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Thymic Origin of Embryonic Intestinal $\gamma/\delta$ T Cells
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Summary
Current evidence suggests both thymic and extrathymic origins for T cells. Studies in mice favor an in situ origin for a prominent population of intestinal intraepithelial lymphocytes that express $\gamma/\delta$ T cell receptor (TCR). This developmental issue is explored in an avian model in which the $\gamma/\delta$ lymphocytes constitute a major T cell subpopulation that is accessible for study during the earliest stages of lymphocyte development. In the chick embryo, cells bearing the $\gamma/\delta$ TCR appear first in the thymus where they reach peak levels on days 14-15 of embryogenesis, just 2 d before $\gamma/\delta$ T cells appear in the intestine. Using two congeneric chick strains, one of which expresses the ov antigen, we studied the origin and kinetics of intestinal colonization by $\gamma/\delta$ T cells. The embryonic $\gamma/\delta^+$ thymocytes homed to the intestine where they survived for months, whereas an embryonic $\gamma/\delta^-$ thymocyte population enriched in thymocyte precursors failed to give rise to intestinal $\gamma/\delta^+$ T cells. Embryonic hemopoietic tissues, bone marrow, and spleen, were also ineffective sources for intestinal $\gamma/\delta^+$ T cells. Intestinal colonization by $\gamma/\delta^+$ thymocytes occurred in two discrete waves in embryos and newly hatched birds. The data indicate that intestinal $\gamma/\delta^+$ T cells in the chicken are primarily thymic migrants that are relatively long-lived.

Abbreviations used in this paper: IEL, intraepithelial lymphocyte; Tx, thymectomized recipient.

Materials and Methods
Animals. Embryos of White Leghorn chicken strain H.B19 were derived from animals kept at our institute's farm in Gipf-Oberfrick.
Figure 1. Embryonic γ/δ thymocytes home to the intestine. Double immunofluorescence staining of frozen tissue section. (A) Thymus from day 16 H.B19ov⁺ embryos. (B and C) Small intestine from day 18 H.B19ov⁻ embryos injected at day 16 with day 14 ov⁺ thymocytes (B) illustrating submucosal lymphoid aggregates (chickens do not have true Peyer’s patches, [8]), (C) correspond to intraepithelial lymphocytes, and (D) small intestine of a 1- to 2-old chicken that was thymectomized 2 d after hatching and immediately injected with 14-d-old ov⁺ splenocytes. (Arrowheads) Donor IEL (ov⁺γ/δ⁺). ×270.

Switzerland. Fertilized eggs were incubated at 38°C and 80% humidity in a ventilated incubator. The H.B19 strain was subdivided into congenic lines (H.B19ov⁺ and H.B19ov⁻). They can be distinguished by the ov antigen which is present on thymocytes and T cells only in H.B19ov⁺ animals, and which is recognized by the mAb 11-A-9 (18–20). The experimental animals were treated according to Swiss government veterinary guidelines.

Injection of Lymphoid Cells into Congenic Chickens. Embryonic day 13 (E13) bone marrow cells, E13 splenocytes, and E14 thymocytes (25 × 10⁶) were injected. Bone marrow and spleen from day 13 embryos do not contain detectable γ/δ⁺ cells. Injections of sorted populations of E14 thymocytes were also performed. In this case, thymocytes from 14-d-old H.B19ov⁺ embryos were suspended in PBS containing 10% FCS, filtered through a nylon sieve (mesh width of 25 μm; Nytal P-25 my, SST, Thal, Switzerland) and centrifuged at 255 g for 7 min. Immunofluorescence staining of these relatively fragile cells was performed in 96-well plates, to avoid repeated centrifugation, using the anti-γ/δ antibody TCR1.
Table 1.  
T Cell Marker Expression by ov* Cells Derived from E14 Thymocyte Transplants in ov− Recipients

<table>
<thead>
<tr>
<th>Days of development</th>
<th>Percent intestinal cells bearing the donor ov antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>γ/δ*</td>
</tr>
<tr>
<td>18</td>
<td>82 ± 7</td>
</tr>
<tr>
<td>19</td>
<td>75 ± 3</td>
</tr>
<tr>
<td>22</td>
<td>74 ± 3</td>
</tr>
<tr>
<td>28</td>
<td>75 ± 3</td>
</tr>
</tbody>
</table>

