A mosaic analysis system with Cre or Tomato expression in the mouse

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Significance

Somatic mutations are the driving force of many age-related diseases such as cancer and hematopoietic failure. A challenge in the field is to evaluate health impact of somatic mutations prior to the appearance of disease symptoms. We describe a genetic tool named MASCOT (mosaic analysis system with Cre or Tomato) for mosaic analysis of somatic mutations that drive clonal hematopoiesis and lymphomagenesis. MASCOT can be applied to mosaic analysis of broad tissue types, and thus provides a valuable tool to aid functional dissection of somatic mutations in studies of development and disease.

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validated the MASCOT system in mosaic analysis of ID3, a transcription regulator with well-characterized roles in lymphopoiesis (22). Mosaic analysis of MASCOT not only confirmed cell-intrinsic roles of ID3 in regulating lymphopoiesis but also revealed a function of ID3 for regulating long-term homeostasis of colonic macrophages. Second, we used the MASCOT method to track the generation and long-term progression of CH induced by deletion of the Ten Eleven Translocation 2 (Tet2) gene (23). We found that CH induced by Tet2 deletion promoted myeloid expansion resembling clonal hematopoiesis of indeterminate potential (CHIP) (24). Third, we carried out mosaic analysis of Tet2/Id3 double mutations and revealed a strong impairment in lymphopoiesis caused by concurrent deletion of Tet and Id3. Fourth, the long-term tracking of mosaic mice revealed development of Tet2-deficient lymphoma, making the mosaic mice a model for studying the transition from CHIP to lymphoma. These proof-of-principle studies demonstrate that MASCOT is an effective tool for modeling CH and for functional dissection of somatic mutations relevant to human diseases.

Results

Construction of MASCOT Reporter Mice. In building a mosaic analysis system for tracking somatic mutations, we sought to satisfy three basic criteria: 1) Somatic mutations can be induced in any tissues at low frequency by tamoxifen treatment. 2) Both mutant and wild-type reference populations are generated concurrently and...
tagged with distinct lineage markers for lineage tracing. 3) The method must be applicable to the study of any single mutation or compound mutations by crossing to preexisting conditional alleles. With these design principles in mind, we have established the MASCOT reporter (Fig. 1A). This reporter contains the CAG (CMV enhancer chicken beta Actin promoter beta-globin first intron) promoter, which drives constitutive expression of either the Tomato marker or the Cre recombinase upon FLP/FRT-mediated recombination. The Cre cDNA is tagged at its 3′ end with irsGFP for lineage tracing of Cre expression. The Tomato and Cre coding sequences are flanked by two distinct pairs of FRT sites (25) with the internal FRT sites hopping over each other. Cre coding sequences are flanked by two distinct pairs of FRT sites (25) with the internal FRT sites hopping over each other. Cre coding sequences are flanked by two distinct pairs of FRT sites (25) with the internal FRT sites hopping over each other.

We then bred the Cot2 line with the R26F/ZsGreen strain (or R26Z) (18) and a modified version of the R26Z strain (or R26z, Materials and Methods) (28), which provide a tamoxifen-inducible FLP recombinase and a Cre-dependent ZsGreen reporter, respectively (SI Appendix, Fig. S2A). Tamoxifen treatment of Cot2+/R26Z mice yielded Tomato-positive and ZsGreen-positive cells that were readily detectable by fluorescent microscopy (Fig. 1B) and flow cytometry (Fig. 1 C and D). Staining with lineage markers confirmed mosaic patterns in multiple cell lineages including HSC, multipotent myeloid progenitors, myeloid, T, and B cells (Fig. 1C). To determine the range of labeling efficiency with the Cot2 reporter in blood cells, we tested varying doses of tamoxifen treatment of Cot2/Cot;R26Z mice (SI Appendix, Fig. S2 B–D). With one round of intraperitoneal delivery of tamoxifen, we typically obtained less than 5% of lineage-labeled cells. However, with three rounds of tamoxifen treatment, we observed up to 20–30% of lineage-labeled cells in the hematopoietic compartment. Among lineage-labeled cells, Tomato+/ZsGreen+ double-positive cells were also detected with expected frequency (assuming Cot2 on homologous chromosomes are independently activated, SI Appendix, Fig. S2D). The frequencies of Tomato+ and ZsGreen+ single-positive cells were also detected with expected frequency (assuming Cot2 on homologous chromosomes are independently activated, SI Appendix, Fig. S2D).

