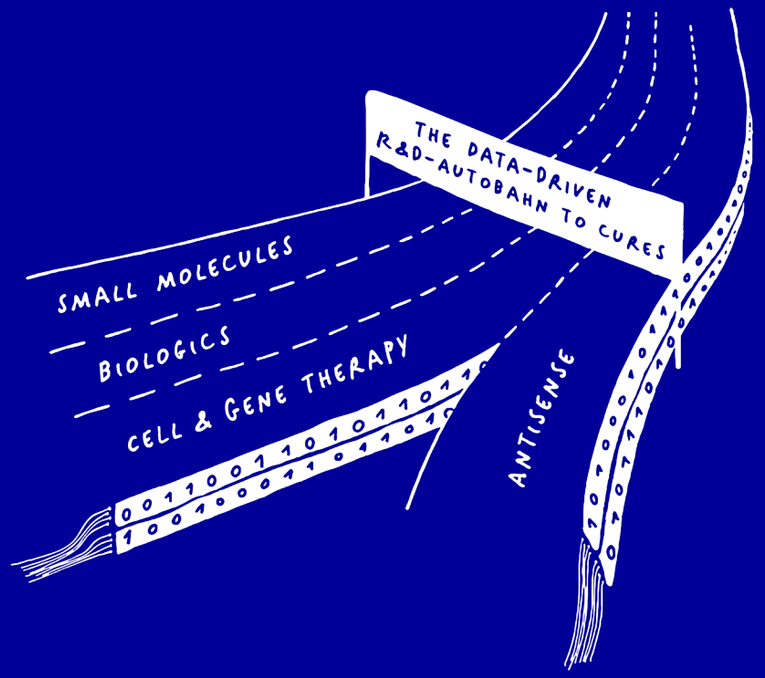
evotec

#RESEARCHNEVERSTOPS

Immuno-Oncology

Harnessing the immune system to fight cancer

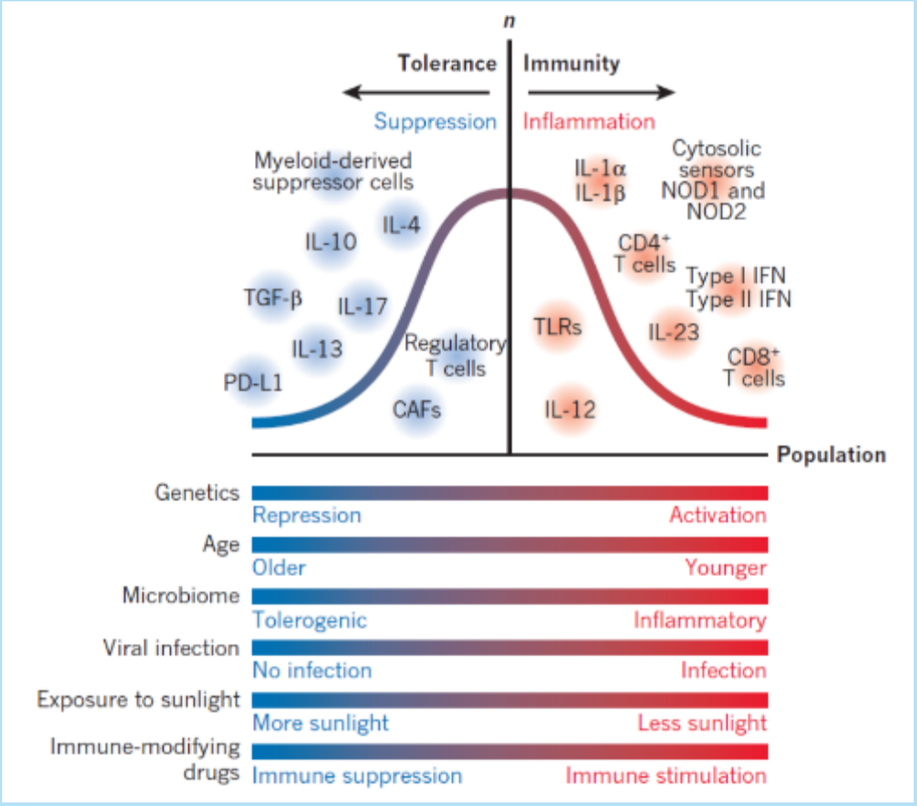


Evotec SE, Immuno-Oncology Therapeutic area, February 2022

The role of the immune system in tumourigenesis

Shifting from Tolerance to Immunity is a multifactorial process

- **Objective of cancer Immunotherapy** is to promote efficient T-cell immunity by shifting from a tolerogenic TME to a pro-inflammatory TME
- **Overcoming Tolerance is:**
  - The pivotal step
  - Most of the time, not achieved by acting on one factor/target (e.g. moderate fraction of patients responding to ICTs (Immune Checkpoint Therapies) around 30%)
- **Shifting the TME to immunity** is leading to strong therapeutic efficacy as observed in patients who respond to ICTs or other immunotherapies

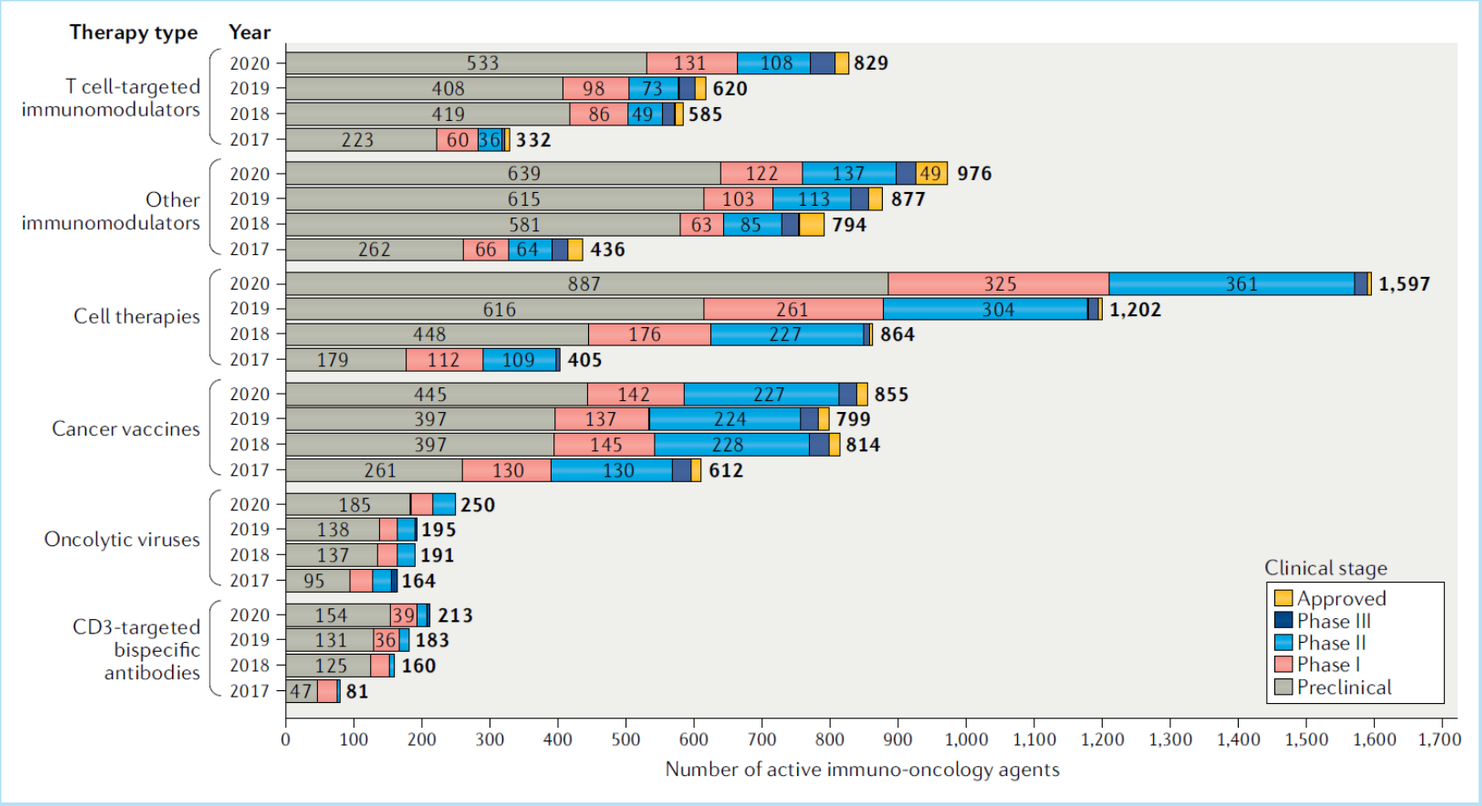


Source: Dan Chen & Ira Mellman Nature 2017
Note: TME (tumour MicroEnvironment)

Immuno-Oncology (IO) drug development is a pillar of cancer treatment

Cancer Immunotherapy is the fastest growing area within Oncology

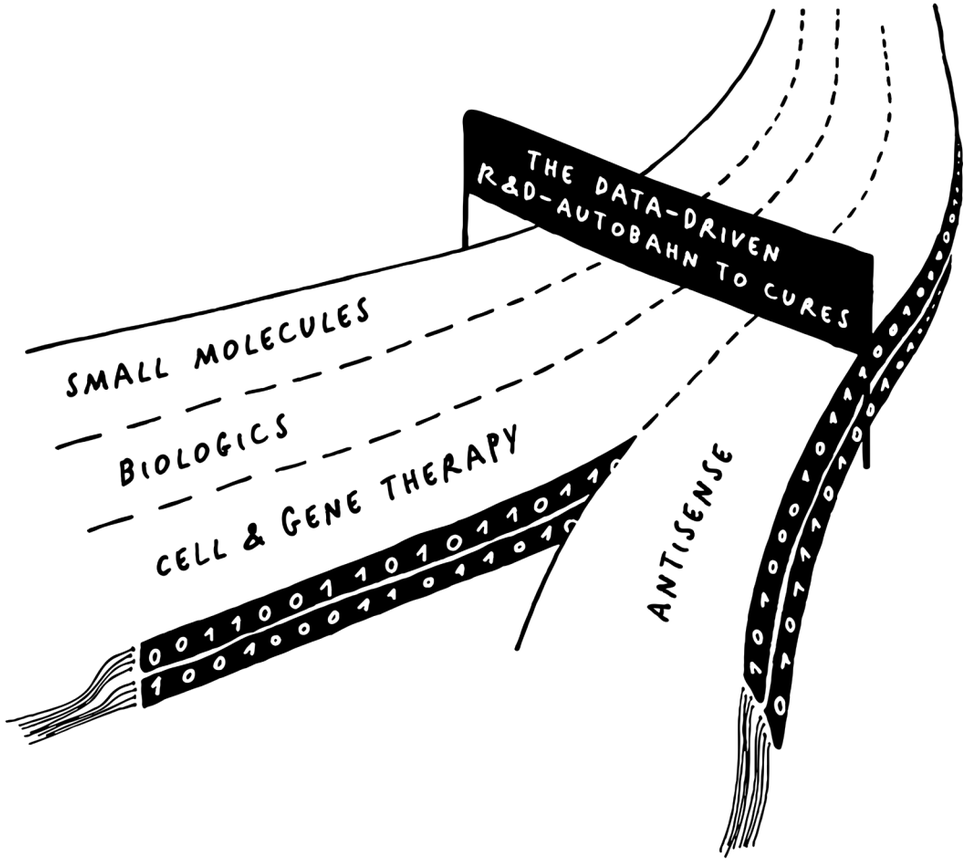
- The number of active IO drugs in development has displayed a **91% increase between 2018 and 2020**
- T-cell targeted immuno-modulators is remaining the top class of IO agents under development (66% of active CT)
- Nevertheless, agents targeting other cell types (NK cells, macrophages, etc.) have increased since 2018 even more than T-cell directed agents
- From 2018 to 2020: **31 approvals by the FDA** for IO drugs
- **Cell therapy** has shown the largest increase from 2017 to 2020 as compared to other IO agents



Source, Upadhaya S. *et al. Nature Rev. Drug Discovery* 2020

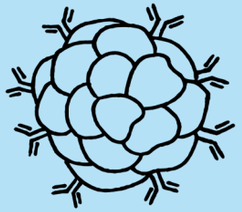
Evotec: a hub for Drug Discovery in a multi-therapeutic modalities fashion

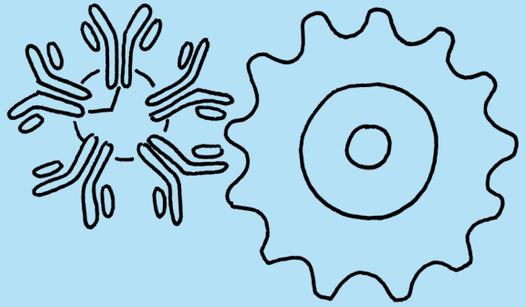
Immuno-Oncology agents are fitting within these different categories



Evotec Immuno-Oncology experts have experience in supporting drug discovery of IO agents for:

- Small molecules
- Antibodies
- Bispecific Antibodies
- ASO (Antisense oligonucleotide)
- Cell Therapy
- Cancer Vaccines
- Biologic therapeutic (e.g. therapeutic protein)



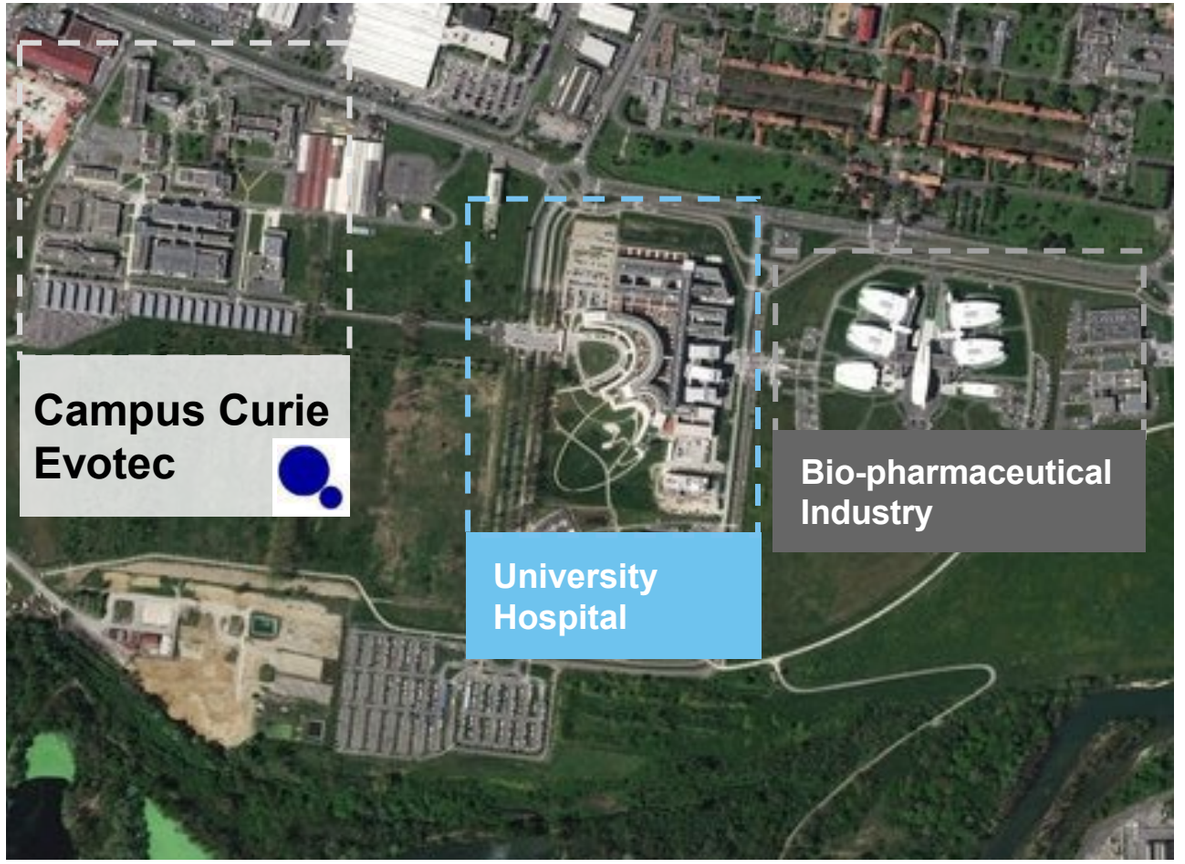


The Evotec-Oncopole Collaboration: Combining Medical and Research Excellence

Accelerated R&D in Oncology through our close working relationship

Oncopole is a highly recognized University hospital (~500M€ public funding)

- Combining medical and research excellence (IUCT, CRCT) in Oncology
- Incubator for midsize pharmaceutical and biotech companies
- **Example of working together:**
  - Kazia (EVT801) supported by Evotec, has started to enroll oncology patients (Nov 2021) for a phase I at Oncopole
  - Exploratory biomarkers evaluation are performed by Evotec



47 persons within the Immuno-Oncology Therapeutic area

Significant and growing expertise overtime

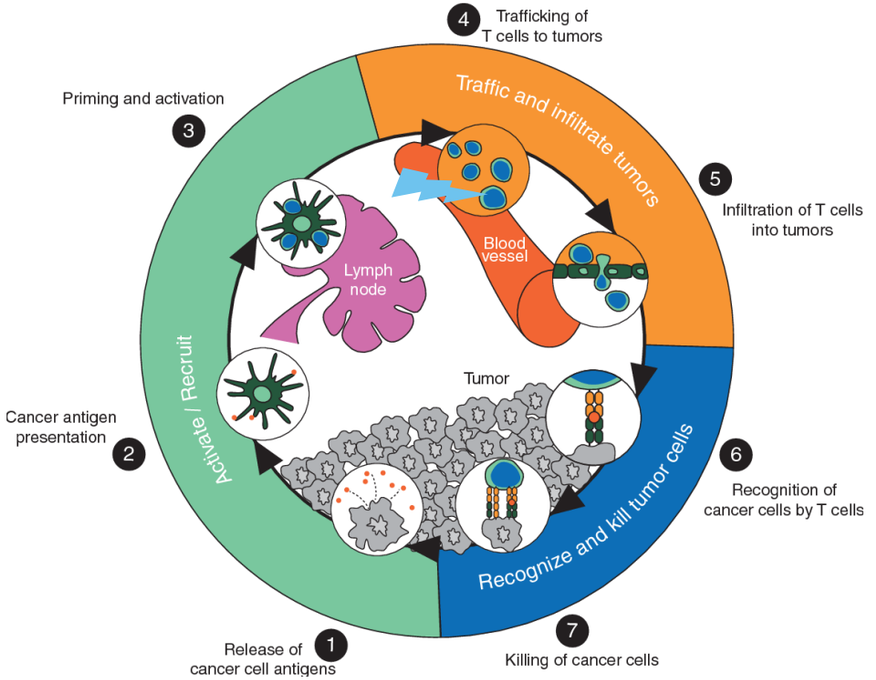
Large panel of expertise & experience in various fields of Immunology and Immuno-Oncology acquired from initial training and/or past experience in academia or in pharmaceutical/biotechnology industry

How we are supporting IO agents Drug Discovery

Of mice and men: a drug discovery continuum including cancer patients samples

- In-depth immunology knowledge on various immune cell types and targets with overall integration within the anti-tumour immune response process
- Development of tailored *in vitro* functional **immunological assays with primary immune cells** for agents evaluation
- ***In vivo* preclinical mouse models** to evaluate IO agents as single agents or as combination therapy with evaluation of therapeutic efficacy and immuno-modulation
- Translational assays using samples from **cancer patients**
- Possibility of genetic editing of primary immune cells by **CRISPR** technology
- **Whole blood** functional assays for on-target biomarker strategy
- Evaluation of **immune-related toxicity** with different animal models

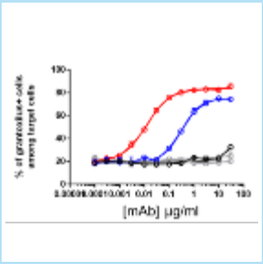
The cancer immunity cycle



Source: Kim JM & Chen DS Annals of Oncology 2016

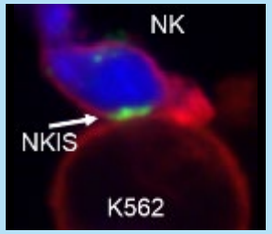
Immuno-Oncology: *in vitro* & *ex vivo* focus

A cutting edge technology platform to make a deep dive in MoA



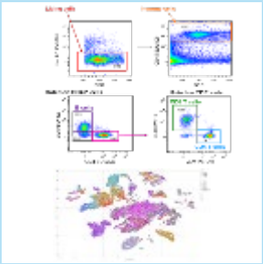
Functional *in vitro* Immunological assays

- Supporting small molecules, biologics and cell therapy programs
- T-cells ($\alpha\beta$ & $\gamma\delta$), Treg, NK cells, B-cells, Neutrophils, M1/M2, Dendritic Cells, MDSCs
- Proliferation, cytokines production, killing, tracking of surface markers, suppression assay



Visualising Immune cells "in action" at the contact of tumour cells

- Evaluation of IO products at the single-cell level monitoring Immunological Synapse
- Quantification of the data using Metamorph software



Filling the gap in drug discovery by accessing cancer patient samples

- Complex flow-cytometry based analyses on fresh human tumour resections
- Functional assays on the blood for target engagement validation, etc.
- Additional technologies for biomarkers identification: scRNAseq, proteomics, metabolomics, etc.

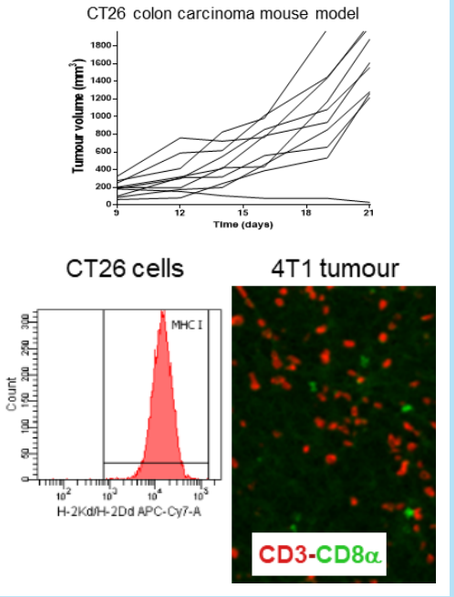
Immuno-oncology: *in vivo* focus

Building together a tailored approach for your drug discovery project

PK Parameter	Intravenous	Oral
Dose (mg/Kg)	1.83 ± 0.04	4.07 ± 0.02
C_0 / C_{max} (ng/mL)	492 ± 25	206 ± 80
T_{max} (h)	-	4
V_{ss} (L/Kg)	6.9 ± 0.3	-
CL (mL/min/Kg)	34.6 ± 0.3	-
Liver Blood Flow (%)	48.1 ± 0.5	-
AUC_{inf} (ng.hr/mL)	880 ± 28	1248 ± 380
Bioavailability (%)	-	64 ± 0.2

PK/PD and toxicity studies

- **PK parameters** (C_{max}, AUC, …) and **PD readouts** with biostatistical support on tumour-bearing mice
- **Compound blood exposure:** bioanalysis (mass spectrometry, ELISA)
- **Determination of:** type and severity of injury, MTD, NOAEL, dose-exposure relationship, etc.



