IL-1α BLOCKADE REDUCES IMMUNE SUPPRESSION IN THE EARLY TUMOR MICRO-ENVIRONMENT

Brenda Melendez

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IL-1α BLOCKADE REDUCES IMMUNE SUPPRESSION IN THE EARLY TUMOR MICROENVIRONMENT

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IL-1α BLOCKADE REDUCES IMMUNE SUPPRESSION IN THE EARLY TUMOR MICROENVIRONMENT

A Dissertation

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for the Degree of

DOCTOR OF PHILOSOPHY

By

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IL-1α Blockade Reduces Immune Suppression in the Early Tumor Micro-Environment

Brenda Melendez, B.S.

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Immunotherapy against melanoma has shown great promise in the clinic for treating advanced-stage patients. However, a major barrier against effective T cell mediated cytotoxicity is immunosuppression in the tumor micro-environment. It has been described that tumors secrete pro-inflammatory cytokines capable of modulating immune responses that favors the growth of tumor cells. Specifically, IL-1 plays a critical role in myeloid cell recruitment and activation, which can in turn inhibit T cell activity in vivo. Moreover, IL-1 is also known to up-regulate immune inhibitory molecules in the tumor micro-environment. To further investigate the effects of IL-1 in melanoma progression, IL-1α was blocked in a highly aggressive pre-clinical B16 melanoma tumor model in three different treatment settings: as a monotherapy, in combination with checkpoint blockade, and in combination with adoptive T cell therapy. In all three settings, IL-1α blockade resulted in tumor reduction and increase in murine survival. This was accompanied by a decrease in myeloid cell tumor infiltration. At early time points following IL-1 α blockade, these myeloid cells also demonstrated partial loss of their immunosuppressive abilities, as supported by a decrease in arginase production and inhibitory molecule expression. Moreover, monocytes demonstrated an increase in co-stimulatory molecules following IL-1 α blockade. In vitro, the myeloid cells’ ability to inhibit T cell cytotoxicity was significantly compromised. These results collectively provide evidence in support of IL-1 α contributing to melanoma immune suppression. Antibody-mediated blockade of IL-1 α improved antitumor responses, suggesting that this modality may improve outcomes of patients undergoing treatment with T-cell based immunotherapies.
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CHAPTER 1:

INTRODUCTION AND BACKGROUND
I. **Immune response**

The study of evolution in a variety of organisms has taught us that several biological mechanisms are conserved and improved through the passage of time. The most basic mechanism of defense is the innate immune system. The innate immune system represents the pinnacle of evolutionary biology, in which generation after generation, we selected the genes and machinery necessary to protect us from pathogens. This response is so versatile and effective that it is conserved across plants, invertebrates, and mammals (1). The innate immune system detects foreign bodies by using receptors encoded in our germline. These receptors activate cell-dependent responses, soluble factors, complement factors, alarmins, cytokines and chemokines, and other molecules responsible for instigating inflammation (2). The innate immune response is triggered by the activation of pattern recognition receptors (PRRs). A subtype of PRRs, toll-like receptors (TLRs), expressed on the surface of resident epithelial cells and recruited hematopoietic cells, sense pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) (3). Other important PRRs are NOD-like receptors, complement receptors and scavenger receptors (4). Once any of these receptors are activated, they start a downstream signal that leads to the secretion of cytokines and chemokines involved in the recruitment of immune cells. Innate immune cells, then, travel to the area of infection (5). Innate cells encompass an array of myeloid and lymphoid cells. They tend to originate from hematopoiesis without antigen receptors. The majority of cells do not develop memory (6). Although, new studies in natural killer cells (NK cells) have introduced the concept of trained immunity based on memory responses by NK cells (7).

a. **Neutrophils**

Neutrophils are the first cells recruited (2). They were considered as short-lived and terminally differentiated phagocytic cells, without any role in the regulation of adaptive
immunity. However, these notions were challenged with newer studies. Observation of circulatory neutrophils in healthy patients showed the average span of neutrophils to be almost a week, more than 10 fold longer than previous estimates (8). Neutrophils were found to be somewhat fluid, acting as myeloid derived suppressor cells (MDSCs) by suppressing T cell expansion and cytokine production (9); therefore, having a role in adaptive immunity.

The main role of neutrophils is as phagocytes. Phagocytosis is the process in which small bacteria are engulfed by neutrophils. It usually occurs within minutes of infection. Large organisms cannot be phagocytosed. Instead, neutrophils release granules that induce death by degrading bacterial and fungal proteins (10). After prolonged exposure to pathogens, neutrophils undergo cell death by apoptosis, necrosis or neutrophil extracellular trap (NET) formation (10). Their death continues the inflammatory response by releasing inflammatory cytokines.

b. Macrophages

Elie Metchnikoff, categorized macrophages as “the phagocytic component of the immune system”. Since the discovery of macrophages, their function has expanded to the phagocytosis of foreign bodies, the clearance of dead cells, production of inflammatory cytokines and participation in homeostasis. Macrophages express an extensive variety of PRRs capable of sensing microorganisms, danger signals, and changes in pH and oxygen concentration (11). After activation, macrophages use several mechanisms to fight infection and eventually stop the immune response. Macrophages have the ability to cross-talk with other neighboring cells by releasing cytokines, chemokines and growth factors. Directly, macrophages can have cell-to-cell interactions via their receptors and gap junctions (12). It has been estimated that humans have approximately 200 billion macrophages (13). Tissue-resident macrophages have a slow turn-over rate under homeostasis. They are established
during embryonic development. They are capable of self-renewal. Blood-circulating monocytes can also differentiate into tissue-resident macrophages (14). Their self-renewal abilities are not shared across all macrophage subtypes. Macrophages derived from the intestine, pancreas, dermis, and heart tend to be replaced by circulating monocytes (Ly6C<sup>Hi</sup>) in a CCR2-dependent way (15). Macrophages have been classically separated into 2 unique categories. M1 (classically activated macrophages) are activated by Th1 cytokines such as IFNγ and bacterial factors. M2 (alternatively activated macrophages) are activated by Th2 cytokines like IL-4 and IL-13. M1 macrophages fight against bacterial function, while, M2 macrophages are part of anti-inflammatory, allergic and tissue repair processes (2). This categorical separation does not hold in vivo due to the presence of both Th1 and Th2 priming factors. Therefore, the newest understanding is that macrophages exist across a spectrum of phenotypes rather than as separate distinct subtypes (16). Macrophages can easily go from one end to the other end of the spectrum with the appropriate stimulating molecules. They also have an epigenetic plasticity (17). Macrophages exposed to pathogens undergo epigenetic reprogramming that establishes “innate immune memory” (18). Their plasticity allows them to be an effective arm of the innate immune response.

c. Myeloid-derived suppressor cells (MDSCs)

As their name states, MDSCs are of myeloid origin. Their main function is to suppress T cell responses. They can be categorized into polymorphonuclear (PMN-MDSC) and monocytic MDSCs. In mice, they can be identified by their Ly6C (monocytic MDSCs) and Ly6G (PMN-MDSCs) expression. In humans, the Ly6C and Ly6G markers do not translate and, thus, the identification of these populations is more complicated. PMN-MDSCs and monocytic MDSCs express CD11b and CD33. Monocytic MDSCs, also, express CD14 and have low levels of MHC-II. PMN-MDSCs, on the other hand, have low CD14, express CD15 and CD66b (19). Since their markers are found in other immune cells,
functional assays are necessary to ascertain their suppressive function. Moreover, neutrophils tend to have similar markers as PMN-MDSCs, so their closeness has been debated.

MDSCs have been heavily studied in mice due to their T cell suppression capabilities. Several mechanism of suppression have been discovered. Bronte and Yang described the ability of MDSCs to modulate angiogenesis and tumor cell motility by the production of metalloproteinases (MMP) and vascular endothelial growth factor (VEGF) (20). Corzo, found that this process is regulated by the transcription factor hypoxia inducible factor (HIF)1α (21). Moreover, MDSCs polarize tissue macrophages into an M2 phenotype, which promotes angiogenesis (22). MDSCs have also been found to suppress NK cell-mediated lysis (23). There are several ways in which MDSCs inhibit T cells. They inhibit antigen-dependent cytokine secretion in T cells (24), induce apoptosis in activated CD8+ T cells via TNF and nitric oxide (NO) (25), secrete immunomodulatory molecules such as TNF-α, H₂O₂, and TGF-β, and release enzymes involved in amino acid metabolism that are used for T cell activation (arginine, tryptophan, and cysteine) (26-28). MDSCs can also induce T regulatory cells (Tregs) (29). Hanson et al, showed how MDSCs disrupted T cell homing to lymph nodes via L-selectin (30).

MDSCs stifle the activation of the adaptive and innate immune response.

d. Adaptive Immune Response

Even though the innate response is effective in eliminating a variety of organisms, the number of molecular patterns it can recognize is limited (31). The ability of pathogens to mutate and evolve forced the development of the adaptive immune response (32). The main feature of the adaptive immune system is the wide receptor repertoire specificity created by somatic recombination of gene segments (31). This mechanism evolved from gene
duplication in early vertebrates to generate highly specific and flexible responses (31). Another key feature, is the ability to gain memory. Cells with antigen-specific receptors can persist for life. T cells and B cells are the two main types of cells in the adaptive immune response.

e. T cells

T cells develop in the thymus from common lymphoid progenitor cells (33). Common lymphoid progenitor cells travel from the bone marrow or fetal liver to the thymus. In the thymus, they begin to expand via IL-7 induction. Mutations in the IL-7 receptor, lead to deficiency in T cells. Expansion of common lymphoid progenitor cells activate Notch-1 and other transcription factors involved in T-cell lineage commitment and up-regulation of the expression of genes responsible for T-cell receptor (TCR) assembly (34). T cells undergo an antigen-independent differentiation process, in which genetic rearrangements create functional genes that encode the α/β chains or the γ/δ chains of the TCR. The TCR loci has arrays of V (variable), D (diversity), and J (joining) segments. β and δ TCR loci contain V, D, and J segments. The rest only have V and J segments. In a serial process, one V, one D (for β, δ), and one J segment are randomly spliced. The spliced recombination is mediated by the V(D)J recombinase, which consists of 2 proteins encoded by the recombinase-activating genes 1 and 2 (RAG1 and RAG2). RAG1 and RAG2 bind to the recombinase signal sequences flanking the edges of V-D-J segments. The structure of chromatin regulates the accessibility of the signal sequence (35). The V(D)J recombinase cuts the DNA at the signal sequences to get hairpin structures. Artemis then cleaves these structures. The DNA breaks are repaired in a process called nonhomologous end-joining, creating a variety of V(D)J combinations. The resulting V(D)J cassette dictates the amino acid sequence and binding specificity of the TCR (35). This is called combinatorial diversity. Junctional diversity, is obtained when bases are added or removed during the repair of
DNA. Created junctional areas encode the region of the antigen-binding pocket of the TCR (35). TCR rearrangements are successful when no stop codons are introduced and a TCR protein can be translated. This turns a pre-T cell to a double-positive T cell. Double positive T cells express both CD4 and CD8. Transition from a double positive T cell to a single positive T cells requires positive and negative selection. Positive selection happens when the TCR binds with low avidity to self-MHC-Peptide complex. Cells that do not bind to self-MHC are eliminated. In negative selection, cells that bind with high avidity to self-MHC are eliminated. Cells that pass either selection differentiate to CD8+ or CD4+ T cells depending on their interaction with MHC class I (CD8) or MHC class II (CD4). Single positive cells exit the thymus and enter into circulation as naïve T cells.

