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ROLES FOR BRAF KINASE ACTIVATING MUTATIONS IN MELANOMA: MICROENVIRONMENTAL IMMUNOSUPPRESSION

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Roles for BRAF Kinase Activating Mutations in Melanoma: MIcroenvironmental Immunosuppression

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Abstract

The BRAF oncogene demonstrates a characteristic mutation (V600E) in a significant fraction of cutaneous melanomas, leading to constitutive activation of the MAP kinase pathway. This genetic lesion endows tumor cells with proliferative and survival advantages, and metastatic melanoma patients treated with the BRAF(V600E)-specific inhibitor, Vemurafenib, have shown dramatic clinical responses. Here, I show that BRAF(V600E) induces transcription of the IL-1α and IL-1β genes in both melanocytes and melanoma cell lines and that this upregulation is specifically abrogated by targeted BRAF(V600E) inhibitors. Furthermore, treatment of melanoma tumor-associated fibroblasts (TAFs) with IL-1α/β significantly enhanced the ability of TAFs to suppress the proliferation and function of melanoma antigen-specific cytotoxic T cells. IL-1α/β treatment of TAFs upregulated multiple immunosuppressive factors, including COX-2 and the PD-1 ligands PD-L1 and PD-L2. Specific BRAF(V600E) inhibitors largely abrogated the ability of melanoma cells to confer T cell-suppressive properties on TAFs. These results support a model in which BRAF(V600E) promotes immune suppression in the melanoma tumor environment through an IL-1-mediated mechanism involving resident stromal fibroblasts. Based on these findings, combination therapies involving targeted BRAF inhibition and T cell-based immunotherapies are warranted.

Chapter 1

General Background

Melanoma

Somatic pigment cells, or melanocytes, can accumulate mutations and transform into malignant melanoma. Pigment cells are found throughout the body. Commonly known to be found in the cutaneous junctional region between the dermis and epidermis, and within hair follicles, these cells also line the mucosal membrane of the intestine, the leptomeninges, and reside in the eye and the inner ear. Melanocytes in all of these tissues can develop into melanoma and transform into lethal malignancies (Figure 1).

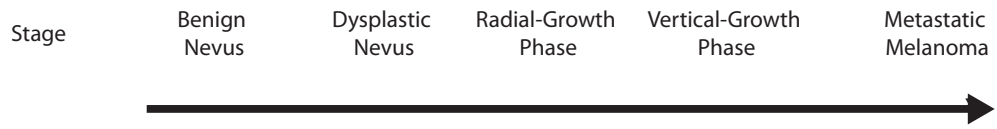
Malignant melanoma spreads throughout the body, establishing itself in vital organs, and ultimately forming large masses, or tumors, that interfere with necessary functions. Because of this behavior, melanoma is a lethal disease. Diagnosis at early stages of progression allows surgical resection of the primary tumor and, if removed prior to metastasis, further risk is highly diminished. In fact, 90% of melanoma cases treated at the earliest primary stage spared the lethality of progressive disease for at least 20 years[1]. However, if surgery or detection is delayed, most cutaneous melanomas metastasize first to draining lymph nodes and may reach almost every site in the body. Patients with stage IV disease with visceral organ metastases have a 5-year median survival rate of only 10%[1]. Multiple clinical trials have demonstrated that melanoma is highly resistant to

chemotherapy; thus, alternative treatment modalities have generally been used to treat patients with this disease. Melanoma is the ultimate cause of death for 8,000-9,000 people in the United States each year[2]. The number of deaths has been increasing despite efforts to promote preventative behaviors, early detection and aggressive therapy.

Treatments for metastatic melanoma either directly target the tumor cells or activate an immune response against the tumor[3]. It is well-known that, for melanoma, conventional alkylating chemotherapy, typically dacarbazine or temozolomide, has low response rates (at best, 15-20%) and is not curative [2,4]. However, immunological interventions can achieve long-lasting durable remissions. Numerous immunological interventions have been conceived and many of them tested, but few have been approved for use by the United States Food and Drug Administration. Only Interkeukin-2 (1998) and Interferon alfa-2b (1995) are approved for use[3,5]. The mechanism of action of both these cytokines is understood to be the activation of tumor infiltration lymphocytes (Til)[6].

Figure 1 Melanoma Progression Schematic Diagram

A benign nevus situated at the dermo-epidermal junction progresses to metastatic melanoma. The process is typified by a stage-wise progression to dysplastic Nevus, a Radial Growth Phase, a Vertical Growth Phase, prior to metastasis[7]. (Adapted from Miller et. al. 2006)


Stage
Benign Nevus
Dysplastic Nevus
Radial-Growth Phase
Vertical-Growth Phase
Metastatic Melanoma

Immunotherapy

Melanoma tumors sometimes contain large numbers of infiltrating immune cells. Of special interest are Til that have the potential to kill tumor cells in specific fashion throughout the entire body. The ability of the immune system to target cancerous cells avails diverse opportunities to reduce patient disease burden, extend life, and even cure melanoma. The ability of the adaptive immune system to increase the rapidity and magnitude of the T cell immune response upon serial exposure to specific targets is presumed to allow for long-term control of cancer cell numbers, in this case, providing a functional cure.

Considering the exceptional ability of an intact immune system to protect humans from pathogenic infection by microorganisms, it is natural to question its apparent failure to control cancer. It is commonly ascribed to the lack of evolutionary pressure to prevent or battle cancer in older age[8]. If so, then the goal of immunotherapy is to understand this natural failure and intervene to access the potential power of anti-cancer immune responses.

With added knowledge of the immune response to pathogenic microorganisms, aberrant responses to self-tissues and tolerance to self-tissue from these robust categorical pathologies and homeostatic tolerance to self, evolutionarily formed, consistent mechanistic knowledge can be gained. Experimentally and clinically, the therapeutic potential of these cells is harnessed through multiple therapeutic strategies, most of which rely on cytotoxic T cells for proximal killing of tumor cells.

Cytotoxic T Lymphocytes

Cytotoxic T lymphocytes are derived from precursor cells that originate in the bone marrow, mature in the thymus and, by expressing CD8, recognize MHC Class I bound peptides via T cell receptors (TCR). As such, this cell can recognize peptides generated inside cells. The TCR is composed of an alpha and a beta chain and functions in a super-complex with CD3 family members, CD8 and a wide variety of accessory molecules that integrate information in the context of TCR ligation to MHC Class I.

The TCR beta chain is composed of three genetic components, the Variable, Junctional and Diversity regions. Through recombination and end-joining deletions and additions, a high-order of diversity is generated in the somatic germline DNA. Limited rearrangement of Variable and Junctional regions occurs in the much more stable alpha chain. These proteins pair to form a unique TCR in each T cell during development in the thymus. In the thymus, the majority of T cells that cannot recognize MHC Class I are destroyed, and subsequently those that remain that bind too well are destroyed. These two processes are termed “Positive and Negative Selection” and precede the appearance of CTLs in any immunological process. The peptides presented in the thymus represent translated coding regions of human DNA; thus, by this process, auto-reactive T cells are destroyed (self), and TCR of unknown peptide specificity (non-self) survive to enter the body. The removal of self-reactive T cells in the thymus is known as “Central Tolerance”. The TCR

repertoire of T cells, which emerge from the thymus, constitutes the range of specificity that can be utilized to detect infected or aberrant cells in the body. These T cells can be primed by Dendritic cells in the lymph nodes or the periphery prior to migration to the tumor site. Though antigen presentation by non hematopoietic cells can tolerize CTLs in the absence of appropriate costimulation[9]. In the case of tumor reactive Til, it is presumed that priming has occurred early in the natural history of carcinogenesis[10]. Thus, the effector phase of the immune response is of proximal importance.

Upon ligation of its TCR, primed melanoma Til have the potential to kill the tumor cell. The two most studied mechanisms are perforin/granzyme-based killing and FAS-FAS Ligand killing[11,12]. By forming an immunological synapse with the tumor cell, CTL exocytose vesicles containing perforin and granzyme are deposited proximal to the tumor cells and form pores to access the target cell's intracellular space, where serine protease activity, triggers caspase dependent apoptosis[12].

The importance of effector T cell function in melanoma immunotherapy is highlighted by the local functional tolerance of melanoma-reactive T cells in the tumor site relative to those circulating in the blood[13]. Stimulation of these tolerized cells reveals defects in the release of perforin and granzyme[14]. In murine models of both spontaneous and carcinogen induced (MCA) cancer, Perforin knockout mice are more susceptible to tumor formation, and B cell lymphomas [15,16,17]. Cytotoxic T lymphocytes were further shown to be the mediator of tumor rejection

sensitive to the loss of perforin in these models[16]. Based on these data, clinical populations were screened and germline Perforin and Fas deficiencies are observed in some lymphoma patients[18].

FASL mediated killing traces a similar pathway as perforin and granzyme, requiring caspase activation, and leading to the death of FAS expressing melanoma cells. However, this mechanism of target killing is less important in isolation than perforin in controlled genetic animal studies where FAS deficient tumors were compared with normal tumor cells[17].

The expression of dominant negative receptors for IFN-γ on tumors cells has a significant effect on tumor immunogenicity, and implantation experiments verify that CD8 mediated immunity is blunted without the direct effects on the cancer cells[19]. Direct anti-melanoma effects of IFN-γ, have been studied in cell lines where consistently, stimulation downregulated the prosurvival and differentiation protein MITF [20]. However, in addition to direct effects on tumor cells, IFN-γ can also enforce the polarization of T cells toward a function phenotype well suited for cellular anti-cancer immune responses. Genetic loss of IFN-γ allows for more rapid spontaneous tumor development. IFN-γ-/- animals (C57BL/6J, BALB/c and 129/SvEv) develop lymphoma and epithelial malignancies at greater rates than wild-type animals[15,21,22]. In an accelerated model of MCA induced cancer with p53 deficiency, IFN-γ is also important in reducing the emergence of tumors[23].

Antigen Presentation

Peptides from 8-11 amino acids in length are processed in the endoplasmic reticulum and loaded on human cells in a process termed “antigen presentation”. This ongoing process results in the production of a trimolecular complex composed of a peptide, an MHC Class I molecule and a Beta-2-microglobulin. The continual production and turnover of these complexes provides a constant presentation of the intracellular protein content of a cell to proximal CTLs. This capability underlies the centrality of CTLs in the response to viral infection, intracellular bacteria and cancer. Through the specific recognition of unique peptides, CTLs possess the ability to mediate specific killing of melanoma cells.

Tumor Associated Antigens

The destruction of cancer cells by CTLs is triggered by antigen recognition. Melanoma cells contain a wide array of antigens that can serve as unique and productive ligands for TCRs. A number of different melanoma tumor antigens are listed in Table 1 from four categories of antigens: Mutation derived, Embryonic, Differentiation and Overexpressed Proteins.

Detailed evaluation of the specificity of melanoma Til have uncovered a wide array of antigens derived from mutated proteins. An example of this approach is utilizes melanoma biopsies where melanoma cancer lines, and Til lines can be expanded *in vitro*.. Cloning of tumor cDNA allows for the selection of Til reactive gene products

and isolation of specific antigens. This approach was used to successfully identify the neoantigen derived from a point mutation in myosin class I gene[24].

Although immune evasion can occur through the loss of antigen presentation to CTLs by the melanoma cells, what is most often observed is the presence of tumor reactive Til that are actively suppressed, mediated by melanoma cells in collaboration with stromal elements of the tumor microenvironment. These two mechanisms by which melanoma can avoid destruction by Til are not mutually exclusive, but rather are progressive and dynamic. The current most effective theory that encompasses this dynamic process is the “Immunoediting theory” [25,26,27].

Table 1 Melanoma Tumor Associated Antigens

Representative MHC Class I associated epitopes demonstrated to be of functional relevant in human melanoma. Category, gene name, HLA allele specificity, peptide sequence, and references study are included.

Category	Gene	Mutation	HLA	Peptide	Reference
Mutation derived	Beta-catenin	T37F	A24	SYLDSGIH**F**	[28]
	N-Ras	Q61R	A01	ILDTAG**R**EEY	[29]
Embryonic	MAGE-3	NA	A01	EVDPTGHSY	[30]
	LAGE-1	NA	A02	SLLMWITQC	[31]
Differentiation	MART-1	NA	A02	EAAGIGILTV	[32]
	TRP-2	NA	A68	EVISCKLIKR	[33]
Overexpressed	hTERT	NA	A0201	ILAKFLHWL	[34]
	MELOE-1	NA	A02	TLNDECWPA	[35]

Immune Evasion

Of equal importance in the design of immunotherapeutic strategies are knowledge of the cancer cell, its physiology and its biological potential. The mutagenic nature of cancer cells allow for the development of variants within a complex field of selective pressures. Immune evasion refers to alterations to the cancer cell which obscure its specific immunogenic antigens from the adaptive immune systems. Many defects in the antigen presentation pathway have been discovered in melanoma and other cancers. Nonsense mutations and loss of functions mutations in HLA molecules, antigenic peptides, TAP, Tapasin, Calreticulin, ERp57, B2M and LMPs are the most common.

Signaling changes that are secondary to specific oncogenic proto-oncogenes or tumor suppressor pathway loss can also downregulate components of this machinery. Downregulation can obtain the same effect: evasion of antigen recognition by Til. However, the specific mutations that underlie these defects in antigen presentation are poorly understood. Yet, stimulation through the IFN-γ receptor can upregulate the antigen presentation machinery and restore tumor immunity in many cases. The presence of IFN-γ secreting CTLs can in fact reverse the adaptation of tumors to evade detection, highlighting the logic in clinical intervention at the stage of immune activation. However, the overall effect of these approaches could still be improved. Other barriers to immunological therapy still exist.

Immunosuppression

The presence of Til predict survival in advanced resected melanoma[36] and in the vertical growth phase of primary melanoma, ovarian and colon cancer CD8+ T cell infiltrates are also a prognostic factor[37,38]. From these observation it is fair to believe that suppressed Til do in fact have some anti tumor activity. Anti-tumor activity which could be unleashed if inhibitory mechanism were blocked[39].

Several human and animal studies have provided evidence that a major barrier to the success of immunotherapy is multiple mechanisms of pre-existing, localized, tumor-induced immune suppression [40,41]. Many of these mechanisms cause downregulation or inhibition of T cell function and are common to multiple cancers, their presence frequently associated with poor patient prognosis [42]. T cell suppression can be manifested directly by tumor cells themselves, either through the secretion of inhibitory cytokines such as IL-10, TGF-β or VEGF, or through membrane expression of co-inhibitory molecules such as the PD-1 ligands PD-L1 or PD-L2 [43]. Alternatively, tumors can secrete factors that serve to recruit and activate inhibitory immune cells such as regulatory T cells, myeloid-derived suppressor cells or tumor-associated macrophages, which can in turn inhibit the function of TIL [44]. Despite the profound suppression of Til in the tumor, the presence of Til is nevertheless beneficial in the control of tumor growth[45].

Numerous mechanisms of CTL suppression have been uncovered which extend beyond the scope of this thesis. I will elaborate on only a limited number of mechanisms of central importance to this thesis.

CoInhibitory molecules

T cell Receptor binding is a dangerous event in humans, as the outcome can be severe tissue damage, possibly life threatening (i.e. Graft-vs-Host Disease, autoimmunity)[46]. Binding cognate peptide in the context of MHC is tightly regulated through the integrations of hundreds of molecules in a dynamic focal synapse between T cells and antigen presenting cells. Interesting antigen-presenting cells in the field of immunosuppression exists within the tumor microenvironment. Melanoma cells are altered-self, so unlike pathogen infected cells.

PD-1

The immune system is well adapted to encounter intracellular pathogens and mediate successful clearance. However, chronic infections have orchestrated a successful evasion of the immune response. One useful model useful for distinguishing functional differences between acute and chronic infections is the Armstrong (acute) and clone 13 (chronic) strains of murine LCMV. In the examination of immune failure against clone 13 infection, investigators in Rafi Ahmed's lab observed that expression of the receptor PD-1 was stably expressed on antigen specific CTLs. PD-1 binds the ligands PD-L1 and PD-L2, which are differentially regulated during the immune response. Binding to ligand triggers the recruitment and activation of the SHP-1 and SHP-2 tyrosine phosphatase, consistent with a role in dampening TCR signaling. The blockade of PD-1, or PD-L1

is sufficient to restore immunity in the clone 13 infection and clear the viral infection and the PD-1+ dysfunctional CTLs are termed “Exhausted”.

Close observation of the PD-1 and PD-L1 knock out mice show mild autoimmune disease. Subsequent experiments in tumor immunity models (B16)[47], (P815)[48], (SCCVII)[49] found that the Til typically expressed PD-1, and the blockade of the receptor or its ligand can restore anti-tumor function. Investigation of the effect of PD-1 on islet specific CTLs at the cellular level have shown by intravital microscopy that PD-1+ tolerized CTLs in lymph nodes fail to engage antigen in the presence of tissue PDL1. Even in a model of autoimmunity, where potentially reactive CTL reside in immunostimulatory lymphonodes, PDL1 on non hematopoetic cell can maintain tolerance to self. Melanoma antigen specific Til express PD-1 in circulation and the tumor microenvironment[50]. Thus methods to interfere with local PD-1 ligands could have a dramatic clinical impact. Clinical trials are underway with antagonistic antibodies to PD-1 and PD-L1, and early results are encouraging in melanoma, leukemia, renal cell carcinoma and lung cancer[48,51,52].

CTLA-4

Very much like PD-1, T cells express CTLA-4 as a checkpoint in the activation progress after T cell receptor ligation. However, CTLA-4 is distinctive in that its role appears to be more transient that PD-1, in that its expression is not stable, but rather maximal early after TCR stimulation. CTLA-4 competes for receptor binding with a critical costimulatory molecule CD28[53]. Both receptors bind CD80 and

CD86 with slightly different affinity[54,55].

When CTLA-4 binds its ligand, it activates of the SHP-1 and SHP-2 tyrosine phosphatases, consistent with a role in dampening TCR signaling, like PD-1. Additionally, CTLA-4 can also bind the phosphatase PP2A which has a greater activity dampening the Pi3K/AKT pathway[56]. The CTLA-4 knockout mouse has a profound autoimmune phenotype, consistent with its role as an inhibitory T cell checkpoint. Blockade of CTLA-4 effectively treats tumors in several disease models. Interestingly blockade of both PD-1 and CTLA-4 increase immune rejection of melanoma cell in the common B16 model[57].

Antibody blockade of CTLA-4 was approved for use in metastatic melanoma as a single agent therapy named Ipilimumab [58,59,60]. Clinical trial in advanced melanoma patients demonstrated an overall survival advantage with Ipilimumab compared to dacarbazine[58]. Interestingly Ipilimumab has serious immune related adverse effects that correlate with clinical activity[61]. These reactions typically are specific for pigmented antigens typified by vitiligo, providing in fact a unambiguous biomarker of drug efficacy.

Microenvironment

Melanoma typically develops in the skin prior to metastasis through the lymph nodes and spread the rest of the body. The microenvironment of a melanoma metastasis can be embedded in any tissue of the body. However, certain cellular

elements of the microenvironment appear to site independent. Migratory immune cells can infiltrate metastatic tumors at any location in the body. So too are blood vessels endothelial cells required and accompanying tumor associated fibroblasts (TAF). Cells in the microenvironment with the exclusion of the cancer cells them selves will be referred to as stromal cells or stromal elements in the thesis.

Discerning the relationship between cells in the tumor microenvironment is a challenge in murine models of cancer. Transplanted tumors grow too quickly and spontaneous models with predictable metastasis are not available, or infeasible. Therefore efforts to delineate relationships between immune cells and stromal elements are more fractured. However, details of the effects of the stromal elements on tumor growth and immune response have been reported in the last decade. Most important are the effects of T cells, as have been discussed, and myeloid cell types, which appear to have an altogether protumorgenic, suppressive effect on T cells. Another important but less studied stromal element is the TAF. These mesenchymal lineage cells have been ascribed functions in chemoresistance, metastasis and immunomodulation.

