The Role of HDAC6 Inhibitors (HDAC6i) on CD47/SIRPa Pathway in Human Melanoma

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Abstract of Thesis

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CD47 is ubiquitously expressed on the surface of cancer cells. Its interaction with SIRPa on macrophages' surfaces inhibits the phagocytic activity of macrophages. Epigenetics has a role to play in cancers that are resistant to immunotherapy. Recent studies show that HDAC6 was a modulator of the expression of CD47 and SIRPa in Melanoma, and macrophages are considered potential targets for cancer immunotherapy. Therefore, further investigation on the modulation of CD47 and macrophage phenotype by epigenetic modifiers is needed.

Herein, a highly specific HDAC6i, Nexturastat A (NextA), was used and has been confirmed to decrease the pro-tumorigenic M2 macrophages phenotype and impact CD47/SIRPa axis in Melanoma. Our results indicated that the combination of NextA and IFNy treatment showed profound changes in the tumor microenvironment (TME), such as a significant downregulation of the expression of CD47 in human Melanoma cells and reduction in SIRPa expression in macrophages after IFNy treatment in different types of cancer cells. This finding could give a potential preclinical efficacy for future studies to use HDAC6is as immunomodulatory agents.

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List of Symbols / Nomenclature

1. HAT: histone acetyltransferase
2. HDAC: histone deacetylase
3. HDAC6is: Histone deacetylase 6 Inhibitors
4. IFNy: Interferon-gamma
5. LPS: Lipo-polysaccharide
6. IL-13: Interleukin 13
7. IL-4: Interleukin 4
8. Nexturastat A: NextA
9. FBS: fetal bovine serum
10. PBS: phosphate-buffered saline
11. qRT-PCR: quantitative real-time polymerase chain reaction

Introduction

Malignant melanoma is the least frequent but most fatal kind of skin cancer. According to the American Cancer Society, it has been reported to have 99,780 predicted new cases, and 7,650 estimated fatalities in the United States alone in 2022 (Siegel, 2022).

Surgery, radiation, and specialized chemotherapies are the most common cancer treatments. Furthermore, a sizable portion of melanoma patients fails to respond to these treatments or, over time, acquire resistance. (Davis LE, 2019).

Immunotherapy is a vital function of the human body to defend itself against tumors (Veillette & Chen, 2018). Immunotherapy aims to ease the patient's immune system in defeating cancer. The $BRAF^{V600E}$ mutation represents 80% of BRAF mutation (Gracia-Hernandez, 2021). The unresponsive percentage to targeted therapies, including BRAF and MEK inhibitors (BRAFi, MEKi, respectively). Immune checkpoint blockade (ICB) is an evolutionary immunotherapy method for melanoma that involves immune checkpoint inhibition and has shown effective results with 50% of melanoma patients with metastatic or advanced melanoma after developing BRAFi resistance (Liu, 2021); only 15-20% of patient who is harboring $BRAF^{V600E}$ did not respond to the therapy (Czarnecka, 2020). Immune-based treatments that target immunosuppressive signaling pathways, such as the combination of ICB and BRAFi, have evolved and are utilized in clinical studies, which have shown some promise for a subgroup of patients (Sharma, 2017).

Phagocytosis is an essential defense process in the body of human beings that is vital for survival (Wang, 2017). It's a process dependent on the endoplasmic reticulum (ER), which undergoes fusion, reaction, regrowth, remodeling, and the resolution phase (Hartenstein, 2019). For phagocytosis to occur, several processes initiate activation after recognizing the pathogens at the affected site or recognizing the presence of a tumor in the human body.

Phagocytes have also been observed to cause inflammatory responses (Reddy et al., 2019). Still, the alternative viewpoint is that phagocytes can generate more than just inflammatory responses, which is biologically possible through reactions of neutrophils and macrophages. This includes using bispecific antibodies, immuno-quiescent bispecific antibodies, epigenetic modifiers, and engineering macrophages using the different antigen receptors and the gamma interferon (Feng, 2019).

