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Fate-mapping mice: new tools and technology for immune discovery

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Abstract

The fate-mapping mouse has become an essential tool in the immunologist's toolbox. Although traditionally used by developmental biologists to trace the origins of cells, immunologists are turning to fate-mapping to better understand the development and function of immune cells. Thus, an expansion in the variety of fate-mapping mouse models has occurred to answer fundamental questions about the immune system. These models are also being combined with new genetic tools to study cancer, infection, and autoimmunity. In this review, we summarize different types of fate-mapping mice and describe emerging technologies that may allow immunologists to leverage this valuable tool and expand our functional knowledge of the immune system.

Keywords

lineage tracing; mouse models; fate mapping; Cre-loxP; immune development

The origins of fate-mapping mice in immunology

Immunologists have long desired the ability to track individual immune cells within an animal to better understand their role in the immune system. Historically, adoptive cell transfers [1] or **bone marrow chimeras** [2-4] were relied upon to track the fates of donor cells in congenically marked recipients. Other methods, such as deuterated water [5,6] or glucose, were used to label certain populations of immune cells to understand how these are produced and maintained in vivo. However, a major limitation of these experimental approaches is that they do not allow the study of distinct populations of immune cells in a physiological manner. As a result, immunologists recently turned their attention to fate-mapping mice to genetically mark and track specific cells of interest in situ.

Fate-mapping mice originated in the developmental biology field, used to understand how cells of different developmental origins contributed to the assembly of organs and tissues. In general, the approach involved using **Cre** recombinase to drive a fluorescent protein that was continually expressed in a specific lineage of cells. Later, geneticists developed

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inducible fate mappers so that the expression of a marker in a certain lineage of cells could be temporally controlled by the researcher. Cell marking using inducible fate-mapping mice was a significant advance in the field because it was more precise, highly reproducible, and long lasting. Thus, it was not long before immunologists took advantage of this robust system to study immune cell lineages. Today, fate-mapping mice are used to study a wide range of functions in many immune cell types.

In this review, we focus on the application of murine models of fate-mapping in immunological studies. Unless otherwise noted, all highlighted studies were conducted in mice. We start with a brief primer on the technical aspects of genetic fate-mapping; specifically, models using inducible systems. We then synthesize recent discoveries in specific cell types and immune functions, as well as in the context of disease, and note opportunities for new frontiers. Lastly, we consider how to streamline workflows when incorporating emerging technologies, and finally highlight the strengths of lineage tracing systems and important caveats when designing experiments.

Lineage tracing in different immune populations

Lineage tracing is the ability to track a cell from the point of its creation. The primary method for lineage tracing is to use a temporally controlled Cre recombinase (see Box 1) under a relevant promoter combined with a **floxed**-stop reporter expressed in a ubiquitously expressed **permissive locus**, such as **Rosa26** (Figure 1) [7], Temporal control can be achieved, for instance, through the expression of a Cre-estrogen receptor (ER)-ligand binding domain fusion protein which is sequestered in the cytoplasm by HSP90 [8,9]. When synthetic estrogen (tamoxifen) is administered, it is bound by the ER fusion protein, resulting in the release of the Cre-ER fusion protein from **HSP90**. The Cre-ER fusion protein then translocates to the nucleus, where it can mediate DNA recombination (Figure 1) [9]. Currently, most mouse models use one of two improved fusion proteins, **CreERT2** [10] or **MerCreMer** [11], that have beenengineered to increase sensitivity to tamoxifen and reduce cytotoxic effects.

To study any immune cell type, a relevant promoter must first be identified, allowing specific cell types or subsets to be followed (Figure 2, Key Figure). Frequently, the reporter is a fluorescent protein, such as the green fluorescent protein (GFP) or red fluorescent protein (RFP), used as tracers for an entire cell population. However, as fate-mapping techniques have matured, reporters have become more specialized. Multiple markers can be produced simultaneously by the same genetic insertion through **bicistronic** expression [12]. Markers can also be made “switchable”, depending on the placement of **LoxP** target sites [13]. For example, **confetti mice** have gene cassettes that allow for the random expression of one to four fluorescent proteins upon gene recombination [14]. The **polylox reporter** has been developed to make **genetic barcodes** to identify individual cells using a combination of 10 equally spaced loxP sites in alternating orientations, such that excisions and inversion recombination events can generate ~600,000 unique genetic identifiers [15,16]. The newer reporters (e.g. confetti [17], polylox) offer the opportunity to study population dynamics within a particular lineage of cells.

All immune cells derive from hematopoietic stem cells (HSCs) in the developmental process of hematopoiesis, giving rise to common myeloid and lymphoid progenitors (Figure 2). Myeloid lineages include erythrocytes, platelets, monocytes, macrophages, and granulocytes. Immune cells of the myeloid lineage are associated with innate immune function. The lymphoid lineage is composed of cells associated with adaptive immune responses, such as B cells and T cells as well as innate lymphoid cells (ILCs). Below is a summary of fate-mapping models currently developed for different lineages of immune cell populations (Figure 2).

Myeloid lineages

Macrophages and monocytes dominate the fate-mapping literature due to their well-known promoters, including *Cx3cr1* [18-36] (non-classical/patrolling), *Csfr1* [19,22,23,33,37] (yolk sacderived) and *Flt3* (fetal-derived) [22,37,38]. Fate-mapping mice have been particularly useful in mapping diverse macrophage subsets in various tissues. For example, $Cx3cr1^{CreERT2}$ mice helped establish that resident synovial and cardiac macrophages were self-renewing [23,24], and that kidney macrophages were derived from yolk sac macrophages [22]. Fate-mapping mice have also been used to define new subsets and functions of monocyte-derived subsets in the brain [25,28,30,35]. As for other myeloid lineage cells, $Runx1^{MerCreMer}$ (embryonic/fetal origin) and $Csf1r^{icre}$ (early erythromyeloid progenitors) mice have revealed tissue-specific genes in mast cells [39]. Granulocytes such as basophils, neutrophils, and eosinophils, have not yet been studied, though promoters have been identified for ancestors of these cells. It is likely only a matter of time before fate-mapping mice are available to study the roles of granulocytes in health and disease.

