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Blockade of Lyn kinase upregulates both canonical and non-canonical TLR-3 pathways in THP-1 monocytes exposed to human cytomegalovirus

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Summary. – Regulation of monocyte response to human cytomegalovirus (HCMV) occurs via activation of receptors that elicit innate antiviral effects and later T-cell responses. Our previous data (Yew *et al.*., 2010) demonstrated that human monocyte scavenger receptor A type 1 (SR-A1) are required for sensing of HCMV by endosomal toll-like receptors (TLRs)-3 and -9, which in turn induce critical pro-inflammatory cytokines. However, it remains unclear which subcellular molecules associated with SR-A1 lead to downstream activation of TLR-3 and/or TLR-9 signaling pathways. Herein we report that Lyn kinase, associated physically and functionally with SR-A for low density lipoprotein (LDL) recognition, acts as a key SR-A1-induced kinase that plays a critical role in TLR-3/9 signal transduction upon HCMV exposure to THP-1 monocytes. We found that disruption of the SR-A1 signal transduction through molecular inhibition by Lyn kinase oligonucleotides not only blocks the activation of downstream TLR-9 pathway but also alters the downstream TLR-3 pathway. In particular, Lyn kinase oligonucleotides resulted in decreased expression of TLR-9-induced tumor necrosis factor alpha (TNF-α) but strongly upregulated canonical TLR-3-induced interferon beta (IFN-β) and non-canonical TLR-3-induced NF-κB-dependent p35 (35kDa) subunit of interleukin 12 (IL-12p35) gene transcription. Thus, the observed shift away from TNF-α to robust IFN-β and IL-12p35 induction may offer opportunities for therapeutic interventions.

Keywords: HCMV; THP-1 monocytes; TLR-3/-9; Lyn kinase; TNF-α; IFN-β; IL-12p35

Introduction

HCMV (the subfamily *Betaherpesvirinae*, the family *Herpesviridae*) is a ubiquitous opportunistic pathogen capable of causing both congenital and acquired infections. HCMV infection is widespread in the general population, but it is usually asymptomatic. However, when primary infection occurs early in pregnancy, HCMV can cause human congenital infection, which affects the developing neurological system *in utero*, thereby leading to sensorineural hearing loss and other neurological sequelae after birth (Landolfo *et al.*, 2003; Abate *et al.*, 2004; Scanga *et al.*, 2006). Human congenital HCMV infection occurs in ~1% of all births but is symptomatic in 10% to 20% of affected infants (Scanga *et al.*, 2006). Moreover, HCMV is a common cause of morbidity and mortality in immunocompromised individuals, including AIDS patients and recipients of bone marrow allografts or of solid organ transplants, but rarely causes disease in immunocompetent people (Bentz *et al.*, 2006). The mechanism of HCMV dissemination remains poorly resolved; however, cells of the innate immune system, which act as the first line of defense, specifically monocytes, are thought to play several key roles in this process (Bentz *et al.*, 2006). Results from *in vitro* and *in vivo* studies suggest that the infectious cycle of HCMV starts with virus binding to the monocyte cell surface receptors and is followed by fusion of the envelope with the plasma membrane with the release of viral structural components into the cell (Abate *et al.*, 2004). Binding and

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Abbreviations: HCMV = human cytomegalovirus; IFN-β = interferon beta; IL-12p35 = p35 (35 kDa) subunit of interleukin 12; IRF3 = interferon regulatory factor 3; LDL = low-density lipoproteins; MYD88 = myeloid differentiation primary response gene (88); NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells; SR-A1 = scavenger receptor A type 1; TLRs = toll-like receptors; TNF-α = tumor necrosis factor alpha; TRIF = TIR-domain-containing adapter-inducing IFN-β

penetration of virus or viral envelope glycoprotein B (gB) or gH triggers a receptor-mediated signal transduction leading to pro-inflammatory cytokine responses in host macrophages, the differentiated counterparts of monocytes (Bentz *et al.*, 2006). Recent evidence from our laboratory further indicates that monocytes upon HCMV exposure undergo cellular activation and the induction of pro-inflammatory cytokines, i.e. TNF-α, IFN-β, and IL-12p35, through SR-A1 and TLRs 3 and 9 (Yew *et al.*, 2010).

TLRs serve as sensors against pathogens that invade the host by recognizing pathogen-associated molecular patterns (Matsushima *et al.*, 2004). Each TLR has been identified to recognize a distinct ligand, supported and confirmed by previous observation of TLR-knockout mice and specific gene modifications. For example, TLR-3 and TLR-9 recognize viral dsRNA and unmethylated consensus DNA sequences, including the CpG motif in bacteria and viruses, respectively (Matsushima *et al.*, 2004). All TLRs mediate signaling through their cytoplasmic TIR domains linked to adaptor proteins, such as MYD-88 and TRIF, which induce the activation of NF-κB and IRF3, respectively. Subsequently, these complexes translocate into the nucleus where they regulate transcription of target genes. For instance, TLR-3 signaling activates TRIF and IRF3, which then mediate IFN-β promoter activation as an innate response to infectious agents (Jiang *et al.*, 2004). Alternatively, TLR-3-mediated TRIF can also signal through TRAF6 and recruit NF-κB p65 to induce cytokine expression (Jiang *et al.*, 2004). In contrast, TLR-9 engagement triggers MYD-88 and NF-κB p65 activation, which regulates gene expression of multiple cytokines, e.g. TNF-α (Tran *et al.*, 2007) and IL-12 (Jiang *et al.*, 2009).

SR-A1, often referred to as CD204, is expressed primarily on the innate immune cells, such as monocytes/macrophages and dendritic cells and functions as a pattern recognition receptor (PRR) (Peiser *et al.*, 2002; Wang *et al.*, 2007). While SR-A1 was initially described to be able to uptake modified LDL through endocytosis, it is also thought to be mainly a trafficking receptor for a wide range of endogenous and microbial ligands, including lipopolysaccharide (LPS) on Gram-negative bacteria (Ishiguro *et al.*, 2001), lipoteichoic acid (LTA) on Gram-positive bacteria (Thomas *et al.*, 2000), apoptotic cells, as well as viral DNA and RNA (Amiel *et al.*, 2008). Subsequent work has demonstrated that the binding of LPS to SR-A on macrophages induces the production of cytokines and nitric oxide (NO), indicating that SR-A might play a pivotal role in host defense mechanism of macrophages (Hampton *et al.*, 1991; Miki *et al.*, 1996).

There is increasing evidence that SR-A1 can function as a carrier or "shuttle" molecule, bringing ligands to endosomal receptors to modulate intracellular signaling pathways. Multiple published reports from a number of groups have speculated that SR-A1 appears to have an essential function in viral dsRNA uptake into the cell via endocytosis, in which SR-A1 may be delivering extracellular viral dsRNA to endosomes to be recognized by TLR-3 (Limmon *et al.*, 2008; DeWitte-Orr *et al.*, 2010). Alternately, MARCO (a member of SR-As) might participate in CpG-ODN delivery to endosomal sites of interaction with TLR9 (Józefowski *et al.*, 2006). Later findings further supported the idea that SR-A1 is required for sensing of HCMV by endosomal TLR-3 and -9, which in turn induce critical pro-inflammatory cytokines (Yew *et al.*, 2010). Thus, the question arises as to whether a subcellular molecule associated with SR-A1 could play a critical role in leading to downstream activation of TLR-3 and/or TLR-9 signaling pathways.

