MERTK INDUCES TOLEROGENIC DENDRITIC CELLS BY METABOLIC REPROGRAMMING

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ABSTRACT

Eden Y Zewdie: MerTK Induces Tolerogenic Dendritic Cells by Metabolic Reprogramming
(Under the direction of H. Shelton Earp III)

Checkpoint inhibitors, specifically anti-PD-1, have shown success in treating metastatic melanoma; however, some patients develop resistance. Dendritic cells (DCs) play a key role in initiating an immune response but in certain circumstances they become ineffective and tolerogenic. We investigated the role of MerTK, a receptor tyrosine kinase responsible for myeloid cell clearance of dead cells, in the regulation of DC function and metabolism in the tumor microenvironment. Anti-PD-1 resistant tumors exhibited increased levels of MerTK^+ DCs. Treating wt DCs with apoptotic dead melanoma cells in vitro resulted in increased MerTK expression, elevated mitochondrial respiration and fatty acid oxidation, and reduced T cell stimulatory capacity, all characteristics of tolerogenic DCs. In contrast, dead cells had only limited effect on the metabolism of MerTK-deficient DCs, which instead maintained an antigen presenting, stimulatory phenotype. The efficacy of anti-PD-1 to slow tumor progression and induce specific T cell infiltration was markedly increased in mice with selective ablation of MerTK in the DC compartment, suggesting the importance of targeting MerTK to modulate DC metabolism and function and enhance anti-PD-1 therapy.
To my parents, I would not have made it this far without your prayers, love, and support. Thank you for your consistent presence in my life.
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This thesis is based on my first author paper, which is in the publication process at *Cancer Immunology Research*, an AACR journal.
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<thead>
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<th>Full Form</th>
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<tbody>
<tr>
<td>DCs</td>
<td>Dendritic Cells</td>
</tr>
<tr>
<td>pDCs</td>
<td>Plasmacytoid DCs</td>
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<tr>
<td>cDCs</td>
<td>Conventional DCs</td>
</tr>
<tr>
<td>moDCs</td>
<td>Monocyte derived DCs</td>
</tr>
<tr>
<td>LCs</td>
<td>Langerhans cells</td>
</tr>
<tr>
<td>TME</td>
<td>Tumor Microenvironment</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed Cell Death Protein 1</td>
</tr>
<tr>
<td>PD-L1</td>
<td>Programmed Death Ligand 1</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte Associated Protein 4</td>
</tr>
<tr>
<td>APCs</td>
<td>Antigen Presenting Cells</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>CMP</td>
<td>Common Myeloid Progenitor</td>
</tr>
<tr>
<td>MDP</td>
<td>Macrophage and DC Progenitor</td>
</tr>
<tr>
<td>CDP</td>
<td>Common DC Progenitor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-Macrophage Colony-Stimulating Factor</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin-4</td>
</tr>
<tr>
<td>IRF8</td>
<td>Interferon Regulatory Factor 8</td>
</tr>
<tr>
<td>FLT3L</td>
<td>Fms-related Tyrosine Kinase 3 Ligand</td>
</tr>
<tr>
<td>BATF3</td>
<td>Basic Leucine Zipper ATF-like Transcription Factor 3</td>
</tr>
<tr>
<td>BCL-6</td>
<td>B-cell Lymphoma 6</td>
</tr>
<tr>
<td>ID2</td>
<td>Inhibitor of DNA Binding</td>
</tr>
<tr>
<td>ZBTB46</td>
<td>Zinc Finger and BTB domain containing 46</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>NFIL3</td>
<td>Nuclear Factor, Interleukin 3</td>
</tr>
<tr>
<td>IRF4</td>
<td>Interferon Regulatory Factor 4</td>
</tr>
<tr>
<td>RBPJ</td>
<td>Recombination Signal Binding Protein For Immunoglobulin Kappa J</td>
</tr>
<tr>
<td>NOTCH2</td>
<td>Notch Receptor 2</td>
</tr>
<tr>
<td>KLF4</td>
<td>Kruppel-like Factor 4</td>
</tr>
<tr>
<td>E2-2</td>
<td>E-protein (transcription factor)</td>
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<tr>
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<td>Interferon Regulatory Factor 7</td>
</tr>
<tr>
<td>CSF1R</td>
<td>Colony Stimulating Factor 1 Receptor</td>
</tr>
<tr>
<td>MAFB</td>
<td>MAF BZIP Transcription Factor B</td>
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<tr>
<td>IL-12</td>
<td>Interleukin-12</td>
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<td>Interferon</td>
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<td>Interleukin-23</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthase</td>
</tr>
<tr>
<td>TAM RTKs</td>
<td>Tyro3, Axl, MerTK Receptor Tyrosine Kinases</td>
</tr>
<tr>
<td>Pros1</td>
<td>Protein S 1</td>
</tr>
<tr>
<td>GAS6</td>
<td>Growth Arrest Specific 6</td>
</tr>
<tr>
<td>PtdSer</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>FAO</td>
<td>Fatty Acid Oxidation</td>
</tr>
<tr>
<td>CPT1</td>
<td>Carnitine Palmitoyl-Transferase I</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>ETO</td>
<td>Etomoxir</td>
</tr>
<tr>
<td>OCR</td>
<td>Oxygen Consumption Rate</td>
</tr>
<tr>
<td>ECAR</td>
<td>Extracellular Acidification Rate</td>
</tr>
<tr>
<td>MDSCs</td>
<td>Myeloid-Derived Suppressor Cells</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver X Receptor</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal Transducer and Activator of Transcription 3</td>
</tr>
<tr>
<td>p38</td>
<td>p38 Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2, 3-dioxygenase</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor beta</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>2-DG</td>
<td>2-deoxy-D-glucose</td>
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INTRODUCTION

1.1 Metastatic Melanoma

Metastatic melanoma is an aggressive cancer with death rates of up to 80% with relation to other skin cancers (Bandarchi et al., 2013). Although there are other skin cancers, melanoma is the most likely to metastasize. Melanoma arises from melanocytes, which produce pigment (melanin), with the majority of them found in the skin and eyes. As such, melanoma occur in any tissue where melanocytes are present; however, the most common is cutaneous melanoma (Sadozai et al., 2017). There have been over 320,000 new melanoma cases globally reported in 2020, with 57000 deaths (Arnold et al., 2022). This global burden gives rise to the need for therapeutic interventions that can increase survival rate. Moreover, with metastatic melanoma being the major cause of death, my objective is to study therapeutic interventions for this specific burden.

Both genetic and environmental factors have been known to contribute to melanoma. Some of the genetic factors include skin complexion and family history of disease, whereas the main environmental factor is mainly UV rays. In addition, sequencing of human melanoma reveals that it carries the highest mutational burden compared to other solid tumor types allowing for potentially effective therapies. Some of the common driver mutations frequented in human melanoma are mutations in BRAF, NRAS, Neurofibromin, Microphthalmia-Associated Transcription Factor, and PTEN;
with the most common being BRAF and loss of PTEN in metastatic melano- 
mamas (Sadozai et al., 2017).

BRAF is part of the RAS-RAF-MEK-ERK pathway and leads to downstream ac-
tivation of cell differentiation and growth transcription factors (Samatar and Poulikakos, 2014). BRAF accounts for 60% of melanoma mutations where the missense mutations of V600E accounts for 80% of BRAF melanoma mutations (Reddy et al., 2017). This particular mutation results in 500-fold increase in BRAF activation in melanocytes (McCain, 2013).

PTEN (Phosphatase and Tensin homolog) is a negative regulator of the PI3K-Akt pathway (Aguissa-Toure and Li, 2012). Loss of PTEN (silencing) in melanocytes results in cell cycle progression, migration, and reduced apoptosis. Although not as common as BRAF mutations in melanoma, PTEN loss is seen in the majority of metastatic melanomas (Reddy et al., 2017). With this in mind, this study focuses on metastatic melanoma with BRAF<sup>V600E</sup> mutation with Pten<sup>−/−</sup>.

With chemotherapy largely ineffective, fortunately new therapeutic approaches for metastatic melanoma have been increased over the past few years. There have been two major approaches. The first is targeted therapies. Due to the high mutation burden, there are various target therapies, some of which are BRAF inhibitors, MEK inhibitors, and mTOR inhibitors. However, these direct targeting therapies are more prone to tumor resistance (Mellman et al., 2023). The second and more recently favored is the use of immunotherapy. Some of which are immune checkpoint inhibitors: cytotoxic T lymphocyte antigen-4 (CTLA-4) and Programmed Cell Death Protein 1 (PD-1) inhibitor (Sadozai et al., 2017).
Targeting immune cells as a form of cancer treatment has shown great success. With that being said, various tumors have different immunotypes. Tumors can be either immune inflamed, immune excluded, or immune desert. Melanoma specifically can exhibit all three immunotypes, and this is highly patient dependent (Mellman et al., 2023).

Metastatic melanoma was highly resistant to therapy until the advent of recent advances targeting the immune system. Anti-PD-1 therapy has been highly successful particularly in patients with immune infiltrated tumor microenvironments (TME). It works by blocking the interaction between Programmed Cell Death Protein 1 (PD-1) receptor, and its ligand Programmed Death Ligand 1 (PD-L1) found on tumor cells and some infiltrating myeloid components; an interaction that dampens anti-tumor immunity (Tumeh et al., 2014). PD-1, which is expressed on T cells, is a vital regulator of T cell activation. Therefore, tumors can express PD-L1 to dampen T cell activation. Therefore, blocking this interaction increases T cell activation (Sadozai et al., 2017).

While remarkable responses occur, less than 50% of metastatic patients respond and many recur over time. A primary reason for failure is adaptive resistance (Sharma et al., 2017), which occurs when tumors that initially respond become resistant for various reasons; among which are reduced antigen presentation or exhaustion of cytotoxic T cell function or both (Nowicki et al., 2018). Therefore, understanding the alterations in antigen presenting cells (APCs) is key to developing approaches to block tumor resistance to immune reactivity.
1.2 Dendritic Cells

Dendritic cells (DCs) are professional antigen presenting cells abundant in the skin and other organs. In the tumor microenvironment (TME), DCs are important for cytotoxic T cell recruitment and function (DeVito et al., 2019). Myeloid cells in general are known to be abundant in the TME of solid cancers (Mellman et al., 2023). Increased infiltration and function of DCs in the TME specifically has been correlated with better patient prognosis (Ma et al., 2013). Particularly in melanoma, DC infiltration is important for the efficacy of immunotherapies, including anti-PD-1 therapy (Wculek et al., 2020).

The significance of DCs, not only in the TME but physiologically arises from their important role as APCs. Antigen presentation is a process by which myeloid cells express exogenous antigens on MHC class I and II molecules leading to activation of adaptive immune cells and stimulate inflammatory responses against the exogenous antigens. In addition, DCs are vital as they also cross present antigens. The main difference being that antigen cross-presentation is the process of loading exogenous antigens on MHC class I molecules after intracellular processing, similar to endogenous antigens. Antigen cross-presentation is especially important in the TME as it is used to prime CD8⁺ T cells (Nierkens et al., 2013).

There are various subsets of DCs mainly based on surface markers. The four main types are plasmacytoid DCs (pDCs), conventional DCs, (cDCs), monocyte derived DCs (moDCs), and Langerhans cells (LCs) (He et al., 2019) (Figure 1) (Krishnaswamy et al., 2018; Satpathy et al., 2012) all of which are CD11c⁺. pDCs primarily release interferons after viral and bacterial exposure, moDCs primarily drive systemic inflammation, and LCs play a role in homeostasis and regulate other immune cell types. Conventional DCs are
further divided into type 1 (cDC1s) and type 2 (cDC2s). Although both type 1 and type 2 conventional DCs process antigens and drive antibody and T cell responses, they are distinct in terms of which T cells they activate (Krishnaswamy et al., 2018). cDC1s mainly contribute to tumor attack and intracellular pathogens. These cells subsets are strong in cross antigen presentation and activate CD8\(^+\) T cells. cDC2s, on the other hand primarily contribute to extracellular pathogen attack and activate CD4\(^+\) T cells (Wculek et al., 2020).

**Figure 1: DC Lineage and Subsets**

**Figure 1** displays DC differentiation from the common myeloid progenitor (CMP) to Macrophage and DC progenitor (MDP) to common DC progenitor (CDP), and monocytes. The differentiation of monocytes is with the help of GM-CSF and IL-4 ((Krishnaswamy et al., 2018), while the differentiation to CDP is through IRF8 (Satpathy
et al., 2012). The specific growth and transcription factors each DC subset depend on are as follows; (cDC1s: FLT3L, GM-CSF, BATF3, IRF8, BCL-6, ID2, ZBTB46, NFIL3), (cDC2s: FLT3L, GM-CSF, IRF4, ID2, RBPJ, NOTCH2, KLF4, ZBTB46), (pDCs: E2-2, IRF7), and (moDCs: CSF1R, GM-CSF, IL-4, KLF4, MAFB) (Wculek et al., 2020). Furthermore, each DC subset commonly produces different cytokines. cDC1s secrete IL-12, IL-6 and type III IFNs. cDC2s secrete IL-6, TNFα, and IL-23. pDCs are known to secrete IFNs, both type I and type II, IL-12, and IL-6. moDCs secrete TNFα, IL-12, IL-23, and iNOS. And finally, LCs secrete IL-23, IL-6, and IL-1β (He et al., 2019). This allows for their specific roles in inflammation mentioned above.

