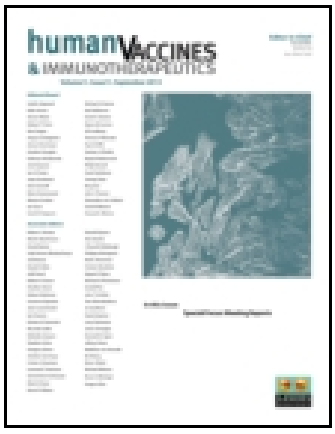




Human Vaccines & Immunotherapeutics
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/khvi20

# Gene expression profiles identify both MyD88-independent and MyD88-dependent pathways involved in the maturation of dendritic cells mediated by heparan sulfate: A novel adjuvant

Meini Wu[a], Haixuan Wang[a], Jiandong Shi[a], Jing Sun[a], Zhiqing Duan[a], Yanhan Li[a], Jianfang Li[a], Ningzhu Hu[a], Yiju Wei[a], Yang Chen[ab] & Yunzhang Hu[a]

[a] Institute of Medical Biology; Chinese Academy of Medical Sciences and Peking Union Medical College; Kunming, China

[b] MOE Key Laboratory of Bioinformatics; Bioinformatics Division/Center for Synthetic & Systems Biology; TNLIST; Tsinghua University; Beijing, China

Published online: 10 Feb 2015.

To cite this article: Meini Wu, Haixuan Wang, Jiandong Shi, Jing Sun, Zhiqing Duan, Yanhan Li, Jianfang Li, Ningzhu Hu, Yiju Wei, Yang Chen & Yunzhang Hu (2014) Gene expression profiles identify both MyD88-independent and MyD88-dependent pathways involved in the maturation of dendritic cells mediated by heparan sulfate: A novel adjuvant, Human Vaccines & Immunotherapeutics, 10:12, 3711-3721, DOI: 10.4161/21645515.2014.980682

To link to this article: http://dx.doi.org/10.4161/21645515.2014.980682

RESEARCH PAPER

Human Vaccines & Immunotherapeutics 10:12, 3711–3721; December 2014; © 2014 Taylor & Francis Group, LLC

# Gene expression profiles identify both MyD88-independent and MyD88-dependent pathways involved in the maturation of dendritic cells mediated by heparan sulfate: A novel adjuvant

Meini Wu[1], Haixuan Wang[1], Jiandong Shi[1], Jing Sun[1], Zhiqing Duan[1], Yanhan Li[1], Jianfang Li[1], Ningzhu Hu[1], Yiju Wei[1], Yang Chen[1,2], and Yunzhang Hu[1,*]

[1]Institute of Medical Biology; Chinese Academy of Medical Sciences and Peking Union Medical College; Kunming, China; [2]MOE Key Laboratory of Bioinformatics; Bioinformatics Division/Center for Synthetic & Systems Biology; TNLIST; Tsinghua University; Beijing, China

**Keywords:** dendritic cells, gene expression profile, heparan sulfate, humoral immune response, toll-like receptor signaling pathway, vaccine adjuvant

**Abbreviations:** HS, heparan sulfate; HBsAg, hepatitis B surface antigen; NF-kB, nuclear factor-kappa B; TRAF3, TNF receptor-associated factor 3; IRF7, interferon regulatory factor 7; MyD88, myeloid differentiation primary response 88; DCs, Dendritic cells; HAV, hepatitis A virus; Rab/Vac, Rabies Vaccine; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes.

The traditional vaccine adjuvant research is mainly based on the trial and error method, and the mechanisms underlying the immune system stimulation remaining largely unknown. We previously demonstrated that heparan sulfate (HS), a TLR-4 ligand and endogenous danger signal, effectively enhanced humoral and cellular immune responses in mice immunized by HBsAg. This study aimed to evaluate whether HS induces better humoral immune responses against inactivated Hepatitis A or Rabies Vaccines, respectively, compared with traditional adjuvants (e.g. Alum and complete Freund's adjuvant). In order to investigate the molecular mechanisms of its adjuvanticity, the gene expression pattern of peripheral blood monocytes derived DCs (dendritic cells) stimulated with HS was analyzed at different times points. Total RNA was hybridized to Agilent SurePrint G3 Human Gene Expression 8 × 60 K one-color oligo-microarray. Through intersection analysis of the microarray results, we found that the Toll-like receptor signaling pathway was significantly activated, and NF-kB, TRAF3 and IRF7 were activated as early as 12 h, and MyD88 was activated at 48 h post-stimulation. Furthermore, the expression of the surface marker CD83 and the co-stimulatory molecules CD80 and CD86 was up-regulated as early as 24 h. Therefore, we speculated that HS-induced human monocyte-derived DC maturation may occur through both MyD88-independent and dependent pathways, but primarily through the former (TRIF pathway). These data provide an important basis for understanding the mechanisms underlying HS enhancement of the immune response.



## Introduction

Vaccine adjuvants play an increasingly important role in most clinically used vaccines; due to the relatively poor immunogenicity of non-live vaccines, co-administration of effective adjuvants is required for their activity. To date, alum is by far the most widely used vaccine adjuvant, even though its mechanisms of action remain a mystery. Hundreds of vaccine adjuvants, including liposomes, emulsions, cytokines, microbial products, mineral salts, microparticles and natural adjuvants,[1] have been studied and some even tested in pre-clinical studies. However, only a handful of adjuvants have been approved for prophylaxis in humans. Alum and the TLR4-agonist monophosphoryl lipid A formulated in alum (AS04) were licensed for human use by the US. Food and Drug Administration (FDA), while AS03, AS04 and the oil-in-water emulsions MF59 have been authorized by the European Medicinal Evaluation Agency.[2] The traditional studies of vaccine adjuvants have been mainly based on the trial and error method, and Janeway famously described adjuvants as 'the dirty little secret of immunologists'.[3,4] Therefore the molecular mechanisms of adjuvanticity has been long considered enigmatic.

Toll-like receptors (TLRs) are pattern recognition receptors present on diverse cell types that recognize conserved molecular motifs of microorganisms like bacteria, virus or fungi.[5] The activation of TLRs by their cognate ligands link innate and adaptive

*Correspondence to: Yunzhang Hu; Email: huyunzhangym@126.com; Yang Chen; Email: yc@mail.tsinghua.edu.cn
Submitted: 05/23/2014; Revised: 07/19/2014; Accepted: 09/12/2014
http://dx.doi.org/10.4161/21645515.2014.980682

immune responses; thus, TLR agonists can be used as vaccine adjuvants to evoke immune response.[6,7] Multiple TLR ligands have been used successfully as adjuvants, including lipoproteins and lipopeptides for TLR-2/1 or TLR-2/6,[8] bacterial lipopolysaccharides for TLR-4,[9] flagellin for TLR-5,[10] double stranded RNA for TLR-3,[11] single stranded RNA genome or oligoribonucleotides for TLR-7 orTLR-8,[12] and bacterial unmethylated CpG DNA for TLR-9.[13]

Dendritic cells (DCs) are potent antigen-presenting cells, which bridge innate and adaptive immunity by providing a link between antigen recognition and antigen processing for presentation to naïve T cells.[14] There are 2 functional stages of DCs: the immature phase for phagocytosing and processing of antigens, and the mature stage for antigen presentation to activated T cells. DC maturation is critical to inducing antigen-specific T lymphocyte responses and controlling T cell differentiation.[15,16] DCs can be activated by pathogens directly, through recognition of pathogen-associated microbial patterns (PAMPs) like endotoxin, peptidoglycan, or unmethylated CpG motifs on Toll-like receptors (TLRs), or indirectly, through recognition of damage associated molecular patterns (DAMPs) like uric acid, ATP, or HMGB-1, released upon tissue damage inflicted by pathogens. This dual activation mechanism is important to understanding the mechanism of action of most adjuvants.

Heparan sulfate (HS), a ubiquitous component of the extracellular matrix, is an endogenous TLR-4 ligand, and has been shown to display beneficial immune stimulatory effects.[17-20] HS is rapidly released from the cell surface and basement membranes, upon tissue damage and inflammation.[21–24] Indeed, HS influences a multitude of processes relevant to inflammation, including the modulation of inflammatory cell maturation and activation, leukocyte adhesion to a stimulated endothelium, chemotaxis and transmigration.[25,26] In addition, it was reported that HS activates murine macrophages [27-29] and induces phenotypic maturation of murine bone marrow-derived dendritic cells[30] to regulate immune responses. We hypothesized that HS may constitute a potential vaccine adjuvant to enhance the immune response induced by poorly immunogenic vaccines. Indeed, we previously demonstrated that HS effectively enhanced immune responses in HBsAg immunized mice.[31] However, it remains unclear how HS triggers the immune response.

Herein, the effect of HS on the immune response in mice immunized by inactivated Hepatitis A or Rabies Vaccines were assessed. In addition, the effect of HS on DCs was investigated in vitro. Furthermore, the gene expression pattern of DCs stimulated by HS was evaluated, in order to explore the possible mechanism by which HS enhances the immune response.

## Results

### The adjuvant effect of HS on HAV or Rabies vaccine-specific humoral immune responses

ELISA was performed to assess HAV-specific antibody responses in mice after co-delivery of inactivated HAV vaccine and HS. As shown in **Fig. 1A**, anti-HAV IgG titers were significantly higher after induction by inactivated HAV vaccine plus 100 μg HS, compared with inactivated HAV vaccine alone ($P < 0.01$). More excitingly, inactivated HAV vaccine co-immunized with 100 μg HS elicited higher antibody titers, compared with inactivated HAV vaccine co-administered with Alum ($P < 0.05$, **Fig. 1A**). Interestingly, the HS adjuvanticity was dose dependent (**Fig. 1A**).

