

Immunomonitoring and full differentiation phenotyping by multiparametric flow cytometry for studies in infectious diseases

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Agenda

- **Imunomonitoramento**
 - Linfócitos T específicos de antígeno
 - Métodos
- **Interesse e aplicações clinicas**
 - Vacinas, imunoterapias, transplantes, infecções, câncer
- **Resultados**
 - COVID-19
 - Biomarcadores de eficácia dos “immune-checkpoints blockers” em imunoterapia do câncer

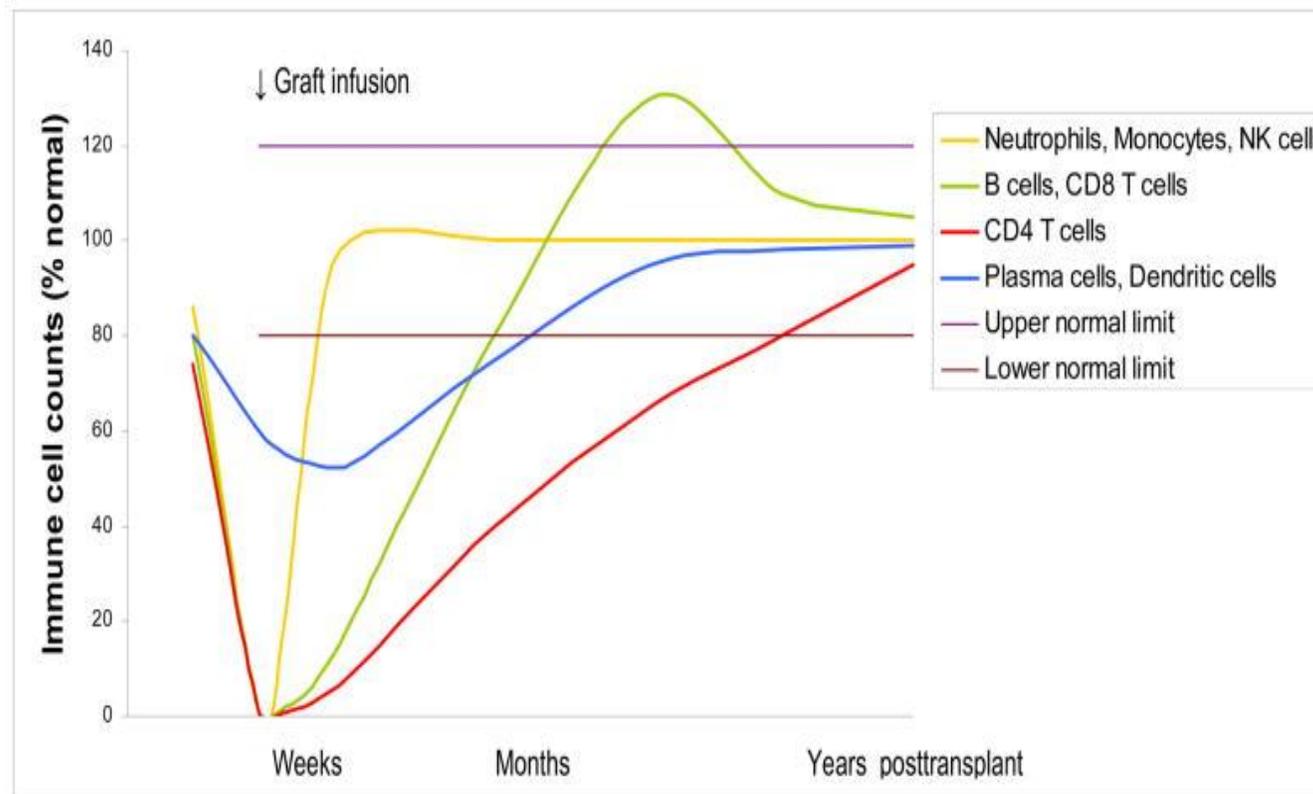
Imunomonitoramento

- Exploração *ex-vivo* do comportamento do sistema imunitário
 - Aspectos quantitativos e qualitativos
 - Situações de imunodepressão
 - inata ou adquirida (infecciosa, imunossupressores,...)
 - Imunoterapias, vacinas
 - Infeções agudas ou crônicas latentes
 - Neoplasias malignas
 - Autoimunidade

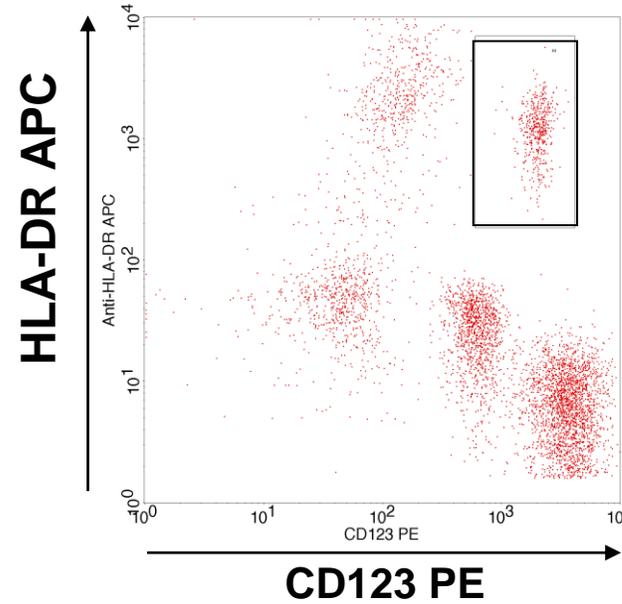
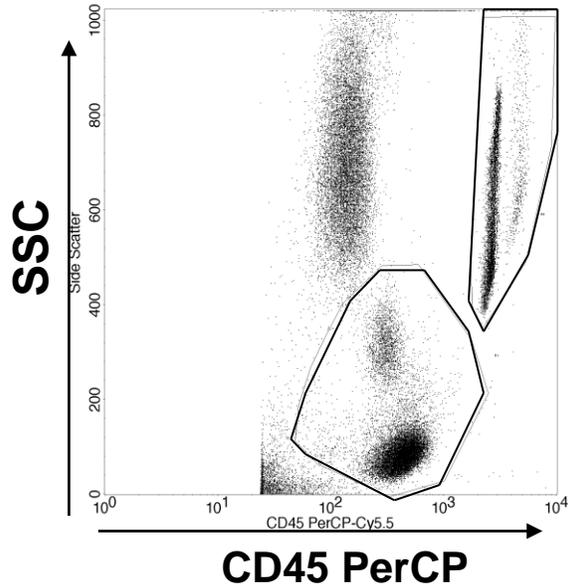
Imunomonitoramento quantitativo

- Rastreamento da quantidade das células imunocompetentes
Linfócitos T, B e NK; monócitos, neutrófilos, células dendríticas,...

Exemplos: Reconstituição imunitária apos transplante de medula óssea,
Imunodeficiência, infecção HIV, tratamento de indução anti-linfocitário nos
transplantes de órgãos,...



Avaliação quantitativa das células dendríticas circulantes (« dendritograma »)



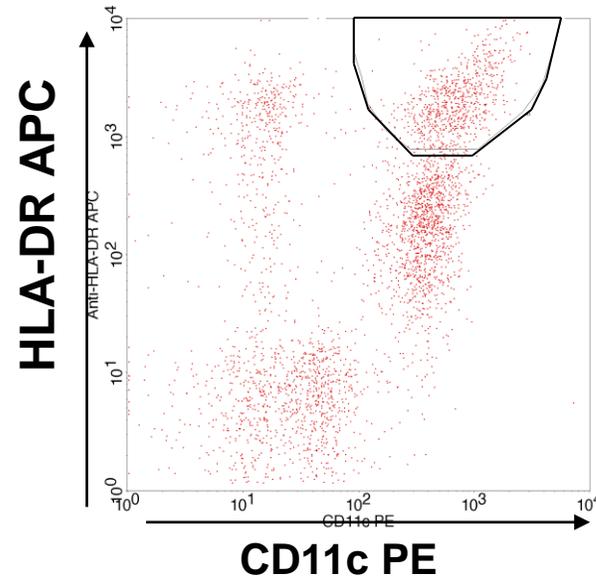
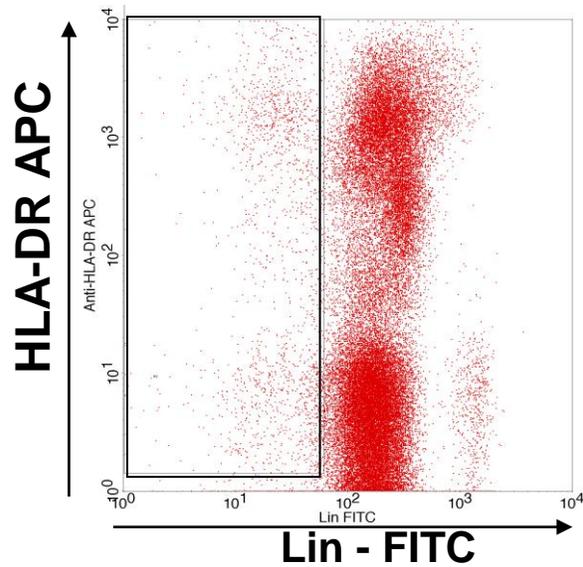
DCs myéloïdes

Decreased Peripheral Dendritic Cell Numbers in Dengue Virus Infection

Marcelo De Carvalho Bittencourt • Jenny Martial •
André Cabié • Laurent Thomas • Raymond Césaire

J Clin Immunol
DOI 10.1007/s10875-011-9592-9

Correlação inversa entre o numero de células dendríticas circulantes e viremia



DCs plasmacytoïdes

Imunomonitoramento das células T específicas de antígenos

Importante no diagnóstico e no acompanhamento de patologias
Correlatos potenciais da eficácia do tratamento ou da progressão da doença

Infecções virais, bacterianas, fúngicas

Neoplasias

antígenos tumorais

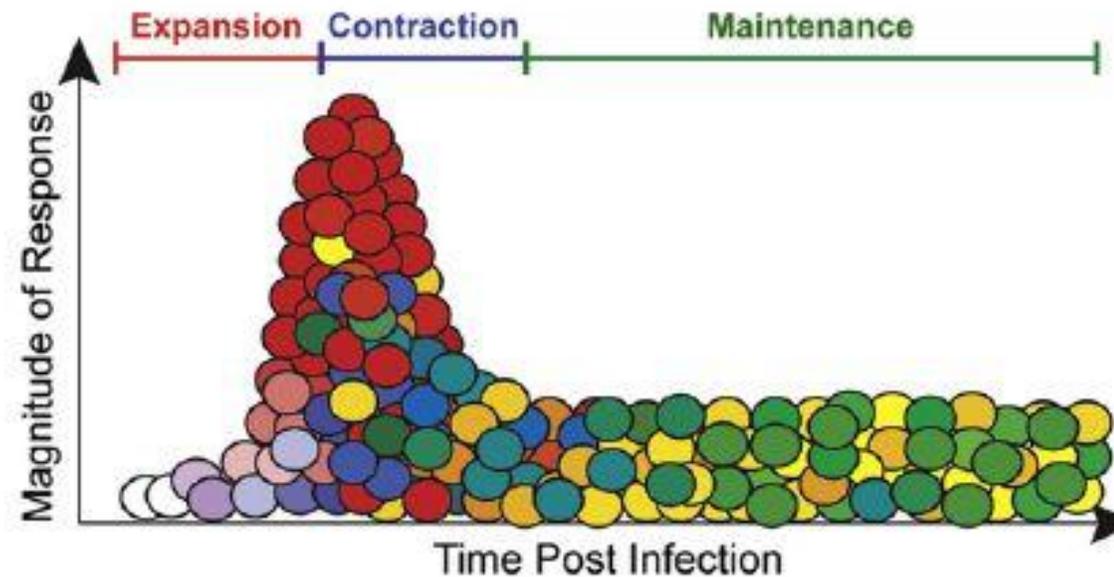
Transplante de órgãos e tecidos

antígenos alogênicos

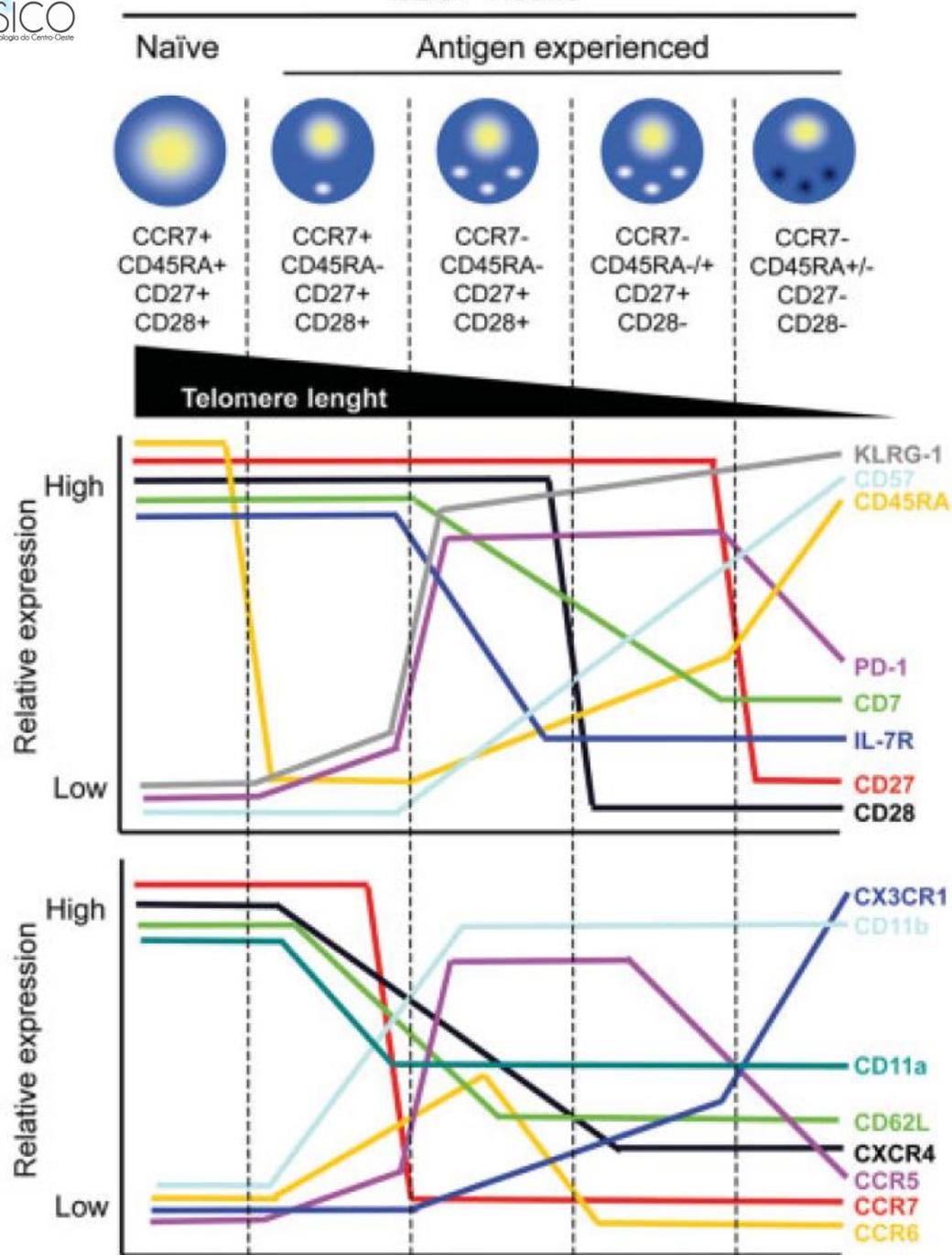
Autoimunidade e Alergia

Fases da imunidade T específica de Ag

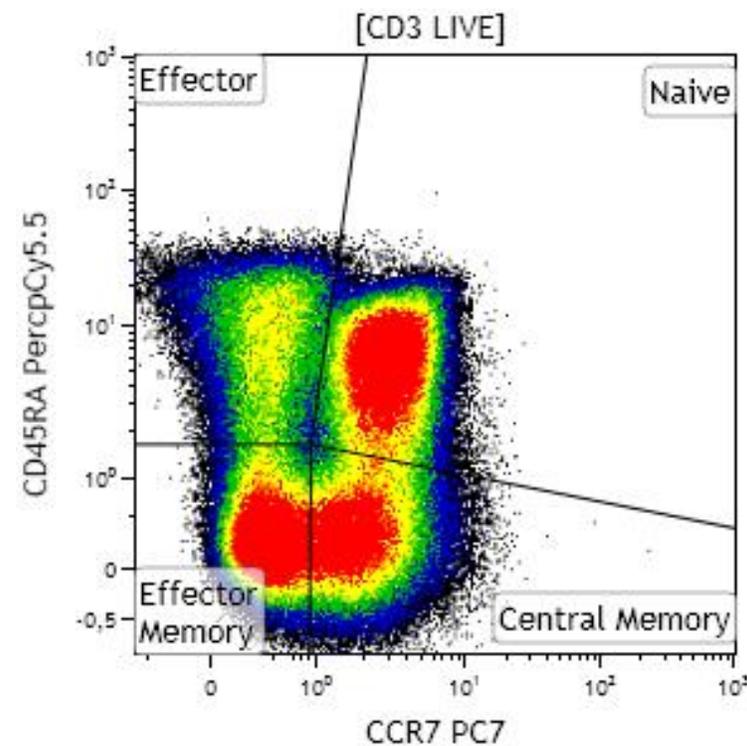
- Reconhecimento específico do antígeno e Ativação
- Diferenciação e expansão clonal
- Controle da infecção e contração clonal
- Memória (heterogeneidade)



CD8+ T cells

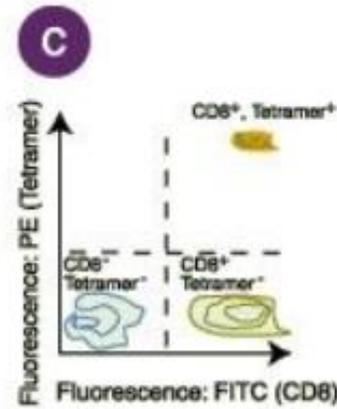
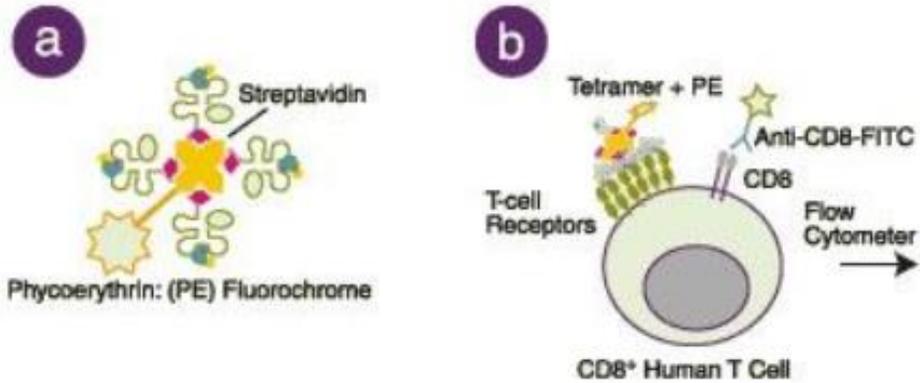


Heterogeneidade das células memória :
 diferentes funções, “homing”, sensibilidades ao antígeno, capacidades proliferativas, etc...

