FOCUSED ULTRASOUND FOUNDATION

Guidelines for immune analysis following FUS treatment

Clinical

Rationale

Local focused ultrasound (FUS) treatment of neoplastic lesions that induces or enhances a systemic anticancer immune response could provide major therapeutic benefits to patients with cancer.

Monitoring the characteristics and temporal evolution of the immune response will provide key information needed to maximize the effectiveness of FUS treatment. This information is necessary to optimize FUS treatment parameters, while further increasing the likelihood of therapeutic success through combination with immunotherapeutic agents or chemotherapies known to have immunostimulatory effects.

The type of assessment and the appropriate assessment time points will likely depend on the mechanism of FUS treatment, such as mechanical v. thermal, the type and location of tumor, availability of biopsy, and the type of combinatorial therapies used.

The primary focus should be on analyzing and documenting the changes in the immune response following FUS treatment to:

1) analyze these changes for the development of more immunocompetent/ less immunosuppressive micro-environment,
2) establish a rationale for the combined treatments regimens including FUS and agents with immunostimulatory effects,
3) identify predictive biomarkers -
    a. static biomarkers, that are present at baseline and can inform patient and treatment selection;
    b. prognostic biomarkers, that are generated upon treatment initiation and can be used to monitor the antitumor immune response.

The proposed guidelines include suggestions for analyses routes and assays. We emphasize the need to perform basic analysis (informed by preclinical work, hypothesis, or drug(s) given) and then save samples for later analysis. These later analyses will be informed, among other factors, by patient outcomes.

General Recommendations

General considerations

1. A clear explanation of statistical methods and of power analysis must be given
2. Analysis will greatly depend on drug used (in combination therapies) and should be optimized accordingly, as a drug may act only on one cell subset, for example
3. Storage of samples is of primary importance and should be addressed before the beginning of the trial to allow:
    a. Running a few very pointed assays/analyses first, before storing remaining samples for later analysis
    b. Later analysis that will be informed by follow-on questions, patient outcomes, clinical data, etc
4. Flow cytometry is imperative for analyses of immune cells. If no facilities/equipment are available at the study site, contract/collaboration with other academic centers or private companies should be set-up, and sample handling/shipping… should be planned before the beginning of the experiments

Design of analyses

The "Supporting information" section of the document provides bases for the proposed recommendation.
There are fundamental questions that studies should be addressing. Is FUS treatment associated with changes in:

- the composition of the tumoral immune infiltrate, phenotype of T-cells and myeloid cells?
- Spatial distribution, infiltration of immune cells?
- T-cells activation/exhaustion status?
- T-cells functions?
- Clonal expansion of T-cells?

It is also important to stress that the design of the analyses will be guided not only by the the fundamental questions that the study should be addressing, that will influence the type and timepoints of necessary samples, but the disease and the type and number samples that can be obtained.

Analyses routes

Below is a suggestion of possible analyses. We recommend in priority histology, RNAseq flow cytometry, TMB and TCR analyses.
For tissue samples, formalin-fixed paraffin-embedded (FFPE) tissue samples will allow stable and durable conservation of the specimens and later analyses such as, H&E, multispectral IHC or RNAseq, and should be prioritized.
Decisions on how to analyze the samples should also be based on the number/frequency of samples available. Flow cytometry and FACS are not infallible and the lost of a sample from a small collection could be critical.
As mentioned above, all the assays described below are not necessary for every trial, and analyses should be designed to answer specific hypothesis driven questions, with samples

stored for later analysis. The amount and type of tissue available will also determine the number of possible assays.
These guidelines are meant to be completed and updated regularly. Other assays that those recommended, such as microarrays for transcriptome analysis, mass cytometry, or mass-based immunolabelling of tissues, and more complete analyses proteins such as metabolomics and proteomics can also be considered.

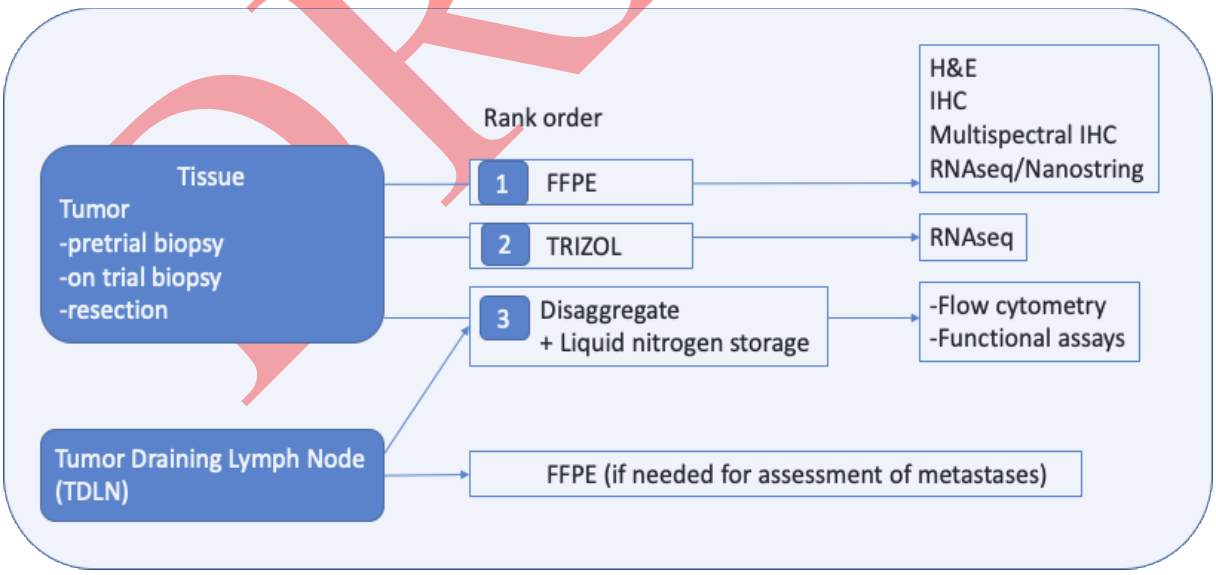
Recommended assays for tumor biopsy or resection:

