Immune regulation by receptors for IgG

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Chapter 7

Highly B lymphocyte-specific tamoxifen inducible transgene expression of CreER^{T2} by using the LC-1 locus BAC vector

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SUMMARY

The generation of cell type specific inducible Cre transgenic mice is the most challenging and limiting part in the development of spatio-temporally controlled knockout mouse models. Here we report the generation and characterization of a B lymphocyte-specific tamoxifen-inducible Cre transgenic mouse strain, LC-1-hCD19-CreER^{T2}. We utilized the human CD19 promoter for expression of the tamoxifen-inducible Cre recombinase (CreER^{T2}) gene, embedded in genomic sequences previously reported to give minimal position effects after transgenesis. Cre recombinase activity was evaluated by cross-breeding the LC-1-hCD19-CreER^{T2} strain with a strain containing a floxed gene widely expressed in the hematopoietic system. Cre activity was only detected in the presence of tamoxifen and was restricted to B lymphocytes. The efficacy of recombination ranged from 27% to 61% in the hemizygous and homozygous mice respectively. In conclusion, the LC-1-hCD19-CreER^{T2} strain is a powerful tool to study gene function specifically in B lymphocytes at any chosen time point in the lifecycle of the mouse.

INTRODUCTION AND RESULTS

B cells belong to the lymphocyte compartment, the main part of the adaptive immune system. Mature B lymphocytes develop from bone marrow progenitor cells in a tightly regulated process and express highly diverse antigen receptors, which are able to recognize a wide variety of foreign compounds. The B lymphocyte repertoire is maintained and renewed during the lifespan of a mammal. Loss of control of these processes can result in the development of autoimmunity, hypersensitivity, impaired protection against pathogens or leukemia (1-3).
B cells are versatile players of the immune system: in addition to secreting antibodies they are also potent antigen-presenting cells and important immune modulators through the secretion of cytokines (4). Throughout a typical immune response activated B cells will secrete antibodies of higher affinity (affinity maturation) and of more potent effector function (IgG, IgA or IgE instead of the IgM isotype). High affinity IgG antibodies are crucial in eliminating pathogens but also in mediating autoimmune pathology. After the pathogen is cleared the majority of the antibody-secreting B cells will undergo apoptosis and long-lived memory B cells are formed that maintain antibody levels in the serum. This multistep process is precisely controlled by a number of genes, many of which act at multiple stages. It is of great importance to study the specific contributions of these genes to B cell function in distinct stages of the immune response or in (auto)immune pathology.
B cell-specific gene inactivation using constitutive Cre-expressing strains (such as the CD19-Cre or mb1-Cre) influences B cell ontogenesis, which might result in altered B lymphocyte repertoire (5). In addition, these models do not allow genetic alterations to be introduced at a well-defined time point of an immune response (such as the recall response) or during autoimmune disease (such as after activation of pathogenic B cells). For these reasons there is a need for transgenic mice that allow efficient B cell-specific somatic mutagenesis in adult mice at a desired time point.
In spatio-temporally controlled systems, the temporal control is provided by transgenic mice expressing the Cre recombinase in an inducible manner (6-8). Alternatively, Cre activity can be controlled on the protein level by fusing the recombinase to a modified ligand-binding domain of the estrogen receptor (ER) (9;10), rendering the fusion protein tamoxifen inducible. The CreER^{T2} system has proven to be the most potent inducible Cre protein with low to non detectable leakiness and high activity *in vivo* (9). We have generated a novel transgenic mouse strain expressing the CreER^{T2} protein exclusively in B cells (see Materials and Methods and Fig. 1).
To obtain B cell-specificity, a 6.3 kb fragment of the human CD19 promoter was used, which has been shown earlier to drive B cell-restricted expression in mice (11) (Fig. 1). The CD19 protein is expressed throughout B cell development from the early pre-B stadium until mature B cells, but not in plasma cells (12). The expression early in B cell development means that all progenitor cells (from immature to mature) will have the recombined locus upon tamoxifen treatment.
The characteristics of inducible constructs in transgenic mice are often influenced by the integration site (7;13). Therefore the knock-in approach is applied using an endogenous promoter to avoid position effects (14). However, recombining the Cre structural gene into the CD19 locus has led to a heterozygous inactivation of the CD19 gene, resulting in decreased

