ISSN 0015-5497, e-ISSN 1734-9168

Folia Biologica (Kraków), vol. 65 (2017), No 1
https://doi.org/10.3409/fb65_1.09

Expression of FOXJ1 and ITGB4 is Activated upon KLH and LTA Stimulation in the DT40 Cell Line

Aleksandra DUNISLAWSKA, Anna SLAWINSKA, Maria SIWEK*

Accepted February 01, 2017 *Published online April 24, 2017* *Published April 28, 2017*

DUNISLAWSKA A., SLAWINSKA A., SIWEK M. 2017. Expression of FOXJ1 and ITGB4 is activated upon KLH and LTA stimulation in the DT40 cell line. Folia Biologica (Kraków) **65**: 09-18.

The immune response is regulated by multiple genes. In chickens, a panel of genes associated with humoral immune responses against Keyhole limpet hemocyanin (KLH), lipopolysaccharide (LPS) and lipoteichoic acid (LTA) was detected on chromosomes 9, 14, 18 and Z. In this study, we analyzed the transcriptional activity of these genes in the DT40 cell line stimulated with KLH, LPS and LTA antigens *in vitro*. mRNA synthesis of the genes of interest was analyzed at 3, 6, 9, and 24 hours post-treatment, using reverse transcription quantitative PCR (RT-qPCR). The results indicate that KLH and LTA antigens were the strongest stimuli of gene expression, whereas LPS treatment did not activate mRNA synthesis of any of the studied genes. Relative analysis of gene expression showed that KLH and LTA treatment triggered up-regulation of forkhead box J1 (*FOXJ1*) and integrin beta 4 (*ITGB4*) genes at 24 hours post-treatment ($P<0.05$). Western blot analysis confirmed expression of *FOXJ1* and *ITGB4* genes by the DT40 cell line also at the protein level. Finally, the functional relations between LTA stimulation and *FOXJ1* and *ITGB4* genes suggests activation of the TLR signaling pathway, but also involvement of scavenger receptors in LTA recognition. This approach provided additional information on transcriptional activity of the genes selected in the genetic association studies.

Key words: chicken, immune response, gene expression, FOXJ1, ITGB4, DT40.

Aleksandra DUNISLAWSKA, Anna SLAWINSKA, Maria SIWEK, Department of Animal Biochemistry and Biotechnology, The Faculty of Animal Breeding and Biology, UTP University of Science and Technology, Mazowiecka 28, 85-084 Bydgoszcz, Poland.
E-mail: aleksandra.dunislawska@utp.edu.pl,
slawinska@utp.edu.pl
siwek@utp.edu.pl

The avian immune system is comprised of the innate and adaptive defense mechanisms that protect the organism against pathogens. The dissection of the molecular background of the immune responses to the broad spectra of antigens is possible by using different antigens. Lipopolysaccharide (LPS) and lipoteichoic acid (LTA) belong to the pathogen-associated molecular patterns (PAMPs) which represent molecules that are associated with specific classes of the microbes, such as Gram-negative (LPS) or Gram-positive (LTA) bacteria (GINSBURG 2002; HEUMANN & ROGER 2002). The molecular motifs of PAMPs are evolutionarily conserved and are recognized by the pattern recognition receptors (PRR) that are part of the innate immune system (AKIRA *et al.* 2006). Therefore, LPS and LTA can reflect the broad spectrum of environmental antigens that are recognized by the mechanisms of the primary immune responses. One of the most useful antigens that allows to determine the overall immunocompetence in chickens includes Keyhole limpet hemocyanin (KLH), an immunogenic glycoprotein commonly used to generate immune responses. The source of KLH is the hemolymph of *Megathura cranulata*, a marine mollusk inhabiting the coast of California. KLH allows to assess the immune function of an individual that had never been exposed to this antigen before, which helps avoiding false positive results of the primary immune response (HARRIS & MARKL 1999). KLH and LTA are considered Th-2 type antigens due to their ability to activate humoral re-

sponses, whereas LPS is a Th-1 type antigen and triggers cellular responses *in vivo* (PARMENTIER *et al.* 2004).

Over the past decade, numerous studies have been carried out in order to dissect the molecular background of the innate (LTA and LPS antigens) and adaptive (KLH antigen) immune responses. They employed different analytical approaches, depending on the technology available at the time and advancement on the poultry genome. Previous studies ranged from quantitative trait loci (QTL) discovery (SIWEK *et al.* 2003; SIWEK *et al.* 2006) and validation (SIWEK *et al.* 2010; SLAWINSKA *et al.* 2011b), meta- and combined QTL analyses (SLAWINSKA & SIWEK 2013), candidate gene *in silico* studies (SLAWINSKA *et al.* 2011a) through an association study of the single nucleotide polymorphisms (SNPs) within the candidate genes with KLH, LPS and LTA titers (BISCARINI *et al.* 2010; SIWEK *et al.* 2015; VAN DER POEL *et al.* 2011). As a result, SIWEK *et al.* (2015) reported that SNPs located in 19 genes were positively associated with the adaptive immune responses to KLH and innate immune responses to LTA and LPS, indicating that they are quantitative trait nucleotides (QTN). However it was unclear whether the selected genes located on the chicken chromosomes GGA9, 14, 18 and Z were actively expressed to mRNA and proteins to fight the pathogen burden. Therefore, we designed an experiment that aimed at the evaluation of gene expression regulation in the chicken DT40 cell line stimulated *in vitro* with KLH, LPS and LTA antigens.

DT40 is a chicken lymphoid cell line derived from a B-cell lymphoma developed in the bursa of Fabricius of a female Leghorn chicken infected with avian leukosis virus (ALV) (BABA *et al.* 1985; BABA & HUMPHRIES 1984). In comparison to native B cells, the DT40 cell line has been transformed by incorporation of the viral *c-myc* locus (BABA *et al.* 1985). DT40 cells were shown to release low levels of virus, but they do not produce significant amounts of cytoplasmic viral polypeptides (BABA *et al.* 1985). Importantly, DT40 cells synthesize and express surface IgM, which confirms their B-lymphocyte lineage. Phenotypically, DT40 cells are small and not as complex as other lymphoid cell lines, corresponding to the lymphoblastoid phenotype of the less differentiated B cells (BABA *et al.* 1985). Cytogenetically, the DT40 cell line may express different levels of mosaicism (CHANG & DELANY 2004) and variation for telomeric array organization (O'HARE & DELANY 2009). Recently published genome sequence data have provided strong evidence that DT40 is a typical transformed cell line with relatively intact genome structure (MOLNAR *et al.* 2014). Taken together, the DT40 cell line has been established as an important cellular model to study recombination, immunoglobulin diversification, gene function as well as gene expression profiling and signaling functions.

