Inaugural dissertation

For obtaining the

doctoral degree

of the

Combined Faculty of Mathematics, Engineering and Natural Sciences

of the

Ruprecht - Karls - University

Heidelberg

Presented by

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born in: Vadodara, India

Oral examination: 29th of June, 2023

APPROACHES TO ADDRESS THE IMMUNOGENICITY AND IMMUNOSUPPRESSIVE FEATURES OF ISOCITRATE DEHYDROGENASE 1 MUTATED GLIOMAS

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NON-DISCLOSURE NOTICE

for Dissertation

"Approaches to address the immunogenicity and immunosuppressive features of isocitrate dehydrogenase 1 mutated gliomas"

by Khwab Sanghvi

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Abstract

The discovery of exploitable tumor-specific targets is central to the development of clinically relevant immunotherapeutic strategies for cancer. Mutations in isocitrate dehydrogenase 1 (IDH1), frequent in diffuse gliomas is one such target. The mutation IDH1R132H (mIDH1) is immunogenic, presented on human MHC-II molecules and induces CD4 T-cell responses in MHC-humanized A2DR1 tumor models as well as IDH1-mutated glioma patients when vaccinated with mIDH1-specific peptide vaccine. mIDH1, however plays the role of a double-edged knife. Its immunogenicity is compensated by the strong immunosuppressive environment orchestrated by the production of oncometabolite 2-HG that is imported by T-cells and leads to a suppression of their activation and proliferation. Inhibition of mIDH1 using small molecule inhibitors (IDH1i) has shown benefit in pre-clinical studies as therapy and has been associated with disease control in early clinical trials. Preclinical studies have also shown the ability of IDH1i to alleviate immunosuppression in the tumor microenvironment (TME). The impact of this inhibition on tumor infiltrating T-cells, however, has not been sufficiently evaluated.

The first part of this work, therefore, aimed at deciphering the effect of IDH1i on tumor-infiltrating T-cell activity and fate in the tumor microenvironment using single-cell RNA and VDJ sequencing of tumor infiltrating immune cells. The lack of suitable murine glioma models where mIDH1 is presented in its native immunogenic capacity on human MHC-II molecules prompted the development of a novel syngeneic mIDH1 glioma model in MHC-humanized A2DR1 mice. Single-cell transcriptomic and T-cell receptor analysis of tumor infiltrating immune cells in IDH1i treated mice revealed an accumulation of infiltrating T-cells potentiated by IDH1i with an increased abundance of CD4 T-cells with a tumor reactive phenotype and a reduction in frequency regulatory T-cells as well as restoration of functional intercellular T-cell communication. Combination treatment of IDH1i and ICB provided a synergistic therapeutic benefit for mIDH1 A2DR1 gliomas. These findings suggest that reduction of 2-HG levels is necessary for enabling a functional anti-tumor immune response which is then exploitable by immune checkpoint blockade and warrants for clinical trials testing the efficacy of IDH1 inhibitors

in combination with adjuvant immunotherapies such as vaccines or immune checkpoint inhibitors in patients with mIDH1 gliomas.

The second part of this work focused on developing a setup for the identification and validation of mIDH1-reactive T-cell receptors (TCR) from IDH1RH-specific vaccinated A2DR1 mice and from the resected lesion of a glioma patient part of the NOA16 mIDH1 peptide vaccine trial. The latter revealed a unique transcriptional signature of mIDH1 reactive CD4 T-cells in the tumor microenvironment characterized by CXCL13 expression. The proof of principle identification of mIDH1 reactive TCRs demonstrates the feasibility of exploiting immune responses against CD4-restricted neo-epitopes as a first step in developing an adoptive TCR-transgenic T-cell therapy for glioma patients

ZUSSAMENFASSUNG

Die Entdeckung verwertbarer tumorspezifischer Zielstrukturen ist von zentraler Bedeutung für die Entwicklung klinisch relevanter immuntherapeutischer Strategien gegen Krebs. Mutationen in der Isocitrat-Dehydrogenase 1 (IDH1), die häufig in diffusen Gliomen vorkommen, sind ein solche Zielstruktur. Die Mutation IDH1R132H (mIDH1) ist immunogen, wird auf menschlichen MHC-II-Molekülen präsentiert und induziert CD4-T-Zell-Reaktionen in MHC-humanisierten A2DR1-Tumormodellen sowie bei IDH1-mutierten Gliompatienten, wenn sie mit einem mIDH1-spezifischen Peptidimpfstoff geimpft werden. mIDH1 spielt jedoch die Rolle eines zweischneidigen Messers. Seine Immunogenität wird durch ein starkes immunsuppressives Umfeld kompensiert, das durch die Produktion von onkometabilem 2-HG entsteht, das von T-Zellen importiert wird und zu einer Unterdrückung ihrer Aktivierung und Proliferation führt. Die Hemmung von mIDH1 durch niedermolekulare Inhibitoren (IDH1i) hat sich in präklinischen Studien als vorteilhaft für die Therapie erwiesen und wurde in frühen klinischen Studien mit der Kontrolle der Krankheit in Verbindung gebracht. Präklinische Studien haben auch gezeigt, dass IDH1i die Fähigkeit besitzt, die Immunsuppression in der Mikroumgebung des Tumors (TME) zu verringern. Die Auswirkungen dieser Hemmung auf die den Tumor infiltrierenden T-Zellen sind jedoch noch nicht ausreichend untersucht worden.

Der erste Teil dieser Arbeit zielte daher darauf ab, die Wirkung von IDH1i auf die Aktivität und das Schicksal von tumorinfiltrierenden T-Zellen in der Tumormikroumgebung mit Hilfe von Einzelzell-RNA und VDJ-Sequenzierung von tumorinfiltrierenden Immunzellen zu entschlüsseln. Das Fehlen geeigneter Maus-Gliom-Modelle, in denen mIDH1 in seiner nativen immunogenen Eigenschaft auf humanen MHC-II-Molekülen präsentiert wird, führte zur Entwicklung eines neuen syngenen mIDH1-Gliom-Modells in MHC-humanisierten A2DR1-Mäusen. Einzelzell-Transkriptom- und T-Zell-Rezeptor-Analysen von tumorinfiltrierenden Immunzellen in IDH1i-behandelten Mäusen zeigten eine Anhäufung von infiltrierenden T-Zellen, die durch IDHi potenziert wurden, mit einer erhöhten Abundanz von CD4-T-Zellen mit einem tumorreaktiven Phänotyp und einer Verringerung der Frequenz regulatorischer T-Zellen sowie einer Störung der funktionellen interzellulären T-Zell-Kommunikation. Die Kombinationsbehandlung von IDH1i und Immun-Checkpoint-Blockade (ICB) bot einen synergistischen therapeutischen

Nutzen für mIDH1-A2DR1-Gliome. Diese Ergebnisse deuten darauf hin, dass die Senkung des 2-HG-Spiegels notwendig ist, um eine funktionelle Anti-Tumor-Immunantwort zu ermöglichen, die dann durch ICB onkoausgenutzt werden kann, und rechtfertigen klinische Studien, in denen die Wirksamkeit von IDH1-Inhibitoren in Kombination mit adjuvanten Immuntherapien wie Impfstoffen oder Immun-Checkpoint-Inhibitoren bei Patienten mit mIDH1-Gliomen getestet wird.

Der zweite Teil dieser Arbeit konzentrierte sich auf die Entwicklung eines Systems zur Identifizierung und Validierung von mIDH1-reaktiven T-Zell Rezeptoren (TCRs) aus IDH1RH-spezifisch geimpften A2DR1-Mäusen und aus der resezierten Läsion eines Gliompatienten im Rahmen der NOA16 mIDH1-Peptidimpfstoffstudie. Letzteres ergab eine einzigartige Transkriptionssignatur von mIDH1-reaktiven CD4-T-Zellen in der Mikroumgebung des Tumors, die durch die Expression von CXCL13 gekennzeichnet ist. Die prinzipielle Identifizierung von mIDH1-reaktiven TCRs zeigt, dass es möglich ist, Immunreaktionen gegen CD4-begrenzte Neo-Epitope als ersten Schritt zur Entwicklung einer adoptiven TCR-transgenen T-Zell-Therapie für Gliompatienten zu nutzen.

ACKNOWLEDGEMENTS

Have you ever done a big solo trip?

Be it an adventurous backpacking trip, a school exchange or a long, unscripted holiday to an adventurous destination. Everyone around you is proud of the idea but cautions you on the idea – what to do, what not to do, where to go and to be prepared for the worst. You are nervous but excited, and naively think it will be a cakewalk as you aren't fazed by anything. You very soon realize you couldn't have been more wrong… enraptured by the vibrant surroundings but you realize your first hotel is unlivable. There is so much to do, so little time. Some experiences make you wish you had done more homework. You are constantly strapped for cash, the language barrier is hard, someone cheated you off your money promising a unique experience because you just didn't know better and to top it all off, you miss your return flight and your camera is stolen. But at the same time, you had an amazing experience filled with crazy stories. Saw sights which made you go "Oh Wow, that's so cool!". Had moments where you jumped in the air, shouting "F**K YES! I did it!". And last but not the least, met some amazing fellow travellers who became lifelong friends. At the end of the trip, you are exhausted but also reinvigorated. You realize you have discovered a whole new version of yourself and have come out stronger than you went in.

Now, if you are reading this and are doing/have done a PhD, this whole rollercoaster would and should sound pretty familiar…..just in a different context!

DKFZ has been a grand destination. The scale, the vitality, the people, the chaos. The perfect Port of call for a once-in-a-lifetime experience (cause you for sure don't want to do a PhD more than once in your life!!)

So first and foremost, I would like to express my immense gratitude to the CEO of the travel agency and also my sponsor for this trip – Prof. Michael Platten. Thank you for giving me this once-in-a-lifetime opportunity (pun intended), the guidance, having trust in my opinions and most importantly for always generously providing the infrastructure to pursue our ideas. My PhD defense and TAC committee – Prof. Offringa, Prof. Stöecklin, Prof. Papavasiliou, Dr. Turcan – for their time and mentorship.

Before every mega trip, one sifts through blogs, YouTube videos to gain knowledge on how to navigate what you have plunged yourself into. My version of travel guidebooks were my 'Lab mamas' – Jana, Katrin and Theresa. They pampered me, taught me and equipped me with the skills I needed for this dive into the unknown. A tour guide can make or break your journey but when you have one whose passion for science is so energetic, you know you will have a good itinerary. You don't always agree with what the tour guide has planned for you but its impelling when they motivate you to Thank you, Lukas for the guidance , for believing in my potential and for always having my back.

To all my colleagues in the Platten Lab(D170), Wick Lab(B320) and the IMU. Some of you were master tour guides, some fellow boat travellers also suffering the sea sickness, some brought comic relief with their drama and spice and some were experiences which gave me fodder for future dinner parties to say, "You won't believe this person I met…". A memorable trip is only possible with this whole combination. A sum of its parts, each of you in a small or big way was instrumental in this journey and I am happy I had the chance, especially the ironically NSFW D170 characteristic coffee chats which produced some wide eyes of shock on unprepared bystanders. There are always, however, special mentions.

On the plane to your destination, there are others also starting the same trip and you realize you are not the only one nervously wondering what's going to happen. The journey may have had different durations, we may have had different paths but we started together and created

a bond. Frederik, you were like the meticulously organized medicine pack I got for free against the demons of the mind.

Sometimes you meet this awe-inspiring fellow traveller who can also be the tour guide. The one who does crazy hikes without breaking a sweat. Miki, your work ethic is envious and always makes me wonder, how the hell you do it. It was an absolute privilege having the opportunity to work with you.

Kyle, Katharina, Ed and Lisa, for the collaboration and the resources. Oh, and yes, fulfilling the role of lab equivalent of travel encyclopaedias in this overarching metaphor.

'Techs' are lab equivalent of 'locals' who give you a lift when you are stranded and feed you with some awesome meals (pun intended, case in point here - the cakes, but in case you didn't get it, it's the metaphor for all the work they do for you). Kristine, Anna, Steffi and Vicky, you were lab lifesavers with all the work help you provided and the entertainment we generated together.

Are you the kind that would love it if their trip felt like a reality travel show? Then come to the Platten/Wick Lab. Nirmeen, for being the one I could bicker with and for the cathartic feeling of complaining together. Matthia, who has been like the big sister whom one looks up to but can also crack a dirty joke with. But on a serious note, to the two of you for the pep talks, for always checking up on me, the constant encouragement and for making me always feel confident about myself. Verena, Mirco, Marie, Sara, Pauline, Alex (Kourtesakis), Ankita, Dennis, Gordon, Jessi, Niklas, Tamara and Yu-Chan for the help and input but more importantly for never a dull moment.

The biggest advantage of a solo trip is the amazing fellow travellers you meet who, where otherwise it may be a momentary fun rendezvous, become the travel companions you always wanted and lifelong friends. They may then become kilometres apart but the impact they had on you stays.

There are some people you meet while travelling that become like family. Shubh and Jenni, the co-conspirators in thrilling chaos (More Shubh than Jenni when it comes to literal chaos..). From the over-enthusiastic arguments which I call healthy discussion and you 'just Khwab talking', to tolerating my never-ending gyan (hindi for wisdom but with pejorative undertone), to coining the phrase 'that's so Khwab', it has been a blast! Frederik and Markus, the ones who are there when you need them and discipline you when you need it. For the night outs, holidays, chill evenings, your invaluable friendship and advise, thank you. You four are life support!

Joël, who I can't thank enough. Like the travelmate who you are also sharing your hostel room with, he is probably the only one who has seen my true face in moments of frustration and the 'behind the scenes' of when you have to put up a fake smile and a 'thumbs up, its all good'.

Imagine a trip, where you find brethren that share a passion for the same niche interest you have. You not only find a group of like-minded weirdos but also get to do things you wouldn't have been able to do alone. To the 'Awesome Potatoes' my theatre group who brought the drama I definitely wanted. Especially Asha, Safak, Deni and Emil, for their constant support, hugs and ears when life didn't feel as forgiving.

No trip is great if you didn't party enough and have a good time. The same is true for a PhD. But you need the right company. Vera, Alex (Kourtesakis), Moritz, Matea, Pablo, Isa, Paul, Franzi, Lisa, Alex (Egl). Some of you were the spark of joy at the end of a dull week, some gossip guilt enablers, some perfect drinking buddies and one an awesome flatmate (and COVID lockdown cellmate).

Ashik, Sakeena, Mahak and Deepti for giving me a taste of India when I missed home.

My landlord and lady – Mr. and Mrs. Lenz. They were like the warm and loving language barrier homestay hosts who end up training you well in explaining your PhD project in German.

As much as having fun is fun on a trip, it becomes more fulfilling when you get an opportunity to become part of a community and do something extra for …The debater, activist, event organizer bug in me 'definitely' didn't have anything to do with me not giving up on the chance to be part of the DKFZ PhD council and the Helmholtz Juniors. Not only were we able to achieve some long-demanded work-life improvement benefits for Doctoral Researchers but I had the amazing privilege of working with people whose passion for working for the community was really inspiring and educational. So, a shout out to my fellow council members – Shubh, Oghuzan, Sam, Michael B and Sonja, my PhD Support Team and my amazing HeJu batchmates.

Then there are the old friends who bear the brunt of the rant and complaining when you can't figure out whether its challenges on the trip or quarter-life crises, often in long tedious text format. But they are also required to respond with sympathy and understanding and can't tell you to suck it up. Eve, Ara, Mukta, Prabhpreet, you did the job brilliantly. Added on top are the special ones, whom you have a regular voice text-based support therapy scene with – Jackie, Asha and Alex (Pirvan). Girls (and boy), that helped!

When you know someone else is going to take a similar trip, you are excited to give all the tips and recommendations. Alina, Arianna who made me first realize how much I enjoyed teaching. And the 'Khwab's LabKids' – Julius and Lea – from students to friends to finally my teachers, I cherished this responsibility. Mr. Julius Michel, there is a reason I kept thanking him towards the end in the acknowledgement. Although a PhD is supposed to be a solo endeavour with people supporting you along the way, he definitely became my partner in crime towards the end. These PhD projects wouldn't have been possible without him. I have told him and repeat here that in my business planner there is a section which you fill at the end of the year mentioning people who have had the most impact on you. You are the person I mention first. Your infectious charm, and perspective towards life and work always made me want to do more and better. Thank you, really, from the bottom of my heart.

And my family, the whole chunk the grandparents and the extended, for rooting hard for this trip to be a success. Mom, Dad, Raa, I love you. Be it when I used the PhD as an excuse for not having time, to cheering the loudest pride at small achievements along the way, you were major pillars of support. I wouldn't have survived this trek which often felt destination less if not for you always unconditionally being there.

And last but not the least, Alcohol. I want to know who the hell has managed a PhD and how without the distracting superpowers of alcohol.

Now that the trip is over, if you asked me whether I would do it all over again…. You would hear a loud, resounding…. 'HELL NO!'. But I had the most exhilarating rollercoaster adventure. As I mentioned at the start, this trip was exhausting but also reinvigorating, so I want to thank my younger self for taking on this challenge.

I have come out learning a lot about myself and being better prepared for upcoming life-changing journeys.

NEW DESTINATIONS AWAIT…….

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2-HG	R-2-Hydroxyglutarate
AHR	Aryl hydrocarbon receptor
AML	Acute myeloid leukemia
APC	Antigen-presenting cell
BSA	Bovine serum albumin
Cas9	CRISPR-associated protein 9
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CGI	CpG islands
CNS	Central nervous system
CRISPR	Clustered regularly interspaced short palindromic repeats
CTL	Cytotoxic T-cells
CTLA-4	Cytotoxic T lymphocyte antigen 4
CXCL	C-X-C motif chemokine
CXCL	Chemokine (C-X-C motif) ligand
DANN	Deoxyribonucleic acid
DKFZ	Deutsches Krebsforschungszentrum
DMSO	Dimethyl Sulfoxide
EGFR	Epidermal growth factor receptor
FBS	Fetal bovine serum
GAM	Glioma-associated myeloid cell
GBM	Glioblastoma
gRNA	Guide ribonucleic acid (tracrRNA + crRNA)
HBSS	Hank's buffered salt solution
HGG	Higher grade gliomas
HLA	Human leukocyte antigen
ICB	Immune checkpoint blockade
IDH1RH-vac	IDH1R132H-specific peptide vaccine
IFNg	Interferon-gamma

IL	Interleukin
InfHA	Influenza hemagglutinin
ISG	Type-I Interferon stimulated gene
LGG	Lower grade gliomas
MDSC	Myeloid-derive suppressor cell
MGMT	O-6-methylguanine-DNA methyltransferase
mIDH	mutant isocitrate dehydrogenase
MOG	Myelin oligodendrocyte protein
NK	Natural killer
ova	Ovalbumin
PBMC	Peripheral blood mononuclear cells
PD1	Programme death 1
RLU	Relative luminescence units
SOC	Standard of Care
TCR	T-cell receptor
TERT	Telomerase reverse transcriptase
TET	Tet methylcytosine hydroxylase
TGFβ	Transforming growth factor β
TIL	Tumor infiltrating leukocyte
TME	Tumor microenvironment
TMZ	Temozolomide
TP53	Tumor suppressor gene p53
UMAP	Uniform manifold approximation and projection
WHO	World Health Organization
wt	Wild type

1 INTRODUCTION

"Cancer is an expansionist disease; it invades through tissues, sets up colonies in hostile landscapes, seeking "sanctuary" in one organ and then immigrating to another. It lives desperately, inventively, fiercely, territorially, cannily, and defensively—at times, as if teaching us how to survive. To confront cancer is to encounter a parallel species, one perhaps more adapted to survival than even we are."

Siddharth Mukherjee, The Emperor of all Maladies

1.1 The role of IDH mutations in glioma

Gliomas are a family of tumors originating in the central nervous system from glial cells in the brain and spinal cord. They account for 30% of all brain and central nervous system tumors and about 81% of all malignant brain tumors [1]. Although having a relatively low incidence rate, they usher significant morbidity and mortality, with adult and pediatric diffuse gliomas being the most aggressive brain tumor types. Where the 5-year relative survival rate for malignant brain and other CNS tumors is 35.6%, the 5-year survival rate for the most common glioma histology – Glioblastoma (~49% of gliomas), is a poor 6.2% [2, 3].

1.1.1 The classification of gliomas

For decades, brain tumors were classified based on their histological features and clinicopathologic correlation. However, this system alone was not sufficient in addressing the diverse morpho-genetic features in this group of tumors. In 2016, the WHO therefore additionally introduced molecular genetic features in their classification[4]. The most up-to-date classification – WHO CNS5 categorizes gliomas into six types – 1. Adult-type diffuse gliomas; 2. pediatric-type diffuse low-grade gliomas; 3. pediatric-type diffuse high-grade gliomas; 4. circumscribed astrocytic gliomas; 5. glioneuronal and neuronal tumors and 6. Ependymomas [5]. The adult-type diffuse gliomas can be categorized into IDH-mutant, 1p/19q codeleted oligodendrogliomas, IDH-mutant non-coldeleted astrocytomas and IDH-wildtype Glioblastoma (GBM). These categorizations are based on molecular and histological features and based on pathological correlates of severity, can be further evaluated as grades I-IV.

In brief, oligodendrogliomas are characteristic of cells with a ‘fried egg appearance’ and Grades 3 and 2 are separated based on the presence and absence of anaplastic features respectively. IDH mutations are a hallmark feature in astrocytomas. Where low-grade astrocytomas are well-differentiated slow-growing tumors, Grade 3 is an aggressive tumor type exhibiting nuclear polymorphisms that often progresses to Grade 4 and are then defined as secondary GBMs [6]. GBMs are characteristic of hypercellularity, microvascular proliferation, nuclear atypia and necrosis [7]. IDH-wildtype GBMs are the most frequent and malignant brain tumor type and are considered primary GBMs

(arising de novo), accounting for 90% of the cases as compared to secondary GBMs and have a dismal overall survival ranging from 12-18 months [2, 8].

The standard of care for gliomas especially GBMs, is surgical resection with concomitant radio and chemotherapy[9] and in recent times, electric field therapy[10]. Primary GBMs are often treated with the alkylating drug Temozolomide (TMZ) which triggers DNA damage-induced tumor cell death [11] whereas treatment of recurrent GBM often employs the use of angiogenic inhibitors such as Bevacizumab against vascular endothelial growth factor-A (VEGF-A) [12].

1.1.2 The genetic and epigenetic landscape of gliomas

The genetic landscape of gliomas is diverse and has been extensively studied. Conventionally, genome-wide trancriptomic profiling of bulk tumor classified glioma into 4 subtypes based on gene expression patterns – mesenchymal, classical, proneural and neural. The clinical significance of this classification however is debatable. The main genetic aberrations in glioma include mutations in IDH1/2, 1p/19q chromosomal co-deletions, ATRX loss, H3F3A alterations, Epidermal growth factor receptor (EGFR) amplification, Cyclin dependent kinase inhibitor 2A (CDKN2A) loss, a combined gain of chromosome 7 and loss of chromosome 10 and TERT (telomerase reverse transcriptase) mutations among others. Overarchingly, alterations at varying degrees of frequencies in 3 critical pathways have been attributed to glioblastoma development. These are the TP53 pathway, the RB1 pathway and PI3K/PTEN pathway. IDH-wildtype GBMs have a higher rate of TERT mutations (72-90%), EGFR amplification (35-45%), 10p or 10q chromosome loss (50 and 70%), CDKN2A/CDKN2B deletion (60%) and a lower rate of TP53 mutations(28-35%). Contrastingly, alterations in TP53 (81%), ATRX mutations (71%), and loss of chromosome arm 19q and 10q (50% and >60% respectively) are characteristic of IDH-mutant GBMs . TERT mutations are less frequent (30%)in IDH-mutant gliomas, with EGFR amplification and PTEN alterations extremely rare but they are closely associated with hypermethylator phenotypes. The relevance of these molecular markers in classifying gliomas has resulted in an integrated diagnostic algorithm shown in Fig.

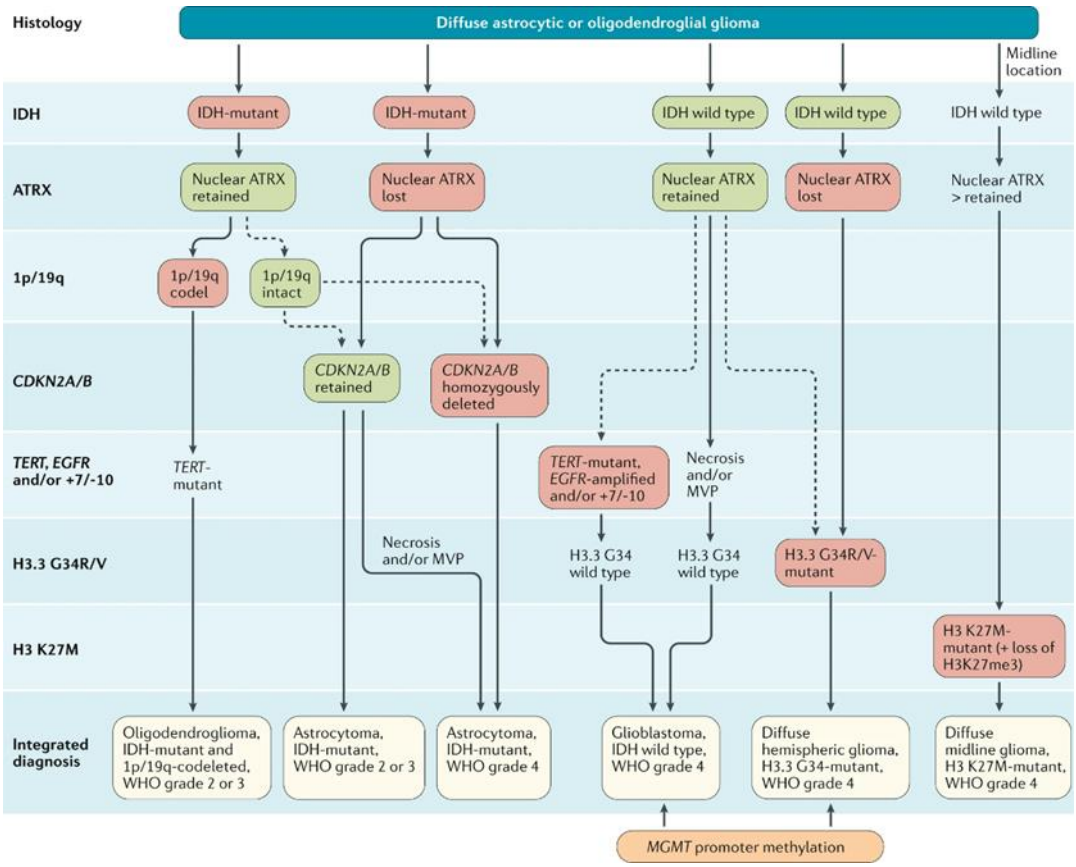

Figure 1.1: A layered approach for the classification of major diffuse gliomas in adults.

An integrated diagnostic algorithm combining histological and molecular features for an integrated diagnosis of glioma sub type. The diagnostically most relevant molecular alterations to each type, are highlighted for their presence or absence in green and red, respectively. Gliomas are additionally graded based on their malignancy by WHO on a scale of 2-4. *(Adapted from the EANO guidelines on the diagnosis and treatment of adult diffuse gliomas [13])*

The layering of molecular marker analyses on histological diagnoses has changed the paradigm in diagnosing the correct glioma subtype and consequently contributed towards improved treatment regimens, but it still does not provide a definitive way to decipher intratumoral heterogeneity. Further refinement in diagnostics has been possible with the emergence of extensive DNA methylation profiling of tumors. Disruption of normal gene regulation is a hallmark of carcinogenesis. Several mechanisms contribute towards this – DNA methylation, alteration in crucial regulatory genes, small and long noncoding RNA expression imbalances to name a few. Additionally, DNA methylation, histone modifications and nucleosome reassembly are epigenetic modifications that play an integral role in gene silencing [14]. Methylation of

cytosine in DNA primarily occurs at dinucleotide CpGs with stretches of frequent CpG sites defined as CpG islands (CGI). Under normal physiological conditions, CGIs at promoter regions are generally unmethylated with methylation occurring as a method of necessary gene repression in certain regions such as inactive X-chromosome and germ-line specific genes[15].