The role DCs play in T cell priming, activation, and overall inflammatory response makes them a good target for immunotherapy. This study does not specifically focus on one DC subtype over the other, but rather the role of DCs in the TME. Specifically, how tumor cells can cause tolerization of DCs. In addition, besides tumor cells, DCs are known to express PD-L1 in the TME, demonstrating the vital role they play for immunotherapies as PD-L1 is the ligand of PD-1 that inhibits T cell activation.

1.3 DC Metabolism and Tolerization

The crucial immunostimulatory role of DCs can be suppressed during tumor evolution, thereby reducing anti-PD-1 efficacy, and allowing for the development of metastasis (Brombacher and Everts, 2020; DeVito et al., 2019). There are various ways cancer cells can suppress DCs. Some of which include inhibition of DC differentiation and activation, TME exclusion, reduction of DC viability, impaired handling of tumor associated antigen, and many more (Wculek et al., 2020). This study focuses on one of
the ways the harsh TME limits inflammatory responses and DC function which is by causing DC metabolic stress (Lee et al., 2020).

DCs exhibit different metabolic pathways depending on activation and maturation states. Activated DCs rely on glycolysis (Biswas, 2015) helping to initiate T cell responses (Patente et al., 2019). In contrast, DCs exhibiting tolerogenic properties, similar to immature DCs (Manicassamy and Pulendran, 2011), display oxidative phosphorylation (Malinarich et al., 2015; Sim et al., 2016). Glycolysis is a process by which glucose is converted into pyruvate in the cytoplasm. This results in rapid energy production. The product, pyruvate can also enter into the Krebs cycle in the mitochondria, which is not a rapid process, but results in greater energy production (He et al., 2019). Tumors are known to favor glycolysis to meet their metabolic needs due to their rapid proliferation, energy expenditure, and the hypoxic TME. This creates a nutrient deficient environment for immune cells in the TME as activated immune cells and tumor cells prefer glycolysis (Moller 2021, O’Sullivan 2019, Buck 2017, Biswas 2015). Therefore, DCs present in the TME are known to have impaired inflammatory response as they block adaptive immunity (Tran Janco et al., 2015).

DC tolerogenic properties have been linked to increased fatty acid oxidation (FAO) (Patente et al., 2019). FAO occurs in the mitochondria, and it is a process by which fatty acids are converted to acyl-CoA by the acyl-CoA synthase enzyme. Acyl-CoA then enters the mitochondria with the help of carnitine palmitoyl- transferase I (CPT I). The resulting acyl-CoA undergoes β-oxidation to produce acetyl-CoA (Sun et al., 2021). Acetyl-CoA then feeds into the Krebs cycle where resulting in oxidative phosphorylation (Figure 2).
Figure 2: FAO and the TCA/Krebs Cycle

Tumor-induced shifts in DC metabolism from glycolysis to FAO results in reduced T cell activation (Zhao et al., 2018), reduced DC antigen cross presentation, and secretion of proinflammatory cytokines (Wculek et al., 2019). Furthermore, the inability of these DCs to efficiently present antigens not only occurs in the tumors but also in the tumor-draining lymph node, further resulting in immunosuppression (Ma et al., 2013). Blocking the increase in FAO and in turn tolerogenic DC functions not only increases immune attack on tumors, but it also increases anti-PD-1 efficacy in melanoma (Zhao et al., 2018), which makes the tackling metabolic reprogramming of DCs crucial. The nomenclature for tolerogenic DCs varies, in this study we emphasize the following properties: a.) impaired CD8+ T cell activation (DeVito et al., 2019; Liu et al., 2009; Norian et al., 2009; Scarlett et
al., 2012), b.) increased T regulatory cell expansion (Dumitriu et al., 2009; Holtzhausen et al., 2015; Kerdidani et al., 2019; Shen et al., 2014), c.) increased FAO (Patente et al., 2019; Zhao et al., 2018), d.) reduced antigen presentation (Svajger and Rozman, 2014), and e.) increased anti-inflammatory properties (DeVito et al., 2019).

To understand metabolic reprogramming and therefore tolerance of DCs, it is important to explore receptors that mediate tolerogenic properties of DCs. One such receptor family are the TAM (Tyro3, Axl, MerTK) receptor tyrosine kinases (RTKs), as they have been shown to disrupt DC activation (Manicassamy and Pulendran, 2011). Importantly, in vitro programming of DCs using dexamethasone (known to generate tolerance) resulted in increased MerTK expression (Li et al., 2023). Therefore, it is significant to study the role of these RTKs in DC metabolism and subsequent tolerance.
1.4 TAM Receptors

TAM receptors are receptor tyrosine kinases expressed in various cell types (Burstyn-Cohen and Maimon, 2019; Graham et al., 2014). Furthermore the majority of the cells express at least two of the TAM receptors and expression of these receptors remains high in adult tissues. These RTKs were some of the last to be discovered and mice with genetic deleitons genetic deleitons of individual TAM RTKs helped understand their function. Deletion of MerTK (Camenisch et al., 1999) was the first to show that MerTK loss produced a hyperinflamatory state. The triple knockout of all three was dramatically hyperinflamatory as well as inferte (Lu et al., 1999; Lu and Lemke, 2001) resulting from a degenerative phenotypes with significant autoimmunity and organ defects (Graham et al., 2014; Lemke and Rothlin, 2008) due to increased DC, macrophage, B cell, and T cell activation (DeRyckere et al., 2023).

The TAM receptors were one of the last receptor tyrosine kinases to be discovered. Axl (UFO) was cloned initially using transfected NIH3T3 cells – fibroblast cell line (Janssen et al., 1991). MerTK, on the other hand, was cloned using phosphotyrosine antibodies in human B-lymphoblastoid Agtl 1 expression library (Graham et al., 1994). Tyro3 (Sky) was cloned from chick erythroblastosis virus oncogene v-sea (Ohashi et al., 1994). The cloning reveled structural similarities grouping them as “TAM” receptor tyrosine kinases.
TAMs are composed of two immunoglobulin domains and two fibronectin type III repeats in tandem, and they signal as dimers (Lemke, 2013; Lemke and Rothlin, 2008).

The TAM receptors are activated by specific ligands, namely Protein S (Pros1) and Growth Arrest Specific 6 (GAS6) that share some structural similarities. Although TAM receptors are similar in structure and share a KWIAIES sequence (Linger et al., 2008) in their kinase domain, there are some differences among these receptors. Namely, while all TAM receptors bind to GAS6, Axl has a stronger affinity, as GAS6 can bind to two separate Axl domains. In addition, only MerTK and Tyro3 bind to Pros1 because of Axl's structural alterations in its extracellular domain (Lew et al., 2014; Tsou et al., 2014). Homodimerization and activation of these receptors leads to downstream signaling involving various pathways including MEK-ERK, p38, and PI3K-AKT pathways (Graham et al., 2014). Both Pros1 and GAS6 bind more favorably to PtdSer compared to phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol. PtdSer binds to the amino-terminus GLA domain of Pros1 and GAS6 in a calcium-dependent manner (Anderson et al., 2003) and the carboxy-terminal domain of Pros1 and GAS6 binds to TAMs for activation (Trevor N. Stitt et al., 1995).

Although there is an abundant presence of PtdSer, TAM activation is initiated by PtdSer on exosomes, PtdSer exposed on apoptotic dead cells (referred to as dead cells in this study) to TAM receptors (Lemke, 2017; Lemke and Rothlin, 2008; Scott et al., 2001) triggers dead cell clearance (Graham et al., 2014; Peeters et al., 2019). Scott et al discovered the importance of MerTK for phagocytosis leading the vast study of efferocytosis. Efferocytosis is the clearance of apoptotic cells and is mainly carried out by myeloid and epithelial cells (Scott et al., 2001; Seitz et al., 2007). This process is very
important for homeostasis since the presence of apoptotic cells can cause tissue toxicity and damage (Burstyn-Cohen and Maimon, 2019). TAM activation and initiation of efferocytosis results in cytoskeletal reorganization that allows for apoptotic cell engulfment (Mao and Finnemann, 2015). Furthermore, efferocytosis is an anti-inflammatory process; therefore, during this process TAMs regulate cytokine production and favor the release of anti-inflammatory cytokines.

TAM receptors also have other physiologic roles including the control of TLR signaling to down-regulate inflammatory cytokines, dampening of NK activation, regulation of T cell proliferation and B cell activation (DeRyckere et al., 2023), all of which result in anti-inflammatory environments. Therefore, in the TME, tumors can take advantage of this physiological immune cell suppression to evade immune cell attack.

Tumor cells can express TAM receptors as well as their ligands, and although TAMs are not significant oncogenic drivers (Graham et al., 2014), TAM receptors are involved in survival signaling through the PI3K-AKT pathway, apoptosis suppression, cancer cell self-renewal, inhibition of growth suppressors, angiogenesis, and senescence evasion (DeRyckere et al., 2023). Extensive studies have shown that increased expression and/or activation of TAM receptors by tumor cells provide a cell intrinsic survival signal adding to resistance to chemotherapy and immunotherapy (DeRyckere et al., 2023; Graham et al., 2014). Moreover, TAM expression in various cancers has been related to poor prognosis (DeRyckere et al., 2023). In melanoma, specifically, Axl and MerTK expression was correlated with expression of genes that favor tumor migration and proliferation which can contribute to metastasis as well as chemoresistance (Dumas et al., 2021; Linger et al., 2013; Sinha et al., 2021). Importantly, TAM expression has been
correlated with immune checkpoint inhibitors in various cancers, including melanoma (Hugo et al., 2016; Jiang et al., 2021).

Although TAM receptors are implicated to be pro-tumoral in various aspects, each of these receptors can have varying functions in different cancers (DeRyckere et al., 2023). Furthermore, TAM receptor tyrosine kinase signaling polarizes innate immune cells toward an anti-inflammatory, tissue reparative response (Graham et al., 2014). TAM receptors have been shown to promote an anti-inflammatory response by inhibiting production of TNF, IL-6, IL-12, and IFNs (Li et al., 2023). In addition, small molecule inhibition of TAM receptors has been shown to increase T cell activation through increase in tumor antigen cross-presentation (Jeon et al., 2022).

Physiologic activation of the TAM receptors leads to homeostatic regulation of the inflammatory function of APCs (Burstyn-Cohen and Maimon, 2019) as humans ingest > 5 billion dead cells per day. This process helps prevent autoimmunity (Carrera Silva et al., 2013; Wallet et al., 2009). There are many PtdSer sources in the TME, some of which include apoptotic cells, tumor-associated endothelial cells, and tumor-derived exosomes (Burstyn-Cohen and Maimon, 2019). This study focuses on PtdSer on dead cells, which are plentiful along with the TAM ligands in the TME. As both tumors and myeloid cells in the TME express TAM receptors, the abundant presence of PtdSer allows for subversion of the normal physiological system and adds to the immunosuppression found in the TME.

Early studies from our group showed reduced growth of tumors in mice deficient in MerTK expression (merkt−/−) that was dependent on CD8+ T cell reactivity (Cook et al., 2013). Our previous work also demonstrated that MerTK+ myeloid-derived suppressor cells (MDSCs) block immune-mediated rejection of syngeneic melanoma models, and
that patients with metastatic melanoma exhibit increased MerTK+ MDSCs in blood (Holtzhausen et al., 2019). We also demonstrated that melanoma cells secrete Protein S (Pros1) again adding to the immunosuppressive TME in a MerTK-dependent manner (Ubil et al., 2018).

DCs also express TAM receptors, and those are expressed at higher levels by tolerized DCs (Giroud et al., 2020; Yi et al., 2009). TAM activation in DCs and other phagocytes favors immunosuppression due to the release of cytokines, chemokines, and mediators that favor an anti-inflammatory response (DeRyckere et al., 2023). Ingestion of dead cells leads to upregulation of PD-L1 (Lee-Sherick et al., 2018) where ingestion through MerTK is most effective in increasing PD-L1 compared to other TAMs (Kasikara et al., 2017). In addition, DCs with tolerogenic phenotypes that are responsible for reduction of T cell responses have been shown to be MerTK dependent in neonatal lungs (Silva-Sanchez et al., 2023). Furthermore, inhibition of MerTK has been shown to favor activation of DCs and T cell stimulation demonstrating its role in DC tolerogenic properties (Wallet et al., 2008). Dead cells hinder DC activation and establish a tolerogenic function (Yi et al., 2009). MerTK induces metabolic reprogramming of immune cells (Peeters et al., 2019). However, the role of MerTK on DC metabolism and tolerization remains to be studied.

