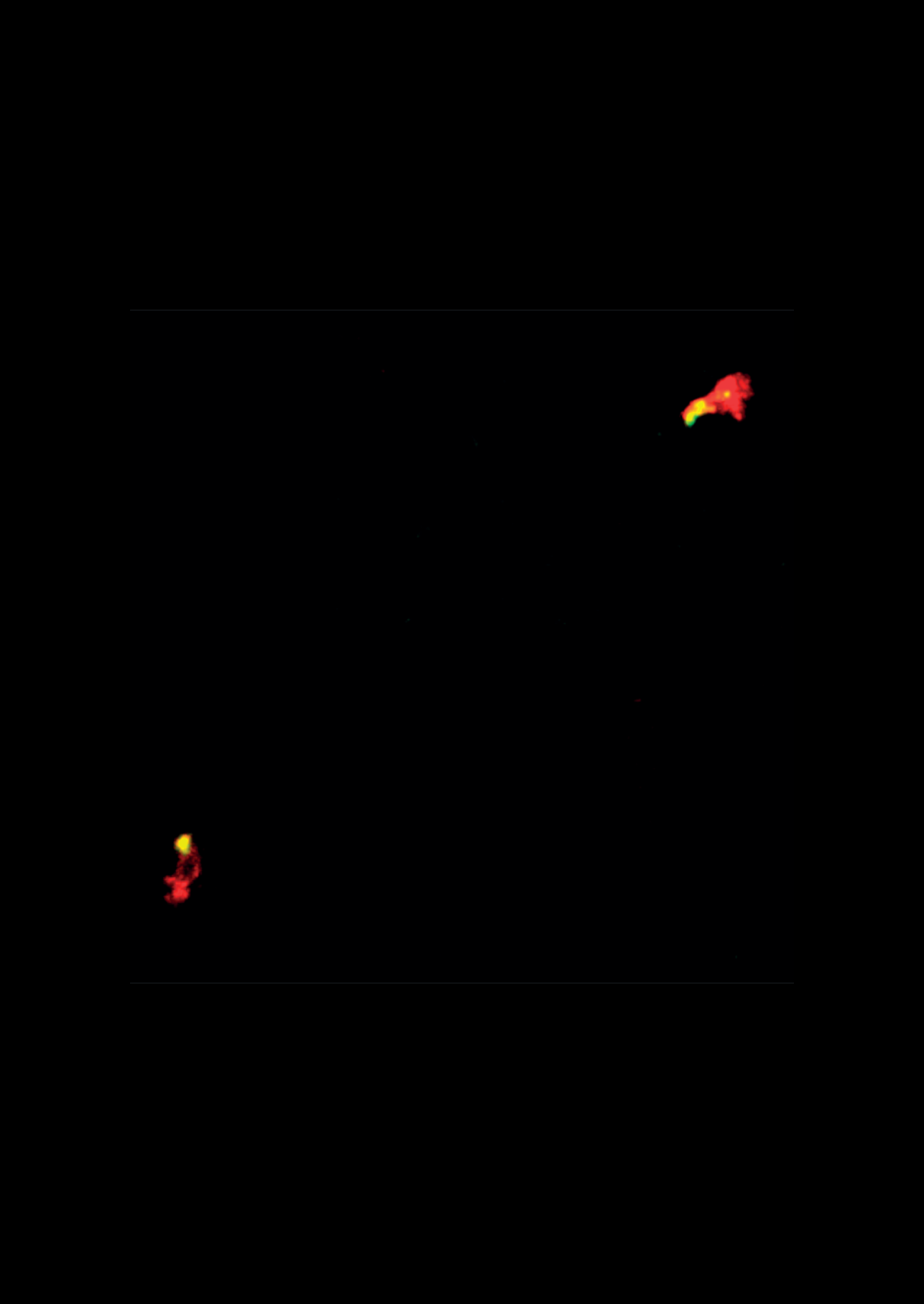

# Chapter 2

## Assessing T lymphocyte function and differentiation by genetically encoded reporter systems

Mirjam E Hoekstra[1], Feline E Dijkgraaf[1], Ton N Schumacher[1,#] and Jan C Rohr[2,#]

*Trends in immunology*, 2015. Jul;36(7):392-400. doi: 10.1016/j.it.2015.05.008

[1] Division of Immunology, The Netherlands Cancer Institute, Amsterdam, The Netherlands

[2] Center for Chronic Immunodeficiency (CCI), University Medical Center Freiburg and University of Freiburg, Freiburg, Germany

# To whom correspondence should be addressed: t.schumacher@nki.nl, jan.rohr@uniklinik-freiburg.de

## ABSTRACT

Upon infection, antigen-specific T lymphocytes become activated, proliferate, differentiate, and acquire various effector functions. Much of our understanding of the molecular mechanisms underlying these processes derives from studies leveraging gene deletion, RNAi, and overexpression approaches. However, these perturbations do not inform on the regulation of gene activity under physiological conditions. Genetic reporter systems that couple biological events to detectable output signals are capable of providing this information. Here, we review the reporter approaches being currently used to investigate various aspects of T cell behavior, and discuss advantages and disadvantages inherent to different designs. We outline emerging applications based on recent advances in other fields, and highlight the potential of synthetic biology and genome engineering to address open questions in the field.

## TOOLS TO STUDY THE LIVES OF T CELLS

T cells have a central role in the elimination of infectious microorganisms and malignant cells. Upon recognition of cognate antigen, T cells become activated, proliferate, and differentiate. Subsequently, a part of the activated T cell population acquires the capacity to travel to sites of infection, where these cells exert effector functions, such as cytokine production and target cell lysis. Following pathogen elimination, most activated T cells undergo apoptosis, leaving behind a small proportion of memory cells that provide rapid protection upon re-encounter of the same pathogen [1]. To study the mechanisms underlying these processes, the expression of a large number of genes has been modulated by knockout, knockdown, and transgenic overexpression. While these approaches have been useful to identify the function of different genes, by themselves they do not reveal whether physiological regulation of their activity in any way controls T cell fate or function. By analogy, a Martian examining regulation of automobile activity by disruption of different functions would rightfully come to the conclusion that both wheels and gas pedal fulfill essential functions, but would fail to distinguish their different roles, as either core components or a critical regulator. To allow such a distinction between core components and regulators within T cell biology, approaches have been developed that probe gene activity and cellular functions in their natural context, through the use of reporter systems.

Here, we provide a comprehensive technical overview of available genetically encoded reporters. We discuss general design principles of reporter systems in live cells, review the application of distinct reporter approaches *in vivo*, as well as their respective advantages and disadvantages, to address outstanding questions in T cell biology. With this aim, we provide guidance with regard to the planning and interpretation of experiments involving genetically encoded reporter systems to probe T cell function. Although not being without limitations, with careful methodological consideration and experimental design, genetically encoded reporters are powerful tools to investigate T lymphocyte biology in an almost unperturbed way. Furthermore, the astonishing progress in synthetic biology and genome engineering will provide scientists with exciting new possibilities to interrogate cellular differentiation and functions in years to come.

## DESIGN PRINCIPLES OF REPORTER SYSTEMS

The variety of available genetically encoded reporter systems can roughly be divided into two main categories: reporters that visualize what a T cell produces, or produced at a given point in time (i.e., gene-expression reporters), and reporters that visualize the signals that a T cell has received (i.e., signaling reporters).