1. Histology: H&E + Multi-spectral IHC [1] (if not possible IHC, fluorescence staining of frozen tissues is another alternative)
2. RNAseq or Nanostring
3. Flow cytometry analysis
4. TCR sequencing and analysis of TCR clonality
5. DNA (WES or other) analysis of tumor and blood samples

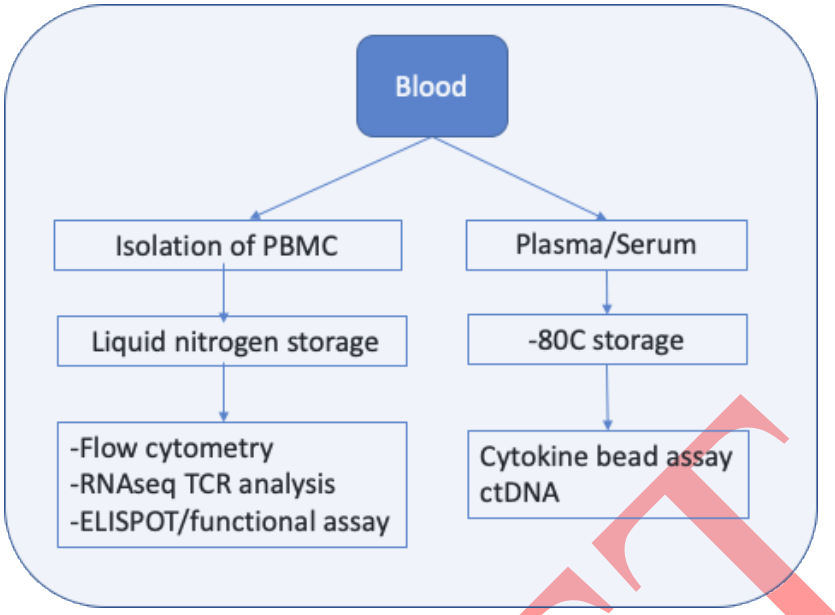
Recommended assays for blood samples

1. Flow cytometry analysis ()
2. Protein assessment of serum sample
3. TCR sequencing and analysis of TCR clonality
4. if Tumor Ag are known, PBMC can be assessed for reactivity by ELISPOT
5. Circulating tumor DNA on blood sample
6. Circulating tumor cells on blood sample

The flow charts below illustrate possible paths for tissues and blood samples processing and analyses



[1] UVa had a core facility that can be available for service for multi-spectral IHC: the Molecular, Immunologic & Translational Sciences (MITS) Core laboratory. Please contact the FUS Foundation for more information.


Blood
Isolation of PBMC
Plasma/Serum
Liquid nitrogen storage
-80C storage
-Flow cytometry
-RNAseq TCR analysis
-ELISPOT/functional assay
Cytokine bead assay
ctDNA

Analysis Recommendations

Analysis of tissue samples

Tumor biopsies (incisional or core needle) should be collected with as many samples as possible at each time point with at least 3-5 biopsies being highly desired. The first 2 biopsy samples should be processed to FFPE and then collect biopsy 3 and greater if desired. Additional tissues should be stored in TRIzol to best preserve tissue quality for for RNA sequencing, but flash freezing is also a viable option.
Otherwise, samples should be preserved in liquid nitrogen (-80°C otherwise) for future interrogation potentially after disaggregation if phenotypic analyses of tissues immune cells using flow cytometry or if functional assays are envisioned (see Appendix 1 for a protocol to isolate and bank viable cell from tissue).

Staining (tumors, in order of priority)

We recommend when possible, multispectral IHC, that can typically allow 7/8 markers simultaneously on a single tissue slide. If not possible, IHC will allow for typically 1-2 markers, as long as they are staining different cells. A pan-cytokeratin marker can also be used to assess tumor location.
Involvement of pathologist from the start of the project will be crucial to ensure rigorous assessments of images.
We recommend:

1. Multispectral IHC (or immunofluorescence on frozen sections, this can provide a lot of info and preserves material with defined T cell/myeloid cell panels) for CD4+ T cells, CD8+ T cells, FOXP3+ T regs, monocyte/macrophage CD68 (CD163 more specific), MHC-I, PDL-1. => their frequency and localization. IHC for H&E. If not using multispectral IHC to combine staining of different cells on the same slide, this will require at least 6 slides for IHC.

If enough material is available,

2. IHC (or immunofluorescence on frozen sections) for T cell activation/phenotypic markers (granzyme B, Ki67), B-cells (CD20) cells => their frequency and localization
3. Staining for necrosis and apoptosis (caspase or TUNEL assay).
4. Staining for DAMPs: HSP (especially if using thermal FUS), calreticulin (translocation to the cellular membrane)
5. Expression for PD1 and PD-L1
6. Tumor type specific markers (e.g. HER2)

Tumor RNAseq

- It is important to stress that RNAseq analyses require intensive quality control procedures to be followed, and that samples should be aggregated in a sequencing run
- When possible RNASeq is recommended as having the broadest evaluation of gene expression. If tumor tissue sample is limiting and/or RNA quality is in question then Nanostring IO360 panel assessment of immune gene signatures is recommended.

- The Foundation does not currently provide access to bioinformatic support so that RNAseq data is analyzed in compatible ways, but this could be envisioned if users are interested

Tumor Genetics including TMB analysis

- DNA sequencing analysis using whole exome or genome sequencing to assess TMB, tumor mutations (eg MHC pathway) and TCR clonality and diversity is recommended.
- Where possible, comparing TCR in tumor and blood pre and post treatment would be a good addition. There seems to be a greater similarity after successful treatment in preclinical models (mice).
- Commercial solutions are available for TCR sequencing and analysis (example Adaptive in Seattle)

Phenotypic analysis of leukocytes

Panels for phenotypic characterization of leukocytes can vary from sites to sites. See the Appendix for panel recommendations. Markers to be used will be guided by the questions at stake and expected effects of FUS. For example, is FUS associated with: DC presence and activation status? T-cells differentiation, proliferation? T-cells function, activation or exhaustion? Changes in myeloid cells population (neutrophils, , granulocytes…)? Increase in the TCR repertoire?

