Immunotherapy and Immune Monitoring

B Cell Monitoring During Anti-B Treatment in Autoimmune Diseases

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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 (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-naive 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

Precursors B Cells Plasmocells

- Pro-B
- Pre-B
- Immature
- Pre-Plasmablast
- Transit
- Mature Plasmablast
- IgM
- IgM/IgD
- Naive Memory Switched
- Mature PC

CD10
CD19
CD20
CD21
CD22
CD24
CD27
CD49d
CD82
CD138
CD267
CD269
CD307a

Phenotypic changes occurring throughout B-cell differentiation:

Plus a number of soluble factors: BAFF, APRIL, TACI...
Validation of Phenotypic / Functional Characterisation of Peripheral B-Cell Subsets

**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

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**Rituximab, Ocrelizumab, Ofatumumab, Obinutuzumab Target CD20+ B Cells**

**CD20- Precursor B Cells and Plasmacells/blasts are Spared**

**Precursors**

- Precursors
- CD20

**B Cells**

- Pre-B
- Pro-B
- Imm. Transit. Mature
- Memory
- Naive
- Mature

**Plasmacells**

- Mostly functional, not phenotypical differentiation

**Long-Lived Plasmablasts**

**Short-Lived Plasmablasts**

**Precursor and Naive B Cells are spared**

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**B-Cell Specific Chemotherapeutic Agents and Their Targets**

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Anti-CD20 Therapeutic Monoclonals

Four Effector Mechanisms to Eliminate B Cells

PMN and Macrophage Phagocytosis

Complement activation (C1q-C4,C5a)

Membrane attack complex (MAC)

Cell lysis

Apoptosis
Homotypic Adhesion

Effector of apoptosis

Macrophage, monocyte or natural killer cell

CD20

B cell

Antibody-dependent cell-mediated cytotoxicity (ADCC)

Antibody-dependent cytotoxicity (CDC)


CD20 therapeutics


to eliminate B cells

Four effector mechanisms

PMN and macrophage phagocytosis

Complement activation (C1q-C4,C5a)

Membrane attack complex (MAC)

Cell lysis

Apoptosis
Homotypic Adhesion

Effector of apoptosis

Macrophage, monocyte or natural killer cell

CD20

B cell

Antibody-dependent cell-mediated cytotoxicity (ADCC)

Antibody-dependent cytotoxicity (CDC)


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 RI (CD64)
- Fc-γ R IIA (CD32A)
- Fc-γ R IIB (CD32B) → The only INHIBITORY member of the group. Binds monomeric IgG with LOW Affinity.
- Fc-γ R IIC (CD32C) Variably expressed on Myeloid and B Cells. Expressed on B cell leukemia/lymphoma cells.
- Fc-γ R IIIA (CD16A) Two isoforms exist (CD16B1 & B2), with different intracytoplasmic domains and propensity to internalize.


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

- 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
- TTP
- Idiopathic Membranous Nephropathy
- IgG4-Related Nephropathies
- Optic Neuritis
- Cryoglobulinemic vasculitis
- Anti-HLA Abs Removal in Transplants
- 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

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
- TTP
- Idiopathic Membranous Nephropathy
- IgG4-Related Nephropathies
- Optic Neuritis
- 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

Cytometrist’s Task

8% 35% 57%

8% 57% 35%

8% 35% 57%

8% 57% 35%

8% 35% 57%

8% 57% 35%

8% 57% 35%

8% 57% 35%

8% 57% 35%

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8% 57% 35%
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.


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

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

<table>
<thead>
<tr>
<th>Basal MD (IQR)</th>
<th>6M MD (IQR)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>% CD3+&lt;/sup&gt;CD45RO&lt;sup&gt;+&lt;/sup&gt;</td>
<td>45 (33.1-51.6)</td>
<td>30.1 (21.1-37.1)</td>
</tr>
<tr>
<td>% CD3+&lt;/sup&gt;CD45RO&lt;sup&gt;+&lt;/sup&gt;</td>
<td>45 (33-52)</td>
<td>61 (50-66)</td>
</tr>
<tr>
<td>AbCD3+&lt;/sup&gt;CD45RO&lt;sup&gt;+&lt;/sup&gt;</td>
<td>282.91 (146.48-845.88)</td>
<td>171.76 (118.84-424.91)</td>
</tr>
<tr>
<td>AbCD3+&lt;/sup&gt;CD45RO&lt;sup&gt;+&lt;/sup&gt;</td>
<td>249.29 (168.15-429.59)</td>
<td>446.4 (230.87-626.35)</td>
</tr>
</tbody>
</table>

Memory T Cells: CD3+ CD45RO<sup>+</sup> ; Naive T Cells: CD3+ CD45 RO<sup>-</sup>

- Percent and absolute Total T Cells do not change remarkably during Rituximab.
- A slight increase of T CD8<sup>+</sup> 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.