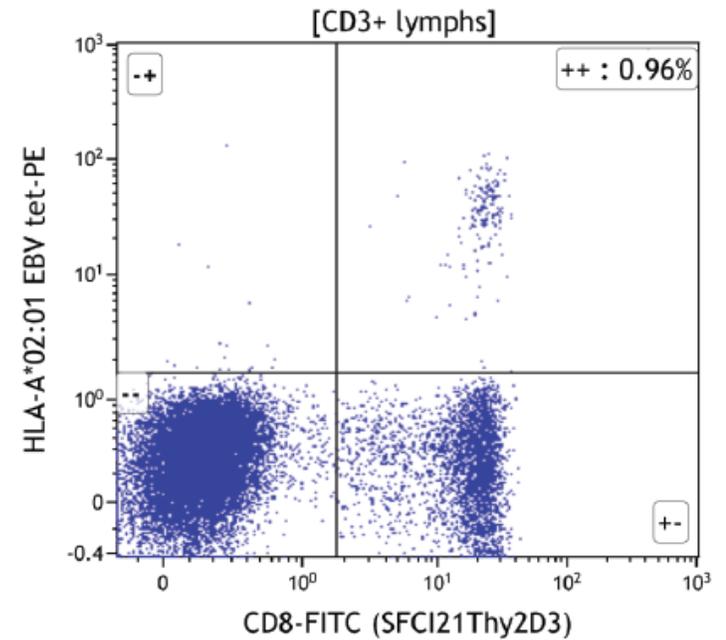
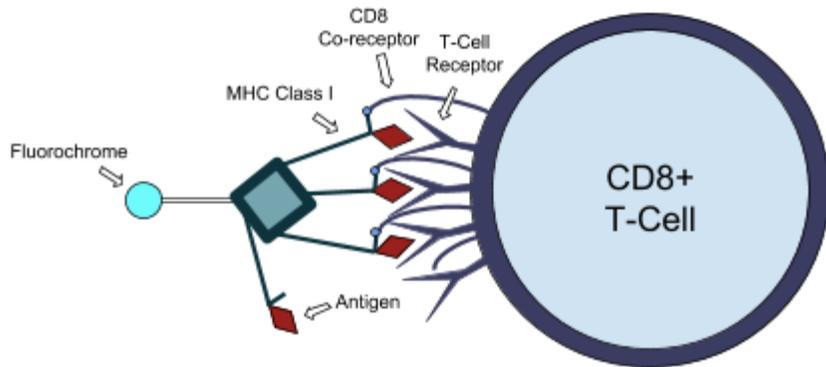


- LT naïves (**T_n**)
- LT memória central (**TCM**)
- LT memória efetora (**TEM**)
- LT memória efetora altamente diferenciada» (**TEMRA**)

Avaliação quantitativa direta dos LT específicos de antígeno usando tetramers



Detection of Epstein Barr Virus-Specific T Cells



HLA-A*02:01 EBV (GLCTLVAML) tetramer-PE (TB-0011-1)

Limitado pelo HLA e peptídeo usado
Avaliação de funções possível

Algumas funções das células T memória importantes para imunidade específica de antígeno

Células raras, ~0,1% dos linfócitos T sanguíneos

Função

Proliferação

Secreção de citocinas/quimiocinas

- IFN- γ , IL-2, TNF- α , IL-17, MIP-1 β ,...

Citotoxicidade

- Lise de células alvo
- Liberação de perforina/granzima B
- Mobilização/degranulação CD107a

Metodologias de avaliação

3 H-thymidine,
Citometria de fluxo (CFSE, ciclo celular)

ELISpot, ELISA

Citometria de fluxo (marcação intracelular)

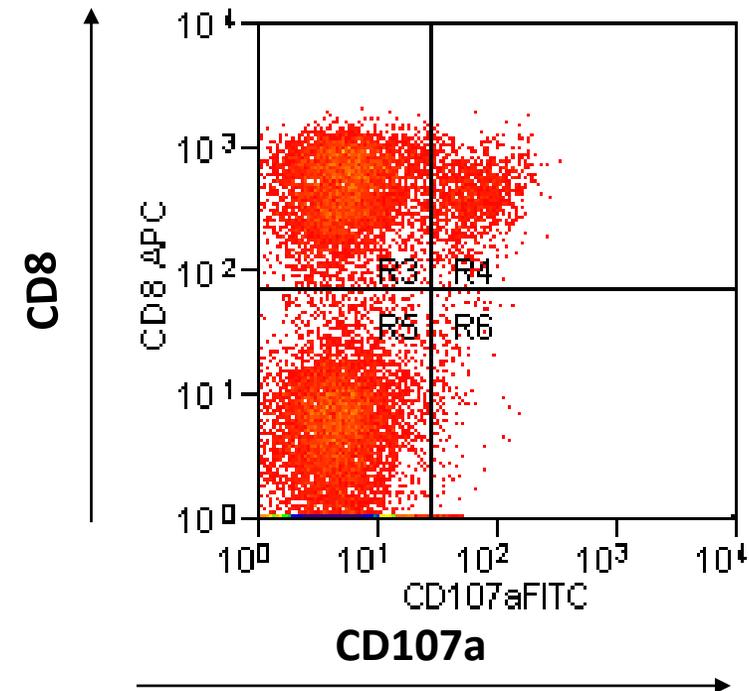
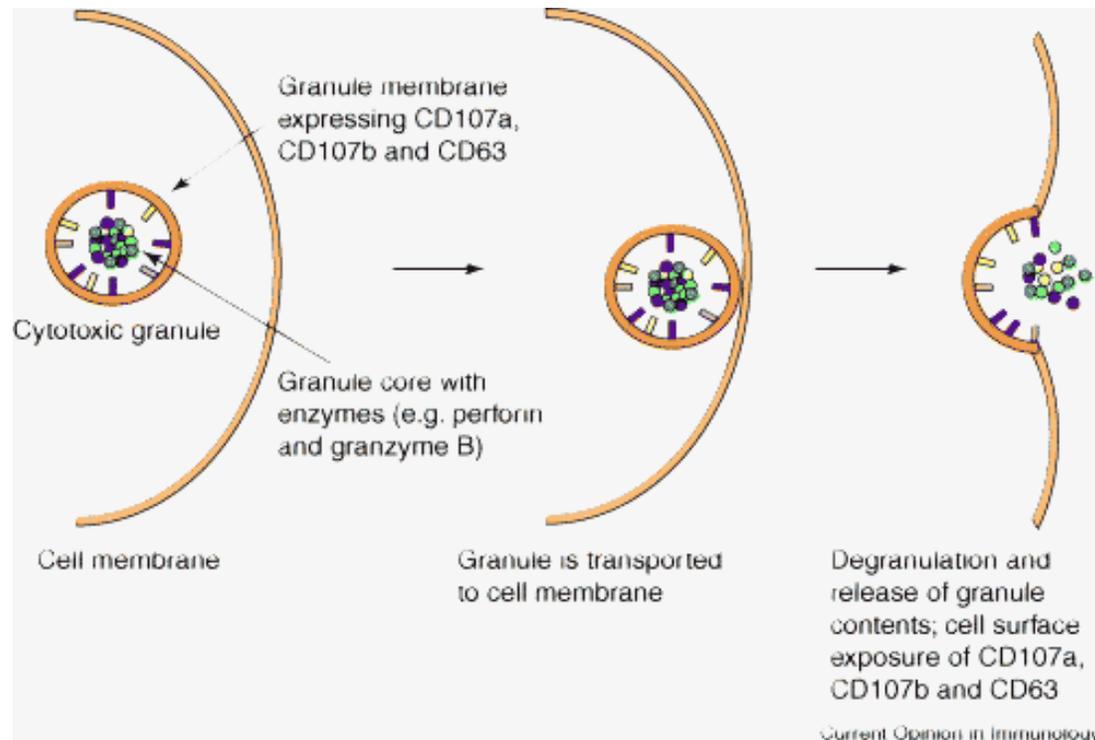
Liberação de ^{51}Cr

Citometria de fluxo , ELISpot

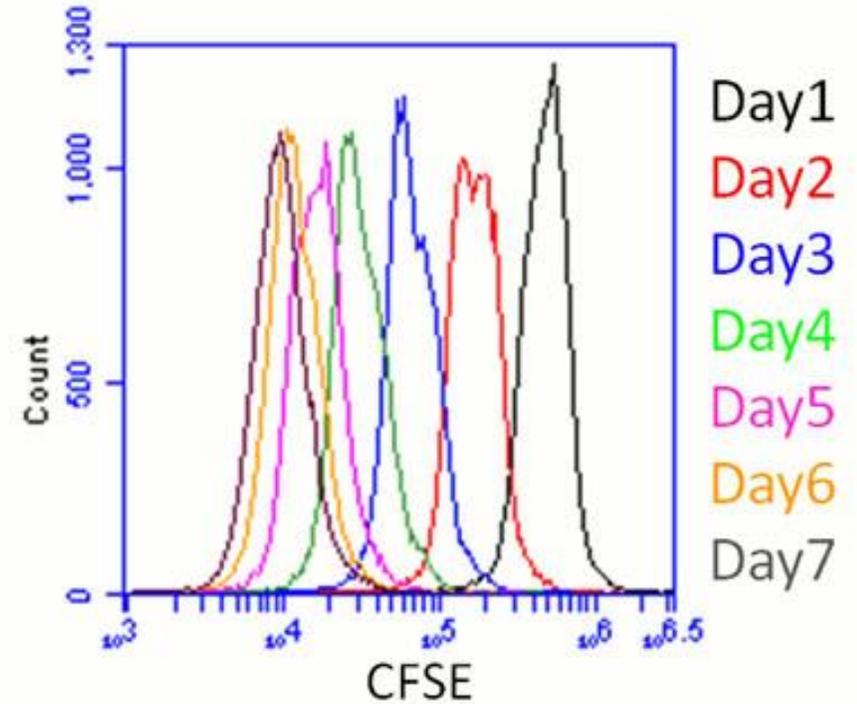
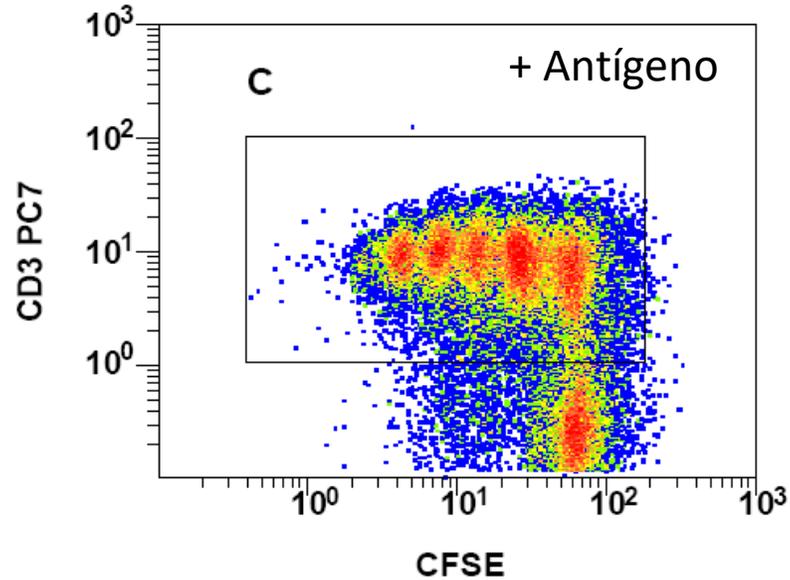
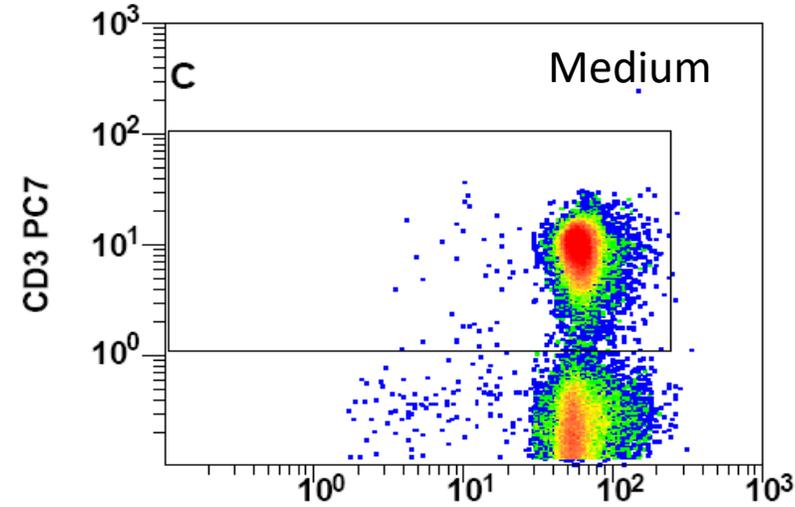
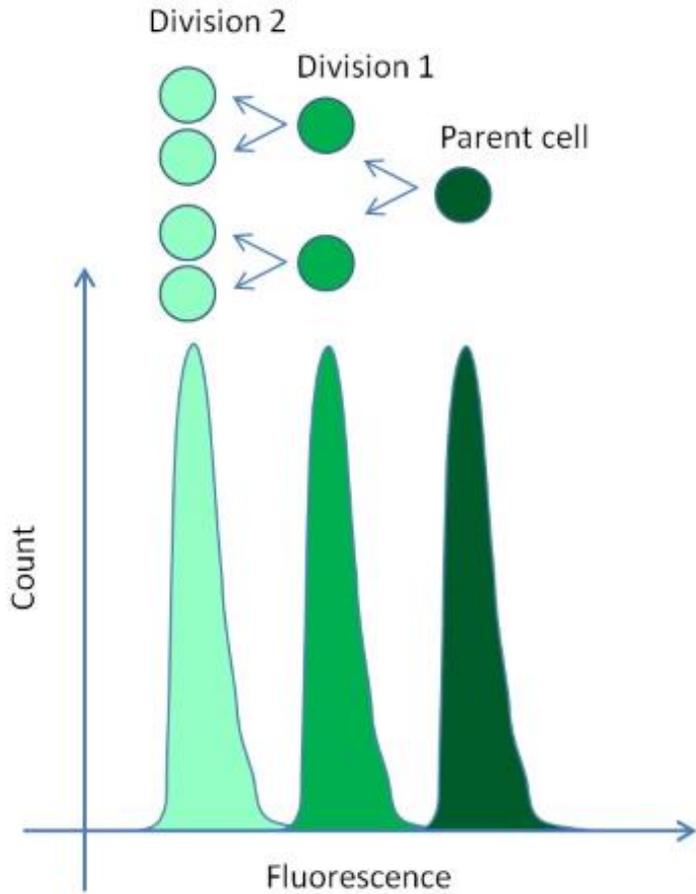
Citometria de fluxo

