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Flow Cytometry for Identification of PRDM1-eYFP Transgenic Mice

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Introduction:

The first transgenic mice were produced in 1974 by Jaenisch and Mintz when they injected viral DNA into blastocyst-stage mouse embryos. After the development of those embryos in recipient females, viral DNA was found in some cells of the offspring, but not germline cells. Stable incorporation of a transgene into germline cells is necessary to allow passage of that gene to subsequent generations through natural breeding. Stable germline incorporation of exogenous DNA in mice was finally accomplished by several groups seven years later, an achievement that made the establishment of sustainable transgenic mouse colonies a reality. (Gordon and Ruddle, 1981; Costantini and Lacy, 1981; Brinster et al., 1981)

Since they were first invented, multiple kinds of transgenic mice have been created. (Wheeler, 2003) The most common type is referred to as a "knockout," in which a gene has been made inoperative. This can be useful for studying the function of a gene by contrasting a normal animal with one in which the gene's function has been lost. Another type of transgenic mouse is often called a "knock-in" because a sequence of DNA has been stably inserted into the genome and is typically expressed in at least some cells of the organism. When that DNA sequence is a protein coding gene the mouse may be capable of expressing a unique protein. These animals are useful for biomedical research or as producers of drugs. Some of the unique proteins expressed in "knock-in" mice include the green fluorescent protein that can be used to identify or track living cells within tissues, enzymes such as phosphoenolpyruvate carboxykinase, which can change muscle cell physiology, and human tissue plasminogen activator that can be harvested and used as a drug to treat stroke patients. (Hanahan et al., 2007; Gordan et al., 1987; Hakimi et al., 2007)

Another transgenic manipulation is the inclusion of a reporter construct into a mouse genome to create reporter mice in which fluorescent or bioluminescent proteins, or biochemical tags, are attached to a protein of interest. This can make the protein of interest detectable within living tissues and cells, and make detection possible when more standard protein detection methods are unavailable (e.g., antibodies) or impractical. A mouse carrying this kind of reporter construct is referred to as a reporter mouse. One such reporter mouse is the B6.Cg-Tg(Prdm1-EYFP)1Mnz/J line.

B6.Cg-Tg(Prdm1-EYFP)1Mnz/J mice contain the *Prdm1* gene promoter linked to the gene for the enhanced yellow fluorescent protein (eYFP). The *Prdm1* gene is expressed in B and T cells only after activation, such as after an animal is infected with a pathogen, or after immunization or vaccination. (Fu et al., 2017; Turner et al., 1994) In these transgenic mice, *Prdm1* expression occurs coincident with *eYFP* expression which makes activated B and T cells fluorescent. Yellow fluoresce is a unique marker that can be used to identify individual *Prdm1*-expressing cells by microscopy or flow cytometry. Flow cytometry is a technique used to analyze the light scatter and fluorescence of individual cells in suspension as measures of relative size, internal structure, DNA content, gene expression, protein expression, membrane potential, and other characteristics. (Shapiro, 2003) A modification of flow cytometry is fluorescence-activated cell sorting, which allows for rapid isolation of distinct cells (e.g., fluorescent) from blood and other mixed populations of cells. The power of this reporter mouse, therefore, is in the ability of its T and B cells to communicate to an investigator when they have participated in an immune response. This gives the researcher the ability to identify individual cells for isolation and analysis.

Animals that are homozygous carriers of transgenes can sometimes suffer from genetic deficiencies. For this reason, breeding of transgenic animals typically involves pairing one hemizygous carrier animal plus one wild type animal of the opposite sex to produce offspring that are approximately 50% hemizygous carriers and 50% non-carriers. In order to identify hemizygous carrier animals for use in experiments or for continued breeding, genetic analysis [e.g., by polymerase chain-reaction (PCR) analysis] is typically performed on tissue samples of each offspring.

