Video Article

The bm12 Inducible Model of Systemic Lupus Erythematosus (SLE) in C57BL/6 Mice

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Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disease with diverse clinical and immunological manifestations. Several spontaneous and inducible animal models mirror common components of human disease, including the bm12 transfer model. Upon transfer of bm12 splenocytes or purified CD4 T cells, C57BL/6 mice rapidly develop large frequencies of T follicular helper cells (Tfh), germinal center (GC) B cells, and plasma cells followed by high levels of circulating anti-nuclear antibodies. Since this model utilizes mice on a pure C57BL/6 background, researchers can quickly and easily study disease progression in transgenic or knockout mouse strains in a relatively short period of time. Here we describe protocols for the induction of the model and the quantitation Tfh, GC B cells, and plasma cells by multi-color flow cytometry. Importantly, these protocols can also be used to characterize disease in most mouse models of SLE and identify Tfh, GC B cells, and plasma cells in other disease models.

Video Link

The video component of this article can be found at https://www.jove.com/video/53319/

Introduction

Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized prototypically by anti-nuclear antibody (ANA) production and glomerulonephritis. Numerous other sequelae, including dermal, cardio-pulmonary, and hepatic lesions are associated with disease in some individuals. Prevalence estimates in the US vary widely, from 150,000-1,500,000[1,2], with particularly high incidence in women and minorities[3]. Although the etiology of SLE has been difficult to discern, it is thought to arise from the interplay of various genetic and environmental factors, which culminate in systemic autoimmunity.

Numerous animal models have been employed to study factors leading to disease onset and progression. Classic mouse models of SLE include genetically predisposed mouse strains including the NZB x NZW F1 model and its NZM derivatives, the MLR/lpr strain, and the BXSB/Yaa strain, and inducible systems, such as the pristane and chronic graft-versus-host disease (cGVHD) models[4]. Early reports of autoantibody production in GVHD models used various mouse strains or hamster strains for parent into F1 transfers[5-8]; more common methods used to study lupus-like disease currently include the DBA/2 parent→(C57BL/6 x DBA/2) F1, and the bm12 transfer model described here. Each model has its own caveats, but they generally share a common set of features that correlate with clinical features of human disease. The most often reported parameters in mouse models include splenomegaly, lymphadenopathy, nephritis, ANA production, and at the cellular level, the expansion of T follicular helper cells (Tfh), germinal center (GC) B cells, and plasma cells.

The inducible bm12 model is achieved by the adoptive transfer of lymphocytes from I-A^bm12 B6(C)-*H2-Ab1^bm12*/KhEgJ (bm12) mice, a strain identical to C57BL/6 except for 3 amino acid substitutions on MHC class II, into I-A^b C57BL/6 (B6) mice. Alloactivation of donor CD4 T cells by recipient APCs leads to cGVHD with symptoms closely resembling SLE. Specifically, these include expansion of donor-derived Tfh, expansion of recipient-derived GC B cells and plasma cells, and production of ANAs including anti-dsDNA, anti-ssDNA, anti-chromatin, and anti-RBC antibodies[9]. Over time, recipient mice develop glomerulonephritis associated with IgG deposits in the glomerular, interstitial, and vascular regions of the kidneys[10]. We have recently shown that, similar to human disease, there is also a critical role for type I IFN in this model[11]. Notably, the defining criteria for human SLE include the development of nephritis compatible with SLE in the presence of anti-dsDNA antibodies[12], both of which are prominent features of this mouse model.

There are several advantages of the bm12 model over the spontaneous models. Classic models that develop SLE-like signs spontaneously rely upon either hybrid mouse strains, inbred mouse strains not on the B6 background, or large genetic loci on the B6 background, which make crossing to knockout or otherwise genetically modified mice difficult and time consuming. With the bm12 inducible model, genetically modified mice can serve as either the donor or recipient, allowing more rapid identification of the cellular compartment in which particular genes may be important for disease. Furthermore, disease development in the bm12 model is much faster, requiring only 2 weeks until the appearance of ANAs, compared to several months for most spontaneous models. Moreover, in contrast to the spontaneous models that develop disease

at different time points, the disease onset and progression in the bm12→B6 model is highly synchronized. This allows for the generation of appropriately sized cohorts that can be used for interventional or therapeutic strategies at any stage of disease development.

What follows is a detailed protocol for initiating SLE-like autoimmunity by the adoptive transfer of bm12 lymphocytes into C57BL/6 mice, or genetic variants on the B6 background. Additionally, we describe a flow cytometric staining protocol for enumerating Tfh, GC B cells, and plasma cells—cell types associated with human disease. Importantly, these protocols can also be used to characterize disease in most mouse models of SLE and identify Tfh, GC B cells and plasma cells in other disease models.

Protocol

Animal work was performed under specific pathogen-free conditions in accordance with guidelines set by the Association for Assessment and Accreditation of Laboratory Animal Care International and our Institutional Animal Care and Use Committee (IACUC).

NOTE: Incorporate mice expressing a congenic marker such as CD45.1 on either donor or recipient animals if possible, because this allows for the monitoring of donor graft efficiency and specific expansion of the donor CD4 T cell population. If considering the use of otherwise genetically modified mice as donor or recipients, ensure the strain is properly backcrossed to the B6 background, or transferred cells may be rejected—this will be addressed in greater detail in the representative results and discussion sections.

NOTE: The following procedures detail volumes for harvesting 4 donor bm12 mice, which should yield enough cells to inject 12-16 mice. Six to twelve week-old bm12 mice yield roughly 100-140 million lymphocytes with approximately 25% CD4 T cells. If starting with different numbers of mice, or different mouse strains, scale volumes up or down accordingly. C57BL/6 mice generally give similar yields with closer to only 20% CD4 T cells.

