TLR5 participates in the TLR4 receptor complex and biases towards MyD88-dependent signaling in environmental lung injury

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Tlr5 participates in Tlr4 signaling

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Abstract

Lung disease causes significant morbidity and mortality, and is exacerbated by environmental injury, e.g. through lipopolysaccharide (LPS) or ozone (O_3). Toll-like receptors (TLRs) orchestrate immune responses to injury by recognizing pathogen- or danger-associated molecular patterns. TLR4, the prototypic receptor for LPS, also mediates inflammation after O_3, triggered by endogenous hyaluronan. Regulation of TLR4 signaling is incompletely understood. TLR5, the flagellin receptor, is expressed in alveolar macrophages, and regulates immune responses to environmental injury. Using *in vivo* animal models of TLR4-mediated inflammations (LPS, O_3, hyaluronan), we show that TLR5 impacts the *in vivo* response to LPS, hyaluronan and O_3. We demonstrate that immune cells of human carriers of a dominant negative TLR5 allele have decreased inflammatory response to O_3 exposure *ex vivo* and LPS exposure *in vitro*. Using primary murine macrophages, we find that TLR5 physically associates with TLR4 and biases TLR4 signaling towards the MyD88 pathway. Our results suggest an updated paradigm for TLR4/TLR5 signaling.

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Introduction

Lung disease is a major contributor to morbidity and mortality worldwide. In the US alone, over 15% of the population suffers from lung disease, at an annual cost of 120,000 deaths, and >$50 billion (1-3). Environmental lung injury, e.g. through inhaled lipopolysaccharide (LPS) or elevated ozone (O_3) levels, exacerbates lung disease (4-7). For example, household LPS levels are directly associated with asthma symptoms and asthma-related health care utilization (7). Also, sound epidemiological studies suggest that for every 10 parts-per-billion (ppb) increase in O_3 levels there is an associated mortality increase of 0.39-0.87% (5, 8). It is therefore imperative to understand the mechanisms of environmentally induced lung injury.

Innate immune activation is a major contributor to lung disease pathogenesis and environmentally-induced exacerbations (9, 10). Toll-like receptors (TLRs) orchestrate the innate immune response to lung injury by recognizing exogenous pathogen- or endogenous danger-associated molecular patterns. TLR4 is the prototypic receptor for LPS (11), which is found in particulate-matter pollution and house dust (7, 12) and is a major contributor to sepsis-induced lung injury (13, 14). TLR4 also mediates inflammation and airway hyperresponsiveness after O_3 exposure (15), triggered by release of the endogenous sugar hyaluronan (16).

Regulation of TLR4 signaling is still incompletely understood. TLR4 can heterodimerize with other TLR like TLR2 and TLR6 (17, 18); in these cases, the partner TLRs serve to expand the TLR4 ligand spectrum. However, until now there has not been evidence of TLR4 interaction with other TLR, that modulates TLR4 signaling in response to its own ligands. TLR4-TLR5 interaction has been reported once (19), wherein TLR4 was shown to promote nitric oxide production after flagellin exposure. We therefore hypothesized that the reciprocal interaction may also be true, i.e. TLR5 participates in TLR4 signaling after environmental lung injury. TLR5, the prototypic receptor for bacterial flagellin, is expressed in alveolar macrophages (20), is induced after injury (21) and

regulates the immune response to injury (22-24). TLR5 plays an important role in immunity and metabolism and has been implicated in processes as varied as asthma (24), antiviral defense (25), ischemia-reperfusion injury (26, 27), radiation–induced injury (22, 28), and regulation of gut immunity (23). Furthermore, known functional genetic polymorphisms in *TLR5* are associated with susceptibility to infections (29-31) and autoimmune disease (32). These findings suggest a clinically relevant role of TLR5 in human immune regulation in the response to injury.

We show that TLR5 deficiency in mice significantly alters the *in vivo* response to TLR4 activators LPS, hyaluronan and O_3. Mechanistically, we show that after ultrapure LPS exposure, TLR5 co-immunoprecipitates with MyD88, TLR4 and LPS. The presence of TLR5 promotes formation of the Myddosome, i.e. association of MyD88 and IRAK4, and biases TLR4 signaling towards the MyD88 pathway. Finally, we demonstrate that human carriers of a dominant-negative TLR5 allele have decreased inflammatory response to O_3 exposure *in vivo* and LPS exposure *in vitro*. Our results thus suggest that TLR5 participates in TLR4 signaling and modulates environmental lung injury in disease-relevant exposures that lead to TLR4 activation.

Results

TLR5 promotes TLR4-mediated inflammation and airway hyperresponsiveness *in vivo*.

We first investigated the effect of TLR5 on TLR4 signaling *in vivo,* by exposing *Tlr5*-deficient mice or wildtype controls to LPS via intraperitoneal administration. As expected, this led to substantial lung inflammation in wildtype mice, which was ameliorated in the absence of TLR5 (**Fig. 1A**). TLR5 deficiency also ameliorated cellular influx and lung injury as evidenced by lung lavage protein (**Fig. 1B**). Furthermore, expression of inflammatory cytokines in the lung was significantly ameliorated in *Tlr5*-deficient mice (**Fig. 1C**). This was largely mirrored in a reduction of inflammatory gene expression in the liver (**Supplementary Fig. 1A**). To ensure that our results were not affected by obesity-, microbiome- or breeding-related immune perturbations, we performed experiments with mice that were either purchased from a commercial vendor (using C57BL/6 as controls) or bred in our NIEHS colony (using wild-type littermates as controls) and treated some mice with neomycin to reduce bacterial burden in the intestinal tract. Our results did not vary regardless of mouse provenance or antibiotic treatment (**Supplementary Fig. 1B**).