Given the fact that Lyn kinase, one of the members of the Src tyrosine kinase, has been reported to be functionally associated with SR-A1 in monocytes (Miki *et al.*, 1996) and involved in many cellular processes including gene differentiation and transcription (Corey *et al.*, 1999), we hypothesized that SR-A1-mediated Lyn kinase recruitment / activation is key to regulating endosomal TLR-3 and TLR-9 signaling mechanisms and subsequent cytokine gene transcription in HCMV-exposed THP-1 monocytes. To test this hypothesis, we evaluated the regulatory role of Lyn kinase and the effect of blocking Lyn kinase signaling by antisense oligonucleotides in THP-1 monocytes upon HCMV exposure. We first observed that mRNA levels of Lyn kinase are significantly increased in THP-1 monocytes within 10 mins after exposure to HCMV assessed by RT-PCR. Furthermore, immunocytochemistry for Lyn kinase revealed that many Lyn-positive cells were seen within 10 mins after HCMV exposure of THP-1 monocytes compared to the control. Importantly, inhibition of Lyn's function by the antisense oligonucleotides prevented the normal rise in TLR-9-induced TNF-α mRNA that occurs in response to HCMV, suggesting that the TLR-9-induced TNF-α is critically regulated by Lyn kinase. The most striking observation was that the transcription of canonical TLR-3-induced IFN-β as well as "non-canonical" TLR-3-induced NF-κB-dependent IL-12p35 were elevated as a result of Lyn kinase inhibition. In retrospect, due to the inhibitory effect of Lyn kinase oligonucleotides, a shift toward an enhanced IFN-β and IL-12p35 induction associated with a decline in TNF-α expression might be caused by an as yet unidentified SR-A1-mediated protein kinase, possibly via the alternative TLR-3 signaling mechanism, upon HCMV exposure to THP-1 monocytes. This report describes a new role of SR-A1-mediated Lyn kinase in HCMV-exposed monocytes and raises a number of interesting opportunities for clinical intervention that might enhance innate antiviral defense and HCMV-specific T-cell responses.

Materials and Methods

Reagents and kits. DMEM, FBS, SYBR Green, Reference Dye for Quantitative PCR, and Protease Inhibitor Cocktail were obtained from Sigma-Aldrich (St. Louis, MO). RPMI 1640 was obtained from Gibco/Invitrogen. RNeasy Mini Kit, Sensiscript Reverse Transcriptase Kit, QIAamp DNA Micro Kit, and QIAquick Gel Extraction Kit were all purchased from Qiagen (Valencia, CA). AmpliTaq Gold with GeneAmp 10X PCR Buffer and $MgCl_2$ solution were from Applied Biosystems (Foster City, CA).

Antibodies. Normal donkey serum and FITC-conjugated AffiniPure donkey anti-mouse IgG antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). Mouse monoclonal antibody [ab6276] to human β-actin and mouse monoclonal antibody [ab1890] to human Lyn kinase were all purchased from ABCAM (Cambridge, MA).

Endo-Porter delivery of antisense oligonucleotides assay. Oligonucleotides complementary to Lyn kinase gene (5′-TT TATACATCCCATATTTCCCGCTC-3′), and missense control oligonucleotides (5′-CCTCTTACCTCAgTTACAATTTATA-3′) were designed and synthesized by Gene Tools (Philomath, OR). Delivery of antisense oligonucleotides with Endo-Porter transfection method was performed according to the manufacturer's protocol. All oligonucleotides were complexed with Endo-Porter delivery reagent and confirmation of delivery was measured by the Western blot analysis. Note that all Endo-Porter transfection experiments were carried out in triplicate and repeated three times.

Cell culture and treatment. Human THP-1 monocytes and human foreskin fibroblasts, purchased from ATCC (Manassas, VA), were grown in RPMI 1640 and DMEM, respectively, with 2 mmol/l L-glutamine, 250 μg/ml amphotericin, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum at 37°C under a humidified condition of 95% air and 5% CO_2. Upon 90% confluence, THP-1 monocytes at passage 10 to 15 were plated at a density of ~10^5 cells/well in 12-well plates with serum-free RPMI 1640 overnight. On the following day, cells were either treated with serum-free medium alone or exposed to HCMV (Toledo) at a multiplicity of infection of 10 throughout the culture period. Antisense oligonucleotide or missense control was added separately to culture media at 5 μmol/l.

RT-PCR (non-quantitative). Total RNA was extracted from cells and treated with DNase. RNA was subjected to reverse transcription. cDNA was then amplified by PCR for 40 cycles (Table 1). All PCR products were separated by electrophoresis in 2% agarose gel. PCR cycles were as follows: initial denaturation at 95°C for 10 mins, followed by 40 cycles of 94°C for 30 secs, 60°C for 30 secs, 72°C for 30 secs and final extension at 72°C for 10 mins.

SYBR Green real-time quantitative PCR. PCR amplifications were performed using an Applied Biosystems 7500 (Carlsbad, CA) sequence detection system. Unless otherwise specified, each reaction mixture contained 10X Gold Buffer, 25 mmol/l $MgCl_2$, 2.5 mmol/l dNTPs, 10X SYBR Green, AmpliTaq Gold polymerase, Reference Dye, dH_2O, DNA template and 10 μmol of each primer. Amplification was performed by initial polymerase

Table 1. PCR Primers

Gene	Accession #	Primers (5′–3′)	Product (bp)
β-actin	NM_001101	FP: AGAAAATCT-GGCACCACACC RP: GGGGTGTTGAAG-GTCTCAAA	142
HCMV-gB	X04606	FP: TGGTCTACAAGCG-CAACATC RP: GCCACGTATTCCG-TATTGCT	130
SR-A1	NM_138715	FP: GACAT-GGAAGCCAACCTCAT RP: CCCTGGACTGAG-GAAAACAA	116
Lyn Kinase	M16038	FP: GCAGAGGGAAT-GGCATACAT RP: CTAGCAAG-GCCAAAATCTGC	116
TLR-3	NM_003265	FP: CCGCCAACT-TCACAAGGTAT RP: AGCTCATTGTGCT-GGAGGTT	130
TLR-9	NM_017442	FP: AATTCCCATCTCTC-CCTGCT RP: TCCTTCACCCCT-TCCTCTTT	135
TRIF	AB093555	FP: ACGCCACTC-CAACTTTCTGT RP: TCAGGTGAGCT-GAACAAGGA	136
IRF3	NM_001571	FP: GATGCACAGCAG-GAGGATTT RP: TCTGCTAAACG-CAACCCTTC	149
MYD88	NM_002468	FP: TGCAGAGCAAG-GAATGTGAC RP: AGGATGCT-GGGGAACTCTTT	121
NF-κB p65	M55643	FP: TGGAGTCT-GGGAAGGATTTG RP: CGAAGCT-GGACAAACACAGA	129
IFN-β	M28622	FP: CATTACCTGAAG-GCCAAGGA RP: AGCAATTGTC-CAGTCCCAGA	150
TNF-α	M10988	FP: GCCCCAATCCCTT-TATTACC RP: CACATTCCTGAATC-CCAGGT	145
IL-12p35	AF101062	FP: GAGGCCTGTTTAC-CATTGGA RP: GCACAGGGCCAT-CATAAAAG	123

FP = forward primer; RP = reverse primer

activation for 10 mins at 95°C, and 40 cycles of denaturation at 95°C for 15 secs, annealing at 60°C for 20 secs and elongation for 30 secs at 72°C. The fluorescence threshold value was calculated using the iCycle system software. The calculation of relative change in mRNA was performed using the delta-delta method (Pfaffl *et al.*., 2001), with normalization for the housekeeping gene β-actin.

