Noninvasive Intravital Imaging of Thymocyte Dynamics in Medaka

Jie Li, Norimasa Iwanami, Vu Quynh Hoa, Makoto Furutani-Seiki and Yousuke Takahama

J Immunol 2007; 179:1605-1615; doi: 10.4049/jimmunol.179.3.1605
http://www.jimmunol.org/content/179/3/1605

Supplementary Material http://www.jimmunol.org/content/suppl/2008/03/12/179.3.1605.DC1
References This article cites 49 articles, 16 of which you can access for free at: http://www.jimmunol.org/content/179/3/1605.full#ref-list-1
Subscription Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription
Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html
Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Noninvasive Intravital Imaging of Thymocyte Dynamics in Medaka

Jie Li,2* Norimasa Iwanami,2* Vu Quynh Hoa,* Makoto Furutani-Seiki, † and Yousuke Takahama3*

In vivo imaging of thymocytes has not been accomplished due to their localization deep within opaque body and high susceptibility to surgical stress. To overcome these problems, medaka is useful because of transparency and ex-uterine development. We report the noninvasive detection of thymocytes in transgenic medaka that express fluorescent protein under the control of immature-lymphocyte-specific rag1. We show that lymphoid progenitor cells colonize the thymus primordium in an anterior-to-posterior orientation-specific manner, revealing that extrathymic anterior components guide prevascular thymus colonization. We also show that developing thymocytes acquire “random walk motility” along with the expression of Ag receptors and coreceptors, suggesting that thymocyte walking is initiated at the developmental stage for repertoire selection. Thus, transgenic medaka enables real-time intravital imaging of thymocytes without surgical invasion. The Journal of Immunology, 2007, 179: 1605–1615.

Thymocyte development through the thymus involves dynamic cellular migration into, within, and out of the thymus (1–3). Static analyses of thymus sections and isolated thymocytes from genetically and pharmaceutically modified animals, along with the logical implications of those results, have revealed the involvement of several molecules including G protein-coupled chemotactic receptors (such as CCR7, CCR9, CXCR4, and S1P1) and adhesion molecules (such as PSGL-1 and VCAM-1) in guiding the relocation of thymocytes during development and selection within multiple environments of the thymus (4–9). However, it is unclear how thymocyte migration is affected or regulated by other components of the intravital environments, including interstitial fluidics and circulating vasculature. Thus, it is tempting to directly visualize how developing thymocytes migrate into, behave within, and egress out of intravital thymic architecture.

To this end, several laboratories have attempted to launch an experimental system in which thymocyte behavior can be time-lapse visualized under a microscope (10). Using two-photon laser-scanning microscopy to visualize fluorescence-labeled thymocytes in reaggregate thymus organ culture (11) and in intact thymus lobe culture, Robey and colleagues (12) showed that a large fraction of cortical thymocytes in intact thymus lobes exhibit random walk motility before positive selection and that the appearance of a thymocyte population displaying rapid and directed migration toward the medulla coincides with positive selection (12). In another study that also used two-photon laser-scanning microscopy to visualize calcium signals in thymocytes that were seeded in thymus slice culture, it was confirmed that most naïve thymocytes are highly motile in the thymus slice culture, and further shown that positively selected thymocytes become immobile and exhibit sustained calcium oscillations (13). Those studies provided a dynamic view of thymocyte behavior in the thymus microenvironment. However, it is important to note that those studies visualized thymocytes in “intact” thymus environments that were “isolated” from the body and that were organ-cultured in vitro. Therefore, those results represented in vitro, not in vivo, behaviors of thymocytes that were removed from interstitial fluidics and circulating vasculature, which are essential components of intravitally intact thymus environments.

To examine the intravital behavior of thymocytes in vivo, our laboratory initially attempted to visualize the thymus in live mice with two-photon microscopy, and immediately realized that it was difficult, if not impossible, to keep the mouse alive while examining endogenous thymus from the chest cavity under a microscope. Consequently, fetal thymus was transplanted into mouse back skin, lethally irradiated, and reconstituted with GFP-expressing bone marrow progenitor cells. It became possible by anesthesia and surgical operation to examine the transplanted intradermal thymus grafts under a microscope, using a technique similar to the intravitally intact thymus lobes (14, 15). In agreement with published results of the isolated thymus in vitro (11–13), we could detect the “random walk motility” of cortical thymocytes in those intradermal thymus lobes in vivo (our unpublished results). However, surgical invasion is known to elicit in vivo stress responses by activating the hypothalamic-pituitary-adrenal axis, thereby increasing systemic glucocorticoid concentration (16, 17), which in turn damages immature thymocytes and causes the involution of the thymus (18, 19). We thus reasoned that the in vivo behavior of mouse thymocytes detected in such intradermal thymus grafts must be severely biased by surgical stress. Moreover, we noticed that the intradermally transplanted thymus lobes were aberrantly vascularized and heavily fibered, precluding the tracking of thymocyte...
dynamics in the thymus environment with physiologically distributed vasculature.

To overcome these problems associated with the use of mouse as model organism, we describe in this study the use of medaka, *Oryzias latipes*, for the intravitral visualization of developing thymocytes. Like zebrafish *Danio rerio* (20–22), medaka is one of the smallest vertebrate species equipped with an adaptive immune system, including the thymus, T lymphocytes, and T cell-mediated cellular immune responses such as allograft rejection (23–25). The small size of the genome (800 Mb in medaka vs 1700 Mb in zebrafish) along with the availability of various genomic resources, including a completed sequence and bacterial artificial chromosome (BAC) library, makes medaka a useful species for genomic analysis and genetic experiments including transgenesis and morpholino antisense oligonucleotide-mediated gene knockdown (26, 27). The availability of various inbred strains is also a powerful feature of medaka over zebrafish (26), especially in studying the immune system such as the development and function of T lymphocytes. The ex-uterine and oviparous development of medaka allows in vivo imaging of fluorescent cells in intact body even during embryogenesis. Most notably, compared with zebrafish, transparency during development and throughout life in many medaka strains is particularly advantageous for the visualization of cellular dynamics in vivo (28).