Macrophages are antigen-presenting and innate immune cells that protect the cells from any foreign insults and engulf particles of different origins (Yuan, 2019). Monocytes and macrophages are characterized by their variety, plasticity, and multiple functions, including antiviral responses, tissue repair, and phagocytosis (Zhang, 2021). Macrophages have been used as mediators to phagocytose, carry, and deliver drugs to tumors (Huang, 2021).

Nevertheless, macrophages significantly regulate the tumor immune microenvironment (TME) (Mantovani, 2017). Traditionally, macrophages can be reprogrammed and polarized into two phenotypes; M1-like macrophages are pro-inflammatory and associated with microbicidal activity, immune response, and tumor

growth inhibition; M2-like macrophages are immunosuppressive that promote and induce tumor growth and are associated with tissue repair (Genin, 2015). Furthermore, macrophage phenotypic programming is triggered by a variety of cytokines.

While IL-4/IL-13 induces the M2 phenotype, inhibiting phagocytic activity, M1 macrophages are activated by LPS and IFNy Watanabe, 2019). IFNy is believed to be potentially helpful for adjuvant immunotherapy for cancer, yet, the potential of IFNy immunotherapy remains unclear (Jorgovanovic, 2020). Therefore, understanding the role of macrophage phenotypes is critical for developing therapies to balance their ratio and maintain their functions. Figure1 exhibit the characteristics of M1 and M2 phenotypes (Russell, 2019).

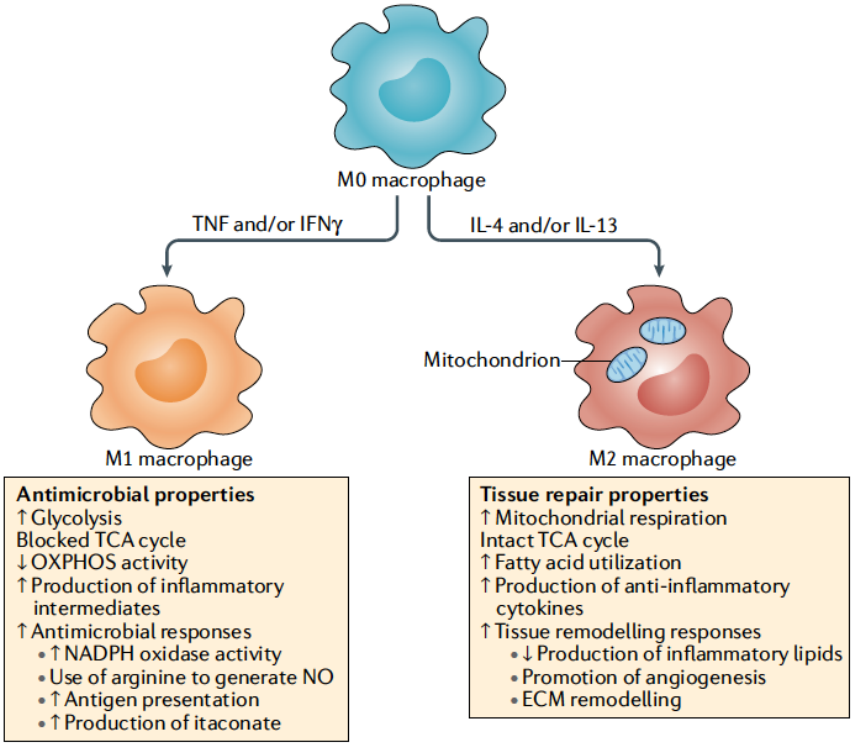

Figure 1. Phenotypic characteristics of the M1-like and M2-like macrophage polarization states (Russell, 2019).

CD47, an integrin-associated protein (IAP), is considered a cancer biomarker; CD47 is frequently over-expressed on the surface of many cancer cells, while SIRPa is a signal-regulatory protein-α that is expressed on macrophages' surface (Yuan, 2019). The interaction of CD47/SIRPa allows the escape of cancer cells from macrophage-mediated phagocytosis by triggering a "don't-eat-me" signal (Takimoto, 2019). Hence, since the phagocytic activity of macrophages is adversely impacted by the CD47/SIRPa pathway, blocking the CD47/SIRPa axis can further enhance the antitumor efficacy of macrophages (Xu, 2019). Figure 2 illustrates the phagocytosis of macrophages by CD47/SIRPa axis before and after blocking the axis with an anti-CD47 antibody (Huang et al, 2020).