Lymphoid lineages

Fate-mapping mice have also been used to study $CD4^+$ and $CD8^+$ T cells. $Cd4^{creERT2}$ mice [40], for example, can be used to track the fates of $CD8^+$ T cells leaving the thymus, since they only express CD4 briefly during thymic development. Similarly, $Cd8^{creERT2}$ [41] mice have been recently developed to map the fates of naïve $CD4^+$ T cells, since CD8 expression is temporally restricted to $CD4^+$ T cells undergoing thymic development. Fate-mapping mice can also be used to label $CD8^+$ and $CD4^+$ T cells during the effector stage of the response to infection. For example, $Klrg1^{cre}$ was used to identify a novel subset of effector $CD8^+$ T cells protecting the host against *Listeria monocytogenes* and cancer in mice [42]. The $Foxp3^{CreERT2}$ driver has been useful in examining how **T regulatory cells** (Tregs) prevent rejection of heart transplants by reducing the numbers of pro-inflammatory cells [43]. Using a $Tbet^{ZsGreen\text{-}T2A\text{-}CreERT2}$ driver, a subset of **T follicular helper cells** (Tfh) were revealed to express the pro-inflammatory cytokine IFN-γ in **germinal centers** (GC) post vaccination with a peptide, AS15 [44]. A report using the $IL\ 17^{cre}$ driver to study **Th17** $CD4^+$ T cells demonstrated context-dependent cellular behavior whereby the experimental autoimmune encephalitis (EAE) disease model of multiple sclerosis resulted in Th17 cells producing non-Th17 cytokines, while fungal *Candida albicans* infection triggered classical Th17 behavior [45]. B cell subsets have also been analyzed. B cell specific drivers revealing the dynamics of B cells in GCs, as well as the ability of B cells to undergo clonal selection and **affinity maturation** following vaccination, include $Aicda^{CreERT2}$ (memory B cells and plasma cells) [46-48] $S1pr2^{CreERT2}$ (gut associated GCs) [48,49] and $Mki67^{CreERT2}$ (GC B

cells) [50]. The *Tbet*$^{ZsGreen\text{-}T2A\text{-}CreERT2}$ driver was also linked to a role of age-associated B cells in driving lupus in mice [51].

Fate mappers have informed our understanding of the development and function of ILCs. *NKp46*^{CreERT2} has allowed the tracking of natural killer (NK) cells to interrogate NK cell memory during toxoplasmosis [52] and murine cytomegalovirus infections [53]. *Cx3cr1*^{CreERT2} (which labels dendritic epidermal T cells) has been used to examine the γδ T cells role in epidermal wound healing [54] and *TCRδ*^{CreER} mice, for assessing the role of γδ T cells in causing psoriasis [55]. Moreover, fate-mapping is especially useful when studying rare immune populations, such as ILCs, which are difficult to detect using flow cytometry [56]. *Id2*^{creERT2} (important for the developmental origin of all ILCs) [57], *Rorc*^{cre} (needed for ILC3) [57] and *Arg1RFP*^{CreERT2} (needed for ILC2) [58] have been used to trace tissue seeding of various ILC populations. Similarly, our understanding of **invariant natural killer T** (iNKT) cell development has been enhanced in studies of *Cd4*^{CreERt2}*xVα14i*^{stop}*FxTraj18KO* (knockout) mice, in which tamoxifen administration marked waves of iNKT cells emerging from the thymus [59]. Although many lymphoid lineages are well represented in the fate-mapping literature, there is still ample room for defining other immune cell populations. For example, mucosal-associated invariant T (**MAIT) cells,** which express *Tcrδ* or *Cd4* during development, might be studied using *Tcrδ*^{CreERT2} or *Cd4*^{CreERT2} mice [60]. As MAIT cells are abundant in humans [60], fate-mapping experiments could potentially help unravel their unknown roles in disease.

Increasing the Breadth and Specificity of Fate-mapping

Currently, most fate-mapping models are designed to track a single population, but appropriately designed model systems can be multiplexed for orthogonal analysis of multiple factors and/or cell types. For example, the Tbiluc mouse displays constitutive T cell-specific green luciferase expression to report location, and NFAT-driven red luciferase to report cell activity [61]. Questions concerning population heterogeneity are well suited to concurrent fate-mapping strategies, as demonstrated by a pancreatic development study, using both Cre-loxP and Dre-roxP recombination to simultaneously track two lineages of β-cells establishing population heterogeneity and linking it to the transcription factor Pft1a [62]. A key to tracking multiple lineages is unique recombinase-target pairs for each population. Therefore, immune cell-specific mouse models using other DNA recombination enzyme systems need to be developed. Such systems include Flp-frt [41], Dre-roxP [63], the more recently developed sCre-sloxP and vCre-vloxP systems [64], as well as CRISPR-Cas9 based systems of gene editing [65]. Moreover, using complimentary induction agents such as tetracycline [66] or photoactivation [67], can also further refine experimental models.

Immunologists have harnessed temporally-controlled Cre recombination to develop numerous fate-mapping mouse models. As a result, lineage tracing can be performed on most immune cell types. The continued development of mouse models that can track multiple cell types simultaneously can thus provide new insights into cellular diversity and function.

Fate-mapping, immune function, and new frontiers

Early immunological applications of fate-mapping mice primarily focused on addressing the stem cell origins of immune cell types [68]. Embryonic and non-embryonic population sources were identified and observed until maturity. A natural extension of these studies was to ask whether functional consequences arose from different developmental origins. Determining the basis of differential behavior is key to understanding how immune cells contribute to homeostatic and disease processes.