Quantitative PCR for HCMV. Viral concentrations in the supernatant of human fibroblast cultures infected with HCMV at 5–7 days post infection were collected using the Millipore® Steriflip® disposable vacuum filtration system (Billerica, MA)with PVDF membrane to remove cellular debris produced during infection of fibroblasts and later using QIAamp DNA Micro Kit for the extraction of total viral DNA, following the manufacturer´s recommendation. HCMV DNA copy number was then determined and detected by the same protocol used in the SYBR Green real-time quantitative PCR, except for the addition of HCMV primers and the HCMV DNA sample. All virus stocks were aliquotted and stored at -80°C until used as viral inocula.

Immunocytochemistry. Upon 10 mins and 1 hr exposure of HCMV to THP-1 monocytes at a multiplicity of infection of 10, cytospin preparations were performed on untreated and treated THP-1 monocytes at a density of ~10^5 cells/ml by centrifugation for 5 mins. Cells were fixed with 4% phosphate-buffered paraformaldehyde for 15 mins at RT. After fixation, the cells were permeabilized with 0.2% Triton X-100 (Sigma, St. Louis, MO) for 5 mins at RT, blocked with normal donkey serum for 30 mins and then incubated with primary mouse anti-human Lyn kinase antibody at a dilution of 1/100 in a moist chamber for 2 hrs at RT. The cells were rinsed with PBS and incubated with secondary fluorescein (FITC)-conjugated donkey anti-mouse IgG for 2 hrs at RT in a dark cabinet. After several washes, coverslips containing cells were mounted onto slides in aqueous mounting medium with anti-fading agents (Biomeda corp., Foster City, CA). Fluorescence digital images were captured using an Olympus BX60 (Melville, NY) microscope attached with Olympus U-PMTVC camera adaptor. Optronics DEI-750 (Goleta, CA) software was used to acquire and analyze images. Each slide was evaluated independently and immuno-positive cells were counted from representative areas of the sections. Results were expressed as the number of positive cells per low power field or the percentage of the total number of cells that were positive for antibody staining.

SDS-PAGE and Western blot analysis. Proteins were separated on a 10% Tris-HCl Ready Gel (Bio-Rad, Hercules, CA), transferred onto nitrocellulose membranes, and incubated with β-actin antibody at a dilution of 1/5000 or Lyn antibody at a dilution of 1/1000 overnight at 4°C. After incubation, the blots were washed 3x for 15 mins in washing buffer (PBS-0.05% Tween20) and incubated with a secondary anti-mouse β-actin or a secondary anti-mouse Lyn antibody coupled to horseradish peroxidase (Vector Labs, Burlingame, CA) for 1 hr at room temperature. Then, the blots were washed 3x for 15 mins in washing buffer, and immunoreactivity was normalized by chemiluminescence (Amersham, ECL+Plus Kit) according to the manufacturer's instructions. The blots were exposed to Kodak scientific imaging films (Rochester, NY) within 1 min for detection.

Statistical analysis. Data were analyzed using the Microsoft Office Excel 2003 and expressed as means ± S.D. where appropriate. Two group comparisons were evaluated using the unpaired Student´s *t*-test. Differences were found significant by the Student´s *t*-test in case $p < 0.05$.

Results

A novel role for Lyn kinase in HCMV exposed THP-1 monocytes

In a previous study (Yew *et al.*, 2010), our group indicated that Lyn kinase is involved in signaling cascades leading to cytokine production in THP-1 monocytes upon exposure of HCMV. To expand on our previous observations that the mRNA levels of Lyn kinase were upregulated in THP-1 monocytes within 1–10 mins but gradually decreased within 1–6 hrs after HCMV exposure as assessed by real time quantitative PCR analysis, we next performed a narrow exposure time-response of HCMV to THP-1 monocytes and found that Lyn kinase mRNA levels exhibit a strong expression at 10 mins compared to 1 hr, as determined by a simple RT-PCR (reverse transcription-polymerase chain reaction) screen (Fig. 1a). Further, immunocytochemistry for Lyn kinase revealed that many Lyn-positive cells were seen at 10 mins post HCMV exposure, whereas only a modest number of THP-1 monocytes were Lyn positive at 1 hr post HCMV exposure (Fig. 1b,c). These results obtained by different techniques convincingly confirm and extend our previous observation that Lyn kinase plays a functional role in HCMV-mediated THP-1 monocytes.

Lyn kinase blockade up-regulates canonical TLR-3-induced cytokines

In accordance with previous studies (Yew *et al.*, 2010), we found that SR-A1 and Lyn kinase are activated simultaneously upon exposure of THP-1 monocytes to HCMV at 10 mins. Furthermore, THP-1 monocytes pretreated with the SR-A/MSR antibody exhibited markedly decreased mRNA levels of Lyn kinase and TLR-3 and TLR-9 as well as their downstream signaling factors at 1 hr**.** This suggests that SR-A1 engagement is functionally associated with Lyn kinase, which is important for activation of TLR-3 and TLR-9 (Yew *et al.*, 2010). In an effort to better delineate the role of Lyn kinase and further validate SR-A1-mediated Lyn kinase signaling mechanisms, we utilized antisense oligonucleotides to block, in a sequence-specific manner, the translation of Lyn kinase mRNA in THP-1 monocytes prior to and during HCMV exposure. Here, there

