2D MXenes with antiviral and immunomodulatory properties: a pilot study against SARS-CoV-2

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1. Materials and methods

MAX Precursor Synthesis

To prepare the MAX precursors, TiC (<2 μm, Alfa Aesar, 99.5%), Mo (-250 mesh, Alfa Aesar, 99.9%), Ta (-325 mesh, Alfa Aesar, 99.97%), Nb (-325 mesh, Beantown Chemicals, 99.99%), Ti (-325 mesh, Alfa Aesar, 99.5%), Al (-325 mesh, Alfa Aesar, 99.5%), and graphite (-325 mesh, Alfa Aesar, 99%) powders were utilized.

For Ti₃AlC₂, a 2:1:1 atomic ratio of TiC:Ti:Al (50 g) was mixed. To produce Mo₂Ti₂AlC₃, a 2:2:1.3:2.7 atomic ratio (10 g) of Mo:Ti:Al:C powders were mixed. To synthesize Ta₄AlC₃, a 4:1:3 atomic ratio (7 g) of Ta:Al:C powders were utilized. For Nb₄AlC₃, a 4:1:1:2:7 atomic ratio (10 g) of Nb:Al:C was used. All aforementioned mixtures were mixed in a 2:1 ball:powder weight ratio using 5 mm alumina balls. The mixtures were ball milled at 60 rpm for 24 hours prior to synthesis.

The high-temperature annealing reactions were conducted in a Carbolite furnace, using heating and cooling rates of 3 °C, along with 200 cm³ min⁻¹ flow of ultra-high purity Ar (99.999%). To synthesize Ti₃AlC₂, the mixture was heated to 1400 °C for 2 h. For Mo₂Ti₂AlC₃, the powders were heated to 1600 °C for 4 h. To produce Ta₄AlC₃, the powders were heated to 1400 °C for 8 h. For Nb₄AlC₃, the mixture was heated to 1650 °C for 4 h. After cooling, the sintered blocks were milled using a TiN-coated bit and sieved through a 400 mesh sieve, resulting in a final particle size <38
μm. All experiments conducted in this study used a single batch of MAX to eliminate any artifacts from variation between MAX synthesis batches.

**MXene Synthesis**

To selectively etch Al from the MAX phases to produce MXenes, HF (Acros Organics, 48-50 wt. %; 29 M), HCl (Fisher Scientific, 37 wt. %; 12 M), and deionized (DI) water (15 MΩ resistivity) was utilized. For the synthesis of all MXenes in this study, Teflon coated stir bars were utilized with at 300 rpm stirring rate. Ti$_3$C$_2$Tx was synthesized by etching 1.0 g Ti$_3$AlC$_2$ in a 1:3:6 volumetric ratio (20 mL total) of HF:H$_2$O:HCl for 24 hours at 35 °C. To produce Mo$_2$Ti$_2$C$_3$Tx, 1.0 g Mo$_2$Ti$_2$AlC$_3$ was added to 20 mL 48-50 wt.% HF for 96 hours at 55 °C. To synthesize Ta$_4$C$_3$Tx, 1.0 g Ta$_4$AlC$_3$ was added to 20 mL 48-50 wt.% HF for 72 hours at 35 °C. Nb$_4$C$_3$Tx was produced by adding 1.0 g of Nb$_4$AlC$_3$ to 20 mL 48-50 wt.% HF and stirred for 120 hours at 35 °C. After the appropriate etching time, the MXenes were washed by centrifugation using DI water. Briefly, the mixtures were added to 150 mL DI H$_2$O, then centrifuged at 3,500 rpm for 10 min. The acidic supernatant (clear) was decanted; the multilayer MXene remained as sediment. New DI H$_2$O was added, the sediment redispersed, and the process repeated. These cycles were repeated eight times to ensure that all residual acid was removed from the multilayer MXene.

