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Oligo form of Hyaluronic acid suppresses TLR3-dependent cytokine expression in a TLR4-dependent manner

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Margaret Youngah Kim

Committee in Charge:

Professor Richard Gallo, Chair
Professor Michael David, Co-chair
Professor Ananda Goldrath

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The thesis of Margaret Youngah Kim is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

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DEDICATION

I would like to dedicate this thesis to my family

and loved ones who have supported me

throμghout my studies

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ABSTRACT OF THE THESIS

Oligo form of Hyaluronic acid suppresses TLR3-dependent cytokine expression in a TLR4-dependent manner

by

Margaret Youngah Kim

Master of Science in Biology

Professor Richard Gallo, Chair

Fragments of Hyaluronan (HA) that are generated after injury have been proposed to be immunomodulatory and act as regulators of inflammation. Previous studies have found that some pro-inflammatory signals of injury are mediated by self-RNA via TLR3 activation. Conversely, HA fragments released after injury may be immunosuppressive, since addition of HA to macrophages before LPS (TLR4 ligand) significantly decreased IL-6, TNFα, and suppressed sepsis response in mice. In this study we investigated how HA may influence inflammation induced by TLR3 ligands. A pure, synthetic small MW form of HA (oligoHA) was added to MHS cells (mouse alveolar macrophage cell line) that were then activated by poly(I:C). ELISA analysis of culture supernatants showed that the presence of oligoHA in co-treatments suppressed IL-6 and TNFα in comparison to poly(I:C) alone. IL-6 mRNA expression was also suppressed as measured by qPCR. To determine how oligoHA acts on macrophages: WT, *Tlr2-/-* or *Tlr4-/-* mouse-derived macrophages were similarly treated with oligoHA and

poly(I:C). *Tlr2-/-* macrophages responded like wild-type (WT) cells and retained suppression of cytokines while *Tlr4-/-* macrophages did not. An increase in Traf1 (TLR negtative regulator) mRNA was observed after oligoHA treatment of WT but not in *Tlr4-/-* mouse macrophages, and oligoHA did not alter cytokine responsiveness in *Traf1-/-* macrophages. Therefore, our results show that oligoHA released after injury is an immunosuppressant that acts through TLR4 and TRAF1 to inhibit TLR3-dependent inflammation. This observation illustrates the complex immunomodulatory action of innate immune signaling and suggests novel approaches for influencing autoimmune disease.

Chapter 1

INNATE IMMUNITY AND HYALURONAN

Innate Immune System:

The innate immune system is the immediate defender against pathogenic invasion. The innate branch of the immune system consists of cells and their receptors that can recognize pathogens to initiate an immediate immune response against them (Akira 2011). Cells of the innate immune system destroy pathogens as well as mediate immune responses to pathogenic infections. The pattern recognition receptors (PRRs) associated with these cells include the Toll-like receptors (TLRs), cytoplasmic helicases like RIG-I, NOD-like receptors (NLRs) and C-type lectin receptors (Akira 2011).

The various cells of the innate immune system are important for the immediate protection of the host, but the protection granted is short-lived in comparison to the adaptive immune system (Akira 2011). Macrophages are phagocytic cells that can recognize and destroy many different pathogens and foreign particles (Galli, Borregaard et al. 2012). Other cells such as leukocytes and dendritic cells also phagocytose pathogens. Innate immune cells not only protect the host by destruction of bacteria but also through the secretion of various cytokines and chemokines that mediate the immune response (Galli, Borregaard et al. 2012).

In the past, the innate immune system was thought to non-specifically recognize pathogens and engulf them. The innate immune system does not grant long-lasting immunity and was considered a “remnant” from ancient times and in higher vertebrates was replaced with the adaptive immune system (Akira 2011). This paradigm has shifted largely due to toll-like receptors (TLRs) and their ability to specifically recognize pathogens and their role in dendritic cell activation. It is now thought that T-cell activation occurs through antigen presentation of activated dendritic cells and the presence of stimulatory cytokines. It is thought that presentation of antigens by activated dendritic cells is necessary for adaptive immune function (Akira 2011).

Toll-like Receptors:

Toll-like receptors are part of the innate immune system that recognizes highly-conserved pathogenic markers of viruses or bacteria also known as “pathogen associated molecular patterns” (PAMPS) (Liew, Xu et al. 2005). TLRs are membrane-bound receptors on the extracellular and endosomal membranes. Plasma membrane-bound receptors include TLRs 1, 2 and 6 which recognize lipoproteins, TLR4 that recognizes LPS (lipopolysaccharide), and TLR5 recognizes flagellin (Hayashi, Smith et al. 2001). The endosomal TLRs are 3, 7, and 9 which recognize double-stranded RNA, single-stranded RNA and unmethylated CPG-DNA (common in bacteria not mammals) respectively (Hemmi, Takeuchi et al. 2000). TLR activation requires the homo or hetero-dimerization of the TLRs for ligand recognition to initiate responses (Takeda and Akira 2004).

TLRs also respond to damage-associated molecular patterns (DAMPS) that are self molecules produced from damage or cell death (Martinon, Petrilli et al. 2006). DAMPs include a variety of different molecules including “ATP, heparin sulfate, High mobility group protein B1 (HMGB1), and S100 proteins” (Modlin 2012). Self RNA has also been shown by several groups to be recognized by TLRs. Lande et. al have shown that plasmacytoid dendritic cells were able to recognize self-RNA when associated with antimicrobial peptide LL-37 via TLR9 (Lande, Gregorio et al. 2007). Our lab has shown that damaged self RNA via UV damage is detected by TLR3 (Bernard, Cowing-Zitron et al. 2012).

Most downstream signaling of TLRs requires the adaptor Myd88 for induction of type-1 interferons and/or inflammatory cytokines, TLR3 being the exception (Kawai, Adachi et al. 1999). TLR3 and 4 are unique in that TLR3 solely relies on the TRIF pathway to induce its cytokines and TLR4 relies on both Myd88 and TRIF (Yamamoto, Sato et al. 2003). Activation of Myd88 induces inflammatory cytokines through NF-κβ and Traf6 while the TRIF pathway (Myd88 independent pathway) activates IRF3 which induces type 1 interferons and is responsible for antiviral activity (Yamamoto, Sato et al. 2003). Toll-like receptors, though integral to innate host defense, have a pathogenic capacity evident in autoimmune and chronic autoinflammatory disease (Liew, Xu et al. 2005).

Pathogenic Capacity and Negative Regulators of TLRs

The septic shock response (endotoxin shock) is an example of the pathogenic capacity of the TLRs where hyperactivation of the innate immune system induces high amounts of proinflammatory cytokines that

can lead to host death. This occurs through high amounts of inflammation leading to tissue injury, systemic oxygen depletion, reduced blood pressure and ultimately multi-organ failure (Nguyen, Rivers et al. 2006).