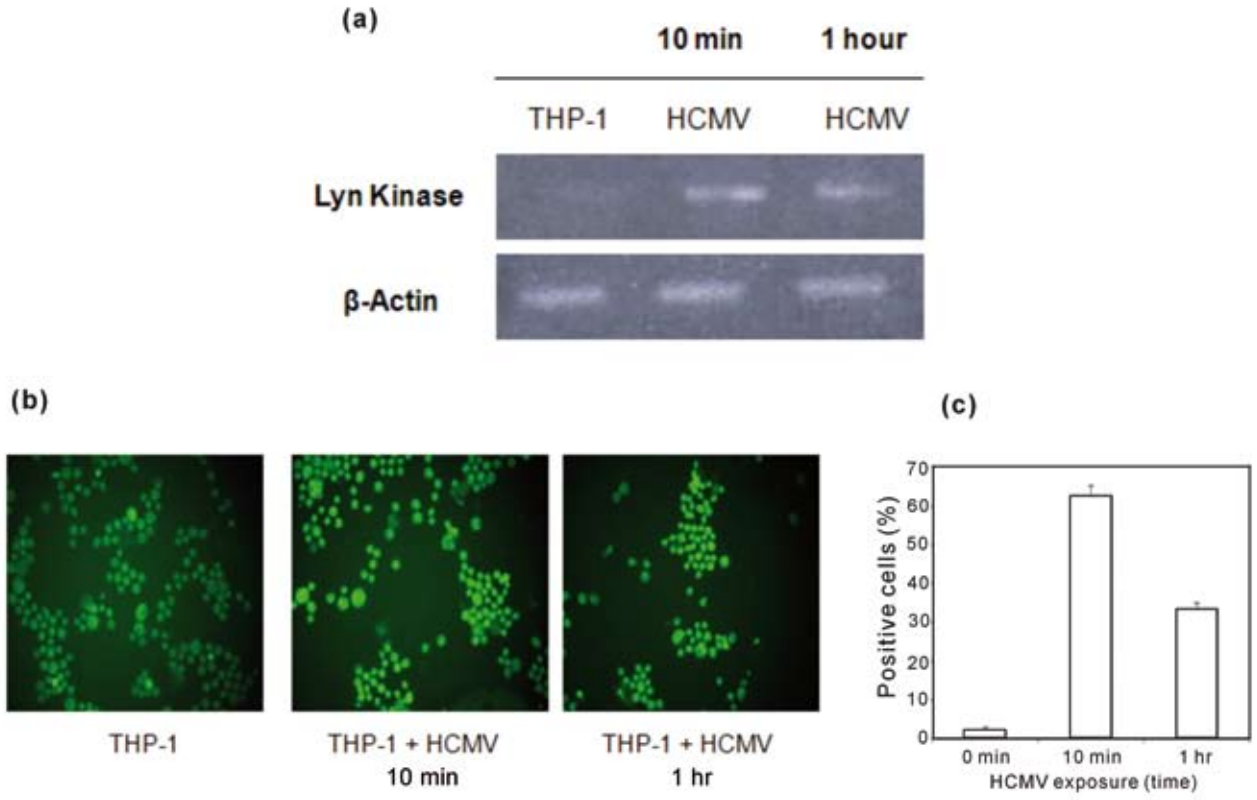

Fig. 1

Induction of Lyn kinase and immunofluorescence detection of Lyn kinase in THP-1 monocytes upon exposition to HCMV

RT-PCR screening for mRNA levels of Lyn kinase expression in THP-1 monocytes upon HCMV exposure. RNA was extracted from THP-1 monocytes upon HCMV exposure within a 10 mins–1 hr time span. All mRNAs were analyzed from the same preparation. Reaction mixtures without reverse transcriptase served as controls for genomic DNA contamination in all cases (data not shown). (a) The gene expression of Lyn kinase mRNAs was weakly present in the baseline THP-1 monocytes but was strongly present in HCMV-exposed THP-1 monocytes at 10 mins and became markedly decreased at 1 hr. β-actin expression is shown for normalization purposes. (b) Similar to the mRNA levels, immunostaining of HCMV-exposed THP-1 monocytes at 10 mins shows marked upregulation of Lyn kinase staining. (c) Quantification of the immunocytochemistry in (b) reflects an increased number of positive cells and an increased percentage of positive cells.

was a dramatic increase in TLR-3 mRNA levels at 1 hr (Fig. 2b). Like TLR-3, its downstream signaling effectors such as TRIF and interferou regulatory factor 3 (IRF3) were found to have the highest expression at 1 hr (Fig. 2c,d). Similarly, IFN-β mRNA (Fig. 2e) also was maximal at the same incubation time. The changes in the levels of these TLR-3 signaling factors may be a key point of "alternative" regulation modulated by an unidentified SR-A1-mediated protein kinase to replace Lyn kinase. No apparent effect on the inhibition of SR-A1 was seen (Fig. 2a); suggesting that SR-A1 is working upstream of Lyn kinase, which is critically regulated by SR-A1.

Lyn kinase blockade down-regulates TLR-9-induced cytokines but up-regulates non-canonical TLR-3-induced cytokines

Our data in Figure 2 shows that the deficiency of Lyn kinase plays an unprecedented role in cytokine expression induced by the TLR-3 signaling pathway upon HCMV exposure of THP-1 monocytes. However, inhibitory effects of Lyn kinase in HCMV-exposed THP-1 monocytes have a direct impact on the TLR-9 signaling pathway. The Lyn kinase oligonucleotides specifically blocked the upregulation of both TLR-9 (Fig. 3a) and TNF-α (Fig. 3d) at 1 hr, suggesting that Lyn kinase is essential for inducing TLR-9 and TNF-α transcription. MYD88 (Fig. 3B), a TLR adapter common to TLR-9 but not TLR-3, was not found to be greatly altered, indicating that it might serve as a rate-limiting factor. Strikingly, NF-kB (Fig. 3c) and IL-12p35 (Fig. 3e) mRNA levels were elevated as a result of Lyn kinase inhibition. Similar to IFN-β mRNA (Fig. 2e), we postulated that the elevated levels of NF-kB and IL-12p35 might be triggered by an unknown SR-A1-mediated enzyme due to the deficiency of Lyn kinase and could represent the abrupt shift of pathway, possibly via the non-canonical TLR-3 signaling mechanism. To confirm the inhibiting effects of the Lyn kinase

