UNIVERSITY OF CALIFORNIA SAN DIEGO

Role of Type-I IFN Experience in Regulating Tumor and Immune Cell Activity

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by

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The thesis of Deepakshi Rajiv Kasat is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2023

DEDICATION

To Mumma, Papa and Drishika.

TABLE OF CONTENTS

Table of Contents v

List of Figures vi

List of Tables vii

LIST OF FIGURES

LIST OF TABLES

Abbreviations

CSC cancer stem cells.

IFN interferon.

IFNAR interferon alpha receptor.

IFNGR interferon gamma receptor.

ISG interferon stimulated genes.

LRCC label-retaining cancer cells.

NaB sodium butyrate.

NSG immunodeficient mice.

STAT signal transducer and activator of transcription.

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Lastly, I want to thank my family and friends for being my pillar of strength in this incredible process.

ABSTRACT OF THE THESIS

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Looking for a cure for cancer has been a priority for scientists for over 250 years now, and with 19.3 million new cases recorded globally in 2020, this is becoming even more urgent. If cancerous tumor cells are developed within our own body, the most effective treatment mechanism would be to redirect the body's natural defenses to identify and destroy these cancerous cells. This study addresses the critical gap in understanding the role of IFN experience in the regulation of cancer stem cell (CSC) marker expression in sarcoma, a rare, aggressive cancer type. Leveraging an MX1-Cre-tdTomato mice reporter model, it explores the effects of IFN exposure on CSC markers' expression, hypothesizing that IFN experience enhances the

stem-like properties of these cells. This research is divided into two areas of focus: bone marrow macrophages and tumor cells derived from the reporter models. While bone marrow macrophages did not show significant results, a notable difference in endocytosis activity between red (IFN-experienced) and non-red (IFN-naïve) spleen macrophages was observed. Type I IFN treatment influenced the growth, proliferation, and CSC marker expression in tumor cells differently based on their IFN experience, with non-red cells becoming more stem-like. The research also revealed a potential role of sodium butyrate in modulating hey1 expression, a gene associated with the Notch signaling pathway and tumor progression. These findings advance our understanding of the complex interplay between IFN treatment, gene expression, and cellular characteristics in tumor biology, which could provide novel therapeutic targets.

Chapter 1

Introduction

Cancer continues to be a significant global health burden, with an increasing need to develop innovative treatment modalities to improve patient outcomes. Immunotherapy has emerged as a promising avenue in cancer research, providing a novel approach to harnessing the body's immune system to recognize and eliminate cancer cells. Among the various immunotherapy strategies, interferons (IFNs) have been the subject of extensive investigation due to their antiviral, immunomodulatory, and antiproliferative properties.

IFNs

IFNs, discovered by Jean Lindenmann and Alick Isaacs, are cytokines that trigger an immune response to a viral infection [1]. There are three main types of IFNs: Type I IFNs include IFN-α (alpha) and IFN-β (beta), among other subtypes. They are produced by almost all cells upon viral infection and play a major role in the innate immune response. These bind to a specific cell surface receptor complex known as the IFN-α receptor (IFNAR) that activates a signal transduction pathway [2]. Type II IFN includes only one member of this class, IFN-γ (gamma), which is secreted by T cells and natural killer (NK) cells. IFN-γ signals through a different receptor, the IFN-γ receptor (IFNGR). It is crucial for adaptive immunity against viral and microbial infections. Type III IFNs include IFN-λ (lambda) IFNs [3]. They are structurally similar to type I IFNs and are involved in the immune response at barrier surfaces.

IFNs play a multifaceted role in the immune system. In response to a pathogen, IFNs are produced and secreted by infected cells. Upon release, they can bind to receptors on neighboring cells, triggering a cascade of intracellular signals that lead to the expression of IFN-stimulated genes (ISGs). These ISGs encode proteins that confer an antiviral state in the cells, inhibiting further spread of the infection. In addition to their antiviral effects, IFNs also have potent immunomodulatory properties. They influence the function of the immune system by affecting the activity of natural killer cells, macrophages, and lymphocytes, and play a key role in the regulation of the immune response. Type I IFNs attach to a receptor that consists of two chains, IFNAR1 and IFNAR2, forming a heterodimer. This binding prompts the associated Janus kinases to become active and initiate the process of phosphorylating signal transducer and activator of transcription 1 (STAT1) and 2 (STAT2). This forms a complex that translocates to the cell nucleus. Once in the nucleus, the STAT complex binds to specific DNA sequences, activating the transcription of ISGs.Type II IFN also works through signal activation of the JAK/STAT pathway using the IFNGR [4].

The role of IFN in cancer has been explored for over 50 years now. Ion Gresser's research in 1969 on anti cancerous properties of exogenous IFN opened doors for exploring these cytokines as a treatment option [5]. The role of IFNs in cancer revolves around their ability to: (i) Inhibiting cell proliferation, (ii) enhancing immune response and (iii) modulating the expression of certain genes involved in cell cycle control, apoptosis, and angiogenesis.

Cancer Stem Cells (CSCs)

CSCs are a small group of cancer cells that possess self-renewal and differentiation capacities, enabling them to initiate and sustain tumor growth. These cells have been implicated in tumor initiation, progression, metastasis, and chemotherapy resistance, making them a critical target for the development of effective cancer treatments because they increase the chances of a relapse as shown in Figure 1.1. [6] CSCs origin has been connected to transformation of either stem cells or differentiated cells, where oncogenes are upregulated and tumor suppressor genes

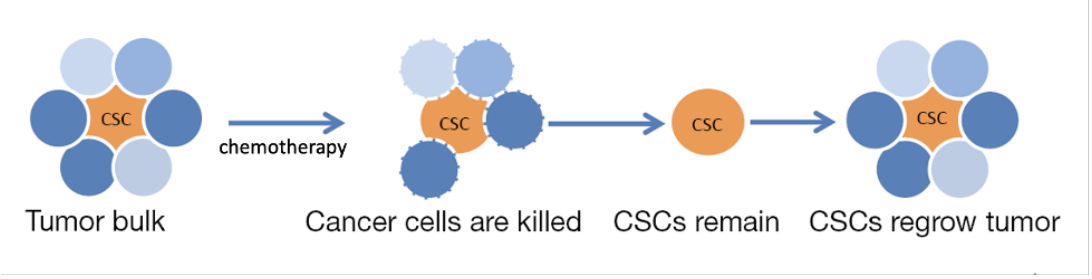

Figure 1.1. CSCs are a critical target: these can survive chemotherapy and increase chances of a relapse.

are downregulated acquiring a CSC property [7].

CSC markers

A number of markers have been identified as potential CSC markers, aiding in their detection and characterization. A few of them along with their role in cancer progression are listed in Table 1.1. [6].

Table 1.1. CSC markers and their role in tumor progression

Marker	Role
CD44 [8]	Proliferation, metastasis and self renewal
CD133 [9]	Sphere forming ability and tumorigenicity
CD326 [10]	Differentiation, proliferation, cell signaling
CD34 [11]	Hematopoietic progenitors, cell adhesion and signaling
CD117 [12]	Cell survival, migration and differentiation
RAE1 [13]	Mitotic checkpoint regulator and increases cell cycle progression
ALDH1A1 [14]	Tumor growth by limiting ROS and reactive aldehydes and initiate carcinogenesis

Anti-CSC role of IFN

Breast cancer: The study by Castiello et al. [15] underscores the importance of endogenous IFN-β signaling in inhibiting the progression of HER2/neu-positive breast cancer and reducing the population of CSCs. They used a murine model with HER2/neu-positive tumors,

where they genetically disrupted the IFN-β signaling pathway. The research showed that the absence of IFN-β signaling led to accelerated tumor growth and increased angiogenesis. In terms of breast CSCs, the absence of IFN-β signaling led to an increase in the CSC population, characterized by the expression of breast CSC marker aldehyde dehydrogenase-1A1. This was further supported by in vitro sphere formation assays that demonstrated an increased ability for CSC self-renewal in the absence of IFN-β signaling. Doherty et al. [16] also studied the role of exogenous type I IFN in triple negative breast CSCs. Their finding concluded that IFNb treatment suppresses CSCs as confirmed by decreased cell migration, reduced ability to form tumor spheres, and the re-expression of CD24. The treatment was observed to induce a less aggressive, more epithelial-like state in cancer cells, marked by the downregulation of proteins like VIMENTIN and SLUG. The study suggests that TNBCs exhibiting CSC-like properties have repressed IFN signaling due to high levels of U-ISGF3, a factor previously connected to resistance against DNA damage. IFN-β treatment appears to mitigate these CSC properties, indicating a potential therapeutic strategy for managing drug-resistant and highly aggressive TNBC tumors.

Glioblastoma: Du et al's paper [17] investigated the effects of treating patient GBM xenolines with human recombinant IFN-α to study glioblastoma CSC proliferation, differentiation, and survival. The results showed that IFN-α treatment significantly inhibited the proliferation of GSCs and reduced stemness. It also restricted sphere forming abilities of these tumor stem cells and prevented them from differentiating. It also stimulated ISG15 expression and transient activation of STAT3.

Colon cancer: Ni et al's research [18] focus on a specific subset of these cells, known as label-retaining cancer cells (LRCC), which are believed to be tumor-initiating cells due to their self-renewing capacity and ability to generate heterogeneous lineages of cancer cells. They resemble CSCs in terms of genes for stemness and marker expression. They found that treatment with IFN-γ selectively induced apoptosis (programmed cell death) in the LRCCs, while non-LRCCs were largely unaffected. They also studied the combined effect of IFNg and

oxaliplatin, suggesting that combining IFNg treatment with chemotherapy could provide an enhanced therapy option.

Pro-CSC role of IFN

Breast cancer: Musella et al's paper [19] showed that KDM1B, an epigenetic regulator, was upregulated with Type I IFN treatment which in turn increased stemness and survival of breast CSCs. By investigating expression of various CSC markers like CD133, CD44 and CD24, they found that the effect of IFN treatment on CSCs was dose and duration dependent. They also concluded that cancer cells can evade therapeutic control by using type I IFN production in response to immunogenic cell death in in vivo and in vitro conditions. They suggested a model that might be promoted by IFN's cytotoxic ability where cancer cells undergoing immunogenic cell death transfer genetic material, including stem-related mRNA, to viable cancer cells, leading to the induction of CSC. Another publication by Qadir et al. established that by knockdown of the STAT1 pathway which reduced CD95/Fas activity and Type I IFN. This resulted in decreased stemness of breast CSCs.

Pancreatic cancer: This research by Zhu et al [20] revealed that type I IFN augments CSC markers' expression like CD24, CD44, and CD133, implying a potential role in promoting CSC in pancreatic ductal adenocarcinoma tumors. They also linked the metastatic and invasiveness of these tumors to CSC marker expression changes. This study offered an intriguing proposition concerning the use of Type I IFN in the treatment of solid tumors, suggesting that IFN$_\alpha$ be administered to patients before chemotherapy commences in a combination therapy setting. This way, it could potentially optimize the effect of subsequent chemotherapy by activating the CSCs first.

Lung cancer: Song et al's [19] study found that IFNγ's impact on non-small cell lung cancer cells is dose-dependent and can either promote or inhibit cancer depending on its concentration. Higher concentrations caused cancer cell apoptosis through the JAK1-STAT1-caspase pathway, whereas lower doses of IFNγ promoted stem-like properties through (ICAM1)-PI3K-

Akt-Notch1 signaling pathway. This was confirmed by blocking ICAM1, which resulted in reduced sphere formation and CSC marker expression. They highlighted the importance of the tumor microenvironment, particularly the level of IFNγ expression, in determining the behavior of cancer cells.

