UNIVERSITY OF CALIFORNIA SAN DIEGO

Engineering exosomes to contain MEK1 as cancer immunotherapy

A Thesis submitted in partial satisfaction of the requirement for the degree

Master of Science

in

Biology

by

Preston Kwankin Lee

Committee in charge:

Professor Jack Bui, Chair
Professor Stephen Hedrick, Co-Chair
Professor Elina Zuniga

2020

The thesis of Preston Lee is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

Co-Chair

_____

Chair

University of California San Diego

2020

Dedication

I dedicate this thesis to my parents, my sister, and my relatives for their love, support, and sacrifices that got me to where I am today.

Table of Contents

List of Figures

Acknowledgements

I am deeply thankful for Dr. Bui for giving me the opportunity to learn and grow in the field of science. His passion, guidance, and patience have helped me gain a better appreciation and curiosity for research. Additionally, he inspires me to grow and improve myself in the future in both research and the medical field.

I would also like to show my sincere appreciation for Steve Searles for becoming my mentor when I first started in the lab. He extended out his hand to help shape me into the researcher I am today and set an example for what it means to be a scientist. It was truly a pleasure learning from him.

I would also not be where I am today without the current and former lab members. Ruth Seelige, Calvin Lee, Allen Washington Jr., Lindsay Charo, Hali Dinh, Isabel Mejin, Melissa Ito, Khang Nguyen, Jarrod Yee, and Lindsay Nguyen. Thank you all for providing me with both mentorship and friendship throughout the process.

Last but not least, I would like to thank my parents for the unconditional love and support. I'll never forget the sacrifices they've made for me. What's in stall for me in the future is still yet to be determined, but I cannot be more grateful for this journey.

ABSTRACT OF THE THESIS

Engineering exosomes to contain MEK1 as cancer immunotherapy

by

Preston Kwankin Lee

Master of Science in Biology

University of California San Diego, 2020

Professor Jack Bui, Chair
Professor Stephen Hedrick, Co-Chair

Exosome-mediated transfer of bioactive cargo among cells is now appreciated as a bonafide method of intercellular communication. In cancer, exosomes have been understood mostly as tumor-promoting factors that are secreted abundantly to promote cell proliferation of non-transformed cells, inhibit anti-tumor immune responses, and facilitate metastasis.

Using mouse progressor and regressor cell lines as a model system, we have identified an immune-stimulating activity of exosomes derived from regressor tumor cells. Our proteomic data revealed that only exosomes from regressor cells contained the protein MAP kinase kinase 1 or MEK1. Notably, simply overexpressing this protein in progressor cells can engineer exosomes to carry MEK1 proteins. These MEK1-containing exosomes can be used therapeutically to induce tumor rejection, which requires the immune system and correlates with an increase in natural killer (NK) cells and M1-type macrophages and a decrease in M2-type macrophages in the tumor. While exosomes have been shown to be taken up by macrophages, their anti-tumor effects are independent of patrolling monocytes, thereby suggesting that exosomes exert their activity in tumor macrophages or classical blood monocytes. Indeed, we have found that exosome treatment of both bone marrow-derived and tumor-derived macrophages can induce the activation of both M1 and M2 genes.

To translate these findings, generating human exosomes with abundant expression of MEK1 is required. Other groups have shown that human telomerase immortalized-mesenchymal stem cells, or hTERT-MSCs, could be engineered to overexpress various genes. hTERT-MSCs have also been approved by the Food and Drug Administration (FDA) for use in patients, so generating exosomes from these cells could lead to a therapeutic reagent that could receive rapid FDA approval. As proof-of-principle, MEK1 was transduced into hTERT-MSCs and shown to be overexpressed in the cytoplasm. Future studies will test exosomes from these human cells and examine the activation of human blood monocytes by human MEK1-containing exosomes.

I.

Introduction

Cancer is a disease that develops when gene mutations accumulate and transform normal cells into uncontrollably dividing cells, resulting in a tumor [1]. Besides the accumulation of intrinsic mutations, intercellular communication between transformed and untransformed cells within and outside the tumor microenvironment contributes to further tumor development [2]. While intercellular communication is essential for normal cell growth and function through direct cell-to-cell contact or mediated by secreted factors, cancer cells co-opt this communication process to create a suitable environment for their own survival [3]. For example, cancer cell-secreted vesicles called exosomes have recently been appreciated to play significant roles in tumor progression [4].

Discovered in 1983, exosomes were previously presumed to be waste disposal units [5]. However, it wasn't until recently that exosomes were appreciated as a means of intercellular communication [6]. Exosomes are 40-150 nm sized vesicles of endosomal origin that participate in cellular homeostasis, activation of intracellular signaling cascades, and immune regulation [7]. Through autocrine and paracrine signaling, exosomes can shuttle biological cargo such as DNA, RNA, and proteins to stimulate a wide range of effects on recipient cells [8, 9]. Specifically, tumor cells secrete significantly more exosomes than do non-transformed cells, indicating that tumor progression relies heavily on the pro-tumorigenic products within tumor-derived (TD) exosomes [10]. A study conducted recently has demonstrated the transfer of oncogenic miRNAs and RAS signaling molecules in prostate cancer cell-derived exosomes as the mechanism for promoting neoplastic transformation of adipose-derived stem cells [11]. Under hypoxic conditions, TD exosomes can also contain pro-angiogenic factors, such

as VEGF, to instigate blood vessel formation for nutrients and cell migration to other sites [12]. Furthermore, TD exosomes exert immune-inhibitory effects, such as stimulating secretion of IL-6 cytokine to promote M2 macrophage polarization via the STAT3-dependent pathway [13]. Abnormal proliferation, angiogenesis, tumor-promoting inflammation, and metastasis described above are all essential hallmarks of cancer, and TD exosomes are key mediators of such processes.

Despite numerous cases supporting the pro-tumorigenic effects of TD exosomes, recent evidence has illuminated their anti-tumor effects via stimulation of the immune system. TD exosomes can display tumor antigens to antigen presenting cells (APCs), thereby activating the immune system and promoting tumor regression [14]. For example, transfer of tumor-associated antigens in exosomes has shown to promote maturation of dendritic cells and cross priming of $CD8^+$ cytotoxic T cells [15]. Moreover, the non-classical monocyte known as patrolling monocytes is another type of APC recently shown to reject tumors via capture of tumor antigens and recruitment of natural killer (NK) cells [16]. A study has implicated patrolling monocytes as a target of TD exosomes to promote tumor clearance [17]. These studies document immunostimulatory effects of TD exosomes and support their use as an immune therapy of cancer.

We previously developed a tumor model system consisting of matched 3' methylcholanthrene (MCA)-induced fibrosarcoma regressor and progressor cell lines that are rejected by the immune system or grow progressively in immune competent mice, respectively [18]. While we identified IL-17D as a secreted cytokine that locally recruit NK cells to promote anti-tumor immunity in regressor tumors, our unpublished

data demonstrated that regressors can induce rejection of contralateral tumors via exosomes containing a regressor-specific protein known as MAP kinase kinase, or MEK1 [18, 19]. Presently, MEK1 is mostly understood as a dual-specific protein kinase that regulates cell proliferation and differentiation via the MAPK/ERK signaling pathway. Since this gene is often mutated in some cancers, FDA-approved MEK1 inhibitors, such as trametinib and cobimetinib, have been developed to counteract the oncogenic effects of the protein [20]. However, when progressor-derived exosomes were engineered to overexpress MEK1 (MEK1-hi exosomes), tumors treated with these exosomes resulted in delayed growth [18]. Interestingly, MEK1 protein in exosomes also promoted anti-tumor immune responses through activation of macrophages via exosomal uptake [18]. Additionally, MEK1-hi exosomes also indirectly recruited NK cells into tumors [18]. Therefore, we hypothesize that MEK1-hi exosomes directly stimulates innate immune responses and subsequently activates adaptive immunity against cancer, thereby inducing tumor regression [18].

Using this unpublished data as the backbone of this study, we collaborated with the Catherine Hedrick lab to investigate the role of patrolling monocytes in mediating the effects of exosomal MEK1, due to their myeloid lineage and ability to recruit NK cells. We further demonstrate that direct uptake of MEK1-expressing exosomes can activate tumor-derived macrophages. Moreover, with substantial evidence proving the anti-tumorigenic effects of exosomal MEK1 in mice, this study generates a human model system using human telomerase immortalized-mesenchymal stem cells (hTERT-MSCs) with forced expression of MEK1 in the cytoplasm. Success in overexpressing MEK in

exosomes will allow future testing of the anti-tumor effects of human MEK1-hi exosomes on human blood monocytes.

II.