Several major players exist in the septic cytokine proinflammatory cascade. IL-6, TNFα, IL1β, and IL-8 are the most strongly associated cytokines to sepsis (Blackwell and Christman 1996). TNF is produced mainly by mononuclear phagocytes. TNF peaks early after the introduction of endotoxin and is a potent inducer of IL-6 and IL-8 (Cannon, Tompkins et al. 1990). Recombinant TNF has also been shown to induce septic-like symptoms (Selby, Hobbs et al. 1987). IL-6 is produced by a variety of cell types and can function as a pyrogen, a substance that induces fever (Dinarello 1989). Peak concentrations of IL-6 after endotoxin introduction is several hours after TNF peak and has been correlated to severity and outcome of septic patients (Selby, Hobbs et al. 1987).

TLR agonists have been found to have the capacity to initiate the septic response in mice. LPS (lipopolysaccharide), a TLR4 agonist, has been shown to be a strong inducer of septic response in mice (Muto, Yamasaki et al. 2009). Poly(I:C) (polyinosinic:polycytidylic acid), the TLR3 agonist, is a synthetic double-stranded RNA that has also been used to induce the septic response (Wen, Lei et al. 2010).Other forms of pathogenic activity of ubiquitous TLR activation include: asthma, lupus, and rheumatoid arthritis (Liew, Xu et al. 2005).

The balance of TLR activity to facilitate host defense while simultaneously preventing pathogenic activity underlies the importance of tight TLR regulation (Liew, Xu et al. 2005). Traf1 and A20 are two examples of negative regulators in the TLR signaling pathway. Traf1 is a negative regulator of the TRIF pathway used by TLR3 and TLR4 which suppresses NF-κβ and IRF3 induced signaling. Traf1 binds to the TIR-domain (Toll/interleukin-1 receptor) of TRIF and is cleaved by a TRIF-induced caspase necessary for cytokine suppression (Vercammen, Staal et al. 2008).

A20 is a more ubiquitous negative regulator activated by LPS and by TNF (Boone, Turer et al. 2004). A20 is expressed in many cell types and its inhibitory function is important in preventing endotoxin shock. A20 has the ability to deubiquitylate Traf6, an important downstream molecule for all TLR signaling. This gives A20 the ability to attenuate not only the Myd88 dependent but also the Myd88-independent pathways (Boone, Turer et al. 2004). Other TLR negative regulators include SOCS1, TOLLIP, PI3K, IRAKM, among others.

Hyaluronan

Hyaluronan (HA) is a high molecular weight glycosaminoglycan composed of repeating disaccharide subunits of D-glucoronic acid and N-acetylglucosamine (figure 1). The sugar is found in highest concentration of soft connective tissue, but is also present in the extracellular matrix of many cell types (Laurent and Fraser 1992). The initial function of HA was mainly thought to be structural, but

this paradigm rapidly changed when receptors to HA and its ability to regulate multiple functions (Laurent and Fraser 1992). Hyaluronan regulation is important both early in development and in various biological processes (e.g. wound healing). Hyaluronan dysfunction during development has shown deficient formations of heart and extremities in chick embryos (Camenisch, Schroeder et al. 2002).

Unlike other glycosaminoglycans, HA is synthesized in the cytoplasm by membrane-bound hyaluronan synthases (HAS) and extruded into the extracellular space where they are found in the million Dalton size range (Saari and Konttinen 1989). Alteration of hyaluronan occurs through hyaluronidases (HYAL) and also thought to breakdown in the presence of reactive oxygen species (Jiang, Liang et al. 2011). Further study has deduced that the function of HA is dependent on its size. It has been shown that fragmented HA mixtures of less than 500kDa induced inflammatory responses, whereas large molecular weight HA mixtures were anti-angiogenic (Deed, Rooney et al. 1997). Clinically, large molecular weight HA has been successfully used as a clinical treatment for arthritis (George 1998).

Hyaluronan has several different binding proteins able to influence immune function such as: CD44, TLRs, etc. CD44 is a widely expressed hyaluronan receptor and plays a role in a variety of different biological mechanisms (Bourguignon, Gilad et al. 2006). HA binding to CD44 has been found during development, inflammation, adaptive immune functions, and tumor growth (Wang and Bourguignon 2006). Mixtures of fragmented HA have been

shown to alter TLR4 signaling by reducing the septic response *in vitro* and *in vivo* in a CD44-dependent manner (Muto, Yamasaki et al. 2009). It has also been shown that CD44 plays an important role in the induction of TLR4 negative regulators (Liang, Jiang et al. 2007). HA has been previously shown to engage a unique receptor complex of CD44 and TLR4 (Taylor, Yamasaki et al. 2007). Hyaluronan fragments have been shown to be DAMPs that bind to TLR2 independently from CD44 and inhibit TLR2 signaling (Scheibner, Lutz et al. 2006).

Previous studies have shown that fragmented HA can modulate TLR4 signaling through a complex with CD44 which reduces the septic response to LPS. Fragmented hyaluronan treatment of C57Bl6 mice was able to rescue treated mice, this was not seen in *CD44-/-* mice (Muto, Yamasaki et al. 2009). Polyinosinic:polycytidylic acid (poly(I:C)) has been shown to also induce inflammatory cytokines associated with the septic response. Though fragmented hyaluronan has been studied, only recently have techniques developed to isolate/synthesize hyaluronan solutions of specific sizes. In this study, we focused on the tetramer form of hyaluronan (oligoHA) on affecting poly(I:C) induced cytokine release.

Chapter 2

RESULTS

Small HA tetramers (oligoHA) suppresses poly(I:C)-induced cytokine secretion in mice

Studies have shown that hyaluronan can bind to TLRs and alter their signaling (Muto, Yamasaki et al. 2009). A mouse model of poly(I:C)-induced shock was used to determine the effects of tetramer oligosaccharides of HA on poly(I:C) signaling. Four groups of C57BL/6 mice (n=4) were injected with 200ul of PBS alone, oligoHA (100μg/mouse) oligoHA and poly(I:C) (50μg/mouse) or poly(I:C). The mice were observed for septic behavioral response: sunken eyes, raised/unkept fur, shivering and slowed movement. Septic behavior response was monitored at 2, 4, 6, and 24 hours after Poly(I:C) injection. PBS and oligoHA injected mice showed no increase in septic behavior compared to either poly(I:C) injected groups (figure 2a). Major differences in response were seen at 4, 6 hours and 24 hours after injection of co-stimulated group compared to poly(I:C) alone injected group (figure 2a). Serum was analyzed by ELISA and showed that oligoHA treated mice had significantly reduced IL-6 (figure 2b) compared to poly(I:C) treated mice after 6h. TNF (figure 2c) was also analyzed and followed a

similar trend to IL-6. These results suggest that oligoHA protects mice from poly(I:C)-induced sepsis.

