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Phyhd1, an *XPhyH-like* homologue, is induced in mouse T cells upon T cell stimulation

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ABSTRACT

We previously identified *XPhyH-like* as a gene whose expression is enhanced in *Xenopus* blood cells during the refractory period, in which *Xenopus* tadpoles transiently lose their tail regenerative ability. Although we hypothesized that some autoreactive immune cells attack tail blastemal cells during the refractory period and *XPhyH-like* expressing immune cells were involved in the process, the nature of cells expressing *XPhyH-like* remain unknown, partly due to the lack of leukocyte markers available in *Xenopus*. In the present study, we used mice to analyze the expression pattern of *XPhyH-like* homologues. When we used quantitative reverse transcription-polymerase chain reaction (RT–PCR) to analyze the expression of mouse *Phyhd1*, an *XPhyH-like* orthologue, and *Phyh*, a *Phyhd1* paralogue, both *Phyhd1* and *Phyh* showed similar tissue-specific expression patterns. The expression pattern in leukocytes, however, differed between *Phyhd1* and *Phyh*; *Phyhd1* was considerably expressed in T cells and B cells. Moreover, the expression of *Phyhd1* in T cells was up-regulated for approximately 3- to 7-times by T cell stimulation 3–4 days after the stimulation, unlike *Phyh*. Our findings suggest that *Phyhd1* and *Phyh* have distinct roles in mouse leukocytes and *Phyhd1* is related to T cell differentiation and/or function of effector T cells.
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1. Introduction

Many animals can regenerate their lost appendages, however the extent of the regenerative ability varies according to the animal species, organs/appendages and developmental stages [1]. Although the molecular mechanisms that define the regenerative ability have been investigated for a long time, the most part of them remains unclear.

The *Xenopus laevis* tadpoles have high ability to regenerate their tails. They can regenerate complete tails within about 1 week after amputation. However the regenerative ability is lost temporarily at certain developmental stages called the 'refractory period' (stage 45–47) [2]. We previously showed that the tail regenerative ability during the refractory period was restored under suppression of the immune responses by immunosuppressant treatments [3]. Moreover knockdown of PU.1, a transcription factor which is essential for the development of some types of immune cells, such as B cells, macrophages and neutrophils, in mice [4], restores the regenerative ability [3]. Therefore, immune response is a critical factor that impairs tail regenerative ability during the refractory period.

In our previous study, aiming at identifying candidate genes related to the impaired tail regenerative ability, we screened for genes whose expression in tail stumps are altered by immunosuppressant (FK-506) treatments during the refractory period, and identified *Xenopus phytanoyl-CoA dioxygenase* (*PhyH*)*-like* (*XPhyH-like*), a paralogue of *Xenopus PhyH* [5]. The expression of *XPhyH-like* in tail stumps increases transiently after amputation during the refractory period and is reduced by FK-506 treatment. Furthermore, expression of *XPhyH-like* in the whole tadpole body is up-regulated during the refractory period and is enriched in blood cell fraction [5]. We hypothesized that some autoreactive immune cells attack tail blastemal cells during the refractory period and *XPhyH-like* expressing immune cells are involved in the process. However the function of *XPhyH-like* and the nature of cells expressing *XPhyH-like* remain unknown. Although it is important for elucidation of the immune responses that impair regeneration to investigate the expression of *XPhyH-like* in leukocytes, the analysis of *Xenopus* leukocytes has many difficulties, because there are few established leukocyte molecular markers and immunological methods to analyze them.

Although mice have limited ability to regenerate their organs and appendages, immune response affects wound healing, the

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phenomenon similar to regeneration. In adult mice, severe injury elicits inflammation, which results in scar formation. However fetuses with only immature immune system as well as several immunodeficient/immune disorder mice strains such as nude mice and *PU.1* null mice show scarless skin repair [6–9]. Nude mice, which lack thymus thus lack almost all T cells, restore the structure and integrity of injured skin without scar formation [9]. *PU.1* null mice, which lack several immune cells because of lacking of *PU.1*, also show scarless wound healing [8]. Moreover MRL-*lpr* mice, which have the lymphoproliferation spontaneous mutation (Fas^{lpr}) and exhibit autoimmune disorders, can heal skin injury rapidly and regenerate injured tissues partially [10]. With these facts, common mechanisms might underlie both of tail regeneration in tadpoles and wound healing in mice. Therefore we planned to analyze the function and the nature of immune cells expressing *XPhyH-like* homologue(s) using mice, in which various methods to analyze leukocytes have been established, instead of *X. laevis*.

Mouse *phytanoyl-CoA dioxygenase domain containing 1* (*Phyhd1*) is a putative orthologue of *XPhyH-like*, its function however is unclear. In the present study, we analyzed the expression of *Phyhd1* in mouse tissues, leukocytes, T cell subpopulations and activated T cells using quantitative reverse transcription-polymerase chain reaction (RT-PCR). We also analyzed the expression of *phytanoyl-CoA hydroxylase* (*Phyh*), a paralogue of *Phyhd1*. The results suggested a possible role of *Phyhd1* but not *Phyh* in differentiation and/or function of effector T cells.

2. Materials and methods

2.1. Mice

For tissue-specific expression analysis, 14–19 weeks old C57BL/6j mice kept under conventional conditions in The University of Tokyo were used. For expression analysis in leukocytes, T cell subpopulations and activated T cells, 8 weeks old male C57BL/6j mice were used. They were purchased from CLEA Japan, Inc. (Tokyo, Japan) and kept under specific pathogen free conditions. All of the manipulations were performed after cervical dislocation or euthanasia by collecting blood from the heart following anesthetizing by Isoflurane. This study was carried out in accordance with the recommendations of the Guidelines for Proper Conduct of Animal Experiments of Science Council of Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of Graduate School of Science, The University of Tokyo (Permission Number: Z 15-5).