By establishing transgenic medaka lines that express enhanced GFP (eGFP) under the control of immature lymphocyte-specific rag1 gene, the present study describes the time-lapse visualization of intravitral behavior of developing thymocytes at single-cell resolution in undisturbed body without surgical stress or anesthetic modification.

**Materials and Methods**

**Transgenic medaka**

The Cab strain of medaka (*Oryzias latipes*) was maintained as described (29) and used for transgenesis. The developmental stage was designated as by Ishiwata (30, 31). A γ-globin RNA polymerase II promoter downstream of the polyadenylation site. The probes for detecting *rag1* expression, or stained with H&E. These single-cell suspensions were resuspended in 2.5 × BSA, 0.5 × I-SceI buffer, 1% rhodamine, and 1 U/µl of I-SceI (New England Biolabs) and injected into medaka eggs at one-cell stage using an Eppendorf-Methode-Hinz 5171 micromanipulator. Embryos around the day of hatching were observed under a fluorescence microscope and those exhibiting eGFP signals in the pharyngeal region were further used to establish *rag1*-egfp-transgenic lines. Three stable transgenic lines were generated from 21 successfully injected eggs.

**Microinjection of morpholino oligonucleotides**

A 50 nM morpholino oligonucleotide RNA covering the start codon of medaka *tbx1* (GGCCATGTCTGCGTCCGTGATGATC) was injected. In control medaka, 0.5% rhodamine dextran (Mw = 10 × 10⁵; Invitrogen Life Technologies) was injected into medaka eggs at one-cell stage. Where indicated, morpholino oligonucleotide RNA for *TC33458* (GGCCATGTCTGCGTCCGTGATGATC) or *TC33458* (GGCCATGTCTGCGTCCGTGATGATC) was injected. In control medaka, 0.5% rhodamine dextran with or without morpholino oligonucleotide RNA for an unrelated gene was maintained as described (32). Adult wild-type medaka (left) and *rag1*-egfp-transgenic (Tg) medaka (right) were observed under a fluorescence microscope. The *rag1*-egfp-transgenic medaka at embryonic stage 37 (7 dpf) was observed for eGFP under a fluorescence microscope. The single-cell suspension was resuspended in 2.5 × BSA, 0.5 × I-SceI buffer, 1% rhodamine, and 1 U/µl of I-SceI (New England Biolabs) and injected into medaka eggs at one-cell stage using an Eppendorf-Methode-Hinz 5171 micromanipulator. Embryos around the day of hatching were observed under a fluorescence microscope and those exhibiting eGFP signals in the pharyngeal region were further used to establish *rag1*-egfp-transgenic lines. Three stable transgenic lines were generated from 21 successfully injected eggs.

**Histological analysis**

Whole medaka bodies and adult medaka kidney were fixed with 4% paraformaldehyde at 4°C for 1 h and embedded with OCT compound (Sakura Tissue-Tek). Serial sections at 10-µm thickness were analyzed for eGFP expression, or stained with H&E. *Ulex europaeus* agglutinin-1 (UEA-1) staining was performed as described (33). Confocal images were acquired with a TCS SP2 laser scanning microscope equipped with a >20 objective lens (1.25-0.75 NA) and argon and helium-neon lasers (Leica Microsystems).

**Flow cytometry analysis and sorting of eGFP-expressing cells**

The single-cell suspensions were resuspended in 2.5 µg/ml propidium iodide. Viable (propidium iodide-negative) cells were analyzed with FACSCalibur and sorted using FACSVantage (BD Bioscience). More than 62% and 99% of eGFP⁺ sorted cells and eGFP⁻ sorted cells were eGFP⁺ and eGFP⁻, respectively. May-Grunwald-Giemsa staining was used to detect nonviable cells.

**FIGURE 1.** Establishment of *rag1*-egfp-transgenic medaka. A, Medaka *rag1* genomic locus (top). The thin bar indicate m flange sequences. Exons of *rag1* coding region are shown in red. The *rag1*-egfp-transgenic construct (bottom) is shown. A 9.6-kb fragment of *rag1* upstream sequence was attached to egfp and polyadenylation sequences. I-SceI sites were added to increase the efficiency of germline transmission (32). B, Adult wild-type medaka (left) and *rag1*-egfp-transgenic (Tg) medaka (right) were observed under a fluorescence microscope. C, The *rag1*-egfp-transgenic medaka at embryonic stage 37 (7 dpf) was observed for eGFP under a fluorescence microscope. D, Adult wild-type medaka (left) and *rag1*-egfp-transgenic medaka (right) were observed under a fluorescence microscope.

*Abbreviations used in this paper: eGFP, enhanced GFP; wpf, weeks postfertilization; dpi, days postfertilization; UEA-1, *Ulex europaeus* agglutinin-1,*
FIGURE 2. eGFP detection of thymocytes in rag1-egfp-transgenic medaka. A. Diagram of the left lobe of young adult (6 wpf) medaka thymus constructed from the analysis of sagittal, transverse, and coronal serial sections. A, anterior; D, dorsal; L, left. B. Sections containing rag1-egfp-transgenic medaka thymus at 6 wpf were examined for eGFP expression (left) and H&E stained (right). Enlarged sections of images are shown at underneath. Scale bar, 20 μm. D, UEA-1 staining (cyan) of medaka sections containing the thymus at 6 and 2 wpf. Scale bar, 20 μm. Red signals represent background transmission. White dashed line indicates localization of the thymus identified by H&E staining and eGFP signals of serial sections. Note that the UEA-1-positive area is detectable in the thymus at 6 wpf but not at 2 wpf. EGFP expression (top) and H&E staining (bottom) of the kidney in 6-wpf rag1-egfp-transgenic medaka. Scale bar, 20 μm. Red signals represent background transmission. Note that the UEA-1-positive area is detectable in the thymus at 6 wpf but not at 2 wpf. E. Quantitative PCR analysis of egfp (○) and rag1 (●) in indicated organs of 6-wpf rag1-egfp-transgenic medaka. The expression in whole body of rag1-egfp-transgenic medaka was normalized to one. Results represent average and SEs of three independent measurements. F, eGFP expression (top) and H&E staining (bottom) of the kidney in 6-wpf rag1-egfp-transgenic medaka. Scale bar, 20 μm. Red signals represent background transmission. G. Quantitative PCR analysis of indicated genes in eGFP+ cells from the kidney (○) or the thymus (●) of 6-wpf rag1-egfp-transgenic medaka. The expression in whole body of rag1-egfp-transgenic medaka was normalized to one. Results represent average and SEs of three independent measurements.
Establishment of rag1-egfp-transgenic medaka

