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Expression efficiency of multiple *Il9* reporter alleles is determined by cell lineage

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Abstract

Generation of allelic gene reporter mice has provided a powerful tool to study gene function *in vivo*. In conjunction with imaging technologies, reporter mouse models facilitate studies of cell lineage tracing, live cell imaging and gene expression in the context of diseases. While there are several advantages to using reporter mice, caution is important to ensure the fidelity of the reporter protein representing the gene of interest. Here we compared the efficiency of two *Il9* reporter strains $Il9^{citrine}$ and $Il9^{GFP}$ in representing IL-9-producing $CD4^+$ T-helper 9 (T_H9) cells. Although both alleles show high specificity in IL-9-expressing populations, we observed that the $Il9^{GFP}$ allele visualized a much larger proportion of the IL-9 producing cells in culture than the $Il9^{citrine}$ reporter allele. In defining the mechanistic basis for these differences, chromatin immunoprecipitation and chromatin accessibility assay showed that the $Il9^{citrine}$ allele was transcriptionally less active in T_H9 cells compared to the wildtype allele. The $Il9^{citrine}$ allele also only captured a fraction of IL-9-expressing bone-marrow-derived mast cells. In contrast, both the $Il9^{citrine}$ and $Il9^{GFP}$ reporter detected *Il9* expression in type 2 innate lymphoid cells (ILC2) at a greater percentage than could be identified by IL-9 intracellular cytokine staining. Taken together, our findings demonstrate that the accuracy of IL-9 reporter mouse models may vary with the cell type being examined. These studies demonstrate the importance of choosing appropriate reporter mouse models that are optimal for detecting the cell type of interest as well as the accuracy of conclusions.

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Introduction:

Cytokines are soluble messenger proteins that allow cell to cell communication among immune system cells and other cells in the body during homeostatic maintenance and inflammatory immune responses. There are various techniques such as ELISA, flow-cytometry and quantitative real-time PCR (qRT-PCR) that facilitate detection of cytokine mRNA and protein in vitro and ex vivo. However, these approaches have limitations for tracking cells in vivo and isolating cytokine-positive cells for functional analyses such as adoptive transfer. To overcome these challenges, reporter mouse models have been developed and widely used for easy detection and the lack of toxicity for the expressing cells.

Transgenic reporter mice represent a powerful approach to understand tissue development in vivo, signals regulating cell-fate decisions, cell lineage tracing and gene function in diseases. In the study of cytokine biology, commonly used approaches in the generation of reporter mouse models for lineage tracing include introduction of internal ribosome entry site (IRES)-reporter under the control of the promoter of target gene or reporter gene knock-in into the first exon of the target gene (1). In the first approach, the target gene remains intact, while in the second approach the target gene is disrupted. Both of these approaches allow for the detection of cells expressing the gene of interest (2). For example, IL-4 is a signature cytokine produced by T_H2 cells that play a fundamental role in pathogenesis of humoral immunity, parasitic infections and allergic inflammatory diseases (3–9). In order to study the role of T_H2 cells and other IL-4 producing cells, several IL-4 reporter mouse models have been generated and each has their advantages and disadvantages. IL-4/GFP-enhanced transcript (4get) mice were one of the first cytokine reporter mice generated to detect IL-4 expression in situ (10). The 4get mice were made by inserting an internal ribosome entry site-green fluorescent protein (IRES-GFP) cassette into the 3’ untranslated region of the *Il4* locus. The IL-4 reporter 4get mice have played an important role in identification of other IL-4 secreting cells populations including natural killer (NK)T cells, basophils, eosinophils and mast cells (11–13). While the 4get mice identified IL-4 protein-expressing cells, the allele also expressed GFP when the allele was in an open and accessible conformation, but no *Il4* mRNA was translated. To identify IL-4 secreting cells, a dual reporter system was generated by using 4get mice and human CD2/IL-4 reporter mice also known as knock-in hCD2 (KN2) mice(14). The KN2 mice were generated by replacing the first exon of mouse *Il4* gene with the huCD2 cassette. The use of dual reporter system allowed identification of IL-4 competent cells that were GFP^+ and IL-4 producing cells that were GFP^+hCD2^+. In a parallel approach to understand the function of IL-4 in vivo, GFP/IL-4 (G4) mice were also generated (15, 16). Here, the first exon and first intron of the *Il4* gene were replaced by the gene encoding enhanced GFP creating mice that are deficient in IL-4 yet allow detection of IL-4 producing cells. Each of the knock-in IL-4 reporter mouse models have proven to be advantageous in studies defining the function of IL-4 in inflammatory diseases (17–19).

Interleukin-9 (IL-9) is another pleotropic cytokine associated with immunity to helminthic parasites and tumors, and a myriad of inflammatory diseases including allergic airway inflammation, and autoimmune disorders (20–24). In allergic airway diseases, IL-9 has been shown to increase accumulation of mast cells and eosinophils in the lung, promote IgE and

type-2 cytokine production, and hyperresponsiveness(25, 26). While the $CD4^+$ T-helper subset called T_H9 cells are predominant producers of IL-9, IL-9 producing cell types also include type-2 innate lymphoid cells (ILC2s), mast cells (MCs) and natural killer T (NKT) cells (26–31). With increasing evidence of the involvement of IL-9 and IL-9 producing cells in immunopathological diseases, several groups have generated IL-9 reporter mouse models to trace IL-9 producing cells in vivo (32, 33). The first IL-9 reporter mice successfully generated were IL-9 fate reporter mice. IL-9 fate reporter mice were made by introducing a first exon knock-in of Cre into an *Il9* BAC (34). Surprisingly, the IL-9 fate reporter, crossed to mice with the *Gt(ROSA)26Sor*$^{tm1(EYFP)}$Cos/J allele, only detected 10% of the IL-9 secreting T cells cultured *in vitro*. Still, the lineage tracer identified IL-9 secreting ILC and T_H9 populations in several airway disease models.

