ISAC
International Society for Advancement of Cytometry
ISAC LETF
Prague Cytometry Workshop 2019
April 12 – 14, 2019, Prague | Czech Republic

Multiparametric analysis of rare events

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Dr. De Biasi is an International **Society for Advancement of Cytometry (ISAC) Marylou Ingram Scholar**

OUTLINE OF THE TALK

- Rare cell analysis: background and keypoints

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- Main problems in the detection of such cells
- Possible solutions: from hardware to software
- Rare cells in the immune system: the case of iNKT cells
- Rare cells in the immune system: the case of CEC

BACKGROUND

- >30 years ago: enumeration of fetal red blood cells in the maternal circulation at a frequency of 1/10,000 to 1/100,000 by Cupp.

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- Now: detection and quantitation of several rare cell populations in blood or bone marrow.

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- Now: detection and quantitation of several rare cell populations in blood or bone marrow.

- Essential tool in the diagnosis and monitoring of hematological cancers and immunological disorders, as well as in the identification of Ag-specific cells.

WARNING

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- The **frequency** of the event of interest, and the **signal-to-noise ratio** of the method used to detect the event are the two most important factors.

KEY POINTS

- ‘‘**Rare-event analysis**’’: detection of events that occur at a frequency of 1 in 1,000 (**0.1%**) or less, although the record claimed in the literature has long stood at 1 cell in 10,000,000 (**0.00001%**) for tumor cells spiked into peripheral blood.

KEY POINTS

- ‘‘**rare-event analysis**,’’: detection of events that occur at a frequency of 1 in 1,000 (**0.1%**) or less, although the record claimed in the literature has long stood at 1 cell in 10,000,000 (**0.00001%**) for tumor cells spiked into peripheral blood.

- Detecting an event at low frequency requires a **high signal-to-noise ratio** and the **acquisition of a large number of events.**

IMMUNOLOGIST'S INTERESTS

- Ag-specific T cells
- NKT and iNKT cells
- Circulating endothelial cells and precursors
- Stem cells (CD34+)
- Particular lymphocytes subpopulations
- Circulating tumor cells
- Polyfunctional assays
- .....

Open pre-analytical questions

- How much blood from patients?

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- Lack of available standardized method

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- Lack of available standardized method
- Enriched or non enriched populations

Enrichment methods for the detection of rare antigen-specific T cells

Direct

MHC-multimers

Pros
- Activation independent
- High specificity

Cons
- Knowledge/availability of MHC/epitope
- Restricted to single epitope specificities
- No functional status of the cells
- Detection of low-affinity cells difficult

T cells detected
- Naive
- Memory
- Treg

Indirect

Cytokine secretion assay

Pros → Isolation of cytokine producing subsets

Cons → Restricted to few selected cytokine producers

T cells detected → Memory

CD154 enrichment (6h)

Pros → Detection of the whole antigen-specific CD4+ T cell response
Fast

Cons → Mainly restricted to CD4+ T cells

T cells detected
- Naive
- Memory

CD137 enrichment (16h)

Pros → Detection of the whole antigen-specific T cell response

Cons
- Not compatible with cytokine analysis
- No differentiation between Treg and Tconv cells

T cells detected
- Naive
- Memory

Open pre-analytical questions

- How much blood from patients
- Lack of available standardized method
- Enriched or non enriched populations
- How many markers/colors

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- How much blood from patients
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Open pre-analytical questions

- How much blood from patients
- Lack of available standardized method
- Enriched or non enriched populations
- How many markers/colours
- How many cells
- Exclusion of doublets, dead cells and debris: use of a DUMP CHANNEL

Number of events to acquire

The Poisson statistic is a discrete probability distribution that:

- Expresses the **probability of a given number of events occurring in a fixed interval of time and/or space** if these events occur with a known average rate and independently of the time since the last event.

- **Can be used for the number of events** in other specified intervals such as distance, area or **volume.**

Counting randomly distributed cells in a certain volume → Poisson statistics applied

Number of events to acquire

A few notes concerning **Poisson** statistics. Consider the general case of enumerating a total of **N events**, of which **R** meet a certain criterion (positives). The proportion of positives, **P=R/N**, will also be the probability of any particular event being observed as positive $0 \leq P \leq 1$, which is clearly related to the random manner in which cells are selected for analysis.

As with all statistical distributions, the **variance, Var**, is a fundamental parameter and, for the binomial, is defined as follows: **Var(R) = NP(1-P)**

The standard deviation, **SD**, is the **square root of the variance**

The **coefficient of variation** (CV) is the **SD expressed as a percentage of the population**: **CV = (SD*100)/R**

These simple equations can now be used to examine some practical situations.

Number of events to acquire

EXAMPLE: Consider a preparation of human peripheral blood mononuclear cells labeled with an antibody to **detect B cells**.
Flow cytometry then indicates that **10%** of the cells present are positive for this marker, so that: **P=0.1** and **P(1-P)=0.09**

If three data sets were collected for	**1,000**, **5,000** and **10,000** events,
we would expect to observe	**100**, **500** and **1,000** positive cells
with variances of	**90**, **450** and **900**, respectively.
Expressed as SD, these would be	**9.5**, **21.2** and **30**
and as CVs	**9.5**, **4.2** and **3**.

Good experimental practice within the biological field usually results in **CVs on the order of 5%.**

Number of events to acquire

Frequency	**10%**		
Events N	**1,000**	**5,000**	**10,000**
Positive R	**100**	**500**	**1,000**
Proportion P	**0.1**	**0.1**	**0.1**
Variance **Var(R) = NP(1-P)**	**90**	**450**	**900**
SD	**9.49**	**21.21**	**30.00**
CV **CV = (SD*100)/R**	**9.49**	**4.24**	**3.00**

Number of events to acquire

Frequency	1%			
events N	1,000	5,000	10,000	100,000
Positive R	10	50	100	1,000
Proportion P	0.01	0.01	0.01	0.01
Variance	9.9	49.5	99.0	990.0
SD	3.15	7.04	9.95	31.46
CV	31.46	14.07	9.95	3.15

Number of events to acquire

Frequency	0.1%		
events N	100,000	1,000,000	*401,000*
Positive R	100	1,000	*401*
Proportion P	0.001	0.001	*0.001*
Variance	99.9	999,0	*400.6*
SD	9.99	31.61	*20.01*
CV	9.99	3.16	*4.99*

Number of events to acquire

Frequency	0.01%		
events N	100,000	1,000,000	10,000,000
Positive R	10	100	1,000
Proportion P	0.0001	0.0001	0.0001
Variance	10.0	100.0	999.9
SD	3.16	10.00	31.62
CV	31.62	10.00	3.16

Numbers of events to acquire

COMMUNICATION TO THE EDITOR

Cytometry PART A
Journal of the International Society for Advancement of Cytometry

ISAC
International Society for Advancement of Cytometry

How Many Events Is Enough? Are You Positive?

Roederer M., Cyt Part A, 73A: 384-385, 2008

- **No reason to fix a threshold** for the number of events below which any frequency must be considered ‘‘negative’’.
- if **adequate negative controls** are set, "positivity’’ can be determined after comparison of the measurements (*i.e.*, positive minus negative, namely positive minus the background), using standard statistical tools to compare the frequencies.
- If T cells from "n" **unvaccinated controls show no activation**, while T cells from "n" vaccinated individuals do, **even low frequencies can be taken as positive**.

Open analytical questions

- Which instrument, and which performances

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- Flow cytometer rates of acquisition

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- Spill over and carry over

Open analytical questions

- Which instrument, and which performances
- Flow cytometer rates of acquisition
- Maximize the signal-to-noise ratio of the cells of interest from the background
- Data acquisition: instrument clean and the background level of noise is below the threshold
- Spill over and carry over
- Adequate software

Gating strategy: useful guidelines

- **Debris** exclusion
- Single/doublets discrimination-> **FSC-A *vs* FSC-H**
- **Dead cells** exclusion-> FSC/SSC *vs.* Viability dye
- **DUMP** channel (cell type of interest *vs.* irrelevant)
- Exclude events during fluidic issues -> **TIME gates**
- Remove **fluorochrome aggregates** -> “Keeper gate”

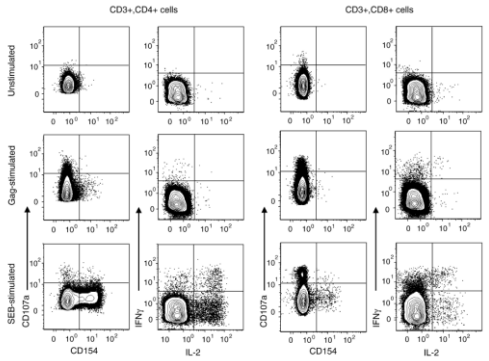
Our previous experience

Polyfunctional analysis of Ag-specific cells

AIDS 2010, **24**:947–957

Cytotoxic granule release dominates gag-specific CD4$^+$ T-cell response in different phases of HIV infection

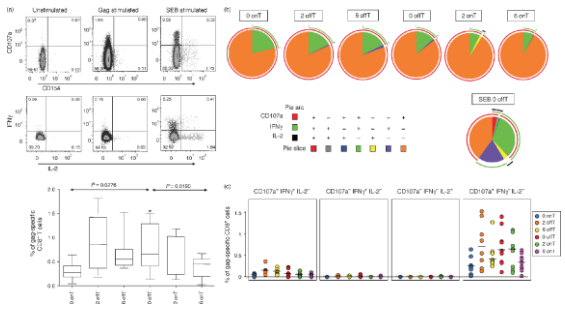
Elisa Nemes[a], Linda Bertoncelli[a], Enrico Lugli[a,b], Marcello Pinti[a], Milena Nasi[a], Lisa Manzini[c], Serena Manzini[a], Francesca Prati[c], Vanni Borghi[c], Andrea Cossarizza[a] and Cristina Mussini[c]



AIDS 2011, **25**:1443–1453

CD4$^+$ T-cell differentiation, regulatory T cells and gag-specific T lymphocytes are unaffected by CD4-guided treatment interruption and therapy resumption

Elisa Nemes[a,*], Enrico Lugli[a,d,*], Linda Bertoncelli[a,*], Milena Nasi[a], Marcello Pinti[a], Serena Manzini[a], Francesca Prati[b], Lisa Manzini[b], Cinzia Del Giovane[c], Roberto D'Amico[c], Andrea Cossarizza[a] and Cristina Mussini[b]

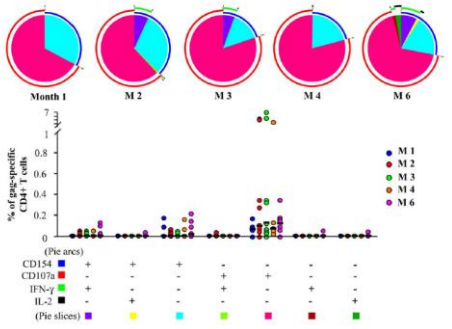


OPEN ACCESS Freely available online

2012 PLOS ONE

T Cell Activation but Not Polyfunctionality after Primary HIV Infection Predicts Control of Viral Load and Length of the Time without Therapy

Andrea Cossarizza[1*☯], Linda Bertoncelli[1☯], Elisa Nemes[1☯¤a], Enrico Lugli[1¤b], Marcello Pinti[2], Milena Nasi[1], Sara De Biasi[1], Lara Gibellini[1], Jonas P. Montagna[1], Marco Vecchia[1], Lisa Manzini[1], Marianna Meschiari[1], Vanni Borghi[3], Giovanni Guaraldi[3,4], Cristina Mussini[1,3]



invariant Natural Killer T (iNKT) cells

- Type I NKT cells; 0.001-3% of human PBMC.
- Expression of **semi-invariant Vα24Jα18 TCR** and a variety of other receptors associated with NK cells.
- Recognition of **glycolipid antigens** presented by the non-classical MHC class I molecule CD1d.
- Divided into **functionally distinct CD4+, CD8+, and CD4-CD8- subsets.**
- Rapid production of large amounts of cytokines upon activation in response to iNKT cell antigen (α-galcer).

↓

regulatory immune functions

MULTIPLE SCLEROSIS (MS)

