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BRAF inhibitors and their immunological effects in malignant melanoma

Dr Rebecca Adams BA BMBCh MRCP*[1], Jack E M Coumbe BSc MSc*[1], Dr Ben G T Coumbe MA MBBS*[1], Dr Jennifer Thomas BA BMBCh MRCP[2], Dr Zena Willsmore[1] MBBS MRCP, Marija Dimitrievska BSc[1], Monica Yasuzawa-Parker BSc[1], Maximilian Hoyle BSc[1], Suhaylah Ingar BSc[1], Jenny L C Geh BMBS BMedSci MSc FRCSEdPLAST[3], Alastair MacKenzie-Ross BA (Hons) MBBChir MA MD (Cantab) FRCS (Plast)[3], Ciaran Healy MD DCH FRCSI FRCS (Plast)[3], Dr Sophie Papa BA MRCP FRCP PhD[4,5], Dr Katie E. Lacy BA MRCP PhD[1], Prof Sophia N Karagiannis BA MS PhD[1,6]

** Equally contributing authors*

[1] St. John's Institute of Dermatology, School of Basic & Medical Biosciences, King's College London, Guy's Hospital, London SE1 9RT, United Kingdom

[2] The Royal Marsden, Downs Road, Sutton, Surrey, United Kingdom

[2] Department of Plastic Surgery at Guy's, King's, and St. Thomas' Hospitals, London, United Kingdom

[4] Department of Medical Oncology, Guy's and St. Thomas' NHS Foundation Trust, London, United Kingdom.

[5] ImmunoEngineering, School of Cancer and Pharmaceutical Sciences, Faculty of Life Sciences and Medicine, King's College London, London, United Kingdom.

[6] Breast Cancer Now Research Unit, School of Cancer & Pharmaceutical Sciences, King's College London, Guy's Cancer Centre, London SE1 9RT, United Kingdom

Correspondence

Sophia N. Karagiannis, PhD, St. John's Institute of Dermatology, School of Basic & Medical Biosciences, King's College London, Guy's Hospital, Tower Wing, 9th Floor, London, SE1 9RT, United Kingdom; Tel: +44[0]20 7188 6355; E-mail: sophia.karagiannis@kcl.ac.uk

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Abstract

Introduction: The treatment of cutaneous melanoma has been revolutionised by the development of small molecule inhibitors targeting the MAPK pathway, including inhibitors of BRAF (BRAFi) and MEK (MEKi), and immune checkpoint blockade antibodies, occurring in tandem. Despite these advances, the 5-year survival rate for patients with advanced melanoma remains only around 50%. Although not designed to alter immune responses within the tumour microenvironment (TME), MAPK pathway inhibitors (MAPKi) exert a range of effects on the host immune compartment which may offer opportunities for therapeutic interventions.

Areas covered: We review the effects of MAPKi especially BRAFi, on the TME, focussing on alterations in inflammatory cytokine secretion, the recruitment of immune cells and their functions, both during response to BRAFi treatment and as resistance develops. We outline potential combinations of MAPKi with established and experimental treatments.

Expert opinion: MAPKi in combination or in sequence with established treatments such as checkpoint inhibitors, anti-angiogenic agents, or new therapies such as adoptive cell therapies, may augment their immunological effects, reverse tumour-associated immune suppression and offer the prospect of longer-lived clinical responses. Refining therapeutic tools at our disposal and embracing "old friends" in the melanoma treatment arsenal, alongside new target identification, may improve the chances of therapeutic success.

Keywords:

BRAF, combination therapy, immune checkpoint inhibitors, MAPK pathway, melanoma, treatment resistance, tumour microenvironment

Article Highlights

- Mutations in the MAPK pathway, the most common of which is the $BRAF^{V600E}$ mutation, contribute to melanoma pathogenesis and progression.
- The development of BRAF inhibitors (BRAFi), and later MEK inhibitors (MEKi), has led to improved survival for patients with melanoma, however, therapeutic resistance typically develops after months of treatment.
- BRAFi can alter the immune response to create a pro-inflammatory tumour microenvironment. BRAFi increase the ratio of effector to immunosuppressive immune cells, increase pro-inflammatory cytokines and reduce cytokines which contribute to the pathogenesis of melanoma, and promote effector cell function by improving antigen presentation and subsequent T cell activation. As BRAFi resistance occurs, these immune changes begin to reverse.
- The effects of BRAFi on host immunity may be harnessed therapeutically to achieve more durable treatment responses.
- Using MAPK pathway inhibitors in combination or sequentially with established or experimental therapies, including checkpoint inhibitor antibodies, may augment the immunological effects of both BRAFi and immunotherapies, improving the effector function of immune cells within the tumour microenvironment and creating more successful and longer lasting treatments for patients with melanoma.

Abbreviations:

BRAFi – BRAF inhibitors

MEKi – MEK inhibitors

ICI – immune checkpoint inhibitors

MAPK – mitogen-activated signalling pathway

MAPKi - mitogen-activated signalling pathway inhibitors

PFS – Progression free survival

TME – tumour microenvironment

OS – overall survival

MDSC – myeloid derived suppressor cells

irAEs – immune related adverse events

trAEs – treatment-related adverse events

T-reg – regulatory T cells

TAMs – tumour associated macrophages

VEGF – vascular endothelial growth factor

APCs – antigen presenting cells

CTLs – cytotoxic T cells

NK cells – natural killer cells

DCs – dendritic cells

MHC-I – major histocompatibility complex

TILs – tumour infiltrating lymphocytes

ACT – adoptive cell therapy

CAR-T – chimeric antigen T-cell therapy

M6PR – mannose 6 phosphate receptor

1. Introduction

The incidence of cutaneous melanoma is increasing, with an estimated 300,000 cases reported globally per annum [1]. Whilst melanoma constitutes less than 5% of all skin cancers diagnosed, it remains the most lethal. Within the last decade there has been significant expansion of the therapeutic arsenal for melanoma with the introduction of molecularly targeted therapies, in the form of MAPK pathway inhibitors, and immune modulatory therapies, in the form of checkpoint inhibitors, occurring in parallel [2]. The MAPK pathway has been identified as a critical signalling cascade underlying disease pathogenesis, with 50% of patients presenting with mutations in the signalling molecule BRAF [3]. Mutations in BRAF lead to constitutive activation of the MAPK pathway, promoting cell proliferation and inhibiting apoptosis, thus driving tumorigenesis [4]. Tumours are tested at diagnosis for BRAF mutations, and BRAF inhibitors (BRAFi) including Vemurafenib, introduced in 2011, and Dabrafenib, approved in 2013, initially demonstrated high efficacy as treatments, with a significant proportion of patients showing profound tumour regression. However, these responses are typically short-lived and development of acquired resistance follows [5,6]. This led to the development of MEK inhibitors (MEKi), which target the MAPK pathway downstream of BRAF. The combination of BRAFi and MEKi is now used as first line treatment for unresectable or metastatic $BRAF^{V600E/K}$ mutant melanoma, leading to greater initial tumour response and delaying MAPK-driven acquired resistance compared with BRAFi treatment alone [7-9]. Although combinations of BRAFi and MEKi improve progression free survival (PFS) when compared to monotherapy [10], therapeutic resistance remains a challenge.

Melanoma is well recognised as the archetypal immunogenic tumour and the interactions between melanoma cells and immune cells within the TME contribute to tumour pathogenesis, invasion and immune evasion [2]. The importance of these interactions has been demonstrated with the success of immune checkpoint inhibition. Immune checkpoint inhibitors (ICI),

developed at the same time as BRAFi, are monoclonal antibodies which target and block the cell surface marker cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or the programme death-1 pathway (PD-1/PD-L1). CTLA-4 is expressed on T cells, including regulatory T cells (T-regs), and inhibits T cell activation through binding of CD80 and CD86 expressed on antigen presenting cells (APC). Blocking this interaction with the antibody Ipilimumab results in T cell activation and enhanced effector function [11]. PD-L1 is highly expressed by tumour and stromal cells within melanoma lesions [12], whilst PD-1 is expressed on the T cell surface [11]. PD-L1/PD-1 interaction leads to negative regulation of T cells. Anti-PD-1 antibodies, such as Nivolumab or Pembrolizumab, and antibodies against PD-L1, such as Atezolizumab, inhibit PD-L1/PD-1 signalling, allowing T cell activation and reducing the number of exhausted T cells within the TME, augmenting anti-tumour immune responses [11,13]. The introduction of ICIs has resulted in significant improvements in PFS in advanced melanoma with up to 50% of patients experiencing unprecedented durable responses at 5 years following treatment with Ipilimumab and Nivolumab [14,15]. The success of ICIs, including in BRAF mutant melanoma [16], demonstrates that the manipulation of the immune system is a valuable therapeutic tool.