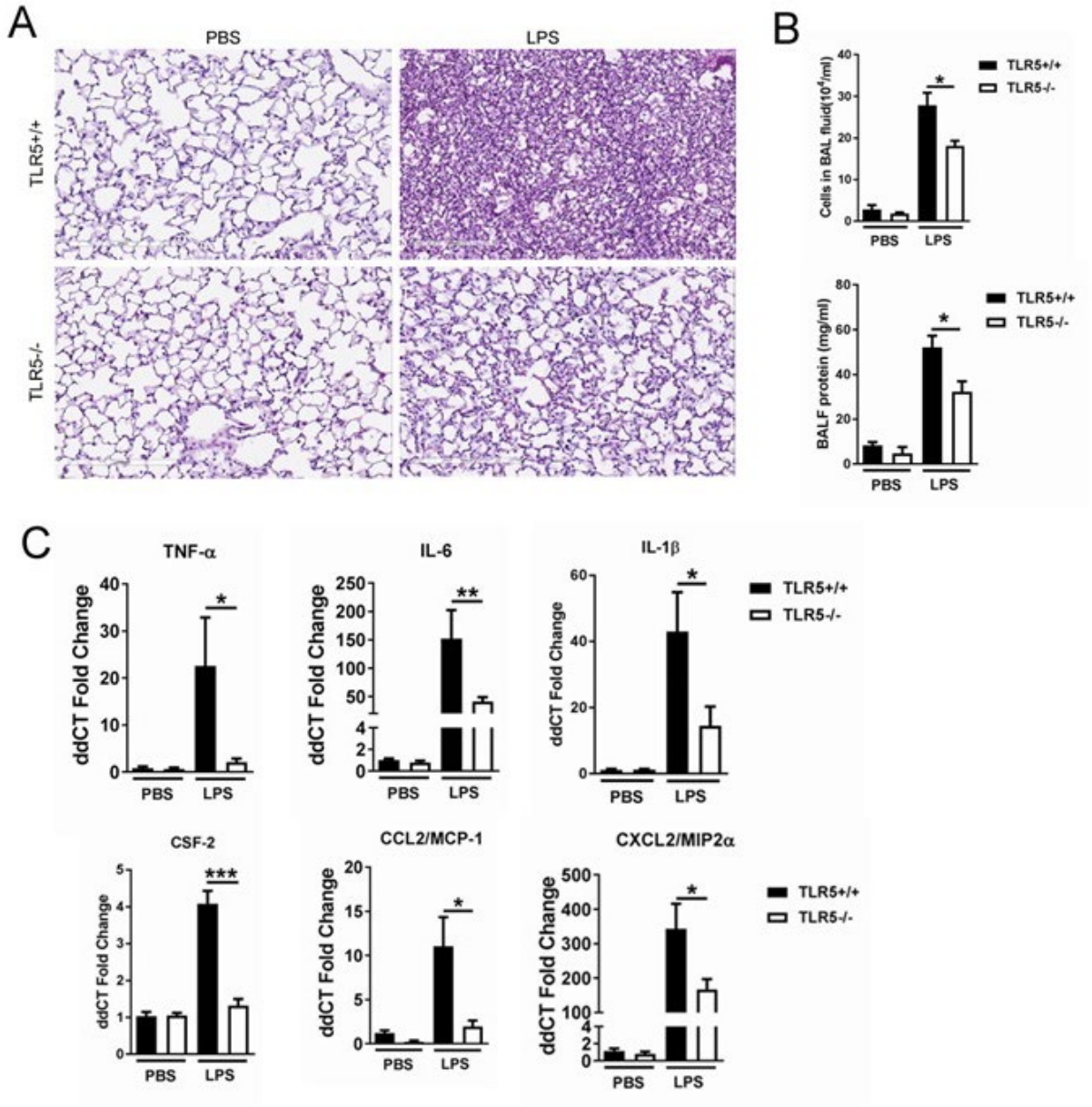

Figure 1. TLR5 deficiency ameliorates the inflammatory lung response to systemic LPS. (**A**) Hematoxylin-Eosin staining of lung sections demonstrates LPS-induced lung injury is ameliorated in Tlr5-deficient ($TLR5^{-/-}$) mice. (**B**) Cellular lung inflammation and lung lavage protein levels are decreased in Tlr5-deficient ($TLR5^{-/-}$) mice. (**C**) Real time quantitative PCR analysis of inflammatory cytokines shows a significant decrease in Tlr5-deficient ($TLR5^{-/-}$) mice. N=5-8 mice per group, experiment repeated twice. Data are represented as mean ± s.e.m. and were analyzed by unpaired t test with Welch's correction * P<0.05 and ** P<0.01 between $TLR5^{+/+}$ and $TLR5^{-/-}$ mice.

To determine if the TLR5 effect on TLR4 signaling has broader biological and clinical relevance in the lung, we explored TLR5-mediated effects on sterile lung injury after exposure to the ambient

pollutant, O_3. O_3 exposure is associated with increased morbidity and mortality in human patients with cardiopulmonary disease (5, 8); furthermore, it is now understood that TLR4 mediates the development of inflammation and airway hyperresponsiveness (AHR) after O_3 exposure (15). We used an O_3 dose that is equivalent to human exposure during a high-O_3 day (33, 34). *Tlr5*-deficient mice had ameliorated airway cytokine expression and almost abolished AHR after O_3 exposure (**Fig. 2A, B**). Because hyaluronan is the endogenous danger-associated molecular pattern that

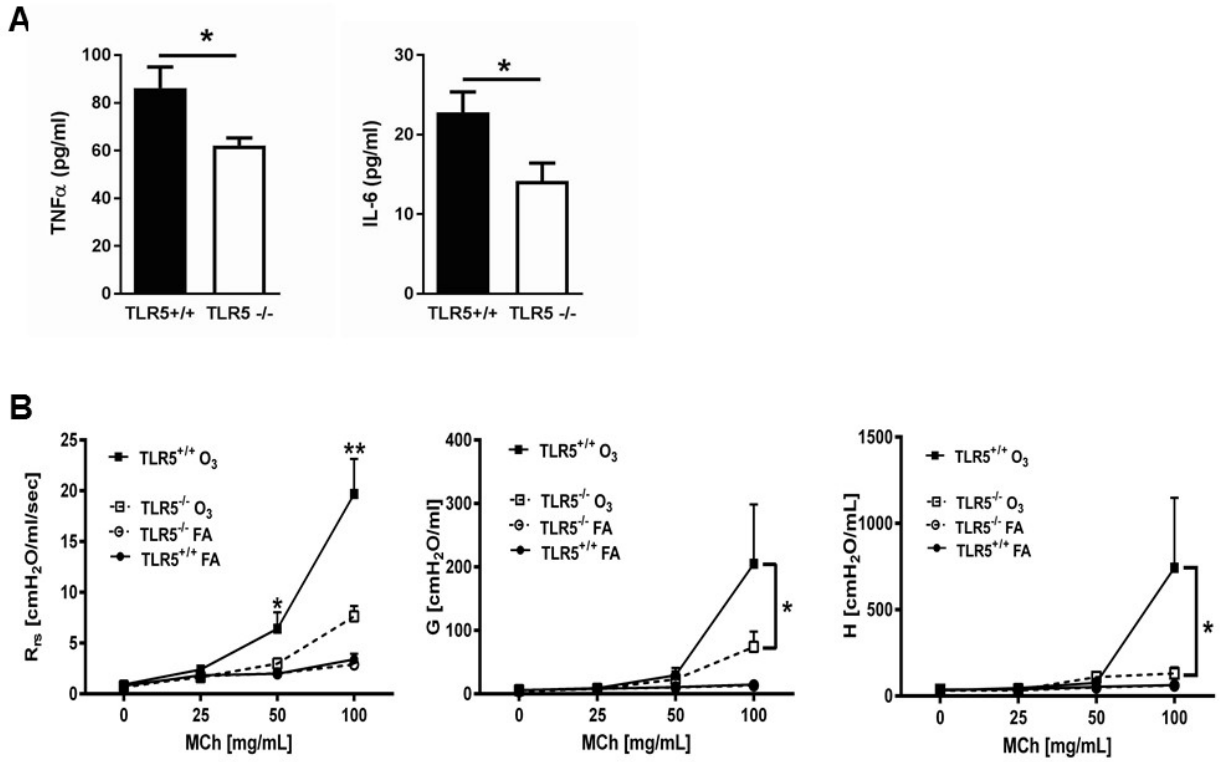

Figure 2. TLR5 deficiency ameliorates the *in vivo* inflammatory response to inhaled O_3. (**A**) TNF-α and IL-6 levels in the lung lavage fluid of *Tlr5*-deficient ($TLR5^{-/-}$) or *Tlr5*-competent ($TLR5^{+/+}$) mice 24 hours after receiving 3 ppm O_3 for 3 hours by inhalation. n = 14 mice for $TLR5^{+/+}$ and n = 12 mice for $TLR5^{-/-}$, experiment repeated twice. (**B**) Airway physiology measurement (total respiratory resistance R_{rs}, tissue damping G and tissue elastance H) to indicated doses of methacholine challenge measured with flexiVent in *Tlr5*-deficient ($TLR5^{-/-}$) or *Tlr5*-competent ($TLR5^{+/+}$) mice 24 hours after 2ppm O_3 or air (FA) exposure. n = 6 for $TLR5^{-/-}$FA and $TLR5^{-/-}O_3$ and n = 7 for $TLR5^{+/+}$FA and $TLR5^{+/+}O_3$, experiment repeated three times. Data are represented as mean ± s.e.m. and were analyzed by unpaired t test with Welch's correction * P<0.05 and ** P<0.01 between $TLR5^{+/+}$ and $TLR5^{-/-}$ mice exposed to O_3

activates TLR4 and mediates the response after O_3 exposure (15, 16), we then investigated the effect of TLR5 on hyaluronan signaling. *Tlr5*-deficient mice had substantially reduced

105 inflammatory gene induction and significantly diminished AHR after instilled ultrapure
106 (pharmaceutical grade) hyaluronan exposure (**Fig. 3A, B**). We then performed a more global