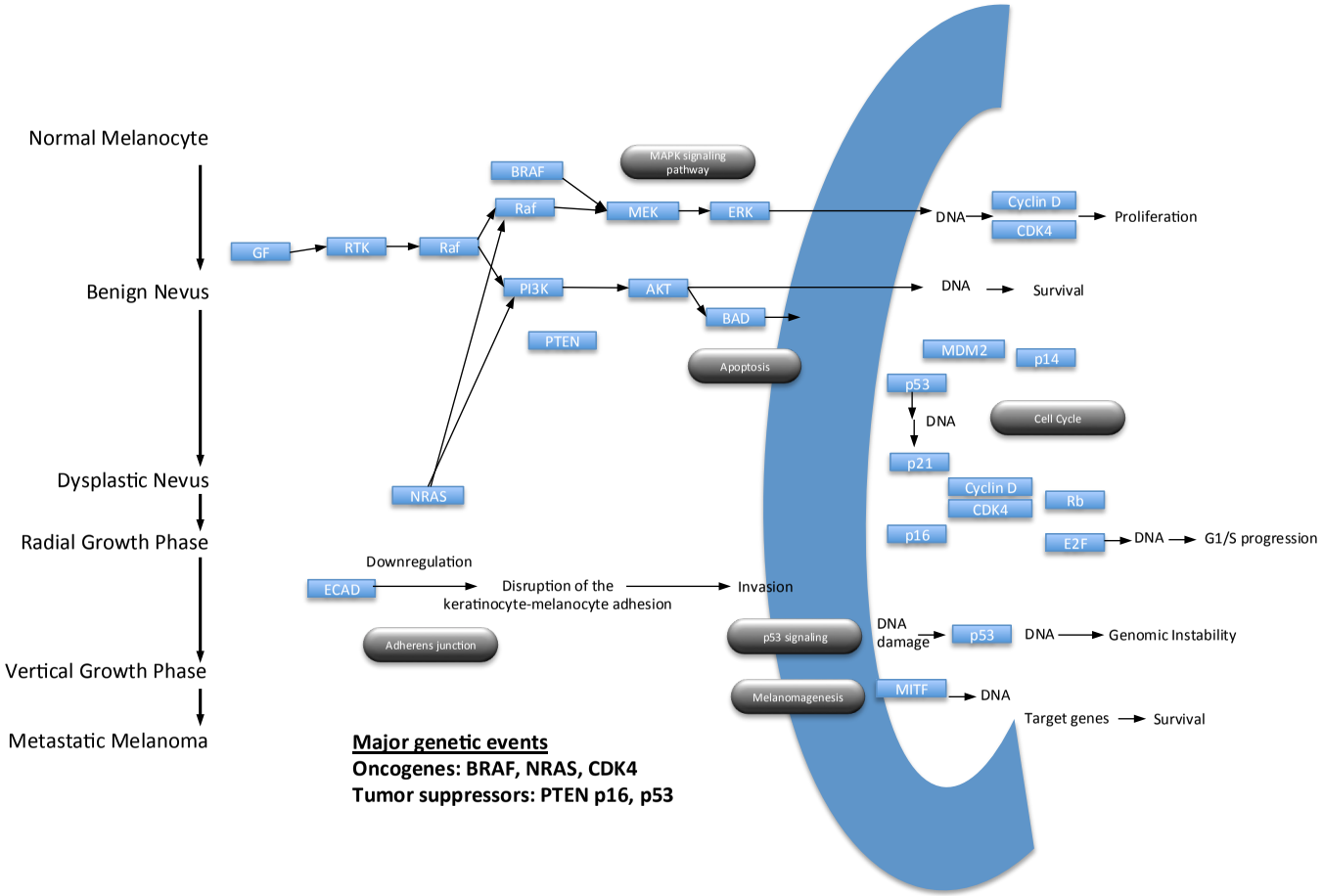
Mutation based therapy

The ultimate realization of molecular cancer medicine is the generation of effective agents that can specifically target cancer cells and that have little to no effect on normal healthy human cells. Small molecules that are structurally designed or discovered with specificity for functionally active mutated proteins are a class of agents currently being developed for clinical use. Few have been successfully generated, in part due to the physical limitations of the chemical space. With the adoption of massively parallel sequencing technologies the set of mutated genes and their respective proteins in cancer cells is expanding rapidly. The dramatic increase in targetable proteins and the ranking of these targets by prevalence will continue to spur efforts to generate these specialized compounds as therapeutic agents.

In melanoma several distinct mutations are commonly observed in two major pathways MEK/MAPK and PI3K/AKT (Figure 2).

Figure 2 Melanoma Signaling Schematic Diagram

Melanoma have activating mutations in genes whose protein products signal through the MEK/MAPK pathway. Additional mutations, of regulator gene losses occurs in order to activat the PI3K/AKT Pathway. On the vertical axis of this illustration are the histological stages of melnaoma progression.


Normal Melanocyte
Benign Nevus
Dysplastic Nevus
Radial Growth Phase
Vertical Growth Phase
Metastatic Melanoma
GF
RTK
Raf
BRAF
Raf
MAPK signaling pathway
MEK
ERK
DNA
Cyclin D
CDK4
Proliferation
PI3K
AKT
DNA
Survival
BAD
PTEN
Apoptosis
MDM2
p14
p53
DNA
Cell Cycle
p21
Cyclin D
CDK4
Rb
p16
E2F
DNA
G1/S progression
NRAS
Downregulation
ECAD
Disruption of the keratinocyte-melanocyte adhesion
Invasion
Adherens junction
p53 signaling
DNA damage
p53
DNA
Genomic Instability
Melanomagenesis
MITF
DNA
Target genes
Survival
Major genetic events
Oncogenes: BRAF, NRAS, CDK4
Tumor suppressors: PTEN p16, p53

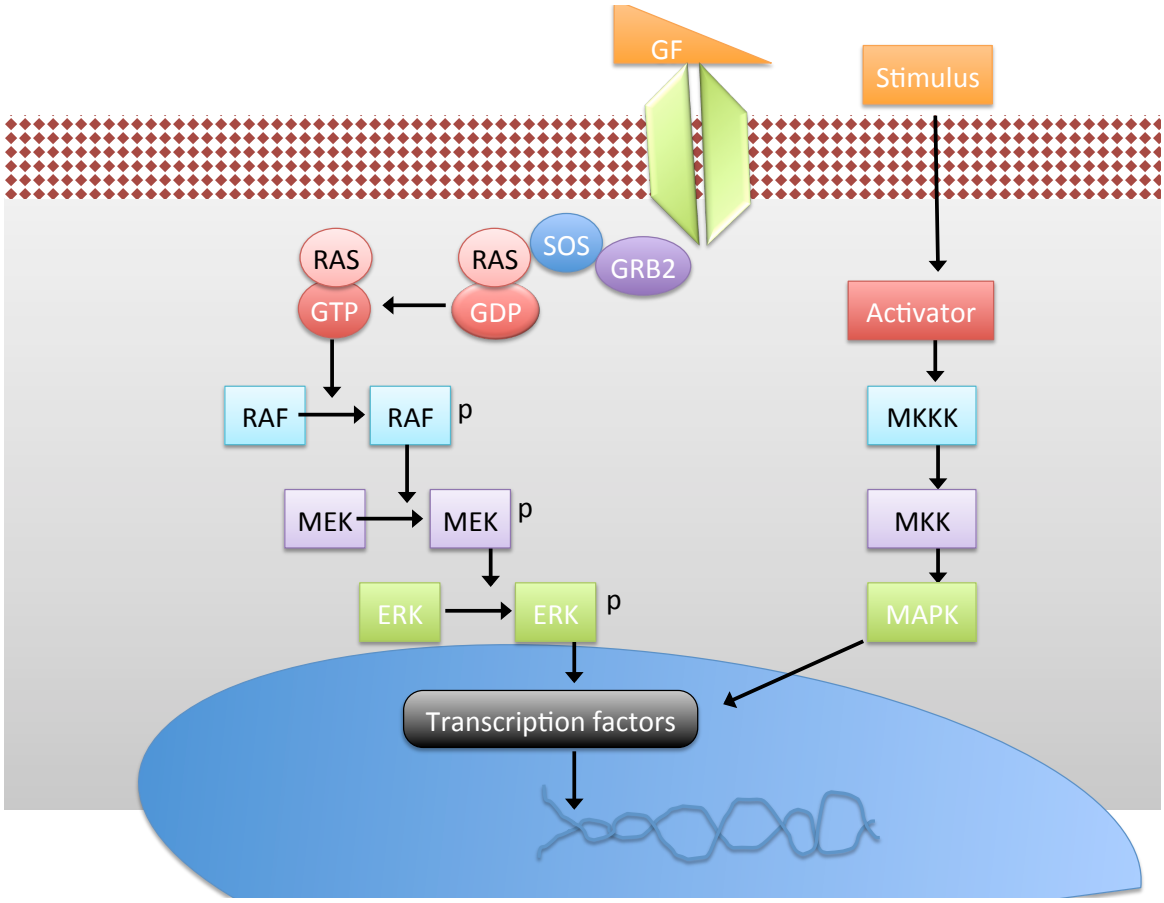
MEK/MAPK Signaling Pathway

The MEK/MAPK signaling pathway is activated by numerous canonical receptor tyrosine kinase (RTK) growth factor receptors. As illustrated in Figure 3, upon binding with ligand, RTKs dimerize, uniting the cytoplasmic tail signaling domains. The tail signaling domain autophosphorylate and cause recruitment and activation of various adaptor-signaling proteins, such as GRB2. The GRB2 associates with RTK domain through its SH2 domain, the SH3 domain of GRB2, binds SOS. RAS activation is regulated by the balance of Guanine-nucleotide-exchange factor (GEF) activity, SOS and inhibitory GTPase-activating protein (GAP) activity, not shown. Ultimately, SOS initiates the MEK/MAPK pathway by activating a membrane-bound RAS family member (N-RAS, H-RAS, K-RAS, RAP, RAL RHEB, RIN or RIT). Figure 3 illustrates the position of GRP2, SOS and RAS upstream of a phosphorylation cascade of RAF, MEK and ERK. Three RAF family members mediate signaling in human cells, A-RAF, BRAF and C-RAF. Upon phosphorylation of ERK1/2, this protein moves into the nucleus and activates specific transcription factors.

The outcome of MEK/MAPK signaling is usually cell proliferation, cell activation or cellular senescence. However, the ultimate effect of the activation of this pathway is highly context dependent, as the state of cellular differentiation and integration of other pathways makes outcomes of MEK/MAPK signaling unpredictable[62].

Figure 3 MEK/MAPK Signaling Pathway

Illustration of the key mediators of the MEK/MAPK signalling pathway originating from growth factor dimerization. This pathway ultimatly activates a large set of transcription factors which alter cell function.


GF
Stimulus
RAS
GTP
RAS
GDP
SOS
GRB2
Activator
RAF
RAF
p
MKKK
MEK
MEK
p
MKK
ERK
ERK
p
MAPK
Transcription factors

BRAF

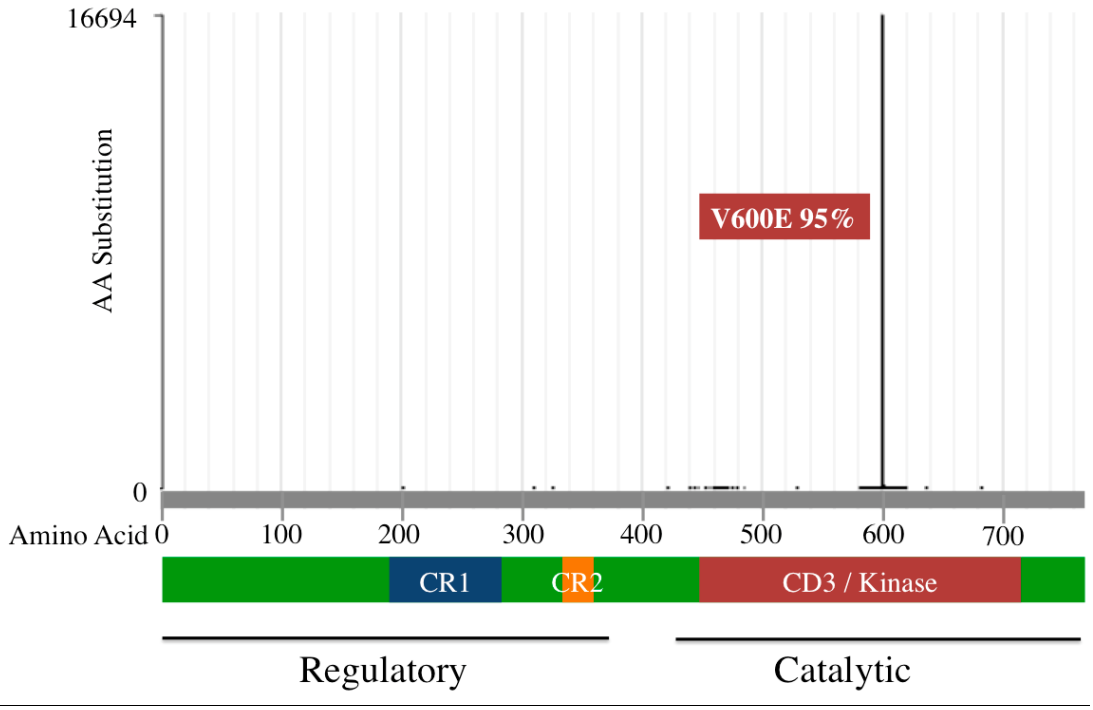
Most melanomas harbor mutations that result in the constitutive activation of the Mitogen-Activated Protein (MAPK) pathway. The appearance of one of these MEK/MAPK activating mutations preclude the appearance of another. The mutual exclusivity of these common activators of the MEK/MAPK pathway suggests functional redundancy.

These mutations have been identified in proximal growth factor receptors and downstream signaling components. In melanoma activating mutations in the RTK, c-Kit, are frequently observed in mucosal melanomas. Mutations in N-RAS are commonly observed in cutaneous melanoma associated with chronic UV exposure (~15-30%). This mutation has the potential to activate both the MEK/MAPK pathway and the PI3K/AKT pathway. Embedded in the MEK/MAPK phosphorylation cascade, the BRAF kinase has activating mutations in 45-50% of human cutaneous melanomas, the vast majority of which result in a substitution of the valine at amino acid position 600 with glutamic acid, termed V600E [63,64]. This mutation, also found to a lesser extent in thyroid, colorectal, ovarian and lung tumors, results in constitutive activation of the MAPK pathway with efficiency at least 2 logs higher than that of wild-type BRAF [65].

BRAF is an 84.4 kD protein, encoded by a gene located on Chromosome 7q34. BRAF has three conserved domains, CR1, CR2 and CR3 as depicted in Figure 4. CR1 and CR2 regulate the binding to target proteins. CR3 is the catalytic domain responsible for phosphorylation of MEK. The BRAF(V600E) mutation in CR3 enhances the kinase activity.

Figure 4 Distribution of BRAF mutations in cancer

The x-axis of this plot represents the amino acid sequence of the human BRAF kinase. Schematic illustration below the axis identifies the corresponding conserved regions (CR) to the linear position of amino acids. Plotted on this histogram are the individual tumor samples (18057) in which BRAF mutations have been observed of a total of 92174 samples (COSMIC database)[66]. The high bias toward mutations at the 600th amino acid is evident[66].


16694
AA Substitution
V600E 95%
0
Amino Acid 0
100
200
300
400
500
600
700
CR1
CR2
CD3 / Kinase
Regulatory
Catalytic

BRAF(V600E) Signaling

In the physiological setting, BRAF(V600) mutations can be found in benign nevi as well as in melanoma. In nevi and melanoma, perhaps surprisingly, the BRAF(V600E) mutation is not associated with increased levels of pERK1/2 *in situ*, nor is the expression of activating N-RAS mutated proteins [67,68,69]. The signaling alterations incurred by the BRAF(V600E) mutation have been investigated, and thus numerous elements of its signaling complexity have been suggested by *in vitro* experiments.

In vitro experiment with BRAF(V600E+) melanoma cells lines definitely and reliably demonstrate the presence and relevance of MEK/MAPK signaling pathway. However, additional roles for BRAF(V600E) have been postulated based on results using RNA interference methodologies or BRAF(V600E) specific inhibitors in melanoma cell lines. Liu et.al demonstrated that NFkB activity is induced by BRAF(V600E)[70]. Estrada et.al. demonstrate a role for BRAF(V600E) in enhancing signaling through p38 MAPK pathway activating JNK [71]. BRAF(V600E) has also been shown to be responsible for the transcription of ERK3, potentiating another alternate MAPK pathway [72]. However, several components of other signaling pathways are unaffected by BRAF(V600E): Nore1[73], 3pK[74], ID1[75], IGF1R[76], IGF1[77], MC1R[78], Notch[79], and methylation[80].

In an effective proteomic study, Old et.al, utilizing MEK inhibition in a BRAF(V600E+) melanoma cell line identified dozens of targets of phosphorylation.

In this screen, the protein MINERVA/FAM129B was demonstrated to be a downstream target of BRAF(V600E) mediated MEK activation. Previously uncharacterized, the authors demonstrated that MINERVA/FAM129B is required for successful cell invasion through collagen.

Functions of BRAF(V600E) in Melanoma

Considering Hanahan and Wienberg's original conception of the six requirements for cancer, research testing the function of the BRAF(V600E) mutation has demonstrated roles for this mutation in several distinct cancer functional phenotypes[81].

Self-sufficiency in growth signal: As the major pathway transducing signals though growth factor receptors it is not surprising that BRAF(V600E) knockdown, specific inhibition or MEK inhibition blunts the proliferation of melanoma. The role for BRAF(V600E) has been establish through single cell and well based tumor growth assays in a number of reports [82,83,84,85,86,87,88,89,90]. Furthermore, growth inhibition BRAF(V600E) signaling interference is confirmed in experiments detecting cell cycle progression [91,92,93]. However, BRAF(V600E) signaling inhibition can result in either G2 cycle arrest[94])[95] or G1 arrest[96,97,98,99,100].

Insensitivity to anti-growth signal: BRAF(V600E) inhibition can lead to growth arrest, for which extraneous growth factors can dominantly rescue [101]. The mechanisms of growth arrest may differentiate the functional role of BRAF(V600E). In melanoma

cell lines, growth arrest through two mechanisms rely on BRAF(V600E), metabolic stress [82,85] and anchorage independent growth [82,97,102,103].

Angiogenesis: In a model of melanoma xenografts, the BRAF(V600E) signaling pathway was essential for proper vascularization of the tumor and subsequent tumor growth [86]. Important for the process of angiogenesis, BRAF(V600E) regulates survival in Hypoxia by upregulating HIF1alpha [104] and transcriptional upregulation VEGF [105].

Tissue Invasion & metastasis: Multiple experimental methodologies in use attempt to isolate distinct cellular actions required for tissue invasion and migration, required for metastasis. In the absence of BRAF(V600E)-induced MEK/MAPK signaling, melanoma cells have reduced capacity for matrigel Invasion [83,84,106,107,108,109], metastatic extravasation *in vivo* [110,111], transformation in soft agar [63,87,108,112], and acquisition of 3D invasion ability through collagen matrix [113,114]. In all these systems, BRAF was important for metastatic behavior.

Resistance to apoptosis Crucial for the progression of melanoma is the resistance to apoptosis. A general dependence of melanoma cell lines on BRAF(V600E) to prevent spontaneous apoptosis, is illustrated by the dearth of studies reporting such evidence [70,87,90,115,116,117,118,119]. Various mechanism of apoptosis induction can occur in melanoma cell lines, but anoikis induced apoptosis appears to reproducibly depend on BRAF(V600E) signaling [116,120,121,122]. Resistance

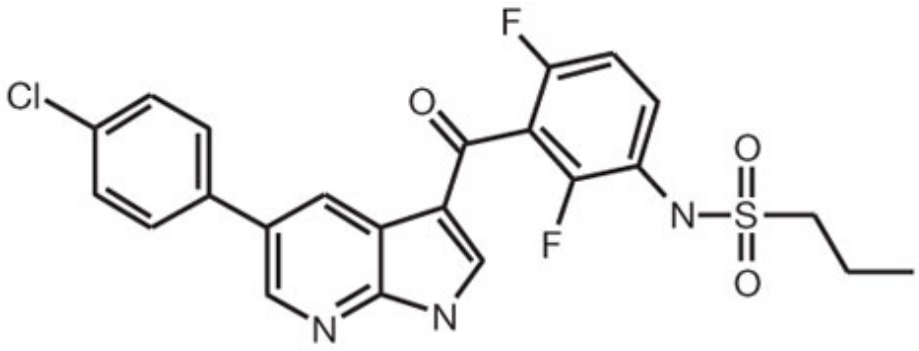
to apoptosis further is shown to rely on the expression of HSP70 and HSP90 [123] by melanoma, and the BRAF(V600E) pathway is known to inhibit expression of the proapoptotic protein Bim [124].

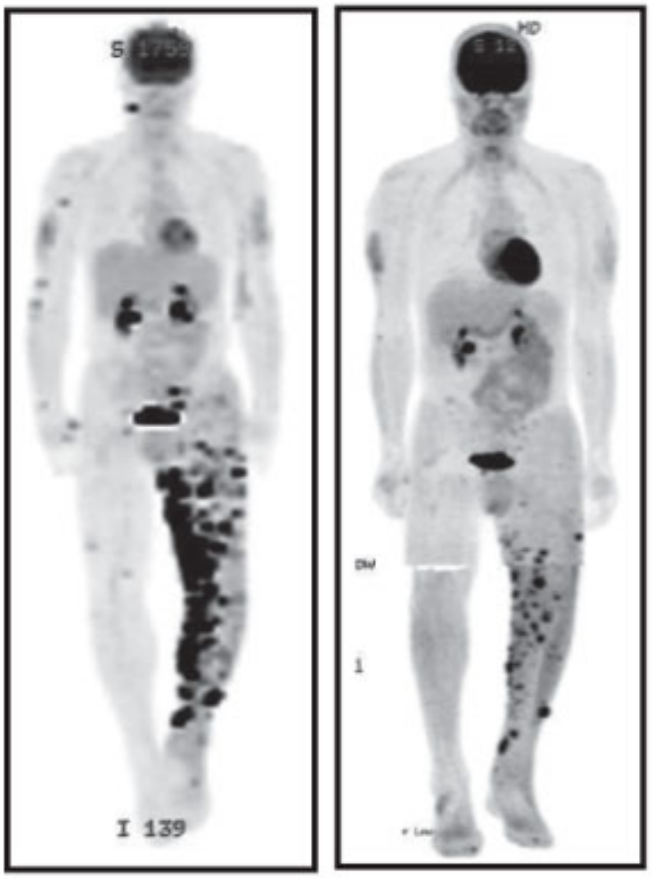
The wide variety of functions that the BRAF(V600E) mutation facilitates in the presently indexed studies suggests that this mutation serves multiple roles in melanoma progression. The reliance on the MEK/MAPK signaling pathway for mutant BRAF provides an opportunity to circumvent its many cellular functions with therapeutic intervention, but with obvious caveats. The presence of the mutation alone fails to predict specific functional dependency upon the activated pathway. Therefore understanding the requirements put on this pathway in patients or even during progression in rodent models of BRAF(V600E)+ melanoma is unreliable.