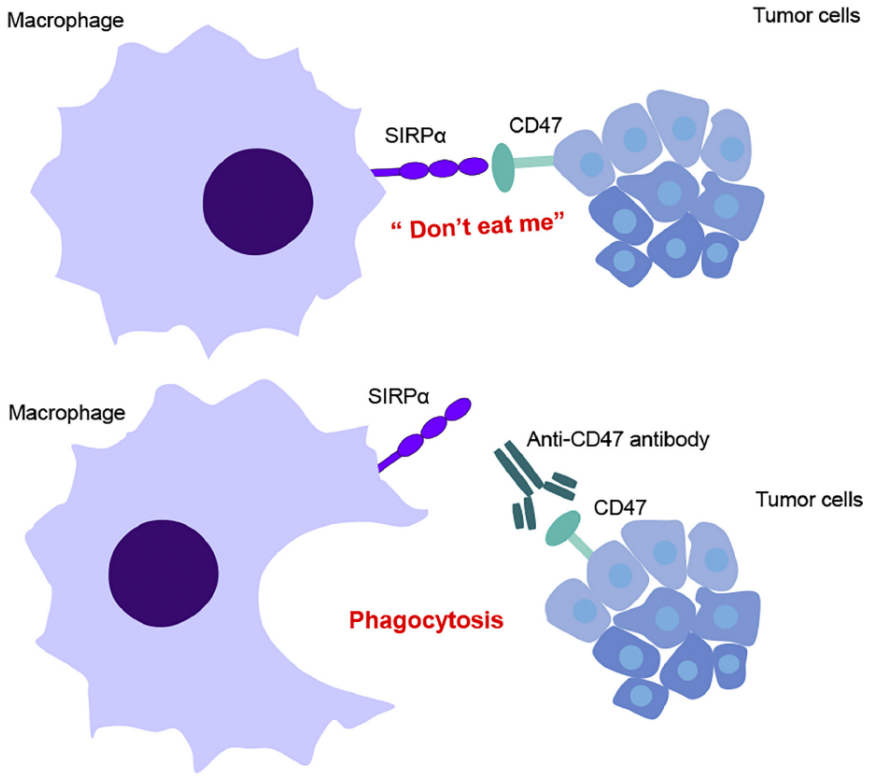

Figure 2. Regulation of phagocytosis by CD47/SIRPa axis. CD47 is overexpressed in most cancer cells, and the binding of CD47/SIRPa sends a "don't eat me" signal, enabling cancer cells to escape macrophage phagocytosis. Blocking of CD47 with an anti-CD47 antibody disables the "don't eat me" signal from the CD47/SIRPa axis, thereby stimulating phagocytosis of cancer cells. (Huang et al, 2020)

Moreover, macrophages are recruited to engulf cancer cells via the interaction of Calreticulin that presents on the surface of cancer cells with LDL-receptor-related protein (LRP) on the macrophages and the engagement of antibody-antigen complexes with the FcR; this pathway is known as an "eat-me signal" (Zhang, 2020).

The checkpoint CD47 blockade defends the body against pathogens by causing cell activation and cross representation of the cyclic GMP-AMP synthesis used in cancer therapy once pathogens and the affected site have been recognized. Bispecific antibodies are involved in antitumor treatment by causing inhibition of growth factors, induction of apoptosis, and hindering proliferation. The bispecific antibodies can also be expressed as macrophages, neutrophils, and eosinophils. Nevertheless, there is a growing interest in epigenetic modifiers' function in controlling immuno-modulatory pathways.

Epigenetic modifiers, on the other hand, work to modulate the process of phagocytosis. This process can be done by helping to ingest foreign particles through the modulation of Histone Deacetylase, which is responsible for transcription and chromatin structure. DNA methylation is a process of epigenetic modifiers that helps control gene expressions (Vymetalkova et al., 2019).