f. T cell activation

T cells are activated after successful TCR-peptide-MHC engagement. CD8+ T cells can recognize peptides between 9-11 amino acids in length bound to MHC class I (HLA-A, HLA-B, and HLA-C). MHC-class I peptides are produced from endogenous protein encoded by either the host or pathogen. CD4+ T cells recognize MHC-II restricted peptides (HLA-DR, HLA-DQ, and HLA-DP). MHC class II molecules are expressed on the surface of antigen presenting cells (APCs). APCs roam in the host sampling environmental antigens and danger signals (35). They phagocytose or endocytose exogenous proteins and then present them on their surface via MHC class II molecules. After APC activation, these cells travel to regional lymph nodes, where they present antigen to T cells. An immunological synapse is formed when the TCR associates with the peptide-MHC complex. Several TCR-associated molecules start clustering at the boundary between T cell and APC (immunological synapse) (36). The CD4/CD8 molecules stabilize the CD3-TCR-MHC complex by binding it to nonpolymorphic regions of MHC. Integrins stabilize the immune synapse.
T cell activation is a three-signal process. Signal one consists of the recognition of the peptide-MHC complex on APCs by the TCR (37). This is an antigen-dependent process that starts the tyrosine phosphorylation of ITAMS located on the cytoplasmic tails of the TCR-CD3 complex, which leads to the initiation of a signaling cascade that activates NFAT and NF-κB pathways, involved in T cell effector function (38). Signal one, alone, may cause T cells to become anergic and apoptotic (39). Signal two is co-stimulation. The most studied co-stimulatory axis is between CD80/CD86 on APCs with CD28 receptor on T cells (40). CD80/CD86-CD28 interactions modulate the scale of T cell response, triggering clonal expansion, differentiation and up-regulation of anti-apoptotic genes (BCL-2 and Bcl-XL) (41). Finally, signal three is cytokine dependent. In 2010, Curtsinger and Mescher, suggested that signals 1 and 2 were not sufficient to sustain clonal expansion, effector function and the creation of memory populations (Figure 1) (42). Cytokines such as IL-12 and Type I IFN, sustain the transcription of factors and regulation of genes involved in differentiation and function. Altogether, all three signals are necessary for optimal T cell response.
Figure 1: Activation of naïve CD8 T cells requires three signals. Ag, co-stimulation, and either IL-12 or IFNα/β. Stimulation with Ag and B7-1 results in extensive proliferation, but survival is compromised and development of effector functions is suboptimal. The small numbers of cells that survive long term are anergic. When either IL-12 or IFNα/β is present, proliferation is comparable but survival is increased, the cells develop strong effector functions, and a protective memory population is formed. Reused by permission from Elsevier Publisher Ltd. Curtsinger, J.M. and M.F. Mescher, Inflammatory cytokines as a third signal for T cell activation. Curr Opin Immunol. Ref 23. Copyright © 2010.
II. Melanoma

Cutaneous melanoma is the deadliest type of skin cancer. It accounts for only 1% of all cases observed. In the United States, it has been estimated that almost 100,000 patients will be diagnosed with melanoma, and 10,000 will succumb to the disease (43). Melanoma occurrence differs greatly among countries, but certain risk factors have been linked to the disease. Racial skin features, sun exposure, gender and age are the most common linking factors in patients. Patients with low levels of melanin in their skin are more sensitive to UV rays, which cause inflammation in the skin. Excessive UV exposure can lead to DNA damage and genetic mutations. The most affected pathways are cell proliferation (BRAF, NRAS, and NF1), growth and metabolism (PTEN, and KIT), and apoptotic resistance (TP53, CDKN2A, and TERT) in melanocytes (44). Current statistics show that incidence rate increases in younger patients (median age of 57 years) compared to other cancers (44). These patients are associated with intermittent sun exposure, BRAF mutation and a low mutation load. Chronical sun exposure is most commonly associated with older patients. These melanomas are driven by BRAF, NF1 and NRAS mutations and have a high mutational load (45). Gender also plays a role, female patients comprise the majority of cases seen in the younger age group. Meanwhile, male patients are more prominent in the older than 55 group (46). Other lesser factors associated with melanoma are pre-existing nevi (commonly known as moles) and family history. Bevona et al showed that 26% of melanoma cases appeared on a pre-existing nevi (47). Only 5-12% of melanomas are inherited (48). The two genes related to inherited melanoma are cyclin-dependent kinase inhibitor 2A (CDKN2A) and cyclin-dependent kinase 4 (CDK4). CDKN2A is a tumor suppressor gene that is involved in the production of p16 and p14 (49). P16 and p14 are both involved in cell cycle regulation (50). CDK4 mutation inhibits cell cycle regulation by p16 (51).
a. **Melanoma treatments**

If caught during early stage, most melanomas are curable with surgery. Melanoma is highly metastatic and in late stages it’s no longer operable and, thus, deadly. The earliest treatment against advanced-stage melanoma was chemotherapy. Dacarbazine was the first chemotherapy drug approved by the FDA to treat metastatic melanoma (52). Dacarbazine induces apoptosis in proliferating cells. Dacarbazine alone achieved less than 5% complete response (CR) rate. For at least 20 years, chemotherapy was the standard of care for late stage melanoma. During the 90’s, studies connected immunology and cancer. Researchers observed that inflammation was greatly increased at the tumor site and there was a consistent presence of infiltrating immune cells (53). Specifically, T cells were linked to anti-tumor response. T cells are immune cells capable of recognizing tumor-specific antigen. After recognition, T cells are activated and are capable of killing tumor cells associated with the corresponding antigen. This discovery led to the design of drugs that stimulated the immune system in patients. In 1995, Interferon α-2b was approved as an adjuvant therapy for treating resected stage II/III melanoma (54). In melanoma, systemic IFNα has an immunomodulatory effect. It can activate T cells, B cells, NK cells, while, inhibiting Tregs and MDSCs (55). It reduces angiogenesis by limiting VEGF expression (56). Also, it increases MHC Class I levels on melanoma cells, leading to increased recognition (57). Alone, IFNα moderately decreases reoccurrence of disease (58). The other cytokine-mediated immunotherapy is Interleukin-2 (IL-2). IL-2 directly increases proliferation of T cells. High-dose IL-2 was FDA approved in 1998 for metastatic melanomas (59). IL-2 has a CR rate of 4% (59). These two immunotherapies paved the way to the development of current immunotherapies. New treatments are capable of producing long lasting responses in metastatic patients.

b. **Checkpoint blockade**
Checkpoint blockade is based on the concept of improving immune response against tumor cells by inhibiting the mechanism responsible for inhibiting T cell activation. Prolonged T cell activation is tightly regulated by several factors most importantly, CTLA-4 and PD-1. Rampant and unchecked T cell activation can cause autoimmunity.

During the 80s, Brunet et al, identified a molecule currently known as CTLA-4. It took almost a decade for its function to be described by Allison’s group in 1995. CTLA-4 is a member of the immunoglobulin superfamily and is solely expressed on T cells (60). CTLA-4 expression is induced upon TCR engagement. Initially, CTLA-4 is expressed intracellularly. After TCR activation and co-stimulation by CD28, CTLA-4 translocates to the surface of T cells, where it competes with its homologous receptor, CD28, for binding against CD80 and CD86 on the surface of APCs (61). CTLA-4 has a higher avidity and affinity with CD80 or CD86 than CD28 leading CTLA-4 to outcompete CD28 (62). Attenuation of downstream CD28 signaling decreases T cell activation and proliferation (63). Mechanistically, CTLA-4 ligation reduces IL-2 production and arrests cell cycle progression (64).

T\(_{\text{regs}}\) constitutively express CTLA-4. Loss of CTLA-4 in T\(_{\text{regs}}\), leads to an abnormal activation of conventional T cells, stressing the role of CTLA-4 in T\(_{\text{reg}}\)-mediated tolerance.

The other prominent T cell inhibitor is programmed cell death protein 1 (PD-1). PD-1 is homologous to CD28 and is involved in inhibiting immune signaling (65) T cells constitutively express PD-1, except naïve T cells. B cells, myeloid dendritic cells, mast cells and Langerhans cells also express PD-1. PD-1 has 2 ligands, PD-L1 and PD-L2. PD-1 ligands are often found on the surface of APCs (66). IFN\(\gamma\) is the main inducer of PD-L1 and PD-L2 (67). PD-1 has an immunoreceptor tyrosine-based inhibition motif (ITIM) and in immunoreceptor tyrosine-based switch motif (ITSM) on its cytoplasmic tail (68). Upon PD-1 activation, ITSM becomes phosphorylated and induces the recruitment of the Src homoly region 2 domain-containing phosphatase 2 (SHP-2). The PD-1-SHP-2 complex can
dephosphorylate CD28, thus inhibiting CD28-CD80/CD86 signaling, resulting in reduced T cell proliferation and cytokine production. PD-1 activity is only relevant during simultaneous T cell activation (68). PD-1 works in maintaining tolerance.

Both anti-CTLA-4 and anti-PD-1 have shown increased patient survival in a variety of cancers. A phase III clinical study showed that anti-PD-1 resulted in better response (44%) compared to CTLA-4 (19%) in melanoma patients. Combination of anti-CTLA-4 and anti-PD-1 has a response of 58% (69).

c. **Peptide vaccines**

Vaccination has proven effective in fighting pathogens (70). Vaccines consists of injecting inactivated forms of microbes to induce an antigen-specific response to protect against future infections. These types of vaccines contain hundreds of proteins; however, only a few offer immunity. Peptide vaccines are a specialized form of vaccine, in which known antigenic-peptides are used to trigger an immune response. To date several peptide vaccines have been designed to stimulate CD8+ T cell cytotoxicity against pathogens. Peptides alone are not sufficient to trigger a robust immune response so adjuvants were developed to improve the immune response to peptide vaccines. The most common adjuvant in the clinic is Freunds complete adjuvant (CFA). CFA contains inactivated and dried mycobacteria. In this manner we can increase humoral and cellular immunity.

Melanoma’s high mutation load makes it easier to find potential candidates to use as a peptide vaccine. For instance, gp100 is a peptide found on a majority of melanoma cells and is currently being used as a peptide vaccine.

III. **Interleukin-1**

The discovery of the first interleukin started from studies trying to isolate endogenous factors responsible for causing fever in patients. Elisha Atkins reported that a protein
appeared in circulation during endotoxin fever and coined the term “endogenous pyrogen” (71). Atkins, Murphy and Wood, went on to study the molecular properties of pyrogen isolated from rabbit neutrophils (72). Eventually, Atkins and Bodel moved to human PBMCs and reported that unlike in rabbit peritoneal cells, pyrogens in human PBMCs were synthesized de novo, and therefore were not present during homeostasis (73). Bodel also showed that pyrogen could also be secreted by monocytic leukemia cells and Hodgkin’s and lymphoma cells (74). In parallel, Dinarello, was, also, trying to isolate the soluble pyrogen. In 1977, his lab isolated pyrogen and named it, human leukocytic pyrogen (LP) (75).

Endogenous pyrogen and human leukocytic pyrogen are different labels to describe the properties of what we now call Interleukin-1. Later findings demonstrated the potent ability of IL-1 to induce a fever with levels as low as 1-10ng/kg (76).

Klampschmidt et al, were the first to propose that individual factors in the supernatant of leukocytes had more than one function (77). Dinarello, showed that human IL-1 could stimulate the production of serum amyloid A (78). Thus, IL-1 became a pleotropic molecule and started cytokine biology.

With the invention of cDNA cloning, scientists were able to identify two distinct molecules with pyrogenic properties. They both had similar molecular weights, but had different isoelectric points. One of the molecules had a pl of 7, which was the recorded pl of IL-1 (79). The other had a pl of 5 (79). We now call pl5 IL-1α and pl7 IL-1β.

a. IL-1α biogenesis

ProIL-1α is the 31Kda precursor to IL-1α. ProIL-1α is customarily found intracellularly and has been described to be active. The precursor is synthesized in association with microtubules (80). ProIL-1α is released when cells undergo necrosis and can then be cleaved by extracellular proteases. In the absence of necrosis, calpain, a calcium-
dependent cysteine protease is also capable of cleaving ProIL-1α into its 17kDa mature form (81).

b. IL-1β biogenesis

Contrary to IL-1α, the precursor of IL-1β is not active and requires further processing for optimal function. ProIL-1β is cleaved by caspase-1. Since both IL-1 precursors lack a signaling peptide, they cannot go through the endoplasmic reticulum-golgi pathway for secretion. During infection or cellular stress, the inflammasome, a 700kDa multi-protein complex is formed to help mediate the cleavage of ProIL-1β. Several types of inflammasomes have been discovered. They all have a distinctive NOD-like receptor (NLR). These are soluble intracellular proteins that survey for foreign bodies. NLRs are multidomain proteins with a tripartite architecture which have a C-terminal region containing a series of leucine-rich repeats, a central region known as the NACHT domain, and an N-terminal effector domain (82). The N-terminal effector domain is responsible for transmitting the signal downstream which prompts caspase activation. NLRs are categorized into several subfamilies. NALP1, NALP2, and NALP3 are known to be the part of caspase-1-related inflammasomes (83). Inflammasomes are triggered by danger signals. IL-1β synthesis and processing requires two signals. The first signal can be initiated by Toll-like receptor (TLR) activation, which results in the production of ProIL-1β. Common activators are LPS, CpG, and resiquimod (84). The second signal can be obtained by the activation of P2X7 receptor by ATP (85). P2X7 activation leads to potassium efflux, plasma-membrane depolarization, cell swelling and disaggregation of the cytoskeletal network (84). Studies have shown that loss of potassium is necessary to trigger caspase-1 (86). Reduction in potassium levels activates calcium-independent phospholipase A2 (iPLA2) (figure 2) (87). Walev, noted that inhibiting iPLA2 stopped IL-1β processing (87). The two signal requirement for IL-1β
synthesis guarantees that inflammation is only induced during infection or tissue injury (84). Dysregulation of this process leads to autoimmune diseases.

