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Hck Tyrosine Kinase Regulates TLR4-Induced TNF and IL-6 Production via AP-1

Maria J. Smolinska,1,2 Theresa H. Page,2 Ania M. Urbaniak, Brenda E. Mutch, and Nicole J. Horwood

The TLRs play a key role in host defense against infection and injury, and mounting evidence suggests that these receptors may also play a role in diseases such as autoimmunity, atherosclerosis, and cancer. Activation of TLRs on macrophages results in the production of multiple soluble mediators including the key inflammatory cytokines, TNF and IL-6. Thus, the intracellular signaling mechanism by which TLRs signal is a subject of great interest. As well as activating the NF-κB and MAPK pathways, TLR engagement leads to tyrosine kinase activation within minutes. Src family kinases (SFKs) are the largest nonreceptor tyrosine kinase family with nine members: Src, Hck, Lyn, Fyn, Fgr, Blk, Lck, Yes, and Ylk. The role of the SFKs in TLR signaling has been an area of much controversy, with conflicting findings between studies using chemical inhibitors and knockout mice. Using primary human macrophages in combination with adenoviral overexpression and small interfering RNA knockdown studies, we show that the SFK, Hck, has a pre-eminence role in LPS/TLR4-induced TNF and IL-6 production. Hck kinase mediates TLR4-induced transcription of both TNF and IL-6 by a mechanism that involves neither the NF-κB nor the MAPK pathways, but rather leads to AP-1 binding with a complex of c-fos and JunD. These data highlight the importance of Hck as an active component in LPS-induced TLR signaling and suggest the possibility of targeting this kinase for the alleviation of excessive inflammation. The Journal of Immunology, 2011, 187: 6043–6051.

The TLR family forms a major part of innate immune defense against infection by recognizing various pathogen-associated molecular patterns, such as LPS, dsRNA, or flagellin (1). TLRs are expressed on many cell types, but it is in cells of the innate immune system that their biology has been most studied. Stimulation of macrophages with TLR ligands such as LPS induces a powerful inflammatory response with the release of numerous cytokines (e.g., TNF, IL-6) and chemokines (e.g., IL-8). TLRs can also respond to the endogenous ligands, the damage-associated molecular patterns, which are by-products of tissue breakdown (2, 3). Thus, despite protecting the host from infection and injury, TLRs can also play a role in pathologies such as septic shock, autoimmune conditions, cancer, and atherosclerosis, in which an overly aggressive and unresolved inflammatory response is seen (4, 5).

The most widely studied TLR signaling pathway, and apparently the most complicated, is that of TLR4, the LPS receptor. LPS signaling requires the recruitment of Toll/IL-1R domain-containing adaptor proteins. These molecules then act via TNFR-associated factor 6 and the IL-1R–associated kinases to activate the MAPK pathways and transcription factors such as NF-κB and AP-1 that regulate the production of TNF, IL-6, and other mediators of the inflammatory response (6). TLR4 also recruits another pair of adaptor molecules: Toll/IL-1–resistance domain-containing adaptor inducing IFN-β and Toll/IL-1–resistance domain-containing adaptor inducing IFN-β-related adapter molecule act via TNFR-associated factor 3 and Tank binding kinase and the IFN regulatory factors to activate an IFN response (7).