Therapeutic efficacy studies

- **Human xenograft models:** s.c. & orthotopic models in **humanized mice** (BRGSF-his and huNOG)
- **Syngeneic tumour models:** s.c. & orthotopic models in immunocompetent mice

- **General evaluation and Clinical pathology:** clinical signs, body weight, food consumption, hematology (RBC and WBC counts)
- **Tumour growth:** digital caliper system, *in vivo* imaging (bioluminescence, fluorescence), X-ray imaging, laser Doppler, quantitative image analysis
- **Analysis of the Tumour Micro-environment (TME):** flow cytometry, IHC
- **Functional T-cells assay:** IFN-γ ELISpot, murine/human cell sorting
- **Broad range of sample analysis** (blood, urine, organs and tumours): gene/mRNA/protein analysis, histology/histopathology/IHC, mass spectrometry (DMPK and metabolite follow up), phosphoprotein analysis (MSD & HTRF technology, western blot, ELISA), custom assay development

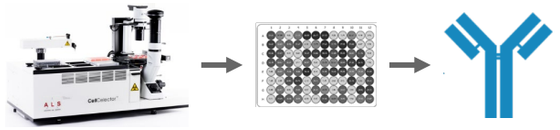
Evotec antibody discovery platforms to support Immuno-Oncology discovery

Fully integrated solutions to develop novel Biologics for Cancer Immunotherapy

In Vivo Antibody Production
Evolved for affinity

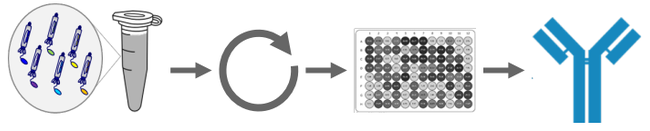
Alloy Therapeutics [1)]

Immunisation with target of interest, followed by harvesting of splenocytes



Hybridoma Technology
State-of-the-art hybridoma generation and high-throughput screening

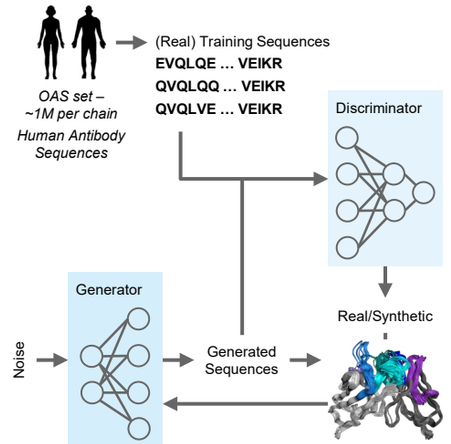
Fully established hybridoma platform



Phage display: immune Antibody Libraries
Phage display lab for the exploration of natural immune repertoires (or naive human library)

Established Phage Display platform

In Vitro Antibody Production
Designed for diversity, humanness & developability



Humanoid Antibody Library (J.HAL^{SM})[2)]
Large, diverse, manufacturable & developable discovery libraries with machine-learned biasing

Available

[1)] https://www.evotec.com/en/invest/news--announcements/p/evotec-partners-with-alloy-therapeutics-to-expand-its-antibody-discovery-platform-6010

[2)] Designing Feature-Controlled Humanoid Antibody Discovery Libraries Using Generative Adversarial Networks: https://www.biorxiv.org/content/10.1101/2020.04.12.024844v2

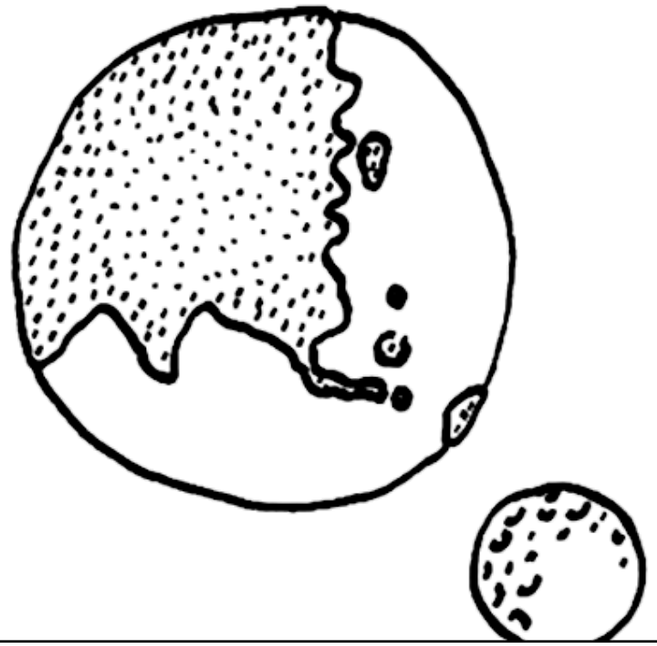
Agenda

Translational Immunological assays with cancer patient samples

Adaptive Immunity

Innate Immunity

Preclinical mouse models



Flow Cytometry at Evotec

A Flow cytometry core facility with powerful instruments

Cell Analysers

BD FACS Canto II
10 parameters (3 lasers)
Plate sampler (96 & 384)

Toulouse and Hamburg

BD Fortessa X20
20 parameters (4 lasers)
Plate sampler (96 & 384)

Toulouse

Biorad ZE5
30 parameters (5 lasers)
Plate sampler (96 & 384) and tube loader

Toulouse

BD Symphony
30 parameters (5 lasers)
Plate sampler (96 & 384)

Toulouse
(Delivered October 2020)

Intellicyt ique
13 parameters (3 lasers)
High-throughput flow cytometer
Plate sampler (96 & 384)

Hamburg

Evotec cell Sorters

BD FACS Melody
9 parameters (3 lasers)
Cell sorter

Hamburg

autoMACS Pro separator
Automated cell isolation
Magnetic cell separation

Toulouse and Hamburg

Other Cell Sorters available in the Toulouse hospital platform

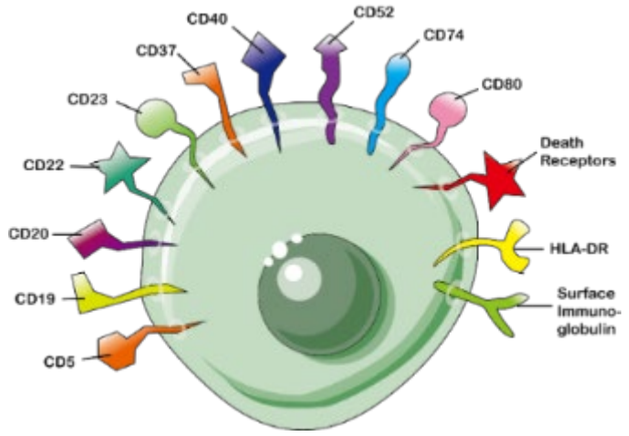
- FACSAria-Sorp (3 lasers)
- FACSAria-II (3 lasers)
- FACSAria-Fusion (5 lasers)

Strong knowledge in Immunology

- Human
- Mouse
- Rat

Complex phenotypic and functional analyses

- FlowJo software
- Diva software

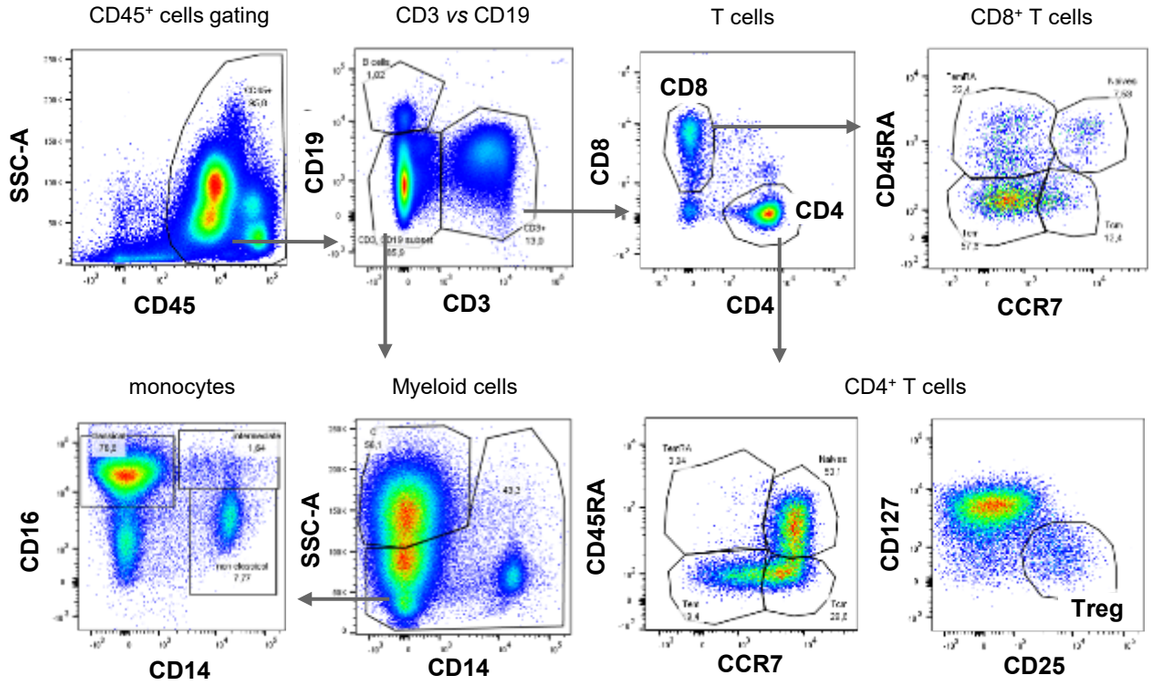


Flow cytometry analysis on blood (healthy donors or cancer patients)

High quality and multi-parametric staining on whole blood or PBMCs

- Staining performed on PBMCs or whole blood
- **Working directly on whole blood:** optimal approach for clinical immunomonitoring as it requires low volume of blood
- Analysis of immune cell subsets within **whole blood functional assays**
- Several flow cytometry panels have been set up (12-18 colors)
- New flow cytometry panels can be developed from scratch as needed for the project
- Possibility to collect and freeze plasma for additional analysis (e.g. proteomics, etc.)

Example of whole blood analysis



Access to fresh and pertinent samples of tumours & circulating blood from patients

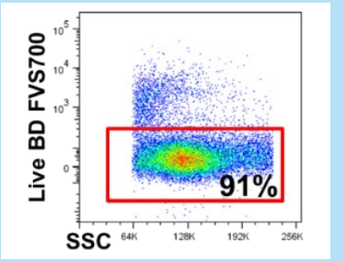
Flow cytometry analysis and single cell mRNA sequencing

Access to healthy tissue, tumour resection & blood

Transport to Evotec (<24h)

Tissue dis-sociation

Living cells

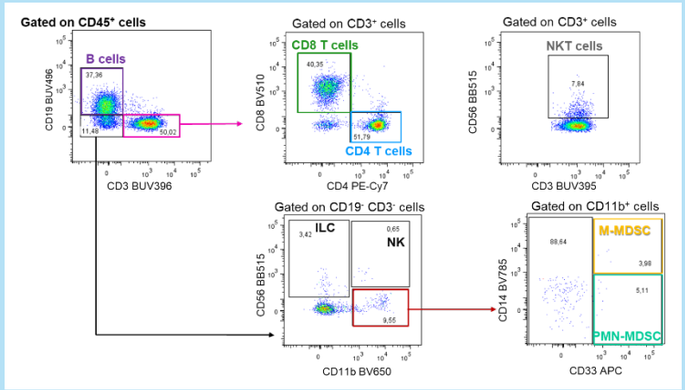


- **Flow cytometry**
- **scRNAseq**
- **3D culture**
- **etc.**

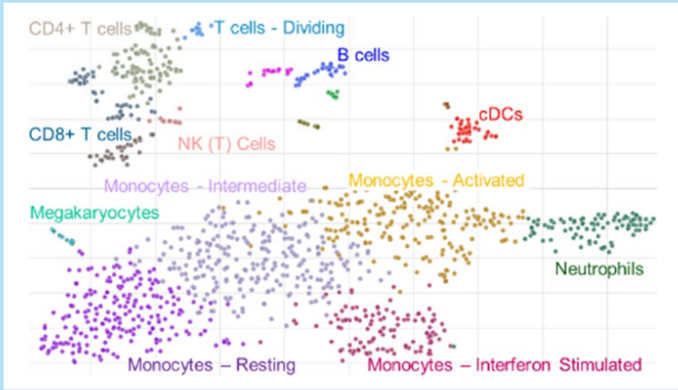
Supernatants

Proteomics

Flow cyto-metry



Single cell mRNA sequencing

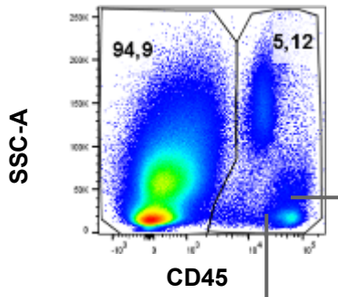


- Well established protocols for analysis of cancer immune phenotypes in patients samples
- Possibility to study the tumour secretome (by ELISA, HTRF, proteomics)

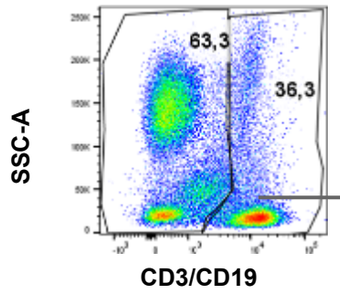
Flow cytometry analysis of patient freshly resected tumour

Flow cytometry analysis of immune cells in a human lung tumour

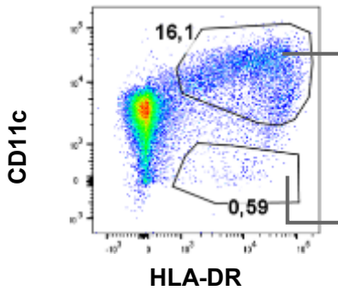
Gated on viable cells



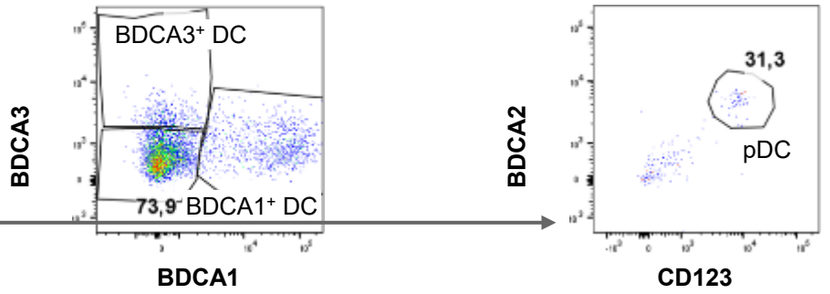
Gated on CD45⁺ cells



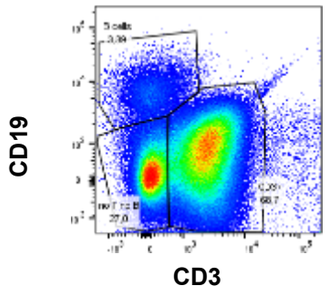
Gated on myeloid cells



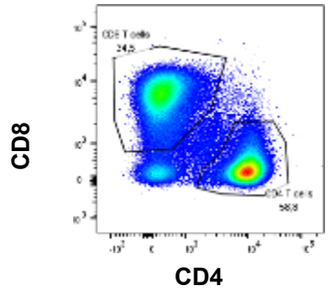
Dendritic cells



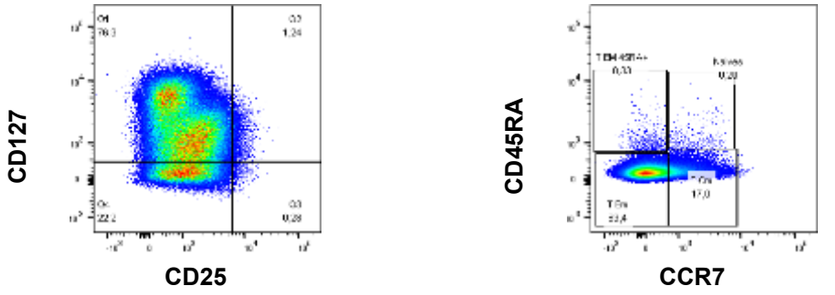
Gated on CD45⁺ cells



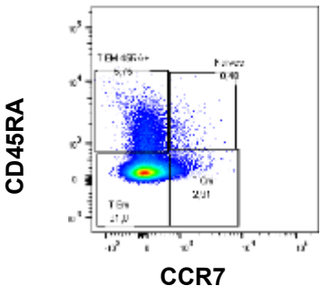
Gated on CD3⁺ cells



CD4⁺ T cells



CD8⁺ T cells



Access to fresh and pertinent samples of blood from patients with hematological malignancies

Well-established workflow for blood samples and clinical associated data

Access to healthy & patient with hematological cancer blood

- Access to specific clinical data
- Follow-up of patients: in 2022
- Access to thematic biobank

Transport to Evotec (<24h)

- Fresh blood
- Serology at day 1 post sampling

Whole blood

Plasma

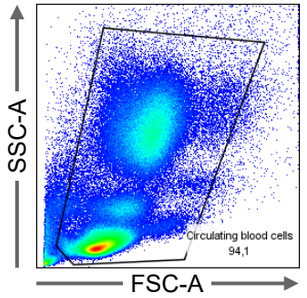
PBMCs

Starting material suitable for phenotypical & functional assays

- Flow cytometry (incl. whole blood staining)
- Live cell imaging (Incucyte)
- scRNAseq
- Cytokine dosage (MSD)
- Metabolomics
- Proteomics
- …

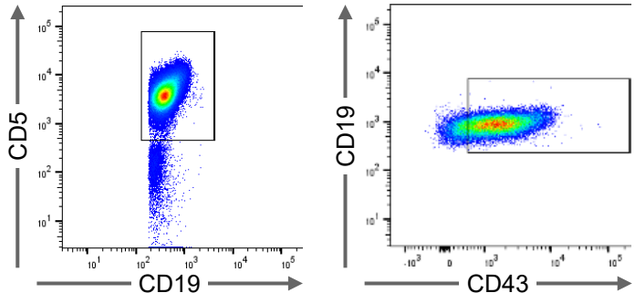
Sample quality
(high viability)

Whole blood staining by FACS



High number of tumour cells
(>50M/patient B-CLL)

B-CLL markers staining on patient-derived PBMCs by FACS

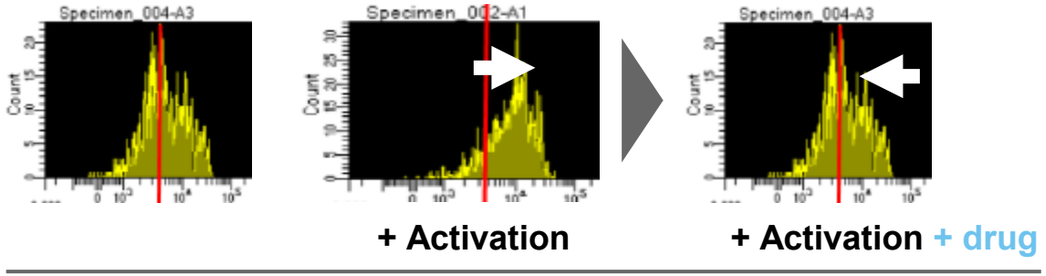


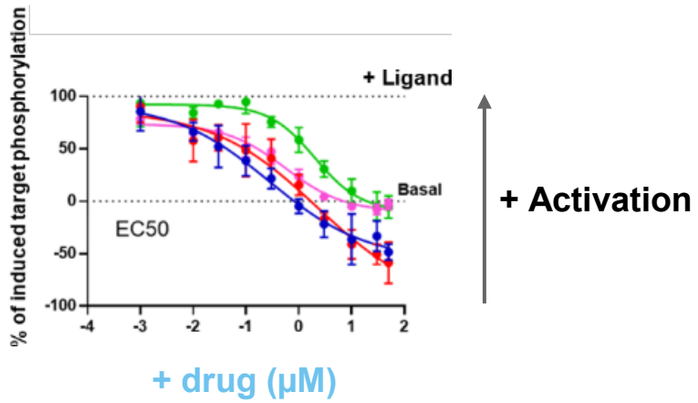
Target Engagement Biomarker assay developed by Evotec and used during EXS21546 clinical trial

FACS-based functional T-cell assay done on whole blood

- **Background:** Develop a target engagement assay to demonstrate that EXS21546 is mechanistically active at the right dose
- **Experimental settings: Flow cytometry analysis**
  - Human whole blood
  - Identify drug efficacy on *ex vivo* activated CD8⁺ T-cells
- **Outcome:** dose-dependent inhibition of activated CD8⁺ T-cells by drug validated in:
  - Blood from healthy subjects
  - Blood from patients with high grade cancer

p-target staining on CD8⁺ T-cells for one healthy donor





Evaluating target engagement on human whole blood (biomarker for the clinic)

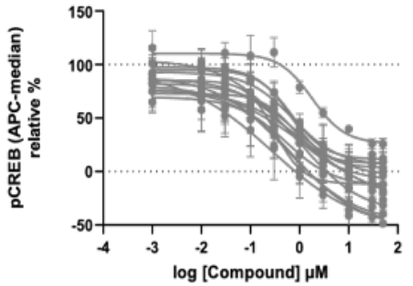
EXS21546, a non-CNS penetrant $A_{2A}R$-selective antagonist for anti-cancer immunotherapy

AACR 2021
Presentation #1731
(Extract)

EXS21546 demonstrates potent target engagement in lung cancer patient blood samples

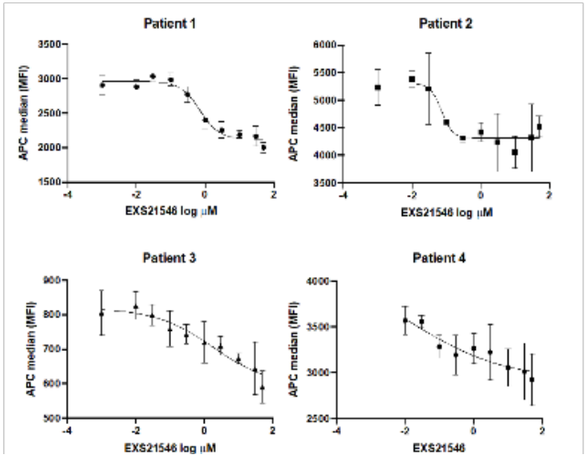
Whole blood was collected by Respiratory Disease Department at Toulouse Cancer Hospital from 4 patients with metastatic lung adenocarcinoma at the time of progression after chemotherapy (n=3, naïve from ICT treatment) or after ICT (pembrolizumab) treatments (n=1). Blood was pre-treated with EXS21546 for 1h prior to stimulation with the adenosine receptor agonist, NECA. Activation of adenosine signaling was measured by quantification of phosphorylated CREB (pCREB) in CD3+CD8+ T-cells in the flow cytometry.

