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Suppression of the immune response potentiates tadpole tail regeneration during the refractory period

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Regenerative ability varies depending on animal species and developmental stage, but the factors that determine this variability remain unclear. Although *Xenopus laevis* tadpole tails possess high regenerative ability, this is transiently lost during the 'refractory period'. Here, we show that tail amputation evokes different immune responses in wound tail stumps between the 'refractory' and 'regeneration' periods: there was delayed or prolonged expression of some immune-related genes in the refractory period, whereas there was no obvious or transient expression of other immune-related genes in the regeneration periods. In addition, immune suppression induced by either immunosuppressant treatment or immune cell depletion by knockdown of *PU.1* significantly restored regenerative ability during the refractory period. These findings indicate that immune responses have a crucial role in determining regenerative ability in *Xenopus* tadpole tails.

KEY WORDS: Regeneration, Immune response, *Xenopus laevis*

INTRODUCTION

Many animal species possess the ability to regenerate their lost appendages, although this ability varies depending on the animal species, organs and appendages, and the developmental stage of the animals (Slack, 2003). Although some molecules are reported to stimulate regenerative abilities in various animals (Whitehead et al., 2005; Kawakami et al., 2006; Adams et al., 2007), the reason for the variable regenerative ability remains a mystery.

Xenopus laevis tadpoles have the ability to regenerate whole tail tissues after tail amputation. Recently, however, *Xenopus* tadpoles were reported to lose their regenerative ability transiently at particular developmental stages (stages 45-47), called the 'refractory period' (Beck et al., 2003). During the refractory period, tadpoles fail to generate a regeneration bud (blastema) and thus fail to regenerate their lost tails (Beck et al., 2003). The refractory period appears between the pre- and post-refractory 'regeneration periods'. We intended to utilize this unique characteristic to analyze the regeneration-specific processes as well as to analyze the molecular basis of the developmental stage-specific regenerative abilities.

In the present study, we compared gene expression profiles in the wound tail stumps between the refractory and regeneration periods using the differential display method, and found that tail amputation evoked different immune responses between these two periods. Furthermore, suppression of the immune response restored regenerative ability during the refractory period, indicating that immune responses play crucial roles in determining the regenerative ability of *Xenopus* tadpole tails.

MATERIALS AND METHODS

Animals

Xenopus laevis tadpoles were kept at 20°C in 0.2% salt water. Stage 39-41, 46-47 and 52-53 tadpoles were used for the pre-refractory regeneration period, refractory period and post-refractory regeneration period, respectively.

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Differential display

The differential display method was performed as described previously (Ishino et al., 2003), using total RNA extracted from wound stumps dissected at 0, 2 and 15 hours post amputation (hpa) during the refractory and post-refractory regeneration periods.

cDNA cloning of *X. laevis FOXP3*

To obtain partial cDNA, degenerate primers were designed for the conserved forkhead domain of *X. laevis FOXP1*, *2* and *4*, and polymerase chain reaction (PCR) was performed with cDNA from thymi of stage 52 tadpoles. The rapid amplification of cDNA ends method (FirstChoice RLM-RACE; Ambion) was performed to obtain the full-length cDNA (GenBank AB359948).

Quantitative reverse transcription (qRT)-PCR and in situ hybridization

For expression analysis using the wound stumps, we collected four sets of wound stumps for pre-refractory regeneration period (*n*=15-16), refractory period (*n*=20-24) and post-refractory regeneration period (*n*=5-6) at 0, 5, 10, 15, 24 and 48 hpa. For developmental expression analysis, we collected four sets of intact tadpoles (*n*=4-5) at 4- to 9-days post fertilization (dpf). qRT-PCR was performed with gene-specific primers. Accession numbers of *X. laevis* chemokine genes were obtained from the Cytokine Family Database (http://cytokine.medic.kumamoto-u.ac.jp/). In situ hybridization on wax sections was performed using standard procedures.

Inhibitor experiments

Tadpoles with tails amputated during the refractory period were exposed to 50 ng/ml Celastrol, 1 μM IKK inhibitor VII, 3 μM FK506, 10 μM cyclosporin A (chemicals were from Calbiochem), or 0.1% dimethyl sulfoxide (vehicle control), in 0.33× De Boer solution. The solutions were refreshed every other day and tail regeneration was checked 7 days post amputation (dpa). The regenerative abilities were classified into four groups: excellent, whole tail structure was regenerated including fin, muscle, notochord and spinal cord; good, whole tail was regenerated, but length was shorter; partial, tail was regenerated, but lacked some tissues or had a curved axis; none, no regeneration was observed.

