Comparative immunophenotyping of peripheral blood T- and NK-cells of patients with diffuse glioma

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1. Hypothesis and Research Question

While the prognosis of many other cancers has been improved by immunotherapeutic approaches that target immune escape mechanisms, immunotherapy has so far not changed the dismal prognosis of glioblastoma patients. Glioblastomas may not only suppress the tumor-directed immune response within the tumor microenvironment but also mediate systemic immunosuppression.

We hypothesized that the immunophenotype of peripheral blood lymphocytes in patients with glioblastoma differs from that in patients with lower-grade glioma (astrocytoma and oligodendroglioma, grade 2 and grade 3) and healthy donors, and that a peripheral immunosuppression may promote more rapid tumor progression, worsening the prognosis in glioblastoma patients. Identifying an abnormal systemic immune status could open new avenues for developing glioblastoma immunotherapies aimed at overcoming immune exhaustion and reinstating immune surveillance.

2. Introduction

Gliomas are the most frequent type of primary malignant brain tumors and are commonly associated with a dismal prognosis. Patients with the most aggressive form, glioblastoma, have an average survival of 14-17 months after diagnosis (Westphal and Lamszus, 2011, Schaff and Mellinghoff, 2023, Omuro and DeAngelis, 2013, Wen et al., 2020). In comparison, lower-grade gliomas have a significantly longer survival of 9-10 years for astrocytoma patients and > 14 years for oligodendroglioma patients (van den Bent and Chang, 2018, van den Bent et al., 2017, Chen et al., 2017, Wang et al., 2023). Since the 2016 WHO classification, diffuse gliomas have been classified by isocitrate dehydrogenase (IDH) mutation status (Wesseling and Capper, 2018, Wang et al., 2023). The newly updated 2021 WHO classification divides gliomas into the following three main groups: glioblastoma, IDH-wildtype (IDHwt); astrocytoma, IDH-mutant (IDHmut); and oligodendroglioma, IDHmut and 1p19q-codeleted (Berger et al., 2022, Wang et al., 2023, WHO Classification of Tumours Board, 2021). The current standard of care for newly diagnosed glioblastomas entails surgical resection, followed by a combination of radiotherapy and chemotherapy (Mirzaei et al., 2017, Wirsching et al., 2016, McKinnon et al., 2021, Stupp et al., 2005). Despite this approach, glioblastomas rapidly recur in most patients.

In a seminal review, Hanahan and Weinberg defined six hallmarks of cancer in general, including resisting cell death, sustaining proliferative signaling, inducing angiogenesis, evading growth suppressors, enabling replicative immortality, and activating invasion and metastases (Hanahan and Weinberg, 2000). All of these tumor cell characteristics likely contribute to the aggressive nature of glioblastomas. As an update to the original six hallmarks, the same authors later also identified deregulation of cellular energetics, genome instability and mutation, tumor-promoting inflammation, and avoiding immune destruction as hallmarks of malignancy (Hanahan and Weinberg, 2011). These factors are all likely to promote glioblastoma progression. The outstanding relevance of the last of these newly recognized hallmarks, avoiding immune destruction, has been shown to be of great clinical significance, as immunological anti-tumor approaches have revolutionized the field of oncology. This advancement has been made possible by the recognition

that T-cell activation can be suppressed by specific signals, such as those mediated by the binding of programmed cell death protein 1 ligand (PD-L1) to its receptor, programmed cell death protein 1 (PD-1), and that tumors take advantage of these mechanisms by inducing T-cell suppression (Okazaki and Honjo, 2006, Okazaki et al., 2002, Iwai et al., 2002, Iwai et al., 2017, Fritz and Lenardo, 2019). By addressing this tumor-mediated suppression of T-cell activation, immune checkpoint therapy has led to a therapeutic breakthrough in several tumor entities, such as melanoma and lung cell carcinoma, including also their distant metastases (Iwai et al., 2017, Anagnostou and Brahmer, 2015, Carlino et al., 2021, Patel and Weiss, 2020, Robert et al., 2015, Horn et al., 2018). Unfortunately, immunotherapy with immune checkpoint inhibitors (ICIs) has so far not resulted in improved outcomes in glioblastoma patients (Miller and DeAngelis, 2020, Reardon et al., 2020). The control of immunosuppression likely differs among different tumor entities and subtypes. Therefore, it is of great importance to phenotype the immune status of patients with glioblastoma in comparison to other glioma subtypes and to characterize how glioblastomas escape immune surveillance. Before summarizing the current knowledge on immune escape mechanisms in glioblastoma, the following sections provide an overview of what is known about immune escape of cancer in general.

1.1. Cancer immunoediting

The concept of cancer immunosurveillance with the immune system repressing growth of cancer is more than a century old (Dunn et al., 2004b). While early anti-tumor immune responses can eliminate tumor cells and provide protection against cancer, they can later also apply selective pressure that shapes further tumor evolution after transformation has occurred. Collectively, these processes, termed cancer immunoediting, encompass three phases: elimination, equilibrium, and escape, ultimately allowing tumors with reduced immunogenicity to emerge and evade immune detection (Dunn et al., 2004a) (Figure 1). The ultimate goal of immunotherapy is to treat cancer by overcoming immune tolerance. The concept of cancer immunoediting suggests that this may be harder for progressed cancers, as compared to early-stage tumors, as they have evolved in an immune-surveilled environment and progressively acquired multiple mechanisms of immune escape.

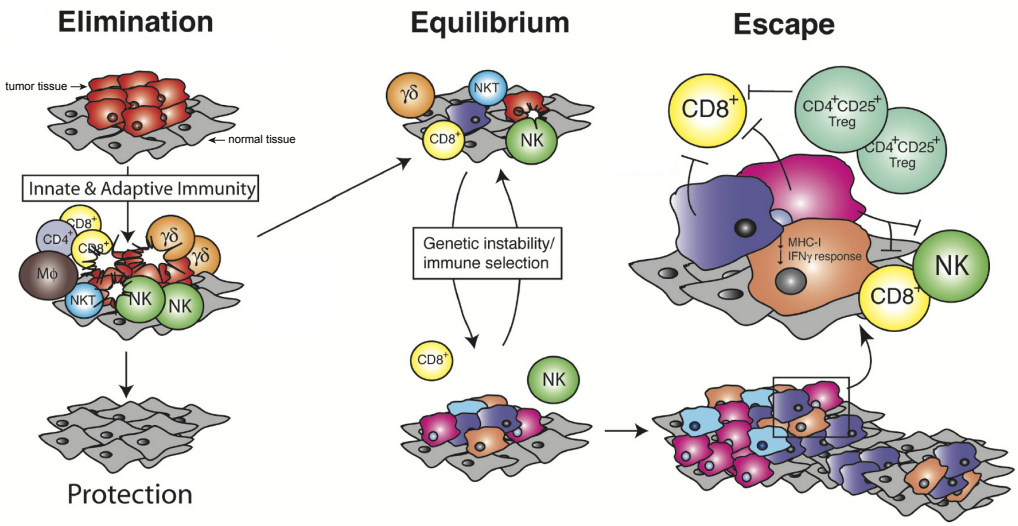


Figure 1. The three phases of cancer immunoediting – elimination, equilibrium, and escape (modified from Dunn *et al.*, 2004a).

1.2. Tumor escape mechanisms – how tumors escape immunosurveillance

Tumor-specific immunosurveillance is mediated through many complex and coordinated mechanisms, among which T-cell–mediated immunity is commonly regarded as a major player. Following the initiation of tumor antigen presentation, T cells are activated in a multi-step process that involves the binding of the T-cell receptor (TCR) to its specific antigen, provision of co-stimulatory signals to enhance the response, and the influence of pro-inflammatory cytokines to amplify and sustain immune activity. In addition, the recruitment of natural killer (NK) cells is critical, as these cells contribute to the direct elimination of tumor cells and support the overall antitumor immune defense. However, the activation of T cells following antigen binding to the TCR does not always result in a robust antitumor response. In some cases, binding may lead to inhibition, preventing an effective immune response, and potentially even inducing immune tolerance. The outcome of this interaction is influenced by a complex interplay of costimulatory and inhibitory signals mediated by surface molecules on both T cells and tumor cells. Additionally, a diverse array of cytokines further shapes and modulates the T-cell activation process, underscoring the intricate balance required for an effective antitumor response. Many malignancies suppress tumor-specific T-cell–mediated immune responses through a plethora of mechanisms which can be grouped into three main categories: inhibitory cell surface receptors, soluble factors, and immunoregulatory cells in the local tumor environment (Wherry, 2011). In addition, tumor cells can escape

recognition by T cells by losing or downregulating Major histocompatibility complex (MHC)-associated antigen presentation.

1.2.1. Inhibitory cell surface receptors

The most intensively studied and clinically targeted inhibitory immune checkpoint molecule is programmed cell death protein 1 (PD-1), which is expressed on the surface of T cells (Mirzaei et al., 2017, Pardoll, 2012, Fecci et al., 2007, Jacobs et al., 2009, Li et al., 2016, Fong et al., 2012, Bloch et al., 2013a, Jiang et al., 2015). Upon binding to its ligand, programmed death-ligand 1 (PD-L1), which is expressed on tumor cells and on antigen-presenting cells such as tumor-associated macrophages (TAMs) and microglial cells, PD-1 suppresses T-cell activity, inhibits T-cell proliferation and induces T-cell apoptosis (Figure 2). This mechanism can effectively downregulate the anti-tumor response that would otherwise be launched by T cells after recognition of tumor antigens through the T-cell receptor. Despite the therapeutic success of ICIs in other malignancies, such as melanoma or lung cancer, administration of neither Pembrolizumab or Nivolumab, both monoclonal antibodies directed against PD-1, has improved overall survival in glioblastoma patients (Reardon et al., 2020, Reardon et al., 2021, Kurz et al., 2018, Mantica et al., 2018, Omuro et al., 2018, Chamberlain and Kim, 2017, Blumenthal et al., 2016, Miller and DeAngelis, 2020).

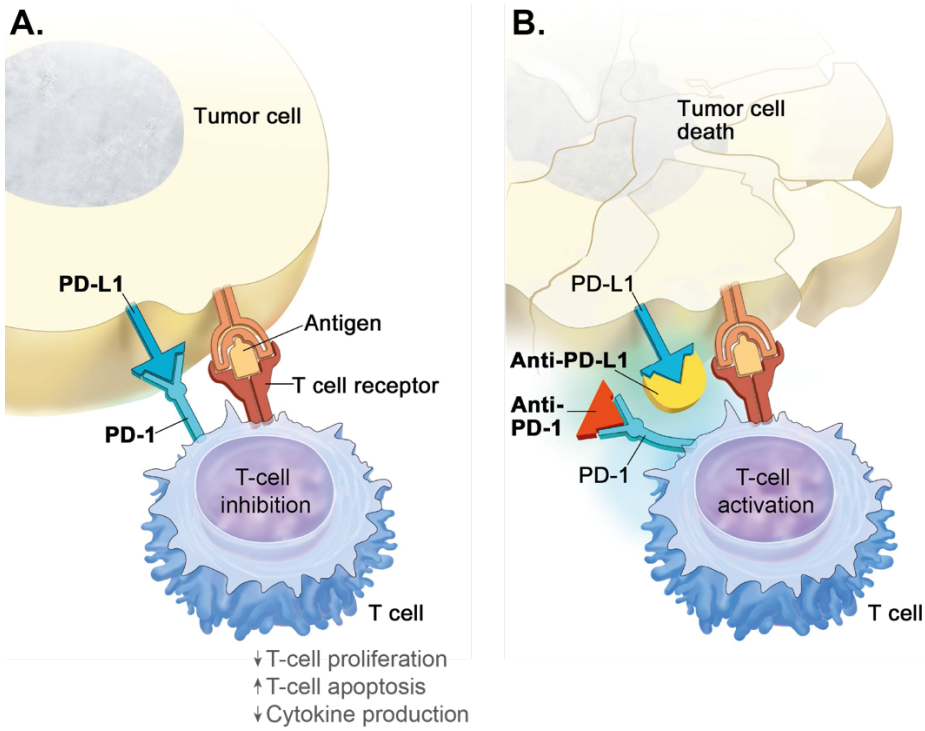


Figure 2. The PD-1/PD-L1 immune checkpoint axis regulates T-cell activity (modified from the National Cancer Institute, 2015). Following tumor antigen recognition by the T-cell receptor, binding of PD-L1 on the tumor cell to PD-1 on the T cell inhibits T-cell activation and effector function (A). Blocking this interaction with an anti–PD-1 antibody restores T-cell activity and promotes tumor-cell destruction (B).

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is another immune checkpoint molecule expressed on T cells. Its ligand CD80/86 is expressed on antigen-presenting cells and binds with greater affinity to CTLA-4 than to CD28, an important co-stimulatory cell surface receptor on T cells. CTLA-4 thus effectively outcompetes CD28 and inhibits the costimulatory CD80/86 signal needed to activate T cells (Pardoll, 2012). CTLA-4 also stimulates regulatory T cells, further contributing to downregulation of the immune response (Pardoll, 2012). While CTLA-4 checkpoint blockade has shown therapeutic success in melanoma (Willsmore et al., 2021, Bagchi et al., 2021), glioblastomas do not appear to respond to this therapeutic approach either (Omuro et al., 2018). Other inhibitory T cell surface molecules include lymphocyte-activation gene 3 (LAG-3), T-cell immunoglobin and mucin-domain containing 3 (TIM-3), B- and T-lymphocyte attenuator (BTLA), and T-cell immunoreceptor with Ig and ITIM domains (TIGIT) (Pardoll, 2012, Jiang et al., 2015, Fecci et al., 2014). LAG-3 amplifies regulatory T cell responses while inhibiting $CD8^+$ effector function (Pardoll, 2012). TIM-3 inhibits T_H1-cell responses (Pardoll, 2012) and drives T-cell differentiation towards the T_H2 phenotype.

1.2.2. Impaired antigen presentation

Tumors can also evade immune detection by impairing antigen presentation through multiple, often converging mechanisms. In addition to the quantitative downregulation or loss of MHC class I molecules on the tumor-cell surface, tumor cells may acquire defects in components of the antigen-processing machinery, such as loss or mutation of β2-microglobulin (B2M), an essential structural component required for the stability and surface expression of MHC class I complexes, or reduced expression and function of the transporter associated with antigen processing (TAP) subunits 1 and 2 (TAP1 and TAP2), which mediate the translocation of antigenic peptides from the cytosol into the endoplasmic reticulum for MHC class I loading. These alterations directly interfere with the assembly and surface expression of peptide-MHC class I complexes, thereby reducing the density and diversity of tumor-derived antigens available for immune surveillance, limiting efficient immune recognition and facilitating immune escape (Dunn et al., 2004a, Seliger et al., 2000).

1.2.3. Soluble factors

Tumors secrete several soluble factors linked to T-cell dysfunction (Wherry, 2011). Interleukin 10 (IL-10) and transforming growth factor beta (TGFβ) are two immunosuppressive cytokines that are capable of silencing effector T cells and inhibiting T-cell proliferation (Li et al., 2016, Bloch et al., 2013a, Jiang et al., 2015, Zisakis et al., 2007, Doucette et al., 2013, Lohr et al., 2011a). TGFβ can also shift naïve T cells to regulatory T cells (Lohr et al., 2011a) and IL-10 further supports this shift (Li et al., 2016). Furthermore, IL-10 can upregulate the expression of the immune checkpoint ligand PD-L1. T_H1 cells secrete pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin 2 (IL-2), interleukin 15 (IL-15), and interferon gamma (IFNγ) (Zisakis et al., 2007). In contrast, T_H2 cells secrete interleukin 4 (IL-4), IL-10, and interleukin 13 (IL-13), which have anti-inflammatory and pro-tumorigenic properties (Zisakis et al., 2007). Therefore, a shift from T_H1 to T_H2 T cells can promote tumor immune escape (Zisakis et al., 2007). Other soluble factors that contribute to immune escape include vascular endothelial growth factor (VEGF), indoleamine 2,3-dioxygenase (IDO), prostaglandin E_2 (PGE_2), and adenosine (Zisakis et al., 2007, Jiang et al., 2015, Dunn et al., 2004a).

1.2.4. Immunoregulatory cells

The induction of regulatory T cells (Tregs) is another mechanism of cancer immune escape. Tregs are an inhibitory subset of T cells that can suppress or downregulate the activation, proliferation and cytokine secretion of effector T cells (Santegoets et al., 2015). Furthermore, Tregs inhibit the expression of co-stimulatory molecules, antigen-presenting molecules, and inflammatory cytokines in antigen-presenting cells (APCs), thus attenuating their ability to stimulate T-cell responses (Wang et al., 2024). IL-2 is not only a pro-inflammatory cytokine but also helps to maintain Tregs (Abbas et al., 2018). Treg differentiation is dependent on IL-10, a potent anti-inflammatory cytokine that inhibits T-cell proliferation (Moore et al., 2001). Tregs are commonly defined by the expression of CD3, CD4, FOXP3 and CD25, and negativity for CD127 (Pardoll, 2012, Fecci et al., 2007, Jacobs et al., 2009, Li et al., 2016, Fong et al., 2012), but can also be defined as $CD3^{+}CD4^{+}CD25^{high}CD127^{low}$ (Mohme et al., 2018). Tregs often present additional surface markers such as CD39, CD73, and killer cell lectin-like receptor subfamily G member 1 (KLRG1)(Santegoets et al., 2015). CD39 and CD73 are both membrane-bound ectonucleotidases that are involved in ATP metabolism (Santegoets et al., 2015). CD39 converts ATP to ADP and AMP, which is further converted to adenosine by CD73. By reducing ATP, which is known to exert pro-inflammatory effects, and by generating adenosine which can suppress T-cell and NK-cell functions, CD39 and CD73 both act as local anti-inflammatory, immunosuppressive immunomodulators (Mohme et al., 2018). This mechanism is often employed by Treg cells but can also be found in other immune cell populations.

In addition to Tregs, myeloid-derived suppressor cells (MDSCs) are immunoregulatory cells that contribute to tumor progression and immune evasion by suppressing T-cell activity (Lin et al., 2024, Codrici et al., 2022, Grabowski et al., 2021). Defined as $CD11b^{+}CD33^{+}HLA\text{-}DR^{-/low}$ (HLA-DR: Human leukocyte antigen-DR) (Grabowski et al., 2021), MDSCs inhibit T-cell activation and proliferation, suppress NK cell function, and promote Treg development (Codrici et al., 2022). MDSCs express arginase, which leads to decreased levels of L-arginine, an amino acid that is needed for normal T-cell function (Grabowski et al., 2021). Additionally, MDSCs secrete nitric oxide and produce reactive oxygen species (ROS), which further suppresses T-cells (Grabowski et al., 2021). They are typically classified into

two main subsets: monocytic MDSCs (M-MDSCs; $CD14^+$) and granuolocytic or polymorphonuclear MDSCs (PMN-MDSCs; $CD15^+$) (Lin et al., 2024, Grabowski et al., 2021). A third category, early stage MDSCs (eMDSCs), has been recently identified as a subtype, whose suppressive capacity appears to be lower than that of other subsets (Lin et al., 2024, Codrici et al., 2022). In glioblastoma specifically, the presence of MDSCs is associated with a poor prognosis (Lin et al., 2024, Codrici et al., 2022, Grabowski et al., 2021). Glioblastomas secrete various factors, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin 6 (IL-6), and VEGF, which drive the recruitment and expansion of MDSCs (Lin et al., 2024). Alongside Tregs and MDSCs, circulating monocytes, the precursors of tissue macrophages, can contribute to immune regulation through cytokine secretion and modulation of peripheral T-cell activity (Yang et al., 2020, Bowman and Joyce, 2014).

1.3. Basics of T-cell immunology

It is important to recognize that T cells are not a homogeneous cell population, but that there are many different types of T cells, which play different roles in anti-tumor immune responses. T-cell subtypes differ in multiple aspects depending on their function, activation, and maturation. These subtypes are not necessarily static but can change into other subtypes, for example, in response to stimuli and their environment. One of the most important ways to define the functionality and activation profile is to classify both $CD4^+$ and $CD8^+$ T cells as either naïve T cells (T_{NV}) or non-naïve cells, including central memory T cells (T_{CM}), transitional memory T cells (T_{TM}), effector memory T-cells (T_{EM}), and terminal effector T cells (T_{TE}). These cells and their memory compartments show progressive differentiation along a continuum with decreasing proliferative potential, self-renewal and multipotency on the one hand, and increasing effector function and antigen dependence on the other hand (Mahnke et al., 2013) (Figure 3).

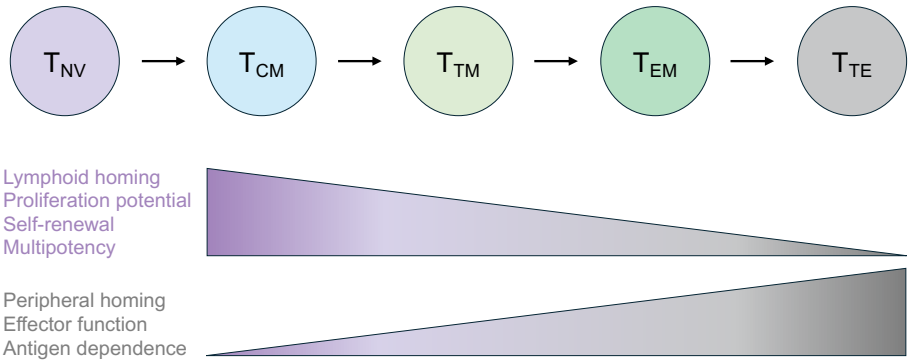


Figure 3. Progressive differentiation of T-cell effector and memory subsets (modified from Mahnke *et al.*, 2013). Naïve T cells (T_{NV}) progressively differentiate into central memory (T_{CM}), transitional memory (T_{TM}), effector memory (T_{EM}), and terminal effector (T_{TE}) subsets. With increasing differentiation, T cells lose lymphoid-homing capacity and proliferative potential while gaining peripheral-homing ability and effector function.

In addition to their differentiation type, T cells can be classified based on cytokine profiles. $CD4^+$ T cells can be broadly divided into conventional $CD4^+$ T cells and Tregs. Conventional $CD4^+$ T cells are divided into the following subsets: T_H1, T_H2, T_H17, non-classical T_H1 (ncT_H1), follicular T-helper (T_{FH}), and gamma delta ($T_{\gamma\delta}$) T cells. $CD4^+$ T_H1 cells, which produce IFNγ, are important for anti-tumor responses, e.g. by priming $CD8^+$ cytotoxic T cells (Dong, 2021, Basu et al., 2021). T_H2 cells are thought to downregulate anti-tumor responses, e.g. by inhibiting T_H1 cells via IL-4 production (Dong, 2021, Basu et al., 2021). Differentiation of naïve T-helper cells into T_H2 cells also requires IL-4 (Dong, 2021). T_H17 cells, characterized by abundant secretion of interleukin 17 (IL-17) and exhibit both pro- and anti-tumorigenic activities (Dong, 2021, Chang, 2019). IL-17 is a pro-inflammatory cytokine that may promote tumor formation by supporting chronic inflammation but can also mediate anti-tumor immunity by recruiting neutrophils and NK cells (Dong, 2021, Chang, 2019). Derived from T_H17 cells, ncT_H1 cells produce IFNγ like classic T_H1 cells, but maintain the T_H17-typical expression of C-C chemokine receptor type 6 (CCR6; CD161) and may be particularly effective in mediating anti-tumor responses (Cosmi et al., 2014, Basdeo et al., 2017). Follicular T-helper cells (T_{FH}) are a T-helper subset distinct from T_H1, T_H2, and T_H17 that are essential for the development and function of germinal centers in lymph nodes and with that are critical for launching B-cell responses (Crotty, 2011, Nurieva et al., 2008). In cancer, T_{FH} cells contribute to the formation of tertiary lymphoid structures through cytokines such as CXCL13 and interleukin 21 (IL-21), supporting local B-cell activation and antitumor immunity that often correlates with improved patient prognosis, e.g. in breast cancer (Xin et al.,

2025). Gamma delta T cells ($T_{\gamma\delta}$) are another distinct subset of T cells that do not require antigen processing. They express IFNγ and IL-17, which may also mediate antitumor responses (Dong, 2021). Mucosal-associated invariant T cells (MAIT) are an additional subset of T cells that display qualities of both innate and adaptive immune responses. Reports of antitumor activity and enrichment in cancers suggest a role for MAIT cells in antitumor immunity (Godfrey et al., 2019).

The functional activity status of T-cell subsets can be evaluated by markers that indicate activated metabolism and proliferation. In addition to metabolic and proliferative markers, HLA-DR, an MHC class II molecule, is widely used as an activation marker on T cells. Expression of HLA-DR on $CD4^+$ and $CD8^+$ T cells reflects recent or ongoing immune activation and has been associated with immune dysfunction in cancer, including glioblastoma (Mohme et al., 2018). Accordingly, HLA-DR represents a relevant marker for assessing activation states within peripheral T-cell populations. CD71 (transferrin receptor 71) is required for iron uptake, which is then utilized in multiple cellular processes, including DNA synthesis and thus cell proliferation (Candelaria et al., 2021). CD98 regulates amino acid transport (Ip and Sethi, 2016) and supports clonal expansion (Cantor and Ginsberg, 2012). Glucose uptake in lymphocytes is regulated by GLUT1 (Lang et al., 2020) which is required for proper T-cell function. CD95 (FAS) is responsible for the induction of apoptosis and is upregulated in proliferating lymphocytes (Chen et al., 2010, Martin-Villalba et al., 2013). Sphingosine-1-phosphate receptor 1 (S1PR1) is an activation marker that is necessary for T cells to exit the lymph nodes (Benechet et al., 2016, Wang et al., 2019, Chongsathidkiet et al., 2018, Chongsathidkiet et al., 2016). Important pro-inflammatory cytokines involved in the anti-tumor response include IFNγ and TNFα. These are not only secreted by T cells but also by other immune cells such as NK cells and macrophages (Conlon et al., 2019, Balkwill, 2006).

1.4. T-cell exhaustion

Another important aspect of T-cell biology is the downregulation of function following a prolonged status of high activity. T cells exhibiting such downregulated effector functions after being continuously activated are defined as exhausted (Mirzaei et al., 2017). Often found in cancer and chronic infections, exhaustion is a

hyporesponsive state where T-cell function deteriorates owing to persistent and excessive antigen exposure and stimulation signals (Mirzaei et al., 2017, Woroniecka et al., 2018a, Wherry and Kurachi, 2015). Exhausted T cells possess inhibitory receptors and produce fewer effector cytokines (Jiang et al., 2015, Zhao et al., 2020, Mohme and Neidert, 2020). Inhibitory checkpoint receptors such as PD-1, CTLA-4, LAG-3, and TIM-3, as well as the ligand PD-L1 are expressed at higher levels by exhausted T cells (Zhao et al., 2020, Mohme and Neidert, 2020, Sayour et al., 2015, Wherry, 2011, Jiang et al., 2015, Fecci et al., 2014), whereas effector cytokines such as IL-2, IFNγ, and TNFα are reduced (Jiang et al., 2015). Exhausted T cells demonstrate a hierarchical loss of function in response to continuous antigen exposure. First, IL-2 production is lost, which in $CD4^+$ T cells can promote a shift away from T_H1 differentiation towards a more tumor-supportive T_H2 phenotype. Next, the production of the pro-inflammatory cytokine TNFα fails. With longer exposure to tumor antigens, IFNγ production is halted. In $CD8^+$ T cells, this progressive dysfunction is characterized by a stepwise loss of proliferative capacity and cytotoxic effector functions, accompanied by sustained expression of inhibitory receptors. Ultimately, T cells are physically depleted by induction of apoptosis, e.g. via the FAS/FAS-ligand pathway or indirectly through PD-1 by inhibiting survival signals (Jiang et al., 2015, Chen et al., 2010, Martin-Villalba et al., 2013). However, exhausted T cells still have the capacity to be reactivated. Overlapping with exhaustion, the more permanent T-cell senescence represents a distinct form of T-cell dysfunction associated with terminal differentiation and reduced proliferative capacity, distinguished by the expression of markers such as CD57 and KLRG1, with partial overlap with inhibitory receptors including TIM-3 and TIGIT (Zhao et al., 2020).

1.5. Immune escape of glioblastomas

It is not entirely understood why current immunotherapeutic approaches have largely failed in glioblastoma patients. A growing body of evidence suggests that glioblastomas induce T-cell dysfunction to evade immune recognition, attack and destruction by T cells (Pearson et al., 2020, Yu et al., 2023). In a recent review, Woroniecka *et al*. (Woroniecka et al., 2018b) presented five categories of T-cell dysfunction that characterize the impaired immune response in glioblastoma patients. Senescence, the first category, results from shortened telomeres, and

describes a hypofunctional state. Due to chronic proliferation as well as damage by reactive oxygen species (ROS), telomeres shorten, and once critical shortening has been reached, cells cease to divide. To bypass this pathway, telomerase can lengthen telomeres, a mechanism often used by tumors, including glioblastoma cells, to resist senescence. However, immune cells are unable to upregulate telomerase and are thus more susceptible to senescence than the tumor itself. T-cell senescence can be defined by $CD57^{+}CD27^{-}CD28^{-}$ expression. Unsurprisingly, the short overall survival of glioblastoma patients has been shown to correlate with high levels of $CD4^{+}CD57^{+}CD28^{-}$ senescent T cells (Woroniecka et al., 2018b). The second category, tolerance, can be further divided into central and peripheral tolerance. Although tumors have only limited influence on central tolerance through negative selection during T-cell development, many pathways exist to induce peripheral tolerance. For example, glioblastomas can induce peripheral T-cell depletion/apoptosis via FAS-ligand expression. Glioblastoma cells also support differentiation into Tregs via several different mechanisms which also contribute to peripheral tolerance (Woroniecka et al., 2018b). Through continuous antigen exposure, T cells can enter a state of anergy, the third category, in which they become unresponsive to their target antigen and inactive. T cells are then unable to produce IL-2, which is required to support proliferation. Glioblastoma patients were shown to exhibit decreased levels of IL-2 in their blood, indicating tumor-associated anergy (Woroniecka et al., 2018b). Similarly, T-cell exhaustion, the fourth category, also results from continuous antigen exposure and describes a hyporesponsive state that is different from T-cell anergy. Many exhaustion markers, including PD-1, CTLA-4, TIM-3, LAG-3, BTLA, 2B4, CD160, TIGIT, and CD39, were shown to be upregulated in peripheral T cells as well as in tumor-infiltrating lymphocytes of glioblastoma patients and to correlate with poorer overall survival (Woroniecka et al., 2018b). T-cell ignorance, the fifth category, refers to a state in which T cells remain naïve because they never encounter or recognize their antigen, typically when it is inaccessible, present at very low levels, or encountered without the inflammatory cues required for activation. Ignorant T cells are naïve and functional. For example, through the loss of S1PR1, T cells are trapped in the bone marrow and are unable to mount a proper peripheral immune response. A higher proportion of ignorant T cells has been reported in patients with glioblastoma (Woroniecka et al., 2018b).