EXS21546 target engagement in healthy donor whole blood samples

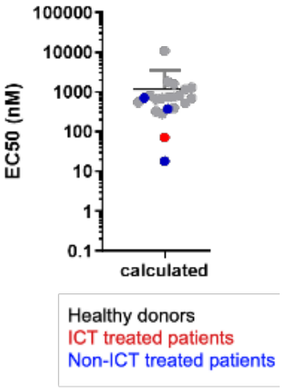


Human p-CREB	EXS21546
Number of donors	15
Calculated EC_{50}	726 nM

EXS21546 target engagement in cancer patient whole blood samples



EXS21546 potency in healthy donors and cancer patient samples

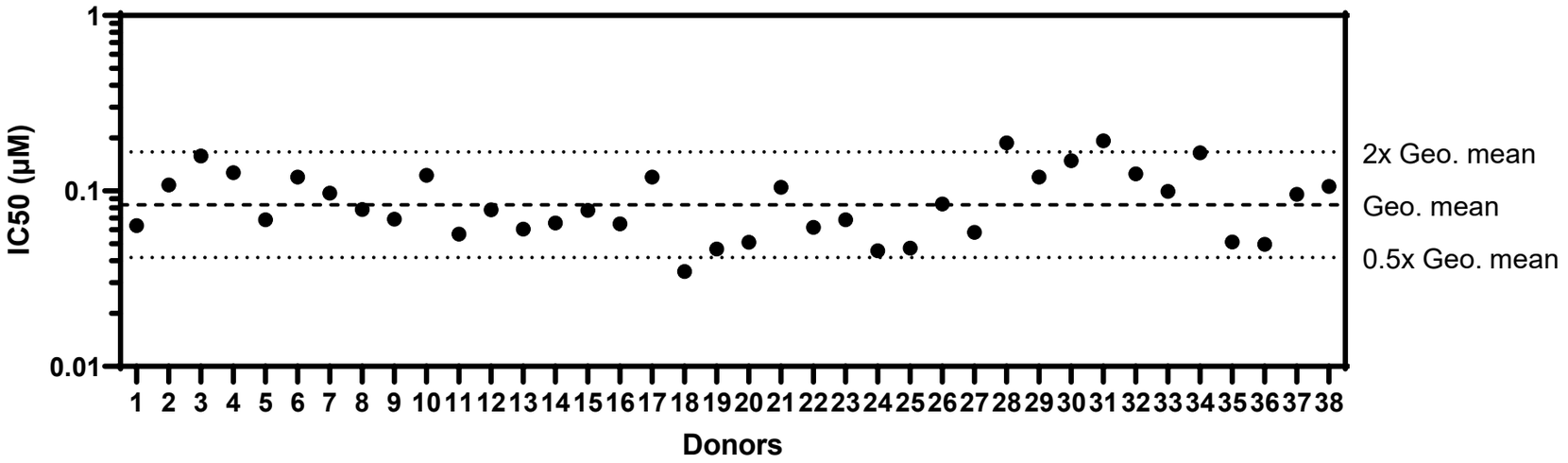


Evaluating T-cell activation on human whole blood

Cumulated IC_{50} values for one reference compound X

- **Protocol**
  - 2-fold diluted human whole blood
  - Coated aCD3 Ab
  - Soluble aCD28 Ab
  - Duration: 24h
  - IL-2 released in supernatant measured by MSD
  - Compound tested in a 8-point semi-log dilution dose study
- **Overall conclusion**
  - Reproducible data across multiple donors
  - IC_{50} values in the range of geo. mean ± 2x geo. mean for >92% of donors

Compound X



Cytokines/chemokines evaluation in cancer patients samples

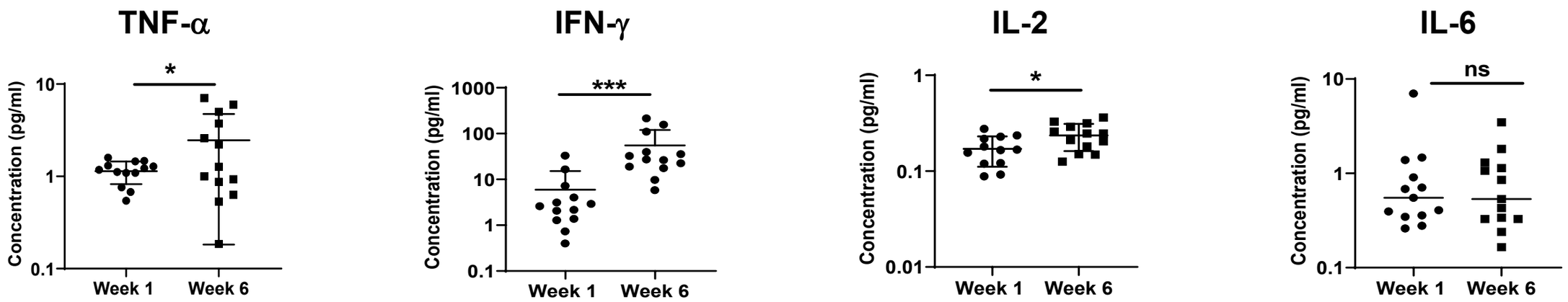
Exploratory immunomonitoring within a Phase 1b clinical trial

Objective: Evaluation of cytokine level by MSD in human plasma samples from patients included in clinical trials

Sector S600

V-PLEX Proinflammatory Panel 1 Human Kit

IFN-γ	IL-2
IL-10	IL-4
IL-12p70	IL-6
IL-13	IL-8
IL-1β	TNF-α



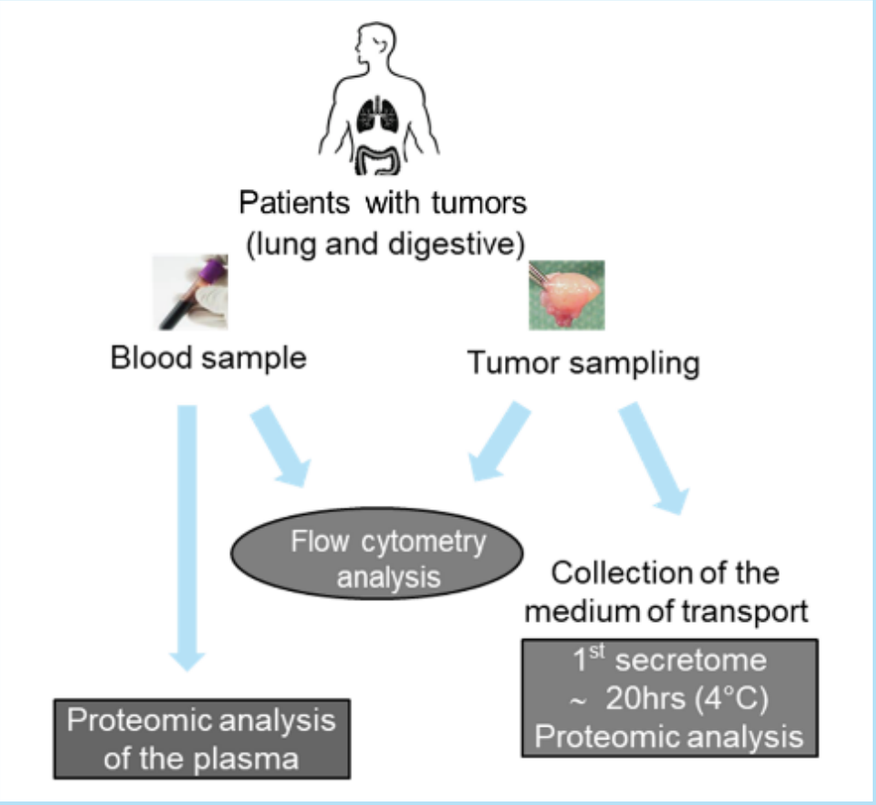
Data from this work were included in Montfort A *et al. Clinical Cancer Research* 2020

Biomarkers identification in freshly isolated patients' samples

Identification of biomarker(s) associated to a tumour phenotype

Analysis of blood and tumour samples coming from the same patient

- Characterisation of the circulating immune cells and of the Tumour Micro Environment by flow cytometry
- Identification of the proteins of the plasma and secreted by the tumours (secreted in the medium of collection of the tumour) from these samples
- Analysis and comparison of the flow cytometry and proteomic data to identify circulating biomarker(s) produced by the tumours and correlate them to the TME phenotype



Pathway investigation in human frozen tumours

Analysis of targets/pathways

Fresh or frozen human tumour sample

Medical data associated to the sample

Metabolite quantification (200 mg)	**Total RNA sequencing** (50 mg)	**Proteome analysis** (50 mg)	**Western blot analysis** (50 mg)	**IHC ISH** (slides)

Characterisation of cancer immunosuppressive pathways correlated with the medical history of the patient

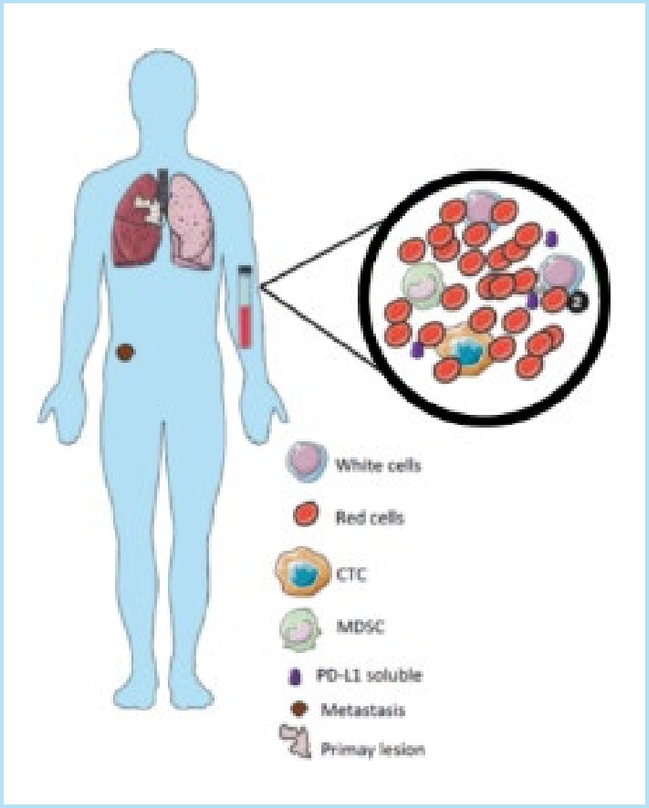
- **Objective:** Evaluate target/pathway markers to establish correlations and validate biomarkers
- **Requirement:** Prospective collection of larger tumour amounts (500mg) from specific cancers

Circulating biomarkers to predict outcome of patients treated with ICT for lung cancer

A case study in collaboration with Oncopole, CRCT and Evotec

IMMUNOPREDICT Trial-NCT02827344[1)]

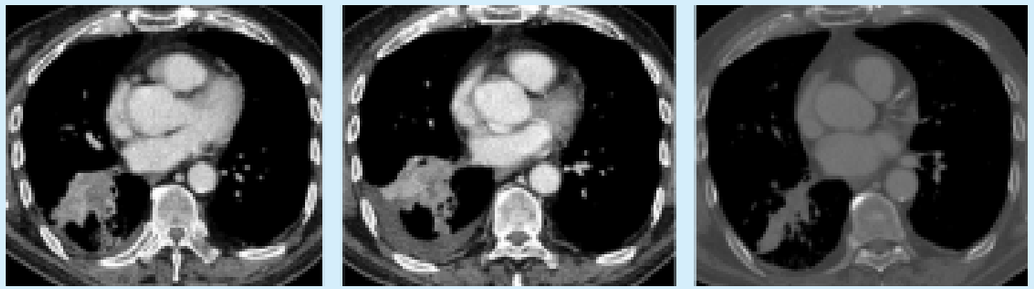
- Clinical database, genetic profile and patient outcome in **a fully annotated prospective cohort of NSCLC patients**
- **Blood sampling during treatment (dose 1/2/4)**
  - Quantification of circulating MDSC/ circulating tumour Cells/ circulating $CD33^{+}/PD\text{-}1^{+}$ were performed at the university
- **Primary tumour/biopsy resection**
  - Gene signature Immuno-onco (Nanostring technology)
  - Histology H&E and PDL-1 labelling



NSCLC patients eligible for immune-checkpoint therapies

ICT Dose 1	**Read-out 1**
Dose 2	**Read-out 2**
Dose 4	**Read-out 3**

Patient classification for response to immune-checkpoint therapies

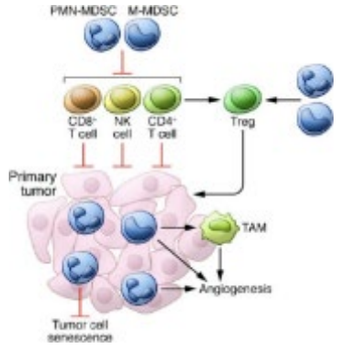


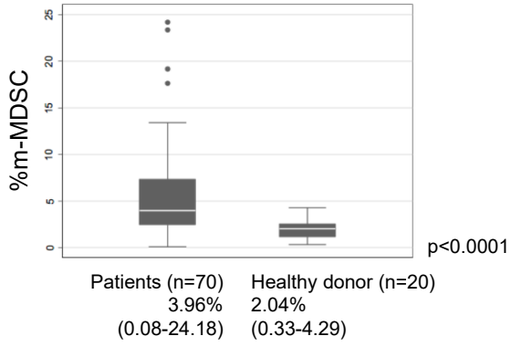
182 patients were integrated into Immunopredict prospective study

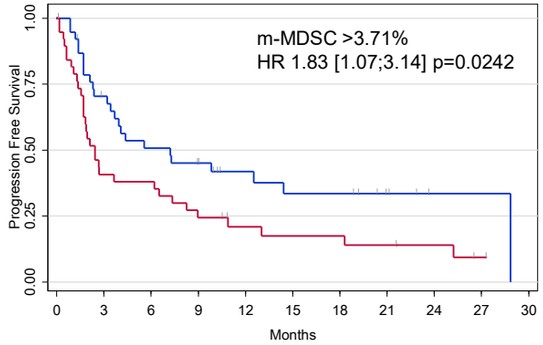
[1)] IMMUNOPREDICT trial NCT02827344 – Pr. J. Mazières, Dr A. Pradines, Dr N. Guibert, Dr M. Delaunay, Pr G. Favre
ICT: Immune Checkpoint Therapy
Marvel and Gabrilovich, J clin invest 2015

Biomarker of activity – circulating immune cells

Quantification of m-MDSC on patients with lung cancer treated with ICT[1)]







Progression free survival as function of %m-MDSC

%m-MDSC<=3.71%
%m-MDSC>3.71%

Analysis of three sub-population of MDSC in 70 patients & 20 healthy donors

- Early stage: e-MDSC
  - $LIN^-/CD14^-/CD15^-/HLADR^-/CD33^+$
- **Monocytic: m-MDSC**
  - $HLADR^{low}/CD14^+/CD11b^+$
- Polymorphonuclear: PMN-MDSC
  - $HLADR^{low}/CD14^-/CD15^+/CD11b^+$

Patients with medium/high progression: visit @ 2 months

	Progression	No progression	
% m-MDSC n= median (Range)	**5.74** 68%>5.74%	**3.45**	p=0.0215

- Higher level of m-MDSC in patients decrease Progression Free Survival
- Patients with medium/high progression @ 2 months have a strong level of m-MDSC
- Progressor patients with m-MDSC > 5.71% is the population of interest for EVT801

[1)] IMMUNOPREDICT trial NCT02827344 – Pr. J. Mazières, Dr A. Pradines, Dr N. Guibert, Dr M. Delaunay, Pr G. Favre
Marvel and Gabrilovich, J clin invest 2015
MDSC: Myeloïd Derived Suppressor Cell

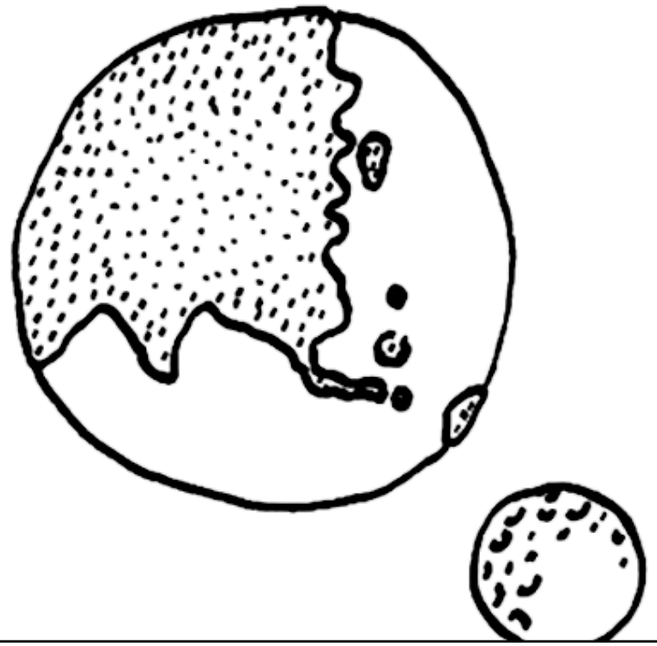
Agenda

Translational Immunological assays with cancer patient samples

Adaptive Immunity

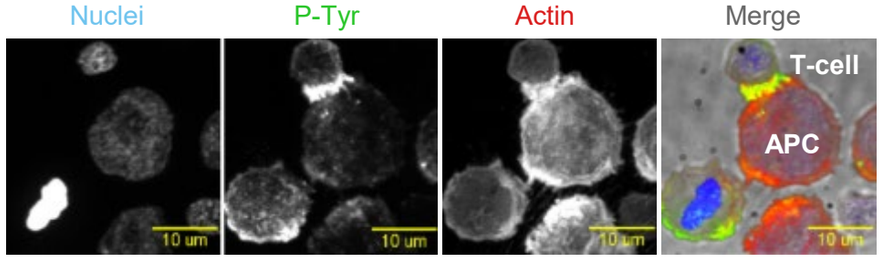
Innate Immunity

Preclinical mouse models



Evaluation of Immunotherapies at the single immune cell level

Visualisation of the human T-cell/APC interaction by confocal microscopy

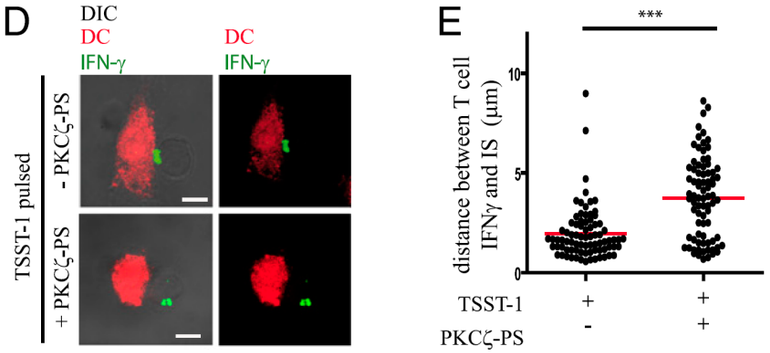


The Immunological Synapse (IS): a specialised interaction between **T-cells & APC** (Antigen Presenting Cells) or **T-cells and tumour cells**

- **Quantification** of the morphological data with the Metamorph software and associated statistical analysis
- **Evaluation of compounds/Ab** in the IO area modulating activation of T-cells when interacting with either APC or tumour cells

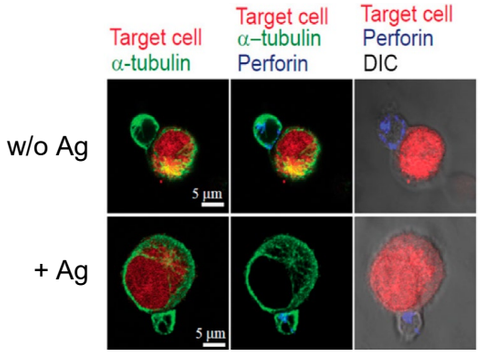
Pharmacological blockade of the PKCζ pathway in T-cell activation

Bertrand F., Esquerré M. *et al.* J Immunol 2010



CTL-mediated killing of tumour cells

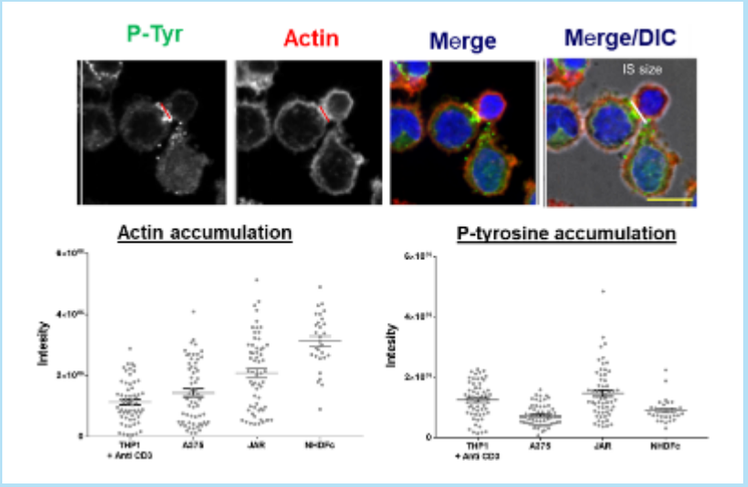
Bertrand F. *et al.* PNAS 2013

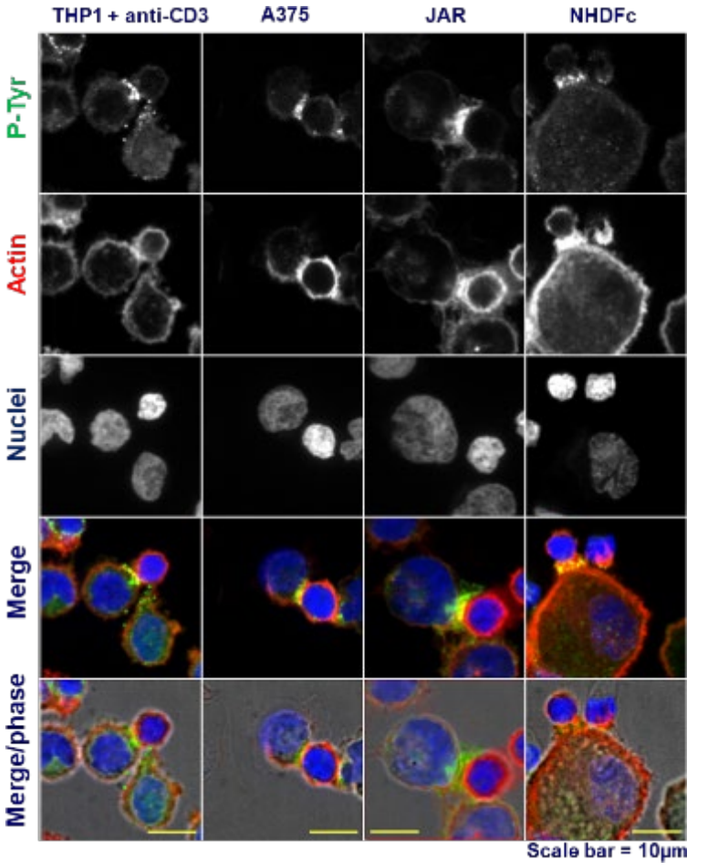


Evaluation of Immunotherapies at the single immune cell level

Case study: T-cells interaction with tumour cells at the single-cell level

- **Co-culture of T-cells with different cancer cell lines** → analysis of early immunological synapse formation after conjugation with tumour cells
  - Images were acquired with a confocal spinning disk microscope, oil immersion objective 100X
  - Settings (Laser power, camera gain) were perform for optimal noise/signal ratio without saturation
  - Acquisition of 30 conjugates per condition for each donor
- IS stability and IS productivity with quantification of the data using Metamorph software (Linescan function)



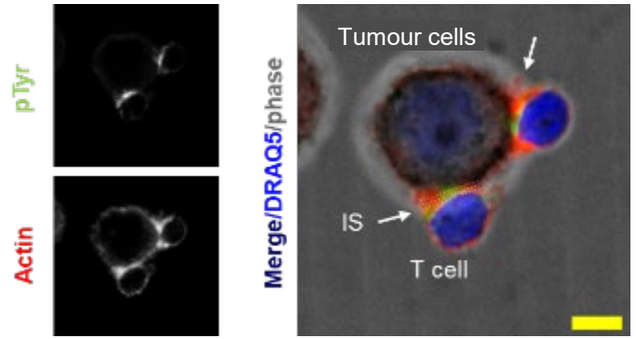


Combining confocal imaging and flow cytometry for in-depth analysis of the Immunological synapse

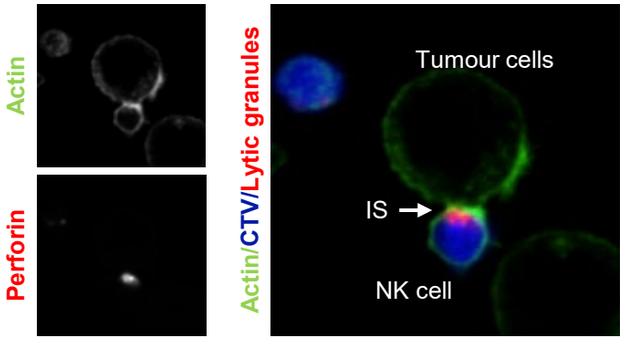
Evaluation of Immune Cell Engagers with T-cells and NK cells

Confocal imaging

IS stability (actin) and productivity (p-Tyr)

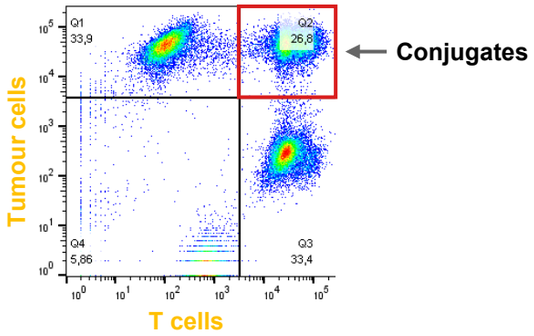


IS lytic function

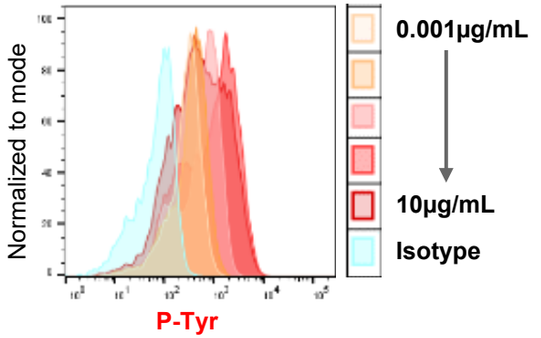


FACS

IS stability % of T cells in conjugate



IS productivity P-Tyr staining in CD8⁺ T cells

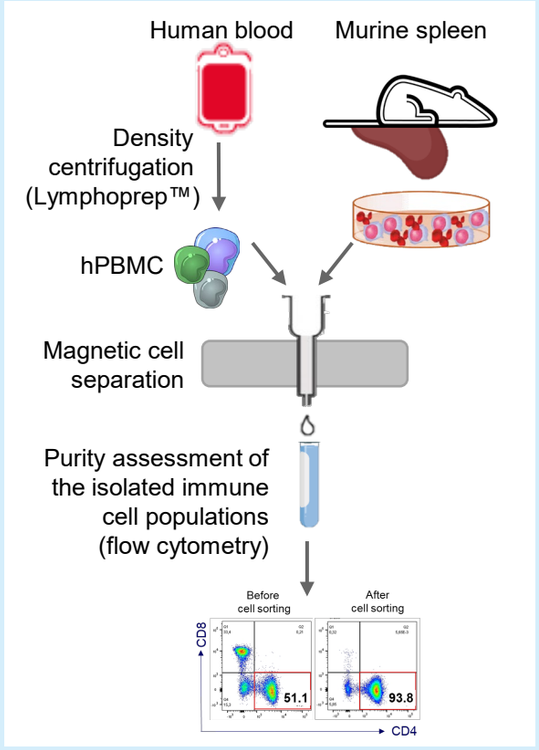


Note: IS (Immunological Synapse)