Host defense

One role of the immune system is to protect the body from infectious disease. The immune response is comprised of multiple immune cell types with unique functions, but even within cell types, diverse behavior is observed. Fate-mapping has provided insights into varying infection responses. By following different fluorescently-labeled populations of dendritic cells (DC) with the *Clec9a*^{Confetti} fate-mapping model, influenza A virus was found to not drive local clonal expansion of conventional dendritic cells (cDCs) [69]. Instead, bone marrow-derived circulating pre-cDCs were rapidly recruited to the lung and were the primary source of cDCs during infection [69]. In another study, the *Cd4*^{creERT2} driver was used to follow CD8^{+} T cells of different developmental origins. CD8^{+} T cells generated early in life were biased towards a short-lived effector fate following *L. monocytogenes* infection, showing that the age at which cells were produced influenced their long-term fate [40]. This CD8^{+} T cell study highlighted how fate-mapping models might be useful for evaluating age-related differences in immune responses across lifespans. Indeed, currently, little is known about the developmental origins of aging immune cells and their contribution to a variety of diseases.

Immunization serves to activate protective immune responses without actual infection, thereby ideally preventing severe disease should infection occur. A mediator of protection is that of antibody production by memory B cells once neutralizing antibody titers decline. Leveraging a GC B cell-specific promoter -- identified by scRNA-seq, *S1pr2*^{creERT2} reporter mice were given tamoxifen to fluorescently label B cells; consequently, cellular behavior could be monitored post-immunization [49]. T cell responses to immunization, particularly tissue-specific responses, are also important for containing infections [70,71], and further insights may be gained about these responses using existing fate-mapping models. Immune priming of HSCs may also be better understood by fate-mapping immune cell populations such as monocytes [72].

Inflammation and tissue repair

Recent work defined the developmental origins of immune cells that mediate inflammation and tissue repair. In particular, fate-mapping mice studies have provided new insights into the roles that tissue-specific macrophages play during responses to, and resolution of injury. Studies showing that cavity macrophages directly contribute to tissue repair have typically relied on genetic deletion of Gata6-expressing cells [73,74]. However, a recently developed fate-mapping strategy demonstrated a more nuanced picture of cavity macrophage behavior. The model used a CD45-driven Dre-rox system to restrict *Gata6*^{iCreERT2} to CD45^{+}

cells, specifically reporting fluorescence in peritoneal cavity macrophages and allowing to track the cavity macrophage response to cryo- and heat-induced injury in the liver; results showed that while cavity macrophages were recruited to the liver parenchyma, they did not penetrate deep into the tissue, suggesting that they did not directly mediate repair in this model [63]. Tissue resident macrophages can be tracked using a *Cx3cr1*^{CreER} mouse to drive the expression of a fluorescent reporter protein [24]. In a study examining cardiac infarction, *Cx3cr1*^{CreER} *x R26*$^{Td/DTR}$ mice were bred to selectively delete tissue-resident cardiac macrophages, demonstrating that tissue-resident cardiac macrophages prevented pathological remodeling after cardiac infarction [24]. Like macrophages, many immune cell types contribute to tissue repair and inflammation regulation, including Tregs [75], and their roles may be further deciphered using fate-mapping models.

Cancer

Immune cells can promote or suppress cancer, depending on context; therefore, balancing their responses is crucial for achieving effective cancer therapies. Fate-mapping mice have been used to understand cancer-driving and protective immune populations. In one example, using a combination of *Cx3cr1*^{CreERT2}, *Lyz2*^{Cre} and *Csf1r*^{CreERT2} mice, the embryonic origin of a cancer-driving macrophage population was determined by fate-mapping embryonic macrophages, which where genetically ablated in a model of metastatic murine epithelial ovarian cancer [19]. Their ablation resulted in reduced tumor growth [19] – an outcome that might eventually be further investigated for creating targeted therapeutics. Similarly, a *FoxP3*^{YFPcre} x *Id2*^{EmGFP} mouse that both reports and over-expresses *Id2*, revealed a genetic basis for the cancer-exacerbating potential of Tregs [76]. In this system, *Id2* was only over-expressed when doxycycline was administered; mice over-expressing Id2 exhibited reduced mouse melanoma B16.F10 tumor growth relative to the control mouse with normal Id2 protein expression, thus identifying another potential candidate drug target in this setting [76]. Moving forward. fate-mapping mice can contribute to further unraveling the heterogeneity of responses to immunomodulators and **checkpoint inhibitors** during immunotherapy, and might provide valuable insight into variable clinical outcomes.

Autoimmunity and allergy

When immune cells aberrantly react to self or innocuous foreign proteins, they can cause disease. Fate-mapping mice can track specific subpopulations of cells that contribute to allergic and/or autoimmune conditions. For example, *Cx3cr1*^{creERT2} and *Cx3cr1*^{cre} mice were used to characterize synovial macrophages using microscopy and single cell sequencing [23]. These synovial lining macrophages were shown to be important in preventing collagen-induced arthritis as evidenced by the increased clinical score reported when the macrophages were ablated in a deleter mouse model restricting diphtheria toxin receptor to *Cx3cr1* expressing cells [23]. In an EAE study, *IL-17*^{Cre} mice were bred to *mTORC1* knockout reporter mice; deleting *mTORC1* led to increased resistance to EAE, as indicated by decreased clinical score and reduced spinal cord immunopathology when compared with mice expressing endogenous mTORC1 This suggested that disease resistance may have been due to metabolic changes in the fate-mapped CD4^{+} Th17 cells, although this remains to be further studied [77]. While B cells [78], CD4^{+} Th2 cells [79] and mast cells

[80] are also important cellular mediators of allergy, fate-mapping mice remain to be used to further explore the different nuances and contributions of these cell types to allergic disease.

Taken together, recent applications of fate-mapping mice have yielded fundamental information about cellular origins and the roles of cell subsets in diverse aspects of immune function; however, there are still new frontiers that remain to be explored using these valuable tools.

Working with fate-mapping mouse models

Experimental design considerations

Inducible Cre-driven models of fate-mapping can reduce the likelihood of issues associated with unexpected Cre expression because Cre activity is restricted in the absence of an induction agent (i.e tamoxifen). However, promoters can display wider or more transient gene expression patterns than anticipated. For instance, even minimal Cre expression can edit at target loxP sites and this so-called “leakiness” results in unintended reporter expression, which causes complications in data interpretation due to the loss of fidelity between reporter expression and specific induction of Cre [81]. Moreover, Cre expression, even in the absence of recombination, can have deleterious effects, including DNA damage and chromosomal abnormalities [82]. Additionally, ERT2-inducible Cre mouse models may show reduced proliferation in rapidly dividing cells, as has been shown for the clonal expansion of T cells [83]. Moreover, the effects of concurrent drug treatments should be considered. For example, ivermectin, an anti-parasitic medication widely used in routine animal husbandry as a treatment for pinworms, can activate ERT2-mediated Cre recombination, thus resulting in fluorescence reporter expression in the absence of tamoxifen administration, an outcome that can confound data interpretation [84].

