SUPPLEMENTARY FIGURE LEGENDS

Figure S1. B cell development and signaling in B cell-*Myd88*$^{-/-}$ mice.

(A) B cell development and maturation in B cell-*Myd88*$^{-/-}$ mice. Spleen and bone marrow cells were isolated from WT and B cell-*Myd88*$^{-/-}$ mice, and stained with fluorescently conjugated antibodies against surface B220, CD93 (cloneAA4.1), CD23, and mIgM, to distinguish mature and immature B cell subpopulations. Absolute B cell numbers (top panel) were calculated for each subpopulation in the spleen and bone marrow, and surface IgM levels (lower panel) were determined by median fluorescent intensity (MFI) within each subpopulation. n = 7-8 mice/group. *P<0.05. (B and C) BCR signaling in B cell-*Myd88*$^{-/-}$ mice. Intracellular calcium mobilization (B), and phospho-ERK1/2 and phospho-AKT (C), were measured by flow cytometry following stimulation with anti-IgM F(ab)'$_2$ for the indicated times. Signaling within each subpopulation was determined using the markers described above, except that mature vs. immature B cells were distinguished by surface CD24 in place of CD93 for p-ERK1/2, and p-AKT analysis. Shown are data from mature B cell populations (follicular and Mz+B1), however, responses within immature B cell populations were also similar between WT and B cell-*Myd88*$^{-/-}$ mice. Mz+B1 B cells from WT and B cell-*Myd88*$^{-/-}$ mice had no detectable p-AKT signal at the time points tested, and are therefore not included here. Data are representative of 3 mice/group. (D) Purified splenic B cells from WT and B cell-*Myd88*$^{-/-}$ mice were left unstimulated (filled grey histogram), or stimulated with 15 μg/ml anti-IgM F(ab) '$_2$ or 500 ng/ml CpG for the indicated times. B cell activation (upper panel) in response to BCR (anti-

IgM) or TLR9 (CpG) agonists was measured by upregulation of surface CD86 on total B cells from WT (solid blue histogram), and B cell-*Myd88*$^{-/-}$ mice (dashed black histogram) 18h after stimulation. B cell proliferation ex vivo (lower panels) was measured by BrdU incorporation (36-48 hours) 48 h after BCR, and TLR9 stimulation of purified splenic B cells from WT (top panels) and B cell-*Myd88*$^{-/-}$ (lower panels) mice. FACS plots for surface CD86 and BrdU positive cells are representative of 3 mice/group.

Figure S2. Antibody responses to a protein antigen mixed with aggregated CpG.

(A) Wild type mice were immunized i.p. with 100 μg OVA mixed together with 25 μg CpG (ODN1826) or with 25 μg of DOTAP-aggregated CpG. (B) Wild type (*Myd88*$^{fl/fl}$), IFNAR1$^{-/-}$ (*Ifnar*$^{-/-}$, *Myd88*$^{fl/fl}$), and DC-*Myd88*$^{-/-}$, IFNAR1$^{-/-}$ (*Myd88*$^{fl/fl}$ CD11c-Cre *Ifnar1*$^{-/-}$) mice were immunized i.p. with 100 μg of OVA mixed with 25 μg of DOTAP-aggregated CpG (ODN1826). (C) Wild type mice reconstituted with bone marrow from either wild type or CD11c-DTR mice were treated with diphtheria toxin to deplete DCs, and then were immunized i.p. with 100 μg OVA mixed together with 25 μg of DOTAP-aggregated CpG (ODN1826). Shown are the titers of anti-OVA antibody of individual mice and the geometrical mean of each mouse group in the primary responses. Similar results (for A-C) were obtained in one additional experiment, respectively. *, $P<0.05$, **, $P<0.01$.

Figure S3. Antibody responses to immunization with VLP-CpG.

(A-C) The primary anti-Qβ antibody responses were compared between indicated groups of mice two weeks after immunized i.p. with 50 μg VLP-CpG. (A) Wild type vs. TCR$\alpha^{-/-}$ mice. Similar results were obtained in one additional experiment. (B) Wild type chimeric mice vs. DC-depleted mice. Similar results were obtained in two additional experiments. (C) C57BL/6 mice vs. Mb1-Cre mice. Shown are the titers of anti-Qβ antibody of individual mice and the geometrical mean of each mouse group in the primary responses. *, $P<0.05$, **, $P<0.01$.