To delaminate the MXenes, and produce a stable colloidal solution, the multilayer MXenes were added to 20 mL of a 5 wt. % TMAOH solution (TMAOH; Sigma Aldrich, 25 wt. % in H$_2$O). The MXenes were stirred for 12 h at 300 rpm at 35 °C. Afterwards, the mixtures were placed into 50 mL centrifugation tubes. DI water was added, and the samples were centrifuged at 10,000 rpm for 20 min. The supernatant (clear) was decanted, with the MXenes redispersed in fresh DI water. This process was
repeated five times to ensure that all excess TMAOH was removed. After the final cycle, the MXenes were fully redispersed in 50 mL DI water, then centrifuged at 3,500 rpm for 10 minutes. The supernatant (dark) was carefully collected, then centrifuged again at 3,500 rpm for 10 min, the supernatant (dark) was collected for use. This procedure ensured that only single-flake MXene remained in the solution. A known volume of this solution was vacuum filtered on Celgard membranes (64 nm pore size, 3501 coated polypropylene), to produce free-standing films for X-ray diffraction (XRD). By measuring the weight of the produced films, the colloidal solution concentration was determined.

**Material Characterization**

XRD patterns of the powders and films were collected on a Rigaku MiniFlex600 (40 kV and 15 mA) diffractometer using Cu Kα radiation. The conditions were as follows: (i) for the MAX powder, step scan 0.02, 3–80 (2θ), step time of 1 s; (ii) for the MXene films, a step scan of 0.03, 3–70 (2θ), step time of 0.5 s was used.

Scanning electron microscopy (SEM) was conducted on a dual-beam focused ion beam (Strata DB235, FEI). The MXene flakes were drop-cast onto an anodized alumina substrate. Pt was deposited onto the flake and substrate to minimize charging. DLS (Zetasizer Nano ZS, Malvern Instruments) was performed to analyze MXene flake size distribution. Three measurements were taken of each sample and the average value was reported.
Cell Culture

Jurkat cells were grown in 75 cm² flasks at 37 °C, 5% CO₂, in DMEM medium (Invitrogen, CA, USA) containing 10% FBS (Invitrogen, CA, USA), and 1% P/S (Invitrogen, CA, USA). The day before exposure to particles, cells were seeded at $10^5$ cells/well in 24-well plates. Vero E6 cells were cultured in high-glucose DMEM medium (Invitrogen, CA, USA) containing 10% FBS (Invitrogen, CA, USA), and 1% P/S (Invitrogen, CA, USA). Cells were maintained at 37 °C, 5% CO₂. For viral infection experiments, African green monkey kidney Vero E6 cell line was purchased from ATCC and maintained in DMEM media containing 10% FBS and 1% antibiotics. Four local SARS-CoV-2 isolates were used in this study and genotype details including GISAID classifications are given in Figure 2B. Viral particles were propagated in Vero E6 cells. All virus-related experiments were performed at Biosafety Level 3 laboratories.

Isolation of PBMCs

PBMCs were harvested from ethylenediaminetetraacetic acid (EDTA)-venous blood from informed healthy donors (25–50 years old) using a Ficoll-Paque (GE Healthcare, CA, USA) standard separation protocol. Informed signed consent was obtained from all the donors. Cell separation and experiments were performed immediately after the blood draw. PBMCs were cultured in 24-well plates in RPMI 1640 medium (Invitrogen, CA, USA), supplemented with 10% heat-inactivated FBS (Invitrogen, CA, USA), 1% P/S (Life Technologies). 1% L-Glutamine (Life Technologies). In addition, EtOH 70% was used as positive control for cell death, while 2 µg/ml LPS (Life Technologies) or 10 µg/ml ConA (Life Technologies) were used as positive control for cell activation.
Flow Cytometer Analysis

Cells were seeded at $10^6$ cells/well in 24-well plates, and the day before the experiment cells were exposed to 50 µg/mL Ti$_3$C$_2$Tx. Cell viability was assessed by means of staining with Fixable Viability Stain 780 (BD Biosciences). EtOH 70% was used as positive control.

