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Depletion of CD20 B cells fails to inhibit relapsing mouse experimental autoimmune encephalomyelitis.

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Running Title: CD20 depletion in EAE

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ABSTRACT

Background. Multiple sclerosis (MS) is often considered to be a CD4, T cell-mediated disease. This is largely based on the capacity of CD4 T cells to induce relapsing experimental autoimmune encephalomyelitis (EAE) in rodents. However, CD4-depletion using a monoclonal antibody was considered unsuccessful and relapsing MS responds well to B cell depletion via CD20 B cell depleting antibodies. The influence of CD20 B cell depletion in relapsing EAE was assessed.

Methods. Relapsing EAE was induced in Biozzi ABH mice. These were treated with CD20-specific (18B12) antibody and the influence on CD45RA-B220 B cell depletion and clinical course was analysed.

Results. Relapsing EAE in Biozzi ABH failed to respond to the marked B cell depletion induced with a CD20-specific antibody. In contrast to CD20 and CD8-specific antibodies, CD4 T cell depletion inhibited EAE.

Conclusion. Spinal cord antigen-induced disease in ABH mice is CD4 T cell-dependent. The lack of influence of CD20 B cell depletion in relapsing EAE, coupled with the relatively marginal and inconsistent results obtained in other mouse studies, suggests that rodents may have limited value in understanding the mechanism occurring following CD20 B cell depletion in humans.

KEYWORDS:

B cells; CD20; experimental autoimmune encephalomyelitis; monoclonal antibody; multiple sclerosis

HIGHLIGHTS

- CD20⁺ B cell depletion did not inhibit relapsing EAE.
- Even when therapeutic, the results following CD20⁺ depletion are marginal and inconsistent between studies in rodents.
- Rodent EAE studies are unlikely to expose the true mechanism of CD20-depleting antibodies in MS

INTRODUCTION

Multiple sclerosis is the major demyelinating disease of the adult central nervous system (Compston & Coles 2008). This is thought by many, to represent a T cell-mediated autoimmune disease, largely based on a significant amount of circumstantial evidence from MS and notably by the induction and response to therapy of experimental autoimmune encephalomyelitis in animals (Martin et al. 2016). However the perceived failure of T cell depletion with CD4 specific antibodies (van Oosten et al 1997) and the robust inhibitory effect of a number CD20-specific B cell depleting antibodies (Bar-Or et al 2008; Hauser et al. 2008, Sorensen et al. 2014, Hauser et al. 2017) in relapsing MS, may perhaps question the central role of T cells as targets to control MS (Baker et al. 2017).

CD20 is a surface marker expressed by B cells from the pre-B cell to the memory B cell phenotypes, which is lost during differentiation into plasma cells (Tedder & Engel 1994). Depletion of these cells from the peripheral B cell pool can inhibit active MS (Cross et al. 2006, Bar-Or et al. 2008). Whilst it has been suggested that CD20-specific mAb act via depletion of a pathogenic T cell subset (Palanichamy et al. 2014, Holley et al 2014), studies in rodents and MS have supported the view that memory B cells act as a central antigen presenting cell to drive T cell autoimmunity (Agahozo et al. 2016, Baker et al. 2017). However, studies using CD20-depleting antibodies in EAE have been variable and may augment or inhibit monophasic chronic EAE in C57BL/6 mice (Matsushita et al. 2008, Weber et al. 2010, Monson et al. 2011). It was hypothesized that therapeutic depletion of B cells would inhibit relapsing EAE, in Biozzi ABH mice (Al-Izki et al. 2012), however surprisingly marked CD20 B cell depletion failed to influence EAE, in contrast to that found in MS.

MATERIALS & METHODS

Animals. Female Biozzi ABH (6-8 weeks) mice were purchased from Harlan UK Ltd (Bicester, UK). These were maintained on a 12h:12h light:dark cycle and were housed and fed with RM1(E) chow and water *ad libitum* as described previously (Al-Izki et al. 2012). These were used according to the United Kingdom, Animals (Scientific procedures) Act 1986, incorporating review by the local Animal Welfare and Ethical Review Body and the United Kingdom Home Office.