CD19 protein levels. Consequently the CD19-Cre knock-in strain provides effective Cre activity in B cells, but due to the decreased CD19 protein levels, these mice show reduced numbers of peritoneal B1 cells (14).
In an attempt to reduce position effects and to preserve the endogenous CD19 activity we employed BAC transgenesis using the LC-1 locus containing BAC clone (7). The LC-1 locus has been identified as a genomic DNA fragment, which allows tightly regulated expression of tetracycline-inducible constructs after transgenic integration (15). The hCD19-CreER^{T2}

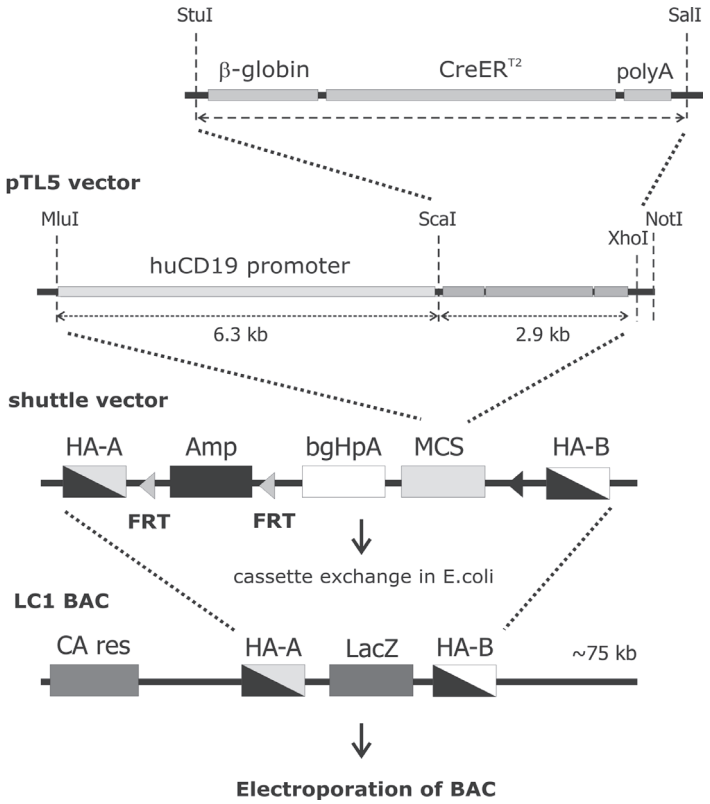

Figure 1. B cell-specific CreER^{T2}-mediated recombination.
Schematic representation of the hCD19-CreER^{T2} construct. The DNA fragment encoding CreER^{T2} was placed downstream of a 6.3 kb fragment of the human CD19 promoter. The construct was then cloned into the multiple cloning site (MCS) of the pE11.F3.M.F. shuttle vector, followed by homologous recombination into the LC-1 BAC clone via lambda red recombination. The linearized BAC clone of 75 kb was used to generate transgenic animals (BAC, bacterial articifial chromosome; MCS, multiple cloning site; FRT, recognition site for Flp recombinase; HA-A and B, homologous arm A and B; bgHpA, bovine growth hormone polyA; Amp, ampicilline; CA, chloramphenicol).

construct was inserted into the LC-1 BAC using Red-mediated homologous recombination in *E. coli* (16). After linearization, the recombinant BAC was injected into pronuclei of fertilized oocytes of C57Bl/6 mice in order to generate transgenic mice (Fig. 1). Two

founders were identified using PCR; and both transmitted the transgene into the germline of the F1 generation. One strain, designated as LC-1-hCD19-CreER^{T2}, provided functional Cre-mediated recombination of target alleles in double transgenic animals. Real time PCR analysis on genomic tail DNA of this line showed that the transgene integrated as a single copy (data not shown).