Hyaluronan oligosaccharide alters macrophage response to poly(I:C) not LPS *in vitro*

In vitro experiments were done to further confirm *in vivo* experimental results. Alveolar macrophage mouse cell lines (MHS cells), were cultured to determine the effect of oligoHA on macrophage cytokine expression. MHS cells were treated with combination of poly(I:C) and/or HA, andIL-6 (figure 3a) and TNF-α (figure 4c) expression was measured. IL-6 and TNFa releases from macrophages were suppressed in 10 μg/ml oligoHA and 10 μg/ml poly(I:C) co-stimulated macrophages compared to the cells treated with poly(I:C) alone . Cytokine mRNA abundance was also measured by quantitative RT-PCR and both IL-6 (figure 4b) and TNF (figure 4d) mRNA were suppressed. These results were consistent with *in vivo* results.

MHS cells were also co-stimulated with LPS (100 ng/ml) and varying concentrations of oligoHA for 24 hours. Supernatant of co-stimulated MHS cells had no observable decrease in IL-6 secretion (figure 3e) . These results showed that oHA could not suppress cytokine release stimulated by LPS treatments .

TLR4 is required for oligoHA to suppress cytokine expression in peritoneal-derived macrophages stimulated with Poly(I:C)

Previous findings have shown that HA is recognized by TLR2 and TLR4 (Jiang, Liang et al. 2011). To further elucidate the receptors involved in the observed phenotype, peritoneal macrophages isolated from *Tlr4-/-* and *Tlr2-/-*

mice were used to observe the differences in poly(I:C)-induced cytokine expressions. Peritoneal macrophages were isolated via peritoneal lavage from both wild-type and knockout mice. Macrophages were plated, cultured, and then treated as previously described. Macrophages from wild-type mice (figure 4a) continued to exhibit suppression of IL-6 in supernatant while *Tlr4-/-* (figure 4b) did not show the same suppression of IL-6. *Tlr2-/-* (figure 4d) macrophages showed same suppression of IL-6 in the supernatant as wild-type (figure 4c) macrophages. These results indicate that the suppression by oligoHA of poly(I:C)-induced cytokine release is dependent on TLR4, not TLR2.

oligoHA induces Traf1 (inhibitor of TLR signaling)

Previous studies have shown that HA can induce negative regulators of TLR signaling (Muto, Yamasaki et al. 2009). To observe whether oligoHA could induce negative regulators of TLR3, MHS cells were treated with 10μg/ml oligoHA at various time points. Induction of negative regulators was measured by RT-qPCR. OligoHA treatment upregulated both Traf1 (figure 5a) and A20 (figure 5b) expression confirming that oligoHA could induce negative regulators of TLR signaling *in vitro* . Wild-type macrophages treated with oligoHA also showed induction of Traf1 (figure 5c) and A20 (figure 5d). These results confirmed that oligoHA treatment could induce negative regulator expression in primary cells

Traf1 is necessary for oligoHA to inhibit TLR3 signaling

Further experiments were performed to elucidate the dependence on Traf1 for oligoHA-mediated cytokine suppression. WT and *Traf1-/-* macrophages were treated similarly with oligoHA and poly(I:C) as in the previous experiments.

Traf1-/- macrophages did not have suppressed cytokine expression of IL-6 (figure 6b) in presence of oligoHA, underlying the involvement of Traf1. This was also shown at the mRNA level, where IL-6 induction was suppressed in wild-type (figure 6c), but not *Traf1-/-* cells (figure 6d). TNF followed a similar trend as IL-6 with suppression in wild-type, but not in the *Traf1-/-* cells by ELISA (figure 6d,e) and qPCR (figure 6f,g). These data s μ guest that Traf1 was involved in oligoHA-mediated suppression of Poly(I:C) stimulated cytokine expressions .

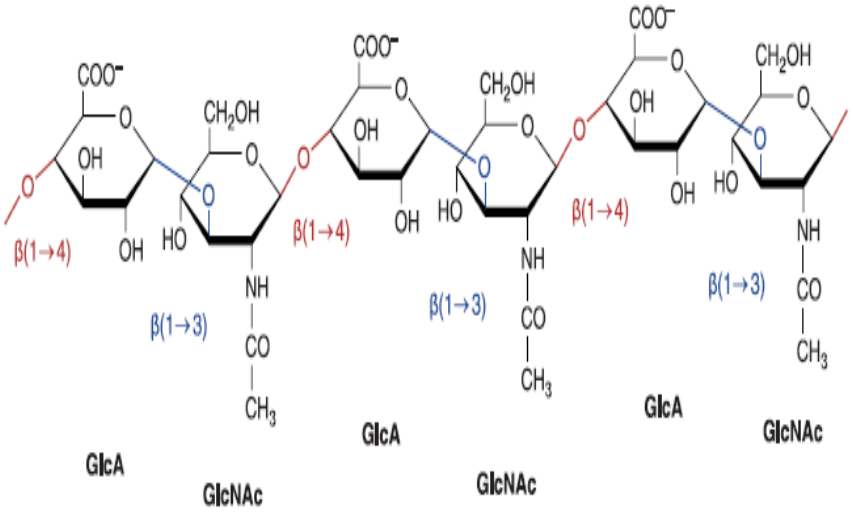

Figure 1. Structure and Function of Hyaluronan
Molecular structure of hyaluronan consisting of D-glucoronic acid and N-acetylglucosamine. Modified from Jian et. al 2007 (Hyaluronan in tissue injury and repair)

