FOCUSED ULTRASOUND FOUNDATION

Guidelines for immune analysis following FUS treatment

Pre-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. diagnostic 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 run a few very pointed assays/analyses first, before storing remaining samples for later analysis.

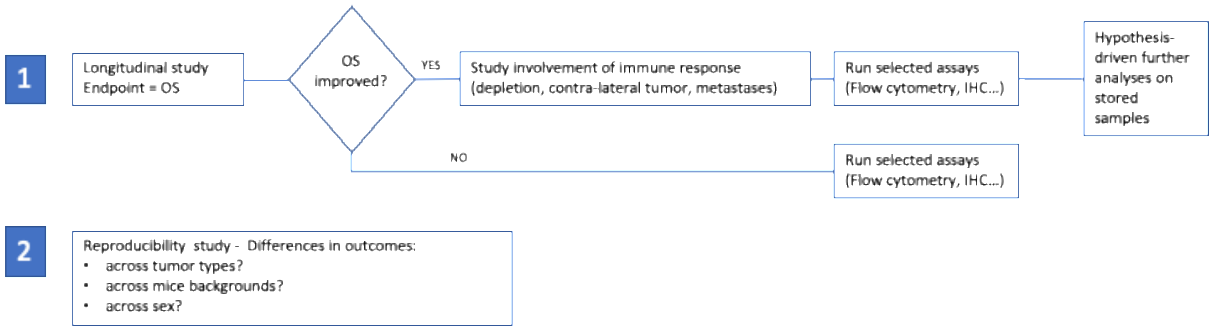
General Recommendations

General considerations

1. A clear explanation of statistical methods and of power analysis must be given to ensure groups are correctly sized.
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 experiment 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, clinical data, etc.
4. Preclinical experiments should be hypothesis driven to drastically reduce analysis needed.
5. Flow cytometry is imperative for analyses of immune cells. If no facilities/equipment is 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 (cf point 3)
6. Rigorous investigation of the reproducibility of the results should include attempts to understand the difference in responses that can originate from three important factors
    a. different tumor types for the disease
    b. different mice backgrounds
    c. when relevant, influence of the sex of the animals.
7. Imaging, which is outside of the scope of these recommendations (Ultrasound, fluorescence, bioluminescence, MRI, CT or other), should be included in the experiment to assess tumor progression/regression/recurrence
8. It is imperative to document FUS treatment parameters, see FUSF Guidelines on Treatment Reporting

Suggestion of Experimental design

1. Perform study with overall survival (OS) as primary endpoint.
2. If OS is improved, validate the involvement of the immune system in the response through the assessment of a systemic response (untreated contralateral tumor and/or metastatic spreading) and /or depletion study (CD8 or CD4 depletion or RAG-KO mice)
3. Run selected assays, typically assessment of CD8/CD4/Treg/TCR clonality
4. Perform hypothesis-driven further analyses on stored samples
5. To rigorously assess the reproducibility of the results, document the difference in responses across different tumor types and/or mice backgrounds for the disease, and when possible and relevant, study the influence of sex on the results. Age may also be a factor of influence and should at minima be reported.



Possible analysis assays

Below is a suggestion of possible analyses. We recommend in priority flow cytometry and multispectral IHC. TCR and RNAsq analyses are also highly suggested, although more complex and costly. See Appendix 6 for description of some of these technologies.