Isolation of immune cell populations in mouse models

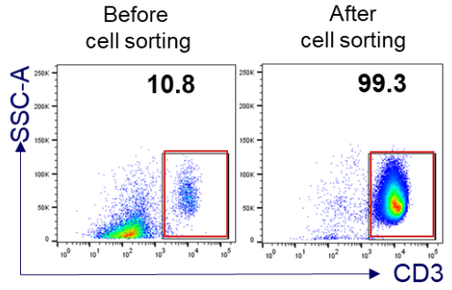
Cell sorting of immune cell populations for functional assays

Protocol

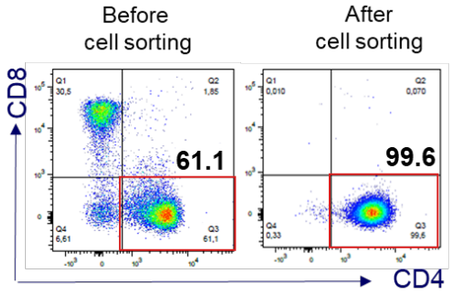


Isolation of highly pure immune cell populations allows *ex vivo* or *in vivo* functional evaluation of the sorted cells

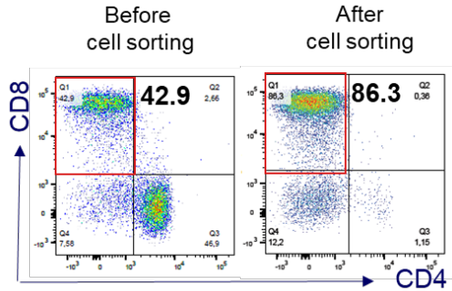
CD3⁺ T-cell sorting on hPBMC



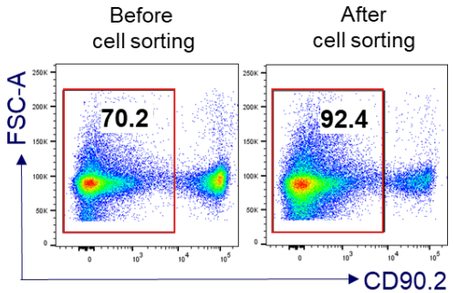
CD4⁺ T-cell sorting on hPBMC



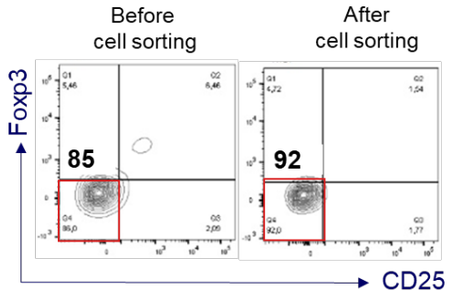
CD8⁺ T-cell sorting on hPBMC



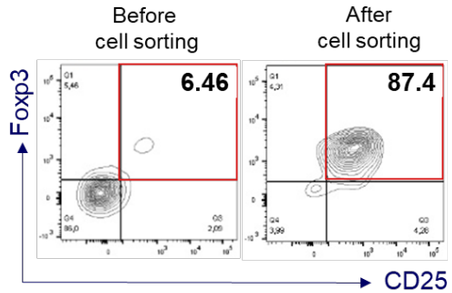
APC (CD90.2⁻) cell sorting on naive mice



T effector (CD4⁺ CD25⁻) cell sorting on naive mice



T regulator (CD4⁺ CD25⁺) cell sorting on naive mice

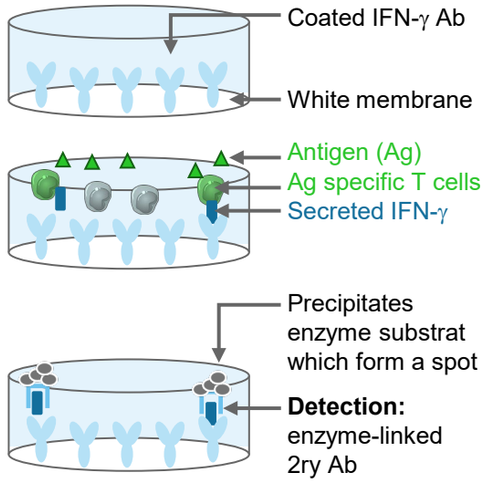


Monitoring of antigen-specific T-cells responses

IFN-γ ELISpot for detection of low-level T-cells responses

Principle

The enzyme-linked immunospot (ELISpot) is a sensitive technique for the detection of cytokine-producing cells at the single cell level. This assay permits the direct enumeration of low-frequency antigen-specific T-cells.

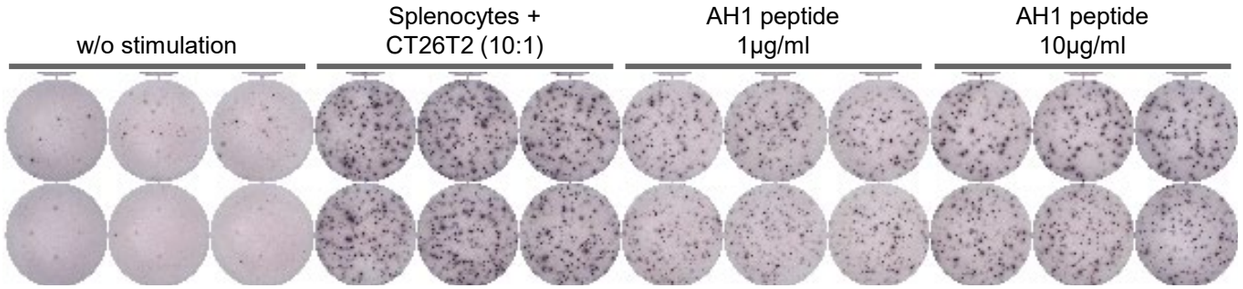


These data indicate a relationship between tumour size & the level of the tumour-specific CD8⁺ T-cell response

Example: CT26 tumour-specific CD8⁺ T-cell responses in αPD-1 mAb-treated mice (study end point: D21)

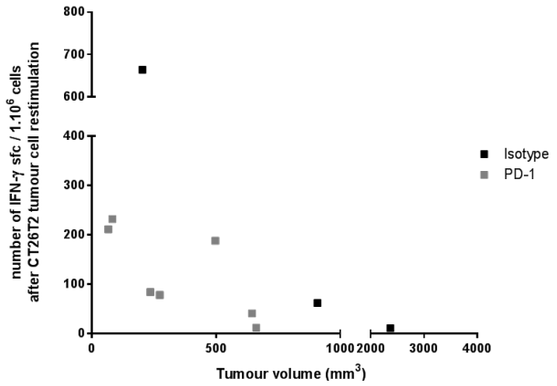
Representative ELISpot images:

AH1= immunodominant antigen of CT26 cells



IFN-γ ELISpot results/ tumour volume correlation:

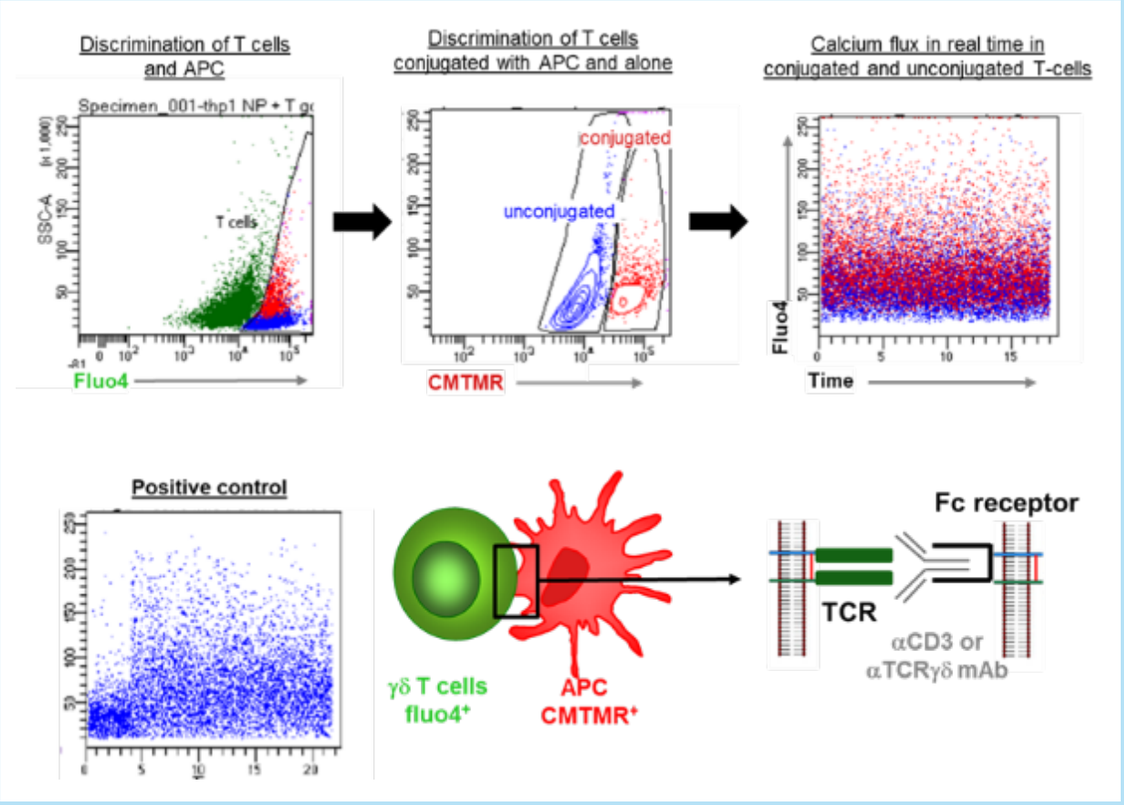
Each dot represents an individual mouse
SFC: Spot Forming Cells



Monitoring early activation/signaling events in T-cells interacting with APC or tumour cells

Case study: following γδT-cells activation by monitoring Ca^{2+} fluxes

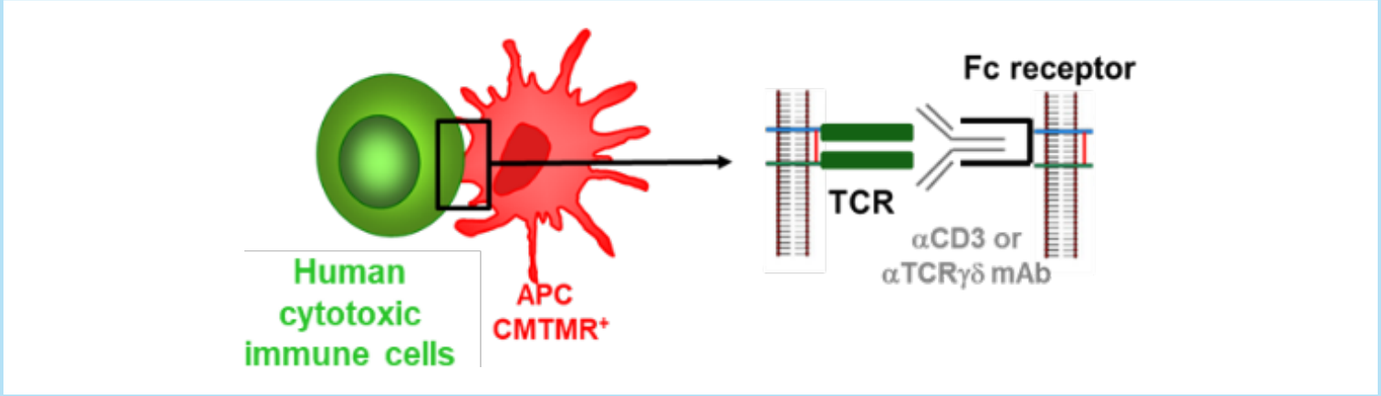
- Possibility to evaluate the potency of immune cell engagers to trigger activation of T-cell or NK cells by measuring Ca^{2+} fluxes by flow cytometry
- **Products could be evaluated on two different features**
  - **Intensity of Ca^{2+} fluxes:** MFI of fluo-4 probe will reflect the level of activation/intracellular signaling triggered by the engager compound
  - Percentage of immune cells fluxing Ca^{2+} will reflect the ability of each product to form **productive Immune cell / target cell conjugates**
- **Highlights from the experimental design**
  - $CMTMR^+$ APC/target (e.g. THP-1 cell lines) loaded or not with anti-CD3 mAb or anti-TCRγδ mAb or their respective isotype control
  - Conjugates formation by short centrifugation
  - After 10 min of conjugation at 37°C, FACS acquisition



Killing assay to evaluate IO compounds

Evaluation of immune cell-mediated killing of tumour cells

- **Killing assays with primary human immune cells and tumour cells as target cells are already setup with**
  - Antibody-pulsed target cells to redirect the killing toward these targets
  - Antigen-specific $CD8^+$ T-cell killing (similar approach than for recall assays with the CEF peptides mixture)
- **Principle of the assay**
  - Use of a cell line loaded with anti-CD3 mAb (here THP-1 cell line)
  - Co-culture with killer cells at different T/E ratio
  - Staining with a viability dye to evaluate the percentage of dead target cells



Note: TME (Tumour MicroEnvironment)

Killing assay to evaluate IO compounds

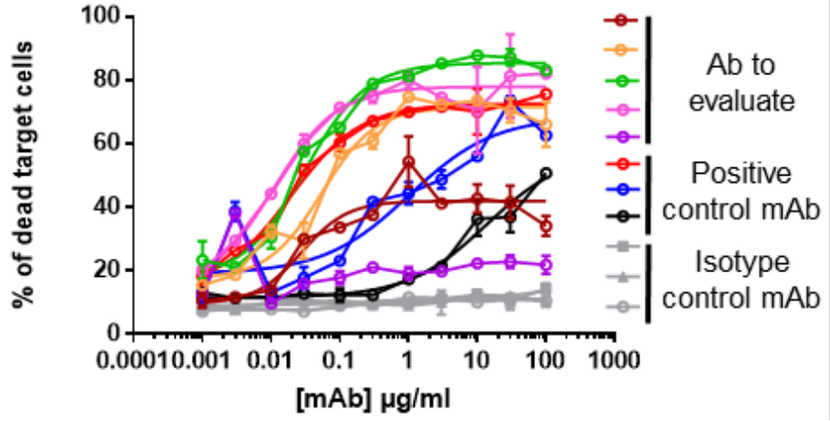
Case study: Ab potency measured with killing assay and CD107a exposure

Several therapeutic antibodies were evaluate in a dose dependent fashion in their ability to boost the T-cell mediated killing of target cells with two different approaches:

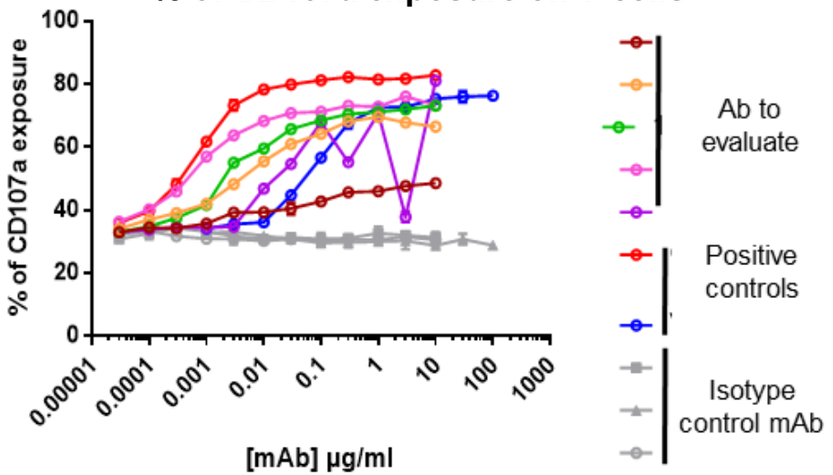
- Killing of target cells
- CD107a exposure on T-cells

→ Both readouts were analysed by flow cytometry

Killing assay: evaluation of % dead target cells

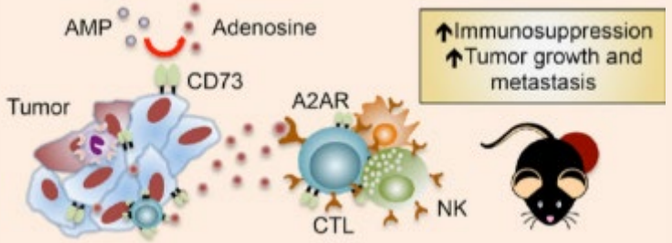


% of CD107a exposure on T-cells

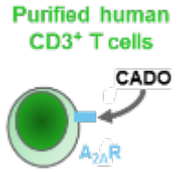


Targeting adenosine immunosuppression

In vitro evaluation of compound antagonist of the $A_{2A}R$ on primary human T-cells



Recovery of IL-2 production by T-cells

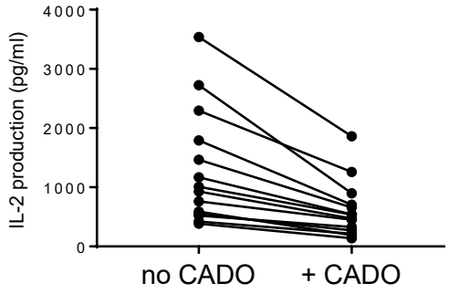


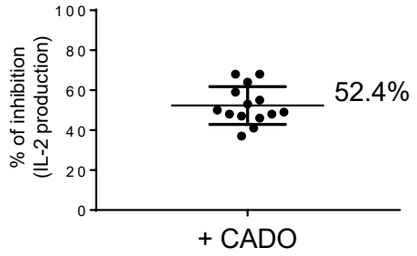
72 hrs

+CD3/CD28 stimulation +/- compounds in dose response

Measurement of IL-2 production by ELISA in culture supernatant

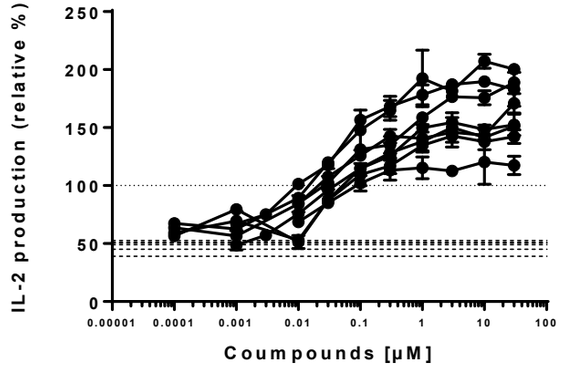
Basal effect of CADO on IL-2 production by T-cells





Results obtained for **16 tested donors** indicate good & robust level of CADO-mediated inhibition, CADO is a stabilized form of Adenosine

Recovery with a reference compound (Preladenant) on 8 donors

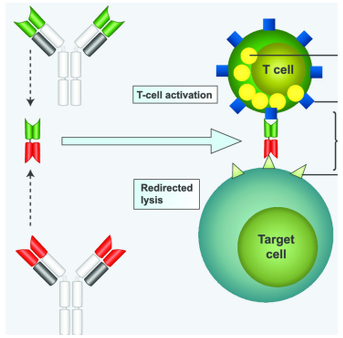


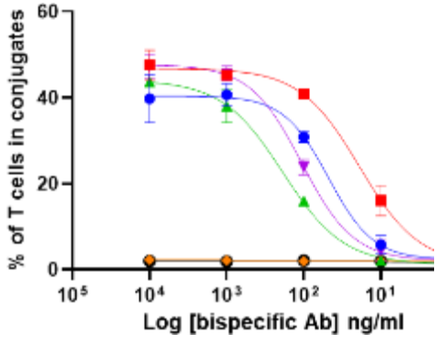
+ CADO (for each donor)

Evaluating bispecific antibodies – T-cell engagers

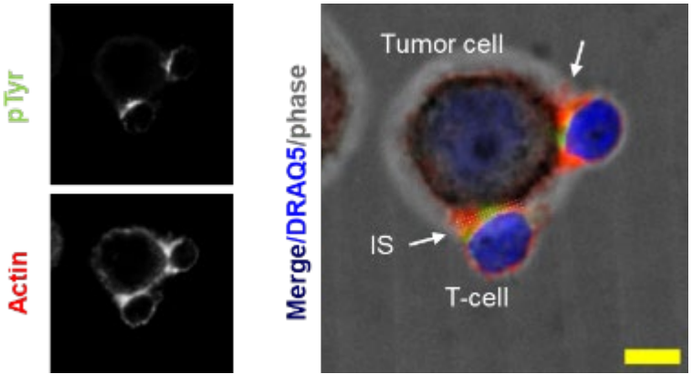
Enabling CD8-mediated killing of tumour cells using bispecifics

- Bispecific antibodies are redirecting CD8⁺ T-cells towards tumour cells expressing the target antigen and inducing activation of the CTL which results in tumour cell killing
- Several type of assays can be used to evaluate the potency of bispecific antibodies:
  - **CD8-mediated killing of tumour cells**
    - Killing assay, GranToxiLux® assay
    - Upregulation of CD107a on CD8⁺ T-cells
  - **T-cell activation features**
    - Cytokines production
    - Activation markers
    - Percentages of T-cells: tumour cells conjugates
    - icCa^{2+} fluxes in T-cells
  - **Visualizing bispecific Abs effect at the Immunological Synapse level**
    - Quantification of the data & signaling pathways





T-cell activation with bispecific Abs



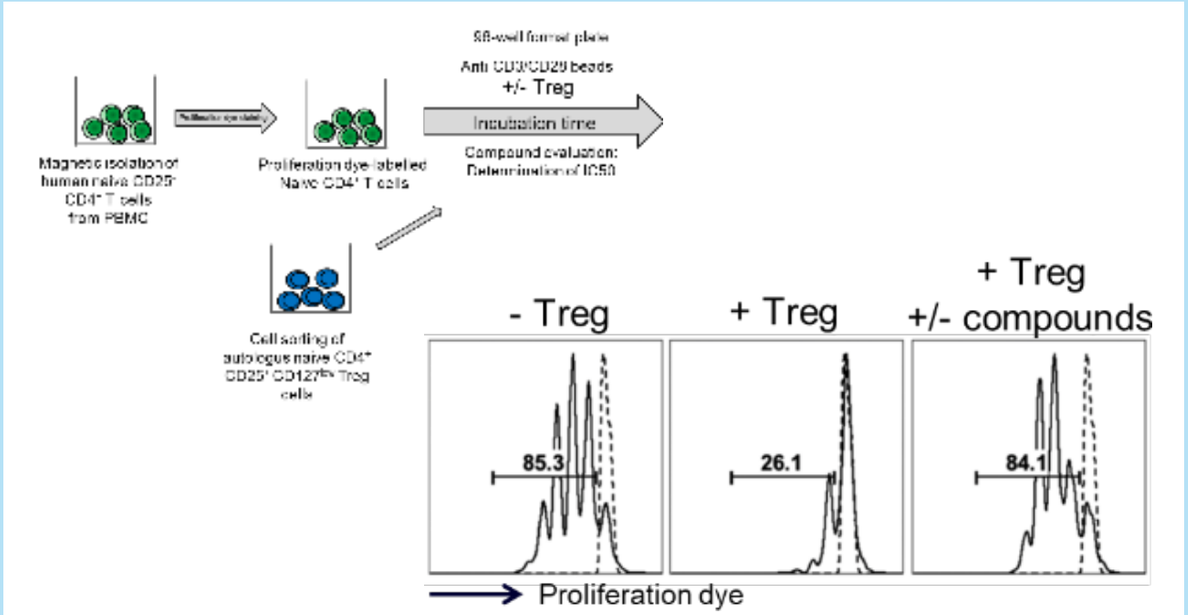
Note: IS (Immunological Synapse), CTL (Cytotoxic T Lymphocytes)
Source: scheme from Baeuerle PA and Reinhardt Cancer Research 2009

