Hematopoietic Stem and Progenitor Cell Activation During Chronic Dermatitis Provoked by Constitutively Active Aryl-Hydrocarbon Receptor Driven by Keratin 14 Promoter

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Received August 1, 2013; accepted November 18, 2013

Polycyclic aromatic hydrocarbons (PAHs) activate aryl-hydrocarbon receptor (AhR). Because PAHs are known as a risk factor for allergic diseases, PAH-induced AhR activation is expected to be involved in the development of the pathology. We previously generated transgenic mice expressing a constitutively active AhR (AhR-CA) under the control of Keratin 14 (K14) promoter (AhR-CA mouse). The mice develop chronic dermatitis with immune imbalance toward Th2 predominance, indicating that the AhR activation driven by K14 promoter provokes allergic response. Because hematopoietic cells actively participate in the development of allergic inflammation, it is important to understand the hematopoietic status under allergic conditions. To clarify how the K14 promoter–driven AhR activation influences hematopoiesis, we analyzed bone marrow and spleen of AhR-CA mice. We verified that AhR-CA was expressed in keratinocytes and thymic epithelial cells but not in hematopoietic cells. The AhR-CA mice with full-blown dermatitis exhibited leukocytosis and skewed differentiation of hematopoietic progenitor cells toward granulocyte-monoocyte lineages. They also showed a significant expansion of short-term hematopoietic stem cells and multipotent progenitors and a subtle reduction in long-term hematopoietic stem cells (LT-HSCs). Their spleens were enlarged and abundantly accumulated hematopoietic stem and progenitor cells. AhR-CA mice at the early stage of dermatitis did not show leukocytosis or splenomegaly but exhibited the granulocyte-monoocyte skewing and the reduction in LT-HSCs. Thus, AhR activation driven by K14 promoter already alters the hematopoietic differentiation and reduces LT-HSCs at the initial stage of dermatitis development. These results suggest that nonhematopoietic exposure to PAHs triggers allergic response and concomitantly affects hematopoiesis.

Key Words: AhR; hematopoietic stem cell; hematopoiesis; allergic inflammation; dermatitis.

Polycyclic aromatic hydrocarbons (PAHs) are major components of air pollutants contained in automobile exhaust, cigarette smoke, and industrial waste. PAHs provoke pleiotropic effects on the cell growth and differentiation. Bone marrow (BM) is one of the vulnerable organs to the toxicity of PAHs, including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Sakai et al., 2003), benzo(a)pyrene (BaP) (Booker and White, 2005), 7,12-dimethylbenza(a)anthracene (DMBA) (N’jai et al., 2010), and benzene (Yoon et al., 2002). Accordingly, systemic and topical exposure to PAHs often results in the disturbance of hematopoiesis. These compounds, in general, activate the aryl-hydrocarbon receptor (AhR) and elicit various adverse effects through AhR-mediated transcription (Marlowe and Puga, 2005; Shimizu et al., 2000). However, PAHs and their metabolites are also reported to activate other signaling pathways, including NF-κB (nuclear factor-kappaB), AP-1 (activator protein-1), and p53 (Li et al., 2004; Solhaug et al., 2004). It seems mandatory to investigate AhR-dependent and -independent pathways separately for the better understanding of the molecular mechanisms how PAHs disturb hematological homeostasis.

Ample epidemiological studies indicate that PAHs are related to the development of allergic diseases (Miller et al., 2010; Nordling et al., 2008). Allergic diseases accompany chronic inflammations mediated by Th2 subtype of CD4+ T lymphocytes. This type of chronic inflammations is characterized by the production of Th2 cytokines including interleukin (IL)-4 and IL-5 (Holgate and Polosa, 2008; Paul and Zhu, 2010). Epithelial cells participate in the development of allergic diseases by secreting Th2-driving cytokines, such as thymic stromal lymphopoietin, IL-25, and IL-33, thereby playing an important role in the initiation of allergic inflammation (Angkasekwinai et al., 2007; Schmitz et al., 2005; Soumelis et al., 2002). Eosinophils, basophils, and mast cells also participate in the allergic diseases by producing Th2 cytokines and Th2-driving cytokines, which amplifies allergic reactions...
(Gessner et al., 2005; Holgate and Polosa, 2008; Paul and Zhu, 2010; Sokol et al., 2008; Wang et al., 2007). Thus, in addition to Th2-skewed immune response, altered activities of epithelial and hematopoietic cells underlie the development of allergic diseases. These contemporary accomplishments lead us to study how each tissue specifically responds to PAHs and how these tissue-specific responses concomitantly contribute to the establishment of allergic diseases.