Three murine models of melanoma have been developed that utilize the BRAF(V600E) mutation[125,126,127]. Although spontaneous, UV and chemically induced murine cancers, including melanoma fail to generate BRAF(V600E) mutation, the controlled expression of this mutated oncoprotein is function *in vivo*. Summarily, these models by Bosenberg, Haluska and Marais, all generate melanomas with different penetrance after initially recapitulating the senescent phenotype of BRAF(V600E)+ nevi. Further work with these models is needed to understand the nature of BRAF(V600E) during the earliest stages of immune surveillance.

Figure 5 Chemical structure of Vemurafenib and Responding Patient

Vemurafenib was developed based on crystallographic modeling techniques to have selective binding to BRAF(V600E) kinase domain. The agent is a potent inhibitor of the BRAF(V600E) kinase. Scans of melanoma tumor glucose metabolism after 2 weeks of daily administration of Vemurafenib. Heavy tumor burden in the lower extremity is highly responsive to Vemurafenib at the metabolic level. Lower figure reproduced with permission of copywrite holder. [128]


F
Cl
O
O
N
S
O
F
N
N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Clinical experience with BRAF(V600E) inhibitors

The preponderance of data suggests an important role for mutant BRAF signaling through the MEK/MAPK signaling pathway in most melanoma, however previous clinical experience with RAF inhibition was disappointing. Sorafenib (BAY 43-9006) is a BRAF inhibitor that failed in phase II trial in advanced melanoma, despite success and approval for hepatocellular and renal cell carcinoma[129]. However, several agents under development are currently in testing to better attack this crucial pathway. The first such agent to receive FDA approval is Vemurafenib[128,130]. The oral daily dosing with this drug at 960 mg twice per day can achieve an 48% response rate.

The overall experience with the remarkable success of Vemurafenib highlight the failure of single agent treatment approaches for melanoma.

Rationale for combination therapy

The successful implementation of immunotherapeutic approaches in melanoma, and the recent advance in targeted inhibition of the BRAF(V600E) mutation avail an opportunity for combination therapies. However, the rational implementation of this drug with the relatively wide varieties of immunotherapy available and with proper consideration for patient selection requires significant understanding of the role of this mutation in the suppression of the anti tumor immune response. There is a great opportunity to serve melanoma patients by developing a knowledge base that can reveal complimentary therapeutic opportunities.

Work by Kawakami established a role for BRAF(V600E) in the secretion of IL-10, IL-6 and VEGF in some melanoma cell lines[105]. These cytokines regulate the priming ability of dendritic cells, and the loss of BRAF(V600E) signaling can diminish their production levels, thus allowing dendritic cells to better prime anti tumor T cell responses. This observation is especially interesting due to the metastatic pattern of melanoma through the lymphatic system. It is possible that poor priming results from the presence BRAF(V600E) induced cytokines in the local priming organ. However, this would be most clinically relevant upon immediate detection of primary lesions where the immune response is still nascent. Even though the priming of T cells toward tumor antigens will occurs prior to metastatic disease, primary melanoma lesions commonly contain tumor reactive Til.

Prior to the discovery of BRAF(V600E) it was known that MAPK/MEK inhibition increases MAA: Mart-1, gp100, Tyr and TRP1[131,132]. Therefore it was not surprising when Boni et. al. reported that inhibition of BRAF(V600E) and MEK inhibition of melanoma cells could upregulate this class of tumor antigen[133]. Increased antigen correlated with more CTL recognition and killing *in vitro*. This data suggests that BRAF(V600E) inhibition can assist in antigen recognition by Til in the tumor microenvironment.

In line with these studies, I extend research in this thesis on the role of BRAF(V600E) regulated cytokines on melanoma tumor associated fibroblasts (TAF) in the tumor microenvironment. I operate under the hypothesis that the BRAF(V600E) mutation is a critical regulator of T cell immune suppression in the melanoma microenvironment.

In doing so, I determined for the first time that IL-1 a and IL-1 b are regulated by the BRAF(V600E) mutation in melanoma cells. I further determined that in collaboration with IL-1 activated TAFs, BRAF(V600E) can suppress Til via PD-1 and Prostaglandin E2. This is the first evidence for BRAF(V600E)-mediated regulation of PD-1 ligands in the melanoma microenvironment, and I demonstrate the treatment of melanoma cells with the drug Vemurafenib blocks this mechanism of immunosuppression.

Chapter 2

BRAF(V600E) as a regulator of immunomodulatory molecules in melanocytic cells

Introduction

Immunosuppression in the tumor microenvironment is categorically different from tolerogenic tissue homeostasis. In normal tissues, the peripheral tolerance is maintained by a variety of mechanisms that are disregulated in autoimmune states. However, even in autoimmune states when disregulation is occurring, the interactions between cells and within cells are governed by normal germline-encoded molecular programs. In otherwise immunologically normal individuals, this disregulation is cellular and molecular but does not involve novel molecular entities. In the tumor, microenvironment novel molecular entities alter the operating rules of the tissues providing for an explicitly unnatural deviation from normal tissue level regulation of the environment. The tumor cell can break “the rules”.

The direct or indirect regulation of immune-related molecules by cancer cells may be mediated by several distinct mutations of obvious importance. This includes the loss of melanoma-associated antigen expression, which permits the evasion from direct killing by cognate cytotoxic T cells frequently found in melanoma lesions. A functionally equivalent mutation in B2M or HLA molecules can exact the same outcome, as can the loss of critical antigen presenting machinery such as TAP1,

TAP2, Tapasin, ERP1 or calreticulin. Indirectly, a tumor cell may increase the production of a secreted factor, which has a suppressive effect upon cytotoxic T cells at higher concentrations. TGF-β and IL-10 are the major examples of these secreted factors. Cytotoxic T cells with the relevant receptors for these factors will have reduced responses to cognate antigens. However, unlike the loss of function mutations leading to immune evasion, the mechanism by which a cancer cell achieves an immunosuppressive microenvironment is not known. Yet, control of tumor growth by the immune system can be impaired by either class of interactions or, by logical extension, local intermediary cells can integrate signals from the tumor-secreted factors and collaborate in the generation of a suppressive microenvironment.

Enhanced expression of gene products is an anticipated outcome of activated oncogenic signaling pathways. Activating mutations in important signaling pathways bypass normal regulation and downstream transcription is enhanced. The genetic mutations in melanoma cells are diverse and deeply integrated at the time of metastasis. The integration of altered signaling required for tumor progression can mask the effect of a single mutations contribution to a cells phenotype. This is perhaps the case for BRAF(V600E). In an effort to define a genetic signature for this mutation, several investigators conducted transcriptional screens with melanoma cell lines [72,75,94,104,134,135,136,137,138,139,140,141,142,143,144,145,146,147,148,149,150].

However, the presence of the common BRAF mutation in metastatic melanoma

cells with integrated signaling networks fails to show a predictive transcriptional signature. Therefore, the contribution of BRAF(V600E) to the transcription of immunoregulatory molecules is not determined by previous studies. One could suggest that the presence of BRAF(V600E) early in the etiology of melanoma necessarily means that its predictive signature is distorted by the adoption of additional mutations. As such, determining general effects of the BRAF(V600E) mutation on the diverse background of a number of unknown other mutations appears to yield data limited to individual melanoma cell lines.

Results and Discussion

BRAF(V600E) Transcriptional Screen

In order to assess the downstream gene transcription profile induced by BRAF(V600E), I developed a lentiviral vector-based system to enforce expression of this oncoprotein in primary human melanocytes, thus mimicking one of the earliest events in melanomagenesis (Figure 6).

Figure 6 Oncogene Expression Vector

A CMV promoter was used to drive expression of either wild-type (wt) BRAF or mutated BRAF(V600E), and eGFP expression driven by a downstream IRES element.

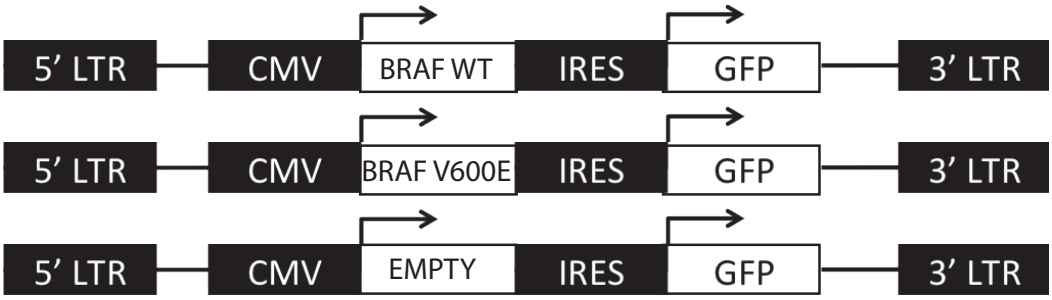

5’ LTR
CMV
BRAF WT
IRES
GFP
3’ LTR
5’ LTR
CMV
BRAF V600E
IRES
GFP
3’ LTR
5’ LTR
CMV
EMPTY
IRES
GFP
3’ LTR

Figure 7 Ectopic expression of BRAF(V600E) in melanocytes

Flow cytometric analysis of green fluorescent protein (GFP) and BRAF expression in dermal melanocytes following transduction with lentiviral- expression vectors BRAF(wt)-IRES-GFP, BRAF(V600E)-IRES-GFP or empty-IRES-GFP. Gated GFP(dim) cells were sorted for use in subsequent studies.

Unstained
Untransduced
GFP vector
BRAF wt vector
BRAF (V600E) vector
BRAF
GFP

As shown in Figure 7, GFP fluorescence correlated with the expression of BRAF protein, as determined by intracellular staining. To control for potential artifacts due to BRAF overexpression, GFPlo cells were collected and analyzed for changes in global gene expression. Cells transduced with empty vector-IRES-eGFP provided a mock control, and comparisons with (wt) BRAF-transduced cells provided a control for V600E-specific gene transcription. Using this system, I analyzed how the introduction of ectopic expression of BRAF(V600E) in melanocytes specifically upregulated the transcription of immunomodulatory genes after 36 hours (Figure 8).

In addition to upregulating the expression of genes previously linked to oncogenic BRAF, including IL-6, IL-8, VEGF, CCL2, DUSP6 and SPRY2, BRAF(V600E) also significantly increased the transcription of IL-1α and IL-1β genes (Figure 8). IL-1 is a major inducer of inflammation in a variety of physiological settings, including fever and wound healing.

Figure 8 Human dermal melanocyte gene expression profiling

Expression levels of selected genes from transduced and sorted human neonatal foreskin-derived dermal melanocytes (36 hours following transduction). Heatmap represents color-coded expression levels for each sample compared to untranduced controls.

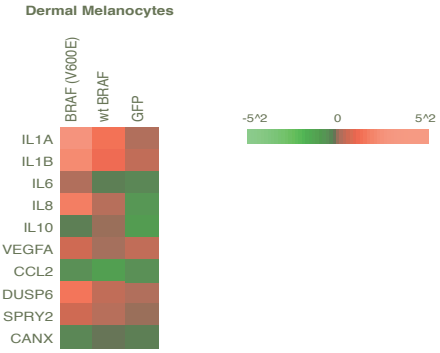
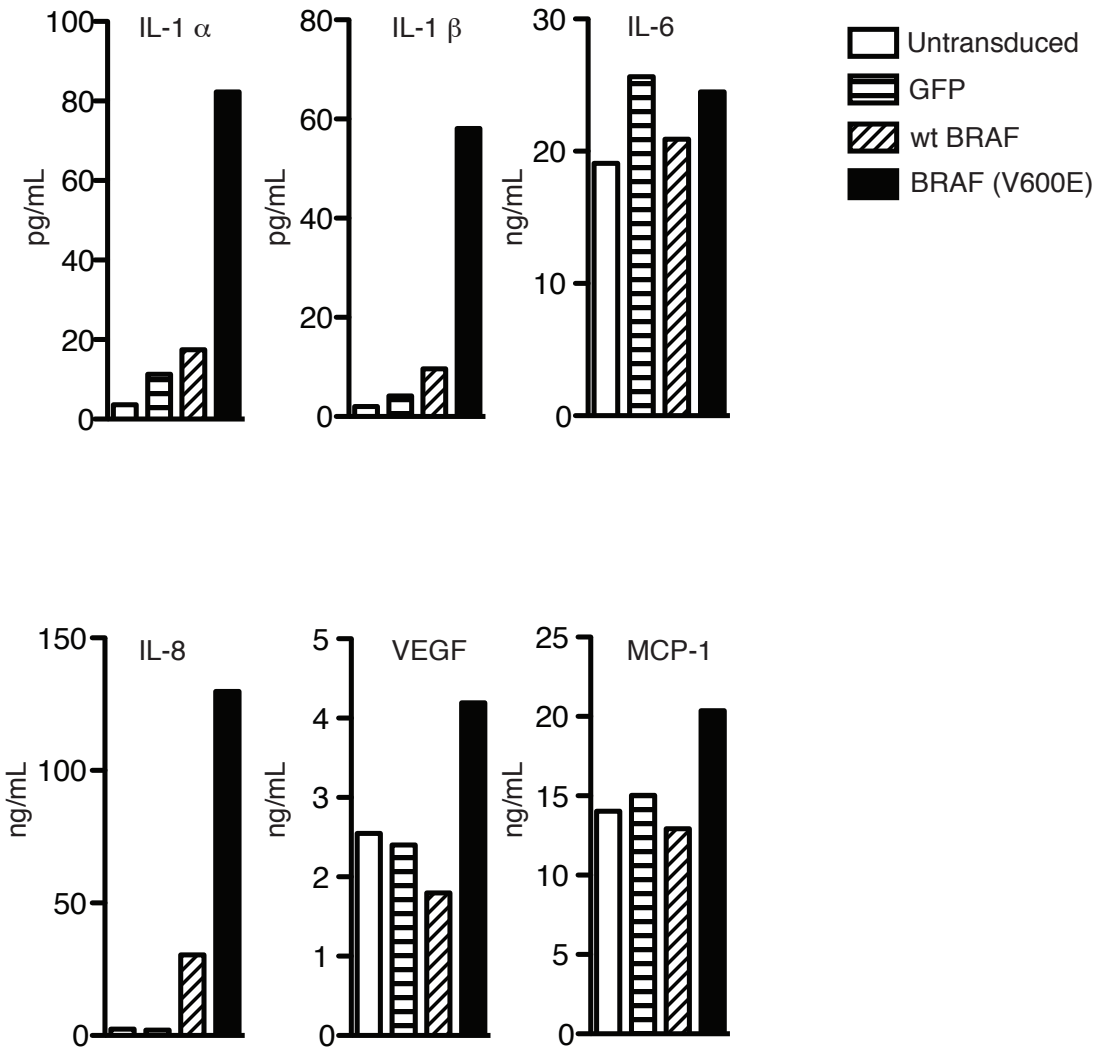

Dermal Melanocytes
BRAF (V600E)
wt BRAF
GFP
IL1A
IL1B
IL6
IL8
IL10
VEGFA
CCL2
DUSP6
SPRY2
CANX
-5^2
0
5^2

Figure 9 Cytokine profiles in supernatants of transduced human dermal melanocyte preparations.

Luminex assay showing cytokine profiles in supernatants of transduced dermal melanocyte preparations cultured for 5 days. Results are representative of 4 independent experiments.

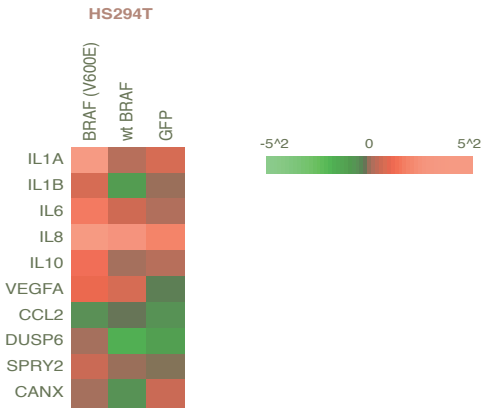

IL-1 α
IL-1 β
IL-6
IL-8
VEGF
MCP-1
pg/mL
ng/mL
Untransduced
GFP
wt BRAF
BRAF (V600E)

As such, I confirmed the effect of gene transcription on cytokine function secretion by analysis of the culture supernatants. After 5-7 days of culture, transduced melanocytes expressing BRAF(V600E) had released substantially more IL-1a, IL-1b, IL-8, VEGF and MCSP but not IL-6 (Figure 9).

To determine whether these results were also relevant for melanoma tumor cells, I performed a similar transduction of the HS294T melanoma cell line, which naturally expresses only (wt) BRAF. As shown in Figure 10, expression of BRAF(V600E) induced similarly high levels of IL-1α gene transcripts compared to HS294T cells transduced with (wt) BRAF or with empty vector. As might be expected, the induced gene expression patterns between primary melanocytes and HS294T cells showed partial overlap; however, the common upregulation of the IL-1α gene in both cell types indicated that oncogenic BRAF(V600E) may be linked to IL-1-mediated inflammation in melanoma.

Figure 10 Gene expression profiling of transduced HS294T cells

Expression levels of selected genes from transduced HS294T cells (24 hours post-transduction). Heatmap represents color-coded expression levels for each sample compared to untranduced controls.


HS294T
BRAF (V600E)
wt BRAF
GFP
IL1A
IL1B
IL6
IL8
IL10
VEGFA
CCL2
DUSP6
SPRY2
CANX
-5^2
0
5^2

Inhibition of BRAF(V600E) abrogates IL-1α and IL-1β production by melanoma cells.

In order to test the hypothesis that BRAF(V600E) was responsible for driving IL-1 production in melanoma, I measured production of IL-1 by BRAF(V600E)-positive melanoma cell lines prior to and following treatment with the BRAF(V600E)-specific inhibitor Vemurafenib (also known as PLX4032, RG7204, or RO5185426). As shown in Figure 11, Vemurafenib (1μM) treatment of the WM793p2 cell line resulted in a progressive reduction in both IL-1α and IL-1β mRNA transcripts, which reached minimum levels within 3-4 hours. Consistent with this result, Vemurafenib treatment reduced IL-1α to nearly undetectable levels at doses as low as 0.1 μM, as demonstrated by intracellular staining and flow cytometric analysis (Figure 12).

In order to determine if this was a more generalized regulatory element of IL-1 transcription on melanoma, I tested four other IL-1-producing melanoma cell lines, three of which were positive for V600E (A375, EB16-MEL and KUL84-MEL), and one which expressed (wt) BRAF (HS294T). As shown in Figure 13, only in the BRAF(V600E)-expressing cell lines was IL-1 production reduced in response to Vemurafenib treatment. This IL-1 inhibition was also confirmed using direct shRNA knockdown of BRAF(V600E) (Figure 16).

Figure 11 Inhibition of BRAF(V600E) abrogates IL-1α and IL-1β production by melanoma cells.

RT-PCR analysis of IL1A, IL1B and GAPDH transcripts in BRAF(V600E)-positive WM793p2 cells at different time points following treatment with 1μM Vemurafenib. Expression levels were normalized to GAPDH expression and adjusted to corresponding baseline samples.

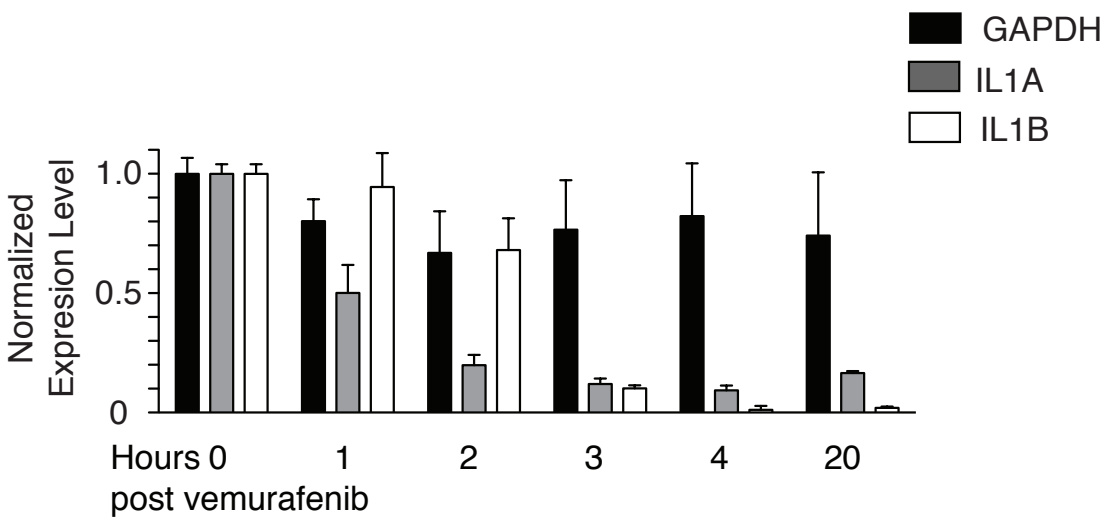

GAPDH
IL1A
IL1B
Normalized
Expresion Level
1.0
0.5
0
Hours 0
1
2
3
4
20
post vemurafenib

Figure 12 Flow cytometric analysis showing intracellular IL-1β expression

Flow cytometric analysis showing intracellular IL-1β expression in live cell gated WM793p2 cells 48 hours following treatment with titrated doses of PLX4032.

