Reprogramming Macrophages with HDAC6 Inhibitors for Anti-cancer Macrophage-based Cell Therapy

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ABSTRACT

Introduction:

Tumor-associated macrophages (TAMs) play a critical role in shaping the tumor microenvironment (TME). Responding to numerous cues from various cell types present in the tumor, TAMs often exhibit a spectrum of phenotypes, often ranging between M1 (inflammatory) and M2 (tumor-supportive) macrophages. The M1/M2 macrophage ratio is a useful parameter that determines tumor suppression or tumor growth. High infiltration of TAMs is usually associated with poor prognosis in several cancer types due to their predisposition towards M2 function. Therefore, reprogramming TAMs to the M1 phenotype is a promising avenue to be explored.

Methodology:

In this study, we reprogrammed bone marrow-derived macrophages (BMDMs) ex-vivo by treating them with HDAC6 inhibitor (HDAC6i) followed by polarization to M1 phenotype and directly implanted them into immunocompetent tumors. We performed immunohistochemistry of tumors for macrophage markers, immunophenotyping of tumor-infiltrated immune cells by flow cytometry, gene expression analysis by quantitative PCR, and immunoblot analyses. We also performed single-cell analyses, including secretome with Isoplexis platform and CD45+ tumor-infiltrated immune cells by single-cell RNA-seq analysis. To further underscore the translatability of macrophage-based cell therapy, we treated humanized NSG-SGM3 mice bearing melanoma PDX tumors with HDAC6i-treated human M1 macrophages.

Results:

Adoptive cell therapy (ACT) with HDAC6i-treated M1 macrophages resulted in significant tumor suppression and prolonged survival compared to other cohorts in the study. ACT macrophages were viable after two weeks of transplantation, and inhibition of HDAC6 rendered them resistant to M2 polarization. Inhibition of HDAC6 suppressed STAT3 activation and subsequent M2 marker Arg1 expression, further underscoring the role of HDAC6 in macrophage plasticity. M2 re-polarization assay further corroborated that HDAC6i-treated M1 macrophages were resistant to change into M2 phenotype. Single-cell secretome analysis by Isoplexis platform revealed polyfunctionality of HDAC6- treated M1 macrophages capable of secreting inflammatory cytokines such as Tnfa and T-cell recruiting chemokine Cxcl10. Histological examination of tumor sections for macrophage phenotypic markers and single-cell transcriptomic analysis of tumor-infiltrated immune cells further corroborated the M1/M2 ratio increase observed by flow cytometry. Proinflammatory gene expression signature from scRNA-seq analysis correlated with better survival in the SKCM dataset. In both immuno-competent SM1 murine melanoma and humanized NSG-SGM3 melanoma models, ACT enhanced anti-tumor immunity by increasing the M1/M2 ratio and infiltration of CD8 effector T-cells shifting the balance towards anti-tumor immunity.

Conclusion:

For the first time, we demonstrate that reprogramming macrophages with class-specific HDAC inhibitors is a viable cell therapy option to treat solid tumors.

INTRODUCTION

Tumor-associated macrophages (TAMs) play an essential role in innate and adaptive antitumor immunity. Dichotomous classification of macrophages according to their inflammatory status into proinflammatory M1 and anti-inflammatory M2 macrophages may be overly simplistic because TAMs can switch between these two phenotypes or may even exhibit a spectrum of hybrid characteristics. High infiltration of TAMs is usually associated with poor prognosis in several cancer types due to their predisposition towards M2 function. Therefore, the M1/M2 macrophage ratio has become an emerging determinant of antitumor immunity. Due to the plastic nature of TAM phenotypes, strategies to alter TAMs into antitumor M1 phenotype or diminish pro-tumor M2 phenotype within the tumor microenvironment (TME) have gained prominence. Therefore, the anticipated outcome of such strategies is to effectively increase the M1/M2 ratio, and a higher M1/M2 ratio has been associated with a favorable outcome in cancer patients.

A comprehensive analysis of cancers in The Cancer Genome Atlas (TCGA) revealed several immune cancer subtypes dominated by macrophages. These immune subtypes vary from being immunologically dormant to immunologically active, suggesting that macrophages play an essential role in shaping the TME. One strategy to boost proinflammatory M1 macrophages in the TME is by transplantation of M1-polarized macrophages into tumors. Autologous and adoptive transplantation of macrophages differentiated from circulating monocytes has been tested in clinical trials to treat immune related conditions and cancers. However, this approach has yielded only modest therapeutic benefits in cancer patients due to the overwhelming immunosuppressive nature of the TME. Moreover, some of these clinical trials were initiated prior to understanding the complexity of macrophage phenotypes, plasticity in response to various cytokines, and the cues from TME.

