In vivo tracking of T cell development, ablation, and engraftment in transgenic zebrafish

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Transgenic zebrafish that express GFP under control of the T cell-specific tyrosine kinase (*lck*) promoter were used to analyze critical aspects of the immune system, including patterns of T cell development and T cell homing after transplant. GFP-labeled T cells could be ablated in larvae by either irradiation or dexamethasone added to the water, illustrating that T cells have evolutionarily conserved responses to chemical and radiation ablation. In transplant experiments, thymocytes from *lck-GFP* fish repopulated the thymus of irradiated wild-type fish only transiently, suggesting that the thymus contains only short-term thymic repopulating cells. By contrast, whole kidney marrow permanently reconstituted the T lymphoid compartment of irradiated wild-type fish, suggesting that long-term thymic repopulating cells reside in the kidney.

The zebrafish has emerged as an important vertebrate genetic model of development. Zebrafish embryos are transparent and develop rapidly *ex utero*, and most organ systems are fully developed by 5 days postfertilization (dpf). Forward genetic screens in the zebrafish have been instrumental in identifying gene mutations that affect development (1–3). Such genetic approaches have been used successfully to identify genes involved in patterning, regeneration, and the development of organs, including heart, eye, and blood (4, 5). Additionally, small-molecule-based screens in the zebrafish have identified chemicals that perturb normal development (6, 7), and drug screens have been proposed to identify compounds that suppress cancer-associated phenotypes (8–10).

Comparisons of fish and mammalian hematopoiesis indicate that the genetic programs underlying vertebrate blood development have been highly conserved throughout evolution (5). Like their mammalian counterparts, fish B cells express Ig proteins, and T cells express T cell receptor components (11); both of which require gene rearrangements mediated by the rag1 and rag2 proteins (12). Moreover, many homologs of the mammalian lymphocyte receptors have been identified in fish, including *CD3* (13) and the *CD8* coreceptor (14). Taken together, these findings suggest that T and B cell development in the fish relies on many of the same molecules and pathways used in mammalian lymphopoiesis (15, 16).

The development and functional anatomy of the thymus are also remarkably similar between fish and humans. The zebrafish thymic rudiment is completely developed by 60 h postfertilization (hpf) and by 68 hpf is colonized by T lymphocyte progenitors, which begin to transcribe *rag1* and *rag2* by 72 hpf (15, 17–19). As in mice and humans, mature T cells localize to the medulla of the adult zebrafish thymus, whereas immature T cells localize to the cortex (20).

The genetics underlying the development of the zebrafish lymphoid system is under active investigation, but functional studies of the zebrafish immune system have been limited by the lack of tools to facilitate the isolation and characterization of different lymphoid cell populations. For example, antibodies against cell lineage markers, such as CD2, CD19, T cell receptors, and Ig proteins, are not available, making the identification of zebrafish T and B cell populations difficult. Also, markers of discrete T cell subpopulations, such as CD3, CD4, and CD8, have yet to be identified in the zebrafish. Because of these limitations, studies in the zebrafish have relied on transgenic technology and on lineage-restricted gene promoters to drive the expression of GFP in specific blood cell populations (21, 22). Transgenic zebrafish lines that express GFP in lymphoid cells have been established with both the *rag1* and *rag2* promoters (23, 24); however, because *rag1* and *rag2* are expressed only in immature T and B cells, such lines cannot be used to track the development and migratory patterns of mature T cells.

Here we describe the generation of a transgenic zebrafish model in which the zebrafish T cell-specific tyrosine kinase (*lck*) gene promoter drives GFP expression in lymphocytes. Using *lck-GFP* and *rag2-GFP* transgenic fish, we identify T and B cell populations in the zebrafish. Using *lck-GFP* transgenic fish, we tracked the development of T cells in the zebrafish from embryogenesis to adulthood, evaluated the sensitivity of T cells in living larvae to chemical and radiation ablation, analyzed T cell homing in transplanted embryos, and assessed the hematopoietic engraftment potential of kidney marrow and thymus cell populations. The results of these experiments provide a foundation for studies of immune responses in the zebrafish.

Materials and Methods

Animals. Zebrafish maintenance, developmental staging, and *in situ* analysis were conducted as described (25, 26). The *cloche* (27) and *bloodless* (28) zebrafish mutant lines, which are deficient in hematopoietic stem cells, and *van gogh*, which lacks the thymus (29, 30), were used to assess T cell-specific expression of the *lck* gene at 4 dpf.

Isolation of cDNA Clones. A degenerate PCR strategy was used to isolate a fragment of the zebrafish *lck* cDNA. A full-length clone was obtained by screening a kidney cDNA library (Fig. 7, which is published as supporting information on the PNAS web site). Protein sequence comparisons were completed by using the Jotun Hein algorithm in the MEGALIGN program of the DNASTAR sequence analysis software package (DNASTAR, Madison, WI).