Similar to IL-9 fate reporter mice, Gerlach et al. utilized *Il9*^{citrine} mice generated by insertion of a citrine coding gene into the first exon of the endogenous *Il9* gene which disrupted expression of endogenous *Il9* but allowed detection of IL-9 producing cells in vivo in an IBD model, but only a fraction of IL-9 secreting cells from T_H9 cultures (35). Similarly, *Il9*^{9er} mice, with enhanced YFP-IRES-Cre knock in to first *Il9* exon, have been used to identify IL-9 producing T cells and ILC populations in acute allergic lung inflammation (36). Using a different approach Licona-Limon et al. generated the interleukin-9 fluorescence enhanced reporter (INFER) mice, *Il9*^{GFP}, by inserting an IRES-EGFP cassette at the 3’ end of the *Il9* gene (37). In vitro T cell differentiation analysis showed that around 80% of the IL-9 secreting population was also positive for the GFP reporter. The *Il9*^{GFP} mice allowed detection of IL-9 secreting ILC and T_H9 populations as well as quantitation of endogenous IL-9 production in a parasite model. Each of the IL-9 reporter mice generated thus far, show fidelity in detecting IL-9 producing cell populations, however, the efficiency in reporting for IL-9 expression varies in different models. Furthermore, the relative efficiency of these reporter mice in accurately detecting IL-9 production in different cell lineages remains unclear. In this study we demonstrate that there are differing efficiencies of *Il9* reporter alleles in detecting IL-9 among distinct cell types.

Materials and methods:

Mice

Exon1-knock-in knock out *Il9*^{tm1Anjm} (*Il9*^{citrine}) mice and INFER *Il9*$^{tm2.1Flv}$ (*Il9*^{GFP}) mice were previously described, were obtained from Dr. Andrew NJ McKenzie’s and Dr. Richard A. Flavell’s laboratory (35, 37). The *Il9*^{citrine} mice are on BALB/cJ genetic background while *Il9*^{GFP} are on C57BL/6J (B6/J), accordingly BALB/cJ, C57BL6J (B6/J) and 129S (129S1/SvlmJ) mice were obtained from Jackson laboratory as background control. All experiments were done with 8–12 week-old mice.

In vitro T-cell differentiation

Naïve CD4 T cells were isolated from mouse spleens using $CD4^+CD62L^+$ T cell isolation kit provided by the supplier (Miltenyi Biotec). Cells were cultured in complete RPMI 1640 media on anti-CD3 (2 Units/ml 145–2C11; BioXCell) coated-plates and soluble anti-CD28 (2.5 μg/ml; BD Pharmingen) under T_H9 polarizing conditions including: hTGF- β1 (2 ng/

ml), IL-4 (20 ng/ml), hIL-2 (50 U/ml), anti-IL-10R (10 μg/ml) and anti-IFN-γ (10 μg/ml) (All cytokines were obtained from PeproTech and antibodies were obtained from BioXcell). On day 3, cells were expanded into fresh media containing the original concentrations of cytokines in the absence of co-stimulatory signals for an additional 2 days. On day 5, mature T_H9 cells were harvested for further analysis.

In vitro bone-marrow derived mast cell (BMMC) generation

To generate bone marrow derived mast cells (BMMCs), bone marrow cells from WT or *Il9*$^{citrine/citrine}$ mice were isolated and red blood cells (RBCs) were lysed using Ammonium-Chloride-Potassium (ACK) lysis buffer (LONZA). Cells were cultured in complete RPMI with IL-3 (10 ng/ml) and stem cell factor (SCF; 30 ng/ml) and allowed to mature for 21 days.

Innate lymphoid cell isolation

For ILC2 isolation, WT and *Il9*$^{citrine/citrine}$ mice were treated intranasally (i.n.) with 0.5 μg of IL-33 for 3 days. On day 4, lungs were harvested, minced and incubated with 0.5 mg/ml of collagenase A (Roche) in Dulbecco's modified Eagle's medium (DMEM) at 37 °C for 45 min. To generate single-cell suspension, digested lung tissue was passed through stainless steel meshed strainers and RBCs were removed by using ACK lysis buffer. Cells were further washed with phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) and filtered through 70 μm nylon mesh to remove debris. For intracellular staining of IL-9 in ILC2 subset, lung cells were re-stimulated with 50 ng ml−1 of IL-33 for 5 hours and treated with monensin for 2 hours. ILC2 were identified as Lineage$^-$ (Lin$^-$), CD45.2+, CD90.2 (Thy1.2)+, Sca-1+, Klrg-1+ and ST2+, and were stained for IL-9. For isolation of ILC2 subset, lung cell suspension was lineage-depleted using biotinylated antibodies including mouse lineage depletion kit and CD11c, CD19, DX5, and NK1.1 from Miltenyi Biotec. Lineage depleted lung cells were further stained for ILC2 markers including CD45.2, 104, BD Horizon; Klrg1, 2F1, BD Horizon; Sca-1, D7, BD Horizon; ST2, U29–93, BD Horizon and sorted on a BD FACS Aria II at the IUSM flow-cytometry core.

Quantitative real-time PCR

Total RNA was isolated from cells using Trizol (Life Technologies). RNA was reverse transcribed according to manufactures directions (Quantabio, Beverly, MA). qRT-PCR was performed with commercially available primers (Life Technologies) with a 7500 Fast-PCR machine (Life Technologies). Gene expression was normalized to housekeeping gene expression (β2-microglobulin). In case of qPCR for ChIP assay, SYBR green master mix (Applied Biosystems) was used for gene expression analysis (38).