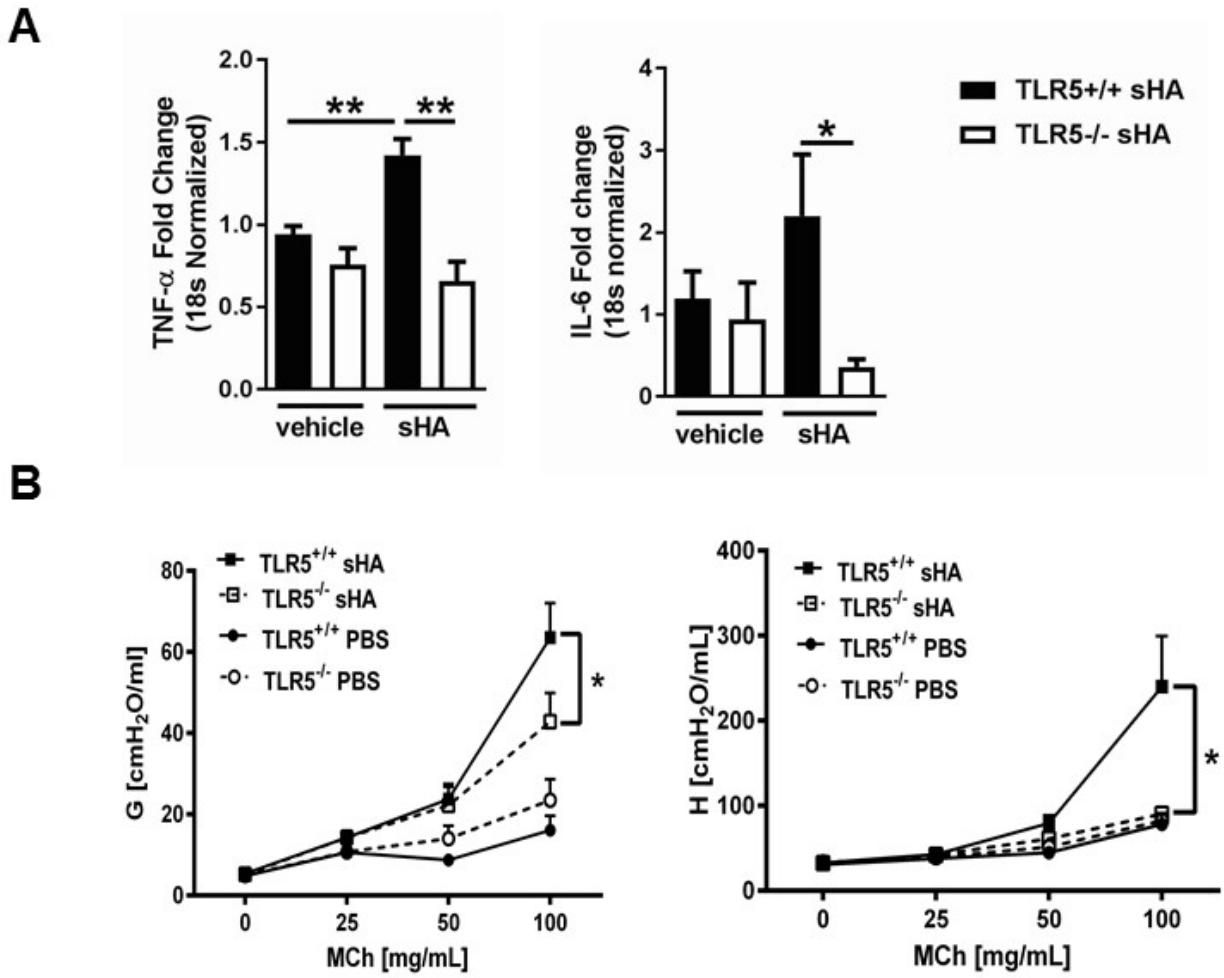

Figure 3. TLR5 deficiency ameliorates the *in vivo* inflammatory response to instilled ultrapure short-fragment hyaluronan (sHA). (**A**) Real time quantitative PCR gene expression of TNF-α and IL-6 in the lung tissues of *Tlr5*-deficient ($TLR5^{-/-}$) or *Tlr5*-competent ($TLR5^{+/+}$) mice 6 hours after exposure to vehicle (PBS) or 50 µl of 3 mg/ml short fragment HA. n = 5 for $TLR5^{-/-}$PBS and $TLR5^{-/-}$sHA and n = 6 for $TLR5^{+/+}$PBS and $TLR5^{+/+}$sHA. Experiment repeated once. (**B**) Airway physiology measurement (tissue damping G and tissue elastance H) to indicated doses of methacholine challenge measured with flexiVent 2 hours after exposure to vehicle (PBS) or 2 mg/ml sHA. n = 5 for $TLR5^{-/-}$PBS and $TLR5^{-/-}$sHA and n = 6 for $TLR5^{+/+}$PBS and $TLR5^{+/+}$sHA, experiment repeated twice. Data are represented as mean ± s.e.m. and were analyzed by unpaired t test with Welch's correction * P<0.05 and ** P<0.01 between $TLR5^{+/+}$ and $TLR5^{-/-}$ mice.

107 analysis of the TLR5 effects on TLR4 signaling. We analyzed gene expression patterns using the
108 NanoString© platform (www.nanostring.com) and utilizing the Mouse Innate Immunity Panel

Codeset (Ns_Mm_Myeloid_v2.0) and focused specifically on 242 genes that were more than 2-fold upregulated after LPS exposure (**Supplementary Table 1 and Supplementary Figure 2**) and sorted them according to magnitude of TLR5 effect. We showed that the presence or absence of functional TLR5 is associated with differential regulation of immune genes in this panel. Interestingly, there was a linear correlation between the magnitude of the TLR5 effect and the proportion of genes that are either published or predicted to be downstream of the NFκB pathway (**Supplementary Figure 2C**): among the genes that were 70-90% upregulated in *Tlr5*-sufficient mice compared to *Tlr5*-deficient mice, almost 90% were in the NFκB pathway, while this proportion fell to 50% among the genes that were no different between genotypes. (R^2=0.89, p=0.0013). This suggested to us that TLR5 may preferentially impact gene expression downstream of MyD88 activation. In aggregate, these results support that TLR5 promotes TLR4 signaling in several models of TLR4 activation through pathogen- or danger-associated molecular patterns (PAMPs or DAMPs, i.e. LPS or hyaluronan respectively) and promotes TLR4-mediated inflammation and airway hyperresponsiveness *in vivo*.

Genetic TLR5 deficiency in humans impacts TLR4 signaling *in vitro* and *ex vivo*.

We then investigated the effect of TLR5 in human TLR4 signaling. In humans, a dominant-negative *TLR5* single nucleotide polymorphism (SNP) (rs5744168, $TLR5^{392STOP}$) (29) is found with a prevalence of 8-10% in Caucasians and 3% in African Americans. We hypothesized that carriers of this SNP may have reduced TLR4-mediated inflammation. We used the Environmental Polymorphisms Registry (35), a NIEHS-supported cohort, to recruit carriers of this allele, as well as "wildtype" controls. Others have reported that whole blood from rs5744168 minor-allele carriers does not differ in the response to LPS compared to "wildtype" (31), and we confirmed this finding (**Supplemental Figure 3A**). We believe this happens because whole blood consists of different

133 cell types, which differentially express TLR5, thereby confounding the effect on TLR4 signaling.

134 We then investigated the effect of this *TLR5* SNP on purified, primary monocyte-derived

135 macrophages. Macrophages from rs5744168 minor-allele carriers had a decreased response to