Isolation and Identification of the Zebrafish *lck* promoter. A genomic zebrafish *lck* clone (84-I6) was identified by screening a PAC library (Incyte, Palo Alto, CA) with the *lck* cDNA probe (*Supporting Text*, which is published as supporting information on the PNAS web site). The 5.5-kb sequence upstream of the *lck* start codon was obtained by sequencing the *lck* PAC clone, and PCR was used to amplify a 3.8-kb genomic fragment extending from the first noncoding exon to the translation initiation site contained within exon 2 (Fig. 1*A*). This fragment was cloned into

Abbreviations: dpf, days postfertilization; FACS, fluorescence-activated cell sorting; *lck*, T cell-specific tyrosine kinase.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY390224).

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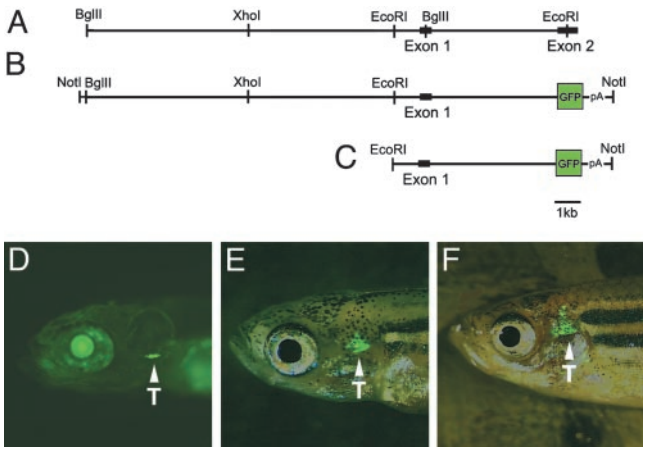

Fig. 1. *lck-GFP* transgenic zebrafish. Diagrams of the genomic DNA sequence comprising the *lck* promoter (*A*) and the GFP construct (*B*) are shown. Enzyme digest sites used for cloning and restriction mapping of the minimal promoter are shown. *lck-GFP* transgenic fish expressing the 5.5-kb *Eco*RI–*Not*I fragment (*C*) are shown at 8 dpf (*D*), 45 dpf (*E*), and 80 dpf (*F*). Arrowheads denote GFP-labeled cells in the thymus (T). The views are lateral with anterior to the left.

a pBluescript vector containing the enhanced GFP transgene (p1x EGFP). The EGFP coding sequence is followed by a SV40 polyadenylation sequence that terminates with a *Not*I restriction digest site.

Restriction enzyme mapping and Southern blot analysis of the *lck* PAC clone identified a 13-kb *Bgl*II genomic fragment that lies immediately upstream of the 3.8-kb fragment. This restriction fragment was cloned upstream of the 3.8-kb genomic p1xEGFP construct.

Generation of Stable Transgenic *lck-GFP* and *rag2-GFP* Transgenic Zebrafish. The *lck* promoter-containing vectors were digested with *Not*I alone or in combination with *Xho*I, *Eco*RI, or *Bgl*II (Fig. 1*B* and *Supporting Text*). Digestion products were resolved on an ethidium bromide-containing agarose gel, and DNA was purified (QIAquick gel extraction kit, Qiagen, Valencia, CA), resuspended in 0.5× Tris-EDTA buffer (pH 8.0) + 100 mM KCl, and injected as described (25). Transgenic *rag2-GFP* fish were generated as reported in refs. 24 and 25.

Immunocytochemistry and RNA *in Situ* Hybridization on Paraffin-Embedded Sections. Paraffin embedding and sectioning, *in situ* hybridization, and immunohistochemical analysis were performed essentially as described in ref. 25. cDNA probes were made by PCR amplification of plasmid DNA containing coding sequences for *rag1*, *rag2*, *IgLC3*, and *lck*, using primers containing T7 and Sp6 promoter sequences (Table 1, which is published as supporting information on the PNAS web site).

Fluorescence-Activated Cell Sorting (FACS) and RT-PCR Analysis. Kidney, thymus, spleen, and whole blood cell suspensions from wild-type AB or transgenic *rag2-GFP* or *lck-GFP* fish were analyzed by FACS based on forward and side scatter and GFP fluorescence as described in refs. 25 and 31. RNA was isolated from GFP-positive and -negative FACS sorted blood cell populations and used in reverse transcription reactions. Complementary DNA samples were analyzed by semiquantitative PCR with primers specific for *lck*, *Ig Light Chain 3* (*IgLC3*), *rag2*, and *β-actin* (Table 2, which is published as supporting information on the PNAS web site).

T Cell Homing in Transplanted Zebrafish Embryos. Whole kidney marrow or whole thymus cells were isolated from 8-week-old transgenic fish, filtered, resuspended in 0.9× PBS + 5% FBS, and injected into the sinus venosus of 2-day-old AB-strain embryos. Approximately 0.5–1.0 × 10^3 kidney marrow cells or thymus cells were injected per embryo (31). T cell homing was assessed by GFP fluorescence at 24 and 48 h posttransplantation.