Antisense morpholino oligonucleotide (MO) experiments

We injected 4.6 nl of 1 mM (PU.1-MO1 and 5mis PU.1-MO1) or 0.75 mM (PU.1-MO2 and 5mis PU.1-MO2) translation-blocking antisense MO (GeneTools) into both blastomeres of 2-cell-stage embryos, essentially as described previously (Iijima et al., 2008; Eisen and Smith, 2008). Injected embryos were maintained at 20°C in 0.1× Steinberg's solution. The tails were amputated at stage 46 and tail regeneration was checked 7 dpa. The

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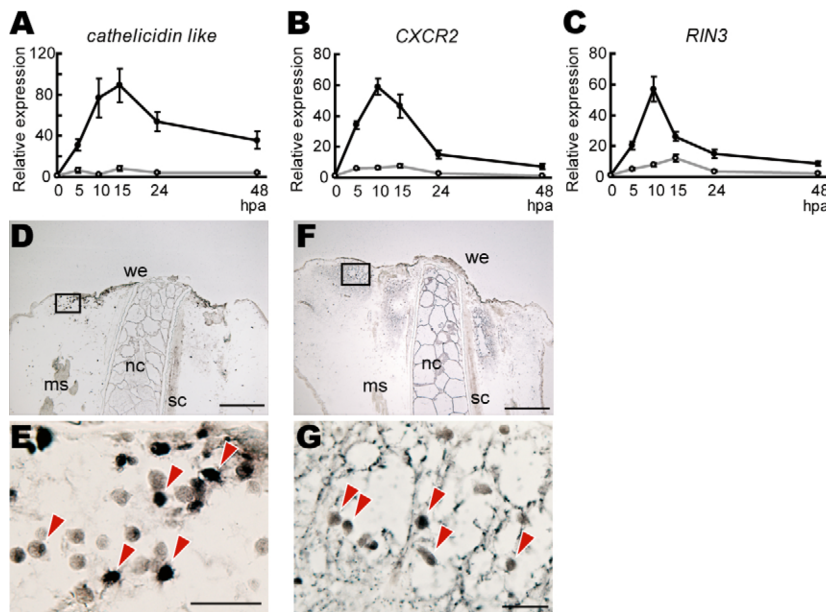

Fig. 1. Expression analysis of genes identified by differential display during the refractory and post-refractory periods. (**A-C**) *cathelicidin-like* (A), *CXCR2* (B) and *RIN3* (C) expression analysis. The relative amounts of transcripts determined by qRT-PCR were obtained by taking the value at 0 hpa as 1 for each refractory period (open circle) and post-refractory regeneration period (solid circle) after normalization relative to *elongation factor 1α* (*EF1α*) transcipt levels. (**D-G**) In situ hybridization for *cathelicidin-like* (D,E) and *CXCR2* (F,G) using the sagittal sections of wound stumps during the post-refractory regeneration period 10 hpa. (E,G) Magnified views of the boxed area in D and F, respectively. Leukocyte-like cells expressing the genes are indicated with arrowheads. ms, muscle; nc, notochord; sc, spinal cord; we, wound epidermis. Scale bars: 300 μm in D,F; 30 μm in E,G.

sequences of MOs used were: PU.1-MO1, 5′-TGTTGTGATATAA-ACACTCCTCGTC-3′; and PU.1-MO2, 5′-ATAGGGGTATGGAGTA-TTCATCACA-3′, which were designed corresponding to –24 to +1 and –49 to –25 of the *PU.1* translation initiation site, respectively; and 5mis PU.1-MO1, 5′-AGTTGAGATAAAAACAGTCCTGGTC-3′; 5mis PU.1-MO2, 5′-ATACGGGTTTGGACTATTGATCAGA-3′, with five mismatches in the PU.1-MO1 and PU.1-MO2, respectively. qRT-PCR was performed with total RNA extracted from 3-4 sets of 8-17 MO-injected or noninjected tadpoles at stage 46.

RESULTS AND DISCUSSION

Comparison of gene expression profiles in wound tail stumps between refractory and post-refractory regeneration periods

To analyze the regenerative processes, we focused on wound epidermis formation, which has important roles in appendage regeneration (Thornton, 1957; Brockes, 1997). In our experimental conditions, wound epidermis coverage completed approximately 8 to 12 hpa (see Fig. S1 in the supplementary material), and proliferating cells appeared approximately 18 to 24 hpa (see Fig. S2 in the supplementary material). Therefore, we intended to compare gene expression profiles in the wound stumps at two time points: one at which the wound epidermis just covered the stump (2 hpa) and the other at which wound epidermis coverage was complete, but proliferating cells had not yet appeared (15 hpa).