Whether HS induces immune memory should be considered an important factor for its development as new vaccine adjuvant. We investigated the duration of HAV-specific long-lasting humoral immune responses in mice immunized by inactivated HAV vaccine plus HS. HAV-specific IgG levels in mice immunized by inactivated HAV vaccine alone declined markedly after peaking at the 8th week after the last immunization (**Fig. 1A**). In contrast, when mice were immunized by inactivated HAV vaccine plus 100 μg HS, the high levels were maintained for 28 weeks of testing (**Fig. 1A**). As expected, HS remarkably enhanced the inactivated HAV vaccine immunogenicity.

In order to evaluate the effect of the HS adjuvant on immune response in mice intramuscularly administered with inactivated Rabies Vaccine, serum Rabies-specific IgG antibody levels were measured after co-administration with HS. HS at 100 μg was tested, as the best dose enhancing HAV immune response, as shown above. Interestingly, higher levels of Rabies -specific IgG were detected in serum samples from mice administered with inactivated Rabies Vaccine /HS taken 2 or 4 weeks after the last immunization, compared with animals administered with inactivated Rabies Vaccine alone ($P < 0.01$, **Fig. 1B**). Importantly, inactivated Rabies Vaccine co-immunized with HS elicited similar antibody titers as with FCA co-administration (**Fig. 1B**) . Therefore, HS is a potential vaccine adjuvant as anticipated, and can be used to enhance Rab/Vac immunogenicity.

### The phenotypic profile of DCs stimulated by HS

To explore the possible mechanisms by which HS enhances the immune response, a cell model of 5-day $CD14^+$ monocyte-derived immature DCs (imDCs) was first established (**Fig. 2A** and **S2A**). The purity of separated $CD14^+$ monocytes was routinely found to be ≥95% which hardly expressed the DC-lineage markers CD1a and CD209(DC-SIGN)(**Fig. 2A**, top row).While the expression of CD1a and CD209 distinctly increased to 88.2% and 93.3%, respectively, and the expression of CD14 was notablely declined to 2.45% after 5-day culture in the presence of GM-CSF and IL-4 (**Fig. 2A**, bottom row).

To determine whether treatment with HS affected DCs maturation, the expression of the surface markers CD80, CD86, CD83, and HLA-DR was analyzed by flow cytometry before and after 24 and 48 hours of HS stimulation, respectively. The expression of CD83 (**Fig. 2B**) that is a cell surface marker predominantly expressed on mature human DCs and co-stimulatory molecules CD80 (**Fig. 2C**) and CD86 (**Fig. 2D**) that aid in T lymphocyte stimulation was increased during maturation, in agreement with previous reports.[18, 30, 32, 33] Specifically, the expression of CD83, CD80 and CD86 was higher at 24 h than at 48 h. However, the expression of HLA-DR (**Fig. S2B**) did not changed overtly.

### Gene expression profile of DCs upon HS stimulation by microarray analysis

The gene expression patterns in DCs was assessed after stimulation with HS for 12, 24 and 48 h. First, the biological significance of differentially expressed genes was investigated separately at each time point using DAVID (Database for Annotation, Visualization and Integrated Discovery). Globally, the GO biological processes (BP) were mostly activated at 12 h, the earliest time point, and no statistically significant GO BP terms were identified at 48 h (**Fig. 3**). The major GO BP terms obtained were (1) response to external stimulus, (2) immune response, (3) response to wound and inflammatory response and (4) chemotaxis and cell chemotaxis at 12 h (**Fig. 3A**), and (1) cellular response to chemical stimulus and (2) cell migration and cell chemotaxis at 24 h (**Fig. 3B**).

To determine which specific biological pathways were differentially affected during the HS stimulation process, we performed a pathway analysis. The most enriched pathways are shown in **Fig. 4** and included cytokine-cytokine receptor interaction, chemokine signaling pathway, NOD-like receptor signaling pathway, MAPK signaling pathway, Toll-like receptor signaling pathway and apoptosis (P value was set at $<0.05$). We were particularly interested in Toll-like receptor signaling pathway because of their potential role in HS adjuvanticity. Interestingly, we found that the Toll-like receptor signaling pathway was significantly activated, well-studied genes of Toll-like receptor signaling pathway were involved such as NF-kB and MyD88 (see supplementary **Table S1**).

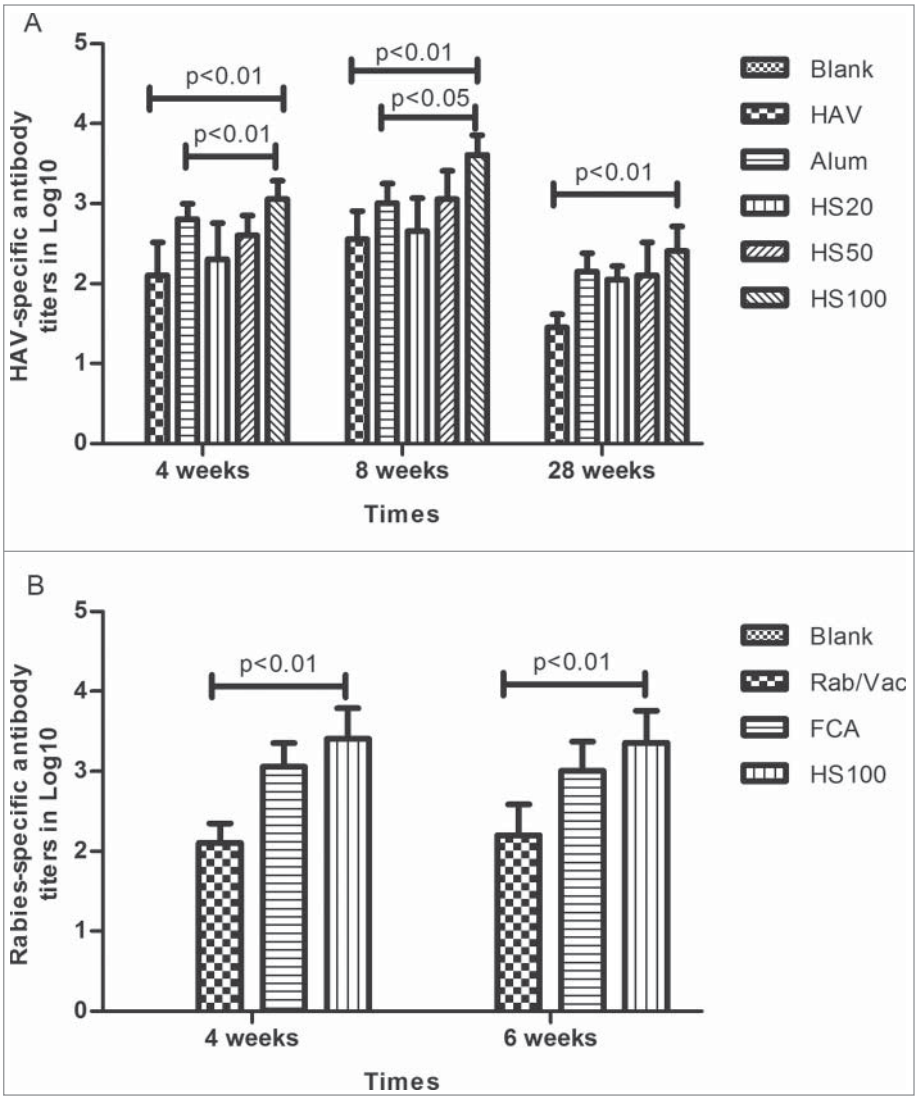



**Figure 1. Immune responses enhanced by HS.** (**A**) HAV-specific antibody titers. At 4, 8, and 28 weeks after the last immunization, mouse serum samples were prepared. HAV-specific IgG was detected by ELISA and presented as log values of the endpoint titers with standard deviations (n = 6). (**B**) Rabies-specific antibody titers. At 2 and 4 weeks after the last immunization, mouse serum samples were obtained. Rabies -specific IgG was detected by ELISA and presented as log values of the endpoint titers with standard deviations (n = 6).

To investigate the Toll-like receptor signaling pathway in detail, the 37 overtly differentially-expressed genes involved in this pathway after stimulation with HS were selected and used to generate a gene network (**Fig. 5**). As shown in **Figure 5**, a number of pro-inflammatory genes such as *il1b*, *il6*, *il12b* and *tnfa* were over-expressed. *Nfkb* and *traf3* were upregulated at 12 h and 48 h post-stimulation. However, *myd88* displayed higher up-regulation at 48 h post-stimulation. *Cd40* and *cd80*, important markers of dendritic cell maturation that aid in T lymphocyte stimulation, were also up-regulated upon stimulation. The expression of a few chemokines (*cxcl10*, *ccl3*, *ccl4* and *ccl5*) was increased as well. On the other hand, HS treatment of dendritic cells negatively modulated the expression of some genes, including *tlr5*, *tlr8*, *akt2*, *akt3*, *cd14*, *fos*, *mapk3* and *mapk2k6*. In particular, the expression of *tlr3* and *tlr7* were down-regulated at early time points, but the trend was reversed with time.

