LUDWIG CANCER RESEARCH

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Monitoring of T Cell immune responses

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ESMO Preceptorship programme
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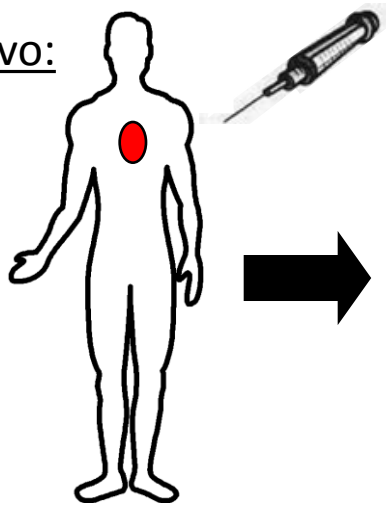
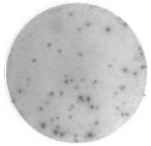
Ex vivo vs. *In vitro* analysis of T cells

Assessment: quantitative qualitative

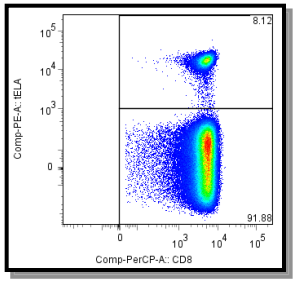
Ex vivo:



Isolation of PBMCs (Ficoll)



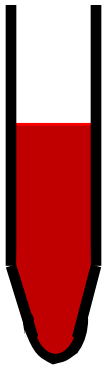
Elispot and multimer analysis



+

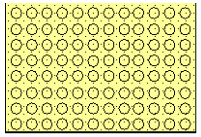
- cellular and molecular phenotype
-function (ICS) and degranulation
- TCR (and clonal distribution)

In vitro stimulation (IVS):



PBMCs

stimulation with peptides & cytokines



1 week +++

T cell frequency 100-1000x ↑ or more

semi-quantitative assessment

qualitative assessment not applicable

Flow Cytometry development to 8, 10 and more colors

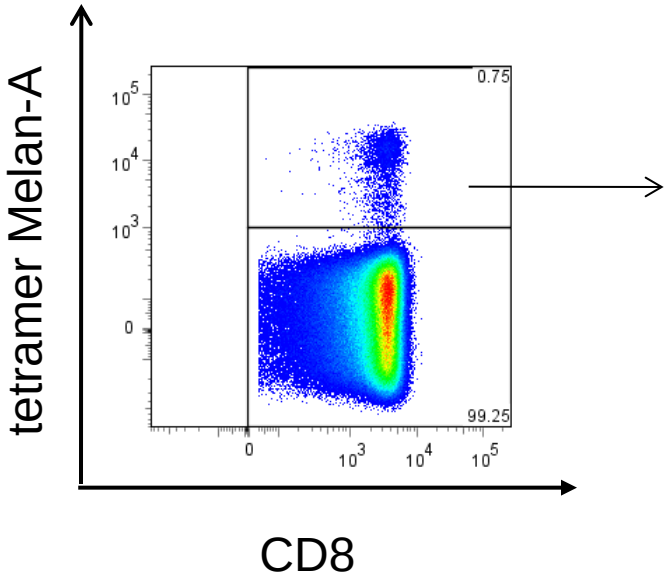
The evolution in Flow Cytometry in the last years allows to get more and more information about the differentiation and functionality on a single cell base.

- **phenotypic characterization (Naïve, CM, EM, EMRA)**: CD45RA, CCR7, CD28, CD27, CD127
- **activation markers**: HLA-DR, CD38, Ki67, Bcl2,….
- **inhibitor receptors (iR)**: PD1, CTLA-4, LAG-3, TIM3,….
- **functionality:** cytokine secretion: IFNγ, TNFα, IL-2, CD107a

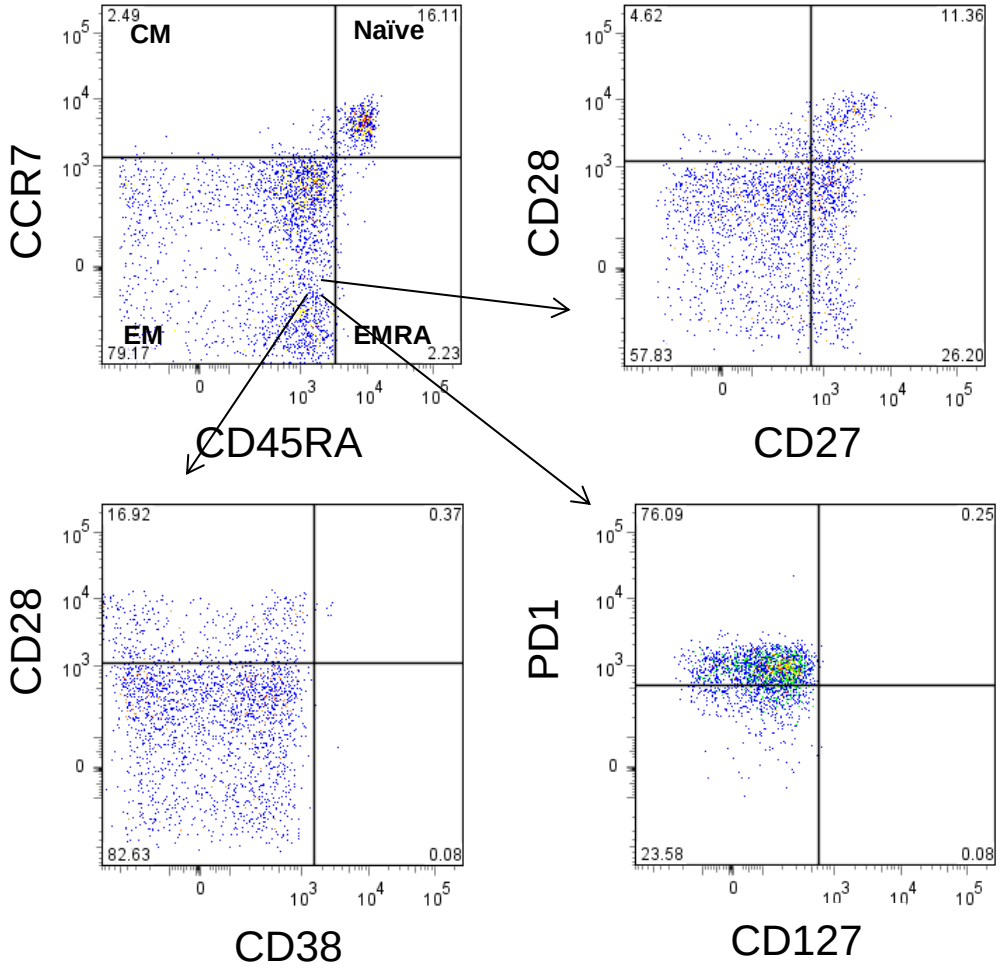
  cytolytic secretion: Perforin, Granzyme A, B and K