Avaliando a citotoxicidade pela expressão de CD107a na membrana

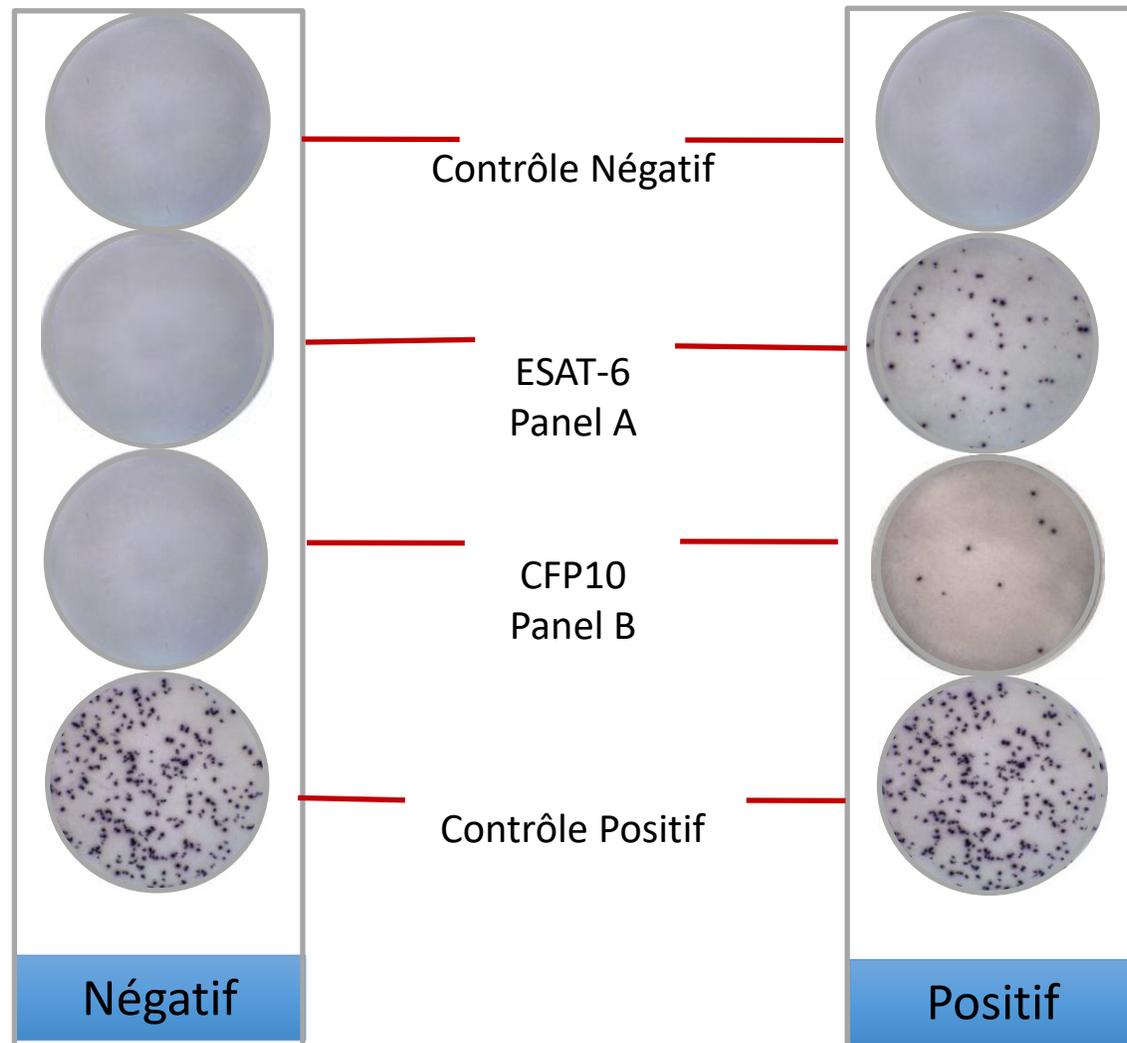
Avalia indiretamente desgranulação, método não radioativo



Avaliando a proliferação pela diluição de CFSE



Diagnosticando a tuberculose latente pela presença de células T memória com ELISpot



T-SPOT.TB™, teste IGRA
« interferon-gamma releasing assay »

Teste quantitativo e semi-qualitativo
(tamanho dos spots proporcional à
quantidade de IFN- γ produzido)

Sem informações sobre o fenótipo das
células produtoras de IFN- γ nem sobre
outras funções

Activation-induced marker « AIM » method

A Cytokine-Independent Approach To Identify Antigen-Specific Human Germinal Center T Follicular Helper Cells and Rare Antigen-Specific CD4⁺ T Cells in Blood

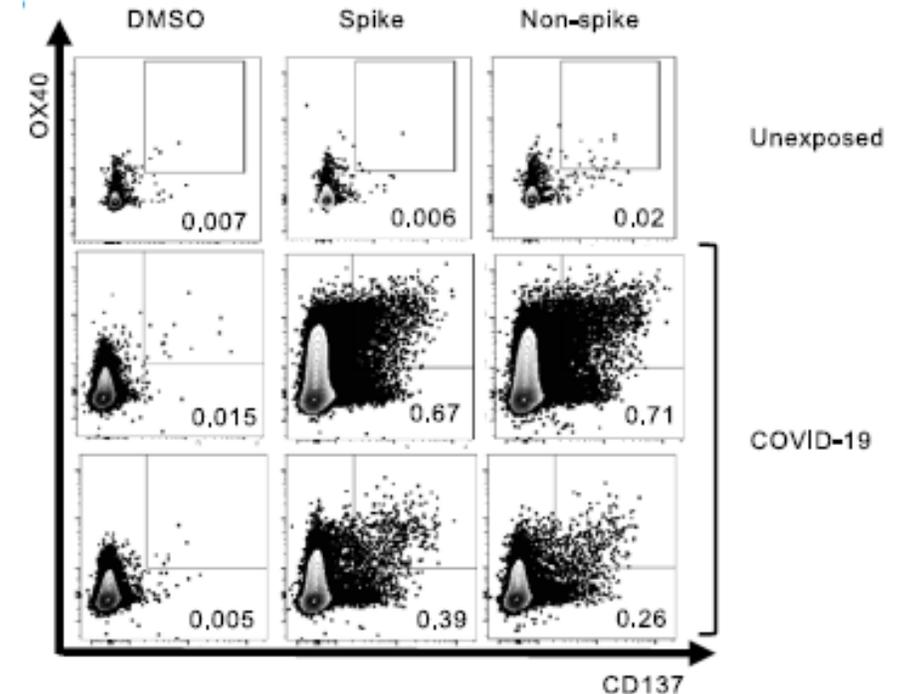
J Immunol 2016; 197:983-993.

Jennifer M. Dan,^{*,†} Cecilia S. Lindestam Arlehamn,^{*} Daniela Weiskopf,^{*}
 Ricardo da Silva Antunes,^{*} Colin Havenar-Daughton,^{*,‡} Samantha M. Reiss,^{*,‡}
 Matthew Brigger,[§] Marcella Bothwell,[§] Alessandro Sette,^{*} and Shane Crotty^{*,†,‡}

Targets of T Cell Responses to SARS-CoV-2 Coronavirus in Humans with COVID-19 Disease and Unexposed Individuals

Grifoni et al., 2020, *Cell* 181, 1489–1501

Upregulation of expression of CD25, CD69, CD134, CD154, CD137...



Avaliação funcional de linfócitos específicos de Ag por citocinas intracelulares

Cultura celular com pools de peptídeos imunodominantes

Apresentação antigênica pelas moléculas HLA de classe I e classe II :

Estimulação dos LT CD4 e CD8 de memória específicos de antígeno

Cultura overnight para produção de citocinas

Acumulação intracitoplasmática (bloqueio da secreção pela BFA)

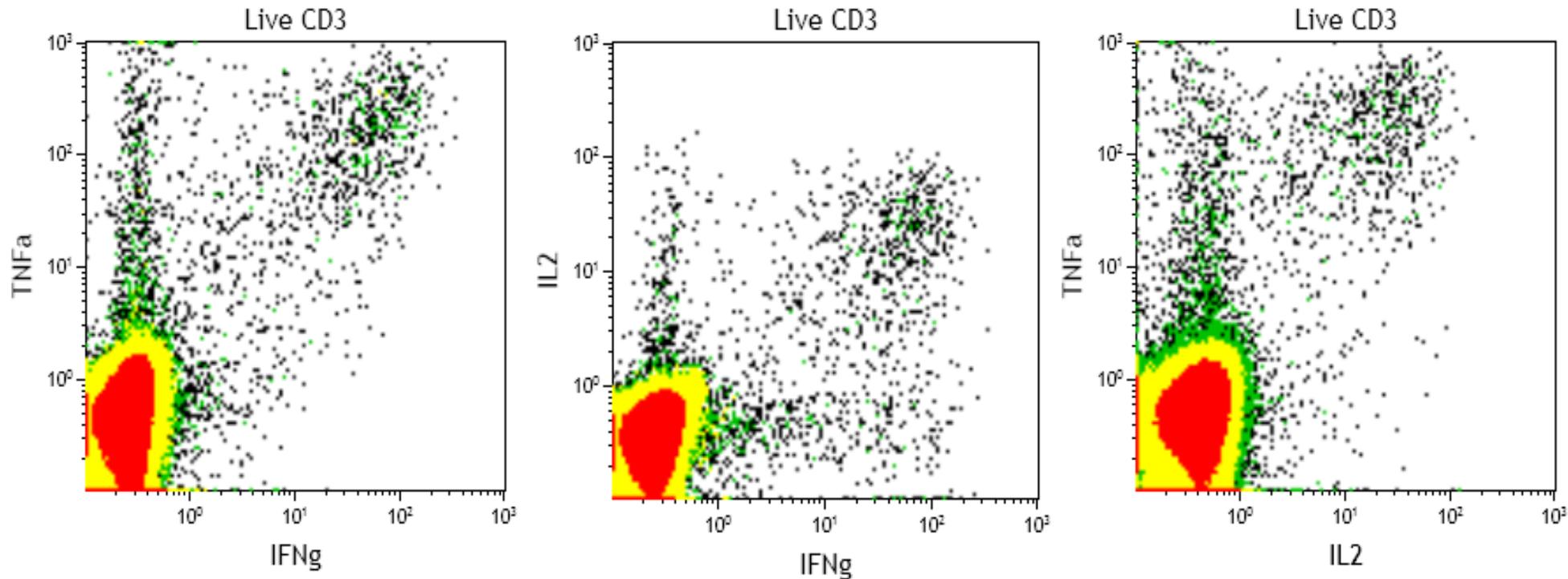
Citometria de Fluxo multiparamétrica e identificação dos LT específicos

Imunofenotipagem de membrana (viabilidade, CD3, CD4, CD8, CD197, CD45RA, CD28, etc...)

Marcação intracelular das citocinas

Exemplo : IFN- γ , TNF- α e IL-2

Avaliação funcional de linfócitos específicos de Ag por citocinas intracelulares

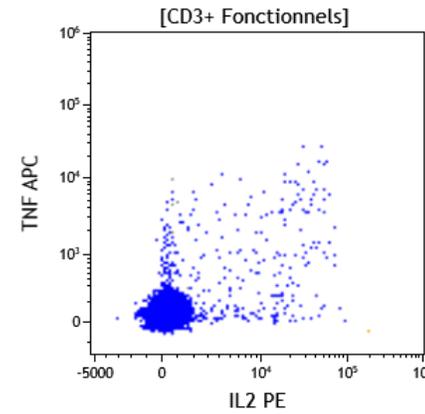
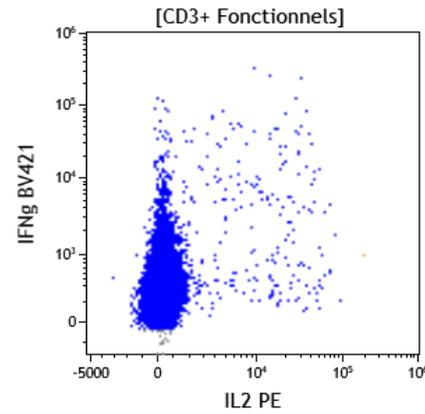
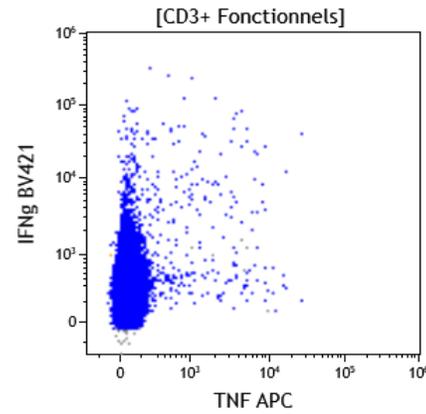
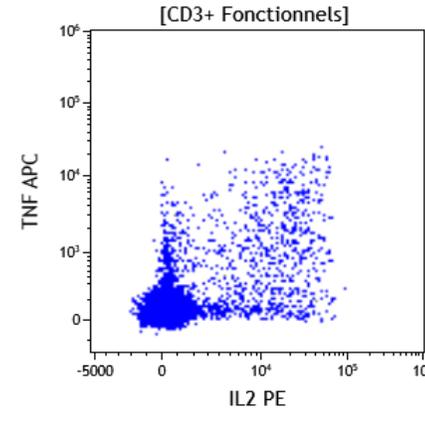
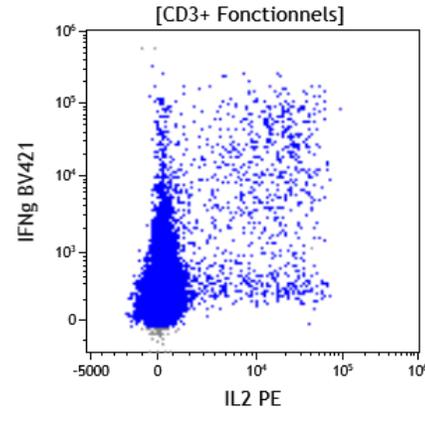
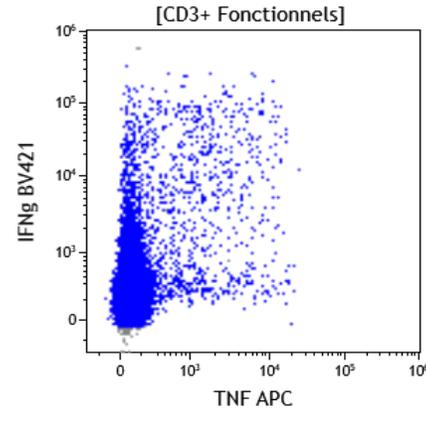
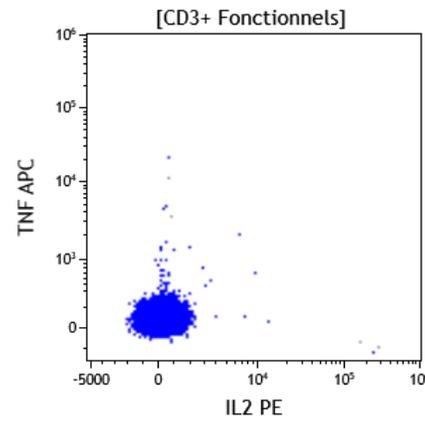
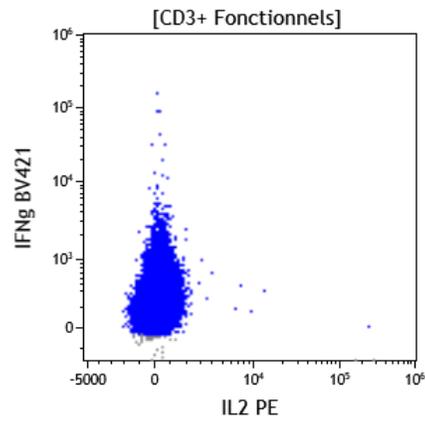
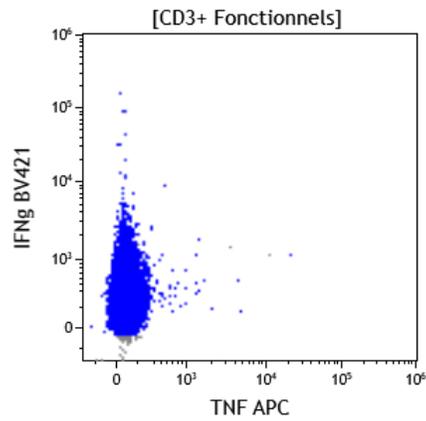
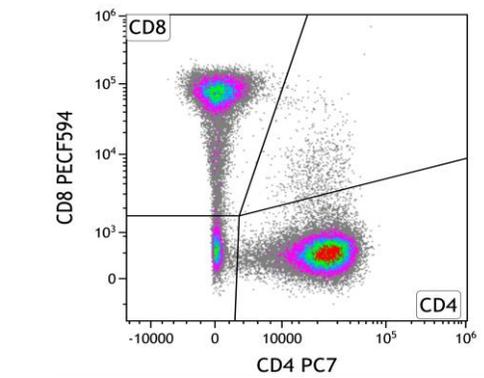
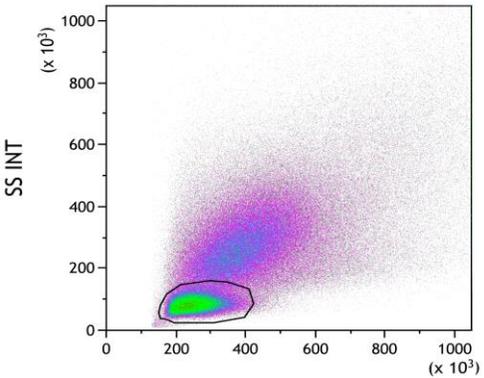


Identificação possível das células T multifuncionais, os mais eficazes contra os antígenos

Comparação de 2 métodos para detectar células T específicas de *M. tuberculosis*

Function	Flow cytometry ICS	ELISPOT
IFN-γ+ total	0.08% (800/10 ⁶ cells)	0.09% (225/2x10 ⁵ cells)
IL-2+ total	0.13% (1300/10 ⁶ cells)	
IFN-γ+ only	0.02% (200/10 ⁶ cells)	
IL2-+ only	0.07% (700/10 ⁶ cells)	
IFN-γ+/IL-2+	0.06% (600/10 ⁶ cells)	

A Citometria de Fluxo permite também a determinação do fenótipo das células T respondendo ao antígeno :
CD4, CD8, memória, exaustão, ...