Although MAPK pathway inhibitors (MAPKi) were not designed to manipulate the immune system, they too, appear to exert a range of immunological effects, which forms the focus of this review. Understanding the interactions between BRAF mutant melanoma cells and immune cells, both during response to MAPKi treatment and as resistance develops, may uncover new therapeutic avenues, to ultimately lead to more sustained responses and improved clinical outcomes. This may include combining new treatments with MAPKi with the aim of identifying new synergistic effects or optimising the combination and sequencing of already successful therapies, including ICIs.

2. The MAPK pathway in melanoma and targeting with inhibitor drugs

The mitogen-activated signalling pathway (MAPK) is a family of intracellular signalling pathways activated through the binding of growth factors to receptor tyrosine kinases (RTK) [17]. Downstream events linked to these pathways include cellular differentiation, proliferation, apoptosis and regulation of both innate and adaptive immune responses [18,19].

The pathway is arranged in three tiers containing sequentially acting serine threonine kinases: MAP kinase kinase kinase (MAPKKK), MAP kinase kinase (MAPKK), and a downstream effector MAP kinase (MAPK) [20]. Sequential phosphorylation of these kinases (outlined in Figure 1) leads to the phosphorylation of downstream targets in the cytosol and nucleus which mediate biological responses [18]. To prevent persistent activation of MAPK, upstream negative feedback occurs. In the absence of RTK-ligand engagement, members of the MAPK pathway remain in their non-phosphorylated, inactive states preventing downstream signalling.

Aberrant activation of the MAPK pathway is a central step in melanoma pathogenesis [21], often driven by activating mutations of RAF (MAPKKK). The RAF family consists of ARAF, BRAF and CRAF. While mutants of ARAF and CRAF are rare, activating mutations of BRAF are found in different malignancies including colorectal cancer, ovarian cancer, lung cancer and melanoma [22]. The most common BRAF mutation, accounting for 90% of melanomas containing MAPK mutations, $BRAF^{V600E}$, remains active in the absence of RAS, resulting in a constitutively active MAPK pathway that can drive aberrant cell growth and proliferation [4,18], and influence the TME [23]. This makes the MAPK pathway a valuable therapeutic target for melanoma.

Selective BRAF inhibitors with a high affinity for the $BRAF^{V600E}$ mutant include Vemurafenib, Dabrafenib and Encorafenib [23]. Preclinical data for Vemurafenib (PLX4032) and Dabrafenib (GSK2118436) demonstrated significant antitumor activity against $BRAF^{V600E}$ mutant melanoma cell lines, including: G1 cell cycle arrest, apoptosis and blockage of ERK

phosphorylation, reducing proliferation [23,24]. Both were also shown to inhibit other $BRAF^{V600}$ mutant cell lines without inhibiting either wild type BRAF or non-V600 BRAF mutants [24,25].

Encorafenib (LGX818), a second-generation BRAFi, produced prolonged target suppression and efficacy compared to first generation BRAFi such as Vemurafenib and Dabrafenib [26]. Studies in xenograft mouse models of $BRAF^{V600E}$ expressing melanoma, revealed that exposure to Vemurafenib, Dabrafenib or Encorafenib resulted in a dose-dependent inhibition of tumour growth and in some cases tumour regression [24,25,27]. Clinical trials of BRAFi monotherapy demonstrated high rates of objective responses, improved PFS and overall survival (OS) when compared to conventional cytotoxic chemotherapy [28]. Despite these results, a median PFS of only 6-7 months was seen on BRAFi monotherapy due to both intrinsic and acquired resistance and subsequent relapse [6,29,30].

Resistance to BRAFi monotherapy is mediated by reactivation of the MAPK pathway which can occur through MAPK-dependent or MAPK-independent mechanisms. Common patterns of resistance include: activating mutations of upstream components (NRAS mutants) and downstream components (MEK mutants) [31,32], activation of non-MAPK growth pathways (phosphatidylinositol-3-kinase/AKT pathway) [33], splice variants of $BRAF^{V600E}$ [34], and enhanced BRAF expression [35]. Vemurafenib, Dabrafenib, and to a lesser extent Encorafenib, can all cause RAS-dependent paradoxical activation of the MAPK pathway, particularly in those tumours with pre-existing RAS mutations [36,37]. Paradoxical activation is associated with hyperactive MAPK signalling resulting in the emergence of hyperproliferative cutaneous events [38]. Resistance may also be precipitated by BRAFi treatment creating an environment which favours the survival of non-$BRAF^{V600}$ cells [36].

Preclinical studies demonstrate that acquired resistance to BRAFi therapy was associated with rapid recovery of MAPK signalling; this suggests that complete pathway inhibition may be

required for therapeutic effect and thus MEK inhibitors (MEKi) are now used as first line treatment in combination with BRAFi. This combination allows further inhibition of the MAPK pathway downstream of BRAF, and thus long-term inhibition of ERK (MAPK). Three phase III clinical trials demonstrated the clinical efficacy of BRAFi/MEKi combination therapy: the COLUMBUS trial compared the combination of Econrafenib + Binimetinib to Vemurafenib or Encorafenib monotherapy; and the COMBI-d and COMBI-v trials compared Trametinib and Dabrafenib combination therapy to Dabrafenib monotherapy. All demonstrated significant and clinically meaningful differences in OS when combinations were used, leading to these combinations to be approved as first line therapy [39,40]. Furthermore, MEKi monotherapy was shown to block BRAFi-induced hyperproliferative cutaneous events in squamous cell carcinoma mouse models, indicating that addition of MEKi may have a role in preventing both BRAFi resistance as well as paradoxical MAPK activation [41].

As well as directly contributing to melanoma cell growth and survival, a constitutively active MAPK pathway contributes to the immunosuppressive TME seen in melanoma tumours. Inhibiting this pathway with BRAFi and MEKi can therefore have immunomodulatory effects, not just by targeting MAPK signalling in melanoma cells, but by causing paradoxical activation in immune and stromal cells within the TME. The immunomodulatory activity of BRAFi may help to overcome the immunosuppressive microenvironment found in BRAF-mutant melanomas [23], an effect reversed with inhibitor resistance.

3. Immunosuppressive environment created by BRAF mutant melanoma

BRAF mutant melanomas can create a TME which promotes both tumour growth and immune evasion by a range of mechanisms, including the secretion of immunosuppressive factors and the recruitment of immunoregulatory cells, outlined in Figure 2A.

BRAF mutant melanomas may recruit immunosuppressive immune cells via the secretion of chemokines, such as CCL2 [42]. The receptor for CCL2, CCR2, is expressed by myeloid derived suppressor cells (MDSCs), regulatory T cells (T-regs) and monocytes, the latter of which are able to differentiate into macrophages upon entry into tumour tissue [43]. Together, these cells aid the establishment of a pro-tumour TME. For example, tumour associated macrophages (TAMs) are skewed towards a more immunoregulatory phenotype in BRAF mutant melanoma, as demonstrated by the expression of marker genes such as *Arg1*, *Chi3l3*, *Mr1* and *Mmp9* [44]. TAMs secrete factors such as: IL-10, which can promote the expansion of T-regs and establish a positive feedback loop with further polarisation of TAMs towards an immunosuppressive phenotype [45]; vascular endothelial growth factor (VEGF), which stimulates tumour growth, angiogenesis and promotes immunosuppressive TAMs [46]; and metalloproteases, which aid tumour growth and invasion [45]. As well as the recruitment of immunosuppressive cells, which secrete regulatory factors such as IL-6, IL-8, IL-10, TGF-β [47,48], cells within the TME demonstrate altered cell-cell signalling, for instance through impaired CD40:CD40L interactions. This prevents the maturation of APCs and CD8+ T cell activation [44]. The resultant effect of the combination of all the above mechanisms is increased immune evasion and therefore enhanced pathogenicity of melanoma lesions.