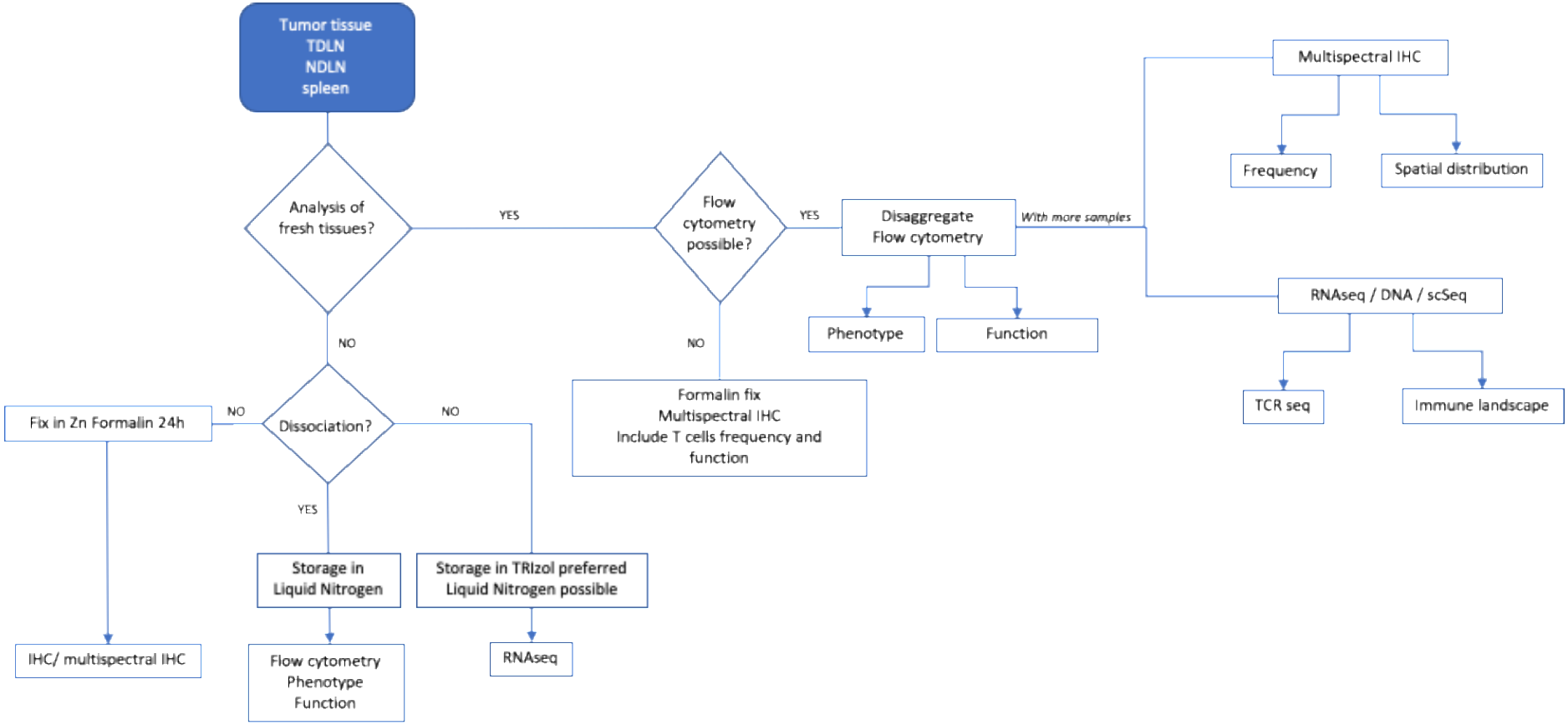
As mentioned above, all the assays described below are not necessary for every project, and analyses should be designed to answer specific hypothesis driven questions, with samples stored for later analysis. The amount of tissue available will also determine the number of possible assays.

1. Flow cytometry analysis of tissue and blood samples
2. H&E + multi-spectral IHC[1] of tumor tissue (if not possible IHC, fluorescence staining of frozen tissues is another alternative)
3. RNA seq of tumor sample
4. TCR sequencing and analysis of TCR clonality
5. Protein dynamic assessment of serum sample
6. DNA (WES or other) analysis of tumor samples (if the model is not documented)

We also discuss in these guidelines two other assays, although they are more relevant for clinical studies and less validated on preclinical models:

7. Circulating tumor cells on blood sample
8. Circulating tumor DNA on blood samples

[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.


Tumor tissue
TDLN
NDLN
spleen
Analysis of fresh tissues?
YES
Flow cytometry possible?
YES
Disaggregate Flow cytometry
With more samples
Multispectral IHC
Frequency
Spatial distribution
Phenotype
Function
RNAseq / DNA / scSeq
TCR seq
Immune landscape
NO
Formalin fix
Multispectral IHC
Include T cells frequency and function
NO
Dissociation?
NO
Fix in Zn Formalin 24h
NO
YES
Storage in Liquid Nitrogen
Storage in TRIzol preferred Liquid Nitrogen possible
IHC/ multispectral IHC
Flow cytometry
Phenotype
Function
RNAseq

Analysis Recommendations

Experimental design

Experimental design starts with the selection of an appropriate tumor model, see Appendix 5 for considerations.

