Toll-like receptor ligand-dependent inflammatory responses in chick skeletal muscle myoblasts

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Abbreviations: BPR, Barred Plymouth Rock; FSL-1, fibroblast-stimulating lipopeptide 1; IL, interleukin; LPS, lipopolysaccharide; MHC, myosin heavy chain; MyD88, Myeloid differentiation primary response gene 88; NF-κB, nuclear factor-κB; TLR, Toll-like receptor; TNF-α, tumor necrosis factor α; UKC, UK Chunky; WL, White Leghorn.

ABSTRACT

Toll-like receptors (TLRs) are a group of sensory receptors which are capable of recognizing a microbial invasion and activating innate immune system responses, including inflammatory responses, in both immune and non-immune cells. However, TLR functions in chick myoblasts, which are myogenic precursor cells contributing to skeletal muscle development and growth, have not been studied. Here, we report the expression patterns of TLR genes as well as TLR ligand-dependent transcriptions of interleukin (IL) genes in primary-cultured chick myoblasts. Almost TLR genes were expressed both in layer and broiler myoblasts but *TLR1A* was detected only in embryonic layer chick myoblasts. Chick TLR1/2 ligands, Pam_3CSK_4 and FSL-1, induced inflammatory ILs in both broiler and layer myoblasts but a TLR4 ligand, lipopolysaccharide, scarcely promoted. This is the first report on TLR ligand-dependent inflammatory responses in chick myoblasts, which may provide useful information to chicken breeding and meat production industries.

Keywords: inflammatory response; interleukin; skeletal muscle myoblast; Toll-like receptor.

1. Introduction

Toll-like receptors (TLRs) are sensory receptors which recognize pathogen-associated molecular patterns (PAMPs) produced by microbial components such as nucleotides, lipopeptides, and lipopolysaccharides (LPSs). Recognition of PAMPs by TLRs is essential for the detection of a microbial invasion and the subsequent activation of the immune system. TLRs are expressed in various immune and non-immune cells, where TLR-dependent signaling pathways rapidly produce inflammatory mediators (Kawai and Akira, 2007). Although general functions of TLRs are evolutionally conserved among multi-cellular organisms, the composition of TLR genes is partially different between mammals and aves (Keestra et al., 2013). Humans have ten TLR genes (*TLR1–10*), while mice have twelve (*Tlr1–9* and *Tlr11–13*) (Roach et al., 2005). Chickens have ten TLR genes, *TLR1A*, *TLR1B*, *TLR2A*, *TLR2B*, *TLR3*, *TLR4*, *TLR5*, *TLR7*, *TLR15*, and *TLR21*. *TLR1A, TLR1B*, and *TLR15* are unique to aves. *TLR21* is shared with fish (Temperley et al., 2008). Therefore, an understanding of the expression patterns and functions of chick TLRs may contribute vastly to the improvement of chicken breeding.

Chick TLRs are able to recognize known TLR ligands and initiate inflammatory responses. The synthetic triacylated lipopeptide, Pam_3CSK_4, is a ligand for the mammalian TLR1/2 heterodimer. The synthetic diacylated lipopeptide, fibroblast-stimulating lipopeptide 1 (FSL-1), is an agonist of the mammalian TLR2/6 heterodimer (Tapping and Tobias, 2003; Wetzler, 2003).

In chickens, both Pam_3CSK_4 and FSL-1 are recognized by the TLR1/2 heterodimer, probably because chickens do not have the TLR6 homolog (Keestra et al., 2007; Higuchi et al., 2008). LPS, a major component of the outer membrane of Gram-negative bacteria, is recognized by mammalian and chick TLR4 (Palsson-McDermott and O'Neill, 2004; Keestra and van Putten, 2008). In mammals, TLR4 forms a complex with a coreceptor MD2 and a lipid scavenger protein CD14 (Lee et al., 2012). Chick CD14 is dissimilar to that of mammals (Wu et al., 2009), and its function is still controversial (Keestra et al., 2013). Ligand-binding to chick TLRs, except TLR3, is considered to activate the adaptor molecule, MyD88, for signal transduction (Keestra et al., 2013). In chickens, Pam_3CSK_4, FSL-1, or LPS induces the expression of inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-8, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α in the bursa of Fabricius (Cheng et al., 2014), spleen (Alkie et al., 2017; Li et al., 2017), primary-cultured splenocytes (St. Paul et al., 2013), heterophils (Kogut et al, 2006), thrombocytes (Ferdous and Scott, 2015; Winkler et al., 2017), cecal tonsil cells (Taha-abdelaziz et al., 2016), and the macrophage cell line MQ-NCSU (Barjesteh et al., 2014; Alkie et al., 2017). LPS also induces nuclear factor (NF)-κB, a transcription factor for regulating immune responses, in the bursa of Fabricius (Cheng et al., 2014) but not in thrombocytes (Winkler et al., 2017). In addition to these inflammatory mediators, IL-10, a feedback negative regulator of inflammation, is known to be induced by LPS in macrophage and is detected in serum from the chickens infected with *Eimeria tenella* (Wu et al., 2016; Qi et al., 2017). Infection of

94 *Cryptosporidium baileyi* to chickens, which causes respiratory disorders
95 (Molina-Lopez et al., 2010), induces a pro-inflammatory cytokine, IL-17,
96 likewise IL-1β and IL-6 in trachea and spleen (Zhao et al., 2014).

97 As mentioned previously, TLR ligand-dependent inflammatory
98 responses in chickens have been reported mostly in immune and
99 hematopoietic cells. The expression and function of chick TLRs in
100 non-immune cells have been rarely investigated. *TLR1B*, *TLR2B*, *TLR3*, and
101 *TLR5* expression has been detected in the skeletal muscle tissues of Line 7_2
102 White Leghorn (Iqbal et al., 2005). However, muscle tissue is composed of a
103 variety of cell types including immune cells. Skeletal muscle consists of a
104 large number of myofibers, which are multi-nucleated myocytes. During
105 muscle development and growth, myogenic precursor cells termed myoblasts
106 proliferate and differentiate into myocytes, which then fuse to existing
107 myofibers (Dumont et al., 2015). Therefore, myoblasts play a central role in
108 skeletal muscle formation, which is of importance to meat production.