Antibodies. Mouse CD4-specific rat IgG2b (YTS191) mAb and mouse CD8-specific rat IgG2b (YTS169) mAb were produced as described previously (O'Neill et al. 1993) and protein G purified using HiTrap™ protein G columns (Pharmacia/GE Healthcare Life Sciences, Amersham, UK) or was purchased from BioXCell (West Lebanon, New Hampshire, USA). 250µg mAb was injected intraperitoneally to deplete CD4 and CD8 T cells for 2-3 weeks (O'Neill et al. 1993). Mouse CD20-specific mouse IgG2a (18B12. IgG2a variant. Hamel et al. 2008) was supplied by Biogen (Cambridge, USA). 250µg mAb, which induced marked depletion of B cells between 1-21 days in BALB/c mice (Hamel et al. 2008), was injected intravenously/mouse on day 8 or day 28. Animals were randomized within cages to control or treatments. Irrelevant rat IgG2b, and mouse IgG2a

mAb do not influence the course of EAE in ABH mice (O'Neill et al. 1993, Al-Izki et al. 2012).

Flow Cytometry. Spleens were removed and single cell suspensions generated using a 40 µm cell strainer. Red cells were lysed by addition of 1 ml lysis buffer (4.14 g 15 mM Ammonium chloride, 0.5 g 10 mM Potassium bicarbonate and 2.5 ml 20 mM EDTA solution in 500 ml distilled water) for a minimum of 1 minute at room temperature. The number of viable cells was determined by trypan blue exclusion method and then 1×10^6 splenocytes were incubated with 1-5µg/ml fluorescent mouse specific antibodies. Terminal cardiac or tail bleeds were collected in heparin-coated tubes (Vet Labs, Pullborough, UK). 50 µl of blood sample was stained with CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7) or CD45RA/B220 (RA3-6B2) specific mAb (Becton Dickinson, Oxford, UK) in 2% heat-inactivated foetal calf serum (Invitrogen, Paisley, UK) in phosphate buffered serum (Invitrogen, Paisley, UK). Cells were incubated for 30 minutes in the dark at room temperature. Red cells were lysed by addition of 1 ml of 1:10 dilution of FACS Lysis Buffer (Becton Dickinson, Oxford, UK). Cells were vortexed and incubated for minimum of 1 minute at room temperature and until solution goes clear red. Cells were fixed by addition of 100 µl Caltag Medium A fixative (Caltag, Buckingham, UK) and incubated for minimum 15 minutes in the dark at room temperature. Samples were analysed by flow cytometry (Becton Dickinson, Oxford, UK).

Induction of experimental autoimmune encephalomyelitis. 6-8 week adult ABH mice were subcutaneously injected with 1mg mouse spinal cord homogenate (SCH) emulsified in Freund's adjuvant containing 60µg *Mycobacterium tuberculosis* H37Ra and *M. butyricum*(8:1) in the flank on day 0 and 7 as described previously (Al-Izki et al. 2012). Relapse was induced by injecting the mice subcutaneously by a further set of injections on day 29 (Al-Izki et al. 2012). Clinical disease was scored: *Normal* = 0; *Fully flaccid tail* = 1; *Impaired righting reflex* = 2; *Hindlimb paresis* = 3; *Complete hindlimb paralysis* = 4 and *Moribund/death* = 5 (Al-Izki et al. 2012). Details of randomization, blinding and sample size calculations and other experimental details relevant to the ARRIVE guidelines have been reported previously (Al-Izki et al. 2012). Use of SCH as immunogen precludes *ex vivo* analysis as SCH-sensitized animals fail to give robust T cell responses to the dominant pathological myelin epitopes.

Statistical analysis. Flow cytometry was analysed by Analysis of Variance with a Bonferroni post hoc test. Results represent the mean maximum ± SEM clinical score or day of onset ± SD were analysed using non-parametric statistics using Sigma plot V11 (Systat Inc, San Jose, USA)

RESULTS

CD20-specific antibody depletes B cell in the blood and spleen.