**Glossary**

**Autofluorescence**: inherent fluorescence emission of biological structures, such as melanin and hemoglobin, upon exposure to light; may spectrally overlap with fluorescence of reporter FPs, thereby limiting sensitivity.
**Fluorescent half-life**: time required for a fluorophore to halve its fluorescent intensity after cessation of excitation.
**Förster resonance energy transfer (FRET)-based probes**: reporter systems that rely on energy transfer between two FPs (a donor and acceptor). Upon excitation of the donor molecule, it emits light of a wavelength that excites the acceptor molecule, which then emits light of a different wavelength. FRET only occurs with appreciable efficiency when both FPs are in close proximity (<10 nm) and, thus, can be used to report on the distance between two molecules.
**Maturation time**: time required to form the fluorophore of an FP upon post-translational protein folding; determines the maximum speed with which an expression-based fluorescent reporter can signal.
**Photoactivation**: the process of activating a substance by means of light.
**Photobleaching**: fluorophore destruction as a consequence of light exposure.
**Photoconversion**: a (permanent) change in fluorescence excitation and emission spectra of a protein by exposure to light of a specific wavelength.
**Phototoxicity**: cellular damage due to light exposure.
**Split FP**: a FP comprising two separate proteins, which by themselves are nonfluorescent, yet form a FP complex upon interaction with each other.

## Design principles of gene-expression reporters

Gene-expression reporters can serve to identify cells expressing a certain gene of interest (GOI) as well as to monitor the kinetics of gene expression. In such gene-expression reporters, a reporter protein is generally expressed under the same transcriptional control elements (promoter, enhancers, etc.) as the gene of interest, thereby aiming to mirror expression of the GOI. Such reporters can be created by inserting a reporter gene flanked by transcriptional control elements of the GOI randomly into the genome (**Box 1**). Alternatively, expression reporters can be created by targeted mutagenesis of the endogenous locus of the GOI, to insert a gene fragment that leads to multicistronic (**Box 2**) expression of the reporter gene and the GOI (**Box 1**). It is important to keep in mind that neither type of reporter system reports on the production and functionality of the protein of interest, but instead both mirror the transcription of the encoding gene. In an alternative expression reporter format, the reporter protein is produced as a single fusion protein with the protein encoded by the GOI. Besides better reflecting protein production, such reporter-fusion proteins allow one to study protein localization and trafficking. As a downside, the physical linkage of reporter and target protein may alter the function or degradation kinetics of the target protein and, thus, by itself induce experimental artifacts [2].

## Design principles of signaling reporters

To identify cells receiving a certain signal or to monitor the activity of the ensuing intracellular signaling cascade, four general types of signaling reporter have been designed (**Figure**

**Box 1 |** Approaches to generate transgenic reporter animals

Transgenic reporter animals can be generated by conventional transgenesis, by BAC transgenesis, or by targeted mutagenesis. In the first two approaches, either a small reporter construct containing defined transcriptional control elements (conventional transgenesis) or a large BAC containing the reporter and the entire locus of the GOI, is injected into germ cell pronuclei, leading to random integration into the genome. Subsequently, these germ cells are injected in pseudopregnant animals, leading to the generation of transgenic pups. Transgenesis is relatively easy and quick to perform. However, the chromosomal position of the integrated transgene and/or BAC influences reporter expression levels and patterns, necessitating the screening of many mice to identify an optimal founder animal for strain generation [99].

In conventional transgenesis, reporter constructs are often based on minimal promoters to drive reporter expression and, thus, do not always quantitatively mirror GOI expression. BAC transgenesis does allow GOI-mirrored reporter expression patterns, since (almost) all the transcriptional regulatory elements are present. However, BAC transgenesis may lead to the undesired co-integration of additional genes located in the large BAC constructs, which can result in confounding phenotypes [100]. Alternatively, reporter genes can be directly targeted into specific endogenous gene loci through homologous recombination. This may either involve insertion into a locus that is not subjected to gene-silencing effects and is relatively easy to target (e.g., *Rosa26* locus) [101], or may involve insertion into the locus of the GOI. A major advantage of the latter is that reporter expression is subject to the same physiological transcriptional control elements as the GOI, thus mirroring target gene expression with regard to timing and cell type specificity. As a downside, reporter introduction can influence the expression of the GOI, for instance by altering its mRNA half-life. In mice, the *Rosa26* locus has become a preferred site for the integration of transgenes and various reporter constructs, because it supports strong, ubiquitous expression of inserted sequences and it is not prone to gene silencing [101].

**Box 2 |** Multicistronic reporter genes

Multicistronic reporter genes allow the synthesis of several individual proteins from a single mRNA. The most commonly used multi-cistronic constructs utilized virus-derived internal ribosome entry site (IRES) sequences, which allow translational initiation within an mRNA sequence, thereby enabling the separate production of two proteins. However, IRES-mediated translation occurs in a 50-cap-independent manner, which is less efficient than 50-cap-dependent protein synthesis [102]. Thus, open reading frames (ORFs) that initiate at the canonical start site and downstream of the IRES sequence are not expressed at an equimolar level, and this can limit the sensitivity of IRES-based reporters. As an alternative to IRES sequences, reporter and target genes can be linked by 2A peptides. These peptides induce the production of two proteins from a single ORF, by inducing a ribosomal skip. 2A-based systems yield equimolar expression levels of both proteins. However, within this system, a short peptide remains attached to the C terminus of the upstream protein, while a proline remains attached to the N terminus of the downstream protein. Depending on the system studied, these additional amino acids may affect protein folding and function [103].

**1**): (i) a reporter gene can be introduced into the locus of an endogenous gene whose expression is upregulated upon stimulation of a particular receptor (i.e., reception of signal A drives expression of the (reporter) gene B). Technically, such reporters use the design of gene-expression reporters, with the main difference that, in the case of signaling reporters, the gene expression observed reflects the activation of a particular signaling pathway; (ii) a potential caveat of the first design principle is that the endogenous loci of many genes

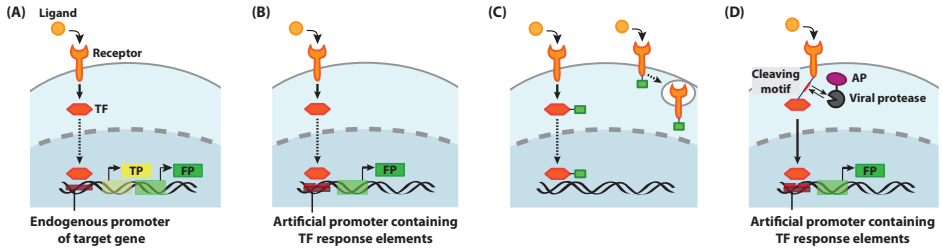


**Figure 1 | Design principles of signaling reporters.** Triggering of cell surface receptors initiates an intracellular signaling cascade that ultimately leads to transcription of target genes. Such signaling events can be detected using different approaches: (**A**) Receptor-triggering can be monitored by placing a reporter sequence under control of the promoter of a target gene that is known to be activated upon ligation of the receptor of interest. (**B**) Alternatively, signaling events can be visualized by reporter expression under control of an artificial promoter comprising responsive elements for a transcription factor that becomes activated as part of a particular signaling pathway. (**C**) For rapid detection of receptor-triggering, either the translocation of signal-specific transcription factors from the cytosol to the nucleus or the internalization of the receptor from the plasma membrane can be monitored. (**D**) If the intracellular signaling cascade or target genes of a receptor are unknown or redundant among multiple receptors, receptor-triggering can be monitored by using synthetic signaling pathways. In one such system, ligand binding to a hybrid cell surface receptor recruits a synthetic adaptor protein with protease activity, which then cleaves off a covalently bound transcription factor from the receptor. Liberation of this transcription factor allows its translocation to the nucleus, where it stimulates reporter gene activity. Abbreviations: AP, adaptor protein; FP, fluorescent protein; TF, transcription factor; TP, target protein.