To assess PBMC activation after treatment with Ti$_3$C$_2$Tx, CD25, CD69, and HLA-DR (PE-conjugated anti-CD25, M-A251 clone; PE-Cy7-conjugated anti-CD69, FN50 clone, BV421-conjugated anti-HLA-DR, G46-6 clone; BD Bioscience) were used as activation markers. Lypopolisaccharides (LPS, 2 µg/mL, Life Technologies) or concanavalin A (ConA, 10 µg/mL, Life Technologies) were used as positive control for cell activation in monocytes and T cells, respectively. Staining was performed in the dark for 20 min. Cells were processed by flow cytometry (BD LSRFortessa™ FACS Canto II, BD Bioscience), and data were analyzed by FlowJo™ Software[1].

PBMC staining and gating strategy by flow cytometry

PBMCs were stained with FITC-conjugated anti-CD3 (UCHT1 clone; BD Biosciences), BV605-conjugated anti-CD4 (RPA-T4 clone; BD Biosciences), BV786-conjugated anti-CD14 (M5E2 clone; BD Biosciences), and BV711-conjugated anti-CD16 (3G8 clone; BD Biosciences).

Gating strategy was performed as described in Figure S6E. Briefly, doublets and cell debris were excluded through the gating strategy and specific subpopulations were determined. Specifically: CD4 T cells (CD3+ CD4+), CD8 T cells (CD3+ CD4-), Classical (C.) monocytes (CD3− CD14+ CD16−), Intermediate (Int.) monocytes (CD3− CD14+ CD16+), Non-classical (N.C.) monocytes (CD3− CD14- CD16+).
Single-cell mass cytometry analysis

Single-cell mass cytometry analysis was performed on isolated PBMCs, collected as described above, seeded at a concentration of $4 \times 10^6$ cells/well in 6-well plates. PBMCs were incubated with 50 μg/mL of Ti$_3$C$_2$Tx for 24 h or left untreated (negative control). LPS at 0.5 μg/mL (Sigma – Aldrich, Missouri, USA) and EtOH 70% were used as positive controls. Six hours prior to the treatment end, cells were co-incubated with 10 μg/mL Brefeldin A (Invitrogen, CA, USA). After the incubation, PBMCs were washed with PBS supplemented with 0.5 M EDTA and 5% Fetal Calf Serum (FCS).

Cells were then barcoded using Cell-ID 20-Plex Pd Barcoding Kit (Fluidigm, CA, USA). The barcoded samples were stained with Cell-ID Cisplatin (1:1000, Fluidigm, CA, USA), Maxpar Human Peripheral Blood Phenotyping and Human Intracellular Cytokine I Panel Kits (Fluidigm, CA, USA) following the manufacturer instructions. To this end, cells were fixed and permeabilized with 1X Fix I Buffer and 1X Barcode Perm Buffer obtaining a uniform cell labeling with the palladium barcode. Thanks to the barcoding process, samples were pooled together and re-suspended into a 5 ml polystyrene round-bottom tube using the Maxpar Cell Staining Buffer.

The surface marker antibody mix (1:100 dilutions for each antibody) was added to the sample. Cells were then carefully mixed and incubated for 30 min at room temperature. At the end of the incubation, samples were washed twice with Maxpar Cell Staining Buffer, and fixed by incubation with 1.6% paraformaldehyde for 10 min. After this, cells were washed twice with Maxpar Perm-S Buffer, centrifuged for 10 min at 1000×g, resuspended in 400 μl of Maxpar Perm-S Buffer, and incubated with cytoplasmic/secerted antibody mix for 30 min (1:100 dilutions for each antibody). Following the incubation, cells were washed twice with Maxpar Cell Staining Buffer, and stained overnight using 0.125 μM Cell-ID Intercalator-Ir solution. The day after,
samples were washed twice with Maxpar Cell Staining Buffer, re-suspended with 2 ml of Maxpar water, and filtered before data acquisition using a 0.22 μm cell strainer cap to remove possible cell clusters or aggregates. Data were analyzed using mass cytometry platform CyTOF2 (Fluidigm Corporation, CA, USA).