In this study, we used the DT40 cell line as a biological model to validate a panel of immune-related genes in their expression upon KLH, LPS and LTA stimulation. We analyzed the gene expression at mRNA level to screen their transcriptional activity in the stimulated cells. The most significantly expressed genes (*FOXJ1* and *ITGB4*) were also analyzed at the protein level.

Material and Methods

Cell line and stimulation

The DT40 cell line (DSMZ, Braunschweig, Germany) was stimulated *in vitro* with KLH, LPS and LTA antigens. The cell line was propagated using Advanced RPMI 1640 medium (Invitrogen, Carlsbad, CA, US) supplemented with 20% Foetal Bovine Serum (Biological Industries, Beit-Haemek, Israel), with 1mM sodium pyruvate, 2 mM L-glutamine, 4.5 g/L glucose, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µM mercaptoethanol, at 37°C and 5% CO_2 atmosphere. Tissue culture tubes (TPP, Trasadingen, Switzerland) were used for cell propagation. Approximately 16 hours before stimulation, the cells were transferred into low FBS medium (Advanced RPMI 1640 supplemented with 2% FBS, 2 mM L-glutamine and 4.5 g/L glucose) to induce the passive stage and make them more prone for treatment. The total number of 5×10^6 cells, with viability of at least 95%, was seeded on a 6-well plate, at the volume of 2 ml. The DT40 cell line was stimulated with 50 µg/ml of a non-pathogenic antigen KLH (Sigma Aldrich, H7017) and 5 µg/ml of the two environmental antigens: LTA from *Staphylococcus aureus* (Sigma Aldrich, L2515) and LPS from *Escherichia coli* (Sigma Aldrich, L2654). The doses of the antigens were selected based on the literature (CASTRILLO *et al.* 2003; HASTY *et al.* 2006; KOHLI *et al.* 2010; REYNOLDS *et al.* 2012) and tested prior to stimulation. The stimulation was carried out at 37ºC and 5% CO_2 atmosphere for 3, 6, 9 and 24 hours.

RNA extraction and RT-qPCR

The cells were harvested directly post-stimulation (1000xg, 5 min) and the total RNA was isolated (EURx, Gdańsk, Poland). cDNA was synthesized using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific/Fermentas, Vilnius, Lithuania), following the manufacturer's recommendations. Prior to qPCR amplification, cDNA was diluted to 70 ng/µl.

Table 1

Candidate genes associated with the innate and/or adaptive humoral immune response to keyhole limpet hemocyanin (KLH), lipopolysaccharide (LPS) and lipoteichoic acid (LTA)

Gene	Description	Chromosome	Association[1]
EPHB1	Eph receptor B1	9	KLH, LPS
FOXJ1	forkhead box J1	18	KLH, LPS, LTA
GPC1	glypican 1	9	KLH, LTA
ITGB4	integrin, beta 4	18	KLH, LTA
JAK2	Janus kinase 2	Z	KLH, LTA, LPS
JMJD6	jumonji domain containing 6	18	KLH, LTA
KLHL6	kelch-like family member 6	9	KLH, LPS, LTA
MAP2K3	mitogen-activated protein kinase kinase 3	14	LTA
MAP2K4	mitogen-activated protein kinase kinase 4	18	KLH
MAPK8IP3	mitogen-activated protein kinase 8 interacting protein 3	14	KLH, LPS, LTA
PRKCB	protein kinase C, beta	14	KLH, LPS, LTA
SOCS1	suppressor of cytokine signaling 1	14	LPS
ST6GAL1	ST6 beta-galactosamide alpha-2,6-sialyltranferase 1	9	KLH, LPS
TNFRSF13B	tumor necrosis factor receptor superfamily, member 13B	14	LTA
TRAF7	TNF receptor-associated factor 7, E3 ubiquitin protein ligase	14	KLH
UNC13D	unc-13 homolog D	18	KLH

[1]Based on association of the SNP markers with immune response to KLH, LPS and LTA. Table adopted from SIWEK *et al.* 2015.

RT-qPCR reactions were conducted at a total volume of 20 µl that included 1x *HOT FIREPol* EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia), 1µM of each of two primers and 4 µl of diluted cDNA. Table 1 shows the list of genes which were analyzed in this study and their association with the immune responses to KLH, LPS and LTA antigens (SIWEK *et al.* 2015).

Oligonucleotide qPCR primers were designed based on cDNA sequences derived from the Ensembl Genome Browser (http://www.ensembl.org). Primer search was facilitated using PrimerExpress 2.0 software (Applied Biosystems, Carlsbad, CA, USA) based on cDNA sequences. Primers were designed to span exon-exon boundaries to eliminate gDNA amplification. Table 2 presents the sequences of the RT-qPCR primers used in this study.

Thermal cycling and recording of the real-time fluorescence emission spectra was performed in LightCycler 480 (Roche Diagnostics, Basel, Switzerland), using the following thermal programme: 15 min pre-incubation at 95°C, amplification (40 cycles): 10 s denaturation at 95°C, 15 s annealing of the primers at 58°C, 30 s primer extension at 72°C (data acquisition step). Melting curve analysis was performed immediately after the amplification protocol under the following conditions: 5 s denaturation at 95°C, 1 min annealing at 65°C and ramping the temperature rapidly from 98 to 40°C (with a ramp rate of 0.11°C/s and continuous data acquisition). Each RT-qPCR reaction was carried out in three individual biological replicates (well as a replicate) and two technical replicates. Standard curves were performed to assess the efficiency of the RT-qPCR reactions.