Ex Vivo Monitoring of CD8 T cells

phenotypic characterization



gated on Melan-A specific CD8 T cells



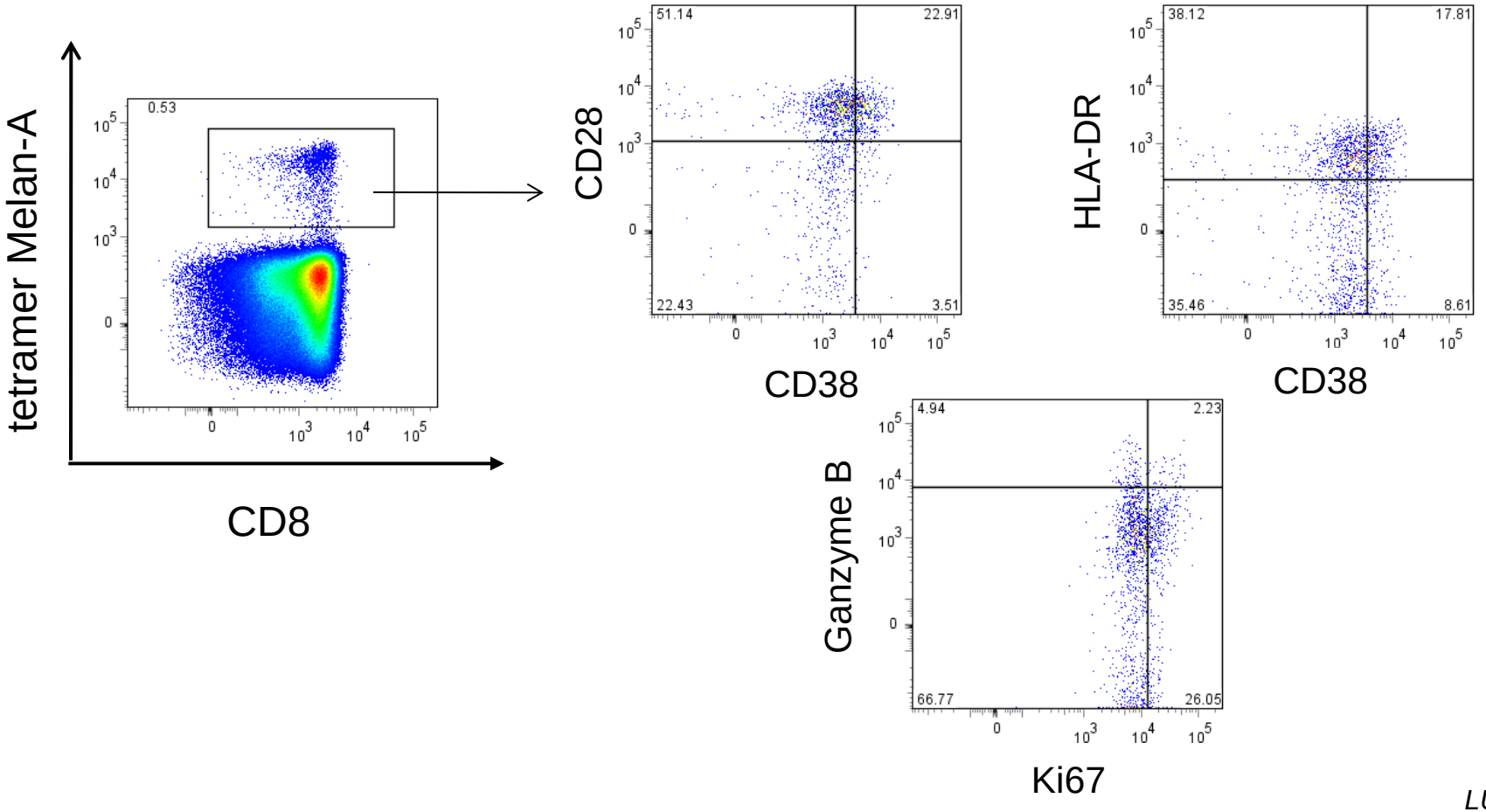
Patient LAU 1106

LUD 00-018
Vaccination with peptide, IFA, CpG

Ex Vivo Monitoring of CD8 T cells

activation markers: HLA-DR, CD38, Ki67

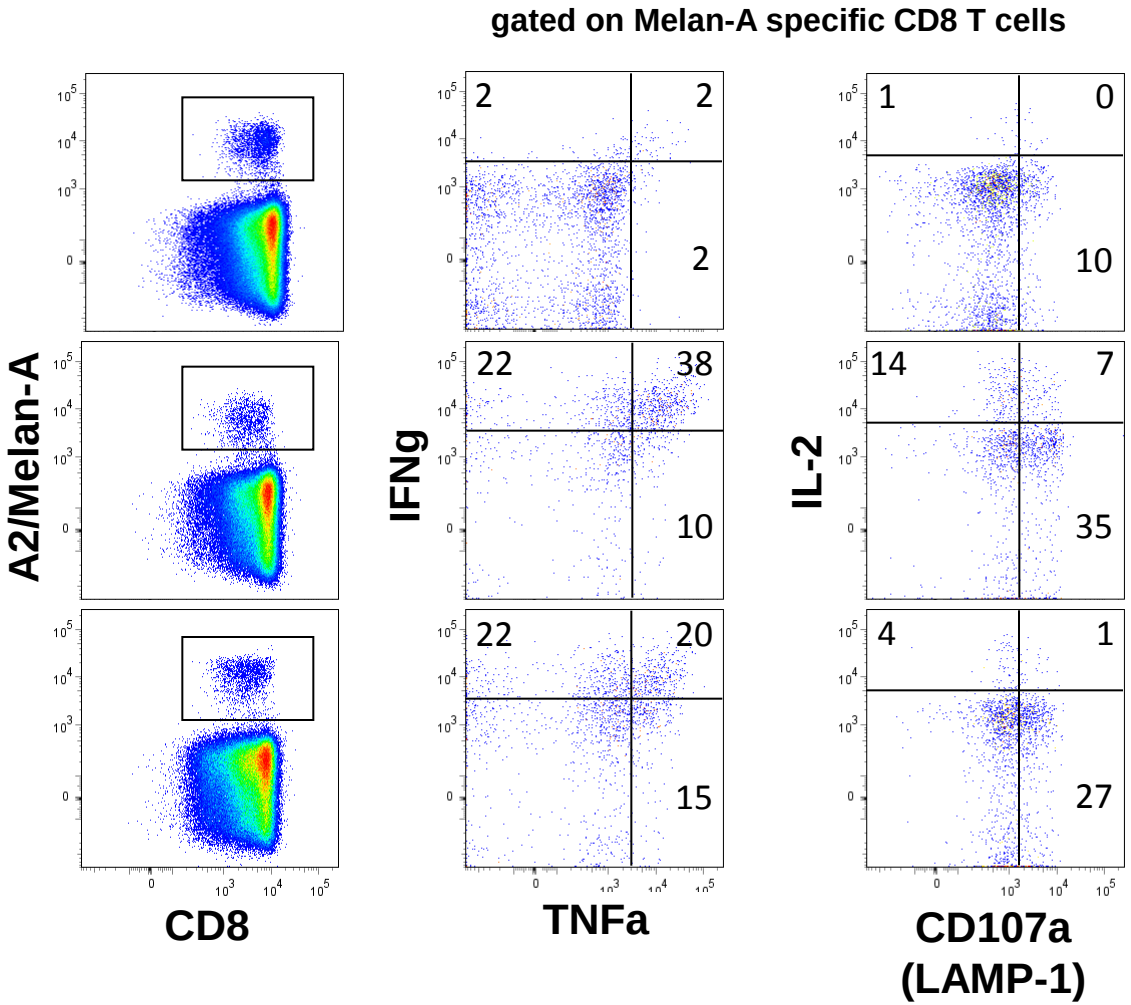
gated on Melan-A specific CD8 T cells



Patient LAU 627

LUD 00-018
Vaccination with peptide, IFA, CpG

Multi-functional antigen-specific CD8 T cells



control

10μM ELA peptide (4h)

PMA / ionomycine (4h)

Patient LAU 1264

LUD 00-018
Vaccination with peptide, IFA, CpG

Parameters for detection of immune responses on T cells

I) Some critical parameters to get good functional responses:

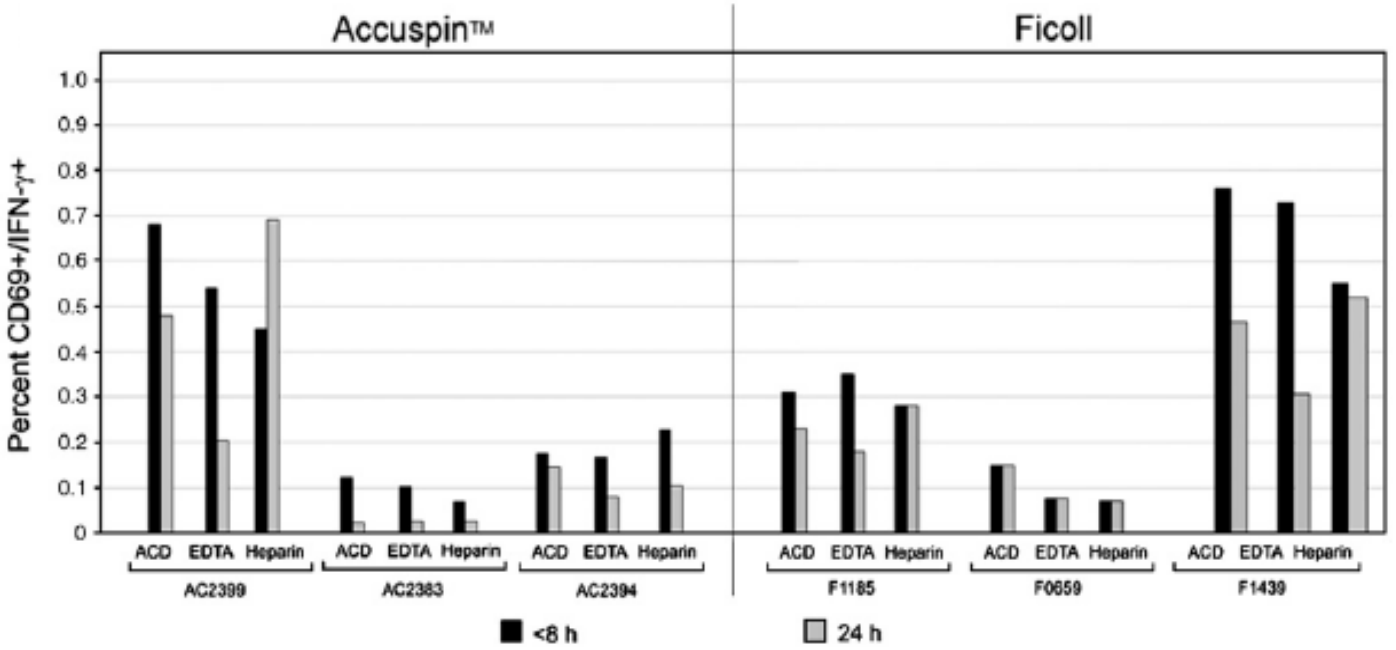
- preparation of the cells (to avoid artefacts, storage)
- conditions of the stimulation (E:T, type of stimulation)
- cell density in the assay

II) Some critical parameters to get good functional analysis:

- choice of the instrument and the fluorochrome to detect specific molecules (surface marker, cytokines, receptors)
- inclusion of a dead cell marker and dump channel (exclusion of specific cell populations)

Functional activity of PBMC isolated at different time points

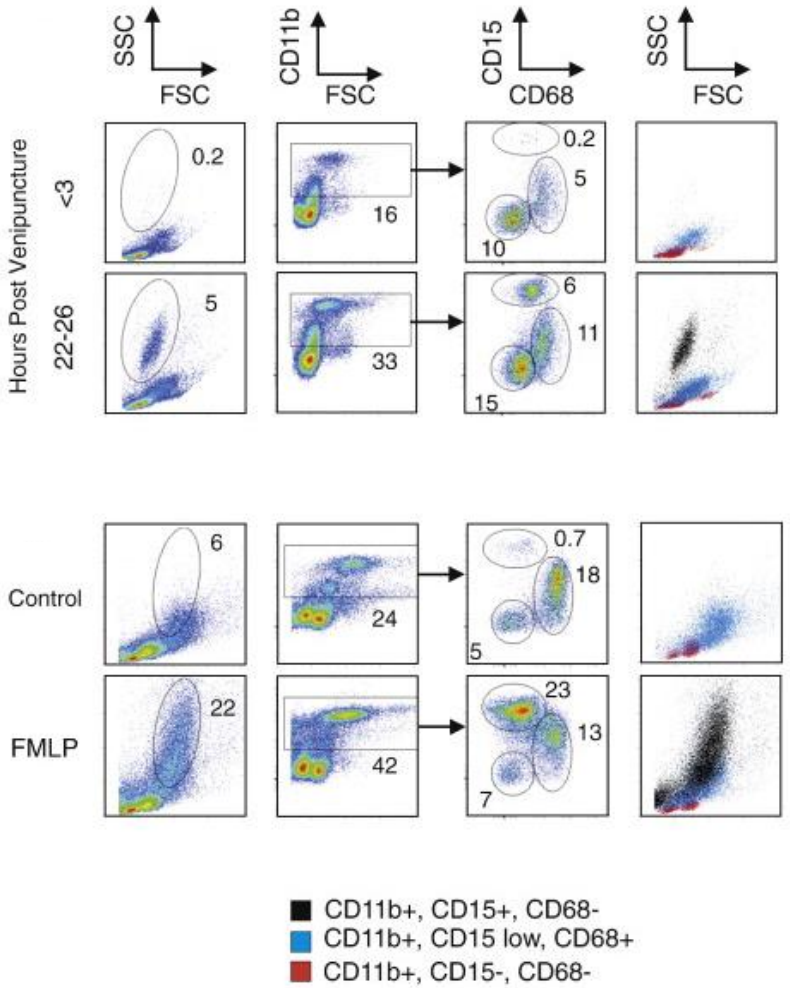
ICS: data gated on CD3+ CD8+ T lymphocytes

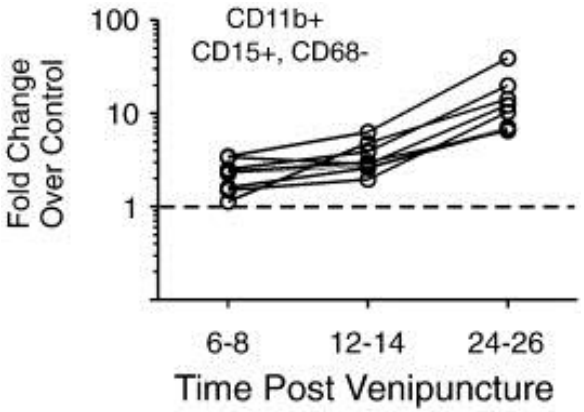


- Functional activity of isolated PBMC decreases with prolonged processing time.

Bull M. et al,(2007) JIM 322

Impact of sample preparation on isolated PBMCs

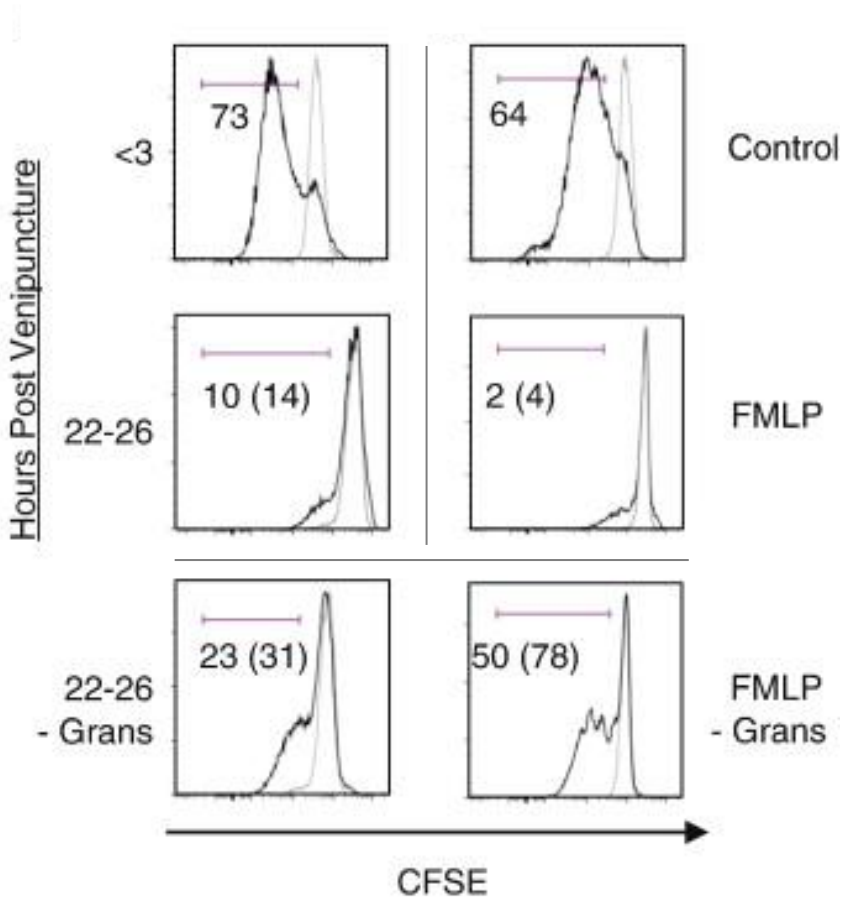




• Prolonged sample preparation leads to enrichment of contaminating granulocytes-like cells (CD11b+, CD15+) in isolated PBMC.

McKenna K. et al, (2009), JIM 341

Inhibition of contaminating CD15+ granulocyte-like cells on T cell proliferation



- CFSE proliferation experiments with PBMCs containing contaminating granulocytes showed reduced proliferation capacity and function of T cells.