In order to decipher the PAH-induced pathogenesis, we have been examining the influences of constitutive AhR activation in epithelial cells by generating transgenic mouse lines that express a constitutively active form of AhR (AhR-CA) under the regulation of Keratin 14 (K14) promoter (Tauchi et al., 2005). The mice develop eczematous dermatitis with multiple characteristic features of atopic dermatitis; for instance, massive mast cell accumulation in the dermis, high serum immunoglobulin E level, and increased secretion of IL-4 and IL-5 from splenic T cells, which are signs of immunological imbalance with Th2 predominance. These results suggest that AhR activation under the control of K14 promoter provokes the local inflammation and the systemic immune imbalance. Because hematopoietic cells actively participate in the development of allergic responses (Gessner et al., 2005; Holgate and Polosa, 2008; Paul and Zhu, 2010; Sokol et al., 2008; Wang et al., 2007), clarifying the hematopoietic status in the AhR-CA mice is beneficial to understand the pathological nature of AhR-provoked allergic conditions. Therefore, in this study, we investigated how the K14 promoter–driven AhR activation affects the hematopoiesis.

We first examined the transgene expression and found that AhR-CA is expressed in keratinocytes and thymic epithelial cells but not in BM cells. Based on this result, we challenged the issue as to how the activation of AhR signaling in nonhematopoietic cells influences the hematopoietic cells in the BM and spleen. AhR-CA mice with full-blown dermatitis had 2 salient features of hematopoietic cells: Firstly, hematopoietic progenitor cells (HPCs) exhibited skewed differentiation into the granulocyte-monocyte lineages in AhR-CA mouse BM, accompanied by marked leukocytosis. Secondly, hematopoietic stem and progenitor cell (HSPC) compartments comprising short-term hematopoietic stem cells (ST-HSCs) and multipotent progenitors (MPPs) showed a dramatic expansion in the BM, whereas long-term HSCs (LT-HSCs) were faintly decreased. Similarly, the HSPC accumulation and the lineage skewing were observed in the AhR-CA mouse spleen that was remarkably enlarged. In contrast, AhR-CA mice at the early stage of dermatitis did not show leukocytosis or splenomegaly but clearly exhibited the granulocyte-monocyte skewing and the reduction in LT-HSCs. These results unequivocally demonstrate that constitutive AhR activation in the epithelial cells, in which the K14 promoter is active, affects HSPCs and promotes the proliferation and differentiation into granulocytes and monocytes.

MATERIALS AND METHODS

Mice. AhR-CA mice (line 239) in the mixed background (C57BL/6, DBA/2, and ICR) and their littermate wild-type mice in the same breeding colony were analyzed at 4 and 9–14 weeks of age. AhR-CA mice of line 239 are maintained by mating the transgenic male mice with wild-type female mice because the transgenic female mice are infertile. The fourth week after birth was selected for the analysis time point when the dermatitis was about to initiate. The 9–14th week was selected for the analysis time point when the dermatitis was fully developed. Both male and female mice were examined. Because no significant differences were observed between males and females, the combined results are shown. All the mice were kept in specific pathogen-free conditions and treated according to the regulations presented in The Standards for Human Care and Use of Laboratory Animals of Tohoku University and Guidelines for Proper Conduct of Animal Experiments by the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

Peripheral blood (PB) was collected from the buffal region and analyzed using a hemocytometer (Nihon Kohden Co).

Flow cytometry. Bone marrow cells were collected from femurs and tibias and used for cytometric analysis and cell sorting (FACS AriA1 and LSR Fortessa, BD Biosciences). The BM cells were incubated with antibodies against Ter119 (TER-119), Mac-1 (M1/70), Gr-1 (RB6-8C5), B220 (RA3-6B2), and CD3e (145-2C11). For lineage-negative (Lin−) cell analyses, BM cells were incubated with biotin-conjugated lineage-positive antibodies, and the lineage-positive cells were removed using streptavidin-coupled conjugate M280 Dynabeads (Invitrogen). The Lin− cells were incubated with antibodies against c-Kit (2B6), Sca-1 (D7), FcγR (93), Flt3 (A2F10), CD34 (RAM34), CD48 (HM48-1), and CD150 (TC15-12F12.2). The CD48 and Sca-1 antibodies were purchased from BioLegend and BD Biosciences, respectively. The other antibodies were purchased from eBioscience. Splenocytes were obtained from whole spleen, hemolyzed, and incubated with the indicated antibodies. The data were analyzed using BD FACSDiva software (BD Biosciences).

Immunoblotting analysis. Thymocytes were collected by crushing thymus into the single cell suspension. The remaining portion of the thymus that still contains thymocytes as well as thymic epithelial cells was used as whole thymus. Skin, whole thymus, thymocytes, and BM cells were homogenized in 2x sample buffer (125mM Tris–HCl [pH 6.8], 10% 2-mercaptoethanol, 4% SDS, 20% glycerol, and bromophenol blue) and boiled at 95°C for 5 min. The samples were loaded on 10% SDS-PAGE and transferred to PVDF membranes (Millipore). The membranes were blocked with 5% skim milk in TBS-T (0.05% Tween 20 in Tris-buffered saline) and reacted with primary antibodies followed by horseradish peroxidase–conjugated secondary antibodies. The band signals were developed with Chemi-Lumi One L (Nacalai Tesque). The primary antibodies used in this study were anti-hemagglutinin (HA) (3F10, Roche), anti-Cyp1a1 (G-18, Santa Cruz Biotechnology), anti-Keratin 14 (AF646, Covance), and anti-A ctin (I-19, Santa Cruz Biotechnology) antibodies.