Mature IL-1β is released by two separate mechanisms. Andrei et al, illustrates how IL-1β is loaded into secretory lysosomes and eventually released in a phospholipase-dependent way (88). Otherwise, IL-1β is externalized by the budding of microvesicles (89).
Figure 2: IL-1β activation. In signal 1, stimulation of innate immune cells (such as macrophages) with any of the various Toll-like receptor (TLR) agonists (such as lipopolysaccharide (LPS), CpG dinucleotides and the lipopeptides Pam3CysSerLys4 (Pam3CSK4) and R848) induces the synthesis of pro-interleukin-1β (pro-IL-1β) and certain inducible components of the inflammasome, such as caspase-11. This signal 'primes' the cells encountering a secondary trigger that stimulates caspase-1 activation, pro-IL-1β and pro-IL-18 cleavage and subsequent mature cytokine release. As such, TLR priming alone is not sufficient for caspase-1 activation. In signal 2, this trigger is in the form of agents that can cause ionic perturbations, specifically potassium efflux. Activating the purinergic P2X7 receptor by ATP is one such example. Other agents that cause
membrane blebbing and pore formation similar to those elicited by P2X7 receptor stimulation include nigericin (a potassium ionophore), maitotoxin (a potent marine toxin that is derived from dinoflagellates) and aerolysin (a pore-forming toxin from *Aeromonas hydrophila*). Potassium depletion mediates IL-1β processing through the activation of calcium-independent phospholipase A2 (iPLA2). TLR priming (for example, by LPS pre-stimulation) of macrophages 'accelerates' caspase-1 processing. In addition, LPS priming results in IL-1β release, which is due to *de novo* synthesis of pro-IL-1β. However, ASC (apoptosis-associated speck-like protein containing a CARD), pro-caspase-1 and pro-IL-18 are constitutively present in large quantities in macrophages and do not require LPS-mediated upregulation. IκB, inhibitor of NF-κB; NF-κB, nuclear factor-κB; NLR, NOD-like receptor. Reused by permission from Springer Nature. Mariathasan, S. and D.M. Monack, Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation. Nat Rev Immunol, 2007. 7(1): p. 31-40. Copyright © 2007.
c. IL-1 signaling

Due to the potent nature of IL-1, there are three main regulatory checkpoints. The first is the control of synthesis and secretion of IL-1 covered in the earlier section (82). The second is the expression levels of the IL-1 family receptors on the surface of cells (90). The last checkpoint, involves the regulation of the downstream signaling after receptor activation (90). Both IL-1α and IL-1β bind to the same receptor, type I IL-1 receptor (IL-1R1).

Successful IL-1 signaling, requires the IL-1/IL-1R1 complex to bind to a second receptor, IL-1 receptor accessory protein (IL-1RAcP). At the receptor level, there exists two inhibitory molecules. IL-1 receptor antagonist (IL-1RA) is a ligand of IL-1R1 and has a similar affinity to IL-1. Binding of IL-1RA to IL-1R1 does not trigger the activation of the receptor. The other is the type II IL-1 receptor (IL-1R2). IL-1R2 lacks the cytoplasmic signaling machinery, so binding of IL-1 with IL-1R2, does not induce the activation of the IL-1 pathway (90).

When IL-1 binds to IL-1R1, IL-1R1 undergoes a conformational change in the first extracellular domain to recruit IL-1RAcP (91). The IL-1R1/IL-1RAcP complex contains conserved cytosolic regions called Toll- and IL-1R1-Like (TIR) domains (92). After activation, myeloid differentiation primary response gene 88 (MYD88) and interleukin-1 receptor-activated protein kinase (IRAK) 4 bind to the TIR domains on the complex (93). IRAK4, then, auto phosphorylates leading to the phosphorylation of IRAK1 and IRAK2 (94). Tumor necrosis factor-associated factor (TRAF) 6 is recruited and oligomerizes with the complex (95). IRAK1, IRAK2 and TRAF6 detach from the receptor complex and are released in the cytoplasm. TRAF6 mediates the attachment of K-63 linked polyubiquitin chains to several IL-1 signaling factors, including IRAK1, transforming growth factor-β (TGF-β)-activated protein kinase-binding protein (TAB2), TAB3 and TGF-β-activated protein kinase (TAK1) (96) (97, 98). Ubiquitination of TAK1 helps it associate with TRAF6 and MEKK3 (99). Pellino homolog (PELI) 1-3 are ubiquitin E3 ligases that bind to IRAK1, 4 and are then
phosphorylated by the IRAKs. The combination of these proteins activate the NF-κB, c-Jun N-terminal kinase (JNK), and p38 MAPK pathways (100, 101). The order of events is still not well understood. NF-κB binds to a conserved motif in several IL-1 responsive genes such as IL-6 (102), IL-8, monocyte chemoattractant protein 1 (MCP1) (103), and cyclooxygenase-2 (COX-2) (104).

The IL-1 signaling pathway is short-lived. IL-1R binds to the adaptor toll-interacting protein (TOLLIP). TOLLIP, then, stops IRAK1 and facilitates the internalization of IL-1R1 to endosomes (105). There are also negative feedback loops in IL-1 signaling. For instance, phosphorylation of TAB1 inhibits TAK1 (106), synthesis of IκBα turns off the NFκB pathway (107), MAPK phosphatase I (MKP1) dephosphorylates MAPKs.

d. IL-1α function

As established earlier, ProIL-1α is biologically functional in the cytosol. During necrosis it acts as an alarmin triggering an immune response. This response can be modulated by the sequestration of ProIL-1α by IL-1R2 (108). This is important in order to differentiate between necrosis and apoptosis. Under normal apoptotic conditions, ProIL-1α is sequestered, to avoid inflammation (109).

IL-1α can start an inflammation loop, wherein IL-1α can start the production of more IL-1α and IL-1β. Membrane-bound IL-1α can induce IL-1 signaling pathway in nearby immune cells (110). IL-1α can induce the expression of other cytokines needed for the recruitment of immune cells to the inflammation site. The cells, in turn, make more IL-1. The IL-1 signaling continues until it's inhibited (110). The paracrine signaling does not only affect immune cells. It has been demonstrated that in systemic sclerosis, fibroblasts produce high levels of IL-1α, which in turn upregulates the expression of IL-6, PDGF-α, IL1R1, and collagen (111).
IL-1α is known to induce other pro-inflammatory cytokines such as COX-2, inducible nitric oxide synthase (iNOS), IL-6, IL-8, and MMPs. Moreover, IL-1α can increase the expression of adhesion molecules on endothelial cells, stromal cells and leukocytes, in order to facilitate cell migration (112).

e. **IL-1β function**

IL-1β has similar effects as IL-1α in the proliferation and differentiation of innate immune cells. However, studies in the past decade have been able to elucidate different functions between the two molecules. Paul et al, has shown that IL-1β can induce the proliferation of naïve and memory CD4+ T cells after antigen recognition (113). For the expansion to take place, T cells must express IL-1R1 on their surface. Members of the IL-1 family can interact with individual STATs to promote a specific phenotype (114). IL-1β in association with STAT3 induces Th17 cells. IL-33 and STAT5 can start a Th2 response, while, IL-18 and STAT4 a Th1 response. Ben-Sasson et al, published the effects of IL-1 in the activation of antigen-specific CD8+ T cells, demonstrating its function as an adjuvant (115).

IL-1β, also, plays a role in hematopoiesis. IL-1β can up-regulate the expression of receptors for colony-stimulating factors (CSFs) on precursor cells (116). In this manner, IL-1β can promote the differentiation and expansion of myeloid-derived suppressor cells (MDSCs), during inflammation (117). MDSCs differentiate into a variety of cells including, macrophages, granulocytes (112).

f. **IL-1 in cancer**

In cancer, IL-1α has several functions. It can induce fever, fenestrations in the vasculature, prostaglandins, pituitary hormones, and collagenases (118). IL-1 can also boost the immune system by increasing the infiltration of leukocytes to the tumor. Due to the
varied functions of IL-1, IL-1 can be either beneficial or disadvantageous in a cancer setting. The ability to increase inflammation can be used by tumor cells to promote malignancy. Cancer cells can produce IL-1 (autocrine) and can also induce IL-1 in other cells in a paracrine fashion. IL-1β has been found in a variety of cancers such as breast, head and neck, colon, melanoma, etc (119). Patients with IL-1β+ tumors have a decreased survival rate. The exact mechanism of how IL-1 promotes malignancy has still not been fully elucidated, although, the consensus is that IL-1 acts indirectly. For example, IL-1 can promote metastases by up-regulating MMPs, VEGF, IL-8, IL-6, TNF-α and TGF-β. In IL-1 knockout mice, melanoma tumors tend to not establish subcutaneously after inoculation. If tumors grow, survival is increased due to less lung metastasis. These studies highlight the importance of IL-1 in angiogenesis, and the extravasation of tumor cells. Saijo et al, demonstrated that Lewis lung carcinoma cells transduced with IL-1β had no significant increase in proliferation in vitro (120). Nevertheless, in vivo, these cells, had a higher tumor growth rate. IHC staining proved that these tumors had more microvessels compared to the control. The increase in angiogenesis was explained by the elevated expression of VEGF, and macrophage-inflammatory protein-2 (MIP-2) (120). In other studies, supernatants from melanoma cell lines (high vs low IL-1) were used to study the effect of IL-1 in endothelial cell permeability (121). Supernatant from the high IL-1 expression cell line, could increase cell permeability and the effect was reversed when using IL-1Ra, the IL-1 agonist. IL-1Ra has also been used to inhibit VEGF production in a colon carcinoma animal model. Another point to note is the importance of tumor-derived IL-1 in the effectivity of IL-1Ra. SMEL (High IL-1) and PMEL (low IL-1) melanoma cells were transduced with IL-1Ra and injected into mice. Only SMEL tumors had a significant reduction in the tumor growth rate. Another animal study, evaluated systemic administration of IL-1Ra to mice injected with human cancer xenografts (122). Similar to the previous study, only tumors that had significant levels of IL-1 responded to the IL-1Ra treatment. These studies suggest the indirect role IL-1 plays
in tumorigenesis and the importance of cross-talk between the tumor and the microenvironment. Lizee et al, described how oncogenes can up-regulate the expression of IL-1 in melanocytes. Our lab showed that mutant B-RAF significantly increased pro-inflammatory cytokines including IL-1α/β, IL-6, and IL-8 (123). Co-culture of recombinant IL-1 with human fibroblasts demonstrated the ability of IL-1 to induce inhibitory molecules on stromal cells, such as PD-L1, PD-L2 and COX-2. Thus, revealing another function of IL-1 on the tumor micro-environment.

In some cancers IL-1α, has anti-tumor properties. Fibrosarcoma cells transduced with IL-1α rarely grow in immune-competent mice. Tumors that grow regress in a mostly CD8+ T cell-mediated fashion. In this scenario, IL-1α acts as an adjuvant for CD8+ T cells (124). Furthermore, increase of adhesion molecules, allows for better cell to cell interactions, improving cytotoxicity.

The dual ability of IL-1 to induce pro- and anti-tumor effects makes it an interesting target to study and modulate. Proper regulation of the expression of IL-1 could tilt the tumor-microenvironment.
CHAPTER 2:

SPECIFIC AIMS
Rationale

Research from our lab demonstrated that melanoma cells up-regulate IL-1 expression via the aberrant activation of the MAPK pathway by mutated B-raf. Culturing IL-1 with tumor-associated fibroblasts resulted in an increase in inhibitory molecules, PD-L1, PD-L2, and COX-2. Therefore, we suggested a potential mechanism on how tumor cells can modulate immunosuppressive factors on neighboring cells via IL-1 induction. Moreover, research has shown that IL-1 is one of the main drivers of innate response, recruiting neutrophils and other immune cells into sites of inflammation. Therefore, we were interested in understanding how IL-1α affected melanoma tumor development. We hypothesized that IL-1α increases immunosuppression in the tumor by promoting the presence of suppressive cells and factors. We tested our hypothesis by answering the following aims:

**Aim 1:** Investigate the effect of IL-1α in the melanoma tumor micro-environment during early tumor development.

**Aim 2:** Evaluate the clinical potential of anti-IL-1α treatment in combination with other immunotherapies in melanoma.