One possible explanation why glioblastomas resist immunotherapeutic approaches is that they are less exposed to systemic immunosurveillance because of their anatomical location within the central nervous system (CNS), beyond the blood-brain barrier (BBB). While the long-held belief that the brain is an immune sanctuary has largely been disproven, it is still considered to some extent immune-privileged due to the tight blood-brain barrier and a unique microenvironment, characterized by myeloid and glial cells and a tightly regulated presence of lymphocytes, including T cells (Phadke et al., 2022, Fares et al., 2021, Westphal et al., 2017, Sampson et al., 2020). Consistent with the partial immune privilege of the CNS, melanoma metastases in the brain respond with lower efficacy than peripheral metastases to immunotherapy(Kluger et al., 2019, Gutzmer et al., 2020). The BBB is partially leaky in both brain metastases and glioblastomas, as evidenced by contrast enhancement on imaging. However, this disruption is likely insufficient to allow efficient penetration of immunotherapeutic drugs into the tumor, and the immunosuppressive CNS microenvironment further counteracts the efficacy of immunotherapy. Notably, circulating tumor cells, although rare, have been identified in the peripheral blood of a small subset patients with glioblastoma (Müller et al., 2014). As glioblastomas only very rarely metastasize outside the brain, this observation could be interpreted as the peripheral immune response being capable of clearing glioblastoma cells in the periphery, but not within the immune-privileged CNS.

Microglia, the resident macrophages of the CNS, exhibit significant immunomodulatory functions (Codrici et al., 2022, Grabowski et al., 2021, Wu and Watabe, 2017) and can contribute to immunosurveillance or failure of immunosurveillance in CNS tumors, including glioblastoma. Under normal conditions, microglia perform innate immunosurveillance and maintains tissue and immune homeostasis within the CNS (Codrici et al., 2022). The classical M1 phenotype mediates pro-inflammatory responses that contribute to anti-tumor immunity through the release of cytokines and ROS (Wu and Watabe, 2017). However, microglia can be polarized into an immunosuppressive, pro-tumor, M2-like phenotype that supports tumor growth, invasion, and immune evasion (Wu and Watabe, 2017). M2 microglia contributes to tumor progression by secreting immunosuppressive factors, such as IL-10 and TGFβ, as well as by facilitating tumor invasion and angiogenesis through the production of matrix metalloproteinases

(MMPs) and VEGF (Wu and Watabe, 2017). However, the M1/M2 polarization paradigm represents an oversimplification of microglial activation, as microglia in glioblastoma exhibits a continuum of functional states rather than discrete phenotypes (Quail and Joyce, 2017). Within the glioblastoma microenvironment, microglia form part of a more complex population of TAMs. These comprise both brain-resident microglia and macrophages derived from circulating monocytes that infiltrate the tumor through a disrupted BBB. Although these populations differ in origin, they share overlapping, predominantly immunosuppressive functions and together constitute the major immune-cell fraction in glioblastoma (Quail and Joyce, 2017). Microglia- and macrophage-derived cytokines such as IL-10 and TGFβ further suppress anti-tumor responses by supporting Treg function (Grabowski et al., 2021, Codrici et al., 2022).

Glioblastoma cells themselves can also evade immune control through cellular alterations affecting antigen presentation and immune recognition. Genetic mechanisms leading to irreversible loss of antigen presentation have been described, including the loss of heterozygosity at the HLA class I locus and B2M region, which are associated with reduced HLA class I expression and shorter overall survival in glioblastoma patients (Yeung et al., 2013). More recently, somatic mutations affecting classical and non-classical HLA genes, as well as genes involved in antigen processing and presentation such as the TAP1/TAP2 and B2M, have been identified in malignant gliomas, with a higher frequency observed in recurrent compared to primary glioblastomas (Schulte et al., 2025). These alterations were associated with reduced intratumoral $CD3^+$ and $CD8^+$ T-cell infiltration, suggesting impaired immune recognition of mutated tumors (Schulte et al., 2025). In addition, mutations affecting stress-induced ligands such as MICA and MICB, which are important for NK cell activation, have been reported and may further contribute to immune evasion (Schulte et al., 2025). However, the overall frequency of HLA and antigen presentation pathway alterations in primary glioblastoma is low, indicating that while tumor-intrinsic antigen presentation defects can contribute to immune escape, they are unlikely to represent a dominant mechanism in most newly diagnosed glioblastomas.

Taken together, glioblastoma employs a complex and highly coordinated network of immune escape mechanisms that converge on profound dysfunction of the adaptive immune response. Beyond tumor cell-intrinsic alterations affecting antigen presentation, glioblastoma promotes multiple, overlapping states of T-cell dysfunction, including exhaustion, senescence, tolerance, anergy, and immune ignorance, as outlined above. These processes are driven by chronic antigen exposure, sustained inhibitory signaling through immune checkpoints, immunosuppressive soluble factors, and the absence of adequate co-stimulatory and inflammatory cues. In parallel, the glioblastoma microenvironment is dominated by immunoregulatory myeloid populations, including microglia, infiltrating macrophages, and MDSCs, which further suppress anti-tumor immunity through cytokine-mediated, metabolic, and cell–cell-dependent mechanisms. Together, these mechanisms provide a plausible, though likely incomplete, explanation for the limited efficacy of current immunotherapeutic approaches in glioblastoma.

Although many immune escape mechanisms in glioblastoma have been identified, their relative contribution, hierarchy, and impact on systemic immunity remain incompletely understood, which has hampered the success of immunotherapeutic strategies. While local immunosuppression within the tumor microenvironment is a hallmark of glioblastoma, increasing evidence indicates that immune dysfunction is not restricted to the tumor site but extends to the systemic immune compartment. Previous studies have demonstrated lymphopenia, impaired T-cell trafficking, and functional inhibition of circulating T cells in patients with glioblastoma, suggesting a clinically relevant role for peripheral immune suppression. Consistent with this notion, immunophenotyping has revealed a pronounced exhaustion signature in tumor-infiltrating lymphocytes, whereas peripheral T cells exhibit a functionally impaired but phenotypically distinct immune profile, characterized by reduced cytokine responsiveness rather than overt exhaustion marker expression (Mohme et al., 2018).

Based on these observations, we hypothesized that systemic alterations of peripheral T-cell subsets and their functional state represent a key component of glioblastoma-associated immune escape. Therefore, the aim of this project was to characterize the peripheral T-cell compartment in a large cohort of glioblastoma

patients in comparison to patients with other gliomas and healthy donors. Using multiparameter flow cytometry, we analyzed T-cell differentiation, activation, exhaustion, metabolic features, and cytokine-associated functional states to gain deeper insight into systemic immune dysregulation in glioblastoma.

3. Materials and Methods

3.1. Sample collection and patient data

Glioma patients scheduled for neurosurgical tumor resection were identified, and blood was drawn from the patients on the day of surgery. Samples were collected in the Laboratory for Brain Tumor Biology of the Department of Neurosurgery at the University Medical Center Hamburg-Eppendorf (Universitätsklinikum Hamburg-Eppendorf, UKE). Blood samples were processed immediately to isolate peripheral blood mononuclear cells (PBMCs). For the healthy donor cohort, pre-selected samples of blood donors aged > 55 years were collected from the blood bank (Blutspende) of the UKE.

The study was approved by the Hamburg Ethics Committee under protocol PV4904, ensuring compliance with the established ethical guidelines for research involving human participants. In accordance with the principles outlined in the Declaration of Helsinki, informed consent was obtained from all participants before their involvement in the study. Confidentiality and data protection were prioritized, with all personal data pseudominized and securely stored to prevent unauthorized access.

The surgically excised tumor specimens were routinely sent to the Department of Neuropathology for histological and molecular diagnostic evaluation. Based on the 2021 WHO classification of CNS tumors, patients were grouped into the following categories (WHO Classification of Tumours Board, 2021):

- Glioblastoma, IDHwt
- astrocytoma, IDHmut, grade 2
- astrocytoma, IDHmut, grade 3
- oligodendroglioma, IDHmut and 1p/19q-codeleted, grade 2
- oligodendroglioma, IDHmut and 1p/19q-codeleted, grade 3

In addition, information on methylation of the O(6)-methylguanine-DNA methyltransferase (MGMT) gene promoter was recorded from the neuropathology report.

Patient charts were used to identify clinical data, including date of birth, sex, and date of surgery. Additional treatments, such as steroids or metformin, as well as pre-surgical blood parameters were also recorded. BrainLab® radiographic pre-surgical cranial magnetic resonance imaging (MRI) data were used to determine tumor size and location. Additionally, through repetitive chart reviews and multiple telephone calls to primary care physicians, follow-up, overall survival, and date of death (if applicable) were recorded.

3.2. PBMC isolation – Ficoll gradient

PBMCs were isolated from EDTA-anticoagulated whole blood using a density gradient centrifugation method (Ficoll gradient). Initially, blood was diluted 1:1 with phosphate-buffered saline (PBS) in a 50 mL tube. 15 mL of LymphoSep™ Lymphocyte Separation Medium (LSM 1077) were added to a separate 50 mL tube. Slowly and at an angle, in order to avoid mixing of layers, the blood-PBS mixture was then carefully pipetted on top of the LymphoSep™ using a serological pipette. Then, the samples were centrifuged at 1200 x g for 20 minutes at room temperature without applying a brake to preserve the integrity of the gradient.

Following centrifugation, the third layer from the bottom, containing PBMCs (above the red blood cells and LymphoSep™ layers), was carefully aspirated using a sterile glass Pasteur pipette and transferred to a new 50 mL tube (Figure 4). The tube was filled with PBS to wash the cells and the suspension was centrifuged at 300 x g for 7 minutes.

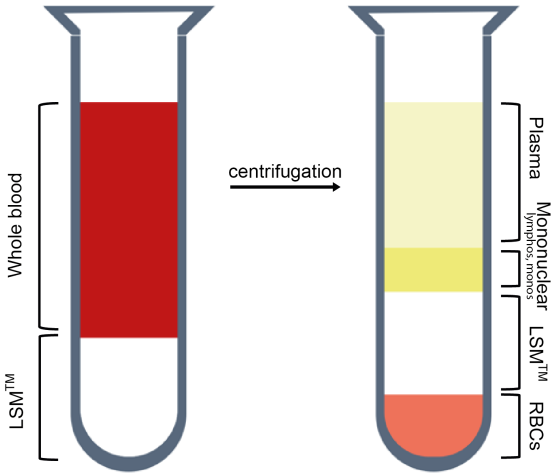


Figure 4. Ficoll gradient for isolation of PBMCs.

The supernatant was aspirated using a sterile glass Pasteur pipette, and the cell pellet was resuspended in 25 mL of PBS. A 10 μL aliquot of the suspension was loaded onto a Neubauer counting chamber with a coverslip for cell counting. The washing steps (centrifugation at 300 x g and aspiration) were repeated to further

purify the cells. The final cell pellet was resuspended in 1 mL freezing medium containing fetal calf serum (FCS) with 10% dimethyl sulfoxide (DMSO) and Roswell Park Memorial Institute (RPMI) medium. Aliquots containing approximately 10-15x10^6 cells were transferred into cryovials, frozen, and stored at -80°C for subsequent use.

3.3. Fluorescence-activated cell sorting (FACS) panels

Flow cytometry employs different lasers to rapidly analyze cells incubated with fluorescently labeled antibodies. The cells are separated into thin capillaries, allowing laser light-induced excitation and detection of multiple fluorescence signals from a high number of single cells (Givan, McKinnon, 2018, Adan et al., 2017, Mousset et al., 2019).

Five fluorescence-activated cell sorting (FACS) panels were used (Table 1). Following antibody titrations and compensation controls (see below) on healthy donor PBMCs, individual markers were exchanged for alternative fluorescence to optimize expression and minimize spectral overlap. See Supplemental Table 1 for the epitope, clone, lot number, company, and dilution of each antibody used.

Table 1. Fluorescence of surface markers in five established FACS panels.

Laser	Fluorescence	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
		T cell exhaustion	Teff/Treg	Th subsets	T cell metabolism	Cytokines
		Epitope	Epitope	Epitope	Epitope	Epitope
405nm	**BV 785**	CD25	CD45RA	CD45RA	CD45RA	**CD45RO (surf)**
	BV 711	KLRG1	KLRG1			
	BV 650	CD127	CD127	CXCR3		TNFa
	BV 605	CD57	CD27	CD161		
	BV 570					
	BV 510	CD4	CD4	CD4	CD4	CD3
	BV 421	BTLA	CD25	CRTH2	FAS	IL-2
488nm	**PerCP-Cy5.5**	CD3	CD3	CD3	CD3	CD4
	FITC, AlexaFluor488	2B4	HLA-DR	Tgd	CD98	IFNg
561nm	**PE-Cy7**	CD56	CD39	CCR4	CD71	**CD56 (surf)**
	PE-Cy5.5					
	PE-Cy5					
	PE-Dazzle	TIGIT	CD28	CXCR5		IL-4
	PE	Tim-3	CD73	CCR6	GLUT1	IL-10
640nm	**APC-Cy7**	Zombie NIR	Zombie NIR	Zombie NIR	Zombie NIR	Zombie NIR
	AlexaFluor700	CD8	CD8	CD8	CD8	**CD8 (surf)**
	APC	PD-1	CCR7	S1PR1	PD-1	IL-17A

With over 200 patients, each with 5 panels and each panel with 10-14 different fluorescent antibody markers it was crucial to minimize total antibody use by optimizing the staining procedures to the lowest antibody concentration. To determine at which concentration/dilution the antibodies were able to reliably stain, a series of antibody titrations was performed for each individual antibody/color combination. The following steps describe the process using the example of CD4 conjugated to BV510.

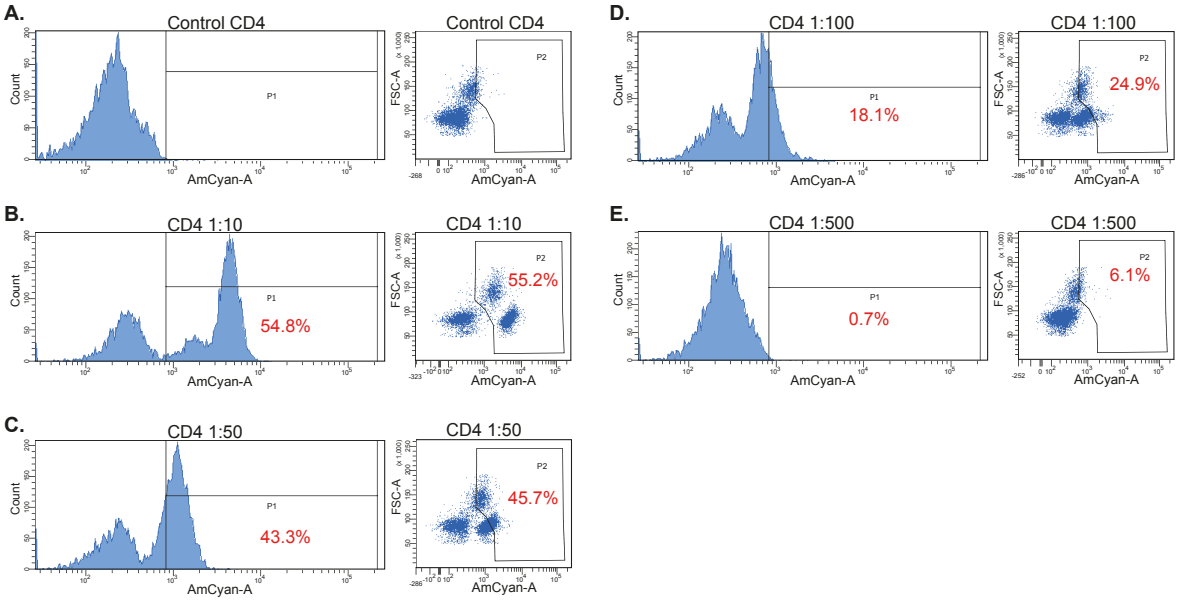


Figure 5. Titration of CD4 with the negative control (**A**) and dilutions of 1:10 (**B**), 1:50 (**C**), 1:100 (**D**), and 1:500 (**E**).

First, a negative, unstained control was measured using PBMCs isolated from healthy donors (Figure 5A). This allowed us to set the zero point. Next, a series of antibody titrations was performed. Here, for example, 1:10, 1:50, 1:100, and 1:500 dilutions were tested (Figure 5B-E). The PBMCs were then stained with the diluted antibodies and analyzed by flow cytometry. With decreasing concentration, the positive (antibody) and negative (control) peaks were harder to differentiate until they ultimately completely merged. For CD4, the dilution used was 1:10. Comparing the vertical “zero bar” from the negative control, a 1:10 dilution clearly eliminated the positive and negative cells. In the next dilution, the zero-line shifted to positive cells, despite the graph showing two distinct populations. This was also seen in the percentage of positive cells; there was a significant drop from 55.2% at the 1:10 dilution to 45.7% at the 1:50 dilution. However, not all titrations were straightforward, as some did not separate neatly into two peaks regardless of concentration. The

titrations were performed individually for each marker. See Supplemental Table 1 for the final dilutions.

After determining the dilutions individually, a FACS derivate compensation was established. For this, the “compensation control” feature of the BD LSRFortessa™ (FACS-Diva Software) was used for each of the five panels. Individually stained PBMCs of each fluorescent color were recorded and gated to fit the positive population. In addition to the negative unstained control, these data were used to create computer-generated compensations. PBMCs from several healthy donors were tested using these compensations, which was then manually adjusted and optimized to fit the specimens. Once functional panels were established, including optimal dilutions and modified compensation controls, one final test was performed on three PBMC samples from glioma patients.

The cell sorter used (BD LSRFortessa™) is part of a core facility at the UKE. To minimize variations between measurements, all samples were measured within a time frame of one week. For this, a coordinated effort was necessary: Each day, 40 PBMC samples were thawed and counted, distributed over five tubes (cell count permitting), activated if necessary, and stained according to the protocols below. This amounted to approximately 200 flow cytometry readings per day.

3.4. Thawing PBMCs

Thawing PBMCs proved to be a challenge, as many cells died during this process. After various attempts, the following protocol was established to most effectively maintain cell viability: cryopreserved cells were thawed by placing the vial in a 37°C water bath for approximately 2 minutes, or until only a small piece of ice remained in the vial. To minimize osmotic shock, 300 µL of cold RPMI medium supplemented with 10% FCS were added dropwise using a 1000 µL pipette, followed by gentle mixing. The cell suspension was then transferred to a new tube, and 1000 µL of cold RMPI + 10% FCS were used to rinse the vial, which was subsequently transferred to a new tube. While the tube was kept on ice, cold RPMI + 10% FCS was slowly added dropwise until the total volume reached 30 mL. The suspension was centrifuged at 350 x g for 6 minutes at 4°C. After carefully removing the supernatant,

the cell pellet was resuspended in 1 mL of cold RMPI + 10% FCS containing DNase (1:1000 dilution), followed by the addition of 9 mL of the same medium to a total volume of 10 mL. Cell viability and concentration were determined by mixing the sample 1:1 with trypan blue and counting the cells using a Neubauer counting chamber. Based on the cell count, the appropriate volume of cell suspension required for flow cytometry was calculated (e.g., 1 mL for a count of 50 cells per quadrant for a total of 500,000 cells per tube).

The cells were transferred to FACS tubes and centrifuged again at 350 x g for 6 minutes. The supernatant was carefully aspirated to avoid disturbing the pellet. The pellets were resuspended in specific volumes and reagents depending on the tube: cells in tubes 1, 2, 3, and 4 were resuspended in 70 μL of FACS buffer containing 2 μL of Fc block and 1:1000 DNase, whereas cells in tube 5 were resuspended in 100 μL of x-vivo medium. Depending on the cell count of unfrozen cells, the yield was not always sufficient to fill all five tubes. If more than 3.3 million cells were thawed, then all tubes could be filled; however, if there were fewer, a hierarchy was established to determine which tubes to prioritize with the existing cells: tubes 1, 2, and 3 each required 600,000 cells per tube, while tube 5 required 1,200,000 cells per tube. Tube 4 required only 300,000 cells/tube. The tubes were filled with thawed cells in the following hierarchical order: tube 1 first, followed by tubes 2, 3, 5, and 4.

3.5. Staining

After aliquoting the cells into their respective tubes, staining was performed. In most tubes, this was carried out directly after thawing. Only in tube 5, the detection of intracellular cytokines required prior stimulation. After staining with the antibody cocktails, the tubes were measured and recorded individually using flow cytometry (BD LSRFortessa™).

Surface staining (tube 1, 2, 3, and 4)

Thawed cells, resuspended in FACS buffer, were prepared for flow cytometry by adding 10 μL of antibody cocktail to each tube. The tubes were vortexed to ensure even distribution of the antibodies and incubated at room temperature in the dark for 45 minutes to prevent photobleaching. After 20 minutes of incubation, the tubes

were mixed again lightly to maintain the cell suspension and optimize the staining. Following incubation, the cells were washed once with 400 μL of FACS buffer containing DNase at a 1:1000 dilution. The suspension was centrifuged at 350 x g for 6 minutes at room temperature and the supernatant was carefully removed to avoid disturbing the pellet. The cells were then resuspended in 150 μL of FACS buffer with DNase (1:1000) and 1 μL of live/dead Zombie APC-Cy7 to label non-viable cells.

Intracellular staining (tube 5)

Thawed cells were resuspended in 100 μL of x-vivo medium and allowed to rest for 4 hours at 37°C in a 5% CO_2 incubator without the lid. The stimulation mixture was prepared as follows.

- 4 μL Phorbol 12-Myristate 13-Acetate (PMA) (40 ng/mL concentration, from a 100 μg/mL stock diluted 1:100 with x-vivo medium)
- 1 μL Ionomycin (1 mg/mL concentration)
- 0.2 μL Brefeldin (6 μg/mL concentration, from a 3 mg/mL stock diluted 1:500)
- 94.8 μL x-vivo medium

100 μL of stimulation mixture was added to each tube, and the cells were incubated for 5 hours at 37°C in a 5% CO_2 incubator, again without the lid. Following stimulation, the cells were washed with 1 mL of PBS and centrifuged at 250 x g for 6 minutes at room temperature. The supernatant was carefully removed, and the cell pellet was resuspended in 70 μL of FACS buffer containing 1:1000 DNase. The 2 μL of Fc block were added to reduce nonspecific binding, and cells were incubated at room temperature for 5 minutes (no washing needed). Surface staining was performed by adding 5.5 μL of antibody cocktail (CD45RO, CD56, CD8a), and tubes were incubated in the dark for 10 minutes at room temperature. Subsequently, 1 μL of live/dead Zombie APC-Cy7 was added, and the cells were incubated for an additional 20 minutes in the dark at room temperature. The cells were then washed with 1 mL of PBS, centrifuged at 350 x g for 6 minutes, and the supernatant was carefully removed. The cells were fixed with 100μL of fixative solution (without DNase) and incubated for 20 minutes in the dark at room temperature. After fixation, the cells were washed twice with 200μL of permeabilization buffer, centrifuged again at 350 x g for 6 minutes each time, and the supernatants carefully removed. Finally,

the cells were resuspended in 70μL of FACS buffer and the intracellular cytokine staining was performed by adding 10.5 μL of cytokine antibody cocktail (CD4, CD3, TNFα, IFNγ, IL-2, IL-4, IL-10, IL-17A) and incubated for 30 minutes in the dark at room temperature. The cells were washed once with 1 mL of permeabilization buffer, and the pellet resuspended in 150μL of FACS buffer (without DNase) for analysis.

3.6. Analysis

3.6.1. Gating strategy

For flow cytometry analysis, gating strategies were used to sequentially identify and define specific cell populations based on the expression of several cellular markers. Gating refers to the first manual and later automated decision process that defines cell populations based on marker expression (Mousset et al., 2019, Verschoor et al., 2015). Figure 6 shows an example of the identification of large T cell populations. An individual gating strategy (Supplemental Figure 1-5) was established for each panel.

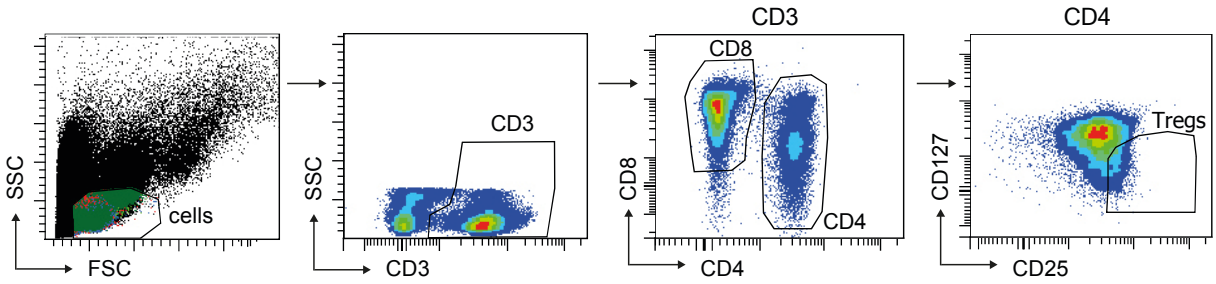


Figure 6. Example of a gating strategy for T-cell subtypes. Plotting of forward and side scatter (FSC and SCC) shows cells and debris. After selecting the cell fraction, staining with CD3 identified all T cells. All $CD3^+$ cells were further divided into $CD4^+$ and $CD8^+$ T cells. Of the $CD4^+$ T cells, Tregs were selected as $CD25^+$ and $CD127^{low}$.

Manual gating was performed using these gating strategies. First, each sample was checked and adjusted individually for compensation. Then, the gates were manually adjusted for each sample before exporting the population counts and hierarchy as CSV files into Excel. These numbers were then inserted into a master table.

3.6.2. Statistical analysis and graphical presentation

Marker expression was analyzed as the proportion (%) of cells expressing the respective marker within each population. Cytokine production was analyzed as the

proportion (%) of cells producing the respective cytokine. Quantitative fluorescence intensity data (mean fluorescence intensity, MFI) were not evaluated. For clarity, the term "expression" will be used to denote the proportion of cells positive for each surface or intracellular marker, whereas the term "production" will refer to the proportion of cells positive for intracellular cytokines. Manual gating counts were imported into GraphPad Prism, which was then used to perform statistical analyses (unpaired t-test) and visualize the data in the form of graphs. In all figures, asterisks indicate the level of statistical significance as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. P values between 0.05 and 0.1 were considered indicative of a statistical trend. STATA software was used to analyze patient characteristics.

Statistical analysis and graphical presentation of both dexamethasone intake and survival data were performed using R. To investigate the effects of dexamethasone intake on immune marker expression, patient data were divided into two groups: those who had received dexamethasone prior to sample collection (“yes”) and those who had not (“no”). For each group, the expression levels of immune markers such, for example $CD8^+$ $PD1^+$, were analyzed, and the median dexamethasone intake was calculated for each group. For survival data, patients were split into the top 30% and bottom 30% of expression for each marker. This approach was used to better capture the potential impact of high and low marker expression. Data were analyzed for the following patient subsets: all gliomas, glioblastomas, primary glioblastomas, and recurrent glioblastomas.

4. Results

4.1. Patient characteristics

Two hundred blood samples from 102 IDHwt glioblastoma patients, 31 IDHmut astrocytoma patients, 16 IDHmut and 1p19q codeleted oligodendroglioma patients, and 51 healthy donors (HD) were examined. Table 2 shows further details of the types of tumors, patient characteristics, and outcomes. The healthy donor group was chosen to age-match the IDHwt patients.

Table 2. Patient characteristics

	HD[1]	IDHwt[2]	IDHmut[3]	1p19q[4]
	n = 51	*n = 102*	*n = 31*	*n = 16*
Age	64.41 (12.49)	60.49 (11.39)	39.97 (9.32)	48.56 (11.35)
Sex				
Female	15 (29.41%)	42 (41.18%)	9 (29.03%)	6 (37.50%)
Male	31 (60.78%)	61 (58.82%)	22 (70.97%)	10 (62.50%)
Diagnosis				
Primary	-	76 (74.51%)	21 (67.74%)	11 (68.75%)
Recurrence	-	26 (25.49%)	10 (32.26%)	5 (31.25%)
MGMT Methylation				
No	-	45 (44.12%)	3 (9.68%)	0 (0.00%)
Yes	-	51 (50.00%)	24 (77.42%)	13 (81.25%)
Unknown	-	6 (5.88%)	4 (12.90%)	3 (18.75%)
Tumor Volume (cm^3)	-	24.70 (24.83)	21.78 (14.68)	26.11 (23.77)
Tumor Location				
Right	-	49 (48.04%)	18 (58.06%)	11 (68.75%)
Left	-	47 (46.08%)	13 (41.94%)	5 (31.25%)
Unknown	-	6 (5.88%)	-	-
Tumor Location II				
Frontal	-	49 (48.04%)	18 (58.06%)	11 (68.75%)
Parietal	-	48 (47.06%)	13 (41.94%)	5 (31.25%)
Unknown	-	5 (4.90%)	-	-
WHO Grading				
2	-	0 (0.00%)	5 (16.13%)	6 (37.50%)
3	-	5 (4.90%)	21 (67.74%)	10 (62.50%)
4	-	97 (95.10%)	5 (16.13%)	0 (0.00%)
Survival				
Dead	-	51 (50.00%)	5 (16.13%)	1 (6.25%)
Alive	-	10 (9.80%)	21 (67.74%)	11 (68.75%)
Unknown	-	41 (41.20%)	5 (16.13%)	4 (25.00%)

[1]HD = healthy donors. Incomplete data for the HD cohort: sex unknown for five patients, age unknown for four patients.
[2]IDHwt = IDH-wildtype glioblastoma.
[3]IDHmut = IDH-mutant astrocytoma.
[4]1p19q = IDH-mutant and 1p19q-codeleted oligodendroglioma

4.2. T-cell populations

No significant differences between CD4⁺, conventional CD4⁺, CD8⁺, Treg, and Tγδ abundance were found in glioblastoma patient blood when compared to HDs (Figure 7). In contrast, NK cells showed a significant decrease in glioblastoma and astrocytoma patients compared with HDs (Figure 7). Across all figures, oligodendrogliomas (1p19q-codeleted) are shown for completeness but were excluded from statistical analysis due to small sample size.