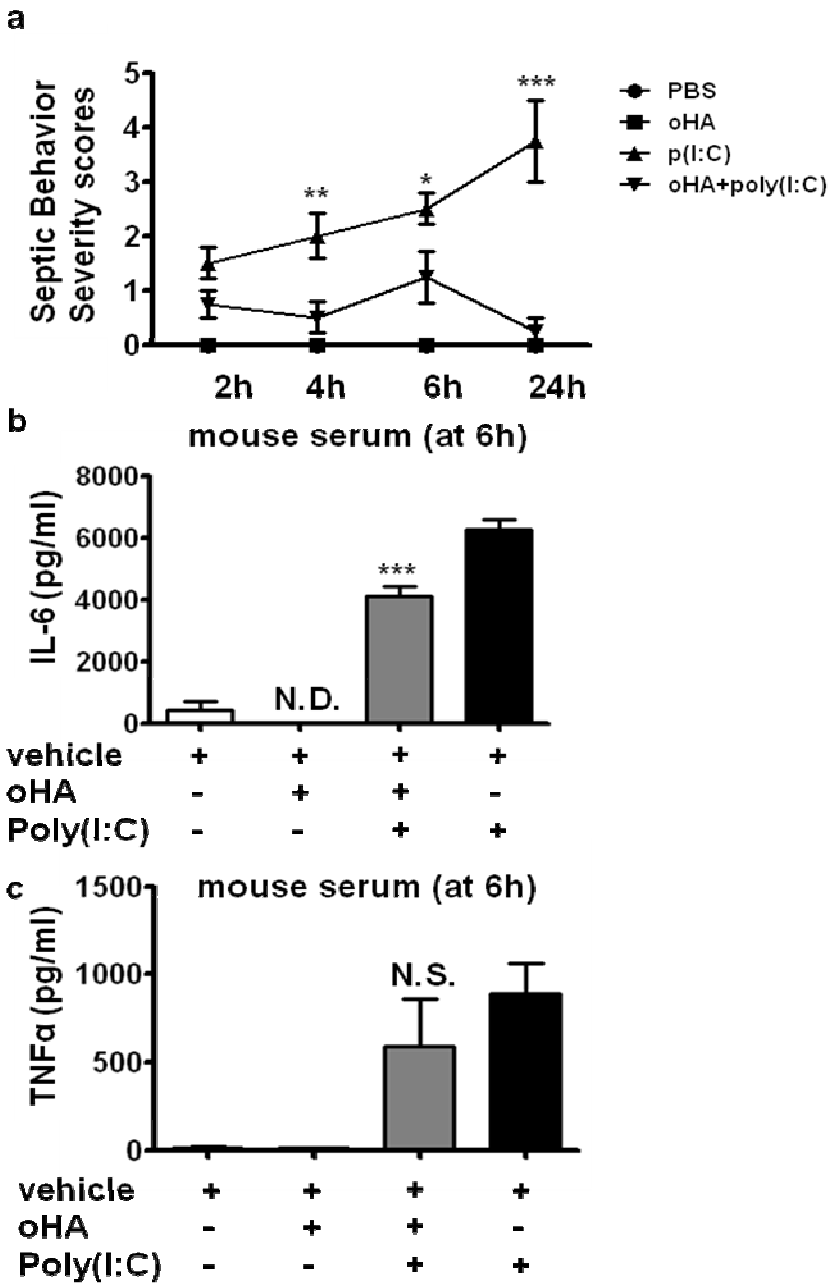

Figure 2. Oligo-HA protects mice from poly(I:C) induced shock response and inhibits cytokine release

(a,b) Mice were injected i.p. with PBS, oHA (100 μg/ mouse), oHA and poly(I:C) (50 μg/ mouse), or poly(I:C). (a) Septic behavior severity scales were scored as described in Materials and Methods, n=4. (b) Serum IL-6 (6h after injection) measured by ELISA. (c) serum TNFα (6h after injection) measured by ELISA. Data are presented as mean ± SEM. *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$, N.D., not detected, N.S., not significant.

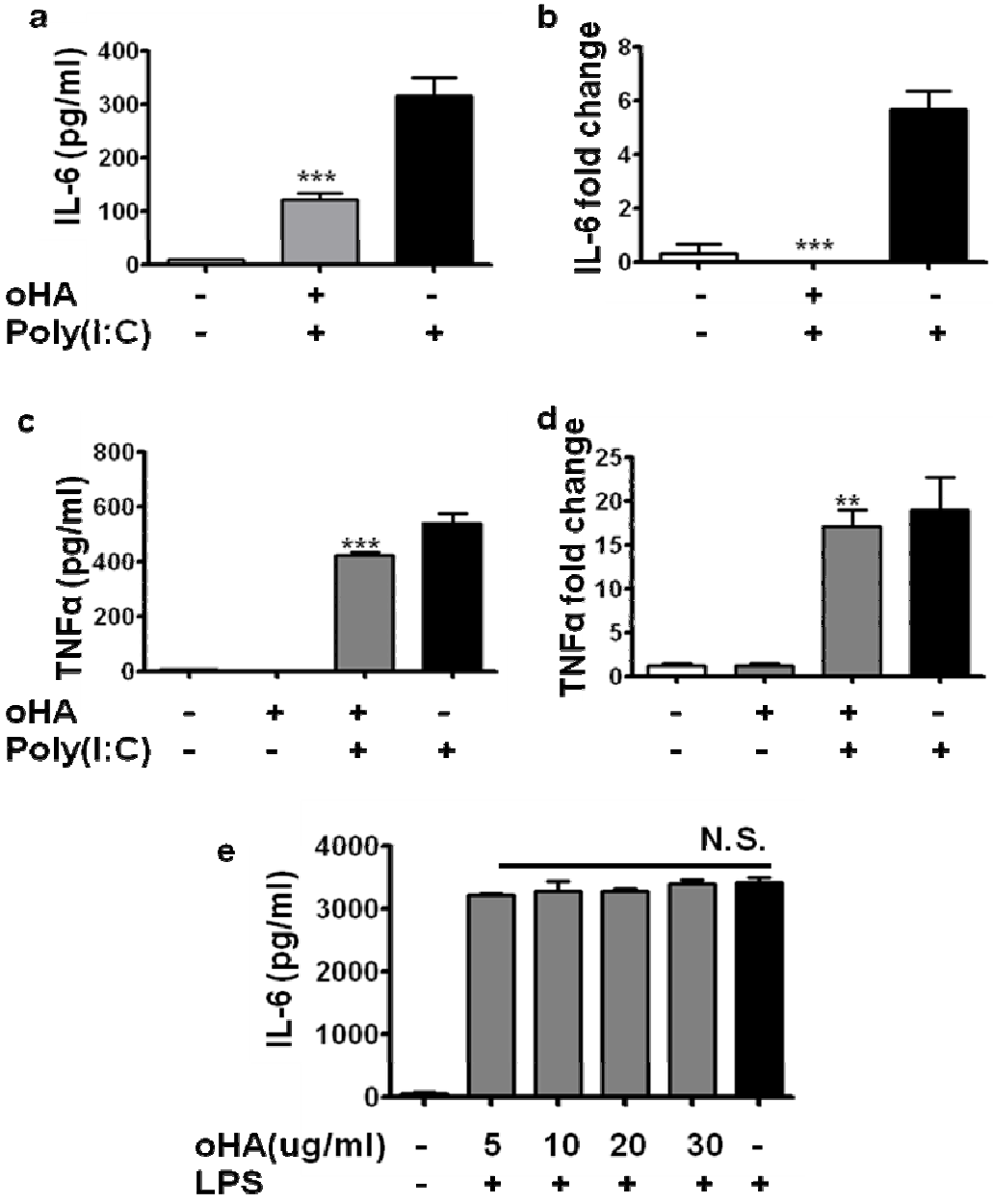

Figure 3. OligoHA suppresses poly(I:C) induced, but not LPS-induced cytokine release in MH-S cells.
(a,b) IL-6 and (c,d) TNF measured by ELISA or by quantitative RT-PCR . Cells were treated with 10μg/ml oHA +10μg/ml poly(I:C), Poly(I:C) or vehicle alone for 24h (ELLISA) or 4h (qPCR). (e) 100ng/ml LPS treatment of MH-S cells treated for 24h IL-6 measured by ELISA. Data are mean ± SEM of three treatments per group and one experiment representative of three independent experiments. *** $p<0.001$. ** $p<0.01$. N.S., not signfiicant.