Reference Levels of Functional B-Cell Subsets in Peripheral Blood

(A Still Debated Issue)

<table>
<thead>
<tr>
<th>Cell Subsets</th>
<th>Phenotype</th>
<th>% of B Cells range (mean)</th>
<th>Abs Val/ µL range (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral B Cells</td>
<td>CD19+ CD20+ CD23+ CD24+</td>
<td>100</td>
<td>70 - 350 (185)</td>
</tr>
<tr>
<td>Immature</td>
<td>CD10+CD19+ CD20+ CD27+ CD38+</td>
<td>1.5 - 10.0 (5.4)</td>
<td>0.3 - 12 (8)</td>
</tr>
<tr>
<td>Transitional</td>
<td>CD10+CD19+ CD20+ CD24+ CD38+</td>
<td>2 - 4 (3)</td>
<td>2 - 11.2 (6)</td>
</tr>
<tr>
<td>Naive</td>
<td>CD10 - CD19+ CD20+ CD27+ CD38</td>
<td>49 - 81 (64)</td>
<td>45 - 165 (100)</td>
</tr>
<tr>
<td>Memory B</td>
<td>CD10- CD19+ CD20+ CD27+ CD38- CD43</td>
<td>14 - 44 (31)</td>
<td>16 - 96 (52)</td>
</tr>
<tr>
<td>Plasmacells (total)</td>
<td>CD10-CD19+ CD20- CD27+ CD38+ CD43+</td>
<td>0.4 - 4.4 (2.1)</td>
<td>* 0.1 - 4.2 (3)</td>
</tr>
<tr>
<td>CD138- Plasmacells</td>
<td>same, CD138- sig cyTg+</td>
<td>57% ± 12 of PC</td>
<td></td>
</tr>
<tr>
<td>CD138+ Plasmacells</td>
<td>same, CD138+ sig cyTg+</td>
<td>43% ± 12 of PC</td>
<td>* &lt; 0.02% of Total Leukocytes</td>
</tr>
</tbody>
</table>

Modified from:

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

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

- 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.

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

<table>
<thead>
<tr>
<th>B-cell subsets</th>
<th>Early B progenitors</th>
<th>Pre-BI</th>
<th>Pre-BII</th>
<th>Immature</th>
<th>Transitional</th>
<th>Early mature</th>
<th>Switched memory</th>
<th>IgM/IgD Marginal Zone</th>
<th>Plasmablast</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<td>-</td>
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<tr>
<td>CD38</td>
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<td>CD24</td>
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<tr>
<td>CD34</td>
<td>+</td>
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<tr>
<td>CD45</td>
<td>+</td>
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<td>-</td>
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<td>+</td>
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<tr>
<td>CD10</td>
<td>+</td>
<td>-</td>
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<tr>
<td>sIgM</td>
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<tr>
<td>sIgD</td>
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</tr>
</tbody>
</table>

Median ± SD: 0 0 0 0 0 0 5.8 2.39 4.46 11.5 6.3 0.0

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

Biological and Pharmacokinetic Comparison Between Major Anti-CD20 MoAbs

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

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.

Sacco KA. Immunotherapy 2018; 10: 713-728.
Pharmacodynamic Profile of Anti-CD20 MoAbs

Non Fc-optimized

A

Complement

Direct

FcyRIIIA

Type I

B

Complement

Direct

FcyRIIIA

C

Complement

Direct

FcyRIIIA

D

Complement

Direct

FcyRIIIA

Fo-optimized


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

Type I

AT10 = Fcγ Blocker Ab. Reduces internalization

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

Discriminating True Target Disappearance from Target Modulation

1) Total disappearance of B Cells under Rituximab.

Cytometrist’s Task

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

Character 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

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 efficiency and in clinical response.


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:

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

SLE:
- doi: 10.3390/jcm7110430

Multiple Sclerosis:

Systemic Sclerosis:

Glomerulonephritis:

Neuromyelitis Optica Sp:

Sjögren’s Syndrome:

Allogeneic Transplantation:
- Ikemiya M. Ther Apher Dial 2017; 21(2): 139-149.

