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Immune Monitoring of Immunotherapy Trials

Considerations for Optimizing Yield of Informative Data

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http://www.mskcc.org/research/immune-monitoring

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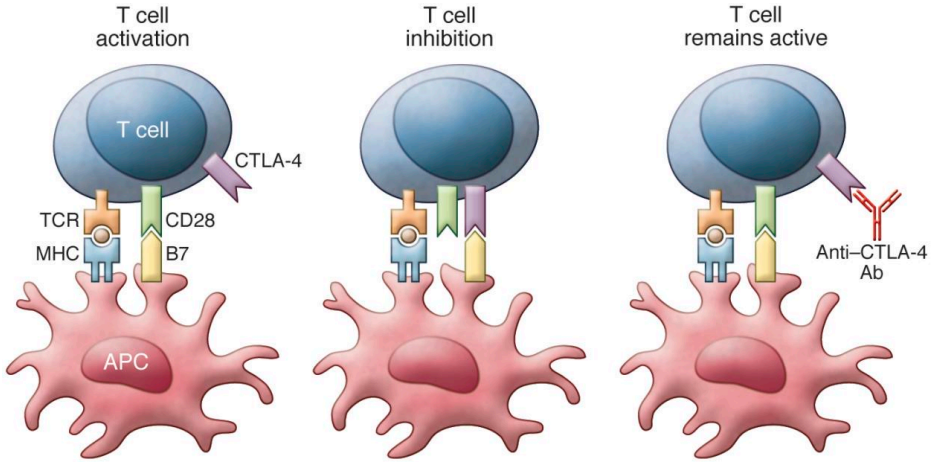
Immune regulatory checkpoint receptors as major targets in cancer therapy

- With better understanding of the stimulatory and inhibitory signals regulating T cell responses and peripheral tolerance, immune regulatory components are now widely targeted in cancer immunotherapy in attempts to activate anti-tumor immune responses, most notably with antibodies such as ipilimumab and nivolumab/pembrolizumab developed to block CTLA-4 and PD-1 inhibitory signals[1]



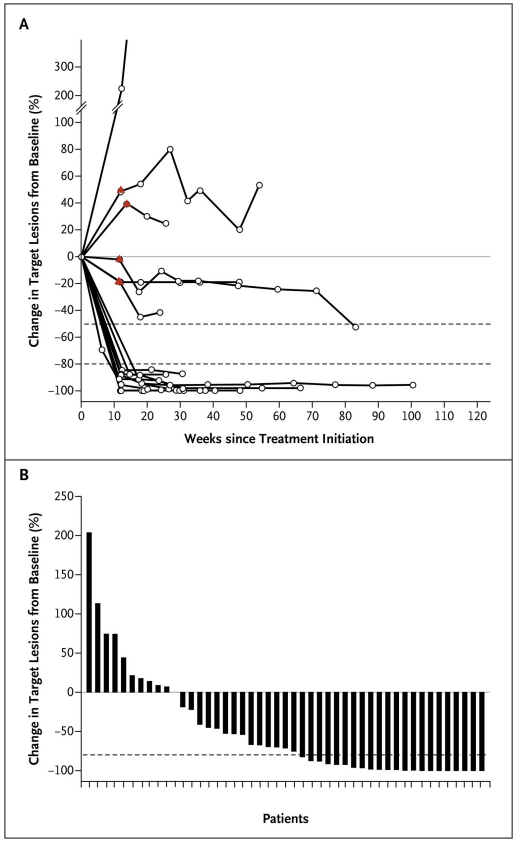
Buchbinder, E., & F.S. Hodi, J Clin Invest **125** (2015): 3377.

1. Leach, D., M. Krummel, J. Allison, Science **271** (1996): 1734.

Impact of checkpoint blockade therapy

- Administration of immune checkpoint inhibitors such as anti-CTLA-4, anti-PD-1, and anti-PD-L1 have resulted in remarkable therapeutic outcomes, resulting in tumor regression and long-term equilibrium or cancer elimination in a large percentage of treated patients

Clinical Activity in Patients Who Received Concurrent Regimen of Nivolumab and Ipilimumab



Wolchok et al., N Engl J Med **369** (2013): 122.

Kaplan–Meier Estimates of Survival

Long-Term Outcomes With Nivolumab Plus Ipilimumab in Melanoma

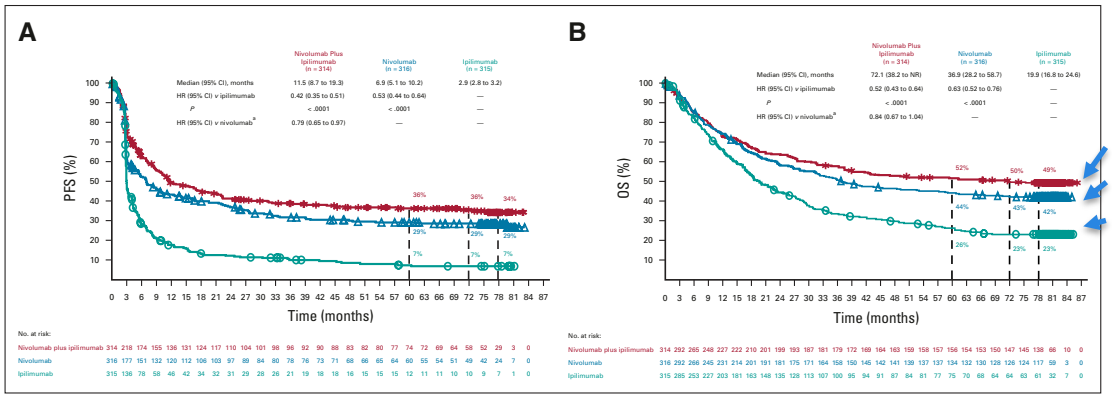


FIG 2. (A) PFS and (B) OS in patients who received nivolumab plus ipilimumab, nivolumab, or ipilimumab. Patients were followed for a minimum of 77 months. All rates are based on the current 6.5-year analysis; rates shown at earlier time points may differ slightly from those of previous reports. [a]Descriptive analysis. HR, hazard ratio; NR, not reached; OS, overall survival; PFS, progression-free survival.

Wolchok et al., J Clin Oncology **40** (2022): 127.

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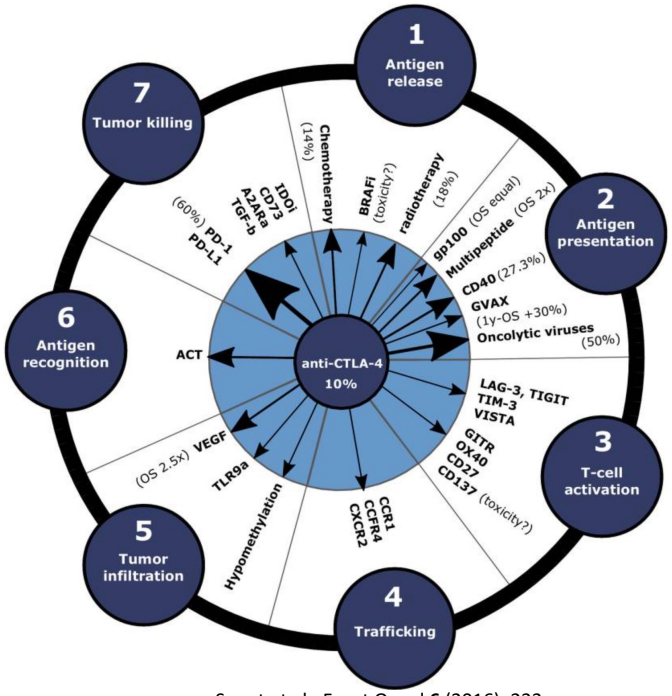
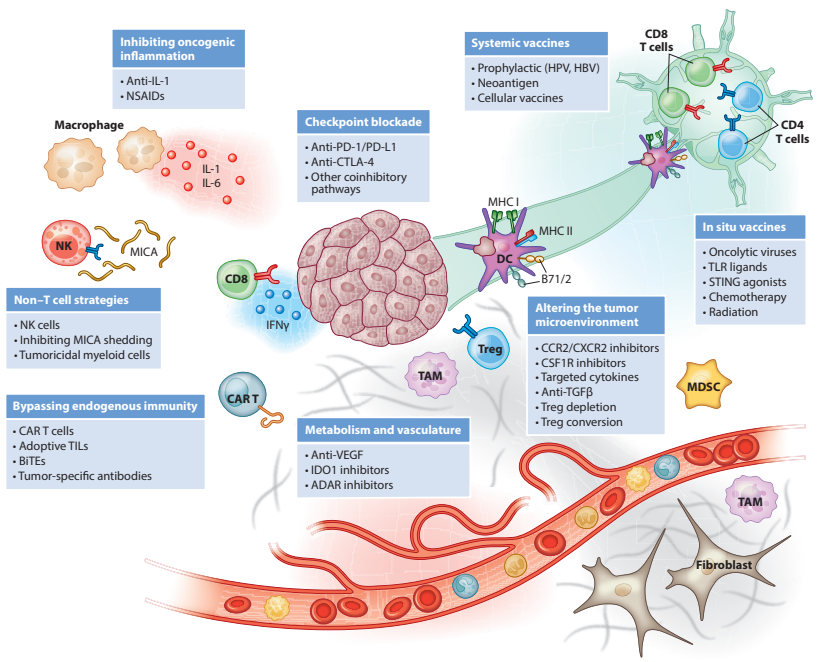
Immunotherapy combinations

- Impressive success and safety profile of immune checkpoint blockade (ICB) therapy rapidly pushed the field of immuno-oncology forward; immunotherapy now assuming critical role as a backbone of many cancer treatment strategies
- Numerous clinical studies ongoing to evaluate various combinations of ICB (anti-CTLA-4, PD-1, LAG-3) with other treatment modalities (e.g., oncolytic viruses, TLR/costimulatory molecule agonists, tumor Ag vaccines, chemotherapy, radiotherapy, Treg/MDSC depletion) to overcome immunosuppressive pathways and improve outcomes



Swart et al., Front Oncol **6** (2016): 233.