To confirm the B cell depleting capabilities of CD20-specific mAb, 250µg 18B12 mouse IgG2 variant was injected into Biozzi ABH mice and blood and splenocytes sampled at baseline 7, 14 and 21 days post-injection and the numbers of B cells assessed (Figure 1). As anticipated a single injection of 250µg mAb depleted the B cells for at least 3 weeks in both the blood (Figure 1A) and spleen (Figure 1B).

Depletion of CD20-specific B cells fails to inhibit EAE

Depletion with CD20-specific mAb after sensitization has been reported to inhibit EAE (Matsushita et al. 2008, Weber et al 2010). Therefore ABH mice were injected with CD20 B cell depleting mAb on day 8 prior to the onset of initial acute EAE (Figure 2A). There was no difference in incidence; day of onset, or the maximum severity of EAE (Figure 2A). In contrast similar treatment with CD4d depleting mAb inhibited the development of EAE, whereas CD8 T cell depletion failed to prevent the development of EAE, similar to CD20 B cell depletion (Figure 2B). This suggested that disease was not responsive to CD20 B cell depletion and was largely driven by the action of CD4 T cells. Furthermore depletion of CD20 B cells did not influence the recovery from paralytic disease (Figure 2B). To assess whether CD20 could inhibit relapsing disease as a more therapeutically relevant situation and to determine whether depletion before the triggering stimulus could inhibit disease, CD20 B cell depletion was initiated before relapse was induced with a further injection of antigen in adjuvant (Figure 2C) and as was found in the initial phase of disease, CD20-depletion failed to inhibit the induced relapse. (Figure 2C). In addition a double dose of CD20 depletion on day 8 and again on day 28, failed to significantly inhibit the incidence, maximal severity or day of onset of an induced relapse (Table 1). Therefore despite marked CD20 B cell depletion there was no inhibition of relapsing EAE in ABH mice.

DISCUSSION

There have been numerous reports of the influence of genetic B cell depletion on EAE, which have supported some involvement of B cells (Agahozo et al. 2016). Likewise there have been a number of reports of CD20-depleting mAb influencing rodent EAE (Agahozo et al. 2016) and this effect can vary depending on the model, immunizing antigen and timing of onset (Figure 3. Matsushita et al. 2008). This can result in augmentation (Matsushita et al. 2008; Weber et al. 2010; Lehmann et al. 2011), inhibition (Matsushita et al. 2008; Weber et al. 2010; Lehmann-Horn et al. 2011; Monson et al. 2011, Anthony et al. 2014) or nothing (Matsushita et al. 2008) as seen here (Figure 2). However, when the data is more critically appraised, it is evident that, even when a therapeutic effect was reported the influence was relatively marginal and is seen by a small delay and/or diminution in the severity of clinical disease, in what is a high optimised system (Figure 3A, B). This contrasts with the essential elimination of disease with agents such as fingolimod, CD4 or CD52 depleting mAb (Al-Izki et al. 2011; von Kutzleben et al. 2017). Perhaps the most compelling inhibition of disease occurred when rituximab was used to inhibit disease in human CD20

transgenic mice (Monson et al. 2011, Figure 3B). However, as that was associated with marked depletion of CD4 T cells (Monson et al. 2011, Figure 3B), that could easily account for any disease inhibition (von Kutzleben et al. 2017) as seen here. Although, T cell depletion does occur following CD20-depleting mAb administration in humans (Palanichamy et al. 2014) the level is modest and well below the level of CD4 T cell depletion that failed to influence MS using CD4 depleting antibodies (van Oosten et al. 1997). This suggests either that there is a very specific subset of important T cells affected or that this activity may be irrelevant to the therapeutic action of CD20 B cell depletion in MS (Baker et al. 2017).