We used the differential display method to detect 36 candidate bands, the intensities of which at 15 hpa differed only during the post-refractory regeneration period, but not during the refractory period, when compared with those at 2 hpa. qRT-PCR confirmed the differential expression of 6 of the 36 candidate genes. cDNA cloning identified three of them as *Xenopus* homologs of *cathelicidin-like*, *CXCR2* and *RIN3*, respectively. Two other genes contained no significant open reading frames and had frequent one-base substitutions, implying their function as non-coding RNAs, and the last gene had an open reading frame, which showed no significant sequence similarity with any other known proteins (data not shown). We focused on the three coding genes for further analysis. Expression of all of these three genes in the wound stump drastically increased until 10 to 15 hpa and then gradually decreased during the post-refractory regeneration period, whereas there were no such obvious changes during the refractory period (Fig. 1A-C). Their expression levels were below the quantification threshold during the pre-refractory regeneration period (data not shown). Next, to identify cell types that express these genes, we performed in situ hybridization using sections of wound stumps at 10 hpa during the post-refractory regeneration period'. *cathelicidin-like* was expressed in leukocyte-like cells, which invade the wound stump (Fig. 1D,E), and *CXCR2* was expressed in both the wound epidermis and in leukocyte-like cells (Fig. 1F,G). In mammals, cathelicidins are antimicrobial peptides expressed in wound epidermis or in neutrophils (Bals and Wilson, 2003). CXCR2 is a receptor for CXC chemokines, which elicit chemotaxis of neutrophils and monocytes that express the receptors (Chuntharapai et al., 1994). Therefore, it is plausible that the induction of *cathelicidin-like* and *CXCR2* expression during the post-refractory regeneration period was due to invasion of the leukocyte-like cells expressing these genes in the wound stump. RIN3 is a RAB5-binding protein that has an important role in endocytosis (Kajiho et al., 2003), and is expressed in monocytes or natural killer cells (Su et al., 2002). *RIN3* expression in the wound stump was, however, not detected by in situ hybridization, possibly due to its low expression level (data not shown).

Tail amputation evokes different immune responses between refractory and pre-/post-refractory regeneration periods

As all of these three genes were related to immune responses, we next compared expression profiles of the other immune-related genes between the refractory and pre-/post-refractory regeneration periods. We analyzed 17 chemokine (CXCLs and CCLs), four chemokine receptor, interleukin-1β (Zou et al., 2000) and the major histocompatibility complex (MHC) genes (Liu et al., 2002; Bos and Waldman, 2006), the sequences of which were available from databases. Expression of seven chemokine genes and *MHC class II* differed between the refractory and post-refractory regeneration periods. Among them, *CXCLb*, *CXCLe* and *CCLf* showed immediate early upregulation (within 5 hours) and subsequent downregulation only during the post-refractory regeneration period, whereas there was no obvious change during

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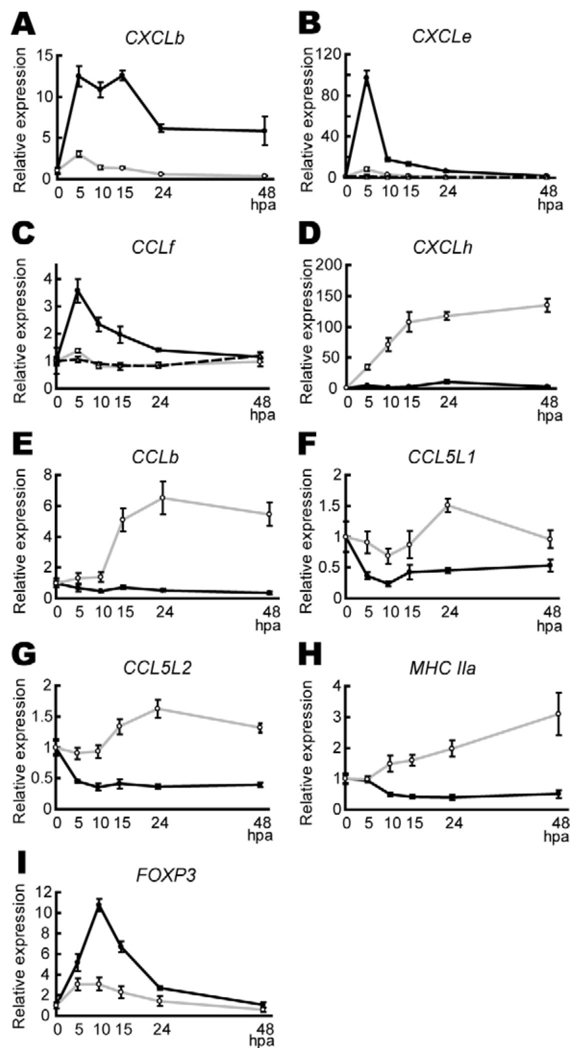