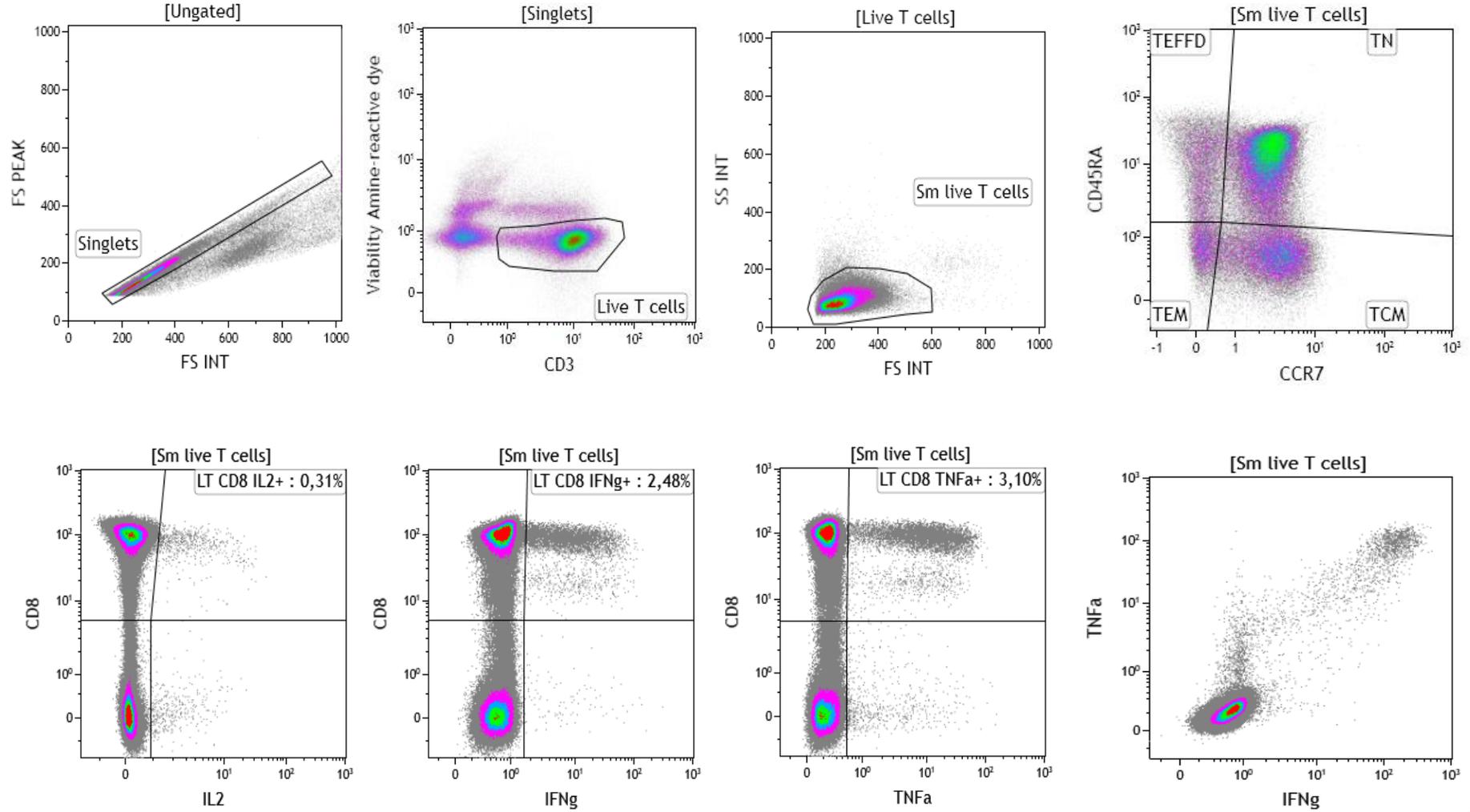


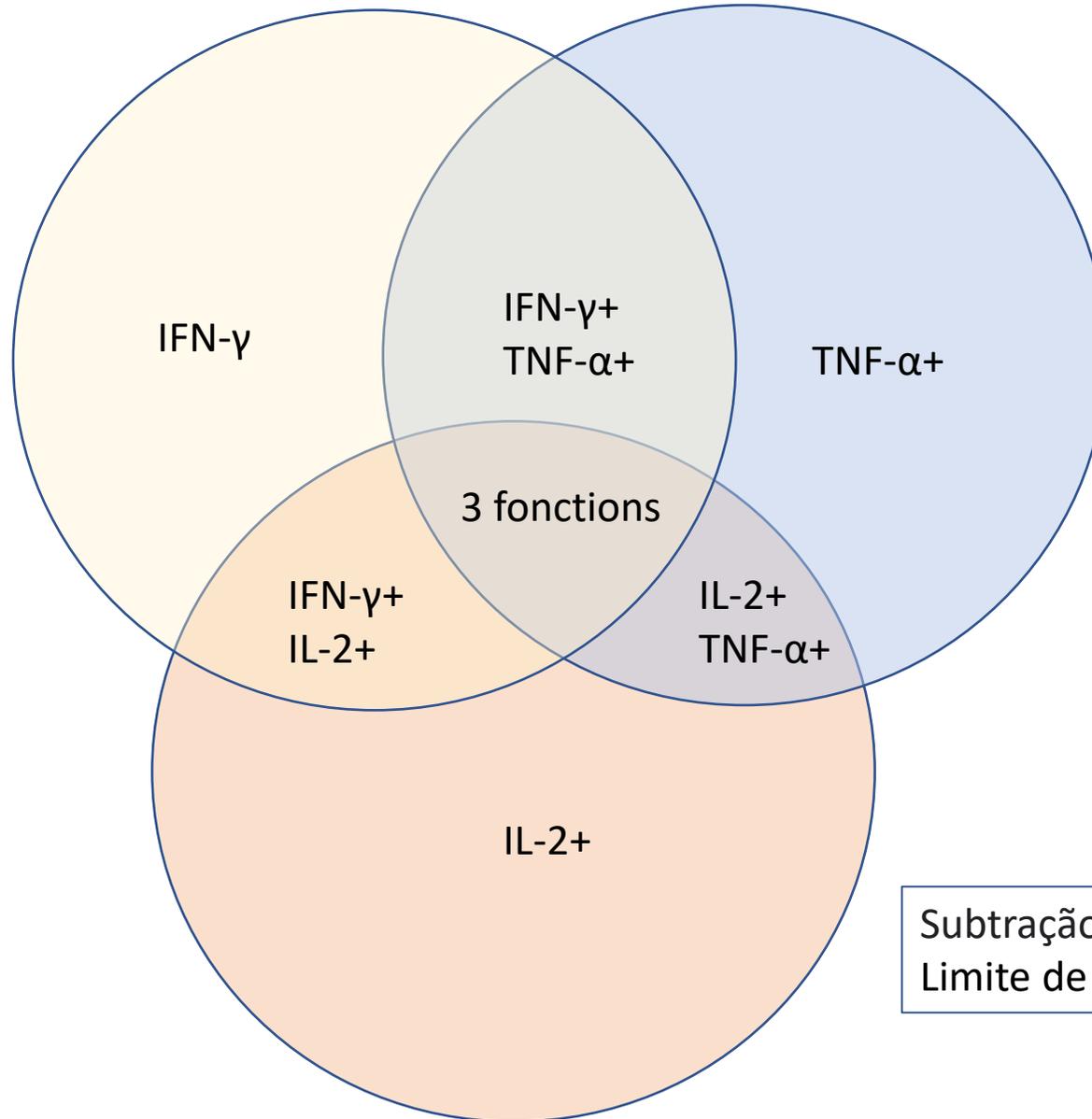
Medium

SARS-COV-2 Spike

SARS-COV-2 Nucleoprotein

Gating et analyse en cytométrie





Operações booleanas e determinação das 7 subpopulações produtoras de citocinas

IFN- γ + monofuncional

TNF- α + monofuncional

IL-2+ monofuncional

IFN- γ +TNF α + bifuncional

IFN- γ +IL2+ bifuncional

TNF- α +IL2+ bifuncional

IFN- γ +TNF- α +IL-2+ trifuncional

Subtração da produção espontânea de cada subpopulação
Limite de positividade : $\Delta > 0,01\%$

Pathogen-Specific T Cell Polyfunctionality Is a Correlate of T Cell Efficacy and Immune Protection

PLOS ONE | DOI:10.1371/journal.pone.0128714 June 5, 2015



Anders Boyd¹, Jorge R. Almeida², Patricia A. Darrah³, Delphine Sauce², Robert A. Seder³, Victor Appay², Guy Gorochov^{2,4,5}, Martin Larsen^{2,4,5*}

“We demonstrate that quantitative assessment of T cell polyfunctionality correlates with T cell efficacy measured as the capacity to kill target cells in vitro and control infection in vivo.”

Polyfunctional and IFN- γ monofunctional human CD4⁺ T cell populations are molecularly distinct

JCI Insight. 2017;2(3):e87499.

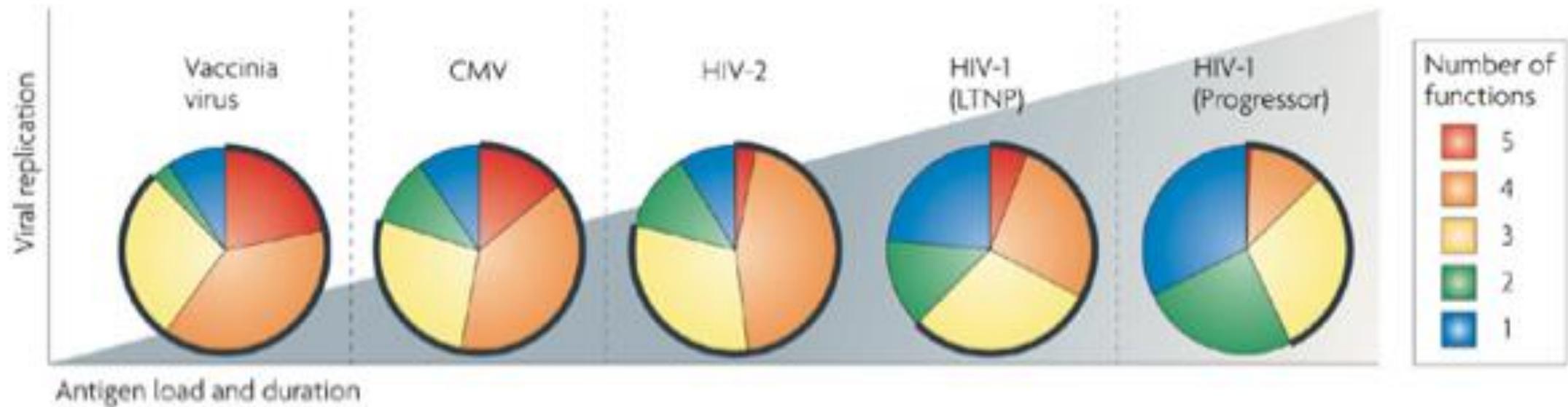
JCI INSIGHT

Julie G. Burel,^{1,2} Simon H. Apte,¹ Penny L. Groves,¹ James S. McCarthy,^{2,3} and Denise L. Doolan^{1,2,4}

“The 14-gene signature revealed in P. falciparum–reactive polyfunctional T cells is associated with cytokine signaling and lymphocyte chemotaxis, and systems biology analysis identified IL-27 as an upstream regulator of the polyfunctional gene signature”

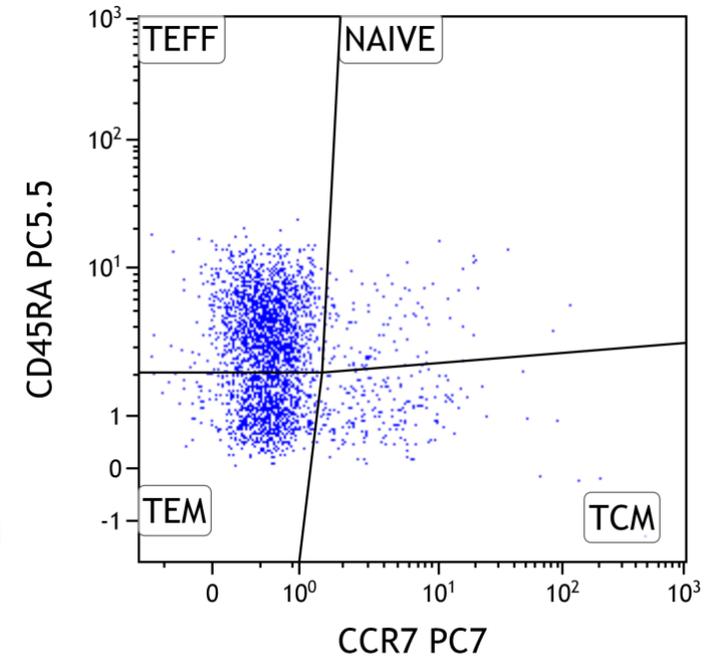
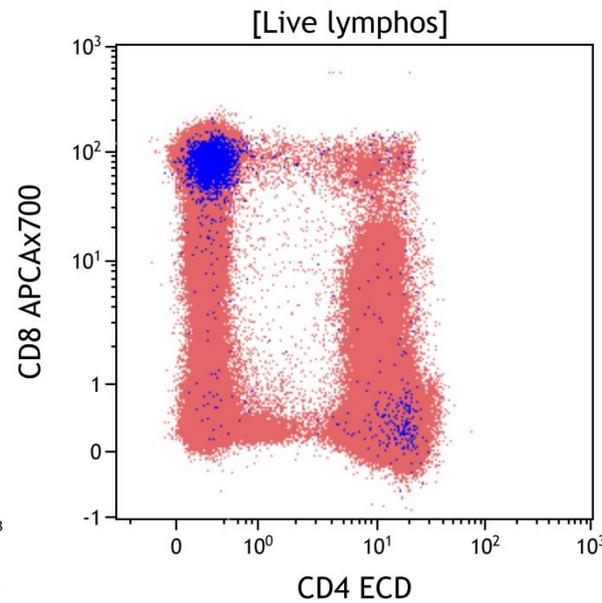
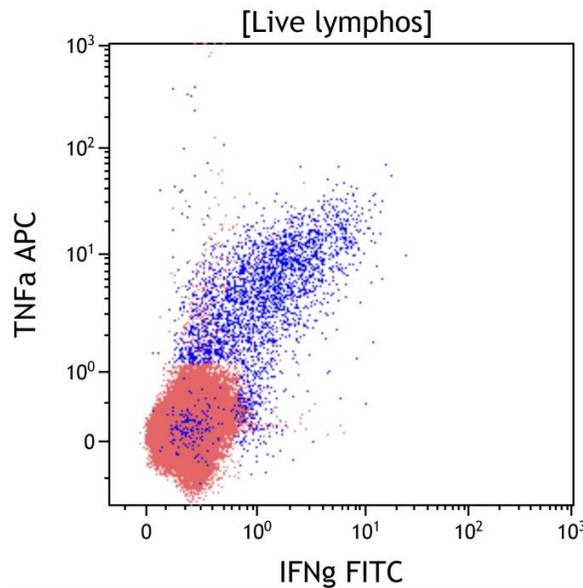
As células T multifuncionais são as mais eficazes no controle das infecções (« **qualidade** » superior) (*Anders Boyd et al, PLoS one 2014*)

Correlação inversa entre carga antigênica e quantidade de LTs multifuncionais (*Roederer et al, Nat Rev Immunol 2008*)



A heterogeneidade das respostas imunes é dependente do antígeno

Diferentes características das células T anti-PSA e anti-hTERT no câncer de próstata