- Chronic progressive inflammatory demyelinating disease affecting the CNS

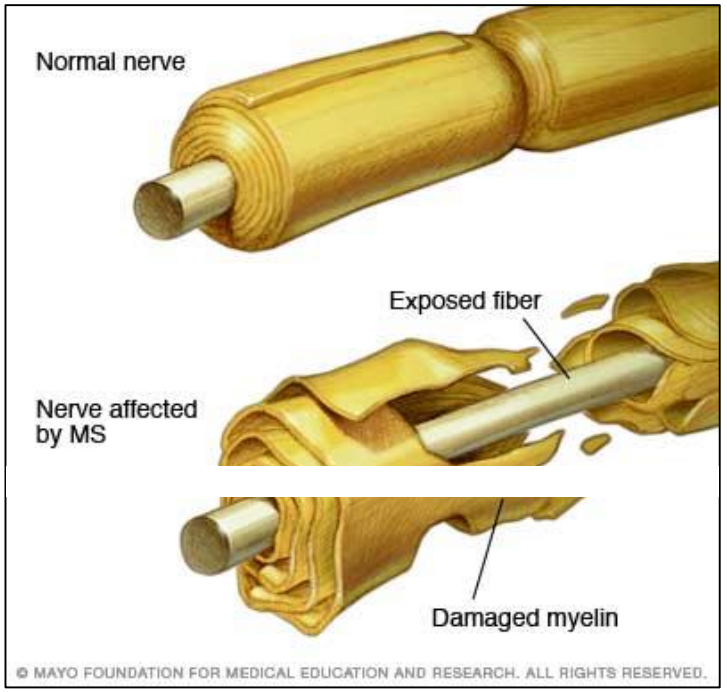
Activated inflammatory cells

↓

Damage to the myelin sheath

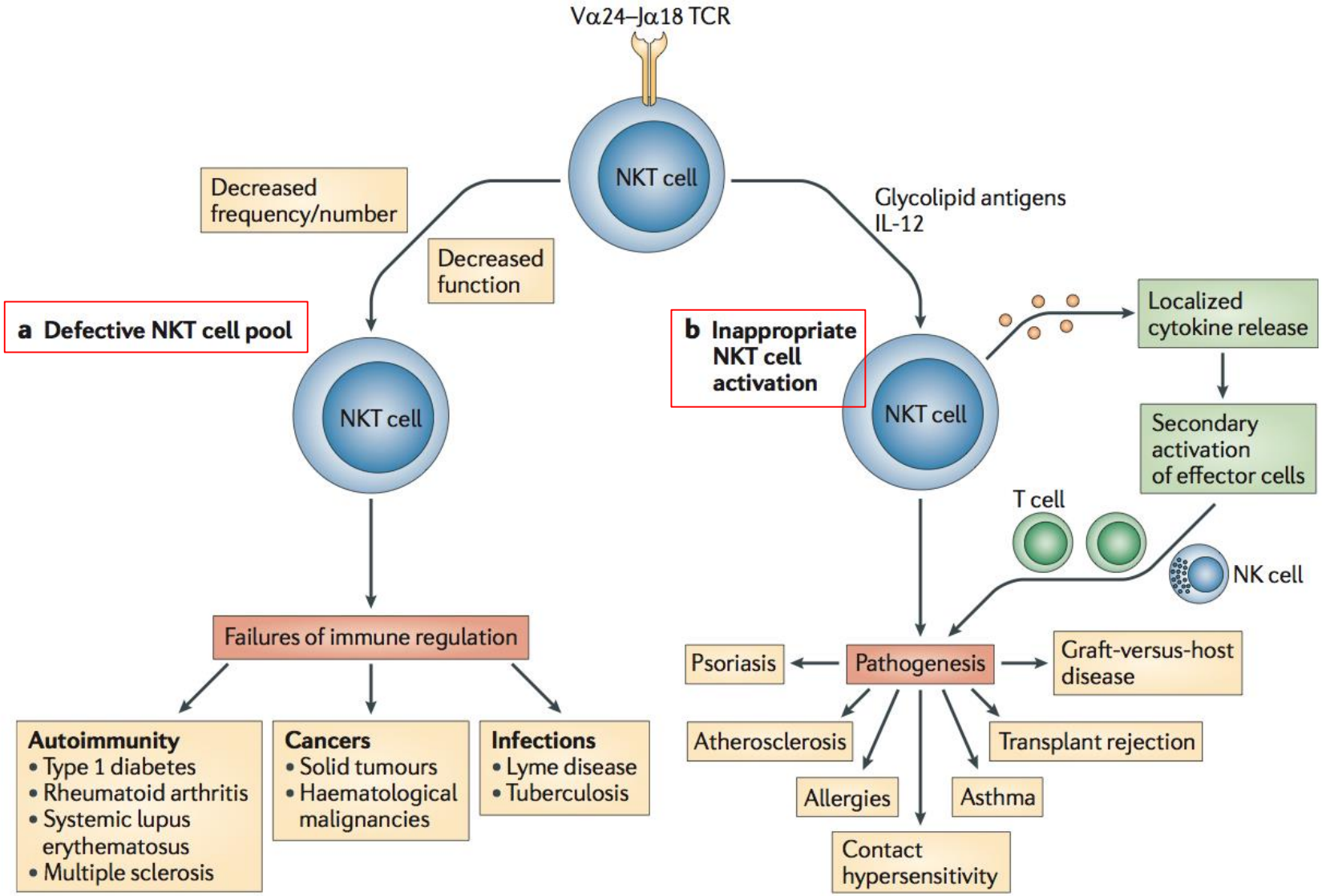
↓

impaired conduction of electrical signals to and from the CNS



- About 2.5-3 million people affected worldwide

NKT cells and MS



Berzins SP., Nat Rev Immunol. 2011

ORIGINAL RESEARCH
published: 30 November 2016
doi: 10.3389/fimmu.2016.00555

iNKT Cells in Secondary Progressive Multiple Sclerosis Patients Display Pro-inflammatory Profiles

Sara De Biasi[1†], Anna Maria Simone[2†], Milena Nasi[1], Elena Bianchini[3], Diana Ferraro[2], Francesca Vitetta[2], Lara Gibellini[1], Marcello Pinti[3], Cinzia Del Giovane[4], Patrizia Sola[2‡] and Andrea Cossarizza[5‡]*

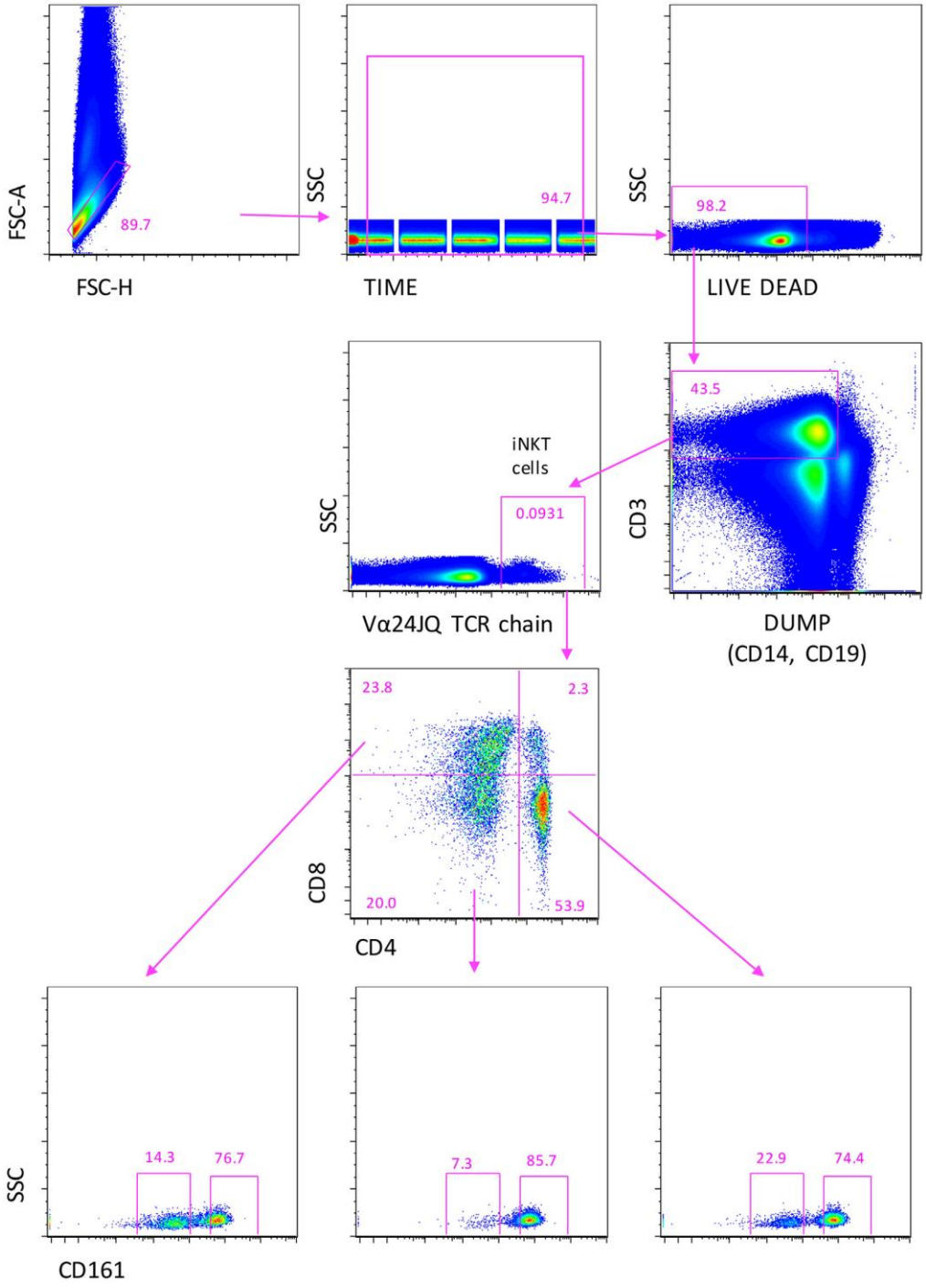
frontiers in Immunology

TABLE 1 | Clinical characteristics of multiple sclerosis patients.

	Total	Newly diagnosed RR (ARR)	Not-active RR (NARR)	RR treated with IFN	RR treated with GA	RR treated with NAT	SP	PP
No. of patients	165	17	19	31	29	25	24	20
Males, *n* (%)	43 (26)	6 (35)	4 (21)	8 (25)	6 (21)	4 (16)	6 (25)	9 (45)
Females, *n* (%)	122 (74)	11 (65)	15 (79)	23 (75)	23 (79)	21 (84)	18 (75)	11 (55)
Age, years[a] range (min; max)	45.1 ± 11.6 (19; 66)	35.5 ± 8.7 (21; 52)	51.1 ± 7.7 (40; 64)	42.9 ± 8.4 (24; 58)	40.7 ± 8.2 (29; 56)	35.5 ± 9.7 (19; 51)	55.7 ± 7.9 (38; 66)	57.2 ± 7.4 (47; 65)
Age at onset, years[a]	33.8 ± 10.1	33.7 ± 9	31.4 ± 9.5	34 ± 8.4	34 ± 9.3	27.9 ± 7.9	34 ± 10.2	43.1 ± 11.7
Disease duration (months)[b]	135.7 ± 107.7	24.5 ± 23.5	237.4 ± 98.5	105.8 ± 78.1	79.4 ± 58.2	90.3 ± 52.7	259.1 ± 104.4	166.7 ± 94.1
Number relapses preceding year[a]	0.2 ± 0.6	0.9 ± 0.6	0.1 ± 0.2	0.1 ± 0.3	0.1 ± 0.4	0.6 ± 1.2	0	0
Severity score[a]	3.2 ± 2.9	2.8 ± 2.7	0.6 ± 0.5	1.6 ± 1.6	2 ± 2.1	3.4 ± 2.5	6.4 ± 2.2	6.2 ± 2.6
EDSS[a]	2.6 ± 2.4	1.3 ± 1.1	1.1 ± 0.7	1.3 ± 1.4	1.1 ± 0.9	2.1 ± 1.3	6.4 ± 1.2	5.2 ± 2
Delta – EDSS (preceding 12 months)[a]	0.1 ± 0.4	2.1 ± 1.3	0 ± 0.3	0 ± 0.4	0 ± 0.1	0 ± 0.4	0.2 ± 0.2	0.2 ± 0.4

[a]*Values expressed as mean ± SD.*
[b]*Values expressed as median ± SD.*

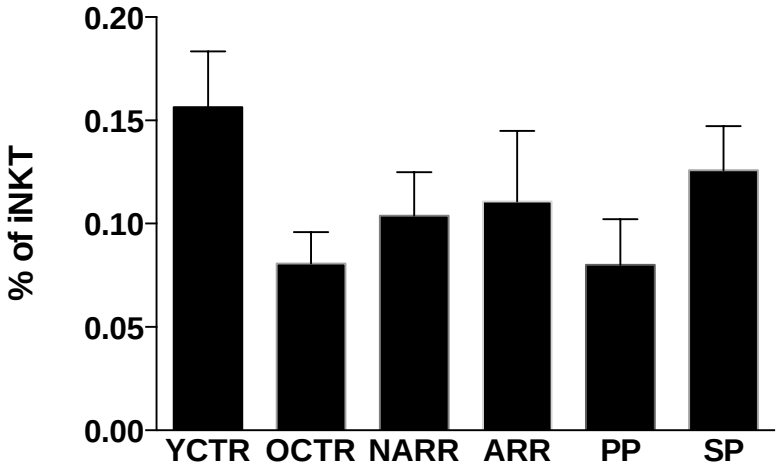
Gating strategy

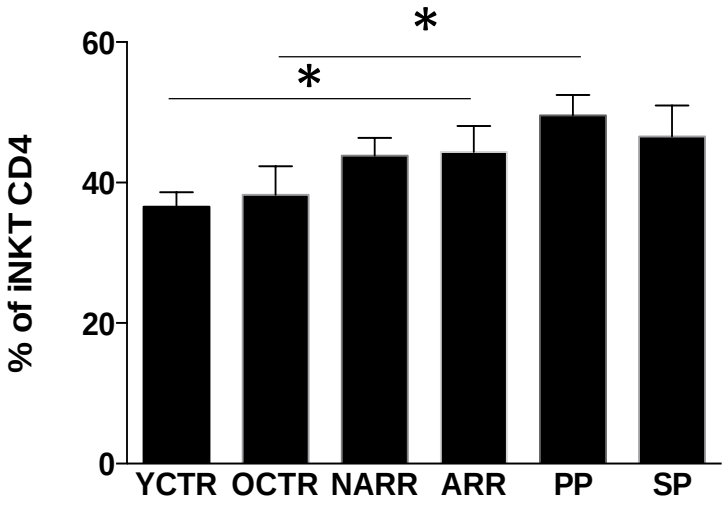


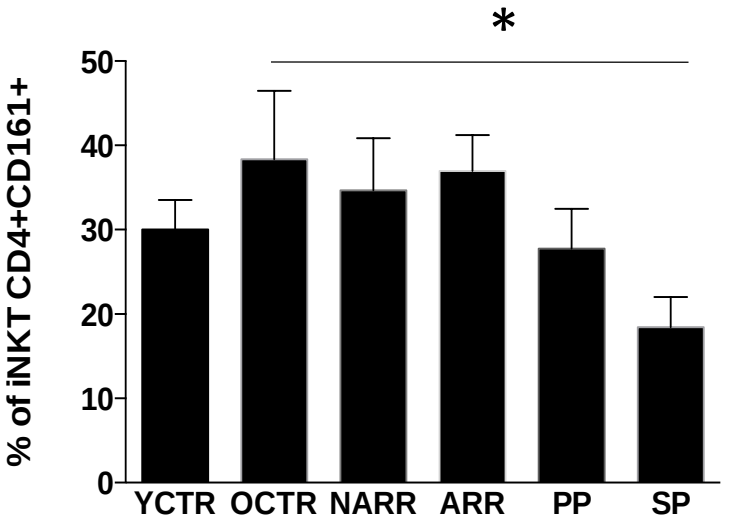
iNKT Cells in Secondary Progressive Multiple Sclerosis Patients Display Pro-inflammatory Profiles

Sara De Biasi[†1], Anna Maria Simone[2†], Milena Nasi[1], Elena Bianchini[3], Diana Ferraro[2], Francesca Vitetta[2], Lara Gibellini[1], Marcello Pinti[3], Cinzia Del Giovane[4], Patrizia Sola[2‡] and Andrea Cossarizza[5‡]*