Fig. 2. Expression profiles of immune-related genes in wound stumps differ between the refractory and pre-/post-refractory regeneration periods. (**A-I**) *CXCLb* (A), *CXCLe* (B), *CCLf* (C), *CXCLh* (D), *CCLb* (E), *CCL5L1* (F), *CCL5L2* (G), *MHC IIa* (H) and *FOXP3* (I) expression analysis. The relative amounts of transcripts determined by qRT-PCR were obtained by taking the value at 0 hpa as 1 for each of the pre-refractory (broken line and open triangle), refractory (gray line and open circle) and post-refractory regeneration (black line and solid circle) periods. Transcript levels were first normalized relative to *EF-1α* transcript levels.

the refractory period (Fig. 2A-C). By contrast, *CXCLh* and *MHC class II* were slowly upregulated only during the refractory period, whereas there was no obvious change or the genes were downregulated during the post-refractory regeneration period (Fig. 2D,H). *CCLb*, *CCL5L1* and *CCL5L2* were upregulated at the later stages (15-24 hpa) during the refractory period and their expression was sustained until 48 hpa, but were immediately (until 5-10 hpa) downregulated during the post-refractory regeneration period (Fig. 2E-G). During the pre-refractory regeneration period, the expression of only two genes, *CXCLe* and *CCLf*, was detected, but no obvious change in expression was observed (Fig. 2B,C). Expression of the other genes did not differ significantly between the refractory and post-refractory regeneration periods or were too low to be detected (data not shown). These findings suggest that the immune responses in the wound stumps differ between the refractory and pre-/post-refractory regeneration periods: prolonged or delayed responses occur during the refractory period, whereas no obvious induction or transient induction occurs during the pre-/post-refractory regeneration periods, respectively.

Treatment with immunosuppressants or injection of antisense MOs for *PU.1* restored regenerative ability during the refractory period

As the immune responses in the refractory period seemed chronic, we hypothesized that these chronic immune responses were responsible for the impaired regenerative ability during this period. To test this, we first examined the effect of four immunosuppressants on regenerative ability during the refractory period. We first used Celastrol, a cell-permeable inhibitor-κB kinase (IKK) inhibitor (Pinna et al., 2004; Lee et al., 2006) because IKK stimulates nuclear factor-κB signaling, which has a pivotal role in immune and inflammatory responses. In untreated groups, almost all of the tadpoles failed to regenerate their lost tails (Fig. 3A,C). By contrast, the Celastrol-treated group significantly recovered their regenerative ability (Fig. 3B,D). A complete tail tissue structure was regenerated in a typical Celastrol-treated tadpole tail (Fig. 3E). We repeated this experiment seven times and obtained six reproducible results (Fig. 3F). Treatment with IKK inhibitor VII (Waelchli et al., 2006) also significantly restored the regenerative ability (Fig. 3F). In addition, treatment with two other immunosuppressants, FK506 and cyclosporin A, which bind to FK-binding protein and cyclophilin, respectively, and inhibit the nuclear factor of activated T cell pathway-dependent cytokine expression (Bierer et al., 1993), also significantly restored the regenerative ability (Fig. 3F). In two experiments (experiment 4 for Celastrol-treated and experiment 10 for cyclosporin A-treated tadpoles; Fig. 3F), the inhibitors failed to restore the regenerative ability, possibly due to physiological conditions or a polymorphism of IKK, cyclophilin, or other target molecules, affecting the efficiency of the inhibitors. FK506 treatment repressed induction of the immune-related genes during the refractory period in amputated tails (see Fig. S3 in the supplementary material), suggesting that the inhibitor actually affected immune responses in *Xenopus* tadpole tails.