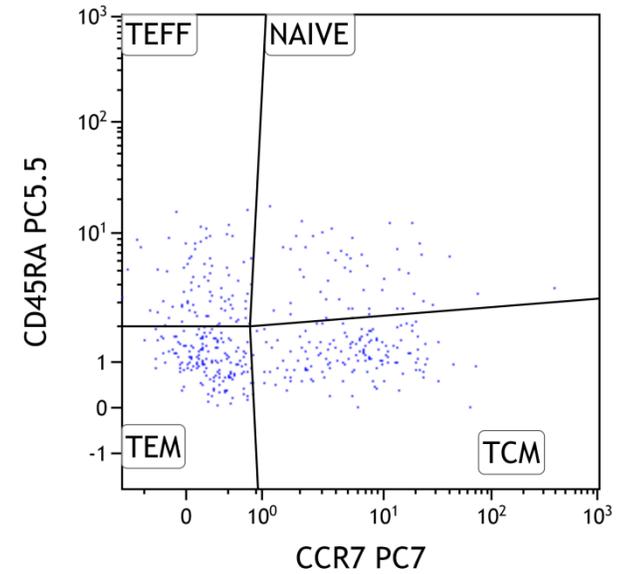
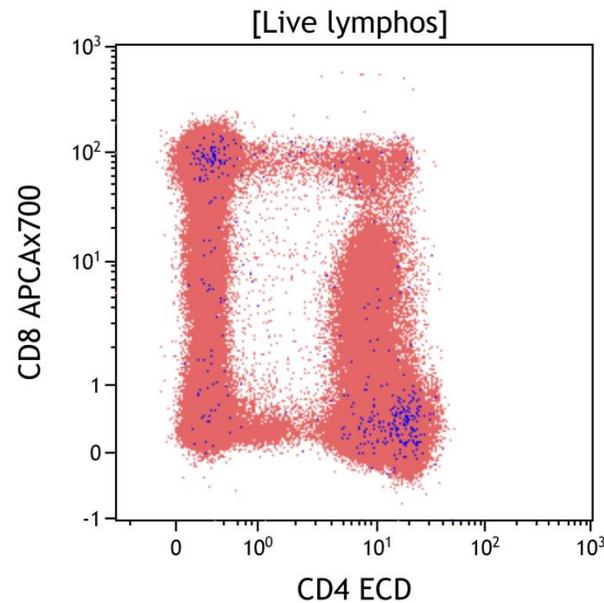
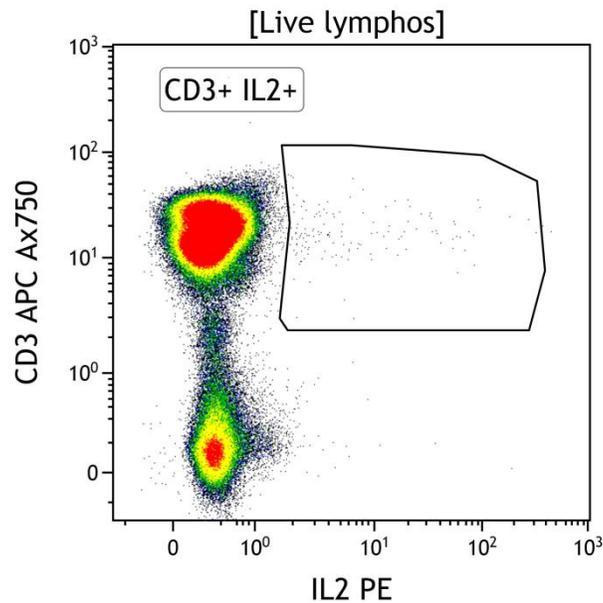
PSA 1.25µg/mL
total CD3+ Ag+: 0.68%

Maioria LT CD8+, TEFF et TEM
Bi-funcionais IFN-γ/TNF-α

Gate	Number	%Gated
All	2,835	100.00
NAIVE	104	3.67
TCM	186	6.56
TEFF	1,616	57.00
TEM	929	32.77

A heterogeneidade das respostas imunes é dependente do antígeno

Diferentes características das células T anti-PSA e anti-hTERT no câncer de próstata



Gate	Number	%Gated
All	426	100.00
NAIVE	53	12.44
TCM	145	34.04
TEFF	83	19.48
TEM	145	34.04

UCPs 5µg/mL

total CD3+ Ag+: 0.1%

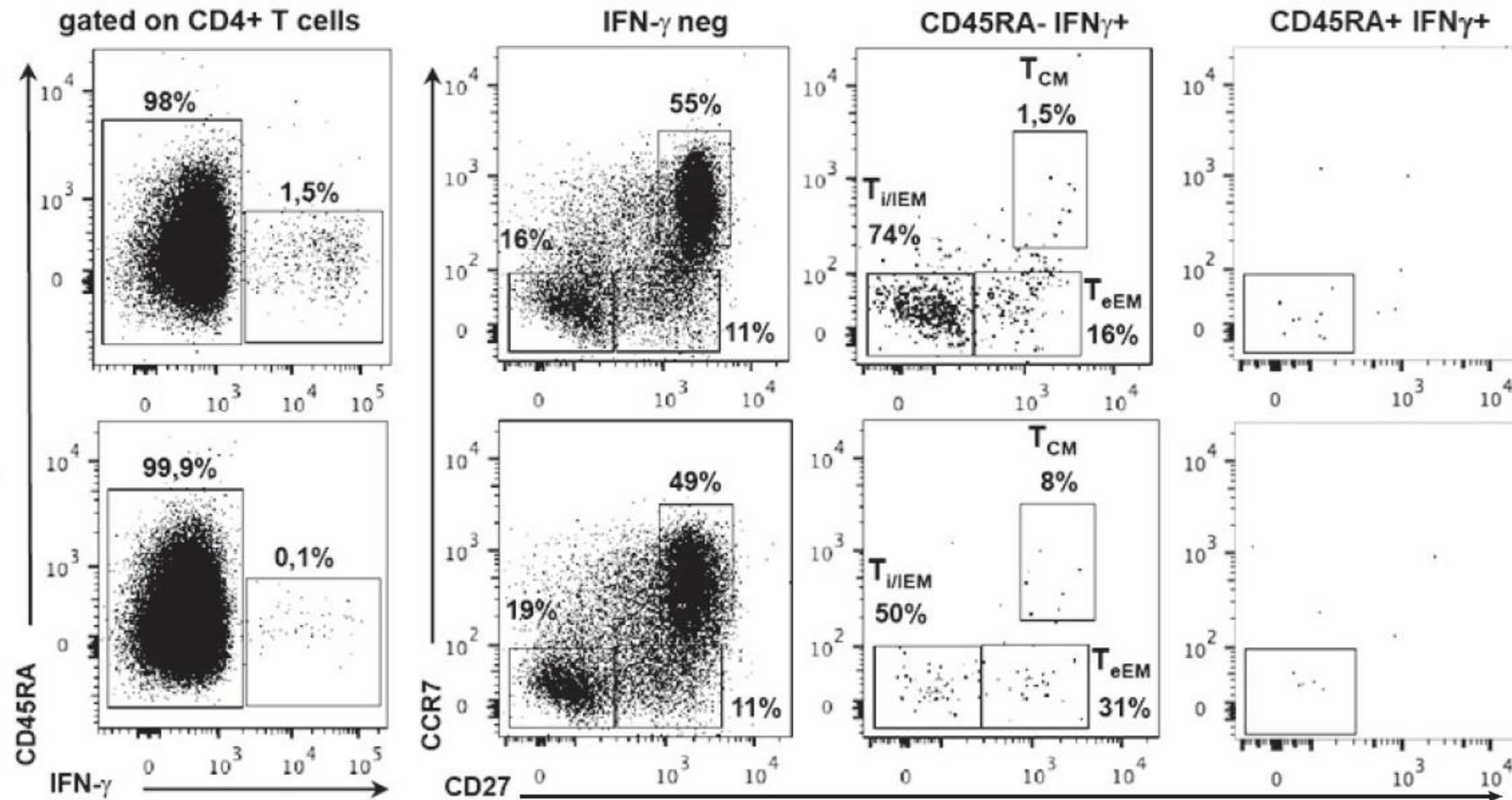
Equivalente LT CD8+ e CD4+, TCM e TEM

Mono-funcionais IL-2, menor quantidade

Phenotypic and Functional Differences between Human Herpesvirus 6- and Human Cytomegalovirus-Specific T Cells

Journal of Virology July 2019 Volume 93 Issue 13 e02321-18

Solène Fastenackels,^a Charles Bayard,^a Martin Larsen,^a Philippe Magnier,^a Pascale Bonnafous,^a Nabila Seddiki,^c Victor Appay,^{a,d} Agnès Gautheret-Dejean,^b Delphine Sauce^a



Healthy coinfecting blood donors.
“Immunological differences in frequency and phenotype of effector/memory and regulatory virus-specific T cells. This suggests that different immune factors are solicited in the control of HHV-6 infection than in that of HCMV infection.”

CD8+ T cells

Naïve

Antigen experienced



CCR7+
CD45RA+
CD27+
CD28+



CCR7+
CD45RA-
CD27+
CD28+



CCR7-
CD45RA-
CD27+
CD28+

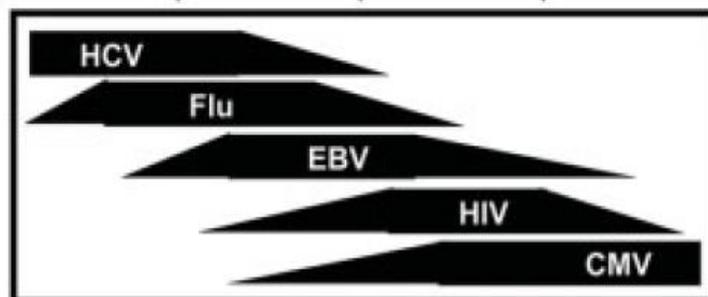
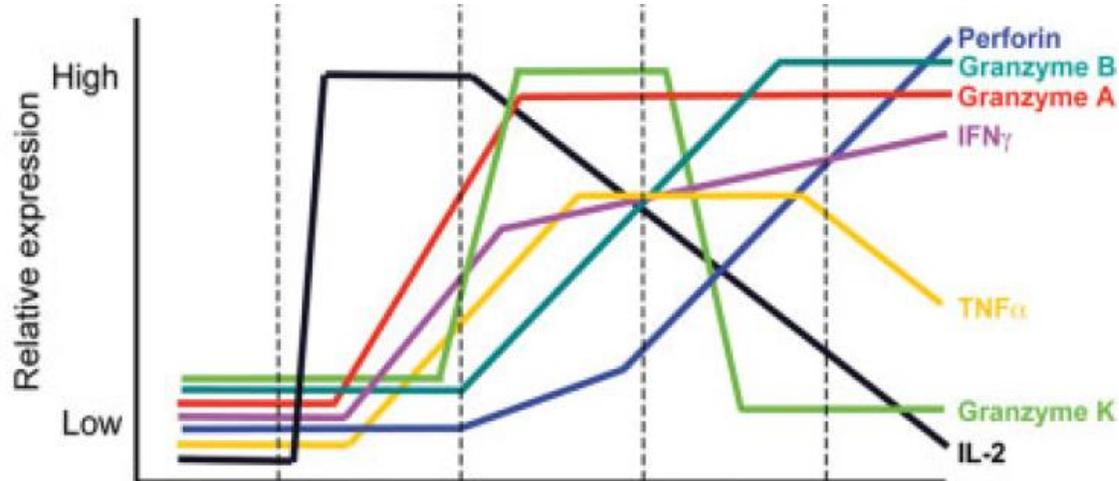


CCR7-
CD45RA+/-
CD27+
CD28-



CCR7-
CD45RA+/-
CD27-
CD28-

Telomere length

Heterogeneidade das células memória

Diferentes funções em relação à diferenciação,

Diferentes perfis com diferentes patógenos

A proporção de diferentes subpopulações de memória é diferente dependendo dos patógenos

(Appay V et al, Nat Med 2002)

A **qualidade** dessas populações também é variável dependendo dos patógenos

os LT CD8+ Teff anti-HIV são predominantemente monofuncionais, mas não os anti-CMV CD8+ Teff (Riou C et al, J Immunol 2012)

Os correlatos de proteção para cada antígeno podem ser diferentes!

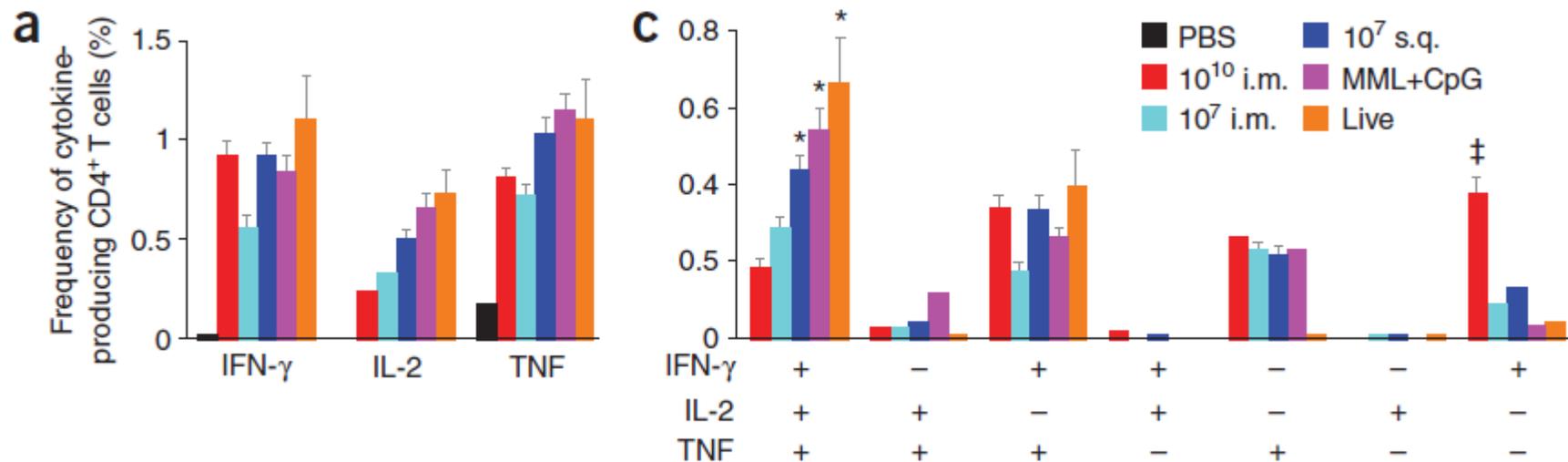
Relevância clínica das células T multifuncionais

Vacinas

Multifunctional T_H1 cells define a correlate of vaccine-mediated protection against *Leishmania major*

NATURE MEDICINE | VOLUME 13 | NUMBER 7 | JULY 2007

Patricia A Darrah¹, Dipti T Patel¹, Paula M De Luca¹, Ross W B Lindsay¹, Dylan F Davey¹, Barbara J Flynn¹, Søren T Hoff², Peter Andersen², Steven G Reed³, Sheldon L Morris⁴, Mario Roederer⁵ & Robert A Seder¹



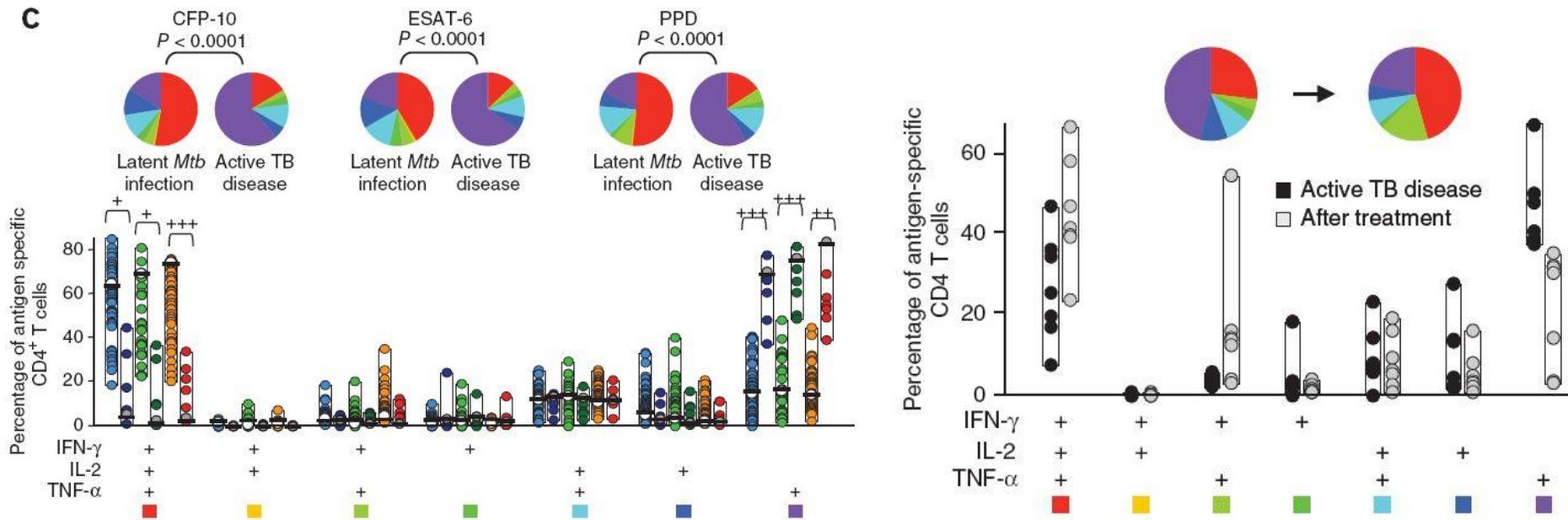
Relevância clínica das células T multifuncionais