It is extremely important to plan in advance the type of analyses that will be performed, as they will determine the amount of required tissue.

It will be difficult to perform flow cytometry and RNAseq on the same tumors for various reasons. The amount of available tissues may not be sufficient. Different tumor types will have different resistance to dissection. Melanoma tumors for example contain few collagen whereas 4T1 breast cancer tumor are much more solid. If there is a poor lymphocyte infiltration, all tumor tissue will have to be used for flow cytometry to produce enough cells to be analyzed. Tumors are sometimes ulcerated, making them less amenable to be cut in pieces.

A pan analysis encompassing flow analysis, FFPE and RNAseq will therefore typically require three different sets of animals.

We recommend to designing experiments for specific assays.

Analysis of tissue samples

Tissue storage

Priority should be given on the analysis of fresh tissue samples, especially for flow cytometry, RNAseq and FFPE (see Appendix 1 for FFPE protocol). Flow cytometry, functional assays and RNAseq can be performed on stored tissue sample, but cell viability, and tissue quality may be degraded when thawing. Mice tissues can be more sensitive to manipulations than human tissues.

For storage, samples should be preserved in liquid nitrogen for future interrogation. When planning on future analysis by flow cytometry or functional assays, tissue dissociation before freezing is recommended (see Appendix 3 for a protocol to isolate and bank viable cell from tissue).

Phenotypic analysis of leukocytes

Analyses of tissues immune cells using flow cytometry analysis is a gold-standard for preclinical studies. Multispectral IHC (or IHC if multispectral is not available) should be used in addition to assess cells distributions within the tumors, but flow cytometry is a more desirable primary endpoint. Tissues to be analyzed include tumor, spleen, draining lymph nodes and non-draining lymph nodes.

Panels for phenotypic characterization of leukocytes can vary from sites to sites. See the Appendix 4 for panel suggestions.
The selection of panels to assess specific subsets of immune cells among myeloid and T-cells will be also dictated by the hypothesis to be tested, such as - is an activation of Tcells or DC is expected, or an increase in the TCR repertoire?

For myeloid cells for example, DC can be interrogated for their frequency and maturation, other subsets can be interrogated for inhibitory checkpoints expression. T cells can be interrogated for their function, proliferation and cytokine expression.

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 for proliferation.

Functional assays

There are different forms of functional assays to be performed on immune cells isolated from the tumoral tissue (gold standard), and we also recommend analyzing the draining and non-draining lymph nodes.

In vitro T lymphocytes and NK cells stimulation can be achieved through:

- If the dominant tumoral antigen (Ag) is known (or when using modified cell line expressing a pseudo tumor antigen such as ovalbumin), T-cells can be activated by culturing with antigen presenting cells that express or have been pulsed with this antigen or an irrelevant peptide (for control)
- Co-culture of T-cells with tumors cells that express the antigen of interest (and antigen-negative control cells)
- Stimulation of T-cells by anti-CD3, such as anti-CD3 monoclonal antibodies or CD3 CD28 stimulation beads. This can be done in combination with MHC-multimer staining to identify the antigen-specific population within those that have been stimulated. Pay careful attention to staining order according to the directions provided by the manufacturer of MHC-multimers.
- Stimulation by stimulatory agents such as PMA-IO cocktail (Phorbol Myristate Acetate/Ionomycin), again in combination with MHC-multimers.

- NK cells function can be assessed by co-culture with RMA cells (B cells that have lost MHC-I expression).

Following stimulation with one of these pathways, several functional assays can be performed:

- Quantitative assessment of expressed cytokines in the supernatant (ELISA, Luminex, MSD, see recommendations of cytokines to analyze below)
- Quantitative assessment of expressed cytokines intra-cellularly if secretion was blocked (for example with brefeldin A, or monensin to prevent exocytosis for NK cells assays) using flow cytometry
- Proliferative capacity of the immune cells after stimulation
- Assessment of T cells cytolysis activity
- Staining with CD107A during the stimulation can also provide an estimation of the effective T-cells immune cell activation and cytotoxic degranulation

If dominant antigens of the tumor have been identified, a tetramer analysis of CD8 and CD4 by flow cytometry can be informative.

