Abrogation of Prostaglandin E\textsubscript{2}/EP4 Signaling Impairs the Development of \textit{rag}^{1+} Lymphoid Precursors in the Thymus of Zebrafish Embryos

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Abrogation of Prostaglandin E\textsubscript{2}/EP4 Signaling Impairs the Development of \textit{rag1}\textsuperscript{+} Lymphoid Precursors in the Thymus of Zebrafish Embryos\textsuperscript{1}

Eduardo J. Villablerna,\textsuperscript{*†} Anna Pistocchi,\textsuperscript{‡} Felipe A. Court,\textsuperscript{§} Franco Cotelli,\textsuperscript{‡} Claudio Bordignon,\textsuperscript{*} Miguel L. Allende,\textsuperscript{¶} Catia Traversari,\textsuperscript{||} and Vincenzo Russo\textsuperscript{2*}

PGE\textsubscript{2} is involved in a wide variety of physiological and pathological processes; however, deciphering its role in early mammalian development has been difficult due to the maternal contribution of PGE\textsubscript{2}. To overcome this limitation we have investigated the role of PGE\textsubscript{2} during T cell development in zebrafish. In this study, we show that zebrafish ep4a, a PGE\textsubscript{2} receptor isoform of EP4, is expressed at 26 h postfertilization in the dorsal aorta-posterior cardinal vein joint region, which has a high homology with the mammal aorta-gonad-mesonephros area and where definitive hemopoiesis arises. Furthermore, it is expressed in the presumptive thymus rudiment by 48 h postfertilization. Supplementation of PGE\textsubscript{2} results in a strong increase in \textit{rag1} levels and cell proliferation in the thymus. In contrast, the inhibition of PGE\textsubscript{2} production, as well as EP4 blockade, abrogates the expression of \textit{rag1} in the thymus and that of the lymphoid precursor marker \textit{ikaros}, not only in the dorsal aorta-posterior cardinal vein joint region but also in the newly identified caudal hemopoietic tissue without affecting early hemopoietic (\textit{scl}, \textit{gata2}) and erythropoietic (\textit{gata1}) markers. These results identify ep4a as the earliest thymus marker and define a novel role for the PGE\textsubscript{2}/EP4 pathway in controlling T cell precursor development in zebrafish. 


Prostaglandins are lipid-derived autocoids produced by membrane arachidonic acid by virtue of the sequential activity of cyclooxygenase (COX)\textsuperscript{1}, COX2, and PG synthases (1, 2). PGE\textsubscript{2} is one of the major COX products, inducing a plethora of effects under a number of physiological conditions. PGE\textsubscript{2} regulates diverse functions of many cell types of the immune system, including dendritic cells, macrophages, and T and B lymphocytes (1, 3, 4). However, in T cells as well as in thymus development little is known about the contribution of PGE\textsubscript{2}. PG bio-synthetic enzymes and receptors have been detected in human, mouse, and rat thymi (5, 6). Thymic cell lines secrete prostaglandins by virtue of the activity of COX1 and COX2 enzymes (7).

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\textsuperscript{2}Address correspondence and reprint requests to Dr. Vincenzo Russo, Cancer Gene Therapy Unit, Scientific Institute H. San Raffaele, Via Olgettina 58 Milan, Italy. E-mail address: v.russo@hsr.it

\textsuperscript{3}Abbreviations used in this paper: COX, cyclooxygenase; AGM, aorta-gonad-mesonephros; CHT, caudal hemopoietic tissue; DA-PCV, dorsal aorta-posterior cardinal vein; DN, double negative; DP, double positive; dpf, days postfertilization; hpf, hours postfertilization; HSC, hemopoietic stem cell; ICM, intermediate cell mass; inh, inhibitor; MO, morpholino oligonucleotide; PCNA, proliferating cell nuclear marker; WISH, whole in situ hybridization.

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produces erythrocytes and some primary macrophages, and definitive hemopoiesis, which gives rise to long-term hemopoietic stem cells (HSCs) capable of unlimited self-renewal that generate all mature hemopoietic lineages (19). In zebrafish, primitive hemopoiesis occurs in the intermediate cell mass (ICM) located in the trunk and in the rostral blood island, whereas definitive hemopoiesis occurs in the dorsal aorta-posterior cardinal vein (DA-PCV) joint region, which is equivalent to the mammal aorta- gonad-mesonephros (AGM) region (18). Recently, an intermediate site of hemopoiesis, the caudal hemopoietic tissue (CHT) has been described (20). This site represents a transitional niche whose function would resemble that of the mammalian fetal liver.