NOTE: Perform all steps at RT and use RT media to avoid heat and cold shock, which can impair long-term lymphocyte viability. Perform tissue-harvesting and tissue-processing steps in a tissue culture hood using aseptic technique. All media in this protocol is IMDM with 10% heat-inactivated FBS, unless indicated otherwise, and will be simply referred to as "complete media."

1. Novel Method for Genotyping bm12 Mice

NOTE: A brief restriction digest-based protocol for genotyping is provided here, as a simple and inexpensive alternative to sequencing, which is currently the only published genotyping method for these mice.

1. Isolate genomic DNA from mouse tails. Please refer to a previous JoVE article for detailed protocols on mouse tail clipping and the generation of cDNA from tail digests[13].
2. Perform a reverse-transcriptase polymerized chain reaction (PCR)[14] to amplify a common 474 bp DNA fragment from MHC-II I-A^b and I-A^{bm12} using primers and thermocycling conditions listed in **Table 1**.
3. Perform restriction digest on ~7 μl of the PCR product using the enzyme PsuI, or one of its isoschizomers (BstX2I, BstYI, MflI, or XhoII), which cuts wild type I-A^b, but not I-A^{bm12} mutant. Follow digest protocol provided by the manufacturer, which will specify a mixture of water, buffer and enzyme, and an incubation of 5 min. to several hours at 37 °C[15].
4. Load digest product and run on 1% polyacrylamide gel with ethidium bromide[14] for 30-45 min. at 150V—though optimal voltage and time settings will vary depending on the PCR gel apparatus used. Visualize DNA bands with a UV illuminator. Representative results are shown in **Figure 1**.
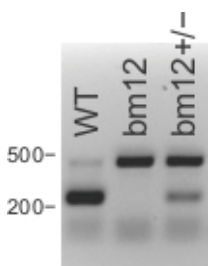
Figure 1. Representative bm12 genotyping results.
To identify mice homozygous for I-A$^{bm12/bm12}$, tail DNA was screened for bm12 by PCR/restriction digest genotyping (Step 1). Homozygous wild type (I-A$^{b/b}$) DNA yields two bands at 227 and 247 bp (visualized as one thick band at ~250 bp); homozygous bm12 (I-A$^{bm12/bm12}$) DNA yields one band at 474 bp; and heterozygous (I-A$^{bm12/b}$) DNA yields two bands at ~250 and 474 bp. Please click here to view a larger version of this figure.

2. Harvesting Donor Cells

1. Sacrifice donor mice using Institutional Animal Care and Use Committee (IACUC)-approved primary and secondary euthanasia methods. For example, euthanize mice by asphyxiation with CO_2 followed by cervical dislocation.
2. Using aseptic technique, harvest spleens and lymph nodes (superficial cervical, mandibular, brachial, axillary, mesenteric, and inguinal) into 15 ml tubes containing 10 ml complete media as in[11-13].
   NOTE: Refer to previous reports for detailed lymph node dissection protocols[16–18]. This model is also successful using only mouse splenocytes for transfer, but the addition of lymph nodes significantly reduces the required number of donor mice.

3. Generate a single-cell suspension by mashing lymphoid tissues collected in step 2.2 through 70 µm cell strainers using the plunger from a syringe. For better yields, do not overload strainers—use ~6 strainers for every 4 mice processed, and rinse strainers frequently while processing. Combine tissues from 4 mice into two 50 ml conical tubes, then centrifuge cells for 5 min at 400 x g and decant supernatant.
4. Resuspend cells from both conical tubes in 50 ml complete media and transfer to new conical tube. Keep this tube at RT.
    NOTE: Fat adheres to the sides of the tube, and if the tube is not replaced, final cellular yields can suffer.

3. Donor Cell Counting

NOTE: To preserve the highest viability, red blood cell (RBC) lysis of the entire sample is not recommended.

1. Mix single cell suspension from step 2.4 well, remove 1 ml and transfer it to a separate 50 ml conical. Set aside remaining, untouched cells (49 ml) at RT while counting.
2. Add 3 ml of an ammonium chloride-based red blood cell lysis buffer to the 1 ml donor cell sample. Mix gently for 1 min. by rocking, then fill to 50 ml with complete media, and centrifuge for 5 min. at 400 x g and decant supernatant.
3. Resuspend RBC-lysed cells in 10 ml complete media and count (*e.g.,* with trypan blue using a hemocytometer). Multiply result by 49—this is the total number of cells remaining in the non-lysed sample that was set aside. Discard RBC-lysed cells.

4. Donor Cell Labeling and/or CD4 T Cell Purification

1. If desired, purify CD4 T cells at this stage, though this is not necessary. Additionally, if desired, label cells with CFSE, as in[19], or other cell tracking dyes, an example of which is shown in **Figure 4** of the representative results section.
    NOTE: For CD4 T cell purification, negative magnetic selection is recommended, as it can achieve high purity and leaves cells untouched with high viability as described in[20]. It is important that endotoxin-free buffers are used; therefore, instead of BSA, make separation buffer with 2% FBS and the recommended concentration of EDTA.