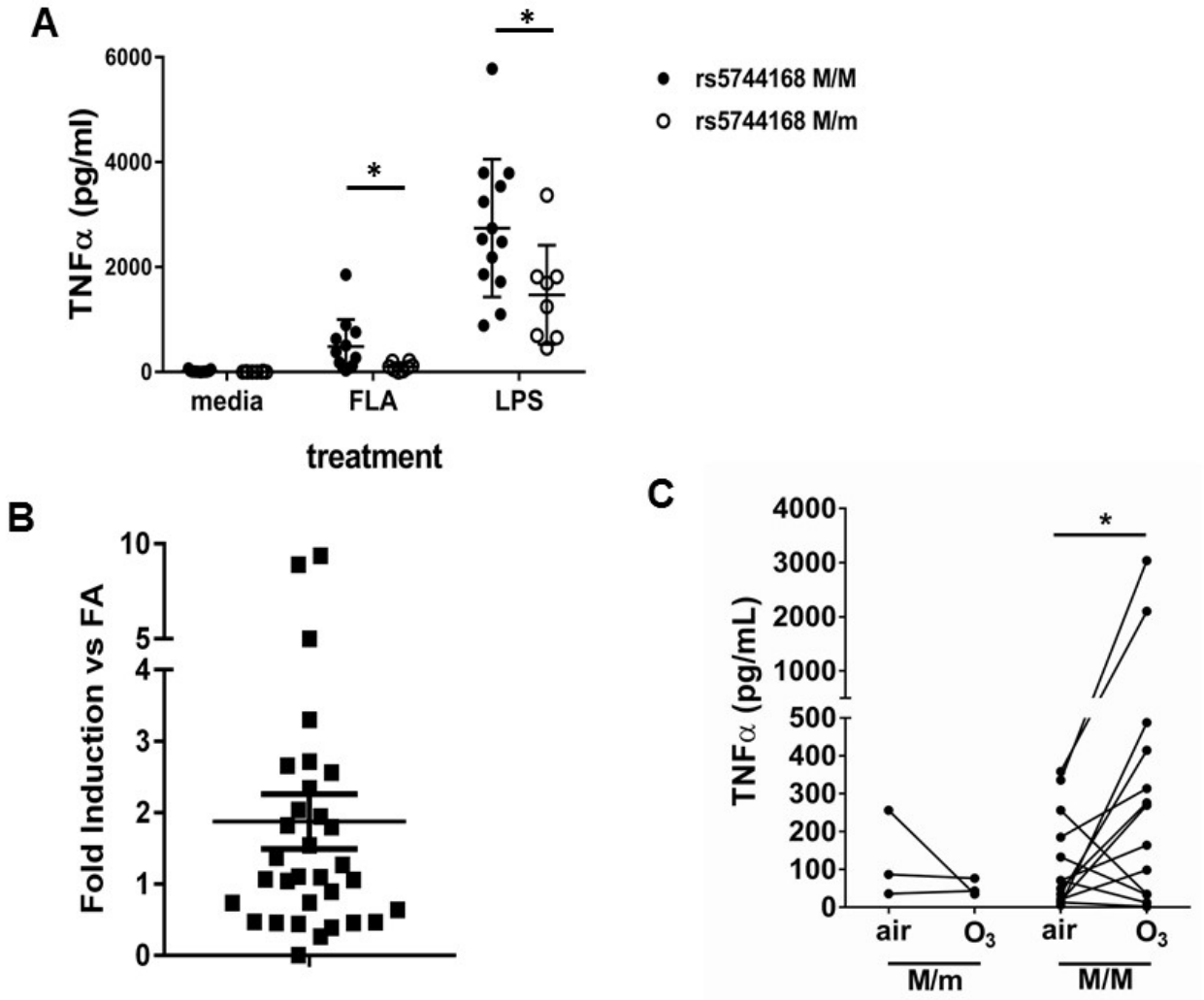

Figure 4. TLR5 participates in TLR4-mediated inflammation in humans. (**A**) TNF-α production by peripheral blood monocyte-derived macrophages from human volunteers either homozygous for the major allele (rs5744168 M/M) or carriers of the minor allele (rs5744168 M/m) for the TLR5 single nucleotide polymorphism rs5744168. Cells were exposed to 10 ng/ml ultrapure LPS or 100 ng/ml ultrapure flagellin for 24 hours and TNF-α levels were analyzed by Duoset ELISA kit. Data are represented as mean ± standard deviation and analyzed by unpaired t test with Welch's correction. N=7-13 individual subjects. (**B**) TLR5 gene expression in alveolar macrophages from human volunteers exposed to 200 ppb O_3 for 135 minutes. N=32 individual subjects. Data are presented as individual values with mean ± s.e.m. and was analyzed by Wilcoxon pairwise signed rank test. (**C**) *Ex-vivo* TNF-α production by human alveolar macrophages after exposure to air or O_3 n=3 minor rs5744168 allele carriers and 20 major allele carriers. Data are represented as individual values and trends and analyzed by Wilcoxon matched-pairs signed rank test. * P<0.05

136 flagellin and ultrapure LPS, but not Pam3CSK4 (**Fig. 4A and Supplemental Figure 3B**), thus

confirming that human *TLR5* genetic variation specifically determines the response to LPS. This was not due to altered expression of TLR4 or CD14, which was not changed by the rs5744168 genotype (**Supplemental Figure 3C**).

We then investigated the effect of TLR5 in O_3-induced inflammation in healthy human volunteers. We exposed human volunteers to O_3, and isolated alveolar macrophages through bronchoscopy 24 hours after exposure, which represents the peak of O_3-induced inflammation and symptoms in humans. *TLR5* expression was modestly increased in alveolar macrophages of human volunteers after O_3 exposure (**Fig. 4B**, p=0.05 by Wilcoxon pairwise signed rank test). There was no association between *TLR5* expression and *TLR4* expression after O_3 exposure. We found, that *TNFα* expression by alveolar macrophages after O_3 exposure was not increased in any of the *TLR5*-deficient individuals (rs5744168 minor-allele carriers), while it was increased in wildtype-allele carriers (**Fig. 4C and Supplementary Fig.3D**).

TLR5 modulates TLR4-dependent signaling.

To investigate the mechanistic role for TLR5 in the response to LPS-induced TLR4 activation, we then compared primary bone marrow derived macrophages (BMDM) from *Tlr5*-deficient and -sufficient mice. *Tlr5*-deficient BMDM had significantly decreased expression of TNFα and IL-6 after ultrapure LPS exposure *in vitro,* by an average of 30-50% (**Fig. 5A**). To ensure that the altered response was not due to LPS contaminants despite the ultrapure preparation, we also assayed *Tlr4*-deficient BMDM and saw no response to ultrapure LPS (**Fig. 5A**). The effect of *Tlr5*-deficiency was also observed when using other sources of Tlr4 activation, such as Monophosphoryl Lipid A (**Supplementary Fig. 4A**). To ascertain that our observation was not due to an off-target effect of genetic *Tlr5* ablation on macrophage biology, we used RAW264.7 cells, a murine macrophage cell line, which are naturally deficient in *Tlr5* (36). We transfected

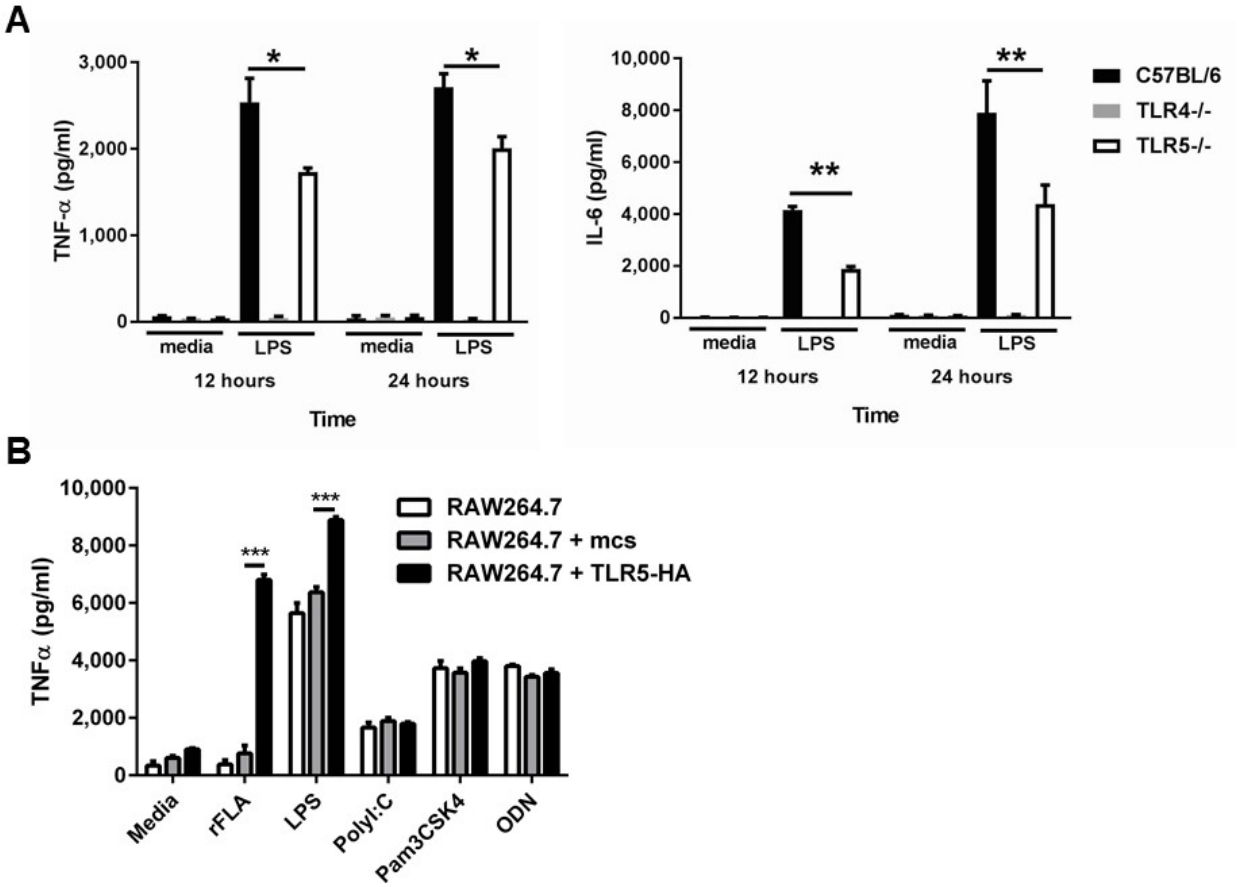

Figure 5. Deficiency of TLR5 reduces ultrapure LPS induced inflammatory cytokine production. (**A**) TNFα and IL-6 production by BMDM from *Tlr5*-deficient (TLR5$^{-/-}$), *Tlr4*-deficient (TLR4$^{-/-}$) and wild-type (C57BL/6) mice after 10 ng/mL ultrapure LPS or vehicle (media) exposure for 12 or 24 hours. N=8 per group, experiment repeated at least 5 times. (**B**) TNF-α secretion by non-transfected (RAW264.7), empty construct transfected (RAW264.7 + mcs) or hemagglutinin-tagged TLR5 construct (TLR5-HA) transfected cells (RAW264.7 + TLR5-HA) after vehicle (media) or TLR5, TLR4, TLR3, TLR2 and TLR9 agonists (100 ng/mL recombinant ultrapure flagellin (rFLA), 10 ng/ml LPS, 10 μg/ml PolyI:C, 1 μg/ml Pam3CSK4, 1μM ODN). N=8 per group, experiment repeated twice. Data are represented as mean ± s.e.m. and were analyzed by two-way analysis of variance (ANOVA) followed by Tukey's post hoc test. * P<0.05, ** P<0.01, *** P<0.001.

these cells with a murine *Tlr5* construct, or empty vector, and noted that the *Tlr5*-transfected cells had significantly higher TNFα expression in response to ultrapure LPS (and the TLR5 ligand flagellin, as expected). The TLR5-dependent effect was specific to LPS exposure, as responses to poly(I:C) (TLR3 ligand), Pam3CSK4 (TLR2 ligand), and ODN (TLR9 ligand) were not affected by the presence of TLR5 (**Fig. 5B**).