Figure S4. Antibody responses to immunization with Fel d1 and VLP-CpG conjugate.

Wild type and B cell-*Myd88*$^{-/-}$ mice were immunized i.p. with 50 μg of Fel d1 and VLP-CpG conjugate. Shown are the titers of anti-Fel d1 antibody (A) and anti-Qβ antibody (B) of individual mice and the geometrical mean of each mouse group in the primary responses. Similar results were obtained in one additional experiment. *, $P<0.05$, **, $P<0.01$.

SUPPLEMENTARY MATERIALS AND METHODS

Cell purification

For purification of B cell subsets, single cell suspensions of splenocytes or peritoneal cells were labeled with fluorescent antibodies and sorted on a MoFlow flow cytometer (DAKO) to obtain follicular B cells ($CD19^+$, $CD93^-$, $CD23^{hi}$, IgM^{low}), marginal zone B cells ($CD19^+$, $CD93^-$, $CD23^{low}$, IgM^{hi}), and peritoneal B cells ($CD19^+$, $F4/80^-$). The purity of the resulting cell preparations was generally more than 98%. Deletion of exon 3 of Myd88 was determined by quantitative PCR of genomic DNA as previously described (Hou et al., 2008).

B cell activation and proliferation

Splenic B cells from WT and B cell-*Myd88*$^{-/-}$ mice were purified by negative selection with mouse anti-CD43 conjugated magnetic beads to deplete all non-B cells (Dynabeads, Invitrogen) according to manufacturers protocol. $1x10^6$ splenic B cells/ml (~95% purity) were left unstimulated, or stimulated with 15 μg/ml anti-IgM $F(ab)'_2$ (Jackson Immunoresearch) or 500 ng/ml CpG (IDT) in complete Iscove's medium containing 10% FBS. 18 hours after stimulation, cells were fixed in 2% paraformaldehyde (PFA) and stained for surface B220 and CD86 with mouse anti-B220-PE-Cy7, and anti-CD86-PE (BD biosciences). For proliferation analysis, splenic B cells were purified, and stimulated as described above, for 48 hours. Cells were incubated overnight with 10 μM BrdU (FITC BrdU flow kit, BD biosciences) 36 hours after stimulation. The following day, cells were and fixed and permeabilized, and stained for surface CD19 (anti-

CD19-APC-cy7, BD biosciences), followed by intracellular staining with anti-BrdU-FITC according to manufacturers instructions. For activation and proliferation experiments, cells were analyzed by flow cytometry on an LSR-II, and all FACS data was analyzed with FlowJo version 8.8.6 (Tree Star software).

B cell development and maturation:

Immature, transitional, and mature B cell populations were identified in the spleen and bone marrow of WT and B cell-*Myd88*$^{-/-}$ mice using fluorescently conjugated antibodies against surface B220, CD23 (BD biosciences), CD93 (clone AA4.1, eBioscience), and IgM F(ab)' monomer (Jackson Immunoresearch). In the spleen, surface CD93 was used to distinguish immature (B220^{+}, CD93^{+}) and mature (B220^{+}, CD93^{-}) B cells. Within the immature B cell population, surface CD23 and IgM levels were used to identify immature-transitional T1 (IgM^{hi}, CD23$^{lo\text{-}neg}$), T2 (IgM^{hi}, CD23$^{hi\text{-}int}$), and T3 (IgM^{lo}, CD23$^{hi\text{-}int}$). Within the mature B cell population, surface CD23 and IgM levels distinguish follicular (IgM$^{lo\text{-}int}$, CD23^{+}) and Marginal zone + B1 B cells (IgM^{hi}, CD23$^{lo\text{-}neg}$). The same markers were used to distinguish immature and pro/pre-B cells (B220^{+}, CD93^{+}), and mature recirculating B cells (B220^{+} CD93^{-}). Surface IgM and CD23 levels within the CD93^{+} population distinguish newly formed immature B cells, BM-T1 (IgM^{hi}, CD23$^{lo\text{-}neg}$) and BM-T2 (IgM^{hi}, CD23^{int}), and pro/pre-B cells (IgM^{-}, CD23^{-}). Absolute cell numbers within each population were back-calculated from total spleen, or total cells/femur and tibia. Surface IgM

levels were obtained from median fluorescence intensity (MFI) within each B cell subpopulation.

