An adaptive signaling network in melanoma inflammatory niches confers tolerance to MAPK signaling inhibition

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INTRODUCTION

Melanoma cells rely heavily on extracellular signal–regulated kinase (ERK)/MAPK signaling as indicated by hyperactivation of this pathway in up to 90% of melanomas. The MAPKKK BRAF is a prominent oncogene in melanoma (Davies et al., 2002), and inhibitors that target BRAFV600E, the most commonly mutated form, are extremely potent, eliciting high response rates (Flaherty et al., 2010; Chapman et al., 2011; Sosman et al., 2012). Despite this, durable responses are rare, and most patients relapse within a year after commencement of treatment (Salama and Flaherty, 2013). Significantly longer responses can be achieved by combining BRAF inhibitors (BRAFi’s) and MEK (MAPK/ERK kinase) inhibitors (MEKi’s), yet the development of drug resistance is still the most common outcome (Long et al., 2016). Acquisition of mutations affecting a variety of components of the RTK–RAS–RAF–MEK–ERK pathway, but also parallel pathways including the PI3K–AKT pathway, enable melanoma cells to resist MAPK signaling inhibition. Moreover, subclones of transformed cells from tumors at distinct anatomical sites, but also within a given tumor, possess different resistance-conferring mutations (Shi et al., 2014; Van Allen et al., 2014; Kemper et al., 2015), and
this inter- and intratumoral heterogeneity poses a formidable obstacle to the development of any salvage therapy. Consequently, focus has recently shifted to defining alterations in intracellular signaling, metabolism, chromatin structure, and gene expression that comprise early (hours to weeks) adaptive responses of cells to MAPK pathway inhibitors, which are reversible (that is independent of acquired mutations) and contribute to the ability of transformed cells to tolerate these therapeutic agents before acquired resistance takes hold (Smith and Wellbrock, 2016). Such adaptive responses can occur in a cancer cell–autonomous fashion (Johannesen et al., 2010; Nazarian et al., 2010; Villani et al., 2010; Poulikakos et al., 2011; Smith et al., 2013; Long et al., 2014). However, it also appears that factors elaborated by stromal and innate immune cells in the tumor microenvironment also enable melanoma cells to tolerate MAPK inhibition (Strausman et al., 2012; Smith et al., 2014; Hirata et al., 2015; Wang et al., 2015). Potentially, compared with mutation–driven events, tumors’ adaptive responses to drugs may be more stereotypical; simultaneously targeting adaptive responses and MAPK signaling might greatly diminish the burden of residual transformed cells, which could otherwise go on to evolve mutations conferring drug resistance (Smith and Wellbrock, 2016).

Importantly, in melanoma patients undergoing MAPK inhibitor treatment, we have shown previously that there is a greater macrophage abundance within the tumors compared with pretreatment (Smith et al., 2014). Macrophages are the major producers of the proinflammatory cytokine TNF, and we and others have shown that TNF not only is important for melanoma growth and invasion, but also contributes to tolerance to MAPK inhibition (Katerinaki et al., 2003; Gray-Schopfer et al., 2007; Smith et al., 2014). However, TNF is not the only proinflammatory cytokine produced by macrophages, and the increased number of macrophages during treatment with MAPK inhibitors might impact drug efficacy through additional factors. One such factor that is closely linked to TNF and produced by macrophages in abundance is IL-1. IL-1 exists as two isoforms, α and β, which both signal via the IL-1 receptor (IL-1R) and the transcription factor NF-κB. However, whereas IL-1α is widely and constitutively expressed and initiates inflammation when passively released from necrotic cells, IL-1β expression is more restricted. Furthermore, unlike IL-1α, the pro-form of IL-1β requires cleavage by caspase 1, which is, in turn, activated by the NLRP3-containing inflammasome, to become active (Garlanda et al., 2013).

Studies on IL-1 expression in established human melanoma cell lines are inconsistent, ranging from constitutive IL-1β expression and secretion only in metastases–derived cells (Okamoto et al., 2010) to constitutive IL-1α and IL-1β expression in the majority of melanoma cell lines independently of disease stage (Qin et al., 2011) and to no IL-1β secretion at all because of lack of expression of one or more inflammasome components (Gehrke et al., 2014). Although these findings do not provide a clear role for IL-1 in isolated melanoma cells in vitro, immunohistochemistry studies imply that IL-1α is uniformly expressed in naevi, primary tumors, and metastases (Qin et al., 2011; Khalili et al., 2012) and, thus, is unrelated to disease progression. In contrast, IL-1β is undetectable in naevi and rarely detected in primary tumors (<10%) but is elevated in metastases (Okamoto et al., 2010; Qin et al., 2011; Khalili et al., 2012; Gehrke et al., 2014). Interestingly, intense IL-1β expression is observed in discrete cells within the tumor, mooted to be melanophages (Gehrke et al., 2014).

A role for host-derived IL-1β, and to a lesser extent IL-1α, in the neovascularization and metastasis of melanoma allografts has been established using recombinant mice (Voronov et al., 2003). Considering that the abundance of macrophages within tumors increases in patients during treatment with MAPK inhibitors and that macrophages can protect melanoma cells from the growth inhibitory effects of MAPK inhibitors (Smith et al., 2014; Wang et al., 2015), we wanted to assess the role of IL-1 signaling in melanoma growth and in the context of MAPK pathway antagonism.

RESULTS

IL-1 and IL-1R1 expression is enriched in the tumor stroma

First, we confirmed the presence of an inflammatory microenvironment in melanoma and detected increased IL1A and IL1B expression in stage–III and stage–IV melanoma patient samples (Fig. 1 A). Up-regulation of IL1B in melanoma was corroborated by microarray data (Talantov et al., 2005) analyzed through the Oncomine platform, demonstrating elevated expression in primary cutaneous melanoma compared with normal skin and benign nevi (Fig. 1 B).

Next, we performed immunohistochemical analysis to assess which cells in the melanoma microenvironment were responsible for the expression of IL-1β. Confirming previous observations (Gehrke et al., 2014), we observed intense staining within discrete cells dispersed throughout tumors (Fig. 1 C, i). Analyzing these specimens for expression of the macrophage markers CD163 and CD68, in combination with normal skin and benign nevi (Fig. 1 B).

