ISBT Science Series (2015) 0, 1–10

1 ORIGINAL PAPER xx-xx-xx 12

Different sensitivities to calcineurin inhibitors between B cells responding to blood group A and B antigens

2 M. Yamashita,[1] Y. Tanaka,[1] H. Tazawa,[2] H. Sakai[1] & H. Ohdan[1]

[1]Department of Gastroenterological and Transplant Surgery, Applied Life Sciences, Institute of Biomedical & Health Sciences, Hiroshima University, Hiroshima, Japan

[2]Department of Surgery, Chugoku Rosai Hospital, Hiroshima, Japan

Background and Objectives We evaluated the phenotypic and functional distinction between B cells with receptors for human blood group A and B determinants in α-galactosyltransferase-deficient ($GalT^{-/-}$) mice.

Materials and Methods $GalT^{-/-}$ mice were immunized with human blood group A or B red blood cells (RBCs), and the anti-A or B anti-bodies (Abs) were determined in their sera. B cells with receptors for group A and B epitopes were identified by the use of synthetic A and B determinants.

Results The anti-A and anti-B Ab levels were comparably elevated in the sera of $GalT^{-/-}$ mice after the immunization with A/B-RBCs. B cells bearing receptors recognizing B epitopes exhibited the $CD5^{dim/-}$ $CD11b^{+}$ B-1b-ell phenotype, whereas those with receptors for A epitopes showed the $CD5^{+}CD11b^{+}$ B-1a-cell phenotype. Myeloid differentiation factor 88 (MyD88) levels increased in B cells with anti-B receptors, indicating a contribution of Toll-like receptor (TLR) signalling, whereas no such elevation was detected in B cells with anti-A receptors. Calcineurin inhibitor (CNI) treatment prevented the elevation of anti-A Ab levels after immunization, but did not affect anti-B Ab levels.

Conclusions These findings indicate different sensitivities of B cells with blood group A and B antigens in the response to CNIs.

Key words: ABO-incompatible transplantation, antibody mediate rejection, B cells, carbohydrates

Introduction

The immunodominant structures of blood group A and B antigens are defined as N-acetyl-D-galactosamine (GalNAc) α1-3(Fuca1-2)Gal and Gala1-3(Fuca1-2)Gal, respectively [1, 2]. Functional A and B alleles encode the A and B transferases, which transfer GalNAc and galactose to the same acceptor H substrates (Fuca1-2Gal), and synthesize the A and B antigenic determinants, respectively. Although the use of ABO-incompatible donor organs is an alternative solution to combat the shortage of donor organs for transplantation, the production of antibodies (Abs) against the blood group A or B carbohydrate epitopes in recipient serum is a major impediment to the success of this approach. Plasmapheresis or plasma exchange, splenectomy, and anti-B-cell immunosuppressant treatment, including rituximab, in the recipient are widely adopted strategies to remove anti-A, anti-B, or both Abs and prevent the Ab-mediated rejection (AMR) of ABO-incompatible organ grafts [3, 4]. Owing to these therapeutic approaches, graft survival in ABO-incompatible organ transplantation is not currently inferior to that in ABO-compatible transplantation [5, 6]. However, the use of non-specific immunosuppressive treatments is likely to increase the incidence of infectious complications such as cytomegalovirus infection [7, 8]. Therefore, optimizing the immunosuppressive regimen might further improve the outcome of ABO-incompatible organ transplantation. For this purpose, identification of the B cell

Correspondence: Hideki Ohdan, Department of Gastroenterological and Transplant Surgery, Applied Life Sciences, Institute of Biomedical & Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. E-mail: hohdan@hiroshima-u.ac.jp

VOXS | 12233 | WILEY | Dispatch: 20.10.15 | CE: Shanthi
Journal Code | Manuscript No. | No. of pages: 10 | PE: Sharmila

clones responding to A/B carbohydrates and determination of their sensitivity to immunosuppressants is required; however, the limited availability of appropriate animal models has thus far been an obstacle in conducting comprehensive evaluations of B cells.

Previous experiments by our group and other researchers showed significant levels of naturally occurring anti-human blood group A-determinant Abs but undetectable levels of anti-human blood group B-determinant Abs in the sera from several strains of mice [9, 10]. The absence of anti-B Abs might be explained by the presence of B-like structures in mouse cells, that is Galα1-3Galβ1-4GlcNAc (Galα1-3Gal) carbohydrate residues, which are completely lacking in human cells because of α-galactosyltransferase deficiency. The inborn expression of Galα1-3Gal in various cells in mice likely maintains B-cell tolerance towards B determinants. Supporting this hypothesis, in this present study, we found that α-galactosyltransferase-deficient ($GalT^{-/-}$) mice, which are completely deficient in Gal epitopes, showed elevated anti-A and anti-B Abs in the sera after immunization with AB-RBCs, thereby resembling the response in blood group O humans. Utilizing these $GalT^{-/-}$ mice as an animal model, we further evaluated the phenotypic and functional distinction between B cells with receptors for human blood group B determinants and those with receptors for A determinants.

Materials and methods

Mice

Balb/c and C57BL/6J (B6) mice were purchased from CLEA Japan (Tokyo, Japan). The $GalT^{-/-}$ mice in the B6 background, which completely lack Gal expression, were kindly provided by Dr. M. Sykes, Massachusetts General Hospital, MA, USA [11]. All the mice were used at 8–12 weeks of age. All experiments were approved by the Institutional Review Board of Hiroshima University and were conducted in accord with the guidelines of the National Institutes of Health (NIH publication No. 86–23, revised 1996).

In vivo immunization with human RBCs

Fresh human peripheral blood samples were collected from volunteers with blood group A, B or AB. Informed consent was obtained from all volunteers in accordance with the Declaration of Helsinki. For *in vivo* immunization, a suspension of 1×10^9 human A- or B-RBCs in 1 ml phosphate-buffered saline (PBS) was injected into the peritoneal cavity (PerC) of each mouse twice in 1-week intervals. When indicated, cyclosporine A (CsA; Novartis, Basel, Switzerland) was administered daily intraperitoneally at 10 mg/kg/day for 4 weeks. One and 2 weeks after commencing CsA treatment, the $GalT^{-/-}$ mice were immunized with human blood group AB-RBCs.