Dougan et al., Ann Rev Cancer Biol **3** (2019): 55-75.

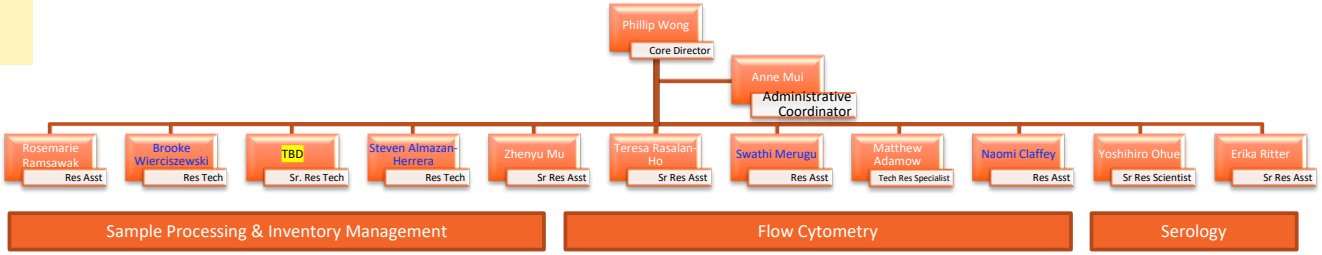
Challenges for immunotherapy

- Understanding mechanisms of resistance to immunotherapy in non-responding patients with different tumor types
- Determining optimal combinations within the vast landscape of immunotherapy options to improve the efficacy of cancer treatment while reducing treatment toxicity
- Predicting clinical response or resistance to specific therapeutic regimens, and likelihood of developing severe adverse events during immunotherapy

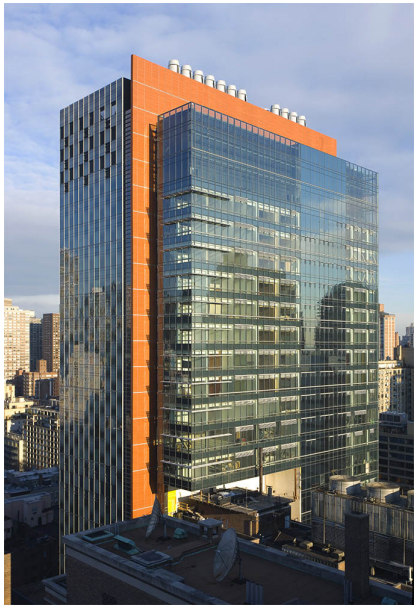
Monitoring of patient immune responses during cancer immunotherapy will be critical to identify biomarkers that may elucidate biologic mechanisms and inform clinical decision making, patient prognosis, and treatment stratification

Immune Monitoring Facility (IMF): Overview

Organizational Structure



Located on 15th fl of Zuckerman Research Center



Aims:

- Provide clinical sample processing, banking, and inventory management capabilities for immune monitoring studies
- Establish cutting edge tools and standardized core technologies to characterize the immune response in clinical trial patients undergoing cancer immunotherapy
- Identify biomarkers that may predict response and/or adverse reactions to therapy to enable appropriate patient stratification for maximal clinical benefit and minimal toxicity

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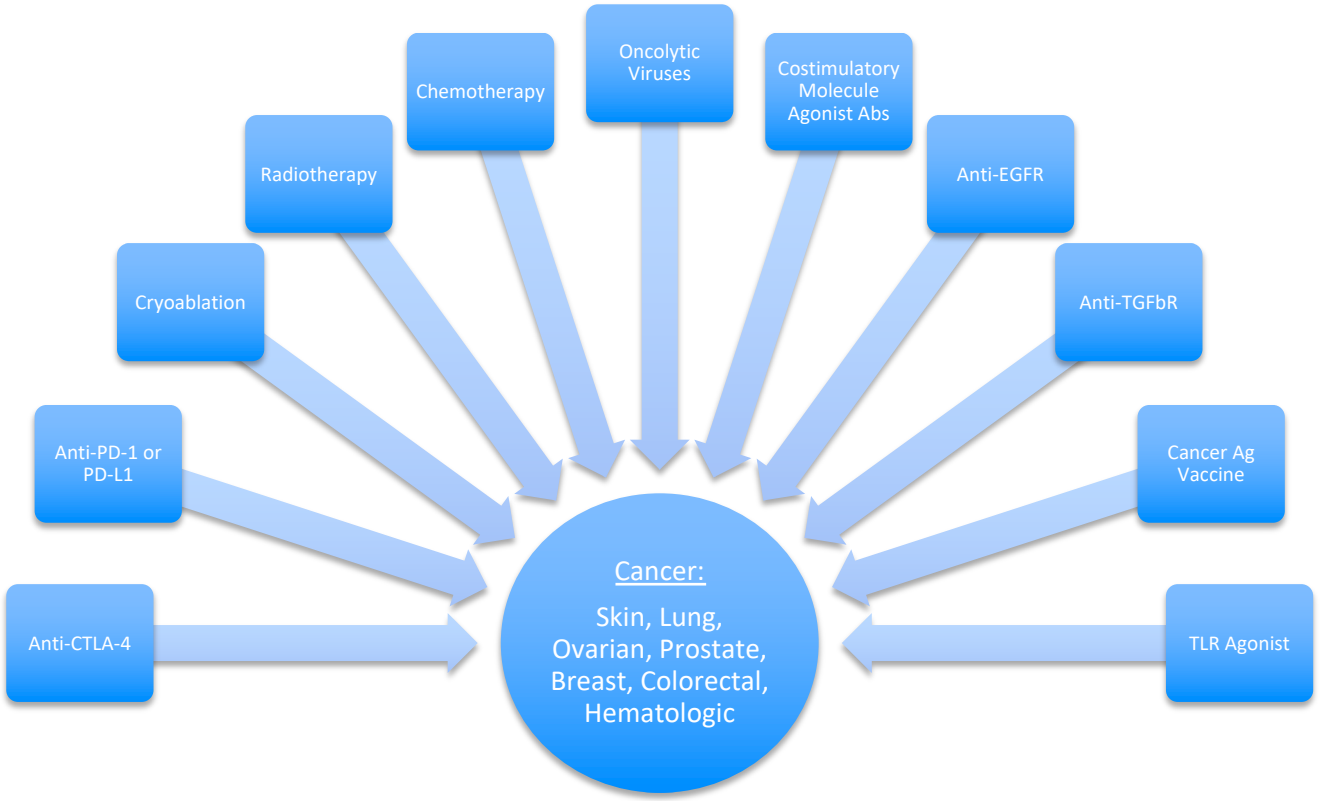
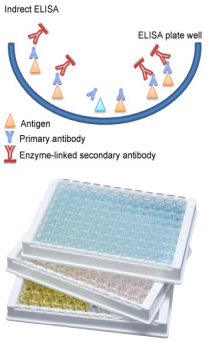
Wide Scope of Immunotherapy Studies Supported

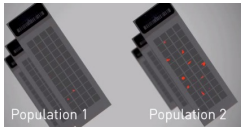


IMF currently supporting >40 active immunotherapy trials investigating treatment of different tumor types with novel combinations of immune checkpoint inhibitors and/or other therapeutic modalities, in collaboration with MSK investigators, LICR, PICI, and industry partners