The expression and the activity of Cre in the LC-1-hCD19-CreER^{T2} strain was evaluated by crossbreeding the strain to mice carrying a conditional ('floxed') allele of the type IIB low affinity IgG Fc receptor, FcγRIIB (P.B., J.S.V. unpublished results). FcγRIIB is broadly expressed in the immune system and cell surface expression of FcγRIIB can be precisely monitored and quantified by cytofluorimetry using a specific monoclonal antibody (K9.361,

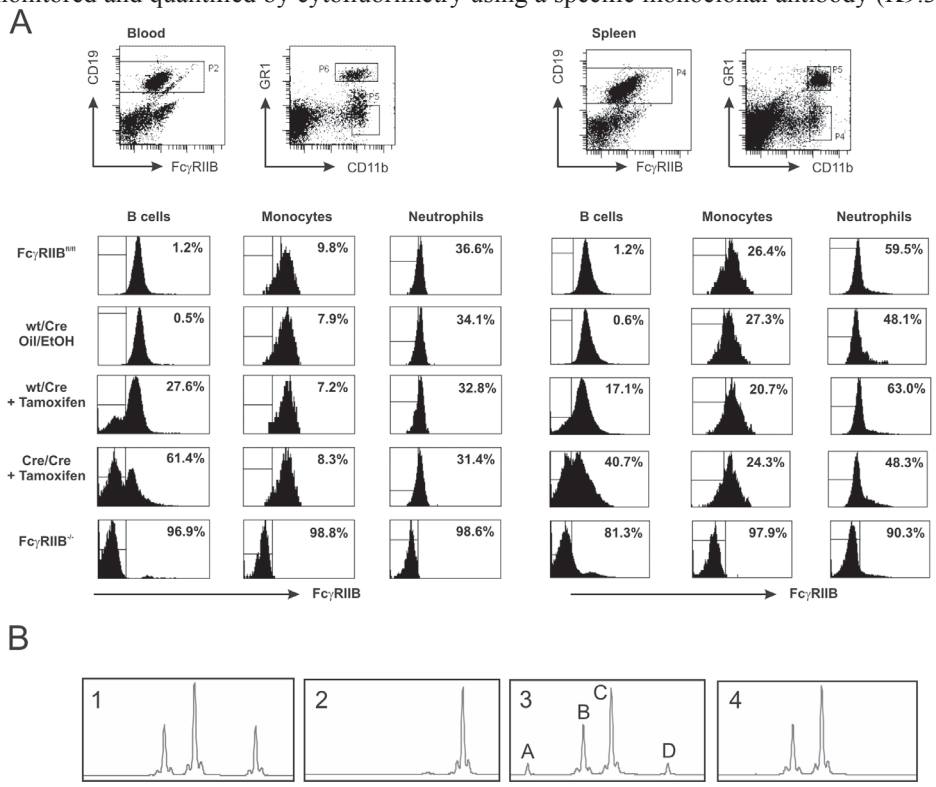

Figure 2. LC-1-hCD19-CreER^{T2} mediated recombination of the floxed FcγRIIB allele.

A: Cytofluorimetric analysis of haemopoietic cells from wt/LC-1-hCD19-CreER^{T2}; FcγRIIB$^{fl/fl}$ mice treated with tamoxifen (2.5mg/day, 5 days). Cell populations in peripheral blood and spleen were identified as follows; B cells: CD19^{+}, macrophages/monocytes: CD11b^{+}GR1^{-}, neutrophils: CD11b^{+}GR1^{+}. Expression of FcγRIIB on the selected populations was analyzed with Alexa488-labelled specific mAb (Ly17.2). Numbers on the histograms indicate percentages of FcγRIIB-deficient cells. (FcγRIIB$^{fl/fl}$, mice with floxed FcγRIIB allele, wt/Cre and Cre/Cre, homozygous and hemizygous LC-1-hCD19-CreER^{T2} mice, respectively)

B: MLPA peaks obtained after capillary electrophoresis. Samples: 1) control DNA from a spleen from a FcγRIIB$^{flox/null}$ mouse 2) control DNA from a spleen from a FcγRIIB$^{null/null}$ mouse 3) DNA from the spleen of a LC-1-hCD19-CreER^{T2}/LC-1-hCD19-CreER^{T2}; FcγRIIB$^{fl/fl}$ after tamoxifen administration. 4) same as 3) except DNA isolated from the kidney. Peaks: Peak A: product of probes C & B (deletion circle), Peak B: product of probes A & B (first loxP), Peak C: product of probes C & D (second loxP), Peak D: product of probes A & D (Null).