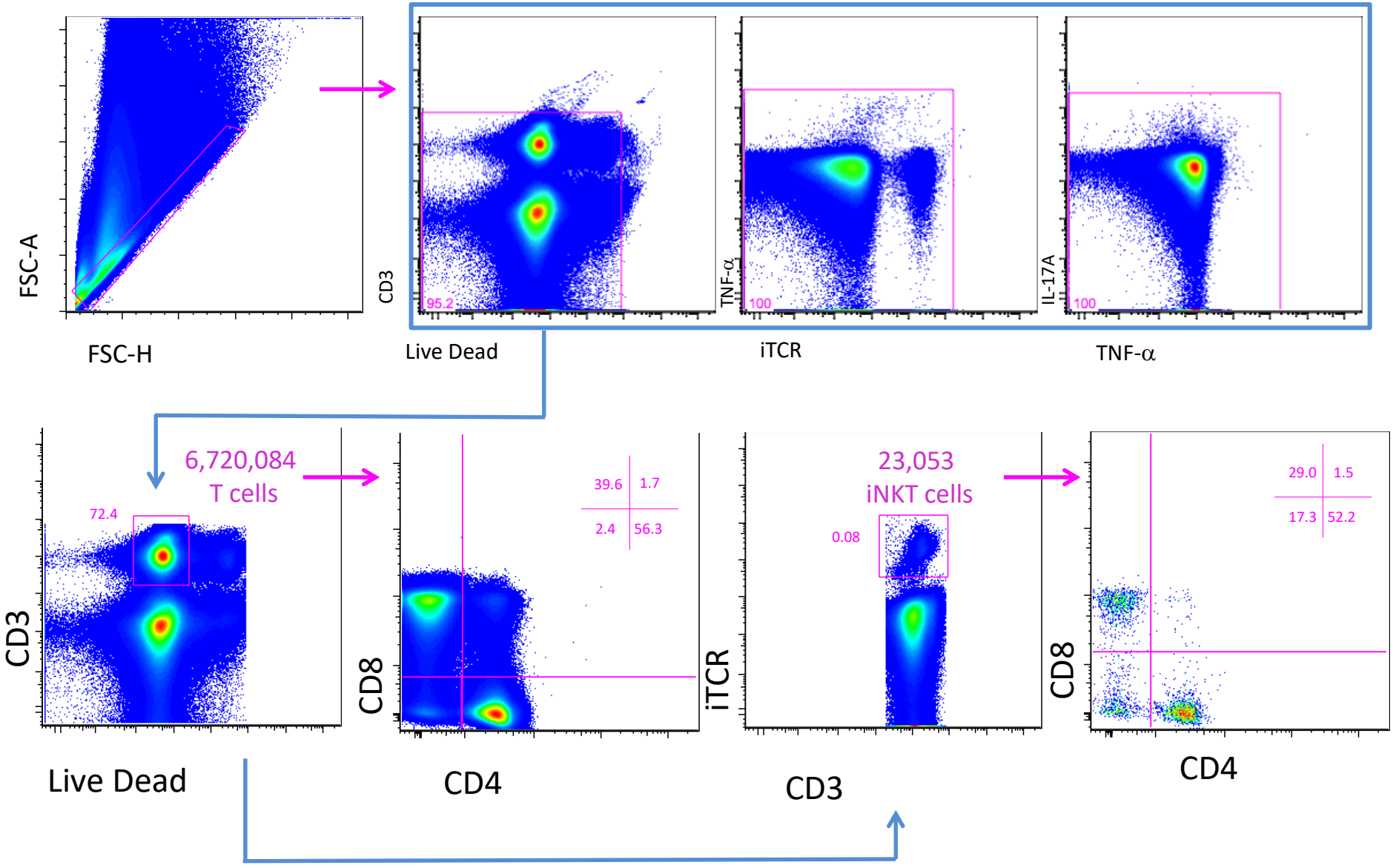
% of iNKT cells does not change among MS patients, but the phenotype changes



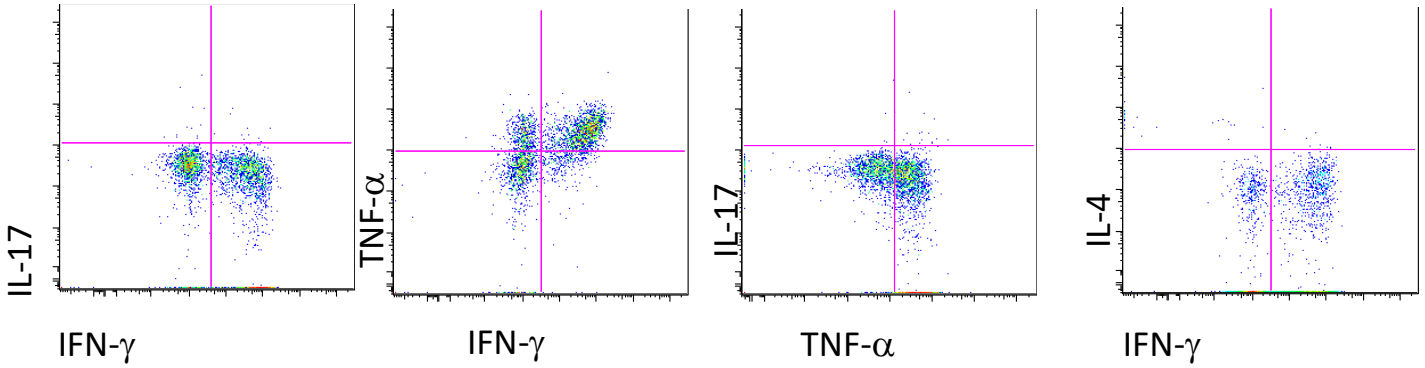




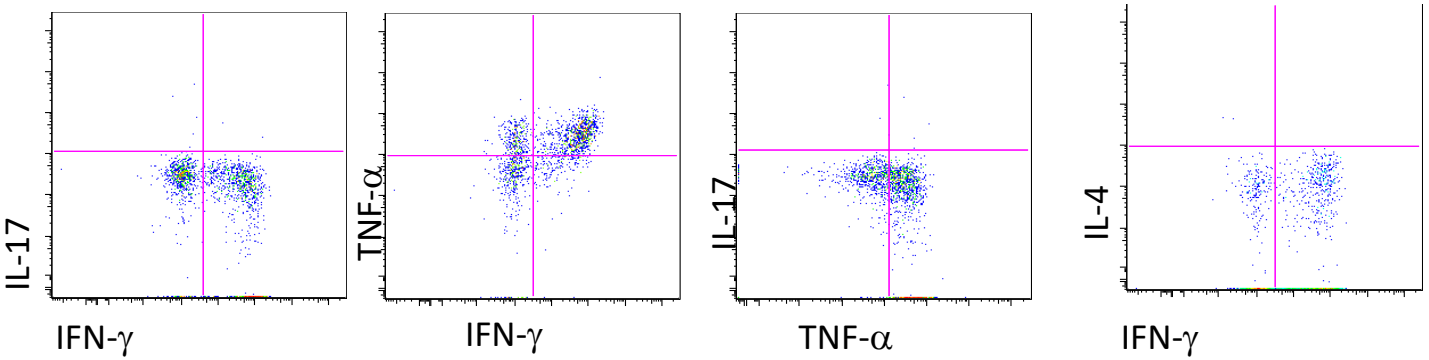
Gating strategy



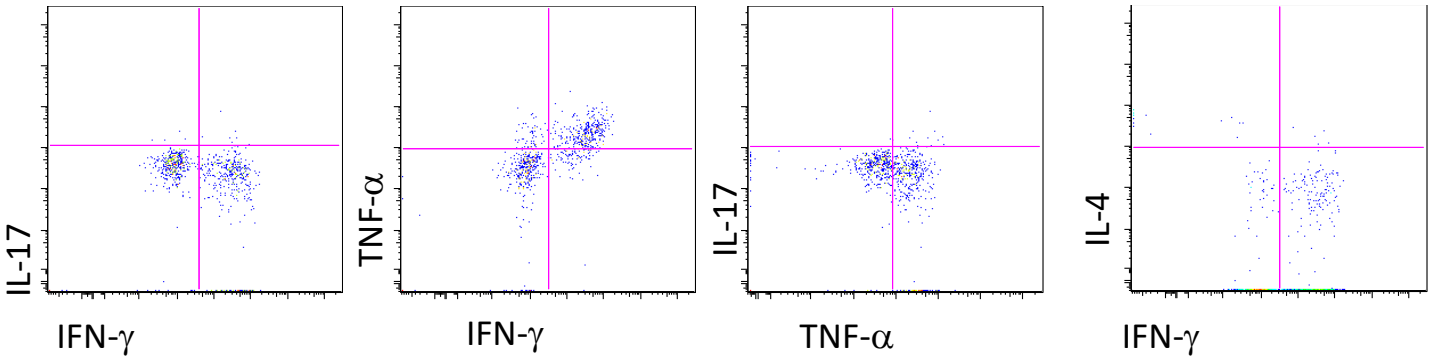
GATED ON CD4+ iNKT CELLS



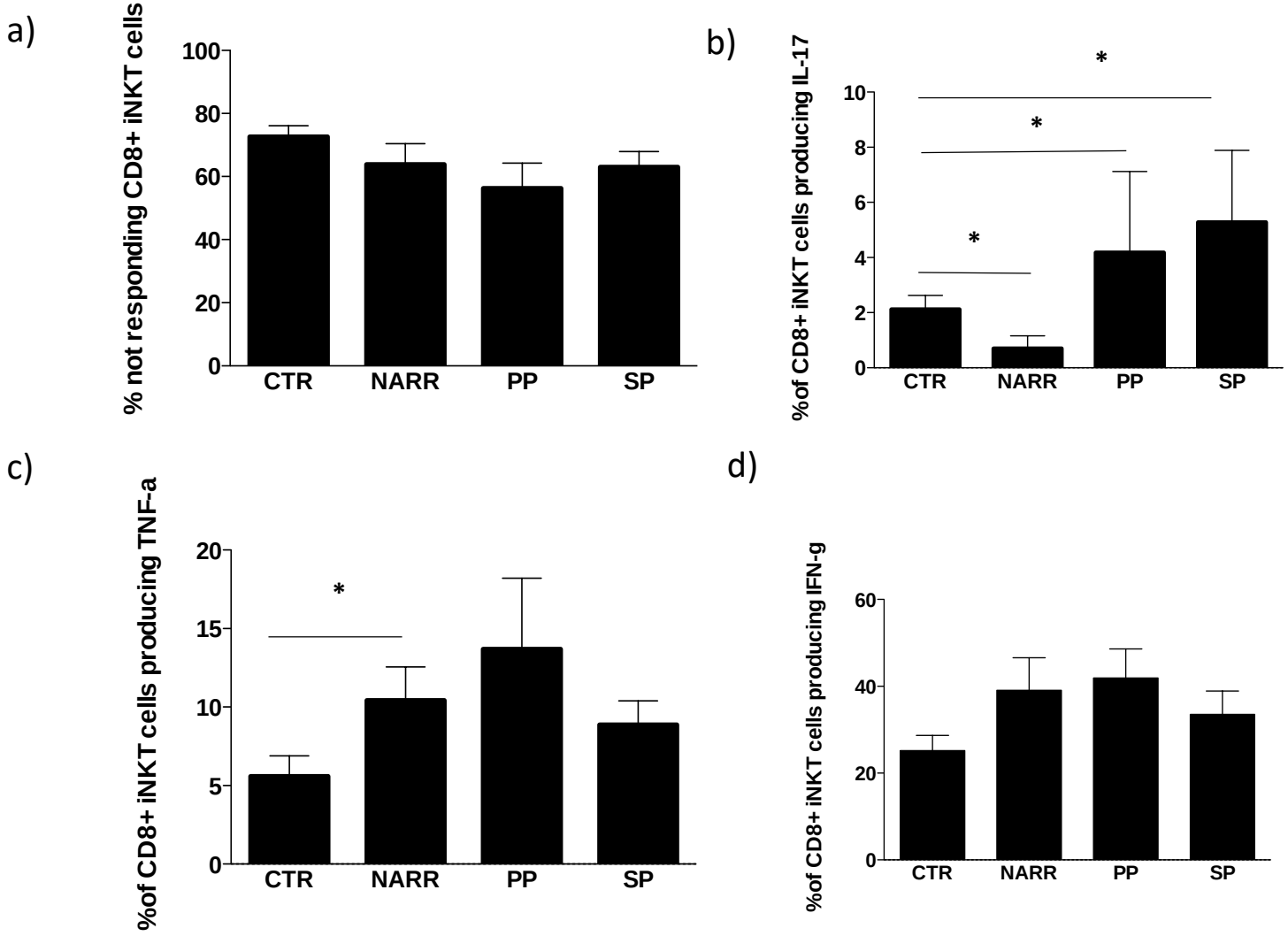
GATED ON CD8+ iNKT CELLS



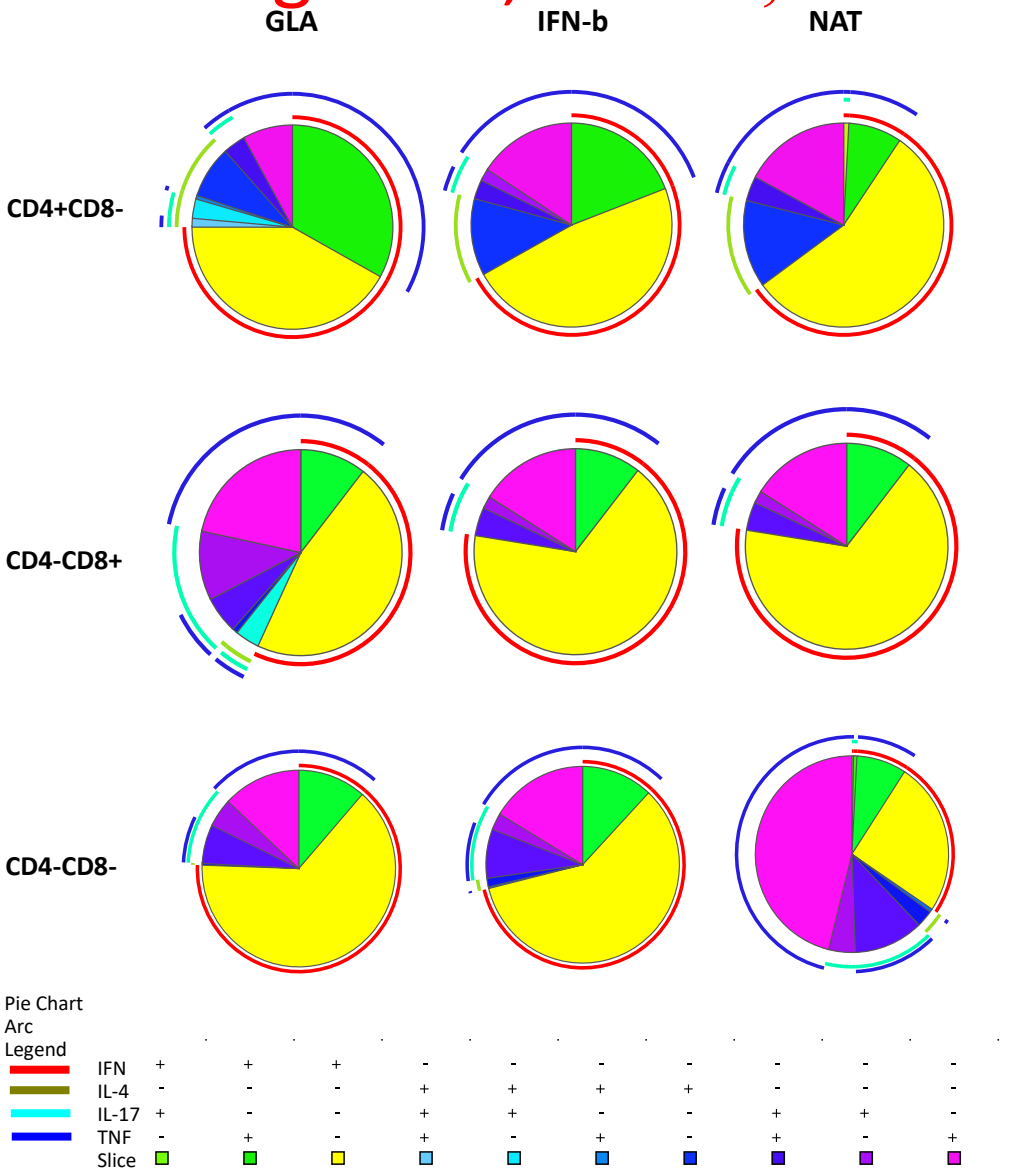
GATED ON CD4-CD8- iNKT CELLS



Cytokine production by CD8+ iNKT cells is skewed toward IL-17 production in MS patients