TLR5 engages with MyD88 after LPS exposure and biases TLR4 signaling towards the MyD88 pathway.

We then interrogated the effect of TLR5 on TLR4 signaling. TLR4 signals through both MyD88 and TRIF pathways, whereas TLR5 signals through MyD88 only (11). We therefore hypothesized that TLR5 may bias the signaling response towards the MyD88 pathway. We found that, in primary murine BMDMs, MyD88 co-immunoprecipitated with TLR5 after ultrapure LPS exposure (**Fig. 6A**). This association was directly dependent upon TLR4 activation, since it was not observed in

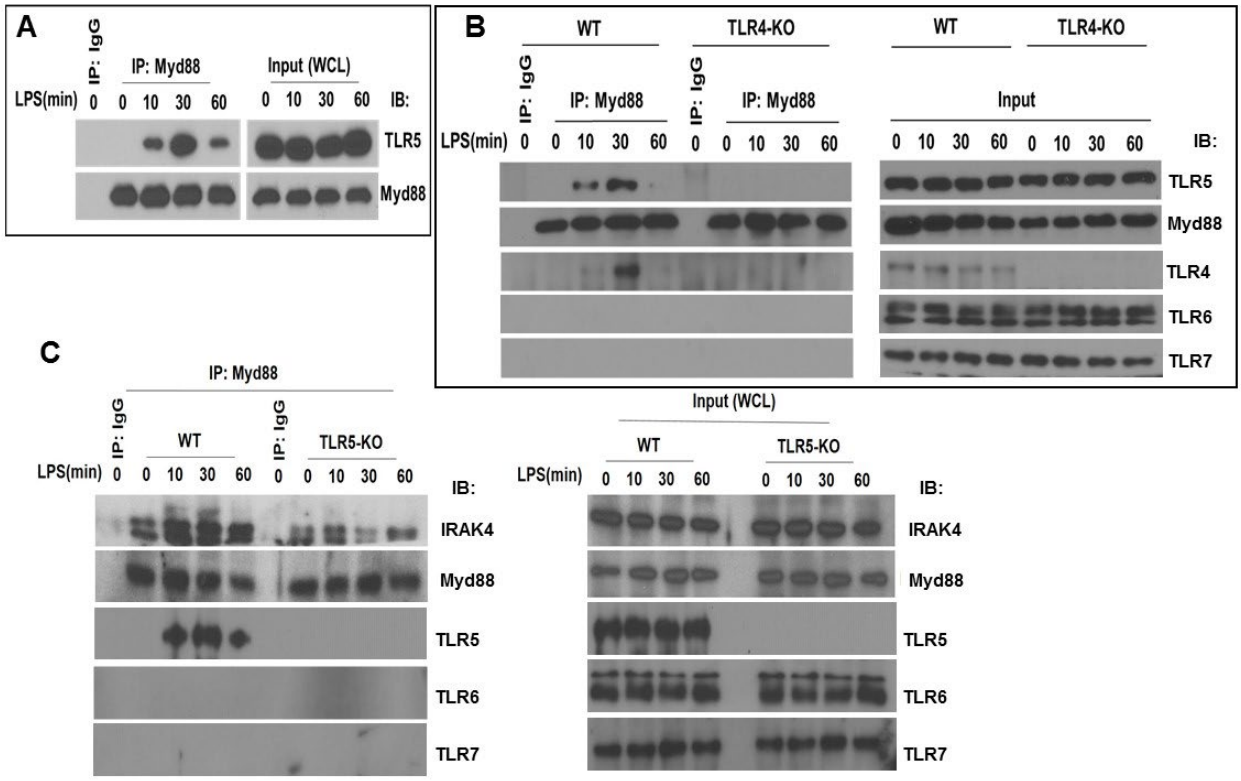

Figure 6. TLR5 engages with MyD88 and promotes Myddosome assembly after TLR4 activation. (**A**) Co-immunoprecipitation of TLR5 with Myd88 in BMDM from C57BL/6J mice after 100 ng/mL LPS exposure for indicated time points. n = 7, experiment was repeated twice. (**B**) immunoprecipitation of TLR5 with Myd88 in BMDM from *Tlr4*-deficient (TLR4-KO) or *Tlr4*-competent (C57BL/6, wildtype WT) mice after 100 ng/mL LPS exposure for indicated time points. Representative of 3 separate experiments. (**C**) Immunoprecipitation of IRAK4 with Myd88 in BMDM from *Tlr5*-deficient (TLR5 KO) or *Tlr5*-competent (C57BL/6, wildtype WT) mice after 100 ng/mL LPS exposure for indicated time points.

Tlr4-deficient primary BMDM (**Fig. 6B**). TLR4, as expected, also immunoprecipitated with MyD88 after LPS exposure (**Fig. 6B**). Furthermore, the association of MyD88 with TLR4 and TLR5 was

specific, since other TLR like TLR6 and TLR7 did not immunoprecipitate with MyD88 after LPS exposure **(Fig. 6B, 6C).** MyD88 signaling occurs through the complexing of Myd88 with IRAK-4 which stabilizes formation of the so-called Myddosome (37). *Tlr5*-deficient BMDM had decreased immunoprecipitation of IRAK-4 with MyD88 **(Fig. 6C)**. There was also significant reduction in the phosphorylation of IKKα/β, IκB, p65, JNK1/2 and ERK1/2 in *Tlr5*-deficient BMDMs compared with wildtype cells during early activation (**Fig. 7A,B, Supplementary Fig. 4B**), which is MyD88- but not TRIF-dependent (38, 39). Nuclear IRF3 (a specific readout of TRIF-dependent signaling (40))