Oral Squamous Cell Carcinoma: Not only did IFNa treatment upregulate expression of ALDH1A1 and CD44, but also that IFNa (primed) exemplified the tumor cytotoxic ability of chemotherapy drugs like Erlotinib and CDDP, suggesting that activating CSCs would make tumor cells more available to chemotherapy drugs. This was shown by Ma et al [21].

Notch pathway and relevant genes (Hey1 and HeyL)

The Notch signaling pathway, largely conserved cell communication system, regulates various aspects of cell differentiation, proliferation, and apoptosis. It plays a crucial role in numerous developmental processes and in the maintenance of adult tissues. The pathway involves the interaction of the Notch receptor (found on the cell surface) with a ligand from a neighboring cell. Once activated by ligand binding, the receptor undergoes a series of cleavages, leading to the release of the Notch intracellular domain, which translocates to the nucleus and regulates gene expression. Both overactivation and downregulation of this pathway seems implicated in various cancers [22].

HEY1 and HEYL are two genes that are part of the Hairy/Enhancer of Split related family of genes, which are direct targets of the Notch signaling pathway. They both encode transcriptional repressors. These repressors play important roles in various developmental processes and can affect the cardiovascular system, skeletal development, and neural development [23] [24].

HEY1 has previously been studied by various researchers, including Xie et al [25] who found that the NOTCH1-HEY1 axis was upregulated in salivary adenoid cystic carcinoma stem cells, and HEY1 was shown to increase cell renewal, proliferative abilities, invasiveness and spreading of tumor. A similar effect was seen in head and neck squamous cell carcinoma stem

cells by Fukusumi et al [26]. HEYL has also shown to increase the metastasising ability of tumor cells in colorectal cancer [27] but there is not much research showing its correlation to cancer stem cells.

Role of epigenetic inhibitors and sodium butyrate (NaB) in cancer

Epigenetic modifications play a critical role in cancer development and progression. These modifications refer to changes in gene expression that do not involve alterations to the underlying DNA sequence, but instead involve changes such as DNA methylation, histone modification, and alterations in non-coding RNA molecules. In the context of cancer, aberrant epigenetic changes can result in the overexpression of oncogenes or the silencing of tumor suppressor genes. For example, hypermethylation of the promoter region of a tumor suppressor gene could lead to its silencing, effectively removing a key check on cell proliferation and potentially leading to the development of cancer. Conversely, hypomethylation of an oncogene could lead to its overexpression, similarly promoting the unchecked growth of cells. Moreover, changes in histone modification, which can influence the structure of the chromatin and hence control gene expression, have also been implicated in cancer. These modifications provide potential targets for cancer therapy, and a growing field of research is focusing on developing epigenetic therapies that can reverse these harmful changes [28].

Sodium butyrate, an epigenetic inhibitor, has a dual role as an histone deacetylase inhibitor and a DNA demethylating agent offering a multi-faceted approach to rectify aberrant epigenetic changes that drive cancer progression. Sodium butyrate inhibits the action of the enzyme histone deacetylase, leading to an increase in the acetylation of histones. This modification relaxes the DNA structure, making it more accessible for transcription, and thus can restore the expression of silenced tumor suppressor genes. By this means, sodium butyrate can potentially slow down or stop the growth of cancer cells. It also has a demethylation role as previously shown in Shin et al's paper [29] where it induced hypomethylation at the promoter of SFRP1/2 in turn reviving its expression in gastric tumor cells. SFRPs are generally inactivated in gastric cancers and their

activation can lead to tumor suppression.

My research

Previous research has explored the relationship between CSCs and the type I and type II IFN response in various cancer types. IFNs have been shown to influence CSC biology and may hold therapeutic potential for targeting these cells. In sarcoma, a rare and heterogeneous group of malignant tumors arising from mesenchymal tissues, CSCs have been implicated in tumor aggressiveness and poor prognosis. However, the role of IFN experience in the regulation of CSC marker expression in sarcoma remains poorly understood.

In this study, I utilized an MX1-Cre tdtomato mice lineage tracing reporter model, as shown in Figure 1.2, to investigate the effects of IFN experience on the expression of CSC markers in sarcoma. The MX1-Cre-tdTomato reporter model was created by breeding MX1-Cre mice with Rosa floxed tdTomato mice. In the MX1-cre genetic system, the Mx1 promoter regulates the Cre recombinase. Mx1-Cre mice are an invaluable tool in studying gene function, as they allow for conditional gene deletion upon activation. This activation is prompted by type I IFNs, which bind to the type I IFN receptor (IFNAR). Upon this binding, the JAK-STAT signaling pathway, specifically the STAT1/STAT2 pathway, is triggered. The activation of this pathway leads to the transcription of the Mx1 promoter, which in turn controls the expression of the Cre recombinase enzyme. This enzyme can then induce the deletion of floxed genes, facilitating the study of their function in a controlled manner [30]. When Mx1-Cre mice are bred with Rosa-floxed-tdTomato mice, an intriguing genetic event occurs. The Rosa-floxed-tdTomato mice have a stop codon sequence located between two loxP sites, preventing the expression of the downstream tdTomato gene. Upon breeding with Mx1-Cre mice and subsequent exposure to IFN, the Cre recombinase controlled by the Mx1 promoter becomes active. This active Cre enzyme identifies and recombines the loxP sites in the Rosa-floxed-tdTomato mice, effectively excising the CAg promoter driven STOP codon that's preventing tdTomato expression. As a result, the tdTomato gene gets expressed, producing a red tdTomato fluorescent protein that labels

the cells of the mice. Consequently, in response to IFN exposure, these mice visually illustrate where the IFN-induced genetic recombination is occurring, by turning those cells red [31]. This model allows for the identification of IFN-experienced cells (red) and IFN-naïve cells (non-red) based on their tdtomato expression. My research was divided into two parts: the first focused on immune cells, specifically bone marrow macrophages, while the second investigated tumor cells derived from these reporter models. Previous research showed that acute IFN exposure typically activates immune cells, but long does it last is not known. My first hypothesis for the immune cells was to see if there are any phenotypic and functional differences between IFN-experienced (red) and IFN-naive (non red) bone marrow macrophages. Likewise, acute IFN exposure leads to increased to decreased CSC activity. My second hypothesis was to see if IFN experienced tumor cells were more stem-like.

By elucidating the relationship between IFN experience and CSC marker expression in sarcoma, my findings may contribute to the development of more effective immunotherapies for this aggressive cancer type. Furthermore, this research may offer insights into the role of IFNs in CSC biology, potentially informing future studies in other cancer types. In the subsequent sections, I will outline the materials and methods employed in my investigation, present the results and engage in a detailed discussion of my findings, and conclude with a summary of the study's implications and directions for future research.

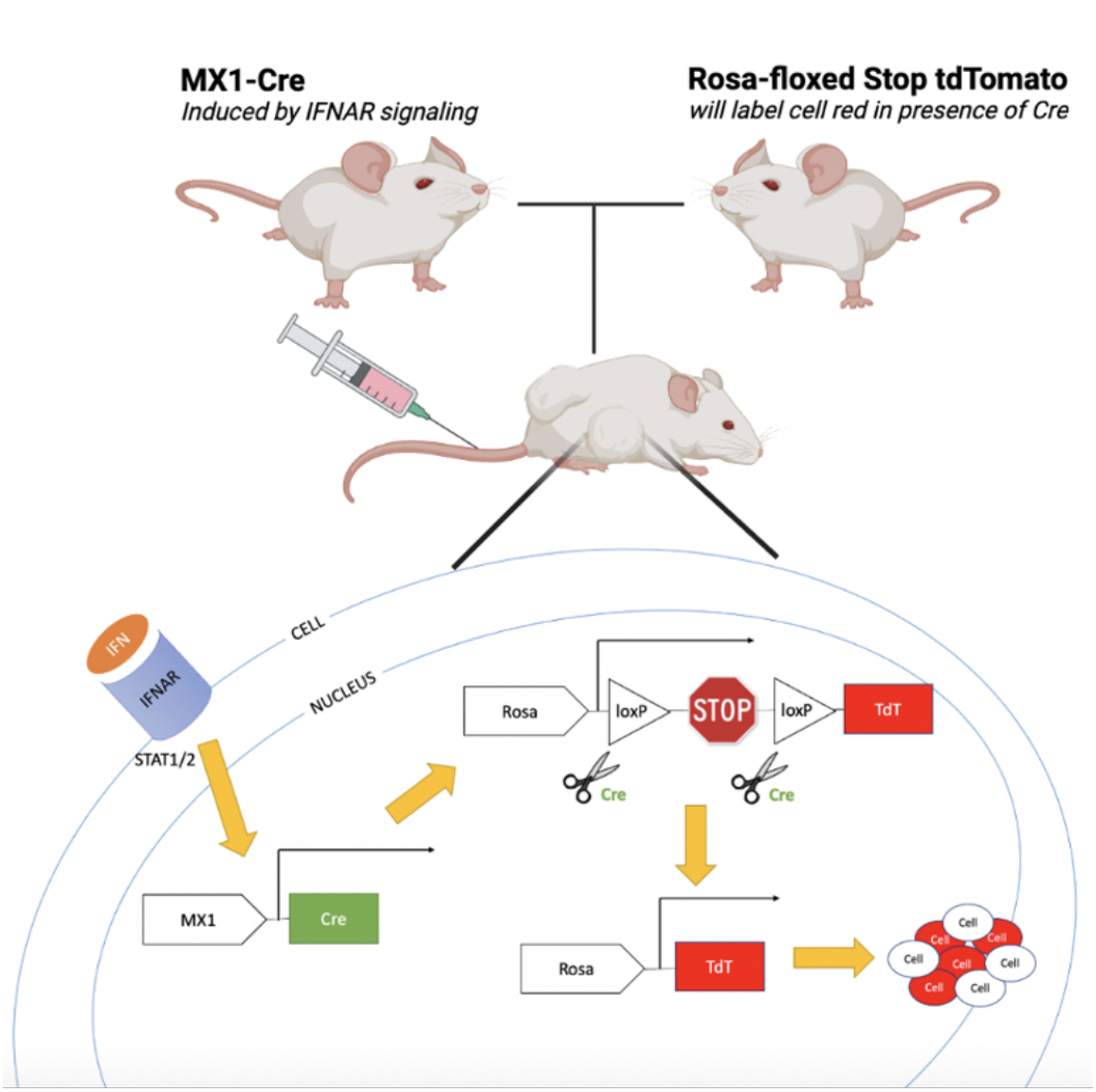

Figure 1.2. Reporter Model for Type I IFN Experience in Sarcoma: MX1-Cre-tdTomato model, derived from the breeding of MX1-Cre mice with Rosa floxed tdTomato mice, allows for the visualization of IFN-experienced (red) and IFN-naive (non-red) cells based on tdTomato expression, providing a powerful tool for investigating the effects of IFN experience on the expression of CSC markers in sarcoma.