Whilst the influence of CD20 depletion on relapsing MS is comparable with the influence of CD52 lymphocyte depletion (Cohen et al. 2012; Hauser et al. 2017), the level of disease inhibition in published studies in rodents is not particularly compelling (Figure 3). Importantly, there is no consistent effect of CD20-specific mAb responsiveness across the rodent models (Figure 3), such as the influence of timing of mAb administration (Matsushita et al. 2008, Weber et al. 2011, Monson et al. 2011). Whilst it has been reported that myelin peptide-induced disease may be relatively B cell-independent compared to myelin protein-induced disease (Weber et al. 2011), in this study we failed to find an influence on protein-induced disease and other studies with myelin-peptide-induced disease has been influenced by CD20 depletion (Matsushita et al. 2008). This aspect is likewise not consistent and creates concern about the translatability of the results for rodents to humans (Baker & Amor 2014). As shown here, relapsing disease, at least in the ABH mouse is clearly not dependent on the action of CD20 B cells, which is consistent with the known CD4 T cell dependence (O'Neill et al. 1993; von Kutzleben et al. 2017). Although people may suggest the influence of CD20-B cell depletion is through the inhibition of antigen-presenting B cells function (Baker et al. 2017), if this were the case perhaps one may expect a more consistent effect in rodents, which is lacking (Figure 3).

However, as MS is human specific (Compston & Coles 2008, Baker & Amor 2014), it may suggest that the central susceptibility issues are restricted to humans. Thus, whilst there is much focus on T cell autoimmunity, it seems that agents that target relapsing MS also have a major action on memory B cells (Baker et al. 2017). This subset is the primary target of persistent Epstein Barr Virus (EBV) infection, thus B cell depletion may be removing a human-specific aetiological risk factor (Baker et al. 2017). As specific-pathogen-free ABH mice develop EAE, without the apparent need for viral susceptibility factors, this perhaps accounts for the lack of marked influence of CD20-depletion in rodents. As such it is of interest that CD20-B cell depletion can be associated with disease inhibition in some marmosets (Kap et al. 2010). There has been suggested to act via targeting B cells infected with an EBV-analogue virus (t'Hart et al. 2013). Further studies to address these aspects are warranted.

In conclusion, we have been unable to demonstrate any value in depleting CD20 B cells on the course of relapsing EAE. Further studies may define whether there is any influence in non-relapsing progressive disease. However, there are clear biology differences between rodent and human B cells, as indicated by the distinct phenotypes and markers during B cell lineage

development (Anderson et al. 2007). Rodents also show differences in response to B cell therapy, this suggests that there may be limited translational value in rodent studies, for this aspect of MS. Furthermore, it may question the value and ability of further rodent studies to uncover a human-relevant mechanism concerning CD20-B cell depleting activity.

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CONFLICTS OF INTEREST: ES. None relevant, DB None relevant, GP None Relevant, UCM Non relevant, GG has received fees for participation in advisory board for Biogen, Novartis. Roche who make CD20-mAb. He has also received travel, consultancy and research funds from other companies including: AbbVie Biotherapeutics, Canbex, Ironwood, Merck, Sanofi-Aventis and Teva.

