 2 µg/mL concentration for 30 minutes at 37 °C and 5% CO_2. To dissolve the DNA scaffold formed during EETs by eosinophils, dry pellets of eosinophils were treated with DNaseI for 30 minutes at 37 °C and 5% CO_2 or DNaseI was added to non-adherent co-cultures to dissolve EET structure in co-culture. DNaseI was pre-diluted 1:50 in 75 µL digestion buffer. After treatment, cells were co-cultured in non-adherent polypropylene tubes in 1 mL CM for 24 or 48 hours. Blocking antibodies, control antibodies and DNaseI are listed in Table 12.

2.2.15 Lysis of eosinophils and inactivation of contents

To expose intracellular granule content of granulocytes, dry pellets of eosinophils were frozen in liquid nitrogen for 1 minute (approach was adapted to the technique published by Mattes et al. [332]). Frozen samples were thawed at 37 °C for 5 minutes. Neutralization of eosinophils content after lysis was carried out by denaturation at 95 °C for 1 hour using a block heater (Stuart). Lysed or heat-inactivated eosinophils were resuspended in CM and co-cultured with CFSE-stained melanoma cells for indicated duration.

2.2.16 Cytospins and HE stainings

Non-adherent 24 hours and 48 hours co-cultures of melanoma cells and eosinophils were carried out as described. After incubation, a maximum of 75,000 cells in 500 µL CM per condition was transferred into the cytospin adapter (Cytospin 2, Shandon) equipped with a glass slide (SuperFrost® Plus miscroscope slides, R. Langenbrinck GmbH) onto which the cells were applied during centrifugation at 19 g for six minutes with low acceleration. Cells were dried for 15 minutes at room temperature and slides were stored at 4 °C (short-term storage) or -20 °C (long-term storage) until further use. For visualization, the cells were stained with hematoxylin and eosin (HE) according to the Autostainer XL protocol provided by the dermatohistopathology department of the University Hospital Würzburg shown in Table 13. Imaging was carried out using the Nikon TI-E microscope or the DM 750 microscope from Leica.

Table 13. HE staining procedure using the Autostainer XL provided by the dermatohistopathology department.

Solution	Duration
warming	10 minutes
100% alcohol	2 minutes
100% alcohol	2 minutes
70% alcohol	1 minutes
washing solution	2 minutes
hematoxylin	5 minutes
HCl-alcohol	2 seconds
washing solution	3 minutes
Scott´s medium	3 minutes
washing solution	3 minutes
eosin	2 minutes
95% alcohol	30 seconds
100% alcohol	2 minutes
100% alcohol	2 minutes
100% alcohol	2 minutes

2.2.17 PMA stimulation experiments

In order to activate eosinophils in co-cultures with melanoma cells, eosinophils or CFSE-stained melanoma cells were pre-incubated with phorbol-12-myristat-13-acetate (PMA). PMA specifically activates protein kinase C (PKC) and correspondingly activates nuclear factor-kappa B (NF-κB) in a dose-dependent manner [333]. Cells were incubated with 50 µL CM containing 50 nM PMA for 2.5 hours at 37 °C. Cells were washed once with CM and co-cultured in 1 mL for non-adherent cultures. For adherent cultures, cells were seeded on a 24-well plate. Once they adhered, cells were treated with 50 nM PMA for 2.5 hours. Subsequently, cells were washed with CM and (co-)cultured for indicated time. Images from adherent cultures were

taken after indicated time points using the Nikon TI-E microscope or the DM 750 microscope from Leica.

Brefeldin A incubation on the other hand, while not disturbing protein synthesis, supposed to disrupt protein secretion by interfering with the Golgi apparatus [334]. Eosinophils or CFSE-stained melanoma cell cultures and co-cultures were treated with 5 mg/mL Brefeldin A in CM for 24 hours. Viability was measured as described above.

2.2.18 Statistical analysis

Analysis of *in vitro* experiments, including assessment of melanoma cell and eosinophil viability in co-cultures, phenotypic characterization of eosinophils, proliferation and cell cycle analysis of melanoma cells, was performed using ANOVA with Bonferroni correction for three or more unmatched groups. Unpaired t-tests were applied for two group comparisons. Statistical analysis of soluble factors and experimental data from melanoma patients prior and during drug administration were analyzed using paired and unpaired t-tests or Mann-Whitney U test was used when normal distribution did not apply. Relative eosinophil counts (REC) of responders and non-responders were compared applying the Mann-Whitney U test. P values < 0.05 were considered significant. Prism (Graph-Pad, version 7) and/or SPSS (IBM, version 28.0) were used for visualizing data.

3   Results

3.1   Correlation of peripheral eosinophil counts, eosinophil-associated soluble mediators and eosinophil functionality with patient response to treatment

3.1.1   Patient cohort and healthy donors

In total, data of 243 melanoma patients were used for correlative studies of peripheral eosinophil counts and eosinophil-secreted markers in response to treatment with targeted therapy or immunotherapy. The median age was 70 years, 115 patients were male (47.3%). Seventeen patients had unresectable stage III disease. The remaining 226 patients were assigned to the categories M1a (9.9%), M1b (24.3%), M1c (35%) and M1d (23.9%) according to the AJCC classification 2017 [335]. Time from pre-treatment blood collection to therapy commencement was 0-63 days. Sixty-seven percent of the patients included in the ECP analysis experienced an objective response (CR and PR). A BRAF-mutation was detected in all patients receiving dual MAPKi. A detailed list of patient's characteristics is presented in Table 1.

3.1.2   High relative eosinophil counts but not pre-treatment ECP concentration is associated with response to targeted therapy

The applicability of peripheral eosinophil counts and serum eosinophilic cationic protein (ECP) as prognostic markers in patients with advanced melanoma was evaluated in a cohort of 52 melanoma patients treated with first-line targeted therapy. Established biomarkers and peripheral blood counts were used for comparative analysis between responders and non-responders (Figure 7, 8). Pre-treatment and on-treatment LDH serum values were significantly higher in non-responders than in responders ($p = 0.005$, $p = 0.010$) (Figure 7A, B). As for granulocyte counts, responders were characterized by significant higher pre-treatment absolute and relative eosinophil counts (AEC $p = 0.0008$, REC $p = 0.05$) as well as higher on-treatment counts (AEC $p = 0.01$, REC $p = 0.008$) compared to non-responders (Figure 7A, B). In contrast, no significant difference in pre-treatment absolute and relative neutrophil counts was observed comparing responders and non-responders (ANC $p = 0.47$, RNC $p = 0.51$). However, on-treatment absolute neutrophil counts tended to be lower in responders compared to the reciprocal group ($p = 0.19$) (Figure 7B). Comparing pre- and on-treatment samples, responders showed a trend towards a decrease of absolute leukocyte counts (ALC $p = 0.05$) and a significant decrease of absolute and relative neutrophil counts (Figure 7C, D). No

significant change in absolute or relative eosinophil counts in responders and non-responders could be observed comparing pre- and on-treatment counts.
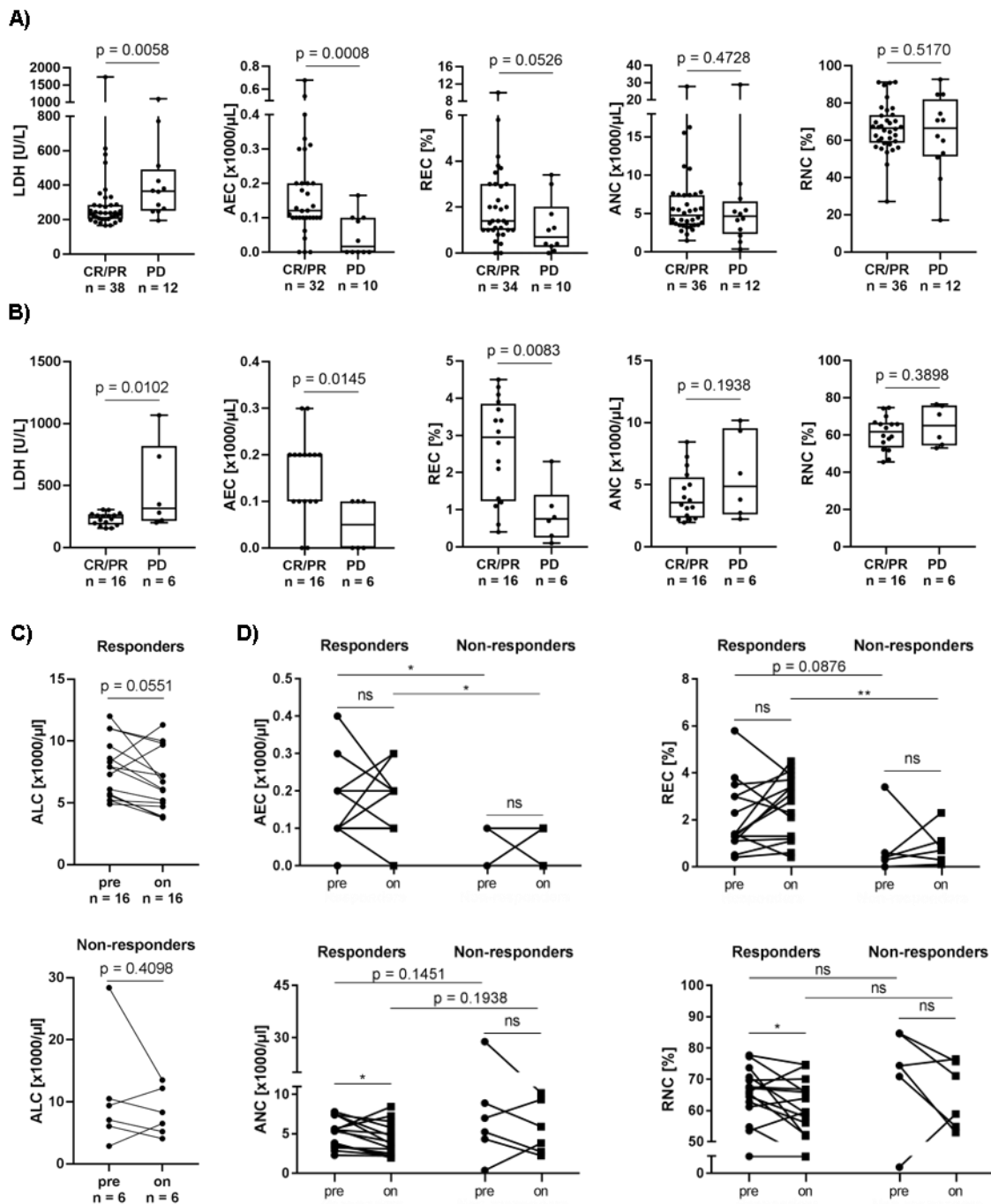

Figure 7. Low LDH and high blood eosinophil counts correlate with therapy response. (A+B) Association of established prognostic markers such as LDH, AEC, REC, ANC and RNC with response to targeted therapy. Comparing depicted clinical blood parameters of responders to non-responders **(A)** prior treatment and **(B)** during drug administration. Responders show significant higher AEC values prior administration compared to non-responders. Box plots show levels of clinical markers (median and the 25th and 75th percentiles; whiskers represent minimal and maximal outliers), as well as individual data points. **(C, D)** Analysis of leukocyte counts in melanoma patients upon targeted therapy. Samples from 16 responders and six non-responders prior (pre) and during (on) treatment. Results are shown as **(C)** absolute leukocyte count (ALC) and **(D)** absolute and relative eosinophil (AEC, REC) and neutrophil count (ANC, RNC). Responders are characterized as CR and PR, non-responders as PD. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$.

Interestingly, in a second independent patient cohort, a high pre-treatment relative eosinophil count disclosed a significant association with response to targeted therapy (p = 0.013) (Figure 8A). As a potential mediator of cytotoxicity by eosinophils, ECP, RANTES, sRAGE, Eotaxin, GM-CSF and APRIL concentration were measured in sera of patients before and during treatment with dual targeted therapy (MAPKi). High pre-treatment ECP levels correlated by trend with non-response to dual targeted therapy (p = 0.12) (Figure 8B, left). On-treatment serum ECP showed no such association (p = 0.59) (Figure 8B, right). Absolute and relative eosinophil counts did not correlate with serum ECP concentration as shown by linear regression (Figure 8C, D).
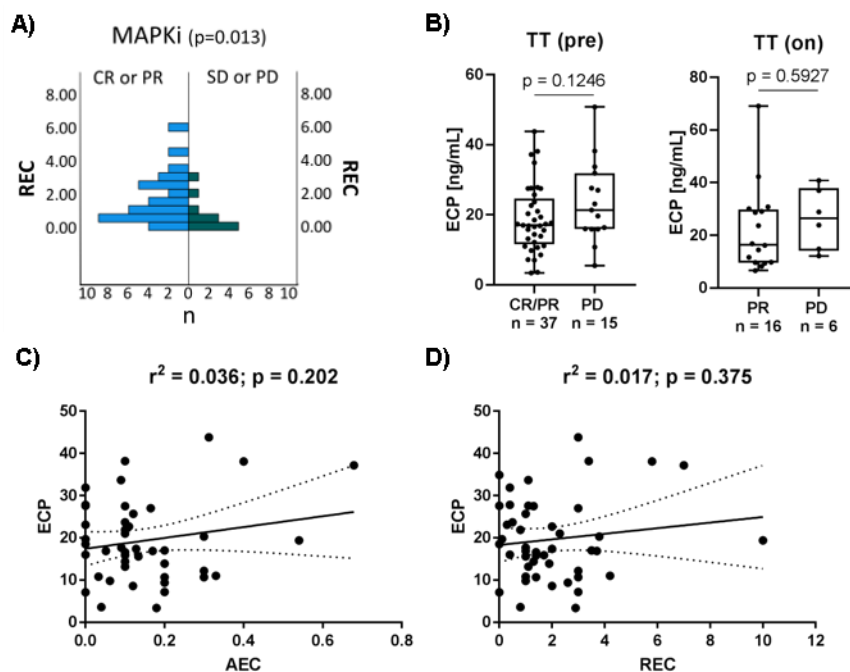
Figure 8. Relative eosinophil counts but not serum ECP concentration is associated with response to targeted therapy. (A) Association of pre-treatment REC with response to dual targeted therapy treatment in an independent patient cohort. In total 50 patients (CR or PR n = 39; SD or PD n = 11) with metastatic melanoma were included in the MAPKi cohort. High REC significantly correlated with better response to MAPKi. Mann-Whitney-U test was used to compare REC in responders and non-responders. **(B)** Comparison of serum ECP concentration (ng/mL) of responders and non-responders (left) prior (TT pre) and (right) during (TT on) targeted therapy. There is a trend towards higher pre-therapeutic values of ECP in melanoma patients with disease progression compared to responders. **(C)+(D)** Correlation of serum ECP concentration with **(C)** absolute eosinophils counts (AEC) and **(D)** relative eosinophil counts (REC) of responders and non-responders to targeted therapy. R square and p-values are displayed from the linear regression analysis.

Pre-treatment proliferation-inducing ligand (APRIL) and on-treatment eotaxin-1 concentrations tended to be higher in non-responders (p = 0.09 and 0.19, respectively) (Figure 9C, E). Unfortunately, no such trend was observed for any of the other markers (Rantes, sRage and GM-CSF) in sera of patients receiving targeted therapy (Figure 9A, B and D).
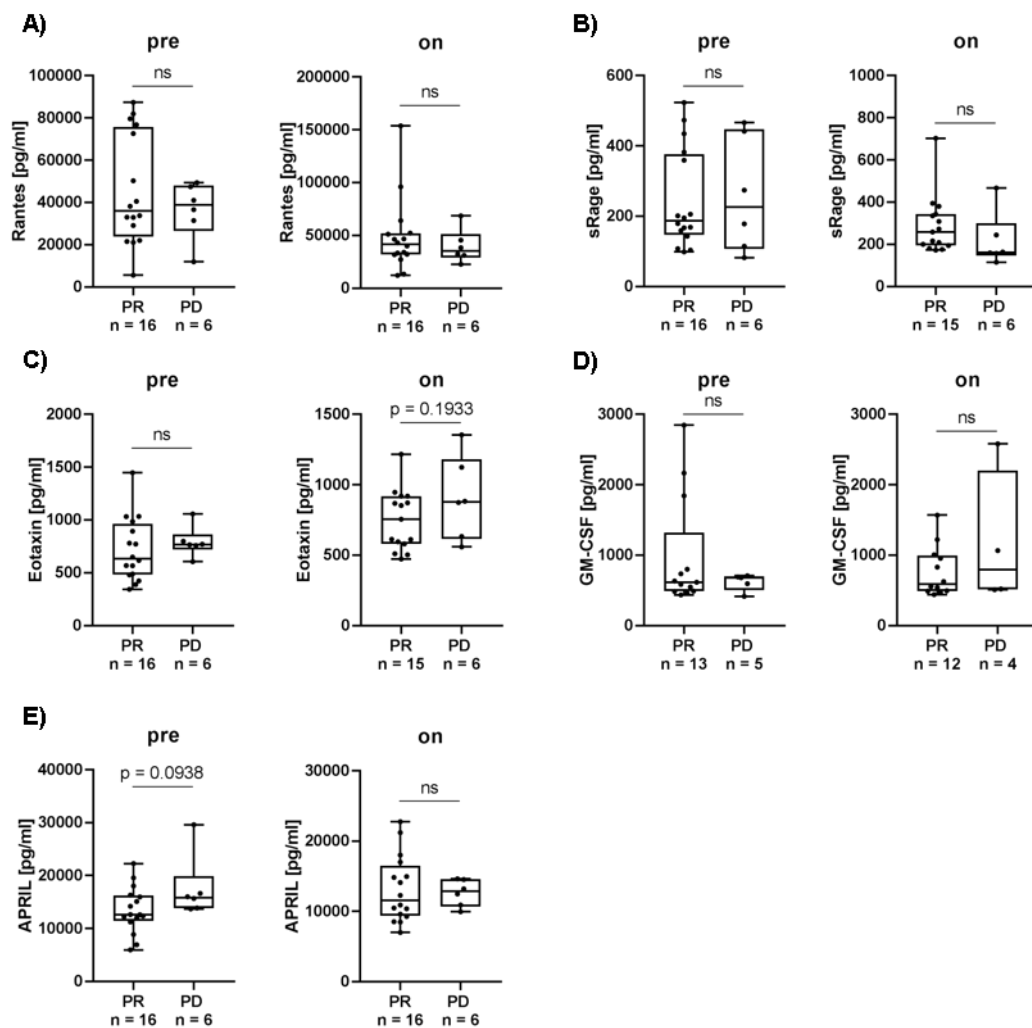
Figure 9. Assessment of eosinophil-related soluble factors in melanoma patient serum prior (pre) and during (on) targeted therapy. Responders are characterized as PR, non-responders as PD. Analysis of **(A)** Rantes (=CCL5), **(B)** sRAGE, **(C)** Eotaxin, **(D)** GM-CSF and **(E)** APRIL before and during drug administration.

To validate the data collected from samples obtained from patients with advanced melanoma treated with targeted therapy, we evaluated peripheral blood counts and serum ECP concentrations in samples from patients receiving immunotherapy. In a cohort of 123 patients, relative eosinophil counts were by trend correlated with response to combination treatment with ipilimumab and nivolumab (p = 0.16) but not with response to PD-1 inhibitor nivolumab alone (p = 0.92) (Figure 10A). Unlike for targeted therapy, we observed a strong correlation between high pre-treatment serum ECP concentration and response to immunotherapy (p = 0.016) (Figure 10B). On-treatment serum ECP concentration did show only a trend towards a correlation with response in this cohort (p = 0.077) (Figure 10B). Pre-treatment comparison of absolute and relative eosinophil counts and ECP concentration revealed a strong correlation (p = 0.005 and p = 0.009, respectively) (Figure 10C). Such correlation could not be reproduced when comparing on-treatment values (Figure 10D). Thus, high pre-treatment ECP

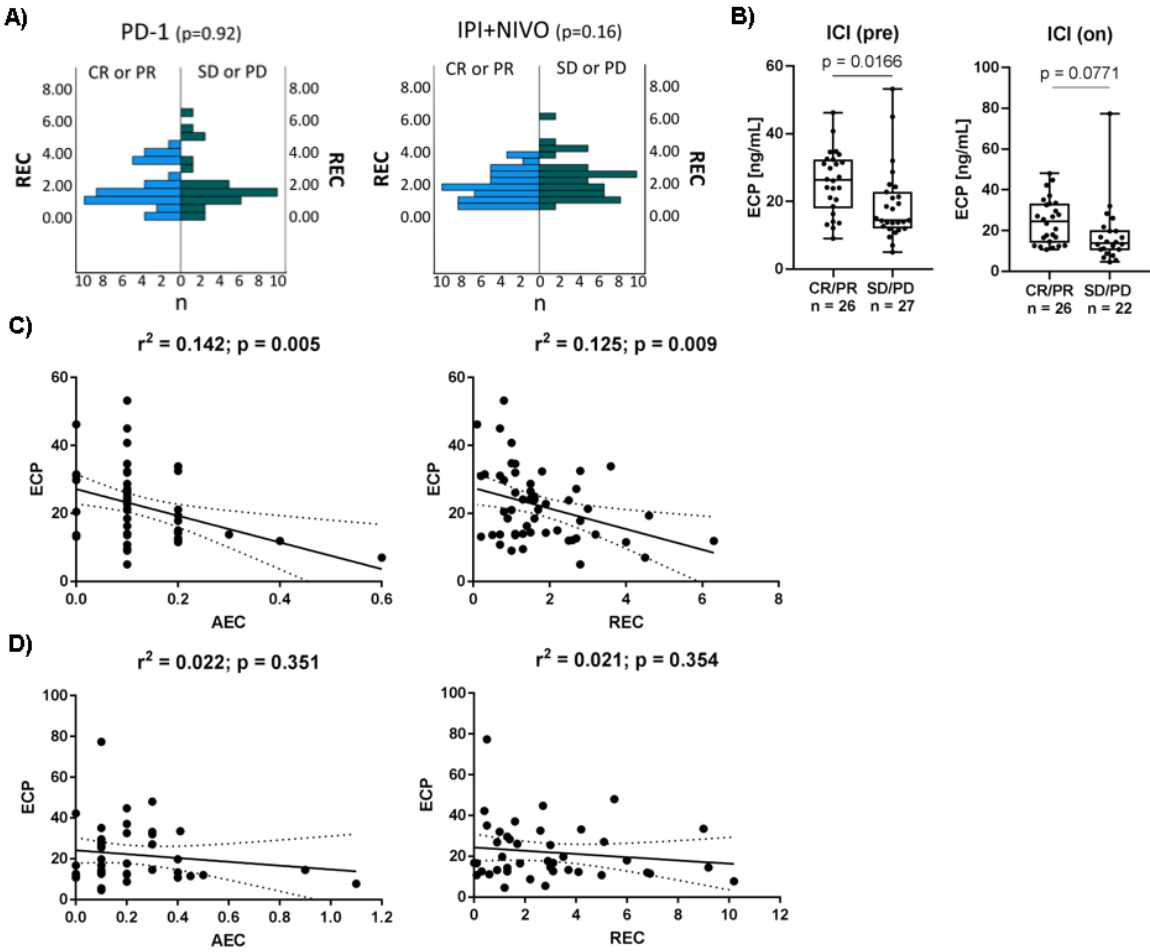concentrations and by trend high relative eosinophil counts are associated with response to immunotherapy.

Figure 10. Association of relative eosinophil counts (REC) and serum ECP with response to immunotherapy. (A) Association of pre-treatment REC with response to immunotherapy (ICI) in an independent patient cohort. In total 59 patients (CR or PR n = 32; SD or PD n = 27) with metastatic melanoma were included in the PD-1 cohort and 64 patients (CR or PR n = 32; SD or PD n = 32) in the combinatorial immunotherapy cohort. REC does not correlate with response to PD-1 treatment and only by trend with ipilimumab and nivolumab as dual therapy. Mann-Whitney-U test was performed to compare REC in responders and non-responders. **(B)** Comparison of serum ECP concentration (ng/mL) of responders and non-responders (left) prior (ICI pre) and (right) during (ICI on) immunotherapy. Responders to ICI show significant higher pre-treatment serum ECP concentration (p = 0.01) compared to non-responders. This is also numerically (p = 0.07) seen during drug administration. **(C)+(D)** Correlation of serum ECP concentration with absolute eosinophils counts (AEC) and relative eosinophil counts (REC) of responders and non-responders **(C)** prior and **(D)** during immunotherapy. R square and p-values are displayed from the linear regression analysis.

3.1.3 Isolation and purification of eosinophils and neutrophils from whole blood

For phenotypical characterization of eosinophils and to investigate the influence of eosinophils on melanoma cell viability, polymorphonuclear cells (PMNs) were harvested from blood received from healthy volunteers, melanoma patients with no evidence of disease or late-stage melanoma patients using gradient centrifugation with subsequent MACS-separation to purify eosinophils and neutrophils. Purity was controlled after isolation and measured by flow cytometry.
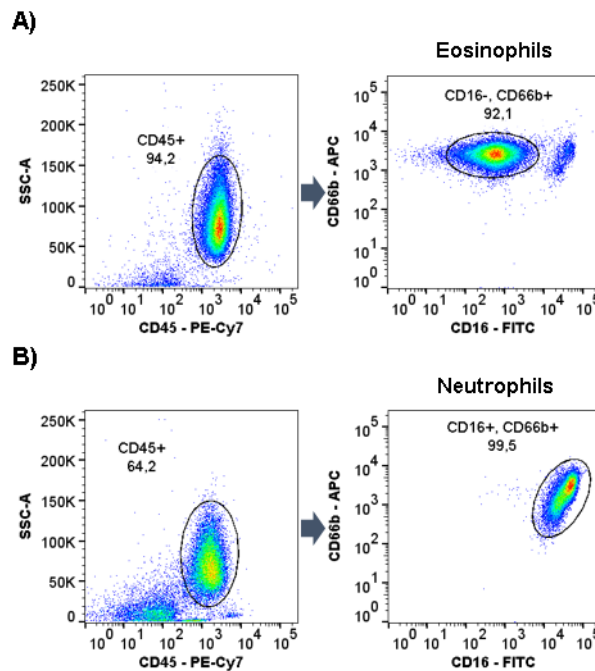
Figure 11. Purity assessment of granulocytes by flow cytometry. Representative gating strategy for **(A)** eosinophils defined as CD45$^+$CD16$^-$CD66b$^+$ and **(B)** neutrophils defined as CD45$^+$CD16$^+$CD66b$^+$. Granulocytes were harvested from whole blood by density centrifugation, erythrocyte lysis and isolation via MACS-separation. Dry pellets of eosinophils and neutrophils were stained with CD16-FITC, CD66b-APC, CD45-PE-Cy7, CD14-PerCP-Cy5.5, CD56-PE, CD3-PE, CD19-PE, CD193-APC-Cy7 anti-human antibodies and purity was determined flow cytometrically. First singlets were gated (not shown), CD45$^+$ cells selected and CD16$^{+/-}$ and CD66b$^+$ cells defined to display purity. Purity control was conducted for every single experiment.

