Supporting Information

Exploration and Evaluation of Therapeutic Efficacy of Drug-Free Macromolecular Therapeutics in Collagen-Induced Rheumatoid Arthritis Mouse Model

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1. Synthesis and characterization of Fab'-MORF1

The 18B12 mAb was used to produce Fab' for synthesis of Fab'-MORF1. Preparation of Fab' from mAb followed an established procedure with adjustments (Figure 2A in main text). First, 10 mg 18B12 mAb was digested into $F(ab')_2$ with 10% (w/w) pepsin (Sigma, St. Louis, MO) in 100 mM citric buffer (pH 4.0). The antibody concentration was 4 mg/ml. This reaction was performed at 37 ^{o}C in water bath for 60 min. The $F(ab')_2$ was purified by 30 kDa cutoff ultra-centrifugal units (Amicon®). Immediately before conjugation, $F(ab')_2$ was reduced to Fab'-SH by 20 mM tris(2-carboxyethyl)phosphine (TCEP, ThermoFisher Scientific, Waltham, MA) in 100 mM citric buffer (pH 5.5). This reaction was performed at 37 ^{o}C for 180 min with antibody fragment concentration of 4 mg ml^{-1}. Again, 10 kDa cutoff ultra-centrifugal units were used to purify Fab' fragment. To prepare the Fab'-MORF1 conjugate, the MORF1 oligo with a 3'-primary amine was reacted with 50x excess $SM(PEG)_2$ (ThermoFisher Scientific, Waltham, MA) to introduce a terminal maleimide group (MORF1-mal). 1.8 mg (200 nmol) MORF1-NH_2 was dissolved in 100 µl phosphate buffered saline (PBS, pH 7.2 and 4.3 mg (10 µmol) $SM(PEG)_2$ was dissolved in 50 µl DMSO. These two solutions were mixed and stirred at room temperature for 2 h and purified by 3 kDa cutoff ultra-centrifugal units. The purified MORF1-mal was conjugated to Fab'-SH *via* a thioether bond to achieve Fab'-MORF1. The feeding ratio of MORF1-mal to Fab' was 1.2 to 1. This reaction was performed at room temperature in pH 6.5 PBS for 3 h and purified by 30 kDa cutoff ultra-centrifugal units. The conjugates were analyzed by size exclusion chromatography (SEC) on an AKTA FPLC system using a Superdex 200 10/300 GL column (GE Healthcare) eluted with PBS (pH 7.2). Bicinchoninic acid (BCA) assay with 18B12 mAb as standard was applied to quantify Fab' content while UV-vis spectrophotometry (NanoDrop ND1000) was used to determine MORF1 content. The absorbance wavelength of MORF1 is 260 nm with extinction coefficient of 278,000 $M^{-1}cm^{-1}$. In the final product, the ratio of MORF1 to Fab' was 1.1 to 1.

2. Synthesis and characterization of P-(MORF2)$_X$

The multivalent P-(MORF2)$_X$ conjugate was synthesized as previously described (Figure 2B in main text). HPMA copolymer grafted with multiple copies of MORF2 was prepared via thiol-ene reaction with optional Cy5.5 labeling. First, HPMA copolymer containing pendent amino groups (P-NH_2) was synthesized by reversible addition-fragmentation chain transfer (RAFT) copolymerization of HPMA with APMA. Optionally, Cy5.5-NHS was used to label P-NH_2. Then polymer precursor containing side chain terminated in maleimide groups (P-mal) was obtained by reaction of P-NH_2 with a heterobifunctional agent, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC). Finally, conjugation was achieved by attaching freshly reduced MORF2 (3′-primary terminated with thiol group) to P-mal.