CD4⁺ regulatory T-cells biology

Inducing and assessing Treg

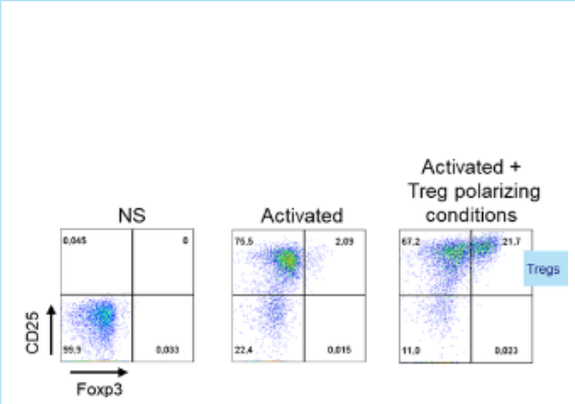
Treg suppression assay

- Possibility to measure proliferation and cytokines production
- Suppressive potential of Treg can be measured with different Treg: Teff ratio



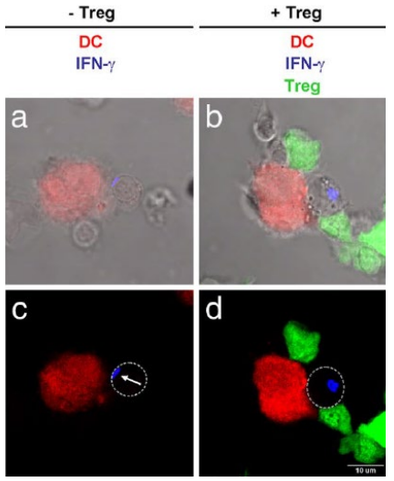
Induction of Treg *in vitro*

- Use of Treg polarising conditions to convert CD4⁺ T-cells to FoxP3⁺ Treg
- Use of these cells in functional *in vitro* assays



Monitoring Treg at the single-cell level

- Analyzing Immunological Synapses of Treg and Teff interacting with a same DC



Esquerre M. *et al.* PNAS 2008

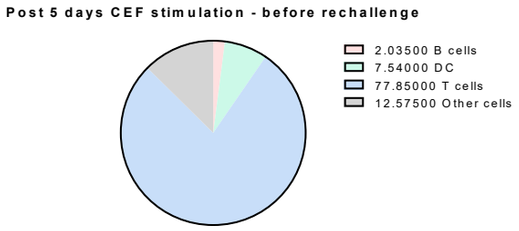
Antigen recall assay with human PBMCs

Recalling Ag-specific memory T-cells for assessing IO agents

Objective: develop functional assays with antigen-specific human T-cells for evaluating IO agents such as immune checkpoint inhibitors (possibility to prepare and work with frozen PBMCs)

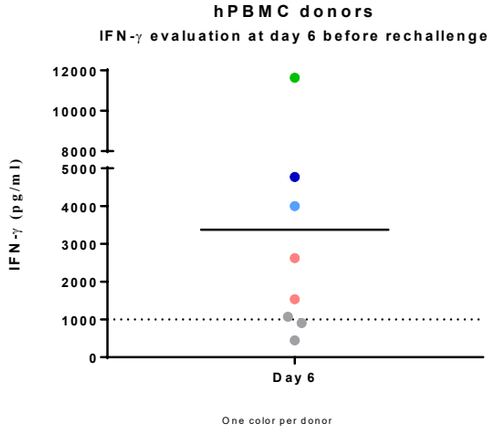
Summary of peptides contained in CEFX Ultra SuperStim Pool

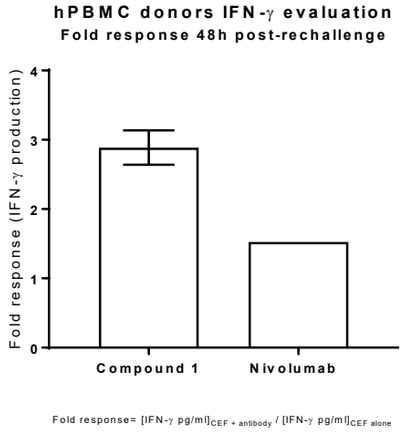
Source	# of peptides
Clostridium tetani	9
Coxsackievirus B4	6
Influenza A virus	16
Helicobacter pylori	9
Human adenovirus 5	11
Human herpesvirus 1	6
Human herpesvirus 2	10
Human herpesvirus 3	5
Human herpesvirus 4	34
Human herpesvirus 5	29
Human herpesvirus 6	3
Human papillomavirus	9
JC polyomavirus	7
Measles virus	3
Rubella virus	4
Toxoplasma gondii	5
Vaccinia virus	10



1000pg/ml is a significant level of IFN-γ production with an Ag-specific stimulation of T-lymphocytes

- Measurement of IFN-γ or other cytokines in culture supernatants
- Evaluation of activation markers by flow cytometry



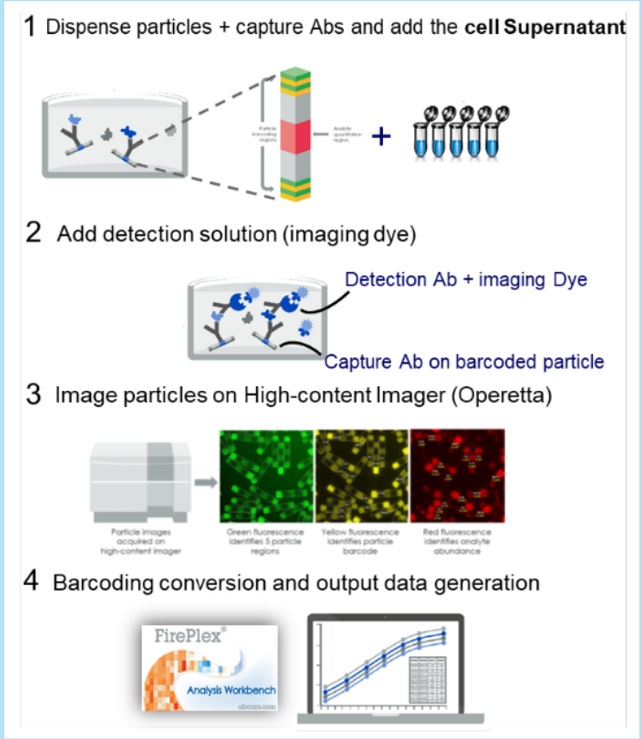


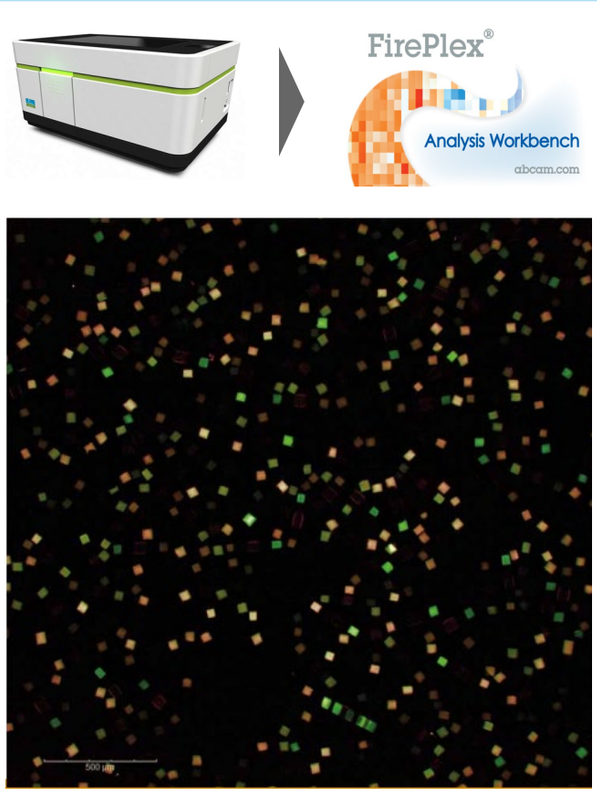
Note: CEFX peptides pool is ordered to JPT Peptides Technologies

Fireplex technology for multiplexed cytokines detection

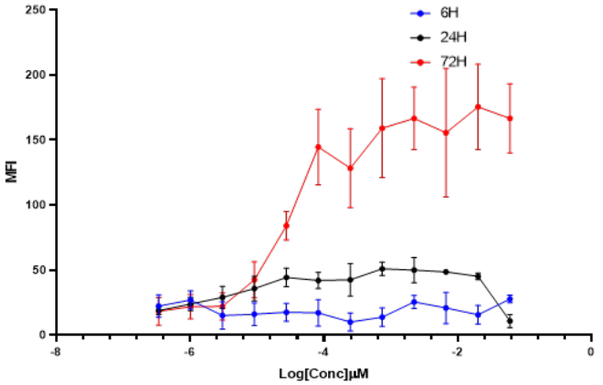
Quantitative measurement of human analytes in cell culture supernatants

FirePlex technology

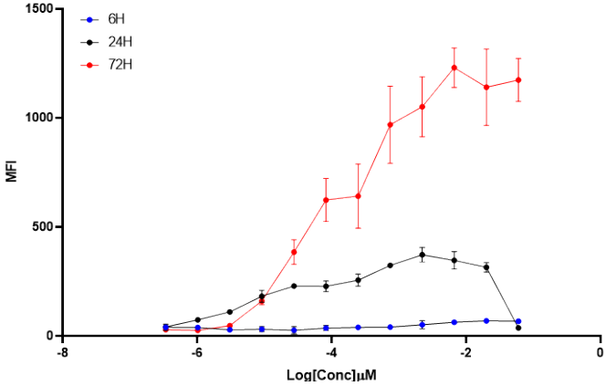




TNFa (1:2)



CXCL 10 (1:2)



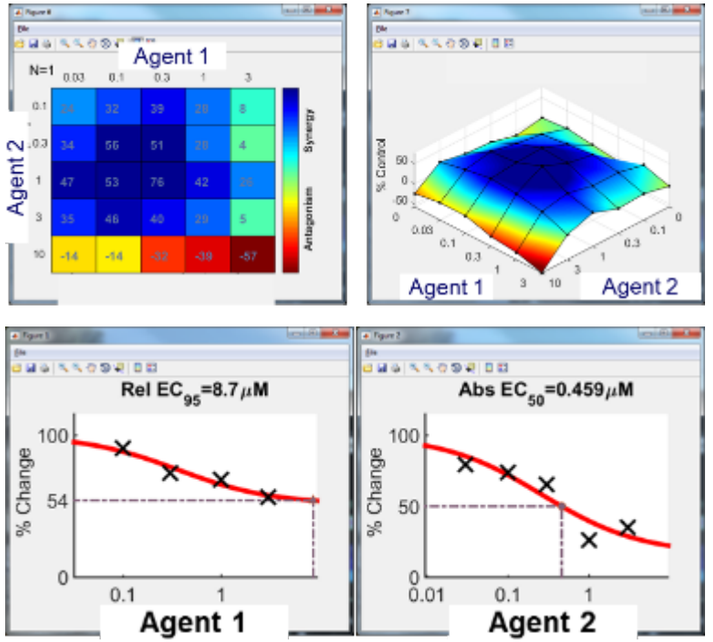
Analyte
Granzyme B
IFN-gamma
IL-1 beta
IL-10
IL-2
IL-6
MIP1b
TNF-alpha

Evaluating synergy between two IO agents with primary human T-cells

Using functional *in vitro* assay for defining optimal synergy among targets

- **Step 1: Start evaluating single agents** in a dose response to calculate the EC_{50} for each product (based on IFN-γ secretion)
  - Possibility to evaluate 8 concentrations to define the range of the EC_{50} for each product
  - Confirm the range of the EC_{50} on 2 donors
  - **Readouts**
    - **IFN-γ secretion by HTRF** in a kinetic fashion
    - **Phenotyping by flow cytometry** on surface activation markers
- **Step 2: Use of Combenefit software to evaluate the synergy** between both targets in a functional immunological assay:
  - 5 concentrations for each agent around the EC_{50} value of each single agent
  - Synergy evaluated on IFN-γ production at the selected day

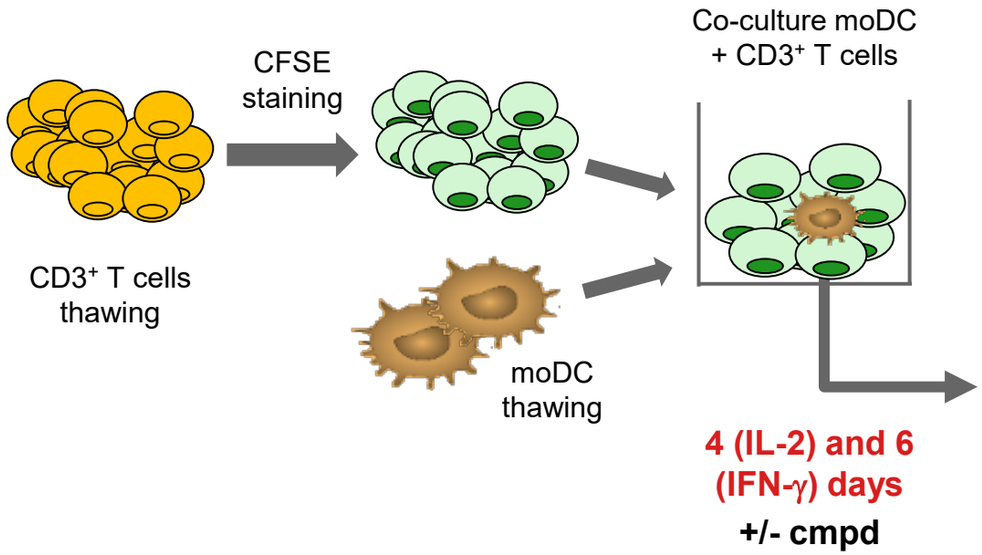
Evotec case study: using primary T-cells, evaluation of the synergy between two IO agents (small molecule and Ab)



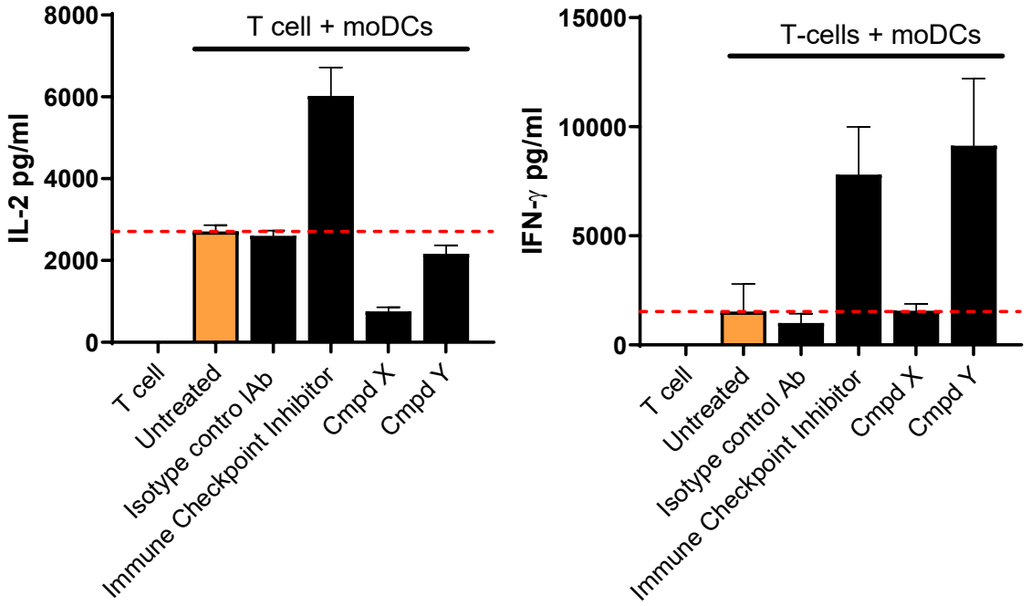
Short-term allogeneic MLR

MLR co-culturing T-cells and Dendritic Cells to evaluate IO compounds

- Performing MLR with allogeneic DCs: T-cells co-culture in a 384w plates format
- Possibility to cryobank T-cells and DCs prior running the assay



Read outs:
IL-2 and IFN-γ secretion
production
by HTRF +
viability by FACS

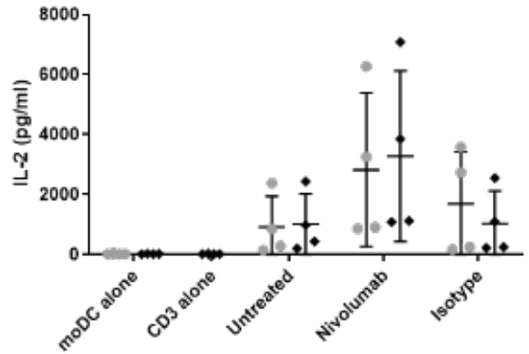


Short-term allogeneic MLR

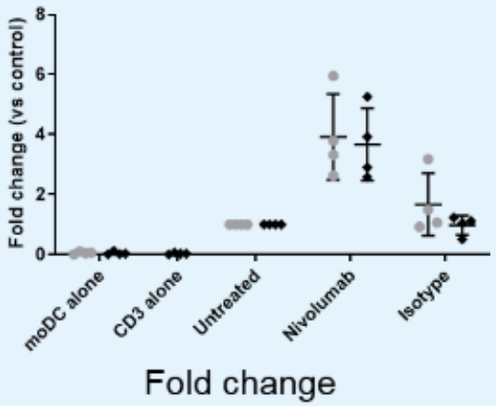
Cytokine release: Pool of 4 MLR

IL-2

IL-2 secretion – Day 6
Pool of 4 MLR

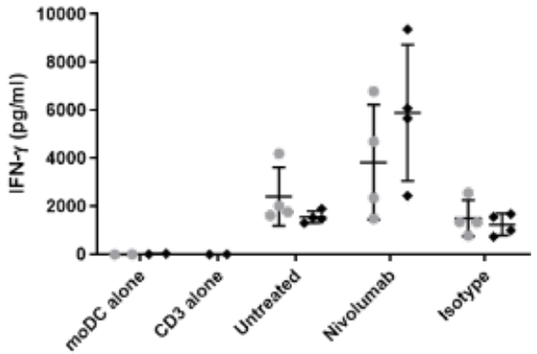


IL-2 secretion – Day 6
Pool of 4 MLR

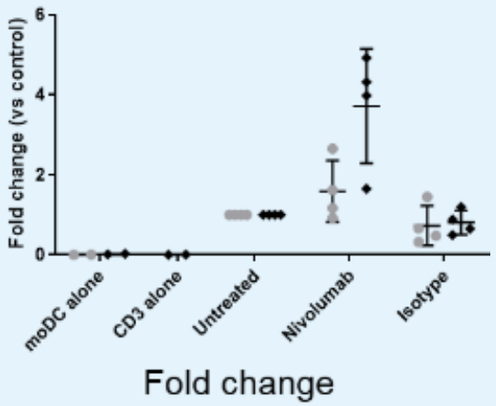


IFN-γ

IFN-γ secretion – Day 6
Pool of 4 MLR



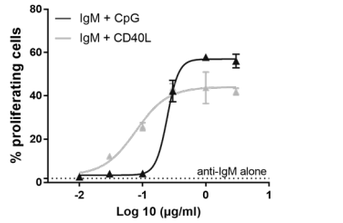
IFN-γ secretion – Day 6
Pool of 4 MLR

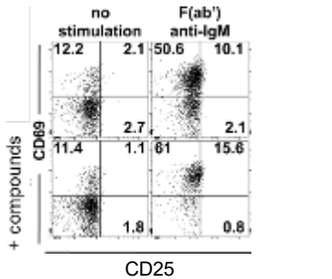


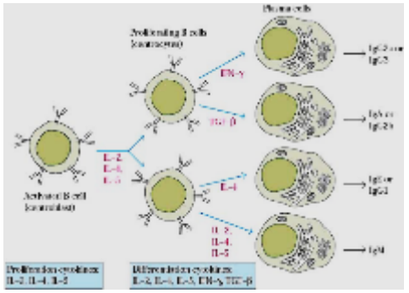
7nM 70nM

B-cells functionality

Expertise to decipher the impact of IO agents on B-lymphocytes







B cell Proliferation	• Proliferation induced with TLRs ligands (CpG, Gardiquimod), CD40L or BCR stimulation with $F_{(ab')}$ anti-IgM • Proliferation readout: CFSE dilution by cytometry
B cell activation	• Polyclonal B cells activation independent of BCR activation: TLRs stimulation (CpG, Gardiquimod) and CD40L • BCR-dependent B cells activation: $F_{(ab')}$ anti-IgM stimulation • Flow cytometry for early activation markers (CD69, CD86) or late activation markers (TACI, CD25)
B cells differ-entiation	• B cells differentiation into plasmablast/plasma cells • Flow cytometry for CD38/CD19 • Cytokine release (IL-6, IL-10, TNF-α)
Ig isotype class switching	• B cell class switch recombination assessment IgM to IgG/IgE (ELISA and flow cytometry)