Mosaicism was also established in nonhematopoietic compartments such as colonic muscle and epithelium (Fig. 1E and SI Appendix, Fig. S2F). Colonic epithelium is made of individual crypts that undergo constant regeneration from stem cells
located at the bottom of each crypt. Study with Confetti mice has demonstrated that any mosaicism introduced into the stem-cell pool will drift toward singularity with time due to competition among stem cells within each crypt (29). Indeed, we found that lineage-labeled crypt cells were converted from isolated patches at 1 wk into uniformly labeled entire crypts at 2 mo post-tamoxifen treatment (Fig. 1E and SI Appendix, Fig. S2F). Thus, lineage tracing of the regeneration process of crypt epithelium with MASCOT produced a similar result as was reported with the Confetti mice (29).

**Mosaic Analysis of Clonal Deletion of the *Id3* Gene.** We next evaluated the effectiveness of MASCOT to induce mutant clones. We chose the *Id3*lox conditional allele to compare the MASCOT method with conventional genetic methods, since the effect of *Id3* deletion on lymphopoiesis has been extensively characterized with both *Id3* germline (22, 30) and conditional knockout mice (31). After crossing *Cot2* and *R26loxp* reporters to the *Id3*lox background, mice were treated with tamoxifen to produce mosaic clones. ZsGreen+ and Tomato+ cells were detected at approximately equal frequency in myeloid, B, and T cells in the blood. Q-PCR analysis of *Id3* expression in sorted B and T cells confirmed the loss of *Id3* expression in ZsGreen+ cells (Fig. 2A). A requirement for *Id3* in T cell receptor-mediated positive selection has been demonstrated in the study of *Id3* germline or conditional knockout mice (22, 31). Therefore, we examined the relative frequency of *Id3*-mutant cells before, during, and immediately after positive selection based on CD69+/TCRγδlow (St0), CD69+/TCRγδhigh (St1), and CD69+/TCRγδhi (St2) staining, respectively (Fig. 2B). A direct comparison between ZsGreen+ cells and Tomato+ cells showed that *Id3* deletion resulted in an increase in the frequency of St1 cells and corresponding decrease of St0 cells (Fig. 2C), indicating a development block during positive selection and a finding consistent with previous reports (22, 31). To gain a broader view of *Id3* function in the development and homeostasis of hematopoietic cells, we examined lineage-labeled cells in secondary lymphoid organs and gut lamina propria 1-y posttamoxifen treatment. Lineage-labeled ZsGreen+ or Tomato+ cells were found at similar frequencies for most hematopoietic lineages examined, including myeloid cells, T cells, B cells, innate lymphoid cells, natural killer T cells, and T regulatory cells (Fig. 2 D and E and SI Appendix, Figs. S3 A and B). One exception observed in this study was gut macrophages, which exhibited a significant increase in the population size of ZsGreen+ cells (Fig. 2E). Intestinal macrophages are a mixture of long-lived cells and newly replenished cells from circulating Ly6C+ monocytes that migrate into the gut (32). To further determine whether the enrichment of lineage-labeled colonic macrophages is a result of developmental regulation or long-term homeostasis, we evaluated mosaic frequency of monocytes at the midpoint of year-long lineage tracking. Numbers of *Id3*-deficient ZsGreen+ monocytes, both short-lived (Ly6Chi) and long-lived (Ly6Clo) either in blood or colon, were similar to those of their Tomato+ wild-type counterparts (Fig. 2F and SI Appendix, Fig. S3C). Thus, the age-dependent accumulation of *Id3*-deficient ZsGreen+ macrophages in colon is unlinked to ongoing replenishment of macrophages from circulating monocytes. It suggests that *Id3* deletion affects long-term homeostasis of preexisting tissue-resident macrophages in the gut. Collectively, our mosaic analysis of the *Id3* conditional allele illustrates the high sensitivity of the MASCOT method in detecting cell-intrinsic defects during development and long-term homeostasis.