We next investigated whether the depletion of immune cells during the refractory period restores the regenerative ability by injecting two kinds of antisense MOs (PU.1-MO1 and MO2) designed for transcription factor PU.1. *PU.1*-null mice show abnormal neutrophil development, delayed T cell development and have no detectable monocytes/macrophages and B cells (McKercher et al., 1996). *Xenopus* embryos injected with MOs were maintained until the refractory period and their tails were amputated. PU.1-MO1- or PU.1-MO2-injected groups showed significantly higher regenerative abilities than control 5mis PU.1-MO1- or 5mis PU.1-MO2-injected, or noninjected groups (Fig. 3G-K). Furthermore, both PU.1-MO1 and PU.1-MO2 injections significantly reduced the expression of *CD45* (Fig. 3L,M), a pan-leukocyte marker (Turpen et al., 1997), at stage 46, indicating that they effectively depleted leukocytes. These results strongly suggest that immune cells that require *PU.1* for their development impair regenerative ability during the refractory period. The recovery of regenerative ability induced by the injection of PU.1-MO was lower than that induced by immunosuppressant treatment, in part because MO injection itself had some negative effects on regenerative ability (Fig. 3J,K; see Fig. S4 in the supplementary material).

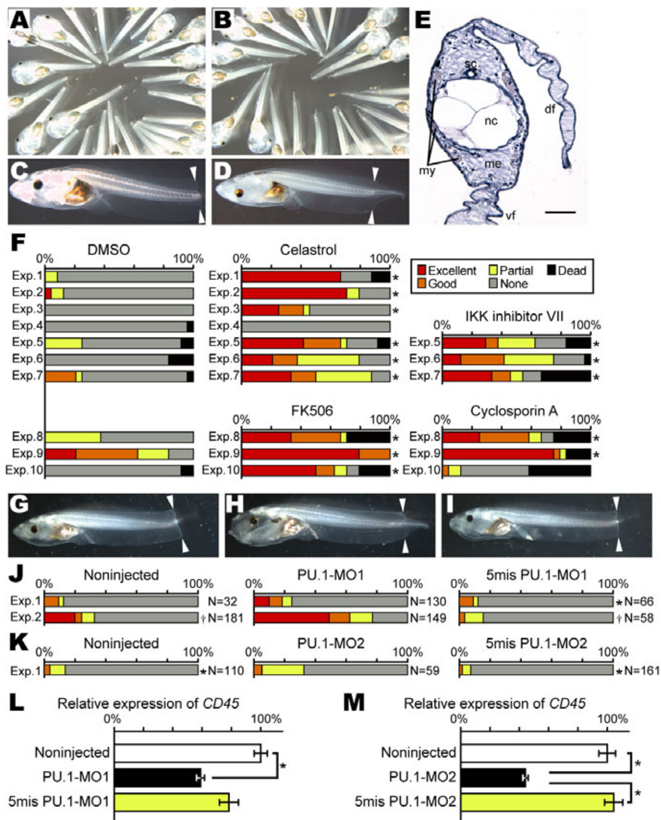

Fig. 3. Immunosuppressant treatment or injection of antisense MOs for *PU.1* restores the regenerative ability during the refractory period. (**A-D**) Vehicle control (A,C) and Celastrol-treated tadpoles (B,D) during the refractory period, observed 7 dpa. Arrowheads indicate the amputation plane. (**E**) Coronal section of a regenerated tail of a Celastrol-treated tadpole. df, dorsal fin; me, mesenchyme; my, myotome; nc, notochord; sc, spinal cord; vf, ventral fin. Scale bar: 50 μm. (**F**) Frequency of regenerative ability (see key) of control and immunosuppressant-treated tadpoles (n=24 in each experiment). The numbers of tadpoles classified in each experiment are presented in Table S1 in the supplementary material. *P<0.003 using χ^2 test (the number of dead tadpoles was excluded). (**G**) A noninjected tadpole with no apparent regeneration. (**H**) A PU.1-MO1-injected tadpole with a completely regenerated tail. (**I**) A 5mis PU.1-MO1-injected tadpole with no apparent regeneration. (**J**,**K**) Frequency of regenerative ability of noninjected, PU.1-MO1-injected and 5mis PU.1-MO1-injected (J), and PU.1-MO2-injected and 5mis PU.1-MO2-injected (K) tadpoles. The number of tadpoles classified in each experiment is presented in Table S2 in the supplementary material. *P<0.05, †$P<1\times10^{-12}$ using χ^2 tests against experimental (PU.1-MO1- or PU.1-MO2-injected) tadpoles. (**L**,**M**) The relative amounts of *CD45* transcripts determined by qRT-PCR were obtained by taking the value of the noninjected group as 100%. Transcript levels were first normalized relative to *EF-1α* transcript levels. *P<0.01, Student's t-test.