To verify purity of isolated granulocytes, singlets and CD45$^+$ cell were gated and granulocytes determined when gating for the granulocytic marker CD66b. CD16 was used to distinguish neutrophils from eosinophils. Representative gating strategy and purity control of freshly isolated eosinophils and neutrophils are shown in Figure 11. Eosinophils were defined as CD45$^+$/CD16$^-$/CD66b$^+$ and neutrophils as CD45$^+$/CD16$^+$/CD66b$^+$. A high purity of >90% was routinely obtained in most samples.

3.1.4 Eosinophil-mediated cytotoxicity is donor-dependent while healthy donor- and patient-derived eosinophils show comparable phenotype

To mechanistically link high eosinophil counts with prolonged survival of patients, we examined cytotoxicity of eosinophils derived from stage IV melanoma patients against a melanoma cell line model, MaMel63a. Cytotoxicity was measured ex vivo before and during targeted therapy (TT) at week 6, 12, 24 and 48 (Figure 12A). Eosinophils derived from healthy donors and patients receiving immunotherapy (ICI) served as control. To display eosinophil-mediated cytotoxicity, MaMel63a cell viability after 24 hours of culture with and without eosinophils was inversely plotted. A cytotoxicity of one means melanoma cell viability was not affected by eosinophils, while a cytotoxicity of two describes a reduction in melanoma cell viability by half compared to melanoma cells not exposed to eosinophils. Healthy donor- and patient-derived eosinophils exert a wide range of donor-dependent cytotoxicity (range 0.96 – 2.35). For healthy donors, cytotoxic values between 1.04 and 2.2 were detected, in patients pre- and on-treatment with targeted therapy the values laid between 1.02 and 2.32 and for eosinophils derived from patients pre- and on-treatment with immunotherapy between 0.96 and 2.35. Similar ranges were observed when addressing healthy donor-cytotoxicity in MaMel51, MaMel80a and MaMel114 (data not shown).

To identify potential similarities or differences in eosinophil phenotypes comparing patient-derived eosinophils with those from healthy donors, we analyzed twelve previously described surface markers on eosinophils from pre-treatment stage IV melanoma patients, healthy donors and stage I and II melanoma patients with no current evidence of disease (Table 12B, C). The investigated molecules included activation (CD69, CD66b) and differentiation markers (Siglec-8), class I and II MHC proteins (HLA-A,-B,-C, HLA-DR), adhesion molecules (CD49d, CD29, CD31) and immunoregulatory receptors (TNFR2, CCR3, PD-L1) [247, 336-337]. A representative gating strategy for eosinophils and their target molecule is shown in Figure 12B. Phenotypically, eosinophils from melanoma patients were comparable to those obtained from healthy donors and stage I/II melanoma patients. No significant difference in the median fluorescence intensity (MFI) for the targeted epitopes on eosinophils (defined as CD16-/CD66b+/CCR3+ cells) was detected (Figure 12C, D).
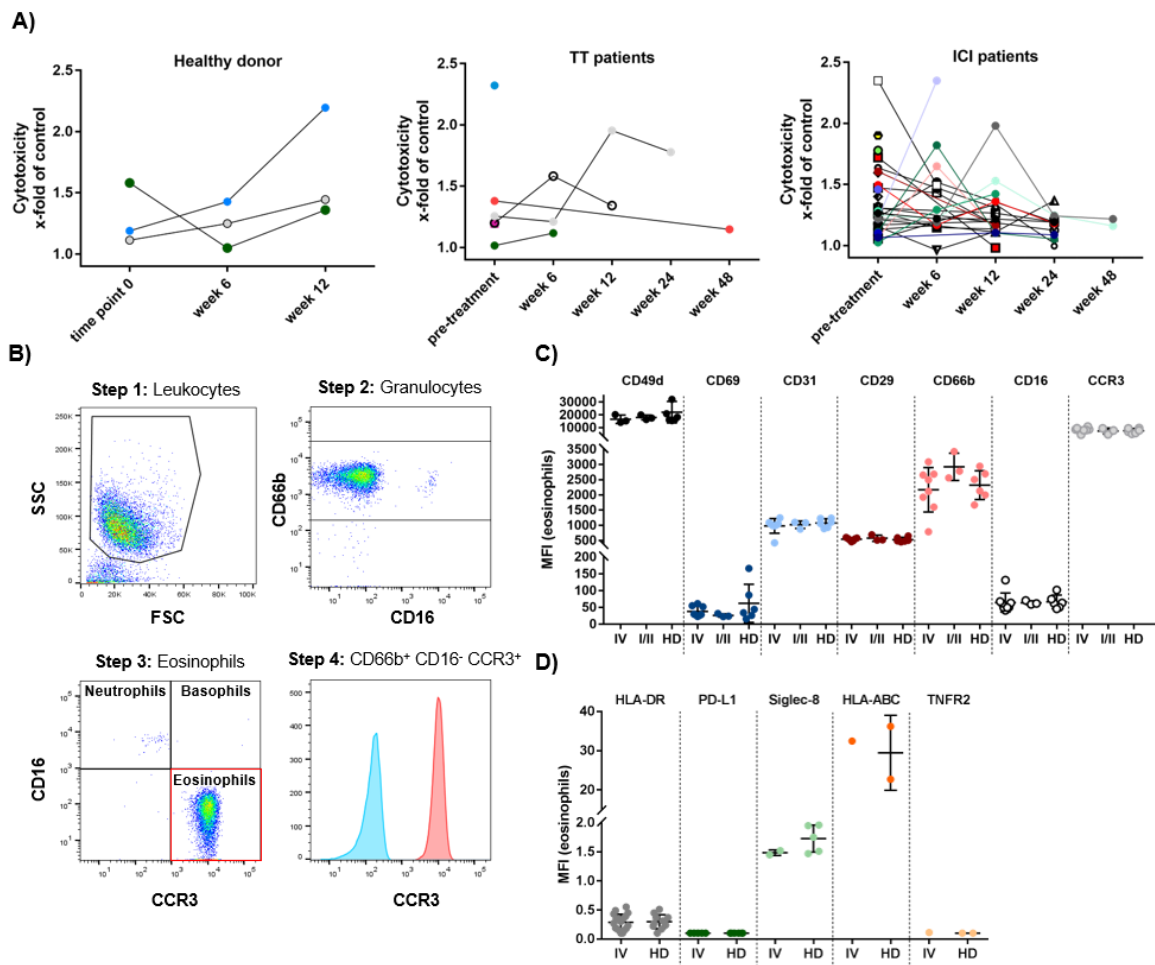
Figure 12. Function and phenotype of peripheral blood eosinophils of melanoma patients. (A) Sequential assessment of cytotoxicity by eosinophils towards melanoma cells. Eosinophils from melanoma patients were isolated prior treatment and during therapy with targeted therapy (TT) or immune checkpoint inhibitors (ICI) at weeks 6, 12, 24 and 48. Non-adherent co-culture of MaMel63a cells and eosinophils serially obtained from healthy donors (HD) or stage IV melanoma patients subsequently receiving therapy in a 1:7.5 ratio for 24 hours. In order to display eosinophil-induced MaMel63a cell death, viability of MaMel63a cells relative to controls (MaMel63a cells cultured alone) was plotted inversely. A high number refers to a high cytotoxic eosinophilic activity. Cytotoxicity x-fold of control is shown. Each symbol represents a healthy donor or patient, respectively. N = 3 healthy donor, n = 6 TT patient and n = 39 ICI patient samples obtained prior to melanoma treatment. **(B-D)** Phenotypic characterization of peripheral blood eosinophils of stage IV and stage I/II melanoma patients compared to healthy donors (HD). **(B)** Dot plots and histogram identifying freshly purified CD66b+ CD16- CCR3+ eosinophils. MFI determination for the expression of **(C)** CD49d, CD69, CD31, CD29, CD66b, CD16 and CCR3 (CD193) and **(D)** HLA-DR, PD-L1, Siglec-8, HLA-A, -B, -C and TNFR2 within freshly purified eosinophils. Each dot represents an individual donor. Mean MFI ± standard deviation (SD) is shown. Analysis included a total of 13 patients with advanced melanoma, six patients with early-stage melanoma and 12 healthy donors. Analysis included a total of 18 patients with advanced melanoma and 11 healthy donors.

3.2 Interaction of eosinophils and melanoma cells

Our data show a strong correlation between blood eosinophil counts with response to melanoma treatment. In the next section, we aimed to explain the relationship of eosinophils with melanoma cells in the context of MAP-kinase inhibition using in vitro tools.

3.2.1 MAP-kinase inhibition in melanoma cell lines

To determine the optimal concentration by which BRAF-inhibitor (BRAFi) or MEK-inhibitor (MEKi) affect cell viability of the BRAFV600E-mutated melanoma cell line MaMel63a, cells were treated with different concentrations of vemurafenib or cobimetinib cultured non-adherently in polypropylene tubes for 24 hours and 48 hours. The effect of the respective treatment on melanoma cell viability was determined using flow cytometry.
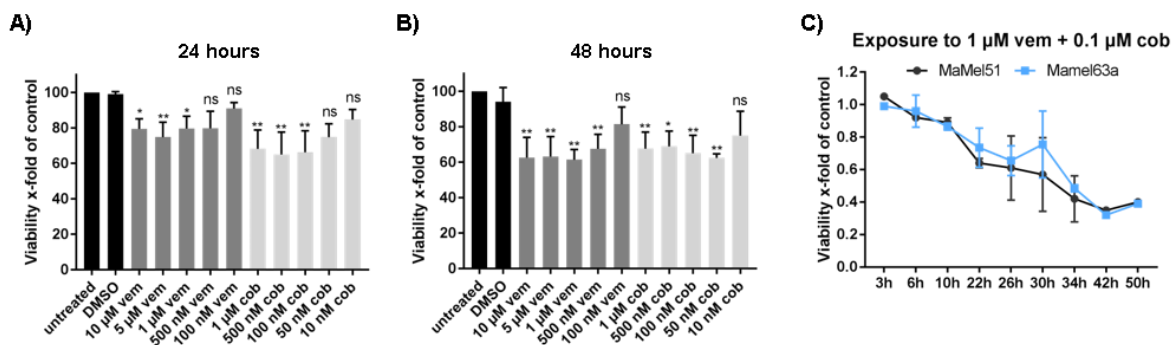
Figure 13. Titration of vemurafenib and cobimetinib for 24 hours and 48 hours. Viability of MaMel63a cells after **(A)** 24 or **(B)** 48 hours treatment with DMSO, vemurafenib or cobimetinib with indicated concentrations. Untreated melanoma cells served as control. Viable cells were defined as Annexin-V$^-$/7-AAD$^-$. Mean percentage of MaMel63a cell viability x-fold changes of control ± standard deviation (SD) is shown for three independent experiments. **(C)** Exposing MaMel51 and MaMel63a cells to a combination of 1 µM vemurafenib and 100 nM cobimetinib for depicted time. Cultures were carried out under non-adherent culture conditions. Viability x-fold of control is shown for one to two independent experiments. ns p > 0.05, * p ≤ 0.05, ** p ≤ 0.01.

Vemurafenib and cobimetinib single treatment significantly reduced MaMel63a cell viability after 24 hours. The strongest effects on cell viability relative to the control were seen when treating with 10 µM (20.48% reduction), 5 µM (25.14% reduction) or 1 µM (20.34% reduction) vemurafenib and 1 µM (31.75% reduction), 500 nM (34.92% reduction) or 100 nM (33.68% reduction) cobimetinib for 24 hours (Figure 13A). Increasing the exposure time to BRAFi and MEKi to 48 hours led to sufficient decrease of MaMel63a viability even when applying 500 nM vemurafenib (32.38% reduction) and 50 nM cobimetinib (37.55% reduction) (Figure 13B). Exposure to BRAFi or MEKi for 48 hours resulted in stronger decrease in MaMel63a viability compared to 24 hours. To avoid drug over-exposure a concentration of 1 µM vemurafenib and 0.1 µM cobimetinib was defined as sufficient and was used for further experiments as monotherapy and/or in combination. Applying 1 µM vemurafenib and 0.1 µM cobimetinib as

combination treatment for non-adherent cultures of MaMel51 and MaMel63a cells, we observed a continuous decrease of cell viability during time for both cell lines (Figure 13C).

3.2.2 The extent of eosinophil-mediated cytotoxicity varies among melanoma and non-melanoma cell lines

Studies report anti-tumoral activity of eosinophils against various cancers [249, 273]. To evaluate whether melanoma cells are susceptible to the tumoricidal function of eosinophils and to examine optimal tumor cell killing by eosinophils, different tumor to effector cell (T:E) ratios were used in co-cultures with MaMel63a cells. Eosinophils were able to decrease the viability of MaMel63a cells in a ratio dependent manner (Figure 14A). Although a ratio of 1:5 (41.16% reduction of viability) was sufficient to significantly reduce MaMel63a cell viability, a ratio of 1:7.5 (49.72% reduction of viability) was declared optimal for functional tests and was used in further analyses. Using a 1:10 ratio did not significantly enhance cytotoxicity. A representative gating for cell viability staining with 7-AAD and Annexin-V-APC is shown in Figure 14B.
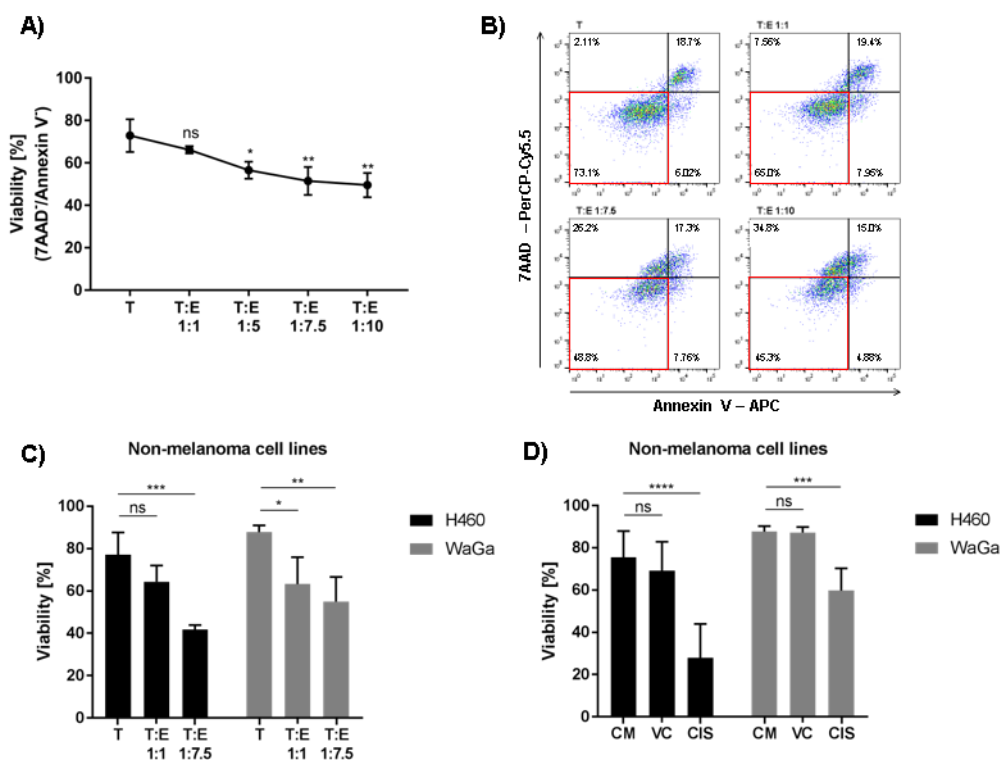

Figure 14. Eosinophil-mediated cytotoxicity is ratio dependent and not restricted to certain cancer types. Non-adherent (co-)cultures of melanoma cell lines (T) with or without eosinophils (E) for 24 hours. **(A)** Eosinophils show a significant target to eosinophil ratio-dependent cytotoxic effect towards MaMel63a cells. A ratio of 1:5 melanoma cells to eosinophils is sufficient to significantly decrease MaMel63a cell viability. Mean percentage of MaMel63a cell viability is shown for three independent experiments. **(B)** Representative viability gating strategy for 7-AAD / Annexin-V staining of 24 hours co-cultures of MaMel63a cells with eosinophils. Viable cells are displayed in red boxes. **(C)** Cytotoxicity assessed in the lung carcinoma cell line, H460 and in the merkel-cell carcinoma cell line, WaGa. Non-adherent (co-)cultures of H460 or WaGa cells with or without eosinophils in a ratio of 1:1 and 1:7.5 cancer cell to effector cell ratio for 24 hours. Eosinophils significantly decrease H460 and WaGa cell viability. A 1:1 ratio is sufficient to significantly impair WaGa cell viability. **(D)** Non-adherent cultures of H460 or WaGa cells with or without combinatory treatment with 1 μM vemurafenib and 100 nM cobimetinib or with 20 μM cisplatin for 24

hours. Both cell lines are unaffected by targeted therapy but cisplatin notably reduces cell viability. Mean percentage of the tumor viability ± standard deviation (SD) is shown for four to eight independent experiments. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

As for non-melanoma cell lines, H460 cells underwent apoptosis upon exposure to eosinophils at a ratio of 1:7.5 (Figure 14C). Interestingly, already a 1:1 target to eosinophil ratio was sufficient to significantly reduce the viability of the Merkel cell carcinoma cell line WaGa (Figure 14C). To analyze the sensitivity of non-melanoma derived cell lines to different drugs, H460 cells and WaGas were exposed to combinatory vemurafenib and cobimetinib treatment or to cisplatin for 24 hours. Both H460 and WaGa are susceptible to cisplatin treatment but not to vemurafenib and cobimetinib after 24 hours (Figure 14D).

We assumed that depending on the eosinophil donor and the used melanoma cell line, eosinophil-mediate cytotoxicity might vary. We therefore assessed the variation of cytotoxicity of the same eosinophils' donor to four additional BRAF-mutated melanoma cell lines (MaMel80a, MaMel51, MaMel06 and MaMel114) (Figure 15A-D). MaMel06 showed the strongest susceptibility to eosinophil exposure, shown by a significant decrease in viability (mean drop of viability: 42.4%) after 24 hours. MaMel63a (mean drop of viability: 23.7%) and MaMel80a cells (mean drop of viability: 21.0%) showed similar reduction in viability when compared to viability of controls (melanoma cells alone). The MaMel51 and MaMel114 cell lines seemed to be least affected by eosinophils (mean drop of viability: 12.9%, and mean drop of viability: 17.1%, respectively) (Figure 15A). Upon eosinophil exposure at a 1:7.5 ratio, MaMel63a and MaMel06 cells showed a significant increase in cells entering early apoptosis and necrosis (Figure 15B, D). Necrosis was also induced in MaMel51 cells and by trend in MaMel80a cells (Figure 15D). None of the examined cell lines showed significant changes in the percentage of cells in late apoptosis (Figure 15C). Examining the viability of melanoma cell lines exposed to eosinophils, we observed a considerable variation in the extent of tumor cell apoptosis and necrosis.
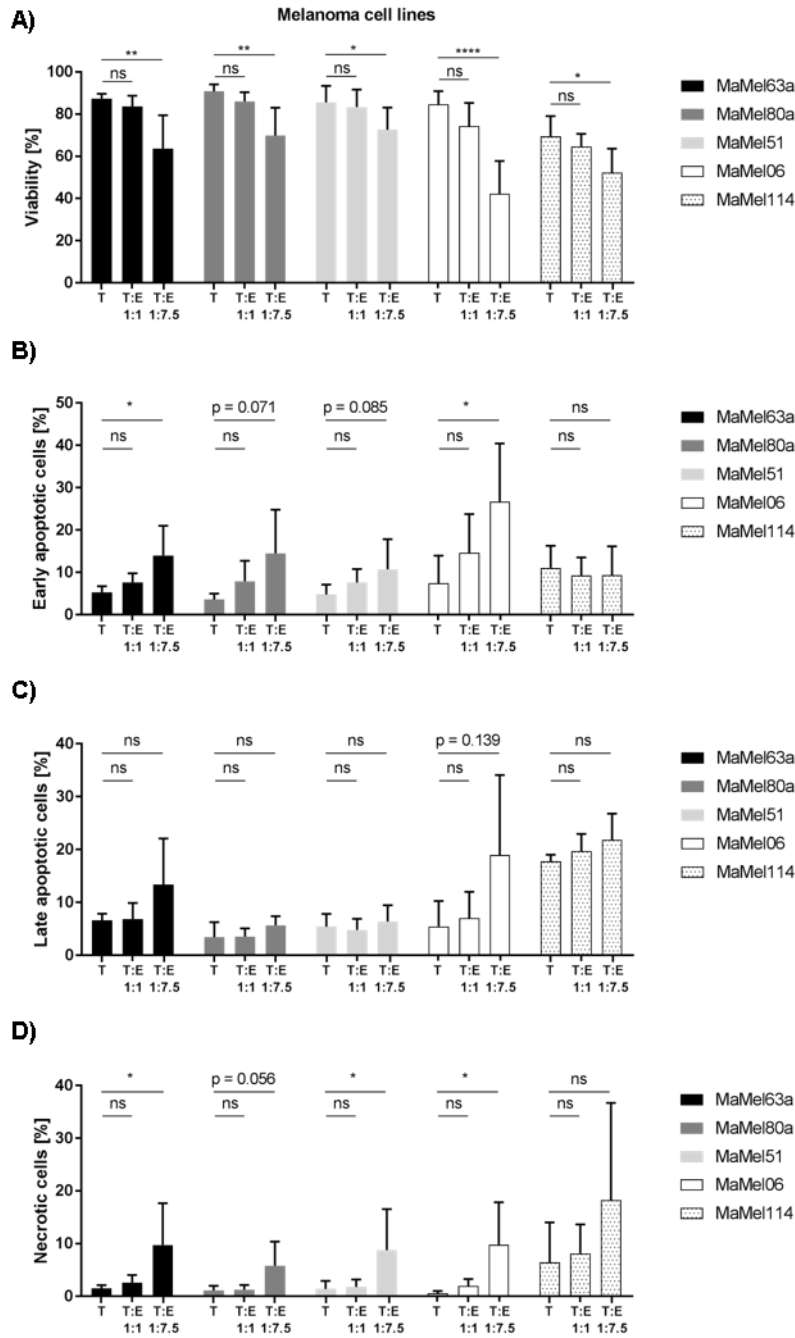
Figure 15. Eosinophil-mediated cytotoxicity induces apoptosis and varies in different melanoma cell lines. Non-adherent (co-)cultures of melanoma cell lines (T) with or without eosinophils (E) for 24 hours. Cytotoxicity assessed in different melanoma cell lines, MaMel63a, MaMel80a, MaMel51, MaMel06 and MaMel114. Mean percentage of **(A)** melanoma cell viability, **(B)** of cells in early apoptosis, **(C)** of cells in late apoptosis, and **(D)** necrotic cells ± standard deviation (SD) is shown for six to eight independent experiments. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

3.2.3 The additive role of therapeutics in eosinophil-melanoma interaction

Combinatory-targeted therapy has been proven superior to monotherapy in clinics regarding tumor growth suppression [148, 159-160]. Additionally, granulocyte subpopulations have been shown to exert different effects on various types of cancers accompanied with distinct clinical outcomes for patients. Tumoricidal properties are attributed to eosinophils, while neutrophils show both anti-tumoral and pro-tumoral effects [249, 258, 272-274]. We evaluated the effects of eosinophils on melanoma cell viability when additionally exposing non-adherent co-cultures to vemurafenib and cobimetinib treatment for 24 hours and 48 hours. Interestingly, the presence of vemurafenib and cobimetinib significantly enhanced the eosinophil-mediated cytotoxicity towards MaMel63a cells when co-cultured in a 1:7.5 ratio for 24 hours (Figure 16A). Cytotoxicity and additional killing effect by BRAF/MEK inhibitor exposure was enhanced when co-culturing for 48 hours (Figure 16B). The strongest additive tumor suppression was observed in vemurafenib-supplemented medium for both 24 hours and 48 hours co-cultures with a mean additive decrease of viability to control of 19.8% and 25.9% respectively. In contrast, cobimetinib and the combination of vemurafenib and cobimetinib after 24 hours significantly induced a mean additive decrease of MaMel63a cell viability of 16.1% and 15.8% respectively (Figure 16A). As for 48 hours culture, only a trend towards additional decrease of melanoma cell viability could be observed when exposing to eosinophils and cobimetinib or a combination of both vemurafenib and cobimetinib.
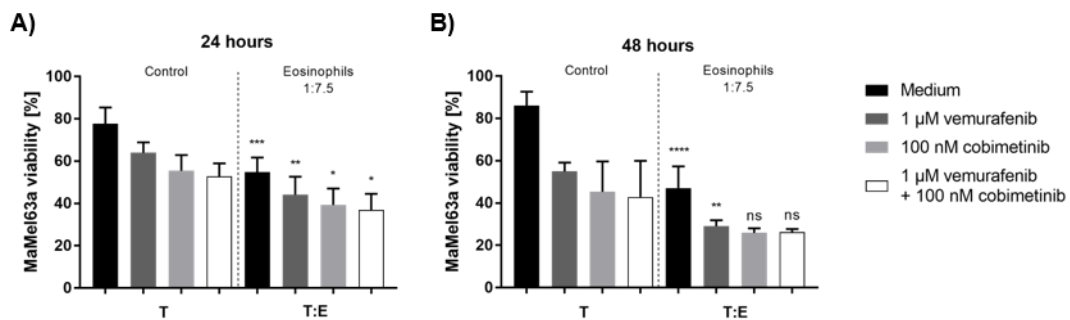

Figure 16. Additive suppressing effect of eosinophils and targeted therapy on MaMel63a cell viability. (Co-)culture of MaMel63a cells with or without freshly purified eosinophils at 1:7.5 ratio (T:E) for **(A)** 24 and **(B)** 48 hours. Cultures were kept in 1 mL CM with or without 1 µM vemurafenib and/or 100 nM cobimetinib. Mean percentage of melanoma cell viability ± SD are shown from three to six independent experiments. Significances compared to respective control without eosinophils are shown. ns p > 0.05, * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001.

3.2.4 Influence of melanoma cells on granulocyte viability

In the previous section, we describe the interference of eosinophils with melanoma cells. We further analyzed how melanoma cells affect granulocyte viability, staining for 7-AAD and Annexin-V after 24 hours or 48 hours non-adherent co-cultures.