To track developing T lymphocytes in the thymus in vivo without surgical invasion, we generated transgenic medaka lines in which immature lymphocytes would express eGFP under the control of cis-elements for the immature lymphocyte-specific rag1. To do so, we isolated genomic clones containing medaka rag1 locus by screening a medaka genomic library. The sequence of a 14.3-kb fragment was ascertained to contain the full-length rag1 (Fig. 1A). The 9.6-kb fragment that is located at the 5′ non-coding region of the rag1 locus and that presumably contains the regulatory sequences for rag1 expression was attached to the egfp encoding sequence followed by the polyadenylation sequence and the I-SceI-sensitive sites (Fig. 1A). This transgenic construct was injected into pronuclei of fertilized medaka eggs. It was found that the transgenic medaka exhibited prominent eGFP expression distinctly in the pharyngeal region (Fig. 1B). The eGFP signal found in the pharyngeal region of the transgenic medaka was similarly localized within the thymus where endogenous rag1 was detected (Fig. 1C). The pharyngeal signals of eGFP and rag1 were synchronously abolished by the morpholino-mediated knockdown of tbx1 (Fig. 1D), a gene essential for pharyngeal arch development including the thymus (35–38). Thus, the eGFP signal detected in the pharyngeal region of rag1-egfp-transgenic medaka enables prominent visualization of the thymus.

Three independently established lines obtained upon injection of the rag1-egfp-transgenic construct showed similar eGFP fluorescence signals in the thymus, and thus we report the results from one of those transgenic lines.

eGFP detection of thymocytes in rag1-egfp-transgenic medaka

By analyzing serial sections of the pharyngeal region, the thymus in young adult medaka at 6 wk postfertilization (wpf) was found to be shaped as illustrated in Fig. 2A, essentially in agreement with a previous description (30). It was found that eGFP+ cells in the pharyngeal region were similarly localized to hematoxylin-rich hemopoietic cells in the thymus (Fig. 2B). At late embryonic stage 36 (6 days postfertilization (dpf)) and stage 37 (7 dpf), the eGFP signal was also detectable in regions anterior and posterior to the thymic mass (Fig. 1C). eGFP+ cells isolated from the thymus-containing pharyngeal

Results

Establishment of rag1-egfp-transgenic medaka
To track developing T lymphocytes in the thymus in vivo without surgical invasion, we generated transgenic medaka lines in which immature lymphocytes would express eGFP under the control of cis-elements for the immature lymphocyte-specific rag1. To do so, we isolated genomic clones containing medaka rag1 locus by screening a medaka genomic library. The sequence of a 14.3-kb fragment was ascertained to contain the full-length rag1 (Fig. 1A). The 9.6-kb fragment that is located at the 5′ non-coding region of the rag1 locus and that presumably contains the regulatory sequences for rag1 expression was attached to the egfp encoding sequence followed by the polyadenylation sequence and the I-SceI-sensitive sites (Fig. 1A). This transgenic construct was injected into pronuclei of fertilized medaka eggs. It was found that the transgenic medaka exhibited prominent eGFP expression distinctly in the pharyngeal region (Fig. 1B). The eGFP signal found in the pharyngeal region of the transgenic medaka was similarly localized within the thymus where endogenous rag1 was detected (Fig. 1C). The pharyngeal signals of eGFP and rag1 were synchronously abolished by the morpholino-mediated knockdown of tbx1 (Fig. 1D), a gene essential for pharyngeal arch development including the thymus (35–38). Thus, the eGFP signal detected in the pharyngeal region of rag1-egfp-transgenic medaka enables prominent visualization of the thymus.

Three independently established lines obtained upon injection of the rag1-egfp-transgenic construct showed similar eGFP fluorescence signals in the thymus, and thus we report the results from one of those transgenic lines.

eGFP detection of thymocytes in rag1-egfp-transgenic medaka
By analyzing serial sections of the pharyngeal region, the thymus in young adult medaka at 6 wk postfertilization (wpf) was found to be shaped as illustrated in Fig. 2A, essentially in agreement with a previous description (30). It was found that eGFP+ cells in the pharyngeal region were similarly localized to hematoxylin-rich hemopoietic cells in the thymus (Fig. 2B). At late embryonic stage 36 (6 days postfertilization (dpf)) and stage 37 (7 dpf), the eGFP signal was also detectable in regions anterior and posterior to the thymic mass (Fig. 1C). eGFP+ cells isolated from the thymus-containing pharyngeal

AB274724; cd4, AB274725; cd8a, AB274726; tcrβ C-region, AB274727; tcrα C-region, AB274728; and igμ C-region, AB274729.
region with a FACS were mostly small and had condensed nuclei and barely detectable cytoplasm, resembling mouse thymocytes (i.e., lymphoid cells in the thymus) rather than thymic epithelial cells or thymic macrophages isolated from mouse thymus (Fig. 2F). Flow cytometry analysis showed that these eGFP+ thymic cells indeed contained two populations, a majority of small cells and a minor population of large cells, identified by forward and side scatter intensities (Fig. 3A), in agreement with the profiles of zebrafish and mammalian thymocytes (22). Thus, eGFP+ cells in the thymus of rag1-egfp-transgenic medaka were identified to be thymocytes. Similar to thymocytes in other species, the majority of thymocytes in medaka were small lymphoid cells, and a minor population of large lymphoblastoid cells was also detectable.

