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Anti-viral immune responses in a primitive lung: characterization and expression analysis of interferon-inducible immunoproteasome subunits LMP2, LMP7 and MECL-1 in a sarcopterygian fish, the Nigerian spotted lungfish (*Protopterus dolloi*)

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Abstract

Lungfishes (Dipnoi) represent the closest ancestor of tetrapods. Dipnoi have dual breathing modes extracting oxygen from water and air. The primitive lungs of lungfishes are exposed to external antigens including viruses. To date, the immune response of lungfishes against viruses has not been investigated. During viral immune responses, cell exposure to type I interferon induces the replacement of the constitutive proteasome with LMP2, LMP7 and MECL-1 beta subunits forming the immunoproteasome and enhancing antigen presentation to MHC class I molecules. In order to study the immune defense system of the lungfish lung, we have characterized for the first time the three immunoproteasome subunits in the sarcopterygian fish, the Nigerian spotted lungfish (*Protopterus dolloi*). LMP2, LMP7 and MECL-1 were identified in *P. dolloi* and their sequences encoded predicted proteins of 216, 275 and 278 amino acids, respectively. The mRNA of these three genes was expressed in multiple tissues, including the lung, with the highest abundance observed in kidney and post-pyloric spleen. *In vitro* stimulation of lungfish lung and kidney primary cell cultures with PolyI:C for 4 and 12 h resulted in increased LMP2, LMP7 and MECL-1 expression in both tissues. These results suggest a central role of these genes in the activation of an antiviral immune response in lungfish. Importantly, they indicate that the primitive lung of the common ancestor of all tetrapods is capable of inducing the expression of these genes in response to viral stimulation.

Keywords

Lungfish; lung; Immunoproteasome; LMP2; LMP7; MECL-1; Virus

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Introduction

Dipnoi fish, such as the African lungfishes, are sarcopterygian or lobed-fin fish with a key phylogenetic position. Recent molecular systematic studies show that they indeed represent the closest ancestor of tetrapods (Amemiya et al., 2013; Liang et al., 2013). The immune system of lungfishes is largely uncharacterized. Lungfishes have a dual mode of living, extracting oxygen from both water and air. This duality means that lungfishes have mucosal surfaces that are the interface between the host and the aquatic and terrestrial environments. The lungs of African lungfishes are primitive and simpler compared to the mammalian lungs but, like their mammalian counterparts, they are exposed to air-borne antigens, including viruses (Fishman et al., 1979). Mammals are able to trap particles by impaction or sedimentation along the branching airways that are subsequently removed by the mucociliary apparatus (Fishman et al., 1979). Lungfishes lack an equivalent mechanism as well as coordinated ciliary apparatus, even though a mucoid surface exists on the pulmonary surface (Fishman et al., 1979).

The antiviral response of all vertebrates relies on the initial activation of the innate immunity and, if needed, the subsequent induction of the adaptive immune system including the presentation of viral antigens via the MHC class I to CD8⁺ T cells. Proteasomes are abundant complex proteases that play a central role in the degradation of full-length proteins and generation of peptides for presentation on MHC class I molecules (Rock et al., 1994). In evolutionary terms, the proteasome was first, and the peptide transporter TAP and MHC class I only adapted to the peptides produced by the proteasome degradation activity (Spaapen and Neefjes, 2012). The 20S proteasomes (the catalytic core of the 26S proteasome) consist of four-ring structure with dyad symmetry ($\alpha\beta\beta\alpha$) and present fourteen distinct structural subunits and six catalytic subunits (two each of the $\beta 1$, $\beta 2$ and $\beta 5$ subunits, also referred as Δ , Z and X) (Coux et al., 1996; Tanaka, 2009). One set of these genes is constitutively expressed by most cell types; the other alternative catalytic subunits denoted $\beta 1i$ (LMP2 or PSMB9), $\beta 2i$ (MECL-1 or PSMB10) and $\beta 5i$ (LMP7 or PSMB8) are constitutively expressed in a number of hematopoietic cells (such as dendritic cells (DCs) and monocyte-derived cells) and are induced in other cell types after exposure to type I interferon (Tanaka, 1994, 2009). These alternative subunits are incorporated into newly assembling complexes, replacing standard subunits, forming immunoproteasomes (Groettrup et al., 2009; Tanaka, 1994) and changing the catalytic activities of these complexes. Immunoproteasomes, in comparison to the constitutive proteasome, are able to cleave faster after hydrophobic and basic amino acid residues and slower after acidic amino acid residues (Driscoll et al., 1993; Gaczynska et al., 1993) creating peptides that preferentially bind MHC class I molecules (Rammensee et al., 1993). Immunoproteasomes are induced by viruses that stimulate high type I interferon response (Khan et al., 2001; Robek et al., 2007).