In cancer, methylation becomes aberrant with hypermethylation at promoter CGIs being a characteristic of gliomas and inducing gene silencing, especially of tumor suppressor genes[16]. The concept of CpG island methylation phenotype (CIMP) was originally proposed in subtypes of colorectal cancer and then described in several tumors [17, 18]. The translation of this signature to refine glioma sub-typing resulted in G-CIMP. Across all morphological glioma grades, G-CIMP positivity is tightly associated with the presence of IDH1 mutations and confers an additional improvement in survival outcomes in these tumors. However, studies have shown that favourable prognoses conferred by G-CIMP positivity are independent of IDH1 mutation status [19, 20]. In recurrent GBM, a small subset of patients progress from the original IDH-mutant low grade glioma with G-CIMP high phenotype to a less favourable G-CIMP low phenotype. A G-CIMP intermediate phenotype has also been characterized that potentially represents the group of tumors in early stage transition from high to low phenotype. A set of predictive biomarker signatures have been found that identify tumors putatively in the ‘risk group’ prone to progression[21]. Wider implementation of this may improve the stratification of recurrent GBM and its management.

Silencing of O(6)-methylguanine-DNA methyltransferase (MGMT) by promoter methylation is another epigenetic phenomenon with significant clinical relevance in gliomas. MGMT is a DNA repair enzyme that protects DNA from damage by alkylating agents such as TMZ, a key chemotherapeutic in GBM treatment. Therefore, its silencing has a strong impact on diminishing tumor cell resistance to alkylating agent therapy. MGMT methylation status is associated with IDH mutation and G-CIMP status, and is used to inform therapeutic decision planning with established prognostic and predictive values in gliomas indicating better survival [22, 23].

1.1.3 IDH1 mutations as a driver of gliomagenesis

The IDH gene encodes for the enzyme isocitrate dehydrogenase, a member of the Citric acid (Krebs cycle) that metabolically converts carbohydrates, proteins and fat in water and carbon dioxide to generate ATP in aerobic organisms. Among its 3 isoforms, IDH2 and IDH3 are found in the mitochondria whereas IDH1 is a cytosolic enzyme, also found in peroxisomes[24]. The pro tumorigenic role of IDH mutations has been implicated in several cancers, such as AML (acute myeloid leukemia), chondrosarcomas, and intrahepatic cholangiocarcinomas to name a few in addition to gliomas [25-28].

IDH mutations are early drivers of gliomagenesis and occur even earlier than TP53 mutations and 1p/19q codeletion. Their diagnostic relevance and ubiquitousness in certain sub-types of glioma (with more than 70% of WHO Grade 2 and 3 gliomas harbouring IDH1 mutations), prompted the WHO to use it as a key glioma classifying marker as previously described[29, 30]. Mutations in IDH are characterized by the non-synonymous substitution of arginine residues (R132 in IDH1 and, R172 and R140 in IDH2) with substitution to histidine (R132H) being the most common[31]. These arginine sites are key residues within the catalytic pocket of the enzyme that is involved with the binding of isocitrate. These substitutions inhibit the wild type enzymatic function by abrogating the ability of the enzyme to bind to its substrate. Consequently, this results in the conversion of alpha-ketoglutarate to R-2-hydroxyglutarate (2-HG), which accumulates within the tumor[32, 33]. 2-HG is the main pathological agent in IDH mutant gliomas that blocks cellular differentiation by inducing epigenetic dysregulation through the inhibition of alpha-ketoglutarate dependent histone and DNA methylases, thereby promoting tumorigenesis [34]. By influencing Hypoxia inducible factor prolyl hydroxylases (HIF-PHDs), a class of dioxygenases, 2-HG can also alter the redox state of the tumor cell[35]. A graphical representation of the cellular processes that are impacted by 2-HG is shown as a part of **Fig. 2**. Additionally, 2-HG is also secreted by glioma cells into the tumor microenvironment where it also accumulates and suppresses infiltrating immune cells[36], which has been described in more detail in upcoming sections.

Over the years, several inhibitors against mutant IDH1(mIDH1) have been developed that act by reducing the cellular production and accumulation of 2-HG. Ivosidenib and

enasidenib (mIDH1 and mIDH2 inhibitors respectively) have been approved by the Food and Drug Administration (FDA) for the treatment of IDH mutant hematological malignancies such as AML [37]. In the context of gliomas, the prototype, AGI-5198 showed *in vivo* therapeutic benefit but owing to poor pharmaceutical properties could not be tested further clinically. However, Phase I clinical studies evaluating ivosidenib (mIDH1 inhibitor) and vorasidenib (mIDH1/2 inhibitor) showed the safety and tolerability of the drug, prolonged stable disease and reduced growth on non-enhancing tumors [38, 39]. Several other inhibitors are currently under clinical investigation of which BAY1436032 and DS-1001 are a few examples[40]. Among these BAY1436032 is an oral small-molecule inhibitor of all known IDH1-R132X mutations. It has shown considerable efficacy in pre-clinical models of AML and glioma[41, 42]. It can penetrate the brain in mouse models of mIDH1 glioma, suppress 2-HG production, enhance survival and induce cellular differentiation[42, 43]. Phase I trials in AML and IDH mutant solid tumors including gliomas met their primary endpoint[44, 45]. These promising results have prompted additional clinical studies evaluating the efficacy of mIDH inhibitors in patients with primary or recurrent IDH mutant lower grade gliomas (LGG) in either treatment naïve settings or combination with other therapies [40].

Mutations in IDH1 are prognostically favourable and are associated with improved overall survival in both GBM and LGG [46]. They are suggested to be more sensitive to radiation therapy and respond better to alkylating agents [30]. They have a close association with clinically beneficial G-CIMP status as previously mentioned and are sufficient for establishing this hypermethylator phenotype through significant epigenomic reprogramming and remodelling of the methylome [47, 48]. IDH1 mutations are, therefore, a double-edged sword. On the one hand, they are the earliest genetic events that drive tumorigenesis but on the other, they yield certain vulnerabilities within tumor cells that make them more susceptible to certain therapeutic interventions. However, being an immunogenic mutation, with intrinsic prognostically favourable downstream metabolic activity and the promising potential of inhibitors, mIDH1 is attributed as a strong target for glioma therapy. A map of the putative ways to target mIDH1 is illunstrated in Fig 2.

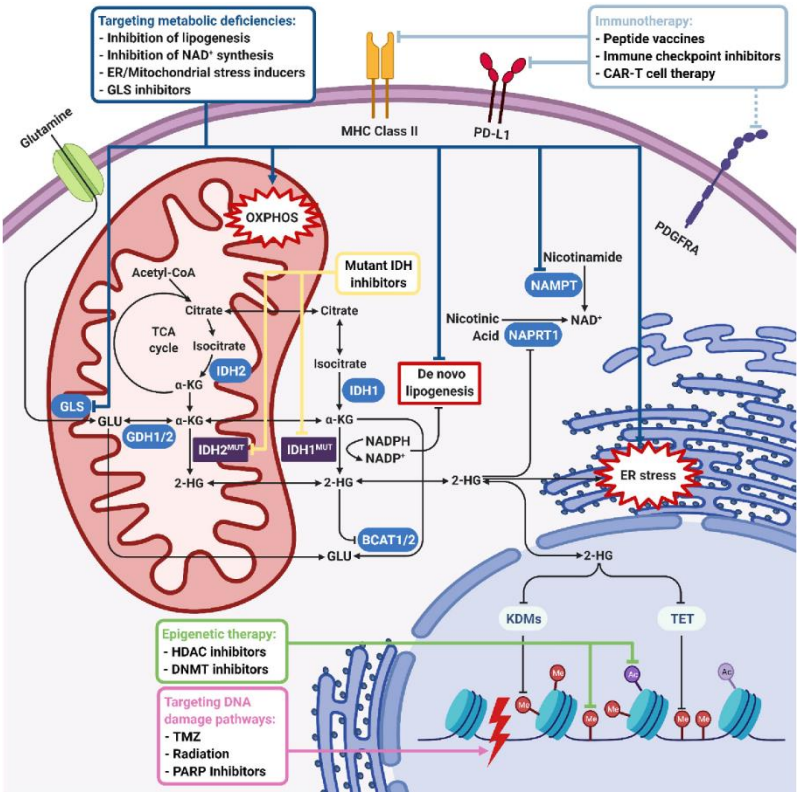

Figure 1.2: Map of therapeutic approaches for IDH mutant gliomas

IDH mutations orchestrate global epigenetic and metabolic changes through the induction of 2-HG accumulation and NADPH depletion. IDH inhibitors can serve as a first-line strategy in reversing the mIDH induced phenotype. The 2-HG cumulation inhibits KDMs and TETs which drives histone and DNA hyermethylation rendering epigenetic enzyme targeting a viable thereapeutic option. In mIDH tumors, oxidative stress is induced by depletion of NADPH which also inhibits *de novo* lipogenesis, making these tumors potentially susceptible to E/mitochondrial stress inducers. Additionally, MGMT promoter methylation which controls DNA damage response, is often associated with IDH mutations, making these tumors sensitive to DNA damage through irradiation, alkylating agents like TMZ and PARP inhibitors. mIDH1 is also an immunogenic epitope that can be targeted through various immunotherapeutic interventions such as peptide vaccines, immune checkpoint inhibition and targeted adoptive T-cell therapies. Moreover, IDH inhibitors also serve to remove the strong immunosuppressive pressure of 2-HG in the glioma immune microenvironment[49].

1.2 How the immune system interacts with glioma

Decades of relentless research have evolved our understanding of cancer as a disease – that it is not merely a mass of autonomous pathological mutant cells but a complex tissue composed of many cell types other than tumor cells that interact with each other. These include components of the extracellular matrix, fibroblasts, surrounding

connective tissue, associated vasculature, signalling molecules and infiltrating immune cells collectively, the tumor microenvironment [50]. The biology of tumors cannot be understood simply by characterizing the tumor cells but require deciphering the contributions of the tumor microenvironment (TME) towards tumorigenesis.

1.2.1 Immunosurveillance in cancer

Even though cancer development hinges on the ability of tumor cells to hijack and exploit the physiological processes of the host, each stage of development is under the pressure of regulation by the immune system. The earliest evidences for the active role of immune system in cancer came from the observation of immune cell infiltrates in histological examinations of tumor tissue, the rapid growth of induced tumors in immune deficient mice and the inefficiency of these tumors to establish secondary tumors when transplanted to syngeneic immune competent hosts [51]. Extensive research over the decades has expanded our understanding of this complex interactome where immune cells constantly surveil the tumor, mount anti-tumor responses and how can they be exploited for therapeutic interventions. Cancer immunoediting proceeds through 3 phases – Elimination, Equilibrium and Escape. Initially transformed cells are eliminated by the immune system. This favours selection for weekly immunogenic tumor cells. A tug of war then ensues where tumors are subjected to constant immunoediting, further selection and consequently get armed with resistance mechanisms leading to a suppressive TME eventually resulting in their escape and outgrowth[52-54].

Recognition of tumor antigens by T-cells aimed at immunologic elimination is a fundamental principle of immunoediting. There is evidence in solid tumors, that it can consequently lead to neoantigen loss. In colorectal and kidney clear cell cancers, when comparing the observed mutational rate of silent mutations from TCGA datasets to the predicted rate of neoantigens from non-silent mutations, fewer neoantigens than predicted were observed[55]. A study on untreated NSCLC (non-small cell lung cancer) reported that patients with high immune cell infiltration displayed enhanced hypermethylation of promoters of gene mutations that encoded predicted neoantigens [56].

Originally, the concept of immunosurveillance became mistakenly synonymous with immunoediting solely as a means of detection and destruction of tumor cells by antigen-specific T-cells. However, tumor associated immune responses can be shaped by the tumor to enhance tumorigenicity and progression. Immunogenic tumors can dismantle components of the immune system aimed at eliminating them. They achieve this by either upregulating factors that regulate and suppress the tumoricidal activity of Natural Killer (NK) cells and cytotoxic T-cells (CTLs) such as immune checkpoint inhibitors (ICIs) [54] or by recruiting or inducing immunosuppressive immune cells such as regulatory T-cells (Treg) and myeloid-derived suppressor cells (MDSCs) [57, 58]. In addition, the chronic activation of innate immune cells and resulting inflammation can facilitate invasion and metastasis by supplying the tumor microenvironment with growth factors, extracellular matrix modifying enzymes and pro-angiogenic factors [59]. Therefore, the modern concept of cancer immunosurveillance emphasizes the dual host protective and tumor-sculpting role of the immune system.

1.2.2 The immune microenvironment in gliomas

Although it is now common knowledge that the immune system is pertinent in shaping tumor fitness, the limited permeability of the blood-brain barrier had historically resulted in a misconception that the CNS immune privilege is an isolation from the immune system. This viewpoint has dramatically changed in the last decade not only owing to the discovery of a functional meningeal lymphatic system that drains CSF and in turn CNS antigens to the deep cervical lymph nodes (LNs)[60] but the change in the status quo on the inability of microglia to present antigens to T-cells with studies showing their ability to induce regulatory T cells [61, 62]. The immune privilege of the CNS is therefore more a case of immunospecialization. Moreover, it has become overwhelmingly clear that glial tumors pilot a highly immunosuppressive micro milieu [63] that drives T-cell exhaustion[64] and results in tumorescape.

Gliomas are extensively infiltrated with myeloid cells which are composed of the CNS-resident microglia and peripheral macrophages collectively called Glioma-associated microglia/macrophages (GAMs)[65]. These myeloid cells are highly plastic and adaptive to specific environmental cues. Macrophages can change their effector mechanisms from an inflammatory anti-tumor phenotype (often called ‘M1’) to an

immunosuppressive, cytoprotective phenotype (‘M2’) [66]. Similarly, microglia can also polarize in response to cues to ‘M1-like’ or ‘M2-like’ functional states. These functional states were described based on a set of differentiating expression markers but lack consensus on a definitive signature of markers and the use of the M1-M2 duality is now discouraged but rather that GAMs exhibit discrete phenotypes within a spectrum [67]. Gene expression profiling of CD11b+ infiltrates (a classical marker for myeloid cells) indicates a lack of classical ‘immune activation’ of GAMs in GBM. The expression of some putative markers of M2 activation is increased and correlates with glioma grade and malignancy [68, 69]. The CD11b+ population in brain infiltrates also includes MDSCs which have natural immunosuppressive functions. Additionally, preclinical models for gliomas do not recapitulate the observation in human GBM. The complexity in distinguishing the cell-types within this heterogenous population makes it, therefore, difficult to refine the definition of GAM phenotypes and their clinical association.

It is hypothesized that leaky vessels, which typically occur in GBM, facilitate T-cell transmigration. The degree of infiltration positively correlates with long-term patient survival. Gliomas are considered immunologically ‘cold’ tumors as they generally exclude functional CTLs from the TME but may contain some dysfunctional CTLs [70-72]. Lack of proper T-cell activation in the TME is attributed to the suppression of anti-tumor responses by cytokines such as Interleukin (IL)-10 and transforming growth factor (TGF)-β secreted by glioma cells. Infiltrating T-cells show decreased expression of activation markers and upregulate immune checkpoint markers such as PD-1, indicating exhaustion.

Tregs are potent suppressors of adaptive immune responses by their ability to prevent the proliferation of any cytokine secreting effector T-cells. Although physiologically necessary to control and resolve immune system activation, gliomas promote the recruitment and accumulation of Tregs, accounting for upto 10% of CD4+ T-cells within the Tumor infiltrating leukocytes (TILs) [73, 74]; by producing huge amounts of indolamine 2,3 dioxygenase (IDO) which further suppress cytotoxic T cell activity[75]. The impact of NK cells on the other hand in glioma control or progression is not well understood. A major hindrance to effective NK-cell mediated killing is the high expression of Major Histocompatibility Complex (MHC) class I molecules on malignant gliomas which inhibit NK-cell activation. However, the presence of activated NK cells in

GBM is associated with a favourable prognosis [76]. Another study showed that activated NK-cells are found more frequently in low grade compared to high grade gliomas indicating that a reduction in the number of activated NK-cells is potentially associated with transition of gliomas from low to high grade [77].

Overall, gliomas harbour a strongly immunosuppressive TME. This is further compounded by their typically low mutational burden and neoantigen levels, limiting the ability of T-cells to recognize the tumor and initiate an anti-tumor response.

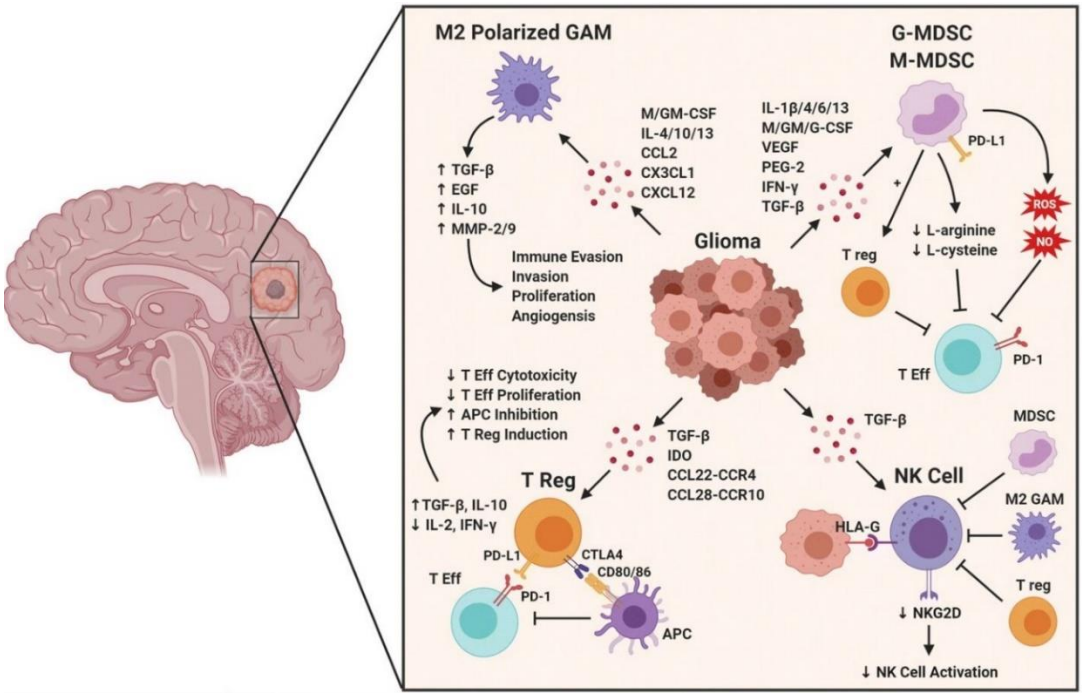

Figure 1.3: A schematic of the immunosuppressive environment exerted by gliomas. Simplified depiction of the glioma and tumor-infiltrating immune cell intercellular communication that drives immunosuppression in the glioma tumor microenvironment. Factors expressed/secreted by gliomas can directly inhibit effector cells and also recruit or drive the differentiation/polarization of other infiltrating immune cells to a tolerogenic phenotype which further suppresses effector cell responses *(ROS: Reactive oxygens species; Teff: effector T-cells; NO:nitric oxide, GM-CSF: Granulocyte-macrophage colony stimulating factor) Figure adapted from [78]*

1.2.3 Impact of 2-HG on the glioma immune microenvironment

The role of the oncometabolite 2-HG produced by IDH mutations in driving gliomagenesis has already been described above. 2-HG however, not only accumulates in the tumor cells but is exported out and can be detected in body fluids [79]. This has prompted investigations into the purpose and consequences of this phenomenon.

Previous studies from my lab and by others have shown that exported 2-HG is imported by immune cells in the TME, primarily T-cells but also myeloid and B-cells leading to direct suppression of T-cell activity and their proliferation. These findings are further supported by the observations that IDH1 mutation confers an immunologically quiescent phenotype onto gliomas. IDH1 mutant gliomas display a lesser abundance of infiltrating T-cells and reduced expression of immune checkpoint programmed death ligand-1 (PDL1) than wt counterparts [80, 81]. This is corroborated by TCGA studies that show IDH1 mutations are associated with reduced expression of CD8+ T-cells, Interferon γ (IFNγ), a key marker of T-cell activation, and altered calcium signalling [82, 83].

The study showed that T-cells in the TME are paracrine targets of 2-HG and its uptake occurs through a sodium-dependent dicarboxylate transport system and directly impairs T-cell activation. Sodium starvation and inhibition of transporter SLC13A3 showed a concentration-dependent decrease in intracellular 2-HG levels in T-cells when exogenously exposed with the latter being able to rescue inactivation partially. 2-HG exposure perturbs the transcriptional activity of Nuclear factor of activated T-cells (NFAT) by inhibiting its nuclear translocation through the interference of calcium influx. The nuclear translocation of NFAT is crucial for T-cell receptor (TCR) downstream signalling and induction of programmed death-1 (PD-1), with the latter showing reduced expression in glioma infiltrating T-cells. Additionally, 2-HG exposure reduced ATP production in T-cells and polyamine biosynthesis, pathways essential for TCR signalling, cell growth and proliferation respectively [80]. Another report showed that transgenic introduction or therapeutic delivery of 2-HG in a preclinical model attenuates the secretion of chemokines C-X-C motif chemokine 9 (CXCL9) and CXCL10 which resulted in reduced CD8+ T-cell infiltration into the tumors. This was orchestrated by suppression of the signal transducer and activator of transcription 1 (STAT1) [83]. Mirroring its tumor intrinsic metabolic consequence, 2-HG triggers HIF-1α protein destabilization, inducing metabolic skewing towards oxidative phosphorylation, reduced T-helper cell 17 (Th17) polarization and an increase in Treg frequency [84].

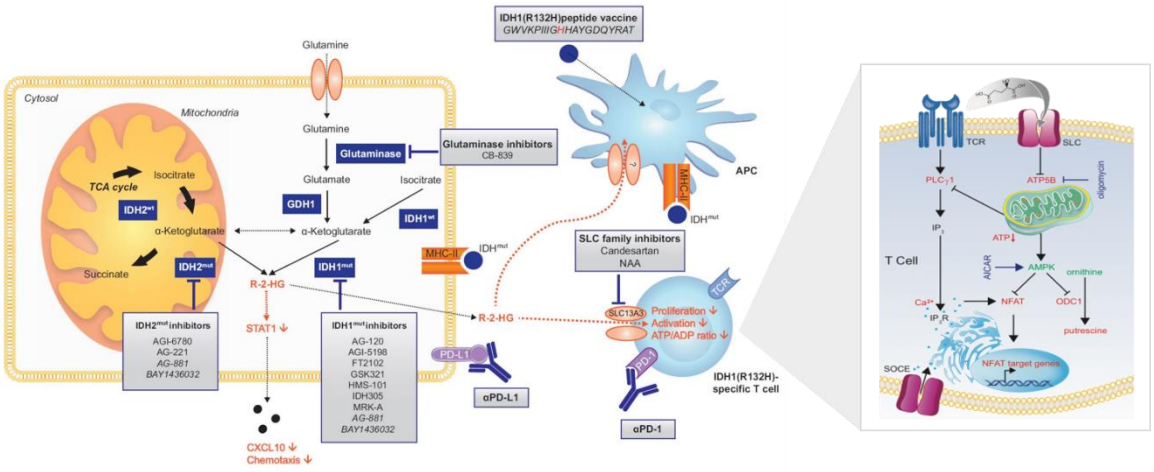

Figure 1.4: Illustration of the immunological impact of 2-HG on T-cells

2-HG produced by mIDH1 is exported into the tumor microenvironment and subsequently imported by infiltrating immune cells by sodium-dependent dicarboxylate transporters. In T-cells, it causes dysregulation of calcium signalling and reduces ATP production which in turn inhibits the activation of NFAT, diminishing TCR downstream signalling and inactivation of T-cells. The reduction in ATP signalling also inhibits polyamine biosynthesis that hurts T-cell proliferation, differentiation and metabolism *(Figure adapted from [36, 85])*

In addition to T-cells, previous work by my colleagues and I suggested an IDH genotype-dependent shaping of GAMs in human HGG towards an immunosuppressive phenotype. Integrated single cell profiling of GAMs in human HGG, showed that IDH mutant HGG derived GAMs displayed a less pronounced downregulation of the microglia homeostatic signature and less marked upregulation of the antigen presentation (AP) signature as compared to IDH wt HGG. Longitudinal analysis of immune infiltrates in an experimental glioma model revealed an enrichment of activated microglia in IDH wt glioma compared to an enrichment of steady-state microglia in IDH mutant glioma. Late stage IDH mutant tumors, harboured macrophages with an attenuated AP signature that could significantly increase suppression of T-cell in *ex vivo* co-cultures compared to IDH wt tumors. This immunosuppressive phenotype of macrophages is driven by 2-HG mediated dysregulation of tryptophan metabolism and subsequent activation of Aryl hydrocarbon receptor (AHR) and its target genes - a master regulator of immunosuppressive cytokines – IL10 , TGFβ and IL-1β [86].

Since 2-HG impairs tumor cell fitness, its hypothesized that the export of 2-HG is maybe a mechanism by which the tumor cell protects itself from the deleterious cell-intrinsic effects of excess intracellular accumulation. The immunosuppressive net with which it then traps immune cells in the TME gives the tumor an additional layer of protection from immunological insult. This creates an interesting dichotomy in IDH mutant gliomas, where they have a better prognosis despite the ability to escape immunological clipping.

1.3 Immunotherapy in Gliomas

Cancer immunotherapy hinges on the exploitation of the immune system in its natural ability to detect and destroy abnormal cells. Historically, the concept of cancer immunotherapy was ideated in the nineteenth century and more rigorously hypothesized in the twentieth century with the conception of the 'cancer immunosurveillance' hypothesis [87] and the detection of anti-tumor immune cell responses[88]. However, the advent of effective chemotherapeutics and radiotherapy as well as the elusiveness of a unifying mechanism prevented its clinical deployment. In the twenty-first century, with the identification of immunotherapeutic targets, the discovery of means of tumor immune escape and the unequivocal clinical evidence of the ability of T-cells to fight cancer, cancer immunotherapy has revolutionized cancer treatment by prolonging patient survival and has now transitioned to a first-line therapy for many cancers[89].

Immune checkpoint blockade (ICB) therapy using antibodies that target immune checkpoints, molecules such as programmed cell death 1 (PD1) and cytotoxic T lymphocyte antigen 4 (CTLA4) that regulate T-cell responses and prevent hyperactivation by driving T-cell exhaustion; have shown remarkable success in metastatic melanoma, non-small cell lung cancer, renal carcinomas and head and neck cancers. [90]. As a result, three classes of immune checkpoint inhibitors (ICI) have received FDA approval for the treatment of many cancer indications - including PD-1 inhibitors (Nivolumab, Pembrolizumab, and Cemiplimab), PDL-1 inhibitors (Atezolimumab, Durvalumab and Avelumab), and CTLA-4 inhibitor (Ipilimumab) [91]. However, not all patients benefit from ICI therapy (20-40% response rate) prompting the need to find effective biomarkers for predicting response. Gene expression analyses,

epigenomics and studies on the spatial organization of these highly heterogenous tumors have shed light and refined predictive biomarkers for response to ICI therapy [92-95]. Additionally many combination therapies with ICIs are being evaluated in clinical trials and have shown efficacy in preclinical models[96, 97]

Gliomas furnish additional challenges for immunotherapy design due to presented features that have led to the belief of them being immunologically 'cold' tumors– a low tumor mutational burden (TMB) with few immunogenic neoantigens[98], a generally low infiltration of T-cells and the release of immunosuppressive factors that can sequester T-cells into the bone marrow and dampen systemic immune responses[99, 100]. Therefore, the approaches, design and application of immunotherapy for gliomas cannot be transplanted one-to-one from other indications but need to be refined to address the challenges manifested by its immune landscape[101]. Immunotherapies that have been evaluated in the treatment of gliomas include ICIs, tumor associated antigen (TAA) and neoantigen targeting vaccines, oncolytic virotherapy, cytokine therapy as well as chimeric antigen receptor (CAR) based adoptive T-cell therapies with mixed outcomes[102].