Eosinophil viability immediately after autoMACS separation and prior (co-)culture was constantly high with a mean viability of 92.3% (Figure 17A, 0h). The viability significantly decreased to a mean viability of 57.8% after 24 hours and 21.2% after 48 hours cultures (Figure 17A). Co-cultures with melanoma cells resulted in a profound increase of viability up to a mean viability of 76.0% after 48 hours compared to eosinophils alone. The increase in eosinophil viability after 48 hours co-culture with MaMel63a cells could not be sustained when adding vemurafenib and cobimetinib to the culture medium. In comparison, neutrophil viability after 24 hours cultures only reached a mean viability of 22.6%. Melanoma cells could not prevent neutrophil apoptosis in culture (Figure 17B). Melanoma cells prolonged survival of eosinophils but not neutrophils in co-culture compared to granulocytes alone (Figure 17A, B). These results emphasize the bi-directional relationship between melanoma cells and granulocytes.
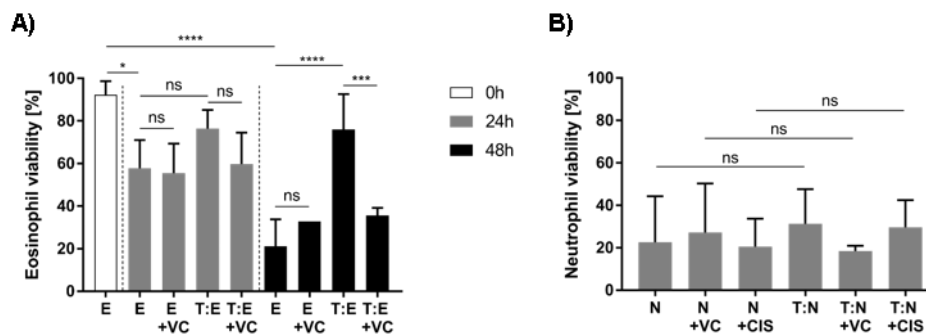

Figure 17. Eosinophil viability increases upon co-culture with melanoma cells, while neutrophils viability is independent of the presence of melanoma cells. (**A**) Non-adherent (co-)culture of eosinophils and MaMel63a cells in complete medium with or without addition of 1µm vemurafenib and 100 nM cobimetinib for 24 and 48 hours. Eosinophil viability was high prior culture and significantly decreased after 24 hours and 48 hours. Eosinophil viability after 48 hours can be rescued when co-culturing with MaMel63a cells. (**B**) Non-adherent (co-)culture of neutrophils and MaMel63a cells in complete medium with or without 1 µM vemurafenib and 100 nM cobimetinib or 20 µM cisplatin for 24 hours. Mean percentage of the granulocyte viability ± standard deviation (SD) is shown for one to nine independent experiments. ns p > 0.05, * p ≤ 0.05, *** p ≤ 0.001, **** p ≤ 0.0001.

3.3 Unraveling the bi-directional relationship between eosinophils and melanoma cells

3.3.1 Eosinophil-mediated cytotoxicity depends on culture condition and is an active process

In order to investigate the interaction of melanoma cells and eosinophils under different culture conditions, co-cultures were carried out under non-adherent culture conditions in polypropylene tubes and under adherent culture condition in 24-well plates allowing adherence to the bottom of the vessel. Adherent MaMel63a cells showed no impairment in viability when exposed to eosinophils (Figure 18A). Switching into non-adherent culture conditions overcomes this effect; eosinophils were able to execute their tumoricidal function towards MaMel63a cells (Figure 18A). Intriguingly, despite the lack of effectiveness of eosinophils towards MaMel63a cell viability under adherent conditions, recording eosinophil viability, MaMel63a cells were able to improve eosinophil survival independent of culture conditions (Figure 18B). In order to verify that the observed eosinophil-mediated cytotoxic effect is an active process, MaMel63a cells were co-cultured with fresh, lysed or heat-inactivated lysed eosinophils, preventing active interaction. Compared to viable eosinophils, lysed eosinophils showed enhanced cytotoxicity towards melanoma cells (Figure 18C). Neutralization of lysed eosinophils contents through heat-inactivation led to inhibition of cytotoxicity towards melanoma cells. Cell cycle and proliferation assays in melanoma cells were performed as an additional functional read-out of non-adherent co-cultures with eosinophils. PI intercalates into the DNA and allows the evaluation of DNA content in distinct cell cycles. PI stainings of 48 hours co-culture with eosinophils showed no change in cell cycle of MaMel63a cells (Figure 18D). Ki-67-specific staining of MaMel63a cell-eosinophil co-culture showed similar proliferation compared to MaMel63a cells cultured alone (Figure 18E). Thus, cytotoxicity is not inducing cell cycle arrest nor does it affect proliferation of MaMel63a cells in non-adherent cultures. Results obtained by cell cycle and proliferation assays could be confirmed by performing colony formation assays. Non-adherent co-cultures of eosinophils and MaMel63a cells for 48 hours and subsequent seeding of MaMel63a cells on a 24-well plate for additional 48 hours showed no impact in melanoma cell density (Figure 18F).
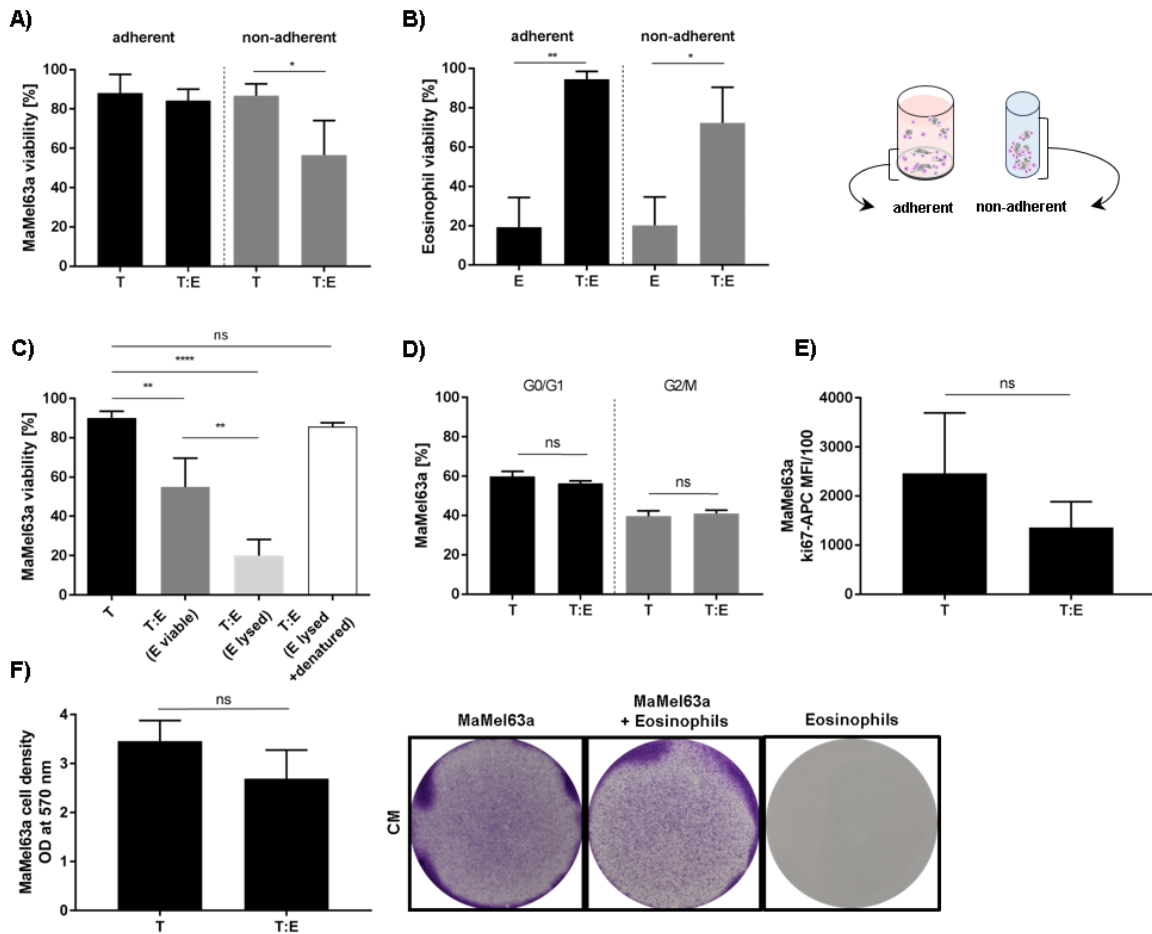
Figure 18. Unraveling the interaction of melanoma cells and blood-derived eosinophils. (A) MaMel63a cells were co-cultured with eosinophils for 48 hours. Separate measurement of viability of adherent (24-well plate) and non-adherent (polypropylene tube) melanoma cells. Cytotoxicity depends on culture condition. Melanoma cell killing by eosinophils is only observed under unattached conditions. **(B)** Displaying the viability of eosinophils for the experimental setup shown in **(A)**. Eosinophil survival in culture was prolonged when co-cultured with melanoma cells. **(C)** Tumoricidal function of eosinophils towards melanoma cells is an active process. Preventing signal transduction in co-cultures by lysis (1-minute liquid nitrogen treatment of eosinophils and subsequent freeze-thaw) and neutralization (heat-inactivation for 1 hour at 95 °C after lysis) of eosinophil content prior culture. Non-adherent co-cultures of MaMel63a cells with or without viable, lysed or neutralized eosinophils for 48 hours. Significant enhancement of cytotoxicity towards MaMel63a cells was observed when cultured with lysed eosinophils. Heat-inactivation of eosinophil content abrogates cytotoxicity towards MaMel63a cells. **(A-C)** Mean percentage of melanoma cell viability ± SD is displayed from two to four independent experiments. **(D+E)** Examining cell cycle and proliferation in MaMel63a cells exposed to eosinophils. PI staining and Ki-67-specific staining of MaMel63a cells in co-cultures with eosinophils after 48 hours. Eosinophils do not affect MaMel63a cell cycle nor proliferation. Mean percentage of MaMel63a cells **(D)** in G0/G1 and G2/M phase and **(E)** positive for Ki-67 is displayed from three independent experiments. **(F)** Assessment of melanoma cell survival using colony formation assay in co-cultures. MaMel63a cells were non-adherently co-cultured with eosinophils for 48 hours. MaMel63a cells were counted and seeded on 6-well plates for additional 48 hours. Co-culture was subsequently stained with crystal violet to visualize cell density (right). The cell density was quantified measuring the absorbance of the crystal violet dye (left). Eosinophils do not affect cell density of MaMel63a. Quantitative crystal violet stainings are shown for three independent experiments. All presented co-cultures were performed using a 1:7.5 melanoma cell (T) to eosinophils (E) ratio. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0005$, **** $p < 0.0001$.

3.3.2 The eosinophil-melanoma relationship relies on cell-cell contact

To unravel the role of physical proximity in the interaction of melanoma cells and eosinophils, co-cultures with conditioned medium and Transwell experiments were carried out. For conditioned medium experiments co-cultures of MaMel63a cells with or without eosinophils as described before were prepared and the supernatant, referred to as conditioned medium (here: CM), was collected in which freshly prepared melanoma cells were cultured for 48 hours. Experiments with conditioned medium containing potential soluble factors from previous co-cultures did not show any effect on MaMel63a cell viability (Figure 19A). However, the beneficial effect of melanoma cells on cultured eosinophils was retained using conditioned medium from previous melanoma cell cultures (Figure 19B). These observations were confirmed by Transwell experiments, separately culturing eosinophils and melanoma cells in Spin-X® tubes carrying a semipermeable membrane, which enables potential communication via soluble mediators like secreted cytokines, while direct cell-cell interaction was disabled. The viability of MaMel63a cells was not affected by separately cultured eosinophils in normal medium or medium containing vemurafenib and cobimetinib (Figure 19C). Thus, eosinophil-mediated cytotoxicity requires cell-cell contact between eosinophils and their target cells.
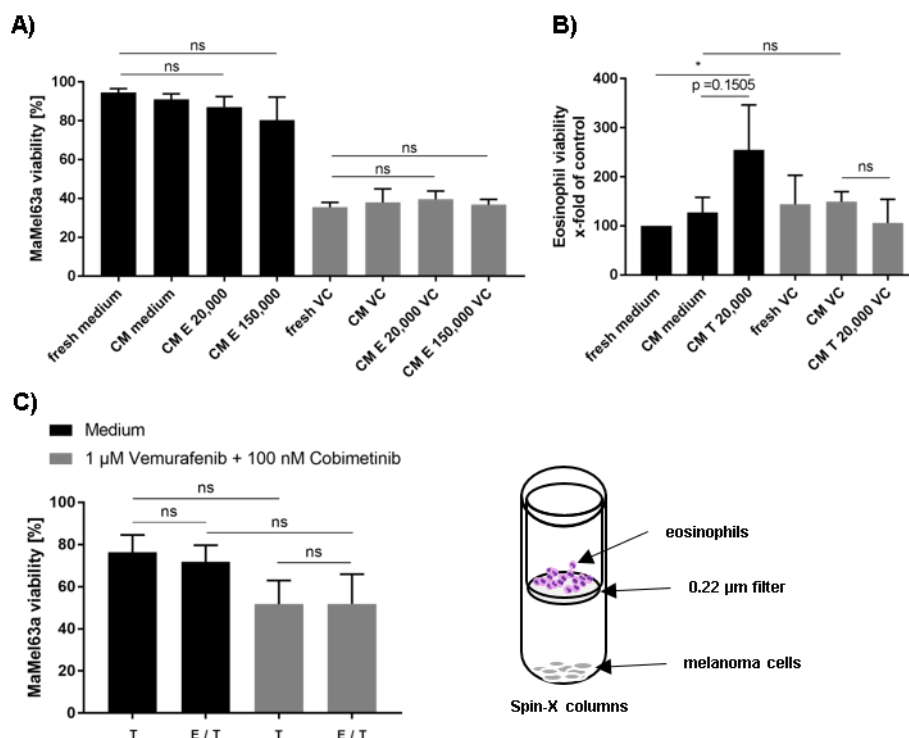

Figure 19. Physical proximity is crucial for the functionality of eosinophils in co-cultures. 48 hours-cultures of **(A)** freshly prepared MaMel63a and **(B)** eosinophils with conditioned medium (here: CM) from previous MaMel63a co-cultures with eosinophils were carried out for 48 hours. Fresh medium with or without addition of 1 µM vemurafenib and 100 nM cobimetinib was used as a control. Mean percentage of melanoma cell viability ± SD are displayed from two to four independent experiments. **(C)** Physical proximity is crucial for the functionality of eosinophils in co-cultures. Separate co-cultures of MaMel63a cells with eosinophils in Spin-X® columns containing a semipermeable membrane (pore size 0.22 µm) for 24 hours. Culture with or without 1 µM vemurafenib and 100 nM cobimetinib. The cytotoxic effect could not be maintained when cells were cultured separately.

In order to visualize the close melanoma cell-eosinophil interaction in vitro, co-cultures were transferred onto a glass slide after 24 hours or 48 hours and stained for HE. Treatment of MaMel63a cells with vemurafenib and cobimetinib decreased the number of cells available on the glass slide (Figure 20A, B). Interestingly, we observed the formation of melanoma cell-eosinophils aggregates in co-cultures both in medium and medium containing vemurafenib and cobimetinib after 24 hours and 48 hours (Figure 20A, B). Eosinophils alone do not accumulate after 24 hours culture but when treated with vemurafenib and cobimetinib or when increasing the culture time to 48 hours (Figure 20C). This observation highlights the close proximity between eosinophils and melanoma cells in vitro.
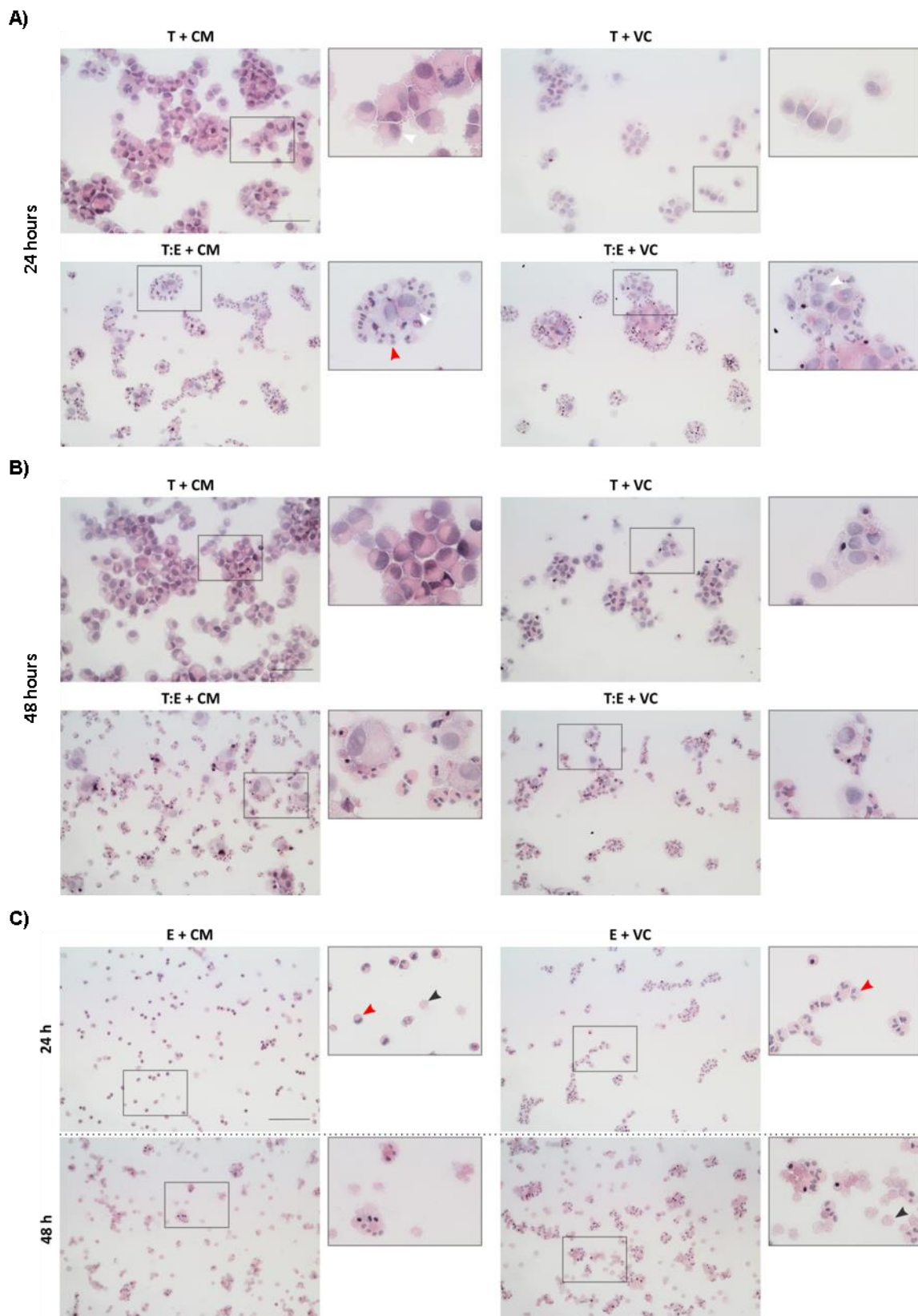
Figure 20. Eosinophils and melanoma cells form aggregates in co-cultures. Cytospin staining with HE for (A) 24 hours and (B) 48 hours non-adherent (co-)culture of MaMel63a (T) and eosinophils (E) in a 1:7.5 ratio in medium (CM) or medium containing 1 μM vemurafenib and 100 nM cobimetinib (VC). (C) Eosinophils alone only form aggregates when cultured in vemurafenib and cobimetinib containing medium after 24 and 48 hours and after 48 hours culture in medium. Black boxes in original image indicate the image section which was used for the magnification (3.2X) shown on the right side of the original image. White arrow points at melanoma cell. Red arrow points at intact eosinophil. Black arrow points at dead eosinophil. Scale bar 100 μm.

3.3.3 Stimulation of eosinophils prior co-cultures with melanoma cells

To examine the effect of pre-activation of granulocytes and their change in functionality upon stimulation in co-culture, eosinophils or melanoma cells were incubated with the stimulant PMA for 2.5 hours prior co-culture (Figure 21). Subsequently, eosinophil-MaMel63a cell co-cultures were carried out for 48 hours in medium. MaMel63a cell viability was not affected by pre-stimulation with PMA before non-adherent and adherent culture (Figure 21A, B). PMA pre-stimulation of eosinophils resulted in significant enhancement of cytotoxicity towards MaMel63a cells relative to untreated cells and only by trend when compared to melanoma cell-eosinophil non-adherent co-culture (p = 0.08) (Figure 21A). As cytotoxicity of eosinophils was intensified upon PMA stimulation, co-cultures with MaMel63a cells were carried out examining the impact of the stimulant on cytotoxicity under adherent conditions.
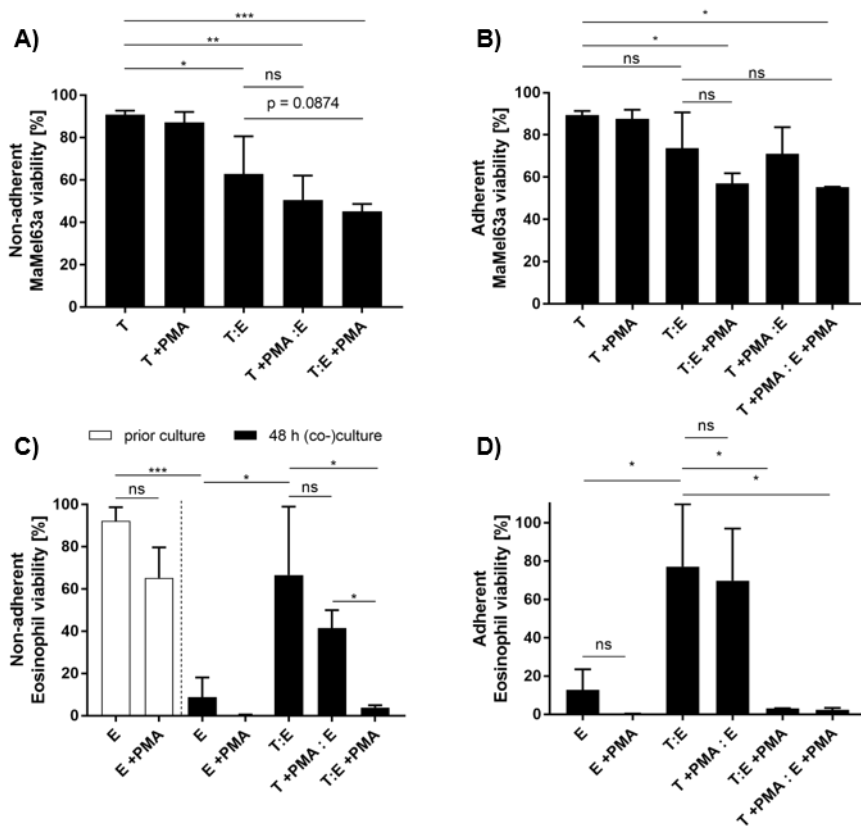

Figure 21. Stimulation of granulocytes in co-cultures. PMA stimulation (50 nM) of eosinophils or melanoma cells for 2.5 hours prior co-culture and subsequent co-culture in medium for 24 hours. **(A)** Viability of MaMel63a cells after 24 hours non-adherent co-culture with eosinophils. PMA does not affect MaMel63a viability. There is a trend towards enhanced eosinophil-mediated cytotoxicity against MaMel63a cells when pre-stimulating melanoma cells or eosinophils with PMA. Mean percentage of MaMel63a cell viability ±SD shown from four to seven independent experiments. **(B)** Viability of MaMel63a cells after 48 hours adherent co-culture with eosinophils. PMA stimulation enables eosinophils to exert their tumoricidal function under adherent culture conditions. Mean percentage of MaMel63a cell viability ±SD shown from two to five independent experiment. **(C)** Eosinophil viability from the same non-adherent culture set up as shown in **(A)**. **(D)** Eosinophil viability from the same non-adherent culture set up as shown in **(B)**. PMA reduces eosinophil viability both in non-adherent and adherent culture setup. The beneficial effect of MaMel63a cells on eosinophil viability was reduced when stimulating eosinophils with PMA prior to co-culture. Mean percentage of eosinophil viability ±SD shown from two to four independent experiments. ns p > 0.05, * p < 0.05, ** p < 0.01, *** p < 0.0005, **** p < 0.0001.

For adherent cultures, when comparing to untreated melanoma cells, eosinophil-mediated cytotoxicity towards MaMel63a cells was enabled when pre-stimulating eosinophils (Figure 21B). Compared to melanoma cell-eosinophil co-culture, pre-stimulation of eosinophils with PMA did not show such an effect. Eosinophil viability significantly decreased during non-adherent and adherent culture (Figure 21C, D). Eosinophil viability decreased by trend when stimulated with PMA prior and after 48 hours non-adherent culture (Figure 21C). MaMel63a cells significantly improved eosinophil survival after 48 hours non-adherent and adherent co-culture (Figure 21C, D). The superior survival of eosinophils co-cultured with MaMel63a cells was significantly dampened when pre-stimulating melanoma cells or eosinophils with PMA (Figure 21C, D).

Next, we analyzed the impact of PMA on melanoma cell morphology. For this purpose, MaMel63a cells were treated with or without PMA for 2.5 hours. Subsequently, cells were either washed (+PMA +wash) or PMA (+PMA) was left in culture for 48 hours. Morphology of MaMel63a cells was examined by imaging cells before, after 2.5 hours culture and after 48 hours adherent culture using a conventional inverse microscope. The cell density increased during culture time (Figure 22).
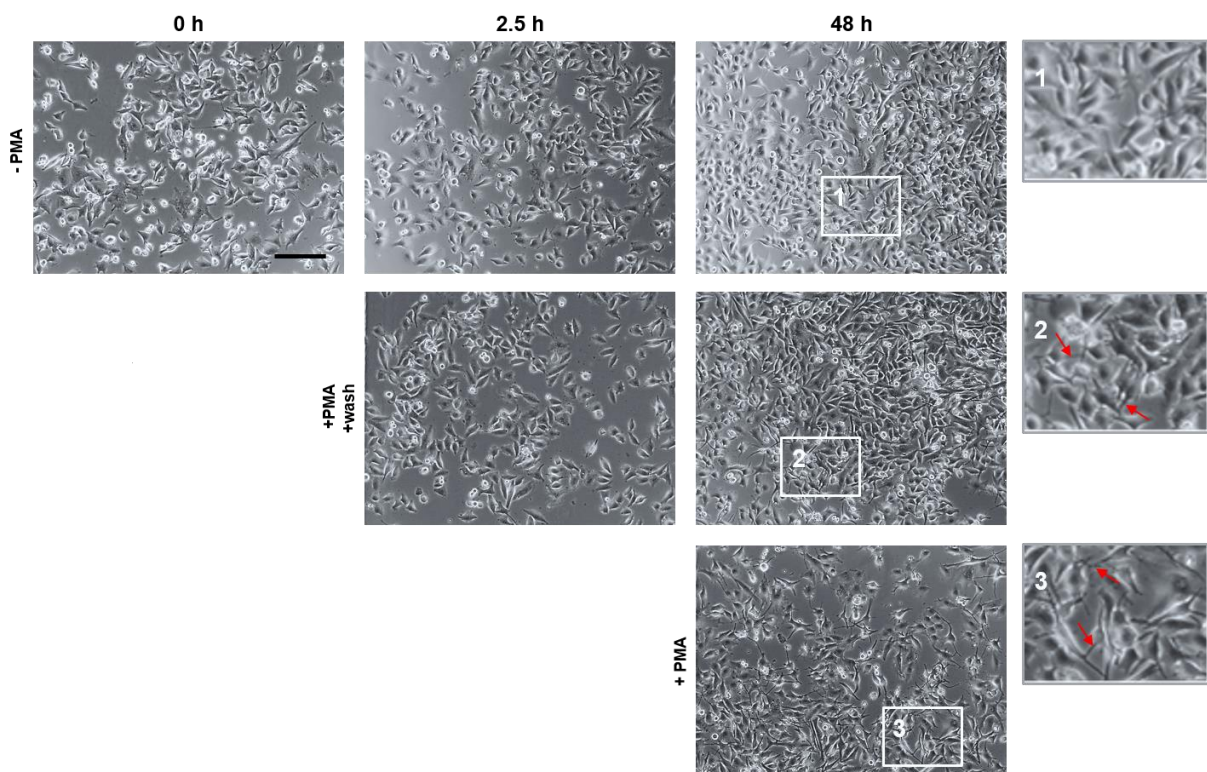

Figure 22. Visualization of the morphological consequence of PMA on MaMel63a cells. Pre-stimulation of MaMel63a cells with 50 nM PMA for 2.5 hours prior culture. After incubation, PMA was either left in medium or washed out once, replaced with fresh medium. Images taken before PMA stimulation, after 2.5 hours and after 48 hours culture in medium with or without PMA using the microscope Leica DMi1. Representative figure from four independent experiments shown. Scale bar 200 μm. Zoom images are 2.4x of original image.