Histological analysis and immunohistochemistry. Tissues were fixed in 3.7% formaldehyde, embedded in paraffin, and sectioned. For the section of BM, the samples were decalcified before the embedding. The sections were stained with hematoxylin and eosin for histological analyses. For the detection of K14 and Cyp1a1, the sections were treated with 3% H2O2 in methanol, washed in distilled water and TBS, and incubated in the blocking solution (Protein Block Serum-Free; X0909, Dako). In the case of K14, antigen retrieval was conducted by autoclaving the samples for 10 min before the H2O2 treatment. After the blocking, the sections were reacted with the primary antibodies against K14 (AF646, Covance) or Cyp1a1 (G-18, Santa Cruz Biotechnology), followed by the reaction with appropriate secondary antibodies. The staining signals were visualized with 3,3-diaminobenzidine tetrahydrochloride (Liquid DAB+ Substrate Chromogen System, Dako), and the sections were counterstained with hematoxylin. The sections of skin, thymus, and BM of each genotype were put through the Substrate Chromogen System, Dako), and the sections were counterstained with hematoxylin.
Real-time quantitative PCR analysis. Total RNA was purified from Lin−Sca-1−c-Kit− (LSK) and Lin−Sca-1−c-Kit− (LK) cells in addition to skin, whole thymus, thymocytes, and BM cells. Complementary DNA was synthesized using SuperScript III reverse transcriptase (Invitrogen). Quantitative PCR was performed on an Applied Biosystems 7300 sequence detector system with Power SYBR Green PCR Master Mix (Applied Biosystems) and THUNDERBIRD SYBR qPCR Mix (Toyobo) for SYBR Green system and with THUNDERBIRD Probe qPCR Mix (Toyobo) for Taqman probe system. The primers utilized in this study were shown in Supplementary Table S1.

Bromodeoxyuridine uptake and cell cycle analysis. Bromodeoxyuridine (BrdU) was ip injected into the mice (1 mg/mouse). After 18 h, Lin− cells were obtained from the mice and stained with the indicated antibodies followed by fixation and staining with BrdU antibodies using BrdU Flow Kit according to the manufacturer’s protocol (BD Pharmingen). To perform cell cycle analysis, Lin− cells were incubated with antibodies against c-Kit, Sca-1, and CD34 and fixed using a Cytofix/Cytoperm Kit (BD Biosciences). The fixed cells were incubated with anti-Ki67 antibodies (SolA15) (eBioscience), stained with Hoechst 33342 (Sigma), and analyzed using flow cytometry. FlowJo software (Tree star) was used for BrdU uptake analysis, and BD FACSDiva software was utilized for cell cycle analysis.

Statistical analysis. The quantitative data are presented as the means ± SD and were analyzed using Student’s t test. p < .05 was considered statistically significant.

RESULTS

AhR-CA Transgene Is Expressed in Skin Keratinocytes and Thymic Epithelial Cells but not in Hematopoietic Cells

We generated the AhR-CA mice by introducing a transgene encoding AhR-CA under K14 promoter regulation and found that the mice spontaneously provoked chronic dermal inflammation (Tauchi et al., 2005). Before going into analysis of hematopoiesis, we examined the transgene expression of AhR-CA mice in hematopoietic cells as well as in skin and thymus, because previous studies described that K14 promoter is active in the basal cell layer of skin and thymic cortical epithelium (Guo et al., 1993; Laufer et al., 1996). In the AhR-CA mice, transgene-derived AhR messenger RNA (mRNA) was detected in skin and whole thymus but not in thymocytes or whole BM cells (Fig. 1A, top panel). Consequently, total AhR mRNA was increased in the former two but not in the latter two of AhR-CA mice (Fig. 1A, middle panel). Among BM cells, we examined hematopoietic cell fractions, ie, LSK and LK cells, and found that these cells did not express detectable amount of the transgene (Fig. 1B, upper panel). Expression levels of the total AhR mRNA in LSK and LK cells were much lower than those of the AhR-CA mouse skin and comparable between the cells of 2 different genotypes (Fig. 1B, lower panel). Thus, AhR-CA mRNA is expressed in skin and thymus but not in thymocytes or BM hematopoietic cells.