Natalizumab induces a decrease in DN iNKT cells producing IL-17, TNF-α, and IFN–γ



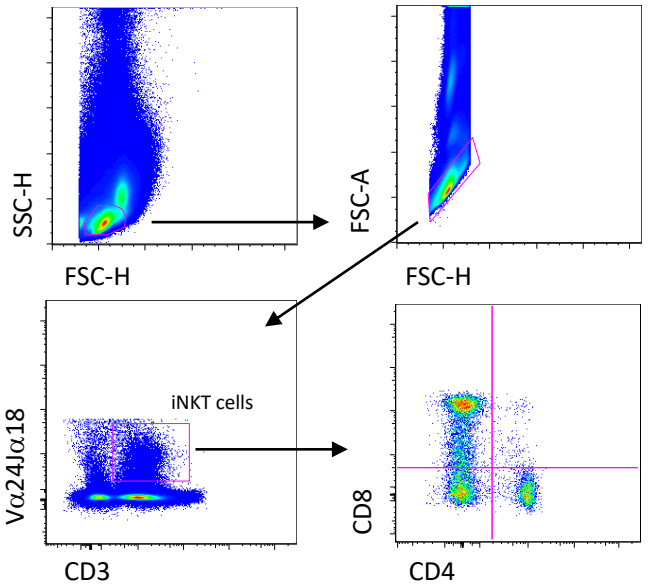
Th1 and Th17 proinflammatory profile characterizes invariant natural killer T cells in virologically suppressed HIV+ patients with low $CD4^+/CD8^+$ ratio

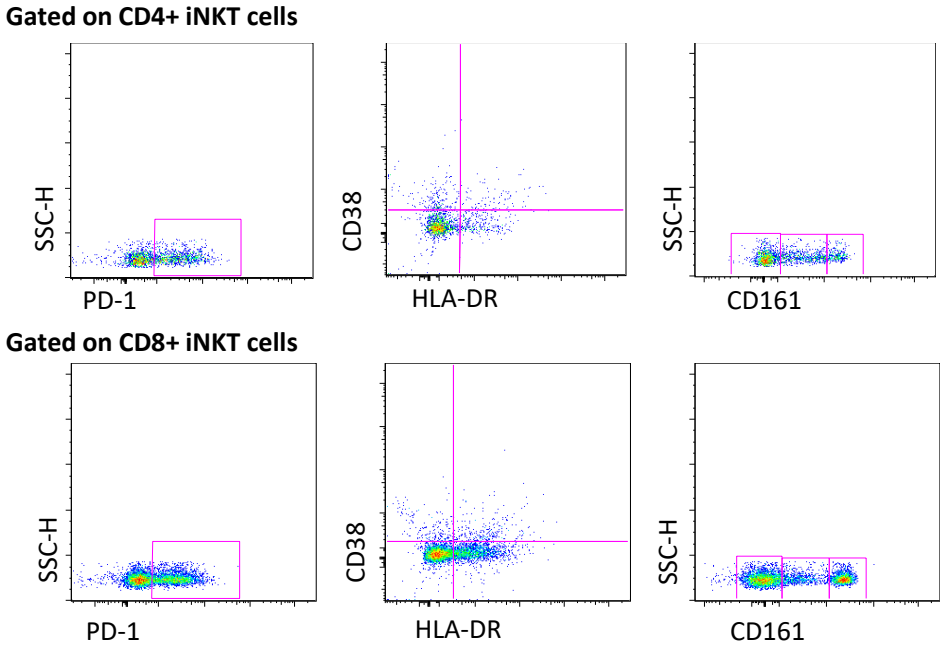
Sara De Biasi[a,*], Elena Bianchini[b,*], Milena Nasi[a], Margherita Digaetano[c], Lara Gibellini[a], Gianluca Carnevale[a], Vanni Borghi[c], Giovanni Guaraldi[c,d], Marcello Pinti[b], Cristina Mussini[a,c] and Andrea Cossarizza[d]

AIDS 2016, **30**:2599–2610

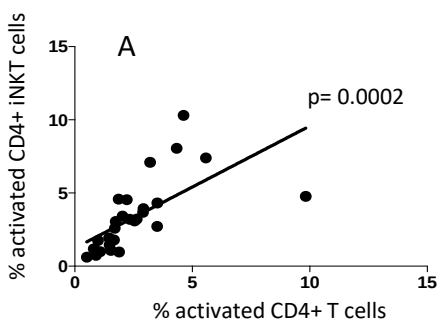
Phenotype of iNKT cells in healthy donors and HIV+ patients with ratio <0.4 (group A) and >1.1 (group B).

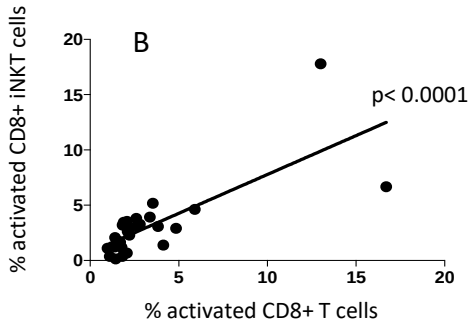
Activation and exhaustion run in parallel in cells of the innate and adaptive immunity

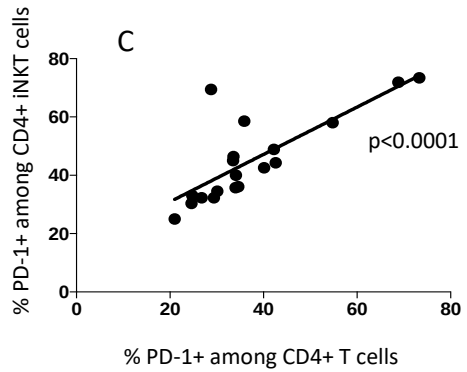


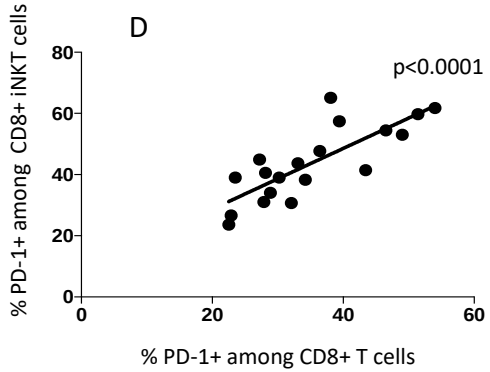


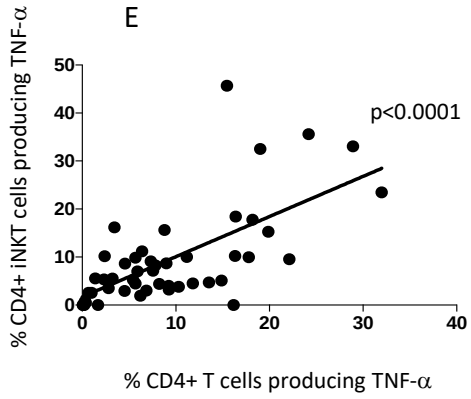
Activation and exhaustion run in parallel in cells of the innate and adaptive immunity

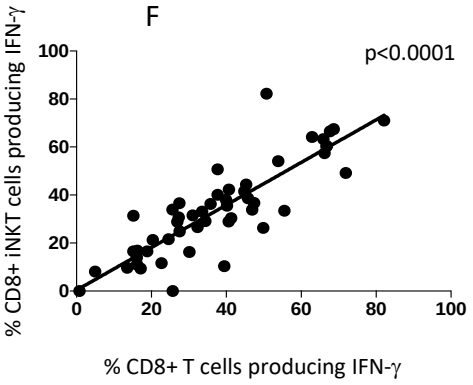












Circulating Endothelial Cells (CEC)
Circulating Endothelial Cell Precursors (EPC)

- **CECs and EPCs are extremely rare events (0.1 – 0.0001% in buffy coat)**
- **Absence of standardized protocol**
- **Lack of unique markers**
- **The needle and the damage done (by the venipuncture)...**

Circulating Endothelial Cells (EPC) and matured Endothelial Cells (CEC)

Diagnostical and prognostical potential
Microvascular disorders
Cancer load

Microvascular Research 79 (2010) 224–228

Contents lists available at ScienceDirect

Microvascular Research

journal homepage: www.elsevier.com/locate/ymvre

Circulating endothelial cells as biomarkers in clinical oncology

Patrizia Mancuso, Francesco Bertolini *

Pitfalls of FACS analysis

1.EPCs and CECs are extremely rare events (0,1 - 0,0001%)

2. Standardized protocol?

3. Lack of relevant markers

Clinical Cancer Research

AACR

Validation of a Standardized Method for Enumerating Circulating Endothelial Cells and Progenitors: Flow Cytometry and Molecular and Ultrastructural Analyses

Patrizia Mancuso, Pierluigi Antoniotti, Jessica Quarna, et al.

Clin Cancer Res 2009;15:267-273.

Nat Protoc. 2007 ; 2(4): 805–810. doi:10.1038/nprot.2007.111.

A protocol for phenotypic detection and enumeration of circulating endothelial cells and circulating progenitor cells in human blood

Dan G Duda[1,3], **Kenneth S Cohen**[2,3], **David T Scadden**[2], and **Rakesh K Jain**[1]

ELSEVIER Journal of Immunological Methods 332 (2008) 31 – 40

www.elsevier.com/locate/jim

Research paper

Quantification of circulating endothelial progenitor cells: A methodological comparison of six flow cytometric approaches

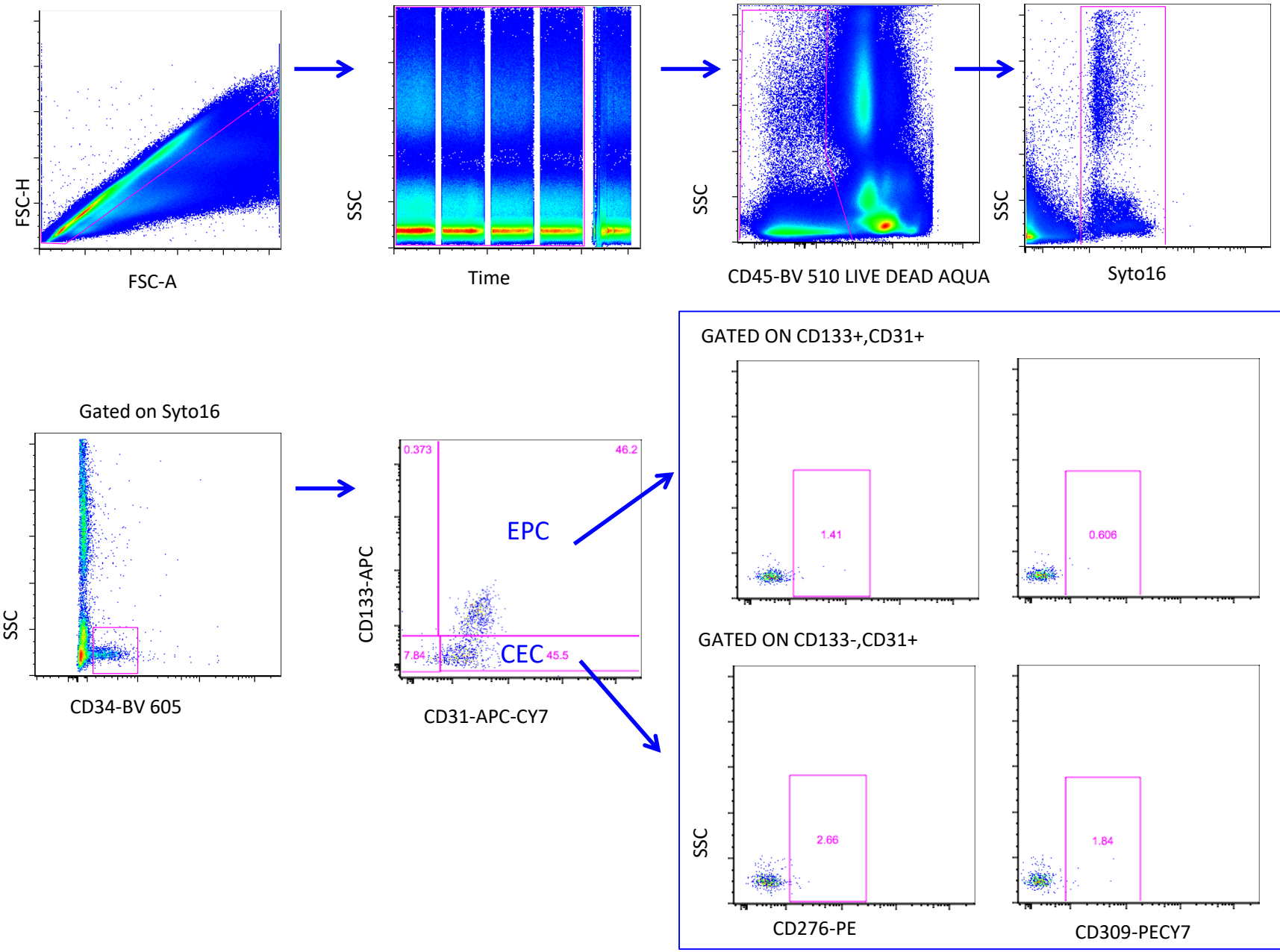
Emeline M.F. Van Craenenbroeck [a,*], Viviane M.A. Conraads [a], Dirk R. Van Bockstaele [b], Steven E. Haine [a], Katrien Vermeulen [b], Viggo F. Van Tendeloo [b], Christiaan J. Vrints [a], Vicky Y. Hoymans [a]