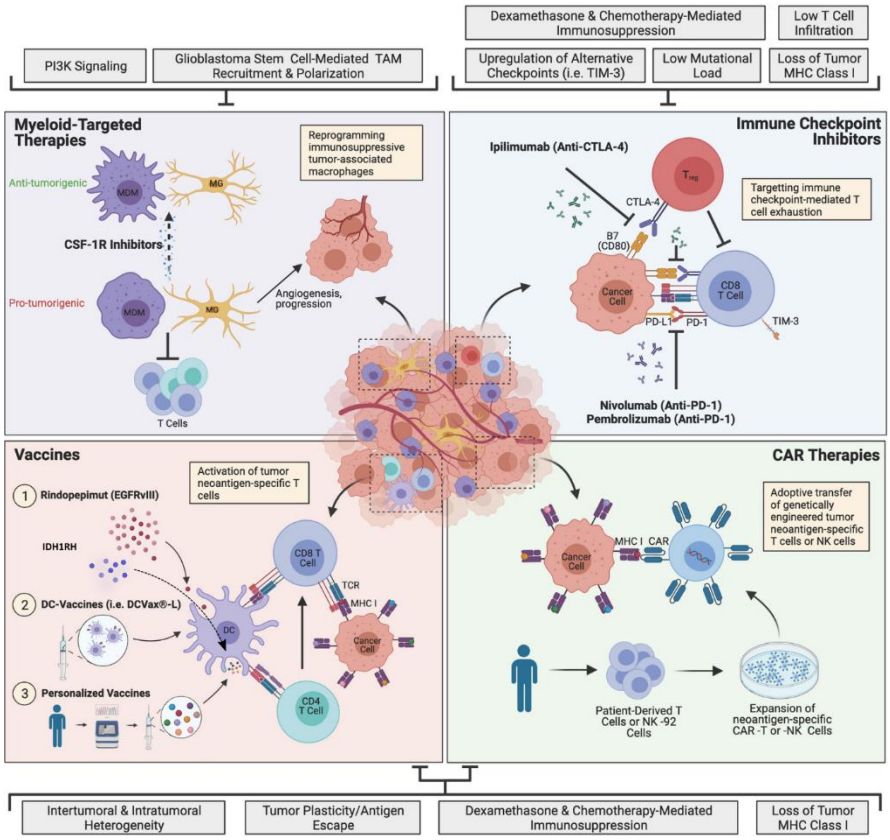

Figure 1.5: Major immunotherapies for gliomas and mechanisms of resistance

Schematic of the landscape of major immunotherapies for glioma and the known mechanisms of resistance. Peptide and dendritic cell (DC) vaccines aim to prime T-cells to neoantigens or tumor associated antigens. Immune checkpoint inhibitors block immune checkpoints such as PD1 and CTLA4 to counteract T-cell exhaustion and induce tumor reactivity. CSF1-R inhibitors are myeloid cell targeting therapies that aim to reprogram pro-tumorigenic and immunosuppressive microglia (MG) and monocyte-derived macrophages (MDMs). Chimeric antigen receptor (CAR) based T-cell therapies involves the adoptive transfer of genetically modified patient autologous T-cells harbouring a neoantigen-specific CAR. Gliomas orchestrate highly immunosuppressive tumor micro environments making them highly resistant to immunotherapies. The grey boxes indicate the major mechanisms of resistance to the respective immunotherapeutic approaches.

1.3.1 The *status quo* of immune checkpoint inhibitor therapy

The use of non-targeted immunotherapies such as ICB in unselected cohorts of patients has generally failed to improve clinical outcomes for gliomas. As an example, in the Checkmate 143 trial, there wasn't a significant survival benefit in nivolumab treated patients compared to bevacizumab treated control group[103]. In Checkmate 498 study, TMZ plus radiotherapy treated cohort showed longer overall survival than patients

treated with PD-1 blockade (plus radiotherapy)[104], and in a trial treating MGMT-methylated gliomas, nivolumab in combination with TMZ and radiotherapy wasn't better than placebo treated groups with chemoradiotherapy[105] However, in studies where PD1 blockade (pembrolizumab) was done in a neoadjuvant setting, for recurrent, surgically resectable tumors, improved overall survival was observed when compared to patients who received only post-surgical adjuvant therapy[106]. In these studies, markers of beneficial immune responses were noted. An early activated cytotoxic CD8 T-cell population was shown to traffic into the tumor which then produces progenitor-exhausted CD8 T-cells. Although promising, this study and another noted that immunosuppressive myeloid cells were still dominant in TME and expressed T-cell suppressive checkpoints which prevent effective T-cell mediated control of the tumor and provide a substantial disadvantage for the curative potential of ICB[107, 108]. Effective ICI therapy in glioma, therefore calls for not only the identification of patients that would meaningfully respond using prognostic biomarkers but also further investigations into the mechanisms of immunosuppressive interplay in the TME and the discovery of means that can make it a more immune-permissive milieu.

1.3.2 Anti-tumor vaccines

In addition to ICI therapy, targeted therapy in glioma has also been pre-clinically and clinically explored. One approach is vaccination to prime T-cells against specific glioma antigens. These could be peptides targeting mutation-derived neoantigens or overexpressed unmutated glioma associated antigens. The efficacy of Rindopepimut - an EGFRvIII targeting peptide vaccine was evaluated in a study of newly diagnosed GBM with validated EGFRvIII expression who had undergone resection and standard-of-care chemo-radio therapy. In vain, the trial had to be terminated due to no benefit in overall survival as compared to control group which received adjuvant TMZ. Interestingly, more than 50% of the patients showed loss of EGFRvIII expression[109]. Other ongoing trials are evaluating the targeting of Wilm's tumor 1(Wm1), Survivin among others as well as mutipeptide vaccine approaches[102]. One such example is the IMA950 trial where a multipeptide vaccine based on antigen expression patterns on the surface of GBM samples was utilized. Patients showed induction of both antigen specfic CD4 and CD8 T-

cell responses and the mean overall survival was 19 months. This potentiated the use of the vaccine in a combination with ICI which is ongoing[110].

Neoantigen vaccines offer an alternative to targeting tumor associated antigens and are potentially considered to be safer due to tumor specific expression and resulting immune targeting. In the NOA16 Phase I clinical trial, the efficacy of a peptide vaccine targeting IDH1RH mutation in IDH1RH-mutated gliomas was evaluated which is discussed in more detail in upcoming sections[111].

A personalized vaccine approach was explored in the pase 1 trail GAPVAC-101. Patients were treated with 2 vaccine combos - APVAC1 and APVAC2, where APVAC1 was based on a premanufactured warehouse of peptides targeting unmutated antigens, APVAC2 targeted neoantigens derived by profiling the patient's genome. Interestingly, APVAC1 elicited CD8 T-cell responses whereas APVAC2 induced CD4 T helper-1 responses against the predicted neoantigens[112]. Personalized vaccines are currently being clinically evaluated in combination with ICI. Outside of peptide vaccines, dendritic cell (DC) based anti-tumor vaccines have also been used in studies as a means of immunotherapy but have primarily not shown promising results[102].

The use of oncolytic viruses is another therapeutic option. Although not vaccines, biral therapy deserves a mention owing to some success in preclinical studies[113]. Such a therapy employs the use of oncolytic viruses that are selectively taken up by the immune system and can trigger an intense adaptive and innate immune response by activating antigen presentation. Although of low adaptability as of now in clinical glioma therapy with the indication of the intratumoral mode of delivery potentially more efficacious [13], the dual benefit of an initial cytotoxic response followed by the establishment of anti-tumor immunity holds promise for use of viral based therapies in the future.

1.3.3 mIDH1 as an immunotherapeutic target

Mutations in IDH1, specifically IDH1R132H (IDHRH) are immunogenic. Previous preclinical studies from my lab had shown that a peptide vaccine targeting IDH1RH could elicit effective anti-tumor response in IDH1RH-mutated tumor bearing A2DR1 mice[114] which are humanized to express human HLA molecules (HLA A2 and HLA DRB1, and knocked out for endogenous murine MHC molecules). This study showed that IDH1RH

is presented on HLADRB1 (MHC-II) and induces CD4 T helper-1 cell responses. Peptide vaccination resulted in abrogation of tumor growth of IDH1RH mutated tumor but not IDH1wt tumors and this therapeutic benefit was lost upond depletion of CD4 T-cells indicating the dependence of CD4 t-cell responses for anti-tumor immunity.

These findings complemented by the ubiquitous expression of IDH1RH in a large subset of gliomas prompted clinical translation in the form of a clinical trial. The resulting NOA16 clinical trial was a multicentre, single-arm, open-label, first-in-humans phase I trial where the safety, tolerability and immunogenicity of the IDH1RH-specific peptide vaccine were evaluated in newly diagnosed WHO (Grade 3 and 4) IDH1RH-mutated astrocytomas. [111] The patients enrolled in the trial were divided into three treatment groups – radiotherapy alone, three cyles of TMZ chemotherapy and combined radiochemotherapy. 32 patients were treated with the vaccine and of the 30 patients who were tested for immunogenicity, 93.3% showed IDH1RH-specifc immune responses (26 of 30 showed IDH1RH-specifc T-cell responses and 28 of 30 showed IDH1RH-specific B-cell responses). The 2 patients who did not moust and IDH1RH-specificimmune response showed progression within 2 years, whereas the patients with immune responses had a two year progression-free rate of 0.82 (95%CI CI 0.623–0.921). Pseudoprogressive disease (PsPD) is an imaging-diagnosed phenomenon in gliomas that's is indicative of inflammation and has prognostic benefit. On comparing the occurrence of PsPD in this study with induced immune responses, an association was observed between PsPD occurrence and onset of IDH1RH-specific responses, where PsPD patients had higher maximal levels of IDH1RH-specifc T-cell responses compared to patients with progressive disease. The success of this trial in meeting its endpoints of safety as well as immunogenicity prompted a follow-up trial – AMPLIFY-NEOVAC., which is a randomized, three-arm, window of opportunity trial to assess the safety, tolerability and immunogenicity of the IDH1RH-specific peptide vaccine in a neoaduvant and adjuvant setting in combination with an anti-PDL1 ICI (Avelumab) in patients with resectable IDH1RH-mutated recurrent astrocytoma and oligodendroglioma [NCT03893903][115]

1.3.4 Towards targeted T-cell therapies

The advent of cellular engineering technologies enabled the development of genetically modified T-cells are weaponized to target tumor specific antigens. Chimeric Antigen Receptors (CAR), are synthetic receptors designed to have an extracellular moiety mostly derived from antibodies with affinities to specific antigens that is linked, via and transmembrane and hinge domains, to intracellular signalling domains that induce T-cell activation. CAR T-cells, as a result, combine the flexibility of Antibody based antigen recognition and the direct cytotoxic ability of T-cells. The main target for which CAR T-cell therapies have been developed for glioma include interleukin (IL)-13 receptor IL13Rα2, Receptor tyrosine-protein kinase ErbB2/HER2 and EGFRvIII[116]. Each of these targets are found overexpressed in a significant proportion of gliomas. Although promising CAR T-cell therapies have not shown significant benefits in the treatment of gliomas [102]

TCR-transgenic T-cell therapies as compared to CAR T-cell therapies, have the advantage of usability in targeting intracellular antigens not expressed on the cell surface but presented by MHC molecules[156]. Additionally, TCR-transgenic T-cell therapy has the advantage of the low epitope density requirement for activation of TCRs compared to CAR T-cells, which is compounded by the higher avidity to their cognate antigens[157]. TCR-trasngenic cell therapies have been evaluated in a number of clinical trials especially targeting melanoma antigens. These therapies have exploited tumor-associated antigens that are overexpressed such as melanoma antigen recognized by T cells 1 (MART-1), and glycoprotein 100 (gp100) and melanoma-associated antigen (MAGE-A) among others. These studies showed some clinical responses but also described the development of several toxicities due to the low basal expression in healthy melanocytes and cross-reactivity with brain expressed depending on the target [156, 158, 159]. However, a newer affinity enhanced gp100 targeting TCR (Tebentafusp) has received clinical approval for a subset of unresectable metastatic melanoma due to overall improved survival [160]. Trial therapies targeting mutation-derive neoantigens include targeting mutated driver genes, such as TP53, KRAS, or PIK3CA, which have shown positive benefits in in vivo studies[161]. In the context of gliomas, several preclinical studies have reported promising results, offering another immunotherapeutic modality for glioma therapy[117, 118].

1.4 Aim of the Study

mIDH1 is a double-edged sword. On one hand, it is an immunogenic target that induces CD4 T-cell responses and is exploitable for glioma immunotherapy. On the other hand, it orchestrates potent immunosuppression by producing 2-HG that inhibits T-cell activation and signalling. IDH1 inhibitors have been shown to be able to overcome this immunosuppression but their exact impact on T-cell immunity hasn't been sufficiently explored. Investigations on the influence of IDH1 inhibition on T-cell transcriptomic states and induction of tumor-reactive phenotypes would shed light on its ability to serve as an agent for cancer immunotherapy

Additionally, the ability of the IDH1RH-specific peptide to induce mutation-specific T-cell responses in patients with IDH1-mutated gliomas paves the way for the development of TCR-transgenic adoptive cell therapies for IDH1-mutated gliomas. This would, however, first require the identification of mutation-reactive TCRs and the development of pipelines to validate them.

A syngeneic immunocompetent glioma model that can effectively present mIDH1 to the immune system doesn't exist. The development of such a model would allow the study of induced anti-tumor immune responses under the influence of .mIDH1 when presented in its native capacity as an immunogen on human MHC-II molecules. Such a tumor model would also serve as an ideal preclinical model to test the efficacy of immunotherapies targeting mIDH1.

To answer these questions, this PhD thesis had the following aims:

1. Development of a syngeneic mIDH1 glioma model in MHC-humanized A2DR1 mice
2. Investigation of the impact of IDH1i on the activation, transcriptional states and intercellular communication of tumor infiltrating T-cells using single cell RNA(scRNA) and single cell VDJ(sc-VDJ) sequencing.
3. Assessing the therapeutic benefit of IDH1i for mIDH1 A2DR1 gliomas as a combinatorial immunotherapy

4. Assessing the suitability of an NFAT based reporter assay for testing TCR reactivity against MHC-II presented epitopes
5. Developing a proof of concept for the identification and validation of mIDH1 reactive TCRs from IDH1RH peptide vaccinated A2DR1 mice and IDH1-mutated glioma patients

Approaches to address the immunogenicity and immunosuppressive features of Isocitrate Dehydrogenase 1 mutated gliomas

2 RESULTS

2-HG promulgates an intense immunosuppressive pressure on TILs in IDH mutant glioma mico environments leading to T-cell inactivation as has been described above. Inhibition of IDH can rescue this T-cell anergy and has been topically explored in various studies. However, the exact nature of the impact of 2HG inhibition on T-cell phenotypes and the transcriptional profiles needs to be deciphered. mIDH1 as a neoepitope is presented on human MHC-II (HLA-DR) alleles. Its presentation by murine MHC-II alleles has not been validated. A syngeneic murine glioma model that can effectively present mIDH1 and induce mIDH1-reactive T-cell response is, therefore. To address these questions, this study has been divided into 3 parts. **Part 1**, describes the generation of a syngeneic MHC-humanized mIDH1 glioma model. **Part 2**, explores the impact of IDH1 inhibition on T-cell states in the TME using single-cell transcriptomic profiling and its therapeutic benefit in this experimental model.

Additionally, the knowledge that mIDH1 induces antigen-specific CD4+ T-cell responses can be exploited for the development of adoptive T-cell therapies. **Part 3** of this work focuses on a proof of concept for the identification and validation of mIDH1-reactive TCRs as a first step in the development of a TCR-transgenic T-cell therapy for mIDH1 gliomas

2.1 Development of a syngeneic mIDH1 glioma model in MHC-humanized mice

Previous studies that demonstrated the substantial immunomodulatory influence of mDIH1 gliomas employed the use of mIDH1 GL261 tumors (C57BL/6 syngeneic) [36, 86]. These models do not provide for the immunogenic potential of mIDH1 that is presented as a neoepitope on human HLA DR molecules.

A2DR1 mice are a genetically modified strain of laboratory mice which have been knocked out for the expression of murine MHC molecules and have human HLA A2 (MHC-I) and HLA DRB1 (MHC-II) knocked in. These mice represent a unique in vivo model where T cell priming and antigen recognition can mimic the human setting allowing the investigation of adaptive immune responses to human neoantigens [119]. Previous work has shown that vaccination with IDH1RH peptide can induce CD4+ T-cell responses and has a therapeutic benefit against mIDH1 tumors in A2DR1 mice [120]. This study however has the limitation that it employed flank tumors that cannot recapitulate the special immunological niche within the brain. This prompted me to develop a syngeneic glioma model in A2DR1 mice as a means to study the immune response in a human physiologically relevant manner where mIDH1 can serve as a potent immunogen.

To generate gliomas in A2DR1 mice, a CRISPR-Cas9 based triple gene knockout approach was utilized as previously described [121]. A schematic of the workflow is show in **Figure 2.1A**. A2DR1 P0 pups were electroporated with guide RNAs to knock out tumor suppressor genes PTEN, p53 and NF1. Success of electroporation was monitored by in vivo bioluminescent imaging (facilitated by the concomitant electroporation of a luciferase encoding plasmid and tumor development was monitored by magnetic resonance imaging (MRI) (data not shown). Between 90-120 days when mice showed signs of neurological deficits, tumors were excised, processed and passaged through NSG mice by subcutaneous flank injections. Tumors were then excised and single cell suspensions were cultured to derive cell lines. *(This part of the workflow was done in collaboration with Michael Kilian and has been published in a report where I am a co-author[117]).*

Tumors obtained from 2 of these electroporated A2DR1 pups were able to sustain tumor cell lines *in vitro.* The ability of these lines to induce brain tumors was ascertained by injecting them intracranially into adult A2DR1 mice **(Figure 2.1B).** Both cell lines were able to induce tumors as seen by MRI. A2DR1 glioma cell line M5 was chosen for further experiments based on growth dynamics.

mIDH1 or IDH1wt overexpression was introduced retrovirally and validated by Western blot and 2-HG measurement **(Figure 2.1C, D).** mIDH1 A2DR1 glioma showed an accumulation of intracellular 2-HG which was absent in IDH1wt overexpressing line or cognate parental M5 A2DR1 glioma. Longitudinal measurement in cell culture supernatant also showed that the lines could export 2-HG which increasingly accumulates over time.

Post genetic modification, the cell lines were re-tested for their ability to induce intracranial tumors as described above **(Figure 2.1E)**. No differences, however, were observed in growth dynamics between mIDH1 and IDH1wt A2DR1 glioma *in vivo* (data not shown). Immunofluorescence (IF) staining of tumor sections revealed infiltration of both CD4 and CD8 T-cells, however, an overall lower infiltration in mIDH1 A2DR1 glioma as compared to IDH1wt was observed **(Figure 2.1F).** This is in line with previous reports which have shown that human mIDH1 gliomas have decreased immune T-cell infiltration compared to wildtype counterparts [80, 81]. Overall these results provide a novel syngeneic experimental glioma model with immune infiltration that can been exploited for studying adaptive immune responses to tumor in a human MHC context.

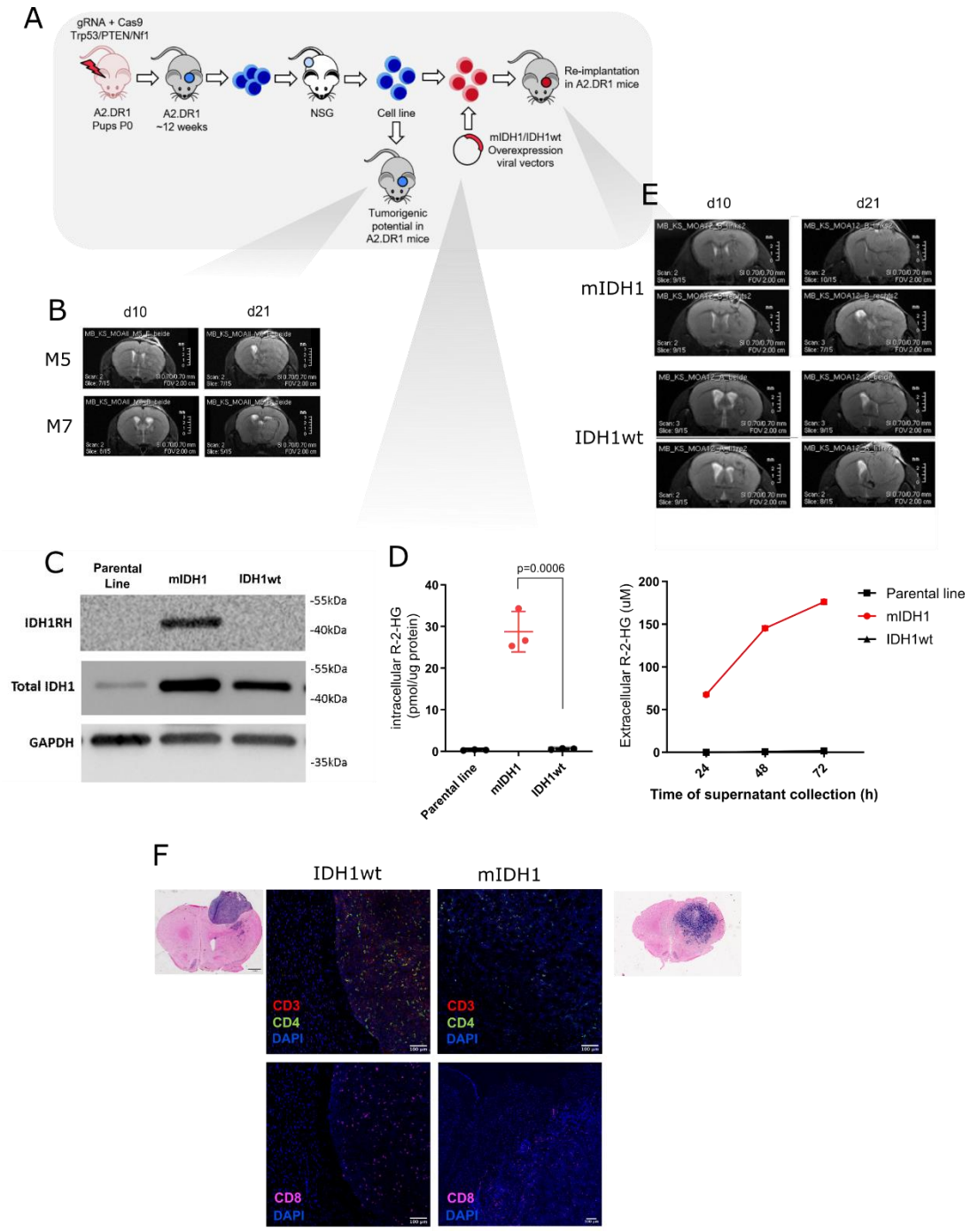

Figure 2.1: A novel syngeneic mIDH1 glioma model in A2DR1 mice.

(A) Workflow schematic or the development of the glioma model. **(B)** Representative MRI of M5 and M7 A2DR1 glioma line on d10 and 21 post tumor inoculation. **(C)** Western blot showing mIDH1 overexpression using an IDH1RH specific antibody. An antibody detecting total IDH1 was used for comparison. GAPDH was used as loading control. **(D)** *In vitro* intracellular 2-HG measurements of cell pellets 48h post culture (left) and longitudinal measurements of secreted 2-HG in the cell culture supernatant. Data plotted as mean ± SD. The statistical significance was assessed using two-tailed student's t test. **(E)** Example MRI of mIDH1 and IDH1wt A2DR1 gliomas orthotopically injected into the brain of adult A2DR1 mice. **(F)** Representative immunofluorescense images of glioma sections from (E). DAPI was used to stain nuclei.

(Part 2)

2.2 Impact of IDH1 inhibition on glioma infiltrating T-cells and its therapeutic benefit in the A2DR1 glioma model

The import of 2-HG by immune cells in the TME leads to the inhibition of T-cell proliferation and activity and the conditioning of myeloid cells to a more immunosuppressive phenotype. This broad-scale immunosuppression is probably the reason why immunotherapies such as immune checkpoint blockade (ICB) have not shown as promising results for glioma treatment as for many other solid tumors. IDH1 inhibitors have been shown to reduce levels of 2-HG accumulation, potentially alleviating the immunosuppressive pressure exerted by mIDH1. IDH1i inhibitors function by blocking the activity of the enzyme. The concentration of inhibitor needed to block mIDH1 is much less than that needed to block IDH1wt activity, avoiding potential cellular toxicity due to the inhibition of a key Krebs cycle enzyme. In a previous study, the survival benefit of BAY146032 IDH1i treatment was assessed in C57B6/J mice bearing syngeneic mIDH1 GL261 orthotopic gliomas[36]. Here IDH1i treatment alone did not increase survival but provided a survival benefit when used in combination with ICB (anti-PD1).

The study, however, did not explore the phenotypic state of TILs upon treatment. Based on these findings, I was interested in evaluating in detail the activation state of T-cells infiltrating the tumor upon IDH1i treatment and whether it can boost T-cell funtion without the aid of additional immunotherapy. BAY146032 IDH1 inhibitor was chosen for this study based on pre-clinical efficacy of this inhibitor in controlling tumors and its current use in clinical trials. The mIDH1 A2DR1 glioma developed as part of this study, provided a suitable experimental model. It had the added benefit of HLA-DRB1 based presentation of mIDH1 as an immunogen which could further improve T-cell responses in an immune permissive TME.

2.2.1 Investigation of T-cell abundance and permissiveness of T-cell activity on IDH1 inhibtion

As a first step I was interested in comparing the difference in T-cell states upon IDH1i treatment to ICB, and whether ICB can at all activate T-cells in such an immunosuppressive TME. Combined single cell RNA (scRNAseq) and VDJ (scVDJseq)

was employed to investigate the transcriptomic profile of tumor infiltrating T-cells. A schematic for the workflow is shown in Figure **2.2**

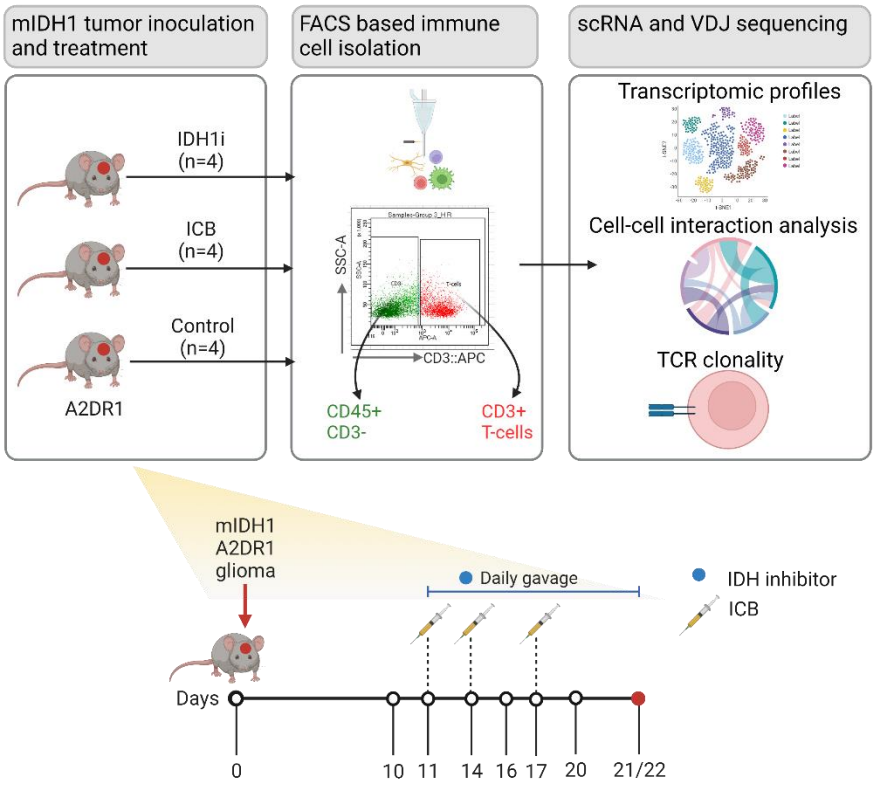

Figure 2.2: Schematic workflow to assess the impact of IDH1 inhibition on TILs in mIDH1 A2DR1 glioma

mIDH1 gliomas were orthotopically injected into the brain of adult A2DR1 mice and were treated with either oral IDH1i, i.p. ICB injections (aPD1+aPDL1+aCTLA4) or as control isotype antibodies and vehicle gavage for 22 days as shown in schematic. Tumor development was validated by MRI. TILs were isolated by Fluorescence activated cell sorting using CD45 and CD3-specific antibodies to separate T-cells from rest of the TILs. Immune cell fractions were then subjected to 10X based single cell RNA and VDJ sequencing. Cells were stained with cell hashing antibodies before FACS to facilitate multiplexing for sequencing workflows.