In addition to the pathways described above, LPS induces the activation of multiple tyrosine kinase families including the Tec family kinases Btk and Bmx (8–11), Pyk2 kinase (12), and Syk (13). Of these, only the Tec family kinases have been conclusively linked with the production of proinflammatory cytokines such as TNF, IL-6, and IL-1 (8, 11). However, the largest tyrosine kinase family activated by TLR4 is the Src family kinases (SFKs), and at least three family members, Hck, Fgr, and Lyn, are rapidly activated by LPS signaling (14–19). Because of conflicting reports, the role that these kinases play in TLR4 signaling is still unclear. Early studies on Hck−/−, Lyn−/−, and Fgr−/− triple-deficient murine macrophages showed no impairment of LPS-induced signaling (ERK and JNK substrate kinase assays, NF-κB consensus sequence binding by EMSA) or cytokine production (TNF, IL-1, and IL-6) (20). Members of the Src kinase family have overlapping functions thought to allow for functional compensation in the absence of some family members; however, because researchers (21–24) were unable to detect the presence of other SFKs within the cells, they concluded that SFKs are not required for TLR signaling. In contrast, other investigators have revealed the existence of an additional six SFKs, Src, Fyn, Lck, Yes, Ylk, Blk (25), and our own studies have shown the presence of six SFK family members in primary human macrophages (26). These data, coupled with reports that double-
knockout $Hck^{-/-}Fgr^{-/-}$ cells also express higher levels of Fyn, Lyn, and Yes than wild-type cells (27), suggest that functional redundancy might have operated in these early experiments. Furthermore, our group and others have shown that the SFK-selective inhibitors PP1 and PP2 inhibit LPS-induced cytokine production (21, 26, 28–30). Thus, an increasing body of apparently contradictory data on the role of SFKs in LPS signaling has now accumulated and deserves to be addressed.

In this study, we have attempted to obtain a more definitive understanding of the role of specific SFKs in LPS/TLR4 signaling and to avoid the use of chemical inhibitors where problems of specificity can be an issue. Using overexpression of each of the six SFKs normally expressed in human macrophages, we found that Hck was by far the most potent at enhancing LPS-induced TNF and IL-6 production. Further experiments using small interfering RNA (siRNA) for Hck resulted in a substantial reduction in both TNF and IL-6 expression. In this article, we investigate how Hck controls gene expression and the signaling pathways and molecules used to make this particular SFK a crucial mediator of LPS-induced cytokine production.

Materials and Methods

Cells and cell culture

RPMI 1640 containing 25 mM L-glutamine, HBSS containing 0.35 g/l sodium bicarbonate (without calcium or magnesium), and cell dissociation solution were purchased from Biowhittaker (Wokingham, Berkshire, U.K.). FCS was from PAA (Yeovil, U.K.) or Labtech (Ringmer, East Sussex, U.K.). All growth media were supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml), supplied as a 100 mM sterile solution from Biowhittaker (Wokingham). PBMCs were prepared from buffy coat fractions of a unit of blood from a single donor using Ficoll-Hypaque (Nycomed, Oslo, Norway). Monocytes were isolated by centrifugal elutiation (>85% purity), as previously described (31), and differentiated in the presence of 100 ng/ml M-CSF (PeproTech, London, U.K.) for 3 d. Nonadherent cells were washed off, and the remaining adherent cells were removed using cell dissociation media, counted, and replated before assay. Cells were pretreated with PP2 (Calbiochem, Nottingham, U.K.) for 30 min before the addition of 10 ng/ml E. coli-derived LPS (Alexa, Exeter, U.K.). All cell culture incubations were performed at 37°C in a humidified atmosphere containing 5% CO2.

Generation of adenoviral vectors and cell infection

Recombinant, replication-deficient, adenoviral constructs encoding wild-type human Src, Hck, Lyn, Fgr, Fyn, and Yes, as well as kinase dead Hck, were created using the AdEasy system as previously described (31). The NF-κB luciferase adenovirus contains four tandem copies of the κ enhancer element located upstream of the firefly luciferase gene (kindly provided by P.B. McCray Jr., University of Iowa, Iowa City, IA), a modification of the pNF-κB reporter vector (BD Clontech). Human TNF-α promoter (-1173 bp) and human IL-6 promoter (-1195 bp) were used to generate TNF $\alpha$ and IL-6 $\beta$ luciferase reporter viruses as described previously (32). M-CSF–derived macrophages were plated in a 96-well plate at $1 \times 10^5$ cells/well and allowed to express adenoviral transgenes overnight before stimulation with LPS for 6 h or other time periods, as stated in the Results. For luciferase assays, cells were subjected to two subsequent rounds of infections: first, with the kinase adenoviral constructs, followed by a 2-h recovery period in complete medium; and second, with luciferase adenoviral constructs 24 h before stimulation.