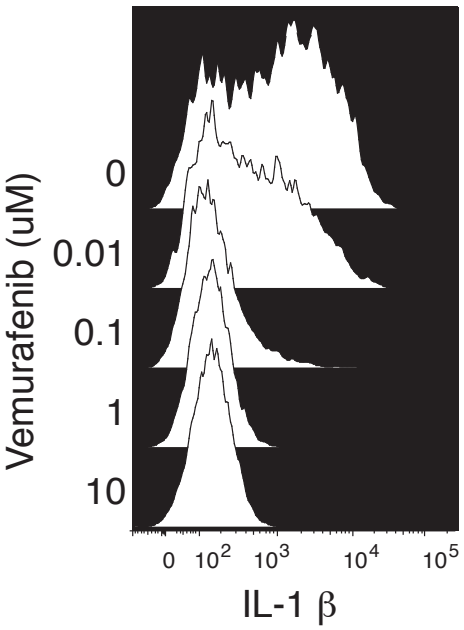
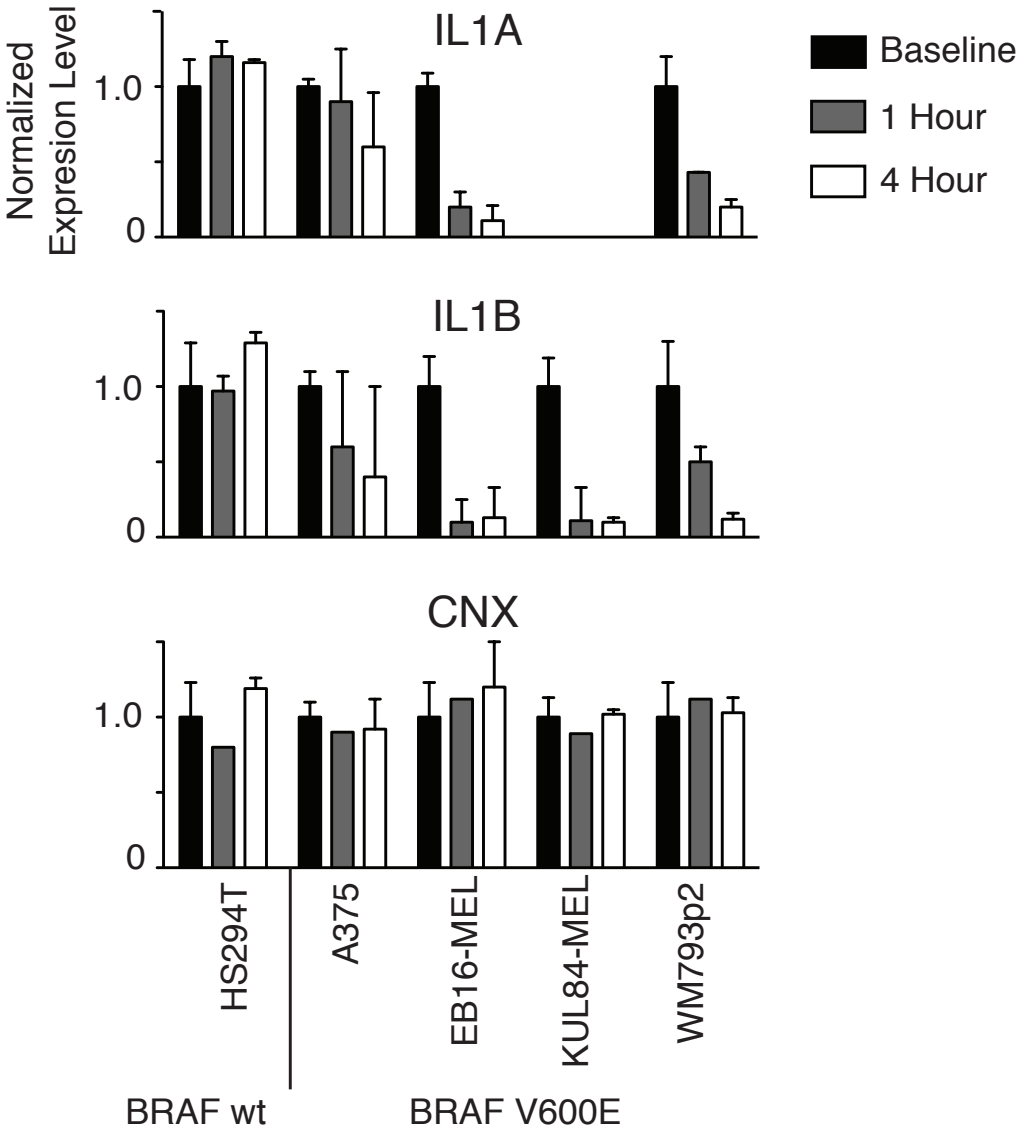

Vemurafenib (uM)
0
0.01
0.1
1
10
0
10^2
10^3
10^4
10^5
IL-1 β

Figure 13 Effect of Vemurafenib on IL1A and IL1B RNA expression in melanoma cell lines

Transcript levels of IL-1α, IL-1β and CNX in five Vemurafenib-treated melanoma cell lines expressing either (wt) BRAF (HS294T) or V600E-mutated BRAF (A375, EB16-MEL, KUL84-MEL, WM793p2). Transcript levels were normalized to GAPDH expression and adjusted to corresponding baseline samples. Data represents 5 experiments with WM793p2 and 3 experiments with other lines.


IL1A
IL1B
CNX
Normalized Expresion Level
1.0
0
Baseline
1 Hour
4 Hour
HS294T
A375
EB16-MEL
KUL84-MEL
WM793p2
BRAF wt
BRAF V600E

Collectively, these results demonstrated that BRAF(V600E) inhibition by Vemurafenib can effectively reduce IL-1 production in a V600E-positive melanoma cell lines at doses <100 times lower than those typically found in Vemurafenib-treated patients (8). Because alternative pathways could be enforcing the production of IL-1 in melanoma cells *in vivo* I evaluated these *in vitro* finding in a xenograft model of melanoma using A375 cell line. Seven days after implantation of 1 million tumor cells in the flank of a NOD/SCID mouse, animals were administered the Vemurafenib analogue PLX4720 by oral gavage. After 3 days of treatment tumors are excised for analysis. Representative H/E staining of vehicle and inhibitor treated tumors are shown to demonstrate the presence of tumor cells, in a fibrotic tumor stroma (Figure 14).

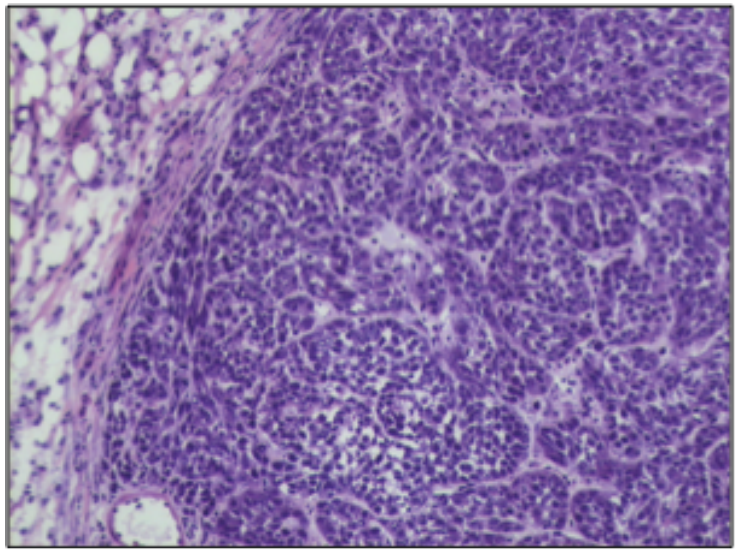
Analysis of human RNA levels in tumor lysates recapitulated my *in vitro* finding, that BRAF(V600E) inhibition arrests the transcription of both IL-1a and IL-1b (Figure 15). Furthermore, consistent with the *in vitro* BRAF expression studies performed in melanocytes, transcription of IL-8 but not that of other control genes was also abrogated. This data shows that BRAF(V600E)-specific inhibitors can block the transcription of IL-1α, IL-1β and IL-8, thus altering the cytokine milieu within the melanoma tumor microenvironment.

Figure 14 Histopathlogy of A375 xenografts

Photos showing H&E staining of representative, A375 xenograft tumor sections excised from NOD/SCID mice. Prior to excision, mice with established tumors were treated for 3 days with either PLX4720 or DMSO vehicle control. Magnification: 4x.

.

Vehicle



PLX4720

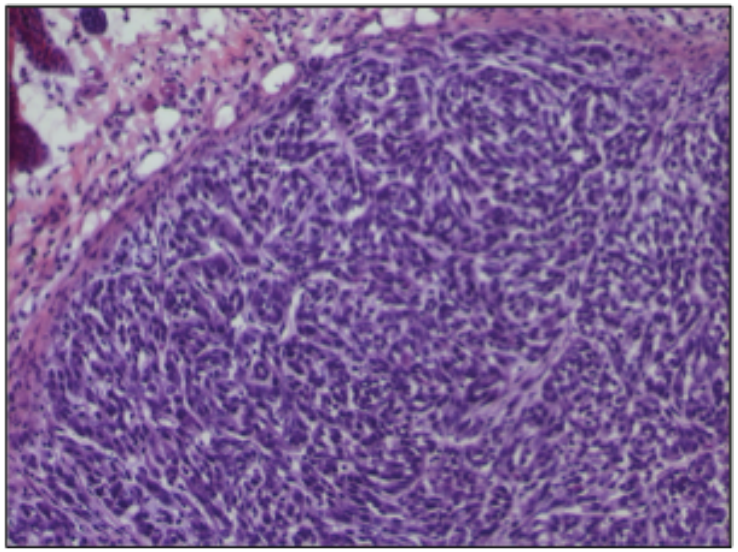


Figure 15 IL1A and IL1B transcriptional suppression *in vivo*

Human IL-1α, IL-1β, IL-8, GRO-α, PD-L1, and PD-L2 transcripts derived from human A375 xenografts implanted into NOD/SCID mice and treated for 3 days with PLX4720 or vehicle. Data shown is the average of 3 mice per group and are representative of 3 separate experiments.

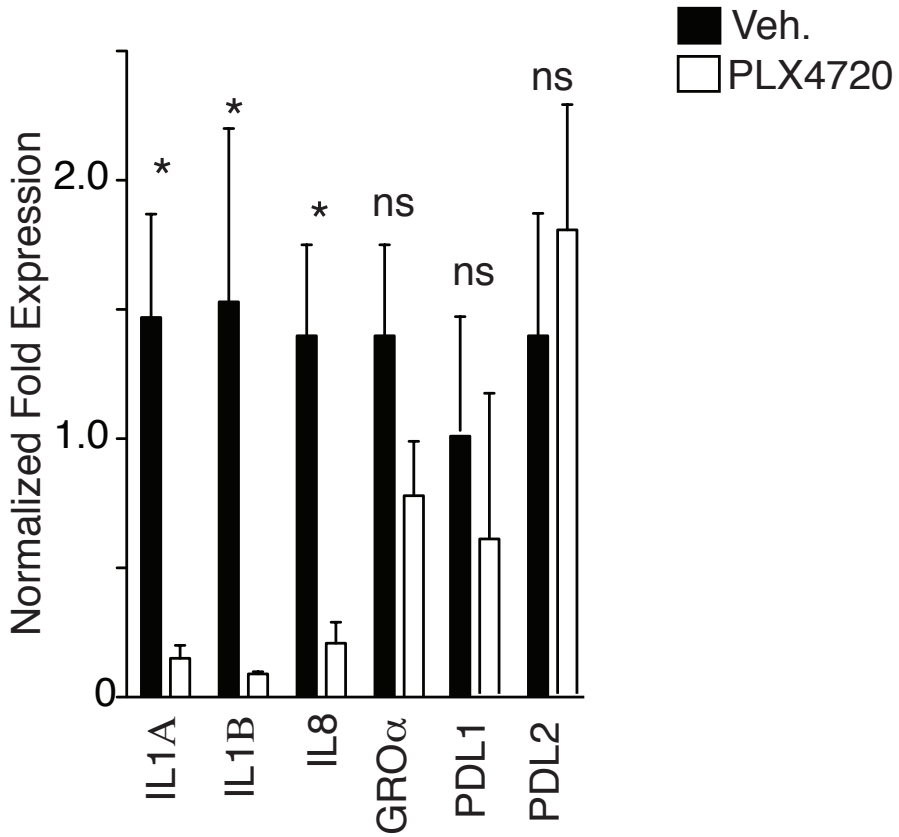
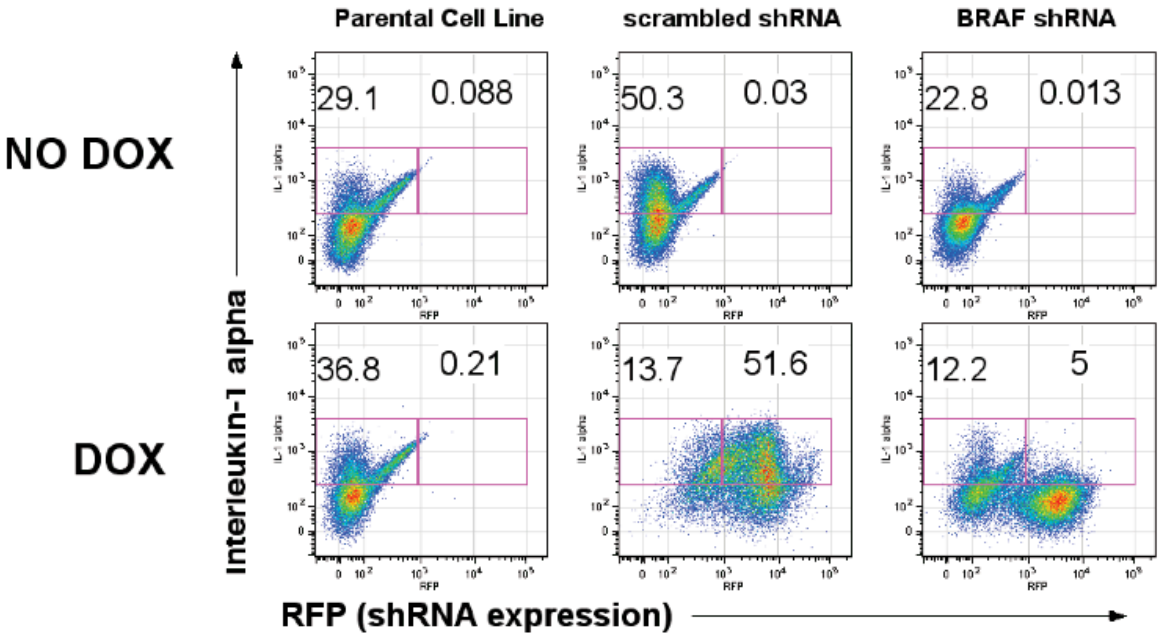

Veh.
PLX4720
Normalized Fold Expression
2.0
1.0
0
*
*
*
ns
ns
ns
IL1A
IL1B
IL8
GROα
PDL1
PDL2

Figure 16 BRAF shRNA expression correlates with loss of IL1α protien *in vitro*

IL-1α staining in cell lines transduced with scrambled shRNA vector, BRAF targeting shRNA vectors or untranduced as indicated. Expression of shRNA is indicated by coexpression of RFP (x-axis) and is induced by treatment with 2 ug/mL Doxycycline (DOX) (Lower). Cells were treated with DOX for 11 days prior to analysis by intracellular staining for IL-1α.


Parental Cell Line
scrambled shRNA
BRAF shRNA
NO DOX
DOX
29.1
0.088
50.3
0.03
22.8
0.013
36.8
0.21
13.7
51.6
12.2
5
Interleukin-1 alpha
RFP (shRNA expression)

IL-1α and IL-1β expression in human melanoma *in situ*

IL-1α and IL-1β expression have long been associated with melanoma; however, wide discrepancies exist in reports of its prevalence as IL-1α or β positivity has ranged from ~10% to 70% of samples analyzed [151,152,153]. The sole ability to upregulate IL-1a and IL-1b RNA is insuffient to generate active IL-1. Additional cleavage of proform IL-1 is required. However, given the role of BRAF(V600E) signaling on the regulation of these cytokine's RNA I sought to test if the BRAF(V600E) mutation is correlated with IL-1 production in patient melanoma samples.

First, using a tissue array generated to represent melanoma different stages of development from a nevus, primary and metastases, I evaluated the frequency of positive staining for IL-1α and IL-1β (Figure 17). Analysis showed that IL-1α is expressed at all stages of melanoma and in benign nevi at a frequency ranging from 63 to 88%, whereas IL-1β is expressed at a lower overall frequency in melanoma (13 to 20%) and not at all in nevi (Table 2). Mutation data is not available for tumors represented on this array, so correlation analysis was not possible, but the frequency of IL-1 expression is greater than that of the BRAF(V600E) mutation, suggesting that other elements could drive the expression of IL-1 in melanoma.

Patient samples with known BRAF mutations were acquired and assembled into additional tissue arrays to directly test if IL-1 is associated with the BRAF(V600E) mutation. However, limited samples prevented a fully powered study, I observed the

presence of IL-1 expression was associated with tumors harboring BRAF(V600E) mutations. Patient tumors with (wt) BRAF express IL-1α and IL-1β both broadly in tumor cells and intensely (Figure 18). This is consistent with the presence of IL-1 expression by melanoma cell lines grown *in vitro* which have (wt) BRAF and also express IL-1.

Figure 17 Range of IL-1α and IL-1β staining in Melanoma

Sections of a melanoma progression tissue array stained with antibodies specific for IL-1α or IL-1β and visualized by Vector-red immunostaining. Red color indicates positive staining for (C-E) IL-1α or (G-I) IL-1β in representative primary and metastatic tumors.

Negative
Low
Medium
High
B
C
D
E
F
G
H
I
30um
IL-1α
IL-1β

Table 2 Prevalence of IL-1α and IL-1β in melanoma

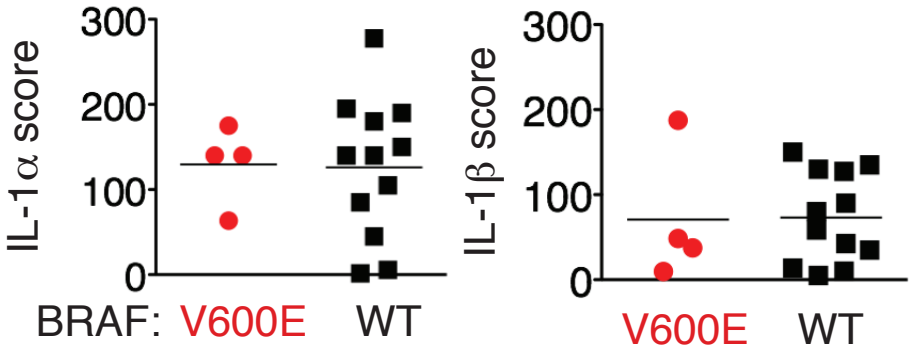
Frequencies of IL-1α and IL-1β staining at different disease stages.

Prevalence of IL-1α and IL-1β in melanoma

Melanocytic Lesion	IL-1 α	IL-1 β
Nevus	22/35 (.63)	0/35 (.00)
Primary	50/57 (.88)	10/49 (.20)
Metastasis	44/55 (.80)	7/55 (.13)

Figure 18 Relationship of BRAF(V600E) mutation and IL-1α or IL-1β levels *in situ*

IL-1α or IL-1β from stage 3 lymph node metastasis of tumors with (red) or without (black) the BRAF(V600E) mutation. Scores indicates the product of the intensity of signal (0-3) and frequency (0-100) of immunoreactive cells.

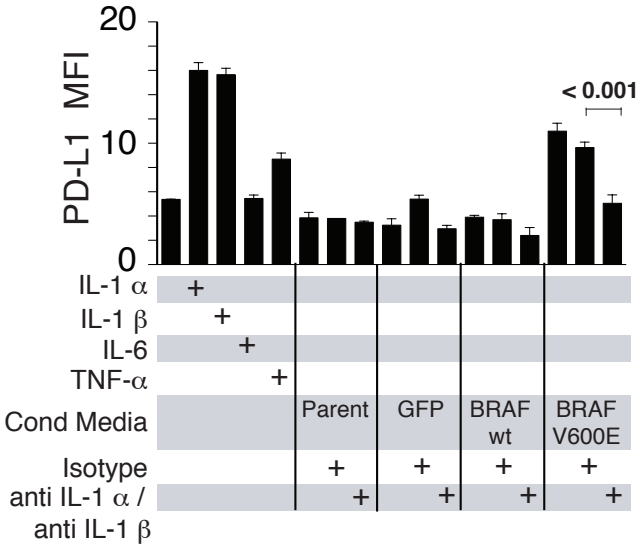

IL-1α score
300
200
100
0
BRAF: V600E WT
IL-1β score
300
200
100
0
V600E WT

The expression of IL-1 in melanoma tumors lacking the BRAF(V600E) mutation suggests that alternative mechanisms of MAPK pathway activation may also induce its expression. Thus, oncogenic BRAF(V600E) is sufficient, but not necessary, to upregulate IL-1 production melanoma.

In early experiments conducted to test the role of BRAF(V600E) expression on T cells suppressive molecules, dermal fibroblasts were treated with culture supernatants as analyzed in Figure 9. I observed that PD-L1 could be specifically regulated by the BRAF(V600E)-induced cell supernatant (Figure 19). The absence of IFN-g and TNF-a, as well as bacterial proteins, suggested to me that another soluble factor could be responsible. The strong evidence for the upregulation of IL-1 by BRAF(V600E) in these samples led me to test the role of this cytokine on the expression of PD-L1 on fibroblasts. Using neutralizing antibodies with the culture supernatants and adding IL-1 in this preliminary experiment indicated that IL-1 was necessary and sufficient for the upregulation PD-L1 on these cells. With these preliminary results, I further attempt to understand the role of BRAF mediated IL-1 on the fibroblast of the melanoma microenvironment, melanoma TAFs.

Figure 19 Regulation of PDL1 on fibroblasts by BRAFV600E transduced melanocytes.