Analysis of Calcium mobilization:

Total splenocytes from WT and B cell-*Myd88*$^{-/-}$ mice were loaded with Indo-1 AM (Molecular Probes) and then stained for immature and mature B cell subpopulations using fluorescently conjugated antibodies against B220, CD93, CD23, and IgM as described above. This combination of antibodies has previously been shown to not perturb early BCR signaling (Gross et al., 2009). Cells were resuspended in RPMI-1640 medium supplemented with 1% BSA and 20 mM HEPES, and warmed for 3 minutes at 37 ^{o}C. Cell indo-1 fluorescence was then acquired on an LSR-II for 30 seconds to establish a baseline recording, and then cells were stimulated with 25 μg/ml anti-IgM F(ab)'$_2$. Events were recorded for an additional 3 minutes, and the median intracellular calcium concentration was derived from the ratio of fluorescence at 405 nm emission to 530 nm emission. Dead cells were excluded by propidium iodide uptake. Data was analyzed with FlowJo version 8.8.6.

Analysis of ERK and AKT phosphorylation:

Total splenocytes were from WT and B cell-*Myd88*$^{-/-}$ mice were resuspended in Iscoves medium supplemented with 1% BSA, 10 mM HEPES, and 50 μM 2-ME, rested at 37 degrees for 30 min, and left unstimulated, or stimulated with 50 μg/ml anti-IgM F(ab)'$_2$ for 3 min (p-ERK) or 30 min (p-AKT). Prior to stimulation,

cells were labeled with anti-IgM-FITC F(ab)' monomer for 10 min at 37 degrees. After stimulation, cells were immediately fixed with 2% PFA (Electron Microscopy Sciences) at the indicated times, and subsequently permeabilized with ice cold 100 % methanol (Electron Microscopy Sciences) for 30 min. Cells were then labeled with phospho-p44/42 (Thr^{202}/Tyr^{204}) rabbit mAB (197G2) or rabbit anti-mouse phospho-AKT (Ser^{473}) (both from Cell Signaling Technology). Cells were then labeled with donkey anti-rabbit IgG-APC (Jackson immunoresearch) as well as fluorescent antibodies against surface B220, CD23, and CD24 (Biolegend) (used in place of CD93 which does not bind after fixation). Cells were analyzed by flow cytometry for intracellular p-ERK or p-AKT levels within each B cell subpopulation.

Generation of bone marrow chimeras and DC depletion

Bone marrow chimeras were generated by injecting bone marrow cells from either CD11c-DTR mice or BoyJ mice into eight weeks old female C57BL/6 mice that were lethally irradiated (900 rad) in a γ-irradiator. Chimerism was examined 7 weeks later, and only mice with greater than 98% of blood myeloid cells coming from the donor mice were used for DC depletion experiments.

To deplete DCs, bone marrow chimeric mice were injected i.p. with 100 ng/mouse diphtheria toxin (Jung et al., 2002) one day before immunization and then every other day until day 11.

REFERENCE

Gross, A.J., Lyandres, J.R., Panigrahi, A.K., Prak, E.T., and DeFranco, A.L. (2009). Developmental acquisition of the Lyn-CD22-SHP-1 inhibitory pathway promotes B cell tolerance. J Immunol *182*, 5382-5392.

Hou, B., Reizis, B., and DeFranco, A.L. (2008). Toll-like receptors activate innate and adaptive immunity by using dendritic cell-intrinsic and -extrinsic mechanisms. Immunity *29*, 272-282.

Jung, S., Unutmaz, D., Wong, P., Sano, G., De los Santos, K., Sparwasser, T., Wu, S., Vuthoori, S., Ko, K., Zavala, F.*, et al.* (2002). In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. Immunity *17*, 211-220.