Identification of Endothelial Cells and Progenitor Cell Subsets in Human Peripheral Blood

Myka L. Estes,[1,2] **Julie A. Mund,**[1,2] **David A. Ingram,**[1,2,3] **and Jamie Case**[1,2]

*Curr.Protoc.Cytom.*52:9.33.1-9.33.11. 2010

Gating strategy for their identification



De Biasi *et al. BMC Medicine* (2015) 13:277
DOI 10.1186/s12916-015-0515-0

Idiopathic pulmonary fibrosis: diagnosis, management and new therapies

BMC Medicine

RESEARCH ARTICLE Open Access

Levels of circulating endothelial cells are low in idiopathic pulmonary fibrosis and are further reduced by anti-fibrotic treatments

Sara De Biasi[1†], Stefania Cerri[2†], Elena Bianchini[3], Lara Gibellini[1], Elisa Persiani[2], Gloria Montanari[2], Fabrizio Luppi[2], Cristiano Matteo Carbonelli[4], Luigi Zucchi[4], Marialuisa Bocchino[5], Alessandro Sanduzzi Zamparelli[5], Carlo Vancheri[6], Giacomo Sgalla[7], Luca Richeldi[7] and Andrea Cossarizza[1,8*]

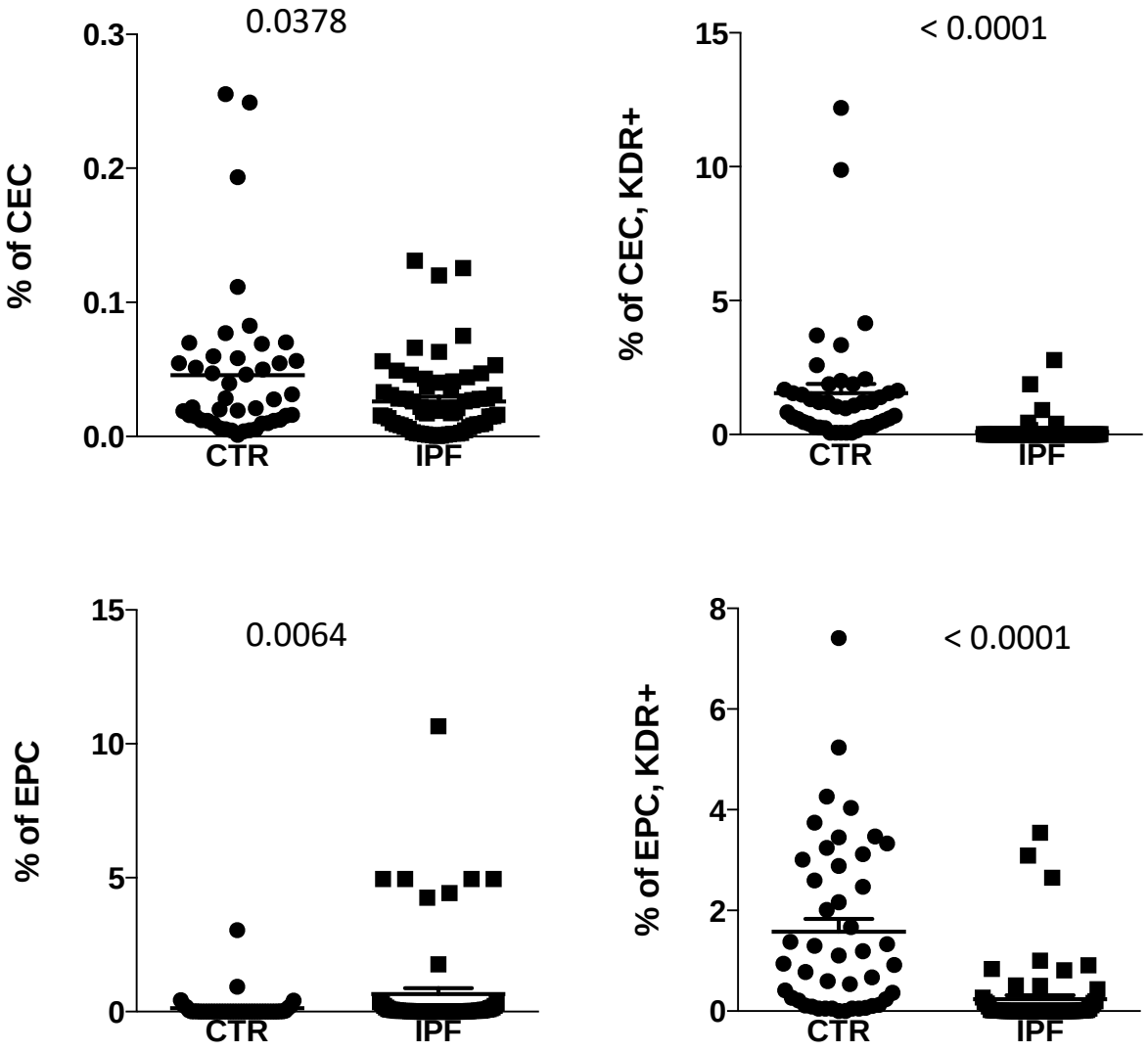
WHICH IS THE ROLE OF CIRCULATING FIBROCYTES, ENDOTHELIAL CELLS AND THEIR PRECURSORS IN THE PATHOGENESIS OF IDIOPATHIC PULMONARY FIBROSIS?

Table 1 Patients' characteristics

	Number	Percentage	Median	IQR
Gender				
Male	53			
Female	14			
Age (years)			74	68.5–77.0
Time from diagnosis (years)			3	2.0–4.5
Smoking history				
Non smoker	19			
Smoker or former smoker	41			
Forced vital capacity (% predicted)		75.0		56.75–93.0
DLCO (% predicted)		41.0		34.0–60.0
GAP stage (%)				
I		32.70		
II		53.10		
III		14.30		
Treatment				
Pirfenidone	18			
Nintedanib	13			
Untreated	26			

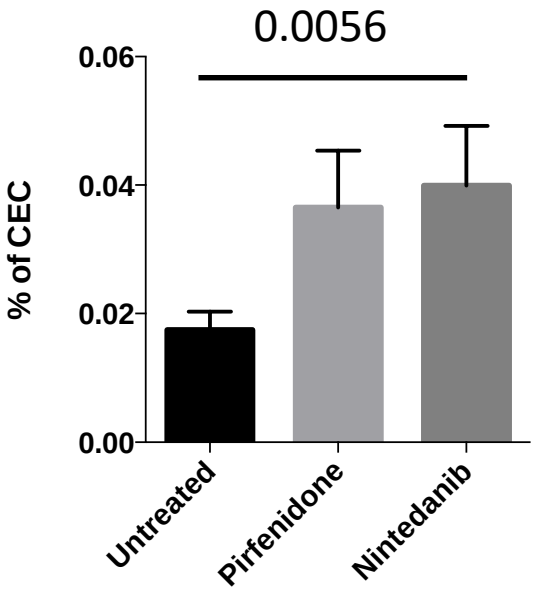
DLCO Diffusing capacity of the lungs for CO_2, *GAP* Gender, Age and Physiology Index, *IQR* interquartile range

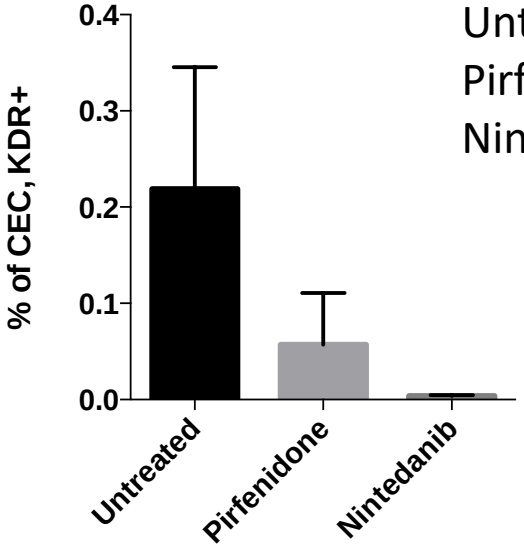
Patients with IPF (67) vs. CTR (45)



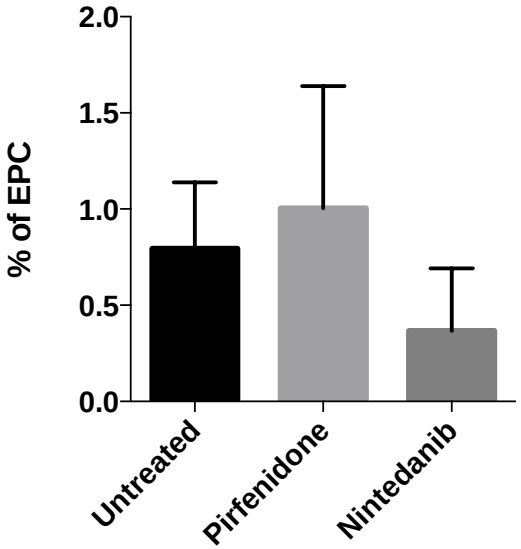
De Biasi et al.,
BMC Medicine 2015

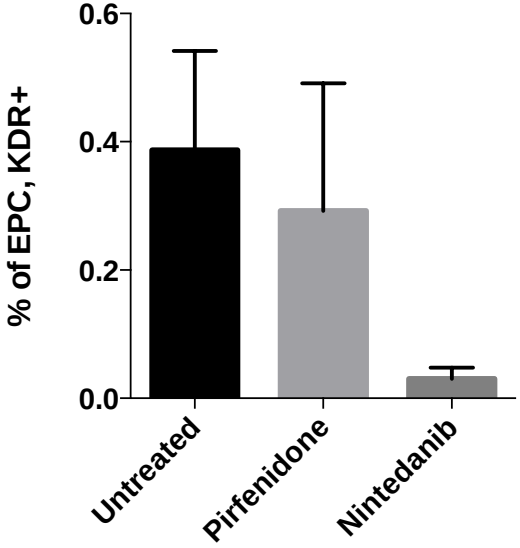
Effects of therapies in Patients with IPF





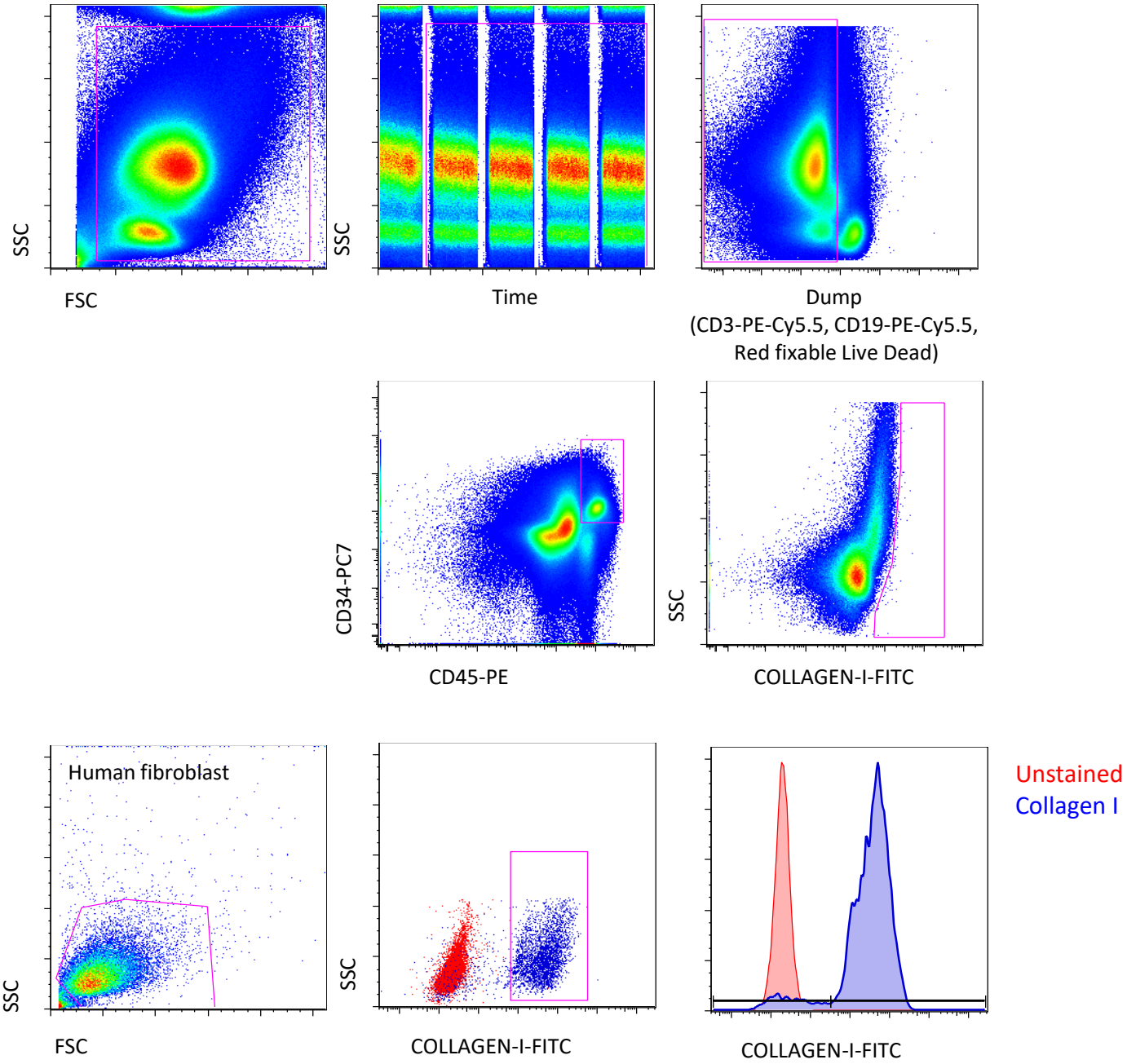
Untreated = 26
Pirfenidone = 18
Nintendanib = 13