We used an anti-IL-1α antibody to demonstrate that blocking IL-1α delays tumor growth. Moreover, we used mass cytometry to illustrate that IL-1α inhibition decreases anti-inflammatory myeloid cells during early tumor progression. Analysis of these cells *ex-vivo* showed that they had a decreased capacity to inhibit T cell activity via Arg-1 and NO. Furthermore, we showed that anti-IL-1α effect is not sustained and eventually the tumor activates alternates pathways to increase myeloid-derived suppressor cells. Results from our first aim indicated that anti-IL-1α could potentially synergize with T-cell-mediated immunotherapies. We hypothesized that the decrease of anti-inflammatory cells in the tumor micro-environment would improve the function of CD8+ T cells in conjunction with checkpoint
blockade or peptide vaccine and T cell therapy. Furthermore, we demonstrated that the combination of anti-IL-1α and anti-PD-1 slowed down tumor growth. Similarly as in anti-IL-1α monotherapy, in the combination group the major changes observed in the tumor microenvironment were in the myeloid cells. Our most promising anti-tumor response, was obtained from combining anti-IL-1α and peptide vaccine with T cell therapy. This combination increased survival of mice by over 3 months. The effect was obtained due to the high CD8+ T cell infiltrate and reduced myeloid cell density during the first week after vaccine treatment.
CHAPTER 3:

IL-1α BLOCKADE
I. Rationale

To evaluate the effects of IL-1α during tumor progression, we contacted the company, XBIOTECH, to use the murine equivalent, Flo1-2a, of their human IL-1α neutralizing antibody. MABp1, is the first true human antibody that targets IL-1α. In a phase 1 clinical trial, MABp1, was found to be well tolerated by patients with no significant side effects. The trial resulted in metastatic cancer patients having stable disease and some partial response after treatment (125).

We treated C57BL/6 mice that had been inoculated with B1-F10 melanoma cells and tracked the changes in the tumor micro-environment, specifically in immune cells. We hypothesized, that in the B16 melanoma mouse model, IL-1α contributed to immunosuppression and inhibition of IL-1α would lead to an increase in the immune response by restructuring the tumor-microenvironment. Understanding how IL-1α orchestrates the infiltration of immune cells into the tumor will help us understand how tumors use IL-1α to evade immune responses. Moreover, the insight gained will help us develop improved therapies for cancer.

II. Results

a. Anti-IL-1α reduces tumor growth in the B16-F10 murine model

As mentioned in the introduction, Weinreich et al, published studies where they determined that inhibition of IL-1 in vivo was only effective against tumors that expressed IL-1. We, determined that our B16 F10 (melanoma) cell line expressed significant levels of IL-1α by western blot (Figure 3A). Based on previous animal studies by Overwijk et al (126), we optimized the number of cells and day of treatment to model a significant tumor growth response. We compared treatment regimens targeting other cytokines such as TNF-α and IFN-γ, and tailored ours as follows: Day 0: tumor inoculation day, Day 3: Start of anti-IL-1α
treatment (100 μg intraperitoneally), mice received treatment every three days until the end of the experiment (Figure 3B). Mice treated at day 3 after tumor inoculation showed signs of tumor delay compared to the control (Figure 3C). Since the B16 model is quite aggressive, we euthanized the mice when the tumor burden reached 200 mm². The spider plots show that in the control, tumors reached the maximum tumor size limit by day 12 (Figure 3C). The majority of mice were euthanized on day 15 and only 20% of the mice survived until day 17 (Figure 3D, E). The treated group, reached its maximum tumor burden by day 15. The majority of mice were sacrificed after day 21. Only 20% of the mice reached day 27. None of the mice were disease free by the end of the experiment.
Figure 3: Anti-IL-1α reduces tumor growth in B16-F10 murine model. (A) Western blot showing Pro-IL-1α and IL-1α expression in METR, MC38, BJAB, and B16 cells. (B) Anti IL-1α (Flo1-2a) treatment schedule (C) Spider plots of tumor measurements per treatment group. (D) Tumor growth curve during the first two weeks of treatment. Data represents mean ± SEM, N=10 (E) Kaplan-Meier tumor survival curve. Data represents at least 3 independent experiments with 8-10 mice per group.
b. Inhibition of IL-1α decreased the myeloid cell infiltrates after one week of treatment

Even though IL-1α blockade did not result in disease free mice, we were encouraged by the moderate anti-tumor response. We believed that studying the tumor micro-environment would give us an insight into how to improve anti-IL-1α therapy in melanoma. Analysis of our growth curves showed tumor size separation after day 6 (Figure 3D). We hypothesized that the most significant changes in immune response occurred in that time frame. We isolated tumors at day 7, after mice received 2 anti-IL-1α treatments (Figure 4A). Immune cells were enriched by gradient density centrifugation and then analyzed by flow cytometry. Our analysis focused on the following immune subsets, T cells (CD45+, CD3+), B cells (CD45+, CD19+), myeloid cells (CD45+, CD11b+), macrophages (CD11b+, F4/80+), monocytes (CD11b+, Ly6C<sup>hi</sup>), and granulocytes (CD11b+, Ly6C<sup>lo</sup>, Ly6G<sup>hi</sup>) (Figure 4C). Tumors on day 7, exhibited a weight difference that we took into consideration for our absolute number calculations (Figure 4B). Compared to the control, IgG, anti-IL-1α tumors consistently had less immune cell infiltrates with an average drop of 30% (Figure 4D). The immune cell decrease was mostly due to significantly less monocyte infiltrates in the first week of tumor development (Figure 4D). T cells increased by at least an average of 35%. Analysis of CD8/CD4 ratios, showed that the spike in numbers was due to increased CD4<sup>+</sup> T cells (Figure 4E). Macrophages decreased by an average of 20%. B cells were not significantly affected by loss of IL-1α. Our findings show that blocking IL-α alters immune cell infiltration in the tumor, as early as the first week of treatment. The main effect was observed in myeloid subtypes congruent with published data on the effects of IL-1α.
Figure 4: Flow cytometric analysis of the immune component of the tumor micro-environment after one week of IL-1α blockade. (A) Treatment schedule of anti-IL-1α. (B) Weight (mg) of collected tumors on day 7. (C) Gating strategy for immune populations in the
tumor micro-environment. (D) Absolute number of immune infiltrates normalized to tumor weight. (E) Absolute number of CD8$^+$ T cells and CD4$^+$ T cells normalized to tumor weight. N=5. Unpaired T-test. Data represents at least 2 independent studies.
c. Inhibition of IL-α promotes pro-inflammatory features in myeloid cells

The first round of animal experiments showed the ability and potential of anti-IL-1α in reconstructing the tumor-microenvironment. To further show the immunosuppressive effects of IL-1α on immune cells, we used mass spectrometry to categorize the immune infiltrates. Enriched immune cells, from day 7, were stained with 30 markers (Table 1) and analyzed using the viSNE algorithm. viSNE allow us to visualize all the immune cell infiltrates in one graph (Figure 5A). cyTOF analysis was performed in FlowJo using the tSNE plug-in. Individual group data was concatenated into a single file to create a visual representation of immune cells across all groups. Additionally, using the concatenated file, we can compare individual groups since the algorithm creates the same map for all conditions. To identify changes in immune subsets, we used the density tool to create density heat maps to highlight clustered populations. viSNE clusters cells according to their similarity in their marker expression profile, thus cells that have similar markers will be clustered together in sub-populations. The further the clusters are from each other, the less they have in common. After the clusters were determined by the algorithm, we manually analyzed each cluster to confirm they were in fact a different subset. We set the cluster threshold at 70 events, meaning clusters that contained 70 cells or less were considered an artifact.

At first glance, we observed that the three main cell types were monocytes, macrophages, and B cells. Percentage comparison of the main populations did not show a major difference between the groups (Figure 5C). However, density plots of each group showed a significant change in subsets of these populations (Figure 5B), specifically in macrophages and monocytes.
<table>
<thead>
<tr>
<th>Marker</th>
<th>Cell type</th>
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<tr>
<td>OX40L</td>
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</tr>
<tr>
<td>CD80</td>
<td>APCs</td>
</tr>
<tr>
<td>CD86b</td>
<td>APCs</td>
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<tr>
<td>MHC Class II</td>
<td>APCs, B cells</td>
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<tr>
<td>PD-L2</td>
<td>APCs, myeloid cells</td>
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<td>IL-1α</td>
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<td>IL-6</td>
<td>APCs, myeloid cells</td>
</tr>
<tr>
<td>CD19</td>
<td>B cells</td>
</tr>
<tr>
<td>B220</td>
<td>B cells</td>
</tr>
<tr>
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<tr>
<td>Ly6G</td>
<td>Granulocytes</td>
</tr>
<tr>
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<td>Immune cells</td>
</tr>
<tr>
<td>F4/80</td>
<td>Macrophages</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>Macrophages</td>
</tr>
<tr>
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<tr>
<td>CD206</td>
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</tr>
<tr>
<td>Ly6C</td>
<td>Monocytes</td>
</tr>
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<tr>
<td>Arg-1</td>
<td>Myeloid cells</td>
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<tr>
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<tr>
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<td>Ki67</td>
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</tr>
<tr>
<td>IFNγ</td>
<td>T cells, myeloid cells, APCs</td>
</tr>
</tbody>
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**Table 1: cyTOF markers.**
A

Subset Name
- CD8
- CD4
- NK cells
- DCs
- B cells
- Macrophages
- Granulocytes
- Monocytes

B

C

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>IgG</th>
<th>Anti-IL-1A</th>
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<tbody>
<tr>
<td>Monocytes</td>
<td>30.4 ± 0.75</td>
<td>31.9 ± 1.46</td>
</tr>
<tr>
<td>Macrophages</td>
<td>22.5 ± 1.59</td>
<td>25.5 ± 1.35</td>
</tr>
<tr>
<td>B cells</td>
<td>21.5 ± 0.93</td>
<td>19 ± 1.28</td>
</tr>
<tr>
<td>CD4⁺ T cells</td>
<td>7.55 ± 1.00</td>
<td>8.79 ± 0.64</td>
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<tr>
<td>CD8⁺ T cells</td>
<td>6.51 ± 0.75</td>
<td>5.25 ± 0.35</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>3.88 ± 0.40</td>
<td>4.19 ± 0.40</td>
</tr>
<tr>
<td>NK cells</td>
<td>2.91 ± 0.35</td>
<td>2.73 ± 0.57</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>1.26 ± 0.24</td>
<td>2 ± 0.45</td>
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</table>
Figure 5: cyTOF analysis of the tumor micro-environment after IL-1α inhibition on day 7. (A) Color coded composite graph of IgG and anti-IL-1α showing major populations identified by viSNE algorithm and manual gating. (B) Pseudocolor density maps of IgG and anti-IL-1α showing differences in subsets of main populations (C) Table showing percent averages ± SEM. Data represents at least 2 independent experiments.
Detailed study of the monocytes, showed 12 unique subset populations. We named them Mo_1 to Mo_12 (Figure 6). After IL-1α inhibition, Mo_1 and Mo_8 subsets increased by 5% and 4%, respectively (Figure 7). Mo_1 was the least differentiated subset, mainly expressing CD11b, Ly6C, and F4/80lo. They express low or no levels of co-stimulatory molecules (CD80, CD86) or MHC-II (Figure 8, 9). Compared to Mo_1, Mo_8 is more active. They have increased expression of co-stimulatory molecules, CD80, CD86 and MHC-II. The Mo_3 subset decreased by 5%. Mo_3 cells express CD11b, Ly6C, CD80, CD86, MHC-II, and produce moderate levels of IL-α and TNF-α. Important to note was that the most active subset, Mo_6, was not impacted by IL-α blockade. Mo_6 was the only subset that expressed Arg-1, IFNγ, IL-1α, IL-6, and TNFα. Moreover, they expressed the proliferation marker, ki67. Neutralization of IL-1α seems to promote the infiltration of non-activated and pro-inflammatory monocytes.
Figure 6: Pseudocolor density map of monocyte subsets in melanoma tumors on day 7. Mo-1 to Mo-12 represent 12 unique monocyte subpopulations identified by viSNE analysis on day 7.
Figure 7: Percentage of monocyte subsets in melanoma tumors on day 7. Percent of individual subset out of CD11b, Ly6C<sup>Hi</sup> cells (monocytes). N=3. Data represents at least 2 independent experiments. Unpaired T-test.
Figure 8: Marker expression intensity heat map of monocyte subsets. Marker expression levels of monocyte subsets in IgG (grey column) and anti-IL-1α (red column) groups. Expression is normalized to the min and max expression per marker. Blue represents the subset with the lowest expression level and red represents the subset with the highest expression per row.
Figure 9: Histogram of the expression profile of monocyte subsets. Marker profile of Mo_1 (least differentiated), Mo_3 (intermediate differentiation), and Mo_6 (highly differentiated). Values represent the min and max MFI per marker ± SEM. Data represents at least 2 independent experiments.
Evaluation of the macrophage compartment led to the identification of 7 subsets (Figure 10A). Three subsets responded to IL-1α inhibition. The Mac_3 subset decreased by an average of 10% compared to the IgG group (Figure 10B). The Mac_3 subset expressed moderate levels of CD11b and F4/80. The Mac_4 subset was reduced by 5%. The Mac_4 cells had the lowest F4/80 expression of all the subsets. They more closely resembled the “M2” phenotype by expressing known M2 markers, CSF-1R, and PD-L2 (Figure 11). The most significant change observed was in the Mac_7 subset, which increased by 15% in the anti-IL-1α group (Figure 10B). Mac_7 cells resemble both M1 and M2 macrophages, signifying that these cells are in the middle of the macrophage differentiation spectrum. They are the most activated cells expressing high levels of M1 markers: CD11b, F4/80, CD11c, CD80, CD86, IFNα, MHC-II, OX-40L, and TNF-α. Moreover, they also express canonical M2 markers such as CD206, Arg-1, and IL-6. It is the only macrophage subset that is actively proliferating at the tumor site.
Figure 10: Macrophage subsets in melanoma tumors on day 7. (A) Pseudocolor density graph of macrophage subsets in IgG and anti-IL-1α groups. (B) Percentage of subsets out of