Cell preparation and flow cytometry (FCM) analyses

After harvesting the spleens (Spls) from mice and erythrocyte lysis with an ammonium chloride/potassium solution, the splenocytes were suspended in PBS. PerC cells were isolated by peritoneal lavage using cold PBS. B cells with receptors for human blood group A and B trisaccharides were detected using fluorescein isothiocyanate (FITC)-conjugated GalNAca1–3Fuca1–2Gal–bovine serum albumin (BSA) (A-BSA: Dextra, Reading, UK), α-l-Fuc-(1→2)-[α-D-Gal-(1→3)]-D-Gal-BSA (B-BSA: Dextra, Reading, UK) and control FITC-conjugated BSA (Roche, Indianapolis, IN). FITC conjugation of A-BSA, B-BSA, and BSA was performed using a SureLINK Fluorescein Labeling Kit (KPL, Gaithersburg, MD, USA). We incubated 10^6 Spl and PerC cells/100 μl with 10 μg/10 μl FITC-A- or B-BSA or control FITC-BSA in medium for 1 h at 4°C. Non-specific Fcγ receptor binding of labelled Abs was blocked by anti-mouse CD16/32 (2·4G2). The cells were further stained with biotin-conjugated anti-mouse IgM (R6-60·2), phycoerythrin (PE)-conjugated anti-mouse CD11b (M1/70), anti-mouse CD19 (1D3) or anti-mouse CD5 (53–7·3) monoclonal antibodies (mAbs) to classify B-cell subsets. The biotinylated mAb was visualized using allophycocyanin–streptavidin. Isotype-matched irrelevant mAbs were used as a control. All mAbs were purchased from BD PharMingen (San Diego, CA, USA). Dead cells were excluded from the analysis based on light scattering and staining with propidium iodide.

Myeloid differentiation factor 88 (MyD88) expression was detected by intracellular staining using the affinity-purified polyclonal goat anti-mouse/rat MyD88 Ab (R&D Systems, Minneapolis, MN, USA) followed by allophycocyanin-conjugated anti-goat IgG secondary Ab (clone SC-2024; R&D Systems, Minneapolis, MN, USA). Cells were fixed and permeabilized in Cytofix/Cytoperm buffers (BD Biosciences) following the manufacturer's protocol, and permeabilized with saponin.

Four-colour FCM was performed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). Data were analysed using FlowJo 7.6.5 software (TreeStar, San Carlos, CA, USA).

Cell sorting

Spl cells were sorted under sterile conditions using a SORPAria cell sorter (Becton Dickinson, Mountain View, CA). Sorted cells were reanalysed for purity on a

FACSCalibur flow cytometer, and were immediately resuspended in culture medium and applied to ELISPOT plates precoated with B-BSA or anti-mouse IgM to determine the frequency of IgM-producing cells.

Enzyme-linked immunosorbent assay (ELISA)

The levels of anti-A or -B-specific Abs in sera were determined by ELISA as described previously [10, 12]. In brief, ELISA plates were coated with 5 μg/ml synthetic A- or B-BSA (Dextra) or control BSA (Roche). Diluted serum samples were incubated in the plates, and bound Abs were detected using horseradish peroxidase-conjugated goat anti-mouse IgG/IgM-specific Abs (IgM: KPL, Guilford, UK, IgG: Jackson ImmunoReserch). Colour development was achieved using 0·1 mg/ml *O*-phenylenediamine (Sigma, St. Louis, MO, USA). The reaction was stopped by the addition of 3 M H_2SO_4, and absorbance was measured at 492 nm.

Enzyme-linked immunospot (ELISPOT) assay

An ELISPOT assay to detect Ab-producing cells was performed as described previously [13]. In brief, nitrocellulose membranes of a 96-well filtration plate (Millipore, Bedford, MA, USA) were coated with 5 μg/ml B-BSA or control BSA for detecting anti-B IgM/IgG-producing cells. Serial dilutions of the cell suspension in Iscove's modified Dulbecco medium (IMDM; Sigma) supplemented with 0·4% BSA, 5 μg/ml insulin (Sigma), 5 μg/ml transferrin (Sigma), 5 ng/ml sodium selenite (Sigma), 50 μM 2-mercaptoethanol (Katayama, Osaka, Japan), and 1 μg/ml gentamicin were added to wells. After 24 h of culture, bound Abs were detected using horseradish peroxidase-conjugated goat anti-mouse IgM/IgG Abs (IgM: KPL, IgG: Jackson ImmunoReserch), followed by colour development with 3-amino-9-ethyl carbazole (Sigma).

In vitro B-cell proliferation assay

The resting B cells were isolated from the splenocytes of untreated Balb/c mice by negative selection using a B-cell isolation kit and an automagnetic-associated cell sorter (MACS) (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. The B cells were labelled with 5 μM of 5-(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA), as described previously [14]. The CFSE-labelled cells were cultured in Roswell Park Memorial Institute (RPMI; Nacalai Tesque, Kyoto, Japan) culture medium, 5 μM 2-mercaptoethanol, 1% HEPES buffer (GIBCO, Grand Island, NY, USA), and 100 IU/ml penicillin/100 μg/ml streptomycin (GIBCO). To differentiate resting B cells to B-1 cells, the B cells were treated with 20 μg/ml of goat anti-mouse IgM F(ab')$_2$ (Jackson Immuno Research Laboratories, West Grove, PA); an analogue of T-cell-independent type 2 (TI-2) antigens (Ags). When indicated, the B cells were treated with a combination of 20 μg/ml of anti-IgM F(ab')$_2$ and 10 μg/ml *Escherichia coli* lipopolysaccharide (LPS; serotype 0111:B4; Sigma), an agonist of Toll-like receptor (TLR)4. To differentiate resting B cells to B-2 cells, the B-cells were activated with irradiated feeder cells of 3T3 fibroblasts transfected with CD40L and B cell activation factor (BAFF) provided by Dr. Daisuke Kitamura, Division of Molecular Biology, Research Institute for Biological Sciences, Tokyo University of Science [15] and 0·02 μg/ml recombinant mouse IL-4 (R&D Systems, Minneapolis, MN, USA). The cultures were incubated for 3 days. The cultivated cells were stained with PE-conjugated CD19 (1D3) or biotinylated CD5 (53-7·3) mAbs. The biotinylated mAbs were visualized using allophycocyanin–streptavidin, followed by 4-colour FCM analyses. Dead cells, identified by light scatter and propidium iodide staining, were excluded from the analyses. The precursor frequency (PF) and mitotic index (MI) were quantitatively estimated using a previously described method [16].

Statistical analysis

The results were statistically analysed using the unpaired Student's *t*-test of means or analysis of variance (ANOVA). A *P*-value of <0.05 was considered statistically significant.

Results

B cells with receptor for blood group B-carbohydrate showed the sIgM^{high} CD19^{+} CD11b^{+} CD5$^{-/dim}$ B-1b phenotype

We attempted to identify B cells with sIgM that binds to B carbohydrate determinants in *GalT*$^{-/-}$ and wild-type (WT) B6 mice immunized with A- or B-RBCs. Cells from the Spl and PerC, where B-1 cells are predominantly located, were stained with FITC-labelled synthetic A-or B-BSA together with anti sIgM mAbs and subjected to FCM analysis.