Figure S1

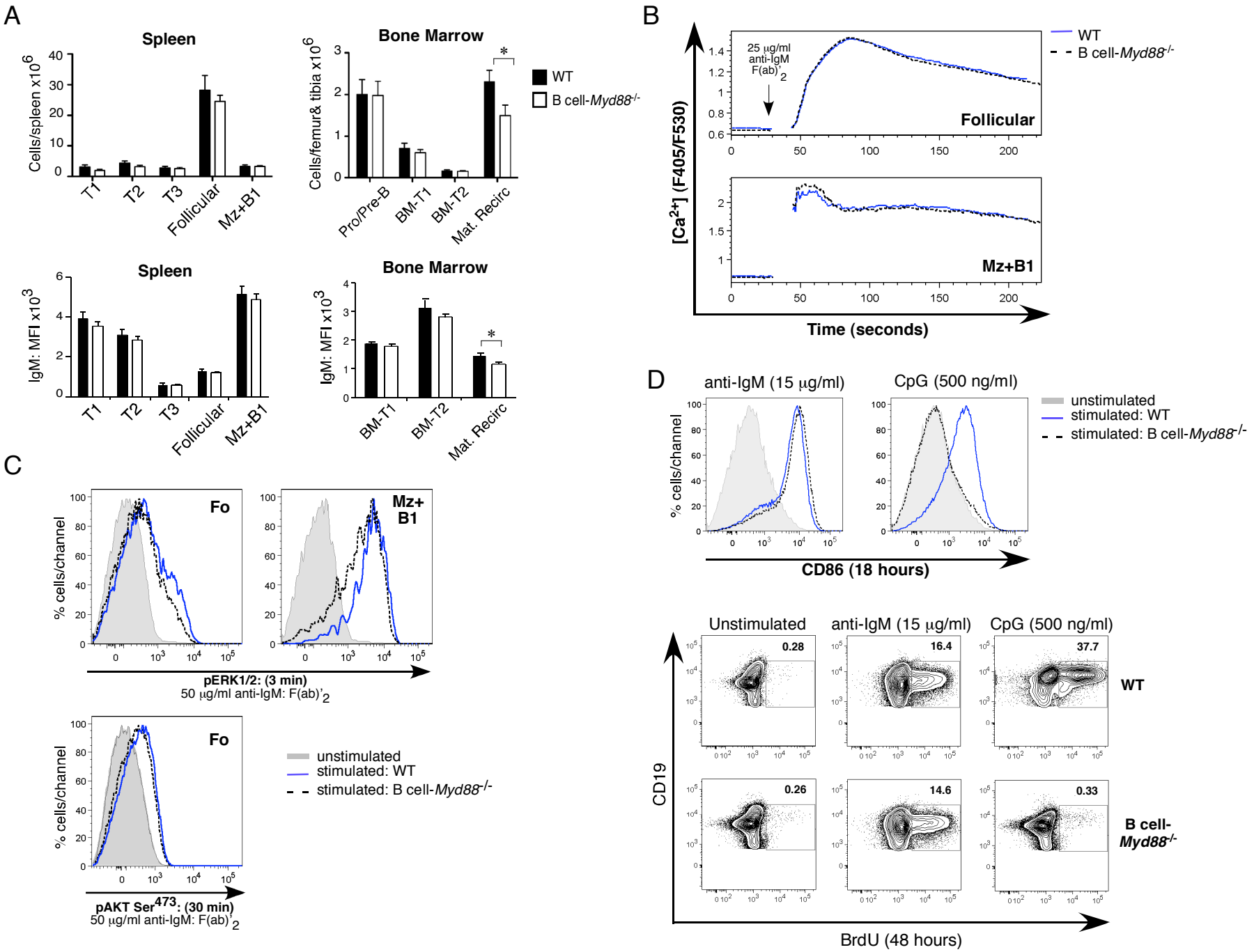

A
Spleen
Cells/spleen x10^6
T1
T2
T3
Follicular
Mz+B1
Bone Marrow
Cells/femur& tibia x10^6
Pro/Pre-B
BM-T1
BM-T2
Mat. Recirc
WT
B cell-Myd88-/-
IgM: MFI x10^3
B
25 µg/ml anti-IgM F(ab)'2
Follicular
Mz+B1
[Ca2+] (F405/F530)
Time (seconds)
C
Fo
Mz+ B1
% cells/channel
pERK1/2: (3 min)
50 µg/ml anti-IgM: F(ab)'2
pAKT Ser473: (30 min)
unstimulated
stimulated: WT
stimulated: B cell-Myd88-/-
D
anti-IgM (15 µg/ml)
CpG (500 ng/ml)
CD86 (18 hours)
Unstimulated
0.28
16.4
37.7
0.26
14.6
0.33
CD19
BrdU (48 hours)