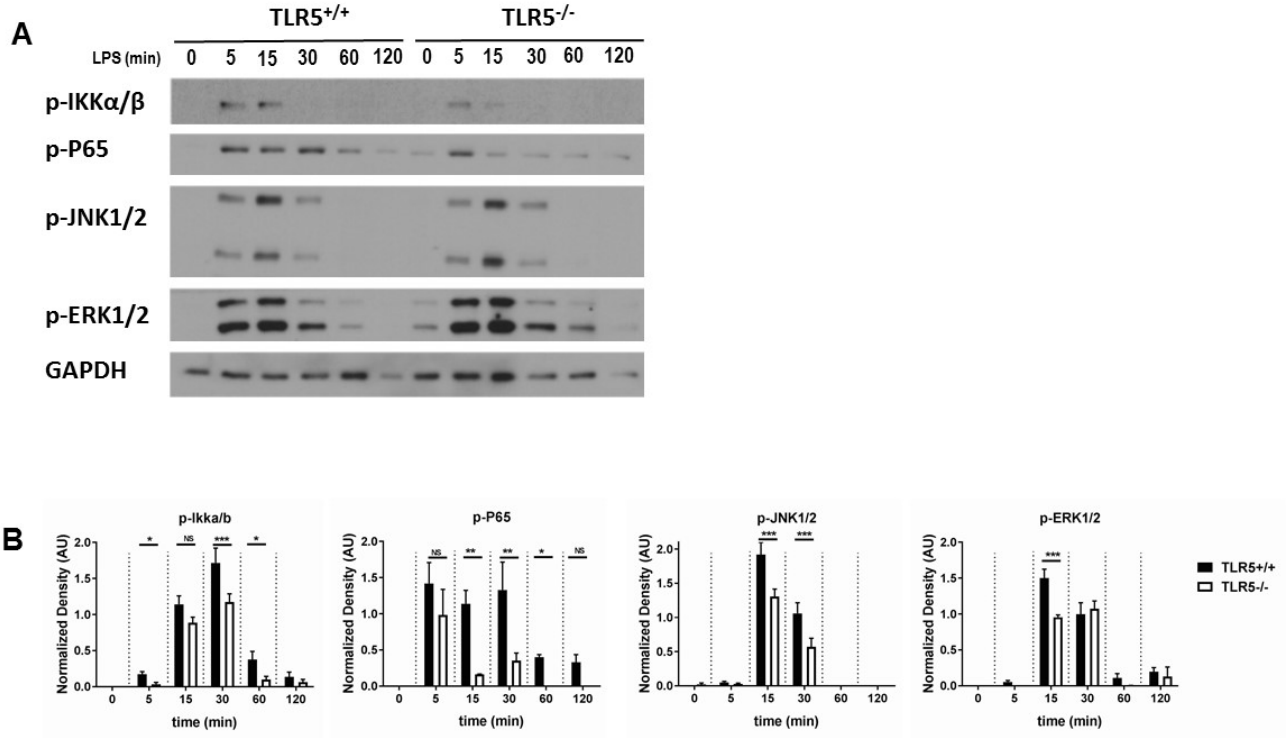

Figure 7. TLR5 promotes MyD88 downstream signaling. (**A**) Western blot analysis of p-P65, p-IKKα/β, p-JNK1/2 and p-ERK1/2 after exposure to 100 ng/mL ultrapure LPS exposure in BMDMs from *Tlr5*-competent (TLR5$^{+/+}$) and *Tlr5*-deficient (TLR5$^{-/-}$) mice. (**B**) Quantification of densitometric analysis of 3 separate blots similar to (3A). Data are represented as mean ± s.e.m. and were analyzed by repeated unpaired t test with Holm-Sidak correction. NS=not significant, * P<0.05, ** P<0.01, *** P<0.001.

was not affected in *Tlr5*-deficient (**Supplementary Fig. 4C**). In aggregate, these results suggest that TLR5 directly interacts with MyD88 after LPS exposure and enhances MyD88-dependent TLR4 signaling by promoting efficient assembly of Myddosome.

TLR5 is part of the TLR4 signaling complex.

We then investigated whether TLR5 participates directly in the TLR4 signaling complex, or whether it affects TLR4 signaling indirectly. We first evaluated whether TLR5 affects TLR4 cell surface expression and trafficking using bone marrow-derived macrophages (BMDM) from genetically deficient or wild-type mice. There were no differences in basal levels of cell surface TLR4 or CD14 between *Tlr5*-deficient and –sufficient BMDM, nor in LPS-induced internalization of TLR4 (**Supplementary Fig. 5A-D**). Because we were unable to find commercially available validated antibodies against TLR4 that could be used in co-immunoprecipitation experiments, we utilized an induced expression system using tagged TLR4 and TLR5 in HEK293 cells and found that TLR4 and TLR5 reciprocally co-immunoprecipitated in transfected HEK293 cells (**Fig. 8A**). We then overexpressed TLR4 and TLR5 in HeLa cells and confirmed their interaction through a

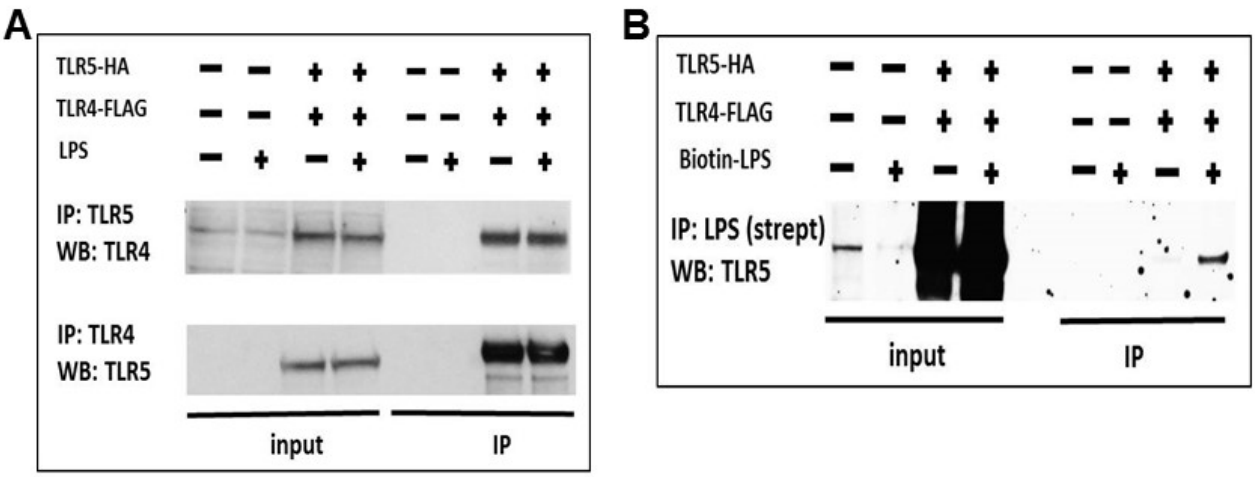

Figure 8. TLR5 participates in TLR4 signaling complex. (**A**) Co-immunoprecipitation of hemagglutinin-tagged TLR5 (TLR5-HA) and FLAG-tagged TLR4 (TLR4-FLAG) in HEK293 cells after 100 ng/mL ultrapure LPS exposure. (**B**) Immunoprecipitation of TLR5 with biotinylated ultrapure LPS (Biotin-LPS) in TLR5-HA and TLR4-FLAG transfected HEK293 cells after 100 ng/mL Biotin-LPS exposure for 15 minutes. Representative of 2 separate experiments.

Proximity Ligation Assay (**Supplementary Fig. 5E**). Furthermore, we exposed TLR5-hemagglutinin tagged expressing RAW264.7 cells to biotin-tagged LPS, and (after thoroughly washing the cells) could co-precipitate TLR5 with LPS (**Fig. 8B**). These results suggest that TLR5

201 directly participates in the TLR4 signaling complex after LPS exposure, and, in aggregate, support

202 a functional interaction of TLR5 with TLR4 in the response to environmental injury.

Discussion

The important novel finding from our work, is that TLR5 heteromerization with TLR4 modulates canonical TLR4 signaling, and biases TLR4 signaling towards MyD88. Recent evidence highlights the role of molecules of the TLR4 receptor complex in modulating TLR4 signaling. For example, elegant work has demonstrated that CD14, which is necessary for LPS binding to TLR4, also controls TLR4 endocytosis after LPS ligation, and thus is necessary for TRIF signaling, which is thought to occur in the endosomal compartment (41). Our work further suggests that TLR4 signaling is modulated by the addition of TLR5 to the receptor complex. Unlike CD14, TLR5 does not appear to regulate LPS-induced internalization of TLR4 (**Supplementary Fig. 5C**). Our findings rather support a model in which TLR5 selectively promotes TLR4/MyD88 signaling at the plasma membrane and is not required for CD14 regulation of the TRIF pathway. Importantly, our work suggests that TLR5 regulation of TLR4 signaling is biologically significant. TLR5-deficient mice had approx. 30-50% decreased cytokine expression in local and systemic LPS models of lung inflammation, while airway hyperresponsiveness after ozone or hyaluronan exposure was significantly reduced, and inflammatory gene induction after hyaluronan exposure was abolished. It is possible that the “fine-tuning”, MyD88-promoting effects of TLR5 are particularly evident in lower-grade inflammation such as ozone- or hyaluronan-induced, which explains the larger impact on TLR5 deficiency on human and murine inflammation after ozone exposure compared to LPS exposure.

Using primary murine macrophages, we demonstrate that, in the physiological state, TLR5 co-immunoprecipitates with MyD88 after ultrapure LPS exposure, but only in the presence of TLR4. This indicates that TLR5 is recruited into the Myddosome assembly, along with TLR4, upon TLR4 activation. Indeed, it has been postulated that the ability of the Myddosome to form 7:4 and 8:4 MyD88:IRAK4 stoichiometries is a potential mechanism through which clusters of activated TLR receptors can be formed and different TLR receptors can be recruited into the same assembly

(42). Higher-order assembly of receptor complexes in lipid raft microdomains is likely to be crucial in the fine-regulation of immune responses (37). Our work suggests that TLR5 may be part of the higher-order receptor assembly that regulates TLR4 signaling. TLR4 is a promiscuous receptor, having been found to heterodimerize with TLR2 and TLR6 (17, 18), but the effect of heteromerization until now has always been to expand the TLR4 ligand spectrum. To our knowledge, this is the first work to demonstrate that TLR heteromerization may serve to modulate canonical TLR signaling.