De Biasi et al.,
BMC Medicine 2015

Circulating fibroblasts



Methods 134–135 (2018) 3–10

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

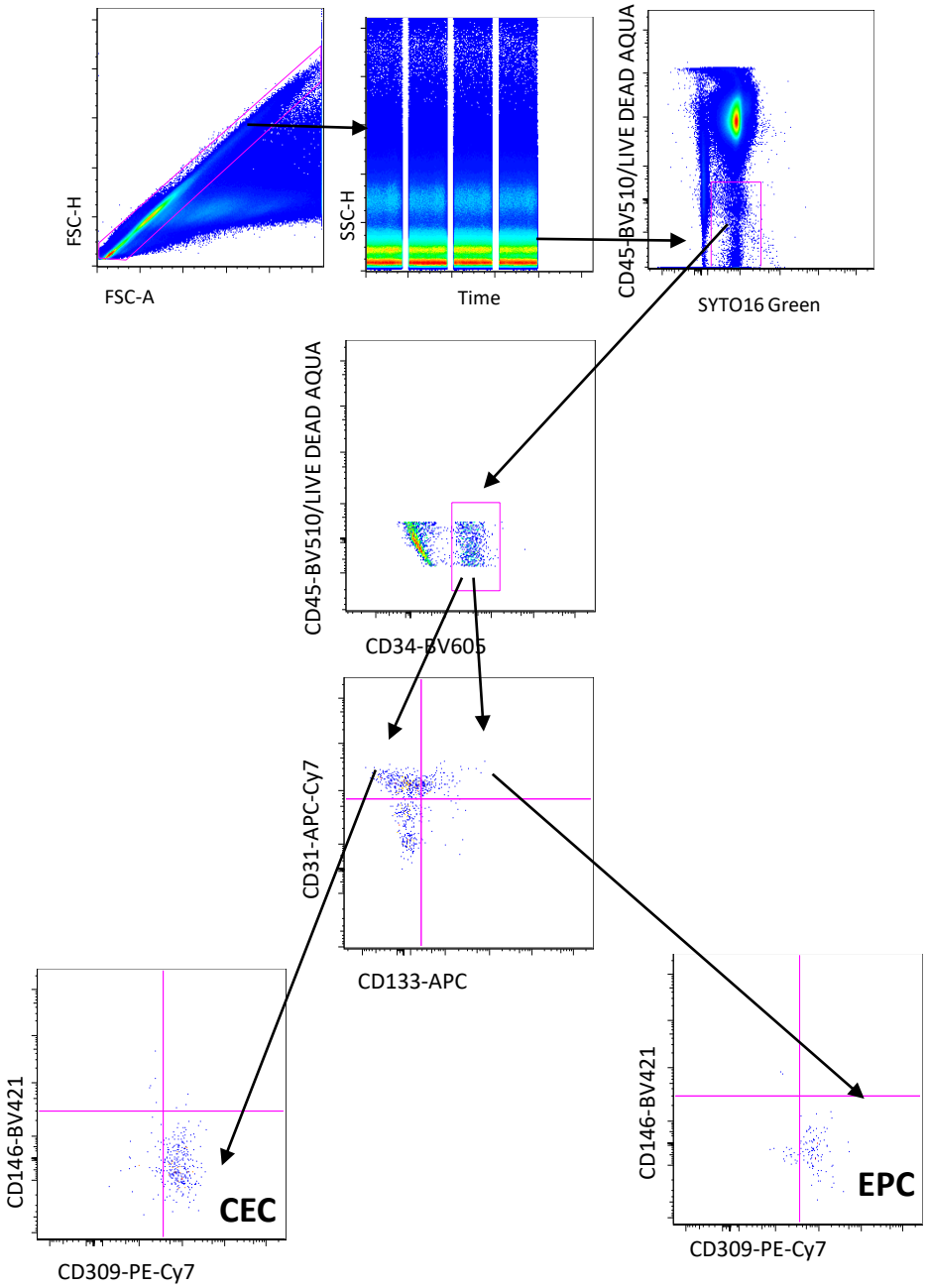
High speed flow cytometry allows the detection of circulating endothelial cells in hemangioblastoma patients

Sara De Biasi [a,1], Lara Gibellini [b,1], Alberto Feletti [c,1], Giacomo Pavesi [c,1], Elena Bianchini [d], Domenico Lo Tartaro [a], Simone Pecorini [d], Anna De Gaetano [d], Rosalberta Pullano [b], Federica Boraldi [a], Milena Nasi [d], Marcello Pinti [a,*,1], Andrea Cossarizza [b,1]

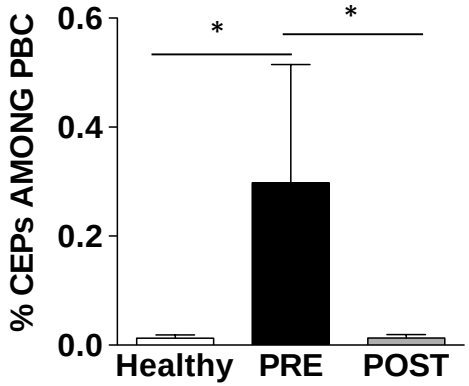
Table 1
Example to calculate the number of acquired events for a rare cell population representing 0.01%.

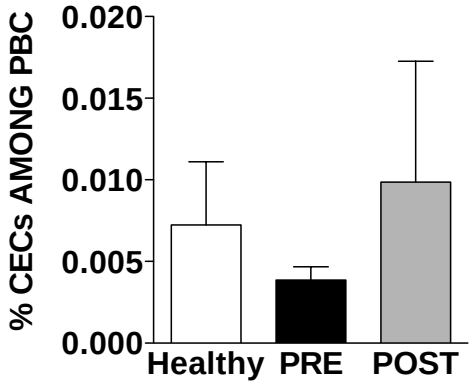
Acquired events **(N)**	100,000	1,000,000	4,010,000	10,000,000
Positive **(R)**	10	100	401	1000
Proportion (**P**)	0.0001	0.0001	0.0001	0.0001
Variance **(Var)**	10.0	100.0	400.6	999.9
Standard deviation **(SD)**	3.16	10.0	20.1	31.62
Coefficient of Variation **(CV)**	31.62	10.00	**4.99**	**3.16**

Experimental conditions with CV below 5%, which is considered satisfactory in rare events analysis by flow cytometry.

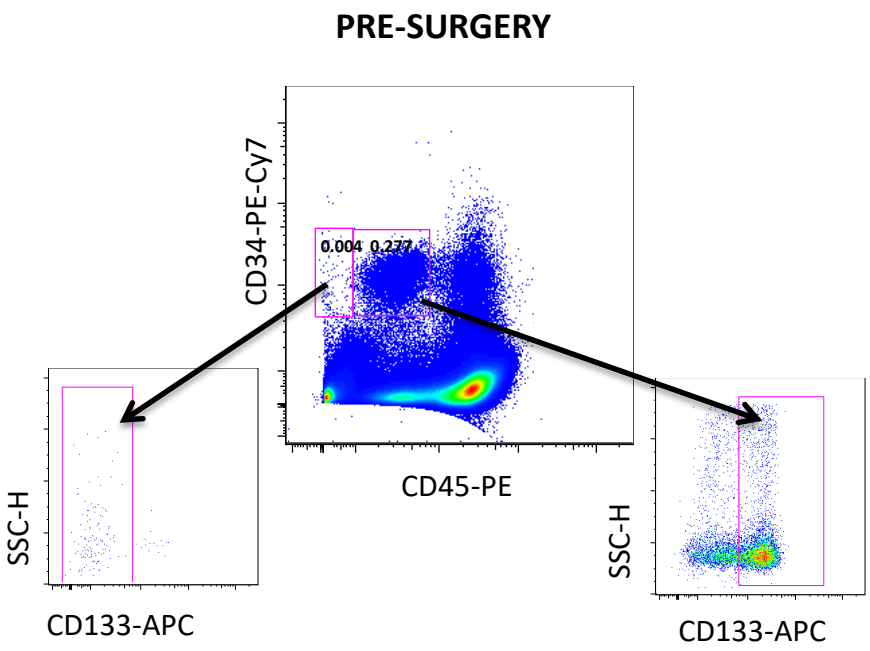


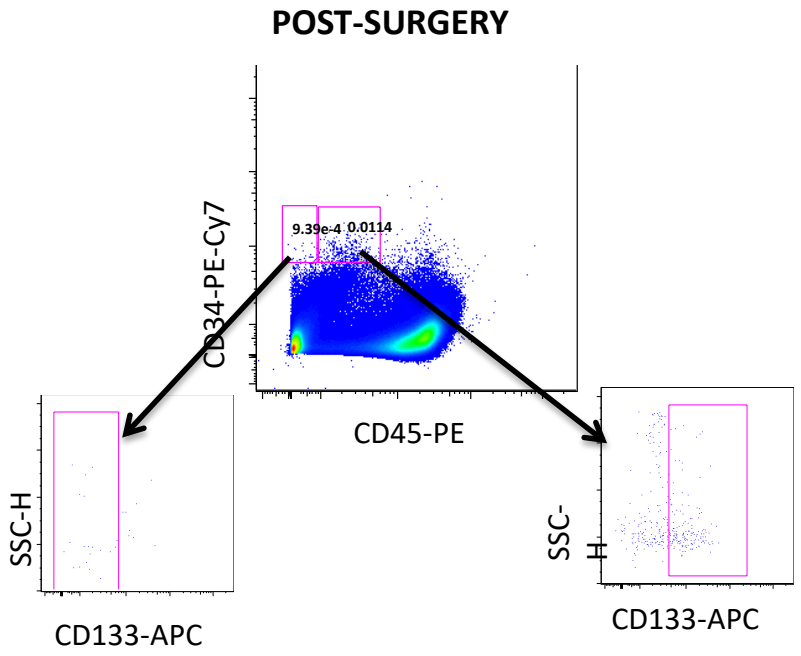
Increased percentage of circulating endothelial cells in hemangioblastoma patients



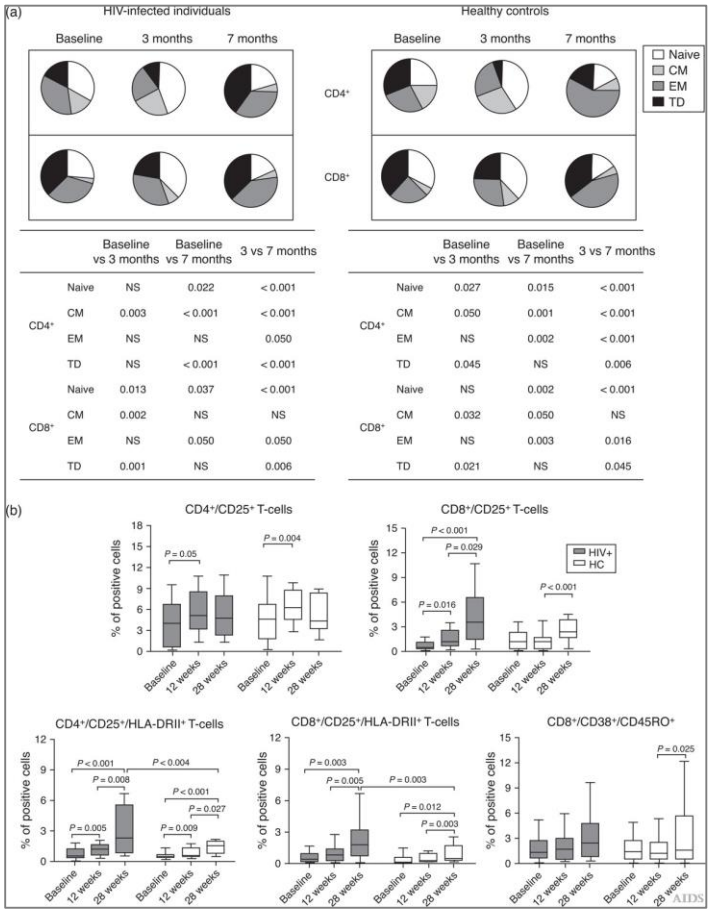


Representative example of CECs and CEPs flow cytometry quantification in blood samples before and one month after surgery.



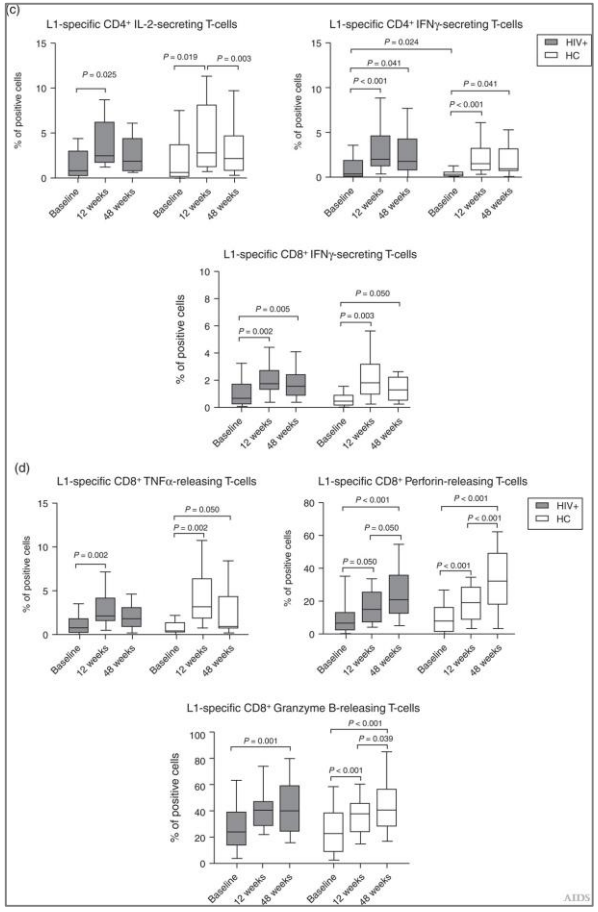


HPV T cell specific response



		Baseline vs 3 months	Baseline vs 7 months	3 vs 7 months
CD4+	Naive	NS	0.022	< 0.001
	CM	0.003	< 0.001	< 0.001
	EM	NS	NS	0.050
	TD	NS	< 0.001	< 0.001
CD8+	Naive	0.013	0.037	< 0.001
	CM	0.002	NS	NS
	EM	NS	0.050	0.050
	TD	0.001	NS	0.006