TAM receptors have been well studied and their vital role in the TME has been shown in different cancers. Consequently, there are various Inhibitors targeting TAMs in approved for different cancers as well as in different phases of the clinical trial process. Some of these are kinase inhibitors targeting multiple receptors, TAM- selective kinase inhibitors, monoclonal antibodies, decoy receptor fusion proteins and many more.
(DeRyckere et al., 2023). Specifically, there are in house small molecule inhibitors made possible with the collaboration of UNC’s Stephen V Frye, PhD. and Xiaodong Wang PhD. such as UNC569 (Christoph et al., 2013) and UNC2025 (Zhang et al., 2014) which have shown potential for leukemia preclinically and UNC1062 (Liu et al., 2013) for metastatic melanoma (Graham et al., 2014). This study uses the small molecule inhibitor, UNC4241, which is specific for MerTK and Tyro3. UNC4241 (trans-4-((2-((4-Fluorophenyl)amino)-5-(5-(pyrrolidin-1-ylmethyl) pyridin-2-yl) pyrimidin-4-yl)amino)cyclohexan-1-ol) was developed, similar to the above listed inhibitors, at UNC Chapel Hill (Holtzhausen et al., 2019).

To understand the relationship between DC metabolic reprogramming and inducing a tolerogenic phenotype in the TME, we conducted a series of in vitro as well as in vivo experiments using genetic and pharmacological approaches to inhibit MerTK. We demonstrate that resistance of a melanoma model to anti-PD-1 therapy correlates with increased MerTK+ DCs, and that MerTK activation by dead melanoma cells stimulates a metabolic switch via activation of STAT3 serine phosphorylation and induction of a key enzyme in FAO, a metabolic switch characteristic of tolerogenic DCs. Treatment of MerTK-replete but not MerTK-deficient DCs with dead cells suppressed T cell proliferation in vitro. BRAFV600E pten−/− melanoma mouse tumors (GEMM6) exhibit reduced growth and enhanced anti-PD-1 efficacy in mice completely lacking MerTK expression and mice in which MerTK-deficiency is specifically targeted to the DC compartment in CRISPR mertkfl/fl B6 mice. Together these findings indicate that targeting MerTK function in DCs is important for reversing the resistance of melanoma tumors to immunotherapy.
METHODS & MATERIALS

2.1 Mice

The original mertk\(^{-/-}\), axl\(^{-/-}\), and tyro3\(^{-/-}\) mice with 129/Sv background (Camenisch et al., 1999) were crossed with C57BL/6 for multiple generations to obtain mertk\(^{-/-}\), axl\(^{-/-}\), and tyro3\(^{-/-}\) mice with C57BL/6 background. C57BL/6J (strain # 000664.), OT-I (003831), OT-II (004194) and CD11c-cre (008068) mice were purchased from The Jackson Labs. LyzM-cre mice (004781) were purchased from The Jackson Labs. LyzM-mertk\(^{-/-}\) were derived in our lab by crossing with Flox MerTK mice (Ubil et al., 2018). CRISPR mertk\(^{fl/fl}\) B6 mice were derived in our lab and crossed with CD11c-cre mice to obtain CD11c-mertk\(^{-/-}\) mice. Mice were genotyped by Transnetyx. Animal studies approved by IACUC (Protocol # 21-089).

2.2 Mass Cytometry

2.2.1 Mass Cytometry Staining

For custom-conjugated antibodies (Table 1), 100 µg of antibody was coupled to Maxpar X8 metal-labeled polymer according to the manufacturer’s protocol (Fluidigm). After conjugation, the metal-labeled antibodies were diluted in Antibody Stabilizer PBS (Candor Bioscience) for long-term storage.
<table>
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<th>Isotype</th>
<th>Antibody Source</th>
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**Table 1:** Antibody clones and sources
After tumor dissociation and RBC lysis as described below, 3x10^6 cells per sample were transferred to 5mL round bottom tubes. Cells were incubated with 300 µL Cell-ID Cisplatin-195PT (Fluidigm) diluted 1:4000 in PBS (Rockland) for 5 min at room temperature (RT), then washed twice in Maxpar Cell Staining Buffer (CSB) (Fluidigm). 50 µL (1:100 dilution) FcR Blocking Reagent (BD Pharmingen) was added to samples and incubated for 10 min at RT before 50 µL extracellular antibody cocktail was added and incubated for 30 min at RT. Cells were then washed twice with CSB and then fixed and permeabilized with eBioscience FoxP3 Transcription Factor Fixation and Permeabilization Buffer for 1 hr at RT, followed by two washes with permeabilization buffer (eBiosciences). 50 µL intracellular antibody cocktail in permeabilization buffer was added and incubated for 30 min at RT, followed by two washes in permeabilization buffer. Cells were fixed in 1.6% methanol-free PFA (Poly Sciences Inc, 18814-10) diluted with Maxpar PBS (Fluidigm) for 1 hr at 4°C. Samples were incubated overnight in Maxpar Fix and Perm Buffer (Fluidigm) with 62.5 nM Cell-ID Intercalator (Fluidigm) containing 191IR and 193IR. Before collection, cells were washed once with CSB, once with Cell Acquisition Solution (CAS) (Fluidigm), passed through a 40µm filter and diluted in CAS containing 10% EQ Calibration Beads (Fluidigm) at 6x10^5 cells/mL before acquisition on a mass cytometer (Helios).

2.2.2 CyTOF Data Analysis

Mass cytometry data were normalized using Fluidigm CyTOF software (v7.0). Individual samples were gated in Cytobank to exclude beads, debris, dead cells, and doublets for further analysis.
2.3 DC Preparation

DCs were isolated from both male and female C57BL/6 mice and Axl<sup>−/−</sup>, MerTK<sup>−/−</sup> as well as Tyro3<sup>−/−</sup> mice with C57BL/6 background ages between 8-12 weeks. Bone marrow was harvested and incubated for 6 days in 37°C with 5% CO<sub>2</sub> in RPMI with 10% FBS, containing 20ng/mL murine GM-CSF and 10ng/mL murine IL-4. Media was changed every 2 days. The resulting bone marrow derived DCs (CD11c cells) were purified using EasySep Dead Cell Removal Kit (STEMCELL Technologies, 17899) and EasySep Mouse CD11c Positive Selection Kit (STEMCELL Technologies, 18780) to isolate CD11c<sup>+</sup> cells, according to the manufacturers’ protocol. DCs prepared from spleen cell suspensions were similarly purified.

2.4 Macrophage and MDSC Preparation

Macrophages and MDSCs were isolated from both male and female C57BL/6 mice and MerTK<sup>−/−</sup> mice with C57BL/6 background ages between 8-12 weeks. Bone marrow was harvested and incubated for 6 days in 37°C with 5% CO<sub>2</sub> in RPMI with 10% FBS, containing 40ng/mL murine M-CSF (Macrophages) or 40 ng/mL GM-CSF, 40ng/mL G-CSF and 40 ng/mL IL6 (MDSCs). Media was changed every 2 days. The resulting bone marrow derived Macrophages or MDSCs (CD11b cells) were purified using EasySep Dead Cell Removal Kit (STEMCELL Technologies, 17899) and EasySep Mouse CD11b Positive Selection Kit (STEMCELL Technologies, 18970) to isolate CD11b<sup>+</sup> cells, according to the manufacturers’ protocol.
2.5 Tumor Cell Lines

GEMM6 melanoma cells (BRAF\textsuperscript{V600E}PTEN\textsuperscript{-/-}) (Holtzhausen et al., 2019), were also engineered to express ovalbumin (Ova; GEMM6-OVA) (Theivanthiran et al., 2020). Cells were not authenticated in the past year. YUMM1.7 cells were obtained from ATCC (CRL-3362). Cell lines were cultured for 2 weeks before each tumor study and passages were kept below 10. Cells were tested for mycoplasma using the Universal Mycoplasma Detection Kit (ATCC, 30-1012K) monthly, and treated with plasmocin (Invivogen, antimpp) as a preventative measure. Cells were not authenticated

2.6 Dead GEMM6 Cell Preparation

Two approaches were used to induce apoptosis in GEMM6 cells. First GEMM6 cells were treated with 50μM staurosporine (Selleckchem, S1421) in DMEM with 10% FBS for 72 hours and then washed 2 times with PBS.

Secondly, GEMM6-OVA cells were incubated with Ova-specific CD8\textsuperscript{+} T cells prepared from the spleen of B6 mice transgenic for the OT-I clonotypic T cell receptor (TCR; C57BL/6-Tg (TcraTcrb)1100Mjb/J). OT-I CD8\textsuperscript{+} T cells were isolated using EasySep Mouse CD8\textsuperscript{+} T cell Isolation Kit (STEMCELL Technologies, 19853) according to the manufacturer’s protocol. Isolated CD8\textsuperscript{+} T cells are then treated with 1mg/mL Ova peptide (SINFEKKL; Sigma S7951) overnight. GEMM6-OVA cells are then co-incubated with stimulated OT-I CD8\textsuperscript{+} T cells at a 1:5 ratio respectively for 5 days, changing media (DMEM with 10% FBS) every two days. The resulting dead GEMM6-OVA cells were then isolated using EasySep Dead Cell Removal Kit (STEMCELL Technologies, 17899)
according to the manufacturer’s protocol, verified as dead by flow cytometry and used to treat CD11c⁺ DCs.

2.7 NanoString: CD11c⁺ DCs

Wt and mertk⁻/⁻ CD11c⁺ DCs were treated with dead GEMM6 cells or left untreated. After culturing, DCs were purified and total RNA prepared using Promega Maxwell 16 MDx (Cat AS3000) and Promega Maxwell 16 LEV simply RNA Cells Kit (Cat AS1270), following TM351 protocol. Total RNA quantity control was done using Thermo Scientific Qubit Flex Fluorometer (Cat Q33327) and Invitrogen Qubit RNA broad range assay kit (Cat Q10211), following MAN0001987 protocol. Total RNA quality was done using Agilent TapeStation 4200 (Cat G2991AA) Agilent RNA ScreenTape Analysis (Cat 5067-5576), following G2991-90020 protocol. NanoString assay was conducted using 50 ng * (100/%DV200) RNA in NanoString nCounter MAX Analysis System (Cat NCT-SYST-LS). The codesets were: nCounter Mouse Myeloid Innate Immunity Panel v2 (Cat# XT-CSO-MMII2-12).

2.8 NanoString: Tumors

Tumors were embedded and kept in 4% formalin for 48 hours then transferred to 70% ethanol for transferring to the NanoString core.

Nucleic acid extraction of total RNA was done using Thermo Scientific KingFisher Flex (Cat 5400610) and Applied Biosystems MagMAX FFPE DNA/RNA Ultra Kit (Cat A31881), following MAN0015906 protocol. Total RNA quantity control was done using
Thermo Scientific Qubit Flex Fluorometer (Cat Q33327) and Invitrogen Qubit RNA broad range assay kit (Cat Q10211), following MAN0001987 protocol. Total RNA quality was done using Agilent TapeStation 4200 (Cat G2991AA) Agilent RNA ScreenTape Analysis (Cat 5067-5576), following G2991-90020 protocol. NanoString assay was conducted using 50 ng * (100/%DV200) RNA in NanoString nCounter MAX Analysis System (Cat NCT-SYST-LS). The codesets were: nCounter Mouse Myeloid Innate Immunity Panel v2 (Cat# XT-CSO-MMII2-12).

Results obtained are determined according to NanoString panels. Pathway score refers to a list of genes involved in a specific pathway in a single score. Cell score refers to the expression of genes involved in identifying a specific cell type.

2.9 Seahorse Assay – Glycolysis Stress Test

CD11c+ DCs (5x10^5/well) were plated in seahorse cell culture plates and incubated for 24 hours in 37°C with 5% CO2. After 24-hours, the plates were then centrifuged, washed one time, and plated with Seahorse XF Base medium with 2 mM glutamine for 1-hour in 37°C without CO2. 100 mM glucose solution, 10 μM oligomycin, and 500 mM 2-DG were loaded in a hydrated cartridge, that was used to calibrate the Seahorse Xfe24 analyzer (Agilent). Extracellular acidification rates by DCs cells were then measured.

2.10 Seahorse Assay – Mitochondrial Stress Test

CD11c+ DCs (5x10^5/well) were plated in seahorse cell culture plates and incubated for 24 hours in 37°C with 5% CO2. The plates were then centrifuged, washed one times,
and plated with Seahorse XF DMEM with 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose for 1-hour at 37°C without CO₂. 15 μM oligomycin, 15 μM FCCP, and 5 μM Rot/AA solution were loaded in a hydrated cartridge, that was used to calibrate the Seahorse Xfe24 analyzer. Oxygen consumption rates by DCs was then measured. For experiments with drug injections, 4 μM Etomoxir or media were used before oligomycin injection.

2.11 CD8⁺ T cell Proliferation Assay

CD11c⁺ DCs (5x10⁵/well) were plated in 24 well plates plus/minus dead GEMM6 cells (2x10⁶/well) and 1mg/mL Ova 257-264 peptide (Sigma, S7951) overnight. DCs were then isolated via CD11c Positive Selection Kit (STEMCELL Technologies, 18780). Purified DCs (5x10⁴) were plated in 96 well plates with naïve CD8⁺ T cells (2x10⁵/well) isolated from the spleen of OT-I mice using EasyStep Mouse Naïve CD8⁺ T cell isolation kit (STEMCELL Technologies, 19858), plus/minus 1mg/mL Ova peptide for 48 hours. Prior to culture OT-I cells were stained with CFSE. Proliferation of OT-I cells, based on CFSE dilution, was measured by flow cytometry and CFSE dilution.