The dorsal part of adult thymus contained an area positively stained with UEA-1 (Fig. 2D), a fucose-binding lectin known to label thymic medulla in mice and rats (39, 40). Like thymic medulla in mice, this UEA-1+ area in medaka thymus was less densely localized with thymocytes than the UEA-1+ area in the thymus and was undetectable during early ontogeny before or at 2 wpf (Fig. 2D), suggesting that the UEA-1+ region in medaka thymus may represent the medullary region of the thymus where positively selected mature thymocytes are accumulated in the thymus.

Other than the thymus, the expression of egfp and rag1 was not very prominent in various organs of adult medaka (Fig. 2E). However, we detected low levels of egfp and rag1 mRNA (Fig. 2E) and scattered distribution of eGFP+ cells (Fig. 2F) in the kidney of adult rag1-egfp-transgenic medaka. Like eGFP+ thymocytes, eGFP+ cells in the kidney were small and hematoxylin-rich, resembling hemopoietic cells (Fig. 2C). Teleost kidney is known to serve as the primary hemopoietic organ, which is equivalent to mammalian bone marrow (20–22, 30). Indeed, eGFP+ cells isolated from adult medaka kidney expressed immature lymphocyte-specific genes such as ikaros and rag1, similar to eGFP+ thymocytes (Fig. 2G).

FIGURE 4. Embryonic thymus colonization in medaka. A, Whole-mount in situ hybridization of ikaros, gata1, and rag1 and eGFP detection of rag1-egfp-transgenic embryos at stage 24 (2 dpf). Arrow denotes the region of intermediate cell mass. B, Sagittal (left) and transverse (right) sections of rag1-egfp-transgenic embryos at stage 27 (2.5 dpf). Enlarged sections are shown under panel. Arrow denotes eGFP+ cells. First somite and the eye are also indicated, based on H&E staining of the section. D, dorsal; DA, dorsal aorta; NC, notochord; R, right; V, ventral. Scale bar, 10 μm. C, Detection of eGFP expression (sagittal section) and whole-mount in situ hybridization of foxn1 of rag1-egfp-transgenic embryos at stage 29 (3 dpf). Enlarged sections are shown in bottom panels. H&E staining of identical regions is also shown. First somite, the ear, and the eye are also indicated, based on H&E staining of the section. May-Grunwald-Giemsa staining of eGFP+ sorted cells from stage 29 (3 dpf) rag1-egfp-transgenic embryos (bottom right). Scale bar, 10 μm for H&E staining and 4 μm for May-Grunwald-Giemsa staining. Arrowheads denote eGFP+ cells, and arrows denote thymic rudiment. D, Quantitative PCR analysis of the expression of egfp, gata1, ikaros, and rag1 in eGFP+ cells and eGFP− cells isolated from stage 27 (2.5 dpf) and stage 29 (3 dpf) rag1-egfp-transgenic embryos. The expression in whole body of rag1-egfp-transgenic medaka was normalized to one. Results represent average and SEs of three independent measurements. E, Dorsal (top) and lateral (middle) views of rag1-egfp-transgenic medaka for eGFP expression (bottom). Scale bar, 20 μm. Arrow (top) denotes the thymus.

FIGURE 5. Pharyngeal eGFP+ cells in rag1-egfp-transgenic medaka at embryonic stage 29 (3 dpf). eGFP expression (in green, left) and H&E staining (right) of the embryos at stage 29 (3 dpf) are shown. eGFP+ cells were detected at the region dorsal to the second and third pharyngeal pouches and ventral to the midbrain (MB)-hindbrain (HB) boundary. Red signals in confocal microscopy images (left) represent background transmission.
FIGURE 6. Intravital visualization of embryonic thymus colonization. A, Schematic illustration of real-time visualization of the thymus of medaka embryos at embryonic stage 32 (4 dpf) and stage 37 (7 dpf). Dechorionated embryos were placed in a drop of Ringer’s solution containing 3% methylcellulose. The thymus is located ∼120 μm deep from the surface of the body. Areas of 187.5 μm square and 7.8 μm depth were time-lapse scanned. B, Embryonic thymus colonization at stage 32 (4 dpf). Arrowheads denote a single cell migrating toward the thymus. The number indicates time (minutes) in the analysis. C, Trajectories of eGFP+ cells migrating toward the fetal thymus at stage 32 (4 dpf). The point of intersection of the horizontal and vertical axes indicates the edge of eGFP+ cell mass of the thymus. Each line shows the data of one cell. Shown are the data of 14 cells measured in 14 independent measurements. D, Velocities of eGFP+ cells that migrated to the embryonic thymus at stage 32 (4 dpf). Time 0 indicates the moment that eGFP+ cells attach to the edge of eGFP+ cell mass of the thymus. Data are 10 independent measurements shown. E, Coronal sections of the thymus at the indicated stages of medaka embryos. The vasculature was labeled with red fluorescent beads. White dashed lines indicate the edge of the thymus. F, Quantitative PCR analysis of the expression of indicated CC chemokines in control dye-treated (●), control morpholino-treated (□), and tbx1–morpholino-treated (○) embryos at indicated stages. Expression of TC38207 (possible ortholog of mouse CCL25), TC33458 (possible ortholog of mouse CCL21), TC33065 (possible ortholog of mouse CCL3), and MF01FSA0338H02 (possible ortholog of mouse CCL20) was measured. Expression levels in control dye-treated embryos at stage 29 (3 dpf) were normalized to one. Results represent average and SEs of three independent measurements. *p < 0.05; **p < 0.001; NS, not significant. G, Detection of eGFP+ cells (top) and whole-mount hybridization of rag1 (bottom) in indicated morpholino-treated rag1-egfp-transgenic medaka at stage 36 (6 dpf). Arrowheads indicate hybridized signals. Red signals in A and B represent background transmission.
FIGURE 7. Intravital visualization of postvascularized embryonic thymus colonization. A, Embryonic thymus colonization at embryonic stage 37 (7 dpf), as shown in Fig. 6B. Results are from two individual measurements are shown. B, Trajectories of eGFP⁺ cells migrating toward the fetal thymus at embryonic stage 37 (7 dpf), as shown in Fig. 6C. Data show 12 trajectories from seven independent measurements. The point of intersection of horizontal and vertical axes indicates the edge of eGFP⁺ cell mass of the thymus. C, Velocities of eGFP⁺ cells that migrated to embryonic thymus at stage 37 (7 dpf), as shown in Fig. 6D. Data are 10 independent measurements are shown. Red signals in A represent background transmission.