4. The immune effects of BRAF inhibition

Although not specifically designed to trigger anti-tumour immune responses, several preclinical and clinical studies (summarised in Table 1) suggest that MAPK pathway inhibition can alter the TME to improve anti-melanoma immune responses. It has been suggested that these effects may contribute to the anti-tumour efficacy of BRAFi. Indeed, immune related adverse events (irAEs), such as vitiligo and panniculitis, have been postulated as a predictive marker of response to BRAFi treatment [49]. The effects of BRAF inhibition on cancer cell

death may lead to the release of danger signals and of cancer-associated antigens. These could enhance anti-tumour immune responses by: 1) increasing inflammation and effector immune cell recruitment, e.g., cytotoxic T lymphocytes (CTLs); 2) altering the balance of cytokines to promote a more pro-inflammatory TME; and 3) enhancing the effector function of such immune cells, for example, by promoting antigen presentation. These effects are outlined in Figure 2B.

4.1 BRAF inhibition and immune cell recruitment

Multiple studies, in samples from both preclinical models of cancer and from patients undergoing BRAFi therapy, have shown that BRAFi promote tumour infiltration by lymphocytes [42,44,47,50,51]. Increased lymphocyte infiltration of melanoma, in particular CD8+ T cells, is correlated with more favourable outcomes [52]. BRAFi treatment has been associated with an increased ratio of cytotoxic CD8+ to CD4+ T cells, the latter of which may include immunosuppressive T-regs [42]. As well as promoting CTL infiltration, one small study looking at sequential melanoma biopsies demonstrated a direct effect of BRAFi on the expansion of T cell populations within the TME [53]. Together, these mechanisms may create a T cell population with a richer and more diverse T cell receptor repertoire [53]. Furthermore, immunosuppressive MDSC populations decrease with BRAFi [44,54,55]. BRAFi cause a reduction in CCL2 secreted by tumour cells [42]. Although CCR2 is expressed on T-regs and MDSCs, it is not expressed on natural killers (NK) cells and CD8+ T cells [42]. The reduction in CCL2 caused by BRAFi, therefore, may support the preferential recruitment of effector cells over immunoregulatory cells, aiding the anti-tumour response. Aside from changes in the CCL2/CCR2 chemokine pathway, BRAFi can also increase the expression of the chemokines CXCL9 and CXCL10, which promote the recruitment of NK cells and T cells [51,56]. Together these effects compound a change in the TME towards one which promotes anti-tumour responses.

Interestingly, the immune cell compartment can also be interrogated to predict response to MAPKi. One study of pre-treatment biopsies of melanomas from patients who subsequently were treated with BRAFi, with or without additions MEKi, demonstrated a correlation between high intratumoural CD8+ T cells with concomitantly low CD163+ myeloid cells, likely representing TAMs, and higher probability of response as well as longer PFS and OS, when compared to those with fewer infiltrating CD8+ T cells and high frequency of TAMs [57].

4.2 Changing the balance to create a more pro-inflammatory and less immunoregulatory TME

As well as altering the immune cell compartment, BRAFi can influence the inflammatory milieu of the TME, upregulating pro-inflammatory cytokines, such as IL-1 and IL-2, IL-15 and IL-18, which promote the activation of T cells and NK cells [51,56], whilst decreasing the secretion of cytokines such as IL-6, which contributes to cancer cell invasion, and IL-8, an important chemokine in melanoma growth, cell motility, invasion and angiogenesis [47,58,59]. BRAFi can reduce VEGF [56,60,61], a strong angiogenic mediator normally secreted by melanoma and tumour-associated stromal cells. Tumour-associated neovasculature is largely abnormal and tortuous. It can restrict the penetration of drugs, impair the infiltration of immune effector cells [62], and increase hypoxia within the TME which promotes immunosuppressive TAMs, T-regs and MDSCs [56,63]. Aside from this, VEGF has a direct effect on melanoma cell growth [64]. The reduced expression of VEGF seen with BRAF inhibition, along with decreased secretion of chemokines that attract “pro-tumour” immune cells, can therefore create a more hospitable environment for effector cell trafficking and effector function against cancer cells [46].

4.3 BRAFi and immune cell function

Not only can BRAFi recruit effector immune cells and create a more favourable environment for their activation and proliferation, but they can also improve the cancer killing mechanisms of such cells. The increased infiltration of CD8+ T cells seen with BRAFi is associated with

tumour shrinkage and necrosis, and improved survival in preclinical models, and BRAFi have been shown to actively improve CTL function, as measured by an increase of granzyme B and perforin, co-localised with CD8+ T cells [47,50]. A key mechanism driving enhanced CTL function is improved antigen presentation: BRAFi promote the maturation of antigen presenting cells, such as TAMs and dendritic cells (DCs), through enhanced CD40:CD40L signalling [44]; BRAFi decreases major histocompatibility complex (MHC-I) internalisation from the melanoma cell surface, thereby increasing the expression of MHC-I on tumour cells [65]; and BRAF inhibition has been reported to lead to increased expression of melanoma specific antigens: Glycoprotein 100 (gp100), Melanoma Antigen Recognized by T cells (MART-1), Dopachrome Tautomerase (DCT) and Tyrosinase related protein 1 (TYRP1), enhancing immune recognition of melanoma cells [47]. Aside from improving CTL function, it has been demonstrated that CD4+ T effector function (through the secretion of IFNγ), and CD4+ cell helper function (via signalling through CD40L), are increased with BRAFi [44].

5. Development of BRAFi resistance – the contribution of immune cells

Although a favourable TME following BRAFi treatment might suggest the creation of an effective immune response to the tumour, as mentioned, BRAFi resistance is common and typically observed clinically after around 6 months of treatment [10]. An important resistance mechanism is the paradoxical activation of MAPK pathways in BRAF wild type cells in the TME, including immune and stromal cells, as outlined in Figure 2C. For example, there is evidence to suggest that tumour-associated fibroblasts can be paradoxically activated by BRAFi and MEKi, leading to matrix remodelling [66] and the secretion of growth factors, such as hepatocyte growth factor (HGF), enabling the survival and growth of melanoma cells [67]. Importantly, due to their abundance in the TME and their pro-tumour functions, TAMs can also be paradoxically activated and are thought to be important contributors to BRAFi

resistance, secreting growth factors such as VEGF, which can in turn cause reactivation of MAPK pathway and enhance tumour growth [68].

As tumours develop resistance to MAPKi, the initial upregulation of melanoma antigens seen on initiation of BRAFi treatment is reversed, and melanoma antigen expression is reduced [69]. This results in reduced CD8+ cell activation and CTL function. Aside from this, the reactivation of MAPK leads to increased expression of PD-L1 on melanoma cells [70], which can impair T cell activation and aid immune evasion. Despite an initial increase in CD8+ T cell infiltration, at the point of progression on BRAFi, patient samples demonstrate a low immune infiltrate [71], and CD8+ T cells with an exhausted phenotype, with increased TIM-3 (T-cell immunoglobulin and mucin domain-containing protein 3) and PD-1 expression [47].

6. Overcoming BRAF resistance

Combinations of BRAFi and MEKi have been used to overcome BRAFi resistance, yet, since both inhibitor types act on the same pathway, resistance still develops by compensatory pathway activation, eventually leading to disease progression. The immunomodulatory effects of MAPKi create the potential for combining BRAFi with treatments which target cancer immunity, to promote a more sustained response to MAPK pathway inhibition, thus prolonging the time to resistance. We outline below potential combinations of both new and established treatments, that could offer the chance of longer-lived responses in patients (summarised in Figure 2D) and have summarised ongoing clinical trials in Table 2.

6.1 Combination with immune checkpoint inhibitors

The rationale for combining BRAFi/MEKi with ICI is attractive given the frequent but short-lived responses to BRAFi/MEKi, and the less predictable but more durable responses seen with ICIs. Preclinical studies have demonstrated that markers of T cell exhaustion, such as PD-1, are upregulated by BRAFi and MEKi. Combined with the fact that these targeted drugs do not

impact negatively on the viability, proliferation or signalling of human lymphocytes, suggests that BRAFi/MEKi could be utilised to prime the tumour and TME for therapy with ICIs [47,72]. Furthermore, there is evidence to suggest that BRAFi may in fact increase the effector function of human lymphocytes, potentially making the TME more amenable to immune activation with ICIs [73]. As discussed, BRAFi can be shown to increase CD8+ T cell tumour infiltration, however this infiltration diminishes with time. Timing of administration of ICIs, may therefore be optimised to coincide with this proinflammatory response to maximise potential anti-tumour efficacy [71]. Studies published to date have focused on concurrent administration of ICI and BRAFi/MEKi, the efficacy of which has been suggested in preclinical models, but in practice has been limited by unacceptable toxicity. [74,75].