Flow cytometric analysis

For cytokine staining, CD4$^+$ T cells were stimulated with Phorbol 12-myristate 13-acetate (PMA, 50ng/ml, Sigma) and ionomycin (1ug/ml, Sigma) for 3 hours followed by monensin (2μM, Biolegend) for total 6 hours at 37ºC. BMMCs were treated with monensin and re-stimulated with IL-33 (50 ng/ml) for 4 hrs. After re-stimulation, cells were washed with

FACS buffer (PBS with 0.5% BSA). CD4+ T, BMMCs or total lung cells were then stained with a fixable viability dye (eFluor780, eBioscience) and surface markers (CD4, GK1.5, Biolgend; CD90.2, 53–2.1, Biolegend; CD117 (c-kit), 104D2, Biolegend; FcεR1a, MAR-1, Biolegend; CD45.2, 104, BD Horizon; Klrg1, 2F1, BD Horizon; Sca-1, D7, BD Horizon; ST2, U29–93, BD Horizon and mouse lineage antibody cocktail, BD Pharmigen) for 30 min at 4 ºC followed by washing and fixation with 4% formaldehyde for 10 min at room temperature. For cytokine staining, cells were then permeabilized with permeabilization buffer (eBioscience) for 30 min at 4 ºC, and stained for cytokines (IL-9; RM9A4, Biolegend) for 30 min at 4 ºC.

Chromatin immunoprecipitation assay

In vitro-differentiated Th cells were activated with anti-CD3 for 3 hours and were crosslinked for 15 min with 1% formaldehyde at RT with rotation. The reaction was quenched by adding 0.125 M glycine and incubated at RT for 5 min. Fixed cells were lysed with cell lysis buffer, followed by nuclear lysis buffer. Nuclei were degraded and chromosomal DNA were fragmented to a size range of 200–500 bp through ultrasonic processor (Vibra-cell). After sonication, the supernatant was diluted 10-fold with ChIP dilution buffer. After pre-clearing, the supernatant was incubated with the ChIP antibodies at 4 °C overnight with rotation. The following day, immunocomplexes were precipitated with Protein Agarose A or G beads at 4 °C for 2–4 h. Immunocomplexes were washed with low salt, high salt, LiCl and two times with TE buffer. After elution followed by reverse crosslinks, DNA was purified and analyzed by qPCR. After normalization to the Input DNA, the amount of output DNA of each target protein was calculated by subtracting that of the IgG control. Quantification of ChIP assay by qPCR was performed using primers as described (38).

Chromatin accessibility assay

Chromatin was isolated from in vitro-cultured T cells and re-stimulated for 3 hours with anti-CD3. Chromatin was digested with nuclease (Nse) mix using the EpiQuik chromatin accessibility assay kit (Epigentek). Isolated chromatin was divided into two, one for nuclease treatment, and another for non-treatment. After incubation at 37 °C for 4 min, reaction was quenched by adding reaction stop solution. Samples were incubated with proteinase K at 65°C for 15 min to degrade any contaminating proteins. DNA was purified followed by qPCR to amplify DNA fragment with primers for *Il9* CNS regions (same as ChIP primers) or Hbb-bs for negative control (F: 5′-gagtggca- cagcatccagggagaaa-3′, R: 5′-ccacaggccagagacagcagccttc-3′) The fold enrichment (FE) was calculated by the formula: FE = 2^(Nse CT − no Nse CT) × 100%.

Statistical analysis

All the data was analyzed using two-tailed Student’s t test to generate p-value. Post hoc Tukey test was used for multiple comparisons. $p \leq 0.05$ was considered statistically significant.

Results:

Il9$^{+/citrine}$ reporter mice inadequately report for IL-9 expression in T_H9 cells differentiated in vitro

To characterize T-helper 9 cells in more detail, we sought to profile IL-9 reporter expression in two distinct IL-9 reporter mice, *Il9*$^{+/citrine}$ or *Il9*$^{+/GFP}$, that have been previously used to identify IL-9-producing cells in disease models. Naïve CD4 T cells from *Il9*$^{+/citrine}$ or *Il9*$^{+/GFP}$ were cultured under T_H9 polarizing conditions for 5 days in vitro and IL-9 or reporter expression was monitored through each day of T_H9 differentiation. Flow-cytometric analysis showed that in the *Il9*$^{+/citrine}$ cultures, there were small populations of IL-9+ and citrine+ cells, which were largely mutually exclusive. Since each allele produces only one of the detected proteins, it suggests there is monoallelic expression of *Il9* alleles within cells (Fig. 1A). There was a large increase in IL-9 staining on day 4, but far less of an increase in citrine expression, with citrine detecting only about a third of the IL-9-producing cells (Fig. 1A). On day 2 and 3 of *Il9*$^{+/GFP}$ cell culture, there were significant amounts of GFP detected in the absence of IL-9 production, suggesting that the reporter is indicating gene competence. By day 4, there is a predominant population of IL-9+ GFP+ cells in the *Il9*$^{+/GFP}$ cell culture (Fig. 1A). To further quantify expression from these alleles, we performed qRT-PCR. The relative amount of GFP transcript expressed by the *Il9*$^{+/GFP}$ allele was about half that of *Il9* (Fig. 1B). However, citrine expression was less than 10% of *Il9* transcript levels (Fig. 1B). In addition to examining reporter expression in T_H9 cells in heterozygous IL-9 reporter mice, we also assessed the reporter expression in reporter allele homozygous mice. Similar to *Il9*$^{+/citrine}$ T_H9 cultures, the *Il9*$^{citrine/citrine}$ cells reported only 7% of the T_H9 culture (Fig. 1C), whereas, *Il9*$^{GFP/GFP}$ cells reported for about half of the T_H9 culture (Fig. 1C). The distinct function of the reporters was not due to differences in the background of the mice as T_H9 cultures from BALB/c mice, C57BL6 mice and 129S mice (which were used to generate the citrine allele (35)). had similar percentages of IL-9+ T_H9 cells (Fig. 1D).