Chapter 2

Results

2.1 Bone marrow macrophages

2.1.1 Differential Expression of CSC Markers in Red and Non-Red Bone Marrow Macrophages

Based on the reporter model, we were able to identify interferon experienced cells as red and interferon naive cells as non red. My study aimed to discern whether there is a significant difference in the expression of CSC markers between red and non-red bone marrow macrophages. Macrophages were first gated for F4/80 and CD11b, both are monocyte/macrophage markers, which confirmed progenitor cells forming macrophages. The observations, as shown in Figure 2.1 were based on the progression of bone marrow macrophages cultured from progenitor cells over seven days. By the seventh day of culture, an interesting pattern emerged (Figure 2.2). Over 80% of the bone marrow macrophages displayed red fluorescence, whereas less than 20% remained non-red. This trend held across all markers examined, with the ratio of red to non-red cells increasing consistently over the seven-day period. A noteworthy finding was that the expression of CSC markers was relatively similar in both red and non-red cells by day seven. This phenomenon might primarily be attributed to the overwhelming majority of red cells by this point in the experiment. A few potential explanations were posited for this outcome. The non-red cells could be experiencing higher rates of cell death, or they could be in the process of converting into red cells. Another possibility is that the presence of red cells might be exerting a

suppressive effect on the non-red cells. Further investigation is required to elucidate the exact mechanisms behind these findings.

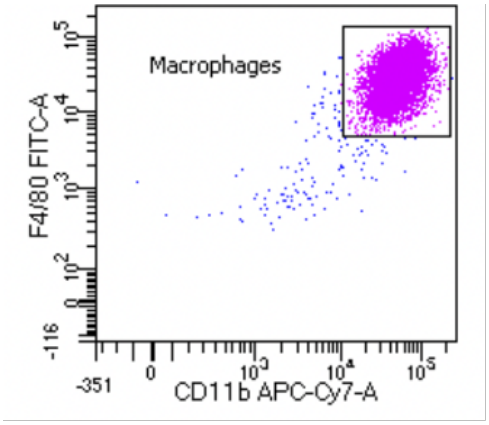

Figure 2.1. Macrophages identified by F4/80+ and CD11b+ gating in flow cytometry.

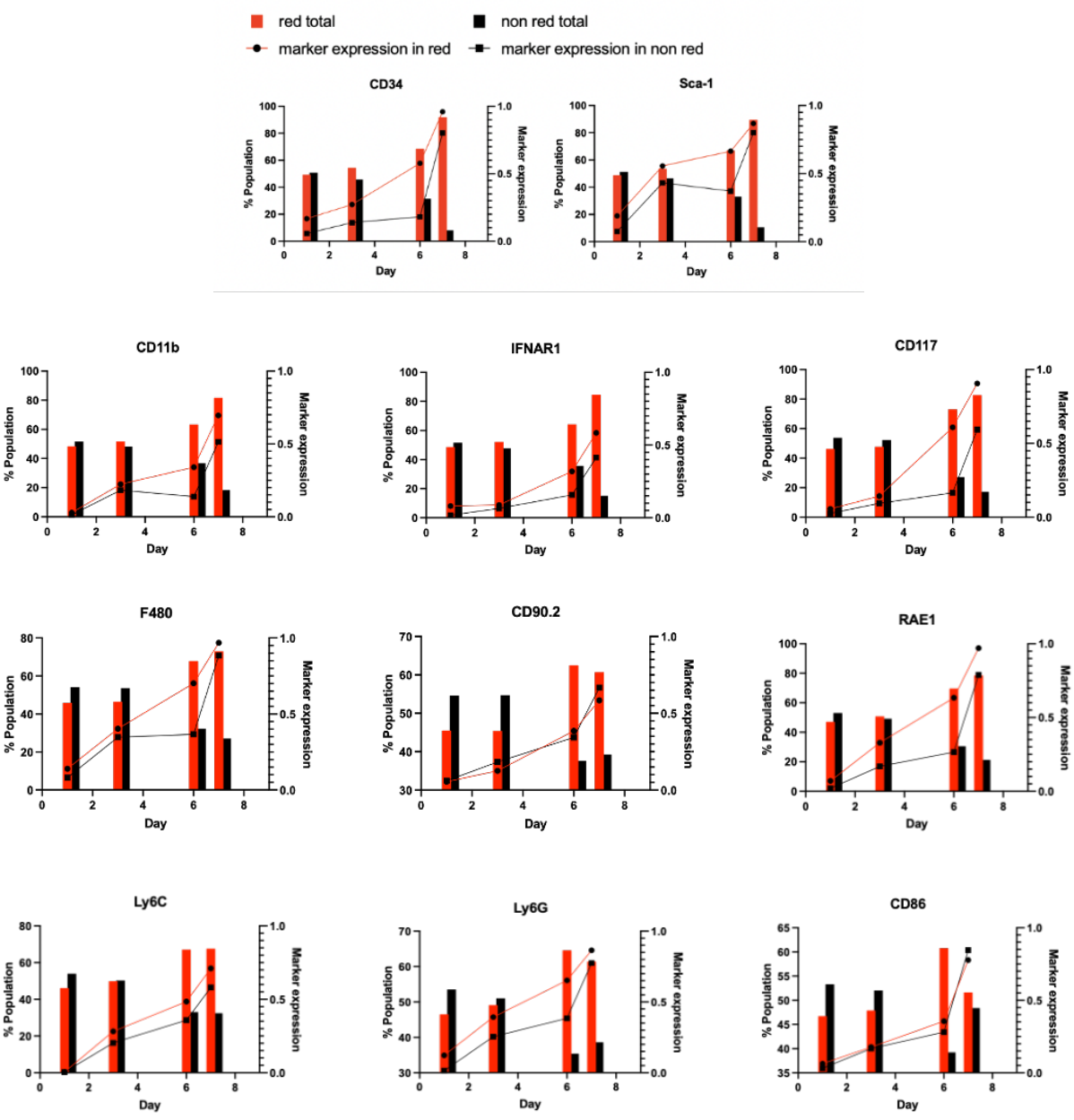

Figure 2.2. Differential Expression and Quantification of CSC Markers in Red and Non-Red Bone Marrow Macrophages: Comparative marker expression (right y-axis) and relative population percentage of red and non-red macrophages (left y-axis) for 11 different markers, providing insights into the cellular dynamics and potential stemness over a week of bone marrow macrophage development.

2.1.2 Influence of Lipopolysaccharide Stimulation on CSC Marker Expression in Red and Non-Red Cells

My hypothesis explored whether IFN experienced (red) and IFN inexperienced (non red) bone marrow macrophages respond differently to a stimulus such as LPS or IFN. LPS is a potent immune cell activator, stimulating a variety of cells, including monocytes, macrophages and B cells to produce cytokines. Activated monocytes, secrete IL-6 and IL-10 upon LPS exposure. To enhance the detectability of cytokines with flow cytometry, Golgi stop was used, allowing for the accumulation of cytokines in the Golgi complex. The experimental design consisted of four treatment groups: untreated, LPS-stimulated, LPS and type I IFN (IFN-I) co-stimulated, and only IFN-I stimulated. The findings, as seen in Figure 2.3, indicate that there was no major difference in marker expression between red and non-red cells across all treatments. Overall, no difference between red and non red cells was seen in response to either stimuli. MHC class II, a macrophage marker, increased upon LPS treatment and IFN treatment for both red and non red cells. CD80 expression increased with higher LPS concentration. Co-stimulation of IFN and LPS improved CD80 expression suggesting that IFN experience might improve LPS stimulation effect. In terms of cytokine production, IL-6 expression did not show very consistent results. Interestingly, no significant differences between red and non-red cells were noted. There was no signficant expression of IL10 in any of the treatment options. LPS stimulation influenced IL-6 expression, which is known to alter the tumor microenvironment by promoting chronic inflammation and suppressing anti-tumor immunity [32], and IL10, which is associated with regulating proliferation, apoptosis and migration in cancer [33]. Based on these results CD80 and MHC-II expression improved with external stimulus, suggesting macrophage activity, but IL6 and IL10 did not seem to be affected by it.

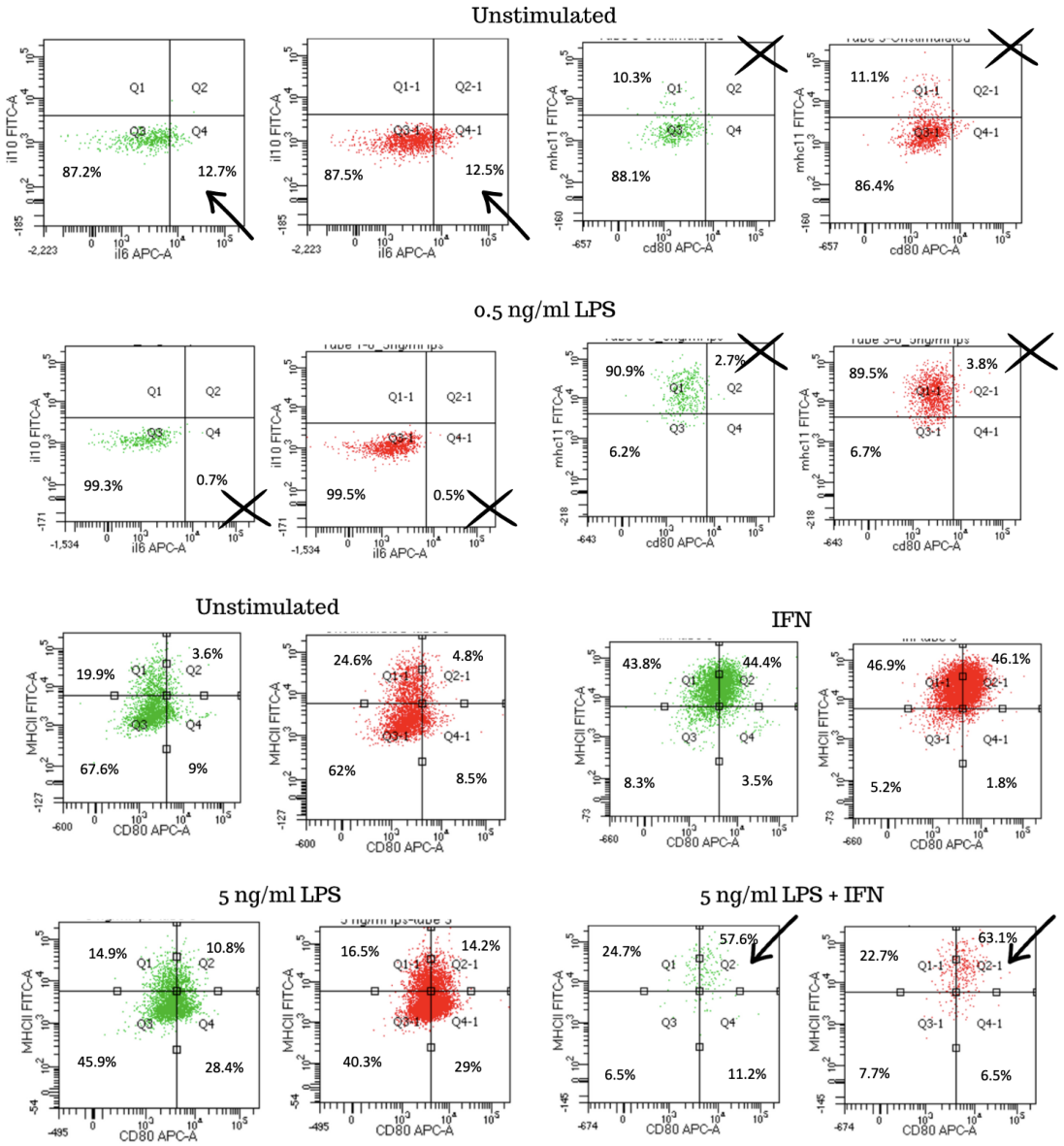

Figure 2.3. CSC Marker Expression and Cytokine Production in Response to LPS and IFN-I Treatments: The graph presents the differential expression of various markers and cytokines across four treatment groups: untreated, LPS-stimulated, LPS and IFN-I co-stimulated, and IFN-I stimulated alone, revealing potential modulations in red and non-red cell phenotypes

2.1.3 Comparing Endocytosis Activity Between Red and Non-Red Bone Marrow and Spleen Macrophages by assessing dextran uptake

Next, I examined the differences in endocytosis activity between red and non-red bone marrow macrophages and extended this analysis to include spleen macrophages. The goal was to determine whether there are differences in the uptake of Dextran-Allophycocyanin (Dextran-APC) among these cells. I utilized bone marrow macrophages harvested on days 7, 8, and 9 and exposed them to varying concentrations of dextran, at different temperatures, and for various durations. The data from flow cytometry analyses (Figure 2.4) revealed no significant difference in the uptake of dextran between red and non-red bone marrow macrophages. One explanation for this could be a transitioning of non-red cells to become recent red cells. The experiment was replicated with spleen macrophages extracted from freshly harvested spleens (treated with $1\mu L$ dextran for 10 and 60 minutes). As seen in Figure 2.5, a consistent difference in dextran uptake between red and non-red spleen macrophages was observed, with a higher uptake occurring in non-red cells compared to red cells. These findings suggest that there might be a discrepancy in endocytic capacity or activity between red and non-red macrophages, potentially attributed to the influence of IFN signaling.