Furthermore, the induction agent tamoxifen, a synthetic estrogen, can also have unintended side effects. Specifically, in pregnant dams, tamoxifen administration can result in embryonic lethality, difficulty in labor, and reduced live birth rates [85]. Therefore, the co-administration of progesterone can reduce some of these side-effects [86]. Also, in males, tamoxifen administration has demonstrated ill effects on reproductive fitness [87]. Generally, long-term exposure to tamoxifen can be quite toxic. Therefore, it is desirable to administer tamoxifen at the lowest effective dose for short time intervals [87], and oral administration can reduce the occurrence of drug delivery-related inflammation in mice [88].

Lastly, fate-mapping models frequently use breeding schemes in which one parent bears the promoter-driven Cre while the other bears the floxed-stop reporter to generate offspring with single copies of each fate-mapping element (Figure 1) [81]. While a single copy of these transgenic elements is usually sufficient for their expression in most cells, cellular marking can be incomplete. For example, green fluorescence driven by TCR$\delta^{creERT2}$ in such transgenic mice varies from 60-90% in splenic $\gamma\delta$ T cells, depending on the genotype of the offspring [89], suggesting that gene copy number may impact reporter expression.

Combining fate-mapping with other experimental tools

There isn't a singular way of experimentally using fate-mapping mice, and taking advantage of current and emerging techniques (Box 2) has many benefits. The specific research question will dictate which techniques are most useful, and oftentimes using a combination of techniques yields additional insights (Figure 3).

High throughput and single-cell sequencing technologies are rapidly-evolving and improved technologies which assess the transcriptional and **epigenetic** state of cells. Many important questions have been addressed with population level **RNA-seq** and **ATAC-seq**, including defining lineage-specific transcription factors relevant to $CD4^+$ T cells in the gut [90]. To correlate gene expression patterns in cells of interest to expression patterns of defined subsets [40], the data collected through these techniques can be compared to curated data sets, such as in the ImmGen database[i] [91-93]. Recent advances using sequencing data to infer cell-cell interactions can be valuable in yielding future insights into the behavior of fate-mapped cells [94]. **Single-cell RNA sequencing (scRNA-seq)** was used in conjunction with a model that fluorescently-barcoded NK cells to identify two distinct differentiation paths for ILC1-like and NK cells [95]. Additionally, multiple mouse models were developed based on scRNA-sequencing that identified *Ms4a3* as a promoter that could efficiently fate-map monocyte lineages [96]. scRNA-seq is currently an expensive endeavor, which can result in having to balance the number of individual cells sequenced and **sequencing depth** [97]. This trade-off can sometimes make it hard to identify populations based on known markers whose genes are expressed at low amounts. CITE-seq [98] was developed in part, to overcome this limitation by using oligonucleotide-conjugated antibodies so that protein expression of markers of cellular identity could be correlated with gene expression during sequencing.

Immunologists have relied on **flow cytometry** for decades because it is an invaluable tool for looking at protein expression at the cellular level. Flow cytometry tracks the phenotype and population dynamics of fate-mapped cells [99]. As cytometric technologies continue to be refined, the number of biomarkers that can be tracked on an individual cell has increased dramatically. The synthesis of high dimensional data has necessitated the adoption of **machine learning** techniques, such as **FlowSOM,** which can help to understand, describe, and predict cellular behavior [100,101]. For example, FlowSOM was used to identify and track the fates of GM-CSF-producing cells in the Frog mouse system [100].

While much has been learned by studying fate-mapped cells in isolation, either in vitro or ex vivo, the ability to see how cellular lineages interact with their natural environment is essential for deepening our understanding of cellular fate and function. Confocal images have helped identify yolk sac-derived mast cells in a $CD5^{creERT2}$ driven fate-mapping mice [54], while clonal dynamics of B cell selection in gut GCs has been visualized using, for instance, the B cell-specific confetti reporter $Aciada^{creERT2}$, coupled with multiphoton imaging [48]. **Light sheet fluorescence microscopy (LSFM)** is also currently being used for lineage tracing by developmental biologists [102,103] and is of great advantage

[i] https://www.immgen.org/

for further answering immunological questions. In addition, the continued development of reporter mice, such as the Tri-modal reporter mouse, which concurrently reports bioluminescence, fluorescence, and **positron emission tomography** can allow for imaging at multiple scales within the same mouse model [104].

The studies highlighted above demonstrate how careful experimental design along with rapid technological advances in high throughput sequencing, cytometry, and imaging are enhancing the power of fate-mapping mouse models to answer questions about immune cell origins, phenotypes, and functions. We anticipate that technological advances will continue to improve the utility of fate-mapping mouse models in future studies.

Concluding remarks

Genetic fate-mapping of immune cells has led to discoveries about the origins of cell lineages, but recently, has been used to further inform on the specific roles of these lineages in health and disease. Additional discoveries in this realm can contribute to our understanding of why and how cellular origins impact immunologic outcomes, but also inform future therapeutic targets in cancer and infection, aid in the design of effective vaccines, and shed light on unexpected treatment outcomes (Box 3. Clinician's Corner). Opportunities exist for developing new tools to better examine the cellular fates of specific responding populations. Engineering viral and bacterial pathogens that express Cre to mark infected cells could also generate insight into host-pathogen interactions, for example for pathogens with immune tropism [105].

In addition, leveraging such highlighted technologies can provide new insights into immune homeostasis and function. As single cell technologies continue to evolve and become more affordable, we posit that they might become the gold standard for understanding the genomics of fate-mapped populations. Technologies that combine aspects of imaging, cytometry, and sequencing, including spatial transcriptomics [106], **scope-seq** [107] and **chip cytometry** [108], are providing new ways to interrogate fate-mapping systems. New analysis techniques, such as **pseudotime analysis** [109], can also push the field toward a deeper understanding of cell function and drive new hypotheses (outstanding questions).