However, eGFP⁺ kidney cells and eGFP⁺ thymocytes expressed significantly different levels of a B cell-specific gene, igµ, and T cell-specific genes, cd4, cd8a, tcrα, and tcrβ (Fig. 2G); igµ was expressed at a higher level in eGFP⁺ kidney cells, whereas cd4, cd8a, tcrα, and tcrβ were expressed at higher levels in eGFP⁺ thymocytes (Fig. 2G). eGFP⁺ cells in adult medaka kidney were a mixture of small cells and large blastoid cells (Fig. 3A). Thus, eGFP⁺ kidney cells in rag1-egfp-transgenic medaka represent rag1-expressing immature lymphoid cells including the cells of B lymphocyte lineage, similar to those in rag2-gfp-transgenic zebrafish (22, 41–43) as well as those in the bone marrow of rag1-gfp-knockin mice (44), rag2-gfp-knockin mice (45), and rag2-gfp-transgenic mice (46). In contrast, eGFP⁺ cells in the medaka thymus are enriched with thymocytes of T lymphocyte lineage.

Embryonic development of rag1-egfp-expressing cells
In teleost species including medaka, embryonic hemopoiesis is first detected at the lateral mesoderm, which forms the accumulation of primary hemopoietic cells at the intermediate cell mass (20, 30, 47). Accordingly, the expression of lymphoid gene ikaros and erythroid gene gata1 was detectable at the intermediate cell mass of stage-24 embryos at 2 days postfertilization (2 dpf) (Fig. 4A). At this stage, no rag1 expression was detectable in rag1-egfp-transgenic medaka in the region of the intermediate cell mass, by in situ hybridization analysis of rag1 as well as by the detection of eGFP⁺ signals (Fig. 4A). eGFP⁺ cells in rag1-egfp-transgenic medaka were first detected at embryonic stage 27 (2.5 dpf) in an abdominal region near the first somite and ventral to the dorsal aorta (Fig. 4B), an area suggested to be the earliest site of definitive hemopoiesis in many vertebrate species including teleosts (47, 48). Interestingly, eGFP⁺ cells in rag1-egfp-transgenic medaka at embryonic stage 29 (3 dpf) were additionally detected in the pharyngeal region anterior to the thymus primordium (the region dorsal to the second and third pharyngeal pouches and ventral to the midbrain-hindbrain boundary) and were not detectable in the thymus primordium, which was identified by the expression of thymic epithelial cell-specific foxn1 (Figs. 4C and Fig. 5). eGFP⁺ cells isolated from embryos at stage 27 (2.5 dpf) and stage 29 (3 dpf) by the cell sorter appeared to be hematoxylin-rich lymphoid cells (Fig. 4C) and expressed ikaros and rag1 but not gata1 (Fig. 4D), suggesting that eGFP⁺ cells in embryonic stage 27 (2.5 dpf) and stage 29 (3 dpf) contained immature lymphoid progenitor cells including prethymic T progenitor cells (prothymocytes). eGFP⁺ viable cells in embryos at stage 27 (2.5 dpf) and stage 29 (3 dpf) exhibited forward and side scatter profiles similar to eGFP⁺ viable cells detectable in adult blood and adult kidney (Fig. 3, A and B), in agreement with the possibility that many of these prethymic eGFP⁺ cells represented immature lymphoid progenitor cells. eGFP⁺ cells in the thymus primordium were first detected at stage 30 (3.5 dpf), and their numbers increased during subsequent embryonic development (Fig. 4E). These results indicate that during embryogenesis in rag1-egfp-transgenic medaka, the detection of eGFP signals enables the visualization of prethymic lymphoid cells and thymus-colonized lymphoid cells.

Intravital visualization of embryonic thymus colonization
To visualize thymus colonization in undisturbed embryos in vivo, medaka eggs were time-lapse monitored under a single-photon confocal laser fluorescence microscope (Fig. 6A). eGFP⁺ cells were detectable in and around the thymus, which was localized ~120 μm from the surface of medaka embryos at stage 32 (4 dpf) (Fig. 6A). Time-lapse images were obtained at 10-s intervals for 10–15 min for six layers (total depth, 7.8 μm) of 187.5 × 187.5 μm² area (Fig. 6A), and the images of the third layer from the surface were subsequently analyzed and are shown in supplemental videos 1 and 2 (Fig. 6, B–D). It was shown that eGFP⁺ cells migrated toward thymus rudiment at an average velocity of 2.30 ± 0.15 μm/min (total cell number measured was 10, total measurement time was 50 min) (Fig. 6D). The cells migrating toward the thymus demonstrated morphological changes during the migration, exhibiting a polarized and extended shape during movement and a nearly round shape during pause as shown in supplemental videos 1 and 2. Interestingly, eGFP⁺ cells immigrated to the thymus only from an anterior edge in an orientation-specific manner (Fig. 6, B and C). The thymus is not yet vascularized at embryonic stage 32 (4 dpf), unlike vascularized thymus at stage 37 (7 dpf) (Fig. 6E). These results suggest that extrathymic tissues anterior to the thymus are involved in guiding lymphoid progenitor cells to the prevascular embryonic thymus.