**Tracking Clonal Hematopoiesis in Tet2 Mosaic Mice.** Next, we tested MASCOT in CH induced by *Tet2* deletion, a phenomenon that has been well-established in both humans and mice (23). *Cot2;R26loxp-;Tet2f/f* mice were produced through intercross of parenteral lines *Cot2;R26loxp-;Tet2f/f* and *Cot2;R26loxp-;Tet2f/f*, where *Cot2* can be either homozygous or heterozygous. Deletion of *Tet2* among the ZsGreen+ cells upon tamoxifen treatment was verified by Q-PCR analysis of *Tet2* mRNA in flow cytometry-sorted splenic CD11b+ myeloid cells (Fig. 3A). We then performed long-term tracking of lineage-labeled cells in a cohort of *Cot2;R26loxp-;Tet2f/f* mice by periodic tail-vein bleeding. We found that the frequency of ZsGreen+ cells in the blood rose gradually with age whereas the frequency of Tomato+ cells remained stable (Fig. 3B and SI Appendix, Fig. S4). Parallel analysis of myeloid and lymphoid lineage cells also revealed differential impacts of *Tet2* deletion on different hematopoietic lineages. The frequency of *Tet2*-deficient clones was significantly higher in the myeloid compartment than in the lymphoid compartment after 30 wk of lineage tracking (Fig. 3C).

Linear regression analysis of the growth curves within this time window further demonstrated differential impact of *Tet2* deletion on individual hematopoietic lineages (Fig. 3D). The expansion rates of lineage-tracked, *Tet2*-deficient myeloid, B, and T cells were 0.73, 0.23, and 0.08% per week, respectively. To determine whether mosaic frequency of blood lymphocytes reflected ongoing lymphopoiesis, we examined the frequency of *Tet2*-deficient T cells in the thymus and spleen. Lineage-labeled T cells in the thymus and spleen during this time window exhibited a similar change in frequency relative to the frequency of lineage-labeled myeloid cells as observed in the blood (Fig. 3E). We next examined clonal expansion in the bone marrow where myelopoiesis occurs. We found that clonal frequency of ZsGreen+ cells was similar among HSC, lin-cKit+ progenitors, and CD11b+ myeloid cells in the bone marrow (Fig. 3F and SI Appendix, Fig. S5). This result confirms that CH in *Tet2*-mosaic mice occurs in HSC and hematopoietic progenitors. Since the frequency of *Tet2*-deficient lymphocytes was significantly less than that of HSC and myeloid cells, we conclude that *Tet2*-deficient HSC promotes myelopoiesis while impairing lymphopoiesis. Collectively, our mosaic analysis confirmed the previous report of TET2 function in restricting HSC/myeloid expansion (11, 33, 34) and indicated a cell-intrinsic requirement of TET2 in lymphoid lineage development.

**Mosaic Analysis of *Id3* and *Tet2* Double Mutations.** To further examine the differential role of *Tet2* in regulating myelopoiesis versus lymphopoiesis, we sought to test whether *Tet2*-dependent lymphopoiesis could be further enhanced by a second mutation. In B cells, *Tet2* has been shown to directly interact with the E2A transcription factor, a key regulator of lymphopoiesis and the canonical target of *Id3* (35). In addition, conditional deletion of *Tet2* and *Id3* genes in T cells led to natural killer T cell lymphoma accompanied by an up-regulation of *Id3* (36). As reported in B cells (35), we found that TET2 and E2A also physically interact with each other in T cells (SI Appendix, Fig. S6). Thus, we tested the genetic interaction between *Tet2* and *Id3* in lymphopoiesis by generating and tracking *Tet2* and *Id3* double-deficient (DKO) mosaic clones in the blood (Fig. 4 A–C and SI Appendix, Fig. S7). Lineage tracking of ZsGreen+ myeloid cells at 30-wk posttamoxifen treatment showed a similar degree of expansion between *Tet2*/*Id3* DKO mosaic mice and *Tet2* single-deficient (SKO) mosaic mice (Fig. 4D). In contrast, lineage tracing revealed a decrease in expansion of ZsGreen+ B cells (Fig. 4E, *P* = 0.0002) and T cells (Fig. 4F, *P* = 0.006) in *Tet2*/*Id3* DKO mosaic mice over that of *Tet2* SKO mosaic mice. Linear regression analysis revealed that lineage-tracked DKO myeloid, B, and T cells expanded 0.66, 0.06, and 0.02% per week, respectively, within the first 32 wk posttamoxifen treatment (Fig. 4G). Based on the difference of observed expansion rates between SKO and DKO mosaic mice (SI Appendix, Fig. S8), we concluded that B and T cell development were further impaired after deletion of both the *Id3* and *Tet2* genes. Because B and T cell numbers were not altered in *Id3* mosaic mice (Fig. 2D), the severe impairment of lymphopoiesis in *Tet2*/*Id3* double-mosaic mice indicated a synergistic interaction between *Tet2* and *Id3* mutations in blocking lymphopoiesis. While further studies are
needed to determine whether this synergistic effect is caused by unchecked E2A activity in the absence of its interaction partners ID3 and TET2, the current finding demonstrates the feasibility of MASCOT for analysis of compound mutations.