Overlapping side effect profiles have caused several initial trials examining the combination of ICI and BRAFi/MEKi to be discontinued early. A phase I study combining Vemurafenib, and Ipilimumab closed to recruitment after six of the first ten patients developed grade 3 hepatic adverse events [76]. A further trial examining the role of doublet therapy of Dabrafenib and Ipilimumab, and triplet therapy of Dabrafenib, Trametinib and Ipilimumab, demonstrated that two of the seven patients receiving triplet therapy developed colitis with intestinal perforation [77]. The lower toxicity of BRAFi when given in combination with MEKi, along with the development of anti-PD-L1/PD-1 agents (themselves associated with lower rates of irAEs than CTLA-4 inhibition), has renewed interest in combination therapy, with a number of clinical trials in progress or recently completed.

The KEYNOTE-022 (NCT02130466) trial compared the combination of Dabrafenib, Trametinib and Pembrolizumab to Dabrafenib, Trametinib and placebo [78]. Whilst statistical significance was not reached for the primary end point of PFS, there was a numerical trend towards improved PFS. Median duration of responses was also numerically longer in triplet therapy than in the placebo arm. However, this was associated with a 58% rate of grade 3-5

treatment-related adverse events (trAEs), which would not typically be accepted in clinical practice [79]. Further to this, the findings of the TRIDeNT (NCT02910700) study, adding Nivolumab to Dabrafenib and Trametinib, showed encouraging efficacy signals at the cost of a 78% rate of grade 3-4 trAEs [80]. The COMBI-I trial (NCT02967692) did not demonstrate any benefit from the addition of the anti-PD-1 monoclonal antibody Spartalizumab to Dabrafenib and Trametinib, with the authors commenting that adverse event management was challenging and frequent dosing adjustments were required [81].

For BRAFi/MEKi/ICI combinations to be safely used in clinical practice there is a need to identify predictive biomarkers for toxicity as well as response. Pre-planned subgroup analyses within the TRILOGY/IMSpire150 trial (NCT0290867) evaluating combinations of Vemurafenib plus Cobimetinib with or without Atezolizumab (Table 2) suggested benefit in those patients with high serum LDH, more than three sites of metastasis and M1c disease, the latter referring to metastatic spread to locations other than the central nervous system [82]. It may be that the benefits of anti-tumour efficacy outweigh those of trAEs in patients with a higher disease burden [78,81]. In addition to improved patient selection, modifications to treatment dose and schedule with "lead in" or priming therapy, whereby BRAFi/MEKi are given prior to ICIs, or "on-off" dosing regimens where BRAFi/MEKi are given for short periods while patients are maintained on ICIs or vice versa, may lead to better tolerated regimens and more durable responses.

As discussed, preclinical studies support the principle of lead in/priming therapy with BRAFi/MEKi prior to ICI therapy. In a phase 1b study (NCT01656642) including a BRAFi/MEKi lead in/priming phase with Vemurafenib and Cobimetinib, followed by the addition of the antibody Atezolizumab (anti-PD-L1), CD4+ T cell proliferation was increased without an increase in T-regs, priming the TME for immunotherapy as demonstrated by increased helper T cells and CTLs in the TME following administration of Atezolizumab [83].

This has been recapitulated in the IMSpire150 trial, which demonstrated an improved median PFS, when Atezolizumab was added after a BRAF/MEKi run in, with little difference in adverse events reported. The US Food and Drug Administration (FDA) have now approved the combination, but it is yet to be approved by the European Medicines Agency (EMA) or the Medicines and Healthcare products Regulatory Agency (MHRA) in the UK [82] (Table 2).

The IMPemBra trial (NCT02625337) examined the impact of short term/intermittent dual MAPK pathway inhibition (Dabrafenib and Trametinib) with Pembrolizumab in the hope of reducing the high frequency of grade 3-4 trAEs in triplet therapy and high rates of treatment discontinuation. This trial demonstrated improved median PFS of patients from 10.6 months in the Pembrolizumab monotherapy arm, to 27.0 months in patients with Pembrolizumab combined with short term intermittent Dabrafenib and Trametinib therapy with improved tolerability compared to traditional triplet therapy [84]. There are ongoing studies exploring the appropriate sequencing of these agents within the DREAMseq (NCT02224781), SECOMBIT (NCT02631447) and NEOTRIO (NCT02858921) studies, and preliminary results have been recently published.

In contrast to preclinical studies, early data from the DREAMSeq trial seem to demonstrate that patients, in fact, benefit from ICI treatment prior to BRAF/MEKi. Their unique trial design enrolled patients to either receive Nivolumab/Ipilmumab or Dabrafenib/Trametinib until disease progression, whereby they switched to the other treatment. Superior OS was seen in the patients receiving ICI first, which became evident at 10 months, with more ongoing responses than patients who started with MAPKi [85]. In addition, preliminary results from the phase 2 SECOMBIT trial, which compared a similar treatment sequencing, appears to support the use of ICI prior to MAPK inhibition [86]. In addition to the treatment arms used in DREAMSeq, SECOMBIT has a third arm, in which patients receive priming with BRAFi/MEKi for eight weeks, before switching to ICI therapy until disease progression, and

switching back to treatment with BRAFi/MEKi thereafter. This third arm also demonstrated better outcomes compared with patients receiving BRAFi/MEKi until disease progression as their first treatment.

It is important to note that the initial advantageous early infiltration of T cells observed with BRAFi is no longer present at the time of disease progression, with a reduced CD8+ T cell population and a more pronounced pro-tumour, anti-inflammatory TAM gene expression profile [50,87,88]. In order to use BRAFi/MEKi to prime the TME for ICI treatment, a shorter priming phase, such as in the third arm of SECOMBIT might be included in trials. In this way, it may be possible to capture the effects of MAPKi at an optimum timepoint for activating the patient's immune response and thus augmenting ICI treatment, but before the onset of resistance.

6.2 Targeting immune cells that contribute to BRAFi resistance: myeloid cells

The importance of the infiltrating myeloid cell compartment in the development of BRAFi resistance has been demonstrated in studies which show how CCL2/CCR2 signalling is restored as BRAFi resistance develops, increasing the numbers of MDSCs and the frequency of TAMs in the TME [89]. Aside from CCL2/CCR2, signalling through colony stimulating factor 1 receptor (CSF1R), expressed on myeloid cells, is essential for their recruitment and maintenance in the TME. Small molecule inhibitors against CSF1R in combination with BRAFi, have demonstrated a reduction in tumour size in preclinical studies [90]. However, anti-CSF1R is less efficacious than BRAFi when used alone and is also expressed on other myeloid cells which may play beneficial roles in anti-tumour immunity [68]. Despite this, there are a two ongoing early phase clinical trials exploring the role of CSF1R inhibitors as monotherapy (NCT02071940, NCT02975700) and two in combination with BRAFi and MEKi (NCT03455764, NCT03101254) (Table 2).

6.3 Angiogenesis and BRAF resistance in melanoma

Angiogenesis is a recognised hallmark of cancer, and within melanoma it is associated with increased aggressiveness and worse prognosis [91]. The importance of VEGF signalling within tumorigenesis has been demonstrated across multiple cancer types including colorectal, ovarian and uterine cancers [91]. Antiangiogenic drugs bind either angiogenic factors (e.g., VEGF-A) or their receptors (e.g., VEGFR1/2). Antiangiogenic agents not only remodel irregular and leaky vessels in cancer lesions, resulting in improved tumour penetration of chemotherapy agents, but may also impact on the TME through relieving tissue hypoxia, which can alter immune cell composition [91]. The first trials involving antiangiogenic drugs within melanoma date back to almost two decades ago, when chemotherapy was the only available treatment for advanced stage disease; these trials often involved small population sizes [91]. Prior to the introduction of targeted and immune checkpoint inhibitors, the FDA, but not the EMA or the MHRA, had approved Bevacizumab, a monoclonal antibody recognising VEGF, plus cytotoxic chemotherapy as a first line treatment for unresectable melanoma [92,93]. There is significant interest in re-examining the role of VEGF in optimising current regimens with BRAFi/MEKi and ICIs. In the setting of BRAFi resistance, it is recognised that BRAFi can lead to the paradoxical activation of the MAPK pathway in non-mutant cells in the TME, including TAMs [68], driving these cells towards immunosuppressive phenotypes and, in the case of TAMs, increasing the secretion of VEGF, which in turn, can reactivate MAPK in melanoma cells [68,94]. Blocking VEGF and its interaction with VEGF-R-expressing cells, in conjunction with BRAFi delayed the time to treatment resistance in a mouse model of melanoma [94].

