MIR-17-92 CLUSTER REGULATION IN DIFFERENTIATED T-CELLS

by

Gary Kohanbash

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This thesis was presented

by

Gary Kohanbash

It was defended on

July 29, 2009

and approved by

Hideho Okada M.D., Ph.D., Associate Professor
Neurological Surgery and Surgery, School of Medicine,
University of Pittsburgh

Todd Reinhart, ScD, Professor, Assistant Chairman and Director of Graduate Programs
Infectious Diseases and Microbiology, Graduate School of Public Health,
University of Pittsburgh

Jeremy Martinson, DPhil, Assistant Professor
Infectious Diseases and Microbiology, Graduate School of Public Health,
University of Pittsburgh

Hideho Okada M.D., Ph.D.

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Gary Kohanbash, M.S.

University of Pittsburgh, 2009

Data from our group and others have demonstrated that tumor-derived factors directly skew T-cell differentiation from an effective tumor fighting Th1 state to a less effective Th2 state, allowing for tumor growth. Why the Th1 response is more effective is largely still unknown. The recently discovered microRNAs (miRNAs) are a large family of small regulatory RNAs that control diverse aspects of cell functions such as cell proliferation, apoptosis, development, differentiation and immune regulation. We thereby sought to examine miRNAs differentially expressed in Th1 and Th2 cells in an effort to better understand the enhanced ability of Th1 cells in tumor immunity. MicroRNA microarray analyses revealed that the miR-17-92 cluster of microRNAs (miR-17-92) is consistently over-expressed in murine Th1 cells compared to Th2 cells. Quantitative RT-PCR confirmed that the miR-17-92 cluster expression was consistently higher in Th1 cells than Th2 cells. Furthermore, disruption of IL-4 signaling through either IL-4 neutralizing antibody or knockout of STAT6 reversed the miR-17-92 cluster suppression in Th2 cells. MiR-17-92 expression correlated with differential proliferation capacity as Th1 cells proliferated at higher levels than Th2 cells, dependent on IL-4 and STAT6. Th1 cells consistently expressed lower levels of anti-proliferative transcription factors E2F1 and E2F2, which are the known targets of miR-17-92. Collectively, our data suggests that the Th2 skewing tumor microenvironment can induce the down-regulation of miR-17-92 expression in CD4+T cells, thereby diminishing the effective proliferation of tumor-specific T cells and tumor

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destruction. This has significant public health relevance as we propose that therapy targeting miR-17-92 cluster may provide enhanced T-cell function and prevent tumor growth.

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1.0   INTRODUCTION

1.1   GLIOBLASTOMA MULTIFORME

Glioblastoma Multiforme (GBM) is the most common and one of the most malignant forms of brain tumors. The primary factors that cause GMBs are still mostly unknown. However these factors lead to epidermal growth factor receptor (EGFR) and phosphatase and tensin homolog (PTEN) mutations with P53 deletions in many cases. Furthermore GBMs are characterized by necrosis, microvascular proliferation and glial cell mitosis [1].

As reviewed by Ohgaki [2], GBM occurrence seems most prevalent in industrialized countries with Caucasians having greater incidence than both African and Asian populations. Limited data is available on causes of GBM however many occupational exposures have been shown to be associated with GMB such as plastics, formaldehyde and lead. Other factors such as smoking and electromagnetic field have shown no association with GMB (in most studies). According to Ohgaki the only factor "unequivocally associate" with GBM is X-irradiation, a therapy used to treat acute lymphoblastic leukemia.

With over 12,000 new cases diagnosed in the United States each year and a median survival time of less than 15 months GBM tumors represent a significant public health problem. One of the most common forms of GMB is the astrocytoma. Of the astrocytomas the World

Health Organization-designated stages of malignancy from I-IV, GBMs represent the highest grade IV tumors [3].

Current treatments for GBM include: chemotherapy, radiotherapy, and surgical resection [4]. However, even the most effective treatments over the past 25 years have at best added 3 months to the 15-month median survival time. There are many challenges in effectively treating GBMs. GBM's malignant nature tumor cells spread to areas throughout the brain and tumor recurrence after resection is typically unavoidable. Additionally, the microvasculature around the brain and the blood brain barrier restricts the passage of many drugs into the brain [3]. For these reason, together with the findings that immune cells do enter the brain, much work has been devoted to immunotherapy, and enhancing the natural response to control the tumor.

1.2 MICRORNA

1.2.1 MicroRNA Biology

MicroRNAs (miRNAs) are endogenous small single-stranded RNA molecules which are 18-24 nucleotides in length [5]. MiRNAs are highly conserved between species and have been identified in plants, animals and viruses [6, 7]. These small RNA are located in various parts of the genome, usually in segments not associated with known genes. Mature miRNA molecules have the ability to repress translation and therefore serve an important role in regulating post transcriptional

activities [8]. There are over 300 microRNAs in the human genome which are predicted to regulated 2/3 of all genes [9, 10].

1.2.2 MiRNA Processing and Function

Genes encoding miRNAs are transcribed by RNA polymerase II into long primary miRNA sequences (pri-miRNAs) with a 5' cap, 3' untranslated region (UTR), and a hairpin sequence that encodes the mature miRNA. The hairpin of the pri-miR is then cleaved by the enzyme Drosha to form precursor microRNAs (pre-miRNAs). Pre-miRNAs and are then transported via Exportin V to the cytoplasm. Once in the cytoplasm dicer, an Rnase III superfamily member cleaves one of the strands and attaches the mature miRNA to an RNA-induced silencing complex (RISC) complex. The full RISC complex (miRNA and RISC) are then able to bind to 3' UTR regions of mRNAs, and inhibit translation. Translational inhibition may occur either through mRNA degradation or translational suppression. When there is complete complementarity of the miRNA to the mRNA 3' UTR, the mRNA is degraded, however, partial complementarity of the miRNA to the 3' UTR sequence results in inhibition of the circularization of the mRNA needed for ribosomal attachment [11].

1.2.3 MiR-17-92 Cluster of MiRNAs

Findings over the past five years strongly support a role for miRNAs in the regulation of crucial processes such as cell proliferation [12], apoptosis [13], development [14], differentiation [15], metabolism [16], and immune regulation [17, 18].

Many of the known microRNAs appear in clusters on single polycistronic transcripts [19]. MiR-17-92 cluster codes for 7 mature miRNAs. Mir17-92 has two paralog clusters, miR-106a cluster and miR-106b cluster [19]. Mir-17-92 and its paralog clusters together consist of miR-17, miR-18, miR-19a, miR-19b, miR-20, miR-25, miR-92, miR-93, miR-106a, and miR-106b [19]. MiRNAs in miR-17-92 clusters are reported to be amplified in various tumor types, such as B-cell lymphoma and lung cancers [5, 20-22]. Recently, these miRNAs have been found to induce proliferation and confer anti-apoptotic function in tumors thereby promoting tumor-progression, and function as oncogenes [6, 20, 21].

Knockout and transgenic studies of the miR-17-92 cluster in mice have demonstrated the importance of this cluster in mammalian biology. While knockout of the mir-17-92 cluster results in immediate post natal death of all progeny, knockout of either or both the miR-106a or miR-106b cluster demonstrated no apparent change in phenotype. However, when the miR-17-92 cluster was knocked out together with miR-106a or 106b cluster the result was embryonic lethality [23]. Further studies have been done in transgenic mice with miR-17-92 overexpressed in lymphocytes through the CD2-CRE in a CRE-Lox system. These mice demonstrated lymphoproliferative disorder and autoimmunity but not cancer [24]. However, transgenic mice overexpressing both miR-17-92 and c-Myc in lymphocytes develop early onset lymphomagenesis disorders. These findings demonstrate a critical role for miR-17-92 cluster in T-cell cell biology.

Many transcription factors have been identified that regulate expression of this cluster, including the E2 transcription factor (E2F) family members [25], c-Myc [5], and signal transducer and activator of transcription-3 (STAT3) [26]. Additionally, miR-17-92 cluster miRNAs have been shown to regulate many genes including: E2F1, E2F2, E2F3, P21, TSP1, CTGF, BIM and

PTEN[5, 25, 27-29]. These genes are all involved in cell cycle regulation, further supporting the importance of miR-17-92 cluster in T-cell biology.

1.3 T-CELL FUNCTION IN CANCER

1.3.1 Relevance of Th1 and Th2 Cell Response in Cancer

T-cell immune responses are classified into distinct effector cell types based on their cytokine-secreting profiles [30-32]. Type-2 T-cells include T-helper (Th2) and T-cytotoxic cells (Tc2), which preferentially secrete interleukin IL-4, IL-5, and IL-10, whereas type-1 T cells [T-helper (Th1) and T-cytotoxic cell (Tc1)] predominantly secrete interferon(IFN)-γ.