2.2 Tumor cells

2.2.1 Growth Differences Between Red and Non-Red Tumor Cells In Vitro and In Vivo

These experiments were focussed on understanding whether red cells exhibit a higher growth rate than non-red cells both in vitro and in vivo. I shifted the focus to tumor cells, given that the earlier investigations with bone marrow macrophages failed to yield significant differences in the phenotypic and functional attributes between red and non-red cells. We used the F68A cell line derived from MX1-cre tdtomato mice, which had previously exhibited differential stemness between red and non-red cells, by enhanced sphere formation, chemore-

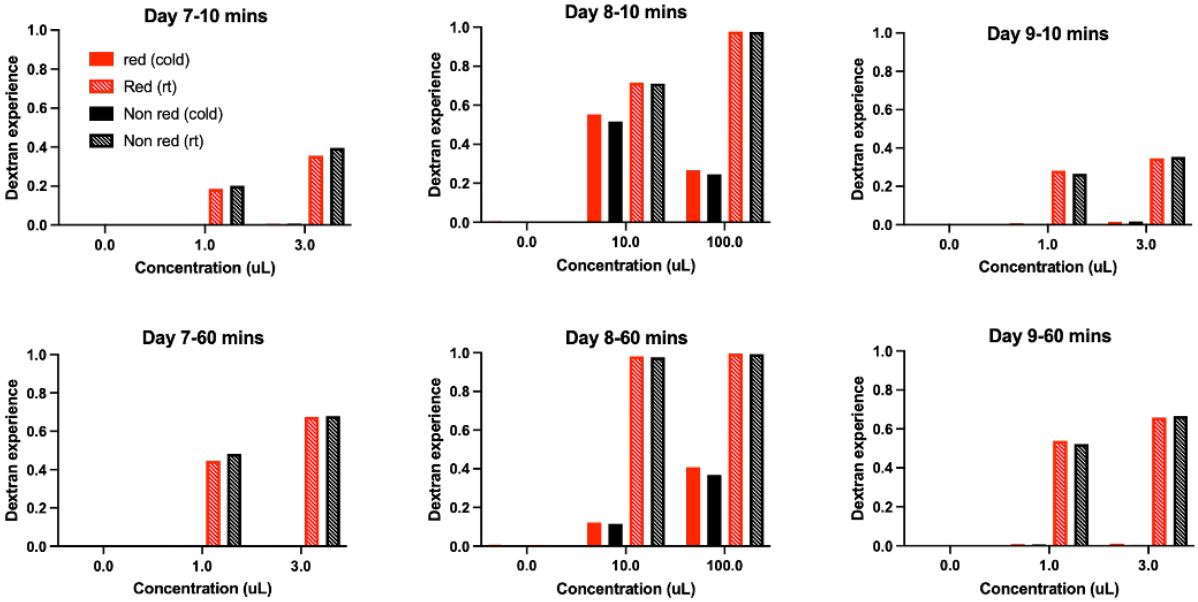

Figure 2.4. Comparative Analysis of Endocytosis in Red and Non-Red Bone Marrow Macrophages: Dextran uptake of red and non-red bone marrow macrophages over days 7, 8, and 9, indicating no significant differences

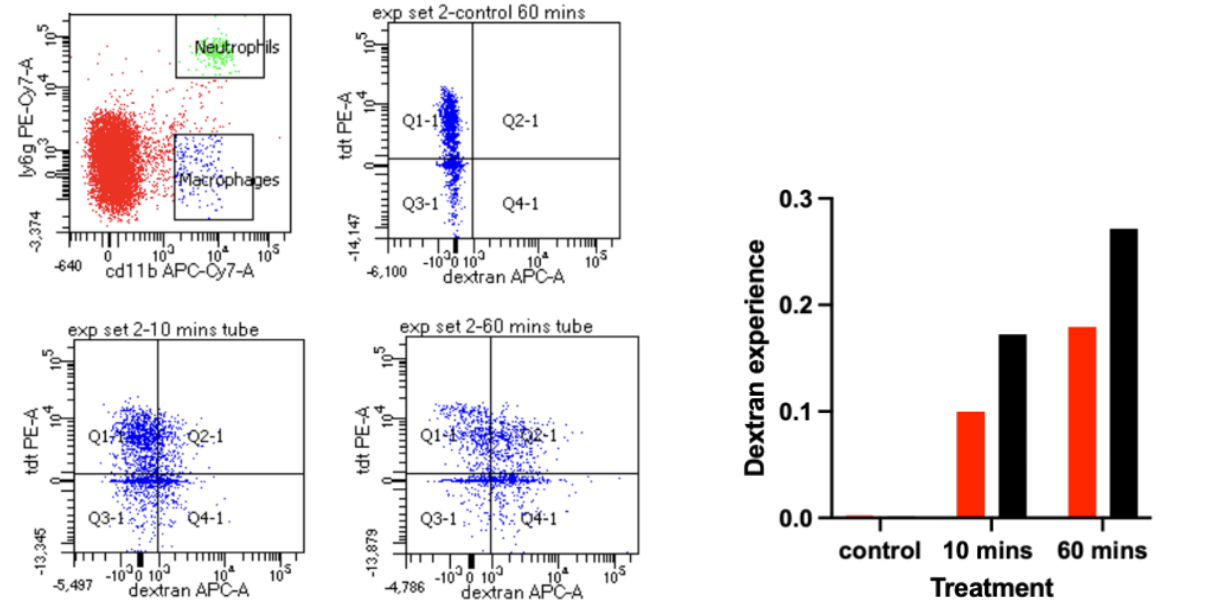

Figure 2.5. Comparative Analysis of Dextran Uptake in Red and Non-Red Spleen Macrophages: flow cytometry gating strategy for macrophage identification from freshly harvested spleens based on CD11b+ and Ly6G- markers, control for 0 ul of dextran uptake and 1 ul dextran uptake at 10 mins and 60 mins. The graph shows a significant difference in dextran uptake between red and non-red spleen macrophages, highlighting potential IFN-related influence on endocytic capacity or activity.

sistance, increased tumor growth, and higher expression of CSC (CSC) markers in red cells. Through in vitro kinetic studies, we observed in Figure 2.6, a more rapid growth of red cells compared to non-red cells, with red cell count nearly doubling per day, while non-red cell growth plateaued after day 5. In vivo, we injected a mix of red and non-red cells (20:80) into MX1-cre tdtomato (wild-type) and (NSG) (immunodeficient) mice, harvested the tumors after 20 days, and monitored the subsequent growth of these harvested cells in vitro for 10 more days. As seen in Figure 2.7, by day 10, we found that the proportion of red cells in harvested tumors from immunodeficient mice increased from 50% to nearly 100%, while non-red cells declined from 50% to almost zero. A similar pattern emerged for wild-type mice, albeit the initial proportion of red cells at harvest after 30 days was higher at 90%, increasing to nearly 100% after 10 more days in vitro. Non-red cells, in contrast, reduced from 10% to near zero. Interestingly, in control in vitro cells, the proportion of red cells increased from 50% on day 10 to 90% by day 20, subsequently reaching a plateau. Faster tumor growth was observed in immunodeficient mice compared to the wild type, suggesting that absence of an immune response facilitates tumor growth. Therefore, these findings corroborate the hypothesis that red cells exhibit a faster growth rate than non-red cells, both in vitro and in vivo, in the context of the F68A cell line.

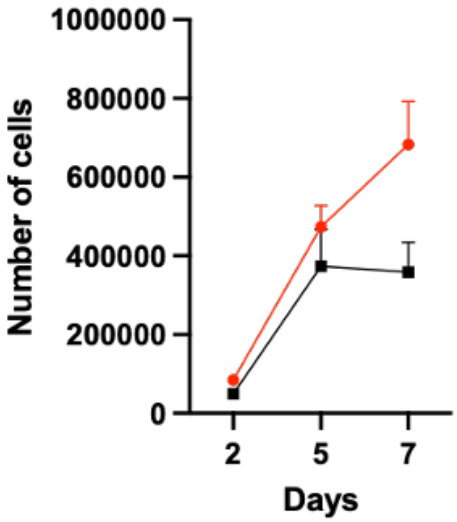

Figure 2.6. Comparative Growth Kinetics of Red and Non-Red Tumor Cells In Vitro: accelerated growth rate of red cells compared to non-red cells in vitro over a week.