CyTOF data analysis was performed as described before[2, 3]. Briefly, background was subtracted and FCS files were normalized and analyzed by Cytobank. Doublets, cell debris, and dead cells were excluded through the gating strategy using Cell-ID Intercalator-Ir and LD. PBMCs subsets and specific subpopulations were determined as portrayed in Figure 1E. Specifically: T cells (CD45+ CD19- CD3+), Naïve Th. Cells (CD3+ CD4+ CD45RA+ CD27+ CD38− HLADR−), Effector Th. cells (CD3+ CD4+ CD45RA+ CD27− CD38− HLADR−), Activated Th. cells (CD3+ CD4+ CD38+ HLADR+), Naïve CT cells (CD3+ CD8+ CD45RA+ CD27+ CD38− HLADR−), Effector CT cells (CD3+ CD8+ CD45RA+ CD27− CD38− HLADR−), Activated CT cells (CD3+ CD8+ CD38+ HLADR+), C. monocytes (CD45+ CD3− CD19− CD20− HLADR+ CD14+), int. monocytes (CD45+ CD3− CD19− CD20− HLADR+ CD14dim CD16+), N.C. monocytes (CD45+ CD3− CD19− CD20− CD11c− CD14− CD16+), mDCs (CD45+ CD3− CD19− CD20− CD14− CD11c− CD123−), pDCs (CD45+ CD3− CD19− CD20− CD14− CD11c− HLADR+ CD123+), NK cells (CD45+ CD3− CD19− CD20− CD14− CD11c− CD38− CD16+), B cells (CD3- CD20+ CD19+), naïve B cells (CD27−), plasma B cells (HLADR- CD38+). The heat map visualization created with Cytobank, allowed the comparison between the marker fluorescence of the treated populations with mean fluorescent intensity towards the untreated control. Moreover, viSNE, a Cytobank tool implemented for cytometry analysis was employed. The t-stochastic neighbor embedding (t-SNE) shows single cells in a two- or three-dimensional plot, on the basis of their relationships. In order to
characterize the viSNE map, 10 cell surface markers were used (i.e. CD3, CD4, CD8a, CD11c, CD14, CD16, CD19, CD20, CD123, and HLA-DR).

Cytokine data were analyzed using the viSNE tool, and displayed as plots of the expression intensity of the studied cytokines (i.e., IL-6, TNF-α, IL-17a, IL17f, IFN-γ, Perforin, and GrB), and heatmaps of mean marker expression ratio for cytokines that displayed modulations upon Ti3C2Tx treatment.

The specific clone of each antibody used for mass cytometry has been provided in Table S3.

Detection of TNF α

TNF-α levels were evaluated in the supernatant of PBMCs treated with Ti3C2Tx (50 µg mL-1) for 24h, or lipopolysaccharide (LPS, a strong immunostimulatory molecule) plus Ti3C2Tx, LPS alone, cell control samples were left untreated. The Cytometric Bead Array (CBA) immunoassay kit (BD Biosciences, USA) was used to quantify TNF-α concentration following the manufacturer instructions. Data were acquired in a BD LRS Fortessa flow cytometry system (BD Biosciences, San Jose, CA, USA) and analyses performed using BD FCAP Array v3.0 software (BD Biosciences, San Jose, CA, USA). The standard curve was determined using a five-parameter logistic (5-PL) equation. The results were based on standard concentration curve and expressed as picogram per milliliter (pg/mL).