A recent paper supported the role or TLR5 in TLR4 signaling, showing similar effects of the rs5744168 minor allele polymorphism on TNFα and IL-8 expression by human monocytes after LPS exposure, and also demonstrating that TLR5 does not influence the gene expression of TLR4 (43), as we also show in our Supplemental Figure 5. By contrast, that paper could not confirm that TLR5 is modifying the NFκB pathway. This apparent discrepancy may be due to different research methodologies: These authors used gene silencing which only resulted in approx. 50% reduction of TLR5 expression, as well as reporter cell lines with transfection-induced TLR5 expression, as opposed to our use of primary cells with genetically knocked-out gene function. Furthermore, the authors of that study did show an effect for TLR5 on NFκB when transfecting with lower (more physiological) doses of TLR5 DNA (43).

In summary, our results suggest a new model of TLR4-TLR5 complex formation in response to the PAMP LPS or the DAMP hyaluronan (**Fig. 9**). We propose that the hitherto accepted model of TLR4 signaling through TRIF and MyD88 rather reflects the TLR5-deficient state. In TLR5-expressing cells, TLR5 participates in a heteromeric higher-order TLR4 receptor complex and potentiates MyD88 signaling by promoting efficient assembly of the Myddosome. This also suggests that exposures that induce TLR5 expression, such as DNA injury, p53 activation (21) or flagellated bacterial infection may in parallel selectively prime the MyD88-dependent pro-inflammatory response to LPS. Since TLR5 signals through MyD88, in a finite TLR4 receptor pool the presence of TLR5/TLR4 higher-order complexes would bias TLR4 signaling towards MyD88. Notably, cell surface expression of TLR4 on immune cells is low (a few hundred or thousand

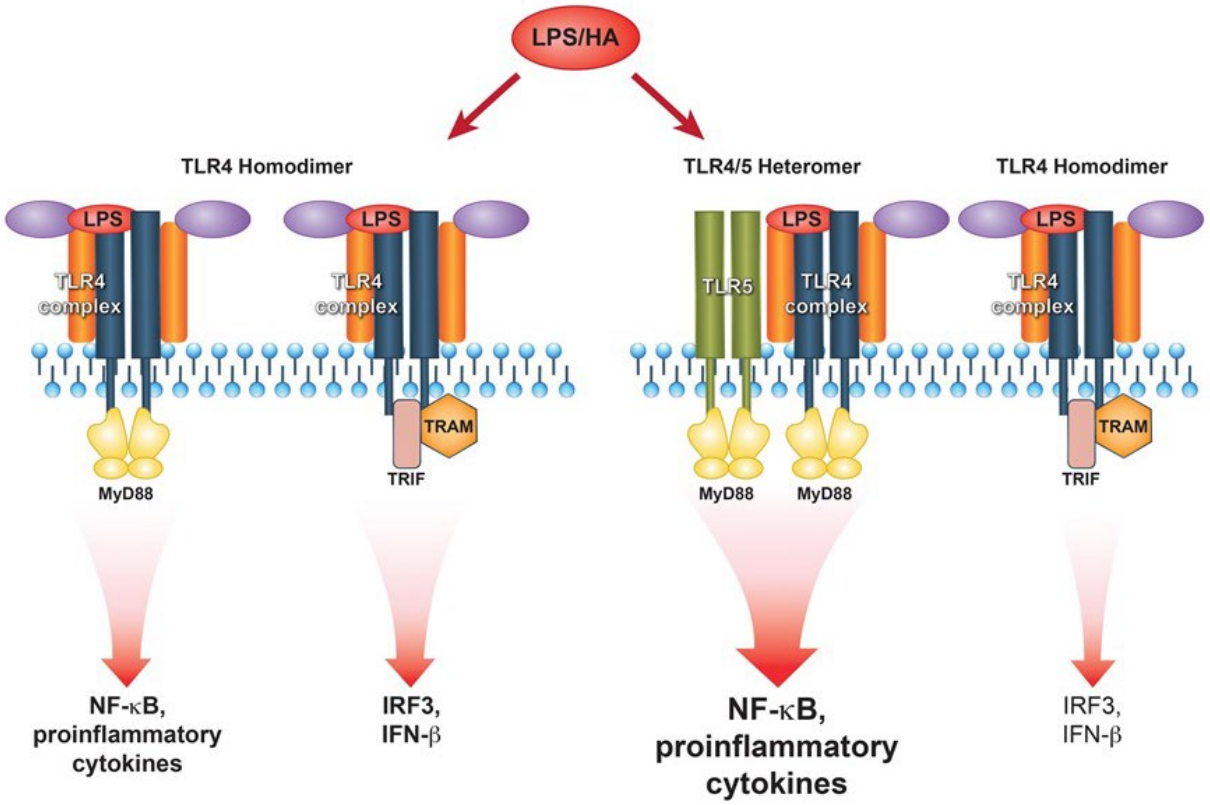

Figure 9. Proposed model of TLR5-TLR4 interaction during environmental lung injury. The current model of canonical TLR4 activation rather applies to the TLR5 deficient status (left panel). In the presence of TLR5 (right panel), TLR5 participates in the TLR4 signaling complex, and promotes signaling downstream the MyD88 pathway.

255 molecules per cell) compared to other TLRs (44, 45), supporting that the presence of relatively

256 few TLR5 receptor molecules may suffice to shift the TLR4 signaling equilibrium towards MyD88.

Methods

Mice:

C57Bl/7J mice and B6.129S1-*Tlr5*^{tm1Flv}/J (TLR5-deficient) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The TLR5-deficient allele was generated in the 129S1 genome and subsequently backcrossed to C57BL/6 before being transferred to the Jackson Laboratory by the donating investigator. When possible, wildtype littermate control mice were used in our study in parallel to commercial wildtype C57BL/6J controls. To ensure that our results were not due to locality-influenced microbiome changes, we repeated experiments in mice that were bred at the NIEHS vivarium, as well as mice purchased from the Jackson Laboratory and studied within 1 week of arrival. In some experiments, mice received neomycin water to control gut microbiome. Results were comparable independent of provenance or antibiotic dosing. Mice were given access to water and chow *ad libitum*, and were maintained at a 12-hour dark-light cycle. No differences in body weight were observed at the ages studied (6-12 weeks old). All experiments are approved by the NIEHS Institutional Animal Care and Use Committee.

Exposures:

Mice received ultrapure *E.coli* O111:B4 LPS (List Biological Labs, Campbell, CA) (50 µl of 1mg/ml in PBS), or control PBS vehicle only, by oropharyngeal aspiration and were phenotyped 24 hours later. For systemic LPS exposure, mice received 10 mg/kg LPS or PBS by intraperitoneal injection and were phenotyped 24 hours later. In other experiments, mice were exposed to 2 ppm ozone for 3 hours, in a chamber with 20 exchanges/hour, 50-65% relative humidity and a temperature of 20-25° C as previously described (16) and were phenotyped 24 hours later. Control mice received filtered air in an identical setup. In other experiments, mice received 50 µl of a 3 mg/ml solution of sonicated, LPS-free, pharmaceutical-grade hyaluronan with an average molecular

weight of 100-300 kDa (derived from Healon, Abbott Laboratories, Abbott Park, IL) or PBS vehicle by retropharyngeal aspiration, and were phenotyped 2 hours later.

Airway physiology measurements:

Airway responsiveness to 25-100 mg/ml methacholine (MCh; Sigma) was measured 24 hours following O_3 or 2 hours following hyaluronan challenge. Briefly, mice were anesthetized with urethane (2 g/kg; Sigma), tracheotomized with a tracheal cannula (Harvard Apparatus) with Luer adapter, and mechanically ventilated on a 42 °C water-heated pad at a rate of 150 breaths/min, a tidal volume of 10 ml/kg and a positive end-expiratory pressure (PEEP) of 3 cm H_2O with a computer-controlled small animal ventilator (FlexiVent, Scireq, Montreal, Canada). To block spontaneous breathing, mice were given pancuronium bromide i.p. (0.8 mg/kg; Sigma-Aldrich) 5min prior to assessment of airway responses. To measure airway responsiveness, a single-frequency forced oscillation waveform, followed by a broadband forced oscillation waveform (matched to the animal breathing frequency) were applied using the Flexiware 7.6 software default mouse inhaled dose-response script. The resulting pressure, volume, and flow signals were fit to either the Single Compartment or Constant Phase model of the lung to obtain total respiratory system resistance (R_{rs}) and elastance (E_{rs}) or Newtonian resistance ($R_{n,}$ generally understood as proximal airway resistance), tissue damping (G, generally understood as peripheral tissue resistance), and tissue elastance (H), respectively(46). The peak response at each dose was averaged and graphed along with the average baseline measurement for each group.