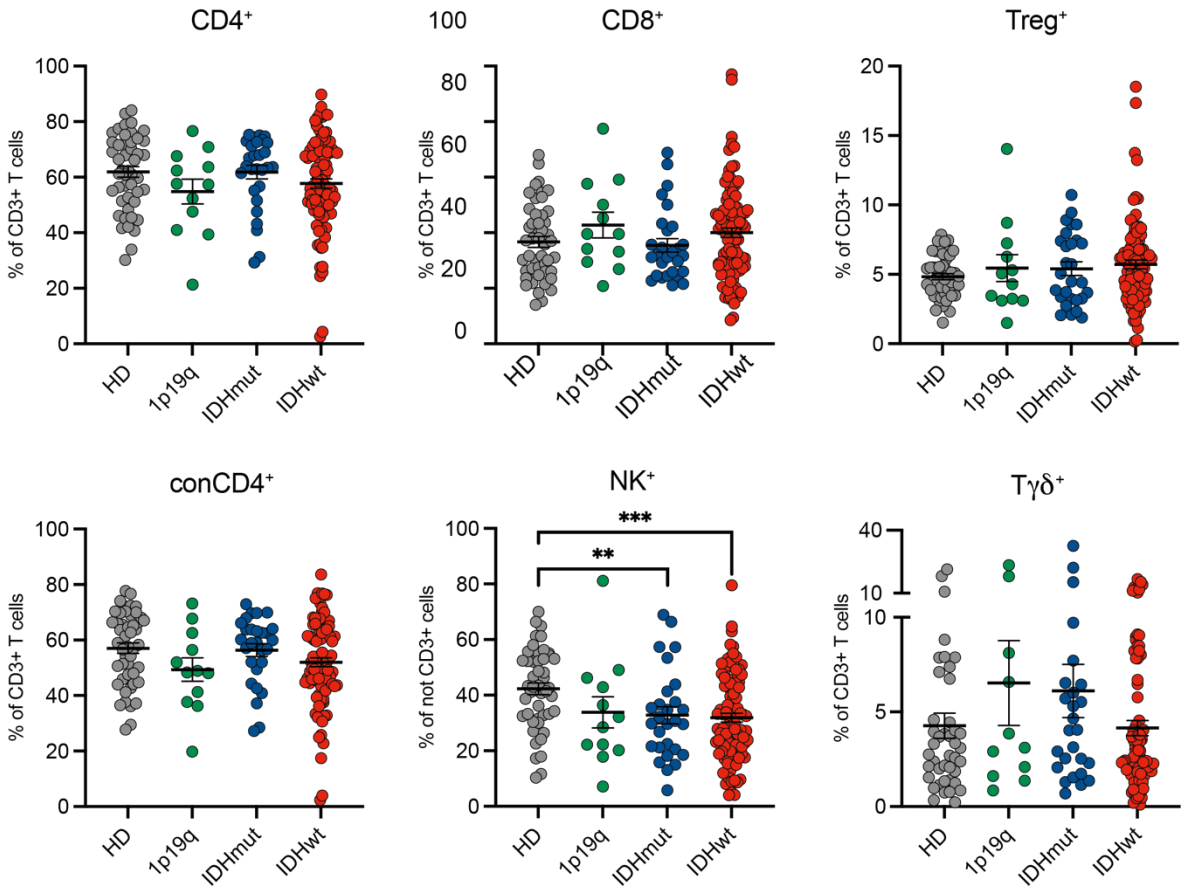


Figure 7. T-cell populations (CD4⁺, CD8⁺, Treg, conventional CD4⁺, NK, and Tγδ⁺), as measured by flow cytometry, in HDs and all glioma cohorts. *NK⁺ cells*: mean HD (42.32) vs. mean IDHwt (31.94), $p = 0.0002$.

For further analysis, the IDHwt glioblastoma cohort was divided into patients with primary and recurrent tumors (Figure 8). In primary (pIDHwt) glioblastomas, but not in recurrent (rIDHwt) tumors, there were significantly fewer NK cells than in HDs. Both CD4⁺ and conventional CD4⁺ cells were significantly reduced in patients with recurrent, but not primary tumors, compared to HDs, while CD8⁺ expression was significantly increased in recurrent tumors. These results show that rIDHwt had

fewer CD4⁺ and more CD8⁺ T cells than both HDs and pIDHwt glioblastomas. Subgroup analyses based on recurrence status (primary vs. recurrent) were performed only for the glioblastoma group. No recurrence- or grade-based stratification was applied to the1p19q-codeleted oligodendrogliomas or the IDHmut astrocytomas owing to the smaller cohort sizes.

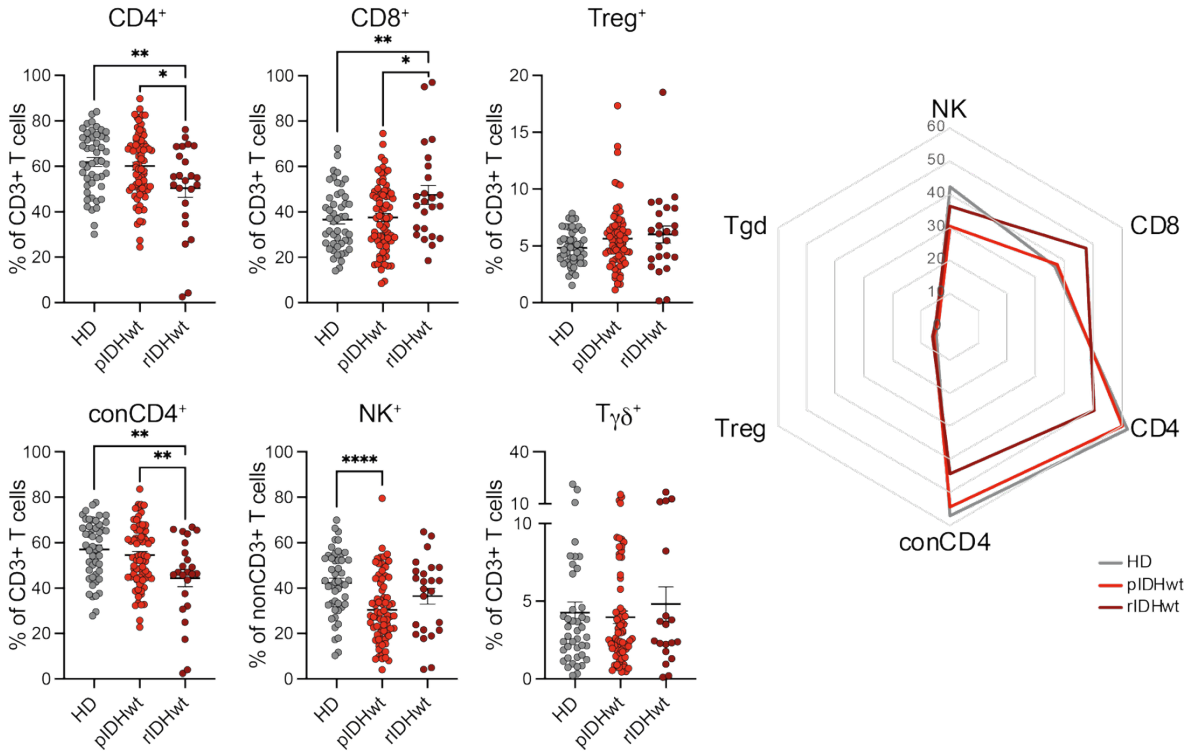


Figure 8. T-cell populations (CD4⁺, CD8⁺, Treg, conventional CD4⁺, NK, and Tγδ⁺), as measured by flow cytometry, in HDs, primary and recurrent IDHwt gliomas. *CD4⁺ cells*: Mean pIDHwt (60.14) vs. mean rIDHwt (50.37), p = 0.0115. *CD8⁺ cells*: mean pIDHwt (37.53) vs. mean rIDHwt (47.48), p = 0.0121.

MAIT cells are a distinct subset of innate-like T cells that differ from conventional CD4⁺ and CD8⁺ T cells in their development and function. They are most commonly CD8⁺, but can also be CD8⁻ and in rare circumstances CD4⁺, which is why they are presented in a separate figure below (Figure 9). MAIT cells were significantly reduced in patients with both IDHwt glioblastomas (Figure 9A) and in primary tumors compared to HDs (Figure 9B).

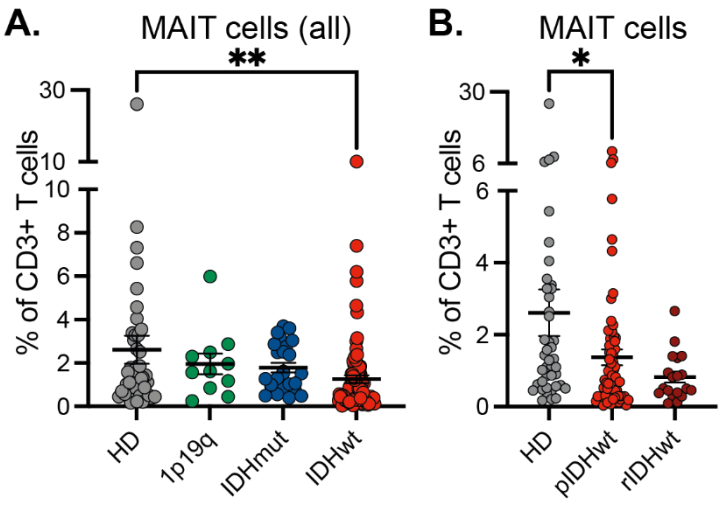


Figure 9. MAIT cells in HD and all glioma cohorts (A), and in primary vs. recurrent IDHwt glioblastomas (B).

4.3. T-cell differentiation/subsets

The gating strategy for the identification of different T-cell differentiation subtypes is shown in Figure 10. Among the $CD4^+$ T-cell differentiation subtypes, early T cells (recently activated cells, beginning to acquire effector functions) were markedly and significantly reduced in glioblastoma when compared to HDs (Figure 11A). For IDHmut astrocytoma patients, the effector RA- fraction of $CD4^+$ T cells (differentiated effector cells lacking CD45RA, often associated with sustained effector function) showed a significant decrease compared to HDs, which was not seen in glioblastoma patients and constitutes a significant difference between astrocytoma and glioblastoma patients (Figure 11A). No significant differences were found in any of the other $CD4^+$ differentiation subtypes.

Among $CD8^+$ T cells, intermediate T cells (transitional cells between early and fully differentiated states) were markedly and significantly reduced in glioblastoma when compared to HDs (Figure 11B). In IDHmut astrocytoma patients, $CD8^+$ T cells showed a significantly increased proportion of naïve (antigen-inexperienced, resting cells ready for activation) and early-like T cells (cells resembling early activated subsets with partial effector features) compared to HDs and also compared to glioblastoma patients (Figure 11B). In addition, there was a slight decrease in the fractions of effector $CD8^+$ RA^+ (terminally differentiated cells with immediate

cytotoxic or helper functions) and $CD8^+$ early T cells in astrocytoma patients compared to HDs (Figure 11B).

Taken together, T-cell differentiation profiles in glioblastoma patients only diverged from those of HDs in the $CD4^+$ early and $CD8^+$ intermediate T cell compartments. Unexpectedly, IDHmut astrocytoma patients exhibited more pronounced alterations in these subsets than glioblastoma patients when compared to HDs, particularly within the $CD8^+$ compartment, including $CD8^+$ naïve, effector RA^+, early, and early-like T cells. This highlights the molecular subtype-specific immunological alterations and the importance of our approach to compare different glioma subtypes rather than limiting the analysis to glioblastoma vs. HDs.

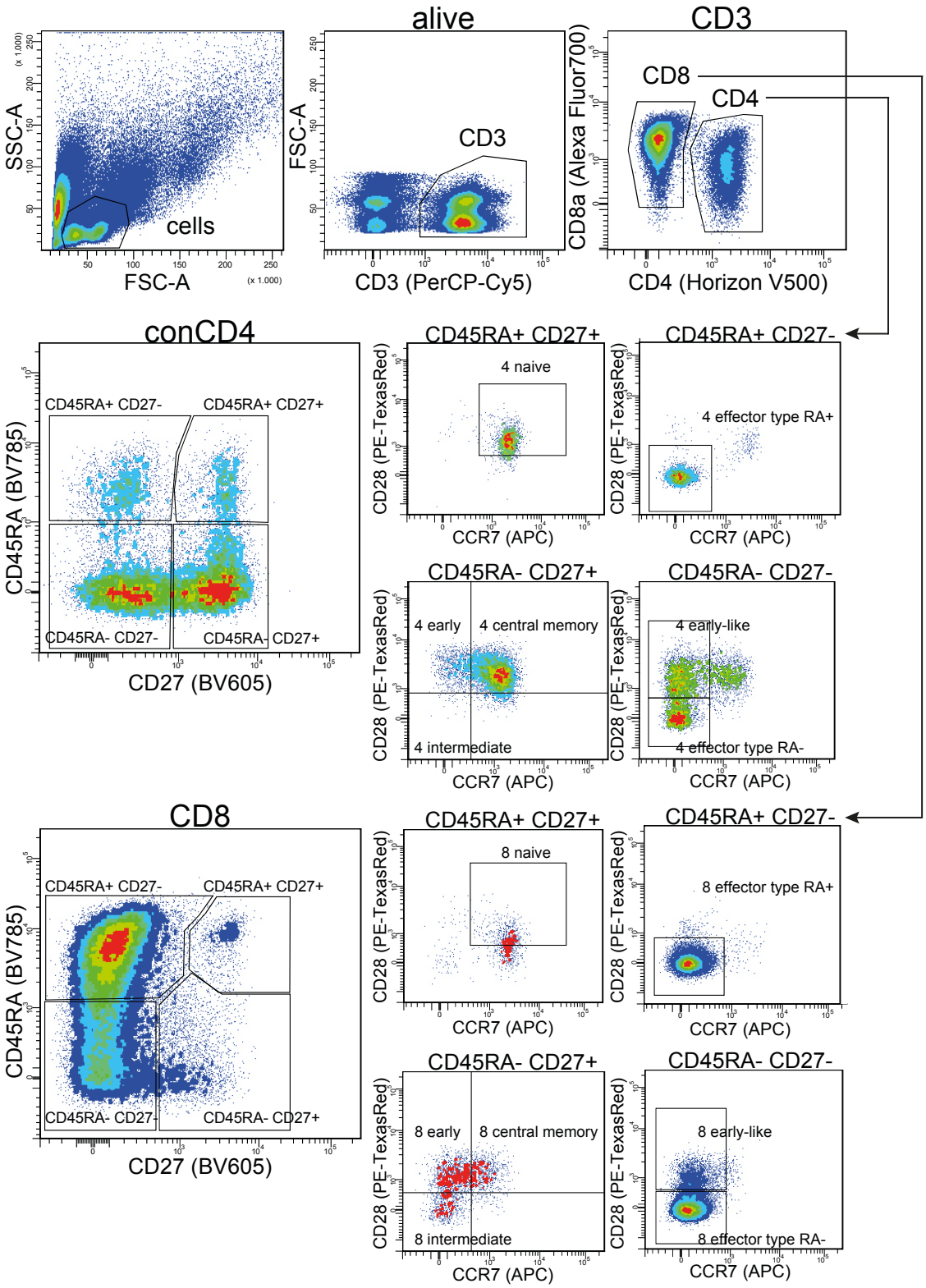


Figure 10. Gating of T-effector subsets.

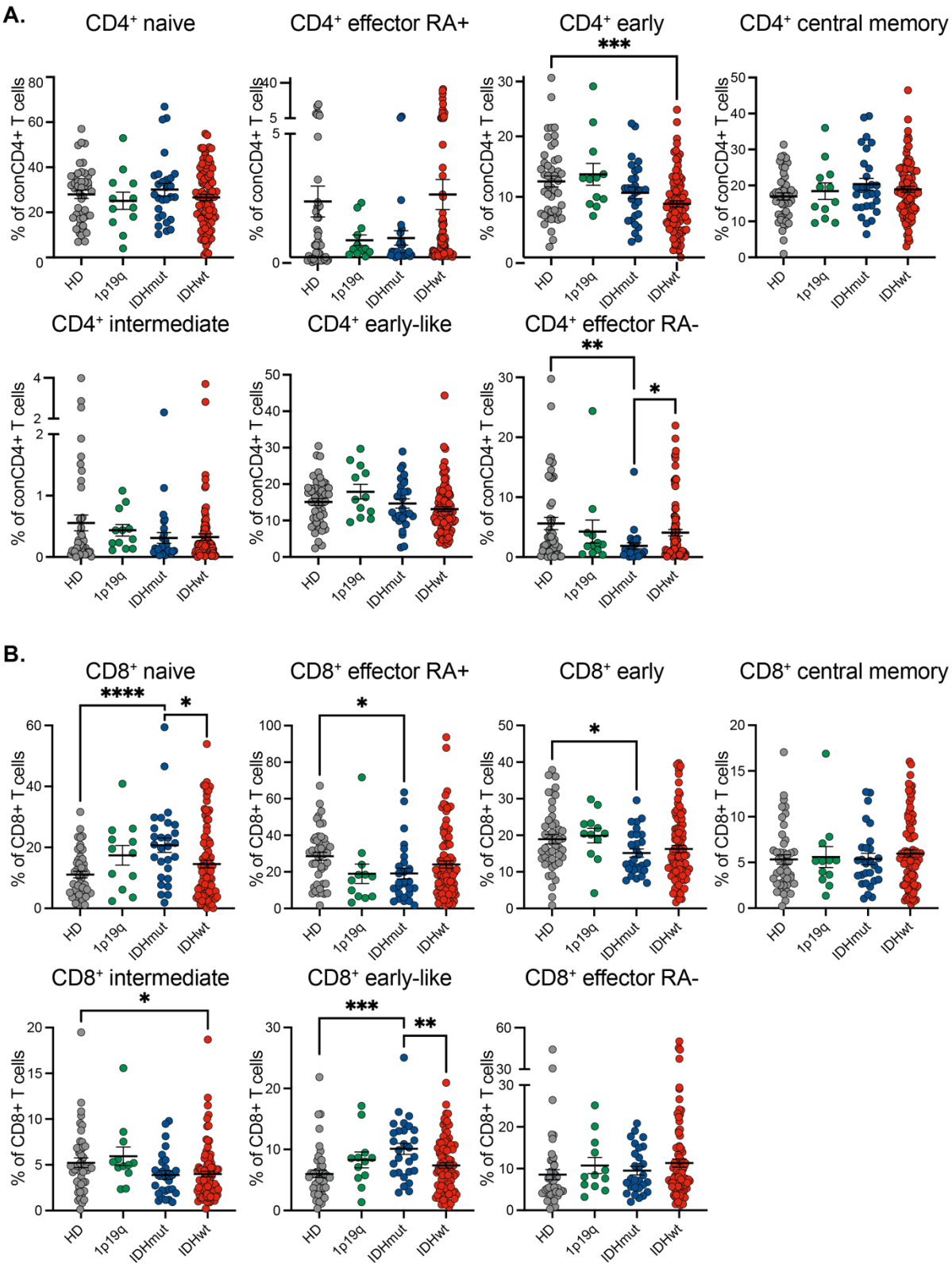


Figure 11. Distribution of T-effector subsets, as measured by flow cytometry, in CD4⁺ T cells (A) and CD8⁺ T cells (B) in healthy donors and all glioma cohorts. *CD4⁺ early*: mean HD (12.57) vs. mean IDHwt (8.870), $p = 0.0002$.

When comparing primary vs. recurrent glioblastomas, we detected significantly fewer $CD4^+$ early and $CD4^+$ early-like T cells in patients with primary tumors vs. HDs, and fewer $CD4^+$ early T cells in primary vs. recurrent tumors (Figure 12A). In contrast, patients with recurrent glioblastomas exhibited a significantly higher proportion of central memory T cells (long-lived cells with high proliferative capacity and lymphoid tissue homing) compared to both HDs and primary glioblastoma patients.

In the $CD8^+$ compartment, naïve cells were enriched in recurrent glioblastoma patients but not in newly diagnosed patients. In contrast, $CD8^+$ early-like and $CD8^+$ effector RA- T cells were significantly increased in patients with primary glioblastomas compared to HDs and to patients with recurrent tumors (Figure 12B).

Collectively, these findings reveal divergent systemic immune profiles between primary and recurrent glioblastoma. Primary tumors show features of immune activation, including increased $CD8^+$ early-like and effector RA- T cells, whereas recurrent tumors are characterized by elevated $CD4^+$ central memory and $CD8^+$ naïve T cells, suggesting a shift toward downregulated immune responses or immune exhaustion. These changes highlight dynamic, stage-specific alterations in T-cell differentiation that may reflect evolving immunosuppressive mechanisms during glioblastoma progression.

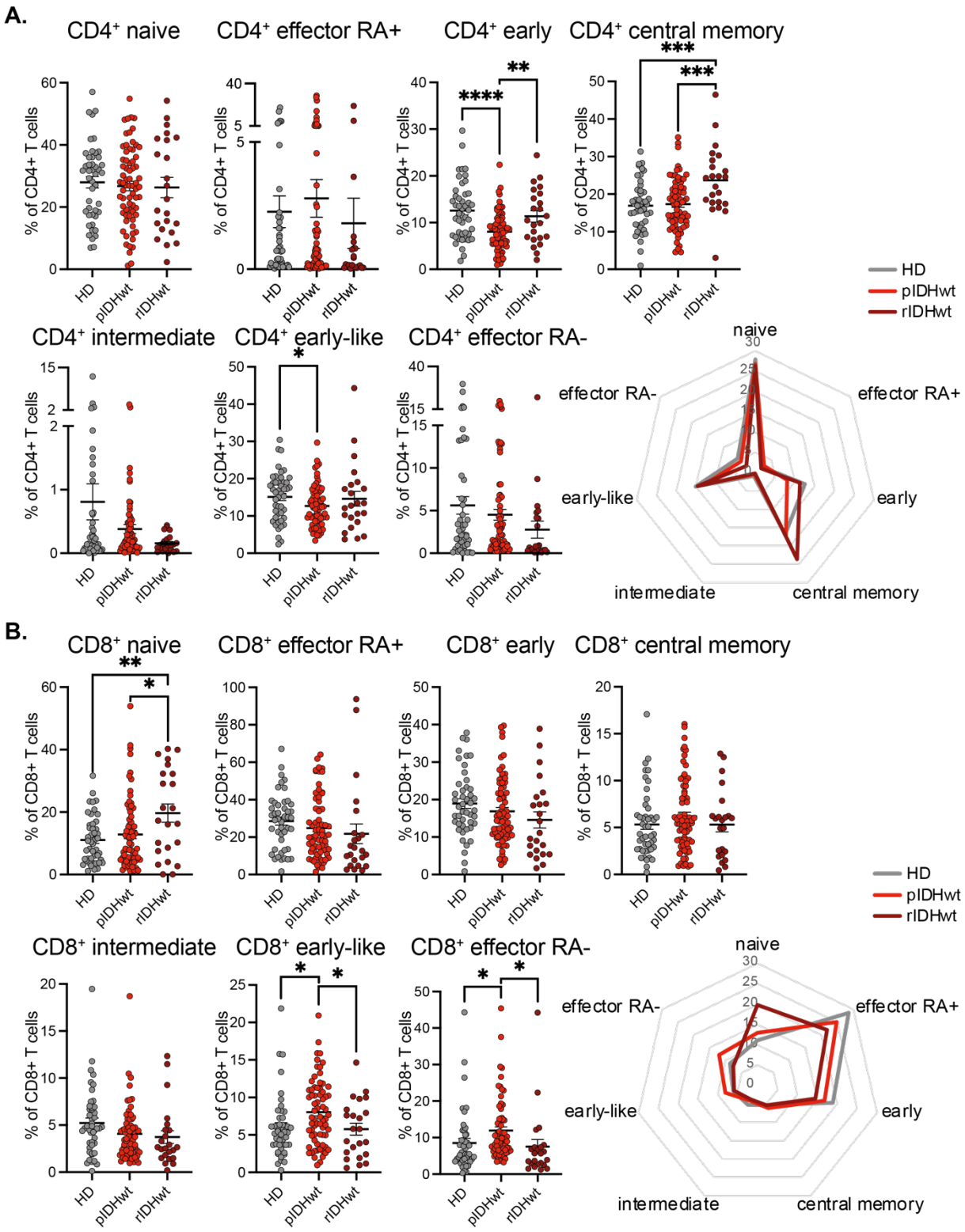


Figure 12. Distribution of T-effector subsets, as measured by flow cytometry, in CD4$^+$ T cells (A) and CD8$^+$ T cells (B) in HD, primary, and recurrent IDHwt gliomas. *CD4$^+$ early*: mean HD (12.57) vs. mean pIDHwt (8.057), $p < 0.0001$; mean pIDHwt (8.057) vs. mean rIDHwt (11.34), $p = 0.0041$. *CD4$^+$ central memory*: mean HD (16.93) vs. mean rIDHwt (23.68), $p = 0.0007$; mean pIDHwt (17.35) vs. mean rIDHwt (23.68), $p = 0.0005$. *CD8$^+$ naïve*: mean HD (11.06) vs. mean rIDHwt (19.67), $p = 0.0014$; mean pIDHwt (12.84) vs. mean rIDHwt (19.67), $p = 0.0186$.

4.4. T-helper cells

Glioblastoma patients showed a striking reduction in the T_{FH} cell population and the non-classical T_H1 population ($CCR6^-$) when compared to HDs and patients with astrocytoma (Figure 13). In contrast, a significant expansion of T_H1 cells ($CCR6^+$) was found in glioblastoma but not in astrocytoma patients. No significant differences were found in the T_H2 and T_H17 T-helper cell subsets between the cohorts.

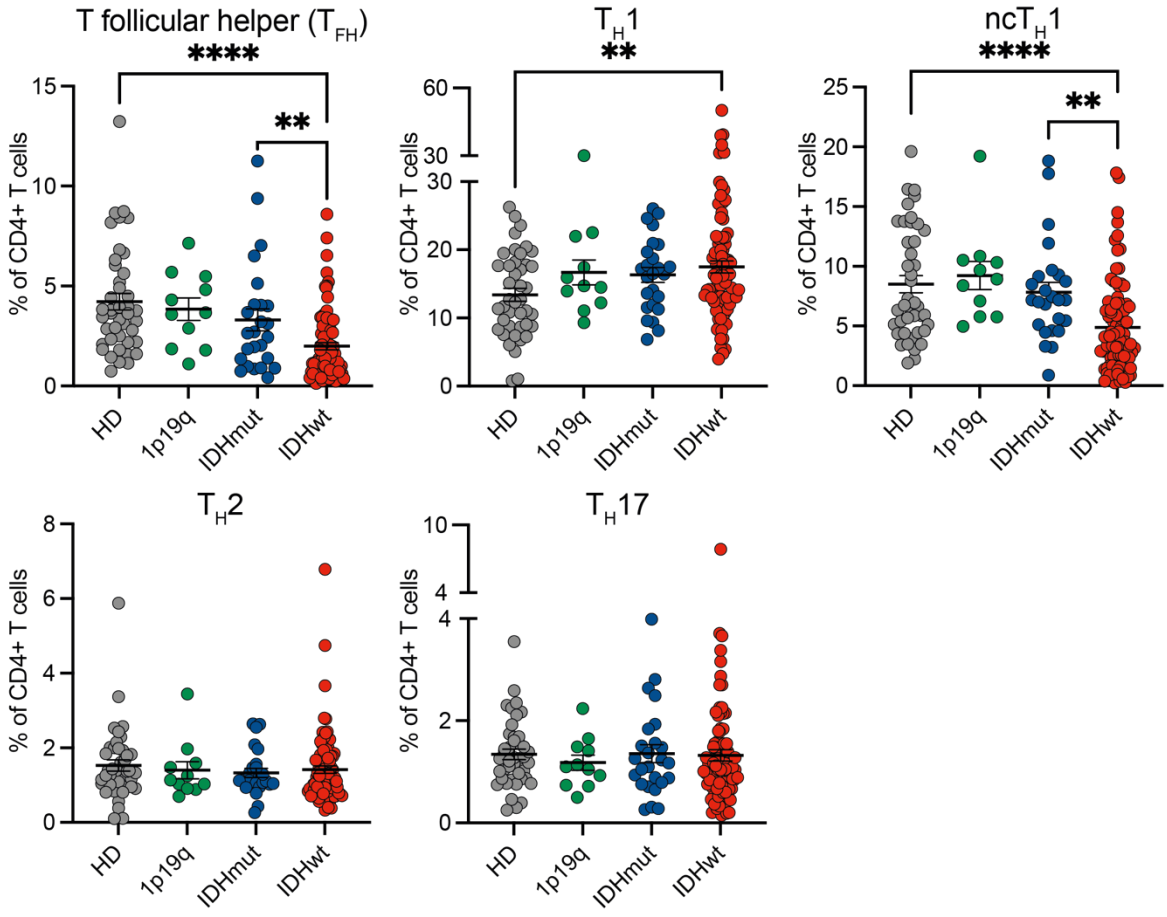


Figure 13. Distribution of T-helper subsets, as measured by flow cytometry, in HD and all glioma subsets. *T_{FH}*: mean HD (4.216) vs. mean IDHwt (1.990), p = < 0.0001; mean IDHmut (3.305) vs. mean IDHwt (1.990), p = 0.0039. *T_H1*: mean HD (13.38) vs. mean IDHwt (17.48), p = 0.0047. *ncT_H1*: mean HD (8.501) vs. mean IDHwt (4.894), p < 0.0001; mean IDHmut (7.816) vs. mean IDHwt (4.894), p = 0.0013.

Subanalysis of IDHwt glioblastoma patients showed that the reduction of T_{FH} cells was significant in both primary and recurrent patients, whereas the reduction of ncT_H1 cells and increase in T_H1 cells only reached significance in primary cases (Figure 14). In addition, while no significant differences had been detectable for T_H17 and T_H2 cells in pooled primary and recurrent patients, the subanalysis revealed a significant decrease in T_H17 T cells in primary glioblastomas compared

to HD, but an increase in recurrent patients, as well as an increase in T_H2 cells in recurrent vs. primary patients (Figure 14).

In summary, these findings show that glioblastoma is associated with a reduction of T_{FH} and ncT_H1 cells, alongside an expansion of classical T_H1 cells, indicating a shift toward a pro-inflammatory but potentially dysregulated T-helper cell profile. These changes suggest elements of both immune activation and immunosuppression, with additional alerted T_H17 and T_H2 dynamics in recurrent tumors, indicating a further shift towards pro-inflammatory (T_H17), as well as potentially tumor-promoting, anti-inflammatory (T_H2) immune responses, further underscoring stage-specific immune modulation.

