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No evidence for immunoproteasomes in chicken lymphoid organs and activated lymphocytes

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Abstract The proteasome is the main protein-degrading machine within the cell, producing ligands for MHC class I molecules. It is a cylindrical multicatalytic protease complex, and the catalytic activity is mediated by the three subunits β1, β2, and β5 which possess caspase-, trypsin-, and chymotrypsin-like activities, respectively. By stimulation with interferon (IFN)-γ the replacement of these subunits by β1i, β2i, and β5i is induced leading to formation of immunoproteasomes with altered proteolytic and antigen processing properties. The genes coding for these immunosubunits are restricted to jawed vertebrates but have so far not been found in the genomes of birds, e.g., chicken, turkey, quail, black grouse and zebra finch. However, the chicken genome sequences are not completely assigned; therefore, we investigated the presence of immunoproteasome on protein level. 20S proteasome was purified from the chicken brain, blood, spleen, and bursa of Fabricius, followed by separation via two-dimensional (2D) gel electrophoresis. We analyzed the protein spots derived from the spleen and brain by mass spectrometry and could identify all 14 proteasomal subunits, but there were no differences detectable in the spot patterns. Moreover, we stimulated the chicken spleen cells with phorbol 12-myristate 13-acetate (PMA) and ionomycin aiming at the induction of immunoproteasome, but in spite of the induction of proliferation and IFN-γ, no evidence for immunoproteasome formation in chicken could be obtained. This result was substantiated by the finding that 20S proteasomes isolated from immune and non-immune tissues showed very similar peptidolytic activities. Taken together, our results indicate that chicken lack immunoproteasomes also on protein level.

Keywords Proteasome · Immunoproteasome · Chicken · Galliformes · MHC locus · Bursa of Fabricius

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Introduction

The proteasome is the major protein-degrading system in the nucleus and the cytoplasm, responsible for the clearance of misfolded proteins, transcription factors, or cell regulators. Proteins are tagged for proteasomal proteolysis by the conjugation to polyubiquitin or the ubiquitin-like modifier FAT10 (Hershko et al. 2000; Miteva et al. 2010; Schmidtke et al. 2014). The main form of proteasome is the so-called 26S proteasome comprised of the cylindric 20S core particle, responsible for the proteolytic activity, capped by the 19S regulator. The 20S proteasome is barrel-shaped and consists of 28 subunits with masses of 20—30 kDa arranged in four rings each comprising seven subunits (Huber et al. 2012). The two outer rings of the cylinder are called the α rings containing subunits α1-7 and the two internal β rings contain subunits β1-7. The β rings possess the proteolytic activity which is mediated by the three different catalytic sites on subunits β1, β2, and β5. Subunit β1 is responsible for the caspase-like activity, β2 for the trypsin-like activity, and β5 for chymotrypsin-like cleavage activity of the proteasome, producing peptides with acidic, basic, and hydrophobic C-terminal residues, respectively (Kniepert and Groettrup 2014). The proteasome is constitutively expressed in most tissues. Through the treatment with the pro-inflammatory cytokines interferon (IFN)-γ or tumor necrosis factor (TNF)-α, a replacement of the three constitutive catalytic subunits by their inducible homologues β1i (PSMB9), β2i

(PSMB10), and β5i (PSMB8) is initiated (Groettrup et al. 1996; Groettrup et al. 1995). This results in the formation of a protease called the immunoproteasome the expression of which is typically restricted to immune cells and has altered proteolytic properties. β1i favors cleavage after hydrophobic residues instead of acidic ones. Further, peptides generated by the proteasome are presented on MHC class I molecules but those originating from the immunoproteasome contain more suitable MHC class I ligands in mice and humans, because the binding groove has a higher binding affinity for peptides with hydrophobic or basic C-termini (Kaufman et al. 1995; Koch et al. 2007; Wallny et al. 2006). In this context, another type of proteasome should be mentioned, the thymoproteasome, which is characterized by the catalytic proteins β1i and β2i as well as β5t and is unique to cortical thymic epithelial cells (cTECs) residing in the cortex of the thymus. Thymoproteasome is critical for the positive selection and maturation of T cells by the production of low affinity MHC class I ligands (Murata et al. 2007).

Although the constitutive proteasome is found in almost all organisms (from plants over fungi to vertebrates), the immunoproteasome is principally found in mammals, fish, amphibians, and reptiles. BLAST search approaches in chicken, turkey, quail, black grouse, and zebra finch failed to detect genes for immuno- and thymoproteasome, suggesting that they have been lost in the lineage of birds (Balakrishnan et al. 2010; Chaves et al. 2009; Shiina et al. 2007; Shiina et al. 2004; Sutoh et al. 2012; Wang et al. 2012). While the genes coding for the catalytic subunits β1i and β5i are present in the class II region of the human and murine MHC loci, sequence searches in chicken genome databases revealed that there are no LMP genes in the avian MHC region, and also the immune subunit β2i could not be identified. All genes located in the chicken MHC locus do have human homologues, e.g., TAP genes, tapasin, complement component C4, but not all human MHC genes are present in chicken and the different gene class regions are organized in a different way (Kaufman 2008). The chicken MHC locus represents a minimized set of genes conserved over the period of evolution between aves and mammals (Kaufman et al. 1999). It can therefore not be ruled out that immunoproteasome genes have been lost from the chicken MHC locus and inserted elsewhere. Such genes may have remained unrecognized due to poor sequence conservation or due to holes in the chicken genome as it is currently known.