Data from our group and others indicate that a type-1 T-cell response is favorable for anti-tumor immunity [33, 34]. Cancers, including GBMs, secrete numerous Type-2 driving cytokines [35-38] that serve to promote tumor proliferation [35, 39, 40], immune escape [41, 42], and skew the T-cell response towards the Type-2 [41-43]. All of these events commonly correlated with poor prognosis in cancer patients [43-45]. However, the exact mechanisms as to why type-1 immune responses are favorable for anti-tumor immunity are largely unknown. We therefore seek to better understand T-cell biology to establish the means to skew the T-cell response toward type-1.

1.3.2 Immunotherapeutic Approaches to GBMs

A major challenge in current immunotherapy for progressive malignant glioma is the systemic suppression of immunity due to chemo-/radiotherapy, tumor elaboration of immunosuppressive substances, and Th2 skewing factors [46-48]. While active immunization for GBM relies on intact host-immune reactivity in order to elicit potent anti-tumor immune responses, it seems promising to generate genetically-modified tumor-specific T-cells *ex vivo*, which are resistant to tumor-mediated immune suppression and possess robust anti-tumor responses. As previously discussed, miR-17-92 miRNAs have the potential to regulate the cell cycle and confer resistance of adoptively transferred T-cells to tumor-derived immunosuppressive factors. Therefore, further characterization of the role of miR-17-92 cluster may provide us with strong bases to develop novel immunotherapy strategies with genetically engineered T cells with improved abilities to mediate anti-tumor effects.

2.0   THESIS AIMS

Enhancing the host immunological response to tumors remains a challenge for glioma researchers. As mentioned previously a Th1 immune response is favorable for anti-tumor immunity. As miRNAs represent a novel class of regulatory molecules we decided to examine the differential expression of miRNAs in Th1 versus the tumor-skewed Th2 cells. We hypothesize that miRNAs overexpressed in Th1 cells may play a critical role in the promoting the anti-tumor response. Therefore, the overall goal of this study was to examine differences in miRNA expression between Th1 and Th2 cells and study immunoregulatory factors present in the tumor microenvironment that may regulate these important miRNA.

2.1   SPECIFIC AIMS

Specific Aim #1: Characterize miRNA expression profile and phenotype of Th1 and Th2 cells.

Hypothesis: We expect to identify miRNA that are upregulated in Th1 over Th2.

Results: Th1 cells demonstrated increased miR-17-92 cluster expression, better ability to proliferate, and decreased expression of anti proliferative factors E2F1 and E2F2 than Th2 cells.

Specific Aim #2: Examine specific mechanisms that regulate miRNA-17-92 expression.

Hypothesis: Type-2 cytokines and their intracellular signaling pathway affect the expression levels of differentially expressed microRNAs.

Results: Suppression of IL-4 or IL-4 signaling resulted in increased miR-17-92 expression and increased proliferative ability of CD4+ T-cells.

3.0   MATERIALS AND METHODS

3.1   REAGENTS

RPMI 1640, FBS, L-glutamine, sodium pyruvate, 2-mercaptoethanol, nonessential amino acids, and penacilin/streptomycin all were obtained from Invitrogen Life Technologies. Recombinant murine (rm) IL-12 was purchased from Cell Sciences Technologies. RmIL-4 and recombinant human IL-2 were purchased from PeproTech. Purified mAbs against IL-12 (C15.6), IFN-γ (R4 – 6A2), IL-4 (11B11), CD3 (145-2C11), were all purchased from BD Pharmingen. All quantitative real time PCR (quantitative RT-PCR) reagents and primers were purchased from Applied Biosystems and analyzed on a BioRad IQ5. WST-1 reagent was purchased from Roche. C57BL/6 mice (5–9 wk of age) and C57BL/6 background STAT6 deficient mice were purchased from The Jackson Laboratory in Bar Harbor, Maine. Animals were handled in the Hillman Cancer Center Animal Facility at University of Pittsburgh per an Institutional Animal Care and Use Committee-approved protocol.

3.2   TH1 AND TH2 CELL CULTURE

Th1 and Th2 cells were differentiated from immunomagnetically-separated CD4+ splenic T-cells. Magnetic activated cell separation (MACS) was carried out using positive selection. Briefly, spleens were minced in complete media, resuspended in red blood cell lysis buffer and stained with immunomagnetically labeled anti-CD4 antibody. Cells were then washed and placed through the magnetic column in 500ul of MACS buffer. The column was then washed 3 times with 3ml of buffer and then removed from the magnet and labeled cells were extracted in 3ml of MACS buffer.

For the differentiation of T-cells, purified $CD4^+$ cells were stimulated in 96 well plates previously coated with 100µl of anti-CD3ε mAb (5µg/ml) for 24 hours at 4°C . Cells were then incubated with irradiated C57BL/6 spleen cells (3000 Rad, 2.3 minutes) as feeder cells. RmIL-12 (ng/ml), rmIFN-γ (ng/ml), anti-IL-4 (ng/ml) mAb and rhIL-2 (100U/ml) was added for Th1 development. Th2 cells were generated from the same CD4+ cell precursors stimulated with anti-CD3 mAb and feeder cells in the presence of rmIL-4 (ng/ml), two anti-IFN-γ mAbs (ng/ml), anti-IL-12 mAb (ng/ml) and rhIL-2 (100U/mL). After 10 days cells were stained for IL-4 and IFN-γ to confirm differentiation. Neutral cell culture included anti-CD3, feeder cells and IL2. For studies involving IL-4 blocking, 12.5ng/ml was used in human experiments and 50ng/ml in murine studies. For FACs analysis, cells were incubated at 4°C for 30 min, washed twice in staining buffer, and fixed in 500 µl of 1% paraformaldehyde in PBS. Cells were stored in the dark at 4°C until analysis. Flow was carried out on the Coulter XL four-color flow cytometer at the flow cytometry core facility of the University of Pittsburgh Cancer Institute.

3.3 MIRNA MICROARRAY

Total RNA was harvested from Th1 and Th2 using the Qiagen RNeasy kit and quality was confirmed with a A260/A280 ratio greater than 1.85. RNA was labeled with either Cy5 (red; Th1) or Cy3 (yellow; Th2) fluorescent dyes. The total RNA samples were next mixed and applied to miRNA array slides prepared by Drs. Ena Wang and Francesco Marincola (the NIH) using robotics for the spotting of 714 murine, human and viral sequences complementary to different mammalian miRNAs, and analyzed with a microarray chip reader. Differentially expressed miRNAs were analyzed by hierarchical clustering of Th1/Th2 pair of miRNA microarray signal. MiRNAs changed with ratio>2fold were considered significant.

3.4 QUANTITATIVE RT-PCR

Total RNA was extracted using the Qiagen RNeasy kit and quality was confirmed with a A260/A280 ration greater than 1.85. RNA (10 ng) was subjected to quantitative RT-PCR analysis using the TaqMan microRNA Reverse Transcription Kit, microRNA Assays (Applied Biosystems), and the Real-Time thermocycler iQ5 (Bio-Rad). The small nucleolar SNO202 was used as the housekeeping small RNA reference gene for all murine samples. All reactions were done in triplicate and relative expression of RNAs was calculated using the $\Delta\Delta C_T$ method.

3.5   PROLIFERATION ASSAYS

For WST-1 proliferation assays 1x 10^4 cells were cultured in a 96 well plate for 24-48 hours in 100ul of complete media. After this time 10ul of WST-1 reagent was added to each well. Cells were incubated at 37^0C, 5% CO_2 for 4 hours, and placed on a shaker for 1 min. The plates were then read on a micro plate reader with a wavelength of 420 nm and a reference at 620 nm.

For CFSE assays 5 x 10^5 immunomagnetically-separated CD4+ splenic T-cells were cultured under Th1 or Th2 polarizing conditions. On day 10 cells were labeled with 0.5uM CFSE and on day 15 cells were then harvested and CFSE dilution was assessed by flow-cytometry.

4.0   AIM 1 RESULTS

4.1   IN VITRO DIFFERENTIATION OF TH1 AND TH2 CELLS

As discussed in the Background section, functional effecter T-cells include those polarized to either Th1 or Th2 phenotypes by environmental instruction. There are also less prevalent functionally-differentiated T-cells such as: Tregs and Th17 [Th17 follow a similar trend in miR-17-92 expression as Th1 (data not shown)].