IMF Capabilities & Services



Tumor Antigen Serology & Protein Microarrays



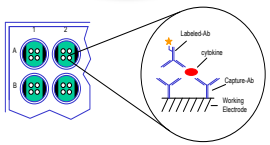
Serum
Plasma

PBMC
TIL

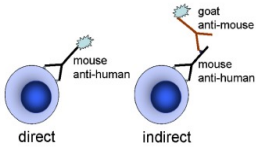
Clinical Sample Mgmt

Whole blood processing & cryopreservation

Cytokine Immunoassays



Flow Cytometry Assays



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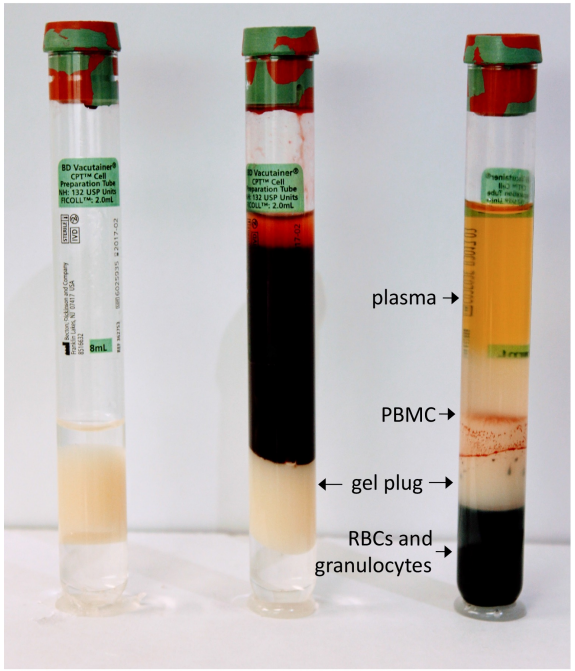
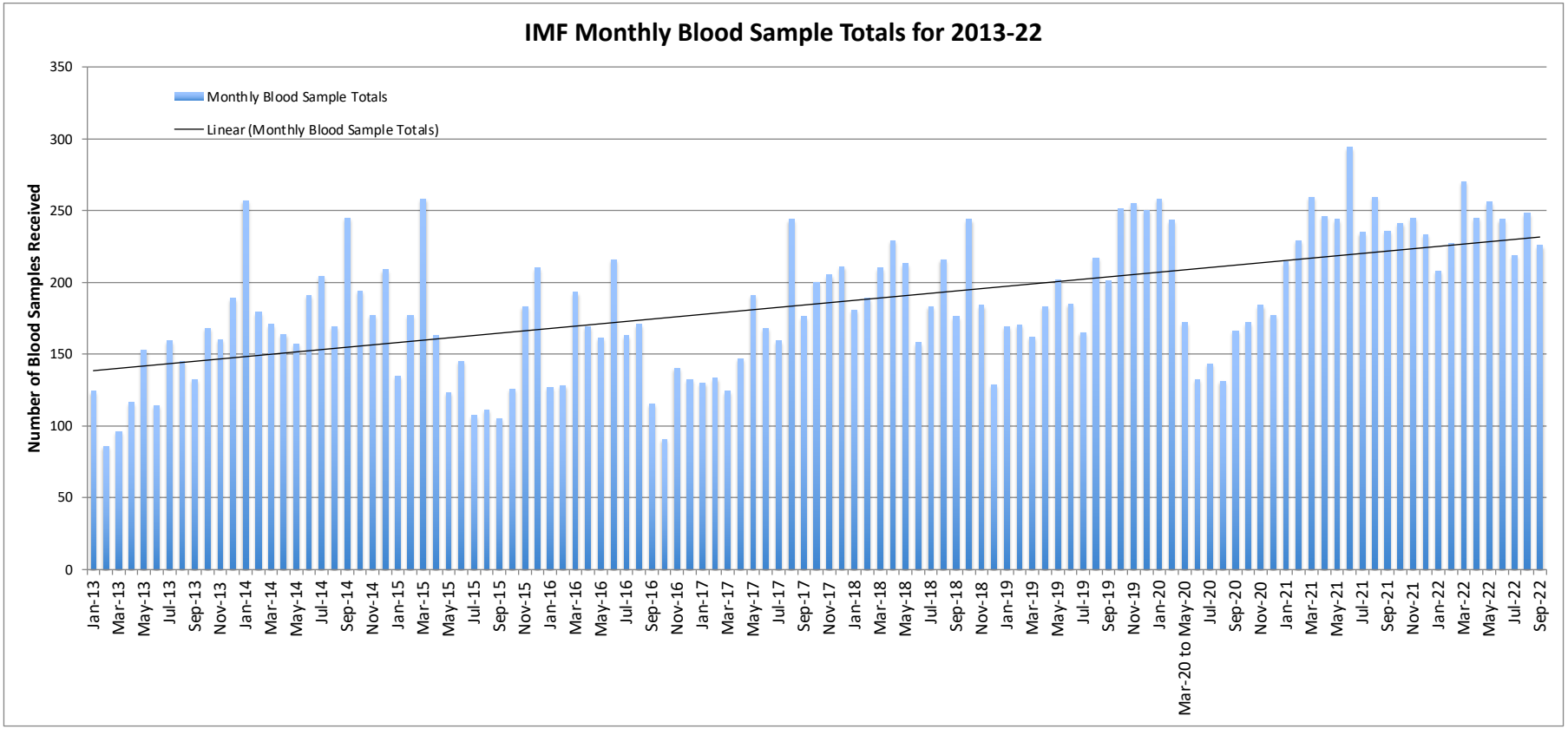
Clinical Sample Banking at MSK IMF

- For peripheral blood, standard processing protocol entails collection of ~32 ml whole blood per patient visit in BD Vacutainer® CPT (Cell Preparation Tubes). These tubes contain an anticoagulant (e.g., Na Heparin) and a FICOLL Hypaque density fluid separated by a polyester gel barrier. This is a convenient, single tube system for the collection of whole blood and the efficient and consistent separation of peripheral blood mononuclear cells (PBMC) immediately upon receipt of samples.

(16x125 mm size, 8 ml draw capacity,
BD Biosciences Cat. 362753)



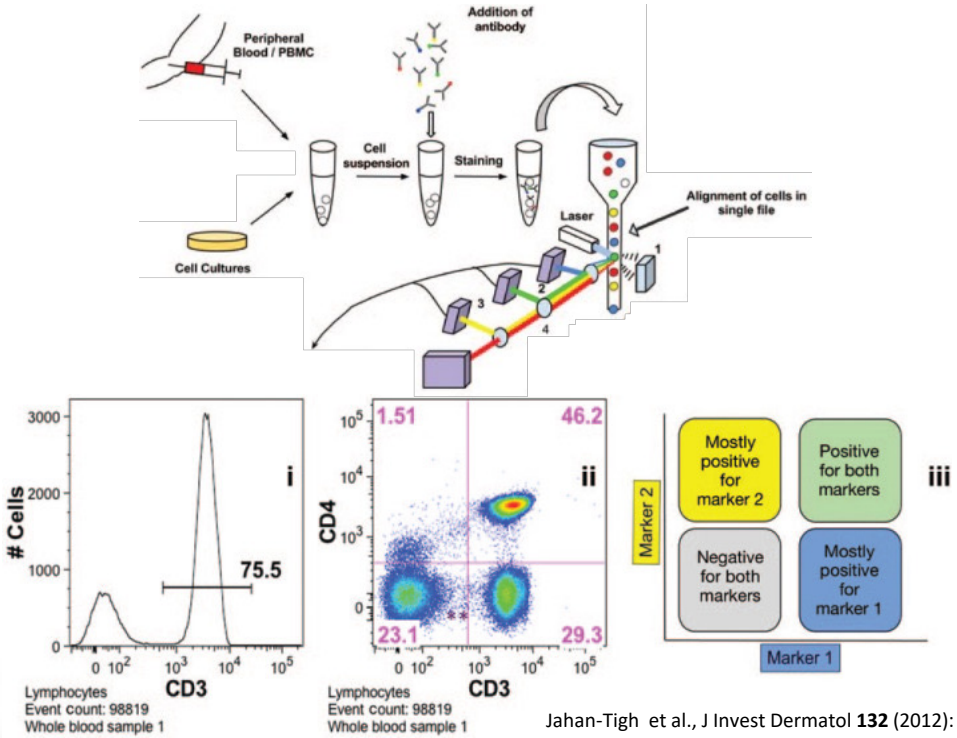
2013-22 Total Clinical Samples Processed by IMF



- Average of 221 blood samples/month (~11/day) for ~47 studies/month for 2020-2022
- Steep dropoff in spring 2020 due to COVID-19 lab shutdown
- PBMC samples stored across 3 LN2 freezers and plasma/serum stored across 5 -80◦C/-20◦C freezers

IMF Assays:
Flow Immunophenotyping & Functional Panels

- Characterize immune cell subsets including T cells and their expression of activation/exhaustion markers pre/post Tx using validated flow panels
- Measure Ag-specific T cell responses and function via intracellular cytokine staining
- Discover novel phenotypes from 28-color phenotyping panels using high dimensional BD FACSymphony (Fortessa X-50) flow cytometer



Jahan-Tigh et al., J Invest Dermatol **132** (2012): 1.