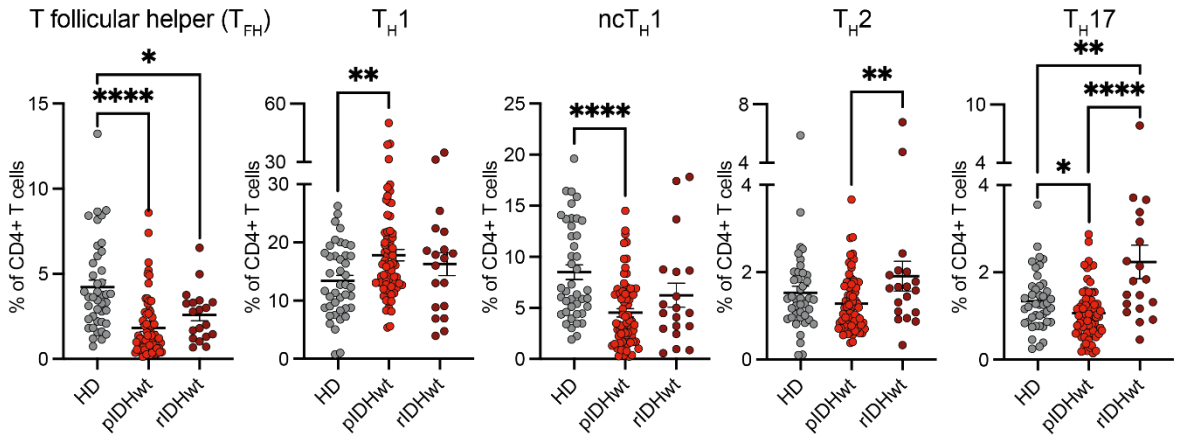


Figure 14. Distribution of T-helper subsets in HD and primary and recurrent IDHwt gliomas as measured by flow cytometry. *T_H2*: mean pIDHwt (1.280) vs. mean rIDHwt (1.908), p = 0.0078. *T_H17*: mean HD (1.344) vs. mean pIDHwt (1.070), p = 0.0262; mean HD (1.344) vs. mean rIDHwt (2.241), p = 0.0039; mean pIDHwt (1.070) vs. mean rIDHwt (2.241), p < 0.0001.

4.5. Cytokines

Beyond determining the differentiation status of circulating T cells, we analyzed the cytokine profiles of $CD4^+$ and $CD8^+$ T cells as well as NK cells by intracellular flow cytometry. Peripheral $CD4^+$ T cells and $CD8^+$ T cells in patients with IDHwt glioblastomas and IDHmut astrocytomas both produced significantly less IFNγ than HDs (Figure 15A-B). Similarly, TNFα production was also reduced in $CD4^+$ and $CD8^+$ T cells for glioblastomas, yet only significantly reduced in $CD8^+$ T cells in astrocytomas (Figure 15A-B). In addition, $CD4^+$ T cells produced higher levels of IL-4 in glioblastomas when compared to HDs (Figure 15A). No significant differences

were found in the production of any of the other cytokines (Figure 15A-B: IL-2, IL-10, IL-17) for these two cell types.

The NK-cell population of glioblastoma patients did not differ from that of HDs in terms of cytokine production (Figure 15C). However, NK cells in astrocytoma patients displayed a significantly lower production of IFNγ and TNFα compared to both HD and glioblastoma patients (Figure 15C).

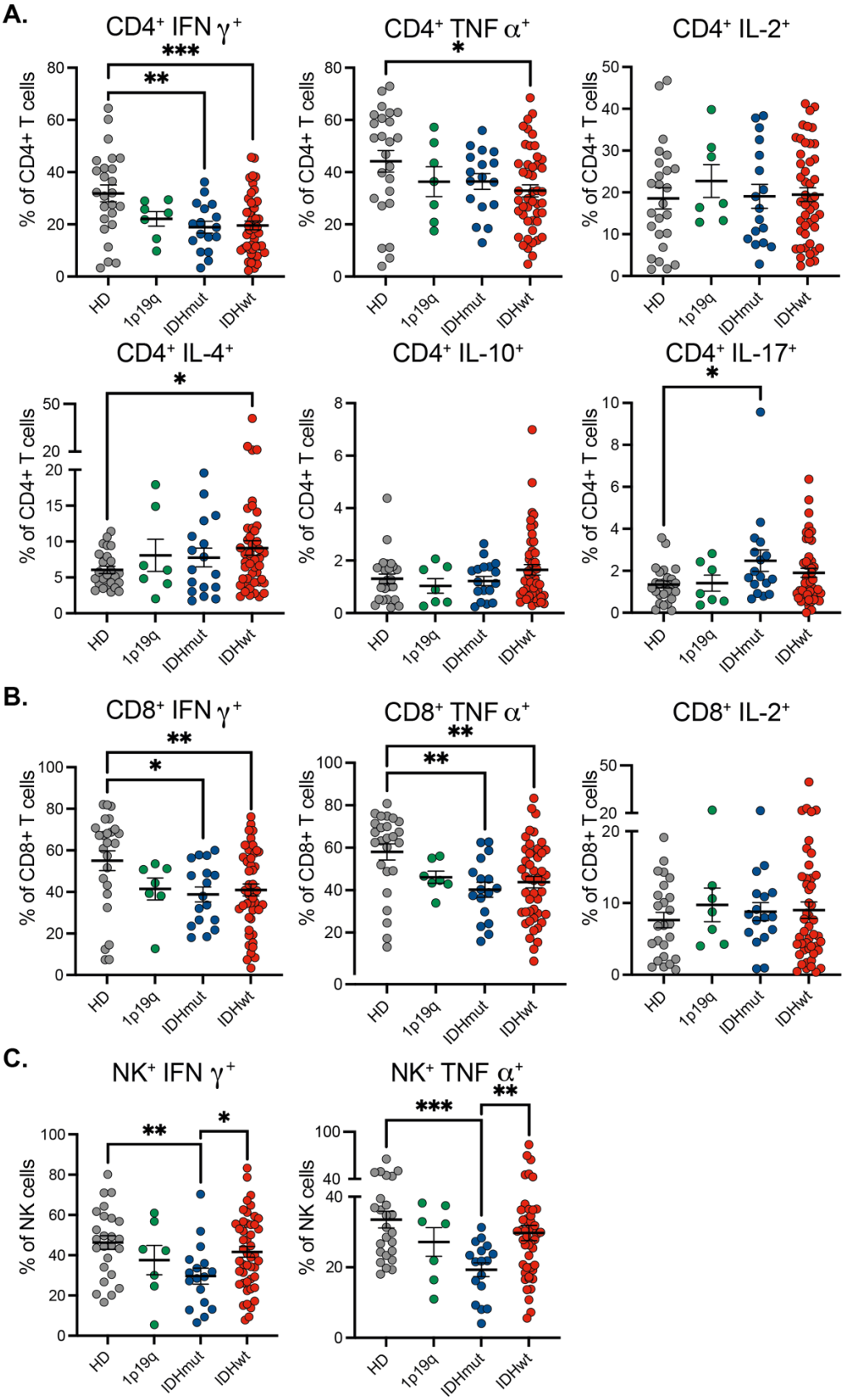


Figure 15. Distribution of intracellular cytokine production, as measured by flow cytometry, in CD4$^+$ T cells (**A**), CD8$^+$ T cells (**B**), and NK cells (**C**) in HD and all glioma cohorts. *CD4$^+$ IFNγ^+*: mean HD (31.86) vs. mean IDHwt (19.57), p = 0.0004. *CD4$^+$ TNFα^+*: mean HD (44.16) vs. mean IDHwt (32.89), p = 0.0118. *CD8$^+$ IFNγ^+*: mean HD (55.03) vs. mean IDHwt (40.95), p = 0.0080. *CD8$^+$ TNFα^+*: mean HD (58.01) vs. mean IDHwt (43.75), p = 0.0028.

Changes similar to those observed in the combined IDHwt cohort (Figure 15) were also detected in the subanalysis of primary and recurrent glioblastomas (Figure 16). Both $CD4^+$ and $CD8^+$ T cells exhibited prominently reduced IFNγ and TNFα production in patients with primary glioblastoma compared to HDs, and recurrent patients showed a similar trend which, however, only reached significance for IFNγ in $CD4^+$ cells and for TNFα in $CD8^+$ T cells (Figure 16A-B). In addition, a significant increase in IL-4, IL-10 and IL-17 was seen in recurrent but not primary glioblastoma patients (Figure 16A).
NK cells only displayed a significant increase in IFNγ in recurrent vs. primary glioblastoma patients (Figure 16C).

Figure 17 summarizes the data shown in Figures 15 and 16 for cytokines in circulating $CD4^+$ and $CD8^+$ T cells and NK cells, for primary and recurrent glioblastomas, and for IDHmut astrocytomas. Figure 17 highlights that IFNγ and TNFα production is reduced in both $CD4^+$ (Figure 17A) and $CD8^+$ T cells (Figure 17B) while IL-4 production is increased in $CD4^+$ cells in glioblastoma patients. NK cells (Figure 17C) from astrocytoma patients show decreased IFNγ and TNFα production, with a similar trend for primary but not recurrent glioblastomas.

Collectively, these findings show a reduction in IFNγ and TNFα production by both $CD4^+$ and $CD8^+$ T cells in glioblastoma and astrocytoma patients, indicating a state of systemic T-cell dysfunction and immunosuppression. In recurrent glioblastoma, increased IL-4, IL-10 and IL-17 production by $CD4^+$ T cells further indicates a shift towards regulatory and pro-inflammatory phenotypes, potentially reflecting chronic immune activation. While NK cells remained largely unchanged in glioblastoma, their reduced cytokine production in astrocytomas and elevated IFNγ in recurrent glioblastomas highlight subtle, context-specific innate immune alterations.

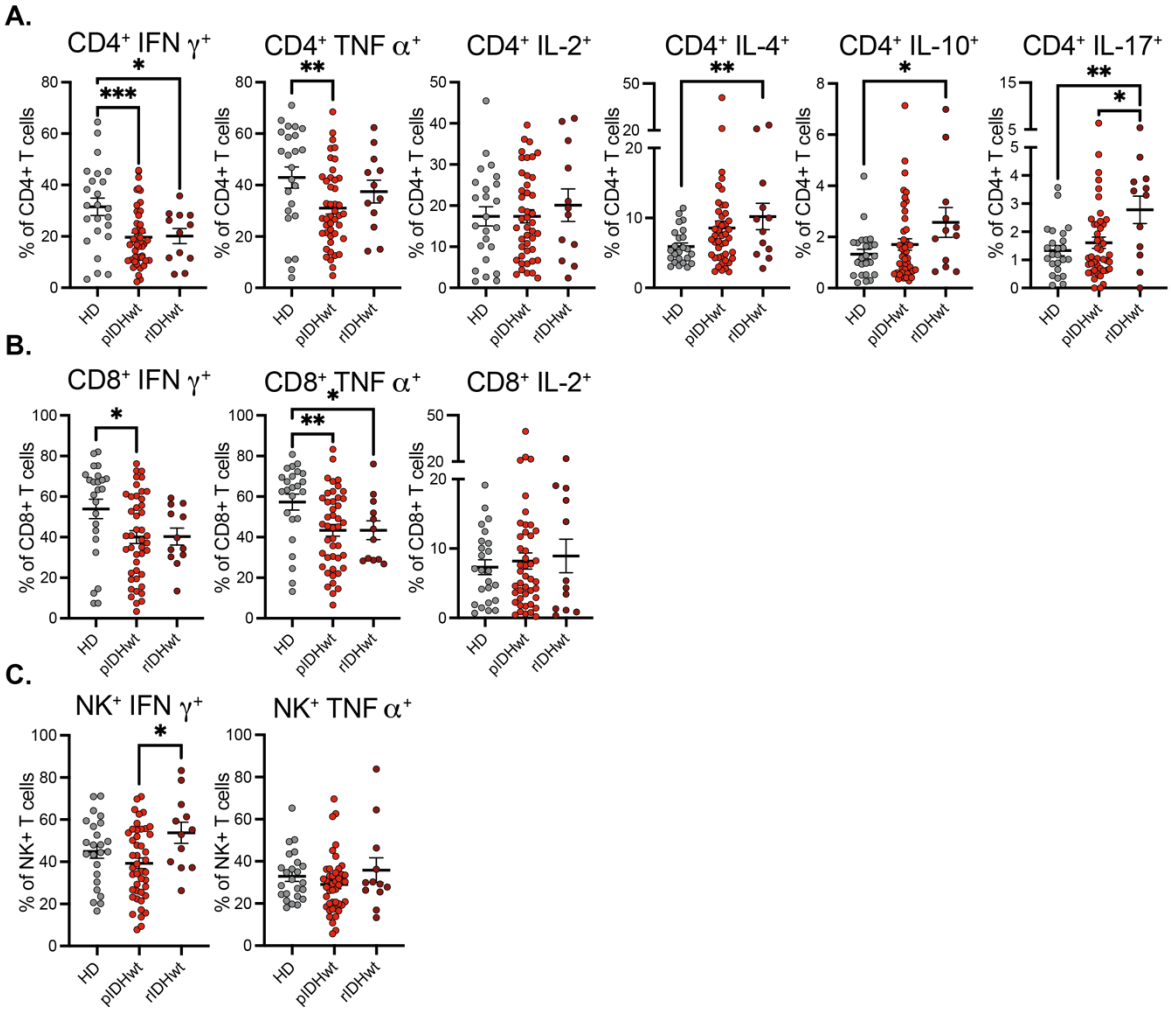


Figure 16. Distribution of intracellular cytokine production, as measured by flow cytometry, for primary and recurrent IDHwt glioblastoma in $CD4^+$ T cells (**A**), $CD8^+$ T cells (**B**), and NK cells (**C**) in HD and primary and recurrent IDHwt glioblastoma. *$CD4^+$ IL-17^+*: mean HD (1.330) vs. mean rIDHwt (2.779), p = 0.0017; mean pIDHwt (1.611) vs. mean rIDHwt (2.779), p = 0.0145.

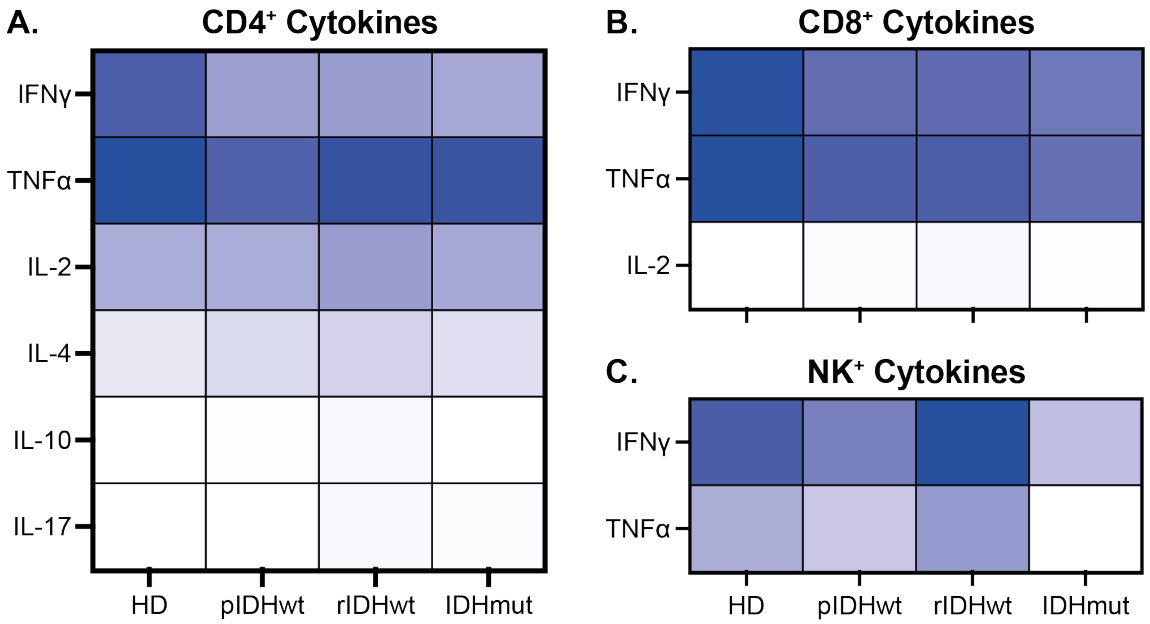


Figure 17. Heat map of intracellular cytokine production in $CD4^+$ T cells (A), $CD8^+$ T cells (B) and NK^+ cells (C) in HD, primary and recurrent IDHwt glioblastoma, and IDHmut astrocytoma.

4.6. Immune checkpoints

The expression of immune checkpoint receptors on T cells and NK cells can profoundly inhibit their activity after engagement with their corresponding ligands. We therefore investigated the expression of key checkpoint molecules by flow cytometry on T cells, including Tregs, and NK cells.

All three T-cell populations, $CD4^+$, $CD8^+$, and $Treg^+$, as well as the NK cells in patients with glioblastoma, showed a significant decrease in two to six T-cell exhaustion markers compared to HDs (Figure 18). $CD4^+$ T cells expressed significantly less PD-1 and BTLA, $CD8^+$ T cells expressed significantly less TIGIT and 2B4, Treg cells expressed significantly less PD-1, TIGIT, BLTA, CD57, KLRG1, and 2B4, while NK cells expressed significantly less TIM-3, TIGIT, and KLRG1. This indicates reduced expression of exhaustion-associated markers across all T-cell populations and NK cells. Astrocytoma patients showed similarly reduced expression of many T-cell exhaustion markers, including PD-1, TIGIT, CD57, and 2B4 on $CD4^+$ and Treg cells, and PD-1, TIGIT, KLRG1, and 2B4 on $CD8^+$ T cells, as well as lower TIM-3 and TIGIT expression on NK cells compared to HDs (Figure 18). When compared to glioblastomas, astrocytoma patients exhibited slightly, yet significantly lower TIGIT expression on $CD8^+$ T cells and lower 2B4 expression on $CD4^+$ T cells (Figure 18).

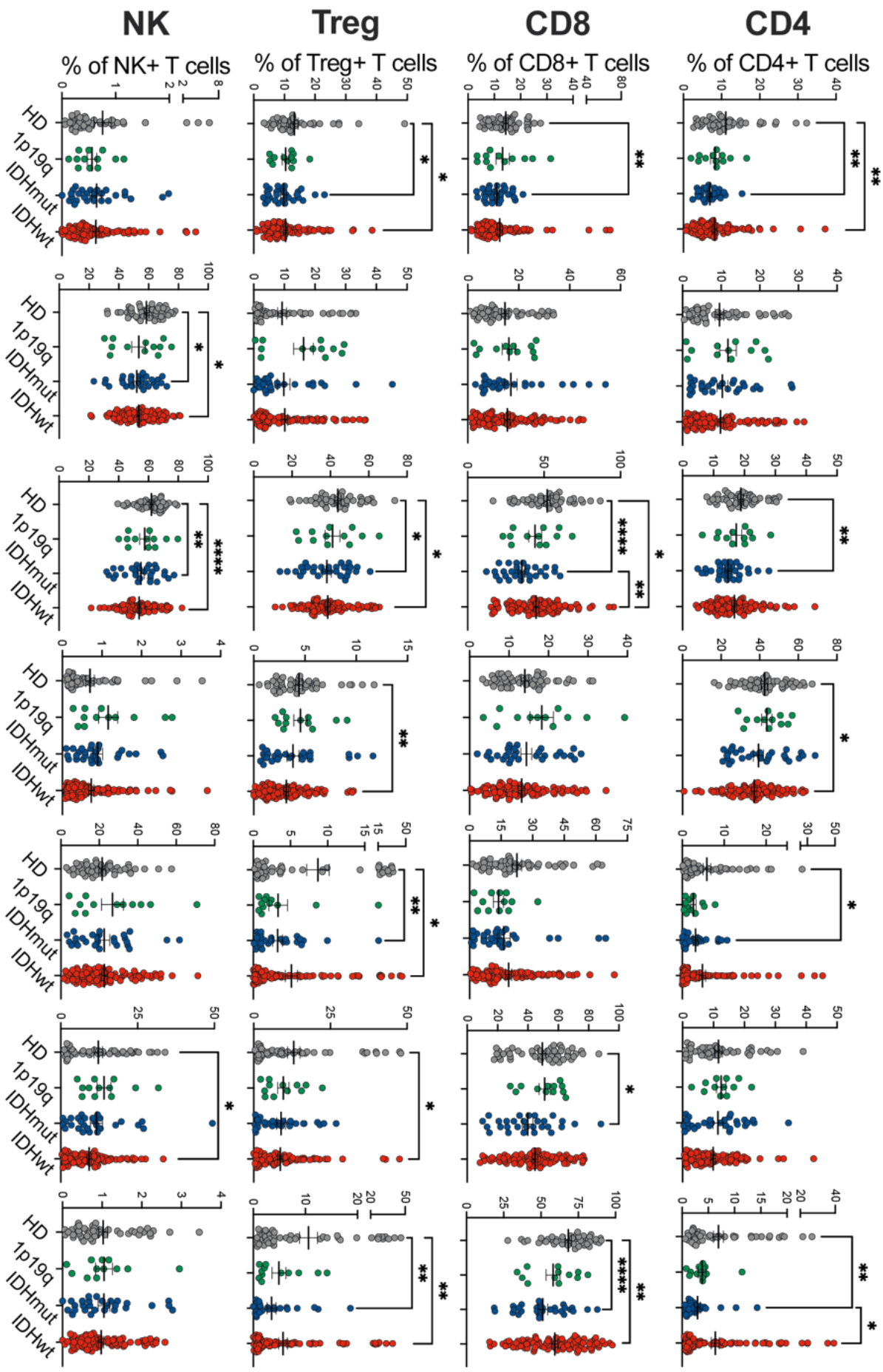

CD4
CD8
Treg
NK
% of CD4+ T cells
% of CD8+ T cells
% of Treg+ T cells
% of NK+ T cells
PD-1
TIM-3
TIGIT
BTLA
CD57
KLRG1
2B4
HD
1p19q
IDHmut
IDHwt

Figure 18. Distribution of T-cell exhaustion markers, as measured by flow cytometry, in $CD4^+$ T cells, $CD8^+$ T cells, Treg cells, and NK cells. *$CD4^+$ PD1*: mean HD (11.11) vs. mean IDHwt (8.204), $p = 0.0092$. *$CD4^+$ BTLA*: mean HD (42.51) vs. mean IDHwt (37.23), $p = 0.0203$. *$CD8^+$ TIGIT*: mean HD (52.01) vs. mean IDHwt (44.80), $p = 0.0142$. *$CD8^+$ 2B4*: mean HD (68.35) vs. mean IDHwt (59.32), $p = 0.0072$. *$Treg^+$ PD1*: mean HD (13.29) vs. IDHwt (10.38), $p = 0.0285$. *$Treg^+$ TIGIT*: mean HD (43.90) vs. mean IDHwt (38.63), $p = 0.0126$. *$Treg^+$ BTLA*: mean HD (4.414) vs. mean IDHwt (3.177), $p = 0.0030$. *$Treg^+$ CD57*: mean HD (8.698) vs. mean IDHwt (5.119), $p = 0.0248$. *$Treg^+$ KLRG1*: mean HD (13.08) vs. mean IDHwt (8.687), $p = 0.0242$. *$Treg^+$ 2B4*: mean HD (10.60) vs. mean IDHwt (5.723), $p = 0.0041$.

Similar to the combined IDHwt glioblastoma cohort, primary glioblastoma patients also showed a significant reduction in the same checkpoints when compared to HDs in the subanalysis (Figure 19): $CD4^+$ PD-1 and BTLA; $CD8^+$ PD-1, TIGIT, and 2B4; $Treg^+$ PD-1, TIGIT, BTLA, CD57, KLRG1, and 2B4; and NK^+ TIM-3 and TIGIT. Patients with recurrent glioblastoma also displayed a reduction in some of these markers compared to HDs, and $Treg^+$ TIGIT exhibited a significant increase in recurrent vs. primary glioblastoma patients. These results indicate that the reduced expression of exhaustion markers on circulating immune cells in glioblastoma patients vs. HDs is not further enhanced in recurrent tumors.

Taken together, these findings show a consistent reduction in the expression of multiple immune checkpoint receptors on $CD4^+$, $CD8^+$, and Treg T cells, as well as NK cells, in patients with IDHwt glioblastoma und IDHmut astrocytoma. This reduction suggests decreased immune exhaustion in the peripheral immune compartment. The similar checkpoint profiles in primary and recurrent glioblastoma further suggest that this phenotype is maintained throughout disease progression.

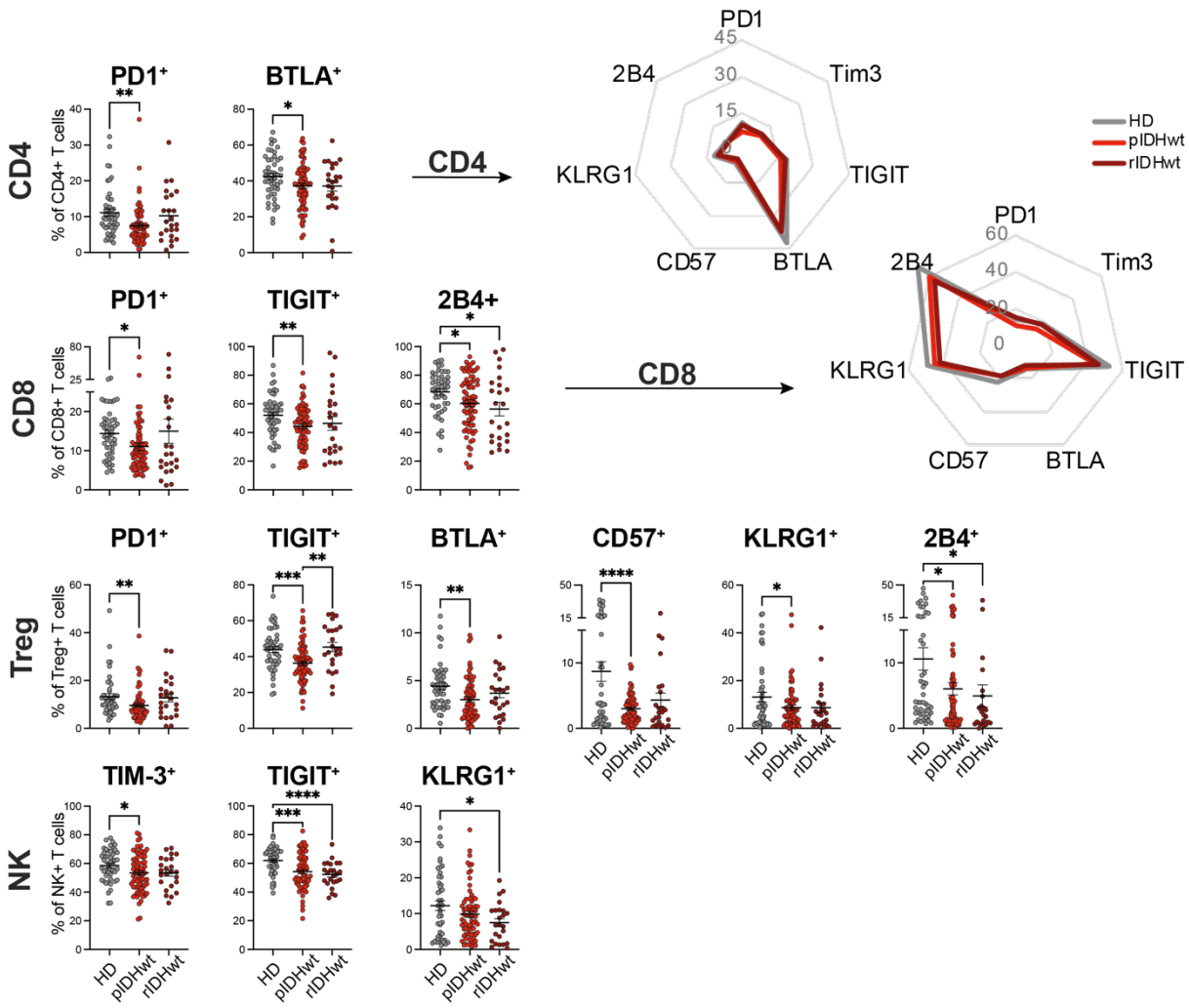


Figure 19. Distribution of T-cell exhaustion markers with significant differences in primary and recurrent IDHwt glioblastoma, as measured by flow cytometry, in $CD4^+$ T cells, $CD8^+$ T cells, Treg cells, and NK cells. Markers without significant differences are not shown. *$Treg^+$ TIGIT*: mean HD (43.90) vs. mean pIDHwt (36.40), $p = 0.0004$; mean pIDHwt (36.40) vs. mean rIDHwt (45.33), $p = 0.0012$.

4.7. Functional and metabolic status

We further assessed the functional activity status of circulating T cells by analyzing markers of metabolism and proliferation. Most strikingly, expression of the amino acid transporter CD98 was strongly increased on both $CD4^+$ and $CD8^+$ T cells in glioblastoma patients and was also increased in astrocytoma patients compared to HDs (Figure 20). The glucose transporter GLUT1 was also mildly increased on $CD4^+$ T cells in glioblastoma and astrocytoma patients and was higher on $CD8^+$ cells in glioblastoma vs. astrocytoma patients. Expression of the apoptosis receptor FAS was reduced on $CD8^+$ T cells in astrocytoma patients compared to HD and also compared to glioblastoma patients.

Expression of the ATP/ADP ectonucleotidase CD39 was moderately increased on $CD4^+$ T cells and Tregs in glioblastoma patients and on $CD8^+$ T cells in astrocytoma patients compared to HDs. The AMP ectonucleotidase CD73 was increased on $CD8^+$ T cells in glioblastoma patients and even more so in astrocytoma patients, while on $CD4^+$ T cells it was only increased in astrocytoma patients. HLA-DR expression was not altered in $CD4^+$ or $CD8^+$ T cells but was found to be increased in Treg cells in glioblastoma patients.