For tissue-specific expression analysis, mice were anesthetized with Isoflurane. The whole blood was collected and then erythrocytes were depleted by treating cells with ACK buffer (150 mM ammonium chloride, 10 mM potassium bicarbonate, and 0.1 mM EDTA disodium salts). The other tissues sampled were kept on dry ice until RNA isolation described later.

2.2. Cell isolation

For expression analysis using leukocytes, splenocytes and peripheral blood leukocytes were prepared by erythrocytes depletion with ACK buffer. The antibodies used were purchased from eBioscience (San Diego, CA) and BioLegend (San Diego, CA). We isolated $CD3\varepsilon^+B220^-$ (T cells), $CD3\varepsilon^-B220^+$ (B cells), $CD3\varepsilon^-B220^-CD11b^+Gr\text{-}1^-$ [monocytes or macrophages (MΦ)], and $CD3\varepsilon^-B220^-CD11b^-Gr\text{-}1^-$ and $CD3\varepsilon^-B220^-CD11b^+Gr\text{-}1^+$ (the other leukocytes) cells, respectively, by using a FACSAria cell sorter (BD Biosciences, San Jose, CA). Doublet or multiplet events were gated out by using forward scatter height and width, and side scatter height and width parameters.

For expression analysis of T cell subpopulations, we isolated $CD3\varepsilon^+CD4^+CD8\alpha^-CD62L^+CD44^-$, $CD3\varepsilon^+CD4^+CD8\alpha^-CD44^+$, $CD3\varepsilon^+CD4^-CD8\alpha^+CD62L^+CD44^-$, $CD3\varepsilon^+CD4^-CD8\alpha^+CD44^+$ cells, respectively, by using a FACSAria cell sorter (BD Biosciences) as described above.

For T cell stimulation experiments, splenocytes were prepared as described above, then $CD4^+$ and $CD8\alpha^+$ cells were enriched from splenocytes by using phycoerythrin-conjugated anti-CD4 antibody or biotin-conjugated anti-CD8α antibody and relevant microbeads-conjugated antibody (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively, and LS column (Miltenyi Biotec). The $CD4^+CD8\alpha^-CD62L^+CD44^-$ and $CD4^-CD8\alpha^+CD62L^+CD44^-$ populations were isolated from enriched $CD4^+$ and $CD8\alpha^+$ cells, respectively, by using a FACSAria cell sorter (BD Biosciences) as described above. Isolated cells were subjected to T cell stimulation described later.

2.3. Isolation of total RNA and complementary DNA (cDNA) synthesis

Total RNA was isolated from tissues using TRIzol® Reagent (Life Technologies, Carlsbad, CA), and from leukocytes using RNeasy Mini Kit (QIAGEN, Hilden, Germany). Total RNA was then reverse transcribed to synthesis cDNA using PrimeScript® RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Otsu, Japan).

2.4. Quantitative RT–PCR

Quantitative RT-PCR was performed using SYBR® *Premix Ex Taq*™ II (Tli RNaseH Plus) (TaKaRa) and LightCycler480® Instrument II (Roche, Basel, Switzerland). The gene specific primers used were: for *hypoxanthine phosphoribosyltransferase* (*HPRT*), forward primer 5′-AGTCCCAGCGTCGTGATTAG-3′ and reverse primer 5′-TCTCGAGCAAGTCTTTCAGTCC-3’; for *β2-microglobulin* (*β2m*), forward primer 5′-CACTGAATTCACCCCCACTGA-3′ and reverse primer 5′-TGTCTCGATCCCAGTAGACGG-3′; for *Phyhd1*, forward primer 5′-TGCCTGAGTCCCTCACAAC-3′ and reverse primer 5′-ACAATCTCACCGATCCTCTGC-3’; for *Phyh*, forward primer 5′-GCCATGGAGCACATTGACAG-3′ and reverse primer 5′-TTGTTAACACCTCCCTCCCAC-3’. These primers were designed to detect all isoforms from each gene predicted in National Center of Biotechnology Information (NCBI) database. The expression levels of *Phyhd1* and *Phyh* were normalized to those of *HPRT* in tissues, and to those of *β2m* in leukocytes.

2.5. T cell culture and stimulation

The wells of 96 well culture plate were coated by 50 μl of 10 μg/ml anti-CD3ε antibody (clone 145.2C11, BioLegend) for overnight at 4 °C. Isolated cells were suspended in RPMI 1640 containing 10% FCS, 2 mM L-Glutamine, 50 μM 2-mercaptoethanol, 10 mM HEPES, 1 mM Sodium Pyruvate, 1x MEM-NEAA (Non Essential Amino Acid; Wako, Osaka, Japan), 100 U/ml Penicillin, 100 μg/ml Streptomycin, at a density of 1×10^6 cells/ml. Two hundreds microliters of cell suspension was placed in a coated well (2×10^5 cells/well) and was added with 0.4 μl of 1 mg/ml anti-CD28 antibody (clone 37.51, BioLegend). The cells were incubated at 37 °C in an atmosphere of 5% CO_2, and then total RNA was extracted as described above.

3. Results

3.1. Expression analysis of Phyhd1 in tissues

In our previous study, we suggested that immune cells expressing *XPhyH-like* might be related to the impaired

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