Source: http://biosiva.50webs.org/plasmacell.htm

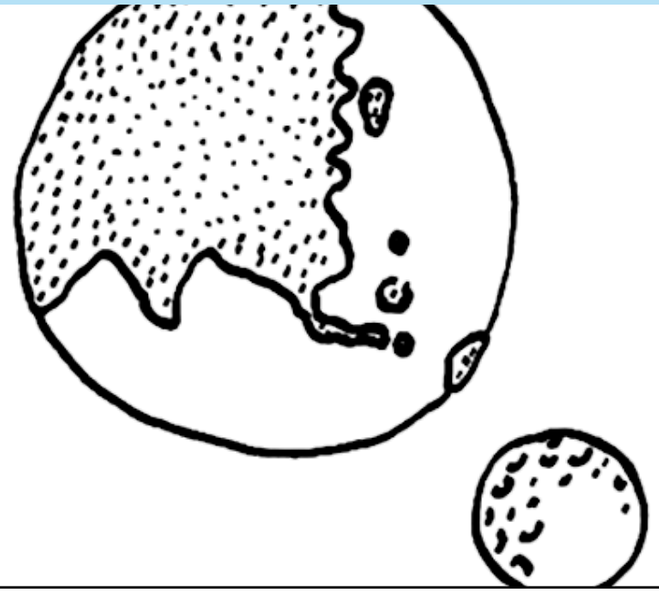
Agenda

Translational Immunological assays with cancer patient samples

Adaptive Immunity

Innate Immunity

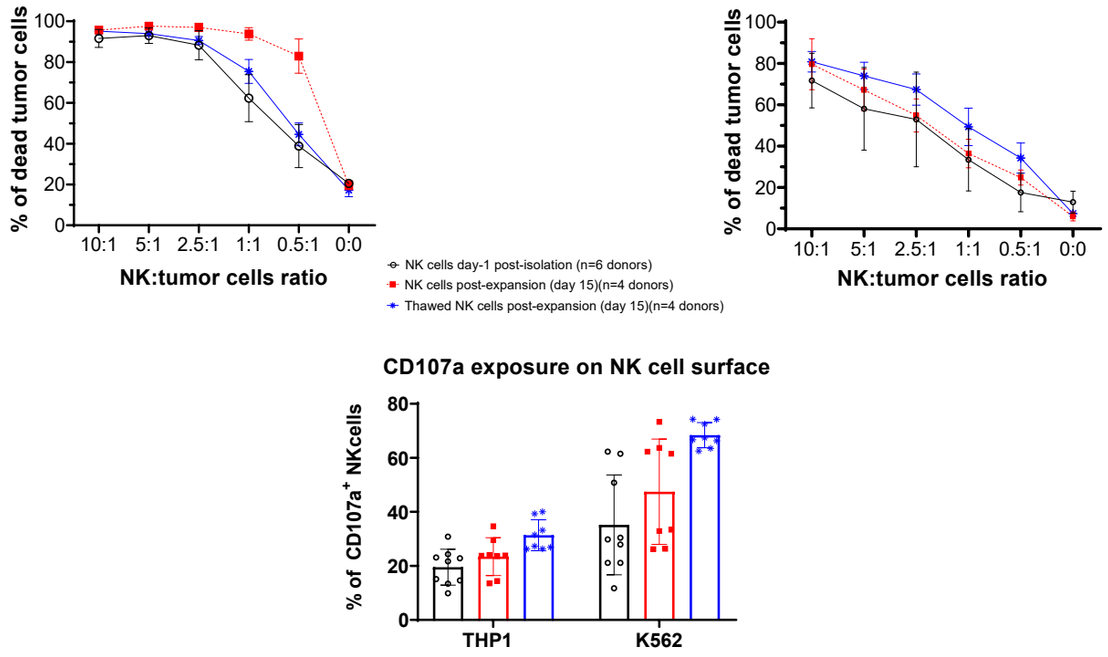
Preclinical mouse models



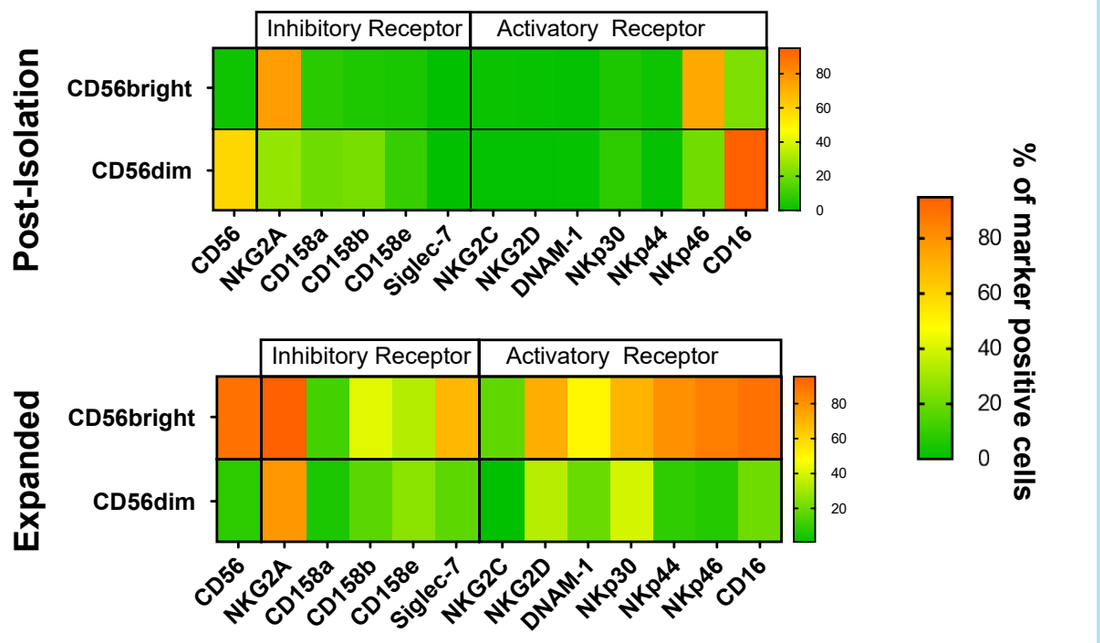
Deciphering NK cells biology

Functional & phenotypical characterisation of blood-derived NK cells

Cytotoxic assay:
NK cells post isolation *versus* post-expansion



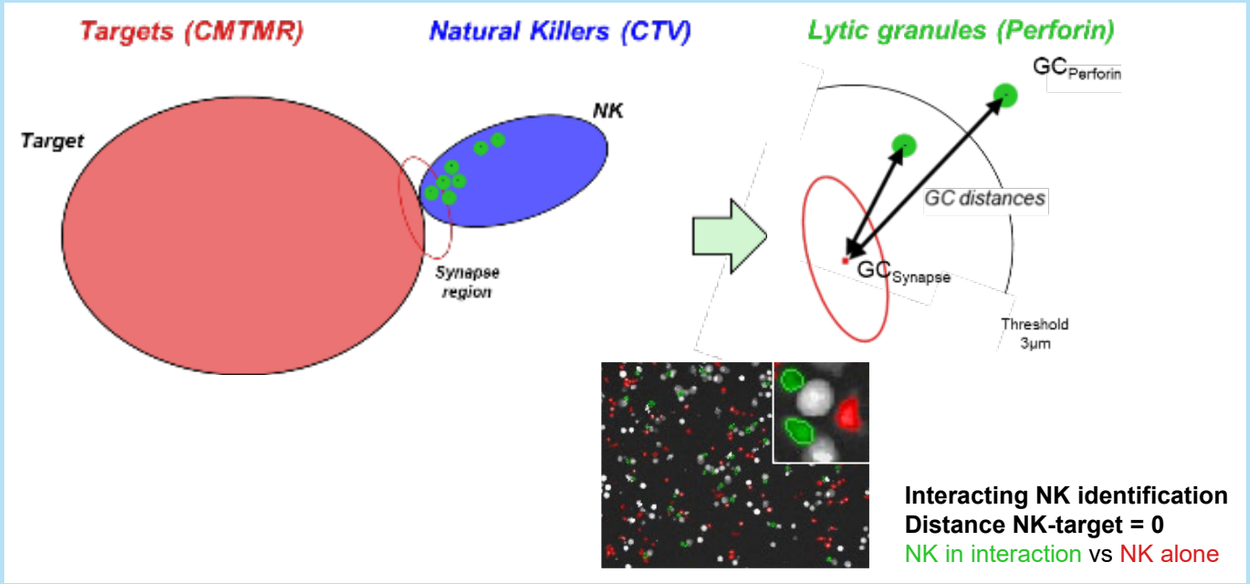
Phenotyping activatory / inhibitory receptors balance using flow cytometry: post isolation *versus* post-expansion



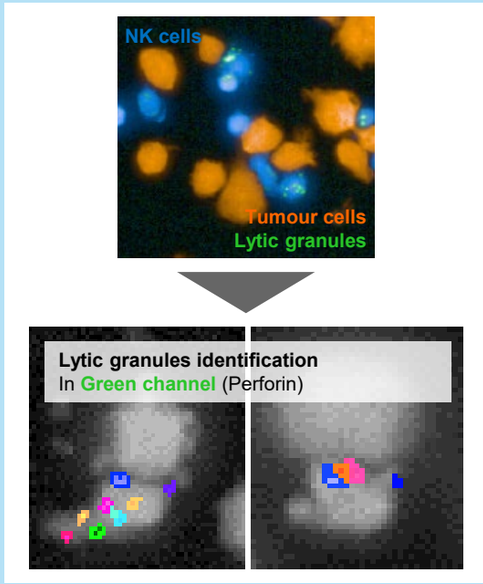
NK cells lytic granules polarisation at the Immunological synapse (IS) with tumour cells

High-throughput confocal imager (Operetta)

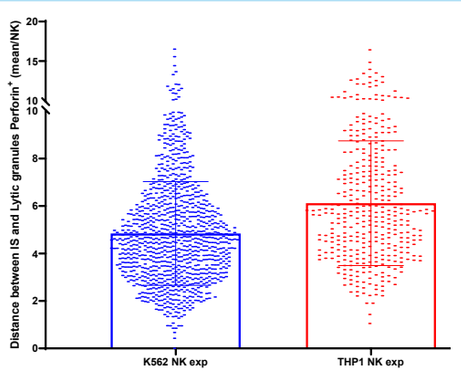
Evaluation of lytic granules (Perforin$^+$) polarisation based on their mean distance to the IS using the Operetta (high-content confocal imaging system)



Interacting NK identification
Distance NK-target = 0
NK in interaction vs NK alone



Distance (µm) between IS and lytic granules (mean/NK cell)



Target cell killing efficiency:
% of apoptotic cells after 4h of co-culture
K562: 50%; **Thp1: 10%**

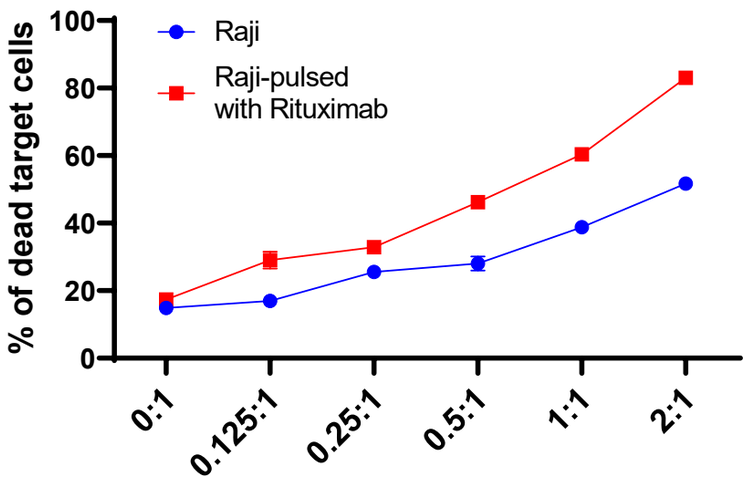
- **Lytic granules polarisation monitoring by Operetta is consistent with target cell killing efficiency** (PoC using 1 sensitive K562 *vs* 1resistant THP-1 line)
- **High-throughput quantitative imaging** with automated collection (384w plate) **providing statistically robust analysis compatible with EC_{50} evaluation**

ADCC assay to evaluate killing of tumour cells

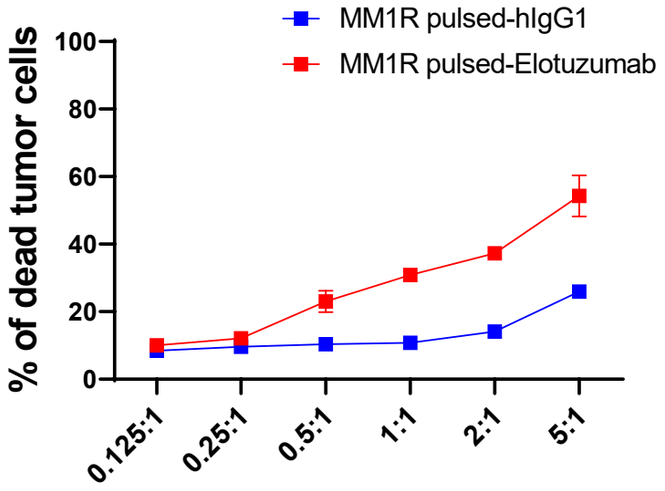
Enhancing NK-cell mediated killing with therapeutic mAb

- **Evaluation of antibodies in their ability to boost the NK-cell mediated ADCC using FACS-based killing assay**
- **Principle of the assay**
  - TAA⁺ Tumour cells were pre-loaded with TAA-targeting mAb
  - Blood-derived NK cells were conjugated to mAb-pulsed tumour cells at different Effector:Target (E:T) ratio
  - Staining with a viability dye to evaluate the percentage of dead target cells

ADCC assay with NK cells and 2 FDA-approved mAb (Rituximab and Elotuzumab) in the liquid tumours space



ADCC assay using Rituximab
(anti-CD20, FDA-approved mAb):
CD20⁺ Raji Lymphoma Cells

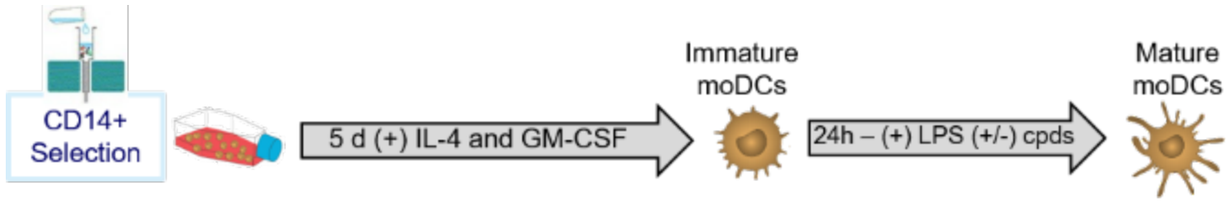


ADCC assay using Elotuzumab
(anti-CS1, FDA-approved mAb, BMS):
CS1⁺ MM.1R Multiple Myeloma Cells

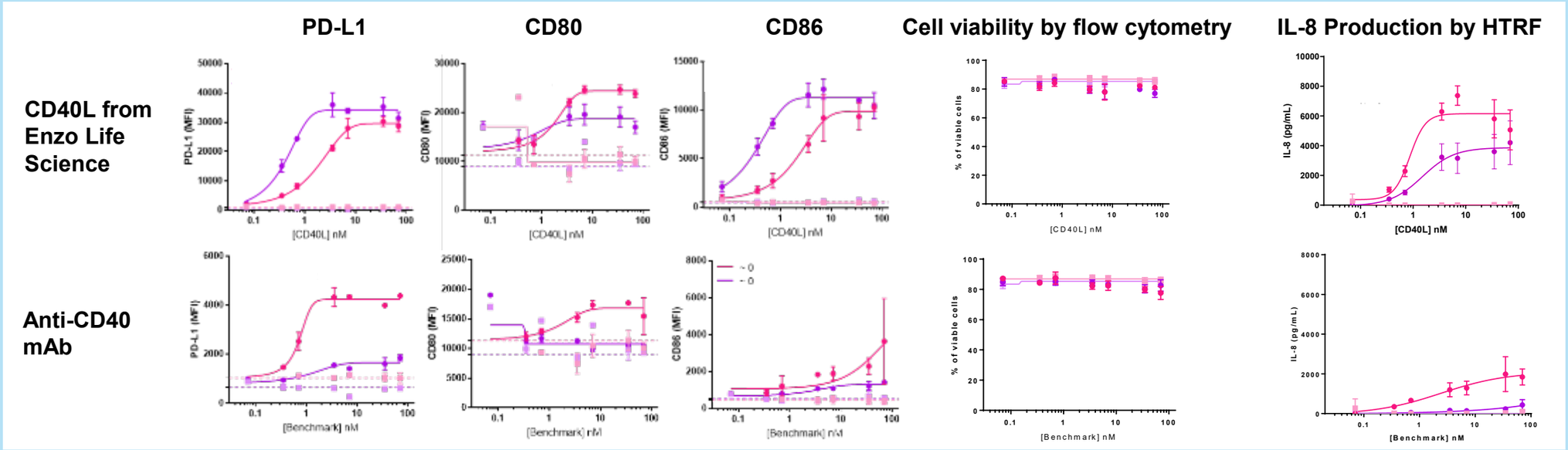
Note: TAA (Tumour Associated Antigen)

Evaluation of biologics on DCs biology

Functional assay on primary human DCs to evaluate compounds (384w)



Activation markers expression by flow Cytometry



Donor 1 Donor 2

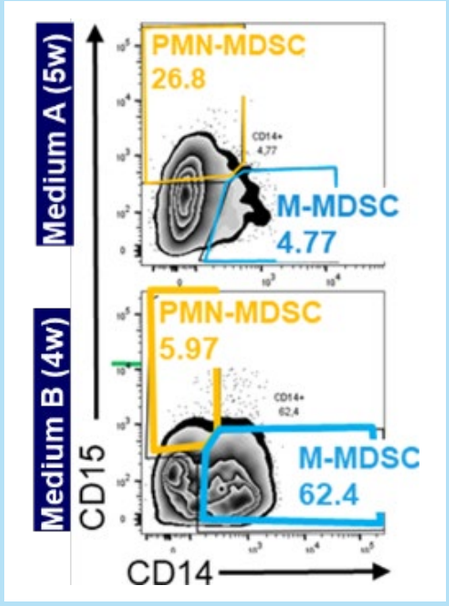
Note: TME (Tumour MicroEnvironment)

Regulatory functions of *in vitro* differentiated MDSCs

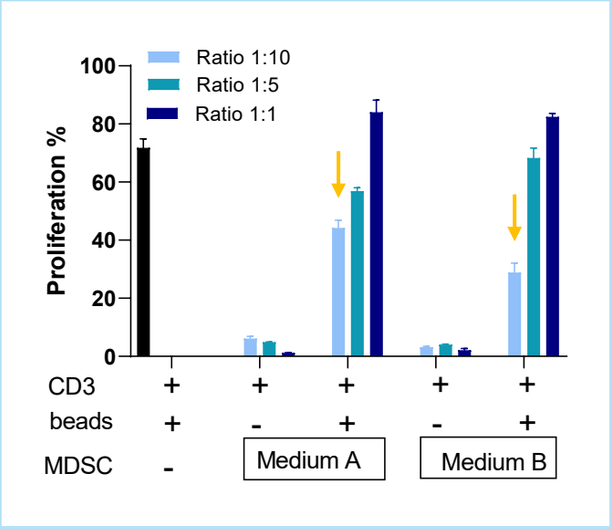
MDSC Phenotype and inhibition of proliferation effect

Cell phenotype analysed by flow cytometry

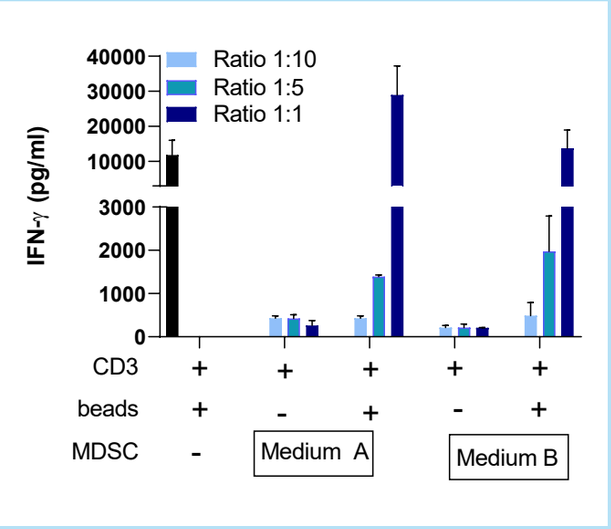
Gated on HLA-DR$^-$ CD11b$^+$ CD33$^+$



MDSC Inhibit T-cell proliferation after 72h co-culture



MDSC Inhibit IFN-γ production in the supernatant after 72h co-culture



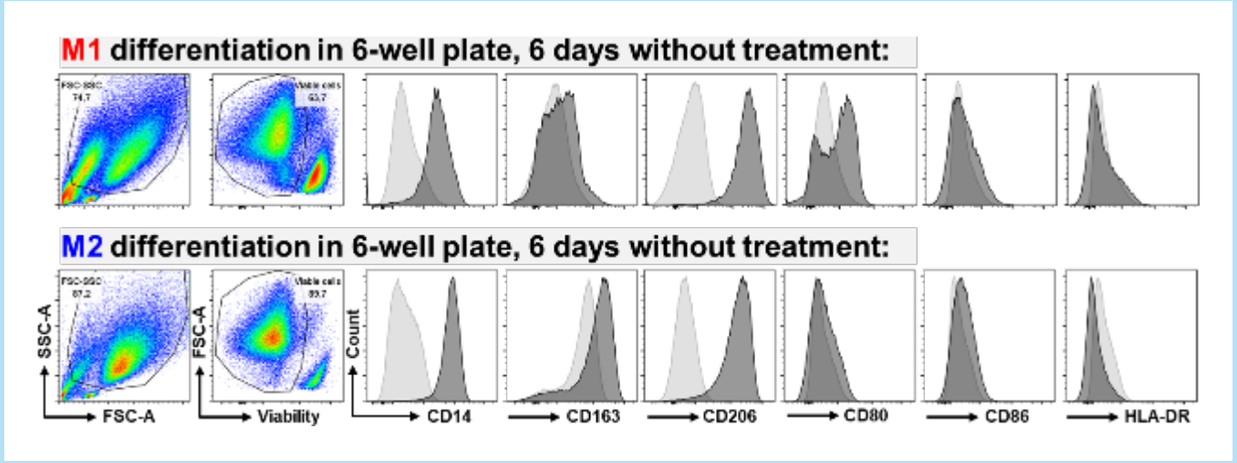
- A differentiation culture method was set up in 2 different mediums allowing to obtain in the culture the 2 MDSC sub-populations: **granulocytics** (PMN) (HLA-DR$^-$CD11b$^+$CD33$^+$**CD15$^+$CD14$^-$**) and **Monocytics** (M) (HLA-DR$^-$CD11b$^+$CD33$^+$**CD15$^-$CD14$^+$**) MDSCs
- Co-culture of the global differentiated cells with allogeneic T cells induced a **dose dependent inhibition of T-cell proliferation and function measured by reduced IFN-γ production** in the supernatant

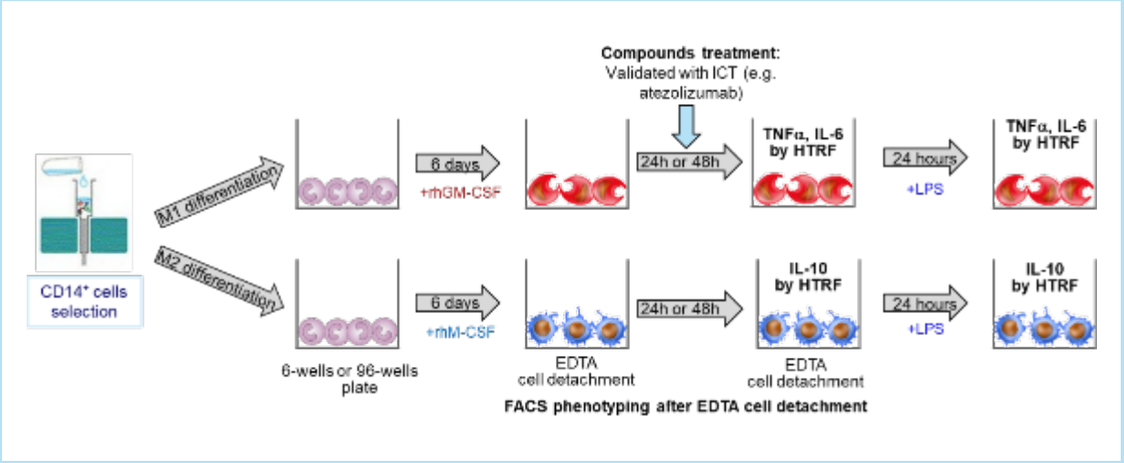
Differentiation of primary macrophages

Effect of compounds on M1 or M2 macrophages differentiated from CD14⁺ cells

Possibility to evaluate compounds (small molecules or biologics)

- Effect on the differentiation process: either the M1 or M2
- Effect on reversion from M2 to M1 for cancer immunotherapy
- Effect on reversion from M1 to M2 for auto-immune diseases and inflammation



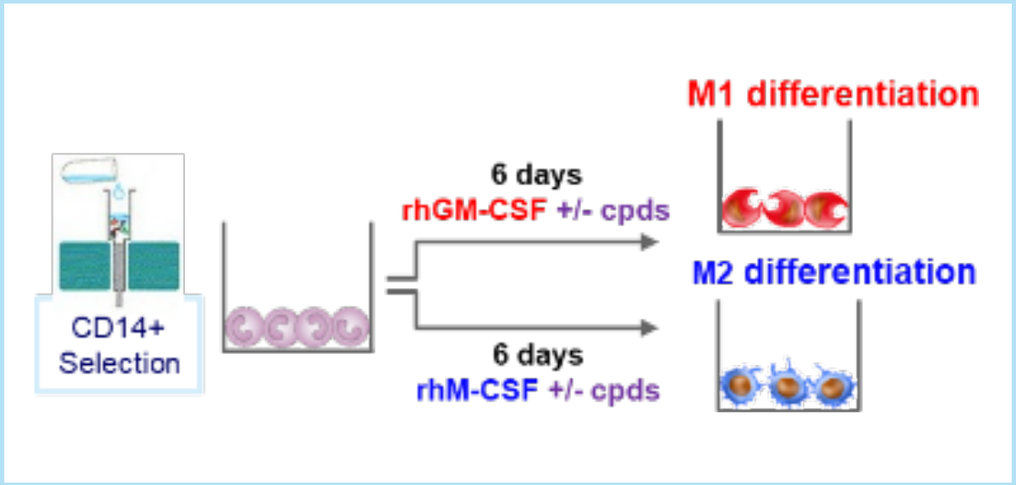


- As described, M1 macrophages express CD14 and CD80 markers and M2 macrophages express CD14 and CD163 markers
- CD206 marker is not discriminant for M1 or M2 characterization