Results

Patrolling monocytes regulate tumor growth at an early timepoint

Due to recent discovery of the role of patrolling monocytes in NK cell recruitment and tumor clearance, presumably via uptake of TD exosomes, we sought to study whether these myeloid-derived immune cells contribute to exosomal MEK1-mediated tumor rejection. In doing so, we hoped to discover the mechanism of how exosome treatment recruits NK cells. Using our 6727 regressor cell lines, we performed two identical growth experiments over the course of 30-40 days to investigate whether patrolling monocytes participate in tumor rejection of highly immunogenic tumor cell lines (Figure 1A). For our patrolling monocyte-deficient mouse models, we utilized mice generally lacking the Nr4a1 gene in all cell types ($Nr4a1^{-/-}$) and mice lacking the E2 super enhancer ($E2^{-/-}$) that specifically regulates the Nr4a1 gene in patrolling monocytes. While tumor sizes varied at the end of the experiments, both trials showed increased tumor growth in $Nr4a1^{-/-}$ and $E2^{-/-}$ mice at an early time point compared to those in WT mice (Figure 1B). Based on these results, we hypothesized that patrolling monocytes are essential for immune regulation only during the early stage of tumor development.

Next, we wanted to characterize the tumors from $Nr4a1^{-/-}$ and $E2^{-/-}$ mice, looking specifically at NK cells, macrophages, $CD4^+$, and $CD8^+$ T cells (Figure 1C). Interestingly, there was no difference in NK cell population among the tumors. The lack of difference in NK cell population suggests that other factors besides patrolling monocytes are responsible for NK cell recruitment in the later stage of the tumor. Furthermore, while tumors from $E2^{-/-}$ mice yielded a slight increase in M1 and slight decrease in M2 macrophage populations relative to those of WT mice, $Nr4a1^{-/-}$ tumors consisted of a drastic increase in M1 macrophages and decrease in M2 macrophages despite having

only 1 sample size. This can be explained by a recent discovery that Nr4a1$^{-/-}$ macrophages were preferentially polarized to exhibit M1-like phenotypes [21]. In regards to T cells, both CD4^{+} and CD8^{+} T cells in Nr4a1$^{-/-}$ tumors were drastically increased, due to the lack of Nr4a1 gene regulating T cell exhaustion. We propose that patrolling monocytes might regulate early onset of tumor progression and that E2$^{-/-}$ mouse model was a more specific system for our studies on the role of patrolling monocytes.

Patrolling monocytes do not mediate the anti-tumor effects of exosomal MEK1

Our data suggested that patrolling monocytes can limit the growth of immunogenic tumor cells at an early timepoint. Next, we wished to further investigate the role of patrolling monocytes in MEK1-hi exosome mediated immune stimulation. We conducted an exosome therapy experiment on WT and E2$^{-/-}$ mice over a 20-day time course, injecting either PBS (control) or MEK1-hi exosomes intratumorally on days 7, 12, and 15 post-tumor transplantation (Figure 2A). Although there was only a minor effect on tumor growth in WT mice, MEK1-hi exosome treatment drastically reduced tumor size in patrolling monocyte-deficient mice relative to no exosome treatment (Figure 2B). Additionally, MEK1-hi exosome treatment slightly reduced tumor weight of WT mice and further decreased tumor weight of E2$^{-/-}$ mice, relative to those without exosome treatment (Figure 2B). Contrary to our hypothesis, these results suggest that MEK1-hi exosomes exert their effects independent of patrolling monocytes.

Next, we analyzed and quantified the tumor-infiltrating immune cells within those tumors using flow cytometry (Figure 2C). Using CD45 as a marker for immune cells, we

found that MEK1-hi exosomes could still induce recruitment of immune cells in $E2^{-/-}$ tumors. While the frequency of NK cells was low among all tumors, MEK1-hi exosomes slightly increased NK cell population in both WT and $E2^{-/-}$ tumors relative to PBS treatment. Aligned with our expectations, lack of patrolling monocytes in $E2^{-/-}$ mice resulted in decreased NK cell population in both PBS-treated and MEK1-hi exosome treated tumors, compared to tumors from WT mice. However, comparing both $E2^{-/-}$ groups, MEK1-hi exosome treatment rescued some infiltration of NK cells, suggesting that NK cells were recruited through other means besides patrolling monocytes.

Since we previously found some effects of MEK1-hi exosomes on promoting M1-like macrophages in tumors, we tested if this effect required patrolling monocytes (Figure 2C). We found that MEK1-hi exosome treatment increased M1 macrophage population compared to no exosome treatment. As expected, we also saw a minor decrease in M2 macrophage population in WT tumors treated with MEK1-hi exosomes compared to those treated with vehicle control. More interestingly, MEK1-hi exosome therapy in $E2^{-/-}$ mice yielded a much greater increase in M1 macrophage population and decrease in M2 macrophage population compared to PBS treatment in $E2^{-/-}$ mice. As a whole, increased M1:M2 ratio in groups treated with MEK1-hi exosomes is consistent with previous findings. However, data from the tumor growth experiment indicated that the anti-tumor effects of MEK1-hi exosomes were independent of patrolling monocytes. Therefore, while patrolling monocytes have been demonstrated to take up exosomes and recruit NK cells in other studies, our study suggests that patrolling monocytes are not involved in the mechanism of MEK1-hi exosome mediated tumor rejection.

MEK1-containing exosomes activate bone marrow-derived macrophages and tumor-derived macrophages

Based on our hypothesis that MEK1-hi exosomes augment polarization states of macrophages, we proceeded to investigate whether MEK1-hi exosomes can induce M1 pro-inflammatory cytokines. Previously, we found that in vitro treatment of bone marrow-derived macrophages (BMDMs) with MEK1-hi versus MEK1-low exosomes led to inconsistent induction of M1 phenotype genes and proteins. Since we found that in vivo treatment with MEK1-hi exosomes resulted in tumor regression correlated with increased M1:M2 ratios inside the tumor, we wished to test if this was due to a direct effect of MEK1-hi exosomes on tumor-derived macrophages.

We previously showed that myeloid-derived monocytes and macrophages in the tumor are responsible for exosomal uptake and hypothesized that MEK1-expressing exosomes induce M1 macrophages while inhibiting M2 macrophages in tumor. To this end, we isolated TD macrophages from 9609 tumors. The macrophages were then treated without exosomes or with CFSE-labeled exosomes in vitro over a 24-hour period (Figure 3A). FACS analysis confirmed the purity and survival of monocyte-derived tumor macrophages based on CD11b expression (Figure 3B). Additionally, all the TD macrophages were $CFSE^+$ when treated with labeled exosomes compared to those without exosome treatment (Figure 3B). These results verified that cells isolated were indeed TD macrophages and that all of them could bind the exosomes.

Next, we proceeded to perform a qPCR analysis on TD macrophages treated with exosomes to study how M1 and M2 gene expression levels were affected. To isolate enough TD macrophages for this experiment, we harvested TD macrophages

from several animals bearing 9609 tumors and pooled the macrophages and re-distributed them across multiple replicates (Figure 3C). TD macrophages were treated without exosomes, with GFP (MEK1-low) exosomes, or MEK1-hi exosomes over 24hrs at various concentrations and number of treatments. Similar to previous experiments on BMDM, qPCR results from TD macrophages varied across experiments (Figure 3D). Despite the variability, we noticed consistent gene upregulation of TNF-α when TD macrophages were treated with exosomal MEK1 relative to those without exosomes. Occasionally, IL-12 gene induction by exosomes was detected. However, both cytokines were induced regardless of MEK1 levels in the exosomes. Averages of M1 and M2 genes from all qPCR results further support TNF-α and IL-12 gene upregulation (Figure 3E). On average, iNOS gene expression was also upregulated by MEK1-low exosomes and further stimulated by MEK1-hi exosomes. Despite the variability, MEK1-expressing exosomes also seemingly activate M2 genes. In conjunction with previous data on BMDM, these results demonstrate that MEK1-expressing exosomes can induce macrophage activity when compared to no treatment.

Human mesenchymal stem cells can be engineered to overexpress MEK1

Next, we proceeded to translate our findings into human models as well. To this end, we generated a new cell line using human telomerase immortalized mesenchymal stem cells (hTERT-MSCs). Since MEK1 was successfully overexpressed in our mouse 9609 fibrosarcoma cell lines via retroviral transduction, we similarly forced MEK1 expression in hTERT-MSCs (MSC-MEK1) via lentiviral transduction. As a control cell line, hTERT-MSCs were separately transduced with GFP (MSC-GFP). Across 3

different primer sets, qPCR data revealed an average of 20-fold increase in MEK1 expression in MSC-MEK1 cells, relative to MEK1 expression in MSC-GFP (Figure 4A). To further validate MEK1 overexpression, we performed western blot to show upregulation of MEK1 protein in the cytoplasm (Figure 4B). However, due to low exosome yield from MSCs compared to tumor cells, we could not perform western blot to confirm MEK1 expression in MSC-derived exosomes. These results demonstrate that human cells can be engineered to highly express MEK1 in the cytoplasm, while overexpression in the exosomes is still yet to be confirmed. Ultimately, exosomes derived from MSC-MEK1 can be tested for their anti-tumor activities on human blood monocytes and for potential clinical applications in the future.