P-NH_2: HPMA (0.5 g, 3.5 mmol) and APMA (33 mg, 0.18 mmol) were weighed into a one-neck flask equipped with a magnetic stir bar. After purge with nitrogen, the monomers were dissolved with 2.5 mL DI H_2O containing 0.1% (v/v) acetic acid. 4-cyanopentanoic acid dithiobenzoate (CPDB) as chain transfer agent (100 µl with conc. 13 mg/ml in methanol) and 4,4′-azobiscyanovaleric acid (V501) as initiator (100 µl with conc. 4.5 mg ml^{-1} in methanol) were added *via* septum using a syringe. The flask was bubbled with N_2 in ice bath for 30 min then was sealed and put into 70 °C oil bath for 20 h. The copolymer was precipitated into acetone. After centrifugation, the copolymer pellets were dissolved in methanol and re-precipitated into acetone to remove unreacted monomers. The average molecular weight and the polydispersity index (PDI) of the conjugates were determined by SEC on an AKTA FPLC system using a Superose 6 HR10/30 column with sodium acetate buffer containing 30% acetonitrile (pH 6.5) as mobile phase. The molecular weight of P-NH_2 is 87.2 kDa with a PDI of 1.14.

End modification: The dithiobenzoate end group was removed by radical-induced modification using excess of 2,2'-azobis(2.4-dimethyl valeronitrile) (V65) in methanol at 55 °C

for 2 h. After precipitation into acetone and centrifugation, white powder was obtained. The polymer was further purified by dialysis (MWCO 6000–8000) against water over 16 h followed by lyophilization. The amine content in the copolymer P-NH_2 was 366 nmol/mg, determined by ninhydrin test.

To fluorescently label copolymer conjugate, 20 μl Cy5.5-*N*-hydroxysuccinimide ester (Cy5.5-NHS) stock solution in dimethyl sulfoxide (DMSO) was added into the solution of copolymer P-NH_2 in 0.1 M $NaHCO_3$ (pH 8.2) with molar ratio of [Cy5.5]:[NH_2] = 1:16 and stirred in dark at room temperature for 2 h. After reaction, the sample was first precipitated into acetone and then purified using PD-10 Sephadex G25 column (GE Healthcare) to remove free dye. To quantify Cy5.5 substitution, P-NH_2-Cy5.5 was dissolved in PBS and the absorbance at 687 nm was determined on a UV–vis spectrophotometer. The amount of Cy5.5 per chain was calculated based on the Cy5.5 standard curve. The remaining amino groups at the side chain termini were converted to maleimide groups (as described above) to yield P-mal-Cy5.5.

P-(MORF2)x conjugate: To conjugate 3′-thio-modified MORF2 to polymer backbone, the pendant amino groups of P-NH_2 were converted to maleimide groups by reaction with SMCC in dimethylformamide (DMF) in the presence of a tertiary amine *N*,*N*-diisopropylethylamine (DIPEA) at room temperature for 3 h (molar ratio of [NH_2]:[SMCC]:[DIPEA] = 1:1.5:3). The maleimide content of the precursor was 276 nmol/mg, measured by modified Ellman's assay.

3′-disulfide amide derived MORF2 was reduced with TCEP (10 mM, pH 7.2) in PBS (pH 7.2) at 37 °C for 60 min to generate free thiol end group. Then the resultant fragment 4-thiolbutanamide and excess TCEP were removed by ultrafiltration (3 kDa MWCO) and washed three times with 10 mM NaH_2PO_4 (pH 5.1). The freshly prepared MORF2-SH was added into P-mal/P-mal-Cy5.5 solution in PBS and kept stirring at room temperature for 3 h. At the end, unreacted MORF2-SH was removed by ultrafiltration (30 kDa MWCO), washed four times with DI water and freeze-dried.

Graft copolymers with different valences of MORF2 were obtained by varying the feed ratio of thiol and maleimide groups. The content of MORF2 in the conjugates P-(MORF2)$_X$ or P-(MORF2)$_X$-Cy5.5 was determined by UV–visible spectrophotometry at 260 nm in 0.1 N HCl, and calculated based on the molar absorptivity of MORF2 (252120 M^{-1} cm^{-1}, provided by the manufacturer Gene Tools). MORF2 valence of P-(MORF2)$_X$ was 9.4 while that of P-Cy5.5-(MORF2)$_X$ was 8.9.