The accumulation of genetic changes affecting both tumor-suppressor genes and oncogenes within cells is considered to cause multi-step carcinogenesis. DNA methylation and post-translational modifications of histones are examples of epigenetic modifications that impact gene expression. Histone proteins can be altered in numerous ways, including acetylation, methylation, phosphorylation, and ubiquitination; acetylation

is one form of histone modification. HATs, histone acetyltransferase, and HDACs are utilized to activate or repress the transcription of genes (Ehrenhofer-Murray, 2004).

Histone deacetylases (HDACs) are enzymes that remove acetyl groups from an amino acid on histone lysine residues, inhibiting gene transcription. (Park, 2020). HDACs are divided into four classes based on the similarities between yeast homology, localization, and amino acid sequence. (Seto, 2016) Class I (HDACs 1–3 and 8) are found within the nucleus; Class II is subdivided into two main classes; class IIa (HDACs 4, 5, 7, and 9), which travel between the nucleus and the cytoplasm, and class IIb (HDACs 6, 10) that are located in the cytoplasm where they can interact with nonhistone proteins. Class III HDACs (SIRT1-7) have no apparent role in the cell cycle. Class IV (HDAC 11) shares similar characteristics with Class I and II yet, has different physiological roles. (Clocchiatti, 2011).

HDAC I, II, and IV are known to be inhibited by pan-HDACi, including sirtinol, vorinostat, and panobinostat. More recent research has exploited class-specific HDACis, such as Nexturastat A (HDAC6i) and MC1568 (class III HDACi), among many others, utilized to minimize the toxic effect of pan-HDAC inhibition (Varayathu, 2021), (Kroesen, 2014).

Table 1. depicts the specificity and current clinical trial status of different pan and selective HDACis. "+" represents inhibitory selectivity. In an empiric fashion, more inhibition is indicated by a greater "+" designation.(Banik, 2019).

	Class I			Class IIb		Class IV		Clinicaltrials.gov
Inhibitor Name	HDAC1	HDAC2	HDAC3	HDAC6	HDAC10	HDAC11		(02/22/2019)
Vorinostat (SAHA, MK0683)	++++	++++	++++	++++	++++	++++	Merck (FDA)	251
Panobinostat (LBH589)	++++	++++	++++	++++	++++	++++	Novartis (FDA)	133
Trichostatin A (TSA)	++++	++++	++++	++++	++++	++++		15
Belinostat (PXD101)	++++	++++	++++	++++	++++	++++	TopoTarget (FDA)	44
LAQ824 (Dacinostat)	++++	++++	++++	++++	++++	++++	Novartis	-
M344	++++	++++	++++	++++	++++	++++		-
AR-42	++++	++++	++++	++++	++++	++++	Arno Theraputics	5
Quisinostat (JNJ-26481585) 2HCl	++++	++++	++++	++	++++	++++		6
RGFP966			++					-
Tubastatin A				+++				-
Nexturastat A				++++				-
SS-2-08				++++				-

Figure 3. Specificity and application of pan and selective HDACis. (Banik, 2019)

HDACis have also been investigated as immune response regulators, modifying macrophage phenotypes and responses to infection (Ghiboub, 2020). It was believed that HDACs' primary function was to alter histones. (Legube, 2003). Yet, HDACs have shown antitumor activity via cell cycle arrest and modulation of immune response and are currently used as anticancer agents (Banik, 2019).

The present thesis aims to evaluate the genetic and pharmacological effect of HDAC6 inhibitors (HDAC6i) on cancer cells and macrophages. Herein, we assess the expression of CD47 and SIRPa and measure the impact of HDAC6i on the CD47/SIRPa pathway. This evaluation might provide a potential therapeutic target to enhance the phagocytic activity of macrophages in tumor therapy.

Materials and Methods

2.1 Cells. Human melanoma cell lines WM164 NT and HDAC6 KD were obtained from Dr. Smalley's Lab at Moffitt Cancer Center (M, P, V, *et al*, 2016). WM793 was purchased from ATCC (Manassas, VA) (Woods, 2015). Melanoma cell lines, WM164 WT, WM9, and WM1361, and Human monocytic THP1 cells were cultured in 6-well plates and grown to 80% confluency (VWR, 353046) in the presence of RPMI 1640 with 10% Fetal Bovine Serum (FBS) (Fisher, Corning: MT35011CV), 1% MEM non-essential amino acids (NEAA) (Corning, 25-025-Cl), and 1% Penicillin-Streptomycin (Corning, 30-002-CI), and incubated overnight at 37°C with 5% CO2. WM793 cells were culture similarly, yet, in the presence of DMEM (Fisher, Corning: 10-017-CV).