The importance of immunoproteasome in antigen presentation has been confirmed in a study where mouse deficient in all three beta subunits of the immunoproteasome showed a markedly reduced MHC class I antigen presentation (Kincaid et al., 2011). An alternative function of the immunoproteasome during oxidative stress condition has been recently proposed. During this condition, the immunoproteasome activity increases preventing aggregation-associated cell death (Pickering et al., 2010).

Phylogenetically, immunoproteasomes are present only in jawed vertebrates. This indicates that they originated from more ancient standard 20S proteasomes concomitant with the emergence of MHC- and T/B-cell-receptor-based adaptive immunity (Kandil et al., 1996). It has been suggested that the genes coding for $\beta 1i$, $\beta 2i$, and $\beta 5i$ subunits arose through two rounds of whole-genome duplication (WGD) (Clark et al., 2000; Danchin et al., 2004;

Flajnik and Kasahara, 2009; Kasahara et al., 1996; Kasahara et al., 1997). Lungfishes have a key phylogenetic position since they are the closest relatives to all tetrapods and represent the transition of vertebrates from water to land (Amemiya et al., 2013; Brinkmann et al., 2004). To date, the immunoproteasome molecules of lungfishes and their role in anti-viral immune responses have not been investigated.

The present study aims to describe the role of the immunoproteasome in the antiviral immune response of the lungfish lung. We report for the first time the immunoproteasome beta subunits of a sarcopterygian fish, the Nigerian spotted lungfish (*P. dolloi*). We provide amino acid sequence comparisons with other vertebrate immunoproteasome subunits and give insights into the function of immunoproteasome in lungfish antiviral responses. By investigating the lung, our studies shed light into the evolution of this mucosal tissue in the immune response against air-borne viral antigens.

Material and Methods

Fish

Juvenile Nigerian spotted lungfish (*P. dolloi*) (9-12 inch total length) were obtained from Segrest farms (Florida, USA) and maintained in 10-gallon aquarium tanks at a constant temperature of 27°C. 10% of the tank water was exchanged daily. Fish were fed frozen earthworms three times a week. Feeding was terminated 48 h before sacrifice. Fish were acclimated to the laboratory conditions for at least 2 wk before in the start of the experiments. Fish were euthanized by adding an overdose of MS-222 (1g/L) to the tank water. Fish were bled from the caudal vein prior to collection of tissue and cell samples. All the experiments conducted in this study were approved by the University of New Mexico IACUC.

Lung histology

Lung tissue samples were obtained from two *P. dolloi* specimens, one adult *Xenopus laevis* specimen and a naïve adult C57BL/6 mouse. Lungs were inflated with 4% paraformaldehyde, fixed overnight at 4°C, transferred to 70% ethanol and then paraffin embedded. Four mm-thick sections were stained with hematoxylin and eosin. Slides were observed under a Zeiss AxioSkop microscope and images were uired with an AxioCam digital camera and the AxioVision software.

Identification and cloning of *P. dolloi* immunoproteasome beta subunits

Initial identification of three immunoproteasome beta molecules in *P. dolloi* was done by 454 pyrosequencing of the transcriptome of the pre-pyloric spleen. Total RNA was isolated from pre-pyloric spleen tissue, and mRNA was isolated from total RNA with the MPG® mRNA Purification Kit (PureBiotech). cDNA was made using the cDNA Synthesis System Kit with random primers (Roche). A cDNA Rapid library was constructed with the GS FLX Titanium Rapid Library Preparation Kit. Emulsion-based (em) PCR amplification of the DNA library was carried out with the GS FLX Titanium LV emPCR Lib-L Kit. Pyrosequencing was conducted using a GS FLX Titanium Sequencing Kit XL+ in a GS FLX+ System. All reagents and protocols used were from Roche 454 Life Sciences, USA. A total of 1 million reads were generated with a mean length of 425 bp. These were assembled into 14,072 contigs with 136,857 singletons, together forming the 454 reads database. The immunoproteasome beta subunits sequences of other vertebrates (available from GenBank) were used to find homologous sequences in the *P. dolloi* 454 reads database using BLAST. Partial sequences were identified as LMP2, LMP7 and MECL-1. The sequences that lacked the 5' UTR were completed by 5' RACE PCR using specific primers (Table I) to obtain the full ORF. The 5' RACE PCR was performed using 5'/3' RACE kit (2nd Generation, Roche)

according to the manufacturer's instructions. All PCR products were ligated into pGEMTeasy (Promega) vector and cloned into TAM1 competent cells (Active Motif). Plasmid DNA was isolated from positive colonies using the QIAprep Miniprep kit (Qiagen) and sequenced with an ABI 7000 sequencer (Applied Biosystems) at the Molecular Biology Core Facility of the Biology Department, University of New Mexico.