### Validation of microarray data by qRT-PCR

As shown in **Figures 4 and 5**, the Toll-like receptor signaling pathway was significantly activated. Furthermore, we found both MyD88-dependent and MyD88-independent pathways were triggered by analyzing the microarray data, In order to validate the microarray results, 13 key genes of the Toll-like receptor signaling pathway involved in both MyD88-dependent and MyD88-independent pathways (*tlr3*,*tlr4*,*tlr5*,*tlr7*, *tlr8*,*myd88*, *traf3*,*irf7*,*nfkb*,*mapk3*,*tnf-a*,*il8* and *ifn-a*) were selected for

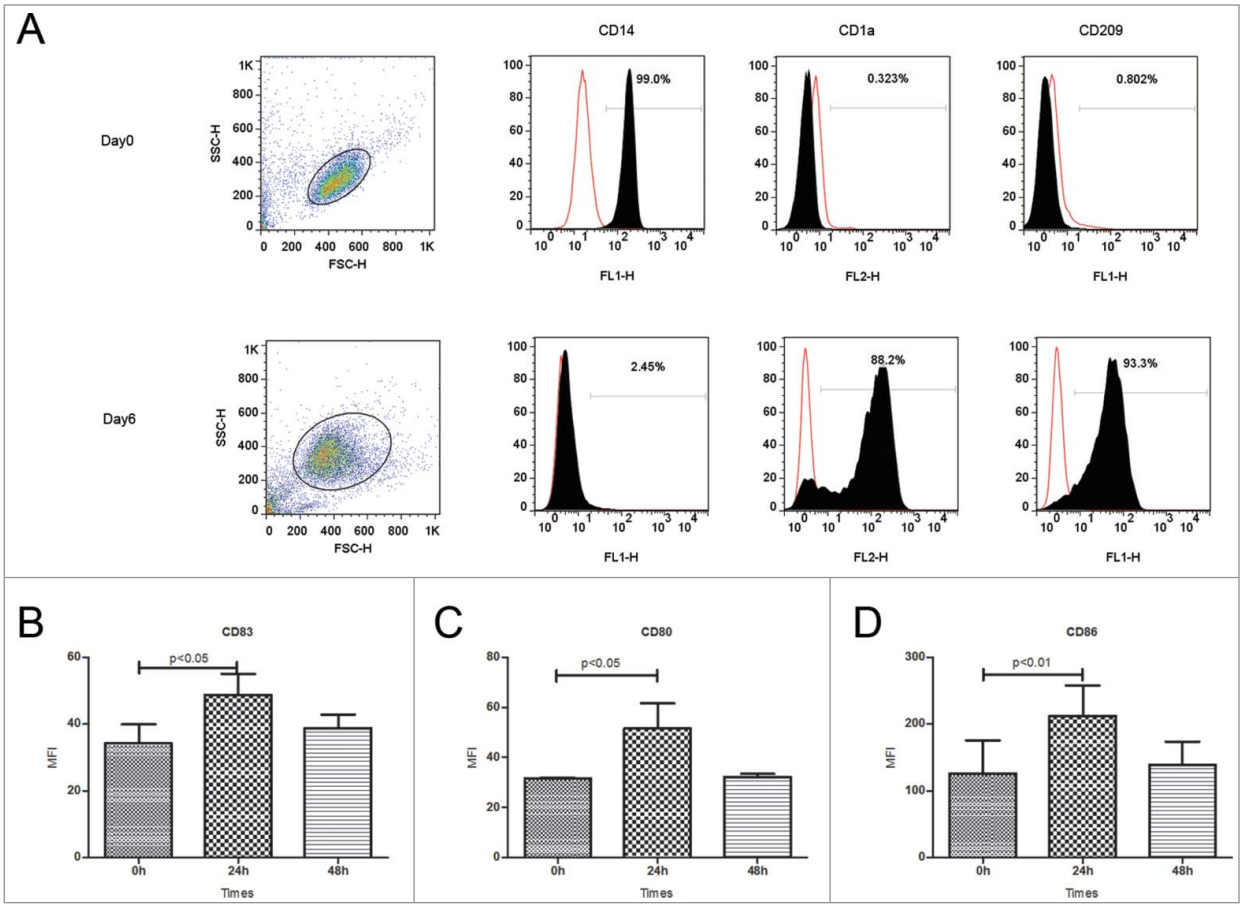



**Figure 2. Expression of DC surface markers measured by FACS analysis at 0, 24 and 48 h.** (**A**) The phenotypic characterization of the cells at day0 (top row) and day6 (bottom row). Data is representative from more than 3 experiments with DC preparations from different donors. (**B**) Expression of the surface marker CD83 was increased after stimulation with HS. (**C**) Expression of the co-stimulatory molecule CD80 was increased after stimulation with HS. (**D**) Expression of the co-stimulatory molecule CD86 was increased after stimulation with HS. Data were obtained from more than 3 experiments with DC preparations from different donors.

analysis by qRT-PCR (**Fig. 6**). The qRT-PCR results were in agreement with the microarray data.

## Discussion

Nowadays, adjuvants have proven to be key vaccine ingredients, which enhance the immunogenicity of vaccines. Although aluminum-containing products have been the most widely used adjuvants in human vaccines for nearly a century, the cellular and molecular mechanisms underlying their actions are surprisingly still unknown.[34,35] It is well accepted that TLRs on APCs are the best characterized receptors in promoting adaptive immune responses.[36] Monophosphoryl lipid A (MPLA), a TLR4 agonist, was the first approved adjuvant capable of driving a robust TH1 response.[37,38] Interestingly, soluble HS fragments derived from HSPG are endogenous danger signals recognized by Toll-like receptor 4 (TLR4).[39] We hypothesized that HS might constitute a potential vaccine adjuvant. Consistently, we previously found that HS effectively enhanced the immune responses in mice immunized by HBsAg.[31]

In this study, we assessed the effects of HS on the inactivated HAV and inactivated Rabies vaccine-specific humoral immune responses in mice. Compared with the immune responses induced by vaccines alone, co-immunization of HS resulted in higher antigen-specific antibody titers (**Fig. 1**). Importantly, HS enhanced the HAV vaccine immunogenicity to a higher degree compared with Alum (**Fig. 1A**). In contrast to immunization with inactivated HAV vaccine alone, higher antibody titers were maintained for a longer duration after co-administration of inactivated HAV vaccine and 100 μg HS (**Fig. 1A**). These findings demonstrated that HS is an effective vaccine adjuvant. Moreover, HS derived from cell basement membrane[19–23] is degradable, indicating that it is safer than exogenous substances as a vaccine adjuvant.

Dendritic cells (DCs) play a key role in the immune system for both the acquired and innate immune responses. DCs maturation induced by proinflammatory cytokines such as IL-1, TNF-α, or IL-6, or by TLR ligands produced by pathogens was shown to be crucial for the activation of naïve T cells.[40] To explore the mechanisms underlying the enhancement by HS of the immune response, monocytes-derived imDCs were