From immunomagnetically isolated murine CD4+ splenic T-cells, Th1 cells were induced by culture in complete media containing IL-2, IL-12 and anti-IL-4 neutralizing antibody for 10 days, whereas Th2 cells were generated in media containing IL-4, neutralizing antibodies against IFN-γ and IL-12. Both groups were stimulated with anti-CD3 antibody and feeder cells on days 0 and 2, and further maintained with hIL2. After 10 days these differentiated T-cells exhibited expected cytokine profiles as shown using flow cytometry (**Figure 1).** Th1 cells predominantly produced IFN-γ but not IL-4, while Th2 cells produced mostly IL-4 consistent with what we expected.
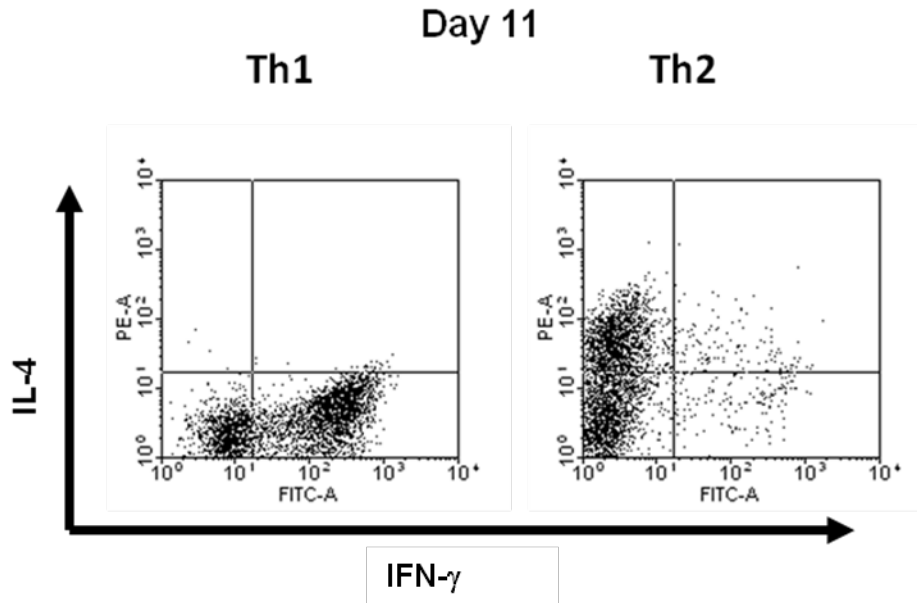
Figure 1: Flow cytometry analysis of Th1 and Th2 differentiated T-cells. Cells were gated on live lymphocyte populations. Intracellular IFN-γ and IL-4 expression was examines for the by Th1 and Th2 cells induced from wild-type CD4^{+} splenic T cells *in vitro*. Flow was carried out to confirm differentiation of all cultured T-cells.

4.2   MICRORNA MICROARRAY OF TH1 AND TH2 CELLS

Total RNA was extracted from day 10 confirmed differentiated CD4+ T-cells. Th1 cell RNA was labeled with Cy5 (red) and Th2 cell RNA were labeled with Cy3 (yellow) and analyzed for miRNA expression by miRNA microarray **(Figure 2)**. As expected of 714 miRNAs, many were differentially expressed between our in-vitro cultured Th1 and Th2 cells. Hierarchical clustering of differentially expressed microRNAs revealed distinct miRNA expression profiles between Th1 and Th2 cells (**Figure 3 and Table 1**). Surprisingly, of the upregulated miRNA, all the miRNA from the miR-17-92 cluster seemed to all be upregulated in Th1 cells. As this miRNA

cluster has been shown to regulate many genes we decided to further examine expression of this

miRNA cluster.
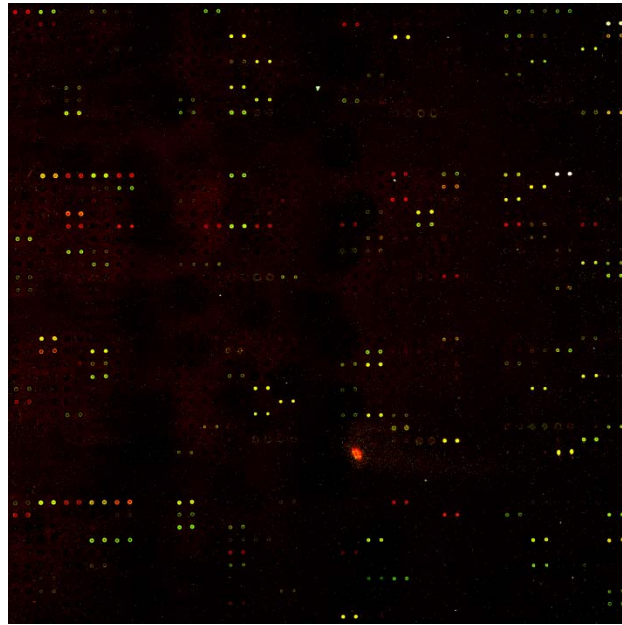
Figure 2: MiRNA microArray Chip. A representative miRNA array chip hybridized with Th1 and Th2 derived total RNA. Total RNA was harvested from Th1 and Th2 cells and labeled with either Cy5 (red; Th1) or Cy3 (yellow; Th2) fluorescent dyes. The total RNA samples were next mixed and applied to miRNA array slides prepared by using robotics for the spotting of 714 murine, human and viral sequences complementary to different mammalian miRNAs. Each dot represents one mature miRNA. Red dots represent miRNAs upregulated in Th1 cells and yellow dots represent miRNAs upregulated in Th2 cells

mmu-miR-494
hsa-miR-494
mmu-mir-690
hsa-miR-30c
hsa-miR-93
hsa-miR-92
hsa-miR-26a
hsa-miR-20b
hsa-miR-106b
hsa-miR-106a
hsa-let-7i
ebv-mir-BART14
mmu-miR-93
kshv-miR-K12-6-5p
hsa-mir-560
hsa-miR-27a
hsa-miR-20b
hsa-miR-19b
mmu-miR-106a
hsa-miR-98
hsa-miR-221
hsa-miR-20a
hsa-miR-195
hsa-miR-17-5p
hsa-let-7a
hsa-let-7b
hsa-miR-506
hsa-miR-378
hsa-miR-19a
hsa-miR-181b
hsa-let-7c
hcmv-miR-UL36-1
mmu-miR-217
hsa-miR-30b
hsa-miR-26b
hsa-miR-21
hsa-miR-200c
mmu-mir-711
hsa-miR-30d
hsa-miR-30a-5p
hsa-miR-23a
hsa-miR-16
hsa-miR-150
hsa-miR-142-5p
hsa-miR-142-3p
hsa-miR-142-3p
hsa-let-7f
hsa-let-7d
hsa-let-7a
rno-miR-352
hsa-mir-602
hsa-miR-495
hsa-let-7d
rno-miR-347
hsa-miR-215
mmu-miR-503
mmu-mir-488
mmu-miR-129-5p
hsa-miR-382
hsa-miR-373
kshv-miR-K12-5
hsa-mir-654
hsa-mir-574

Figure 3: Hierarchal clustering of Th1 vs. Th2 differentially expressed miRNAs. Differentially expressed miRNAs were analyzed by hierarchical clustering. Red indicates upregulation in Th1 cell RNA and green represents up-regulation in Th2 cell RNA. MiRNAs changed with ratio>2 fold are shown.
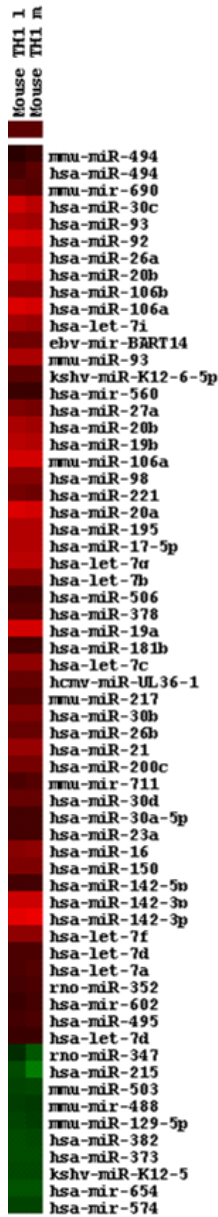
Table 1: MiRNA overexpression in Th1/Th2 cells. MiRNA were ranked in terms of relative fold expression of Th1/Th2 cells. Arrows indicate members of the miR-17-92 cluster. miRNAs with a relative expression of >2 fold are shown

NAME	Mouse Th1/Th2
hsa-miR-142-3p	3.86
→ hsa-miR-92	3.66
→ hsa-miR-20a	3.66
→ hsa-miR-106a	3.642
→ mmu-miR-106a	3.561
hsa-miR-30c	3.551
→ hsa-miR-19a	3.492
→ hsa-miR-20b	3.451
hsa-miR-142-3p	3.355
hsa-let-7g	3.173
→ hsa-miR-19b	3.134
→ hsa-miR-17-5p	3.06
hsa-miR-195	3.048
→ mmu-miR-93	2.917
hsa-miR-26a	2.882
hsa-miR-93	2.87
→ hsa-miR-20b	2.864
hsa-let-7i	2.704
→ hsa-miR-21	2.566
hsa-let-7f	2.433
hsa-let-7c	2.408
→ hsa-miR-106b	2.369
hsa-miR-98	2.35

