Gene dose matters: Considerations for the use of inducible CD4-CreER\textsuperscript{12} mouse lines

A growing body of evidence suggests that Cre recombinase can be toxic to immune cells in various experimental settings [1-5]. Cre recombinase toxicity is dependent on the level of Cre activity and may also interfere with cell proliferation [6, 7].

Here, we compared two different published tamoxifen-inducible CD4-CreER\textsuperscript{12} mouse lines for their suitability to study the dynamics of T-follicular helper (Tfh) cell responses in vivo. Our data underscore that under certain circumstances inducible Cre toxicity (tamoxifen application results in translocation of preformed CreER\textsuperscript{12} to the nucleus) interferes with cell survival and, therefore, necessitates careful interpretation of experimental data and the inclusion of appropriate controls. Interestingly, our data indicate that low expression of CreER\textsuperscript{12} can still allow for efficient recombination in proliferating lymphocytes without causing excessive cell loss due to Cre toxicity.

The first CD4-CreER\textsuperscript{12} mouse line (Tg(Cd4-Cre/ERT2)11Gnri) we tested has been constructed as a transgene (TG) [8], while the second line (Cd4\textsuperscript{m1(cre/ERT2)Thbu}) has been constructed as a knock-in (KI) [9] (Fig. 1A). Both mouse lines, which were additionally crossed to homozygous Rosa\textsuperscript{26}stop-bal-YFP reporter alleles, were immunized with the hapten-protein conjugate NP-KLH in alum adjuvant to elicit Tfh cell differentiation, revealed efficient induction of YFP on day 7 following tamoxifen application on day 3 and 4. YFP expression was specific for CD4\textsuperscript{+} T cells and was not expressed in CD8\textsuperscript{+} T cells (Fig. 1B, Supporting Information Fig. 1). The frequency of YFP\textsuperscript{+} cells was reproducibly higher among CD4\textsuperscript{+} T cells derived from the TG mouse line as compared to cells derived from the KI mouse line. We therefore preagated on YFP-positive cells for normalization purposes. We next assessed Tfh cell frequencies 1 week after immunization. Surprisingly, we could hardly detect any CXCR5\textsuperscript{hi}PD-1\textsuperscript{hi} Tfh cells among the Cre-recombined (YFP\textsuperscript{hi}) TG cells, but normal frequencies among the KI cells. Moreover, we observed that activated (CD44\textsuperscript{hi}CD62L\textsuperscript{lo}) cell frequencies were significantly reduced among YFP\textsuperscript{+} CD4\textsuperscript{+} T cells in the TG strain. Since Tfh cells are activated CD4\textsuperscript{+} T cells, we next gated on the remaining activated YFP\textsuperscript{+} T cells in the TG strain and found that Tfh cell frequencies among activated CD4\textsuperscript{+} T cells were similar to those found in KI mice. This clearly indicated that the reduced Tfh cell frequencies were not the result of a Tfh cell defect, but rather a problem due to absence of activated CD4\textsuperscript{+} T cells. We obtained similar findings in experiments with altered tamoxifen application and analysis time points. For example, reduced frequencies of activated CD4\textsuperscript{+} T cells were also evident in the TG, but not the KI line, after tamoxifen gavage on day 6 and 7 and analysis on day 14 following NP-KLH/alum immunization (data not shown). Notably, similar findings were also obtained when we administered tamoxifen during acute LCMV infection at days 3 and 4 and analyzed the CD4\textsuperscript{+} T cells from TG and KI mice on day 7 (data not shown). We reasoned that one explanation for the different effect of tamoxifen treatment was Cre toxicity, since cells derived from TG mice likely contain several copies of Cre [4], which may result in higher Cre expression levels as compared to the heterozygous KI strain that only contained one allele of the Cd4-CreER\textsuperscript{12} gene. Indeed, measurement of Cre mRNA levels in CD4\textsuperscript{+} T cells of untreated TG and KI mice revealed very high levels of Cre mRNA in the TG strain (Fig. 1D). In line with this, we did not observe any overt signs of Cre toxicity in the KI line, as frequencies of the analyzed subsets among YFP\textsuperscript{+} CD4\textsuperscript{+} T cells were comparable to those among CD4\textsuperscript{+} T cells derived from WT mice in the same experiment (data not shown).

In summary, we would like to emphasize that CreER\textsuperscript{12} systems provide unique opportunities for addressing important biological questions that could not be easily answered before (time-stamping, temporally-guided inactivation of genes, etc.). However, given the complexities of these sophisticated biological systems, it is imperative to carefully design experiments, knowing the caveats of the systems, and using the appropriate controls, namely CreER\textsuperscript{12}-expressing mice lacking loxP-flanked alleles. Heterozygous loxP-flanked alleles should only be used as control if the gene of interest is not haploinsufficient. While the TG CD4-CreER\textsuperscript{12} mouse line may not be well-suited for studies on activated CD4\textsuperscript{+} T cells, it may still be useful for recombining conditional alleles in nonactivated T cells. We would further like to point out that even if frequencies of YFP-expressing cells in our experiments...
Figure 1. Comparison of two different CD4-CreERT2 mouse lines reveals effects of copy number-dependent Cre-mediated toxicity on activated T-helper cells. (A) Mouse genetics and experimental design. Mice were anesthetized by isoflurane inhalation and subcutaneously immunized in the hock with 20 μg NP-KLH in alum per mouse (10 μg per each hind limb). On day 3 and 4 post immunization, mice received a dose of 5 mg tamoxifen in corn oil (150 μL) by intragastric gavage twice daily. Seven days after immunization, mice were sacrificed and popliteal lymph nodes were analyzed by flow cytometry. Mixed cohorts of 8 to 10-week-old male and female mice (5 WT mice, 4 TG mice, 5 KI mice) were used for this experiment and all mice were analyzed on the same day. The data are representative of two independent experiments performed with two to five mice per condition per experiment for the given tamoxifen treatment and analysis time points. (B) YFP expression in CD4+ and CD8+ T cells derived from popliteal lymph nodes of mice treated as described in (A). Gated on live CD19– cells. (C) Representative plots showing frequencies of CXCR5hiPD-1hi Tfh cells and activated (CD44hiCD62Llo)CD4+ T cells derived from popliteal lymph nodes of TG and KI mice treated as described in (A). Data are quantified in bar graphs (mean ± SEM), each dot representing one mouse, and statistical significance was determined with the Mann-Whitney test (*p < 0.05, **p < 0.01, ***p < 0.001). (D) Cre mRNA expression in CD4+ T cells isolated from spleen and peripheral lymph nodes of unimmunized TG and KI mice (four TG and four KI female mice, 8 weeks old) that had not received tamoxifen was determined by qRT-PCR (normalized to β-actin). The data are representative of two independent experiments performed with two to four mice per condition per experiment.

appear lower in the KI compared to the TG line, this does not necessarily reflect the CreER72 recombination efficiency for other loxP-flanked alleles of interest, which in our hands can be up to almost 100% with the KI line (data not shown). Thus, the efficiency of Cre-mediated recombination should be carefully determined for each individual conditional allele. Future work should aim at carefully characterizing and optimizing CreER72 expression and its potential toxic effects after tamoxifen
treatment, for example, by determining the optimal amount of CreERT2 per cell compartment and how this may be affected by different promoters. Titration of tamoxifen may also provide benefits. Novel modified Cre recombinase that exhibits reduced toxicity, for example, by reducing its half-life, may also be designed or CreERT2 constructs may be driven by alternative or weaker gene loci.

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