Immunotherapy and Immune Monitoring

B Cell Monitoring During Anti-B Treatment in Autoimmune Diseases

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The Changing Scenario of Immunosuppressive Therapy (1)

1960's-1980's

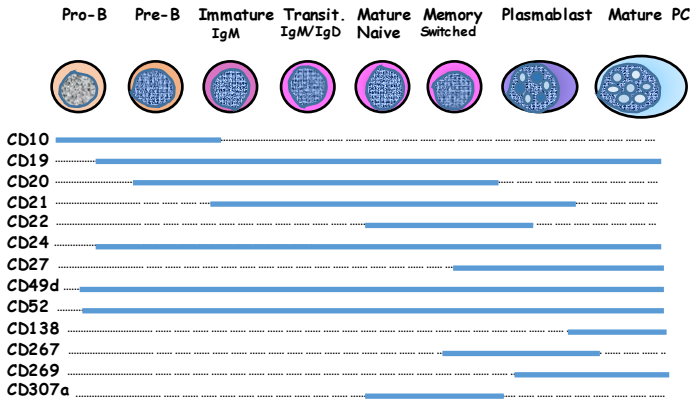
The T Cell-dependence of autoreactive and adaptive immune responses as a central concept in classical immunology
Development of therapeutic strategies to control alloimmune and autoimmune reactions, in which T Cells and their soluble products were the major target
Aims: Restoration of self-tolerance in autoimmune diseases and 'Immunological Resetting' in alloimmune reactions such as transplant rejection.
The Price to be Paid: Over-Immunosuppression, organ toxicity and increased incidence of neoplasms

The Changing Scenario of Immunosuppressive Therapy (2)

End 1990's to ~ 2005

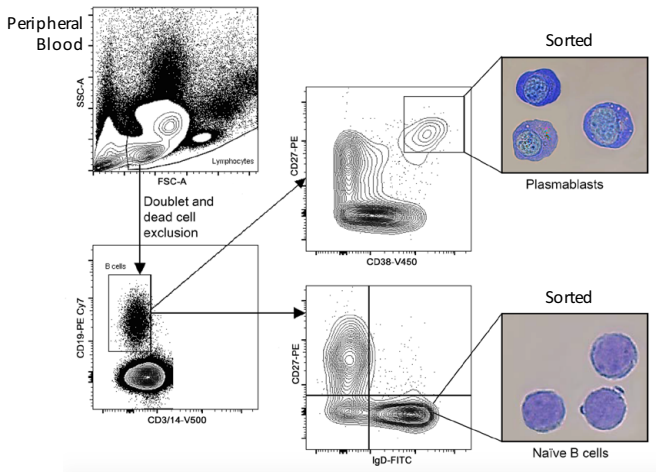
The almost incidental evidence that Rituximab, used to treat lymphoproliferative disorders, can also improve autoimmune diseases.
Development of the rationale of anti-B-Cell regimens in the treatment of autoimmune diseases and alloimmunity. Evidence that CD20 is a suitable target for MoAbs.
Aims: Depleting the reservoir of 'pathogenic antibodies', Disrupting the ectopic germinal centers in target organs, Favoring the repopulation by Ag-naïve B-Cells.
The Price to be Paid: The need of repeated cycles of anti-CD20 therapy; Some diseases seem resistant to Rituximab anyway (i.e. SLE); Not all B-Cells are nasty (i.e. B-Regs exist); Just one target may not be enough.

Phenotypic Changes Occurring Throughout B-Cell Differentiation



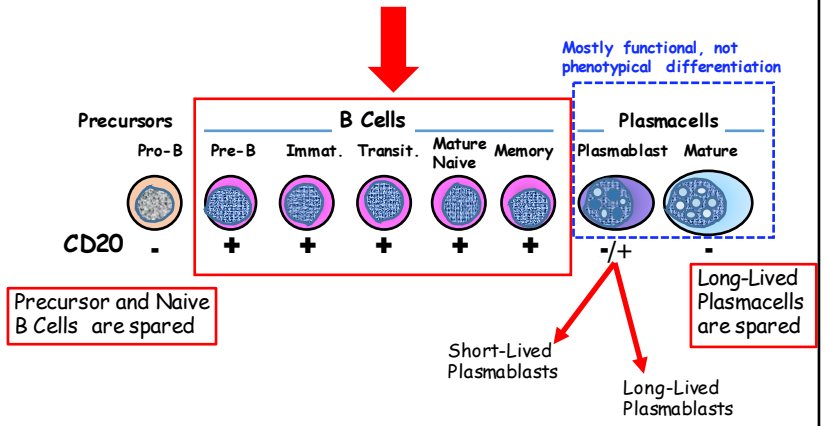
Plus a number of soluble factors: BAFF, APRIL, TACI...

Validation of Phenotipic / Functional Characterisation of Peripheral B-Cell Subsets



Stathopoulos P. JCI Insight 2017; 2(17): e94263

Rituximab, Ocrelizumab, Ofatumumab, Obinutuzumab Target CD20+ B Cells
CD20- Precursor B Cells and Plasmacells/blasts are Spared



Clinical Use of Therapeutic Monoclonals
What Must Happen – What Can Happen

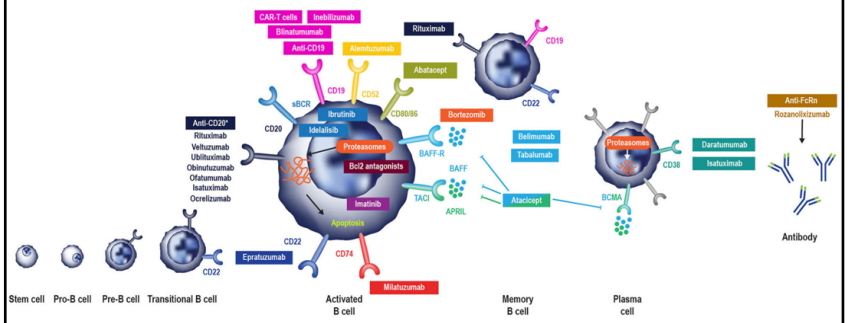
THE ASSESSMENT OF THE TARGET ANTIGEN FEATURES IS A PREREQUISITE OF ANY TREATMENT WITH MoAbs

- The target Cells **must express** the relevant antigen ***(some exceptions)***.
- The target Cells **must disappear** during the treatment.
- Antigen **modulation** must be distinguished from target cell **disappearance** and properly ruled out.
- Blood cells can be indicators also for MoAbs acting on solid organ targets.
- Consider the presence of **antigen-negative malignant cell subclones**.

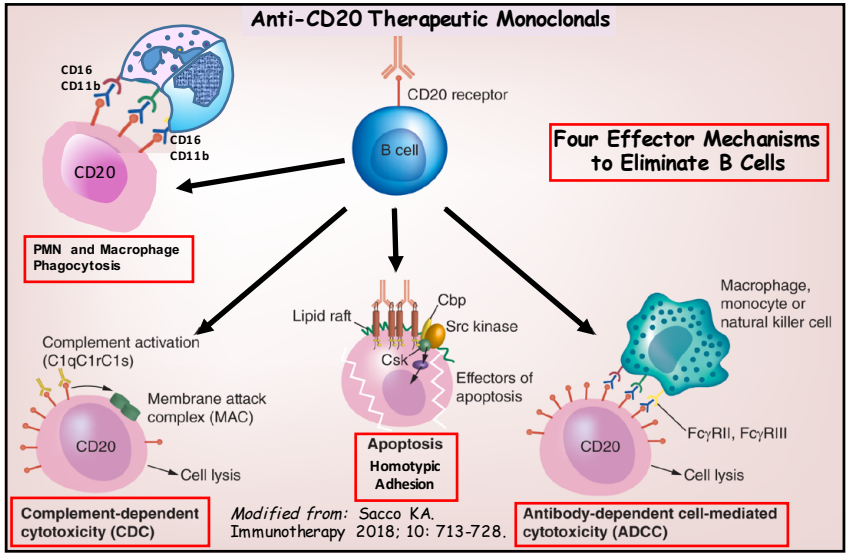
Cytometrist's Tasks:

- Make a baseline assessment of the relevant Antigen on target Cells
- Set the appropriate reagent protocol to assess MoAb efficacy
- Set protocols to distinguish cell Disappearance from Ag Modulation
- Extend the baseline phenotyping to disclose variant subclones

B-Cell Specific Chemotherapeutic Agents and Their Targets



Srivastava S. Clin Med (Lond) 2016; 16(6): 571-576.