Mice bearing orthotopically injected mIDH1 A2DR1 gliomas in the brain were treated with IDH1 inhibitor BAY146032 by oral gavage. Immune checkpoint blockade (ICB) was used as a comparative immunotherapy. Owing to the highly immunosuppressive nature of mIDH1gliomas, a triple ICB was used with *in vivo* compatible blocking antibodies against PD1, PDL1 and CTLA4. TILs were isolated from the tumors via FACS. Leukocytes

were identified as CD45+ and CD3+ T-cells were further separated to enrich them owing to relatively low numbers of infiltrating T-cells. The two cell fractions were then subjected to scRNAseq and scVDJseq using the 10x platform. scRNAseq would provide a map of the transcriptomic profiles in the TILs while scVDJseq allowed the inference of TCR clonality and its clonal distribution with the T-cell subtypes.

Seurat analysis of scRNAseq of glioma infiltrating T-cells revealed several transcriptionally distinct clusters corresponding to different celltype and state (**Figure 2.3A**). Cluster identification was educated by cell phenotype defining markers as well as conventionally known markers for T-cell activation, exhaustion and cytotoxicity [122, 123](**Supplementary Figure 8.1**). The cytotoxic signature was defined by the average expression of Granzyme A (Gzma), Granzyme B (Gzmb) and Perforin 1(Prf1); the exhaustion signature by PD1, HAVCR2 and lymphocyte activation gene 3 (Lag3). Naïve T-cells were identified by the expression of lymphocyte enhancer binding factor 1 (Lef1), selectin L (Sell), transcription factor 7 (TCF7) and C-C-C chemokine receptor 7 (CCR7) CD40L expression was used to ascertain CD4 T-cell activation (**Figure 2.3C**). Interestingly, a cluster defined by the expression of Type I Interferon stimulated genes (ISG) such as Irf7, Usp18, Ifitm3, Ifitm1, Oasl1 and Isg20 was also identified. This cluster contained both CD4 and CD8 T-cells.

IDH1 inhibition resulted in an overall increased abundance of tumor infiltrating CD4 and CD8 T-cells compared to ICB or control (**Figure 2.3B**.). On comparing between treatments, a significant increase in the frequency of cytotoxic CD8 T-cells was observed on IDH1i compared to control. Similarly, IDH1i increased the abundance of transitional and exhausted CD4 T-cells and resulted in a reduced frequency of Tregs (**Figure 2.3D**). Exhaustion markers such as PD1 are indicators of tumor reactivity [124, 125], therefore, the exhausted CD4 T-cells in this dataset indicate a population of tumor reactive T-cells that are markedly increased on IDH1 inhibition. ISG T-cells were also more frequent in IDH1i TILs.

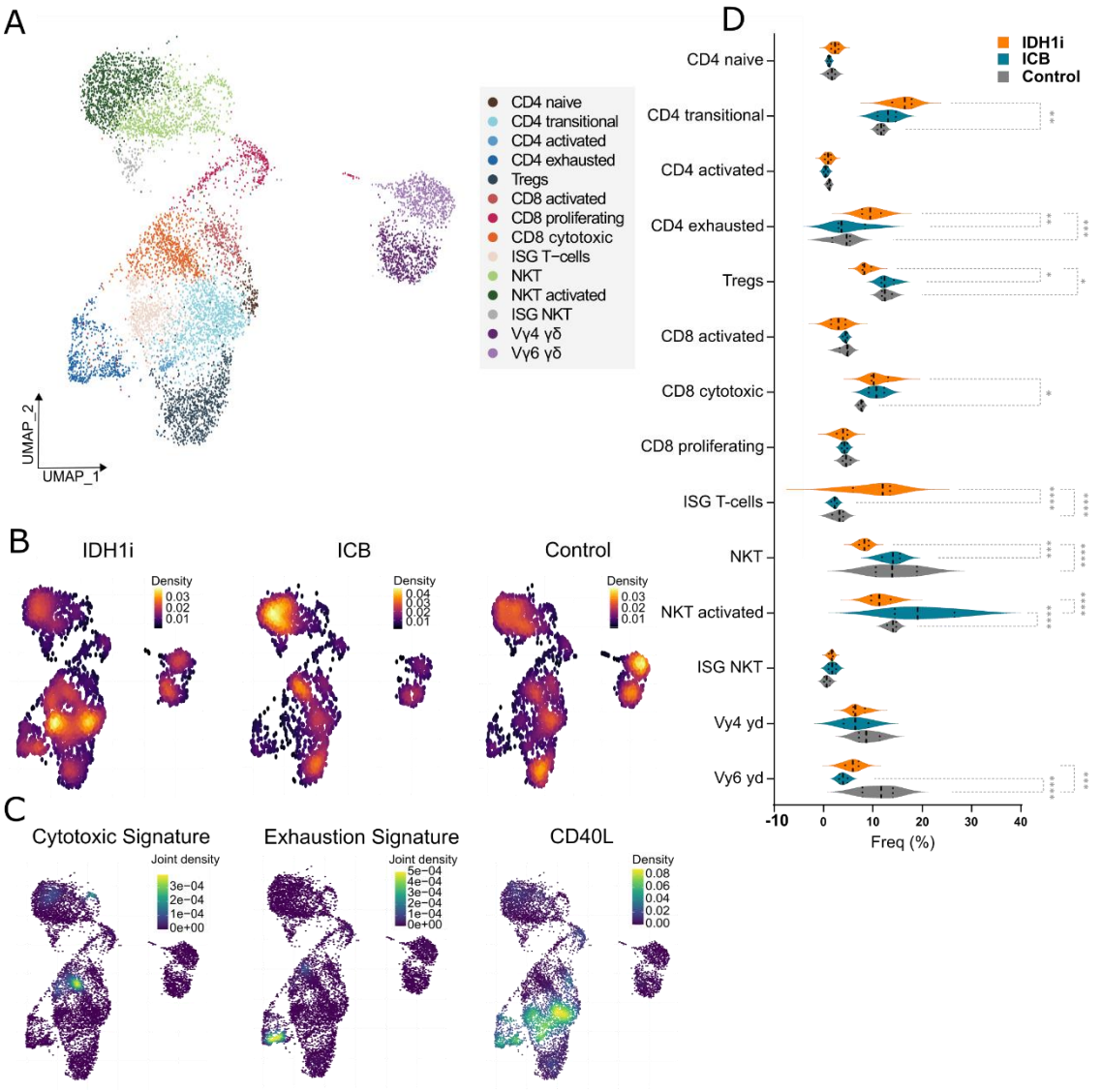

Figure 2.3:IDH1i leads to an increase in abundance of putative tumor reactive T-cells in mIDH1 A2DR1 glioma.

(A) Uniform manifold approximation and projection (UMAP) plot of molecular clusters of T-cells colour coded for cell types based on the expression of cell type-enriched gene signatures (NKT, Natural Killer-like T cells; γδ, gamma delta (gd) T-cells. **(B)** Density plot showing cellular abundance within clusters as defined in (A) in individual treatments. **(C)** Expression of cytotoxic and exhaustion gene signature as well as expression of CD40L as a marker for CD4 T-cell activation within clusters as defined in (A). **(D)** Violin plot comparing the frequency of cells within each cluster as defined in (A) between treatments. The statistical significance was assessed using one-way ANOVA followed by Tukey's test (****:p<0.0001; ***: p<0.001; **: p<0.01; *:p<0.05)

Taken together, these results validate the establishment of an immune permissive TME on the inhibition of IDH1. Interestingly, the phenotypic distribution of T-cells in ICB treated mice was similar to Control treatment. Levels of cytotoxic T-cells was higher than

in Control treatment and comparable to IDH1i but ICB failed to induce infiltration and activation of CD4 T-cells possibly due to the strong immunosuppression by 2-HG as a consequence of IDH1 mutation. Additionally, significantly higher levels of activated NKT cells were found in ICB treated mice.

2.2.2 Impact of IDH1 Inhibition on TCR Clonality and Distribution

scVDJ sequencing was employed to identify the TCR sequences of tumor infiltrating T-cells. The proportion of the top 5, as well as top 10 TCR clonotypes among all identified clonotypes within each treatment, was higher on IDH1i compared to Control as well as ICB indicating T-cell expansion.

Combining scRNA sequencing with scVDJ sequencing allowed the mapping of the TCR identity of a T-cell to its transcriptomic identity. Looking at the broad distribution of TCR clonotypes based on their clonal proportion showed that large clonotypes were mostly found within cytotoxic CD8 and exhausted CD4 T-cells. Taking a closer look at the distribution of the top 20 clonotypes from each treatment within the different transcriptomic clusters showed that in IDHi treated mice, there was a greater proportion of top clonotypes within CD4 T-cells, mainly within exhausted CD4 T cells. Whereas, in ICB treated mice, most top clonotypes were found within cytotoxic CD8 T-cells. Conversely, in control treated mice, many of the top clonotypes were found within Tregs indicating their strong immunosuppressive presence in untreated mIDH1 gliomas. Clonotypes that were prominent in Tregs were different than the clonotypes prominent in exhausted CD4 T-cells. In IDH1i and ICB treated mice none of the top 20 clonotypes were present within Tregs indicating that the immunotherapies were able to blunt the expansion of immunosuppressive Tregs in the TME.

Metrics such as Shannon's entropy index reveal the diversity of TCR within each sample population. It takes into account both the sample richness and the degree of unevenness in the frequencies of CDR3 aa sequences. IDH1i promulgated a greater diversity of TCRs compared to control treatment in tumor infiltrating T cells prompted by its immune permissive benefit. Interestingly ICB treatment also resulted in a high TCR diversity owing to a comparable high abundance of cytotoxic CD8 T-cells. These findings show that IDH1i facilitates the expansion of tumor reactive CD4 T-cells

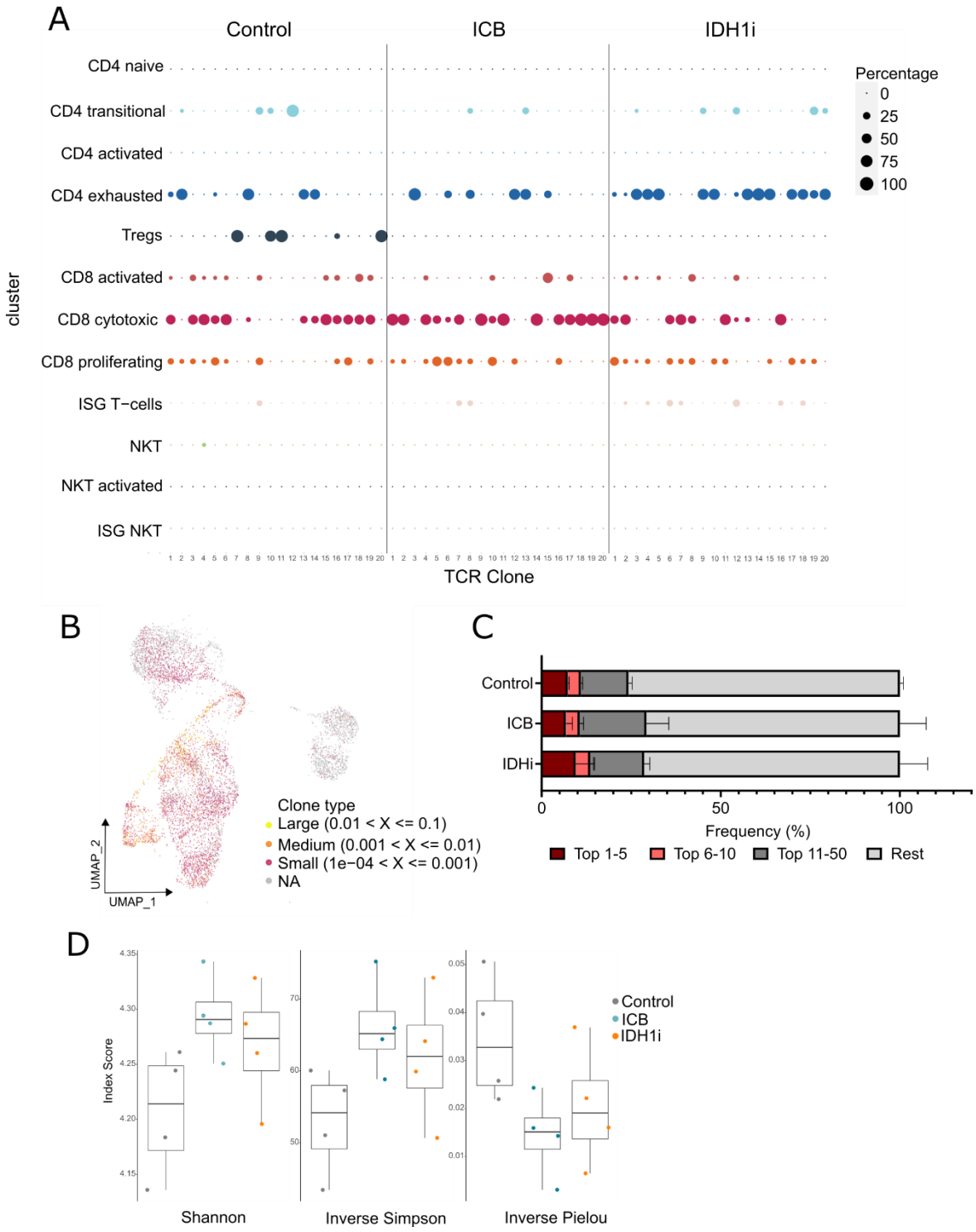

Figure 2.4: TCR analysis indicates an expansion of inferred CD4+ tumor reactive T-cells on IDH1 inhibition

(A) Bubble plot mapping of top 20 TCR clonotypes defined by scVDJ sequencing onto each transcriptomic cluster defined in Figure 23 compared between treatments. **(B)** UMAP plot showing the color coded distribution of TCR clonotypes based on their clonal proportion within T-cell clusters. **(C)**TCR clonality as a measure of the frequency of TCR clone groups among all TCRs within a mouse sample compared between treatments. Data are plotted as mean ± SD. **(D)** Diversity metrics for TCRs from each sample

compared between treatments. For Shannon's diversity index and Inverse Simpson index, a higher index score means greater diversity, whereas for the Inverse Pielou index higher score means less diversity.

TCRs with a high sequence similarity can potentially indicate reactivity to similar antigenic sequences. A TCR superclustering analysis using the *tcrdist3* software package[126] was hence performed. This tool compares biochemical similarity of TCRs to form groups - that can allow quantification of functionally similar TCRs across individuals. To this end, TCRs were grouped based on the sequence similarity of their TCR beta (TCRb) chains yielding a map of superclusters as a result (**Figure 2.5A**). The distribution of the TCRs within a TCRb supercluster based on their transcriptomic identity was then analysed to identify whether there is an enrichment of specific T-cell clusters within a TCRb supercluster. As a TCRb supertype could be composed of TCRs from different treatment groups due to their sequence similarity, this distribution was then compared between treatments (**Figure 2.5B**). TCRb superclusters were either enriched for specific transcriptomic T cell clusters or were composed of T-cells from several different T-cell clusters. TCRb superclusters that had an enrichment were either composed mainly of cytotxic CD8 T-cells or exhausted CD4 T-cells . This enrichment indicates that there are groups of TCRs within these T-cell clusters that show a high degree of sequence similarity hinting towards clonal expansion within these T-cell clusters. Interestingly, on comparing treatment groups, most superclusters that are composed mainly of TCRs from IDH1i treated mice were enriched for exhausted CD4 T-cells (Supercluster 2, 28, 36, and 45), with a few enriched for cytotoxic CD8 T-cells (Supercluster 11 and 57). This further indicates that IDH1i treatment facilitates the tumor reactivity of T-cells, and these putative reactive TCR clonotypes then expand.

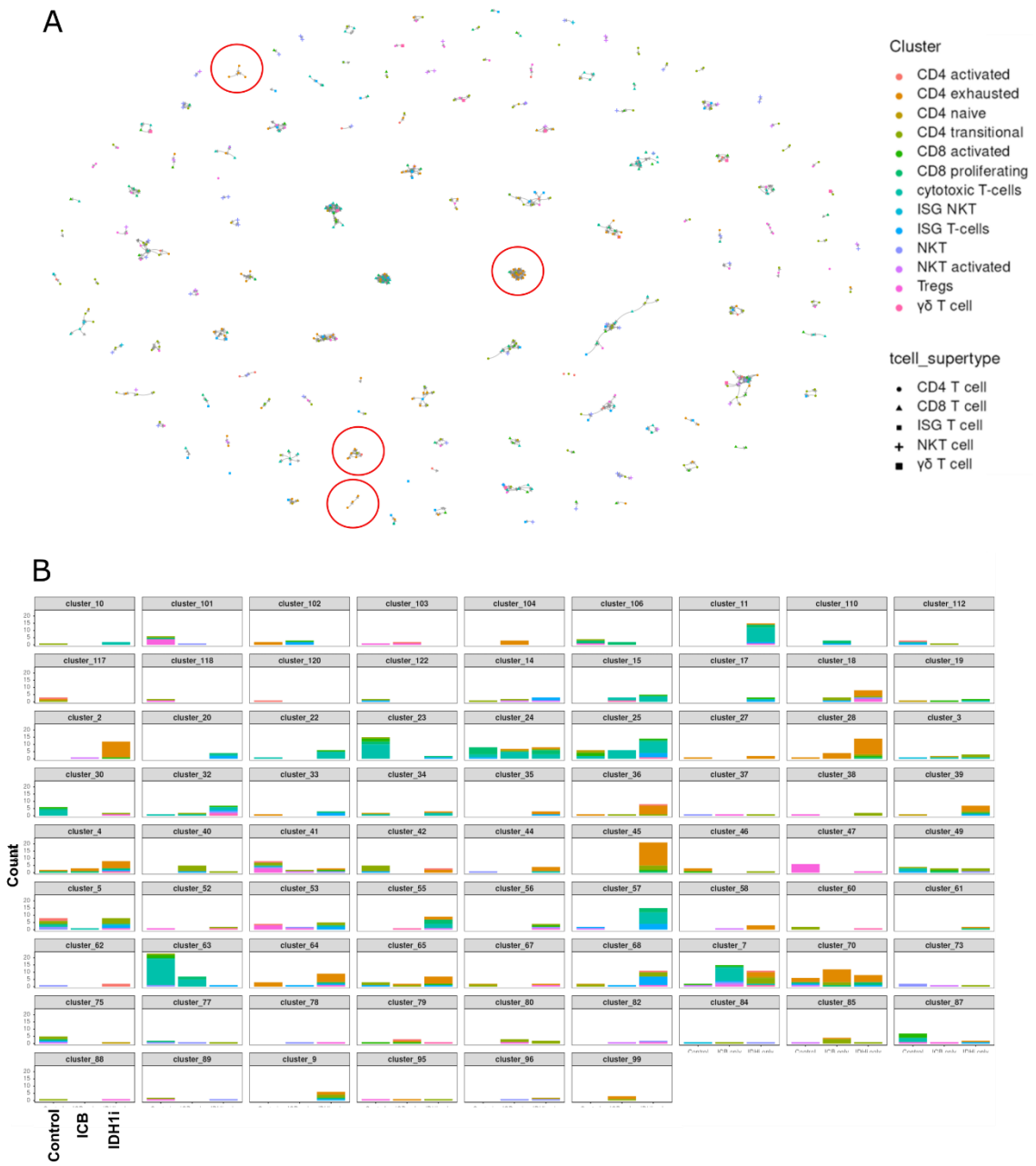

Figure 2.5: TCRbeta superclusters overrepresented in IDHi treated mice are mostly composed of exhausted CD4 T-cells

(A) Map of TCR beta (TCRb) superclusters obtained by grouping TCRs based on the sequence similarity of their TCRb chains. TCRs within each supercluster are color coded based on the transcriptomic identity of the T-cell. Superclusters enriched for exhausted CD4 T-cells are circled. **(B)** Distribution of TCRs composed within each supercluster between treatments. Each stacked column within a supercluster represents the count of TCRs belonging to that supercluster found in the respective treatment group. The stacking within a column represents the distribution of the TCRs of that supertype based on their transcriptomic identity.

2.2.3 Intercellular communication on IDH1 inhibition

Intercellular communication is integral for the induction and modulation of tumor-reactive immune responses. Chemokine signalling recruits T-cells into the TME, where antigen-presenting myeloid cells prime and activate T-cells against tumor antigens. Resulting cytokine secretion further leads to the activation of T-cells and other innate immune cells and drives T-cell expansion. At the same time, the modulation by the tumor cells of myeloid cells to immunosuppressive phenotypes can lead to T-cell tolerance. Therefore, an intercellular communication evaluation can give insight into the most relevant and activated signalling pathways in a given context and the interacting partners that drive that signalling. Here, the aim was to investigate which signalling pathways are the most affected upon IDH1i treatment and identify the differential network of T-cell interactions in the TME.

Receptor-ligand analysis is a powerful bioinformatic tool to study communication between different cell types within a sample set. To infer intercellular interactions, these analyses take into account the gene expression of known receptors and ligands by combining information on the number and combination of such molecules expressed and the degree of expression of these molecules relative to each other. The publicly available CellChatDB algorithm was utilized for such analyses

To enable the exploration of cell-cell interactions between tumor infiltrating myeloid cells (brain resident and circulating) and T-cells and other infiltrating lymphocytes, the scRNA seq data from the FACS sorted CD45+CD3+ (T-cells) and CD45+CD3–(others) was integrated. Seurat informed molecular clustering of cells was done again and cell types were inferred based on the expression of cell type defining canonical markers. Clustering analysis identified transcriptionally distinct clusters of microglia, monocyte/macrophages (Mo/Mϕ), Dendritic cells (DCs), NK cells and of course T-cells, and the presence of B-cells and granulocytes (**Figure 2.6A)**. Since the data was integrated from 2 -separate FACS sorted populations, inference on differences in relative abundance between cell types would be misleading. However, inference on the relative abundance between different treatment groups revealed the increased abundance of T-cells as expected and, interestingly, a decreased presence of a sub-population of macrophages (Mo/Mϕ1) on IDH1i treatment **Figure 2.6B).**

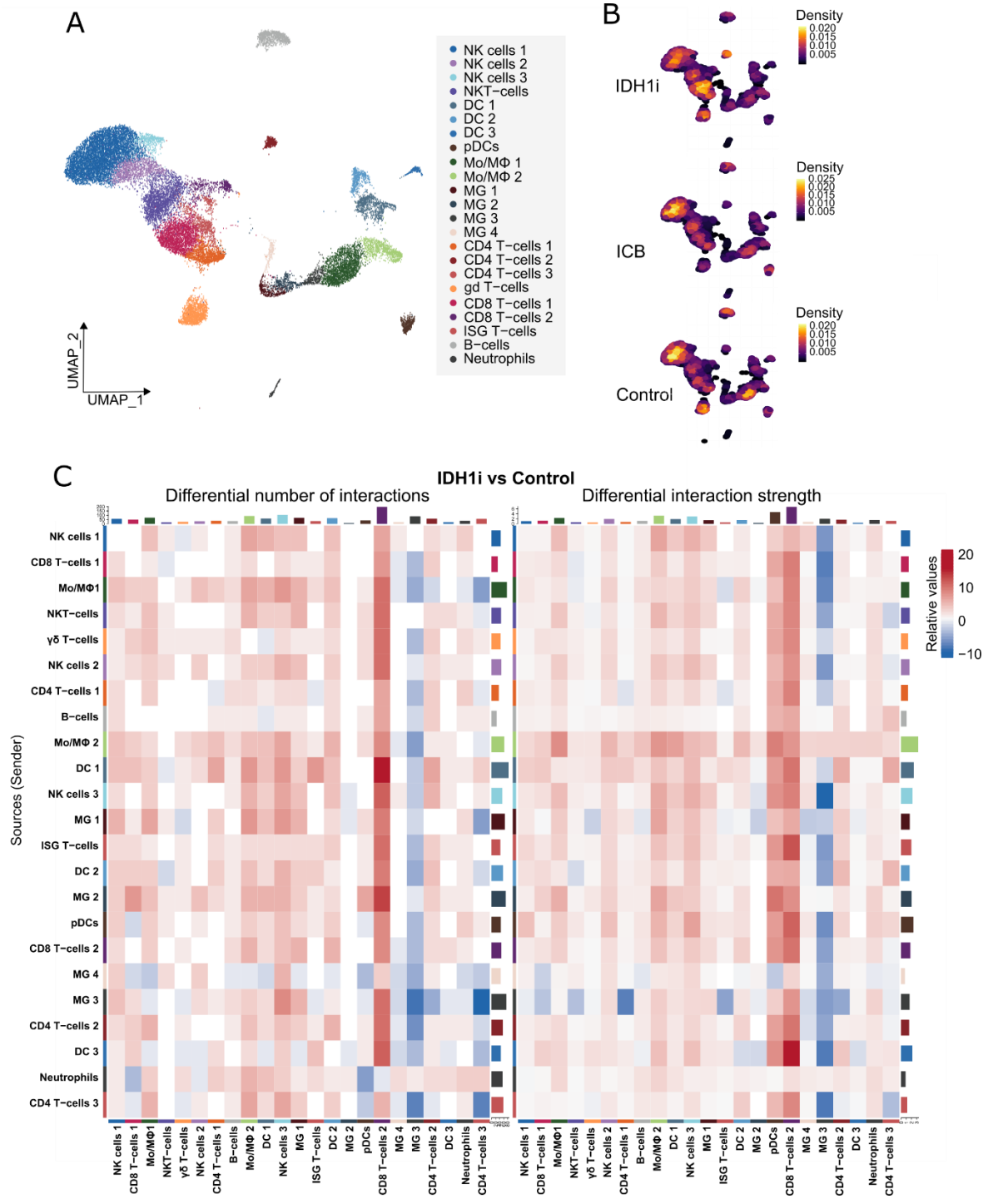

Figure 2.6: IDH1i boosts intercellular communication among immune subsets

(A) Integrated UMAP of CD45+CD3+ and CD45+CD3- TILs from mIDH1+ A2DR1 glioma depicting colour coded molecular clusters based on the clustering output of the Seurat algorithm. Cell type assignment was conducted on the basis of the expression of cell type-enriched gene signatures. **(B)** Density plot showing cellular abundance within clusters as defined in (A) in individual treatments. **(C)** CellChatDB derived heatmap visualizing the bioinformatically inferred cell-cell. communication between clusters in IDH1i relative to control treatment. The left side displays the inferred number of differential interactions based on the number of known receptors/ligands expressed

within clusters. The right side displays inferred interaction strength between clusters based on the level of expression of interacting partners.

Receptor ligand analysis revealed an overall increased intercellular communication on IDH1i treatment compared to control **(Figure 2.6C)** counterparts and ICB (**Supplementary Figure 5.2)**. Both the inferred differential number of interactions and their strength were heightened. Interactions targeting CD8 T-cells 2 showed the most up-regulation, with heightened signalling from almost all other cell-types. Contrary to expectation, the same was not true for CD4 T-cells . However, this analysis was a summary of overall interactions and, considering that PD-1+ CD4 T-cells were more frequent upon IDH1 inhibition, prompted a closer look at specific differentially regulated pathways. Since an increased intercellular communication wasn't inferred in ICB compared to control counterparts (Supplementary Figure 5.2), I focused only on IDH1i treated and control groups for further analyses.