Luminex analysis

To evaluate the impact of MXenes on cytokine release by PBMCs, cells were incubated for 24 h with 50 µg/mL of Ti3C2Tx. ConA, 4 µg/mL (Sigma) and LPS, 2 µg/mL (Sigma) were used as positive controls, while samples incubated with medium
alone were used as negative controls. Supernatants were collected and analysed by Luminex technology using Bio-Plex Pro Human Chemokine 40-plex Panel (Bio-Rad) to measure C-C Motif Chemokine Ligand (CCL) 21 (CCL21), chemokine (C-X-C motif) ligand (CXCL) 13 (CXCL13), CCL27, CXCL5, CCL11, CCL24, CCL26, C-X3-C Motif Chemokine Ligand 1 (CX3CL1), CXCL6, granulocyte macrophage-colony stimulating factor (GM-CSF), CXCL1, CXCL2, CCL1, interferon gamma (IFN-γ), interleukin (IL)-1β, IL-2, IL-4, IL-6, CXCL8, IL-10, IL-16, CXCL10, CXCL11, CCL2, CCL8, CCL7, CCL13, CCL22, macrophage migration inhibitory factor (MIF), CXCL9, CCL3, CCL15, CCL20, CCL19, CCL23, CXCL16, CXCL12, CCL17, CCL25 and tumor necrosis factor (TNF)-α. 5-parameter-Logistic regressions with a power low variance weighing were calculated for each cytokine standard with a recovery range of 70-130% using Bioplex Manager V6.2 (BioRad). Concentration falling within the recovery range, expressed in pg/ml were extrapolated from the median fluorescence intensity of each cytokine bead set. For analytes above or below the standard recovery ranges, upper and lower limits of quantification computed from the standard curves were substituted. Data were then Log2 transformed and compared across experiments by fitting a general ANOVA model with contrast between groups; p values were corrected using Benjamini and Hochberg false discovery rate, FDR; statistically significant p value cut-off was set at FDR p < 0.05.

**Molecular Docking**

Water and ligand molecules were removed from protein structures using UCSF Chimera Software Version 1.14[4]. All protein structures were downloaded from the Protein Data Bank. Protein models and Ti₃C₂T₃ ligand were converted to PDBQT file format using parameters defined by default in the AUTODOCK 4.0[5] program, and
blind docking calculations were made for each protein by defining grid box maximum and grid space 1 Å. Since all conformations are very close to each other (± 0.01 kcal / mol), the first conformation was taken as basis in all analyzes. Discovery Studio's academic version was used for the preparation of the visuals of the calculations and additional calculations[6].

**Viral Infection**

Vero E6 cells were seeded onto 96-well plates at confluency. Cells were incubated with MXenes (at different dilutions from a stock solution of 1.1 mg/mL) and SARS-CoV-2 (MOI 0.1). Cell viability was obtained after 48 h by using crystal violet staining. Briefly, cells were first incubated in 10% formaldehyde. Following this decontamination, plates were washed under running water and stained with Crystal Violet (Sigma) for 20 min. EtOH was added in order to solubilize the stain and absorbance values were obtained at 495 nm wavelength. Percentage of cell viability was obtained following normalization against untreated control cells.

**qRT-PCR analysis**

Following treatment, cell culture supernatants were removed and RNA was isolated via MPLC Total Nucleic Acid Isolation Kit (Roche) using automated MagNA Pure LC Instrument (Roche). One-step qRT-PCR was performed using the Transcriptor One-Step RT-PCR Kit (Roche). A standard curve was constructed in order to calculate viral copies per µL. Primer sequences (2019-nCoV_N1) were obtained from the Division of Viral Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA.
LC-MS/MS Analysis

Vero E6 cells were incubated with Ti₃C₂Tx (50 µg/mL) in 6 well-plates for 24 h. Subsequently, cells were washed with cold PBS and cell pellets were obtained. Total protein concentration was obtained using BCA assay (Thermo). LC-MS/MS analysis was performed at the proteomic facility of Acibadem Labmed (Turkey). Protein profiling was performed using nano Acquity ultra performance liquid chromatography coupled with LC-MS/MS system on Synapt instrument (Waters) as described before[7, 8].