Functional Heterogeneity of Cellular Fc-γ Receptor Family in Humans

Well known variations in cell expression patterns and different affinities with monomeric IgG

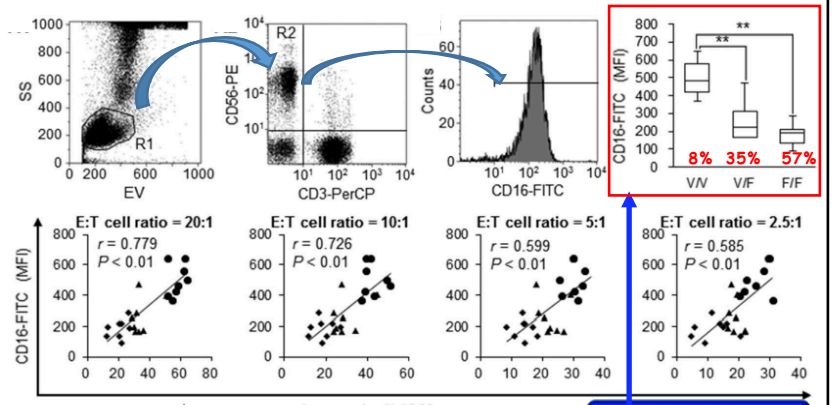
- Fc-γ R I (CD64)
- Fc-γ R IIA (CD32A)
- Fc-γ R IIB (CD32B)
- Fc-γ R IIC (CD32C)
- Fc-γ R IIIA (CD16A)
- Fc-γ R IIIB (CD16B)

→ The only INHIBITORY member of the group.
Binds monomeric IgG with LOW Affinity.
Variably expressed on Myeloid and B Cells.
Expressed on B cell leukemia/lymphoma cells.
Two Isoforms exist (CD32B1 & B2), with different intracytoplasmic domains and propensity to internalize.

Stopforth RJ. J Clin Immunol 2016; 36 (Suppl.1): S88-S94.

FcγRIIIa-V158F Genotype Influences the FcγR Density on NK Cells and ADCC Efficiency

(A possible explanation of the individual variability in clinical response to Rituximab)



- Moraru M. J Immunol 2015; 195: 1676-1684.
- Kim SH. JAMA Neurol 2015; 72: 989-995.
- Oboshi W. Human Immunology 2016; 77: 165-171.
- Sacco KA. Immunotherapy 2018; 10: 713-728.

Cytometrist's Task

B-Cell Depletion Therapies (With Anti-CD20) Continue to Expand in the Treatment of Immune-Mediated Diseases

Approved Usages

- Severe Rheumatoid Arthritis (Anti-TNF failures)
- ANCA-mediated vasculitis
- Granulomatosis with Polyangiitis (Wegener) and Microscopic Polyangiitis
- Relapsing-Remitting MS
- Primary Progressive MS
- Renal and Extra-Renal SLE
- ITP
- Idiopathic Membranous Nephropathy
- IgG4-Related Nephropathies
- Optic Neuromyelitis
- Cryoglobulinemic vasculitis
- Anti-HLA Abs Removal in Transplants

Other Applications (Literature)

- Sjogren's Syndrome
- Scleroderma
- Myositis
- Anti-Phospholipid Syndrome
- MuSK-Mediated Myasthenia Gravis
- TTP
- Autoimmune Hemolytic Anemia
- Inflammatory bowel disease
- Chronic Graft-versus-Host disease
- Pemphigus - Blistering skin diseases
- Pulmonary hypertension
- Hepatitis C Cryoglobulinemia
- IgM-associated polyneuropathy
- Uveitis
- Autoimmune paraneoplastic syndromes
- .....

Rationale for B-Cell Depletion in Autoimmune Diseases: The Good and Bad

- Although a direct ligand is unknown, CD20 is a stimulatory receptor in B Cells.
- Disruption of pathogenic (Auto/Allo)antibody production (mainly IgM).
- B lymphocytes act as Ag-presenting cells in T-Cell activation.
- B Cells generate ectopic germinal centers and produce inflammatory cytokines.
- 'Pathogenic Abs' often do not change (are they really pathogenic?).
- B Cells also include regulatory subsets (Bregs, CD5+ CD25+ IL-10+).
- Peripheral blood B Cell populations may not reflect B Cell homing in spleen and target affected organs (*B Cell location → Resistance to Rtx?*).
- Acting on a single molecule may be not enough: The biological redundancy may require the addition of more downstream actions
- Very wide individual variability.

- Cassia M. Expert Rev Clin Immunol 2017; 13: 951-962.
- Cambridge G. Journal of Autoimmunity 2014; 50: 67-76
- Baker D. EBioMedicine 2017; 16: 41-50

Rituximab Reduces Memory T Cells at 3 and 6 Months in SLE Patients

Anti-CD20 influences the immune system beyond hitting its cell target

	BASAL MD (IQR)	6M MD (IQR)	P-Value
% $CD3^+CD45R0^+$	45 (33.1–51.6)	30.1 (21.1–37.1)	<0.001
% $CD3^+CD45R0^-$	45 (33–52)	61 (50–66)	<0.001
Abs $CD3^+CD45R0^+$	282.91 (146.48–845.88)	171.76 (118.84–424.91)	0.214
Abs $CD3^+CD45R0^{-}$	249.29 (168.15–429.59)	446.4 (236.87–628.35)	0.035

Memory T Cells: CD3+ CD45R0+ ; Naïve T Cells: CD3+ CD45 R0-

- Percent and absolute Total T Cells do not change remarkably during Rituximab.
- A slight increase of T CD8+ seems correlated with the trigger of cytotoxicity.
- Some CD20 is expressed also in a fraction of T Cells.
- The abrupt disappearance of B Cells in secondary lymphoid organs induces a homeostatic rearrangement of T Cell homing.
- Absolute T-Reg levels increase at 6 months, whereas activated T Cell decrease.

Sentis A. Immunobiology 2017; 222: 620-630.

Anti-CD20 Therapies Remove Memory B-Cells in Autoimmune Diseases

DISEASE	PATHOGENIC ANTIBODIES
Rheumatoid Arthritis	Citrullinated Cyclic Peptides, RF
Systemic Lupus Erythematosus	ANA, dsDNA, Ro
ANCA-Mediated Vasculitis, Polyangiitis	pANCA, cANCA
Membranous Nephropathy	PLA2R1
IgG4-Related Nephopathies	IgG4+ Plasmacells*
Dense Deposits - C3 Nephritis	Anti-Complement Factors H / I
Optic Neuromyelitis	Aquaporin-4
Pre-Transplant Immunisation	HLA Class I and Class II
MuSK-Mediated Myasthenia Gravis	Muscle-Specific Tyrosine Kinase Abs
Myasthenia Gravis	Acetylcholine Receptor Motor End Plate
Pemphigus	Keratinocyte Desmoglein 1 and 3

- Anti-CD20 therapies can stop the production of pathogenic antibodies when the auto-Ab producing reservoirs are **within the Ag-primed CD27+ Memory B-Cells** and short-lived Plasmablasts.
- The reappearance of Memory B-Cells can be thus taken as a sign of impending relapse and can herald the re-synthesis of pathogenic auto-Ab in some cases.

- Hiepe F. Nat Rev Rheumatol 2011; 7: 170-178.
- Cambridge G. J Autoimmun 2016; 70: 22-30.