contain multiple transcriptional control elements (i.e., enhancers, silencers, or binding sites for different transcription factors) that are regulated by distinct signaling pathways. By definition, the existence of such multiple levels of control will negatively affect the specificity of the reporter system for a particular signal. In such cases, the specificity of reporter systems may be improved by placing a reporter gene under control of an artificial promoter containing only binding sites for a single transcription factor (TF) that is selectively activated by the receptor for a signal of interest. In this way, reporter expression can be specifically tailored to a particular receptor or signaling pathway; (iii) given the time span required for transcription and translation of the reporter protein, the genetic reporter systems outlined above exhibit a considerable delay between input and output signals. More rapid reporting on signals can, for example, be achieved by using the intracellular translocation of stably expressed reporter proteins as a readout. To this end, reporters may be expressed as fusion proteins with either the receptor of interest or a specific transcription factor. Thus, such fusion proteins enable monitoring of ligand-triggered internalization of cell surface receptors or the translocation of transcription factors into the nucleus, respectively. Alternatively, Förster resonance energy transfer (FRET) based systems (see **Glossary**) can be used for the rapid detection of receptor-triggering [3]; for example, when a donor fluorescent protein (FP) is fused to the receptor and an acceptor FP to a receptor-specific adaptor molecule. While these systems report much faster than the transcription- and/or translation-based reporters, their output signal will

usually be weaker since no signal amplification occurs; (iv) If downstream signaling pathways or target genes of a particular receptor are shared with other receptors or are unknown, the receptor design principles outlined above will not be suitable. This issue may be overcome by coupling the receptor of interest to a synthetic and, hence, private signaling cascade that drives reporter expression. An intriguing example of this class of reporter systems is formed by the TANGO system, which uses ligand-triggered cleavage of a bacterial transcription factor fused to the receptor of interest [4]. Once released, this transcription factor then drives expression of the reporter gene. This system has enabled the *in vivo* identification of cells receiving dopamine and sphingosine 1 phosphate signals [5,6].

## REPORTER PROTEIN CONSIDERATIONS

### Reporter types

The most widely used type of reporter proteins in immunological studies is formed by FPs derived from genes originally isolated from, among others, corals, that have been engineered to optimize aspects such as expression level, brightness, and emission spectrum. FPs are often preferred over other reporter proteins (such as enzyme-based reporters; e.g., β-galactosidase) because of the availability of variants with many different spectra, with different maturation rates and half-lives, and because no exogenous co-factors are required for their function. In addition, variants of FPs, such as photoactivatable FPs [2] and split FPs [7], have been developed that allow further control over reporter activation. As a downside, FPs require exogenous illumination for their visualization, which may cause phototoxicity and photobleaching of the reporter as well as cellular autofluorescence. An additional limitation applying to all reporter proteins is their potential immunogenicity when used *in vivo* (**Box 3**).

**Box 3 |** Immunogenicity

Since reporter proteins are applied in species other than those in which they are physiologically expressed, they may be regarded as 'foreign' by the immune system and thereby induce rejection of reporter-expressing cells. The immunogenicity of a given reporter protein depends on the major histocompatibility complex (MHC) repertoire of the host and, therefore, can vary considerably between mouse strains [104]. Over the past years, immunogenic properties of a small number of reporter proteins have been demonstrated, including the widely used eGFP and Dsred [105–107]. Reporter immunogenicity is most relevant in adoptive transfer experiments of reporter-producing cells into nonreporter-expressing hosts, but may in theory also occur in systems that use reporter gene expression under tightly controlled inducible promoters [108]. Although rarely reported, the potential immunogenicity of reporter proteins should be considered when planning experiments.

### Reporter signaling kinetics

Different biological systems display distinct signaling kinetics, and the choice of reporter should be tailored to the biological question being studied. For example, early activation

events after T cell receptor-triggering occur within seconds to minutes, requiring a rapid reporter 'on-time' in case one wants to understand the timing of the activating event. Vice versa, in case one wishes to study when a certain signaling event ceases, a rapid 'turn-off time' is essential. Finally, if one wants to determine whether a cell received a signal at some earlier point in time, or perhaps many cell generations ago, reporter systems with very slow turn-off times are required.

## Strategies to influence on-time and turn-off time

As mentioned above, one strategy to investigate the timing of rapid biological phenomena is formed by the monitoring of the translocation of a constitutively expressed reporter protein fused to the protein of interest. Alternatively, FRET-based probes also provide an immediate readout, as well as split FP when fused to interacting protein domains. For rapid reporting on gene expression, the *de novo* transcription of rapidly maturing fluorescent reporter proteins may be used, such as the yellow FP (YFP) variant Venus, which matures in approximately 7 min [8]. Analysis of the cessation of a particular biological function requires the turn-off time of the reporter to mirror the turn-off time of the protein of interest. To accomplish this, the lifetime of reporter proteins can be adjusted through incorporation of destabilization signals [9,10]. For example, addition of a PEST motif decreases the half-life of GFP from 5 days to 3 h [10]. An inherent disadvantage of this approach is that it also diminishes reporter signal intensity.

To achieve the continuous labeling of cells that persists after expression of the gene of interest has ceased, the use of reporter fusion proteins has also proven useful. For example, reporter proteins fused to histone-2B (H2B) proteins, have a substantially increased half-life compared with free GFP, and can be detectable for months [11,12]. However, when cells divide, dilution of the reporter protein leads to a reduction in signal, a property that can be used to selectively visualize quiescent cell populations [11,12]. In cases in which it is desirable to identify reporter activation in cells 'many generations ago', systems that create permanent, heritable alterations are required. Such systems typically use inducible genetic recombination, leading to persistent reporter activation. This can be accomplished, for instance, by inducing a Cre-recombinase through a signal of interest, which then excises a floxed stop-cassette upstream of a reporter sequence [13]. Furthermore, with the development of clustered regularly interspaced short palindromic repeats (CRISPR) technology, many alternative strategies may be pursued (**Box 4**; see also Concluding remarks).

If it is desired to distinguish between cells that are actively expressing a GOI and cells that have previously expressed the GOI, classical gene-expression reporters and Cre-recombinase-based reporters can be combined; for example, by simultaneous expression of a FP and a Cre- recombinase in the locus of a GOI. While the FP serves as a reporter for ongoing GOI expression, the Cre-recombinase can mediate the excision of a stop cassette upstream of a second FP. The latter can then be used to visualize cells that expressed

the GOI at any point in time. Alternatively, 'fluorescent timers' can be used as reporters to discriminate between cells with ongoing or prior expression of a GOI. These FPs spontaneously change their emission spectrum over time, with conversion rates varying from several minutes up to days, depending on the fluorophore, oxygen concentration, and temperature [14].