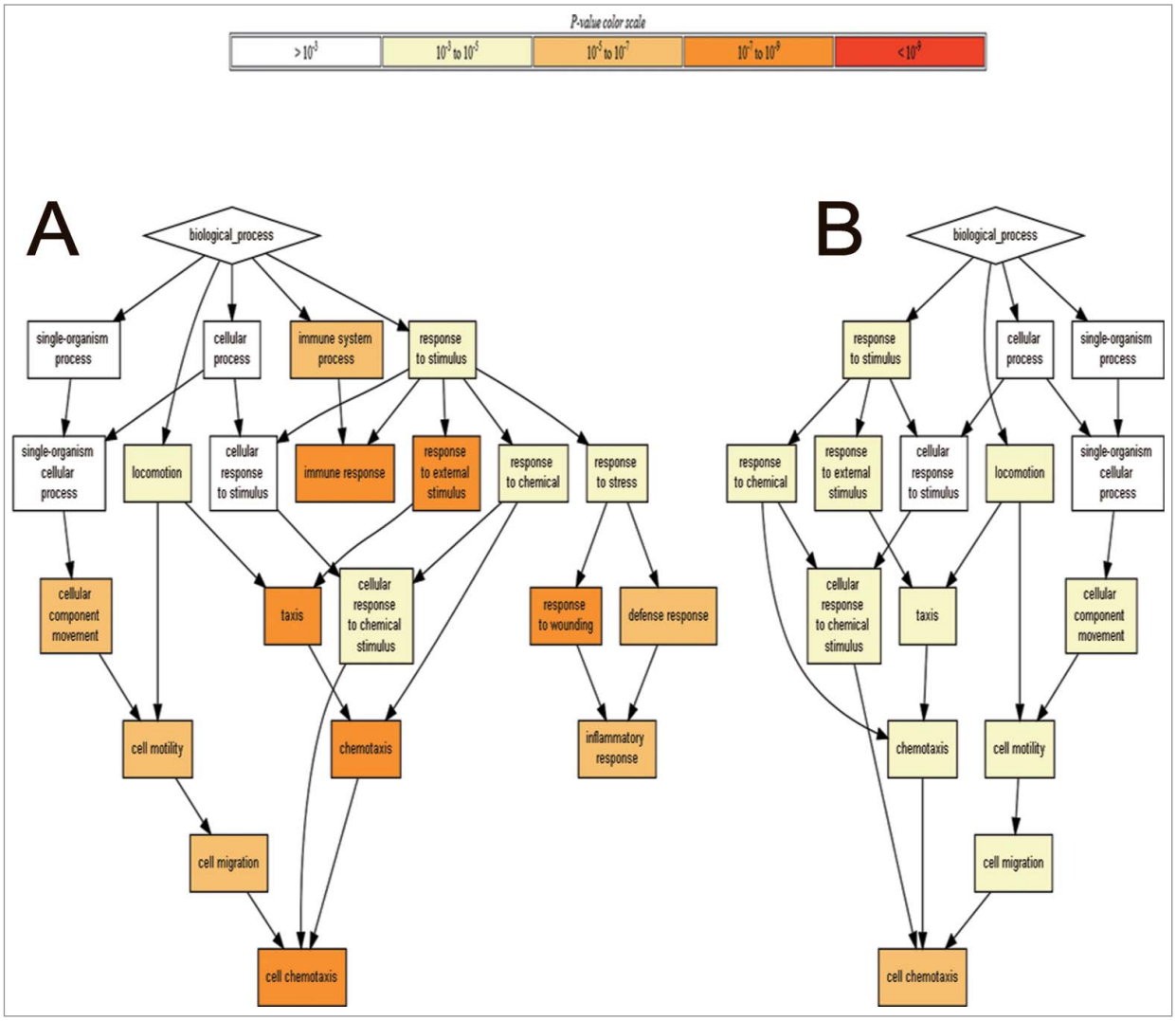



**Figure 3. Biological process enrichment of differential expressed genes at 12 and 24 h.** DEGs at (**A**)12 h and (**B**)24 h were used for biological process enrichment with Gorilla, an online gene ontology analyzer. The color bar shows the P value scale.

stimulated by HS in vitro and DC gene expression profiles were analyzed at different time points. As shown above, HS stimulated DCs maturation by up-regulating the expression of the surface marker CD83 (**Fig. 2B**) and the co-stimulatory molecules CD80 and CD86 (**Fig. 2C and D**) that aid in T-lymphocyte stimulation, in agreement with previous reports.[18, 30,32,33,41-43]

To further characterize the underlying mechanisms of HS adjuvanticity, the gene expression profiles of DCs exposed to HS were analyzed at different time points. GO classification showed that the most enriched GO terms included immune response, response to external stimulus, response to wound and inflammatory response, cellular response to chemical stimulus, chemotaxis and cell chemotaxis (**Fig. 3**). In addition, analysis of KEGG pathways revealed that the most enriched pathways included cytokine-cytokine receptor interaction, chemokine signaling pathway, NOD-like receptor signaling pathway, MAPK signaling pathway, Toll-like receptor signaling pathway and apoptosis (**Fig. 4**). We were particularly interested in Toll-like receptor signaling because of their potential role in HS adjuvanticity.

TLR4 signaling pathway can be activated by 2 adaptors: MyD88 and TRIF/TRAM, which triggers MyD88-dependent and MyD88-independent pathways, respectively.[44] Therefore, there are 2 distinct corresponding outcomes: the former leads to expression of proinflammatory genes such as TNF, IL-1β, IL-6, IL-12 through activation of NF-kB,[45] while the latter leads to IFN regulatory factor 3/7 (IRF3/7)-mediated expression of type I IFNs (IFN-α and IFN-β) for anti-viral responses[46,47] and IFN-inducible chemokines like CXCLl0, CCL5, and CCL2.[44,48] As shown above, NF-kB, TRAF3 and IRF7 were activated as early as 12 h, and MyD88 was activated at 48 h post-stimulation (**Figs. 5 and 6**). Furthermore, CD40, CD80 and CD86 were upregulated at early time points (**Figs. 2 and 5**). These findings suggested that the HS-induced human monocytes-derived DCs maturation may occur through both MyD88-dependent and

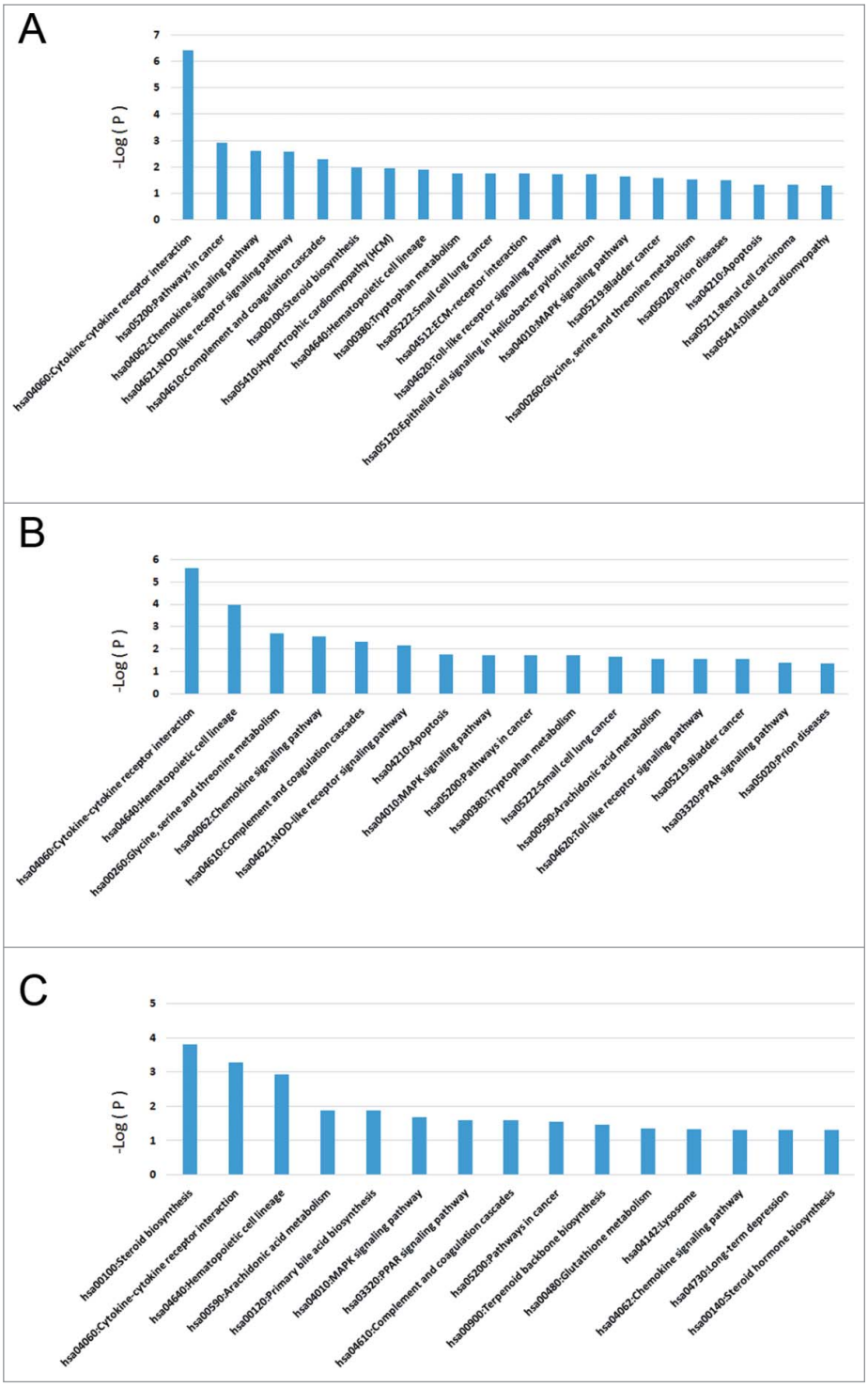



**Figure 4. KEGG pathways enrichment of differential expressed genes after HS induction.** DEGs at (**A**) 12 h, (**B**) 24 h, and (**C**) 48 h were used for KEGG pathway analysis with the DAVID functional enrichment database. Pathways with P value less than 0.05 are shown.

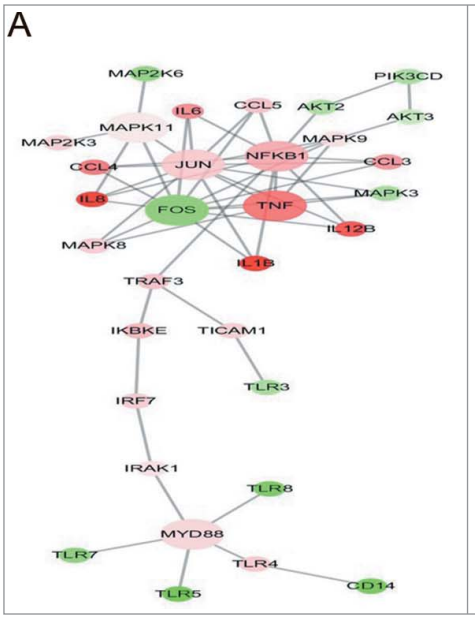
MyD88-independent pathways, but primarily through the MyD88-independent (TRIF) pathway, in accordance with other reports. For instance, it was reported that both MyD88 and TRIF pathways downstream of TLR4 can lead to functional DC maturation,[49-52] which is critical to link the innate and adaptive immune cascades. It has also been reported that HS-induced inflammation is executed predominantly through the IRF3 pathway, a MyD88-independent/TRIF pathway, which is distinct from that induced by LPS.[53] We demonstrated that a number of pro-inflammatory genes such as *il1b*, *il6*, *il12b* and *tnfa* and chemokines such as *cxcl10*, *ccl3*, *ccl4* and *ccl5* were overexpressed; these genes are crucial for fighting infection and establishing immunity.[54,55] Both MyD88 and TRIF signaling pathways were shown to be essential for effective adjuvanticity of a TLR4 agonist in inducing a Th1 immune response characterized by $CD4^+$ T cells producing IFN-γ, TNF, and IL-2.[56] Interestingly, TRIF activation is considered necessary and sufficient for effective adjuvant activity of TLR4 agonists.[57–60] Additionally, the adjuvant effect of LPS in inducing $CD8^+$ T-cell responses was shown to be dependent on both MyD88 and TRIF.[61]

In conclusion, we demonstrated that HS has potent effects as vaccine adjuvant when co-immunized with inactivated vaccines in mice. To further explore the underlying mechanism of HS adjuvanticity, the transcriptional expression patterns of DCs exposed to HS was assessed. To the best of our knowledge, this is the first study using microarray assays to investigate the effects of HS on the immune system, and assess the effects of HS on human monocytes-derived DCs. From the microarray results, we found numerous differentially expressed genes after HS treatment at different time points and analyzed them using functional enrichment analysis. Our results provide a strong basis for further exploration and help better understand the adjuvanticity mechanisms of HS. However, more studies should be carried out to further evaluate HS, as a novel vaccine adjuvant.