Reference Levels of Functional B-Cell Subsets in Peripheral Blood

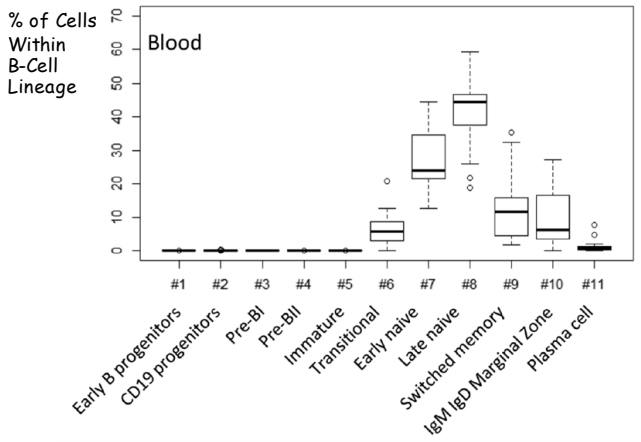
(A Still Debated Issue)

B Cell Subsets	Phenotype	% of B Cells range (mean)	Abs Value/µL range (mean)
Peripheral B Cells	**CD19+ CD20+ CD23+ CD24+**	**100**	**70 - 350 (185)**
Immature	CD10+ CD19+ CD20+ CD27- CD38+	1.5 - 10.0 (5.4)	0.1 - 12 (8)
Transitional	CD10+ CD19+ CD20+ CD24++ CD38++	2 - 4 (3)	2 - 11.2 (6)
Naive	CD10- CD19+ CD20+ CD27- CD38-	49 - 81 (64)	45 - 165 (100)
Memory B	CD10- CD19+ CD20+ CD27+ CD38- CD43-	14 - 44 (31)	16 - 96 (52)
Plasmacells (total)	CD10- CD19+ CD20- CD27++ CD38++ CD43+	0.4 - 4.4 (2.1)*	0.1 - 4.2 (3)
CD138- Plasmacells	same, CD138- slg+ cylg+	57% ± 12 of PC	-
CD138+ Plasmacells	same, CD138+ slg- cylg+	43% ± 12 of PC	-

* < 0.02% of Total Leukocytes

Modified from:
- Perez-Andres M. Cytometry Part B 2010; 78B (Suppl. 1): S47-S60.
- Caraux A. Haematologica 2010; 95: 1016 - 1020.

Eleven Maturation Stages of B Cells Can Be Detected in Peripheral Blood



Carrion C. Cytometry Part B (Clinical Cytometry) 2019; 96B: 30–38

Eleven Maturation Stages of B Cells Can Be Detected in Peripheral Blood

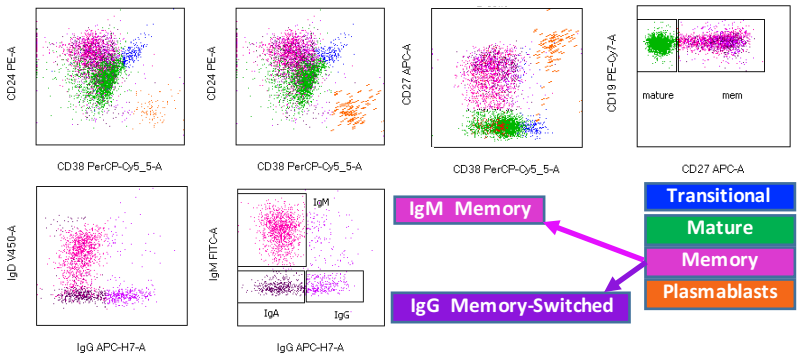
Peripheral B Cell Subsets Defined by a 9-Color Immunophenotyping Panel:
CD19 / CD38 / CD24 / CD34 / CD45 / CD10 / sIgM / sIgD / CD27

B-cell subsets	#1 Early B progenitors	#2 CD19 B progenitors	#3 Pre-BI	#4 Pre-BII	#5 Immature	#6 Transitional	#7 Early naive	#8 Late naive	#9 Switched memory	#10 IgM IgD marginal zone	#11 Plasma cells
CD19	–	+	+	+	+	+	+	+	+	+	+
CD38	hi	hi	hi	hi	hi	hi	low	low	low	low	++++
CD24	low	hi	hi	hi	hi	hi	pos	pos	hi	hi	–
CD34	+	+	–	–	–	–	–	–	–	–	–
CD45	low	int	int	int	int	int	hi	hi	hi	hi	low
CD10	hi	hi	hi	low	low	+/–	–	–	–	–	–
IgM	–	–	–	– – – +	– – + +	– + + +	+	+	–	hi	–
IgD	–	–	–	–	– – +	– + +	+	+	–	+	–
CD27	–/low	–/low	–/low	–/low	–/low	–/low	–	low	+	+	+
Median %	0	0	0	0	0	5.8	23.9	44.6	11.5	6.3	0.
± SD	*0*	*0*	*0*	*0*	*0*	*5.3*	*8.8*	*11.2*	*10.6*	*9.0*	*2.*

(Modified) from Carrion C. Cytometry Part B (Clinical Cytometry) 2019; 96B: 30–38

'Carsetti' 8-Color B Cell Panel

V450	V500	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7
IgD	CD45	IgM	CD24	CD38	CD19	CD27	IgG



Courtesy of F. Capolunghi and R. Carsetti - 2019

Biological and Pharmacokinetic Comparison Between Major Anti-CD20 MoAbs

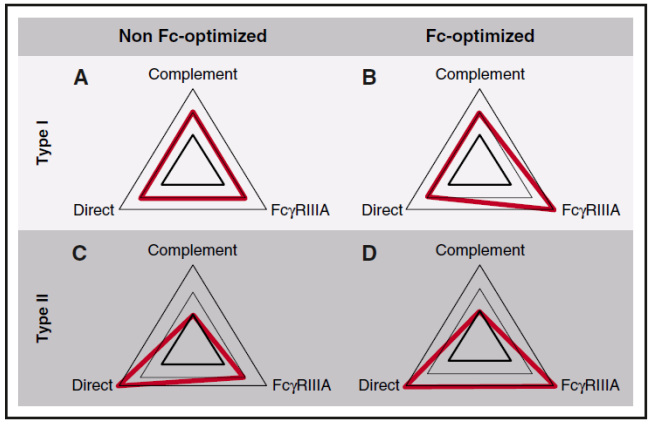
ANTI-CD20 Monoclonal	Rituximab	Ocrelizumab	Ofatumumab	Obinutuzumab
Elimination Half-Life (days)	RA: 18, Other 22-23	26	17.6	24
ADCC	+	++	++	+++
CDC	+++	+++	+++	+
APOPTOSIS	+/-	+/-	+/-	++ direct
PHAGOCYTOSIS	+	+	+	+++
INTERNALIZATION	+++	++ var	++ var	+/-

But...

- Anti-CD20 pharmacokinetics in B-Cell malignancies and Autoimmunity is not comparable.
- IgG catabolism may vary in different diseases (i.e. faster clearance in SLE than in RA).
- The immune competence of the host is a major variable.

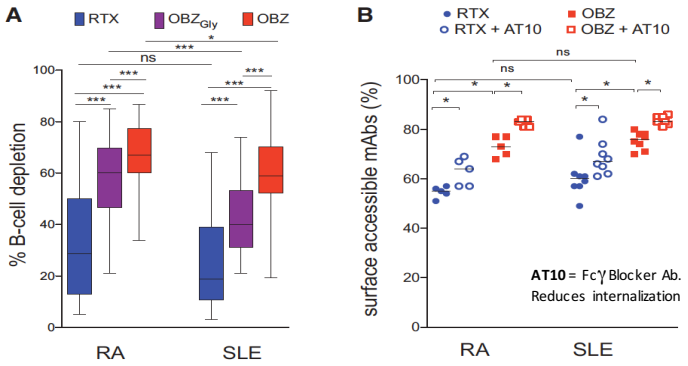
- Golay J. Blood 2013; 122: 3482-3491.
- Reddy V. Drug Discovery Today 2016; 21: 1330-1338.
- Reddy V. Rheumatology 2017; 56: 1227-1237.
- Sacco KA. Immunotherapy 2018; 10: 713-728.

Pharmacodynamic Profile of Anti-CD20 MoAbs



Cartron G & Watier H. Blood 2017; 130(5): 581-589.

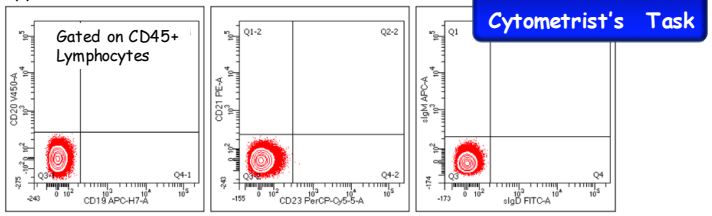
B-Cell depletion, Internalization and CDC Elicited by Anti-CD20 MoAbs in RA and SLE Patients



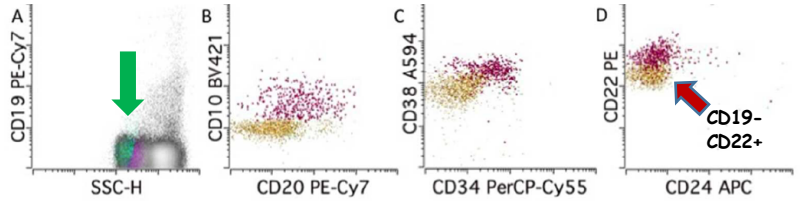
Reddy V. Rheumatology 2017; 56: 1227-1237.

