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TLR-MyD88-signaling blockades inhibit refractory B-1b cell immune responses to transplant-related glycan antigens

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Abbreviations

TI, T cell-independent; Ags, antigens; Gal, Galα1,3Galβ1,4GlcNAc; TLRs, Toll-like receptors; BCRs, B cell receptors; CNI, calcineurin inhibitor; NFATc1, nuclear factor of activated T-cells,

cytoplasmic 1; ABOi, ABO-incompatible; B6, C57BL/6J; GalT-KO, α-galactosyltransferase knock-out; CFSE, 5 μM 5-(6)-carboxyfluorescein diacetate succinimidyl ester; LPS, lipopolysaccharide; RBCs, red blood cells; PBS, phosphate-buffered saline; CsA, cyclosporine A; PerC, Peritoneal cavity; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; APC, allophycocyanin; PE, phycoerythrin; PI, propidium iodide, ELISA, Enzyme-linked immunosorbent assay; ELISPOT, Enzyme-Linked Immuno spot; SEM, standard error of the mean; WT, wild-type; BTK, Bruton's tyrosine kinase; HDAC, histone deacetylase;

Abstract

Refractory B cell responses to T cell-independent (TI) carbohydrate antigens (Ags) are critical drivers of rejection reactions to ABO-incompatible allogeneic grafts and xenogeneic grafts from other species. To explore the biological significance of crosstalk between Toll-like receptors (TLRs) and B cell receptors (BCRs) in the TI B cell immunity, we here used MyD88-, TRIF-, and α-galactosyltransferase-deficient mice to study B cell phenotypes and functional properties during TI transplant-related glycan-Ag exposure. BCR stimulation alone induced differentiation into $CD5^{high}$ (B-1a) cells, which were highly sensitive to a calcineurin inhibitor (CNI), while co-stimulation of TLRs and BCRs induced differentiation into $CD5^{dim}$ (B-1b) cells in MyD88-dependent and CNI-resistant manners. MyD88-dependent TLR stimulation in B-1b cells enhanced downstream factors in the BCR–calcineurin pathway, including a nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1). TLR inhibitor together with CNI abrogated refractory B-1b cell immune responses against the ABO-blood group Ags, while blocking both BCRs and TLRs–MyD88 by using Bruton's tyrosine kinase inhibitor and histone deacetylase inhibitor abrogated refractory B-1b cell immune responses against Gal-glycan Ags. Thus, this study provides a rationale for a novel therapeutic approach to overcome refractory transplant-related anti-glycan Ab production by blocking both BCR and TLRs–MyD88 signals.

1 | INTRODUCTION

Humoral responses to T cell-independent (TI) carbohydrate antigens (Ags) are critical drivers of the adverse reactions to ABO-incompatible (ABOi) organ transplantation and the rejection of xenogeneic grafts from other species [1,2]. Antibodies (Abs) to those TI antigens are thought to be mainly produced by distinguished B cell subpopulations, B-1 cells, which mainly express germline-encoded antigen-specific B cell receptors (BCRs) that have limited diversity and are enriched for specificities that recognize a variety of carbohydrate residues [3]. In addition to BCR ligation, the activation of pattern-recognition receptors, including Toll-like receptors (TLRs), on B-1 cells is also thought to be important for their immune responses, i.e. coordinated stimulation of TLR and BCR signaling has been proven to activates B cells and triggers the rapid induction of TI Ag-specific IgM responses [4,5]. Although simultaneous or sequential engagement of BCRs and TLRs likely alter and fine tune B cell activation [6], the details of the crosstalk between those signals in regulating the functions of B cells in TI humoral immunity have been poorly defined.

The immunodominant structures of blood group A and B antigens are N-acetyl-D-galactosamine (GalNAc)α1-3(Fucα1-2)Gal and Galα1-3(Fucα1-2)Gal, respectively [7,8], whereas those of xenoantigens are Galα1,3Galβ1,4GlcNAc(Gal) carbohydrate residues. We have previously demonstrated that B cells with receptors recognizing blood group A carbohydrate residues show the $sIgM^{+}CD11b^{+}CD5^{+}$ (B-1a) phenotype [9,10], whereas B cells with receptors for xenoantigen Gal carbohydrate residues show the $sIgM^{+}CD11b^{+}CD5^{-}$ (B-1b) phenotype [11,12]. Phenotypically, B-1a and B-1b cells are essentially identical, being distinguished only by the presence or absence of the CD5 marker. Although previous data demonstrated a different pattern of V_H family usage in B-1b versus B-1a cells in mice, suggesting divergence in the repertoires and functions of the B-1a and B-1b subsets [13,14], functionally, no differences between the two populations have been clearly identified.

Both B-1a and B-1b cells are known to express TLRs, except for TLR5 [15], and respond to TLR ligation, while TLR8 is thought to be non-functional in mice [16]. Surface receptors such as TLR1, TLR2, TLR4, and TLR6, as well as the intracellular receptors such as TLR3, TLR7, TLR8, and TLR9, are expressed in mouse B-1 cells [15]. Although certain TLR ligands such as CpG1668, LPS, R848, MALP2, and Pam3CSK4 can selectively promote B-1 cell differentiation [15], the precise roles of these TLR-signaling molecules in B cell activation remain to be elucidated. Previously, we showed that upon the *in vitro* ligation of TLR4 by lipopolysaccharide (LPS) together with BCR crosslinking induced by anti-IgM $F(ab')_2$, an analog of TI-2 Ags, resting B cells differentiated phenotypically to B-1b cells in mice, whereas resting B cells differentiated to B-1a cells by anti-IgM $F(ab')_2$-induced BCR crosslinking alone [9,17]. Calcineurin inhibitor (CNI) completely blocked differentiation into B-1a cells, but not B-1b cells, consistent with a fact that anti-Gal Ab production is hard to control by conventional immunosuppressants, when compared to anti-blood group A Ab production [11,17]. In this study, we investigated the role and molecular mechanism of TLR- and BCR-signals in B-1a/B-1b cell activation responding to the TI transplant-related glycan-Ags in mice.

2 | MATERIALS AND METHODS

2.1 | Mice

BALB/c and C57BL/6J (B6) mice were purchased from CLEA Japan (Tokyo, Japan). α-galactosyltransferase knock-out (GalT-KO) mice in the B6 background, which completely lack Galα1,3Galβ1,4GlcNAc carbohydrate residues, were kindly provided by Dr. M. Sykes, Columbia University, NY, USA [18]. The *Myd88*$^{-/-}$ and *Trif*$^{-/-}$ mice in the B6 background were purchased from Oriental Bio Service (Kyoto, Japan). NOD-*Cg-Rag1*^{tm1Mom} *IL2rg*$^{tm1wjl/SzJ}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed in the animal facility of Hiroshima University, in a pathogen-free environment and were used at an age of 8–10 weeks.

2.2 | *In vitro* B-cell proliferation assay

Resting B cells were isolated from the splenocytes of untreated B6, TRIF KO, MyD88-KO, and BALB/c mice by negative selection using a B Cell Isolation Kit and an automagnetic-associated cell sorter (Miltenyi Biotec, Auburn, CA, USA). The B cells were labeled with 5 μM 5-(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA), as described previously [19]. The CFSE-labeled cells were cultured in Roswell Park Memorial Institute (Nacalai Tesque, Kyoto, Japan) culture medium, 5 μM 2-mercaptoethanol, 1% HEPES buffer (Gibco), and 100 IU/ml penicillin/100 μg/ml streptomycin (Gibco). Where indicated, the B cells were treated with a combination of 10 μg/ml anti-IgM F(ab′)$_2$ (Jackson ImmunoResearch Laboratories) and 1 or 10 μg/ml LPS (*E. coli* 0111:B4, Sigma), 300 ng/ml Pam3CSK4 for TLR1/2 (Pam3CysSerLys4, InvivoGen, San Diego, CA, USA), 10 μg/ml poly(I:C) low-molecular weight (LMW) for TLR3 (InvivoGen), 10 μg/ml poly(I:C) high-molecular weight (HMW) for TLR3 (InvivoGen), 10 μg/ml LPS-EK for TLR4 (*E. coli* K12, InvivoGen), 100 ng/ml MALP2 for TLR6/2 (Pam2CGDPKHPKSF, InvivoGen), 10 μg/ml R848

for TLR7 (Resiquimod, InvivoGen), and 5 nmol/ml CpG-ODN for TLR9 (ODN1862, InvivoGen). The cultured cells were stained with PE-conjugated CD19 (1D3, BD Pharmingen, San Diego, CA, USA) or biotinylated CD5 (53-7.3, BD Pharmingen) monoclonal Abs (mAbs). The biotinylated mAbs were visualized using allophycocyanin–streptavidin, followed by 4-color FCM analyses.

