2. Materials & Methods

2.1 Ethical approvals and signed informed consents of Glioblastoma patients.

The study on T cells of Glioblastoma patients has an IRB approval No. 0482-21-HMO from Hadassah Hebrew University Medical Center, Jerusalem 91120, Israel.
All the Glioblastoma patients signed informed consents.

2.2 The Glioblastoma Patients

Glioblastoma Patient 1

The clinical information of Glioblastoma Patient 1 is summarized in Supplementary Table 1A. Our findings regarding the T cells of this patient are summarized in Table 1B, described in the 'Results', and shown in Figs. 1A-F, Figs. 2A-J, Figs. 3A-D, and Figs. 4A-G. Glioblastoma Patient 1 is an adult who presented with behavioral changes and headaches in August 2022. A left frontal mass lesion was diagnosed based on CT and MRI scans. On August 17, 2022, the patient underwent an image-guided left frontal craniotomy. A 5-Aminolevulinic Acid-guided resection with electrophysiological monitoring was performed, resulting in a gross total resection. Pathological findings confirmed Glioblastoma IDH wild type. The patient recovered quickly with no residual neurological deficits and was discharged home. Adjuvant therapy, including external beam radiotherapy and concomitant Temozolomide, was initiated. A follow-up MRI on December 26, 2022, showed a small nodular recurrence at the lateral aspect of the resection bed. Following a tumor board discussion, the patient was scheduled for re-craniotomy and safe resection of the nodular recurrence. During anesthesia induction for this surgery, peripheral blood was drained for the present immunotherapy study. Second-line therapy included Avastin after the second surgery. The patient remained clinically well until March 2024 when the patient was hospitalized in the Department of Neurology with a generalized seizure and acute confusional state, likely secondary to disease progression in the cerebellum with suspected leptomeningeal dissemination.

The patient expired in hospice due to aspiration pneumonia on April 15, 2024, at 19 months since the initial diagnosis.

Glioblastoma Patient 2

The clinical information of Glioblastoma Patient 2 is summarized in Supplementary Table 2A. Our findings regarding the T cells of this patient are summarized in Supplementary Table 2B, described in the 'Results', and shown in Figs. 5A-L, Figs. 6A-G, and Figs. 7A-H.

Glioblastoma Patient 2 is an adult who presented with a frontal headache followed by a sudden onset of incontinence. Neurological examination revealed right hemiparesis, motor dysphasia, and drowsiness. MRI data sets showed a 70mm mass lesion in the left fronto-temporal region with associated mass effect and severe perilesional vasogenic edema, indicative of a high-grade glioma. On January 31, 2023, the patient underwent an image-guided left fronto-temporal craniotomy. A 5-Aminolevulinic Acid-guided resection with electrophysiological monitoring was performed, resulting in a gross total resection. The postoperative course was uneventful, with improvements in hemiparesis and speech functions. Pathological findings confirmed Glioblastoma IDH wild type. Adjuvant therapy, including external beam radiotherapy and concomitant Temozolomide, was initiated. Treatment continued with Avastin and Tumor Treating Fields (TTF) until her last clinical follow-up on April 16, 2024.

Glioblastoma Patient 3

The clinical information of Glioblastoma Patient 3 is summarized in Supplementary Table 3A. Our findings regarding the T cells of this patient are summarized in Supplementary Table 3B, described in the 'Results', and shown in Figs. 8A-J.

Glioblastoma Patient 3 is an adult who presented in May 2023 with confusion, anxiety, aggressive behavior, and headaches. Neurological examination revealed some minor cognitive loss. A right temporal mass lesion was diagnosed based on CT and MRI scans. On May 17, 2023, the patient underwent an image-guided left frontal craniotomy. A 5-Aminolevulinic Acid-guided resection with electrophysiological monitoring was

performed, resulting in a gross total resection. Pathological findings confirmed Glioblastoma IDH wild type. The patient recovered quickly with no residual neurological deficits and was discharged home. Adjuvant therapy, including external beam radiotherapy and concomitant Temozolomide was initiated.