Discriminating True Target Disappearance from Target Modulation

1) Total disappearance of B Cells under **Rituximab**.



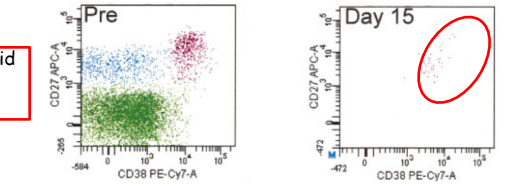
2) After **CART-19** treatment: Disappearance of CD19+ cells and expansion of CD19- CD22+.



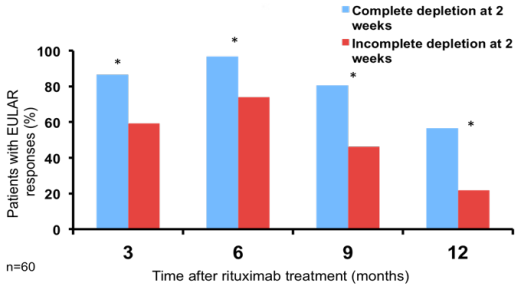
Cherian S. Cytometry Part B (Clin Cytom) 2018; 94: 112-120.

Early Experience of B-Cell Monitoring in Rituximab-Treated RA Patients

Rheumatoid Arthritis Patients

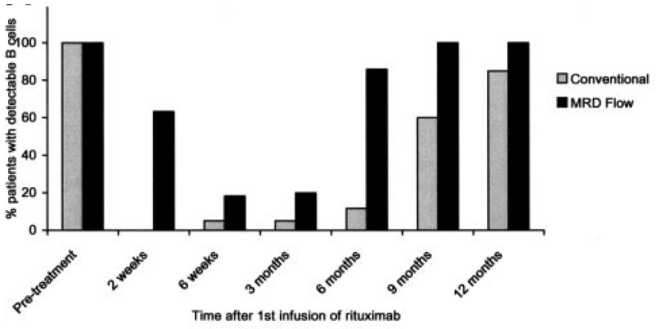


Persistence of Plasmacells at Day 15 Predicts Clinical Non-Response



Dass S, Rawstron AC et al. Arthritis Rheum 2008; 58: 2993-2999.

Persistence of Plasmacells at Day 15 Predicts Clinical Non-Response
But the Flow Cytometric Technique Makes the Difference



- High variability in early B-Cell monitoring studies due to the insensitive FCM techniques used
- Using MRD-like FCM techniques has made it possible to lower by 2-Log the sensitivity.
- Variability in B-Cell depletion effficiency and in clinical response.

Dass S, Rawstron AC et al. Arthritis Rheum 2008; 58: 2993-2999.

With the increasing usage of Rituximab in the treatment of autoimmune disorders **MEMORY B-Cells** have been identified as a reliable indicator of disease course:

Reduced Memory B-Cells →Remission. **Increased Memory B-Cells →Impending Relapse**

Rheumatoid Arthritis: Calero I. Rheum Dis Clin N Am 2010; 36: 325-343.
Dass S. Arthritis Rheum 2008; 58: 2993-2999.
Becerra E. Clin Exp Immunol 2017; 190: 372-383.
Roll P. Arthritis Rheum 2008; 58: 1566-1575.

Juvenile RA: Marasco E. Arthritis Rheumatol 2018; 70: 606-615.

SLE: Cassia M. Expert Rev Clin Immunol 2017; 13: 951-962.
Reddy V. Arthritis Research & Therapy 2013, 15(Supp 1): S2 1-16.
Reddy V. Rheumatology 2017; 56: 1227-1237.
Vital EM. Arthritis Rheum 2011; 63: 3038-3047.

Multiple Sclerosis: Baker D. EBioMedicine 2017; 16: 41-50.
Greenfield AL. Ann Neurol 2018; 83: 13-26.

Systemic Sclerosis: Gernert M. Arthritis Research & Therapy 2019; 21: 106.

1

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Neuromyelitis Optica Sp: Kim SH. JAMA Neurol 2015; 72(9): 989-995.
Lebrun C. Neurol Ther 2018; 7: 373-383.

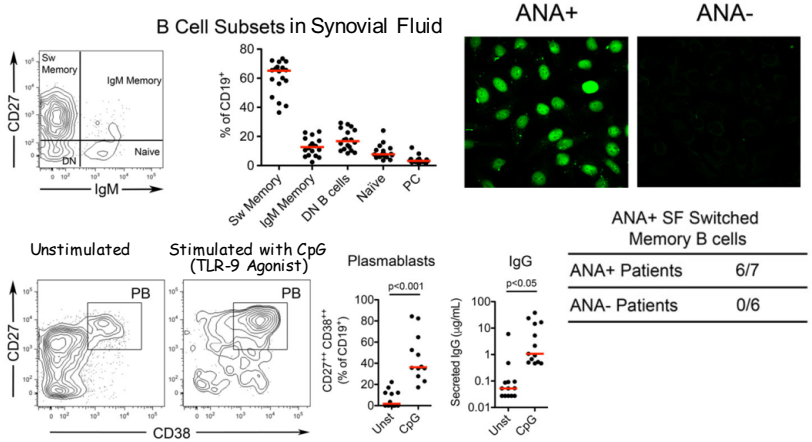
Sjögren's Syndrome: Mariette X. N Engl J Med 2018; 378: 931-939.

Glomerulonephritis: Leibler C. J Clin Med 2018; 7: 430; doi:10.3390/jcm7110430
Colucci M. J Am Soc Nephrol 2016; 27: 1811-1822.
Iijima K. Clin Exp Nephrol 2017; 21: 193-202.

Allogeneic Transplantation: Beausang JF. J Transl Med 2017; 15: 9; doi: 10.1186/s12967-017-1118-7
Ikemiyagi M. Ther Apher Dial 2017; 21(2): 139-149.

2

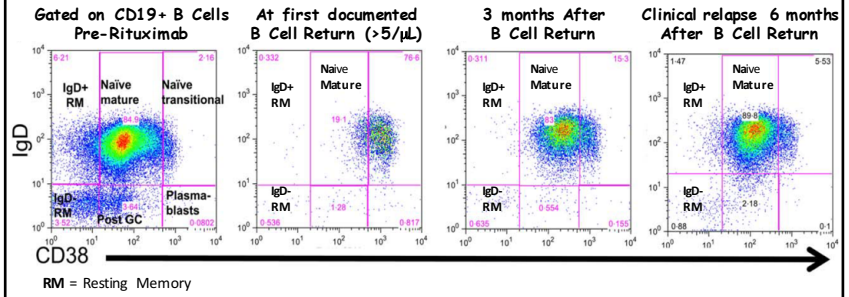
Switched Memory B Cells in Inflamed Joints in JRA Patients Produce ANAs



ANA+ SF Switched Memory B cells	
ANA+ Patients	6/7
ANA- Patients	0/6

Marasco E. Arthritis and Rheumatology 2018; 70(4): 606-615.

Kinetics of B-Cell Subsets in RA Patients Treated with Rituximab
Different Technical Approaches Can Be Used

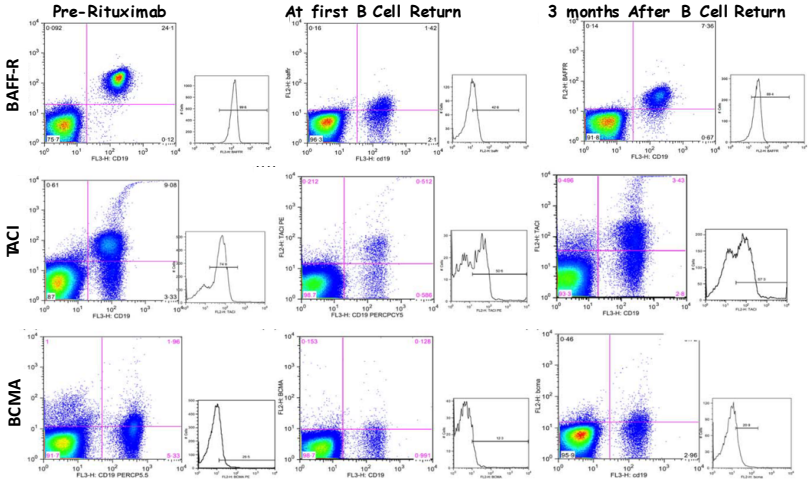


RM = Resting Memory

- 'Adequate' B Cell depletion defined as CD19+ Cells < 5/μL.
- 'B Cell Return' (repopulation) defined as CD19+ Cells again > 5/μL.
- Study performed on Ficoll-Hypaque mononuclears.