Such a prevascular and orientation-specific guidance of thymus colonization may be at least partially mediated by the combination

5 The online version of this article contains supplemental material.
of chemokines because the expression of several CC chemokine
genes such as TC38207 (possible ortholog of mouse CCL25 with
34.4% identity in amino acids) and TC33065 (possible ortholog of
mouse CCL3 with 27.7% identity in amino acids) was significantly
abolished by the morpholino-mediated knockdown of tbx1 (Fig.
6F), which controls the development of pharyngeal arches includ-
ing the thymus primordium (35–38) also shown in Fig. 1D.
Morpholino-mediated knockdown of either TC38207 or TC33065
reduced the accumulation of rag1-expressing cells in the thymus
at stage 36 (6 dpf) (Fig. 6G), indicating that TC38207 and TC33065
are involved in embryonic thymus development. TC38207 appeared
to be expressed in pharyngeal region that contained the thymus (Fig.
6H), in agreement with the possibility that TC38207-encoded chemokine is involved in embryonic thymus colonization.
Whole-mount in situ hybridization of TC33458 expression (Fig.
6H) and TC33065 expression (data not shown) gave weak and
late-appearing signals at a region neighboring stage 37 (7 dpf)
thyms and gave no specific signals so far, respectively.
In contrast to the stage 32 (4 dpf) prevascular thymus, the
postvascularized thymus at stage 37 (7 dpf) was seeded with
eGFP⁺ cells from multiple directions (Fig. 7A and B). The average
velocity of thymic immigration at stage 37 (7 dpf) was
7.09 ± 0.34 μm/min (total cell number measured was 10, total
measurement time was 50 min), which was approximately three
times higher than the velocity at stage 32 (4 dpf) shown in sup-
plemental videos 3 and 4 (Fig. 7C). These results indicate that the
postvascularized thymus is seeded with lymphoid progenitor cells
via multiple routes, perhaps via the entry through the circulation.

**Intravital visualization of thymocyte behavior**

We finally examined the in situ motility of thymocytes within the
thymus in an undisturbed body. Medaka at 1 wpf (7 dpf, the day

![FIGURE 8. Intravital visualization of thymocyte behavior in medaka. A, Schematic illustration of real-time visualization of young adult thymus at 6 wpf. The thymus is located ~230 μm deep from the surface of the body. Areas of 150.25 μm square and 2.6 μm depth were time-lapse scanned. B, Thymocyte motility at indicated ages. Arrows and arrowheads denote time-lapse motility of a single cell in the thymus. The Number indicates time (minutes) in the analysis. Scale bar, 4 μm. C, Frequency of motile thymocytes at indicated ages. Results shown are the mean ± SEs of the frequency of cells with velocities higher than 2 μm/min for 114 cells at 1 wpf shown in three independent video clips, 200 cells at 2 wpf in three independent video clips, and 200 cells at 6 wpf in three independent video clips. D, Distribution of thymocyte velocity at 6 wpf. Results shown are of 200 cells from three independent video clips. E, Velocities of thymocytes at indicated ages. Data shown are of 10 independent measurements. F, Trajectories of thymocytes at indicated ages. Each plot represents superimposed trajectories of 50 thymocytes (each line represents one cell) over a 3-min time span. G, Morphologies of eGFP⁺ thymocytes at indicated ages. Fluorescence images (top) and the images obtained by May-Grunwald-Giemsa staining (bottom) are shown. H, Quantitative PCR analysis of the expression of indicated genes in eGFP⁺ thymocytes at indicated ages. Expression levels in whole body of 6-wpf rag1-egfp-transgenic medaka were normalized to one. Results represent the average and SEs of three independent measurements.**
of hatching), 2 wpf (infancy), and 6 wpf (young adult) were placed in Ringer’s solution containing 3% methylcellulose, and eGFP+ cells were time-lapse traced under a single-photon confocal laser fluorescence microscope (Fig. 8A). eGFP+ thymocytes at 6 wpf were detectable at a depth of ~230 μm from the body surface (Fig. 8A). Time-lapse images were obtained at 10-s intervals for three layers (total depth: 2.6 μm) of 150.25 × 150.25 μm2 area (Fig. 8A), and the images of the middle layer were analyzed subsequently and shown in supplemental videos 5 and 6 (Fig. 8, B–F).5 The results showed that 29.1 ± 1.1% of eGFP+ thymocytes at 6 wpf in the UEA-1− cortex-like area, resembling the thymic cortex, exhibited motility at velocities >2 μm/min (n = 200 cells) (Fig. 8C). The velocity of motile cells was 8.8 ± 0.9 μm/min (total cell number measured was 10, total measurement time was 33.3 min) (Fig. 8D), and many motile cells paused between movements (Fig. 8E). The direction of thymocyte movement appeared random and was not uniform (Fig. 8F). A small fraction of moving cells exhibited motility at velocities higher than 12 μm/min as shown in supplemental video 7 (Fig. 8D).5 These results indicate that a considerable fraction of thymocytes in intravital adult medaka exhibit random-walk motility, as detected in vivo at single-cell resolution without any surgical invasion, by means of conventional confocal microscopy with a single-photon laser. Simply placing viable medaka in water containing viscous methylcellulose enabled time-lapse and intravital imaging of eGFP+ thymocytes in vivo without anesthesia. Oviparous development of medaka further allowed time-lapse and intravital imaging of eGFP+ lymphoid progenitor cells traveling toward the thymus during embryogenesis. Thus, we have established hitherto unaccomplished imaging of the single-cell dynamics of intravital thymocytes without surgical invasion or anesthetic modification.