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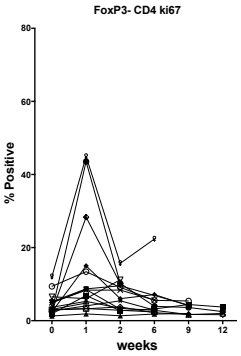
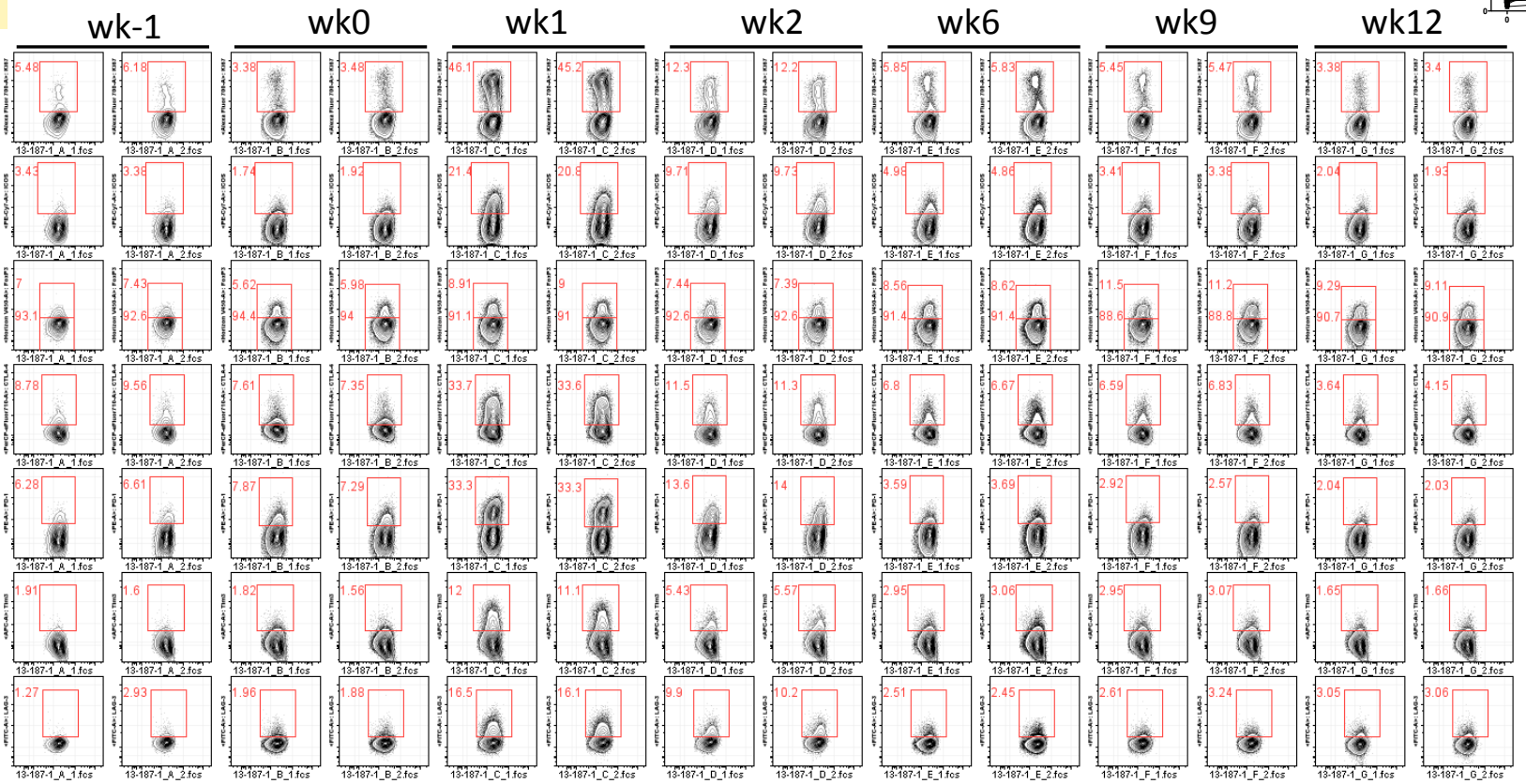
Analysis of changes in T cell phenotype in patients after checkpoint blockade





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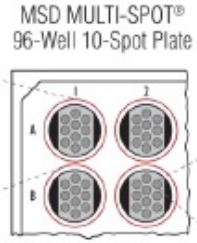
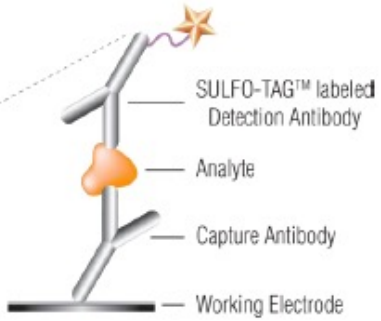
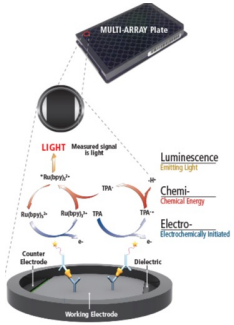
IMF Assays:
Multiplex Cytokine Immunoassays

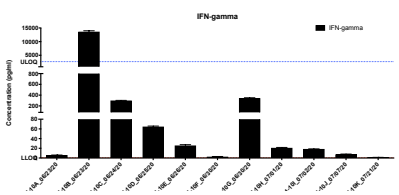
- Meso Scale Discovery multiplex assay platform
  - Highly sensitive quantitation of cytokines and other soluble protein biomarkers in serum, plasma, CSF, & cell culture supernatant via electrochemiluminescence detection
  - Primary MSD 10-plex human V-Plex Th1/Th2 cytokine/chemokine panels include:
    - IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-α
    - GM-CSF, IL-1α, IL-5, IL-7, IL-12/IL-23p40, IL-15, IL-16, IL-17A, TNF-β, VEGF-A
    - Eotaxin, Eotaxin-3, IL-8 (HA), IP-10, MCP-1, MCP-4, MDC, MIP-1α, MIP-1β, TARC
    - Other panels and individual analytes available from MSD

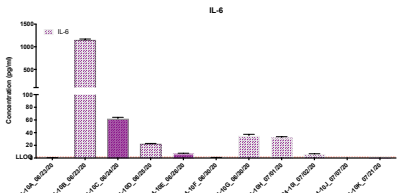
1. IFN-γ
2. IL-1β
3. IL-2
4. IL-4
5. IL-6
6. IL-8
7. IL-10
8. IL-12p70
9. IL-13
10. TNF-α

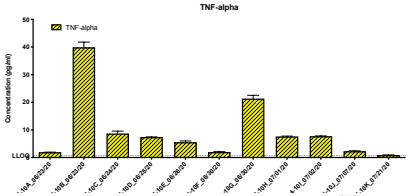












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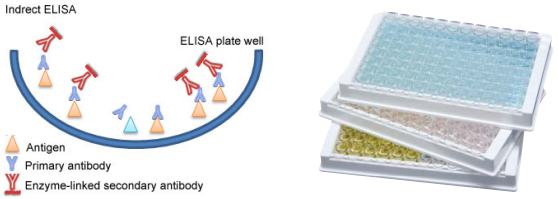
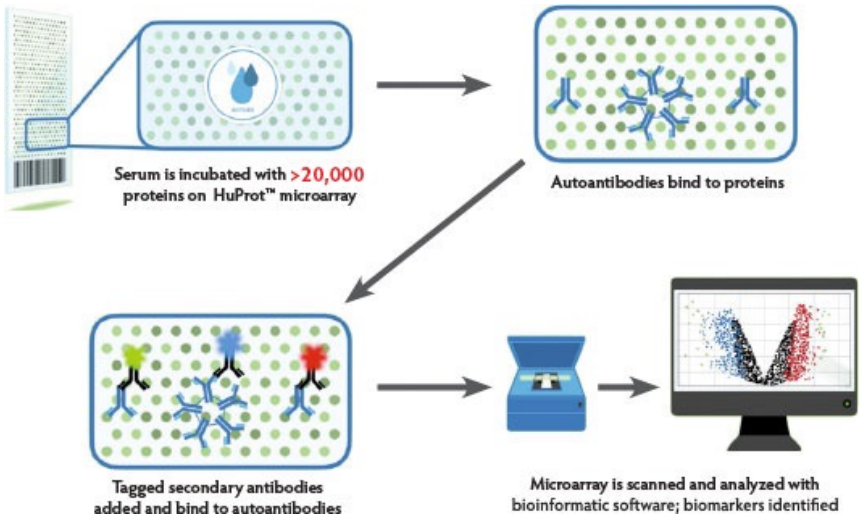
IMF Assays: Serology

