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Cre Driver Mice Targeting Macrophages

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Abstract

The Cre/loxP system is a widely applied technology for site-specific genetic manipulation in mice. This system allows for deletion of the genes of interest in specific cells, tissues and the whole organism to generate a diversity of conditional knockout mouse strains. Additionally, the Cre/loxP system is useful for development of cell- and tissue-specific reporter mice for lineage tracing, and cell-specific conditional depletion models in mice. Recently, the Cre/loxP technique was extensively adopted to characterize the monocyte/macrophage biology in mouse models. Compared to other relatively homogenous immune cell types such as neutrophils, mast cells and basophils, monocytes/macrophages represent a highly heterogeneous population which lack specific markers or transcriptional factors. Though great efforts have been made towards establishing macrophage-specific Cre driver mice in the past decade, all of the current available strains are not perfect with regards to their depletion efficiency and targeting specificity for endogenous macrophages. Here we overview the commonly used Cre driver mouse strains targeting macrophages and discuss their major applications and limitations.

Keywords

Macrophages; Monocytes; Cre/loxP; Macrophage reporter mice; Macrophage-specific conditional knockout

1. Introduction

Cre/loxP system is a site-specific genetic modulation technique which has been extensively applied to create gene deletions, insertions, inversions and translocations at the specified DNA sites in mice (1). This system contains two major components: the enzyme Cre recombinase and the loxP sites. The Cre recombinase, which is originally derived from P1 bacteriophage, specifically recognizes the loxP sites, the 34-base pair asymmetric DNA sequences with directionality. Depending on the orientation of the two loxP sites, Cre

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recombinase either excises or inverts the transgene sequences inserted between the two loxP sites. Therefore, the Cre/loxP system allows for making a series of DNA sequence rearrangements, which helps to build one of the most versatile genetic tools in mice (2,3).

The major application of Cre/loxP system is generation of cell- or tissue-specific conditional knockout alleles in mice (4). This is achieved by knocking in two loxP sites at distant introns within the gene of interest, and such a knock-in strain is then crossed with another mouse strain that contains the Cre recombinase transgene under the direction of a cell or tissue-specific gene promoter. In the specific cells or tissues expressing Cre, the gene of interest will then be excised leading to a conditional gene knockout. As an example shown in Figure 1, when the myelomonoytic cell-specific Lysozyme M (LysM)-Cre mice are crossed with homozygous loxP-flanked transgenic strain of interest (gene A), mice heterozygous for the loxP allele will be generated in the first generation. The experimental mice, homozygous for the loxP-flanked gene A with Cre activity, will be obtained by further crossing the heterozygous mice back to the homozygous loxP-flanked mice. In the $LysM^+$ myelomonoytic cells of the experimental mice, the transgene A will be knocked out while $LysM^-$ cells remain unaffected. This type of cell- or tissue-specific gene knockout models is very helpful for a precise understanding of any genes of interest in a given cell type or tissue. When fusing the Cre recombinase gene with the gene encoding estrogen receptor (ER), such Cre driver mice can be used for a temporal control of loxP-flanked transgene expression upon administration of tamoxifen, the estrogen antagonist (5). This is particularly critical when the gene knockout in specific cells or tissues causes embryonic lethality.

In addition, the Cre/loxP system is widely utilized for development of mouse strains with conditional gene activation/overexpression, reporter mice for cell tracking or lineage tracing, and cell-specific depletion models (6). For example, to generate a conditional gene activation strain as shown in Figure 2, the transcriptional stop sequence, such as a polyadenylation signal, is inserted upstream of the gene of interest (gene B) to prevent the transgene expression. As the “stop” sequence is flanked by the two loxP sites (“lox-stop-lox” cassette), when crossing this strain with *LysM-Cre* transgenic mice, Cre-mediated deletion of the “stop” sequence drives the expression of gene B only in $LysM^+$ myelomonoytic cells. This gene activation or overexpression model offers a gain-of-function approach to precisely dissect the function of any genes of interest in a specific cell type or tissue.