Finally, new fate-mapping mouse models can also exploit emerging genetic tools, such as CRISPR-Cas9 gene editing [110-112], **polycistronic expression** systems [113] and intersectional approaches [114] . For instance, improved reporters that can reveal cellular function and identity [115] may help deepen our understanding of biological processes in cell lineages. Implementing genetic barcoding [111,116] in cells of interest can also help uncover lineage diversity during homeostasis or disease. We eagerly anticipate the rapid development of additional fate-mapping tools combined with emerging technologies to enhance our fundamental knowledge of how cell origin can influence immune cell fates and functions.

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Glossary

Affinity maturation
biochemical process B cells undergo to produce higher affinity antibodies

ATAC-seq
high throughput sequencing technique assessing chromatin accessibility

Bicistronic
translation of two genes from one mRNA transcript

Bone marrow chimera
mouse model in which the immune compartment is reconstituted with donor mouse stem cells

Checkpoint inhibitor
immunotherapeutic drug targeting immune regulators (checkpoints)

Chip cytometry
immobilized cell arrays for single cell analysis

Confetti mouse
reporter mouse stochastically expressing different fluorescent proteins

Cre
Bacteriophage P1-derived site-specfic DNA recombinase

Epigenetic
regulation of gene expression without changing DNA sequence

ERT2
triple mutant of estrogen receptor with high specificity for tamoxifen

Fate-mapping
method used to study how the origin of cells influences their trajectory

Flow cytometry
technique that rapidly analyzes cells for parameters such as size, granularity, and protein expression

FlowSOM
self-organized map algorithm used on flow cytometry data

Floxed
flanked by two loxP sequences

Genetic barcoding

marking unique identifiers with short DNA sequences

Germinal center
location within a lymphoid organ follicle where high affinity antibodies are produced by B cells

HSP90
molecular chaperone aiding in protein folding

Invariant natural killer T cell (iNKT cell)
NKT cell lymphoid population recognizing specific lipids

Light sheet fluorescent microscopy (LSFM)
excitation occurs in a plane perpendicular to the observational direction

LoxP
the DNA sequence is a target for Cre

Machine Learning
computer systems that learn and adapt by using algorithms to analyze data patterns

MerCreMer
fusion protein of Cre flanked by two modified ER binding domains

MAIT cell
innate like T cell bearing an invariant TCR

Multi-photon microscopy
using near-infrared light to excite fluorescent molecules with 2+ photons to image deep into animal tissue while minimizing damage

Permissive locus
gene region allowing genetic insertion without transgene silencing or dysregulation of neighboring genes. In this review, different from transcriptionally permissive

Polycistronic expression
translation of multiple genes from a single mRNA transcript

Polylox reporter
system whereby Cre drives the generation of unique genetic barcodes

Positron emission tomography (PET)
functional imaging technique using radioactive substances

Pseudotime analysis
dynamic analysis ordering cells along a lineage based on gene expression profiles

RNA-seq
high throughput sequencing analyzing transcriptional profiles

Rosa26
permissive gene locus for ubiquitous expression

SCOPE-seq
sequencing method merging scRNAseq and live cell imaging

Sequencing depth
average number of times nucleotides are read in high throughput sequencing

scRNA-seq
high throughput sequencing analyzing transcriptional profiles of individual cells

tSNE
statistical method for visualizing high-dimensional data

T follicular helper cells
$CD4^+$ T cells that aid B cell maturation

Tregs
immunosuppressive $CD4^+$ T cells

Th17 cells
$CD4^+$ T cells producing IL-17

UMAP
dimension reduction algorithm for high parameter data

2A
self-cleaving peptide sequences placed between genes of interest; used to generate individual, instead of fusion proteins

3DISCO
tissue-clearing method for 3D imaging of entire organs without sectioning

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Outstanding Questions

- How can we integrate multiple fate-mapping modalities to mark more specialized populations in mice? By combining several types of drivers and reporters, multiple populations might be tracked simultaneously.
- Many immune cell types have been described using fate-mapping mice, but we still lack mouse models for many rare cell types. What new mice might be designed to lineage-trace other immune cell types/subtypes? The field currently lacks fate-mapping mice for certain populations of granulocytes, ILCs, and MAIT cell.
- How can we leverage imaging techniques in conjunction with fate-mapping mice, such as multiphoton imaging, to better understand the developmental origins of tissue distribution during homeostasis and disease?
- How can viruses or bacteria expressing Cre be utilized to mark and track infected cells in mice?
- Can we apply previously created fate-mapping mice to answer new questions related to infection and cancer, particularly to understand the developmental origins of the heterogeneous response to treatments and vaccines?

Highlights Box

- Fate-mapping mice have revealed the developmental origins of multiple types of immune cells.
- When combined with technologies such as scRNAseq, multiphoton imaging, and multiparameter flow cytometry, fate-mapping mice can define novel cell populations.
- When utilized in the context of infection and cancer, fate-mapping mice can both aid in understanding immune cell responses and help uncover new putative therapeutic targets that are unique to cells of specific developmental origins.
- Emerging fate-mapping models take advantage of newer genetic tools, such as cellular barcoding and stochastic multi-color reporters, thus allowing further resolution of the dynamics of immune cell populations in mice.

Box 1.

Marking a moment in space and time

Successful fate-mapping requires tracking the trajectory of a cell from its origins. Most methods of lineage tracing rely on temporally controlled site-specific recombination (SSR) systems in conjunction with appropriate reporter constructs.

Site-specific recombination (SSR) systems.

SSR systems integrate DNA recombinases and their target DNA sequences into the genome of the species of interest (e.g., mice) for the purpose of editing genetic information. The most used recombinase-target pair is Cre-loxP, which is derived from the P1 bacteriophage. The placement and orientation of the flanking target sequences dictate the type of gene editing that occurs: if a region of interest is flanked by two targeting sequences in the same orientation, that region of interest is excised; if the two targeting sequences are in opposite orientation, the region will be inverted. In the case of Cre-loxP, this flanking process is commonly referred to as floxing. By linking the expression of the recombinase to a cell-specific promoter, you can restrict deletion (or expression) of genes to a specific population of interest [81].