## Materials and Methods

### Animal immunization

Six to 8-week-old female ICR mice (18–22g) were purchased from the Institute of Medical Biology, the Chinese Academy of Medical Sciences & Peking Union Medical College. All animal experiments were in conformity with the Office of Laboratory




B

| Gene | HS-12h | HS-24h | HS-48h | Degree | BC |
|---|---|---|---|---|---|
| NFKB1 | 1.911010066 | 1.356508827 | 0.733886082 | 11 | 0.61469534 |
| TRAF3 | 1.367389357 | 0.983819029 | 0.854887818 | 3 | 0.51182796 |
| IKBKE | 1.579081663 | 1.32950357 | 1.808034257 | 2 | 0.39569892 |
| IRF7 | 1.107463969 | 0.719632248 | 0.69050526 | 2 | 0.36129032 |
| IRAK1 | 0.660217989 | 0.667999076 | 1.31074909 | 2 | 0.32258065 |
| MYD88 | 0.865334654 | 1.197173686 | 1.971670378 | 5 | 0.29892473 |
| MAPK11 | 0.472282535 | 1.292389274 | 1.273190714 | 5 | 0.12706093 |
| TNF | 3.399874751 | 2.250028162 | 2.270479854 | 6 | 0.11415771 |
| IL8 | 5.056557623 | 3.919352773 | 2.209151198 | 4 | 0.11200717 |
| JUN | 1.17730558 | 0.716116226 | -0.1717766 | 12 | 0.07706093 |
| FOS | -1.576293616 | -1.035250531 | -1.373590545 | 12 | 0.07706093 |
| TLR4 | 1.190310449 | 0.223529128 | 0.945111728 | 2 | 0.06451613 |
| TICAM1 | 1.07378552 | 0.950084142 | 0.742028869 | 2 | 0.06451613 |
| AKT3 | -0.610878186 | -1.084944795 | -0.904424711 | 2 | 0.0311828 |
| AKT2 | -0.977466732 | -1.090115591 | -2.306899699 | 2 | 0.0311828 |
| IL1B | 5.944865863 | 3.872489021 | 2.222944124 | 3 | 0.00878136 |
| IL12B | 4.380353319 | 5.287487945 | 5.67920228 | 3 | 0.00878136 |
| CCL4 | 3.273753364 | 1.260823877 | 1.099130327 | 3 | 0.00878136 |
| IL6 | 2.752313211 | 1.336484198 | -0.152392728 | 3 | 0.00878136 |
| CCL3 | 2.490687504 | 1.534952039 | 1.399099852 | 3 | 0.00878136 |
| CCL5 | 1.343709906 | 0.718851904 | -0.864195632 | 3 | 0.00878136 |
| PIK3CD | -1.024003355 | -0.631705867 | -0.490478301 | 2 | 0.00107527 |
| MAPK8 | 1.112240138 | 1.015216361 | 0.575918305 | 3 | 0.00017921 |
| MAPK9 | 0.66883723 | 0.738907885 | 1.21846955 | 3 | 0.00017921 |
| MAPK3 | -1.072791128 | -0.605519162 | 0.770933272 | 3 | 0.00017921 |
| MAP2K3 | 1.005225242 | 0.237885711 | 0.43352135 | 1 | 0 |
| TLR3 | -1.061693349 | 0.210508207 | 0.852440031 | 1 | 0 |
| MAP2K6 | -1.397698892 | -1.415240255 | -2.007268797 | 1 | 0 |
| TLR7 | -1.512648128 | -1.733792372 | 1.783618321 | 1 | 0 |
| TLR8 | -1.919532549 | -1.346162968 | 0.071878124 | 1 | 0 |
| CD14 | -2.089713806 | -2.741201355 | -1.366437924 | 1 | 0 |
| TLR5 | -3.10742178 | -2.387029344 | -1.686526488 | 1 | 0 |
| CD40 | 2.458143379 | 2.162124119 | 0.713278137 | #N/A | #N/A |
| CD80 | 2.454653018 | 1.90141453 | 0.924089369 | #N/A | #N/A |
| CXCL10 | 2.087119551 | 1.937039056 | 1.69714727 | #N/A | #N/A |
| TOLLIP | 1.609907906 | 1.174071642 | 1.579537379 | #N/A | #N/A |
| IFNAR1 | 0.877610353 | 1.21506186 | 1.032296649 | #N/A | #N/A |

**Figure 5. Protein-protein interaction network of the Toll-like receptor signaling pathway induced by HS.** (**A**) DEGs enriched in Toll-like receptor signaling pathway were used to build a protein-protein interaction network. The red nodes indicate up-regulated gene expression while green nodes indicate downregulated gene expression. The larger nodes represent genes with the higher degree, which show that these genes have more neighbor genes and are defined as key genes of the network. (**B**) This table was used to provide more information for panel A. The columns 'HS-12', 'HS-24' and 'HS-48' show the time series fold changes of each gene in the network by separately comparing 12 h, 24 h, and 48 h to 0 h. The columns 'degree' and 'BC' (betweenness centrality) show 2 topological properties of each gene in network, which were well-used for indicting the importance of each gene in the network. In general, higher degree or betweenness centrality means that gene is more important of in the network. The definition of degree and betweenness centrality are described in method. The last 5 genes (from CD40 to IFNAR1) are no value of degree and betweenness centrality for disconnecting with the network.



Animal Management of Yunnan Province, China. To evaluate the adjuvanticity, mice were randomly divided into 10 groups (n = 6). Five groups were immunized subcutaneously with inactivated Hepatitis A Vaccine (640 EU/ml, Institute of Medical Biology) alone or co-immunized with Alum, or different doses HS (20 μg, 50 μg and 100 μg). The remaining groups received intramuscular administration of inactivated Rabies Vaccine (Hamster Kidney Cell, Hissen) alone or with FCA, or 100 μg HS. The different treatment groups are summarized in **Table 1**. Blood samples were obtained from tail veins at different time points after the last immunization. The mouse immunization and sample analysis schedules are shown in **Figure S1A and S1B**.

### Enzyme-linked immunosorbent assay (ELISA)

Levels of specific IgG antibody were measured by ELISA, according to the technical guide for KPL ELISA-protocols. Briefly, 96-well flat-bottom plates (Costar, USA) were coated with HAV or Rabies antigens, respectively, in coating solution (1×, KPL) at 4°C overnight. Then, plates were washed 4 times with washing solution (1×, KPL). Next, the wells were blocked with the blocking solution (1×BSA, KPL) at 37°C for 1 h. Mouse serum samples were submitted to a 2-fold serial dilution in blocking solution (starting at 1:20) and 100 μl were added to each well. After incubation at 37°C for 1 h, plates were washed 4 times and incubated with 1:1000 diluted HRP-labeled goat anti-mouse IgG antibody (KPL) at 37°C for 1 h. After a final wash, 100 μl of freshly prepared substrate (ABTS substrate solution A and peroxidase solution B, mixed at equal volumes) were dispensed into each well followed by 15 min incubation. 2M $H_2SO_4$ was used to stop the reaction and optical density (OD) was measured at 405/625 nm on a Multscan ELISA plate reader (BIO-RAD).