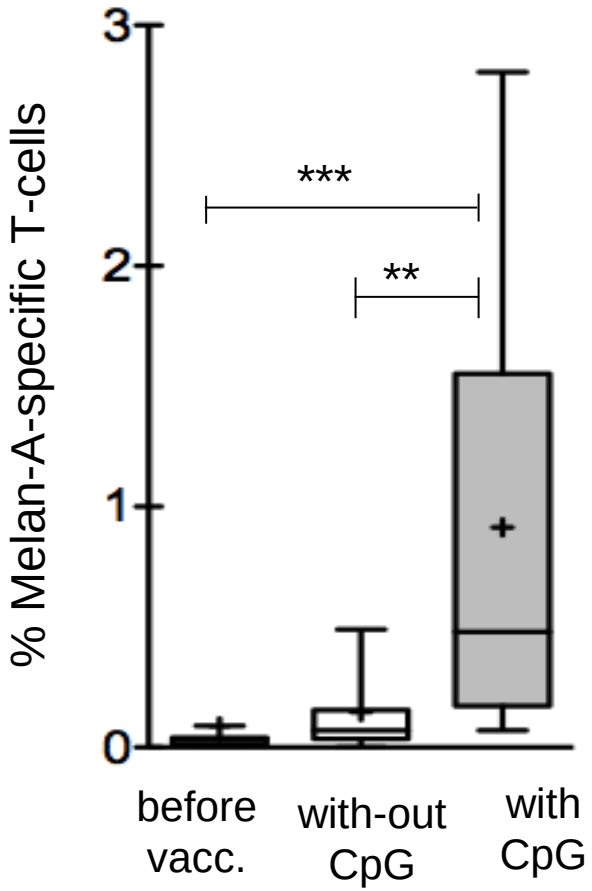
McKenna K. et al, (2009), JIM 341

Analysis of antigen-specific CD8 T cells of stage III and IV melanoma patients

High numbers of Melan-A specific CD8 T cells induced by s.c. vaccinations with CpG + Montanide + peptide

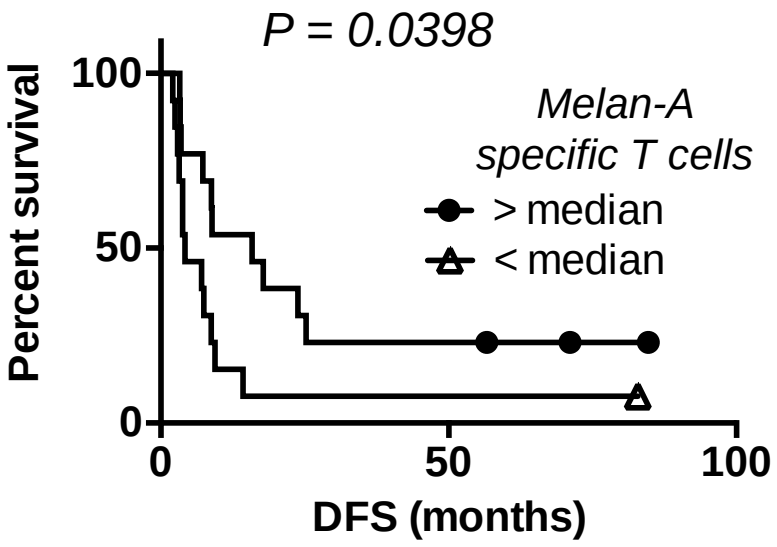
Peptide vaccination with the adjuvant **CpG 7909 (PF-3512676)** induced very strong Melan-A specific $CD8^+$ T cell responses as compared to vaccination without CpG (the data without CpG are from the previous trial LUD 96-010).

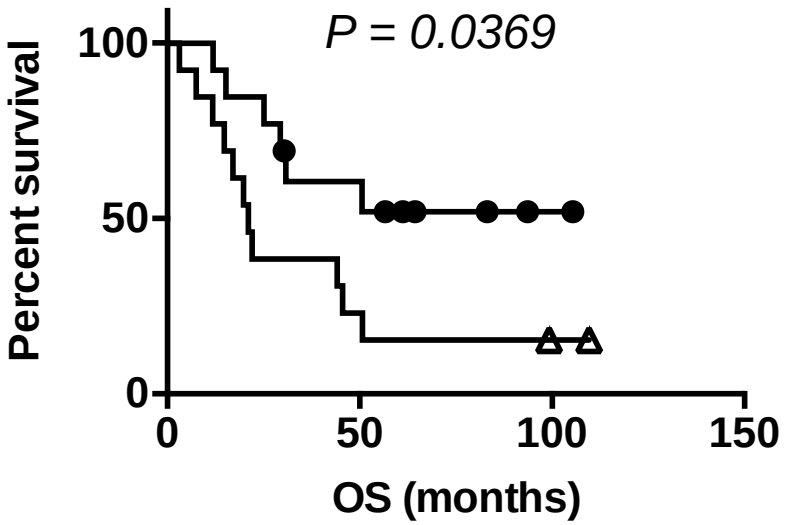
PBMC were analyzed directly *ex vivo* using tetramers.



Baumgaertner P. et al. , IJC 2012

Melan-A specific T cells correlated with patient survival



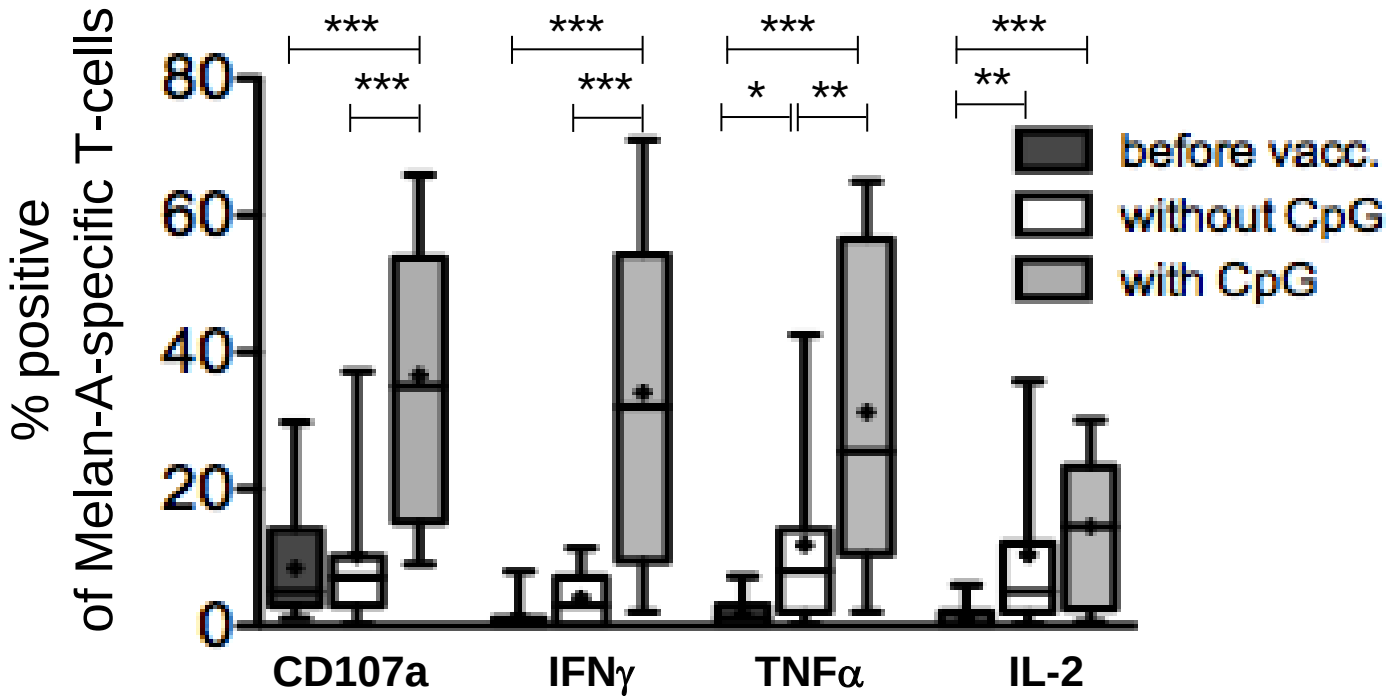


n =18 patients

Patients with Melan-A specific T cells above the median value showed a better **D**isease **F**ree **S**urvival and **O**verall **S**urvival.

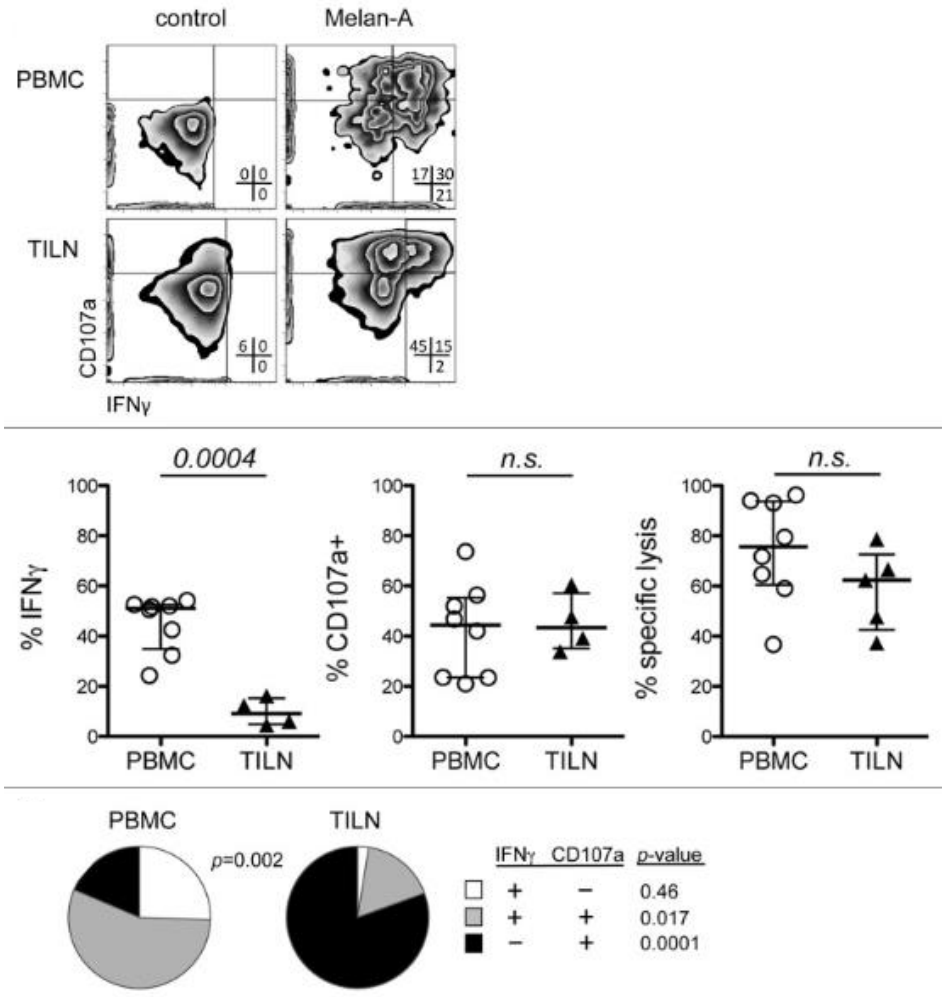
Baumgaertner P. et al. , IJC 2012

Multi-functionality of Melan-A specific CD8 T cells



Baumgaertner P. et al. , IJC 2012

Reduced functionality of Melan-A specific CD8 T cells in TILN compared to PBMCs

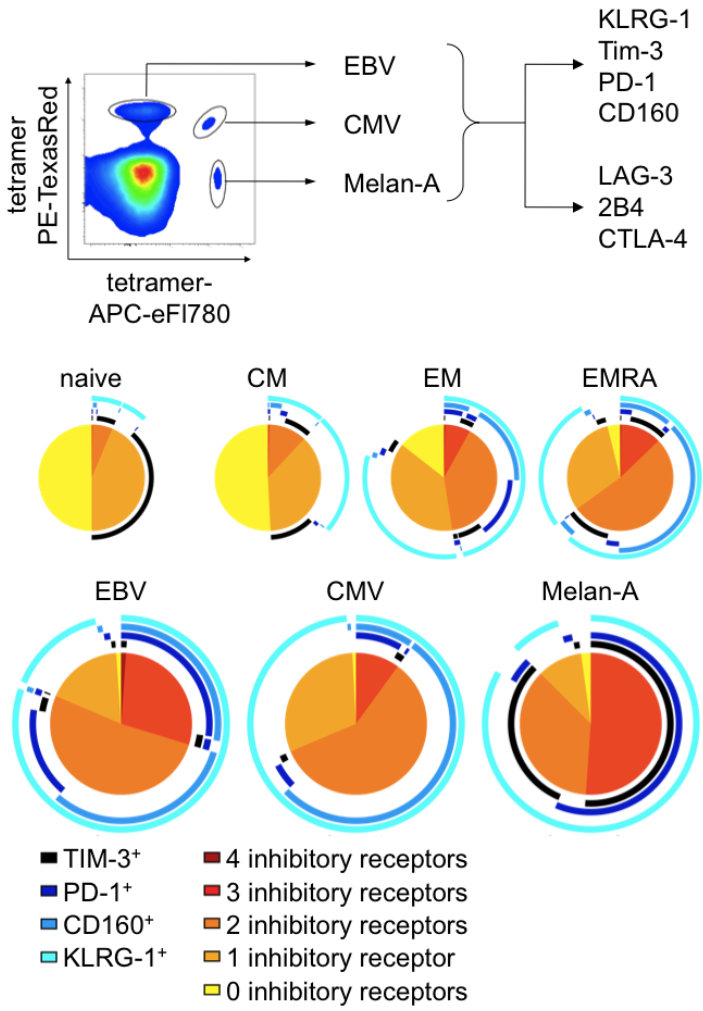


- TILN-derived Melan-A specific $CD8^+$ T-cells exhibit decreased IFNγ production.