In the present study we have investigated the role of PGE2/EP4 signaling in thymus development in vivo. We show ep4a expression in the thymus rudiment by 48 hpf and in the AGM, where definitive hemopoiesis arises, by 26 hpf. Additionally, we show expression in the thymus rudiment by 48 hpf and in the AGM, where definitive hemopoiesis occurs in the dorsal aorta-posterior cardinal vein (DA-PCV) joint region, which is equivalent to the mammal aorta- gonad-mesonephros (AGM) region (18). Recently, an intermediate site of hemopoiesis, the caudal hemopoietic tissue (CHT) has been described (20). This site represents a transitional niche whose function would resemble that of the mammalian fetal liver.

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The Materials and Methods section is as follows:

### Materials and Methods

#### Zebrafish colony

Zebrafish were bred and maintained as described (21). Cloche mutants and fltl:/GFP transgenic line were provided by Drs. M. Schorpp (Max-Planck Institute of Immunobiology, Freiburg, Germany) and F. Cotelli (University of Milan, Milan, Italy), respectively.

#### ep2, ep4a, and ep4b cDNA cloning

By using the human EP-4 protein (GenBank accession no. AAA36434; previously missnamed EP2; Ref. 22) as a query, we searched the zebrafish expressed sequence tag database and identified three complete cDNA clones (National Center for Biotechnology Information accession nos. DQ286580, XM685848, and DQ202321). The protein sequences of these clones were aligned and showed high similarity to that of the mammalian PGE2 receptor EP4 (DQ202321 and XM685848) and EP2 (DQ286580). Homology search was conducted using the Basic Local Alignment Search Tool (BLAST) (23). Ep4a (DQ202321) and EP2 (DQ286580) have previously been described (24).

#### In situ hybridization, RT-PCR analysis and morpholinos

Whole-mount in situ hybridization was performed as described (25). Analysis of the expression of ep4a by RT-PCR was performed using total RNA from the developmental stages 24 hpf, 48 hpf, 3 days postfertilization (dpf), and 4 dpf and from ovaries extracted from adult fish. RT-PCR was conducted using the following primers: 5’-TGCTCAATCCCGCTTGTTGTC and 4 dpf and from ovaries extracted from adult fish. RT-PCR was conducted using the following primers: 5’-TGCTCAATCCCGCTTGTTGTC and 5’-CCGGGTTTGGTCTTGCTGATGAAT-3’ (mp4a-SGR1), 5’-ATCGTCTCTATGCCACGCCACTCT-3’ (mp4b-SGF1) and 5’-TCCGGGTTTGGTCTTGCTGATGAAT-3’ (mp4b-SGF1), and 5’-TCCGGGTTTGGTCTTGCTGATGAAT-3’ (mp4b-SGF1). The protein sequences of these clones were aligned and showed high similarity to that of the mammalian PGE2 receptor EP4 (DQ202321 and XM685848) and EP2 (DQ286580). Homology search was conducted using the Basic Local Alignment Search Tool (BLAST) (23). Ep4a (DQ202321) and EP2 (DQ286580) have previously been described (24).

#### Immunohistochemistry

Dechorionated zebrafish embryos were collected and fixed overnight at 4°C in a solution of 4% paraformaldehyde in PBS containing 1% Tween 20 (PBT solution). Embryos were permeabilized by proteinase K treatment (25 μg/ml) for 1 h and then fixed. Samples were washed in PBT solution and blocked with 10% sheep serum at room temperature. Primary and secondary Abs were added overnight at 4°C in PBT solution. Ab dilution was 1/200 (Alexa Fluor 488 and mouse anti-proliferating cell nuclear Ag (PCNA); Santa Cruz Biotechnology).