109 In human myoblasts, *TLR3*, *TLR4*, and *TLR7* are expressed, and the
110 synthetic double-strand RNA as a TLR3 ligand promotes IL-6 expression
111 (Tournadre et al, 2010; Kim et al, 2013). In the murine myoblast cell line
112 C2C12, *Tlr1–7* are expressed, and Pam_3CSK_4 or LPS induces IL-6, TNF-α,
113 and NF-κB (Frost et al., 2002; Frost et al., 2006). These studies strongly
114 suggest that the TLR-dependent pathway in chick myoblasts may be
115 involved in generating inflammatory responses. Acute and chronic
116 inflammatory changes in the breast meat of broiler chickens have been
117 increasingly reported (Kuttappan et al., 2013). However, the pathogenesis of

these serious lesions still remains unclear. In this study, primary-cultured chick myoblasts were utilized to investigate the expression patterns of TLR genes and related ligand-dependent inflammatory responses. A better understanding of TLRs in chick myoblasts may provide interesting insights into skeletal muscle tissue inflammation.

2. Materials and methods

2.1. Isolation, culture, and stimulation of chick myoblasts

All experimental procedures were conducted in accordance with the Regulations for Animal Experimentation of Shinshu University, and the animal protocol was approved by the Committee for Animal Experiments of Shinshu University. Spleens were extirpated from two-day-old chickens. Skeletal muscle myoblasts were isolated from the leg muscles of E10 chick embryos and two-day-old chickens as previously described (Takaya et al., 2017). The myoblasts were cultured on dishes coated with collagen type I-C (Cellmatrix; Nitta Gelatin, Osaka, Japan) at 37°C with 5% CO_2 throughout the experiment. Undifferentiated myoblasts were maintained in a growth medium (GM) consisting of RPMI1640 (Nacalai, Osaka, Japan), 20% fetal bovine serum (FBS) (HyClone; GE Healthcare, UT, USA), 1% non-essential amino acids (Wako, Osaka, Japan), 1% chicken embryo extract (US Biological, MA, USA), and 2 ng/ml basic fibroblast growth factor (Wako). In order to initiate myotube formation, 1.0×10^5 of myoblasts were seeded on 30-mm dishes, then myogenic differentiation was induced by replacing GM with a differentiation medium consisting of DMEM and 2% FBS with TLR ligands on the next day.

Pam_3CSK_4 (tripamitoyl-*S*-(bis(palmitoyloxy)propyl)-Cys-Ser-$(Lys)_3$-Lys; Novus Biologicals, CO, USA), FSL-1 (*S*-(2,3-bispalmitoyloxypropyl)-Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe; Adipogen, CA, USA), or LPS from *Escherichia coli* O55:B5 (L2637;

148 Sigma-Aldrich, MO, USA) were dissolved in sterile water. For the purpose of
149 administrating TLR ligands, 2.0×10^5 of myoblasts in GM were seeded on
150 60-mm dishes and stimulated with Pam_3CSK_4, FSL-1, or LPS on the next
151 day. Equal volume of sterile water was administered as a negative control in
152 each experiment.
153
154 *2.2. RT-PCR*
155 Total RNA of myoblasts and spleens were isolated using TRIzol
156 Reagent (Thermo Fisher Scientific, MA, USA) and reverse transcribed using
157 ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). cDNAs were
158 amplified by PCR (40 cycles), subjected to agarose gel electrophoresis, and
159 stained with ethidium bromide to visualize PCR products. Quantitative
160 real-time RT-PCR (qPCR) was performed using GoTaq qPCR Master Mix
161 (Promega, WI, USA) with StepOne Real-Time PCR System (Thermo Fisher
162 Scientific). The amount of each transcript was normalized to that of 16S
163 ribosomal RNA. The results were expressed as fold-change. Primer
164 sequences are described in Table 1.
165
166 *2.3. Histocytochemistry*
167 For myosin heavy chain (MHC) staining, myoblasts were fixed with
168 2% paraformaldehyde, permeabilized using 0.2% triton, and immunostained
169 with mouse monoclonal anti-MHC antibody MF20 (R&D Systems, MN, USA).
170 Cell nuclei were visualized by DAPI staining. Phase-contrast and fluorescent
171 images were taken and layered using EVOS FL Auto microscope

(AMAFD1000; Thermo Fisher Scientific). The ratio of MHC^+ cells were expressed as the number of nuclei in all MHC^+ cells divided by the total number of nuclei. Fusion indexes were expressed as the number of nuclei in multinuclear MHC^+ myotubes divided by the total number of nuclei.

2.4. Statistical analysis

The results were presented as mean ± standard error. Statistical comparisons were performed using unpaired two-tailed Student's *t* tests. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Expression patterns of TLRs in chick myoblasts

Primary-cultured myoblasts were isolated from E10 embryos or two-day-old (D2) chickens of White Leghorn (WL) layer chicken, Barred Plymouth Rock (BPR) dual-purpose chicken, and UK Chunky (UKC) broiler chicken. The expression of ten TLR genes (*TLR1A*, *TLR1B*, *TLR2A*, *TLR2B*, *TLR3*, *TLR4*, *TLR5*, *TLR7*, *TLR15*, and *TLR21*) in the myoblasts were examined by RT-PCR. At E10, all TLR cDNAs were detected in WL myoblasts but *TLR1A* was not observed in BPR and UKC myoblasts. However at D2, *TLR1A* expression disappeared in WL myoblasts and was slightly detected in UKC myoblasts (Fig. 1A, myoblast). Since both WL and UKC spleens at D2 distinctly expressed *TLR1A* (Fig. 1A, spleen), *TLR1A* transcription in myoblasts may be epigenetically regulated during muscle development from E10 to D2, and its regulation seems to be different among chicken breeds.