Sequence analysis

HMM (hidden Markov model) analysis was performed and six-frame translations of the sequences in the 454 reads database were scanned with HMMER version 3.1b1 (<http://hmmmer.org>) against SUPERFAMILY version 1.75 hidden Markov models (Gough et al., 2001). SCOP (Murzin et al., 1995) superfamily, family and domain assignments were also carried out.

Prediction of the open reading frame (ORF) was performed with the programs BLAST (Altschul et al., 1990) and the ExPASy proteomics server (ca.expasy.org/). Multiple sequence alignments were generated using CLUSTAL W (<http://align.genome.jp/>) (Chenna et al., 2003). Phylogenetic tree was constructed from generated alignments using the Neighbour-Joining (NJ) method within the software MEGA 4 (Kumar et al., 2004) and was bootstrapped 1000 times. To obtain the identity of the sequences, the software MatGat 2.02 (Campanella et al., 2003) was used.

Tissue samples

Pre-pyloric spleen, post-pyloric spleen, gut, kidney and lung tissue were collected from freshly killed healthy fish and stored in RNA later (Ambion). For RNA extraction the tissue (50 mg) was homogenized in TRIzol (Invitrogen) using tungsten carbide beads (3 mm, Qiagen) following the manufacturer's instructions. The resulting RNA pellets were rinsed in 500 μ l 80% ethanol, air-dried and resuspended in RNase free H₂O. The RNA was stored at -80°C until required for cDNA synthesis. cDNA synthesis was performed using 1 μ g of total RNA, which was denatured (65°C , 5 min) in the presence of 1 μ l of oligo-dT17, 1 μ l dNTP (deoxynucleoside triphosphate mix 10 mM each (Promega) and RNA/DNA free water (Sigma) in a volume of 13 μ l. The RNA was then cooled on ice, and synthesis carried out using 1 μ l Superscript III enzyme reverse transcriptase (Invitrogen) in the presence of 5 μ l of 5x first strand buffer, 1 μ l 0.1 M DTT, made up to a final volume of 25 μ l with water, and incubated at 55°C for 1 h. The resultant cDNA was diluted to a final volume of 50 μ l in RNA/DNA free water (Sigma) and stored at -20°C .

In vitro stimulation

Lungs and kidneys were collected aseptically from two fish, and placed in 5 ml of DMEM/F medium (Thermo Scientific) containing 100 IU/ml penicillin (Invitrogen) 100 μ g/ml streptomycin and 10 IU/mL heparin (Sigma). Tissues were finely minced (about 100 times) using sterile scissors and the small pieces were then pushed through a 100- μ m cell strainer. The cell suspensions were then centrifuged at 300 g for 10 min, and washed three times with DMEM/F without heparin. Cells were counted and 5×10^5 cells/well were seeded into 24 well plates ready for treatment. Kidney and lung primary cell cultures were incubated with polyinosinic:polycytidylic acid (PolyI:C; Sigma, 50 μ g/mL) or left untreated (control) for 4, 12, 24 and 48 h in triplicates. After the incubation periods, total RNA was extracted from cells using RNeasy Micro Kit (Qiagen). In order to collect the RNA from both adherent and non-adherent cells plates were first centrifuged (400 g, 10 min). After removing the culture supernatant, the RNA isolation buffer was added directly to the well and vigorously pipetted up and down prior to RNA extraction. To confirm that stimulation was successful, the expression of the viral-inducible protein Mx-1 (KF241956) was examined in the PolyI:C stimulated samples compared to control samples.

Quantitative real-time PCR (qPCR) analyses of *P. dolloi* immunoproteasome subunits expression

qPCR was used to determine the abundance of LMP2, LMP7 and MECL-1 transcripts in *P. dolloi* tissues and in primary lung and kidney cell culture. The qPCRs were performed in triplicate and each contained 3 μ l of a diluted cDNA template, 12.5 μ l of Power SYBR Green PCR master mix (2 $\times$ Applied Biosystems), and 100 nM forward and reverse primers in a 25 μ l reaction volume. The amplification profile consisted of an initial denaturation step at 95°C for 10 min, and then 30 cycles of 95°C for 15 s and 60°C for 1 min followed by melting (dissociation stage) from 72°C to 95°C in an ABI Prism 7000 (Applied Biosystems) sequence detection system. A negative control (no template) reaction was also performed for each primer pair. A sample from the serial dilution was run on a 2% agarose gel and stained with RedGel Nucleic Acid Stain (Biotium) and viewed under UV light to confirm that a band of the correct size was amplified. A melting curve for each PCR was determined by reading fluorescence at every degree between 72°C and 95°C to ensure only a single product had been amplified. *P. dolloi* elongation factor EF-1 α was used as control gene for normalization of expression.