In both the WT *GalT*$^{+/+}$ *and GalT*$^{-/-}$ mice, the frequency of cells with sIgM that bound to B-BSA (B-BSA-binding B cells) in the PerC was higher than that in the Spl (Fig. 1a and b). The frequencies B-BSA-binding B cells in both the Spl and PerC of *GalT*$^{-/-}$ mice were higher than those of WT mice. To characterize the phenotype of B cells recognizing B-determinants, B-BSA-binding B cells in the immunized *GalT*$^{-/-}$ mice were selected by gating and were assessed based on the expression of various surface markers with multicolour FCM analysis. Consistent with our previous findings [10], A-BSA-binding B cells showed an

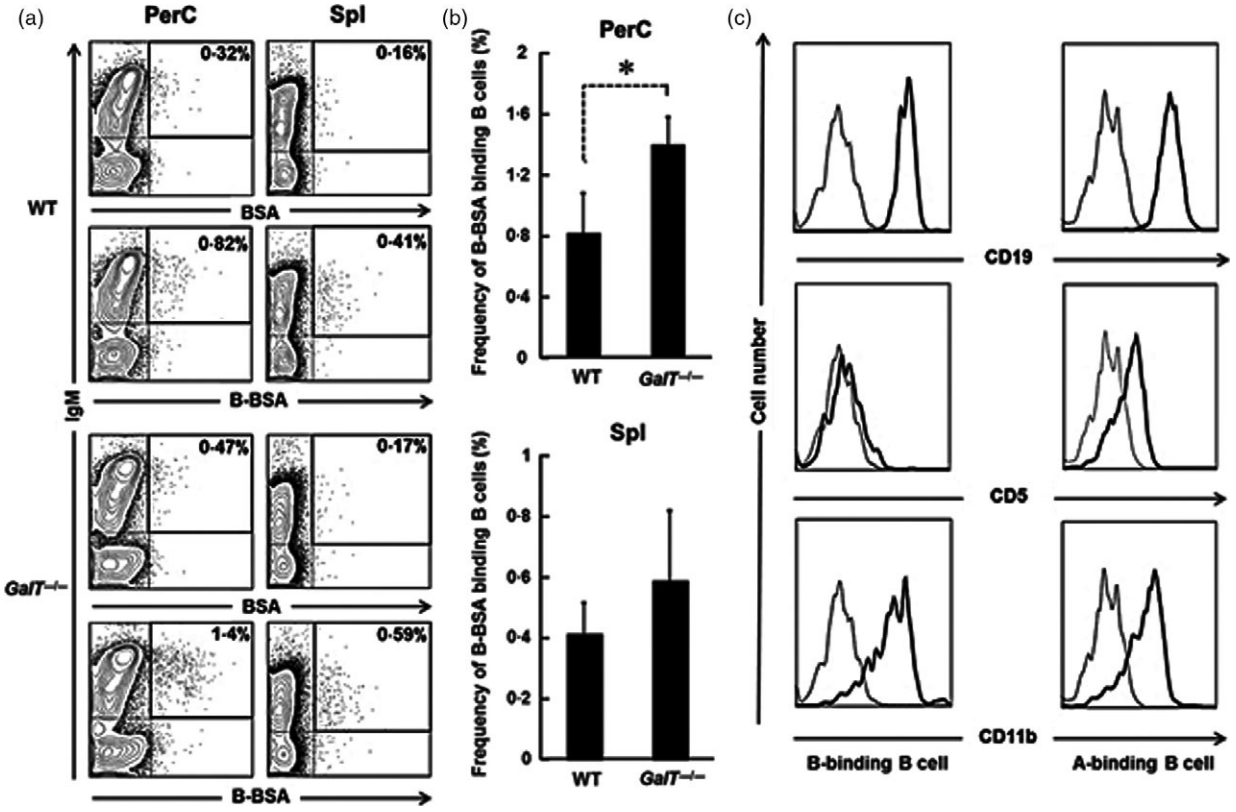


Fig. 1 Phenotypic and functional properties of B cells with receptors for blood group B carbohydrate determinants in mice. B6 *GalT*$^{-/-}$ (n = 5) and B6 wild-type (WT; n = 5) mice were immunized with human blood group B-RBCs (1 × 10^9/mouse) once a week four times. To determine the proportion of B cells with receptors for group B carbohydrates, PerC and Spl cells were prepared from B6 *GalT*$^{-/-}$ and B6 WT mice at 7 days after the last immunization. The cells were stained with either FITC-labelled B-BSA or control FITC-labelled BSA together with biotinylated anti-mouse IgM mAb, along with various PE-conjugated mAbs (anti-CD5, CD19, or CD11b mAb). To evaluate the statistical significance between groups, 10^6 PerC cells and 10^6 Spl cells were collected for each sample. (a) Representative FCM results of B-BSA-binding B cells are shown. The percentages in the figure represent the proportion among total IgM$^+$ cells. (b) The frequency of B-BSA-binding B cells was significantly increased in the PerC in B6 *GalT*$^{-/-}$ mice. *P < 0·05 compared to the data from B6 WT mice. (c) B-BSA-binding sIgM$^+$ B cells were selected by gating and analysed for the expression of various B-cell markers. Dotted lines represent negative control staining with isotype-matched Abs. The results were consistent in three independent experiments.

IgM^{high}, CD19$^+$, CD11b$^+$ and CD5$^+$ phenotype, which is consistent with the properties of the B-1a like subpopulation (Fig. 1c). By contrast, B-BSA-binding B cells in the PerC showed an IgM^{high}, CD19$^+$, CD11b$^+$, and CD5$^{-/dim}$ phenotype, which is consistent with the properties of the B-1b-like subpopulation. Thus, B cells responding to blood group B epitopes were phenotypically distinct from B-cells responding to blood group A epitopes.

In response to immunization with A-RBCs (via intraperitoneal injection of 1 × 10^9 RBCs), the serum levels of anti-A Abs were remarkably elevated in both WT and *GalT*$^{-/-}$ mice (Fig. 2a). In contrast, the elevation of serum levels of anti-B Abs in WT mice never reached the same level as those in *GalT*$^{-/-}$ mice in response to immunization with B-RBCs. Consistently, an ELISPOT assay conducted 14 days after the immunization showed that the frequency of anti-B Ab-producing cells (both IgM and IgG) in the Spl was significantly higher in *GalT*$^{-/-}$ mice than that in WT mice (Fig. 2b). The combined FCM sorting and ELISPOT assay revealed that anti-B Ab (IgM)-producing cells were greatly enriched in the sorted B-BSA-binding IgM$^+$ population and were undetectable in the sorted B-BSA-nonbinding IgM$^+$ population (Fig. 3), demonstrating that the B-BSA-binding Spl cells included all anti-B Ab-producing cells. This result verified the specificity of the B-BSA ligand for the corresponding receptors on B cells. Hence, *GalT*$^{-/-}$ mice are preferable to WT mice for investigating B cells that recognize B-determinants.