**Figure 11: Marker expression intensity heat map of macrophage subsets.** Marker expression of macrophage subsets in IgG (grey column) and anti-IL-1α (red column) groups. Expression is normalized to the min and max expression per marker. Blue represents the subset with the lowest expression level and red represents the subset with the highest expression per row.
Figure 12: Histograms of the phenotypic profile of macrophage subsets. Marker profile of Mac_1 (least differentiated), Mac_3 (int. differentiation), and Mac_7 (highly differentiated). Values represent the min and max MFI per marker ± SEM. Data represents at least 2 independent experiments.
d. IL-1α blockade decreases CD8+T cell infiltrates

Tumors treated with anti-IL-α had reduced CD8+ T cell infiltrates. cyTOF analysis identified 2 CD8+ sub-populations (Figure 13A) differentiated by their Ly6C expression (Figure 13C). We did not see an increase in T H1 response by IL-1α neutralization based on the CD8+ infiltrates’ lack of IFNγ, TNF-α, and Granzyme B (Figure 13D). Contrary to CD8+ T cells, CD4+ T cells increased with lack of IL-1α. Similarly to CD8+ T cells, CD4+ T cells were separated into 2 subtypes, CD4_1 and CD4_2. CD4_2 cells have a slight increase in CD4 and TIGIT expression compared to CD4_1 (Figure 13E). TIGIT has been shown to act an inhibitory molecule in CD4 effector T cells. Neither subset had intracellular FoxP3. Blocking IL-1α does not induce IFN-γ and TNF-α production in CD4_1 and CD4_2 cells (Figure 13F). Overall, the total T cell infiltrate increases with IL-1α inhibition, compromised by a majority of CD4+ T cells.
A

B

C

Normalized To Mode

CD8_1

CD8_2

CD4_1

CD4_2

Percent of CD3+ cells

Percent of CD3+ cells

Percent of CD3+ cells

Percent of CD3+ cells

1.17±0.41

213±12.23
Figure 13: IL-1α blockade decreases CD8⁺ T cell infiltrates in melanoma tumors on day 7. (A) Pseudocolor density map of T cell subsets. (B) Percentage of CD8 and CD4 subsets. (C) Ly6C expression in CD8_1 (blue) and CD8_2 (red). (D) Expression of CD8⁺ T cell activating markers in subsets ± SEM. N=3. Unpaired T-test. Data represents at least 2 independent experiments.
Neutralization of IL-1α modulates B cell subsets

Analysis of the B cell subsets showed that 5 distinct populations were present at the tumor site (Figure 14A). These subsets were differentiated by their level of CD19, B220 and MHC-II. Subset B_1, increased by at least 10% in tumors treated with anti-IL-1α. B_1, is the least activated B cell, having the lowest expression of CD19, B220 and MHC-II. It has no co-stimulatory molecules, and production of cytokines such as TNF-α, IFNγ and IL-α. The most activated B cell subtype was the one of two subpopulations that decreased in the treated group. B_5 has the highest expression of CD19, B220, and MHC-II, and shows signs of slight up-regulation of OX-40L, TNF-α, and IL-α. Likewise, the other reduced population was B_3, which has a similar expression profile as B_5. More markers are needed to elucidate the function of B cells in the tumor micro-environment after IL-1α inhibition.
Figure 14: Neutralization of IL-1α modulates B cell subset composition on day 7. (A) Pseudocolor density map of B cell subsets. (B) Percentage of B cell subsets. (C) Marker expression histograms of B cell subsets. Values represent the lowest (blue) and highest (purple) MFI ± SEM. N=3. Unpaired T-test. Data represents at least 2 independent experiments.
f. Myeloid cells isolated from tumors treated with anti-IL-1α are inefficient in suppressing T cells

Flow cytometry and cyTOF analysis showed that lack of IL-1α predominantly affects myeloid cells. Specifically, IL-1α blockade increased non- or low-differentiated myeloid cells and decreased M2-like macrophages. M2 macrophages and MDSCs inhibit T cell function. Therefore we wanted to assess if myeloid cells (CD11b⁺) treated with anti-IL-1α were less capable in reducing T cell activation. Since we cannot measure their ability to suppress T cells in vivo, we isolated myeloid cells from both control and treated groups and measured their arginase and NO activity, by using an arginase colorimetric assay and the griess assay, respectively. Inhibition of IL-1α significantly decreased the production of both arginase and NO in isolated myeloid cells (Figure 15). To further prove their decreased T cell inhibiting ability, we co-cultured isolated myeloid cells with p-mel T cells for three days and then used those T cells in a tumor cell killing assay (Figure 17). The tumor cell killing assay measures caspase 3 expression. Caspase 3 cleavage is activated during cell apoptosis, so increase in caspase 3 is directly proportional to increase in tumor cell apoptosis. The assay demonstrated that myeloid cells isolated from tumors treated with anti-IL-1α affected T cell activity to a lesser degree which led to an increase in tumor killing. Thus, from our data we can infer that IL-1α increases immunosuppression in B16 tumors by restructuring the tumor-microenvironment, specifically myeloid cells.
Figure 15: IL-1α inhibition decreases Arg-1 and NO in myeloid cells isolated from day 7 tumors. CD11b+ positive cells were sorted from tumors. Cell lysates and supernatants were collected to measure Arg-1 and NO using an arginase colorimetric assay (arginase) and the griess reaction assay (NO). N=5. Unpaired T-test. Data represents 2 independent experiments.
Figure 16: Gating strategy for isolating myeloid cells in the tumor. Immune cells were isolated from tumors at day 7. Enriched immune cells were sorted on CD45 and CD11b positive cells.
Figure 17: Tumor killing assay experimental procedure. Enriched immune cells from tumors were sorted on CD11b positive cells. After sorting cells were cultured with p-mel T cells for 3 days. On day 2, DDAO stained B16 cells were plated on a 96 well plate. On day 3, p-mel T cell/myeloid cell mixture was added to B16 plate. After 3 hours, cells were stained for caspase 3 and analyzed by flow cytometry.
Figure 18: Tumor killing assay shows myeloid cells isolated from tumor treated with anti-IL-1α have less effect on tumor killing. Enriched immune cells from tumors were sorted on CD11b positive cells. After sorting cells were cultured with p-mel T cells for 3 days. On day 2, DDAO stained B16 cells were plated on a 96 well plate. On day 3, p-mel T cell/myeloid cell mixture was added to B16 plate. After 3 hours, cells were stained for caspase 3 and analyzed by flow cytometry. N=3. Unpaired T-test. Data represents at least 2 independent experiments.
e. Anti-IL-1α effect is not sustainable

Our animal experiments show that inhibition of IL-1α modulates anti-tumor response by decreasing the infiltration of monocytes into the tumor micro-environment during the first week of treatment. Since, we did not see any disease free mice, we inferred that IL-1α effect was short-lived and, eventually, the tumor microenvironment reverts to an immunosuppressive state, even though anti-IL-1α treatment is administered until the end. We were able to show this by analyzing mice at the end stage of the experiment. When comparing the myeloid population at day 7 and at termination day, we can see that the positive effect seen at day 7 is gone (Figure 19). At termination, the myeloid composition looks similar in both groups. There is no difference in monocytes and granulocytes. Likewise, we see a similar effect in macrophages at termination day (Figure 20). Furthermore, on termination day, Arg-1 and NO expression returns to similar levels seen in the control during day 7 (Figure 21). At some point, tumors activate alternative pathways that induce immunosuppression in the tumor micro-environment.
Figure 19: Percentage of monocytes and granulocytes out of total myeloid cells at day 7 and termination day. Pseudocolor plot shows that at day 7 there’s a decrease in monocytes and increase in granulocytes due to anti-IL-1α. The effect is eradicated at termination day. Percent ± SEM. Data represents at least 2 independent experiments.
Figure 20: Percentage of macrophages out of total myeloid cells on day 7. Anti-IL-1α promotes a decrease in macrophages. The effect is gone by termination day. Percent ± SEM. Data represents at least 2 independent experiments.
Figure 21: Arg-1 and NO production are similar at termination day in IgG and anti-IL-1α. Myeloid cells isolated from IgG and anti-IL-1α have similar production of Arg-1 and NO at termination day. N=3. Unpaired t-test. Data represents at least 2 independent experiments.
III. Discussion

Current FDA approved immunotherapies have been shown to have success in metastatic melanoma patients. We know that tumor-mediated immunosuppression decreases the effectivity of these treatments in patients. Studies have shown that melanoma tumors express IL-1. We know that IL-1 coordinates a variety of pathways that leads to an increase in immunosuppression in the tumor-microenvironment. Thus, we wanted to study how IL-1α altered immune cells in the tumor. We used, Flo1-2α, the murine equivalent of MapB1 to treat B16 tumors in C57BL/6 mice. Tumors were treated on day 3 and thereafter every 3 days until the end of the experiment. As a monotherapy, anti-IL-1α, reduced tumor growth rate and increased mice survival by 10 days compared to IgG. Tumor growth is controlled by a variety of mechanism including, increased tumor cell proliferation, angiogenesis, and reduced immune response. In this project we focus on the immune response. Anti-IL-1α decreased immune infiltrates at the tumor site and changed immune cell percentages. The most evident change was observed in monocytes. Monocyte infiltrates decreased by half compared to IgG group. When we analyzed the different monocyte subsets in the treated group, we observed an increase in non-activated monocytes, Mo_1. Non-activated monocytes expressed CD11b and Ly6C. They had no antigen presentation co-stimulatory molecules and did not produce pro-inflammatory cytokines. The most differentiated monocyte subsets, Mo_5, Mo_6, and Mo_7 were not affected by anti-IL-1α. Unlike monocytes, highly active macrophages were the most affected by lack of IL-1α. Mac_7, expressed markers inducing both pro-inflammatory and anti-inflammatory responses. We deduced that Mac_7 subset was in the middle of the macrophage differentiation spectrum thus expressing markers from both extremes. Finally, we saw a decrease in Mac_4. Based on the marker expression, we can classify Mac_4 as an “M2”
macrophage involved in anti-inflammatory responses. Mac_4 cells expressed PD-L2, which is involved in the activation of PD-1 in T cells, resulting in T cell inhibition. Anti-IL-1α increased T cell infiltrates at day 7 via increase of CD4^+ T cells. These T cells did not exhibit Th1-associated cytokines such as IFNγ, TNF-α, and Granzyme β production. Important to note, was that CD8^+ T cell percentage decreased. Their function was also not changed by anti-IL-1α. The last immune population we analyzed was B cells. Anti-IL-1α did not alter the number of B cells but did affect their composition. TNF-α producing B cells were reduced in the treated group. At day 7, there were no B cell subsets producing co-stimulatory molecules CD80, and CD86. Unfortunately, we needed more markers to explain the function of B cells during early tumor progression.