Evaluating the overall participation of different cell types in multiple signalling pathways, revealed a global intensification of intercellular signalling on IDH1i treatment **(Figure 2.7A).** A method for bioinformatically evaluating the strength of signalling in a pathway relative to another is an information flow analysis, which infers the top regulated pathways based on overall information flow in a signalling pathway – a measure of the perceived strength of cumulative signalling taking all interacting cell types into account. This analysis revealed that the top upregulated pathways were associated with immune cell activation and included signalling axes involved in immune cell recruitment (CXCL), T-cell activation (LCK (indicative of TCR signalling), CD40, CD86, MHC-II) as well as T-cell exhaustion (PDL1), with the latter a marker for tumor-reactivity **(**Figure 2.7**B)** . A deeper look was then taken into the network of intercellular interactions in signalling pathways relevant to T-cell activation and effector response **(Figure 2.7C).** All 4 pathways evaluated (Type-II Interferon signalling, TNFa, CD40L and PDL1 signalling pathways) displayed a global intensification of intercellular communication network activity. Additionally, dependent on pathway IDH1i treatment induced active signalling in cell types not initially participating (or signalling was dampened) in control treated mice. Interestingly the CD40L pathway interaction network identified the new involvement of

CD4 T-cells 3 sub-population which along with CD4 T-cells 2 corresponds to the CD4-exhausted cluster (PD1+) from the T-cell specific clustering analysis.

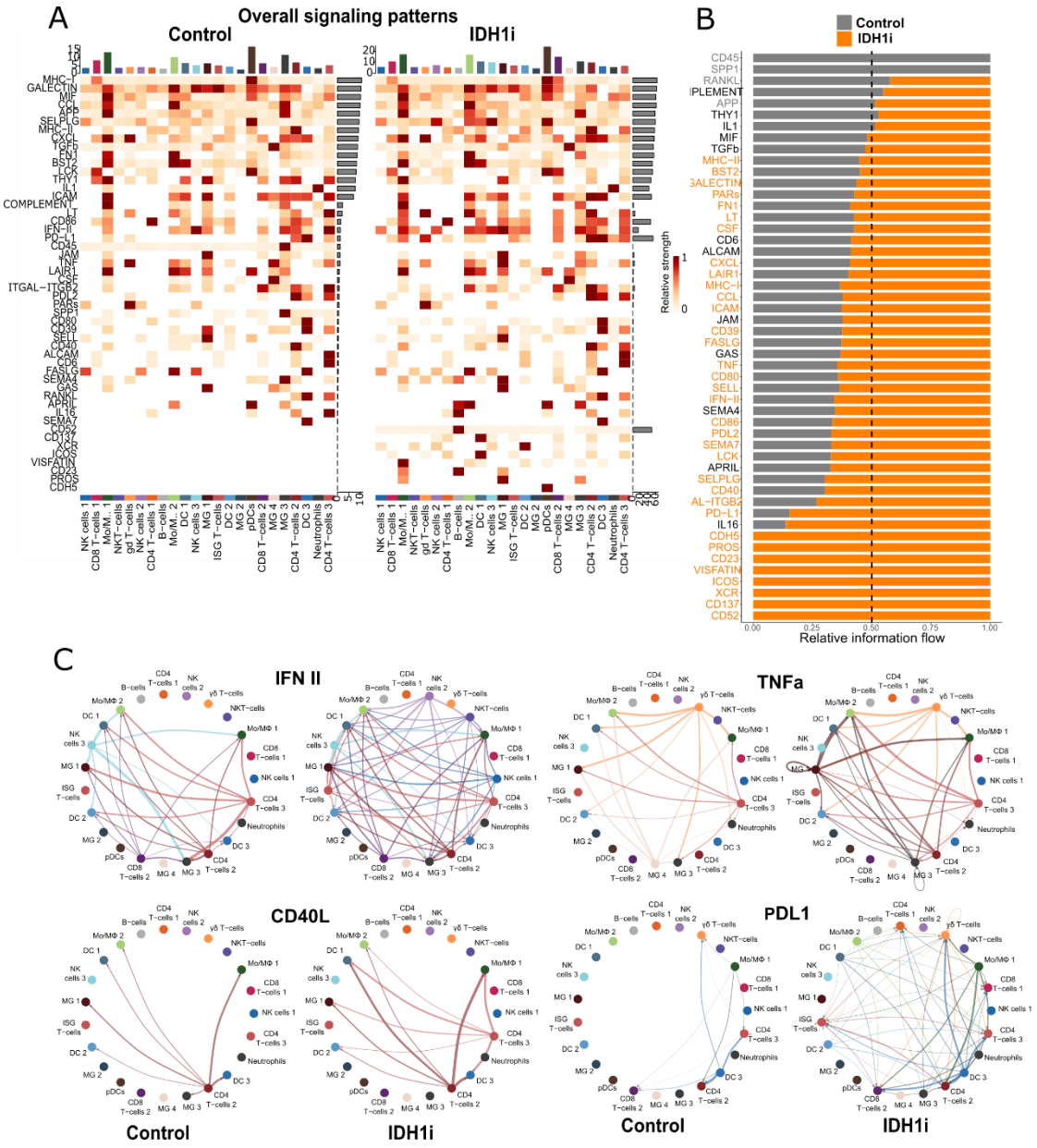

Figure 2.7: IDH1i facilitates functional T-cell interactions

(A) Heatmap visualizing the overall strength of signalling patterns in relevant signalling pathways in each cell cluster on IDH1i compared to control treatment. **(B)**Information flow chart ranking top regulated pathways based on differences in overall information flow within the inferred signalling networks between IDH1i and control. The bottom signalling pathways coloured in orange were more enriched in IDH1i. **(C)** Circle plots visualising the cell communication network between cell clusters mediated by type II Interferon (IFN II), TNFa, CD40L and PDL1 signaling pathways. The size of each circle is proportional to the number of cells in that given cluster. The thickness of arrows is

proportional to the strength of interaction between interacting cell types mediated by the corresponding signalling pathway.

Overall, these analyses reveal that IDH1 inhibition ameliorates the dampening of T-cell signalling as induced by 2-HG and permits activation of T-cells as well as their tumor reactivity as inferred by an increased signalling in the PDL1 axis and the increased frequency of and clonality of tumor infiltrating PD1+ T-cells.

2.2.4 The therapeutic benefit of IDH1 inhibition and in combination with ICB in mIDH1 A2DR1 gliomas

Based on the above described findings that IDH1i can indeed induce tumor reactive T-cells, I was interested in assessing its therapeutic benefit in mIDH1 A2DR1 gliomas. The observations, that even though ICB doesn't promote CD4 T-cell response, it induces infiltration of cytotoxic T-cells allowed me to hypothesize that combination therapy of ICB and IDH1i may have synergistic therapeutic benefit. To this aim, mIDH1 A2DR1 gliomas were orthotopically injected into A2DR1 mice brains and mice were treated as described above. Treatment was initiated on d10 post tumor inoculation when tumors were first visible as assessed by MRI. Tumor growth was evaluated by successive MRI on d16 and d21 and the size of tumors measured.

IDH1i treatment was capable of significantly abrogating tumor growth compared to the control treatment **(Figure 2.8A).** Additionally, combination treatment of IDH1i and ICB further abrogated tumor growth and as hypothesized provided a synergistic therapeutic benefit. Individual tumor growth curves are shown in **Figure 2.8B**. On the other hand, ICB treatment failed to provide statistically significant therapeutic benefits as compared to the control treatment. The size of tumors, however, was on average slightly smaller. This was an interesting observation as even though ICB treatment resulted in an increased abundance of cytotoxic T-cells in the TME as compared to the control treatment, it didn't lead to abrogation of tumor growth. This indicated that effective anti-tumor responses in these tumors are largely CD4 T-cell driven, which are only induced upon IDH1i treatment.

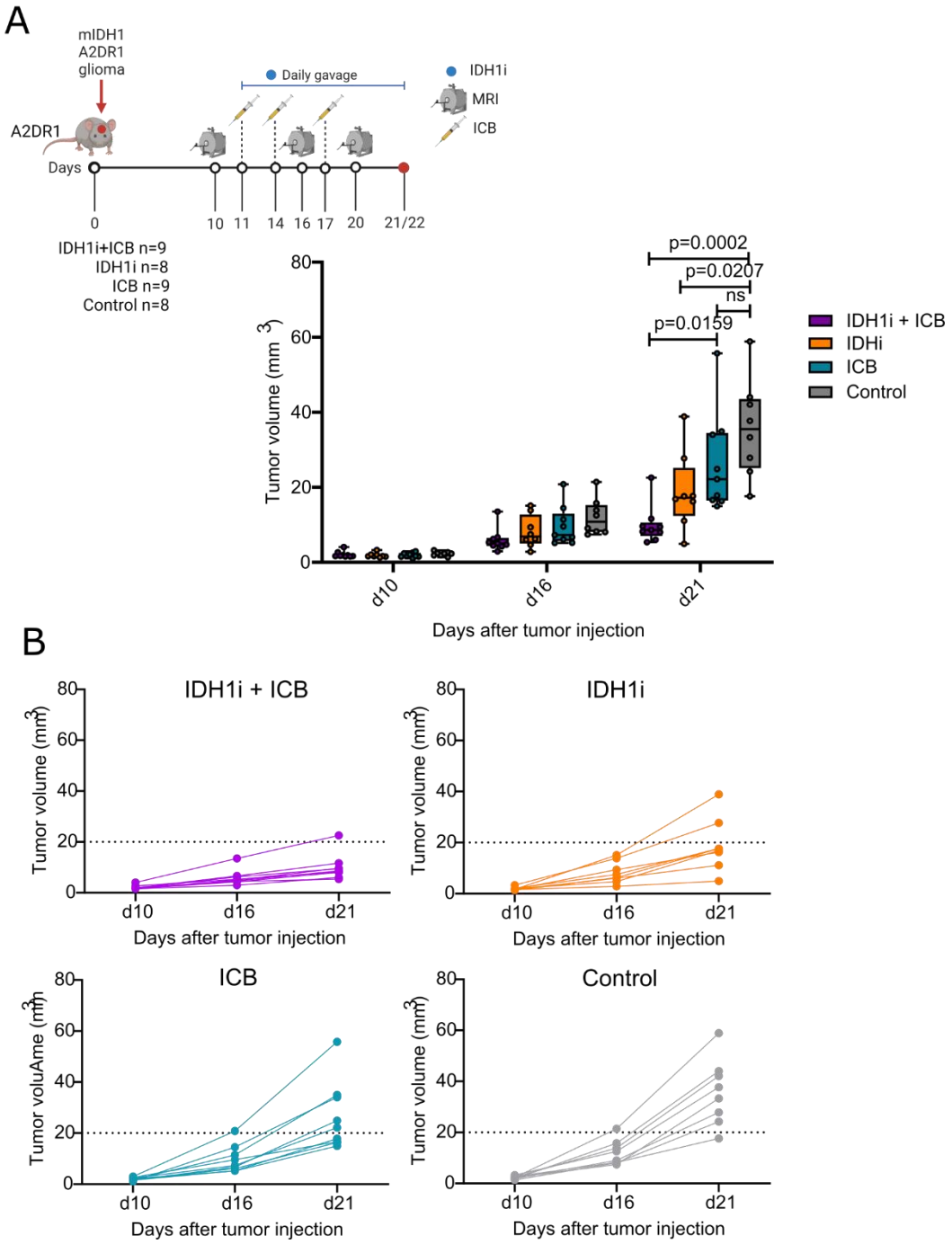

Figure 2.8: Syngeneic therapeutic benefit of IDH1i and ICB treatment in mIDH1 A2DR1 glioma model.

(A) Longitudinal volumetry of tumors comparing tumor growth in A2DR1 mice orthotopically injected with mIDH1 A2DR1 between each treatment arm. Tumor growth in mice was measured by MRI from d10, d16 and d21 post tumor inoculation.. A schematic of the treatment scheme is shown as inlay and was same as that utilized for transcriptomic profiling of TILs with the additional inclusion of a combination treatment arm. Number of animals used in each treatment arm is shown in inlay. Data is plotted as box and whisker plots Statistical testing was done using On way ANOVA followed by Tukey's test. **(B)** Individual tumor growth curves for each treatment arm

(part 3)

2.3 A proof of concept for the identification and validation of mIDH1-reactive TCRs

mIDH1is immunogenic, and a previous study had shown that a neoepitope of IDH1R132H (IDH1RH) mutation is presented on HLADRB1 and vaccination in A2DR1 mice with a peptide coding for IDH1RH can induce mutation specific CD4 T-cell responses. In this study, vaccination was able to abrogate the growth of IDH1RH mutated A2DR1 sarcomas and the therapeutic benefit was provided by mutation reactive CD4 T-cells as depletion of CD4 T-cells eliminated tumor control. Therefore, it was pertinent to establish a method for identifying and validating mIDH1 reactive TCRs that could be used as a first step in the putative development of TCR-transgenic adoptive T-cell immunotherapies against IDH1 mutated gliomas and other solid tumors. The following results serve as a 'proof of principle' for the identification of mIDH1 reactive TCRs and by extension other neoepitopes presented on MHC-II molecules

2.3.1 Identification of mIDH1 reactive TCRs in peptide-vaccinated A2DR1 mice

To identify mIDH1 specific TCRs, A2DR1 mice were vaccinated with an IDH1RH-specific peptide vaccine (IDH1RH-vac) with a boost on d10. On d21, spleens were excised and splenocytes were isolated. An IDH1RH-specifc T-cell was then generated to enrich IDH1RH-reactive T-cells as previously described[114]. A schematic of the pipeline is shown in **Figure 2.9A**. IFNg+ CD4 T-cells based on an IFNg secretion Catch assay (**Figure 2.9B)** where the T-cell line was stimulated with IDH1RH-vac were purified by FACS (data now shown) and subjected to 10x based single cell VDJ (scVDJ) sequencing. Analysis of the TCR repertoire identified the top TCR clonotypes where the combined frequency of the top three clonotypes made up ~80% of the entire TCR repertoire indicating that IDH1RH-reactive T-cells were highly clonal (**Figure 2.9C**).

For TCR delivery, novel S/MAR (scaffold/matrix attachment region) based gene therapy vectors were utilized which allow for the extrachromosomal replication of the vector within eukaryotic cells, with demonstrated persistence[127]. S/MAR vectors overcome some of the disadvantages of viral vectors such as random integration and silencing; and are potentially easier to translate to the clinic. The validation of neoepitope reactive

TCRs requires a robust assay sensitive enough to identify TCRs with a potentially poor affinity that are however reactive to the neoepitope. Since IDH1-vac induces CD4 T-cell response, it was necessary to utilize an assay which can test for TCRs reacting to MHC-II-presented epitopes as compared to the more commonly used CD8 T-cell based cytotoxicity assays against MHC-I epitopes. I, therefore set up an NFAT reporter based luciferase assay for screening candidate TCRs using TCR deficient Jurkat76 cell line as host for TCR delivery co-cultured with peptide-loaded peripheral blood mononuclear cells(PBMCs) that served as antigen presenting cells (APCs) (**Figure 2.9D**). Stimulation of TCR leads to the dephosphorylation of NFAT and its translocation to the nucleus where it interacts with other proteins and leads to the activation of multiple genes involved in T-cell activation and response[128]. In this assay, NFAT was exploited due to its early response to TCR stimulation to drive the expression of NanoLuc luciferase (nLuc) which has enhanced stability and lower background activity as compared to traditionally used luciferase.[129]. Bioluminescence imaging was then used as a readout for degree of TCR activation in response to peptide-MHC complex stimulation. The assay was optimized for the amount of TCR delivered for optimum TCR expression and co-culture conditions to increase assay sensitivity (data not shown).

Top TCRs identified from the scVDJ seq were cloned into S/MAR vectors and co-delivered with the NFAT-nLUC reporter vector into Jurkat76 cells. The success of TCR delivery was validated by checking the surface expression levels of TCR by flow cytometry (**Figure 2.9E**) As mouse TCRbeta (TCRb) chain specific antibody was used as the TCR vectors were designed to contain mouse TCRb constant chain for easy detection in human cell hosts. Their reactivity to IDH1RH was probed using the NFAT-reporter based assay as described above. Reactivity to (myelin oligondendrocyte protein) MOG peptide known to be presented on MHC-II served as a negative control reference. A publicly known TCR against Influenza Hemagglutinin (InfHa) which is reactive to HLA DRB1 presented InfHA peptide was used as a control TCR for the assay. TCR1 was reactive to IDH1RH 20mer (**Figure 2.9F),** which is used as IDH1-vac as well as to a IDH1RH 15mer peptide peptide harbouring the mutation, albeit with lower levels of reactivity. TCR2 showed minimal reactivity to IDH1RH.

These results show that a scTCR sequencing based platform can identify IDH1RH-specific TCRs from vaccinated mice and by extension potential TCRs against other neopitopes that can drive T-cell responses when used as a vaccine candidate. Additionally, these results validated the suitability of an NFAT based reporter assay for testing the reactivity of CD4 TCRs against MHC-II presented antigens.

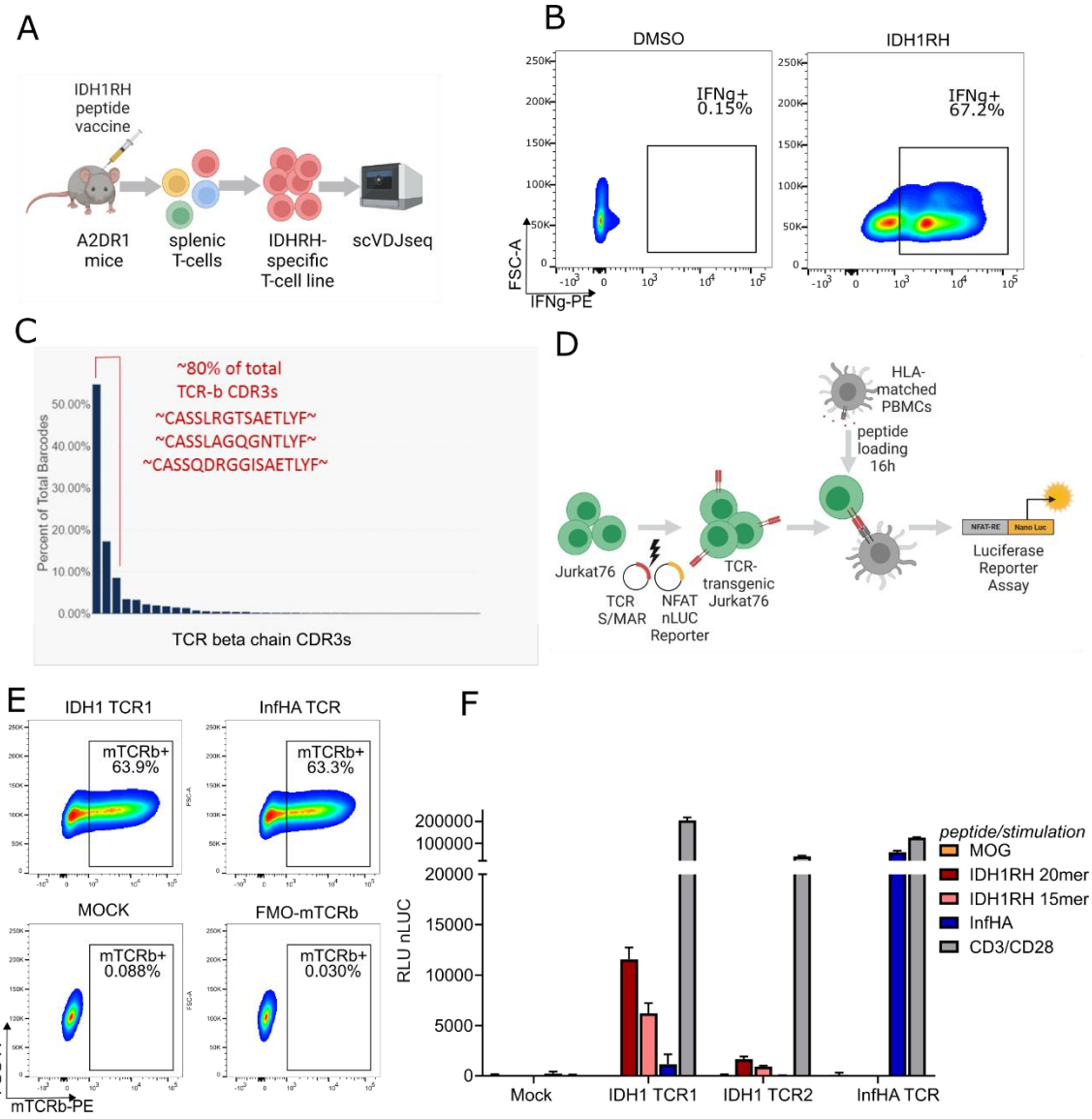

Figure 2.9: scTCR sequencing based identification of IDHRH reactive TCRs induced by IDH1RH-vac in A2DR1 mice and validation using an NFAT-based reporter assay

(A) Schematic workflow for the identification of IDH1RH-reactive TCRs from from A2DR1 mice vaccinated with IDH1-vac. **(B)**Flow cytometry plots depicting IFNg secreting CD4 T-cells on stimulation of the IDH1RH T-cell line with IDH1RH peptide and detection of IFNg using an IFNg secretion catch assay. DMSO was used as control stimulation. **(C)**

Frequency of top TCR clonotypes in the IDH1RH-specific T-cell line obtained by scRNA sequencing. **(D)**Schematic for the NFAT based luciferase assay for validation of TCR reactivity**. (E)** Flow cytometry showing the detection of surface expression of delivered TCRs in Jurkat76 cells. Mock refers to no TCR delivered.FMO was used to detect background signal *(FMO, fluorescence minus one)* **(F)** NFAT based luciferase reporter assay showing TCR reactivity of top 2 TCRs. TCRs were overexpressed in TCR-deficient Jurkat76 cells and co-cultured with IDH1RH peptide loaded HLA DRB1+ donor PBMCs. MOG peptide was used as negative control. CD3/CD28 stimulation was used as positive control. Influenza Hemagglutinin (InfHA) peptide presented on HLA DRB1 was used to check for non-specificty. Known TCR reactive to InfHA peptide was used as assay control. Data depicted as mean ± SD of 3 technical replicates. Representative of three independent experiments. Luminescence measurements were normalized to background. *(RLU, relative luminescence units*)

2.3.2 Identification of mIDH1-specific TCR in a peptide-vaccinated IDH1-mutated glioma patient

(Results from work I performed as part of this section have been published in **Platten, M. et al. Nature 592, 463–468 (2021)** in which I am a co-author. The associated figure has been adapted from the manuscript[111])

The NOA16, Phase I, first in human clinical trial aimed to assess the safety of an IDH1R132H-specific peptide vaccine (IDH1RH-vac) in newly diagnosed mIDH1+ (IDH1R132H+) glioblastoma patients and vaccine induced responses [111]. 26 of the 30 patients tested for immunogenicity showed IDH1RH-vac induced peripheral T-cell responses across multiple HLA-alleles. The occurrence of Pseudoporgression (PsPD) as diagnosed by brain imaging is an indicator of intratumoral inflammatory reactions and was associated with the onset of IDH1RH-vac induced peripheral T-cell responses. Patients with PsPD also had higher maximal levels of peripheral IDH1RH-vac induced T cell immune responses as compared to patients who had progressive disease. These observations prompted the question of whether mIDH1-specific T-cell responses can be observed within the tumor.

Among patients with PsPD, only patient (ID08) who underwent resection of the PsPD lesion was, therefore, the only patient and available tissue material to test specific T-cell responses in the tumor. Lesion infiltrating leukocytes (LILs) of patient ID08 were isolated from the resection and purified by FACS. CD45+CD3+ T-cells were then subjected to

combined scRNAseq and sdVDJseq using the 10x platform to determine the transcriptional phenotype of T-cells within the LILs and their TCR identity. Clusters were identified based on the expression of cell-type determining canonical markers and additional cluster defining genes (**Figure 2.10A**). CD4 T-cells were focused upon as preclinical data has shown that IDH1RH-vac induces CD4 T-cell responses and CD8 T-cells are not reactive to IDH1RH [120]. Three clusters of CD4 T-cells were identified within the LILs, CD40L CD4 T-cells, CXCL13 CD4 T-cells and regulatory T cells based on cluster defining genes. CD40L is a marker for activated CD4 T-cells. Interestingly, CXCL13+ T-cells have been implicated in being important for antitumor immunity in previous reports. Plotting the expression of CXCL13 within the T-cell clusters showed that it was expressed exclusively in one CD4 T-cell cluster and one CD8 T-cell cluster (RSG13 CD8) (**Figure 2.10B**).

scVDJ sequencing allowed the identification of the TCR repertoire of T-cells within the LILs. scRNA sequencing combined with scVDJ sequencing was used to map the TCR identity of a T-cell to its transcriptomic identity. The distribution of top CD8 and CD4 TCR clonotypes among defined T-cell clusters was analysed (**Figure 2.10C,D**). Both CD40L CD4 T-cell and CXCL13 CD4 T-cell clusters had dominance of one TCR (TCR14). The top three most abundant TCR clonotypes within the CD4 T-cell TCR repertoire, as well as the fifth most abundant (TCR11-13, TCR15), were expressed mainly in regulatory T cells. On the other hand, TCR14 which was the fourth most abundant TCR clonotype within the CD4 T-cell TCR repertoire was minimally expressed in regulatory T cells. This was a highly interesting observation and identified a potential reactive TCR.

The reactivity of the top five CD4 TCRs to IDH1RH was then probed using the NFAT based luciferase reporter assay as set up in the previous section (**Figure 2.10E**). The TCRs were cloned into S/MAR vectors and overexpressed in TCR-deficient human Jurkat76 cells by electroporation. TCR-transgenic Jurkat76 cells were then co-cultured with IDH1RH peptide loaded autologous PBMCs and luciferase expression was subsequently measured by bioluminescence as a readout for TCR activation.. Reactivity to MOG peptide served as negative control reference. InfHA reactive TCR was used as an assay control (using peptide loaded HLA DRB1+ donor PBMCs for co-culture with TCR-transgenic Jurkat76 cells). As hypothesized, TCR14 was reactive to IDH1RH whereas the other TCRs were not-reactive. Overall, these results indicate that the clonal expansion

of IDH1RH-reactive CD4 T-cells that infiltrated into the lesion was induced upon IDH1RH-vac. Additionally, these results show that a pipeline of combined scRNA and scVDJ sequencing can be used to identify mIDH1-specific TCRs in glioma patients and hint at CXCL13 being a putative marker for neoantigen reactive CD4 T-cells.