In order to investigate the presence of immunoproteasome in chicken on the protein level, we purified 20S proteasomes from different organs and analyzed them by two-dimensional (2D) gel electrophoresis followed by mass spectrometric analysis. Typical organs in chicken where immunoproteasome would be suspected are the spleen, thymus, and bursa of Fabricius, while it is assumed that the brain exclusively expresses constitutive proteasome and whole blood might be a mixture of both proteasomes, in which the constitutive one predominates. In contrast to the other organs, the bursa of Fabricius is a primary lymphoid organ for the production of B cells which is unique to birds. In humans, the differentiation of B cells occurs in the bone marrow whereas birds use the bursa of Fabricius, which is located dorsal to the cloaca (Rodriguez-Mendez et al. 2010). During embryonic days 4 to 8, the bursa is formed and afterwards colonized by B cells. Until sexual maturity at 4–6 months, it is the place of B cell differentiation. Subsequently, the bursa regresses completely and the B cells migrate to secondary lymphatic organs and the blood, from then on representing the peripheral B cell pool (Mustonen et al. 2001).

The goal of this study was to investigate the presence of constitutive and immunoproteasome in chicken on protein level. To this aim, 20S proteasomes were purified from different lymphatic organs in comparison to non-lymphatic organs and proteasomes were analyzed by 2D gel electrophoresis. Further, we determined the three different catalytic activities of the purified chicken proteasomes.

Methods

Purification of 20S proteasome from chicken organs

The purification of 20S proteasome from the brain, whole blood, spleen, bursa of Fabricius, and thymus, retrieved from laying hens and broiler chickens, was performed as previously described (Basler and Groettrup 2012). Shortly, organs were lysed and homogenized and then centrifuged to remove cell debris. Overnight, negatively charged proteins were bound to DEAE Sephacel™ (GE Healthcare), washed the next day, and eluted using 500 mM KCl buffer, 10 mM HEPES, and 5 mM $MgCl_2$, pH 7.2. Fractions containing protein were pooled and precipitated with 35 % ammonium sulfate; subsequently, the supernatant was adjusted to 80 % ammonium sulfate; after centrifugation, the protein pellet was resolved and separated via a sucrose gradient (15–40 %) using an ultracentrifuge. Fractions which possess proteolytic activity were pooled and finally purified by FPLC (ÄKTA, GE Healthcare) using a Resource™ Q 1-ml column (Amersham Pharmacia Biotech). Purifications were tested for proteasome activity, protein concentration, and purity by 2D gel electrophoresis. All animal work was conducted according to the German guidelines and was approved by the ethics committee of Regierungspräsidium Freiburg, Germany.

2D gel electrophoresis

Purified chicken 20S proteasome was separated in a non-equilibrium pH gradient gel electrophoresis/sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to previous descriptions (Groettrup et al. 1996).

Briefly, 60 μg of purified proteasome was precipitated using 10 % trichloroacetic acid and washed with ice-cold acetone and 100 % ethanol. The precipitated proteasome was dissolved in 80 μl sample buffer (9.5 M urea, 2 % NP-40, 5 % Servalyt® ampholines, pH 3–10 (Serva, Heidelberg, Germany), 0.3 % SDS, and 5 % 2-mercaptoethanol) during overnight shaking at a temperature of 28 °C. Samples were loaded on 12-cm gel rods and were separated in a 2D gel apparatus (BioRad) along a pH gradient (anode: 0.01 M H_3PO_4; cathode: 0.02 M NaOH). For the second dimension, the gel rods were equilibrated and fixed to the top of a 15 % SDS-polyacrylamide gel and afterwards 1100 Vh were applied overnight and gels were stained with Coomassie blue.

Mass spectrometric analysis of purified chicken 20S proteasome

Protein spots of interest were excised from the gels, and the pieces were incubated at 56 °C in 50 mM ammonium bicarbonate containing 10 mM DTT. After 1 h, the solution was removed and 50 mM iodoacetamide diluted in 50 mM ammonium bicarbonate were added for another hour at room temperature. Before dehydration with 3/2 acetonitrile/MilliQ, gel pieces were washed in MilliQ water and were incubated for 30 min in 50 mM ammonium bicarbonate. Once the gels lost their color, they were incubated for 10 min with pure acetonitrile and were subsequently air dried. Gel pieces were rehydrated with cold 50 mM ammonium bicarbonate containing 10 ng/μl trypsin (45 min at 4 °C). To digest the proteins, the gel pieces were covered with 50 mM ammonium bicarbonate and were incubated overnight at 37 °C. The peptides were eluted by adding 3/2 acetonitrile/0.1 % trifluoroacetic acid for 1 h at room temperature and were dried in a vacuum centrifuge. Finally, the samples were dissolved and were analyzed by nano-LC-ESI-MS/MS using a Thermo LTQ Orbitrap Discovery with Eksigent 2D nano-HPLC. Subsequent identification of proteins was achieved by searching the Swiss-Prot database (UniProtKB/Swiss-Prot sequences).