2.2 Macrophage treatment and polarization. THP1 monocytes were cultured in RPMI 1640 with 10% Fetal Bovine Serum (FBS), 1% MEM non-essential amino acids (NEAA) (Corning, 25-025-Cl), and 1% Penicillin-Streptomycin (Corning, 30-002-CI) and grown to 70–80% confluency. The cells were treated with PMA for 24 hours to be polarized to M0 macrophages. Cells were pretreated with 10,5, and 2.5 μM HDAC6i and kept in a humidified incubator at 37°C with 5% CO2 for 2 hours before polarization. 100 ng/mL of lipopolysaccharide (LPS) (Sigma-Aldrich, L2880), 50ng/mL IFNy (Biolegend, 575304), were added to the THP1 cell culture to polarize it into M1-like macrophages and incubated for a total of 24 hours. To polarize THP1 cells into M2-like macrophages, a 20 ng/mL IL-13 (Biolegend, 575902) and 20 ng/mL IL-4 (Peprotech, 214-14) were added and incubated for 24 hours.

2.3 HDAC6i. The HDAC6 selective inhibitor NexturastatA (NextA) was purchased from StarWise Pharmaceuticals, Madison, Wisconsin, USA. NextA was reconstituted according to the manufacturer's protocol and stored in aliquots at 80 °C. NextA was diluted using media to the concentrations 2.5, 5, and 10 μM that were used for each specific experiment and added to the appropriate well of the cell plate. Treated cells were treated with NextA before IFNy treatment and incubated under the conditions indicated above for 2,4,8,12, and 24 hours.

2.4 Reagents and plasmids. The recombinant cytokines human IL-13 (575902), and human IFNy (575304), were purchased from Biolegend. Human IL-4 (214-14) was purchased from Peprotech, Lipopolysaccharide (LPS) (L2880), and PMA (P1585-1MG) were purchased from Sigma-Aldrich. Primers ACTB, CD47, and GAPDH (Invitrogen,10336022), and SIRPa (Qiagen, 249900) used for qRT-PCR were designed by Origene as follows:

Human ACTB:

Fwd: CACCATTGGCAATGAGCGGTTC

Rev: AGGTCTTTGCGGATGTCCACGT

Human CD47 primer

sense primer, 5′-GGCAATGACGAAGGAGGTTA-3′;

antisense primer, 5′-ATCCGGTGGTATGGATGAGA-3′

GAPDH:

Fwd: GTCTCCTCTGACTTCAACAGCG

Rev: ACCACCCTGTTGCTGTAGCCAA

2.5 Quantitative real-time RT-PCR. Melanoma cells were plated at 3 x 10 6 per 35mm well and cultured under the conditions detailed above. The RNA was extracted from the cells using TRIzol® (Ambion, TRIzol® LS Reagent, 10296-010). Concisely, cells were homogenized in TRIzol and stored in -80 overnight; the RNA was extracted by 200ul of chloroform and allowed to homogenize for 3min at room temperature. The aqueous top layer is separated, and a 1:1 ratio to TRIzol of isopropyl alcohol is added.

The precipitated RNA from the aqueous layer is washed with 75% ethanol (in DEPC-treated water). The ethanol is discarded, and the cells are suspended in 50ul of DEPC water and resuspended for use in downstream applications.

The quantification of RNA was done using spectrophotometrically via Nanodrop. The cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, 1708890). The mRNA was quantified using a real-time PCR detection system (Bio-Rad) and iQ SYBR Green Supermix (Bio-Rad, 1708880). Briefly, 5X iQ SYBR Green Supermix (1ul Template (Bio-Rad iScript cDNA Synthesis Kit), 1ul of the primer mixed with Nuclease-free water, and 3Xul Nuclease-free water (VWR, 10220-398) were carried out on the ice to prepare the reaction mix. 10ul of the reaction is loaded in triplicate in 96-wells plate, sealed, spun at 300 *xg* for 3-4 minutes, and placed in the Real-Time PCR Machine (CFX96 qPCR System). Each experiment was replicated at least three times to get the average for each condition.