Disruption of exon-1 region in *Il9* gene in *Il9*$^{citrine/citrine}$ reporter mice reduced transcriptional activity at the *Il9* locus in T_H9 cells

The decreased ability of the citrine allele to report for IL-9 production suggested that targeting the exon1 region at *Il9* locus may have interfered with regulation of the reporter allele. Recent studies have identified and reported conserved non-coding sequence (CNS) regions across the *Il9* locus that play an important role in *Il9* gene transcription and regulation (38, 39) (Figure 2A). To define how targeting the first exon of *Il9* affected gene activity, we measured chromatin accessibility across the conserved noncoding sequence (CNS) regions at the *Il9* locus in both BALB/c and *Il9*$^{citrine/citrine}$ mice (38). The *Il9* locus in T_H9 cells cultured from *Il9*$^{citrine/citrine}$ mice was much less accessible than the wild-type allele (Figure 2B). To determine whether the disruption of the exon-1 region of the *Il9* locus in *Il9*^{citrine} mice affected transcriptional activity, we performed chromatin immunoprecipitation assays to assess histone modifications at the intronic, exonic and conserved CNS regions of the *Il9* locus in BALB/c, C57BL/6, 129S, *Il9*^{citrine} and *Il9*^{GFP} Th9 cultures. H3K27 acetylation, a mark of open chromatin was similar among Th9 cells from BALB/c, C57BL/6, 129S and *Il9*^{GFP} although it was considerably lower in the *Il9*^{citrine}

cultures (Fig. 2C-D). H3K4 trimethylation, a mark of active transcription demonstrated a similar pattern in the wild type strains, but was lower in the *Il9*^{GFP} Th9 cells, and almost undetectable in the *Il9*^{citrine} cultures (Fig. 2C-D). The pattern of RNA pol II binding across the locus varied greatly among the wild type strains suggesting strain-specific patterns of pol II pausing on the allele (Fig. 2C-D). The *Il9*^{GFP} Th9 cells showed a unique peak in the 3' end, suggesting a link to the IRES element (Fig. 2D). In contrast, the *Il9*^{citrine} Th9 cells had barely detectable RNA pol II binding (Fig. 2C). Together these findings demonstrated that disruption of the exon-1 region of *Il9* gene impaired transcriptional activity of the *Il9*^{citrine} reporter allele in T_H9 cells.

In contrast to T_H9 and mast cells, *Il9*$^{citrine/citrine}$ reporter mice demonstrated higher IL-9 reporter expression in ILC2 population compared to wild-type allele.

In addition to T_H9 cells, mast cells and ILC2s have also been described as potent IL-9 producers (28, 33, 40). To investigate whether *Il9*$^{citrine/citrine}$ mice differentially reported for other IL-9 producing cells we looked at IL-9 and reporter expression in bone-marrow derived mast cells (BMMCs) re-stimulated with IL-33. As in T_H9 cells, *Il9*$^{citrine/citrine}$ reported for less than half of the IL-9 production in ckit$^+$ FcεR1α^+ BMMCs cultured in vitro (Figure 3). We were unable to detect consistent IL-9 production in mast cell cultures from wild-type and *Il9*$^{GFP/GFP}$ mice on the C57BL/6 background in these studies.

In order to examine whether the *Il9*$^{citrine/citrine}$ and *Il9*$^{GFP/GFP}$ allele exhibited a similar pattern of reporter expression in ILC2 populations, *Il9*$^{citrine/citrine}$ and *Il9*$^{GFP/GFP}$ mice along with their respective wild-type background (BALB/c and C57BL/6) mice were intranasally challenged with recombinant IL-33 for 3 days (Figure 4A). On day 4, lungs were harvested and samples were analyzed for IL-9 or reporter expression in the ILC2 population defined as Lin$^-$ CD45.2$^+$ Sca1$^+$ Klrg1$^+$ ST2$^+$ Thy1.2$^+$ cells. Using flow-cytometry, we observed that in contrast to T_H9 and mast cells, both *Il9*$^{citrine/citrine}$ and *Il9*$^{GFP/GFP}$ mice reported higher percentages of IL-9-producing ILC2 cells than wild-type mice, although the increase in *Il9*$^{GFP/GFP}$ mice was not significant (Figure 4B-D). We then sorted ILC2 populations from the lungs of *Il9*$^{+/citrine}$ *and Il9*^{GFP} mice intranasally challenged with rIL-33 for 3 days and measured transcript level expression of IL-9 and the respective reporter and compared expression to in vitro derived Th9 cells examined in Fig. 1 and 2. Comparing the ratio of reporter:*Il9* gene expression in T_H9 and ILC2 populations in the respective reporter mice, we observed that the *Il9*^{citrine} allele preferentially reported for IL-9 in ILC2 cells when compared to Th9 cells, and the *Il9*^{GFP} allele trended in that direction (Figure 4E). Importantly, the *Il9*^{citrine} allele in ILC2s reported approximately 100 times more expression than in the T_H9 population, whereas the *Il9*^{GFP} allele showed differences that were more modest and not statistically significant. These findings indicate that there are important differences in how a reporter allele identifies cytokine expression in distinct cells types and that knock-in to a coding sequence or modifying the target gene locus has the potential to interfere with locus expression. This is an important caveat in interpreting experiments using various reporter mice.

Discussion:

Reporter mice serve as a remarkable tool to identify gene function and detect cells expressing the gene of interest in vivo with minimal toxic effects. However, the fidelity of the reporter to identify populations of cells expressing the gene of interest and the efficiency in reporting for gene expression needs to be defined for each model as well as cell type. In this study we examined the fidelity and efficiency of two IL-9 reporter mice in identifying the IL-9 secreting T cells cultured in vitro. Our results showed that although both *Il9*^{GFP} and *Il9*^{citrine} mice identified IL-9 secreting cells, the *Il9*^{GFP} mice detected a majority of the IL-9-producing T cells whereas the *Il9*^{citrine} mice identified ~20% of the IL-9 secreting T cells in vitro. Thus, different approaches for targeting a gene of interest can result in differing efficiencies of reporting.