- Detection of antigen-specific B cell antibody responses by ELISA
  - Shared tumor Ags: NY-ESO-1, Melan-A, Mage-1, Mage-4, Mage-10, CT-7, CT-10, CT-45, CT-46, CT-47, CXorf48, Gage2, Rab38, Sage1, SOX2, SSX1, SSX2, SSX4, p53, UBQLN2, TRAG-3, and DHFR (neg control)
  - SARS-CoV-2 (COVID-19) Spike/RBD/Nucleocapsid (IgG/IgA/IgM)



- High content protein microarrays for seromics analyses
  - CDI arrays contain >20,000 full-length purified human proteins (~80% of human proteome) spotted on glass slides for characterization of autoAb reactivities in serum samples that may change after treatment and may be associated with clinical response/drug toxicity



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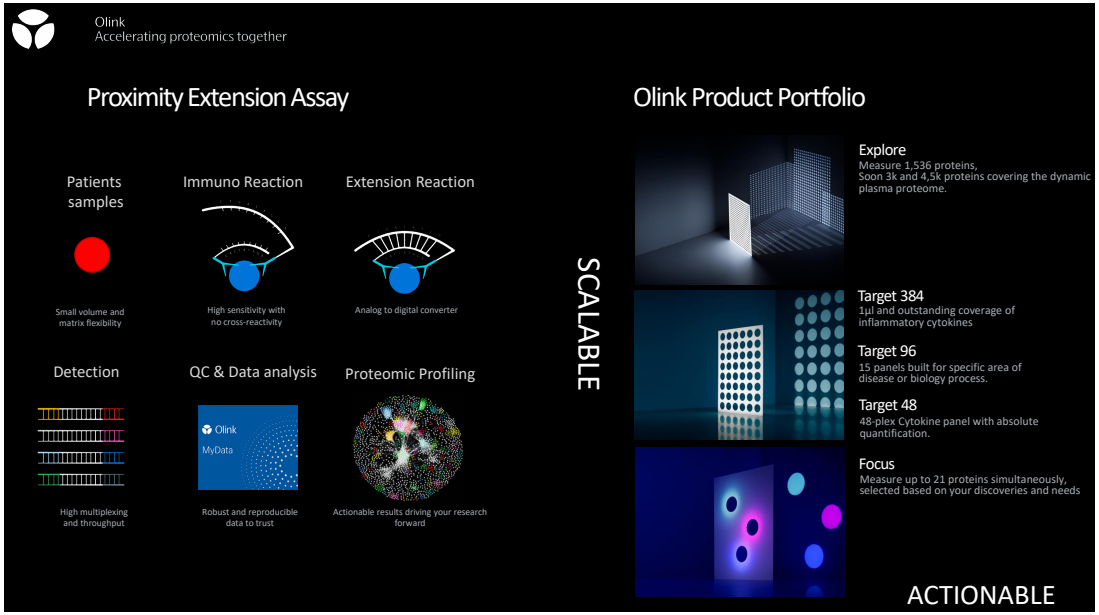
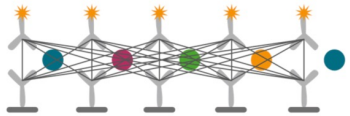
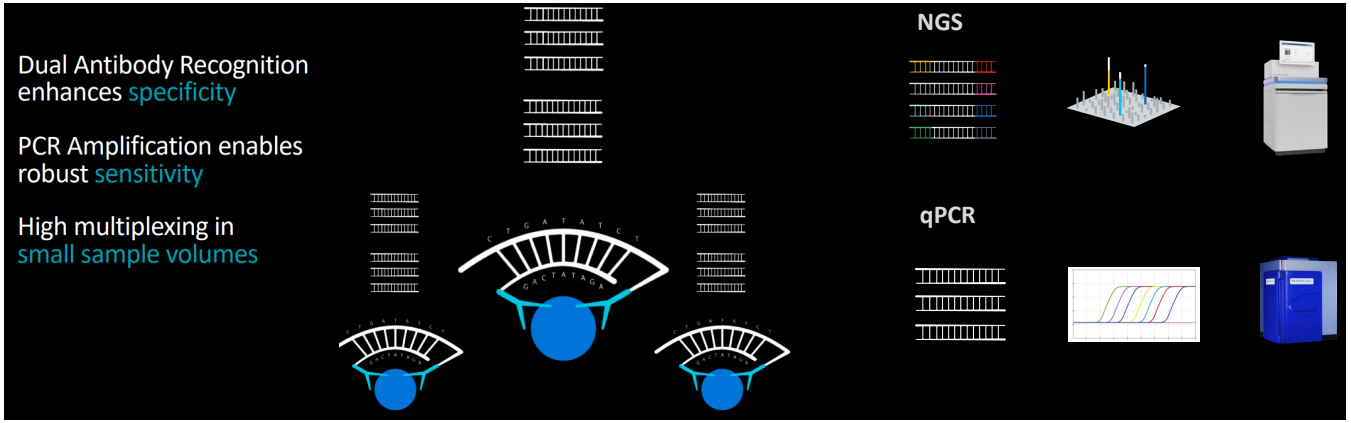
Technologies Being Evaluated: Olink





A) Conventional immunoassays: cross-reactivity due to unspecific binding of antibodies limits the degree of multiplexing.

B) Olink's technology: unique DNA oligo sequences report only matched DNA-pairs (e.g. 1A+1B). Cross-reactive events are not detected.



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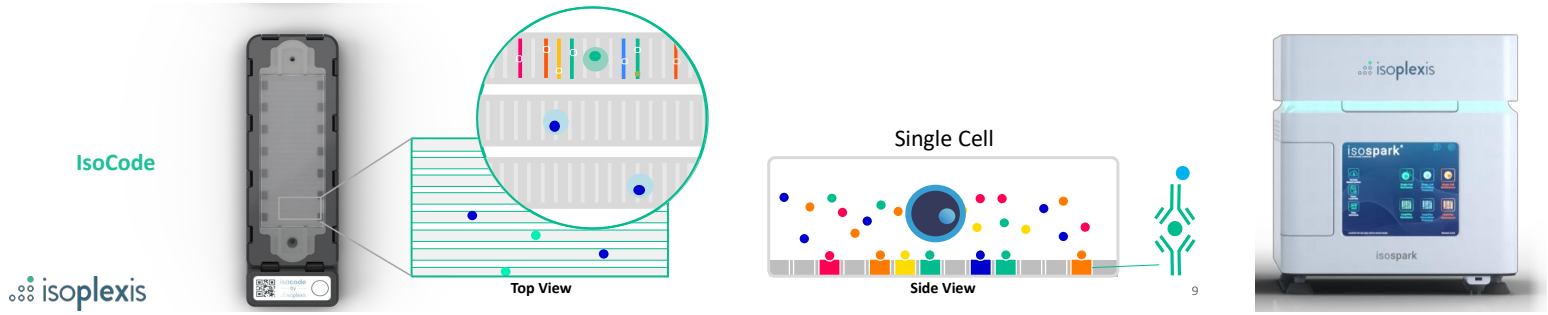
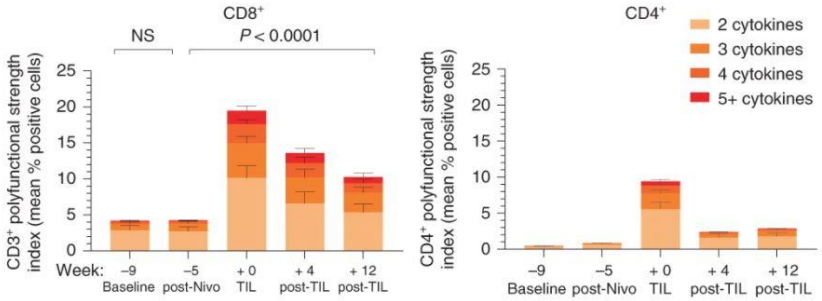
Technologies Being Evaluated: IsoPlexis



Functional Immune Landscaping

Accelerate the ability to clarify lead candidate choice and durable biomarkers using the proteomic secretome from each single cell to accelerate path to higher efficacy with targeted immune therapies.