**Modeling Lymphomagenesis with MASCOT.** In human studies, CH driven by Tet2 mutations has been linked to the development of AML (1, 3, 4). However, a recent study of pediatric patients with germline TET2 loss-of-function revealed development of lymphoma even though hematopoiesis of patient-derived stem cells displayed a skewing toward the myeloid lineage (37). We evaluated whether introduction of Tet2 deficiency in ZsGreen-labeled CD11b+ myeloid cells posttamoxifen treatment (Fig. 5A) exhibited splenomegaly and/or enlargement of lymph nodes (Fig. 5 D and E and SI Appendix, Fig. S9D). Histologic analysis also identified tumor masses in the liver (Fig. 5F). Fluorescence microscopy analysis of enlarged lymph nodes indicated high content of proliferating cells based on Ki67 staining and high content of Tet2 mutant cells based on ZsGreen expression (Fig. 5G). Fluorescence-activated cell sorter (FACS) analysis further confirmed high content of ZsGreen+ B cells (Fig. 5H) and the blasting phenotype of the B cells in the enlarged lymph nodes (SI Appendix, Fig. S9E). These findings complement a recent report that conditional deletion of Tet2 together with repeated immunization promotes B cell lymphomagenesis (38). However, many of our mosaic mice did not show tumor masses at the time of euthanasia even though they exhibited B cell expansion after 1 y of lineage tracking (SI Appendix, Fig. S9D). To test the possibility that the intact immune system in the mosaic mice suppresses malignant growth of the expanded B cells, we adoptively transferred splenocytes from two of the Tet2/Id3 mosaic mice exhibiting B cell expansion but without visible tumor masses into Rag2-deficient mice. Although unfractionated splenocytes were used in the transfer, both donors demonstrated exclusive expansion of B cells in the Rag2 hosts (Fig. 5 I and J), with development of aggressive lymphoma involving multiple organs, including liver and kidney (Fig. 5K). RNA analysis of tumor samples confirmed
clones in both mitotically active (e.g., blood and epithelium) and labeled daughter clones (17), MASCOT can produce mosaic MADM method which requires mitosis to produce differentially improvement over the MASTR method. In comparison with the separate marker to label a reference population represents an lineage development and long-term homeostasis. The use of a in identifying cell-intrinsic gene function in the contexts of

Discussion

Our mosaic analysis of Id3 and Tet2, either individually or in combination, demonstrated the utility of the MASCOT method in identifying cell-intrinsic gene function in the contexts of lineage development and long-term homeostasis. The use of a separate marker to label a reference population represents an improvement over the MASTR method. In comparison with the MADM method which requires mitosis to produce differentially labeled daughter clones (17), MASCOT can produce mosaic clones in both mitotically active (e.g., blood and epithelium) and quiescent tissues (e.g., muscle). Although the ratio between Tomato-labeled cells and ZsGreen-labeled cells is stochastically determined based on the binary outcomes of Flp/FRT-mediated recombination, it is fixed upon completion of tamoxifen treatment in any given animal. Thus, any changes in the relative ratio among sister lineages at different timepoints during longitudinal tracking would provide a sensitive readout for cell-intrinsic functions. In addition to confirming previous observations relating to the roles of Id3 and Tet2 in lymphopoiesis and clonal hematopoiesis, the MASCOT method enabled us to: 1) uncover a cell-intrinsic function of Id3 in regulating homeostasis of gut-resident macrophages; 2) define differential roles of Tet2 in myeloid vs. lymphoid lineage development; 3) analyze genetic interaction between Id3 and Tet2 in lymphopoiesis; and 4) establish a lymphoma model based on long-term progression from CH. Thus, MASCOT provides a useful genetic tool for dissecting cell-intrinsic phenotypes in developmental and cancer studies.

The mosaic analysis described here allowed us to simulate a broader window of CH driven by Tet2 deletion, from the initial expansion of mutation cells at a very young age to lymphoma development at much older ages. It is important to point out that mutations generated in the MASCOT mice involve a small number of cells whereas most CH are a result of clonal expansion from a single mutant. Despite this, at the population level, our study revealed that Tet2 deletion promoted expansion of the myeloid compartment, an outcome consistent with the role of TET2 in restricting HSC expansion demonstrated using the bone marrow chimeric approach (11, 33, 34). Our model also indicates that although Tet2 deletion initially suppresses lymphoid expansion, it promotes lymphomagenesis at a later stage. Previous studies of Tet2 germline knockout mice have observed high frequency of myeloid malignancies (39) and human chronic myelomonocytic leukemia-like disease (11). More recent study of B-lineage-specific Tet2 knockout reported Tet2-deficient B cell lymphoma in aged mice (38, 40). An important difference between our MASCOT mouse model and these other Tet2-knockout studies is the presence of an intact immune system in our Tet2-mosaic mice. The relatively low frequency of tumor incidence observed in our Tet2-mosaic mice suggests that an active immune surveillance is still in place even after an overt myeloid and lymphoid expansion. Thus, the MASCOT method could potentially be used as an immunocompetent model for preclinical investigation of lymphomagenesis and lymphoma treatment.