Culture supernatants from melanocytes that were stably transduced to express either wt or mutated BRAF and then exposed these supernatants to fibroblasts overnight. Flow cytometric analysis revealed that only conditioned media from BRAF(V600E)-transduced melanocytes, but not those transduced with (wt) BRAF or GFP vectors, were capable of upregulating surface expression of PD-L1 (Figure 5A). Importantly, IL-1α/β antibody blockade abrogated this PD-L1 expression, demonstrating that IL-1 is the sole mediator of PD-1 ligand upregulation on TAFs induced by BRAF(V600E).


20
10
0
PD-L1 MFI
< 0.001
IL-1 α
IL-1 β
IL-6
TNF-α
Cond Media
Parent
GFP
BRAF wt
BRAF V600E
Isotype
anti IL-1 α / anti IL-1 β

Chapter 3

BRAF(V600E) regulated soluble factors signal to tumor associated fibroblasts

Introduction

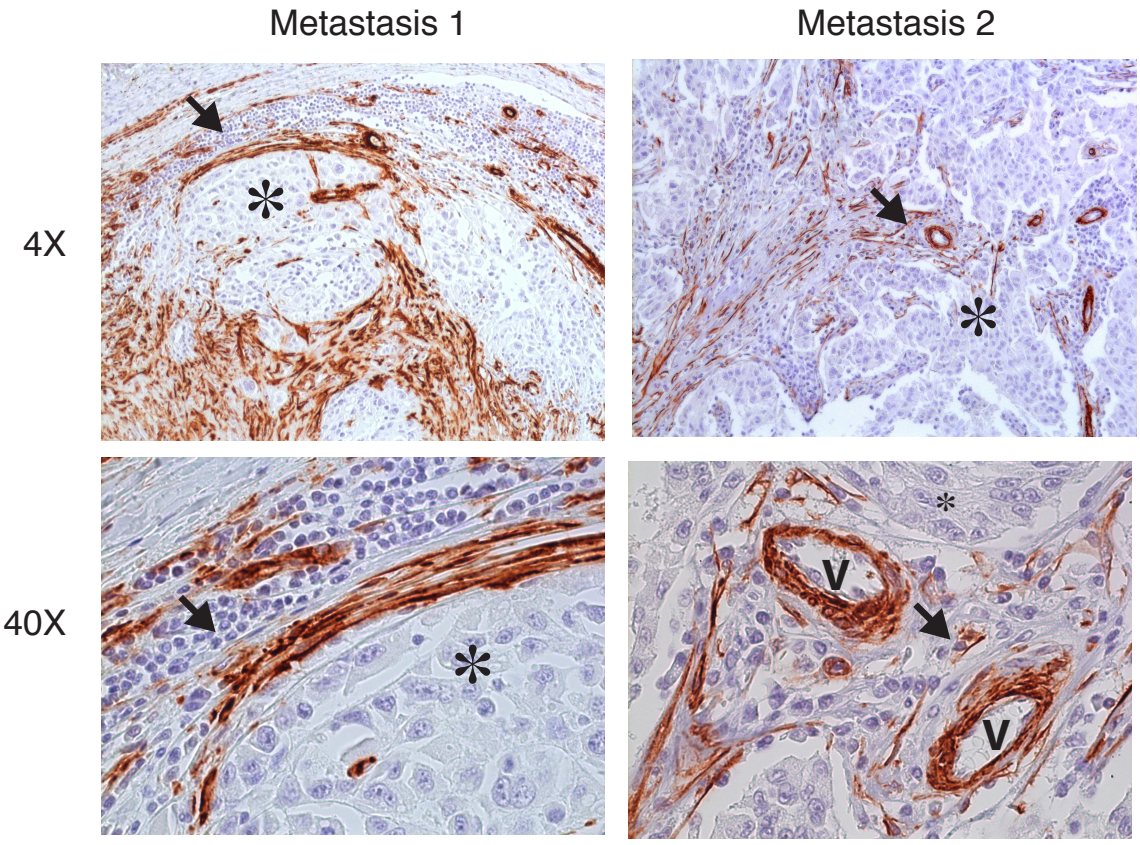
In this chapter, I address the immunomodulatory role of BRAF in the tumor microenvironment by following up the conclusion of the previous chapter. BRAF(V600E) upregulates IL-1α and IL-1β transcription, and conversely, blocking this oncogene arrests cytokine production. Having preliminary data that IL-1 can regulate PD-L1 on dermal fibroblasts, I focus my attention on the fibroblasts of the melanoma microenvironment, melanoma TAFs. These cells have been implicated as immunomodulatory in cancers *in vivo* and *in vitro*. However, the role of IL-1 on these cells has not been described. However, *in vitro* culture experiments with NIH3T3 fibroblast cell lines and melanoma cell lines have implicated IL-1 signaling from melanoma to fibroblasts as a dominant factor in this setting. With access to surgically-removed patient metastasis, I conduct my study of these cells in the melanoma microenvironment with primary cells.

I explored the hypothesis that IL-1 production within the melanoma tumor microenvironment could regulate T cells through interactions with resident stromal fibroblasts. In melanoma tumor samples containing TIL, lymphocytes are frequently

found in close proximity to phenotypic or smooth muscle actin (SMA)-a positive TAFs, which line tumor vasculature and form physical barriers between TIL and tumor cells (Figure 20).

Figure 20 Melanoma Tumor Associated Fibroblasts *in situ*

Tissue sections from two representative melanoma metastases labeled with anti-α-SMA antibody and visualized with peroxidase immunostaining. Red-brown color shows staining of α-SMA-positive tumor-associated fibroblasts (TAF), asterisks denote tumor cells, arrows indicate tumor infiltrating lymphocytes (TIL), and 'V' denotes tumor vasculature.


Metastasis 1
Metastasis 2
4X
40X
V
V

Results and Discussion

To investigate whether fibroblasts derived from melanoma tumors demonstrated IL-1-dependent expression of immunosuppressive molecules, TAFs were isolated from cultured digests of melanoma metastases by CD90 bead positive selection or FACS based cell sorting [154,155,156] (Figure 20).

Global Transcriptional analysis of IL-1 signaling in melanoma TAFs

I performed a global transcriptional analysis of TAFs treated with IL-1, with the primary aim of being to identify candidate immunomodulators that might mediate the immune modulation of T cells. For this experiment, TAFs were isolated and purified from three different melanoma patient tumors derived from metastases of lymph node, lung and soft tissue (Figure 20). The isolated TAFs were treated with IL-1α in culture for 24 hours, and mRNA was isolated for gene expression microarray analysis. The purity of CD90 selection for the discrimination of myeloid and endothelial cells is indicated by the high signals for CD90, a-SMA, Fibroblast Specific Protein (FSP), and as signals for the endothelial marker CD31, myeloid marker CD11b being equivalent to the T cell marker CD3 delta.

IL-1α treatment induced the transcription of 197 genes with a False Discovery Rate (FDR) of 0.01, common to all three TAFs (Figure 21). These included a number of genes with immune-related functions, including multiple chemokines as well as several cytokines which are included in the larger sets of cytokines and chemokines

in Figures 23 and 24. Interestingly, many of these genes have been previously associated with melanoma, suggesting that IL-1 treated TAFs may act as signal amplifiers for melanoma-associated gene products IL-8, IL-6, IL-1α and IL-1β.

Importantly, a number of upregulated genes were potential mediators of T cell suppression (Table 3). For conformational studies, I focused on three genes: COX-2 and the PD-1 ligands PD-L1 and PD-L2 (Figure 21). These genes were among the most highly upregulated and are known to exert powerful suppressive effects on T cells, and their mechanisms of action have been relatively well-characterized in multiple cancer types [157,158].

Figure 21 Cultured Melanoma TAF micrograph

Cells isolated by CD90 expression after 1 week in culture post tumor digest. Cells cultured 3-5 weeks in total. Tumor-associated fibroblast (TAF) cultures were derived from three metastatic melanoma biopsies from three distinct anatomical sties: lymph node, lung and soft tissue.

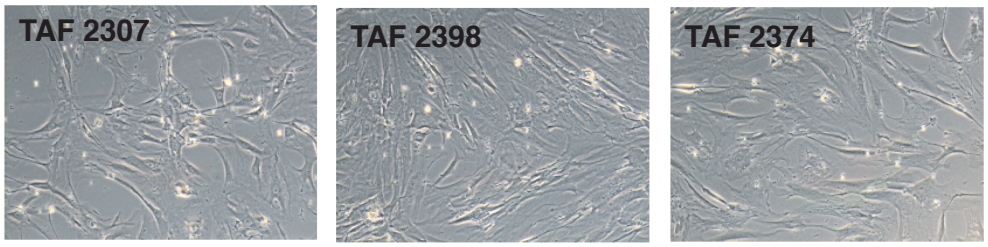

TAF 2307
TAF 2398
TAF 2374

Figure 22 Unsupervised clustering of IL-1α regulated genes in three TAFs derived from metastatic melanoma patients.

Cultured TAFs were exposed overnight to IL-1α, and treated and untreated cells were subjected to Affymatrix gene expression analysis. Heatmap displays the 197 most differentially expressed genes selected at FDR 0.01. Genes have been standardized at +- 2 standard deviations, with a scale of log values indicated in the color key.

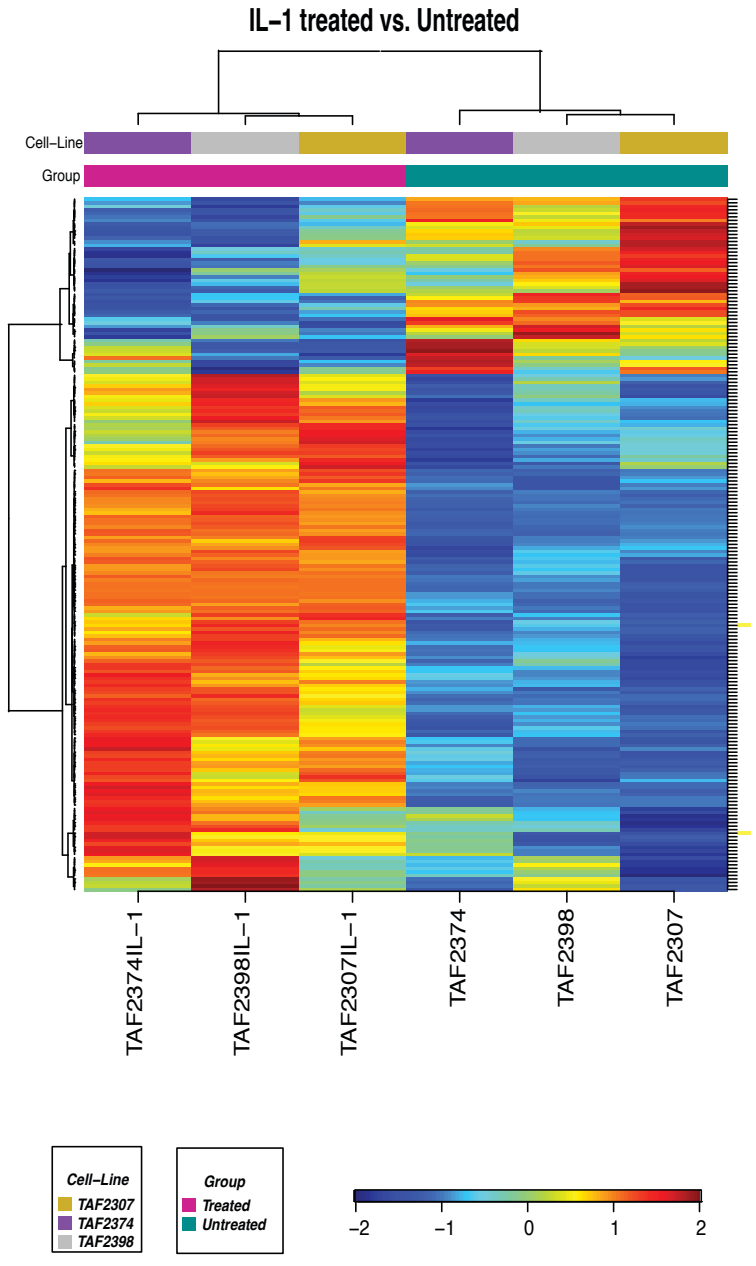

IL−1 treated vs. Untreated
Cell−Line
Group
TAF2374IL−1
TAF2398IL−1
TAF2307IL−1
TAF2374
TAF2398
TAF2307
Cell−Line
TAF2307
TAF2374
TAF2398
Group
Treated
Untreated
−2
−1
0
1
2

Figure 23 Ad hoc analysis of TAF lineage marker

Relative gene expression levels of Fibroblast (CD90, FAP, α-SMA), melanocytic lineage (Tyrosinase), T cell (CD3D), myeloid dendritic (CD11c), endothelial (CD31) lineage-related genes as determined by Affymetrix gene expression analysis in three melanoma TAF cell lines. Wide lines indicate Mean, error bars indicate 1 SEM.

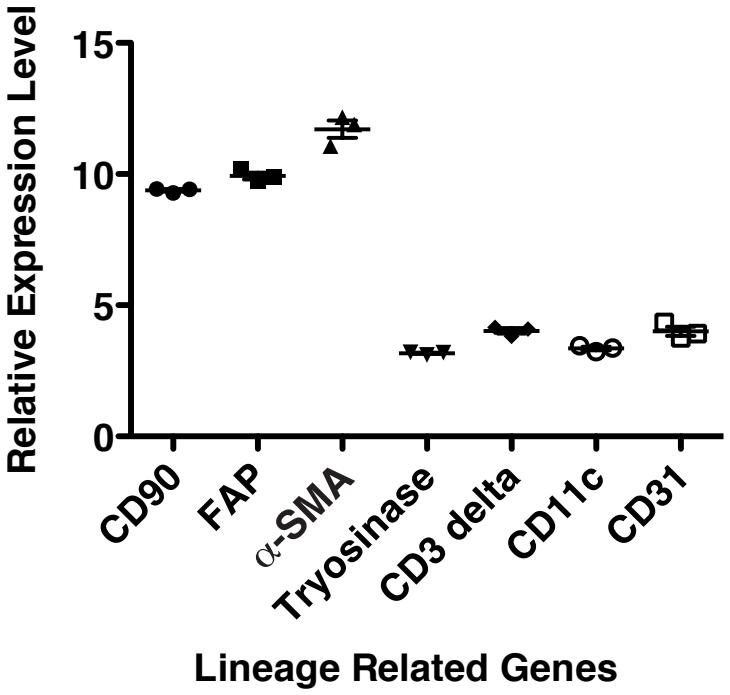

Relative Expression Level
15
10
5
0
CD90
FAP
α-SMA
Tryosinase
CD3 delta
CD11c
CD31
Lineage Related Genes

Figure 24 Regulation of TAF cytokines by IL-1α

Selection of cytokine genes upregulated by three melanoma TAFs after 24 hrs in response to IL-1α. The t-stat indicates the level of upregulation of the probe ID signal in response to the cytokine treatment.

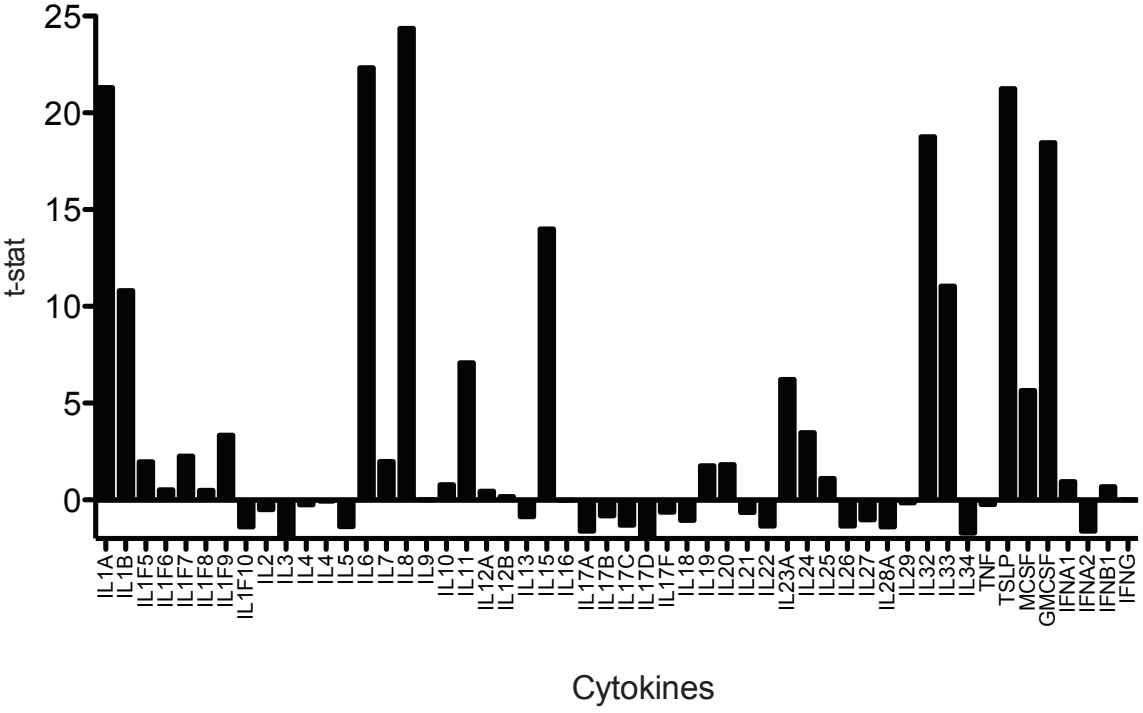

25
20
15
10
5
0
t-stat
IL1A
IL1B
IL1F5
IL1F6
IL1F7
IL1F8
IL1F9
IL1F10
IL2
IL3
IL4
IL4
IL5
IL6
IL7
IL8
IL9
IL10
IL11
IL12A
IL12B
IL13
IL15
IL16
IL17A
IL17B
IL17C
IL17D
IL17F
IL18
IL19
IL20
IL21
IL22
IL23A
IL24
IL25
IL26
IL27
IL28A
IL29
IL32
IL33
IL34
TNF
TSLP
MCSF
GMCSF
IFNA1
IFNA2
IFNB1
IFNG
Cytokines

Figure 25 Regulation of TAF Chemokines by IL-1α

Selection of chemokine genes upregulated by three melanoma TAFs after 24hrs in response to IL-1α. The t-stat indicates the level of upregulation of the probe ID signal in response to the cytokine treatment.

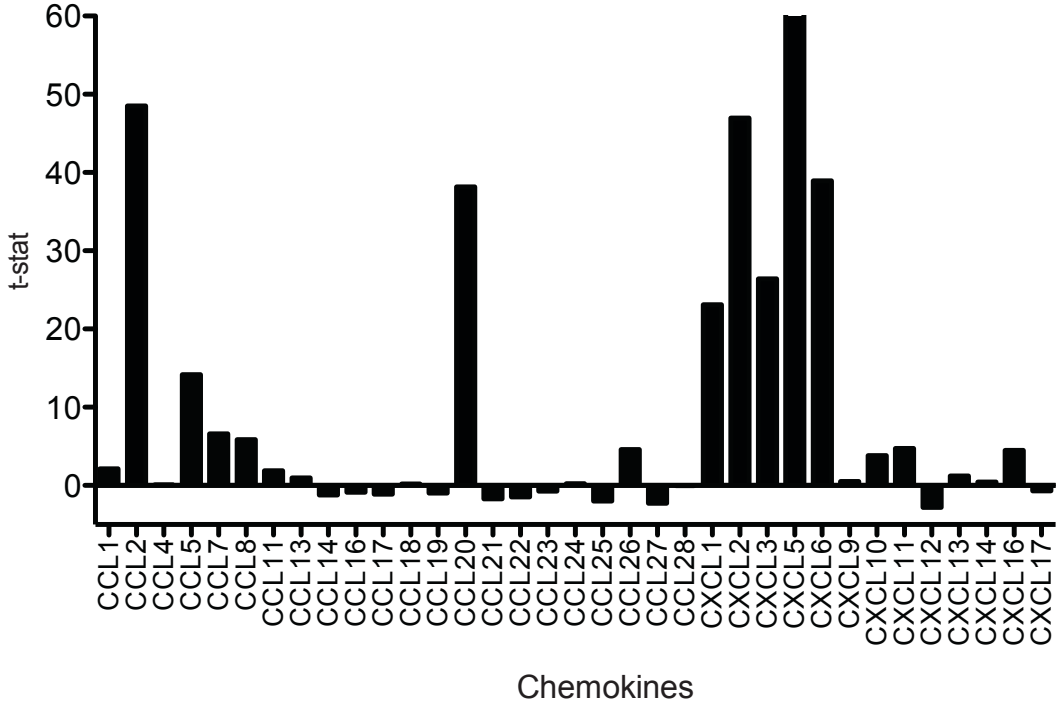

60
50
40
30
20
10
0
t-stat
CCL1
CCL2
CCL4
CCL5
CCL7
CCL8
CCL11
CCL13
CCL14
CCL16
CCL17
CCL18
CCL19
CCL20
CCL21
CCL22
CCL23
CCL24
CCL25
CCL26
CCL27
CCL28
CXCL1
CXCL2
CXCL3
CXCL5
CXCL6
CXCL9
CXCL10
CXCL11
CXCL12
CXCL13
CXCL14
CXCL16
CXCL17
Chemokines

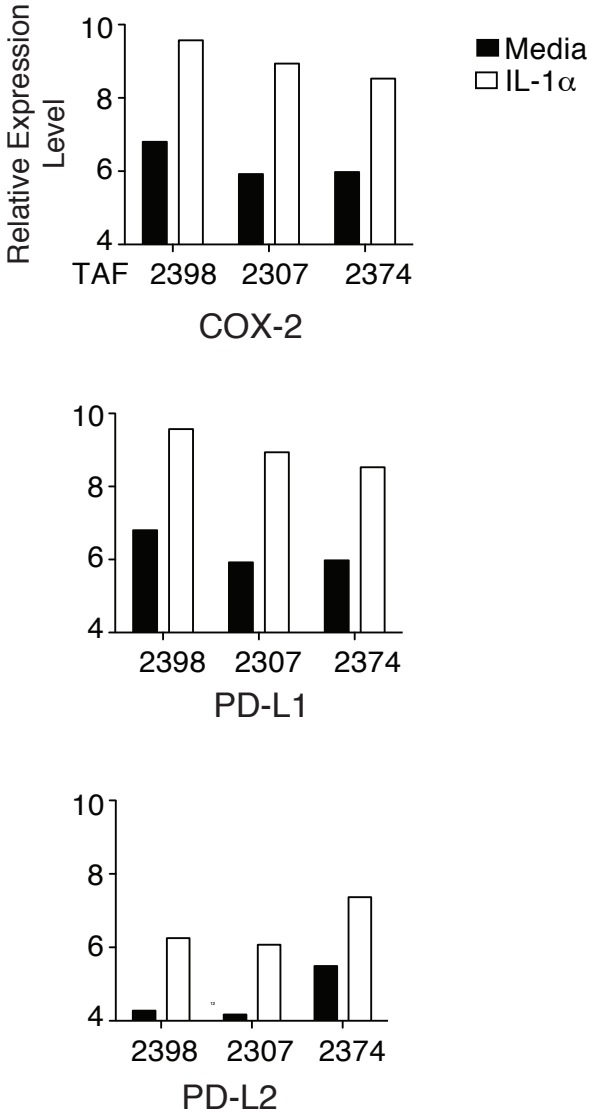
Table 3 List of immunosuppressive factors significantly upregulated in melanoma TAF in response to IL-1α

Selection of immunosuppressive genes upregulated by three melanoma TAFs after 24 hours in response to IL-1α. The t-stat indicates the level of upregulation of the probe ID signal in response to the cytokine treatment. [159,160,161,162,163,164,165,166,167].