Towards reprogramming the TME, several groups, including us, have shown that intervention of specific histone deacetylases (HDACs) using small molecule inhibitors, such as HDAC6 inhibitors (HDAC6is), has immunomodulatory effects, particularly on macrophages. The above resulted in a paradigm shift from historical usage of pan-HDAC inhibitors for their cytotoxicity towards highly specific and isoform-selective HDAC inhibitors capable of regulating precise immune-related pathways. In this study, we designed a strategy to effectively reprogram macrophages towards proinflammatory M1 phenotype as a cell therapy modality. Our goal was to enhance antitumor immunity by treating M1 macrophages ex-vivo with HDAC6is followed by intratumor adoptive cell transplantation. Intratumoral transplanted macrophages were viable, sustained M1 phenotype post-transplantation, and enhanced antitumor immunity by increasing the infiltration of CD8 effector T-cells into the TME. Moreover, adoptive cell therapy increased the M1/M2 ratio by polarizing the host macrophages towards the M1 phenotype. Furthermore, ex vivo inhibition of HDAC6 in M1 macrophages attenuated STAT3 mediated M2 polarization in the hostile TME, thereby sustaining the M1 phenotype of transplanted macrophages. Similar results were observed in the SM1 syngeneic murine melanoma models and humanized NSG-SGM3 mice with melanoma patient-derived xenograft (PDX) tumors resulting in diminished tumor growth. Our study further validates the immunomodulatory effects of HDAC6 inhibitors and demonstrates the effectiveness of macrophage-based cell therapy to treat solid tumors.

M1/M2 macrophage ratio is an indicator of the immune status of the TME in melanoma

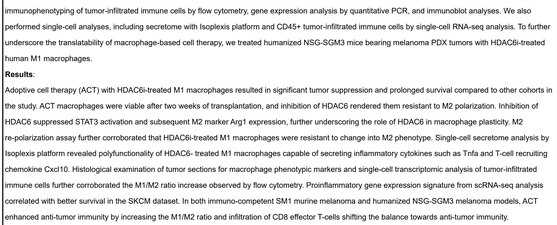

Fig. 1 | M1/M2 ratio reflects the immune status of the tumor microenvironment.
A-F, Kaplan Meier survival analysis of key M1 macrophages genes is associated with better survival in melanoma patients (SKCM) (TCGA, n=470). **G** & **H**, Cibersortx analysis of scRNA-seq data (GSE120575) from metastatic melanoma patients (n=48 tumor biopsies) treated with anti-PD1 and/or anti-CTLA4 immune checkpoint blockade therapy. **I**, Growth chart of immune-competent SM1 murine melanoma tumors in C57BL/6 mice. **J**, Negative correlation between SM1 murine melanoma tumor volume and tumor-associated M1 macrophages. **K**, Positive correlation between the tumor volume and tumor-associated M2 macrophages. **L**, Negative correlation between the tumor volume and M1/M2 macrophage ratio.

HDAC6 inhibitor suppressed M2 macrophage phenotype and function.