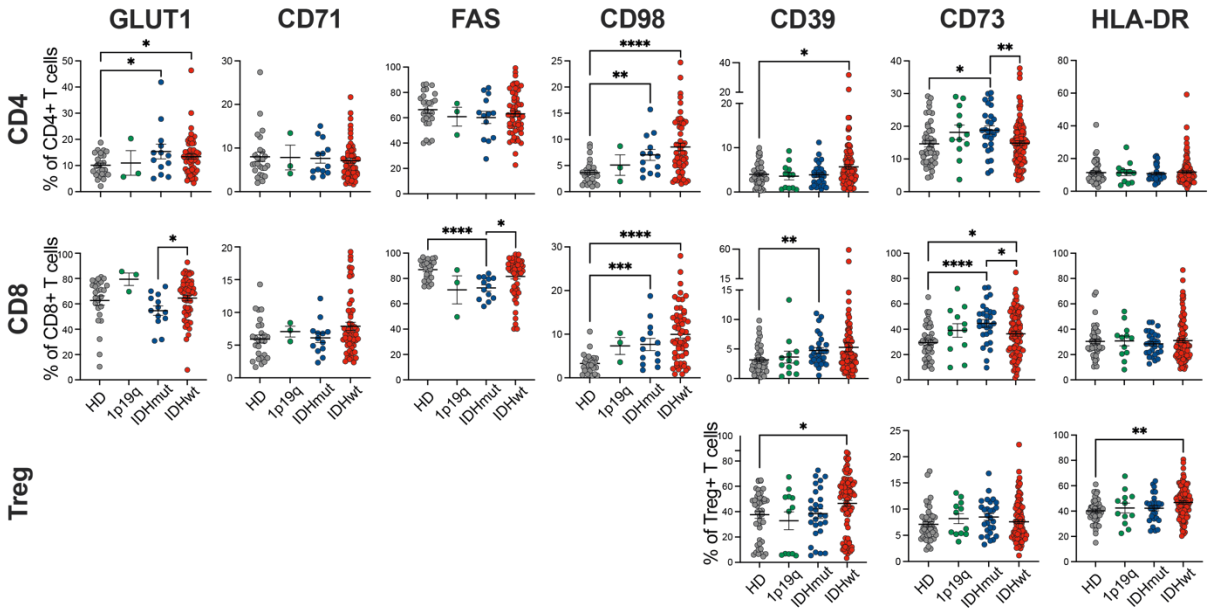


Figure 20. Distribution of metabolic immunomodulation and activation markers in T cells in HD and all glioma cohorts, as measured by flow cytometry. *$CD4^+$ CD98*: mean HD (3.645) vs. mean IDHwt (8.553), p = < 0.0001. *$CD4^+$ CD39*: mean HD (3.979) vs. mean IDHwt (5.678), p = 0.0277. *$CD8^+$ CD98*: mean HD (3.30) vs. mean IDHwt (9.962), p = < 0.0001. *$CD8^+$ CD73*: mean HD (29.50) vs. mean IDHwt (36.36), p = 0.0215; mean IDHmut (44.61) vs. mean IDHwt (36.36), p = 0.0283. *$Treg^+$ CD39*: mean HD (37.61) vs. mean IDHwt (46.54), p = 0.0229. *$Treg^+$ HLA-DR*: mean HD (40.20) vs. mean IDHwt (46.79), p = 0.0020.

Subanalysis of the IDHwt glioblastoma cohort revealed several striking differences between primary and recurrent glioblastomas (Figure 21). Elevated expression of CD39 as well as HLA-DR on $CD4^+$, $CD8^+$ and regulatory T cells were significant only in recurrent but not in primary glioblastoma patients. Conversely, CD73 expression on $CD8^+$ T cells was only elevated in primary glioblastoma patients vs. HDs. GLUT1 expression was only increased on $CD4^+$ T cells in primary glioblastoma patients, whereas FAS was increased on CD4 T cells in recurrent vs. primary patients. While the proportion of $CD4^+$ and $CD8^+$ T cells that expressed the transferrin receptor CD71 did not differ between the complete cohort and HDs

(Figure 20), CD71 expressing $CD4^+$ T cells were significantly increased in recurrent vs. primary glioblastoma patients, whereas CD71 positive $CD8^+$ T cells were increased in primary glioblastoma patients vs. HDs (Figure 21). CD98 expression on $CD4^+$ and $CD8^+$ T cells was similarly increased in primary and recurrent glioblastomas. Taken together, these data show that the metabolic activity of T cells in recurrent glioblastoma patients differs greatly from that in primary glioblastoma patients.

In summary, these findings show that T cells from glioblastoma and astrocytoma patients exhibit increased metabolic activity, as evidenced by elevated expression of CD98 and GLUT1, suggesting a state of immune activation. However, concurrent upregulation of immunoregulatory markers such as CD39, CD73, and HLA-DR, particularly in recurrent glioblastoma, points to a complex immune state involving both activation and compensatory immunosuppressive mechanisms.

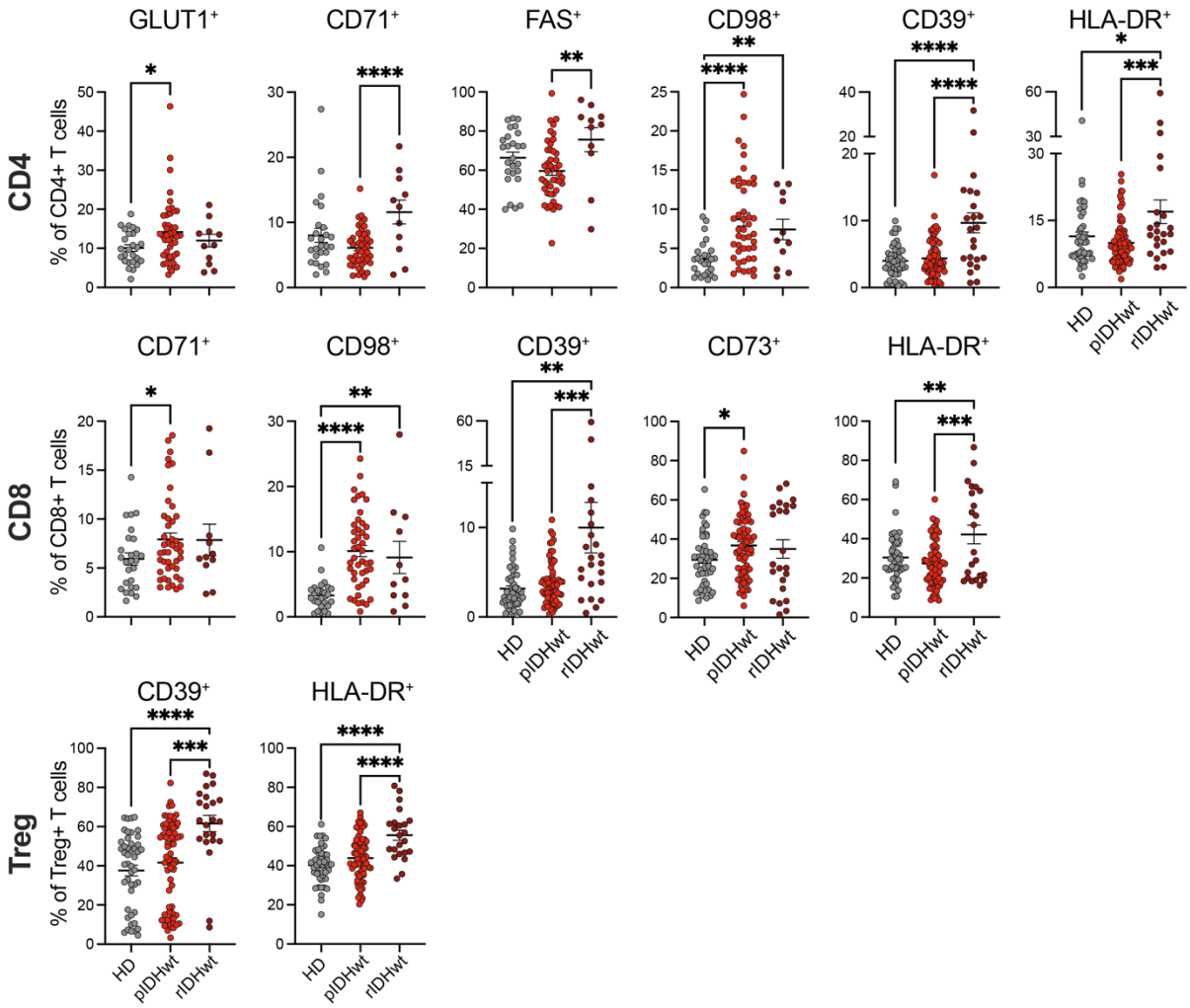


Figure 21. Distribution of metabolic immunomodulation and activation markers in T cells in HD and primary and recurrent IDHwt glioblastoma compared to HDs, as measured by flow cytometry. Markers without significant differences are not shown. *$CD4^+$ CD71*: mean pIDHwt (6.108) vs. mean rIDHwt (11.59), p = < 0.0001. *$CD4^+$ FAS*: mean pIDHwt (59.63) vs. mean rIDHwt (75.59), p = 0.0049. *$CD4^+$ CD39*: mean pIDHwt (4.370) vs. mean rIDHwt (9.660), p = < 0.0001. *$CD4^+$ HLA-DR*: mean pIDHwt (10.01) vs. mean rIDHwt (16.97), p = 0.0003. *$CD8^+$ CD39*: mean pIDHwt (3.741) vs. mean rIDHwt (9.990), p = 0.0003. *$CD8^+$ HLA-DR*: mean pIDHwt (27.35) vs. mean rIDHwt (42.17), p = 0.0001. *$Treg^+$ CD39*: mean pIDHwt (41.60) vs. mean rIDHwt (61.59), p = 0.0002. *$Treg^+$ HLA-DR*: mean pIDHwt (43.91) vs. mean rIDHwt (55.53), p = < 0.0001.

4.8. Summary of main findings

Table 3 summarizes the main peripheral immune abnormalities in glioblastoma patients (combined cohort) compared to healthy controls, and table 4 summarizes the main abnormalities in recurrent glioblastoma patients compared to primary glioblastoma patients. For clarity, the main peripheral immune differences between all, primary, and recurrent glioblastoma patients in comparison with HDs are visualized in Figure 22, and differences between recurrent vs. primary glioblastoma

patients are further highlighted in Figure 23. Only immune parameters showing statistically significant differences are included in both figures.

Notably, the most prominent differences were seen in markers of metabolism, together with alterations in immune checkpoint expression, cytokine production, and helper T-cell subsets. Compared to HDs, glioblastoma patients exhibited a metabolically activated peripheral T-cell phenotype, reflected by increased expression of metabolic and activation-associated markers such as GLUT1, CD98, CD73, CD39, and HLA-DR across multiple lymphocyte subsets (Table 3, Figure 22). This metabolic activation coincided with reduced production of pro-inflammatory cytokines, including IFNγ and TNFα, shifts in $CD4^+$ T-helper cell composition with reduced T_{FH} and ncT_H1 cells and increased T_H1 and IL-4–producing cells, as well as decreased expression of inhibitory checkpoint receptors on $CD4^+$, $CD8^+$ NK, and regulatory T-cell populations. In addition, glioblastoma patients showed reduced frequencies of NK and MAIT cells, indicating additional alterations of innate-like immune compartments.

Importantly, recurrent glioblastoma patients displayed further changes compared with primary glioblastoma patients, characterized by increased expression of metabolic and activation markers such as CD71, CD39, HLA-DR, and FAS on $CD4^+$, $CD8^+$ and regulatory T cells, alongside shifts in T-cell differentiation toward central memory, T_H2, and T_H17 phenotypes (Table 4, Figure 23). These changes were further reflected in cytotoxic and effector-associated compartments, including altered $CD8^+$ T-cell subset distributions and increased IFNγ production by NK cells, suggesting ongoing immune stimulation in the recurrent setting.

Taken together, these findings indicate that peripheral T cells in glioblastoma patients have undergone prior and sustained activation, as evidenced by enhanced metabolic and activation marker expression, but concurrently display features of functional impairment, including reduced effector cytokine production and altered differentiation states. This pattern is consistent with a state of immune dysfunction rather than effective anti-tumor immunity and becomes more pronounced with disease recurrence.

Table 3. Main peripheral immune abnormalities in IDHwt glioblastoma patients compared with HDs.

	$CD4^+$	**$CD8^+$**	**Other cell populations**
T-cell population			↓**NK** (-24.53%) ↓**MAIT** (-51.88%)
T-cell differentiation	↓early (T_{TM}) (-29.44%)	↓int. (T_{TE}) (-23.78%)	
T-helper	↓T_{FH} (-52.80%) ↑T_H1 (+30.64%) ↓ncT_H1 (-42.43%)		
Cytokines	↓IFNγ (-38.58%) ↓TNFα (-25.52%) ↑IL-4 (+50.51%)	↓ IFNγ (-25.59%) ↓ TNFα (-24.58%)	
Checkpoints	↓PD1 (-26.16%) ↓BTLA (-12.42%)	↓TIGIT (-13.86%) ↓2B4 (-13.21%)	**Treg:** ↓PD-1 (-21.90%) ↓TIGIT (-12.00%) ↓CD57 (-41.15%) ↓2B4 (-46.01%) ↓BTLA (-28.02%) ↓KLRG1 (-33.59%) **NK:** ↓TIGIT (-13.29%) ↓TIM-3 (-8.86%) ↓KLRG1 (-24.20%)
Function & Metabolism	↑GLUT1 (+32.64%) ↑CD98 (+134.65%) ↑CD39 (+42.70%)	↑CD98 (+201.88%) ↑CD73 (+23.25%)	**Treg:** ↑CD39 (+23.74%) ↑HLA-DR (+16.39%)

Table 4. Main peripheral immune abnormalities in recurrent IDHwt glioblastoma patients compared to primary IDHwt glioblastoma patients.

	$CD4^+$	**$CD8^+$**	**Other cell populations**
T-cell population	↓CD4 (-16.25%) ↓conCD4 (-18.64%)	↑CD8 (+26.51)%	
T-cell differentiation	↑T_{CM} (+36.48%)	↑T_{NV} (+53.19%) ↓Tearly-like (-28.24%) ↓eff RA- (-36.87%)	
T-helper	↑T_H2 (+49.06%) ↑T_H17 (+109.44%)		
Cytokines	↑IL-17 (+72.50%)		**NK:** ↑IFNγ (+36.84%)
Checkpoints			**Treg:** ↑TIGIT (+24.53%)
Function & Metabolism	↑CD71 (+89.75%) ↑FAS (+26.77%) ↑CD39 (+121.05%) ↑HLA-DR (+69.53%)	↑CD39 (+167.04%) ↑HLA-DR (+54.19%)	**Treg:** ↑CD39 (+48.05%) ↑HLA-DR (+26.46)

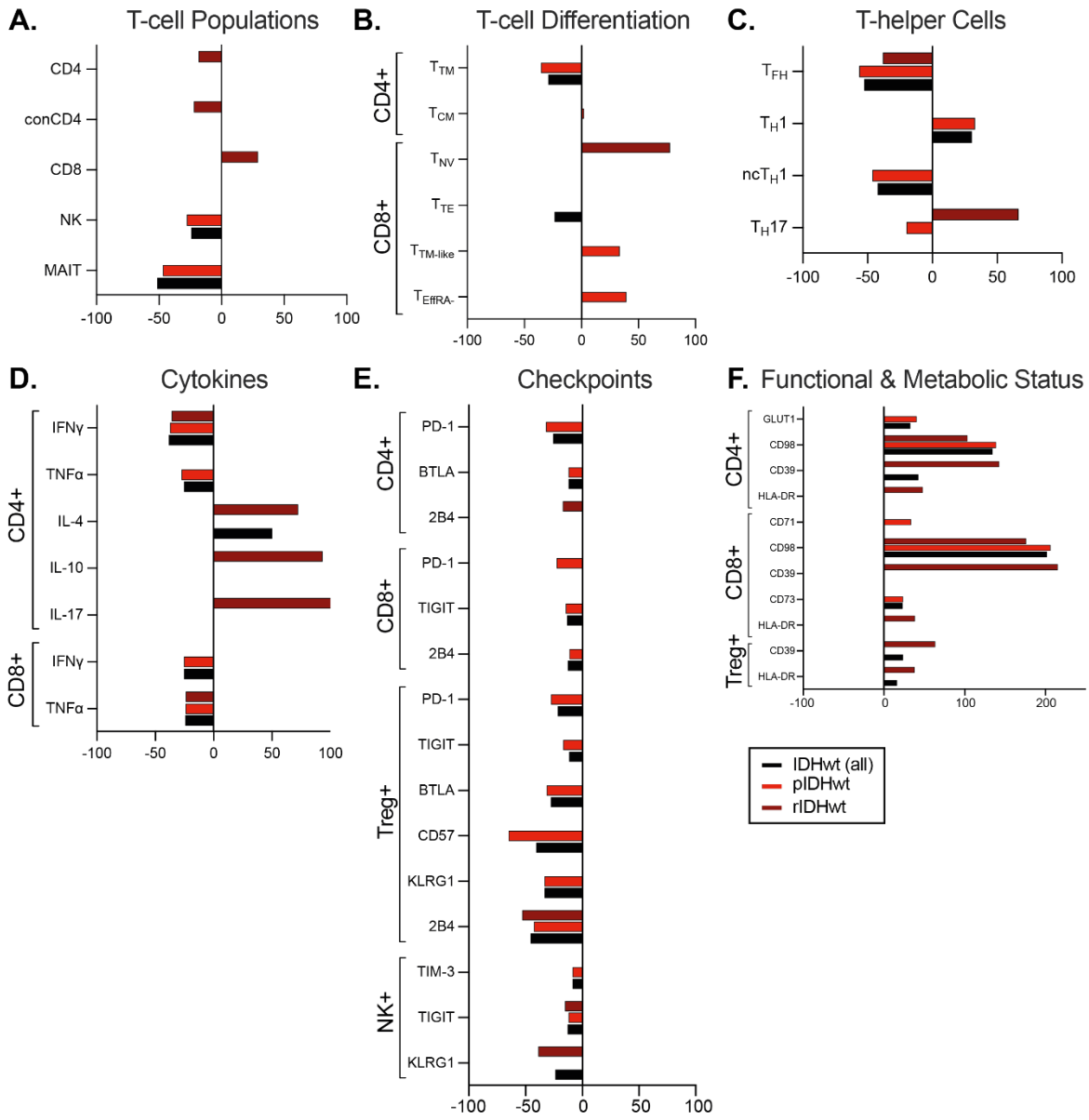


Figure 22. Main peripheral immune abnormalities between all IDHwt, primary IDHwt, and recurrent IDHwt glioblastoma, each compared to HDs. Shown are the alterations in T-cell populations (A), T-cell differentiation (B), T-helper subsets (C), cytokine profiles (D), immune checkpoint expression (E), and T-cell functional and metabolic status (F).

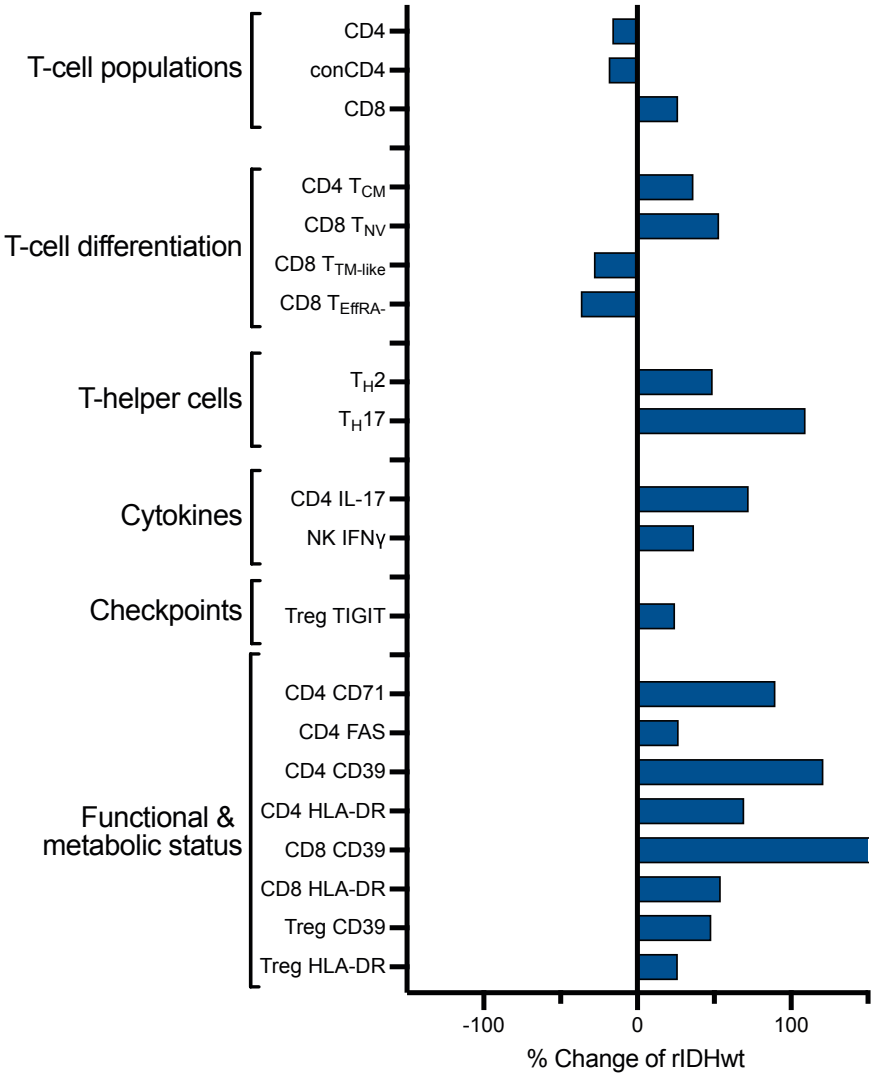


Figure 23. Main peripheral immune abnormalities in recurrent IDHwt glioblastoma patients, as compared to primary IDHwt glioblastoma patients.

4.9. Dexamethasone

Dexamethasone is frequently administered to glioblastoma patients for the management of peritumoral edema and increased intracranial pressure. However, corticosteroids are well known to exert immunosuppressive effects, including reductions in T-cell numbers and alterations in $CD4^+/CD8^+$ T-cell ratios, making it important to account for potential steroid-associated effects when analyzing peripheral immune profiles.

Overall, peripheral immune marker expression was largely comparable between glioblastoma patients taking preoperative dexamethasone and those not taking preoperative dexamethasone. The only significant difference observed was a

reduction in for T_{FH} $CD4^+$ T cells in patients treated with dexamethasone (Supplemental Figure 6), indicating that preoperative dexamethasone treatment did not exert a major effect on the peripheral immune phenotype in this cohort.

4.10. Overall survival

To evaluate the prognostic relevance of immune cell phenotype alterations, we analyzed the survival of glioblastoma patients in relation to all flow cytometry markers assessed. For survival analyses, patients were divided into the top 30% and bottom 30% of expression for each marker (see Methods). Of the T-cell markers that showed significant differences in expression when compared with healthy donors, only six were associated with a significant difference ($p < 0.05$) in survival of either the entire glioblastoma patient cohort and/or patients with primary or recurrent tumors (Figure 24). In addition, four markers showed trends toward an association with survival that did not reach significance ($p < 0.1$).

In IDHwt glioblastomas (combined primary and recurrent), high $CD8^+TIGIT^+$ and Treg HLA-DR^+ expression were significantly associated with shorter patient survival (Figure 24A). For $CD8^+CD73^+$, there was a trend toward an association with prolonged survival, although it did not reach statistical significance ($p = 0.053$). When only primary glioblastomas were analyzed, $CD8^+TIGIT^+$ and $CD8^+CD73^+$, were prognostically significant (Figure 24B). In addition, high levels of Treg $CD57^+$ cells were associated with shorter survival in primary glioblastoma patients. In recurrent glioblastomas, higher levels of conventional $CD4^+$ cells were significantly associated with patient survival (Figure 24C). $CD4^+IFN\gamma$, $CD4^+$IL-4, and NK $KLRG1^+$ cells showed trends toward a negative association with survival that did, however, not reach statistical significance ($p = 0.08$, $p = 0.08$, and $p = 0.089$, respectively; Figure 24C).

Given the differences in the peripheral immune phenotypes observed between primary and recurrent glioblastoma, we additionally explored whether immune markers that differed significantly between these two disease stages were associated with differences in survival. No statistically significant survival differences were observed for any of the markers analyzed in this comparison, although $CD4^+$IL-17 ($p = 0.081$) and $CD4^+FAS^+$ ($p = 0.062$), both upregulated in

glioblastoma, showed trends toward shorter overall survival with higher expression, whereas $CD8^+$ naïve (p = 0.058), also upregulated in glioblastoma, showed a trend toward longer overall survival with higher expression. However, these trends did not reach statistical significance (data not shown).

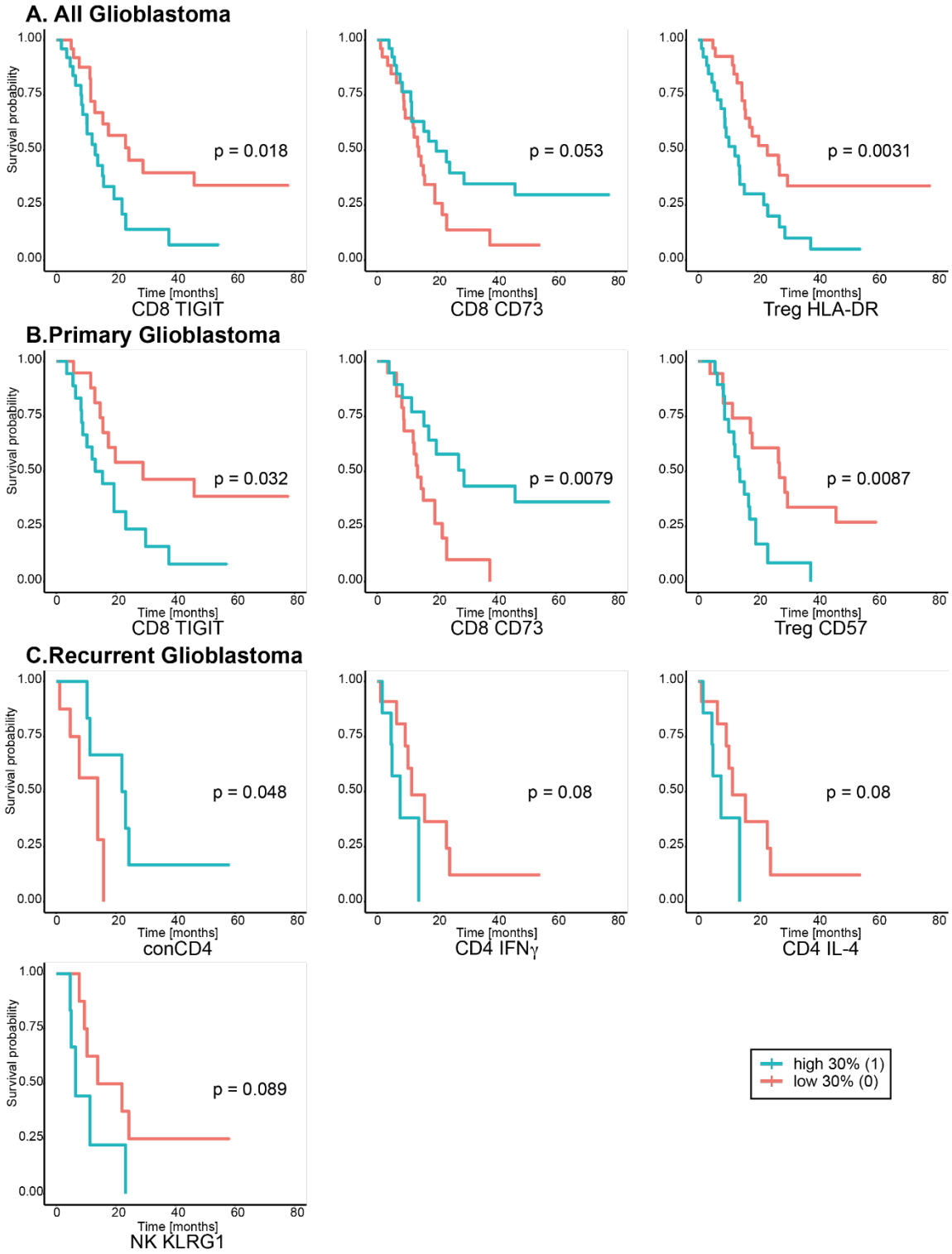


Figure 24. Survival analysis of patients with either high (top 30%) or low (bottom 30%) expression of immune markers that were significantly different between HDs and all IDHwt glioblastoma (A), HDs and primary IDHwt glioblastoma (B), or HDs and recurrent IDHwt glioblastoma (C). Only markers with significant differences between glioblastoma (all,

primary, or recurrent) and HDs were initially considered, and survival comparisons with *p*-values > 0.1 were excluded. The two survival curves represent the highest and lowest 30% for each marker, respectively. Only markers that were significantly different between glioblastoma (all, primary, or recurrent) and HDs were initially considered. Differences in survival between the top 30% and bottom 30% expression groups were excluded if the *p*-value exceeded 0.1.

4.10.1. Association of peripheral immune marker expression with overall survival

Survival-associated differences identified in the preceding analyses were subsequently examined in relation to the corresponding peripheral immune marker expression levels. Figure 25 first illustrates the relationship between overall survival and peripheral immune marker expression in the combined IDHwt glioblastoma cohort. Table 5 provides a summary overview of all immune markers associated with differences in survival, including the direction of regulation in glioblastoma patients compared with HDs. This enables survival-associated expression patterns to be presented alongside disease-related immune alterations. Figures 26 and 27 extend this analysis by visualizing peripheral immune marker expression separately in primary and recurrent glioblastoma patients. Figure 27 highlights markers exhibiting more pronounced expression differences between primary and recurrent disease stages. In all figures, circles are used as a visual aid to indicate the relative position of patients with higher and lower marker expression contributing to the survival differences. A detailed interpretation of these associations is provided in the discussion.

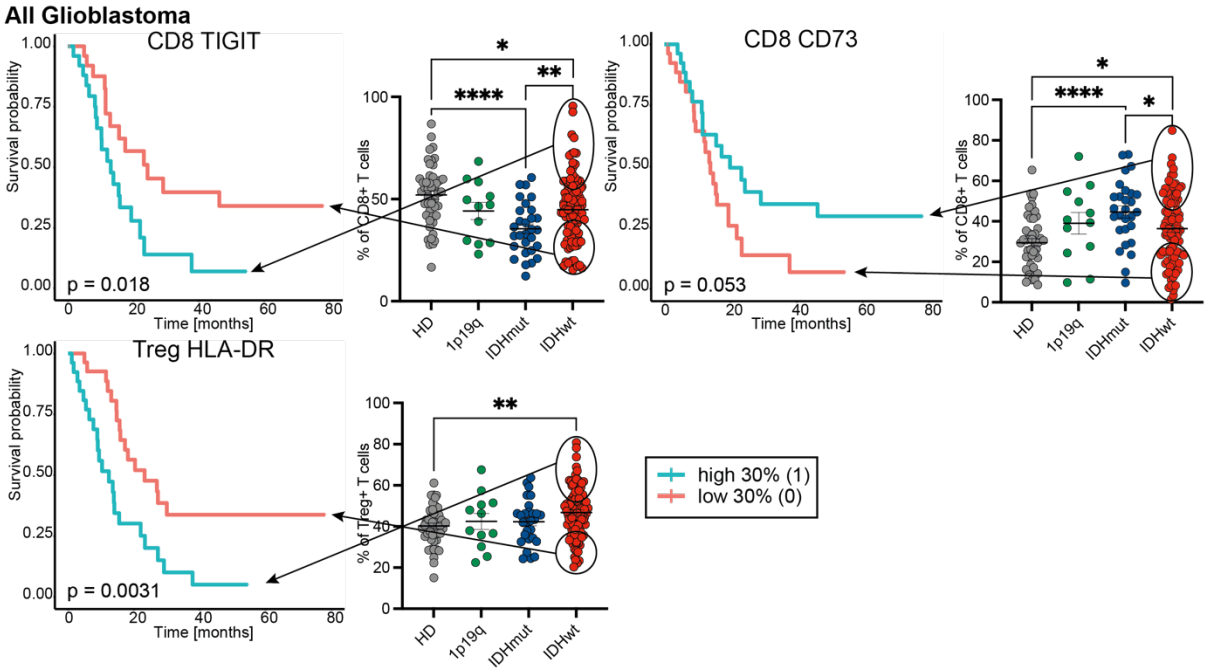


Figure 25. Comparison of survival data from Figure 24 (A) with the corresponding marker expression in peripheral blood for all IDHwt glioblastomas. Circling is an approximation of the upper and lower 30% values.