Results

Most patients with NRAS or BRAF mutations have a poorer prognosis due to the high aggressiveness of both mutations' tumors. Targeted immunotherapies for both mutations could help melanoma patients' overall survival (Randic, 2021). In this study, CD47 expression was evaluated in different melanoma cell lines based on the different mutations. WM164, WM793, and WM9 are $BRAF^{V600E}$ mutated that cause the activation of MEK and ERK signaling pathways (Kretschmer, 2018). Additionally, WM9 expresses PTEN hemizygous deletion, and is a wild type for N-RAS, c-KIT, and CDK4. WM1361 is in the N-RAS gene that contains a Q61R mutation; this cell line is activated due to UV and radiation exposure causing cell production and division (Li, 2016).

A real-time PCR was conducted to evaluate the effect of IFNy on CD47 expression. The human melanoma WM164 NT and HDAC6 KD cell lines were treated with IFNy (100ug/ml) and incubated for different time points, 2, 4, 8, 12, & 24 hours, to determine the kinetics is for CD47 after stimulation with the cytokine. The best time point that had the highest IFNy stimulation was 12hrs.

In untreated cells without IFNy stimulation, CD47 expression was similar in both HDAC6 NT and KD cells. However, after treating cells with IFNy, an up-regulation of CD47 in the mRNA level was observed at 8 and 12 hours; no up-regulation in the absence of HDAC6, and no change in HDAC6 KD cells as shown in Figure 4.

To uncover the influence of NextA on CD47 expression at the mRNA level, qRT-PCR analysis was conducted for multiple human melanoma cells. The cells were pretreated with 5 and 10μM of NextA, HDAC6 inhibitor, for 18hrs to demonstrate IFNy impact on inducing CD47 expression.

The treatment after that combined with or without 100ng/ml IFNy for 12hrs for human melanoma cells and 24hrs for macrophages, as illustrated in Figure 5. NextA remarkably inhibited CD47 up-regulation in mRNA level in combination with IFNy as noticed by qRT-PCR analysis for WM793 and WM1361, while an increase in the CD47 expression in WM164 (WT) and no change in WM9.

To evaluate SIRPa expression in THP1-derived macrophages, the cells were pretreated cancer cells with 5 μM NextA for 18hrs, then stimulated by IFNy for another 24 hrs. The SIRPa expression that was up-regulated by IFNy combined with NextA treatment was partially reduced, as shown in Figure 6.

GAPDH and ACTB were used as negative controls in melanoma in all cell lines and THP1 derived macrophages and the results showed no change in the expression of CD47 and SIRPa, no shown data.

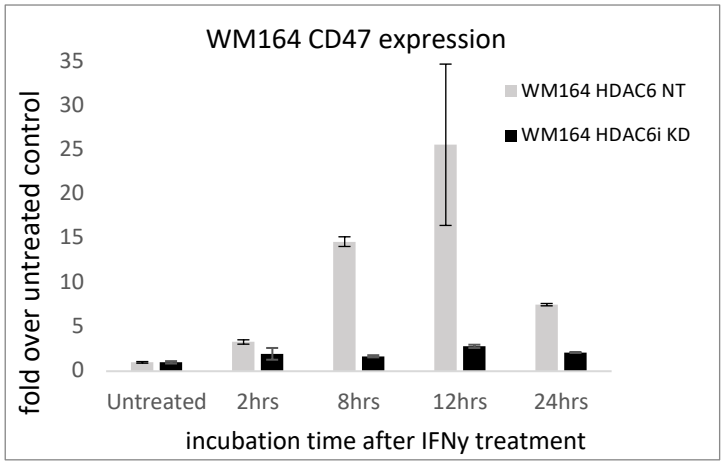

Figure 4. WM164 NT and HDAC6 KD kinetic. The graph shows the quantification of qRT-PCR for WM164 NT and HDAC6 KD cells treated with IFNy (100ug/ml).