To determine which cells within the tumor might be responding to IL-1 stimulation, we performed immunohistochemical analysis for IL-1R1 expression in specimens taken from patient skin metastases. Importantly, IL-1R1 expression was not detectable in melanoma cells. Instead, we observed receptor expression both in endothelial cells (Fig. 1 D) and in fusiform stromal cells (Fig. 1 D, ii and iii, indicated by arrowheads), which coexpress α-smooth muscle actin (α-SMA; Fig. 1 D, iii and vi), revealing them to be melanoma–associated fibroblasts. Thus, stromal cells are the principal candidates responding to IL-1 signaling in melanoma.

To corroborate our findings from immunohistochemical analyses, we analyzed a panel of human melanoma cell
lines for the expression of both the IL-1β precursor protein and IL-1R1 by Western blot analysis (further details on the mutational status and origin of the melanoma cells used are outlined in Table S1). In agreement with our findings in melanoma biopsies, we found that established melanoma cell lines express only very low levels of IL-1R1 if any, whereas human foreskin fibroblasts (HFFs) expressed high levels of IL-1R1 (Fig. 1 E). Furthermore, IL-1β precursor protein expression in melanoma cells was negligible (Fig. 1 E), and these cells did not secrete the active, cleaved form of the IL-1β protein (Fig. 1 F). Also, fibroblasts did not express or secrete IL-1β (Fig. 1, E and F). However, as expected, activated macrophages (activated with IFN-γ and LPS) express the precleaved protein (Fig. 1 E) and secrete the active form (Fig. 1 F). This supports the candidacy of macrophages as the primary source of IL-1β in the melanoma microenvironment, in line with
Our observations in melanoma biopsies. Interestingly, although ordinarily undetectable, we observed IL-1α precursor protein expression in melanoma cells infected with mycoplasma (unpublished data). This suggests that, under normal growth conditions, melanoma cells do not produce significant amounts of precleaved IL-1α, and yet, they have the capacity to do so when stressed.

**Stromal IL-1–IL-1R1 signaling contributes to melanoma growth**

In line with the increase in IL1B observed in stage-III and stage-IV melanoma (Fig. 1 A) and a role for macrophages as the predominant source and fibroblasts as potential recipients of the cytokine signal, we found an increase in expression of the pan-macrophage marker CD68 and the cancer-associated fibroblast marker SMA in patient melanoma samples compared with normal skin (Fig. 2 A). Moreover, by analyzing melanoma samples for SMA and both CD163 and CD68 expression using immunohistochemistry, we observed fibroblasts and macrophages localized together in bands of connective tissue traversing melanoma metastases taken from skin and lung (Fig. 2 B). Thus, melanoma tumors appear to contain IL-1α-signaling inflammatory niches, a configuration where cross talk between macrophages and stromal cells may be optimized.

To test the importance of IL-1 signaling within the host stroma for melanoma growth, we injected 4434 BrafV600E melanoma cells, derived from melanoma-bearing BrafV600E mice (Dhomen et al., 2009), into either syngeneic control (IL-1r1fl/fl) or recently generated IL-1r1−/− mice (Abdulaal et al., 2016), and then compared these with normal skin (Fig. 2 A). Moreover, tumors in IL-1r1−/− mice grew significantly slower than tumors in control mice, resulting in a profound reduction in tumor size at 28 d after injection (Fig. 2 C). This finding confirms a role for IL-1 signaling in melanoma growth (Voronov et al., 2003) and, furthermore, reveals that a major part of the tumor growth support relies on IL-1 signaling in the host stroma. Monocyte numbers were previously shown to be normal in IL-1R1−/− mice (Abdulaal et al., 2016), and we now show that bone marrow mononuclear cells derived from IL-1R1−/− mice can be induced to differentiate ex vivo into macrophages comparable with bone marrow mononuclear cells derived from control mice (Fig. 2 D). We further show that these macrophages both express and secrete levels of IL-1α comparable with macrophages derived from control mice when stimulated with LPS and IFN-γ (Fig. 2 E). Moreover, immunohistochemical analysis to detect IBA1/ AIF1 indicated comparable infiltration by macrophages into tumors that grew in IL-1R1−/− mice as compared with control mice, as immunohistochemical analysis to detect SMA indicated comparable recruitment of fibroblasts (Fig. 2 F). Thus, differences in macrophage and fibroblast recruitment to tumors growing in IL-1R1−/− compared with control mice is not responsible for the difference observed in tumor growth, implicating a deficiency in stromal IL-1 responsiveness for the reduction in tumor growth.

**Melanoma cells induce IL-1β production by macrophages**

To dissect the cross talk occurring among melanoma cells, macrophages, and fibroblasts in the tumor, we set up an in vitro system using conditioned media from melanoma cells and macrophages (Fig. 3 A). Because we found that melanoma cells do not produce significant amounts of IL-1α themselves (Fig. 1 E) and melanoma cells have previously been shown to stimulate monocyte differentiation into macrophages (Wang et al., 2012), we hypothesized that melanoma cells might trigger IL-1β production and secretion in macrophages. To test this, we cultured human monocytes isolated from peripheral blood in melanoma cell–conditioned medium (Mel-CM) for 7 d (Fig. 3 A). During this time, the morphology of the monocytes became strikingly different to those left untreated, cultured with M-CSF, or cultured in media taken from normal human melanocyte (NHM) cells. In contrast to these control-treated cells that had a typical round fried egg morphology, Mel-CM–treated cells adopted an elongated and dendritic morphology (Fig. 3 B), as described previously (Wang et al., 2012). Moreover, the macrophages displayed high expression of both the precleaved and cleaved IL-1β protein 24 h after the end of the 7-d differentiation phase in Mel-CM (Fig. 3 C). This correlated with high IL-1β secretion by the macrophages, and both the protein expression and secretion were still detectable 48 h after the 7-d differentiation phase (Fig. 3 C). The persistence in IL-1β production even in the absence of Mel-CM suggests that the macrophages may be permanently differentiated. We also found that mouse bone marrow mononuclear cells treated with M-CSF, and thus differentiated into macrophages, also expressed and secreted IL-1β when incubated for a further 24 h in conditioned media from 4434 mouse melanoma cells (Fig. 3 D), whereas cells incubated in conditioned media from untransformed 3T3 cells expressed and secreted low levels of IL-1β (Fig. 3 D). Notably, this phenomenon was consistent in macrophages from both IL-1R1β/β and IL-1R1−/− mice. These findings point to melanoma cells playing a role in stimulating monocytes to adopt a proinflammatory macrophage phenotype, which results in IL-1β production, among other factors.