4.3 QUANTITATIVE RT-PCR OF MATURE MIRNA ISOLATED FROM TH1 AND TH2 CELLS

To confirm expression of miR-17-92 as seen in the microRNA microarray we performed quantitative RT-PCR for each of the mature miRNA in the miR-17-92 cluster **(Figure 4).**
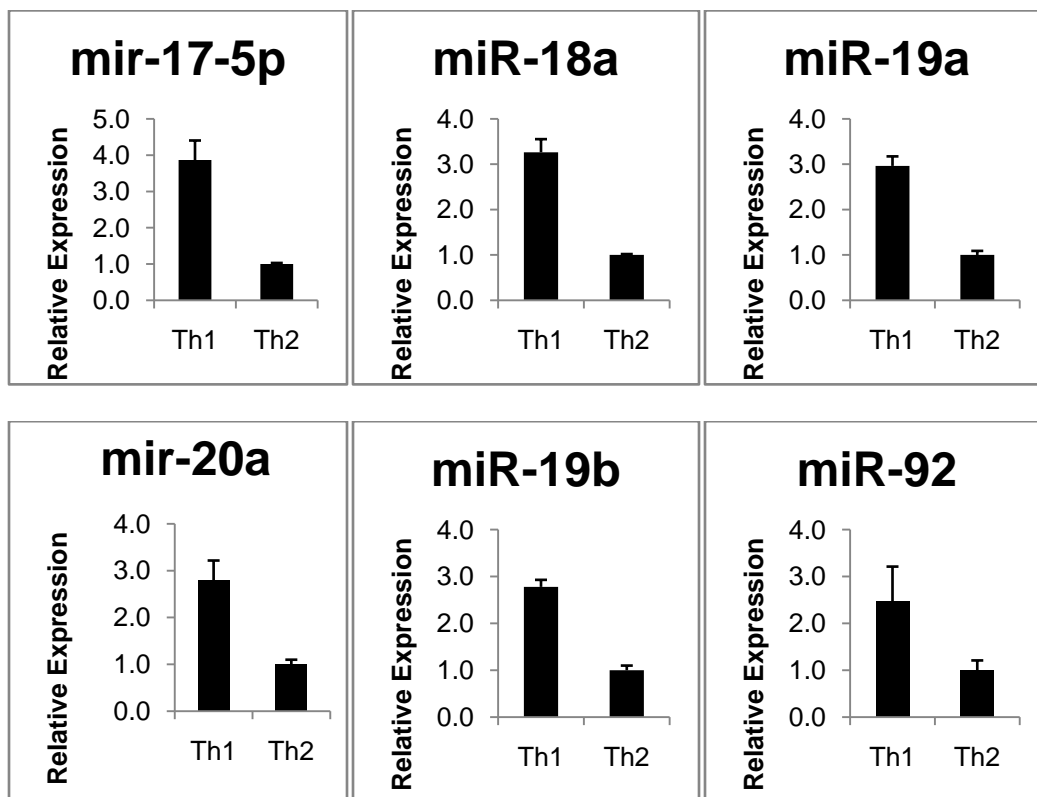
Figure 4: Quantitative RT-PCR of all miRNA from the miR-17-92 cluster. Data represent relative expression of mature microRNA expression of each miRNA from Th1 or Th2 cell RNA. SNO202 was used as the internal control and $2^{\Delta\Delta CT}$ method was used to examine expression relative to the Th2 cell value. Error bars indicate the standard deviation. Columns indicate the mean of 2 separate experiments, each experiment with 3 wells and Error Bars indicate standard deviation across these 2 experiment. Statistical analysis was carried out on graphpad prism using the student t test. All values were significant with a p<.01 for miR-92 and p<.0001 for all others.

We thus concluded that relative expression of all miRNAs from miR-17-92 cluster were consistently upregulated in Th1 vs. Th2.

4.4   EXPRESSION OF MIR-17-92 PARALOG CLUSTERS

As mentioned previously, miR-17-92 cluster has 2 paralog clusters: miR-106a-92 and miR-106b-25. These paralog clusters target similar mRNAs as miR-17-92 cluster [49] . If Th2 cells overexpressed the 2 paralog clusters and Th1 cells overexpressed miR-17-92 cluster similar mRNAs would be suppressed in both cell types and no resulting change in phenotype would be expected. However if these 2 paralog clusters were overexpressed in Th1 we could expect to see an amplified response and further downregulation of mIR-17-92 targets. To establish if these paralog microRNA clusters were overexpressed in our Th1 versus Th2 cells we next ran quantitative RT-PCR for multiple miRNAs in each of these clusters. Representative for these paralog clusters are miR-106a and miR106b (**Figure 5**).
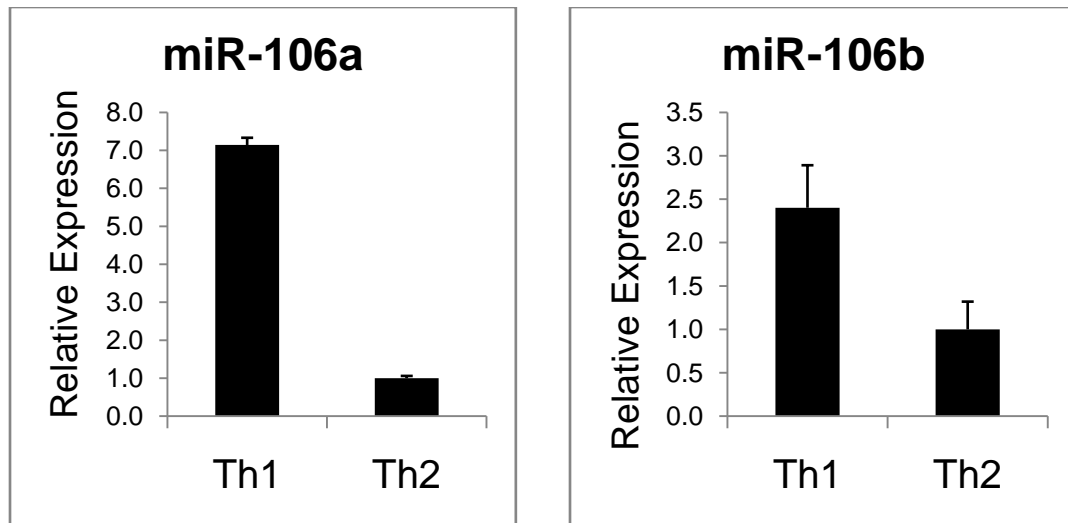
Figure 5: Quantitative RT-PCR of miR-106a and miR-106b expression in Th1 and Th2 cultured cells. Data represent relative expression of each mature miRNA from Th1 or Th2 cell RNA. SNO202 was used as the internal control and $2^{\Delta\Delta CT}$ method was used to examine expression relative to the Th2 cell value. Data is representative of 2 experiments. Data represent the mean of a single experiment containing triplicate samples for quantitative RT-PCR. Error Bars indicate standard deviation of the triplicate samples. Statistical analysis was carried out on graphpad prism using the student t test. Samples are significant with a p<.001 for miR-16a and a p<.05 for miR106b.

These data demonstrate that the paralog clusters of miRNA were also overexpressed in Th1 cells over Th2 and that the effect of the miR-17-92 cluster can be enhances [49]. Despite the similar trend relative expression levels seemed varied between the miR-17-92 cluster and its paralog clusters. Because these miRNA are on separate chromosomes it is likely that other factors also participate in their regulation.

4.5   PROLIFERATION OF TH1 AND TH2 CELLS IN CULTURE

4.5.1   CFSE Staining of Th1 and Th2 Cells

As previously discussed, miR-17-92 and its paralog clusters have been predicted and demonstrated to regulate many genes involved in proliferation and the cell cycle [12-14]. We thus next sought to examine if proliferation of our Th1 and Th2 cells correlated with miR-17-92 cluster expression using a CFSE proliferation assay **(Figure 6).**
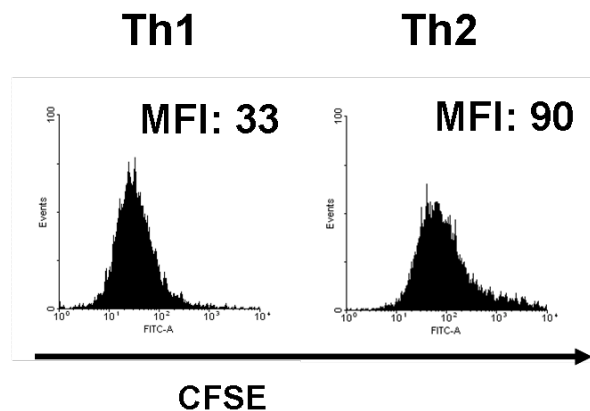
Figure 6: Proliferative ability of Th1 vs. Th2 cells examined through CFSE assay. immunomagnetically-separated CD4$^+$ splenic T cells derived from Wild-type mice and then cultured with Th1 or Th2 cytokine conditions on day 10 cells were labeled with 0.5μM of CFSE on day 15, cells were harvested and CFSE dilution was assessed by flow-cytometry.