The AhR-CA transgene expression was also assessed at protein levels. Because the AhR-CA protein is linked to HA tag (Fig. 1C), we utilized an anti-HA antibody for the detection of AhR-CA. The anti-HA antibody detected the transgene protein in whole cell extracts of skin and thymus from AhR-CA mice (Fig. 1D, HA sh). In contrast, no bands were observed in whole cell extracts of thymocytes or BM cells even in the long-exposure film (Fig. 1D, HA lo). To validate whether AhR-CA was constitutively active in the tissues where it is expressed in AhR-CA mice, we examined the expression of Cyp1a1, which is one of typical AhR target genes. The mRNA levels of Cyp1a1 were elevated in the skin and thymus of AhR-CA mice but not in the thymocytes or BM cells (Fig. 1A, bottom panel), which was precisely reflected in Cyp1a1 protein levels (Fig. 1D, Cyp1a1). These results verify that the hematopoietic cells and thymocytes do not express the AhR-CA transgene.

To clarify the distribution of AhR-CA-expressing cells in skin and thymus of AhR-CA mice, we conducted immunohistochemistry using anti-Cyp1a1 antibody so that we could detect the cells where AhR-CA was substantially functioning. In agreement with the results of immunoblot analysis, Cyp1a1-positive signals were distributed in the keratinocytes of skin and thymic cortical cells of AhR-CA mice (Fig. 1E, panels a–h). We then compared the distribution of Cyp1a1-positive cells with that of K14-positive cells. In the skin, almost all layers of keratinocytes appeared positive for K14 expression, especially in AhR-CA mice (Fig. 1E, panels i–n), although K14 has been known to localize in the basal layer keratinocytes. The distribution patterns of Cyp1a1 and K14 were very similar in AhR-CA mouse skin (Fig. 1E, panels b, d, l, and n). In AhR-CA mouse thymus, K14-positive cells existed in the cortical and medullary regions, and their distribution in the cortical region was similar to that of Cyp1a1-positive cells (Fig. 1E, panels f, h, p, and r). This corresponds to the previous report that the K14 promoter is active in the thymic cortical epithelial cells (Lauffer et al., 1996). The K14-positive cells were increased in the cortical region of AhR-CA mouse thymus compared with that of wild-type mouse thymus (Fig. 1E, panels o–r), suggesting that AhR-CA expression promotes the proliferation of thymic cortical epithelial cells.

In the immunohistochemical examination of the BM, we detected no Cyp1a1-positive cells (Fig. 1E, panels i and j), which suggests that AhR-CA was not expressed in either hematopoietic cells or stromal cells of AhR-CA mouse BM. This was supported by the observation that K14 staining was not detected above the background level in the BM, regardless of genotypes (Fig. 1E, panels s and t). Faint staining for K14 in the BM was interpreted as nonspecific because no bands were detected in the immunoblot analysis (Fig. 1D, K14). These results show that in the AhR-CA mice, the AhR pathway is activated in keratinocytes and thymic cortical epithelial cells but not in BM cells, including stromal cells, and thereby indicate that the BM microenvironment for hematopoietic cells is comparable between AhR-CA and wild-type mice. Thus, AhR-CA mice exhibit genetic activation of AhR in extra-BM tissues and serve as a good model for our analysis to assess hematopoietic alterations under the condition in which AhR is constitutively active in nonhematopoietic cells.
AhR-CA transgene is expressed in skin and thymus but not in hematopoietic cells. A, Expression levels of transgene (Tg)-derived AhR, total AhR, and Cyp1a1 in skin, whole thymus, thymocytes, and whole bone marrow (BM) cells (n = 3–4). Thymus, whole thymus; T, thymocytes; BM, whole BM cells. B, Expression levels of Tg-derived AhR and total AhR in Lin−Sca-1−c-Kit+ (LSK) and Lin−Sca-1−c-Kit+ (LK) cells (n = 3–5). All the samples were quantified against the same standard curve, and each expression level was normalized to Gapdh expression. Data represent the means ± SD. C, Structure of AhR-CA protein expressed from the transgene. The AhR-CA lacks PAS-B domain, a ligand-binding domain, and hemagglutinin (HA) tag is added at the C-terminus. bHLH, basic helix-loop-helix. D, Immunoblot analysis of whole cell extracts from skin, whole thymus, thymocytes, and whole BM cells. AhR-CA protein was detected by using anti-HA antibody. HA sh and HA lo indicate short- and long-exposure results of HA detection, respectively. Cyp1a1 and K14 were detected by using each specific antibody. Actin was detected as a loading control. Tg, AhR-CA mice. E, Immunohistochemical detection of Keratin 14 (K14) and Cyp1a1. Boxed areas in upper panels are shown at a higher magnification in lower panels for skin and thymus. Black arrows indicate the stained cells for Cyp1a1 and K14 in panels h and r, respectively. The scale bar corresponds to 20 μm in panels c, d, m, and n; 50 μm in panels a, b, k, l, g–j, and q–t; and 100 μm in panels e, f, o, and p.
Hematopoietic Stem cell Response to AhR-mediated Dermatitis