The mechanisms for the functional differences between reporter alleles are still not entirely clear. Although the *Il9*^{citrine} allele maintains specificity for expression in T_H9 cells (35, 41), it is likely that the *Il9*^{citrine} allele disrupts a regulatory element that contributes to *Il9* expression in T cells and mast cells, consistent with the altered chromatin modifications observed at the *Il9*^{citrine} allele. Deletion of the *Il9* enhancer region CNS −25 was similarly crucial for *Il9* gene regulation in T cells and mast cells but not in the ILC2 population (38, 39). Thus, these findings indicate that the first exon of the *Il9* locus may be important in transcriptional regulation of *Il9* in T cells. In contrast to the lack of expression of the *Il9*^{citrine} allele in T cells, ILC2 cells show enhanced reporter protein and mRNA compared to IL-9 protein and *Il9* mRNA for both reporter strains. The differences in protein could result from differences in protein half-lives of reporter versus cytokine, and of fluorescent proteins among cell types. The differences in mRNA might result from altered regulation of the gene, but it is also possible that the gene encoding the fluorescent protein confers greater stability to the mRNA in ILC2 cells. These distinctions would require more detailed analyses.

The expression pattern observed in the *Il9*^{citrine} mice is similar to the previously generated *Il9*^{Cre} fate-reporter mice where only 10% of IL-9 secreting T cells were identified by the IL-9 fate-reporter mice (34). Both *Il9*^{citrine} and IL-9 fate reporter mice were generated by knocking in the reporter into the first exon of *Il9* gene. Consistent with these observations, Schwartz et. al. noted substantial differences between gene expression patterns observed in IL-9 fate reporter mice compared to *Il9*^{GFP} mice in a papain-induced allergic airway disease model (42). These identified differences in expression efficiency among *Il9* reporter allele across cell lineages requires a reexamination of conclusions regarding the relative contributions of cells producing IL-9. In models of airway inflammation and rheumatoid arthritis it was concluded that ILC2s are the primary producer of IL-9 (34, 43, 44). The IL-9 fate reporter and *Il9*^{citrine} mice used in these studies more efficiently identifies IL-9 expression in ILC2 than T_H9 cells, potentially underrepresenting the role of T_H9 cells. Until IL-9 conditional mutant mice are used to selectively remove IL-9 production in specific populations the definitive answer to the major or required source of IL-9 will be unclear. More than likely the cellular source of IL-9 will vary depending on the model and type of inflammation.

Another potential concern in reporter alleles is elimination of the endogenous gene, as occurs in the *Il9*^{citrine} mice, if there is positive feedback on gene expression. Indeed, in mast cells IL-9 can amplify its own production as well as the production of other type 2 cytokines (45). However, in multiple experiments with Th9 cultures where IL-9 is blocked during culture or supplemental IL-9 is added during culture, we have not observed any changes in IL-9 production at the end of the culture (not shown). Thus, the reduced IL-9 production in the *Il9*^{citrine} Th9 cultures is unlikely to be from a lack of a positive feedback from IL-9 itself.

The *Il9*^{citrine} allele is clearly less active. Compared to either BALB/c or 129S control cells the allele was less accessible, had less H3K27 acetylation and less H3K4 trimethylation. These markers of active genes also correlated with less RNA pol II binding. In contrast, the *Il9*^{GFP} allele had normal H3K27 acetylation. The decreased H3K4 trimethylation at the *Il9*^{GFP} allele was surprising given the expression of the allele. However, this might be a result of IRES effects on the rest of the gene. In fact, we observed unique peaks at exon 5 for RNA pol II binding in the *Il9*^{GFP} allele that support a role for the IRES in altering transcription patterns at the *Il9* locus. Together these results suggest that reporter alleles, regardless of targeting strategy, likely alter the chromatin structure of the allele, and that inadvertently targeting regulatory elements or inserting IRES sequences will both have effects.

Reporter alleles can also help define the monoallelic expression observed in some cytokine genes. For example, Hollander et. al, first reported monoallelic expression of IL-2 in mature thymocytes and T cell populations identified using polymorphisms in alleles (46, 47). Interestingly Naramura et al., showed that CD4 T cells cultured from IL-2-GFP^{ki} mice (GFP knocked into *Il2* locus) failed to exhibit monoallelic expression or allelic exclusion (48). These observations suggest that gene regulatory elements including exons, introns, promoter and enhancer regions can influence allelic expression in specific cells types. The *Il9*^{citrine} T_H9 cultures early in differentiation do show early exclusion, showing preferential expression of either IL-9 or citrine. However, at the later stages of differentiation there are double-positive cells, suggesting that exclusion is not complete. It is possible that while gaining competence for expression, there is monoallelic expression, but as the cell becomes more differentiated, both alleles are expressed. Importantly, the early exclusion is not observed in the *Il9*^{GFP} allele since both IL-9 and GFP can be generated from the same allele.

There is also the question of whether alleles report strictly for expression, or also for competence of the allele, as observed in 4get mice (10). In the *Il9*^{GFP} allele, generated with an IRES insertion like the 4get allele, there are large populations of GFP+ cells during early T_H9 differentiation, when IL-9 protein secretion is absent. That observation parallels data from the 4get mice that showed GFP expression where there was no *Il4* mRNA. This parallel suggests that competency of the locus is being reported and that the allele might be useful in analysis of changes in chromatin structure at the *Il9* locus.