Expression of T cell markers was determined by double immunofluorescence staining of gut frozen sections. Each slide contained a section of the duodenum, a section of the ileum, and a section located around the caecal tonsils. Counting was performed on two slides for each animal. 200 ov* intestinal cells were counted per slide. Data are expressed in percent ov* intestinal cells and correspond to the mean of three animals. Variability corresponds to SE. 0 is defined as <0.1%, i.e., no ov* γ/δ* T cells were detected in analyzed slides.

and then fluorescein-coupled anti-mouse Ig antibody (Silenus, Hawthorn, Australia). Stained and unstained thymocytes were resuspended in 10% FCS/PBS and sorted using a cell sorter (FACStar plus®, Becton Dickinson & Co., Mountain View, CA).

These hemopoietic cells were injected into a large vein at the end of the airsac of 16-d-old H.B19ov− embryos. Alternatively, cells (20 × 10⁶) were injected into the jugular vein of H.B19ov− chicks just after thymectomy on the second day after hatching. After killing, the neck region of thymectomized chickens was disected and examined for remnants that would indicate incomplete thymectomy. These cases in which thymic remnants were detected were excluded from this analysis. Control animals were H.B19ov− recipients that were not thymectomized. The different recipients received no irradiation or drug treatment.

Analysis of donor cells in the intestine was performed by FACS® analysis and by two-color immunofluorescence staining of frozen tissue sections. For FACSscan® analysis of intestinal lymphocytes, the small intestine was extensively washed with PBS containing 4 g/liter glucose, miniced into small pieces at 4°C, washed 10 min at room temperature with 2 mM DTT/4 g/liter glucose plus PBS, and lymphocytes were detached in 3 mM EDTA/2 mM DTT/4 g/liter glucose plus PBS at 37°C for 15 min (21). Cells were then pelleted by centrifugation and stained.

Immunolabelling. ov, γ/δ α/β, CD3, CD8, and CD4 antigens were detected using 11-A-9, TCR1, TCR2, CT3, CT8, and CT4 mAbs, respectively (14, 18, 22–25). Except for 11-A-9, which was a mouse IgM (18, 19), all mAbs were mouse IgG1. Second antibodies were fluorescein-labeled sheep anti-mouse IgM and PE or Texas red-coupled anti-mouse IgG1 antibody (Southern Biotechnology Associates, Birmingham, AL). Controls were performed using second antibodies alone and also with tissues of noninjected individuals from the H.B19ov− strain.

Frozen sections of embryonic organs were cut to a thickness of 5 μm on a cryostat (E. Leitz, Wetzlar, Germany), fixed with acetone, rehydrated in PBS containing 1% BSA.

Results

H.B19ov+ chickens express ov antigen on the surface of most hemopoietic precursors during embryonic life, but only on T lineage cells and their precursors postnatally (18). Chickens of the congenic strain H.B19ov−, which do not express the ov antigen (18–20), were employed as recipients in these experiments. On day 14 of embryogenesis, ~25% of all thymocytes are γ/δ+ and <3% are α/β+, all of these being ov+ in the H.B19ov+ strain (23 and data not shown). Injection of day 14 H.B19ov+ thymocytes into 15- or 16-d-old H.B19ov− embryos led to the appearance of γ/δ+ T cells of the donor ov+ type in the small intestine within 2 d after injection (Fig. 1). The injected thymocytes homed to the intestine and the spleen, but neither thymus nor bursa were colonized at significant levels (Figs. 1 and 2 and data not shown). The percentage of donor γ/δ+ T cells in the intestine remained relatively constant until the end of embryogenesis (days 18, 19, and 20).

Donor ov+ E14 thymocytes homing to the gut expressed CD3 and >75% of these were identifiable as γ/δ+ (Table 1).