Becerra E. Clin Exp Immunol 2017; 190: 372-383.

RA Relapse Seems Independent of B-Cell BAFF/BAFF-R System



Becerra E. Clin Exp Immunol 2017; 190: 372-383.

The NEW ENGLAND JOURNAL of MEDICINE
N Engl J Med 2008; 359: 242-251
ORIGINAL ARTICLE

Rituximab and Intravenous Immune Globulin for Desensitization during Renal Transplantation

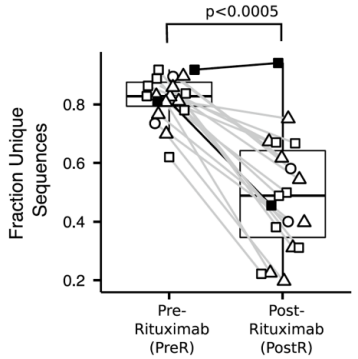
Ashley A. Vo, Pharm.D., Marina Lukovsky, Pharm.D., Mieko Toyoda, Ph.D.,

- Rituximab plus i.v. Immunoglobulins or Plasma Exchange and Immuno-suppressors allows the clearance of Anti-HLA alloantibodies, that impact negatively on Transplant compatibility and outcome.
- This treatment is indicated in highly immunized recipients and in ABO-incompatible transplantations.
- Rituximab interferes however with pre-transplant FCM Cross-Matching, and requires the treatment of donor cells with Pronase or Blocking Abs.

- Szewczyk K. Human Immunology 2016; 77: 449-455.
- Alheim M. Human Immunology 2018; 79: 132-135.

Cytometrist's Task

B Cell Repertoires During HLA Desensitization with Rituximab in Renal Tx Candidates



- Memory B cells are the repository of anti-HLA Alloantibodies, and can quickly differentiate into Ab-secreting PC upon antigen re-exposure.
- Pre-Transplant Rituximab induces the reduction of anti-HLA panel-reactive antibodies (cPRA).
- Unique sequences are long reduced by Rituximab in alloreactive B cells.
- Conversely, no changes are induced by IVIG administration.

Beausang JF. J Transl Med 2017; 15:9; doi: 10.1186/s12967-017-1118-7

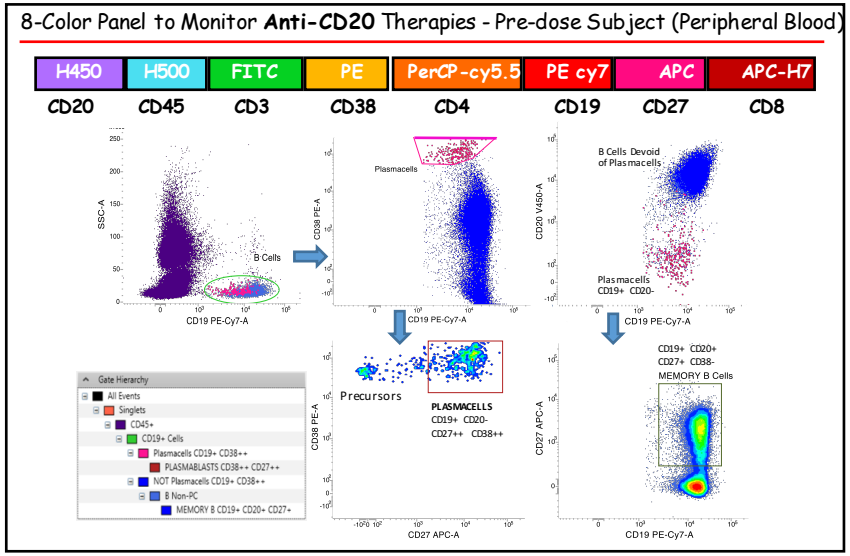
Research article Related Commentary, page 1803

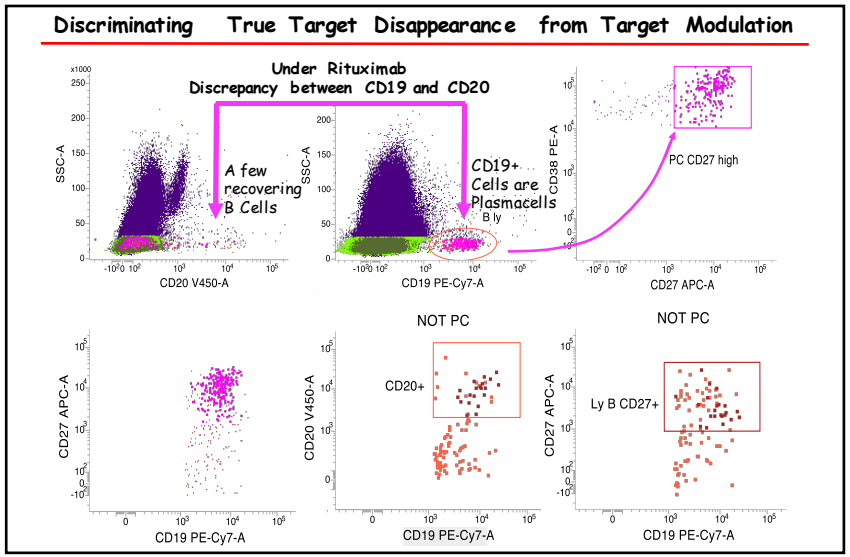
Identification of a B cell signature associated with renal transplant tolerance in humans

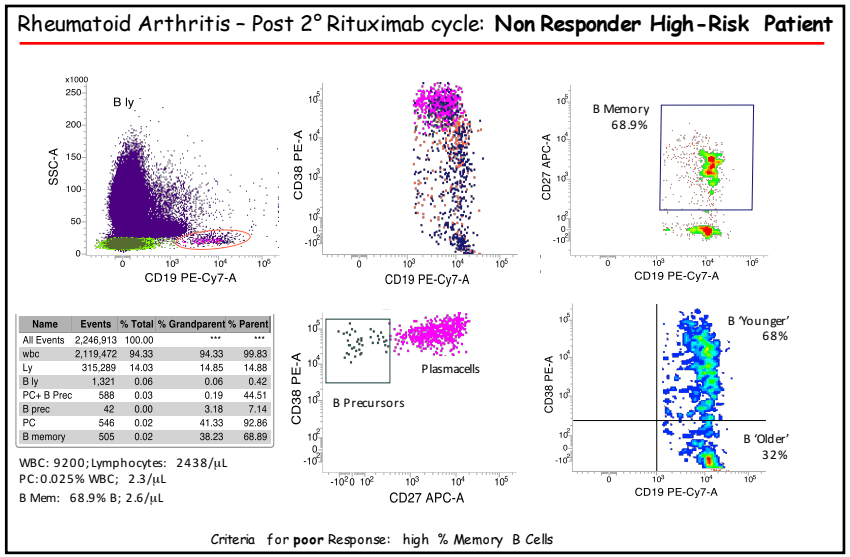
Kenneth A. Newell,[1] Adam Asare,[2,3] Allan D. Kirk,[1] Trang D. Gisler,[2,3] Kasia Bourcier,[2,3]

- A set of 3 B cell differentiation genes distinguishes tolerant from non-tolerant subjects.
- Spontaneous operational tolerance to kidney allograft is associated with an **increased number of circulating naïve and transitional B cells**, suggesting a critical role for these B cells subsets in the regulation of alloimmune response.
- Naïve B cells are poor Ag-presenting cells and induce tolerance by orienting T cells into Tregs.

Newell KA. J Clin Invest 2010; 120: 1836-1847.