Interestingly, PMA stimulation induced morphological changes of melanoma cells after 48 hours, including an elongated cell shape, and increased (no quantification available) the amount of visible pseudopodia formation indicated by the red arrows in Figure 22.

Additionally, morphology of the PMA pre-stimulated adherent eosinophil-MaMel63a cell co-culture in 24-well plate was examined after 48 hours. MaMel63a cells alone (T) displayed an even cell distribution cultured adherently for 48 hours in medium (Figure 23). Pre-stimulation with PMA (T+PMA) led to a trend towards increased cell-detachment relative to the untreated control. As shown previously (Figure 22), MaMel63a cells adapt a more elongated cell shape when exposed to PMA and develop filopodial structures (indicated by red arrows). Adherent co-culture of MaMel63a cells with eosinophils (T:E 1:7.5) revealed an even distribution of MaMel63a cells on the surface surrounded by eosinophils. Interestingly, stimulation of eosinophils prior co-culture with MaMel63a cells (T:E +PMA) drastically decreased the amount of MaMel63a cell spread / density. MaMel63a cells displayed an elongated cell shape similar to those observed in the T +PMA-condition (indicated by red arrows). Eosinophils accumulated around the melanoma cells. PMA stimulation of MaMel63a cells prior to co-culture with eosinophils (T +PMA:E) also reduced MaMel63a distribution. The strongest cell reduction upon PMA exposure was observed when pre-stimulating MaMel63a and eosinophils (T +PMA:E +PMA) prior co-culture. Interestingly, eosinophils alone (E) showed even and single cell distribution in the 24-well plates. PMA stimulation of eosinophils resulted in the accumulation and the formation of cell clumps in both cultures with and without melanoma cells (Figure 23; E +PMA, T:E +PMA and T +PMA:E +PMA).
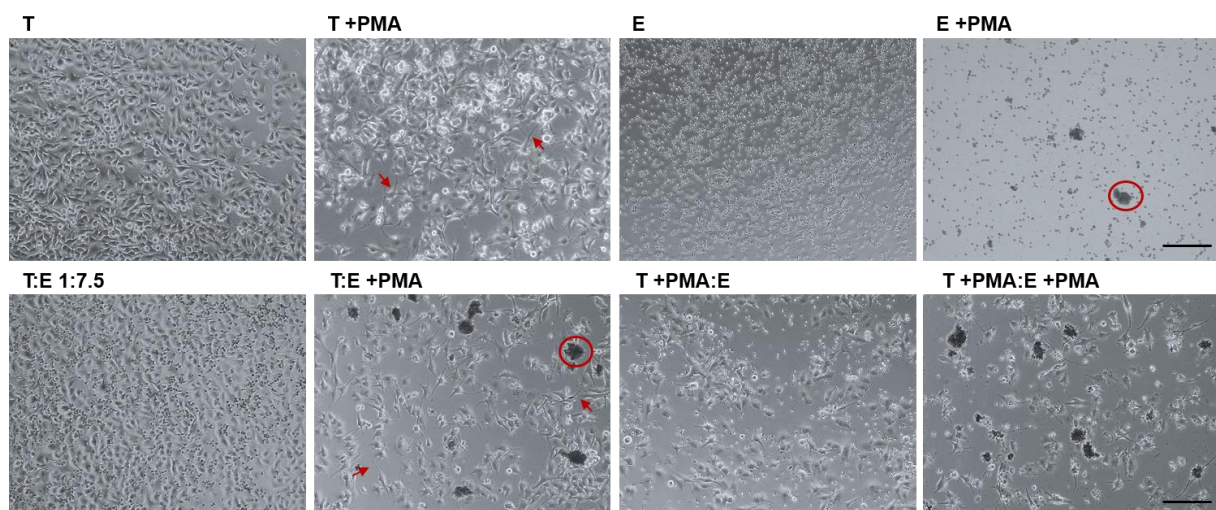

Figure 23. Visualization of the morphological consequence of PMA in eosinophil-MaMel63a co-culture. Pre-stimulation of MaMel63a cells or eosinophils with 50 nM PMA for 2.5 hours prior culture. After incubation, PMA was washed out and replaced with fresh medium. Images taken after 48 hours culture in medium with or without PMA using the microscope Leica DMi1. T = MaMel63a, E = eosinophils. Red arrows highlight prolonged morphological shape of MaMel63a cells after PMA stimulation. Red circle point at eosinophil aggregates formed after PMA stimulation. Scale bar 200 µm.

3.3.4 Inhibition of intracellular transport using Brefeldin A

Under *in vivo* inflammatory conditions, granulocytes are recruited to the site of action and release their toxic granules. Previous data have shown cytotoxicity of eosinophils towards melanoma cells, which led to a significant decrease of melanoma cell viability after co-culture. We hypothesize that granules exposed by eosinophils may at least be partially responsible for the drop of melanoma cell viability as they contain eosinophil proteases and ROS [338]. To block the transport of eosinophil granules to the Golgi apparatus, MaMel63a cells and eosinophils were non-adherently co-cultured in medium containing 5 µg/mL Brefeldin A for 24 hours. After incubation, 7-AAD and Annexin-V FACS staining determined the viability of both cell types. Brefeldin A decreased MaMel63a cell viability by 6.5% after 24 hours but cytotoxicity was not affected by treatment (Figure 24A). Interestingly, eosinophil viability decreased from 50.5% to 14.9% when treated with Brefeldin A for 24 hours (Figure 24B). Melanoma cells were unable to prevent eosinophil apoptosis in co-cultures with Brefeldin A. Despite the drop of eosinophil viability after treatment, Brefeldin A did not prevent or enhance the toxic functionality of eosinophils in this setup as shown in the unaffected viability of MaMel63a cells.
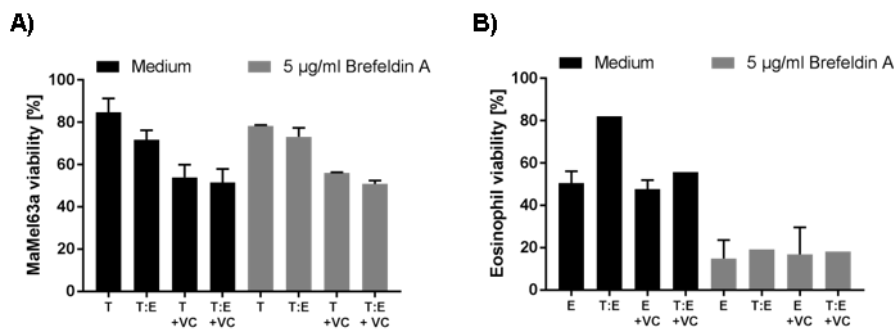

Figure 24. Prevention of protein transport through the Golgi apparatus by Brefeldin A. Co-culture of CFSE-stained MaMel63a cells and eosinophils under non-adherent culture conditions for 24 hours in medium with or without 5 µg/mL Brefeldin A. Viability of **(A)** MaMel63a cells and **(B)** eosinophils is shown staining for 7-AAD and Annexin V. **(A)** Brefeldin A treatment shows no impact in melanoma cell viability. Eosinophil-mediated toxicity could not be prevented nor increased by Brefeldin A. **(B)** Treatment with Brefeldin A drastically decreases eosinophil viability after 24 hours. Mean percentage ± SD of cell viability from one to two independent experiments is shown.

3.3.5 Melanoma cells affect expression of markers for migration and activation of eosinophils in co-culture

As melanoma cells affect eosinophil survival in vitro and vice versa, we wondered whether the bi-directional relationship is accompanied by regulation of eosinophil-specific markers by co-cultures with MaMel63a cells. We phenotypically characterized healthy donor-derived eosinophils before and after co-culture. Expression of the early activation marker, CD69, HLA-DR, PD-L1 and TNFR2 on eosinophils increased during culture for 48 hours, relative to the MFI of controls (here: E 0 h) (Figure 25). The increase of expression of the above-mentioned

antigens was prevented when co-culturing eosinophils with MaMel63a cells for 48 hours. Unlike neutrophils, eosinophils express low levels of CD16. Interestingly, during culture for 48 hours, CD16 expression significantly increased. Amplified expression of CD16 was significantly inhibited in co-cultures with MaMel63a cells. In contrast, activation and migration marker CD66b and CCR3 expression decreased during culture after 48 hours relative to the control (here: E 0 h) (Figure 25). Downregulation of CD66b expression on eosinophils after 48 hours of culture was significantly prevented upon co-culture with MaMel63a cells. As for HLA-A, B, C and CD31, we observed decreasing expression during 24 hours and 48 hours of culture, which were not affected by co-culture with MaMel63a cells.
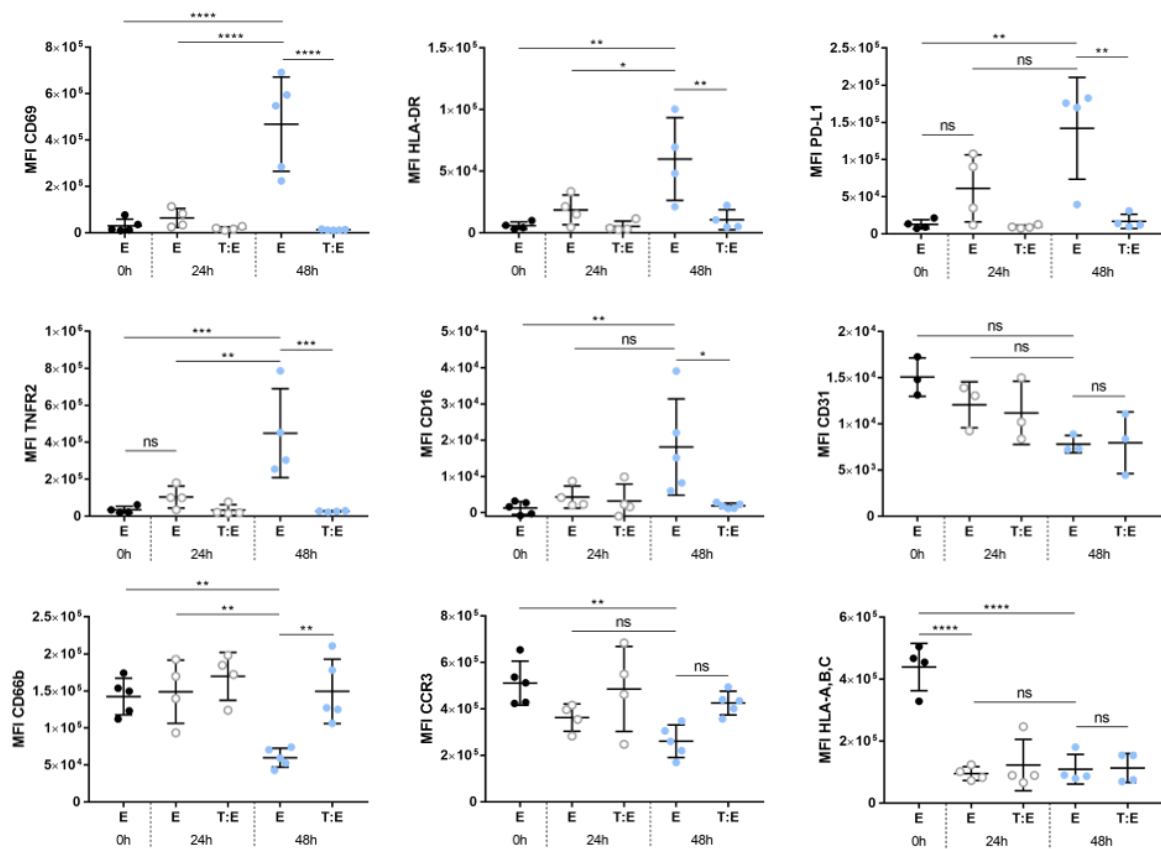

Figure 25. In vitro phenotypic characterization of eosinophils in co-culture with melanoma cells. Phenotypic characterization of peripheral blood eosinophils from healthy donors in (co-)culture with or without MaMel63a cells. Phenotypic epitopes were analyzed at time points 0 hours, 24 hours and 48 hours using flow cytometry. The MFI of the target epitopes on eosinophils were plotted and the expression levels at time point 0 hours were compared to expression after 24 hours and 48 hours (co-)culture. MaMel63a cells appear to be regulating surface marker expression of eosinophils after 48-hour co-culture. The MFI ± standard deviation (SD) is shown from three to five independent experiments. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.3.6 Identifying the driving force for the eosinophil-mediated cytotoxicity in co-cultures with melanoma cells

Studies propose the existence of an immunological synapse, upon which granulocytes secrete soluble factors to their close environment after contacting surrounding cells, exposing them to toxic or favoring mediators [270]. Performing blocking experiments, we explored several target molecules, such as CD11a, CD11b, CD18, CD54 (ICAM-1) and CD49d, which have been described as potential candidates for eosinophil-melanoma cell interaction. MaMel63a cells or freshly isolated eosinophils were pre-incubated with the indicated blocking antibodies prior co-culture. None of the tested target molecules appeared to be involved in eosinophil cytotoxicity since blocking antibodies against these receptors showed no effect (Figure 26A). To explore extracellular eosinophil traps (EETs) as a mechanism used by eosinophil to exert their tumoricidal function in the interaction with MaMel63a cells, DNaseI was added to the co-culture to destroy the DNA network formed during EETosis. DNaseI treatment in in vitro co-culture assays did not affect cytotoxicity by eosinophils. No relationship between EET DNA scaffold formation and cytotoxicity was found (Figure 26B).
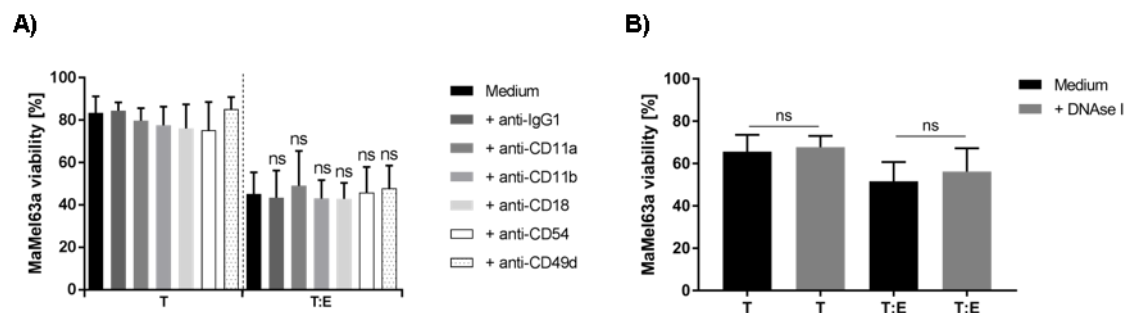

Figure 26. Blocking of potential target molecules and dissolving potential EET-DNA structures in co-cultures. (A) Pre-treatment of MaMel63a or eosinophils with anti-IgG1, anti-CD11a, anti-CD11b, anti-CD18, anti-CD54 (ICAM-1) or anti-CD49d. Subsequent co-culture at a ratio 1:7.5 for 48 hours in CM. None of the investigated target molecules are driving cytotoxicity by eosinophils. **(B)** Dissolving DNA-containing EETs in co-culture. Non-adherent co-culture of MaMel63a cells and eosinophils for 24 hours in medium or medium containing DNaseI. Mean percentage viability of MaMel63a cells ± SD is shown from three to six independent experiments. ns $p > 0.05$.

4   Discussion

Despite tremendous achievements in the treatment of advanced melanoma with improved clinical outcomes and increasing knowledge on its biology, there is still an urgent need of prognostic and predictive biomarkers. As easily accessible, blood eosinophils might be a potential biomarker in advanced melanoma. However, their reliability is yet to be clarified.
In this study, we investigated the relevance of blood eosinophils and their soluble mediators in late-stage melanoma patients receiving first-line targeted therapy or immunotherapy. To clarify the link between blood eosinophils, eosinophil-mediated cytotoxicity, tumor rejection and clinical response to melanoma treatment, we functionally and phenotypically characterized blood eosinophils. In vitro experiments helped unravelling the close communication between blood eosinophils and melanoma cells and supported our patient-derived data.

Numerous studies have demonstrated the pleiotropic role of eosinophilic granulocytes in various diseases. Among others, eosinophils could be assigned both pro- and anti-tumoral properties. Therefore, it is not surprising that patients' prognoses can be improved or worse depending on the presence of eosinophils in blood and tumor tissue [248, 261, 285-290]. While Hopkin`s lymphoma [339-340], cervical or larynx cancer patients [341-342] with tumor-associated tissue eosinophilia (TATE) are at a higher risk of poor outcomes, 5-year survival rate, OS and disease-free survival was improved in patients with colon carcinoma when eosinophil infiltration into the tumor and blood eosinophil counts was high [288, 343]. A positive correlation between the clinical outcome and high eosinophil counts in the peripheral blood could also be demonstrated in patients with breast cancer, renal cell cancer and melanoma [294, 344-345]. Elevated baseline LDH concentration in serum of advanced melanoma patients is a robust prognostic and predictive marker, and has been correlated with a worse clinical outcome, independent of the therapeutic choice [346-347]. By converting pyruvate into lactate, it causes local acidosis and suppresses immune effector cells like CD8+ T cells and natural killer cells, which might explain its correlation with increased disease progression, poor response and patient survival [347]. Thus, any new biomarker must be compared to LDH. Analyzing peripheral blood eosinophil counts and other prognostic markers in a homogeneous patient cohort, we found that low levels of LDH, high AEC and high REC were associated with response to targeted therapy before and after treatment, which corresponds to previous observations [294, 296]. Importantly, in an independent cohort, we were able to demonstrate that high pre-treatment REC are highly associated with a favorable treatment response. However, it should not be underestimated that despite eosinophils being linked to enhanced survival in melanoma patients, their counts are associated with immune-related adverse events (irAEs) that occur during immunotherapy [298]. In fact, tissue damage has been

attributed to eosinophilia as shown in asthma, atopic dermatitis and other skin-related diseases. Eosinophilia can be triggered either by intrinsic, e.g., by mutations in hematopoietic stem cells, or by extrinsic factors, e.g., due to release of cytokines like IL-5 or GM-CSF by tumor cells or immune cells and therapeutic agents [348]. There is an increasing demand for models predicting risk-benefit ratios compared to single clinical endpoints as the clinical landscape begins to shift toward personalized therapies [321]. However, we did not collect any data on treatment-related adverse events. AEC of melanoma patients has been reported to change drastically during immunotherapy [300, 349]. Analyzing peripheral blood counts of patients receiving immunotherapy, we showed that REC did not correlate with response to PD-1 monotherapy but by trend with combinatory treatment with ipilimumab and nivolumab. As a result of our research, REC prior to targeted therapy now has a place in the list of biomarkers for predicting outcomes before treatment is initiated, a finding that is more relevant to clinical practice than on-treatment changes.

As a counterpart to eosinophils, studies describe a confusing relationship of neutrophils and cancer [257]. Solid tumors, including melanoma and gastric carcinoma, are highly infiltrated by neutrophils, making up to 80% of the infiltrate [350]. On the one hand, neutrophils induce tumor cell killing by production and release of toxic reactive oxygen species (ROS) and apoptosis through FAS expression, referred to as an anti-tumorigenic N1-phenotype, also characterized by high levels of TNF-α [350-351]. However, most clinical reports rather link high peripheral neutrophil counts and abundance of neutrophils within a tumor with tumor progression and poor clinical prognosis [352]. In melanoma, overall survival was worse in patients with high neutrophil-to-lymphocyte ratio (NLR) [353-354]. Referred to as a pro-tumorigenic N2-phenotype accompanied by immunosuppression, promoting tumor growth by neutrophils is partially driven by the production of proteinases like neutrophil elastase and MMP9 [350, 355-356]. Entry of tumor cells into the vasculature is mediated by the production of proangiogenic factors like the vascular epithelial growth factor (VEGF) by neutrophils, facilitating metastasis [357]. A decrease in ANC and RNC during treatment was associated with a positive response to targeted therapy, supporting these observations.

Upon exposure to chemoattractant like eotaxin-1 (CCL11), eosinophils migrate into inflamed tissue [267, 358]. A study by Gebhardt et al. showed decreasing serum concentrations of eotaxin-1 in melanoma patients that show non-response to immunotherapy [300]. Serum eotaxin-1 levels before drug administration in our cohort were similar between responders and non-responders, while eotaxin-1 during treatment was by trend higher in non-responders. As we associated non-response to targeted therapy with lower blood eosinophil counts, we assume that higher on-treatment eotaxin-1 in sera of non-responders might be compensatory

mechanism to overcome the lack of eosinophils in this patient group. Our cohort lacked in statistical power, as we the cohort size for eotaxin-1 was too small (in total n = 22). The involvement of APRIL in proliferation and tumor survival has been shown in various cancers [359]. Data have pointed at granulocytes but also cancerous cells as a source of APRIL [360]. APRIL expression was associated with tumor aggression and worse clinical outcome in both solid and non-solid tumors [360]. In accordance with these observations, we showed by trend higher APRIL concentrations in sera from patients without response to targeted therapy. No association with response was observed for other markers like RANTES, sRAGE or GM-CSF in our study. Eosinophils either degranulate while maintaining their integrity through activation, e.g., by alarmins, or initiate cytolysis. In both cases, eosinophils release their cytotoxic granules, which induce oxidative stress through ROS production mediated by EPX, triggering apoptosis and necrosis in the target cell, or they generate pores in the membrane of the target, thus promoting an influx of further cytotoxic mediators [361]. The latter is induced by ECP and EDN [248, 267, 303]. Further, we investigated the relevance of ECP as a prognostic serum marker in patients receiving targeted therapy as first-line treatment. Among other toxic granules secreted by eosinophils, ECP exerts cytotoxicity on cancer cells in vitro [362]. Its potential as a prognostic marker for malignancies such as melanoma has been proposed by Krückel et al. [313]. Serum ECP levels coincided with enhanced risk for disease progression. Interestingly, serum ECP of a heterogeneous study cohort inversely correlated with overall survival, contrary to expectations [313]. We could show a trend towards lower serum ECP concentrations in patients responding to targeted therapy. ECP levels did not correlate with AEC or REC. However, pre-treatment serum ECP levels were significantly higher in responders receiving either monotherapy with PD-1 inhibitor or combination therapy with CLTA4-inhibitor compared to non-responders. Thus, indicating higher eosinophil activity in this patient subgroup. Additionally, pre-treatment but not on-treatment ECP negatively correlated with AEC and REC. As peripheral blood eosinophil counts is higher in patients with melanoma responding to immunotherapy, we assume that the inverse correlation to ECP indicates eosinophil degranulation rather than cytolysis [294, 363]. In contrast, cytolysis, followed by rupture of the cell membrane releasing cellular content into the surrounding, might decrease the amount of eosinophils observable in the peripheral blood. Considering all available data, including our study, based on its predictive ability, serum ECP may be used as a biomarker to select first-line therapy.

While anti-tumor cytotoxicity is not yet well understood, it is evident that eosinophils both in blood and tissue affect tumor progression in some malignancies [248]. A study conducted in mice showed increased tumor incidence with partial or complete eosinophil deficiency. In vitro experiments displayed direct eosinophil-mediated cytotoxicity towards fibrosarcoma cells used

in their study [364]. The beneficial effect of blood eosinophils on a patient's outcome may be explained by direct eosinophil cytotoxicity towards melanoma cells. We demonstrated that different melanoma and non-melanoma cell lines were efficiently induced to succumb to apoptosis and necrosis by freshly isolated blood-derived eosinophils. In addition, we found that the cytotoxicity was dose dependent; melanoma cells showed higher levels of apoptosis when more eosinophils are added to the co-culture. However, no association was found between cytotoxicity and clinical outcome [274]. Eosinophils are susceptible to cytokines and chemoattractants released by innate and adaptive immune cells [248]. A study by Carretero et al. disclosed the pivotal role of eosinophils in coordinating the cytotoxic function of CD8+ T cells towards tumor cells and their infiltration into the tumor [275]. The important crosstalk between eosinophils and T cells was not taken in account in this project. Our in vitro experiments indicate the independence of eosinophil-mediated cytotoxicity of CD8+ T cell presence as shown by the induction of apoptosis and necrosis by eosinophils in co-cultured tumor cells. This fact might open clinical therapeutic approaches targeting eosinophil function and availability combined with enhanced T cell activity. However, treatment-related toxicity (irAE) and eosinophil-mediated tissue damage should not be underestimated as previously discussed.

Eosinophils are reported to exert their cytotoxic function against colon carcinoma utilizing the CD11a/CD18 (LFA-1) axis [272]. The beneficial inhibition of tumor growth in vitro in Colo-205 and in the colons of mice was abolished by neutralizing CD18 [306, 365]. While we investigated several suggested receptors, including LFA-1, which might mediate the close interaction between eosinophils and melanoma cells, none of the tested blocking antibodies prevented cytotoxicity towards melanoma cells [274].

Another mechanism through which granulocytes may exert their function is by forming eosinophil extracellular traps (EETs) or neutrophil extracellular traps (NETs) [366-369]. These extracellular traps consist of a DNA scaffold decorated with antimicrobial peptides and proteases and are expelled from eosinophils or neutrophils upon activation. In 2018, Albrengues et al. elucidated the link between continuous inflammation and the recruitment of neutrophils to the inflammatory site and subsequent appearance of NET formation in a mouse model [370]. Their study described the ability of NET-associated neutrophil elastase (NE) and matrix metalloproteinase 9 (MMP9) to awaken dormant cancer cells and essentially induce tumor progression. Resolving the DNA scaffold of EETs by DNAseI treatment in co-cultures with melanoma cells did not prevent eosinophil-mediated cytotoxicity. However, by simply dissolving the DNA scaffold, the release of proteases and other cytotoxic mediators is not inhibited. In fact, eosinophil extracellular traps are decorated with cytotoxic ECP and other

eosinophil-secreted granules like MBP [348]. Thus, DNAseI treatment might not be sufficient to prevent EETs and eosinophil-mediated cytotoxicity in co-cultures.