Our data illustrates how blocking IL-1α induces an increase in non-activated cells and decrease in anti-inflammatory cells. Thus, we can conclude that the tumor microenvironment favors an anti-tumor response during the first week of tumor growth. Furthermore, we wanted to show that blocking IL-1α in the tumor micro-environment reduced the immunosuppressive function in myeloid cells. To do this, we isolated myeloid cells from tumors and measured their production of Arg-1 and NO. Studies have shown that Arg-1 inhibits T cell function by diminishing arginine, an amino acid necessary for T cell activation. NO is involved in T cell apoptosis. Quantification of Arg-1 and NO showed that myeloid cells isolated from IgG tumors, had higher levels of these molecules compared to tumors treated with anti-IL-1α. We can infer that IgG myeloid cells have a higher capacity to inhibit T cell activation. We tested this by using the tumor killing assay. We co-cultured myeloid cells, isolated from both groups, and p-mel T cells for 3 days in the presence of gp100 peptide, necessary for T cell activation. We, then, used this mixture to induce B16 killing, which was measured by the increase of caspase 3 cleavage in tumor cells. We noted that myeloid cells isolated from tumors treated with anti-IL-1α, had a lesser ability to inhibit T
cell activation. Therefore, one possible mechanism of action of anti-IL-1α is the reduction of immunosuppressive cells, allowing proper T cell activation and expansion.

The reduction of myeloid cells by inhibiting IL-α is temporary. When we analyzed tumors at the termination day, we observed no differences in monocytes and macrophages, even though we administered anti-IL-1α until the end of the experiment. This could be attributed to tumors over-expressing IL-1α so the antibody dosage is not enough to neutralize all available IL-1α. Also, cancer cells could be activating alternative pathways to drive immunosuppression, which are IL-1α independent.

IV. Material and Methods

a. Mice and tumor cells

Animal experiments performed in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas MD Anderson Cancer Center. 6 to 12 wk old female C57BL/6 mice were purchased from Charles River Frederick research model facility (Bethesda, MD). B16.F10 gp100+ is a spontaneous C57BL/6 melanoma obtained from the National Cancer Institute tumor repository and maintained in RPMI 1640 with 10% FBS, 100 μg ml\(^{-1}\) streptomycin and 100 μg ml\(^{-1}\) penicillin (Invitrogen).

b. Animal Experiments

Antibody against the secreted form of IL-1 α, Flo1-2a, was provided by XBioTech. Rat IgG2a, κ was purchased from BioXcell. C57BL/6 mice were inoculated with 3 x 10^5 B16.F10 gp100+ cells s.c. on the belly. Three days later mice were treated with 100 μg of Flo1-2a (i.p), or 100 μg IgG2a and, thereafter, 3x a week until end of experiment. Tumors were measured using calipers and collected at determined time points for tumor micro-environment analysis. For survival experiments, mice were sacrificed when tumors reached 200 mm\(^2\) or became too ulcerated.

c. Flow cytometry and CyTOF analysis
Single cell suspensions were prepared by carefully smashing tumors on a 70 μm strainer using a plunger from a 3 ml syringe. Cells were kept at 4°C in PBS with 2% FBS until needed. Intracellular staining was performed using the cytofix/cytoperm kit (BD Bioscience) according to the manufacturer’s protocol. The following antibodies were used during flow cytometry analysis, CD45, CD11b, CD3, CD19, CD11c, CD4, CD8, Ly6C, and Ly6G. Alternatively, cells were stained for CyTOF analysis. Briefly, surface markers were stained in 0.5% BSA-PBS for 1 hr at RT. After washing, cells were stained with 25 μM cisplatin for 1 min. Cells were fixed, permeabilized and stained with intracellular markers for 1 hr at RT, washed and then stained with 250nM IR-intercalator. Cells analyzed by Helios mass cytometer.

d. Arginase and NO assays

CD11b+ cells were isolated from tumor and spleen. Arginase activity was measured using the BioVision Arginase Activity Colorimetric assay kit. The kit indirectly measures arginase by first reacting arginine with arginase producing an intermediate that reacts with OxiRed probe. We measure the absorbance of the samples at 570nm and then use an equation to calculate arginase activity. Sample arginase activity = H2O2 amount from standard curve / (reaction time X sample volume added into the reaction well) X Dilution factor. NO was measured using the Griess reagent system from promega. NO is measured by quantifying one of its products, nitrate. The Griess Reagent system uses sulfanilamide and N-1-naphthylethylenediamine dihydrochloride (NED) under acidic conditions. Sulfanilamide and NED compete for nitrite. Absorbance was measured at 520nm and concentration was calculated using a nitrate standard curve.

e. Tumor killing assay

CD11b+ cells were isolated from tumors and spleen. CD11b+ cells were quickly co-cultured with p-mel T cells isolated from P-Mel-1 TCR/Thy1.1 mice at a 1:1 ratio with 1ng/ml
of gp100 peptide to activate the T cells. After 2 days, B16 cells were stained with DDAO and plated on a 96 well plate. On day 3, CD11b/Tcell co-culture was added to B16 cells for 3 hours and then cells were stained for caspase 3. Cells were analyzed by flow cytometry.

f. Statistical Analysis

All results are expressed as (mean ± SEM). Animal group size was (n=10) unless otherwise indicated. All experiments were repeated at least twice with comparable results. Data was analyzed using paired and non-paired t-test where appropriate and differences were determined significant at (P<0.05). Difference in tumor size among several treatments was evaluated using variance ANOVA. To compare survival, we used the Kaplan-Meier method and log-rank test. Graphs were created using GraphPad Prism 6 software.
CHAPTER 4: ANTI-IL-1α IN COMBINATION WITH CHECKPOINT BLOCKADE AND PEPTIDE VACCINE
I. Rationale

Data from our first experiments show that Flo1-2a reduces immunosuppression in the tumor micro-environment. This effect is seen during early tumor progression and dissipates as the tumor grows. Therefore, we hypothesized that Flo1-2a can be used as a pre-treatment to improve immunotherapy. We believe that reduction of myeloid cells by anti-IL-1α will improve the efficacy of T cell-mediated immunotherapies. We picked two different T-cell mediated immunotherapies. The first, anti-PD-1, has proven moderately effective in treating metastatic melanoma patients. The second one is a peptide vaccine plus T cell therapy. This peptide vaccine has been extensively studied by Dr. Overwijk’s lab in B16 melanoma models. The peptide vaccine consists of a combination of naïve p-mel T cells, IL-2, gp100, anti-CD40, and imiquimod (TLR-7 agonist) (127). From now on we will call this combination, covax. In this model, IL-2 induces the proliferation of T cells. gp100 activates naïve p-mel T cells. Anti-CD40, activates APCs. Lastly, imiquimod activates the innate immune response via TLR pathways. Together, the treatment activates both the adaptive and innate immune response, producing a strong anti-tumor effect. In the B16 model, both immunotherapies do not result in disease free mice. We believe, that adding Flo1-2a will increase mice survival.

II. Results

a. Combination of Anti-IL-1α and anti-PD1 increases survival in B16 tumor bearing mice.

Similarly to our monotherapy experiments, we started anti-IL-1α and anti-PD1 treatment at day 3 and continued every 3 days until the termination of the experiment (Figure 22A). We noted that anti-IL-1α alone and anti-PD-1α alone produced similar results (Figure 22B). We saw no difference in mice survival between both monotherapies (Figure 22C). The combination increased survival by 10 days. Compared to the IgG group, the
combination of anti-IL-1α and anti-PD-1 increased survival by 2 weeks. Moreover, 20% of the mice survived over 1 month. Analysis of the tumor micro-environment, at day 7, in the combination group showed similar infiltration pattern of myeloid cells as seen in the anti-IL-1α monotherapy group. For instance, the absolute number of monocytes decreased by half when we added anti-IL-1α to the anti-PD-1 group (Figure 23). We also noted an increase in granulocytes in the combination group. On the other hand, we saw a decrease in total T cells in the combination group. Our data shows that, as expected, Flo1-2a decreased monocytes even in the presence of anti-PD-1.
Figure 22: Anti-IL-1α and anti-PD-1 delay tumor growth. (A) Treatment schedule. Flo1-2a and anti-PD-1 treatment started at day 3 via (IP) injection. (B) Spider plots of tumor size from each animal. (C) Kaplan-Meier tumor survival curve. Data represents at least 2 independent experiments.
Figure 23: Anti-IL-1α and anti-PD1 treatment modulates immune infiltration on day 7.

Monocyte and granulocyte infiltration retains the same pattern seen as anti-IL-1α monotherapy. N=3. Unpaired T-test. Data represents at least 2 independent experiments.
b. Combining anti-IL-1α and anti-PD-1 alters macrophage subsets.

Analysis of macrophages, show 11 distinct sub-populations (Figure 25). In the combination group, we observe a decrease in non-activated macrophages represented by Mac_2 (Figure 26). Moreover, there was a decrease in Mac_4, which are macrophages that have high levels of MHC-II, but do not have co-stimulatory molecules on their surface (Figure 27, 28). We also noted a decrease in Mac_10, which most closely resembles “M2” macrophages by expressing PD-L2, CD206 and Arg-1. Mac_7 and Mac_11 represent subsets that are in between “M1” and “M2” phenotypes. They, both express, varying levels of antigen presentation-associated molecules and anti-inflammatory molecules. These two subsets increase in the combination group. IL-1α and PD-1 blockade promotes the increase in pro-inflammatory macrophages and decreases of anti-inflammatory macrophages.
Figure 24: Anti-IL-1α and anti-PD-1 modulate macrophage function on day 7. (A)
Composite viSNE map of IgG, anti-IL-1α, anti-PD1, and anti-IL-1α and anti-PD-1 groups showing the distribution of immune cells in the tumor microenvironment.
Figure 25: Pseudocolor map of macrophage subsets on day 7. viSNE analysis identified 11 distinct macrophage populations.
Figure 26: Macrophage subset percentages in anti-IL-1α and anti-PD-1 treated tumors on day 7. N=3. Unpaired T-test. Data represents at least 2 independent experiments.
Figure 27: Marker expression intensity heat map of macrophage subsets. Marker expression intensity of macrophage subsets in IgG (grey column), anti-IL-1α (red column), anti-PD-1 (green column), and anti-IL-1α + anti-PD-1 (blue column) groups. Expression is normalized to the min and max expression per marker. Blue represents the subset with the lowest expression level and red represents the subset with the highest expression per row.
Figure 28: Marker expression histograms of macrophage subsets found in anti-IL-1α and anti-PD-1 group. Blockade of IL-1α and PD-1 promotes pro-inflammatory
macrophages, while reducing anti-inflammatory macrophages. Values show min and max
values ± SEM. Data represents at least 2 independent experiments.
c. Combining anti-IL-1α and anti-PD-1 decreases non-activated monocytes.