We recommend to study at least these populations:

1. Effector/ suppressor cells including CD4+ and CD8+ Tcells, NK cells
2. APC (antigen presenting cells) including DCs, macrophages (M1 and M2)
3. Suppressive immune cells: monocytes and granulocytes (and MDSC variants thereof), M2 macrophages; FoxP3+ Treg CD4+ T cells
4. B-cells
5. Immune phenotype, activation: T cell subsets: naive, central memory and effector memory; CD44; CD62L; CD38, T-bet, Tox, TCF7
6. gamma delta T cells
7. Expression of Immune checkpoint receptors and ligands: e.g. PD-1, PD-L1; Tim3, Gal9, Lag3, MHC class II, CD39.

These staining should be combined with viability stain to exclude dead cells. Ki67 should also be stained to assess proliferation.

Functional assays

It will be important to identify relevant time points to perform these analyses. It might be expected some acute cytokine production in response to FUS, but also some durable changes due to tumor ablation, and these two categories would likely have different cytokines.

There are several types of functional assays, we propose below recommendations for analysis of cytokines production after stimulation of isolated immune cells. Analysis of cells activation status by flow cytometry is also possible. Cytokines panels will be different for different cell types, such as DC and T cells. What cytokines to be analyzed will also be dictated by the hypothesis to be tested. We are suggesting below a few cytokines' panels:

- Cytokines panels:

    - Recommendations for commercial panels such as:
        - For Luminex Luminex technology (example for mouse): https://www.thermofisher.com/order/catalog/product/EPX260-26088-901?SID=srch-srp-EPX260-26088-901
        - For MSD technology (example): https://www.thermofisher.com/order/catalog/product/LHC6005M#/LHC6005M
    - Or specific list of cytokines to be assessed, for example:
        - T cell panel cytokines: IFN-g, TNF-a, IL-2, IL-4, IL-6, IL-17A, IL-10
        - T cell panel cytolytic proteins: Granzyme A and B, Perforin, sFasL
        - T cell chemokines to span IF-stimulated genes, (e.g. Cxcl9),
        - T cell related adhesion proteins, CD84, Adgre1.
- DAMPS, such as HMGB1

Analysis of blood samples

Samples should be preserved at -80°C for future interrogation (see Appendix for a protocol to process and bank blood samples for PBMC and plasma).

PBMC will be characterized by phenotyping (flow cytometry) and functional assay. Cytokines in plasma will be quantified using technologies such as Luminex or MSD.
In a second level of analysis, proteomics, exosomes, circulating DNA can be assessed, if such information are relevant for the research question.

Phenotypic analysis of leukocytes

Refer to the phenotypic analysis of leukocytes section in the tissue sample analysis section for recommendations on populations to be analyzed.

Functional assays

It will be important to identify relevant time points to perform these analyses. It might be expected some acute cytokine production in response to FUS, but also some durable changes due to tumor ablation, and these two categories would likely have different cytokines.
There are several types of functional assays, we propose below recommendations for analysis of cytokines production after stimulation of PBMC. Analysis of cells activation status by flow cytometry is also possible. Cytokines panels will be different for different cell types, such as DC and T cells. What cytokines to be analyzed will also be dictated by the hypothesis to be tested. We are suggesting below a few cytokines panels:

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        - For MSD technology (example): https://www.thermofisher.com/order/catalog/product/LHC6005M#/LHC6005M

    - Or specific list of cytokines to be assessed, for example:
        - T cell panel cytokines: IFN-g, TNF-a, IL-2, IL-4, IL-6, IL-17A, IL-10
        - T cell panel cytolytic proteins: Granzyme A and B, Perforin, sFasL
        - T cell chemokines to span IF-stimulated genes, (e.g. Cxcl9),
        - T cell related adhesion proteins, CD84, Adgre1.
- DAMPS, such as HMGB1

Circulating tumor cells

- CellSearch is recommended for tumors that have well been characterized to have a high degree of circulating tumor cells such as breast and prostate cancer.

Tumor DNA present in plasma samples

- This is an emerging space and depending on the tumor type and analysis should be evaluated at the time for inclusion.
- Tumor types with high TMB or well characterized tumor mutations may consider including a BCT-Streck tube collection for isolating and banking the plasma for cfDNA/ctDNA analysis.
- Technology is still in development and we suggest following recommendation by the BloodPAC consortium that is developing standardization and published Minimum Technical Data Element (MTDE) for ecommendations for required preanalytic attributes and detailed workflow[2]