Exploring the interaction of eosinophils and melanoma cells, we could show that eosinophil-induced apoptosis in melanoma depends on the close cell–cell proximity and could be induced neither by separate co-culture nor by eosinophil conditioned medium. The observation is in line with the cytotoxicity of blood eosinophils against a colon carcinoma cell line, showing the necessity of direct contact between eosinophils and their targets to induce apoptosis [306]. The dependence of effector-target cell function on cellular adhesion was also manifested in a study conducted using *Schistosoma mansoni*, a parasitic larvae, as a bait for eosinophil cytotoxicity [371]. The initial co-culture of melanoma cells was performed in a non-adherent environment in order to define the melanoma cell interaction. This imitates the interaction of circulating tumor cells (CTCs) with peripheral blood eosinophils. Bloodstream CTCs are frequently found in melanoma tumors, which exhibit high metastatic potential [372-374]. It is noteworthy that activated eosinophils robustly reduced pulmonary metastasis and improved tumor rejection by improving T cell infiltration in melanoma mouse models [275, 279, 307, 332]. The beneficial effect of high circulating eosinophil counts in melanoma patients could be explained by the CTCs being exposed to eosinophils and activating the bloodstream eosinophil killing mechanism [274]. This observation is supported by our in vitro experiments showing that eosinophils, in combination with targeted therapy, significantly and additively reduce melanoma cell viability under non-adherent conditions. Thus, patients with advanced melanoma may benefit from the cooperative cytotoxic action of eosinophils and targeted therapy. We hypothesize that as a result of being exposed to targeted therapy, melanoma cells release stress signals, which might enhance the effectiveness of eosinophils for causing melanoma cells to undergo apoptosis. This could explain the additive cytotoxic effect of eosinophils and targeted therapy in vitro [375-376]. Interestingly, targeted therapy induced aggregation of eosinophils alone after 24 h. It is possible that targeted therapy induces degranulation and/or adhesion in eosinophils and might lead to the expression of an "eat-me" signal on melanoma cells [274]. However, while eosinophils in tissue are easily detected by H&E staining in colorectal cancer, eosinophils are rarely found in melanoma metastasis as confirmed after consultation with the in-house histology of der dermatology clinics UKW Würzburg [247].

The effects of eosinophils on cancer are variable, depending on the location and circumstances [249]. By switching from non-adherent cultures to adherent conditions, rather mimicking solid tumor environments, eosinophils were unable to induce apoptosis in melanoma cells [274].

Melanoma cells' adherence properties may be affected by different surface structures, which may alter survival signals to resist eosinophil-mediated cytotoxicity [377].

According to a study conducted in the lab of L. Erpenbeck apoptosis through formation of neutrophil extracellular traps (NETosis) by neutrophils activated with LPS depends on adhesion and substrate elasticity [378]. Further studies demonstrated the ability of physical factors, like tissue stiffness [379-380], to modulate macrophages [381-382], antigen-presenting dendritic cells [383] and mesenchymal stem cells (MSCs) phenotype [384-385] and influence cell functionality, migration and chemotaxis of neutrophils [386-389]. It is possible that also eosinophil-mediated cytotoxicity is subject to such physical influence, thus changing functionality under adherent conditions. Experiments with differently coated glass slides for co-cultures might further unravel the dependence of certain physical conditions in co-cultures. Such coating could include varies degrees of Poly-L-lysine (PLL) or Poly-L-lysine-grafted-polyethylene glycol (PLL-g-PEG). While PLL-coating promotes adhesion, PEG-coating prevents adhesion of cells or proteins to the surface [378]. However, we did not perform further experiments since we focused on eosinophil function and phenotype.

We hypothesized that phenotype and cytotoxic potential of blood-derived eosinophils differ between healthy donors and melanoma patients. Eosinophils derived from allergic donors display higher cytotoxic potential, which might be explained by enhanced activity or increased expression of adhesion receptors [272, 390]. Screening for various eosinophil markers, we can show that pre-treatment blood eosinophils of late-stage melanoma patients and controls show comparable expression patterns. Activation markers like CD69 and CD66b and adhesion molecules like CD29 and CD31 are similarly expressed in the compared cohorts [274]. Without ruling out potential differences in other markers that were not assessed in this study, we assume that phenotypical changes and differences in eosinophils might happen during treatment and/or during direct contact with melanoma cells. The latter is supported by the downregulation of CD69 and upregulation of CD66b after in vitro co-cultures [274]. Moreover, we found that eosinophils are significantly more viable when co-cultured with melanoma cells, suggesting a bidirectional interaction. CD66b was described to promote cellular adhesion of eosinophils and expression can be upregulated in vitro by IL-5 [391]. IL-5 or chemoattractants such as GM-CSF are also able to induce expression of CD69, CD16 and HLA-DR in vitro [392-394]. We have observed that eosinophils and melanoma cells can form aggregates, which might be modulated by the upregulation of CD66b. By modulating eosinophil activation, melanoma cells seem to prevent metastasis inhibition, while allowing and regulating eosinophil adhesion and improving their survival in vitro [274].

Under in vivo inflammatory conditions, granulocytes are recruited to the site of action and release their toxic content upon activation. Activation of eosinophils through various mediators like IL-5, GM-CSF but also phorbol-12-myristate-13-acetate (PMA) or the lipopolysaccharide (LPS) is known to alter eosinophil functionality. As for neutrophils, PMA and LPS induce NETosis and ROS generation. Three-fold increase of leukotriene C4 release and thus enhanced cytotoxicity was reported by eosinophils stimulated with calcium ionophore [260]. Stimulation of eosinophils with PMA showed only a trend towards increased cytotoxicity towards melanoma cells for both non-adherent and adherent culture conditions compared to untreated co-cultures. The superior survival of eosinophils co-cultured with MaMel63a cells was significantly dampened when pre-stimulating eosinophils with PMA. This observation might be explained by the decrease of eosinophils viability upon PMA stimulation and their subsequent degranulation with exposure of cytotoxic granule contents [395]. Filopodia modulate cell adhesion and migration and contain accumulated beta-1 integrin [396-397]. Fascin, an actin-bundling cytoskeletal protein is localized in these filopodial structures, and is found upregulated in metastatic breast cancer correlating with poor clinical outcome [398]. Stimulation of melanoma cells with PMA resulted in formation of filopodia. We speculated an enhancement of adhesion of melanoma cells to the flask surface and potentially even to eosinophils under adherent conditions upon PMA stimulation, which might explain the trend towards increased cytotoxicity.

Aggregation of eosinophils and tumor cells has been subject of a study based on ultrastructural observations published by Caruso et al., hinting at an intimate cross-talk between these two cell subtypes [282]. Additionally, eosinophilia has been shown attracted by necrotic cells and was observed in the capsule region of solid tumors [376, 399]. Attraction of eosinophils in necrotic areas might be caused by local hypoxia. By releasing cytotoxic granules, cytokines and chemotactic factors, eosinophils could potentially induce recruitment of more eosinophils resulting in local eosinophil accumulation [376]. As a result of IL-33 stimulation, eosinophils and cancer cells formed stable aggregates in a melanoma mouse model [270]. Another study using a melanoma mouse model reported that eosinophil accumulation in solid tumor was partially limited to the necrotic and capsule regions [376]. There are several reported mechanisms describing degranulation, one of which involves cytolysis [400-401]. Enhanced eosinophil viability in co-culture with melanoma cells might be caused by immune synapse-like mechanism [274]. Andreone et al. proposed such a mechanism was involved in the interaction of eosinophils and tumor cells [270]. Aggregation of these two cell subtypes potentially stimulates the production of GM-CSF by melanoma cells. GM-CSF is produced by a variety of cells including epithelial cells and numerous types of cancer, and is able to prolong the survival of eosinophils in a co-culture [260, 402-405]. This positive effect on eosinophil viability was

also seen in other studies in co-cultures with glioblastoma multiforme or conjunctival fibroblasts [406-407]. In vitro, we demonstrated that intact eosinophils mediate induced apoptosis in melanoma cells. The exposure to released eosinophil content by lysis induced enhanced melanoma cell destruction, which could be prevented by heat-inactivation [274]. This observation is in accordance with a study showing the toxicity of eosinophil content towards B16 melanoma cells [332]. Taken together, our data show that melanoma cells exert a regulatory function towards eosinophils and their interaction is an active process [274].

5 Conclusion

Taken together, we demonstrated that patients with advanced melanoma with high eosinophil counts prior to targeted therapy initiation are highly responsive to targeted therapy. However, their ECP serum concentrations and functional differences in peripheral eosinophils were not related to their response. In contrast, relative eosinophil counts and high ECP serum concentrations prior immunotherapy initiation were associated with response to immunotherapy. Our results are consistent with previous studies linking eosinophil blood counts to better survival in melanoma patients. In vitro functional assays demonstrated close and active interaction between peripheral eosinophils and melanoma cells. The interaction with melanoma cells seemed bidirectional and depended on various factors like the eosinophil donor, providence of adherence and the type of melanoma. Melanoma cells are significantly apoptotic and necrotic when treated with eosinophils, which can be augmented with BRAF-plus-MEK inhibitors. Although the mechanism of this additive effect remains a mystery, our data suggest eosinophils are a potential prognostic biomarker worth further study. In addition, we provide insight into how eosinophils and their secreted molecules control melanoma in a multifaceted, treatment-dependent manner.

6 References

1. Pedersen, L.; Jemec, G. B., Mechanical properties and barrier function of healthy human skin. *Acta Derm Venereol* **2006,** *86* (4), 308-11. DOI: 10.2340/00015555-0080.
2. Kanwar, A. J., Skin barrier function. *Indian J Med Res* **2018,** *147* (1), 117-118. DOI: 10.4103/0971-5916.232013.
3. Besaratinia, A., et al., Wavelength dependence of ultraviolet radiation-induced DNA damage as determined by laser irradiation suggests that cyclobutane pyrimidine dimers are the principal DNA lesions produced by terrestrial sunlight. *FASEB J* **2011,** *25* (9), 3079-91. DOI: 10.1096/fj.11-187336.
4. Runger, T. M., How different wavelengths of the ultraviolet spectrum contribute to skin carcinogenesis: the role of cellular damage responses. *J Invest Dermatol* **2007,** *127* (9), 2103-5. DOI: 10.1038/sj.jid.5700988.
5. Maverakis, E., et al., Light, including ultraviolet. *J Autoimmun* **2010,** *34* (3), J247-57. DOI: 10.1016/j.jaut.2009.11.011.
6. Khan, A. Q., et al., Roles of UVA radiation and DNA damage responses in melanoma pathogenesis. *Environ Mol Mutagen* **2018,** *59* (5), 438-460. DOI: 10.1002/em.22176.
7. Foundation, S. C. UV Radiation & Your Skin. https://www.skincancer.org/risk-factors/uv-radiation/#:~:text=Two%20types%20of%20UV%20light,is%20associated%20with%20skin%20burning. (accessed January 4, 2021).
8. D'Orazio, J., et al., UV radiation and the skin. *Int J Mol Sci* **2013,** *14* (6), 12222-48. DOI: 10.3390/ijms140612222.
9. Arisi, M., et al., Sun Exposure and Melanoma, Certainties and Weaknesses of the Present Knowledge. *Front Med (Lausanne)* **2018,** *5*, 235. DOI: 10.3389/fmed.2018.00235.
10. Lippke, J. A., et al., Distribution of UV light-induced damage in a defined sequence of human DNA: detection of alkaline-sensitive lesions at pyrimidine nucleoside-cytidine sequences. *Proc Natl Acad Sci U S A* **1981,** *78* (6), 3388-92. DOI: 10.1073/pnas.78.6.3388.
11. Lo, H. L., et al., Differential biologic effects of CPD and 6-4PP UV-induced DNA damage on the induction of apoptosis and cell-cycle arrest. *BMC Cancer* **2005,** *5*, 135. DOI: 10.1186/1471-2407-5-135.
12. Mitchell, D. L.; Nairn, R. S., The biology of the (6-4) photoproduct. *Photochem Photobiol* **1989,** *49* (6), 805-19. DOI: 10.1111/j.1751-1097.1989.tb05578.x.
13. de Gruijl, F. R., Photocarcinogenesis: UVA vs. UVB radiation. *Skin Pharmacol Appl Skin Physiol* **2002,** *15* (5), 316-20. DOI: 10.1159/000064535.
14. de Jager, T. L., et al., Ultraviolet Light Induced Generation of Reactive Oxygen Species. *Adv Exp Med Biol* **2017,** *996*, 15-23. DOI: 10.1007/978-3-319-56017-5_2.
15. Sample, A.; He, Y. Y., Mechanisms and prevention of UV-induced melanoma. *Photodermatol Photoimmunol Photomed* **2018,** *34* (1), 13-24. DOI: 10.1111/phpp.12329.
16. MacKie, R. M.; Aitchison, T., Severe sunburn and subsequent risk of primary cutaneous malignant melanoma in scotland. *Br J Cancer* **1982,** *46* (6), 955-60. DOI: 10.1038/bjc.1982.307.
17. Marks, R., Epidemiology of melanoma. *Clin Exp Dermatol* **2000,** *25* (6), 459-63. DOI: 10.1046/j.1365-2230.2000.00693.x.
18. Parkin, D. M., et al., 13. Cancers attributable to solar (ultraviolet) radiation exposure in the UK in 2010. *Br J Cancer* **2011,** *105 Suppl 2*, S66-9. DOI: 10.1038/bjc.2011.486.
19. Whiteman, D. C., et al., Childhood sun exposure as a risk factor for melanoma: a systematic review of epidemiologic studies. *Cancer Causes Control* **2001,** *12* (1), 69-82. DOI: 10.1023/a:1008980919928.
20. Kolarsick, P. A. J., et al., Anatomy and Physiology of the Skin. *Journal of the Dermatology Nurses' Association* **2011,** *3* (4), 203-213. DOI: 10.1097/JDN.0b013e3182274a98.
21. Erickson, C. A.; Reedy, M. V., Neural crest development: the interplay between morphogenesis and cell differentiation. *Curr Top Dev Biol* **1998,** *40*, 177-209. DOI: 10.1016/s0070-2153(08)60367-1.
22. Plouhinec, J. L., et al., Pax3 and Zic1 trigger the early neural crest gene regulatory network by the direct activation of multiple key neural crest specifiers. *Dev Biol* **2014,** *386* (2), 461-72. DOI: 10.1016/j.ydbio.2013.12.010.
23. White, R. M.; Zon, L. I., Melanocytes in development, regeneration, and cancer. *Cell Stem Cell* **2008,** *3* (3), 242-52. DOI: 10.1016/j.stem.2008.08.005.
24. Aim at melanoma foundation What Are The Stages of Melanoma? https://www.aimatmelanoma.org/stages-of-melanoma/ (accessed July 5, 2022).
25. Goldstein, A. M., et al., Features associated with germline CDKN2A mutations: a GenoMEL study of melanoma-prone families from three continents. *J Med Genet* **2007,** *44* (2), 99-106. DOI: 10.1136/jmg.2006.043802.
26. Ransohoff, K. J., et al., Familial skin cancer syndromes: Increased melanoma risk. *J Am Acad Dermatol* **2016,** *74* (3), 423-34; quiz 435-6. DOI: 10.1016/j.jaad.2015.09.070.
27. Read, J., et al., Melanoma genetics. *J Med Genet* **2016,** *53* (1), 1-14. DOI: 10.1136/jmedgenet-2015-103150.
28. Davies, H., et al., Mutations of the BRAF gene in human cancer. *Nature* **2002,** *417* (6892), 949-54. DOI: 10.1038/nature00766.
29. Peyssonnaux, C.; Eychene, A., The Raf/MEK/ERK pathway: new concepts of activation. *Biol Cell* **2001,** *93* (1-2), 53-62. DOI: 10.1016/s0248-4900(01)01125-x.
30. Qi, M.; Elion, E. A., MAP kinase pathways. *J Cell Sci* **2005,** *118* (Pt 16), 3569-72. DOI: 10.1242/jcs.02470.

31. Albino, A. P., et al., Transforming ras genes from human melanoma: a manifestation of tumour heterogeneity? *Nature* **1984,** *308* (5954), 69-72. DOI: 10.1038/308069a0.

32. Hodis, E., et al., A landscape of driver mutations in melanoma. *Cell* **2012,** *150* (2), 251-63. DOI: 10.1016/j.cell.2012.06.024.

33. Roberts, P. J.; Der, C. J., Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. *Oncogene* **2007,** *26* (22), 3291-310. DOI: 10.1038/sj.onc.1210422.

34. Samatar, A. A.; Poulikakos, P. I., Targeting RAS-ERK signalling in cancer: promises and challenges. *Nat Rev Drug Discov* **2014,** *13* (12), 928-42. DOI: 10.1038/nrd4281.

35. Hagemann, C.; Rapp, U. R., Isotype-specific functions of Raf kinases. *Exp Cell Res* **1999,** *253* (1), 34-46. DOI: 10.1006/excr.1999.4689.

36. Wellbrock, C., et al., The RAF proteins take centre stage. *Nature Reviews Molecular Cell Biology* **2004,** *5* (11), 875-885. DOI: 10.1038/nrm1498.

37. Rajakulendran, T., et al., A dimerization-dependent mechanism drives RAF catalytic activation. *Nature* **2009,** *461* (7263), 542-5. DOI: 10.1038/nature08314.

38. Lavoie, H.; Therrien, M., Regulation of RAF protein kinases in ERK signalling. *Nat Rev Mol Cell Biol* **2015,** *16* (5), 281-98. DOI: 10.1038/nrm3979.

39. Cheng, L., et al., Molecular testing for BRAF mutations to inform melanoma treatment decisions: a move toward precision medicine. *Mod Pathol* **2018,** *31* (1), 24-38. DOI: 10.1038/modpathol.2017.104.

40. Pavey, S., et al., Microarray expression profiling in melanoma reveals a BRAF mutation signature. *Oncogene* **2004,** *23* (23), 4060-7. DOI: 10.1038/sj.onc.1207563.

41. Becchetti, A., et al., Dynamics and physiological meaning of complexes between ion channels and integrin receptors: the case of Kv11.1. *Am J Physiol Cell Physiol* **2022,** *322* (6), C1138-C1150. DOI: 10.1152/ajpcell.00107.2022.

42. Karoulia, Z., et al., An Integrated Model of RAF Inhibitor Action Predicts Inhibitor Activity against Oncogenic BRAF Signaling. *Cancer Cell* **2016,** *30* (3), 485-498. DOI: 10.1016/j.ccell.2016.06.024.

43. Yao, Z., et al., BRAF Mutants Evade ERK-Dependent Feedback by Different Mechanisms that Determine Their Sensitivity to Pharmacologic Inhibition. *Cancer Cell* **2015,** *28* (3), 370-83. DOI: 10.1016/j.ccell.2015.08.001.

44. Wan, P. T., et al., Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell* **2004,** *116* (6), 855-67. DOI: 10.1016/s0092-8674(04)00215-6.

45. Long, G. V., et al., Prognostic and clinicopathologic associations of oncogenic BRAF in metastatic melanoma. *J Clin Oncol* **2011,** *29* (10), 1239-46. DOI: 10.1200/JCO.2010.32.4327.

46. Lovly, C. M., et al., Routine multiplex mutational profiling of melanomas enables enrollment in genotype-driven therapeutic trials. *PLoS One* **2012,** *7* (4), e35309. DOI: 10.1371/journal.pone.0035309.

47. Malkhasyan, K. A., et al., The clinical characteristics of melanoma with BRAF V600R mutation: a case series study. *Melanoma Res* **2020,** *30* (1), 107-112. DOI: 10.1097/CMR.0000000000000630.

48. Clancy, C., et al., BRAF mutation is associated with distinct clinicopathological characteristics in colorectal cancer: a systematic review and meta-analysis. *Colorectal Dis* **2013,** *15* (12), e711-8. DOI: 10.1111/codi.12427.

49. Ikenoue, T., et al., Functional analysis of mutations within the kinase activation segment of B-Raf in human colorectal tumors. *Cancer Res* **2003,** *63* (23), 8132-7.

50. Rajagopalan, H., et al., Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. *Nature* **2002,** *418* (6901), 934. DOI: 10.1038/418934a.

51. Singer, G., et al., Mutations in BRAF and KRAS characterize the development of low-grade ovarian serous carcinoma. *J Natl Cancer Inst* **2003,** *95* (6), 484-6. DOI: 10.1093/jnci/95.6.484.

52. Tejpar, S., et al., Prognostic and predictive biomarkers in resected colon cancer: current status and future perspectives for integrating genomics into biomarker discovery. *Oncologist* **2010,** *15* (4), 390-404. DOI: 10.1634/theoncologist.2009-0233.

53. Duffy, K.; Grossman, D., The dysplastic nevus: from historical perspective to management in the modern era: part I. Historical, histologic, and clinical aspects. *J Am Acad Dermatol* **2012,** *67* (1), 1 e1-16; quiz 17-8. DOI: 10.1016/j.jaad.2012.02.047.

54. Tucker, M. A., et al., Clinically recognized dysplastic nevi. A central risk factor for cutaneous melanoma. *JAMA* **1997,** *277* (18), 1439-44.

55. Dhomen, N., et al., Oncogenic Braf induces melanocyte senescence and melanoma in mice. *Cancer Cell* **2009,** *15* (4), 294-303. DOI: 10.1016/j.ccr.2009.02.022.

56. Michaloglou, C., et al., BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature* **2005,** *436* (7051), 720-4. DOI: 10.1038/nature03890.

57. Pollock, P. M., et al., High frequency of BRAF mutations in nevi. *Nat Genet* **2003,** *33* (1), 19-20. DOI: 10.1038/ng1054.

58. Shain, A. H., et al., The Genetic Evolution of Melanoma from Precursor Lesions. *New England Journal of Medicine* **2015,** *373* (20), 1926-1936. DOI: 10.1056/NEJMoa1502583.

59. Bandarchi, B., et al., From melanocyte to metastatic malignant melanoma. *Dermatology research and practice* **2010,** *2010.* DOI: 10.1155/2010/583748.

60. Bastian, B. C., The molecular pathology of melanoma: an integrated taxonomy of melanocytic neoplasia. *Annu Rev Pathol* **2014,** *9*, 239-71. DOI: 10.1146/annurev-pathol-012513-104658.

61. Belanger, F., et al., A majority of human melanoma cell lines exhibits an S phase-specific defect in excision of UV-induced DNA photoproducts. *PLoS One* **2014,** *9* (1), e85294. DOI: 10.1371/journal.pone.0085294.

62. Bowden, N. A., et al., Nucleotide excision repair gene expression after Cisplatin treatment in melanoma. *Cancer Res* **2010,** *70* (20), 7918-26. DOI: 10.1158/0008-5472.CAN-10-0161.

63. Daniotti, M., et al., BRAF alterations are associated with complex mutational profiles in malignant melanoma. *Oncogene* **2004,** *23* (35), 5968-77. DOI: 10.1038/sj.onc.1207780.

64. Dankort, D., et al., Braf(V600E) cooperates with Pten loss to induce metastatic melanoma. *Nat Genet* **2009,** *41* (5), 544-52. DOI: 10.1038/ng.356.

65. Goel, V. K., et al., Examination of mutations in BRAF, NRAS, and PTEN in primary cutaneous melanoma. *J Invest Dermatol* **2006,** *126* (1), 154-60. DOI: 10.1038/sj.jid.5700026.

66. Karim, R. Z., et al., Reduced p16 and increased cyclin D1 and pRb expression are correlated with progression in cutaneous melanocytic tumors. *Int J Surg Pathol* **2009,** *17* (5), 361-7. DOI: 10.1177/1066896909336177.

67. Nagore, E., et al., Analysis of the CDKN2A and CDK4 genes and HLA-DR and HLA-DQ alleles in two Spanish familial melanoma kindreds. *Acta Derm Venereol* **2000,** *80* (6), 440-2. DOI: 10.1080/000155500300012936.

68. Vezzoni, R., et al., Is There More Than One Road to Nevus-Associated Melanoma? *Dermatol Pract Concept* **2020,** *10* (2), e2020028. DOI: 10.5826/dpc.1002a28.

69. Celebi, J. T., et al., Identification of PTEN mutations in metastatic melanoma specimens. *J Med Genet* **2000,** *37* (9), 653-7. DOI: 10.1136/jmg.37.9.653.

70. Kanoko, M., et al., Expression of p53 protein in melanoma progression. *J Dermatol Sci* **1996,** *12* (2), 97-103. DOI: 10.1016/0923-1811(95)00465-3.

71. Weiss, J., et al., Mutation and expression of TP53 in malignant melanomas. *Recent Results Cancer Res* **1995,** *139*, 137-54. DOI: 10.1007/978-3-642-78771-3_10.

72. Alexandrov, L. B., et al., Signatures of mutational processes in human cancer. *Nature* **2013,** *500* (7463), 415-21. DOI: 10.1038/nature12477.

73. Berger, M. F., et al., Melanoma genome sequencing reveals frequent PREX2 mutations. *Nature* **2012,** *485* (7399), 502-6. DOI: 10.1038/nature11071.

74. Davis, E. J., et al., Melanoma: What do all the mutations mean? *Cancer* **2018,** *124* (17), 3490-3499. DOI: 10.1002/cncr.31345.

75. Pleasance, E. D., et al., A comprehensive catalogue of somatic mutations from a human cancer genome. *Nature* **2010,** *463* (7278), 191-6. DOI: 10.1038/nature08658.

76. Bastian, B. C., et al., Classifying melanocytic tumors based on DNA copy number changes. *Am J Pathol* **2003,** *163* (5), 1765-70. DOI: 10.1016/S0002-9440(10)63536-5.

77. Curtin, J. A., et al., Distinct sets of genetic alterations in melanoma. *N Engl J Med* **2005,** *353* (20), 2135-47. DOI: 10.1056/NEJMoa050092.

78. Rivers, J. K., Is there more than one road to melanoma? *Lancet* **2004,** *363* (9410), 728-30. DOI: 10.1016/S0140-6736(04)15649-3.

79. Whiteman, D. C., et al., p53 expression and risk factors for cutaneous melanoma: a case-control study. *Int J Cancer* **1998,** *77* (6), 843-8. DOI: 10.1002/(sici)1097-0215(19980911)77:6<843::aid-ijc8>3.0.co;2-u.

80. Whiteman, D. C., et al., Melanocytic nevi, solar keratoses, and divergent pathways to cutaneous melanoma. *J Natl Cancer Inst* **2003,** *95* (11), 806-12. DOI: 10.1093/jnci/95.11.806.

81. Shain, A. H.; Bastian, B. C., From melanocytes to melanomas. *Nat Rev Cancer* **2016,** *16* (6), 345-58. DOI: 10.1038/nrc.2016.37.

82. Maldonado, J. L., et al., Determinants of BRAF mutations in primary melanomas. *J Natl Cancer Inst* **2003,** *95* (24), 1878-90. DOI: 10.1093/jnci/djg123.

83. Zalaudek, I., et al., Frequency of dermoscopic nevus subtypes by age and body site: a cross-sectional study. *Arch Dermatol* **2011,** *147* (6), 663-70. DOI: 10.1001/archdermatol.2011.149.

84. Bataille, V., et al., The association between naevi and melanoma in populations with different levels of sun exposure: a joint case-control study of melanoma in the UK and Australia. *Br J Cancer* **1998,** *77* (3), 505-10. DOI: 10.1038/bjc.1998.81.