Functional Annotation

For the analyses the proteins with \( p<0.05 \) and fold change \((\text{FC}) \geq 1\) based on \(\log_2\) expression value were assigned as differentially expressed. Gene Ontology (GO) is used to annotate the proteins[9]. The three sub-ontologies of biological process (BP), cellular component (CC), and molecular function (MF) were identified. To obtain a comprehensive functional annotation, different databases were searched. The ontology enrichment and pathway analyses were performed using the DAVID database[10]. Metabolic pathways based on available databases such as KEGG[11] and Reactome[12] were also used to describe the pathway enrichment analyses. The protein-protein interactions network was predicted via String database[13]. The interaction score was set to \( \geq 0.4 \), and the PPI (Protein-Protein Interaction) networks were created. BiomaRt R package was used to combine the datasets[14].

Statistical Analysis

All values are expressed as mean ± ST.D. Comparison between groups was performed by one-way ANOVA, followed by a Tukey’s post hoc multiple comparisons where data
were normally distributed. At least three independent samples were analyzed. A value of $p < 0.05$ was considered significant.

2. Supplementary Figures

Figure S1. Evaluation of the effect of Ti$_3$C$_2$Tx on SARS-CoV-2 infection *in silico*. *In silico* molecular docking analysis of Ti$_3$C$_2$Tx against different viral protein domains (6VWW, 6LU7, 6M0J, 6M03, 6VXX and 6VYB) was performed and types of interactions and amino acid residues involved during these interactions were identified.

Figure S2. Functional annotation of Ti$_3$C$_2$Tx treated Vero E6 cells. Vero E6 cells were treated with Ti$_3$C$_2$Tx (50 µg/ml for 4 h) and cellular total proteins were isolated for LC-MS/MS analysis. GO annotation was performed for A) biological process (BP) and B) molecular function (MF).

Supplementary Figure 3. Full reactome of Ti$_3$C$_2$Tx treated cells. Vero E6 cells were treated with Ti$_3$C$_2$Tx and cellular total proteins were isolated for LC-MS/MS analysis. Genome-wide overview of the Reactome pathway analysis was performed and the whole reactome image is represented. Scale bar represents p value from 0 to 0.05.

Supplementary Figure 4. Functional annotation of Ti$_3$C$_2$Tx treated Vero E6 cells. Vero E6 cells were treated with Ti$_3$C$_2$Tx and cellular total proteins were isolated for LC-MS/MS analysis. Network analysis was performed in order to dissect the functional protein associations by the String program for the significantly overlapping proteins.
with the SARS-CoV-2 protein interaction map reported in the study by Gordon et al [15]. The jointed lines represent the predicted protein-protein connections and correlations among the network. Differentially expressed proteins of Ti₃C₂T₇ treatment shows numerous connections between the proteins.

**Figure S5. Viability on Jurkat cells and gating strategy on peripheral blood mononuclear cells.** Flow cytometry analysis on Jurkat cells and PBMCs. Jurkat cells were treated with 12.5, 25 and 50 µg/ml of Ti₃C₂T₇, while PBMCs were treated with the selected concentration of 50 µg/mL of Ti₃C₂T₇ for 24 h or left untreated (Unt). EtOH 70% was used as a positive control to induce cell death. LPS (2 µg/mL) and ConA (10 µg/mL) were used as positive controls for cell differentiation analysis. A) Jurkat cells were stained with Live/Dead staining and cell death was evaluated and expressed as percentage of total cell number. B) Representative flow cytometric dot plot of Jurkat cells. C) Histogram plots showing live and dead Jurkat cells. D) Pseudocolor plot showing the gating strategy applied to flow cytometry analyses of PBMCs. E) Representative flow cytometric dot plot of PBMCs untreated (Unt) or treated with: EtOH 70%, LPS (2 µg/mL), ConA (10 µg/mL), or Ti₃C₂T₇ (50 µg/mL). Data are presented as mean ± ST.D. of three independent samples.