Distorted Lineage Commitment Toward Granulocytes and Monocytes in AhR-CA Mice

To elucidate how the K14 promoter–driven AhR activation affects hematopoiesis, we first examined PB and BM cells in AhR-CA mice at 9–14 weeks after birth when the dermatitis had fully developed (Fig. 2A). The PB count of AhR-CA mice showed a marked increase in leukocytes with a reduction in erythrocytes, hemoglobin, and hematocrit compared with wild-type mice (Table 1). To clarify the hematopoietic alterations underlying the leukocytosis, we analyzed BM composition with flow cytometry. Whereas BM cellularity was comparable between AhR-CA and wild-type mice (Fig. 2B), Mac-1^+Gr-1^+ cells were increased and Ter119^- and CD3e^+ cells were decreased in AhR-CA mice compared with wild-type mice (Fig. 2C). Consistently, among the lineage-committed progenitor cells in AhR-CA mice, granulocyte-monocyte progenitors (GMPs) were dramatically increased, whereas common myeloid progenitors (CMPs) and megakaryocyte-erythroid progenitors (MEPs) were reduced (Figs. 2D and 2E). These results indicate that hematopoietic differentiation is distorted to the granulocyte and monocyte lineages in the AhR-CA mice.

### Table 1

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<th>Wild-type</th>
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<tr>
<td><strong>WBC (10^2/µl)</strong></td>
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<td>215 ± 152</td>
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<td><strong>RBC (10^2/µl)</strong></td>
<td>948 ± 48</td>
<td>862 ± 88</td>
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<td><strong>HGB (g/dl)</strong></td>
<td>15.1 ± 0.6</td>
<td>13.9 ± 1.4</td>
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<td><strong>HCT (%)</strong></td>
<td>44.6 ± 2.7</td>
<td>41.5 ± 4.4</td>
<td>&lt; .03</td>
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<tr>
<td><strong>MCV (fl)</strong></td>
<td>47.1 ± 1.8</td>
<td>48.2 ± 2.4</td>
<td>ns</td>
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<tr>
<td><strong>MCH (pg)</strong></td>
<td>15.9 ± 0.5</td>
<td>16.1 ± 0.8</td>
<td>ns</td>
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<tr>
<td><strong>PLT (10^4/µl)</strong></td>
<td>89 ± 19</td>
<td>78 ± 22</td>
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Values represent the means ± SD of wild-type (n = 15) and AhR-CA (n = 14) mice.

Abbreviations: HCT, hematocrit; HGB, hemoglobin; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; ns, not significant; PLT, platelet; RBC, red blood cell; WBC, white blood cell.

**HSC Expansion and Proliferation in AhR-CA Mice**

Inflammatory signals have been shown to activate HSCs and change their behavior, particularly their maintenance and differentiation (Chen et al., 2010; Esplin et al., 2011; Odouro et al., 2012). Because AhR-CA mice display severe dermatitis...
accompanied by increased gene expression of cytokines and chemokines in skin (Tauchi et al., 2005), we surmise that HSCs are responding to the AhR-mediated inflammation.

To delineate this point, we next examined HSC fraction in the AhR-CA mouse BM and found that LSK and LK cells were increased in AhR-CA mice (Fig. 3A). Among the LSK subfractions, the LSK Flt3−CD34− cells, which we referred to as an HSC fraction in this study, exhibited a prominent expansion (Figs. 3B and 3C). The LSK Flt3+CD34+ cells, comprising more mature cells than the LSK Flt3−CD34− cells, were also increased. These results suggest the notion that HSC maintenance and differentiation are modified in AhR-CA mice.

To clarify the underlying mechanisms for the expansion of HSC fraction, we examined BrdU incorporation for quantifying DNA-synthesizing cells and performed Ki67-Hoechst 33342 staining to analyze cell cycle status. We examined LSK

![Fig. 3](https://academic.oup.com/toxsci/article-abstract/138/1/47/1671702)
CD34+ cells as a representative HSC fraction, as this simplifies the multi-fluorescence flow cytometric analysis. Notably, the LSK CD34+ fraction contained more BrdU-positive cells in AhR-CA mice than in wild-type mice (Figs. 3D and 3E). Ki67-Hoechst 33342 staining revealed that the LSK CD34+ fraction in AhR-CA mice contained a slightly but significantly lower ratio of quiescent (G0) cells than in wild-type mice (Fig. 3F). The percentage of G1 cells in LSK CD34+ fraction was conversely increased in AhR-CA mice. These results raise the possibility that the maintenance of HSC quiescence is attenuated in AhR-CA mice.