In-vivo functional assay can also be performed but are more complex to carry. They involve injecting the animals with brefeldin A to block cytokine secretion, and then quantifying intracellular cytokines in various immune cell subsets.

What cytokines should be analyzed will also be dictated by the hypothesis to be tested. We are suggesting below a few cytokines panels:

- Recommendations for commercial panels such as:
  - For 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 for mouse): https://www.mesoscale.com/en/products/v-plex-mouse-cytokine-29-plex-kit-k15267d/
- 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.

Tissue staining

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. When possible, staining of endothelial cells and lymphatics can also be informative.

Some free software for image analyses, such as QuPath[2], are available, but image interpretation such as assessment of the spatial distribution of immune cells, and selection of metrics for qualitative or quantitative analyses of these images, are not trivial issues. We strongly recommend involving pathologists from the beginning of the projects. A typical metrics for mice tissue analysis is to count the number of positive cells per mm^2, which will allow for an overall assessment of the level of changes induced by a treatment.

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, myeloid marker, MHC-I. => their frequency and localization. Slide for H&E. This will require several 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 TUNNEL 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

For the analysis of gene expression

- 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.
- RNAseq analysis will require collaboration with bioinformaticians

For the analysis of T-cells Repertoire (TCR):

- It is possible to do TCR sequencing from RNA

Analysis of DNA

For the analysis of T-cells Repertoire (TCR):

- Where possible, compare TCR in tumor pre and post treatment. There seems to be a greater similarity after successful treatment in preclinical models (mice).
- If not, a sequencing of the TCR will be required. Commercial solutions are available for TCR sequencing and analysis (example Adaptive in Seattle)

A DNA sequencing analysis may be useful is the tumor genetics has not yet been published:

- DNA sequencing analysis using whole exome or genome sequencing to assess TMB, tumor mutations (eg MHC pathway) and TCR clonality and diversity is recommended.

[2] https://qupath.github.io

Analysis of blood samples

Samples should be preserved at -80°C for future interrogation (see Appendix 2 for a protocol to process and bank blood samples for PBMC and plasma)

We recommend 2 assays for small animal blood samples:

- phenotypic analysis of leukocytes as the main analysis
- quantification of plasma cytokines

We discuss below circulating tumor cells and tumor DNA but recommend keeping these assays for clinical studies. Other standard blood biochemistry assays could be useful, such as LDH or transaminases.

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.

Plasma cytokines

Technologies mentioned above for quantification of cytokines (Luminex, MSD) can be used to quantify plasma cytokines.

- We suggest to focus on a few circulating cytokines, including IFN-1, IL-1, IL-6

Circulating tumor cells

This assay is available, although we don't yet recommend its use in small animals. Metastatic spreading can be assessed by other ways, such as imaging, or direct analysis of known metastatic sites on harvested organs.

- Flow cytometry can be used to enumerate CTC in preclinical models, especially if the tumor cell expresses a fluorescent marker such as GFP.
- CellSearch, which is FDA approved, can also be used. It has been used to assess CTC in murine models of breast and prostate cancers, but it will be necessary to adapt the human CTC assay for use with small blood volumes (Kitz 2018).

Tumor DNA present in plasma samples

This assay is available, although we don't yet recommend its use in small animals.

- This is an emerging space and depending on the tumor type and analysis should be evaluated at the time for inclusion, although this may end up being irrelevant preclinically due to very small sample size
- For clinical trials, 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. Whether this will be relevant for preclinical FUS research remain to be investigated.

Timepoints for Preclinical studies

These time points are recommendations only and should not limit the analyses. If drugs are used in combination with FUS, a guide to estimate relevant timepoints should come from the drug PK.
The sequence of the combination should also be considered when selecting time points, depending on whether drug treatment is administered before, simultaneously or after FUS, and could lead for example to collection before pre-treatment, before priming FUS, or after FUS but before other therapy, when possible.