PCR analysis is the benchmark technique used to identify transgene carrier animals. (Bonaparte et al., 2013) In this technique, a mouse's DNA is first extracted from a tissue sample. The isolated DNA is then incubated with DNA polymerase, DNA primers, and other reagents under reaction conditions that are optimized for DNA synthesis. Because the primers target specific DNA sequence for synthesis by DNA polymerase, and synthesized products increase 2-fold in number with each cycle of temperature changes, multiple reaction cycles can result in more than a million-fold amplification of a specific DNA sequence. Because of this, the amplified DNA can be easily detected using electrophoresis or other techniques. In the case of the eYFP animal, PCR generates a DNA product of 400 base pairs whereas no product is produced from the tissue of non-carrier animals. Although the PCR technique is widely used, it is both costly and time-consuming (i.e. days) to perform, with commercial costs exceeding $5.00 per sample. (Mouse Genotype)

The goal of this study was to determine if flow cytometry could be used as a less costly and more time-efficient technique for identifying eYFP transgene carrier offspring at the University of Northern Colorado (UNC). Because some T and B cells in eYFP transgenic mice fluoresce yellow, and because T and B cells are found in blood, we predicted that B6.Cg-

Tg(Prdm1-EYFP)1Mnz/J mice would have detectable eYFP-expressing cells in their blood. We show here that blood leukocytes of eYFP animals (identified by PCR) contained a significantly larger percentage of yellow fluorescent cells (eYFP+) than did blood leukocytes from wild type C57Bl/6J mice. These results were generated in our laboratory within six hours for a cost of $7.85 per animal. By contrast, PCR analysis cost $16.00 per animal and required approximately 20 hours of labor over three days. We conclude that flow cytometry for identifying eYPF animals is both more time- and cost-effective than PCR at UNC.

Methods/Materials:

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (National Research Council, 1996). The protocol (#1911B) was approved by the Institutional Animal Care and Use Committee at UNC. All efforts were made to minimize suffering.

Animals

Breeding stocks of wild type C57Bl/6J and B6.Cg-Tg(Prdm1-EYFP)1Mnz/J mice were obtained from Jackson Laboratories and maintained at the Animal Research Facility at UNC, on a 12-hour (7 AM/7 PM) light/dark cycle. Individual breeder cages were established containing one adult (≥6 weeks of age) wild type C57Bl/6J mouse and one adult hemizygous B6.Cg-Tg(Prdm1-EYFP)1Mnz/J mouse of the opposite sex. Male and female offspring were weaned and separated at ≥3 weeks of age. All animals were housed in Optimice® cages containing Tek-Fresh bedding (Envigo, 7099) and provided with food (Envigo, Rodent Diet 2016) and deionized water ad libitum. A total of 17 adult animals (aged six weeks to one year) were randomly

selected from among the available populations for use in this study and included 10 wild type mice (two males and eight females) and seven eYFP transgenic mice (three males and four females).

Blood collection and flow cytometric analysis

Blood was collected from mice using the method of Golde et al. (2005). Erythrocytes were removed from blood using the ACK lysis method. (Bossuyt et al., 1997) Approximately 100,000 leukocytes per sample were prepared for flow cytometry in Staining Buffer (BioLegend, Cat. #420201) as previously described. (Mbow et al, 2001) Fluorescence and light scatter data were collected using an Attune NxT V6 flow cytometer. (Thermo) In some samples, leukocytes were identified by their expression of CD45, a marker expressed on all leukocytes. (Shapiro, 2003) Briefly, these samples were prepared by incubating with anti-CD16/32 (BioLegend, Cat. #101302) to block non-specific binding of other antibodies, followed by staining with APC-anti-CD45 (BioLegend, Cat. #103111, 0.05 mcg for 15 minutes). CD45 expression was analyzed using data collected in the RL1 fluorescence channel. (Figure 2A) CD45-expressing populations were then identified within the multiple populations evident by light scatter (forward scatter versus side scatter, Figure 2B). The largest population of blood leukocytes in mice is comprised of lymphocytes. (O'Connel et al., 2015) Subsequently, for all samples, eYFP data (collected in the BL1 fluorescence channel) was collected for single cells contained within the light scatter lymphocyte gate. (Figures 2C and 2D) The number of lymphocytes analyzed ranged 2300-30,000 per sample (mean of 11,400). Two metrics were analyzed: 1) the percent of lymphocytes expressing eYFP (above a pre-established level based on control eYFP- cells, Figure 2E) and 2) the geometric mean of fluorescence intensity (MFI) for eYFP+ lymphocytes.