85. Black, W. C., Residual dysplastic and other nevi in superficial spreading melanoma. Clinical correlations and association with sun damage. *Cancer* **1988,** *62* (1), 163-73. DOI: 10.1002/1097-0142(19880701)62:1<163::aid-cncr2820620126>3.0.co;2-n.

86. Cymerman, R. M., et al., De Novo vs Nevus-Associated Melanomas: Differences in Associations With Prognostic Indicators and Survival. *J Natl Cancer Inst* **2016,** *108* (10). DOI: 10.1093/jnci/djw121.

87. Lazovich, D., et al., Indoor tanning and risk of melanoma: a case-control study in a highly exposed population. *Cancer Epidemiol Biomarkers Prev* **2010,** *19* (6), 1557-68. DOI: 10.1158/1055-9965.EPI-09-1249.

88. Shitara, D., et al., Nevus-associated melanomas: clinicopathologic features. *Am J Clin Pathol* **2014,** *142* (4), 485-91. DOI: 10.1309/AJCP4L5CJGKTJVDD.

89. Stolz, W., et al., Association of early malignant melanoma with nevocytic nevi. *Cancer* **1989,** *63* (3), 550-5. DOI: 10.1002/1097-0142(19890201)63:3<550::aid-cncr2820630325>3.0.co;2-a.

90. Ellerhorst, J. A., et al., Clinical correlates of NRAS and BRAF mutations in primary human melanoma. *Clin Cancer Res* **2011,** *17* (2), 229-35. DOI: 10.1158/1078-0432.CCR-10-2276.

91. Hayward, N. K., et al., Whole-genome landscapes of major melanoma subtypes. *Nature* **2017,** *545* (7653), 175-180. DOI: 10.1038/nature22071.

92. Bastian, B. C., et al., Gene amplifications characterize acral melanoma and permit the detection of occult tumor cells in the surrounding skin. *Cancer Res* **2000,** *60* (7), 1968-73.

93. Helwick, C., Melanoma Mutations: What You Need to Know. *The ASCO post, Harborside Press* **2017.**

94. Van Raamsdonk, C. D., et al., Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. *Nature* **2009,** *457* (7229), 599-602. DOI: 10.1038/nature07586.
95. Van Raamsdonk, C. D., et al., Mutations in GNA11 in uveal melanoma. *N Engl J Med* **2010,** *363* (23), 2191-9. DOI: 10.1056/NEJMoa1000584.
96. Zuidervaart, W., et al., Activation of the MAPK pathway is a common event in uveal melanomas although it rarely occurs through mutation of BRAF or RAS. *Br J Cancer* **2005,** *92* (11), 2032-8. DOI: 10.1038/sj.bjc.6602598.
97. Cancer Genome Atlas, N., Genomic Classification of Cutaneous Melanoma. *Cell* **2015,** *161* (7), 1681-96. DOI: 10.1016/j.cell.2015.05.044.
98. Lens, M. B.; Dawes, M., Global perspectives of contemporary epidemiological trends of cutaneous malignant melanoma. *Br J Dermatol* **2004,** *150* (2), 179-85. DOI: 10.1111/j.1365-2133.2004.05708.x.
99. Linos, E., et al., Increasing burden of melanoma in the United States. *J Invest Dermatol* **2009,** *129* (7), 1666-74. DOI: 10.1038/jid.2008.423.
100. Clark, W. H., Jr., et al., The histogenesis and biologic behavior of primary human malignant melanomas of the skin. *Cancer Res* **1969,** *29* (3), 705-27.
101. Clark, W. H., Jr., et al., A study of tumor progression: the precursor lesions of superficial spreading and nodular melanoma. *Hum Pathol* **1984,** *15* (12), 1147-65. DOI: 10.1016/s0046-8177(84)80310-x.
102. Mitchell, T. C., et al., 66 - Melanoma. In *Abeloff's Clinical Oncology (Sixth Edition)*, Niederhuber, J. E.; Armitage, J. O.; Kastan, M. B.; Doroshow, J. H.; Tepper, J. E., Eds. Elsevier: Philadelphia, 2020; pp 1034-1051.e2. https://doi.org/10.1016/B978-0-323-47674-4.00066-9.
103. McGovern, V. J., et al., The classification of malignant melanoma and its histologic reporting. *Cancer* **1973,** *32* (6), 1446-57. DOI: 10.1002/1097-0142(197312)32:6<1446::aid-cncr2820320623>3.0.co;2-8.
104. Marchetti, M. A., et al., Screening for Acral Lentiginous Melanoma in Dark-Skinned Individuals. *JAMA Dermatol* **2015,** *151* (10), 1055-6. DOI: 10.1001/jamadermatol.2015.1347.
105. Krantz, B. A., et al., Uveal melanoma: epidemiology, etiology, and treatment of primary disease. *Clin Ophthalmol* **2017,** *11*, 279-289. DOI: 10.2147/OPTH.S89591.
106. Gershenwald, J. E.; Scolyer, R. A., Melanoma Staging: American Joint Committee on Cancer (AJCC) 8th Edition and Beyond. *Annals of Surgical Oncology* **2018,** *25* (8), 2105-2110. DOI: 10.1245/s10434-018-6513-7.
107. Amin MB, G. F., Edge SB, Compton CC, Gershenwald JE, Brookland RK, Meyer L, Gress DM, Byrd DR, Winchester DP, *AJCC Cancer Staging Manual* 8th ed.; Springer: New York, 2017.
108. Leitlinienprogramm Onkologie (Deutsche Krebsgesellschaft, D. K.; AWMF) Diagnostik, Therapie und Nachsorge des Melanoms. http://www.leitlinienprogramm-onkologie.de/leitlinien/melanom/ (accessed July 6, 2022).
109. Torre, L. A., et al., Global cancer statistics, 2012. *CA Cancer J Clin* **2015,** *65* (2), 87-108. DOI: 10.3322/caac.21262.
110. Harpio, R.; Einarsson, R., S100 proteins as cancer biomarkers with focus on S100B in malignant melanoma. *Clin Biochem* **2004,** *37* (7), 512-8. DOI: 10.1016/j.clinbiochem.2004.05.012.
111. Henze, G., et al., Serum S100--a marker for disease monitoring in metastatic melanoma. *Dermatology* **1997,** *194* (3), 208-12. DOI: 10.1159/000246103.
112. Agarwala, S. S., et al., LDH correlation with survival in advanced melanoma from two large, randomised trials (Oblimersen GM301 and EORTC 18951). *Eur J Cancer* **2009,** *45* (10), 1807-14. DOI: 10.1016/j.ejca.2009.04.016.
113. Gogas, H., et al., Biomarkers in melanoma. *Ann Oncol* **2009,** *20 Suppl 6*, vi8-13. DOI: 10.1093/annonc/mdp251.
114. Balch, C. M., et al., Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma. *J Clin Oncol* **2001,** *19* (16), 3635-48. DOI: 10.1200/JCO.2001.19.16.3635.
115. Diem, S., et al., Serum lactate dehydrogenase as an early marker for outcome in patients treated with anti-PD-1 therapy in metastatic melanoma. *Br J Cancer* **2016,** *114* (3), 256-61. DOI: 10.1038/bjc.2015.467.
116. Wagner, N. B., et al., S100B and LDH as early prognostic markers for response and overall survival in melanoma patients treated with anti-PD-1 or combined anti-PD-1 plus anti-CTLA-4 antibodies. *British Journal of Cancer* **2018,** *119* (3), 339-346. DOI: 10.1038/s41416-018-0167-x.
117. Griewank, K. G., et al., Genetic alterations and personalized medicine in melanoma: progress and future prospects. *J Natl Cancer Inst* **2014,** *106* (2), djt435. DOI: 10.1093/jnci/djt435.
118. Klein, O., et al., BRAF inhibitor activity in V600R metastatic melanoma. *Eur J Cancer* **2013,** *49* (5), 1073-9. DOI: 10.1016/j.ejca.2012.11.004.
119. Griewank, K. G.; Schilling, B., Next-Generation Sequencing to Guide Treatment of Advanced Melanoma. *American Journal of Clinical Dermatology* **2017,** *18* (3), 303-310. DOI: 10.1007/s40257-017-0260-6.
120. Domingues, B., et al., Melanoma treatment in review. *Immunotargets Ther* **2018,** *7*, 35-49. DOI: 10.2147/ITT.S134842.
121. Lui, P., et al., Treatments for metastatic melanoma: synthesis of evidence from randomized trials. *Cancer Treat Rev* **2007,** *33* (8), 665-80. DOI: 10.1016/j.ctrv.2007.06.004.
122. Su, P. J., et al., Biochemotherapy with carmustine, cisplatin, dacarbazine, tamoxifen and low-dose interleukin-2 for patients with metastatic malignant melanoma. *Chang Gung Med J* **2011,** *34* (5), 478-86.
123. Wilson, M. A.; Schuchter, L. M., Chemotherapy for Melanoma. *Cancer Treat Res* **2016,** *167*, 209-29. DOI: 10.1007/978-3-319-22539-5_8.
124. Xing, Y., et al., Contemporary diagnostic imaging modalities for the staging and surveillance of melanoma patients: a meta-analysis. *J Natl Cancer Inst* **2011,** *103* (2), 129-42. DOI: 10.1093/jnci/djq455.

125. Dinnes, J., et al., Ultrasound, CT, MRI, or PET-CT for staging and re-staging of adults with cutaneous melanoma. *Cochrane Database Syst Rev* **2019,** *7*, CD012806. DOI: 10.1002/14651858.CD012806.pub2.

126. Carvajal, R. D., et al., KIT as a therapeutic target in metastatic melanoma. *JAMA* **2011,** *305* (22), 2327-34. DOI: 10.1001/jama.2011.746.

127. Buchbinder, E. I.; Desai, A., CTLA-4 and PD-1 Pathways: Similarities, Differences, and Implications of Their Inhibition. *Am J Clin Oncol* **2016,** *39* (1), 98-106. DOI: 10.1097/COC.0000000000000239.

128. Chen, L.; Flies, D. B., Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol* **2013,** *13* (4), 227-42. DOI: 10.1038/nri3405.

129. Squibb, B.-M., Yervoy (ipilimumab)[package insert]. *Princeton, NJ, Bristol-Myers Squibb* **2018**.

130. Ramos-Casals, M., et al., Immune-related adverse events of checkpoint inhibitors. *Nature Reviews Disease Primers* **2020,** *6* (1), 38. DOI: 10.1038/s41572-020-0160-6.

131. Hwang, J. R., et al., Recent insights of T cell receptor-mediated signaling pathways for T cell activation and development. *Exp Mol Med* **2020,** *52* (5), 750-761. DOI: 10.1038/s12276-020-0435-8.

132. Hersh, E. M., et al., A phase II multicenter study of ipilimumab with or without dacarbazine in chemotherapy-naive patients with advanced melanoma. *Invest New Drugs* **2011,** *29* (3), 489-98. DOI: 10.1007/s10637-009-9376-8.

133. Hodi, F. S., et al., Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* **2010,** *363* (8), 711-23. DOI: 10.1056/NEJMoa1003466.

134. Robert, C., et al., Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* **2011,** *364* (26), 2517-26. DOI: 10.1056/NEJMoa1104621.

135. O'Day, S., et al., A phase III, randomized, double-blind, multicenter study comparing monotherapy with ipilimumab or gp100 peptide vaccine and the combination in patients with previously treated, unresectable stage III or IV melanoma. *Journal of Clinical Oncology* **2010,** *28* (18_suppl), 4-4. DOI: 10.1200/jco.2010.28.18_suppl.4.

136. Schadendorf, D., et al., Pooled Analysis of Long-Term Survival Data From Phase II and Phase III Trials of Ipilimumab in Unresectable or Metastatic Melanoma. *J Clin Oncol* **2015,** *33* (17), 1889-94. DOI: 10.1200/JCO.2014.56.2736.

137. Callahan, M. K., et al., Immunomodulatory therapy for melanoma: ipilimumab and beyond. *Clin Dermatol* **2013,** *31* (2), 191-9. DOI: 10.1016/j.clindermatol.2012.08.006.

138. Robert, C., et al., Nivolumab in previously untreated melanoma without BRAF mutation. *N Engl J Med* **2015,** *372* (4), 320-30. DOI: 10.1056/NEJMoa1412082.

139. Larkin, J., et al., Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. *N Engl J Med* **2015,** *373* (1), 23-34. DOI: 10.1056/NEJMoa1504030.

140. Juneja, V. R., et al., PD-L1 on tumor cells is sufficient for immune evasion in immunogenic tumors and inhibits CD8 T cell cytotoxicity. *J Exp Med* **2017,** *214* (4), 895-904. DOI: 10.1084/jem.20160801.

141. Gadiot, J., et al., Overall survival and PD-L1 expression in metastasized malignant melanoma. *Cancer* **2011,** *117* (10), 2192-201. DOI: 10.1002/cncr.25747.

142. Sunshine, J. C., et al., PD-L1 Expression in Melanoma: A Quantitative Immunohistochemical Antibody Comparison. *Clin Cancer Res* **2017,** *23* (16), 4938-4944. DOI: 10.1158/1078-0432.CCR-16-1821.

143. Yang, J., et al., A pooled analysis of the prognostic value of PD-L1 in melanoma: evidence from 1062 patients. *Cancer Cell Int* **2020,** *20*, 96. DOI: 10.1186/s12935-020-01187-x.

144. Yun, S., et al., Clinicopathological and prognostic significance of programmed death ligand 1 expression in Korean melanoma patients. *J Cancer* **2019,** *10* (13), 3070-3078. DOI: 10.7150/jca.30573.

145. Zhao, T., et al., Prognostic value of PD-L1 expression in tumor infiltrating immune cells in cancers: A meta-analysis. *PLoS One* **2017,** *12* (4), e0176822. DOI: 10.1371/journal.pone.0176822.

146. Schadendorf, D., et al., Melanoma. *Lancet* **2018,** *392* (10151), 971-984. DOI: 10.1016/S0140-6736(18)31559-9.

147. Larkin, J., et al., Five-Year Survival with Combined Nivolumab and Ipilimumab in Advanced Melanoma. *N Engl J Med* **2019,** *381* (16), 1535-1546. DOI: 10.1056/NEJMoa1910836.

148. Robert, C., et al., Improved overall survival in melanoma with combined dabrafenib and trametinib. *N Engl J Med* **2015,** *372* (1), 30-9. DOI: 10.1056/NEJMoa1412690.

149. Sumimoto, H., et al., The BRAF-MAPK signaling pathway is essential for cancer-immune evasion in human melanoma cells. *J Exp Med* **2006,** *203* (7), 1651-6. DOI: 10.1084/jem.20051848.

150. Proietti, I., et al., BRAF Inhibitors: Molecular Targeting and Immunomodulatory Actions. *Cancers (Basel)* **2020,** *12* (7). DOI: 10.3390/cancers12071823.

151. Kim, G., et al., FDA approval summary: vemurafenib for treatment of unresectable or metastatic melanoma with the BRAFV600E mutation. *Clin Cancer Res* **2014,** *20* (19), 4994-5000. DOI: 10.1158/1078-0432.CCR-14-0776.

152. Chapman, P. B., et al., Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* **2011,** *364* (26), 2507-16. DOI: 10.1056/NEJMoa1103782.

153. Bollag, G., et al., Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. *Nature* **2010,** *467* (7315), 596-9. DOI: 10.1038/nature09454.

154. Joseph, E. W., et al., The RAF inhibitor PLX4032 inhibits ERK signaling and tumor cell proliferation in a V600E BRAF-selective manner. *Proc Natl Acad Sci U S A* **2010,** *107* (33), 14903-8. DOI: 10.1073/pnas.1008990107.

155. Tsai, J., et al., Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity. *Proc Natl Acad Sci U S A* **2008,** *105* (8), 3041-6. DOI: 10.1073/pnas.0711741105.

156. Hauschild, A., et al., Long-term outcomes in patients with BRAF V600-mutant metastatic melanoma receiving dabrafenib monotherapy: Analysis from phase 2 and 3 clinical trials. *Eur J Cancer* **2020,** *125*, 114-120. DOI: 10.1016/j.ejca.2019.10.033.

157. Hauschild, A., et al., Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-label, phase 3 randomised controlled trial. *Lancet* **2012,** *380* (9839), 358-65. DOI: 10.1016/S0140-6736(12)60868-X.

158. Hauschild, A., et al., An update on BREAK-3, a phase III, randomized trial: Dabrafenib (DAB) versus dacarbazine (DTIC) in patients with BRAF V600E-positive mutation metastatic melanoma (MM). *Journal of Clinical Oncology* **2013,** *31* (15_suppl), 9013-9013. DOI: 10.1200/jco.2013.31.15_suppl.9013.

159. Larkin, J., et al., Combined vemurafenib and cobimetinib in BRAF-mutated melanoma. *N Engl J Med* **2014,** *371* (20), 1867-76. DOI: 10.1056/NEJMoa1408868.

160. Long, G. V., et al., Dabrafenib and trametinib versus dabrafenib and placebo for Val600 BRAF-mutant melanoma: a multicentre, double-blind, phase 3 randomised controlled trial. *Lancet* **2015,** *386* (9992), 444-51. DOI: 10.1016/S0140-6736(15)60898-4.

161. Delord, J. P., et al., Phase I Dose-Escalation and -Expansion Study of the BRAF Inhibitor Encorafenib (LGX818) in Metastatic BRAF-Mutant Melanoma. *Clin Cancer Res* **2017,** *23* (18), 5339-5348. DOI: 10.1158/1078-0432.CCR-16-2923.

162. Dummer, R., et al., Overall survival in patients with BRAF-mutant melanoma receiving encorafenib plus binimetinib versus vemurafenib or encorafenib (COLUMBUS): a multicentre, open-label, randomised, phase 3 trial. *Lancet Oncol* **2018,** *19* (10), 1315-1327. DOI: 10.1016/S1470-2045(18)30497-2.

163. Dummer, R., et al., Encorafenib plus binimetinib versus vemurafenib or encorafenib in patients with BRAF-mutant melanoma (COLUMBUS): a multicentre, open-label, randomised phase 3 trial. *Lancet Oncol* **2018,** *19* (5), 603-615. DOI: 10.1016/S1470-2045(18)30142-6.

164. Schilling, B., et al., Differential influence of vemurafenib and dabrafenib on patients' lymphocytes despite similar clinical efficacy in melanoma. *Ann Oncol* **2014,** *25* (3), 747-53. DOI: 10.1093/annonc/mdt587.

165. Frederick, D. T., et al., BRAF inhibition is associated with enhanced melanoma antigen expression and a more favorable tumor microenvironment in patients with metastatic melanoma. *Clin Cancer Res* **2013,** *19* (5), 1225-31. DOI: 10.1158/1078-0432.CCR-12-1630.

166. Boni, A., et al., Selective BRAFV600E inhibition enhances T-cell recognition of melanoma without affecting lymphocyte function. *Cancer Res* **2010,** *70* (13), 5213-9. DOI: 10.1158/0008-5472.CAN-10-0118.

167. Comin-Anduix, B., et al., The oncogenic BRAF kinase inhibitor PLX4032/RG7204 does not affect the viability or function of human lymphocytes across a wide range of concentrations. *Clin Cancer Res* **2010,** *16* (24), 6040-8. DOI: 10.1158/1078-0432.CCR-10-1911.

168. Gibney, G. T., et al., Paradoxical oncogenesis--the long-term effects of BRAF inhibition in melanoma. *Nat Rev Clin Oncol* **2013,** *10* (7), 390-9. DOI: 10.1038/nrclinonc.2013.83.

169. Lo, R. S., Combinatorial therapies to overcome B-RAF inhibitor resistance in melanomas. *Pharmacogenomics* **2012,** *13* (2), 125-8. DOI: 10.2217/pgs.11.166.

170. Hamid, O., et al., Efficacy, Safety, and Tolerability of Approved Combination BRAF and MEK Inhibitor Regimens for BRAF-Mutant Melanoma. *Cancers (Basel)* **2019,** *11* (11). DOI: 10.3390/cancers11111642.

171. Ugurel, S., et al., Survival of patients with advanced metastatic melanoma: The impact of MAP kinase pathway inhibition and immune checkpoint inhibition - Update 2019. *Eur J Cancer* **2020,** *130*, 126-138. DOI: 10.1016/j.ejca.2020.02.021.

172. World Health Organization, T. G. C. O. Melanoma of skin. https://gco.iarc.fr/today/data/factsheets/cancers/16-Melanoma-of-skin-fact-sheet.pdf (accessed July 6, 2022).

173. Erdmann, F., et al., International trends in the incidence of malignant melanoma 1953-2008--are recent generations at higher or lower risk? *Int J Cancer* **2013,** *132* (2), 385-400. DOI: 10.1002/ijc.27616.

174. Sacchetto, L., et al., Trends in incidence of thick, thin and in situ melanoma in Europe. *Eur J Cancer* **2018,** *92*, 108-118. DOI: 10.1016/j.ejca.2017.12.024.

175. Breitbart, E. W., et al., Systematic skin cancer screening in Northern Germany. *J Am Acad Dermatol* **2012,** *66* (2), 201-11. DOI: 10.1016/j.jaad.2010.11.016.

176. Kruger, S., et al., Epidemiologic evidence for the role of melanocytic nevi as risk markers and direct precursors of cutaneous malignant melanoma. Results of a case control study in melanoma patients and nonmelanoma control subjects. *J Am Acad Dermatol* **1992,** *26* (6), 920-6. DOI: 10.1016/0190-9622(92)70133-z.

177. Goss, P. E., et al., Challenges to effective cancer control in China, India, and Russia. *Lancet Oncol* **2014,** *15* (5), 489-538. DOI: 10.1016/S1470-2045(14)70029-4.

178. Howlader, N., Noone, A.M., Krapcho, M., Neyman, N., Aminou, R., Waldron, W., Altekruse, S.F., Kosary, C.L., Ruhl, J., Tatalovich, Z., Cho, H., Mariotto, A., Eisner, M.P., Lewis, D.R., Chen, H.S., Feuer, E.J., Cronin, K.A. and Edwards, B.K., SEER Cancer Statistics Review, 1975-2008. **2011.**

179. Society, A. C. Key Statistics for Melanoma Skin Cancer. https://www.cancer.org/cancer/melanoma-skin-cancer/about/key-statistics.html (accessed July 7, 2022).

180. Lozano, R., et al., Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* **2012,** *380* (9859), 2095-128. DOI: 10.1016/S0140-6736(12)61728-0.

181. Lin, J. Y.; Fisher, D. E., Melanocyte biology and skin pigmentation. *Nature* **2007,** *445* (7130), 843-50. DOI: 10.1038/nature05660.

182. Roider, E. M.; Fisher, D. E., Red Hair, Light Skin, and UV-Independent Risk for Melanoma Development in Humans. *JAMA Dermatol* **2016,** *152* (7), 751-3. DOI: 10.1001/jamadermatol.2016.0524.

183. Tanaka, H., et al., The Pro-Oxidant Activity of Pheomelanin is Significantly Enhanced by UVA Irradiation: Benzothiazole Moieties Are More Reactive than Benzothiazine Moieties. *Int J Mol Sci* **2018,** *19* (10). DOI: 10.3390/ijms19102889.
184. Valverde, P., et al., Variants of the melanocyte-stimulating hormone receptor gene are associated with red hair and fair skin in humans. *Nat Genet* **1995,** *11* (3), 328-30. DOI: 10.1038/ng1195-328.
185. Hamid, O., et al., Five-year survival outcomes for patients with advanced melanoma treated with pembrolizumab in KEYNOTE-001. *Ann Oncol* **2019,** *30* (4), 582-588. DOI: 10.1093/annonc/mdz011.
186. Robert, C., et al., Five-Year Outcomes with Dabrafenib plus Trametinib in Metastatic Melanoma. *N Engl J Med* **2019,** *381* (7), 626-636. DOI: 10.1056/NEJMoa1904059.
187. Miller, K. D., et al., Cancer treatment and survivorship statistics, 2019. *CA Cancer J Clin* **2019,** *69* (5), 363-385. DOI: 10.3322/caac.21565.
188. McCain, J., The MAPK (ERK) Pathway: Investigational Combinations for the Treatment Of BRAF-Mutated Metastatic Melanoma. *P T* **2013,** *38* (2), 96-108.
189. Farolfi, A., et al., Ipilimumab in advanced melanoma: reports of long-lasting responses. *Melanoma Res* **2012,** *22* (3), 263-70. DOI: 10.1097/CMR.0b013e328353e65c.
190. Ottensmeier, C., et al. In *Ipilimumab produces durable objective responses in patients with previously treated, advanced melanoma: results from a phase III trial*, Annals of Oncology, OXFORD UNIV PRESS GREAT CLARENDON ST, OXFORD OX2 6DP, ENGLAND: 2010; pp 401-402.
191. Flaherty, K. T., et al., Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med* **2010,** *363* (9), 809-19. DOI: 10.1056/NEJMoa1002011.
192. Puzanov, I., et al., Biological challenges of BRAF inhibitor therapy. *Mol Oncol* **2011,** *5* (2), 116-23. DOI: 10.1016/j.molonc.2011.01.005.
193. Eggermont, A. M., et al., Prolonged Survival in Stage III Melanoma with Ipilimumab Adjuvant Therapy. *N Engl J Med* **2016,** *375* (19), 1845-1855. DOI: 10.1056/NEJMoa1611299.
194. Eggermont, A. M., et al., Adjuvant ipilimumab versus placebo after complete resection of high-risk stage III melanoma (EORTC 18071): a randomised, double-blind, phase 3 trial. *Lancet Oncol* **2015,** *16* (5), 522-30. DOI: 10.1016/S1470-2045(15)70122-1.
195. Ribas, A., et al., Association of Pembrolizumab With Tumor Response and Survival Among Patients With Advanced Melanoma. *JAMA* **2016,** *315* (15), 1600-9. DOI: 10.1001/jama.2016.4059.
196. Syn, N. L., et al., De-novo and acquired resistance to immune checkpoint targeting. *Lancet Oncol* **2017,** *18* (12), e731-e741. DOI: 10.1016/S1470-2045(17)30607-1.
197. Sharma, P., et al., Primary, Adaptive, and Acquired Resistance to Cancer Immunotherapy. *Cell* **2017,** *168* (4), 707-723. DOI: 10.1016/j.cell.2017.01.017.
198. Dunn, G. P., et al., IFN unresponsiveness in LNCaP cells due to the lack of JAK1 gene expression. *Cancer Res* **2005,** *65* (8), 3447-53. DOI: 10.1158/0008-5472.CAN-04-4316.
199. Sucker, A., et al., Acquired IFNγ resistance impairs anti-tumor immunity and gives rise to T-cell-resistant melanoma lesions. *Nature Communications* **2017,** *8* (1), 15440. DOI: 10.1038/ncomms15440.
200. Zaretsky, J. M., et al., Mutations Associated with Acquired Resistance to PD-1 Blockade in Melanoma. *N Engl J Med* **2016,** *375* (9), 819-29. DOI: 10.1056/NEJMoa1604958.
201. Marincola, F. M., et al., Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Adv Immunol* **2000,** *74*, 181-273. DOI: 10.1016/s0065-2776(08)60911-6.
202. Ritz, U.; Seliger, B., The transporter associated with antigen processing (TAP): structural integrity, expression, function, and its clinical relevance. *Mol Med* **2001,** *7* (3), 149-58.
203. Sucker, A., et al., Genetic evolution of T-cell resistance in the course of melanoma progression. *Clin Cancer Res* **2014,** *20* (24), 6593-604. DOI: 10.1158/1078-0432.CCR-14-0567.
204. Garrido, F., et al., The urgent need to recover MHC class I in cancers for effective immunotherapy. *Curr Opin Immunol* **2016,** *39*, 44-51. DOI: 10.1016/j.coi.2015.12.007.
205. Lee, J. H., et al., Transcriptional downregulation of MHC class I and melanoma de- differentiation in resistance to PD-1 inhibition. *Nat Commun* **2020,** *11* (1), 1897. DOI: 10.1038/s41467-020-15726-7.
206. Anagnostou, V., et al., Evolution of Neoantigen Landscape during Immune Checkpoint Blockade in Non-Small Cell Lung Cancer. *Cancer Discov* **2017,** *7* (3), 264-276. DOI: 10.1158/2159-8290.CD-16-0828.
207. Lauss, M., et al., Mutational and putative neoantigen load predict clinical benefit of adoptive T cell therapy in melanoma. *Nature Communications* **2017,** *8* (1), 1738. DOI: 10.1038/s41467-017-01460-0.
208. Becker, J. C., et al., Immune-suppressive properties of the tumor microenvironment. *Cancer Immunol Immunother* **2013,** *62* (7), 1137-48. DOI: 10.1007/s00262-013-1434-6.
209. Umansky, V.; Sevko, A., Melanoma-induced immunosuppression and its neutralization. *Semin Cancer Biol* **2012,** *22* (4), 319-26. DOI: 10.1016/j.semcancer.2012.02.003.
210. Whiteside, T. L., What are regulatory T cells (Treg) regulating in cancer and why? *Semin Cancer Biol* **2012,** *22* (4), 327-34. DOI: 10.1016/j.semcancer.2012.03.004.
211. Sakaguchi, S., et al., Regulatory T Cells and Immune Tolerance. *Cell* **2008,** *133* (5), 775-787. DOI: https://doi.org/10.1016/j.cell.2008.05.009.
212. Sundstedt, A., et al., Role for IL-10 in suppression mediated by peptide-induced regulatory T cells in vivo. *J Immunol* **2003,** *170* (3), 1240-8. DOI: 10.4049/jimmunol.170.3.1240.
213. Meyer, C., et al., Frequencies of circulating MDSC correlate with clinical outcome of melanoma patients treated with ipilimumab. *Cancer Immunol Immunother* **2014,** *63* (3), 247-57. DOI: 10.1007/s00262-013-1508-5.