Following a similar strategy, the myelomonoytic cell-specific reporter strains can be developed if the transgene B (Figure 2) is a visible marker, such as green fluorescent protein (GFP), red fluorescent protein (RFP), tdTomato fluorescent protein and LacZ (6). The reporter mice are good tools for spatial and temporal tracking of the specific cells in vivo, and also benefit cell lineage tracing to determine the developmental origins of specific cell types since the Cre-mediated reporter gene expression is irreversible from ancestor to descendant cells. Moreover, the strategy shown in Figure 2 also helps to generate cell-specific conditional depletion models if the transgene B is diphtheria toxin receptor (DTR) gene or other similar constructs (7). In this model, Cre-induced deletion of the “lox-stop-lox” cassette leads to expression of the DTR gene which in turn forms an inducible ablation system specific for $LysM^+$ cells upon receiving diphtheria toxin (DT). The cell depletion

models facilitate us to understand the systemic functions of a given cell type in physiological and pathological conditions.

One of the major limitations of Cre/loxP system is its off-target effects. It has been frequently reported that many Cre transgenic mouse strains express Cre recombinase in non-target cells or tissues. Such off-target effects may confound the results from gene conditional knockout, reporter and cell depletion models, as well as other Cre/loxP-based genetic tools. Thus, a thorough analysis of each Cre transgenic strain with regards to their Cre sensitivity (efficiency) and expression pattern (specificity) should be carefully performed and recorded. To this end, the Jackson Laboratory (USA) has developed a Cre portal which aims to provide the scientific community with a comprehensive well-characterized Cre transgenic mouse strains and high throughput data related to these strains (2).

The currently available Cre driver mice targeting macrophages are primarily designed based on the monocyte/macrophage markers such as LysM, colony-stimulating factor 1 receptor (CSF1R or CD115), CD11b, F4/80 and CX3C chemokine receptor 1 (CX3CR1) (8), although none of them are perfectly specific. On the basis of these Cre driver mice, a diversity of conditional gene knockout strains, reporter strains and cell depletion strains were accordingly developed towards elucidating the macrophage lineage development in prenatal and adult life, as well as their functional contribution to tissue homeostasis and pathological processes in mice. The main applications and specificity of these Cre driver mouse strains are described below. For each strain, its number (s) recorded in the Mouse Genome Informatics (MGI) database (http://www.informatics.jax.org/) is also included and more information about these strains can be obtained from this database.

2. Overview of The Commonly Used Cre Driver Mice for Macrophages

2.1 *LysM-Cre* (MGI: 1934631)

Lysozymes are antimicrobial enzymes widely produced by the innate immune cells in eukaryota. As a type of glycoside hydrolase, lysozymes function to cleave the peptidoglycan of bacterial cell wall as their antimicrobial mechanism (9). In mice, there are two forms of lysozyme genes, *Lyz1* and *Lyz2*, which encode lysozyme P (LysP) and lysozyme M (LysM), respectively. While LysP is expressed in the Paneth cells of the small intestine, LysM is exclusively expressed in myelomonoytic cells including monocytes, macrophages and granulocytes in mice (10). LysM is therefore served as a marker for myelomonoytic cells.

In 1999, Clausen et al. generated the *LysM-Cre* mouse by targeted inserting the Cre cDNA into the endogenous *LysM* gene locus (11). When crossing the *LysM-Cre* mice with two mouse strains with different loxP-flanked targeted genes (*β-polymerase* and transcription factor *RX5*), there was a highly efficient gene depletion in mature macrophages and granulocytes isolated from peritoneal cavity or derived from bone marrow (BM). No depletion was detected in lymphoid cells such as B and T lymphocytes (11). Later on, Faust et al. Created a *LysM*-reporter strain by knocking the enhanced GFP (*EGFP*) gene into the *LysM* gene locus together with a targeting vector, which contains a neomycin resistant (*neo*) gene flanked by loxP sites (12). Using this reporter strain, the $EGFP^+$ cells were confirmed to be mostly myelomonocytic cells in peripheral blood, peritoneal cavity and BM (12). A

further cross of this *LsyM*-reporter strain with Cre-expressing mice, Cre-mediated removal of the loxP-flanked neo gene led to generation of *LysM*-deficient mice in the offspring (13). In these *LysM*-deficient mice, a prolonged and more robust inflammatory response was observed compared to the wild type (WT) controls when challenged by normally non-pathogenic bacteria. This suggested a critical role of myelomonocytic cells in a timely elimination of bacteria and their products which elicit immune responses (13).