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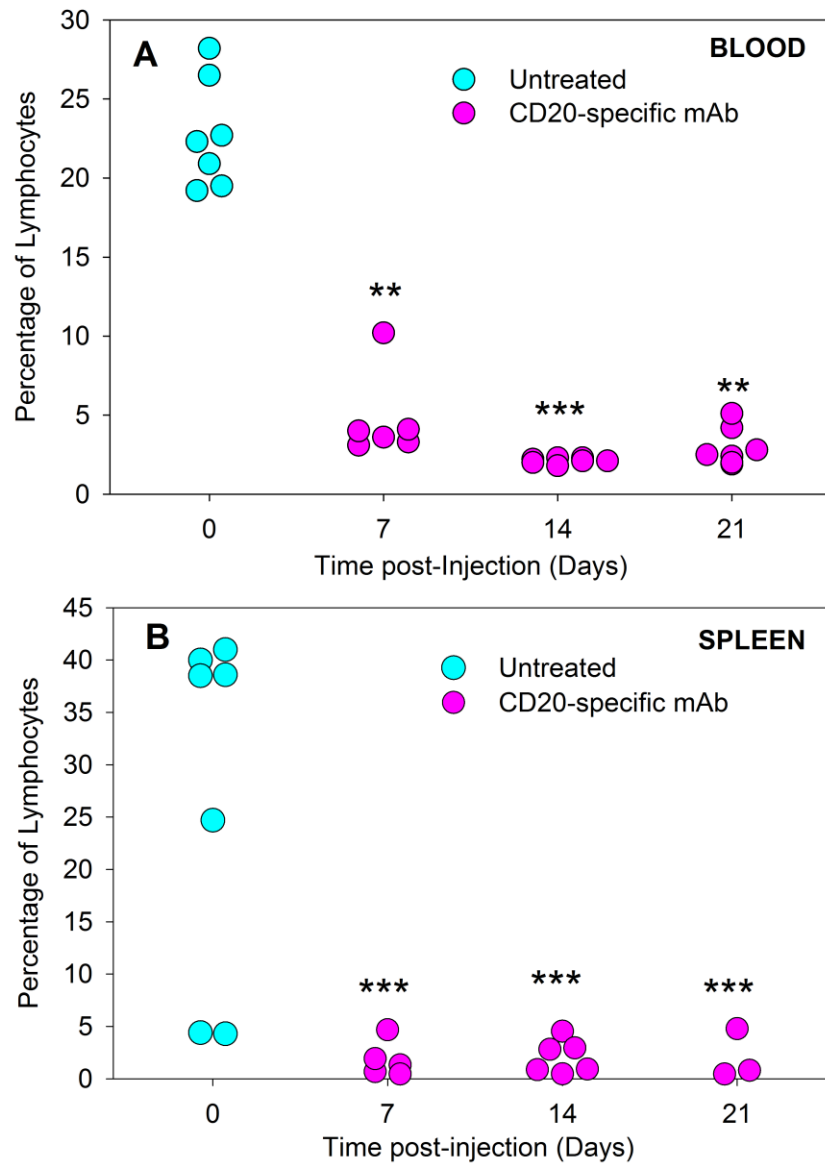
Weber MS, Prod'homme T, Patarroyo JC, Molnarfi N, Karnezis T, Lehmann-Horn K, Danilenko DM, Eastham-Anderson J, Slavin AJ, Linington C, Bernard CC, Martin F, Zamvil SS. B cell activation influences T cell polarization and outcome of anti-CD20 B cell depletion in central nervous system autoimmunity. *Ann Neurol.* 2010. 68:369-383.

TABLE 1. CD20 B cell depletion does not inhibit relapsing EAE in ABH mice.