In following experiments, E10 myoblasts were utilized to investigate TLR functions because expression of *TLR1A* was greatly different between WL and UKC. Transcriptions of TLRs and their adaptors, CD14, MD2 (*LY96*), and MyD88, in myoblasts were quantified by qPCR (Fig. 1B). *TLR1A* was not detected in UKC myoblasts also by qPCR. The mRNA levels of *TLR1B*, *TLR2A*, and *TLR7* were significantly lower in UKC myoblasts compared to WL myoblasts. However, the levels of TLR adaptors were almost equal between WL and UKC myoblasts. As shown in Fig. 1C, expression patterns

206 of TLR genes in UKC myoblasts were not significantly changed during
207 myogenic differentiation. Even following the induction of differentiation, the
208 levels of TLRs, excluding that of *TLR3,* were almost the same on
209 differentiation day 0 and on day 2 in UKC myoblasts. These results suggest
210 that *TLR1A* transcription may be silenced in muscular lineage cells during
211 embryonic myotube formation in broiler chickens.
212
213 *3.2. TLR ligands induce ILs in chick myoblasts*
214 In UKC myoblasts at E10, *TLR1A* was not expressed and the levels
215 of *TLR1B* and *TLR2A* were significantly lower than those in WL myoblasts.
216 Pam_3CSK_4 and FSL-1 are ligands for chick TLR1/2 heterodimer (Keestra et
217 al., 2007; Higuchi et al., 2008) and known to induce ILs in immune cells. To
218 compare TLR1/2-dependent induction of ILs between WL and UKC
219 myoblasts, E10 myoblasts were stimulated with 10 ng/ml Pam_3CSK_4 or 100
220 ng/ml FSL-1 for 4 hours, following which transcriptions of IL-1β (*IL1B*), IL-6
221 (*IL6*), IL-8 (*IL8L1* and *IL8L2*), IL-10 (*IL10*), and IL-17 (*IL17A* and *IL17F*)
222 were quantified by qPCR (Fig. 2). Both Pam_3CSK_4 and FSL-1 upregulated
223 transcriptions of inflammatory interleukins, *IL1B*, *IL6*, *IL8L1*, and *IL8L2*, in
224 the UKC myoblasts in a manner similar to that in WL myoblasts. However,
225 neither Pam_3CSK_4 nor FSL-1 increased transcriptions of a suppressive IL,
226 *IL10*, and a pro-inflammatory IL, *IL17A*, in both myoblasts. *IL17F*
227 expression was not detected in any samples at all (data not shown). These
228 results demonstrate that UKC myoblasts, regardless of zero or low

transcriptions of *TLR1A*, *TLR1B*, and *TLR2A*, are able to recognize Pam_3CSK_4 and FSL-1 to induce inflammatory ILs.

Next, dose-dependent effects of Pam_3CSK_4 and FSL-1 on IL transcriptions in WL and UKC myoblasts at E10 were examined. The myoblasts were challenged by 1, 10, and 100 ng/ml of Pam_3CSK_4 or FSL-1 for 4 hours (Fig. 3). Both Pam_3CSK_4 and FSL-1 dose-dependently induced *IL1B*, *IL6*, and *IL8L2* transcriptions with no significant differences between WL and UKC myoblasts. At the doses of 10 and 100 ng/ml, the levels of induction by Pam_3CSK_4 were more than ten times higher than those by FSL-1, indicating that chick myoblasts are more sensitive to Pam_3CSK_4 compared to FSL-1 and its sensitivity is independent of TLR1A.

To investigate the time course of TLR ligand-dependent inflammatory responses, WL and UKC myoblasts at E10 were stimulated with 100 ng/ml Pam_3CSK_4, 100 ng/ml FSL-1, or 1 μg/ml LPS and harvested 1, 2, 4, and 8 hours after the stimulation. Transcriptions of *IL1B*, *IL6*, *IL8L2*, TNF-α (*LITAF*), and NF-κB (*NFKB1*) were quantified by qPCR (Fig. 4). Both in WL and UKC myoblasts, *IL1B* levels revealed >1000- and >200-fold increases 2 hours after the Pam_3CSK_4 and FSL-1 stimulation, respectively. However, LPS administration showed only a maximum of around 10-fold increase in *IL1B* expression. Similarly, *IL6* levels peaked 2 hours after the stimulation with Pam_3CSK_4 (122-fold in WL and 218-fold in UKC), FSL-1 (19.1-fold in WL and 33.2-fold in UKC), or LPS (3.3-fold in WL and 4.3-fold in UKC). Peak *IL8L2* levels approximately 4 hours after the treatments were as follows; Pam_3CSK_4 (415-fold in WL and 497-fold in UKC), FSL-1 (59.8-fold

253 in WL and 82.1-fold in UKC), and LPS (3.3-fold in WL and 5.2-fold in UKC).
254 *IL1B*, *IL6*, and *IL8L2* transcriptions were rapidly increased by a significant
255 amount due to Pam_3CSK_4 but were hardly enhanced by LPS. Remarkably,
256 the levels of *LITAF* and *NFKB1* were not altered by stimulation with either
257 Pam_3CSK_4, FSL-1 or LPS both in WL and UKC myoblasts.
258
259 *3.3. TLR ligands do not induce differentiation of chick myoblasts*
260 IL-6 is also known as a myokine (Serrano et al., 2008), and a TLR
261 adaptor, MyD88, promotes myotube formation in mice (Hindi et al., 2017).
262 Since Pam_3CSK_4 and FSL-1 enhanced *IL6* transcription in myoblasts, their
263 effects on myogenic differentiation were evaluated. UKC myoblasts at E10
264 were induced to differentiate with 100 ng/ml of Pam_3CSK_4 or FSL-1. First,
265 inductions of ILs by Pam_3CSK_4 or FSL-1 during differentiation were
266 confirmed. qPCR results showed that both Pam_3CSK_4 and FSL-1 promote
267 *IL1B* and *IL6* transcriptions also in differentiating condition (Fig. 5A and B).
268 On differentiation day 2, MHC^+ myotube formation of the stimulated
269 myoblasts was measured by immunostaining (Fig. 5C). The number of $DAPI^+$
270 cell nuclei was not reduced by Pam_3CSK_4 or FSL-1, indicating that these
271 TLR ligands did not cause cell death. The ratio of MHC^+ differentiated
272 myocytes were significantly decreased by Pam_3CSK_4 but not by FSL-1.
273 Formation of multinuclear myotubes was not affected by Pam_3CSK_4 or
274 FSL-1 (Fig. 5D). These results demonstrate that TLR1/2 ligands do not
275 facilitate myogenic differentiation of chick myoblasts.
276

4. Discussion

This is the first study to report that chick myoblasts express TLR genes and produce ILs in response to TLR ligands. Interestingly, *TLR1A* gene was clearly expressed only in embryonic layer WL myoblasts. *TLR1A* expression was scarcely detected in broiler UKC myoblasts both at E10 and D2 and disappeared even in WL myoblasts after hatching. Since both WL and UKC spleens express all TLR genes, *TLR1A* transcription may be considered as being epigenetically silenced specifically in myoblasts. Embryonic UKC myoblasts also exhibited lower transcriptional levels of *TLR1B*, *TLR2A*, and *TLR7* compared to those of WL myoblasts. In order to improve meat production, broiler chickens have been crossbred through many generations. Microarray analyses indicated that gene expression profiles in skeletal muscle tissues are different between broiler and layer chickens (Zheng et al., 2009). This suggests that transcriptions of TLR genes in UKC myoblasts may have been genetically modified by long-term breeding. On the other hand, the levels of TLR adaptors, *CD14*, *LY96*, and *MYD88*, were almost equal between WL and UKC myoblasts. Transcriptome analyses of myoblasts will clarify such differential gene transcriptions involved in the TLR signaling pathway among chicken breeds.