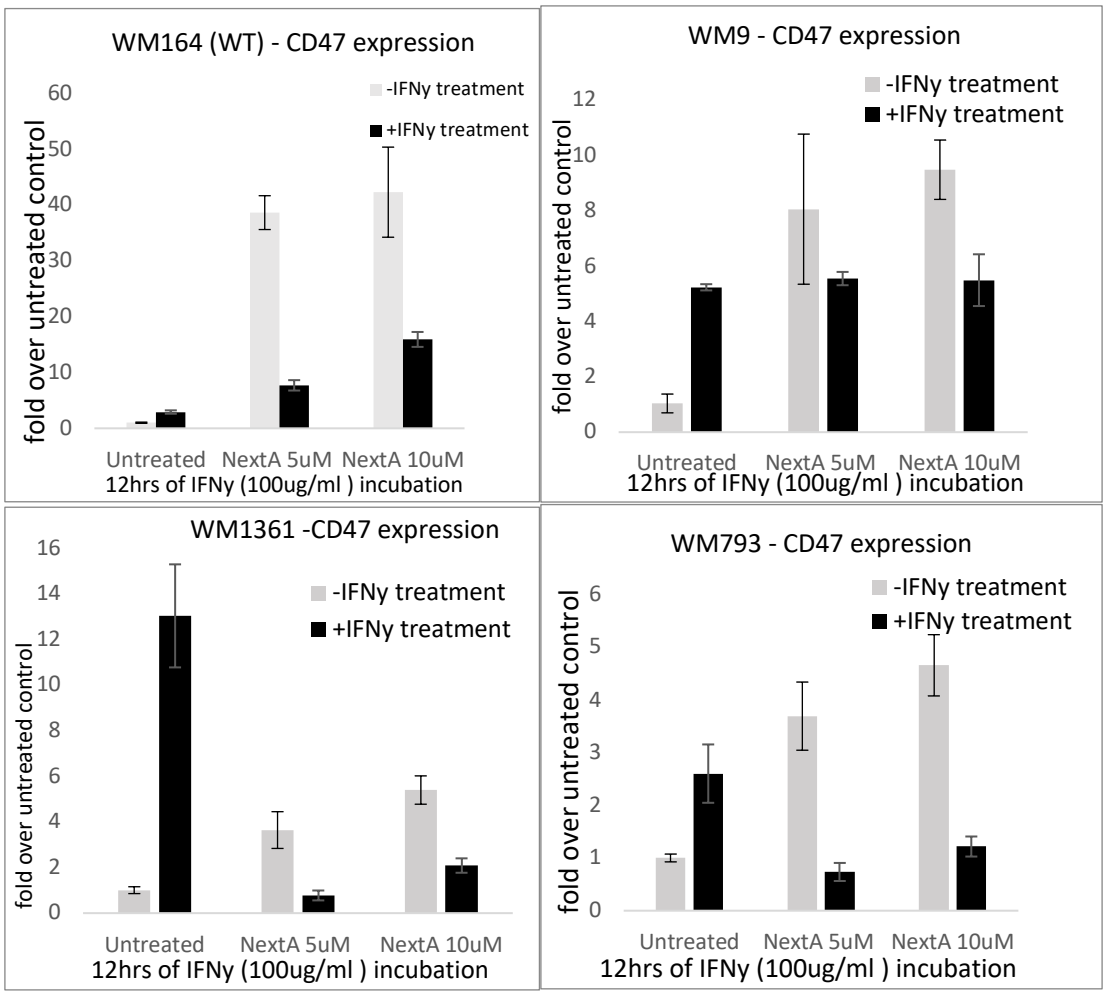

Figure 5. NextA abrogates the up-regulation of CD47 by IFNy by qRT-PCR analysis in human melanoma WM164 (WT), WM9, WM793, and WM1361 cell lines. The mRNA level of CD47 was detected by qRT-PCR.