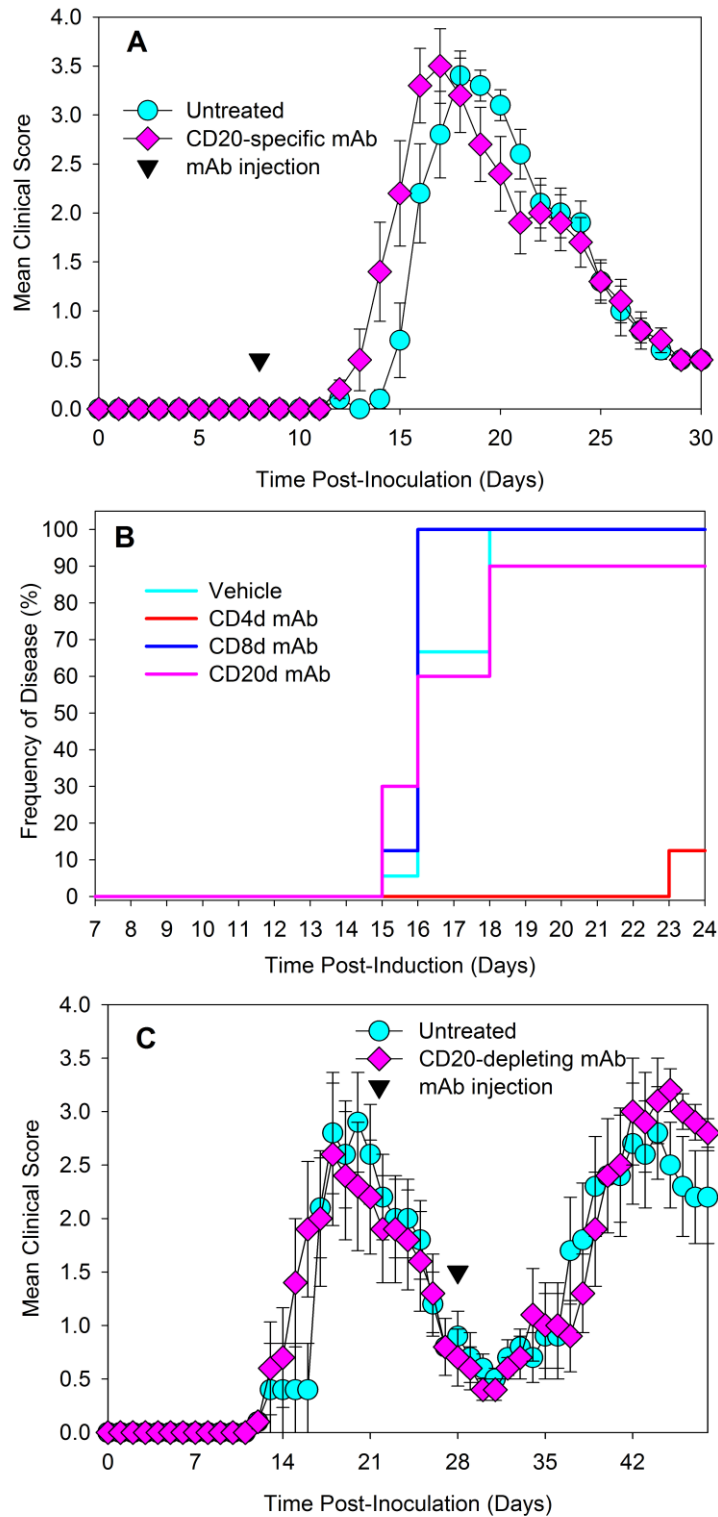
Treatment	Day	No. Relapse	Group Score	EAE Score	Onset Day
Control	v, v	9/9	3.2 ± 0.2	3.2 ± 0.2	35.6 ± 1.6
CD20 mAb	8, v	7/7	3.7 ± 0.1	3.7 ± 0.1	35.0 ± 0.6
CD20 mAb	v, 28	5/5	3.3 ± 0.2	3.3 ± 0.2	34.4 ± 0.5
CD20 mAb	8, 28	5/6	3.1 ± 0.5	3.6 ± 0.2	38.8 ± 5.9

Biozzi ABH mice were injected with spinal cord homogenate on day 0 and 7 with spinal cord homogenate in Freund's adjuvant and repeated on day 29 to induce a relapse. (A) Animals were injected with vehicle (v) or 250µg CD20 depleting mAb on day 8, 28 or day 8 and 28. The results represent the mean maximal clinical score ± SEM, the mean maximal clinical score of animals with relapse ± SEM (EAE score) and day of onset of relapse ± SD. There was no statistically significant differences between the CD20 mAb groups and the control group (Mann Whitney U)