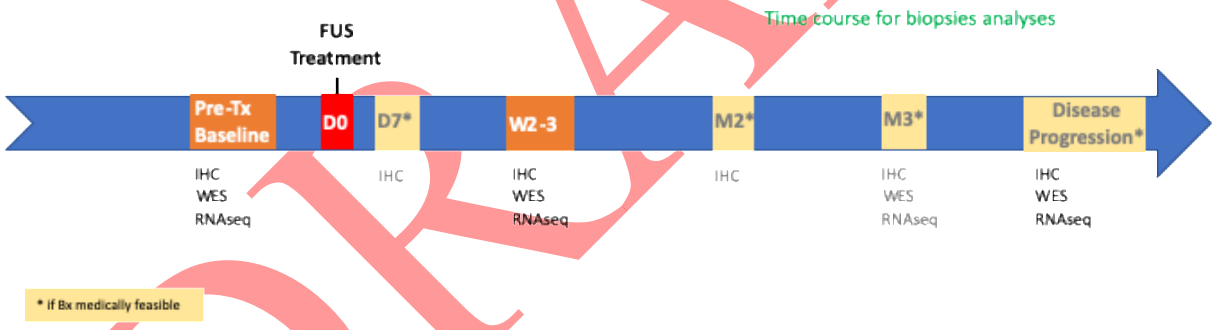
2 https://www.bloodpac.org/new-page

Timepoints for Clinical studies

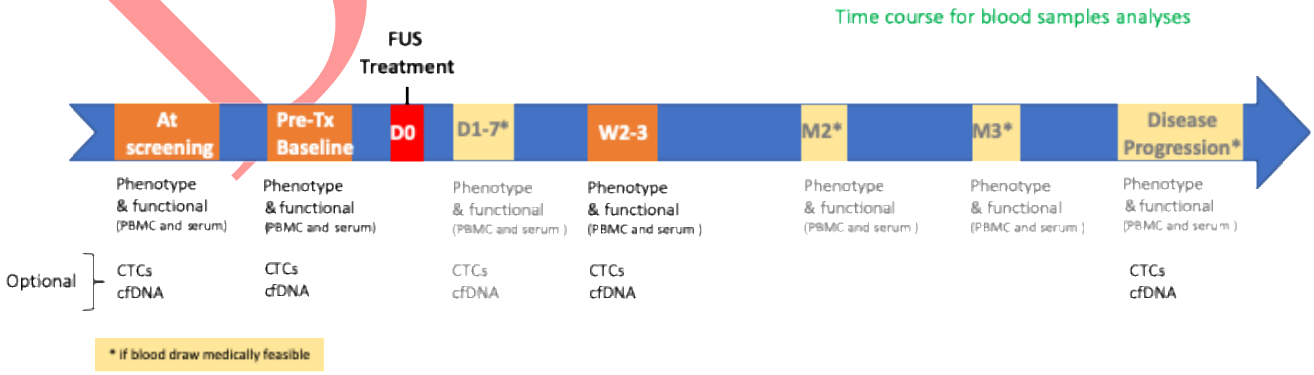
These time points are recommendations only and should not limit the analyses. Blood draws and biopsy timing should be informed by drug/FUS dosing/treatment schedule and pharmacokinetics.
A few points can be considered to determine an optimized schedule:

- Pre-treatment sample as a requirement for baseline. Archival tissue may be used if obtained proximal to time of treatment, typically within 6 weeks of treatment initiation, and without intervening therapies.
- If the treatment consists in a combination of FUS and IO, it may be relevant to piggyback on infusion, with blood samples just before IO infusion sessions.
- The number of IO infusions depend on the drug.
- The response to IO is most often seen between the 1st and 3rd dose
- Time points should be driven by drug PK: typically max after 3-4 doses
- After beginning of a response to IO or after max PK, it is possible to schedule longer intervals between blood draws
- An acute 24h timepoint for blood sample analyses can be helpful for gauging the inflammatory response to FUS

Recommendations for tissue biopsies analyses



Recommendations for blood samples analyses



Supporting information

The supporting information provide bases for the proposed recommendations.

Overview

Certain tumors are primed and ready to respond to treatment with an immunotherapy, and others are not. Cancer patients who benefit from checkpoint inhibitor immunotherapy are characterized as having "hot" tumors, whereas those who do not respond to treatment are characterized as having "cold" tumors. Several biomarkers have been proposed to classify a tumor as "hot" versus "cold," including programmed cell death ligand-1 (PD-L1) expression, tumor mutation burden (Rizvi 2015), interferon-gamma expression (Ayers 2017), tumor immune signature (Chen 2016), fecal microbiome profile (Gopalakrishnan 2018), and the extent of the CD8 T-cell infiltrate (Hegde et al, 2016). These biomarkers have an association with the CD8 T-cell infiltrate (Maby 2015; Thompson 2017; Roy and Trinchieri 2017). Several clinical studies have shown that clinical response to anti-PD-1 treatment is correlated with the pre-treatment number or the percentage of intra-tumoral CD8 cells (Tumeh 2014; Chen 2016; Johnson 2016). Therefore, larger numbers of intra-tumoral CD8 cells may be a useful biomarker to identify "hot" (CD8 high) tumors.

Analysis of tumor samples

Tumor biopsies (incisional or core needle) should be collected with as many samples as possible at each time point with at least 3-5 biopsies being highly desired. The collection procedure is described for formalin fixed paraffin embedded (FFPE) as that gives the ability to perform IHC and nucleic acid analyses. FFPE tissue prevents the ability to evaluate tumor immune infiltrate by flow cytometry or other methods or any opportunities for emerging methods such as single cell analysis. It can be determined to collect the first 2 biopsy samples and process as FFPE and then collect biopsy 3 and greater as flash frozen if desired. Frozen tissue does preserve RNA quality better and can lead to better results for RNA sequencing as compared to FFPE. If sufficiently large amount of tissues can be available, it can also be determined to divide into FFPE, flash frozen (or TRIzol) for RNAseq, and disaggregated cells for future flow/functional analysis.

Immunohistochemistry (IHC) of tumor samples

The first step in analyzing tumor samples will be to assess the quality of the sample using H&E. After confirming sample quality, the next step is staining or IHC of a limited subset of cells to provide general information on the immune infiltrate.

There is no consensus on the identity of biomarkers to predict responses. For colorectal cancer for example, an immunoscore based on the densities of CD3, CD8, CD45RO, CD20, and FOXP3 T cells at the center and the invasive margin of metastases have been reported to be a reliable estimate of the risk of recurrence in patients with colon cancer (Mlecnik 2017*)*. For patients treated with immunotherapies, immunoscore still needs to be defined, although sub-classification of tumor types may be predictive of the response (Smyth 2016). Evaluating the

presence of cytotoxic CD8 T cells in the tumor is a critical parameter and at a minimum characterizing the level of CD8 cell infiltrate by IHC before and after treatment is important.

Thermal treatments result in a central zone that undergoes coagulative necrosis, surrounded by a transitional zone with possible invasion of inflammatory cells and T lymphocytes. The ratio of cytotoxic T cells (mostly CD8+) to regulatory T cells (FOXP3+) could also indicate the local tonus.
It has been reported that in the peripheral zone, the presence of DC and the balance between necrosis and apoptosis, the latter being considered a more immunogenic cell death, are likely to dictate the extent of the antitumor immune response (Chu 2014).
Adjuvanticity, the presence of danger signals that activate the effector cells of the innate system, is an important component of the response, as apoptosis or antigen release without exposure of DAMPs (danger-associated molecular patterns) may lead to tolerogenic effect.

RNA analysis of tumor samples

The expression of mutated or ectopic proteins and peptides specific to malignant tissues can be indicative of anti-tumor immunity and can be assessed by RNAseq. RNAseq analysis, together with CIBERSORT, GSEA or other algorithms can provide guidance for further more specific staining or IHC. Analysis pathways should include inflammatory genes, tumor immune signatures (e.g. IFN), immune checkpoint genes, and oncogenes. If tumor sample or quality is limiting an alternative analysis is to utilize the Nanostring IO360 immune profiling panel.