Western blot

Total protein was isolated at each time point post-stimulation. DT40 cells were washed with cold PBS, and then centrifuged (8000 rpm, 10 min at 4°C). PBS was removed and RIPA lysis buffer was added. Cell lysis was carried out for 30 min. Protein concentration was assessed using the BCA Protein Assay Kit (Thermo Scientific Pierce, Rockford, IL, USA), according to the manufacturer's recommendations. Prior to electrophoresis, protein concentration was standardized to 30 ng and the samples were thermally denatured at 70°C for 10 min. Electrophoretic separation of the proteins was done with Bolt 4-12% Bis-Tris-Plus Gels (Life Technologies, Carlsbad, CA, USA). After electrophoresis, proteins were transferred from the gel to a membrane in a semi-dry transfer. The membrane was blocked with 1% BSA. Membranes were probed with rabbit polyclonal antibodies recognizing ITGB4 (NBP2-16975, Novus Biologicals, Littleton, USA) and FOXJ1 (LS-C6957, LifeSpan BioSciences, Seattle, USA). The reference protein in both cases was alpha tubulin (11224-1-AP, Proteintech, Chicago,USA). Membranes were then stained with horseradish peroxidase conjugated with the anti-rabbit IgG. Chemiluminescent signal detection was done with G:Box (Syngene, Cambridge, UK).

Table 2

RT-qPCR oligonucleotide assays for the genes of interest analyzed in gene expression study

Gene	Gene ID	Primer sequences (5'→ 3')[1]	Amplicon (bp)
EPHB1	396177	F: TGACCGATGATGACTACAAGTCTGA R: CCACCAGCGAAACAATGAAGA	96
FOXJ1[1]	770009	F: GCAGAACTCCATCCGACACA R: TCTTGAAGGCCCCGTTCATC	140
GPC1	424770	F: GGACATCACCAAGCCAGATATGA R: CGTCACTCGCATCTTGGAAA	119
ITGB4[1]	417374	F: TGCAAGGACAAGATTGGCTG R: GGGTAGTCCTGCTTGGTGTCAT	182
JAK2	374199	F: GCTACAGTGCAGGTCGTAGGAA R: TGGTCTAACCTCTCTTTGTGTTTCTG	100
JMJD6	417355	F: CCGTCACAACTACTGCGAGA R: CAGGACTACGGGCTTGTACG	136
KLHL6	424762	F: GGTTGAAGCCAAATGCATCA R: GCCCCACCCACAACATAAAT	63
MAP2K3	416496	F: CGGCTGTGTGCCGTTTC R: TTGGAATCTTGCTTCTTGTCCAT	62
MAP2K4	417312	F: ATGGCGCCGGAAAGGATA R: CGTCTGAGCGGACGTCATAG	61
MAPK8IP3	426986	F: AAATGGCCCGGGTTCTGA R: TCACGGGAAGCTCTGATCATC	100
PRKCB	416567	F: CGACCACTGCGGCTCACT R: TCATCATGCAGGTGTCACATTTC	68
SOCS1	416630	F: CAGCAGACAATGCAGTTGCA R: CGTGCCGGAGGGTCAAG	55
ST6GAL1	396169	F: AAATCTGCCAACAGCCAAAAA R: GCTGGGCCTGTTGGACAA	64
TNFRSF13B	770275	F: TGTGCGAGTCTGCAGTCTGTAA R: ACACACTGTGCTGCAGCTGAT	86
TRAF7	416555	F: CACACCAGATCAAACCAAAGTCTT R: CATGCTCCACACCCTGAGAGA	64
UNC13D	417369	F: GGTGAAGAGCATGGAGGAAAAT R: GAGATCTCCTATCACCTCCAAAAGG	67
UB[2]	396425	F: GGGATGCAGATCTTCGTGAAA R: CTTGCCAGCAAAGATCAACCTT	147

[1] lower dose of primers (0.75 μM); for the remaining primers, the primer concentration in qPCR reaction was 1 μM;
[2] Ubiquitin C (UB), reference gene (DE BOEVER *et al*. 2008).

Statistical analysis

The main effects of stimulation of the DT40 cell line were estimated using the least square means method implemented in JMP Pro 10.0.2 software (SAS Institute, Cary, NC, USA). Treatment (KLH, LPS, LTA or untreated) and time of the stimulation (3 h, 6 h, 9 h or 24 h) as well as their interaction were fitted in the model. Analyses were performed separately for each treatment using dCt values (Ct target – Ct reference). The relative quantification analysis of RT-qPCR data was performed by calculating fold induction of the genes using the ddCt method (LIVAK & SCHMITTGEN 2001) and ubiquitin as a reference gene. The significance of the expression data was determined with Student's *t*-test ($P<0.05$) using JMP Pro 10.0.2 software (SAS Institute, Cary, NC, USA). The automated web service BioGraph (LIEKENS *et al.* 2011) was used to determine the functional relations between the gene expression data of the target genes and the biomedical literature sources.

Results

The significance of treatment (KLH, LPS, LTA or untreated control) and/or time of stimulation (3, 6, 9 or 24 hours) as the main effects influencing gene expression in the DT40 cell line are presented in Table 3. Briefly, mRNA expression of six genes (out of 16) was influenced by the time of stimulation (*EPHB1*, *FOXJ1*, *ITGB4*, *PRKCB*, *ST6GAL1* and *UNC13D*) and six genes – by the applied stimuli (*FOXJ1*, *ITGB4*, *JAK2*, *JMJD6*, *MAPK8IP3* and *PRKCB*) ($P<0.05$). Expression of two genes (*FOXJ1* and *MAPK8IP3*) was affected by time and treatment interaction.