As CFSE labeled cells proliferate CFSE intensity is diluted and mean florescent intensity (MFI) of is decreased [50]. Here we demonstrate that Th1 cells exhibited increased growth relative to Th2 cultured cells.

4.5.2 WST-1 Proliferation Assay of Th1 and Th2 Cells

To further examine the proliferative capacity of Th1 cells compared to Th2 cells we next performed a WST-1 proliferation assay. Briefly, day 10-differentiated proliferating cells were cultured overnight and then incubated in the presence of WST-1 reagent for 4 hours. Absorbance of each wells were then measured on an ELISA plate reader **(Figure 7).**
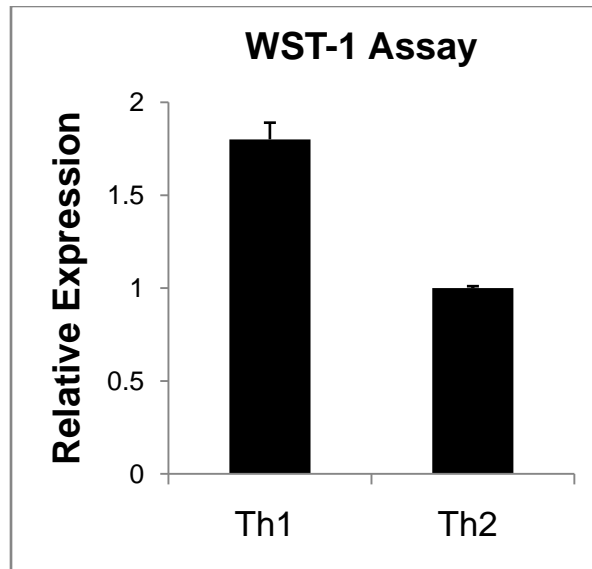
Figure 7: WST-1 Assay of Th1 and Th2 cultured cells. 1x 10^4 cells were cultured in a 96 well plate for 24-48 hours in 100ul of complete media. After this time 10ul of WST-1 reagent was added to each well. Cells were incubated at 37^0C, 5% CO_2 for 4 hours, and placed on a shaker for 1 min. The plates were then read on a micro plate reader with a wavelength of 420 nm. Columns represent the mean of 2 separate Th1 and Th2 cultures, each run in quadruplicate; error bars represent standard deviation of all 8 samples. Statistical analysis was carried out on graphpad prism using the student t test. Values are significant with a p<.01

WST-1 absorbance directly correlates with proliferation and cell viability. To this end Th1 and Th2 cell proliferation followed a similar trend with miR-17-92 expression, further suggesting the importance of miR-17-92 in Th1 and Th2 cells.

4.6   EXPRESSION OF E2F'S IN TH1 AND TH2

The E2F family members are predicted targets of miR-17-92. E2Fs are transcription factors known to be important in regulating many cell cycle genes [51]. Specifically, E2F1 and E2F2 are known negative regulators of T-cell proliferation. We therefore proposed that miR-17-92 may be downregulating the E2F pathways in Th1 cells resulting in the observed enhanced proliferation over Th2. We collected protein lysates from Th1 and Th2 cells and ran a western blot to examine E2F1 and E2F2 expression **(Figure 8).**
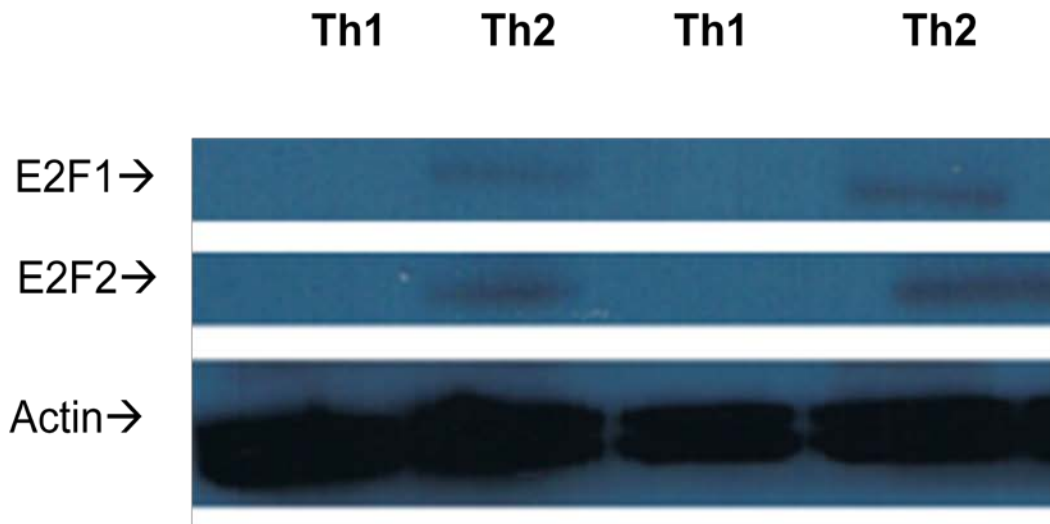
Figure 8: Western blot Analysis of E2F1 and E2F2 from Th1 and Th2 protein lysates. Protein fractions were extracted from day 10-confirmed Th1 and Th2 cells on 2 independent occasions. Twenty micrograms of protein from each sample were then loaded onto 3 separate 10% SDS-PAGE gels(one gel for each antibody). Proteins were transferred to a Polyvinylidene fluoride (PVDF) membrane and each membrane was immunostained for E2F1, E2F2 or b-Actin primary antibody followed by horseradish peroxidase HRP conjugated secondary antibody and exposed using Millipore western blot exposure reagent.

Our results demonstrate that as expected Th1 cells with upregulated miR-17-92 have lower expression of E2F1 and E2F2 molecules. This supports our hypothesis that miR-17-92 promotes better proliferation of differentiated T-cells.

5.0 AIM 1 CONCLUSION

Our conclusion for Aim 1 is that many miRNAs are differentially expressed between Th1 and Th2 cells. Of these differentially expressed miRNA, the miR-17-92 cluster and its paralog clusters all were overexpressed in Th1 cultured cells relative to Th2 cultured cells. Since these miRNAs have been implicated in cell growth, we also demonstrated that overexpression of these miRNAs correlated with higher proliferation in Th1 versus Th2 cells and reduced expression of the anti-proliferative transcription factors E2F1 and E2F2.

6.0 AIM 2 RESULTS

6.1 BLOCKING IL-4 REGULATED THE EXPRESSION OF MIR-17-92 CLUSTER

After demonstrating the difference in miR-17-92 cluster expression between Th1 and Th2 cells we next aimed to determine factors that may regulate the cluster. After addition of IL-4 neutralizing antibodies to neutral cell cultures RNA was extracted from these cells and quantitative RT-PCR was used to examine expression of the first and last microRNA in the miR-17-92 cluster **(Figure 9).**
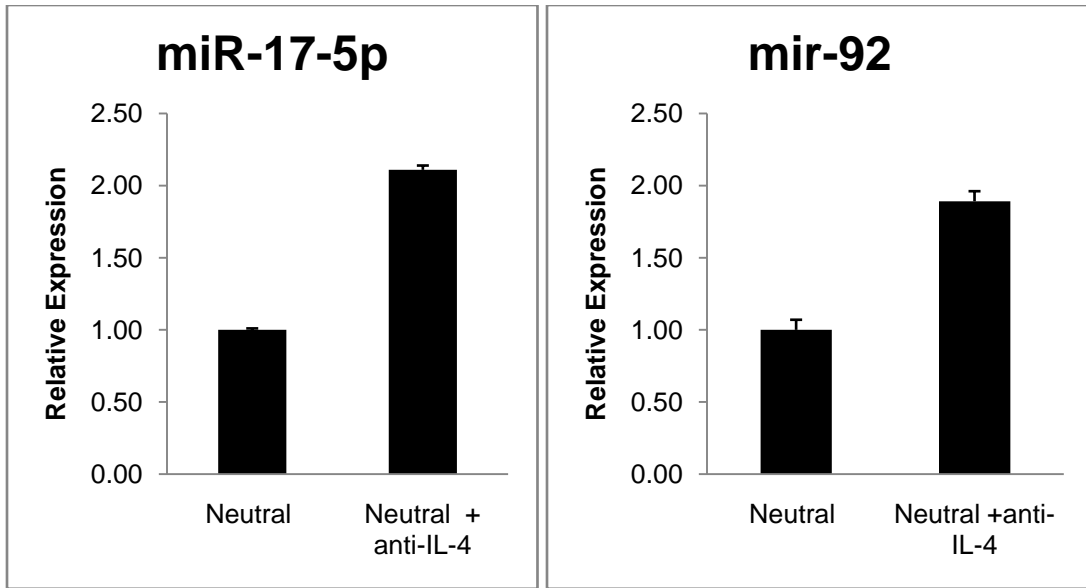
Figure 9: MiR-17-5p and miR-92 expression on neutral CD4+ T-cells after addition of IL-4 neutralizing antibody. Neutral treated (anti-CD3, feeder cells, and hIL2) cells were cultured from immunomagnetically isolated CD4+ T-cells with 5μg/ml plated anti-CD3, feeder cells and 100U/mL hIL2. Two and one half μg/mL of anti-IL-4 was added to the appropriate wells and cultured for 5 days prior to extraction of RNA. Quantitative RT-PCR data is representative of 2 identical experiments. Columns represent the mean of triplicates from a single experiment and error bars represent standard deviation. Statistical analysis was carried out on graphpad prism using the student t test. MiR-17-5p and miR-92 were significant with p<.001 and p<.005, respectively.