A number of clinical trials are studying this further (NCT01495988, NCT03175432) [91]. There have also been initial promising results within clinical trials combining immunotherapy and antiangiogenic agents which are now progressing to phase III studies (NCT04356729, NCT02681549, NCT03239145) [95,96] (Table 2). Exploring combinations of inhibitors such

as anti-VEGF, may still reveal novel mechanisms in the future that may help understand BRAFi resistance.

6.4 Enhancing Immune Effector Function: Adoptive Cell Therapies

Personalised cell therapy targeting tumour-associated antigens with expanded tumour-infiltrating lymphocytes (TILs) has shown some promise in metastatic melanoma since the early 1990s [97]. Adoptive cell therapy (ACT) of T cells, involving the allogenic transplant of TILs, and CAR-T therapy, whereby genetically modified T cells expressing novel T cell receptors, chimeric antigen receptors (CAR) are expanded and reinfused into the patient, have both shown promise in treating haematological malignancies but efficacy is yet to be realised in solid tumours, including melanoma [98].

In the latest Phase 2 study examining ACT using TILs in patients with treatment refractory melanoma, 36% patients had a partial response (22 of 66), with two patients having a complete response [99]. The safety profile was comparable to lymphodepleting chemotherapy, and this has led to studies evaluating combinations of ACT with other treatments. MAPKi have been suggested to enhance the clinical efficacy of ACT. In a preclinical study, BRAFi was shown to induce the upregulation of the mannose 6 phosphate receptor (M6PR) in a dose dependent fashion in both BRAFi sensitive and resistant melanoma cells. M6PR increases cancer cell uptake of granzyme B, a main component of the cytotoxic activity of CTLs. Thus, it has been suggested that it may be beneficial to treat with BRAFi prior to TIL therapy, as a means of enhancing ACT-mediated elimination of tumour cells [100]. A clinical study of 13 patients with checkpoint inhibitor resistant melanoma evaluated priming treatment with Vemurafenib prior to ACT with TILs. This study demonstrated significant clinical responses (one complete, eight partial responses, three patients with stable disease) and no unexpected toxicity [101]. While this may serve as proof of principle, larger trials with longer follow up times are required

in order to identify patients most likely to benefit, identify the most appropriate point of intervention and to establish durability of responses.

There has been significant interest in examining the role of CTL populations in anti-cancer therapy, however other cell types within the TME, including NK cells, can also engender tumour cell destruction. NK cells are of particular interest since their effector functions are not impaired as BRAFi resistance develops, and indeed their ability to lyse melanoma cells can increase in models of BRAFi-resistant melanoma [102,103]. Boosting the effector function of NK cells may delay BRAFi resistance and increase an effective anti-tumour response. The TLR7 agonist, imiquimod, already used for the topical treatment of non-melanoma skin cancers, has been shown to increase the activity of both NK cells and T cells in a murine model of melanoma [51] and this is currently being tested in clinical trials as an adjuvant to melanoma vaccine therapies and checkpoint inhibitors (NCT0436423, NCT04401995).

Aside from ACT, several large scale clinical trials using CAR-T cells for melanoma are on-going as a second line therapy after treatment failure with ICI or BRAFi/MEKi, however, the use of CAR-T in BRAFi resistant melanoma remains underexplored [104].

7. Conclusion

Although designed to impact on the growth, proliferation and survival of melanoma cells, MAPK pathway small molecule inhibitors such as BRAFi in clinical use for the treatment of BRAF-mutant melanoma can influence the immune composition of the TME. With immunotherapy dominating the landscape of melanoma therapeutics development, it is worth understanding how BRAFi can manipulate the immune response and how new and established treatments may be used sequentially or in combination to take advantage of cancer vulnerabilities and improve outcomes for patients.

8. Expert Opinion:

The past two decades have witnessed an unprecedented expansion in our understanding of the biology of melanoma, underpinning the development of new therapeutic classes: targeted oncogene inhibitors and immune checkpoint inhibitors. These developments have significantly improved the historically-low five-year survival rates in advanced melanoma. Within cancer therapeutics, however, the development of resistance is a frustrating, yet unsurprising, phenomenon, given the selection pressure that therapies apply on cancer cells.

The success of ICIs has highlighted the importance of understanding the dynamic interactions between tumour cells and the immune component of the TME. Research has largely focused on the role of BRAF as an oncogenic target, however there is evidence that MAPK inhibition significantly impacts on the TME by its effects in both immune and non-immune cells. In the next decade, research will reveal previously-unappreciated immune mechanisms which accompany response and resistance to BRAFi. These will enable us to better select treatment combinations or refine the sequence and timing of administering therapeutic agents to unlock more durable responses for our patients.

BRAFi initially leads to profound tumour regression, however responses are short lived. This contrasts with ICI, where responses are less predictable, but more durable. The approach within current clinical practice suggests that these treatments are mutually exclusive as first line therapy, with debate over which treatments should be used first. New evidence suggests that ICIs may prolong survival in patients with BRAF-mutant melanoma over MAPK inhibition [16], however, it does not follow that BRAFi and MEKi should be relegated to second line therapies, especially given that there are still significant limitations to ICI therapy. There remains a degree of unpredictability about which patients will respond to ICIs, and whether responses to treatment differ between patient cohorts. For example, patients of female sex and increasing age appear to derive less benefit from ICI than their younger and male counterparts

[105,106], whilst at the same time BRAFi appear to have comparable efficacy across age groups [107]. An arsenal of multiple first line therapies and patient stratification may help ensure more patients, from multiple cohorts, respond to treatment. In addition to this, it is important to understand how MAPKi can modulate the immune system, and with the correct timing, could be successfully used as an adjuvant not only to ICIs, but with other therapies too.

It is possible that different regimens of existing drugs, or better stratification of patients receiving them, may lead to better outcomes quicker than the development of novel therapies. For example, since BRAFi resistance in melanoma can lead to the upregulation of VEGF pathways, and VEGF inhibitors have shown efficacy in a range of solid tumours including renal, urological and ovarian cancers, it may be timely to reconsider the use of VEGF inhibitors alongside BRAFi. Early trials in melanoma were disappointing, a better understanding of why these therapies failed and how new treatments interact with them, may reveal how best to use them in the future. To take the first point, since the early initial trials of VEGF inhibitors, it has been demonstrated that the expression of VEGF decreases with aging [108]. This has been shown to reduce responses to anti-VEGF treatments in older patients, while the age-related proangiogenic factor secreted frizzled-related protein 2 (sFRP2) may be responsible for driving angiogenesis in older aged groups. Thus, better stratification of patients may lead to more successful implementation of tailored anti-angiogenic therapies and combinations. Alongside, since BRAFi resistance is characterised by the paradoxical increased secretion of VEGF, there is an opportunity for VEGF or other targeted anti-angiogenic inhibitors to work synergistically with a treatment which had not been discovered when VEGF inhibitors were initially trialled.

The success of ICIs has invariably led a focus on how to improve and augment the clinical efficacy of this class of drug, including how MAPKi can be used alongside ICIs. Increased understanding in how both MAPKi and ICIs alter the TME may provide answers to the current clinical problems arriving from combination treatment, namely: how can toxicity be overcome

and how can sequencing be optimised to prolong patient survival? New trial design, as seen in SECOMBIT and DREAMSeq, whereby different sequences of therapy are compared, will be invaluable in answering some of these questions. As well as clinical outcomes, the examination of biomarkers and biopsies throughout these studies will enable a better insight into the synergistic immune mechanisms that could underpin successful combinations of the two most revolutionary melanoma treatments of the decade.

The success of ICIs may represent the end of the beginning for drug discovery in melanoma rather than the beginning of the end. In cancers where ICI have failed, clinical trials re-evaluating old drugs, as well as those testing new therapies abound. In contrast, the focus within melanoma treatment appears to be optimising ICI regimens when we know that responses are unpredictable and not universal. However, refining and augmenting established treatment strategies, such as MAPK pathway inhibition hold significant merit.