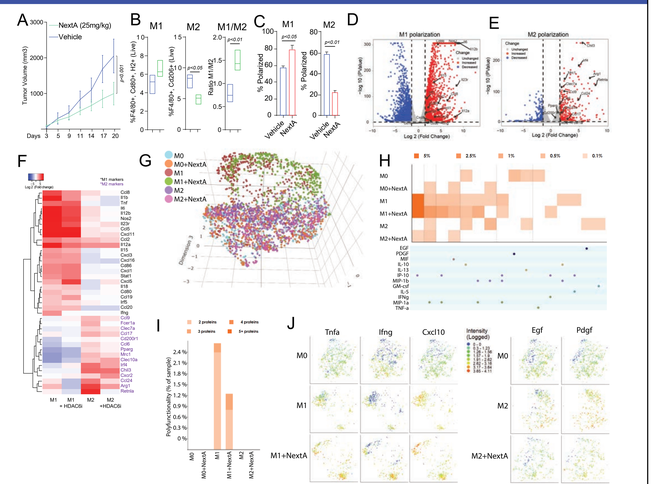

Fig. 2 | HDAC6 inhibition modulates M2 macrophage phenotype.
A, Tumor growth chart of syngeneic SM1 murine melanoma tumors in C57BL/6 mice treated with intraperitoneal (IP) injection of 25mg/kg HDAC6 inhibitor, NexturastatA (NextA) or vehicle. **B**, Tumor-associated M1, M2 macrophages and M1/M2 ratio in vehicle and NextA treated mice bearing SM1 murine melanoma tumors as a fraction of F4/80+ Cd80+ and F4/80+ Cd206+ macrophages, respectively. **C**, Polarization efficiency of murine bone marrow-derived macrophages (BMDMs) to M1 and M2 phenotypes after treatment with HDAC6i, NextA (5μM) determined by flow cytometry. Volcano plot showing fold-change and p-value for the comparisons of vehicle M1 versus M0 (**D**) and vehicle M2 versus M0 macrophages (**E**). The significance level was determined by log2 fold changes ≥ 1.5 (upregulation/increased) or ≤-1.5 (downregulation/decreased) and p-value < 0.05. Differentially expressed genes are depicted in blue and red, where known M1 markers and M2 markers are labeled in figures D and E, respectively. **F**, Heatmap of known markers for classically activated M1 and M2-like macrophage markers. Differential expression for HDAC6 inhibition versus vehicle was performed and M1 markers (in black) and M2 markers (in purple) were represented using the log 2 transformed fold changes relative to vehicle M0. **G**, UMAP analysis of BMDMs at a single cell resolution separated the cells into defined clusters based on their secretome profile. **H**, Polyfunctionality heatmap of M1 macrophages secreting more than one cytokine/chemokine compared to other phenotypes. **I**, Polyfunctionality strength index representing M1 and M1+NextA BMDMs secreting more than two proteins. **J**, 2-D tsne plots of each cell are represented as colored dots where blue indicates low expression and red indicates high expression. Proinflammatory cytokines Tnfa, Ifng, and T-cell recruiting chemokine Ip-10(Cxcl10) are elevated in M1+NextA, whereas growth factors Egf and Pdgf secreted by M2 phenotype are decreased with NextA treatment.

STAT3 mediated M2 phenotype is suppressed with HDAC6 inhibition.

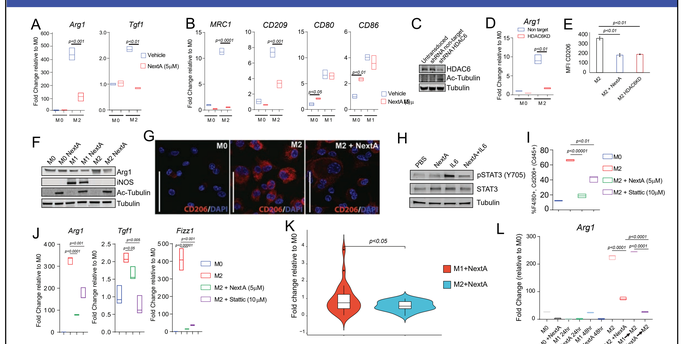

Fig. 3 | HDAC6 inhibition suppresses macrophage polarization towards the M2 phenotype.
A, mRNA expression levels of M2 markers Arg1 and Tgfb1 by qRT-PCR in murine BMDMs. **B**, mRNA expression levels of M2 markers MRC1 (CD206) and CD209, M1 markers CD80 and CD86 in human macrophages derived from Thp1 monocytic cell line. **C**, Immunoblot analysis of shRNA mediated knockdown of Hdac6 in immortalized murine macrophage cell line BMA3.1A7. **D**, mRNA expression levels of M2 marker Arg1 by qRT-PCR in non-target and Hdac6 knockdown (HDAC6KD) BMA3.1A7 cells by q-PCR. **E**, Mean fluorescence intensity (MFI) of CD206 in HDAC6KD BMA3.1A7 murine M2 macrophages analyzed by flow cytometry. **F**, Immunoblot analysis of BMDM macrophages for M1 (iNOS) and M2 (Arg1) markers. Tubulin is loading control and acetyl-tubulin as markers for HDAC6 inhibition **G**. Immunofluorescence analysis M2 marker CD206 in naïve and M2 polarized BMDMs with or without NextA treatment. **H**, Immunoblot analysis of IL6 mediated STAT3 phosphorylation in RAW macrophages treated with NextA or vehicle. **I**, Flow cytometry analysis of BMDM derived M2 macrophages as a percentage of Cd45+ cells treated with HDAC6 inhibitor, NextA (5μM) and STAT3 inhibitor, Stattic (10μM). **J**, mRNA expression of Arg1, Tfgb1, Fizz1 by qRT-PCR analysis in bone marrow-derived M2 macrophages treated with NextA and Stattic. **K**, Macrophage repolarization assay, mRNA expression analysis of Arg1 by qRT-PCR in M1 macrophages exposed to M2 polarizing cytokines.