### Generation of monocyte-derived DCs

Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood of healthy donors by Ficoll-Hypaque gradient centrifugation. Blood samples were obtained from the Yunnan Kunming Blood Center, after approval by the local Medical Ethics Committee. $CD14^+$ monocytes were purified using the MACS CD14 isolation kit (Miltenyi Biotec), according to the manufacturer's instructions, and cell purity was more than 95%, as measured by flow cytometry. The separated cells were cultured in fresh RPMI 1640 complete medium (HyClone) supplemented with 10% fetal calf serum (Gbico), 1% penicillin/streptomycin, 1000 U/ml GM-CSF (R&D Systems, Minneapolis, MN, USA) and 500 U/ml IL-4 (R&D Systems), at 1–1.5 × $10^6$ cells/ml in 6-well plates for 5 d at 37°C, in a 5% $CO_2$ humidified incubator. Half of the medium was renewed at the third day and GM-CSF and IL-4 were added to initial concentrations. On day 6, some of the immature dendritic cells (imDCs) were further incubated with heparan sulfate (10 μg/ml, Sigma Aldrich). The DCs were harvested at 0, 12, 24 and 48 hours (h)

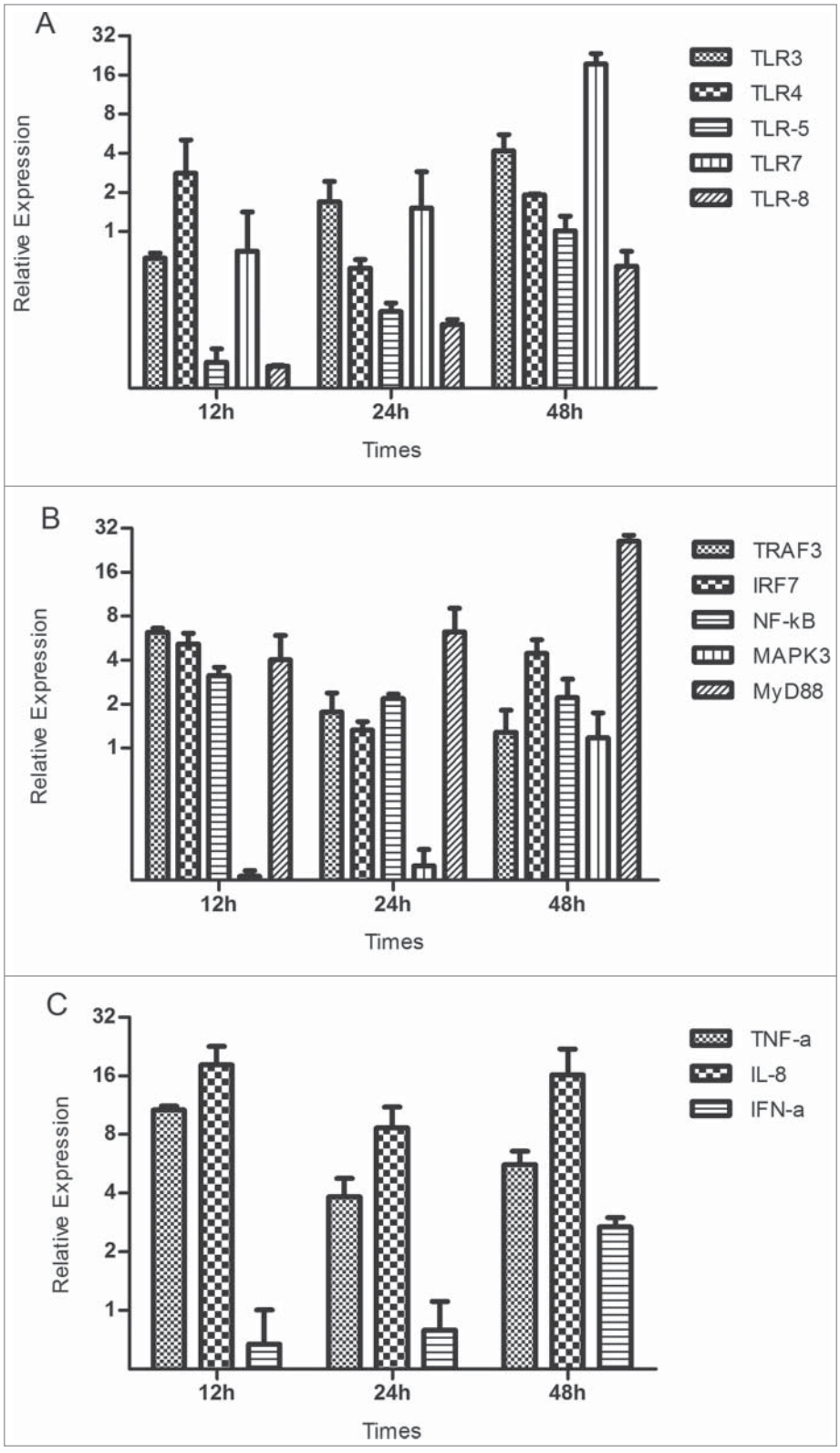



**Figure 6. Verification of microarray analyses by quantitative real-time PCR.** Relative expression of mRNAs of 13 key Toll-like receptor signaling pathway genes at 3 time points (12, 24 and 48 h post-stimulation), normalized to the reference gene gapdh and the reference sample at 0 h by the $2^{-\Delta\Delta Ct}$ method. (**A**) Toll like receptors (*tlr3*, *4*, *5*, *7* and *8*). (**B**) Adaptors and transcriptional factors (*traf3*, *irf7*, *myd88*, *nfkb*, *mapk3*). (**C**) Inflammatory cytokines (*Il8*, *ifna* and *tnfa*).

after addition of heparan sulfate. DC generation, stimulation and detection scheme is shown in **Figure S2A**.

### Flow cytometry

Surface markers of DCs were examined by flow cytometry on a FACSCALIBUR (BD Biosciences) with the FlowJo software, using a panel of monoclonal antibodies: anti-CD14-FITC (clone M5E2, BD PharMingen), anti-CD83-FITC (clone HB15E, BD PharMingen), anti-CD209- FITC (DC-SIGN; clone DCN46, BD PharMingen), anti-CD80-FITC (clone L307.4, BD PharMingen), anti-CD86-PE (clone FUN-1, BD PharMingen), anti-HLA-DR- FITC (clone l243, BD PharMingen), and anti-CD1a-PE (clone hi149, BD PharMingen). Mouse isotype controls were IgG1κ-FITC (clone MOPC-21, BD PharMingen), IgG1κ-PE (clone MOPC-21, BD PharMingen), IgG2ɜκ-FITC (clone G155–178, BD PharMingen), and IgG2bκ-FITC (clone 27–35, BD PharMingen). After antibody binding at 4°C for 30 min and 2 washings with PBS, the cells were fixed with 4% paraformaldehyde. Finally, cells were gated according to their forward and side scatter profiles, and surface marker expression of the large and granular cell population was analyzed using the FlowJo software.

### Preparation of RNA and microarray analysis

Total RNA was isolated from DCs pooled from 9 healthy donor blood samples after stimulation with HS (pooling of samples from 9 donors reduced individual variations) for 0, 12, 24, and 48 h, using the mirVana™ RNA Isolation Kit (Applied Biosystem, AM1556). RNA was purified by the QIAGEN RNAeasy mini kit (QIAGEN), according to the RNeasy Mini Protocol. RNA quality, concentration, and integrity were assessed on an Agilent 2100 Bioanalyzer. One-color Cy3 RNA labeling, array hybridization to Agilent SurePrint G3 8 × 60 K Human Gene Expression Arrays (Agilent Technologies), data collection, and analysis were performed at Oebiotech Biotechnology Corporation (Shanghai,China), according to Agilent protocols.

**Table 1.** Animal groups and immunogens

| Groups | Antigen | Dose | Adjuvant | Dose | Injection Routs | Injection times | Number |
|---|---|---|---|---|---|---|---|
| Inactivated Hepatitis A Vaccine | | | | | | | |
| Blank | / | / | / | / | / | / | 6 |
| HAV | Inactivated HAV | 20 μl | / | / | subcutaneous | 1 | 6 |
| Alum | Inactivated HAV | 20 μl | Alum | 300 μg | subcutaneous | 1 | 6 |
| HS20 | Inactivated HAV | 20 μl | HS | 20 μg | subcutaneous | 1 | 6 |
| HS50 | Inactivated HAV | 20 μl | HS | 50 μg | subcutaneous | 1 | 6 |
| HS100 | Inactivated HAV | 20 μl | HS | 100 μg | subcutaneous | 1 | 6 |
| Inactivated Rabies Vaccine | | | | | | | |
| Blank | / | / | / | / | / | / | 6 |
| Rab/Vac | Inactivated Rab/Vac | 50 μl | / | / | intramuscular | 3 | 6 |
| FCA | Inactivated Rab/Vac | 50 μl | FCA | 50 μl | intramuscular | 3 | 6 |
| HS100 | Inactivated Rab/Vac | 50 μl | HS | 100 μg | intramuscular | 3 | 6 |

HAV: inactivated hepatitis A vaccine; Rab/Vac: inactivated rabies vaccine; Alum: aluminum hydroxide; HS: heparan sulfate; FCA: Freund's complete adjuvant.



**Functional enrichment of differentially expressed genes (DEGs)**

DEGs at 12, 24 and 48 h were used for biological process enrichment with Gorilla, an online gene ontology analyzer. In addition, DEGs were used for pathway enrichment with DAVID functional annotation bioinformatics microarray.

**Construction and analysis of protein-protein interaction network of Toll-like receptor signaling pathway induced by HS**

The interactions between genes were constructed by protein-protein interaction database Human Protein Reference database (HPRD, v8.0). The node degree and betweenness centrality of DEGs were analyzed by Network Analyzer, a plug-in of Cytoscape 3.0.2. For the network, degree of a gene i in the network is the number of edges linked to the gene. And betweenness centrality of a gene i is defined as $b_i = \left(\sum_{s\neq i\neq t} \frac{\varphi_{st}(i)}{\varphi_{st}}\right) / \left(\frac{(N-1)(N-2)}{2}\right)$, where s and t are genes in the network different from gene i, $\varphi_{st}$ is the number of shortest paths from s to t, $\varphi_{st}(i)$ is the number of shortest paths from s to t that n lies on, and N is the total number of genes in the network.

**Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)**

To validate the results of the microarray analysis, 13 key genes of the Toll-like receptor signaling pathway were selected for analysis by quantitative real-time/ reverse-transcription polymerase chain reaction (qRT-PCR). Total RNA was isolated from DCs obtained from 6 additional healthy donors, and stimulated with HS for 0, 12, 24, and 48 h. The gene-specific primer pairs were selected from the PrimerBank database (http://pga.mgh.harvard.edu/primerbank/index.html) (**Table S2**).[62–64] First strand cDNA was synthesized from 5–10 μg RNA using GoScript™ Reverse Transcription System (Promega), according to manufacturers' protocol. Gene expression was quantified by GoTaq® qPCR Master Mix (Promega) on a CFX96™ Real-Time PCR Detection System (Applied Biosystems), according to manufacturer's instructions. Differences in expression were determined by the relative quantification method; threshold cycle [Ct] values were normalized with the endogenous control GAPDH to generate ΔCt, and the difference between ΔCt value of the sample (at 12, 24, and 48 h) and that of the reference sample (at 0 h) was calculated as ΔΔCt. The fold change was calculated based on relative quantity (RQ), with $RQ = 2^{-\Delta\Delta Ct}$.

**Statistical analysis**

Data are presented as mean ± standard deviation (SD) and statistical analyses were performed with professional statistical computer software SPSS (SPSS, USA). Differences between groups were determined using a t test. Significance level was set at $P < 0.05$.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

### Funding

This study was supported by grants from the National High Technology Research and Development Program (863 Program) of China (Grant No. 2012AA02A406) and the National Natural Science Foundation of China (NSFC, Grant No.31301044); Innovation Team Project of Yunnan province of China (Grant No. 2011CI140); and Applied and Fundamental Research program of Yunnan Province (Grant No. 2013FA025 and Grant No.2013FZ133). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

### Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

### References

1. Carl RA, Kristina KP, Mangala R and Steven GR. Adjuvants for human vaccines. Curr Opin Immunol 2012; 23:1-6
2. Lamine Mbow M, De Gregorio E, Valiante NM, Rappuoli R. New adjuvants for human vaccines. Curr Opin Immunol 2010; 22:411-6; PMID:20466528; http://dx.doi.org/10.1016/j.coi.2010.04.004
3. Janeway CA Jr. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol 1989; 54(Pt 1):1-13;

PMID:2700931; http://dx.doi.org/10.1101/SQB.1989.054.01.003
4. Brewer JM, Alexander J. Cytokines and the mechanisms of action of vaccine adjuvants. Cytokines Cell Mol Ther 1997; 3:233-46; PMID:9740352
5. Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol 2003; 21:335-76; PMID:12524386; http://dx.doi.org/10.1146/annurev.immunol.21.120601.141126
6. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol 2004; 5 (October (10)):987-95; PMID:15454922; http://dx.doi.org/10.1038/ni1112
7. van Duin D, Medzhitov R, Shaw AC. Triggering TLR signaling in vaccination. Trends Immunol 2006; 27 (January (1)):49-55; PMID:16310411; http://dx.doi.org/10.1016/j.it.2005.11.005
8. Takeda K, Takeuchi O, Akira S. Recognition of lipopeptides by toll-like receptors. J Endotoxin Res 2002; 8 (6):459-63; PMID:12697090; http://dx.doi.org/10.1179/096805102125001073
9. Cluff CW, Baldridge JR, Stover AG, Evans JT, Johnson DA, Lacy MJ, Clawson VG, Yorgensen VM, Johnson CL, Livesay MT, et al. Synthetic toll-like receptor 4 agonists stimulate innate resistance to infectious challenge. Infect Immun 2005; 73(May (5)):3044-52; PMID:15845512; http://dx.doi.org/10.1128/IAI.73.5.3044-3052.2005
10. Huleatt JW, Jacobs AR, Tang J, Desai P, Kopp EB, Huang Y, Song L, Nakaar V, Powell TJ. Vaccination with recombinant fusion proteins incorporating Toll-like receptor ligands induces rapid cellular and humoral immunity. Vaccine 2007; 25(January 8 (4)):763-75; PMID:16968658; http://dx.doi.org/10.1016/j.vaccine.2006.08.013
11. Trumpfheller C, Caskey M, Nchinda G, Longhi MP, Mizenina O, Huang Y, Schlesinger SJ, Colonna M, Steinman RM. The microbialmimic poly IC induces durable and protective CD4+ T cell immunity together with a dendritic cell targeted vaccine. Proc Natl Acad Sci USA 2008; 105(February 19 (7)):2574-9; PMID:18256187; http://dx.doi.org/10.1073/pnas.0711976105
12. Wille-Reece U, Flynn BJ, Lore K, Koup RA, Kedl RM, Mattapallil JJ, Weiss WR, Roederer M, Seder RA. HIV Gag protein conjugated to a Toll-like receptor 7/8 agonist improves the magnitude and quality of Th1 and CD8+ T cell responses in nonhuman primates. Proc Natl Acad Sci USA 2005; 102(October 18 (42)):15190-4; PMID:16219698; http://dx.doi.org/10.1073/pnas.0507484102
13. Waag DM, McCluskie MJ, Zhang N, Krieg AM. A CpG oligonucleotide can protect mice from a low aerosol challenge dose of Burkholderia mallei. Infect Immun 2006; 74(March (3)):1944-8; PMID:16495571; http://dx.doi.org/10.1128/IAI.74.3.1944-1948.2006
14. Bendelac A, Medzhitov R. Adjuvants of immunity: harnessing innate immunity to promote adaptive immunity. J Exp Med 2002; 195:F19-23; PMID:11877490; http://dx.doi.org/10.1084/jem.20020073
15. Cella M, Sallusto F, Lanzavecchia A. Origin, maturation and antigen presenting function of dendritic cells. Curr Opin Immunol 1997; 9:10-16; PMID:9039784; http://dx.doi.org/10.1016/S0952-7915(97)80153-7
16. Abbas AK, Murphy KM, Sher A. Functional diversity of helper T lymphocytes. Nature 1996; 383:787-93; PMID:8893001; http://dx.doi.org/10.1038/383787a0
17. Wrenshall LE, Platt JL, Stevens ET, Wight TN, Miller JD. Propagation and control of T-cell responses by heparan sulfate-bound IL-2. J. Immunol 2003; 170 (11): 5470-4; PMID:12759423; http://dx.doi.org/10.4049/jimmunol.170.11.5470
18. Brennan TV, Lin L, Huang X, Cardona D M, Li Z, Dredge K, Yang Y. Heparan sulfate, an endogenous TLR4 agonist, promotes acute GVHD after allogeneic stem cell transplantation. Blood 2012; 120(14); 2899-908; PMID:22760779; http://dx.doi.org/10.1182/blood-2011-07-368720
19. Ihrcke NS, Wrenshall LE, Lindman BJ, Platt JL. Role of heparan sulfate in immune system-blood vessel interactions. Immunol 1993; Today 14:500; PMID:8274190
20. Gilat D, Cahalon L, Hershkoviz R, Lider O. Interplay of T cells and cytokines in the context of enzymatically modified extracellular matrix. Immunol 1996; Today 17:16; PMID:8652044
21. Kato M, Wang H, Kainulainen V, Fitzgerald ML, Ledbetter S, Ornitz DM, Bernfield M. Physiological degradation converts the soluble syndecan-1 ectodomain from an inhibitor to a potent activator of FGF-2. Nat Med 1998; 4:691; PMID:9623978
22. Subramanian SV, Fitzgerald ML, Bernfield M. Regulated shedding of syndecan-1 and -4 ectodomains by thrombin and growth factor receptor activation. J Biol Chem 1997; 272:14713; PMID:9169435
23. Platt JL, Dalmasso AP, Lindman BJ, Ihrcke NS, Bach FH. The role of C5a and Ab in the release of heparan sulfate from endothelial cells. Eur J Immunol 1991; 21:2887; PMID:1936126; http://dx.doi.org/10.1002/eji.1830211135
24. Ihrcke NS, Parker W, Reissner KJ, Platt JL. Regulation of platelet heparanase during inflammation: role of pH and proteinases. J Cell Physiol 1998; 175:255; PMID:9572470; http://dx.doi.org/10.1002/(SICI)1097-4652(199806)175:3%3c255::AID-JCP3%3e3.0.CO;2-N
25. Gotte M. Syndecans in inflammation. FASEB J 2003; 17, 575-91; PMID:12665470; http://dx.doi.org/10.1096/fj.02-0739rev
26. Johnston B, Butcher EC. Chemokines in rapid leukocyte adhesion triggering and migration. Semin Immunol 2002; 14:83-92; PMID:11978080; http://dx.doi.org/10.1006/smim.2001.0345
27. Wrenshall LE, Cerra FB, Singh RK, Platt JL. Heparan sulfate initiates signals in murine macrophages leading to divergent biological outcomes. J Immunol 1995; 154:871; PMID:7814890
28. Wrenshall LE, Cerra FB, Carlson A, Bach FH, Platt JL. Regulation of murine splenocyte responses by heparan sulfate. J Immunol 1991; 147:455; PMID:1830063
29. Wrenshall LE, Stevens RB, Cerra FB, Platt JL. Modulation of macrophage and B cell function by glycosaminoglycans. J Leukocyte Biol 1999; 66:391; PMID:10496308
30. Kodaira Y, Nair SK, Wrenshall LE, Gilboa E, Platt JL. Phenotypic and functional maturation of dendritic cells mediated by heparan sulfate. J Immunol 2000; 165(3):1599-604; PMID:10903769; http://dx.doi.org/10.4049/jimmunol.165.3.1599
31. Lan Y, Wang H-X, Wu MN, Hu N-Z, Hu Y-Z. Effect of low molecular weight heparan sulfate, an endogenous danger signal, as an immunological adjuvant. Chin J Biologicals 2011; January 24(1):5-9
32. Granucci F, Ferrero E, Foti M, Aggujaro D, Vettoretto K, Ricciardi-Castagnoli P. Early events in dendritic cell maturation induced by LPS. Microbes Infect 1999; 1:1079; PMID:10572310; http://dx.doi.org/10.1016/S1286-4579(99)00209-9
33. Sparwasser T, Koch ES, Vabulas RM, Heeg K, Lipford GB, Ellwart JW, Wagner H. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. Eur J Immunol 1998; 28:2045; PMID:9645386; http://dx.doi.org/10.1002/(SICI)1521-4141(199806)28:06%3c2045::AID-IMMU2045%3e3.0.CO;2-8
34. Mosca F, Tritto E, Muzzi A, Monaci E, Bagnoli F, Iavarone C, O'Hagan D, Rappuoli R, De Gregorio E. Molecular and cellular signatures of human vaccine adjuvants. Proc Natl Acad Sci USA 2008; 105(30), 10501-6; PMID:18650390; http://dx.doi.org/10.1073/pnas.0804699105
35. De Gregorio E, Tritto E, Rappuoli R. Alum adjuvanticity: unraveling a century old mystery. Eur J Immunol 2008; 38(8):2068-71; PMID:18651701; http://dx.doi.org/10.1002/eji.200838648
36. Iwasaki A, Medzhitov R. Regulation of adaptive immunity by the innate immune system. Science 2010; 327 (5963):291-5; PMID:20075244; http://dx.doi.org/10.1126/science.1183021
37. Casella CR, Mitchell TC. Putting endotoxin to work for us:monophosphoryl lipid A as a safe and effective vaccine adjuvant. Cell Mol Life Sci 2008; 65:3231-40; PMID:18668203; http://dx.doi.org/10.1007/s00018-008-8228-6
38. Cluff CW. Monophosphoryl lipidA(MPL) as an adjuvant for anti-cancer vaccines: clinical results. Adv Exp Med Biol 2010; 667:111-23; PMID:20665204; http://dx.doi.org/10.1007/978-1-4419-1603-7_10
39. Piccinini AM, Midwood KS. DAMPening inflammation by modulating TLR signalling. Mediat Inflamm 2010; pii: 672395.Epub 2010 Jul 13; PMID:20706656; http://dx.doi.org/10.1155/2010/672395
40. Kis-Toth K, Tsokos GC. Dendritic cell function in lupus: Independent contributors or victims of aberrant immune regulation. Autoimmunity 2010; 43(2):121-30; PMID:20102311; http://dx.doi.org/10.3109/08916930903214041
41. Schaefer L, Babelova A, Kiss E, Hausser HJ, Baliova M, Krzyzankova M, Marsche G, Young MF, Mihalik D, Gotte M, et al. The matrix component biglycan is proinflammatory and signals through Toll-like receptors 4 and 2 in macrophages. J Clin Invest 2005; 115:2223-33; PMID:16025156; http://dx.doi.org/10.1172/JCI23755
42. Termeer C, Benedix F, Sleeman J, Fieber C, Voith U, Ahrens T, Miyake K, Freudenberg M, Galanos C, Simon JC. Oligosaccharides of Hyaluronan activate dendritic cells via toll-like receptor 4. J Exp Med 2002; 195:99-111; PMID:11781369; http://dx.doi.org/10.1084/jem.20001858
43. Johnson GB, Brunn GJ, Kodaira Y, Platt JL. Receptor-mediated monitoring of tissue well-being via detection of soluble heparan sulfate by Toll-like receptor 4. J Immunol 2002; 168:5233-9; PMID:11994480; http://dx.doi.org/10.4049/jimmunol.168.10.5233
44. Kawai T, Akira S. Pathogen recognition with Toll-like receptors. Curr Opin Immunol 2005; 17:338-44
45. Kawai T, Adachi O, Ogawa T, Takeda K, Akira S. Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity 1999; 11:115-22; PMID:10435584; http://dx.doi.org/10.1016/S1074-7613(00)80086-2
46. Akira S, Takeda K. Functions of toll-like receptors: lessons from KO mice. C R Biol 2004; 327:581-9; PMID:15330257
47. Akira S1, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006; Feb 24;124 (4):783-801; PMID:16497588; http://dx.doi.org/10.1016/j.cell.2006.02.015
48. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, Akira S. Role of adaptor TRIF in the MyD88-independent Toll-like receptor signaling pathway. Science 2003; 301:640-3; PMID:12855817; http://dx.doi.org/10.1126/science.1087262
49. Kaisho T, Takeuchi O, Kawai T, Hoshino K, Akira S. Endotoxin-induced maturation of MyD88-deficient dendritic cells. J Immunol 2001; 166(9):5688-94; PMID:11313410; http://dx.doi.org/10.4049/jimmunol.166.9.5688
50. Kolanowski ST, Dieker MC, Lissenberg-Thunnissen SN, van Schijndel GM, van Ham SM, Brinke AT. TLR4-mediated pro-inflammatory dendritic cell differentiation in humans requires the combined action of MyD88 and TRIF. Innate Immun 2014; 20(4):423-30; PMID:23941760; http://dx.doi.org/10.1177/1753425913498626
51. Shen H, Tesar BM, Walker WE, Goldstein DR. Dual signaling of MyD88 and TRIF is critical for maximal TLR4-induced dendritic cell maturation. J Immunol 2008; 181:1849-58; PMID:18641322; http://dx.doi.org/10.4049/jimmunol.181.3.1849