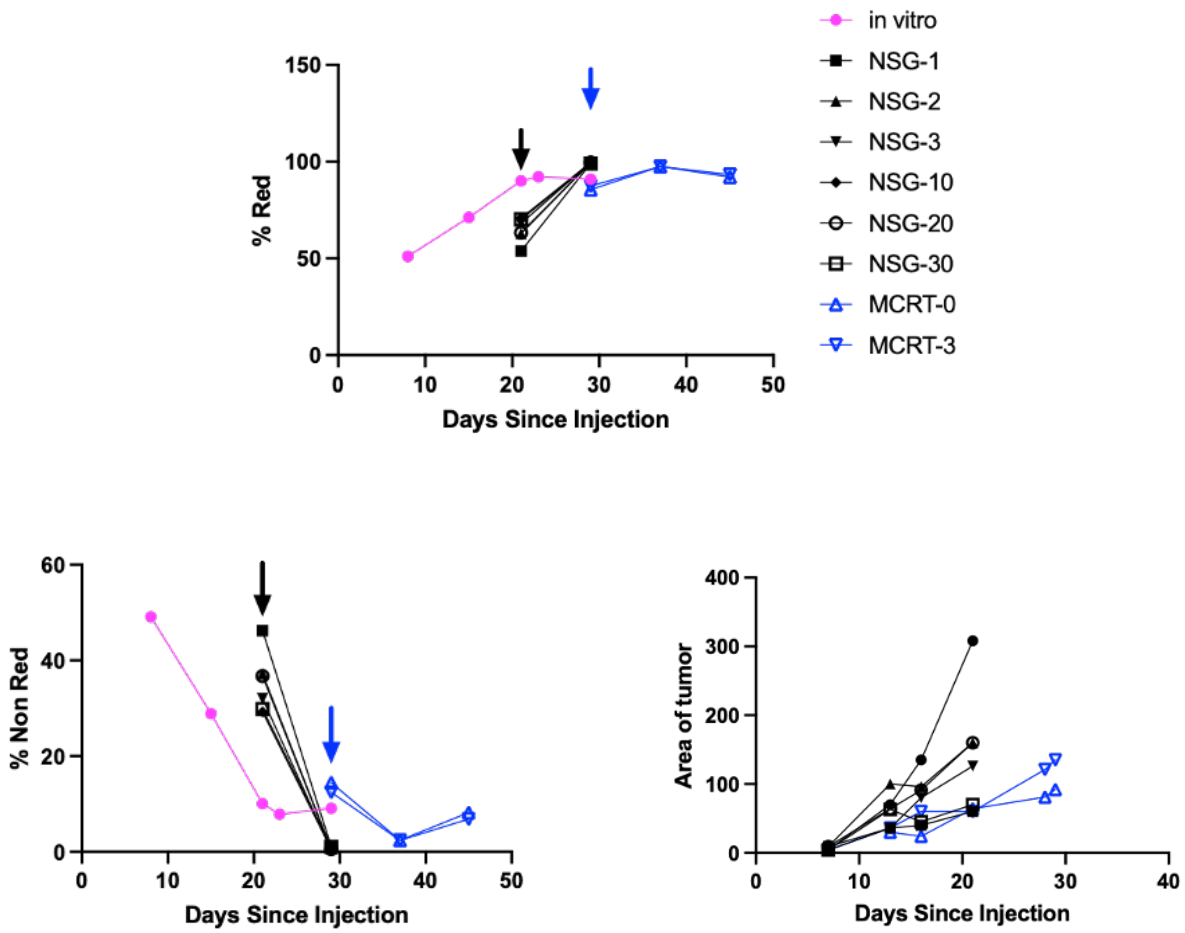

Figure 2.7. Comparative Growth Kinetics of Red and Non-Red Tumor Cells In Vivo: in vivo experiment results, indicating an increasing proportion of red cells in harvested tumors over time in both immunodeficient (NSG) and wild-type mice, and a subsequent decrease in non red cells. The final graph highlights the average tumor area expansion over time noted in the absence of an immune response.

2.2.2 Type I IFN Impact on Tumor Growth, Proliferation, and CSC Marker Expression in Red and Non-Red Cells

The hypothesis was to ascertain whether treatment with Type I IFN could alter tumor growth, proliferation, and CSC marker expression in red and non-red cells, potentially rendering non-red cells more stem-like. The kinetic analysis of sorted F68A red and non-red cells for in vitro tumor growth demonstrated, as in Figure 2.8, that IFN treatment (1000U/ml) does not enhance tumor growth in red cells, but it does significantly increase tumor growth in non-red

cells compared to untreated control. Through cell proliferation BrdU analysis, performed after a 45-minute treatment of unsorted F68A cells with BrdU, we found more proliferating red cells than non-red ones (Figure 2.9). However, IFN treatment led to reduced proliferation in red cells, while a slight increase in non-red cell proliferation was seen at a concentration of 1000U/ml IFNa. A higher IFN concentration significantly reduced non-red cell proliferation, suggesting a concentration-dependent response to IFN treatment, as has already been seen in previous research [34].

To understand the impact of IFN treatment on CSC marker expression, we compared red and non-red cells with and without IFN treatment over a week. Our results as shown in Figure 2.10 were:

- Sca1 expression was higher without IFNb treatment in both cell types, but overall higher in red cells.
- CD90.1 expression increased with IFNb treatment, more noticeably in non-red cells, but was overall lower in red cells.
- No significant difference was found in CD95, CD105, and CD44 expression. However, overall, CD95 was lower and CD105 was higher in red cells.
- MHC1 expression decreased with IFNb treatment in red cells but was overall higher in this group.
- Galectin9 expression was slightly higher with IFNb treatment, but overall lower in red cells.
- CD24 expression slightly increased in red cells with IFNb treatment and was overall higher in red cells.

There is a lot of heterogeneity seen in these results for marker expression. These findings suggest that IFN treatment can enhance tumor growth and modulate the expression of certain CSC

markers, potentially leading to a more stem-like phenotype in non-red cells. However, it might be a dosage dependent effect.

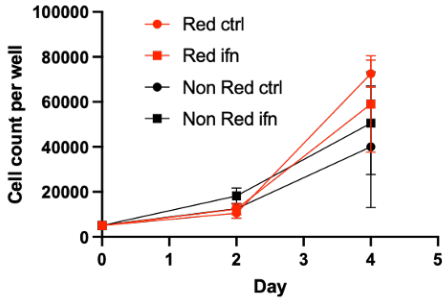

Figure 2.8. Type I IFN Impact on Tumor Growth: growth kinetics of red and non-red cells under IFN treatment, showing increased growth in non-red cells

2.2.3 Differential Expression of Hey1 and HeyL in Type I IFN-Treated F68A Cells

The hypothesis tested in this study was that F68A cells treated with Type I IFN in vitro would show differences in hey1 and heyL gene expression. The aim of the investigation was to examine gene expression variances between red and non-red cells, from the list of differentially expressed genes generated by RNA sequencing analysis previously done in the lab (Figure 2.11). By quantitative PCR, we confirmed that hey1 and heyL genes were upregulated nearly four times more in red cells as compared to non-red cells. Hey1 and heyL genes stood out because of their status as target genes of the Notch signaling pathway, a pathway previously linked with CSCs in literature. The Notch signaling pathway is known to contribute significantly to tumor initiation, progression, invasion, and metastasis by directly or indirectly interacting with other signaling pathways. The multitude of factors within Notch signaling, such as ligands, receptors, signal transducers, and effectors, can have manifold effects [35]. Upon treating red and non-red cells with 1000U/ml of Type I IFN, we observed a four-fold increase in hey1 and heyL expression in red cells. Conversely, upon treatment of non-red cells with varying IFN concentrations, hey1 expression notably increased at 1000 U/ml but declined with higher concentrations, hinting at a dose-dependent impact of IFN on CSCs. The expression of heyL in non-red cells remained

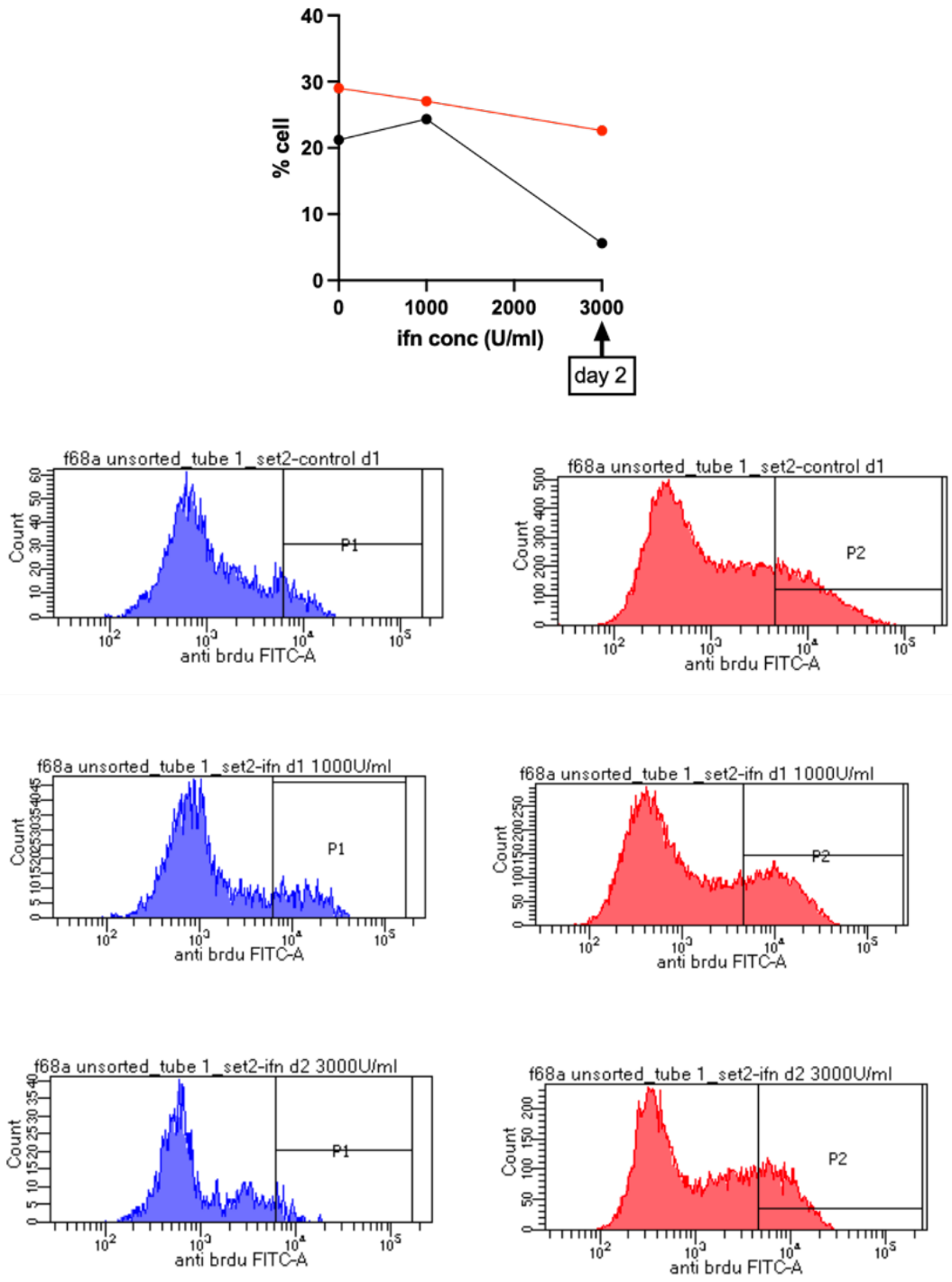

Figure 2.9. Type I IFN Impact on Tumor Proliferation in Red and Non-Red Tumor Cells: BrdU proliferation analysis and flow cytometry histograms, highlighting a decrease in proliferation of red cells and a concentration-dependent increase in non-red cells with IFN treatment.