Tuberculose

TNF- α + LT monofuncional correlaciona-se com a forma de TB ativa, aparecimento de LT polifuncional após tratamento eficaz

Dominant TNF- α + Mycobacterium tuberculosis-specific CD4+ T cell responses discriminate between latent infection and active disease. Harari *et al*, Nat Med 2011

Citometria de fluxo multiparamétrica útil para diagnóstico e monitoramento de pacientes



Relevância clínica das células T multifuncionais

Câncer

Polyfunctional CD4⁺ T cells are essential for eradicating advanced B-cell lymphoma after chemotherapy *BLOOD*, 13 SEPTEMBER 2012 • VOLUME 120, NUMBER 11

Zhi-Chun Ding,¹ Lei Huang,² Bruce R. Blazar,³ Hideo Yagita,⁴ Andrew L. Mellor,^{2,5} David H. Munn,^{1,6} and Gang Zhou^{1,5}

Functional T Cell Responses to Tumor Antigens in Breast Cancer Patients Have a Distinct Phenotype and Cytokine Signature¹

J Immunol 2007; 179:2627-2633

Margaret Inokuma,* Corazon dela Rosa,[†] Charles Schmitt,[‡] Perry Haaland,[‡] Janet Siebert,[§] Douglas Petry,* MengXiang Tang,* Maria A. Suni,* Smita A. Ghanekar,* Daiva Gladding,* John F. Dunne,* Vernon C. Maino,* Mary L. Disis,[†] and Holden T. Maecker^{2*}

Human Antitumor CD8⁺ T Cells Producing Th1 Polycytokines Show Superior Antigen Sensitivity and Tumor Recognition *J Immunol* 2012; 189:598-605

Susanne Wilde,* Daniel Sommermeyer,[†] Matthias Leisegang,[†] Bernhard Frankenberger,* Barbara Mosetter,* Wolfgang Uckert,^{†,‡} and Dolores J. Schendel^{*,§}

Relevância clínica das células T multifuncionais

Infecções virais

Multiple-Cytokine-Producing Antiviral CD4 T Cells Are Functionally Superior to Single-Cytokine-Producing Cells[∇]

Sunil Kannanganat, Chris Ibegbu, Lakshmi Chennareddi, Harriet L. Robinson, and Rama Rao Amara*

JOURNAL OF VIROLOGY, Aug. 2007, p. 8468–8476

HIV nonprogressors preferentially maintain highly functional HIV-specific CD8⁺ T cells

BLOOD, 15 JUNE 2008 • VOLUME 107, NUMBER 12

Michael R. Betts, Martha C. Nason, Sadie M. West, Stephen C. De Rosa, Stephen A. Migueles, Jonathan Abraham, Michael M. Lederman, Jose M. Benito, Paul A. Goepfert, Mark Connors, Mario Roederer, and Richard A. Koup

Transplantes

Polyfunctional T-Cell Signatures to Predict Protection from Cytomegalovirus after Lung Transplantation

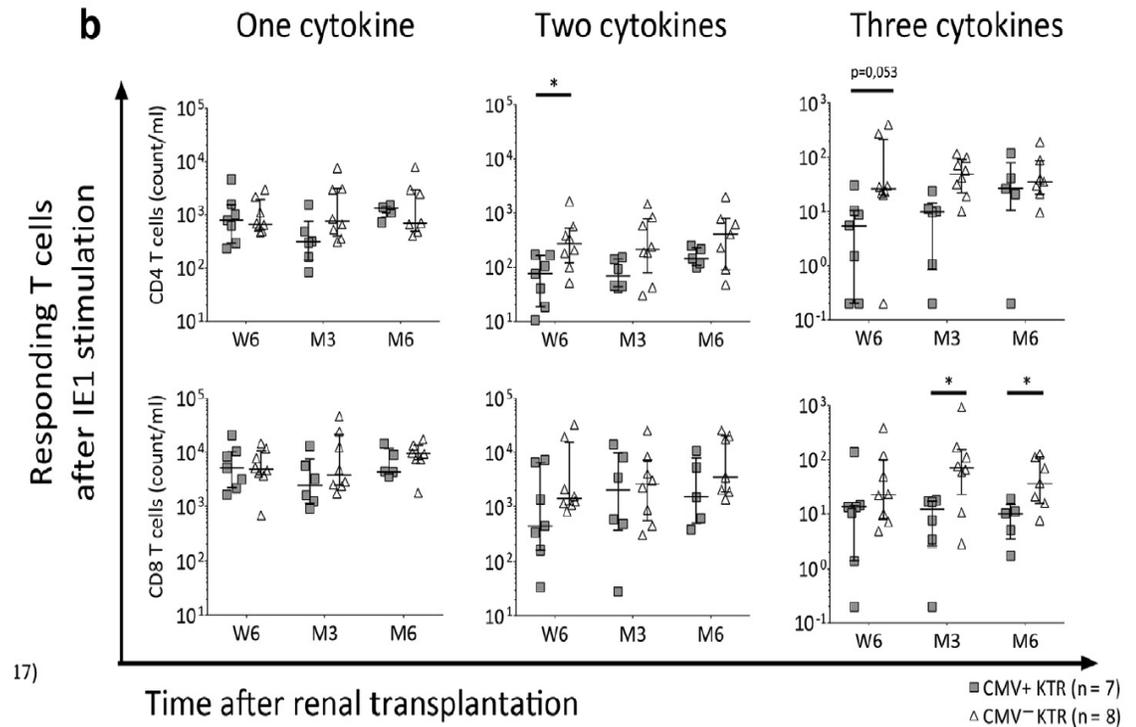
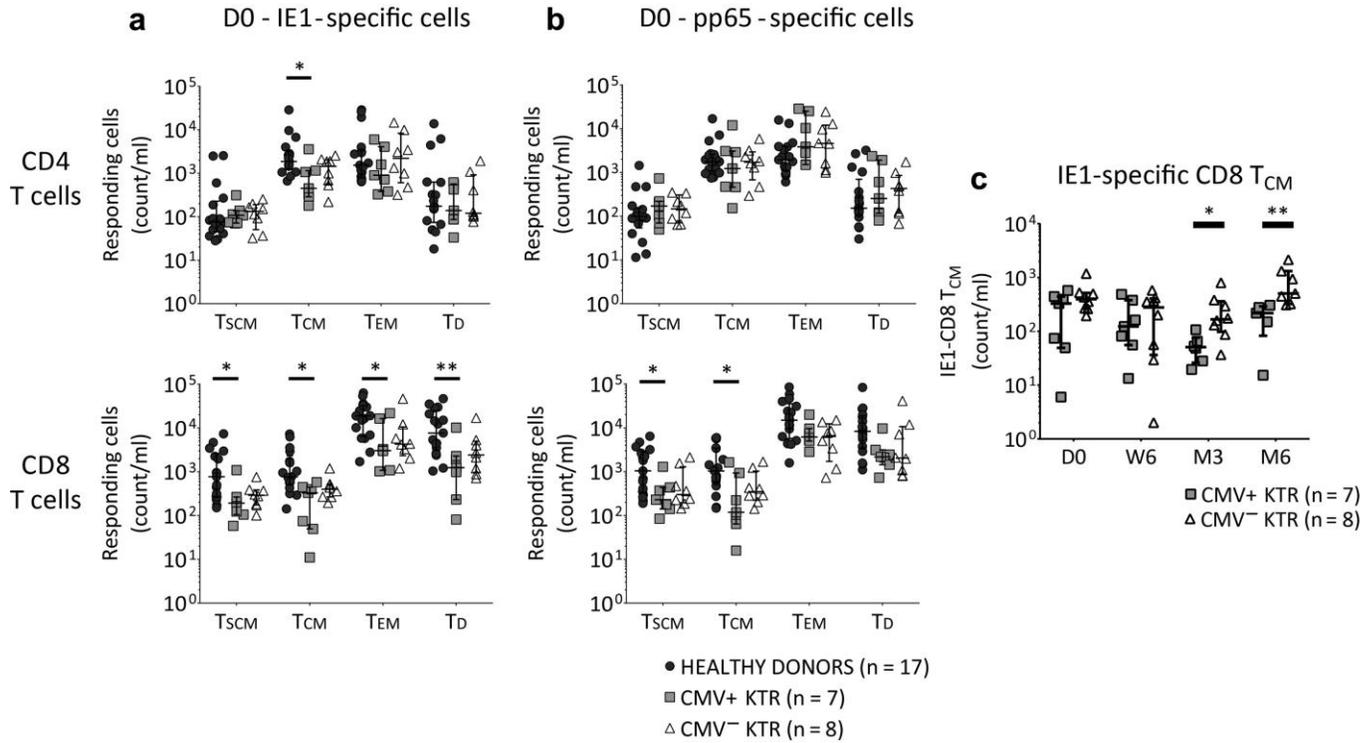
Laurie D. Snyder^{1*}, Cliburn Chan^{2*}, Darongsae Kwon^{2*}, John S. Yi³, Jessica A. Martissa¹, C. Ashley Finlen Copeland¹, Robyn J. Osborne³, Sara D. Sparks³, Scott M. Palmer^{1‡}, and Kent J. Weinhold^{3,4‡}

An Early Immediate Early Protein IE-1-Specific T-Cell Polyfunctionality Is Associated With a Better Control of Cytomegalovirus Reactivation in Kidney Transplantation

Manon Dekeyser¹, Marc Ladrière¹,
Sandra Audonnet², Luc Frimat¹ and Marcelo De
Carvalho Bittencourt²

¹Department of Nephrology and Kidney Transplantation, CHRU Nancy, Nancy, France; and ²Laboratory of Immunology, Nancy-tomique, Pôle Laboratoires, CHRU Nancy, Nancy, France

Kidney International Reports (2017) 2, 486–492



Evaluating Cellular Polyfunctionality with a Novel Polyfunctionality Index

Plos One (2012) | Volume 7 | Issue 7 | e42403

Martin Larsen^{1,2*}, Delphine Sauce^{1,2}, Laurent Arnaud^{1,3}, Solène Fastenackels^{1,2}, Victor Appay^{1,2,4}, Guy Gorochov^{1,2,4}

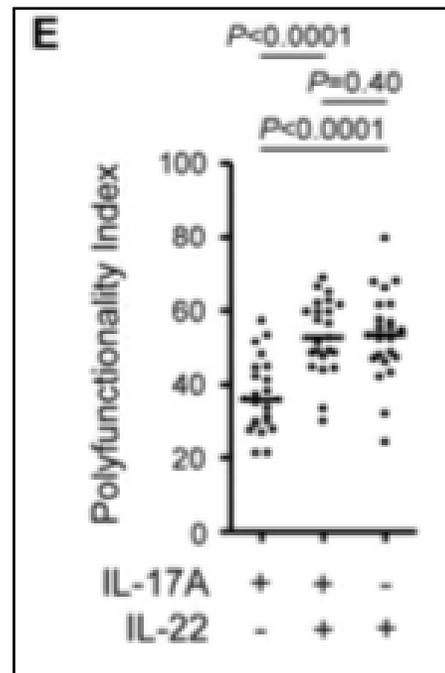
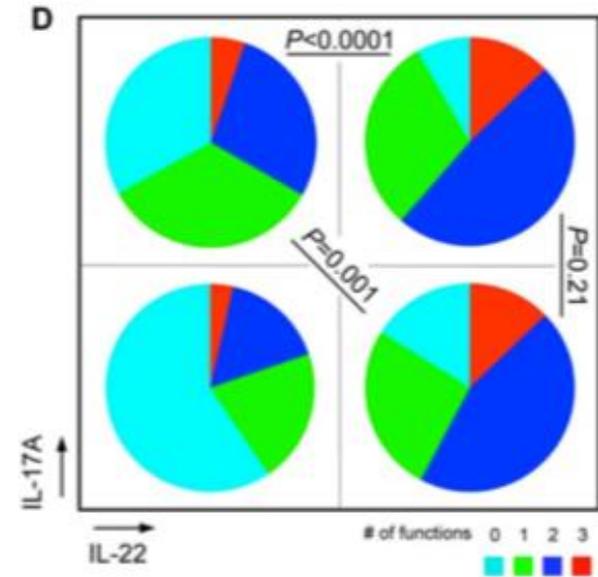
Polyfunctionality Index

$$= \sum_{i=1}^n Fi \cdot \left(\frac{i}{n}\right)^q$$

n : numero de funções
(ex. 3 : IFN- γ , TNF- α , IL-2)

Fi : frequência (%) de células apresentando i funções

q : ponderação de cada Fi



Redução a um parâmetro
Comparação estatística mais fácil

An Early Immediate Early Protein IE-1–Specific T-Cell Polyfunctionality Is Associated With a Better Control of Cytomegalovirus Reactivation in Kidney Transplantation

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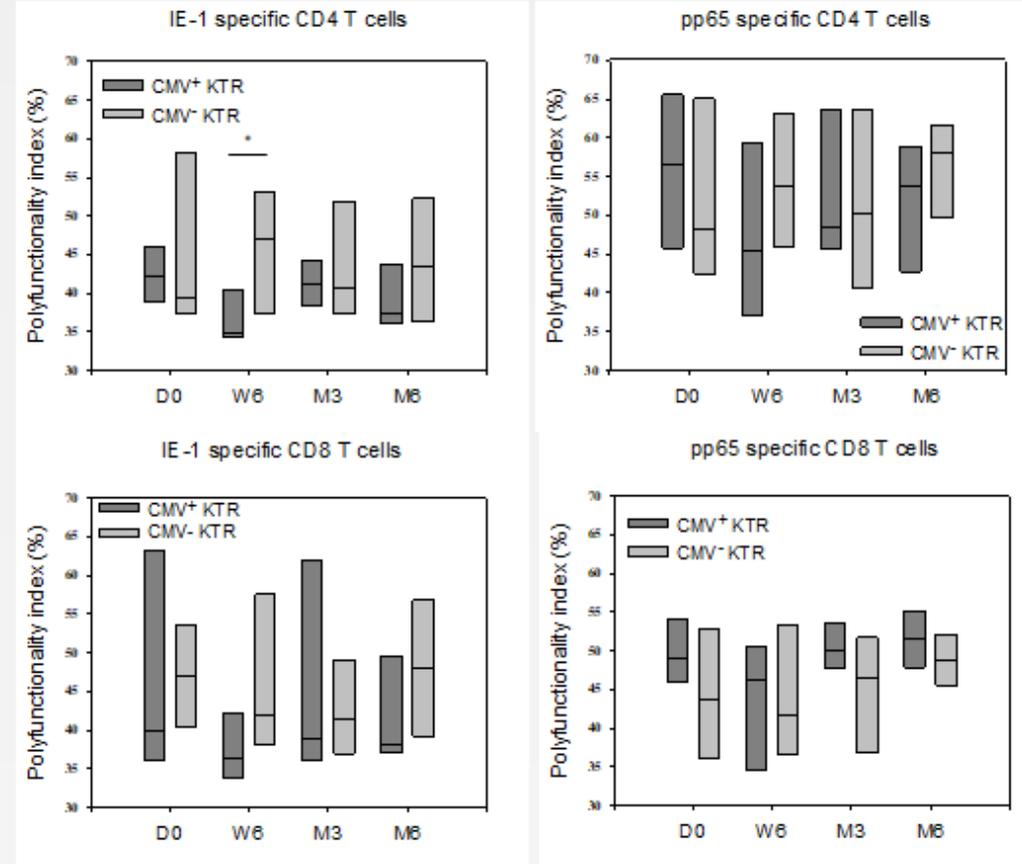


Figure 3. Quality of anti-CMV T cells as evaluated by the Polyfunctionality index (Larsen M et al, *PloS One* 2012). At the 6th week post-transplantation, the polyfunctionality index of IE-1 specific CD4 T cells was superior in KTR with no symptomatic CMV reactivation. Median and the 25th and 75th percentiles of polyfunctionality index in each group. *p<0.05.