The relative expression level of the genes was determined using the Pfaffl method (Pfaffl, 2001). Efficiency of the amplification was determined for each primer pair using serial 10 fold dilutions of pooled cDNA, performed on the same plate as the experimental samples. The efficiency was calculated as $E = 10^{-1/s}$ where s is the slope generated from the serial dilutions, when Log dilution is plotted against Δ CT (threshold cycle number). Primers were designed to have a T_m of 55°C, and where possible, to cross an exon-exon junction to avoid amplification of genomic DNA. Exon-intron junction sites were determined by comparing the *P. dolloi* cDNA with genomic sequence for orthologous genes from other vertebrates obtained from Ensembl (<http://ensembl.org/>). The sequences of all the PCR products amplified by qPCR were further confirmed by cloning.

Statistical analysis

Data is presented as mean $\pm$ standard error. qPCR measurements were analyzed by t-test by comparing values with either the EF-1 α control or the non-infected control. One-way ANOVA analysis followed by Tukey's posthoc test was used to identify expression differences among tissues. p-values <0.05 were considered significant. All statistical analyses were performed using Prism GraphPad version 5.0.

Results

Comparative histology of vertebrate lungs

In order to recapitulate the major morphological and histological differences present in the lung of different vertebrate groups, we performed histological examinations of lung tissue from our model species (lungfish), and two tetrapods, one amphibian and one mammal.

As previously described in other *Protopterus sp.*, the primitive lungs of *P. dolloi* are formed by a pair of fibromuscular air sacs forming a common chamber underneath the glottis. The lungs do not contain alveoli, but numerous small air chambers protrude from a central hollow tube. The walls of the air chambers contain capillaries lymphatic vessels, connective tissue, nerve fibers and numerous mesenchymal cells (Fig 1A).

Compared to other tetrapods *Xenopus* (and amphibians in general) have some of the simplest lungs. As in the lungfish, *Xenopus* lungs do not contain alveoli and the parenchyma shows low degree of partitioning, as in the lungfish. The respiratory portion of *Xenopus* lung consists of large air sacs, which contain a close network of capillaries in their walls. The air

sacs are lined by a continuous layer of respiratory epithelium. The cells forming this epithelium are termed 'pneumonocytes' (or pneumocyte) as they appear to be homologues of the alveolar cells in mammalian lung (Meban, 1973). Similar to lungfishes, amphibians have only one type of pneumocyte that has a free surface with short microvilli but no cilia (García, 1995) (Fig 1B).

The lungs of the mouse are covered by visceral pleura and consist of an undivided left lung and a right lung divided into four lobes. The primary bronchi are the only bronchi in the mouse that contain cartilage and are lined by respiratory epithelium, a ciliated pseudostratified columnar epithelium interspersed with goblet cells. Subsequent branches of the bronchial tree are the smaller intrapulmonary bronchi, the terminal bronchioles, and the respiratory bronchioles. The terminal bronchioles transition directly into the alveolar ducts. Respiratory bronchioles have saccular outpocketing in their wall called alveoli (Treuting and Dintzis, 2011). The lungs of mice possess two line of defense against airborne substances: the mucociliary escalator and alveolar macrophage (Green et al., 1977) (Fig 1C).

Sequence analysis of lungfish immunoproteasome subunits

HMM analysis of *P. dolloi* 454 resulted in the discovery of 9 α subunits and 10 β subunits of the 20S proteasome (Supplementary Table I). Only matches with e-value better than 10^{-5} were retained. Within the β subunits we were able to identify the 7 constitutive subunits ($\beta 1$ - $\beta 7$) and the three immunoproteasome subunits ($\beta 1i$, $\beta 2i$ and $\beta 5i$). HMM study did not show the presence of multiple forms for any of the β subunits. This initial search permitted the identification of two partial sequences and one full-length sequence denoted as LMP2, LMP7 and MECL-1. The lungfish LMP2 sequence (KF241953) is 1204 bp and contains a 5'-UTR of 119 bp, an open reading frame of 648 bp encoding an ORF of 216 amino acids and a 3'-UTR of 437 bp. Since the N-terminal region is believed to be proteolytically removed before assembling into proteasome, this region often shows poor conservation (Nonaka et al., 1997) and therefore only the amino acid sequences of the mature peptide of LMP2, LMP7 and MECL-1 were used for the sequence analysis. The amino acid sequence of the mature peptide shows high identity to other characterized LMP2 sequences (e.g., 67.8% to coelacanth LPM2 and 66.3% to *Xenopus* LMP2) (Table II). The LMP7 sequence of lungfish (KF241954) is 1056 bp and contains a 5'-UTR of 8 bp, an open reading frame of 825 bp encoding an ORF of 275 amino acids and a 3'-UTR of 223 bp. The lungfish LMP7 mature peptide amino acid sequence shows high identity to the other vertebrate LMP7 sequences, in particular it shares 82.5% and 78.8% amino acid identity with shark and coelacanth LMP7 respectively (Table IIA-C). The lungfish MECL-1 sequence (KF241955) is 1456 bp long containing a 5'-UTR of 147 bp and an open reading frame of 834 bp. It encodes an ORF of 278 amino acids and a 3'-UTR of 475 bp. The MECL-1 amino acid sequence shows high identity to that of other vertebrates. For instance, lungfish MECL-1 mature peptide is 75.9% identical to coelacanth MECL-1 and 72% to shark MECL-1 (Table II). To further compare lungfish immunoproteasome subunits with other available LMP2, LMP7 and MECL-1 sequences, a multiple alignment was performed (Fig 2) showing the presence of a conserved proteasome beta type domain in each sequence (beta type 6, 5 and 7 respectively) and a signal peptide. In addition, two cysteine residues were conserved in the LMP2 sequences of all species examined. In LMP7, two cysteine residues were also conserved among all vertebrates, whereas four conserved cysteine residues are conserved in MECL-1.