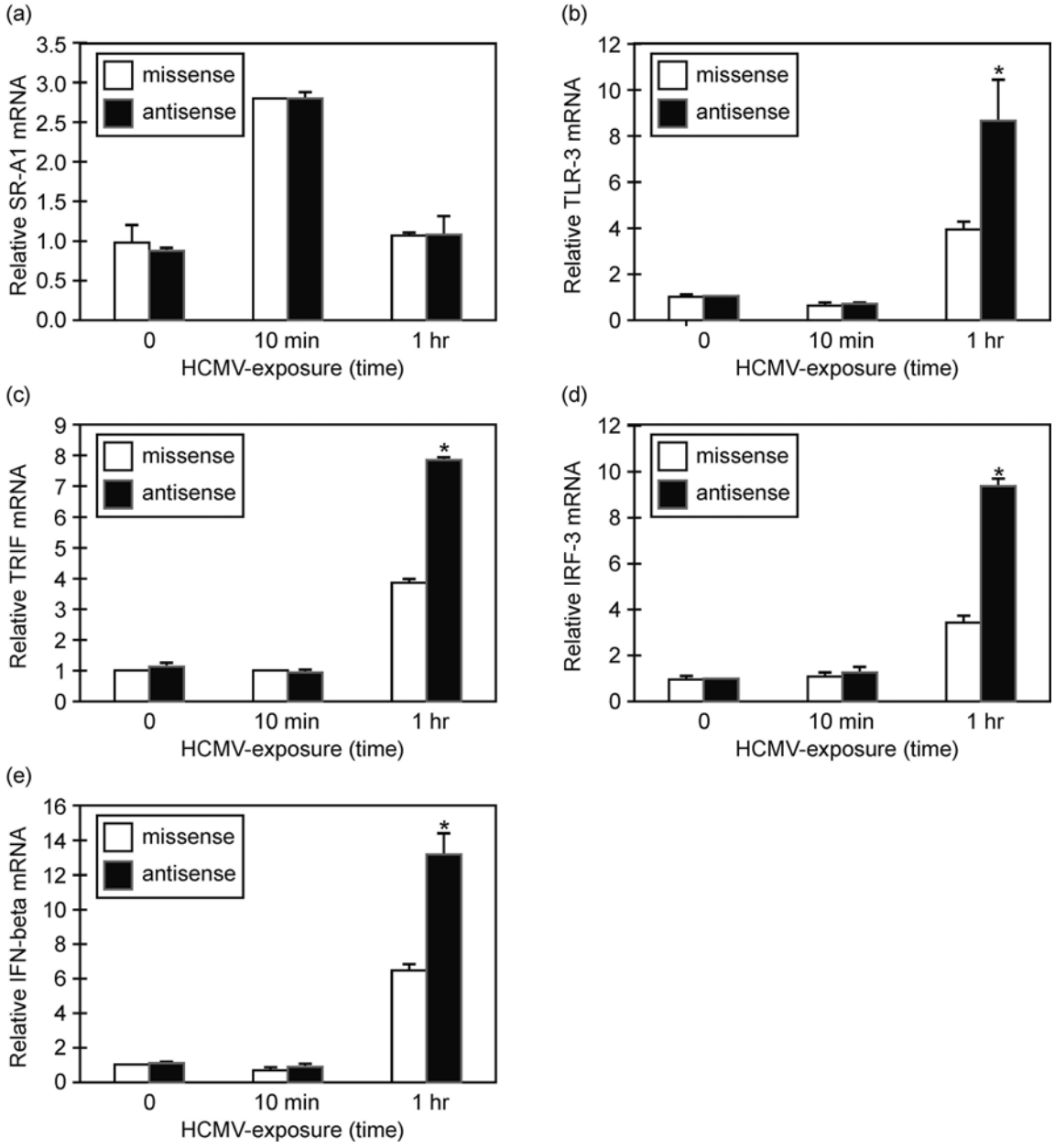

Fig. 2

Effect of blockade of Lyn kinase with antisense oligonucleotide on TLR-3 signaling pathway in THP-1 monocytes

(a) Lyn kinase missense oligonucleotides had no apparent effect on SR-A1, suggesting that SR-A1 is working upstream of Lyn kinase effects. Conversely, TLR-3 (b) and its downstream factors, such as TRIF (*c*), IRF3 (d), and IFN-β (e) showed marked elevation of mRNA levels in response to Lyn kinase missense oligonucleotides, particularly at 1 hr (means ± SD, n = 4). *$p < 0.05$ compared with control at 1 hr; empty columns = missense; full columns = Lyn kinase oligonucleotides.

oligonucleotides, Western blot analysis was performed in antisense oligonucleotide-treated and sense-treated controls, confirming sequence-specific decreases in target Lyn kinases by antisense oligonucleotides (Fig. 4).

Taken together with our previous findings (Yew *et al.*, 2010), a model for the proposed integrated role of SR-A1-mediated Lyn kinase, which might function as an intracellular signaling messenger to induce activation of endosomal TLR-3 and TLR-9 signaling pathways, thereby leading to transcription of cytokine genes, is depicted in Fig. 5. The diagram illustrates a sequence of events whereby HCMV is initially recognized by SR-A1. The interaction between

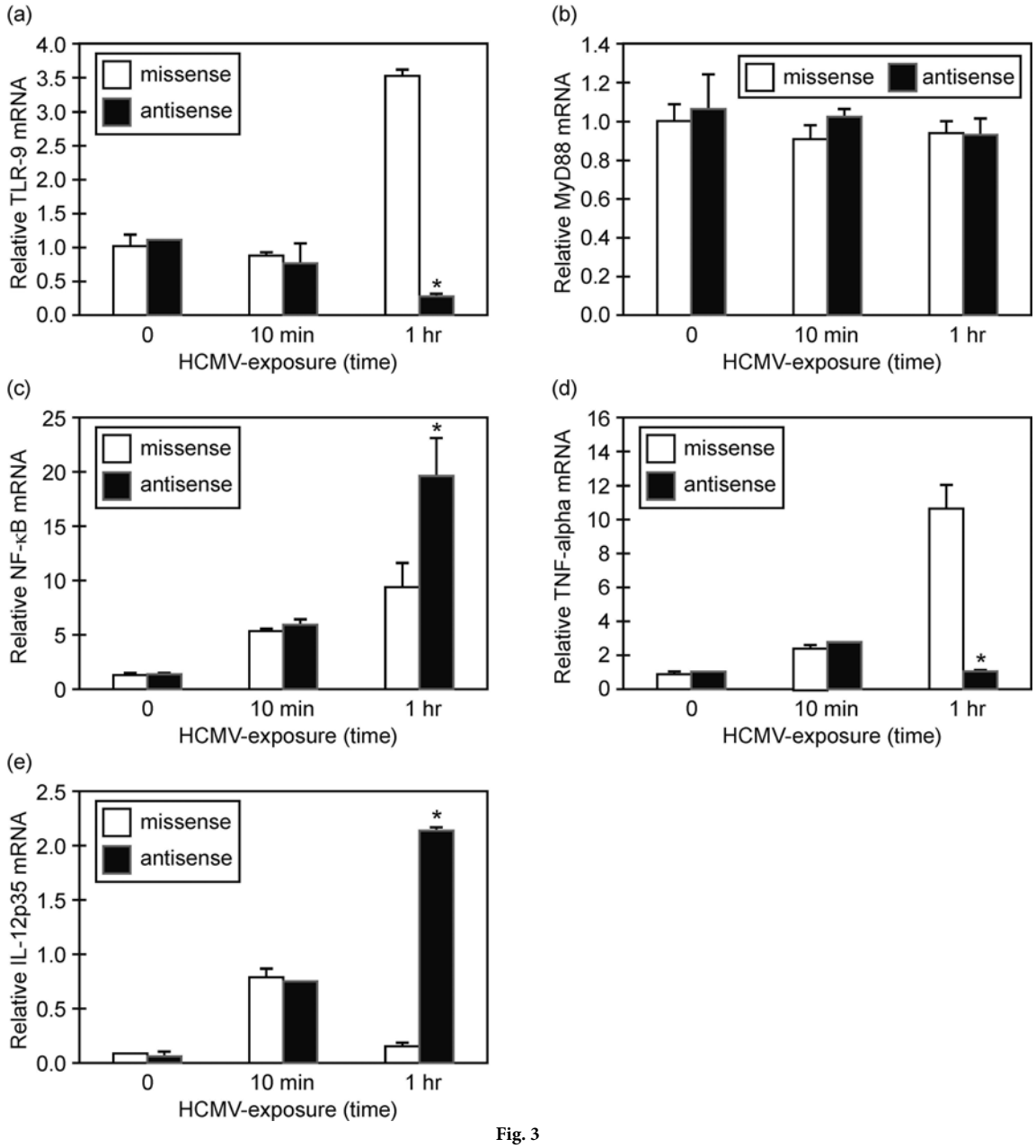

Fig. 3

Effect of blockade of Lyn kinase with antisense oligonucleotide on TLR-9 signaling pathway in THP-1 monocytes