To further address this point, we analyzed the expression of 8 cell cycle–related genes in LSK cells. p57, p18, and Cyclin G2 were significantly decreased in AhR-CA mouse LSK cells (Fig. 3G). This supports our notion that quiescent HSCs are reduced in AhR-CA mice, because downregulation of either p57 or p18 gene promotes the cell cycle entry of HSCs (Matsumoto et al., 2011; Yuan et al., 2004; Zou et al., 2011) and because Cyclin G2 blocks the cell cycle progression (Bennin et al., 2002). Among 3 proliferation-associated genes, Cyclin D1, Cyclin E1, and Ki67, the latter two were significantly upregulated in the LSK cells of AhR-CA mice. Combining the results of cell cycle analysis and the gene expression profile, we suggest that HSCs in AhR-CA mice are activated and driven into proliferation, producing many HPCs, which may be advantageous for the replenishment of inflammatory cells.

Increased ST-HSC/MPP Proliferation in AhR-CA Mice

To closely assess how HSCs are influenced under the AhR-mediated inflammatory milieu, we investigated the activity and property of long-term HSCs (LT-HSCs). The LSK Flt3+CD34− subfractions were further fractionated using the SLAM family CD48 and CD150 surface markers (Kiel et al., 2005). The LT-HSCs defined as LSK Flt3+CD34−CD48+CD150+ cells seemed to decrease in AhR-CA mice compared with wild-type mice, whereas LSK Flt3+CD34−CD48−CD150− cells, mostly corresponding to ST-HSCs and MPPs, were dramatically expanded (Figs. 4A and 4B). Based on the elaborate fractionation results, we conclude that the HSC expansion in AhR-CA mice is attributed to the increased ST-HSCs and MPPs.

We next investigated BrdU uptake in LSK CD150+ fraction as a representative LT-HSC fraction. In AhR-CA mice, BrdU uptake was subtly promoted in the LSK CD150+ fraction and remarkably enhanced in LSK CD150− fraction that includes ST-HSCs and MPPs (Figs. 4C and 4D). These results imply that LT-HSCs are activated under the influence from the AhR-induced dermatitis and produce the ST-HSCs and MPPs, which become highly proliferative, leading to the accumulation of HSC fraction in AhR-CA mice.

Abnormal HSPC Accumulation in AhR-CA Mouse Spleen

Inflammatory signals accumulate and/or expand HSPCs in spleen and make them differentiate into inflammatory cells, which then exacerbate the inflammation (Griseri et al., 2012). To examine whether inflammatory signals originated by the AhR activation in extra-BM cells induce an accumulation of HSPCs in spleen, we analyzed the splenocyte composition of AhR-CA mice. Upon calculation of the HSPC frequency in the spleen, lineage-positive cells were not removed in this analysis.

AhR-CA mice developed severe splenomegaly (Fig. 5A), and HSPCs defined as Sca-1−c-Kit+ and Sca-1−c-Kit− fractions massively accumulated in AhR-CA mice versus wild-type mice (Figs. 5B and 5C). Among the Sca-1−c-Kit+ cells, the Sca-1−c-Kit+FcyRIIcCD34+ cells that correspond to GMPs

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**FIG. 4.** Expansion and enhanced proliferation of short-term hematopoietic stem cells and multipotent progenitors in AhR-CA mice. A and B, Analysis of Lin−Sca-1−c-Kit+(LSK) Flt3+CD34− subfractions using CD48 and CD150 surface markers. Representative contour plots (A) and cell numbers of subpopulations according to CD150 and CD48 surface marker status in LSK Flt3+CD34− cells (B) are shown. Data represent the means ± SD (n = 4–6). C and D, Bromodeoxyuridine (BrdU) uptake of LSK CD150− and CD150+ cells. Representative histograms (C) and frequencies of BrdU-positive cells (D) are shown for LSK CD150− and CD150+ cells. Data represent the means ± SD (n = 5–6). All the experiments were performed at least 3 times. *p < .05 and **p < .005.
were significantly expanded compared with the other subfractions (Figs. 5B and 5D). It should be noted that the skewing toward granulocyte-monocyte lineage was also evident because of increased Mac-1+Gr-1+ cells and decreased CD3e+ cells (Fig. 5E). These results suggest that the AhR-mediated inflammatory signals provoke the accumulation of HSPCs in spleen as well as in BM, which causes the excessive production of inflammatory cells. The massive production of inflammatory cells may be one of the aggravating factors of the local inflammation in AhR-CA mice.

Skewed Differentiation and LT-HSC Reduction at Early Stage of Dermatitis

To clarify the relation between the development of dermatitis and hematopoietic alterations, we analyzed AhR-CA mice at 4 weeks of age when dermatitis did not reach the full-blown stage. At 4 weeks after birth, inflammatory cells had already started to infiltrate into the skin in AhR-CA mice, judging from the increased cell density in the dermis, but the epithelial layer still appeared normal; there were no hyperkeratinization or apparent acanthosis (Fig. 6A).