**Melanoma cells initiate an IL-1α-mediated cross talk between macrophages and fibroblasts that is disrupted by IL-1R1 ablation**

With fibroblasts being the potential recipients of the IL-1 signal in the melanoma microenvironment, we wished to assess the effects of IL-1R1 activation in fibroblasts. For this, we profiled the secretome of fibroblasts stably overexpressing IL-1 using a cytokine antibody array and observed profoundly increased levels of GROα, IL-6, and IL-8 (Fig. 4 A). In line with these data, when we treated human fibroblasts with recombinant IL-1β over a 6-h time course, we observed up-regulation of GROα, IL-6, and IL-8 proteins, accom-
panied by NF-κB phosphorylation (Fig. 4 B). Because we hypothesized that macrophages trigger IL-1R1 signaling in fibroblasts, we next tested the ability of the Mel-CM–treated macrophages to stimulate these cells (Fig. 3 A). As observed with isolated IL-1β, we found that fibroblasts cultured in conditioned media taken from Mel-CM–differentiated macrophages showed a strong induction in expression of IL-6, IL-8, and GROα (Fig. 4 C). Importantly, this expression was inhibited using an IL-1β–neutralizing antibody, indicating that the induction of IL-6, IL-8, and GROα was dependent on macrophage-derived IL-1β. Of note, macrophages that had been cultured in NHM-conditioned media were not able to stimulate cytokine production in fibroblasts, doubtless because of the lack of IL-1β production (Fig. 3 C and Fig. 4 C).

These data clearly demonstrate that fibroblasts can respond to IL-1β with the production of growth factors such...
as GROα, and this response would be abolished in the absence of an intact IL-1R1. In line with this, we found that GROα expression was significantly reduced in tumor sections obtained from Il-1r1−/− mice compared with Il-1r1fl/fl mice (Fig. 4 D, i and ii). Real-time quantitative PCR (qPCR) analysis to measure Groα mRNA expression also confirmed this result (Fig. 4 E). Additionally, GROα was frequently coexpressed in SMA-expressing cells in Il-1r1fl/fl mice (Fig. 4 D, iii), implicating fibroblasts as the primary source of GROα in melanoma.

**IL-1β expression correlates with IL-8 and GROα expression in melanoma**

Altogether, these data provide evidence for a relay of signals among melanoma cells, macrophages, and fibroblasts in the melanoma microenvironment. As IL-6, IL-8, and GROα have already been demonstrated to be important cytokines for melanoma growth and progression (Schadendorf et al., 1993; Bar-Eli, 1999; Haghnegahdar et al., 2000; Huang et al., 2002; von Felbert et al., 2005; Varney et al., 2006), this provides a link between IL-1 signaling in the stroma and melanoma growth support. In line with this, we observed a marked increase in IL8 and GROα mRNA expression in human melanoma samples (Fig. 5, A and B). The expression of both IL8 and GROα in primary melanomas correlated strongly with I1B expression (Fig. 5, C and D), consistent with their expression being largely dependent on I1B expression. Immunohistochemical analysis of GROα expression in specimens taken from patient skin metastases revealed that fibroblasts are one of the major producers of GROα in melanoma (Fig. 5 E, i–iii), substantiating earlier observations in mouse tumors (Fig. 4 D).

**IL-1β–activated fibroblasts confer tolerance to BRAF/MEK combination therapy through NF-κB and BCL2**

As mentioned earlier, we had previously detected increased macrophage abundance in BRAFV600E–positive melanomas from patients that had been treated with BRAF and MEK inhibitors.
for 10–14 d (Smith et al., 2014). As we had identified macrophages as a crucial source of IL-1–induced growth support signals, we wanted to analyze IL-1 expression in these patient samples (for further patient details, see Table S2). This analysis revealed a decrease in IL1A expression in patients on treatment compared with pretreatment (Fig. 6 A, left), consistent with previous observations and with IL1A being a MAPK signaling target (Khalili et al., 2012). However, we detected a clear increase in IL1B expression in the majority of samples from patients on treatment compared with pretreatment (Fig. 6 A, right), consistent with our previous finding of increased macrophage abundance in patient tumors on treatment (Smith et al., 2014). Real-time qPCR analysis also demonstrated increased Il1b mRNA in 4434-derived mouse allograft tumors treated with MEKi (Fig. 6 B).

The increase in macrophage abundance and IL1B expression on treatment could contribute to the adaptive response of melanoma cells that promotes treatment tolerance (Smith and Wellbrock, 2016). However, in contrast to TNF, which has been shown to directly prevent melanoma cell death in the presence of BRAF signaling inhibition, IL–1β cannot prevent cell death when BRAF signaling is inhibited (Gray-Schopfer et al., 2007; Smith et al., 2014). Nonetheless, because macrophage-derived IL–1β can activate fibroblasts to produce cytokines that could hypothetically protect against MAPK inhibitors, we subsequently examined the ability of melanoma cells exposed to fibroblast-conditioned media (Fib–CM) pretreated with IL–1β (IL–1β–Fib–CM; Fig. 6 C) to tolerate the BRAFi vemurafenib, the pan-RAF inhibitor RAF265, the MEKi selumetinib, or, indeed, a combination of these therapeutics. In line with previously published work and a role for secreted factors in ERK reactivation upstream of MEK (Straussman et al., 2012), we found that A375 cells cultured in medium from unstimulated fibroblasts were protected to an extent against BRAF inhibition, but the factors present in the medium were not sufficient to protect from a combination of BRAF and MEKi treatment (Fig. 6 D). However, A375 cells cultured in IL–1β–Fib–CM were protected not only from BRAF inhibition, but also from BRAF/MEKi combination (Fig. 6 D). Moreover, IL–1β–Fib–CM also protected A375 cells from pan-RAF inhibition (Fig. 6 E). Similar effects were observed in WM266-4 and 4434 melanoma cell lines (Fig. 6 F).