T Cell Ablation by γ-Irradiation. Six-day-old *rag2-GFP* and *lck-GFP* transgenic embryos were given a whole-body dose of γ-irradiation (15 Gy from a ^{137}Cs source) and analyzed for GFP expression within the thymus at 1, 2, and 3 days posttreatment.

T Cell Ablation by Dexamethasone Treatment. Five-day-old *rag2-GFP* and *lck-GFP* transgenic embryos were treated with dexamethasone [250 $\mu g \cdot ml^{-1}$ (1% ethanol), 100 $\mu g \cdot ml^{-1}$ (0.4% ethanol), or 25 $\mu g \cdot ml^{-1}$ (0.1% ethanol)] or with ethanol vehicle alone (1%, 0.4%, and 0.1%) in egg water (60 $mg \cdot liter^{-1}$ instant ocean in distilled water containing methylene blue). Fish were analyzed at 1, 2, and 3 days posttreatment for T cell ablation as detected by loss of GFP fluorescence in the thymus.

Hematopoietic Cell Transplantation into Adult Zebrafish. Nonirradiated and irradiated (23 Gy from a ^{137}Cs source 2 days before transplantation) 2-month-old AB wild-type fish were injected i.p. with either whole kidney marrow cells (3 × 10^5) or cells isolated from the thymi (1.0–1.5 × 10^6) of transgenic *rag2-GFP* and *lck-GFP* fish. Cells were resuspended in 0.9× PBS + 5% FBS, and 5 μl was injected i.p. with a 10-μl Hamilton syringe. Radiation doses of 20–23 Gy were chosen because these are sublethal, have been shown to be immunoablative in adult zebrafish (D.T., A. Winzeler, H. M. Stern, E. A. Mayhall, D.M.L., J. L. Kutok, A.T.L., and L.I.Z., unpublished data), and were used successfully for engraftment of T cell leukemias into irradiated fish (25).

Results

***lck* mRNA Expression Is Confined to T Cells Throughout Development.** Developmental expression of *lck*, *rag1*, and *rag2* RNA was analyzed by using whole-mount *in situ* hybridization. *lck* mRNA expression was first detected in T cells located in the bilateral thymic lobes by 3 dpf (Fig. 8 *C* and *G*, which is published as supporting information on the PNAS web site), and by 7dpf, the number of *lck*-positive T cells in the thymus had increased (Fig. 8 *D* and *H*). The pattern of *lck* expression was similar to that of *rag2* (Fig. 8 *A*, *B*, *E*, and *F*), but *lck* was not detected in the epithelial cells of the olfactory placode, as has been described for *rag1* and *rag2* (17, 23, 24), nor was *lck* detected in *cloche*, *bloodless*, or *van gogh* zebrafish mutants (Fig. 9, which is published as supporting information on the PNAS web site, and data not shown), all of which have defects resulting in the absence of T cells or the thymus (29, 30, 32).

To further analyze the anatomic subcompartments of the adult zebrafish thymus, we analyzed thymic sections from 3-month-old fish by RNA *in situ* hybridization for *lck*, *rag1*, *rag2*, and *T cell receptor alpha* (*TCR-α*) expression. *lck* transcripts were detected in cells throughout the thymus (Fig. 10, which is published as supporting information on the PNAS web site), whereas *rag1* and *rag2* transcripts were selectively expressed in the cortex, and *TCR-α* was predominantly localized to cells found in the medulla and the corticomedullary junction (20) (Fig. 10).

Generation of Stable *lck-GFP* Transgenic Zebrafish. A 17-kb genomic fragment 5′ to the *lck* translation start codon (Fig. 1 *A* and *B*) was found to drive expression of GFP in the T cells of transiently injected embryos (data not shown). To identify the minimal promoter fragment that is required for T cell-specific expression, we truncated the GFP-containing DNA construct, using *Not*I

with *Xho*I, *Eco*RI, or *Bgl*II (Fig. 1*B*). The 11-kb *Xho*I/*Not*I and the 5.5-kb *Eco*RI/*Not*I fragments drove GFP expression in the thymic compartment, whereas the 3.8-kb *Bgl*II/*Not*I fragment was inactive (data not shown). Four stable *lck-GFP* transgenic zebrafish lines expressing the 5.5-kb *Eco*RI/*Not*I restriction fragment were generated (Fig. 1*C* and *Supporting Text*), two of which showed strong T cell-specific expression and were used for subsequent analyses (Fig. 1 *D–F*).