- **Unique Superpowered Biology:** Detect subsets of superpowered immune cells
- **Uniquely Predictive**: 50+ uniquely correlative data sets
- **Gold Standard:** Leading tool for single-cell multiplexed cytokine profiling
- **Highly Multiplexed:** Targets 30+ cytokines per immune cell
- **Fully Automated ELISA Workflow**
- **Consistency:** 20% CV
- **Sensitive:** ~2-2000 pg/ml
- **Widely Published in Biomarkers & Discovery**



Creelan B.C. et al *Nat Med* **27,** 1410–1418 (2021)

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Challenges of Immune Monitoring

- Current biomarker assay technologies can provide a wealth of information with increasingly high dimensional data output but the complex results generated can frequently be difficult to interpret and compare between laboratories

- Large numbers of clinical samples and big data sets being collected across multiple technology platforms require careful oversight to ensure reliability of results

- Some contributors to poor data quality:
  - Lack of recognition of proper upstream sample handling and delivery logistics as a critical requirement for biomarker accuracy, stability, and reproducibility
  - Non-adherence to standardized protocols or lack of standardized instruments/ materials/ reagents/ analysts during sample collection or assay runs to remove sources of variability

Upfront study planning to minimize issues above is highly recommended to increase usefulness and accuracy of the data that is obtained

Study Planning Considerations

- What questions are being asked and what biomarker assays would be most informative to perform to address those questions?
  - Determine the validated assays available, the cost and feasibility of running these assays with the samples collected
  - If budget and samples are limiting, can prioritize assays such that results from one may inform the utility of the next
  - Technologies can evolve so allow flexibility in clinical protocol in terms of the specific assays to be performed, but still good to have a general idea of areas of investigation and the sample collection requirements entailed
  - Investigators sometimes request research sample banking without plans for specific assays but this risks suboptimal sample preparation – some assays may not be possible under certain sample collection conditions (e.g., sensitive functional assays)

Study Planning Considerations

- What sample types should be interrogated, how much, and at what time points?
  - Peripheral blood is most accessible, will allow characterization of circulating immune cells and systemic Ab/cytokine profiles although may not reflect local tumor response
  - Plasma vs. serum – only one or the other is needed for most applications but be consistent; some analytes can only be measured accurately in one type of matrix; platelet-poor plasma is required for analytes (e.g., PDGF) that may be nonspecifically released by activated platelets in whole blood during sample collection and processing
  - Tumor biopsies are useful for tumor microenvironment analyses such as IHC, exome sequencing, gene expression profiling, or extraction of TILs for flow & TCR repertoire analysis (but core biopsies often have insufficient material for flow)
  - Volume of sample needed will depend on the number and types of downstream assays
    - Standard blood collection volumes of ~32 ml blood (4 tubes) at IMF sufficient for most applications
  - Sample collection should occur at rationally selected visits/time points
    - Ideal to have >1 baseline sample if possible as reference points to confirm pre-treatment data, perform repeat or additional timepoint runs if needed, or to test in multiple assays
    - Data from single (non-serial) timepoint biopsies can be difficult to interpret w/o add'l controls
    - Early frequent sampling (wks 1-3 post-dosing) to detect immediate PD effects (cytokines, cellular activation), with longer interval sampling at limited downstream time points (e.g., every 3 wks up to 12 wks), perhaps following dosing schedule, disease progression, or interesting clinical observations
  - May not be feasible or necessary to collect research blood at every possible visit
    - Blood draw limits and impact on patient
    - Local lab freezer storage space limitations; may need to consider offsite storage options

Study Planning Considerations

- How should the samples be collected and handled for the assays being considered?
  - Sample viability, yields, and biomarker stability can be highly dependent upon the **temperature** and **time elapsed** between sample collection and delivery from the clinic to the lab for processing; poor sample integrity or low yields will lead to inconclusive data
    - Optimal preservation of cell viability, phenotype, and function when blood is kept at RT (or 4°C if measuring cytokines) and processed asap (no longer than 8-24 hrs after collection, will depend on the assays being used)
  - Type of anticoagulant (e.g., heparin vs. EDTA) used in blood tubes can be important
    - Heparin may be best for evaluating cellular function, while EDTA may be better for DNA sequencing applications
  - Functional assays that may entail cell stimulation for response readouts can require greater amounts of material and more stringent sample processing requirements (tighter time frame between blood draw and processing)
  - Can consider alternative collection tube types (PAXgene, Streck Cyto-Chex) that are designed for maintaining long-term integrity of samples for the relevant downstream applications (need to validate for specific markers assessed)

CYTO-CHEX® BCT
BLOOD COLLECTION TUBE

A direct-draw blood collection tube used for immunophenotyping of white blood cells by flow cytometry.

Convenient

- Minimizes the adverse effects of time, storage and transport conditions on sample integrity
- Maintains cellular morphology and surface antigen expression
- Samples are stable at room temperature for up to 14 days prior to analysis for convenient transport and storage
- Allows samples to be batched to improve laboratory efficiency

Effects of blood sample handling procedures (temperature and time delay to processing) on measurable inflammatory markers

Plasma

Table 2
Inflammatory markers measured in plasma from blood stored at different conditions before centrifugation

	4 °C			RT			35 °C		
Plasma	4 h	24 h	48 h	4 h	24 h	48 h	4 h	24 h	48 h
IL-1b	1.0	1.1	1.6	3.4*	3.1*	5.6*	1.9*	1.3*	1.9*
IL-2	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-4	1.3	0.8	1.2	1.2	0.8	1.2	1.9	1.0	1.0
IL-5	1.1*	1.1	1.0	2.1*	2.4*	3.2*	1.2	1.1	0.9
IL-6	0.9	1.1	1.2	1.2	1.4*	1.9*	1.2	1.2	0.9
IL-8	1.0	1.0	1.0	1.0	3.7*	31.6*	1.0	14.7*	30.4*
IL-10	1.2	1.0	1.2	2.1	1.5	1.6	1.2	1.0	1.1
IL-12	1.3	1.0	0.9	4.5	2.2	2.8*	1.6	1.1	1.1
IL-17	2.2*	1.7	1.9*	6.4*	5.9*	7.2*	2.9*	3.3*	2.5*
IL-18	1.1	1.1	1.1	1.3*	10.5*	19.5*	1.5*	3.3*	2.7*
sIL-6ra	1.1	1.1	1.1	1.0	1.1	1.0	1.1	1.0	0.9*
IFN-g	1.4	1.2	1.1	1.8*	1.3	1.3	1.6	0.9	1.1
TNF-a	1.0	1.0	0.9	1.4*	1.4	2.0*	1.3	1.2	1.0
TNF-b	1.7*	1.1	1.4*	5.3*	4.6*	5.0*	2.7*	3.7*	2.8
MCP-1	1.0	1.3	1.3	1.5*	1.5	1.5*	1.1	1.0	1.1
TGF-b	1.1	1.0	1.0	1.5	1.0	2.3*	1.1	1.0	1.0
MIP-1a	1.4	1.4	1.2*	2.9*	4.1*	4.4*	1.4	1.9*	2.1
MIP-1b	1.1	1.2*	1.3*	1.7*	2.4*	2.7*	1.1	1.7	1.5
MMP-9	0.9	1.2	1.2	1.8*	3.1*	3.7*	1.5*	2.0*	1.9*
TREM-1	1.0	1.0	1.0	2.3*	2.4*	2.6*	1.0	1.0	1.0
BDNF	1.6*	2.6	2.7*	15.4*	18.5*	25.8*	4.1*	8.8*	7.3*
GM-CSF	1.4*	1.0	1.0	2.3*	2.0*	2.3*	1.1	1.0	1.1
NT-4	1.0	1.0	1.0	1.9*	2.1*	1.7*	1.0	1.1	1.0
NT-3	1.1	1.1	1.1	4.1	1.7*	4.9*	2.7	2.0*	1.1
sTNF RI	1.4	1.3*	1.4	1.9*	2.1*	2.4*	1.4*	1.6*	1.6*
MIF	1.4*	1.4*	2.0*	1.5*	1.9*	2.7*	1.4*	1.7*	1.9*
RANTES	1.6*	1.4	1.5*	1.3*	1.6*	1.8*	1.9*	1.5*	1.2*
CRP	1.5*	1.2	1.3*	2.0*	1.9*	1.9*	1.7*	1.9*	1.8*
mean	1.3	1.2	1.3	2.8	3.2	5.4	1.6	2.3	2.7

All values are multiples of concentrations of control samples. *indicates $p<0.05$ calculated with Wilcoxon's signed rank sum test. nd = not detectable.