Table 5. Immune markers of particular interest: significantly altered immune markers with prominent differences in survival between the upper and lower 30% of marker expression.

IDHwt (all)		**Direction of regulation (glioblastoma vs. HD)**	**Prognosis in patients with high expression**
CD8 TIGIT$^+$ (p = 0.0018)	Checkpoint marker; exhaustion	↓	worse
CD8 CD73$^+$ (p = 0.053)	ATP metabolism; reduces T-cell activation and cytotoxicity	↑	better
Treg HLA-DR$^+$ (p = 0.0031)	Activation marker	↑	worse
Primary IDHwt			
CD4 IL-17$^+$ (p = 0.081)	Immune activating, when overstimmulated chronic inflammation	↑	worse
CD8 naive (p = 0.058)	Immune activating	↑	better
CD8 TIGIT$^+$ (p = 0.032)	Checkpoint marker; exhaustion	↓	worse
CD8 CD73$^+$ (p = 0.0079)	ATP metabolism; reduces T-cell activation and cytotoxicity	↑	better
Treg CD57$^+$ (p = 0.0087)	Checkpoint marker; exhaustion	↓	worse
Recurrent IDHwt			
conCD4$^+$ (p = 0.048)	Helper T cells	↓	better
CD4 IFNγ^+ (p = 0.08)	Effector function, shift towards T_H1	↓	worse
CD4 IL-4$^+$ (p = 0.08)	Shift towards T_H2	↑	worse
CD4 FAS$^+$ (p = 0.062)	Apoptosis	↑	worse
NK KLRG1$^+$ (p = 0.089)	Checkpoint markers; exhaustion	↓	worse

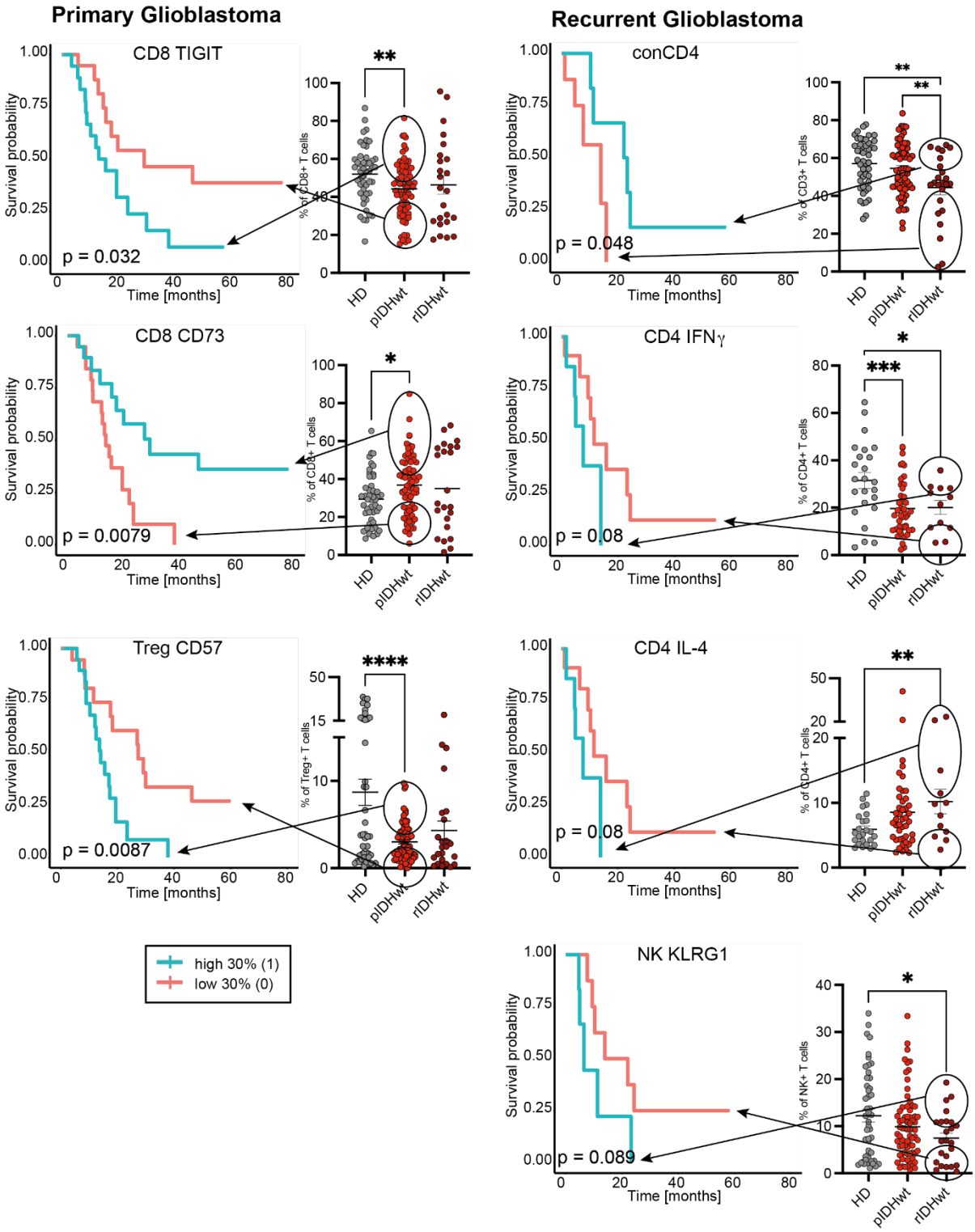


Figure 26. Comparison of survival data from Figure 24 (B and C) with the corresponding marker expression in peripheral blood for primary (left column) and recurrent (right column) IDHwt glioblastoma. The circling is an approximation of the upper and lower 30%.

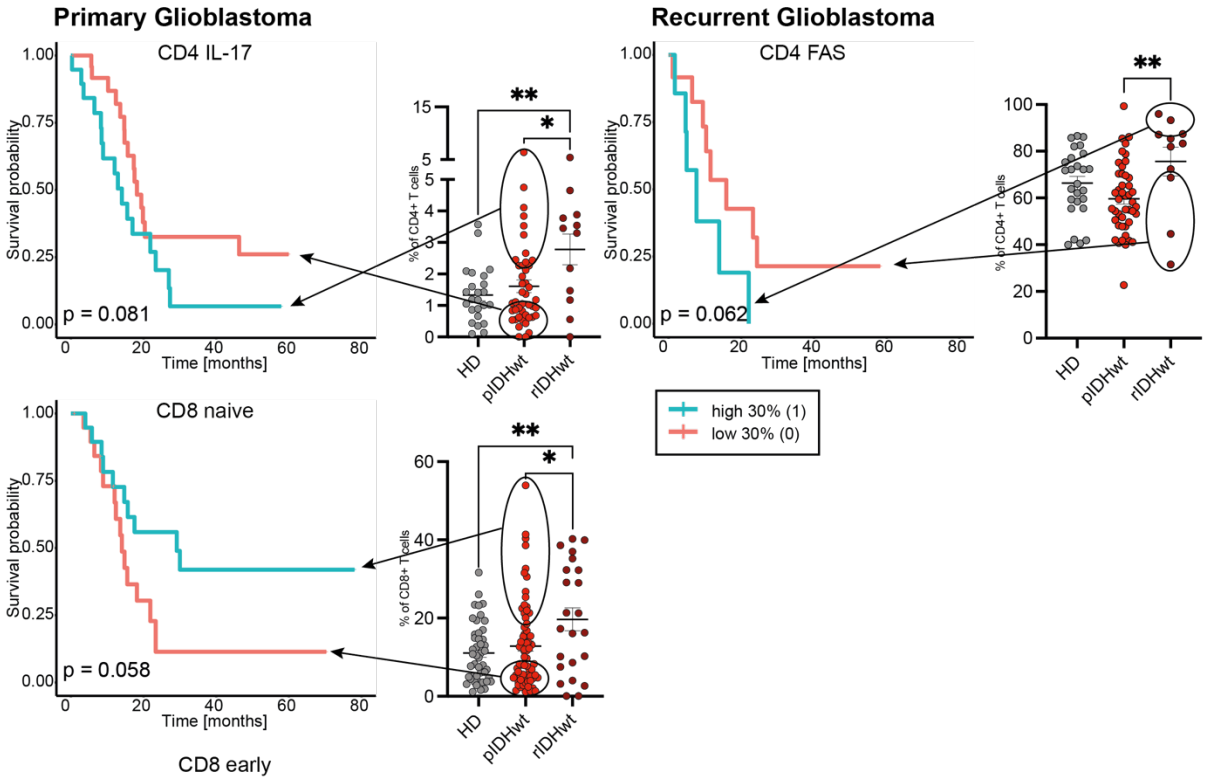


Figure 27. Comparison of survival data with the corresponding marker expression in the peripheral blood for primary (left column) and recurrent (right column) IDHwt glioblastoma for markers with prominent (p < 0.1) differences between primary and recurrent IDHwt glioblastoma (not when compared with HDs). The circling is an approximation of the upper and lower 30%.

5. Discussion

5.1. Peripheral immune phenotype of gliomas

In this study, we found profound alterations in the peripheral immune status of patients with glioblastoma. Some of these changes point to increased immune activation with less T-cell exhaustion, more T_H1 cells, and increased T-cell metabolic activity. On the other hand, there is ample evidence of a suppressed or less effective peripheral immunity, with fewer NK, MAIT, T_{FH} and non-classic T_H1 cells, more activated and less exhausted Tregs, increased ATP metabolism, as well as production of less proinflammatory and more anti-inflammatory cytokines (Table 6).

Table 6. The peripheral immune status of glioblastoma patients compared to healthy donors is characterized by simultaneous immune activation and immune suppression.

Immune activation	Immune suppression
↓ exhaustion ($CD4^+$, $CD8^+$) ↑ T_H1 cells ↑ metabolism (GLUT1 on CD4+ cells; $CD98^+$ on $CD4^+$ and $CD8^+$ cells)	↓ NK cells ↓ MAIT cells ↓ T_{FH} cells ↓ non-classic T_H1 cells ↓ exhaustion (Tregs) ↑ ATP metabolism (CD39 on $CD4^+$ cells and Tregs, CD73 on $CD8^+$ cells) ↓ pro-inflammatory cytokines (IFNγ, TNFα) ↑ anti-inflammatory cytokines (IL-4) with a shift towards T_H2

Our subgroup analysis of primary and recurrent glioblastoma patients showed similar shifts, indicating increased immune activation on the one hand and immune suppression on the other hand in both subgroups. However, there were also some significant differences in peripheral immune status between primary and recurrent glioblastoma (Table 7), indicating additional immune alterations along glioblastoma progression. On the side of immune activation, recurrent glioblastoma patients exhibited an increase in $CD8^+$ T cells, which are crucial for cytotoxic anti-tumor responses, compared to primary glioblastoma patients. Additionally, the increase in T_H17 cells, along with elevated levels of the pro-inflammatory cytokine IL-17, suggests a further increase in inflammation. The increased expression of HLA-DR in both $CD4^+$ and $CD8^+$ T cells indicates amplified antigen presentation and T-cell activation. Increased Treg exhaustion, as indicated by an increase in TIGIT

expression, may impair the suppressive capacity of Treg cells and thus tilt the balance towards immune activation.

Parallel to these findings of increased immune activation, several markers of immune suppression were also more prevalent in patients with recurrent glioblastoma than in patients with primary glioblastoma. An overall reduction in both $CD4^+$ and conventional $CD4^+$ T cells reflects a diminished T-helper cell pool. Increased expression of CD39 in $CD4^+$, $CD8^+$, and Treg subsets suggests enhanced ATP metabolism, which has been associated with an immunosuppressive extracellular adenosine environment, favoring tumor immune evasion (Allard et al., 2017). Elevated HLA-DR on Tregs implies an activated regulatory phenotype, which may counteract the observed Treg exhaustion and further suppress antitumor immunity. Additionally, a decline in central memory $CD4^+$ T cells indicates a reduced capacity to sustain long-term immunosurveillance. A shift towards T_H2 differentiation, which we found in recurrent glioblastoma, is also known to be associated with a less effective immune response than the T_H1 phenotype (Basu et al., 2021). These results highlight the dynamic and in part contradictory nature of peripheral immune responses in patients with recurrent glioblastoma. While certain aspects of immune activation suggest an ongoing antitumor response, simultaneous immune suppression may undermine its effectiveness, creating a permissive environment for tumor progression.

Table 7. The peripheral immune status of recurrent IDHwt glioblastoma patients compared to that of primary IDHwt glioblastoma is characterized by distinct immune activation and immune suppression.

Immune activation	Immune suppression
↑$CD8^+$ cells ↑T_H2 cells ↑T_H17 cells ↑pro-inflammatory cytokines (IL-17) ↑exhaustion (Tregs: TIGIT) ↑HLA-DR ($CD4^+$ and $CD8^+$ cells) ↑$CD4^+$ T_{NV} ↓$CD4^+$ early (T_{TE})	↓$CD4^+$ cells, ↓conCD4$^+$ ↑ATP metabolism (CD39 on $CD4^+$, $CD8^+$, and Treg cells) ↑HLA-DR (Tregs) ↓$CD4^+$ T_{CM}

There are only a few other publications reporting the peripheral immune status of glioblastoma patients (Mohme et al., 2018, Woroniecka et al., 2018a, Shimato et al., 2012, Shen et al., 2021, Mostafa et al., 2016, Fecci et al., 2006). Similar to our data,

all of these studies described significant alterations, yet with differences in the details. A previous report from our laboratory described a smaller cohort of 40 glioblastoma patients and 15 healthy donors, with a less detailed analysis of the peripheral immune phenotype and a stronger focus on tumor-infiltrating lymphocytes (TILs) (Mohme et al., 2018). Similar to our current results, this previous study revealed a profound suppression in the peripheral immune response, for example, with a similar reduction in inflammatory cytokine production (IFNγ, TNFα) in peripheral T cells. Moreover, we here found that PD-1 expression on both $CD4^+$ and $CD8^+$ T cells as well as Treg cells was reduced in primary glioblastoma patients (Figure 19), which was also observed in the prior report (Mohme et al., 2018). Extending beyond the previous results, we now detected decreased expression of other exhaustion markers, such as TIGIT, 2B4, and BTLA (Figure 18, Figure 19, Figure 22) especially in primary glioblastoma patients, suggesting a common mechanism of immune checkpoint downregulation on peripheral T cells. The previous study showed a trend towards an increase in PD-1 expression in recurrent vs. primary glioblastoma patients, and this trend was also observed here, although not significant (Figure 19) (Mohme et al., 2018). Of note, in the previous study we had compared TILs to peripheral T cells and found a striking increase of several immune checkpoints, such as PD-1 and TIM-3, on TILs compared to peripheral T cells in glioblastoma patients. Taken together, the decreased expression of multiple immune checkpoints on peripheral T cells in conjunction with reduced cytokine production indicates systemic T cell dysfunction and may be linked to the accumulation of exhausted T cells in the tumor microenvironment. Alternatively, the reduced expression of exhaustion markers in the periphery could also be due to T-cell sequestration in the bone marrow or lymph nodes (Mohme et al., 2018), a mechanism suggested by Chongsathidkiet *et al*. (Chongsathidkiet et al., 2018).

Other studies on the peripheral immune status in glioblastoma patients have either focused on a few specific types of immune cells, such as e.g. peripheral $CD4^+$ T cells, or on a smaller number of specific markers in smaller patient cohorts. No other study has attempted a comprehensive characterization of the peripheral immune status in a large number of patients, as presented here. In contrast to our current study, Shen *et al*. (Shen et al., 2021) reported increased levels of the checkpoint markers LAG-3, TIM-3, and BTLA in both TILs and PBMCs (in $CD4^+$ and $CD8^+$) of

glioblastoma patients. However, similar to our findings this was not confirmed in a study by Woroniecka *et al*., who instead observed that T-cell exhaustion was primarily confined to TILs rather than peripheral T cells (Woroniecka et al., 2018a).

Shimato *et al*. (Shimato et al., 2012) investigated cytokine expression and described a modest decrease in IFNγ secretion and an increase in IL-5 production in stimulated peripheral $CD4^+$ T cells of 13 primary glioblastoma patients, and interpreted this data as indicating a predominance of T_H2 immune responses. While we did not directly investigate IL-5, we also found a decrease in the T_H1 cytokine IFNγ in $CD8^+$ and $CD4^+$ T cells and an increase in another T_H2 cytokine, IL-4, in $CD4^+$ cells. In recurrent glioblastoma, we also observed an increase in T_H2 cells (Figure 14). Thus, our results are in agreement with Shimato *et al*., indicating that an important part of the systemic immune abnormality in glioblastoma patients is a shift towards T_H2 cells, which mediate less effective antitumor responses than T_H1 cells (Shimato et al., 2012).

Fecci *et al*. (Fecci et al., 2006) found a decrease in Treg numbers in 20 glioblastoma patients, which was not observed in our data. We did, however, also detect alterations of peripheral Tregs, including increased expression of CD39, suggesting enhanced ATP metabolism and immunosuppressive potential, as well as reduced expression of checkpoint marker expression on Tregs, such as lower PD-1 expression (Figures 18 and 19). In line with these findings, Mostafa *et al*. (Mostafa et al., 2016) detected higher expression of CD39 on peripheral Tregs, indicating increased immunosuppressive functions of these cells.

Consistent with our results, Shen *et al*. (Shen et al., 2021) and Mostafa *et al*. (Mostafa et al., 2016) both detected reduced numbers of peripheral NK cells in 36 and 51 primary glioblastoma patients, respectively. In addition, we found reduced TIGIT expression on NK cells (Figures 18 and 19), indicating altered activation or differentiation states of peripheral NK cells in glioblastoma patients.

In summary, our data on the peripheral immune status of glioblastoma patients confirm and extend some, but not all findings obtained in previous studies. In particular, our observation that at several immune pathways, such as T_H17/IL-17

and T_H2 cytokine responses, are activated in glioblastoma patients is novel and has not been previously reported. Despite this partial immune activation, it is obvious from the rapid progression and high mortality rates of glioblastoma patients that the net balance of side-by-side immune activation and immune suppression still results in a failure of an effective immune response against the tumor.

In order to identify the most critical components of the immune response in glioblastoma patients, we analyzed the association of immune markers with patient survival. Among the studies discussed above, Mostafa *et al*. are the only authors to have linked peripheral blood immune phenotypes to overall survival (Mostafa et al., 2016). However, their analysis was limited to a small number of immune parameters, whereas we performed a comprehensive survival analysis across a broad range of peripheral immune features. Specifically, we compared survival between patients with the highest and lowest expression of selected immune marker parameters that had shown either statistically significant differences ($p < 0.05$) or non-significant trends ($p < 0.1$) between glioblastoma and HDs, as well as between primary and recurrent glioblastomas. Of all the T-cell parameters that differed significantly between HDs and glioblastomas (primary and recurrent combined), only three showed either statistically significant survival differences or non-significant trends toward survival differences ($p < 0.1$) (Figure 24A). Further analysis subdividing the group of all glioblastoma patients into primary and recurrent glioblastomas, revealed five additional markers showing statistically significant survival differences or non-significant survival trends, including one marker in primary glioblastoma and four in recurrent glioblastomas (Figure 24B-C). Three additional markers were found to have non-significant trends toward survival differences between primary and recurrent glioblastomas. Therefore, these eleven markers are likely to be clinically relevant for the immune escape of glioblastomas. Table 5 lists these eleven markers. For a more detailed interpretation of these eleven markers Figures 25-27 juxtapose immune marker expression with survival data. The following sections discuss each of these markers of interest in detail, separated by cell types (CD8, CD4, Treg, and NK cells).

On $CD8^+$ T cells, TIGIT expression was identified as associated with survival in the analysis of all glioblastomas and in the subgroup of primary glioblastomas. TIGIT is

a checkpoint marker that indicates T cell exhaustion. As expected, a low prevalence of TIGIT expressing CD8 T cells was associated with improved survival (Table 5, Figure 25). Another marker on CD8 T cells that had a significant prognostic impact in both cohorts was CD73, a marker for ATP metabolism. By degrading AMP to adenosine, CD73 is thought to reduce inflammation, T-cell activation and cytotoxicity against tumor cells (Allard et al., 2017). We would expect a lower expression to correspond with improved survival, as has been previously reported in melanoma (Shevchenko et al., 2020). However, we found the opposite to be true (Table 5, Figure 25). There is no good explanation for this unexpected observation, except that, in contrast to current thinking, elevated ATP metabolism may also indicate higher T-cell activity. We further found that higher levels of $CD8^+$ naïve cells were associated with a longer survival in patients with primary glioblastomas (Table 5, Figure 27). Naïve T cells are responsible for immune surveillance and can differentiate into effector cells once activated (Mousset et al., 2019). Elevated $CD8^+$ naïve cells may thus indicate a better availability and reserve for an active immune response to tumor cell antigens, against which no immune responses have been mounted yet.

In primary glioblastoma patients, $CD4^+$ T cells expressing the proinflammatory cytokine IL-17 were associated with survival. Despite its protective capacity, IL-17 can lead to chronic inflammation and cancer progression when overstimulated (Amatya et al., 2017). We found a trend toward prolonged survival in patients with lower $CD4^+$ IL-17 production (Table 5, Figure 27), pointing towards overstimulation and thus less effective immune responses for those patients with higher production. In recurrent glioblastoma, our data showed significantly longer survival for patients with the highest numbers of conventional $CD4^+$ T cells (Table 5, Figure 26). This cell subset, encompassing all helper T cells, is essential for a coordinated immune response, and a higher number of CD4 cells indicates a stronger immune response. CD4-derived IFNγ is generally associated with a shift towards T_H1, and therefore a more effective anti-tumor response (Dong, 2021, Basu et al., 2021). However, our data showed a trend toward prolonged survival for patients with low $CD4^+$ IFNγ in the recurrent glioblastoma cohort (Table 5, Figure 26), which did not fit with the expected function. A stronger T_H1 response usually suggests a better immune response, but the data indicate that this may not necessarily correlate with better

survival, possibly due to the inflammatory damage or exhaustion seen with chronic activation. Another marker that showed a trend toward differences in survival in recurrent glioblastoma patients was $CD4^+$ IL-4. These cells are indicative of a shift towards a T_H2 response. Our finding of a tendency for prolonged survival in patients with low numbers of IL-4 producing $CD4^+$ cells (Table 5, Figure 26) is consistent with the immunosuppressive role of T_H2 cells. Moreover, we detected a trend toward prolonged survival in recurrent glioblastoma patients with low concentrations of $CD4^+$ cells expressing FAS (Table 5, Figure 26). FAS is a receptor involved in apoptosis (Chen et al., 2010, Martin-Villalba et al., 2013), and when expressed on $CD4^+$ cells, it typically leads to immunosuppressive effects by eliminating T cells and reducing excessive immune responses. Reduced FAS expression may allow for prolonged survival of $CD4^+$ T cells and thereby support a more robust immune response.

HLD-DR, an MHC class II molecule, plays an important role in antigen presentation and immune activation (Jones et al., 2006). Expression of HLA-DR on Tregs indicates activation and strong immunosuppressive potential (Machicote et al., 2018), which is corroborated by our finding that survival is highly significantly prolonged in patients with low Treg HLA-DR expression on Tregs in the combined glioblastoma cohort (Table 5, Figure 25). Another marker relevant for Treg function is CD57, a checkpoint and exhaustion marker (Zhao et al., 2020), which we have also found to be negatively associated with survival in primary glioblastoma patients (Table 5, Figure 26). CD57 expression on Tregs usually distinguishes less active inhibitory cells and therefore indicates less immune inhibition. A possible explanation for the negative survival association may be that these less dysfunctional Tregs help to maintain a balanced immune response and that perhaps a pathological, futile shift of immune activity is being suppressed through this cell subset.

The last of the eleven markers, KLRG1 on NK cells, showed a trend toward an association with shorter survival in patients with recurrent glioblastomas (Tabel 5, Figure 26). KLRG1 is a marker of terminal differentiation and senescence of NK cells and T cells (Zhang et al., 2024, Zhao et al., 2020). Accordingly, increased

KLRG1 expression on NK cells may reflect reduced proliferative capacity and contribute to impaired anti-tumor immunity and shorter patient survival.

Considering the above-summarized immune status of glioblastoma patients, these 11 markers of particular interest point in the same direction of higher but less effective immune activation. These markers also represent possible targets for immunotherapy as well as possible mechanisms for improving the efficacy of existing immunotherapeutic agents. Among these, TIGIT expression on $CD8^+$ T cells appears particularly relevant, as TIGIT represents a targetable immune checkpoint with emerging therapeutic relevance in oncology (Harjunpää and Guillerey, 2020, Chauvin and Zarour, 2020, Rousseau et al., 2023). Although TIGIT expression was reduced in glioblastoma patients when compared to HDs, higher TIGIT expression within the glioblastoma cohort was associated with shorter survival, indicating a context-dependent prognostic role. This suggests that TIGIT-targeted approaches may hold therapeutic potential in selected glioblastoma patients. It is noteworthy that immune checkpoints targeted in effective classic immunotherapy in other malignancies, but not in glioblastoma (for example, PD-1 and CTLA), are not among the 11 markers of particular interest identified here, as they were neither upregulated in glioblastoma patients when compared to healthy donors nor associated with patient survival.

5.2. Peripheral immune phenotype in other malignancies

To further evaluate our results, it is important to compare our results to data on the peripheral immune status in patients with other malignancies that have more favorable prognoses and/or better responses to immunotherapy. Many studies have investigated the peripheral immune status of patients with a variety of different cancers, some of which attempted to correlate selected immune markers with patient prognosis. Fewer studies have provided an in-depth analysis of the peripheral immune status of cancer patients as detailed as we did here. The following studies were selected because they either represent seminal work or use similar flow cytometry-based profiling of peripheral immune cells.

In metastatic colorectal cancer, Tada *et al.* (Tada et al., 2016) used a multicolor flow cytometry panel, albeit less extensive than the panel used in our study, to analyze circulating immune cell subsets and found that low effector memory $CD4^+$ and $CD8^+$ T cells, as well as elevated MDSCs, correlated with shorter progression-free survival. Although we observed changes within the differentiation compartments, including effector subsets, such as a decrease in CD4+ early and CD8+ intermediate T cells in glioblastoma when compared to HDs (Figure 11), and fewer CD4+ early and early-like T cells alongside an increase in CD8+ early-like and effector RA- subsets in primary glioblastoma (Figure 12), we found no correlation between these subsets and overall survival.

Brooks *et al.* (Brooks et al., 2012) characterized peripheral blood monocytes in endometrial cancer and observed an altered phenotype with increased IL-12 and decreased IL-10 secretion, although no analysis with respect to survival was performed. Although monocytes were not analyzed in our study, this finding is relevant in the context of systemic immune modulation in cancer. In contrast, we focused on T and NK cells rather than monocytes and found elevated $CD4^+$ IL-10 production in recurrent glioblastoma (Figure 16) but detected no association with survival.

Batalha *et al.* (Batalha et al., 2021) examined peripheral blood T cell populations and found an expanded Treg population in patients with breast cancer compared to healthy individuals. They reported a distinct peripheral Treg population with a particularly strong suppressive phenotype and upregulated CD39, CTLA-4, and TIGIT. Interestingly, this Treg population clonally overlapped with tumor-infiltrating Tregs, suggesting that these peripheral Tregs can infiltrate the tumor microenvironment (TME). They also reported that peripheral T cells secreted less pro-inflammatory cytokines (for example, IL-2, IFNγ, IL-21, TNFα, and IL-4), and expressed more FAS, both of which correlated with poorer overall survival. Additionally, the shift of effector CD8+ T cells toward more differentiated subsets was associated with an unfavorable prognosis. In our study, Tregs similarly expressed higher CD39, whereas TIGIT expression was reduced in all glioblastoma patients but increased in recurrent disease, in line with the upregulation reported by Batalha *et al.* (Figures 18 and 19, Tables 3 and 4). Consistent with their findings,

we observed reduced $CD4^+$ and $CD8^+$ IFNγ and reduced $CD8^+$ TNFα production, but, in contrast, increased $CD4^+$ IL-4 with no changes in IL-2 (Figure 15, Table 3). Furthermore, we also found $CD4^+$ FAS expression to be elevated in recurrent glioblastoma (Figure 21, Table 4). Higher CD8+ TIGIT, as well as increased $CD4^+$ IFNγ and IL-4 expression, were associated with worse survival (Table 7), contrasting the finding of Batalha *et al.*, who linked reduced cytokine production, but not TIGIT expression, to poorer survival.