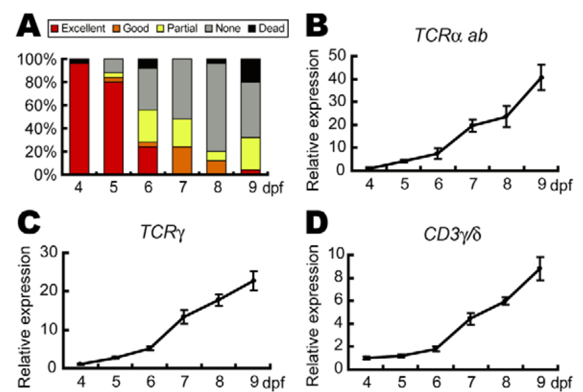

Fig. 4. The decrease in regenerative ability correlates with the T cell population development. (**A**) Frequency of regenerative ability (see key) of 4 to 9 dpf tadpoles (n=25 in each experiment). The numbers of tadpoles classified in each experiment are presented in Table S3 in the supplementary material. (**B-D**) *TCRα* (B), *TCRγ* (C) and *CD3γ/δ* (D) expression analysis. The relative amounts of transcripts obtained by qRT-PCR were determined by taking the value at 4 dpf as 1, after normalization with *ornithine decarboxylase 1*.

Finally, to investigate the possible relationship between regenerative ability and immune system development in *X. laevis* tadpoles, we examined the development of the T cell population around the onset of the refractory period. We quantified by qRT-PCR the amounts of transcripts for T cell markers: T cell antigen receptor (TCR) α and γ subunits (Haire et al., 2002) and the CD3γ/δ subunit (Dzialo and Cooper, 1988), using total RNAs extracted from the whole intact tadpoles at 4 to 9 dpf. In our experimental condition, the refractory period started around 6 dpf (Fig. 4A). The timings of *TCRα*, *TCRγ* and *CD3γ/δ* induction (Fig. 4B,C,D, respectively) and the decrease in regenerative ability largely coincided (around 6 dpf; Fig. 4A), suggesting that the development of the immune cell population is related to the decrease in regenerative ability during the refractory period.

As tadpoles regain their regenerative ability at the post-refractory regeneration period, we also hypothesized that 'regulatory T cells' that suppress the function of various immune cells (Piccirillo, 2008) are related to this period. To test this, we analyzed expression of the *X. laevis* homolog of *FOXP3*, a key regulatory gene of regulatory T cells (Hori et al., 2003). qRT-PCR revealed that transient *FOXP3* induction in the wound stump was more prominent in the post-refractory regeneration period than in the refractory period (Fig. 2I), implying that regulatory T cells play a role in regaining the regenerative ability during the post-refractory regeneration period. Taken together, it is plausible that there are only a few immune cells that impair the regenerative ability during the pre-refractory regeneration period, whereas such immune cells develop to impair the ability during the refractory period, and then an immune regulatory system is established, which again permits regeneration, during the post-refractory regeneration period (see Fig. S5 in the supplementary material).

Although there has been significant discussion on the possible link between immune responses and the regenerative abilities in some animal species (Arai et al., 1998; Mescher and Neff, 2005; Kanao and Miyachi, 2006), our research provides the first direct evidence for this link in *X. laevis* tadpole tails, and provides a clue for understanding not only developmental stage-specific regenerative ability, but also the molecular mechanisms of early epimorphic regenerative processes.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/14/2323/DC1

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References

Adams, D. S., Masi, A. and Levin, M. (2007). H^+ pump-dependent changes in membrane voltage are an early mechanism necessary and sufficient to induce *Xenopus* tail regeneration. *Development* **134**, 1323-1335.

Arai, T., Kawasaki, K., Kubo, T. and Natori, S. (1998). Cloning of cDNA for regenectin, a humoral C-type lectin of Periplaneta americana, and expression of the regenectin gene during leg regeneration. *Insect Biochem. Mol. Biol.* **28**, 987-994.

Bals, R. and Wilson, J. M. (2003). Cathelicidins – a family of multifunctional antimicrobial peptides. *Cell. Mol. Life Sci.* **60**, 711-720.

Beck, C. W., Christen, B. and Slack, J. M. (2003). Molecular pathways needed for regeneration of spinal cord and muscle in a vertebrate. *Dev. Cell* **5**, 429-439.

Bierer, B. E., Holländer, G., Fruman, D. and Burakoff, S. J. (1993). Cyclosporin A and FK506: molecular mechanisms of immunosuppression and probes for transplantation biology. *Curr. Opin. Immunol.* **5**, 763-773.