Recommendations for tissues harvest and analysis

Tissue biopsies should include at least tumor and draining lymph node. When possible, include spleen, non-draining lymph nodes, blood.

It is important to note that:

- Each time point will require separate resections.
- Each modality (flow cytometry, IHC/H&E, RNAseq, TCR/TMB) will likely require separate resections

Careful planning is therefore required to adjust the number of animals, and it can be assumed that the FUS treatment's throughout will have to be considered when deciding how many animals to treat. If the FUS system allow for treatment of large cohort of animals, a longitudinal analysis with several timepoints may be feasible. If the number of treated animals is restricted, we suggest dedicating experiments to specific time points, and recommend including at least two timepoints:

- An acute time point D1-D3 post treatment when much of the inflammation will occur
- A later time point W1-W2, after an adaptative response will have been mounted

Other time will have to be defined specifically for the study, depending on the model characteristics, response to treatment, etc.

In the following chart, we indicated in orange the two recommended time points, and in beige possible others.

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 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 tumoral CD8 cells (Tumeh 2014; Chen 2016; Johnson 2016). Therefore, larger numbers of tumoral CD8 cells may be a useful biomarker to identify "hot" (CD8 high) tumors.

Analysis of tumor samples

Frozen tissue does preserve RNA quality better and can lead to better results for RNA sequencing.

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 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 Nanostring immune profiling panels (some cancer specific panels are available for mice studies and include immune response).

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. Many preclinical models have now been characterized in terms of TMB and TCR, and each particular mode will have merits/demerits that may drive immune analyses.

If unknown, 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.

Assessment of TCR clonality and diversity before and after treatment has been shown to be a parameter that is associated with immune responsiveness (Yost 2019).

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.

Analysis of blood samples

For murine blood samples, RBC can be separated from PBMC by a lysis protocol or density-based centrifugation (e.g. Lymphoprep), and serum can be collected separately (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 can also be analyzed after stimulation of PBMCs with selected compounds, including as 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 -70°C 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 Pre-clinical studies

For tumor samples, timepoints should include

- Acute time point, 1-7 days 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

If studying tumor neoantigen and clonal evolution, more frequent collection would be ideal, but may not be possible from the same animal, unless needle aspirates could be collected.

Similarly, for blood sample, timepoints should include:

- Acute time point, day 1 if possible post-treatment to assess the short-term (inflammatory) response, especially assessment of inflammatory cytokines (Luminex or equivalent)
- A 2-3 weeks sample for when an expanding immune response can be expected

Other timepoints, when feasible, may include pre-treatment sample on the first day of drug administration (Cycle 1, day 1 pre-infusion) or FUS treatment, and later timepoints to monitor the evolution of the response if the animal survives.

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Appendix 1 - Tumor tissue processing (FFPE)

Below are recommendation for processing of tissue for FFPE:

- In order to keep time from tissue acquisition to fixation as short as possible, samples should be placed in 10% neutral buffered formalin (NBF) within 15 minutes of the harvest. 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 2 –Viable cell isolation from spleen and blood samples

This protocol was provided by the Bullock laboratory at UVa

Harvest spleen

Give 500ul tribromoethanol to euthanize mouse and cervical dislocation to confirm death
Remove spleen from animal and place in 5ml MCTL in 15ml conical
Homogenize spleen and filter through cell strainer mesh into 15ml conical
Rinse homogenizer with 5ml MCTL and pour through cell strainer mesh
Spin down for 5 minutes, 1200 rpm
Pipette off supernatant

Harvest blood

Warm mouse's tail and slice laterally across vein with a sterile scalpel
Collect blood with a heparinized capillary tube and deposit into 1-2ml MCTL
Spin down for 5 minutes, 1200 rpm
Pipette off supernatant

RBC lysis

Resuspend pellet in 1-2ml RBC lysis
Swirl contents periodically to mix
Incubate at room temperature for 2 minutes (spleen; other tissues) or 3-5 minutes (blood, until the sample goes from opaque to translucent). Any longer will lead to lymphocyte cytolysis.
Quench RBC reaction by filling tube to the top with PBS or media
Spin down for 5 minutes, 1200 rpm
Dump off supernatant and resuspend pellet for staining, plating, etc.