Same method was used to isolate CD11c⁺ cells from GEMM6-OVA tumor bearing mice and were co-incubated with OT-I naïve CD8⁺ T cells. CD11c⁺ cells were not pre-treated with ova peptide as they were isolated from GEMM6-OVA tumors.
2.12 Treg Differentiation Assay

CD11c$^+$ DCs (5x10$^5$/well) were plated in 24 well plates plus/minus dead GEMM6 cells (2x10$^6$/well) and 1mg/mL Ova 323-339 peptide (Sigma, O1641) overnight. DCs were then isolated via CD11c Positive Selection Kit (STEMCELL Technologies, 18780). Purified DCs (5x10$^4$) were plated in 96 well plates treated with 200pg/ml TGF-β1 protein (R&D Systems, 7666-MB) and co-incubated with naïve CD4$^+$ T cells (2x10$^5$/well) isolated from the spleen of OT-II mice using EasyStep Mouse Naïve CD4$^+$ T cell isolation kit (STEMCELL Technologies, 19765), plus/minus 1mg/mL Ova peptide for 5 days. Differentiation was then measured by flow cytometry.

The same method was used to isolate CD11c$^+$ cells from GEMM6-OVA tumor bearing mice and were co-incubated with OT-II naïve CD4$^+$ T cells. CD11c$^+$ cells were not pre-treated with Ova peptide as they were isolated from GEMM6-OVA tumors.

2.13 Flow Cytometry

Antibodies: anti-CD103 (BioLegend Cat# 121414), -CD11c (BioLegend Cat# 117311), -CD11b (BioLegend Cat# 101225), -CD24 (BioLegend Cat# 108105), -CD272 (BioLegend Cat# 139107), -CD3 (BioLegend Cat# 100227), -CD64 (BioLegend Cat# 305017), -CD8 (BioLegend Cat# 100708), -CD4 (BioLegend Cat# 116004), -FoxP3 (BioLegend Cat# 126403), -F4/80 (BioLegend Cat# 157308), -Axl (Thermo Fisher Scientific Cat# 12-1087-42), -Tyro3 (R&D Systems Cat# FAB759V), -MerTK (Thermo Fisher Scientific Cat# 56-5751-82), -MHCII (BD Biosciences Cat# 563414), Live/Dead (Thermo Fisher Scientific Cat# L34976), CFSE (Life Technologies Cat# C34554) were
purchased and stained cells fixed with 4% formaldehyde were ran on an Attune NXT or BD Fortessa and analyzed using FlowJo software. Dead cells were excluded in the analysis by staining with Live/Dead purchased from Life Technologies. For Annexin V staining, cells were resuspended Annexin V Binding Buffer (BioLegend, Cat# 422201) plus FITC-Annexin V (BioLegend, Cat# 640905) and PI (BioLegend, Cat# 421301) and incubated for 15 minutes. Cells are then washed with Annexin V Binding Buffer and analyzed as above.

2.14 RT-PCR

RNA from cells was purified using the RNeasy Plus Mini Kit (Qiagen, 74136) and reverse transcribed to cDNA using SuperScript IV VILO Master Mix (Thermo Fisher, 11756050). PowerUp SYBER Green Master Mix (Thermo Fisher, A25742) or TaqMan Fast Advanced Master Mix (Thermo Fisher, 4444557) were used for q-PCR depending on the primers. RT-PCR was then performed using Bio-Rad RT-PCR system. Taqman probes used: GAPDH (Mm99999915_g1), MerTK (Mm00434920_m1), Axl (Mm00437221_m1), Tyro3 (Mm00444547_m1).
2.15 Primers

*Actinβ* Forward: 5' GGCTGTATTCCCCCTCCATCG 3'
Reverse: 5' CCAGTTGGTAACAAATGCCATGT 3'
*Cpt1a* Forward: 5' CTCAGTTGGAGCGACTCTTCA 3'
Reverse: 5' GGCCTCTGTGGTACACGACAA 3'
*Lxrβ* Forward: 5' ATGTCTTCCCCCACAAGTTCT 3'
Reverse: 5' GACCACGATGTAGGCAGAC 3'
*Tnfa* Forward: 5' AGGAGGAGTCTGCGAAGAAGA 3'
Reverse: 5' GGCAGTGGAACCACATAACTCG 3'
*Tgfβ1* Forward: 5' AGACCACATCAGCATTGAGTG 3'
Reverse: 5' GGTGGCAACGAATGTAGCTGT 3'
*Infδ* Forward: 5' TCTCCAGAAACCCTCCTGCTG 3'
Reverse: 5' TCAGCGGATTCATCTGCTTCG 3'
*Il12* Forward: 5' CGTGCTCATGGCTGGTGCAAAG 3'
Reverse: 5' GAACACATGCCCACTTGCTG 3'
*Ido1* Forward: 5' CAGGCCAGAGCAGCATCTTC 3'
Reverse: 5' GCCAGCCTCGTTTTATTTCC 3'
*tgfβ* Forward: 5' CTCCCCTGGGCTTCTAGTGC 3'
Reverse: 5' GCCTTAGTTTGACAGGATCTG 3'
2.16 Western Blotting

Cells were lysed using Laemmli Sample Buffer 2x (Bio Rad, 1610731) and run on SDS-PAGE, transferred to a PVDF membrane, and incubated overnight with a primary antibody at 4°C on a shaker. Blots were then developed using ECL (Pierce, 34095) and imaging was carried out using a Bio-Rad ChemiDoc XRS+ Imaging System for analyses. The same blot was used for each experiment. Antibodies were stripped using a stripping buffer (Thermo Scientific, 21059).

Immunoprecipitation of MerTK was done by first treating cells with PerVanadate for 15 minutes (20mM Sodium Orthovanadate (Sigma, 567540) and 0.3% Hydrogen Peroxide (Sigma, H1009)) then lysed using a lysis buffer (1% NP-40 (Thermo Fisher, 85124)) 50mM Tris-HCl pH7.5 (BP1756-100), 150mM NaCl (Sigma, S9888), 0.25%deoxycholate (Sigma, 89904), 1mM EDTA (Sigma, E9884), 1mM EGTA (Fisher Scientific, 41-005-0gm), 5% Glycerol (Sigma, G5516), and 20mM Beta-Glycerophosphate (Sigma, G5422)). Lysed cells are then incubated with 25µL Protein A Agarose Beads (Santa Cruz, sc-2001) and 5µL N14 MerTK antibody (Rockland Scientific) at 4°C tumbling overnight. Beads were then washed 3 times before adding 25µL Sample Buffer (Bio-Rad, 161073). The samples were then run on SDS-PAGE and Western blotting was carried out as described.

Antibodies: anti-STAT3 (Cell Signaling Technology Cat# 9139), -phospho-STAT3 serine (Cell Signaling Technology Cat# 9136), -phospho-STAT3 tyrosine (Cell Signaling Technology Cat# 9145), -p38 (Cell Signaling Technology Cat# 9212), phospho-p38 (Cell Signaling Technology Cat# 9211), -β-actin (Cell Signaling Technology Cat# 58169), -p-Tyr (Cell Signaling Technology Cat# 9411).
2.17 Seahorse Study in DCs Isolated from Tumor Bearing Mice

Eight to 12-week-old wt and Mertk\(^{-/-}\) mice received GEMM6 tumor via intraperitoneal (i.p.) injection and then were treated with either 250\(\mu\)g anti-IgG (Bio X Cell, BE0093) or 250\(\mu\)g anti-PD-1 (Bio X Cell, BE0146) every 3 days once tumors were palpable. Once tumors reached 1\(\text{cm}^3\) volume, spleens were harvested and CD11c\(^+\) DCs isolated to conduct Seahorse mitochondrial stress test assay. Serum samples were also obtained.

2.18 Meso Scale Discovery (MSD)

Terminal blood samples were collected from GEMM6 tumor bearing wt and mertk\(^{-/-}\) mice were treated with either 250\(\mu\)g anti-IgG or 250\(\mu\)g anti-PD-1. Serum cytokine levels were measured using a U-PLEX TH1/TH2 Combo (MSD, K15071K-1) kit according to the manufacturer’s protocol.

2.19 Tumor Studies

Eight to 12-week-old LyzM-cre and LyzM-mertk\(^{-/-}\) mice were injected (subcutaneous injection - s.q.) with GEMM6 tumor cells. Once tumors were palpable, mice were treated with either 250\(\mu\)g anti-IgG (Bio X Cell, BE0093) or 250\(\mu\)g anti-PD-1 (Bio X Cell, BE0146) every 3 days.

Eight to 12-week-old CD11c-cre and CD11c-mertk\(^{-/-}\) mice were injected s.q. with YUMM1.7. Once tumors were palpable, mice were treated with either 250\(\mu\)g anti-IgG (Bio X Cell, BE0093) or 250\(\mu\)g anti-PD-1 (Bio X Cell, BE0146) every 3 days.
2.20 GEMM6-OVA Tetramer Staining

Eight to 12-week-old CD11c-cre and CD11c-mertk<sup>Δ</sup> mice received GEMM6-OVA (GEMM6 cell line expressing full length ovalbumin - Gift from the Hanks lab, Duke University (Theivanthiran et al., 2020)) tumor via s.q. and then were treated with either 250μg anti-IgG (Bio X Cell, BE0093) or 250μg anti-PD-1 (Bio X Cell, BE0146) every 3 days once tumors were palpable. Lymph nodes were harvested, and a single cell suspension was created by pushing nodes through a cell strainer. Cells were stained with CT-CD8a antibody to block non-specific binding (ThermoFisher MA5-17594) followed by staining with CD8 (clone KT15) (ThermoFisher MA5-16759), Live/Dead fixable Near-IR dye (ThermoFisher L10119), CD45 (BD Biosciences 564279), CD3 (BioLegend 100241), and H-2K(b) SIINFEKL tetramer (NIH Tetramer Core).

2.21 IFN-γ ELiSpot

BMDCs were stimulated with dead cells (1:4 ratio), 300nM UNC4241 or 50μM ETO as indicated and 1μg/ml SIINFEKL peptide overnight. DCs were then washed and plated at 10,000 per well. 40,000 CD8 T cells purified from the spleens of OT-1 mice (C57BL/6-Tg(TcraTcrb)1100Mjb/J Strain # 003831, Jackson Labs) for 3 days. The assay was performed according to manufacturer’s protocol (Mabtech 3321-4HPT-2) and the plate was read and analyzed using an AID EliSpot plate reader.
2.22 Tumor Analyses of CD8 Cell Infiltrate

Eight to 12-week-old CD11c-cre and CD11c-\textit{mertk}\textsuperscript{-/-} mice were injected i.p. with GEMM6 tumor cells. Once tumors were palpable, mice were treated with either saline, 250μg anti-PD-1 (Bio X Cell, BE0146), 100μM Etomoxir (MilliporeSigma, E1905-25mg), or a combination of 250μg anti-PD-1 and 100μM ETO, every 3 days. Tumors upon reaching 1cm\textsuperscript{3} volume, were harvested, fixed in 10% formalin, paraffin embedded, and 5μm sections prepared. Sections were stained with hematoxylin and eosin and digitally imaged with the Aperio AT2 (Leica Biosystems) using 20x objective. Alternatively, sections were examined via immunofluorescence (IF). Here sections were dewaxed in Bond Dewax solution (AR9222) and hydrated in Bond Wash solution (AR9590). Slides were then incubated with a rabbit anti-CD8 antibody (98941, Cell Signaling Technology) and binding detected with an HRP-conjugated goat anti-rabbit secondary antibody (MP-7451, Vector Laboratories). Tyramide Signal Amplification (TSA) system with Cy5 (SAT705A001EA, Akoya Biosciences) was used for visualization. Stained slides were counterstained with Hoechst 33258 (H3569, Invitrogen) and mounted with ProLong Gold antifade reagent (P36930, Life Technologies). Positive and negative controls (no primary antibody) were included. Slides were digitized using the Aperio ScanScope FL (Leica Biosystems). The digital images were captured in each channel by 20x objective (0.468 μm/pixel resolution) using line-scan camera technology. The adjacent 1 mm stripes captured across the entire slide were aligned into a contiguous digital image by an image composer.
2.22.1 Quantitative Image Analysis

Slides were scanned with a Versa whole slide imager (Leica Biosystems) using the 20X objective. A boarded veterinary pathologist completed all quantitative analyses as part of the UNC Pathology Services Core using Definiens Architect XD 64 2.7.0. Tissues were detected using 290,000 um\(^2\) minimum tissue size and 7253 brightness, and 0.4 homogeneity thresholds. Cell nuclei in the DAPI channel were identified using a stain threshold of 0.1 and average size of 38.14 um\(^2\). Partial nuclei were excluded if they were smaller than 10 um\(^2\). Positive CD8 staining thresholds were set at 7353.3, 17528.9, and 25386.7 for low, medium, and brightly staining nuclei, respectively. Data output from the analysis included percent positive cells, H score, and positive cell density. The algorithm was validated using a relative percent positive assessment on randomly selected slides.