B cells responding to blood group B-antigens express MyD88

Several gram-negative bacteria express histo-blood group antigens in their LPS *O*-chain [17, 18]. Previous chemical and serological investigations have demonstrated that the strong blood group activity of *E. coli* O86 is derived from

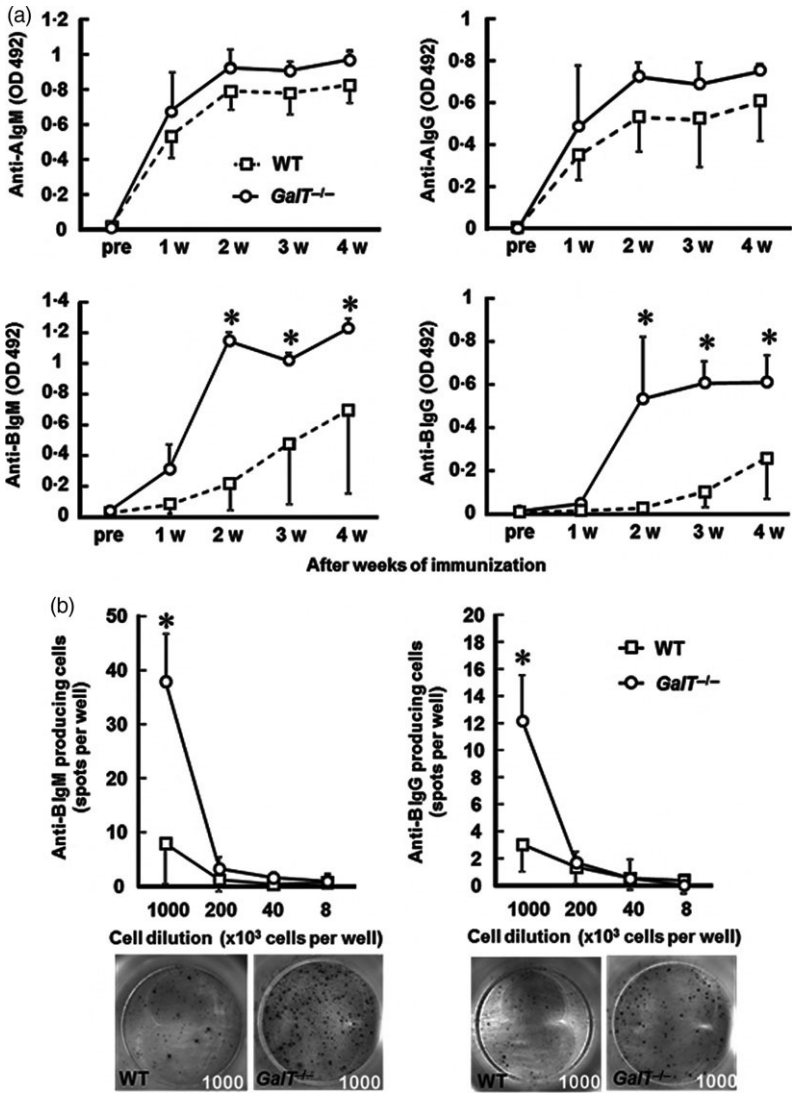


Fig. 2 The anti-A and anti-B Ab levels were comparably elevated in the sera of *GalT*$^{-/-}$ mice after immunization with A/B-RBCs. The B6 *GalT*$^{-/-}$ mice ($n = 5$) and WT mice ($n = 5$) were immunized with human blood group A or B-RBCs (1×10^9/mouse) once a week four times. (a) The serum anti-A- and anti-B-specific IgM and IgG levels, were respectively, determined using ELISA at 4 weeks after immunization. The kinetics of the serum anti-A and anti-B IgM/IgG titres in B6 *GalT*$^{-/-}$ and WT mice are shown (5 × -diluted serum was used). (b) To quantify the frequency of anti-B Ab-producing cells in the Spl, we performed an ELISPOT assay. The Spl cells were seeded in 96-well filtration plate coated with either 5 μg/ml B-BSA or control BSA. Representative images of ELISPOT wells and the frequency of Anti-B Ab-producing cells are shown. The frequency of anti-B-Ab-producing cells was determined based on the average of the number of red plaques in duplicate wells of serially diluted cells. Number in each picture refers to the 1000 cells seeded per well. The results shown are the average values ± SEM for the individual groups. *GalT*$^{-/-}$ mice $n = 5$; WT mice $n = 5$. $^*P < 0{\cdot}05$.

LPS containing the blood group B trisaccharide partial structure [19, 20]. This fact prompted us to investigate whether blood group B carbohydrate determinants activate TLR4, which recognizes LPS from gram-negative bacteria, resulting in activation of the MyD88-dependent signalling pathway. For this purpose, *GalT*$^{-/-}$ mice were immunized with either A-RBCs or B-RBCs (via intraperitoneal injection of 1×10^9 RBCs) twice in 1-week intervals. FCM analyses revealed strong MyD88 expression in B-BSA-binding B cells in the Spl cells of *GalT*$^{-/-}$ mice immunized with B-RBCs, whereas MyD88 was barely detectable in A-BSA-binding B cells in the Spl cells of *GalT*$^{-/-}$ mice immunized with A-RBCs at 12 h after the second immunization (Fig. 4). Hence, B cells responding to blood group B-antigens were likely activated via TLR4-MyD88 signalling, distinguishing them from B cells responding to blood group A-antigens.