Serum

Table 3
Inflammatory markers measured in serum from blood stored at different conditions before centrifugation

	4 °C			RT			35 °C		
Serum	4 h	24 h	48 h	4 h	24 h	48 h	4 h	24 h	48 h
IL-1b	0.9	1.3	1.9*	1.6*	12.6*	216.1*	19.9*	1006.6*	2659.8*
IL-2	nd	nd	nd	nd	nd	nd	nd	nd	nd
IL-4	0.7	0.9	0.7	1.1	0.8	1.0	0.9	1.3	1.1
IL-5	1.0	1.3*	1.6*	1.8*	1.4*	1.5	1.9	1.7*	1.8*
IL-6	1.1	0.9	1.2	1.5	4.9	407.0*	4.8*	2602.1*	3069.2*
IL-8	1.0	1.0	1.0	1.0	794.3*	1718.4*	887.8*	1750.4*	1703.2*
IL-10	1.0	1.3	1.1	1.6*	1.2	3.5*	1.7*	98.5*	173.3*
IL-12	1.0	1.2	2.1*	1.7*	1.0	2.8*	1.9	7.1*	5.7*
IL-17	1.7	4.4*	4.3*	4.2*	5.1*	6.2*	4.5*	6.1*	8.4*
IL-18	1.1	1.2	1.4	1.3	1.7*	2.1*	1.3	1.3	1.4*
sIL-6ra	1.1	1.1	1.2	1.2	1.3	1.3*	1.2	1.0	1.0
IFN-g	1.1	1.3	2.2*	1.4	1.4	2.1*	1.4*	1.8	1.8
TNF-a	1.6	1.5	1.5	1.6*	2.5*	15.7*	5.8*	73.9*	74.7*
TNF-b	1.1	1.7	1.9	3.0*	3.2*	1.9*	2.8*	3.6*	3.2*
MCP-1	1.2*	1.8*	2.1*	1.9*	4.0*	17.3*	2.6*	21.9*	59.6*
TGF-b	1.1	2.3*	2.3*	2.5*	1.3*	1.9	2.1*	1.1	1.7*
MIP-1a	1.5*	2.8*	3.7*	3.5*	10.3*	100.4*	30.5*	544.1*	759.1*
MIP-1b	1.3*	2.2*	2.6*	2.8*	5.8*	27.1*	14.6*	56.0*	57.0*
MMP-9	1.3*	3.2*	4.0*	9.1*	18.0*	21.5*	18.9*	19.3*	20.4*
TREM-1	1.1	1.2*	1.4*	1.4	1.3	2.2*	2.3	1.7*	1.9*
BDNF	2.7*	18.0*	21.9*	23.2*	23.9*	28.1*	26.3*	20.5*	21.0*
GM-CSF	1.0	1.2	1.3*	1.5*	1.8*	7.9*	1.5*	4.4*	17.6*
NT-4	1.0	1.0	1.0	1.8*	1.2	2.2*	1.5*	3.2*	3.2*
NT-3	1.6	2.6*	5.5*	8.5*	8.4*	9.2*	7.1*	6.0*	10.2*
sTNF RI	1.2*	1.5*	2.0*	1.9*	2.2*	2.5*	2.0*	2.4*	3.8*
MIF	0.8	1.0	1.3	1.0	2.3*	2.0*	1.7*	1.7	1.7*
RANTES	1.0*	1.3*	1.1*	1.2*	1.1*	1.3*	1.1*	1.1*	1.2*
CRP	1.2*	1.2*	1.2*	1.2*	1.3*	1.4*	1.3*	1.2*	1.3*
mean	1.2	2.2	2.7	3.1	33.9	96.5	38.9	231.1	320.9

All values are multiples of concentrations of control samples. *indicates $p<0.05$ calculated with Wilcoxon's signed rank sum test. nd = not detectable.

Skogstrand et al., *J Immunol Methods* **336** (2008): 78-84.

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Recommended procedures for blood sample processing for optimal PBMC in T cell assays

Table 2. Recommended procedures in processing blood samples for T cell assays.

Step	Recommendations	Level of evidence	Major unknowns	T cell assays used for validation	References
Blood drawing	Fasting state	E		n.a.	[1–20,71,72]
	Same time of the day	E		Whole blood IFN-γ/IL-10 ELISA	[21,22]
	Vacuum tubes or syringes	D		IFN-γ ELISPOT, class I MMrs	[23]
	✓ Sodium or lithium heparin	C		IFN-γ ELISPOT, IFN-γ ICS	[24–26]
	Minimum delay in processing (8–12 h)	C		IFN-γ ELISPOT	[23,24,27–30]
	Room tewmperature storage, gentle agitation	D		IFN-γ ELISPOT, class I MMrs	[23,25,29,32]
	Dilution if storage > 8 h	C		IFN-γ ELISPOT, class I MMrs	[23,29]
Blood shipping	Insulated containers	B		IFN-γ ELISPOT	[33,34]
PBMC preparation	Ficoll ± Leucosep® or CPT™ tubes	D		IFN-γ ELISPOT, class I MMrs	[23,36–38]
			Ficoll preparations?		[35]
	Washes with media containing human serum	D		IFN-γ ELISPOT, class I MMrs	[23]
			Whole blood *versus* PBMCs?		[26,40–42]
PBMC freezing			Freezing media?		[24,53,54]
			Medium temperature?		[35,47,56]
			Caspase inhibitors?		[55]
	PBMC concentration $\leq 3 \times 10^7$/ml	E		n.a.	[53]
	Cooling rate 1°C/min down to −70°C	E		n.a.	
	Prompt transfer to liquid nitrogen (24–72 h)	E		n.a.	[24,53]
	Storage temperature ≤ −132°C (vapour or liquid nitrogen)	E		n.a.	[24,53]
PBMC shipping	Liquid nitrogen (vapour or liquid phase)	D		IFN-γ ELISPOT, IFN-γ ICS	[24]
Long-term preservation	Better if < 6 months	D		IFN-γ ICS	[57]
PBMC thawing	Rapid, 37°C thawing temperature	E		n.a.	[53]
	15–50 ml thawing volumes	E		n.a.	[53]
	280–450 ***g***, 5–10 min centrifugation	E		n.a.	[53]
	DNase treatment if clumping	C		[^{3}H]-TdR, cytokine ELISA	[58]
	1–12 h PBMC resting	C		IFN-γ ELISPOT	[27,46,59,60]
T cell assays	Human serum-supplemented media	E		n.a.	
	Serum-free media	B		IFN-γ ELISPOT	[54]
			Addition of low-dose cytokines (e.g. IL-7)?		[33,34,54]
	High quality protein or peptide antigens	A		[^{3}H]-TdR	[62–67]
	Positive controls: recall antigens and polyclonal stimuli	E		n.a.	

[^{3}H]-TdR: thymidine incorporation; ICS: intracellular cytokine staining; MMrs: human leucocyte antigen multimers; n.a.: not available; PBMC: peripheral blood mononuclear cells; IL: interleukin; IFN: interferon; ELISPOT: enzyme-linked immunospot; ELISA: enzyme-linked immunosorbent assay.

Mallone et al., *Clin Exp Immunol* **163** (2011): 33-49.

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Refrigeration of whole blood and delayed PBMC processing negatively impacts viable lymphocyte recoveries and downstream cellular assays

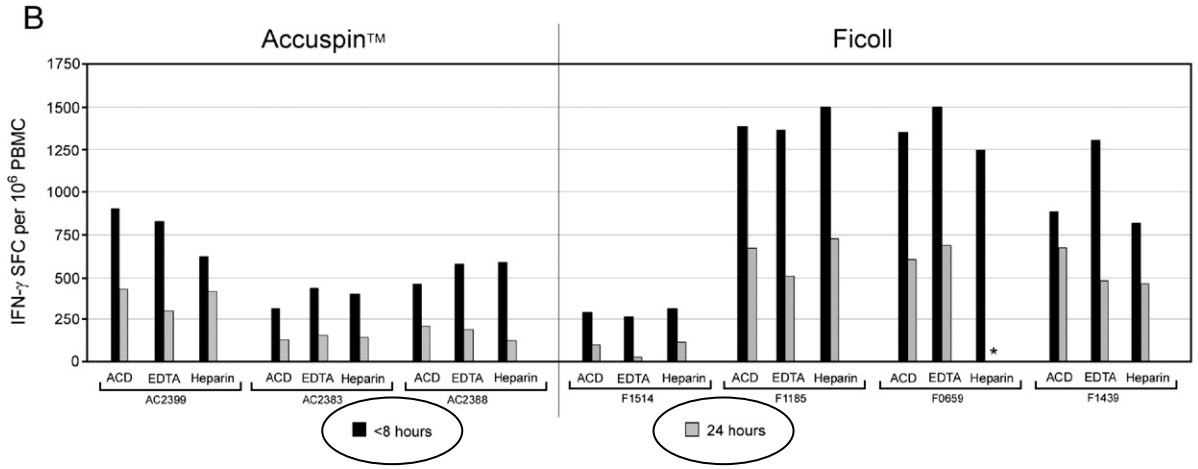
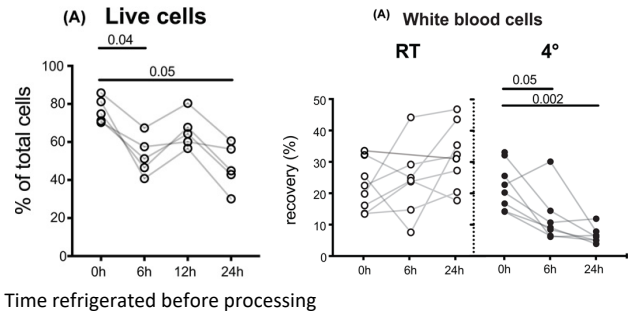
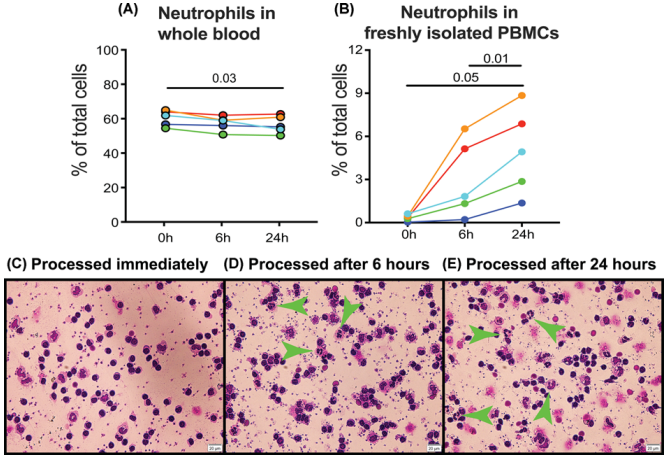
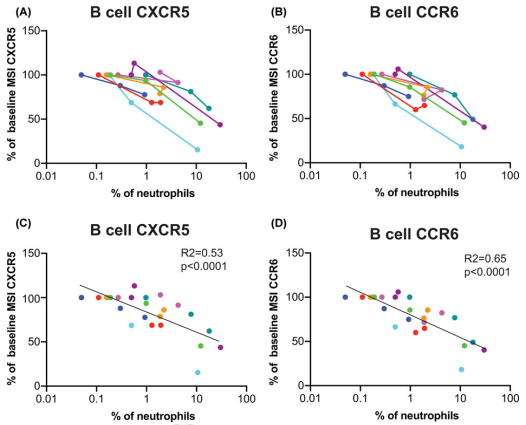