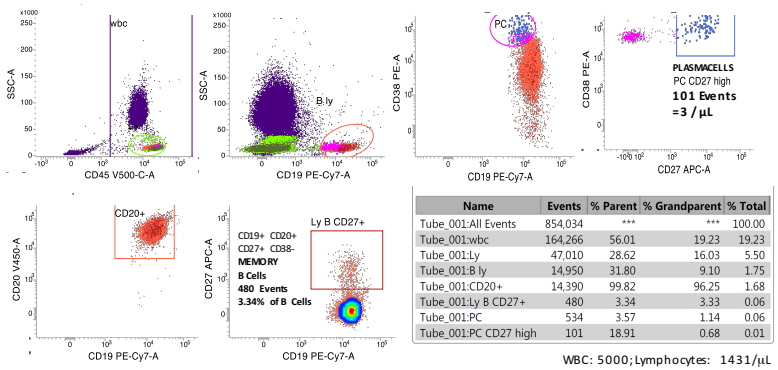






Name	Events	% Total	% Grandparent	% Parent
All Events	2,246,913	100.00	***	***
wbc	2,119,472	94.33	94.33	99.83
Ly	315,289	14.03	14.85	14.88
B ly	1,321	0.06	0.06	0.42
PC+ B Prec	588	0.03	0.19	44.51
B prec	42	0.00	3.18	7.14
PC	546	0.02	41.33	92.86
B memory	505	0.02	38.23	68.89

Rheumatoid Arthritis – Post Rituximab: **Good Responder Patient**

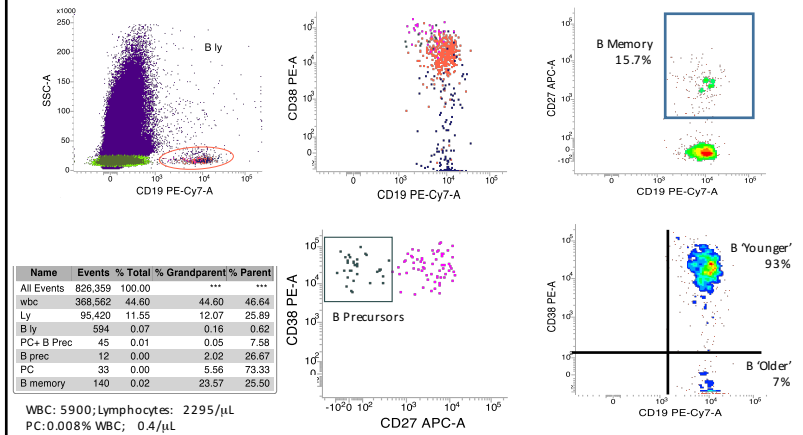


Name	Events	% Parent	% Grandparent	% Total
Tube_001:All Events	854,034	***	***	100.00
Tube_001:wbc	164,266	56.01	19.23	19.23
Tube_001:Ly	47,010	28.62	16.03	5.50
Tube_001:B ly	14,950	31.80	9.10	1.75
Tube_001:CD20+	14,390	99.82	96.25	1.68
Tube_001:Ly B CD27+	480	3.34	3.33	0.06
Tube_001:PC	534	3.57	1.14	0.06
Tube_001:PC CD27 high	101	18.91	0.68	0.01

WBC: 5000; Lymphocytes: 1431/µL
PC: 0.06% WBC; 3/µL
B Mem: 3.34% B; 15/µL

Criteria for Good Response: Low % and abs Memory B Cells - Plasmacells < 3 /µL

Rheumatoid Arthritis – Post 1st Rituximab cycle: **Good Responder Low-Risk Patient**

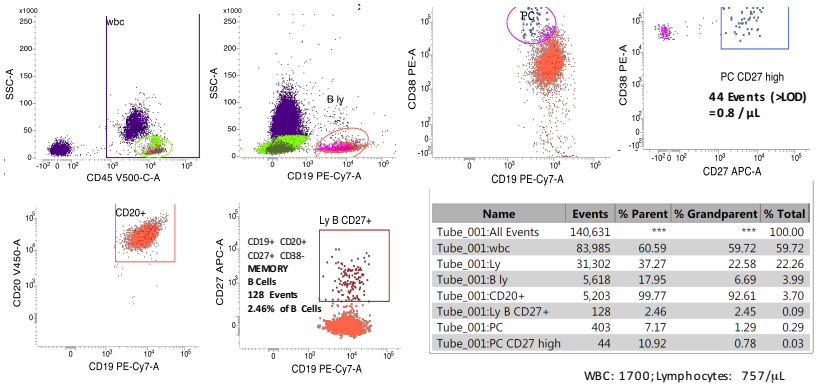


Name	Events	% Total	% Grandparent	% Parent
All Events	826,359	100.00	***	***
wbc	368,562	44.60	44.60	46.64
Ly	95,420	11.55	12.07	25.89
B ly	594	0.07	0.16	0.62
PC+ B Prec	45	0.01	0.05	7.58
B prec	12	0.00	2.02	26.67
PC	33	0.00	5.56	73.33
B memory	140	0.02	23.57	25.50

WBC: 5900; Lymphocytes: 2295/µL
PC: 0.008% WBC; 0.4/µL
B Mem: 23.5% B; 3.3/µL

Criteria for **good** Response: Low % and abs CD27+ Memory B Cells - Very low % and abs Plasmacells;

Systemic Sclerosis – Post Rituximab: **Good Responder Patient**

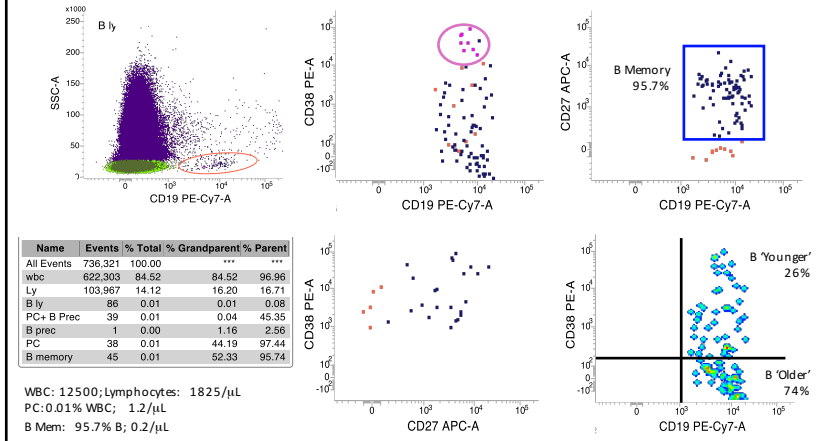


Name	Events	% Parent	% Grandparent	% Total
Tube_001:All Events	140,631	***	***	100.00
Tube_001:wbc	83,985	60.59	59.72	59.72
Tube_001:Ly	31,302	37.27	22.58	22.26
Tube_001:B ly	5,618	17.95	6.69	3.99
Tube_001:CD20+	5,203	99.77	92.61	3.70
Tube_001:Ly B CD27+	128	2.46	2.45	0.09
Tube_001:PC	403	7.17	1.29	0.29
Tube_001:PC CD27 high	44	10.92	0.78	0.03

WBC: 1700; Lymphocytes: 757/µL
PC: 0.05% WBC; 0.8/µL
B Mem: 2.4% B; 3.3/µL

Criteria for **good** Response: Very low % and abs Memory B Cells - Very low abs Plasmacells

Rheumatoid Arthritis – Post 1st Rituximab cycle: **Poor Responder High-Risk Patient**



Name	Events	% Total	% Grandparent	% Parent
All Events	736,321	100.00	***	***
wbc	622,303	84.52	84.52	96.96
Ly	103,967	14.12	16.20	16.71
B ly	86	0.01	0.01	0.08
PC+ B Prec	39	0.01	0.04	45.35
B prec	1	0.00	1.16	2.56
PC	38	0.01	44.19	97.44
B memory	45	0.01	52.33	95.74

WBC: 12500; Lymphocytes: 1825/µL
PC: 0.01% WBC; 1.2/µL
B Mem: 95.7% B; 0.2/µL

Criteria for **poor** Response: Very high % CD27+ Memory B Cells

Rheumatoid Arthritis – B-Cell Subsets in 29 Patients Treated with Rituximab

Mean ± SD	N Ctrl	Non Responder	Responder	p
	n. 15	n. 13	n. 16	
CD19+CD20+%	12 (± 6)	1.4 (± 2.0)	5.1 (± 9)	ns
Absolute B cells /µL	216 (± 147)	36 (± 53)	103 (± 198)	ns
Memory B cells %	36 (± 13)	39.5 (± 30)	13.8 (± 15)	0.003
Absolute memory B cells /µL	58 (± 70)	4 (± 5.6)	9.1 (± 19.2)	ns
B cell Naive %	64 (± 13)	62 (± 30.6)	86.2 (± 15)	0.003
Absolute naive B cells /µL	144 (± 41)	31 (± 49)	95.1 (± 181)	ns
Plasma cells%	0.02 (± 0.02)	0.1 (± 0.3)	0.02 (± 0.01)	ns
Memory B cells CD38 neg %	53.5 (± 10)	51 (± 16.3)	42.5 (± 19)	0.079
		Mean DAS28: **4.8**	Mean DAS28: **2.69**	

- Higher percent levels of Memory B-Cells are reproducibly found in non-responder RA patients treated with Rituximab.
- Measurements were made after 3-8 months from the last RTX dose.