3. B cell depletion analysis

Cells were collected from spleen, lymph nodes, bone marrow and blood on day -4, 0, 30, 60, 90 and 120. The cells were stained with anti-B220-FITC and anti-IgG-PE Cy7 and analyzed by flow cytometry (see main text for details). Data from day 60 are depicted in Figure 4; data from day -4, 0, 30, 90, and 120 are shown below (Figures S1-S5).

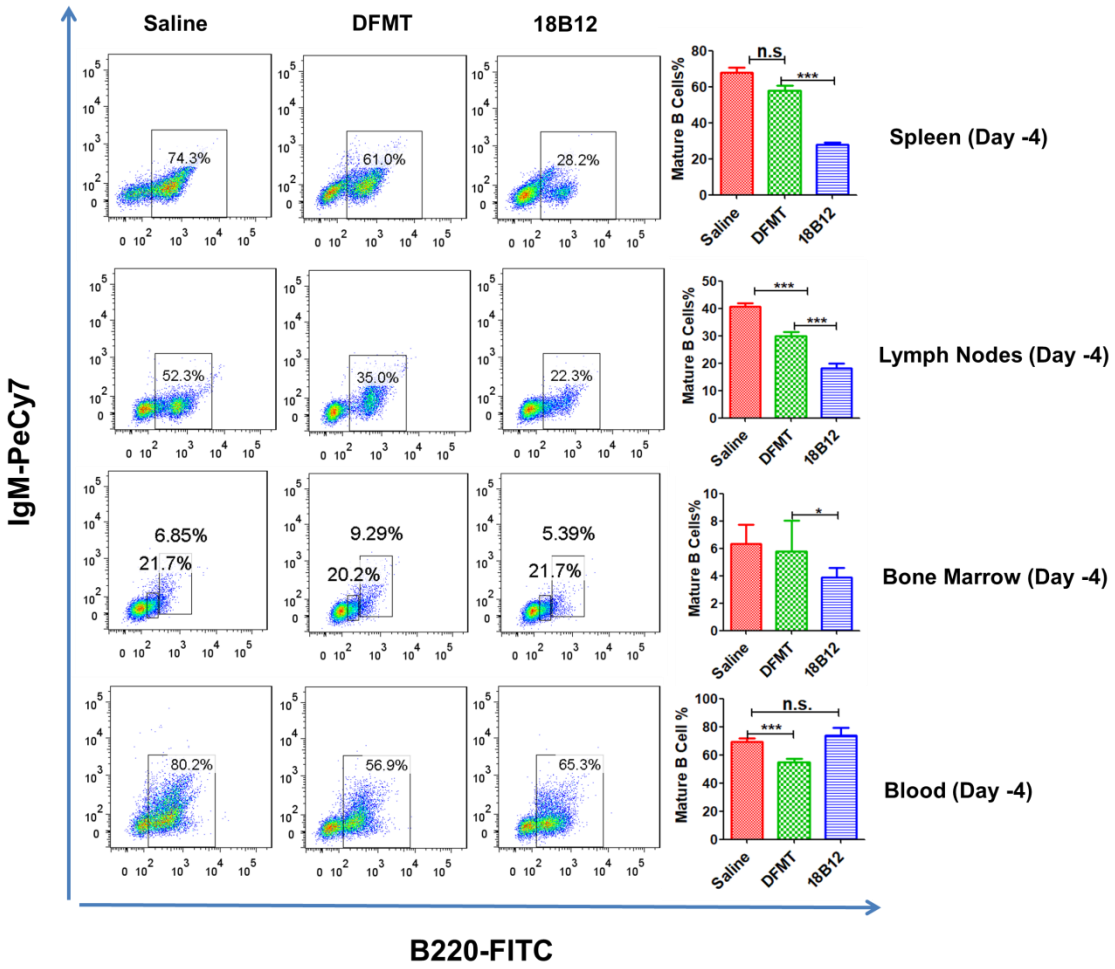

Figure S1. Mature B cell percentage in tissues collected on day -4. At day -4 (three days after first treatment administration), spleen, lymph nodes, bone marrow and blood were collected for cell staining followed by flow cytometry to determine mature B cell percentage. Both, DFMT and 18B12 mAb treatments began to show depletion effect in cells from spleen and lymph nodes though 18B12 mAb had a higher B cell depletion level. 18B12 mAb treatment also showed efficiency in cells from bone marrow while DFMT treatment showed positive difference in cells from blood.