BRAF inhibition resulted in loss of ERK phosphorylation, but this was rescued when cells were cultured in media taken from either unstimulated fibroblasts or IL–1β–activated fibroblasts (Fig. 6 D), as previously described (Straussman et al., 2012). However, ERK reactivation was not observed when melanoma cells were treated with BRAF/MEKi combination therapy (Fig. 6 D) and, similarly, when treated with...
a pan-RAF inhibitor (Fig. 6 E). Thus, our data confirm that fibroblasts can protect melanoma cells from BRAF inhibition through reactivation of the MAPK pathway. However, we demonstrate that, when activated by IL-1β, fibroblasts can protect melanoma cells from MEK inhibition through an ERK-independent mechanism.

To more closely model heterotypic cell interactions in the tumor microenvironment, we cultured A375 and WM266-4 melanoma cells in media taken from fibroblasts that had themselves previously been cultured in conditioned media taken from Mel-CM–differentiated macrophages. We found that the melanoma cells were indeed protected against BRAF and MEK inhibition (Fig. 6 G), although protection was lost if macrophage-conditioned medium was preincubated with IL-1β–neutralizing antibody or IL-1 receptor antagonist (IL-1RA; Fig. 6 G), further confirming a role for macrophage secretion of IL-1β in protecting melanoma cells against MAPK inhibitors.

We further confirmed that IL-1β–Fib-CM–induced tolerance to MAPK antagonism is not PI3K dependent, as IL-1β–Fib-CM also protected A375 cells from a BRAF/MEK/AKT inhibitor combination (Fig. 7 A). This effect was also observed in WM266-4 cells (Fig. 7 B) and 4434 cells (Fig. 7 C). Next, we analyzed how IL-1β–Fib-CM enables melanoma cells to overcome MAPK inhibition in an ERK-independent manner. We found that NF-κB p65 phosphorylation and BCL2 expression were increased in melanoma cells treated with IL-1β–Fib-CM (Fig. 7 D).
Importantly, this was not affected by BRAF/MEKi combination treatment (Fig. 7 D). The fact that an IkB kinase inhibitor (Fig. 7 E) or a BCL2 inhibitor (Fig. 7 F) could overcome the protective effect conferred by IL-1β–Fib-CM suggests that NF-κB activation and BCL2 up-regulation contribute to the survival signals.

IL-1β–activated fibroblasts protect melanoma cells from MAPK inhibition by signaling through the CXCR2 receptor

To test the importance of IL-1β–mediated stromal signals in conferring tolerance to MAPK inhibition in vivo, we again injected 4434 into Il-1r1fl/fl and Il-1r1−/− mice and analyzed tumor growth in the presence of MEKi. Whereas MEK inhib-
bition in control mice resulted in ∼24% reduction in tumor growth, ∼81% reduction was observed in Il-1r1−/− mice treated with MEKi (Fig. 8 A), clearly demonstrating that stromal IL-1 signals are important in promoting tolerance to MAPK inhibition in melanoma.

Next, we wished to dissect the stromal signaling that IL-1 induces to promote MAPK inhibitor tolerance. As we have shown that IL-1β stimulates IL-6, IL-8, and GROα production in fibroblasts, these cytokines could potentially contribute to the stromal-derived tolerance. However, we found that IL-6 induced growth inhibition in melanoma cells (unpublished data) and was therefore deemed an unlikely candidate. GROα and IL-8 are both ligands for the CXCR2 receptor, so to assess whether CXCR2 plays a role in the inflammatory niche–mediated tolerance, we used A375 cells in which receptor expression is depleted by expression of a CXCR2 targeting shRNA (A375 CXCR2 knockdown [CXCR2KD] cells; Fig. 8 B). Whereas IL-1β–Fib-CM offered significant protection against BRAF/MEK combination treatment in A375 cells, in A375 CXCR2KD cells, this protection was lost (Fig. 8 C). A375 CXCR2KD cells grew at a slightly slower rate than A375 cells (Fig. 8 C), which could be linked to a basal growth-promoting role of CXCR2 signaling (Schadendorf et al., 1993; Singh et al., 1994; Haghnegahdar et al., 2000).

Given the potential role for CXCR2 in the inflammatory niche–mediated tolerance and because IL1B expression was up-regulated in tumor biopsies from patients after 10–14 d of treatment with BRAF and MEKi's, we analyzed these tumors for CXCR2 and its ligands GROα and IL8. We observed an increase in CXCR2 and GROα expression (Fig. 8 D). However, we only found a slight increase in IL8 expression and, in several cases, even a reduction in IL8 expression in patients on treatment (Fig. 8 D), which confirms previous observations (Sannamed et al., 2014; Wilmott et al.,...
This renders IL-8 an unlikely candidate for the stimulation of CXCR2 in the presence of MAPK antagonists. Furthermore, qPCR analysis revealed increased Groα mRNA in MEKi-treated A375 allografts compared with vehicle-treated controls (Fig. 8 E, left), and specifically, mouse Groα mRNA and not human Groα mRNA was up-regulated in MEKi-treated A375 xenografts (Fig. 8 E, right; Smith et al., 2013), confirming the tumor stroma as the source of Groα.

In line with these findings, Groα and IL-8 were able to protect melanoma cells from BRAFi- and MEKi-induced death. Addition of IL-8, Groα, or a combination of both in the presence of BRAFi and MEKi increased the 50% effective concentration by approximately fourfold (from 0.01 to 0.04 µM), ninefold (from 0.01 to 0.09 µM), and 25-fold (from 0.01 to 0.25 µM), respectively, in A375 cells (Fig. 9 A). Therefore, Groα conferred more protection than IL-8, but the combination of the two cytokines offered the best protection, confirming our finding that Groα is the more likely candidate for the stimulation of CXCR2. This protective effect was lost in A375 CXCR2KD cells (Fig. 9 B). As anticipated, treatment with IL-1β alone did not confer any protection from BRAF/MEKi-induced cell death in A375 cells (Fig. 9 C). Groα, IL-8, and a combination of Groα and IL-8 also protected WM266-4 and 4434 cells with similar effect (Fig. 9, D and E), although IL-8 did not appear to offer any protection to WM266-4 cells.