Bos, D. H. and Waldman, B. (2006). Polymorphism, natural selection, and structural modeling of class Ia MHC in the African clawed frog (*Xenopus laevis*). *Immunogenetics* **58**, 433-442.

Brockes, J. P. (1997). Amphibian limb regeneration: rebuilding a complex structure. *Science* **276**, 81-87.

Chuntharapai, A., Lee, J., Hébert, C. A. and Kim, K. J. (1994). Monoclonal antibodies detect different distribution patterns of IL-8 receptor A and IL-8 receptor B on human peripheral blood leukocytes. *J. Immunol.* **153**, 5682-5688.

Dzialo, R. C. and Cooper, M. D. (1997). An amphibian CD3 homologue of the mammalian CD3 gamma and delta genes. *Eur. J. Immunol.* **27**, 1640-1647.

Eisen, J. S. and Smith, J. C. (2008). Controlling morpholino experiments: don't stop making antisense. *Development* **135**, 1735-1743.

Haire, R. N., Kitzan Haindfield, M. K., Turpen, J. B. and Litman, G. W. (2002). Structure and diversity of T-lymphocyte antigen receptors alpha and gamma in *Xenopus*. *Immunogenetics* **54**, 431-438.

Hori, S., Nomura, T. and Sakaguchi, S. (2003). Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299**, 1057-1061.

Iijima, R., Kunieda, T., Yamaguchi, S., Kamigaki, H., Fujii-Taira, I., Sekimizu, K., Kubo, T., Natori, S. and Homma, K. J. (2008). The extracellular adenosine deaminase growth factor, ADGF/CECR1, plays a role in *Xenopus* embryogenesis via the adenosine/P1 receptor. *J. Biol. Chem.* **283**, 2255-2264.

Ishino, T., Shirai, M., Kunieda, T., Sekimizu, K., Natori, S. and Kubo, T. (2003). Identification of genes induced in regenerating *Xenopus* tadpole tails by using the differential display method. *Dev. Dyn.* **226**, 317-325.

Kajiho, H., Saito, K., Tsujita, K., Kontani, K., Araki, Y., Kurosu, H. and Katada, T. (2003). RIN3: a novel Rab5 GEF interacting with amphiphysin II involved in the early endocytic pathway. *J. Cell Sci.* **116**, 4159-4168.

Kanao, T. and Miyachi, Y. (2006). Lymphangiogenesis promotes lens destruction and subsequent lens regeneration in the newt eyeball, and both processes can be accelerated by transplantation of dendritic cells. *Dev. Biol.* **290**, 118-124.

Kawakami, Y., Rodriguez Esteban, C., Raya, M., Kawakami, H., Martí, M., Dubova, I. and Izpisúa Belmonte, J. C. (2006). Wnt/beta-catenin signaling regulates vertebrate limb regeneration. *Genes Dev.* **20**, 3232-3237.

Lee, J. H., Koo, T. H., Yoon, H., Jung, H. S., Jin, H. Z., Lee, K., Hong, Y. S. and Lee, J. J. (2006). Inhibition of NF-κB activation through targeting IκB kinase by celastrol, a quinone methide triterpenoid. *Biochem. Pharmacol.* **72**, 1311-1321.

Liu, Y., Kasahara, M., Rumfelt, L. L. and Flajnik, M. F. (2002). *Xenopus* class II A genes: studies of genetics, polymorphism, and expression. *Dev. Comp. Immunol.* **26**, 735-750.

McKercher, S. R., Torbett, B. E., Anderson, K. L., Henkel, G. W., Vestal, D. J., Baribault, H., Klemsz, M., Feeney, A. J., Wu, G. E., Paige, C. J. et al. (1996). Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *EMBO J.* **15**, 5647-5658.

Mescher, A. L. and Neff, A. W. (2005). Regenerative capacity and the developing immune system. *Adv. Biochem. Eng. Biotechnol.* **93**, 39-66.

Piccirillo, C. A. (2008). Regulatory T cells in health and disease. *Cytokine* **43**, 395-401.

Pinna, G. F., Fiorucci, M., Reimund, J. M., Taquet, N., Arondel, Y. and Muller, C. D. (2004). Celastrol inhibits pro-inflammatory cytokine secretion in Crohn's disease biopsies. *Biochem. Biophys. Res. Commun.* **322**, 778-786.

Slack, J. M. (2003). Regeneration research today. *Dev. Dyn.* **226**, 162-166.