#### Pharmacological experiments

Commercially available PGE2 and PGG2, COX2-inh (NS-398), COX1-inh (SC-560), and indomethacin (COX1/2-inh) were from Cayman Chemical. The selective COX2-inh SC-236 was provided by Drs. G. Folco and C. Bucellati, University of Milan, Milan, Italy. Butaprost and AH23848 were from Sigma-Aldrich. We supplemented wild-type embryos with 50 μM PGE2, 10–50 μM PGG2, 10 μM AH23848, and 25–50 μM of COX inh.

### Results

Ep4a is expressed by hemopoietic/lymphoid tissues during embryonic zebrafish development

Recently two orthologues of the human PGE2 receptors EP2 and EP4 have been described in zebrafish (24). We sought to identify the entire repertoire of zebrafish EP receptors to examine their expression and role in thymus development. By using the tBLASTn search algorithm of the National Center for Biotechnology Information we obtained a sequence orthologous to human ep2 and two sequences orthologous to human ep4 named ep4a and ep4b. Zebrafish ep1 and ep3 orthologues were not found. We detected ep2 and ep4a transcripts by RT-PCR at 24 hpf and up to 4 dpf, whereas ep4b became undetectable at 48 hpf and re-expressed at 4 dpf (data not shown). This differential expression pattern suggests nonredundant functions between these two ep4 isoforms.

We then investigated whether these genes were expressed in the thymus rudiment during zebrafish development by WISH. At 48 hpf ep4a was expressed in a bilateral anatomic structure where at later stages the thymus anlage appears (Fig. 1, a–c). In contrast, the other EP receptors were not detected in this position (data not shown). The earliest known thymus markers, namely ikaros and foxn1, begin to be expressed at 3 dpf (12, 15, 16). Therefore, we analyzed ep4a expression at this stage and later on. At 3.5, 4, and 5 dpf ep4a expression was maintained in the presumptive thymus anlage (Fig. 1, d–f). To better define whether ep4a was expressed within the thymus, we performed double WISH analysis at the 5-dpf stage using the T cell precursor markers rag1 and ep4a. The double labeling showed a colocalization of both mRNAs in the thymus area, thus indicating that ep4a was indeed expressed in the thymus (Fig. 1e, purple staining, black arrowheads). Because the thymus is composed of an epithelial compartment and immigrant lymphoid progenitors that start to colonize the thymic rudiment by 65 hpf (27, 28), we sought to distinguish whether ep4a was expressed in one or both thymic cellular components. To address this issue, we took advantage of the cloche mutant line, which is devoid of endothelial cells, thus lacking T cell precursors within the thymus (29). WISH analysis showed that ep4a expression in the thymus of these mutants was considerably weaker than in wild-type embryos although not absent (Fig. 1f), demonstrating that ep4a was expressed in both thymic cellular components. Because it has been proposed that lymphoid precursors seeding the thymus arise from the AGM
HSCs (19), we looked for ep4a expression in this site. By 26 hpf ep4a was detected in the AGM (Fig. 1g) with an expression pattern resembling that reported for c-myb (16). In contrast, ep2 and ep4b were not detected (data not shown).

Taken together, these data indicate that ep4a is the earliest thymus marker identified to date. The expression of ep4a in sites of hemopoiesis (i.e., AGM) suggests a role for ep4a in T cell development.

PGE$_2$ treatment acting via EP4a increases rag1 expression in the thymus

PGE$_2$ is the naturally produced ligand of EP receptors. Moreover, it has been shown that zebrafish embryos produce PGE$_2$ during development (30). To investigate the role of PGE$_2$/EP4 signaling in the thymus and/or T cell development, we treated zebrafish embryos at 3 dpf when T cell precursors start to colonize the thymus, with PGE$_2$. Embryos were fixed after 24 h of incubation and the T cell precursor marker rag1 was visualized by WISH. Thymi of PGE$_2$-treated embryos showed a stronger rag1 expression than either control or DMSO-treated embryos (Fig. 2, a and c). To provide objective criteria and statistical backing to these differences of expression, we randomly took several images of the thymus of untreated (n = 7), DMSO-treated (n = 7), and PGE$_2$-treated (n = 8) embryos. After alignment of the thymus images, the mean pixel intensity was obtained by using the MATLAB software. This analysis showed an increase of the area highly expressing the rag1 transcript in PGE$_2$-treated embryos compared with untreated or DMSO-treated embryos (Fig. 2a, middle column). Three-dimensional analysis confirmed an increase of pixel intensity in the rag1-treated thymus (Fig. 2a, right column). The increase of intensity between controls and PGE$_2$-treated embryos was statistically significant (Fig. 2b; ***, p < 0.005). In contrast, the 24-h treatment with either COX1-inh (SC-560) or COX2-inh (SC-236), which blocks the endogenous PGE$_2$ synthesis, did not allow rag1 expression in the thymus (Fig. 2c). In agreement with these results, images from thymi treated with the COX2-inh (n = 4) resulted in flat, three-dimensional pseudocolor graphs indicating levels of expression similar to background (Fig. 2a, middle and right columns). Again, the decrease of rag1 expression was statistically significant (Fig. 2b, ***, p < 0.005).