Despite the lack of *TLR1A* expression, UKC myoblasts produced inflammatory ILs in response to the TLR1/2 ligand, Pam_3CSK_4, at the same level as that of WL myoblasts. Reactivity of the TLR1B/2A heterodimer to Pam_3CSK_4 is several times higher than that of TLR1A/2A (Higuchi et al.,

2008). Thus, in UKC myoblasts, TLR1B may compensate TLR1A to recognize TLR1/2 ligands and transduce its signaling. Another TLR1/2 ligand, FSL-1, was also able to induce inflammatory ILs in myoblasts but its effects were relatively weak compared to those of Pam_3CSK_4. Pam_3CSK_4 and FSL-1 differentially induced ILs in murine macrophages (Long et al., 2009) and chick splenocytes (St Paul et al., 2013) in a manner similar to that seen in chick myoblasts. These studies suggest that the response kinetics of TLR1/2 may be different among ligands. Remarkably, neither Pam_3CSK_4 nor FSL-1 upregulated suppressive IL-10 and pro-inflammatory IL-17A in myoblasts. A previous study correspondingly reported that Pam_3CSK_4 does not induce IL-10 and IL-17A in chick $CD4^+$ T cells (St Paul et al., 2012). In mammals, TLR ligands activate T cells together with T cell receptor (TCR) signaling. Lack of TCR stimulation may be a reason why Pam_3CSK_4 and FSL-1 did not induce IL-10 and IL-17 in chick myoblasts.

A TLR4 ligand, LPS, hardly induced inflammatory ILs in chick myoblasts compared to Pam_3CSK_4 and FSL-1. However, preceding studies described LPS-induced IL-1β, IL-6, and IL-10 in chick macrophages (Wu et al., 2016; Qi et al., 2017). In mammals, LPS is recognized by TLR4/CD14/MD2 complex which activates the MyD88/Toll-IL-1 receptor (TIR) domain-containing adaptor protein (TIRAP) pathway and the TIR domain-containing adaptor inducing interferon (TRIF)/TRIF-related adaptor molecule (TRAM) pathway (O'Neill and Bowie, 2007). Chickens carry the MyD88 and TRIF homologs, but not the well-conserved CD14 and TRAM homologs (Wu et al., 2009; Keestra and van Putten, 2008). It is possible to

speculate that, in chickens, functional alternatives of CD14 and TRAM serves in immune cells but not in myoblasts, which may partly be the reason for LPS not initiating inflammatory responses in chick myoblasts.

Although Pam_3CSK_4 or LPS may induce TNF-α and NF-κB transcriptions in murine C2C12 myoblasts (Frost et al., 2002; Frost et al., 2006), neither Pam_3CSK_4, FSL-1, nor LPS upregulated TNF-α and NF-κB in chick myoblasts. TNF-α and NF-κB transcriptions were elevated in the bursa of Fabricius of LPS-challenged chickens (Cheng et al., 2014) while phosphorylated NF-κB was not elevated in LPS-treated thrombocytes (Winkler et al., 2017). It is still unclear whether chick TLR1/2 ligands enhance NF-κB expression in other types of cells. Inflammatory gene transcriptions such as ILs are regulated in part by nuclear translocation of transcription factors including NF-κB (Kawai and Akira, 2007). In chick myoblasts, TLR1/2 ligands might induce inflammatory ILs by mediating the nuclear translocation of NF-κB.

IL-6, which is induced by Pam_3CSK_4 and FSL-1 in chick myoblasts, has been known to function as a myokine (Serrano et al., 2008). IL-6 alters the expression of myogenic transcription factors by modulating nicotinamide phosphoribosyltransferase (Nampt) in chick myoblasts (Krzysik-Walker et al., 2011). In addition, chick TLR1/2 ligands are believed to activate MyD88 which induces ILs (Keestra et al., 2013). MyD88 promotes murine myotube formation with a modest increase of myogenin (Hindi et al., 2017). These findings caused us to examine the effects of TLR ligands on the differentiation of chick myoblasts. However, both Pam_3CSK_4 and FSL-1 did

not facilitate myogenic differentiation into MHC^+ myocytes and multinuclear myotubes. In differentiating condition, Pam_3CSK_4 strongly facilitated IL-1β expression and mildly suppressed myogenic differentiation. It has been reported that IL-1β delays progression of myogenesis by upregulating a Notch ligand Jagged1 in human myogenic cells (Nagata et al., 2017). Probably also in chick myoblasts, Pam_3CSK_4-induced IL-1β attenuated differentiation into MHC^+ myocytes.

Recent studies have described acute or chronic inflammatory lesions with the degeneration and regeneration of myofibers in the breast muscle of broiler chickens (Kuttappan et al., 2013). However, the causes of such myopathic damages have not been clarified. In the mouse model of Duchenne muscular dystrophy or dysferlinopathy, leaky dystrophin- or dysferlin-deficient myofibers release the endogenous TLR ligands recognized by TLRs on muscle cells, which promote IL production during disease progression (Uaesoontrachoon et al., 2013; Henriques-Pons et al., 2014). These findings support the hypothesis that the TLR signaling pathway in chick myoblasts or myofibers may contribute to the inflammation observed in broiler breast muscle. The present study examined the effects of three ligands of TLR1/2 and TLR4 on chick myoblasts. In order to better understand chick skeletal muscle pathogenesis more precisely, mechanisms underlying the functioning of remaining TLRs and their ligands should be elucidated via further studies.

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555 **Figure legends**

556

557 **Fig. 1.** (A) RT-PCR (40 cycles) results of TLR gene transcriptions in UKC,
558 BPR, and WL myoblasts and spleens. (B and C) qPCR results of TLR and
559 adaptor gene transcriptions in UKC and WL myoblasts at E10 (B) and that
560 in UKC myoblasts at differentiation day 0 and 2 (C). The data is expressed
561 as fold-change ± standard error of the mean, of three independent
562 experiments, each carried out in duplicate. The mean value in WL myoblasts
563 (B) or UKC myoblasts at day 0 (C) was set at 1.0 for each gene. ND, not
564 detected. *, $p < 0.05$; **, $p < 0.01$ vs WL.