- In contrast, the degranulation capacity was comparable to their PBMC-derived counterparts.

Devevre E. et al. , Oncoimmunology 2012

Multi-tetramer staining assessing co-expression of inhibitory receptors

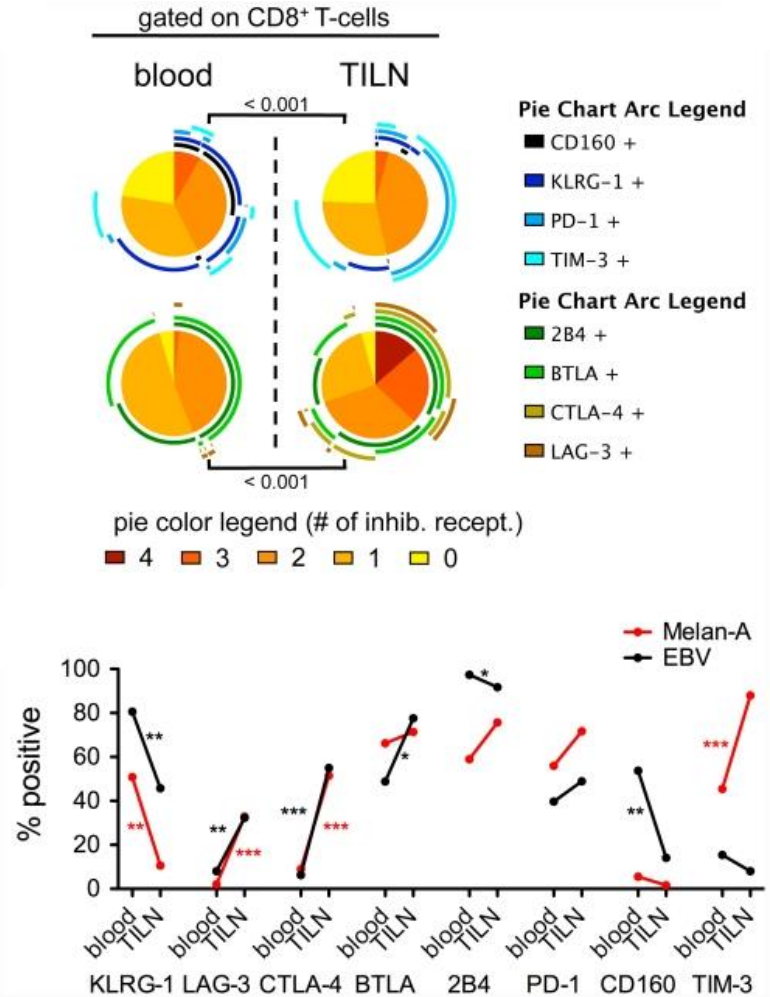


- staining with tetramers binding to EBV-, Melan-A- or CMV- specific CD8 T cells (combinatorial tetramer staining)

- T cells were analyzed for co-expression of 7 inhibitory receptors (iR):
KLRG-1/ TIM-3/ PD-1/ CD160
or
LAG-3/ 2B4/ CTLA-4

Baitsch L. et al. , JCI 2011

Expression of inhibitory receptors by CD8 T cells derived from blood and TILNs

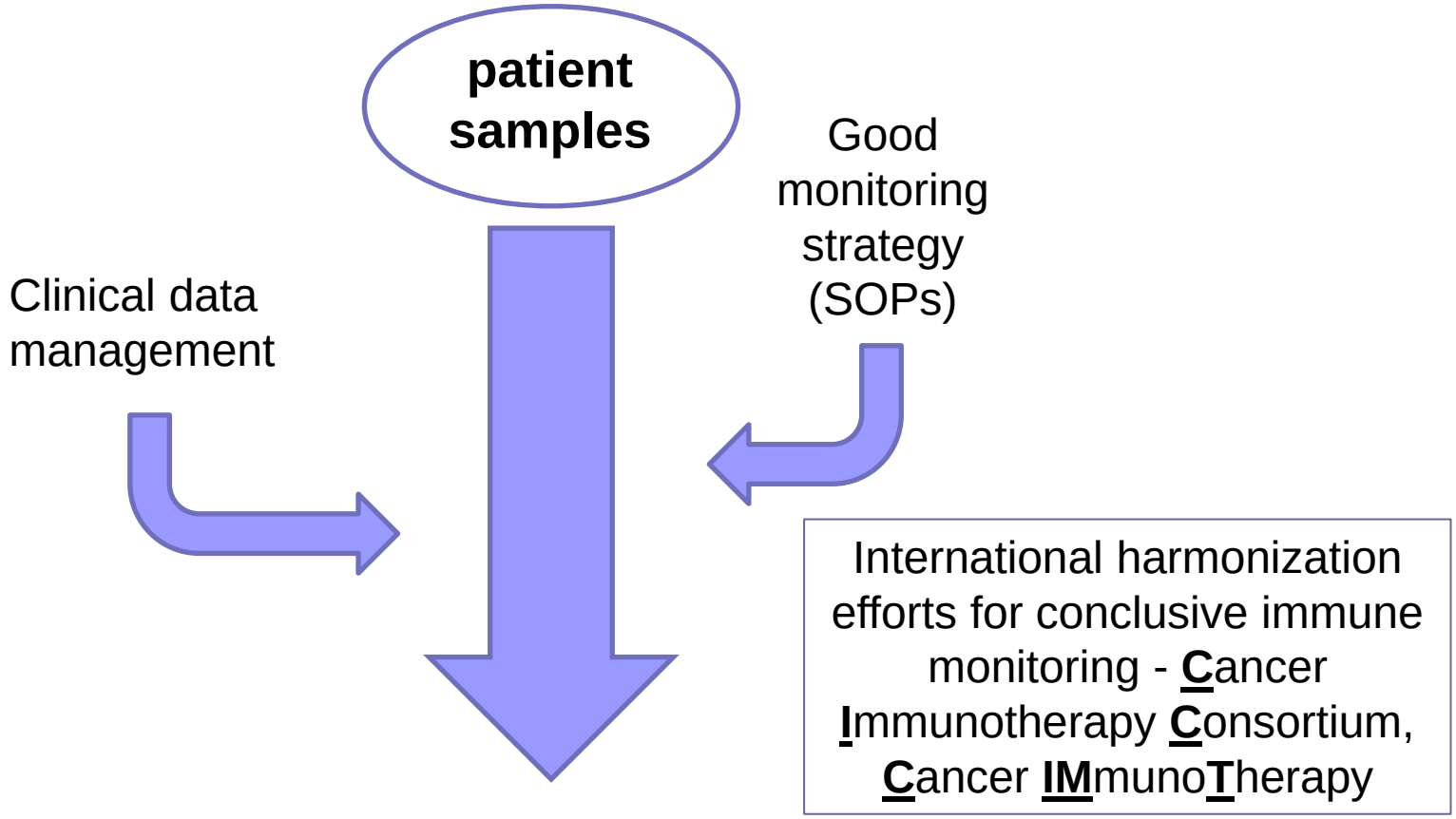


- co-expression analysis of total CD8 T cells
- positivity of Melan-A- and EBV-specific CD8 T cells for the inhibitory receptors

Blood samples were from patients vaccinated either with CpG or without CpG

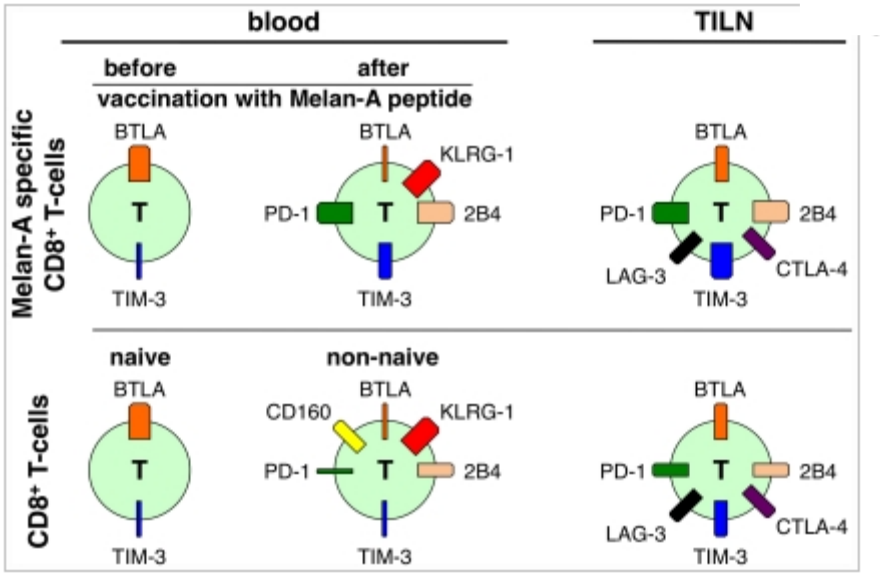
Baitsch L. et al. , JCI 2011

Key elements of immune monitoring



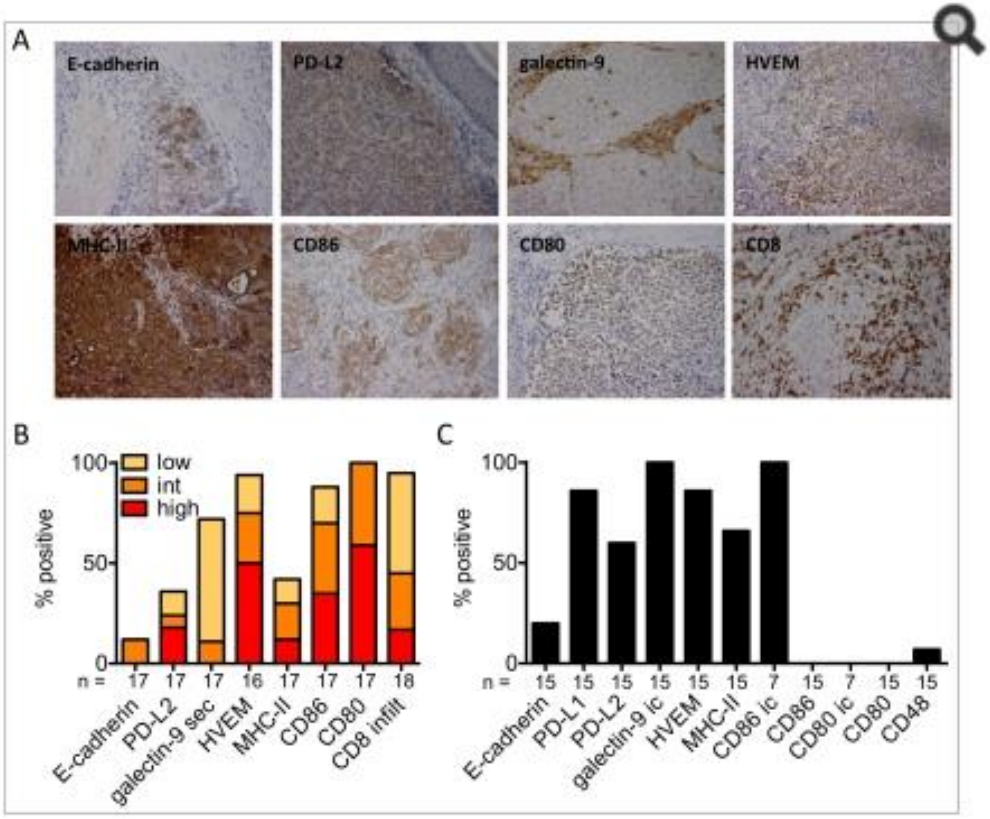
→ **Comprehensive clinical phase 1 trials**