5. Injection of Donor bm12 Cells

1. After counting donor lymphocytes in step 3 (and purified or labeled as in step 4, as desired), centrifuge cells for 5 min at 400 x g. Decant supernatant and resuspend cells in PBS at 120 million lymphocytes per ml (or 30 million purified CD4 T cells per ml). Transfer cells to a sterile 5 ml round-bottom tube, or other sterile tube that easily accommodates a 1 ml syringe fitted with a 27.5 G x 13 mm needle.
2. Prior to injection, set aside a small sample of donor cells from step 5.1 at 4 °C for flow staining to determine the percentage of CD4 T cells within donor samples. Stain these samples as described in Step 8 using the minimal antibody panel (**Table 2**).
    NOTE: If cells from different mouse strains are used as separate donors, this is an important consideration, and if CD4 T cell percentages vary substantially, purification may be required.
3. Mix cells gently, but thoroughly. This can be done by pipetting cells up and down using a 1 ml syringe without an attached needle. After mixing, draw cells into the 1 ml syringe. Attach needle after removing any air bubbles. Keeping the needle off while priming the syringe helps maintain cell viability.
4. Inject 250 µl per mouse (which is equal to 30 million lymphocytes, or 7.5 million purified CD4 T cells per mouse) intraperitoneally, as described in[21].
    NOTE: In experiments shown here and in our prior work[11], each mouse is injected with 30 million total lymphocytes from bm12 donors, rather than the 100 million total splenocytes traditionally used. In unpublished data from our lab, no difference was observed in serum anti-dsDNA at day 14 following injections of 30 or 100 million lymphocytes per mouse. While this significantly reduces the number of mice needed for experiments, the development of nephritis with this number of cells has not been assessed.

6. Determine Grafting Efficiency

NOTE 1: This section will describe how to determine the degree of donor cell grafting in the recipient at day 3 in order to identify any mice which may have received suboptimal injections (*e.g.,* the graft in one mouse is <10% of that seen in all other mice from the same group). These data can also help determine whether cells from a genetically modified mouse strain are rejected at later time points (for details, see representative results section, **Figure 6**).

NOTE 2: This section is only possible if donors and recipients are from mice on different congenic backgrounds, *e.g.,* when using CD45.1 bm12 donors and CD45.2 C57BL/6 recipients, or when donor cells are labeled with a cell tracking dye. Importantly, at 3 days post-injection, CD4 T cells have undergone minimal expansion (see representative results section, **Figure 4**), so differences observed in the degree of grafting are due to variability in injections, not expansion.

1. Anesthetize mice with 4% isoflurane or other IACUC-approved method. Test rear foot reflexes to ensure mouse is properly anesthetized before proceeding to the blood draw.
2. Harvest 100-200 µl blood using an IACUC-approved method, such as retro-orbital puncture as in[22]. Collect blood into individual 0.5 ml microcentrifuge tubes containing an anti-coagulant, such as the plasma collection tubes referenced in the materials table, which contain lyophilized dipotassium EDTA. After blood collection, apply gentle pressure to the mouse eye using a sterile towel or gauze to ensure the bleeding stops, then place the mouse back in its home cage where it will remain until the final tissue harvest (Step 7).
    NOTE: Do not leave mice unattended until mice have regained sufficient consciousness to maintain ventral recumbency, and do not return mice to the company of others until fully recovered.
3. Bring blood volume to 500 µl with 21 °C PBS and transfer to conical-bottom microcentrifuge tube, then slowly underlay 200 µl of 21 °C high density cell separation solution with a 200 µl pipet, being careful to minimize mixing between the two phases (see Materials Table for recommended solutions). Centrifuge cells at 700 x g for 20 min at 20-25 °C with centrifuge brake set to low.

4. Remove top layer containing lymphocytes with a 1 ml pipet and transfer to a new microcentrifuge tube containing 800 µl cold complete media. Gently vortex to mix cells. Centrifuge cells at 700 x g for 5 min at 4 °C and decant supernatant.
5. Resuspend cells in 200 µl complete media, transfer to a 96-well U-bottom plate, and stain for flow cytometry as described in Step 8 using a minimal antibody panel (**Table 2**) to determine the relative abundance of the CD4 T cell graft as a percentage of PBMCs.

7. Final Tissue Harvest

NOTE: The experiments described in the results section were harvested 14 days after injection of donor cells (or in some cases less time), as they focus on the initial development of Tfh cells and plasma cells; however, since this model is a chronic GVHD model of SLE, disease can be monitored at much later time points. The optimal timeframe will depend upon the research question posed in each individual experiment.