Table 3

Significance of the effect of time of stimulation, type of antigen and their interaction on the gene expression regulation in chicken DT40 cell line stimulated by KLH, LPS or LTA *in vitro*

Gene	Time	Treatment	Time x Treatment
EPHB1	0.0111	ns	ns
FOXJ1	<0.0001	0.0147	0.0199
GPC1	ns	ns	ns
ITGB4	<0.0001	0.0060	ns
JAK2	ns	0.0029	ns
JMJD6	ns	0.0010	ns
KLHL6	ns	ns	ns
MAP2K3	ns	ns	ns
MAP2K4	ns	ns	ns
MAPK8IP3	ns	0.0025	0.0008
PRKCB	<0.0001	0.0043	ns
SOCS1	ns	ns	ns
ST6GAL1	0.0040	ns	ns
TNFRSF13B	ns	ns	ns
TRAF7	ns	ns	ns
UNC13D	0.0389	ns	ns

Significance of the effects calculated using least square means method implemented in the JMP Pro 10.0.2 software (SAS Institute, Cary, NC, USA). Significance threshold at $P<0.05$; ns (not significant) $P<0.05$ Time: duration of the stimulation (3 h, 6 h, 9 h or 24 h); Treatment: KLH (50 μg/ml), LPS (5 μg/ml) or LTA (5 μg/ml).

Comparison of the fold induction between control and treatment groups at each time point showed that there were no differences in the mRNA expression level in most of the genes, except *MAPK8IP3* at 6 and 24 hours, *FOXJ1* at 24 hours and *ITGB4* at 24 hours ($P<0.05$). *FOXJ1* and *ITGB4* had the highest values of fold induction, therefore, their expression was analyzed also at the protein level. Figures 1 and 2 present changes in gene expression at mRNA level upon stimulation with KLH (Fig. 1) and LTA (Fig. 2). LPS did not regulate mRNA expression of the analyzed genes ($P<0.05$). Western blot analysis of ITGB4 and FOXJ1 proteins confirmed their expression at the protein level after stimulation with LTA and KLH antigens (Fig. 3). Modification of FOXJ1 and ITGB4 expression is detectable during all time points of LTA stimulation. In case of KLH stimulation, expression of the FOXJ1 protein changed after 9 hours of treatment. KLH and LTA antigens did not activate gene expression at the same time at mRNA and protein levels.

Discussion

In this study we analyzed the expression of 16 genes associated with the humoral immune responses against KLH (SpAb), LPS (NAb) and LTA (NAb) in chickens. We used the DT40 cell line to analyze the direct impact of the KLH, LPS and LTA antigens on gene activation in immune cells. In this model, stimulation with KLH and LTA antigens significantly up-regulated mRNA expression of two genes: forkhead box J1 (*FOXJ1*) and integrin beta 4 (*ITGB4*), 24 hours post-stimulation.

LTA is a PAMP derived from Gram-positive bacteria found in *Lactobacillus*, *Streptococcus* and *Staphylococcus* strains, which often interacts with the intestinal immune cells located in the gut-associated lymphoid tissue (GALT). Chickens, like other vertebrates, develop a complicated microbiome during their lifespan, which modulates their immune responses (SEKIROV *et al.* 2010). Bursal lymphoma, the source of the DT40 cell line, was generated in the bursa of Fabricius, an organ located in close proximity to the intestinal microbiota and GALT. Therefore, bursal lymphocytes used in this study were likely to have encountered LTA antigens in the past and have developed sensitive recognizing mechanisms. In animals, LTA is a molecule recognized by evolutionarily conserved Toll-like receptors (TLR), which are expressed by B cells in addition to B cell receptors (BCR). These receptors allow B lymphocytes to integrate both innate and adaptive immune responses (RAWLINGS *et al.* 2012). The DT40 cell line was previously reported to express high levels of TLRs (TLR1 and TLR 2 in particular, involved in LTA recognition) (KOGUT *et al.* 2005) which explains the reaction of cells to the lower dose of the antigen (5 ug/ml LTA vs. 50 ug/ml KLH).