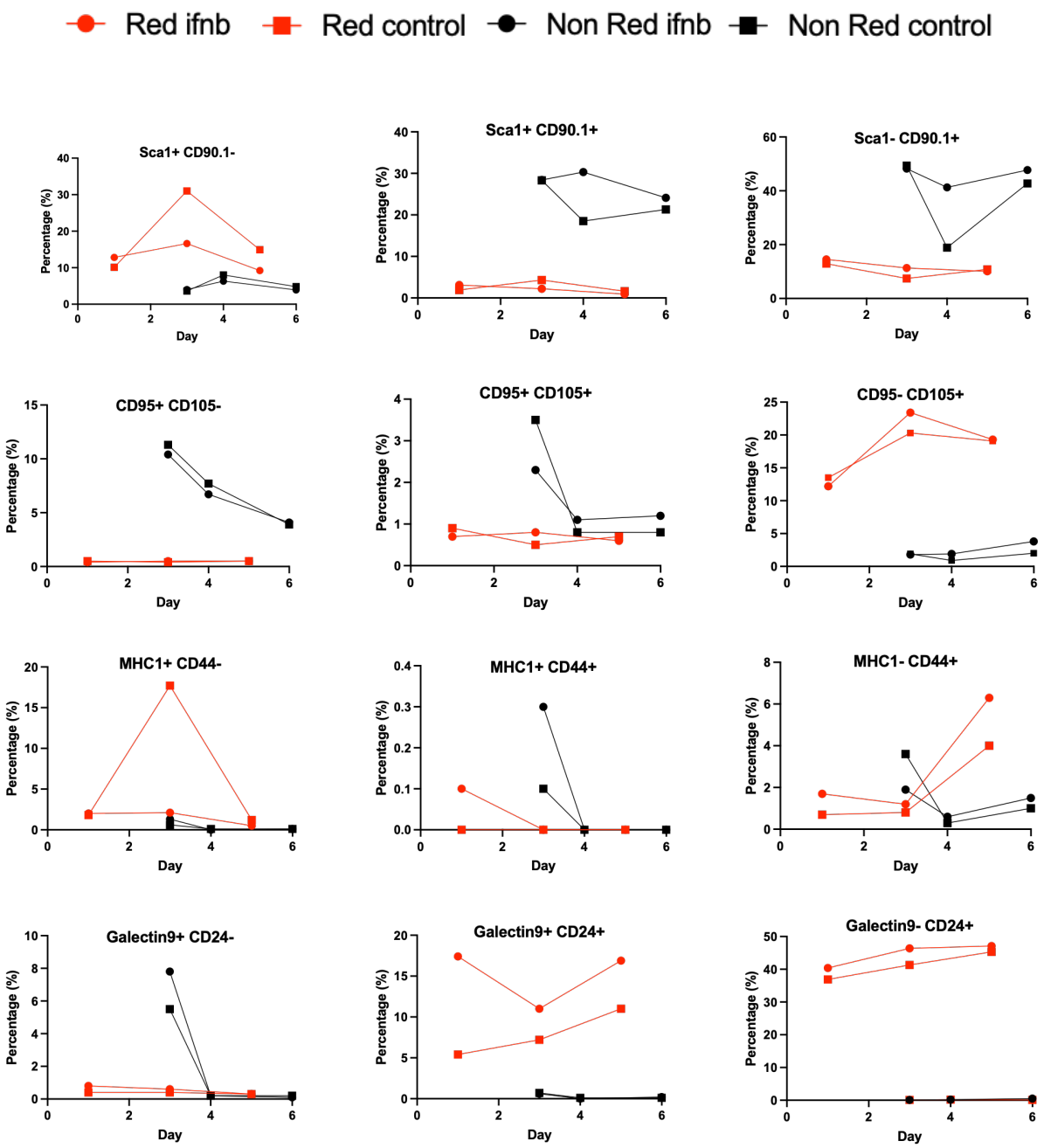

Figure 2.10. Type I IFN Impact on CSC Marker Expression in Red and Non-Red Tumor Cells: changes in CSC marker expression following IFN treatment, pointing to a potential shift towards a more stem-like phenotype in non-red cells.

largely constant with varying IFN experience, suggesting that this gene might not be directly responsive to exogenous IFN experience.

We also examined other genes such as sca1 and cd90.1. Sca1 was upregulated at 1000U/ml IFN treatment but slightly reduced at high doses. Considering sca1 is a CSC marker, these findings further support the hypothesis that IFN treatment, at a lower concentration, encourages a more stem-like phenotype in non-red cells. In conclusion, hey1 displayed an intriguing relationship with type I IFN, particularly based on the change in hey1 expression in non-red cells upon in vitro IFN treatment. This suggests a possible correlation between IFN experience and the expression of hey1 in CSCs, positioning hey1 as a potential target gene for silencing.

2.2.4 Effect of epigenetic inhibitor, NaB, on Hey1 Expression in F68A Cells

The hypothesis was that treatment of F68A cells with NaB would increase hey1 expression in both red and non-red cells. NaB is known as a histone deacetylase inhibitor and has also been found to induce demethylation in the promoter region of SFRP1/2, thereby retaining its expression in human gastric cancer cells [36]. There is also research that shows that hypomethylation of the Hey1 promoter region contributes to tumor proliferation in glioblastomas [36]. In my study, I treated red and non-red F68A cells with varying concentrations of NaB over a period of 24 and 48 hours, and subsequently examined the expression of hey1 by qPCR. The results, as in Figure 2.12, reveal a significant upregulation of hey1 expression in NaB treated red cells, especially at 2mM concentration on day 2. Similarly, hey1 expression in non-red cells also increased at the 2mM NaB treatment after 48 hours. In conclusion, my results affirm my initial hypothesis. The increased expression of hey1 in both cell types upon treatment with NaB could present an alternative model for further studies aimed at increasing the stem-like properties of non-red cells.

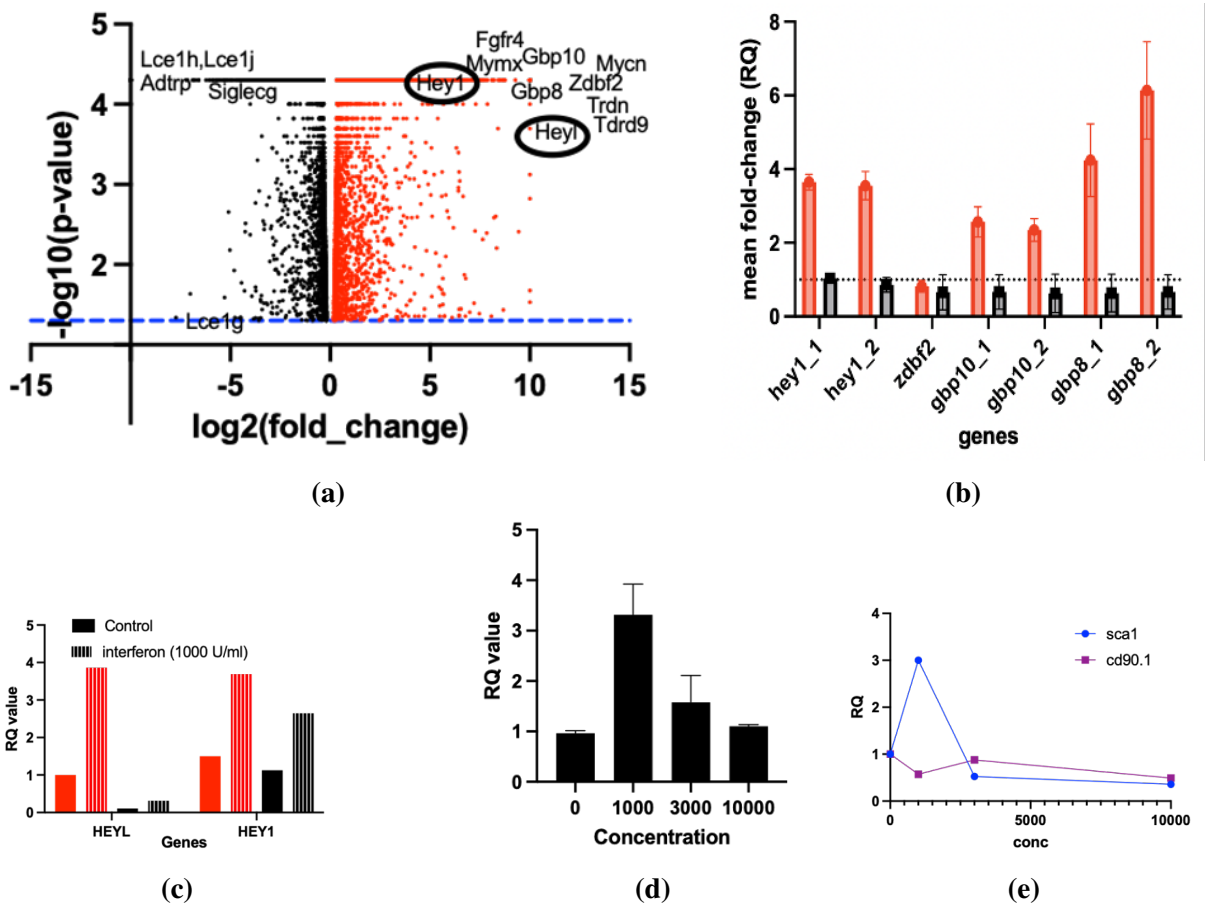

Figure 2.11. Differential Gene Expression in Red and Non-Red Cells Under IFN Treatment: (a) highlights the significantly upregulated genes in red and non-red cells, as revealed by differential gene expression analysis. (b) confirms via qPCR the upregulation of certain genes, including hey1 and heyL, in red cells. (c) demonstrates increased expression of hey1 and heyL, key genes in the Notch signaling pathway, upon IFN treatment. (d) showcases the dose-dependent response of hey1 expression in non-red cells to varying concentrations of IFN, with increased expression at 1000U/ml and a decrease at higher concentrations. (e) displays the expression of CSC markers sca1 and cd90.1 in non-red cells under IFN treatment, hinting at a potential shift towards a more stem-like phenotype at lower IFN concentrations.

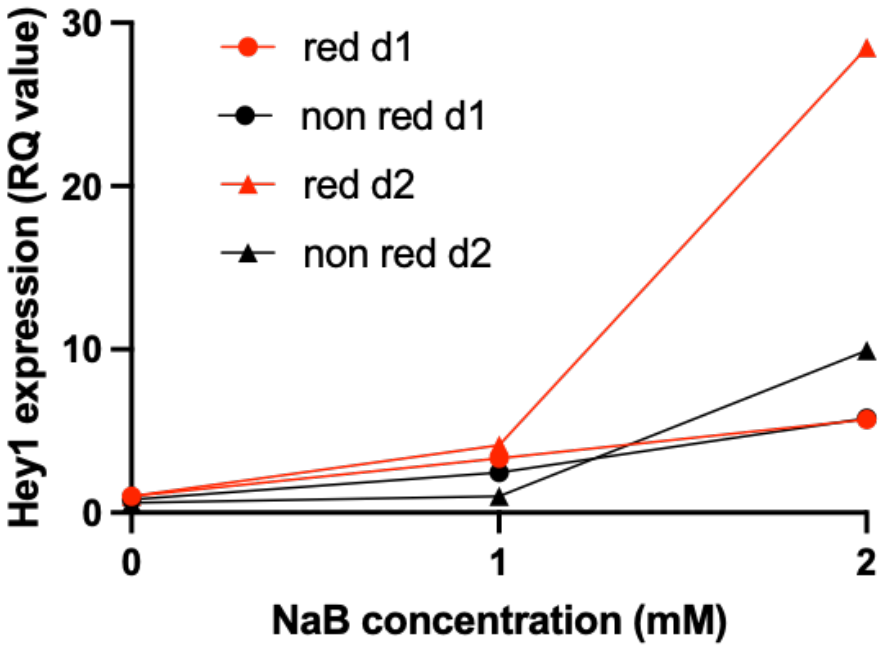

Figure 2.12. NaB Effects on Hey1 Expression in Red and Non-Red Cells. This graph displays the increased expression of Hey1 in both red and non-red cells under treatment with varying concentrations of NaB, an epigenetic inhibitor, over a period of 24 and 48 hours. Higher Hey1 expression is notably observed at 2mM concentration in both cell types.

Chapter 3

Discussion

Bone marrow macrophages

We used a lineage tracing reporter model that helps identify interferon experienced cells as red and interferon naive cells as non red. The presented study investigates the expression of CSC markers in red and non-red bone marrow macrophages (BMM) as well as the differential responses of these cell populations to lipopolysaccharide (LPS) stimulation. Additionally, the study explores differences in endocytosis activity between red and non-red BMMs and spleen macrophages. My results showed that by day seven of culture, the majority (over 80%) of BMMs were red fluorescent, leaving less than 20% as non-red. Despite this imbalance, the expression of CSC markers remained relatively similar in both populations. This unexpected result could be due to higher cell mortality rates in the non-red population, their conversion into red cells, or a suppressive effect exerted by the red cells on their non-red counterparts. These findings invite further investigation to better understand the underlying mechanisms and their potential implications. Regarding the influence of LPS stimulation, our results did not consistently indicate a difference in CSC marker expression between red and non-red cells. Interestingly, LPS and IFN did modulate the expression of MHC-II and CD80, both of which are macrophage markers. These results suggest that LPS stimulation might modify the macrophage activity which might be enhanced with an IFN costimulation.