To simplify and extend the analysis to a larger number of embryos, we set up a quantitative WISH assay. Embryos were placed into three arbitrary categories (low/null, normal, and high; Fig. 3) depending on the level of rag1 expression in the thymus. In different experiments, untreated embryos showed some variability in the expression of a given phenotype (normal, range 35.7–51.7%; null/low, range 14.7–38%; and high, range 25–44%). Therefore, the results were normalized by reporting in Table I the ratio of the percentage of embryos detected in treated and untreated embryos for each category. The quantitative WISH analysis confirmed the results obtained by using the MATLAB software. As reported in Table I, embryos treated with PGE$_2$ for 24 h showed a 1.8-fold increase of the high rag1 phenotype (treated to untreated ratio was 46.2/26.5%), while the number of embryos with null/low phenotype decreased (treated to untreated ratio was 15.3/21.9%). The treatment with either COX inhibitor resulted in a complete null/low phenotype due to the whole lack of rag1 signal in the thymus. Similar results were obtained using different COX inhibitors (NS-398 and indomethacin; data not shown). In addition, the response to COX2-inh was dose dependent (Table I). No such modifications were observed in DMSO-treated embryos. Furthermore, incubation of zebrafish embryos with other prostaglandins like PGD$_2$ did not influence rag1 expression (Table I), indicating that the increase of rag1 expression was PGE$_2$-dependent.

The role of PGE$_2$ was further supported by rescue experiments. As shown in Table I, PGE$_2$ was able to revert the phenotype elicited by COX2-inh, inducing a decrease of the null/low phenotype from 4.6 to 2.5 (treated to untreated ratio from 100/21.8% to 54/
21.8%) and a partial increase of the high category from 0 to 0.2 (treated to untreated ratio from 0/26.5% to 6.25/26.5%). More importantly, we observed a rescue of the normal phenotype, which went from 0% for COX2-inh alone (treated to untreated ratio

FIGURE 2. PGE2, COX2-inh, and the EP4 antagonist AH23848 differentially affect rag1 expression in the thymus of zebrafish embryos. a, Pseudocolor surface plots of the mean signal intensity (scale, 0–140) for rag1 mRNA in the thymi of untreated (UT; n = 7) and DMSO- (n = 7), PGE2- (n = 8), AH23848- (n = 8), and COX2-inh-treated (n = 4) embryos. The left column shows a representative WISH image of the thymus, the middle column represents the pseudocolor surface plot of the mean signal intensity obtained by concatenating individual images in the Z dimension, and the right column is a three-dimensional array showing the mean signal intensity in the Z axis. After PGE2 exposure the area highly expressing rag1 (red) is increased. In contrast, the intensity is reduced after the treatment with AH23848 (EP4 antagonist) and is completely absent following the incubation with COX2-inh. b, Graph representing the mean intensity of the rag1 signal collected from control or treated embryos. Rag1 expression in the thymus was increased by PGE2 and reduced by treatments with AH23848 and COX2-inh. Asterisks indicate a statistically significant difference between treated embryos and controls (untreated (UT) and DMSO-treated embryos) based on Student’s t-test. *, p < 0.05; **, p < 0.005. c, Representative WISH images showing rag1 expression in the thymus of control (UT) and treated embryos. Embryos were treated with PGE2, COX1/2-inh, or COX2-inh for 24 h. PGE2-treated embryos show an increase in the rag1 signal, whereas COX1/2- or COX2-inh treated embryos show a complete inhibition of rag1 expression. Each inset is a magnification of the thymus.