(a) The increase in TLR-9 mRNA levels of THP-1 monocytes at 1 hr in response to HCMV is completely ablated by the missense Lyn kinase oligonucleotides. (b) MYD88, which serves as a universal adapter protein for all TLRs, but TLR-3, to activate transcription factor NF-κB, was not found to be significantly altered by the missense Lyn kinase oligonucleotides. (c-e) Interestingly, missense Lyn kinase oligonucleotides treatment dramatically enhances levels of NF-κB p65 (c) and IL-12p35 (e) at 1 hr. Unlike upregulations of NF-κB p65 and IL-12p35 mRNA, TNF-α mRNA level (d) was downregulated back to baseline (means ± SD, n = 4). *p <0.05 compared with control at 1 hr; empty columns = missense; full columns = Lyn kinase oligonucleotides.

HCMV and SR-A1 triggers activation of Lyn kinase, which in turn serves as an intracellular signaling effector to regulate the activation of the endosomal TLR-9 pathway. The normal activation of NF-κB p65 through MYD-88 by TLR-9, which later induces enhanced TNF-α expression in HCMV-exposed THP-1 monocytes, was not seen. Conversely, when Lyn kinase is blocked, TRIF-mediated IRF3 leading to IFN-β activation triggered by TLR-3 was found to be strongly upregulated upon THP-1 monocyte exposure to HCMV. Thus, we hypothesized that an unidentified SR-A1-mediated kinase exists, comes into play when there is a defect in Lyn kinase activity, and triggers an alternative TLR-3-mediated

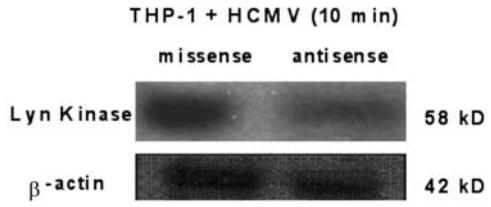

Fig. 4

Western blot analysis for Lyn kinase in HCMV-exposed THP-1 monocytes with Lyn kinase antisense oligonucleotides and missense control

SDS-PAGE and Western blot analysis confirms the inhibitory effects of the antisense oligonucleotide on Lyn kinase levels (n = 3). β-actin served as control for equal loading.

NF-κB p65 induction of IL-12p35. Hence, the observed shift away from TNF-α to a robust effect of IFN-β and IL-12p35 in HCMV-exposed monocytes by Lyn blockade may provide new immunotherapeutic intervention for HCMV congenital infection.

Discussion

Despite a better understanding of the HCMV infection in congenitally infected infants due to recent advances in antenatal and perinatal screening and neuroimaging, late-onset deafness and mental retardation are still unpredictable. During HCMV infection, monocytes and their derivative cells, which represent the first line of defense, are thought to participate in innate immune response by restricting viral dissemination. Experimental studies indicate that recognition or binding of HCMV particles, which can take place either on the cell surface (Hampton *et al.*, 1991; Peiser *et al.*, 2002; Abate *et al.*, 2004; DeWitte-Orr *et al.*, 2010) or within intracellular endosome (Jiang *et al.*, 2004; Matsushima *et al.*, 2004; Jiang *et al.*, 2009), is an early and potent regulatory signal for the activation of pro-inflammatory cytokines. Monocyte-derived cytokines are essential in triggering T-cell response.

The exact mechanism of action, by which HCMV-encoded proteins interact with immune cells and induce

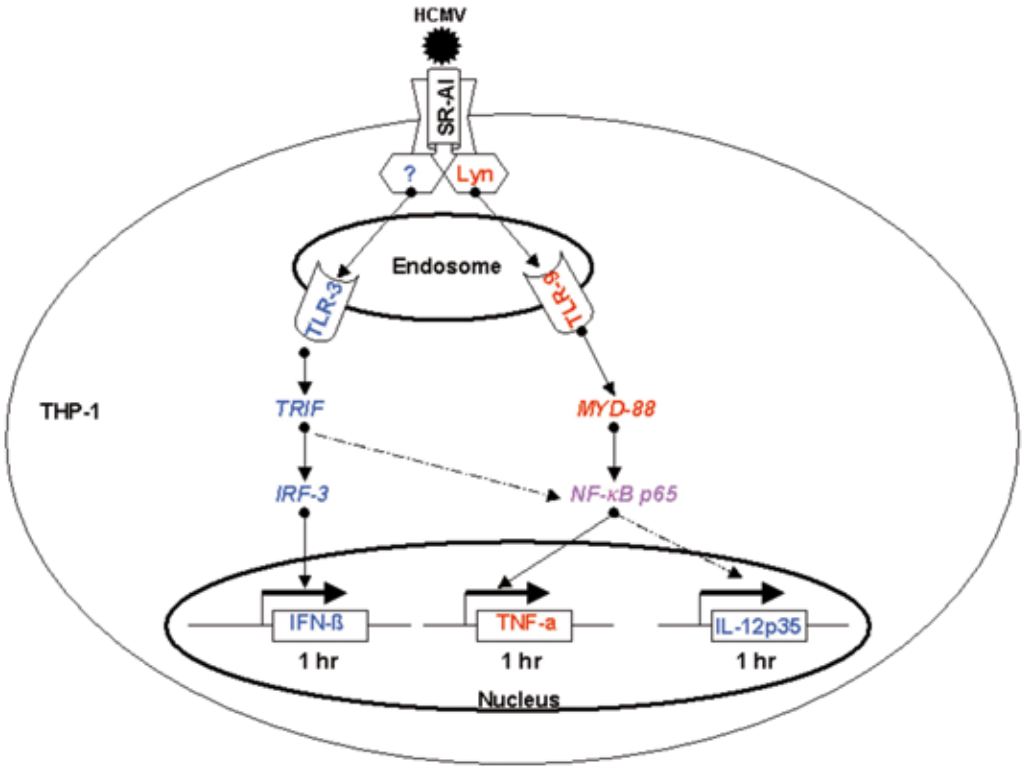

Fig. 5

Schematic diagram of hypothesized interrelationship of SR-A1 and TLR-3/-9 in HCMV-exposed THP-1 monocytes