**CXCR2 inhibition synergizes with MEK inhibition in vivo to significantly reduce tumor growth**

Our data emphasize that IL-1β cannot directly induce tolerance to MAPK inhibition in melanoma cells but requires signaling through CXCR2. Furthermore, we found that
CXCR2 expression is up-regulated in the majority of tumors in patients on treatment with MAPK inhibitors (Fig. 8 D). Thus, pharmacologically inhibiting CXCR2 signaling represents an attractive therapeutic approach that would prevent IL-1β-activated fibroblasts from protecting melanoma cells from MAPK inhibition. Indeed, using the potent and highly selective CXCR2 inhibitor SB225002 (Bento et al., 2008; Manjavachi et al., 2010) resulted in a significant loss of IL-1β–Fib-CM–mediated protection from BRAF/MEKi combination treatment in A375 (Fig. 10 A), WM266-4 (Fig. 10 B), and also 4434 (Fig. 10 C) cells. In line with this, CXCR2 inhibition blocked the IL-1β–Fib-CM–induced p65 phosphorylation and BCL2 up-regulation (Fig. 10 D). Together, these data suggest that interfering with CXCR2 signaling could be very effective in improving responses to MAPK inhibitor therapy.

Thus, to test the effect of CXCR2 inhibition in vivo, we again used the 4434 allograft melanoma model. We treated 4434 allograft–bearing mice with an MEKi alone or in combination with the CXCR2 inhibitor sch-527123 (navarixin), which has been optimized for clinical use (Holz et al., 2010; Nair et al., 2012). A significant reduction in tumor growth was observed in mice treated with the combination of navarixin and MEKi compared with either treatment alone (Fig. 10 E). This suggests that targeting CXCR2 in combination with MAPK signaling could improve initial responses to MAPK inhibitors in melanoma patients.
The biggest hurdle yet to be overcome for the treatment of disseminated melanoma using targeted therapies is the emergence of resistant disease. It is clear from our data and previous studies (Straussman et al., 2012; Smith et al., 2014; Hirata et al., 2015; Wang et al., 2015) that innate immune cells and stromal cells of the melanoma microenvironment play a role in this process in allowing melanoma cells to rapidly tolerate MAPK signaling inhibition before genetic mechanisms of resistance are acquired. We show that macrophages and fibroblasts are located in inflammatory niches in melanomas and are responsible for elevated IL-1 signaling in the melanoma stroma. We show that cross talk between melanoma cells, macrophages, and fibroblasts initiates an IL-1 signaling cascade that generates a CXCR2-stimulating secretome, which ultimately leads to enhanced melanoma cell survival in the presence of MAPK signaling inhibitors.

Monocyte differentiation into macrophages is regulated by several cytokines, including but not limited to M-CSF/CSF-1 (Wang et al., 2012). Typically, production and release of IL-1β by macrophages requires activation of NF-κB by cytokine or Toll-like receptor signaling to induce gene expression and, subsequently, activation of the inflammasomes by pathogen-associated or damage-associated molecular patterns to stimulate secretion (Garlanda et al., 2013). Analysis of the secretome of melanoma cells has revealed several soluble factors including cytokines such as M-CSF/CSF-1, CCL2, IFN-γ, IL-6, GM-CSF, leukemia inhibitory factor, and vascular endothelial growth factor A, as well as ligands for Toll-like receptors such as high mobility group box proteins and heat shock proteins, which could participate in driving the differentiation of monocytes to macrophages and/or stimulate IL-1β expression (unpublished data; Ohanna et al., 2011; Wang et al., 2012; Obenauf et al., 2015), whereas reactive oxygen species generated by metabolically active melanoma cells or damage-associated molecular patterns released by dying melanoma cells could all potentially activate the inflammasomes of macrophages. The action of the various factors together in a single secretome is very complex, and indeed, they play a re-

**DISCUSSION**

The biggest hurdle yet to be overcome for the treatment of disseminated melanoma using targeted therapies is the emergence of resistant disease. It is clear from our data and previous studies (Straussman et al., 2012; Smith et al., 2014; Hirata et al., 2015; Wang et al., 2015) that innate immune cells and stromal cells of the melanoma microenvironment play a role in this process in allowing melanoma cells to rapidly tolerate MAPK signaling inhibition before genetic mechanisms of resistance are acquired. We show that macrophages and fibroblasts are located in inflammatory niches in melanomas and are responsible for elevated IL-1 signaling in the melanoma stroma. We show that cross talk between melanoma cells, macrophages, and fibroblasts initiates an IL-1 signaling cascade that generates a CXCR2-stimulating secretome, which ultimately leads to enhanced melanoma cell survival in the presence of MAPK signaling inhibition, via BCL2 up-regulation (Fig. 10 F). We show that blocking IL-1R1 signaling or CXCR2 signaling synergizes effectively with MEK inhibition in vivo, suggesting this as a means to delay the onset of resistance that presently too frequently occurs in melanoma patients.
It is known from previous studies that the cells of the tumor microenvironment are themselves influenced by MAPK signaling inhibition (Hirata et al., 2015; Wang et al., 2015). We have previously observed a marked increase in the number of macrophages in human tumor biopsies from patients on treatment with vemurafenib or a combination of dabrafenib and trametinib, compared with pretreatment (Smith et al., 2014). This may potentially explain the increase in IL-1 signaling we also observed in the tumor biopsies from patients on treatment compared with pretreatment. Therefore, macrophages may be recruited to melanomas upon MAPK inhibitor treatment, which allows for a relatively quick development of drug tolerance through IL-1 signaling activation and subsequent stimulation of fibroblasts. This suggests that targeting this mechanism in combination with MAPK inhibitor therapy may result in a much more potent response in patients.

Previous work has demonstrated the importance of CXCR2 signaling for growth in mouse transplantation melanoma models (Singh et al., 2009a,b) and other cancer models (Tazzyman et al., 2011). To our knowledge, however, CXCR2 signaling has not been previously implicated in promoting tolerance to MAPK signaling inhibition in melanoma; thus, we describe a novel mechanism by which cells can tolerate MAPK therapy. IL-8 has been shown in vitro to stimulate tumor activity in a preclinical model for colon cancer (Ning et al., 2012) and slowed growth and antagonized metastasis in a tumor angiogenesis (Voronov et al., 2003), which is critical for tumor growth. IL-1 has also been implicated in immunosuppression in the tumor microenvironment through PDL1 induction in fibroblasts (Khalili et al., 2012). Therefore, IL-1 signaling could also be a promising candidate to target therapeutically. IL-1 blockade is used to treat a multitude of inflammatory diseases (Dinarello et al., 2012) and is generally well tolerated in patients (Mertens and Singh, 2009; Galloway et al., 2011). Clinical experience with IL-1R1 and CXCR2 antagonists should expedite translation of our findings.