2.3 | Western blot analysis

Spleen B cells were stimulated as indicated, and >5 × 10^6 cells were centrifuged and resuspended in lysis buffer (Sigma–Aldrich) on ice for 30 min. The lysates were mixed with sodium dodecyl sulfate (SDS) sample loading buffer and boiled at 95°C for 10 min. Proteins were separated by 8% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The following Abs were used as primary Abs: mouse anti-nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) mAb (7A6, Santa Cruz Biotechnology, Inc. Dallas, Texas, USA), mouse anti-NFκB p52 and p100 mAbs (C-5, Santa Cruz Biotechnology), affinity purified rabbit polyclonal anti-NFκB p65 Ab (sc-372, Santa Cruz Biotechnology), and mouse anti-β-Actin mAb (M177-3, Medical & Biological Laboratories Co., Ltd. Nagoya, Japan). Protein levels were detected with an HRP-conjugated secondary anti-mouse/rabbit IgG Ab (GE Healthcare UK Ltd, Buckinghamshire, UK) using the ECL Prime Kit (GE Healthcare UK, Ltd).

2.4 | *In vivo* study

For *in vivo* immunization, a suspension of 1 × 10^9 human blood group A-erythrocytes (A-RBCs) or 1 × 10^7 rabbit RBCs in 0.5 ml phosphate-buffered saline (PBS) was injected into the peritoneal cavity (PerC) of each mouse twice, at 1-week intervals. Where indicated, cyclosporine A (CsA; which was kindly provided by Novartis, Basel, Switzerland) was intraperitoneally administered daily at 10 mg/kg/day for 3 consecutive weeks (from 1 week before the first immunization to 1

week after the last immunization). The TLR4 signaling antagonist eritoran, which was kindly provided by Eisai Inc., (Andover, MA, USA), was intraperitoneally administered at a dose of 10 mg/kg in PBS per mouse on the day of immunization with human RBCs. Bruton's tyrosine kinase inhibitor, ibrutinib (PCI-32765; Chem Scene, Monmouth Junction, NJ, USA) and the histone deacetylase inhibitor, panobinostat (Chem Scene) were dissolved in dimethyl sulfoxide and diluted in corn oil. In mice immunized with rabbit RBCs, CsA (10 mg/kg/day), eritoran (10 mg/kg/day), MyD88 homodimerization-inhibitory peptide (Novus Biologicals, Littleton, CO, USA) diluted in PBS (5 mg/mg/day), ibrutinib (20 mg/kg/day), and panobinostat (1 mg/kg/day), or a vehicle control, were intraperitoneally administered daily for 3 consecutive weeks, from 1 week before the first immunization to 1 week after the last immunization. Among the PerC cells and splenocytes of the immunized mice, B cells with receptors for blood group A and Gal trisaccharides were detected using fluorescein isothiocyanate (FITC)-conjugated GalNAcα1-3Fucα1-2Gal-bovine serum albumin (BSA) (A-BSA: Dextra, Reading, UK), Galα1-3Galβ1-4GlcNAc-3-BSA (Gal-BSA: Dextra), and control FITC-conjugated BSA (Roche, Indianapolis, IN) (7, 8). The levels of anti-A or -Gal-specific Abs or total immunoglobulin in sera were determined by enzyme-linked immunosorbent assay (ELISA) [11]. Enzyme-linked immune-spot (ELISPOT) assays were performed to detect Ab-producing cells in the spleen [20].

2.5 | Statistical analyses

Data were analyzed with Prism 7 software (GraphPad). Experimental groups were compared using a two-tailed Student's unpaired *t*-test or the Mann–Whitney U test. *F*-testing was conducted before Student's *t*-test to compare the variance of two samples. To analyze multiple group data, one-way ANOVA test followed by Tukey's test was performed. $P < 0.05$ were considered statistically significant.

3 | RESULTS

3.1 | Contributions of TLR–MyD88 signaling to CNI-resistance in B-1b cells

Utilizing an *in vitro* TI B cell-differentiation model in mice, we explored downstream molecules in TLR signaling- as well as TRIF- and MyD88-dependent pathways, both of which are mediated by TLR4-signaling. CFSE-stained resting B cells isolated from the splenocytes of wild-type (WT) B6, TRIF KO, and MyD88 KO mice were stimulated *in vitro* with anti-IgM $F(ab')_2$ for 3 days in the presence or absence of LPS. Proliferating B cells from WT mice differentiated into $CD5^-$ or $CD5^+$ cells in the presence or absence of LPS, respectively (Figure 1A). CsA remarkably inhibited $CD5^+$ B cell differentiation with the stimulus of anti-IgM $F(ab')_2$ alone but exerted less suppressive effect on $CD5^-$ B cell differentiation with anti-IgM $F(ab')_2$ and LPS stimulation (Figure 1B). The use of tacrolimus, another CNI, lead to similar results, i.e. the stimulus of LPS also induced resistance to tacrolimus (Figure S1). In mice deficient in either TRIF or MyD88, B cells differentiated to $CD5^+$ B cells by anti-IgM $F(ab')_2$ alone, which was completely inhibited by CsA, similar to the results observed with WT mice. In TRIF-deficient mice, B cells predominantly differentiated to $CD5^-$ B cells with anti-IgM $F(ab')_2$ and LPS stimulation, which was partially inhibited by CsA. In MyD88-deficient mice, B cells exclusively differentiated to $CD5^+$ B cells even after stimulation with both anti-IgM $F(ab')_2$ and LPS, which was completely inhibited by CsA.

We further assessed the phenotypic properties and CsA-sensitivity of proliferating B cells from WT mice in response to anti-IgM $F(ab')_2$ treatment together with agonists of various TLR subtypes (Pam3CSK4 for TLR1/2, poly(I:C) for TLR3, LPS for TLR4, MALP2 for TLR6/2, R848 for TLR7, and CpG-ODN for TLR9). B cells stimulated with anti-IgM $F(ab')_2$ and poly(I:C), which induces TLR3–TRIF signaling independent of MyD88 signaling, differentiated into $CD5^+$ cells. However, treatment with anti-IgM $F(ab')_2$ and other TLR agonists that activate MyD88

signaling, induced differentiation of B cells into $CD5^-$ cells, demonstrating the dependence of MyD88 signaling on differentiation into B-1b-like $CD5^-$ cells (Figure 1C). The differentiation of B-1a-like $CD5^+$ cells induced by MyD88-independent TLR3 activation was abrogated by CsA, while that of B-1b-like $CD5^-$ cells induced by MyD88-dependent TLR agonists were resistant to CsA (Figure 1D). Collectively, these findings indicated that $CD5^-$ B cell differentiation and CsA resistance in response to TI stimuli depend on TLR–MyD88 signaling in B cells.

Next, we investigated the possible association between BCR- and TLR-signaling pathways in TI B cells. BCR signaling requires activities of calcineurin, NFATc1, and non-canonical NF-κB subunits (such as p100 and p52) [21-23], while TLR signaling induces activation of the canonical NF-κB p65 subunit [24]. Western blot analysis showed that BCR crosslinking by anti-IgM $F(ab')_2$ activated the non-canonical NF-κB subunits p100 and p52. TLR and BCR co-stimulation enhanced not only the canonical NF-κB p65 subunit, but also the non-canonical NF-κB subunits p100 and p52, indicating that TLR signaling enhanced the activity of the BCR-signaling pathway (Figure 1E). Co-stimulation of BCR and TLR enhanced NFATc1 expression in B cells from WT mice, but not in MyD88-deficient B cells (Figure 1E), demonstrating that the inhibitory effect of CNI was disabled by activation of MyD88-dependent TLR signaling. Consistently, eritoran (E5564; Eisai), a synthetic TLR4 antagonist, in combination with CsA treatment markedly inhibited differentiation into B-1b-like $CD5^-$ cells and their proliferation induced by anti-IgM $F(ab')_2$ and LPS (Figure S2).