Localization of GFP-Labeled Lymphocytes in *lck-GFP* and *rag2-GFP* Transgenic Zebrafish. Because the location of T and B cell populations in adult zebrafish is largely unknown, we used anti-GFP immunostaining to identify sites of T and B cell accumulation in paraffin-embedded sections of adult transgenic fish. GFP-positive lymphocytes were found in the thymus (Fig. 11 *A* and *G*, which is published as supporting information on the PNAS web site), kidney (Fig. 11 *B* and *H*), and spleen (Fig. 11*F* and data not shown) of both *lck-GFP* and *rag2-GFP* adult fish. In adult *lck-GFP* transgenic fish, such cells were also detected in the gastrointestinal lining (Fig. 11*C*), the esophagus, gills, at the base of the olfactory epithelium (Fig. 11*D*), and surrounding regressing oocytes in the ovary (Fig. 11*E*). Except for the nose, none of these sites in *rag2-GFP* fish contained GFP-labeled cells (Fig. 11 *I–K*), indicating that immature T and B lymphocytes do not develop in or migrate to these tissues. In the nasal epithelium *rag2-GFP*-positive cells were histologically of nonlymphoid origin, and their location near the surface of the nasal epithelium did not overlap with that of *lck-GFP*-positive cells. We therefore conclude that the GFP signal in the nasal epithelium of *rag2-GFP* adult fish emanates from nonlymphoid tissues (Fig. 11*J*).

***lck-GFP* Expression Identifies Mature T Cells in the Kidney.** Although the dogma in mammals is that T cells develop in the thymus, it is unknown whether T cell development occurs in tissues other than the thymus of the adult zebrafish. Given that the fish lack bone marrow and that the kidney is the primary site of definitive hematopoiesis in the fish, we wanted to determine whether immature T cells reside in the kidney marrow.

Suspensions of kidney cells consisted of ≈29% lymphocytes (Fig. 2 *A* and *C*). GFP-positive cells were largely confined to the lymphoid cell compartment as defined by Traver *et al.* (31) for both the *rag2-GFP* and *lck-GFP* transgenic kidney (Fig. 2 *B* and *D*). GFP-positive lymphocytes represented a much higher percentage of the total kidney lymphoid cells in *rag2-GFP* compared with *lck-GFP* transgenic fish (mean 38.8 ± 8.8% vs. mean 18.4 ± 5.9%, respectively), suggesting that *rag2*-expression identifies a distinct subset of lymphocytes in the kidney. RT-PCR analysis of FACS-sorted GFP-positive and GFP-negative kidney cells showed that GFP-labeled cells from *rag2-GFP* fish express both *rag2* and *IgLC3*, but not *lck*, whereas GFP-labeled cells from *lck-GFP* fish do not express either *IgLC3* or *rag2* (Fig. 3). By contrast, GFP-negative blood cells in the *rag2-GFP* kidney express *lck* (Fig. 3) and low levels of *IgLC3* when RT-PCR cycle number is increased (data not shown). Taken together, these results indicate that GFP-labeled kidney cells in *rag2-GFP* fish are B cells, whereas those in *lck-GFP* fish are mature T cells.

FACS analysis of *rag2-GFP* and *lck-GFP* thymocytes revealed that the thymus harbors ≈73% lymphocytes (Fig. 2 *E* and *G*), with two distinct subpopulations of T cells (Fig. 2 *F* and *H*). Both subpopulations showed essentially the same granularity based on side scatter but differed markedly in cell size based on forward scatter. This finding suggests that zebrafish lymphocytes may change in size as they mature, in agreement with the positive correlation between lymphocyte size and maturational stage of development in mammals.

Whole blood and spleen samples from 10-week-old *lck-GFP* fish were analyzed for GFP expression by FACS. The spleen contained a mean of 3.2 ± 1.0% GFP-positive T cells, compared