Inhibitory receptor co-expression according to differentiation status and physical location

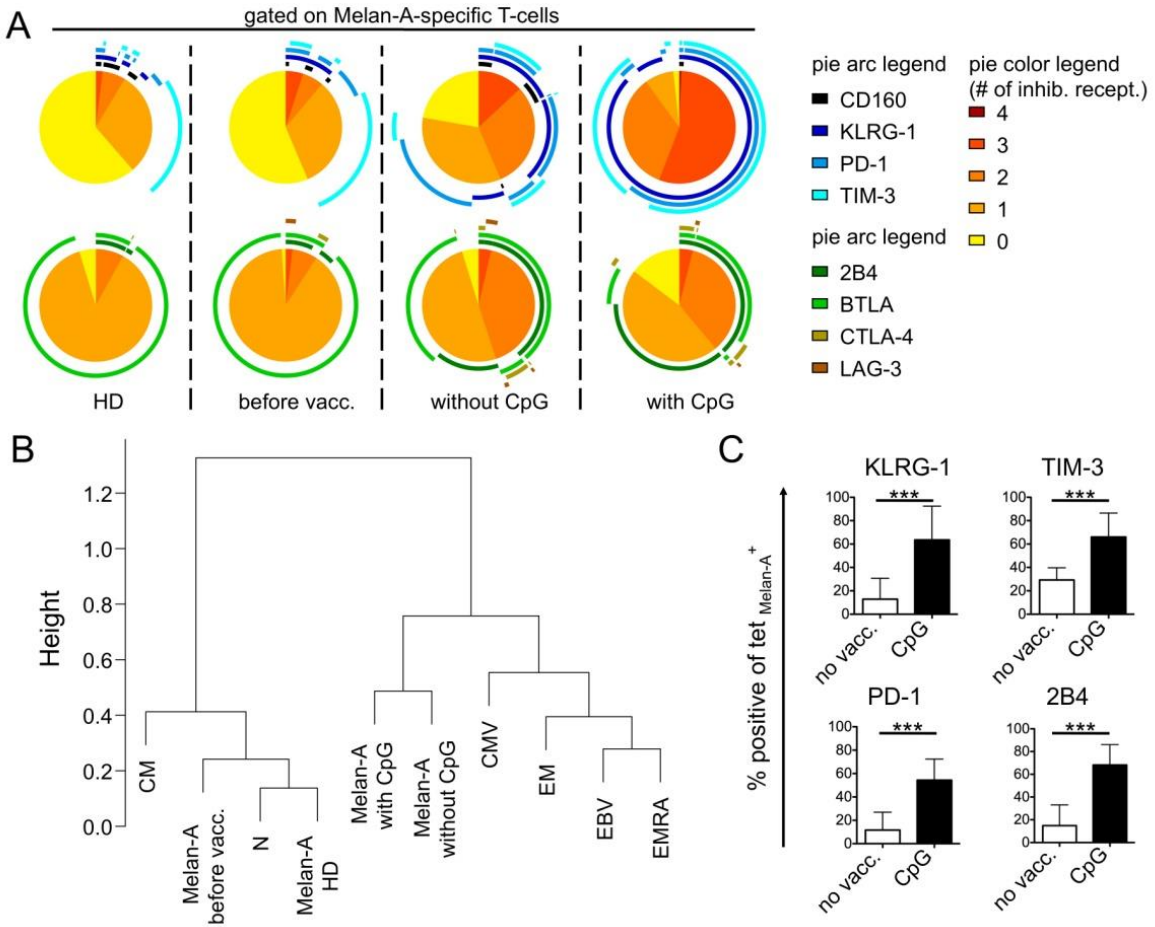


- Naive cells express BTLA and TIM-3.
- After peptide vaccination, Melan-A specific T-cells upregulate KLRG-1, 2B4, TIM-3 and PD-1, while they downregulate BTLA.
- Total CD8 T-cells upregulate similar inhibitory receptors, but less PD-1 and TIM-3. They also express CD160, which is not expressed by tumor-specific T-cells.
- In TILN, both total CD8 T-cells, which are to a large extent tumor-specific, and Melan-A specific T-cells downregulate KLRG-1 (and in total CD8 T-cells CD160) and concomitantly express more PD-1, LAG-3, TIM-3 and CTLA-4.

Expression of ligands of inhibitory receptors in melanoma metastases and by melanoma cell lines



- Paraffin-embedded tumor sections from 16-18 tumors were stained by immunohistochemistry for seven inhibitory receptors and CD8.
- (A) Representative stainings (×200) for each ligand investigated.
- (B) Summary of immunohistochemical stainings represented as percent of positive samples.
- (C) Summary of expression by melanoma cell lines on the surface or intracellular (ic) as percent of positive cell lines.


A
gated on Melan-A-specific T-cells
HD
before vacc.
without CpG
with CpG
pie arc legend
CD160
KLRG-1
PD-1
TIM-3
pie arc legend
2B4
BTLA
CTLA-4
LAG-3
pie color legend
(# of inhib. recept.)
4
3
2
1
0
B
Height
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CM
Melan-A before vacc.
N
Melan-A HD
Melan-A with CpG
Melan-A without CpG
CMV
EM
EBV
EMRA
C
KLRG-1
TIM-3
PD-1
2B4
% positive of tet Melan-A+
***
no vacc.
CpG
100
80
60
40
20
0

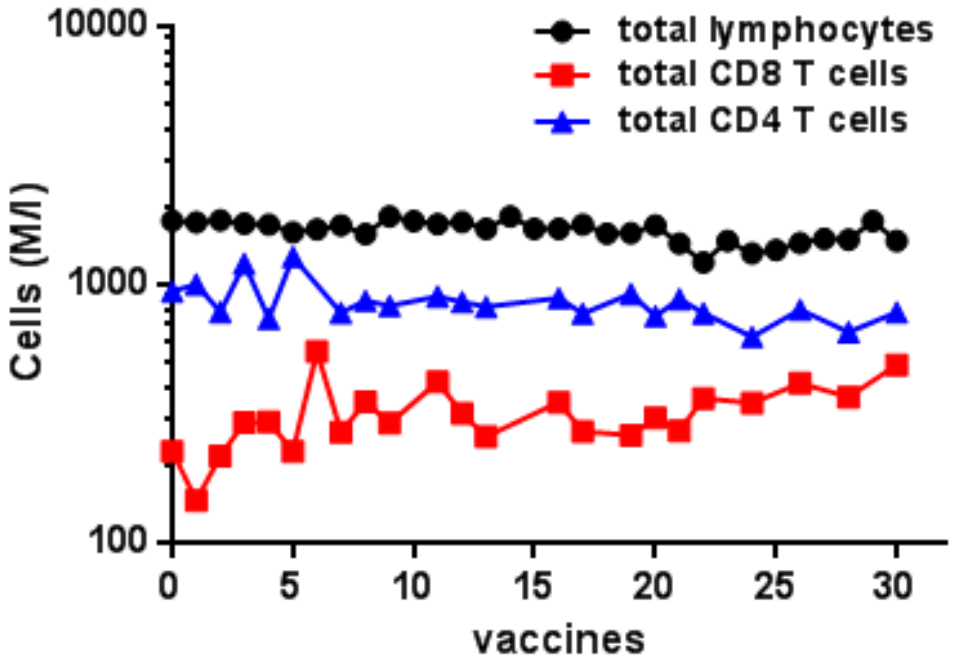
Cancer vaccine trial LUD 00-018

- 29 HLA-A2 positive patients with stage 3/4 melanoma
- Patients received up to 8 cycles of 4 monthly vaccines containing

  ⟶ CpG + Montanide + tumor-antigen-peptides (Melan-A/MART-1 -/+ tyrosinase peptides)

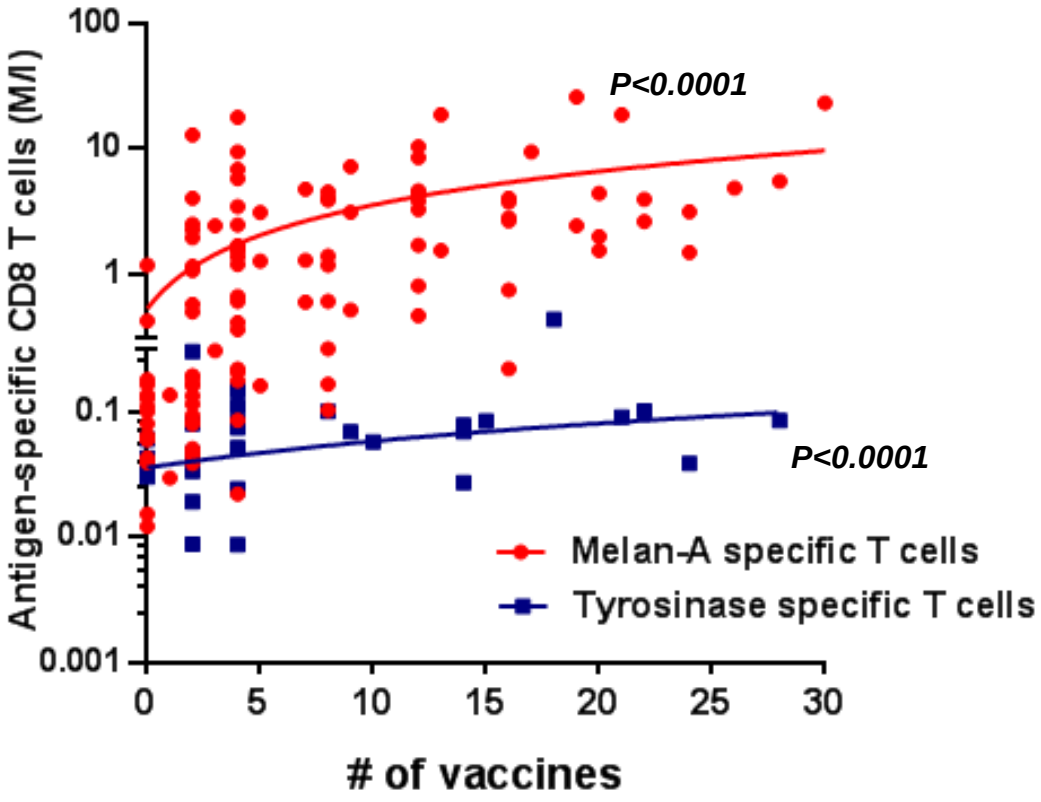
- 26/26 evaluable patients generated high numbers of Melan-A specific CD8 T cells which secreted IFNγ in 24 patients

Total lymphocyte numbers remained stable,



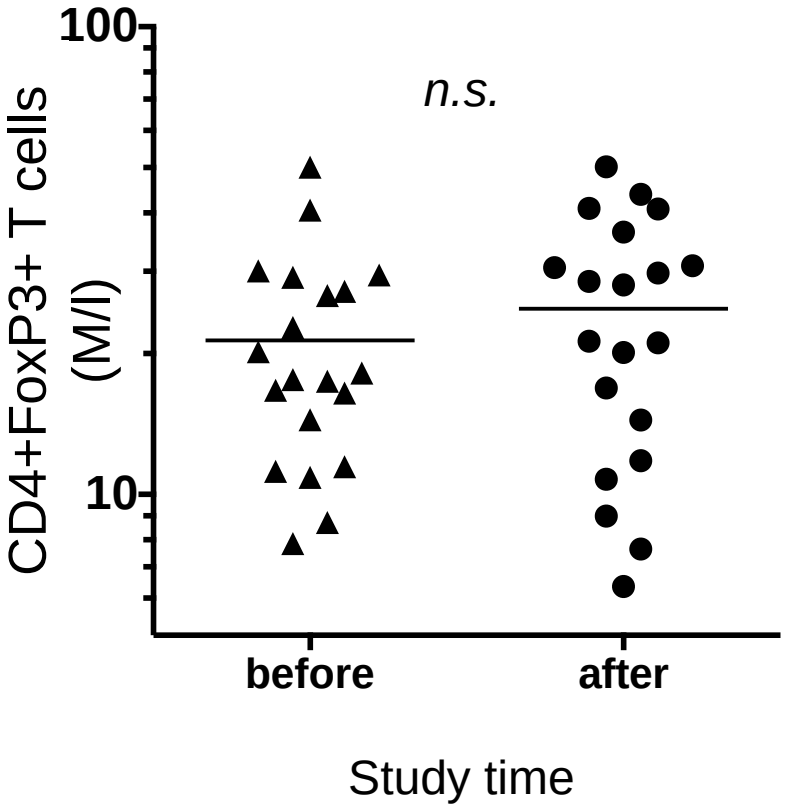
- The total numbers of lymphocytes, CD8 and CD4 T cells remained stable over time and after many vaccinations.