565

566 **Fig. 2.** qPCR results of IL gene transcriptions in the UKC and WL myoblasts
567 at E10 stimulated with 10 ng/ml Pam_3CSK_4 (A) or 100 ng/ml FSL-1 (B) for 4
568 hours. The data is expressed as fold-change ± standard error of the mean, of
569 three independent experiments, each carried out in duplicate. The mean
570 value of the non-stimulated (control) WL myoblasts was set at 1.0 for each
571 gene. NS, no significant difference compared to WL.

572

573 **Fig. 3.** qPCR results of IL gene transcriptions in the WL and UKC myoblasts
574 at E10 stimulated with 1, 10, or 100 ng/ml of Pam_3CSK_4 (A) or FSL-1 (B) for
575 4 hours. The data is expressed as fold-change ± standard error of the mean,
576 of three or four independent experiments, each carried out in duplicate. The
577 mean value of the non-stimulated (0 ng/ml) WL myoblasts was set at 1.0 for
578 each gene. NS, no significant difference compared to WL.

579

580 **Fig. 4.** qPCR results of IL gene transcriptions in the WL and UKC myoblasts
581 at E10 stimulated with 100 ng/ml Pam_3CSK_4, 100 ng/ml FSL-1, or 1 μg/ml
582 LPS for 1, 2, 4, or 8 hours. The data is expressed as fold-change. The mean
583 value of the non-stimulated (0 hour) WL myoblasts was set at 1.0 for each
584 gene.

585

586 **Fig. 5.** qPCR results of IL gene transcriptions in the UKC myoblasts at E10
587 stimulated with 100 ng/ml of Pam_3CSK_4 (A) or FSL-1 (B) in differentiating
588 condition for 4 or 48 hours. The data is expressed as fold-change ± standard
589 error of the mean, of three independent experiments, each carried out in
590 duplicate. The mean value of the non-stimulated (control) myoblasts at 4
591 hour was set at 1.0 for each gene. NS, no significant difference compared to
592 control. (C) Representative images of MHC and DAPI staining of the UKC
593 myoblasts described in (A) and (B) at 2 days after induction of myogenic
594 differentiation. Scale bar, 200 μm. (D) Quantifications of the relative
595 numbers of $DAPI^+$ nuclei (left panel), the ratio of MHC^+ myocytes (middle
596 panel), and fusion index as the ratio of MHC^+ multinuclear myotubes (right
597 panel). The data is expressed as mean value ± standard error of the mean, of
598 five independent experiments. *, $p < 0.05$ vs. control.

599

600 **Table 1** Primer sequences for RT-PCR.

601

Gene	Sequence (5'-3')	Reference
16S	ACCTATTTGACTCCCTCAACCA AAGTTTACGCCGTAGGAGGATAGGTT	Fujimura et al., 2008
CD14	CCGCATCTTTAACTTGTCCA GTTGCGGCTGAGGAAGAG	This study
IL1B	ACTGGGCATCAAGGGCTACA GCTGTCCAGGCGGTAGAAGA	Cheng et al., 2014
IL6	AGCAAAACACCTGTTACATTTCT AGTCTGGCTGCTGGACATTT	Cheng et al., 2015
IL8L1	AACTCCGATGCCAGTG TTGGTGTCTGCCTTGT	Qi et al., 2017
IL8L2	CCAAGCACACCTCTCTTCCA GCAAGGTAGGACGCTGGTAA	St Paul et al., 2013
IL10	CGCTGTCACCGCTTCTTCA CGTCTCCTTGATCTGCTTGATG	Qi et al., 2017
IL17A	CATGGGATTACAGGATCGATGA GCGGCACTGGGCATCA	Connerton et al., 2018
IL17F	TGACCCTGCCTCTAGGATGATC GGGTCCTCATCGAGCCTGTA	Connerton et al., 2018
LITAF	GCCCTTCCTGTAACCAGATG ACACGACAGCCAAGTCAACG	Cheng et al., 2014
LY96	GTAACAACAAAGGCAGAA AGAAAAATCCACTGACTCC	Karaffova et al., 2017
MYD88	GAGGATGGTGGTCGTCATTT GTCTTGCACTTGACCGGAAT	This study
NFKB1	TCAACGCAGGACCTAAAGACAT GCAGATAGCCAAGTTCAGGATG	Cheng et al., 2014
TLR1A	AGCGTTCCTAAATTCCTGT TGCATCCAAAATCAAAGCAA	This study
TLR1B	TCCCAAATGGTTTTTGGAAC TTTTAGAAGGCAACGGCACT	This study
TLR2A	GTCAGTGTATTTCCACAATG CCAAAAAGCCTGAAGTTGTTC	This study

TLR2B	CATGCAAACCAGTCACAATCTCTG CACCTTGGTATTTTCAACTGTGA	This study
TLR3	TCAGTACATTTGTAACACCCCGCC GGCGTCATAATCAAACACTCC	St Paul et al., 2012
TLR4	TGCCATCCCAACCCAACCACAG ACACCCACTGAGCAGCACCAA	St Paul et al., 2012
TLR5	TTCTTGCAACCTCACAGGTGTTCC CAGGTCCAAGACACGAAGATT	St Paul et al., 2012
TLR7	ATCAGCACAGGGATGGAAAG TTGAGTTCTGGAGGCATAGC	Brownlie et al., 2009
TLR15	TTGATGGGCTGTGGTATGTG CGTGCTCGCTGTATGAAATG	Brownlie et al., 2009
TLR21	CAACCTGAGCAGCCTGTACG GGATGGACCGCAGCATGTTC	Brownlie et al., 2009