**Box 4 |** CRISPR technology

Over the past few years, CRISPR-associated technology has become an extraordinarily powerful tool for genetic engineering. In its most commonly used form, expression of Cas9 and a short guide RNA (sgRNA) homologous to a specific genomic sequence of interest is used to create double-strand breaks (DSB) at the intended locus. Exploiting the relatively error-prone nonhomologous end joining mechanism (NHEJ), this may allow inactivation of a locus of interest by introduction of random mutations. Alternatively, by providing a DNA template homologous to the sequence flanking the DSB, homology-directed repair (HDR) can occur instead of NHEJ, thereby allowing the targeted introduction of desired genetic alterations, such as specific point mutations, short DNA sequences (such as LoxP-sites), and even larger DNA elements, such as, for example, reporter genes.

Compared with conventional gene targeting approaches, CRISPR technology is simple, versatile, and efficient. Furthermore, by co-introduction of different guide RNAs, multiple genomic loci can be targeted simultaneously. CRISPR-mediated genome engineering can be performed in zygotes and, thus, does not require embryonic stem cell technology, which considerably accelerates the generation of transgenic reporter animals.

An addition to serving as a genome-engineering tool to introduce reporter genes into the genome, CRISPRs may also be used as an active component of a reporter system, for example by fusing endonuclease-inactive Cas9 variants to other proteins, such as transcriptional activators and/or repressors or chromatin-modifying enzymes [109,110].

## Additional levels of control

If one desires to study GOI expression only when a cell is at a given site, or if one wants to monitor whether a signal is received within a given time window, it is desirable to incorporate external spatial or temporal control within the reporter system. Such control can be achieved by putting reporter systems in a 'reporting state' via administration of exogenous substances: for example, tetracycline-inducible systems are based on recombinant transcription factors that initiate (tet-on) or terminate (tet-off) transcription upon binding to tetracycline, thus allowing for reversible temporal control of expression of reporter components [15,16]. To obtain inducible and irreversible control of reporter expression, the Cre-Lox system can be modified by fusing the Cre-recombinase to a steroid receptor (e.g., estrogen receptor T2 (ERT2)), which causes Cre to remain inactive until the receptor ligand (e.g., tamoxifen) becomes available.

Finally, light-induced activation or photoconversion of reporter proteins, such as Kaede, may be used to facilitate cell migration studies [17]. While Kaede-expressing cells constitutively emit green light upon excitation, this emission can be photoconverted to red fluorescence by exposure to violet light. This system has enabled the measurement of *in vivo*

immune cell trafficking by photoconversion of cells located in one inguinal lymph node and analysis of their migration to other lymphoid organs [18].

## THE USE OF GENETIC REPORTERS IN T CELL BIOLOGY

Upon activation, T cells diversify along multiple axes that influence their phenotype, effector functions, transcription factor expression profile, trafficking pattern, proliferative capacity, and their ability to survive long-term. Here, we discuss how genetic reporter systems have contributed to our understanding of these different aspects of 'the lives of T cells'.

### Investigating T cell activation

T cell activation requires the recognition of cognate antigen by the T cell receptor (TCR), which activates downstream signaling events within minutes after TCR ligation. Systems based on inducible expression of reporter genes generally do not have the temporal resolution to mirror such rapidly occurring events. However, as the TCR becomes internalized upon triggering, constitutively expressed TCR$\alpha$-GFP fusion proteins can be used to microscopically detect T cell activation at an early stage *in vivo* [19]. Alternatively, since intracellular $Ca^{2+}$ levels increase shortly after TCR triggering, calcium sensors can be used to rapidly report on T cell activation [20]. This has been accomplished using a FRET-based reporter encoding a fusion protein of cyan FP (CFP) and cpCitrine linked by the $Ca^{2+}$-binding protein troponin C. At low calcium concentrations, the conformation of troponin C spatially separates CFP and cpCitrine, preventing FRET. Increasing $Ca^{2+}$ levels induce troponin C to undergo a conformational change, resulting in FRET between the two proteins. This reporter system has been used to visualize encephalitogenic T cell activation *in vivo* [21].

A third type of reporter for early T cell activation utilizes the transcription factor nuclear factor of activated T cells (NFAT). Upon TCR triggering, NFAT rapidly translocates from the cytosol to the nucleus to activate expression of multiple genes [22]. This translocation can be visualized using NFAT-FP fusion proteins in combination with a constitutively marked nucleus (e.g., H2B-FP fusions). Adoptive transfer experiments using NFAT-YFP/H2B-mCherry T cells [23] or NFAT-EGFP/H2B-mRFP T cells [24] have revealed that T cell activation occurs in both stationary and moving effector T cells in central nervous system autoimmunity [23] and antitumor responses [24].

Exploiting the biological function of NFAT as a transcription factor, reporters have also been created that harbor a synthetic promoter containing multiple NFAT-binding sites followed by a reporter gene. In this setting, TCR-triggered NFAT translocation to the nucleus activates the reporter. While the on-rates of these reporters are generally too slow to study early TCR signaling events, they do enable the identification of antigen-specific T cells within a polyclonal T cell population when used in transgenic animals [25,26].

While available T cell activation reporters identify activated T cells, most of them do not measure the strength of the TCR signal perceived. A way to accomplish this is to report on the activation of a target gene that has distinct expression levels depending on TCR signal strength, such as Nr4a1 [encoding nuclear receptor 77 (Nur77)]. In bacterial artificial chromosome (BAC) transgenic mice expressing a GFP-Cre fusion protein from the *Nr4a1* locus, TCR signal strength was reflected by GFP expression levels [27]. This approach was used to demonstrate that regulatory T cells (Treg) and invariant natural killer T (iNKT) cells receive stronger TCR signals compared with conventional T cells during their development [27]. Furthermore, this system was also used to show that the expression of certain tumor necrosis factor receptor superfamily members directly correlates with TCR signal strength in Treg cell progenitors [28], and that naïve $CD8^+$ T cells with heightened self-reactivity display enhanced reactivity toward foreign antigens [29]. The Cre-recombinase included in the reporter was not used in these studies, but could be beneficial in future fate mapping experiments.

## INVESTIGATING T CELL SUBSET DIFFERENTIATION

T cells can differentiate into several different subsets that are characterized by the expression of different transcription factors. Such subsets are, for example, Th1, Th2, Th17, and Treg cells, whose prototypical transcription factors are T-bet, GATA-3, retinoid-acid receptor-related orphan receptor (ROR)-γt, and forkhead box protein (FoxP)-3, respectively. Given that these subset-specific transcription factors are located intracellularly, conventional identification of T cell subsets usually requires intracellular staining after cellular permeabilization, a procedure that is not compatible with subsequent cell tracing. However, linking the expression of these transcription factors to reporter genes has been demonstrated to overcome this obstacle.

To report on Th1 cells, a transgenic reporter mouse strain containing a ZsGreen reporter inserted into the *T-bet* locus has been generated. This reporter has been used to monitor the differentiation of $CD4^+$ T cells into Th1 cells *in vivo* and was instrumental in demonstrating that T-bet represses expression of Th2-related genes [30]. Similarly, GATA3-GPF [31] and RORγt-eGFP [32] reporter mice have been created, which have been used to study CD4 lineage differentiation into Th2 and Th17 subsets [33–38].

To study the *in vivo* formation and fate of Treg cells, numerous FoxP3 reporters have been described, including reporters in which FP expression is placed under the control of *Foxp3* regulatory elements [39], Foxp3-reporter fusion proteins [40], and bicistronic Foxp3/reporter expression cassettes [41–44]. For fate-mapping experiments and to investigate the degree of Treg lineage commitment, mouse strains that can discriminate between cells actively expressing FoxP3 and cells that have previously expressed Foxp3 have also been

generated. This has been accomplished by crossing mice carrying a YFP-Cre fusion protein [45], an eGFP-CRE-ERT2 fusion protein [46], or IRES-separated GFP and Cre [47] in the *Foxp3* locus, with a strain carrying a floxed stop-FP cassette. These reporters have been used to demonstrate that the Treg cell population shows remarkably stable lineage commitment under physiologic and inflammatory conditions.