Figure S2

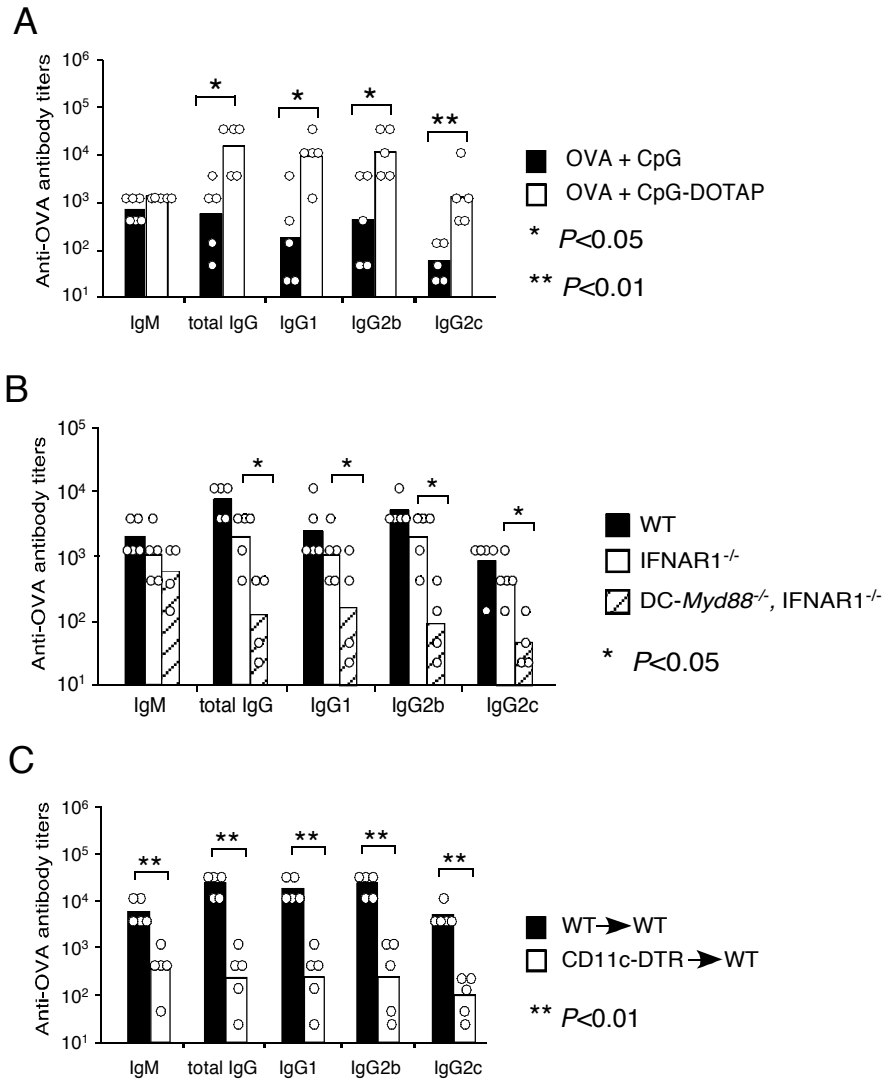

A
Anti-OVA antibody titers
IgM
total IgG
IgG1
IgG2b
IgG2c
OVA + CpG
OVA + CpG-DOTAP
* P<0.05
** P<0.01
B
WT
IFNAR1-/-
DC-Myd88-/-, IFNAR1-/-
* P<0.05
C
WT→WT
CD11c-DTR→WT
** P<0.01