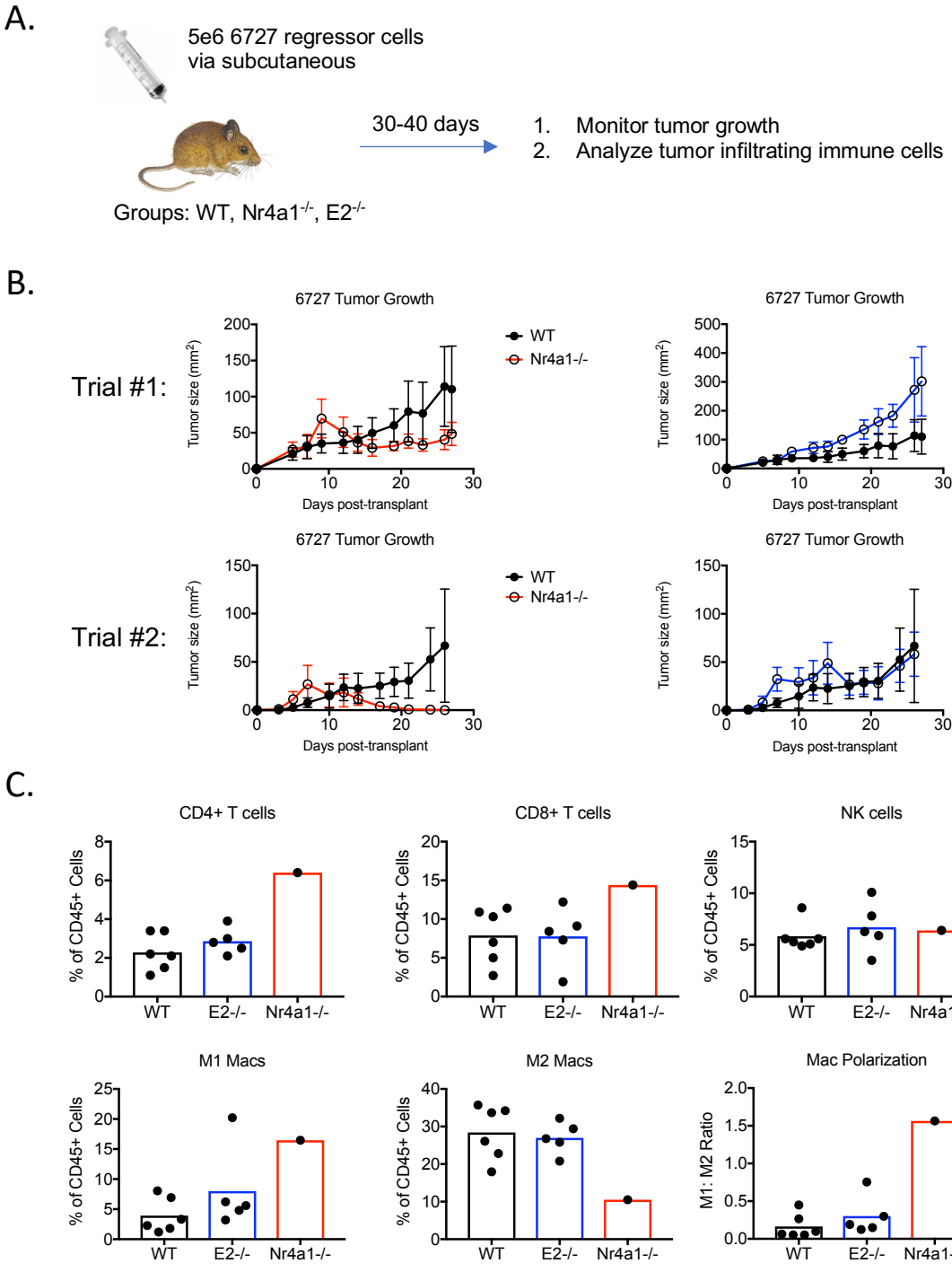


Figure 1. Patrolling monocytes regulate tumor growth at an early timepoint. (A) Experimental design depicting subcutaneous injection of 5e6 6727 regressor tumor cells over the course of 30 to 40 days. Two trials were performed (n=5 or 6). (B) 6727 regressor tumor growth comparing WT, Nr4a1$^{-/-}$, and E2$^{-/-}$ mice. (C) Immuno-analysis of the percentage of CD4^{+} T cells, CD8^{+} T cells, NK cells, M1 macrophages, M2 macrophages, and M1:M2 ratio within 6727 regressor tumors grown in WT, Nr4a1$^{-/-}$, or E2$^{-/-}$.

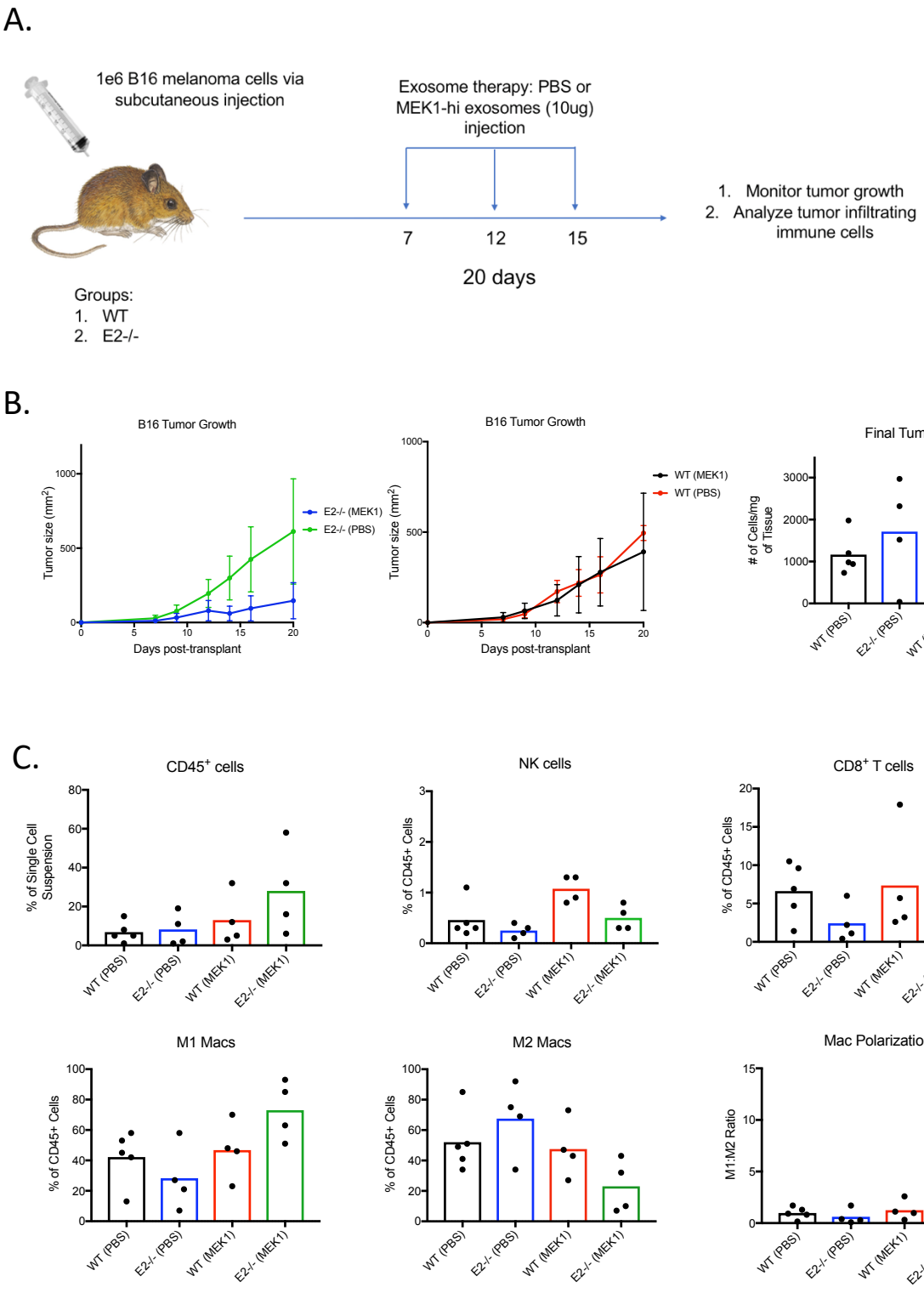
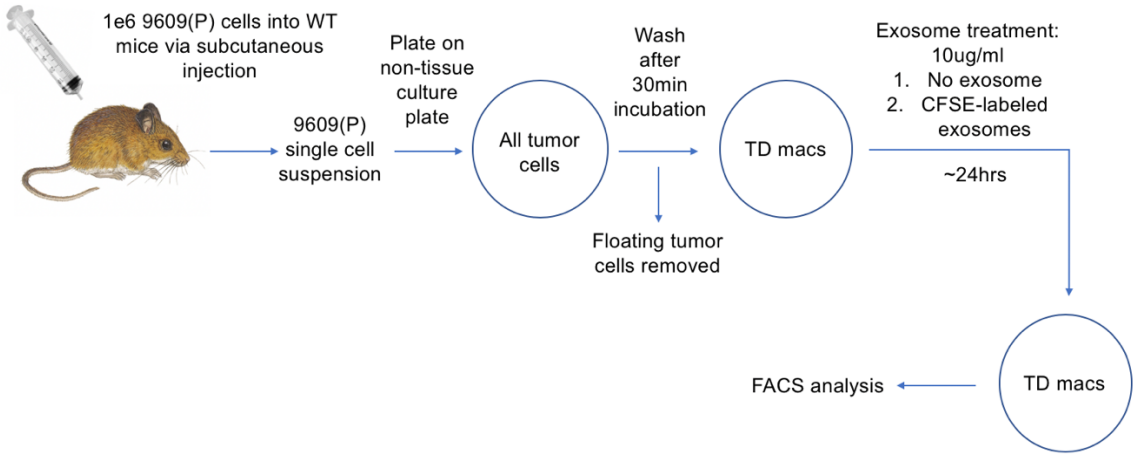