Follow-up MRI on July 29, 2023, at 1 month post completion of irradiation, showed a suspected tumor recurrence around the resection cavity with involvement of the right hippocampus. Tumor board discussion advised re-operation; however, the patient declined surgical options and preferred to continue medical therapy with oral Temozolomide. As of April 29th, 2024, the patient is still under neuro-oncological surveillance and therapy with a poor functional status.

Glioblastoma Patient 4

The clinical information of Glioblastoma Patient 4 is summarized in Supplementary Table 4A. Our findings regarding the T cells of this patient are summarized in Supplementary Table 4B, described in Supplementary file called Glioblastoma Patient 4, and shown in the respective Supplementary Figs. 1A-D, 2 and 3A-K.

Glioblastoma Patient 4 is an adult who presented back in April 2021 with word-finding difficulties. CT and MRI scans revealed an oval lesion at the base of the left temporal lobe measuring 23 mm by 21 mm with a moderate amount of surrounding edema. A radiological diagnosis of suspected high-grade glioma was made, leading to the patient undergoing an image-guided left temporal craniotomy assisted by A 5-Aminolevulinic Acid-guided resection. Postoperative MRI showed gross total resection, and the postoperative course was uneventful.

Pathological findings confirmed Glioblastoma IDH wild type. Adjuvant therapy, including external beam radiotherapy and concomitant Temozolomide, was initiated. Treatment continued with Temozolomide and TTF. Additionally, the patient received 'Personalized tumor neoantigen-specific vaccination' in a private clinic in Germany (this treatment was remote, independent and prior to the current study).

On May 2023, the patient experienced reading difficulties, prompting an urgent MRI on May 28th, 2023, which revealed recurrent tumor at the surgical bed with similar size

and shape. After a detailed discussion with the patient, we proceeded with a recraniotomy on May 31, 2023. During anesthesia induction for this surgery, peripheral blood was drained for the present immunotherapy study. Once again, a careful A 5-Aminolevulinic Acid-guided resection of the tumor was conducted, resulting in gross total resection. The patient opted to continue the investigational immunotherapy protocol in Germany. Since then, the patient was lost to our clinical and radiological follow-up. The patient died in 2025.

2.3 Neurotransmitters and Neuropeptides.

The following Neurotransmitters and Neuropeptides were used in the present study: Glutamate (Sigma-Aldrich, Israel), Dopamine (Sigma-Aldrich), GnRH-II (LHRH-II) (Bachem, Switzerland), and Neuropeptide Y (Bachem) All of them were used at a final concentration of 10^-8M (i.e., 10nM). In each experiment, for each Neurotransmitter or Neuropeptide, freshly made serial 1:10 dilutions from much higher conc. stocks (kept frozen in -20C or -70C) were prepared few minutes before use. In most experiments, each Neurotransmitter and Neuropeptide was diluted to 10^-7M, and then diluted again 1:10 when it was added to the T cells which were suspended in their regular growth medium, to reach the final desired concentration of 10^-8M.

2.4 Separating T cells from blood of human subjects.

The T cells of all the Glioblastoma patients were separated from their blood samples (55-45ml) soon after blood withdrawal, by 'STEMMCELL RosetteSep™ Human T cell enrichment cocktail' – Cat. No. 15061, according to the manufacturer instructions. This RosetteSep™ T Cell Enrichment Cocktail is designed to isolate T cells from whole blood by negative selection. Unwanted cells are targeted for removal with Tetrameric Antibody Complexes recognizing non-T cells and glycophorin A on red blood cells. The product information and directions for use of this T cell purification method can be found in https://cdn.stemcell.com/media/files/pis/28570-PIS_1_3_1.pdf, and in *https://www.stemcell.com/products/rosettesep-human-t-cell-enrichment-cocktail.html*.

2.5 Evaluating the size and granularity of T cells of Glioblastoma patients and healthy control subjects

Basic flow cytometry was used to evaluate the size (forward scatter) and granularity (side scatter) of the T cells for each Glioblastoma patient or healthy control subject.

2.6 Immunofluorescence staining of the immune checkpoint inhibitory receptors: PD-1, LAG-3, Tim-3, TIGIT, and CD160 in the T cells of the Glioblastoma patients.