		Baseline vs 3 months	Baseline vs 7 months	3 vs 7 months
CD4+	Naive	0.027	0.015	< 0.001
	CM	0.050	0.001	< 0.001
	EM	NS	0.002	< 0.001
	TD	0.045	NS	0.006
CD8+	Naive	NS	0.002	< 0.001
	CM	0.032	0.050	NS
	EM	NS	0.003	0.016
	TD	0.021	NS	0.045



ART-treated
HIV-infected young adults

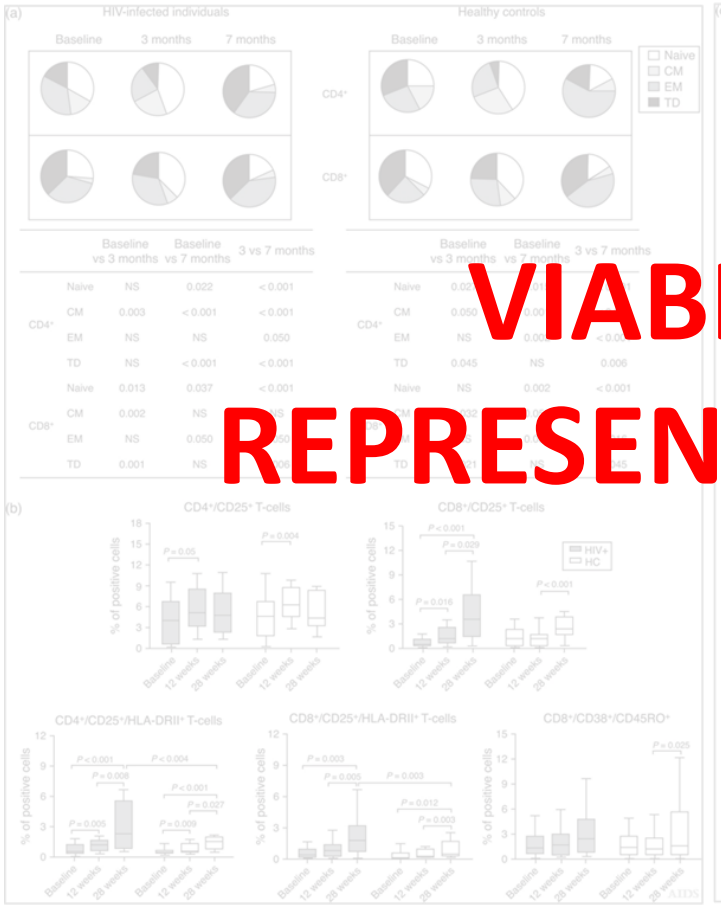
[Human papilloma virus vaccination induces strong human papilloma virus specific cell-mediated immune responses in HIV-infected adolescents and young adults]

Rainone, Veronica; Giacomet, Vania; Penagini, Francesca; Fabiano, Valentina; Calascibetta, Francesca; Mameli, Chiara; Pisanelli, Stefania; Zuccotti, Gian Vincenzo; Clerici, Mario; Trabattoni, Daria

AIDS 29(6):739-743, March 27th, 2015.

doi: 10.1097/QAD.0000000000000597

HPV T cell specific response



ART-treated HIV-infected young adults

VIABILITY MARKER?

REPRESENTATIVE DOT PLOTS?

Human papilloma virus vaccination induces strong human papilloma virus specific cell-mediated immune responses in HIV-infected adolescents and young adults

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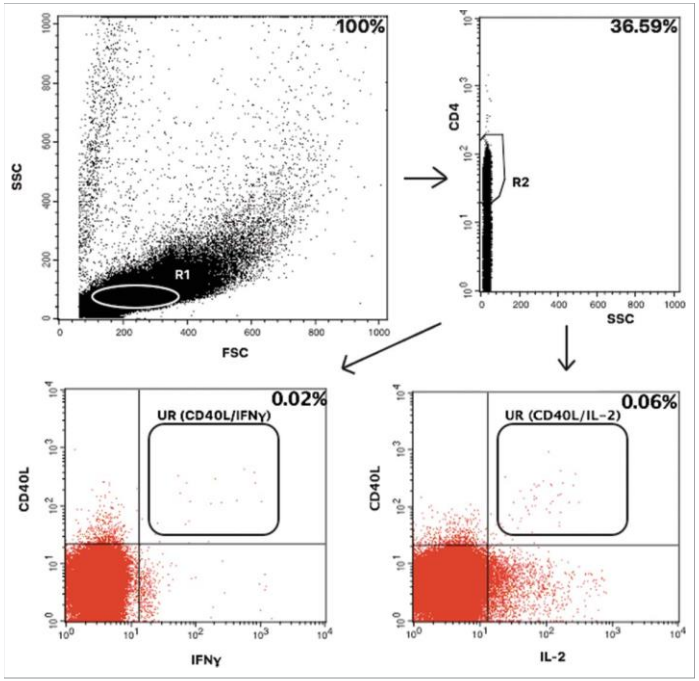
Cellular immunogenicity of human papillomavirus vaccines Cervarix and Gardasil in adults with HIV infection

Maria Zurek Munk-Madsen, Lars Toft, Tina Kube, Rolf Richter, Lars Ostergaard, Ole S. Søgaard, Martin Tolstrup & Andreas M. Kaufmann

ABSTRACT

Human papillomavirus (HPV) infection is a frequent cause of malignant and non-malignant disease, in particular among persons with HIV. HPV serotype-specific anti L1 antibodies protect against HPV infection but little is known about prophylactic HPV vaccine-induced cell-mediated immunity against HPV in high-risk individuals. We recently showed that both HPV vaccines (Gardasil® and Cervarix®) induce solid, serological immune responses in HIV-infected persons. This study aimed to characterize HPV-specific CD4 T cells in HIV-infected HPV-vaccine recipients, T cell responses being critical for B cell activation and antibody-isotype switching. Thirty HIV-infected patients on long-term antiretroviral treatment (ART) received 3 doses of either Cervarix (n = 15) or Gardasil (n = 15) vaccine at month 0, 1.5 and 6. Cryopreserved peripheral blood mononuclear cells (PBMC) from baseline, 7 and 12 months were subjected to 24-hour stimulation with specific pools of HPV L1-peptides (HPV6, 11, 16, 18, 31 and 45) and HPV E6/E7-peptide pools (HPV6/11 and HPV16/18). Fluorescence-activated cell sorting with intracellular staining (IC-FACS) against CD4, CD154, IL-2, and IFNγ was performed. Frequencies (%) of HPV-antigen specific CD4+ T cells (CD154^{+}/IL-2^{+} or CD154^{+}/ IFNγ^{+}) were determined. Both HPV-vaccines significantly and comparably enhanced cell-mediated vaccine L1 antigen-specific immunity in HIV-positive adults receiving ART therapy at month 7 and 12 after first vaccine dose. This suggests that the vaccines induce CD4 T cellular memory despite HIV-induced immune compromisation.

HUMAN VACCINES & IMMUNOTHERAPEUTICS
2018, VOL. 14, NO. 4, 909–916



100,000-160,000 CD4+ acquired

Cellular immunogenicity of human papillomavirus vaccines Cervarix and Gardasil in adults with HIV infection

Maria Zurek Munk-Madsen, Lars Toft, Tina Kube, Rolf Richter, Lars Ostergaard, Ole S. Søgaard, Martin Tolstrup & Andreas M. Kaufmann

VIABILITY MARKER?

Number of event acquired?

ABSTRACT

Human papillomavirus (HPV) infection is a frequent cause of malignant and non-malignant disease, in particular among persons with HIV. HPV serotype-specific anti L1 antibodies protect against HPV infection but little is known about prophylactic HPV vaccine induced cell-mediated immunity against HPV in high-risk individuals. We [illegible] serological immune [illegible] T cells in HIV-infected [illegible] antibody-isotype switching. Thirty HIV-infected patients on long-term antiretroviral treatment (ART) received 3 doses of either Cervarix (n D 15) or Gardasil (n D 15) vaccine at month 0, 1.5 and 6. Cryopreserved peripheral blood mononuclear cells (PBMC) from baseline, 7 and 12 months were subjected to 24-hour stimulation with specific pools of HPV L1-peptides (HPV6, 11, 16, 18, 31 and 45) and HPV E6/E7-peptide pools (HPV6/11 and HPV16/18). Fluorescence-activated cell sorting with intracellular staining (ICFACS) against CD4, CD154, IL-2, and IFNg was performed. Frequencies (%) of HPV-antigen specific CD4C T cells ($CD154^C$/$IL\text{-}2^C$ or $CD154^C$/ $IFNg^C$) were determined. Both HPV-vaccines significantly and comparably enhanced cell-mediated vaccine L1 antigen-specific immunity in HIV-positive adults receiving ART therapy at month 7 and 12 after first vaccine dose. This suggests that the vaccines induce CD4 T cellular memory despite HIV-induced immune compromisation.

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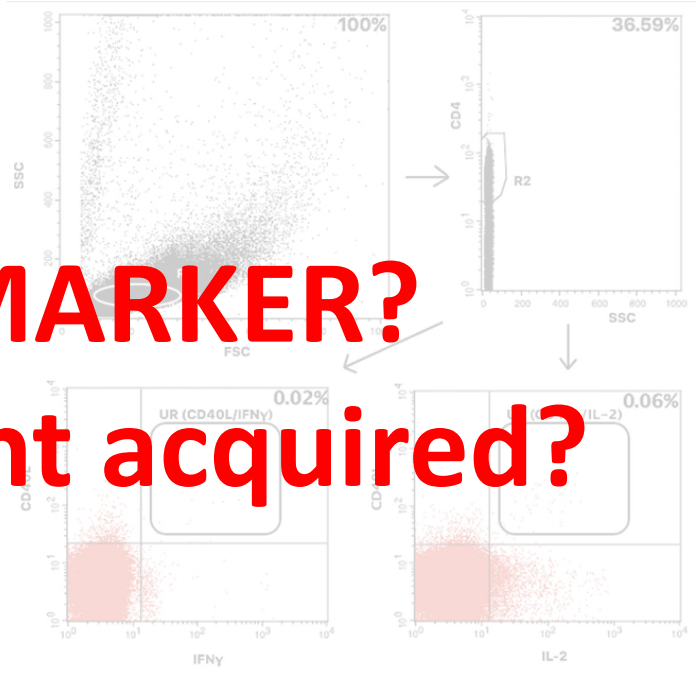