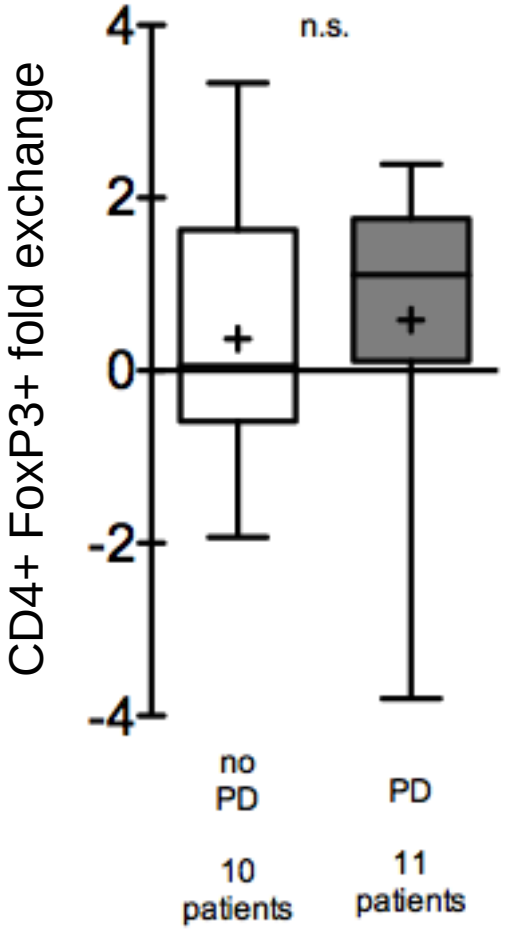
The numbers of Melan-A and Tyrosinase specific CD8 T cells increased



- Melan-A specific CD8$^+$ T cell numbers increased very strongly (*P<0.0001).*
- Tyrosinase specific CD8$^+$ T cell numbers also increased (*P<0.0001)*.

Total number of CD4+ Tregs did not change significant during the study time, but...

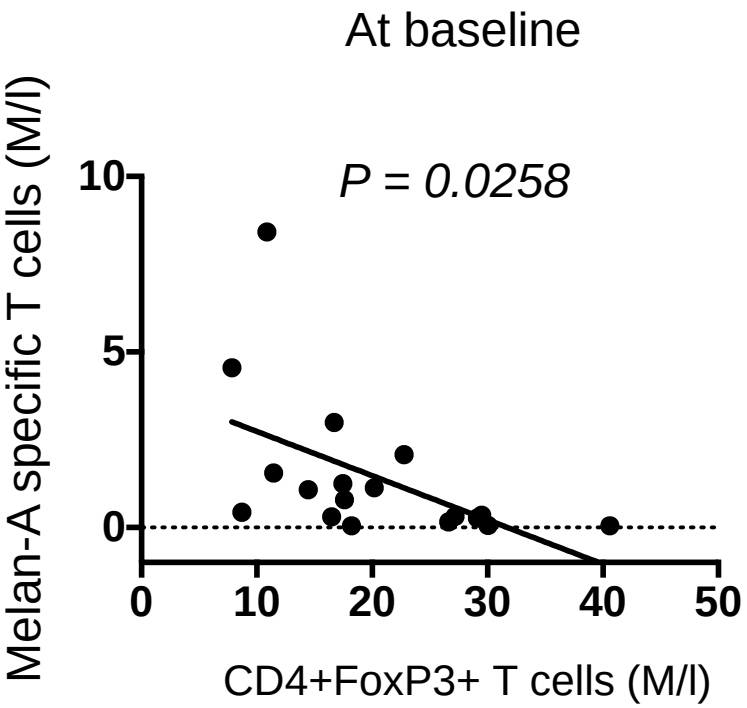


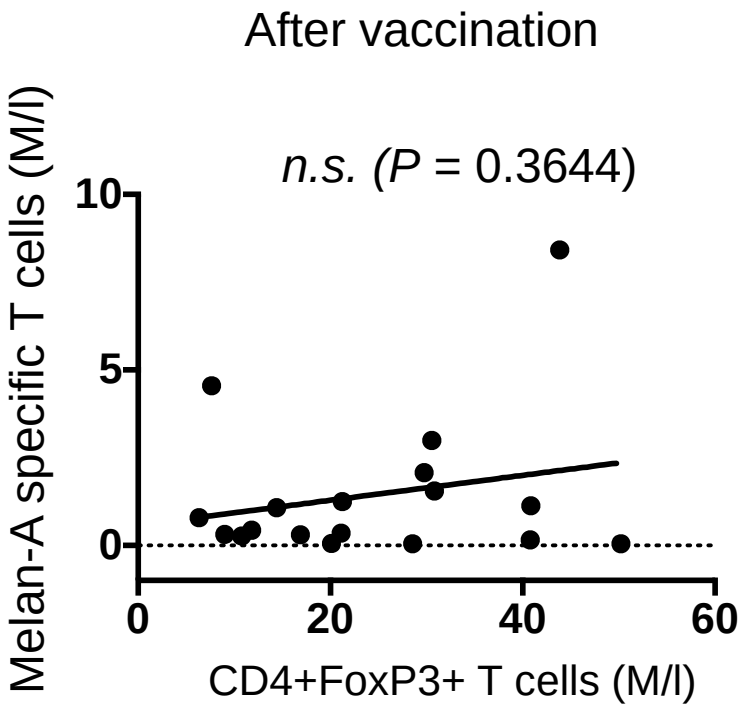


Change from before to after vaccination

… low Treg numbers at baseline

predicted enhanced CD8 T cell responses

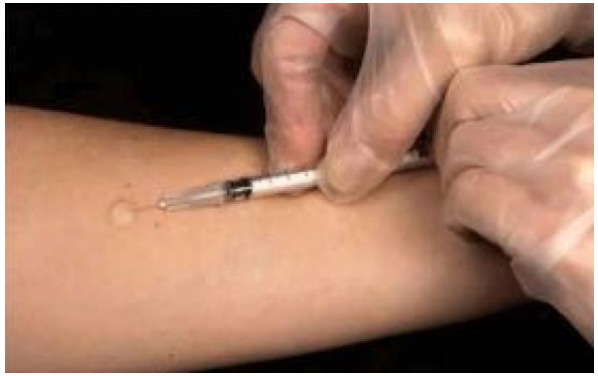


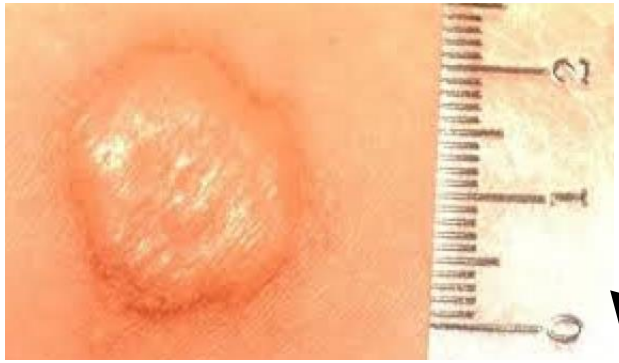


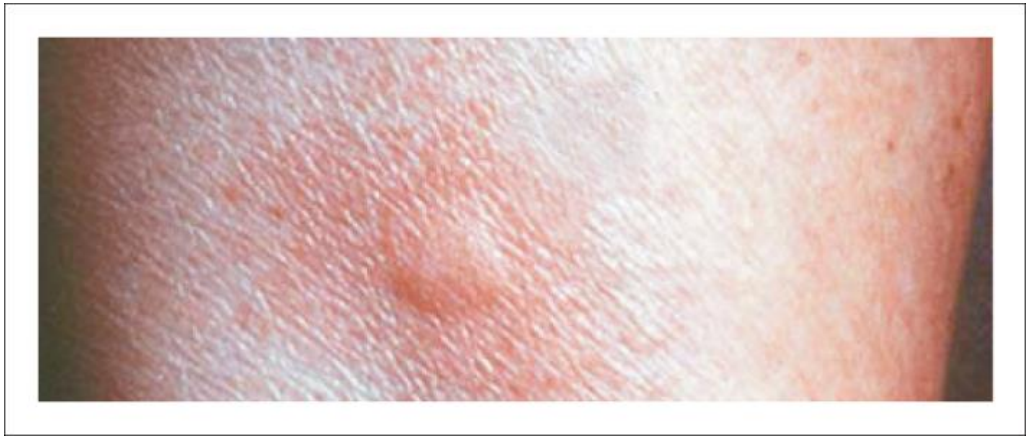
DTH - **D**elayed **T**ype **H**ypersensitivity reactions (Melan-A)

A method to test tissue homing and function of antigen-specific CD8 T cells in vivo

Intradermal injection of 10 µg Melan-A peptide in DMSO/ PBS





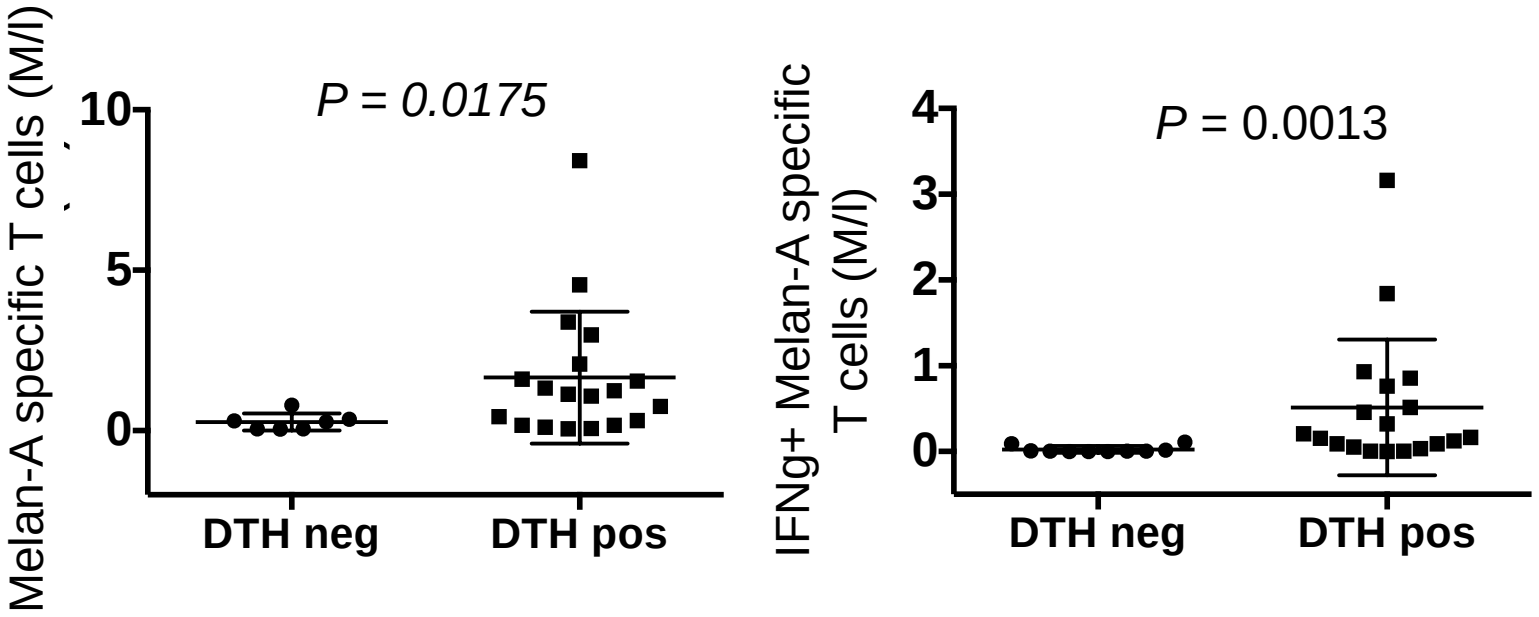


Positive DTH after 48 hours (> 4 mm of diameter with induration)