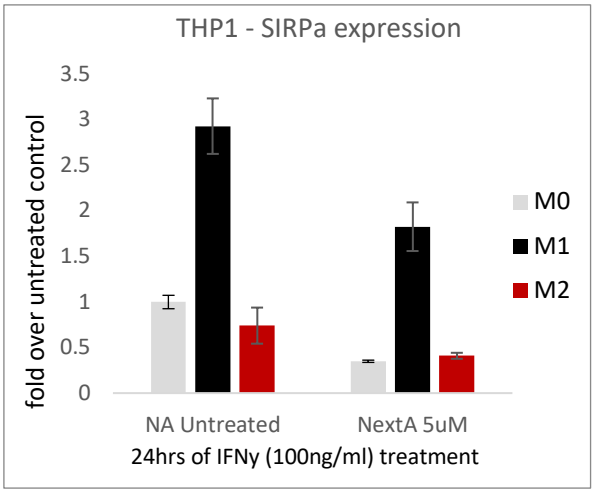

Figure 6. NextA decreases the up-regulation of SIRPa by IFNy by qRT-PCR analysis in THP1-derived macrophages.

Discussion and Conclusion

In this study, an *in vitro* assessment was conducted to evaluate the role of HDAC6i on the CD47/SIRPa pathway in Melanoma. The expression of CD47 was evaluated in human melanoma cell lines, WM164, WM9, WM1361, and WM793, and SIRPa in THP-1-derived macrophages in the combination and absence of IFNy by qRT-PCR analysis.

IFNy has an efficient role in inducing the expression of immune checkpoint receptors/genes in cancer cells (Jorgovanovic, 2020). Based on the results from this study, the expression of CD47 at different time points following IFNy stimulation of WM164 NT and HDAC6 KD cells was examined under IFNy stimulation, and HDAC6 KD cells were used as control. A maximum downregulation in the CD47 expression in the NT cells occurred at 12 hrs, and no change in the HDAC6 KD cells, as shown in Figure 4.

Epigenetic modifiers, including HDAC6i, appear to be therapeutic modulators in Melanoma's tumor microenvironment (TME) (Woan, 2015). HDAC6i modulates the expression of the CD47/SIRPa axis and the macrophage phenotype in Melanoma and enhances the antitumor efficacy of macrophages (Zhang, 2020).

Since CD47 is over-expressed on the surface of cancer cells, this study aimed to evaluate the expression of CD47 induced by IFNy in four human melanoma cell lines. Among the four human melanoma cell lines, WM164 (WT), WM9, WM793, and WM1361, the combined treatment of HDAC6i, NextA (5μM), induced by IFNy markedly increased CD47 expression in WM1361 at 12hrs followed by WM379 cell lines.

Although WM9 revealed no change in the CD47 expression, WM164 (WT) cell line showed an increase in CD47 expression with the combined treatment;s peculation might be attributed to the inefficient stimulating of IFNy in CD47 expression as represented in Figure 5.

It was reported that the expression of CD47 has increased when induced by IFNy independently in the mRNA level due to the activation of different pathways, yet further investigation might be needed (Ye, 2021). Also, the treatment with NextA independently shows an increase in CD47 expression; this has been proved in an ongoing *in vivo* study in our lab, yet no data has shown.

Additionally, IFNy donates to cancer immunotherapy effectiveness and is known to have antiviral, antitumor, and immunomodulatory functions. Moreover, IFNy produces pro-tumorigenic and antitumor immunity in the tumor microenvironment, such as reprogramming macrophages phenotype to either M1 or M2 phenotype (Jorgovanovic, 2020).

THP1 monocytes were differentiated and polarized into M1 and M2. It was detected that IFNy had increased SIRPa expression by 2-fold in M1 macrophages and had almost no effect on the M2 phenotype. Nevertheless, after treatment with NextA, the M1 phenotype was decreased by 1-fold, and no change in the anti-inflammatory M2 macrophage phenotype; this could demonstrate that NextA may impact the inhibition of the M2 phenotype, as shown in Figure 6. These results might provide optimal results for future immunotherapy targeting immune checkpoints.

In conclusion, the genetic and pharmacological inhibition of HDAC6 inhibitor induced by IFNy was evaluated and resulted in downregulating the expression of CD47 of melanoma cells *in vitro* and inhibiting the anti-inflammatory M2 macrophage phenotype with a slight impact on the pro-inflammatory M1 macrophages phenotype, since M1 has a more robust phagocytic activity. This study could be helpful for future immunotherapy targeting the CD47/SIRPa pathway.

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