In anti-IL-1α treated tumors, we observed the increase of non-activated monocytes. However, in tumors treated with the anti-IL-1α and checkpoint blockade, we observe a preference for activated monocytes instead of non-activated. viSNE analysis separated monocytes into 7 subsets (Figure 29A). Mo_1 and Mo_6 were the two subsets that were reduced in the combination group (Figure 29B). Mo_1 are non-activated monocytes, while Mo_6 are monocytes with MHC-II expression, only. The Mo_3 subset increased in the combination group. They are pro-inflammatory cells that produce TNF-α and IL-1α.
Figure 29: IL-1α and PD-1 blockade decreases non-activated monocytes on day 7. (A) Pseudocolor density map showing 7 monocyte subsets. (B) Monocyte subset percentage (C) Marker expression of monocyte subsets found in tumors treated with anti-IL-1α and anti-PD-1. Values show the min and max MFI ± SEM. N=3. Unpaired T-test. Data represents at least 2 independent experiments.
I. Combining anti-IL-1α and anti-PD-1 decreases CD8⁺ Ly6C⁺ T cells.

viSNE analysis segregated CD8⁺ T cells into 3 subtypes (Figure 30A). CD8_1 and CD8_2 subsets expressed moderate levels of Ly6C (Figure 30C). These two subpopulations were decreased in the combination group (Figure 30B). CD8_1 and CD8_2 are naïve T cells that do not express IFNγ and TNF-α. Anti-IL-1α and anti-PD-1 increased the CD8_3 subset in the tumor micro-environment. CD8_3 is the only subset that does not express Ly6C. The function of Ly6C on T cells is still not completely clear. It has been associated with T cell homing to the lymph nodes (128). Crosslinking of Ly6C, decreases IL-2 production by T cells. Therefore, we can infer that reduction of Ly6C⁺ T cells increases IL-2 availability, which in turn promotes proliferation in CD8⁺ T cells.
Figure 30: IL-1α and PD-1 blockade promotes CD8⁺LY6C⁻ T cells on day 7. (A)
Pseudocolor density map showing 3 CD8⁺ T cell subsets. (B) CD8⁺ T cell subset percentages. (C) Marker expression histograms of CD8⁺ T cell subsets. Values represent the low (blue) and highest (red) value ± SEM. N=3. Unpaired T-test. Data represents at least 2 independent experiments.
II. Combining anti-IL-1α and anti-PD-1 decreases CD4$^+$ TIGIT$^+$ T cells on day 7.

viSNE analysis separated CD4$^+$ T cells into 5 different sub-populations (Figure 31A). Only two subpopulations changed due to the blockade. Inhibition of IL-1α and PD-1 increased TNF-α producing CD4$^+$ T cells (Figure 31C). The most significant change was in CD4_3 cells. CD4_3 cells express inhibitory receptor, TIGIT. TIGIT signaling blocks NF-κB, PI3K and MAPK pathways (129). Studies have shown that presence of CD4$^+$ TIGIT$^+$ cells correlates with poor clinical outcome in melanoma patients due to their potent immunosuppressive ability (130). In our model, checkpoint blockade with anti-IL-1α induces CD4$^+$ T cells.
Figure 31: Anti-IL-1α and anti-PD-1 reduce CD4+ TIGIT+ T cells. (A) Pseudocolor density map showing 5 CD4+ T cell subpopulations. (B) CD4 subtype percentage out of total T cells. (C) Marker expression histograms. Values show low (blue) and max (red) MFI per marker ± SEM. N=3. Unpaired T-test. Data represents at least 2 independent experiments.
III. Combining anti-IL-1α with peptide vaccine and T cell therapy.

In our previous animal experiments, we were able to show that decreasing myeloid cells via IL-1α inhibition improved the anti-tumor response of anti-PD-1. In that model, we activated endogenous T cells by blocking, inhibitory receptor, PD-1. We then wanted to combine anti-IL-1α with a peptide vaccine treatment, optimized by Overwijk’s lab. Dr. Overwijk has specialized in using peptide vaccines with naïve T cells to treat B16 tumor cells. We decided to first pre-treat with anti-IL-1α before starting vaccine protocol (Figure 32A). We wanted to reduce myeloid cells first before adding exogenous T cells into the mice. We hypothesized that pre-treatment would significantly improve T cell activation. We injected tumor cells on day 0 and started anti-IL-1α treatment on day 3 and continued every 3 days for 21 days (Figure 32A). Vaccine treatment started on day 6. The vaccine consisted of naïve p-mel T cells, IL-2, for T cell proliferation, gp100, for T cell activation, anti-CD40, to activate APCs, and imiquimod, to activate the innate response. The combination of IL-2, anti-CD40, and imiquimod will be called covax. Survival experiments showed a significant increase in mice survival compared to peptide vaccine with P-mel T cells (Figure 32B). Mice survived for more than three months in the combination group. The most startling effect of the combined treatment was the significant tumor growth delay. Figure 32C shows pictures of the tumors from p-mel + gp100 + covax and anti-IL-1α + p-mel + gp100 + covax groups. It is evident that on day 40 the combination group has barely palpable tumors. Contrary to the peptide vaccine alone group, there is no sign of ulceration in the anti-IL-1α and peptide vaccine group. Due to the small size of the tumors, cyTOF analysis was not feasible. We switched to 18 color flow to analyze the tumor micro-environment after one week of treatment (day 14). After one week of vaccination, analysis of the tumor micro-environment showed that 80% of immune cells are CD8⁺ T cells in the combination group (Figure 34A). Myeloid cells barely infiltrate the tumor. 70% of the CD8⁺ T cells produce TNF-α (Figure 34B). The dramatic reduction in myeloid cells does not last. Analysis of the tumor
microenvironment on day 21 shows the re-population of myeloid cells (Figure 35). At this stage, we do not observe large percentages of monocytes. But as we have seen in the monotherapy, as the tumors become non-responsive to treatment they start increasing myeloid infiltrates in the tumor, specifically monocytes.
Figure 32: IL-1α blockade and peptide vaccine with naïve CD8+ T cells extends mice survival to over 3 months. (A) Treatment schedule (B) Kaplan-Meier curve showing increased survival in the P-mel + covax + anti-IL-1α group compared to the P-mel + covax group. (C) Pictures of tumors on day 40. Combination group shows no ulceration at this time point.
Figure 3: Flow cytometric analysis of the tumor micro-environment showing reduced myeloid infiltration. Tumor isolated on day 14 were stained and analyzed using flow cytometry. Data represents at least 2 independent experiments.
Figure 34: Immune response of anti-IL-1α and peptide vaccine consists of predominantly TNF-α producing CD8+ T cells. (A) Percent of CD8+ T cells present in the tumor on day 14 in each group (B) Plots showing TNF-α and Ly6C expression in CD8+ T
cells isolated from each group. Values show MFI ± SEM. Data represents at least 2
independent experiments.
Figure 35: Myeloid cells infiltrate the tumor after two weeks of anti-IL-1α and peptide vaccine treatment. Tumors were isolated on day 21 and analyzed by flow cytometry to identify monocytes and granulocytes. Values show MFI ± SEM. Data represents at least 2 independent experiments.
III. Discussion

Chapter 3 of this dissertation, shows the potential of IL-1α blockade as a treatment in metastatic melanoma. In chapter 4, we further prove the advantages of inhibiting IL-1α before treating with T-cell mediated immunotherapies. Our studies with checkpoint blockade and anti-IL-1α demonstrated the combined effect in increased survival of mice compared to the control group. Analysis of the tumor micro-environment showed a decrease in non-activated monocytes and an increase of pro-inflammatory monocytes. This is the opposite effect seen in chapter 3. We believe this change is due to anti-PD-1-mediated responses. Pro-inflammatory monocytes are major sources of TNF-α. TNF-α has dual roles in tumor progression. TNF-α can activate the NFκB pathway leading to increased proliferation in cells. Thus, TNF-α can increase the expansion of immune cells and tumor cells, equally. A proper balance of TNF-α production is required to maintain an anti-tumor response.

Blockade of IL-1α and PD-1, also increases pro-inflammatory macrophages. These cells secrete IFNγ, TNF-α, IL-1α and IL-6. These cytokines induce Th1 and Th2 responses in T cells. Moreover, we were able to study macrophages in hybrid states. These macrophages do not conform to the classical M1 vs M2 phenotype. They expressed both pro and anti-inflammatory molecules. Inhibition of PD-1 and IL-1α decreased CD8+ T cell infiltration while increasing CD4+ T cells. Specifically, CD8+, Ly6C+ subsets were reduced in the tumor micro-environment. Furthermore, blockade promoted the expansion of CD4+ TNF-α+ T cells, while decreasing an immunosuppressive subset identified by their TIGIT expression. Taken together, we can see that inhibiting IL-α even in the presence of anti-PD1 skews the tumor micro-environment to induce an anti-tumor response. In both models, we see a decrease in monocytes cells. Our data suggests that monocytes are a key driver of immunosuppression in our model.

Our last model, anti-IL-1α and peptide vaccine with naïve T cells, illustrates why we studied the effects of IL-1α on the tumor micro-environment. Analysis of immune filtration
showed that in the combination group mostly CD8+T infiltrated the tumor. The effect was
temporary but enough to increase survival by a month.

IV. Material and Methods

Animal Experiments

Anti PD-1 antibody (clone 29F.1A12) and Rat IgG2a, κ were purchased from BioXcell. The synthetic H-2D\textsuperscript{b} restricted gp100\textsuperscript{25-33} peptide (KVPRNQDWL) was a gift from Dr. Willem Overwijk’s lab. C57BL/6 mice were inoculated with 3 x 10\textsuperscript{5} B16.F10 gp100+ cells s.c. on the belly. Three d later mice were treated with 100 μg of Flo1-2a (i.p), 200 μg Anti PD-1 (i.p) or 100 μg IgG2a and, thereafter, 3x a week until end of experiment. For the peptide vaccine experiment, on d 6 after tumor inoculation, mice were injected with 1000 naïve P-mel 1 T cells (i.v) and were vaccinated with one s.c injection in each flank of PBS containing 100 μg of hgp100 and 50 μg of anti CD40. 5% Imiquimod cream was applied topically on the vaccination site. 100,000 UI of hrIL-2 was injected (i.p) on d 0 and then 2x a d for 2 d for a total of 500,000 UI. Tumors were measured using calipers and collected at determined time points for tumor micro-environment analysis. For survival experiments, mice were sacrificed when tumors reached 200 mm\textsuperscript{2} or became too ulcerated.

18 color flow cytometry

Single cell suspensions were prepared by carefully smashing tumors on a 70 μm strainer using a plunger from a 3 ml syringe. Cells were kept at 4°C in PBS with 2% FBS until needed. The following antibodies were used during flow cytometry analysis CD45, CD3, CD8, CD11b, CD11c, CD19, CD25, CD49b, CD103, Arg-1, CD206, FoxP3, F4/80, Ly6C, and Ly6G. Surface markers were stained in 2% FBS-PBS for 1 hr at RT. After washing, cells were fixed and permeabilized using the cytofix/cytoperm kit (BD Bioscience)
according to the manufacturer’s protocol. Intracellular markers were stained for 1 hr at RT, washed and then fixed with 1.6% paraformaldehyde. Cells were analyzed by X-20 fortessa.

**Statistical Analysis**

All results are expressed as (mean ± SEM). Animal group size was (n=10) unless otherwise indicated. All experiments were repeated at least twice with comparable results. Data was analyzed using non-paired t-test where appropriate and differences were determined significant at (P<0.05). To compare survival, we used the Kaplan-Meier method and log-rank test. Graphs were created using GraphPad Prism 6 software.
CHAPTER 5: DISCUSSION