Tail tissue collection and polymerase chain reaction analysis

Tail digestion solution was composed of 50 mM Tris (pH 8.0) with 50 mM EDTA, 100 mM NaCl, and 1% (v/v) SDS. Tail salts were composed of 4.21 M NaCl, 0.63 M KCl, and 10 mM Tris (pH 8.0). Tail tissue was collected from each mouse using the method of Sysol et al. (2013). Tail tissue was incubated at 55° C overnight in tail digestion solution. Supernatant was isolated from each sample by centrifugation (10,000 x g), diluted to 0.71X with tail salts, and incubated at 4° C for four hours. The DNA was precipitated with two volumes of 100% ethanol at -20° C for one hour, and the pellet was subsequently dissolved in water. This DNA was used directly in PCR.

PCR was performed at UNC as described previously (Worley et al., 2015), and PCR products were visualized in 1.8% agarose gels using ethidium bromide epifluorescence. PCR primer sequences for mouse beta actin were obtained from RTPrimerDB.org (pair ID #2848) and for the eYFP transgene from Jackson Laboratories (#15730 and #15731). The sequences were: 1) beta actin forward (ATG CTC CCC GGG CTG TAT), 2) beta actin reverse (CAT AGG AGT CCT TCT GAC CCA TTC), 3) transgene forward (TTC CAC AGC TCT GAG GGT CT), 4) transgene reverse (CGG TGG TGC AGA TGA ACT T).

Statistical analysis

Statistical analysis of flow cytometry data (% eYFP or MFI) was performed using SigmaPlot 14. (Systat Software, Inc.) Shapiro-Wilk tests were used to determine if data sets varied significantly from the pattern expected of normally-distributed data. Statistical hypothesis testing was performed using 2-way analysis of variance (ANOVA) with day of analysis and transgene carrier status (positive or negative by PCR) as group variables. Differences between means were considered significant at $P < 0.05$.

Google search

The Google internet search engine was used to identify commercial services for genotyping mice (keywords: pcr, genotyping, cost) on May 5, 2021. About 11,000,000 results were generated. The first result that was not an advertisement but was a commercial provider of PCR services (indicated by a title of "mouse genotype") was selected as a source of information on the cost and time required for performing mouse genotype analysis by PCR. (Mouse Genotyping)

Results

Transgene expression analysis

By PCR analysis, seven mice were found to be transgene carriers (identification of a 400 bp transgene band in an electrophoretic gel) and 10 were found to be wild type (absence of transgene band). All 17 animals were found to carry the beta actin gene (174 bp band). Representative PCR results are shown in Figure 1. Flow cytometric analysis (see gating strategy, Figure 2) demonstrated that blood lymphocytes from wild type animals contained an average of 0.18% eYPF+ cells, whereas the blood lymphocytes from transgene carrier animals contained a significantly larger fraction that averaged 1.54%. (Figure 3) For this metric, there was no overlap of data points between the two groups of mice. None of the wild type animals contained more than 0.42% eYFP+ blood lymphocytes, and none of the transgene carrier animals contained less than 0.70% eYFP+ cells. Our flow cytometric analysis also demonstrated that the eYFP+ MFI values were significantly different between wild type (mean of 252) and transgene carrier animals (mean of 514), but the range of data points for these two groups overlapped. (Figure 3)

Cost analysis

The cost of genotyping tail tissue by PCR using a commercially available service was determined by extracting information from MouseGenotype.com. (Google search, see Methods) On May 5, 2021, the list price per sample was indicated as $5.50, not including shipping, with results returned within 24-72 hours after the receipt of samples. Shipping costs for a Flat Rate Envelope ($7.40, adequate for 20 samples) were obtained from the United States Postal Service website (USPS.com) with an estimated delivery time of 1-3 days. Thus, the cost per animal when using a commercial genotyping service was $5.87 for a batch of 20 tail tissue samples. The estimated time required for analysis was 3-7 days including tissue processing at UNC (one day), shipping, and PCR followed by transmittal of the data.

At UNC, the per animal cost of analysis was estimated for 20 animals per batch. PCR analysis was typically completed within 20 hours over the course of three days. The cost of labor was based on a standard $15.00 per hour wage that is used for undergraduate research assistants. Therefore, labor contributed $15.00 to the per animal cost of PCR analysis ($15/hr × 20 hrs ÷ 20 mice). Reagents for DNA isolation, PCR, and gel electrophoresis included various buffers, enzymes, and other chemicals (see Methods) and disposable plastics that were estimated to cost $1.00/mouse. Thus, the estimated cost of the PCR method at UNC was $16.00 per mouse.