3.2 | Association of TLR stimulation with CNI-resistant B-1b cell immune response *in vivo*

Utilizing an *in vivo* mouse model, we investigated the influence of TLR stimuli on the phenotypic properties of B cells responding to A-RBCs and their sensitivity to CsA; BALB/c mice were immunized with A-RBCs with or without LPS injection. B cells expressing the anti-A receptor can

be detected using FITC-labeled synthetic A-carbohydrate antigens, conjugated with BSA. We found that phenotypically distinct IgM^{high} and IgM^{dim} subsets were detected among A-BSA^+ B cells. Whereas both subsets expressed CD11b (Figure S3), CD5 expression differed: the IgM^{high} subset showed the $CD5^{high}$ B-1a phenotype, whereas the IgM^{dim} subset showed the $CD5^{dim}$ B-1b phenotype (Figure 2B, 2D). This variance in CD5 expression on B cells positive for the anti-A receptor was consistently observed regardless of LPS and CsA injection. LPS stimulation together with A-RBC sensitization enhanced the frequency of the A-BSA^+ IgM^{dim} subset and decreased the frequency of the A-BSA^+ IgM^{high} subset (Figure 2A). Thus, the LPS stimulation shifted the dominant responder toward A-RBC stimulus from $CD5^{high}$ B-1a cells to $CD5^{dim}$ B-1b cells. CsA treatment resulted in the expansion of $CD5^{dim}$ B-1b cells, regardless of LPS stimulation (Figure 2C). Two weeks after immunization with A-RBCs alone, the serum anti-A IgM levels in mice treated with CsA were significantly lower than those of the untreated mice, while the anti-A IgG levels showed similar trends, although the difference did not reach statistical significance (Figure 2E). LPS injection elevated the levels of both anti-A IgM and anti-A IgG (even in CsA-treated mice) to similar levels to those of untreated mice at the same time point after A-RBC immunization, indicating that LPS stimulation eliminated the inhibitory effects of CsA on anti-A Ab production.

In separate experiments, we explored whether the A-BSA^+ IgM^{high} $CD5^+$ B-1a and/or the A-BSA^+ IgM^{dim} $CD5^-$ B-1b subsets were actually precursors of Anti-A Ab-secreting cells. We adoptively transferred either A-BSA^+ IgM^{high} cells, A-BSA^+ IgM^{dim}, or A-BSA^- IgM^+ cells isolated from the peritoneal cavity of donor BALB/c mice, which were immunized with A-RBCs, into recipient NOD-Cg-*Rag1*^{tm1Mom}*IL2rg*$^{tm1wjl/SzJ}$ mice, followed by a second stimulation with A-RBCs. The serum levels of anti-A Abs in the recipient mice were quantified 2weeks after the adoptive transfer. While anti-A Abs were not detectable in the sera of control recipients of A-BSA^- IgM^+

cells, anti-A IgM/IgG were detected in the sera of both recipients of A-BSA$^+$ IgM^{high} cells and those of A-BSA$^+$ IgM^{dim} cells (Figure S4), indicating that A-BSA$^+$ cells were precursors of anti-A Abs secreting cells, regardless of their expression of CD5.

3.3 | Dependence of TLR4–MyD88 signaling on the LPS-induced disability of CNI to inhibit anti-glycan Ab production *in vivo*

To assess the role of MyD88 signaling in anti-A Ab production, we compared anti-A Ab production in mice lacking MyD88 signals to that in WT mice 2 weeks after LPS administration and A-RBC immunization. The serum anti-A Ab levels of MyD88-deficient mice were markedly lower than those in WT mice (Figure 3A). As expected, LPS stimulation elevated anti-A Ab levels even under CsA treatment in WT mice (Figure 3B). In contrast, injection of eritoran completely inhibited the elevation in anti-A Ab levels.

3.4 | Combined blocking of BCRs and TLRs–MyD88 in B cell immune response against xenogeneic Gal glycan Ags

To address a question as to whether blocking the TLR–MyD88 pathway also inhibits the activation of B-1b cells responding to xenoantigen Gal carbohydrate residues, GalT-KO mice were prepared by two immunizations with xenogeneic rabbit RBCs expressing abundant Gal Ags. A week after the second immunization, the proportion of B-1b cells expressing anti-Gal receptors in the peritoneal cavity, the serum anti-Gal Ab levels, and the frequency of anti-Gal Ab-producing cells in the spleen significantly increased in the GalT-KO mice (Figure 4B–E). We daily administered the eritoran together with CsA for 3 consecutive weeks. To directly target MyD88, MyD88 homodimerization-inhibitory peptides (a MyD88-inhibitor) were similarly injected together with CsA. In the peritoneal cavity, CsA treatment alone did not influence the proportion

of either $CD11b^{+}CD5^{+}$ B-1a cells or $CD11b^{+}CD5^{-}$ B-1b cells (Figure 4A). CsA-treatment with either eritoran or the MyD88 inhibitor reduced the proportion of both B cell subclasses, and treatment with both inhibitors reduced their proportions further. Thus, MyD88 signaling may play a significant role in the homeostasis of both B-1 cell subclass, consistent with previous evidence that mature B-1 cells are maintained by self-renewal, which requires canonical NF-κB signals [25-27]. CsA in combination with either eritoran or the MyD88 inhibitor displayed synergistic effects in reducing the proportion of B cells with anti-Gal receptors in both the peritoneal cavity and spleen (Figure 4B, 4C), whereas treatment with either eritoran or the MyD88 inhibitor alone did not (Figure S5A, S5B), suggesting that Gal Ags can potently activate B-1b cells through both the BCR and TLR–MyD88 pathways. Anti-Gal Ab-producing cells in the spleen were also reduced by combination treatment, leading to inhibited serum anti-Gal Ab levels (Figure 4D, 4E). A combination of all the three agents (CsA, eritoran, and MyD88 inhibitor), which block BCR and all TLR subtypes (except for TLR3, which induces TRIF signaling), inhibited anti-Gal Ab production more than combined treatment with two agents (CsA and either eritoran or the MyD88 inhibitor) (Figure 4D, 4E). These findings suggested that not only TLR4-MyD88, but the other TLR subtypes and/or TRIF signaling via TLR4 might partially contribute to B-1b cell responses against Gal epitopes. Those combination therapies did not affect the total serum immunoglobulin levels in mice. Weight loss in the mice was not observed during the treatment period (Figure S6A, S6B). Thus, our findings provide a rationale for novel therapeutic approach to regulate refractory anti-glycan Ab production by blocking both BCR and TLR-MyD88 signals.

Finally, we examined the effect of clinically available agents capable of potently inhibiting both BCR and MyD88 signals on the abrogation of anti-Gal responses in our mouse model. Bruton's tyrosine kinase (BTK) inhibitor (ibrutinib) was used to inhibit BCR and TLR4–MyD88 signals and a histone deacetylase (HDAC) inhibitor (panobinostat) was used to inhibit

MyD88 signals. The drug ibrutinib is used to target B cell malignancies refractory to standard chemotherapy due to abundant BCR–BTK signaling, which leads to NF-κB activation in such tumors [28,29]. Ibrutinib can potently inhibit BCR and TLR4–MyD88 signaling, both of which require BTK [30]. Panobinostat is an HDAC inhibitor that is useful therapeutically in treating hematologic cancers by regulating MyD88 activity [31]. Treatment with ibrutinib or panobinostat alone significantly inhibited anti-Gal production. No additive effect of CsA was observed on ibrutinib-based inhibition of anti-Gal Ab production. However, combined treatment with ibrutinib and panobinostat abolished anti-Gal Ab production, while total immunoglobulin levels were maintained (Figure 5A, 5B and Figure S5C, S5D).