Our results show that many eGFP+ thymocytes in the UEA-1− cortex-like area of the thymus in young adult medaka are motile at velocities higher than 2 μm/min. Robey and her colleagues (12) showed that a vast majority of mouse cortical thymocytes in intact thymus cultures are motile at velocities higher than 3 μm/min. The observed behavior of most motile thymocytes, including randomly directional movement, wide range of velocities, and occasional pauses between movements, is alike between medaka (this study) and mouse (12), indicating that the random-walk motility of a considerable fraction of thymocytes is a behavior that is shared by multiple vertebrate species and is likely important for the developmental regulation of thymocytes. Accordingly, this random-walk motility may represent the search by newly generated TCR+ CD4+CD8+ thymocytes for the interaction with peptide/MHC complexes that determine developmental fate of TCR+ CD4+CD8+ thymocytes through positive and negative selection, as previously suggested (1, 2, 12). We also detected a minor population of highly motile thymocytes having velocities higher than 12 μm/min, possibly representing positively selected thymocytes that are moving toward the medulla (12). Because of technical difficulty to define the UEA-1− region in the intravital medaka thymus, this study did not address whether the movement of highly motile thymocytes might be directional toward the UEA-1− medulla-like region and how thymocyte motility in the UEA-1− medulla-like region might be different from that in the UEA-1− cortex-like region.

In contrast, the frequency of motile thymocytes was much lower in medaka (29%) than in mouse (95%) (12). This finding could be due to the difference in frequency of TCR+ CD4+CD8+ thymocytes, which undergo positive and negative selection, between the two species, and/or to ontogenic differences between 6 wpf medaka and 4.5–5.5-wk-old bone marrow chimera mice. It is also possible that the difference may be attributed to thermokinetic differences in cellular motility due to different body temperatures (28°C in medaka vs 37°C in mouse) and/or other technical differences in experimental conditions (for example, intravital condition in medaka vs tissue culture condition in mouse). Nonetheless, it should be emphasized that our results show for the first time the behavior of intravital thymocytes in vivo without surgical invasion and without anesthetic modification.

Discussion

In this study, we found that eGFP is specifically detectable in lymphoid cells including thymocytes and lymphoid progenitor cells in rag1-egfp-transgenic medaka. Because medaka is a vertebrate species with exceptional transparency, the preparation of rag1-egfp-transgenic medaka has enabled visualization of thymocytes in vivo at single-cell resolution without any surgical invasion, by means of conventional confocal microscopy with a single-photon laser. Simply placing viable medaka in water containing viscous methylcellulose enabled time-lapse and intravital imaging of eGFP+ thymocytes in vivo without anesthesia. Oviparous development of medaka further allowed time-lapse and intravital imaging of eGFP+ lymphoid progenitor cells traveling toward the thymus during embryogenesis. Thus, we have established hitherto unaccomplished imaging of the single-cell dynamics of intravital thymocytes without surgical invasion or anesthetic modification.

The results showed that 29.1 ± 1.1% of eGFP+ thymocytes at 6 wpf in the UEA-1− cortex-like area of the thymus in young adult medaka are motile at velocities higher than 2 μm/min. Robey and her colleagues (12) showed that a vast majority of mouse cortical thymocytes in intact thymus cultures are motile at velocities higher than 3 μm/min. The observed behavior of most motile thymocytes, including randomly directional movement, wide range of velocities, and occasional pauses between movements, is alike between medaka (this study) and mouse (12), indicating that the random-walk motility of a considerable fraction of thymocytes is a behavior that is shared by multiple vertebrate species and is likely important for the developmental regulation of thymocytes. Accordingly, this random-walk motility may represent the search by newly generated TCR+ CD4+CD8+ thymocytes for the interaction with peptide/MHC complexes that determine developmental fate of TCR+ CD4+CD8+ thymocytes through positive and negative selection, as previously suggested (1, 2, 12). We also detected a minor population of highly motile thymocytes having velocities higher than 12 μm/min, possibly representing positively selected thymocytes that are moving toward the medulla (12). Because of technical difficulty to define the UEA-1− region in the intravital medaka thymus, this study did not address whether the movement of highly motile thymocytes might be directional toward the UEA-1− medulla-like region and how thymocyte motility in the UEA-1− medulla-like region might be different from that in the UEA-1− cortex-like region.

In contrast, the frequency of motile thymocytes was much lower in medaka (29%) than in mouse (95%) (12). This finding could be due to the difference in frequency of TCR+ CD4+CD8+ thymocytes, which undergo positive and negative selection, between the two species, and/or to ontogenic differences between 6 wpf medaka and 4.5–5.5-wk-old bone marrow chimera mice. It is also possible that the difference may be attributed to thermokinetic differences in cellular motility due to different body temperatures (28°C in medaka vs 37°C in mouse) and/or other technical differences in experimental conditions (for example, intravital condition in medaka vs tissue culture condition in mouse). Nonetheless, it should be emphasized that our results show for the first time the behavior of intravital thymocytes in vivo without surgical invasion and without anesthetic modification.
The results of noninvasive intravital imaging also show that thymocyte motility is initiated during ontogeny. Thymocytes in baby medaka on the day of hatching (3 days after the initial thymus colonization) were nonmotile, whereas thymocytes at 1 wk after hatching (2 wpf) contained randomly walking cells, similar to young adult thymocytes at 6 wpf. The developmental acquisition of random-walk motility by thymocytes during ontogeny coincided with the expression of Ag-receptor genes \( tcra \) and \( tcrb \) as well as coexpressor genes \( cd4 \) and \( cd8a \) in the thymocytes. The expression of \( TCR \), \( CD4 \), and \( CD8 \) by developing thymocytes during ontogeny and the concomitant acquisition of thymocyte motility suggest that \( TCR \), \( CD4 \), \( CD8 \) thymocytes may be nonmotile, and their development into \( TCR-CD4-CD8- \) thymocytes initiates the random-walk motility for \( TCR \)-mediated positive and negative selection, further supporting the possibility that the random-walk motility is involved in repertoire selection of \( TCR-CD4-CD8- \) thymocytes.