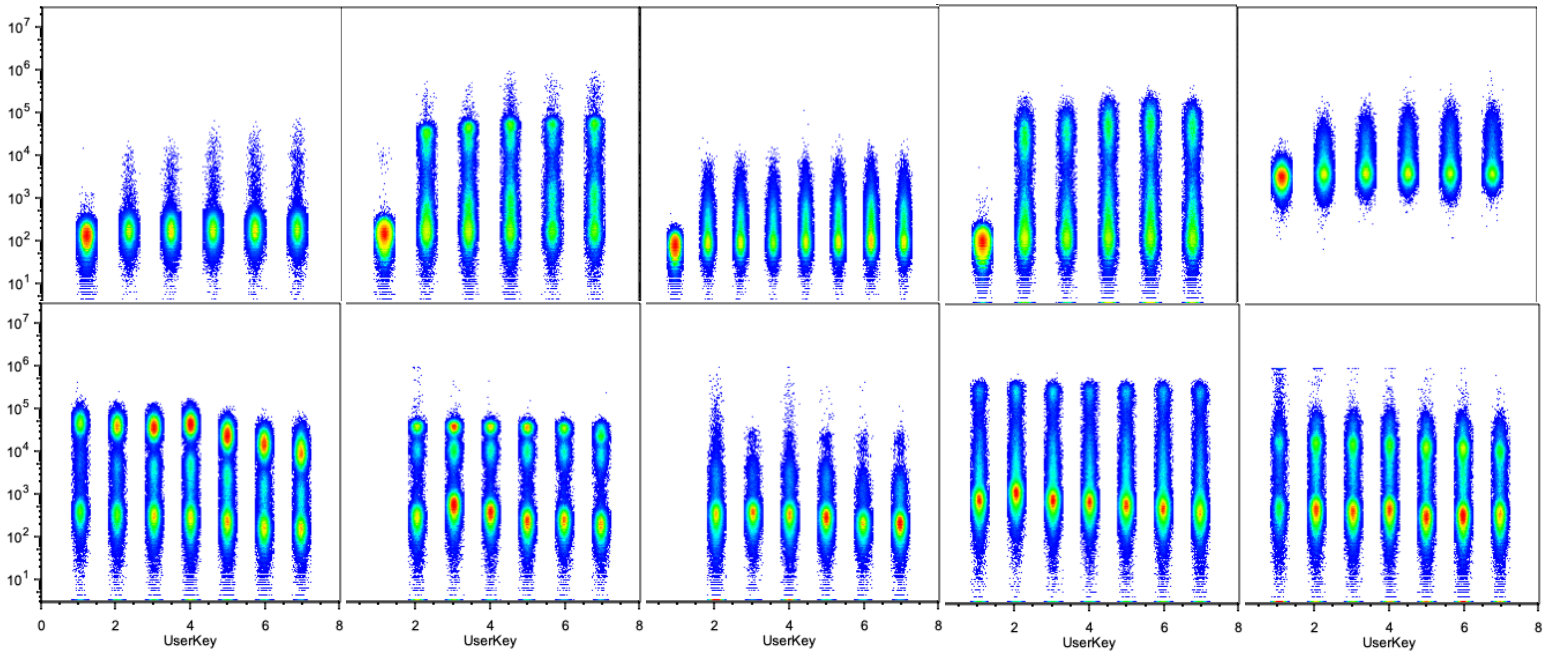
100,000-160,000 CD4+ acquired

Methods

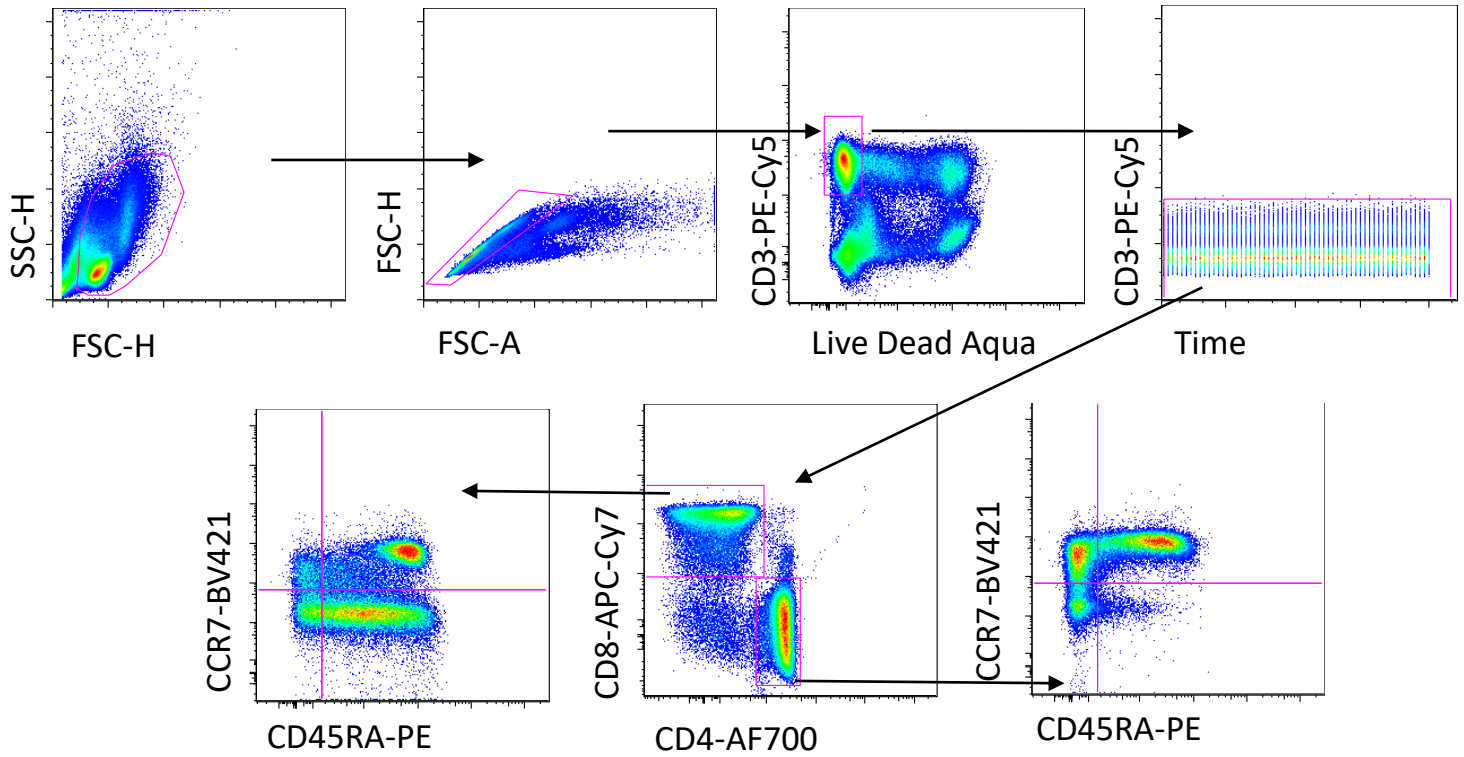
- 30 mL of blood from patients and healthy subjects before the vaccine administration and after 1 year;
- PBMCs isolation and storage in liquid nitrogen;
- Thawed PBMCs; rested for 16hrs and then stimulated for 16hrs with 1μg/ml of HPV16 L1 and HPV18 L1 (JPT) and 1μg/ml of anti-CD28; Brefeldin A added in the culture media. Stimulation with anti-CD3/CD28 (1μg/ml) used as positive control;

TARGET	LABEL	
LIVE DEAD	AQUA	RT
CCR7	BV421	37°C
CD4	AF700	RT
CD8	APC-CY7	RT
CD45RA	PE	RT
CD3	PE-CY5	INTRA
IFN-G	FITC	INTRA
TNF-A	BV605	INTRA
IL-2	APC	INTRA
IL-17	PE-CY7	INTRA

Antibody titration



Gating strategy



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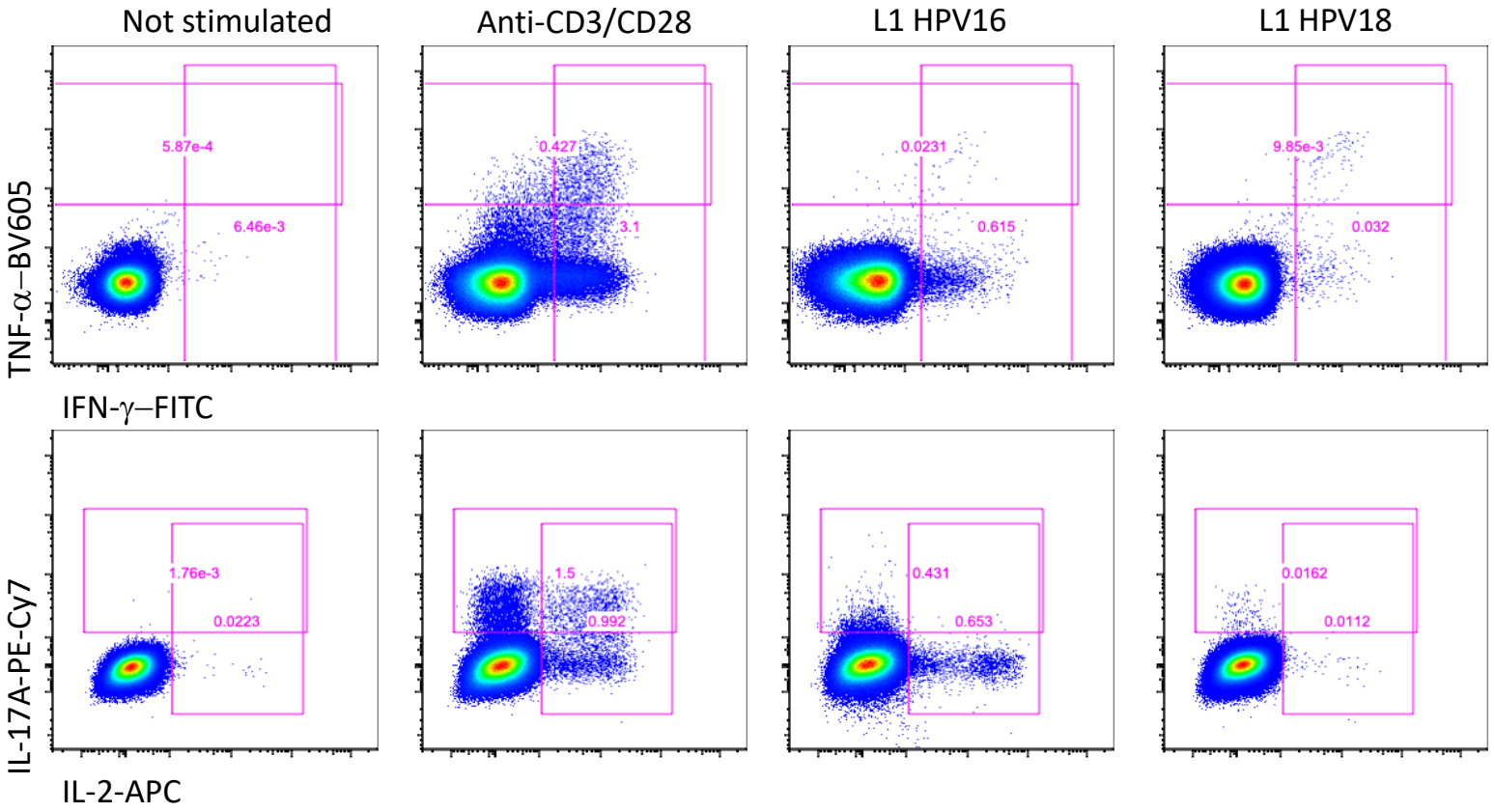
Gating strategy



Unpublished

CONCLUSIONS

- Studying rare cells requires careful attention, optimal methodologies in all phases, including collection of biological samples, adequate software and hardware.

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- I have shown you some examples (besides Ag-specific cells) that could be of interest for immunologists.

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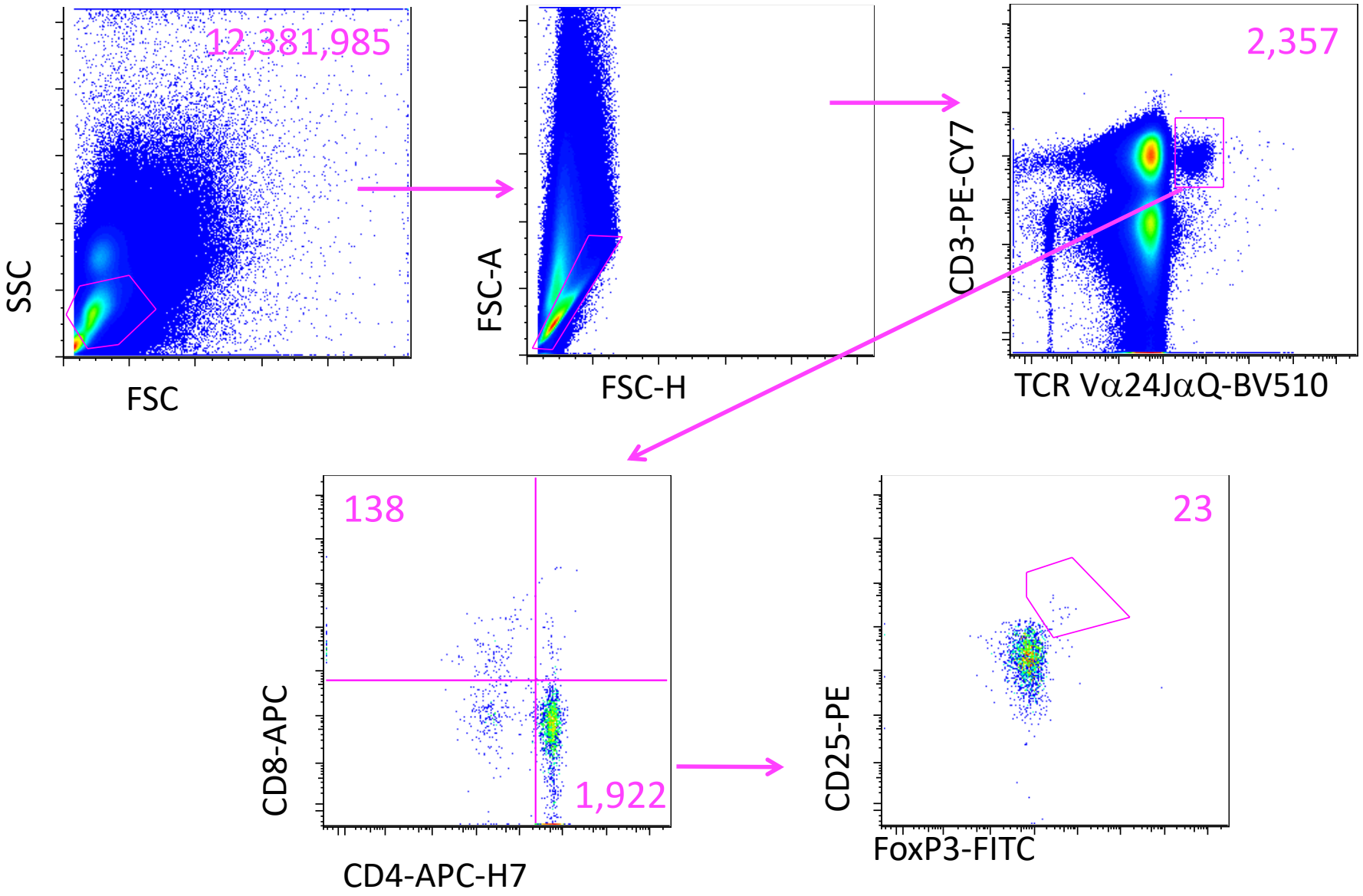
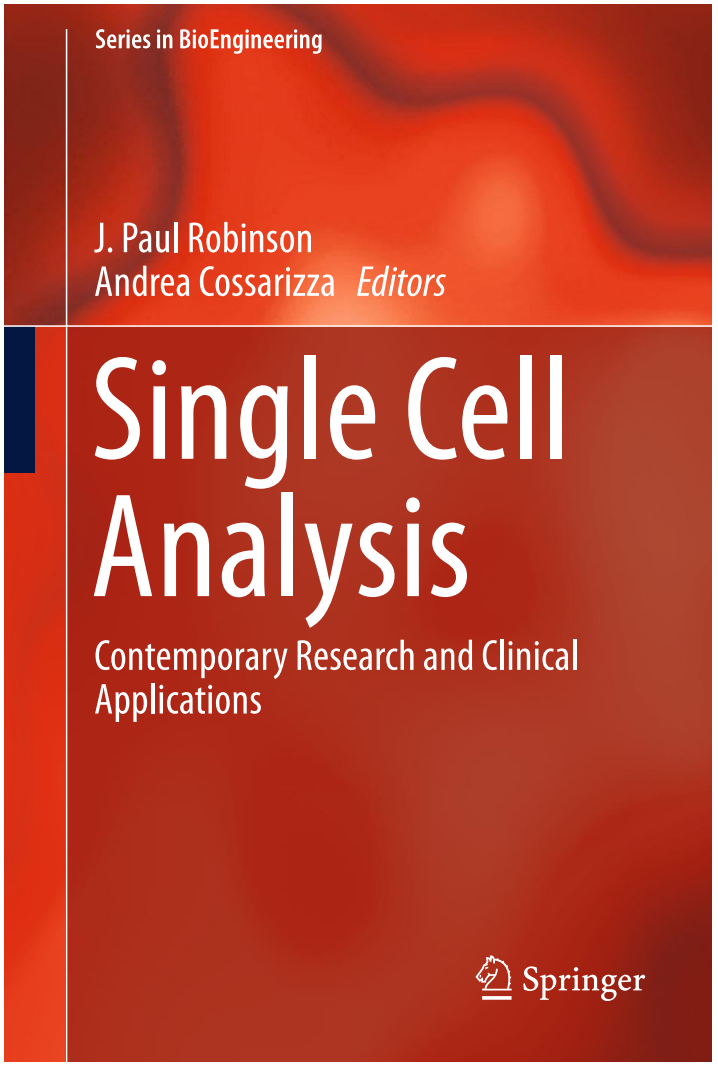
- Studying rare cells requires careful attention, optimal methodologies in all phases, including collection of biological samples, adequate software and hardware.
- I have shown you some examples (besides Ag-specific cells) that could be of interest for immunologists.
- "Next generation" instruments that work at a very high speed and sensitivity are now allowing an easy detection and analysis of such cells.

Near to the limit…



Acknowledgments

If you want to know more…



Eur. J. Immunol. 2017. 00: 1–212 DOI: 10.1002/eji.201646632

European Journal of Immunology

Guidelines for the use of flow cytometry and cell sorting in immunological studies

Cossarizza et al. J immunol 2018