These data suggest that blockade of endogenously produced IL-4 is sufficient to upregulate miR-17-92 expression by approximately 50%, and IL-4 produced in the tumor microenvironment can potentially cause a decrease in miR-17-92 cluster expression.

6.2   MIR-17-92 EXPRESSION IN STAT6 DEFICIENT T-CELLS

To further illustrate the effect of IL-4 signaling on miR-17-92 cluster expression we next isolated CD4+ T-cells from mice deficient of the critical IL-4 signaling molecule, STAT6. After isolation

we cultured these cells in Th1- or Th2-skewing conditions for 10 days. Quantitative RT-PCR analysis from these cells demonstrated that STAT6 deficient Th2 cultured cells exhibited no reduction in either miR-17-5p expression **(Figure 10)**.
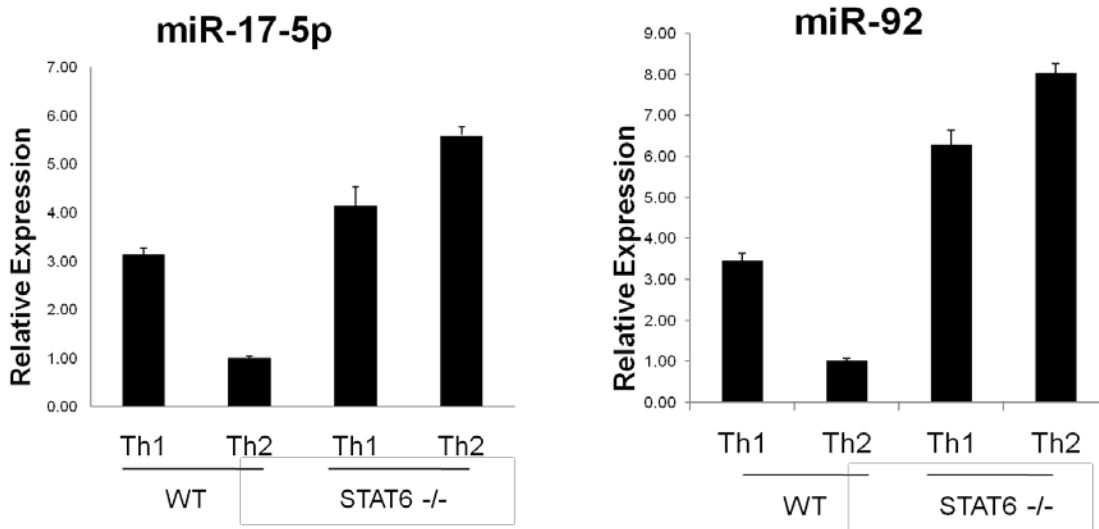
Figure 10: Down-regulation of miR-17-5 and miR-92 in Th2 cultured cells is STAT6-dependent. Th1 and Th2 cultured cells were induced from CD4+ T cells isolated from either wild-type or STAT6 knockout mouse-derived splenocytes. Total RNA was extracted from each cells and quantitative RT-PCR was performed using specific primers against miR-17-5p. Columns represent the mean of triplicates from a single experiment and error bars represent standard deviation. Statistical analysis was carried out on graphpad prism using Bonferroni's multiple comparison test. All values were significantly different from each other column with a p<.001.

These data further supports our hypothesis that IL-4 signaling is responsible for the suppression of miR-17-92 cluster in T-cells. IL-4 suppression of miR-17-92 potentially occurs *in vivo* where tumor-derived factors result in the secretion of IL-4, and promotion of a Th2-skewed ineffective response.

6.3   DOSE RESPONSE OF CD4+ T-CELLS TO IL-4

To determine if the dose of IL-4 plays a role in the suppression of miR-17-92 cluster we next treated CD4+ T-cells with increasing doses of IL-4 at either 0ng/ml, 10ng/ml, 50ng/ml or 100ng/ml and measured miR-17-5p expression with quantitative RT-PCR **(Figure 11).**

mIR-17-5p Expression
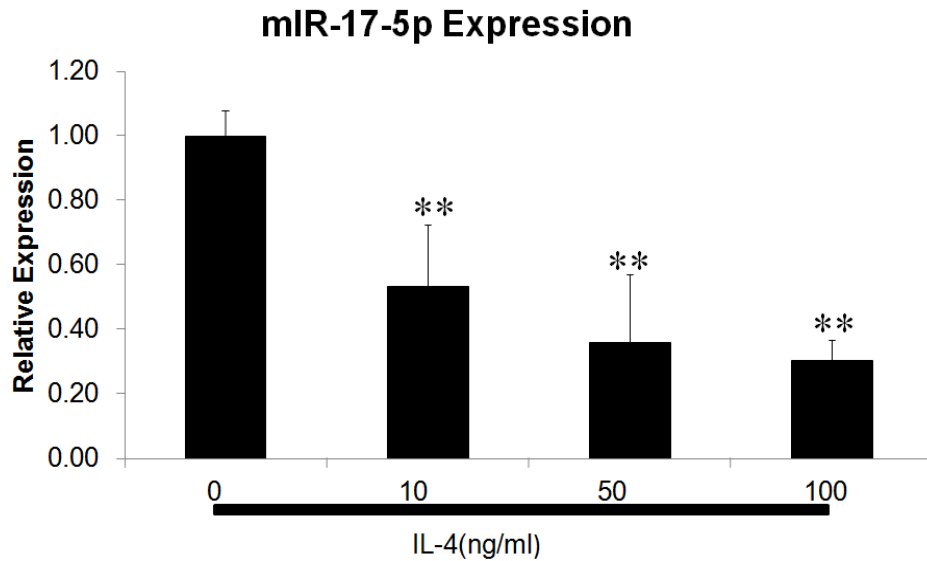
Figure 11: Dose response of miR-17-5p to IL-4 treatment. Neutral treated (anti-CD3, feeder cells, and hIL2) cells were cultured with anti-CD3, feeder cells, and hIL-2 and varying amounts of IL-4 for 5 day. RNA was then extracted and analyzed by quantitative RT-PCR for miR-17-5p expression. Columns represent mean of a single experiment carried out in triplicate. **indicates $P<.001$ using ANOVA test. Curve was further analyzed using post test for linear trend (graphpad prism) and was significant ($p<.001$)

These data demonstrate that the mIR-17-92 suppression occurs in a dose-dependent manner and that partial blockade of IL-4 should be sufficient to enhance T-cell microRNA levels.

6.4   NEUTRALIZATION OF IL-4 ENHANCES CD4+ T-CELL PROLIFERATION

To further examine our hypothesis that IL-4 regulation of miR-17-92 and the E2F pathways control proliferation of CD4+ T-cells through E2F signaling we next evaluated proliferation of non differentiated T-cells in the presence of IL-4-neutralizing antibody **(Figure 12).**
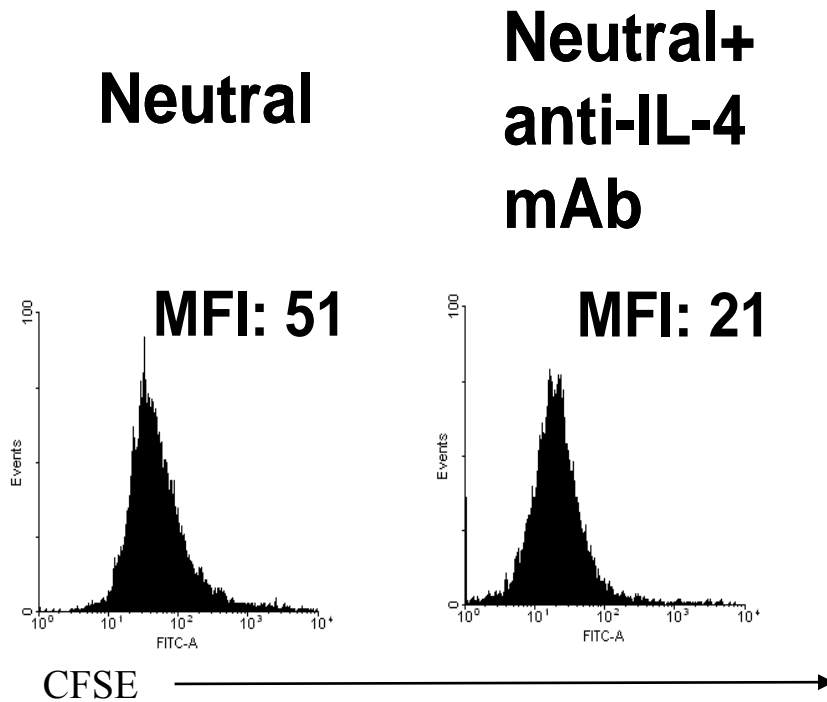
Figure 12: Proliferation of CD4+ cells in the presence of IL-4 neutralizing antibody examined through a CFSE assay. Immunomagnetically-separated CD4+ splenic T-cells derived from wild-type mice and then cultured with or without IL-4 neutralizing antibody in neutral conditions (anti-CD3, feeder cells, and hIL2). On day 10 cells were labeled with 0.5μM of CFSE. On day 15, cells were harvested and CFSE dilution was assessed by flow-cytometry.