Su, A. I., Cooke, M. P., Ching, K. A., Hakak, Y., Walker, J. R., Wiltshire, T., Orth, A. P., Vega, R. G., Sapinoso, L. M., Moqrich, A. et al. (2002). Large-scale analysis of the human and mouse transcriptomes. *Proc. Natl. Acad. Sci. USA* **99**, 4465-4470.

Thornton, C. S. (1957). The effect of apical cap removal on limb regeneration in Amblystoma larvae. *J. Exp. Zool.* **134**, 357-381.

Turpen, J. B., Kelley, C. M., Mead, P. E. and Zon, L. I. (1997). Bipotential primitive-definitive hematopoietic progenitors in the vertebrate embryo. *Immunity* **7**, 325-334.

Waelchli, R., Bollbuck, B., Bruns, C., Buhl, T., Eder, J., Feifel, R., Hersperger, R., Janser, P., Revesz, L., Zerwes, H. G. et al. (2006). Design and preparation of 2-benzamido-pyrimidines as inhibitors of IKK. *Bioorg. Med. Chem. Lett.* **16**, 108-112.

Whitehead, G. G., Makino, S., Lien, C. L. and Keating, M. T. (2005). *fgf20* is essential for initiating zebrafish fin regeneration. *Science* **310**, 1957-1960.

Zou, J., Bird, S., Minter, R., Horton, J., Cunningham, C. and Secombes, C. J. (2000). Molecular cloning of the gene for interleukin-1beta from *Xenopus laevis* and analysis of expression in vivo and in vitro. *Immunogenetics* **51**, 332-338.

Table S1. The number of tadpoles classified according to their regenerative abilities in inhibitor treatment experiments

Treatment	Regenerative ability	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6	Experiment 7
DMSO	Excellent	0	1	0	0	0	0	0
	Good	0	0	0	0	0	0	5
	Partial	2	2	0	0	6	0	1
	None	22	21	24	23	16	20	17
	Dead	0	0	0	1	2	4	1
	Sum	24	24	24	24	24	24	24
Cela	Excellent	16	17	6	0	10	5	8
	Good	0	0	4	0	6	4	4
	Partial	0	2	1	0	1	10	9
	None	5	5	13	24	5	5	3
	Dead	3	0	0	0	2	0	0
	Sum	24	24	24	24	24	24	24
IKK-VII	Excellent					7	3	8
	Good					2	7	3
	Partial					6	8	2
	None					5	5	3
	Dead					4	1	8
	Sum					24	24	24

Treatment	Regenerative ability	Experiment 8	Experiment 9	Experiment 10
DMSO	Excellent	0	5	0
	Good	0	10	0
	Partial	9	5	0
	None	15	4	22
	Dead	0	0	2
	Sum	24	24	24
CsA	Excellent	6	18	0
	Good	8	1	1
	Partial	2	1	2
	None	2	0	11
	Dead	6	4	10
	Sum	24	24	24
FK506	Excellent	8	19	12
	Good	8	5	3
	Partial	1	0	2
	None	0	0	2
	Dead	7	0	5
	Sum	24	24	24

Cela, 50 ng/ml Celastrol-treated group; IKK-VII, 1 μM IKK inhibitor VII-treated group; CsA, 10 μM cyclosporin A-treated group; FK506, 3 μM FK506-treated group.

Table S2. The number of tadpoles classified according to their regenerative abilities in antisense morpholino oligonucleotide experiments

Treatment	Regenerative ability	Experiment 1	Experiment 2
Noninjected	Excellent	0	36
	Good	3	8
	Partial	1	15
	None	28	122
	Sum	32	181
5mis PU.1-MO1	Excellent	0	0
	Good	6	2
	Partial	2	7
	None	58	49
	Sum	66	58
PU.1-MO1	Excellent	13	73
	Good	11	20
	Partial	8	22
	None	98	34
	Sum	130	149
Noninjected	Excellent	0	3
	Good	4	16
	Partial	11	32
	None	95	177
	Sum	110	228
5mis PU.1-MO2	Excellent	0	0
	Good	3	2
	Partial	9	4
	None	149	188
	Sum	161	194
PU.1-MO2	Excellent	0	3
	Good	3	5
	Partial	16	23
	None	40	132
	Sum	59	163

Table S3. The number of tadpoles classified according to their regenerative abilities at 4 to 9 dpf tadpole stage

Regenerative ability	4 dpf	5 dpf	6 dpf	7 dpf	8 dpf	9 dpf
Excellent	24	20	6	0	0	1
Good	0	1	1	6	3	0
Partial	0	1	7	6	2	7
None	0	3	9	13	19	12
Dead	1	0	2	0	1	5
Sum	25	25	25	25	25	25