Temporal control of expression.

The selection of the promoter is crucial to effective lineage tracing. Restricting reporter expression to a specific cell type is often not sufficient for experimental success; one must also control when the reporter is turned on and off. Temporal expression can be achieved through various mechanisms, but a popular mechanism is drug induction. The most common in fate-mapping studies is the tamoxifen-estrogen receptor (ER) system, which relies on the Cre-ER fusion protein being sequestered in the cytoplasm unless tamoxifen is administered [117]. Other mechanisms that can provide temporal control are doxycycline-reverse tetracycline transactivator (rtTA) systems or light-induced split-Cre systems, where Cre is split into N- and C-terminal halves that can only make functional Cre when expressing cells are exposed to light (e.g. CRY2) [118].

Choosing a reporter.

Probably the most frequently used type of reporter in SSR systems is a simple floxed-stop-fluorescent protein. Another popular option is an enzyme like β-galactosidase or luciferase. Through bicistronic expression (via **2A** sequence or similar), reporters can have multiple components to simultaneously report multiple colors or report on function. Other reporters switch colors upon recombination [13]. Reporters that allow for stochastic marking, either by randomly marking with several colors [14] or by generating a unique genetic barcode [15,16], are useful for tracking multiple clonal populations simultaneously.

Box 2.

A fate mapper's toolbox

For maximal discovery, fate-mapping models should take advantage of cutting-edge scientific technologies and methods:

Sequencing technologies.

RNA sequencing comprehensively describes the transcriptome of a cellular population, while ATAC sequencing is a method to identify areas of open chromatin. Both of these technologies are now routinely being applied to single cells, most frequently using the 10x genomics platform [119]. Additionally, for those interested in clonal populations of B and T cells, receptor identification and transcriptional profiling can be assessed simultaneously at the individual cell level. The genomics field is continually developing new computational algorithms and machine learning techniques to identify important signaling networks and underlying regulatory mechanisms.

High parameter flow cytometry:

Cytometry is a workhorse of immunology research. Optical advances, including full spectrum cytometry, now allow for the analysis of upwards of twenty-five parameters in any given sample for the identification of cell types and subpopulations with unique functions [120]. While conventional one- and two-dimensional analysis can result in useful findings, additional interpretation (and visual representation) of high-dimensional data utilizes dimensional reduction algorithms, such as FlowSOM, as well as **t-SNE** and **UMAP** [101].

Mathematical modeling:

Experiments designed to track turnover of cellular populations over time yield data that can be used to construct mathematical models describing kinetics of expansion, contraction, differentiation, and survival. These models can, in turn, be used to form hypotheses about cellular behavior that can then be experimentally tested.

Imaging Technologies:

Many of the experimental tools used by immunologists rely on the removal of cells from their native environment. Use of fate-mapping models with newer imaging modalities can provide essential information about how populations of interest act within tissues. Multiphoton techniques take advantage of high powered near-infrared lasers and 2+ photon absorption, allowing for deep imaging within tissues [121]. Alternatively, whole tissues can be imaged at high resolution by newer methods of tissue clearance (e,g.**3DISCO**) [122], in conjunction with confocal and light sheet microscopy. Non-invasive techniques such as a positron emission tomography (PET) scan can be used to track marked populations over time within the same animal [123].

Box 3.

Clinician's Corner

- Fate-mapping mice track defined populations of cells with specificity that is not achievable in humans. The singular method of fate-mapping within humans involves administering deuterated water, which only allows proliferation to be tracked broadly.
- By marking specific populations, fate-mapping mice present an ideal model for studying heterogeneity in immune cell responses with high clinical relevance. For example, fate-mapping mice may help uncover why some patients respond to immunomodulators or checkpoint inhibitors, while others do not. This approach is especially powerful when combined with emerging techniques such as barcoding individual cells.
- Findings from fate-mapping mice can be verified in humans. Tools, such as sequencing and imaging, may reveal markers and tissue specific properties that might be correlated to human samples.
- Results from fate-mapping mice studies can also have translational benefit in designing new therapeutics. Once the dynamics of disease-causing populations are defined, drugs may then be designed and tested.