Importantly, there must be continued investment and research into new druggable targets for melanoma. The discovery of new drugs will contribute to the transformation of therapeutic strategies for melanoma patients we have witnessed over the past decade. Revisiting old therapies in the context of recent conceptual and translational advances, may unearth combinations or sequential therapeutic protocols that can improve patient care and outcomes immeasurably.

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*** The latest clinical trial data studying the sequencing of ICIs and MAPKi when combined.**

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Figure Legends

Figure 1: The mitogen-activated signalling pathway – MAPK, from left to right: **A)** normal function of MAPK pathway signalling; **B)** $BRAF^{V600E}$ mutation leading to constituently activated MAPK signalling and enhanced pathogenesis of melanoma; **C)** targeting of MAPK pathway, with BRAFi and MEKi, reducing melanoma pathogenicity; **D)** mechanisms by which resistance can develop. Created with BioRender.com.

Figure 2: Potential immunological effects of BRAFi, BRAFi resistance and mechanisms to therapeutically enhance BRAFi: A) The immunosuppressive TME created in BRAF mutant melanoma includes the recruitment and expansion of immunosuppressive cells, effector cells with reduced cancer killing potency and the secretion of factors by immune cells which can increase cancer growth and angiogenesis; **B)** The pro-inflammatory TME created by BRAFi includes increased effector immune cell recruitment and expansion, reduced immunosuppressive immune cell recruitment, and increased ability of immune effector cells to trigger cancer killing; **C)** As BRAFi resistance develops, the reversal of BRAFi immune effects is observed; **D)** There are multiple mechanisms by which BRAFi resistance can be overcome using current and future therapies. These include depleting immunosuppressive cells such as tumour associated macrophages (TAMs), targeting angiogenesis and growth promoting vascular endothelial growth factor (VEGF), targeting immune exhaustion molecules with checkpoint inhibitors (PD-1/PD-L1), and enhancing cancer killing with adoptive cell therapy (ACT), including the use of chimeric antigen receptor T cell therapies (CAR-T), as well as stimulating effector cells, for example by using toll-like receptor agonists (TLR). Created with BioRender.com.

Table 1

BRAFi effect of immune function	Findings	Model Type	Papers/Refs
Immune stimulatory factors	Increased mRNA expression of IL-12a, IL-12b, IL-15 IL-18	*In vivo* mouse model	Bellmann et al [51]
	Increased mRNA expression of chemokines CCL2, CCL3, CCL4, CXCL9, CXCL10	*In vivo* mouse model	Bellmann et al [51]
	Increased production of IFNγ	*In vivo* mouse model	Ho et al [44]
Immunosuppressive factors	Decreased IL-6, IL-8	Tissue samples from patients	Frederick et al [47]
	Decreased PD-L1, IL-1, IL-8, VEGFA	*In vitro* human cell lines and *in vivo* mouse model	Liu et al [61]
	Decreased IL-7, CX3CL1, GCSF, CXCL1, TGFa2, IL-8, VEGF, IFNα	Human cell lines	Whipple et al [56]
	Decreased VEGF	Tissue samples from patients	Liu et al [60]
Immune cell infiltration	Increased TILs	Tissue samples from patients *In vivo* mouse model Tissue samples from patients *In vivo* mouse model + one patient follow up throughout treatment *In vivo* mouse model	Frederick et al [47] Knight et al [42] Wilmott et al [50] Cooper et al [75] Ho et al [44]
	Increased tumour infiltrating NK cells	*In vivo* mouse model *In vivo* mouse model	Bellmann et al [51] Knight et al [42]
	Increased CD8:CD4 ratio in TILs	*In vivo* mouse model + one patient follow up throughout treatment	Cooper [75]
	Decreased T-regs	*In vivo* mouse model	Knight et al [42]
	Decreased MDSCs	Blood samples from patients *In vivo* mouse model	Schilling et al [54,55] Ho et al [44]
Immune cell function	Increased melanoma antigen expression MART1, TYRP1, TYRP2, GP100 Increased MHC class I and II	Tissue samples from patients *In vivo* mouse model *In vitro* human cell lines and *in vivo* mouse model	Frederick et al [47] Bellmann et al [51] Liu et al [61]
		Human cell lines Human cell lines	Whipple et al [56] Bradley et al [65]

	Increased makers of T cell cytotoxicity: increased perforin, granzyme B	Tissue samples from patients Tissue samples from patients	Frederick et al [47] Wilmott et al [50]
	Increased maturation of APCs	*In vivo* mouse model	Ho et al [44]
	Increased expression of CD40L on CD4	*In vivo* mouse model	Ho et al [44]
Immune cells exhaustion	Increased PD-1, PD-L1 and TIM3	Tissue samples from patients	Frederick et al [47]
	Increased PD-L1 and PD-L2	*In vivo* mouse model	Cooper et al [75]

Table 1: BRAFi effect on immune function: preclinical data of the effect of BRAFi on the tumour microenvironment, specifically changes in immune mechanisms and pathways. TILS - tumour infiltrating lymphocytes; NK cells – natural killer cells; T-regs – regulatory T cells; MDSCs – myeloid derived suppressor cells; APCs- antigen presenting cells

Table 2

	Study (NCT)	Phase	Clinical Setting	Line of Treatment	Arms	Primary and Secondary End Points	Outcome
BRAFi/MEKi and ICI – Upfront Combination	Ribas et al (73)	1	$BRAF^{V600E}$ mutant positive metastatic melanoma	1st Line	Arm 1 (n=6): Vemurafenib (high dose) run in + Ipilimumab Arm 2 (n=6): Vemurafenib (low dose) + Ipilimumab	Evaluate safety and dose administration	Discontinued due to hepatotoxicity
	Minor et al (74) NCT01767454	1/2	$BRAF^{V600E}$ mutant positive unresectable or metastatic melanoma	Unspecified	Arm 1: Dabrafenib + Ipilimumab Arm 2: Dabrafenib + Trametinib + Ipilimumab	Evaluate safety of combinations	Discontinued due to intestinal perforation (n=2) in the triplet arm
	KEYNOTE-022 NCT02130466	1/2	Metastatic melanoma	Unspecified	BRAF mutant: Dabrafenib + Trametinib + Pembrolizumab BRAF wild type ($BRAF^{WT}$) continuous administration: Trametinib 1 month run in + Dabrafenib + Pembrolizumab $BRAF^{WT}$ intermittent administration: Trametinib intermittent + Pembrolizumab	Number of participants with dose limiting toxicities Objective response rate in those without the $BRAF^{V600E/K}$ mutations Progression free survival in those with the mutation	Phase 1: ORR 67% in BRAF-mutant Phase 2: (Dabrafenib + Trametinib + Pembrolizumab versus Dabrafenib + Trametinib + placebo) in BRAF mutant: ORR 63% versus 72% 73% of patients with grade 3-4 treatment related toxicities
	TRIDeNT NCT02910700	2	$BRAF^{V600E}$ mutant positive unresectable or metastatic melanoma (stage III/stage IV)	Unspecified	*Binimetinib: MEK 1/2 inhibitor* Arm 1: Dabrafenib + Trametinib + Nivolumab Arm 2: Trametinib + Nivolumab Arm 3: Binimetinib + Encorafenib + Nivolumab	Objective Response Rate Secondary: Incidence of adverse events Complete response Partial response	ORR 91% 21% discontinued study due to toxicity (hepatitis + nephritis)
	COMBI-I, NCT02967692	3, part 1	Previously untreated patients with unresectable locally advanced disease or metastatic melanoma $BRAF^{V600E}$ mutant positive	1st Line	*Spartiluzimab: anti-PD-1 antibody* Arm 1: Spartiluzimab + Dabrafenib + Trametinib Arm 2: Pembroluzimab + Dabrafenib + Trametinib	Safety run in Part 1 – evidence of dose limiting toxicities Biomarker cohort Randomised progression free survival	ORR 100% 22% discontinued Spartilizumab due to toxicity (hepatitis + transaminitis)

