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Rituximab induces clonal expansion of IgG memory B-cells in patients with inflammatory central nervous system demyelination

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ABSTRACT

Rituximab, a monoclonal B-cell cytolytic antibody, has beneficial effects in patients with inflammatory demyelinating diseases. So far, little data exists on B-cell subset recovery after rituximab treatment in inflammatory demyelinating diseases of the central nervous system (CNS). To elucidate whether rituximab promotes qualitative changes in the IgG memory B-cell repertoire we performed a single cell analysis in three patients with CNS demyelination. We did not observe any qualitative changes but detected an increased clonal expansion in the IgG memory B-cell compartment after treatment, indicating that a single course of rituximab does not eliminate specific IgG memory B-cells.

1. Introduction

Rituximab is a B-cell cytolytic chimeric IgG1 monoclonal antibody that targets the CD20 molecule, expressed on pre-B and mature B-cells, until their differentiation into plasma cells. Rituximab causes B-cell depletion by antibody-dependent cell-mediated cytotoxicity (ADCC), complement-mediated lysis and induction of apoptosis. Mainly peripheral B-cells are affected, whereas germinal-center B-cells and, in particular, marginal-zone B-cells were found to be resistant to killing (Edwards and Cambridge, 2006; Gong et al., 2005).

Rituximab has already been proven to have beneficial effects in patients with inflammatory demyelinating disorders (Bar-Or et al., 2008; Cree et al., 2005; Dalakas et al., 2009; Hauser et al., 2008; Kim et al., 2013; Kim et al., 2011; Monson et al., 2005), but so far little data exists on B cell subset recovery after rituximab treatment in inflammatory demyelinating diseases of the central nervous system (CNS). We previously reported that the therapeutic effect of rituximab in anti-myelin associated glycoprotein (MAG) neuropathy depends on efficient depletion of non-circulating B-cells and reconfiguration of the B-cell memory compartment (Maurer et al., 2012). In this study we could demonstrate that the long-term immunomodulatory effects of rituximab in anti-MAG neuropathy are mediated by the sustained reduction of expanded autoreactive IgM memory B-cells. Patients who responded to rituximab showed a reduction in the clonal expanded IgM memory B-cell compartment after therapy, which was associated with clinical disease remission. Interestingly, the frequency of clonal expanded IgG memory B-cells slightly increased after rituximab therapy. To prove whether this finding is unique to the anti-MAG-neuropathy patients, we additionally examined the peripheral IgG gene repertoire in patients with inflammatory demyelinating disorders of the CNS during rituximab therapy. We have analyzed one patient with aquaporin-4 (AQP4) antibody positive neuromyelitis optica (NMO), one patient with AQP4-antibody negative recurrent transverse myelitis and one patient with primary progressive multiple sclerosis (PPMS).

2. Materials and methods

2.1. Patients

The study was approved by the institutional review board of Medical University of Innsbruck (study no. UN3041 257/4.8) and all participants gave written informed consent. One patient with NMO (patient 1), one patient with recurrent myelitis (patient 2) and one patient with PPMS (patient 3) were included in this observational study (Table 1). Patients were treated with rituximab at a dosage of 375 mg/m^2 and 100–250 mg prednisolone was administered *i.v.* prior to each rituximab application. Patient 1 and patient 2 were recruited from a recently published study

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Table 1
Demographical, clinical and immunological characteristics of patients.

	Patient 1	Patient 2	Patient 3
Gender	Female	Male	Female
Age (Years)	20	21	46
Diagnosis	Neuromyelitis optica	Recurrent myelitis	Primary progressive multiple sclerosis
Disease duration before therapy (years)	4.3	0.6	5.0
Time from first to second sample (months)	5	7	9
Percentage of total B-cells before therapy	6.8	10.0	12.4
Percentage of IgG memory B cells before therapy	0.80	0.90	1.60
Percentage of total B-cells after therapy	0.7	2.0	0.6
Percentage of IgG memory B cells after therapy	0.05	0.10	0.05
AQP4-antibody titer before therapy	1:20,480	0	0
AQP4-antibody titer after therapy	1:20,480	0	0
Serum BAFF ng/ml before therapy	0.64	0.5	0.58
Serum BAFF ng/ml after therapy	3.45	5.11	2.55

on the effect of rituximab on patients with NMO spectrum disorders (NMOSD) from Medical University of Innsbruck (Gredler et al., 2013). Serum AQP4-antibody and B-cell activating factor (BAFF) levels were measured as previously described (Gredler et al., 2013; Mader et al., 2010).