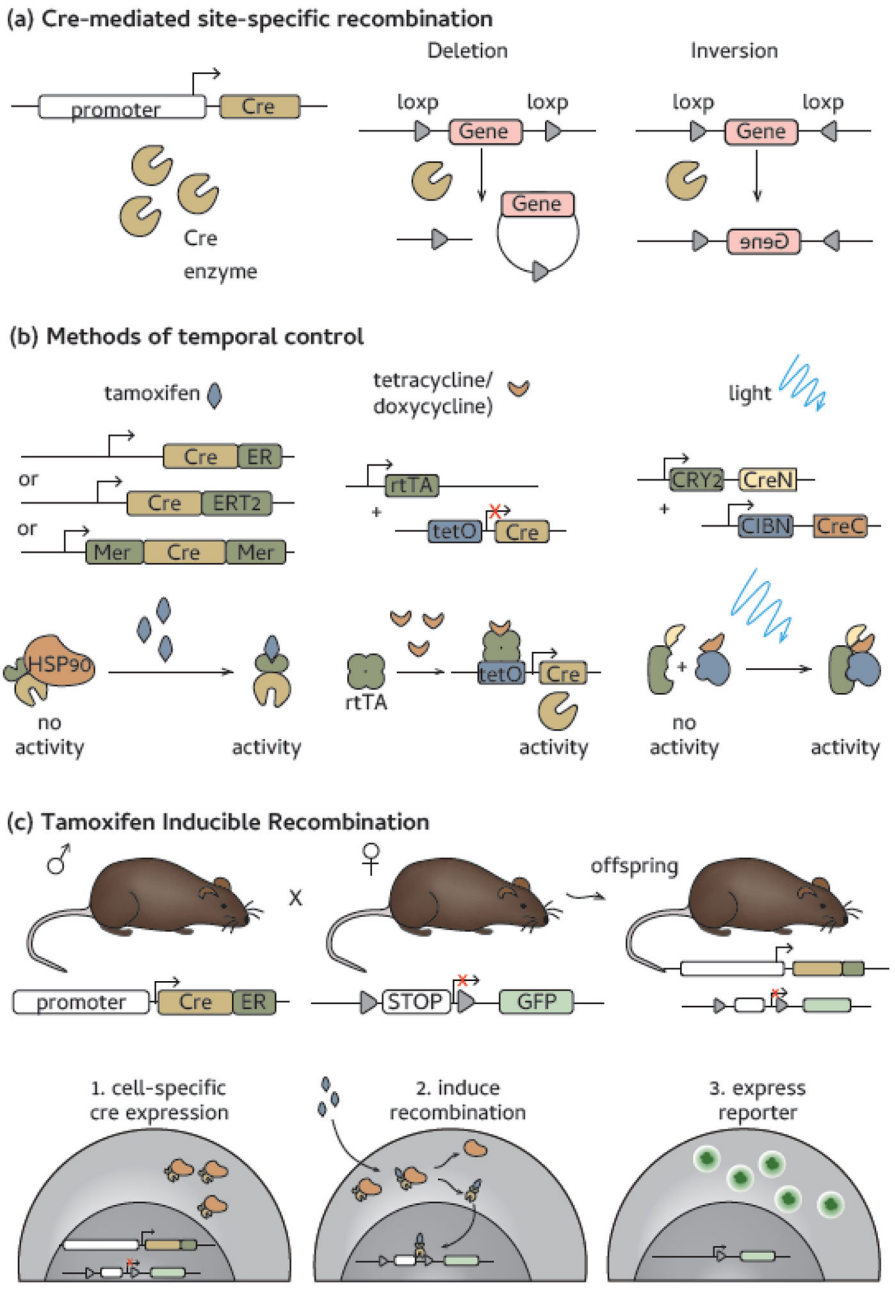

Figure 1. How fate-mapping mice are made.

(a) Cre cassettes can be placed under the control of cell specific promoters. Cre is a site-specific recombinase that recognizes loxP sites as targets for DNA cleavage. Recognition of loxP sites in the same direction result in gene cassette deletion, while gene inversion occurs when loxP sites are placed in opposing directions. (b) Temporal expression is key for most current fate-mapping models. Tamoxifen mediated induction is a common method of inductions and is accomplished through one of three drivers: Cre-estrogen receptor (ER), Cre-ERT2 [10] or MerCreMer [11]. Other methods of temporal control include tetracycline induction [124] or split-Cre constructs that can be activated by a protein conformational change induced by light exposure [118]. (c) A fully functional fate-mapping model is often

made by crossing driver and reporter lines to make a F1 generation with each transgene. In offspring, (1) Cre expression is cell specific, upon addition of tamoxifen, (2) the Cre-ER fusion protein translocates to the nucleus and mediates the recombination event and this (3) allows the reporter protein(s) to be expressed in a cell specific manner [10].

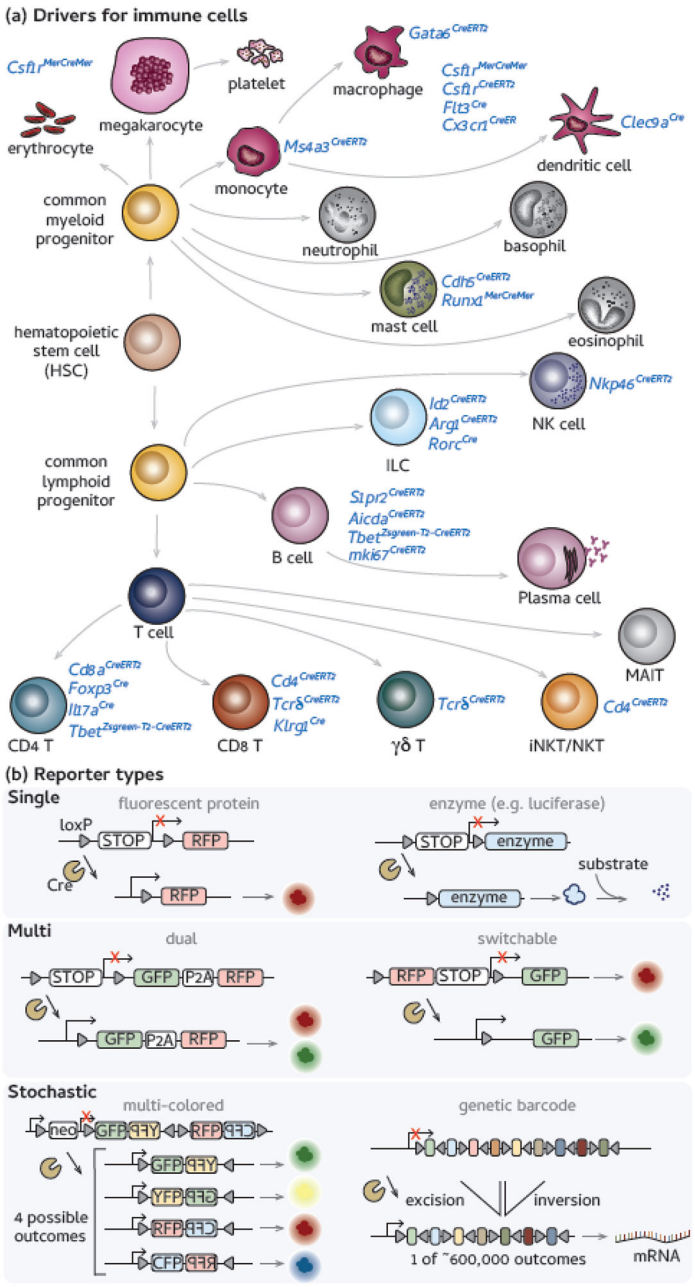

Figure 2 (Key Figure). Examples of current immune cell-specific drivers and reporter options.