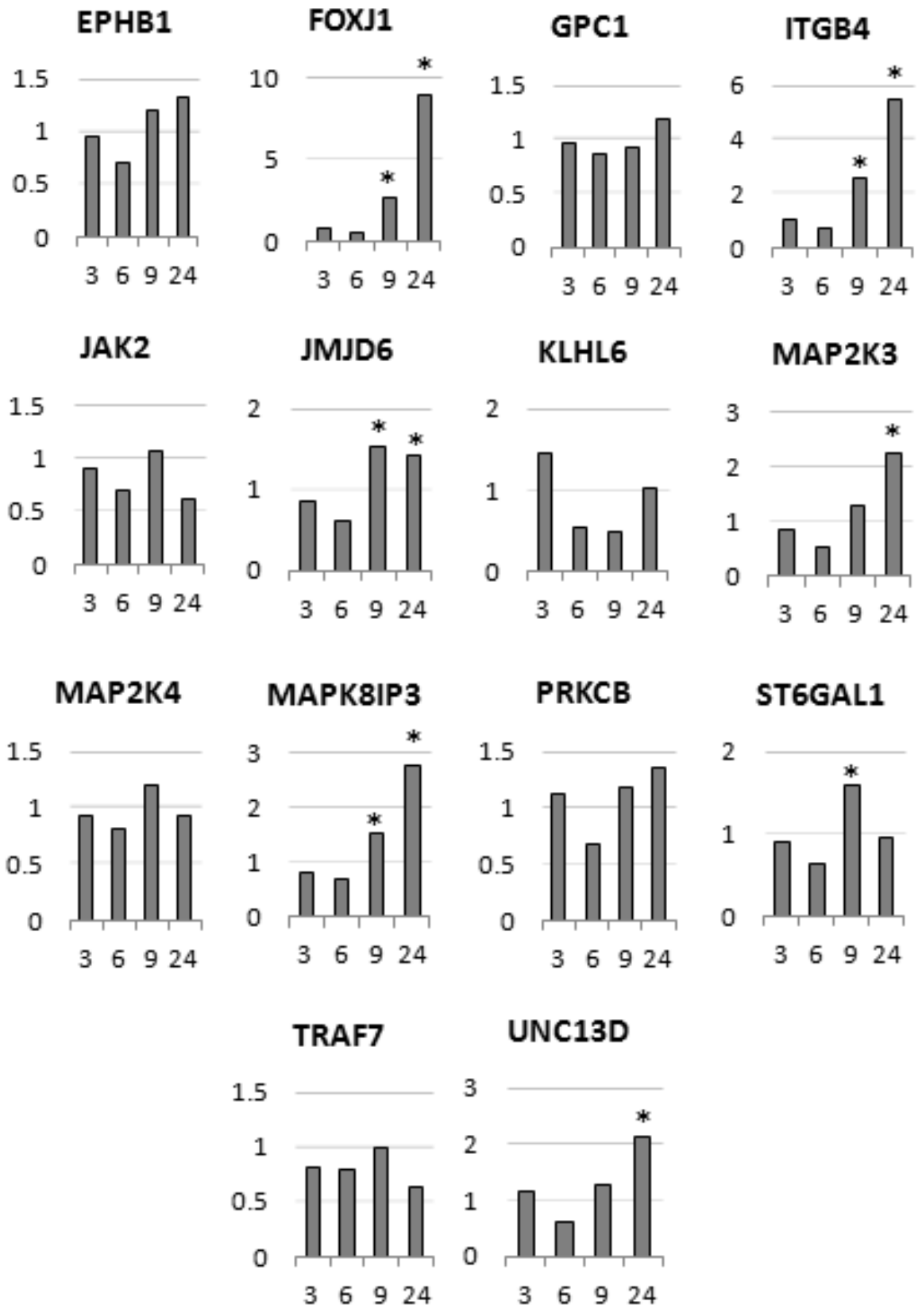

Fig. 1. Gene expression at the mRNA level. Relative gene expression of genes in the DT40 cell line stimulated with KLH (50 μg/ml) at mRNA level. mRNA gene expression was analyzed with the RT-qPCR method and fold induction was calculated with the ddCt method (LIVAK & SCHMITTGEN 2001) using ubiquitin C (UB) as a reference gene. Graphs (n=3) represent fold induction in arbitrary units. Statistical analysis was performed by comparing the significance of the time of stimulation with the control group by Student's *t*-test; * for $P<0.05$.

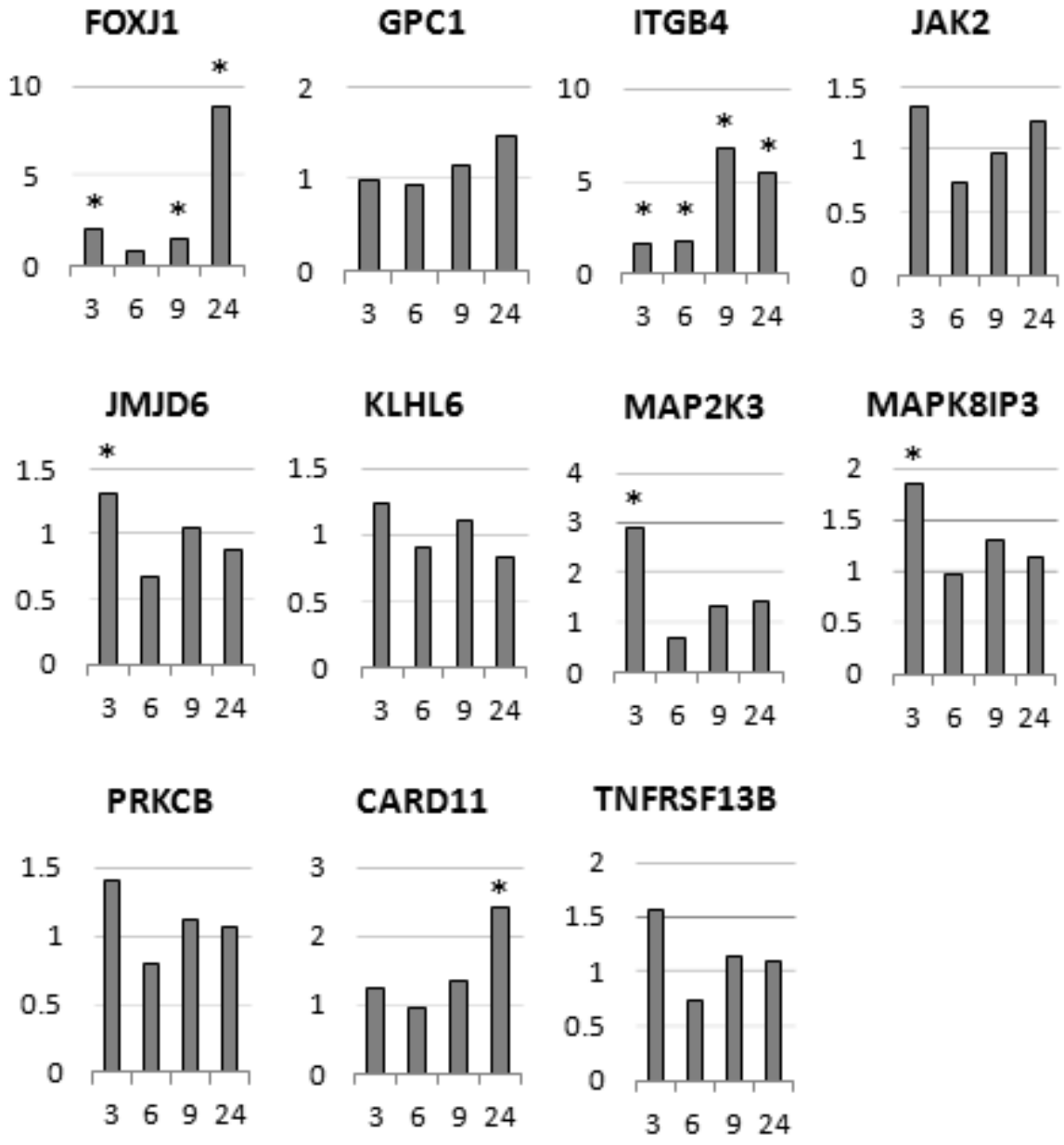

Fig. 2. Gene expression at the mRNA level. Relative gene expression of genes in the DT40 cell line stimulated with LTA (5 µg/ml) at mRNA level. mRNA gene expression was analyzed with the RT-qPCR method and fold induction was calculated with the ddCt method (LIVAK & SCHMITTGEN 2001) using ubiquitin C (UB) as a reference gene. Graphs (n=3) represent fold induction in arbitrary units. Statistical analysis was performed by comparing the significance of the time of stimulation with the control group by Student's *t*-test; * for $P<0.05$.