602

Figure 1

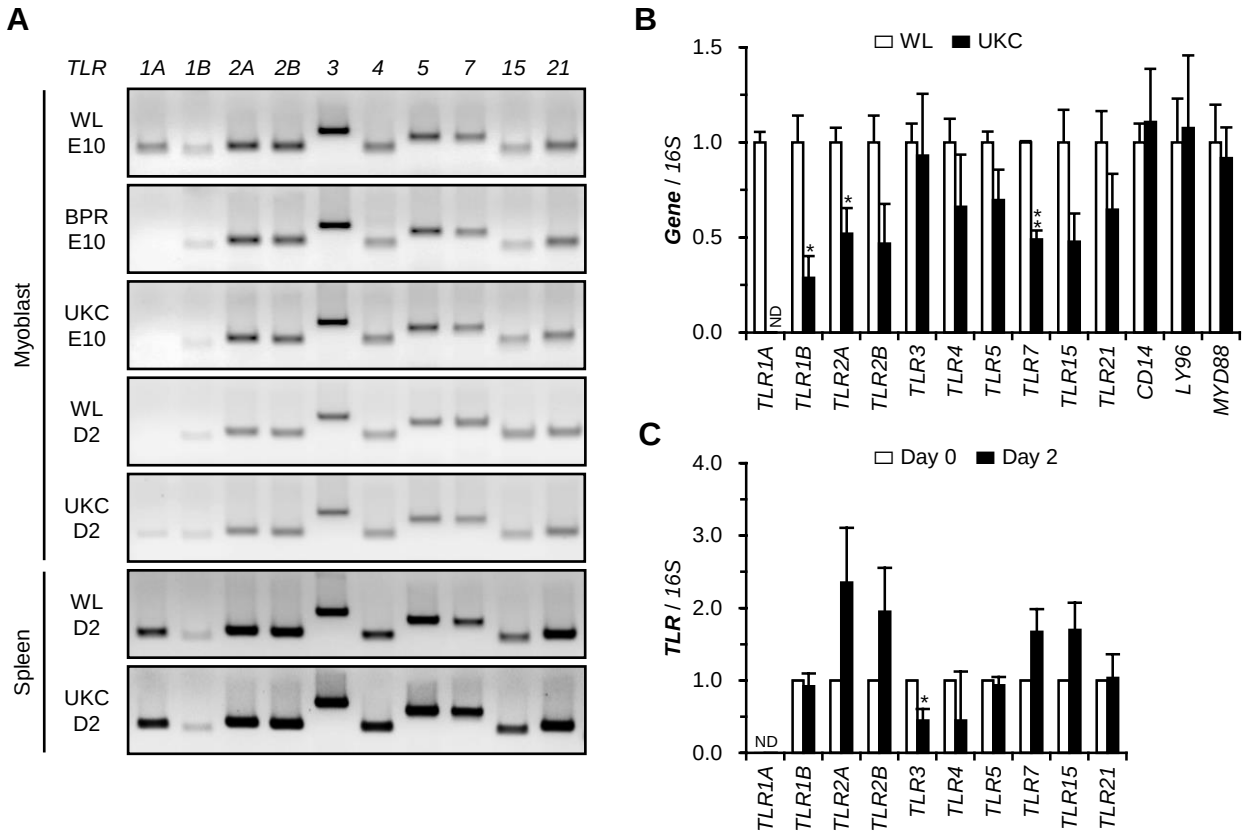

A
TLR 1A 1B 2A 2B 3 4 5 7 15 21
Myoblast
WL E10
BPR E10
UKC E10
WL D2
UKC D2
Spleen
WL D2
UKC D2
B
WL UKC
Gene / 16S
1.5
1.0
0.5
0.0
ND
*
*
**
TLR1A TLR1B TLR2A TLR2B TLR3 TLR4 TLR5 TLR7 TLR15 TLR21 CD14 LY96 MYD88
C
Day 0 Day 2
TLR / 16S
4.0
3.0
2.0
1.0
0.0
ND
*
TLR1A TLR1B TLR2A TLR2B TLR3 TLR4 TLR5 TLR7 TLR15 TLR21

Figure 2

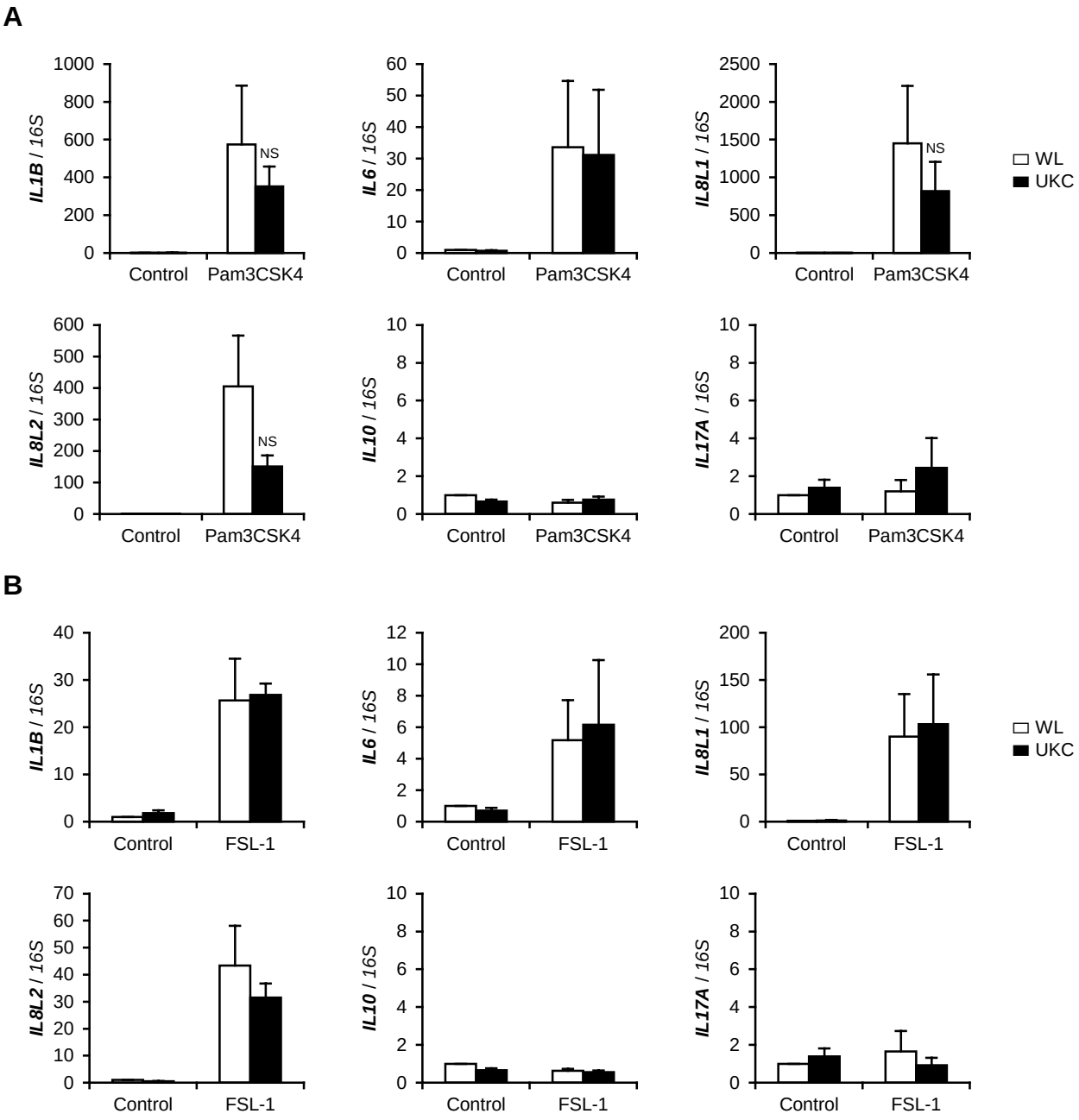
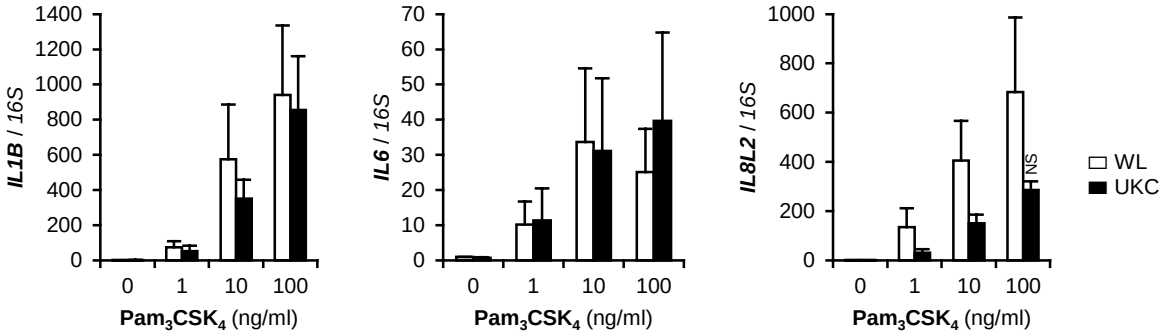