Figure 2. Survival pattern of donor γ/δ+ cells in intestine. Day 16 H.B19ov+ embryos were injected with 25 × 10⁶ day 14 H.B19ov+ thymocytes and killed at various times after injection. (Arrow) Hatching at day 21. Data are expressed as percent γ/δ+ IEL that express the donor ov antigen marker and correspond to the mean of two to four animals derived from three independent experiments. Analysis was performed in intestine (□) and thymus (▲). These results were obtained from two-color immunofluorescence analysis of tissue sections throughout development. Each slide contained a section of the duodenum, a section of the ileum, and a section located around the caecal tonsils. Counting was performed on two slides for each animal. 200 and 500 γ/δ IEL were counted per slide of embryonic intestine and newborn chick, respectively. By day 22 (after hatching) these results were confirmed by FACSscan® analysis. Error bars correspond to SE.
Figure 3. Quantitation of ileal γ/δ+ T cells during development. Ileal γ/δ+ T cells were counted on frozen gut sections stained by immunofluorescence. Taking into account the length of the ileum, the area of the ileum sections, and the thickness of these sections, the total number of ileal γ/δ+ lymphocytes was calculated for the different stages of development. (□) Control animals; (■) chickens thymectomized on the second day after hatching; and (▲) ileal ov+ γ/δ+ T cells of H.B19ov- animals injected with 25 x 10⁶ day 14 H.B19ov+ thymocytes (see Fig. 2). Day 21 corresponds to hatching.

1. Approximately 40% of the ov+ γ/δ+ T cells in the intestine were CD8+ and the remainder were CD4-CD8-, a phenotypic distribution similar to that of γ/δ T cells in the adult intestinal mucosa (15) which has also been observed in human intraepithelial mucosa (26-27). As expected at this early embryonic stage, significant homing of ov+ ot/fl+ E14 thymocytes to the gut was not observed. The donor γ/δ T cells persisted in the recipient intestine beyond 75 d, although a dramatic decrease in the percentage of donor intestinal γ/δ+ T cells occurred around hatching (Fig. 2). This decrease might result from dilution by the intestinal arrival of host γ/δ+ T cells. The quantitative analysis of ileal γ/δ+ T cells during their development, which revealed a significant increase in the total number of cells occurring in the first days after hatching (Fig. 3), favored this hypothesis. Alternatively, a loss of γ/δ+ IEL due to the onset of digestive function could theoretically account for the observed decrease.

To analyze the origin of the posthatch emigrants to the intestine, we injected lymphoid cells into normal and thymectomized chickens after hatching (Table 2). Injection of 14-d-old H.B19ov+ embryonic thymocytes into 2-d-old H.B19ov- thymectomized chickens led to the development of >20% ov+ cells among the γ/δ+ T cell population in the intestine during the first month after the injection. Injection of the same number of ov+ thymocytes into 2-d-old, non-thymectomized chickens led to only 3–9% of intestinal γ/δ+ T cells being of the donor type. These results suggest that cells derived from the recipient's thymus compete with the homing of the donor thymocytes. Moreover, γ/δ thymocytes from either 14-d-old H.B19ov+ embryos or 2-d-old H.B19ov+ chicks colonized the intestine of 2-d-old H.B19ov- chicks with the same efficiency (data not shown). The progeny of stem cells of the second wave of thymus colonization (days 12–13 of embryogenesis) mature around the time of hatching (7–8 d later; [17, 28]). Thus, this second wave of γ/δ+ T cells homing to the intestine after hatching also appears to be thymus derived. In further support of this interpretation, injection of 35 x 10⁶ 14-d-old H.B19ov+ thymocytes into H.B19ov- chickens at 1 wk of age, contributed a very small proportion of intestinal γ/δ+ T cells (0.5 ± 0.5%).