Preparation of spleen single cell suspensions and LPS treatment

Dissected spleens from laying hens or broiler chickens were collected in 10 ml cell culture medium (IMDM GlutaMAX supplemented with 10 % FCS, 1 % penicillin-streptomycin and 1 % gentamicin, Gibco® Life Technologies, Karlsruhe, Germany) on ice. The single cell suspension was prepared by gently pushing the organ through a sterile steel grid; afterwards, cells were rinsed through a 70-μm cell strainer (BD Falcon) and were washed twice with PBS (137 mM NaCl, 2.68 mM KCl, 8.1 mM $Na_2HPO_4{\cdot}2H_2O$ and 1.47 mM KH_2PO_4, pH 7.2). Finally, cells were resuspended in culture medium and one half was directly spun down (1300×*g* for 10 min) and the cell pellet was stored at −20 °C, while the other half was seeded into 15-cm^2 cell culture dishes and was incubated with 10 ng/ml phorbol 12-myristate 13-acetate (PMA) and 0.5 μg/ml ionomycin (Sigma-Aldrich), 10 μg/ml concanavalin A (Sigma-Aldrich), or 3 μg/ml lipopolysaccharides (LPS from *Escherichia coli* 0111:B4, Sigma-Aldrich). Every day, cell proliferation was analyzed by carboxyfluorescein succinimidyl ester (CFSE) dilution assay and flow cytometry. The upregulation of IFN-γ was monitored by quantitative real-time RT-PCR using the primers ACTGGACAGA GAGAAATGAGAA and GTCGTTCATCGGGAGCTTGG. For normalization, the GAPDH primers GAAGGCTGGGGC TCATCTGAA and ATCATACTTGGCTGGTTTCTCC were used at an annealing temperature of 62 °C. After 3 days, stimulated cells were harvested and stored at −20 °C until the purification of 20S proteasome (see above).

Proteasome activity assay

Five hundred nanogram of purified chicken proteasome was incubated with a fluorogenic peptide substrate at 37 °C, and fluorescence was measured as an indication of proteolytic cleavage at 360 nm excitation and 465 nm emission for the fluorophore 7-amino-4-methylcoumarin (AMC) and at 340 nm excitation and 405 nm emission for the fluorophore β-naphthylamide (βNA). Fluorogenic substrates (Suc-LLVY-AMC, Bz-VGR-AMC, Ac-YVAD-AMC, Z-LLE-βNA; Bachem) were dissolved in DMSO and used at a final concentration of 100 μM. Activity assays were performed as previously described (Basler and Groettrup 2012). Using the proteasome inhibitor MG-132, proteasome and inhibitor were preincubated for 30 min at 37 °C before adding the respective fluorogenic substrates. Fluorescence signals are given as signal above the autofluorescence of each substrate. Data are shown as mean±SD of triplicates or two experiments performed in triplicate.

Results

Identification of chicken proteasome subunits in different organs or cell types

Purified 20S proteasome from the 18-month-old chicken brain and spleen was separated by 2D non-equilibrium pH gradient gel electrophoresis (NEPHGE)-SDS-PAGE, and protein spots were visualized by Coomassie staining. As shown in the 2D gel in Fig. 1a, the different chicken proteasomes possess high similarity in the numbers and migratory positions of protein spots and the proteasome subunits are distributed over the same isoelectric point (pI) as for human and murine

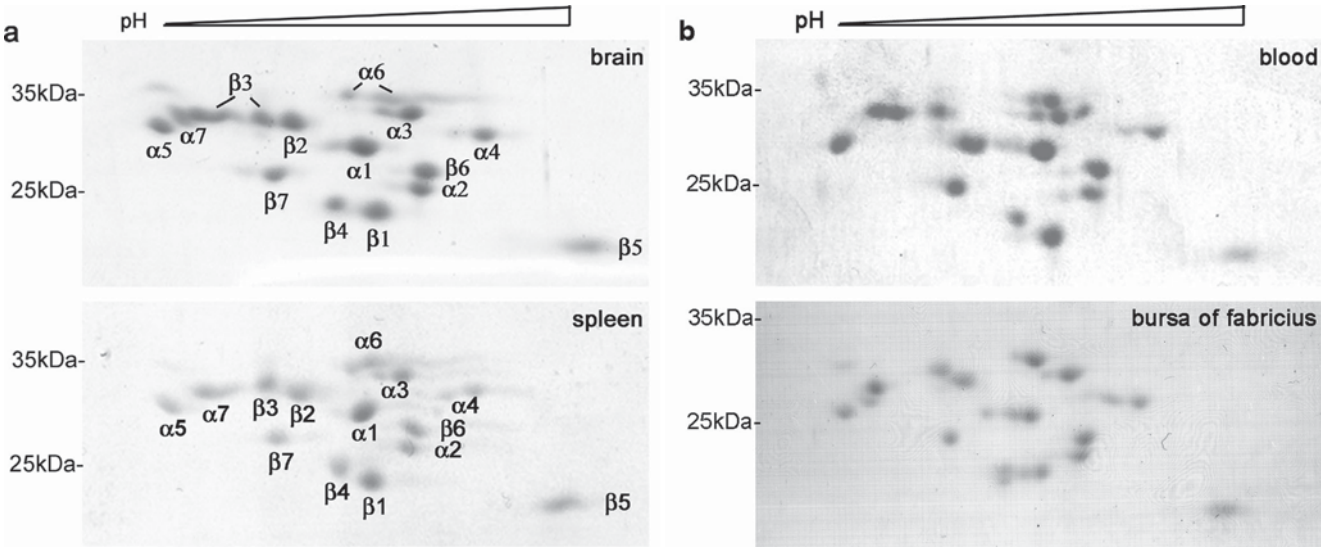

Fig. 1 Two dimensional gel electrophoresis and mass spectrometric analysis of purified chicken 20S proteasomes. 20S proteasome purified from the 18 month old chicken **a** brain and spleen and **b** blood and 7 week old bursa of Fabricius as indicated were separated by two dimensional non equilibrium pH gradient gel electrophoresis (NEPHGE)/SDS PAGE, and proteasome subunits were visualized by Coomassie blue staining. **a** For the identification of the subunits, 18 visible spots were excised and analyzed by nano LC ESI MS/MS. Some subunits were identified in two nearby spots, e.g., α3, α6, α4, and β3 which might be a sign of post translational modifications changing the migratory properties. **b** Purifications of 20S proteasome from whole blood and the bursa of Fabricius. In general, 20S proteasome from all four tissues showed similar protein spot patterns