HDAC6 inhibitor treated M1 macrophage based adoptive cell therapy.

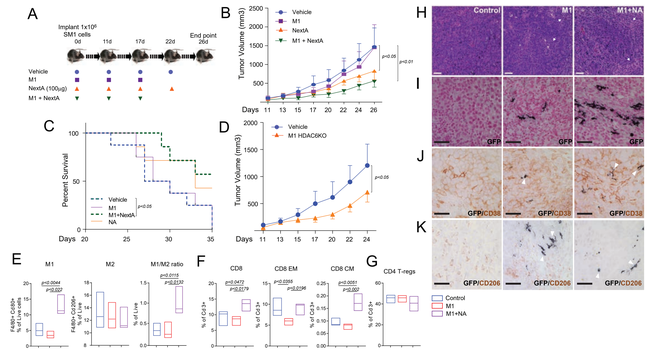

Fig. 4 | Adoptive cell therapy of HDAC6 inhibitor treated M1 macrophages diminish melanoma tumor growth inimmunocompetent mice. A, Schematic workflow of macrophage adoptive cell therapy (ACT) in SM1 murine melanoma model. **B**, Tumor growth chart of SM1 murine model treated with vehicle (PBS), intratumor implantation of M1 macrophages, NextA (100ug) and M1 macrophages pretreated with NextA (5μM) ex-vivo. **C**, Survival analysis of mice treated with macrophage adoptive cell therapy. **D**, Tumor growth chart of SM1 murine melanoma tumors with vehicle (PBS) or bone marrow-derived M1 macrophages from HDAC6KO mouse. **E**, Flow cytometry based immunophenotyping of M1, M2 macrophages as a fraction of F4/80+ Cd80+ and F4/80+ Cd206+ macrophages respectively and M1/M2 ratio in SM1 murine melanoma tumors treated with adoptive cell therapy. **F**, CD8 T-cells as a fraction of Cd3+ cells. **G**, CD4 T-cells and T-regs as a fraction of Cd3+ cells. **H**, Analysis of SM1 murine melanoma tumors treated with vehicle control, M1 macrophages or HDAC6 inhibitor treated M1 macrophages (M1+NextA) by H&E staining and **I**, immunohistochemistry staining to detect GFP expressing macrophages, GFP and CD38 expressing macrophages **J**, GFP and CD206 expressing M2 macrophages **K**.

Single cell studies of tumor infiltrated immune cells.