2.2. Flow cytometry and single cell analysis

Single cell analysis was performed the same way as for the anti-MAG neuropathy patients as previously described (Maurer et al., 2012). In brief, frozen peripheral blood mononuclear cells (PBMC) were thawed in FACS buffer (2 mM EDTA, 5% FCS, 20 μg/ml DNAse [Roche] in PBS). After thawing, the PBMCs were strained (0.22 μm cell strainer; BD) and centrifuged for 15 min at 150 g and 4 °C. The cells were incubated with PE-conjugated anti-CD27 clone M-T271, PE-Cy7-conjugated anti-CD19 clone SJ25C1, biotin-conjugated anti-IgG clone G18-145, and FITC-conjugated streptavidin (all BD) and with component H (Invitrogen) for dead cell discrimination. Single $CD19^+$ $CD27^+$ IgG^+ memory B-cells were purified by flow cytometric cell sorting using a Facs Aria cell sorter and Diva software (both BD). Single cell sorting was directly into a 96-well plate (thermoquick PCR-Plate; Greiner bioOne) with 20 μl OneStep RT-PCR reaction mix (Qiagen) supplemented with 0.5 μM each of *Igh* variable region-specific forward and IgG reverse primers per well (Tiller et al., 2008). Directly after sorting plates were incubated for 60 min at 50 °C for reverse transcription. After the reverse transcription step, DNA polymerase was activated with an initial step of 15 min at 95 °C. Amplification of the resulting cDNA of *Igh* was performed at an annealing temperature of 58 °C for 45 s, an elongation temperature of 72 °C for 1 min, and a denaturation temperature of 94 °C for 30 s for 48 cycles. Nontemplate controls were included to exclude any contaminations in the master mix.

To reduce PCR error rates, we used the HotStar Taq DNA polymerase (Qiagen) with an error rate of 2×10^{-5} per nucleotide and cycle. PCR amplicons were separated by electrophoresis on a 1.2% agarose gel. To avoid cross contaminations on the gel, PCR products were physically separated from each other. Bands with the correct mass (approximately 500 bp) were excised using individual sterile scalpels; DNA was extracted (Qiagen MiniElute Gel Extraction Kit) and sequenced. The sequenced variable region of the amplified *Igh* was corrected with CLC Main Workbench and analyzed on the international immunogenetic information system (Brochet et al., 2008; Giudicelli et al., 2011). The variable region was analyzed for VH, DH, and JH usage; somatic hypermutations (SHM); CDR3 length; pI; and clonal expansion.

2.3. Sequence analysis

Clonal expansions were determined by similar CDR3 amino acid sequences and V_H, D_H, and J_H usage in the IgG memory B-cell pool. The length of the CDR3 was defined as the number of amino acids from the third position after the cysteine motive of the CDR3 to the tryptophan amino acid. For pI analysis, we used the entire amino acid sequence of the CDR3 and calculated it with the tool on ExPASy proteomics server (Gasteiger et al., 2003). The V_H, D_H, and J_H gene usage was weight balanced by the number of sequences derived from each donor to avoid tempering the result by different amount of sequences due to clonal expansion or PCR efficacy. For the SHM analysis, sequences were first blasted against the germline sequences in the database of IMGT.

2.4. Statistical analysis

The two-tailed Fisher exact test was used to compare frequencies of V_H, D_H and J_H gene family usage and the size of clonal expansions. All analyses are based on individual sequences, and data in figures, in which error bars are shown, are presented as mean ± SEM. Since we analyzed pooled sequence data, a repeated-observation analysis was not applied. A *P* value less than 0.05 was considered significant.

3. Results

3.1. No qualitative changes in the IgG gene repertoire after rituximab infusion

To determine whether rituximab induces qualitative changes in the peripheral IgG memory B-cell repertoire, we amplified and sequenced Ig heavy chain (*Igh*) genes of single sorted IgG memory B-cells ($CD19^+$ $CD27^+$ IgG^+) before and 4–9 months after therapy (Table 1). The percentage of the B-cell and the IgG memory B-cell pool within the peripheral lymphocyte compartment declined dramatically after rituximab therapy (Table 1). Nevertheless, we could sort sufficient B-cells for single cell analysis after therapy. Sequence analysis revealed no major differences in the V_H, D_H and J_H gene family usage between the patients (Fig. 1). Additionally, we could not find differences in the gene family usage of our patient cohort and the anti-MAG neuropathy patients and demographically matched healthy blood donors (Maurer et al., 2012). Furthermore, we could not find any significant differences in V_H, D_H and J_H gene usage before and after rituximab therapy (Fig. 1 and Supplementary Table). Next, we examined heavy chain characteristics of the complementary-determining region 3 (CDR3), as long and positively charged CDR3 regions have been associated with antibody-mediated autoreactivity (Wardemann et al., 2003; Yurasov et al., 2005). Again, we could not detect significant differences in length and isoelectric point (PI) after rituximab therapy in our patients. These data are compatible with our previously reported findings in rituximab-treated patients with anti-MAG neuropathy, at least for the IgG memory compartment (Maurer et al., 2012). We conclude that rituximab does not promote qualitative changes in the IgG memory compartment in patients with inflammatory demyelination.