proteasome. In complete brain tissue of the mouse, only constitutive 20S proteasome is visible in Coomassie-stained 2D gels (Kremer et al. 2010). In contrast to the chicken spleen, in the mouse spleen, about equal amounts of constitutive 20S proteasome and 20S immunoproteasome was found and the immunoproteasome subunits β1i (LMP2), β2i (MECL-1), and β5i (LMP7) were readily visible on Coomassie-stained 2D gels and easily distinguished from the constitutive subunits β1, β2, and β5 due to marked differences in molecular mass and/or isoelectric point (Groettrup et al. 1996; Khan et al. 2001; Kremer et al. 2010). To identify the subunits of chicken 20S proteasome from the brain and spleen and to exclude that putative immunosubunits migrated equally as constitutive ones, 18 spots were excised from 2D gels and were analyzed by mass spectrometry. We were able to identify all 14 different subunits of the chicken 20S proteasome including the catalytically active subunits β2 and β5, but not the third catalytic subunit β1 (Table 1 and Fig. 1a). There is no available sequence for chicken β1 in the database; therefore, we first blasted murine β1 versus the chicken ESTs database (PubMed) and identified the cDNA sequence BX933198.1, which has all highly conserved amino acids typical for the catalytically active subunit β1 (Huber et al. 2012). By MS analysis, β5 was identified as hypothetical protein RCJMB04 14i9 (Swiss-Prot database), which is 100 % identical to proteasome subunit alpha type-5 [*Gallus gallus*] (NCBI Reference Sequence: NP 001026578.1; refseq protein database). In Fig. 1a, the 2D gels are depicted with the assignments of the identified proteasome subunits. Not all values for the molecular weights and isoelectric points of the subunits are conforming with the spot distribution in the 2D gel, but this is not unexpected because they were only predicted and not experimentally determined (compare Table 1 to Fig. 1a). All 14 identified proteasome subunits were in line with previously published data of an MS analysis performed with 20S proteasome from chicken skeletal muscle (Hayter et al. 2005). So far, we could conclude that in contrast to the human and the mouse, the brain and spleen from chicken revealed virtually identical protein spot patterns in their 20S proteasomes.

To further address this issue, 2D gel electrophoresis of 20S proteasomes from the chicken whole blood (18 months) and bursa of Fabricius (7 weeks) was performed in order to determine the migratory pattern of these 20S proteasome subunits. Whole blood is expected to mainly contain constitutive proteasome due to the predominance of erythrocytes, while the bursa of Fabricius consists mostly of B cells and therefore is suspected to express immunoproteasome. Spot patterns from both samples showed high similarity, and MS analysis revealed also no difference in protein composition (Fig. 1b). Finally, the 20S proteasome from the thymus and brain of the same 10-week-old chicken was compared in a 2D gel analysis. The brain sample served as an example of constitutive proteasome, and the thymus, which is the place of T cell selection until sexual maturity, is supposed to express mostly immunoproteasome. Here, as well, no differences in

Table 1 Identification of 20S proteasome subunits by mass spectrometry (coverage indicated in percent for brain (top) and spleen (bottom))

Subunit	Accession	Description	MW (kDa)	Calc. pI	Coverage
α1	F1NEQ6	Proteasome subunit alpha type (fragment) [*Gallus gallus*] PSMA6	27.7	6.55	47.8 % 36.6 %
α2	E1C006	Proteasome subunit alpha type [*Gallus gallus*] PSMA2	25.9	7.49	54.3 % 48.7 %
α3	F1NC02	Proteasome subunit alpha type (fragment) [Gallus gallus] PSMA4	29.5	7.33	60.5 % 59.7 %
α4	F1NWM1	Proteasome subunit alpha type 7 (fragment) [*Gallus gallus*] PSMA7	24.3	8.7	58.3 % 59.7 %
α5	Q5ZJX9	Proteasome subunit alpha type [*Gallus gallus]* RCJMB04_14i9/PSMA5	26.4	4.82	63.5 % 61.0 %
α6	F1NFI8	Proteasome subunit alpha type [*Gallus gallus*] PSMA1	29.3	6.54	51.7 % 73.9 %
α7	Q5ZLI2	Proteasome subunit alpha type [*Gallus gallus*] PSMA3	28.5	5.05	21.6 % 27.8 %
β1	BX933198.1	*Gallus gallus* finished cDNA, clone ChEST511c20	27.2	5.91	52.1 % 50.6 %
β2	Q7ZT63	Proteasome subunit beta type [*Gallus gallus*] cpsmb7	29.9	6.4	46.2 % 42.6 %
β3	E1BYW9	Proteasome subunit beta type [*Gallus gallus*] PSMB3	23.1	5.41	40.0 % 36.6 %
β4	F1NZ80	Proteasome subunit beta type (fragment) [*Gallus gallus*] PSMB2	23.7	7.97	58.6 % 58.1 %
β5	P34065	Proteasome subunit beta type 5 (fragment) [*Gallus gallus*] PSMB5	27	8.68	45.3 % 49.2 %
β6	Q6JLB2	Proteasome subunit beta type [*Gallus gallus*] PSMB1	26	6.89	44.7 % 35.9 %
β7	H9L0U6	Proteasome subunit beta type [*Gallus gallus*] PSMB4	28.1	5.97	38.0 % 36.4 %

the spot distribution were observed and this was also confirmed by MS analysis (Fig. 2). This implicates that chicken expresses constitutive proteasome, but apparently does not produce immunoproteasome in lymphoid tissues.