4 | DISCUSSION

BCR triggering of B cells leads to strong induction of NFATc1, which is required for optimal proliferation and survival of B-1a cells [21,32]. The activation of calcineurin by BCR crosslinking dephosphorylates and activates NFATc1[21,33], suggesting that B-1a cell activation is solely due to BCR-signals that can be inhibited by CNI. Consistently, in this study, B cells stimulated with anti-IgM $F(ab')_2$ *in vitro* and B cells primed with blood group-A carbohydrate antigens *in vivo* differentiated into $CD5^{high}$ B-1a cells showing high susceptibility to CNI-induced suppression. We also found that B-1b differentiation was implicated in both BCR and TLR–MyD88 signaling. Focusing on the association of TLR stimuli with BCR signals, a recent study demonstrated that BCR signaling by itself activates the non-canonical NF-κB p100 pathway [22], whereas LPS triggers TLR4 and BCR signaling, and activates both the canonical NF-κB p65 and non-canonical NF-κB p100 pathways. Non-canonical NF-κB p100 deficiency impairs immune responses of the BCR to TI-2 antigens, but does not affect TLR responses [23]. Here, we demonstrated that TLR agonists induced signals in $CD5^{-}$ B-1b cells via the MyD88 pathway, consequently enhancing NF-κB p65 and BCR signaling by activating NFATc1 and the non-canonical NF-κB subunit p100, a downstream factor of calcineurin, demonstrating that CNI cannot inhibit B-1b cell activation (Figure 6). In addition to the mechanism of CNI resistance in B-1b cells demonstrated in this study, it was previously demonstrated that calcineurin negatively regulates TLR signaling by inhibiting MyD88, suggesting that CNI activates TLR signaling [34,35]. These findings also support evidence of CNI resistance induced by MyD88 signaling activation in B-1b cells.

It is well established that B-1 cells (in contrast to B-2 cells) are generated more abundantly from fetal liver than from the bone marrow [36] and are maintained by self-renewal [25,26]. Two different models of the origin of B-1 cells have been proposed, including the lineage hypothesis [37,38] and the induced-differentiation hypothesis [39,40]. The model examined in this study

was based more on the induced-differentiation hypothesis: the intensity of TLR–MyD88–NF-kB signaling in immature/naive B cells activated by TI Ags affected the fate of those cells developing into B-1a or B-1b cells. Even in previous reports supporting the lineage hypothesis, it is argued that distinct NF-kB signaling plays a critical role in development into either B-1 or B-2 cells: the canonical NF-kB pathway is not needed for B-1 cell development [27]. Moreover, excessive NF-κB signaling in transitional B cell stage gives rise to a complete absence of peritoneal B-1a cells, while pro/pre-, immature, and mature follicular B cells develop normally, revealing a requirement for intact NF-κB signaling during this stage of B-1 cell development, especially in B-1a cells [25,41]. Recently, it has been demonstrated that TLR activation of B-1a cells results in downregulation of the BCR inhibitory protein CD5, making the B cells phenotypically become 'B-1b' like [42]. Consistently, B-intrinsic TLR signaling has been proven to be critical for the differentiation of B-1a cells into antibody secreting cells in the context of infection with influenza virus and Salmonella typhimurium in mice [43]. Our finding of B-1b differentiation induced by BCR and TLR–MyD88 signaling supports the model of coordinated integration of TLR/BCR signaling in regulating B-1 responses describe above.

Naturally occurring anti-Gal Abs in the serum, which initiate hyper-acute rejection, are a major barrier to the xenotransplantation of pig organs into humans [2,44]. Although GalT-KO pigs lacking Gal epitopes have facilitated longer survival of nonhuman primate recipients with less immune modulation [45-48], acute vascular rejection and delayed graft rejection of xenografts (even from GalT-KO pigs) are caused by induced Abs against TI glycan Ags other than the Gal epitope (non-Gal Ags), such as N-glycolylneuraminic acid epitopes [49-51]. It has been demonstrated that conventional immunosuppressive drugs can hardly prevent anti-glycan Ab-mediated xenograft rejections [11,12,49,52]. Therefore, the exploration of a therapeutic strategy for inhibiting refractory B cell immune responses (not only against Gal, but also non-Gal TI glycan Ags) has become a major

area of interest for the success of xenotransplantation. We demonstrated that Ag-induced B-1b cell development requires TLR–MyD88 signaling, indicating that xenogeneic glycan Ags can activate TLR–MyD88 pathways that induce B-1b cell immune responses conferring resistance to CNI. Blocking TLR–MyD88 signaling provoked an inhibitory effect of CNI on B-1b cells responding to xenogeneic Ags, suggesting that the TLR–MyD88 pathway can serve as a therapeutic target for preventing/curing anti-glycan Ab-mediated xenograft rejection.

In the field of B cell oncology, genetic mutations in BCR and MyD88 that induce constitutive NF-κB activation can promote malignant cell survival in the activated B cell-like subtype of diffuse large B cell lymphomas, which are refractory to standard chemotherapy regimens [28,29,53]. Recently, BTK and HDAC inhibitors have been suggested to possess therapeutic potential for treating these types of tumors [54]. The data of the current study indicate that clinically available inhibitors targeting BCR and TLR–MyD88 signaling can be repurposed for inhibiting B-1b cells responding to transplant-related TI glycan Ags (Figure 6). It has been known that one of the characteristic adverse effects of BTK and HDAC inhibitors is myelosuppression/pancytopenia, which necessitates the tight tuning of these drugs. Since it is likely that the optimal doses of BTK and HDAC inhibitors as anti-neoplastic drugs differ from those for inhibiting B cell responses, further studies might be needed to define the doses required for their application as immunosuppressants.

In conclusion, the present study demonstrated that blocking both BCRs and TLRs abrogated refractory B-1b cell immune responses against ABO-blood group and xenogeneic Gal-glycan Ags, overcoming refractory transplant-related anti-glycan Ab production.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article.

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Figure legends

Figure 1. TLR–MyD88 signaling promotes CsA resistance by activating NFATc1 in B-1b cells.

(**A–D**) CFSE-stained B cells from C57BL/6 (B6), TRIF-KO (B6), MyD88-KO (B6), and BALB/c mouse splenocytes were stimulated *in vitro* with anti-IgM $F(ab')_2$ with/without each TLR-agonist. The cells were stained with CD19 and CD5 mAbs. Nonspecific FcγR binding of labeled mAbs was blocked using anti CD16/32 mAb. Dead cells, identified by light scattering and PI staining, were excluded from the analyses. Isotype-matched irrelevant mAbs were used as negative controls. Representative contour plots of live $CD19^+$ cells. (**A**) Percentage of $CD5^-$ cells among proliferating B cells ($CFSE^-$ cells) stimulated with anti-IgM $F(ab')_2$ with/without LPS were compared (n = 4/group). (**B**) Sensitivity to CsA in proliferating B cells stimulated with anti-IgM $F(ab')_2$ with/without LPS (n = 4/group). (**C**) Percentage of $CD5^+$ and $CD5^-$ cells and (**D**) sensitivity to CsA in proliferating B cells from BALB/c mice stimulated with various TLR-agonist subtypes and anti-IgM $F(ab')_2$. Each TLR agonists: TRIF-dependent: poly(I:C) low and high molecular weight (LMW/HMW) for TLR3; MyD88-dependent: Pam3CSK4 for TLR1/2, MALP2 for TLR6/2, R848 for TLR7, and CpG-ODN for TLR9; both MyD88- and TRIF-dependent: LPS-KE for TLR4. (**E**) Western blots showing NFATc1, NF-κB p100/p52, and p65 in differentiated B cells stimulated with anti-IgM $F(ab')_2$, with/without LPS. The data represent the mean ± SEM for at least three independent experiments. Results were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. NS, not significant

Figure 2. CsA-resistant B-1b cells with anti-glycan Ag receptors dominantly respond to corresponding Ags after TLR-stimulation *in vivo.*

(**A–E**) BALB/c mice were immunized with human group A-RBCs alone or together with LPS twice at 1-week intervals, (**A, B**) without or (**C, D**) with CsA treatment. The proportion of (PerC) B cells with group-A carbohydrate receptors at 1 week after the last immunization was determined by staining with FITC-labeled A-BSA or control FITC-labeled BSA, together with APC-IgM and PE-CD5. The frequencies of A-BSA-FITC^{+}IgM^{high} (R1) and A-BSA-FITC^{+}IgM^{dim} (R2) cells were compared. CD5 expression was compared between R1 and R2 cells. Dotted lines represent negative-control staining with isotype-matched Abs. Results were consistent in at least three independent experiments. (**E**) Serum anti-A IgM and IgG levels 1 week after the last immunization, measured by ELISA (n = 5/group). Average ± SEM values are shown for each group. Results were analyzed by one-way ANOVA followed by Tukey's test (A–**D**) or a two-tailed, unpaired Student's *t* test (**E**) *F*-testing was conducted before Student's *t*-test to compare the variance of two samples. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001

Figure 3. LPS-dependent inhibition of the ability of CsA to block anti-glycan Ab production depends on TLR4–MyD88 signaling *in vivo*.