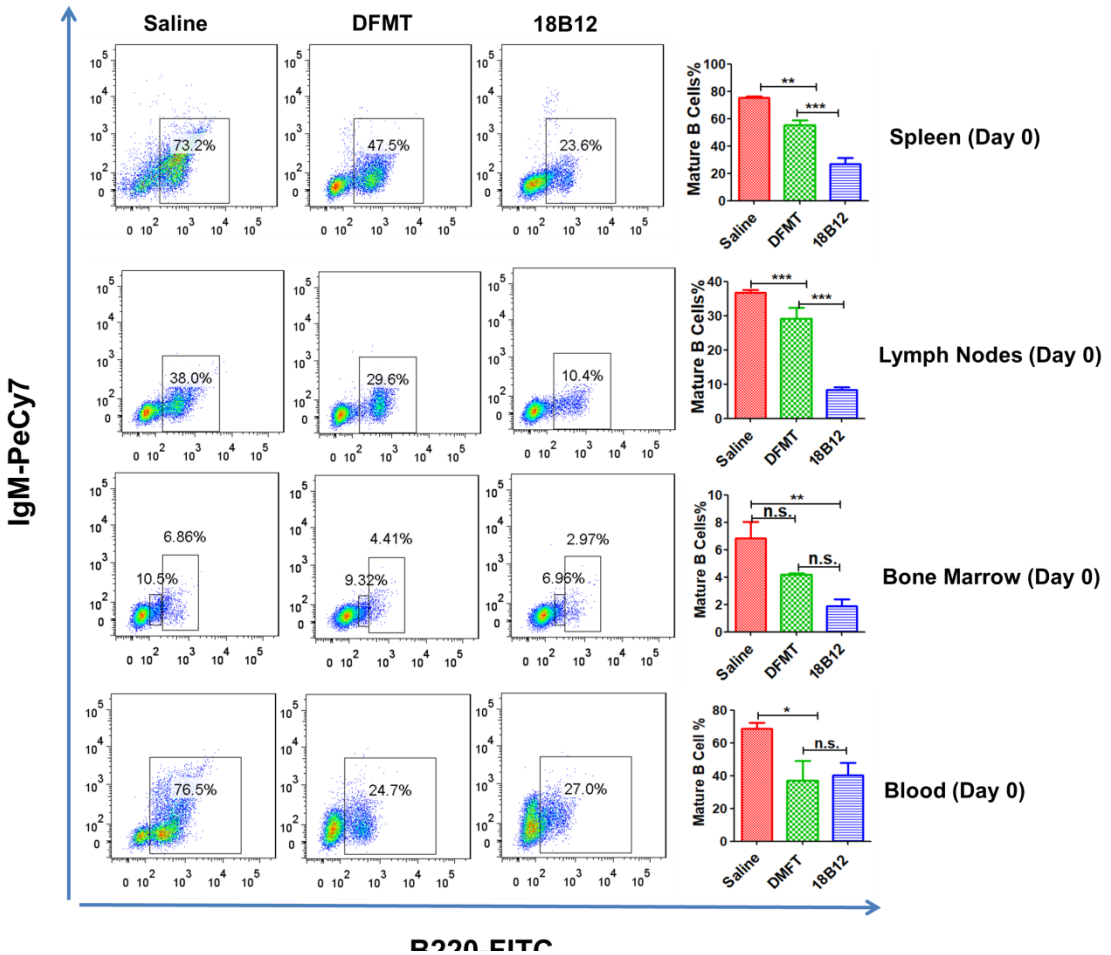

Figure S2. Mature B cell percentage in tissues collected on day 0. Spleen, lymph nodes, bone marrow and blood were collected for cell staining followed by flow cytometry to determine mature B cell percentage. At seven days after first treatment administration, DFMT killed part of mature B cells in the spleen, lymph nodes, and blood. While the mature B cell portions killed by DFMT were smaller in spleen and lymph nodes, it achieved comparable depletion levels with 18B12 mAb treatment in blood.