To determine the relationship between LMP2, LMP7 and MECL-1 a phylogenetic tree, using mature peptide amino acid sequences, was constructed using software MEGA 4 (Tamura et al., 2007). The phylogenetic tree (Fig 3) clearly shows that the LMP2, LMP7 and MECL-1 cluster into three robust branches, indicating that these three genes from lungfish

are counterparts of the same genes from mammals and other tetrapods. Trout and zebrafish LMP2 sequences group together, while shark and coelacanth sequences show an intermediate degree of clustering between teleost fish and tetrapods. Lungfish LMP2 sequence appears more divergent and forms a separate out-group. On the other hand, *P. dolloi* LMP7 clusters with shark LMP7, whilst the other vertebrate sequences form a different group with tetrapod LMP7 forming a subgroup and teleosts a different subgroup. Lungfish MECL-1, differentially from LMP2 and LMP7, closely clusters with the coelacanth MECL-1 sequence and groups with shark and tetrapod sequences whilst teleost MECL-1 form a different group.

Constitutive expression of immunoproteasome subunits in lungfish tissues

The expression of lungfish LMP2, LMP7 and MECL-1 was examined in five different tissues (pre-pyloric spleen, post-pyloric spleen, gut, kidney and lung) from healthy fish (n=4) by qPCR. The relative expression of each gene was normalized first with the housekeeping genes and then presented relative to the expression in pre-pyloric spleen (the tissue that generally had the lowest expression) that was given an arbitrary value of 1. The three immunoproteasome genes examined were expressed in all the tissues studied with highest expression levels found in kidney and post-pyloric spleen (Fig 4). In the lung, the constitutive expression levels were approximately 3 times lower (LMP2), 5.5 times lower (LMP7) and 4 times lower (MECL-1) than those found in the kidney.

***In vitro* modulation of immunoproteasome expression following PolyI:C stimulation**

Primary cell suspensions from lung tissue were obtained in order to perform the *in vitro* stimulation studies. Lung cell suspensions were observed under a light microscope prior to and after plating. Cell suspensions consisted primarily of epithelial cells, muscle cells and fibroblast-like cells. Abundant large dark pigment-containing cells (putative melanomacrophages) were also part of the suspension. Some immune cells including lymphocytes, macrophages and granulocytes could be observed and accounted for approximately 10% of the total cell numbers. During *in vitro* culture, some cell death and degranulation of pigmented cells occurred during the first 24 h of the experiment. A mixture of adherent cells and cells in suspension could be observed throughout the length of the experiment with no signs of bacterial contamination. RNA was isolated from primary lung and kidney cell cultures stimulated with either PolyI:C or left untreated (control). A kinetics study was performed for both tissues, with expression being monitored at four different time points. PolyI:C is structurally similar to double stranded RNA present in some viruses and it is able to mimic a viral infection. To confirm a positive response to the *in vitro* immunostimulation, the expression of the viral-inducible protein Mx-1 (which is known to be up regulated during double-stranded virus infection) was measured in every sample. Mx-1 expression was induced in both lung and kidney cells upon PolyI:C treatment (Supplementary Fig. I).

Generally speaking all three genes behaved in a similar fashion during the *in vitro* stimulation. In lung primary cell cultures, the immunoproteasome subunits were found to be up regulated 4 h and 12 h following PolyI:C stimulation, with no significant changes found at the later time points (Fig 5A). In the kidney cells, 4 h and 12 h after PolyI:C stimulation, the expression of the three immunoproteasome subunits was found to be significantly up regulated, whilst a down regulation was found 24h after stimulation. At 48h no changes in the expression of LMP2, LMP7 and MECL-1 were observed (Fig 5B).