	TRILOGY, NCT02908672 (IMspire150)	3 (double blind, randomised)	Previously untreated patients with unresectable locally advanced disease or metastatic melanoma $BRAF^{V600E}$ mutant positive	1st Line	Placebo Arm: Vemurafenib + Cobimetinib (n=256) Experimental Arm: Vemurafenib + Cobimetinib + Atezolizumab (n=258)	Progression Free Survival Secondary: Percentage of participants with objective response rate Duration of response Overall Survival	Active HR = 0.72 (p=0.025)
BRAFi/MEKi and ICI – Lead In	NCT01656642	1b (open label)	$BRAF^{V600E}$ mutant positive melanoma	1st Line	Arm 1: Vemurafenib + Cobimetinib 1 month run in followed by Atezolizumab Arm 2: Vemurafenib + Cobimetinib + Atezolizumab	Percentage of Participants with Dose Limiting Toxicities Percentage of participants with adverse events Secondary: Pharmacokinetics Measurements Percentage of Participants with Objective Response	Enrolment complete ORR 85.3% 44.1% of patients with grade 3-4 treatment related adverse events
	NCT02027961	1	$BRAF^{V600E}$ mutant positive	Unspecified	*Durvalumab: anti-PD-L1 antibody* BRAF mutant: Dabrafenib + Trametinib + Durvalumab $BRAF^{WT}$ concurrent: Trametinib + Durvalumab $BRAF^{WT}$ sequential: Trametinib → Durvalumab	Number of participants with dose limiting toxicities and treatment related adverse events Secondary: Percentage of Participants with Objective Response Duration of Response Progression Free Survival	ORR 76% in BRAF mutant ORR 21% in $BRAF^{WT}$ concurrent ORR 50% $BRAF^{WT}$ sequential 39% of patients with grade 3-4 treatment related toxicities in BRAF – mutant, 40% in $BRAF^{WT}$concurrent, 17% in $BRAF^{WT}$sequential
BRAFi/MEKi and ICI – Cycling/Sequencing	IMPemBra (NCT02625337)	2	Stage IV $BRAF^{V600E}$ or $BRAF^{V600K}$ positive metastatic melanoma	First Line (n=32)	Arm 1: Pembrolizumab Arm 2: Pembrolizumab + Dabrafenib + Trametinib (short) Arm 3: Pembrolizumab + Dabrafenib + Trametinib (intermediate) Arm 4: Pembrolizumab + Dabrafenib + Trametinib (long)	Safety of different schemes of continuous/intermittent Dabrefinib + Trametinib during treatment with Pembrolizumab Feasibility of different schemes of continuous/intermittent Dabrefinib-Trametinib Secondary: Determine PFS Determine rates of response Determine long term toxicities	After a median follow-up of 17.4 months, the median PFS of patients treated with PEM monotherapy was 10.6 months compared to 27.0 months for patients treated with PEM and short-term/intermittent Dabrafenib + trametinib (p = 0.13)
	DREAMseq (NCT02224781)	3	Locally advanced unresectable melanoma or stage IV melanoma	Unspecified	Arm A: Nivolumab + Ipilimumab (induction), Nivolumab (maintenance) Arm B: Dabrafenib + Trametinib, followed by Nivolumab + Ipilimumab	Overall survival rate Secondary: Progression Free Survival Objective Response Rate Toxicity Rate for irAEs	Active, recruiting

					Arm C: Dabrafenib + Trametinib Arm D: Nivolumab + Ipilimumab (induction), Nivolumab (maintenance)		
	SECOMBIT (NCT 02631447)	2	Metastatic melanoma with theBRAF^{V600E} mutation	Unspecified	*BRAFi: LGX818* *MEKi: MEK162* *PD = progressive disease* Arm A: LGX818 + MEK162 until PD, followed by Nivolumab + Ipilimumab Arm B: Nivolumab + Ipilimumab until PD, followed by LGX818 + MEK162 Arm C: LGX818 + MEK162 for 8 weeks, Nivolumab + Ipilimumab until PD, then LGX818 + MEK162	Overall Survival Secondary: Total Progression Free Survival, best overall response rate and duration of response	Active, not recruiting
	NEOTRIO (NCT02858921)	2	BRAF^{V600} mutant resectable Stage III B/ III C melanoma	Unspecified	Three Arms: Arm 1: Dabrafenib + Trametinib, then Pembrolizumab Arm 2: Dabrafenib + Trametinib + Pembrolizumab (6w), then Pembrolizumab Arm 3: Pembrolizumab	Pathological Response Rate Secondary: Objective Clinical Response Rate Relapse Free Survival Overall Survival	Active, not recruiting
BRAFi/MEKi and antiangiogenic agent	NCT01495988	2	Stage IV BRAF^{V600E} positive melanoma	1st Line	Arm 1: Vemurafenib + Cobimetinib + Placebo Arm 2: Vemurafenib + Cobimetinib + Bevacizumab	Maximum tolerated dose Median Progression Free Survival Secondary: Overall Survival + Response Rates	Terminated – slow accrual/toxicity
	NCT03175432	2	Untreated melanoma with brain metastasis	1st Line	Arm 1: Bevacizumab + Atezoluzimab + Placebo Arm 2: Bevacizumab + Atezolizumab + Cobimetinib	Objective intracranial response rate Safety and tolerability of the arms in trial Secondary: Incidence of adverse events Overall response rates Duration of response	Active, recruiting
BRAFi/MEKi and cell-based therapy	NCT03455764	1 + 2	Advanced melanoma BRAF$^{V600E/K}$ positive	2nd Line (following BRAFi/MEKi treatment)	*Lacnotuzumab (MCS110): Colony-stimulating factor-1 (CSF-1) inhibitor antibody*	Dose Limiting Toxicity Secondary: Overall Response Rate Complete Response Rate	Active, not recruiting

					Lacnotuzumab + Dabrafenib + Trametinib		
	NCT03101254	1 + 2	Advanced melanoma $BRAF^{V600E/K}$ positive	1st Line	*LY3022855: Colony-stimulating factor-1 receptor (CSF-1R) inhibitor* LY3022855 + Vemurafenib and Cobimetinib	Progression Free Survival Secondary: Side Effects from Therapy Overall Response Rate	Active, not recruiting

Table 2: Clinical trials of combination therapies in metastatic melanoma with BRAFi/MEKi with another therapeutic intervention.