Immune stimulation of chicken spleen cells

Given that immunoproteasome expression can be induced by inflammatory cytokines like IFN-γ and TNF-α, chicken splenocytes were incubated with different stimuli, thereby promoting the immunoproteasome expression if the corresponding genes were present in the avian genome. The induction of IFN-γ was assessed 24 h after stimulation by real-time RT-PCR, and the highest amount of IFN-γ mRNA was recorded in spleen cells treated with PMA/ionomycin and concanavalin A, while LPS had no effect (Fig. 3b). During stimulation, we observed that PMA/ionomycin treatment resulted in cell proliferation as an indication of an ongoing inflammatory response. This was shown by CFSE dilution assay with spleen cells which were analyzed 72 h after stimulation (Fig. 3a).

After 3 days of stimulation with PMA/ionomycin, spleen cells were harvested for 20S proteasome purification and were analyzed by 2D gel electrophoresis in comparison to unstimulated spleen cells. The separation by 2D gel electrophoresis of unstimulated versus stimulated samples displayed the same subunit patterns after Coomassie blue-staining (Fig. 3c). Comparing Fig. 3c to Figs. 1 and 2, this protein pattern is similar to that before described for the bursa of Fabricius, brain, and whole blood cells. Interestingly, a new spot appeared on the 2D gel with the same molecular weight as β5, but a bit less alkaline, which is maybe due to phosphorylation or acetylation (Moiseeva et al. 2013) of the subunit. Taken together, it appears that stimulation of spleen cells with PMA/ionomycin, which induced lymphocyte proliferation, did not provide any evidence for the induction of immunoproteasomes.

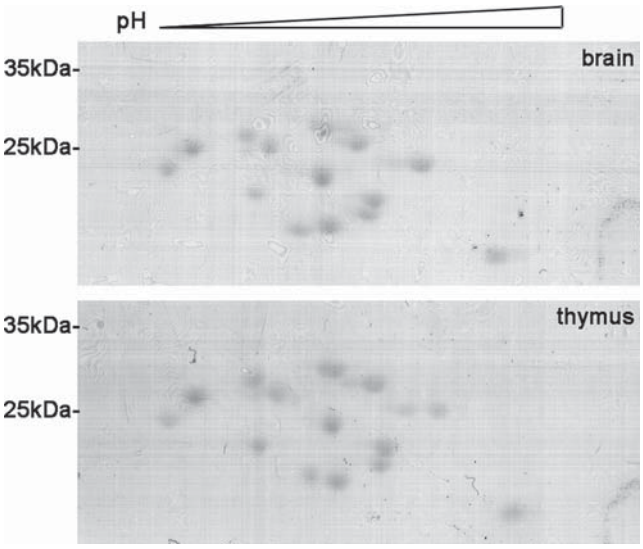

Fig. 2 Two dimensional gel electrophoresis of 20S proteasomes purified from the chicken brain and thymus. The brain and thymus were taken from the same 10 week old chicken, and purified 20S proteasome was separated by NEPHGE/SDS PAGE. The protein spots were visualized by Coomassie blue staining

Proteolytic activity of chicken proteasome from different organs and cell types

Typically, the constitutive proteasome in humans and mice has three catalytically active subunits, which possess different cleavage properties, in particular, trypsin-, caspase-, and chymotrypsin-like activities. To compare the proteolytic cleavage of all purified 20S proteasomes from the different chicken tissues, the respective 20S proteasomes were incubated with the fluorogenic substrates Suc-LLVY-AMC, Bz-VGR-AMC, Ac-YVAD-AMC, and Z-LLE-βNA, to follow chymotrypsin-, trypsin-, and caspase-like activities, respectively, by measuring the fluorescence of the free leaving groups AMC and βNA. In general, the chicken proteasomes from different organs possessed all the three tested proteolytic activities to a similar extent (Fig. 4a). Notably, for the whole blood 20S proteasome, the trypsin-like activity was nearly twice as high compared to the other proteasomes and proteolytic activities. The chymotrypsin- and caspase-like activities were similar between proteasomes from the brain, whole blood, bursa of Fabricius, spleen, or thymus, while the human 20S immunoproteasome purified from the T cell lymphoblastoid line LCL721-145 (Salter and Cresswell 1986), which was tested in parallel, showed a strongly reduced caspase-like activity as would be expected for the replacement of β1 with β1i (LMP2) (Khan et al. 2001). In contrary, 20S constitutive proteasome from the β1i- and β5i-deficient T cell lymphoblastoid line LCL721-174 (Salter and Cresswell 1986) showed very high caspase-like cleavage activity.