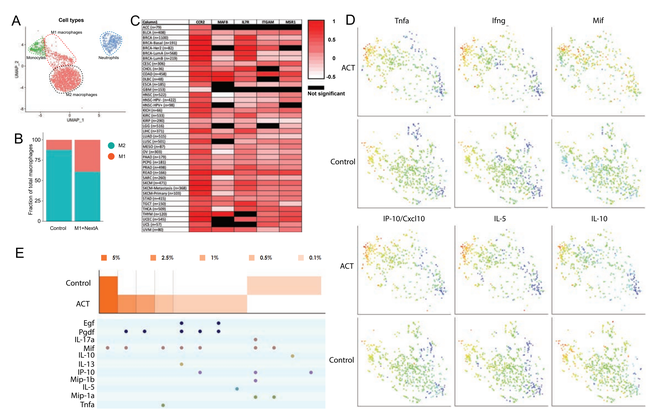

Fig. 5 | Single-cell analyses of CD45 (RNA-seq) and F4/80 (Isoplexis) sorted tumor infiltrated immune cells.
A, UMAP cluster analysis of aggregated immune cells by SingleR cell annotation revealing three major immune cell populations. **B**, Percentage of M1 and M2 macrophages in control and M1+NextA treated tumors. **C**, a Heat map of M1 gene signature expression in different cancer types in TCGA database. High expression is indicated by red, and low expression is indicated by white. Not significant expression in that cancer is indicated by black. **D**, Tumor associated macrophages were sorted for F4/80 and analyzed on Isoplexis platform for innate immune single cell secretome. Increased expression of proinflammatory cytokines Tnfa, Ifng, Mif in macrophages isolated from tumors treated with macrophage adoptive cell therapy compared to control. Also, increased secretion of Ip-10 /Cxcl10 which recruits CD8 T-cells along with immunostimulatory Il-5 by tumor macrophages treated with ACT compared to Control (treated with PBS). Finally, immunoregulatory Il-10 secretion is reduced by macrophages treated with ACT compared to Control suggesting a lower propensity of tumor macrophages to polarize toward M2 phneotype. **E**, Increased percentage of polyfunctional tumor macrophages in ACT group compared to the control group suggesting a robust anti-tumor response with HDAC6 inhibitor treated macrophage ACT.

Macrophage ACT in NSG-SGM3 melanoma PDX model

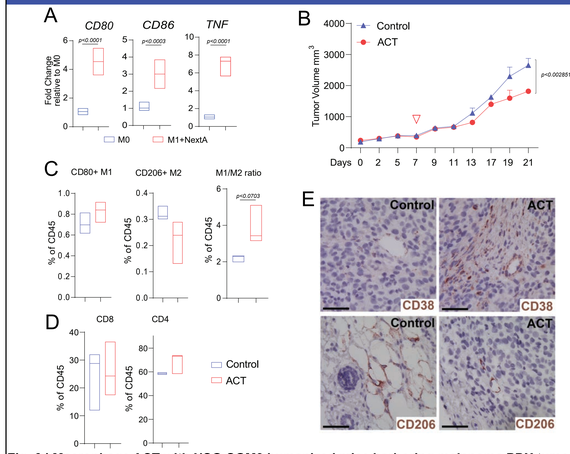

Fig. 6 | Macrophage ACT with NSG-SGM3 humanized mice harboring melanoma PDX tumor. A, Validation of M1 genes in human monocyte-derived macrophages. **B**, Tumor growth kinetics of melanoma PDX tumors in NSG-SGM3 mice in control and ACT treatment groups. **C**, Immunopheno-typing of tumor infiltrated immune cells by flow cytometry. **D**, Immunohistochemistry analysis of PDX tumors post ACT therapy for M1 macrophages (CD38) and M2 macrophages (CD206).

CONCLUSIONS

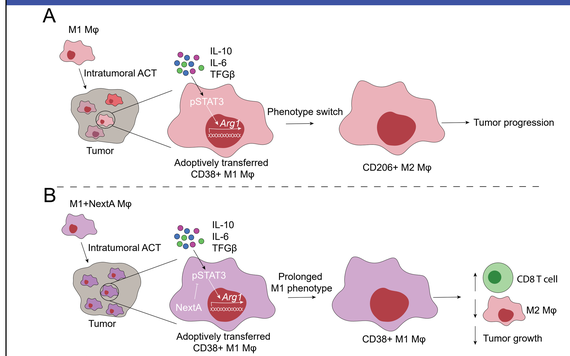

Fig. 7 | HDAC6 inhibitor treated M1 macrophages improve antitumor immunity with adoptive cell therapy. A, M1 macrophages upon intratumor transplantation are influenced by immunosuppre-ssive and anti-inflammatory cytokines to change towards tumor promoting M2 phenotype. **B**, On the other hand, M1 macrophages treated with HDAC6 inhibitor, Nexturastat A (NextA) are resistant to changing towards M2 phenotype due to deactivation of STAT3 signaling. Therefore, M1+NextA retain M1 phenotype in the TME and improver antitumor immunity by increasing the M1/M2 ratio and infiltration of CD8 effector T-cells.

- For the first time, we demonstrated that HDAC6i treated macrophage ACT is effective as anti-tumor therapy.
- HDAC6 inhibition in macrophages suppressed STAT3 mediated M2 polarization and sustained M1 phenotype.
- Transplanted macrophages survived and retained M1 phenotype in the TME as evidenced by increase in the M1/M2 ratio in SM1 murine melanoma model.
- Macrophage ACT increase infiltration of effector, effector memory and central memory CD8 T-cells.
- Histology and scRNA-seq analyses validated the flow cytometry data of increased M1/M2 ratio.
- Single cell secretome analysis further validated the anti-tumor activity of transplanted macrophages
- Finally, observations in murine melanoma model with macrophage ACT were recapitulated in NSG-SGM3 mice bearing human PDX tumors further underscoring the potential of macrophage based ACT as an anti-tumor therapy.

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