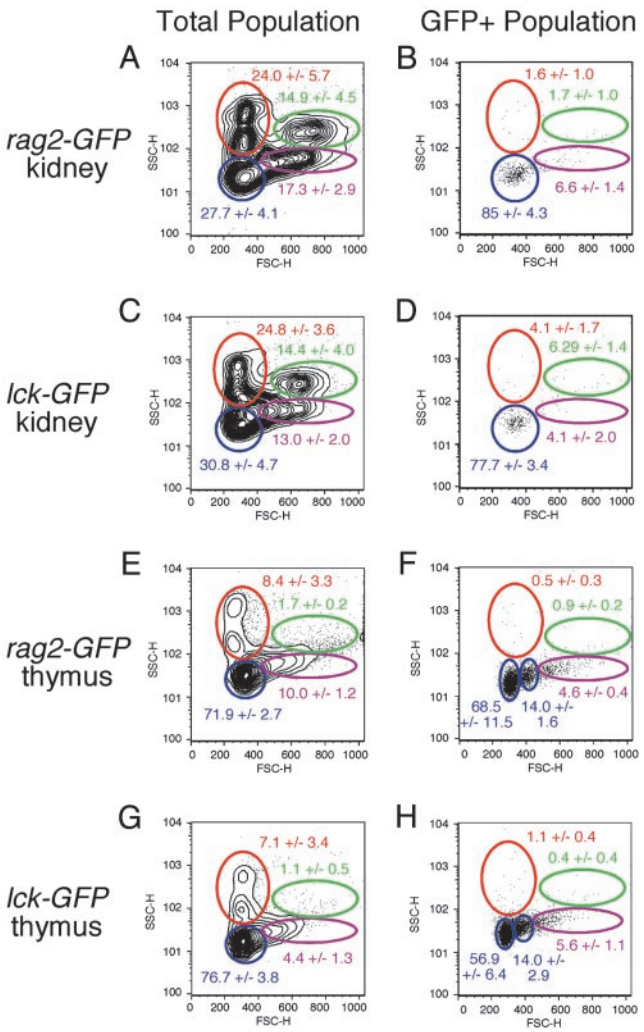

Fig. 2. FACS analysis of cells from the kidney (*A–D*) and thymus (*E–H*) of *rag2-GFP* and *lck-GFP* transgenic zebrafish. Gated populations of erythrocytes (red), lymphocytes (blue), granulocytes and monocytes (green), and blood cell precursors (purple) are outlined. Populations of cells within each gate are described as percentages of total live cells (±1 SD; $n = 24$ for *A*; $n = 18$ for *C*; and $n = 8$ for *B*, *D*, and *E–H*). Cell size is represented by forward scatter (FSC), and granularity is represented by side scatter (SSC). GFP-positive cells in the progenitor gate in thymus samples became confined to the lymphoid gate upon reanalysis, confirming that GFP-labeled populations in the progenitor gate are predominately lymphoid in origin.

with 0.07% in whole blood (data not shown), indicating that only a minority of T cells are in circulation at any given time, as in humans and mice.

GFP-Labeled T Cells Home to the Thymus After Transplantation. Knowledge of the homing properties of transplanted zebrafish lymphocytes will be important for successful use of this model in studies of the immune system. Thymocytes isolated from adult *lck-GFP* fish homed to the thymus when injected into 2-day-old embryos (Fig. 4 *A* and *B*). Twenty-four hours after injection into the sinus venosus, the GFP-labeled cells were found in the thymus, in the circulation (Movies 1 and 2, which are published as supporting information on the PNAS web site), and in a region of the tail (Fig. 4*C*) where macrophages reside and hematopoietic activity takes place (32). These results indicate that a subset of thymocytes have the capacity to enter circulation and home to the thymus of 3-day-old zebrafish. Transplantation experi-

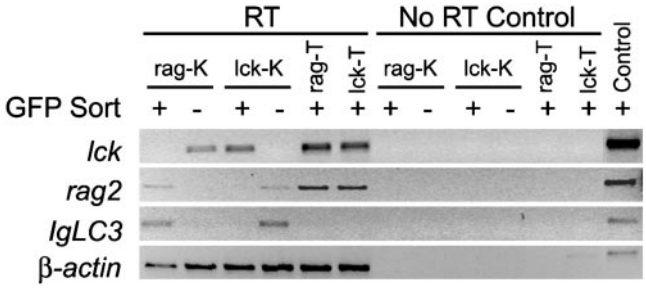

Fig. 3. Semiquantitative RT-PCR analysis of FACS-sorted blood cell populations in the kidney (K) and thymus (T) of *lck-GFP* and *rag2-GFP* transgenic fish. GFP-positive (+) and -negative (−) blood cell populations are shown. Results for ''No RT Control'' show absence of genomic DNA contamination in samples. The *β-actin* PCR control was completed on genomic DNA. Because the *β-actin* primers span an intron, PCR amplifies a 100-bp larger fragment than seen in RT samples.

ments in adult fish confirm that GFP-labeled T cells can home to the thymus, kidney, and peripheral sites of T cell accumulation (*Supporting Text*).

Thymic Engraftment Requires Ablation of the Immune System. To visualize T cell ablation, *lck-GFP* larvae were treated with a single dose of γ-irradiation (15 Gy) administered at 6 dpf. By 8 dpf, the nonirradiated control fish retained GFP-labeled T cells (Fig. 5*A*), whereas fish treated with γ-irradiation had lost them (Fig. 5*B*). T cells from adult fish were also radiation-sensitive and could be ablated with γ-irradiation (*Supporting Text*).