Ly17.2-specific). Wt/LC-1-hCD19-CreER^{T2}; FcγRIIB$^{fl/fl}$ mice were treated orally with tamoxifen (2.5 mg/day) (as described in Materials and Methods) for 5 days. Five days after the treatment 25-33% of circulating B cells lost FcγRIIB expression, which was monitored by cytofluorimetry (Fig. 2A).
Recombination events were not observed in the absence of tamoxifen or in other haematopoietic cells, such as neutrophils or macrophages (Fig. 2A). Recombination efficacy in bone marrow (BM) or peritoneal B cells was somewhat lower (~12% and 7-18%, respectively) (data not shown). This could be explained by the continuous efflux of newly formed B cells from the BM and the lower turnover rate of peritoneal B1 cells (17;18). Six LC-1-hCD19-CreER^{T2}; FcγRIIB$^{fl/fl}$ mice were monitored for a longer period of time after tamoxifen treatment and the percentages of FcγRIIB-deficient blood B cells were determined at the indicated time points (Fig. 3A). The number of FcγRIIB-deficient B cells strongly decreased after 3 weeks of the treatment, probably due to efflux of newly formed wild-type B cells from the bone marrow. Fourteen weeks after the five day period of tamoxifen administration 5% of the circulating B cells were still FcγRIIB-deficient (Fig. 3A). By increasing the dose of tamoxifen (5 mg/day, 5 days or 2.5 mg/day, 10 days) no marked increase of Cre activity was observed indicating that with 2.5 mg/day, maximal Cre activity was reached (data not shown). In LC-1-hCD19-CreER^{T2}/LC-1-hCD19-CreER^{T2}; FcγRIIB$^{fl/fl}$ mice recombination efficacy reached 60-64% due to the presence of two copies of the transgene but a marked reduction of B cell numbers was observed compared to all other groups (Fig. 3B). Since homozygous and hemizygous Cre mice showed similar blood B cell numbers before treatment with tamoxifen this reduction is most likely due to Cre toxicity as reported previously (14) and not related to effects of the integration site of the transgene. The recombination efficacy values deduced from the cytofluorimetry data (Fig. 2A) were confirmed using a recently published MLPA-based method (19) with DNA samples isolated from the spleen (Fig. 2B).
In addition to the cross with FcγRIIB$^{fl/fl}$ mice the LC-1-hCD19-CreER^{T2} was crossed with the

