



Universiteit  
Leiden  
The Netherlands

## Targeting the humoral immune system of patients with rheumatoid arthritis

Teng, Y.K.O.

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# Chapter 7

## CD20 epitope masking by rituximab

Teng YKO  
Ioan-Facsinay A  
van Laar JM

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Rituximab, an anti-CD20 B-cell depleting monoclonal antibody (mAb), is increasingly used in the treatment of refractory autoimmune diseases, such as rheumatoid arthritis<sup>1,2</sup> and systemic lupus erythematosus<sup>3</sup>. Mouse anti-human mAb against the pan-B-cell markers CD20 and/or CD19 are generally used for flow cytometric and immunohistochemical analyses of blood and tissue to assess the extent of B-cell depletion. Teeling *et al.* recently reported that all mouse anti-human CD20 mAb, including rituximab, bind the large extracellular loop of the CD20 membrane protein<sup>4</sup>. Therefore, it is conceivable that cellular analyses of rituximab-treated patients using anti-CD20 antibodies are influenced by masking of the CD20 epitope by rituximab. In order to investigate this, we performed a flow cytometric competition assay with rituximab and a commonly used mouse anti-human mAb against CD20.

Peripheral blood mononuclear cells from 4 healthy volunteers were obtained through isolation over a Ficoll gradient and freshly processed for 2 sets of experiments, as follows. In one experiment, samples were preincubated with 0.67 mg/ml rituximab, washed vigorously in phosphate buffered saline-1% bovine serum albumin, and then stained with increasing concentrations of fluorescein isothiocyanate (FITC)-conjugated anti-CD20 (clone 2H7) in combination with an optimal concentration of phyco-erythrin (PE)-conjugated anti-CD19 (clone H1B19) (all from Becton Dickinson, San Jose, CA). In the second experiment, samples were stained with optimal concentrations of FITC-conjugated anti-CD20 (clone 2H7) and PE-conjugated anti-CD19 (clone H1B19) and subsequently incubated with increasing concentrations of rituximab. Thereafter, samples were vigorously washed, and data were collected using a FACSCalibur flow cytometer and analyzed with the Flow Jo software program (Tree Star, Ashland, OR).

Preincubation with rituximab before flow cytometric staining led to a significant decrease in the number of CD20+, CD19+ B-cells (from a mean  $\pm$  SEM of  $91 \pm 3.1\%$  to  $12 \pm 2.6\%$ ;  $P=0.004$ ) and a significant reduction in the mean fluorescence intensity (MFI) of CD20 (from  $25 \pm 3.2$  to  $4.4 \pm 0.6$ ;  $P=0.024$ ), even with a large surplus of clone 2H7 mAb. When B-cells were first double-stained for CD19 and CD20 and then incubated with increasing concentrations of rituximab, CD19+ B-cells gradually lost their CD20 expression (MFI  $17 \pm 2.4$  without rituximab and  $6.4 \pm 1.0$  after incubation with 2.67 mg/ml rituximab;

$P=0.017$ ). Importantly, the percentages of CD19+ B-cells in the pre-rituximab incubation experiments compared with the post-rituximab incubation experiments were constant ( $6.1 \pm 2.1\%$  and  $6.8 \pm 1.8\%$ , respectively;  $P=0.34$ ).

The effective competition of rituximab against cell-bound anti-CD20 antibody and the failure of increasing concentrations of 2H7 to dislodge rituximab after binding to B-cells indicate that rituximab binds with higher affinity to the CD20 epitope than does 2H7. Our findings suggest that data based on CD20 staining of B-cells, as in Gunnarsson and colleagues' study, should be interpreted with caution. Importantly, CD19 expression on B-cells was not influenced by rituximab, and therefore, studies using rituximab should include pan-B-cell markers, such as CD19, CD79a, or CD22, to corroborate data on CD20 expression and/or cytoplasmatic CD20 staining.

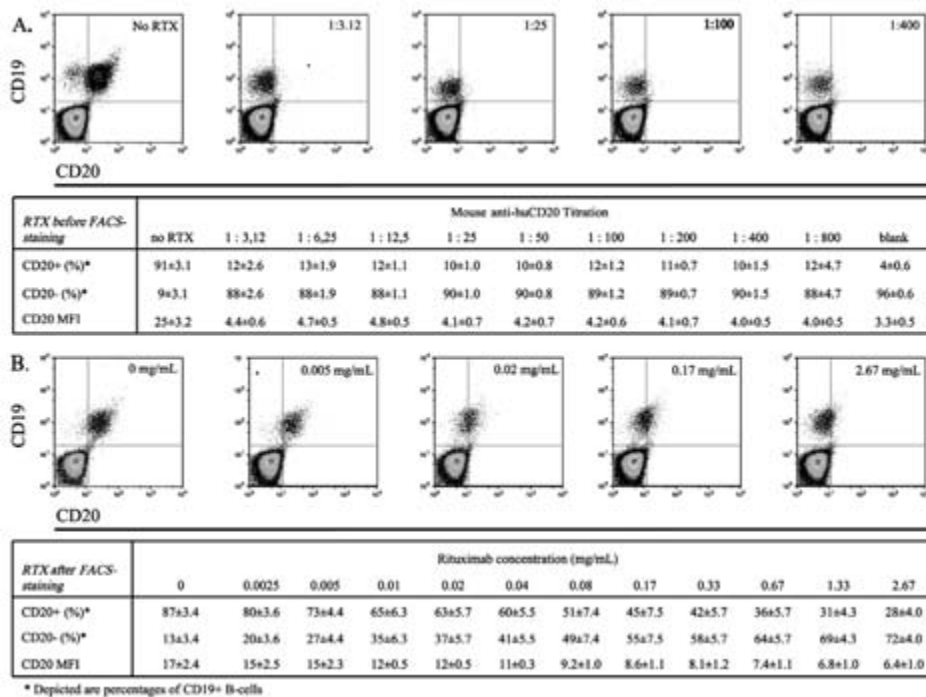


Figure 1

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