(a) Simplified diagram of hematopoiesis and cell lineages of the mouse immune system. In blue are the drivers that have been used to perform fate-mapping on specific cell types with corresponding references: megakaryocytes [125]; macrophages [2,18-37,63,126]; monocytes [96]; dendritic cells [69]; mast cells [39,86]; natural killer (NK) cells [52,53,95]; ILCs [57,58]; B cells [46-51]; $CD4^+$T cells [41,43-45,77,127]; $CD8^+$ T cells [40,42,99]; γδ T cells [54,55,89,128]; invariant natural killer T (iNKT) cells [59]. (b) Reporter constructs are usually inserted into a permissive locus. Reporters can be classified into three major types: single reporters, such as those that express a single fluorescent protein or an enzyme; multi reporters, such as those that bicistronically express two proteins [12] or are switchable [13];

and stochastic reporters in which recombination leads to random expression of a fluorescent marker (confetti) [14] or a genetic barcode (polylox) [15,16].

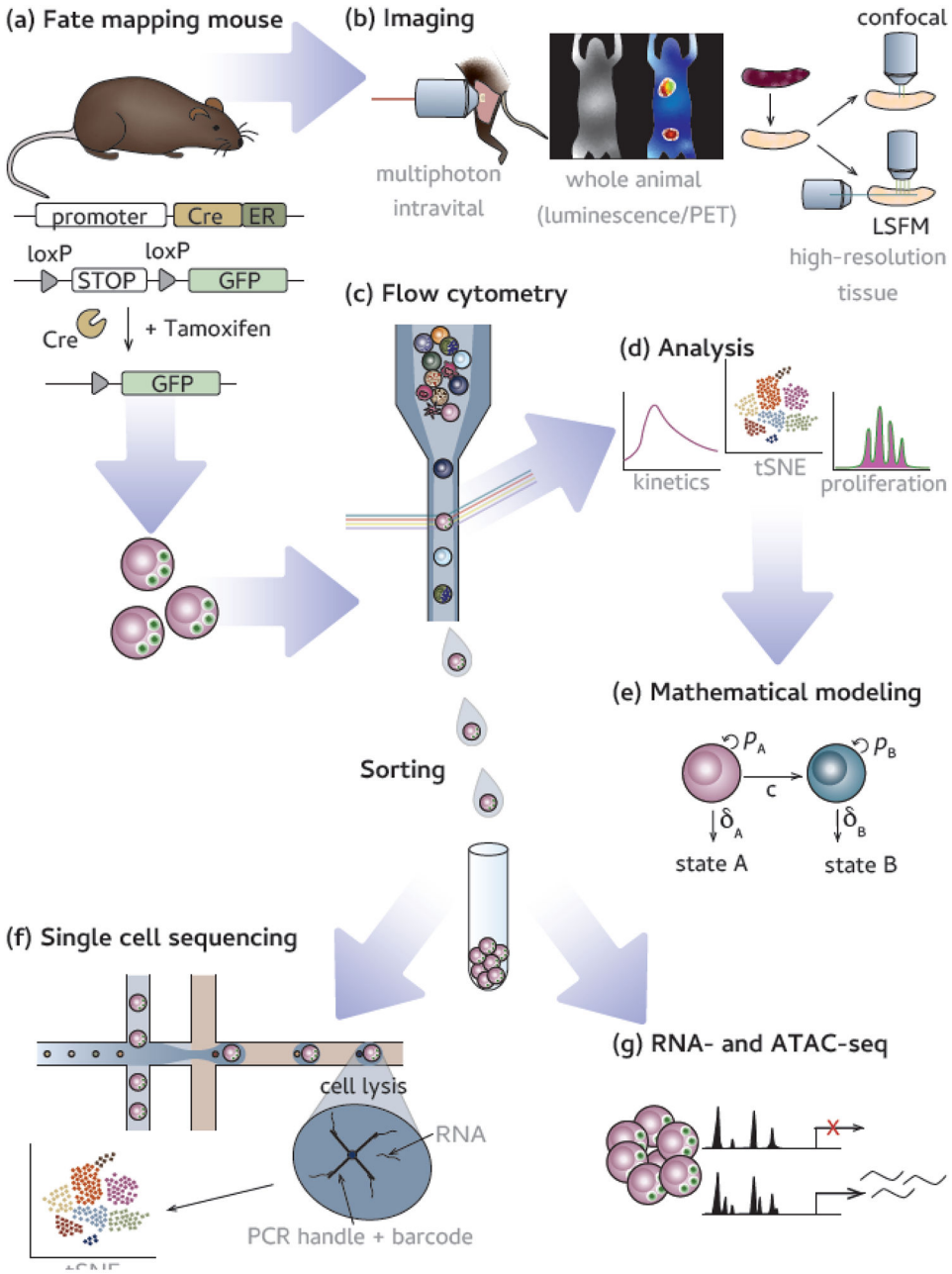

Figure 3. Examples of experimental techniques that are compatible with fate-mapping mice.

(a) Fate-mapping begins with the temporal expression of a reporter allows for fate-mapping. Following fate-mapping, researchers can use many tools to understand how cellular origin can dictate phenotype and function. (b) Imaging technologies are a powerful collection of tools to assess in situ localization and interactions with other cell types. (left) Multiphoton imaging is used to perform intravital imaging deep into animal tissues of interest [121] (an example is live imaging of cellular interactions and dynamics in lymph nodes); (middle) whole animal imaging, either by luminescence or positron emission tomography (PET) modalities, allows for non-invasive longitudinal study; (right) advances in tissue clearance (e.g. 3DISCO) [122] improve high resolution microscopy of tissues with techniques such

as confocal and light sheet fluorescent microscopy (LSFM). (c) Flow cytometry uses laser excitation to measure fluorescence of labeled markers on cells. (d) Traditional and high parameter analyses can be performed via flow cytometry data. (e) Mathematical modeling uses accumulated data to model cellular behavior. Flow cytometry can also sort cells, based on specific marker expression, for a variety of downstream technologies such as (f) single cell sequencing analysis in which an individual cell's transcripts are associated with a unique barcode and (e) bulk RNA and ATAC sequencing in which cellular populations are sequences for transcriptional (RNA) or chromatin accessibility (ATAC) information [94,129].