Since the creation of this *LysM-Cre* strain, it has been extensively applied to develop reporter mice for in vivo tracking or lineage tracing of macrophages and other myelomonocytic cells in normal and diseased conditions. For example, in the myelomonocytic cell reporter mice generated through crossing *LysM-Cre* mice with *ROSA26-flox-stop-flox-EYFP (Rosa26-LSL-EYFP)* mice, yellow fluorescent protein (YFP) was found to be expressed not only in myelomonocytic cells, but also in a fraction of long-term hematopoietic stem cells (LT-HSCs) as well as megakaryocyte/erythrocyte progenitor (MEP) cells suggesting a universal expression of *LysM* at the hematopoietic stem/progenitor cell (HSPC) stages in mice. Differentiation of HSPCs towards myelomonocytic cells is presumably as a default while their differentiation to other lineages might be dominated by additional transcriptional factors (14).

A series of macrophage-specific conditional gene knockout mice were generated by crossing the *LysM-Cre* line with different “floxed” strains, which highly accelerate identification of numerous myeloid genes which play key roles in a variety of inflammatory disorders such as infections, autoimmune diseases, fibrosis and cancer progression (15–19). As well, the *LysM-Cre* strain was also crossed with loxP-flanked DTR mice to develop the inducible macrophage depletion model-*LysM-Cre/iDTR* line to characterize the systemic functions of endogenous macrophages in wound healing, fibrotic diseases, arterial hypertension and vascular dysfunction in mouse models (20–23).

In spite of a high efficiency of *LysM-Cre* strain in manipulating endogenous macrophages, LysM is not a specific marker for macrophages. In addition to monocytes and mature macrophages, LysM is also expressed in most granulocytes and few $CD11c^+$ dendritic cells (DCs), as well as a small percentage of non-hematopoietic cells such as type II lung alveolar cells in mice (12,23). In a comparative analysis of multiple myeloid cell-specific Cre reporter strains, about 60–80% neutrophils in spleen, peripheral blood and BM were shown as $LysM^+$ (8). For mature macrophage populations, about 90–100% alveolar macrophages and peritoneal macrophages were $LysM^+$, whereas only 40% of spleen red pulp or spleen marginal zone macrophages had LysM-Cre activity implicating the heterogeneous origins of tissue resident macrophages (8). Overall, the *LysM-Cre* mouse strain is a broadly used tool to investigate the endogenous myelomonocytic cells.

2.2 *Csf1r-Cre* (MGI: 4429470)

CSF1R, also known as macrophage colony-stimulating factor receptor (M-CSFR), and CD115, is the receptor for the major monocyte/macrophage lineage differentiation factor CSF1. It is a tyrosine kinase encoded by the proto-oncogene *c-fms* and expressed in all monocytic cells including monocytes and macrophages in mice.

Prior to generation of the *Csf1r-Cre* strain, the *Csf1r-EGFP* reporter mice (MacGreen mice) had been created and were successfully utilized for tracing the endogenous macrophages in various tissues (24). The *Csf1r-Cre* line was developed in 2010 which was used to identify the role of signal transducer and activator of transcription 3 (STAT3) in macrophages (25). In the conditional *STAT3*-knockout mice generated via mating the *Csf1-Cre* strain with the $STAT3^{flox/flox}$ strain, spontaneous colitis and tumor formation in the inflamed colon and cecum were detected indicating a key contribution of myeloid signaling to malignancy in mouse models (25).

With this *Csf1r-Cre* strain, several groups generated macrophage reporter mice by crossing the *Csf1r-Cre* mice with *Rosa26-LSL-YFP* mice to determine the macrophage lineage development during organogenesis in the mouse prenatal stage (26–28). From these lineage tracing studies, the yolk-sac macrophages, originated from yolk-sac-derived erythro-myeloid progenitors, were shown to give rise to some $F4/80^{bright}$ tissue resident macrophages such as liver Kupffer cells, epidermal Langerhans cells and microglial cells (27,28). In mouse models of wound healing, autoimmune encephalitis and breast carcinoma, the roles of macrophage-associated factors such as neuropilin-1, porcupine, Wnt family member 7B (Wnt 7B) and vascular endothelial growth factor receptor 1 (VEGFR1), were characterized with their respective *Csf1r*-specific gene knockout strains (29–33).