Current advancements in technology and science have facilitated the design of improved therapies to treat metastatic melanoma. Clinical trials have demonstrated the effectiveness of immunotherapies in treating a wide range of cancer patients, especially treatments that boost the activity of endogenous CD8⁺ T cells. Nevertheless, a major barrier against effective T-cell mediated treatments is the rise of immunosuppressive cells and factors in the tumor micro-environment mediated by the tumor. Our lab, published a tumor-dependent mechanism in which abnormal activation of the MAPK pathway, via the induction of mutated B-raf, led to the increase of NFκB, a modulator of IL-1 (123). The tumor-derived IL-1 can then increase inhibitory molecules on neighboring cells in a paracrine fashion, potentially inhibiting T cell cytotoxicity. The purpose of my project was to understand how IL-1α altered the architecture of the tumor micro-environment. We hypothesized that blocking IL-1α would lead to a decrease in immunosuppressive factors, improving T cell response. To gain insight into IL-1α function in tumor development, we contacted XBioTech. They currently synthesize an anti-human and anti-mouse IL-1α neutralizing antibody. Animal studies in aim 1, demonstrated that blocking IL-1α led to a delay in tumor development. Due to the malignancy of B16-F10, we did not expect to see any disease free mice. IL-1α blockade prolonged mouse survival by ten days. The increase in survival and reduced growth rate can be attributed to several functions of IL-1α in melanoma. First, IL-1α up-regulates VEGF, which promotes tumor angiogenesis (131). Increase in vasculature leads to an increase in oxygen and metabolic factors necessary for optimal cell growth and proliferation. Moreover, IL-1 directly impacts the expression of adhesion molecules and metalloproteinases involved in the process of tumor invasion (132). Increased expression of integrins on the surface of tumor cells, allow cancer cells to more efficiently enter into circulation and seed in other areas. Lastly, IL-1α increases other growth factors that can activate cell growth and proliferation pathways, such
as IL-8, IL-6, TNF-α and TGF-β (132). Thus reducing available IL-1α limits the tumor’s ability to grow and expand, as demonstrated by our animal studies. However, due to an innate redundancy in growth and proliferation pathways, tumors can activate other pathways to overcome the effects of inhibiting IL-1α. Therefore, it is possible for some tumors to be more sensitive to IL-1α blockade. Survival experiments showed that after six days of treatment, tumor sizes started diverging in the anti-IL-α group. Certain tumors responded to anti-IL-1α treatment and displayed a slower tumor growth rate, while others continued growing at a rapid rate. The control group, which was injected with IgG, rarely survived past two weeks after initial tumor inoculation. Aside from the effects of IL-1α on cancer growth and proliferation, we were interested on the effects of IL-1α on infiltrating immune cells during early tumor development. Of most interest were the changes in infiltrating myeloid cells, since IL-1 expression has been associated with the increase of MDSCs in the tumor (133). We used flow cytometry and mass cytometry to phenotype all immune cells on day seven. As stated earlier, size discrepancies were apparent after day 6, so we chose to analyze the tumor microenvironment after one week of treatment to try to understand how immune cells respond to lack of IL-1α, while the tumor is still developing. Mass cytomometry or cyTOF allows the use of 35 surface and intracellular antibodies simultaneously. Contrary to flow cytometry, the use of metal conjugates in lieu of fluorochromes, leads to a significant reduction in background. Our findings demonstrated that IL-1α neutralization, results in a decrease in immune cell infiltrates, identified by their expression of CD45. Further analysis, showed that mainly myeloid cell trafficking was affected, consistent with published data. This effect on myeloid cells may be explained by a decreased expression of known chemokines involved in myeloid cell recruitment, such as, CXCL1 (neutrophils), CXCL14 (monocytes), and CXCL13 (macrophages) (134). Focusing on specific myeloid subsets, monocyte infiltration is drastically reduced. The monocyte composition changed favoring non-activated monocytes and monocytes expressing antigen presentation molecules: CD80, CD86 and MHC-II. This shift
may be due to a loss of anti-inflammatory cytokines needed for suppressive monocyte polarization, like IL-6, CCL2, CXCL1, and CXCL10 (134). Moreover, we found an increase in TNF-α expression across several cells when treated with anti-IL-1α. TNF-α induces the differentiation of myeloid cells into pro-inflammatory cells (135). Thus, reduced IL-1α could directly induce TNF-α production in immune cells which in turn can promote a pro-inflammatory response. Similarly, macrophages displayed a propensity to express and produce factors involved in a Th1 response. Specifically certain subsets had an increase production of IL-1α and TNF-α, which are known cytokines linked to M1 macrophages. Interestingly, there were macrophage subsets that exhibited both M1 and M2 markers. This can be explained by the plasticity of macrophages. When encountering Th1 and Th2 signals, macrophages can quickly move across the differentiation spectrum (16). On day 7, the Mac_7 subset expressed M2-associated molecules such as Arg-1, and CD206, while also, expressing M1-associated markers MHC-II, CD80, CD86 and TNF-α. Finally, the last observed changed in myeloid cells was the increase of granulocytes. Research has shown that in most cancers, granulocyte presence correlates with a poor prognosis. Specifically, an increase in the neutrophil to lymphocyte ratio is associated with worse outcomes in solid tumors (136). Early decrease in neutrophil to lymphocyte ratio due to targeted treatment leads to favorable outcomes. Thus, reduction in granulocytes would be an advantageous result. However, our data shows the opposite result. Tumors treated with anti-IL-1α have an increase in granulocytes. We observed no difference, in their surface marker profile, between granulocytes in the control group versus the treated group. Anti-IL-1α granulocytes exhibited higher TNF-α levels, hinting at the possibility of N1 neutrophils. Neutrophils and PMN-MDSCs have similar phenotypes and can only be distinguished by extensive functional studies in vitro. Analogous to macrophages, neutrophils can be commonly categorized into N1 and N2 neutrophils. N1 neutrophils express elevated levels of TNF-α and ICAM1α and have tumor killing abilities (137). Alternatively, N2 neutrophils produce IL-6, CCL2, CXCL1 and CXCL10,
iNOS, and Arg-1. N2 neutrophils have been linked to help in tumor initiation, growth, proliferating and metastatic spread (137). Based on the elevated levels of TNF-α in myeloid cells treated with anti-IL-1α, we can speculate that the infiltrating granulocytes can be N1 neutrophils. Ex-vivo analysis of immunosuppressive factors in myeloid cells, identified by CD11b, showed a decrease in arginase-1 and nitric oxide production, suggesting that the majority of anti-IL-1α myeloid cells have reduced immunosuppressive abilities. Moreover, we directly demonstrated that these cells have a decreased capacity in inhibiting T cell function and activity, in vitro. T cell analysis shows that on day 7, IL-1α inhibition increases T cell infiltration, specifically CD4+ T cells. Neither CD8+ nor CD4+ T cells expressed Th1-related factors such as IFN-γ, Granzyme B, and TNF-α. Thus, on day 7, IL-1α blockade does not elicit a Th1 response in T cells. It could be possible that at a later time point, we can observe the induction of these factors, or that in our model T cell response is not Th1-dependent. Based on our studies IL-1α blockade is not sufficient to mount a robust immune response. It is clear, that suppressive myeloid cell reduction is not enough to obtain a lasting anti-tumor effect. Finally, analysis of terminal day tumors, showed that the changes observed in the tumor micro-environment by inhibiting IL-1α are not sustained. Termination day tumors in both control and treated groups have similar myeloid cell compositions, even though anti-IL-1α is administered until the end of the experiment. It’s possible that tumors over-express IL-1α so the antibody dosage is not enough to neutralize all available IL-1α. In the future, testing progressive escalating doses of anti-IL-1α could be beneficial in prolonging the anti-tumor response. Cancer cells could be activating alternative pathways to drive immunosuppression, which are IL-1α independent. This immune evasion mechanism is commonly seen in patients that have undergone targeted therapy. For instance, patients treated with B-raf inhibitor exhibit abnormal up-regulation of other genes in the MAPK pathway which leads to increased proliferation and survival. Similarly, tumors could be up-regulating other cytokines including IL-6, IL-8, and IL-10 which are also involved in the recruitment of myeloid cells into the tumor.
Taking into consideration all these factors, to induce long lasting anti-tumor effects, it is necessary to combine IL-1α blockade with other therapies. In aim 2, we test the synergy of IL-1α blockade with T-cell mediated immunotherapies.

The second animal model tested the joint effect of anti-IL-1α with anti-PD-1. The decrease in myeloid cells, due to IL-1α inhibition combined with the improved T cell activity, due to anti-PD-1, furthered delayed tumor growth, compared to anti-IL-1α monotherapy alone. Twenty percent of mice survived for a month. Analysis of the tumor micro-environment painted a similar picture as seen in the monotherapy setting. Overall myeloid cells decreased in the combination group, as expected. This change was due to a decrease in the monocyte population. The remaining monocytic cells presented pro-inflammatory molecules on their surface. The combination of anti-IL-1α and anti-PD-1 increased the differentiation of monocytes, based on their increased number of markers displayed. This could be partially due to the unintended activation of monocytes by anti-PD-1. Studies have shown that certain macrophages and monocytes express PD-1 on their surface (139). Ligation of the PD-1 receptor may have started an activation cascade that converted monocytes into activated pro-inflammatory monocytes or macrophages. This could also explain the unexpected rise of macrophages in the combination group. Joint activity of anti-IL-1α and anti-PD-1 produced an environment that favored M1 macrophage activation and proliferation. Startlingly, there was no strong T\textsubscript{H1} T cell response in tumors treated with the combination. Anti-PD-1 works by preventing T cell activity inhibition, so we were expecting the presence of activated T\textsubscript{H1} T cells. However, at this time point both CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells lacked IFN-γ, and Granzyme B production. Based on this and data from our previous model, it would be valuable to test T cell activity at different time points to understand the kinetics of T cell activation in the absence of IL-1α. From our findings, it is evident that additional factors are needed to prime and activate T cells. Therefore, in our last model we combined anti-IL-1α with a peptide vaccine and naïve
CD8$^+$ p-mel T cells. The peptide vaccine contained the peptide, gp100, which can prime p-mel$^+$ T cells, anti-CD40, increases T cell priming by activating APCs, IL-2, induces T cell proliferation and imiquimod, a TLR agonist that increases APC activity. Contrary to our anti-PD-1 and anti-IL-1α model, we started anti-IL-1α treatment three days before the vaccine day, this resulted in a significant decrease of myeloid cell infiltration after one week of vaccine treatment. With this combination, mice survival was extended for a month compared to the peptide vaccine alone. Tumor growth was significantly delayed. Pictures of tumors on day 40 show barely palpable tumors with no ulceration. Analysis of the tumor micro-environment after one week of vaccine, showed that almost 80% of immune cells were CD8$^+$ T cells. Consistent with a Th1 response, CD8$^+$ T cells expressed high levels of TNF-α and IFN-γ. We saw no significant difference in IFN-γ levels, but saw an increase in TNF-α, which was the most common up-regulated molecule across all cells. As in the earlier models, TNF-α plays an important role in the conversion of the tumor micro-environment into a less immunosuppressive surrounding. Studies have shown that low levels of IL-1 can induce local inflammation which leads to an immune response; however, high levels of IL-1 can instead induce anti-inflammatory responses to attenuate the increased immune response (110). So, low levels of IL-1α can directly increase TNF-α production in cells in a paracrine fashion resulting in a favorable anti-tumor response. This point seems critical in patient care since depending on the expression of IL-1 in tumors, the anti-IL-1 dosage might have to be optimized in a patient to patient basis to obtain satisfactory results.

Our data suggests that anti-IL-1α has potential in delaying tumor growth when combined with the appropriate therapy in patients. Nevertheless, there a few things to consider. In our most promising model, we obtained almost complete inhibition of myeloid cell trafficking into the tumor, which greatly increased T cell presence, but most importantly TNF-α and other Th1 cytokines. Unregulated synthesis of these cytokines could induce
autoimmune diseases in patients. Therefore, in patients it would be appropriate to combine anti-IL-1α with CAR T cell therapy, to avoid having to add the extra immune modulating factors. In some cases, patients would have to be administered steroids to decrease the cytokine storm. Moreover reduction of anti-inflammatory myeloid cells potentially reduce factors involved in angiogenesis, tumor growth and survival. Due to this, IL-1α blockade is more pertinent when tumors are just starting to grow to limit several pro-tumor mechanisms simultaneously. Anti-IL-1α could be administered to patients that have just undergone tumor resection, to avoid possible recurrence and further metastases. For advanced solid tumors, IL-1α monotherapy is not sufficient and would have to be combined with other T cell-mediated immunotherapies. Patients undergoing ACT could be administered with anti-IL-1α after whole-body irradiation to prevent the trafficking of suppressive cells to the tumor site before T cell infusion.

This project started by trying to understand how IL-1α modulates immune response during tumor development. In this manuscript, we suggest that the anti-tumor effect is mostly myeloid cell driven. Lack of IL-1α promoted the differentiation of pro-inflammatory monocytes and macrophages, which are involved in T cell priming and direct cancer cell killing. The reduction in suppressive myeloid cells led to an increase in anti-tumor response. Most importantly, we were able to wield the capabilities of anti-IL-1α to limit immunosuppression to improve checkpoint blockade and peptide vaccine immunotherapies in mice. Further studies with other immunotherapies would give us a better insight into which treatments synergize with anti-IL-1α blockade. For instance, we have only explored anti-PD-1, future studies could focus on treating mice with anti-IL-1α and anti-CTLA-4. We would expect an even better anti-tumor response since anti-CTLA-4 also blocks the activation of Tregs. Another possible treatment route is targeted therapy. Melanoma cells over express oncogenic B-raf and MEK. Combining anti-IL-1α with B-raf and MEK inhibitors could potentially increase the activity of T
cells due to the increase in available stimulating peptides. Finally, to improve our phenotypic profile, we could combine genetic profiling based on single cell RNA-seq. In this manner, we could identify which genes are up- or down- regulated with IL-1α blockade. This could give us a hint into other possible targets to combine with anti-IL-1α.
References


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