LPS/TLR4 signalling impaired the inhibitory effects of CsA on anti-IgM F(ab')$_2$-induced B-cell proliferation

We previously demonstrated that mouse's resting splenic B cells treated with anti-IgM F(ab')$_2$, an analogue of TI-2 Ags, differentiated into CD5$^+$ B cells *in vitro*, whereas those treated with CD40L and IL-4, which provide thymus-dependent inductive signals, differentiated into CD5$^-$ B-cells [21]. We also observed that a clinically relevant concentration of CsA (100 ng/ml) completely blocked CD5$^+$ B-cell proliferation but did not inhibit CD5$^-$ B-cell proliferation. Prior to treatments with anti-IgM F(ab')$_2$ or BAFF/CD40L-expressing feeder cells and IL-4, resting B cells were labelled with CFSE, thereby enabling phenotypic analysis of the proliferating cells by multicolour FCM analysis. As previously demonstrated,

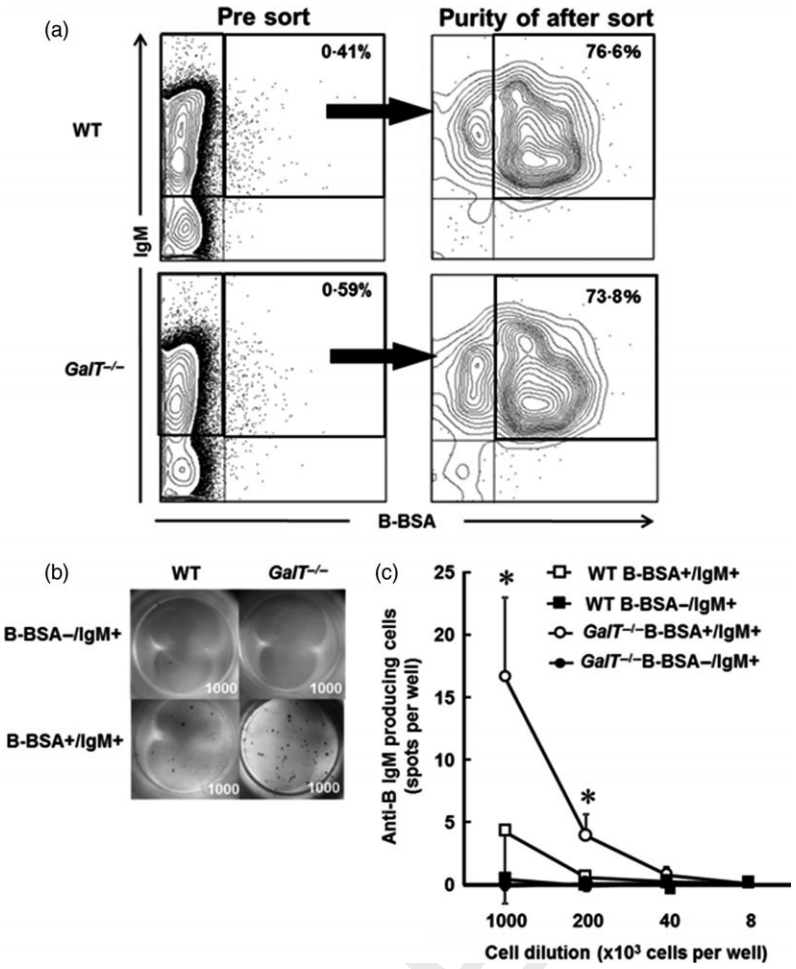


Fig. 3 Anti-B Ab-producing cells were greatly enriched in the sorted B-BSA-binding Spl B cells. The Spl cells were prepared from $GalT^{-/-}$ mice ($n = 5$) and WT mice ($n = 5$) after immunization with human blood group B-RBC (1×10^9/mouse) once a week four times. (a) The pooled cells were stained with FITC-labelled B-BSA or control FITC-labelled BSA together with biotinylated anti-mouse IgM mAb. B-BSA-binding B cells were sorted under sterile conditions. The sorted cells were reanalysed for purity. (b) The sorted B-BSA$^-$/IgM$^+$ and B-BSA$^+$/IgM$^+$ cells were seeded in a 96-well filtration plate coated with 5 μg/ml B-BSA or control BSA. Representative images of ELISPOT wells are shown. Number in each picture refers to the 1000 cells seeded per well. (c) The results of the frequency of anti-B Ab-producing cells shown are the average $\pm$ SEM calculated from the number of red spots in tetracate wells. $^*P < 0{\cdot}05$ between B-BSA$^-$/IgM$^+$ and B-BSA$^+$/IgM$^+$ cells in each of $GalT^{-/-}$ mice and B6 WT mice.

CD19$^+$ B cells from Balb/c mouse splenocytes stimulated with anti-IgM F(ab')$_2$ or with BAFF/CD40L/IL-4 vigorously proliferated after 3 days in culture, but only the former developed into CD5$^+$ B cells, consistent with the B-1a-cell phenotype. The addition of CsA at the start of culture significantly inhibited the B-cell proliferation induced by anti-IgM F(ab')$_2$ in a dose-dependent manner; however, it had no effect on BAFF/CD40L/IL-4-induced B-cell proliferation. Moreover, CD19$^+$ B-cells stimulated with anti-IgM F(ab')$_2$ together with LPS, which provided TLR4-signalling, differentiated into CD5^{dim} B cells, consistent with the B-1b cell phenotype (Fig. 5a). The addition of CsA also inhibited the B cell proliferation induced by both anti-IgM F(ab')$_2$ and LPS, but the inhibitory effect was inferior to that observed with respect to B cell proliferation induced by anti-IgM F(ab')$_2$ alone (Fig. 5b).

CsA inhibited anti-A Ab production, but not anti-B Ab production

To investigate the resistance to CsA's inhibitory effects on Ab production in response to blood group B-antigens, which likely activates B-1b cells via TLR4-MyD88 signaling, $GalT^{-/-}$ mice were daily administered intraperitoneally with CsA at 10 mg/kg/day for 4 weeks. One and 2 weeks after commencing CsA treatment, $GalT^{-/-}$ mice were immunized with human blood group AB-RBCs. The CsA treatment completely prevented the elevation of anti-A Ab serum levels, even after immunization with AB-RBCs; however, it did not have any effect on anti-B Ab levels (Fig. 6). This result indicates that the sensitivity of CsA to B cells responding to B-Ags markedly differs from that of B cells responding to A-Ags.

Discussion

Mature B cells are heterogeneous and belong to four subsets in mice: follicular (FO), marginal zone (MZ), CD5$^+$ B-1a and CD5$^-$ B-1b. FO B cells recirculate in the blood among the lymphoid follicles and mount Ab responses against T-cell-dependent protein Ags [22]. MZ B cells, which are localized proximal to the marginal sinus of the Spl, as well as B-1a and B-1b cells, which are most abundant in the peritoneal and pleural cavities, generate