Our results demonstrate that in the presence of IL-4-neutralizing antibody there is increase in CD4+ T-cell proliferation. Additionally, cells treated with IL-4-neutralizing antibody had decreased expression of E2F1 and E2F2 as determined by western blot (data not shown).

These results support our hypothesis that enhanced CD4+ T-cell proliferation is associated with increased miR-17-92 cluster expression.

6.5   STAT6 DEFICIENT CD4+ T-CELLS ENHANCE TH2 PROLIFERATION

To further examine our hypothesis we next examined proliferation of day FACs 10 confirmed Th1 and Th2 cells derived from either wild type C57BL/6 mice or STAT6 deficient mice (**Figure 13).**
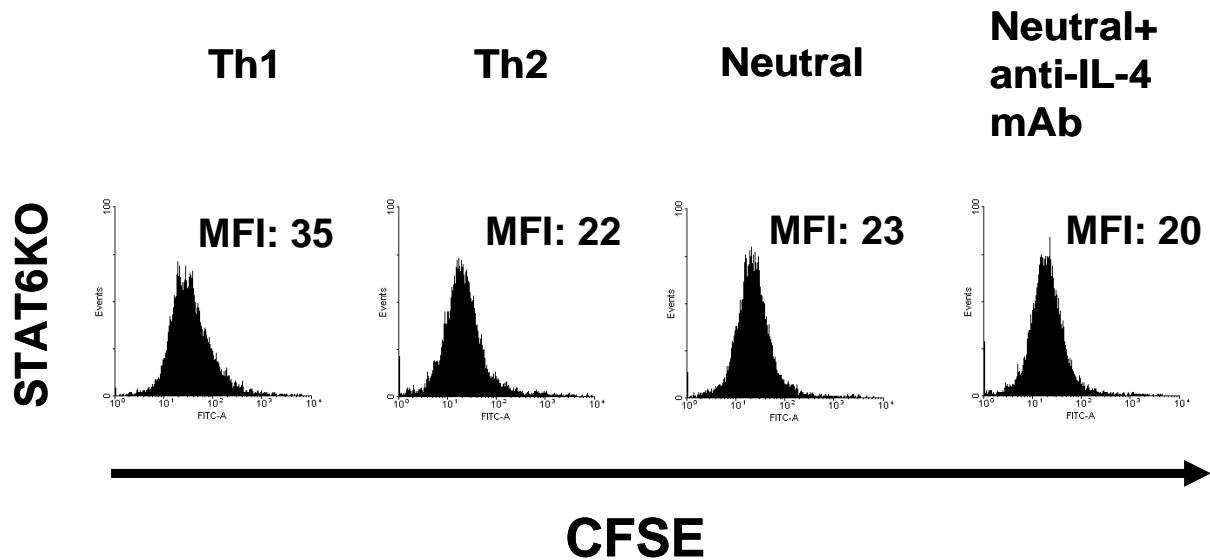
Figure 13: STAT6 deficient Th2 cells have enhanced proliferation that is not enhanced by neutralization of IL-4. Immunomagnetically-separated CD4+ splenic T cells derived from Wild-type or STAT6 -/- mouse CD4+ cells were labeled with 0.5μM CFSE and then cultured under Th1, Th2 Neutral, or Neutral+anti-IL-4 mAb cytokine conditions as described in the methods. At day 15, cells were harvested and CFSE dilution was assessed by flow-cytometry.

Our results demonstrate that unlike wild-type mice, STAT6-deficient mice exhibit increased proliferation of CD4+ splenocytes cultured in Th2 conditions relative to those in Th1 culture conditions. Additionally, this increased proliferation is not accelerated by neutralization of IL-4. These data further support our hypothesis that miR-17-92 cluster is involved in T-cell proliferation and regulated by IL-4.

7.0 AIM 2 CONCLUSION

Based on the present data we conclude that IL-4 suppresses miR-17-92 through a STAT6 dependant pathway in a dose dependent manner. Furthermore, enhanced proliferation of Th1 cells vs. Th2 cells is dependent on the IL-4 and STAT6 signaling pathway as STAT6 deficient mice and addition of IL-4 neutralizing ab resulted in an increase in proliferation of cells cultured under Th2 cytokine conditions.

8.0   DISCUSSION

Based on the present data we conclude that miR-17-92 is more highly expressed in the Th1 cells phenotype as compared to Th2 cells. We have shown that in Th2 cells IL-4 suppresses this miR-17-92 through the STAT6 signaling pathway. Furthermore, as miR-17-92 expression is predicted to regulate the cell cycle, we have shown that miR-17-92 cluster expression correlated with cell proliferation, and that predicted miR-17-92 targets E2F1 and E2F2 were downregulated. We propose a model in which IL-4 from Th2 skewing cells or from the tumor environment [52] are able to decrease the proliferative ability of T-cells and how Th1 skewing conditions can increase the proliferative ability(**Figure 14**).
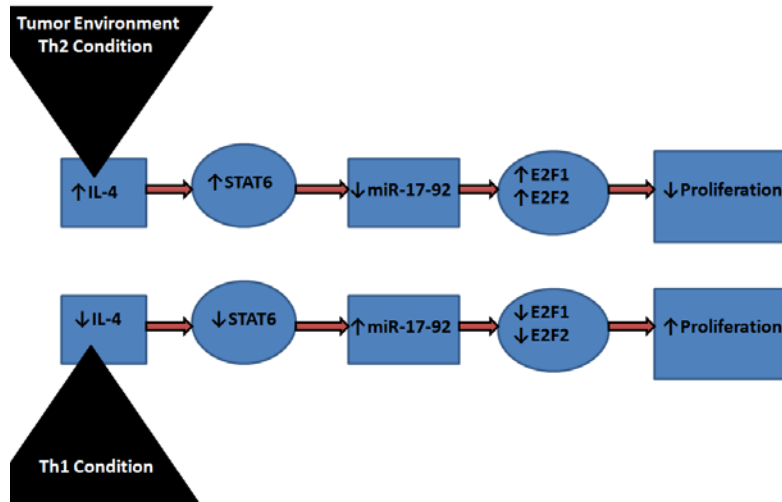
Figure 14: Model of miR-17-92 signaling pathway. Based on our current data we propose that IL-4 from Th2 skewing conditions such as the tumor environment downregulates miR-17-92 through the STAT6 pathway. This downregulation of mIR-17-92 results in upregulation of anti proliferative E2F1 and E2F2 molecules resulting in decreased proliferation relative to Th1. Conversely Th1 conditions lack activation of STAT6 and therefore have upregulation of miR-17-92, decreased E2F1 and E2F2, and increase proliferation relative to Th2.

To be able to potentially regulate this cluster, it is important to better understand how IL-4-STAT6 controls miR-17-92 expression. Although we are uncertain of the mechanism in which IL-4-STAT6 regulates miR-17-92 cluster, we have identified a potential STAT6 binding site between miR-19a and miR20a. We acknowledge that transcription factors bind and regulate at the promotor regions of genes, however, this may represent a novel method of transcriptional regulation. In this regulation, the transcription factor STAT6 could bind directly to the miR-17-92 cluster and blocks transcription. There are however no known reports of STAT molecules inhibiting translation. We will also examine the effect of STAT6 on other factors that have the ability to regulate miR-17-92 expression at its promotor.

The Th1 and Th2 cells used in our model were induced *in vitro* from murine CD4+ cells. While this allowed for a very controlled system to study differentiated T-cells we acknowledge that there are inherent issues that must be addressed in the future. The ability of IL-4, a single cytokine to skew miR-17-92 expression raises the possibility that other factors from the tumor environments may interfere and regulate miR-17-92 expression. Another concern of our current system is the use of only one animal strain. This raises the concern that a single strain might be unique and not representative. Ideally we would like to examine our system in multiple animal strains and eventually in human Th1 and Th2 cells.