At UNC, flow cytometric analysis was typically completed within six hours over the course of one day with a per animal cost of $7.85. For a batch of 20 samples, this included a labor cost of $4.50/mouse ($15/hr × 6 hrs ÷ 20 mice), a reagent/supply cost of $2.85/mouse (e.g., buffers, disposable plastics), and a flow cytometer use fee of $0.50/mouse. This cost estimate did not include the expense of staining for CD45 expression. This cost analysis revealed that,

when performed at UNC, the flow cytometric method could be performed for approximately half the cost of the PCR method.

Discussion

Using flow cytometric analysis of blood lymphocytes, we determined that transgene carrier animals contained a significantly higher percentage of eYFP+ cells than did wild type animals. (Figure 3) Although the sample sizes were small (10 and seven for wild type and transgene carriers, respectively), the data ranges of the two groups for eYFP expression did not overlap, suggesting the potential for a robust metric for distinguishing between these two groups. We propose to use >0.5% eYFP+ blood lymphocytes as a criterion for identifying transgene carrier animals. Given that percent eYFP+ data for wild type mice was approximately normally distributed, and the prediction of the Central Limit Theorem that 99.7% of the values of a population will fall within three standard deviations (SD) of the mean, we suggest that the upper limit of the three SD range for percent eYFP+ data for wild type single lymphocytes (0.52%) is adequate as a point below which samples can be reasonably judged to have originated from wild type animals. (Armbruster and Pry, 2008) It therefore follows that samples with values above the mean plus three SD can be reasonably judged to be from transgene carrier animals. In contrast, the data ranges of the two groups for MFI did overlap. Therefore, this metric has less advantage for distinguishing between wild type and transgene carrier mice than does the percent eYFP metric.

In this study, the average fraction of blood lymphocytes expressing eYFP was approximately 1.5%. We have found no other published study that quantified this population in B6.Cg-Tg(Prdm1-EYFP)1Mnz/J mice. T and B cells make up approximately 90% of blood lymphocytes in mice. (Ridge et al., 2019) *Prdm1* is not expressed in naive or memory B cells.

(Angelin-Duclos et al., 2000) *Prdm1* is expressed in plasma cells and plasmablasts, but only plasmablasts are found in blood. (Wols 2005) *Prdm1* is expressed in activated and memory T cells (Fu et al., 2017), but the majority of CD4+ (65%) and CD8+ (75%) cells in blood are naive (Oughton et al., 1995). This suggests that a small number of blood lymphocytes would be expected to express eYFP in these transgenic animals. Future studies in this laboratory will determine the phenotypes of eYFP+ cells in mouse blood.

The estimated per animal costs for the two procedures, when performed at UNC, are subject to increase under different scenarios. For example, reducing the number of animals per batch would increase the per animal cost for both the PCR and flow cytometry methods primarily because the amount of time, and labor cost, required to complete the procedures for a batch of samples would be reduced only minimally. The cost of the flow cytometry method would be increased by approximately $0.63 per animal if each blood sample was stained with anti-CD45 antibody. Doing so could increase confidence in identifying lymphocytes and therefore reduce the potential error in estimating eYFP expression on those cells. Similarly, use of a cell viability dye (e.g., propidium iodide, Sytox) could be used to improve discrimination of dead cells (with potentially low eYFP expression levels), and this would increase the per animal cost by approximately $0.40. Addition of these different staining steps to the flow cytometry protocol would inflate the estimated cost of the technique to greater than the cost of PCR analysis performed at a commercial laboratory, but it would still be lower than the cost of performing the PCR technique at UNC. Given that the eYFP expression of blood lymphocytes from four of the 10 control animals was greater than 0.2%, unexpectedly high when little or none should be detected, additional improvements in this technique may be warranted. Using more costly approaches may improve the fidelity of detecting truly eYFP positive or negative cells,

but, as we have shown here, using unstained cells is sufficient for identifying transgene carrier mice with the lowest cost overall.

Conclusion

We conclude that flow cytometry is an adequate method for identifying eYFP transgene-carrier animals, and we estimate that 0.52% eYFP+ is the limit of detection when analyzing single blood lymphocytes from B6.Cg-Tg(Prdm1-EYFP)1Mnz/J mice. Moreover, when performed at UNC, the flow cytometry method was less costly in time and labor by approximately half when compared to the PCR method.