(**A, B**) Anti-A IgM and IgG values in sera at 1 week after the last immunization in each group were measured by ELISA. The data were determined by optical-density measurements (492 nm) of background BSA-binding Abs subtracted from those of A-BSA binding Abs. (**A**) C57BL/6 (WT) or MyD88-KO mice were immunized with human group-A red blood cells (RBCs) together with LPS twice at 1-week intervals (n = 5/ group). (**B**) BALB/c mice were immunized with human group A-RBCs alone (LPS [–]) or together with LPS (LPS[+]) twice at 1-week intervals under treatment with CsA alone , or treatment with CsA and a single dose of eritoran (TLR4-i) 2 h before each immunization (n = 5/group). The average ± SEM for the individual groups are shown. The results were analyzed by a two-tailed Mann–Whitney U test (**A**) or an unpaired Student's

t-test (**B**). *F*-testing was conducted before Student's *t*-test to compare the variance of two samples. **P* < 0.05, ***P* < 0.01

Figure 4. Combined blocking of BCR and TLR–MyD88 pathways to regulate refractory anti-Galα1,3Galβ1,4GlcNAc Ab production.

(**A–E**) GalT-KO mice were immunized with rabbit RBCs twice at 1-week intervals. CsA, eritoran (TLR4-i), and MyD88-i were administered daily for 3 consecutive weeks, from 1 week before the first immunization to 1 week after the last immunization (n =5/group). (**A**) Ratios of B cell subsets ($IgM^+CD11b^+CD5^+$ B-1a, $IgM^+CD11b^+CD5^-$ B-1b, and $IgM^+CD11b^-CD5^-$ B-2 cells) in the peritoneal cavity were analyzed by flow cytometry. (**B, C**) To determine the proportion of B cells with receptors for Gal epitopes, PerC and spleen cells were stained with either FITC- Gal-BSA or control FITC- BSA, together with APC-IgM. (**D**) Anti-Gal Ab-producing cells in the spleen of each mouse were detected by performing ELISPOT assays. (**E**) The serum anti-Gal IgM and IgG values 7 days after the last immunization were measured by ELISA. Representative FACS analysis and ELISPOT images are shown. The average values ± SEM for the individual groups are shown. Results were analyzed by one-way ANOVA test followed by Tukey's test (**A–C**), or by an unpaired two-tailed Student's *t*-test (**D, E**). *F*-testing was conducted before Student's *t*-test to compare the variance of two samples. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001

Figure 5. Combined blocking of BCR and TLR–MyD88 pathways by BTK and HDAC inhibitors abrogates refractory anti-Gal Ab production.

(**A, B**) GalT-KO mice were immunized with rabbit RBCs twice at 1-week intervals under the indicated treatment. Each agent was intraperitoneally administered daily for 3 consecutive weeks, from 1 week before the first immunization to 1 week after the last immunization (CsA: 10

mg/[kg•day], Bruton's tyrosine kinase (BTK) inhibitor (ibrutinib): 20 mg/[kg•day], and histone deacetylase (HDAC) inhibitor (panobinostat): 1 mg/[kg•day]; n = 4/group). (**A**) Anti-Gal Ab-producing cells in mouse splenocytes 7 days after the last immunization were detected in ELISPOT assays. Mouse splenocytes were seeded at the indicated densities. Representative images of ELISPOT wells are shown for each group. (**B**) The serum anti-Gal IgM and IgG concentrations 7 days after the last immunization were measured by ELISA. The average values ± SEM for individual groups are shown. Results were analyzed by one-way ANOVA followed by Tukey's test (**B**), or by an unpaired, two-tailed Student's *t*-test (**A**). *F*-testing was conducted before Student's *t*-test to compare the variance of two samples. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001

Figure 6. Blocking both BCRs and TLRs abrogated refractory B-1b cell immune responses against refractory transplant-related anti-glycan Ags.

TLR–MyD88 signaling enhances BCR signaling by activating NFATc1, a factor functioning downstream of calcineurin activity. Calcineurin activation by BCR crosslinking with T cell-independent (TI) carbohydrate Ags dephosphorylates and activates NFATc1, which is associated with recombination-activating gene (RAG) expression and is required for optimal proliferation and survival of B-1a cells. B-1a cell activation is solely due to BCR signals that can be inhibited by a calcineurin inhibitor (CNI). BCR-signaling by itself activates the non-canonical NF-κB p100 pathway. TLR–MyD88 signals induce B-1b cell differentiation and enhance both canonical NF-κB p65 and BCR signaling (by activating NFATc1) and non-canonical NF-κB p100/p52, a downstream factor of the calcineurin–NFATc1 pathway, indicating that CNI cannot inhibit B-1b cells induced by MyD88 signaling activation. TLR4 inhibition suppressed NF-κB p65, NFATc1, and NF-κB p100/p52. Combined CNI and TLR4 inhibition suppressed

CNI-resistant B-1b activation. Bruton's tyrosine kinase (BTK) is an essential element of BCR signaling. TLR4–MyD88 activation also requires BTK. The combination of a potent BTK inhibitor and a histone deacetylase (HDAC) inhibitor to inhibit MyD88 signals abrogates refractory B-1b cell responses to TI carbohydrate Ags.