We acknowledge that some of our experiments were only done one time and therefore lack statistical proof. However we believe that taken together our data strongly illustrates the role of IL-4-STAT6 in miR-17-92 expression and that this pathway reflects the proliferative ability of CD4+ T-cells.

Future work is needed to demonstrate a direct linkage between miR-17-92 and T-cell proliferation. Our results together with the results of others strongly suggest that miR-17-92 overexpression will enhance T-cell proliferation. Our future studies will aim at overexpressing and knocking down of miR-17-92 to determine how proliferation is affected by this cluster. Furthermore, we will examine other potentially therapeutic benefits of this cluster, including resistance to activation induced cell death, chemotherapeutic responses and TGF-β suppression.

One of the major limitations to cancer immunotherapy is the short duration of T-cell survival after adoptive transfer [53]. Therefore we hypothesize that transgene-mediated expression of miR17-92 will promote the survival and function of anti-tumor T cells. Our future goal will be to test the ability of miR-17-92 cluster to provide better T-cell immunity in GMB patients in a clinical trial. We propose that overexpression of miR-17-92 cluster in T-cells of GBM patients

alone or in combination with other immunotherapy strategies will lead to an enhance immune response, able to fight the tumor.

9.0 FUTURE DIRECTIONS

9.1 MURINE STUDIES

9.1.1 Mir-17-92 Expression from Tumor Bearing Mice

To further our findings in an *in vivo* system we will examine the miR-17-92 expression in CD4+ T-cells from tumor bearing mice. In order to skew the systemic immune response and have sufficient numbers of T-cells for analysis we will use an invasive melanoma model tumor. One million B16 tumor cells will be inoculated into the right flank of C57BL/6 mice. After 2 weeks we will harvest the spleen, isolate CD4+ cells with negative immuno-magnetic separation and examine miR-17-92 expression. We will also confirm using an ELISA for IFN-γ whether the cells are Th1 or Th2 skewed phenotype cells. Our hypothesis is that miR-17-92 expression will be downregulated and these cells will be Th2 skewed.

9.1.2 Overexpression of MiR-17-92 in CD4+ T-cells

To show that miR-17-92 cluster directly affects proliferation and function of CD4+ T-cells we will next produce stable miR-17-92 overexpressing CD4+ T-cells. For this we will use nucleofection of 2 plasmids in a transposon/ transposase system. We have cloned the entire miR-17-92 gene segments under a CMV promotor and GFP under its own SV40 promotor between

two IR/DR site (sites needed for transposase). With an Amaxa neucleofection kit we will nucleofect our miR-17-92 plasmid together with another plasmid encoding the transposase enzyme. This will allow us to create stable miR-17-92 overexpressing cells. These cells will be selected using GFP and miR-17-92 overexpression will be confirmed. We will then examine a variety of functional tests on these cells including: WST-1 proliferation assay, chemotherapy drug resistance assay, activation induced cell death/apoptosis assay, and other in vitro assays. We will also examine the effect of Th1 and Th2 skewing under various cytokine conditions. Our hypothesis is that miR-17-92 overexpression in CD4+ T-cells will skew Th1 and that these cells will proliferate better in suppressive and inhibitory conditions.

9.1.3 Adoptive Therapy of MiR-17-92 Overexpressing T-cells

As T-cell suppression remains a potent mechanism of tumor immune escape and because Th1 skewed T-cells has been shown to be preferential in tumor immunity over Th2 we predict miR-17-92 overexpressing cells will restrict tumor growth and provide increased protection against the tumor. To test this hypothesis we will evaluate the effect of adoptively transferred T-cells in protection against a GL261 tumor. Using a Hamilton syringe, 1×10^5 GL261 cells will be stereotactically injected through an entry site at the bregma, 3 mm to the right of sagittal suture and 4 mm below the surface of the skull of anesthetized mice using a stereotactic frame. On day 10, 5 mice per group will receive an i.v. injection with 2×10^7 miR-17-92 overexpressing or control transduced CD4+ cells, and cultured for 9 days with 100 U/ml of hIL-2. Mice will be closely monitored for any neurological signs, or any signs of weakness or malaise, which are considered to be an endpoint and mice will be sacrificed. Additionally some mice will be sacrificed 6 days after adoptive transfer, brain infiltrating lymphocytes (BILs) will be isolated

and GFP+ cells will be analyzed for proliferation and apoptosis between the two groups. We hypothesize that animals with miR-17-92 overexpressing CD4+ T-cells will have longer survival than those without miR-17-92 overexpression. Additionally the adoptively transferred miR-17-92 CD4+ cells will have better ability to proliferate and decreased levels of apoptosis than control cells.

9.2   HUMAN STUDIES

9.2.1   Mir17-92 Expression in Human Th1 vs. th2

To determine if the data obtained from mouse Th1 and Th2 cells are relevant to the human immune system, we will induce Th1 and Th2 cells from human PBMC *in vitro* and examine the miR-17 cluster expression.   Human Th1 and Th2 cells will be generated from naïve CD4^{+}CD45RA^{+} T cells as described previously[54]. Briefly, naïve CD4^{+}CD45RA^{+} T cells will be isolated using naïve CD4^{+} T cell isolation kit (Myltenyi Biotec) and be stimulated with plate-bound anti-CD3 (1 μg/ml; clone OKT3) and anti-CD28 (2 μg/ml; clone 15E8; BioLegends) and rIL-2 (50 units/ml; Peprotech). For Th1 differentiation, rIL-12 (2.5 ng/ml; R&D Systems), anti-IL-4 mAb (5 μg/ml; clone MP4–25D2; BD Biosciences), and anti-IL-10 mAb (5 μg/ml; clone JES3–9D7; BioLegends) will be added. For Th2 differentiation, rIL-4 (12.5 ng/ml; R&D Systems), anti-IFN-γ mAb (5 μg/ml; clone B-B1; BioLegends), and anti-IL-10 mAb (5 μg/ml; clone JES3–9D7; BioLegends) will be added. After 4 days, the cells will be expanded under the same conditions in the absence of anti-CD3 or anti-CD28.

Total RNA will be isolated from resting Th1/Th2 cells or cells that will have been activated for 4 h with PMA/ionomycin after 14, 21, or 28 days of differentiation, and quantitative RT-PCR will be carried out with primers for miRNAs in miR-17-92 cluster. Following analysis of miR-17-92 cluster we will examine the proliferative ability of these T-cells with a WST-1 and a CFSE assay.

9.2.2 MiR-17-92 Expression in GBM patient CD4+ T-cells

To further examine the role of miR-17-92 cluster we next will obtain frozen PBMCs from glioma patients as well as healthy donors. From these cells we will isolate CD4+ cells. A fraction of the cells will be stimulated with anti-CD3 (OKT3 or UCHT1) used for human IFN-γ ELISA to determine whether these cells are Th1 or Th2 skewed. RNA will then be extracted and miR-17-92 cluster expression will be analyzed from both ex vivo CD4+ cells and stimulated CD4+ cells. We hypothesize that GBM patient CD4+ cells are Th2 skewed and that miR-17-92 will be down regulated.

9.2.3 Overexpression of MiR-17-92 in Human CD4+ T-cells

As described in 9.1.2 we will utilize a transposon/transposase system to examine miR-17-92 overexpression in murine CD4+ T-cells. We will use the same systems to overexpress mIR-17-92 in human CD4+ T-cells. Human CD4+ T-cells will be isolated from fresh PBMCs using negative magnetic bead selection. Cells will be stimulated for 24 hours followed by neucleofection. We will continue culture for 7 days and then confirm stable expression of miR-17-92 expression and functional analysis of these cells.

9.3   OTHER FUTURE PLANS

All future plans mentioned to this point focused on the role of Th1 and Th2 CD4+ T-cells. Differentiated Tc1 vs. Tc2 CD8+ T-cells may follow a similar trend to Th1 vs. Th2 and also have improved proliferative ability. For this reason all our experiments and future plans will be adapted for Tc1 and Tc2 and CD8+ human and murine cells.

Furthermore our methods of transduction may also include lenti viral vector system which we are currently working on. This will allow for a less invasive method of transfection. The reason we have chosen the transposon/transposase based system is for its relative safety in patient use.

Although not mentioned, in addition to overexpression studies we would like to do knockdown studies. In these studies we will use anti-sense microRNA to bind to the miR-17-92 cluster block the miR-17-92 function. We will expect to see decreased proliferation and opposite phenotype as when we overexpress the same miRNA.

We have also obtained mice with miR-17-92 flanked by lox-p sites and Lck-Cre mice. This will allow us to overexpress miR-17-92 cluster in T cells of mice and will hopefully lead to the development of more projects.

Finally in these experiments we have been examining miR-17-92 cluster expression as a whole which contains 7 mature microRNAs. Before miR-17-92 can be used in patients we will examine each mature miRNA individually in different combinations to find the most effective strategy of bettering T-cells

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