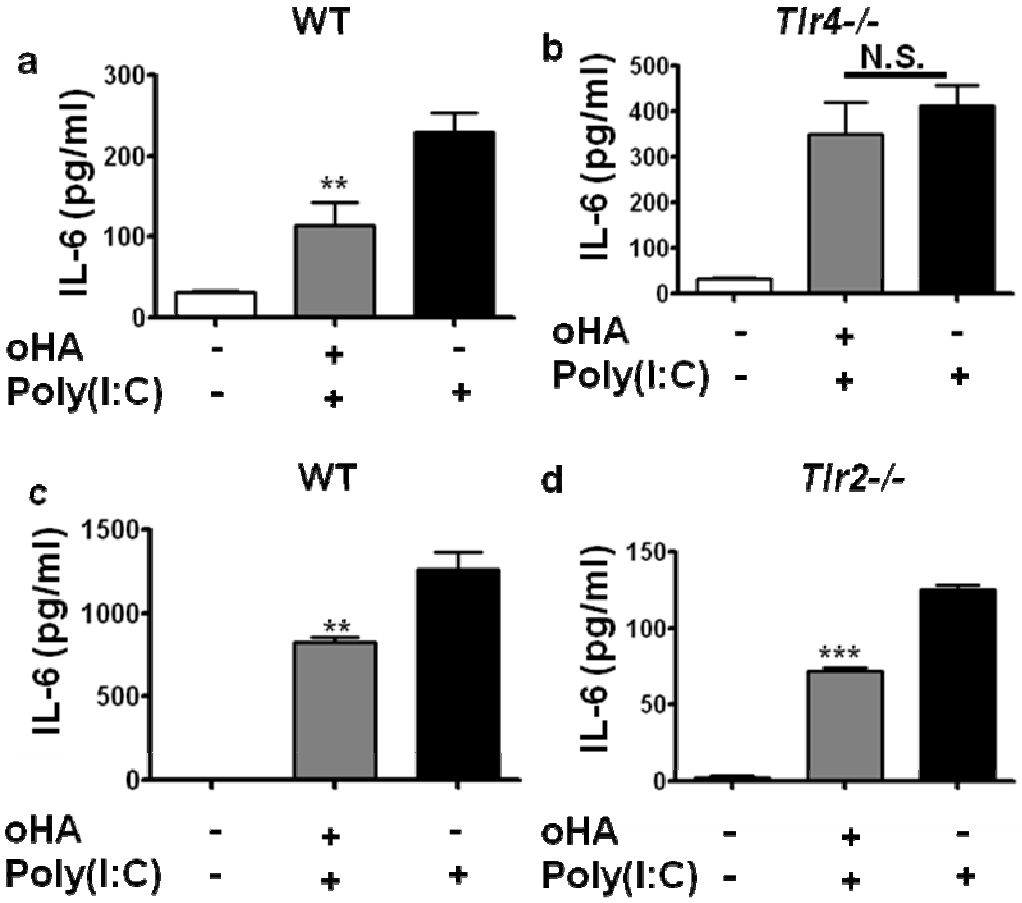

Figure 4. TLR4 is required for oHA to suppress cytokine secretion and expression in peritoneal macrophages

(a-d) IL-6 measured by ELISA in the cultured medium of (a,c) C57Bl6, (b) *Tlr4-/-* and (d) *Tlr2-/-* peritoneal macrophages. Macrophages were treated with 10µg/ml oHA+10µg/ml poly(I:C) , Poly(I:C) alone or vehicle (PBS) for 24h before media collection. Data are mean ± SEM of three treatments per group and one experiment representative of three independent experiments. ** $p<0.01$, N.S: not significant.

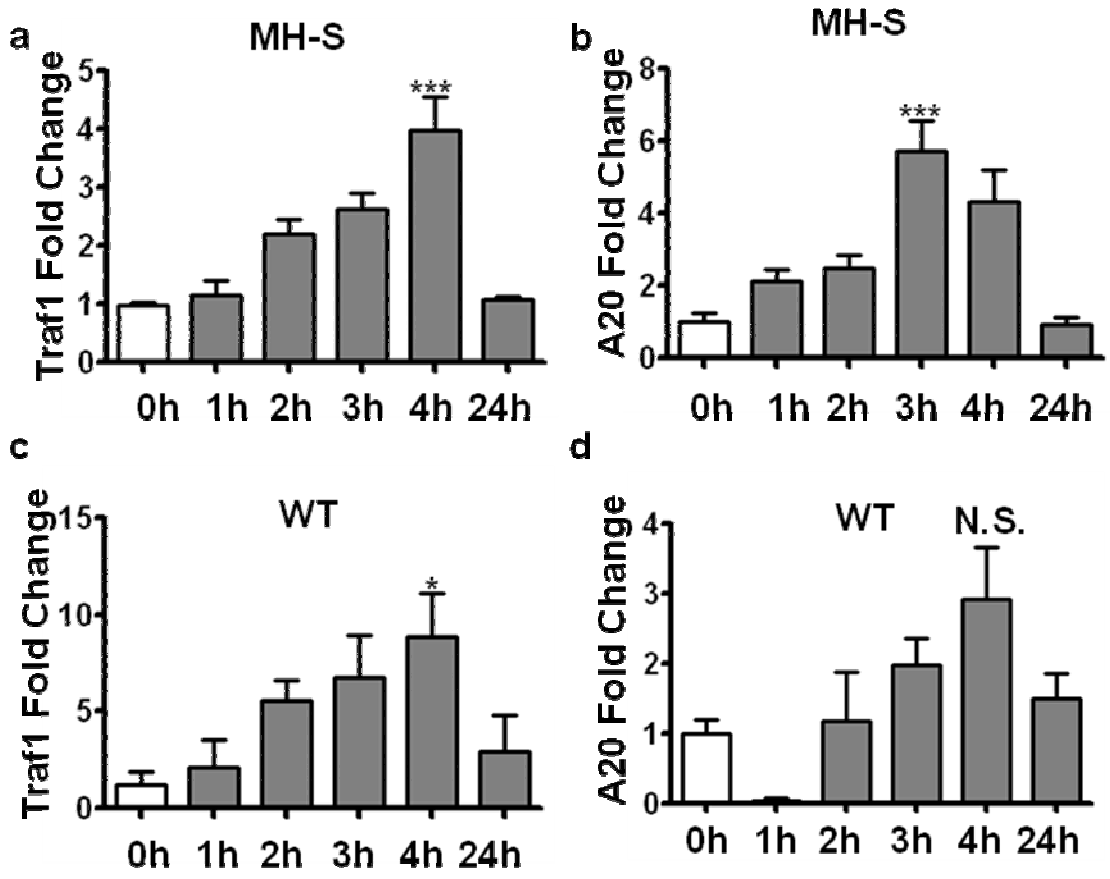

Figure 5. OligoHA induces inhibitors of TLR signaling in control but not *Tlr4-/-* cells.
(a,b) MH-S cells and (c,d) WT (C57Bl/6) peritoneal macrophages were treated with 10µg/ml oHA or vehicle control for indicated time points. Traf1 and A20 mRNA expressions were assessed by quantitative RT-PCR. Data are shown as relative expression compared to untreated macrophages (0 h). *:*P<0.05;* ***:*p<0.001*; N.S: not significant