Irradiation not only ablates the thymic cell compartment but also causes ablation of hemato-lymphoid cells in the kidney (D.T., A. Winzeler, H. M. Stern, G. A. Mayhall, D.M.L., J. L. Kutok, A.T.L., and L.I.Z., unpublished data). Thus, we wanted to test whether hematopoietic cell engraftment requires that recipients be irradiated before transplantation. *rag2-GFP* kidney marrow (3×10^5 cells) was injected into sublethally irradiated and nonirradiated recipient fish. Fluorescent microscopy enabled us to visualize reconstitution of the lymphoid compartment through GFP expression by thymic T cells. Kidney marrow cells from the *rag2-GFP* fish failed to engraft into nonirradiated

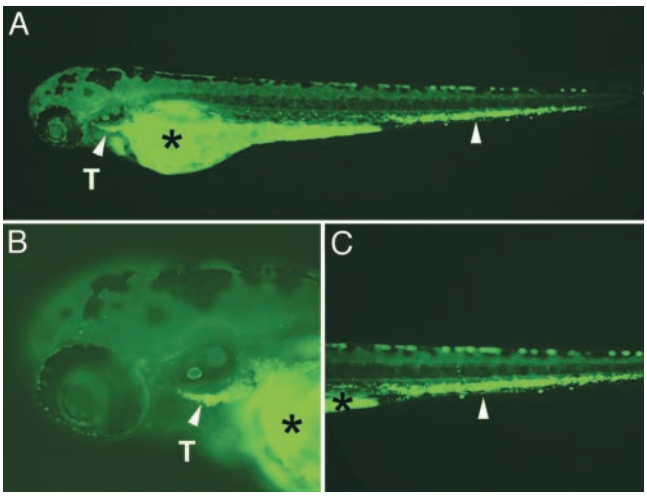

Fig. 4. GFP-labeled thymic T cells obtained from adult *lck-GFP* transgenic fish home to the thymus of transplanted embryos. (*A*) GFP fluorescent microscopic images of a 4-day-old transplanted embryo (anterior to the left and dorsal to the top) are shown. (*B* and *C*) Enlarged views of the head and tail region, respectively. Arrowheads denote GFP-labeled cells in the thymus (T) and in the tail region. Asterisks denote autofluorescence of the yolk sac.

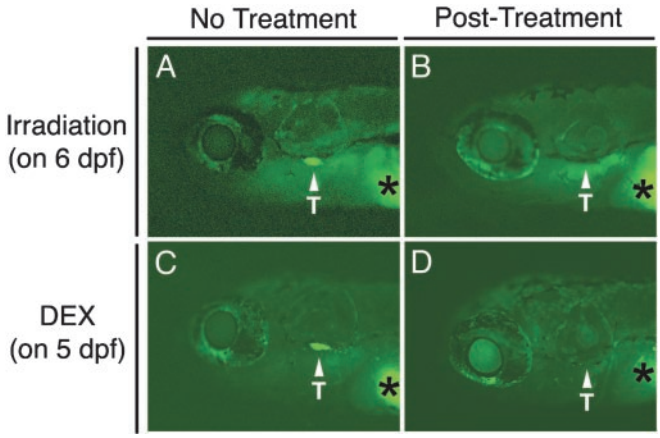

Fig. 5. GFP-labeled T cells in 8-day-old *lck-GFP* fish are ablated in response to γ-irradiation or dexamethasone treatment. (*A*) Nonirradiated control fish. (*B*) Fish 2 days postirradiation. (*C*) Control fish with 0.4% ethanol. (*D*) Fish 3 days after treatment with 100 $\mu g \cdot ml^{-1}$ dexamethasone (DEX). Asterisks denote autofluorescence of the yolk sac. Arrowheads denote GFP-labeled cells in the thymus (T). The views are lateral with anterior to the left.

recipient fish analyzed at 14 and 25 days postinjection (0 of 12), but engrafted well into γ-irradiated recipient fish (10 of 10) (Fig. 12, which is published as supporting information on the PNAS web site).

T Cells Can Be Ablated by Dexamethasone. To test whether zebrafish thymocytes are susceptible to chemical ablative agents, we treated *lck-GFP* transgenic fish with 250 $\mu g \cdot ml^{-1}$ or 100 $\mu g \cdot ml^{-1}$ of dexamethasone at 5 dpf. Control larvae treated with the ethanol vehicle retained GFP-labeled T cells at 8 dpf (Fig. 5*C*), whereas all surviving dexamethasone-treated larvae lacked these cells in the thymus when analyzed at 8 dpf (Fig. 5*D*). Lower doses of dexamethasone were able to ablate GFP-labeled T cells in transgenic larvae (Table 3 and *Supporting Text*, which are published as supporting information on the PNAS web site). These results indicate that 5- to 8-day-old zebrafish embryos are able to absorb chemicals from the water and that immature GFP-labeled T cells are responsive to chemical ablation by dexamethasone.