Discussion

Vertebrates transitioned from water to land about 400 million years ago (Clack, 2005). During this evolution, their respiratory organs have undergone major adaptational changes. The acquisition of lungs and an air-breathing system implied new exposure to air-borne pathogens, including viruses. Lungfishes are the closest relative to all tetrapods and have primitive lungs that are in direct contact with air-born viruses. Using *P. dolloi* as a model allowed us to study the antiviral immune response in a primitive vertebrate lung. African lungfishes are obligatory air breathers and their lungs derived from a diverticulum of their air bladder (Burggren and Johansen, 1986; Johansen, 1970). The lungs of African lungfish consist of fibromuscular walls with air sacs for gas exchange but no alveoli (Fishman et al., 1979). Despite the absence of resident population of phagocytes, corresponding to alveolar macrophages in mammals, the lungs of African lungfish are able to respond to stimuli and phagocytic cells can be observed mostly by the infiltration of macrophages from the respiratory endothelium and epithelium (Fishman et al., 1979). In mammals, exposure to viruses such as the flu, results in formation of inducible bronchus-associated lymphoid tissue (iBALT) (Moyron-Quiroz et al., 2004). BALT is a submucosal lymphoid aggregate found beneath the epithelium at bronchi branches within the lower respiratory tract (Sminia et al., 1989). Our histological examination of *P. dolloi* lung did not reveal the presence of BALT. However, since no *in vivo* viral infections were performed, we cannot rule out the potential appearance of iBALT-like structures in lungfish.

The proteasome is responsible for recognition and degradation of protein substrates modified with polyubiquitin chains. In mice, the small protein fragments generated by the proteasome can be used by MHC class I molecules for the display of antigens to the immune system (van Deventer and Neefjes, 2010). The three active beta subunits of a constitutive proteasome are PSMB5 (X), PSMB6 (Δ), and PSMB7 (Z) and they present, respectively, chymotrypsin-like, caspase-like, and trypsin-like proteinase activities. These subunits are replaced by type I interferon inducible beta subunits with proteinase activities known as PSMB8 (LMP7), PSMB9 (LMP2), and PSMB10 (MECL-1), respectively, resulting in the formation of the immunoproteasome (Tanoka and Kasahara, 1998). Prior works have indicated that the immunoproteasome promotes the generation of relevant peptides for presentation by MHC class I molecules; and *in vitro* experiments demonstrated that immunoproteasomes produce different peptides than those produced by standard proteasomes playing a key role in antigen processing and recognition by T-lymphocytes (Kloetzel, 2001; Tanoka and Kasahara, 1998).

In this study we have characterized the LMP2, LMP7 and MECL-1 genes in the African lungfish *P. dolloi*. LMP2, LMP7 and MECL-1 amino acid sequences showed the presence of a signal peptide and a conserved motif (proteasome beta type 6, 5 and 7 respectively) that characterizes members of the N-terminal nucleophile (Ntn)-hydrolase superfamily (involved in peptide bond hydrolysis). From these phylogenetic analyses it appears that lungfish LMP2, LMP7 and MECL-1 display different levels of divergence when compared to their corresponding vertebrate sequences. The latter is evidence by: 1) lungfish LMP2 has diverged from the rest of the vertebrate LMP2s since the group formed by elasmobranch, teleost, coelacanth and tetrapod LMP2 sequences does not cluster with its lungfish counterpart 2) Lungfish and shark LMP7 are, in turn, closely related, the coelacanth sequence being closer to the teleost LMP7 and separated from the tetrapod LMP7 group 3) Finally, *P. dolloi* MECL-1 is more closely related to the coelacanth MECL-1, the other lobbed-fin fish species here analyzed. Altogether, these results suggest that lungfish LMP2, LMP7 and MECL-1 have experienced different evolutionary pressures leading to complex phylogenetic relationships with the rest of the vertebrate immunoproteasome subunits.

LMP2, LMP7 and MECL-1 were constitutively expressed in all the tissues examined with the highest expression found in the kidney and post-pyloric spleen. Constitutive expression was also found in the lung although at lower levels. This result confirms the central role of these molecules in the lungfish immune response. The modulation of the three immunoproteasome subunits was studied *in vitro* under PolyI:C stimulation. PolyI:C is a double stranded RNA molecule (dsRNA) recognized by the toll-like receptor 3 (TLR-3) which activates the NF- κ B pathway (Alexopoulou et al., 2001) leading to the induction of an antiviral response and up regulating genes such as Mx-1 and IFN γ (Collet et al., 2004; Goodbourn et al., 2000; Tumpey et al., 2007).