Our results have demonstrated that different approaches to targeting the *Il9* locus in the generation of a reporter allele result in reporters that have efficiencies that vary with cell type. This is an important consideration in selecting alleles for the studies of specific cell types and presents caveats for studies where positive cells could not be identified. In these

situations, it is possible that technical issues with a reporter allele could be responsible for negative results. Whether this paradigm is restricted to the *Il9* locus, or whether it is broadly applicable to other reporter alleles will be defined as studies with these reagents continues.

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Abbreviations:

IL-9	interleukin-9
T_H9	T-helper 9
ILC2	type 2 innate lymphoid cells
BMMCs	bone-marrow derived mast cells
CNS	conserved non-coding sequence
IRES-GFP	internal ribosome entry site-green fluorescent protein
INFER	interleukin-nine fluorescent reporter

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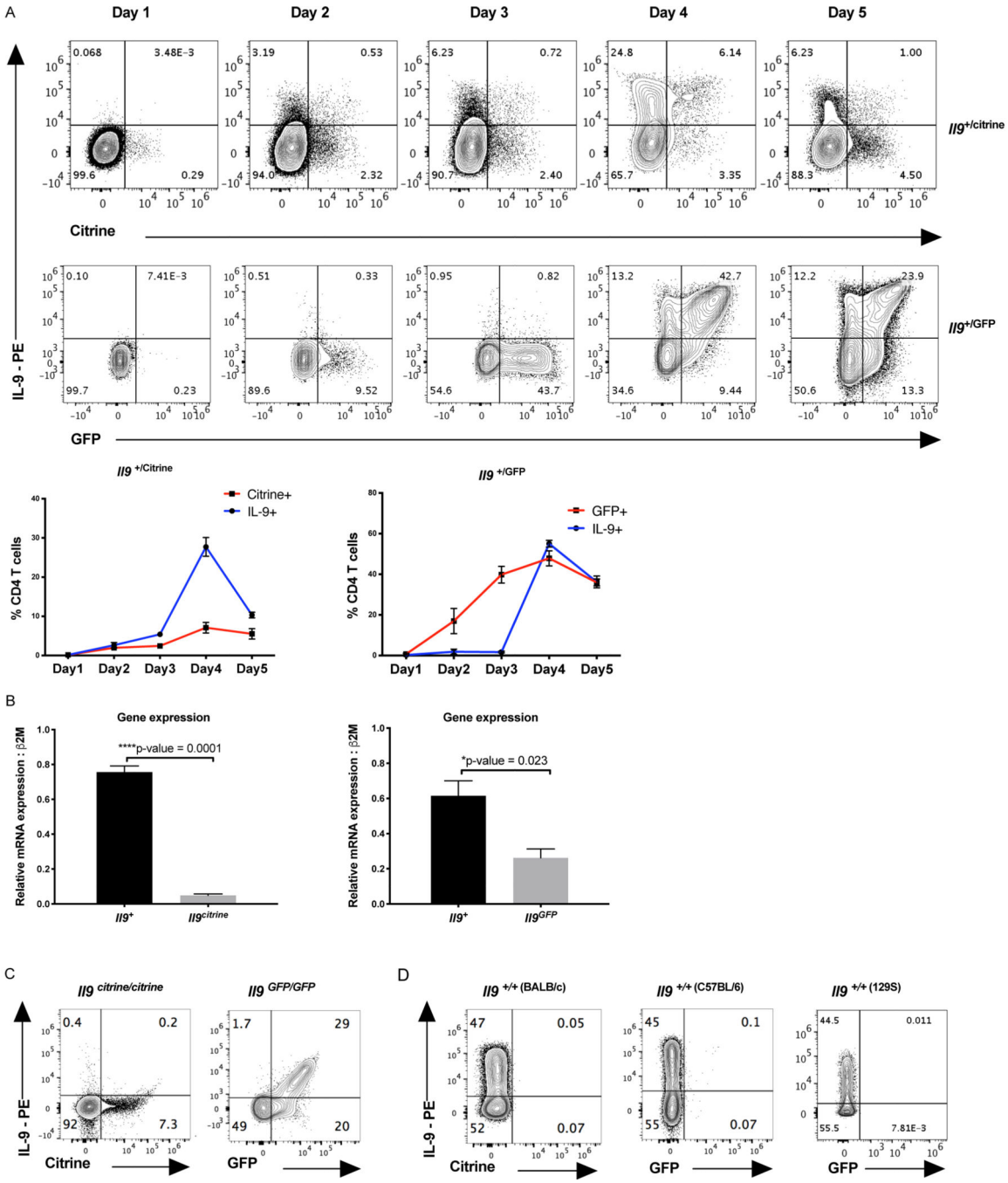

Figure 1: Profile of fluorescent protein expression in *Il9* reporter mice

A. Reporter expression measured over a period of 5 days during T_H9 differentiation in vitro from *Il9*$^{+/citrine}$ or *Il9*$^{+/GFP}$ mice. B. mRNA expression of *Il9* and respective reporter (citrine or GFP) measured on day 4 of T_H9 differentiation. C. IL-9 reporter expression by day 5 T_H9 cells cultured *in vitro* from in *Il9*$^{citrine/citrine}$ and *Il9*$^{GFP/GFP}$ mice. D. IL-9 production by day 5 T_H9 cells cultured *in vitro* from BALB/c, C57BL6 and 129S mice Data are mean ± SEM of 3 mice per experiment and representative of at least two independent experiments. *p<0.05.