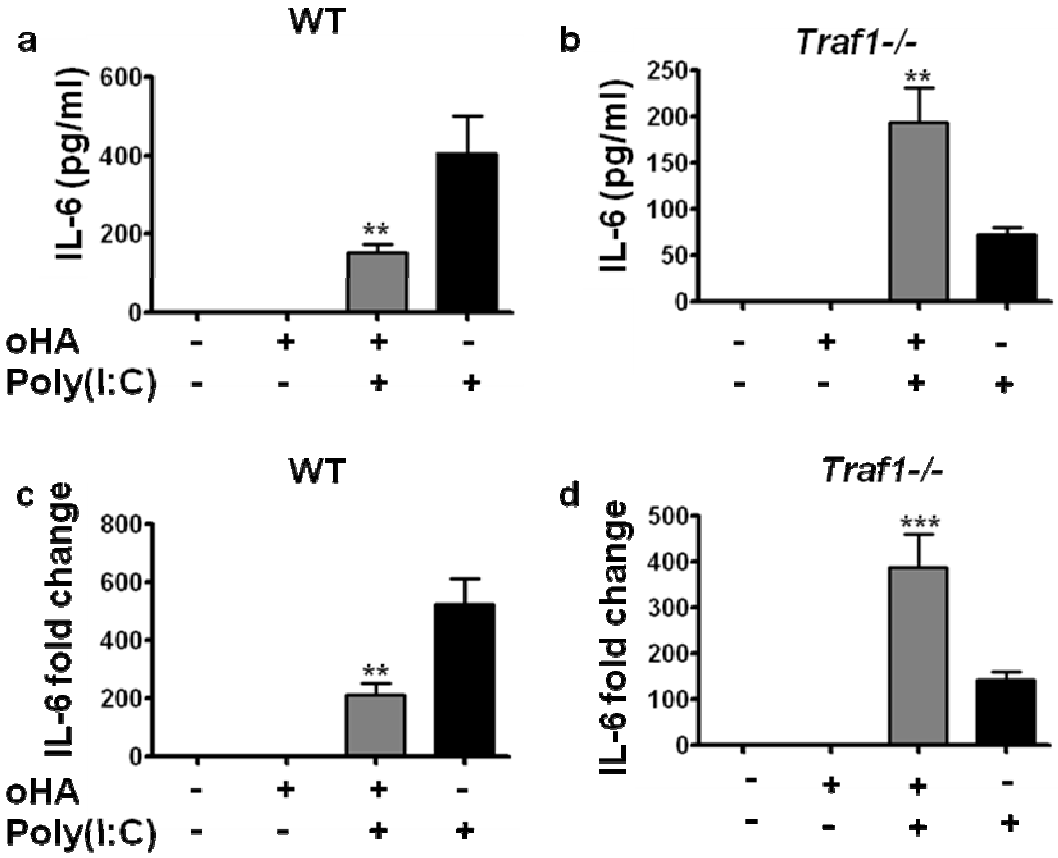

Figure 6. Traf1 is necessary for oHA to suppress TLR3 signaling
Peritoneal macrophages from (a,c,e,g) wild-type, (b,d,f,h)*Traf1-/-* , were co-treated with oHA and poly(I:C). IL-6 (a,b) and TNF (e,f) measured by ELISA in cultured medium. IL-6 (b,d) and TNF (f,h) assessed by Quantitative RT-PCR after4h. Quantitative RT-PCR data are shown as relative expression compared to vehicle treated macrophages. Data are presented as mean ± SEM of 3 samples per group of one experiment representative of three independent experiments. . * $p<0.05$. ** $p<0.01$. ***$p<0.001$. N.S: not significant.

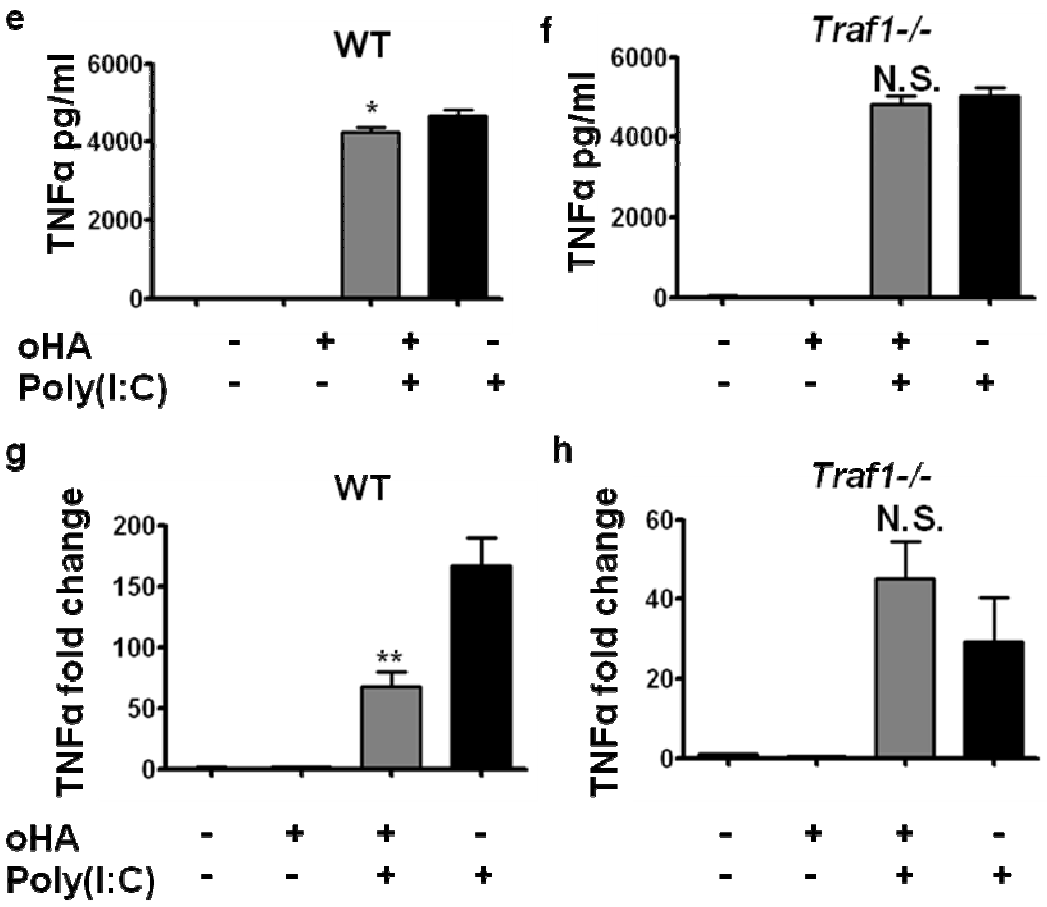

Figure 6 continued

Chapter 3

DISCUSSION

Septic shock remains to be a large problem in medicine with mortality rates of close to 50% of those affected by severe sepsis (Nguyen, Rivers et al. 2006). This study demonstrates that oligoHA protects mice from poly(I:C)-induced septic shock by suppressing cytokine secretion in serum. Data suggests that inhibition of poly(I:C)-induced cytokine expression is due to the induction of Traf1 which inhibits the TRIF pathway after caspase processing (Vercammen, Staal et al. 2008). The absence of Traf1 diminished the observed phenotype and no longer reduced cytokine upregulation in macrophages. Double-stranded RNA has been shown to be a marker for cell damage, and poly(I:C) has been used as a mimic for cell damage (Lin, Fang et al. 2011). Therefore, oligoHA may be responsible for regulating host responses to wounding and inhibit the septic response through induction of Traf1.