FIGURE 3. Arbitrary categories of rag1 expression in thymi. Representative WISH images showing the different levels of rag1 expression detected in untreated embryos. The three arbitrary categories null/low, normal, and high were defined according to the different levels of rag1 expression in the thymus.

Table 1. WISH analysis of rag1 expression in the thymus of treated embryos

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Null/Low</th>
<th>Normal</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGE2 (n = 119)</td>
<td>0.5</td>
<td>0.9</td>
<td>1.8</td>
</tr>
<tr>
<td>COX1/2-inh (n = 50)</td>
<td>4.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>COX2-inh (25 μM) (n = 51)</td>
<td>2.6</td>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>COX2-inh (50 μM) (n = 51)</td>
<td>4.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>COX1-inh plus PGE2 (n = 50)</td>
<td>4.1</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>COX2-inh plus PGE2 (n = 49)</td>
<td>2.5</td>
<td>0.8</td>
<td>0.2</td>
</tr>
<tr>
<td>PGD2 (n = 21)</td>
<td>0.8</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td>AH23848 (n = 42)</td>
<td>1.9</td>
<td>0.6</td>
<td>0.2</td>
</tr>
<tr>
<td>DMSO (n = 70)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>EP4-MO1 (n = 102)</td>
<td>2.8</td>
<td>0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>EP4-MO2 (n = 74)</td>
<td>4.3</td>
<td>0.9</td>
<td>0.5</td>
</tr>
<tr>
<td>Ctrl-MOs (n = 75)</td>
<td>1.2</td>
<td>1.3</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Embryos were injected with morpholino at 1-cell stage, or treated with the different compounds at 3 dpf. Rag1 expression was analyzed at 4 dpf. n, Number of treated embryos.

The level of rag1 expression was scored as null/low, normal, or high in accordance with Fig. 3. The results were normalized by reporting the ratio of the percentages of positive embryos detected in treated (T) and in untreated (UT) animals for each category.
Of COX1-inh was only slightly affected by the PGE2 addition (Table I) to 41.6% by adding PGE2 (treated to untreated ratio 41.6/51.6% = 0.8 in Table I), almost reaching the level detected in untreated embryos. On the contrary, the activity of COX1-inh was only slightly affected by the PGE2 addition (Table I). Thus, a direct role of the COX2/PGE2 pathway in regulating ragl expression in the thymus is demonstrated.

Because EP4 was the only PGE2 receptor detected in the thymus, we sought to verify whether PGE2 activity was indeed exerted via the EP4 receptor. To address this question we used the EP2 agonist butaprost and the EP4-specific antagonist AH23848. Butaprost treatment for 24 h did not increase ragl expression, in accordance with the absence of ep2 transcripts in the thymus (data not shown). In contrast, embryos treated with AH23848 showed a marked inhibition of ragl transcripts (Table I). The MATLAB analysis of thymi from embryos treated with the AH23848 (n = 8) demonstrated a statistically significant reduction of ragl expression compared with controls (Fig. 2, a and b, ∗, p < 0.05). To confirm this inhibition, we genetically blocked ep4a by performing MO knockdown experiments (31). Because the treatment with 0.26 pmol/embryo results in gastrulation defects (24), we tested lower concentrations to identify the dose (i.e., 0.13 pmol/embryo) allowing a normal gastrulation process. The injection of either ep4a-MO1 or ep4a-MO2 at 0.13 pmol/embryo caused an increase of 2.8- and 4.3-fold, respectively, of embryos with a null/low ragl expression category in comparison with untreated embryos (treated to untreated ratio was 70/39% and 26/66%, respectively). As expected, the control MOs did not increase this category (Table I).

To verify whether the absence of ragl+ cells within the thymus upon EP4 inhibition was due to the induction of apoptosis, we performed TUNEL experiments. The thymi of 5 dpf embryos treated with PGE2, AH23848, or left untreated showed a similar number of apoptotic cells (data not shown), demonstrating that the decrease of ragl-expressing cells in the thymus upon EP4 inhibition was not due to programmed cell death. Based on this we can conclude that the PGE2/EP4 signaling in the thymus controls the expression of ragl.