DNA analysis of tumor samples and peripheral blood mononuclear cells (normal tissue)

The antigenicity of the tumors, the presence of antigenic determinants absent in non-malignant cells and tissues, can be indicative of anti-tumor immunity. DNA sequencing using whole exome, whole genome or targeted panels of tumor samples and PBMC to assess normal tissue can be used to evaluate tumor mutational burden, tumor genetics such as mutation or loss of MHC pathway genes, for T cells receptor (TCR) TCR repertoire assessment and other genetic alterations.
Assessing tumor mutational burden (TMB) as a biomarker for response to immunotherapy is predicated on the concept that more mutations yield more T cell-recognized tumor neoantigens, and is supported by clinical evidence (Buttner 2019). Several next-generation sequencing (NGS) approaches exist to assess TMB, with target region ranging from genome-wide analysis (whole genome sequencing (WGS)) to whole exome sequencing (WES, covering the entire coding regions of genes in the genome) and large targeted gene panels. WES is considered the gold standard for measuring TMB because it offers high breadth of coverage compared with gene panels. Additionally, assessment TCR clonality and diversity before and after treatment has been shown to be a parameter that is associated with immune responsiveness (Yost 2019).

Analysis of blood samples

Blood should be separated into PBMC and serum (the latter for cytokine analysis and potentially circulating DNA/exosomes).

Circulating immune cells by flow cytometry

A baseline assessment, following isolation of mononuclear cells from peripheral blood collected at selected time points during therapy (or from tumor biopsies after dissociation) should include analysis of circulating T cells and antigen presenting cells, combined with viability stain to exclude dead cells. The use of multiparameter immune panels analyzing peripheral blood mononuclear cells (PBMCs) has been reported using such methods as mass cytometry (Cytof). Immune cell populations that have been associated with immunotherapy response include T cell subsets for both CD8 and CD4 cells (activation and exhaustion makers), gamma delta T cells, B cells and monocytic cell types such as myeloid derived suppressor cells (MDSCs) (Hartmann 2019).

The activation status of the PBMCs should also be analyzed after stimulation of PBMCs with selected compounds, including PAMPS such as pIC, cytokines such as IFN-1 or specific activation of T-cells with a CD3+CD28, analysis of cytokines production in the supernatant (different technology available such as Luminex or MSD assays), or analysis of cells activation status by flow cytometry. Cytokines panels will be different for different cell types, DC and T cells.

Serum samples

A baseline serum sample at screening and pre-infusion on the day of treatment should be collected to ensure sufficient pre-treatment samples for analysis. On treatment serum samples should be collected to assess the effects of treatment and can be used to assess circulating cytokines, proteins and antibodies. Serum should be collected and processed as quickly as possible and within an hour or two of collection. Serum should be aliquoted into cryovials and frozen at -70oC or colder when possible.

RNAseq of PBMC

There's a strong argument to be made for RNAseq on PBMC for TCR analysis to help understand whether you have clonal expansion.

Circulating tumor cells

Circulating tumor cells (CTCs) should be analyzed to ensure that the treatment has no adverse effect in tumor types reported to have a high degree of CTCs (breast, prostate and colorectal cancer). Different technologies are available (Banko 2019), based on immunocapture. Cellsearch is FDA approved for advanced breast, prostate, and colorectal cancers using EpCAM expression for cell selection. Other techniques based on size or density separation are also available but lack clinical validation.

Tumor DNA present in plasma samples

Isolation of cell-free DNA (cfDNA) from liquid biopsies for assessment of circulating tumor DNA (ctDNA) is attractive because of the less invasive nature of sample acquisition and greater opportunity for obtaining frequent samples. However, variable data exist for concordance between solid (tumor) and liquid (plasma) samples at the clinical level, with studies demonstrating that the sensitivity of detecting mutations from cfDNA is decreased compared with solid tumor samples for both WES and gene

panels. The correlation between TMB assessed from tissue and circulating free DNA is greater for WES than for panel. There remains a need for clinical validation studies, but the TMB assessment and/or other tumor mutation assessment from liquid biopsies is feasible and may serve as biomarker for baseline characteristics and/or a surrogate marker of antitumor activity.

Timepoints for Clinical studies

The ability to get the on-trial biopsy is going to be dependent upon 1) physician concerns 2) disease type (e.g. melanoma vs GBM) 3) patient consent and medical feasibility.

For clinical studies, medical feasibility will often limit the number of biopsies. We suggest a minimum of two biopsies, baseline and after treatment, and when possible add in acute and at progression.

For tumor samples, timepoints should include

- Pre-treatment sample as a requirement for baseline. Archival tissue may be used if obtained proximal to time of treatment and without intervening therapies.
- If medically feasible, acute time point, 1-7 days post-treatment to assess the short-term (inflammatory) response; 72 hours has been interesting in preclinical studies
- The majority of clinical studies with radiotherapies have assessed the on-treatment effects of treatment after 2 cycles of study intervention (Cycle 2) and generally aim to obtain the biopsy between day 2 and 10. With FUS as a single treatment, we recommend a 2-3 weeks timepoints, that may have to be adjusted.
- A monthly sample would then be ideal to monitor the evolution of the response. If not possible, a sample within a few months (3-4 months) after the treatment, especially to assess changes in disease that lead to disease advancement.

Generally, when possible efforts should be taken to obtain the baseline and on treatment biopsies from the same lesion and from a lesion that has not been treated with FUS.

Similarly, for blood sample, timepoints should include:

- Pre-treatment sample as a requirement for baseline. It is advisable to obtain a sample at screening and before treatment on the first day drug administration (Cycle 1, day 1 pre-infusion) or FUS treatment. This ensures that if a sample is missed or compromised in processing that a baseline sample exists to evaluate.
- Acute time point, day 1 if possible post-treatment to assess the short-term (inflammatory) response; 72 hours has been interesting in preclinical studies
- A 2-3 weeks sample for when an expanding immune response can be expected
- Weekly/monthly samples depending on the protocol to monitor the evolution of the response. If not possible, a sample within a few months (3-4 months) after the treatment
- A blood sample at the time of disease progression is also of interest.