Immunofluorescence staining of the immune checkpoint inhibitory receptors expressed on the cell surface of T cells of Glioblastoma patients: PD-1, LAG-3, Tim-3, TIGIT, and CD160, was performed with the following specific fluorescently-coupled antibodies:
Alexa Fluor 488 anti-human CD279 (PD-1) (Biolegend Cat. No. BLG-329935),
APC anti-human CD223 (LAG-3) (Biolegend Cat. No. BLG-369211),
PE anti-human CD366 (Tim-3) (Biolegend Cat. No. BLG-345005),
APC anti-human TIGIT (VSTM3) (Biolegend Cat. No. BLG-372705),
PerCP/Cyanine5.5 anti-human CD160 (Biolegend Cat. No. BLG-341209), and
PE-anti-human CD152 (CTLA-4) (Biolegend Cat. No. BLG-369603).

2.7 Immunofluorescence staining of CD3, CD3zeta, CD4, and CD8 in T cells of Glioblastoma patients.

The immunofluorescence staining of CD3ε^+, CD3zeta (CD3ζ^+), CD4$^+$, and CD8$^+$ T cells was done by specific immunostaining with the following antibodies:
PE-Mouse anti-human CD3ε chain (BD Pharmingen Cat. No. 561803),
PE-Mouse anti-human CD3ζ chain (CD247) (BD Pharmingen Cat. No. 558448),
PE-Mouse anti-human CD4 (BD Pharmingen Cat. No. 555347), and
PE-Mouse anti-human CD8 (BD Pharmingen Cat. No. 555635).

2.8 Co-culture of human Glioblastoma cells and T cells of Glioblastoma patients, and thereafter follow-up and analysis by the Incucyte technology.

We prepared co-cultures of human Glioblastoma cells and T cells of Glioblastoma patients, in wells of 48-well microtiter plates. The wells contained the two types of cells, as described below.

1) Adherent human Glioblastoma cells (U87 cell line). These cells grew in tissue culture in MEM medium + 10% Fetal Calf Serum (FSC), 1% Non-essential amino acids, 1% Glutamine, 1% Sodium Pyruvate and 1% antibiotics, in wells of 12 wells microtiter plates. Two days before the onset of each co-culture experiment, the human Glioblastoma cells were detached from the wells in which the grew, washed, counted , suspended in fresh medium and seeded in wells of a new 48 well plates, usually at a conc. of ~5000 cells/200μl/well. Then, the Glioblastoma cells were placed in an Incucyte incubator for 2-3 days before the T cells of the Glioblastoma patients were added. In these initial days, before the onset of the real co-culture experiment, the Glioblastoma cells were not measured or photographed by the Incucyte technology.

2) T cells of Glioblastoma patients. The patient's T cells were purified from the patient's blood samples (withdrawn before brain surgery), allowed to rest in a tissue culture incubator for 24 hr. after purification, and then either untreated or pre-treated Ex Vivo (for 24 hrs.) with selected Neurotransmitters and Neuropeptides, all at a final conc. of 10^-8M (prepared freshly in each experiment, just before the usage, by serial dilutions from stocks (of much higher concentration, which were kept frozen or refrigerated). In most experiments 20,000 T cells (untreated or treated) were added to sets of replicate microtiter already containing the human Glioblastoma cells. Thus, the ratio of T cells to Glioblastoma cells was usually ~4:1. Each experimental group consisted of at least four replicate wells.

From then onwards, we set the Incucyte follow-up program to measure continuously the adherent Glioblastoma cells in nine fields of each single well, and to photograph the cells in each well every 3 hours, for 5-7 days. Few summary words about the Incucyte device and technology are written below in part 2.9.

The quantitative data analysis was done automatically by the Incucyte build-in NeuroTrack analysis software. The quantitative kinetic curves were also drawn by the Incucyte software based on the analyzed data. The additional later calculations of the averages, SD, and p values, and the drawing of the histograms at select time points, were done in Excel, after exporting the raw data from the Incucyte to Excel.

2.9 The Incucyte® Live-Cell Analysis System.

The Incucyte® Live-Cell Analysis System (Sartorius) is designed to capture efficiently cellular changes in the incubator, as well as all possibilities and modes for its use in various biological purposes, can be found at https://www.sartorius.com/en/products/live-cell-imaging-analysis.