### PGE2 induces cell proliferation in the zebrafish thymus

The Ragl signal in the thymus of PGE2-treated embryos was more widespread than in control siblings (Figs. 2 a and 3). This hybridization pattern suggests that the increase in ragl expression is not likely due to enhanced transcription in single cells. Therefore, we sought to ascertain whether the PGE2-induced increase of ragl expression was due to either an enlargement of thymus, and therefore an increase in the total number of epithelial cells, or to an increased number of ragl+ cells. Indeed, in vitro experiments have shown that PGE2 protects human thymocytes from apoptosis (32) and induces thymocyte differentiation from DN to DP cells (7). We first tested whether the PGE2/EP4 pathway was able to settle the thymus architecture. The epithelial component of the thymus did not undergo significant changes in size upon PGE2 treatment as evaluated by using foxn1, a specific marker for the epithelial compartment of the zebrafish thymus (15) (Fig. 4, a and b). These results were further confirmed by treating embryos with either the EP4 antagonist AH23848 or the COX-inh or by injecting the EP4-MOs (Fig. 4, c–f). We then tested whether PGE2 could favor T cell precursor differentiation by inducing ragl expression. To this end we provided PGE2 to zebrafish embryos for 12 or 24 h at 48 hpf when ep4a but not ragl is expressed. No changes in ragl expression kinetics could be detected by WISH (data not shown), suggesting that PGE2 was not favoring thymus differentiation by inducing the premature expression of ragl. We next investigated whether, upon PGE2 exposure, the proliferation rate of T cell precursors was increased. By using the PCNA we observed that PGE2-treated embryos showed PCNA+ cells in the thymus (Fig. 4h), whereas the control and AH23848-treated embryos did not (Fig. 4, g and i). Taken together, these data indicate that PGE2, acting via the EP4 receptor, increases the number of ragl-expressing cells as well as cell proliferation in the zebrafish thymus.

### Blocking PGE2/EP4 signaling interferes with early lymphoid precursor development

We detected ep4a expression at 26 hpf in the AGM, where definitive hemopoiesis arises (Fig. 1g). Therefore, we hypothesized that, in EP4-lacking embryos, the absence of ragl-expressing cells in the thymus could be due to the impairment of T cell progenitor differentiation in hemopoietic sites. The origin of the lymphoid progenitors that seed the zebrafish thymus is unknown. However, a population of ikaros+ cells present in the ventral wall of the dorsal aorta is thought to be the most likely source (16). At 24 hpf ikaros-expressing cells can be detected in the DA-PCV joint region. At 48 hpf those cells can be detected throughout the pharyngeal arches and later they can be detected in the thymus (16).

To investigate the role of the PGE2/EP4 pathway in controlling ikaros+ cell differentiation, we injected embryos with either ep4a-MO1 or ep4a-MO2. This led to a marked and statistically significant reduction of the expression of ikaros in the AGM at 24 hpf (Fig. 5, a and b, and data not shown). At 5 dpf we observed a strong reduction of the expression of ikaros in ep4a-MO2-injected embryos in a region resembling the recently described CHT (Fig. 5, f and g; arrowheads). Accordingly, the addition of either the EP4 antagonist (i.e., AH23848) or PGE2 partially reduced or modestly increased the level of ikaros expression in the AGM (Fig. 5, c–e). The absence of ikaros+ HSCs in the AGM and the CHT upon inhibition of the PGE2/EP4 pathway was not due to apoptotic cell death as evaluated by TUNEL experiments (data not shown). These results demonstrate that the PGE2/EP4 pathway acts upstream of the differentiation of ragl+ cells, affecting the differentiation of ikaros-expressing cells in the AGM and in the CHT.
we demonstrate that the absence of EP4-lacking embryos (data not shown). Thus, a statistically significant reduction of MO2-injected embryos was not significantly altered, whereas a EP4 inhibition was not due to defects in vascular tube or dorsal fli1 expression in the AGM (bi), demonstrating that **ep4a** was indeed expressed in the thymus. Thus, to our knowledge expression in the ICM of either **ep4a**-MO1- or MO2-injected embryos (**g**) do not express **ikaros** in the CHT. **h** and **i**, fli1::GFP transgenic lines that express GFP in developing blood vessels were injected with **ep4a**-MO1. Both control embryos (**b**) as well as **ep4a**-MO1-injected embryos (**i**) show normal blood vessel development. **d**, Dorsal aorta; **v.t.**, vascular tube. **j**, Representative WISH images from one experiment showing normal and reduced expression of **ikaros**, **gata1**, and **scl** in the ICM of **EP4-MO1**-injected embryos. Percentages of normal and reduced expression are reported. **k**, Graphs showing the results obtained from three independent experiments. **EP4-MO1**- and **EP4-MO2**-injected embryos showed a significant reduction in **ikaros** expression (**p < 0.005; n = 96**), whereas **gata1** and **scl** were not significantly affected. UT, Untreated.