ELISA

Macrophages were plated at $5 \times 10^5$ cells/well in a 96-well plate and stimulated with 10 ng/ml LPS. Supernatants were harvested after 18 h and examined for concentrations of TNF, IL-6, and IL-10 by ELISA (R&D Systems, Minneapolis, MN).

**FIGURE 1.** Overexpression of SFKs in primary human macrophages causes changes in LPS-induced production of TNF and IL-6. A, A total of $1 \times 10^7$/ml primary human M-CSF–derived macrophages was infected with increasing moi (0, 25, 50, or 100) of the indicated adenoviral constructs for the expression of either SFKs or Pyk2. Cells were lysed and examined for the presence of the relevant kinases by Western blotting. The lower panel for each kinase shows β-actin as a loading control. Cells infected with 100 moi of each virus were stimulated for 18 h with 10 ng/ml LPS, and culture supernatants were examined for TNF (B) and IL-6 (C) production by ELISA. Results are shown as percentage change relative to Pyk2-transfected cells and represent the combined data from between three and nine separate donors ± SEM. Statistical analysis was performed using the paired Student t test ($^*p < 0.05$, **$p < 0.01$, ***$p < 0.001$).
assays were performed by incubating 5 × 10^4 cpm) [32P]-ATP–labeled, double-stranded NF-

Western blotting

Cells were lysed on ice in Tris pH 7.5, 150 mM NaCl, 1 mM sodium orthovanadate, supplemented with protease inhibitor mixture and containing 1% Nonidet P-40 and insoluble debris removed by centrifugation. Whole-cell lysates were separated by SDS-PAGE followed by electro-transfer onto polyvinyl difluoride membrane (Millipore). Abs against p38, p42/44, and p46/54 MAPKs were from New England Biolabs (Hitchin, U.K.), and β-actin Ab was obtained from Sigma (Poole, U.K.). IκBα Ab, tubulin, Stat3, Hsp90, and Fos and Jun Abs were obtained from Santa Cruz. Abs against SFKs were purchased from Santa Cruz, Upstate, or Cell Signaling. Blots were developed using ECL, according to manufacturer’s instructions (Amersham International, Chalfont, U.K.).

EMSA

Nuclear extracts (10 μg) were mixed with 5× binding buffer and 1 ml (5 × 10^10 cpm) [32P]-labeled, double-stranded NF-κB consensus probe (5'- AGTTGAGGGACTTTCCCAGGC-3') or AP-1 consensus probe (5'- CGCTTGATGAGTCAGCCGGAA-3'; both from Promega, Southampton, U.K.) with nuclease-free water to a total volume of 10 μl. The reactions were incubated at room temperature with shaking for 30 min. Supershift assays were performed by incubating 5 μg nuclear extract (derived from macrophages stimulated with 10 ng/ml LPS for 60 min) with or without 2 μg of the AP-1 Ab for 30 min at room temperature. Immediately after, 1 μl of the [32P] probe and 1× EMSA binding buffer (Promega) were added to the nuclear extract at total reaction volume of 15 μl, and the samples were incubated for 20 min at room temperature. Abs for JunB (C-11) mouse mAb, JunD (329) rabbit pAb, c-Fos (6-2H-2F) mouse mAb, and c-Jun (N) rabbit pAb were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Excess unlabeled competitor DNA was added to control samples. The reaction was terminated by the addition of 1 μl loading buffer, and samples were loaded onto a nondenaturing 5% polyacrylamide gel. Gels were dried and exposed to Hyperfilm ECL (Amersham Biosciences) at −70°C and developed using an AGFA Curix 60 Film Processor (Agfa/Gevaert, Brentford, U.K.).

RNA isolation and real-time PCR

Total RNA was isolated from 5 × 10^5 cells using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. Predesigned “Assays on Demand” primer/probe combinations for TNF, IL-6, and GAPDH were purchased from Applied Biosystems. Quantitative RT-PCRs were performed in a final volume of 10 μl using the SuperScript III One-Step RT-PCR system with Platinum Taq DNA polymerase (Invitrogen, Paisley, U.K.) according to the manufacturer’s instructions. The reactions, carried out with 1 μl total RNA, were performed on a Qiagen RG-6000 machine. The reverse transcription was performed at 50°C for 20 min before inactivation of the reverse transcriptase at 95°C for 2 min. PCRs were performed at 95°C for 12 s, followed by 57°C for 25 s for a total of 45 cycles. Obtained values were normalized to GAPDH, to account for variability in the initial concentration of RNA and the conversion efficiency of the reverse transcription reaction. Relative quantitation was performed using the comparative ΔΔCt method according to the manufacturer’s instructions. The data were analyzed using Rotor Gene 6000 Series Software 1.7.