Therefore, the *Csf1r-Cre* strain is a good tool for fate mapping of macrophages in development and identification of macrophage regulatory genes essential for both steady status and inflammatory conditions. The *Csf1r-Cre*-based gene deletion was detected in macrophages of various tissues including liver, spleen, intestine, heart, kidney, and muscle with a high efficiency in mice. However, the specificity of *Csf1r-Cre* is not high and Cre-mediated deletion could be detected in DCs, granulocytes and T lymphocytes (25).

2.3 *CD11b-Cre* (MGI: 3577104; 3629092)

CD11b, also known as integrin alpha M (ITGAM), is an integrin family member which pairs with CD18 to form the heterodimeric integrin alpha M beta-2 (αMβ2) molecule (macrophage-1 antigen, Mac-1). It is widely expressed in monocytes, macrophages, granulocytes and natural killer (NK) cells. Functionally, CD11b regulates leukocyte adhesion and migration which is thus a key molecule in inflammatory responses.

In 2005, Ferron et al. created the *CD11b-Cre* mouse strain aiming to establish the conditional gene ablation in the hematopoietic myeloid-osteoclast lineage (34). In this study, the *CD11b*-reporter line was generated by crossing the *CD11b-Cre* strain with the Z/EG (*lacZ/EGFP*) double reporter strain to determine the Cre specificity. As expected, the Cre activity was detected in most peritoneal macrophages, and a large portion of macrophages and granulocytes in BM and spleen. In addition, mature osteoclast cells derived from BM and spleen progenitors of the reporter mice were mostly $EGFP^{+}$ (34).

Afterwards, the *CD11b-Cre* strain was applied for identifying the key myeloid genes through its cross with the respective loxP-flanked transgenic mice. In particular, a plethora of conditional knockout models were constructed to study the microglia-associated factors such as prostaglandin E2 (PGE2) receptors EP2 and EP4, inhibitor of nuclear factor kappa-B

kinase subunit beta (IKK-β), brain-derived neurotrophic factor (BDNF), progranulin and superoxide dismutase (SOD1) in induction of Alzheimer's disease, autoimmune encephalitis, hyperalgesia, neuroinflammation, amyotrophic lateral sclerosis (ALS) and hypertension in mice (35–40). Using the *CD11b-Cre* mice, the roles of serine/threonine-protein kinase/endoribonuclease IRE1a, myeloid differentiation primary response 88 (Myd88), huntingtin, cathepsin K were also characterized in inflammatory responses, Huntington's disease, bone formation and other pathological processes in tissue macrophages (41–44).

In comparison to other Cre driver mice targeting macrophages, the *CD11b-Cre* strain appeared to have a relatively low efficiency (less than 50%) to mark macrophages in bronchoalveolar lavage (BAL), peritoneal cavity, spleen and BM (8). As well, the CD11b-Cre activity was detected not only in monocytes and macrophages, but also in neutrophils and DCs suggesting a low level of specificity for macrophages (8). Lastly, this Cre transgenic strain was reported to be an unreliable line as inconsistent deletion among littermates had been observed (8).

2.4 *F4/80-Cre* (MGI: 2429642)

F4/80, also known as EGF-like module-containing mucin-like hormone receptor-like 1 (EMR1), is encoded by *adhesion G protein-coupled receptor E1* (*ADGRE1*) gene in mice. As a cell surface glycoprotein, F4/80 is expressed in tissue macrophages such as liver Kupffer cells and the spleen red pulp macrophages in mice (45).

The *F4/80-Cre* strain was generated in 2002 in which the Cre recombinase cDNA is introduced into the first coding exon of *F4/80* (46). This line has been crossed with loxP-flanked *sphingosine-1-phosphate receptor 1* (*S1PR1*) and *ferroportin* (*Fpn1*) transgenic mice to determine their respective roles in peritonitis and iron homeostasis (47,48). As well, the *F4/80-Cre* line was used to create reporter mice to monitor the interstitial lung macrophage development after crossing with *Rosa26-LSL-Tdtomato* strain (49).

As the F4/80-Cre activity was revealed to be associated with limited types of tissue macrophages such as peritoneal macrophages (8), this strain was not often applied for developing genetic manipulation models compared to other Cre driver mice for macrophages.