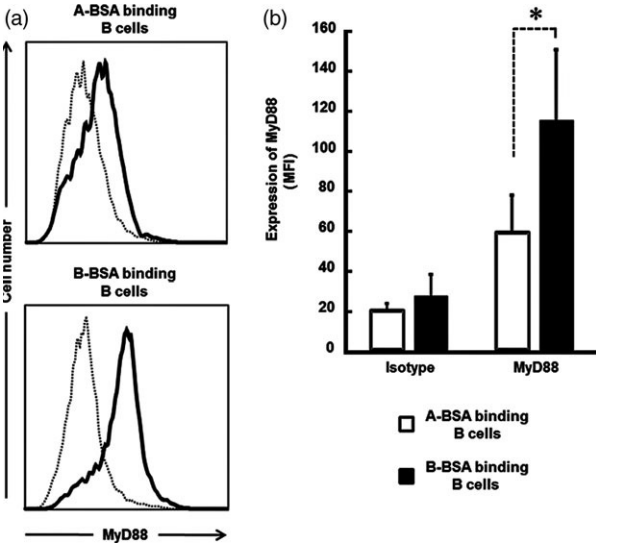


Fig. 4 MyD88 showed strong expression in B-BSA-binding B cells in the Spl cells of *GalT*$^{-/-}$ mice immunized with B-RBCs. B6 *GalT*$^{-/-}$ mice were immunized with human blood group A-RBCs (n = 5) or B-RBCs (n = 5) (1×10^9/mouse) once a week twice. Twelve hours after the last immunization, the Spl cells were prepared. (a) The expression of MyD88 was determined in A-BSA- and B-BSA-binding B cells by intracellular staining. Dotted lines represent negative control staining with isotype-matched Abs. (b) The levels of MyD88 expression are presented as median fluorescence intensity (MFI). Average values ± SEM for the individual groups are shown. *GalT*$^{-/-}$ mice, n = 5; WT mice, n = 5.

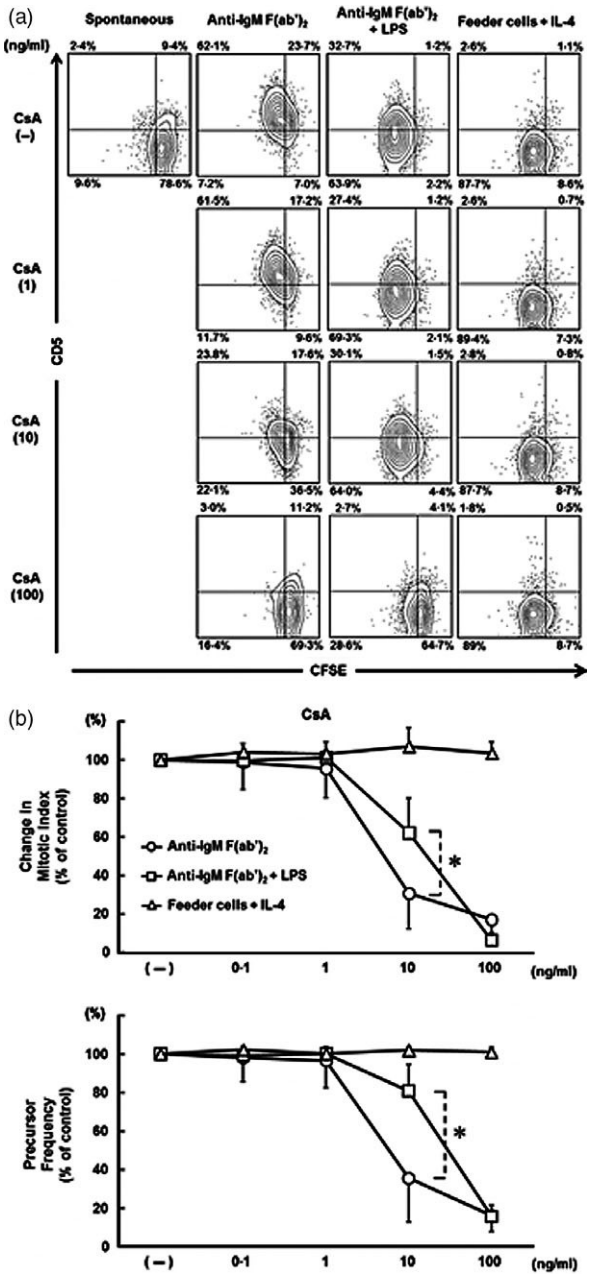
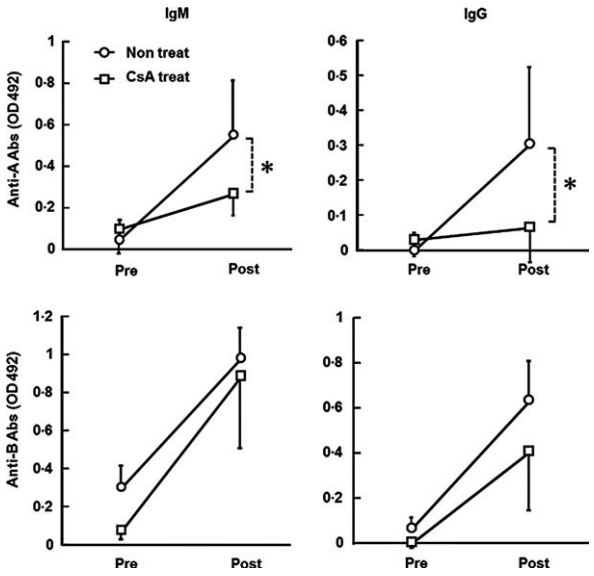


Fig. 5 LPS/TLR4 signalling impaired the inhibitory effects of CsA on anti-IgM F(ab')$_2$-induced B-cell proliferation. The CFSE-labelled resting B cells from the untreated Balb/c mice were cultured in the presence of either soluble F(ab')$_2$ fragments of anti-IgM, as an analogue of TI-2 Ags, or BAFF/CD40L-expressing feeder cells and IL-4, which provide thymus-dependent inductive signals, for 3 days. As indicated, LPS was further added to induce TLR signalling. CsA was added to the culture medium at various doses. The cultivated cells were stained with PE-conjugated CD19 and biotinylated CD5 mAb. The biotinylated mAbs were visualized using allophycocyanin–streptavidin. (a) The relationship between the concentration of CsA and its inhibitory effect on CD5$^+$ B-1a-cell differentiation induced by anti-IgM F(ab')$_2$ fragments, CD5^{dim} B-1b-cell differentiation induced by anti-IgM F(ab')$_2$ fragments plus LPS, and CD5$^-$ B-2 cell differentiation induced by BAFF/CD40L-expressing feeder cells and IL-4. Representative contour plots obtained by FCM analysis are shown. The percentages of the total number of cultivated cells in each quadrant are shown. (b) The percentage (relative to the control) of the mitotic index and precursor frequency in each B-cell differentiation subclass in the presence of various doses of CsA. Average values ± SEM of five independent experiments for the individual groups are shown.