In addition, strains expressing a diphtheria toxin receptor (DTR)-GFP fusion protein under the control of the Foxp3 promoter have been created, which not only enable the identification of Treg cells, but also their inducible deletion via administration of diphtheria toxin [48–50], a strategy that has been used to explore the role of many other cell types [51,52]. For a more detailed and comprehensive overview of Treg cell reporters, we refer the reader to the excellent review by Jeremiah and Liston [53].

## Monitoring T cell expansion and death

T cell expansion reflects the net value of proliferation and apoptosis, because some T cells may still proliferate while others already undergo apoptosis. Analysis of the contribution of both processes to T cell expansion observed is made possible by several types of reporter system.

T cell proliferation can, for example, be monitored using reporter proteins whose half-lives are substantially longer than the timeframe in which proliferation occurs. If such proteins are expressed in a cell before, but not during, division, half of the reporter proteins will be inherited by each daughter cell, leading to halving of reporter intensity upon each cell division. This approach has been utilized in a mouse model in which cells can be induced to express a highly stable H2B-GFP fusion by doxycycline administration. Upon withdrawal of doxycycline, flow cytometric analysis of H2B-GFP expression levels is used to determine the number of divisions a cell has undergone [12]. While this inducible H2B-GFP system has not yet been used to investigate clonal expansion of mature T cells, a similar approach has been used to monitor proliferation of T cells during thymic development, in this case using a Tcrd-H2Be-GFP knock-in reporter strain [54]. In these mice, an expression cassette encoding IRES-H2B-GFP was introduced in the 3' untranslated region (UTR) of the *Tcrd* locus, which is transcribed during early T cell development but excised during TCR$\alpha$-chain VJ-recombination. Consequently, proliferation of T cells directly after positive selection could be monitored. Using this setup, it was shown that $CD4^+$ and $CD8^+$ T cells hardly divide following positive selection, whereas iNKT cells undergo a phase of intense proliferation [54]. A major advantage of the H2B-GFP system over conventional methods to measure cell proliferation, such as carboxyfluorescein succinimidyl ester (CFSE) labeling, is that this system can report on the number of divisions a cell has undergone without a requirement for prior in vitro labeling and adoptive transfer of cells.

Alternatively, proliferation-associated cell cycle progression can be studied using the fluorescent ubiquitination-based cell cycle indicator (Fucci) reporter system. In this system,

green and red FPs are fused to protein domains that cause rapid degradation at different stages of the cell cycle, leading to oscillation of red and green signals in dividing cells [55]. Fucci transgenic mice have recently been used to show how signal integration by TCR, co-stimulatory receptors, and cytokines influences the kinetics and phenotypic diversification of T cell responses upon infection [56,57].

The direct detection of T cell death is complicated by the rapid clearance of apoptotic cells *in vivo* [58]. Thus, reporter activation has to occur early during the apoptotic cascade. An early event in the apoptotic cascade is the activation of caspase-3, and the proteolytic activity of this caspase has successfully been used to detect apoptotic cells [59]. To this end, a FRET-based reporter was created that comprises CFP and YFP linked by a caspase-3 cleavage sequence. Upon caspase-3-mediated cleavage of the peptide linker, FRET between the two FPs is lost. This approach has enabled the direct identification of apoptotic cells *in vivo*, and has been used to show that contraction of the antigen-specific T cell response is regulated by antigen availability [59]. However, also with this reporter, the detection of apoptotic cells is limited to the relatively short time interval between onset of apoptosis and clearance of the cellular remnants, thereby providing a likely underestimate of the extent of apoptosis.

## Monitoring T cell effector functions

### Cytokine reporters

Activated T cells can functionally diversify through acquisition of multiple different cytokine expression profiles, and analysis of cytokine expression constitutes the area within T cell research in which reporter systems have been used most extensively. To date, more than 20 different mouse strains that report on expression of cytokines, such as interleukin (IL)-2, IL-4, IL-7, IL-9, IL-10, IL-17, IL-22, interferon (IFN)-β, IFNγ and transforming growth factor (TGF)-β have been generated [60–75]. In an ideal case, activity of these expression reporters reflects the levels of cytokine produced. However, many cytokine mRNAs are tightly regulated and short lived, and, therefore, it can be difficult to achieve detectable levels of reporter output with systems that are under control of all physiological regulatory elements. To increase the strength of the reporter signal, many cytokine reporters contain mRNA-stabilizing polyadenylation sequences, such as the bovine growth hormone (bgh) polyA or SV40 polyA sequences. For example, the bicistronic YFP-enhanced transcript for IFNγ (Yeti) knock-in transgenic mouse [73,76] carries a bgh polyA signal in the 3' UTR of the *Ifng* gene to delay reporter mRNA degradation. However, since the reporter gene is transcribed from the same mRNA as the endogenous *Ifng* gene, stabilization of the reporter mRNA also influences the expression pattern of IFNγ. This problem is most profound in homozygous Yeti mice, which die prematurely due to elevated IFNγ levels [77], but T cells from heterozygous Yeti mice also show increased IFNγ production upon activation compared with wild type T cells [78]. To circumvent these problems associated with exogenous polyA signals, improved cytokine

reporters containing endogenous 3' UTRs have been generated, such as the 'GREAT' mice that also report on IFNγ expression [74].

### Cytotoxicity reporters

Cytotoxic T cells (CTLs) kill target cells by releasing toxic molecules, such as granzyme B (GrzB), and several gene-expression reporters for GrzB have been developed to mark CTLs [79,80]. A more direct appreciation of the cytolytic function requires cytotoxin-reporter fusion molecules, which enable the visualization of the cellular distribution and excretion of cytotoxins. In this case, the choice of fluorochrome requires particular consideration since the low pH in cytotoxin-containing granules can profoundly decrease fluorescence intensity. While yellow and green FPs are particularly sensitive to low pH, many blue, red, and far-red variants are less pH dependent and, thus, can be used for this purpose [81]. Recently, a transgenic mouse expressing a GrzB-tdTomato fusion protein was developed (GZMB-Tom-KI) that enabled the analysis of the distribution of GrzB-containing vesicles at different steps of CTL–target cell interactions [82]. Alternatively, combinations of pH-stable and pH-sensitive FP-cytotoxin-fusion proteins (which only become fluorescent once lytic granules fuse with the plasma membrane) have been used in in vitro studies to trace lytic granules continuously throughout exocytosis [83–85].

## Single cell fate-mapping reporters

Most of the available reporter systems can only be utilized to track the behavior of populations of cells. To understand cell fate at the single cell level, approaches such as cellular barcoding and allotype markers have been exploited to demonstrate, for instance, that naïve T cell activation is highly efficient [86] and to demonstrate that individual naïve T cells are extremely heterogeneous in the amount and type of offspring that they produce [87,88]. Similar approaches, based on the combinatorial expression of multiple FPs, enabling one to label individual cells with distinctive color combinations, have also been developed. The first of these, which received considerable attention under the names 'brainbow' and 'confetti', utilizes Cre-Lox recombination to stochastically induce stable expression of one out of two to four FPs localized in the reporter cassette [89,90]. Furthermore, integration of multiple transgene copies allows for the combinatorial expression of these FPs, creating almost 100 hues that can be distinguished by microscopy. These approaches have been used to visualize individual neurons in the brain [89], for lineage tracing of intestinal stem cells [90], and for fate mapping of skin-resident Langerhans cells [91] and lymph node follicular dendritic cells [92].