The Kidney Marrow Contains Long-Term Thymic Repopulating Cells. Next we asked whether long-term thymic repopulating cells reside in the kidney marrow or the thymus. Kidney marrow (3×10^5 cells) and thymic cell (1.0 to 1.5×10^6 cells) populations from *lck-GFP* fish were transplanted into 2-month-old irradiated recipient fish. GFP-positive cells were detected in the thymus of both transplant groups by 14 days posttransplantation (Fig. 6 *A* and *C*). Seven of ten fish transplanted with *lck-GFP* kidney marrow showed strong expression of GFP-positive T cells in the thymus by 14 days posttransplantation, compared with only 4 of 11 fish transplanted with cells from the thymus, indicating weak T cell reconstitution. By 24 days posttransplantation, the GFP-positive T cells could no longer be detected in fish transplanted with thymic cells (Fig. 6*B*), whereas fish given whole kidney marrow maintained GFP expression for greater than 6 months (Fig. 6*D*), the last time point at which the fish were analyzed.

Discussion

We have taken advantage of the fact that *lck* is expressed in both immature and mature T cells, and that the *rag1* and *rag2* genes are expressed in immature B and T cells, to characterize the zebrafish lymphoid system by using *lck-GFP* and *rag2-GFP* transgenic lines. GFP expression in the *lck-GFP* transgenic line

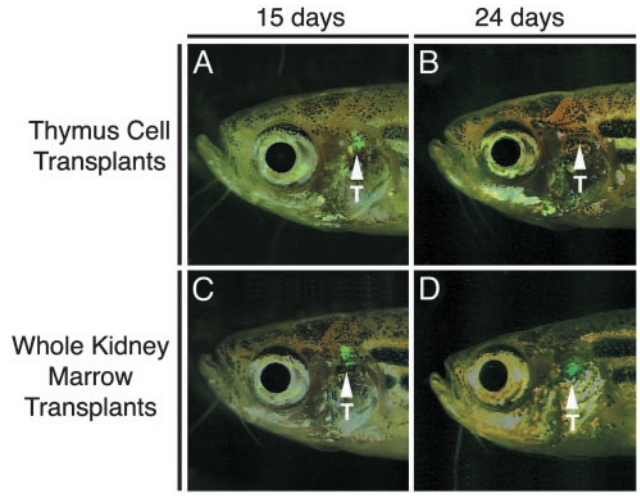

Fig. 6. Lymphopoiesis is fully reconstituted in irradiated adult recipients transplanted with *lck-GFP* kidney marrow. Transplants consisted of thymus cells (1.5×10^6 cells, *A* and *B*) or whole kidney marrow (3×10^5 cells, *C* and *D*) at 15 or 24 days posttransplantation. The views are lateral with anterior to the left. Arrowheads denote the location of the thymus (T).

faithfully recapitulates endogenous *lck* mRNA expression during embryogenesis. We show that *lck* is expressed in thymic T cells as early as 3 dpf and in both immature cortical and mature medullary thymocytes of adult fish. Similarly, *lck-GFP* transgenic fish have GFP-expressing T cells found in the thymus by 4 dpf and on into adulthood. Immunohistochemical studies of *rag2-GFP* transgenic fish reveal that GFP staining is detected in both the cortical and medullary regions of the thymus. This finding contrasts with the restricted expression of *rag1* and *rag2* mRNA expression in immature cortical thymocytes (20). These results can be explained by the stability of the GFP fluorophore and indicate that GFP protein expression may persist longer than the highly labile rag1 and rag2 proteins. Similar results have been reported in *rag2-GFP* transgenic mice (33).

T cells have been identified in the thymus; however, until our study, additional sites of T cell accumulation had not, to our knowledge, been identified in the adult zebrafish. We used GFP-immunostaining of paraffin-embedded sections to identify *lck-GFP*-positive T cell populations in peripheral sites including the intestine, esophagus, ovary, and the base of the olfactory epithelium. In support of the olfactory placode being a preferred site of T cell accumulation in fish, Myc-transformed T cells have been shown to home to this region when transplanted into irradiated recipients (25). Given that the olfactory epithelium, esophagus, and intestine are constantly exposed to foreign microbes, and that mice and humans have T cell accumulation in these sites as well (34–36), it is not surprising that mature T cells would be present at these sites in the zebrafish. By contrast, GFP-positive cells from the *rag2-GFP* fish were not found in peripheral organs, with the exception of GFP-labeled epithelial cells in the olfactory bulb. Taken together, these results indicate that GFP-labeled lymphoid cells from the *rag2-GFP* line are likely restricted to thymocytes, and B cells in the kidney. In contrast to previous reports identifying the pancreas as a lymphoid organ during embryogenesis (37), we failed to detect GFP-labeled lymphocytes in the pancreas of developing fry or adult *rag2-GFP* and *lck-GFP* transgenic zebrafish (data not shown). This discrepancy may be due to a short transit time of developing B cells in the pancreas. If the time from onset of *rag* transcription to exit of the developing B cells from the pancreas is shorter than the time required for the GFP protein to mature, fluorescence would not be expected to be detectable in the pancreas. A second possibility is that the *rag2* promoter used in our transgenic zebrafish lines is missing a critical regulatory element required for *rag2* expression in immature pancreatic B cells. Finally, it is possible that B cells do not reside in the pancreas. Further experiments are needed to resolve this discrepancy.