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Appendix 1 –Viable cell isolation from tissue

Equipment/Supplies

1. 5mL and 10mL sterile pipettes, individually wrapped
2. 50mL polypropylene tubes (sterile)
3. 1.8mL cryovials
4. 100 micron cell strainer
5. Controlled rate freezing containers (Mr.Frosty) with isopropanol solution
6. -80°c Freezer
7. Hemocytometer
8. Rotating shaker
9. Centrifuge with swinging bucket rotor
10. Scalpel #10
11. Petri dish sterile

Reagents

1. RPMI media
2. Fetal Bovine Serum (Gibco)
3. Dulbecco's Phosphate-Buffered Saline
4. DMSO (Cat# D2650, Sigma)
5. Trypan Blue
6. Enzyme digestion mixture(kept at -20C):

RPMI media containing 1mg /ml Collagenase type 4 (Worthington Corporation). 10ug/mL DNAse I (Worthington Corporation) and 2.5U/mL Hyaluronidase (Worthington Corporation)

7. 100x Antibiotic and Antimycotic (Invitrogen)

Tissue collection

a. Transport a tube of cold RPMI on wet ice to the collection site (antibiotic and Antimycotic can be added if needed)
b. Place tissues into the RPMI media and transport directly to the laboratory on ice.

Tissue Processing

a. Chop tissue into small pieces using a sterile scalpel in RPMI medium in a petri dish
b. Collect single cells by passing the cell suspension through a 100 micron cell strainer in 50 ml conical tube
c. Add 20 mL of 10 % FBS/PBS into the cell strainer. *Remove any tissue sample that did not pass through the cell strainer into a new 50mL tube.*
d. Pellet the pass-through suspension at 300xg for 10 minutes.
e. Remove supernatant, and gently flick the tube to resuspend cell pellet in 10% FBS/PBS 10 ml.
f. Take a small aliquot~50-100 ul for cell counting and determine number of cryovials needed (5-10 millions/cryovial). During counting, pellet the cells at 300xg for 10 minutes.
g. Ensure that the cryovials are labeled with the Patient ID (PTID), time point, type of specimen, amount of cells and "Non-digest material"
h. Divide number of cryovials by 2 and use this number for amount of FBS (ml) to resuspend the cell pellet

i. Obtain the same volume (step h) of 20% DMSO in FBS and add the solution slowly (while gently swirling the tube) into the cell suspension
j. Aliquot 1ml of the suspension into each cryovial and place the cryovials in Mr. Frosty and leave it in -80°c for overnight or for a minimum of 12 hours and a maximum of 24 hours.
k. Store the cryovials in -80°c until shipment (within 2 weeks) or transfer to liquid nitrogen storage for long term storage.
l. Add 20 mL or a 10X volume of enzyme digestion mixture to tissue sample that did not pass through the cell strainer (step c).
m. Add FBS and antibiotic and antimycotic solution into the cell suspension to the final concentration of 0.1% and 0.01%, respectively.
n. Place the tube in a rotating shaker at room temperature overnight.
o. Isolate viable cells again by repeating step b – k.
p. label samples with “digest day 1” time point.
q. If tissue is still visible in the cell strainer, transfer the samples to new tube and repeat the cell isolation again for another day and label samples with “digest day 2”.

Appendix 2 - Tumor biopsy processing (FFPE)

The collection technique of the fresh tumor biopsy samples will be dependent on the site-preferred technique to obtain incisional or punch biopsy tumor samples to provide sufficient tissue for at least 3 – 5 samples (preferred 16 gauge core size and tissue sample in the range of 3 to 5 mm). Fine needle aspirates are NOT acceptable.

- In order to keep time from tissue acquisition to fixation as short as possible, these samples should be placed in 10% neutral buffered formalin (NBF) within 15 minutes of the procedure. Make sure to document the fixation time.
- The samples should be kept in 10% NBF for at least 6 hours and no longer than 72 hours.
- Following formalin fixation, tissue samples should be paraffin embedded according to established in house protocols.
- Paraffin embedded samples should be placed into cassettes and labeled.
- Tissue acquisition and processing must be documented including cold ischemic time - defined as the time between removal of the tissue until formalin fixation - and duration of formalin fixation of the samples.

Appendix 3 – Instructions for processing blood samples for PBMC and plasma: Isolation and cryopreservation

SCOPE AND APPLICATION

This procedure describes the isolation of peripheral blood lymphocytes (PBL) and plasma from blood obtained from human subjects.

DEFINITIONS

RCF – relative centrifugation unit (also known as g force) measurement of the force applied to a sample within a centrifuge. This can be calculated from the speed (RPM) and the rotational radius (cm) using the following calculation: g = RCF = 0.00001118 (r) (N2)
where g = Relative centrifuge force, r = rotational radius (centimeters, cm), N = rotating speed (revolutions per minute, r/min).

REQUIRED MATERIALS, APPARATUS, AND RESOURCES

Equipment/Supplies

1. 50mL Leucosep tubes
2. Ficoll-Paque PLUS (Amersham Biosciences, Cat. # 17-1440-03)
3. 5mL and 10mL sterile pipettes, individually wrapped
4. 50mL polypropylene tubes (sterile)
5. 15mL polypropylene tubes (sterile)
6. 1.8mL cryotube vials
7. Controlled rate freezing containers (Nalgene “Mr. Frostys”)
8. -80ºC Freezer
9. Hemocytometer for cell counting or any cell counter apparatus

Reagents

1. Fetal Bovine Serum (Gibco)
2. Dulbecco’s Phosphate-Buffered Saline
3. DMSO (Cat# D2650, endotoxin tested, Sigma)
4. Dye for cell counting

PROCEDURE

The desired quantity of peripheral blood leukocytes (PBL) is around 8 million cell per vial plus 2 vials for flow cytometry (FACS) at around 2.0-2.4 million cells per vial.

A. Preparation of Ficoll paque /leucosep tube

1) Pipette 15ml of Ficoll into each of the 50ml Leucosep tubes.
2) Centrifuge at 1000 RCF for 30s, then store in the dark at room temperature.