2.5 *CX3CR1-Cre* (MGI: 5311737; 5467983; 5450813; 5467985)

CX3C chemokine receptor 1 (CX3CR1), also called fractalkine receptor or G-protein coupled receptor 13 (GPR13), is the receptor for chemokine CX3CL1 and exclusively expressed in the mononuclear phagocyte system. In 2013, two groups reported their generation of *Cx3cr1-Cre* and *Cx3cr1-CreER* mice with constitutive or tamoxifen-inducible Cre recombinase inserted in the *Cx3cr1* loci (50,51). The *Cx3cr1-CreER* line is particularly useful as it allows for a temporal control of the transgene knockout/activation in macrophages.

There are three main applications for the *Cx3cr1-Cre* and *Cx3cr1-CreER* strains. First, these strains can be crossed with fluorescent reporters such as *Rosa26-LSL-YFP*, *Rosa26-LSL-*

RFP, *Rosa26-LSL-GFP* and *Rosa26-LSL-Tdtomato* lines to generate reporter mice for fate mapping of fetal monocytes/macrophages during prenatal development and the adult macrophages in heart, ileum, colon, peritoneal cavity and liver during tissue homeostasis (50,52–57). Second, by mating the *Cx3cr1-Cre* or *Cx3cr1-CreER* strain with loxP-flanked transgenic mice, a diversity of lines of macrophage-specific conditional knockout mice were developed. These mice advanced our knowledge about the macrophage-associated genes such as TGF-beta activated kinase 1 (TAK1), ubiquitin specific peptidase 18 (Usp18), transmembrane protein 16F (TMEM16F), tumor necrosis factor alpha (TNFα) and channelrhodopsin-2 (ChR2) in maintenance of brain and heart functions and their involvement in inflammation and autoimmune disorders (58–65). Lastly, through crossing with the *Rosa26*^{iDTR} strain, the *Cx3cr1-Cre* and *Cx3cr1-CreER* mice were also utilized to build macrophage-specific conditional depletion models in which the macrophages can be ablated upon injection of DT toxin. These models showed a high efficiency in depleting microglia and intestinal macrophages in mice (52,66).

With regards to the specificity, although *Cx3cr1-Cre* does not significantly mark neutrophils, this strain provides deletion in mast cells and DCs in addition to monocytes/macrophages (8,53). The deletion efficiency was high for peritoneal macrophages (70–80%), but relatively low (40–60%) for BAL and splenic macrophages, as well as peripheral blood monocytes (8).

3. Concluding Remarks

In this chapter, we mainly discussed the commonly used Cre driver mice targeting macrophages. Besides these strains, there are some other myeloid transgenes specific for certain subpopulations of tissue macrophages, which had also been applied to generate Cre transgenic mice. For example, the c-type lectin CD207a (langerin) is exclusively expressed in epidermal macrophages (Langerhans cells) and the *CD207a-Cre* strain was accordingly created for studying the endogenous Langerhans cells in mice (67,68). Furthermore, in examining macrophage development at the early prenatal stages, several Cre transgenic mice under the control of promoters of hematopoietic cell differentiation genes such as runt-related transcription factor 1 (Runx1) and FMS-like tyrosine kinase 3 (Flt3), were also applied (28,69,70).

Macrophages are a highly heterogeneous and plastic myeloid cell population in both steady status and pathological conditions. Their gene expression patterns dynamically change during the prenatal development, adult tissue homeostasis and in various inflammatory diseases. Moreover, such gene expression profiles are largely overlapped among monocytes/macrophages, granulocytes and DCs due to their close lineage relationship. Therefore, a Cre-transgenic line perfectly specific for macrophages is intrinsically impossible. The usage of these Cre driver mouse lines must be accompanied by other technical analyses such as flow cytometry, immunostaining and functional genomics before drawing any affirmative conclusions about the macrophage biology.