Malignant melanoma has long been recognized as an immune-controlled tumor; in recent years, it has been shown to be particularly responsive to immune checkpoint inhibitors. In analyzing the peripheral $CD8^+$ T cells of melanoma patients treated with anti-PD-1 therapy, Manabe *et al.* (Manabe et al., 2021) found that patients who responded to treatment showed a significant increase in functional $CD8^+PD\text{-}1^+$ peripheral T cells that were able to produce cytokines such as IL-2, TNFα, and/or IFNγ, pointing towards a less exhausted immune phenotype in patients who responded well to treatment. Among melanoma patients treated with CTLA-4 inhibitors, those with higher levels of Tregs showed increased overall survival (Buder-Bakhaya and Hassel, 2018). Since CTLA-4 is expressed on both regulatory and effector T cells, CTLA-4 blockade primarily enhances T-cell activation and priming, while its effects on Tregs are context-dependent. Increased levels of Tregs in treatment responders may reflect a secondary expansion of Tregs in the context of enhanced overall immune activation, rather than a loss of regulatory function (Buder-Bakhaya and Hassel, 2018). In contrast, high expression of PD-L1 in peripheral T cells in patients treated with CTLA-4 inhibitors was correlated with poorer outcomes (Buder-Bakhaya and Hassel, 2018). In our study, $CD4^+$ PD-1 expression in peripheral T cells was decreased in glioblastoma patients (Figure 18, Table 3), and we observed reduced $CD4^+$ and $CD8^+$ IFNγ and reduced $CD8^+$ TNFα production (Figure 15, Table 3), consistent with a less effective peripheral immune response. This is consistent with the findings of Manabe *et al.*, where clinical responders were characterized by increased $CD8^+PD\text{-}1^+$ expression and increased cytokine production, whereas non-responders lacked this phenotype. The absence of such a PD-1+ peripheral T-cell signature in glioblastoma patients may partly explain the markedly poorer prognosis and limited responsiveness to immune checkpoint blockade compared to melanoma. Furthermore, although higher Treg

levels were linked to improved survival in melanoma under CTLA-4 blockade, we observed reduced checkpoint expression in Tregs (Figure 18, Table 3), while in primary glioblastoma, higher Treg $CD57^+$ expression was associated with worse survival (Table 5).

When comparing the peripheral immune phenotypes reported across the studies discussed above with our data on the peripheral immune status of glioblastoma patients, several similarities are noted. These include a generally reduced production of T cell effector cytokines, particularly IFNγ, as well as the upregulation of inhibitory or regulatory markers in the combined glioblastoma cohort, such as CD39 on Tregs and increased expression of IL-4 in $CD4^+$ T cells, compared to HDs, with increased IL-10 expression observed specifically in recurrent glioblastoma patients. Despite these overlaps, glioblastoma patients lack a clear expansion of regulatory T cells and show reduced expression of immune checkpoint molecules within peripheral T-cell compartments. It remains unclear whether this absence of peripheral regulatory expansion and checkpoint expression contributes to impaired immunosurveillance and the rapid disease progression characteristic of glioblastoma. These differences may be due to the strongly immunosuppressive properties of the CNS tumor environment, reflecting a brain-specific, evolutionary conserved immune regulatory mechanism that limits inflammation and tissue damage, rather than intrinsic tumor immunogenicity, as proposed for intracranial tumors and other CNS pathologies (Lorrey et al., 2023).

5.3. Limitations of the study

First, there were several noteworthy technical limitations in our study. We used frozen PBMCs, and despite various attempts to optimize the thawing process, many cells were lost; thus, not every patient sample could be used for all six panels. Although compensation controls were set before the measurement of the samples, manual compensation was still needed and was thus subject to human error. The same pertains to gating itself, as the global strategy had to be slightly adjusted to fit each sample and its variances. We obtained complete blood cell counts from glioblastoma patients, but not from healthy donors. Therefore, we were unable to

determine if the absolute numbers of the studied cell populations differed between glioblastoma patients and healthy donors and could only compare the ratios.

Second, clinical variables may be confounding factors. We did control for a factor that we deemed very important, namely dexamethasone treatment. Many glioma patients receive corticosteroids to treat tumor-associated brain edema, often before surgery, which we suspected could alter the parameters measured in this study since corticosteroids exert strong systemic immunosuppressive effects and have been shown to substantially alter presurgical peripheral immune cell profiles in glioma patients (Bracci et al., 2022). For example, perioperative steroids have been found to upregulate co-inhibitory receptors (Jansen et al., 2021). However, except for reduced T_{FH} cells in patients who received dexamethasone, we found no significant differences in marker expression between patients with or without dexamethasone medication in our data (Supplemental Figure 6). Other potentially confounding factors, such as age, sex, coagulation status, infections, or performance indices (e.g. Karnofsky Index, ECOG) were not analyzed due to the limited cohort size.

Despite several significant differences between populations, we identified no single marker that would allow an unequivocally clear distinction between glioma patients and healthy donors. As can be seen in the figures, there often was a high marker variability among the individual samples in all four studied populations, and despite the detected significant differences between the four patient groups, no single marker or marker combination could clearly define any of the groups.

5.4. Local immune response

The goal of our study was to characterize the peripheral immune status of glioma patients. The systemic response is only a small part of the overall antitumor response, as it ultimately takes place at the tumor site itself. While studying the local anti-glioblastoma immune response was not part of this study, putting our data on the systemic immune status in glioblastoma patients in context with previously obtained information on local antitumor responses is very important. In the following section, I review published data on local immune responses and how this may relate

to our findings. In tumors of the central nervous system, many types of immune cells, including macrophages, natural killer cells, dendritic cells, and T- and B-cells, have been found to infiltrate tumors with varying abundances and functions (Domingues et al., 2016, Ghouzlani et al., 2021, Bloch et al., 2013b, Jiang et al., 2015).

5.4.1. Tumor-infiltrating lymphocytes: T-helper cells ($CD4^+$) and cytotoxic T cells ($CD8^+$)

Tumor-infiltrating lymphocytes (TILs) in glioblastomas consist predominantly of $CD3^+$ T cells, including cytotoxic $CD8^+$ T cells, $CD4^+$ helper T-cell subsets, and regulatory T cells. Multiple studies have demonstrated that higher levels of T-cell infiltration, particularly $CD8^+$ TILs, are associated with prolonged survival in glioblastoma patients (Mohme and Neidert, 2020, Kim et al., 2012, Lohr et al., 2011b, Kmiecik et al., 2013). Increased densities of $CD3^+$ and $CD8^+$ TILs have been linked to improved clinical outcome, whereas immunosuppressive T-cell subsets are more frequently observed in aggressive disease (Kim et al., 2012, Lohr et al., 2011b, Kmiecik et al., 2013, Sayour et al., 2015).

$CD4^+$ T cells within the TME comprise functionally diverse subsets that can either support anti-tumor immunity or promote immunosuppression. T_H1-polarized $CD4^+$ cells produce IFNγ and shift the local immune response towards pro-inflammatory, anti-tumoral immune responses, including macrophage polarization towards an M1 phenotype, while T_H2-polarized $CD4^+$ cells secrete cytokines such as IL-4, IL-5 and IL-13, which favor anti-inflammatory, pro-tumorigenic M2 response. Consistent with this, glioblastoma tumors are characterized by a predominance of type 2–associated immune responses within the TME, including increased expression of T_H2-associated cytokines and reduced d T_H1-associated activity (Domingues et al., 2016).

Directly correlating peripheral immune phenotypes with TIL composition remains challenging, as the relationship between circulating immune cells and their counterparts within the TME is complex and not necessarily linear. Rather than focusing on absolute cell numbers, functional characteristics of immune cells may provide more informative insights into glioblastoma-associated immune

dysregulation. These considerations are addressed in the context of specific findings in the sections that follow.

While we did not observe differences in the relative frequencies of total peripheral T cells in the combined glioblastoma cohort, we did find that an increase in conCD4$^+$ T cells in recurrent glioblastoma was associated with improved survival (Figure 27), similar to the above-mentioned survival benefit of increased TILs in glioblastomas. Our data showed a decrease in CD4$^+$ and an increase in CD8$^+$ T cells in recurrent glioblastoma when compared to primary glioblastoma (Figure 5, Table 4), although it remains unclear how these changes in the peripheral blood relate to TILs and functional states within the TME. Importantly, our observation of reduced IFNγ in both CD4$^+$ and CD8$^+$ T cells, together with the increase of IL-4 production in CD4$^+$ T cells in the periphery (Figures 15 and 16, Table 3), mirrors the T_H1-to-T_H2–skewed immune signatures reported for TILs in glioblastoma, rather than reflecting differences in absolute T-cell infiltration (Domingues et al., 2016).

5.4.2. Exhaustion

One of our main findings was reduced peripheral T-cell exhaustion in both CD4$^+$ and CD8$^+$ T cells, in particular less PD-1 and BTLA expression on CD4$^+$ T cells and less TIGIT, and 2B4 expression on CD8$^+$ T cells in glioblastoma patients compared to HDs (Figures 18 and 19, Table 3). This finding is notable because it contrasts with the well-established phenotype of pronounced T-cell exhaustion within the glioblastoma TME.

TILs in glioblastoma and in most other solid cancers have been shown to exhibit an upregulation of co-inhibitory receptors (immune checkpoints), such as PD-1, CTLA-4, TIGIT, CD39, LAG-3, 2B4, and TIM-3, indicating a state of T-cell exhaustion (Jansen et al., 2021, Ghouzlani et al., 2021). Importantly, this exhausted phenotype has been described specifically within the TME and therefore differs fundamentally from the peripheral T-cell compartment analyzed in the present study. The TME of glioblastoma is characterized by pronounced T-cell exhaustion, with particularly prominent expression of inhibitory receptors such as BTLA, CD39, and TIM-3 on TILs (Mohme and Neidert, 2020, Ghouzlani et al., 2021, Davidson et al., 2019). Despite evidence of activation-associated features, these T cells remain functionally

impaired, suggesting a dysfunctional or incomplete activation state rather than effective antitumor immunity (Davidson et al., 2019).

Consistent with this exhausted TME, increased activity of inhibitory checkpoint pathways, such as the PD-1/PD-L1 axis, has been associated with poorer overall survival in glioblastoma patients (Mohme and Neidert, 2020). Another exhaustion marker, TIM-3 and its ligand galectin-9 (GAL-9), is upregulated in TILs, and high levels of GAL-9 are associated with shorter survival (Ghouzlani et al., 2021). Higher levels of CTLA-4, a checkpoint molecule expressed on Tregs that suppresses T-cell activation, have also been linked to poorer prognosis in glioblastoma patients (Ghouzlani et al., 2021).

Importantly, PD-1 expression was significantly higher in both $CD4^+$ and $CD8^+$ TILs in glioblastoma patients than in PBMCs of both glioblastoma patients and HDs (Davidson et al., 2019), indicating that T-cell exhaustion within the TME does not necessarily mirror the exhaustion phenotype observed in the peripheral blood. Similarly, Woroniecka *et al*. (Woroniecka et al., 2018a) demonstrated that TILs in glioblastoma patients had less IFNγ, IL-2, and TNFα after stimulation than peripheral blood lymphocytes (PBLs) of both glioblastoma patients and HDs. T cells expressing multiple checkpoint markers (for example, PD-1^+, TIM-3^+, and LAG-3^+) do not produce the above-mentioned cytokines (Woroniecka et al., 2018a), indicating a spectrum of T-cell exhaustion.

For further in-depth analysis, Woroniecka *et al*. (Woroniecka et al., 2018a) examined PD-1 expression in combination with other exhaustion markers and found that the expression of multiple markers indicated a more dysfunctional T-cell status. In contrast, when PD-1 was expressed alone, T cells remained functional. This dual role suggests that PD-1 may reflect recent activation when expressed alone, but contributes to exhaustion when co-expressed with additional inhibitory receptors (Woroniecka et al., 2018a). Therefore, functionality must be combined with phenotypic expression to determine the state of exhaustion correctly (Woroniecka et al., 2018a).

In summary, our data reveal an apparent disconnect between the strongly exhausted T-cell phenotype observed within the glioblastoma TME, as reported by others, and the reduced expression of exhaustion markers detected in peripheral T cells, as described here. One possible explanation is the preferential recruitment or retention of highly exhausted, checkpoint-expressing T cells within the tumor, resulting in a relative depletion of such cells from the circulation. Alternatively, the reduced expression of exhaustion markers in peripheral T cells may reflect a state of incomplete or dysfunctional activation rather than a preserved effector function. In either case, our findings suggest that reduced peripheral T-cell exhaustion does not translate into effective antitumor immunity, as evidenced by the persistent immune dysfunction within the TME and the poor clinical outcome of glioblastoma patients.

5.4.3. Regulatory T cells

We observed a decrease in Treg exhaustion (less PD-1, TIGIT, CD57, 2B4, BTLA, and KLRG1) accompanied by enhanced Treg activation (e.g. more CD39 and HLD-DR) in the peripheral blood of glioblastoma patients (Figure 18 and 20, Table 3). Tregs are abundant among glioblastoma TILs (Domingues et al., 2016). These tumor-infiltrating Tregs are active, shifting the local immune response towards immunosuppression by inducing M2 phenotypic TAMs (Domingues et al., 2016) and expressing immunosuppressive cytokines such as IL-10 and TGFβ (Mohme and Neidert, 2020). Mohme *et al*. (Mohme and Neidert, 2020) described a correlation between tumor grade and Treg infiltration, with lower numbers of Tregs corresponding to less aggressive gliomas. This has also been validated in mouse models (Domingues et al., 2016). Although originally thought to be predictive of overall survival, studies have shown that the number of Tregs does not accurately predict the survival of glioblastoma patients (Thomas et al., 2015). The Treg/T-cell ratio has also been shown to be increased, especially in high-grade glioblastoma (Fecci et al., 2006) and to be a prognostic factor in glioblastoma, in contrast to the mere number of Tregs (Sayour et al., 2015). For example, recurrent glioblastoma has a higher Treg/T-cell ratio than primary glioblastoma (Sayour et al., 2015) and a high number of Tregs has been correlated with earlier recurrence of glioblastoma (Mohme and Neidert, 2020). The apparent critical role of glioblastoma-infiltrating Tregs is mirrored by our findings of decreased peripheral Treg exhaustion and

increased Treg activation, and it is tempting to speculate that an alteration of the peripheral Treg status directly affects local immune responses.

5.4.4. Natural killer cells

In addition to the T-cell changes, we also observed many differences in NK cells. In the peripheral blood of glioblastoma patients, NK cells were less abundant but showed reduced expression of exhaustion markers such as TIM-3, TIGIT, and KLGR1. Additionally, we found an increase in IFNγ production in recurrent disease, pointing towards a more activated phenotype. In the TME, NK cells use perforins and granzymes to kill tumor cells (Ghouzlani et al., 2021, Domingues et al., 2016). In addition, NK cells use expression of CD95 (FAS) and TNFα on its surface to induce tumor cell apoptosis, magnifying their anti-tumor capacities (Domingues et al., 2016). Thus, greater infiltration of NK cells into the tumor is correlated with a more effective immune response (Ghouzlani et al., 2021). However, few and often less functional NK cells tend to infiltrate gliomas, as other immunosuppressive mechanisms aim to hinder their immune response (Ghouzlani et al., 2021, Domingues et al., 2016). The reduced numbers of circulating NK cells we found in glioblastoma patients may reduce the pool of available NK cells and result in reduced NK-cell infiltration into the TME. Alternatively, peripheral reduction may reflect increased NK-cell recruitment into tumors.

5.4.5. Tumor-associated macrophages

Tumor-associated macrophages are a heterogeneous population comprising resident brain microglia and infiltrating monocyte-derived macrophages that accumulate within the tumor microenvironment of gliomas, and inhibit immune reactions by releasing various growth factors and cytokines (Ghouzlani et al., 2021, Domingues et al., 2016). Recruitment of TAMs is mediated by the CC-chemokine ligands 2 (CCL2), 7 (CCL7), and 22 (CCL22) that are secreted by glioblastoma tumor cells. These chemokines also mediate recruitment of Tregs and MDSCs (Jacobs et al., 2009, Crane et al., 2012, Chang et al., 2016, Jiang et al., 2015, Raychaudhuri et al., 2011, Speiser et al., 2016). TAMs use several mechanisms to induce immunosuppression including secretion of immune-inhibitory cytokines such as TGFβ, downregulation of proinflammatory cytokines such as TNFα, IL-1β, and

IL-6, as well as induction of suppressive surface molecules such as FAS (See et al., 2015, Domingues et al., 2016). They also lack the co-stimulatory molecules required for T-cell activation (Domingues et al., 2016).

Although this concept has recently been challenged, previous studies have grouped macrophages into pro-inflammatory, anti-tumor M1 or anti-inflammatory, pro-tumor M2 phenotypes (Domingues et al., 2016). Pro-inflammatory M1 macrophages are induced by IFNγ, TNFα, IL-12, IL-23, and low levels of IL-10, and in turn produce IL-1, TNFα, and IL-6 (Domingues et al., 2016). On the other hand, M2 macrophages are induced by the immunosuppressive cytokines IL-4 and IL-13 and secrete anti-inflammatory cytokines, shifting the local immune response towards a T_H2 response (Domingues et al., 2016). A shift towards either the M1 or M2 phenotype depends on the local microenvironment. TAMs are usually of the M2 phenotype, supporting tumor growth within the TME through T-cell suppression via IL-10 and TGFβ secretion (Jiang et al., 2015). In addition to these cytokines, M2-type TAMs express inhibitory ligands such as PD-L1 and FAS-L that induce T-cell exhaustion and apoptosis, while promoting the expansion of regulatory T cells (See et al., 2015, Domingues et al., 2016, Ghouzlani et al., 2021). Together, these interactions within the TME reinforce local immunosuppression and facilitate tumor immune evasion.

It is unclear how TAMs are affected by changes in systemic immune responses. It is possible that the reduced production of INFγ and TNFα and increased production of IL-4 by peripheral immune cells, which we describe in the study presented here, may contribute to a shift towards M2-type macrophages in the TME. However, it may be even more plausible that M2-polarized macrophages themselves contribute to peripheral immunosuppression rather than merely reflecting it. Soluble factors released by M2-type macrophages can enter the circulation and are thought to dampen systemic T-cell activation and effector function, further contributing to peripheral immune dysfunction (Ghouzlani et al., 2021, Domingues et al., 2016, See et al., 2015). Although speculative, it is indeed possible that TAMs contribute to the peripheral immune changes observed in this study. For example, the lower IFNγ and TNFα production in $CD4^+$ T cells we observed (Figures 15 and 16, Table 3) could be the result of TAM-released IL-10 and TGFβ. These cytokines may have also

contributed to the shift towards Th2, as shown by our increase in IL-4 production in circulating $CD4^+$ T cells (Figures 15 and 16, Table 3).

5.4.6. Dendritic cells

We did not evaluate dendritic cells (DCs) in the peripheral blood, where they are not typically found except as rare circulating precursors (Mellman and Steinman, 2001). DCs are antigen-presenting cells that, when functioning normally, can induce tumor-specific T-cell activation by presenting tumor cell antigens to T cells and releasing cytokines and chemokines after binding of presented tumor antigens to the TCR. In mouse models, DCs from gliomas lack costimulatory signals and are thus unable to stimulate T cells and promote the generation of Tregs, creating an immunosuppressive environment (Ghouzlani et al., 2021, Domingues et al., 2016). It is unclear whether any of the altered peripheral immune features described here have an impact on creating such a local anergy.

In summary, all these published findings about TILs and TAMs in the TME and thoughts about how those may relate to our data of the peripheral immune status, demonstrate a profound difference between the local and peripheral immune status in glioblastoma patients. Nevertheless, it is very unlikely that they are completely independent but rather interact with influences in either direction. Indeed, emerging evidence suggests that glioblastoma itself drives profound systemic immune derangements, including T-cell lymphopenia, lymphoid organ atrophy, and bone-marrow sequestration, through tumor-derived soluble factors and CNS-mediated neuroimmune signaling (Lorrey et al., 2023, Puviindran et al., 2025). These mechanisms likely connect the TME with the peripheral immune system in a bidirectional manner: cytokines and metabolites produced by TILs and TAMs can enter the circulation to dampen systemic immune activation, while peripheral immune dysfunction limits the recruitment and effector function of T cells that could otherwise infiltrate the TME and control the tumor (Puviindran et al., 2025). To further address questions regarding the interactions between local and systemic immune responses, future studies should ideally examine matched peripheral blood and tumor samples from the same patient.

5.5. Immunotherapy in glioma

The ultimate goal of studying immune responses in glioblastoma is to develop more effective immunotherapy.

5.5.1. Limited effects of immune checkpoint inhibitors in glioma patients

Despite breakthroughs in cancer treatment with immune checkpoint inhibitors, especially in melanoma patients (Tawbi et al., 2022), immune checkpoint inhibitors have only shown limited efficacy in patients with glioblastoma (Brahm et al., 2020, Jansen et al., 2021). No improvement of overall survival has been observed in glioma patients, even when combining anti-PD-1 with anti-CTLA-4 approaches or in adjuvant therapeutic settings (Ghouzlani et al., 2021). Here, we describe PD-1 to be less expressed by peripheral T cells of glioblastoma patients and therefore hypothesize that the decreased expression of this important immune checkpoint receptor may explain the lack of efficacy of immunotherapy with PD-1 inhibition. Similarly, Shen *et al*. argued that immune checkpoint inhibitors may not elicit a sufficient immune response in glioblastoma patients due to low levels of PD-1/PD-L1 expression in the glioma microenvironment (Shen et al., 2021). The poor efficacy of immune checkpoint inhibitors in glioblastoma patients may also be due to the expression of multiple other checkpoint receptors such as TIM-3, LAG-3, BLTA, 2B4, CD160, CD39, and TIGIT, which are present in the extensively exhausted TILs of glioblastoma patients (Woroniecka et al., 2018a). In contrast, we found decreased expression of exhaustion markers such as BTLA, 2B4, and TIGIT in peripheral $CD4^+$ and $CD8^+$ T cells in the peripheral blood (Figures 18 and 19, Table 3), again indicating the previously mentioned discrepancy between local and systemic immune responses.

5.5.2. Immune distinction of brain malignancies

The discrepancy between an ineffective local tumor response and our findings of peripheral immune activation, albeit dysfunctional, in glioblastoma may be due to the unique anatomic and immunological aspects of the CNS. I therefore would like to discuss the potential of the impact of the organ specificity in gliomas with potential avenues to improve therapeutic outcomes.

Interestingly, patients with brain metastases such as melanoma have shown promising responses to immune checkpoint inhibitors (Kluger et al., 2019, Jansen et al., 2021), suggesting that indeed the tumor immunology is different in brain metastases as opposed to primary brain tumors. While the long-held belief that the brain is an immune sanctuary or an immune-privileged organ has now been disproven, the above-mentioned (partial) efficacy of immunotherapy of brain metastases is just one case in point; it is still considered immune-distinct due to the tight blood-brain barrier and a unique microenvironment (Phadke et al., 2022, Fares et al., 2021, Westphal et al., 2017, Sampson et al., 2020, Ghouzlani et al., 2021, Jansen et al., 2021). Both the blood-brain barrier and blood-cerebrospinal fluid barrier must be overcome to gain access to the brain (Jansen et al., 2021). Immune surveillance in the brain occurs through the most abundant cell populations of microglia, tissue-resident macrophages, dendritic cells, and B- and T- cells (Ghouzlani et al., 2021). Cytokines, either pro- or anti-inflammatory, were initially believed to lack the ability to surpass the blood brain barrier. However, it has now been shown that cytokines can penetrate either through compromised sections or through transport systems (Ghouzlani et al., 2021). Despite evidence of immune responses in the brain, these processes have been shown to occur at a much slower rate than those in other organs (Ghouzlani et al., 2021).

In the search for treatment options for gliomas, many drugs have failed because of the inability to cross the blood-brain barrier (Ghouzlani et al., 2021). Though anti PD-1 antibodies were initially believed to fail because of this phenomenon, passage into the brain has now been shown to be possible by backpacking onto PD-1 or CTLA-4 on peripheral lymphocytes. After passing through the blood-brain barrier, the antibodies can bind to TILs to perform their intended functions (Ghouzlani et al., 2021). CTLA-4 antibodies, however, have not been shown to cross the blood–brain barrier. To circumnavigate this problem, attempts to combine antibodies with nanotechnology to penetrate the blood-brain barrier in mice have been successful and have been shown to correlate with increased survival and activation of the local immune system (Ghouzlani et al., 2021). Antigen presentation in the CNS occurs in a different manner than in the periphery, and because of limited space, immune cells have limited access to the brain parenchyma; thus, antigens drain passively through the glymphatic system where mixing with cerebral spinal fluid (CSF) occurs.

While CSF drains back through arachnoid granulations, immune cells are further drained into the deep cervical lymph nodes, where T-cell activation through antigen-presenting cells can then occur (Jansen et al., 2021). Activation of the immune system through antigen presentation can occur through (g)lymphatic drainage or interaction with glioblastoma cells outside of the brain parenchyma. The latter, however, is much less likely, as only anecdotal evidence of extracranial metastases of glioblastoma exist (Jansen et al., 2021). Thus, the idea of antigenic ignorance has arisen, referring to the low chance of tumor antigens being presented to T cells. This occurs because of the low mutational burden and limited activity of APCs in the deep brain parenchyma (Jansen et al., 2021).

5.5.3. Improving efficacy of immune checkpoint inhibitors in glioma patients

Effective cancer immunotherapy consists of both normalization, the notion of overcoming malignancy-induced immune escape, commonly through immune checkpoint inhibitors, and enhancement of the immune response, such as priming effector responses or enabling antigen presentation (Jansen et al., 2021). As mentioned above, combining immune checkpoint inhibitor antibodies with nanotechnology to penetrate the blood-brain barrier has shown promising results in mouse models, pointing to the positive effects of normalization of immune response in glioblastoma once drugs successfully reach the CNS. CD96 is often expressed alongside immune checkpoints, such as PD-1, CLTA-4, TIGIT, and TIM-3, and may potentially act synergistically (Ghouzlani et al., 2021). High CD96 expression has been shown to correlate with worse outcomes and may therefore be another possible blockade target (Ghouzlani et al., 2021). A synergy has also been suggested between PD-1, TIM-3, and LAG-3 by Woroniecka *et al*. (Woroniecka et al., 2018a), who found that the expression of TIM-3 and LAG-3 only occurs alongside PD-1 expression. In murine models, it has been shown that by increasing lymphatic drainage to the cervical lymph nodes, a more effective T-cell anti-glioblastoma response can be mounted (Jansen et al., 2021), thus overcoming antigenic ignorance. Additionally, identifying mechanisms to lower the threshold for T-cell activation both in the periphery and CNS may lead to further pathways of immune enhancement (Jansen et al., 2021). Blocking TIGIT is another promising strategy for enhancing immunity. TIGIT is upregulated in Treg TILs, and through its blockage, Tregs have been observed to modify pro-inflammatory T cells (Jansen et

al., 2021). However, other studies have shown blockage of TIGIT without changes in overall survival in a glioblastoma mouse model (Ghouzlani et al., 2021). In mouse models, the combination of anti-TIGIT and anti-PD-1 resulted in greater overall survival (Ghouzlani et al., 2021). Thus, future trials should aim not only to normalize anti-tumor immune responses but also to incorporate immune-enhancing aspects to optimize efficacy.

Developing successful immunotherapies for glioblastoma will depend on a complete understanding of the systemic and local immune responses that are likely, as mentioned above, interdependent. We hope that this careful characterization of the systemic immune status of glioblastoma patients presented here will ultimately be helpful towards this goal.

6. Conclusion

Peripheral immune profiling in glioblastoma patients revealed profound systemic alterations with features of both immune activation and suppression. Increased T-cell metabolic activity and reduced T-cell exhaustion point to immune activation, while diminished pro-inflammatory cytokines, activated and less exhausted Tregs, and reduced NK cells reflect immune suppression. Recurrent glioblastomas showed even more pronounced abnormalities, including a shift toward T_H2 responses, pointing to increasing immune dysfunction with disease progression. Several parameters correlated with patient survival. Higher $CD8^+CD73^+$ expression and increased conventional $CD4^+$ T cells were associated with better outcomes, whereas elevated $CD8^+TIGIT^+$ and Treg HLA-DR correlated with poorer prognosis. These findings suggest that the dismal prognosis of glioblastoma patients, with a failure of anti-tumor immunity, may result from an imbalance of systemic immune activation and suppression. Targeting this systemic immune dysfunction may provide novel therapeutic opportunities to restore effective anti-tumor immunity in glioblastoma patients. Markers correlating with survival, such as TIGIT, may be of special interest.

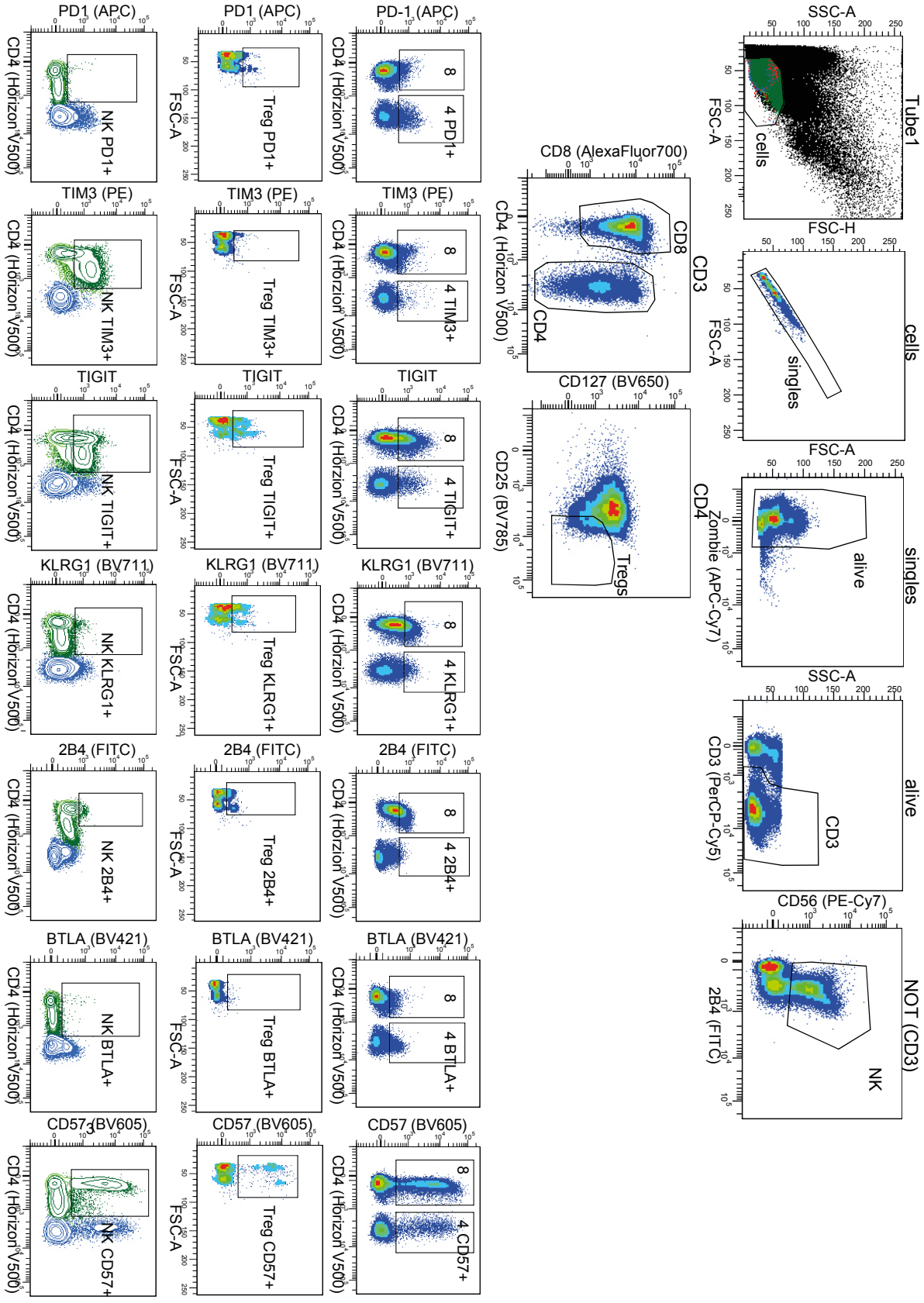
Periphere Immunprofile bei Glioblastom Patient:innen zeigten tiefgreifende Veränderungen mit Merkmalen von sowohl Immunaktivierung als auch Immunsuppression. Eine erhöhte Stoffwechselaktivität der T-Zellen und eine verringerte T-Zell-Erschöpfung deuten auf eine Immunaktivierung hin, während verminderte proinflammatorische Zytokine, aktivierte und weniger erschöpfte Tregs und weniger NK-Zellen eine Immunsuppression widerspiegeln. Glioblastomrezidive zeigten noch ausgeprägtere Anomalien, darunter eine Verschiebung hin zu T_H2-Reaktionen, was auf eine zunehmende Immunschwäche bei Tumorprogression hindeutet. Mehrere Parameter korrelierten mit Patient:innen Überleben. Eine höhere $CD8^+CD73^+$ Expression und mehr konventionelle $CD4^+$ T-Zellen waren mit längerem Überleben assoziiert, während erhöhte $CD8^+TIGIT^+$ und Treg HLA-DR Expression mit einer schlechteren Prognose korrelierten. Diese Ergebnisse deuten darauf hin, dass die unzureichenden Antitumorimmunität und damit schlechte Prognose von Glioblastomen möglicherweise auf ein Ungleichgewicht zwischen systemischer Immunaktivierung und -suppression zurückzuführen sind. Die gezielte Modulation dieses Ungleichgewichts könnte neue Therapieansätze eröffnen.