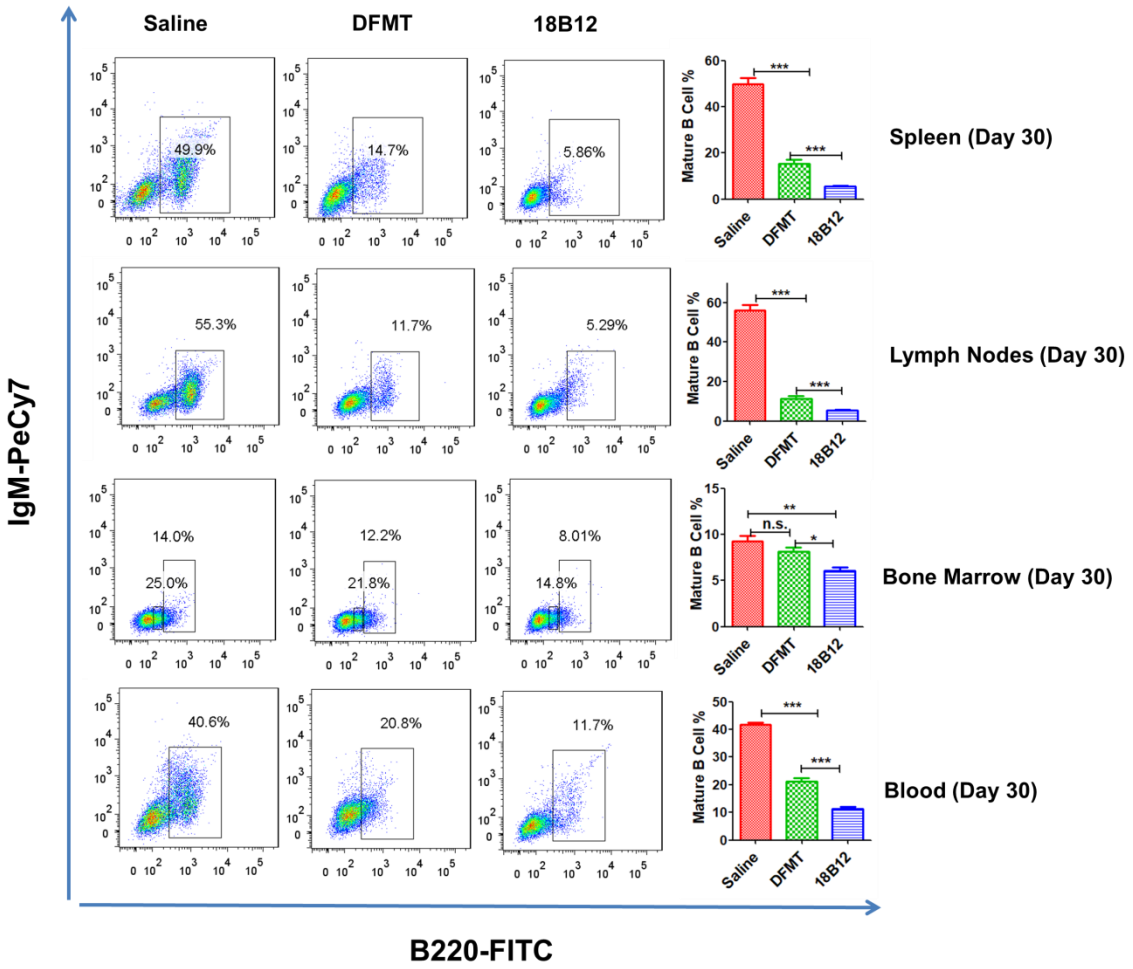

Figure S3. Mature B cell percentage in tissues collected on day 30. Spleen, lymph nodes, bone marrow and blood were collected for cell staining followed by flow cytometry to determine mature B cell percentage. At this time point, four treatments have been administrated. DFMT treatments achieved its maximum B cell depletion level during the 120 days experiment. Except in bone marrow, DFMT treatments killed 50%-80% mature B cells in spleen, lymph nodes and blood. The difference in depletion levels between DFMT and 18B12 mAb treatments was reduced considerably though it still existed.