We conclude that host cell activity in the melanoma microenvironment must be considered to develop the most effective therapeutic strategy for treating melanoma. Our study illustrates that a complex web of paracrine signals relayed between heterotypic cells within the tumor promotes treatment tolerance. We propose that targeting this network in parallel with MAPK inhibition would not only be extremely effective in reducing tumor growth, but also delay relapse in melanoma patients.

**MATERIALS AND METHODS**

**Cell culture**

All human melanoma cell lines and the 4434 BrafV600E mouse melanoma cell line (Table S1) as well as immortalized HFF cells (a gift from P. Caswell, The University of Manchester, Manchester, England, UK) were maintained in DMEM with L-glutamine, pyruvate, and sodium bicarbonate (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) and 1% penicillin/streptomycin solution (Sigma-Aldrich). PBMCs from healthy donors were isolated from leukocyte cones (National Institute for Health Research Blood and Transplant) by subjecting to density gradient centrifugation using Ficoll Paque Plus (GE Healthcare).
for 50 min at 400 RCF PBMCs were transferred to flasks in serum-free RPMI 1640 Glutamax medium (Thermo Fisher Scientific) for 1 h at 37°C to allow enrichment of monocytes by adherence to tissue culture plastic. After differentiation to macrophages (see the Monocyte differentiation into macrophages section), cells were maintained in RPMI 1640 Glutamax medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution. NHM cells were maintained in medium 254 (Thermo Fisher Scientific) supplemented with 1% human melanocyte growth supplement (Thermo Fisher Scientific). A375 CXCR2KD cells were generated by transfection (Lipofectamine; Invitrogen) using a previously described shRNA vector (Acosta et al., 2008), and clones were subsequently selected using puromycin. All cells were maintained under standard conditions at 37°C with 5% CO₂.

**Patient samples**

Patients with BRAF<sup>V600E</sup>-positive metastatic melanoma were treated with either a BRAFi or a combination of BRAFis and MEKi (details outlined in Table S2). All patients gave their consent for tissue acquisition according to an MD Anderson’s Institutional Review Board–approved protocol. Tumor biopsies were obtained before treatment (day 0), at 10–14 d on treatment, and/or at the time of progression, if applicable. Two commercially available cDNA arrays, MERT101 and MERT102 (OriGene), were analyzed for the expression of various genes in stage-III and stage-IV melanomas. The arrays consisted of cDNA derived from stage-III and stage-IV (n = 39) melanomas, staged according to the revised tumor nodes metastasis classification with minimum stage grouping (Balch et al., 2009), and from normal skin (n = 8). The expression in normal skin was set to one. β-Actin expression in each sample was used to normalize relative gene expression. Both these cDNA samples and the patient pretreatment and on-treatment cDNA samples had to be preamplified before qPCR analysis because of the low amount of cDNA provided. The cDNA samples were preamplified using the TaqMan PreAmp Master Mix kit (PN4384267; Applied Biosystems) using the following reaction mix: 25 µl of preamp master mix, 12.5 µl cDNA, and 12.5 µl of pooled primers (2.5 µl of each primer at 3 µM) in a 50-µl total reaction volume. All genes were amplified in the same reaction to ensure consistent preamplification. Samples were amplified using a G-Storm thermal cycler (GR1 Ltd) and the following cycling conditions: 95°C for 15 s and 10 cycles at 60°C for 4 min. After preamplification, the reaction mix was diluted fivefold to generate a usable stock for qPCR.

**RNA isolation and qPCR analysis**

RNA was isolated from samples using TRIzol (QIAGEN). cDNA was synthesized from RNA using the Omniscript reverse transcription kit (QIAGEN) according to the manufacturer’s instructions. Amplification of specific PCR products was detected using the SensiMix SYBR No-ROX kit (Bioline), an Mx3000P system (Agilent Technologies), and the following cycling conditions: 95°C for 10 min and 40 cycles at 95°C for 30 s, 55°C for 45 s, and 72°C for 45 s.

The following primer sequences were used for qPCR analysis: for human genes β-actin forward, 5′-GCAAGCAGGAGTATGACGAG-3′ and reverse, 5′-CAAATAAAGCCA TGCCAATC-3′; IL1A forward, 5′-AATGACGCCCTC AATCAAG-3′ and reverse, 5′-TGGGATTCAGCTGAC TCTCC-3′; TNFA forward, 5′-TCAGAGGCGTGTGAC TCTCAT-3′ and reverse, 5′-GAGGGTGACCTGGTTGCT GT-GG-3′; IL10 forward, 5′-AAGACCCAGACATCAAGG CG-3′ and reverse, 5′-CACGGGCTTTGCTTGGTTTTT-3′; CD68 forward, 5′-TCAGGTGTTGATCTGACAG-3′ and reverse, 5′-AGGTGGACAGCTGGTGAAG-3′; SMA forward, 5′-ACCCACATGTCACCATCTA-3′ and reverse, 5′-GAAGGAATTACGCACGTCAG-3′; IL8 forward, 5′-GCTCGGTGTTGCGCAAACGAT-3′ and reverse, 5′-CTC TGACCCAGGTTTCTCTT-3′; CXCR2 forward, 5′-GCTCTTTTCGAGGCACACT-3′ and reverse, 5′-ACC AGTGGAACATGAGG-3′; IL1B (Quantitect QT00021385; QIAGEN) and GROα (Quantitect QT00199752; QIA GEN). For mouse genes, Gapdh forward, 5′-TCTCCC TCACAATTTCATCCAG-3′ and reverse, 5′-GGG TCGACGCAACTTTATTGATGG-3′; Groa (Quantitect QT00199752; QIAGEN); Il1b forward, 5′-ATGGCAACT GTTCCCTGAACACTCAACT-3′ and reverse, 5′-CAGGAC AGGTATATCTCCTTTCTT-3′.

**Gene expression analysis using the Oncomine platform**

The Oncomine dataset used in this study was the Talanton melanoma dataset (Talantov et al., 2005) containing 70 samples: 7 skin, 18 benign melanocytic skin nevi, and 45 cutaneous melanoma samples. The threshold settings were set as: P-value = 1E⁻⁴, fold-change = 2, and gene rank = top 10%. The dataset was exported from Oncomine and analyzed in Prism (GraphPad Software).