2.23 MTT Assay and Reagents

24Hr treated CD11c\(^+\) cells were plated and incubated in 37°C with 5% CO\(_2\) for 3 hours with 50 µL MTT reagent, then with 150 µL MTT solvent for 15 minutes (abcam, ab211091). Absorbance was then read at 590 nm. Reagents: 20 nmol/L Stattic (Selleckchem, S7024), 300 nmol/L UNC4241 (Holtzhausen et al., 2019), 1 µmol/L p38 MAPK Inhibitor XIX Skepinone-L (Calbiochem 506174), 4 µmol/L Etomoxir (MilliporeSigma, E1905-25mg), 50 µmol/L IDO inhibitor Epacadostat (INCB024360) (Selleckchem, S7910).
2.24 Statistical Analysis

GraphPad Prism 9 was used for all statistical analysis. Specific tests used are stated in figure legends. In summary, unpaired t-test is used to analyze *in vitro* data, to compare means of control and treatment groups. For *in vivo* tumor data analysis, a specific week is taken to analyze significance using unpaired t-test. Significance is determined by a $P$ value of less than 0.05.
3.1 Resistance to anti-PD-1 therapy correlates with increased tumor infiltrating MerTK+ DCs

To analyze the effect of anti-PD-1 treatment on TME resistance, we monitored GEMM6 melanoma tumor growth (Holtzhausen et al., 2019), with CyTOF-based immune cell profiling of C57BL/6 (B6) wt tumor bearing mice treated with either anti-PD-1 or IgG isotype control antibody, at harvest times, early (day 7, sensitive) and late (day 21, when tumors were developing resistance) (Zhao et al., 2018) (Figure 3A). Increased MerTK+ DCs were detected in tumors harvested on day 21 from anti-PD-1 compared to other groups (Figure 3B), suggesting an association between MerTK+ DCs and anti-PD-1 treatment resistance. Axl and Tyro3 did not change significantly (Figure 4A), suggesting this effect is specific to MerTK.
Figure 3: **MerTK expression in DCs is increased in Anti-PD-1 resistant GEMM6 tumors. Dead cell treated DCs have reduced inflammatory RNA expression.**

A) GEMM6 tumor bearing mice schematic where mice are treated with either anti-PD-1 or anti-IgG and harvested at Day 7 or Day 21, a time of emerging anti-PD-1 resistance. B) CyTOF analysis of MerTK expression levels of DCs according to harvest time points, where MerTK expression is increased in DCs in TME as anti-PD-1 resistance is observed. The impact of dead cell treatment on DC RNA as shown through (C) Nanostring RNA analysis of genes involved in antigen presentation and interferon signaling of CD11c⁺ cells treated with and without dead GEMM6 cells and (D) Nanostring gene expression analysis of inflammatory cytokines of CD11c⁺ cells treated with and without dead GEMM6 cells, where dead cell treatment favors anti-inflammatory RNA expression. N=3 where all data are replicated independently ± SEM. Unpaired t-test: *P<0.05, **P<0.01, ***P<0.001 each compared to control groups.
Since dead cells (PtdSer externalized) can induce MerTK expression and activation (Birge et al., 2016), and are abundant in the TME, we tested the direct effect of dead cells on the pro-inflammatory properties of purified CD11c\(^+\) wt bone marrow derived DCs (CD11c\(^+\) cells). The purification successfully selected CD11c\(^+\) DCs (**Figure 4B**) which were treated with and without unmanipulated or staurosporine-induced apoptotic GEMM6 cells (referred to as “Dead cells”) for 24 hours. CD11c\(^+\) cells were re-isolated, to make sure GEMM6 RNA did not contribute to the Nanostring immune transcript panel analysis. RNA from dead GEMM6 cell treated CD11c\(^+\) cells exhibited reduced transcripts defining antigen presentation and interferon signaling pathways. The score reflects reduced expression of genes related to antigen presentation and interferon signaling (**Figure 5C**). Specifically, dead cell treatment produced substantial reductions in IL-12, IFN-\(\gamma\), and TNF\(\alpha\) inflammatory cytokines. (**Figure 3D**).
Figure 4: *Axl and Tyro3 expression are not altered on DCs in anti-IgG or anti-PD-1 treated tumors.*

A) CyTOF analysis of Axl and Tyro3 expression levels of DCs according to harvest time points. B) DC population after CD11c^+ cell isolation from WT and MerTK^-/^- mice, determined by flow, to confirm the majority of cells selected with CD11c^+ cell isolation are DCs. N=3 where all data are replicated independently ± SEM. Unpaired t-test: *P<0.05 each compared to control groups.
3.2 TAM receptors modulate DC metabolic reprogramming

To assess if DC metabolism is modulated in a TAM-dependent manner, CD11c+ cells were prepared from wt mice, or B6 mice deficient in the TAM receptors Axl (axl−/−), Tyro3 (tyro3−/−), and MerTK (mertk−/−) (Figure 5A & 5B). Since DC activation is dependent on glycolysis, we first evaluated glycolysis in unmanipulated CD11c+ cells via Seahorse. Absence of individual TAM receptors did not significantly affect the DC glycolytic rate as determined by the extracellular acidification rate (ECAR) (Figure 6A). On the other hand, lack of TAM receptors reduced DC mitochondrial respiration as measured by oxygen consumption rate (OCR) (Figure 6B). MerTK-deficiency resulted in the greatest reduction in OCR compared to CD11c+ cells from axl−/−, tyro3−/−, or wt mice. Similar results were seen with DCs purified from the spleen of the respective B6 lines (Figure 7A & 7B). Baseline glycolysis was similar for each genotype; both mertk−/− and tyro3−/− splenic DCs exhibited significant OCR reduction.
Figure 5: TAM receptor expression on CD11c\(^+\) and CD11b\(^+\) cells.
A) TAM receptor RT-PCR analysis of bone marrow derived wt, axl\(^{-/-}\), mertk\(^{-/-}\), and tyro3\(^{-/-}\) CD11c\(^+\) cells. B) Flow Cytometry analysis of bone marrow derived wt and mertk\(^{-/-}\) CD11c\(^+\) cells. N=3 where all data are replicated independently ± SEM. Unpaired t-test: *P<0.05, **P<0.01, ***P<0.001 each compared to control groups.

To assess oxidative phosphorylation, wt CD11c\(^+\) cells were treated with dead GEMM6 cells which increased OCR in both wt CD11c\(^+\) cells (Figure 6C) and purified wt splenic CD11c\(^+\) cells (Figure 7C). Dead GEMM6 cells alone exhibited no OCR (Figure 7D) and therefore did not contribute to the increase. To determine if the method of killing had an impact, we tried three different ways of killing GEMM6 cells; a). Staurosporine killed with magnetic selection of annexin V\(^+\) cells and washed (DSS), b). Staurosporine
killed without magnetic selection of annexin V+ cells (DSU), and c). T cell killing of tumor cells to mimic in vivo tumor death in the tumor microenvironment (DT). Each method resulted in more than 80% GEMM6 cell death and a similar, significant increase in oxygen consumption rate in dead cell-treated CD11c+ cells (Figure 8A & 8B). GEMM6 killed with staurosporine without annexin V selection were used for subsequent experiments. Addition of the ligands GAS6 and Protein S, to dead cell did not alter the results (Figure 7E) presumably as ligands are already saturated due to serum presence in the media.

Dead GEMM6 cells also significantly reduced DC glycolysis, that was marked by a decreased ECAR. Notably, the effects of dead GEMM6 cells on ECAR were similar to that seen when DCs were treated with 2-deoxy-D-glucose (2-DG), a known glycolysis inhibitor (Figure 7F).

We next explored MerTK effects on DC metabolic reprogramming using CD11c+ cells from wt and mertk−/− mice. Treatment of wt CD11c+ cells with dead GEMM6 cells increased OCR (Figure 6D). While dead GEMM6 cells modestly increased OCR in mertk−/− CD11c+ cells, the effect was significantly reduced compared to wt CD11c+ cells (Figure 6D) suggesting that MerTK plays a regulatory role in DC mitochondrial respiration.

FAO has been linked to DC tolerization (Malinarich et al., 2015; Zhao et al., 2018), and therefore, we explored this pathway using etomoxir (ETO) which inhibits a key enzyme, carnitine palmitoyl transferase I (CPT1a), that allows fatty acid products to enter mitochondrial respiration. ETO lowered OCR for all conditions (Figure 6D) although ETO did not completely abrogate OCR as there are pathways other than FAO contributing to mitochondrial respiration. Interestingly, OCR in mertk−/− CD11c+ cells were even lower with ETO compared to media alone but not completely abrogated, suggesting that neither ETO
inhibition nor MerTK are sole regulators of mitochondrial respiration. Other factors, for example, potentially Tyro3 may play a compensatory role, as seen through their impact on OCR of CD11c⁺ cells (Figure 6B). ETO treatment did lower dead cell induced OCR, indicating that dead cells increase CD11c⁺ cell mitochondrial respiration through increased FAO.
Figure 6: TAM receptors metabolically reprogram DCs to utilize oxidative phosphorylation. 
A) Seahorse glycolysis analysis of wt, axl<sup>-/-</sup>, mertk<sup>-/-</sup>, and tyro3<sup>-/-</sup> CD11c<sup>+</sup> cells, does not show significant changes in glycolysis. 
B) Seahorse mitochondrial respiration analysis of wt, axl<sup>-/-</sup>, mertk<sup>-/-</sup>, and tyro3<sup>-/-</sup> CD11c<sup>+</sup> cells; lack of MerTK causes significant reduction in DC mitochondrial respiration. 
C) Seahorse mitochondrial respiration analysis of wt CD11c<sup>+</sup> cells with and without 24-hour dead GEMM6 cell treatment at a 1:4 ratio CD11c<sup>+</sup> cells to dead GEMM6 cells; dead cell treatment increased DC mitochondrial respiration. 
D) Seahorse mitochondrial respiration analysis of wt and mertk<sup>-/-</sup> CD11c<sup>+</sup> cells with and without ETO as well as 24-hour dead GEMM6 cell treatment at a 1:4 ratio CD11c<sup>+</sup> cells to dead GEMM6 cells; lack of MerTK resulted in similar metabolic effect as inhibition of FAO in DCs. 
N=3 where all data are replicated independently ± SEM. Unpaired t-test: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 each compared to control groups.
Figure 7: TAM receptors and their ligands have varying effects on glycolysis and oxidative phosphorylation.

A) Seahorse glycolysis analysis of spleen derived wt, axl−/−, mertk−/−, and tyro3−/− CD11c+ cells. B) Seahorse mitochondrial respiration analysis of spleen derived wt, axl−/−, mertk−/−, and tyro3−/− CD11c+ cells. C&D) Seahorse mitochondrial respiration analysis of spleen derived, and bone marrow derived wt CD11c+ cells with and without 24-hour dead GEMM6 cell treatment at a 1:4 ratio CD11c+ cells to dead GEMM6 cells showing that dead cells do not contribute to the increase in mitochondrial respiration of DCs. A,B,C) demonstrate the similarity between splenic and bone marrow derived DCs. E) Seahorse mitochondrial respiration of wt CD11c+ cells, 5 µg/mL Pros1 treated wt CD11c+ cells and 200 ng/mL GAS6 treated wt CD11c+ cells, with and without 24-hour dead GEMM6 cell treatment at a 1:1 ratio CD11c+ cells to dead GEMM6 cells, demonstrating ligand saturation. F) Seahorse glycolysis analysis of bone marrow derived wt CD11c+ cells with and without 2-DG as well as 24-hour dead GEMM6 cell treatment at a 1:4 ratio CD11c+ cells to dead GEMM6 cells showing the impact of dead cells on DC glycolysis. N=3 where all data are replicated independently ± SEM. Unpaired t-test: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 each compared to control groups.
Figure 8: The method of tumor cell killing does not affect subsequent downstream assays.
A) Seahorse mitochondrial respiration analysis and comparison of wt CD11c^+ cells with and without 24-hour dead GEMM6 cell treatment (DSS – staurosporine killed Annexin V selected, DSU – staurosporine killed without Annexin V selection, and DT – T cell killed GEMM6) at a 1:4 ratio CD11c^+ cells to dead GEMM6 cells. B) Flow cytometry Annexin V analysis of live GEMM6 cells (UT), DSS, DSU, and DT. N=3 where all data are replicated independently ± SEM. Unpaired t-test: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 each compared to control groups.

To determine if MerTK regulated FAO in other myeloid cells as it does in DCs, we investigated FAO in MDSCs and Macrophages. Using the same protocols, we measured OCR in these cells with and without ETO. Figure 9A shows an overall prominent reduction in OCR for mertk^-/- vs wt MDSCs. Interestingly, the addition of dead GEMM6 cells or ETO does not further reduce OCR. On the other hand, Macrophages seem to behave a similar manner to DCs, where the addition of dead GEMM6 cells increases
OCR overall (Figure 6D). Although OCR is reduced with mertk−/− or ETO inhibition, we do not observe the same rescue when treated with dead GEMM6 cells (Figure 9B). This suggests different pathways are responsible for MDSC and Macrophage metabolism. Dead cell action in Macrophages and DCs differ somewhat in magnitude.