To further characterize the five different chicken proteasomes, we used the well-known broad spectrum proteasome inhibitor MG-132 which inhibits all activities of the 20S proteasome. At a concentration of 10 μM, MG-132 led to a marked decrease of the cleavage of Suc-LLVY-AMC substrate by human constitutive and immunoproteasome and

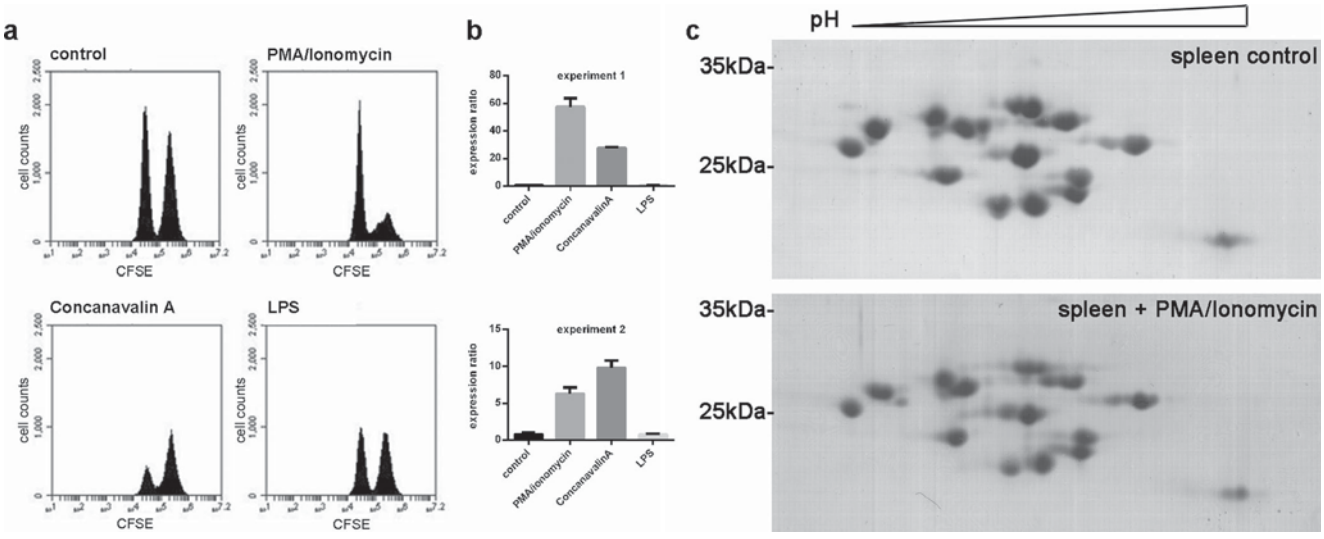

Fig. 3 Stimulation of a chicken spleen single cell suspension with PMA/ionomycin, concanavalin A and lipopolysaccharide (LPS). **a** Proliferation of spleen cells was investigated by CFSE dilution assay 48 h after adding the indicated stimuli. **b** The induction of INF γ mRNA was analyzed 24 h after stimulation of spleen cells. Data are shown as mean±SD of tripli cates normalized to GAPDH from two different experiments. **c** Two dimensional gel electrophoresis of 20S proteasomes purified from the chicken spleen single cell suspension either left unstimulated or stimulated. Seven spleens were dissected from 7 week old chicken, followed by the preparation of single cell suspensions. One half of the cells was not stimulated and was immediately frozen. The other half was seeded for stimulation with 10 ng/ml PMA and 0.5 μg/ml ionomycin and was incubated for 3 days at 37 °C. Proteasome subunits were separated by NEPHGE/SDS PAGE and were visualized by Coomassie blue staining