FIGURE 2. Kinase activity is required for Hck-mediated changes in LPS-induced cytokine production. A, Primary human macrophages were stimulated for various times with 10 ng/ml LPS. Cell lysates were subjected to immunoprecipitation with either an isotype-matched control Ab (Ab) or SFK (Hck, Lyn, or Src)-specific Abs. The presence of phosphorylated forms of each kinase was detected by Western blotting with a phospho-specific Ab for each SFK. Blots were stripped and reprobed for total SFK expression as a loading control. B, Primary human macrophages were uninfected (0) or infected with 25, 50, or 100 moi of kinase dead Hck (Hck KE). After 48 h, cell lysates were examined for Hck expression by Western blotting. Blots were stripped and reprobed with actin as a loading control. Primary human macrophages were either uninfected or infected with 100 moi of adenoviral constructs containing either Pyk2 or kinase dead Hck (Hck KE). Cells were stimulated for 18 h with 10 ng/ml LPS, and cytokine content of supernatants was measured for the presence of TNF (C) or IL-6 (D) by ELISA. Results are shown as mean (± SEM) values of percentage change in cytokine levels relative to Pyk2 and represent combined data from at least three separate experiments.

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RNA interference

For targeted protein knockdown using RNA interference, 3 × 10^6 3-d M-CSF–differentiated macrophages were nucleofected with targeting siRNA or control oligonucleotides (siControlTM D-001206-13) and human Hck duplex D-003141-06 (Dharmacon, Chicago, IL) at concentration ranges of 100–200 nM using the Human Monocyte Nucleofector Kit (Amaxa Biosystems, Cologne, Germany), according to the manufacturer’s instructions. After transfection, cells were incubated in the presence of M-CSF for 72 h. Protein knockdown was subsequently assessed by Western blotting.

Reporter assays

Macrophages in a 96-well plate were infected with reporter viruses. After 12 h, cells were either unstimulated or treated with LPS for 6 h, then washed in ice-cold PBS and lysed in 100 ml 0.65% Nonidet P-40 chloramphenicol acetyl transferase lysis buffer. Fifty milliliters lysate was transferred to the luminometer plates, and 120 ml assay buffer (25 mM Tris pH 7.8, 8 mM MgCl₂, 15% glycerol, 1 mM EDTA, 1% Triton X-100, 0.5 mM ATP, 1 mM DTT) was added. Thirty milliliters of 1.5 mM beetle luciferin was added to the mixture before measuring the photon release using the Wallac MicroBeta Tri Lux luminometer (GMI, Ramsey, MN) machine and software. The results were recorded in relative luciferase units.

Statistical analysis

Data in each group were expressed as means ± SEM. The level of statistical significance was established using the paired Student t test.

Results

Overexpression of Hck potently enhances LPS-induced TNF and IL-6 production

Our previous study showed that human macrophages express multiple SFKs, and that inhibition of one or more of these are the likely underlying mechanism for the inhibitory effect of PP2 on TLR-induced cytokine production (26). Because PP2 acts by inhibiting all SFK family members, the role of individual SFKs in TLR4 signaling was investigated to understand whether all SFKs were equally involved in TLR signaling.

Recombinant adenoviral vectors were generated encoding each of the SFKs expressed in human primary macrophages: Hck, Lyn, Fgr, Fyn, Src, and Yes. Infection of macrophages with each of these constructs independently produced a dose-dependent increase in the expression of the subject kinase over the endogenous level (Fig. 3).
Variation in band intensity on the Western blots reflects the efficiency of individual Abs and was not a reflection of the comparative expression of the different kinases.