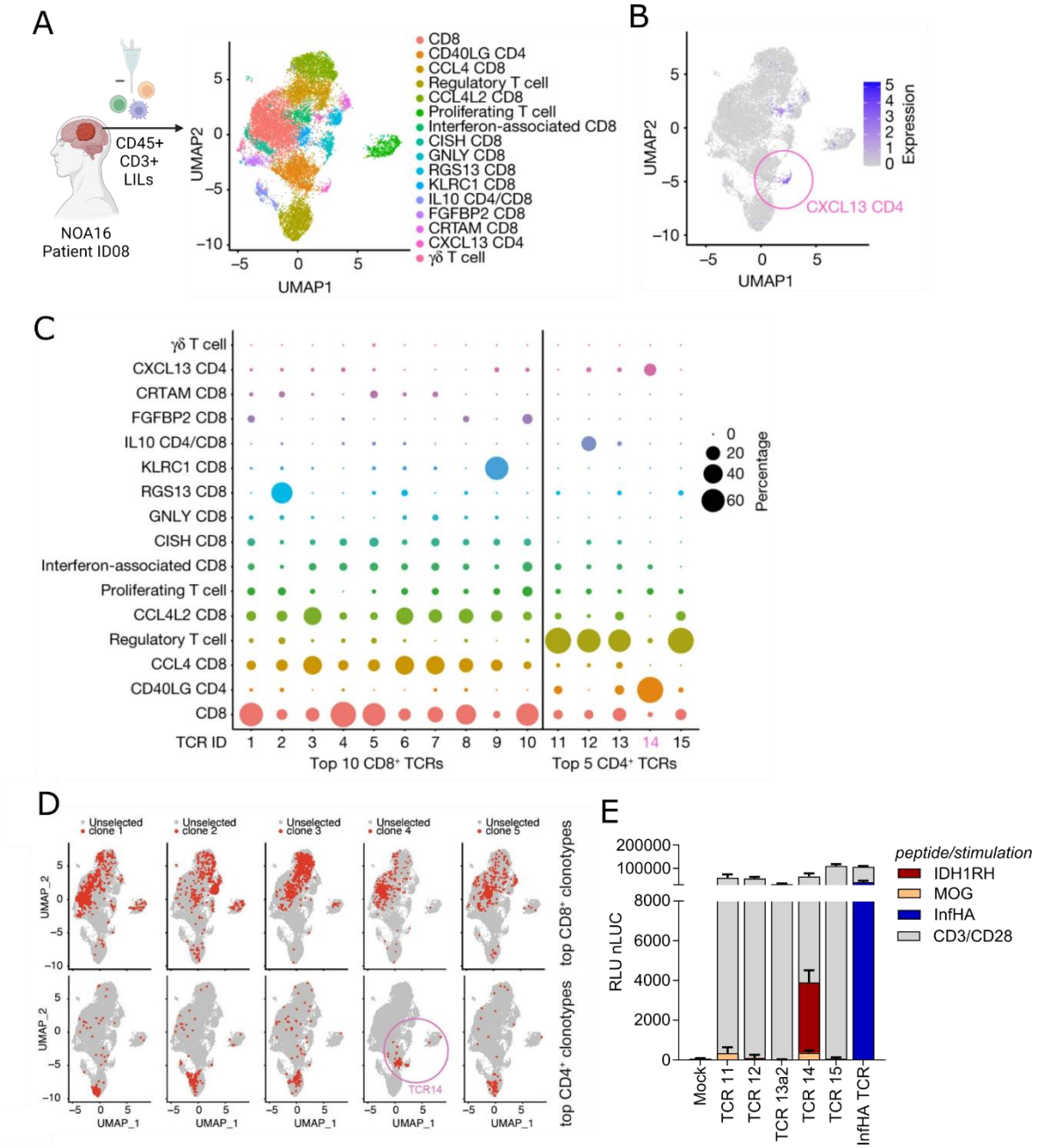

Figure 2.10: Combined scRNA and VDJ sequencing identifies IDH1RH reactive TCR induced by IDH1RH-vac in a glioma patient and is defined by the expression of CXCL13

(A) UMAP plot depicting transcriptionally distinct T-cell clusters as defined by single cell transcriptomics of PsPD LILs from Patient ID08 color coded for cell types based on cluster defining genes. **(B)** CXCL13 expression in LILs from patient ID08 within clusters as defined

in (A). CXCL13 expression in CD4 T-cells has been circled. **(C)** Bubble plot mapping of top 20 CD8 TCR clonotypes and Top 5 CD4 TCR clonotypes as defined by scVDJ sequencing onto each transcriptomic cluster defined in (A). **(D)** UMAP plot showing the expression of top 5 CD8 and CD4 TCR clonotypes within T-cell clusters. Expression of TCR14 has been additionally indicated. **(E)** NFAT based luciferase reporter assay showing TCR reactivity of top 5 CD4 TCRs. TCRs were overexpressed in TCR-deficient Jurkat76 cells and co-cultured with IDH1RH peptide loaded autologous PBMCs. MOG peptide was used as a negative control. CD3/CD28 stimulation was used as a positive control. As assay control a known TCR reactive to Influenza Hemagglutinin (InfHA) peptide presented on HLA DRB1 was co-cultured with HLA DRB1+ PBMCs loaded with corresponding peptide. Mock refers to no TCR. Data depicted as mean ± SD of 4 technical replicates. Representative of three independent experiments. *(RLU, relative luminescence units ; nLUC, nano luciferase*) *(Assitance in bioinformatic analyses for this part of the work was provided by Chin Leng Tan)*

Approaches to address the immunogenicity and immunosuppressive features of Isocitrate Dehydrogenase 1 mutated gliomas

3 DISCUSSION

3.1 The benefit of a syngeneic mIDH1 A2DR1 glioma model

Experimental models for glioma either use exogenous inoculation of tumor lines syngeneic to common strains of mice in laboratories or genetically induce tumors [130]. These models, however, cannot mimic antigen presentation as in humans and are therefore limited in their use for understanding human neoepitope-specific immune responses. Orthotopic xenograft models are often employed to study the tumorigeneis of, and preclinical drug testing on human derived tumors. However, such studies require the use of genetically modified mouse strains that are either deficient or limited in the immune system to avoid allogeneic rejection of human tumors, making them unsuitable for studying immune responses[131]. Adoptive cell therapies can be used to supplement this, but the lack of a robust native immune system prevents detailed investigation into the complex cellular interaction within the TME and the impact of other immune cell types on T-cell responses to tumors.

The development of a syngeneic mIDH1 glioma in MHC-humanized A2DR1 mice was pursued to overcome these limitations. Using CRISPR-Cas9 based approach targeting several tumor suppressor genes, tumors were induced in P0 A2DR1 pups. Cell lines established from these tumors were then modified to overexpress mIDH1 and IDHiwt, and their *in vivo* tumorigencity was tested. Immune infiltration of both CD4 and CD8 T-

cells was observed with reduced infiltration in mIDH1 tumors mirroring clinical observations[82]. Thus, the model facilitates the study of adaptive immune responses to mIDH1 gliomas in a humanized context where mIDH1 is available as an immunogen presented on HLA DRB1 (MHC-II).

3.2 Insights from the impact of IDH1i on glioma infiltrating T-cells

Immunosuppression is a hallmark feature of gliomas. The weapon of choice for mIDH1 gliomas is 2-HG. Interpreting the impact of 2-HG in a creative way, one can postulate that a small group of criminal pro-tumorigenic cells developed a weapon when they mutated IDH1. The sharpness of this blade in inflicting metabolic and epigenetic damage allowed these cells to proliferate and become gliomas. However, these cells were not proficient in using this weapon and the self-harm it caused diminished their notoriety among the community of gliomas. These mIDH1 gliomas learnt a new trick. By brandishing this weapon and showing it off to their new, more civil-minded and nosey neighbours, the immune cells, they could not only fend off most of the policing but also convert some of them to facilitate their crime organization further!

Several reports have characterised the immunosuppressive damage that 2-HG inflicts on tumor infiltrating immune cells[36, 83, 86]. The study by Bunse et al., additionally showed improved survival of mIDH1 GL261glioma-bearing mice upon treatment with IDH1i in combination with anti-PD1 ICB. This observation provided a rationale for a more descriptive analysis of the transcriptional state of tumor infiltrating T-cells upon IDH1 inhibition and whether the consequent depletion of 2-HG can restore T-cell function. With the aid of scRNA and scVDJ seq, and utilizing the syngeneic mIDH1 A2DR1 glioma model, an increased abundance of tumor infiltrating T-cells was observed upon 2-HG depletion. CD4 CD40L, PD1+ CD4 (CD4 exhausted), and CD8 cytotoxic T-cells were enriched upon, and Tregs depleted upon IDH1 inhibition compared to control and ICB treated, except CD8 cytotoxic cells, which were also abundant in ICB treated mice. These observations highlighted activation and tumor experience of infiltrating T-cells when 2-HG was depleted in the TME. TCR analysis , further identified the top TCR clonotypes to be mostly CD4 exhausted cells in 2-HG depleted TME.

The role of CD4 T-cells in eliciting and sustaining anti-tumor responses, although largely neglected, has gained traction in recent years. Studies first reported that immunogenic tumour mutations in the 'mutanomes' of preclinical mouse tumour models largely induced a CD4 T cell response[132] and that personalized neopitope vaccines using long peptides in melanoma patients primarily induced tumour-specific responses in CD4 rather than CD8 T cells[133]. Additionally, studies have highlighted the role of CD4 T-cells in enhancing anti-tumor CD8 T-cell immune response[134, 135]. Although a checkpoint molecule and a marker for exhaustion, many studies have established the relevance of PD1 as a marker for identifying CD8 tumour reactive T-cells. However, recent studies have also shown that high expression of PD1 identifies tumor.reactive CD4 T-cells in mouse tumor models, as well as in multiple human solid tumors [124, 136] . These studies help inform that the enriched CD4 exhausted T-cell subset upon 2-HG depletion represents putative tumor reactive T-cells in mIDH1 glioma.

A deeper look at inferred intercellular communication using receptor-ligand analyses revealed that IDH1 inhibition increased overall intercellular communication and pathways that were most upregulated immune cell recruitment, T-cell activation and effector cytokine production. Network analyses revealed that the significant interactors in upregulated pathways such as TNFa, type II Interferon, CD40L signalling and PDL1 signalling axis were CD4 exhausted T-cell, further corroborating that the depletion of 2-HG enables effective T-cell communication with myeloid cell in the TMEand restores immune function. Further analyses are, however needed to decipher which myeloid interacting partners are most relevant in inducing anti-tumor CD4 T-cell responses. A recent study reported that neoadjuvant PD1 blockade in GBM patients improved T-cell infiltration, which they postulated as a potential effect of conventional type 1 dendritic cell activation. However, a, sustained high expression immune checkpoints on tumor associated macrophages prevented optimal T-cell activation[107]. A deeper look therefore on network signalling in TILs in ICB treated mice, may educate whether T-cell inhibitory interactions from immunosuppressive myeloid cells are curtailed upon IDH1 inhibition.

Finally, based on the observation that even though ICB did not induce CD4 T-cell activation, it augmented CD8 cytotoxic T-cell infiltration, we investigated the therapeutic effect of each monotherapy as well as combination therapy of IDH1i and

ICB. IDH1i monotherapy abrogated tumor growth and interestingly synergized with ICB when ICB monotherapy was not successful when compared to control treated mice. These observations enable two conclusions – Firstly, CD4 T-cell responses are needed for effective tumor control of mIDH1 gliomas. Second, in the presence of 2-HG, ICB cannot restore T-cell function of suppressed T-cells, but in an immune permissive TME mediated by IDH1 inhibition, it can augment the activity of PD1+ CD4 T-cells and orchestrate effective tumor control. These conclusions are depicted as a schematic illustration in **Figure 3.1**

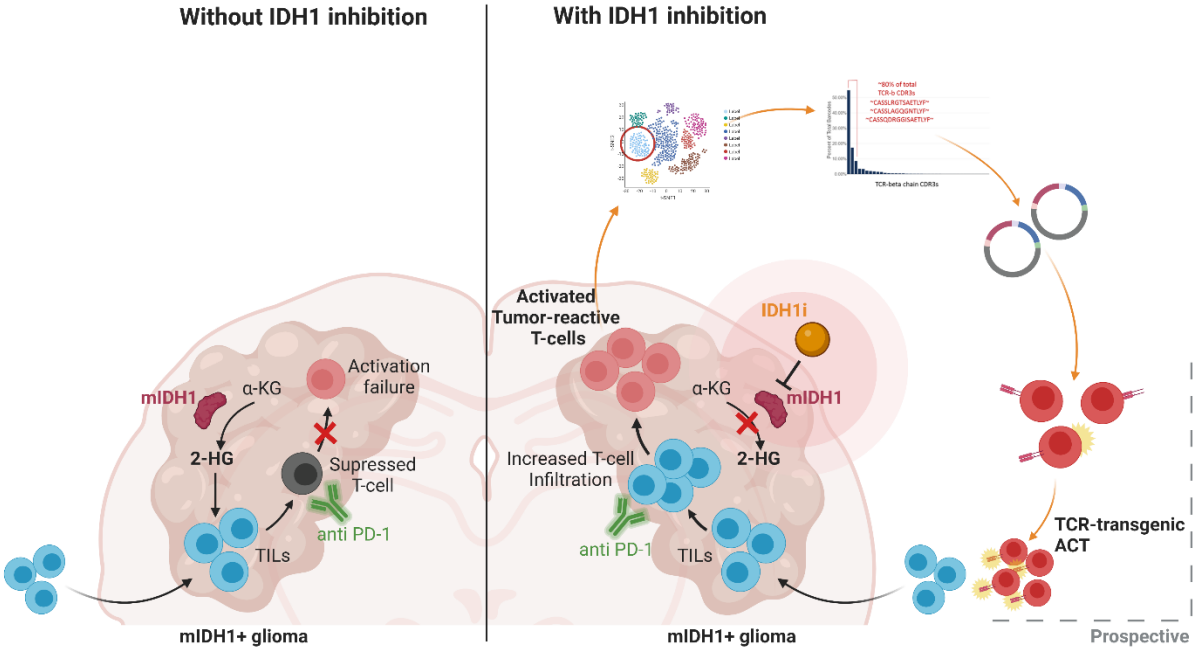

Figure 3.1: Impact of IDH1i on tumor infiltrating T-cells in mIDH1 glioma

A graphic abstract illustrating the fate of T-cells in the TME in the presence and absence of IDH1 inhibition. In the natural setting, T-cells infiltrating the TME encounter 2-HG and import it leading that actuates signalling dysfunction and suppresses the T-cells. ICB given in such a setting fails to restore T-cell function and induce anti-tumor response as T-cells are unable to overcome the immunosuppressive pressure of 2-HG . Upon IDH1 inhibition, the 2-HG induced immunosuppressive barrier is lifted and infiltrating T-cells can get activated in response to glioma specific neo-antigens such as mIDH1. Combinatorial ICB in this setting can then effectively target T-cell exhaustion and induce potent anti-tumor responses and a synergistic therapeutic benefit Prospectively single cell transcriptomic assessment can identify neoantigen-reactive T-cells. Subsequent TCR identification and validation can educate a tumor-reactive transcriptional signature that can be exploited to develop transgenic T-cell therapies.

While this study was being undertaken, two other studies were independently published that reported similar results on the induction of immune responses in the glioma TME upon IDH1 inhibtion. Using a mouse neurosphere (NS) based glioma model, the first study by *Kadiyala et al.* [137] showed an increase in median overall survival of mIDH1 glioma bearing mice when IDH1i was given as monotherapy or combined with irradiation and TMZ. This survival benefit was not realized in IDH1wt glioma bearing mice. IDH1i resulted in an upregulation of PDL1 expression on mIDH1 mouse NSs in comparison to IDHi1wt NSs and the authors attributed it to lower PDL1 methylation levels due to the inhibition of 2-HG. This is consistent with human gliomas where in the native setting, mIDH1 gliomas have a lower PDL1 expression compared to IDH1wt gliomas and hypermethylation of the CD274 gene (gene annotation for PDL1)[138]. The induced expression of PDL1 on mIDH1-mouse NS prompted the authors to investigate the therapeutic efficacy of IDH1i (with adjuvant irradiation/TMZ as standard of care (SOC)) in combination with anti-PDL1 ICB and tumor regression in 60% of the mice was observed. In concordance with my findings, they identified an increase in CD8 T-cell infiltration. Using a mIDH1 gliomas harbouring a surrogate tumor MHC-I restricted antigen ovalbumin (Ova), they further demonstrated recruitment and activation of antigen presenting DCs in the triple treatment, an increase in frequency of antigen specific CD8+ T-cells and reduced accumulation of immunosuppressive MDSCs, Tregs and M2-like macrophages. Additionally, the triple treatment reduced the exhaustion of T-cells and favoured the generation of memory CD8 T-cell reponse. Although this report was consistent with my observations on the induction of T-cell reponses upon IDH1i treatment and its synergism with ICB, this report had two major limitations. First, using a model, highly immunogenic antigen such as Ova [132] didn't address the impact of IDHi on anti-tumor T-cell responses to intrinsic tumor-specific antigens that are not as immunogenic. Secondly, the use of a mIDH1 mouse-NS glioma model with mouse MHC molecules, where mIDH1 itself is not presented in an immunogenic capacity, limited the induction of potential mIDH1 specific immune responses. This limitation is also probably why they did not observe enhanced recruitment of Cd4 T-cells or their activation upon IDh1 inhibition or combination therapy.

The second study by *Chuntova et al.* [139] addressed these limitations by using an orthotopic mIDH1 glioma model in A2DR1 mice (independently developed to work presented in this thesis) and reported suppression of tumor growth *in vivo* upon IDH1 inhibition. In contrast to observations by *Kadiyala et al.,* this study did not observe changes in the tumor infiltrating myeloid cell population but observed increased recruitment of CD4 T-cells. Depletion of either CD4 or CD8 T-cells abrogated the therapeutic benefit of IDH1i. Additionally, consistent with my observations, they observed a survival benefit when IDH1i was combined with anti-PDL1 ICB, but whether this was better than IDH1i monotherapy could not be delineated as IDH1i monotherapy in this experimental cohort failed.

The work presented in this thesis corroborates the observations of both these studies but, additionally with the aid of single-cell transcriptomic analyses, includes a broader non-biased investigation of T-cell states, signalling and fate in the TME upon inhibition of 2-HG. The above-described studies used either AGI-598 or AG-120 IDHI inhibitors. The former was not tested clinically due to poor clinical druggability and other disadvantages. AG-120, structurally similar to AGI-5198, has been shown to have a favourable safety profile for non-enhancing IDH1-R132H gliomas and is being further clinincall evaluated[140]. In the presented thesis, BAY1436032 was used., which is highly selective and bioavailable. In a Phase I study targeting mIDH1 solid tumors, it was well tolerated and showed durable objective responses in a small subset of subjects with LGG [45]. Taken together, the work presented in this thesis as well as the above-mentioned studies support the testing of IDH1 inhibition with adjuvant immunotherapies as targeted therapy for glioma patients.

Such a therapy would additionally benefit from IDH1i combinatiorial therapy, as removing the ‘2-HG wall’ would potentiate their anti-tumor response and prevent dysfunction.

3.3 Identification of mIDH1-reactive TCRs as a first step in the development of mIDH1 targeting T-cell therapies

The rationale for the identification of mIDH1-reactive TCRs and their validation came from the need for a transcriptional signature that putatively identifies antigen-specific T-cells. Identifying such a signature can educate the prospective development of TCR-transgenic adoptive T-cell therapy for IDH1 mutated gliomas to exploit further the previously described immunogenicity of mIDH1 [111, 114]

Given the diversity and size of the TCR repertoire and the dependence on its recognition of a peptide-MHC complex, identifying antigen-specific TCRs is akin to finding a 'needle in the haystack'. It is postulated that one TCR can recognize up to 106 different epitopes and that at the same time one epitope can be identified by several different TCRs [141, 142]. Most frequently, T-cells are isolated directly from the tumor or from patient PBMCs. Human antigen-specific TCRs identification is facilitated by first priming the T-cells in vitro with a known antigen[143, 144], by using peptide-pulsed APC libraries [145, 146] or by genetic modification of APCs with tandem mini genes encoding an array of epitopes[147]. This is followed by sequencing of the alpha (TRAC) and beta chains (TRBC) of enriched antigen-specific cells due to antigen stimulated clonal expansion. Fluorescent-labelled Peptide-MHC multimer complexes can ease the identification of antigen-specific TCRs by adding another layer of selectivity[148]. The latter, however is largely restricted to MHC-I presented antigens and cognate CD8 TCRs due to the difficulty of manufacturing stable peptide-MHC-II multimers for all candidate epitopes presently. Additionally the multimer method only identifies high-affinity TCRs[149] but reports have shown that affinity to antigen does not impact the entry of low affinity CD4 T-cells into primary immune response and their maintenance[150] Although these methods are highly useful for identifying antigen-reactive TCRs against unknown antigens in an unbiased approach, they are explicitly dependent on the pre-existence of antigen-reactive T-cells in the assayed biomaterial which may be found in extremely low frequencies and escape enrichment. Identification of MHC-II epitope specific TCRs faces another hurdle due to the variable length of epitopes that can be presented on MHC-II and the inefficiency of MHC-II binding prediction algorithms [151]. MHC-humanized mouse models provide a suitable alternative to test immunogenicity of neoantigens to induce CD4 T-cell reponses by long peptide vaccinations encoding the mutation and can

overcome the challenge of epitope promiscuity as has been shown in several studies [114, 117, 152, 153] allowing for putative CD4 TCR identification.

By exploiting this approach, I first explored the possibility of identifying antigen-specific TCRs from mIDH1 peptide vaccinated A2DR1 mice. By exploiting a T-cell line that was enriched for antigen-reactive T-cells and employing scVDJ sequencing, putative TCRs were identified and a high clonality of the TCR repertoire was observed. However, validating these TCRs as the next step, required the setup of an assay that could probe the reactivity of TCRs from CD4 T-cells against MHC-II presented epitopes. Conventionally TCR testing, primarily for CD8 TCRs has employed the use of cytotoxicity assays which are unsuitable for CD4 TCRs or flow cytometry based approaches detecting extracellular T-cell activation markers such as CD69 and CD137 or effector cytokine expression (IFNg, TNFa)[154]

Using the NFAT reporter assay, a mIDH1 reactive TCR was successfully identified from vaccinated A2DR1 mice. This discovery, helped confirm the suitability of the NFAT reporter assay for testing the antigen-specificity of TCRs identified by single cell sequencing for mIDH1. *This approach was published as a part of a Chapter on TCR discovery authored by my colleagues and me in the book Methods in Enzymology, Volume 629, 2019[155]* . This assay by extension, can be used to test TCR reactivity to other MHC-II presented antigens One such example is the identification of mutant Capicua Transcriptional Repressor(CIC) reactive TCRs which employed the above developed assay[117]. CIC inactivating hotspot mutations are found in around 70% of 1p19q co-deleted oligodendrogliomas and of these 5-10% harbour a mutation (CICRR15W) that is immunogenic and induces CD4 T-cell responses to peptide vaccination. Additionally, the NFAT reporter assay's higher throughput compared to flow cytometry based testing facilitates its suitability for testing TCR reactivity in an antigen-agnostic setting. Such approaches are based on the identification of TCRs from T-cells bearing predictive transcriptional signatures without prior knowledge of epitope, where reactivity testing of TCRs needs to be assayed against a large number of predicted neoantigens [156] Further refinement of this assay is however warranted to improve sensitivity.

Validated mIDH1-reactive TCR in turn provides a tool to test the therapeutic potential of a TCR-transgenic adoptive cell therapy. A number of transgenic T-cell therapies have been evaluated mostly targeting melanoma antigens[157]. These approaches, however, are focused on MHC-I antigens with known HLA restrictions. One exception is MHC-II restricted TCR therapy targeting MAGE-A3, which showed promising clinical results[158]. This preference is based on the cytotoxic potential of CD8 T-cells and the assumption that effective TCR therapy requires the use of cytotoxic T-cells. Previous studies have reported a mechanistically crucial role of CD4+ T cells for the induction of cytotoxic CD8 T-cell responses against tumors or the activation of IFNg-releasing macrophages [159, 160] In the context of glioma a recent preclinical study showed the efficacy of an intraventricularly administered TCR therapy targeting a MHC-II restricted antigen of mutant CIC and showed iproved survival in synergism with ICB.

The identification of mIDH1-reactive TCRs from immunized mice and the promising preclinical results of glioma neoantigen targeting transgenic TCR cell therapy, prompted the identification of mIDH1-reactive TCRs from IDH1-mutated glioma patients immunized with a mIDH1 targeting peptide vaccine. In the NOA16 Phase I trial durable mIDH1-specific T-cell responses were observed as described above. One patient underwent resection of the diagnosed PsPD lesion providing an opportunity to identify mIDH1 reactive T-cells from the lesion infiltrating leukocytes (LILs). Using a combined scRNA and scVDJ sequencing approach, TCRs were identified and mapped onto defined transcriptionally distinct T-cell clusters. This approach identified that majority of the top CD4 T-cell TCR clonotypes were predominantly found in the Treg compartment. One TCR clonotype, the fourth most abundant in the LILs among CD4 clonotypes, had a distinct CXCL13+ transcriptional signature. CXCL13 is a chemokine that exclusively binds the receptor CXCR5 expressed on B-cell, follicular T helper cells and follicular cytotoxic T cells; and plays a major role in immune cell recruitment, activation and regulation of adaptive immune responses at sites of chronic inflammation[161]. Historically, CXCL13 was described as an exacerbator of autoimmune conditions by the recruitment of B-cells and the development of aberrant tertiary lymphoid structures at the sites of inflammation[161-163]. In the context of cancer, investigating identified tertiary lymphoid structures showed the crucial involvement of the CXCL13-CXCR5 axis. CXCL13 signalling has been shown to recruit T-cells to these structures, and the presence of

tertiary lymphoid structures prognostically associates with long term survival in lung cancer patients and improves response to ICB in mealnoma[164, 165]. Recent evidence also showed that expression of CXCL13 by tumor infiltrating lymphocytes in lung cancer predicts response to anti-PD1 ICB [158]; in ovarian cancer, TLS formation was promoted by CXCL13-producing CD4 T cells, which improved survival in a mouse model by enhancing CD8 T-cell infiltration[166]. On testing the top CD4 TCRs from the NOA16 patient ID08, mIDH1 reactivity of TCR14 was observed, corresponding to the CXCL13+ T-cell subset. This result also identifies CXCL13 as a putative biomarker in gliomas for anti-tumor T-cell responses. Further validation of this signature in additional patient cohorts would, however, be needed to confirm the relevance of CXCL13 as a biomarker for immunotherapy response in gliomas.

The therapeutic translation of identified antigen-reactive TCRs further requires optimization of the TCR for improved efficacy in the clinic. One approach, which was also employed in this work substitutes human constant chains of both TRAC and TRBC with murine constant chains. This prevents the mispairing of transgenic TCRs with endogenous TCRs and resulting toxicities due to the creation of new TCRs with potential cross reactivity in the clinical setting[167]. Additionally, constructing TCRs with an extra disulphide bond in the murine constant regions increased TCR stability at the membrane as previously reported [168]. The use of S/MAR based gene therapy vectors in turn, overcomes some of the limitations of traditional viral vectors and facilitates ease of translation.

In summary the use of a biomarker educated identification of mIDH1-reactive TCRs and subsequent validation presents a first step in a pipeline for the development of a TCR-transgenic T-cell therapy for IDH1 mutated gliomas **(Figure 3.2)**

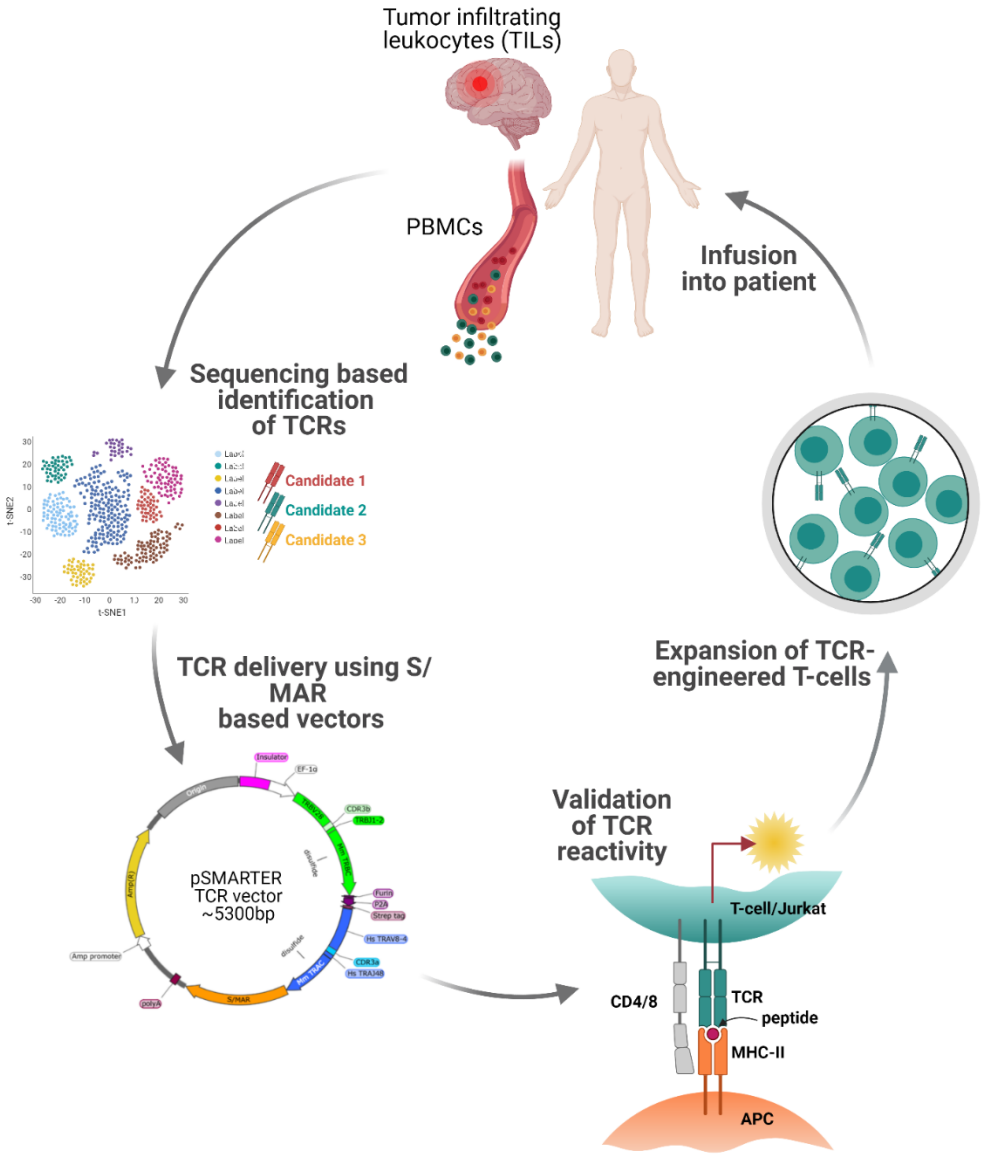

Figure 3.2: Pipeline for the development of a mIDH1-specific TCR transgenic adoptive cell therapy

Single-cell VDJ sequencing based identification of TCRs , educated by putative tumor-reactive signatures can filter the number of TCRs that need to be validated. S/MAR based delivery of TCRs provides ease of translation and NFAT reporter based screening facilitates increased throughput of TCR validation. Patient autologous T-cell CliniMACS Prodigy like automated cell processing platforms then enable scalable GMP-compliant manufacturing of cell therapy products

3.4 Conclusion and Outlook

The presented study was designed to address the dual immunogenic and immunosuppressive features of IDH1 mutations with the aim of finding approaches for more effective immunotherapies for IDH1 mutated gliomas.

mIDH1 gliomas display potent immunosuppression leading to the dismal performance of currently tested immunotherapies. The production of 2-HG that inactivates infiltrating T-cells is possibly a contributing cause of immunotherapy failure. By employing single-cell sequencing technologies, this study showed that T-cell function is restored upon IDH1 inhibition. It further revealed that CD4 T-cells drove consequent immune responses and identified putative tumor-reactive CD4 T-cells. Additionally, when combined with ICB, a synergistic therapeutic benefit and reduced tumor growth were observed in a syngenic mIDH1 MHc-humanized A2DR1 glioma model compared to IDH1i monotherapy. These findings suggest that the reduction of 2-HG levels is necessary for enabling a functional anti-tumor immune response which is then exploitable by immune checkpoint blockade. In turn, they support clinical trials testing the efficacy of IDH1 inhibitors in combination with adjuvant immunotherapies such as vaccines or immune checkpoint inhibitors in patients with IDH mutant gliomas

Owing to the immunogenicity of mIDH1, this study further identified mIDH1-reactive TCRs from A2DR1 mice and an IDH1-mutated glioma patient treated with a mIDH1-specific peptide vaccine. An association between CXCL13 expression and mIDH1-reactivity was observed. This proof of principle identification of mIDH1 reactive TCRs demonstrates the feasibility of exploiting immune responses against CD4-restricted neo-epitopes as a first step in developing an adoptive TCR-transgenic T cell therapy for glioma patients. However, the limitation of using TIls due to dependence on tissue availability prompts the need to establish a pipeline for identifying mIDH1-reactive TCRs from peripheral blood. Such an approach is currently being explored by colleagues in the lab.