52. Hoebe K, Janssen EM, Kim SO, Alexopoulou L, Flavell RA, Han J, Beutler B. Upregulation of costimulatory molecules induced by lipopolysaccharide and double-stranded RNA occurs by Trif-dependent and Trif-independent pathways. Nat Immunol 2003; 4:1223-9; PMID:14625548; http://dx.doi.org/10.1038/ni1010
53. Akbarshahi H, Axelsson JB, Said K, Malmstrom A, Fischer H, Andersson R. TLR4 dependent heparan sulphate-induced pancreatic inflammatory response is IRF3-mediated. J Transl Med 2011; 9:219; PMID:22188870; http://dx.doi.org/10.1186/1479-5876-9-219
54. Gautier G, Humbert M, Deauvieau F, Scuiller M, Hiscott J, Bates EEM, Trinchieri G, Caux C, Garrone P. A type I interferon autocrine-paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells. J Exp Med 2005; 201:1435-46; PMID:15851485; http://dx.doi.org/10.1084/jem.20041964
55. Groom JR, Luster AD. CXCR3 in T cell function. Exp Cell Res 2011; 317:620-31; PMID:21376175; http://dx.doi.org/10.1016/j.yexcr.2010.12.017
56. Orr MT, Duthie MS, Windish HP, Lucas EA, Guderian JA, Hudson TE, Coler RN. MyD88 and TRIF synergistic interaction is required for TH1-cell polarization with a synthetic TLR4 agonist adjuvant. Eur J Immunol 2013; 43 (9):2398-408; PMID:23716300; http://dx.doi.org/10.1002/eji.201243124
57. Mata-Haro V, Cekic C, Martin M, Chilton PM, Casella CR, Mitchell TC. The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. Science 2007; 316:1628-32; PMID:17569868; http://dx.doi.org/10.1126/science.1138963
58. Cekic C, Casella CR, Eaves CA, Matsuzawa A, Ichijo H, Mitchell TC. Selective activation of the p38 MAPK pathway by synthetic monophosphoryl lipid A. J Biol Chem 2009; 284:31982-91; PMID:19759006; http://dx.doi.org/10.1074/jbc.M109.046383
59. Persing DH, Coler RN, Lacy MJ, Johnson DA, Baldridge JR, Hershberg RM, Reed SG. Taking toll: lipid Amimetics as adjuvants and immunomodulators. Trends Microbiol 2002; 10:S32-7; PMID:12377566; http://dx.doi.org/10.1016/S0966-842X(02)02426-5
60. Gandhapudi SK, Chilton PM, Mitchell TC. TRIF is required for TLR4 mediated adjuvant effects on T cell clonal expansion. PLoS One 2013; 8(2):e56855; PMID:23457630; http://dx.doi.org/10.1371/journal.pone.0056855
61. Rhee EG, Kelley RP, Agarwal I, Lynch DM, La Porte A, Simmons NL, Clark SL, Barouch DH. TLR4 ligands augment antigen-specific CD8+ T lymphocyte responses elicited by a viral vaccine vector. J Virol 2010; 84:10413-9; PMID:20631129; http://dx.doi.org/10.1128/JVI.00928-10
62. Athanasia S, Xiaowei W, Huajun W, Brian S. PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification. Nucl Acids Res 2010; 38:D792-9; PMID:19906719; http://dx.doi.org/10.1093/nar/gkp1005
63. Athanasia S, Xiaowei W, Huajun W, Stefan D, Tara T, Brian S. A comprehensive collection of experimentally validated primers for Polymerase chain reaction quantitation of murine transcript abundance. BMC Genomics 2008; 9:633; PMID:19108745; http://dx.doi.org/10.1186/1471-2164-9-633
64. Xiaowei W, Brian S. A PCR primer bank for quantitative gene expression analysis. Nucleic Acids Res 2003; 31(24):e154; 1-8; PMID:14654707; http://dx.doi.org/10.1093/nar/gng154