1. At a predetermined time point after injection of bm12 donor cells (step 5.4), sacrifice mice using an IACUC-approved method of euthanasia. Asphyxiation with CO_2 followed by exsanguination is recommended. Cervical dislocation can function as a secondary method of euthanasia, but this may reduce the blood draw yield.
2. Wet the abdomen of the mouse lightly with a spray bottle containing 70% ethanol. Make a small, superficial incision with surgical scissors approximately 1 cm above the genitalia. Draw back the skin of the abdomen toward the sternum, being careful to keep the peritoneal fascia intact.
   NOTE: Diseased mice usually present with 0.5-3 ml ascites at day 14, which, although it has not yet been well characterized, can be measured and analyzed as an additional disease parameter.
3. Fit a 5 ml syringe with an 18 G needle. Insert the needle into the lower right quadrant of the abdomen with the needle directed up toward the animal's head and at 15° angle to the plane of the fascia. Position the needle tip near the cecum to help prevent the needle from getting clogged with intestine while aspirating ascites.
4. Carefully rotate the mouse on its side, then slowly draw ascites into the syringe. Once ascites has been recovered, remove the syringe and record the aspirate volume, based upon the volumetric markings on the side of the syringe.
5. Discharge ascites into a 5 ml round-bottom tube and store on ice for later processing.
6. Harvest blood via draw from the inferior vena cava (IVC) essentially as in[22]. Using dull forceps, gently move intestines to the left side of the mouse, uncovering the IVC. Insert a 27.5 G needle fitted to a 1 ml syringe into the IVC and slowly draw 400-500 µl of blood.
7. To minimize hemolysis, slowly inject blood into a 0.5 ml microcentrifuge serum or plasma collection tube. For later serum analysis of ANAs by ELISA, keep blood on ice.
8. Dissect and obtain additional relevant tissues (spleen and, if desired, lymph nodes and kidneys, particularly if collecting at later time points and glomerulonephritis will be scored). Remove spleen by gently placing the intestines back toward the right side of the animal, and pulling on the pancreas, which is the spleen's primary connective tissue.
9. Remove any pancreas remaining, then weigh spleens on a high precision balance immediately after dissection, as gross splenomegaly is a commonly reported parameter in mouse models of SLE. Place lymphoid tissues into individual 1.5 ml tubes filled with 1ml complete media on ice. Fix kidneys in 10% neutral buffered formalin or snap freeze kidneys for later histology as in[23].
10. Centrifuge blood within 2 hr of collection for 3 min. at 10,000 x g (4 °C). Remove serum and store at -80 °C for later analysis of ANA by ELISA. Refer to previous reports for detailed ANA ELISA protocols[24,25]. Store serum in multiple 10-20 µl aliquots to minimize freeze/thaw cycles, and allow a greater number of future assays.
11. Centrifuge ascites 400 x g for 5 min. Remove the supernatant with a 1 ml pipet and aliquot into several 0.5 ml tubes. Freeze supernatant at -80 °C for later analyses of anti-nuclear antibodies (ANAs) or other soluble inflammatory mediators.
12. Resuspend cellular fraction in approximately 1 ml complete media and transfer 200 µl to a 96-well U-bottom plate for flow staining (as in Step 8).
13. Mash each spleen through 70 µm cell strainers into separate tubes and rinse with complete media. Resuspend splenocytes with 1 ml cold RBC lysis buffer for 1 min. and mix gently by rocking. Bring volume to 10 ml with cold complete media and centrifuge for 5 min at 400 x g and decant supernatant.
14. Resuspend cell pellet in 5 ml complete media and count using a hemocytometer. Adjust volume such that 200 µl of complete media contains 1-3 million cells. Begin staining for flow cytometry (Step 8) using an extended panel (**Table 2**) to quantify donor CD4 T cell and recipient B cell differentiation and expansion.
   NOTE: Depending upon what laser, photomultiplier tube (PMT), and filter set combinations are available, separating the T and B cell analysis panel into multiple panels may be necessary.

8. Flow Staining

1. Transfer 1-3 million splenocytes in 200 µl complete media into individual 5 ml round-bottom tubes, or into separate wells of a 96-well U-bottom plate.
   NOTE: Using a 96-well plate is an efficient way to stain multiple samples, but take care to plate samples in every other well in order to prevent cross-contamination. One 96-well plate can accordingly hold 24 samples.
2. Centrifuge plate at 500 x g for 3 min., then flick supernatant from plate into appropriate (biohazard) container.
3. Resuspend the cell pellets with 100 µl of flow buffer (1% FBS in PBS) containing a fixable viability dye at the manufacturer's recommended concentration and purified anti-CD16/anti-CD32 antibody cocktail at 1 µg/ml. Incubate for 10 min at 20-25 °C, then add 100 µl cold complete media to quench viability dye.
   NOTE: Splenocytes from diseased mice usually contain relatively high numbers of dead or dying cells; therefore, the inclusion of a viability dye is recommended to avoid non-specific antibody labeling of dying cells, resulting in cleaner, more reliable data. Similarly, the anti-CD16/anti-CD32 cocktail is included to block non-specific fluorescently-labeled antibody binding by Fc receptors.
4. Centrifuge plate at 500 x g for 3 min., then flick supernatant from plate into biohazard container. Resuspend cells in 200 µl flow buffer. Centrifuge plate at 500 x g for 3 min., then flick supernatant from plate into biohazard container.
5. Resuspend cells in 50 µl flow buffer containing antibody cocktail (**Table 2**). Incubate for 20 min at 4 °C, then add 150 µl flow buffer. Repeat wash step 8.4.

6. Resuspend cells with 100 µl 2% paraformaldehyde in PBS. Incubate for 30 min at 4 °C, then add 100 µl flow buffer. Centrifuge plate at 500 x g for 3 min., then flick supernatant from plate into biohazard container.
7. Resuspend in 200 µl flow buffer, transfer to flow tube inserts or standard flow tubes, add an additional 100-200 µl flow buffer, and store at 4 °C in the dark until acquiring on flow cytometer.
8. Acquire on a flow cytometer equipped with the appropriate lasers and PMTs for the chosen antibody panels within several days of staining. Record forward scatter width and/or height in addition to forward scatter area, side scatter area, and area of the fluorescent parameters utilized. For reliable results, acquire ≥1,000 donor cells in each WT sample, or an equivalent number of total lymphocytes in genetically modified or otherwise manipulated mice that show minimal expansion of donor cells, where collection of so many events may not be feasible. NOTE: Flow cytometry analyses are described in detail in the representative results section (**Figures 3**-**6**).

Representative Results

Diseased mice develop splenomegaly in as little as 14 days, exhibiting spleens 2-3 times the size of healthy mice in terms of mass and cellularity (**Figure 2**).

Splenocytes are sequentially gated on light scatter (FSC-A by SSC-A), elimination of doublets (FSC-W or -H by FSC-A), viable cells (low staining of viability dye), and CD4$^+$TCRβ$^+$ (**Figure 3A**). Donor cells are distinguished from recipient cells based on CD45.1 and CD45.2 (**Figure 3B,** bottom left). Donor cells predominantly adopt a T follicular helper (Tfh) cell phenotype, as characterized by the upregulation of PD-1, CXCR5, Bcl-6, and ICOS (**Figure 3B, C**). A portion of the recipient CD4 T cell population also differentiates into Tfh (**Figure 3B,** bottom right). After an initial die-off and/or migration of transferred cells, the expansion of donor-derived Tfh is logarithmic, reaching 10-20 million cells in the spleens of mice 14 days after injection (**Figure 3D**).