4 MATERIALS AND METHODS

(*Methods pertaining to parts of this work which were published have been adapted from the manuscripts. I was a co-author in these publications and authored those sections)*

4.1 *In vivo* experiments

4.1.1 Mice

A2DR1 mice transgenic for HLA-A*0201 HLA-DRA*0101 HLA-DRB1*0101 and devoid of all mouse MHC molecules (C57BL/6-Tg(HLA-DRA*0101,HLA-DRB1*0101)^{1Dmz} Tg(HLA-A/H2-D/B2M)^{1Bpe} H2-Ab^{1tm1Doi} B2m^{tm1Unc}H2-D1^{tm1Bpe}) (for *in vivo* vaccination experiments and *in vivo* glioma experiments) and were provided by M. Berard (Insitute Pasteur) [119] and were bred in-house at the DKFZ animal facility. 8-16wk old mice were used for experiments and sex and age matched . NSG mice (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) [169] (for *in vivo* tumor passaging) were obtained from Jackson laboratories and were bred in-house at the DKFZ animal facility.

Mice were housed under specific pathogen-free conditions (SOPF) at the animal facility of the DKFZ, Heidelberg. All experiments were performed according to the rules of the German Welfare Act and were licensed by the regional authorities in Karlsruhe, Germany (G-251/18; G-264/18; DKFZ268)

4.1.2 Generation of A2DR1 gliomas

(The development of A2DR1 glioma lines has has been pulished in Kilian et al., Clin Cancer Res, (2022) , in which I am a co-author and helped perform these experiments)

A CRISPR-Cas9–based triple gene knockout approach was utilized for the generation of A2DR1 gliomas as described previously[121]. In brief, A2DR1 P0 pups were electroporated with guide RNAs targeting p53, NF1, and Pten genes cloned into pX330 plasmids (Addgene). pT2K IRES-luciferase plasmid was electroporated to enable detection of successful electroporation. P0 A2DR1 mice were anaesthetized with 2% isoflurane and injected with 1 µg DNA in 1 µL into and delivered laterally with electric square pulses using forceps-like electrodes [35 mV (VZ), 50 ms-on, 950 ms-off, 5 pulses]. Bioluminescence imaging of luciferase activity (IVIS) in the brain was performed 7 days after electroporation to confirm delivery of the vectors. Tumor growth in mice was checked by magnetic resonance imaging (MRI) two times (6 and 12weeks) after electroporation. Tumors were excised when mice showed signs of neurologic deficit (between 90 and 120 days). Excised tumors were mashed through a 100-µm cell strainer and passaged through NSG mice by subcutaneous injection into the flank of NSG mice. Upon reaching 1 cm in diameter, tumors were excised, and a single-cell suspension was prepared by mashing them through a 100µm cel strainer). To obtain tumor cell lines , cells were cultured in DMEM supplemented with 10% Fetal bovine serum (FBS) and 5% penicillin/streptomycin (P/S) for a minimum of three passages to remove contaminating stromal cells. Tumors from two mice yielded *in vitro* viable cell lines. The resulting A2DR1 glioma cell lines named M5 and M7 were tested for their potential to establish in vivo brain tumors by intracranially injecting them into adult A2DR1 mice (as described in Section 4.1.3) and tumor growth evaluated by MRI. Based on the growth characteristic A2DR1 glioma line M5 was selected for further experiments.

4.1.3 Intracranial tumor cell line inoculation

$1x10^5$ tumor cells (either A2DR1 glioma M5 or mIDH1 A2DR1 glioma) were diluted in 2µl PBS and stereotactically injected using a Hamilton microsyringe driven by a stereotactic device (Stoetling), into the right hemisphere of 12 to 14wk old male A2DR1 mice (injection coordinates: 2 mm right lateral of the bregma and 1 mm anterior to the coronal suture) at an injection depth of 3mm below the dural surface). The injection was

performed under anaesthesia and mice were supplied analgesics subcutaneously for 2 days post surgery. Mice were controlled regularly for tumor-related symptoms and were sacrificed when experiment stop criteria were met or on signs of neurological deficit.

4.1.4 Treatment with IDH1i

A2DR1mice inoculated with intracranial mIDH1 A2DR1 gliomas were administered with a daily oral gavage of IDH1 inhibitor BAY1436032 (150 mg/kg). The inhibitor was dissolved in 30% HP Beta CD (in water)(Sigma H107) and pH adjusted (pH 7-8) and administered in a single dose of 250ul. 30%HP Beta CD gavage served as vehicle in control. For timeline and combination with ICB see **Figure 2.8.**

4.1.5 Treatment with Immune checkpoint blockade

A2DR1mice inoculated with intracranial mIDH1 A2DR1 gliomas were treated intraperitoneally with anti-PD1, anti-PDL1 and anti-CTLA4 antibodies or corresponding istotype antibodies in 200ul PBS wvery 3 days for a total of 3 doses. All antibodies were obtained from Bioxcell. For dosage see **Table 4.1.**

Table 4.1: Dosage of ICB and antibodies used

Antibody (clone)	Amount /dose	Product Code	Corresponding isotype clone	Amount /dose	Product Code
aPD1 (RMP-14)	250ug	BE0146, Bioxcell	**2A3 (iso PD1)**	250ug	BE0089, Bioxcell
aCTLA4 (9D9)	100ug	Be0164, Bioxcell	**MCP-11 (iso CTLA4)**	100ug	BE0086, Bioxcell
aPDL1 (10F. 9G2)	200ug	BE0101, Bioxcell	**LTF2 (iso PDL1)**	200ug	BE0090, Bioxcell

4.1.6 MRI

MRI was performed at the MRI facility, department of neuroradiology, University Hospital Heidelberg (9.4 T Bruker Biospec 9/20).

For imaging, mice were first anaesthetized with 1% to 2% isoflurane (in air). And lesion detection was done using a standard T2-weighted sequence. The frequency and timeline of measurements were dependent on the experiment and has been referred to in corresponding sections. Tumor volume was manually calculated by segmentation using Bruker ParaVision software.

4.1.7 Vaccination of mice with peptides

A2DR1 were vaccinated with 100 µg of IDH1R132H (p123-142) peptide emulsified in Montanide-ISA51 (Seppic) at an equal volume to 1mg/ml. Mice were injected subcutaneously into the lateral pectoral regions with 2 injections of 50ul each. Mice were additionally injected with 300 ng rmGM-CSF (Peprotech) in PBS subcutaneously between injection sites and Aldara 5% imiquimod cream (Meda Pharma) was applied at the shaved injection site as adjuvant. Mice were given a booster vaccination after 10 days with no additional rmGM-CSF application. Mice were sacrificed after 21 days

4.2 Cell culture

4.2.1 Isolation of TILs from brain tumors

A2DR1 glioma bearing A2DR1 mice were anaesthetized and perfused with 20 mL PBS. The right hemispheres of the brains were then extracted and digested with 50 µg/mL Liberase (Sigma) in HBSS for 30 minutes at 37°C and successively mashed through 100-µm and 70-µm cell strainers to obtain a single-cell suspension. Myelin removal was done using a 30% continuous percoll gradient (GE healthcare).

4.2.2 Isolation of splenocytes

IDH1RH vaccinated A2DR1 mice were sacrificed by cervical dislocation and the spleens were excised. Spleens were then mashed through a 70-µm cell strainer to yield a single cell suspension. Erythrocytes were lysed using 2ml/spleen of ACK lysis buffer (Gibco).

Cells were washed with PBS and were filtered again through a 70-µm cell strainer and then resuspended in TCPM culture medium.

4.2.3 Generation of IDH1RH-specific T-cell line

The generation of antigen-specifc T-cell line was done as previously described (See Ref [120]. In brief, splenocytes from A2DR1 mice vaccinated with IDH1R132H (p123–142)(IDH1RH peptide) as described above, were stimulated with 10 µg/ml of IDH1RH. Medium was exchanged after 7 days, and supplemented with 3% (v/v) ConA supplement (gift from S. Eichmueller) and 15 mM α-methylmonnopyranoside (α-MM, Sigma-Aldrich) weekly. After 4 weeks, cells were restimulated with autologous splenocytes (from naive A2DR1 mice) that were irradiated with 30 Gy and loaded with 2 µg/ ml IDH1RH peptide in medium supplemented with ConA supplement α-MM. Cells were assessed for polarized morphology to induction of clonal expansion.

4.2.4 Isolation of LILs

Freshly resected lesion tissue of NOA16 Patient ID08 was dissected into small pieces (approx.. 2 × 2 mm), transferred to HBSS and successively mashed through 100-µm, 70-µm and 40-µm cell strainers to create a single cell suspension. Cells were intermittently washed with HBSS between every step. Finally resuspended in PBS+0.04% BSA and used for FACS.

For fluorescence-activated cell sorting (FACS) of LILs, patient tissue was dissected into small pieces, transferred to HBSS (Sigma Aldrich) and strained successively through 100-µm, 70-µm and 40-µm cell strainers with intermittent washes with HBSS to obtain a single-cell suspension.

4.2.5 Isolation of PBMCs

PBMCs from heparinized blood of NOA16 Patient ID08 or Buff Coats of HLADRB1 donors were obtained from the Immune monitoring Unit, NCT, Heidelberg. The process of isolation, in brief is describd. PBMCs were isolated by density-gradient centrifugation (800g without brake at RT) of blood diluted with phosphate-buffered saline (PBS) after loading onto Biocoll Separation Solution (Biochrom) and using Leucosep tubes (Greiner Bio-One). Isolated PBMCs were then cryopreserved in 50% freezing medium A (60% X-

Vivo 20, 40% FBS) and 50% medium B (80% FBS, 20% DMSO) and stored in liquid nitrogen at −140 °C until analysis.

4.3 Generation of transgenic cells

4.3.1 Generation of IDH1RH expressing A2DR1 glioma cell line

Full-length cDNA of human IDH1RH or IDH1 wild type was obtained from S.Pusch(DKFZ, Heidelberg) and was introduced into a modified retroviral vector pMXs-IRES-BsdR (Cell Biolabs, Inc) using the Gateway cloning system. A2.DR1 glioma cells were transduced with the resultant viral vector using FuGene HD transfection reagent (Promega) according to the manufacturer's protocol. Cells were selected with 9 µg/mL blasticidin (Sigma-Aldrich) 72 hours after transfection.

4.3.2 Cloning of TCRs

The alpha and beta VDJ fragments of the variable region of the TCR as identified by scVDJ sequencing were purchased from Twist biosciences as synthetic gene fragments compatible with BsaI-mediated Golden Gate Assembly cloning. The TCRs were cloned into an S/MAR sequence-bearing expression vector (pSMARTer)that allows extrachromosomal replication of the vector in eukaryotic cells. This vector was designed to harbour mouse alpha and beta constant TCR regions and a p2a self-cleaving peptide linker. This linker facilitates the cleaving of the gene product into separate alpha and beta polypeptide chains of the TCR. and was obtained from E. Green (DKFZ, Heidelberg) The The alpha and beta variable fragments were cloned into the expression vector via single-step Golden Gate assembly followed by trasnformation into NEB5-alpha-competent E coli (NEB). Colonies were screened for the transgene by ampicillin resistance, and endotoxin-free plasmids were purified using NucleoBond Extra Maxi EF kit (Macherey-Nagel) and used for transfection.The same process was used for both mouse and human derived TCRs and the publicly known InfHA reactive TCR.

4.3.3 TCR delivery into Jurkat76 cells

Jurkat76 were transfected with the above cloned S/MAR based TCR expression vectors using electroporation (Neon Transfection system, ThermoFisher Scientific). A nano-luciferase-based NFAT reporter vector (pDONR, with 4× NFAT-response elements) was

co-delivered to enable TCR reactivity testing using the NFAT reporter assay. 6µg TCR expression vector with 3µg NFAT reporter vector was used per electroporation with 2 × 10^6 cells with Neon 100-µl.tips and E2 buffer. Cells were prepared according to the manufacturer's protocol and electroporated with 1,325 V, 10 ms, 3 pulses based on the manufacturer's recommendation. For mock electroporation only the NFAT reporter vector was used. Post electroporation, cells were immediately transferred to pre-warmed antibiotic-free RPM1 1640 medium containing 10% FBS and rested for 48h. Surface expression levels of TCR were then verified by Flow cytometry.

4.4 *In vitro* assays

4.4.1 TCR testing using NFAT luciferase reporter assay

As antigen presenting cells, patient autologous PBMCs or HLADRB1 typed donor PBMCS were used as indicated. Cells were thawed in X-VIVO 15 medium (Lonza) containing 50 U/ml benzonase (Sigma-Aldrich) and rested for 12-16h before setup of co-culture in falcon tubes stored vertically to prevent adherence of cells PBMCs were then seeded into 96-well white-opaque tissue culture-treated plates (Falcon) at 1.5 × 10^5 cells per well, and peptides were loaded at a final concentration of 10 µg/ml in a total volume of 150 µl for 16h. The human IDH1RH(p123-142) peptide used for immunization was used as stimulant. MOG (p35–55) at equal concentrations and PBS+1% DMSO (vehicle) at equal volume were used as negative controls and InfHA HLADRB101 presented peptide (.PKYVKQNTLKLAT) was used for assay control and to test non-specific TCR reactivity. TCR transgenic Jurkat 76 cells were collected and subsequently co-cultured with cognate peptide-loaded PBMCs for 6h at a 1:1 ratio. Human T-cell TransAct beads (Miltenyi)(CD3/CD28) were used as positive control stimulation. Nano-luciferase induction, indicative of TCR activation, was measured using the Nano-Glo Luciferase assay system (Promega) according to the manufacturer's protocol. Luminescence signal detection was done using PHERAstar FS plate reader (BMG Labtech). Measured signals were first normalized by background subtraction using blank wells and then with non-specific background signal using wells containing only unstimulated Jurkat76 cells.

4.4.2 IFNg secretion capture assay

To enrich for T-cell clones reactive to IDH1RH for subsequent scVDJ sequencing, the generated IDH1RH-specific T-cell line was re-stimulated with autologous splenocytes from naïve A2DR1 mice loaded with IDH1RH (10ug/ml) and IFNg secretion was detected using a commercial available IFNg secretion assay kit (IFN-γ Secretion Assay Cell Enrichment and Detection Kit (PE); mouse, 130-090-517; Miltenyi Biotec). Cells were treated and stained according to manufacturer's protocol. In brief, cells were labelled with IFNg catch reagent. The secreted IFNg caught on the cell surface after a secretion period was labelled with a PE-conjugated antibody. Stimulation with DMSO served as negative control and stimulation with 1 µg/ml staphylococcus-derived enterotoxin B (SEB, Sigma-Aldrich) as positive control. The cells were then stained for FACS and sorted.

4.5 Flow cytometry

A common protocol was used for staining of cells for flow cytometry-based experiments. For extracellular staining, cells were stained by resuspension in PBS supplemented with surface antibodies (100 µl/1x106 cells) and incubated for 1h at 4°C in the dark. In case of staining of murine cells, the staining mix was supplemented with mouseCD16/32 for blocking of Fc-gamma receptors. In the case of human cells, blocking before extracellular staining was done using 10% human serum (in PBS) for 10min.

For validating delivery of TCR vectors and surface expression of TCRs in Jurkat76, cells were stained PE-conjugated anti-mouse TCRbeta Constant region (H57-597, Biolegend) and APC conjugated anti-human CD3 (OKT3, Biolegend) and Fixable Viabilty Dye eFluor780 (eBioscience; 65-0865)

Flow cytometry was performed on FACSCanto II (BD Biosciences). Compensation for spectral overlap was done by using OneComp compensation Beads (eBioscience) stained with antibodies used in respective experiments. Data were analyzed using FlowJo V10.

4.5.1 Fluorescence-activated cell sorting (FACS)

FACS was performed on FACSAria II using BD FACSDiva Software and the 100 µm nozzle. Cells were sorted in the 1.5 Drop Pure Sort Mode at an event rate of 2,000-3,000 events/sec. Sorted cells were collected into tubes precoated with sterile 10% BSA (Roth 8076.4) to prevent sticking to plastic.

For sorting TILs from A2DR1 glioma-bearing mice, cells were stained before sorting with Hashing antibodies (TotalSeqC mouse, Biolegend) to allow multiplexing for scRNA sequencing (TotalSeqC hashing antibodies, Biolegend). Cells were gated on size, singularity and viability. Cells were first gated for CD45 (BV510) expression and then subsequent CD3 (APC) expression. CD45+CD3+ cells and CD45+CD3– cells were then sorted as 2 separate populations.

For sorting of IFNg secreting cells of the IDH1RH-specific T-cell line, cells were stained after the IFNg secretion assay with BV711 conjugated anti-mouse CD3(17A2, Biolegend), BV421 conjugated anti-mouse CD4 (RM4-5, Biolegend) and PerCp Cy5.5 conjugated anti-mouse CD8(53-6.7, Biolegend) and Fixable Viabilty Dye eFluor780 (eBioscience; 65-0865). Cells were also stained with Hashing antibodies (TotalSeqC mouse, Biolegend) to allow multiplexing for scVDJ sequencing. Cells were gated on size, singularity and viability. CD4+ IFNg+ T-cells were sorted for subsequent scVDJ sequencing.

For sorting of LILs from NOA16 Patient ID08, cells were extracellularly stained with the following antibodies: eFluor 450 conjugated anti-human CD45(clone 2D1, ebioscience), PE conjugated anti-human CD3 (HIT3a, BioLegend) and Fixable Viabilty Dye eFluor780 (eBioscience; 65-0865)

Cells were gated on size, singularity and viability and sorted into CD45+CD3+ and CD45+CD3− cell populations.

4.6 Single cell RNA and TCR sequencing

4.6.1 Capture and library construction

The 10x platform was used for the single-cell capture and downstream library generation of FACS sorted cells. The Chromium Single Cell V(D)J Reagent kit v1 chemistry was utilized (10x Genomics; PN-1000006, PN-1000020, PN-1000005, PN-120262) according to the manufacturer's protocol. Since samples were stained with TotalSeq C hashtag antibodies (BioLegend)for multiplexing, it allowed them to be combined before single cell capture. The constructed scVDJ library , scGEX libraries and feature barcode libraris (for hashing antibodies) were combined and multiplex sequenced on HiSeq4000 platform (Illumina).

4.6.2 Data integration and analyses

Quality control and normalization

scRNA data were processed using the CellRanger pipeline (version 6.0) to the GRChg38 reference genome with all default settings. All cells which had unique feature counts over 2,500 or less than 200 as well as >10% mitochondrial counts were excluded from the downstream analysis. The Seurat pipeline was used on filtered libraries for further analyses[170].The multiplexed fractions were demultiplexed and using the HTODemux command with default settings[171], cells were classified as Hashtag+ singlets, doublets or unassigned cells. Cells missing a assigned hashtag (unassigned cells) or doublets were removed. In further analyses, only cells classified as singlets were used. Gene expression was normalized using Seurat's LogNormalization() and highly variable genes were identified by using the FindVariableFeatures(). VDJ data was added using the combineExpression() function using the scRepertoire package V.1.3.1. The amino acid sequence (CTaa) was used for clonotype calling. The tcrdist3 software package was used for TCR-superclustering [126]

Filtered matrices from scRNA seq libraries from CD45+CD3+ and CD45+CD3–sorted cell fractions for each of the treatment groups were merged using Seurat's merge(). Subsequently, groups were combined to perform downstream integration.

Data integration

CD45+CD3+ and CD45+CD3–sorted cell fractions were integrated by using the Harmony V0.1.0 package according to the published vignette. Normalization was done by LogNormalize() and FindVariableFeatures generating 5000 Variable features. Subsequently batch effect-associated features as well as immune-receptor variable genes -

(Fos|Jun|Gm|Rps|Rpl|Atf3|Zfp36|AY|Egr|Malat1|Xist|Hsp|mt-|Hist|Socs3|Lars2|Trav|Trad|Traj|Trbv|Trbd|Trbj|Trgv|Trgd|Trgj|Trdv|Trdd|Trdj)

were filtered from the variable features for subsequent ScaleData() and the RunPCA function with npcs=50. Integration of the respective datasets was achieved by using the Harmony V0.1.0 package with the following function and parameters: RunHarmony(object ,c("orig.ident", max.iter.harmony =15, max.iter.cluster=30, dims.use= 1:30, epsilon.cluster = -Inf, epsilon.harmony = -Inf).

The generated Harmony reductions were used for further clustering. Hamony Dims 1:30 were used for FindClusters(..., resolution = c(0.5, 0.7, 0.9, 1.0, 1.2, 1.4, 1.5, 1.6, 1.8, 2.0)), FindNeighbors() and RunUMAP.

Cell-cell interaction analysis

cell-cell communication was assessed using Cellchat package[172] (https://github.com/sqjin/CellChat) . First Cellchat objects were created for each group separately. Subsequently using cellchat's following functions cell-cell interactions were calculated -

identifyOverExpressedGenes(),

identifyOverExpressedInteractions(),

computeCommunProb() and

filterCommunication().

To compare the treatment groups, CellChat objects were merged using cellchat's mergeCellChat() following the 'Comparison analysis of multiple datasets' vignette. Dat visualizations were done using ggplot2 package.

4.7 Western Blot

Total protein from cultured A2DR1 glioma cell lines (mIDH1, IDH1wt and M5(parental) A2dR1 glioma) was extracted by cell lysis using ice-cold TRIS-HCl, 50 mM, pH 8.0 supplemented with 150 mM NaCl, 1% Nonidet P-40 (Genaxxon Bioscience), 10 mM EDTA, 200 mM dithiothreitol (Carl Roth), 100 mM phenyl-methylsulphonyl fluoride (PMSF), phosphatase inhibitor cocktails 2 and 3 (1:100, Roche), and cOmpleteTM (1:50, Roche) for 20 min and subsequently centrifuged to pellet debris. Concentration of total protein in cell lysates was measured via the Bio-Rad Bradford protein assay (Bio-Rad) 10 µg of protein was then diluted in Laemmli sample buffer and denatured for 5mins at 95 °C and separated electrophoretically on acrylamide-polyacrylamide SDS-containing gels. Blotting onto nitrocellulose membranes was done by wet blot for 1 h. and subsequently blocked with 5% milk powder in 0.5 M TBS, pH 7.4, 1.5 M NaCl, 0.05 % Tween 20. For detecting mIDH1, membranes were incubated with primary mouse anti-IDH1R132H (1:500, H09, Dianova), rat anti-panIDH1 (1:500, W09, Dianova) overnight. Goat anti-GAPDH (1:5,000, Linaris) was used as loading control. Membranes were then washed

and incubated with secondary antibodies for 1h in blocking buffer. Secondary horseradish peroxidase-conjugated antibodies were anti-rat (1:1000, Dako), anti-mouse (1:5,000, GE Healthcare), and anti-goat (1:5,000, Santa Cruz Biotechnology). Detection of signal was done by Chemiluminescent development using ECL(Amersham).

4.8 2-HG measurements

A2DR1 glioma cell lines (mIDH1, IDH1wt and M5 (parental) A2DR1 glioma) were cultured in 6-well tissue culture plates at a density of 1x105 cells /well for 48h and for 24,48 and72h for intracellular and extracellular measurements respectively. The levels of 2-HG in cell pellets (intracellular) and collected cell culture supernatant (extracellular) were measured enzymatically and normalized to total protein content as previously described. The measurements were performed by S. Pusch (DKFZ, Heidelberg) as described previously[173]

4.9 Histology

Brains from perfused mIDH1 or IDH1wt A2.DR1 glioma-bearing mice were cryopreserved for histology in Tissue-Tek OCT medium (Sakura) and stored at −80 °C. Preserved brains were cut into 6 to 8µm sections using a cryotome (Leika).

For Hematoxylin and eosin (H&E) staining, fixation of 8-µm slides was achieved with Roti-Histofix 4.5%. H&E staining was performed using hematoxylin and bluing reagent for 4 minutes.

For immunofluorescence staining, slides were first air dried and then fixed with ice-cold methanol and subsequently blocked for 2 hours with normal goat serum (Sigma). Incubation with respective primary antibodies was done overnight in blocking buffer. and Finally, slides were mounted using DAPI-containing mounting medium (Invitrogen). Image acquisition was performed within 24 hours on a Cellobserver (Zeiss) or LSM700 confocal microscope (Zeiss). Semiquantitative evaluation of the infiltration and density of TILs in tissue sections (CD3+, CD4+ and CD8+) was performed by overall impression and judged for their presence or absence. Contrast and brightness of acquired images were linearly optimized using ImageJ software.

4.10 Graphical represention and statistics

Analysis of scRNA and scVDJ seq has been described above. Statistical analyses of all other experiments were performed in GraphPadPrism (v9) with an unpaired, two-tailed Student's t-test or one-way ANOVA and Kruskal-Wallis test combined with Tukeys's correction for multiple testing. P values have been indicated on the graphs. P <0.05 was considered significant (*:p<0.05, **: p<0.01, *** :p<0.001, ****: p<0.0001).

Figures were prepared using BioRender software and Inkscape vector graphics software.

4.11 Materials

4.11.1 Peptides

All peptides used were synthesized at the DKFZ. Lyophilized peptides were reconstituted in 100% DMSO and diluted to a final concentration of 10 mg/ml with PBS. The final DMSO concentration was 10%.