<table>
<thead>
<tr>
<th>Recipient age</th>
<th>E14 thymocytes</th>
<th>Donor-derived γ/δ+ T cells after injection of:</th>
<th>E13 bone marrow cells</th>
<th>E13 splenocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tx</td>
<td>Control</td>
<td>Tx</td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>1 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23 ± 6</td>
<td>9 ± 4</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>30 ± 8</td>
<td>3 ± 2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20 ± 6</td>
<td>3 ± 2</td>
<td>0.5 ± 0.5</td>
<td>0</td>
<td>0.5 ± 0.5</td>
</tr>
<tr>
<td>8</td>
<td>8 ± 5</td>
<td>ND</td>
<td>0.5 ± 0.5</td>
<td>0.5 ± 0.5</td>
</tr>
</tbody>
</table>

Data correspond to the percent γ/δ IEL that express the donor ov alloantigen marker. Each point corresponds to the mean of two to four animals. E13 and E14 correspond to 13- and 14-d-old embryos. γ/δ donor cells were analyzed by two-color immunofluorescence staining of intestine sections. Each slide contained a section of the duodenum, a section of the ileum, and a section located around the coecal tonsils. Counting was performed on two slides for each animal. 200 γ/δ IEL were counted per slide. Variability corresponds to SE. 0 is defined as <0.1%; no ov+ γ/δ+ T cells were detected in analyzed slides. These results were confirmed by FACScan® analysis.

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might differentiate extrathymically into \( \gamma/\delta \) T cells, donor thymocytes from 14-d-old embryos were separated into \( \gamma/\delta^- \) and \( \gamma/\delta^+ \) populations. To yield an enrichment of thymocyte precursors, embryos at this development stage were chosen as donors since the second wave of precursor cells enters the thymus between embryonic days 12–14 (see Fig. 4). When sorted \( \gamma/\delta^- \) or \( \gamma/\delta^+ \) thymocytes were injected into 16-d-old embryos, the \( \gamma/\delta^+ \) thymocytes gave rise to \( \gamma/\delta^- \) intestinal lymphocytes of the donor type while the \( \gamma/\delta^- \) thymocyte population did not (Table 3). Indeed, when H.B19ov- chickens were injected with \( \gamma/\delta^+ \) thymocytes, donor intestinal \( \gamma/\delta^+ \) T cells were still undetectable 2 mo after hatching.

**Discussion**

These results provide direct evidence for population of intestinal epithelium by emigrating \( \gamma/\delta \) thymocytes. Our data indicate that the homing of \( \gamma/\delta \) T cells from the thymus might be relevant to the intestine occurs in a developmental pattern that follows the scheme of thymus colonization by waves of precursor stem cells. In chick-quail chimera, three waves of precursor cells have been shown to enter the thymus during embryonic life as outlined in Fig. 4 (28). Within the thymus, each in turn gives rise to a wave of progeny T cells that includes both \( \gamma/\delta \) and \( \alpha/\beta \) subpopulations (17). Using congenic strains of chickens, one of which expresses the marker ov antigen, we found that \( \gamma/\delta \) T cells in the first two developmental waves of embryonic thymocytes migrate to the intestine where they populate the epithelial lining. These thymus-derived \( \gamma/\delta^+ \) IEL apparently can survive for long periods of time in this environment. In our cell transfer experiments, embryonic \( \gamma/\delta^- \) thymocytes rapidly populated the intestines of \( \gamma/\delta^- \) embryonic recipients, where they were found to persist into young adulthood. While the present experiments did not explore the fate of \( \gamma/\delta^- \) thymocytes in subsequent waves or the survival of the initial migrants beyond 75 d of age, the data suggest that, once established, this long-lived population \( \gamma/\delta^- \) IEL may require minimal replenishment. Since \( \alpha/\beta^+ \) cells do not appear before E19–20 in intestine and represent <3% of E14 thymocytes (16, 23), the experiments performed here, involving injection of E14 thymocytes, do not allow analysis of the origin of \( \alpha/\beta^- \) intestinal cells.