Figures and figure legends

Figure 1. Representative agarose electrophoretic gel data. Identification of a transgene carrier animal was obtained with the appearance of a 400 bp band in lanes marked TG primers. Appearance of a 174 bp band in lanes marked Actin primers indicates the presence of genomic DNA within the sample and confirms a successful reaction.

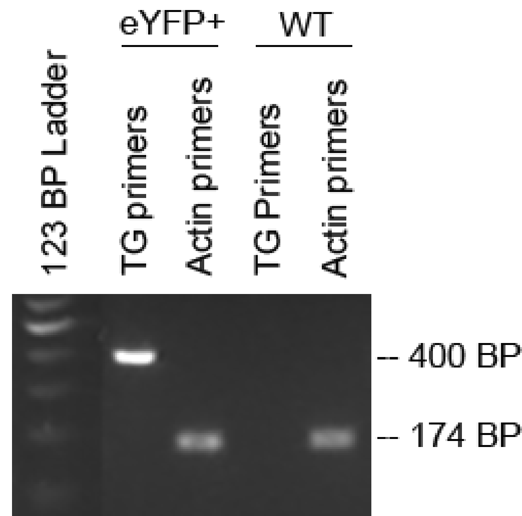


Figure 2. Cell populations and gating strategy for flow cytometric analysis of blood leukocyte eYFP expression. Representative data are shown for blood leukocytes that were isolated and analyzed by flow cytometry as described in Methods. **A.** Stained CD45+ cells (shown in blue) were identified by fluorescence as distinct from CD45 negative cells (red). **B.** Different light scatter populations of stained CD45+ cells (shown in blue – gated from panel A.) were identified, the largest of which is lymphocytes (70-80% of blood leukocytes; O'Connell et al., 2015). Control cells showing the CD45 negative populations are shown in red. **C.** Different light scatter populations of unstained cells are shown with a region identifying lymphocytes and their percentage as a fraction of total events. **D.** A single cell gate was established for the lymphocytes identified in Panel C. **E & F.** A region was established to identify eYFP+ single lymphocytes. The bounds of the region were set to include approximately 0.20% of single lymphocytes from wild type animals (Panel E). All eYFP transgenic animals showed >0.70% of cells within this region (Panel F, also see Figure 3).