CFSE-labeling of transferred lymphocytes demonstrated that donor CD4 T cells differentiate into Tfh early after activation; essentially all divided cells observed at days 3, 7, and 14 upregulated CXCR5 and PD-1 (**Figure 4**). The proliferation peak profiles also suggest that a relatively low percentage of donor CD4 T cells underwent alloactivation and division. By day 14, most of the detectable donor cells are those that have divided beyond the maximum number of divisions measurable by CFSE.

The expansion of donor-derived Tfh is accompanied by a corresponding accumulation of endogenous GC B cells and plasma cells (**Figure 5A**). Plasma cell accumulation in the spleen is delayed compared to that of Tfh, exhibiting no increase over naïve animals on days 3 or 7 (**Figure 5B**). Accordingly, anti-nuclear antibodies are not readily detectable prior to day 9 (data not shown), but can be reliably quantified on day 14[11].

Through the use of congenic markers, we have observed rejection of donor cells in multiple strains. While this is a common problem when mice are not sufficiently backcrossed to the C57BL/6 background, we also observed rejection when bm12 cells were transferred into B6.PL-*Thy1^a*/CyJ (CD90.1) mice that are generally considered to be fully backcrossed. Therefore, mice are routinely screened at day ~3 by flow staining blood samples to assess the efficiency of the initial CD4 T cell graft; we know from CFSE proliferation experiments that grafted cells have expanded very little at this early time point. These results are then compared to results obtained from the day 14 harvest. In an example case of rejection, 30 million CD45.1$^+$ bm12 lymphocytes were transferred into *Cardif$^{-/-}$* mice which had been backcrossed to C57BL/6 mice for 12 generations. At day 5, all mice displayed equivalent grafting (2-3% of circulating CD4 T cells), but by day 14, bm12 donor cells were completely eliminated from the genetically modified recipient (**Figure 6**).
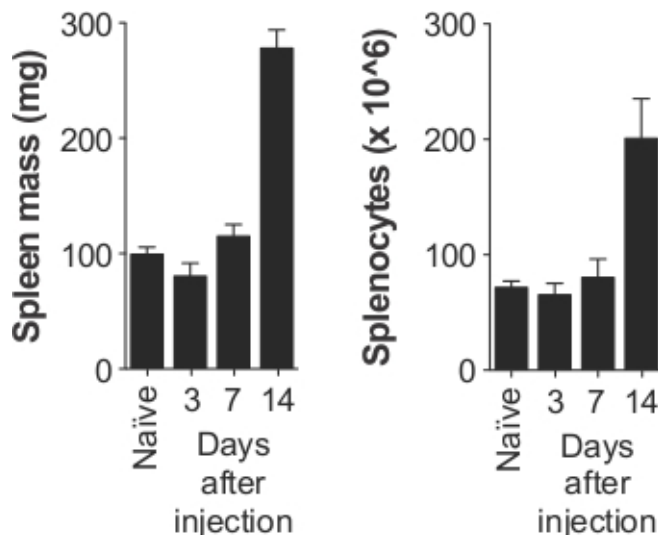