At this early stage of dermatitis, there were no significant changes in the PB count and the whole BM cellularity between AhR-CA and wild-type mice (Table 2 and Fig. 6B). In contrast, the lineage skewing of BM cells was already apparent in 4-week-old AhR-CA mice: decrease and increase in Ter119+ cells and in Mac-1+Gr-1+ cells, respectively (Fig. 6C). In HSPC fractions, the cell number of each subfraction was almost comparable between AhR-CA mice and wild-type mice (Figs. 6D and 6E). However, fractionation of the LSK Flt3−CD34− population with CD48 and CD150 surface markers revealed that LT-HSCs, LSK Flt3−CD34−CD48−CD150− fraction, were significantly reduced in AhR-CA mice at 4 weeks of age compared with wild-type mice (Figs. 6D and 6E). This reduction was remarkable compared with that observed in AhR-CA mice with full-blown dermatitis (Fig. 4B). In contrast, the increase of LSK Flt3−CD34−CD48+CD150− cells clearly observed in AhR-CA mice with full-blown dermatitis was not apparent in
the young AhR-CA mice. As for the spleen, splenomegaly and HSPC accumulation were not observed (Figs. 6H–J).

Therefore, the lineage skewing toward granulocytes and monocytes and the reduction in LT-HSCs are early events during the course of dermatitis development in AhR-CA mice, suggesting that AhR activation in K14-promoter-active cells might induce factors directly causing these phenotypes. In contrast, the changes in the PB count and the expansion of HSPC subfractions in BM and spleen emerged in the late stage of the dermatitis development, which implies that secondary signals might be involved in these phenotypes. These results show that AhR activation in K14-promoter-active cells, majorly...
AhR-activating ligand, results in the suppression of reconstitution activity of LT-HSCs (Sakai et al., 2003), suggesting that AhR activation in HSCs impairs the HSC function. This notion is supported by the BM analysis of AhR-deficient mice (Singh et al., 2011). AhR-deficient mice exhibit an expansion of HSC fractions with higher proliferative ability, and the competitive repopulation study shows that functional LT-HSCs are richly contained in the expanded HSC fractions (Singh et al., 2011), which indicates that inhibition of AhR function strengthens the HSC function. Furthermore, AhR antagonists are identified as means to induce an ex vivo expansion of human HSCs (Boitano et al., 2010). Thus, the aforementioned effect of systematically administered TCDD is likely attributed to the cell-autonomous function of AhR in HSCs. In contrast, our analysis focuses on non-cell-autonomous effects of AhR activation on hematopoiesis. We have presented evidence that AhR activation in K14-promoter-active cells modifies hematopoiesis, which is very different from the consequence of direct AhR activation in hematopoietic cells.

As to other AhR-activating ligands, such as DMBA and BaP, more complex effects are generated on hematopoiesis. For example, DMBA is shown to disrupt lymphoid and myeloid progenitor activity (N’jai et al., 2010). Chronic dermal application of BaP causes anemia without apparent leukocytosis in NZB/WF1 mice that are genetically prone to autoimmune diseases (Booker and White, 2005). These effects on hematopoietic cells might be the mixture of AhR-dependent and -independent effects. In this study, we focused on the AhR-dependent pathway by utilizing AhR-CA molecule instead of exogenous AhR ligands. Thus, the hematopoietic phenotypes observed in this study can be interpreted as the AhR-dependent components of the PAH-induced pathology.

When we executed transcriptome analyses of the AhR-CA mouse skin that was before the emergence of apparent inflammation, we found that genes encoding various cytokines and chemokines are abundantly expressed in the skin (Tauchi et al., 2005). We envisage that the keratinocyte-derived signals lead to the infiltration of inflammatory cells to the skin as an early event during the initiation of inflammation. Thus, cytokines derived from the migratory inflammatory cells may be responsible for the secondary signals to activate HSPCs in AhR-CA mice. In addition, because the keratinocytes are reported to secrete cytokines that can activate HSPCs (Uchi et al., 2000), we assume that certain humoral factors secreted from keratinocytes through the AhR-dependent transcriptional program may also provoke the HSPC response. Supporting this possibility, the lineage skewing and LT-HSC response are already detectable in AhR-CA mice at the initial stage of the dermatitis when the secondary signals from inflammatory cells are not expected to be active enough. Because AhR-CA seems to be expressed in several other epithelial cells in addition to those of skin and thymus, according to the previous study (Wang et al., 1997), further investigations are needed to elucidate what signaling is important for the HSPC response in AhR-CA mice.