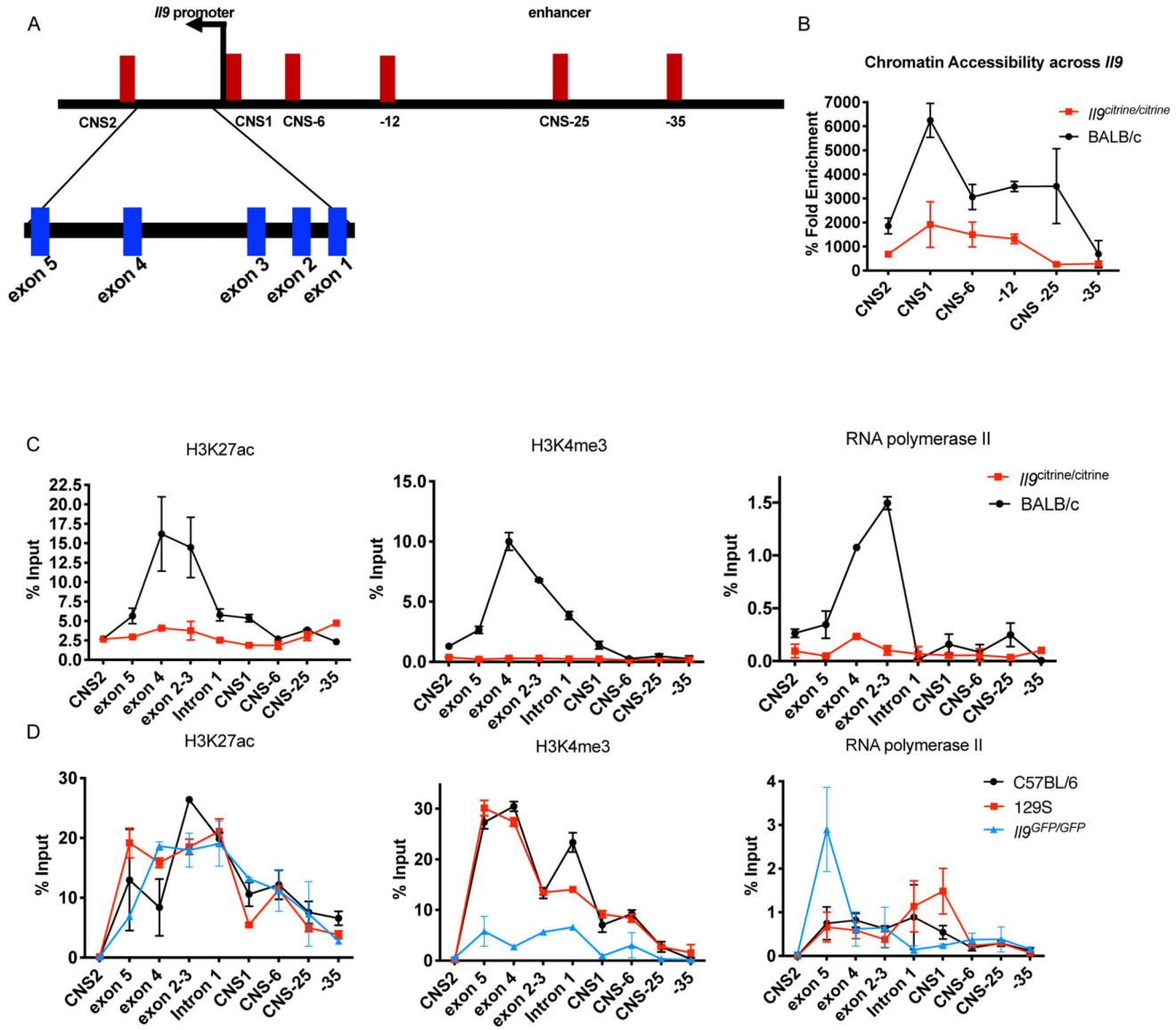

Figure 2: Disruption of *Il9* exon 1 impaired transcriptional activity

A. Schematic of conserved non-coding sequences (CNS) at the *Il9* locus. (B-F) Naive CD4+ T cells from wild-type or *Il9*^{citrine} mice were cultured in T_H9 conditions for 5 days and restimulated with PMA and ionomycin for 3 hours upon harvest on day 5. B. Chromatin accessibility assessed at *Il9* locus in BALB/c and *Il9*$^{citrine/citrine}$ allele. C. Relative amounts of H3K27ac, H3K4me3 and RNA polymerase-II at *Il9* locus in T_H9 cells examined ChIP-qPCR in BALB/c and *Il9*$^{citrine/citrine}$ mice. D. Relative amounts of H3K27ac, H3K4me3 and RNA polymerase-II at *Il9* locus in T_H9 cells examined ChIP-qPCR in C57BL6, 129S and *Il9*$^{GFP/GFP}$ mice. Data are mean ± SEM of 3 mice per experiment and representative of two independent experiments.

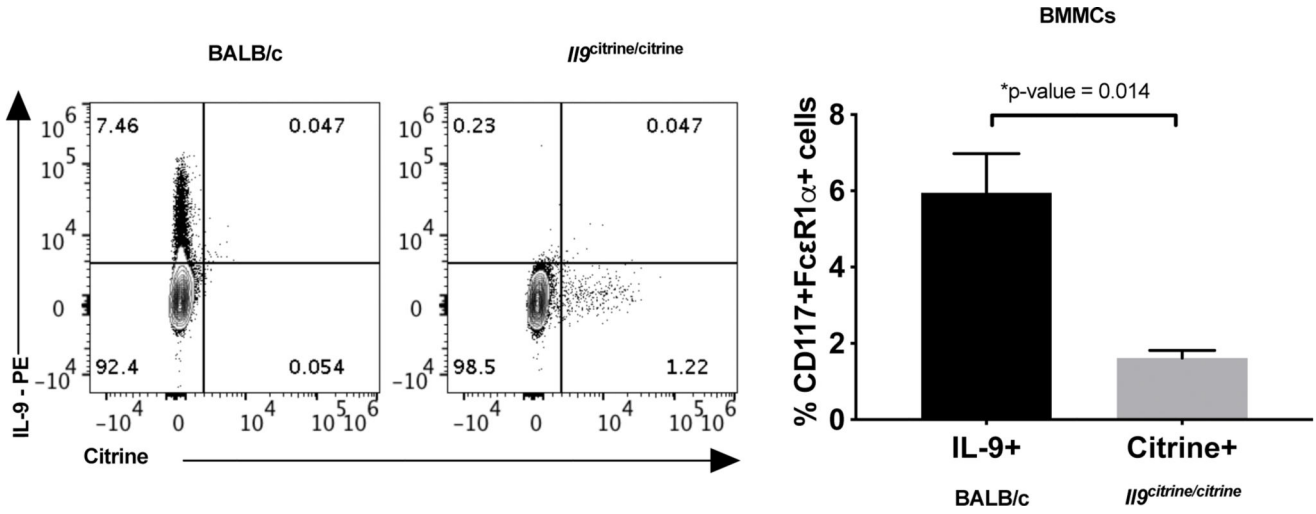

Figure 3: *Il9* reporter expression in BMMCs compared.