Titration experiments to establish the most effective multiplicity of infection (moi) of these viruses in relation to their effects on LPS-induced cytokine production revealed that a dose of 100 moi was the most effective for all the SFKs (Supplemental Fig. 1). The effect of overexpression of the individual SFKs on LPS-induced cytokine production was examined for each SFK individually. Fig. 1B shows combined data from multiple donors, and is expressed as the percentage increase relative to the control Pyk2 construct. As previously shown, although Pyk2 is an LPS-activated tyrosine kinase, its overexpression has no effect on LPS-induced cytokine expression (8). Accordingly, cytokine production from these cells was comparable with uninfected cells, thus discounting any effect of the viral infection itself or that of simply overexpressing any tyrosine kinase (Fig. 1A). As shown in Figs. 1B and 2C, the overexpression of individual SFKs produced varied effects on TNF and IL-6 expression. Hck overexpression resulted in the most potent effect, significantly increasing TNF and IL-6 production by nearly 4- and 7-fold, respectively. Overexpression of Lyn, Fgr, and Fyn also significantly increased TNF production, although this was a weaker effect, producing only a 1.5- to 2-fold increase. These constructs also produced a similar effect on IL-6 production, although this was not statistically significant (Fig. 1C). The Yes construct had no effect on TNF production and a small, although significant, effect on IL-6, whereas the Src construct had no effect on TNF production but curiously significantly inhibited IL-6 production.

Effects of Hck on cytokine production require kinase activity

Given the clear differences between the different SFKs on LPS-induced cytokine production, we chose to investigate this in more detail. Because Hck caused the strongest enhancement of cytokine production, studies concentrated on this SFK family member. LPS-mediated activation of Lyn was also examined because it represented the next most active kinase, and Src because it had no effect on TNF and actually inhibited IL-6 expression. All three kinases were activated by LPS, albeit with different kinetics (Fig. 2A). As shown previously, Hck activity was induced within 5 min (26). Phosphorylation of Lyn showed similar kinetics, whereas Src phosphorylation was delayed until 20–40 min. An adenoviral construct containing the kinase inactive form of Hck, K269E, was made; this has previously been reported to act as a dominant-negative form of the kinase (33). HckK269E was well expressed in macrophages (Fig. 2B), but, in contrast with wild-type Hck, had no effect on LPS-induced cytokine production (Fig. 2C, 2D). These data show that Hck kinase activity is required for the enhancing effect of Hck overexpression on TNF and IL-6 production. However, HckK269E did not act as a classical "dominant-negative" by inhibiting TNF production because it simply prevented the superinduction of TNF seen with the wild-type Hck.

siRNA knockdown of Hck inhibits LPS-induced cytokine production

The data above indicated that Hck is the dominant SFK family member in mediating LPS-induced cytokine production. To further confirm a role for this kinase, siRNA knockdown of the Hck protein was undertaken. As shown in Fig. 3A, 48 h after nucleofection with an siRNA oligo, Hck expression was dose-dependently reduced in human macrophages, although it should be noted that siRNA knockdown does not lead to complete ablation of the kinase and some residual Hck activity may remain. The targeting of Hck had no effect on the expression of Lyn, the closest SFK family member to Hck at the amino acid level, or on the levels of tubulin within the cells.

**FIGURE 4.** LPS-induced MAPK activity is not influenced by Hck. A, Primary human macrophages were either uninfected or infected with adenoviral constructs expressing wild-type Pyk2 (Pyk2wt), Hck (Hckwt), or kinase dead Hck (Hck KE). Cells were stimulated for the times indicated with 10 ng/ml LPS. The same Western blots were sequentially probed for the phosphorylated forms of p38, p42/44, or p46/54 MAPKs. The Western blots were lastly probed with β-actin as a loading control. B, Primary human macrophages were subjected to siRNA-mediated knockdown using either random, scrambled oligos as control (R) or specific anti-Hck oligos (Hck). Cells were stimulated with 10 ng/ml LPS for the indicated times, and p38, p42/44, and p46/54 activity were monitored by Western blotting. Data shown are representative of at least three separate experiments.
LPS-mediated activation of cells depleted of Hck using siRNA showed a significant reduction in TNF and IL-6 protein production by 50% (Fig. 3B). Hck knockdown produced a similar reduction in the LPS-induced expression of TNF and IL-6 at the mRNA level (Fig. 3C). To further establish how Hck was regulating cytokine mRNA, we used reporter gene constructs driven by the 5′ promoter region of either the TNF or IL-6 genes. Reducing Hck protein expression decreased LPS responsiveness in human macrophages for both the TNF and IL-6 5′ promoter-driven luciferase reporter constructs, indicating that Hck was able to regulate these genes at the level of transcription (Fig. 3D).