Proximity Ligation Assays (PLA, Duolink)

TLR4-FLAG M2 or MyD88-V5 and TLR5-HA were overexpressed in HeLa cells grown on glass coverslips. 24h post-transfection the cells were fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature (RT) and blocked with 10% normal goat serum for 1h at RT. The

cells were next permeabilized with 0.1% TritonX-100 in goat serum for 15 minutes at RT and incubated with primary antibodies (dilution 1:1000) against epitope-tags overnight: rabbit anti-FLAG M2 (Cell Signaling), mouse anti-V5 (Invitrogen) and mouse anti-HA (Sigma). Duolink, based on in situ proximity ligation assay (PLA), was performed according to manufacturer instructions (Sigma).

Bone Marrow Derived Macrophage (BMDM)

Bone marrow was collected from tibias and femurs of wildtype *and Tlr5*-deficient mice and cell single preparations were made. Growth media for maturation of BMDM consisted of DMEM-F12 containing 10mM L-glutamine, 10% embryonic stem cell qualified fetal bovine serum, 1% antibiotic and antifungal mix and 30 ng/mL murine M-CSF. Cells were cultivated in an incubator at 37°C, 5% CO_2 for up to 7 days with media change every 48 hours after first media change 72 hours after platting. *Tlr5*-deficient BMDM were evaluated for responsiveness to flagellin and were found to be unresponsive (Supplementary Fig. 6)

Flow Cytometry

In order to access cell surface expression of TLR4, Wildtype, *Tlr4*-deficient or *Tlr5*-deficient BMDM were harvested, washed with PBS and were exposed to ultrapure LPS for 0,15,30, 60 or 90 minutes. Cells were washed with cold PBS and gently lifted from the culture dishes using a cell lifter. Cells numbers were estimated and cells were aliquoted in 1 x 10^6 cells per tube in the FACS buffer (0.5% BSA, 0.1% NaN3, and 2mM EDTA in PBS). Cells were blocked for 20 minutes on ice in a blocking solution (FACS buffer, 10% species specific serum, and 1% FCR block). Cells were stained using APC anti-mouse CD284 (TLR4) Antibody (clone SA15-21), anti-CD14 Antibody (Biolegend) or isotype controls for 30 minutes on ice. Cells were washed two times with 1mL FACS buffer after staining, suspended in 500 uL FACS buffer containing 1mg/mL propidium iodide (to identify dead cells) and analyzed on a BD FACSAria II equipment.

333

334 **Western Blotting**

335 Wildtype or *Tlr5*-deficient BMDM were harvested, washed once with cold PBS, and lysed for 30
336 minutes at 4°C in 1% TritonX-100, 20 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM $MgCl_2$, 2mM
337 EGTA, protease and phosphatase inhibitors (Roche). Cellular debris was removed by
338 centrifugation at 16,000rcf for 10 min. For immunoblotting, cell extracts were fractionated by SDS-
339 PAGE and transferred to Immobilon-P transfer membranes (Millipore), using either a wet transfer
340 apparatus (Bio-Rad) or with a dry transfer system (iBlot) from Invitrogen. Immunoblot analysis
341 was performed, and the bands were visualized with HRP-coupled goat anti-rabbit, goat anti
342 mouse, or donkey anti-goat Ig as appropriate (Rockland), using the ECL Western blotting
343 detection system (GE Healthcare). Protein levels were equilibrated with the Protein Assay
344 Reagent (Bio-Rad).

345

346 **Co-immunoprecipitation**

347 For coimmunoprecipitations, cells were harvested, washed once with cold PBS, and lysed in a
348 TritonX-100-containing buffer (0.5% TritonX-100, 20 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM
349 $MgCl_2$, 2mM EGTA, protease and phosphatase inhibitors (Roche). Cell extracts were incubated
350 with 1 µg of Ab (anti-HA, Sigma) or normal IgG (negative control) for 2 h, followed by incubation
351 for 12 h with 30 µl of protein G-Sepharose beads (prewashed and resuspended in lysis buffer at
352 a 1:1 ratio). After incubations, the beads were washed four times with lysis buffer, separated by
353 SDS-PAGE, and analyzed by immunoblotting. For TLR4, TLR5, TLR6 and TLR7 antibodies,
354 blocking agent was 5% BSA and antibody dilution was 1:10,000. For Myd88 antibody, blocking
355 agent was 5% milk and antibody dilution was 1:1,000. Antibodies used were TLR4: Cat#482300
356 (Life Technology). TLR5: Cat#PA1-41139 (Invitrogen), TLR6: Cat#AF1533 (R&D Systems),
357 TLR7: Cat# MAB7156 (R&D Systems), Myd88: Cat#4283 (cell signaling)

358

359 **ELISA Assays**

360 ELISA assay was performed using either R and D Duoset assay kits or Luminex multiplex assays
361 according to manufacturer recommendations.

362

363 **Human Ozone Exposure, Alveolar Macrophage Isolation, Culture**

364 The complete details of the human exposure studies and the subject characteristics were
365 previously published(47). After obtaining informed consent through a Duke University Institutional
366 Review Board approved protocol, healthy human subjects were exposed to filtered air and ozone
367 (200 parts per billion) in a crossover challenge designed study. Exposures were for 135 minutes,
368 during which participants alternated between resting and walking on a treadmill at 2-3 mph to
369 mimic an individual performing mildly strenuous activity under ambient conditions. Ozone was
370 created from a 100% O2 source by cold plasma corona discharge (Ozotech, Yreka CA), and
371 mixed with filtered air before addition to chamber and was continuously monitored. The order of
372 filtered air or ozone exposure were randomized for every participant, with at least a 21-day
373 washout period. Approximately 20 hours after exposure, participants underwent a flexible
374 bronchoscopy with bronchoalveolar lavage. Following bronchial alveolar lavage, human alveolar
375 macrophages were isolated. After red cell lysis and counting, the macrophages were re-
376 suspended in media (RPMI1640 with 10% heat-inactivated FBS, 100 units/ml penicillin, and 100
377 µg/ml streptomycin) and plated in a 24 well plate at a density of 200,000 cells per well. The cells
378 were maintained in a CO2 incubator at 37º C for 2 hours. After 2 hours, the media was replaced
379 to remove non-adherent cells and then 2 hours later, the supernatant was collected and the cells
380 were harvested for RNA. RNA extraction was performed using the Fourth Edition Qiagen Protocol
381 (Qiagen, RNeasy Mini Kit, 4th edition, Valencia, CA), followed by DNase treatment (DNase I,
382 Ambion, Austin, TX) and cDNA synthesis (BioRad). RT-PCR was performed on an ABI SDS 7500
383 (Applied Biosystems) using SYBR Green Reagent (Clontec Laboratories Inc., Mountain View,
384 CA). TLR5 expression was determined in comparison to the 18-s RNA housekeeping gene and

the data reported as fold change over the matched filter air sample for each individual subject. The following primers were used for RT-PCR: 18s (Fwd: GTAACCCGTTGAACCCCATT, Rev: CCATCCAATCGGTAGTAGCG); TLR4 (Fwd: GGCCATTGCTGCCAACAT, Rev: CAACAATCA CCTTTCGGCTTTT), TLR5 (Fwd: TGTATGCACTGTCACTCTGACTCTGT, Rev: AGCCCCGGAACTTTGTGACT). Human TNF-α was measured from the cell supernatants via ELISA (MAX Standard Set kit, BioLegend) according to manufactures instructions. Readings were taken using BMG LABTECH Omega (Software Version 1.20). In a second study, participants were invited according to their rs5744168 genotype. Peripheral blood was drawn, and monocyte-derived macrophages isolated after 7 days in culture and exposed to ultrapure LPS or flagellin as indicated.

Statistical Analyses

Data are represented as mean ± s.e.m. and were analyzed depending on experimental design by either analysis of variance (one-way or two-way ANOVA) followed by Tukey's post hoc test or by unpaired t test with Welch's or Holm-Sidak correction as appropriate. Tlr5 gene expression and TNF-α production data from ozone expose human volunteer macrophages is presented as individual values and analyzed by Wilcoxon pairwise signed rank test.

Study approval

All clinical studies described in this work were approved by the Institutional Review Boards of the NIEHS and Duke University respectively. Written informed consent was received from all participants prior to inclusion in the described studies.

Author Contributions

SG and SH conceived the work, designed experiments, analyzed data and wrote the manuscript with input from all co-authors. SH, CJG, JS, XM, VPS, JMC, KB, CL, WQ, JA, KMG and RMT performed experiments and contributed in data analysis as well manuscript writing. WMF, RMT, JWH, XL, MBF contributed in manuscript writing and provided critical input for improving the study. All authors read and approved final version of the manuscript.

Salik Hussain, Collin G Johnson and Joseph Sciurba contributed equally in performing experiments.

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