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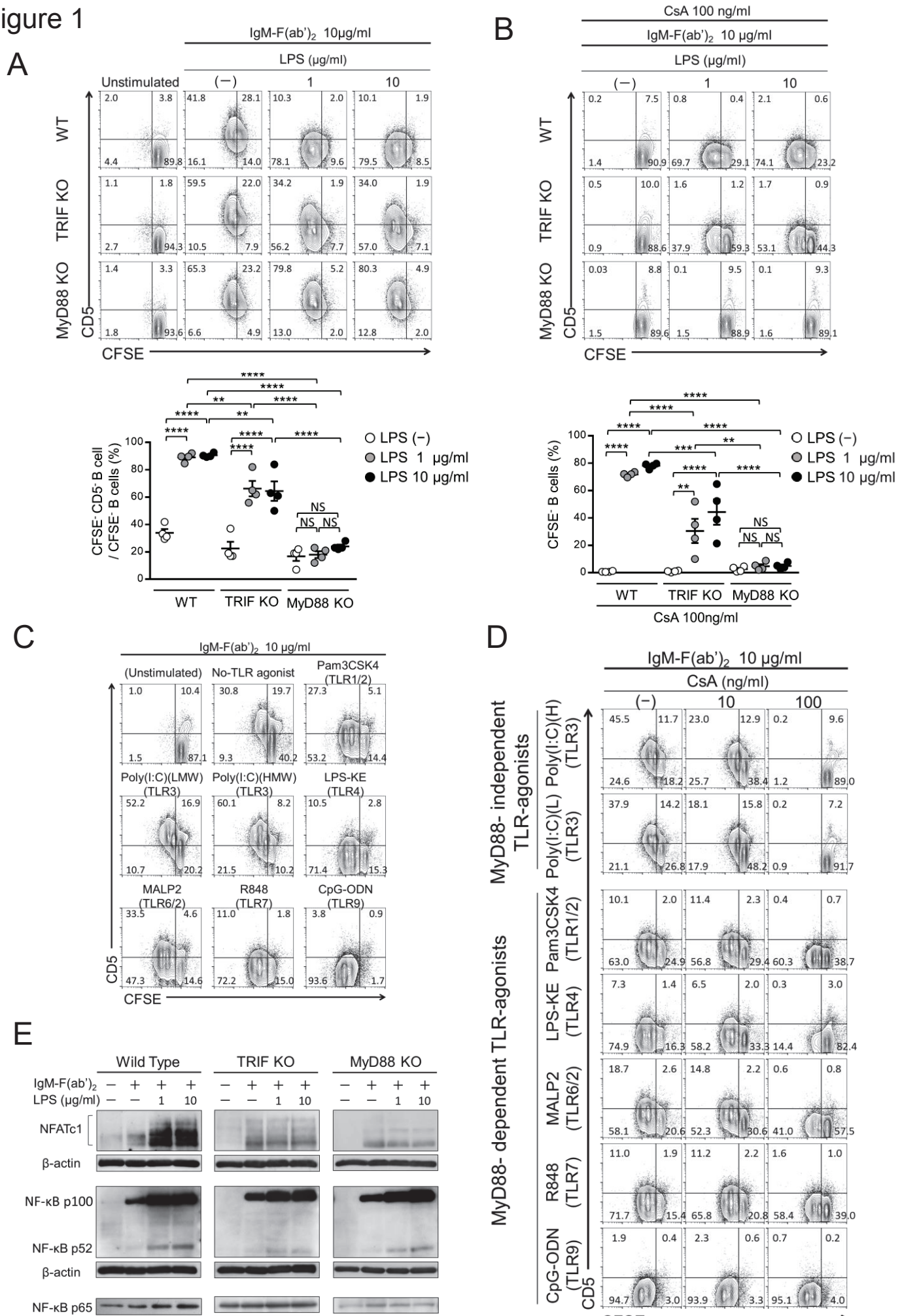
Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure 1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WT
TRIF KO
MyD88 KO
CD5
CFSE
CFSE- CD5- B cell / CFSE- B cells (%)
LPS (−)
LPS 1 μg/ml
LPS 10 μg/ml
NS
WT
TRIF KO
MyD88 KO
B
CsA 100 ng/ml
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LPS (μg/ml)
CFSE- B cells (%)
CsA 100ng/ml
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(Unstimulated)
No-TLR agonist
Pam3CSK4 (TLR1/2)
Poly(I:C)(LMW) (TLR3)
Poly(I:C)(HMW) (TLR3)
LPS-KE (TLR4)
MALP2 (TLR6/2)
R848 (TLR7)
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MyD88- independent TLR-agonists
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CpG-ODN (TLR9)
E
Wild Type
TRIF KO
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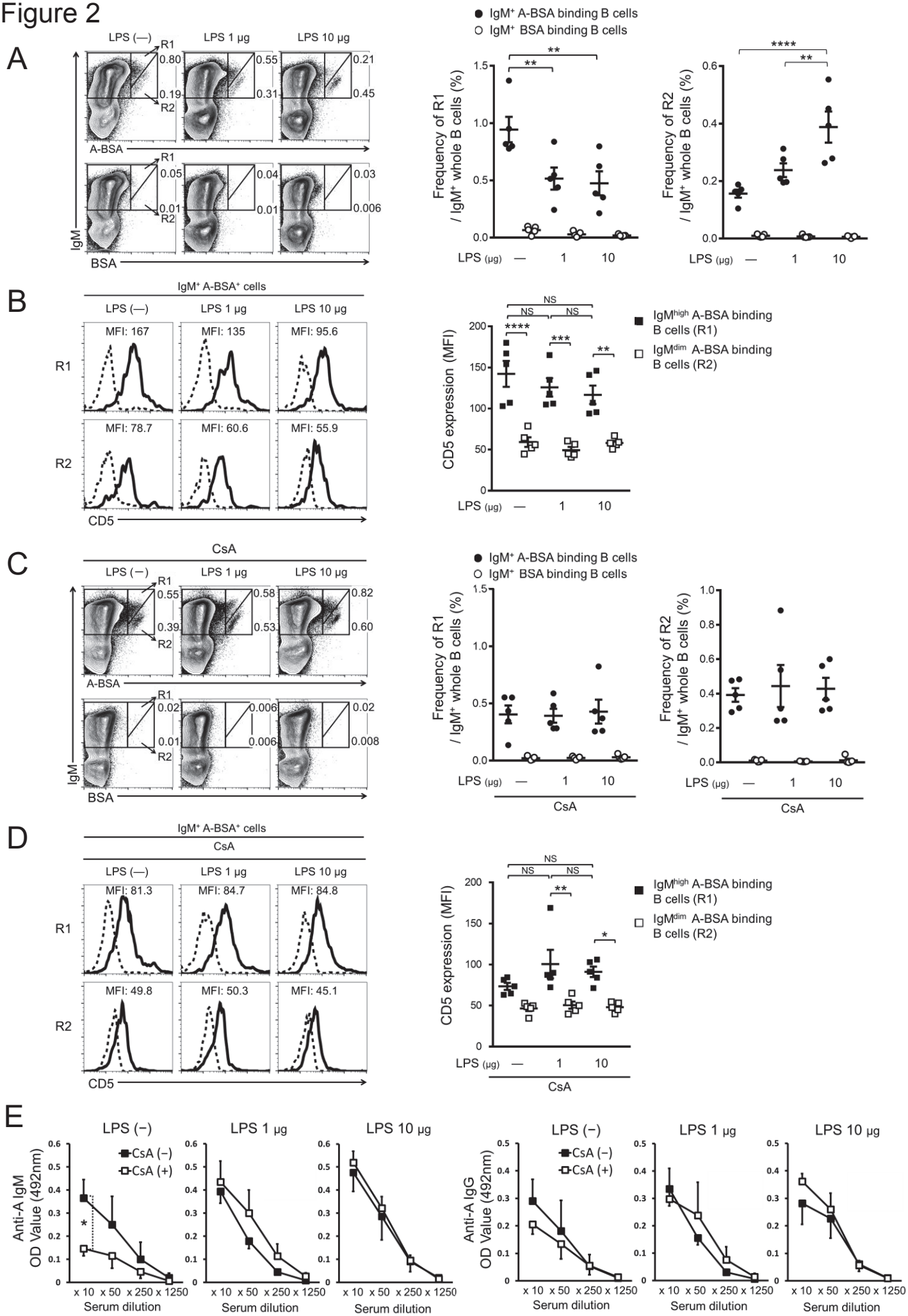
NF-κB p65