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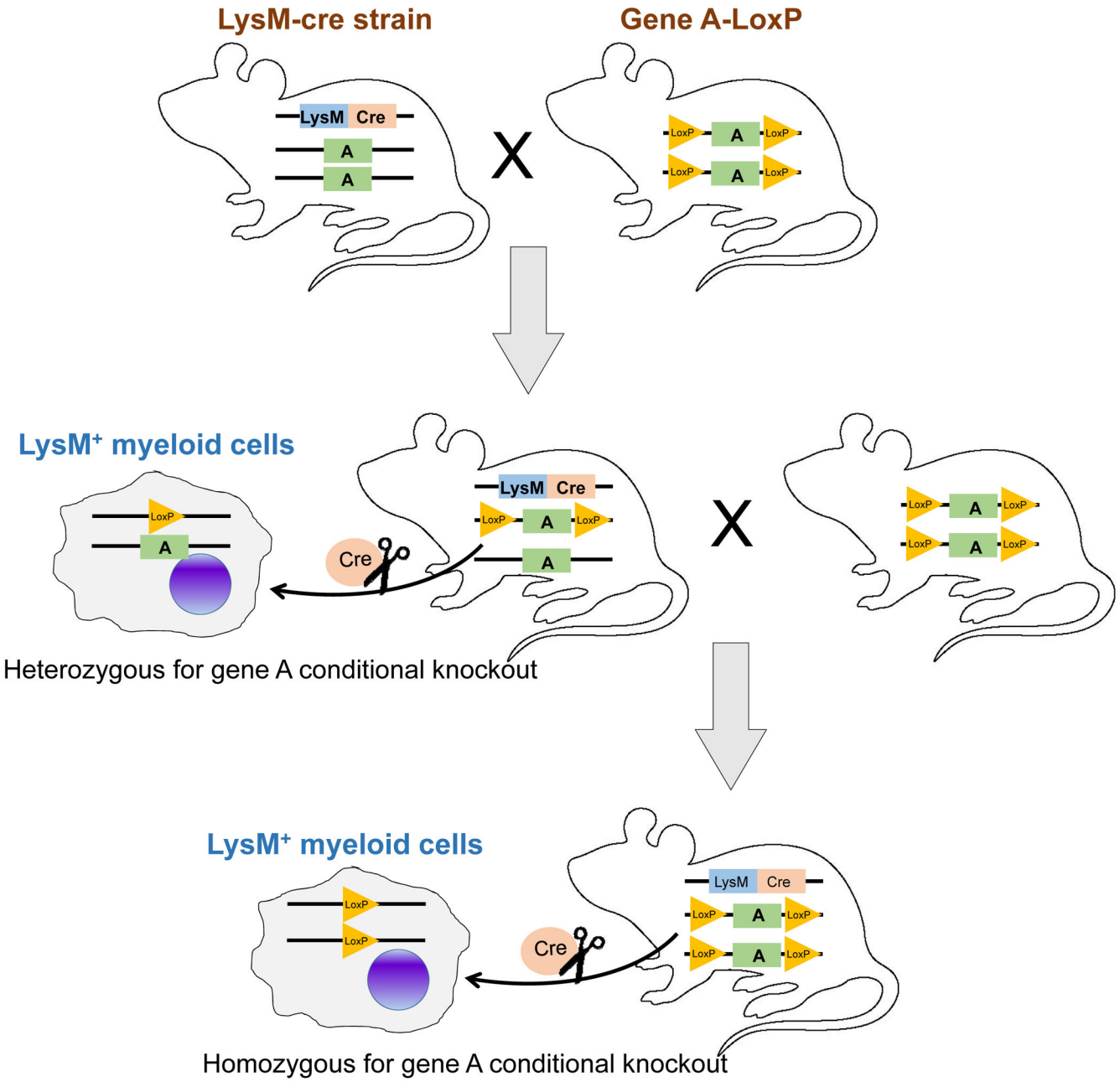

Figure 1. Generation of LysM$^+$ cell-specific conditional knockout mice using Cre/loxP technology.

LysM-Cre transgenic mouse is first crossed with the mouse homozygous for loxP-flanked gene *A*. Approximately 50% of the offspring will be heterozygous for the loxP-flanked gene *A* and hemizygous/heterozygous for the *Cre* transgene. In this F1 generation, the gene *A* is heterozygous knockout specific for LysM$^+$ cells. A further cross of this F1 generation with the parental mice homozygous for loxP-flanked gene *A* will lead to generation of about 25% of the progeny (experimental mice) homozygous for the loxP-flanked gene *A* and hemizygous/heterozygous for the *Cre* transgene. In this F2 generation of mice, Cre-mediated excision causes the homozygous knockout for gene *A* specifically in LysM$^+$ cells. Other F2

littermates, such as mice homozygous for loxP with no *Cre* transgene, and mice heterozygous for *loxP* and hemizygous/heterozygous for the *Cre* transgene, can serve as controls.