**Figure S6. Viability of PBMCs and monocytes.** Cell viability and activation staining analysis by flow cytometry and CyTOF. Cells were treated with Ti₃C₂T₇ (50 µg/mL) for 24 h or left untreated (Unt). EtOH 70% was used as a positive control to induce cell death. Cells were stained with Fixable Viability Staining 780 to evaluate necrosis. A) Representative flow cytometric analysis for cell viability staining of PBMCs. B) Bar graph of cisplatin (LD) mean marker expression ratio for all CD45+ PBMCs. C) Bar
graph of cisplatin (LD) mean marker expression ratio for all T cell, monocyte, DC, NK, and B cell subpopulations. Heatmaps and viSNE plots were generated on the concatenated files. Data are presented as mean ± ST.D. of three independent samples.

**D)** Representative flow cytometric analysis (left panel) and histogram plot (right panel) for cell viability staining of CD4 T cells. **E)** Representative flow cytometric analysis (left panel) and histogram plot (right panel) for cell viability staining of CD8 T cells. **F)** Representative flow cytometric analysis for cell viability staining of monocytes. **G)** Representative flow cytometric analysis (left panel) and histogram plot (right panel) for cell viability staining of C. monocytes. **H)** Representative flow cytometric analysis (left panel) and histogram plot (right panel) for cell viability staining of Int. monocytes. **I)** Representative flow cytometric analysis (left panel) and histogram plot (right panel) for cell viability staining of N.C. monocytes. Data are presented as mean ± ST.D. of three independent samples.

**Figure S7. Analysis of activation on peripheral blood mononuclear cells and monocytes.** Cell activation staining analysis by flow cytometry. Cells were treated with 50 µg/ml Ti3C2Tx for 24h or left untreated (Unt). LPS 2 µg/ml was used as a positive control for PBMCs while ConA 10 µg/ml was used as positive control for T cells. To evaluate cell activation, PBMCs and monocytes were stained with CD25-PE and CD69-PE-Cy7. **A)** Representative flow cytometric analysis for cell activation staining of PBMCs. **B)** Representative flow cytometric analysis (left panel) and histogram plot (right panel) for cell activation staining of CD4 T cells. **C)** Representative flow cytometric analysis (left panel) and histogram plot (right panel) for cell activation staining of CD8 T cells. **D)** Representative flow cytometric analysis (left panel) and histogram plot (right panel) for cell activation staining of monocytes. **E)** Representative...
flow cytometric analysis (left panel) and histogram plot (right panel) for cell activation staining of C. monocytes. F) Representative flow cytometric analysis (left panel) and histogram plot (right panel) for cell activation staining of Int. monocytes. G) Representative flow cytometric analysis (left panel) and histogram plot (right panel) for cell activation staining of N.C. monocytes. H) Representative flow cytometric analysis for CD25-PE staining in PBMCs treated with 50 µg/mL of Ti₃C₂Tx. Cell activation was expressed as % of positive cells. I) TNF-α concentration measured in the supernatant of PBMCs treated with Ti₃C₂Tx (50 µg/mL) using BD Cytometric Bead Array(CBA) Kit. Data are presented as mean ± SD of three independent samples. *, p < 0.05; **, p < 0.01; ***, p < 0.001; **** p < 0.0001 (Ordinary one-way ANOVA).

**Figure S8.** Analysis of HLA-DR on peripheral blood mononuclear cells and monocytes. HLA-DR staining analysis by flow cytometry. Cells were treated with 50 µg/ml Ti₃C₂Tx for 24h or left untreated (Unt). LPS 2 µg/ml and ConA 10 µg/ml were used as positive control. A) PBMCs were stained with BV421-HLA-DR, and HLA-DR expression was evaluated in PBMCs, CD4 T cells, and CD8 T cells. Results are expressed as percentage of total cell number. B) Representative flow cytometric analysis for HLA-DR staining of PBMCs, CD4 T cells, and CD8 T cells. C) Histogram plots showing HLA-DR staining of PBMCs, CD4 T cells, and CD8 T cells. D) Monocytes were stained with BV421 HLA-DR and HLA-DR expression was evaluated in monocytes as well in monocyte subpopulations (i.e., C. monocytes, Int. monocytes and N.C. monocytes). Results are expressed as percentage of total cell number. E) Representative flow cytometric analysis for HLA-DR staining of monocytes and monocyte subpopulations (i.e., C. monocytes, Int. monocytes and N.C. monocytes).
F) Histogram plots showing HLA-DR staining of monocytes and monocyte subpopulations (i.e., C. monocytes, Int. monocytes and N.C. monocytes).