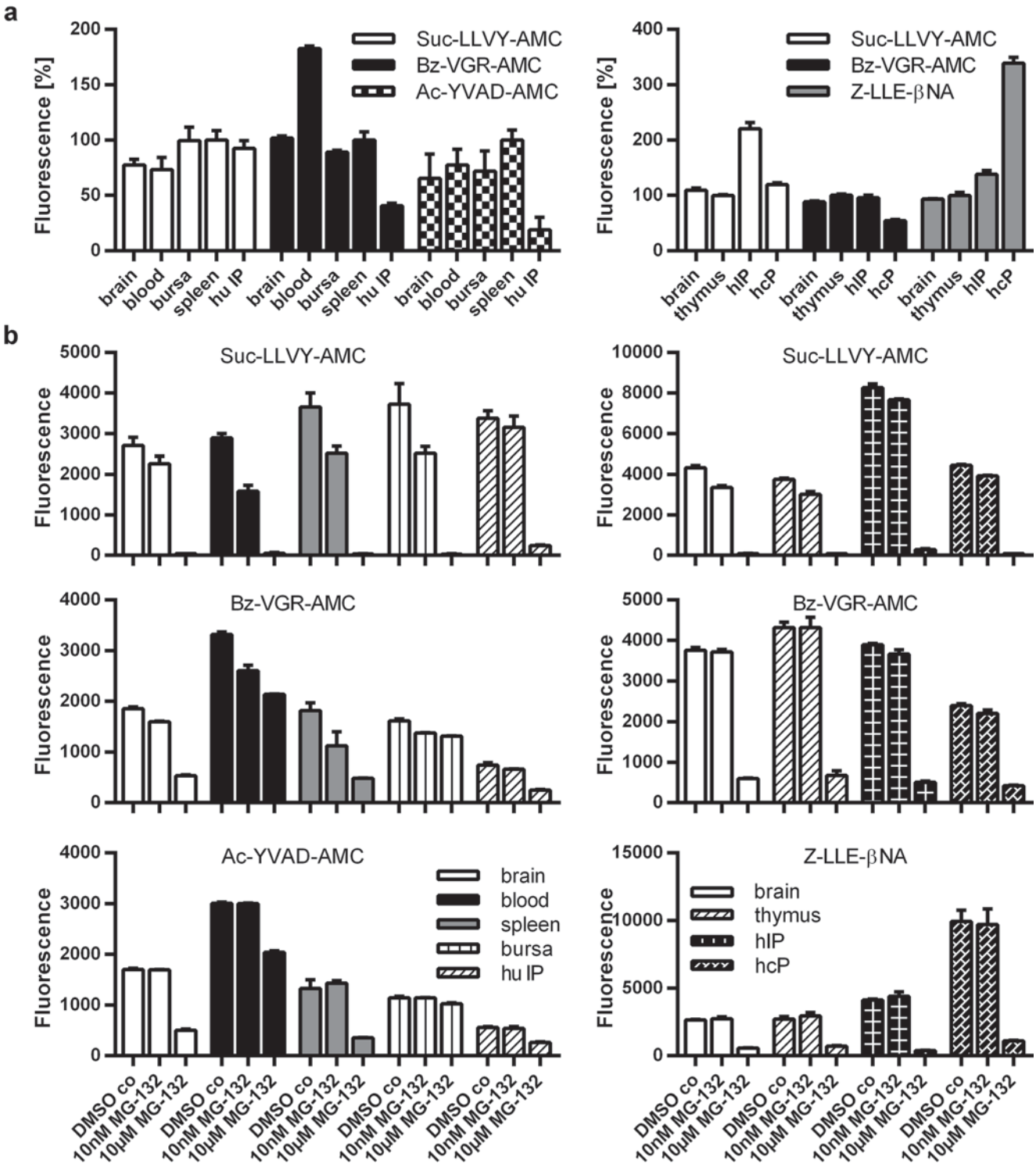

Fig. 4 Proteolytic activity of chicken 20S proteasomes purified from the brain, whole blood, bursa of Fabricius, spleen, and thymus in comparison to human immunoproteasome (*hIP*) and human constitutive proteasome (*hcP*). **a** 20S proteasomes were incubated for 1 h at 37 °C with the fluorogenic peptide substrates Suc LLVY AMC, Bz VGR AMC, Ac YVAD AMC, and Z LLE βNA to follow their chymotrypsin , trypsin , and caspase like cleavage properties, respectively. The substrate hydrolysis was measured in triplicate; fluorescence is depicted as signal above background (substrate without proteasome) and normalized to spleen (*left panel*) or thymus (*right panel*). **b** The experiment was performed as described in (**a**), but before adding the substrates, 20S proteasomes were incubated for 30 min with the indicated concentrations of the proteasome inhibitor MG 132. The *upper panel* shows chymotrypsin like activity, the *middle panel* trypsin like activity, and in the *lower panel*, the caspase like cleavage activity is depicted. The assay was performed in triplicate and fluorescence signals are given as signal above background. Data are shown as mean±SD of triplicates

chicken proteasomes, while the inhibition of the cleavage of Bz-VGR-AMC, Ac-YVAD-AMC, and Z-LLE-βNA substrates was somewhat less prominent (Fig. 4b).

Discussion

In jawed vertebrates, the immunoproteasome is expressed constitutively in lymphocytes and monocytes or can be induced by IFN-γ treatment. Together with MHC- and T/B cell receptor-based adaptive immunity, the immunoproteasome evolved and seemed to disappear in the lineage of aves (Sutoh et al. 2012). With the aim of investigating the presence of the constitutive and immunoproteasome in chicken, we purified 20S proteasomes from different lymphoid and non-lymphoid organs. Furthermore, the subunits of these proteasomes were separated by 2D gel electrophoresis and the protein spots from proteasome purified from the brain and spleen were identified by mass spectrometry. All spots between 20 and 30 kDa were matched to proteasomal subunits, indicating the purity of the preparations, and altogether, 14 subunits were identified in chicken as already found in humans, mice, and rats (Claverol et al. 2002; Froment et al. 2005; Schmidt et al. 2006). Actually, the number of excised spots exceeded that of the proteasome subunits by 4, indicating that some proteins might be post-translationally modified, e.g., by glycosylation, phosphorylation, acetylation, or deamination and therefore have different properties, e.g., a different isoelectric point. This was confirmed by the finding that some subunits were found in two juxtaposed protein spots, e.g., α3, α6, α4, and β3. The identification of all 14 subunits and also the organization of protein spots on the 2D gel, which depends on the molecular weight and isoelectric point, are in line with the publication of Hayter et al. (2005). They showed the 2D gel analysis of 20S proteasome purified from chicken skeletal muscle and also identified the proteasomal subunits by a combination of MALDI-TOF peptide mass fingerprinting and de novo sequencing by MS/MS analysis, but had problems with the assignment because the chicken genome sequences for the proteasomal subunits were not available at that time (Hayter et al. 2005). We found that the protein spot pattern of 20S proteasome purified from the brain was similar to that of the spleen, although it is expected that constitutive proteasome is found in the brain and immunoproteasome in the spleen, which can be easily detected due to obvious and consistent differences in the spot arrangement in the 2D gel (Groettrup et al. 1996). After exhaustive MS analysis of deviant protein spots, we concluded that neither for the brain and spleen nor for the whole blood, bursa of Fabricius, and thymus could any difference be detected, indicating that immunoproteasome is not expressed in chicken.