T-cell-independent responses [23]. Although CD5 expression is the phenotypic criterion used to distinguish B-1a from B-1b subsets, several other biological distinctions exist between these cell populations. Defects in BCR signalling more profoundly affect B-1a-cell development [24]. Furthermore, CD19 expression has been shown to be essential for the development of B-1a- but not B-1b- cells [25]. Nevertheless, both the B-1a and B-1b cell subsets seem to require unique activation signals and to react with distinct response patterns. A previous study also demonstrated a different pattern of V_H family usage in B-



11 **Fig. 6** CsA inhibited anti-A Ab production, but not anti-B Ab production in *GalT*$^{-/-}$ mice. *GalT*$^{-/-}$ mice were intraperitoneally administered CsA at 10 mg/kg daily for 4 weeks. One and 2 weeks after commencing the CsA treatment, *GalT*$^{-/-}$ mice were immunized with human blood group AB-RBCs. The levels of anti-A and anti-B Abs (IgM/IgG) in the sera of the mice were determined by ELISA. The results shown are the average values ± SEM for the individual groups. CsA-treated mice, $n = 5$; untreated control mice, $n = 5$. *$P < 0{\cdot}05$.

1b cells compared with B-1a cells in mice, suggesting divergence in the repertoires and functions of the B-1a and B-1b subsets [26, 27]. This study revealed that B cells with receptors for the blood group B epitope phenotypically belong to the B-1b-cell subset, which is distinct from B-1a cells with receptors for the blood group A epitope, in *GalT*$^{-/-}$ mice with the ability to respond to both blood group A and B antigens.

Previous investigations have demonstrated that the strong blood group activity of *E. coli* O86 is derived from LPS containing the blood group B trisaccharide partial structure [19, 20]. It is likely that blood group B carbohydrate determinants activate TLR4, which recognizes LPSs. In this study, we found strong expression of MyD88 in B-BSA-binding B cells in the Spl cells of *GalT*$^{-/-}$ mice immunized with B-RBCs, but MyD88 was barely detectable in A-BSA-binding B cells in the Spl cells of *GalT*$^{-/-}$ mice immunized with A-RBCs (Fig. 4). These findings suggest that B cells responding to blood group B-antigens are activated via TLR4-MyD88 signalling, distinguishing them from B cells responding to blood group A-antigens. Both B-1a and B-1b cells are known to express TLRs and respond to ligation of TLRs. The surface expression of TLR2 and TLR4 and the intracellular expression of TLR7 and TLR9 are high in mouse B-1 cells [28]. Although certain TLR ligands such as CpG1668, LPS, R848, and Pam3CSK4 can selectively promote B-1-cell differentiation [29], the precise roles of these TLR-signalling molecules in B-cell activation remain to be elucidated. Upon the ligation of TLR4 by LPS together with BCR crosslinking induced by anti-IgM F(ab')$_2$, resting B cells were here found to phenotypically differentiated into CD5$^-$ B-1b cells, whereas resting B cells differentiated into CD5$^+$ B-1a cells by anti-IgM F(ab')$_2$-induced BCR crosslinking without ligation of TLR4 by LPS (Fig. 5). All of these findings seem to be consistent with the hypothesis that B cells with BCR for blood group B-antigens are activated via TLR4-MyD88 signalling together with BCR-crosslinking, distinguishing them from B cells with BCR for A-antigens, which are activated only via BCR-crosslinking.

The ontogenic and functional properties of different B-1-cell subsets are of great interest in achieving a comprehensive understanding of B-cell biology. B-1a cells are drastically reduced or absent in strains with impaired classical nuclear factor-kappa B (NF-κB) signalling, whereas B-1b cells are less affected [30]. This difference in the dependence on NF-κB signalling between B-1a cells and B-1b-cells could possibly explain the observed difference between B-1b cells responding to B-Ags and B-1a-cells responding to A-Ags in the sensitivity to CsA, since BCR- and BAFF-induced signalling to NF-κB is known to contribute to B-cell immune responses [31]. A recent report demonstrated that calcineurin inhibitors (CNIs) inhibit the protein phosphatase activity of calcineurin, leading to suppression of both of the nuclear translocation of NFAT and NF-κB phosphorylation as well as inhibition of T-cell activation [32]. Because it is also likely that CNIs block NF-κB activation, even in B cells, this would explain why CsA inhibited anti-A Ab production via NF-κB-dependent B-1a-cell activation, but not anti-B Ab production via NF-κB-independent B-1b cell activation.

To investigate the clinical relevance of our findings described above, we analysed the data from the Japanese Transplantation Registry for ABO-incompatible living-donor kidney transplantation. Among the whole population, we selected patients that had been treated with a CsA-based immunosuppressive regimen without pan-B-cell inhibition by either rituximab or splenectomy, and then analysed the graft survival rates. The results showed that 15 years after transplantation, the graft survival rate of recipients with B-antigen disparate grafts was significantly worse than that of recipients with A-antigen disparate grafts (Fig. S1), which likely reflects the difference in the sensitivity to CsA of B cells responding to blood group A and B antigens. However, there was no difference in the graft survival rate among the recipients treated with rituximab, which can deplete both B-1a and B-1b cells expressing CD20 (data not shown). Thus, the decision to omit rituximab from an immunosuppressive

regimen for recipients with B-antigen disparate grafts should be carefully considered.

In conclusion, we found that B cells with receptors for blood group B-carbohydrate show the sIgM^{high} CD19^{+} CD11b^{+} CD5$^{-/dim}$ B-1b cell phenotype in mice, whereas B cells with receptors for blood group A-carbohydrate show the sIgM^{high} CD19^{+} CD11b^{+} CD5^{+} B-1a-cell phenotype. Furthermore, B cells responding to blood group B-antigens express MyD88, suggesting the involvement of TLR signalling in Ab production against blood group B-antigens. Consistently, CsA inhibited anti-A Ab production, but not anti-B Ab production. To confirm these results, further studies with human B cells should be conducted.

Acknowledgements

We thank Drs. Kentaro Ide, Toshimitsu Irei for advice and encouragement; Ms Yuka Igarashi, and Yuko Ishida for technical assistance. This work was carried out at the Research Facilities for Laboratory Animal Science, Natural Science Center for Basic Research and Development (N-BARD) and the Analysis Center of Life Science, Hiroshima University. This work was supported by a Japan Society for the Promotion of Science Grant-in-Aid for Scientific Research (A) (15H02555).

5 Author contributions

HO was designed the experiment. HO and MY and YT analysed data and wrote the paper. MY and HT and HS performed experiment. YT performed flow sorting. All authors reviewed the manuscript.

Conflict of interest

The authors have declared no conflicts of interest.

Disclosures

The authors have no other conflicting of financial interests.