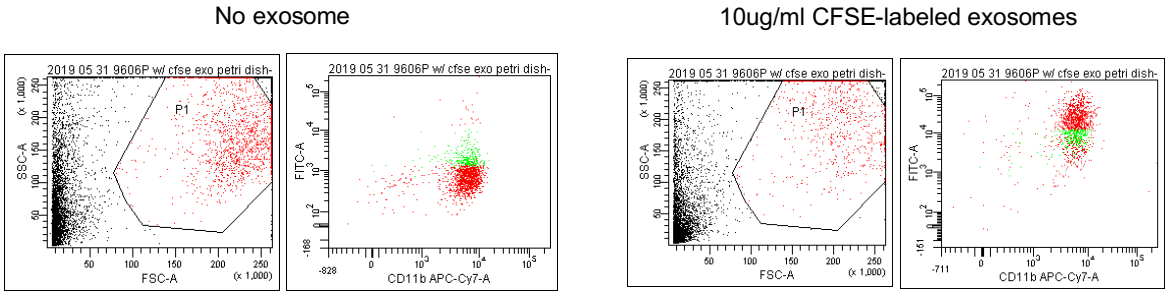
Figure 2. Patrolling monocytes do not mediate the anti-tumor effects of exosomal MEK1. (A) Experimental design showing subcutaneous injection of 1e6 B16 melanoma cells. Tumors were injected intratumorally with either PBS or 10ug of MEK1-hi exosomes on days 7, 12, and 15. (B) Tumor growth, weight, and (C) immuno-analysis of tumor infiltrating CD45^{+} cells, NK cells, CD8^{+} T cells, and macrophages comparing WT tumors with E2$^{-/-}$ tumors injected with PBS or MEK1-hi exosomes (n=5).

Figure 3. MEK1-containing exosomes activate bone marrow-derived macrophages and tumor-derived macrophages. (A) Design of experiment showing injection of 1e6 9609 parent tumor cells and isolation of TD macrophages. The isolated macrophages were treated either without exosomes or with 10ug of CFSE-labeled exosomes in vitro. (B) FACS analysis showing survival and purity of TD macrophage treated with CFSE-labeled exosomes using CD11b marker. Analysis of CFSE expression indicating exosomal uptake by TD macrophages. (C) Schematic illustrating isolation of TD macrophages from 9609(P) tumors (n=5). TD macrophages were treated without exosomes, with GFP (control) exosomes, or with MEK1-hi exosomes over a 24hr period before qPCR analysis. (D) Individual and (E) average qPCR data showing M1 and M2 gene expression of 9609 TD macrophages with or without exosome treatment. TNF-α, iNOS, and IL-12 represent M1 genes. Ym, Arg1, and Gas3 represent M2 genes. Expression levels were normalized to mouse 18S transcripts.

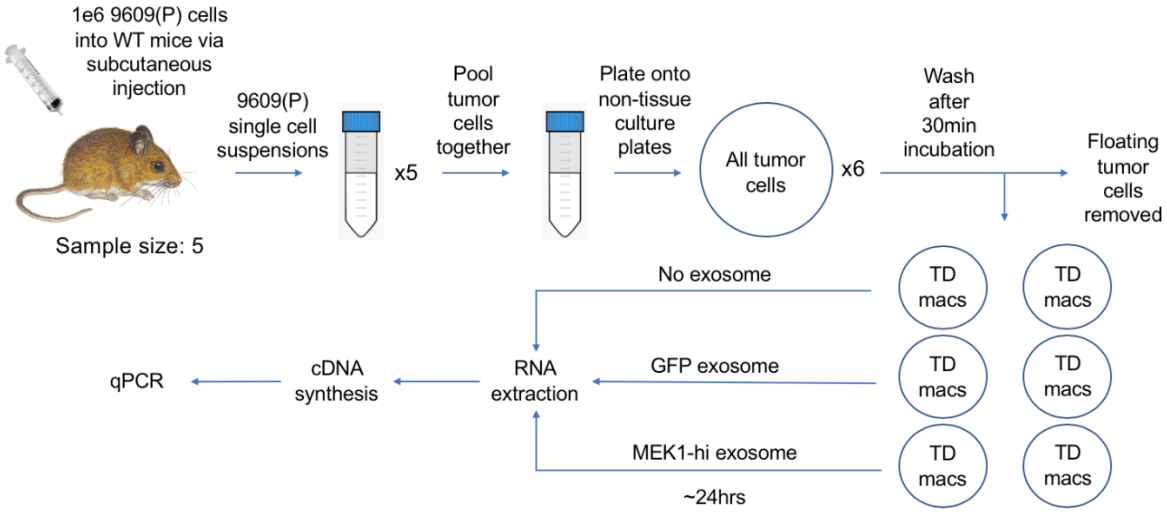
A.


1e6 9609(P) cells into WT mice via subcutaneous injection
9609(P) single cell suspension
Plate on non-tissue culture plate
All tumor cells
Wash after 30min incubation
Floating tumor cells removed
TD macs
Exosome treatment:
10ug/ml
1. No exosome
2. CFSE-labeled exosomes
~24hrs
TD macs
FACS analysis

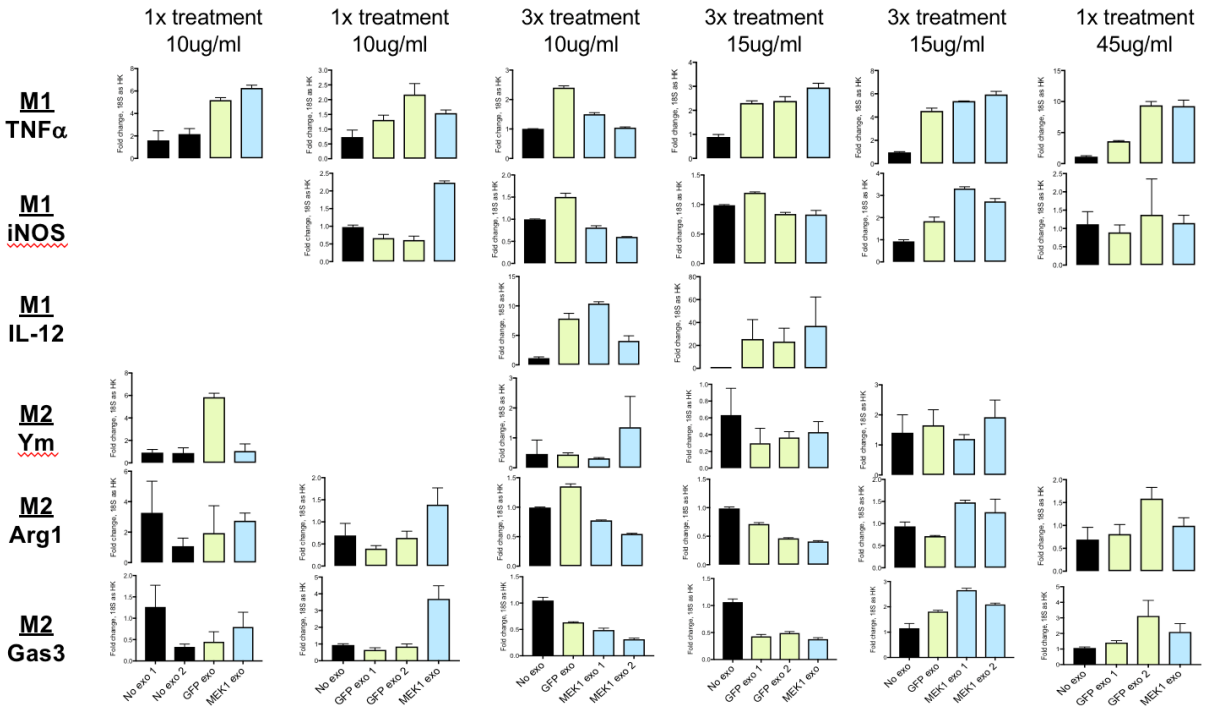
B.