It is known that the function of HA is dependent on its molecular size, but specific function has not been correlated with particular size (Jiang, Liang et al. 2011). Intermediate sizes of HA, 200-500kDa, have been shown to induce inflammatory cytokines (Yamasaki, Muto et al. 2009). Small oligosaccharide fragments (tetra- and hexasaccharide) have been shown to induce

immunophenotypic maturation of human monocyte-derived dendritic cells (Jiang, Liang et al. 2011). Fragmented HA has also been shown to be a “danger singal” associated with sterile injury (Yamasaki, Muto et al. 2009). We used the four sugar (tetramer) form of HA to determine its effects on TLR signaling and found that oligoHA suppresses the cytokine response *in vivo* and *in vitro* which may imply other effects on wounding.

The ability of oligoHA to induce negative regulator Traf1 and suppress cytokine induction under wounding conditions has therapeutic implications, especially in relation to the skin. During wounding, fragments of HA, including oligoHA, reside locally where damage initially occurs and may limit inflammation. This may benefit the host in regulating local inflammation in response to the wound in order to prevent overactivation of the innate immune system and excessive local damage.

In summary, data confirms the ability of oligoHA in altering the inflammatory response to poly(I:C) in a TLR4 dependent manner. The suppression seems to be dependent on the induction of Traf1, a negative regulator of TLR3. These results show the capacity of oligoHA in altering the immune response to wounding and the septic response. Therefore, oligoHA may be an important player in regulating host inflammatory responses to injury.

Implications for the Future

Te study of oligoHA can also be further defined for areas of interest beyond the mouse model of septic shock. Therapeutic use of oligoHA could be

administered intravenously or the peritoneal cavity to reduce inflammatory cytokines and improve chances of survival. Though the implications of the therapeutic use of oligoHA in reducing the inflammatory response are vast, more work in understanding the wound healing process must be done. Septic shock is an example of the overactivation of the inflammatory response that is detrimental to the host, but too little inflammation can delay wound healing (Lin, Fang et al. 2011).

Lin et al. have shown that ubiquitous suppression of the inflammatory response is also detrimental in host wound healing via impaired recruitment of myeloid cells (Lin, Fang et al. 2011). The impaired recruitment of myeloid cells is due to decreased chemokine expression due to reduced inflammation. Understanding the role that HA sizes plays for both inflammation and wound healing will be integral in uncovering more thereapeutic uses for HA. Furthermore, greater understanding of the mechanisms of inflammation wound healing is necessary before oligoHA can be used a therapeutic agent.

Chapter 4

MATERIALS AND METHODS

Materials and Methods

The mouse alveolar cell line MH-S was purchased from American Type Culture Collection (ATCC, catalog CRL-2019). Cells were maintained in RPMI1640 media which contained 10% heat-inactivated fetal calf serum (FCS), penicillin/streptomycin (100 units/ml and 50 mg/ml, respectively), and 50uM 2-Mercaptoethanol. Oligo hyaluronan (tetrasaccharide) was purchased from Hyalose. HA preparations did not absorb at 260 and 280nm which shows no DNA or protein contaminations. Poly(I:C) was obtained from Invitrogen Carlsbad, CA . C57Bl/6J mice were purchased from Jackson Laboratories. TLR4-deficient mice (TLR4-/-) were originally generated by someone. Traf1-deficient mice (Traf1-/-) were originally generated by someone. Age and sex-matched animals were used for experiments.

Endotoxic Shock studies

Age and sex-matched female wild-type mice were used. Mice were injected with 100µg/mouse HA or PBS intraperitoneally (i.p.) as a pretreatment. One hour after pretreatment, mice were then injected with 100µg/mouse poly(I:C) or PBS i.p. to induce shock.

Peritoneal macrophage isolation

Age and sex-matched wild-type, TLR2-/-, TLR4-/-, and Traf1-/- were injected i.p. with 3% thioglycolate. After 72 hours peritoneal macrophages were collected by peritoneal lavage and plated on to 24-well flat bottom plates at 30 x 10^6 cell/well in 10% FCS RPMI1640 media. Cells were treated once they reached 80% confluency; supernatants and cells were collected after treatments.

***In vitro*/*Ex Vivo* cell stimulation**

Macrophages were grown to 80% confluence in 24-well flat bottom plates (Corning Incorporated Life Sciences, Lowell, MA). Cells were initially grown in 10% FCS media which was removed from cells and replaced with low serum media (1% FCS) containing either poly(I:C) or HA at the indicated concentrations and time points. After stimulation, cells were allowed to incubate after which media was collected and stored at -20˚C until analysis. RNA was extracted from cells after supernatant collection using TRIzol reagent (Invitrogen Carlsbad, CA) and stored at -20˚C until cDNA was made stored at 4˚C.

Quantitative PCR

Quantitative RT-PCR was used to determine the induction of IL-6, TNF-α, Traf1, A20 mRNA after stimulation with oligo-HA and poly(I:C) or oligo-HA alone, cDNA was synthesized as specified by iScript cDNA synthesis kit (BioRad) instructions. TaqMan Gene Expression Assays were used to analyze expression of IL-6, TNF-α, Traf1, and A20 per instructions. Gapdh mRNA was used as an internal control to validate RNA for each sample. All data are presented as normalized data against each control (mean non-stimulated cells).

Analysis of supernatants and sera by ELISA

Supernatant or mouse serum was analyzed for species-specific IL-6 and TNF-a through ELISA (BioRad)

Statistical analysis

One-way ANOVA with Turkey post-test was used to determine significance in the experiments, analyzed through Graphpad Prism 5.

REFERENCES

Akira, S. (2011). "Innate immunity and adjuvants." Philos Trans R Soc Lond B Biol Sci **366**(1579): 2748-55.

Bernard, J. J., C. Cowing-Zitron, T. Nakatsuji, B. Muehleisen, J. Muto, A. W. Borkowski, L. Martinez, E. L. Greidinger, B. D. Yu and R. L. Gallo (2012). "Ultraviolet radiation damages self noncoding RNA and is detected by TLR3." Nat Med.

Blackwell, T. S. and J. W. Christman (1996). "Sepsis and cytokines: current status." Br J Anaesth **77**(1): 110-7.

Boone, D. L., E. E. Turer, E. G. Lee, R. C. Ahmad, M. T. Wheeler, C. Tsui, P. Hurley, M. Chien, S. Chai, O. Hitotsumatsu, E. McNally, C. Pickart and A. Ma (2004). "The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses." Nat Immunol **5**(10): 1052-60.