\( eGFP^+ \) lymphoid cells in \( rag1-egfp \)-transgenic medaka were first detected in ontogeny at stage 27 (2.5 dpf, before thymus formation) in the region near the first somite and ventral to the dorsal aorta, an area suggested to be the earliest site of definitive hematopoiesis in many vertebrate species including teleosts (47, 48). By embryonic stage 29 (3 dpf), \( eGFP^+ \) cells were also detected in the pharyngeal region anterior to the thymus primordium, which was positive for thymic epithelial cell-specific \( foxn1 \) but was still not colonized by \( eGFP^+ \) thymocytes. \( eGFP^+ \) lymphoid cells in embryos at stage 29 (3 dpf) likely represent immature lymphoid progenitor cells including prothymocytes because these cells expressed \( ikaros \) and \( rag1 \) but not \( gata1 \), \( tcra \), \( tcrb \), \( cd4 \), \( cd8a \), or \( igα \) (Fig. 4D and data not shown). The nature of the pharyngeal region anterior to the thymus is still unclear, but may represent a prethymic reservoir for thymus-colonizing prothymocytes. Unlike \( eGFP^+ \) cells near the dorsal aorta detectable at stage 27 (2.5 dpf), \( eGFP^+ \) cells in the region anterior to the thymus detectable at stage 29 (3 dpf) were susceptible to \( tbx1 \)-morpholino (data not shown), suggesting that this prethymic reservoir may be localized in \( tbx1 \)-dependent pharyngeal tissues. Colonization of medaka thymus by \( eGFP^+ \) lymphoid progenitor cells was first detected at stage 30 (3.5 dpf), when the thymus is still not vascularized. Whether lymphoid progenitor cells generated at the region near the first somite and ventral to the dorsal aorta indeed migrate to the pharyngeal region anterior to the thymus and whether lymphoid cells found at the pharyngeal region anterior to the thymus primordium migrate to the thymus are also unclear.

Time-lapse detection of embryonic thymus revealed that prevascular colonization of the thymus primordium is an orientation-specific event that occurs in an anterior-to-posterior manner toward the thymus. It is thus possible that similar to prevascular thymus colonization in mouse embryos, prevascular thymus colonization in medaka is mediated by multiple chemokines, and that at least one chemokine expressed in a region anterior to and outside the thymus primordium may guide the chemotaxis toward the thymus (33). Indeed, the \( tbx1 \)-morpholino-mediated defect of pharyngeal arch development, including thymus organogenesis and thymus colonization (35–38), resulted in the loss of at least two chemokine genes (\( TC38207 \), possible ortholog of mouse \( CCL25 \), and \( TC33065 \), possible ortholog of mouse \( CCL3 \)) and morpholino-mediated knockdown of either \( TC38207 \) or \( TC33065 \) reduced the accumulation of \( rag1 \)-expressing cells in embryonic thymus. It was also found that \( TC38207 \) was expressed in pharyngeal region that contained the thymus. These results suggest that \( TC38207 \) and \( TC33065 \) are involved in directing embryonic thymus colonization.

Possible involvement of a chemokine expressed in a region anterior to and outside the thymus primordium agrees with the results in mice showing the coordination between Gcm2-dependent parathyroid and Foxn1-dependent thymic primordia in establishing \( CCL21/CCL7- \) and \( CCL25/CCL9 \)-mediated chemokine guidance essential for prevascular fetal thymus colonization (33). Vascularization of the thymus was first detected in ontogeny at stage 37 (7 dpf), at which thymus seeding was detected from multiple directions and at threefold higher velocity than earlier prevascular colonization. Thus, our results support the possibility that thymus seeding is mediated by at least two different and developmentally regulated molecular mechanisms, initially via the chemokine-dependent anterior-specific route and later via the multidirectional entry perhaps through the circulation.

In the three lines of \( rag1-egfp \)-transgenic medaka prepared in this study, prominent \( eGFP \) expression was specifically detectable in lymphoid cells, including thymocytes and lymphoid progenitor cells. Additional and prominent expression of \( eGFP \) was detected in olfactory tissues (Fig. 9), as previously reported in \( rag1-egfp \)-transgenic zebrafish (49). It may be also interesting to note that the 9.6-kb fragment that is located at the 5’ non-coding region of medaka \( rag1 \) locus was sufficient to specifically direct the expression of downstream \( egfp \) in a fidelity reasonable to the expression profile of endogenous \( rag1 \). In a study using \( rag1-egfp- \)transgenic zebrafish (50), 1.5- to 8.1-kb fragments of the 5’ region of the \( rag1 \) translation initiation site directed the expression of GFP reporter gene in both lymphoid and nonlymphoid cells (50). Thus, the medaka 9.6-kb fragment likely contains negative regulatory elements that are required to restrict \( rag1 \) expression to appropriate tissues and that are absent in the zebrafish 8.1-kb fragment.

Taken together, we have established the noninvasive intravital imaging of thymocyte dynamics in medaka. Noninvasive imaging of intravital thymocytes at single-cell resolution demonstrated the developmental acquisition of random-walk behavior by thymocytes and the orientation-specific seeding of embryonic thymocytes, and will likely lead to further understanding of hitherto unknown mechanisms for immune system development including repertoire formation.

Acknowledgments
We thank Akihito Yassouka for pKan’ plasmid; Akihiro Momoi, Cunlan Liu, Yu Lei, and Fumi Saito for technical assistance; Michio Tomura and Kenji Usui for initial attempt of time-lapse analysis of thymocyte motility in mice; and Graham Anderson, Masahiko Hibi, Masato Kinoshita, Hiroshi Nakase, Takeshi Nitta, and Tomoou Ueno for reading the manuscript.

Disclosures
The authors have no financial conflict of interest.

References


169: 4354 – 4361.