Together, the diagram illustrates a sequence event whereby HCMV is recognized by SR-A1 and triggers activation of Lyn kinase, which serves as a shuttle molecule for the endosomal TLR-9 pathway. The TLR-9-induced TNF-α is critically regulated by Lyn kinase. However, the transcriptions of canonical TLR-3-induced IFN-β as well as "non-canonical" TLR-3-induced NF-κB-dependent IL-12p35 may possibly be regulated by an unknown SR-A1-mediated protein kinase upon HCMV exposure to THP-1 monocytes.

innate immune response, has not been fully elucidated, but several receptors and protein kinases have been reported to be essential in the early HCMV-induced pro-inflammatory cytokine production leading to a very robust T-cell response. These receptors and kinases include SR-A1 (Limmon *et al.*, 2008; DeWitte-Orr *et al.*, 2010), TLR-3 (Tabeta *et al.*, 2004), TLR-9 (Tabeta *et al.*, 2004; Iversen *et al.*, 2009), Src kinases (Baron *et al.*, 2008), and RIP1-interacting protein (Mack *et al.*, 2008). Overwhelming evidence has accumulated that ligand binding to SR-As triggers activation of intracellular signaling through phosphorylation of phospholipase C (PLC)-γ1, phosphoinositide 3 (PI3)-kinase, and protein kinase C (PKC), which lead to cytokine induction and production (Limmon *et al.*., 2008).

Given the supportive role of Lyn kinase associated with SR-A1 in monocytes (Miki *et al.*, 1996) in many cellular processes including gene differentiation and transcription (Corey *et al.*, 1999), and that SR-A1 has been shown to deliver extracellular viral dsRNA to endosomes (DeWitte-Orr *et al.*, 2010), we sought to determine whether Lyn kinase may mediate a similar effect in HCMV-exposed THP-1 monocytes. Earlier, our group suggested an involvement of SR-A-mediated Lyn kinase in cytokine induction by HCMV-exposed THP-1 monocytes (Yew *et al.*, 2010) as assessed by real time quantitative PCR analysis. Here, we have shown by several different techniques that there is a dramatic increase in Lyn kinase mRNA levels, suggesting that Lyn kinase is an enzyme with a potentially novel function that we observed in the THP-1 monocytes HCMV exposed to HCMV. These results are in agreement with a previous study conducted by Cheung and co-workers (Cheung *et al.*, 2008), who have demonstrated that concomitant activation of Lyn kinase is required for HIV-1 envelope glycoprotein gp120-induced cytokine production in primary human monocyte-derived macrophages.

We previously reported that SR-A1 along with the expression of Lyn kinase is required for sensing HCMV by endosomal TLR-3 and TLR-9, which in turn induce critical pro-inflammatory cytokines (Yew *et al.*, 2010). We found that SR-A1-mediated Lyn kinase blocked by antisense oligonucleotides resulted in greatly decreased mRNA levels of both TLR-9 and TNF-α. The necessity of Lyn kinase for TLR-9 is consistent with recent reports from the Sanjuan group (Sanjuan *et al.*, 2006) that knockdown of Lyn kinase expression or the use of specific kinase inhibitors blocked TLR-9-dependent signaling and cytokine secretion, providing evidence that Lyn tyrosine phosphorylation is an upstream requirement for the activation of TLR-9. Therefore, it seems plausible that the SR-A1-induced Lyn kinase may be a key mediator in regulating the activation of endosomal TLR-9 pathway. In addition, the Sanjuan group also found that knockdown of Lyn kinase resulted in a significant decrease in cellular spreading, adhesion, and motility.

However, the inhibitory effects of Lyn kinase in HCMV-exposed THP-1 monocytes point to an unexpected but unprecedented role for the TLR-3 signaling pathway. We found that the transcriptions of canonical TLR-3-induced IFN-β as well as non-canonical TLR-3-induced NF-κB-dependent IL-12p35 were elevated as a result of Lyn kinase inhibition. The elevated levels of TLR-3-induced IFN-β and IL-12p35 might be triggered by an unidentified SR-A1-mediated enzyme, which replaces the Lyn kinase and could represent the abrupt shift of pathway, possibly via the non-canonical TLR-3 signaling pathway. In agreement with these *in vitro* experiments, Keck and Freudenberg recently reported that Lyn-deficient mice produced higher amounts of pro-inflammatory cytokines than did wild-type mice after injection of LPS (Keck *et al.*, 2010). Other evidence also indicates that Lyn ablation in B-cells induced an enhanced MAP kinase activation (Chan *et al.*, 1997), which may serve as a potential target kinase for future exploration. This possibility is supported by the fact that the regulation of protein phosphorylation requires a balance in the activity of protein kinases and protein phosphatases.

Taken collectively with our previous observations (Yew *et al.*, 2010), the current findings establish a novel paradigm of interaction between SR-A1-mediated Lyn kinase and two endosomal pattern-recognition receptors, TLR-3 and TLR-9, resulting in an inflammatory response. Fig. 5 depicts a hypothetical model of association of SR-A1-mediated Lyn kinase with TLR-9 that accommodates our data. In this study, we provide the first evidence that Lyn kinase is an upstream mediator of the TLR-9 signaling pathway and a key determinant for the regulation of TLR-9 and TNF-α expression in THP-1 monocytes upon HCMV exposure. The model indicates that when Lyn kinase activity is reduced, induction of an unknown SR-A1-mediated enzyme leads to the activation of TRIF and NF-κB to regulate a robust IFN-β and IL-12p35 cytokine response, respectively. The substantial induction of NF-κB-dependent IL-12p35 in response to Lyn kinase inhibition appears to signal via a non-canonical TLR-3 signaling mechanism. Future studies investigating the mechanisms, by which the unidentified SR-A1-mediated kinase interacts directly with TLR-3 or indirectly, through an intermediate adapter, should prove informative and indicate promising areas for further investigation. Ultimately, greater understanding of the mechanisms behind the potential importance of Lyn kinase in TLR-3/-9 signaling will hopefully lead to the development of novel therapeutic regimes targeting HCMV-specific T-cell responses against HCMV disease.

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References

Abate DA, Watanabe S, Mocarski ES (2004): Major human cytomegalovirus structural protein pp65 (ppUL83) prevents interferon response factor 3 activation in the interferon response. J. Virol. 78, 10995–1006. doi.org/10.1128/JVI.78.20.10995–11006.2004

Amiel E, Alonso A, Uematsu S, Akira S, Poynter ME, Berwin B (2008): Pivotal Advance: Toll-like receptor regulation of scavenger receptor-A-mediated phagocytosis. J. Leukoc. Biol. 85, 595–605. doi.org/10.1189/jlb.1008631

Baron M, Davignon JL (2008): Inhibition of IFN-gamma-induced STAT1 tyrosine phosphorylation by human CMV is mediated by SHP2. J. Immunol. 181, 5530–5536.