Figure S3

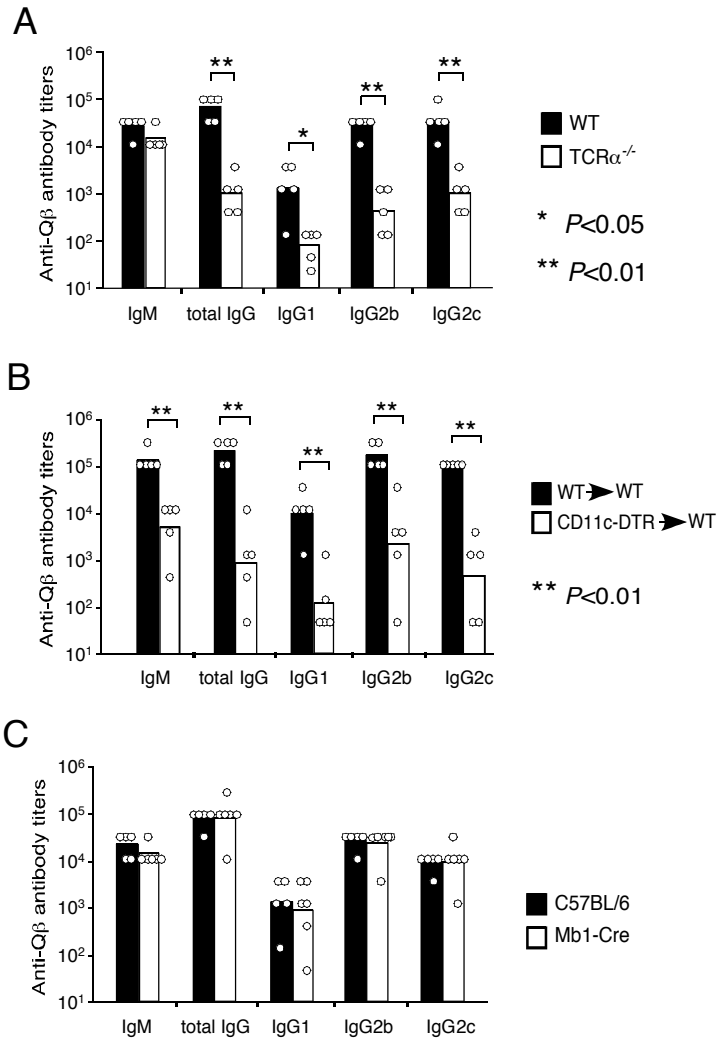
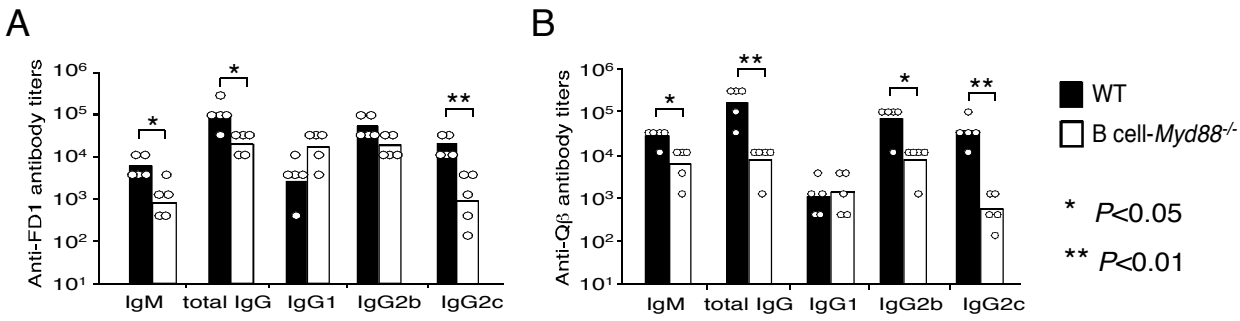

A
Anti-Qβ antibody titers
10^6
10^5
10^4
10^3
10^2
10^1
**
*
**
**
IgM
total IgG
IgG1
IgG2b
IgG2c
WT
TCRα-/-
* P<0.05
** P<0.01
B
Anti-Qβ antibody titers
**
**
**
**
**
IgM
total IgG
IgG1
IgG2b
IgG2c
WT➔WT
CD11c-DTR➔WT
** P<0.01
C
Anti-Qβ antibody titers
IgM
total IgG
IgG1
IgG2b
IgG2c
C57BL/6
Mb1-Cre

Figure S4


A
B
Anti-FD1 antibody titers
Anti-Qβ antibody titers
IgM
total IgG
IgG1
IgG2b
IgG2c
WT
B cell-Myd88-/-
* P<0.05
** P<0.01