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

B Cell Subsets in Synovial Fluid

Unstimulated
Stimulated with CpG
(TLR-9 Agonist)

Unstimulated
Stimulated with CpG
(TLR-9 Agonist)

ANA+ SF Switched Memory B cells

ANA+ Patients: 0/7
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

- '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.


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

- Pre-Rituximab
- At first B Cell Return
- 3 months After B Cell Return
- Clinical relapse 6 months After B Cell Return


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.

Identification of a B cell signature associated with renal transplant tolerance in humans
Kenneth A. Newell,1 Adam Asano,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 cell subsets in the regulation of alloimmune response.
- Naïve B cells are poor Ag-presenting cells and induce tolerance by orienting T cells into Tregs.


Rheumatoid Arthritis - Post 2nd Rituximab cycle: Non Responder High-Risk Patient

Criteria for poor response: high % Memory B Cells
**Rheumatoid Arthritis - Post Rituximab: Good Responder Patient**

Criteria for Good Response: Low % and abs Memory B Cells - Plasmacells $< 3/\mu$L

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

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

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

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**

Criteria for poor Response: Very high % CD27+ Memory B Cells
### Rheumatoid Arthritis – B-Cell Subsets in 29 Patients Treated with Rituximab

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

Mean ± SD: 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.

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

### Conflicting Biomarkers and Endpoints for Anti-CD20 Treatments in Autoimmunity

<table>
<thead>
<tr>
<th>DISEASE</th>
<th>TARGET VALUES</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rheumatoid Arthritis/SLE</td>
<td>&lt;0.01 B Cells/µL (MRD)</td>
<td>Doss S, 2008; Calero I, 2010; Stradner MH, 2016; Md Yusof MY, 2017</td>
</tr>
<tr>
<td>IgG4-Related Diseases</td>
<td>‘Disappearance’ of IgG4+ Plasmablasts</td>
<td>Perugino CA, 2017</td>
</tr>
<tr>
<td>Optic Neuromyelitis</td>
<td>CD27+ memory B Cells &lt;0.05% of mononuclears</td>
<td>Collongues N, 2016; Cohen M, 2017</td>
</tr>
</tbody>
</table>

**References**

Dass S, 2008
Calero I, 2010
Stradner MH, 2016
Md Yusof MY, 2017
Pozdziek A, 2016
Perugino CA, 2017
Collongues N, 2016; Cohen M, 2017

**Conflicting Biomarkers and Endpoints for Anti-CD20 Treatments in Autoimmunity**

**RA Induction dose:** 375 mg/m² b.s. x 4 times

**Maintenance dose:** 375 mg/m² b.s.

IF CD27+ Memory B-Cells are > 0.05% of peripheral Mononuclear Cells AND T CD4+ cells are > 250/microliter, the Rituximab treatment is approved.

**Markers Antibody panel can be reimbursed**

8 Markers Antibody panel can be reimbursed
**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 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++ Plasmacyte 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)

---

**The ISCCA Protocol for High-Resolution Monitoring of B-Cell Subsets in Peripheral Blood in Patients Treated with Anti-CD20 Therapeutic Monoclonals**

- **Anti-CD20** (1 to 4 Boluses)
  - **TEST at Baseline (Pre-Dose)**
  - **TEST after 3 Months**
    - if CD19< 0.1/µL
      - re-TEST after 2 more Months
    - and re-TEST after 1 more Month
  - Consider a new cycle or a switch to other MoAbs

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**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)


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**The ISCCA Protocol for High-Resolution Monitoring of B-Cell Subsets in Peripheral Blood in Patients Treated with Anti-CD20 Therapeutic Monoclonals**

- **Anti-CD20** (1 to 4 Boluses)
  - **TEST at Baseline (Pre-Dose)**
  - **TEST after 3 Months**
    - if CD19> 0.3-0.5/µL (Subsetting Becomes Informative)
    - and re-TEST after 1 more Month
    - if CD19> 1/µL and Memory B>65%
      - re-TEST after 2 more Months

---

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

- Staining mixture: CD19 / CD20 / CD27 / CD38 / CD45 (+ sIgM & sIgG)
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- Timepoints for tests: Baseline, 3 Mo, Long-Term... (Depending on drug schedule and also very patient-dependent)
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 naive 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 Lineage

- 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.

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.

Common Causes of Secondary Antibody Deficiencies

Secondary antibody deficiencies are 30 times more common than primary deficiencies.


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

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,3 Arianna Gatti,1 Alfredo Maria Larrat,3 Paola M.I. Faggioli3