On the contrary, KLH antigen is derived from a marine organism, geographically and ecologically distant from the environment of chickens. NOSSAL and RIEDEL (1989) reported that only 1% of the total splenic B cell repertoire could produce IgG1 with KLH binding capability prior to KLH immunization. The proportion of KLH-competent B cells changed after immunization of the mice with KLH, which resulted in the sudden emergence of B lymphocytes that could form clones excreting anti-KLH IgG1. Similarly, HERSH and DYRE (1974) studied human peripheral blood leukocytes and KLH-stimulated cultured lymphocytes and concluded that only 1-13% of unimmunized lymphocytes and 0-80% of the immunized ones possessed the ability to bind KLH.

FOXJ1 belongs to the forkhead box (FOX) transcription factor gene family that plays a role in developmental, metabolic and immunoregulatory processes (COFFER & BURGERING 2004; JONSSON & PENG 2005). With respect to immune functions, expression of *FOXJ1* was found in the naïve T cells,

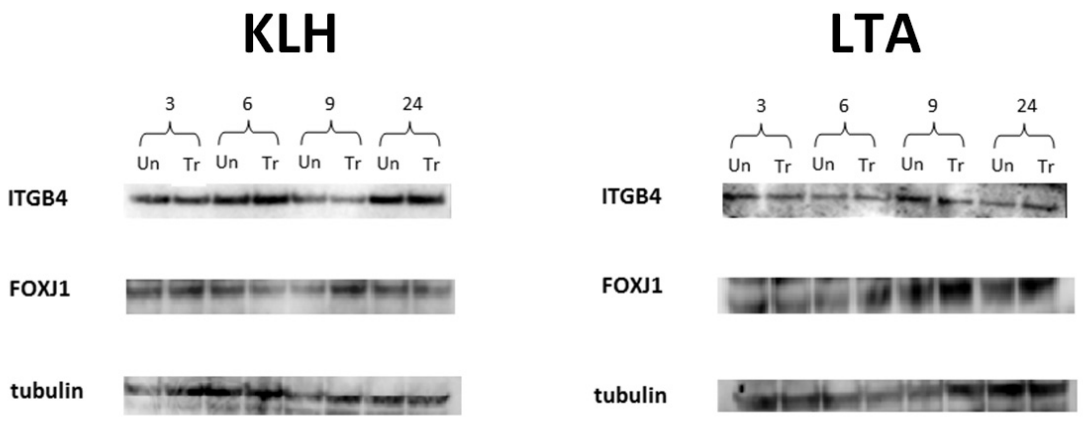

Fig. 3. *FOXJ1* and *ITGB4* expression at the protein level. Relative gene expression of *FOXJ1* and *ITGB4* in the DT40 cell line stimulated with KLH (50 µg/ml) and LTA (5 µg/ml) at the protein level. Protein analysis was based on the Western blot method using tubulin as a reference protein. Samples treated (Tr) with 50 µg/ml of KLH or 5 µg/ml of LTA antigens were compared to untreated (Un) protein at each time point – 3, 6, 9 and 24 hours.

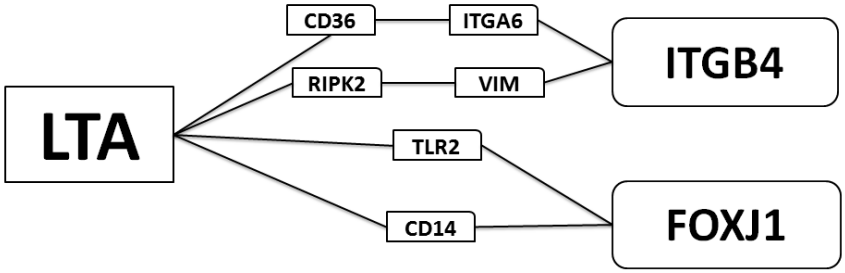

Fig. 4. Functional analysis of downstream signaling pathway leading to transcription activation of *ITGB4* and *FOXJ1* genes in the DT40 cell line stimulated with LTA. Adapted from BioGraph (LIEKENS *et al.* 2011).

where it prevents T-cells from spontaneous activation by inhibiting the NF-kB pathway (LIN *et al.* 2004). The FOXJ1 protein also has a critical regulatory role in B cells in which it inhibits autoimmune humoral responses through inhibition of NF-kB pathway genes (LIN *et al.* 2004). In this study, the expression of *FOXJ1* was robustly stimulated by LTA and KLH antigens, indicates its role in humoral responses to Th-2 type antigens. Analysis of the functional relations between the gene and the stimuli (Fig. 4) indicated that the expression of *FOXJ1* is activated in response to LTA through cluster of differentiation 14 (CD14) and TLR2. This suggests the role of *FOXJ1* in innate immunity mediated through PRRs.

ITGB4 is an integrin, a group of heterodimer transmembrane receptors that provide bidirectional signaling between adherent cells or extracellular matrix and the cytoskeleton (HYNES 2002; TAKADA *et al.* 2007). Integrins consist of non-covalently linked α (extracellular) and β (intracellular) subunits. Intracellular activation (inside-out signaling) is triggered through G-protein-coupled receptor, whereas binding of the external ligands, such as laminin or collagen, triggers an outside-in signaling route. The *ITGB4* gene encodes the β4 cytoplasmic subunit, which in humans is usually linked with the α6 subunit and binds laminin. LIU *et al.* (2012) reported that impaired expression of *ITGB4* is associated with down-regulation of the MHCII and inhibition of antigen presentation in the human bronchial epithelial cell line. Furthermore, integrins are involved in B-1 cell migration *in vivo*, directly coordinated by TLR signaling (HA *et al*. 2006). Our study also proved that *ITGB4* is involved in the immune response to LTA and KLH

antigens, 24 hours post-treatment, suggesting that it is a late-expressed gene. Functional relations analysis between LTA treatment and *ITGB4* expression (Fig. 4) indicated that the activation of *ITGB4* is mediated by induction of the *CD36* molecule and integrin alpha 6 (*ITGA6*). Both integrins and the CD36 molecule were reported to take part in cytoadherence (DAVIS *et al.* 2013). CD36 belongs to the scavenger receptors that have evolved with the innate immunity systems as primitive receptors able to recognize specific components of bacterial cell walls (SILVERSTEIN & FEBBRAIO 2009). These findings suggest a hypothesis that there may be another LTA-recognizing innate receptor on B cells as an alternative to TLR signaling. Such evolutionary adaptations for hijacking of an innate immune receptor have been reported in the recently discovered staphylococcal strategy to overcome TLR2 recognition (WATANABE *et al.* 2007).