The current application of MASCOT relies on R26CreER to induce mosaicism at low frequency in nearly all tissue types and thus offers broad applications. While the study presented here focused on hematopoietic system, mosaic mice created by MASCOT may also be used for examining cell-intrinsic function in other organs, such as the colon (Fig. 1E), where lineage-marked colon epithelial clones are readily detectable with fluorescent microscopy. In comparison to the widely used CreER or MxCre system (15, 16), MASCOT adds an internal reference population that allows more accurate quantification of any change in clonal size within the same tissue. On the flip side, the current version of the MASCOT design requires more rounds of breeding than the inducible CreER and MxCre systems for experimental execution. Therefore, the latter ones would be the first choice in experiments when the goal is simply to generate lineage-marked mutant cells. The burden of multiple rounds of breeding associated with MASCOT could be reduced in the future by engineering MASCOT and R26CreER into the same locus. Another difference between the inducible Cre systems and our MASCOT systems is that Cre is transiently expressed in the inducible system and constitutively expressed in the mosaic analysis among mutant cells generated. One cannot rule out the possibility that Cre-associated toxicity may interfere with experimental readout. Therefore, it is critical to test the mosaic analysis system
Fig. 5. Lymphomagenesis in Tet2 and Tet2/Id3 mosaic mice after long-term tracking. (A) Representative lineage tracking of a Tet2 single and a Tet2/Id3 double-knockout mosaic mouse that underwent abrupt B cell expansion after long-term lineage tracking. (B) Tomato+ and ZsGreen+ cells in myeloid, B, and T cell compartments of Tet2 SKO mosaic mice are independently calculated as fold changes between the beginning (<1 mo) and the endpoint (>16 mo) of lineage tracking (n = 9, paired t test). (C) Comparison of labeling frequency of ZsGreen+ cells between 30 wk and the endpoints (over 16 mo) of lineage tracking (same group as in B, n = 9, paired t test). (D) Gross phenotypes observed in 6 mice out of 27 mice upon euthanasia. (E) Representative gross phenotypes of spleen are shown for a Tet2 SKO and a Tet2/Id3 DKO mosaic mouse in comparison with the spleen of a nonmosaic control mouse (Left). (F) Liver with white nodules was sectioned and stained with H&E (hematoxylin and eosin), with the insert showing a border area between lymphocytes and hepatocytes (red arrow). (G) Immunofluorescent image of a frozen section from an enlarged lymph node analyzed for ZsGreen (Left) and Ki67 expression (Right). DAPI channel is included in the Ki67 image. (H) Representative FACS analysis of myeloid (CD11b+) and B (B220+) cells in the blood and spleen of a mouse with tumors (n = 6). (I and J) Blood tracking after adoptive transfer of total splenocytes from two independent mosaic donors (recipients n = 7). Total B and T cell count (I) and lineage frequency of B cells (J) were tracked for 21 wk before terminal analysis when the mice became visibly sick. (K) H&E staining of tumor infiltrates in the liver and kidney at 21 wk.
on the wild-type background prior to using it in the mosaic analysis of mutant clones. One more caveat of using R26^{FloPoER} is that dominant clones arising in one cell lineage may interfere with mosaic analysis of other cell types in the same mice, particularly during long-term tracing of tumorigenesis. In our study of Tet2 mosaic mice, the fast-growing tumor types may prevent further study of other tumor types that develop in a slower pace. This issue could be addressed in the future by combining MASCOT with lineage-specific FlpoER strains that restrict mosaic analysis to designated tissue types.

In summary, the MASCOT method enables easy tracking and retrieving of mutant cells from different tissues at any point during lineage tracing for quantitative assessment of cell-intrinsic phenotypes. With a large number of loxp-based conditional strains already produced in the field, MASCOT-based mosaic analysis provides another valuable tool to aid functional dissection of somatically acquired mutations in studies of development and disease.