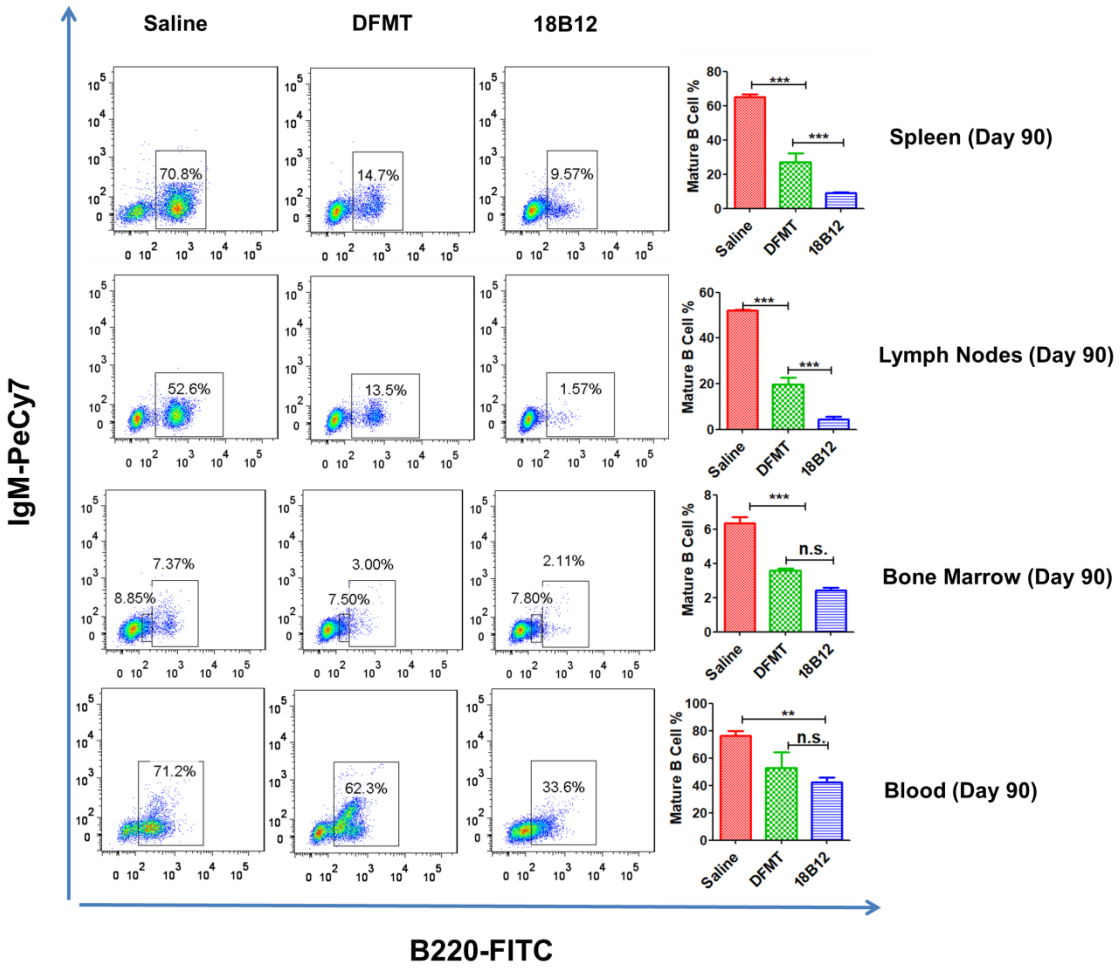

Figure S4. Mature B cell percentage in tissues collected on day 90. Spleen, lymph nodes, bone marrow and blood were collected for cell staining followed by flow cytometry to determine mature B cell percentage. At this time point, all six treatments have been administered. The mature B cell percentages from spleen and lymph nodes in DFMT treated group reversed back slightly but still kept relatively low, indicating the depletion effect was still effective (~60% depleted). In bone marrow and blood, about 40% mature B cells were depleted by DFMT treatment.