B. PBL isolation, aliquoting and freezing

1) Pipette whole blood onto membrane frit of the Leucosep tube. (No more than 30ml of whole blood in each Leucosep tube)

2) Centrifuge at 1000 RCF for 10 minutes, brake off
3) If possible, pipette and save up to 12 mL of plasma from the top layer. Place plasma in 15 ml conical tube and place in -80ºC.
4) Pipette away the buffy-coat PBL inter-phase layer and the overlaying plasma with a 5ml pipette. (You may have to gently scrape the side of the tube with the pipette tip to remove adherent PBL, and be careful that you do not pipette any of the underlying Ficoll.)
5) Transfer buffy-coat into 2 new 50ml tubes and fill each to 45 ml with PBS + 10% FBS.
6) Centrifuge the 50ml tubes with the buffy-coat at 250 RCF for 10 minutes
7) Carefully decant the supernatant into a container with bleach (beware of splashing bleach) and re-suspend the pellets containing the PBL in a total of 25ml of PBS in a single 50ml tube.
8) Keep a small aliquot for counting (see SOP#BTRF-7 for cell counting)
9) Centrifuge at 250 RCF for 10 minutes
10) Determine the number of cryovials needed (see below, Data analysis/ calculations)
11) Decant supernatant into bleach.
12) Count cryovials and divide the # by 2. This is the number of mL of FBS to re-suspend the cell pellet
13) Re-suspend pellet in FBS.
14) Draw 1ml of the suspension.
15) Place ¼ of above into a new 15ml tube.
16) Place ¾ back into the original 50ml tube.
17) Discard pipette.
18) Draw 1ml of FBS.
19) Place ¼ into re-suspension (original 50mL tube).
20) Place ¾ into the 15ml tube containing ¼ of cell suspension
21) Using a new pipette, obtain the same number of ml as step 12 of 20% DMSO in FBS.
22) Add drop wise (while gently swirling the tube) to the re-suspension in the 50ml tube.
23) Place 1ml of the re-suspension into each cryovial.
24) Add 1ml of 20 % DMSO/FBS into the 15ml tube containg 1 ml of cell suspension (drop wise/swirl)
25) Pipette the cell suspension to two FACS vials, 1 ml each.
26) Place the cryovials + FACS vials in a controlled rate freezing container
27) Place the freezing container in -80º C freezer for at least an overnight period (12 hours), then transfer to liquid nitrogen freezer.

DATA ANALYSIS/CALCULATIONS

A (total cells)/ 8,000,000 = B (number of vials you want)
[A / (.5 x B)] x 0.25 = C (Total number of PBL in both FACS vials)
A – C = D (number of lymphocytes left after FACS lymphocytes removed)
D / B = number of lymphocytes in each regular vial

Appendix 4 – Panels for ImmunoPhenotyping of Human Cells

Panels for phenotypic characterization of human leukocytes can vary from laboratories to laboratories. Extremely detailed guidelines have been published in Eur. J. Immunology in 2019 (Cossarizza et al., ref [7]), that contain comprehensive sections of all major immune cell types with helpful Tables detailing phenotypes in murine and human cells. These guidelines are however too detailed for the purpose of these recommendations, and we suggest as a starting point the panels proposed by The Human Immune Phenotyping Consortium (HIPC) that was developed by the Federation of Clinical Immunology Societies (FOCIS). These panels were published in 2016 (Finak et al. ref [6]). Tables below are reproduced from this publication.

These suggestions of panels will be updated regularly.

Panels for human leukocytes

	T cell	Treg	B cell	DC/mono/NK	Th1/2/17
FITC	dead	dead	dead	dead	dead
PE	CCR7 (150503)	CD25 (2A3)	CD24 (ML5)	CD56 (B159)	CXCR3 (1C6/CXCR3)
PerCP-Cy5.5	CD4 (SK3)	CD4 (SK3)	CD19 (SJ25C1)	CD123 (7G3)	CD4 (SK3)
PE-Cy7	CD45RA (L48)	CCR4 (1G1)	CD27 (M-T271)	CD11c (B-LY6)	CCR6 (11A9)
APC	CD38 (HIT2)	CD127 (HIL-7R-M21)	CD38 (HIT2)	CD16 (B73.1)	CD38 (HIT2)
APC-H7	CD8 (SK1)	CD45RO (UCHL1)	CD20 (2H7)	CD3+19+20 (SK7, SJ25C1, 2H7)	CD8 (SK1)
V450	CD3 (UCHT1)	CD3 (UCHT1)	CD3 (UCHT1)	CD14 (MPHIP9)	CD3 (UCHT1)
V500	HLA-DR (G46-6)	HLA-DR (G46-6)	IgD (IA6-2)	HLA-DR (G46-6)	HLA-DR (G46-6)

Table 1. The HIPC antibody panel, specificities and clones.