Methods

Animal Procedures.

Mouse strains and housing. The MASCOT reporter strain was produced in the Duke Transgenic Mouse Facility. The Tet2^{Cre} conditional allele (Jax mice # 017573 Tet2^{Cre/1}^1), the R26^{G2aCre} reporter (Jax mice # 007906, also known as Ai6), and the Flpo^{Th} strain (Jax mice # 019016) were purchased from the Jackson Laboratory. The Id3 conditional allele (31) was maintained in our breeding colony. All experimental mice were housed and bred in SPF barrier facilities managed by the Division of Laboratory Animal Research at Duke University. Animal protocols for the reported studies were reviewed and approved by Duke University Institutional Animal Care and Use Committee.

Modification of the R26^{G2aCre} strain. The original R26^{G2aCre} strain contains a pair of FRT sites flanking the reporter, in addition to the flox-stop cassette (Fig. S2). To avoid inadvertent deletion of the Zsgreen by Flp-mediated recombination, we mutated the downstream FRT site with Crispr-CAS9 microinjection of the guide RNA and CAS9 constructs into fertilized embryos. Leukocytes were analyzed on either Canto or FortessaFlow cytometer after lysing red blood cells and staining with CD3, CD24, CD11b, CD11c, F4/80, CD64, MHCII) for final analysis on a flow cytometer (FortessaX20, BD).

Blood tracking of mosaic clones. Approximately 10 μl of blood were collected from tail vein by bleeding with a 21-gauge needle at a frequency no more than once per week. Leukocytes were analyzed on either Canto or FortessaFlow cytometer after lysing red blood cells and staining with anti-CD45, B220, CD19, CD11b, CD11c, and CD3 antibodies in a single tube using multiple color analysis. Adoptive transfer. Spleens from donor mice were mechanically dissociated into single-cell suspensions and treated with red blood cell lysing buffer. After washing with 1x phosphate-buffered saline (PBS) twice, live nucleated cells were counted and resuspended at 2.5x 10^6 cells/mL in 1x PBS. 5x 10^6 cells in 200 μL were transferred into each Ragu2^{−/−} mouse via tail-vein injection.

**Immunofluorescence Microscopy.** Tissues were fixed in 4% paraformaldehyde for 24 h, dehydrated in 30% sucrose for 24 h, embedded and frozen in OCT cutting compound, and then cut into 8-μm frozen sections. Primary antibody antisense LacZ (abcam ab643100) and anti-Ki-67 (Abcam ab15580) were incubated at 4 °C for 24 h and then washed three times with 1x PBS. Slides were incubated with the secondary antibodies Alexa Fluor 647-conjugated goat anti-rat (e Bioscience, A-21247) and goat anti-rabbit antibody (Invitrogen, A32733) for 24 h at 4 °C in dark. After washing for three times, the slides were mounted with DAPI-containing mounting medium (VECTORLAB, H-1200). Images were collected with a Zeiss Axio imager.

**Q-PCR.** Splenocytes were stained with anti-CD3, anti-B220, and anti-CD11b antibodies before sorting for Tomato^−, Zsgreen^−, or double-negative populations. Cell pellets were lysed with RTzol (Invitrogen, 15596018). RNA was isolated with Zymo Direct-zol RNA Kits (Zymo Research, 2050), and cDNA was prepared with SuperScript III Cells Direct cDNA Synthesis Kit (Invitrogen, 18008020). Fast SYBR Green kits (Thermo, 4385612) were used for Q-PCR primers. Primers were 5′-gttgagctttttccggag-3′ for Id3_F and 5′-gctgcctggttctggta-3′ for Id3_R.

**Isolation of Intestinal Lamina Propria Lymphocytes.** The colon was dissected free of the fat and connective tissue, cut open longitudinally, washed clean, and cut into 1-cm small pieces. Intraepithelial lymphocytes were removed by washing with ethylenediaminetetraacetic acid (EDTA)-containing buffer for 10 min each. The resulting tissue was digested in prewarmed Collagenase VIII (Sigma, C2139) and DNase I (Sigma, DN25) buffer at 37 °C for 50 min. Lamina propria lymphocytes were isolated with 40 and 80% Percoll (Sigma, GE17-0891-01), and stained with a mixture of lineage antibodies (CD45, CD90, CD3, CD24, CD11b, CD11c, F4/80, CD64, MHCII) for final analysis on a flow cytometer (FortessaX20, BD).

**Data Availability.** All study data are included in the article and SI Appendix.

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