**Monocyte differentiation into macrophages**

After thorough washing, monocytes were incubated for 7 d in RPMI 1640 Glutamax medium with 10% fetal bovine serum and 1% penicillin/streptomycin solution supplemented with 100 ng/ml human M-CSF or Mel-CM to stimulate macrophage differentiation. To produce Mel-CM, melanoma cells were incubated in RPMI 1640 Glutamax medium for 72 h. Dead cells in the media were pelleted by centrifugation for 5 min at 200 RCF; and the media was subsequently filtered through a 0.45-µm filter. Conditioned media was diluted fourfold in fresh media before adding to culture flasks containing monocytes. On day 3 of incubation, 10 ml of fresh media (media supplemented with M-CSF or Mel-CM) was added to the culture flasks. Macrophages were detached by incubating with Accutase solution (Sigma-Aldrich) for 15 min followed by scraping and were subsequently seeded in tissue culture–coated plates. Cells were allowed to recover overnight before beginning assays. For the IFN-γ and LPS
stimulation of macrophages, used as a positive control for IL-1β production in Fig. 1 (E and F), differentiated macrophages were stimulated with 100 ng/ml of human recombinant IFN-γ (PeproTech) for 24 h and then 20 ng/ml bacterial LPS (Sigma-Aldrich) for a further 24 h, which was directly added to the IFN-γ-supplemented media.

ELISA

The level of IL-1β secretion by Mel-CM–treated macrophages 24 and 48 h after differentiation (when the cells were no longer in Mel-CM) and also by mouse Mel-CM–treated macrophages 24 h after stimulation was quantified with a Duoset ELISA (R&D Systems) according to the manufacturer’s instructions.

IL-1β signaling blockade in fibroblasts and melanoma cell functional assay

HFF cells were cultured in conditioned media taken from NHM macrophages (NHM-Mφ), A375-Mφ, WM266-4-Mφ, WM164-Mφ, and MM485-Mφ 24 h after the 7-d differentiation period (when the cells were no longer in conditioned media). The media was supplemented with 1 µg/ml goat IgG control (R&D Systems), 1 µg/ml IL-1β neutralizing antibody (R&D systems), or 1 µg/ml IL-1RA (PeproTech) overnight. The next morning, the cells were incubated in fresh media for 5 h, which was subsequently added to melanoma cells plated in 12-well plates with 1% DMSO (Sigma-Aldrich) or 0.5 µM both PLX4032 (Selleck Chemicals) and selumetinib (Selleck Chemicals). Then, HFF cell lysates were taken to analyze the expression of IL-6, IL-8, and GROα. After 48 h, melanoma cell survival was assayed by crystal violet staining (outlined in the Drug dose–response analysis and survival assays section).

Melanoma cell survival assay with Fib-CM

Fully confluent fibroblasts plated in T162 flasks were treated overnight with either fresh media or fresh media supplemented with 100 ng/ml human recombinant IL-1β (PeproTech). The next morning, the cells were incubated in fresh media for 5 h, which was subsequently added to melanoma cells plated in either 6- or 12-well plates for 48 h with various inhibitors. The reagents used for these experiments were 1% DMSO (Sigma-Aldrich), 1 µM PLX4032 (Selleck Chemicals), 1 µM RAF265 (Selleck Chemicals), or 0.5 µM both PLX4032 and selumetinib (Selleck Chemicals). When the duotherapy treatment (PLX4032 and selumetinib) was also used in combination with either MK–2206, SB 225002, Bay 11–7082, or obatoclax, the concentration of each drug used was: 1 µM MK–2206 (Selleck Chemicals), 0.5 µM SB 225002 (Alfa Aesar), 0.2 µM Bay 11–7082 (Sigma–Aldrich), and 0.2 µM obatoclax (Selleck Chemicals). These concentrations were also used when these inhibitors were used as single agents. For each drug treatment, melanoma cells were cultured in non–conditioned media, conditioned media taken from unstimulated fibroblasts, or conditioned media taken from fibroblasts previously stimulated with IL-1β. Then, cell survival was assayed by crystal violet staining (outlined in the next section).

Drug dose–response analysis and survival assays

For drug dose–responses assays, cells were plated in 96-well plates and treated with serial dilutions of PLX4032 (Selleck Chemicals) and selumetinib (Selleck Chemicals) for 72 h. For the melanoma cell survival assay with Fib-CM, melanoma cells were plated in either 6- or 12-well plates for 48 h with the various inhibitors as indicated in the figure legends. Cell survival was assayed by fixing and staining cells with 0.5% crystal violet in 4% formaldehyde. Survival was quantified by measuring the absorbance of the solubilized dye (in 2% SDS in PBS) at an optical density of 595 nm.

Cytokine array

IMR–90 human diploid fibroblasts were transduced with empty MSCV-puro retroviral vector or vector encoding IL-1A as previously described (Acosta et al., 2013). IMR–90 cells were selected with puromycin 48 h after infection at a final concentration of 0.5 mg/ml for 1 wk. For the antibody array, supernatant was harvested from cells and passed through a 0.2-µm filter to remove cells before being incubated with cytokine V arrays (RayBiotech) according to the manufacturer’s instructions. Signal on the membrane was developed using enhanced chemiluminescence and scanned. Scanned images were quantified using ImageJ software (National Institutes of Health).