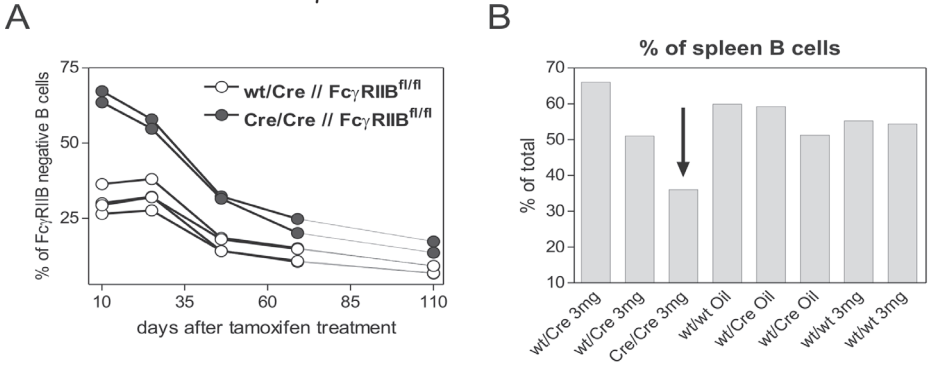

Figure 3. Follow up of LC-1-hCD19-CreER^{T2}-mediated recombination in time.
A: wt/LC-1-hCD19-CreER^{T2}; FcγRIIB$^{fl/fl}$ or LC-1-hCD19-CreER^{T2}/LC-1-hCD19-CreER^{T2}; FcγRIIB$^{fl/fl}$ mice were treated with tamoxifen (2.5 mg/day, 5 days) and were followed and recombination efficacy of FcγRIIB on B cells was determined by cytofluorimetry at the indicated time points.
B: B cell numbers in the spleen after treatment with tamoxifen. LC-1-hCD19-CreER^{T2}/LC-1-hCD19-CreER^{T2} mice show decreased B cell numbers after tamoxifen treatment (arrow).

Z/EG double reporter mouse strain (20). In this Cre reporter strain the transgene consists of the ubiquitous CGAGS promoter driving a lacZ reporter unit flanked by loxP sites followed by an EGFP reporter gene. Upon Cre activity the lacZ gene, functioning as a stop cassette, preventing expression of the downstream EGFP gene, is removed and EGFP is expressed, allowing detection of recombination events by cytofluorimetry. We compared the efficacy of recombination in Wt/LC-1-hCD19-CreER^{T2}; Z/EG mice with ROSA26-CreER^{T2}; Z/EG mice, which provide ubiquitous inducible Cre activity (21). We observed B cell-specific GFP expression in tamoxifen treated (5 mg/day, 5 days) LC1-hCD19-CreER^{T2}; Z/EG animals but not in mice treated with vehicle only (Fig. 4). The percentage of GFP positive cells, however, was very low, ranging from 1.85 - 2% blood B cells and 0.6 - 1.5% splenic B cells. Intraperitoneal injection of vehicle (oil) only also resulted in a relative decrease of B cell numbers suggesting that the oil has an effect on the B cell compartment. Increasing the dose of tamoxifen above 5 mg/mouse did not further improve efficacy but was toxic to the mice. About 7.45% of the circulating B cells from tamoxifen-treated ROSA26-CreER^{T2}; Z/EG mice were GFP positive, while recombination also occurred in non-B cells as expected (Fig. 4) (21).

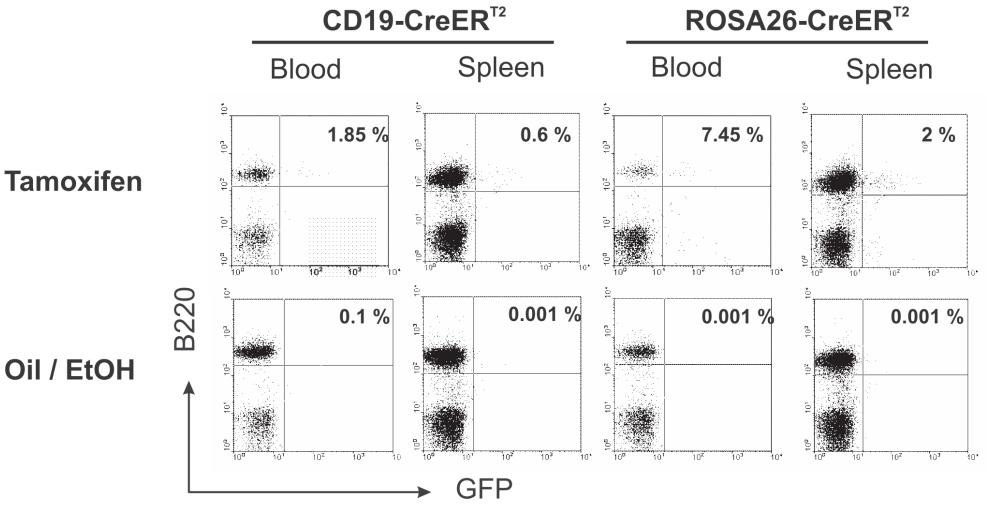

Figure 4. In vivo analysis of LC-1-hCD19-CreER^{T2} mediated activity using the Z/EG strain.
Double transgenic animals (left panels: LC-1-hCD19-CreER^{T2}; Z/EG, right panels ROSA26-CreER^{T2}; Z/EG) were treated with tamoxifen (5 mg/day, 5 days) or as control with sunflower oil/ethanol only orally for 5 consecutive days and were sacrificed 5 days after the last administration day. Peripheral blood and splenic cells were subjected to cytofluorimetric analysis. B cells were stained with the B220 Ab. Green fluorescent signal (GFP) indicates that recombination occurred in the reporter gene. Percentages of GFP positive B cells are indicated in the upper right corner. Data are representative of three independent experiments.