Fig. 3. CMV-specific T cell responses detected by IFN-γ ELISpot in 11 representative donors, stratified by high (A) and low to intermediate (B) IFN-γ SFC frequencies. Blood was taken with one of three anticoagulants (ACD, EDTA, Heparin), and PBMC were isolated by Ficoll centrifugation or with Accuspin tubes. Cryopreservation of PBMC occurred within 8 or 24 h of venipuncture, as indicated. Data shown are background subtracted. *Sample not available.







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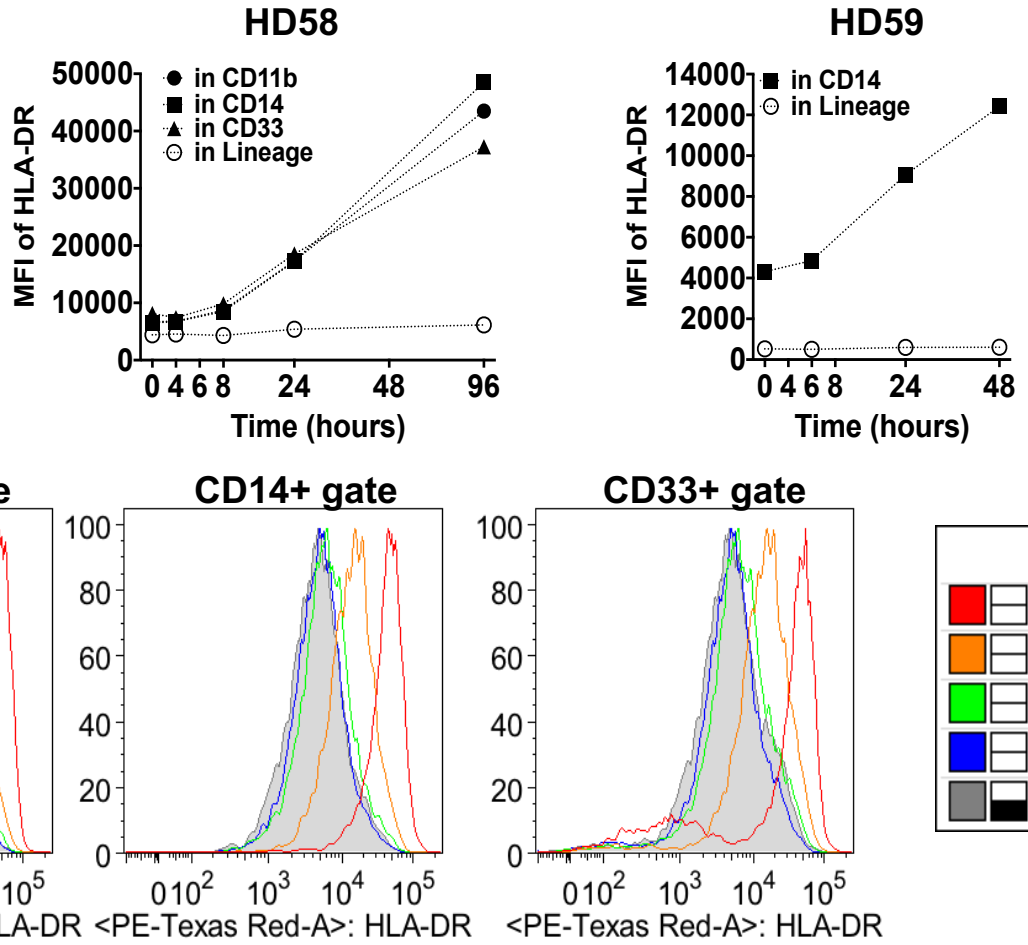
Bull et al., *J Immunol Methods* **322** (2007): 57-69.
Jerram et al., *Bioscience Reports* **41** (2021): 1-15.

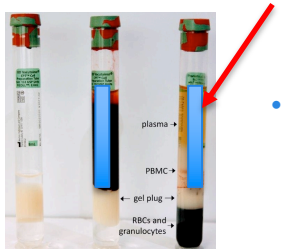
Lack of stability of HLA-DR expression in peripheral blood monocytes for MDSC enumeration



Surface HLA-DR expression on monocytes increases over length of time of blood is kept at RT prior to whole blood processing for PBMC isolation and MDSC analysis

Study Planning Considerations

- Feasibility of implementing the proper sample handling methods?
  - Single- vs. multi-site study
    - Centralized sample processing at a single site (potential delayed processing of offsite samples) vs. a harmonized sample collection method across multiple sites (potential variability across labs)
    - Timing of patient dosing and sample collection to avoid delivery outside of processing lab hours
  - Sample processing & cryopreservation capabilities of central or local lab(s)
    - Experienced laboratory staff that can adhere to established SOP
    - Centrifuges for PBMC separation, TC hoods, controlled rate cooling, temperature monitored LN2 freezers for PBMC storage
    - Ability to count viable cells is critical for optimal freezing concentrations and may provide information re: abnormally low or high cell yields that may be disease- or treatment-related
  - Sample delivery logistics and tracking/inventory management
    - Clear instructions in lab manual regarding collection and transport of samples, proper documentation of sample collection times, clear labeling of collection tubes, proper protection of tubes from breakage, maintenance of correct temperature during transit, active communication between clinic and lab to track samples
    - Maintenance of an accurate clinical sample inventory database with sample IDs, dates of collection/processing, amounts, freezer/box/aliquot locations, documentation of any protocol deviations or sample observations to help with interpretation of data after sample testing



Biomarker Assay Considerations

- Timing of sample analysis
  - Immediate analysis of fresh rather than frozen samples may be more optimal for certain biomarkers and sample types (e.g., TILs) but batch-to-batch run variability may arise when comparing multiple assay runs
  - Interim analysis after a set number of samples are banked may be useful not only for early analysis of biomarker data but also to evaluate quality of banked samples, but need to be aware of sample aliquot numbers at each visit to allow for repeat testing at end of study with all timepoints included for comparison in a single experiment
  - Batched sample analysis at end of study in a single central lab by designated analyst is ideal to minimize inter-lab, inter-assay, and inter-operator variability
- Fit-for-purpose assay validation
  - Verify instrument performance & perform routine maintenance/calibration
  - Understand specificity/sensitivity of assay and limits of quantitation
  - Define positivity and acceptance criteria for results
  - Characterize assay robustness under various sample storage and assay conditions
  - Evaluate potential effects of residual therapeutic drug on assay
    - Non-specific binding of therapeutic Ab to protein microarrays – run assay using drug product alone
    - Interference of therapeutic with staining Abs in flow
  - Assess inter-operator, intra- and inter-assay precision, longitudinal variability of assay
  - Generate quality controls to run in each assay to monitor consistency of assay performance
  - Draft standard operating procedures and data reporting formats, training of analysts
- Rigorous data review after sample testing, bioinformatics support useful

Summary

- Immune monitoring of clinical studies using current assay technologies has the potential to provide both mechanistic insights into the immune response during cancer immunotherapy and to identify clinically relevant biomarkers
- Consistency of implementation of an optimized sample collection and sample testing methodology is critical to ensure accuracy of data
- Biomarker assay requirements and identification of preanalytical factors impacting biomarker stability can inform sample collection strategy
- Proper sample handling and removal of sources of assay variability will enhance the reliability and interpretability of results

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