A
IL1B / 16S
IL6 / 16S
IL8L1 / 16S
IL8L2 / 16S
IL10 / 16S
IL17A / 16S
NS
Control
Pam3CSK4
WL
UKC
B
FSL-1

Figure 3

A


IL1B / 16S
1400
1200
1000
800
600
400
200
0
0 1 10 100
Pam3CSK4 (ng/ml)
IL6 / 16S
70
60
50
40
30
20
10
0
0 1 10 100
Pam3CSK4 (ng/ml)
IL8L2 / 16S
1000
800
600
400
200
0
NS
0 1 10 100
Pam3CSK4 (ng/ml)
WL
UKC

B

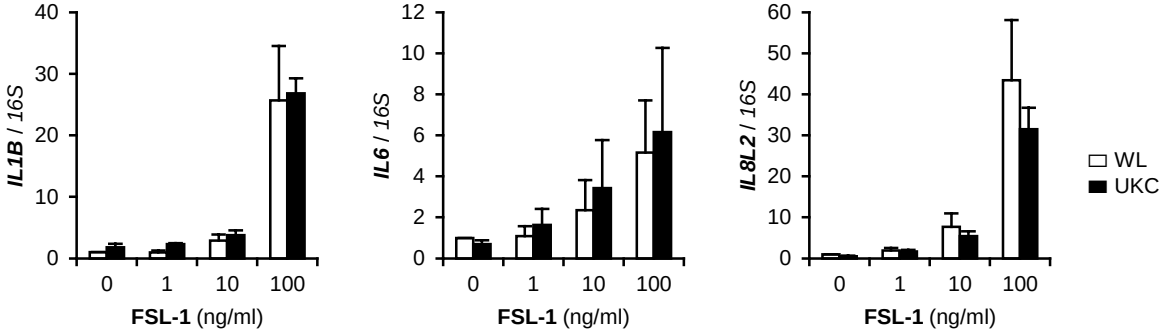

IL1B / 16S
40
30
20
10
0
0 1 10 100
FSL-1 (ng/ml)
IL6 / 16S
12
10
8
6
4
2
0
0 1 10 100
FSL-1 (ng/ml)
IL8L2 / 16S
60
50
40
30
20
10
0
0 1 10 100
FSL-1 (ng/ml)
WL
UKC