DTH responses to tumor-antigen injection

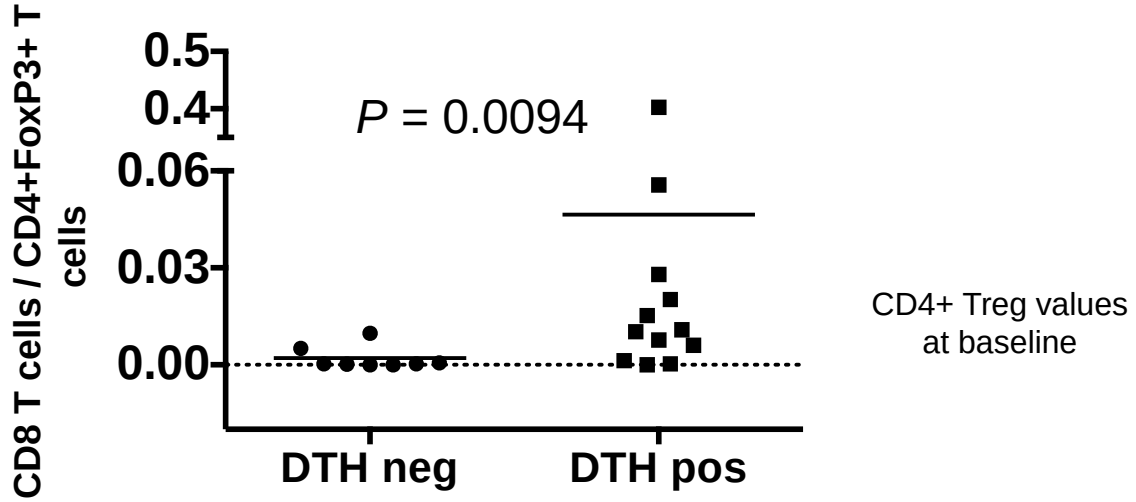
	Stage III (n = 21)	Stage IV (n = 8)
At least 1 positive DTH during the study		
Yes	13	6
No	8	2
Increase of DTH reaction during the study		
Yes	11	4
No	7	2
Not Assessable	3	2
Median number of vaccinations before the first positive DTH (range)	4 (13 patients assessable)	4 (only 1 patient assessable)

DTH responses and Melan-A specific T cells



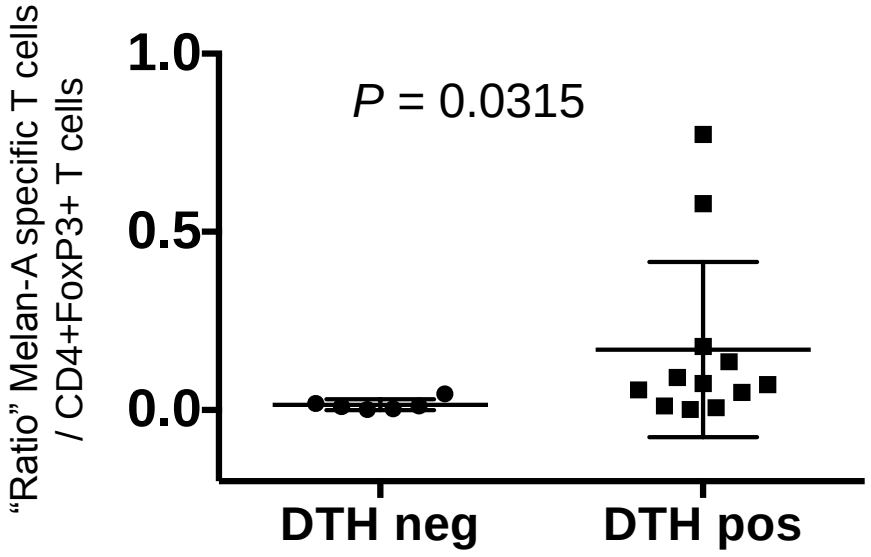
Positive DTH responses correlated with
increased numbers of Melan-A specific T cells and their IFNγ production.

DTH responses and the «ratio» between CD8 T cells and CD4+ Tregs



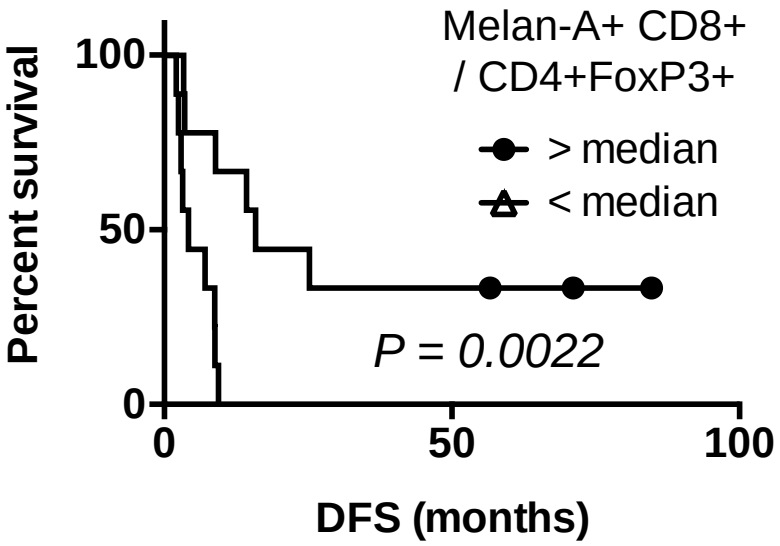
The ratio between CD8 T cells and CD4+ Tregs correlates with positive DTH responses.

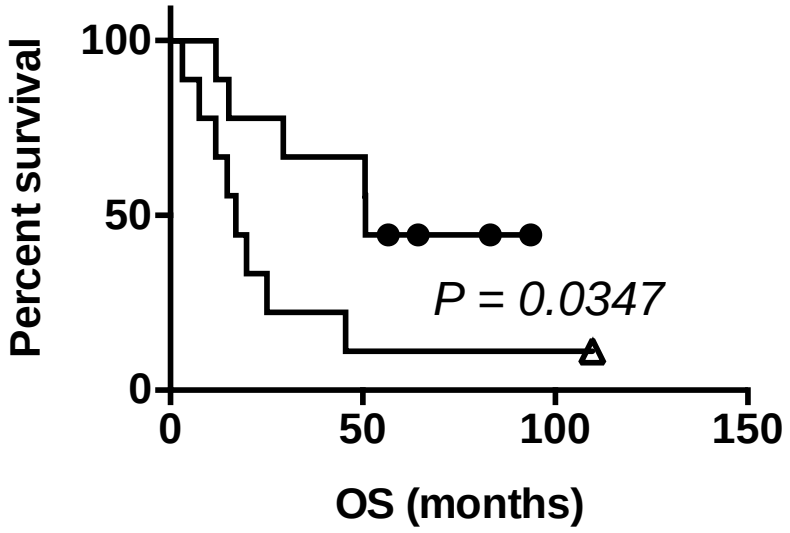
DTH responses and Melan-A+ CD8+ effector T cell/Treg ratios



The ratio "Melan-A specific CD8+ T cells / CD4+ Tregs" correlated with positive DTH reaction.

The ratio of Melan-A specific T cells / Treg cells correlated with patient survival

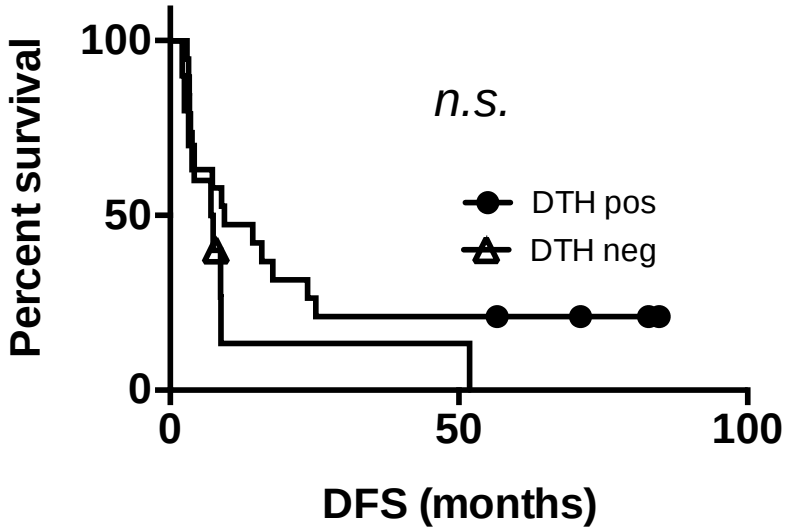


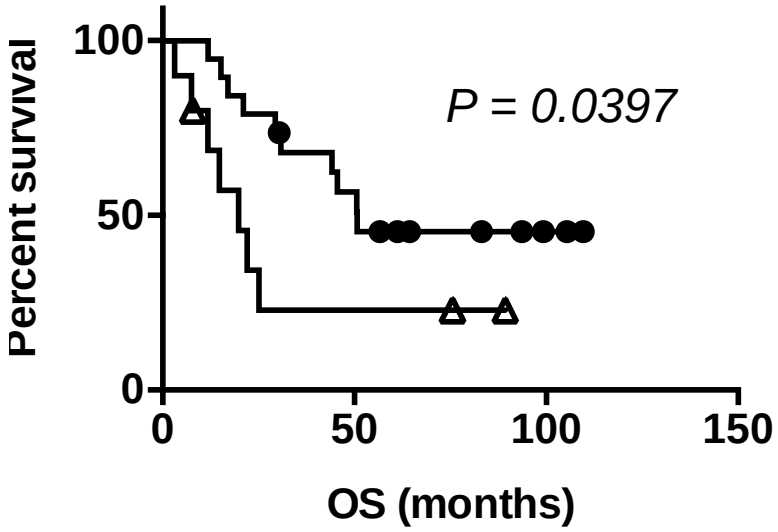


n =18 patients

Patients with ratio “Melan-A+ CD8+ T cells / CD4+FoxP3+ T cells” above the median value showed better **D**isease **F**ree **S**urvival and **O**verall **S**urvival.