Human and mouse IDH1RH sequences are similar in the region spanning the R132H mutation (amino acid substitution replacing arginin with histidine at position 132) for the peptides used except p122 (serine in human and threonine in mouse). Vaccination experiments were done with IDH1RH (20mer) and in vitro stimulation was done with IDH1RH (20mer) and IDH1RH (15mer) as indicated

Table 4.2: Peptide sequences

Name	Ingredient
IDH1RH (20mer) p123-142	GWVKPIIIGHHAYGDQYRAT
IDH1RH (15mer) p122-136	SGWVKPIIIGRHAYG
MOG p35-55	MEVGWYRSPFSRVVHLYRNGK
InfHA 307-319 (HLADRB101)	PKYVKQNTLKLAT

4.11.2 Cell culture media

Table 4.3: Cell culture media composition

Name	Ingredient	Specification
A2DR1 glioma culture medium	DMEM high-glucose	Sigma-Aldrich; D6429
	>10% FBS	Sigma-Aldrich; F0804
	>100 U/ml penicillin	Sigma-Aldrich; P4333
	> 0.1 mg/ml streptomycin	Sigma-Aldrich; P4333
	>0.09% Blasticidin	
Jurkat76 culture medium	RPMI-1640	PAN Biotech; P04-16500
	>10% FBS	Sigma-Aldrich; F0804
	>100 U/ml penicillin	Sigma-Aldrich; P4333
	> 0.1 mg/ml streptomycin	Sigma-Aldrich; P4333
PBMC thawing medium	X-Vivio 15	Lonza
	>50U/ml Benzonase	Sigma-Aldrich
NFAT reporter assay co-culture medium	X-Vivio 15	Lonza
T cell proliferation medium (TCPM)	RPMI-1640	PAN Biotech; P04-16500
	> 2 mM L-glutamine	ThermoFisher; 25030081
	> 10% FBS	Sigma-Aldrich; F0804
	> 100 U/mL penicillin	Sigma-Aldrich; P4333
	> 0.1 mg/mL streptomycin	Sigma-Aldrich; P4333
	> 25 mM HEPES pH 7.4	Sigma-Aldrich; H0887
	> 1 mM sodium pyruvate	Sigma-Aldrich; S8636
	> 0.1 mM NEAA	Lonza; 13-114E
	> 5 x 10^{-5} M 2-mercaptoethanol	Sigma-Aldrich; M6250
Standard cell freezing medium	RPMI-1640	PAN Biotech; P04-16500
	>10% DMSO	Roth

PBMC freezing medium			
-	Freezing Medium A	60% X-VIVO 20 40% DMSO	Lonza Roth
-	Freezing Medium B	80% FBS 20% DMSO	Sigma-Aldrich; Roth

4.11.3 Buffers

Table 4.4: Composition of buffers

Name	Ingredient	Specification
ACK lysis buffer, pH 7.2	VE H_2O	
	> 0.15 M NH_4Cl	Carl Roth; 5470
	> 10 mM $KHCO_3$	Carl Roth; P748.1
	> 0.1 mM Na_2EDTA	AppliChem; A2937
FACS staining buffer	1X PBS	Sigma-Aldrich; D8537
	> 3% FBS	Sigma-Aldrich; F0804
	> 2mM EDTA	AppliChem; A3562.1000
FACS sorting buffer	1X PBS	Sigma-Aldrich; D8537
	> 0.04%BSA	Roth; 8076.4
Cytokine reconstruction buffer	Milli-Q H_2O >0.1% BSA	Roth; 8076.4
IFNg secretion assay buffer	1x PBS >0.5% BSA	Sigma-Aldrich; D8537 Roth; 8076.4

	>2mM EDTA	AppliChem; A3562.1000
Histology Wash buffer	1x PBS	
	>0.1% Tween20	
Protein lysis buffer	TRIS-HCL, 50mM, pH8.0	Roth
	>150mM NaCl	J.T. Baker
	>1% Nondiet P-40	Genaxxon Biosciences
	>10mM EDTA	AppliChem
	>200mM ditiothreitol	Roth
	>100µM PMSF	Sigma-Aldrich
	>1:50 Complete	Roche
Western Blot Washing Buffer (TBST)	0.5M Tris Based Slaine (TBS). pH7.4	Roth
	>1.5M NaCl	Roth
	>0.05% Tween20	Roth
Western Blot Blocking buffer	0.5M Tris.Based Saline (TBS) pH7.4	Roth
	>1.5M NaCl	Roth
	>0.05% Tween20	Roth
	>5% milk powder	Roth

5 SUPPLEMENTARY

5.1 Supplementary Figures

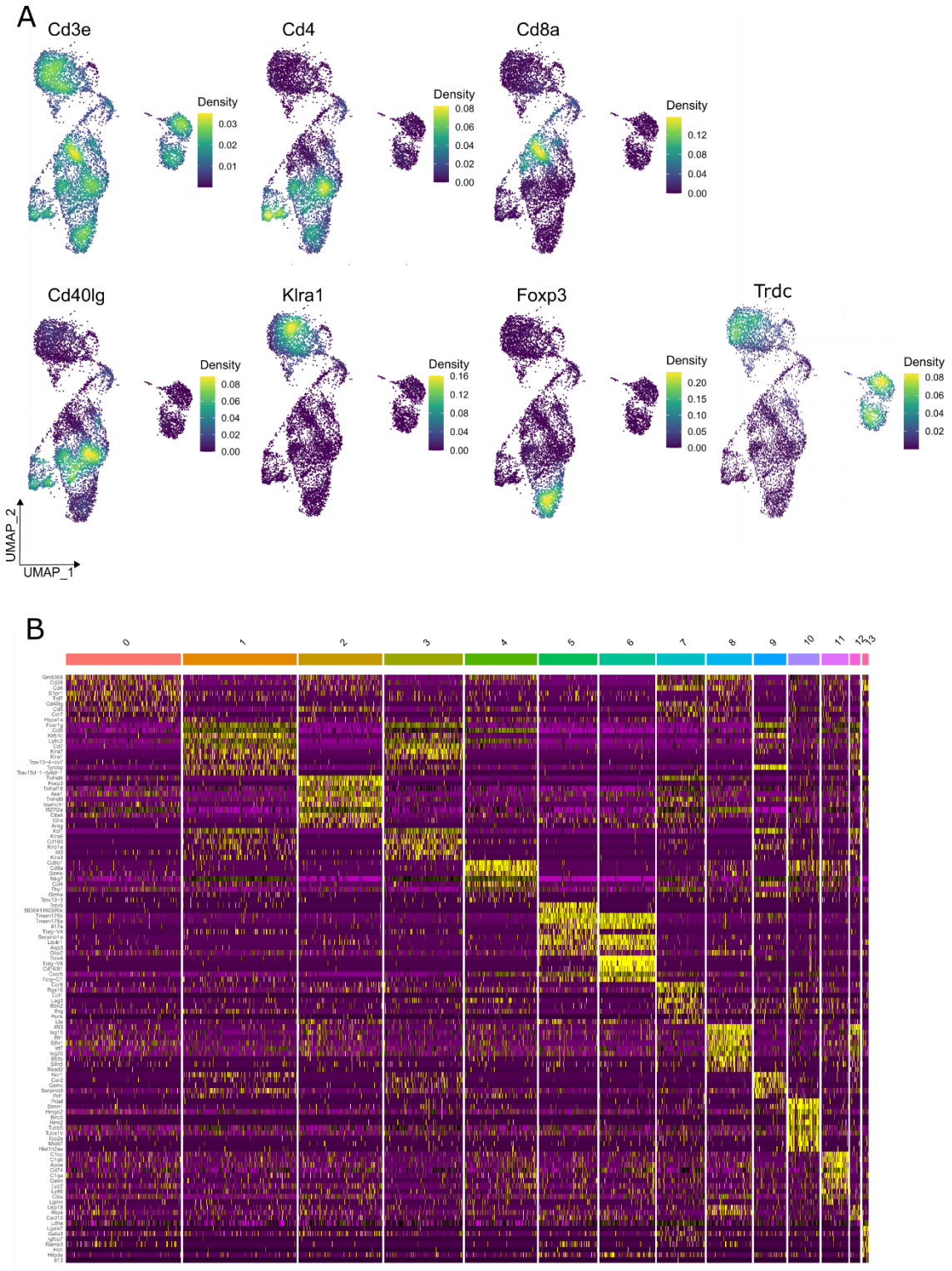

Figure 5.1:Differential gene expression analysis of identified molecular clusters of T-cells in mIDH1 A2DR1 glioma bearing A2DR1 mice

(A) Expression of canonical markers in scRNA seq identified molecular clusters of mIDH1 A2DR1 glioma infiltratingT-cells. **(B)** Differential gene expression between identified clusters

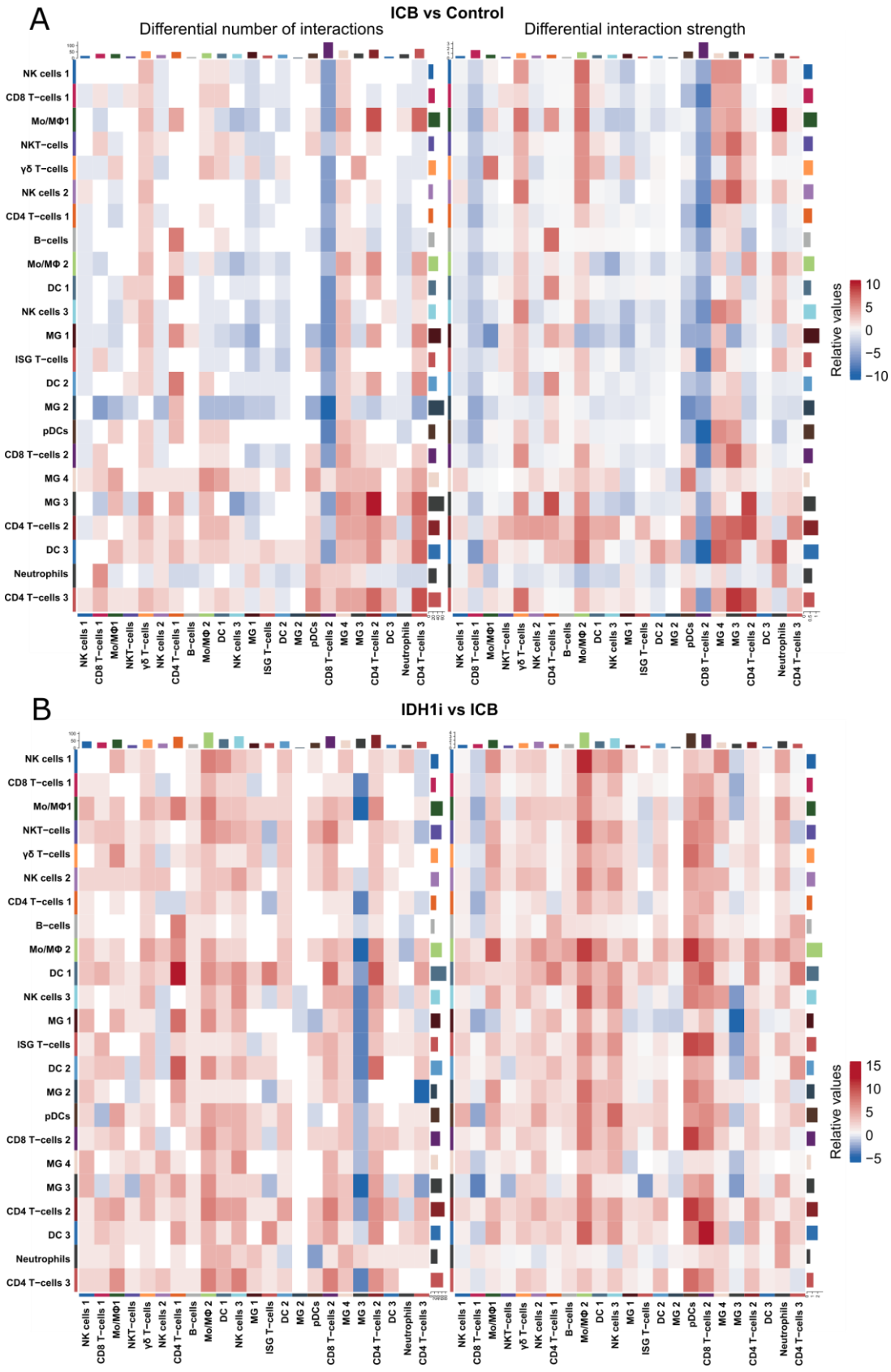

Figure 5.2: Receptor ligand analysis heat maps

CellChatDB derived heatmap visualizing the bioinformatically inferred cell-cell. communication between clusters in **(A)** ICB relative to control treatment and **(B)** IDH1i relative to ICB treatment

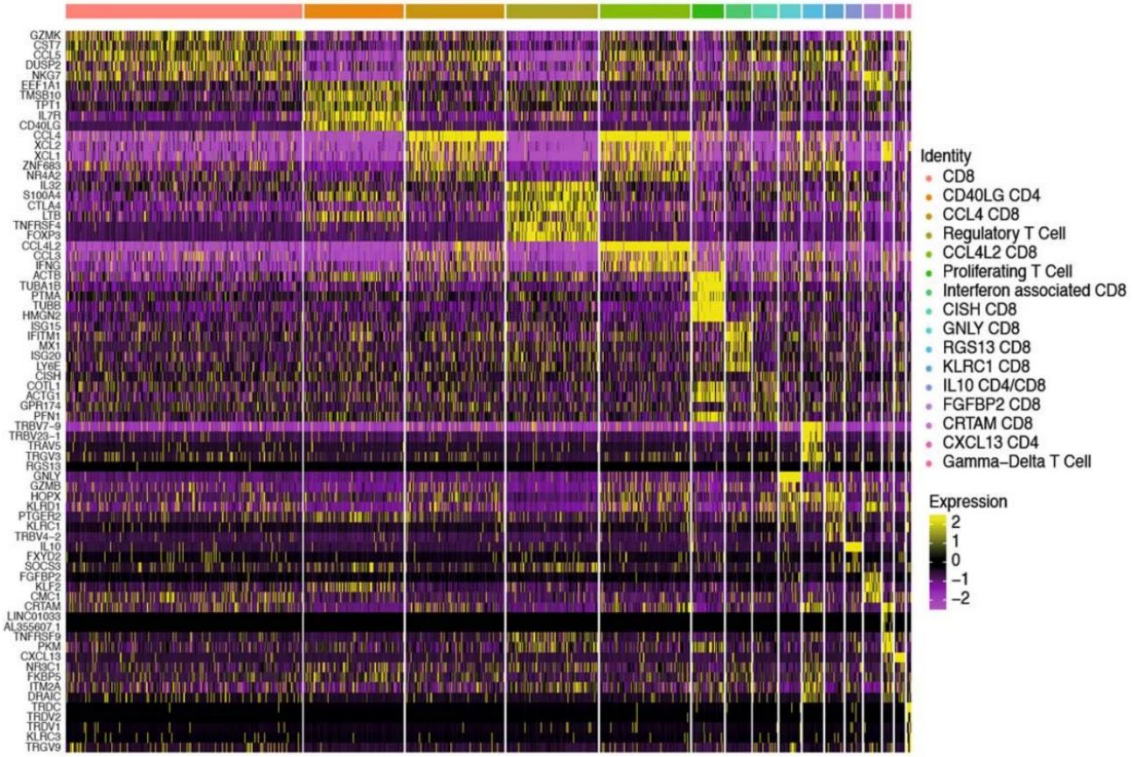

Figure 5.3: Differential gene expression of identified molecular clusters of LILs in NOA16 patient ID08

5.2 Vector Maps

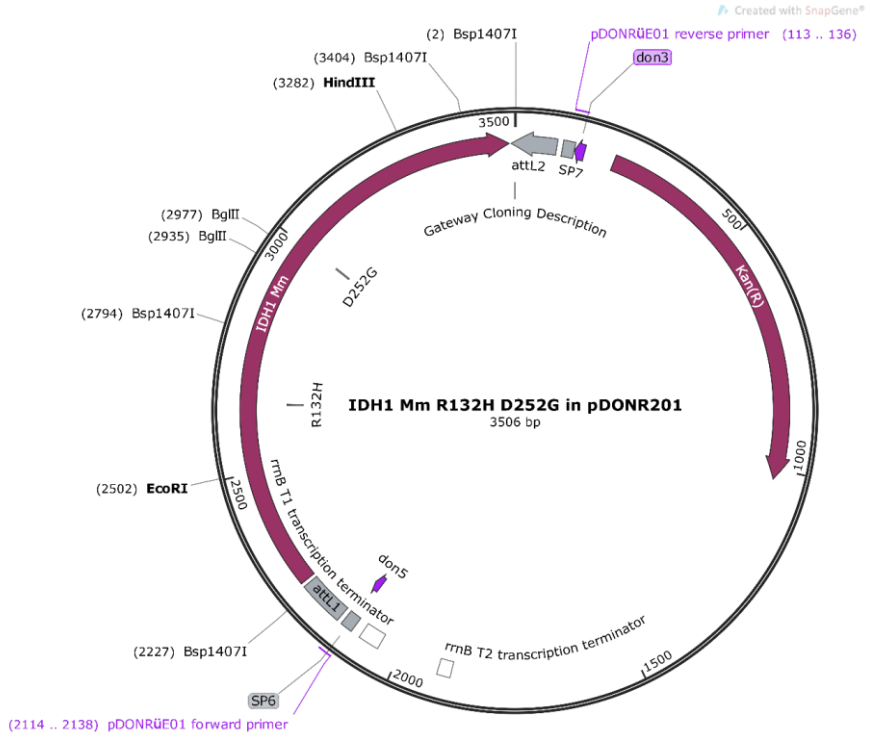

Figure 5.4: Donor vector for generation of mIDH1 and IDH1wt retroviral expression vectors

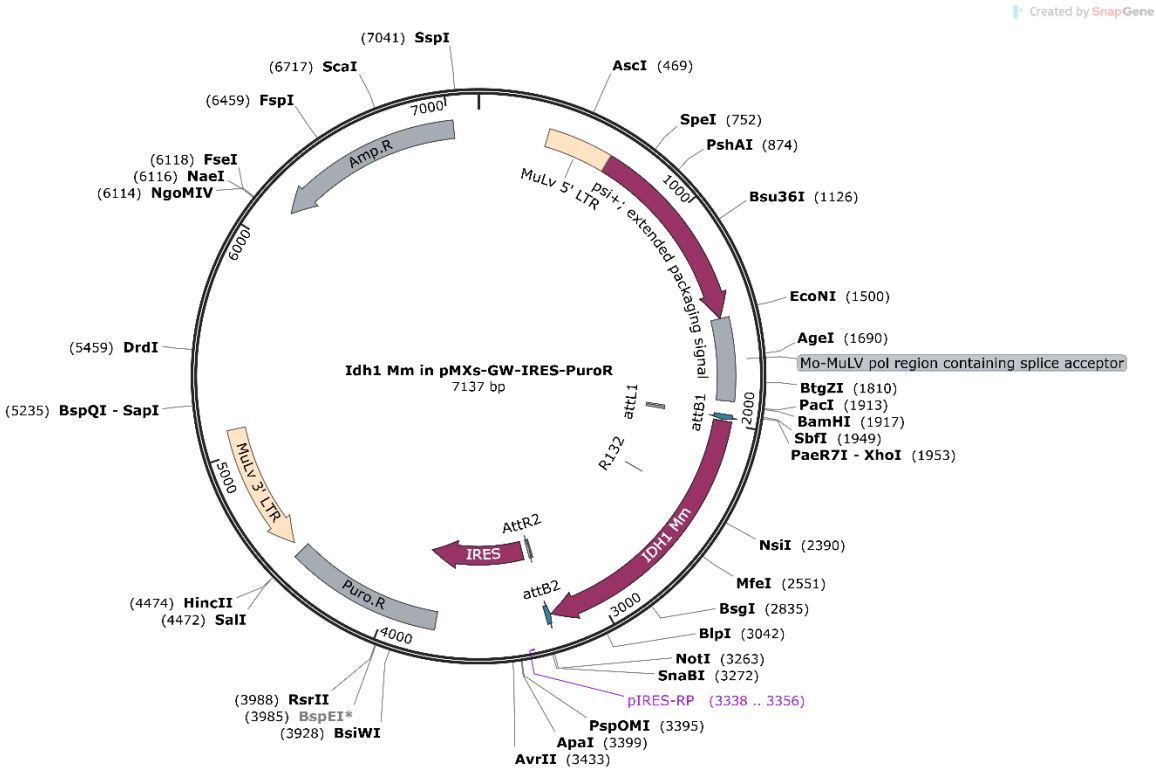

Figure 5.5: mIDH1 and IDH1wt Retroviral expression vector

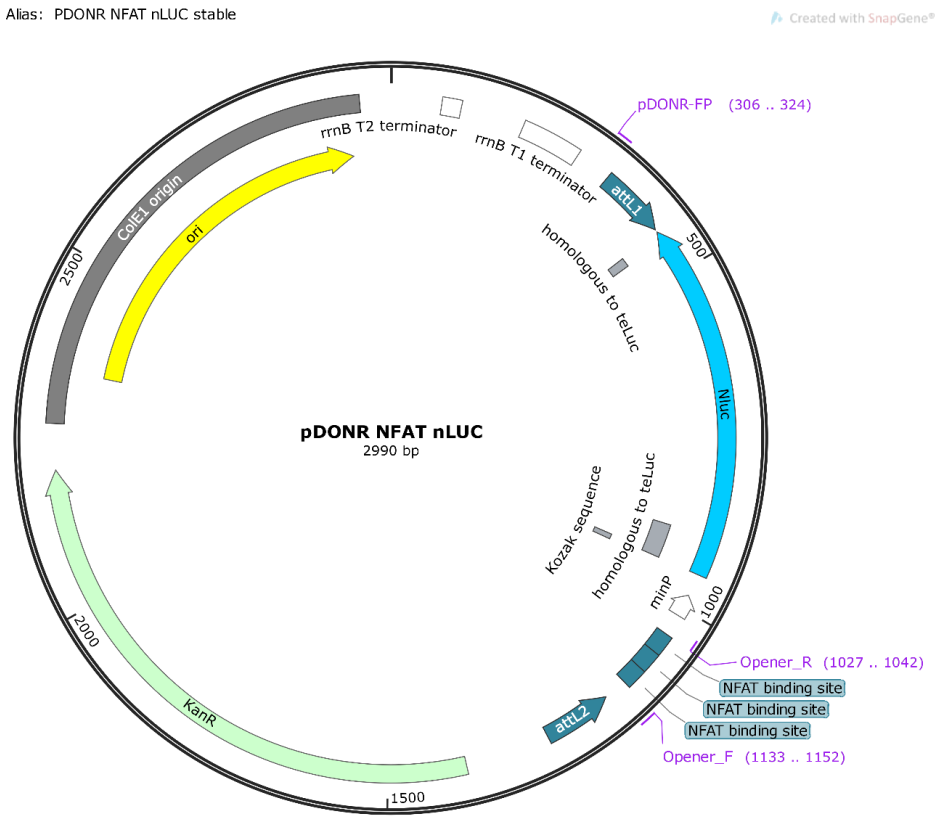

Figure 5.6: NFAT-nLUC reporter vector

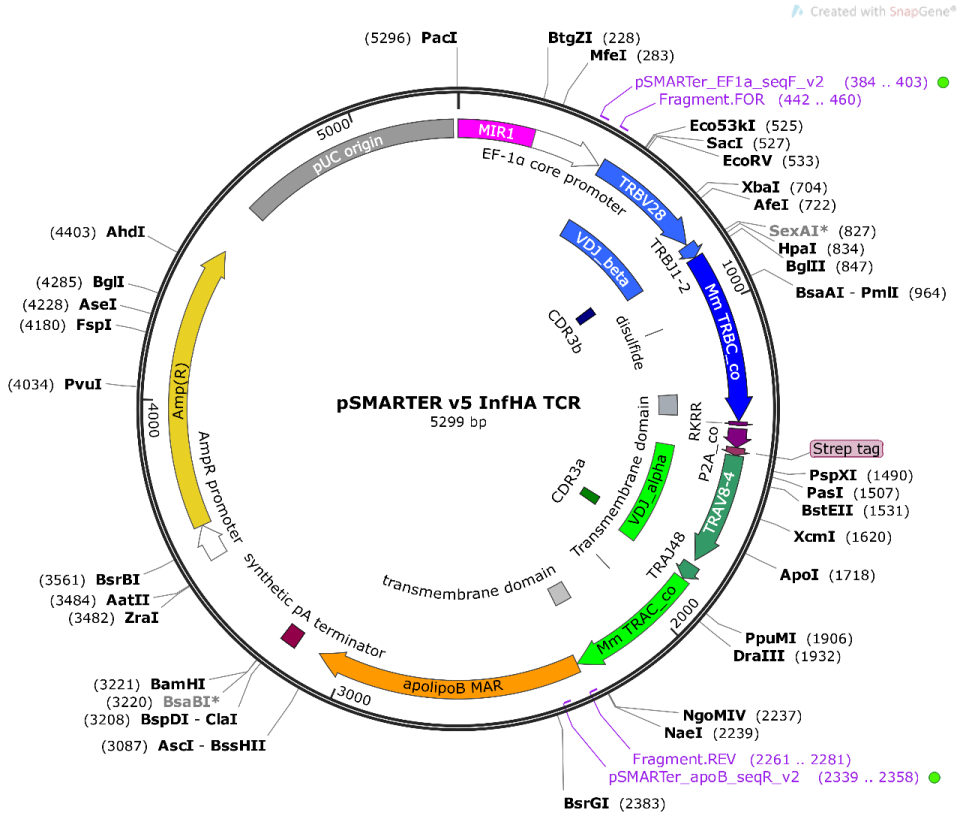

Figure 5.7: pSMARTER v5 TCR expression vector

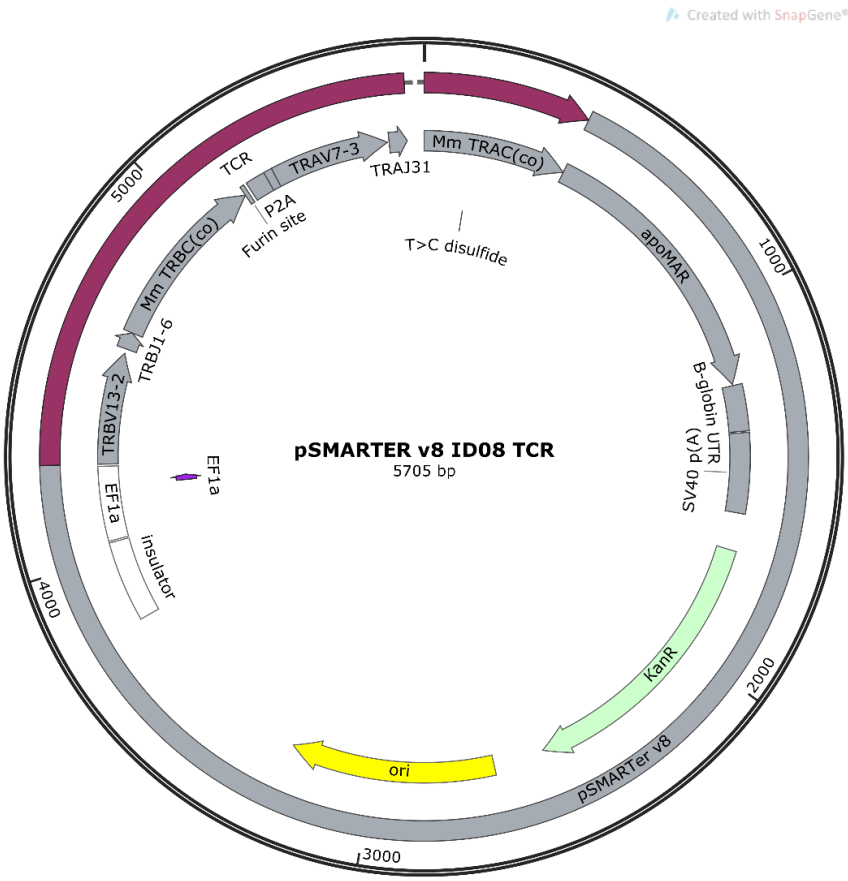

Figure 5.8: pSMARTER v8 TCR expression vector

Approaches to address the immunogenicity and immunosuppressive features of Isocitrate Dehydrogenase 1 mutated gliomas

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