Figure 2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R2
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IgM
BSA
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Frequency of R1 / IgM+ whole B cells (%)
Frequency of R2 / IgM+ whole B cells (%)
LPS (μg)
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MFI: 135
MFI: 95.6
MFI: 78.7
MFI: 60.6
MFI: 55.9
CD5
CD5 expression (MFI)
NS
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IgMdim A-BSA binding B cells (R2)
C
CsA
D
MFI: 81.3
MFI: 84.7
MFI: 84.8
MFI: 49.8
MFI: 50.3
MFI: 45.1
E
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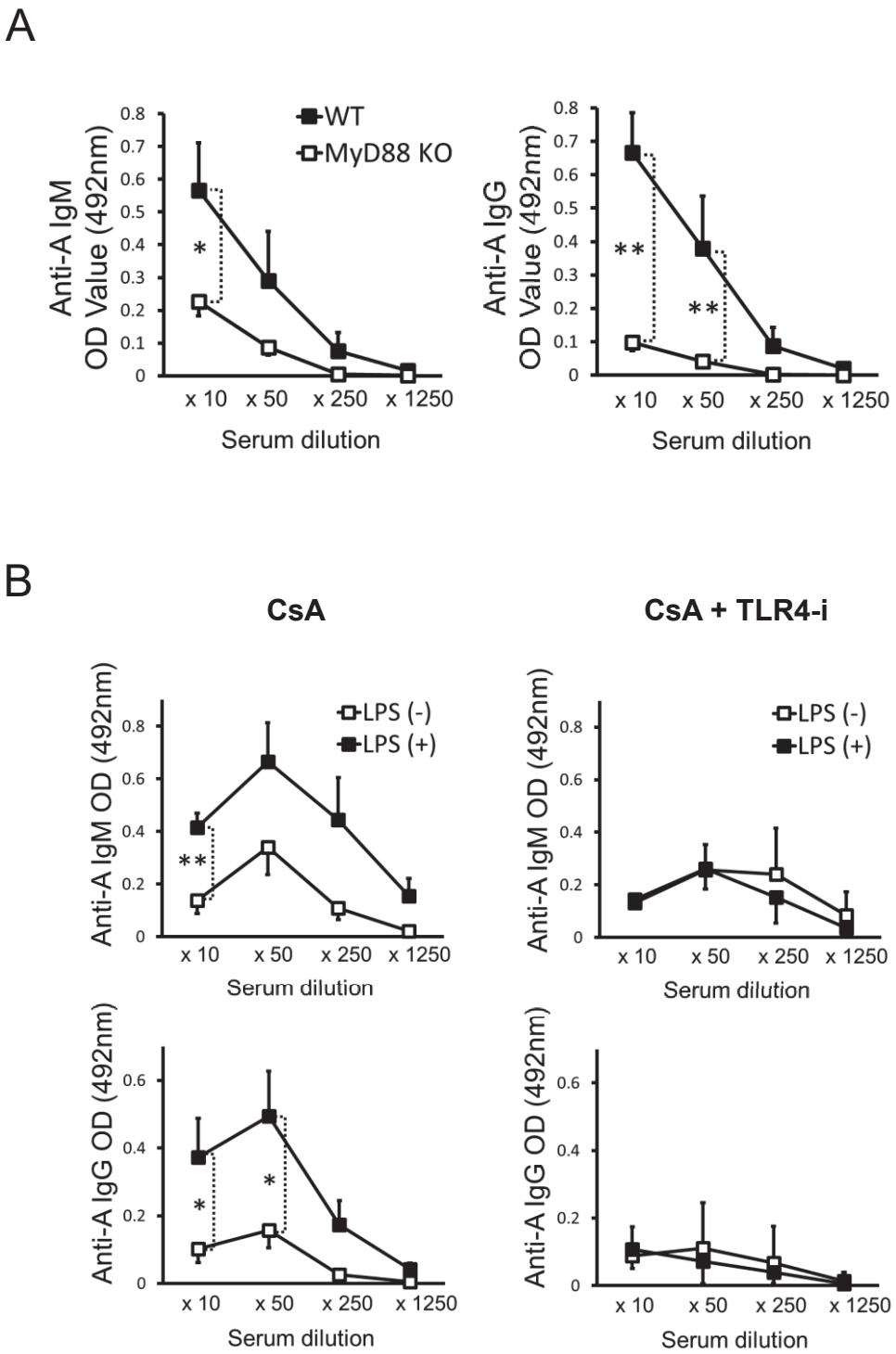
Anti-A IgG OD Value (492nm)
CsA (−)
CsA (+)
x 10 x 50 x 250 x 1250
Serum dilution

Figure 3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WT
MyD88 KO
Anti-A IgM
OD Value (492nm)
Anti-A IgG
OD Value (492nm)
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0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
**
**
x 10
x 50
x 250
x 1250
Serum dilution
B
CsA
CsA + TLR4-i
LPS (-)
LPS (+)
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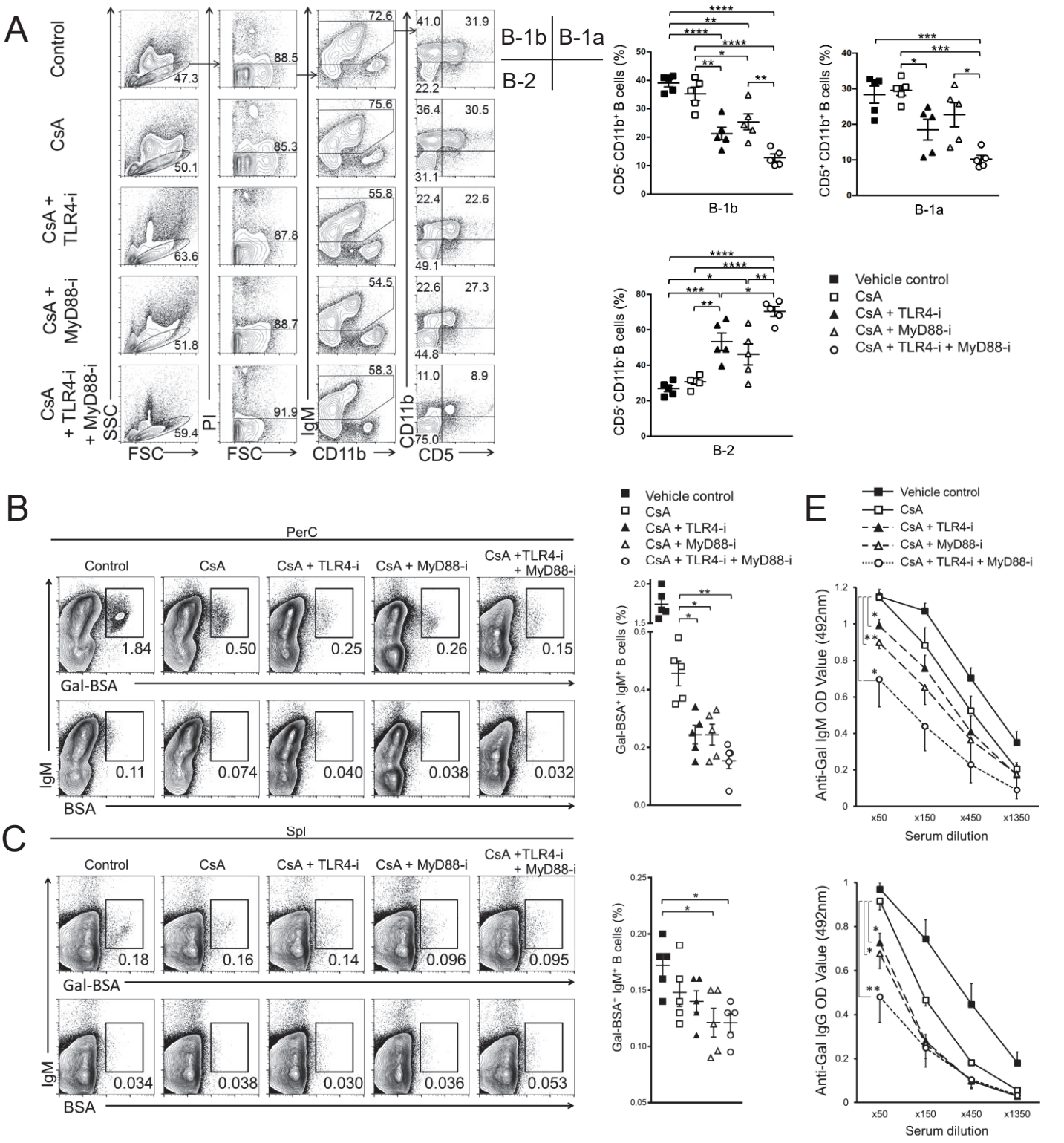
Anti-A IgG OD (492nm)
**
*
*
x 10
x 50
x 250
x 1250
Serum dilution