Macrophage generation and function in Il-1r1−/− mice

Isolation and stimulation. Il-1r1−/− flox control and knock-out mice have been previously described (Abdulaal et al., 2016) and were provided by A. Waisman (University of Mainz, Mainz, Germany), W. Muller, and E. Pinteaux (The University of Manchester, Manchester, England, UK). Bone marrow cell suspensions were collected from femurs and tibias of 8–15-wk-old mice by flushing with complete DMEM (10% FBS and 1% penicillin/streptomycin solution) using Myjector U–100 insulin syringes with 29G × 0.5 needles. Cell aggregates were resuspended by gentle pipetting, and the solution was passed through a 40-µm nylon web. After centrifugation, cells were resuspended in complete DMEM supplemented with 15% 1-929 cell–conditioned medium (as a source of M-CSF) to induce macrophage differentiation. Cells were seeded on 12- or 6-well ultra-low attachment surface plates (Corning) and cultured in a humidified incubator at 37°C and 5% CO2. At day 7, differentiated macrophages were washed and incubated with complete DMEM (control) or 100 ng/ml LPS (Sigma-Aldrich) and 50 ng/ml IFN-γ (PeproTech) or with 4434 melanoma– or NIH3T3 fibroblast–conditioned supernatant. After 24 h, cells were washed and incubated with complete DMEM for 24 h. Macrophage-conditioned media was collected and analyzed by ELISA to detect mature secreted IL-1β as described in the ELISA section.
Flow cytometry. Single-cell suspensions of bone marrow mononuclear cells were analyzed on a FACS. Cells suspensions were pelleted, washed twice, and resuspended in magnetic-activated cell-sorting solution (PBS containing 10% FBS and 1 mM EDTA). A trypan blue exclusion viability test was performed to discriminate dead from live cells. For surface staining, cells were first incubated with anti-mouse FcR antibody (mouse serum blocker FcR; BioRad Laboratories) for 20 min at 4°C. Then, mononuclear cells were stained with the following antibodies from BD, conjugated to either FITC or PE: CD115-PE (1:80) and F4/80-FITC (1:100). Flow cytometry analysis was performed with a FACScan instrument (BD) and analyzed using FlowJo software (Tree Star).

Mouse allograft model

4434 subcutaneous implantation was performed as previously described (Smith et al., 2014). Treatment commenced when tumors reached 100 mm³, and mice were randomly assigned into groups. Both drugs were prepared in 8:1:1 (vol/vol) water/ethanol/Cremophor EL (Sebolt-Leopold et al., 1999). The CXCR2 inhibitor navarixin (SCH 527123; MK-7123) was dosed at 30 mg/kg at 0.1 ml/10 g body weight, and the MEK1 PD184352 was dosed at 25 mg/kg (Thermo Fisher Scientific), IL-1R1 (1:1,000; Sigma-Aldrich), β-tubulin (1:5,000; Santa Cruz Biotechnology, Inc.), ERK2 (1:5,000; Santa Cruz Biotechnology, Inc.), pp65 (1:1,000; Cell Signaling Technology), p65 (1:1,000; Cell Signaling Technology), and BCL2 (1:1,000; Cell Signaling Technology). Anti–rabbit IgG-HRP (1:5,000) and anti–mouse IgG-HRP (1:5,000) were obtained from GE Healthcare, and anti–goat IgG-HRP (1:2,000) was obtained from Santa Cruz Biotechnology, Inc. The primary antibodies used for immunohistochemical analysis included anti–IL-1β (goat polyclonal; 1:50; R&D Systems), anti–IL-1R1 (goat polyclonal; 1:50; R&D Systems), anti–CD68 (clone KP1; mouse; 1:300; Dako), anti–CD163 (clone 10D6; mouse; 1:50; Thermo Fisher Scientific), anti–SOX10 (goat polyclonal; 1:120; Santa Cruz Biotechnology, Inc.), anti–IBA-1 (rabbit polyclonal; 1:300; Wako Pure Chemical Industries), anti–SMA (clone 1A4; mouse; 1:200; Thermo Fisher Scientific), and anti–GROα (rabbit polyclonal; 1:50; Proteintech) followed by appropriate detection systems.

Statistical analysis

Data were analyzed using a one-way ANOVA followed by a posthoc test (Dunn’s or Tukey’s multiple comparisons, as indicated in the figure legends), a Mann-Whitney test, or a Student’s t test, as indicated, using Prism (version 6; GraphPad Software). Pearson correlation was used to analyze associated gene expression.

Online supplemental material

Table S1 shows the origin and mutational status of human melanoma cell lines used in this study. Table S2 shows patient characteristics.

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Immunoblotting and immunohistochemistry

Cells were lysed in SDS lysis buffer and analyzed using standard Western blotting protocols. Scanned Western blot images were quantified using ImageJ software. Formalin-fixed paraffin-embedded tissue blocks used for this study were retrieved from the archive of the Department of Pathology, Spedali Civili di Brescia. Human tissues included primary cutaneous melanoma and skin and lung metastasis of primary cutaneous melanoma. 4 µm–thick tissue sections were used for immunohistochemical staining. For double and triple staining, after completing the first immune reaction, the second reaction was visualized using Mach 4 MR-AP (Biocare Medical), followed by Ferrangi Blue. For triple staining, the third reaction was revealed using a REAL Detection System (Alkaline Phosphatase/RED Rabbit/Mouse; Dako).

Antibodies

The antibodies used for immunoblot analysis included IL-1β (1:1,000; R&D Systems), GROα (1:1,000; Thermo Fisher Scientific), IL-6 (1:1,000; R&D Systems), IL-8 (1:1,000; R&D Systems), IL-1R1 (1:1,000; R&D Systems), phosphorylated ERK (pERK; 1:5,000; Sigma-Aldrich), β-tubulin (1:5,000; Santa Cruz Biotechnology, Inc.), ERK2 (1:5,000; Santa Cruz Biotechnology, Inc.), pp65 (1:1,000; Cell Signaling Technology), p65 (1:1,000; Cell Signaling Technology), and BCL2 (1:1,000; Cell Signaling Technology). Anti–rabbit IgG-HRP (1:5,000) and anti–mouse IgG-HRP (1:5,000) were obtained from GE Healthcare, and anti–goat IgG-HRP (1:2,000) was obtained from Santa Cruz Biotechnology, Inc. The primary antibodies used for immunohistochemical analysis included anti–IL-1β (goat polyclonal; 1:50; R&D Systems), anti–IL-1R1 (goat polyclonal; 1:50; R&D Systems), anti–CD68 (clone KP1; mouse; 1:300; Dako), anti–CD163 (clone 10D6; mouse; 1:50; Thermo Fisher Scientific), anti–SOX10 (goat polyclonal; 1:120; Santa Cruz Biotechnology, Inc.), anti–IBA-1 (rabbit polyclonal; 1:300; Wako Pure Chemical Industries), anti–SMA (clone 1A4; mouse; 1:200; Thermo Fisher Scientific), and anti–GROα (rabbit polyclonal; 1:50; Proteintech) followed by appropriate detection systems.
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References