The details of the Incucyte® Neurotrack Analysis Software Module we used, that allows users to automatically process Incucyte® images and analyze the dynamics of cells of the nervous system, with or without labels in living cells, in each well of a tissue culture well plate, can be found in https://www.sartorius.com/en/products/live-cell-imaging-analysis/live-cell-analysis-software/incucyte-neurotrack-analysis-software.

The Incucyte® Neurotrack algorithm masks and quantifies each image and returns neuronal cell metrics including neurite length, branch points, cell body area, and number of cell clusters. These metrics are visualized in full time course plots for each well in tissue culture well plates.

2.10 Measuring the In Vitro migration of T cells of Glioblastoma patients towards the medium in which human Glioblastoma cells grew in tissue culture.

We used the Boyden Chamber Transwell migration assay (Transwell 5.0 lm, 6,5 mm insert, 24 well, Costar Corning NY, USA) to measure the extent of In Vitro migration of T cells of Glioblastoma Patient 4, either untreated or pre-treated with Dopamine, Neuropeptide Y or Glutamate + Neuropeptide Y, towards the medium in which the human Glioblastoma cells (U87) grew continuously in tissue culture.

We assumed (but did not prove) that this medium in which the Glioblastoma cells grew

continuously, contained various tumor-specific factors that these Glioblastoma cells secreted, that could inhibit the migration of T cells, and wanted to test if pre-treatment of the T cells with some of the selected Neurotransmitters and/or Neuropeptides would overcome this inhibition.

Note, in most studies using Boyden Chamber Transwell migration assay, the number of cells typically placed in the upper chambers of a 24-well Transwell insert with a 5.0 μm pore size is generally 5 x 10^5 cells, in a volume of 100μL to 500μL of serum-free medium. In our experiments in this study, we did not have a sufficiently high number of T cells of Glioblastoma Patients 1, 2 and 3 to perform migration assays (in addition to all the other experiments we performed with them). We only tested the T cells of Glioblastoma Patient 4 in a migration assay, and had to put in the upper chambers only 100,000 T cells in 100 μL /well in serum-free medium. The T cells of Glioblastoma Patient 4 in the upper wells of this assay (in duplicates) were untreated or pre-treated with either Dopamine, Neuropeptide Y, or Glutamate + Neuropeptide Y (all at 10^-8M, for 24 hr. before the migration assay). The lower chambers of all the wells contained only 600μL of medium in which human U87 Glioblastoma cells grew for several prior days, without anything else. The migration assay was run for 4 h. Then, the upper chambers were very removed gently, the 600μL medium of the lower chambers containing the migrating T cells, and were transferred into clean test tubes. Then, the no. of migrating T cells in each tube was counted by the FACS option of counting the number of cells (even if they are not stained with a fluorescence dye) within a given sample during a desired pre-determined time, which we fixed for 1 min.

It is noteworthy that in few of our previous studies we used the Boyden Chamber Transwell migration assay successfully, to test the chemotactic migration of human T cells (10, 14, 15), and the effects of selected Neurotransmitters and/or neuropeptides on their migration. In one of these studies (10) we tested the T cells of few patients with Head and Neck Cancer patients for three types of migration: spontaneous migration, chemotactic migration towards SDF-1, and migration towards tissue culture medium containing pieces of the patient's own surgically-removed Head and Neck

Cancer tissue, which were kept in tissue culture for a few days after their removal from the patients. Here too, this growth medium, in which the patient's own Head and Neck Cancer tissue was kept in tissue culture for few days prior to the migration assay, was assumed to contain some tumor-specific factors secreted by the cancer cells, that could suppress T cells. The scheme and details of the assay, as well as the remarkable good results, are described in (10).

2.11 Statistical analysis.

For each functional experiment, the mean and SD of each experimental group was calculated. Then, multiple comparisons, t-tests, and p values were calculated for comparing all replicates of the untreated T cells, with all replicates of T cells treated with any given Neurotransmitter, Neuropeptide, or their combination.

The p values, for each functional experiment performed with T cells of each human individual tested herein, are specified in the figures and in some cases also in the main body of the results.