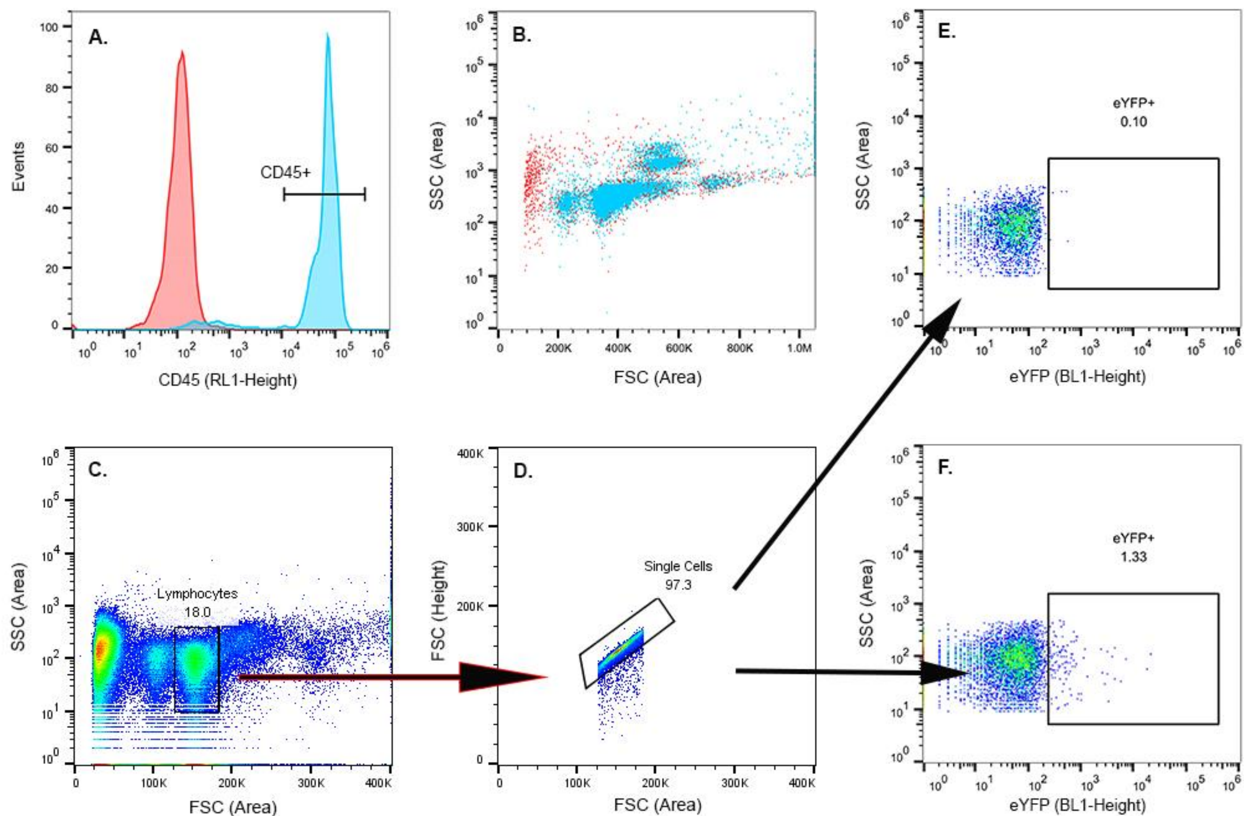
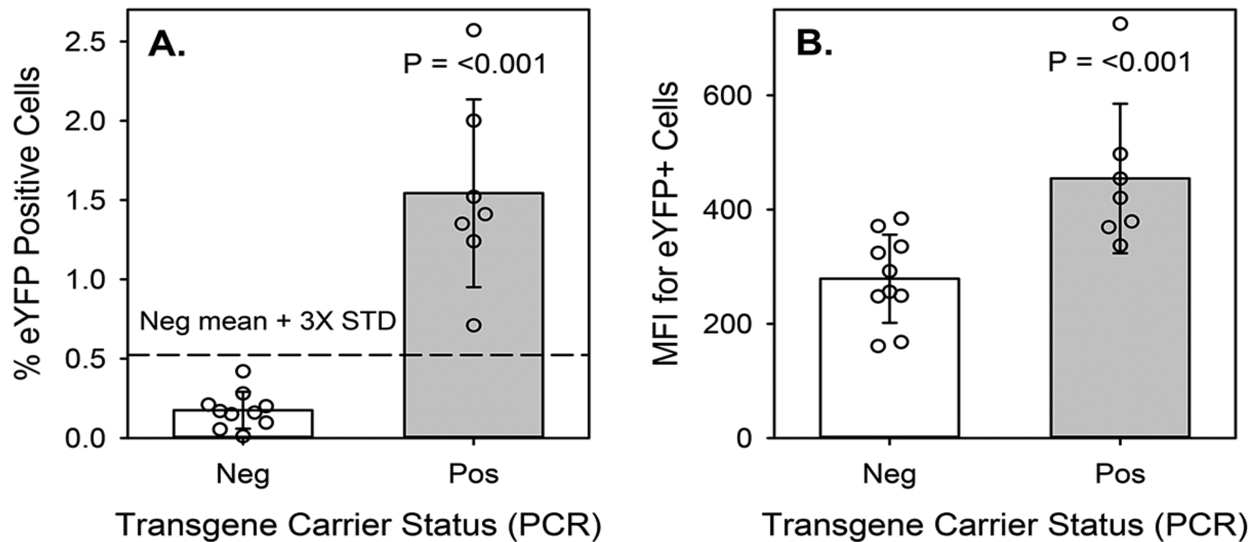


Figure 3. Transgene carrier status correlates with eYFP fluorescence. Blood leukocytes were isolated and analyzed by flow cytometry as described in Methods. Data in each panel represent means ± STD for 7-10 animals per group: wild type (Neg) or eYFP transgenic (Pos). **A.** The percentage of single lymphocytes falling within the eYFP region (defined in Figure 2, Panel E). The dashed line represents the estimated limit of detection for eYFP+ animals. **B.** The mean fluorescence intensity (MFI) of eYFP fluorescence for single eYFP+ lymphocytes per sample.



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