DTH reactions correlated with patient survival

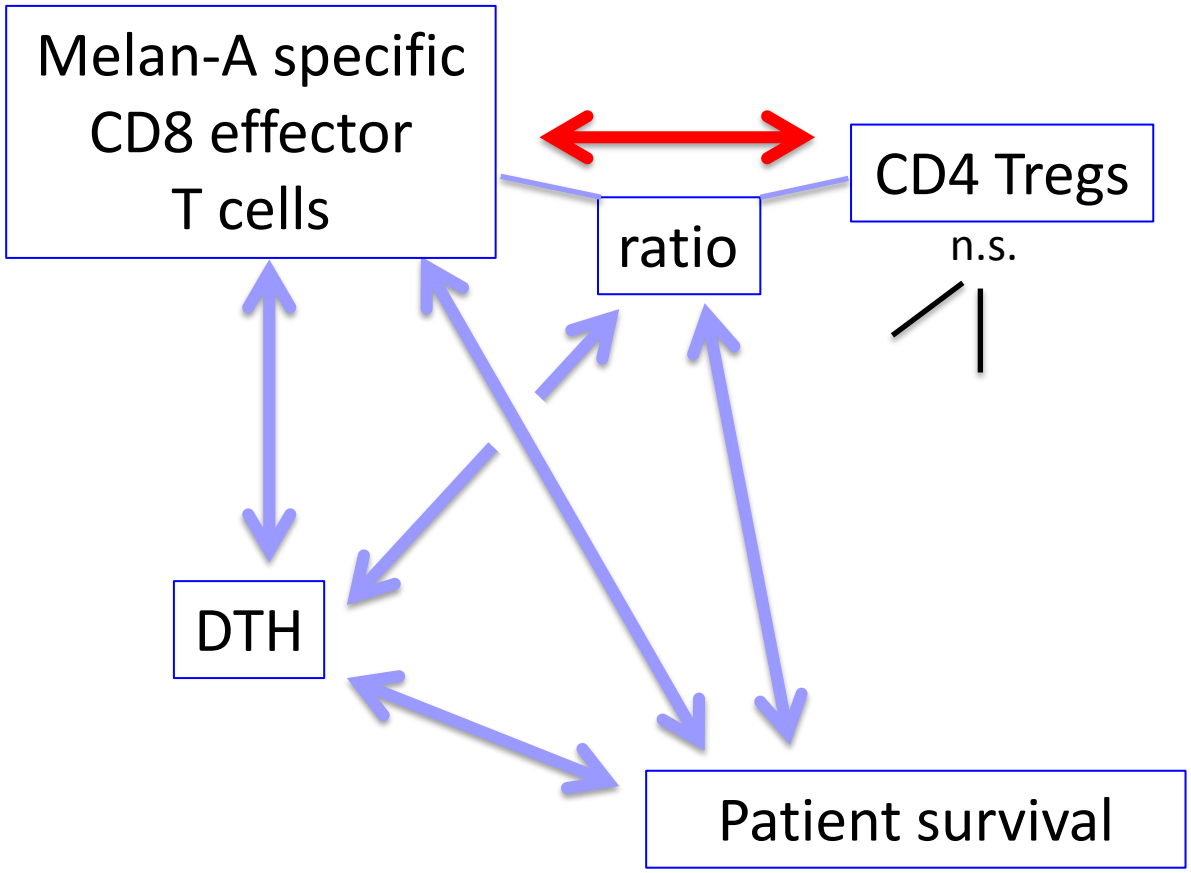




n = 24 patients

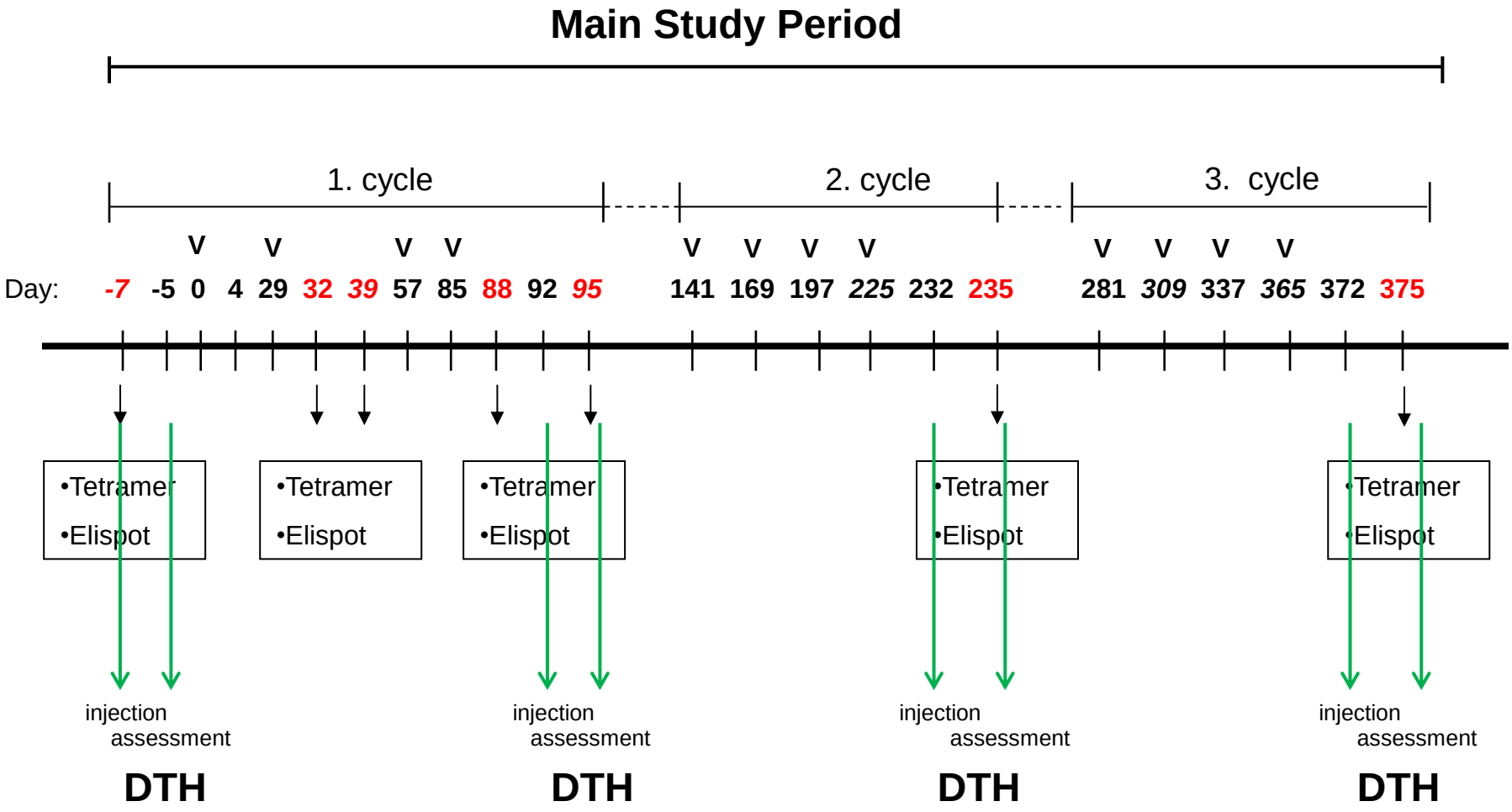
Patients with positive DTH had significantly longer **O**verall **S**urvival
(and a trend for longer **D**isease **F**ree **S**urvival)

Conclusions



- The number of Melan-A specific CD8+ T cells (and their function) correlated with positive DTH reactions and patient survival.
- High numbers of Melan-A specific T cells and low CD4+ Tregs at baseline predicted long Disease Free Survival and Overall Survival.
- The numbers of CD4+ Treg cells had no direct impact on patient survival.

Schedule of vaccination

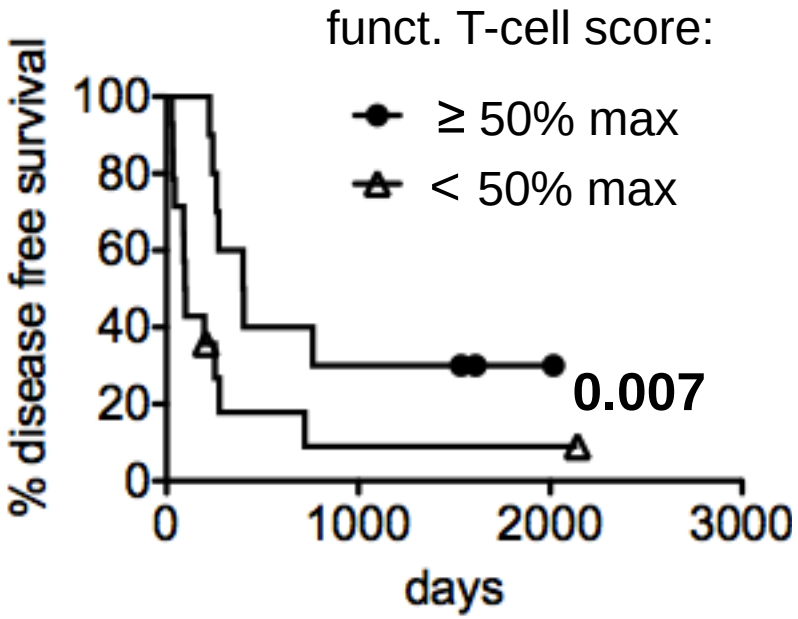


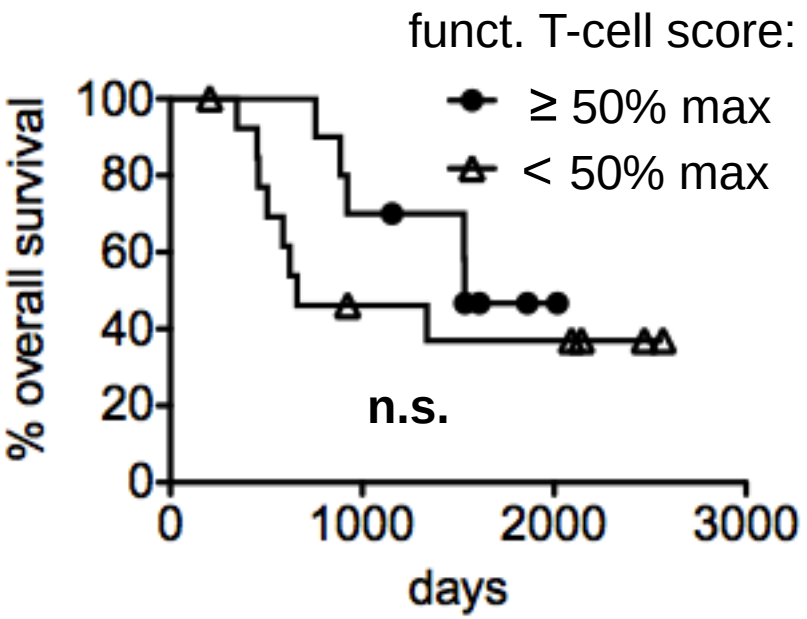
Additional analysis

with the aim to identify possible relationships between

- Tumor-specific effector CD8 T cells in circulation
- Tregs in circulation
- Tissue homing of T cells

Multi-functionality of Melan-A specific CD8 T cells





• Patients with multi-functional T cell response showed increased disease free survival.

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LUDWIG CANCER RESEARCH

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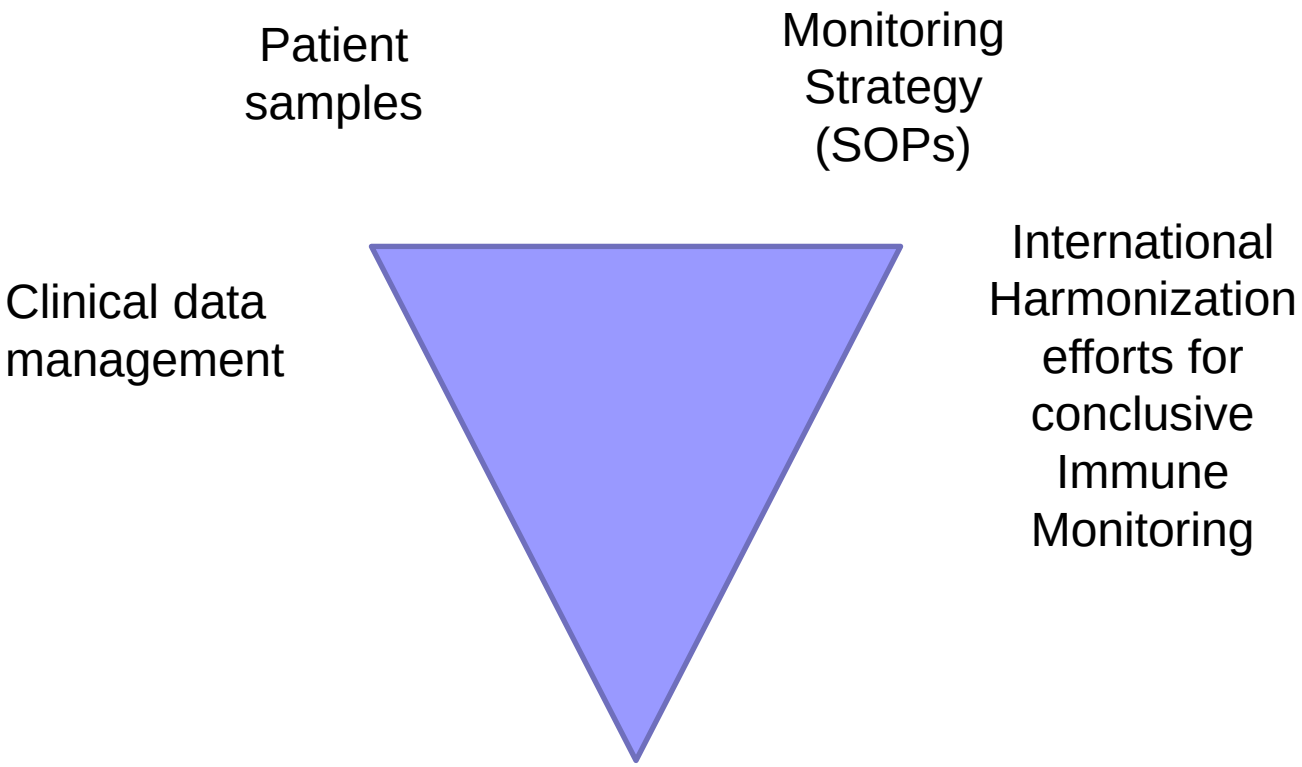
Immune monitoring of patient samples



→ Comprehensive clinical phase 1 trials