Bourguignon, L. Y., E. Gilad, A. Brightman, F. Diedrich and P. Singleton (2006). "Hyaluronan-CD44 interaction with leukemia-associated RhoGEF and epidermal growth factor receptor promotes Rho/Ras co-activation, phospholipase C epsilon-Ca2+ signaling, and cytoskeleton modification in head and neck squamous cell carcinoma cells." J Biol Chem **281**(20): 14026-40.

Camenisch, T. D., J. A. Schroeder, J. Bradley, S. E. Klewer and J. A. McDonald (2002). "Heart-valve mesenchyme formation is dependent on hyaluronan-augmented activation of ErbB2-ErbB3 receptors." Nat Med **8**(8): 850-5.

Cannon, J. G., R. G. Tompkins, J. A. Gelfand, H. R. Michie, G. G. Stanford, J. W. van der Meer, S. Endres, G. Lonnemann, J. Corsetti, B. Chernow and et al. (1990). "Circulating interleukin-1 and tumor necrosis factor in septic shock and experimental endotoxin fever." J Infect Dis **161**(1): 79-84.

Deed, R., P. Rooney, P. Kumar, J. D. Norton, J. Smith, A. J. Freemont and S. Kumar (1997). "Early-response gene signalling is induced by angiogenic oligosaccharides of hyaluronan in endothelial cells. Inhibition by non-angiogenic, high-molecular-weight hyaluronan." Int J Cancer **71**(2): 251-6.

Dinarello, C. A. (1989). "The endogenous pyrogens in host-defense interactions." Hosp Pract (Off Ed) **24**(11): 111-5, 118, 121 passim.

Galli, S. J., N. Borregaard and T. A. Wynn (2012). "Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils." Nat Immunol **12**(11): 1035-44.

George, E. (1998). "Intra-articular hyaluronan treatment for osteoarthritis." Ann Rheum Dis **57**(11): 637-40.

Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill and A. Aderem (2001). "The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5." Nature **410**(6832): 1099-103.

Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda and S. Akira (2000). "A Toll-like receptor recognizes bacterial DNA." Nature **408**(6813): 740-5.

Jiang, D., J. Liang and P. W. Noble (2011). "Hyaluronan as an immune regulator in human diseases." Physiol Rev **91**(1): 221-64.

Kawai, T., O. Adachi, T. Ogawa, K. Takeda and S. Akira (1999). "Unresponsiveness of MyD88-deficient mice to endotoxin." Immunity **11**(1): 115-22.

Lande, R., J. Gregorio, V. Facchinetti, B. Chatterjee, Y. H. Wang, B. Homey, W. Cao, B. Su, F. O. Nestle, T. Zal, I. Mellman, J. M. Schroder, Y. J. Liu and M. Gilliet (2007). "Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide." Nature **449**(7162): 564-9.

Laurent, T. C. and J. R. Fraser (1992). "Hyaluronan." FASEB J **6**(7): 2397-404.

Liang, J., D. Jiang, J. Griffith, S. Yu, J. Fan, X. Zhao, R. Bucala and P. W. Noble (2007). "CD44 is a negative regulator of acute pulmonary inflammation and lipopolysaccharide-TLR signaling in mouse macrophages." J Immunol **178**(4): 2469-75.

Liew, F. Y., D. Xu, E. K. Brint and L. A. O'Neill (2005). "Negative regulation of toll-like receptor-mediated immune responses." Nat Rev Immunol **5**(6): 446-58.

Lin, Q., D. Fang, J. Fang, X. Ren, X. Yang, F. Wen and S. B. Su (2011). "Impaired wound healing with defective expression of chemokines and recruitment of myeloid cells in TLR3-deficient mice." J Immunol **186**(6): 3710-7.

Martinon, F., V. Petrilli, A. Mayor, A. Tardivel and J. Tschopp (2006). "Gout-associated uric acid crystals activate the NALP3 inflammasome." Nature **440**(7081): 237-41.

Modlin, R. L. (2012). "Innate immunity: ignored for decades, but not forgotten." J Invest Dermatol **132**(3 Pt 2): 882-6.

Muto, J., K. Yamasaki, K. R. Taylor and R. L. Gallo (2009). "Engagement of CD44 by hyaluronan suppresses TLR4 signaling and the septic response to LPS." Mol Immunol **47**(2-3): 449-56.

Nguyen, H. B., E. P. Rivers, F. M. Abrahamian, G. J. Moran, E. Abraham, S. Trzeciak, D. T. Huang, T. Osborn, D. Stevens and D. A. Talan (2006). "Severe sepsis and septic shock: review of the literature and emergency department management guidelines." Ann Emerg Med **48**(1): 28-54.

Saari, H. and Y. T. Konttinen (1989). "Determination of synovial fluid hyaluronate concentration and polymerisation by high performance liquid chromatography." Ann Rheum Dis **48**(7): 565-70.

Scheibner, K. A., M. A. Lutz, S. Boodoo, M. J. Fenton, J. D. Powell and M. R. Horton (2006). "Hyaluronan fragments act as an endogenous danger signal by engaging TLR2." J Immunol **177**(2): 1272-81.

Selby, P., S. Hobbs, C. Viner, E. Jackson, A. Jones, D. Newell, A. H. Calvert, T. McElwain, K. Fearon, J. Humphreys and et al. (1987). "Tumour necrosis factor in man: clinical and biological observations." Br J Cancer **56**(6): 803-8.

Takeda, K. and S. Akira (2004). "TLR signaling pathways." Semin Immunol **16**(1): 3-9.

Taylor, K. R., K. Yamasaki, K. A. Radek, A. Di Nardo, H. Goodarzi, D. Golenbock, B. Beutler and R. L. Gallo (2007). "Recognition of hyaluronan released in sterile injury involves a unique receptor complex dependent on Toll-like receptor 4, CD44, and MD-2." J Biol Chem **282**(25): 18265-75.

Vercammen, E., J. Staal and R. Beyaert (2008). "Sensing of viral infection and activation of innate immunity by toll-like receptor 3." Clin Microbiol Rev **21**(1): 13-25.

Wang, S. J. and L. Y. Bourguignon (2006). "Hyaluronan and the interaction between CD44 and epidermal growth factor receptor in oncogenic

signaling and chemotherapy resistance in head and neck cancer." Arch Otolaryngol Head Neck Surg **132**(7): 771-8.

Wen, H., Y. Lei, S. Y. Eun and J. P. Ting (2010). "Plexin-A4-semaphorin 3A signaling is required for Toll-like receptor- and sepsis-induced cytokine storm." J Exp Med **207**(13): 2943-57.

Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda and S. Akira (2003). "Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway." Science **301**(5633): 640-3.

Yamasaki, K., J. Muto, K. R. Taylor, A. L. Cogen, D. Audish, J. Bertin, E. P. Grant, A. J. Coyle, A. Misaghi, H. M. Hoffman and R. L. Gallo (2009). "NLRP3/cryopyrin is necessary for interleukin-1beta (IL-1beta) release in response to hyaluronan, an endogenous trigger of inflammation in response to injury." J Biol Chem **284**(19): 12762-71.