7. Supplements

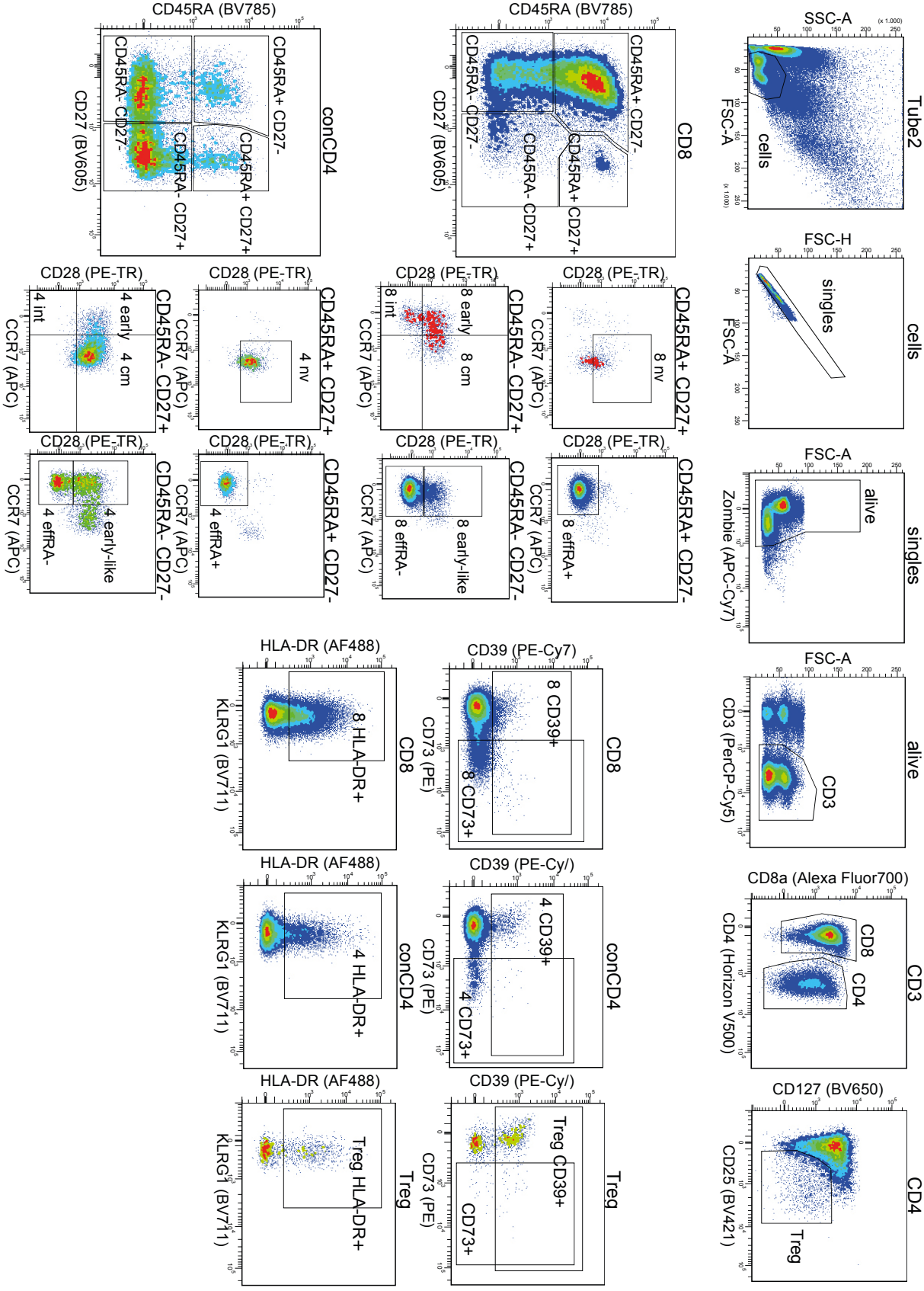
Supplemental Table 1. Epitope, clone, lot number, company, and dilution of fluorochromes.

Fluorochrome	Epitope	Clone	Lot Number	Company	Dilution
BV 785	CD25	BC96	B232830	Biolegend	01:10
BV 785	CD45RA	HI100	B246560	Biolegend	01:30
BV 785	CD45RO	UCHL1	B247718	Biolegend	01:50
BV 711	KLRG1	2F1/KLRG1	B239161	Biolegend	01:10
BV 650	CD127	A019D5	B238890	Biolegend	01:30
BV 650	CXCR3	G025H7	B225762	Biolegend	01:10
BV 650	TNFα	MAb11	B241926	Biolegend	01:10
BV 605	CD57	QA17A04	B259702	Biolegend	01:20
BV 605	CD27	O323	B241914	Biolegend	01:30
BV 605	CD161	HP-3G10	B245750	Biolegend	01:20
BV 510	CD4	RPA-T4	B251573	Biolegend	01:30
BV 510	CD3	UCTH1	B243078	Biolegend	01:20
BV 421	BTLA	MIH26	B238296	Biolegend	01:20
BV 421	CD25	BC96	B249792	Biolegend	01:30
BV 421	CRTH2	BM16	B245754	Biolegend	01:10
BV 421	CD95 (Fas)	DX2	B224424	Biolegend	01:30
BV 421	IL-2	MQ1-17H12	B254111	Biolegend	01:10
PerCP-Cy5.5	CD3	UCHT1	B249759	Biolegend	01:50
PerCP-Cy5.5	CD4	RPA-T4	B257495	Biolegend	01:10
FITC	2B4	C1.7	B177799	Biolegend	01:20
FITC	HLA-DR	L243	B246766	Biolegend	1:30
FITC	Tgd	11F2	8005745	BD	01:20
FITC	CD98	MEM-108	B236685	Biolegend	01:20
AlexaFluor488	IFNγ	4S.B3	B251617	Biolegend	01:30
PE-Cy7	CD56	5.1H11	B256222	Biolegend	01:50
PE-Cy7	CD39	A1	B251864	Biolegend	01:30
PE-Cy7	CCR4	L291H4	B236410	Biolegend	01:30
PE-Cy7	CD71	CY1G4	B231313	Biolegend	01:20
PE-Dazzle	TIGIT	A15153G	B254055	Biolegend	01:30
PE-Dazzle	CD28	CD28.2	B242368	Biolegend	1:100
PE-Dazzle	CXCR5	J252D4	B226045	Biolegend	01:30
PE-Dazzle	IL-4	MP4-25D2	B202705	Biolegend	01:30
PE	Tim-3	F38-2E2	B204713	Biolegend	01:10
PE	CD73	AD2	B248074	Biolegend	01:50
PE	CCR6	G034E3	B240450	Biolegend	01:30
PE	GLUT1	202915	AAQT0417111	R&D	01:10
PE	IL-10	JES3-9D7	B244023	Biolegend	01:10

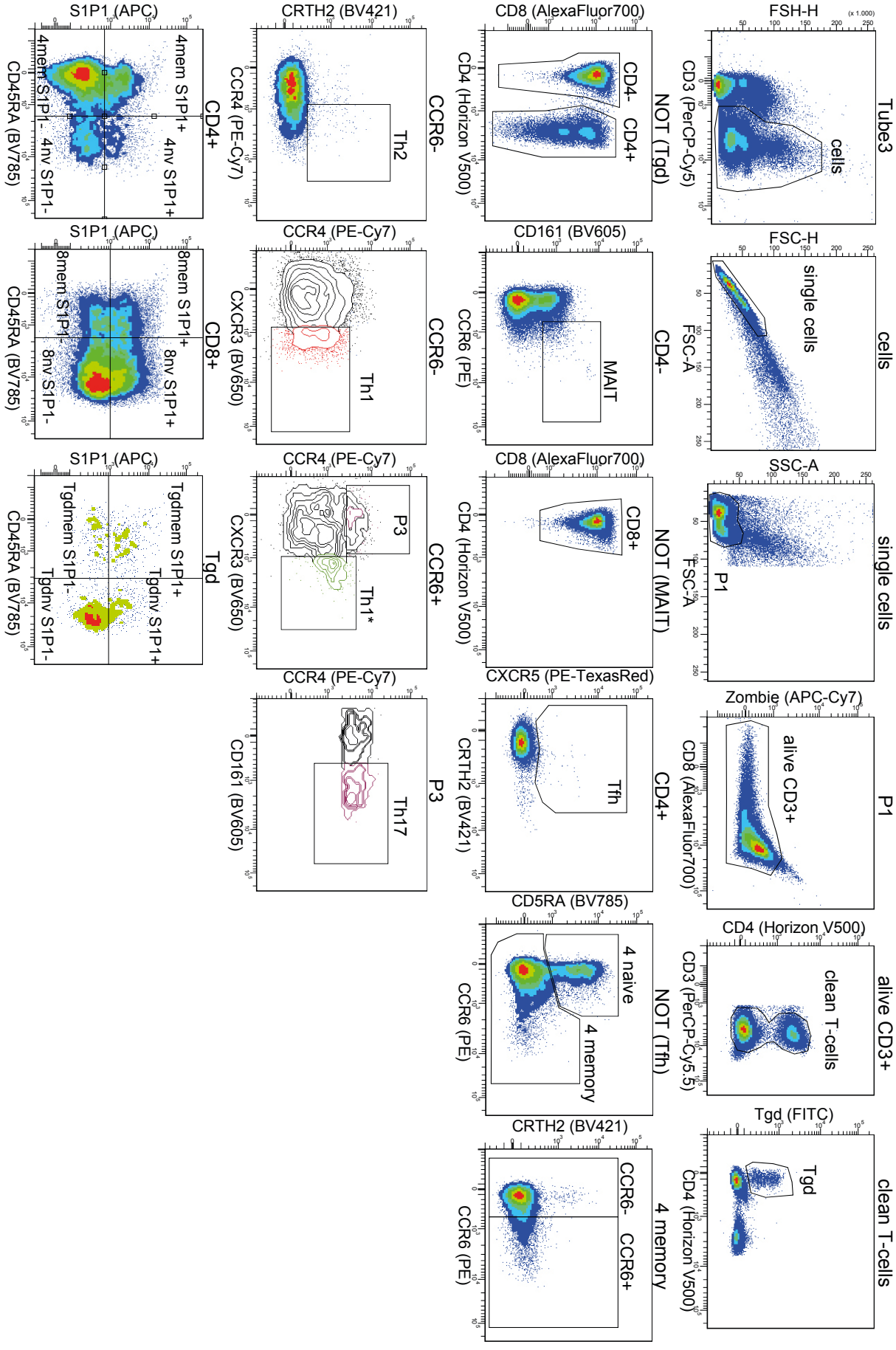
APC-Cy7	Zombie NIR	-	B258274	Biolegend	01:10
AlexaFluor700	CD8a	RPA-T8	B210113	Biolegend	1:100
APC	PD-1	EH12.2H7	B240811	Biolegend	01:10
APC	CCR7	G043H7	B249565	Biolegend	01:20
APC	IL-17A	BL168	B234864	Biolegend	01:10
APC	S1PR1	SW4GYPP	-	eBio	01:20



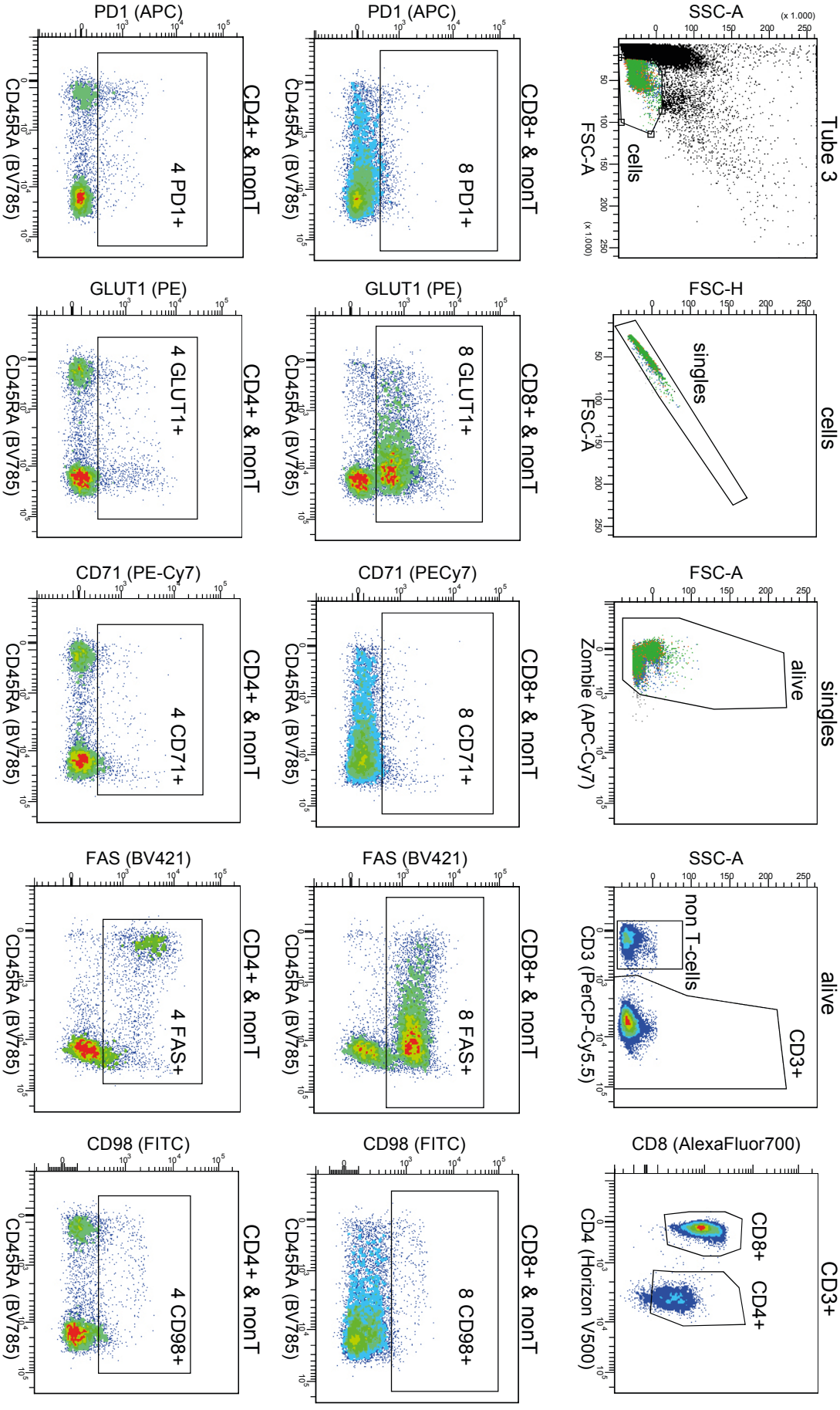
Supplemental Figure 1. Gating strategy for Panel 1.



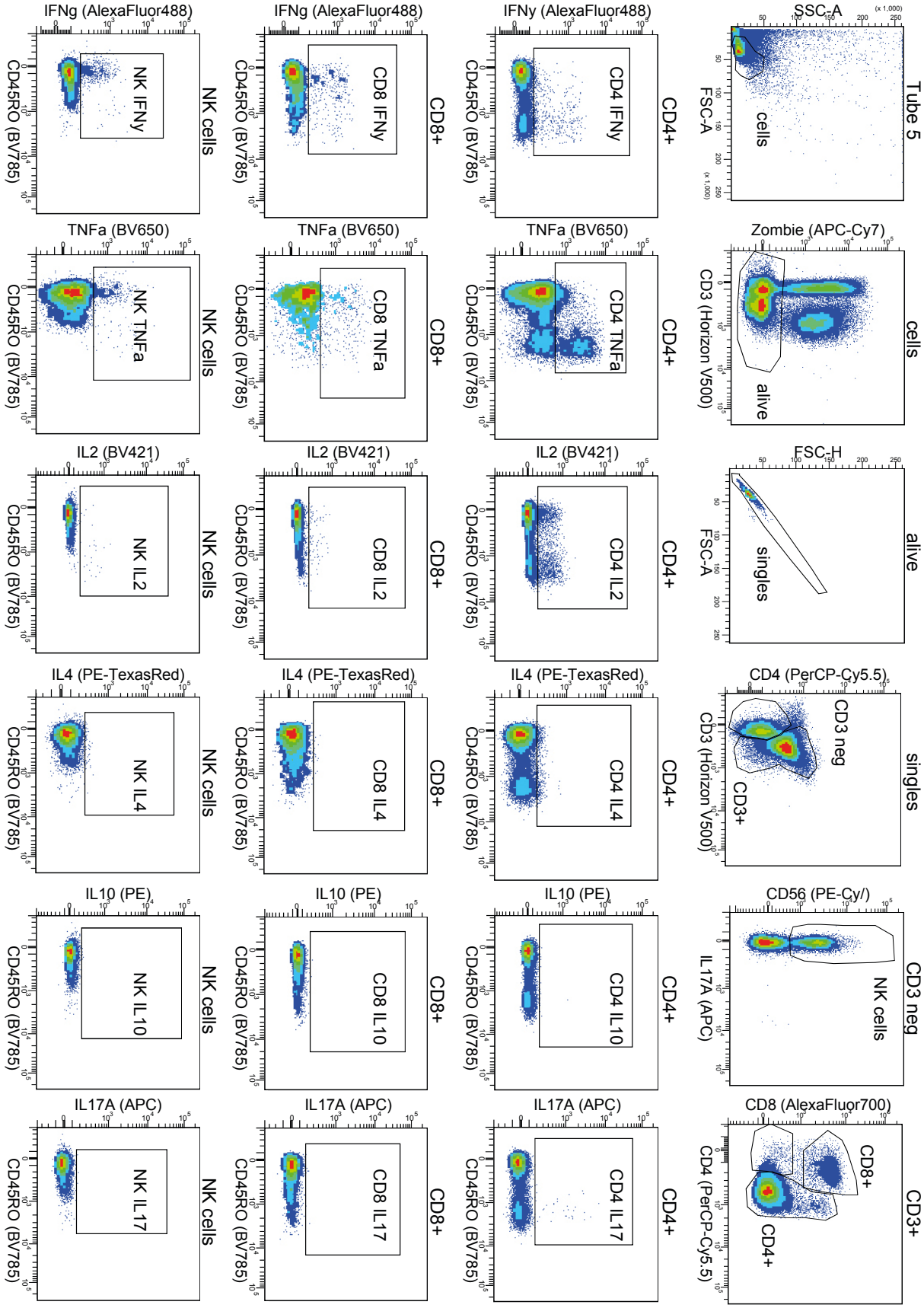
Supplemental Figure 2. Gating strategy for Panel 2.



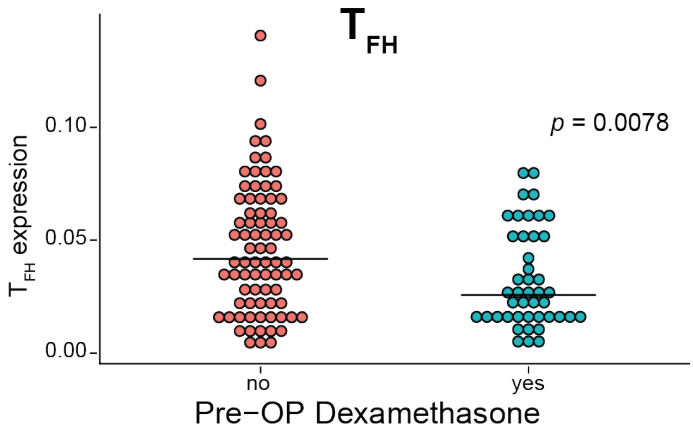
Supplemental Figure 3. Gating strategy for Panel 3.



Supplemental Figure 4. Gating strategy for Panel 4.



Supplemental Figure 5. Gating strategy for Panel 5.



Supplemental Figure 6. Reduced T_{FH} expression in patients receiving pre-operative dexamethasone. Comparison of circulating T_{FH} cell expression in patients who did (yes) and did not (no) receive pre-operative dexamethasone. Among all immune markers analyzed, only T_{FH} cells showed a significant difference, with lower expression observed in the dexamethasone group ($p = 0.0078$).

8. References

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9. Abbreviations

Abbreviation	Definition
APCs	antigen presenting cells
B2M	β2-microglobulin
BBB	blood brain barrier
BTLA	B- and T-lymphocyte attenuator
CCR6	C-C chemokine receptor type 6
CNS	central nervous system
CSF	cerebral spinal fluid
CTLA-4	cytotoxic T-lymphocyte-associated protein
DCs	dendritic cells
DMSO	dimethyl sulfoxide
eMDSCs	early myeloid-derived suppressor cells
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
GAL-9	galectin-9
GM-CSF	granulocyte-macrophage colony-stimulating factor
HD	healthy donors
HLA-DR	human leukocyte antigen – DR isotype
ICI	immune checkpoint inhibitor
IDH	isocitrate dehydrogenase
IDHmut	isocitrate dehydrogenase mutant
IDO	Indoleamine 2,3-dioxygenase
IDHwt	isocitrate dehydrogenase wildtype
IFNγ	interferon gamma
IL-10	interleukin 10
IL-13	interleukin 13
IL-15	interleukin 15
IL-17	interleukin 17
IL-2	interleukin 2
IL-4	interleukin 4
IL-6	interleukin 6
KLRG1	killer cell lectin-like receptor subfamily G member 1
LAG-3	lymphocyte-activation gene 3
LSM	Lymphocyte Separation Medium
M-MDSCs	monocytic myeloid-derived suppressor cells
MAIT	mucosa-associated invariant T cells
MDSCs	myeloid-derived suppressor cells
MGMT	O(6)-methylguanine-DNA methyltransferase
MHC	major histocompatibility complex
MMPs	metalloproteinases
MRI	magnetic resonance imagaing
ncT_H1	non-classical T_H1 cells

NK	natural killer
PBL	peripheral blood lymphocytes
PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PD-1	programmed cell death protein 1
PD-L1	programmed cell death ligand 1
PGE_2	prostaglandin E_2
PMN-MDSCs	polymorpohnuclear myeloid-derived suppressor cells
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
S1PR1	sphingosine-1-phosphate receptor 1
TAMs	tumor-associated macrophages
TAP	transporter associated with antigen processing
TAP1	transporter associated with antigen processing subunit 1
TAP2	transporter associated with antigen processing subunit 1
T_{CM}	central memory T cells
TCR	T-cell receptor
T_{EM}	effector memory T cells
T_{FH}	follicular T-helper cells
$T_{\gamma\delta}$	gamma delta T cells
TGFβ	transforming growth factor beta
TIGIT	T-cell immunoreceptor with Ig and ITIM domains
TILs	tumor infiltrating lymphocytes
TIM-3	T-cell immunoglobin and mucin-domain containing 3
TME	tumor microenvironment
TNFα	transforming growth factor alpha
T_{NV}	naïve T cells
Tregs	regulatory T cells
T_{TE}	terminal effector T cells
T_{TM}	transitional memory T cells
UKE	Universitätsklinikum Hamburg-Eppendorf
VEGF	vascular endothelial growth factor

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Figure 17. Heat map of intracellular cytokine production in $CD4^+$ T cells (A), $CD8^+$ T cells (B) and NK^+ cells (C) in HD, primary and recurrent IDHwt glioblastoma, and IDHmut astrocytoma.

Figure 18. Distribution of T-cell exhaustion markers, as measured by flow cytometry, in $CD4^+$ T cells, in $CD8^+$ T cells, in Treg cells, and in NK cells.

Figure 19. Distribution of T-cell exhaustion markers with significant differences in primary and recurrent IDHwt glioblastoma, as measured by flow cytometry, in $CD4^+$ T cells, in $CD8^+$ T cells, in Treg cells, and in NK cells.

Figure 20. Distribution of metabolic immunomodulation and activation markers in T cells in HD and all glioma cohorts, as measured by flow cytometry.

Figure 21. Distribution of metabolic immunomodulation and activation markers in T cells in HD, primary and recurrent IDHwt glioblastoma compared to HDs, as measured by flow cytometry.

Figure 22. Main peripheral immune abnormalities between all IDHwt, primary IDHwt, and recurrent IDHwt glioblastoma, each compared to HDs. Shown are the alterations in T-cell populations (A), T-cell differentiation (B), T-helper subsets (C), cytokine profiles (D), immune checkpoint expression (E), and T-cell functional and metabolic status (F).

Figure 23. Main peripheral immune abnormalities in recurrent IDHwt glioblastoma patients, as compared to primary IDHwt glioblastoma patients.

Figure 24. Survival analysis of patients showing prominent differences ($p < 0.1$) between the top 30% and bottom 30% expression levels of immune markers that were significantly altered between HDs and all IDHwt glioblastoma (A), primary IDHwt glioblastoma (B), or recurrent IDHwt glioblastoma (C). The two survival curves represent the highest and lowest 30% for each marker, respectively.

Figure 25. Comparison of survival data from Figure 24 (A) with the corresponding marker expression in peripheral blood for all IDHwt glioblastomas. Circling is an approximation of the upper and lower 30% values.

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Figure 27. Comparison of survival data with the corresponding marker expression in the peripheral blood for primary (left column) and recurrent (right column) IDHwt glioblastoma for markers with prominent ($p < 0.1$) differences between primary and recurrent IDHwt glioblastoma (not when compared with HDs). The circling is an approximation of the upper and lower 30%.

Supplemental Figure 1. Gating strategy for Panel 1

Supplemental Figure 2. Gating strategy for Panel 2

Supplemental Figure 3. Gating strategy for Panel 3

Supplemental Figure 4. Gating strategy for Panel 4

Supplemental Figure 5. Gating strategy for Panel 5

Supplemental Figure 6. Reduced T_{FH} expression in patients receiving pre-operative dexamethasone.

11. List of Tables

12. Related Publications

Submitted for publication:

Maire CL, Ryba A, Djabali Y, Müller C, Fita K, Spohn M, Yabo Y, Rünger A, Reetz M, Drexler R, Meyerhöfer N, Simnica D, Oubounyt M, Gallus M, Giang MH, Kornahrens jr W, Glau L, Mazgaeen L, Zolotareva O, Burankova Y, Riecken K, Nejo T, Dirksen P, Freitas JA, Schulte S, Piffko A, Dierlamm J, Peine S, Akyüz N, Bockmayr M, Schüller U, Dilthey A, Peters W, Neidert MC, Dührsen L, Westphal M, Ricklefs FL, Baumbach J, Binder M, Monje M, Okada H, Blumenthal DB, Tolosa E, Alawi M, Lamszus K, Bischof O, Heiland DH, Brown M, Mohme M. Neuroimmune crosstalk suppresses systemic T cell function in glioblastoma. Manuscript submitted to *Nature*.

13. Declaration of Own Contribution

This doctoral thesis was conducted within the framework of the research project on the characterization of the peripheral immune response in glioblastomas in the Department of Neurosurgery in the Brain Tumor Biology Laboratory under the supervision of Prof. Dr. Katrin Lamszus and PD. Dr. Malte Mohme.

The overall concept of the study, including the definition of the scientific objectives, selection of immune parameters, and experimental design, was developed by my supervisors, with significant input from me along the progression of the project. While some blood samples were already present in the Department's bio bank, I also collected additional blood samples from patients. Clinical data connected to the samples were collated by me. For the existing samples, I extended the existing data, e.g. by calling patients or the patients' medical providers, to complete data on further clinical courses. For the new samples, I collected all data myself.

All experimental work, including cell isolation, flow cytometric analyses, and functional assays, was performed independently by me. I received technical assistance from Cecile Maire, Katharina Kolbe, Mareike Holz, Sarah Matula, and Svenja Zapf in learning all laboratory techniques.

Data collection, processing, statistical analysis, and figure preparation were carried out independently by me, with guidance by PD. Dr. Malte Mohme. The interpretation of the data and the discussion of the results in the scientific context were developed jointly with my supervisors.

The written thesis, including all figures and all chapters (Introduction, Methods, Results, Discussion, and Conclusion), was prepared and compiled solely by me. Contributions from my supervisors and collaborators were limited to scientific advice, feedback, and editorial comments.

14. Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe, insbesondere ohne entgeltliche Hilfe von Vermittlungs- und Beratungsdiensten, verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe. Das gilt insbesondere auch für alle Informationen aus Internetquellen.

Soweit beim Verfassen der Dissertation KI-basierte Tools („Chatbots“) verwendet wurden, versichere ich ausdrücklich, den daraus generierten Anteil deutlich kenntlich gemacht zu haben. Die „Stellungnahme des Präsidiums der Deutschen Forschungsgemeinschaft (DFG) zum Einfluss generativer Modelle für die Text- und Bilderstellung auf die Wissenschaften und das Förderhandeln der DFG“ aus September 2023 wurde dabei beachtet.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Ich erkläre mich damit einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Datum Unterschrift

15. Acknowledgments

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