Gatti A. Presented at ESCCA 2019 - Bergen

The Degree of B-Cell Depletion in Autoimmune Diseases: Is it Important?

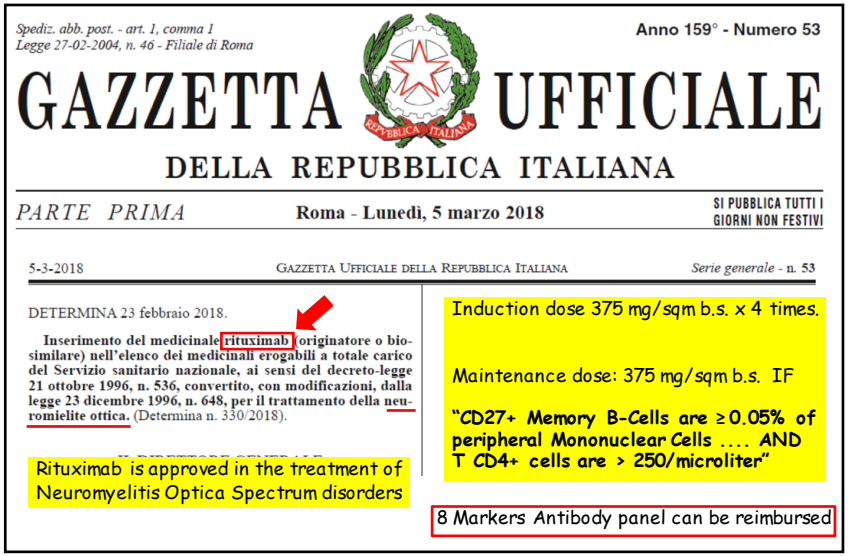
- Incomplete B-Cell depletion following Anti-CD20 MoAbs is associated with poor clinical response in both SLE and RA.
- Disease-specific mechanisms of Anti-CD20 MoAb accelerated clearance have been demonstrated in SLE.
- Enhanced and more prolonged B-Cell depletion can be achieved using additional doses of Anti-CD20 MoAbs, with a better clinical response.
- Therefore, achieving a complete and durable B-Cell depletion will improve clinical response in both SLE and RA.

➔ The problem is: **HOW ACCURATELY CAN ONE DEFINE 'PROFOUND' B-CELL DEPLETION ?**

Reddy V. Rheumatology 2017; 56: 1227-1237.

Conflicting Biomarkers and Endpoints for Anti-CD20 Treatments in Autoimmunity

DISEASE	TARGET VALUES	REFERENCES
Rheumatoid Arthritis SLE	<0.01 B Cells/µL (MRD) After the 1st dose. Plasmacells <2.85%.	Dass S, 2008 Calero I, 2010 Stradner MH, 2016 Md Yusof MY, 2017
Membranous Nephropathy	'Disappearance' of CD27++ Plasmablasts	Pozdzik A, 2016
IgG4-Related Diseases	'Disappearance' of IgG4+ Plasmablasts	Perugino CA, 2017
Optic Neuromyelitis	CD27+ memory B Cells <0.05% of mononuclears	Collongues N, 2016 Cohen M, 2017



Spediz. abb. post. - art. 1, comma 1
Legge 27-02-2004, n. 46 - Filiale di Roma

Anno 159° - Numero 53

GAZZETTA UFFICIALE DELLA REPUBBLICA ITALIANA

PARTE PRIMA — Roma - Lunedì, 5 marzo 2018 — SI PUBBLICA TUTTI I GIORNI NON FESTIVI

5-3-2018 GAZZETTA UFFICIALE DELLA REPUBBLICA ITALIANA Serie generale - n. 53

DETERMINA 23 febbraio 2018.

Inserimento del medicinale rituximab (originatore o biosimilare) nell'elenco dei medicinali erogabili a totale carico del Servizio sanitario nazionale, ai sensi del decreto-legge 21 ottobre 1996, n. 536, convertito, con modificazioni, dalla legge 23 dicembre 1996, n. 648, per il trattamento della neuromielite ottica. (Determina n. 330/2018).

Induction dose 375 mg/sqm b.s. x 4 times.

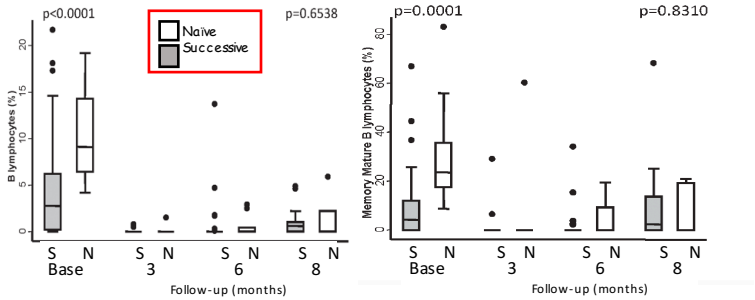
Maintenance dose: 375 mg/sqm b.s. IF

"CD27+ Memory B-Cells are ≥ 0.05% of peripheral Mononuclear Cells .... AND T CD4+ cells are > 250/microliter"

Rituximab is approved in the treatment of Neuromyelitis Optica Spectrum disorders

8 Markers Antibody panel can be reimbursed

Same Behavior of Total and Memory B-Cell Kinetics in Rituximab-Naive and Rituximab-Treated RA Patients, Despite Different Baseline Levels



A threshold of **5 B-Cells/µL** is proposed to proceed with B-Cell subsetting during the follow-up *(but just 100,000 cells acquired)*

- Becerra E. Clinical and Experimental Immunology 2017; 190: 372 - 383.
- Lòpez J. Clin Exp Rheumatol 2019; 37(4): 615-622.

A Unifying B Cell Counting Procedure to Monitor Anti-CD20 Therapies

- Staining mixture: **CD19 / CD20 / CD27 / CD38 / CD45** (+ sIgM & sIgG)
- Optional counterstaining with CD3 / CD4 / CD8 (+ CD16 / CD56)

- Acquire cells as for MRD studies (i.e. ≥ **1,000,000** total clean events to ensure at least a **0.01%** LLOQ). ***Subsetting requires more events !***

- Have a Full Blood Count with **Absolute Lymphocytes/µL**.
- Record Percent and Absolute B Cell (**CD19+**) values.

- Record **CD19+ CD20- CD27++ CD38++ Plasmacell** Absolute count and as Percentage of **total leukocytes**.
- Record **CD19+ CD20+ CD27+ CD38- Memory B Cell** Absolute count and as Percentage of **total B Cells**.

- Timepoints for tests: **Baseline, 3 Mo, Long-Term...**
(Depending on drug schedule and also **very** patient-dependent)

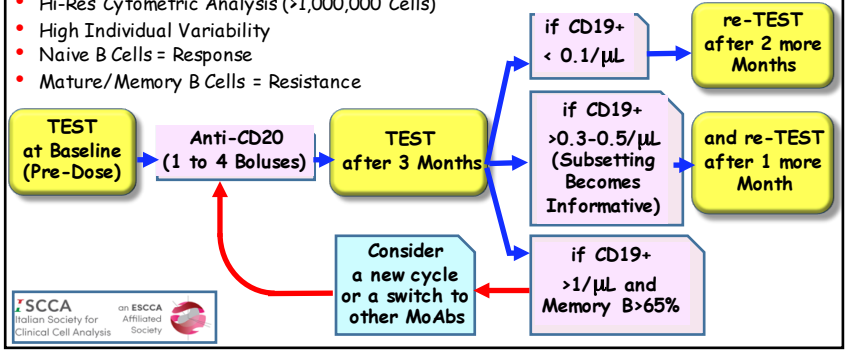
ISCCA Italian Society for Clinical Cell Analysis — an ESCCA Affiliated Society

The ISCCA Protocol for High-Resolution Monitoring of B-Cell Subsets in Peripheral Blood

in Patients Treated with Anti-CD20 Therapeutic Monoclonals

V450	V500c	FITC	PE	PerCP-cy5.5	PE cy7	APC	APC-H7
CD20	CD45	CD4 +anti-IgM	CD38	CD3	CD19	CD27	CD8 +anti-IgG