Figure 4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CsA + TLR4-i
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SSC
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PI
IgM
CD11b
CD5
B-1b
B-1a
B-2
CD5⁻ CD11b⁺ B cells (%)
CD5⁺ CD11b⁺ B cells (%)
CD5⁻ CD11b⁻ B cells (%)
Vehicle control
CsA
CsA + TLR4-i
CsA + MyD88-i
CsA + TLR4-i + MyD88-i
B
PerC
Gal-BSA
BSA
Gal-BSA⁺ IgM⁺ B cells (%)
E
Anti-Gal IgM OD Value (492nm)
Anti-Gal IgG OD Value (492nm)
Serum dilution
x50
x150
x450
x1350
C
Spl
D
Anti-Gal IgM producing cells
Anti-Gal IgG producing cells
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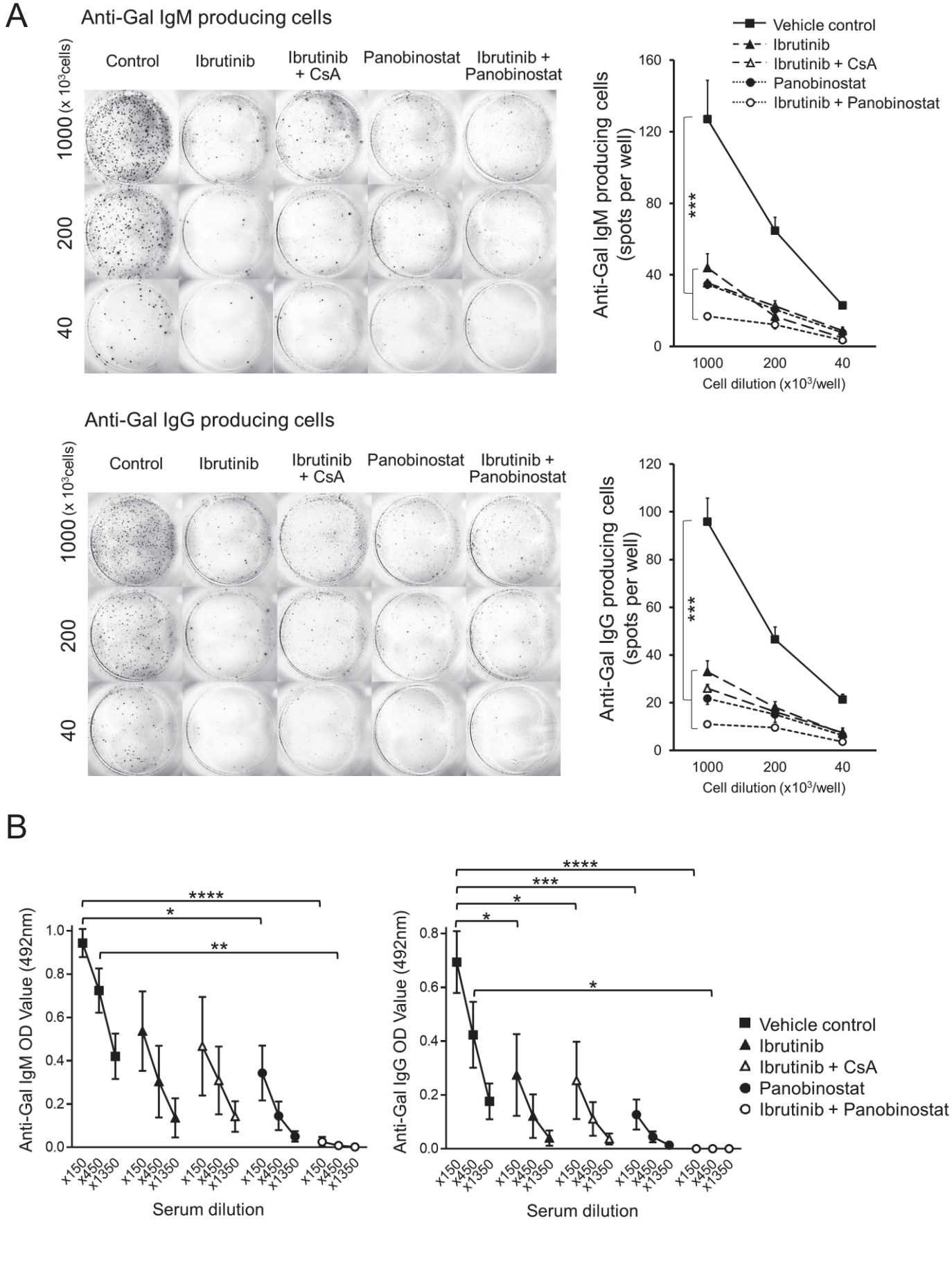
40
Anti-Gal IgM producing cells (spots per well)
Anti-Gal IgG producing cells (spots per well)
Cell dilution (x10³/well)

Figure 5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Control
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Panobinostat
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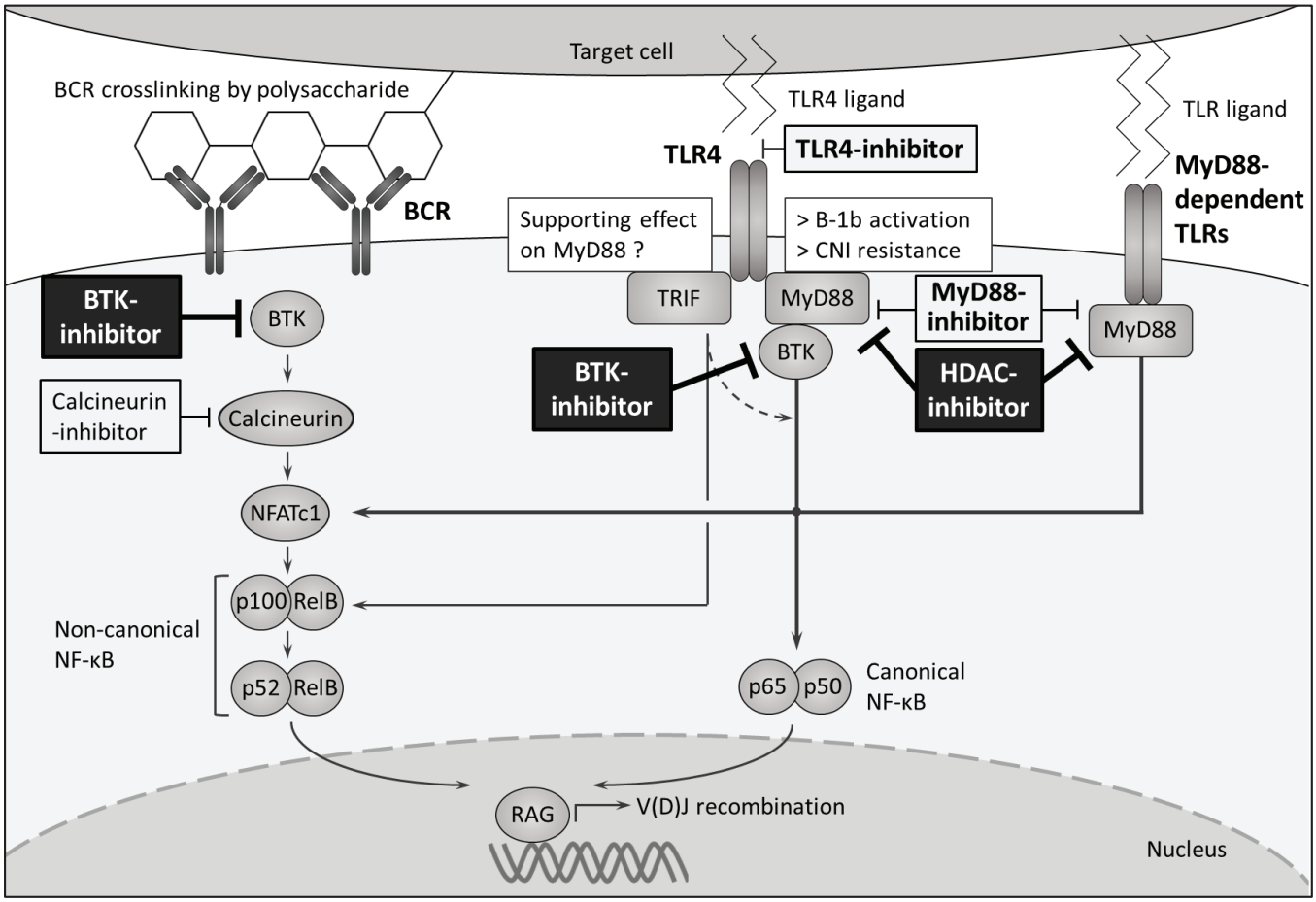
1000 (x 10³cells)
200
40
Vehicle control
Ibrutinib
Ibrutinib + CsA
Panobinostat
Ibrutinib + Panobinostat
Anti-Gal IgM producing cells (spots per well)
***
Cell dilution (x10³/well)
Anti-Gal IgG producing cells
Anti-Gal IgG producing cells (spots per well)
B
Anti-Gal IgM OD Value (492nm)
Anti-Gal IgG OD Value (492nm)
Serum dilution
x150
x450
x1350

Figure 6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> CNI resistance
TRIF
MyD88
MyD88-inhibitor
MyD88
BTK-inhibitor
BTK
BTK-inhibitor
BTK
HDAC-inhibitor
Calcineurin -inhibitor
Calcineurin
NFATc1
p100
RelB
Non-canonical NF-κB
p52
RelB
p65
p50
Canonical NF-κB
RAG
V(D)J recombination
Nucleus