Figure 9: Metabolic reprogramming in mertk−/− MDSCs and Macrophages are distinct from that in DCs.
A&B) Seahorse mitochondrial respiration analysis of wt and mertk−/− MDSCs and Macrophages with and without ETO as well as 24-hour dead GEMM6 cell treatment at a 1:4 ratio CD11c+ cells to dead GEMM6 cells, where clear differences in metabolism among myeloid cells with MerTK inhibition is observed. N=3 where all data are replicated independently ± SEM. Unpaired t-test: *P<0.05, **P<0.01, ***P<0.001 each compared to control groups.
3.3 MerTK is induced via LXR and increases CPT1a expression in a p38-STAT3 dependent manner

MerTK-mediated efferocytosis induces Mertk gene transcription in professional phagocytes (Burstyn-Cohen and Maimon, 2019), a “feed-forward” mechanism, presumably to compensate for MerTK surface clearing. Wt CD11c+ cells treated with dead GEMM6 cells also demonstrate increased Mertk mRNA (Figure 10A); as expected, dead cell treated mertk−/− CD11c+ cells showed no increased in Mertk mRNA (Figure 10A). To establish MerTK kinase participation in feed forward induction, we pretreated cells with UNC4241, a selective MerTK and Tyro3 tyrosine kinase inhibitor (Holtzhausen et al., 2019); UNC4241 inhibited dead cell induction of MerTK (Figure 10A). Oxysterols from efferocytosed apoptotic cells activate LXR-β, a nuclear receptor, which transcriptionally stimulates MerTK expression (Kidani and Bensinger, 2012; N et al., 2009). We treated wt CD11c+ cells with GW 3965, an LXR agonist, and dead GEMM6 cells. Figure 3B shows both MerTK and LXR-β were similarly increased with either GW 3965 or dead GEMM6 cells, suggesting that MerTK can both regulate and be regulated by LXR-β.

To investigate mechanisms, we explored the effect of dead cell induced MerTK signaling on CPT1a (Figure 10C). Cpt1a mRNA increased with dead cell treatment of wt CD11c+ cells; this was not seen with dead cell treatment of mertk−/− CD11c+ cells or in wt CD11c+ cells pre-treated with UNC4241 (Figure 10C). Cpt1a mRNA expression increased in CD11c+ cells pre-treated with ETO with and without dead GEMM6 cells. We speculate that this might be due to a negative feedback mechanism as ETO blocks CPT1a protein function. These results suggest that MerTK activation increased CD11c+ cell CPT1a enhancing FAO.
Figure 10: **MerTK signals through p38 and Stat3 to regulate CPT1a and DC metabolism.**

**A)** RT-PCR analysis of MerTK expression of wt, *merk*−/−, and UNC4241 treated CD11c+ cells. **B)** RT-PCR analysis of MerTK and LXR-β expression of wt and GW treated wt CD11c+ cells. **C)** RT-PCR analysis of *Cpt1a* expression of wt, *merk*−/−, UNC4241, and ETO treated CD11c+ cells. **D)** Western analysis of wt, *merk*−/−, UNC4241, and Stattic treated CD11c+ cells. **E&F)** RT-PCR analysis of *Cpt1a* expression and seahorse mitochondrial respiration analysis of wt, static, and UNC4241 treated wt CD11c+ cells. (A), (B), (C), and (E) all with and without 24-hour dead GEMM6 cell treatment at a 1:4 ratio CD11c+ cells to dead GEMM6 cells (D) 3-hour and 10-minute dead GEMM6 cell treatment and (F) 1:1 ratio CD11c+ cells to dead GEMM6 cells. **G)** Depiction of MerTK induction and downstream phosphorylation cascade upon activation in DCs demonstrating increased *Mertk*, *Lxr*-β, and *Cpt1a* expression in DCs with dead cell treatment resulting in the phosphorylation of p38 and STAT3 to induce FAO. N=3 where all data are replicated independently ± SEM. Unpaired t-test: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 each compared to control groups.
We previously show that STAT3 serine phosphorylation was involved in MerTK immunosuppressive activity in MDSCs (Holtzhausen et al., 2019) and STAT3 is a known regulator of CPT1 expression (Zhang et al., 2020). To investigate the dead cell-MerTK-STAT3 axis, we used wt CD11c+ cells, mertk−/− CD11c+ cells, and UNC4241 treated wt CD11c+ cells. Western blotting revealed an increase in STAT3 serine phosphorylation with dead cell treatment of wt CD11c+ cells (Figure 10D). This was diminished in mertk−/− CD11c+ cells and was abrogated with UNC4241 treated wt CD11c+ cells. Tyrosine phosphorylation of STAT3 was maintained with dead cell treated mertk−/− CD11c+ cells while we observe a reduction with UNC4241 CD11c+ cells treated with dead cells. A possible explanation is that UNC4241 inhibits both MerTK and Tyro3. Stattic (a known STAT3 inhibitor) treated CD11c+ cells with and without dead cells did not increase either serine or tyrosine phosphorylation of STAT3 (Figure 10D). The loss of MerTK tyrosine phosphorylation with UNC4241 was confirmed (Figure 11B).

MerTK cannot directly phosphorylate the serine site of STAT3. We investigated if p38 is the relevant serine kinase. p38 tyrosine phosphorylation increased within 10-minute of dead cell treatment of wt but this effect was attenuated in mertk−/− CD11c+ cells (Figure 10D). The increase in p38 phosphorylation in wt CD11c+ cells was inhibited by UNC4241 (Figure 10D). STAT3 serine phosphorylation was decreased with pre-treatment with a p38 MAPK inhibitor prior to dead GEMM6 cell treatment (Figure 11C) suggesting that p38 is upstream of STAT3 and downstream of MerTK.

STAT3 activation can increase FAO via direct induction of CPT1 expression (Zhang et al., 2020). Dead cell activation of CD11c+ cell Cpt1a mRNA expression was abrogated by pre-treatment of CD11c+ cells with Stattic (Figure 10E) as was increased
mitochondrial respiration (Figure 5F). UNC4241 also significantly reduced OCR in dead cell treated CD11c<sup>+</sup> cells. The reduced OCR in wt CD11c<sup>+</sup> cells was not due to toxic effects of Stattic and UNC4241 (Figure 11A). The results with p38 and STAT3 inhibitors indicate that dead cell activated MerTK leads to p38 serine phosphorylation of STAT3, CPT1a induction, and increased FAO in DCs (Figure 10G).

**Figure 11: Dead cells induce p38 signaling.**

A) MTT analysis of wt CD11c<sup>+</sup> cells treated with UNC4241, Stattic, p38 inhibitor, ETO, and IDO inhibitor. B) Western MerTK tyrosine phosphorylation analysis of wt and UNC4241 treated wt CD11c<sup>+</sup> cells. C) Western analysis of wt and p38 inhibitor treated wt CD11c<sup>+</sup> cells with and without 10-minute dead GEMM6 cell treatment at a 1:4 ratio CD11c<sup>+</sup> cells to dead GEMM6 cells. N=3 where all data are replicated independently ± SEM. Unpaired t-test: *P<0.05 each compared to control groups.
3.4 MerTK metabolic reprogramming hinders DC function in vitro

DCs have a central role activating, expanding, and promoting subset differentiation of T cells (Fu and Jiang, 2018). To evaluate specific DC function, we obtained CD8⁺ T cells from OT-I mice, which are specific for Ova peptide. CD11c⁺ cells were treated with 1mg/mL Ova peptide plus/minus dead GEMM6 cells for 24 hours, washed, and then co-cultured with naïve OT-I CD8⁺ T cells labeled with CFSE to measure T cell proliferation (Figure 13A). Wt CD11c⁺ cells exhibited peptide specific CD8⁺ T cell proliferation that was suppressed by co-culture with dead cell treated DCs (showing the tolerizing phenotype induced by dead cells) (Figure 12A). In comparison, co-culture with mertk⁻/⁻ CD11c⁺ cells stimulated increased OT-I CD8⁺ T cell proliferation; this effect was not suppressed by dead GEMM6 cells, suggesting abrogation of the tolerizing phenotype (Figure 12A). UNC4241 and ETO treated wt CD11c⁺ cells exhibited an increased stimulatory capacity, mimicking the mertk⁻/⁻ CD11c⁺ cells and also limited the suppressive effect of dead GEMM6 cells. These results suggest that the absence or inhibition of MerTK or FAO rescues the stimulatory DC function induced by dead cells.

To further probe antigen specific priming capability, we conducted IFN-γ EliSpot analysis. The results (Figure 12B) recapitulate the directionality of the effects on CD8⁺ T cell proliferation. T cell co-culture of Ova-stimulated wt CD11c⁺ cells increased T cell IFN-γ production, and that increase was suppressed by dead cell treatment; in contrast, mertk⁻/⁻ CD11c⁺ cell stimulation of T cell IFN-γ was not as significantly suppressed by dead cells. Again, using wt CD11c⁺ cells that were subjected to either MerTK inhibition (UNC4241) or CPT1a inhibition (ETO) inflammatory changes were seen, mimicking the effect of MerTK deletion.
Figure 12: MerTK loss increases antigen presentation resulting in amplified CD8⁺ T cell proliferation and decreased Treg differentiation.

A) OT-I CD8⁺ T cell proliferation analysis using flow cytometry via co-incubation with wt, mertk⁻/⁻, UNC4241, and ETO treated CD11c⁺ cells with and without 24-hour dead GEMM6 cell treatment at a 1:4 ratio CD11c⁺ cells to dead GEMM6 cells. B) IFNγ EliSpot spot number and quantification for OT-I CD8⁺ T cell co-incubation with wt, mertk⁻/⁻, UNC4241, and ETO treated CD11c⁺ cells with and without 24-hour dead GEMM6 cell treatment at a 1:4 ratio CD11c⁺ cells to dead GEMM6 cells. A&B) demonstrate the tolerizing phenotype of DCs through the decrease in CD8⁺ T cell proliferation and magnitude of response with dead cell treatment, which is abrogated with MerTK and FAO inhibition. C) OT-II Treg Differentiation analysis using flow cytometry via co-incubation with wt, mertk⁻/⁻, UNC4241, and ETO treated CD11c⁺ cells with and without 24-hour dead GEMM6 cell treatment at a 1:4 ratio CD11c⁺ cells to dead GEMM6 cells. D) RT-PCR analysis of Ido and Tgfβ expression of wt, mertk⁻/⁻, UNC4241, ETO, and IDO inhibitor treated CD11c⁺ cells with and without 24-hour dead GEMM6 cell treatment at a 1:4 ratio, CD11c⁺ cells to dead GEMM6 cells. C&D) demonstrate the tolerizing phenotype of DCs through the increase in Treg differentiation and Ido and Tgfβ expression with dead cell treatment and reversal with MerTK deletion. N=3 where all data are replicated independently ± SEM. Unpaired t-test: *P<0.05, **P<0.01, ***P<0.001 each compared to control groups unless otherwise indicated by significance lines.

To evaluate DC tolerogenic properties, we treated CD11c⁺ cells with Ova peptide plus/minus dead cells for 24 hours, washed, and co-cultured them with naïve OT-II CD4⁺ T cells specific for Ova. DC induced T regulatory cell (Treg) differentiation was tested and was analyzed using flow (Figure 13B). Figure 12C shows a significant reduction in Treg differentiation when co-incubated with mertk⁻/⁻ CD11c⁺ cells compared to wt CD11c⁺ cells. Dead cell treatment of wt CD11c⁺ cells increased Treg differentiation to a greater extent than did co-culture with dead cell treated mertk⁻/⁻ CD11c⁺ cells. An additional method of reducing DC induced Treg differentiation was tested, IDO inhibition. IDO inhibitor treated wt CD11c⁺ cells with dead cell treatment resulted in the same level of reduced Treg differentiation as did mertk⁻/⁻ CD11c⁺ cells with dead cell treatment, signifying that inhibition of MerTK alone is sufficient to reduce the tolerizing effect of dead cell treatment. Contrary to CD8⁺ T cell proliferation, treatment of CD11c⁺ cells with UNC4241 or ETO with and without dead cells did not result in a significant difference in Treg differentiation compared to wt CD11c⁺ cells. Further experiments will be needed to determine whether off-target inhibitory effects of UNC4241 could counteract MerTK inhibition, or whether
MerTK regulates Treg differentiation in a kinase independent manner. In addition, Treg differentiation, although reliant on MerTK, may not be dependent on FAO in CD11c+ cells as we do not see a difference with ETO treated CD11c+ cells.