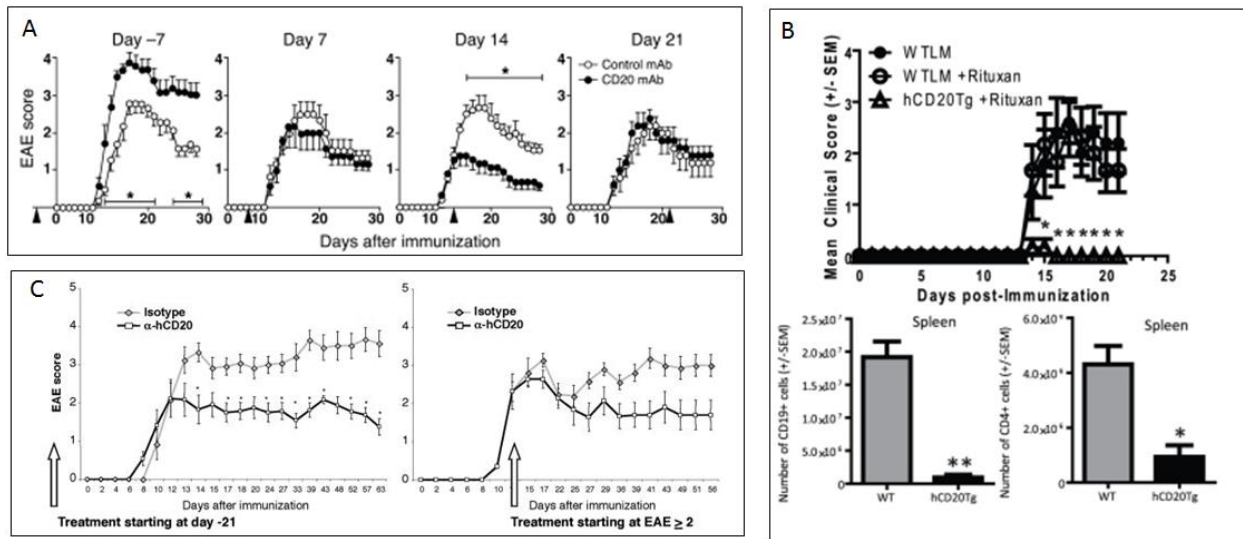
Figure 1. Depletion of B cells by CD20-specific mAb in Biozzi ABH mice.



Following the injection of 250 μ g CD20-specific mAb i.v. (A) Peripheral blood lymphocytes and (B) splenocytes were stained to detect B220 positive B cells within gated lymphocytes. The results represent individual results ($n= 3-7$). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ compared to untreated animals.

Figure 2. CD20 B cell depletion does not inhibit clinical EAE

Animals were injected with spinal cord homogenate on (A-C) day 0 and 7 with spinal cord homogenate in Freund's adjuvant and (C) repeated on day 29 to induce a relapse. (A) Animals were untreated ($n = 10$) or were injected with $250\mu\text{g}$ CD20 depleting mAb on day 8 ($n = 10$) or were (B) treated with vehicle (PBS, $n = 18$) with CD4 depleting (CD4d, $n=16$), CD8 depleting (CD8d $n=8$) or CD20 depleting (CD20d, $n=10$) mAb. (C) Animals were untreated ($n=9$) or were injected with $250\mu\text{g}$ CD20 depleting mAb i.v. on day 28 ($n = 9$). The results represent (A, C) The mean daily clinical score \pm SEM or (B) Kaplan Meier survival curve showing the onset of disease

Figure 3. Influence of CD20 B cell depletion in rodent encephalomyelitis

(A) Wildtype C57BL/6 mice were immunized with MOG³⁵⁻⁵⁵ peptide were injected with mouse CD20 mouse IgG2c B cell depleting mAb at various times before and after immunization. Reproduced with permission from American Association of Clinical Investigation. Matsushita et al. 2008. *J. Clin Invest.* doi:10.1172/JCI36030. (B) Human CD20 transgenic C57BL/6 mice were immunized with recombinant MOG protein and treated with human CD20 reactive chimeric human IgG1 antibody. Spleens were harvested and stained with CD19 or CD4 specific antibodies. Reproduced under the Creative Commons Attribution License. PLoSOne Monson et al. 2011 DoI 10.1371/journal.pone.0017103. (C) Human CD20 transgenic C57BL/6 mice were immunized with recombinant MOG protein and treated with human CD20 reactive mouse IgG2a antibody at weekly intervals from day-21 or following onset. Reproduced with permission from John Wiley & Sons. *Annal Neurol.* Weber et al. 2010. doi: 10.1002/ana.22081.