A second strategy to achieve multicolored cell populations uses lentiviral gene ontology (LeGO) vectors encoding red, green, or blue FP [93,94]. Simultaneous transduction with these three vectors provides individual cells with a stochastic and heritable combination of different FPs [93,94]. This approach, named red-green-blue (RGB) marking, has been

used to track the differentiation of hematopoietic stem cells *in vivo* [93]. To our knowledge, neither of the two FP-based methods described above have been used to track individual T lymphocytes and their offspring, and development of non-invasive technology to follow the fate of many individual cells in parallel remains an important priority.

### Investigating cell memory formation

Genetic reporters have been used to investigate the formation of T cell memory, particularly regarding the question of whether memory T cells are derived from effector cells or directly from naïve T cells, without prior acquisition of effector functions. To this end, reporters that link the expression of a gene expressed at a specific T cell differentiation state (e.g., effector phase) to continuous reporter gene expression have been designed. This principle has been used in a transgenic mouse carrying a human *GRZB* promoter-controlled Cre-recombinase. Expression of GRZB drives Cre-mediated excision of a floxed stop-cassette that blocks the expressing of a placental alkaline phosphatase (PLAP) reporter gene [79]. In this system, both cells actively expressing GRZB and cells that have previously expressed GrzB are stably marked by PLAP expression. This approach has been used to demonstrate that memory cells can derive from GrzB-expressing (effector) T cells [79]. These observations were confirmed in a second transgenic reporter system in which an inducible Cre-recombinase was controlled by the murine *GrzB* locus [80]. Similar approaches have been taken to investigate whether IFNγ-producing T cells can become memory cells. To this purpose, two mouse models were generated, in which either the first exon of the *Ifng* gene was replaced by a *Thy1.1* (CD90.1) reporter gene, or in which an IRES-Thy1.1-construct was knocked into the endogenous *Ifng* locus [95]. Using these mice, it was demonstrated that IFNγ-expressing T cells can also differentiate into memory cells.

To understand which factors may determine whether individual T cells survive long term, a mouse strain has been generated that reports on the expression of the anti-apoptotic molecule B cell lymphoma 2 (Bcl-2) [96]. In this strain, a *YFP* gene was inserted into the translation initiation site of the *Bcl2* locus. Using this model, a subset of effector $CD8^+$ T cells was found to express high levels of Bcl-2, which correlated with the ability of those cells to persist long term [96].

## CONCLUDING REMARKS

Genetically encoded reporter systems have proven to be highly versatile tools to monitor the activation, function, and differentiation of T cells during immune responses. However, several aspects in (T cell) biology have been difficult to investigate with currently available reporters. For example, to date, it is difficult to translate the strength of a signal that a T cell has received into a heritable output signal. Specifically, most published reporter systems

have used digital output signals (on/off). However, because for many biological systems (i.e., stress responses) gene expression levels are tuned based on the strength of an input signal, reporter systems providing graded or analog output signals proportional to the intensity of the input signal would be desirable. Studies in yeast have revealed that analog reporter output can be achieved through the concerted action of (i) the low affinity of a given transcription factor to its binding site; (ii) high competition among transcription factor binding sites across the genome; and (iii) low cooperativity of transcription factor binding [97]. While to our knowledge, this has not been translated to reporter systems used in T lymphocytes, it paves the way for novel reporter systems yielding graded output signals. Mirroring such an analog input signal by a graded heritable output signal (i.e., 'analog memory') will be even more challenging [98].

A second aspect in T cell biology that has been difficult to study thus far is the effect of T cell-secreted factors on neighboring cells *in vivo*. Such systems would be of value by complementing our understanding of T cell activity under different conditions to the effects of these activities on the surrounding tissue. Development of such reporters is challenging, since the downstream receptor-signaling pathways of most T cell secreted factors are (or are currently thought to be) triggered by several factors. Conceivably, the use of reporter systems that use private, synthetic signaling pathways, such as the TANGO system, will provide inroads to address questions along these lines. Alternatively, application of Boolean logic through 'multiplexed genome engineering' could be an option to further increase specificity of a reporter system for a signal interest. For instance, if only a combination of two gene transcripts is sufficiently specific for a signal, an 'AND' strategy could be designed, in which concomitant expression of red and green FPs inserted in the two respective loci is used as a readout. By the same token, 'NOT' strategies may be designed, in which a signal of interest is revealed by transcription of FP 1 but not 2. Such multiplexed signaling reporters may not only be used to reveal the influence of T cells on their surroundings, but also be of significant use in other biological systems that are based on cellular crosstalk.

## REFERENCES


1. Murphy, K. et al. (2008) *Janeway's Immunobiology*. (7th edn), Garland Science
2. Chudakov, D.M. et al. (2010) Fluorescent proteins and their applications in imaging living cells and tissues. *Physiol. Rev.* 90, 1103–1163
3. Ibraheem, A. and Campbell, R.E. (2010) Designs and applications of fluorescent protein-based biosensors. *Curr. Opin. Chem. Biol.* 14, 30–36
4. Barnea, G. et al. (2008) The genetic design of signaling cascades to record receptor activation. *Proc. Natl. Acad. Sci. U.S.A.* 105, 64–69
5. Inagaki, H.K. et al. (2012) Visualizing neuromodulation in vivo: TANGO-mapping of dopamine signaling reveals appetite control of sugar sensing. *Cell* 148, 583–595
6. Kono, M. et al. (2014) Sphingosine-1-phosphate receptor 1 reporter mice reveal receptor activation sites in vivo. *J. Clin. Invest.* 124, 2076–2086
7. Kerppola, T.K. (2008) Bimolecular fluorescence complementation (BiFC) analysis as a probe of protein interactions in living cells. *Annu. Rev. Biophys.* 37, 465–487
8. Yu, J. et al. (2006) Probing gene expression in live cells, one protein molecule at a time. *Science* 311, 1600–1603
9. Lemay, N. et al. (2008) The role of the tight-turn, broken hydrogen bonding, Glu222 and Arg96 in the post-translational green fluorescent protein chromophore formation. *Chem. Phys.* 348, 152–160
10. Li, X. et al. (1998) Generation of destabilized green fluorescent protein as a transcription reporter. *J. Biol. Chem.* 273, 34970–34975
11. Tumbar, T. et al. (2004) Defining the epithelial stem cell niche in skin. *Science* 303, 359–363
12. Foudi, A. et al. (2009) Analysis of histone 2B-GFP retention reveals slowly cycling hematopoietic stem cells. *Nat. Biotechnol.* 27, 84–90
13. Sauer, B. (1998) Inducible gene targeting in mice using the Cre/lox system. *Methods* 14, 381–392
14. Subach, F.V. et al. (2009) Monomeric fluorescent timers that change color from blue to red report on cellular trafficking. *Nat. Chem. Biol.* 5, 118–126
15. Gossen, M. and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. U.S.A.* 89, 5547–5551
16. Gossen, M. and Bujard, H. (1995) Efficacy of tetracycline-controlled gene expression is influenced by cell type: commentary. *Biotechniques* 19, 213–216 discussion 216–217
17. Ando, R. et al. (2002) An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. *Proc. Natl. Acad. Sci. U.S.A.* 99, 12651–12656
18. Tomura, M. et al. (2008) Monitoring cellular movement in vivo with photoconvertible fluorescence protein "Kaede" transgenic mice. *Proc. Natl. Acad. Sci. U.S.A.* 105, 10871–10876
19. Friedman, R. et al. (2010) Real-time analysis of T cell receptors in naive cells in vitro and in vivo reveals flexibility in synapse and signaling dynamics. *J. Exp. Med.* 207, 2733–2749
20. Vig, M. and Kinet, J.P. (2009) Calcium signaling in immune cells. *Nat. Immunol.* 10, 21–27
21. Mues, M. et al. (2013) Real-time in vivo analysis of T cell activation in the central nervous system using a genetically encoded calcium indicator. *Nat. Med.* 19, 778–783
22. Macian, F. (2005) NFAT proteins: key regulators of T-cell development and function. *Nat. Rev. Immunol.* 5, 472–484
23. Lodygin, D. et al. (2013) A combination of fluorescent NFAT and H2B sensors uncovers dynamics of T cell activation in real time during CNS autoimmunity. *Nat. Med.* 19, 784–790