To further challenge the proposed lack of immunoproteasomes in chicken, spleen cells were stimulated with PMA/ionomycin aiming at the induction of immunoproteasome. For chicken erythrocytes, thrombocytes as well as bone-marrow-derived DCs, macrophages, and monocyte-derived macrophages, it was already shown that they react to immune stimuli by, e.g., up- or downregulation of chemokine receptors and induction of IFN-γ (Morera et al. 2011; St Paul et al. 2012a; St Paul et al. 2012b; St Paul et al. 2012c; Wu and Kaiser 2011). We could confirm that chicken spleen cells incubated with PMA/ionomycin and concanavalin A showed an induction of IFN-γ mRNA and the stimulation was further documented by a strong cell proliferation after PMA/ionomycin treatment. Despite the proliferation of the spleen cells which is a reliable sign for lymphocyte activation, the subunit composition of 20S proteasome purified from stimulated cells looked almost identical to that of unstimulated cells.

Since we could not observe any reasonable hint for the presence of immunoproteasome in chicken by 2D gel analysis, our next approach was to check the catalytic activities of the different proteasomes. Human and murine constitutive proteasome has three different proteolytic sites for chymotrypsin-, trypsin-, and caspase-like activities. Upon the induction of the immunoproteasome, the proteolytic properties change and thereby the caspase-like activity is dramatically decreased. The change of the cleavage properties was followed by the use of three fluorogenic proteasomal substrates with hydrophobic (Suc-LLVY-AMC), basic (Bz-VGR-AMC), and acidic (Ac-YVAD-AMC, Z-LLE-βNA) amino acid residues in the P1 position. The chymotrypsin-like cleavage activity examined by the substrate Suc-LLVY-AMC showed no significant change between chicken proteasomes from lymphoid and non-lymphoid tissues, and the same was observed for the caspase-like activity (Ac-YVAD-AMC, Z-LLE-βNA). Since the caspase-like activity is strongly downregulated upon the replacement of constitutive proteasomes by immunoproteasomes in the mouse and human (Groettrup et al. 1996), the absence of such a change when comparing proteasome from the chicken brain and spleen already strongly suggests that no immunoproteasome is present in the chicken spleen, bursa of Fabricius, or thymus. After the duck genome was reported last year, we blasted the available sequences for the immunosubunits β1i, β2i, and β5i as well as the thymus-specific subunit β5t, but these subunits were not detectable as already reported for Galliformes and zebra finch (Huang et al. 2013).

In humans and mice, the MHC class I molecule presents with a higher affinity for short peptides with hydrophobic or sometimes basic amino acid side chains at the carboxyl terminus. The production of such peptides is additionally increased through the induction of the immunoproteasome, implicating an important role of the immunoproteasome in the cytotoxic T

lymphocyte responses. Consistently, chicken which lack the immunoproteasome in their genome and on protein level possesses MHC class I molecules, which accommodate peptides with acidic C-terminal anchor residues. One could hence argue that in chicken, the caspase-like activity of constitutive proteasomes does not need to be downregulated for optimal production of MHC class I ligands.

Recently, however, new functions of the immunoproteasome, which are unrelated to antigen processing, have been discovered. The immunoproteasome was shown to support the development of inflammatory T helper cells of the Th1 and Th17 type and to promote the production of pro-inflammatory cytokine responses (Moebius et al. 2010; Muchamuel et al. 2009). The inhibitor ONX0914 (formerly named PR-957) irreversibly blocks the activity of the immunoproteasome subunit β5i in mice and humans. It suppresses the production of pro-inflammatory cytokines and the differentiation of Th1 and Th17 T helper cells while regulatory T cells are promoted and Th2 cells remain unaffected (Kalim et al. 2012; Muchamuel et al. 2009). In different mouse models, the treatment with ONX0914 suppressed autoimmune disease like rheumatoid arthritis, diabetes (Muchamuel et al. 2009), colitis (Basler et al. 2010), systemic lupus erythematosus (Ichikawa et al. 2012), and experimental autoimmune encephalomyelitis (Basler et al. 2014). Additionally, this is affirmed by reports that in humans, mutations in the gene encoding for β5i are associated with autoinflammatory disease (Agarwal et al. 2010; Arima et al. 2011; Kitamura et al. 2011). Apparently, the latter regulatory functions of immunoproteasomes are not required in the chicken immune system.

Another interesting aspect is that the whole lineage of birds seems to lack thymoproteasome, because the thymus-specific subunit β5t is missing in the genome (Sutoh et al. 2012). This 20S thymoproteasome which is only expressed by cortical thymic epithelial cells (cTECs) but not medullary TECs has changed catalytic properties and produces peptides that bind with a lower affinity to MHC class I molecules. The different proteasome species in cTECs and mTECs were proposed to generate differences in the pools of negatively and positively selecting MHC class I ligands needed for efficient T cell selection. Mice with a deletion of the subunit β5t were still able to perform the early selection of T cells but these animals formed a small and immune incompetent repertoire of $CD8^+$ T cells which was reduced in number to 20–30 % and were therefore hypersensitive to influenza virus infections (Nitta et al. 2010). The question arises, how do chickens or birds compensate for the lack of immuno- and thymoproteasome and what does this mean to our understanding of the function of these proteasomes in thymic selection. Clearly, a more detailed study of T cell selection in birds is warranted.

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