Panel	Population Name	Reliability	Corresponding Markers
T-cell	CD8 Activated	–	CD3+/CD8+/CD4− /CD38+/HLADR+
T-cell	CD4 Activated	+	CD3+/CD8−/CD4+/CD38+/HLADR+
T-cell	CD4 Central Memory	–	CD3+/CD8−/CD4+/CCR7+/CD45RA−
T-cell	CD8 Central Memory	–	CD3+/CD8+/CD4−/CCR7+/CD45RA−
T-cell	CD4 Effector	+	CD3+/CD8−/CD4+/CCR7−/CD45RA+
T-cell	CD8 Effector	+	CD3+/CD8+/CD4−/CCR7−/CD45RA+
T-cell	CD4 Effector Memory	+	CD3+/CD8−/CD4+/CCR7−/CD45RA−
T-cell	CD8 Effector Memory	–	CD3+/CD8+/CD4−/CCR7−/CD45RA−
T-cell	CD4 Naïve	+	CD3+/CD8−/CD4+/CCR7+/CD45RA+
	CD8 Naïve	+	CD3+/CD8+/CD4−/CCR7+/CD45RA+
B-cell	IgD−/CD27−	–	CD3−/CD19+/CD20+/IgD−/CD27−
B-cell	Transitional	+	CD3−/CD19+/CD20+
B-cell	Plasmablasts	–	CD3−/CD19+/CD20−/$Cd24^{high}$/$CD38^{high}$
B-cell	Naïve B	+	CD3−/CD19+/CD20+/CD27−/IgD+
B-cell	Memory IgD+	+	CD3−/CD19+/CD20+/IgD+/CD27+/IgD+
B-cell	CD19	+	CD3−/CD19+
B-cell	CD20	+	CD3−/CD20+
B-cell	Memory IgD-	+	CD3−/CD19+/CD20+/CD27+/IgD−
T-regulatory	Total T-regulatory	+	CD3+/CD4+/CD8-/LoCD127/HiCD25/CCR4+ (as % of CD4)
T-regulatory	Memory T-regulatory	+	CD3+/CD4+/CD8−/LoCD127/HiCD25/CCR4+/CD45RO+ (as % of total Treg)
T-regulatory	Naïve T-regulatory	+	CD3+/CD4+/CD8−/LoCD127/HiCD25/CCR4+/CD45RO− (as % of total Treg)
T-regulatory	CCR4-/CD45RO−	–	CD3+/CD4+/CD8−/LoCD127/HiCD25/CCR4−/CD45RO− (as % of parent)
T-regulatory	CCR4-CD45RO+	–	CD3+/CD4+/CD8−/LoCD127/HiCD25/CCR4−CD45RO+ (as % of parent)
T-regulatory	CCR4-HLADR−	+	CD3+/CD4+/CD8−/LoCD127/HiCD25/CCR4−HLADR− (as % of parent)
T-regulatory	CCR4-/HLADR+	–	CD3+/CD4+/CD8−/LoCD127/HiCD25/CCR4−/HLADR+ (as % of parent)
T-regulatory	CCR4+/CD45RO−	–	CD3+/CD4+/CD8−/LoCD127/HiCD25/CCR4+/CD45RO− (as % of parent)
T-regulatory	CCR4+/HLADR+	+	CD3+/CD4+/CD8−/LoCD127/HiCD25/CCR4+/HLADR+ (as % of parent)
T-regulatory	Total CD4	+	CD3+/CD4+/CD8− (as % of parent)
T-regulatory	LoCD127/HiCD25	+	CD3+/CD4+/CD8−/LoCD127/HiCD25 (as % of parent)
T-regulatory	Activated	+	CD3+/CD4+/CD8−/LoCD127/HiCD25/CCR4+/HLADR+ (as % of total Treg)
DC/Mono/NK	CD11c-/CD123-	–	CD11c−/CD123−
DC/Mono/NK	CD11c-/CD123+	+	CD11c−/CD123+
DC/Mono/NK	CD11c+/CD123−	+	CD11c+/CD123−
DC/Mono/NK	CD11c+/CD123+	–	CD11c+/CD123+
DC/Mono/NK	CD14+/CD16+	–	CD14+/CD16+
DC/Mono/NK	CD16-/CD56+	+	CD16−/CD56+
DC/Mono/NK	CD16+/CD56-	–	CD16+/CD56−
DC/Mono/NK	CD16+/CD56+	+	CD16+/CD56+
DC/Mono/NK	HLADR+	–	HLADR+
DC/Mono/NK	Lin-CD14−	+	Lin-CD14−
DC/Mono/NK	Lin−/CD14+	+	Lin−/CD14+
DC/Mono/NK	CD16−/CD56−	–	CD16−/CD56−

Table 2. Cell populations evaluated by the HIPC panels. evaluated in the study, showing their common names and phenotypes (live, lymphocye, and singlet gates are not listed). Cell populations which could be reliably detected by automated gating in a panel are marked with a "+" in the "reliable" column, while those that were unreliable are marked with a "−". We did not evalute the Th1/Th2/Th17 panel as it was determined early on in preliminary analysis that the panel was too variable to be reliable.

In addition, we suggest thee following panels to identify myeloid-derived suppressor cells (MDSC), based on a 2019 publication (Cassetta et al, ref [8]):

- Human polymorphonuclear MDSC (PMN-MDSC): $CD66b^{+}CD15^{+}CD14^{-/dim}CD33^{dim}HLA\text{-}DR^{-}$
- Human mono-cytic MDSC (M-MDSC) : human: $CD11b^{+}HLA\text{-}DR^{-}CD14^{+}CD15^{-}$

Appendix 5 – Technologies available for assessments

This appendix, describing some of techniques for biological assessments, is not exhaustive and currently restricted to some of the main technologies described in the guidelines. This will be regularly completed.

Technology	Description and Outputs	Commment
Flow cytometry	Optical technique that classifies cells on the basis of properties such as size, granularity and the presence of signature proteins labelled with fluorescent antibodies. 'Flow' refers to the mechanics of the technique: cells flow in single-file past a series of lasers and detectors, which read them as they pass.	Pro : fast, it can sort more than 10,000 cells per second Con : visible-light spectrum limits most experiments to no more than a dozen or so protein markers
Multispectral IHC	Similar to immunohistochemistry (IHC), where labelled antibody are used to identify antigens by selective binding, multispectral IHC uses specific imaging systems to with multispectral unmixing strategies to allow for simultaneous staining with up to 7/8 markers. These multidimensional data allow for investigation related to tissue architecture, spatial distribution of multiple cell phenotypes, and co-expression of signaling and cell cycle marker.	Pro: Compared to conventional IHC, allow for simcal capturing of more markers Staining can be performed on FFPE tumor tissues
Mass cytometry (often called CyTOF)	Blends flow cytometry with mass spectrometry, using metal-conjugated antibodies to boost the number of detectable markers to 50 or so. This technology is particularly good for exploration. Once mass cytometry reveals which cells and molecules to focus on, a flow cytometer can often prove useful for follow-up analysis	Pro : Lots of simultaneous marker Con: Less rapid than flow cytometry, 1000 cells/s Cells are destroyed during the process Use many more antibodies per sample, Cost
RNA-seq	Conventional 'bulk' methods of RNA sequencing (RNA-seq) that process hundreds of thousands of cells at a time and average out the differences Bulk analyses typically investigate how gene expression differs between two or more treatment conditions.	Pipelines exist (algorithms work best for each step and how they should be run) Complex analyses, Need support of bioinformatician

single-cell RNA sequencing (scRNA-seq).	Single cells techiques are often aiming to identify new cell types or states or reconstruct developmental cellular pathways. With the single single-cell RNA sequencing (scRNA-seq), the outputs can be detection of subpopulations, identification of clusters of similar cells (uisng dimensionality reduction), spatial subpopulation positioning, pseudo-time analysis (infer sequence of gene-expression changes that accompany cellular differentiation	Complex process of scRNA-seq data analysis Need support of bioinformatician (computational biologists) Appropriate methods can be “very data-set dependent” Con : Cost