Figure 2. Spleen growth kinetics after injection of bm12 lymphocytes. Spleens were weighed on a high precision balance directly after excision (left). Live cell numbers were determined by counting with a hemocytometer using trypan blue to exclude dead cells (right). Results are depicted as mean ± SEM, where n = 9, 4, 3, and 6, respectively. Please click here to view a larger version of this figure.
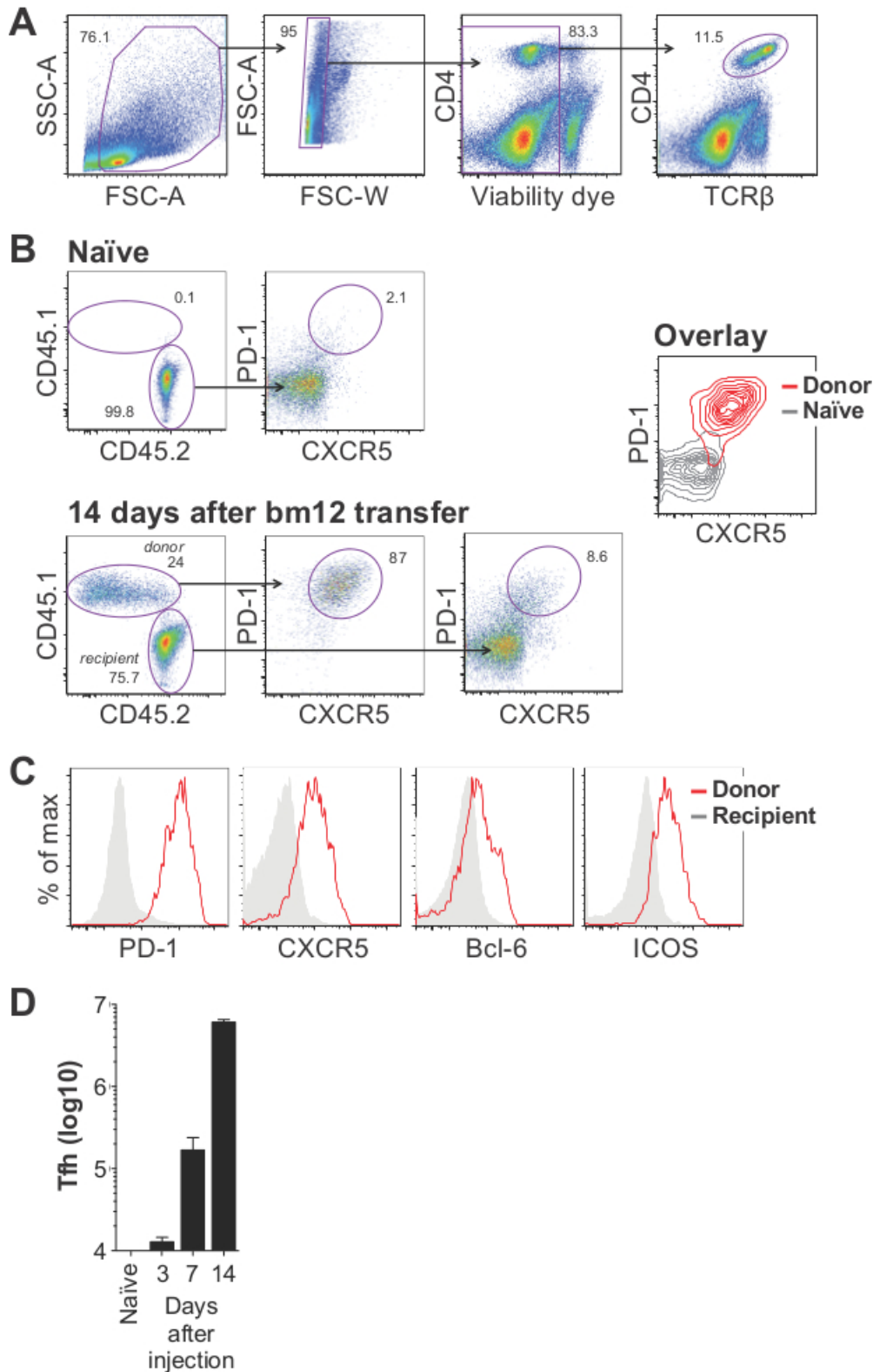
Figure 3. Analysis of T follicular helper cell expansion in the bm12 model of SLE. CD45.1[+] bm12 lymphocytes were transferred into C57BL/6 recipients and spleens were analyzed 14 days later. (**A**) Representative gating strategy showing "lymphocytes" (first panel), "single cells" (second panel), "live cells" (third panel), and "CD4 T cells" (fourth panel). (**B**) Donor cells are distinguished from recipient CD4 T cells by CD45.1 and CD45.2 staining and analyzed for expression of PD-1 and CXCR5. (**C**) Donor cells adopt Tfh phenotype, as indicated by the upregulation of several proteins commonly associated with Tfh. (**D**) Typical results showing the expansion of donor-derived Tfh (defined as CD4^{+}CD45.1^{+}PD-1^{+}CXCR5^{+} live cells) at days 3, 7, and 14 post transfer. Results are depicted as mean ±SEM, where n = 5, 4, 3, and 6, respectively. Please click here to view a larger version of this figure.
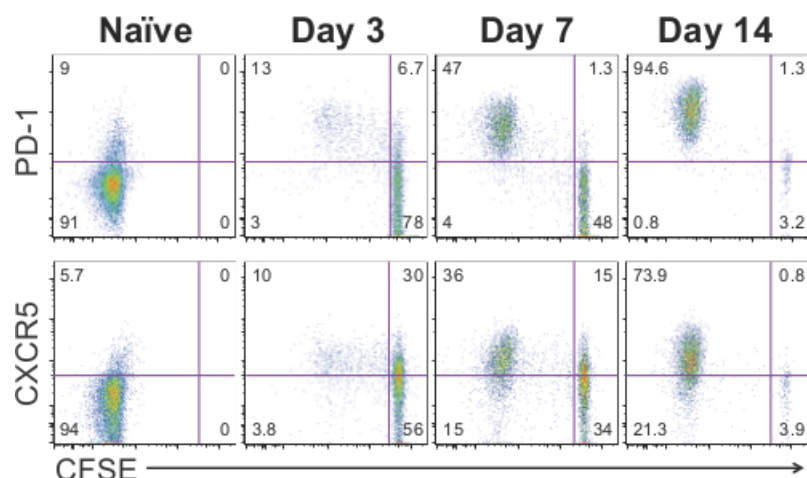
Figure 4. PD-1 and CXCR5 are upregulated on dividing cells. Bm12 cells were labeled with CFSE prior to injection into C57BL/6 recipients. Representative flow plots are shown for donor CD4 T cells at 3, 7, and 14 days after injection. Please click here to view a larger version of this figure.
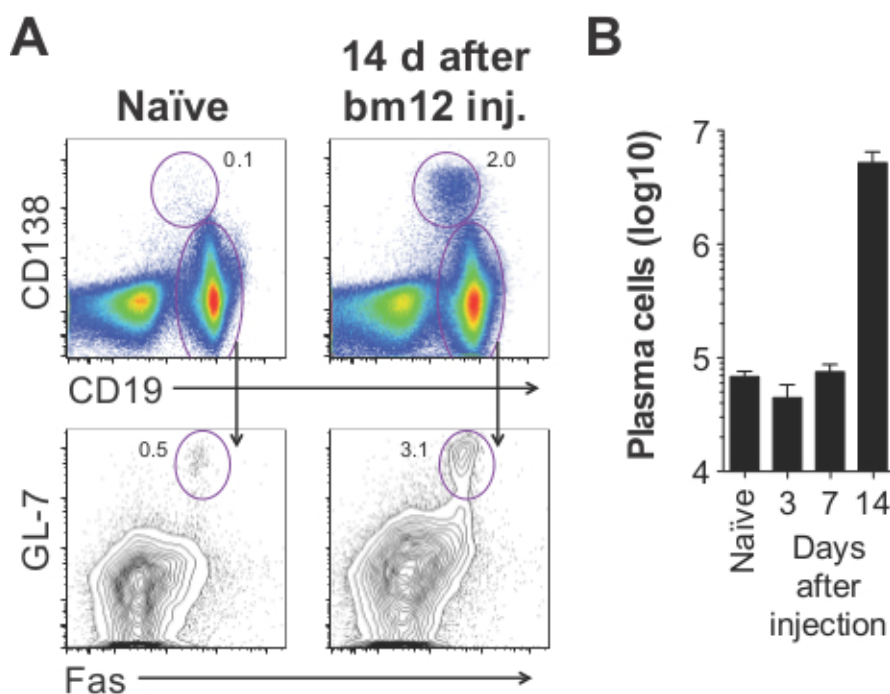
Figure 5. Expansion of splenic plasma cells and GC B cells. (A) Representative flow plots from naïve mouse spleens, or those from mice 14 days after CD45.1$^+$ bm12 transfer. Plasma cells are defined as CD138$^+$CD19^{low} live cells (top panels). GC B cells are a subset of CD19$^+$ B cells, which express GL-7 and Fas (bottom panels). **(B)** Quantitave data showing the accumulation of splenic plasma cells over time. Results are depicted as mean ± SEM, where n = 5, 4, 3, and 6, respectively. Please click here to view a larger version of this figure.
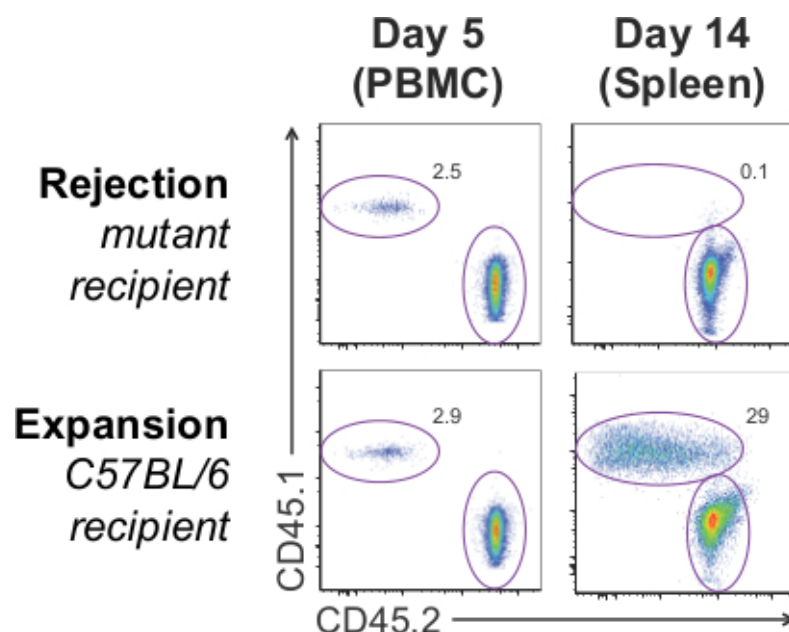
Figure 6. Bm12 grafts are rejected by some genetically modified recipient mice. CD45.1$^+$ bm12 lymphocytes were transferred into either genetically modified mice (top panels) or C57BL/6 (bottom panels) recipient mice. Mice were bled at 5 days post injection and assessed for graft efficiency. Splenocytes from the same mice were analyzed 14 days later. Please click here to view a larger version of this figure.