No exosome
10ug/ml CFSE-labeled exosomes
2019 05 31 9606P w/ cfse exo petri dish-
P1
SSC-A
FSC-A
FITC-A
CD11b APC-Cy7-A

C.


1e6 9609(P) cells into WT mice via subcutaneous injection
Sample size: 5
9609(P) single cell suspensions
x5
Pool tumor cells together
Plate onto non-tissue culture plates
All tumor cells
x6
Wash after 30min incubation
Floating tumor cells removed
No exosome
GFP exosome
MEK1-hi exosome
~24hrs
TD macs
RNA extraction
cDNA synthesis
qPCR

D.



E.

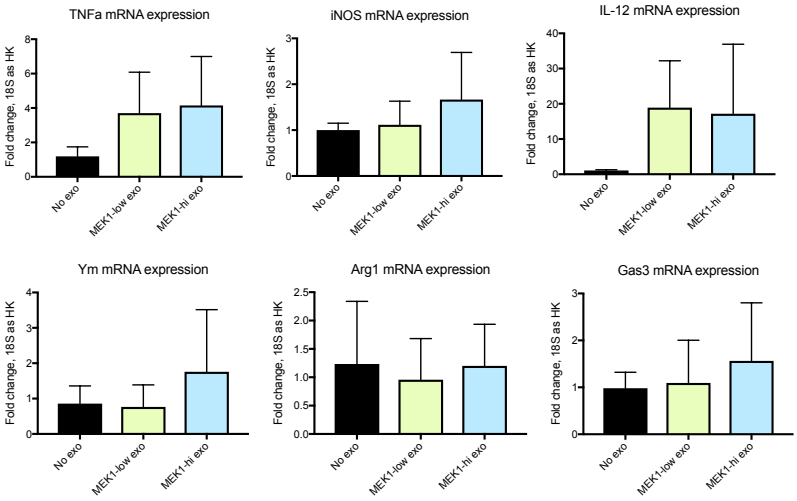


Figure 3. MEK1-containing exosomes activate bone marrow-derived macrophages and tumor-derived macrophages, Continued. (A) Design of experiment showing injection of 1e6 9609 parent tumor cells and isolation of TD macrophages. The isolated macrophages were treated either without exosomes or with 10ug of CFSE-labeled exosomes in vitro. (B) FACS analysis showing survival and purity of TD macrophage treated with CFSE-labeled exosomes using CD11b marker. Analysis of CFSE expression indicating exosomal uptake by TD macrophages. (C) Schematic illustrating isolation of TD macrophages from 9609(P) tumors (n=5). TD macrophages were treated without exosomes, with GFP (control) exosomes, or with MEK1-hi exosomes over a 24hr period before qPCR analysis. (D) Individual and (E) average qPCR data showing M1 and M2 gene expression of 9609 TD macrophages with or without exosome treatment. TNF-α, iNOS, and IL-12 represent M1 genes. Ym, Arg1, and Gas3 represent M2 genes. Expression levels were normalized to mouse 18S transcripts.

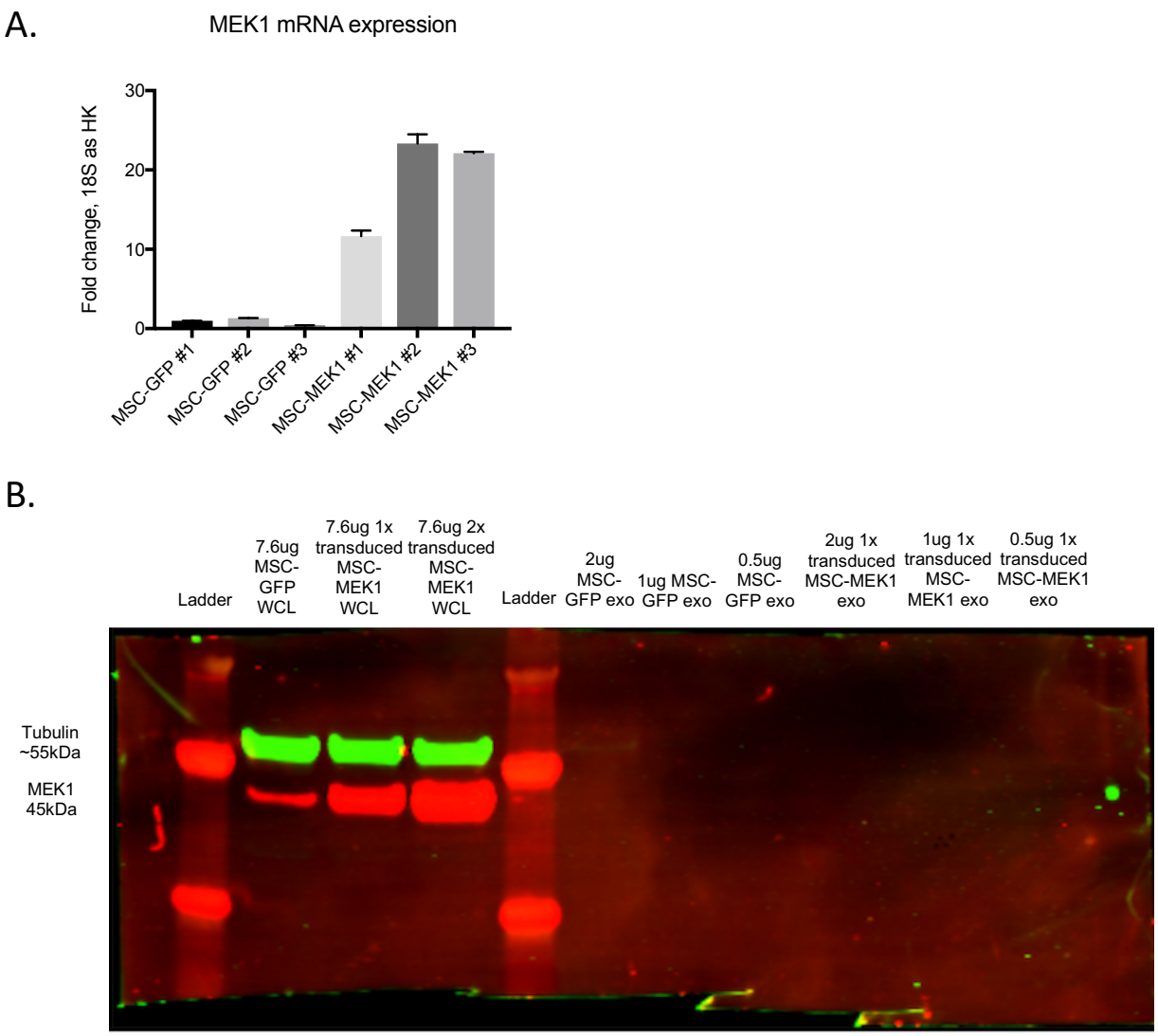


Figure 4. Human mesenchymal stem cells can be engineered to overexpress MEK1. (A) qPCR analysis comparing MEK1 expression between GFP-transduced MSCs and MEK1-transduced MSCs. 3 different human MEK1 primers were tested. Expression level were normalized to mouse 18S transcripts. (B) Western blot comparing MEK1 protein expression in the cytoplasm and exosomes between MSC-GFP and MSC-MEK1. Tubulin was used as loading control.

III.

Discussion

This thesis describes a novel view of MEK1 protein and the therapeutic capabilities of exosomes in cancer treatment. Current knowledge on MEK1 is generally limited to its role in the MAPK/ERK pathway as a cell-intrinsic modulator for cell growth and survival [22]. Mutations of this gene often upregulates the pathway and leads to tumorigenesis of many cancers [22]. Naturally, suppression of MEK1 halts the growth cycle, thereby impeding tumor progression [23]. Generally, this has been the dogma for the function of MEK1. Therefore, it was astonishing to discover that MEK1 overexpression in tumors and in tumor-derived exosomes hindered tumor growth. More remarkably, administration of exosomal MEK1 directly into tumors enhanced anti-tumor immunity against cancer, further contradicting the oncogenic view of MEK1. While MEK1 has inherent properties of cell proliferation, we discovered that MEK1 proteins in exosomes have cell-extrinsic properties to stimulate both the innate and adaptive immune system [18]. Specifically, inoculation of MEK1-hi exosomes yielded increased infiltration of immune cells, particularly M1 macrophages, NK cells, and T cells. M1-like macrophages are known to have anti-tumor properties, and studies have shown that macrophages can phagocytose TD exosomes to promote a shift in polarization states to target cancer more efficiently [24]. In our previous work, monocyte-derived macrophages were shown to be the only cells that engulf MEK1-hi exosomes, thereby suggesting a direct activation of macrophages against cancer. In turn, increased macrophages and NK cells within the tumor can prime T cells of the adaptive immune system to enhance tumor clearance. However, our current studies found inconsistency among M1 and M2 gene activation, and our data did not support the hypothesis that exosomal MEK1 overexpression drives tumor-associated macrophages to have M1-like

phenotype or inhibit M2 polarization. Instead, they implicate that macrophages are stimulated merely by the presence of exosomes. On the other hand, other M1 and M2 genes may have been affected on a grander scale.