These results show that C57Bl/6 LC-1-hCD19-CreER^{T2} transgenic mice can provide efficient tamoxifen dependent Cre activity exclusively in B cells. B cell-specific inducible Cre expressing mice (CreED30) have been generated previously using the CreER^{T} system on (C57Bl6/CBA)F2 background (6). In these mice the immunoglobulin heavy chain enhancer

(Eμ) was combined with the SV40 early minimal promoter to drive expression of Cre specifically in B cells. Probably owing to the less sensitive $CreER^{T}$ system, for efficient induction of Cre activity these mice required high doses of 4-OH-tamoxifen, a derivative of tamoxifen that is 30-100 times more effective than the tamoxifen used in the experiments described here (22). Moreover, low levels of recombination were observed even in the absence of 4-OH-tamoxifen (6). Most importantly, Cre activity was lost during back-crossing of the CreED30 mice onto the C57Bl/6 background (Thomas Wunderlich, Ari Waisman, personal communication).

The LC-1-hCD19-$CreER^{T2}$ single copy transgene provides about 30% recombination exclusively in B cells upon tamoxifen administration. Cre recombination can be increased up to 60% by increasing gene dosage using homozygous transgenic mice. Although 100% deletion of the target locus is sometimes desirable, in many cases, the presence of a mixed population of wild-type and recombined cells may be advantageous by allowing direct comparison of the wild-type/mutant cells *in vivo*.

The poor results of the LC-1-hCD19-$CreER^{T2}$ with the Z/EG reporter, confirmed with the ubiquitously expressed ROSA26-$CreER^{T2}$, suggest that (some) Cre reporter strains are of limited use for a body wide validation of (inducible) Cre mouse strains. This is most likely because of the restricted activity of the promoter of the reporter gene, although cell type-dependent accessibility of the reporter locus for Cre recombinase might also play a role as suggested by the difference in the percentage of GFP positive B cells between C57Bl/6 LC-1-hCD19-$CreER^{T2}$; Z/EG (2%) and ROSA26-$CreER^{T2}$; Z/EG mice (7.5%).

Taken together, we have established a novel transgenic mouse strain on C57Bl/6 background, LC-1-hCD19-$CreER^{T2}$, that provides tamoxifen inducible B cell-specific Cre activity. No recombination in non-B cells or in the absence of tamoxifen was observed. Our data suggest that the LC-1-BAC provides effective insulation and can be used to reduce genomic position effects on the function of $CreER^{T2}$-based transgenes. The LC-1-hCD19-$CreER^{T2}$ mouse strain can serve as a useful tool for efficient somatic mutagenesis in B lymphocytes at any chosen time point in the lifecycle of the mouse.