References

1 Galili U, Buehler J, Shohet SB, *et al.*: The human natural anti-Gal IgG. III. The subtlety of immune tolerance in man as demonstrated by crossreactivity between natural anti-Gal and anti-B antibodies. *J Exp Med* 1987; **165**:693–704
2 Galili U, Ishida H, Tanabe K, *et al.*: Anti-gal A/B, a novel anti-blood group antibody identified in recipients of abo-incompatible kidney allografts. *Transplantation* 2002; **74**:1574–1580
3 Takahashi K, Saito K, Takahara S, *et al.*: Excellent long-term outcome of ABO-incompatible living donor kidney transplantation in Japan. *Am J Transplant* 2004; **4**:1089–1096
4 Wilpert J, Fischer K-G, Pisarski P, *et al.*: Long-term outcome of ABO-incompatible living donor kidney transplantation based on antigen-specific desensitization. An observational comparative analysis. *Nephrol Dial Transplant* 2010; **25**:3778–3786
5 Fuchinoue S, Ishii Y, Sawada T, *et al.*: The 5-year outcome of ABO-incompatible kidney transplantation with rituximab induction. *Transplantation* 2011; **91**:853–857
6 Takahashi K, Saito K: ABO-incompatible kidney transplantation. *Transplant Rev* 2013; **27**:1–8
7 Egli A, Binggeli S, Bodaghi S, *et al.*: Cytomegalovirus and polyomavirus BK posttransplant. *Nephrol Dial Transplant* 2007; **22**(Suppl 8):viii72–viii82
8 Habicht A, Broker V, Blume C, *et al.*: Increase of infectious complications in ABO-incompatible kidney transplant recipients–a single centre experience. *Nephrol Dial Transplant* 2011; **26**:4124–4131
9 Néron S, Lemieu R: Type 2 T-cell-independent murine immune response to the human AB0 blood group antigens. *Vox Sang* 1994; **67**:68–74
10 Zhou W, Ohdan H, Tanaka Y, *et al.*: NOD/SCID mice engrafted with human peripheral blood lymphocytes can be a model for investigating B cells responding to blood group A carbohydrate determinant. *Transpl Immunol* 2003; **12**:9–18
11 Thall AD, Malý P, Lowe JB: Oocyte Galα1, 3Gal epitopes implicated in sperm adhesion to the zona pellucida glycoprotein ZP3 are not required for fertilization in the mouse. *J Biol Chem* 1995; **270**:21437–21440
12 Ohdan H, Yang Y-G, Shimizu A, *et al.*: Mixed chimerism induced without lethal conditioning prevents T cell–and anti-Galα1, 3Gal–mediated graft rejection. *J Clin Invest* 1999; **104**:281–290
13 Tanaka Y, Ohdan H, Zhou W, *et al.*: Enzyme-linked immunospot assay for detecting cells secreting antibodies against human blood group A epitopes. *Transplant Proc* 2003; **35**:555–556
14 Tanaka Y, Ohdan H, Onoe T, *et al.*: Low incidence of acute rejection after living-donor liver transplantation: Immunologic analyses by mixed lymphocyte reaction using a carboxyfluorescein diacetate succinimidyl ester labeling technique. *Transplantation* 2005; **79**:1262–1267
15 Nojima T, Haniuda K, Moutai T, *et al.*: In-vitro derived germinal centre B cells differentially generate memory B or plasma cells *in vivo*. *Nat Commun* 2011; **2**:465
16 Tanaka Y, Ohdan H, Onoe T, *et al.*: Multiparameter flow cytometric approach for simultaneous evaluation of proliferation and cytokine-secreting activity in T cells responding to allostimulation. *Immunol Invest* 2004; **33**:309–324
17 Rasko DA, Wang G, Palcic MM, *et al.*: Cloning and characterization of the α (1, 3/4) fucosyltransferase of Helicobacter pylori. *J Biol Chem* 2000; **275**:4988–4994
18 Shao J, Zhang J, Kowal P, *et al.*: Overexpression and biochemical characterization of β-1, 3-N-acetylgalactosaminyltransferase LgtD from Haemophilus influenzae strain Rd. *Biochem Biophys Res Commun* 2002; **295**:1–8
19 Springer GF, Horton RE, Forbes M: Origin of anti-human blood group B agglutinins in white leghorn chicks. *J Exp Med* 1959; **110**:221–244

20 Kochibe N, Iseki S: Immunochemical studies on bacterial blood group substances. *Jpn J Microbiol* 1968; **12**:403–411
21 Irei T, Ohdan H, Zhou W, *et al.*: The persistent elimination of B cells responding to blood group A carbohydrates by synthetic group A carbohydrates and B-1 cell differentiation blockade: novel concept in preventing antibody-mediated rejection in ABO-incompatible transplantation. *Blood* 2007; **110**:4567–4575
22 Martin F, Oliver AM, Kearney JF: Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity* 2001; **14**:617–629
23 Alugupalli KR, Gerstein RM: Divide and conquer: division of labor by B-1 B cells. *Immunity* 2005; **23**:1–2
24 Hardy RR, Hayakawa K: B cell development pathways. *Annu Rev Immunol* 2001; **19**:595–621
25 Haas KM, Poe JC, Steeber DA, *et al.*: B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to S. pneumoniae. *Immunity* 2005; **23**:7–18
26 Kantor AB, Merrill CE, Herzenberg LA, *et al.*: An unbiased analysis of V (H)-DJ (H) sequences from B-1a, B-1b, and conventional B cells. *J Immunol* 1997; **158**:1175–1186
27 Alugupalli KR, Leong JM, Woodland RT, *et al.*: B1b lymphocytes confer T cell-independent long-lasting immunity. *Immunity* 2004; **21**:379–390
28 Gururajan M, Jacob J, Pulendran B: Toll-like receptor expression and responsiveness of distinct murine splenic and mucosal B-cell subsets. *PLoS ONE* 2007; **2**:e863
29 Genestier L, Taillardet M, Mondiere P, *et al.*: TLR agonists selectively promote terminal plasma cell differentiation of B cell subsets specialized in thymus-independent responses. *J Immunol* 2007; **178**:7779–7786
30 Jacque E, Schweighoffer E, Visekruna A, *et al.*: IKK-induced NF-κB1 p105 proteolysis is critical for B cell antibody responses to T cell–dependent antigen. *J Exp Med* 2014; **211**:2085–2101
31 Shinners NP, Carlesso G, Castro I, *et al.*: Bruton's tyrosine kinase mediates NF-κB activation and B cell survival by B cell-activating factor receptor of the TNF-R family. *J Immunol* 2007; **179**:3872–3880
32 Vafadari R, Kraaijeveld R, Weimar W, *et al.*: Tacrolimus inhibits NF-κB activation in peripheral human T cells. *PLoS ONE* 2013; **8**:e60784

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Graft survival rate in patients of ABO incompatible living-donor kidney transplantation.