Whereas macrophages directly interact with exosomal MEK1, NK cells were shown to be indirectly recruited through a mechanism still yet to be discovered. Recent studies have revealed patrolling monocytes as important leukocytes capable of secretion chemokines to recruit NK cells upon induction by TD exosomes. [16, 17]. Contrary to these findings, our study revealed that exosomal MEK1 delays tumor growth independent of patrolling monocytes, and MEK1-induced recruitment of NK cells is still unknown. However, crosstalk between macrophages and NK cells are known to be involved in anti-tumor immunity, through macrophage-secreted chemokines and cytokines [25]. Studies have shown that MIP-1α or CXCL10 were known macrophage-secreted chemokines that enlist NK cells to directly kill cancer cells in the tumor [25]. Therefore, it is plausible that increased NK cell population within the tumor is due to macrophage activation upon treatment of exosomal MEK1, but still independent of patrolling monocytes.

Our study also brought to light the limitations of MEK1 inhibitors. Over the past decade, only a few MEK1 inhibitors have been FDA-approved, such as trametinib and cobimetinib [26]. These inhibitors are often used in combination with other drugs, such as BRAF inhibitor to treat melanoma or anti-PD1 to treat lung cancer [27]. However, clinical studies demonstrated only minimal effects of MEK1 inhibitors against tumors and unveiled some challenges impeding full efficacy of the drugs [28]. Since we found that MEK1-hi exosomes could induce tumor rejection, our studies suggest that the use

of MEK1 inhibitors could paradoxically limit tumor immunity mediated by immune-stimulatory exosomes. In fact, a recent study has shown that downregulation of MEK1 within macrophages actually promotes M2 macrophage polarization to exhibit reparative properties, thus enhancing tumorigenesis [29]. This correlates with our data that MEK1-hi exosome treatment not only required MEK1 activity but also increased and decreased M1 and M2 macrophage population, respectively. This observation implicates the anti-tumor effects of MEK1 on macrophage polarization. Ultimately, our observations highlight some limitations of MEK1 inhibitors and demonstrate the importance of exosomal MEK1 in immune activation.

While this newly discovered function of MEK1 is only supported by our mouse models, our data suggests human cells can be augmented to overexpress MEK1. Interest has grown recently in the therapeutic values of MSC-derived exosomes in cancer therapy. A recent study achieved success in targeting KRAS mutation in pancreatic cancer through delivery of siRNA or shRNA in MSC-derived exosomes [30]. In addition, MSC-derived exosomes are not only protected from immune targeting but also do not exhibit cytotoxic effects harmful for the body as opposed to delivery through other foreign carriers [31]. General safety and effectiveness of MSC-derived exosomes will prove useful for our study of exosomal MEK1 in humans. While our data proved MEK1 overexpression in the cytoplasm of our human MSC cell line, detection of MEK1 protein in the exosomes proved difficult due to the low exosome yield. Unlike tumor cells, normal cells release exosomes in lower abundance. Therefore, other exosome isolation techniques need to be considered and implemented to account for a different cell line.

In the near future, success in generating human MSCs to overexpress MEK1 in the exosomes will allow further examination of this new role of MEK1 in anti-tumor immunity.

To fully comprehend the mechanism of how exosomal MEK1 triggers immune response, future studies should focus on the changes in macrophages upon treatment of MEK1-hi exosomes through RNA-seq experiments. We propose that exosomal MEK1 uptake increases the ability of macrophages as antigen presenting cells to recruit NK cells and prime T cells against cancer. Further testing will also determine the effects of MSC-derived exosomes expressing MEK1 on human blood monocytes to investigate whether MEK1-mediated immunity can be replicated in humans. Understanding the mechanism of action and human applications of exosomal MEK1 will bring forth a new immunotherapeutic treatment against cancer.

IV.

Materials and Methods

Cell culture and cell line generation

9609 MCA sarcoma cells, 6727 MCA sarcoma cells, and B16 melanoma cells were cultured in RPMI 1640 medium along with 10% fetal bovine serum, 1% MEM non-essential amino acids, 1% sodium pyruvate, 1% 200mM L-glutamine, 1% penicillin streptomycin, 0.5% sodium bicarbonate, and 0.3% beta-mercaptoethanol (Gibco by Life Technologies).

Human telomerase reverse transcriptase-immortalized mesenchymal stem cells (hTERT-MSCs) were obtained from the Klemke laboratory and cultured in alpha-MEM medium with 16.5% fetal bovine serum, 1% 1M HEPES, 1% 200mM L-glutamine, and 1% antibiotic antimycotic (Gibco by Life Technologies). hTERT-MSCs were transduced with human MAP2K1 mammalian gene expression lentiviral vector and treated with puromycin to eliminate cells without the vector.

Mice

Wildtype mice with C57BL/6 background used for all mouse experiments were bred in-house. $E2^{-/-}$ and $Nr4a1^{-/-}$ mice were provided by the Catherine Lynn Hedrick laboratory and were further bred in-house. All experiments were conducted using male mice.

Exosome isolation and CFSE exosome staining

Cells were grown to 40-50% confluency before replacing media with exosome-depleted media, made via ultracentrifugation of fetal bovine serum at 100,000 xg for 70 minutes before addition to media. After 48 hours of culture, conditioned media was

collected and centrifuged at 500 xg for 10 minutes to remove dead cells, followed by ultracentrifugation at 20,000 xg for 20 minutes at 4°C to remove cellular debris. Supernatant was then collected and centrifuged at 100,000 xg for 70 minutes at 4°C, resulting in an exosome pellet. Exosomes were resuspended in HBSS (w/ $Ca^{2+}Mg^{2+}$) before undergoing an additional spin with the previous settings to purify exosomes from soluble proteins. CFSE labeling was done using CellTrace™ CFSE Cell Proliferation Kit (Thermofisher). Pellet was resuspended in HBSS (w/ $Ca^{2+}Mg^{2+}$) with CFSE dye at a concentration of 25 uM. Exosomes were incubated in the dark for 30 mins at 37°C and centrifuged at 100,000 xg for 70 minutes at 4°C. Lastly, exosome pellet was resuspended in HBSS (w/ $Ca^{2+}Mg^{2+}$) and frozen for future use.

CFSE fluorescence analysis on beads

To confirm exosomes were fully labeled with CFSE dye, 1 uL of 4 um diameter aldehyde/sulfate latex beads (Thermo Fisher) were used with 1 ug to 10 ug of exosomes. HBSS was added to reach a volume of 30 uL, following with a 30-minute incubation at room temperature. Another 170 uL of HBSS was added, and samples were incubated while spinning for another 2.5 hours at room temperature. Samples were then treated with 110 uL of 1M glycine and incubated while spinning for 30 minutes at room temperature. Samples then underwent 2 sets of washes with PBS (w/ 0.5% BSA) before resuspended in FACS staining buffer for analysis.

Bicinchoninic acid assay (BCA)

To determine protein concentration of exosomes, bovine serum albumin (BSA) is prepared at 2 mg/ml with 6 serial dilutions to set up the standard curve ranging from 1,000 mg/ml to 15.6 mg/ml. BSA and samples of interest were added at 25 ul. BCA working reagents (WR) A and B are mixed fresh at a ratio of 50:1 (WR A:B) and added at a 1:8 ratio of sample to WR. Samples were incubated at 37°C for 30 minutes before analysis under spectrophotometer.

Injections of tumor cells and exosomes in mice

In preparation for injections, all tumor cells were trypsinized at 37°C for 3 to 5 minutes and washed 3 times with HBSS with Ca^{2+} and Mg^{2+}. Progressors and regressors were subcutaneously injected at $1x10^6$ cells and $5x10^6$ cells, respectively. All exosome experiments in vivo were done via intratumoral injection at a concentration of 0.1 ug/ul.

Tumor growth experiments

For all tumor growth experiments, measurements were taking every 2 or 3 days starting on day 3 or 4 post tumor transplantation. The cross sections of tumors were measured; the average and standard deviation within the sample group were determined and plotted onto an xy graph to follow the progression of tumor growth.