A Longitudinal Study of Immune Cells in Severe COVID-19 Patients

 [Didier Payen](#)¹,  [Maxime Cravat](#)²,  [Hadil Maadadi](#)³,  [Carole Didelot](#)⁴,  [Lydia Prosic](#)⁴,  [Claire Dupuis](#)⁵,  [Marie-Reine Losser](#)^{3,6†} and  [Marcelo De Carvalho Bittencourt](#)^{2,4,7*†}

Estudo COVIDIMM, Nancy CHRU

Imunomonitoramento de 15 pacientes com Covid-19 em terapia intensiva

- Expressão de HLA-DR por monócitos
- Subpopulações de linfócitos T, B e NK
- Avaliação da resposta celular (LT anti-Sars-Cov-2) em 5 pacientes

Resumo dos resultados de células T do estudo COVIDIMM

Antígenos Spike mais imunogênicos do que o antígeno Nucleoprotein
Presença de uma boa resposta multifuncional dos LT CD4 específicos
anti-SARS-CoV-2

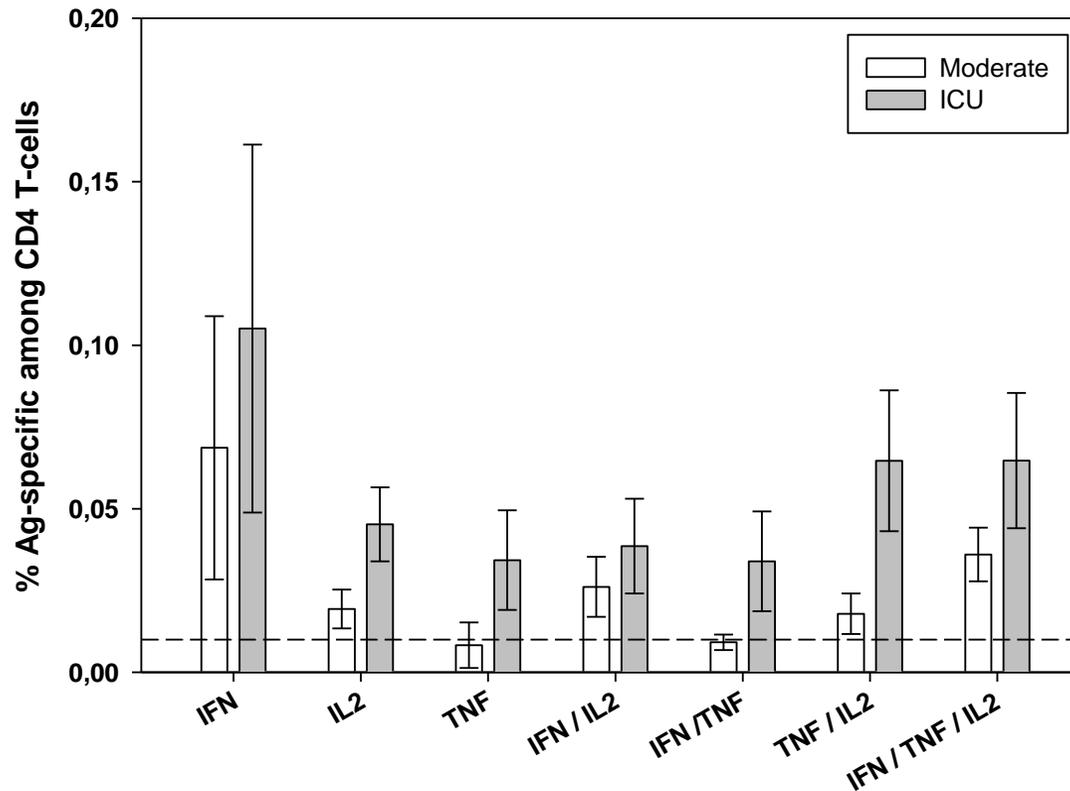
Resposta mais fraca e predominantemente monofuncional dos LT
CD8 específicos

Resposta dos LT CD8 anti-infecciosa global preservada

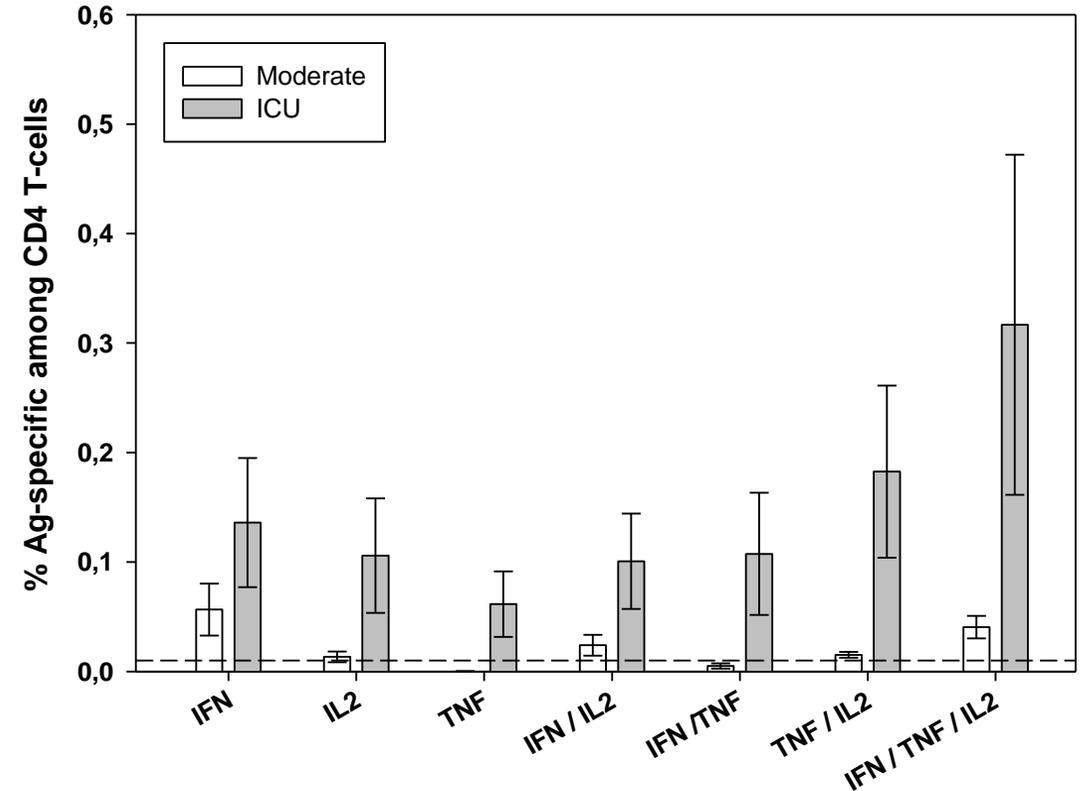
Comparação casos moderados vs. severos

LT CD4

Nucleoprotein-specific CD4 T-cells



Spike1-specific CD4 T-cells

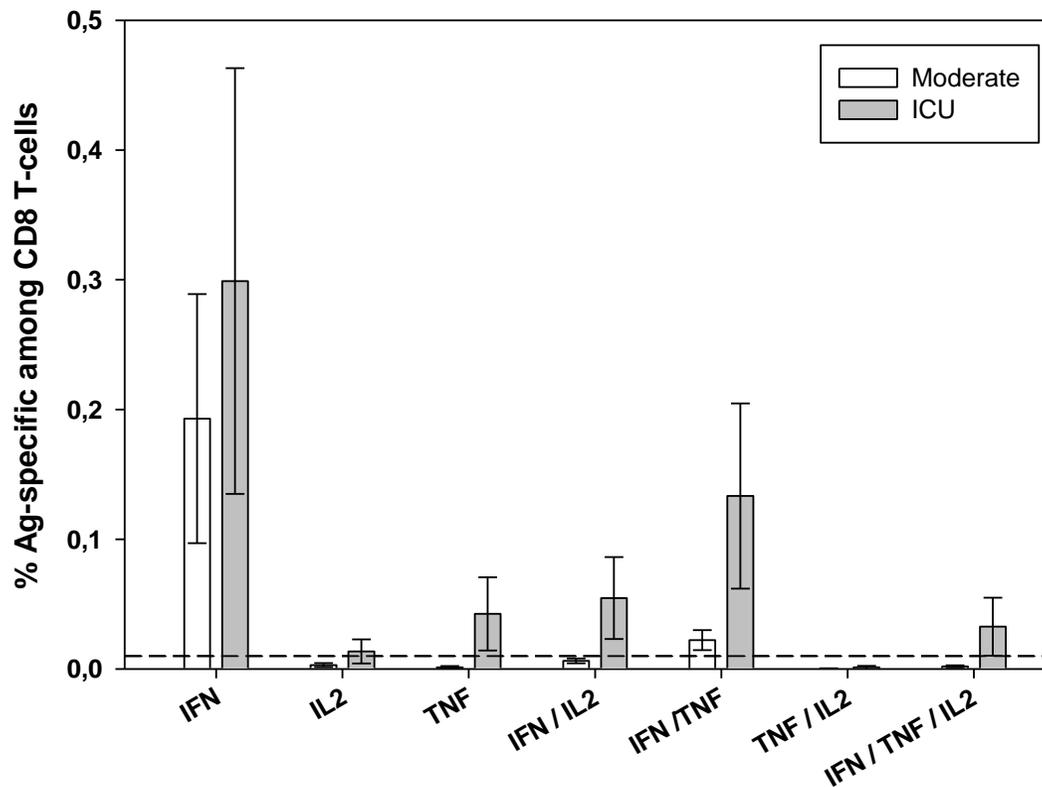


n : 11 (each moderate and ICU)

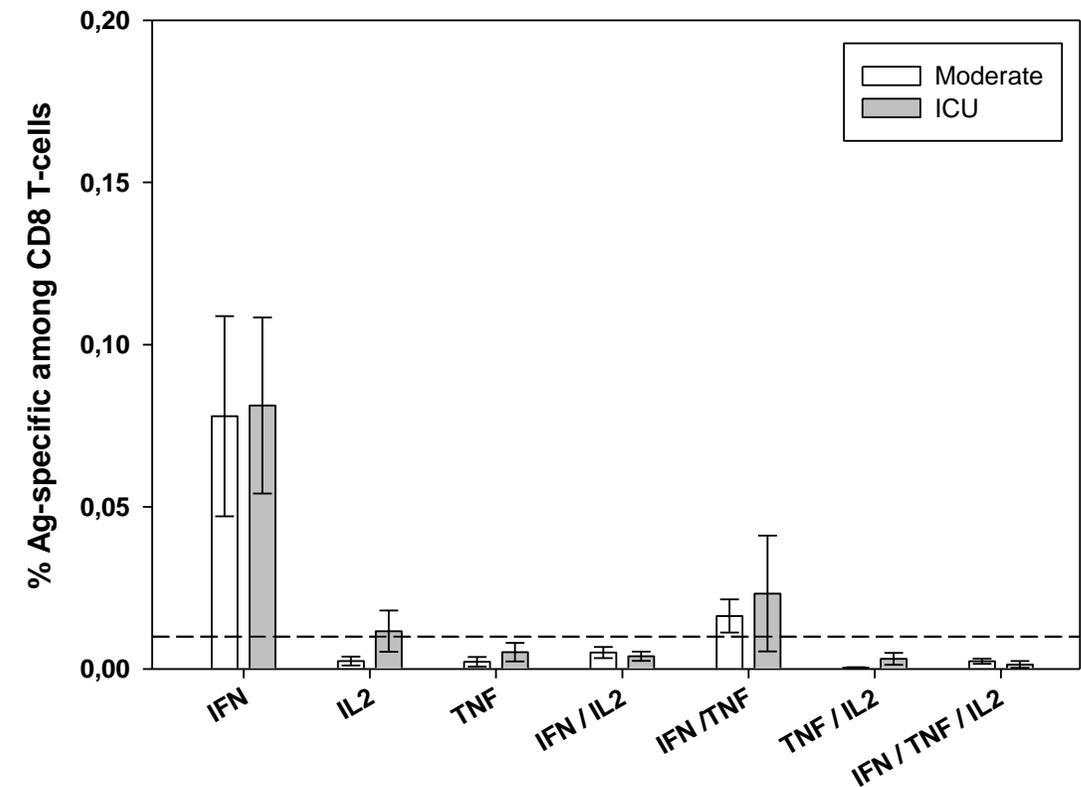
Comparação casos moderados vs. severos

LT CD8

Nucleoprotein-specific CD8 T-cells



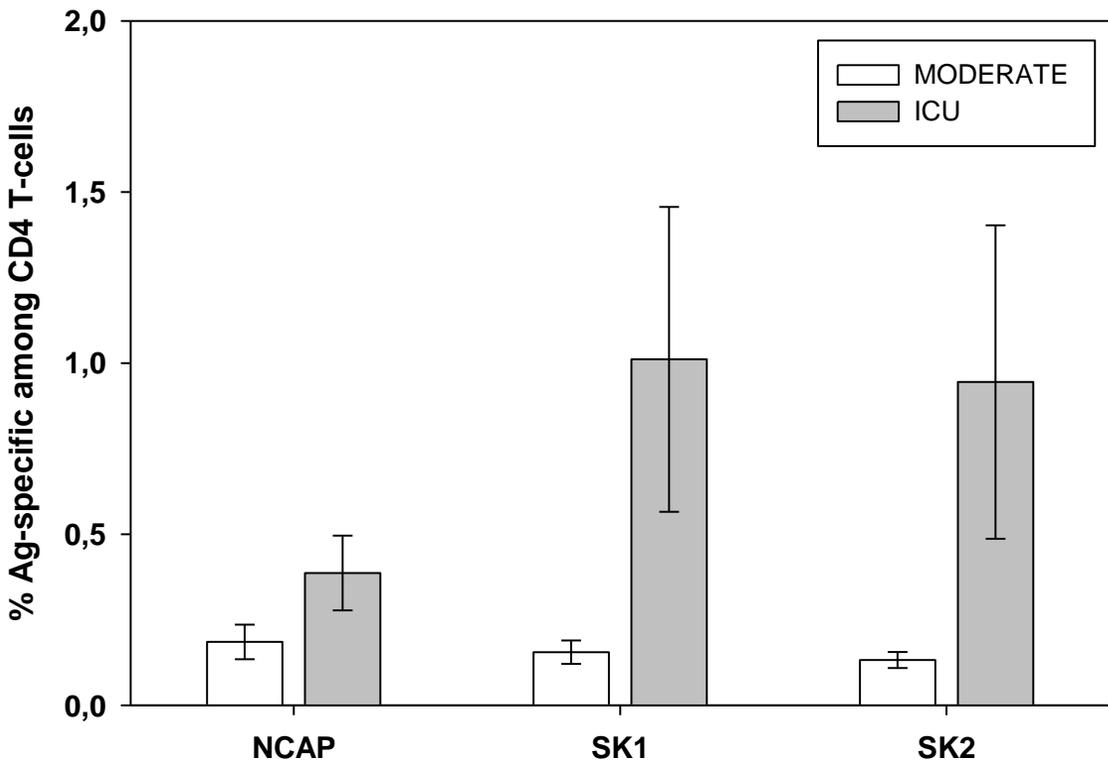
Spike1-specific CD8 T-cells



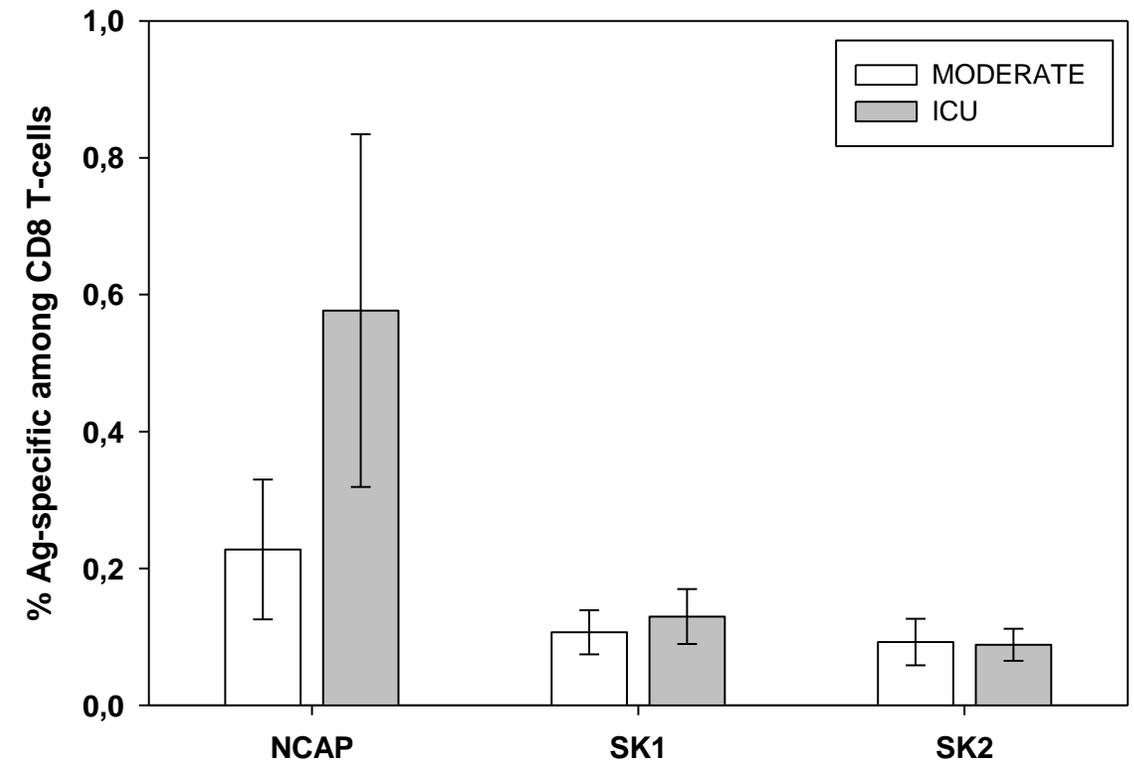
n : 11 (each moderate and ICU)