To investigate whether **ep4a** could interfere with the development of the vascular tube and dorsal aorta, we took advantage of the fli1::GFP transgenic line (33) whose blood vessels constitutively express GFP. The expression of GFP was intact in embryos treated with PGE2, AH23848, or **EP4-MO1** both at 24 hpf and 5 dpf (Fig. 5, **h** and **i**, and data not shown). Furthermore, the analysis of vessel and artery formation by using WISH for **flk-1** and en, respectively, did not show any difference between control and EP4-lacking embryos (data not shown). Thus, we demonstrate that the absence of **ikaros**+ cells mediated by EP4 inhibition was not due to defects in vascular tube or dorsal aorta specification.

To confirm the specific role of the PGE2/EP4 pathway in lymphoid cell differentiation, we analyzed the expression of the HSC markers **scl**, **gata1**, and **gata2** at 24 hpf following the injection with **ep4a**-MO1, **ep4a**-MO2, or MOs control. An example of the expression patterns (i.e., normal and reduced) observed upon the treatments (e.g., **ep4a**-MO1) is reported in Fig. 5j. The expression of **scl**, **gata1**, and **gata2** in the ICM of either **ep4a**-MO1- or **ep4a**-MO2-injected embryos was not significantly altered, whereas a statistically significant reduction of **ikaros** expression was observed (Fig. 5k and data not shown). Thus, the involvement of **ep4a** in lymphoid precursor development is indicated.

In conclusion, these results indicate that the PGE2/EP4 pathway acts downstream of the activation of **gata2** in the differentiation of a nonerythropoietic, lymphoid-specific (**ikaros**+) precursor.

**Discussion**

In this report, we demonstrate that the PGE2/EP4 pathway is involved in lymphoid precursor development in zebrafish. Indeed, the inhibition of PGE2 production abrogated **rag1** expression within the thymus, whereas its addition induced an increase of **rag1**-expressing cells. Importantly, we demonstrate pharmacologically and genetically that **rag1** induction occurs specifically via the EP4 receptor. Interestingly, we find another **ep4** isoform (i.e., **ep4b**) that is not expressed in the AGM or the thymus (data not shown), which suggests different roles for the two EP4 isoforms.

In zebrafish, the thymic anlage is first detectable by 60 hpf by ultrastructural analysis (18). At this time, T cell precursors begin to seed the thymus (13, 28). **ikaros** is the first lymphoid-related gene detected in the thymus (16, 34) at 72 hpf. Later on (92 hpf), developing thymocytes begin to express **rag1** and **rag2** (27, 34). Interestingly, by 48 hpf we found that **ep4a** was expressed bilaterally in the presumptive thymus rudiment. Later, the expression was maintained and colocalized with **rag1**, demonstrating that **ep4a** was indeed expressed in the thymus. Thus, to our knowledge **ep4a** becomes the earliest thymus marker described to date, useful for tracking thymus development in zebrafish.