My experiment for the endocytosis activity revealed a distinct difference in dextran uptake

between red and non-red spleen macrophages, with non-red cells displaying a higher uptake rate. This disparity in endocytic activity might be a consequence of IFN signaling influences and suggests that red and non-red macrophages may have different functional roles within the immune system. The observed difference in endocytosis activity among spleen macrophages provides yet another interesting experiment to repeat and follow the reason for this difference in the future. Given these findings, it would be insightful to further understand the molecular pathways involved in these processes, which could provide novel therapeutic targets for cancer treatment.

However, the limitations of the present study should be acknowledged. The significant reduction in non-red BMM population by day 7 of culture could have confounded our observations and interpretations. Further, our experiments with LPS stimulation did not yield consistent differences in CSC marker expression between red and non-red cells. In light of these limitations, future studies should consider experimental designs that can better maintain cell population balance and improve the consistency of treatment responses.

Tumor Cells

Our findings shed light on tumor cell growth dynamics and the effects of type I IFN treatment in red and non-red tumor cells. As hypothesized, the red cells exhibited a higher growth rate than non-red cells, both in vitro and in vivo. Kinetic studies highlighted the rapid proliferation of red cells in contrast to non-red cells, which plateaued after day 5. The enhancement of tumor growth in immunodeficient mice versus wild type hinted at the role of immune response in regulating tumor progression, which appears to favor the rapid overtake of red over non red in vivo.

On the question of type I IFN's influence on tumor growth, proliferation, and CSC marker expression, our results point to a differential impact. While IFN treatment did not augment tumor growth in red cells, it increased tumor growth in non-red cells, potentially rendering them more stem-like. Furthermore, IFN treatment led to a decrease in proliferation of red cells,

whereas a slight increase was observed in non-red cell proliferation at a moderate dose of IFNα. This should be repeated and could be an exciting finding if it true. Interestingly, higher IFN concentrations led to reduced non-red cell proliferation, which is in line with prior research suggesting a concentration-dependent response to IFN treatment. Consistent with Song et al. and Musella et al. [34], our results seem to suggest a complex interplay between IFN levels and the resulting biological responses. Lower levels of IFN could confer CSC-like properties through various pathways, while higher levels could induce apoptosis or not result in CSC accumulation. Our data also underscore the necessity of understanding the intricate balance of IFN dosing and timing for optimal outcomes in different cancer cell lines.

Regarding the impact of IFN treatment on CSC marker expression, our results painted a nuanced picture. While Sca1 and CD90.1, which are CSC markers [37] [38], expressions were modulated by IFN treatment, no major differences were observed in CD95, CD105, and CD44 expressions. Additionally, changes were observed in MHC1, Galectin9, and CD24 expressions with IFN treatment, more pronounced in red cells. These results hint at the selective modulation of certain CSC markers by IFN treatment. However, our study is not without limitations. The high concentration of IFN used in this study (10000U/ml) might have impacted our findings that could emerge better at lower concentrations. Therefore, future studies could benefit from exploring a broader range of IFN concentrations.

The role of Notch signaling pathway in tumor initiation, progression, and metastasis is well-established. Past research has demonstrated that suppressing this pathway, and specifically genes such as notch1, notch2, and hey1, can potentially mitigate tumor stemness in renal cell carcinoma [38]. Upon in vitro treatment of red and non-red cells with Type I IFN, we observed a notable increase in the expression of hey1 and heyL genes, particularly in red cells. This supports our hypothesis that Type I IFN could induce changes in these genes, potentially impacting CSC phenotype. We also observed a dose-dependent response to IFN treatment in non-red cells. This concentration-based response mirrors our previous results and corroborates with the existing literature where lower doses of IFN treatment were found to enhance stem-like characteristics

in cells. This is in line with studies demonstrating a dual role of IFN, where low doses could promote stemness while high doses induced differentiation or apoptosis, reflecting its complex interaction with cell signaling pathways.

Given NaB's known role in inducing demethylation in the promoter region of SFRP1/2 in human gastric cancer cells and the link between hypomethylation of the Hey1 promoter region and tumor proliferation in glioblastomas, we hypothesized that NaB could also modulate hey1 expression in our cell types. Our results corroborate this hypothesis, showing a significant upregulation of hey1 expression in NaB-treated red cells and non-red cells, particularly at a 2mM concentration after 2 days. These findings hold significant potential for targeted therapeutics in cancer treatment. However, it is imperative to address the limitations of this study as well. Our study would have benefited from multiple repetitions of the experiments to ensure more reproducible data. Furthermore, the concentration of NaB treatment warrants optimization for maximum efficacy. Future studies should also examine the in vivo effects of NaB and Type I IFN treatments, which would provide more comprehensive insights into their influence on tumor growth and progression. Ultimately, the delineation of the mechanisms by which Type I IFN and NaB influence gene expression and CSC phenotype could revolutionize our understanding of CSC biology and offer novel therapeutic targets.

Future Directions

Increased Endocytosis/Phagocytosis of Non-red Macrophages: My initial results suggest increased endocytosis/phagocytosis in non-red macrophages derived from the spleen. While these findings are intriguing, it is essential to confirm these observations in subsequent experiments.

Role of Hey1 and HeyL in Cell Growth: The involvement of hey1 and heyL genes in the growth of F68A red and non-red cells emerged as a key finding in my research. Future experiments should aim to determine whether these genes are indeed necessary or sufficient to promote cell growth. This could be achieved through gene silencing experiments in red cells and overexpression in non-red cells, employing techniques such as siRNA-mediated knockdown or

CRISPR/Cas9 gene editing for silencing, and plasmid or viral vector-based gene delivery for overexpression. The outcomes of these experiments would clarify the roles of these genes in regulating the growth and potential stem-like characteristics of the cells.

Regulation of Hey1 and HeyL by NaB: NaB's potential role as an epigenetic regulator emerged as a fascinating area of our study. Future investigations should confirm and further explore its effects on hey1 and heyL expression. These investigations could include a range of NaB concentrations to better understand its dose-dependent effects, coupled with time-course studies to investigate the temporal dynamics of these changes.

Overall, the next steps of this research is to provide a more detailed picture of the interplay between IFN treatment, gene expression, and cellular characteristics in the context of tumor biology. This multifaceted approach promises to contribute valuable insights to the field and guide the development of more effective cancer therapies.

Chapter 4

Materials and Methods

Cell Lines

F68A cell lines were maintained by passaging cells when 80% confluent. This was done by trypsinizing the cells for 5 mins at 37°C and washing twice with HBSS. They were then reseeded in fresh medium and placed at 37°C in the incubator. Excess cells were frozen down by resuspending in CR-10 media and 10% DMSO in cryovials for future use.

Media Preparation (CR-10 media)

500 ml of RPMI 1640 medium (ThermoFisher Scientific) was used as a base for the culture for bone marrow macrophages and tumor cells. This was supplemented with 5 ml of 200mM sodium pyruvate, 5 ml of 200mM L-glutamine, 2.5 ml of 7.5% sodium bicarbonate, 0.5 ml of 55mM b=mercaptoethanol and 5 ml non essential amino acids. 50 ml of fetal bovine serum and 500 ul of ciprofloxacin as mycoplasma contamination control were added.

Harvesting bone marrow progenitors from mice

MX1-cre tdt mice aged 6-12 weeks were housed and maintained in accordance with institutional guidelines. Bone marrow progenitors were harvested. Mice were euthanized, and the hind limbs were aseptically removed. The femur and tibia were separated and cleaned of muscle and connective tissues. The epiphyses of both bones were cut, and bone marrow was

flushed with cold sterile HBSS using a 25g needle attached to a 3-5 mL syringe into a 70 μm filter atop a conical tube. Bone marrow cells were collected, centrifuged at 200-500g for 5 minutes at 4°C, and subjected to red blood cell lysis.

Red Blood Cells lysis

The cells were incubated with Ammonium-Chloride-Potassium (ACK) buffer for 10 mins at 36C and vortexed every 5 minutes. They were then washed twice with 1 ml PBS and 3 ml PBS respectively at 500g for 5 mins and lastly with 3 ml PBS at 100g for 10 mins.

Genotyping red and non red cells

ACK lysed cells were resuspended in 200 ul facs buffer and analyzed under the flow cytometer for expression of PE tdtomato.

Bone Marrow Progenitor Culture

After red blood cell lysis, bone marrow cells were resuspended in complete media and counted. The cell density was adjusted to 2.5 x 10^6 cells/ml, and recombinant mouse M-CSF was added at a concentration of 25 ng/ml. Cells were aliquoted into non-tissue culture-treated dishes and cultured at 37°C with 5% CO2. The media was changed, and M-CSF was replenished every 2-3 days. Bone marrow progenitor cells were ready for functional assays on day 6 and remained suitable for use up to day 10.

Antibodies and flow cytometry for bone marrow macrophages

Bone marrow macrophages were trypsinized and distributed across fluorescence-activated cell sorting tubes with appropriate stains. The cells were stained for 20 mins at 4C under dark conditions and then washed twice with facs staining buffer (2% FBS, 0.1% sodium azide, 1x PBS),. The cells were then resuspended in 1:1000 7-aminoactinomycin D live-dead staining buffer and were analyzed on a BD FACsCanto. Table 2 shows all the marker panels used in the experiments conducted throughout using flow cytometry.

Table 4.1. Staining marker panel for flow cytometry

Tube	Bone marrow macrophages staining				
	FITC	**PE**	**APC**	**APC-CY7**	**PE-CY7**
1.	CD34	tdT	IFNAR1	CD11b	Sca1
2.	CD117	tdT	IFNAR1	CD90.2	F4/80
3.	RAE1	tdT	CD86	Ly6C	Ly6G
	Bone marrow macrophages LPS treatment				
1.	IL10	tdT	IL6	CD11b	F4/80
2.	RAE1	tdT	IL2/IL23	CD11b	F4/80
3.	MHC-II	tdT	CD80	CD11b	F4/80
4.	Rat	tdT	Rat	CD11b	F4/80
	IFN titration				
1.	MHC-II	tdT	CD80	CD11b	F4/80
2.	rat	tdT	rat	CD11b	F4/80
	Dextran treatment				
Bone Marrow		tdT	dextran		
Spleen	MHC-II	tdT	dextran	Ly6G	CD11b
	Tumor cells (F68A) IFN treatment				
1.	Sca1	tdT	CD95	CD90.2	CD105
2.	MHC-I	tdT	Galectin-9	CD44	CD24

Stimulating bone marrow macrophages with lipopolysaccharide

Media was replenished in 6,7 and 8 days old grown bone marrow macrophages were then subjected to varying concentrations (0 ng/ml, 0.5 ng/ml and 5 ng/ml) of lipopolysaccharide. The cells were treated with 100 units/ml of IFN gamma and Brefeldin A (1:1000) before being incubated overnight.