Finally, the results of Western blot confirmed expression of FOXJ1 and ITGB4 in DT40 cells upon KLH and LTA stimulation. Western blot analysis has been used primarily as a qualitative method, indicating the presence of the analyzed protein in order to validate the results of qPCR. However, activation of protein expression upon KLH and LTA treatment was not as unequivocal as in the case of mRNA. This can be explained by the different mechanisms that regulate mRNA and protein expression. Due to post-transcriptional and post-translational modifications, the protein amount does not necessarily correspond to the amount of mRNA (VOGEL & MARCOTTE 2013).

In conclusion, in this study we developed an *in vitro* model for testing transcriptional activity of genes associated with the innate and adaptive humoral responses towards KLH, LPS and LTA in chicken. The tested panel of 16 genes was selected based on prior investigations of the genetic background of the immune response in chickens. This report presents expression validation of genes previously analyzed at the DNA level. KLH and LTA antigens strongly activated mRNA expression of *FOXJ1* and *ITGB4* genes at 24 hours post-stimulation. Western blot confirmed the expression of these genes at the protein level. Reversely, LPS, was not a potent stimuli of the genes of interest. Finally, this approach provided additional information on transcriptional activity of genes selected in genetic association studies.

Acknowledgements

The authors are thankful to Ewa NIESYN and Soraia VIEIRA for their excellent assistance with laboratory work.

References

AKIRA S., UEMATSU S., TAKEUCHI O. 2006. Pathogen recognition and innate immunity. Cell **124**: 783-801.

BABA T.W., GIROIR B.P., HUMPHRIES E.H. 1985. Cell lines derived from avian lymphomas exhibit two distinct phenotypes. Virology **144**: 139-151.

BABA T.W., HUMPHRIES E.H. 1984. Differential response to avian leukosis virus infection exhibited by two chicken lines. Virology **135**: 181-188.

BISCARINI F., BOVENHUIS H., VAN ARENDONK J.A., PARMENTIER H.K., JUNGERIUS A.P., VAN DER POEL J.J. 2010. Across-line SNP association study of innate and adaptive immune response in laying hens. Anim. Genet. **41**: 26-38.

CASTRILLO A., JOSEPH S.B., VAIDYA S.A., HABERLAND M., FOGELMAN A.M., CHENG G., TONTONOZ P. 2003. Crosstalk between LXR and Toll-like Receptor Signaling Mediates Becterial and Viral Antagonism of Cholesterol Metabolism. Molecular Cell **12**: 805-816.

CHANG H., DELANY ME. 2004. Karyotype stability of the DT40 chicken B cell line: macrochromosome variation and cytogenetic mosaicism. Chromosome Res. **12**: 299-307.

COFFER P.J., BURGERING B.M. 2004. Forkhead-box transcription factors and their role in the immune system. Nat. Rev. Immunol. **4**: 889-899.

DAVIS S.P., LEE K., GILLRIE M.R., ROA L., AMREIN M., HO M. 2013. CD36 recruits alpha(5)beta(1) integrin to promote cytoadherence of P. falciparum-infected erythrocytes. PLoS Pathog. **9**: e1003590.

DE BOEVER S., VANGESTEL P., DE BACKER S., CROUBELS S.U. 2008. Identification and validation of housekeeping genes as internal control for gene expression in an intravenous LPS inflammation model in chickens. Vet. Immunol. Immunop. **122**: 312-317.

GINSBURG I. 2002. Role of lipoteichoic acid in infection and inflammation. The Lancet **2**: 171-179.

HA S.A., TSUJI M., SUZUKI K., MEEK B., YASUDA N., KAISHO T., FAGARASAN S. 2006. Regulation of B1 cell migration by signals through Toll-like receptors. J. Exp. Med. **203**: 2541-2550.

HARRIS J.R., MARKL J. 1999. Keyhole limpet hemocyanin (KLH): a biomedical review. Micron **30**: 597-623.

HASTY D.L., MARON-SUDAI S., COX K.H., NAGORNA T., RUIZ-BUSTOS E., LOSI E., COURTNEY H.S., MAHROUS E.A., LEE R., OFEK I. 2006. Monocyte and Macrophage Activation by Lipoteichoic Acid is Independent of Alanine and is Potentiated by Hemoglobin. J. Immunol. **176**: 5567-5576.

HERSH E.M., DYRE S.E. 1974. Cells binding the antigen keyhole limpet haemocyanin in the peripheral blood and in the lymphocyte cultures of non-immune and immunized human subjects. Clin. Exp. Immunol. **17**: 299-309.

HEUMANN D., ROGER T. 2002. Initial responses to endotoxins and Gram-negative bacteria. Clin. Chim. Acta **323**: 59-72.

*Partially supported by grants NN 311 5586 40 and UMO-2014/13/B/NZ9/02123 from the National Science Centre in Cracow (Poland). The research was performed using equipment purchased within the frame of "Implementation of the 2nd stage of Regional Centre of Innovation", a project co-financed by the European Regional Development Fund under the Regional Operational Program for Kujawsko-Pomorskie Voivodeship for the years 2007-2013.

HYNES R.O. 2002. Integrins: bidirectional, allosteric signaling machines. Cell **110**: 673-687.

JONSSON H., PENG S.L. 2005. Forkhead transcription factors in immunology. Cell. Mol. Life Sci. **62**: 397-409.

KOGUT M.H., IQBAL M., HE H., PHILBIN V., KAISER P., SMITH A. 2005. Expression and function of Toll-like receptors in chicken heterophils. Dev. Comp. Immunol. **29**: 791-807.