In addition, we examined *ldo* and *Tgfβ* RNA levels (Figure 10D) to further confirm DC tolerogenic properties with dead cell treatment. Both *ldo* and *Tgfβ* mRNA expression significantly increase when *wt* CD11c+ cells are treated with dead cells. This is abrogated with MerTK inhibition, both with and without dead cell treatment. *ldo* and *Tgfβ* mRNA expression did not change with *wt* CD11c+ cells treated with either UNC4241 or ETO; however, the increase in *ldo* and *Tgfβ* mRNA expression was blocked with the treatment of dead GEMM6 cells in *wt* CD11c+ cells pre-treated with UNC4241 or ETO.
Figure 13: Flow Cytometry representation of in vitro CD8$^+$ T cell proliferation and Treg differentiation from Figure 12.
A) OT-I CD8$^+$ T cell proliferation analysis using flow cytometry via co-incubation with wt, mertk$^{-/-}$, UNC4241, and ETO treated CD11c$^+$ cells with and without 24-hour dead GEMM6 cell treatment at a 1:4 ratio CD11c$^+$ cells to dead GEMM6 cells. B) OT-II Treg Differentiation analysis using flow cytometry via co-incubation with wt, mertk$^{-/-}$, UNC4241, and ETO treated CD11c$^+$ cells with and without 24-hour dead GEMM6 cell treatment at a 1:4 ratio CD11c$^+$ cells to dead GEMM6 cells. N=3 where all data are replicated independently.
3.5 Characterizing DC function in wt and mertk<sup>−/−</sup> mice in vivo

To understand the impact of MerTK inhibition on DC metabolism in vivo, wt and mertk<sup>−/−</sup> GEMM6 tumor-bearing mice were treated with either anti-PD-1 or IgG. A significant reduction in tumor volume and an increased survival rate (Figure 14A) was detected in mertk<sup>−/−</sup> versus wt mice treated with anti-PD-1. Once tumors reached maximum measurement of 1cm<sup>3</sup>, (to simulate the time of anti-PD-1 resistance Figure 3B), splenic DCs were purified, and mitochondrial respiration measured. As shown in Figure 14B, splenic CD11c<sup>+</sup> cells from wt mice treated with anti-PD-1 showed a significant increase in OCR compared to other treatment groups. This increase in oxidative phosphorylation in splenic CD11c<sup>+</sup> cells correlated with anti-PD-1 resistance suggesting that the DC metabolic switch may contribute to adaptive resistance.

Additional in vivo serum correlates included an increase in serum IL-10, a tolerogenic cytokine (Wculek et al., 2020), found in anti-PD-1 treated wt but not mertk<sup>−/−</sup> mice (Figure 14C, left). In contrast, an inflammatory cytokine, IL-12p70, was increased in the serum of tumor-bearing mertk<sup>−/−</sup> mice treated with anti-PD-1 or IgG compared to wt mice treated with anti-PD-1 (Figure 14C, right).

Since there was no tumor to assess from mertk<sup>−/−</sup> mice treated with anti-PD-1 at end of study, to test intratumoral RNA levels, we repeated the study and harvested tumors from wt and mertk<sup>−/−</sup> mice 15 days post-anti-PD-1 or IgG treatment. Tumors in mertk<sup>−/−</sup> mice treated with either anti-PD-1 or IgG had elevated levels of Il-12, Tnfa, and Ifnγ RNA expression compared to wt mice (Figure 14D). These data demonstrate that absence of MerTK expression favors a pro-inflammatory tumor-specific response.
Figure 14: **MerTK loss decreases tumor growth and augments PD-1 inhibition through DC metabolic reprogramming and altered cytokine production.**

A) Tumor volume in mm$^3$ (unpaired t-test done on Day 33) and survival rate of GEMM6 tumor bearing wt and mertk$^{-/-}$ mice with and without anti-PD-1 treatment, where genetic knockout of MerTK significantly reduces tumor volume with anti-PD-1 treatment. B) Seahorse mitochondrial respiration of spleen derived wt, and mertk$^{-/-}$ CD11c$^+$ cells with and without anti-PD-1 treatment, where wt anti-PD-1 treatment results in increased mitochondrial respiration of splenic DCs. C) Serum IL-10 and IL-12p70 cytokine MSD analysis of GEMM6 tumor bearing wt and mertk$^{-/-}$ with and without anti-PD-1 treatment demonstrating a correlation between tumor growth and the type of serum cytokine increase. D) RT-PCR analysis of intratumoral cytokine expression of tumors harvested at day 15 from wt and mertk$^{-/-}$ mice with and without anti-PD-1 treatment. **C&D** demonstrate the inflammatory phenotype that results with MerTK knockout along with anti-PD-1 treatment. N=5-9 ± SEM. Unpaired t-test: *P<0.05, **P<0.01, ***P<0.001 each compared to control groups unless otherwise indicated by significance lines.
To obtain a more complete picture of immune-related RNA expression, tumors from untreated wt and \textit{mertk}^{-/-} mice were harvested at day 24 and RNA was analyzed using a Nanostring 700-gene immune panel. \textbf{Figure 15A & 15B} shows the lower growth of GEMM6 tumors in \textit{mertk}^{-/-} mice and the increase in genes indicative of antigen presentation (pathway score) even though the overall DC transcript score indicated similar DC numbers.

\textbf{Figure 15: MerTK loss inhibits tumor growth and increases antigen presentation without altering DC numbers.} 
\textbf{A)} Tumor volume in mm$^3$ (unpaired t-test done on Day 24) \textbf{(B)} and Nanostring analysis of antigen presentation genes and number of DCs of tumors harvested from \textit{wt} and \textit{mertk}^{-/-} mice at Day 23 after GEMM6 tumor injection (N=5-6), where genes responsible for antigen presentation are increased overall in tumors of \textit{mertk}^{-/-} mice without any changes to DC number. All data are ± SEM. Unpaired t-test: *\textit{P}<0.05, **\textit{P}<0.01, ***\textit{P}<0.001 each compared to control groups.
To understand the impact of the myeloid compartment on GEMM6 tumor growth, we utilized previously derived LyzM-cre and LyzM-mertk<sup>−/−</sup> mice (Ubil et al., 2018) confirming MerTK loss in CD11b<sup>+</sup> and CD11c<sup>+</sup> cells (Abram et al., 2014) (Figure 16A). Reduced tumor growth (Figure 16B) was similar in direction to that in Figure 7A, showing reduced tumor volume in LyzM-mertk<sup>−/−</sup> mice with and without anti-PD-1 signifying total loss of MerTK in the myeloid compartment is effective even though the metabolic regulatory mechanism may be different in CD11b<sup>+</sup> and CD11c<sup>+</sup> cells.

![Image of Figure 16A and Figure 16B]

**Figure 16: Loss of MerTK in myeloid cells decreases tumor growth and is augmented by anti-PD-1 treatment.**

A) Flow Cytometry analysis of splenic CD11c<sup>+</sup> and CD11b<sup>+</sup> cell TAM protein levels of LyzM-cre and LyzM-mertk<sup>−/−</sup> mice. N=3 where all data are replicated independently ± SEM

B) Tumor volume in mm<sup>3</sup> of GEMM6 tumor bearing LyzM-cre and LyzM-mertk<sup>−/−</sup> mice with and without anti-PD-1 treatment, Unpaired t-test done on Day 33, demonstrating similar tumor volume pattern as the whole MerTK knockout mice, signifying the importance of MerTK inhibition in myeloid cells. N=6 ± SEM. Unpaired t-test: *P<0.05 each compared to control groups.
3.6 Deletion of MerTK specifically in CD11c+ cells helps in reversing anti-PD-1 resistance

The above experiments were performed with the original (Camenisch et al., 1999) MerTK knockout mice. To define the in vivo effects of selectively targeting MerTK-deficiency to the DC compartment, our recently established CRISPR mertk^{fl/fl} B6 mice were crossed to CD11c-cre B6 mice (CD11c-mertk^{-/-}). Splenic DCs prepared from CD11c-mertk^{-/-} mice had reduced Mertk mRNA and surface protein relative to control (CD11c-cre mice) (Figure 17A & 17B). Seahorse analysis of FAO of CD11c+ cells from control and CD11c-mertk^{-/-} mice were similar to those from the original mouse (Figure 6D). In addition, CD11c-mertk^{-/-} CD11c+ cells treated with dead cells lost the increase in OCR (Figure 18A) with presumably the same mechanism as induction of Cpt1a mRNA expression by dead cells was observed in control but not CD11c-mertk^{-/-} CD11c+ cells (Figure 18B).
Figure 17: TAM receptor expression on CD11c<sup>+</sup> and CD11b<sup>+</sup> cells.
A&B) RT-PCR and Flow Cytometry analysis of splenic CD11c<sup>+</sup> TAM RNA expression and protein levels from CD11c-cre and CD11c-<i>mertk</i>−/− mice. N=3 with ± SEM, where all data are replicated independently. Unpaired t-test: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 each compared to control groups.

With respect to tumor progression, GEMM6 tumors grew similarly in the new CRISPR-derived mice (Figure 14A vs Figure 18C left), with tumor size reduced ~50% in anti-IgG treated CD11c-<i>mertk</i>−/− versus control mice, and the further reduction in anti-PD-1 treated CD11c-<i>mertk</i>−/−. To better investigate the in vivo mechanism, we inhibited CPT1a function with ETO. ETO treatment and MerTK-deficiency in DCs slowed tumor growth similarly by ~50% (anti-PD-1 again also slowed growth by ~50%) (Figure 18C right). Thus, as single “agents” ETO and CD11c specific MerTK deletion slowed growth ostensibly with a one common pathway, inhibition of FAO. The addition of ETO in the CD11c-<i>mertk</i>−/− mice was not additive, inhibition of FAO as anti-PD-1 treated CD11c-
mertk^- mice were near maximally inhibited. Interestingly, ETO addition to anti-PD-1 in control mice did not replicate the dramatic inhibition of anti-PD-1 in CD11c-mertk^- mice suggesting that CD11c specific MerTK deletion has additional mechanism of enhancing anti-PD-1 revealed by IHC assessment of the T cell infiltrate in the GEMM6 TME. Figure 8D shows, although not significant, a slight increase in CD8^+ T cell population with untreated CD11c-mertk^- and in control mice treated with anti-PD-1. However, there was a significant increase in CD8^+ T cell infiltration in CD11c-mertk^- mice treated with anti-PD-1, the most effective therapy combination. Adding ETO was less effective in altering T cell infiltration.
Figure 18: DC-specific deletion of MerTK is sufficient to reduce tumor growth through reduced fatty acid oxidation.

A) Seahorse mitochondrial respiration analysis of bone marrow derived CD11c-cre and CD11c-mertk⁻/⁻ CD11c⁺ cells, with and without 24-hour dead GEMM6 cell treatment at a 1:4 ratio CD11c⁺ cells to dead GEMM6 cells. B) RT-PCR analysis of Cpt1a expression of bone marrow derived CD11c-cre and CD11c-mertk⁻/⁻ CD11c⁺ cells. N=3 where all data are replicated independently ± SEM. A&B) demonstrate the similarity of DC metabolism and Cpt1a expression between two mouse models used (mertk⁻/⁻ and CD11c-mertk⁻/⁻). C) GEMM6 tumor volume in mm³ (where N=14-16 for left and N=6-10 for right) for CD11c-cre and CD11c-mertk⁻/⁻ mice with and without anti-PD-1 and ETO treatment (Unpaired t-test done on Day 27). D) CD8 IHC staining of GEMM6 tumors from CD11c-cre and CD11c-mertk⁻/⁻ mice, with and without anti-PD-1 and ETO treatment where yellow is low and red is high (CD8 staining levels), and blue is DAPI nuclear staining, N=6-13 ± SEM. C&D) show the impact of DC specific MerTK knockout along with anti-PD-1 treatment on reducing tumor volume and increasing CD8⁺ T cell tumor infiltration. Unpaired t-test: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 each compared to control groups.

To obtain additional confirmation of antigen presentation specificity in the context of CD11c-mertk⁻/⁻, we used GEMM6-OVA tumor bearing mice treated with either anti-PD-1 or IgG. At Day 20, tumor draining lymph nodes and tumors were harvested. The lymph nodes were then stained with Ova-specific CD8 tetramers. The results (Figure 19A), as expected, showed a significant increase in CD8⁺ Ova Tetramer (H-2K(b) SIINFEKL) in the control CD11c-cre with anti-PD-1 treatment. The untreated CD11c-mertk⁻/⁻ mice showed tetramer levels equivalent to the anti PD-1 treated control mice. Tetramer⁺ T cells increased further to the highest level with anti-PD-1 treatment of the CD11c-mertk⁻/⁻ mice.
With respect to intratumoral antigen processing, CD11c+ cells were isolated from the harvested GEMM6-OVA tumors where they would have encountered and digested Ova protein *in vivo*. The isolated CD11c+ cells were either co-incubated with OT-I naïve CD8+ T cells or OT-II naïve CD4+ T cells to measure CD8+ T cell proliferation and Treg differentiation respectively. There was no added peptide as all peptide had been ingested *in vivo*. **Figure 19B & 19C,** demonstrate a significant increase in CD8+ T cell proliferation with CD11c-cre tumors with anti-PD-1 treatment. An equivalent stimulation was seen from the CD11c-mer*tk−/−* CD11c+ cells even without anti-PD-1; treatment with anti-PD-1 provided an additional increment. There is reduced Treg differentiation in anti-PD-1 treated control tumor bearing mice, while CD11c-mer*tk−/−* CD11c+ cells show this reduction in Treg differentiation with and without anti-PD-1. These functional assays further confirm the reversal of DC tolerogenic properties with selective elimination of MerTK in CD11c+ cells (**Figure 20A & 20B**).
Figure 19: DC specific deletion of MerTK and anti-PD-1 therapy increases antigen specific CD8+ T cells and results in specific antigen presentation changes.