**TABLE 2**

**Peripheral Blood Counts in 4-Week-Old Mice**

<table>
<thead>
<tr>
<th>Wild-type</th>
<th>AhR-CA</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (10^9/µl)</td>
<td>62±28</td>
<td>77±47</td>
</tr>
<tr>
<td>RBC (10^12/µl)</td>
<td>848±51</td>
<td>892±85</td>
</tr>
<tr>
<td>HGB (g/dl)</td>
<td>12.7±0.8</td>
<td>12.4±0.7</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>50.4±2.1</td>
<td>48.3±3.8</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>15.0±0.9</td>
<td>14.0±1.1</td>
</tr>
<tr>
<td>PLT (10^9/µl)</td>
<td>80±8</td>
<td>77±8</td>
</tr>
</tbody>
</table>

Values represent the means ± SD of wild-type (n = 5) and AhR-CA (n = 6) mice.

Abbreviations: HCT, hematocrit; HGB, hemoglobin; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; ns, not significant; PLT, platelet; RBC, red blood cell; WBC, white blood cell.

in keratinocytes and thymic epithelial cells, has a substantial influence on the hematopoietic cells before the full development of inflammatory milieu.

**DISCUSSION**

This study demonstrates that constitutive AhR activation under the control of K14 promoter has substantial impacts on HSPCs. We found that AhR-CA mice expressed the transgene-derived AhR-CA in keratinocytes and thymic cortical epithelial cells but not in BM cells, either stromal cells or hematopoietic cells. Thus, we used the mice as a unique model for analyzing hematopoietic response to PAH exposure in nonhematopoietic cells. A novel feature of this study is examining the hematopoietic response to the AhR activation without any exogenous chemicals, thereby excluding nonspecific AhR-independent effects that are often triggered by the PAH exposure. Our analysis has classified the hematopoietic phenotypes of the AhR-CA mice into 2 categories, early events and late events in terms of each onset. The skewing of differentiation and the decrease in the LT-HSC fraction size are early events, being already initiated before the full development of dermatitis. In contrast, the leukocytosis and the HSPC expansion are late events, emerging in the late stage of the dermatitis development. We surmise that the former are caused by the direct influence of the AhR activation in K14-promoter-active cells, whereas the latter are involved in the secondary responses to dermatitis. These hematopoietic alterations, resulting in the increased production of inflammatory cells, seemingly contribute to the exacerbation and amplification of the inflammatory milieu in AhR-CA mice. This study has clarified the comprehensive view of the pathological conditions provoked by AhR activation in K14-promoter-active cells, particularly in keratinocytes, which often serve as the first-line defense against PAHs, and thymic cortical epithelial cells.

Diverse contributions of AhR and its ligands to hematopoiesis have been reported. Administration of TCDD, a well-known
In allergic inflammation, aberrant accumulation of eosinophils, basophils, and mast cells into local inflammatory sites is responsible for the chronicity (Holgate and Polosa, 2008; Paul and Zhu, 2010). These cells are rapidly provided from HPCs both in the BM (Li et al., 2005; Penncok and Grecnis, 2004) and periphery (Blanchet and McNagny, 2009; Saenz et al., 2010) when the HPCs are stimulated by inflammatory signals. Importantly, the HPCs producing granulocytes and monocytes, defined as Sca-1−c-Kit−FcyγR−CD34+ cells (Iwasaki and Akashi, 2007), are expanded in BM and spleen of AhR-CA mice. These cells may act to exacerbate the inflammatory response through excessive supply of eosinophils, basophils, and mast cells. Indeed, numerous mast cells are infiltrated into the dermal layers of AhR-CA mice (Tauchi et al., 2005). Thus, we conclude that the accumulation and expansion of HPCs that prefer to differentiate into granulocyte-monocyte lineages contribute to the pathology of allergic inflammation in AhR-CA mice.

Prolonged exposure to inflammatory signals has been reported to alter the LT-HSC characteristics. Upon exposure to continuous inflammatory signals, LT-HSCs acquire a preference for the myeloid differentiation, which is related to the exacerbation of inflammation through the excessive supply of inflammatory cells (Esplin et al., 2011; Griseri et al., 2012; Oduro et al., 2012). In addition, LT-HSCs under the chronic inflammatory conditions deteriorate the maintenance of stemness (Chen et al., 2010; Esplin et al., 2011). We found in this study the slight reduction in LT-HSCs under the AhR-mediated dermatitis, but the property and function of the LT-HSCs still remain to be elucidated. Characterization of LT-HSCs in AhR-CA mice will provide an important clue to the better understanding of the PAH exposure effects on hematopoiesis. A solid conclusion as to the influence of AhR-mediated inflammation on LT-HSCs awaits further analyses such as BM transplantation.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

**FUNDING**

Japan Society for the Promotion of Science KAKENHI (24249015 to M.Y., 24390075 to H.M.); Ministry of Education, Culture, Sports, Science and Technology-Japan KAKENHI (23116002 to H.M.); Naito Foundation (M.Y.); Takeda Scientific Foundation (H.M. and M.Y.); and the Core Research for Evolutionary Science and Technology from the JST (H.M. and M.Y.).

**ACKNOWLEDGMENTS**

We thank Ms. Eriko Naganuma and the Biomedical Research Core at the Tohoku University for technical support.

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