Phagocytosis assay

Dextran APC beads were resuspended in PBS at 0.6 mg/ml. 5 bone marrow macrophage plates were pre-cooled at 4°C in the cold room. The plates were treated as follows: half the plates were treated with 0 ul, 10 ul and 100 ul of dextran APC beads for 10 mins and 60 mins respectively at room temperature, whereas the other half were treated under the same conditions at 4°C. These were then washed twice with 2 ml cold PBS. After being treated with 0.5 ml trypsin and incubated at 37°C for 15 mins, the detached macrophages were resuspended in facs tubes, washed twice with facs buffer and analyzed by flow cytometry.

Harvesting macrophages from spleen

In this study, spleens were harvested for further analysis. The spleen was carefully dissected and placed into a dish containing 2 mL of ACK lysing buffer. The spleen tissue was then gently mashed between two frosted slides to dissociate the cells. The resulting spleen mash and buffer mixture were collected and passed through a 70 μm filter to remove debris and tissue fragments. The filter was washed, and the sample was centrifuged to pellet the cells. Optionally, the washing and centrifugation steps could be repeated for further purification. Finally, the cell pellet was resuspended in PBS, yielding a single cell suspension suitable for phagocytosis assay analysis.

Harvesting mice ear and skin for fibroblasts

A small section, 2-3 mm, of the pinna's edge were cut from MX1-cre tdt mice using sharp scissors. The harvested mouse ear was sterilized by sequential immersion in two tubes containing 2.5 mL of ethanol for 2 minutes each. The ear tissue was then minced using sterile razor blades and scissors into approximately 1 mm x 1 mm pieces and incubated in a 5 mL tube containing 0.5 mL of sterile 0.25% trypsin, topped off with 2 mL of trypsin. The tissue was incubated in a 37°C water bath for 10 minutes, after which the trypsin was neutralized with an equal volume of complete media. The tissue suspension was centrifuged at 500g for 5 minutes at 4°C. The cell pellet was resuspended in complete media for further culture.

For mouse skin fibroblast isolation, fur was removed using Nair hair removal cream, and a section of the skin was excised. Skin tissue was treated with either collagenase for 15 minutes. The subsequent protocol was the same as that used for mouse ear fibroblast isolation.

Preparing tumor cells for injection

Tumor cells were prepared for subcutaneous injection into mice while ensuring high cell viability and minimal impact on the growth phenotype. Approximately 10 million tumor cells were harvested from three 70% confluent T175 flasks for injection into five mice. The cells were detached using trypsin and resuspended in complete media before being successively transferred between flasks to create a single cell suspension. Cells were then pelleted and resuspended in room temperature CR-10 medium, followed by a series of washes with ice-cold HBSS containing calcium/magnesium but without phenol red. The final cell pellet was resuspended in 1 mL of the HBSS solution. The cell suspension was then diluted to a concentration of 5 million cells/mL, and 200 μL of this suspension, equivalent to 1 million cells, was injected subcutaneously into the right flank of each mouse as soon as possible after preparation. Red and non red F68A cells in the ratio of 20:80 were injected in wild type and immunodeficient mice.

Tumor harvest

Tumors were harvested from mice that were sacrificed by cervical dislocation. The mice were sterilized by spraying with ethanol before making an incision at the garrot to expose

the tumor on the right flank. Tumor tissue was carefully separated from the surrounding fat and placed in a petri dish containing 5 mL HBSS. Razor blades, sterilized with 70% ethanol, were used to mince the tumor into small 1mm x 1mm pieces. The tumor fragments were then homogenized with media to obtain a single cell suspension. Subsequently a digestion buffer of 1mg/ml Collagenase Type I, 1mg/ml Soybean Trypsin Inhibitor and HBSS (with calcium and magnesium) was added, and the mixture was incubated for 35 minutes to further dissociate the tumor tissue. After incubation, the sample was centrifuged for 5 minutes at 1200 rpm, and the resulting cell pellet was resuspended in CR-10 media. The cell suspension was then passed through a 70 μm filter to remove debris and large aggregates, yielding a single cell suspension suitable for further analyses.

Treatment with NaB

F68A red and non red cells were seeded at 40,000 cells per well in a 6 well plate. Once 70% confluent, they were treated with varying concentrations of NaB: 0mM, 1mM, 2mM and 2.5mM for one and two days. The cells were then trypsinized and prepared for flow cytometry as described previously.

Treating cells with varying IFN concentrations

F68A red and non red cells were seeded at 40,000 cells per well in a 6 well plate. Once 70% confluent, they were treated with varying concentrations of type I IFN: 0 U/ml, 1000 U/ml, 3000 U/ml, 5000 U/ml, 8000 U/ml and 10000 U/ml for one day. The cells were then trypsinized and prepared for flow cytometry as described previously.

RNA extraction, cdna synthesis and quantitative real-time PCR

RNA extraction was performed using Trizol (Invitrogen). The starting material was homogenized in 1 mL of Trizol, and 0.2 mL of chloroform was added per mL of Trizol used. The samples were shaken vigorously and allowed to sit at room temperature for 2-3 minutes. The samples

were then centrifuged at 10,000g for 10 minutes at 4°C, and the aqueous phase was carefully transferred to a new sterile RNase-free tube. An equal volume of 100% RNA-free ethanol was added to the aqueous phase, and the mixture was loaded onto an RNeasy column (Qiagen) for purification following the manufacturer's protocol using RW1 and RPE buffer.

cDNA synthesis was performed using 10 μL of extracted RNA. A reverse transcriptase mix was prepared, containing 2 μL of 10x RT buffer, 0.8 μL of 25x dNTPs, 2 μL of 10x RT random primers, 1 μL of multiscribe reverse transcriptase and 4.2 μL of RNase-free water. The 10 μL of 2x reverse transcriptase mix was added to the 10 μL of extracted RNA, and the samples were incubated at room temperature for 10 minutes, followed by incubation at 37°C for 2 hours and 85°C for 5 minutes for cDNA synthesis in the thermocycler.

Required dilutions of cDNA were prepared (5ng/ml). 1:100 dilution of the stock primer solutions were used. Master mixes with 10ul SYBR green, 200 nM forward and reverse primers, 8 ul cdna and rnase free water to make a total volume of 20 ul per sample were prepared. These were then subjected to a reaction of: 10 min at 95C (denaturation), 40 cycles of 10 seconds each at 95C (denature) and 60 mins at 60C (anneal/extension) in QuantStudio Real-Time PCR system.

Primer design

The primers shown in Table 3 were designed on NCBI's primer design tool and ordered from Integrated DNA Technologies IDT.

BrdU assay

BrdU staining was performed using the Invitrogen BrdU staining kit (Thermo Fisher Scientific) according to the manufacturer's instructions. BrdU was thawed and diluted with sterile PBS at 1X to prepare a working concentration of 1 mM. 10^5 to 10^8 dividing cells were labeled with 10 μM BrdU for 45 minutes at 37°C. After incubation, cells were harvested and washed with 2 mL of Flow Cytometry Staining Buffer, followed by centrifugation at 300-400xg for 5 minutes at room temperature.

Table 4.2. Primers used to verify gene expression in red and non red tumor cells.

Gene	Forward Primer	Reverse Primer	Detected
Hey1_1	GCCACTGCAGTTAACTCCTCC	GCCGAACTCAAGTTTCCATTCT	Y
Hey1_2	GCAGTTAACTCCTCCTTGCCC	GCCGAACTCAAGTTTCCATTCTC	Y
Zdbf2_1	TCCGCTCCGCTCCTCC	CTTGACAAGCATCTCCGTGG	N
Zdbf2_1	CTCCGCTCCGCTCCTCC	TCTTGACAAGCATCTCCGTGG	Y
Gbp10_1	GCTGATGCAGGAGAGAGAGC	AGGAAAGCCTTTTGATCCTTCAG	Y
Gbp10_2	CTGATGCAGGAGAGAGAGCAG	GGAAAGCCTTTTGATCCTTCAGC	Y
Gbp8_1	AGTGAGCCTGAGGAGGCAG	GCCAACGTAGATGAATCTGGTC	Y
Gbp8_2	GTGAGCCTGAGGAGGCAG	CAACGTAGATGAATCTGGTCCC	Y
Tdrd9_1	CTGGTTCACCATCGGCAAGA	TCTGACTGAGTGACCTCCCG	N
Tdrd9_2	CGGCAAGACGGTGACCAATG	TCCTCTGACTGAGTGACCTCCC	N
Lce1g_1	TGCTACCCTTCATATTGCTCCT	ACAGCCCCCAGAACCCAG	N
Lce1g_2	GCTACCCTTCATATTGCTCCTG	CCCCAGAACCCAGGCTA	N
Lce1h_1	TCCATTCACTGGCTGACTGAG	TGTGAGTGTTCAGGAGCAAGA	N
Lce1h_2	ATCCATTCACTGGCTGACTGAG	TGTGAGTGTTCAGGAGCAAGAT	N
Siglecg_1	TGGGGACTCTGGACACTACA	GAGGATTTCCAACAACAGCACA	N
Siglecg_2	TGGGGACTCTGGACACTACA	AGGATTTCCAACAACAGCACA	N
Sca1_1	ACCCCTCCCTCTTCAGGATG	GCTGCACAGATAAAACCTAGCA	Y
Sca1_2	AACCCCTCCCTCTTCAGGAT	CTGCACAGATAAAACCTAGCAGC	Y
Cd90.1_1	CAAGTCGGAACTCTTGGCAC	GGACACCTGCAAGACTGAGA	Y
Cs90.1_2	ATCCAAGTCGGAACTCTTGGC	GGACACCTGCAAGACTGAGAG	Y

For fixation and intracellular staining with Anti-BrdU, DNase I solution was thawed on ice, and a working solution was prepared by adding 300 μL of DNase I solution to 700 μL of Flow Cytometry Staining Buffer. Cells were resuspended in 1X BrdU Staining Buffer working solution and incubated for 15 minutes at room temperature in the dark. After washing with Flow Cytometry Staining Buffer, cells were incubated with DNase I working solution for 1 hour at 37°C in the dark. Cells were subsequently washed twice and half of them were incubated with 5 μL of Anti-BrdU fluorochrome-conjugated antibody for 20-30 minutes at room temperature in the dark. Finally, cells were washed twice with Flow Cytometry Staining Buffer before analysis. Data was then acquired on the flow cytometer.

Cell counting for studying cell kinetics

Cell counting was performed using a hemocytometer following standard procedures. Cells were harvested and centrifuged at 500g for 5 minutes at room temperature. The cell pellet was resuspended in an appropriate volume of culture media or buffer. The cell suspension was gently mixed to ensure a homogeneous distribution of cells.The cell suspension was further diluted (if necessary) to obtain a countable density. $10\mu L$ of the cell suspension was mixed with equal volume of 0.4% trypan blue dye was loaded into the hemocytometer chamber and cells were counted in the 4 large squares. The following equation was used to calculate cell concentration per ml= $(Averagenumberofcellsperlargesquare)x(1/2)x(10^4)$. The total cell count was obtained by multiplying the cell concentration by the volume of the cell suspension.

Statistical Significance

The findings to assess the phenotypical and functional variations between red and non-red bone marrow macrophages failed to reveal any statistically significant difference between the red and non red groups. Statistical differences between these two groups were were tested with a two tailed test. However, the study's second hypothesis pertaining to tumor cells gave more promising results. Both in vitro and in vivo growth studies along with the results for Hey1

and NaB experiments demonstrated a statistically significant difference between red and non red tumor cells. This observation was verified through the t-test for two group studies and a one-way ANOVA analyses for grouped studies using the GraphPad Prism Software. p¡0.05 was considered to be statistically significant.

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