24. Marangoni, F. et al. (2013) The transcription factor NFAT exhibits signal memory during serial T cell interactions with antigen-presenting cells. *Immunity* 38, 237–249
25. Hooijberg, E. et al. (2000) NFAT-controlled expression of GFP permits visualization and isolation of antigen-stimulated primary human T cells. *Blood* 96, 459–466
26. Ponomarev, V. et al. (2001) Imaging TCR-dependent NFAT-mediated T-cell activation with positron emission tomography in vivo. *Neoplasia* 3, 480–488
27. Moran, A. et al. (2011) T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *J. Exp. Med.* 208, 1279–1289
28. Mahmud,S.A.etal.(2014)Costimulationviathetumor-necrosisfactor receptor superfamily couples TCR signal strength to the thymic differentiation of regulatory T cells. *Nat. Immunol.* 15, 473–481
29. Fulton, R.B. et al. (2015) The TCR's sensitivity to self peptide-MHC dictates the ability of naive CD8(+) T cells to respond to foreign antigens. *Nat. Immunol.* 16, 107–117
30. Zhu, J. et al. (2012) The transcription factor T-bet is induced by multiple pathways and prevents an endogenous Th2 cell program during Th1 cell responses. *Immunity* 37, 660–673
31. Grote, D. et al. (2006) Pax 2/8-regulated Gata 3 expression is necessary for morphogenesis and guidance of the nephric duct in the developing kidney. *Development* 133, 53–61
32. Eberl, G. et al. (2004) An essential function for the nuclear receptor RORgamma(t) in the generation of fetal lymphoid tissue inducer cells. *Nat. Immunol.* 5, 64–73
33. Gimferrer, I. et al. (2011) Regulation of GATA-3 expression during CD4 lineage differentiation. *J. Immunol.* 186, 3892–3898
34. Ivanov, I.I. et al. (2006) The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126, 1121–1133
35. Nowak, E.C. et al. (2009) IL-9 as a mediator of Th17-driven inflammatory disease. *J. Exp. Med.* 206, 1653–1660
36. Sharma, M.D. et al. (2009) Indoleamine 2,3-dioxygenase controls conversion of Foxp3+ Tregs to TH17-like cells in tumor-draining lymph nodes. *Blood* 113, 6102–6111
37. Shaw, M.H. et al. (2012) Microbiota-induced IL-1beta, but not IL-6, is critical for the development of steady-state TH17 cells in the intestine. *J. Exp. Med.* 209, 251–258
38. Chalmin, F. et al. (2012) Stat3 and Gfi-1 transcription factors control Th17 cell immunosuppressive activity via the regulation of ectonucleotidase expression. *Immunity* 36, 362–373
39. Kuczma, M. et al. (2009) Foxp3-deficient regulatory T cells do not revert into conventional effector CD4+ T cells but constitute a unique cell subset. *J. Immunol.* 183, 3731–3741
40. Fontenot, J.D. et al. (2005) Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22, 329–341
41. Wan, Y.Y. and Flavell, R.A. (2005) Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. *Proc. Natl. Acad. Sci. U.S.A.* 102, 5126–5131
42. Bettelli, E. et al. (2006) Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441, 235–238
43. Haribhai, D. et al. (2007) Regulatory T cells dynamically control the primary immune response to foreign antigen. *J. Immunol.* 178, 2961–2972
44. Wang, Y. et al. (2008) Th2 lymphoproliferative disorder of LatY136F mutant mice unfolds independently of TCR-MHC engagement and is insensitive to the action of Foxp3+ regulatory T cells. *J. Immunol.* 180, 1565–1575
45. Rubtsov, Y.P. et al. (2008) Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* 28, 546–558
46. Rubtsov, Y.P. et al. (2010) Stability of the regulatory T cell lineage in vivo. *Science* 329, 1667–1671

47. Zhou, X. et al. (2008) Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity. *J. Exp. Med.* 205, 1983–1991
48. Kim, J.M. et al. (2007) Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat. Immunol.* 8, 191–197
49. Lahl, K. et al. (2007) Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. *J. Exp. Med.* 204, 57–63
50. Feuerer, M. et al. (2009) How punctual ablation of regulatory T cells unleashes an autoimmune lesion within the pancreatic islets. *Immunity* 31, 654–664
51. Jung, S. et al. (2002) In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. *Immunity* 17, 211–220
52. Saito, M. et al. (2001) Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. *Nat. Biotechnol.* 19, 746–750
53. Jeremiah, N.M. and Liston, A. (2011) Genetic tools for analysis of FoxP3+ regulatory T cells in vivo. *Methods Mol. Biol.* 707, 105–118
54. Fohse, L. et al. (2013) Differential postselection proliferation dynamics of alphabeta T cells, Foxp3+ regulatory T cells, and invariant NKT cells monitored by genetic pulse labeling. *J. Immunol.* 191, 2384–2392
55. Sakaue-Sawano, A. et al. (2008) Visualizing spatiotemporal dynamics of multicellular cell-cycle progression. *Cell* 132, 487–498
56. Marchingo, J.M. et al. (2014) T cell signaling. Antigen affinity, costimulation, and cytokine inputs sum linearly to amplify T cell expansion. *Science* 346, 1123–1127
57. Kinjyo, I. et al. (2015) Real-time tracking of cell cycle progression during CD8+ effector and memory T-cell differentiation. *Nat. Commun.* 6, 6301
58. Bahl, K. et al. (2010) Analysis of apoptosis of memory T cells and dendritic cells during the early stages of viral infection or exposure to toll-like receptor agonists. *J. Virol.* 84, 4866–4877
59. Garrod, K.R. et al. (2012) Dissecting T cell contraction in vivo using a genetically encoded reporter of apoptosis. *Cell Rep.* 2, 1438–1447
60. Saparov, A. et al. (1999) Interleukin-2 expression by a subpopulation of primary T cells is linked to enhanced memory/effector function. *Immunity* 11, 271–280
61. Yui, M.A. et al. (2001) A new regulatory region of the IL-2 locus that confers position-independent transgene expression. *J. Immunol.* 166, 1730–1739
62. Amado, I.F. et al. (2013) IL-2 coordinates IL-2-producing and regulatory T cell interplay. *J. Exp. Med.* 210, 2707–2720
63. Kumar, S. et al. (2005) A cytokine promoter/yellow fluorescent protein reporter transgene serves as an early activation marker of lymphocyte subsets. *Cell. Immunol.* 237, 131–140
64. Mohrs, K. et al. (2005) A two-step process for cytokine production revealed by IL-4 dual-reporter mice. *Immunity* 23, 419–429
65. Repass, J.F. et al. (2009) IL7-hCD25 and IL7-Cre BAC transgenic mouse lines: new tools for analysis of IL-7 expressing cells. *Genesis* 47, 281–287
66. Shalapour, S. et al. (2010) Commensal microflora and interferon-gamma promote steady-state interleukin-7 production in vivo. *Eur. J. Immunol.* 40, 2391–2400
67. Gerlach, K. et al. (2014) TH9 cells that express the transcription factor PU.1 drive T cell-mediated colitis via IL-9 receptor signaling in intestinal epithelial cells. *Nat. Immunol.* 15, 676–686
68. Bouabe, H. (2012) Cytokine reporter mice: the special case of IL-10. Scand. *J. Immunol.* 75, 553–567