KOHLI R.M., MAUL R.W., GUMINSKI A.F., MCCLURE R.L., GAJULA K.S., SARIBASAK H., MCMAHON M.A., SILICIANO R.F., GEARHART P.J., STIVERS J.T. 2010. Local sequence targeting in the AID/APOBEC family differentially impacts retrovirus restriction and antibody diversification. J. Biol. Chem. **285**: 40956-40964.

LIEKENS A.M., DE KNIJF J., DAELEMANS W., GOETHALS B., DE RIJK P., DEL-FAVERO J. 2011. BioGraph: unsupervised biomedical knowledge discovery via automated hypothesis generation. Genome biol. **12**: R57.

LIN L., SPOOR M.S., GERTH A.J., BRODY S.L., PENG S.L. 2004. Modulation of Th1 activation and inflammation by the NF-kappaB repressor Foxj1. Science **303**: 1017-1020.

LIU C., QIN X.Q., XIANG Y., LIU H.J., LIU Y., LIU C.X. 2012. Downregulation of ITGB4 expression on airway epithelial cells regulates the local inflammation response. Regulatory Peptides **177**: S26.

LIVAK K.J., SCHMITTGEN T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods **25**: 402-408.

MOLNAR J., POTI A., PIPEK O., KRZYSTANEK M., KANU N., SWANTON C., TUSNADY G.E., SZALLASI Z., CSABAI I., SZUTS D. 2014. The genome of the chicken DT40 bursal lymphoma cell line. G3 **4**: 2231-2240.

NOSSAL G.J., RIEDEL C. 1989. Sudden appearance of anti-protein IgG1-forming cell precursors early during primary immunization. Proc. Natl. Acad. Sci. U. S. A. **86**: 4679-4683.

O'HARE T.H., DELANY M.E. 2009. Genetic variation exists for telomeric array organization within and among the genomes of normal, immortalized, and transformed chicken systems. Chromosome Res. **17**: 947-964.

PARMENTIER H.K., VAN DEN KIEBOOM W.J., NIEUWLAND M.G., DE VRIES REILINGH G., HANGALAPURA B.N., SAVELKOUL H.F., LAMMERS A. 2004. Differential effects of lipopolysaccharide and lipoteichoic acid on the primary antibody response to keyhole limpet hemocyanin of chickens selected for high or low antibody responses to sheep red blood cells. Poultry Sci. **83**: 1133-1139.

RAWLINGS D.J., SCHWARTZ M.A., JACKSON S.W., MAYER-BAHLBURG A. 2012. Integration of B cell responses through Toll-like receptors and antigen receptors. Nat. Rev. Immunol. **12**: 282-294.

REYNOLDS J.M., MARTINEZ G.J., CHUNG Y., DONG Ch. 2012. Toll-like receptor 4 signaling in T cells promotes autoimmune inflammation. Proc. Natl. Acad. Sci. U. S. A. **109**: 13064-13069.

SEKIROV I., RUSSELL S.L., ANTUNES L.C., FINLAY B.B. 2010. Gut microbiota in health and disease. Physiol. Rev. **90**: 859-904.

SILVERSTEIN R.L., FEBBRAIO M. 2009. CD36, a scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior. Sci. Signal. **2**: re3.

SIWEK M., BUITENHUIS A.J., CORNELISSEN S.J., NIEUWLAND M.G., BOVENHUIS H., CROOIJMANS R.P., GROENEN M.A., DE VRIES-REILINGH G., PARMENTIER H.K., VAN DER POEL J.J. 2003. Detection of different quantitative trait loci for antibody responses to keyhole lympet hemocyanin and Mycobacterium butyricum in two unrelated populations of laying hens. Poultry Sci. **82**: 1845-1852.

SIWEK M., BUITENHUIS B., CORNELISSEN S., NIEUWLAND M., KNOL E.F., CROOIJMANS R., GROENEN M., PARMENTIER H., VAN DER POEL J. 2006. Detection of QTL for innate: non-specific antibody levels binding LPS and LTA in two independent populations of laying hens. Dev. Comp. Immunol. **30**: 659-666.

SIWEK M., SLAWINSKA A., NIEUWLAND M., WITKOWSKI A., ZIEBA G., MINOZZI G., KNOL E.F., BEDNARCZYK M. 2010. A quantitative trait locus for a primary antibody response to keyhole limpet hemocyanin on chicken chromosome 14-confirmation and candidate gene approach. Poultry Sci. **89**: 1850-1857.

SIWEK M., SLAWINSKA A., RYDZANICZ M., WESOLY J., FRASZCZAK M., SUCHOCKI T., SKIBA K., SZYDA J. 2015. Identification of candidate genes and mutations in QTL regions for immune responses in chicken. Anim. Genet. **46**: 247-254.

SLAWINSKA A., SIWEK M. 2013. Meta – and combined – QTL analysis of different experiments on immune traits in chickens. J. Appl. Genet. **54**: 483-487.

SLAWINSKA A., WITKOWSKI A., BEDNARCZYK M., SIWEK M. 2011a. In silico analysis of candidate genes associated with humoral innate immune response in chicken. BMC Proc. **5**: Suppl 4, S36.

SLAWINSKA A., WITKOWSKI A., NIEUWLAND M., MINOZZI G., BEDNARCZYK M., SIWEK M. 2011b. Quantitative trait loci associated with the humoral innate immune response in chickens were confirmed in a cross between Green-Legged Partridgelike and White Leghorn. Poultry Sci. **90**: 1909-1915.

TAKADA Y., YE X., SIMON S. 2007. The integrins. Genome Biol. **8**: 215.

VAN DER POEL J.J., BISCARINI F., RODENBURG B.T., VAN ARENDONK J.A., PARMENTIER H.K., JUNGERIUS A.P., BOVENHUIS H. 2011. Across-line SNP association study for (innate) immune and behavioral traits in laying hens. BMC Proc. **5**: Suppl 4, S18.

WATANABE I., ICHIKI M., SHIRATSCHU A., NAKANISHI Y. 2007. TLR2-mediated survival of Staphylococcus aureus in macrophages: a novel bacterial strategy against host innate immunity. J. Immunol. **178**: 4917-4925.

VOGEL Ch., MARCOTTE E.M. 2013. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nat. Rev. Genet. **13**: 227-232.