In mammals, T cell development occurs predominantly in the thymus. However, the presence of *rag2-GFP*-positive cells in the kidney prompted us to question whether a portion of these cells are immature T cells. RT-PCR analysis of FACS sorted GFP-positive and -negative kidney cells revealed that *rag2*-positive lymphocytes express *IgLC3* but not *lck*, indicating that these cells are B cells. By contrast, we found that *lck*-positive cells in the kidney fail to express B cell markers or the *rag* genes, indicating that these cells are likely mature T cells. Based on RT-PCR analysis of fluorescence-activated cell-sorted GFP-labeled cell populations from the kidney and GFP immunostaining of paraffin-embedded sections, we conclude that the thymus is the only site of T cell development in the zebrafish, and that B cell development occurs in the kidney marrow.

The thymus contains T cells at various stages of development. To assess whether a subset or all of the thymocytes are able to home to the thymus, we transplanted thymus cells from *lck-GFP* transgenic fish into 2-day-old embryos. Only a subset of GFP-labeled thymocytes is able to enter circulation and home to the thymus of transplanted 3-day-old embryos by 24 h posttransplantation. The remaining GFP-labeled cells entered circulation or became confined to a region of the tail. These results indicate that a subset of thymocytes are able to home back to the thymus, a process that can be easily visualized by fluorescence microscopy. Using *lck-GFP* transgenic fish, we will be able to assess whether immature thymocytes and mature T cells have different homing potential. Finally, these experiments suggest that the thymus of 3-day-old fish expresses receptors and/or chemoattractants that facilitate the migration of immature T cell progenitors back into the thymus.

Using *lck-GFP* transgenic fish, we show that GFP-labeled T cells are responsive to irradiation and chemical ablation in a similar manner as has been described in mouse and man. In mice, dexamethasone is administered by i.p. injection, with ≈90% of thymocytes dying by 2 days posttreatment (38, 39). Our experiments show that the zebrafish absorb the steroid from the water and respond to treatment within 3 days. Because fish must pass water over the gills to obtain oxygen and because the zebrafish thymus remains contiguous with the gill arches throughout development until 15 weeks postfertilization (20), the thymus is likely constantly exposed to environmental agents, and thus, thymocytes may directly absorb dexamethasone from the water.

In mice, long-term engraftment of adult bone marrow cells requires ablation of the host hematopoietic system. However, it was unknown whether transplant engraftment in fish would be similar. Nonirradiated adult fish transplanted with *lck-GFP* marrow failed to develop GFP-labeled T cells in the thymus by 25 days postinjection. By contrast, whole kidney marrow cells isolated from *lck-GFP* fish were able to reconstitute lymphopoiesis in recipient fish after a sublethal dose of irradiation for >6 months posttransplantation. Previously, long-term reconstitution of the erythroid lineage was reported in zebrafish embryos transplanted with kidney marrow (31). To our knowledge, our results are the first demonstration of long-term reconstitution of a hematopoietic cell lineage in irradiated adult zebrafish transplanted with whole kidney marrow. Taken together, these results suggest that either donor cells were rejected in nonirradiated recipients or that engraftment of donor hematopoietic cells must overcome competition with endogenous cells. In the latter case, hematopoietic engraftment likely occurs in irradiated fish because of the opening of niche space in the kidney.

To determine whether the thymus contains T cell progenitors that have the capacity to reconstitute T cell lymphopoiesis in the thymus, we injected irradiated adult wild-type fish with thymocytes from *lck-GFP* fish. T cell reconstitution of the thymic compartment was only transient, indicating that progenitor cells home to the thymus and differentiate into mature T cells, but these progenitor cells lack the capacity for self-renewal. Similar results have been reported in mice, in that bone marrow transplantation into irradiated recipient mice led to full reconstitution of the immune system (40). However, transplantation of thymus cells could only transiently reconstitute the thymic compartment (41). Our experiments in the zebrafish indicate that hematopoietic cells in the kidney marrow give rise to T cell progenitors that migrate to the thymus throughout life. It is these committed thymic progenitor cell populations that give rise to mature T cells in the thymus.

The results of these experiments provide a foundation for studies of immune responses in the zebrafish and illustrate both the remarkable evolutionary conservation of T cell immunobiology and the power of the zebrafish to visualize T cell responses *in vivo* through the use of GFP transgenic technology. Given that the response to chemical and radiation-induced T cell ablation can be easily monitored by loss of GFP-labeled T cells in the thymus, our *lck-GFP* transgenic zebrafish lines represent a new platform for the development of high-throughput *in vivo*-based drug screens designed to identify new T cell ablative agents. Finally, the *lck-GFP* transgenic zebrafish model will be useful for studying the genetics underlying T cell development and for developing the next generation of *in vivo* genetic screens designed to dissect genetic pathways that affect T cell homing and activation.

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