Gene Name	Symbol	t-stat	Probe ID
COX 2	PTGS2	23.08	204748_at
PD-L1	CD274	20.36	227458_at
PD-L2	PDCD1LG2	9.07	224399_at
Leukemia Inhibitory Factor	LIF	10.36	205266_at
CD200 molecule	CD200	6.93	209582_s_at
Inhibin, beta A	INHBA	5.67	204926_at
Thrombospondin 1	THBS1	5.49	201109_s_at
MHC, class I G	HLA-G	4.28	211530_x_at
5'-nucleotidase, ecto (CD73)	NT5E	3.20	1553995_a_at
stanniocalcin 1	STC1	18.54	204595_s_at
Thymic Stromal Lymphopoietin	TSLP	21.25	235737_at

Figure 26 Immunosuppressive TAF transcription common to Lung, Soft tissue and Lymph node metastasis

Normalized relative transcriptional expression levels of COX-2, PD-L1 and PD-L2 in 24 h IL-1α treated or untreated TAFs, as analyzed by the Affymetrix gene expression array

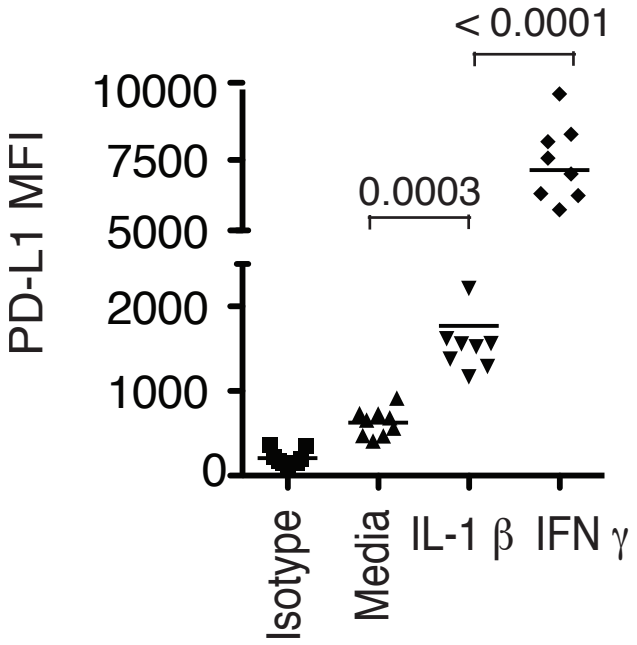

Relative Expression Level
10
8
6
4
TAF 2398 2307 2374
COX-2
Media
IL-1α
10
8
6
4
2398 2307 2374
PD-L1
10
8
6
4
2398 2307 2374
PD-L2

Confirmation of PD-1 Ligand and COX 2 induction on IL-1 treated melanoma TAFs

I tested for the augmented expression of the three gene products in response to IL-1 treatment at the protein level with multiple, independently-derived melanoma TAFs. Western blot analysis showed increased levels of COX-2 protein (Figure 27), and flow cytometric analysis demonstrated increased TAF surface expression levels of both PD-L1 and PD-L2 following IL-1α treatment (Figure 22 and 23). IL-1α and IL-1β were both shown to be very potent inducers of PD-1 ligand expression, demonstrating activity at concentrations as low as 1 pg/ml and inducing protein expression as early as 6 hours after treatment(Figures 28 and 29). Furthermore, although IL-1α/β was not as effective as IFN-γ at inducing expression of PD-L1, it was equally effective at inducing expression of the higher affinity PD-1 ligand PD-L2 in 9 of 9 TAFs analyzed (Figures 22). Collectively, these results demonstrate that TAFs exposed to low concentrations of IL-1α/β respond by rapidly stimulating the production of at least three molecules known to directly induce T cell suppression.

Figure 27 IL-1 and IFN-g induce PD-L1 and PD-L2 on melanoma TAFs

Surface expression of PD-1 ligands PD-L1 and PD-L2 on TAFs 24 hours after treatment with IL-1α or IFN-γ, as determined by flow cytometry. Data from nine different melanoma-derived TAF lines are shown. Geometric mean fluorescent intensity (MFI) was determined using immuno Flow cytometry. Asterisks indicate statistical significance ($P < 0.05$); ns, not significant.


< 0.0001
10000
7500
0.0003
5000
2000
1000
0
PD-L1 MFI
Isotype
Media
IL-1 β
IFN γ

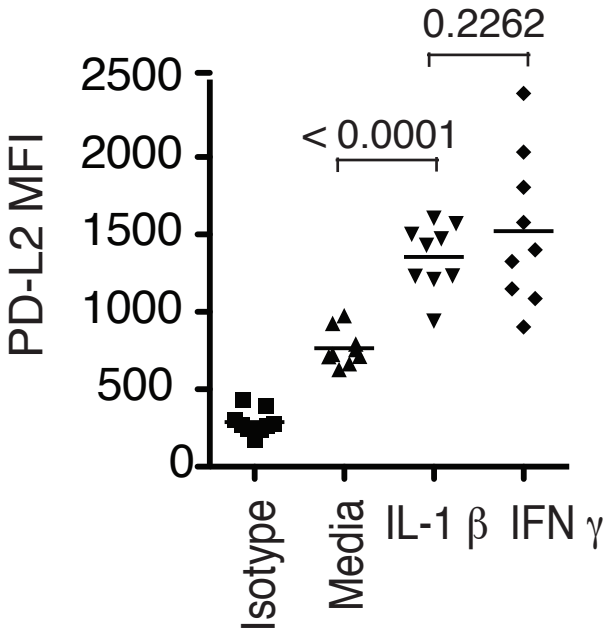

0.2262
2500
2000
< 0.0001
1500
1000
500
0
PD-L2 MFI
Isotype
Media
IL-1 β
IFN γ

Figure 28 Regulation of COX-2 by IL-1 in melanoma TAFs

Western blot analysis showing COX-2 and β-actin protein expression in four additional patient-derived TAF lines, prior to and 24 hours following treatment with IL-1α.

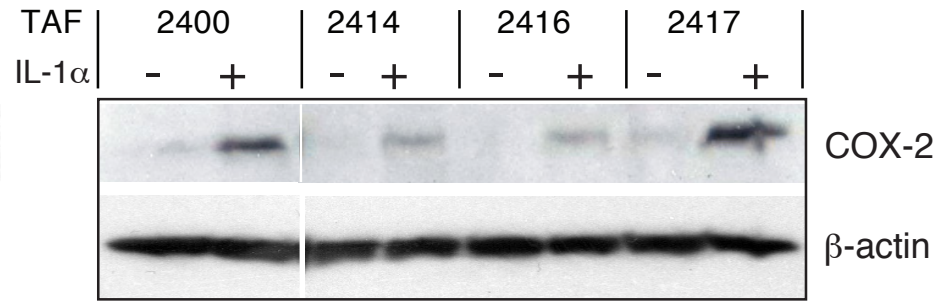

TAF
2400
2414
2416
2417
IL-1α
-
+
-
+
-
+
-
+
COX-2
β-actin

Figure 29 Phenotype similarities between Dermal Fibroblasts and melanoma TAFs

Expression levels of PD-L1, PD-L2, MHC Class I and MHC Class II by normal human fibroblasts and melanoma TAFs after 24 hour exposure to IL-1β or IFN-γ. Also indicated are basal expression levels of CD90 and MCSP.

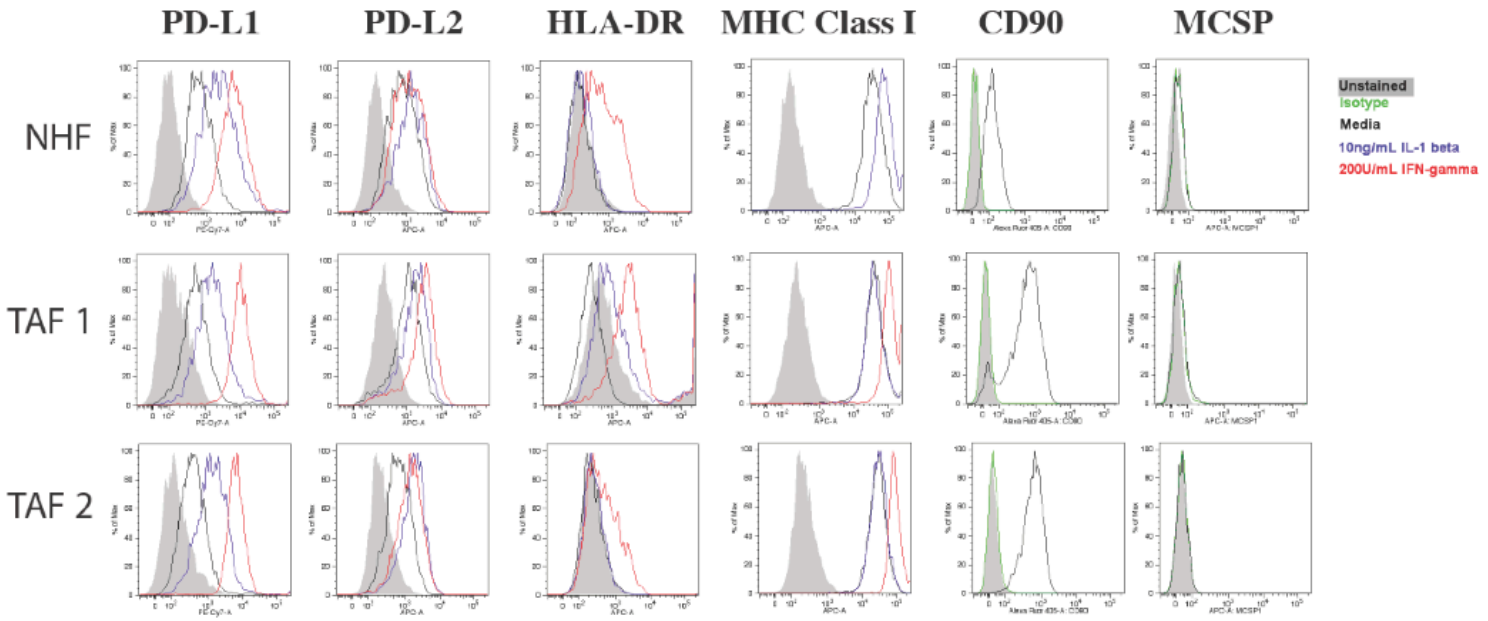

PD-L1
PD-L2
HLA-DR
MHC Class I
CD90
MCSP
NHF
TAF 1
TAF 2
Unstained
Isotype
Media
10ng/mL IL-1 beta
200U/mL IFN-gamma

Figure 30 Kinetics of B7-H1 expression after IL-1 treatment

Expression of PD-L1 on dermal fibroblasts at the indicated time after treatment with 100 pg/mL IL-1β. Signal was measured by FACS staining.

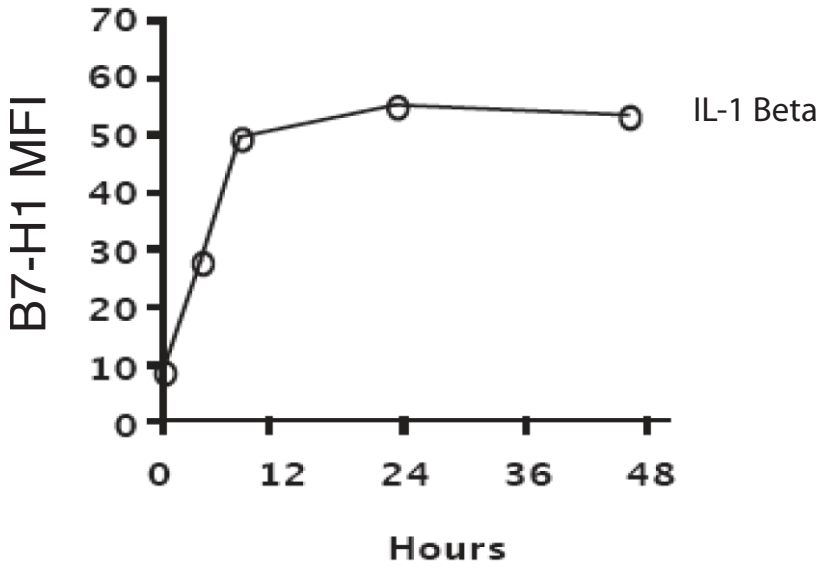
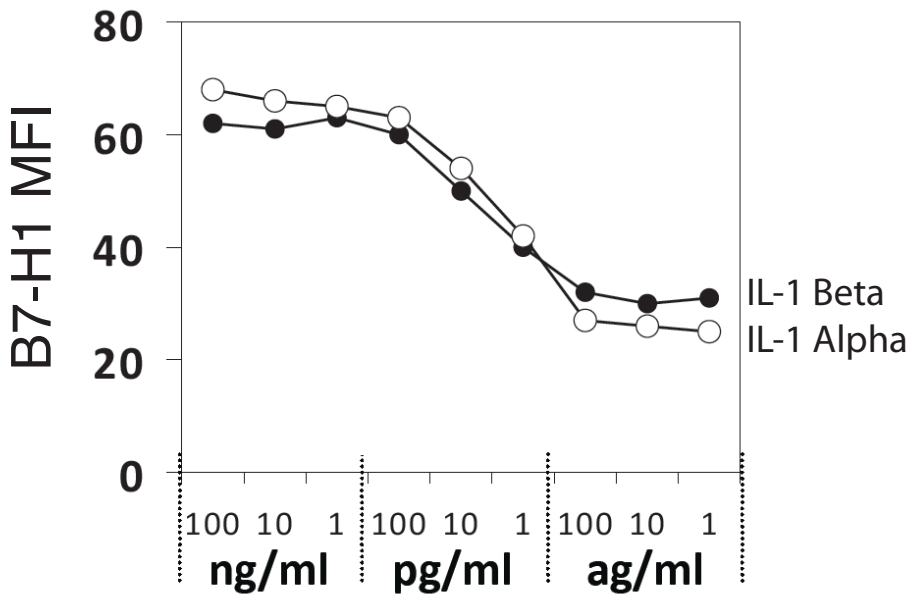

B7-H1 MFI
70
60
50
40
30
20
10
0
0
12
24
36
48
Hours
IL-1 Beta

Figure 31 Dose range of IL-1 regulation of PD-L1

Expression of surface PD-L1 after 24 hours of treatment with IL-1α or IL-1β. Signal was measured by FACS staining.


B7-H1 MFI
80
60
40
20
0
100 10 1
100 10 1
100 10 1
ng/ml
pg/ml
ag/ml
IL-1 Beta
IL-1 Alpha

Chapter 4

Vemurafenib relieves CTL dysfunction in the presence of tumor associated fibroblasts

Introduction

Considering the importance of TIL for mediating tumor regressions in melanoma patients [168,169], I next tested whether cultured fibroblasts and TAFs were capable of suppressing CD8+ T cell function and whether IL-1 could impact this suppression. Further I set out to determine the contribution of IL-1 induced PD-1 ligands on PD-1+ melanoma Til. The ready availability of COX-2 inhibitors allowed me also to address the contribution of the COX-2/PGE2 mechanism suggested in experiments from the previous chapter.

Results and Discussion

Interleukin-1 activated TAF mediated suppression

I tested the ability of fibroblasts to suppress HLA-A*0201-restricted MART-1-specific CD8+ TIL in the context of direct presentation, thus in the absence of any other cell types to isolate the IL-1 response. Dermal fibroblasts were co-cultured with HLA-matched, MART-1 or control dermal fibroblasts that were either untreated or pre-

stimulated with IL-1β for 24 hours. In order to measure an integrated function of TCR signaling, I assessed the presence of the protein Ki-67 in the Mart-1 specific CTLs. Ki-67 is a protein uniquely associated with the cell cycle, being upregulated in G1 phase and rapidly degraded during cytokinesis[170]. Its utility in measurement of cycling cells is well-established in oncology and immunological fields of study.

IL-1β pre-treatment reduced TIL cell cycle entry (Ki-67+ cells) by approximately 50% in MART-1 tetramer-positive CTLs after 3 days of culture (Figure 33). Similar results were observed after 5 days. Thus, in the context of direct presentation of antigen to CTLs, IL-1 treated fibroblasts can suppress antigen induced proliferation.

If this suppressive effect is mediated by altered antigen presentation, or limited to direct presentation, this assay was elaborated to include antigen pulsed third-party APCs (irradiated PD-1L- B cells). Similar results were observed in this assay, as co-cultured IL-1β stimulated fibroblasts exerted a similarly suppressive influence on MART-1-specific TIL in the presence of additional antigenic stimulation from a third party cell (Figure 33).

In order to assess the effect of fibroblasts on cytotoxic granule release I measured the amount of CD107 that emerges on the surface of the T cells. CD107 is a molecule that is typically restricted to intracellular vesicles that contain perforin and granzyme. However, upon fusion of these vesicles with the membrane during the release of the cytotoxic molecules, CD107a is transiently present on the surface of

the cells. Pulsing the culture media with fluorescently conjugated antibody to CD107a allowed me to quantify the number of cells that degranulated in response to the pulsed peptide, thus establishing any modulators of cytotoxic function in my cellular system. Although suppression could be observed with peptide pulsed cells with intact endogenous antigen presentation machinery, for the experiment and those that follow, I used a special antigen-presenting cell line known as T2. T2 cells lacking TAP1 and thus cannot load their MHC Class I (HLA-A201) with endogenous peptide. As such, pulsed cells contain only the experimental peptide, allowing more uniform and consistent assay conditions.

As shown in Figure 32, Mart-1 containing TIL lines had increased degranulation in the presence of MART-1 peptide pulsed T2 cells as compared to control HIV derived peptides. I observed suppression of their response to MART-1 antigen in the presence of IL-1β pretreated fibroblasts, compared to untreated fibroblasts; similar results were observed with IL-1α. Suppression of proximal cytotoxic function is a profound defect in the melanoma microenvironment and has been ascribed as a function of PD-1ligation.

In a third assay for CTL function, the release of IFN-γ in response to antigen stimulation, melanoma TAFs from 6 different patient tumors were evaluated. As previous described, these cells were tested for suppressive function toward MART-1-specific TIL exposed to MART-1 peptide-pulsed T2 stimulator cells. Whereas untreated TAFs demonstrated some suppression of TIL cytokine production, IL-1α

pretreatment reduced IFN-γ production by ~5-fold (Figure 34). Furthermore, antibody-mediated neutralization of IL-1α/β abrogated the suppressive effect of IL-1 in combination with TAFs (Figure 34).

Figure 32 Dermal Fibroblasts suppress Til cytotoxic degranulation

Frequency of CD107a-positive MART-1 reactive TIL following co-culture with MART-1 peptide-pulsed dermal fibroblasts pretreated with or without IL-1α, as determined by flow cytometry. Data from 2 different melanoma patient TIL are shown, and are representative of 2 independent experiments.