We selected these two tissues for the following reasons: i) the lung because of its importance in the transition of vertebrates from water to land; ii) the kidney because it had the highest constitutive expression of the immunoproteasome genes and because it is known as one of the main hematopoietic organs in lungfish (Delaney et al., 1976; Jordan and Speidel, 1931).

In the present study we demonstrated that *in vitro* stimulation of *P. dolloi* lung cells with PolyI:C induced a type I interferon response and the activation of the immunoproteasome beta subunits. The up regulation of LMP2, LMP7 and MECL-1 was observed after 4 and 12 h following stimulation. Lungfish kidney cells showed an induction of LMP2, LMP7 and MECL-1 genes at 4 and 12 h following stimulation indicating that the fish was able to mount an immune response against viral infection. Differentially from lung cells a significant suppression of the genes studied was observed at 24h following stimulation, whilst at 48h no modification of expression was found. Previous studies in teleost fish have demonstrated the induction of immunoproteasomes during a viral infection (Hansen and La Patra, 2002; Hansen et al., 2012; Jørgensen et al., 2006). Previous studies in mice lungs have demonstrated the ability of mammals to respond to IFN γ stimulation via the expression of the immunoproteasome subunits (Barton et al., 2002).

Altogether, our results demonstrate the conserved presence of the immunoproteasome system in the lungfish lung and its involvement in the response against viral antigens *in vitro*. The present study suggests that the acquisition of a dual breathing mode by Dipnoi involved the ability for their primitive lungs to mount adaptive immune responses against viral antigens via the immunoproteasome pathway. The identification of naturally occurring viral infections in Dipnoi will allow to further test this hypothesis *in vivo*.

Conclusions

In the present study we have cloned and characterized three immunoproteasome beta subunits in the African lungfish *P. dolloi*. These genes showed the canonical proteasome beta type conserved domain indicating their role in peptide bond hydrolysis. Tissue distribution of LMP2, LMP7 and MECL-1 showed that these genes were expressed in all the tissues studied, including the lung. *In vitro* immune stimulation resulted in increased LMP2, LMP7 and MECL-1 expression in both lung and kidney cells indicating an important role of these molecules in immune response against viral infections in lungfish and shedding light on the evolution of lung immunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

We describe three immunoproteasome beta subunits (LMP2, LMP7 and MECL-1) in lungfish. Gene structure is conserved in vertebrates. Responses to PolyI:C immunostimulation have been analyzed by qPCR over time. The primitive lung of lungfishes responds to viral antigens via the immunoproteasome pathway.

Figure 1.

Comparative histology of vertebrate lungs. A) Lung of the lungfish *P. dolloi*, B) Lung of the amphibian *X. laevis*, C) Lung of the mouse *Mus musculus*. AS: air sacs; Fmw: fibromuscular wall; Cap: capillaries; clct: central layer of connective tissue; bv: blood vessel; tb: terminal bronchiole; av: alveoli.

Figure 2.

A) Alignment of the LMP2 amino acid sequences of lungfish (KF241953), human (CAG46457), *Xenopus* (NP_001079339), trout (ADM95871), zebrafish (NP_571466), coelacanth (ENSLACG00000014279) and shark (AAL59852). Residues constituting the proteasome beta type 6 are highlighted with a solid bar and those forming the signal peptide are highlighted with a dash bar above the sequence. “*” indicate identical amino acids between all sequences, whilst “:” and “.” show conservative substitutions. “+” indicate fully conserved cysteine. B) Alignment of the LMP7 amino acid sequences of lungfish (KF241954), human (CAA47026), *Xenopus* (NP_001084323), trout (BAD89554), zebrafish (AAB87679), coelacanth (ENSLACG00000014047) and shark (BAA10933). Residues constituting the proteasome beta type 5 are highlighted with a bar above the sequence. “*” Indicates identical amino acids between all sequences, whilst “:” and “.” show conservative substitutions. “+” indicate fully conserved cysteine. C) Alignment of the MECL-1 amino acid sequences of lungfish (KF241955), human (CAG33263), *Xenopus* (NP_001079861), trout (ADM95869), zebrafish (AAI65548), coelacanth (ENSLACG00000013849) and shark (DR783428). Residues constituting the proteasome beta type 7 are highlighted with a bar above the sequence. “*” Indicates identical amino acids between all sequences, whilst “:” and “.” show conservative substitutions. “+” indicate fully conserved cysteine.

Figure 3.