69. Croxford, A.L. et al. (2009) Cutting edge: an IL-17F-CreEYFP reporter mouse allows fate mapping of Th17 cells. *J. Immunol.* 182, 1237–1241
70. Lee, Y.K. et al. (2009) Late developmental plasticity in the T helper 17 lineage. *Immunity* 30, 92–107
71. Ahlfors, H. et al. (2014) IL-22 fate reporter reveals origin and control of IL-22 production in homeostasis and infection. *J. Immunol.* 193, 4602–4613
72. Scheu, S. et al. (2008) Visualization of IFNbeta production by plasmacytoid versus conventional dendritic cells under specific stimulation conditions in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 105, 20416–20421
73. Stetson, D. et al. (2003) Constitutive cytokine mRNAs mark natural killer (NK) and NK T cells poised for rapid effector function. *J. Exp. Med.* 198, 1069–1076
74. Reinhardt, R. et al. (2009) Cytokine-secreting follicular T cells shape the antibody repertoire. *Nat. Immunol.* 10, 385–393
75. Li, M.O. et al. (2007) T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. *Immunity* 26, 579–591
76. Matsuda, J.L. et al. (2003) Mouse V alpha 14i natural killer T cells are resistant to cytokine polarization in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 100, 8395–8400
77. Reinhardt, R.L. et al. (2015) A novel model for IFN-gamma-mediated autoinflammatory syndromes. *J. Immunol.* 194, 2358–2368
78. Beuneu, H. et al. (2010) Visualizing the functional diversification of CD8+ T cell responses in lymph nodes. *Immunity* 33, 412–423
79. Jacob, J. and Baltimore, D. (1999) Modelling T-cell memory by genetic marking of memory T cells in vivo. *Nature* 399, 593–597
80. Bannard, O. et al. (2009) Secondary replicative function of CD8+ T cells that had developed an effector phenotype. *Science* 323, 505–509
81. Chudakov, D. et al. (2010) Fluorescent proteins and their applications in imaging living cells and tissues. *Physiol. Rev.* 90, 1103–1163
82. Mouchacca, P. et al. (2013) Visualization of cytolytic T cell differentiation and granule exocytosis with T cells from mice expressing active fluorescent granzyme B. *PLoS ONE* 8, e67239
83. Bird, C. et al. (2010) Use of granzyme B-based fluorescent protein reporters to monitor granzyme distribution and granule integrity in live cells. *Biol. Chem.* 391, 999–1004
84. Martina, J. et al. (2011) Imaging of lytic granule exocytosis in CD8+ cytotoxic T lymphocytes reveals a modified form of full fusion. *Cell. Immunol.* 271, 267–279
85. Liu, D. et al. (2011) Two modes of lytic granule fusion during degranulation by natural killer cells. Immunol. *Cell Biol.* 89, 728–738
86. van Heijst, J.W. et al. (2009) Recruitment of antigen-specific CD8+ T cells in response to infection is markedly efficient. *Science* 325, 1265–1269
87. Gerlach, C. et al. (2013) Heterogeneous differentiation patterns of individual CD8+ T cells. *Science* 340, 635–639
88. Buchholz, V.R. et al. (2013) Disparate individual fates compose robust CD8+ T cell immunity. *Science* 340, 630–635
89. Livet, J. et al. (2007) Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. *Nature* 450, 56–62
90. Snippert, H.J. et al. (2010) Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. *Cell* 143, 134–144

91. Ghigo, C. et al. (2013) Multicolor fate mapping of Langerhans cell homeostasis. *J. Exp. Med.* 210, 1657–1664
92. Jarjour, M. et al. (2014) Fate mapping reveals origin and dynamics of lymph node follicular dendritic cells. *J. Exp. Med.* 211, 1109–1122
93. Weber, K. et al. (2011) RGB marking facilitates multicolor clonal cell tracking. *Nat. Med.* 17, 504–509
94. Weber, K. et al. (2012) RGB marking with lentiviral vectors for multicolor clonal cell tracking. *Nat. Protoc.* 7, 839–849
95. Harrington, L.E. et al. (2008) Memory CD4 T cells emerge from effector T-cell progenitors. *Nature* 452, 356–360
96. Dunkle, A. et al. (2013) Transfer of CD8+ T cell memory using Bcl-2 as a marker. *J. Immunol.* 190, 940–947
97. Stewart-Ornstein, J. et al. (2013) Msn2 coordinates a stoichiometric gene expression program. *Curr. Biol.* 23, 2336–2345
98. Siuti, P. et al. (2013) Synthetic circuits integrating logic and memory in living cells. *Nat. Biotechnol.* 31, 448–452
99. Haruyama, N. et al. (2009) Overview: engineering transgenic constructs and mice. *Curr. Protoc. Cell Biol.* 19, 19.10.1–19.10.9
100. Croxford, A. and Buch, T. (2011) Cytokine reporter mice in immunological research: perspectives and lessons learned. *Immunology* 132, 1–8
101. Zambrowicz, B.P. et al. (1997) Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proc. Natl. Acad. Sci. U.S.A.* 94, 3789–3794
102. Mizuguchi, H. et al. (2000) IRES-dependent second gene expression is significantly lower than cap-dependent first gene expression in a bicistronic vector. *Mol. Ther.* 1, 376–382
103. Trichas, G. et al. (2008) Use of the viral 2A peptide for bicistronic expression in transgenic mice. *BMC Biol.* 6, 40
104. Buus, S. et al. (1987) The relation between major histocompatibility complex (MHC) restriction and the capacity of Ia to bind immunogenic peptides. *Science* 235, 1353–1358
105. Rosenzweig, M. et al. (2001) Induction of cytotoxic T lymphocyte and antibody responses to enhanced green fluorescent protein following transplantation of transduced CD34(+) hematopoietic cells. *Blood* 97, 1951–1959
106. Stripecke, R. et al. (1999) Immune response to green fluorescent protein: implications for gene therapy. *Gene Ther.* 6, 1305–1312
107. Davey, G. et al. (2013) Identification of a MHC I-restricted epitope of DsRed in C57BL/6 mice. *Mol. Immunol.* 53, 450–452
108. Mantovani, A. (2000) Investigating T-cell memory. *Nature* 407, 40
109. Cheng, A.W. et al. (2013) Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell Res.* 23, 1163–1171
110. Mali, P. et al. (2013) CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering. *Nat. Biotechnol.* 31, 833–838