214. Schilling, B.; Paschen, A., Immunological consequences of selective BRAF inhibitors in malignant melanoma: Neutralization of myeloid-derived suppressor cells. *Oncoimmunology* **2013,** *2* (8), e25218. DOI: 10.4161/onci.25218.

215. Highfill, S. L., et al., Disruption of CXCR2-mediated MDSC tumor trafficking enhances anti-PD1 efficacy. *Sci Transl Med* **2014,** *6* (237), 237ra67. DOI: 10.1126/scitranslmed.3007974.

216. Van Allen, E. M., et al., The genetic landscape of clinical resistance to RAF inhibition in metastatic melanoma. *Cancer Discov* **2014,** *4* (1), 94-109. DOI: 10.1158/2159-8290.CD-13-0617.

217. Johnson, D. B., et al., Acquired BRAF inhibitor resistance: A multicenter meta-analysis of the spectrum and frequencies, clinical behaviour, and phenotypic associations of resistance mechanisms. *Eur J Cancer* **2015,** *51* (18), 2792-9. DOI: 10.1016/j.ejca.2015.08.022.

218. Nazarian, R., et al., Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. *Nature* **2010,** *468* (7326), 973-7. DOI: 10.1038/nature09626.

219. Poulikakos, P. I., et al., RAF inhibitor resistance is mediated by dimerization of aberrantly spliced BRAF(V600E). *Nature* **2011,** *480* (7377), 387-90. DOI: 10.1038/nature10662.

220. Shi, H., et al., Melanoma whole-exome sequencing identifies (V600E)B-RAF amplification-mediated acquired B-RAF inhibitor resistance. *Nat Commun* **2012,** *3*, 724. DOI: 10.1038/ncomms1727.

221. Wagle, N., et al., Dissecting therapeutic resistance to RAF inhibition in melanoma by tumor genomic profiling. *J Clin Oncol* **2011,** *29* (22), 3085-96. DOI: 10.1200/JCO.2010.33.2312.

222. Lau, E.; Ronai, Z., RAF-isotype switching: from B to C through PDE. *Nat Struct Mol Biol* **2011,** *18* (5), 517-8. DOI: 10.1038/nsmb.2063.

223. Alcala, A. M.; Flaherty, K. T., BRAF inhibitors for the treatment of metastatic melanoma: clinical trials and mechanisms of resistance. *Clin Cancer Res* **2012,** *18* (1), 33-9. DOI: 10.1158/1078-0432.CCR-11-0997.

224. Busca, R., et al., Ras mediates the cAMP-dependent activation of extracellular signal-regulated kinases (ERKs) in melanocytes. *EMBO J* **2000,** *19* (12), 2900-10. DOI: 10.1093/emboj/19.12.2900.

225. Dumaz, N., et al., In melanoma, RAS mutations are accompanied by switching signaling from BRAF to CRAF and disrupted cyclic AMP signaling. *Cancer Res* **2006,** *66* (19), 9483-91. DOI: 10.1158/0008-5472.CAN-05-4227.

226. Paraiso, K. H., et al., Recovery of phospho-ERK activity allows melanoma cells to escape from BRAF inhibitor therapy. *Br J Cancer* **2010,** *102* (12), 1724-30. DOI: 10.1038/sj.bjc.6605714.

227. Long, G. V., et al., Increased MAPK reactivation in early resistance to dabrafenib/trametinib combination therapy of BRAF-mutant metastatic melanoma. *Nat Commun* **2014,** *5*, 5694. DOI: 10.1038/ncomms6694.

228. LoRusso, P. M., et al., Pilot Trial of Selecting Molecularly Guided Therapy for Patients with Non-V600 BRAF-Mutant Metastatic Melanoma: Experience of the SU2C/MRA Melanoma Dream Team. *Mol Cancer Ther* **2015,** *14* (8), 1962-71. DOI: 10.1158/1535-7163.MCT-15-0153.

229. Paraiso, K. H., et al., PTEN loss confers BRAF inhibitor resistance to melanoma cells through the suppression of BIM expression. *Cancer Res* **2011,** *71* (7), 2750-60. DOI: 10.1158/0008-5472.CAN-10-2954.

230. Xing, F., et al., Concurrent loss of the PTEN and RB1 tumor suppressors attenuates RAF dependence in melanomas harboring (V600E)BRAF. *Oncogene* **2012,** *31* (4), 446-57. DOI: 10.1038/onc.2011.250.

231. Whittaker, S. R., et al., A genome-scale RNA interference screen implicates NF1 loss in resistance to RAF inhibition. *Cancer Discov* **2013,** *3* (3), 350-62. DOI: 10.1158/2159-8290.CD-12-0470.

232. Wheler, J., et al., Next generation sequencing of exceptional responders with BRAF-mutant melanoma: implications for sensitivity and resistance. *BMC Cancer* **2015,** *15*, 61. DOI: 10.1186/s12885-015-1029-z.

233. Czarnecka, A. M., et al., Targeted Therapy in Melanoma and Mechanisms of Resistance. *Int J Mol Sci* **2020,** *21* (13). DOI: 10.3390/ijms21134576.

234. Olbryt, M., et al., Genetic Profiling of Advanced Melanoma: Candidate Mutations for Predicting Sensitivity and Resistance to Targeted Therapy. *Targeted Oncology* **2020,** *15* (1), 101-113. DOI: 10.1007/s11523-020-00695-0.

235. Kozar, I., et al., Many ways to resistance: How melanoma cells evade targeted therapies. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* **2019,** *1871* (2), 313-322. DOI: https://doi.org/10.1016/j.bbcan.2019.02.002.

236. Yuan, J., et al., Novel technologies and emerging biomarkers for personalized cancer immunotherapy. *J Immunother Cancer* **2016,** *4*, 3. DOI: 10.1186/s40425-016-0107-3.

237. Pantel, K.; Speicher, M. R., The biology of circulating tumor cells. *Oncogene* **2016,** *35* (10), 1216-24. DOI: 10.1038/onc.2015.192.

238. Perez-Callejo, D., et al., Liquid biopsy based biomarkers in non-small cell lung cancer for diagnosis and treatment monitoring. *Transl Lung Cancer Res* **2016,** *5* (5), 455-465. DOI: 10.21037/tlcr.2016.10.07.

239. Pantel, K.; Alix-Panabieres, C., Real-time liquid biopsy in cancer patients: fact or fiction? *Cancer Res* **2013,** *73* (21), 6384-8. DOI: 10.1158/0008-5472.CAN-13-2030.

240. Huang, A. C., et al., T-cell invigoration to tumour burden ratio associated with anti-PD-1 response. *Nature* **2017,** *545* (7652), 60-65. DOI: 10.1038/nature22079.

241. Martens, A., et al., Baseline Peripheral Blood Biomarkers Associated with Clinical Outcome of Advanced Melanoma Patients Treated with Ipilimumab. *Clin Cancer Res* **2016,** *22* (12), 2908-18. DOI: 10.1158/1078-0432.CCR-15-2412.

242. Weide, B., et al., Baseline Biomarkers for Outcome of Melanoma Patients Treated with Pembrolizumab. *Clin Cancer Res* **2016,** *22* (22), 5487-5496. DOI: 10.1158/1078-0432.CCR-16-0127.

243. Mukherji, B., Immunology of melanoma. *Clin Dermatol* **2013,** *31* (2), 156-65. DOI: 10.1016/j.clindermatol.2012.08.017.

244.   Gatault, S., et al., Involvement of eosinophils in the anti-tumor response. *Cancer Immunol Immunother* **2012,** *61* (9), 1527-34. DOI: 10.1007/s00262-012-1288-3.

245.   Gerloni, M.; Zanetti, M., CD4 T cells in tumor immunity. *Springer Semin Immunopathol* **2005,** *27* (1), 37-48. DOI: 10.1007/s00281-004-0193-z.

246.   St Paul, M.; Ohashi, P. S., The Roles of CD8(+) T Cell Subsets in Antitumor Immunity. *Trends Cell Biol* **2020,** *30* (9), 695-704. DOI: 10.1016/j.tcb.2020.06.003.

247.   Varricchi, G., et al., Eosinophils: The unsung heroes in cancer? *Oncoimmunology* **2018,** *7* (2), e1393134. DOI: 10.1080/2162402X.2017.1393134.

248.   Davis, B. P.; Rothenberg, M. E., Eosinophils and cancer. *Cancer immunology research* **2014,** *2* (1), 1-8. DOI: 10.1158/2326-6066.CIR-13-0196.

249.   Reichman, H., et al., Emerging Roles for Eosinophils in the Tumor Microenvironment. *Trends Cancer* **2016,** *2* (11), 664-675. DOI: 10.1016/j.trecan.2016.10.002.

250.   Rosenberg, H. F., et al., Eosinophils: changing perspectives in health and disease. *Nat Rev Immunol* **2013,** *13* (1), 9-22. DOI: 10.1038/nri3341.

251.   Uhm, T. G., et al., Eosinophil development, regulation of eosinophil-specific genes, and role of eosinophils in the pathogenesis of asthma. *Allergy Asthma Immunol Res* **2012,** *4* (2), 68-79. DOI: 10.4168/aair.2012.4.2.68.

252.   Wolf, A. A., et al., The Ontogeny of Monocyte Subsets. *Front Immunol* **2019,** *10*, 1642. DOI: 10.3389/fimmu.2019.01642.

253.   Yanez, A., et al., Granulocyte-Monocyte Progenitors and Monocyte-Dendritic Cell Progenitors Independently Produce Functionally Distinct Monocytes. *Immunity* **2017,** *47* (5), 890-902 e4. DOI: 10.1016/j.immuni.2017.10.021.

254.   Fiedler, K.; Brunner, C., The role of transcription factors in the guidance of granulopoiesis. *Am J Blood Res* **2012,** *2* (1), 57-65.

255.   Willebrand, R.; Voehringer, D., Regulation of eosinophil development and survival. *Curr Opin Hematol* **2017,** *24* (1), 9-15. DOI: 10.1097/MOH.0000000000000293.

256.   Fulkerson, P. C., Transcription Factors in Eosinophil Development and As Therapeutic Targets. *Front Med (Lausanne)* **2017,** *4*, 115. DOI: 10.3389/fmed.2017.00115.

257.   Mayadas, T. N., et al., The multifaceted functions of neutrophils. *Annu Rev Pathol* **2014,** *9*, 181-218. DOI: 10.1146/annurev-pathol-020712-164023.

258.   Shah, K., et al., The emerging roles of eosinophils in mucosal homeostasis. *Mucosal Immunol* **2020,** *13* (4), 574-583. DOI: 10.1038/s41385-020-0281-y.

259.   Kita, H., Eosinophils: multifaceted biological properties and roles in health and disease. *Immunol Rev* **2011,** *242* (1), 161-77. DOI: 10.1111/j.1600-065X.2011.01026.x.

260.   Rothenberg, M. E., et al., Eosinophils cocultured with endothelial cells have increased survival and functional properties. *Science* **1987,** *237* (4815), 645-7. DOI: 10.1126/science.3110954.

261.   Davoine, F.; Lacy, P., Eosinophil cytokines, chemokines, and growth factors: emerging roles in immunity. *Front Immunol* **2014,** *5*, 570. DOI: 10.3389/fimmu.2014.00570.

262.   Chen, F., et al., Characteristics and Role of Neutrophil Extracellular Traps in Asthma. *Inflammation* **2022,** *45* (1), 6-13. DOI: 10.1007/s10753-021-01526-8.

263.   Kim, K., et al., Terminally Differentiating Eosinophils Express Neutrophil Primary Granule Proteins as well as Eosinophil-specific Granule Proteins in a Temporal Manner. *Immune Netw* **2017,** *17* (6), 410-423. DOI: 10.4110/in.2017.17.6.410.

264.   Dricu, A., et al., A synthetic peptide derived from the human eosinophil-derived neurotoxin induces apoptosis in Kaposi's sarcoma cells. *Anticancer research* **2004,** *24* (3a), 1427-32.

265.   Hamann, K. J., et al., The molecular biology of eosinophil granule proteins. *International archives of allergy and applied immunology* **1991,** *94* (1-4), 202-9. DOI: 10.1159/000235362.

266.   Pereira, M. C., et al., The role of eosinophils and eosinophil cationic protein in oral cancer: a review. *Arch Oral Biol* **2011,** *56* (4), 353-8. DOI: 10.1016/j.archoralbio.2010.10.015.

267.   Grisaru-Tal, S., et al., A new dawn for eosinophils in the tumour microenvironment. *Nature Reviews Cancer* **2020,** *20* (10), 594-607. DOI: 10.1038/s41568-020-0283-9.

268.   Jacobsen, E. A., et al., Allergic pulmonary inflammation in mice is dependent on eosinophil-induced recruitment of effector T cells. *J Exp Med* **2008,** *205* (3), 699-710. DOI: 10.1084/jem.20071840.

269.   Lotfi, R., et al., Eosinophils oxidize damage-associated molecular pattern molecules derived from stressed cells. *J Immunol* **2009,** *183* (8), 5023-31. DOI: 10.4049/jimmunol.0900504.

270.   Andreone, S., et al., IL-33 Promotes CD11b/CD18-Mediated Adhesion of Eosinophils to Cancer Cells and Synapse-Polarized Degranulation Leading to Tumor Cell Killing. *Cancers (Basel)* **2019,** *11* (11). DOI: 10.3390/cancers11111664.

271.   Barthel, S. R., et al., Roles of integrin activation in eosinophil function and the eosinophilic inflammation of asthma. *Journal of leukocyte biology* **2008,** *83* (1), 1-12. DOI: 10.1189/jlb.0607344.

272.   Gatault, S., et al., IL-18 Is Involved in Eosinophil-Mediated Tumoricidal Activity against a Colon Carcinoma Cell Line by Upregulating LFA-1 and ICAM-1. *J Immunol* **2015,** *195* (5), 2483-92. DOI: 10.4049/jimmunol.1402914.

273.   Reichman, H., et al., Activated Eosinophils Exert Antitumorigenic Activities in Colorectal Cancer. *Cancer immunology research* **2019,** *7* (3), 388-400. DOI: 10.1158/2326-6066.CIR-18-0494.

274.   Wendlinger, S., et al., Blood Eosinophils Are Associated with Efficacy of Targeted Therapy in Patients with Advanced Melanoma. *Cancers (Basel)* **2022,** *14* (9). DOI: 10.3390/cancers14092294.

275. Carretero, R., et al., Eosinophils orchestrate cancer rejection by normalizing tumor vessels and enhancing infiltration of CD8(+) T cells. *Nat Immunol* **2015,** *16* (6), 609-17. DOI: 10.1038/ni.3159.
276. Huang, L.; Appleton, J. A., Eosinophils in Helminth Infection: Defenders and Dupes. *Trends Parasitol* **2016,** *32* (10), 798-807. DOI: 10.1016/j.pt.2016.05.004.
277. Lavinskiene, S., et al., Functional activity of peripheral blood eosinophils in allergen-induced late-phase airway inflammation in asthma patients. *Journal of inflammation* **2015,** *12*, 25. DOI: 10.1186/s12950-015-0065-4.
278. Maizels, R. M.; Allen, J. E., Immunology. Eosinophils forestall obesity. *Science* **2011,** *332* (6026), 186-7. DOI: 10.1126/science.1205313.
279. Ikutani, M., et al., Identification of innate IL-5-producing cells and their role in lung eosinophil regulation and antitumor immunity. *J Immunol* **2012,** *188* (2), 703-13. DOI: 10.4049/jimmunol.1101270.
280. Rosenberg, H. F.; Domachowske, J. B., Eosinophils, eosinophil ribonucleases, and their role in host defense against respiratory virus pathogens. *Journal of leukocyte biology* **2001,** *70* (5), 691-8.
281. Caruso, R. A., et al., Degranulation patterns of eosinophils in advanced gastric carcinoma: an electron microscopic study. *Ultrastructural pathology* **2005,** *29* (1), 29-36. DOI: 10.1080/019131290882303.
282. Caruso, R. A., et al., Ultrastructural descriptions of heterotypic aggregation between eosinophils and tumor cells in human gastric carcinomas. *Ultrastructural pathology* **2011,** *35* (4), 145-9. DOI: 10.3109/01913123.2011.578233.
283. Ieni, A., et al., Mast Cell Interaction with Neutrophils in Human Gastric Carcinomas: Ultrastructural Observations. *Analytical cellular pathology* **2016,** *2016*, 6891971. DOI: 10.1155/2016/6891971.
284. Reinbach, G., Über das Verhalten der Leukocyten bei malignen Tumoren. *Archiv für Klinische Chirurgie* **1893,** *46*, 486-562.
285. von Wasielewski, R., et al., Tissue eosinophilia correlates strongly with poor prognosis in nodular sclerosing Hodgkin's disease, allowing for known prognostic factors. *Blood* **2000,** *95* (4), 1207-13.
286. Samoszuk, M., Eosinophils and human cancer. *Histol Histopathol* **1997,** *12* (3), 807-12.
287. Prizment, A. E., et al., Inverse association of eosinophil count with colorectal cancer incidence: atherosclerosis risk in communities study. *Cancer Epidemiol Biomarkers Prev* **2011,** *20* (9), 1861-4. DOI: 10.1158/1055-9965.EPI-11-0360.
288. Prizment, A. E., et al., Tumor eosinophil infiltration and improved survival of colorectal cancer patients: Iowa Women's Health Study. *Mod Pathol* **2016,** *29* (5), 516-27. DOI: 10.1038/modpathol.2016.42.
289. Luna-More, S., et al., Neutral and acid mucins and eosinophil and argyrophil crystalloids in carcinoma and atypical adenomatous hyperplasia of the prostate. *Pathology, research and practice* **1997,** *193* (4), 291-8. DOI: 10.1016/S0344-0338(97)80006-4.
290. McNeel, D. G., et al., A transient increase in eosinophils is associated with prolonged survival in men with metastatic castration-resistant prostate cancer who receive sipuleucel-T. *Cancer immunology research* **2014,** *2* (10), 988-99. DOI: 10.1158/2326-6066.CIR-14-0073.
291. Herbst, R. S., et al., Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature* **2014,** *515* (7528), 563-7. DOI: 10.1038/nature14011.
292. Meng, X., et al., Predictive biomarkers in PD-1/PD-L1 checkpoint blockade immunotherapy. *Cancer Treat Rev* **2015,** *41* (10), 868-76. DOI: 10.1016/j.ctrv.2015.11.001.
293. Teng, M. W., et al., Classifying Cancers Based on T-cell Infiltration and PD-L1. *Cancer Res* **2015,** *75* (11), 2139-45. DOI: 10.1158/0008-5472.CAN-15-0255.
294. Moreira, A., et al., Eosinophilic count as a biomarker for prognosis of melanoma patients and its importance in the response to immunotherapy. *Immunotherapy* **2017,** *9* (2), 115-121. DOI: 10.2217/imt-2016-0138.
295. Bernard-Tessier, A., et al., Immune-related eosinophilia induced by anti-programmed death 1 or death-ligand 1 antibodies. *Eur J Cancer* **2017,** *81*, 135-137. DOI: 10.1016/j.ejca.2017.05.017.
296. Delyon, J., et al., Experience in daily practice with ipilimumab for the treatment of patients with metastatic melanoma: an early increase in lymphocyte and eosinophil counts is associated with improved survival. *Ann Oncol* **2013,** *24* (6), 1697-703. DOI: 10.1093/annonc/mdt027.
297. Ferrucci, P. F., et al., Baseline relative eosinophil count as a predictive biomarker for ipilimumab treatment in advanced melanoma. *Oncotarget* **2017,** *8* (45), 79809-79815. DOI: 10.18632/oncotarget.19748.
298. Giommoni, E., et al., Eosinophil Count as Predictive Biomarker of Immune-Related Adverse Events (irAEs) in Immune Checkpoint Inhibitors (ICIs) Therapies in Oncological Patients. *Immuno* **2021,** *1* (3), 253-263.
299. Bristol, J. A., et al., In vitro and in vivo activities of an oncolytic adenoviral vector designed to express GM-CSF. *Molecular therapy : the journal of the American Society of Gene Therapy* **2003,** *7* (6), 755-64. DOI: 10.1016/s1525-0016(03)00103-5.
300. Gebhardt, C., et al., Myeloid Cells and Related Chronic Inflammatory Factors as Novel Predictive Markers in Melanoma Treatment with Ipilimumab. *Clin Cancer Res* **2015,** *21* (24), 5453-9. DOI: 10.1158/1078-0432.CCR-15-0676.
301. Huland, E.; Huland, H., Tumor-associated eosinophilia in interleukin-2-treated patients: evidence of toxic eosinophil degranulation on bladder cancer cells. *Journal of cancer research and clinical oncology* **1992,** *118* (6), 463-7. DOI: 10.1007/BF01629431.
302. Sosman, J. A., et al., Evidence for eosinophil activation in cancer patients receiving recombinant interleukin-4: effects of interleukin-4 alone and following interleukin-2 administration. *Clin Cancer Res* **1995,** *1* (8), 805-12.
303. Simon, S. C. S., et al., Opposing roles of eosinophils in cancer. *Cancer Immunol Immunother* **2019,** *68* (5), 823-833. DOI: 10.1007/s00262-018-2255-4.

304. Macdonald, D., et al., Interleukin-2 treatment-associated eosinophilia is mediated by interleukin-5 production. *British journal of haematology* **1990,** *76* (2), 168-73. DOI: 10.1111/j.1365-2141.1990.tb07867.x.

305. van Haelst Pisani, C., et al., Administration of interleukin-2 (IL-2) results in increased plasma concentrations of IL-5 and eosinophilia in patients with cancer. *Blood* **1991,** *78* (6), 1538-44.

306. Legrand, F., et al., Human eosinophils exert TNF-alpha and granzyme A-mediated tumoricidal activity toward colon carcinoma cells. *J Immunol* **2010,** *185* (12), 7443-51. DOI: 10.4049/jimmunol.1000446.

307. Lucarini, V., et al., IL-33 restricts tumor growth and inhibits pulmonary metastasis in melanoma-bearing mice through eosinophils. *Oncoimmunology* **2017,** *6* (6), e1317420. DOI: 10.1080/2162402X.2017.1317420.

308. Furbert-Harris, P. M., et al., Activated eosinophils upregulate the metastasis suppressor molecule E-cadherin on prostate tumor cells. *Cellular and molecular biology* **2003,** *49* (7), 1009-16.

309. Kanda, A., et al., Th2-activated eosinophils release Th1 cytokines that modulate allergic inflammation. *Allergology international : official journal of the Japanese Society of Allergology* **2015,** *64 Suppl*, S71-3. DOI: 10.1016/j.alit.2015.03.006.

310. Spencer, L. A., et al., Human eosinophils constitutively express multiple Th1, Th2, and immunoregulatory cytokines that are secreted rapidly and differentially. *Journal of leukocyte biology* **2009,** *85* (1), 117-23. DOI: 10.1189/jlb.0108058.

311. Woerly, G., et al., Expression of CD28 and CD86 by human eosinophils and role in the secretion of type 1 cytokines (interleukin 2 and interferon gamma): inhibition by immunoglobulin a complexes. *J Exp Med* **1999,** *190* (4), 487-95. DOI: 10.1084/jem.190.4.487.

312. Legrand, F., et al., A functional gammadeltaTCR/CD3 complex distinct from gammadeltaT cells is expressed by human eosinophils. *PLoS One* **2009,** *4* (6), e5926. DOI: 10.1371/journal.pone.0005926.

313. Kruckel, A., et al., Eosinophil-cationic protein - a novel liquid prognostic biomarker in melanoma. *BMC Cancer* **2019,** *19* (1), 207. DOI: 10.1186/s12885-019-5384-z.

314. Park, Y. J., et al., Plasma eosinophil cationic protein, interleukin-5, and ECP/Eo count ratio in patients with various eosinophilic diseases. *Annals of clinical and laboratory science* **2006,** *36* (3), 262-6.

315. Trulson, A., et al., The eosinophil granule proteins in serum, but not the oxidative metabolism of the blood eosinophils, are increased in cancer. *British journal of haematology* **1997,** *98* (2), 312-4. DOI: 10.1046/j.1365-2141.1997.2203035.x.

316. Bobbio-Pallavicini E, et al., Study of release of eosinophil cationic proteins (ECP and EPX) in the hypereosinophilic syndrome (HES) and other hypereosinophilic conditions. *Panminerva Med* **1998,** *40* (3), 186-90.

317. Fujitaka, M., et al., Significance of the eosinophil cationic protein/eosinophil count ratio in asthmatic patients: its relationship to disease severity. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology* **2001,** *86* (3), 323-9. DOI: 10.1016/S1081-1206(10)63307-8.