- *+ Full Blood Count for Absolute Lymphocyte Levels*
- Hi-Res Cytometric Analysis (>1,000,000 Cells)
- High Individual Variability
- Naive B Cells = Response
- Mature/Memory B Cells = Resistance



ISCCA Italian Society for Clinical Cell Analysis — an ESCCA Affiliated Society

Sistema Socio Sanitario
Regione Lombardia
ASST Ovest Milanese
U.O.C. Centro ImmunoTrasfusionale (Direttore Dott. B. Brando)

Sistema Qualità Certificato UNI EN ISO 9001-2008

Sig. [redacted]
Sesso M Data Nascita: [redacted] Id.:19008045 Cod.Fiscale: [redacted]
Provenienza: 71111 MAG - 71111 - Reumatologia

Data di Stampa: 26/02/2019 Ore: 10:05 Pag. 1 / 7 Richiesta: **361032** del: 21/02/2019 **Routine**

Esame	*Esito*	*U.M.*	*Valori Riferimento*
Centro Immuno Trasfusionale			
Sg--LINFOCITI CD3 (T)			
CD3 (T) %	**64**	%	60 - 86
CD3 (T) #	**0.83**	10^3/µL	0.70 - 2.40
Sg--LINFOCITI CD 19 (B)			
CD19 (B) %	**0.9** <	%	5.00 - 22.00
CD19 (B) #	**0.01** <	10^3/µL	0.1000 - 0.5000
Comparto Plasmacellulare			
Plasmacellule%	**0.01**	%	0.000 - 0.020
Plasmacellule microL	**0.97**	/µL	0.000 - 3.000
Comparto B ly CD19+CD20+			
B precursori	**0.01**	%	<0.01
LY B memory%	**2** <	%	10.0 - 35.0
LY B memory microL	**0.23** <	/µL	10.00 - 175.00

Lessons Learned From B-Cell Subset Monitoring in RA and Autoimmune Disorders

- The degree of B-Cell depletion is the factor that is best correlated to the clinical response in Anti-CD20 treated patients.
- '**Adequate B-Cell depletion**' must be defined with High-Resolution Flow Cytometry (i.e. < 5 B Cells /μL may be still **TOO MUCH**).
- The longer the duration of the B-Cell depletion, the better the clinical response.
- Relapse can occur despite low circulating memory B cells, suggesting that long-lived memory B-Cells (and possibly CD20- plasma cells) can act as the possible repository of disease memory, with relapse after B-Cell return.
- B-Cell repopulation recapitulates the physiological ontogeny, with an increased release of transitional and naïve B cells.
- No differences in B-Cell repopulation in therapy-naive patients and after multiple cycles.
- Serum level of soluble BAFF increase after Rituximab treatment, but the role of BAFF/BAFF-R, TACI and BCMA analysis in this setting is still unclear.

Reconstitution of B-Cells After Rituximab Depletion Mimics the Ontogeny of B-Cell Lineag

- RA Patients at the first Rituximab course (**naïve patients**) vs Patients at **successive Rituximab cycles**.
- Studied at Baseline, 3rd, 6th and 8th month of each cycle.
- Naïve patients have higher Total and Memory B-Cell % at baseline.
- In naïve patients the Transitional B-Cell% at baseline correlates with disease activity.
- However, recovery of B-Cell subsets after Rituximab is similar.
- Multiple Rituximab cycles do not induce cumulative effects on B-Cell subpopulations and recovery rates.

Lòpez J. Clin Exp Rheumatol 2018; Dec 19. [e-published ahead of print]

Immune Deficiency Following Therapies with Anti-CD20 MoAbs

- In some clinical protocols (both for Lymphoproliferative disorders and Autoimmune diseases) patients are treated with very prolonged cycles of Anti-CD20 MoAbs → **Concern for Over-Immunosuppression**.
- **Disease**-specific and **Treatment**-specific factors cooperate in generating post-MoAb hypogammaglobulinemia.
- Post-MoAb B-Cell reconstitution by naive cells causes a delay in the recovery of endogenous Ig production.
- Low Pre-therapy Ig, Lymphocyte and B-Cell levels are strong risk factors for the development of Post-MoAb immune deficiency.
- Recurrent infections associated with low levels of Ig and reduced B-Cell effector subsets should be taken as indicators to start i.v. Ig replacement therapy.

Sacco KA. Immunotherapy 2018; 10: 713-728.

Immune Deficiency Following Therapies with Anti-CD20 MoAbs (2)

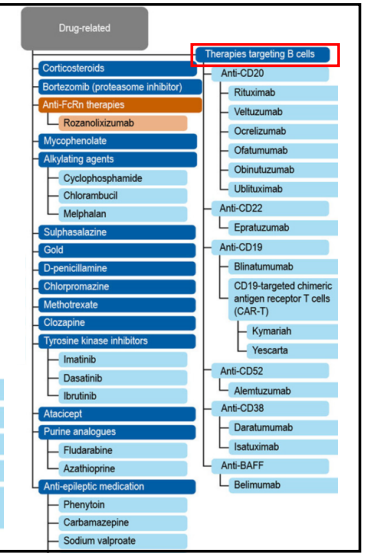
- 243 Patients treated with average 6 g Rituximab for systemic vasculitis and other multisystem autoimmune diseases were followed for 42 months.
- Moderate to severe hypogammaglobulinemia (IgG < 500 mg dL) occurred in 63 cases (26%).
- In a half of them IgG concentration improved spontaneously at treatment discontinuation.
- IgG replacement therapy was initiated in 12 (4.2%), who had reduced Ig levels before treatment.

Roberts DM. Journal of Autoimmunity 2015; 57: 60-65.

Common Causes of Secondary Antibody Deficiencies

Secondary antibody deficiencies are 30 times more common than primary deficiencies

Patel SY.
Front Immunol 2019; 10:33.
doi: 10.3389/fimmu.2019.00033

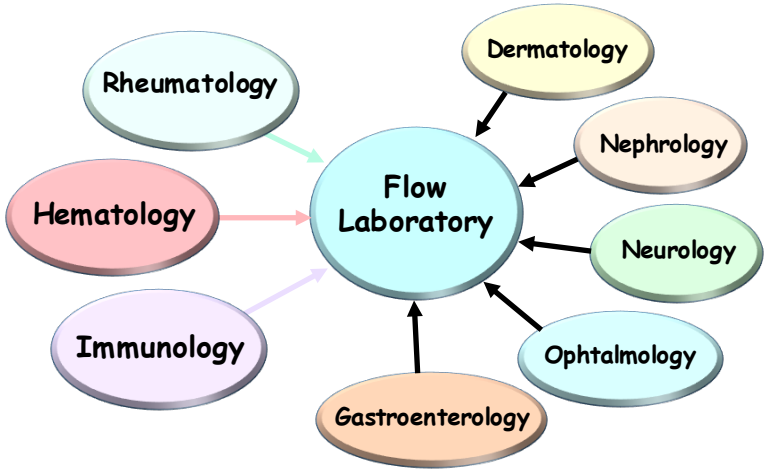


B-Cell Depleting Immunotherapies in Autoimmunity and Malignancies: Open Questions

- Anti-CD20 of various generations are DIFFERENT DRUGS and have different effects on the cell targets depending on their interaction with effector cell Fcγ Receptors.
- The interaction with Fcγ Receptors may have activatory or inhibitory effects: Need to manage and orient these opposite effects.
- Engineering of the Ig Fc fragments can modify such interactions, thus favoring certain effector functions.
- Further studies are needed to better understand the role of sIgM+ memory B-Cells and sIgG+ memory-switched B-Cells (different meaning in monitoring autoimmune diseases ?)

Conclusions Immune Monitoring of Anti-CD20 Monoclonals

Keep in Close Contact With Our Clinical Colleagues And Be of Help



Recent References

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Monitoring anti-B cell immunotherapies in autoimmune diseases: Go with the flow. A Position Paper of the Italian Society for Clinical Cell Analysis (ISCCA)

Bruno Brando,[1] Arianna Gatti,[1] Alfredo Maria Lurati,[2] Paola M.L. Faggioli[2]

Open Access at: http://www.beyond-rheumatology.org/index.php/br/article/view/26/40

Cytometry: Part B - Clinical Cytometry

Gatti A, Buccisano F, Scupoli MT and Brando B.

THE ISCCA FLOW PROTOCOL FOR THE MONITORING OF ANTI-CD20 THERAPIES IN AUTOIMMUNE DISORDERS

Cytometry: Part B - Clinical Cytometry 2020, Accepted, in press