Phylogenetic tree showing the evolutionary relationship of the immunoproteasome beta subunits molecules in vertebrates. The tree was constructed using the Neighbour-Joining method in MEGA 4 and was bootstrapped 10,000 times. GenBank accession numbers are as follows: LMP2 lungfish (KF241953), human (CAG46457), *Xenopus* (NP_001079339), trout (ADM95871), zebrafish (NP_571466), coelacanth (ENSLACG00000014279) and shark (AAL59852); LMP7 lungfish (KF241954), human (CAA47026), *Xenopus* (NP_001084323), trout (BAD89554), zebrafish (AAB87679), coelacanth (ENSLACG00000014047) and shark (BAA10933); MECL-1 lungfish (KF241955), human (CAG33263), *Xenopus* (NP_001079861), trout (ADM95869), zebrafish (AAI65548), coelacanth (ENSLACG00000013849) and shark (DR783428).

Figure 4.

Tissue distribution of A) LMP2, B) LM7 and C) MECL-1 expression in control Nigerian spotted lungfish (*P. dolloi*) (n=6). The relative expression of each gene was normalized first with the house keeping genes, and then divided by the average expression level in the tissue with the lowest value. Bars represent means $\pm$ standard error of four fish. Different letters represent statistically significant groups after Tukey's test ($p < 0.05$).

Figure 5. Fold change of LMP2, LMP7 and MECL-1 expression in A) lung primary cell culture, B) kidney primary cell culture of Nigerian spotted lungfish at 4, 12, 24 and 48 h following stimulation with PolyI:C compared to samples from cells untreated (control). Bars represent means $\pm$ standard error of four fish. Different letters represent statistically significant groups after Tukey's test ($p < 0.05$).

Table I
Primers used in this study

Gene	Primer	Sequence (5'-3')	Application
LMP2	LMP2R1	GGAACAGTAGATCTTGTCATGG	5' RACE
	LMP2R2	GACTACTCCTCCATCAAACACC	5' RACE
	LMP2R3	GACTCCAAGAGGTCATCCATGG	5' RACE
	LMP2F0	CCATGACAAGATCTACTGTTCC	qPCR
	LMP2R0	CCATATTTGTGGTCCCAACCTGC	qPCR
LMP7	LMP7R1	GCCTACTGAAAGTCCCATTCC	5' RACE&qPCR
	LMP7R2	GCAGCTGAGACAGAGATTTCTTCC	5' RACE
	LMP7R3	CCAGACATGGTACCCAGAAGG	5' RACE
	LMP7F0	CCTTCTGGGTACCATGTCTGG	qPCR
MECL-1	MECL-1F0	CCTAACATCTATTGTTGTGGTGC	qPCR
	MECL-1R0	GGATGAATGCTGTAGATGTGAGG	qPCR
Mx-1	Mx-1F0	GCTTTGTCAGGAGTAGGACTGC	qPCR
	Mx-1R0	CTTCTGATGGGCTTGTAAGTTCC	qPCR
EF-1 α	EF1aF	GGAAAGTCTCTCGAGGCAAGG	qPCR
	EF1aR	CCATACCAGGTTTCAAGACACC	qPCR

Amino acid sequence identity (%) of lungfish immunoproteasome molecules with other vertebrates immunoproteasome molecules.

Table II

LMP2	1	2	3	4	5	6	7
1. Lungfish		66.3	67.3	63.3	62.3	67.8	64.3
2. Human	85.9		71.4	67.3	67.3	73.4	70.4
3. Xenopus	83.4	87.9		64.3	63.8	67.8	66.8
4. Zebrafish	78.4	84.4	83.9		81.9	70.9	73.4
5. Trout	81.9	84.9	82.9	92.5		69.3	70.4
6. Coelacanth	84.4	89.4	87.4	85.9	86.9		72.4
7. Shark	80.9	85.4	84.9	86.9	85.9	85.9	
A							
LMP7	1	2	3	4	5	6	7
1. Lungfish		78.3	75.1	78.8	77.2	78.8	82.5
2. Human	87.8		82	82.5	81	83.6	76.7
3. Xenopus	85.2	89.9		79.9	79.9	79.9	74.6
4. Zebrafish	92.1	92.1	88.9		95.2	88.9	77.8
5. Trout	88.9	89.9	88.4	96.8		85.7	78.3
6. Coelacanth	91	93.7	91	96.3	93.7		79.9
7. Shark	93.7	89.9	87.3	91	89.4	91.5	
B							
MECL-1	1	2	3	4	5	6	7
1. Lungfish		71.6	58.8	64.5	63.9	72	75.9
2. Human	88.5		50	60.1	57.4	64.2	67.3
3. Xenopus	68.5	63.8		48.7	48.3	54.9	59.2
4. Zebrafish	82	78.7	62.1		86.3	58.5	60.3
5. Trout	82.5	77.6	60.4	95.1		57	58.3
6. Shark	84.9	79.2	67.2	74.5	74		71.9
7. Coelacanth	85.4	79.9	70.6	75.4	74.9	83.9	

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