Primer Name	Sequence (5' - 3')		Annealing Temperature
Bm12F	CGTGGTCCCCGCTGTCCCCC		*65 °C
Bm12R	GGGCAGAGGGCAGAGGTGAG		
Segment	Number of cycles	Temperature	Duration
1	1	95 °C	2 min.
2	40	95 °C	15 sec.
		*65 − 0.5 °C/step	15 sec.
		72 °C	45 sec.
3	1	72 °C	10 min.

Table 1: Primers and thermocycling conditions for bm12 genotyping. Optimal thermocycling conditions will depend upon the exact reagents and instruments used. These conditions have been optimized for a thermocycler capable of changing annealing temperature at every step, though the protocol may also work using a static annealing temperature.

| Day 3: grafting efficiency | | | Day 14: T & B cell analyses | | | Recommended |
Antigen	Fluorochrome		Antigen	Fluorochrome		long pass
na	BUV395		GL-7-biotin	SA-BUV395		na
CD4	BV421		CD138	BV421		na
na	BV510		CD4	BV510		505
na	BV605		TCRβ	BV605		595
CD45.1	BV711		CD45.1	BV711		685
CD45.2	FITC		CD45.2	FITC		505
na	PE		PD-1	PE		575
na	PerCP		CD19	PerCP		635
na	PE-Cy7		Fas	PE-Cy7		735
na	APC		CXCR5	APC		na
na	ef780		Live/Dead	ef780		755

*These will depend upon the particular configuration of your flow cytometer

Table 2: Antibody panels for splenic Tfh, GC B and plasma cell identification by flow cytometry. Presented are panels we have used successfully for assessing graft efficiency at day 3 (left) and the analysis of T and B cells at day 14 after bm12 cell injection (middle). These have been optimized for an LSRII with 5 lasers (355, 405, 488, 561, and 640 nm). Recommended filter sets for each fluorochrome are listed (right), though these may not be the best option for every machine. Depending upon what laser, PMT, and filter set combinations are available, separating the T and B cell analysis panel into multiple panels may be necessary.

Discussion

The bm12 inducible model is a relatively easy and efficient way to study the cellular and molecular processes of SLE. Chronic activation of adoptively transferred CD4 T cells directed against self antigens leads to the accumulation of Tfh, GC B cells, and plasma cells which can be measured by flow cytometry, as described here. Future studies using this model can quickly and easily interrogate the role of candidate genes and novel therapies in the autoimmune germinal center processes which resemble those occurring in patients with SLE, and ultimately govern the pathological accumulation of autoantibodies. Furthermore, the flow cytometric analysis described here can be used to study additional mouse models that involve the development of immunoglobulins including, but not limited to autoimmunity, infection, and allergy.