Hck is not required for LPS-induced MAPK or NF-κB activation

Within the TNF and IL-6 promoter regions, there are binding sites for numerous transcription factors including NF-κB, AP-1, Ets,

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**FIGURE 5.** LPS-induced NF-κB activity is not influenced by Hck. A, Primary human macrophages were either uninfected or infected with adenoviral constructs expressing wild-type Pyk2, Src, or Hck. Cells were stimulated for various times with 10 ng/ml LPS, and the presence of the phosphorylated form of IκBα was assessed by Western blotting. Western blots for Hsp-90 are shown as loading controls. B, Primary human macrophages were infected with a κB tandem repeat luciferase reporter gene adenoviral construct. Infected cells were rested for 2 h before being infected with either Pyk, AdSrc, or AdHck. Cells were then rested overnight and stimulated with LPS (10 ng/ml) for 6 h. Promoter activity was assessed by monitoring luciferase activity. Data are shown as mean (± SEM) percentage activity relative to Pyk2 and represent combined data from three separate donors. C, Primary human macrophages were either uninfected or infected with adenoviral constructs expressing wild-type Src or Hck. Cells were stimulated for various times with 10 ng/ml LPS. A total of 10 μg nuclear protein was incubated with consensus probes for NF-κB and subjected to electromobility shift assay. Scan shown represents a single representative example of at least three separate experiments. Primary human macrophages were subjected to gene knockdown with either random, scrambled oligos (R) or specific anti-Hck oligos (Hck). D, Cells were stimulated for various times with 10 ng/ml LPS, and the presence of the phosphorylated form of IκBα was assessed by Western blotting. Western blots for Stat 3 are shown as loading controls. E, Cells were infected with a luciferase reporter gene adenoviral construct containing consensus κB sites in its promoter region. After stimulation with LPS (6 h), promoter activity was assessed by monitoring luciferase activity. Data are shown as mean (± SEM) percentage activity relative to random control and represent combined data from three separate donors. F, Cells were stimulated for various times with 10 ng/ml LPS. A total of 10 μg nuclear protein was incubated with consensus probes for NF-κB and subjected to EMSA. Scan shown represents a single representative example of at least three separate experiments.
C/EBP, and AMP-dependent transcription factor (34, 35). Because Hck appeared to be necessary for LPS-induced cytokine production, the role of Hck in a number of key downstream signaling pathways was examined. Overexpression of wild-type Hck protein (Fig. 4A) or siRNA-mediated knockdown of Hck protein (Fig. 4B) in primary human macrophages did not result in significant changes in the activation of the p42/44 Erk, p38, or p46/54 JNK MAPK families. Minor changes in phosphorylation level of p38 after Hck overexpression were inconsistent and not considered significant.

The transcription factor NF-κB is known to be required for both the expression of TNF and IL-6 in human macrophages (31, 36). Assessment of the LPS activation of NF-κB was determined by IκBα degradation after Hck overexpression or Hck knockdown (Fig. 5A, 5D). Activation of IκBα was detected in both cases as shown by the IκBα phosphorylation at 20 min followed by restoration at 2 h (Fig. 5A) and by the phosphorylation-induced degradation at 30 min and restoration by 1 h (Fig. 5D). Furthermore, EMSA (Fig. 5B, 5E) and NF-κB–driven luciferase reporter gene assay (Fig. 5C, 5F) showed that neither Hck overexpression (Fig. 5A–C) nor Hck siRNA knockdown (Fig. 5D–F) had any effect on the activation of this transcription factor. Although the control in Fig. 5F appears higher, there is no difference in the fold increase in binding relative to the zero control for the random or Hck siRNAs, respectively. Additional experiments were performed using all of the other SFK viruses in conjunction with the NF-κB reporter in human macrophages and likewise showed no effect on reporter activity (data not shown).