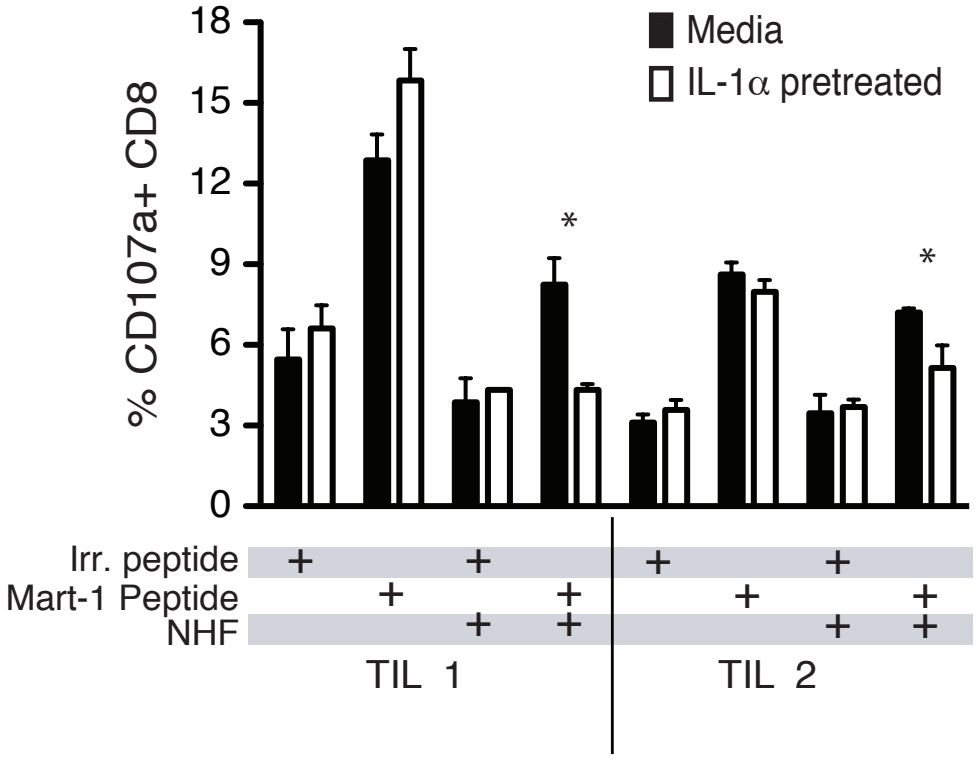
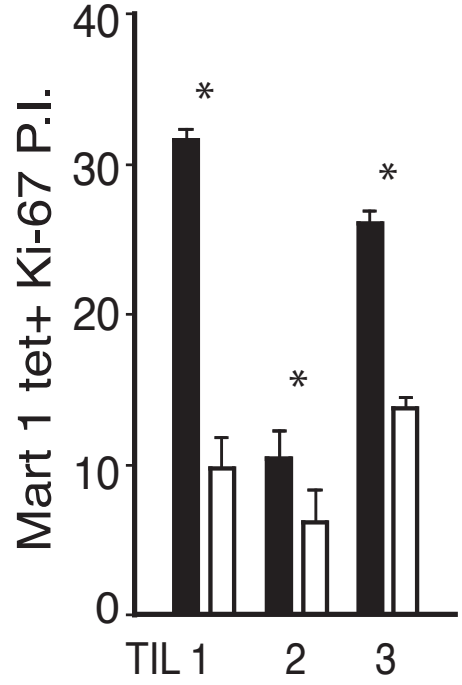

Media
IL-1α pretreated
% CD107a+ CD8
18
15
12
9
6
3
0
*
*
Irr. peptide
Mart-1 Peptide
NHF
TIL 1
TIL 2

Figure 33 IL-1α activated fibroblasts suppress Til proliferation with indirect antigen presentation.

Figure 3.3 (B) Frequency of Ki67-positive MART-1 reactive TIL after 3 days of co-culture with MART-1 peptide-pulsed HLA-A2+ dermal fibroblasts pretreated with or without IL-1α, as determined by flow cytometry. Data from 3 melanoma patient TIL are shown, and are representative of at least 5 independent experiments. IL-1 treatment White bars, media Black bars.

Frequency of Ki67-positive MART-1 reactive TIL after 3 days of co-culture with MART-1 peptide-pulsed antigen presenting cells (APC) consisted of third-party, irradiated B cells, and HLA-A2+ dermal fibroblasts pretreated with or without IL-1α, as determined by flow cytometry. Data from 3 melanoma patient TIL are shown, and are representative of at least 5 independent experiments. IL-1 treatment White bars, media Black bars.


Mart 1 tet+ Ki-67 P.I.
40
30
20
10
0
*
*
*
TIL 1
2
3

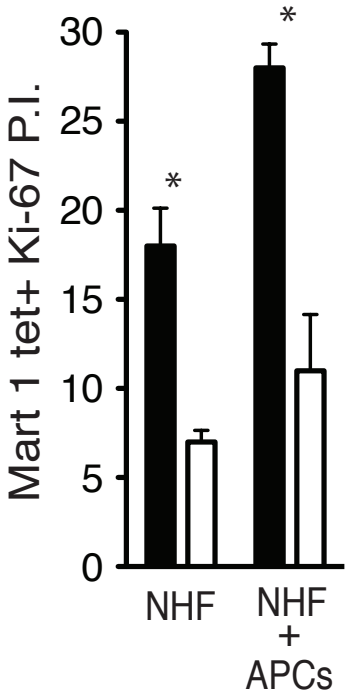
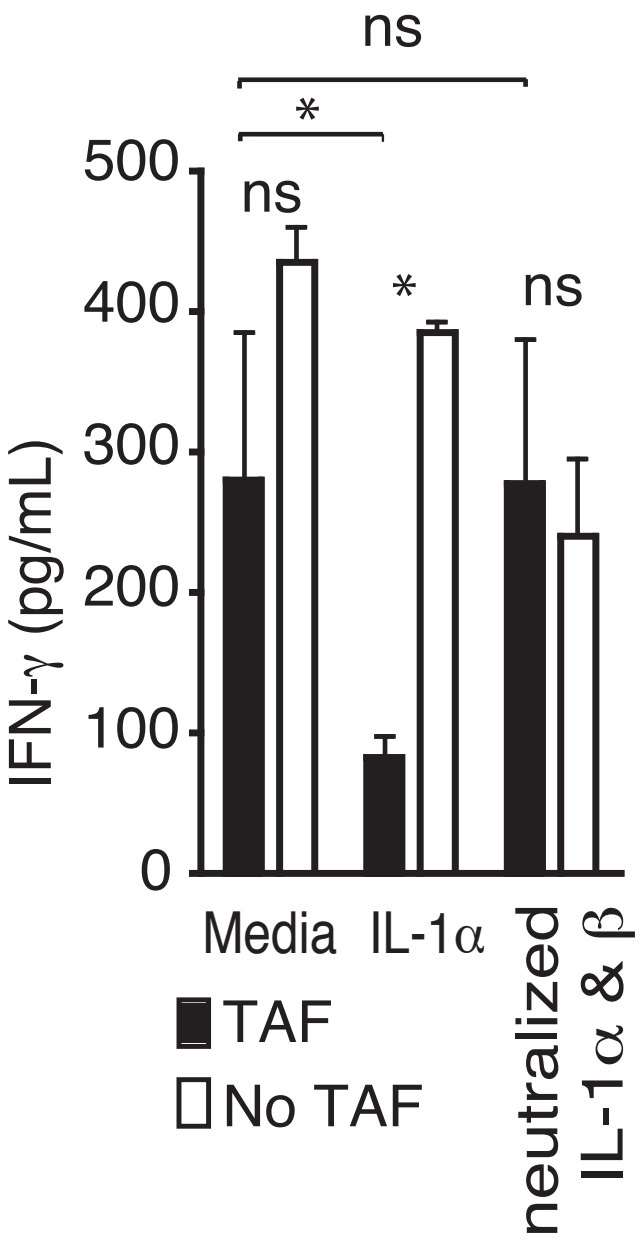

Mart 1 tet+ Ki-67 P.I.
30
25
20
15
10
5
0
*
*
NHF
NHF + APCs

Figure 34 IL-1 activated melanoma TAFs suppress antigen induced release of IFNg by melanoma Til

Interferon-γ release by TIL stimulated with MART-1 peptide-pulsed T2 cells in the presence or absence of untreated or IL-1α treated melanoma TAFs, with or without the addition of IL-1 neutralizing antibodies. Data are representative of six different TAF lines analyzed and three experimental replicates. Asterisks indicate statistical significance ($P < 0.05$); ns, not significant.


ns
*
ns
*
ns
500
400
300
200
100
0
IFN-γ (pg/mL)
Media
IL-1α
neutralized IL-1α & β
TAF
No TAF

BRAF(V600E) inhibitor effects on melanoma activated TAF mediated suppression

I next assessed whether PD-1 ligand upregulation by TAFs can be directly attributed to IL-1 production induced by BRAF(V600E). In order to establish this linkage, I first tested whether pharmacologic BRAF(V600E) inhibition could relieve TAF-mediated T cell suppression, I exposed six different melanoma-derived TAFs to supernatants from BRAF(V600E)-positive melanoma cell lines that were either untreated or treated with Vemurafenib. Following overnight exposure to these supernatants, TAFs were co-cultured with MART-1-specific TIL expressing PD-1 (Figure 38) and MART-1 peptide pulsed T2 target cells for 18 hours. The following day, culture supernatants were assayed for IFN-γ production. As shown in Figure 37, BRAF(V600E) inhibition by Vemurafenib resulted in a dramatic augmentation of T cell IFN-γ production in all six TAFs analyzed, supporting the notion that BRAF(V600E) could induce functional TIL suppression mediated through TAFs.

To ascertain whether IL-1α/β, COX-2 or PD-1 ligands played a role in mediating this suppression, I next performed a similar experiment in the presence or absence of IL-1α/β or PD-L1/PD-L2 antibody blockade, or the COX-2 inhibitor NS398. Antibody neutralization of IL-1α/β in melanoma cell line supernatants partly relieved the MART-1 TIL functional suppression observed in co-cultures with TAFs (Figure 37). In addition, antibody neutralization of PD-1 ligands and COX-2 also partly relieved TAF-mediated suppression, whereas combined neutralization of IL-1α/β and PD-1 ligands with COX-2 inhibition could further improve T cell function in two of the three

melanoma supernatants tested. Although there was some variability in the extent of T cell suppression mediated by different TAF lines, overall these data are consistent with a role for IL-1 mediating suppression through TAFs by upregulation of PD-1 ligands and COX-2.

By contrast, neutralization of IL-1α/β in supernatants from the same melanoma cell lines pretreated with Vemurafenib had no significant effects on TIL function, nor did PD-1 ligand blockade or COX-2 inhibition facilitate increased cytokine secretion by T cells. These results indicate that BRAF(V600E) inhibition can relieve suppression of T cell function mediated by TAFs expressing PD-1 ligands and COX-2, at least in part through reducing IL-1α/β production by melanoma cells. Taken together, these experiments support a mechanism of IL-1 induced TAF-mediated immune suppression that is sensitive to V600E-specific inhibition.

IL-1α signaling upregulates expression of immunosuppressive genes in melanoma-derived TAFs, which results in CTL functional suppression. Understanding the basic mechanisms of IL-1 induced suppression by TAFs can inform more general clinical strategies to improve immunotherapies.

Figure 35 BRAF(V600E) can induce T-cell suppression through IL-1 mediated upregulation of PD-1 ligands and COX-2 on TAFs.

Interferon-γ release by T2-stimulated MART-1 reactive TIL in the presence of melanoma patient-derived TAFs previously exposed to conditioned media from BRAF(V600E) mutant-expressing melanoma cell lines that were either untreated or treated with PLX4032. Three melanoma TAF lines were pre-treated with conditioned media from untreated or PLX4032-treated melanoma cell lines (WM793p2, EB16-MEL and KUL84-MEL), in the presence of either IL-1α/β blocking antibodies or the COX-2 inhibitor NS398. Pre-conditioned TAFs were then incubated with TIL and MART-1 peptide-pulsed T2 cells in the presence of isotype control antibody or antibodies specific for PD-L1 and PD-L2. P values represent results for paired t-tests.

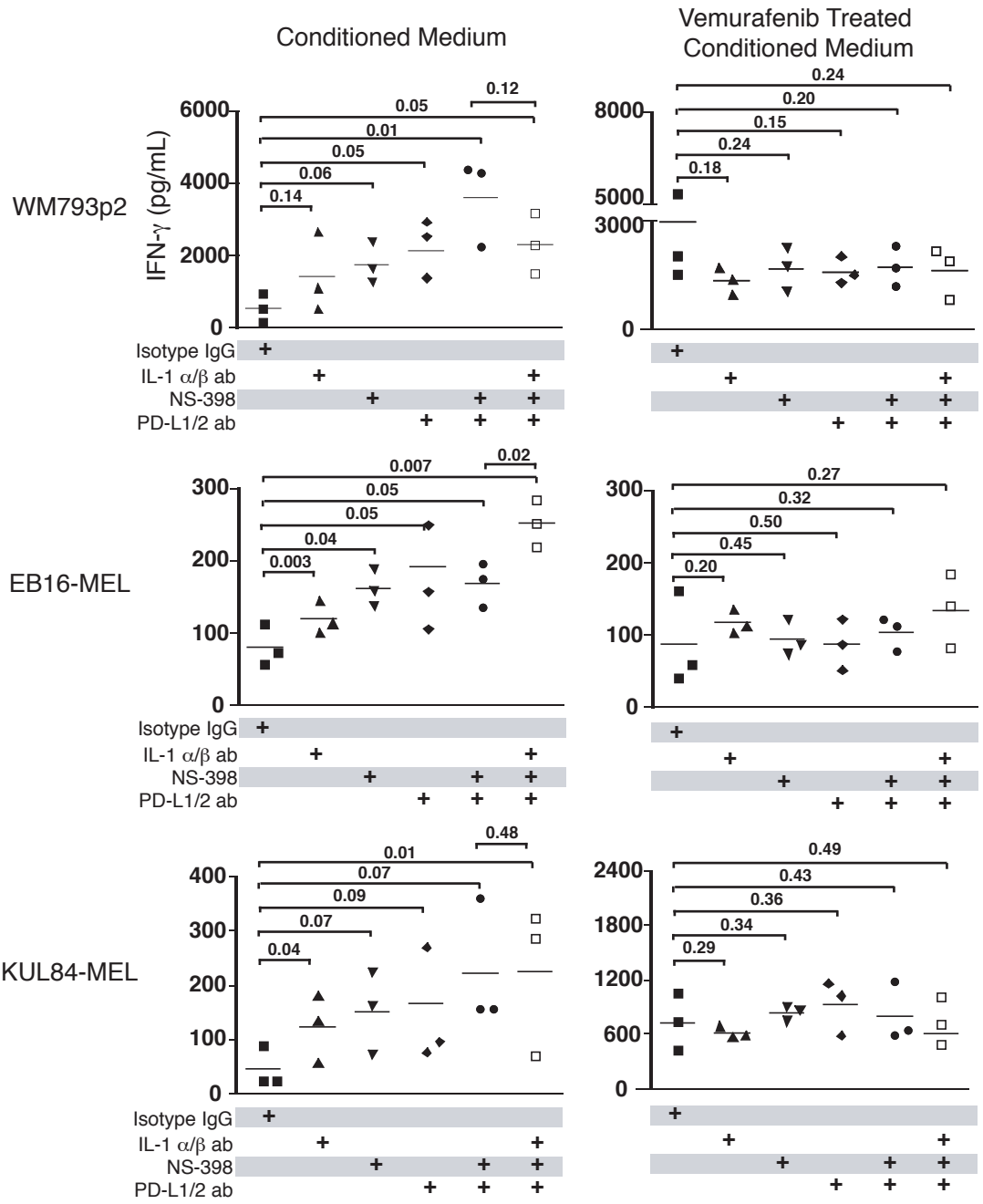

Conditioned Medium
Vemurafenib Treated Conditioned Medium
WM793p2
EB16-MEL
KUL84-MEL
IFN-γ (pg/mL)
Isotype IgG
IL-1 α/β ab
NS-398
PD-L1/2 ab

Figure 36 Til suppression by TAFs activated by melanoma supernatants is releived by BRAF(V600E) inhibition

Interferon-γ release by T2-stimulated MART-1 reactive TIL in the presence of melanoma patient-derived TAFs previously exposed to conditioned media from BRAF(V600E) mutant-expressing melanoma cell lines that were either untreated or treated with PLX4032. Results from five different TAF lines are shown.

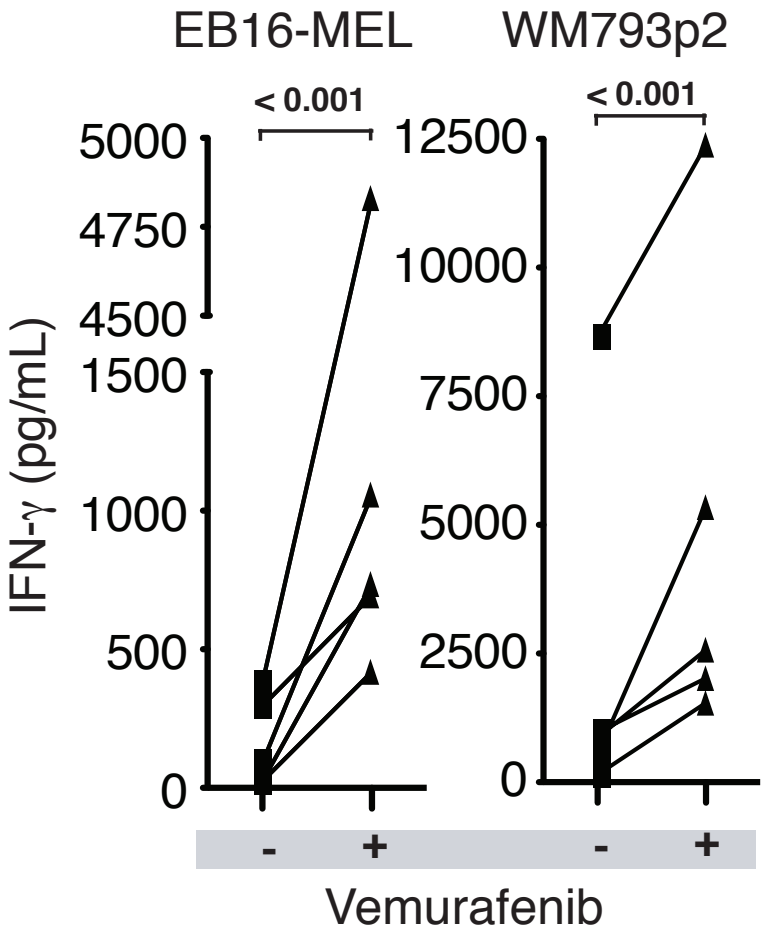
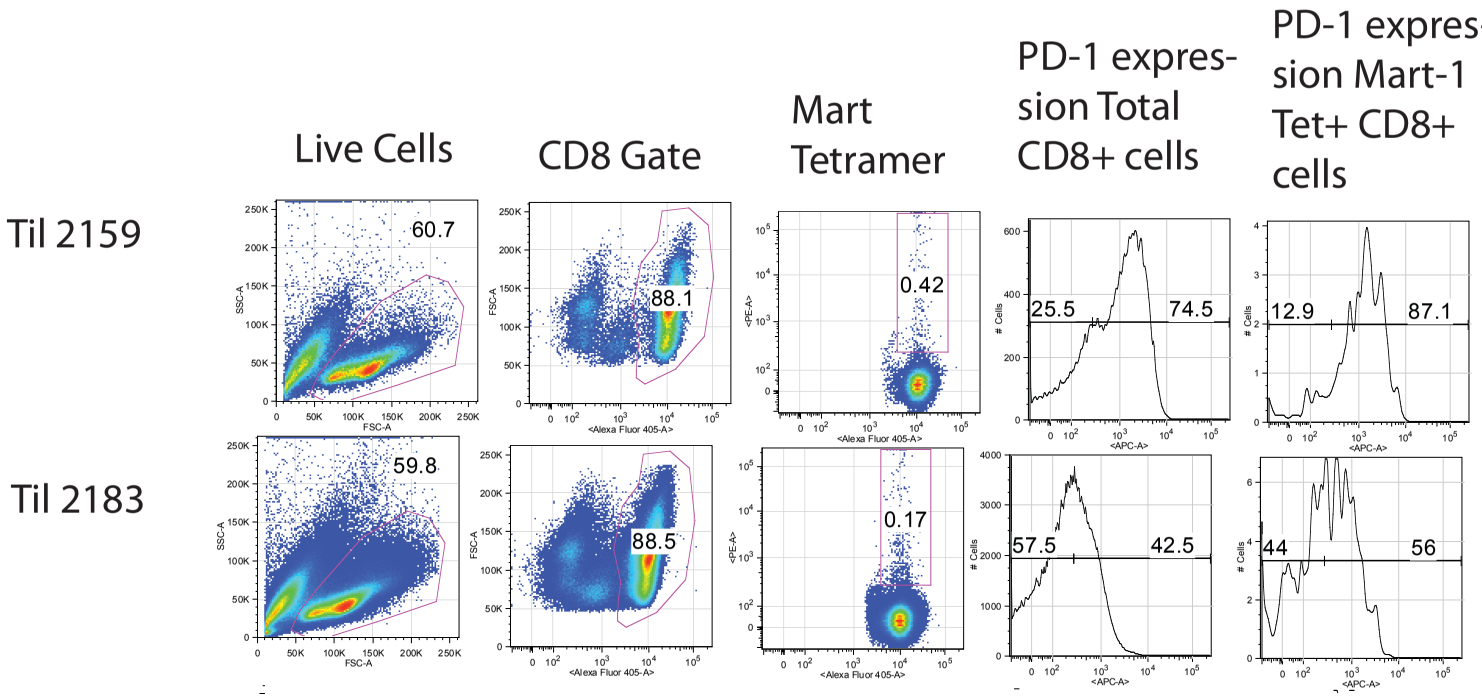

EB16-MEL
WM793p2
< 0.001
< 0.001
5000
4750
4500
1500
1000
500
0
12500
10000
7500
5000
2500
0
IFN-γ (pg/mL)
-
+
-
+
Vemurafenib

Figure 37 Expression of PD-1 on antigen specific melanoma Til

Flow cytometric analysis of two different TIL cultures (2159 and 2183) showing surface expression levels of PD-1 on total live CD8+ T cells and on MART-1 tetramer binding CD8+ T cells.