Like all animal models of human disease, this model also has its limitations. Given the speed with which disease develops and its magnitude, not all genes involved in the development of SLE are likely to be necessary for pathogenicity in this model. Additionally, care must be taken to rule out graft rejection when data suggest minimal expansion of Tfh in genetically modified recipients. Congenic markers should be used to confirm the presence of donor cells at harvest especially since recipient cells can also differentiate into Tfh (**Figure 3B**), which could otherwise mask the absence of donor cells. Using congenic markers, we observed complete elimination of donor cells that evidently harbored rejection antigens by day 14 (**Figure 6**). We have successfully used CD45.1[+] bm12 and CD45.2[+] bm12 mice as donors, and CD45.1[+] BoyJ (B6.SJL-*Ptprc*[a] *Pepc*[b]/ BoyJ) and CD45.2[+] C57BL/6 mice as recipients (**Figures 3, 6, 7,** and unpublished data). However, wild type congenic CD90.1^{+} cells from the B6.PL-*Thy1*[a]/CyJ mouse strain do not graft well, nor do CD90.2^{+} bm12 cells graft well in a CD90.1[+] recipient (unpublished data), a phenomenon that is evidently not unique to this model[26].

Either C57BL/6 or bm12 mice can serve as the donor or the recipient, as initially reported by Morris et al.[9] However, in our lab we find the transfer of bm12 cells into B6 mice produces more consistent expansion of T cell and B cell populations at day 14. Furthermore, donor and recipient mice from different groups should be gender- and age-matched. Although experiments reported in the first description of the bm12 model found no significant difference in any disease parameter between male→male and female→female transfers[9], male→female transfers are not advised, as male antigen (H-Y) expressed by transferred CD4 T cells may induce graft rejection in female recipients[27].

When choosing antibodies and fluorochromes for congenic markers, it should be noted that donor cells become positive for the recipient congenic marker to varying degrees, such that a CD45.2[−] gate would not constitute the entire CD45.1[+] graft. The phenomenon is clearly illustrated in a comparison of CD45.2 expression by CD45.1[+] naïve, CD45.1[+] donor, and CD45.2[+] recipient CD4 T cells (**Figure 7**). Notably, donor cells also seem to upregulate expression of their own congenic marker, compared to naïve controls (**Figure 7**). Similar results are also seen with CD45.2[+] bm12 transfer into CD45.1[+] BoyJ mice (data not shown). Presumably, acquisition of recipient CD45 results from trogocytosis[28] by activated CD4 T cells following repeated interactions with recipient B cells. In fact, the bm12 model may prove a useful tool in studying the process of trogocytosis *in vivo*.
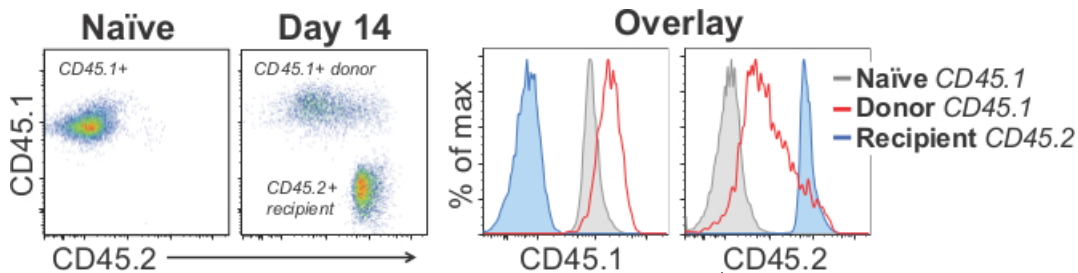
Figure 7. Donor cells acquire recipient CD45 congenic marker. CD45.1[+] bm12 lymphocytes were transferred into CD45.2[+] C57BL/6 recipients. Donor, recipient, and naïve (CD45.1[+]) CD4 T cells are assessed for CD45.1 and CD45.2 expression 14 days after transfer. Please click here to view a larger version of this figure.

It has been shown that the transfer of purified bm12 CD4 T cells into C57BL/7 mice is sufficient to initiate disease[29]; however, equivalent disease develops when whole lymphocytes are transferred, and purification is not necessarily required to study disease dependence upon T cell-intrinsic gene expression. Eisenberg and colleagues, clearly demonstrated that the antibody-producing cell in the bm12 model is almost exclusively of recipient origin[30]. Furthermore, the requirement for recipient CD4 T cells[10] is limited to the 'nurturing' of B cells during their development, which can be off-set by the addition of exogenous IL-4[31], although our data indicate that recipient T cells may participate somewhat in the germinal center response, as some of them do develop a Tfh phenotype (**Figure 3B**, bottom right).

One critical decision that must be made for every bm12 experiment is when to harvest tissues for analyses. Of course the decision depends upon exactly what factors are important to the current study, which may vary. The experiments described here take up to 14 days, as they focus on the initial development of Tfh cells and plasma cells; however, since this model is a chronic GVHD model of SLE, disease can be monitored much longer. In fact, certain clinical features of SLE, including glomerulonephritis, develop later. Proteinuria has been detected as early as 2 weeks post-injection, but reaches peak levels at 4-8 weeks post-injection[10,31]. Additionally, the flow cytometric analyses described here have been optimized for experiments lasting 2 weeks. We find a considerable number of GC B cells and plasma cells at this time point; although, given additional time, a large percentage of ANA-secreting plasma cells may reside in the bone marrow[32]. Moreover, the plasma cells identified by this flow staining panel likely also include plasmablasts—in order to distinguish between these two cell types, additional antibodies would be required. Tfh expansion presumably reaches a peak at some point after the 14-day window on which we have focused. However, to our knowledge, no studies have reported bm12 donor cell number beyond 2 weeks, or used congenic markers to track adoptively transferred cells.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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