Tumor harvest and immune-analysis by flow cytometry

Tumors were grown for a duration of 20 to 30 days depending on the experiment. During harvest, tumors were weighted, diced, and incubated while shaking in HBSS (w/ $Ca^{2+}Mg^{2+}$) with 10% collagenase for 30 minutes at 37°C. Samples were spun down, resuspended in 10mL cold HBSS (w/ $Ca^{2+}Mg^{2+}$), and filtered through 70 μM cell strainers into new conical tubes. Cells were centrifuged to single cell suspensions and incubated with antibodies: 1:100 anti-CD45 (Biolegend, Clone 30-F11), 1:100 anti-F4/80 (Biolegend, Clone BM8), 1:100 anti-CD11b (Biolegend, Clone M1/70), 1:200 anti-MHC-II (Biolegend, Clone M5/114 15.2), 1:100 anti-CD3 (Biolegend, Clone 17A.2), 1:100 anti-CD4 (Biolegend, Clone GK1.5), 1:100 anti-CD8 (Biolegend, Clone 53-6.7), 1:100 anti-NK1.1 (Biolegend, Clone PK136), 1:100 anti-CD45.2 (Biolegend, Clone 104), 1:200 anti-Ly6G (Biolegend, Clone 1A8), and 1:100 anti-Ly6C (Biolegend, Clone HK1.4). Cells were stained with 1:500 7- Aminoactinomycin D (7AAD) to identify live/dead cells.

Tumor-derived macrophage isolation, exosome treatment, and flow cytometry analysis

Tumors were grown between 10 and 20 days before harvest using the same method as mentioned above. Once samples were filtered and spun down to a single cell suspensions, pellets were resuspended in media and plated onto 35 mm x 10 mm non-tissue culture treated plates. After 30 minutes of incubation at 37°C, media was removed along with tumor cells, and TD macrophages were washed in HBSS twice before replenishing with media. TD macrophages were treated with exosomes at a concentration of 15 ug/ml and incubated overnight. After 24 hours, cell identity, survival,

and exosome uptake were analyzed using flow cytometry. Macrophages were washed twice in HBSS (w/o Ca^{2+} Mg^{2+}), trypsinized, and scrapped before centrifuging in FACS buffer. Cells were incubated with FACS buffer (w/ Fc block) and either 1:100 anti-F4/80 (Biolegend, Clone BM8) or 1:250 anti-CD11b (Biolegend, Clone M1/70) for 10 minutes. Cells were resuspended in FACS staining buffer with 1:500 7AAD to isolate dead cells.

RNA isolation, cDNA synthesis, real-time qPCR

All RNA isolations were performed using TRIzol reagent® (Life Technologies), and High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to synthesize cDNA. Samples were run using a veriti thermal cycler (Applied Biosystems) under the following conditions: 10 minutes at 25°C, 120 minutes at 37°C, and 5 minutes at 85°C. Real time quantitative polymerase chain reaction was performed using SYBR Green PCR Master Mix (Applied Biosystems). Gene expressions were measured with QuantStudio Real-Time PCR System (Applied Biosystems) and analyzed using the $2^{-\Delta\Delta Ct}$ method. Mouse 18S (m18S) was used as the housekeeping gene for normalization, with the primer sequences being: m18S forward 5'-GTAACCCGTTGAACCCCATT-3', m18S reverse 5'-CCATCCAATCGGTAGTAGCG-3'. Primers of interest have the following sequences: mTNFa forward 5'-GGCAGGTCTACTTTGGAGTCA-3', mTNFa reverse 5'-ACATTCGAGGCTCCAGTGAATT-3', miNos2 forward 5'-GTTCTCAGCCCAACAATACAAGA-3', miNos2 reverse 5'-GTGGACGGGTCGATGTCAC-3', mIL-12 forward 5'-AGACCCTGCCCATTGAACTG-3', mIL-12 reverse 5'-GGCGGGTCTGGTTTGATGAT-3', mArg1 forward 5'-CAAGACAGGGCTCCTTTCAG-3', mArg1 reverse 5'-CACCTCCTCTGCTGTCTTCC-3',

human MAP2K1 #1 (hMAP2K1 #1) forward 5'-TCTGCAGTTAACGGGACCAG-3', hMAP2K1 #1 reverse 5'-AGCTCTAGCTCCTCCAGCTT-3', hMAP2K1 #2 forward 5'-CTCTGCAGTTAACGGGACCA-3', hMAP2K1 #2 reverse 5'-CTCCCACCTTCTGCTTCTGG-3', hMAP2K1 #3 forward 5'-CTGCAGTTAACGGGACCAGC-3', hMAP2K1 #3 reverse 5'-CAAGCTCTAGCTCCTCCAGC-3'.

Western blot

Whole cell lysate is obtained from cells at 80-90% confluency. After 3 washes with HBSS (w/wo $Ca^{2+}Mg^{2+}$), cells were incubated in RIPA buffer with 1:200 proteinase inhibitor (Sigma #8340) for 10 mins on ice. Lysate is then spun at max speed for 10 minutes at 4°C.

Lysate and exosome samples were mixed with 1x Laemmli loading buffer and 10% BME before loaded onto SDS-PAGE. PVDF membrane was briefly soaked in MeOH before transfer into equilibrium buffer until proteins were fractionated. Gel was then transferred onto the membrane and into the transfer-apparatus (Bio-rad). Membrane was then quickly transferred into block buffer for 30 minutes to 1 hour before incubating in primary (1°) antibodies: anti-mouse tubulin (1:1000), anti-mouse ß-actin (1:1000), anti-rabbit CD3 (1:1000), and anti-rabbit MEK1 (1:1000). After overnight incubation at 4°C, membrane was washed 3 times with wash buffer for 10 minutes each. For 30 minutes in the dark, membrane was then soaked in blocking buffer with secondary (2°) antibodies: goat anti-rabbit and goat anti-mouse (1:10,000). After 3

washes for 10 minutes each, membrane was soaked in PBS to be analyzed using a western blot detection system (Licor).

References

1. Loeb, L. A., Loeb, K. R., & Anderson, J. P. (2003). Multiple mutations and cancer. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(3), 776–781. https://doi.org/10.1073/pnas.0334858100

2. Mittelbrunn, M., & Sánchez-Madrid, F. (2012). Intercellular communication: Diverse structures for exchange of genetic information. *Nature Reviews. Molecular Cell Biology*, *13*(5), 328–335. https://doi.org/10.1038/nrm3335

3. Schwager, S. C., Taufalele, P. V., & Reinhart-King, C. A. (2018). Cell–Cell Mechanical Communication in Cancer. *Cellular and Molecular Bioengineering*, *12*(1), 1–14. https://doi.org/10.1007/s12195-018-00564-x

4. Becker, A., Thakur, B. K., Weiss, J. M., Kim, H. S., Peinado, H., & Lyden, D. (2016). Extracellular vesicles in cancer: Cell-to-cell mediators of metastasis. *Cancer Cell*, *30*(6), 836–848. https://doi.org/10.1016/j.ccell.2016.10.009

5. Harding, C. V., Heuser, J. E., & Stahl, P. D. (2013). Exosomes: Looking back three decades and into the future. *The Journal of Cell Biology*, *200*(4), 367–371. https://doi.org/10.1083/jcb.201212113

6. Xiao, Y., Zhong, J., Zhong, B., Huang, J., Jiang, L., Jiang, Y., Yuan, J., Sun, J., Dai, L., Yang, C., Li, Z., Wang, J., & Zhong, T. (2020). Exosomes as potential sources of biomarkers in colorectal cancer. *Cancer Letters*, *476*, 13–22. https://doi.org/10.1016/j.canlet.2020.01.033

7. Théry, C. (2011). Exosomes: Secreted vesicles and intercellular communications. *F1000 Biology Reports*, *3*. https://doi.org/10.3410/B3-15

8. Xiao, D., Barry, S., Kmetz, D., Egger, M., Pan, J., Rai, S. N., Qu, J., McMasters, K. M., & Hao, H. (2016). Melanoma cell-derived exosomes promote epithelial-mesenchymal transition in primary melanocytes through paracrine/autocrine signaling in the tumor microenvironment. *Cancer Letters*, *376*(2), 318–327. https://doi.org/10.1016/j.canlet.2016.03.050

9. Statello, L., Maugeri, M., Garre, E., Nawaz, M., Wahlgren, J., Papadimitriou, A., Lundqvist, C., Lindfors, L., Collén, A., Sunnerhagen, P., Ragusa, M., Purrello, M., Di Pietro, C., Tigue, N., & Valadi, H. (2018). Identification of RNA-binding proteins in exosomes capable of interacting with different types of RNA: RBP-facilitated transport of RNAs into exosomes. *PLoS ONE*, *13*(4). https://doi.org/10.1371/journal.pone.0195969