A) CD8 tetramer Ova positive cells from tumor draining lymph nodes of GEMM6-OVA bearing CD11c-cre and CD11c-merTK−/− mice with and without anti-PD-1 treatment along with Flow Cytometry representation, where DC specific MerTK knockout along with anti-PD-1 treatment results in the highest number of CD8+ T cells in the tumor draining lymph node. B&C) CD8+ T cell proliferation and Treg differentiation analysis with OT-I and OT-II derived T cell co-incubation with CD11c+ cells isolated from GEMM6-OVA tumors of CD11c-cre and CD11c-merTK−/− mice with and without Anti-PD-1 treatment (no added Ova peptide). DC specific MerTK knockout along with anti-PD-1 treatment abrogates the tolerized phenotype (heightened T cell proliferation and reduction in Treg differentiation) than the control group. N=5-9 ± SEM. Unpaired t-test: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 each compared to control groups.
Figure 20: Flow Cytometry representation of ex vivo CD8$^+$ T cell proliferation and Treg differentiation from Figure 19.

A) CD8$^+$ T cell proliferation and (B) Treg differentiation analysis with OT-I and OT-II derived T cell co-incubation with CD11c$^+$ cells isolated from GEMM6-OVA tumors of CD11c-cre and CD11c-merTK$^{-/-}$ mice with and without Anti-PD-1 treatment, where DC specific MerTK knockout along with anti-PD-1 treatment abrogates the tolerized phenotype seen in the control group. N=5-9 ± SEM. Unpaired t-test: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 each compared to control groups.

To understand the generalizability of these results, we investigated another melanoma model, YUMM1.7 (Meeth et al., 2016). Like GEMM6, addition of dead YUMM1.7 cells (Figure 21A) increased OCR in control CD11c-cre CD11c$^+$ cells and OCR was reduced in CD11c-merTK$^{-/-}$ CD11c$^+$ cells treated with dead YUMM1.7. However, the magnitude of inhibition is less compared to CD11c-merTK$^{-/-}$ CD11c$^+$ cells treated with dead
GEMM6 cells (Figure 18A). In addition, CD11c-cre and CD11c-

merk⁻/⁻ YUMM1.7 tumor bearing mice were analyzed with and without anti-PD-1 treatment. Tumor volume was decreased in untreated CD11c-merk⁻/⁻ mice when compared to control mice. Addition of anti-PD-1 in both CD11c-cre and CD11c-merk⁻/⁻ tumor bearing mice resulted in a significant tumor reduction as compared to control tumor bearing mice (Figure 21B). The YUMM1.7 model is more responsive to anti-PD-1 than GEMM6 and so the effect of MerTK elimination in CD11c⁺ cells, while present, is less striking.
Figure 21: **Loss of MerTK reduces YUMM1.7 tumor growth and is augmented by anti-PD-1 treatment.**

A) Seahorse mitochondrial respiration analysis of CD11c-cre and CD11c-*mertk*−/− CD11c+ cells with and without 24-hour dead YUMM1.7 cell treatment at a 1:4 ratio CD11c+ cells to dead YUMM1.7 cells. N=3 with ± SEM, where all data are replicated independently. B) Tumor volume in mm³ of YUMM1.7 tumor bearing CD11c-cre and CD11c-*mertk*−/− mice (N=6 with ± SEM) with and without anti-PD-1 treatment, Unpaired t-test done on Day 30. **A&B** demonstrate the similarities between the two melanoma tumor cell lines. Unpaired t-test: *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 each compared to control groups.
DISCUSSION

Anti-PD-1 therapy revolutionized the field of immunotherapy and has proven effective in multiple cancer types, particularly melanoma, yet some patients fail to respond, and many become resistant. Finding underlying mechanisms causing primary and adaptive resistance and biomarkers thereof is crucial to improve survival. We have previously examined the role of MerTK in melanoma tumor cells (Keating et al., 2010; Linger et al., 2013) and in the TME where its normal anti-inflammatory myeloid mechanisms are subverted enhancing the immunosuppressive milieu (Cook et al., 2013; Holtzhausen et al., 2019; Ubil et al., 2018). Since both bridging protein ligand and apoptotic cell externalized PtdSer are present in the TME, MerTK will be consistently activated in, for example, MerTK-positive MDSCs and tumor associated macrophages (31, 32).

DCs in the TME are critical in facilitating the immune response but could assume a tolerogenic phenotype (Schmidt et al., 2012; Svajger and Rozman, 2014). Our initial mouse experiments showed MerTK+ DCs increased at later times during anti-PD-1 treatment. To understand the impact, dead GEMM6 cells were co-cultured with purified bone marrow derived DCs; RNA analysis showed substantially reduced antigen presentation and interferon signaling pathway transcripts as well as significantly suppressing Il-12b, Ifnγ, and Tnfα RNAs. Each result suggested dead cell signaling established a tolerized DC phenotype, consistent with earlier studies (Wallet et al., 2009;
This MerTK-dependent efferocytosis leads to metabolism of dead cells to oxysterols (Madenspacher et al., 2020), their binding to LXR and enhancement of MerTK transcription. Our experiments confirmed that DCs treated with dead cells induce both Lxr-β and Mertk mRNAs.

Activation of DCs relies on glycolysis for immunogenic activity, maturation, migration (Brombacher and Everts, 2020) and secretion of inflammatory cytokines (Wculek et al., 2020). Alternatively, for DCs, increased oxidative phosphorylation, specifically FAO results in a tolerogenic phenotype (Malinarich et al., 2015; Zhao et al., 2018). To elucidate the role of MerTK (and other TAM RTKs), DCs derived from wt, mertk−/−, axl−/−, and tyro3−/− mice were treated with dead GEMM6 cells. Oxidative phosphorylation was increased upon treatment of wt DCs with dead cells (Figure 6). Oxidative phosphorylation was reduced in mertk−/− DCs at baseline, and the increase with dead cells was markedly attenuated in mertk−/− DCs. Axl or Tyro3 deletion also attenuated oxidative phosphorylation but to a smaller degree. Cpt1a, the gateway enzyme in FAO metabolic control, was significantly increased in DCs treated with dead cells; Cpt1a induction was not seen in DCs isolated from mertk−/− mice and was blocked in wt DCs pretreated with the MerTK kinase inhibitor UNC4241.

Mechanism studies in DCs revealed that MerTK activation stimulated p38 STAT3 serine phosphorylation, a known control point in macrophages (Ubil et al., 2018) and MDSCs (Holtzhausen et al., 2019). Directly blocking STAT3 has been shown to increase DC maturation (Nefedova et al., 2005). This signaling cascade appears to be one path to MerTK regulation of DC metabolism through Cpt1a induction and subsequent FAO enhancement. Blockade high in the cascade with UNC4241 and towards the nuclear
effector stage by blocking STAT3 activation via Stattic appear to substantiate the
cascade.

The consequences of MerTK-regulated changes in DC metabolism in vitro are
substantial. Figure 1C demonstrates MerTK-dependent binding of dead cells suppressed
antigen presentation and interferon RNAs but also limited the capacity of DCs to stimulate
CD8+ T cells (Figure 12A & 12B) and enhanced Treg differentiation and Ido/Tgfβ mRNA
expression (Figure 12C & 12D). These tolerogenic DC activities were abrogated in mertk−/−
DCs, or UNC4241 treated wt DCs. Abrogation of the FAO DC phenotype by MerTK
inhibition reduced the metabolic change and enhanced CD8+ T cell proliferation and IFN-
y production, as well as reduced Treg differentiation and Ido/Tgfβ mRNA expression.

The impact of MerTK on DC metabolism and function was also evident in vivo in
terms of GEMM6 tumor growth and the efficacy of anti-PD-1 therapy. In the original
untreated mertk−/− mice, tumor growth was reduced by ~50% equivalent to the effect of
anti-PD-1 in wt mice. Furthermore, tumor progression was nearly eliminated in mertk−/−
mice treated with anti-PD-1. Reduced growth in mertk−/− mice treated with anti-PD-1
correlated with reduced levels of serum IL-10 and increased serum IL-12p70. A similar
trend was seen in analysis of intratumoral cytokine mRNA with the inflammatory
cytokines: IL-12, TNFα, and IFN-γ significantly increased with MerTK deletion and
addition of anti-PD-1.

While not as extensively investigated, we demonstrated subtle pathway
differences in OCR control between DCs, MDSCs and Macrophages (Figure 9) but each,
upon deletion of MerTK had an overall reduction of OCR. Complete myeloid cell loss of
MerTK in the LyzM-*mertk* mice presumably reduced several myeloid immunosuppressive actions and slowed growth.

To directly determine the *in vivo* effects of MerTK-deficiency in the DC compartment, we established and employed CD11c-*mertk*−/− B6 mice. Here, CRISPR was used to introduce *mertk*fl/fl directly into the B6 genotype. This was fortuitous as a recent publication suggests that a mixed genotype between B6 and 129 strains in our original *mertk*−/− mouse may account for some decreased tumor growth phenotypes (Akalu et al., 2022). Notably, in this study, GEMM6 tumor progression *in vivo* was similarly affected in *mertk*−/− and new CD11c-*mertk*−/− mice; *in vitro* studies of DCs isolated from *mertk*−/− and CD11c-*mertk*−/− mice exhibited similar metabolic events upon treatment with dead cells. In this model, loss of MerTK selectively in the CD11c+ cell compartment was sufficient to have a substantial growth slowing and anti-PD-1 enhancing effect.

In addition to growth retardation, enhancement of anti-PD-1 efficacy, alteration in cytokines in an immunostimulatory direction, and reduction of immunosuppressive modes in MerTK deleted mice, we detected several other correlates of selective actions of MerTK deletion in DCs, namely T cell infiltration and specific evidence of antigen presentation changes. First immunofluorescence analysis of T cell infiltration into GEMM6 tumors showed that the number of T cells was highest in the tumor bearing CD11c-*mertk*−/− mice treated with anti-PD-1 compared to other groups. ETO paralysis of CPT1a and subsequent lack of increased FAO in CD11c-*mertk*−/− mice, did not add to the increase in T cell infiltration even with anti-PD-1; MerTK deletion in DCs appears to be sufficient. The tumor studies conducted show promising results. Although we do not observe a complete
abrogation, we were able to limit tumor growth for a long period. This is clinically relevant as it translates to possible prolonged disease stability in patients.

To test antigen presentation more directly than RNA transcript analysis, we used ovalbumin expressing GEMM6 tumors (Figures 19A-C). First, we observed increased CD8+ OVA-Tet+ T cells in tumor draining lymph nodes of control CD11c-cre mice with anti-PD-1 treatment. CD11c-mertk−/− mice, even without anti-PD1, had Tetramer+ CD8+ T cells equivalent to the treated control mice; the number was further increased in the anti-PD-1 treated CD11c-mertk−/− mice. Second, we isolated CD11c+ cells from the GEMM6-OVA tumors and co-cultured with OT-I naïve CD8+ T cells or OT-II naïve CD4+ T cells. These cells were not treated nor was any peptide added to the co-cultures. The highest stimulation of CD8+ T cell proliferation and lowest level of Treg differentiation in the co-culture was accomplished by DCs from the CD11c-mertk−/− mice treated with anti-PD-1. These results confirm the specific suppression of antigen presentation in the TME with tolerizing DCs obtained from control mice without treatment and the reduction in the tolerizing phenotype with the deletion of MerTK specifically in the DC compartment of the CRISPR-derived mouse. This result strongly suggests the rescue of tolerogenic properties of DCs with MerTK inhibition and anti-PD-1 treatment.

The role of MerTK in DC tolerization shown in this study is one aspect of the multifactorial and complex effect of TAM RTKs on the immunosuppressive TME. Our experiments and those of others make progress by using models and isolating individual components of the pathophysiology of normal systems subverted by disease evolution. A recent publication nicely defined a physiologic role of MerTK+ tolerizing DCs in neonatal lung development (Silva-Sanchez et al., 2023). An influx of MerTK+ DCs from the bone
marrow occurs in the first week of mouse lung development and ingest lung cells dying as the developing organ is remodeled; these apoptotic ingesting DCs prevent inflammatory damage. When this period is over MerTK+ DC infiltration subsides. The complexity of the TAM RTK physiology and pathophysiology undoubtedly varies in an organ, context specific manner as well as by which TAM member plays the dominant role. This manuscript showed some effect on DC metabolism by both Axl and Tyro3, but clearly in this model, in this tumor, MerTK was the most relevant. Therefore, understanding the TAM RTK role in a specific tumor or context is not just an academic pursuit as clinical trials of small molecules and biologics targeting the family are in progress and the therapeutic/toxicity ratios will begin to emerge.
CONCLUSION

The important and wide use of anti-PD-1 therapy for metastatic melanoma is being compromised by both primary and tumor adaptive resistance. In summary, we present evidence that dead cells in the TME can induce MerTK expression in DCs, reprogramming DC metabolism to an oxidative state potentiating a tolerizing phenotype. The resulting tolerized DCs may contribute to anti-PD-1 resistance, identifying MerTK and other DC metabolism regulators as targets for clinical studies aimed at augmenting immunotherapy.
REFERENCES