**FIGURE 6.** LPS-mediated AP-1 activity is regulated by Hck. Primary human macrophages were either uninfected or infected with AdHck (A), or transfected with random, scrambled oligos (R), or Hck-specific siRNA oligos (B). Cells were stimulated for various times with 10 ng/ml LPS and 10 μg nuclear extract subjected to EMSA with consensus probes for AP-1. C. Monocytes were stimulated with LPS (10 ng/ml) for 1 h, and 5 μg nuclear extract was subjected to EMSA with consensus probes for AP-1. A supershift assay was performed with Abs against c-Jun, c-Fos, JunB, and JunD. As a control, a competitor wild-type nonradiolabeled AP-1 oligonucleotide was added in excess into one of the samples.

**Hck is required for LPS-induced AP-1 activation**

Although NF-κB is considered to be the dominant transcription factor controlling TNF and IL-6 expression in response to LPS, both genes also contain sites for the transcription factor AP-1. We therefore investigated whether, as the predominant SFK in LPS signaling, Hck was able to influence LPS-induced AP-1 activity. EMSA assay of AP-1 activity showed that Hck overexpression enhanced the LPS-induced activation of this transcription factor (Fig. 6A). Conversely, Hck siRNA knockdown reduced AP-1 EMSA activity (Fig. 6B). Furthermore, analysis of the AP-1 components by supershift assay revealed that c-Fos and JunD were present in the complex, but not JunB or c-Jun (Fig. 6C). In the presence of an AP-1 unlabeled probe, named as competitor in this study, there was no accumulation of protein–DNA complexes. We have previously shown that knockdown of JunD in primary human macrophages leads to a decrease in TNF, IL-6, and IL-10, thus highlighting the importance of AP-1 signaling in the production of these cytokines (37).

**Discussion**

Although it has long been described that LPS/TLR4 is able to activate SFKs, the role of this family of kinases in controlling LPS-induced cytokine production has been a matter of much disagreement. In particular, the key study of Meng and Lowell (20) appeared to effectively disprove any significant role for the SFKs in macrophage function by claiming that deletion of the only three SFKs in murine macrophages, Hck, Lyn, and Fgr, had no effect on LPS-induced cytokine production; indeed, the mice appeared surprisingly normal. Nevertheless, studies by ourselves and others have shown that other SFKs (i.e., Src, Fyn, and Yes) are also expressed in both murine and human macrophages (14, 26), and it is therefore possible that other family members would have compensated for the loss of Hck, Lyn, and Fgr, thereby masking the role of SFKs in TLR4 signaling.

In this study, both overexpression and siRNA knockdown, we demonstrate that Hck has an important role in the LPS regulation of TNF and IL-6 production. A key point to distinguish these studies from those of Meng and Lowell (20) is that short-term siRNA knockdown in terminally differentiated cells such as the human macrophage is unlikely to be compensated for by changes in the expression of other SFK family members. Indeed, in Hck knockdown cells, there was no change in expression of Lyn, the closest family member to Hck, and one of three other SFKs (Lyn, Fyn, and Fgr) capable of contributing to LPS signaling (Figs. 1, 3A).

Although siRNA-mediated knockdown of Hck reduced its expression, there was still significant residual cytokine production possibly because of the presence of residual Hck kinase activity in the cells. An alternative possibility is that there is active functional redundancy with other SFKs (Lyn, Fyn, Fgr) expressed in human macrophages. Unfortunately, siRNA knockdown does not allow for the complete ablation of Hck protein expression; therefore, a combined approach to knockdown of all SFKs in human macrophages is unlikely to add further clarity to the exact requirement for each kinase. As cross-SFK redundancy has been observed in several systems and mice deficient in SFK generally only show a phenotype when more than one kinase is knocked out (38, 39), it is not altogether surprising that there is still some cytokine expression in Hck-deficient cells. This potential redundancy would explain why Hck+/−/Fgr−−/− murine macrophages, for example, still respond to LPS, especially when one considers that increased levels of Fyn, Lyn, and Yes are found in these cells (40). In support of this “redundancy hypothesis,” treatment of primary...
human macrophages with PP2, at concentrations that would inhibit all SFK family members present within the cells, has a much more profound effect on LPS-induced cytokine production (26). We have observed similar potential redundancy between the Tec family tyrosine kinases, Btk, Bmx, and Tec, in the regulation of TNF production (8, 11).