Bentz GL, Jarquin-Pardo M, Chan G, Smith MS, Sinzger C, Yurochko AD (2006): Human cytomegalovirus (HCMV) infection of endothelial cells promotes naive monocyte extravasation and transfer of productive virus to enhance hematogenous dissemination of HCMV. J. Virol. 80, 11539–1155. doi.org/10.1128/JVI.01016–06

Chan VW, Meng F, Soriano P, DeFranco AL, Lowell CA (1997): Characterization of the B lymphocyte populations in Lyn–deficient mice and the role of Lyn in signal initiation and down–regulation. Immunity 7, 69–81. doi.org/10.1016/S1074–7613(00)80511–7

Cheung R, Ravyn V, Wang L, Ptasznik A, Collman RG (2008): Signaling mechanism of HIV-1 gp120 and virion-induced IL-1beta release in primary human macrophages. J. Immunol. 180, 6675–6684.

Corey SJ, Anderson SM (1999): Src-related protein tyrosine kinases in hematopoiesis. Blood 93, 1–14.

DeWitte-Orr SJ, Collins SE, Bauer CM, Bowdish DM, Mossman KL (2010): An accessory to the Trinity: SR-As are essential pathogen sensors of extracellular dsRNA, mediating entry and leading to subsequent type I IFN responses. PLoS Pathog. 6, e1000829. doi.org/10.1371/journal.ppat.1000829

Hampton RY, Golenbock DT, Penman M, Krieger M, Raetz CR (1991): Recognition and plasma clearance of endotoxin by scavenger receptors. Nature 352, 342–344. doi.org/10.1038/352342a0

Ishiguro T, Naito M, Yamamoto T, Hasegawa G, Gejyo F, Mitsuyama M, Suzuki H, Kodama T (2001): Role of macrophage scavenger receptors in response to Listeria monocytogenes infection in mice. Am. J. Pathol. 158, 179–188. doi.org/10.1016/S0002–9440(10)63956–9

Iversen AC, Steinkjer B, Nilsen N, Bohnhorst J, Moen SH, Vik R, Stephens P, Thomas DW, Benedict CA, Espevik T (2009): A proviral role for CpG in cytomegalovirus infection. J. Immunol. 182, 5672–5681. doi.org/10.4049/jimmunol.0801268

Jiang W, Sun R, Zhou R, Wei H, Tian Z (2009): TLR–9 activation aggravates concanavalin A-induced hepatitis via promoting accumulation and activation of liver CD4+ NKT cells. J. Immunol. 182, 3768–3774. doi.org/10.4049/jimmunol.0800973

Jiang Z, Mak TW, Sen G, Li X (2004): Toll-like receptor 3-mediated activation of NF-kappaB and IRF3 diverges at Toll-IL-1 receptor domain-containing adapter inducing IFN-beta. Proc. Natl. Acad. Sci. USA 101, 3533–3538. doi.org/10.1073/pnas.0308496101

Józefowski S, Sulahian TH, Arredouani M, Kobzik L (2006): Role of scavenger receptor MARCO in macrophage responses to CpG oligodeoxynucleotides. J. Leukoc. Biol. 80, 870–879.

Keck S, Freudenberg M, Huber M (2010): Activation of murine macrophages via TLR2 and TLR4 is negatively regulated by a Lyn/PI3K module and promoted by SHIP1. J. Immunol. 184, 5809–5818. doi.org/10.4049/jimmunol.0901423

Landolfo S, Gariglio M, Gribaudo G, Lembo D (2003): The human cytomegalovirus. Pharmacol. Ther. 98, 269–297. doi.org/10.1016/S0163–7258(03)00034–2

Limmon GV, Arredouani M, McCann KL, Corn Minor RA, Kobzik L, Imani F (2008): Scavenger receptor class-A is a novel cell surface receptor for double-stranded RNA. FASEB J. 22, 159–167. doi.org/10.1096/fj.07–8348com

Mack C, Sickmann A, Lembo D, Brune W (2008): Inhibition of proinflammatory and innate immune signaling pathways by a cytomegalovirus RIP1-interacting protein. Proc. Natl. Acad. Sci. USA 105, 3094–3099. doi.org/10.1073/pnas.0800168105

Matsushima H, Yamada N, Matsue H, Shimada S (2004): TLR3-, TLR7-, and TLR9-mediated production of proinflammatory cytokines and chemokines from murine connective tissue type skin-derived mast cells but not from bone marrow-derived mast cells. J. Immunol. 173, 531–541.

Miki S, Tsukada S, Nakamura Y, Aimoto S, Hojo H, Sato B, Yamamoto M, Miki Y (1996): Functional and possible physical association of scavenger receptor with cytoplasmic tyrosine kinase Lyn in monocytic THP-1-derived macrophages. FEBS Lett. 399, 241–144. doi.org/10.1016/S0014–5793(96)01332–4

Peiser L, Mukhopadhyay S, Gordon S (2002): Scavenger receptors in innate immunity. Curr. Opin. Immunol. 14, 23–128. doi.org/10.1016/S0952–7915(01)00307–7

Pfaffl MW (2001): A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45. doi.org/10.1093/nar/29.9.e45

Sanjuan MA, Rao N, Lai KT, Gu Y, Sun S, Fuchs A, Fung-Leung WP, Colonna M, Karlsson L (2006): CpG-induced tyrosine phosphorylation occurs via a TLR9-independent mechanism and is required for cytokine secretion. J. Cell Biol. 172, 1057–1068. doi.org/10.1083/jcb.200508058

Scanga L, Chaing S, Powell C, Aylsworth AS, Harrell LJ, Henshaw NG, Civalier CJ, Thorne LB, Weck K, Booker J, Gulley ML (2006): Diagnosis of human congenital cytomegalovirus infection by amplification of viral DNA from dried blood spots on perinatal cards. J. Mol. Diagn. 8, 240–245. doi.org/10.2353/jmoldx.2006.050075

Tabeta K, Georgel P, Janssen E, Du X, Hoebe K, Crozat K, Mudd S, Shamel L, Sovath S, Goode J, Alexopoulou L, Flavell RA, Beutler B (2004): Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. Proc. Natl. Acad. Sci. USA 101, 3516–3521. doi.org/10.1073/pnas.0400525101

Thomas CA, Li Y, Kodama T, Suzuki H, Silverstein SC, El Khoury J (2000): Protection from lethal gram-positive infection by macrophage scavenger receptor-dependent phagocytosis. J. Exp. Med. 191, 147–156. doi.org/10.1084/jem.191.1.147

Tran N, Koch A, Berkels R, Boehm O, Zacharowski PA, Baumgarten G, Knuefermann P, Schott M, Kanczkowski W, Bornstein SR, Lightman SL, Zacharowski K (2007): Toll-like receptor 9 expression in murine and human adrenal glands and possible implications during inflammation. J. Clin. Endocrinol. Metab. 92, 2773–2783. doi.org/10.1210/jc.2006–2697

Wang XY, Facciponte J, Chen X, Subjeck JR, Repasky EA (2007): Scavenger receptor-A negatively regulates antitumor immunity. Cancer Res. 67, 4996–5002. doi.org/10.1158/0008–5472.CAN–06–3138

Yew KH, Carsten B, Harrison C (2010): Scavenger receptor A1 is required for sensing HCMV by endosomal TLR-3/-9 in monocytic THP-1 cells. Mol. Immunol. 47, 883–893. doi.org/10.1016/j.molimm.2009.10.009