Comparação casos moderados vs. severos

SARS-CoV2-specific CD4 T-cells



SARS-CoV2-specific CD8 T-cells



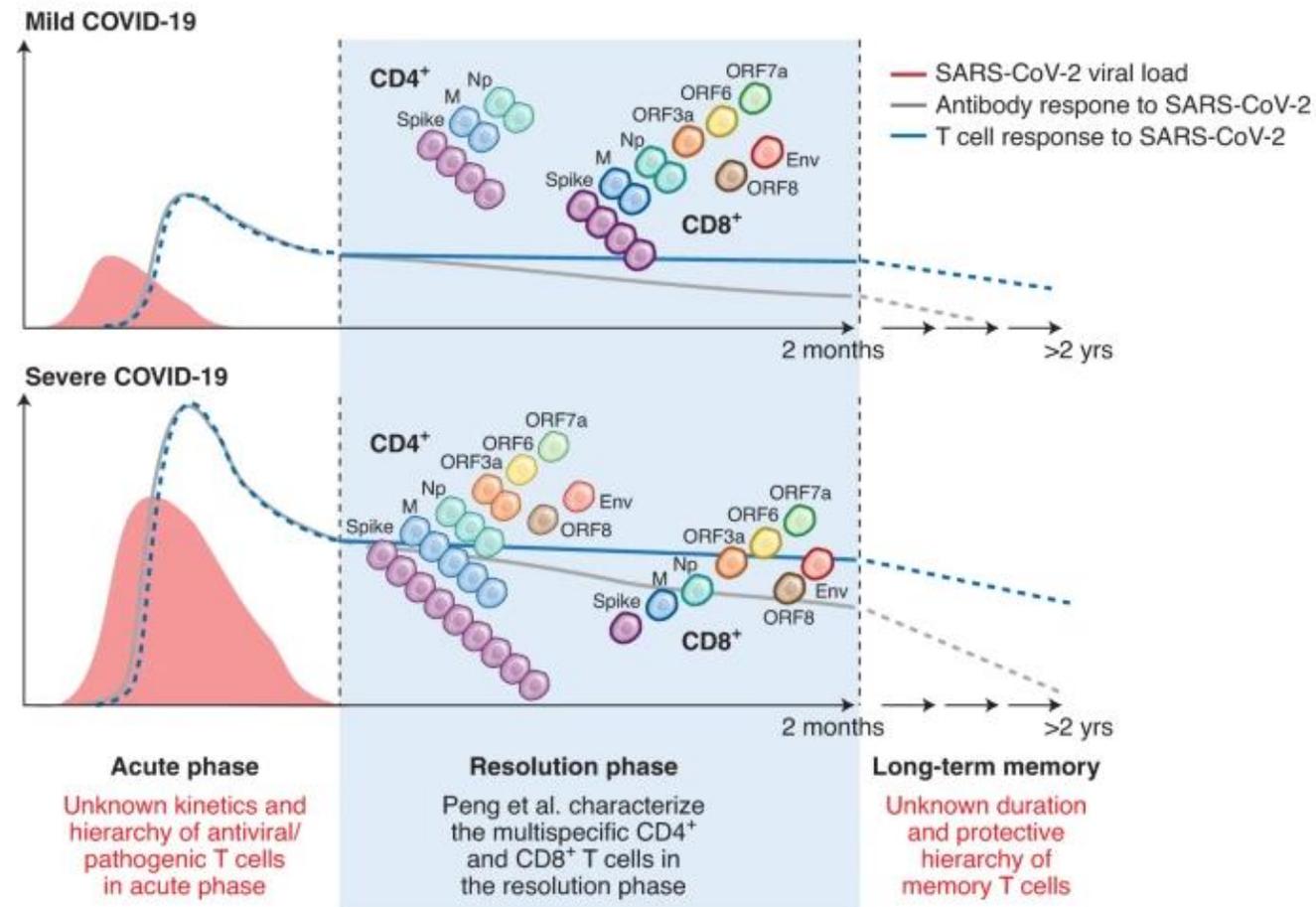
n : 11 (each moderate and ICU)

Broad and strong memory CD4⁺ and CD8⁺ T cells induced by SARS-CoV-2 in UK convalescent individuals following COVID-19

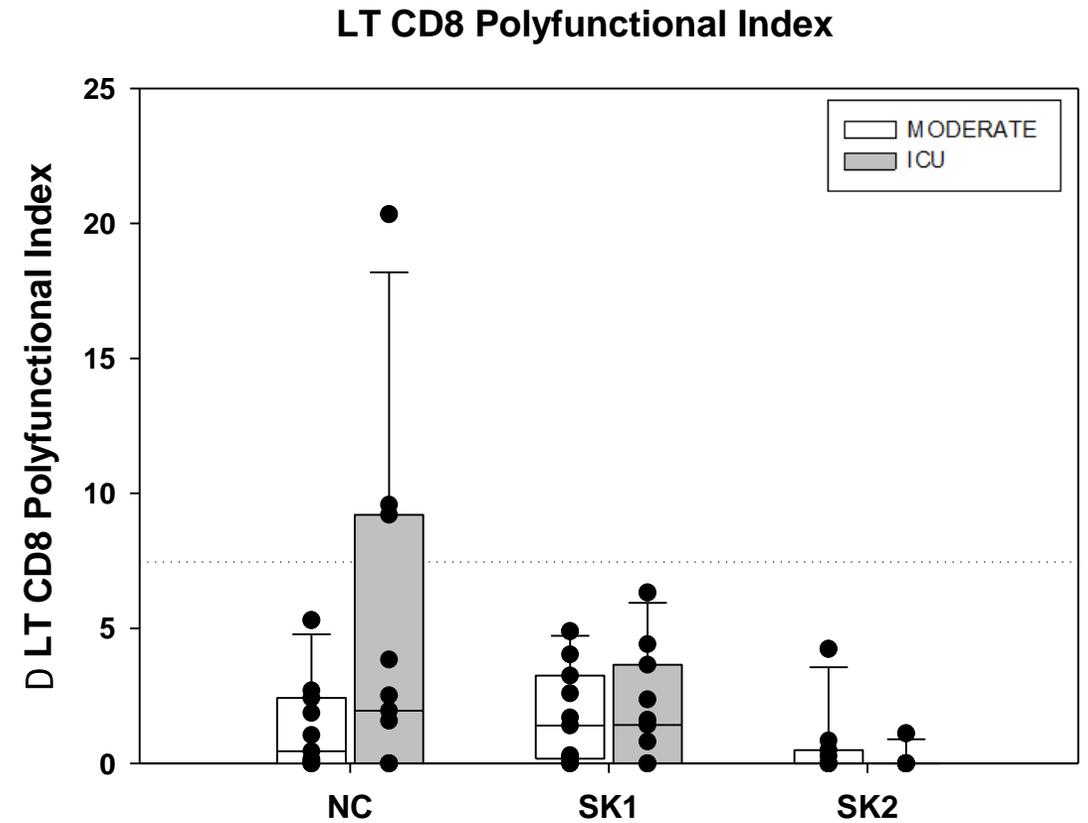
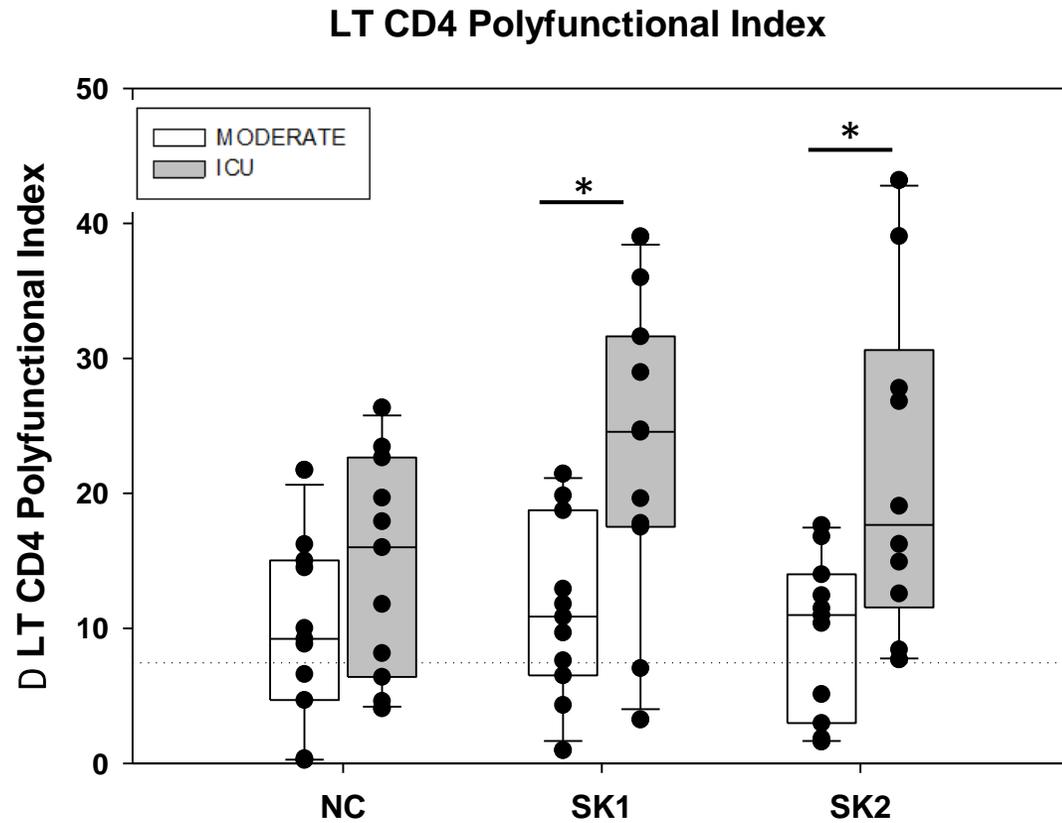
Yanchun Peng, Alexander J. Mentzer, [...] Tao Dong 

Nature Immunology 21, 1336–1345(2020) | [Cite this article](#)

Fig. 1: Frequency and specificity of T cells in the resolution phase of mild or severe COVID-19.

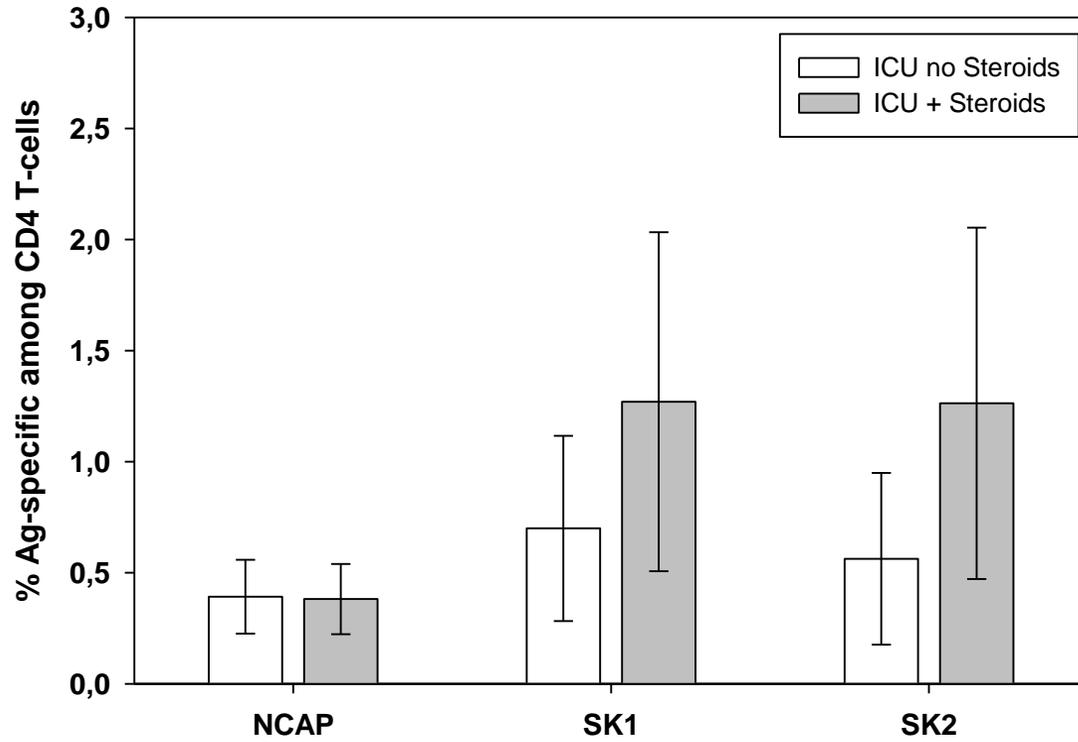


Comparação casos moderados vs. severos

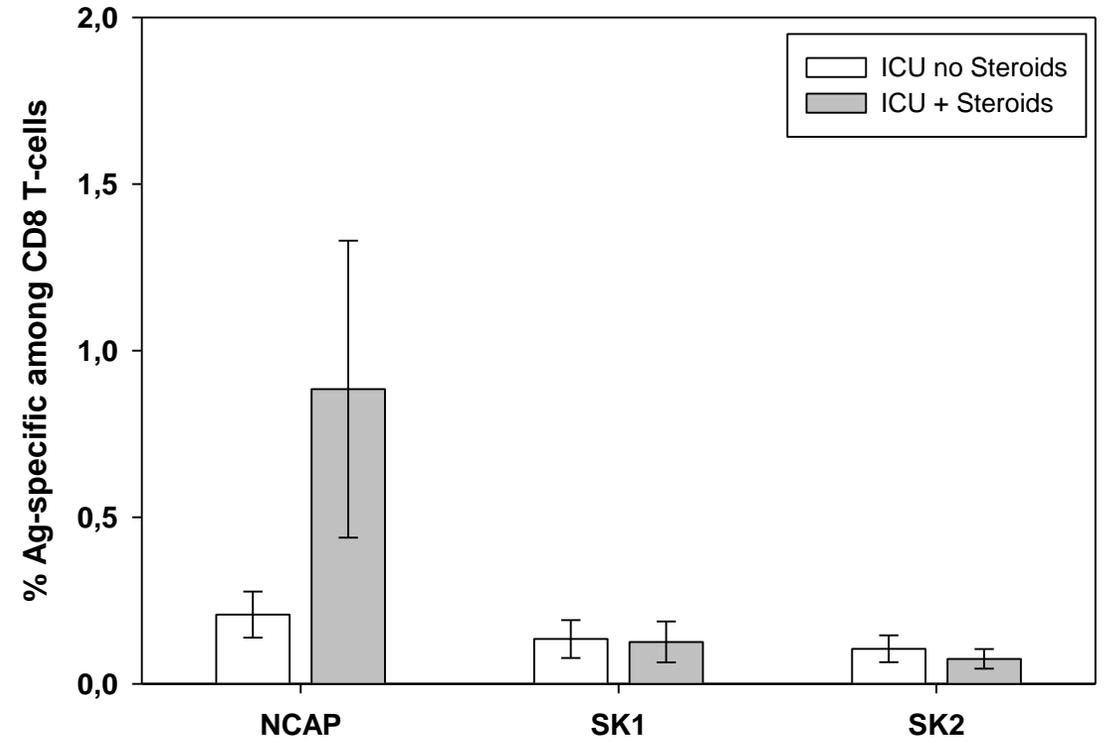


n : 11 (each moderate and ICU)

SARS-CoV2-specific CD4 T-cells



SARS-CoV2-specific CD8 T-cells



n : 5 ICU no steroids and 6 ICU+Steroids

Proposição para o imunomonitoramento da COVID-19

Imunidade celular (ICS, IGRA com Elispot)

Imunidade humoral IgG + IgM, mas também IgA (salivar?)

Good IgA Bad IgG in SARS-CoV-2 Infection?

FREE

Marie C Béné ✉, Marcelo de Carvalho Bittencourt, Marion Eveillard,
Yannick Le Bris

Clinical Infectious Diseases, Volume 71, Issue 15, 1 August 2020, Pages 897–898,
<https://doi.org/10.1093/cid/ciaa426>

Perspectivas

Medicina personalizada

Identificação de antígenos em doenças autoimunes, infecciosas, neoplásicas

Correlação com evolução clínica

Estudo das respostas pos-vacinais

Caracterização extensa de células T memória

- Citômetros de Fluxo com > 30 parâmetros

- Classificação celular e sequenciamento do TCR (“single-cell sequencing”)

- Determinação da afinidade pelo antígeno

- Utilização para imunoterapia celular

Merci de votre attention

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<http://www.scoop.it/t/immune-monitoring-1>

http://publicationslist.org/m_de_carvalho_bittencourt