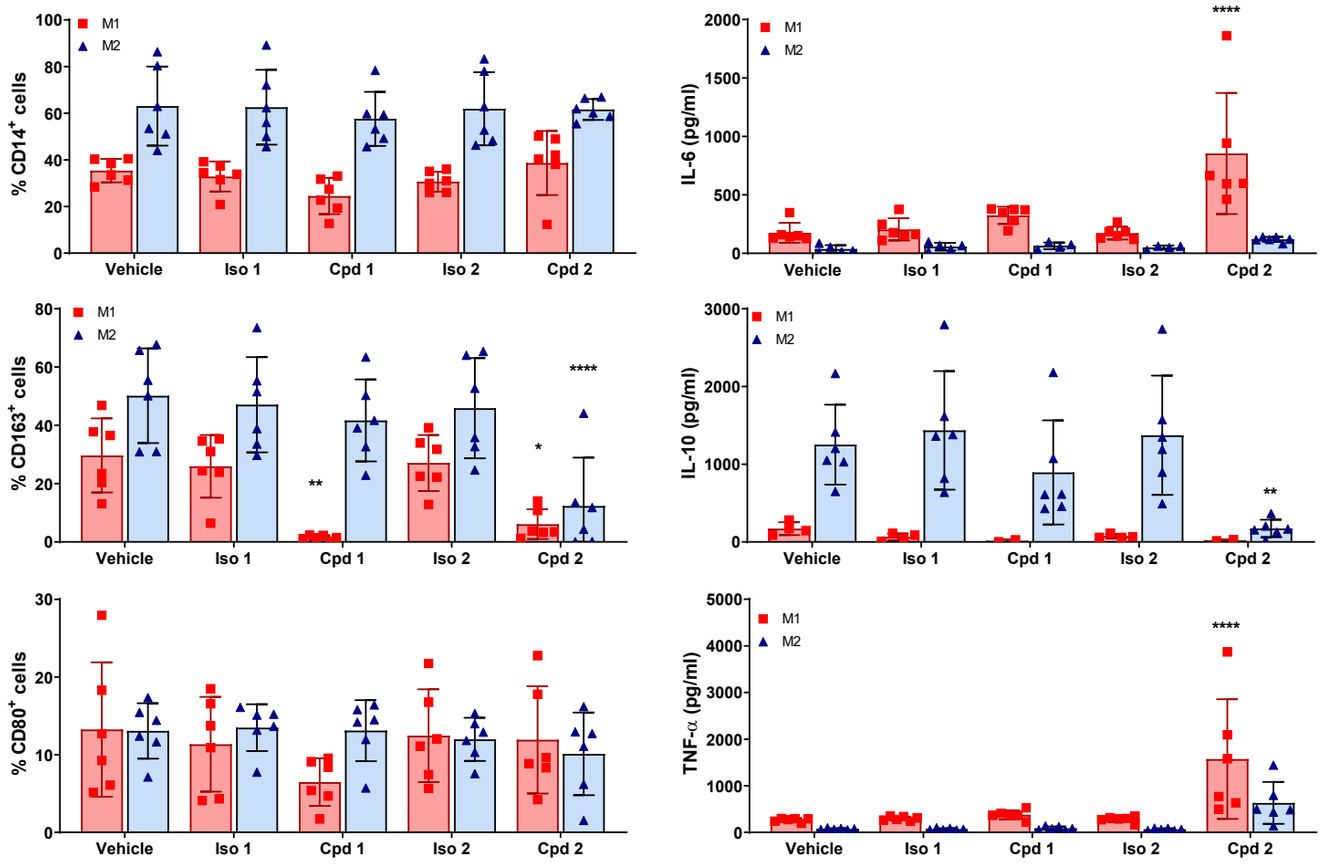
Differentiation of primary macrophages

Effect of compounds on M1 and M2 macrophages differentiation from $CD14^+$ cells

Study design



Compound addition during monocytes differentiation induces a skewing of M2 macrophage toward an M1 phenotype (decrease of CD163 expression and IL-10 production)



Bar graph represents the percentage of expressing population among live cells
Pool of 6 donors – 3 independent studies_ Compounds used at 70nM
Statistics: Two-way Anova Dunnett's multiple comparison test *p<0.05, ** p<0.005 ***p<0.0005, ****P<0.0001

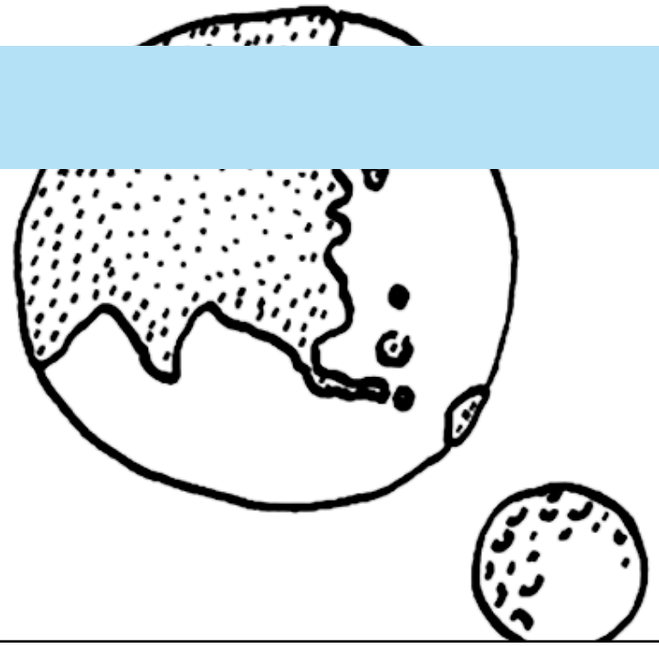
Agenda

Translational Immunological assays with cancer patient samples

Adaptive Immunity

Innate Immunity

Preclinical mouse models



Syngeneic tumour mouse models

Preclinical models with a functionally intact immune system (immunocompetent mice)

Advantages of syngeneic models

- Integrity of the **tumour microenvironment**: immune populations, stroma/fibroblasts (orthotopic implantation)
- Responses to **standard of care therapy** and **ICTs** (αPD-1, αCTLA4 and αPD-L1 available for combination studies)
- **Reduced cost** of the mice and **Rapid** expansion of tumour cell lines (suitable when large group numbers is required)

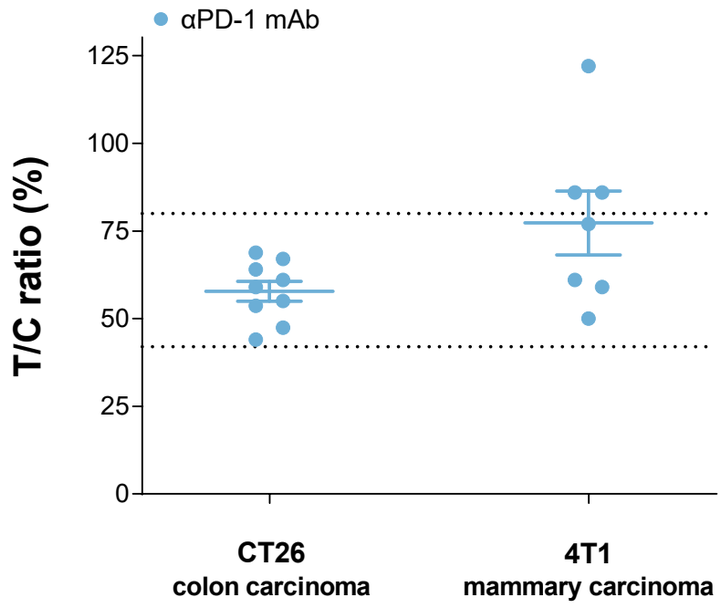
Orthotopic and subcutaneous models

Cancer	Cell	Inoculation site	Treatment with ICTS
Colorectal	CT26	s.c., colon (spleen for liver metastasis)	αPD-(L)1/ αCTLA-4
Colorectal	MC38	s.c.	αPD-(L)1/ αCTLA-4
Breast	4T1	s.c. (mammary fat pad)	αPD-1/ αCTLA-4
Pulmonary	LL2	s.c. and lung (transpleural implantation)	–
Melanoma	B16	s.c. and skin (intradermal; id)	–
Renal cell carcinoma	Renca	renal subcapsule	–
Pancreatic adenocarcinoma	Panc02	pancreas	αPD-1
Hepatoma	BNL-R3	s.c.	–
Fibrosarcoma	MCA205	s.c.	αPD-L1/ αPD-1
Lymphoma	EG7-OVA	s.c	αPD-L1
Ovarian	ID8	s.c.	–

Reliable models for combination studies with ICTs

Interstudy variability in response to ICT as single agent in 2 different syngeneic models

αPD-1 anti-tumour response variability across studies in CT26 and 4T1 syngeneic models



Models	Description	Nb of studies	Mean T/C value (%)
CT26	Colon carcinoma (s.c.)	8	56%
4T1	Mammary carcinoma (s.c.)	7	77%

T/C>80%
42%<T/C<80%
T/C<42%

T/C ratio (%) = (mean treated-tumour weight / mean control-tumour weight) x 100

The MC38 colon carcinoma model

Combining ICTs enhances anti-tumoural response

Study design

MC38 cancer cells (s.c.) in C57Bl6/J mice (n=11-12/group)

Mice randomization based on tumour volume

Treatment with ICTs (i.p., 10 mg/kg)

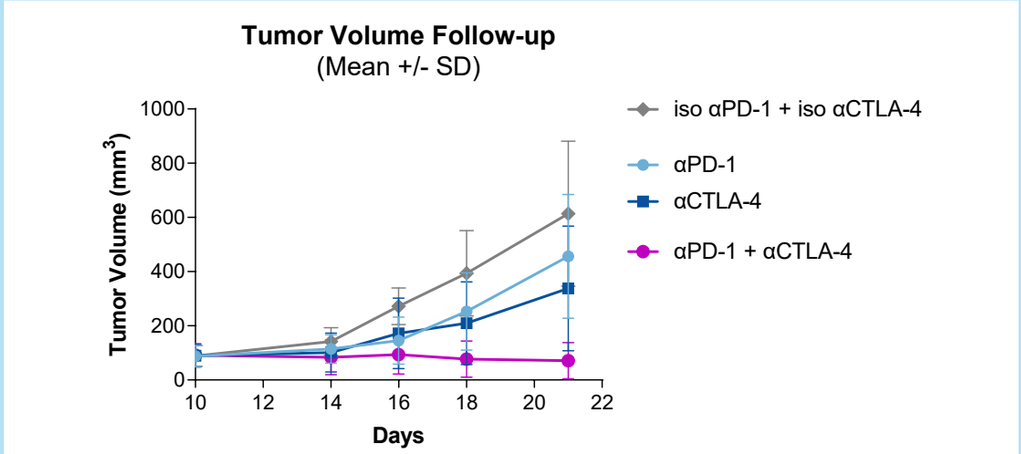
D0 D10 D14 D18 D24

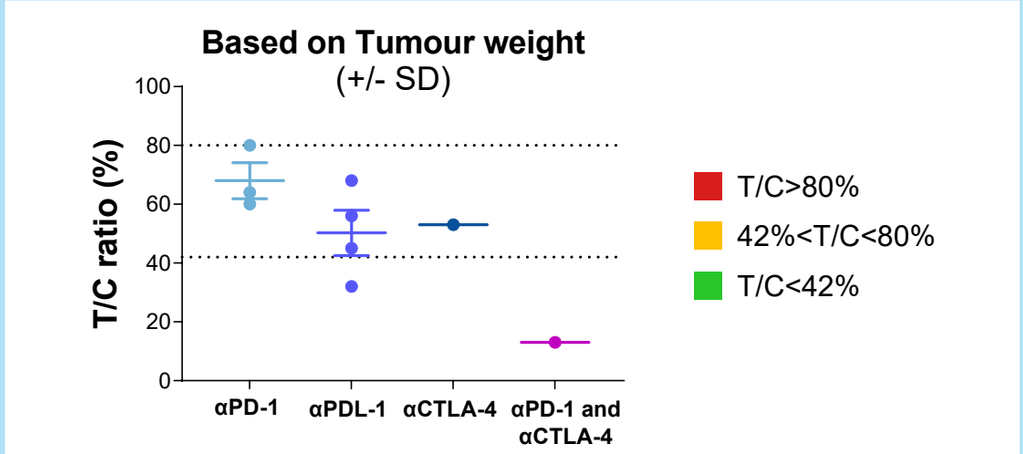
Time-course follow up of mice body weight and tumour volume (3x/week)

Endpoint tumour:

- Weight
- Immune cells infiltration (flow cytometry)

MC38 is sensitive to ICT mono-therapy and combination αCTLA-4 plus αPD-1 ICTs enhances anti-tumour efficacy



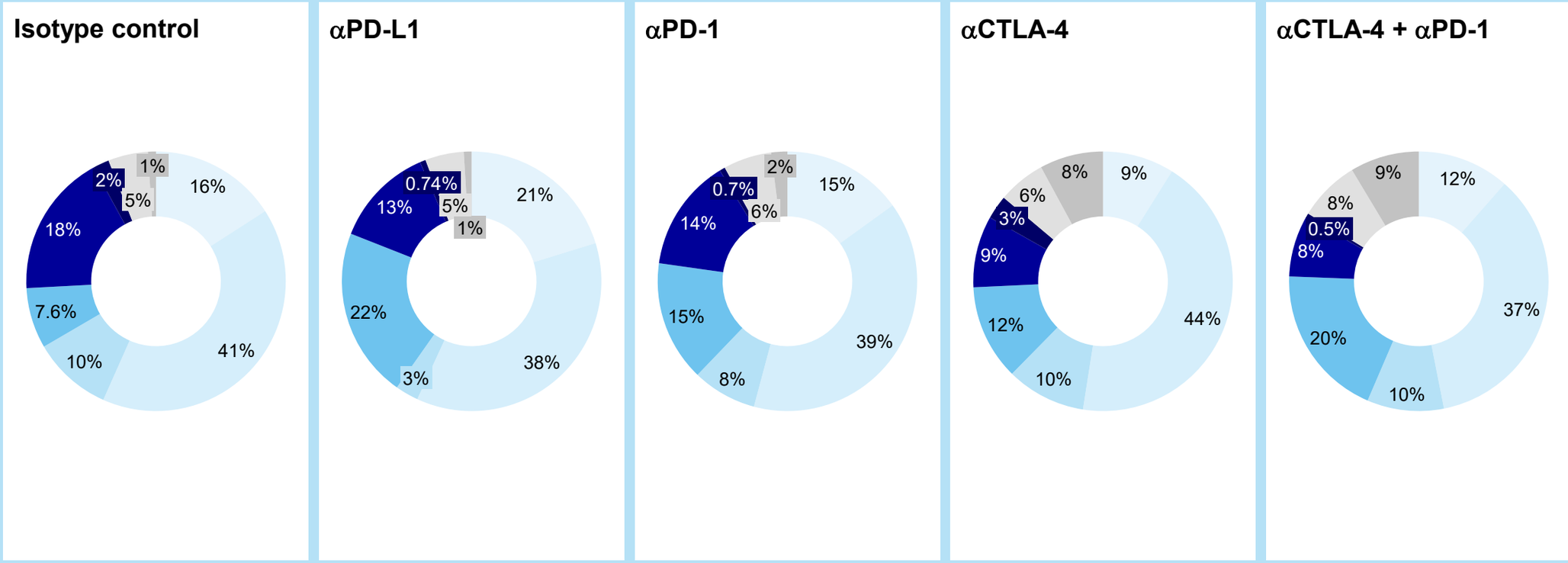


The MC38 colon carcinoma model

Efficacy of ICTs is associated to marked increase in CD8+ T-cells plus M1 macrophages

Single ICT increases CD8+ T-cells or antitumour M1 macrophages. Combined ICT elicits marked tumour infiltration of both cell types.

Evaluation of immune cell infiltrates within the tumour (flow cytometry), in Percent



DC Monocytes MDSC/Neutro M1 M2 B cells CD4 T cells CD8 T cells

The CT26 colon carcinoma mouse model

Benchmarking single ICT response to select optimal immunotherapy combination

Study design

CT26 cancer cells (s.c.) in Balb/c (n=10-15/group)

Mice randomization based on tumour volume

Treatment with ICTs (i.p., 10 mg/kg, Q10Dx2-3)

D0 — D10 — D25

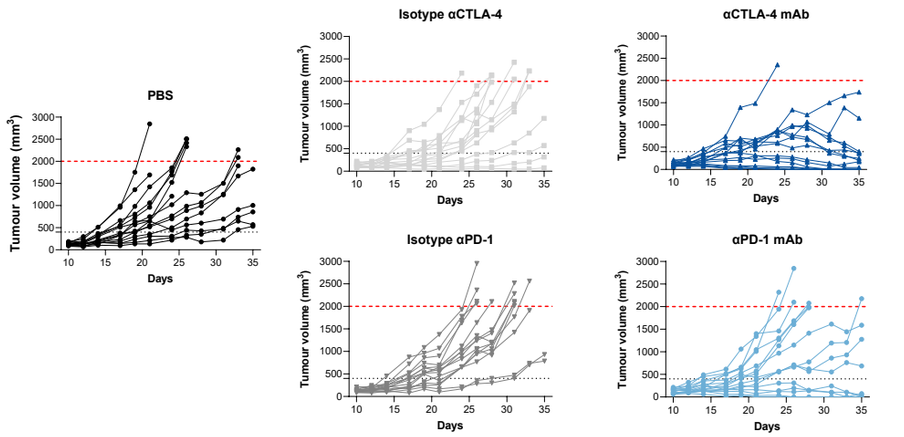
Time-course follow up of mice body weight and tumour volume (3x/week)

Endpoint tumour:

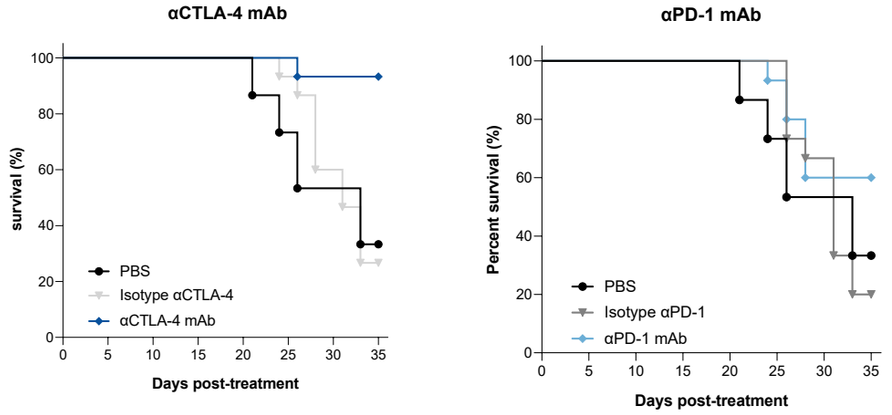
- Weight
- Immune cells infiltration (flow cytometry)

αCTLA-4 monotherapy markedly improves survival whereas αPD-1 mediates low anti-tumour efficacy

Time course of tumour growth (individual data)



Survival

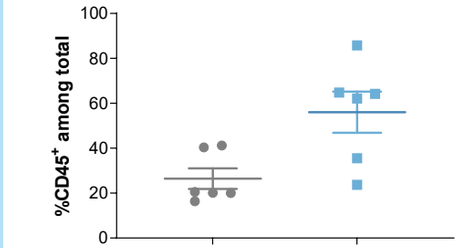


The CT26 colon carcinoma mouse model

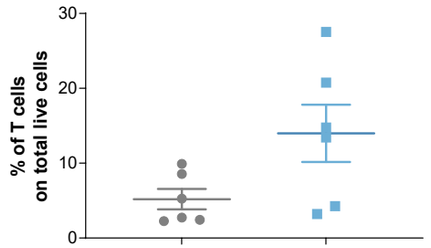
Increase in T-cells infiltration elicited by αPD-1 ICT is related to antitumour effect

αPD-1 elicits an increase in immune cells infiltration (particularly CD8⁺ T-cells) which is correlated to tumour weight

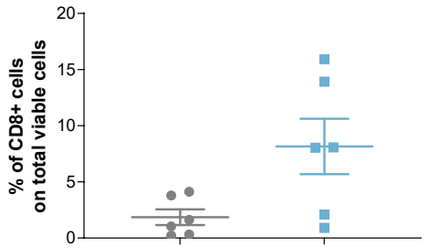
Hematopoïetic cells
(among total viable cells)



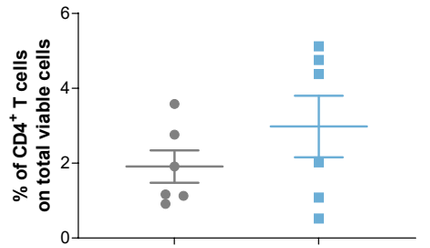
T lymphocytes
(among total viable cells)



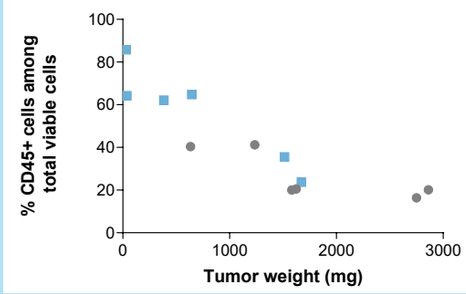
CD8⁺ T lymphocytes
(among total viable cells)



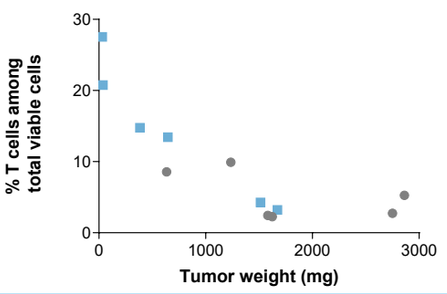
CD4⁺ T lymphocytes
(among total viable cells)



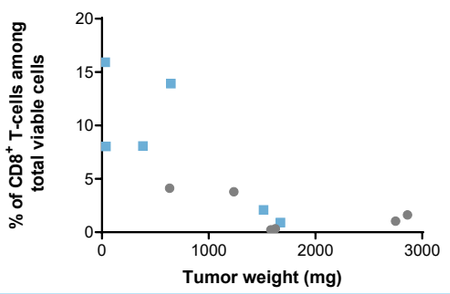
Correlation % CD45⁺ cells and tumour weight



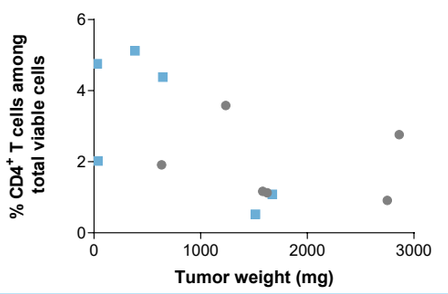
Correlation % T-cells and tumour weight



Correlation % CD8⁺ T-cells and tumour weight



Correlation % CD4⁺ T-cells and tumour weight



Isotype control αPD-1

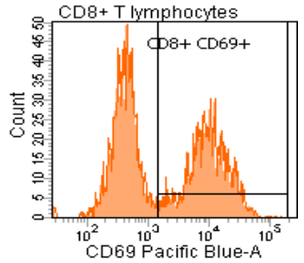
The CT26 colon carcinoma mouse model

αPD-1 drives antitumour immunity through modulation of both the T cell & myeloid compartments

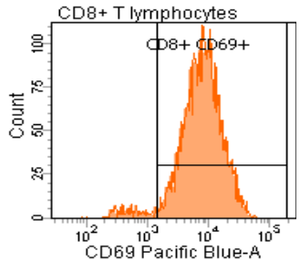
Blockade of the PD-1/PD-L1 pathway is associated to an increase in activated CD8⁺ T-cells and M1 polarization of macrophages into the TME

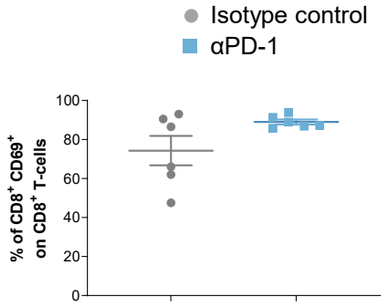
CD8⁺ CD69⁺ T cells (among CD8⁺ T cells)

Isotype control group



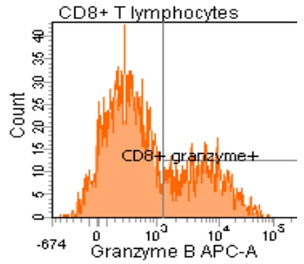
αPD-1 mAb



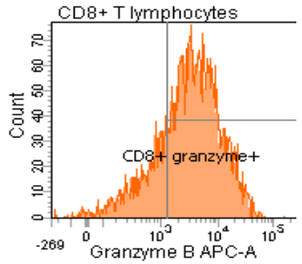


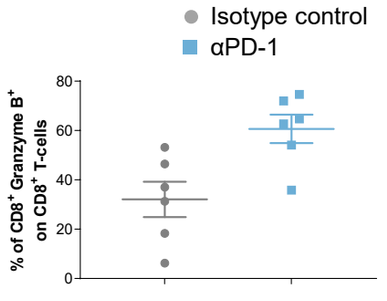
CD8⁺ granzyme B+ T cells (among CD8⁺ T cells)