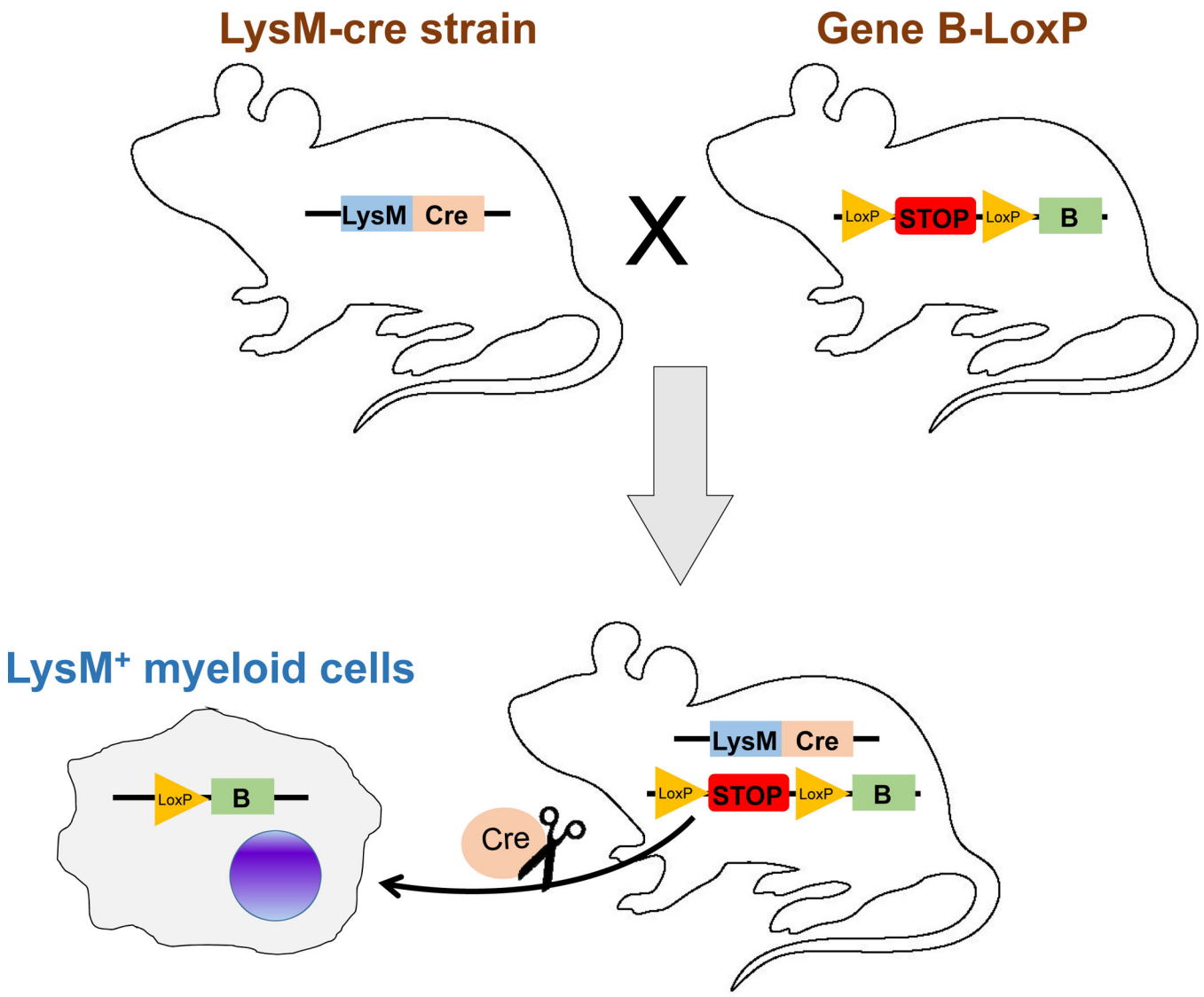

Figure 2. Application of Cre/loxP system for generation of mouse lines with gene conditional activation.

The gene *B*-loxP transgenic mouse is created by inserting a loxP-flanked transcriptional "STOP" sequence between the promoter and the gene *B* coding sequence, which prevents the expression of gene *B*. To cross this gene *B*-loxP transgenic mouse with *LysM-Cre* mouse will generate the progeny with gene *B* specifically expressed in $LysM^+$ cells. Such a system helps to develop cell-specific reporter mice, gene activation/overexpression and conditional depletion mouse lines.