Figure 4

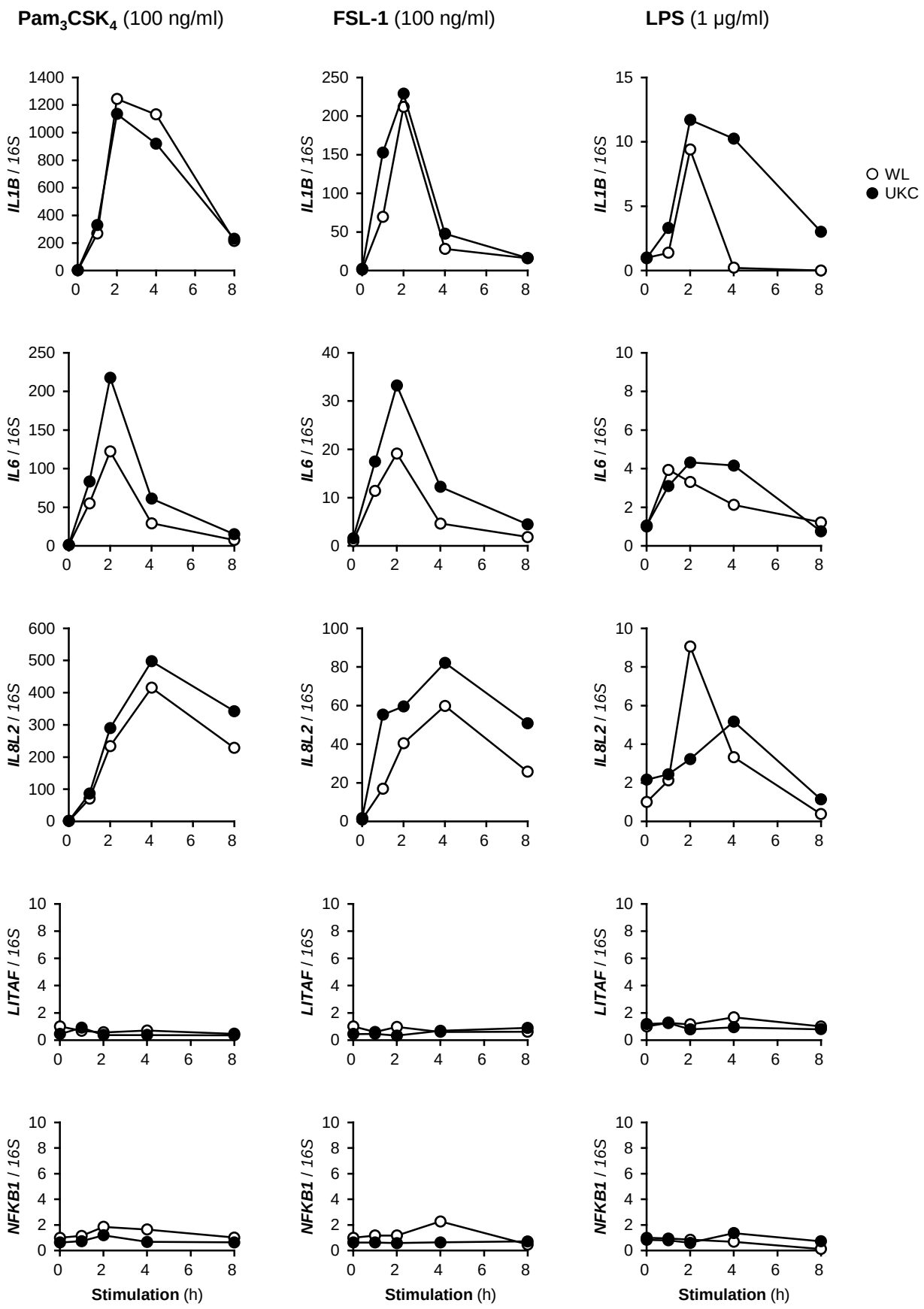
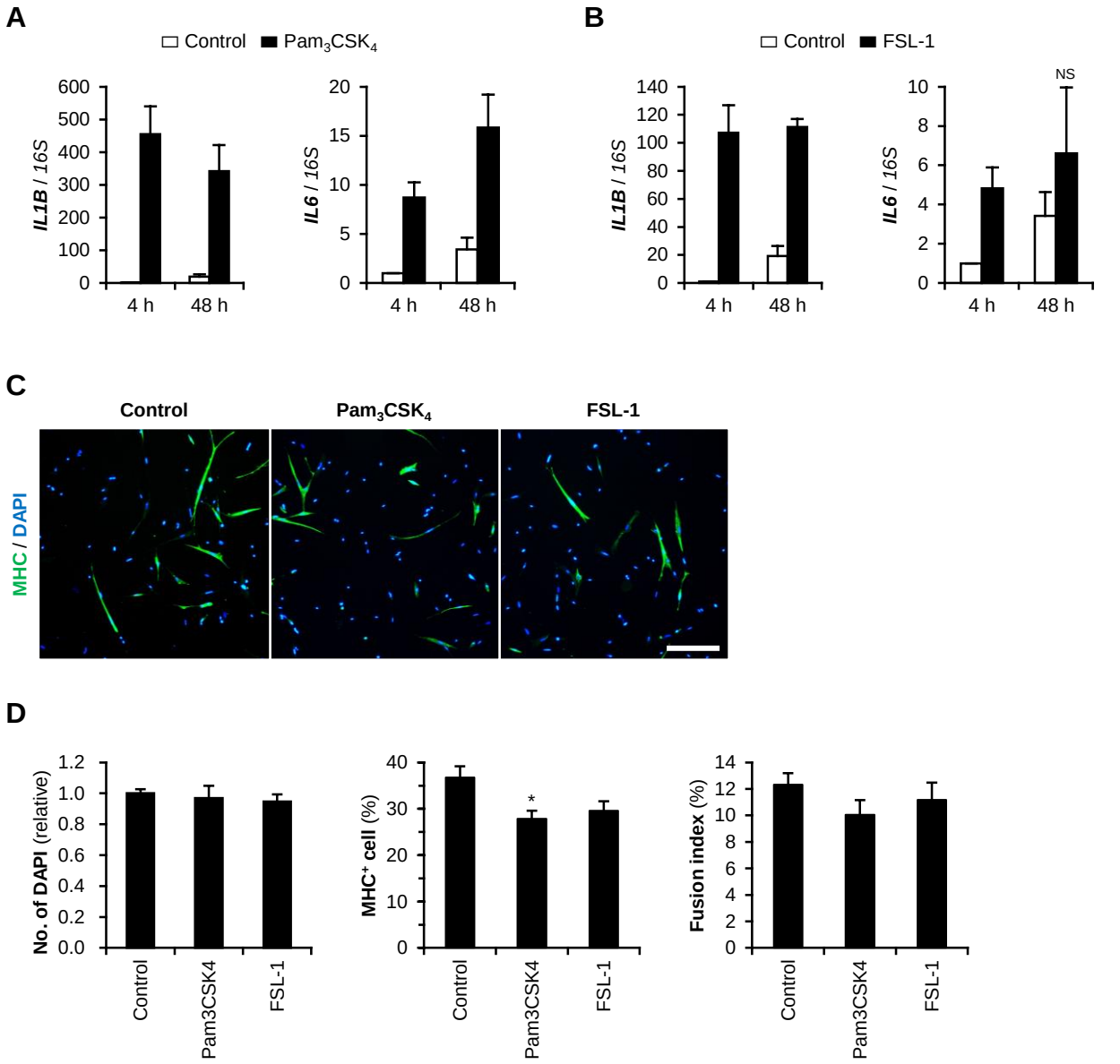

Pam3CSK4 (100 ng/ml)
FSL-1 (100 ng/ml)
LPS (1 µg/ml)
IL1B / 16S
IL6 / 16S
IL8L2 / 16S
LITAF / 16S
NFKB1 / 16S
WL
UKC
Stimulation (h)

Figure 5


A
Control
Pam3CSK4
IL1B / 16S
IL6 / 16S
4 h
48 h
B
Control
FSL-1
NS
C
Control
Pam3CSK4
FSL-1
MHC / DAPI
D
No. of DAPI (relative)
MHC+ cell (%)
Fusion index (%)
Control
Pam3CSK4
FSL-1