Isotype control group

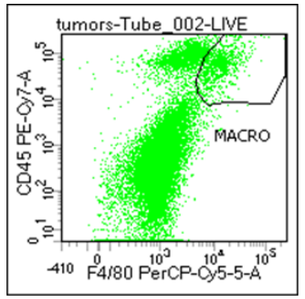


αPD-1 mAb

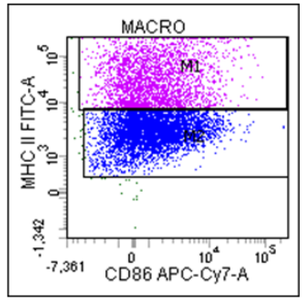


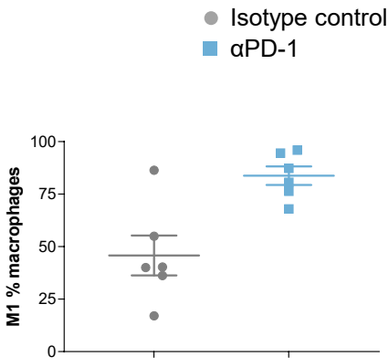


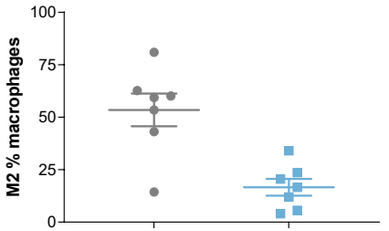
M1/M2 macrophages (among macrophages)



● Macrophages = F4/80⁺ cells







● M1 macrophages = CD86+MHC-2^{high} cells
● M2 macrophages = CD86+MHC-2^{int} cells

The MCA205 sarcoma model

Model allowing assessment of combination cancer therapies with αPD-1 ICT

Study design

MCA205 cancer cells (s.c.) in C57Bl6/J mice (n=10/group)

Mice randomization based on tumour volume

Treatment with ICT (i.p., 10 mg/kg)

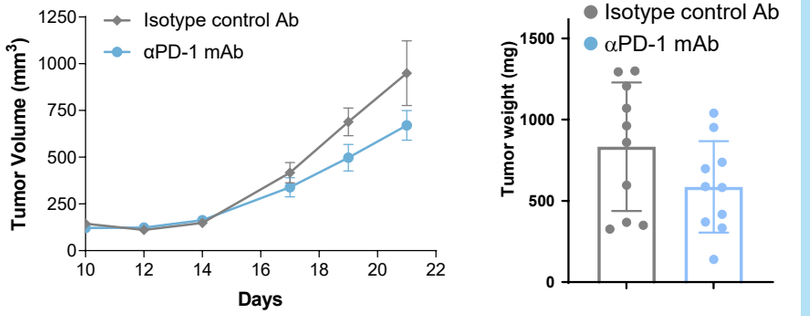
D0 D10 D13 D16 D19 D21

Time-course follow up of mice body weight and tumour volume (3x/week)

Endpoint tumour:

- Weight
- Immune cells infiltration (flow cytometry)

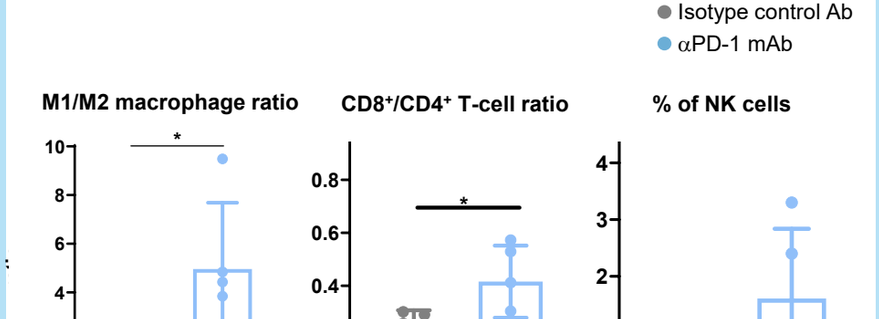
αPD-1 monotherapy mediates low anti-tumour efficacy



Groups	T/C ratio
αPD-1 mAb dose 1	67%
αPD-1 mAb dose 2	70%

T/C>80%
42%<T/C<80%
T/C<42%

αPD-1 enhances CD8⁺/CD4⁺ T-cell & M1/M2 macro-phage ratio as well as NK cells tumour infiltration



The 4T1 breast cancer mouse model

Model allowing assessment of combination cancer therapies with αPD-1 or αCTLA-4 ICT

Study design

4T1 cancer cells (s.c.)
in Balb/c (n=10/group)

Mice randomization based on tumour volume

Treatment with ICTs (i.p., 10 mg/kg)

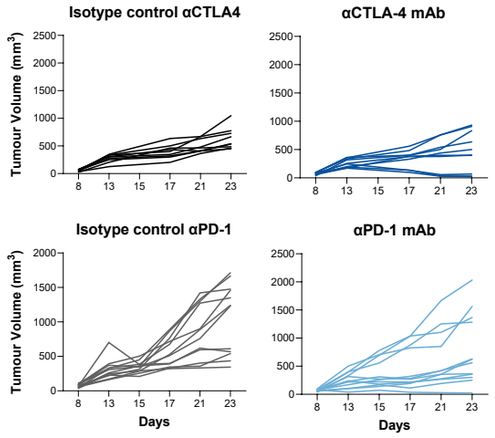
D0 D8 D13 D18 D23

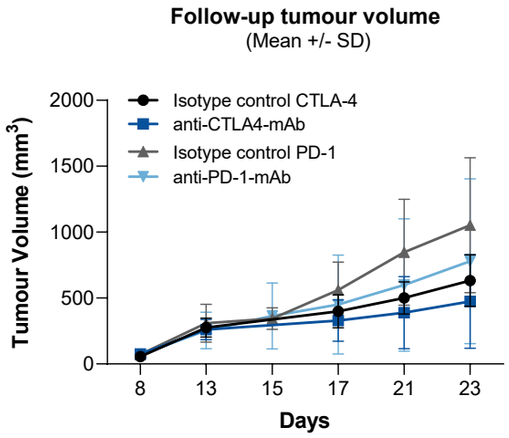
Time-course follow up of
mice body weight and tumour volume (3x/week)

Endpoint tumour:

- Weight
- Immune cells infiltration (flow cytometry) data not shown

αPD-1 or αCTLA-4 monotherapy mediates low anti-tumour efficacy





Groups	T/C ratio
αPD-1 mAb	77%
αCTLA-4 mAb	54%

T/C>80%
42%<T/C<80%
T/C<42%

The EG7-OVA lymphoma cancer mouse model

Model allowing assessment of combination cancer therapies with αPD-L1 ICT

Study design

EG7-OVA cancer cells
(s.c.) in C57Bl6 (n=11/group)

Mice randomization
based on body weight

Treatment with ICT (i.p., 12.5 mg/kg)

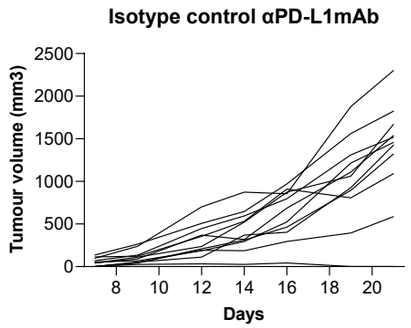
D0 D5 D8 D11 D14 D17 D22

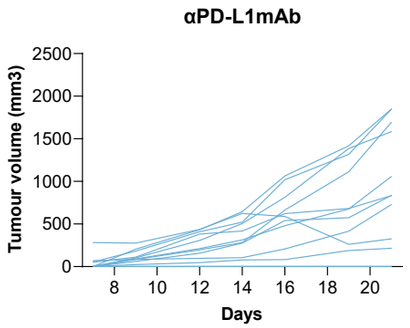
Time-course follow up of
mice body weight and tumour volume (3x/week)

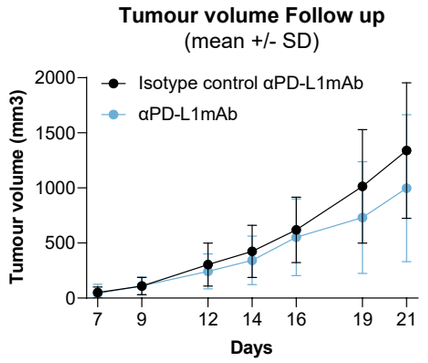
Endpoint tumour:

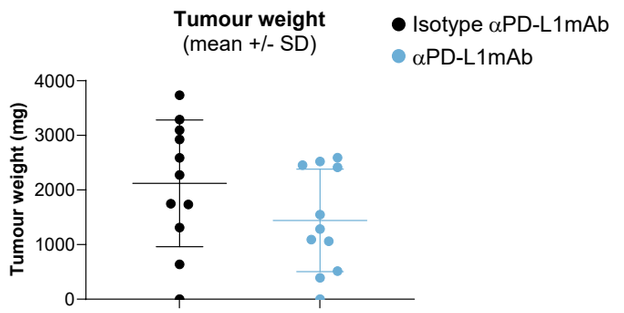
- Weight
- Immune cells infiltration (flow cytometry) data not shown

αPD-L1 monotherapy mediates low anti-tumour efficacy









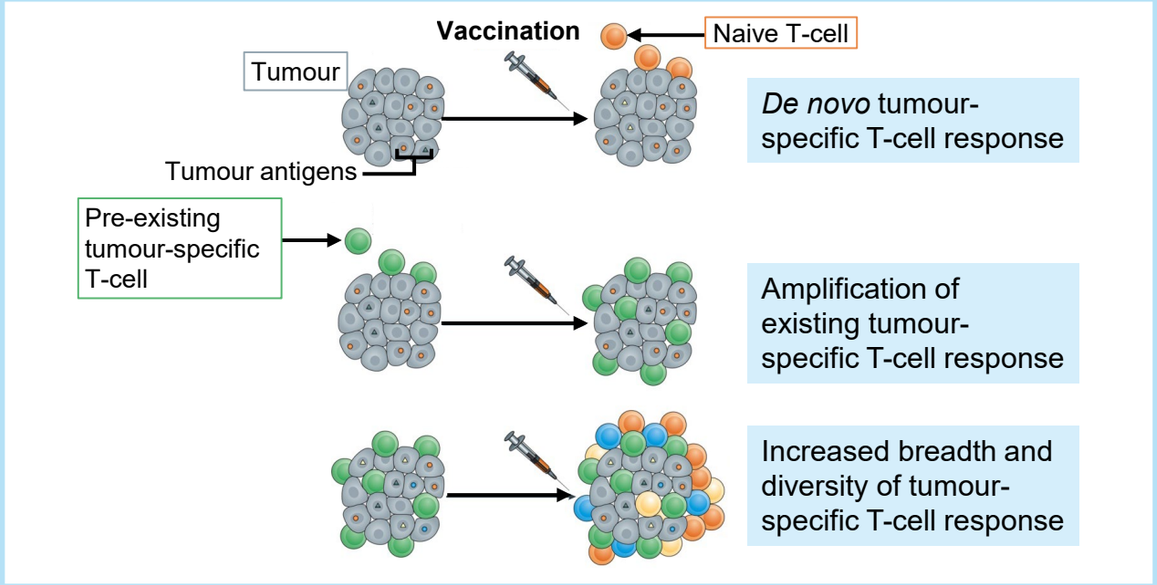
Group	Studies	T/C ratio
αPD-L1 mAb	N=2	77%

T/C>80%
42%<T/C<80%
T/C<42%

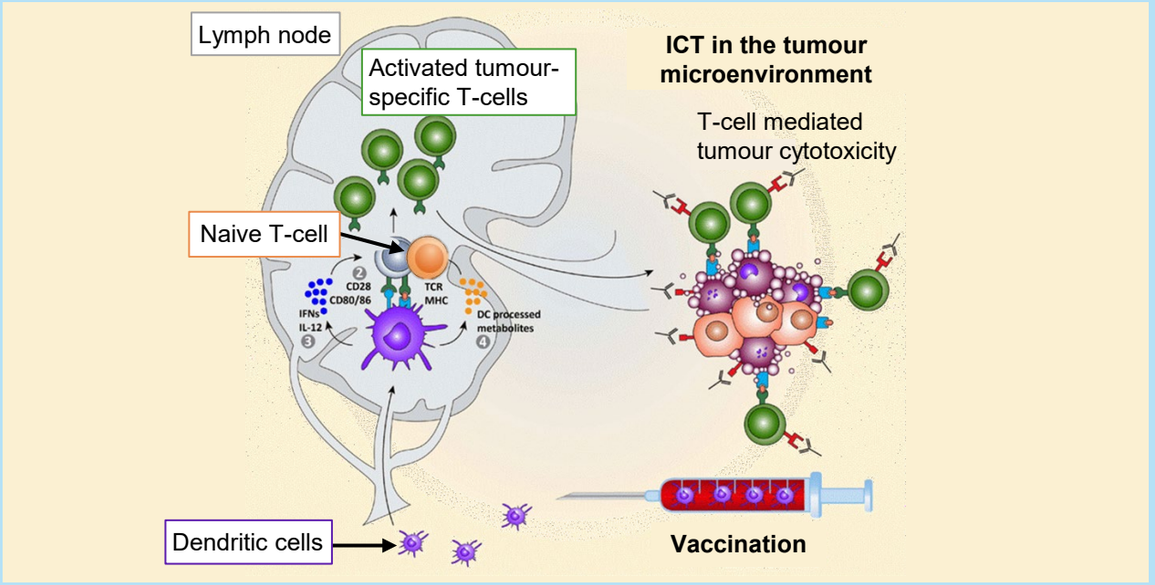
Therapeutic cancer vaccines

Vaccine immunogenicity: a critical step for anti-tumour response

Cancer vaccination includes a wide range of approaches to generate or amplify (or a combination of both) antitumour immunity



Cancer vaccine may be also an effective combinatorial partner with ICT
(given their potential to both generate new antigen-specific T cell responses against tumour cells and amplify existing responses)



Regardless of the approaches, efficacy of cancer vaccines relies on induction of an immune response to vaccination

Adapted from Zhuting Hu *et al., Nature Review Immunology*, 2018, **18**: 168, and Vasaturo Angela *et al., Frontiers in Immunology*, 2013, **4**:417

Immunogenicity assessment to OVA vaccine

Model to evaluate potency of adjuvants on specific CD8⁺ T-cell response to OVA vaccination

Study design

C57/Bl6

Immunization (OVA/CpG)

D0 — 7 days

IFN-γ ELISpot (on splenocytes)

Treatment	Admin. route	Restimulation
Vehicle	i.d	OVA 257-264 OVA 323-339
OVA + CpG	i.d	

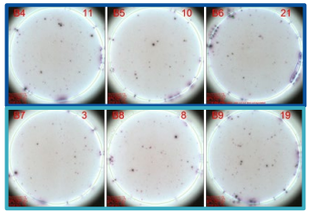
Vaccination induces activation of OVA-specific CD8⁺ T-cells (no CD4⁺ T-cells response)

Quantification of IFN-γ producing T cells (ELISpot):

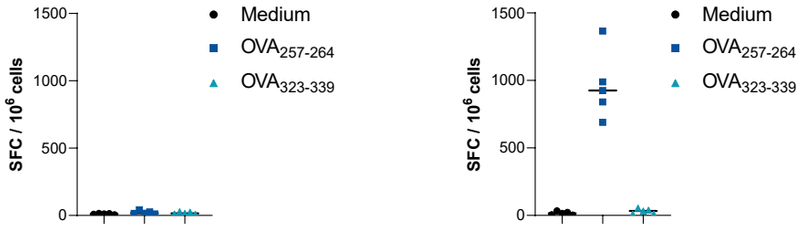
Representative ELISpot images:

Restimulation: OVA 257-264
CD8 specific peptide

Restimulation: OVA 323-339
CD4 specific peptide



Quantification:
Results are expressed as median of triplicat IFN-γ secreting cells, measured as spot forming cells (SFC) per million splenocytes



Immunogenicity assessment to OVA vaccine

Model to evaluate potency of adjuvants on specific Ab response to OVA vaccination

Study design

C57/Bl6

Immunization (OVA/CpG)

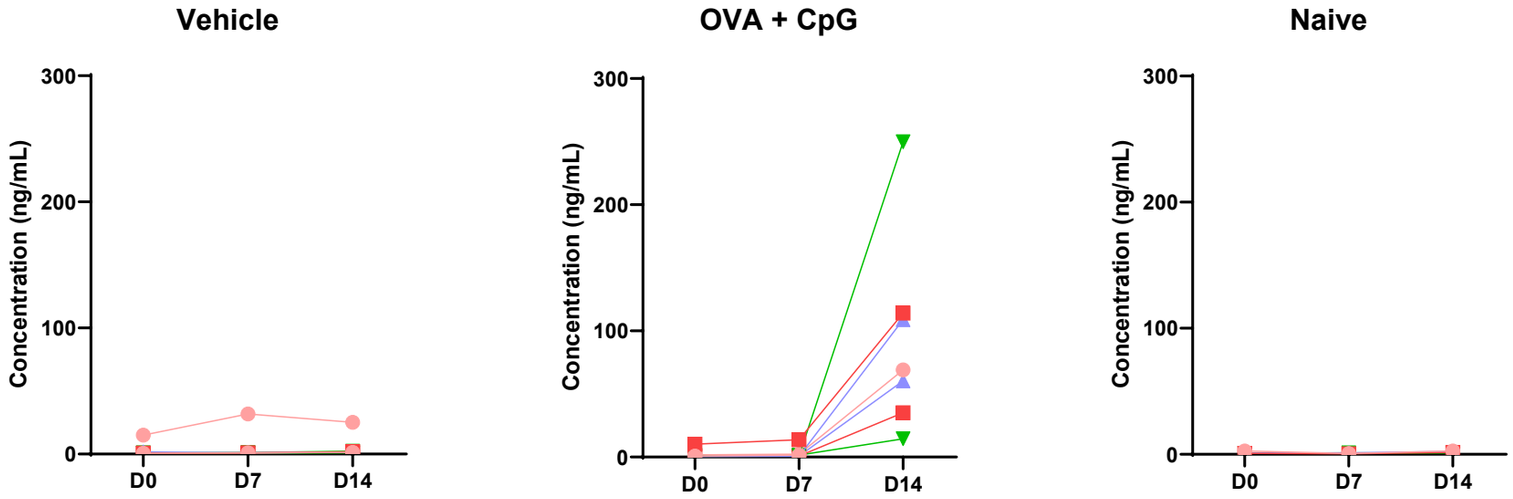
D0 D7 14 days

IgG anti-OVA in house ELISA (blood)

Treatment	Admin. route
Vehicle	i.d
OVA + CpG	i.d
Naive	–

OVA vaccination induced the production of specific anti-OVA IgG in the serum

Quantification of specific IgG anti-OVA by anin house developed ELISA method:



Immunogenicity assessment to candidate vaccines

Model to evaluate/optimize specific CD8⁺ T-cell response to candidate vaccines

Study design

CD1 outbred mice (n=8/group)

Immunization

D0 — 14 days

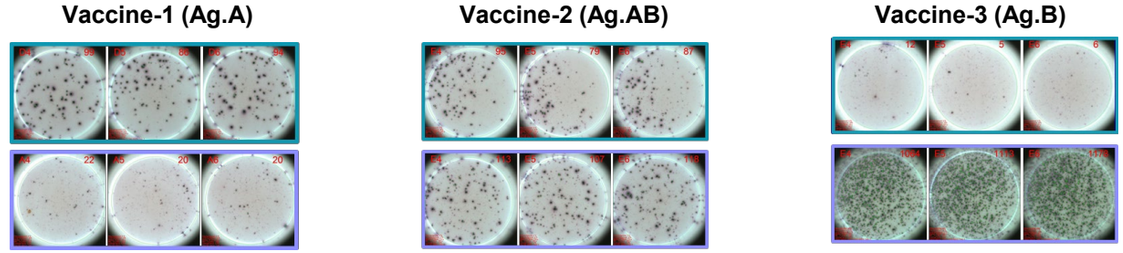
IFN-γ ELISpot
(on splenocytes)

Treatment	Admin. route	Restimulation
Vaccine_1 (Ag.A)	i.m	Pool Ag.A or Pool Ag.B
Vaccine_2 (Ag.AB)	i.m	
Vaccine_3 (Ag.B)	i.m	

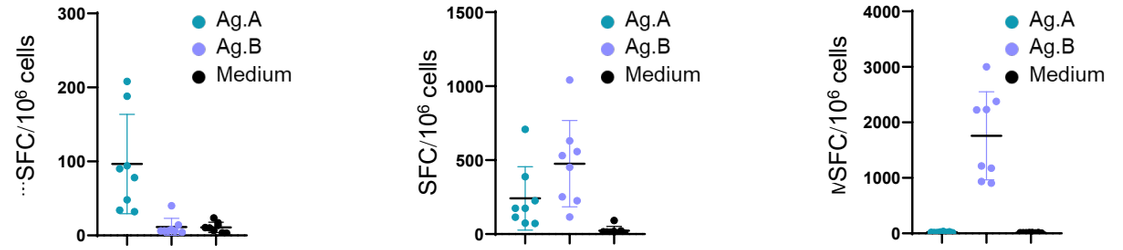
Vaccination induces development of Ag-specific CD8⁺ T-cells

IFN- g producing CD8⁺ T-cells in response to a specific Ag

Representative ELISpot images:



Quantification:
Results are expressed as median of triplicat IFN-γ secreting cells, measured as spot forming cells (SFC) per million splenocytes



evotec

#RESEARCHNEVERSTOPS

Your contact:

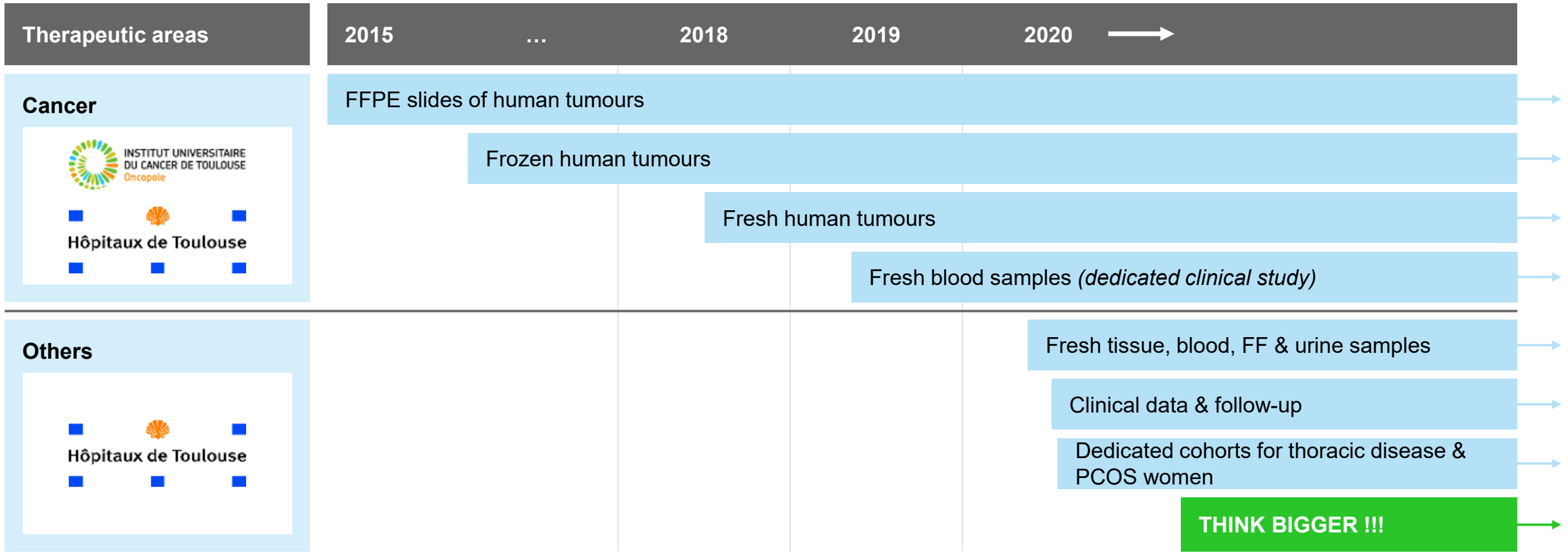
Business Development
114 Innovation Drive, Milton Park, Abingdon
Oxfordshire OX14 4RZ, UK

T: +44.(0)1235.86 15 61
F: +44.(0)1235.86 31 39
info@evotec.com

Backup slides

Translational Biomarkers group has strongly progressed to develop challenging projects

Clinical biomarkers expertise on patient samples



FFPE: Formalin-Fixed Paraffin-Embedded; FF: Follicular Fluids; PCOS: Polycystic Ovarian Syndrom; MTA: Material Transfer Agreement