318. Stelmach, I., et al., The ECP/Eo count ratio in children with asthma. *The Journal of asthma : official journal of the Association for the Care of Asthma* **2004,** *41* (5), 539-46. DOI: 10.1081/jas-120037654.

319. Zetter, B. R., Angiogenesis and tumor metastasis. *Annu Rev Med* **1998,** *49*, 407-24. DOI: 10.1146/annurev.med.49.1.407.

320. Xing, Y., et al., CCL11-induced eosinophils inhibit the formation of blood vessels and cause tumor necrosis. *Genes Cells* **2016,** *21* (6), 624-38. DOI: 10.1111/gtc.12371.

321. Scanvion, Q., et al., Moderate-to-severe eosinophilia induced by treatment with immune checkpoint inhibitors: 37 cases from a national reference center for hypereosinophilic syndromes and the French pharmacovigilance database. *Oncoimmunology* **2020,** *9* (1), 1722022. DOI: 10.1080/2162402X.2020.1722022.

322. Ugurel, S., et al., B-RAF and N-RAS mutations are preserved during short time in vitro propagation and differentially impact prognosis. *PLoS One* **2007,** *2* (2), e236. DOI: 10.1371/journal.pone.0000236.

323. Peterson, C. G., et al., Radioimmunoassay of human eosinophil cationic protein (ECP) by an improved method. Establishment of normal levels in serum and turnover in vivo. *Clin Exp Allergy* **1991,** *21* (5), 561-7. DOI: 10.1111/j.1365-2222.1991.tb00847.x.

324. Gerdes, J., et al., Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. *J Immunol* **1984,** *133* (4), 1710-5.

325. Ohsie, S. J., et al., Immunohistochemical characteristics of melanoma. *J Cutan Pathol* **2008,** *35* (5), 433-44. DOI: 10.1111/j.1600-0560.2007.00891.x.

326. Dowsett, M., et al., Assessment of Ki67 in breast cancer: recommendations from the International Ki67 in Breast Cancer working group. *J Natl Cancer Inst* **2011,** *103* (22), 1656-64. DOI: 10.1093/jnci/djr393.

327. Gerdes, J., et al., Prognostic relevance of tumour-cell growth fraction in malignant non-Hodgkin's lymphomas. *Lancet* **1987,** *2* (8556), 448-9. DOI: 10.1016/s0140-6736(87)90977-9.

328. Richards-Taylor, S., et al., The assessment of Ki-67 as a prognostic marker in neuroendocrine tumours: a systematic review and meta-analysis. *J Clin Pathol* **2016,** *69* (7), 612-8. DOI: 10.1136/jclinpath-2015-203340.

329. Sun, X.; Kaufman, P. D., Ki-67: more than a proliferation marker. *Chromosoma* **2018,** *127* (2), 175-186. DOI: 10.1007/s00412-018-0659-8.

330. Gimotty, P. A., et al., Biologic and prognostic significance of dermal Ki67 expression, mitoses, and tumorigenicity in thin invasive cutaneous melanoma. *J Clin Oncol* **2005,** *23* (31), 8048-56. DOI: 10.1200/JCO.2005.02.0735.

331. Ladstein, R. G., et al., Ki-67 expression is superior to mitotic count and novel proliferation markers PHH3, MCM4 and mitosin as a prognostic factor in thick cutaneous melanoma. *BMC Cancer* **2010,** *10,* 140. DOI: 10.1186/1471-2407-10-140.

332. Mattes, J., et al., Immunotherapy of cytotoxic T cell-resistant tumors by T helper 2 cells: an eotaxin and STAT6-dependent process. *J Exp Med* **2003,** *197* (3), 387-93. DOI: 10.1084/jem.20021683.

333. Hellweg, C. E., et al., Activation of nuclear factor kappa B by different agents: influence of culture conditions in a cell-based assay. *Ann N Y Acad Sci* **2006,** *1091,* 191-204. DOI: 10.1196/annals.1378.066.

334. Fujiwara, T., et al., Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. *J Biol Chem* **1988,** *263* (34), 18545-52.

335. Gershenwald, J. E., et al., Melanoma staging: Evidence-based changes in the American Joint Committee on Cancer eighth edition cancer staging manual. *CA Cancer J Clin* **2017,** *67* (6), 472-492. DOI: 10.3322/caac.21409.

336. Bochner, B. S., Siglec-8 on human eosinophils and mast cells, and Siglec-F on murine eosinophils, are functionally related inhibitory receptors. *Clin Exp Allergy* **2009,** *39* (3), 317-24. DOI: 10.1111/j.1365-2222.2008.03173.x.

337. Lucey, D. R., et al., Mature human eosinophils have the capacity to express HLA-DR. *Proc Natl Acad Sci U S A* **1989,** *86* (4), 1348-51. DOI: 10.1073/pnas.86.4.1348.

338. Walsh, G. M., Eosinophil granule proteins and their role in disease. *Curr Opin Hematol* **2001,** *8* (1), 28-33. DOI: 10.1097/00062752-200101000-00006.

339. Enblad, G., et al., Infiltration of eosinophils in Hodgkin's disease involved lymph nodes predicts prognosis. *Hematological oncology* **1993,** *11* (4), 187-93. DOI: 10.1002/hon.2900110404.

340. Von Wasielewski, R., et al., Tissue eosinophilia correlates strongly with poor prognosis in nodular sclerosing Hodgkin's disease, allowing for known prognostic factors. *Blood, The Journal of the American Society of Hematology* **2000,** *95* (4), 1207-1213.

341. Thompson, A. C., et al., Tumor-associated tissue eosinophilia and long-term prognosis for carcinoma of the larynx. *American journal of surgery* **1994,** *168* (5), 469-71. DOI: 10.1016/s0002-9610(05)80102-3.

342. van Driel, W. J., et al., Tumor-associated eosinophilic infiltrate of cervical cancer is indicative for a less effective immune response. *Hum Pathol* **1996,** *27* (9), 904-11. DOI: 10.1016/s0046-8177(96)90216-6.

343. Wei, Y., et al., The impacts of pretreatment circulating eosinophils and basophils on prognosis of stage Ⅰ–Ⅲ colorectal cancer. *Asia-Pacific Journal of Clinical Oncology* **2018,** *14* (5), e243-e251.

344. Ownby, H. E., et al., Peripheral lymphocyte and eosinophil counts as indicators of prognosis in primary breast cancer. *Cancer* **1983,** *52* (1), 126-30. DOI: 10.1002/1097-0142(19830701)52:1<126::aid-cncr2820520123>3.0.co;2-y.

345. Wang, H. K., et al., Eosinophil percentage elevation as a prognostic factor for overall survival in patients with metastatic renal cell carcinoma treated with tyrosine kinase inhibitor. *Oncotarget* **2016,** *7* (42), 68943-68953. DOI: 10.18632/oncotarget.12126.

346. Knispel, S., et al., Outcome of melanoma patients with elevated LDH treated with first-line targeted therapy or PD-1-based immune checkpoint inhibition. *Eur J Cancer* **2021,** *148,* 61-75. DOI: 10.1016/j.ejca.2021.01.034.

347. Petrelli, F., et al., Prognostic and predictive role of elevated lactate dehydrogenase in patients with melanoma treated with immunotherapy and BRAF inhibitors: a systematic review and meta-analysis. *Melanoma Res* **2019,** *29* (1), 1-12. DOI: 10.1097/CMR.0000000000000520.

348. Radonjic-Hoesli, S., et al., Eosinophils in skin diseases. *Semin Immunopathol* **2021,** *43* (3), 393-409. DOI: 10.1007/s00281-021-00868-7.

349. Gaba, L., et al., Changes in blood eosinophilia during anti-PD1 therapy as a predictor of long term disease control in metastatic melanoma. *Journal of Clinical Oncology* **2015,** *33* (15_suppl), 9069-9069. DOI: 10.1200/jco.2015.33.15_suppl.9069.

350. Masucci, M. T., et al., Tumor Associated Neutrophils. Their Role in Tumorigenesis, Metastasis, Prognosis and Therapy. *Frontiers in oncology* **2019,** *9,* 1146. DOI: 10.3389/fonc.2019.01146.

351. Granot, Z., et al., Tumor entrained neutrophils inhibit seeding in the premetastatic lung. *Cancer Cell* **2011,** *20* (3), 300-14. DOI: 10.1016/j.ccr.2011.08.012.

352. Jensen, H. K., et al., Presence of intratumoral neutrophils is an independent prognostic factor in localized renal cell carcinoma. *J Clin Oncol* **2009,** *27* (28), 4709-17. DOI: 10.1200/JCO.2008.18.9498.

353. Cohen, J. T., et al., Is the neutrophil-to-lymphocyte ratio a useful prognostic indicator in melanoma patients? *Melanoma management* **2020,** *7* (3), MMT47. DOI: 10.2217/mmt-2020-0006.

354. Jensen, T. O., et al., Intratumoral neutrophils and plasmacytoid dendritic cells indicate poor prognosis and are associated with pSTAT3 expression in AJCC stage I/II melanoma. *Cancer* **2012,** *118* (9), 2476-85. DOI: 10.1002/cncr.26511.

355. Coussens, L. M., et al., MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell* **2000,** *103* (3), 481-90. DOI: 10.1016/s0092-8674(00)00139-2.

356. Houghton, A. M., et al., Neutrophil elastase-mediated degradation of IRS-1 accelerates lung tumor growth. *Nature medicine* **2010,** *16* (2), 219-23. DOI: 10.1038/nm.2084.

357. Huh, S. J., et al., Transiently entrapped circulating tumor cells interact with neutrophils to facilitate lung metastasis development. *Cancer Res* **2010,** *70* (14), 6071-82. DOI: 10.1158/0008-5472.CAN-09-4442.

358. Rankin, S. M., et al., Eotaxin and eosinophil recruitment: implications for human disease. *Molecular medicine today* **2000,** *6* (1), 20-7. DOI: 10.1016/s1357-4310(99)01635-4.

359. Vincent, F. B., et al., The BAFF/APRIL system: emerging functions beyond B cell biology and autoimmunity. *Cytokine & growth factor reviews* **2013,** *24* (3), 203-15. DOI: 10.1016/j.cytogfr.2013.04.003.

360. Ratajczak-Wrona, W., et al., A Proliferation-Inducing Ligand Regulation in Polymorphonuclear Neutrophils by Panax ginseng. *Archivum immunologiae et therapiae experimentalis* **2020,** *68* (6), 32. DOI: 10.1007/s00005-020-00597-z.
361. Young, J. D., et al., Mechanism of membrane damage mediated by human eosinophil cationic protein. *Nature* **1986,** *321* (6070), 613-6. DOI: 10.1038/321613a0.
362. PO, D. E. L., et al., Effect of eosinophil cationic protein on human oral squamous carcinoma cell viability. *Molecular and clinical oncology* **2015,** *3* (2), 353-356. DOI: 10.3892/mco.2014.477.
363. Simon, S. C. S., et al., Eosinophil accumulation predicts response to melanoma treatment with immune checkpoint inhibitors. *Oncoimmunology* **2020,** *9* (1), 1727116. DOI: 10.1080/2162402X.2020.1727116.
364. Simson, L., et al., Regulation of carcinogenesis by IL-5 and CCL11: a potential role for eosinophils in tumor immune surveillance. *J Immunol* **2007,** *178* (7), 4222-9. DOI: 10.4049/jimmunol.178.7.4222.
365. Salcedo, R., et al., MyD88-mediated signaling prevents development of adenocarcinomas of the colon: role of interleukin 18. *J Exp Med* **2010,** *207* (8), 1625-36. DOI: 10.1084/jem.20100199.
366. Gaida, M. M., et al., Polymorphonuclear neutrophils promote dyshesion of tumor cells and elastase-mediated degradation of E-cadherin in pancreatic tumors. *European journal of immunology* **2012,** *42* (12), 3369-80. DOI: 10.1002/eji.201242628.
367. Mehner, C., et al., Tumor cell-produced matrix metalloproteinase 9 (MMP-9) drives malignant progression and metastasis of basal-like triple negative breast cancer. *Oncotarget* **2014,** *5* (9), 2736-49. DOI: 10.18632/oncotarget.1932.
368. Mukherjee, M., et al., Eosinophil Extracellular Traps and Inflammatory Pathologies-Untangling the Web! *Front Immunol* **2018,** *9*, 2763. DOI: 10.3389/fimmu.2018.02763.
369. Neubert, E., et al., Serum and Serum Albumin Inhibit in vitro Formation of Neutrophil Extracellular Traps (NETs). *Front Immunol* **2019,** *10*, 12. DOI: 10.3389/fimmu.2019.00012.
370. Albrengues, J., et al., Neutrophil extracellular traps produced during inflammation awaken dormant cancer cells in mice. *Science* **2018,** *361* (6409). DOI: 10.1126/science.aao4227.
371. Nutten, S., et al., Selectin and Lewis(x) are required as co-receptors in antibody-dependent cell-mediated cytotoxicity of human eosinophils to Schistosoma mansoni schistosomula. *European journal of immunology* **1999,** *29* (3), 799-808. DOI: 10.1002/(SICI)1521-4141(199903)29:03<799::AID-IMMU799>3.0.CO;2-1.
372. Meng, S., et al., Circulating tumor cells in patients with breast cancer dormancy. *Clin Cancer Res* **2004,** *10* (24), 8152-62. DOI: 10.1158/1078-0432.CCR-04-1110.
373. Muller, V., et al., Circulating tumor cells in breast cancer: correlation to bone marrow micrometastases, heterogeneous response to systemic therapy and low proliferative activity. *Clin Cancer Res* **2005,** *11* (10), 3678-85. DOI: 10.1158/1078-0432.CCR-04-2469.
374. Seyfried, T. N.; Huysentruyt, L. C., On the origin of cancer metastasis. *Critical reviews in oncogenesis* **2013,** *18* (1-2), 43-73. DOI: 10.1615/critrevoncog.v18.i1-2.40.
375. Balkwill, F.; Mantovani, A., Inflammation and cancer: back to Virchow? *Lancet* **2001,** *357* (9255), 539-45. DOI: 10.1016/S0140-6736(00)04046-0.
376. Cormier, S. A., et al., Pivotal Advance: eosinophil infiltration of solid tumors is an early and persistent inflammatory host response. *Journal of leukocyte biology* **2006,** *79* (6), 1131-9. DOI: 10.1189/jlb.0106027.
377. Marsavela, G., et al., Melanoma circulating tumor cells: Benefits and challenges required for clinical application. *Cancer letters* **2018,** *424*, 1-8. DOI: 10.1016/j.canlet.2018.03.013.
378. Erpenbeck, L., et al., Effect of Adhesion and Substrate Elasticity on Neutrophil Extracellular Trap Formation. *Front Immunol* **2019,** *10*, 2320. DOI: 10.3389/fimmu.2019.02320.
379. Discher, D. E., et al., Tissue cells feel and respond to the stiffness of their substrate. *Science* **2005,** *310* (5751), 1139-43. DOI: 10.1126/science.1116995.
380. Rehfeldt, F., et al., Cell responses to the mechanochemical microenvironment--implications for regenerative medicine and drug delivery. *Advanced drug delivery reviews* **2007,** *59* (13), 1329-39. DOI: 10.1016/j.addr.2007.08.007.
381. Adlerz, K. M., et al., Substrate elasticity regulates the behavior of human monocyte-derived macrophages. *European biophysics journal : EBJ* **2016,** *45* (4), 301-9. DOI: 10.1007/s00249-015-1096-8.
382. Okamoto, T., et al., Reduced substrate stiffness promotes M2-like macrophage activation and enhances peroxisome proliferator-activated receptor gamma expression. *Exp Cell Res* **2018,** *367* (2), 264-273. DOI: 10.1016/j.yexcr.2018.04.005.
383. Mennens, S. F. B., et al., Substrate stiffness influences phenotype and function of human antigen-presenting dendritic cells. *Scientific reports* **2017,** *7* (1), 17511. DOI: 10.1038/s41598-017-17787-z.
384. Engler, A. J., et al., Matrix elasticity directs stem cell lineage specification. *Cell* **2006,** *126* (4), 677-89. DOI: 10.1016/j.cell.2006.06.044.
385. Lee-Thedieck, C., et al., Impact of substrate elasticity on human hematopoietic stem and progenitor cell adhesion and motility. *J Cell Sci* **2012,** *125* (Pt 16), 3765-75. DOI: 10.1242/jcs.095596.
386. Jannat, R. A., et al., Neutrophil adhesion and chemotaxis depend on substrate mechanics. *Journal of physics. Condensed matter : an Institute of Physics journal* **2010,** *22* (19), 194117. DOI: 10.1088/0953-8984/22/19/194117.
387. Oakes, P. W., et al., Neutrophil morphology and migration are affected by substrate elasticity. *Blood* **2009,** *114* (7), 1387-95. DOI: 10.1182/blood-2008-11-191445.
388. Stroka, K. M.; Aranda-Espinoza, H., Neutrophils display biphasic relationship between migration and substrate stiffness. *Cell motility and the cytoskeleton* **2009,** *66* (6), 328-41. DOI: 10.1002/cm.20363.

389. Stroka, K. M.; Aranda-Espinoza, H., Endothelial cell substrate stiffness influences neutrophil transmigration via myosin light chain kinase-dependent cell contraction. *Blood* **2011,** *118* (6), 1632-40. DOI: 10.1182/blood-2010-11-321125.

390. Grisaru-Tal, S., et al., A new dawn for eosinophils in the tumour microenvironment. *Nature reviews. Cancer* **2020,** *20* (10), 594-607. DOI: 10.1038/s41568-020-0283-9.

391. Yoon, J., et al., CD66b regulates adhesion and activation of human eosinophils. *J Immunol* **2007,** *179* (12), 8454-62. DOI: 10.4049/jimmunol.179.12.8454.

392. Hansel, T. T., et al., Induction and function of eosinophil intercellular adhesion molecule-1 and HLA-DR. *J Immunol* **1992,** *149* (6), 2130-6.

393. Johansson, M. W., Activation states of blood eosinophils in asthma. *Clin Exp Allergy* **2014,** *44* (4), 482-98. DOI: 10.1111/cea.12292.

394. Weller, P. F., et al., Accessory cell function of human eosinophils. HLA-DR-dependent, MHC-restricted antigen-presentation and IL-1 alpha expression. *J Immunol* **1993,** *150* (6), 2554-62.

395. Clark, K., et al., Eosinophil degranulation in the allergic lung of mice primarily occurs in the airway lumen. *Journal of leukocyte biology* **2004,** *75* (6), 1001-9. DOI: 10.1189/jlb.0803391.

396. Arjonen, A., et al., Filopodia and adhesion in cancer cell motility. *Cell adhesion & migration* **2011,** *5* (5), 421-30. DOI: 10.4161/cam.5.5.17723.

397. Jacquemet, G., et al., Filopodia in cell adhesion, 3D migration and cancer cell invasion. *Current opinion in cell biology* **2015,** *36*, 23-31. DOI: 10.1016/j.ceb.2015.06.007.

398. Al-Alwan, M., et al., Fascin is a key regulator of breast cancer invasion that acts via the modification of metastasis-associated molecules. *PLoS One* **2011,** *6* (11), e27339. DOI: 10.1371/journal.pone.0027339.

399. Stenfeldt, A. L.; Wenneras, C., Danger signals derived from stressed and necrotic epithelial cells activate human eosinophils. *Immunology* **2004,** *112* (4), 605-14. DOI: 10.1111/j.1365-2567.2004.01906.x.

400. Radonjic-Hoesli, S., et al., Adhesion-induced eosinophil cytolysis requires the receptor-interacting protein kinase 3 (RIPK3)-mixed lineage kinase-like (MLKL) signaling pathway, which is counterregulated by autophagy. *J Allergy Clin Immunol* **2017,** *140* (6), 1632-1642. DOI: 10.1016/j.jaci.2017.01.044.

401. Spencer, L. A., et al., Eosinophil secretion of granule-derived cytokines. *Front Immunol* **2014,** *5*, 496. DOI: 10.3389/fimmu.2014.00496.

402. Griffin, J. D., et al., The biology of GM-CSF: regulation of production and interaction with its receptor. *International journal of cell cloning* **1990,** *8 Suppl 1*, 35-44; discussion 44-5. DOI: 10.1002/stem.5530080705.

403. Hamilton, J. A., GM-CSF in inflammation. *J Exp Med* **2020,** *217* (1). DOI: 10.1084/jem.20190945.

404. Zhang, S., et al., Cell cultures from bronchial subepithelial myofibroblasts enhance eosinophil survival in vitro. *The European respiratory journal* **1996,** *9* (9), 1839-46. DOI: 10.1183/09031936.96.09091839.

405. R, O. D., An update on GM-CSF and its potential role in melanoma management. *Melanoma management* **2020,** *7* (3), MMT49. DOI: 10.2217/mmt-2020-0011.

406. Curran, C. S., et al., GM-CSF production by glioblastoma cells has a functional role in eosinophil survival, activation, and growth factor production for enhanced tumor cell proliferation. *J Immunol* **2011,** *187* (3), 1254-63. DOI: 10.4049/jimmunol.1001965.

407. Solomon, A., et al., Conjunctival fibroblasts enhance the survival and functional activity of peripheral blood eosinophils in vitro. *Investigative ophthalmology & visual science* **2000,** *41* (5), 1038-44.

Appendix

I.   Abbreviations

7-AAD	7-aminoactinomycin
AE	adverse events
AJCC	American Joint Committee on Cancer
APC	antigen-presenting cell
BRAF	B-Raf proto-oncogene, serine/threonine kinase
BRAFi	B-Raf proto-oncogene, serine/threonine kinase inhibitor
BOR	best overall response
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCND1	cyclin D1
CCL5	CC-chemokine ligand 5; also called RANTES
cDNA	complementary DNA
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CPD	cyclobutene pyrimidine dimers
CDK2	cyclin-dependent kinase 2
CDKN2A	cyclin-dependent kinase inhibitor 2A
CD	cluster of differentiation
CO_2	carbon dioxide
CSD	chronically sun damaged
CXCL	chemokinie (C-X-C motif) ligand
DMSO	dimethyl sulfoxide
DNA	desoxcyribonucleic acid
DPBS	dulbecco´s phosphate buffered saline
ECP	eosinophil cationic protein
ECT	electrochemotherapy
EDN	eosinophil-derived neurotoxin
EDTA	ethylenediamine tetraacetic acid
EPO	eosinophil peroxidase
ER	endoplasmic reticulum
ERK	extracellular regulated MAP kinase
FDA	Food and Drug Administration
FCS	fetal calf serum
GNA11	G protein subunit alpha 11
GNAQ	G protein subunit alpha q
HLA	human leukocyte antigen
IFN-γ	interferon-γ
IL	interleukin
IHES	idiopathic hypereosinophilic syndrome
KIT	KIT proto-oncogene receptor tyrosine kinase
LDH	lactate dehydrogenase
LEAF	low endotoxin, acid-free
MBP	major basic protein
MC1R	melanocortin-1 receptor
MDSC	myeloid-derived suppressor cells
MHC	major histocompatibility complex
MITF	micrphthalamia-associated transcription factor
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase

NaCl	natrium chloride
NCSC	neural crest stem cells
NER	nucleotide excision repair
NRAS	neuroblastoma ras viral oncogene homolog, proto-oncogene, GTPase
OR	overall response
OS	overall survival
PD-1/PD-L1	programmed cell death-1/-ligand 1
PFS	progression-free survival
PKA	protein kinase
PMA	Phorbol-12-myristate-13-acetate
PTEN	phosphatase and tensin homolog
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute Medium
ROS	reactive oxygen species
RT	room temperature
SLNB	sentinel lymph node biopsy
TAP	transporter associated with antigen processing
TCR	T cell receptor
TERT	telomerase reverse transcriptase
TNF	tumor necrosis factor
TP53	tumor protein p53
T-VEC	Talimogene Laherparepvec
6-4PP	6-4 photoproduct
UV	ultraviolet
VCAM-1	vascular cell adhesion molecule 1

II.   List of figures

Figure 26. Blocking of potential target molecules and dissolving potential EET-DNA structures in co-cultures.

III. List of tables

IV.   Affidavit / Eidestattliche Erklärung

Affidavit

I hereby confirm that my thesis entitled **"Function of Peripheral Blood Eosinophils in Melanoma"** is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor similar form.

Place, Date   Signature

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation **"Funktion der Eosinophilen Granulozyten aus dem peripheren Blut im Melanom"** eigenständig, d.h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ort, Datum   Unterschrift

V.   Curriculum Vitae

VI. Publications and conference contributions

<u>**Publication**</u>

2017 - 2022: During the PhD at the Department of Dermatology and the University Hospital Würzburg:

Wendlinger, S.; Wohlfarth, J.; Kreft, S.; Siedel, C.; Kilian, T.; Dischinger, U.; Heppt, M.V.; Wistuba-Hamprecht, K.; Meier, F.; Goebeler, M.; Schadendorf, D.; Gesierich, A.; Kosnopfel, C.; Schilling, B. Blood Eosinophils Are Associated with Efficacy of Targeted Therapy in Patients with Advanced Melanoma. *Cancers* **2022**, *14*, 2294. https://doi.org/10.3390/cancers14092294

Note: With the consent of the journal *Cancers*, the use and modification of the tables and figures from the above-mentioned publication used in this thesis does not require explicit permission.

2013: During the practical semester at the Institute of Clinical Neuroimmunology, Hospital Grosshadern, Ludwig-Maximilians-University Munich:

Hoffmann, F.S.; Kuhn, P.H.; Laurent, S.A.; Hauck, S.M.; Berer, K.; **Wendlinger, S.A.**; Krumbholz, M.; Khademi, M.; Olsson, T.; Dreyling, M.; Pfister, H.W.; Alexander, T.; Hiepe, F.; Kümpfel, T.; Crawford, H.C.; Wekerle, H.; Hohlfeld, R.; Lichtenthaler, S.F.; Meinl, E. The immunoregulator soluble TACI is released by ADAM10 and reflects B cell activation in autoimmunity. *J Immunol.* **2015**, 194(2):542-52. doi: 10.4049/jimmunol.1402070.

<u>**Conference contributions**</u>

03/2018	45th Annual Conference organized by the Arbeitsgemeinschaft Dermatologische Forschung (ADF), Zürich, Switzerland Contribution: Poster "Interaction of Neutrophils and Melanoma in the Context of MAP-Kinase Inhibition"
09/2018	28th German Skin Cancer Conference organized by the Arbeitsgemeinschaft Dermatologische Onkologie (ADO), Stuttgart, Germany Contribution: ePoster "Interaktion von Melanomzellen und Granulozyten im Kontext der MAP-Kinase Inhibition"
10/2018	13th International GSLS Student Symposium "EUREKA!", organized by the Doctoral Researchers of the Graduate School of Life Sciences, Würzburg, Germany Contribution: Poster "Interaction of Granulocytes and Melanoma in the Context of MAP-Kinase Inhibition"
10/2020	International GSLS Student Symposium "EUREKA!", organized by the Doctoral Researchers of the Graduate School of Life Sciences, Würzburg, Germany Contribution: Poster "Eosinophils and Neutrophils in Melanoma – a Tale of Good and Evil"

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