**Table 3. Intestinal Homing of Sorted Congenic \( \gamma/\delta^+ \) or \( \gamma/\delta^- \) Thymocytes Injected Intravenously**

<table>
<thead>
<tr>
<th>No. of cells injected</th>
<th>( \gamma/\delta^+ ) thymocytes</th>
<th>( \gamma/\delta^- ) thymocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( 1.5 \times 10^6 )</td>
<td>( 3 \times 10^6 )</td>
</tr>
<tr>
<td>Intestinal colonization by ( \gamma/\delta ) T cells/no. of recipients</td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>0/3</td>
<td></td>
</tr>
</tbody>
</table>

*Sorted \( \gamma/\delta^- \) and \( \gamma/\delta^+ \) thymocytes from 14-d-old H.B19ov+ embryos were injected in 16-d-old H.B19ov- embryos. Embryos were killed at day 18 and \( \gamma/\delta \) donor cells were analyzed by two-color immunofluorescence staining of intestine sections. Each slide contained a section of the duodenum, a section of the ileum, and a section located around the coecal tonsils. Counting was performed on three slides for each animal. 500 \( \gamma/\delta \) IEL were counted per slide. By day 22 (after hatching) these results were confirmed by FACScan analysis. Embryos injected with \( \gamma/\delta^- \) thymocytes presented 13 and 5% of \( \gamma/\delta \) IEL of the donor type. No \( \gamma/\delta^- \) donor cells were detected in embryos injected with \( \gamma/\delta^+ \) thymocytes.*

![Figure 4](image_url)
(Table 3), no γ/δ+ T cell progeny were found subsequently, whereas the γ/δ+ thymocytes efficiently populated the intestinal epithelium. We conclude from these observations that the thymus is the prime source of γ/δ+ IELs in the chicken, at least early in development when the question of cell origin can be experimentally addressed most incisively.

It is interesting that ~25% of the IELs of donor thymocyte origin were noted to be CD3+ in tissue sections, but negative for both γ/δ and α/β TCR (Table 1). In these experiments, the entire thymocyte population of 14-d-old embryos was infused in the donor inoculum. The cytoplasmic CD3+/TCR- lymphocytes in the chicken have been characterized in other studies as NK cells (29). Thus, the avian thymus may contain precursors of both T and NK cells, as has been proposed for mice and humans (30). Although the rearrangement of TCR genes to become a T cell would appear to require microenvironmental cues provided in the thymus, without the need for TCR gene rearrangement, NK cell progeny can be generated in extrathymic tissues (30, 31).

The selective homing of γ/δ T cells to the intestinal epithelium appears to be a highly conserved property of these cells since this pattern of localization is seen in birds, mammals, and amphibians (2, 32), suggesting an important role in defense of this epithelial barrier. Whereas our experimental results indicate that the thymus is the major source of these cells in the chicken, studies in mice suggest that many γ/δ+ IEL are extrathymic in origin. In irradiated thymectomized recipients, the γ/δ IEL population can be restored by infusion of bone marrow or fetal (E15) liver cells (9–12), and γ/δ T cells are also acquired in the intestine and skin of nude mice with severe thymic hypoplasia (33–35). However, it is difficult to be certain of an extrathymic origin for the γ/δ IEL in these experimental models, because the thymic generation of γ/δ T cells begins on day 12 of embryonic life, and the nude mouse has a small vestigial thymus with apparent lymphopoietic function (36). Rearrangements of the Vγ5 gene have been identified by PCR technique in the liver and intestine as early as embryonic day 11, but it is not known that these rearrangements are productive (37). The similarity of the Vγ5/Vδ1 repertoire in the gut and thymus of mice may imply that most intestinal γ/δ T cells are also thymus derived in the embryonic mouse (38).

In sheep, as in the chicken, the thymus also appears to be the primary source of γ/δ IEL. Thymectomy performed in utero, just a few days after departure of the first thymic emigrants, results in severe and persistent depletion of the γ/δ T cells in lambs (39). Intestinal γ/δ T cells were virtually absent in the thymectomized lambs from birth through the first year of life, in contrast to the abundant population of intestinal γ/δ T cells in normal animals. It is interesting to note that γ/δ cells are predominant IEL in both chicken and sheep, whereas they represent 50% of IEL in mice and no more than 10–15% of IEL in human (2). One possibility is that these numerical differences in γ/δ IEL abundance may reflect differences in the proportion of extrathymic versus thymic-derived γ/δ IEL in these species. Alternatively, the complementary nature of the results in the sheep model and our data in the chicken may suggest the need for reappraisal of the hypothesis of an extrathymic origin for γ/δ T cells during mouse and human development.

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