Figure S9. Ti$_3$C$_2$Tx impact on peripheral blood mononuclear cell subpopulations at single-cell level. Blood from healthy donors was drawn, and PBMCs were isolated and incubated with 50 µg/ml Ti$_3$C$_2$Tx for 24h and stained for mass cytometry analysis. LPS 2 µg/ml was used as a positive control. A-G) Bar graphs reporting the immune response to Ti$_3$C$_2$T$_x$ of three biological replicates for A) IL-6, B) TNF-α, C) IFN-γ, D) Perforin, E) GrB, F) IL-17a, and B) IL-17f and immune subpopulations identified. Data are presented as mean ± ST.D. of three independent samples. Statistical differences: *p<0.05; **p<0.01; ***p<0.001 (One-way ANOVA and Tukey’s multiple comparison test).

Figure S10. Immune cell subpopulations gating strategy. Dot plots showing the gating strategy used for the identification of the different immune cell subpopulations by CyTOF.

References

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Figure S1

6VWW

- van der Waals
- Metal-Acceptor
- Alkyl
- Pi-Alkyl

6LU7

- Unfavorable Bump
- Metal-Acceptor
- Alkyl

6M03

- Metal-Acceptor
- Alkyl
- Pi-Alkyl

6VXX

- Alkyl
- Pi-Alkyl
- Unfavorable Bump

6M0J

- Unfavorable Bump
- Metal-Acceptor
- Alkyl
- Pi-Alkyl

6VYB

- Unfavorable Bump
- Metal-Acceptor
- Alkyl
Figure S2

A  Biological Process

B  Molecular Function

- DNA replication-dependent nucleosome assembly
- DNA replication-independent nucleosome assembly
- DNA-templated transcription initiation
- negative regulation of megakaryocyte differentiation
- protein heterotetramerization
- intracellular protein transport
- transport
- regulation of transcription DNA-templated
- poly(A) RNA binding
- DNA binding
- ATP binding
- GTP binding
- zinc ion binding
- calcium ion binding
- chromatin binding
- GTPase activity
Figure S6

A

B

C

D

E

F

G

H

I

Figure S6
Figure S7

A

B

C

D

E

F

G

H

I

**Experimental Results**

**Analysis**

- **Figure S7A**: CD69 and CD25 expression levels in PBMCs under different treatments.
- **Figure S7B**: CD69 and CD25 expression in CD4 T cells.
- **Figure S7C**: CD69 and CD25 expression in CD8 T cells.
- **Figure S7D**: CD69 and CD25 expression in monocytes.
- **Figure S7E**: CD69 and CD25 expression in C. Monocytes.
- **Figure S7F**: CD69 and CD25 expression in Int. Monocytes.
- **Figure S7G**: CD69 and CD25 expression in N.C. Monocytes.
- **Figure S7H**: CD69 expression as a percentage of positive cells.
- **Figure S7I**: TNF-α levels in different conditions.

**Statistical Analysis**

- *p < 0.05
- **p < 0.01
- ***p < 0.001
- ****p < 0.0001

**Notes**

- Untreated
- LPS
- Ti3C2Tx
- Ti3C2Tx + LPS

**Conclusion**

- The treatment with Ti3C2Tx + LPS significantly increased CD69 expression compared to untreated and LPS alone.
- TNF-α levels were also higher in the Ti3C2Tx + LPS condition compared to other treatments.
Figure S9

A

IL-6 mean intensity

B

TNF-α mean intensity

C

IFN-γ mean intensity

D

Perforin mean intensity

E

GrB mean intensity

F

IL-17a mean intensity

G

IL-17f mean intensity