10. Whiteside, T. L. (2016). Tumor-derived exosomes and their role in cancer progression. *Advances in Clinical Chemistry*, *74*, 103–141. https://doi.org/10.1016/bs.acc.2015.12.005

11. Abd Elmageed, Z. Y., Yang, Y., Thomas, R., Ranjan, M., Mondal, D., Moroz, K., Fang, Z., Rezk, B. M., Moparty, K., Sikka, S. C., Sartor, O., & Abdel-Mageed, A. B. (2014). Neoplastic reprogramming of patient-derived adipose stem cells by prostate cancer cell-associated exosomes. *Stem Cells (Dayton, Ohio)*, *32*(4), 983–997. https://doi.org/10.1002/stem.1619

12. Kucharzewska, P., Christianson, H. C., Welch, J. E., Svensson, K. J., Fredlund, E., Ringnér, M., Mörgelin, M., Bourseau-Guilmain, E., Bengzon, J., & Belting, M. (2013). Exosomes reflect the hypoxic status of glioma cells and mediate hypoxia-dependent activation of vascular cells during tumor development. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(18), 7312–7317. https://doi.org/10.1073/pnas.1220998110

13. Bretz, N. P., Ridinger, J., Rupp, A.-K., Rimbach, K., Keller, S., Rupp, C., Marmé, F., Umansky, L., Umansky, V., Eigenbrod, T., Sammar, M., & Altevogt, P. (2013). Body Fluid Exosomes Promote Secretion of Inflammatory Cytokines in Monocytic Cells via Toll-like Receptor Signaling. *The Journal of Biological Chemistry*, *288*(51), 36691–36702. https://doi.org/10.1074/jbc.M113.512806

14. Gutiérrez-Vázquez, C., Villarroya-Beltri, C., Mittelbrunn, M., & Sánchez-Madrid, F. (2013). Transfer of extracellular vesicles during immune cell-cell interactions. *Immunological Reviews*, *251*(1), 125–142. https://doi.org/10.1111/imr.12013

15. Wolfers, J., Lozier, A., Raposo, G., Regnault, A., Théry, C., Masurier, C., Flament, C., Pouzieux, S., Faure, F., Tursz, T., Angevin, E., Amigorena, S., & Zitvogel, L. (2001). Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nature Medicine*, *7*(3), 297–303. https://doi.org/10.1038/85438

16. Hanna, R. N., Cekic, C., Sag, D., Tacke, R., Thomas, G. D., Nowyhed, H., Herrley, E., Rasquinha, N., McArdle, S., Wu, R., Peluso, E., Metzger, D., Ichinose, H., Shaked, I., Chodaczek, G., Biswas, S. K., & Hedrick, C. C. (2015). Patrolling Monocytes Control Tumor Metastasis to the Lung. *Science (New York, N.Y.)*, *350*(6263), 985–990. https://doi.org/10.1126/science.aac9407

17. Plebanek, M. P., Angeloni, N. L., Vinokour, E., Li, J., Henkin, A., Martinez-Marin, D., Filleur, S., Bhowmick, R., Henkin, J., Miller, S. D., Ifergan, I., Lee, Y., Osman, I., Thaxton, C. S., & Volpert, O. V. (2017). Pre-metastatic cancer exosomes induce immune surveillance by patrolling monocytes at the metastatic niche. *Nature Communications*, *8*(1), 1319. https://doi.org/10.1038/s41467-017-01433-3

18. Searles, S. C. (2018). *Exosomes and cell-cell fusion in cancer* [UC San Diego]. PhD Dissertation. https://escholarship.org/uc/item/5jj2q5kp

19. O'Sullivan, T., Saddawi-Konefka, R., Gross, E., Tran, M., Mayfield, S. P., Ikeda, H., & Bui, J. D. (2014). Interleukin-17D mediates tumor rejection through recruitment of natural killer cells. *Cell Reports*, *7*(4), 989–998. https://doi.org/10.1016/j.celrep.2014.03.073

20. Lulli, D., Carbone, M. L., & Pastore, S. (2017). The MEK Inhibitors Trametinib and Cobimetinib Induce a Type I Interferon Response in Human Keratinocytes. *International Journal of Molecular Sciences*, *18*(10). https://doi.org/10.3390/ijms18102227

21. Hanna, R. N., Shaked, I., Hubbeling, H. G., Punt, J. A., Wu, R., Herrley, E., Zaugg, C., Pei, H., Geissmann, F., Ley, K., & Hedrick, C. C. (2012). NR4A1 (Nur77) Deletion Polarizes Macrophages Towards an Inflammatory Phenotype and Increases Atherosclerosis. *Circulation Research*, *110*(3), 416–427. https://doi.org/10.1161/CIRCRESAHA.111.253377

22. McCubrey, J. A., Steelman, L. S., Chappell, W. H., Abrams, S. L., Wong, E. WT., Chang, F., Lehmann, B., Terrian, D. M., Milella, M., Tafuri, A., Stivala, F., Libra, M., Basecke, J., Evangelisti, C., Martelli, A. M., & Franklin, R. A. (2007). ROLES OF THE RAF/MEK/ERK PATHWAY IN CELL GROWTH, MALIGNANT TRANSFORMATION AND DRUG RESISTANCE. *Biochimica et Biophysica Acta*, *1773*(8), 1263–1284. https://doi.org/10.1016/j.bbamcr.2006.10.001

23. Tanaka, R., Tomosugi, M., Sakai, T., & Sowa, Y. (2016). MEK Inhibitor Suppresses Expression of the miR-17-92 Cluster with G1-Phase Arrest in HT-29 Human Colon Cancer Cells and MIA PaCa-2 Pancreatic Cancer Cells. *Anticancer Research*, *36*(9), 4537–4543.

24. Xiao, M., Zhang, J., Chen, W., & Chen, W. (2018). M1-like tumor-associated macrophages activated by exosome-transferred THBS1 promote malignant migration in oral squamous cell carcinoma. *Journal of Experimental & Clinical Cancer Research : CR*, *37*. https://doi.org/10.1186/s13046-018-0815-2

25. Glas, R., Franksson, L., Une, C., Eloranta, M.-L., Öhlén, C., Örn, A., & Kärre, K. (2000). Recruitment and Activation of Natural Killer (Nk) Cells in Vivo Determined by the Target Cell Phenotype. *The Journal of Experimental Medicine*, *191*(1), 129–138.

26. Cheng, Y., & Tian, H. (2017). Current Development Status of MEK Inhibitors. *Molecules : A Journal of Synthetic Chemistry and Natural Product Chemistry*, *22*(10). https://doi.org/10.3390/molecules22101551

27. Lee, J. W., Zhang, Y., Eoh, K. J., Sharma, R., Sanmamed, M. F., Wu, J., Choi, J., Park, H. S., Iwasaki, A., Kaftan, E., Chen, L., Papadimitrakopoulou, V., Herbst, R. S., & Koo, J. S. (2019). The Combination of MEK inhibitor with Immunomodulatory Antibodies Targeting PD-1 and PD-L1 Results in Prolonged Survival in Kras/p53-

Driven Lung Cancer. *Journal of Thoracic Oncology : Official Publication of the International Association for the Study of Lung Cancer*, *14*(6), 1046–1060. https://doi.org/10.1016/j.jtho.2019.02.004

28. Zhao, Y., & Adjei, A. A. (2014). The clinical development of MEK inhibitors. *Nature Reviews. Clinical Oncology*, *11*(7), 385–400. https://doi.org/10.1038/nrclinonc.2014.83

29. Long, M. E., Eddy, W. E., Gong, K.-Q., Lovelace-Macon, L. L., McMahan, R. S., Charron, J., Liles, W. C., & Manicone, A. M. (2017). MEK1/2 Inhibition Promotes Macrophage Reparative Properties. *Journal of Immunology (Baltimore, Md. : 1950)*, *198*(2), 862–872. https://doi.org/10.4049/jimmunol.1601059

30. Kamerkar, S., LeBleu, V. S., Sugimoto, H., Yang, S., Ruivo, C. F., Melo, S. A., Lee, J. J., & Kalluri, R. (2017). Exosomes Facilitate Therapeutic Targeting of Oncogenic Kras in Pancreatic Cancer. *Nature*, *546*(7659), 498–503. https://doi.org/10.1038/nature22341

31. Mendt, M., Kamerkar, S., Sugimoto, H., McAndrews, K. M., Wu, C.-C., Gagea, M., Yang, S., Blanko, E. V. R., Peng, Q., Ma, X., Marszalek, J. R., Maitra, A., Yee, C., Rezvani, K., Shpall, E., LeBleu, V. S., & Kalluri, R. (n.d.). Generation and testing of clinical-grade exosomes for pancreatic cancer. *JCI Insight*, *3*(8). https://doi.org/10.1172/jci.insight.99263