NCT = clinicaltrials.gov number

Figure 1

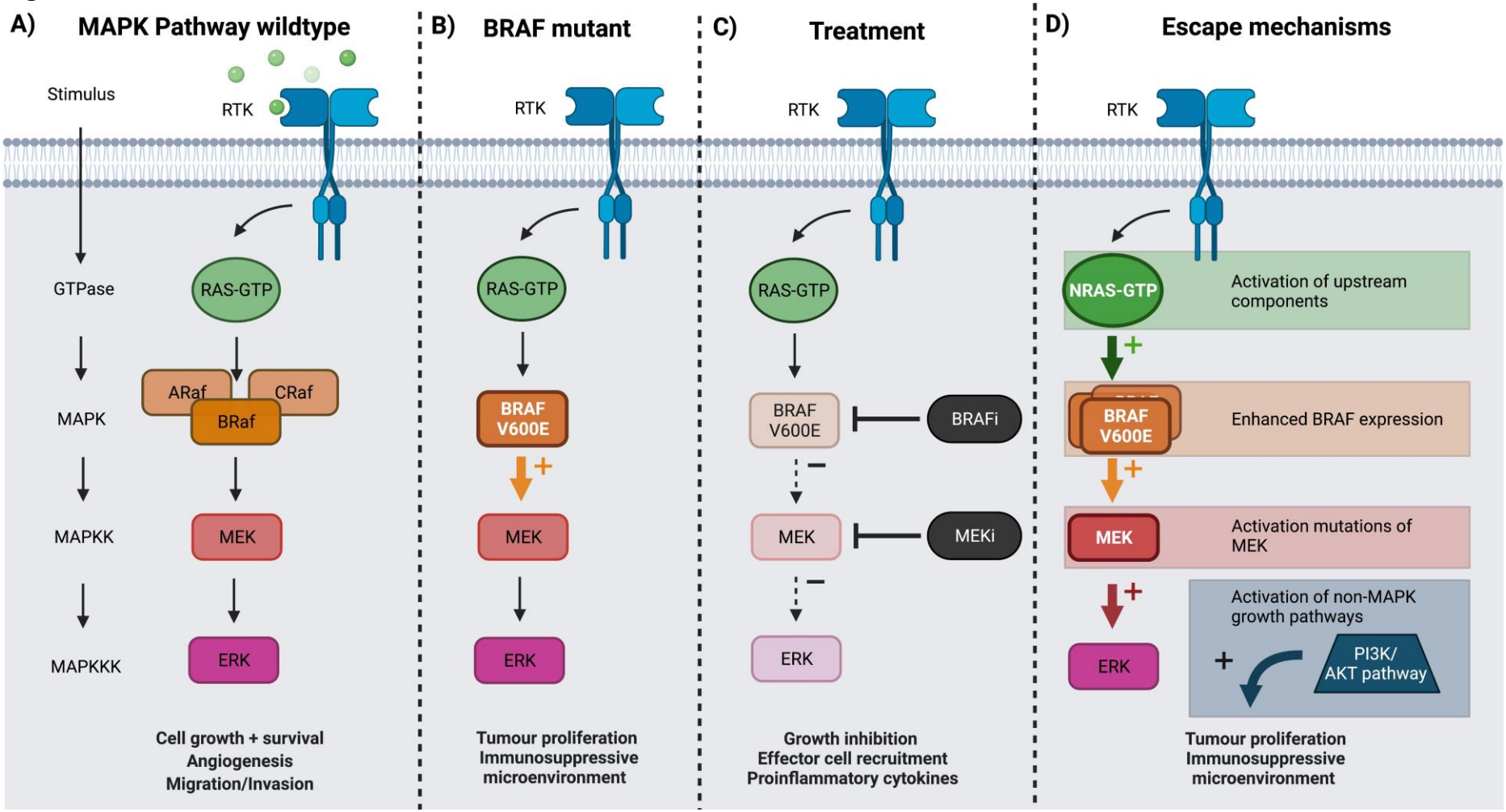
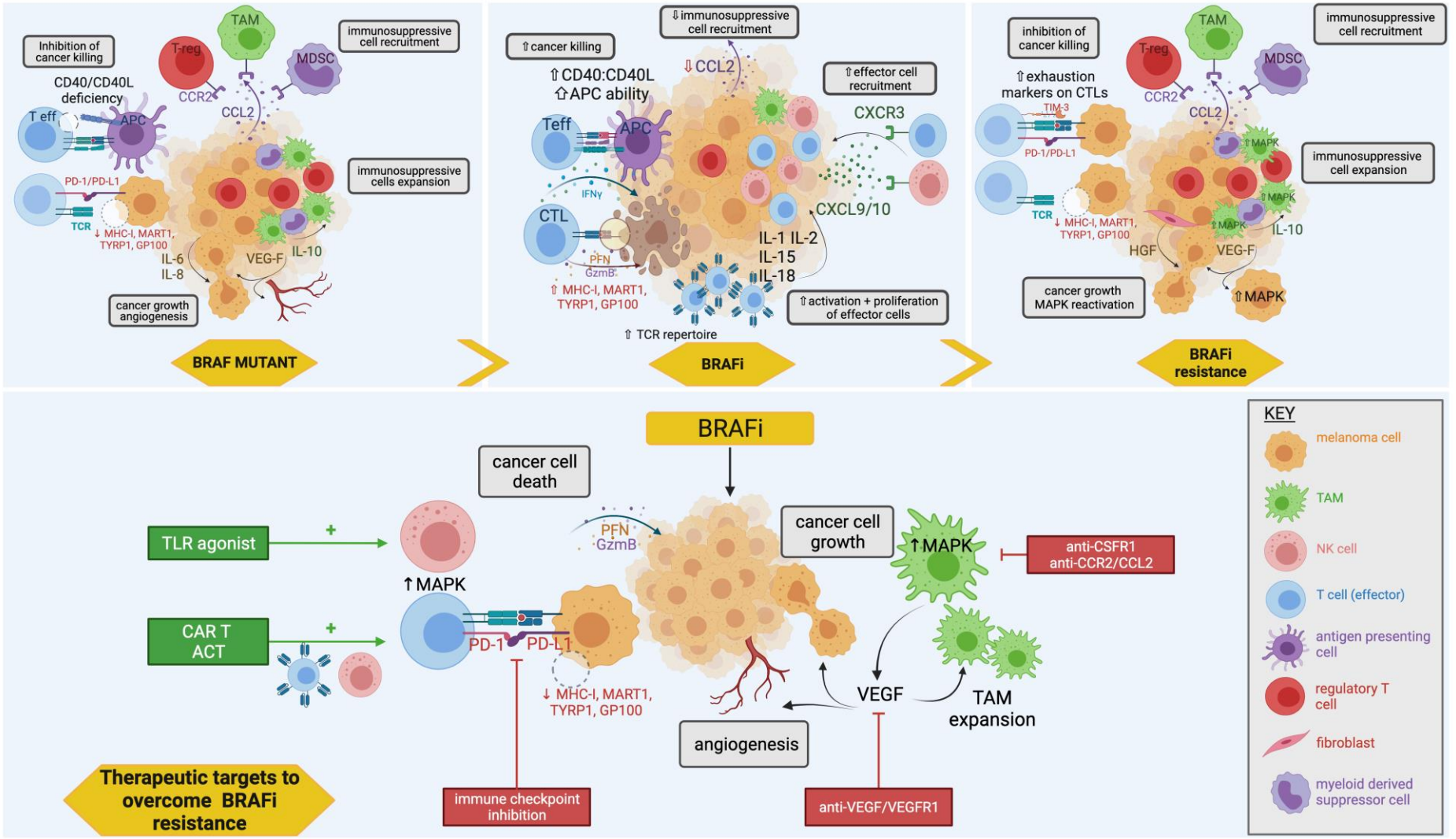

A)
MAPK Pathway wildtype
Stimulus
RTK
GTPase
RAS-GTP
MAPK
ARaf
CRaf
BRaf
MAPKK
MEK
MAPKKK
ERK
Cell growth + survival
Angiogenesis
Migration/Invasion
B)
BRAF mutant
RTK
RAS-GTP
BRAF V600E
MEK
ERK
Tumour proliferation
Immunosuppressive microenvironment
C)
Treatment
RTK
RAS-GTP
BRAF V600E
BRAFi
MEK
MEKi
ERK
Growth inhibition
Effector cell recruitment
Proinflammatory cytokines
D)
Escape mechanisms
RTK
NRAS-GTP
Activation of upstream components
BRAF V600E
Enhanced BRAF expression
MEK
Activation mutations of MEK
Activation of non-MAPK growth pathways
PI3K/ AKT pathway
ERK
Tumour proliferation
Immunosuppressive microenvironment

Figure 2


Inhibition of cancer killing
CD40/CD40L deficiency
T eff
APC
TAM
T-reg
MDSC
CCR2
CCL2
immunosuppressive cell recruitment
immunosuppressive cells expansion
PD-1/PD-L1
TCR
↓ MHC-I, MART1, TYRP1, GP100
IL-6
IL-8
VEG-F
IL-10
cancer growth angiogenesis
BRAF MUTANT
⇧cancer killing
⇩immunosuppressive cell recruitment
⇧CD40:CD40L
⇧APC ability
⇩CCL2
⇧effector cell recruitment
Teff
CXCR3
IFNγ
CTL
CXCL9/10
PFN
GzmB
IL-1 IL-2
IL-15
IL-18
⇧ MHC-I, MART1, TYRP1, GP100
⇧activation + proliferation of effector cells
⇧ TCR repertoire
BRAFi
inhibition of cancer killing
⇧exhaustion markers on CTLs
TIM-3
PD-1/PD-L1
TCR
↓ MHC-I, MART1, TYRP1, GP100
CCR2
CCL2
TAM
T-reg
MDSC
immunosuppressive cell recruitment
immunosuppressive cell expansion
⇧MAPK
HGF
VEG-F
IL-10
cancer growth MAPK reactivation
BRAFi resistance
BRAFi
cancer cell death
TLR agonist
CAR T ACT
↑MAPK
PD-1
PD-L1
PFN
GzmB
↓ MHC-I, MART1, TYRP1, GP100
cancer cell growth
↑MAPK
anti-CSFR1 anti-CCR2/CCL2
VEGF
TAM expansion
angiogenesis
Therapeutic targets to overcome BRAFi resistance
immune checkpoint inhibition
anti-VEGF/VEGFR1
KEY
melanoma cell
TAM
NK cell
T cell (effector)
antigen presenting cell
regulatory T cell
fibroblast
myeloid derived suppressor cell