For greater purity, isolation of PMBC via lymphoprep is recommended:
Pellet blood, resuspend in 2ml MCTL then underlay with 2ml lympholyteM.
Centrifuge according to manufacturer's directions for tube size.
Use pipette to harvest interface containing lymphocytes and monocytes.

Appendix 3 – Isolation of Tumor Infiltrating Lymphocytes (TILs) from Solid Tumor

This protocol was provided by the Bullock laboratory at UVa

Preparation

Remove Lympholyte M (Cedarlane Labs, CL5035) from fridge, shake to ensure homogenous distribution, and allow to warm to room temperature

Harvest tissue

Give 500ul tribromoethanol to euthanize mouse and cervical dislocation to confirm death
Remove tumor from animal
Place tumor in weigh boat or sterile dish
Thoroughly mince tumor with scissors
NOTE: depending on the tumors, you may need to just cut into a few smaller pieces, as if you mince thoroughly the pieces it will be much harder to homogenize
Transfer tumor to 15ml conical with 4ml MCTL (or 2mL 1x PBS)
If necessary, rinse dish with 1ml media/buffer to ensure full recovery
Mechanically homogenize tumor using dounce homogenizer and filter through 70um or 100um cell strainer mesh into 50ml conical
Rinse homogenizer with 5ml MCTL (or 5mL 1x PBS) and pipette/pour through cell strainer mesh
Spin down for 5 minutes, 1200 rpm
Pipette/pour off supernatant

Lymphoprep

Resuspend pellet in 10ml 1x PBS

- Must use serum free media

Underlay with 10ml room temperature Lympholyte Mouse
NOTE: Lympholyte should be kept sterile so this underlay step can either be performed in the tissue culture hood or appropriate amount can be transferred to a new bottle for use outside the hood
Centrifuge at 2200 RPM for 20 minutes, 0 brake, 0 acceleration
Remove tube carefully and pipette out interface into 50mL conical (avoid the pellet)
Fill conical with 1x PBS and centrifuge at 1500 for 10 minutes
Dump off supernatant and resuspend pellet for count/staining/culture, etc.

Notes

Tissue digestion with collagenase/DNAse/hyaluronidase or accutase can be done to facilitate release of TILs from tumor tissue, although mincing the tumor may work quite well without tissue digestion.
Using MCTL in the early homogenization steps is best for maintaining cells, however 1x PBS can be substituted.
MCTL: RPMI1640+10% FCS, non-essential and essential amino acids; sodium pyruvate. HEPES and 2ME.

Appendix 4 – Panels for ImmunoPhenotyping of Murine Cells

Panels for phenotypic characterization of leukocytes can vary from sites to sites. Extremly 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 manufacturers such as Biorad:
https://www.bio-rad-antibodies.com/murine-no-compensation-panels.html?JSESSIONID_STERLING=39E8172191672FEDBC118F74ADE2E1F6.ecommerce1&evCntryLang=US-en&cntry=US&thirdPartyCookieEnabled=true

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

- Murine polymorphonuclear MDSC (PMN-MDSC): $CD11b^{+}Ly6G^{+}Ly6C^{lo}$
- Murine mono-cytic MDSC (M-MDSC) : murine : $CD11b^{+}Ly6G^{-}Ly6C^{hi}$

Appendix 5 – Considerations for selection of preclinical models

Several aspects have to be considered when selecting a preclinical tumor model, among them:

- Syngeneic immunocompetent murine model v. genetically engineered mouse models (GEMM) v. patient derived xenotransplant (PDX) models into immunodeficient mice
- Implantable v. inducible tumors
- If implantable, heterotopic and orthotopic implantation
- Natural immune repertoire of the tumor
- Myelopoiesis induced by the model
- Vascularization of the tumor
- Stromal compartment of the tumor (dense or not, composition …)
- Metastatic capabilities of the model
- Sensitivity to drugs (such as immune checkpoint inhibitors) when used in combination with FUS

Appendix 6 – 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	Pipelines exist (algorithms work best for each step and how they should be run) Complex analyses, Need support of bioinformatician

	Bulk analyses typically investigate how gene expression differs between two or more treatment conditions.	
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