Live Cells
CD8 Gate
Mart Tetramer
PD-1 expression Total CD8+ cells
PD-1 expression Mart-1 Tet+ CD8+ cells
Til 2159
Til 2183
60.7
88.1
0.42
25.5
74.5
12.9
87.1
59.8
88.5
0.17
57.5
42.5
44
56
SSC-A
FSC-A
<Alexa Fluor 405-A>
<PE-A>
<APC-A>
Cells

Chapter 5

General discussion and Future directions

Discussion

As a wholly embedded member of the MEK-MAPK signaling pathway, the study of activated BRAF, via the V600E mutation, is largely a surrogate for the study of this pathway as itself. However, with the unique distinction of specific inhibition with mutation specific small molecule inhibitors.

I set out with the hypothesis that this mutation could manifest an immunomodulatory role in the melanoma tumor microenvironment. I observed that ectopic BRAF(V600E) expression in human melanocytes upregulated the expression of IL-1α and IL-1β, I found that IL-1 production in a subset of human melanoma cell lines was also under the control of this same oncogenic protein. I further delineated the frequency of IL-1α and IL-1β production in melanoma patient samples, determining that the majority of tumors contain this cytokine. Additionally, I observed that the expression of IL-1 by melanoma does not require the BRAF(V600E) mutation. This set of data indicates that the BRAF(V600E) mutation is sufficient but not necessary of IL-1 production by melanoma.

As an immune modulatory cytokine with pleotropic functions, I defined IL-1α and IL-1β signaling as regulator of immune suppression as manifested by melanoma tumor-associated stromal fibroblast cells (TAFs). This suppression was exerted upon melanoma antigen specific CTLs in part by COX-2, PD-L1 and PD-L2. In doing so I have delineates a novel link between oncogene activation in tumors and the resulting downstream effects on inflammation and immune suppression within the melanoma tumor microenvironment.

Constitutive activation of the MAPK pathway through oncogene mutations or amplifications has long been recognized as a hallmark common to many different cancer types [171]. Such genetic lesions provide cancer cells with a number of distinct advantages that promote tumor growth *in vivo*, including increased proliferation, reduced apoptosis, extended survival, and augmented metastatic potential [70,82,86]. Examples include KRAS, found to be mutated in lung carcinomas, colorectal [172] and >80% of pancreatic cancers [173], as well and PDGFRA and CKIT, which are often mutated in hematopoetic neoplasia and GIST [174,175]. Although BRAF(V600E) is the most common genetic lesion in cutaneous melanoma, NRAS [176] is mutated in a further ~20% of cases, and CKIT and GNAQ/GNA11 have been shown to frequently contain activating mutations in acral lentiginous and uveal melanomas, respectively [177]. The frequent occurrence of these mutations, combined with their propensity to provide tumor cells with a survival advantage, has made them highly favorable targets for the development of therapeutic, small molecule inhibitors to fight cancer [178].

Over the past decade, a number of small molecule inhibitors that target elements of the MAPK pathway and its downstream targets MEK and ERK have been tested in experimental clinical trials for cancer patients. Although these agents often show a high degree of efficacy *in vitro* or in xenogeneic animal models, clinical response rates have been modest. For example, therapy with a selective MAPK/extracellular signal-related kinase (ERK) kinase (MEK) inhibitor AZD6244 demonstrated only a 12%-40% response rate among BRAF(V600E)-positive melanoma patients [179,180]. The lack of specificity of many of these drugs have often been cited as a major problem that not only limits their clinical efficacy, but also leads to severe off-target effects such as cytotoxicity to immune cells that can result in lymphopenia and increased frequency of pathogen infections [181,182,183]. Since T cells require the MAPK pathway for antigen recognition and anti-tumor function, they are particularly sensitive to these off-target effects [184,185]. Furthermore, strong evidence has been accumulating that the immune system can make a critical contribution to antitumor responses even in the context of non-immunotherapeutic treatments [186,187,188,189], with the emerging paradigm being that an intact immune system contributes significantly to the outcome of treatment, and may be critical for clearance of drug-resistant tumor cells and for prevention of recurrences [190].

More recent generations of kinase-targeted agents have shown a much higher degree of specificity for the mutated forms of oncogenic kinases found only in tumor

cells, and importantly also show significantly less harmful effects on immune cells [191]. Inhibitors that specifically target mutated BRAF(V600E), such as Vemurafenib and GSK2118436, have shown remarkable efficacy in the clinic and in phase II/phase III trials, inducing responses in the majority of melanoma patients harboring the V600E mutation [130]. However, despite encouraging results, responses to BRAF(V600E) inhibition are relatively short-lived, and disease recurrence of inhibitor-resistant tumors occurs in nearly all treated patients [192]. These clinical findings have led many to propose combining BRAF(V600E) inhibition with immunotherapies to increase response rates, and our data suggests that this combination approach may show therapeutic synergy [105,191,193].

Our study identifies BRAF(V600E)-induced IL-1 as being a key mediator of immune suppression in melanoma. Unlike other cytokines which can impact T cells directly, IL-1 reinforces immune suppression indirectly through the stimulation of melanoma TAFs. A number of recent studies have highlighted the importance of stromal TAFs in promoting tumor cell survival and evasion of NK cell-mediated antitumor immunity [194]. Our results are experimentally consistent with these findings and show that IL-1α and β can induce melanoma TAFs to directly inhibit the antitumor function of melanoma antigen-specific CD8+ T cells. Gene expression analysis showed that this IL-1-mediated suppression is likely mediated by a host of factors known to affect T cell function as well as other lymphocytes, (Table 3), including but not limited to TAF expression of PD-1 ligands PD-L1 and PD-L2 and COX-2. Importantly, the location of TAFs within the architecture of the melanoma tumor

microenvironment, frequently lining tumor vasculature and/or forming a physical barrier between TIL and tumor cells, suggest that TAFs are ideally located to mediate immune cell suppression *in vivo*.

The most crucial aspect of this study is the finding that pharmacologic inhibition of BRAF(V600E) in melanoma cells can relieve TAF-mediated suppression of immune cell function and largely restore antitumor T cell responses. Although study of a cohort of BRAF(V600E) inhibitor-treated patients will be required to confirm these findings *in vivo*, collectively these results have important clinical implications that strongly support the notion of combining BRAF(V600E)-specific inhibitors with immune-based therapies. In particular, it will be important to test the prediction that patients treated with V600E inhibitors show a reduction in tumor-mediated IL-1 production that may be linked to reduced COX-2 or PD-1 ligand expression by TAFs and improved activity of tumor-specific T cells. The emerging link between MAPK pathway activation and immune suppression, combined with the lack of off-target effects shown by mutated kinase-targeted agents, suggests that such combination approaches may show therapeutic synergy and result in significantly better and more durable clinical responses in V600E-positive melanoma patients. Furthermore, our findings also support the notion that patients harboring non-BRAF mutated tumors may benefit from treatments that combine immunotherapy with IL-1 blockade.

Future directions

The connection of oncogenes and immunosuppressive mechanisms in the tumor microenvironment has the potential to guide clinical practice in the future. The data collected in the progress of my work supplies several more questions calling for experimental validation. The future directions I outline based on my work all follow the direct line of evidence generated by my work under the hypothesis that BRAF V600E regulates immunosuppression of the melanoma microenvironment.

At the most basic level, it is important to test the effects of BRAF V600E inhibition on serial biopsies of patients for immunomodulatory factors including IL-1α/β, PD-L1, PD-L2 and COX-2 both before and after treatment. This will ascertain if in patients these mechanisms are robust to stand in the complex milieu of the variety and heterogeneous patient melanoma patient population. Confirmation of these mechanisms in treated patients is strong validation of the data in this thesis.

More speculatively, patient selection based on IL-1α/β level in the tumor could be a biomarker of those who would benefit most from combination therapy with BRAF V600E inhibitors and activating immunotherapies, such as high dose IL-2 or adoptive cellular therapy with Til or TCR modified cells. Or, as it is a finding in this thesis that IL-1α/β activated melanoma TAFs increase transcription of several known immunosuppressive molecules at the RNA level these could serve a role as biomarkers. However, it was beyond the scope of this work to determine the validity

of these elements in functional experiments. But given that these gene products were upregulated in TAFs from different patients and metastatic sites, it suggests that an immunosuppressive role of IL-1 signaling in TAFs is not restricted to one tissue site or patient. This weighs in favor of further efforts to understand the role of these molecules, perhaps as secondary indicators for patients with BRAF V600E / IL-1α/β axis mediated suppression of the melanoma immune response.

Recently development murine models of spontaneous melanoma that are sensitive to BRAF V600E inhibitors offer another opportunity to evaluate the role of IL-1 production during the early development of melanoma. Crossing these animals with IL-1R1 knockout animals will be a powerful direction to further develop the finding of my work. A deeper understanding of the role of sterile inflammation in the progression of cancer is valuable in the development of adjuvant therapies to prevent reoccurrences secondary to successful immunological interventions. This work can only be carried out in complete physiological systems such as those developed in mice where immunological memory can be model. This is far outside the scope of the proximal effector functions evaluated *in vitro* in the course of my work.

In the process of phenotyping melanoma TAFs derived from patient biopsy material, it became clear to me the immunological variations at the basal level. Although PD-1 ligands and COX-2 are consistently upregulated with IL-1α/β treatment, other molecules are only induced by IFN-γ. In fact melanoma TAFs can be bisected by

the ability to induce MHC Class II in response to IFN-γ. This suggests a significantly different immunological role in the presence of activated T cells. As my work was focused on the role of TAFs in a chronically suppressive melanoma microenvironment where IFN-γ is necessarily absent, and my focus was on the role of MHC Class I restricted CTLs this observation was not followed up. In fact the diversity of immune modulatory possibility of fibroblasts is vast and future studies to define these in the melanoma microenvironment would be quite productive.

Chapter 6

Material and Methods

Cell Culture and Transduction

WM793p2, A375 and T2 cells were cultured in RPMI 1640 medium (GIBCO Grand Island, NY) containing 10% fetal bovine serum (GIBCO), 10 IU/mL penicillin (Cellgrow Manassas, VA) and 10 mg/mL streptomycin (Cellgrow) and maintained at 37°C in 5% CO2. The EB16-MEL and KUL84-MEL cell lines, which were kindly provided by Etienne De Plaen (Ludwig Institute for Cancer Research, Brussels), were cultured in IMDM medium (GIBCO) containing 20% fetal bovine serum (GIBCO), 10 IU/mL penicillin (Cellgrow) and 10 mg/mL streptomycin (Cellgrow). Dermal Cell preparations were obtained from Sciencell (Carlsbad, CA) and cultured in the provided Melanocyte Medium (Sciencell). Primary neonatal epidermal melanocytes were obtained from ATCC and cultured in Dermal Cell Basal Medium (ATCC). Patient biopsy derived TIL and TAFs were available with institutional IRB approval and patient informed consent. TIL or tumor digests were maintained in RPMI-1640 medium (GIBCO) containing 10% fetal bovine serum (GIBCO), 10 IU/mL penicillin (Cellgrow Manassas, VA), 10ug/mL streptomycin (Cellgrow) and supplemented with 200 IU/mL of IL-2 (Prometheus) unless otherwise indicated [195]. TAFs were isolated from mixed tumor cell cultures based on CD90 positivity and MCSP negativity by cell sorting. TAFs were isolated from melanoma biopsies from lymph nodes, soft tissue, lung, brain and chest wall.

BRAF(V600E) and wt mutant plasmids were obtained from R. Marais [196]. Genes were cloned into pDonor 222 (Invitrogen) by standard methodologies. These constructs were then sequenced and cloned into a self-inactivating bicistronic lentivirus expression vector (PLV401) containing the CMV promoter via LR reactions (Invitrogen). Lentivirus was generated by Lipofectamine 2000 transfection of the packaging cell line, 293-T METR, with packaging plasmids containing pΔR8.91, CMV-pVSVG, and the indicated expression vectors. Viral supernatants were collected at 48 hours and concentrated by ultracentrifugation. Dermal cell preparations and Melanocytes were transduced at MOI of ~10, or by viral titration followed by selection of equivalently transduced lines based on GFP expression. Experiments using melanocytes transduced with BRAF(V600E) and control expression vectors were carried out between 2 and 10 days after transduction.

Melanoma Xenograft

NOD/SCID mice were obtained from Jackson Laboratory. Mice were maintained in accordance with the institutional guidelines of MD Anderson Cancer center. Melanoma tumors were generated by subcutaneous injection of 10 million A375 cells on Day 0. Seven days after tumor cell inoculation, animals were treated with PLX4720 (100 mg/kg bodyweight), administered by gavage. This was repeated for two subsequent days. Vehicle solution contained 3% DMSO and 1% methylcellulose was used as a control treatment. Harvested tumors were divided in half for histology and transcriptional analysis. Experiments used 3 mice per group.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from cultured melanoma cell lines and A375 xenografts using the RNAqueous NA isolation kit from Ambion. One step RT-PCR was conducted using iScript (Bio-RAD) according to the manufacturer's instructions. Primer sequences used are as followed:

IL1aF: 5'TGGCCCACTCCATGAAGGCTGC;

IL1aR: 5'GTCATTGGCGATGGCGATGGCCTCCAGG;

IL1bF: 5'GCTTATGTGCACGATGCACCTG;

IL1bR: 5'TCCTGTCCCTGGAGGTGGAGAG;

GAPDHF: AGAAGGCTGGGGCTCATTTG;

GAPDHR: AGGGGCCATCCACAGTCTTC;

CNXF: GCGTTGTGGGGCAGATGAT;

CNXR CCGGTTGAGGTGCATCAGT.

Exclusively for analysis of human transcripts from A375 xenografts, the primer sequences used are as follows:

IL1aF: 5'GAGGCCATCGCCAATGACTCAGAG;

IL1aR: 5'CAGCCGTGAGGTACTGATCATTGG;

IL1bF: 5'GGACCTGGACCTCTGCCCTCTGGATGGCG;

IL1bR: 5'GTACAGGTGCATCGTGCACATAAGCC;

CXCL8F: 5'CTGCAGCTCTGTGTGAAGGTGCAG;

CXCL8R: 5’GGTCCAGACAGAGCTCTCTTCCATC;

CXCL1F: 5’CGCGCTGCTCTCTCCGCCGCC;

CXCL1R: 5’GTCCGGGGGACTTCACGTTCACAC;

PDL1F: 5’CCACCACCACCAATTCCAAGAG;

PDL1R: 5’CGGAAGATGAATGTCAGTGCTACACC;

PDL2F: 5’GGACCCATCCAACTTGGCTG;

PDL2R: 5’CACTTCCCTCTTTGTTGTGGTGACAG;

BACTINF: 5’CGAGGCCCAGAGCAAGAGAG;

BACTINR: 5’CGGTTGGCCTTAGGGTTCAG.

Reactions were analyzed using a BIO-RAD CFX96 thermocycler and Ct values normalized to untreated samples relative to GAPDH or B-actin expression using the ΔΔCt method.

Microarray analysis and statistical methods

Transduced dermal cell preparations were sorted based on GFP expression. TAFs were cultured with 2 ng/mL IL-1α (PeproTech Inc. Rocky Hill, NJ) or in regular media for 24 hours, detached from culture plates and stored at -80 as cell pellets until total RNA extraction. Total RNA was extracted using RNeasy RNA extraction kit (Qiagen) and tested for quality by RIN analysis after product separation using an Agilent 2100 Bioanalyser (Santa Clara, CA). RNA was prepared and hybridized on Affymetrix Human Genome U133A 2.0 Array (Santa Clara, CA) by Expression

Analyses (Durham, NC). Expression data was normalized based on Bland-Altman (M-versus-A) pairwise plots, density plots, and boxplots.

In TAFs, to identify differentially-expressed genes between untreated and IL-1 treated groups, I applied modified paired two-sample t-tests using Limma package. The beta-uniform mixture (BUM) model, described by Pounds and Morris [197], was used to control false discovery rate (FDR). Heatmap displays of the 197 most differentially expressed genes selected at FDR 0.01 have been standardized and at ±2 standard deviations for display purpose (The scale of the values is indicated in the color key). All of the tests in Figures are 1-sided t-tests, asterisks indicate p-values of <0.05, if not explicitly provided. In some tests 1-sided t-tests are paired, and indicated as such.

Western Blotting

Cell lysates were prepared from cultured TAFs. Protein content was normalized using the BCA method (Thermo-Fischer Rockford, IL). Protein samples were separated on 10% SDS-PAGE and transferred to PVDF membrane (BIO-RAD Hercules, CA). Membranes were blocked in 5% nonfat milk and incubated with primary anti COX-2 at 1:1000 dilution overnight at 4°C. Washed membranes were incubated with appropriate HRP-conjugated secondary antibodies for 2 hours at RT prior to chemiluminescent detection (GE Healthcare, Buckinghamshire, UK).

Flow cytometric analyses

Analysis of surface antigens on melanoma cell lines and melanocytes was carried out by standard flow cytometry methods. Antibodies were obtained from the indicated suppliers, From: PD-L1 (M1H1), PD-L2 (MIH18), AF647 labeled anti-rabbit IgG fabs (eBiosciences San Diego, CA); CD8 (RPA-T8), Ki-67 (B56), CD25 (M-A251), IL-1α (AS5), IL-1β (AS10), CD90 (5E10), (BD Biosciences); PD-1 (EH12.2H7) (Biolegend San Diego, CA); MCSP (EP-1) (Miltenyi Biotec,Bergisch Gladbach, Germany) and BRAF (EP152Y) (Epitomics Burlingame, CA). Intracellular antigens were stained after fixation and permeabilization using Foxp3 intracellular staining Kit (ebiosciences). Stained cells were analyzed using a FACScanto II flow cytometer, or a FacsCaliber flow cytometer (BD Biosciences San Jose CA). Data was analyzed using Flowjo analysis software (Treestar Ashland OR).

Cytokine detection

Supernatants from cell lines or co-cultures were collected and aliquots stored at -20°C prior to detection of IFN-γ, IL-1α, IL-1β, by standard ELISA methods according to manufacturer's instructions (R&D Systems Minneapolis, MN). For some experiments supernatants were analyzed for multiple cytokines (IL-1α, IL-1β, IL-8, IL-6, MCP-1, CXCL-1, IFN-γ and TNF-α) by multiplex Luminex assays according to manufacturer's instructions (Millipore Bedford, MA).

TIL suppression assay

Foreskin-derived dermal cell preparations or melanoma TAFs were plated into 96-well flat bottom plates and cultured until 80% confluent. Interleukin-1 α/β (1 ng/ml) or conditioned medium was added overnight. To test direct presentation, wells were washed prior to Mart-1 27L (AAGIGILTV) peptide pulsing (100 nM, 3hrs, 37°C, serum free medium). For third-party cell stimulation, T2 cells or irradiated CD40L-activated B cells were pulsed with peptide and added with 1E5 Til at a 1:1 ratio to cultures. Proliferation was inferred by expression of cell cycle protein Ki-67 in the Mart-1 tetramer binding CD8 T cells. Supernatants were collected at the indicated times during the coculture for IFN-γ analysis as described above. Melanoma cell line conditioned medium was obtained from confluent cell cultures in T150 flasks containing 13 mL of medium after 24 hours with and without treatment with 1 mM Vemurafenib (Plexxikon Berkeley, CA). This treatment condition did not significantly alter the cell number/ flask. Medium was centrifuged and 0.22 mM filtered prior to use in assays. Blockade of PD-L1 and PD-L2 was achieved with the use of purified Azide free PD-L1 (M1H1), PD-L2 (MIH18) antibodies (BD Biosciences) at 1.5 mg/mL doses. The COX-2 inhibitor NS398 (Cayman Chemicals Ann Arbor, Michigan) and DMSO vehicle controls were used at the time of IL-1 treatment at 50 mM. IL-1α and IL-1β neutralizing antibodies (R&D Systems) were used at 20ug/mL each; isotype antibody (R&D Systems) controls were included where indicated.

Tissue Collection and Mutational Analysis

Additional tissue was available for analysis on a subset of patients in the IHC analysis of IL-1α/β expression. From tissue at MDACC, patients either had results of Clinical Laboratory Improvement Amendment (CLIA) certified pyrosequencing of BRAF (exon 15) and NRAS (codons 12, 13, 61). For the remaining patients at MDACC who did not have CLIA mutational analysis done formalin-fixed paraffin embedded (FFPE) tissues were analyzed. The MDACC Biospecimen Core Facility extracted DNA from samples with at least 50% tumor content. Mass-spectroscopy genotyping for BRAF (exon 15) and NRAS (codons 12, 13, 61) mutations was performed as previously described. Patients without a BRAF or NRAS mutation were classified as wild-type (wt/wt).

Immunohistochemistry and Scoring method

IHC staining was conducted as previously described using primary IL-1α (LS-B1581, 1:100) (LifeSpan Biosciences Seattle, WA) and IL-1β (sc-7884, 1:50) (Santa Cruz Biotechnology Santa Cruz, CA). Cores were scored as positive or negative for IL-1α or IL-1β immunoreactivity by three individuals. The percentage of staining cells in each core and the intensity of staining was scored from 1-3. The product of both values was used to determine a summary staining score. Scores were compiled and then reported

BRAF Knock Down Cell lines

WM793p2 cells lines were transduced with lentiviral particles to introduce the pTRIPz construct from Open Bio Systems containing either a nonsilencing shRNAmir sequence (RHS4743), or a BRAF targeting shRNAmir with the BRAF antisense sequence, (V2THS_262034) CAGATGAAGATCATCGAAA. Cells were cultured in 2 ug/mL Doxycycline for 11 or 14 days and IL-1a measured by intracellular staining and FACs analysis, as described in main text.

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