By using the cloche mutant lacking the entire hemopoietic cell lineage (29), we demonstrated that **ep4a** was expressed in both the thymic epithelium as well as in T cell precursors, a finding consistent with previous in vitro studies in mammals (5, 6). The idea that **ep4a** is expressed by T cell precursors was supported by the strong reduction of **ep4a** expression observed in the cloche mutant as well as by the detection of **ep4a** expression in the DA-PCV joint region, where definitive hemopoiesis arises. Further studies will be needed to clarify the role of **ep4a** in the epithelial cells of the thymus.
In agreement with these findings, pharmacological and genetic inhibition of EP4 reduced ragl expression in the thymus without evidence of cell death, whereas the treatment of embryos with PGE$_2$ induced cell proliferation in the thymus (Fig. 4h). Whether proliferating cells belong to the thymic epithelium, the T cell lineage or both remains unclear. The fact that the inhibition of EP4 induced a partial reduction of ragl while we observed a complete reduction in embryos incubated with COX-inh could be due to the lower doses of EP4-MOs (0.13 pmol/embryo) used to preserve the gastrulation process (24). Alternatively, we may hypothesize a role for other PG derivatives in T cell development. In an in vitro murine model it has been shown that the effect of the COX2-inh NS-398 in T cell development is COX2- and PGE$_2$-independent; indeed, PGE$_2$ was not able to reverse the effect of NS-398 in fetal thymic organ culture (8). In our system the effect of COX1-inh was poorly reversed while that of COX2 was rescued by PGE$_2$, suggesting that the COX1-inh could act in a PGE$_2$-independent manner while the effect of COX2-inh was mainly due to the inhibition of PGE$_2$. However, we cannot exclude the possibility that the activity of COX1-inh, used at lower concentration, could be reverted by PGE$_2$.

Moreover, a pharmacological and genetic blockade of the EP4 receptor recapitulated the inhibition of ragl seen with COX2-inh, further confirming a PGE$_2$-dependent mechanism in the regulation of ragl-expressing cells in the thymus.

In this study, we consistently found ep4a expression in the DA-PCV joint region at 26 hpf (Fig. 1g), where definitive hemopoiesis arises. Inhibition of EP4 abolished the expression of the lymphoid precursor marker ikaros in the AGM and at 5 dpf in the CHT. Additional experiments using transgenic fluorescent reporter lines and/or cell transplants will elucidate the direct lineage relationship between ikaros-expressing cells in the DA-PCV joint region and CHT and the ragl-expressing cells in the thymus. The inhibition of ikaros expression mediated by EP4 blockade indicates that the PGE$_2$/EP4 pathway might play a novel role in hemopoiesis, specifically in lymphopoiesis. This hypothesis was confirmed by dissecting the role of the PGE$_2$/EP4 pathway at different steps of hemopoietic specification. scl and gata2 are genes encoding essential transcription factors for vertebrate hemopoietic specification (19). In zebrafish, the ablation of scl results in loss of primitive and definitive hemopoiesis (35). In contrast, GATA1-null mice and GATA1-lacking cell lines have defective erythroid gene expression (36, 37). Moreover, in zebrafish embryos it has been shown that gata1 regulates the myeloiderythroid fate decision (38). Our results demonstrate that loss of ep4a does not significantly affect the expression of scl and gata2, which are located upstream of ikaros, as well as the expression of the erythroid progenitor marker gata1 (Fig. 4, h and i, and data not shown), thus confirming that EP4 is dispensable for the early steps of HSC specification.

Although PGE$_2$ signaling has been shown to promote thymocyte differentiation and protection from apoptosis (7, 32), its role and the mechanism by which it is involved in T cell development in vivo are completely unknown. Differing from our results in mor- pholino knockout embryos, studies performed in COX1/2 double-knockout mice have shown that PGE$_2$ seems to play a marginal role in T cell development (8). However, in both systems stronger effects were observed when chemical inhibitors were used. These observations suggest a greater PGE$_2$ dependence for T cell development in mammals than in zebrafish, perhaps because of a lower level of redundancy; alternatively, it may reflect on the difference of the experimental systems used (i.e., the in vivo model for zebrafish or the ex vivo model for the murine fetal thymic organ cultures).

Our studies provide the first evidence that PGE$_2$ signaling through the EP4 receptor regulates lymphocyte precursors homoeostasis in vivo. Understanding the detailed mechanism of PGE$_2$ signaling in zebrafish T cell development may provide significant insights into how PGE$_2$ regulates the immune response in mammals and how it may influence the onset of immune system diseases such as autoimmunity or hemological malignancies. In contrast, this study suggests that the expansion/differentiation of ragl$^+$ cells in the thymus could be a mechanism used to generate mature T cells during in differentiation, a process associated with the production of relevant amounts of PGE$_2$.

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Disclosures

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References