METHODS

Generation of transgenic construct and mice

The gene encoding the fusion protein CreER^{T2} was placed as a SalI/StuI fragment downstream of the 6.3 kb fragment of the human CD19 promoter in the cosmid vector pTL5 (constructs were kind gifts from Drs. Pierre Chambon and Rudi Hendriks respectively). The hCD19-CreER^{T2} was removed as a MluI/NotI fragment and cloned into the shuttle vector pE11. F3.M.F., which contains short homologous sequences to the LC1 locus. EL 250 cells cells carrying the LC-1-BAC (E11-LC-1 BAC) were transformed with the vector linearized with ApaI and homologous recombination events were enriched by positive selection with ampicillin (16). Subsequently the FRT-flanked resistance cassette was excised by temporarily activating the Flp recombinase cloned under the control of a heat sensitive promoter (Kai Schönig, unpublished results). The 65 kb long BAC clone was linearized with NotI, purified by gel elution and used for pronuclear injections into fertilized C57Bl/6 oocytes (Charles River, Netherlands). Injection resulted in two independent founder lines that were identified with PCR using primers annealing for the Cre gene (Cre fwd: GCC TGC ATT ACC GGT CGA TGC AAC GA, Cre rev: GTG GCA GAT GGC GCG GCA ACA CCA TT). The LC-1-hCD19-CreER^{T2} strain was maintained heterozygous by breeding to wild type C57Bl/6 mice (Charles River, the Netherlands). The ROSA26-CreER^{T2} mice were kindly provided by Dr. Anton Berns (NKI). The Z/EG reporter strain (kindly provided by Dr. Corine Lobe) and mice with a conditional FcγRIIB allele (P.B., J.S.V., unpublished results) were used to characterize Cre activity. Mice were used at 8-14 weeks of age. All strains were housed under specific pathogen free condition at the animal facility of the Leiden University Medical Center. All experiments were approved by the local committee for laboratory animal welfare.

Real Time PCR

Cre copy number estimation was done on genomic mouse tail DNA with the UPL real time PCR system (The Universal Probe Library, Roche) using an independent genomic locus as a control in each sample. Primers for Cre detection were: Cre(qPCR)-Fw: TTTTGCCGGGTCAGAAAA, Cre(qPCR)-Rev: GGCGCGAGTTGATAGCTG (UPL Probe #87). Primers for the independent locus: Dedd, Dedd SNP Fw: AACGTGGACTCATCCGAAAT, Dedd SNP Rev: CCTCACCAGCTCGTCTCTTC (UPL Probe #81).

eMLPA

The efficacy of the *Cre* recombinase on the FcγRIIB locus was tested with a variant of MLPA called extension-MLPA (eMLPA) as described by Leonhard et al. (19). Primers for eMLPA: for the 5' loxP site: MLPA-5'-2b-A: GGGTTCCCTAAGGGTTGGACATCCCCATCTC AGGAGAGGAAGGGCAACACGCGGCAT, MLPA-3'-2b-B:CGTGTATCTTGGATACCT GTTGCGCTCTAGATTGGATCTTGCTGGCGC (with a 5' phosphor group). Primers for the 3' loxP site: MLPA-5'-2b-C: GGGTTCCCTAAGGGTTGGACAAGCTTATCGATAA GCTTCGAGGGACC,MLPA-3'-2b-D: GGGTTATTGAATATGATCGGAATTCCTGCA GCCCGGTCTAGATTGGATCTTGCTGGCGC (with a 5' phosphor group). Calculations of recombination efficacy were based on the percentage of B cells in the spleen as determined by FACS analysis.

Tamoxifen preparation
Tamoxifen (Sigma T5648) was dissolved in sunflower oil / ethanol (10:1) at a concentration of 100 mg/ml by sonication. The emulsion was further diluted with sunflower oil / ethanol to a concentration of 20 mg/ml and stored in aliquots at –20 °C.

Tamoxifen administration
After thawing, tamoxifen aliquots were placed at 55 °C for 5 minutes before administration. Mice were administered varying amounts (0.5 mg – 10 mg) of tamoxifen emulsion orally using a feeding needle for 5 consecutive days and sacrificed on the fifth day after the last administration day. As vehicle control, mice were given sunflower oil / ethanol emulsion only.

Cytofluorimetry
Single cell suspensions were prepared from spleen and bone marrow and stained with the following antibodies and conjugates: CD45 (B220)-PE, CD3-PE, GR1-PE, IgM-biotin, CD19-biotin, SA-FITC (Pharmingen), CD11b-APC (eBioscience) and F4/80-biotin, SA-PE (Serotec). The mAb against FcγRIIB (clone K9.361, Ly17.2-specific) was a kind gift from Dr. Shozu Izui. Blood and peritoneal cells were stained similarly; red blood cells were lysed after staining (Blood Lysis Buffer, BD Biosciences).

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