IL-9 and citrine expression in BMMCs derived from wild-type or *Il9*$^{citrine/citrine}$ mice. Data are mean ± SEM of 3 mice per experiment and representative of two independent experiments. *p<0.05.

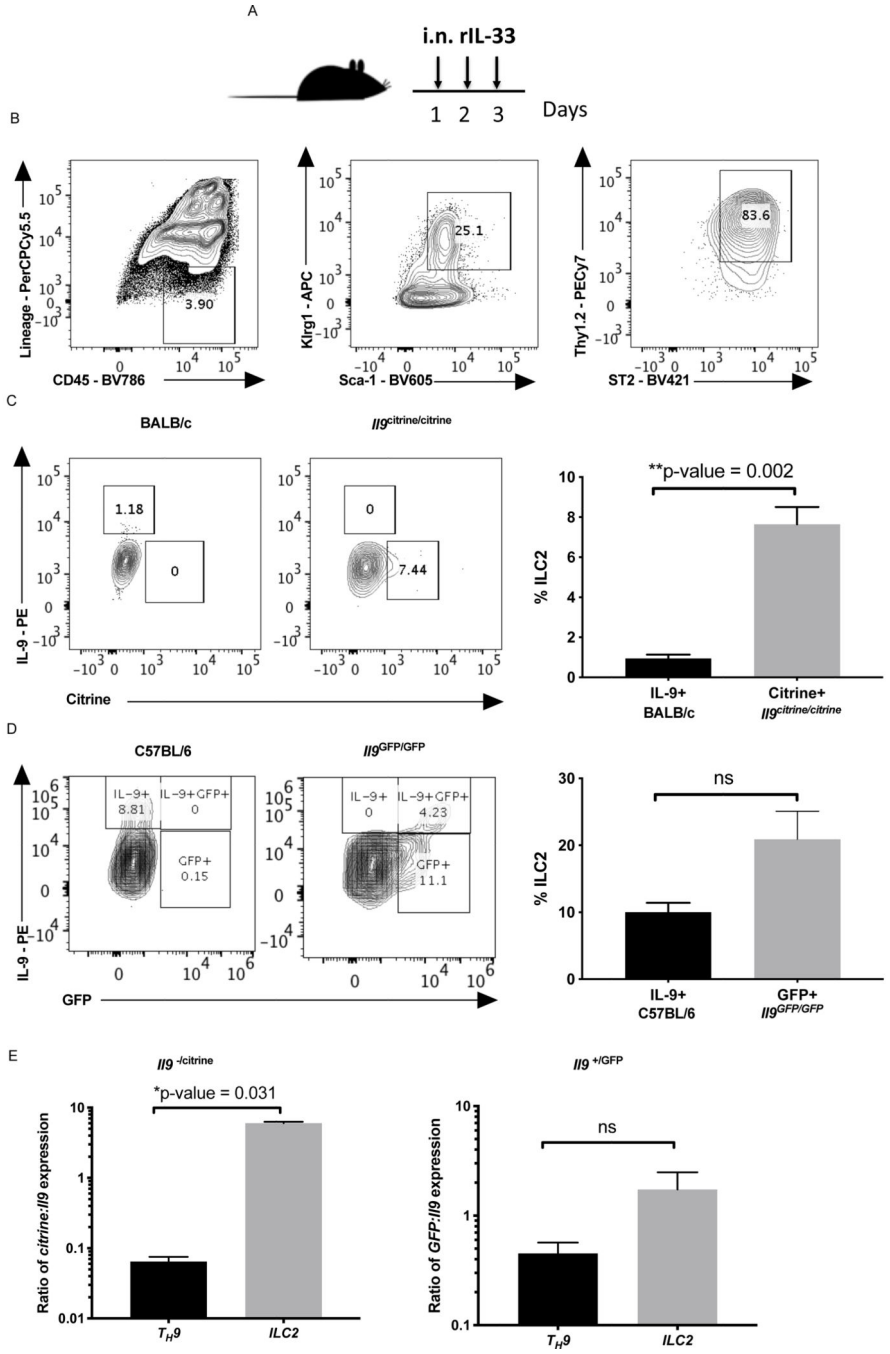

Figure 4: *Il9*$^{citrine/citrine}$ and *Il9*$^{GFP/GFP}$ mice selectively reported IL-9 production in ILC2s.

A. Schematic of rIL-33 intranasal challenge in *Il9*$^{+/citrine}$ mice for generation and detection of lung ILC2 population. B. Gating strategy for identification of ILC2 population in the lung defined as Lineage^{-}CD45^{+}Klrg1^{+}Sca1^{+}Thy1.2^{+}ST2^{+}. C. Detection of IL-9 and citrine reporter production in lung ILC2s. D. Detection of IL-9 and GFP reporter production in lung ILC2s. E. Ratio of *reporter:Il9* allelic expression in T_H9 cells (as generated in Fig 1) and

ILC2 subsets. Data are mean ± SEM of 3 mice per experiment and representative of two independent experiments. *p<0.05.