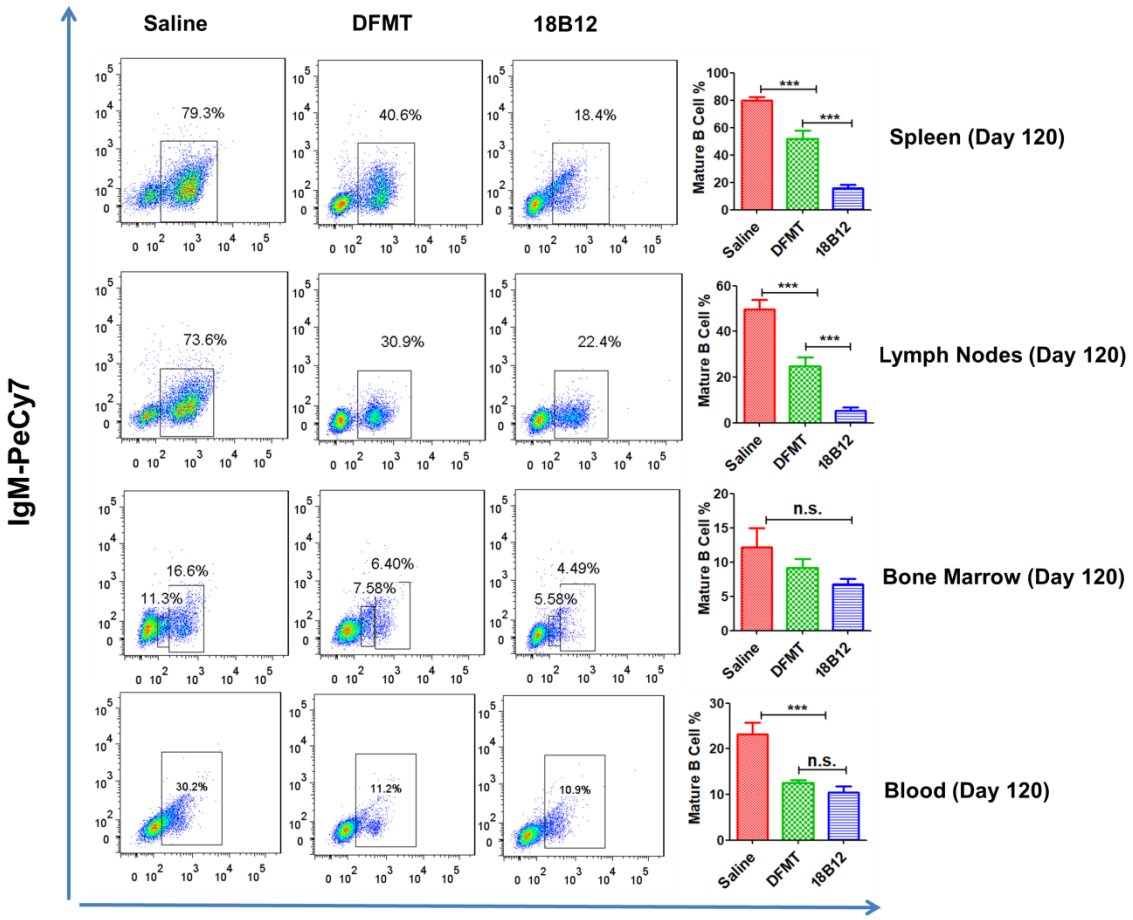

Figure S5. Mature B cell percentage in tissues collected on day 120. Spleen, lymph nodes, bone marrow and blood were collected for cell staining followed by flow cytometry to determine mature B cell percentage. At this time point, it has been 50 days since the last treatment. B cells were depleted about 50% by DFMT in lymph nodes and blood while the depletion level was around 25% in the spleen. No statistically significant difference was observed in bone marrow between the treatment groups. 18B12 mAb treatment kept good depletion levels due a relatively long half-life.