Given the number of SFKs that could substitute for Hck, plus the presence of residual kinase expression in the knockdown cells, one might be surprised that the effect on cytokine production was as large as observed. However, studies presented in Fig. 1 suggest that Hck is the preferred kinase used by LPS/TLR4 in macrophages, although it should be noted that it is not the only SFK affecting cytokines levels after LPS stimulation. These observations are supported by previous studies in murine primary cells and cell lines where the overexpression of constitutively active Hck resulted in higher LPS-induced TNF production (41, 42). Hck knockin mice are also hypersensitive to endotoxemia, and their macrophages released exaggerated amounts of TNF-α and other inflammatory mediators (42).

Although our studies have shown Hck to be dominant in the regulation of TNF and IL-6, other SFKs clearly have their own unique profiles of effect on cytokine expression (Fig. 1). This could not simply be accounted for by different efficacies of expression of the constructs used in this study, because all the kinases were highly expressed over endogenous levels. Moreover, our finding that Src overexpression had a suppressive effect on IL-6 production, whereas overexpression of Pyk2 had no effect, shows that the data cannot be simply dismissed as an artifact of just overexpressing a tyrosine kinase. How Hck and Src act so differently on cytokine expression is unclear. Experiments in this laboratory with a kinase dead version of Src (Src K295E) have shown that, in common with Hck (Fig. 2), kinase activity is required for this function (data not shown); but it is interesting to note that the kinetics of LPS-mediated activation of these two kinases was quite different, with Hck being phosphorylated much faster than Src. Different SFKs have also been shown to recognize different substrates (43), and this combined with the sequential regulation of Hck and Src may explain why different SFK family members play distinct roles within a single cell type. A recent publication by Keck et al. (44) demonstrated that the ablation of Lyn in murine macrophages actually resulted in increased TNF, IL-6, and IFNα/β, and there is an exacerbated autoimmunity in these mice (45). Thus, the lack of effect in the triple SFK knockouts may be because of not only functional redundancy, but may also be attributed to the kinases having both positive and negative effects on cytokine production.

The studies reported in this article would explain the effect of the SFK inhibitors PP1/PP2 on LPS function and imply that it is not due to the inhibition of other targets of these drugs (21–24, 26, 46, 47). Moreover, like these drugs, Hck knockdown had no detectable effect on NF-κB or MAPK activation. Rather, we observed that Hck was upstream of the transcription factor AP-1. The regulation of AP-1 would provide a credible mechanism for the function of Hck because this transcription factor is thought to be required for the expression of both TNF and IL-6 (48, 49). However, the mechanism by which Hck regulates AP-1 is unclear. Active AP-1 dimers can form as a result of the association of a variety of members of the Fos and Jun family, and as shown in Fig. 6C, LPS stimulation of primary human macrophages induces DNA binding of c-fos and c-jun to an AP-1 consensus sequence. This finding confirms our previously reported data showing that knockdown of JunD in primary human macrophages inhibits LPS-induced production of both TNF and IL-6 (37). LPS stimulation of primary human macrophages may be able to induce production of c-Fos:JunD heterodimers that mediate the transcription of both TNF and IL-6 genes. The ability of Hck to influence AP-1 dimer DNA binding activity might therefore explain its ability to modulate cytokine production.

Furthermore, our data, in agreement with findings in dendritic cells (50), reveals that Hck does not lie upstream of any of the MAPK enzymes. Other kinases able to modulate AP-1 components have been described and include protein kinase A, cdc2, and protein kinase C (PKC). PKCδ, a reported SFK substrate, is thought to be capable of activating AP-1 components including JunD (51–53). Hence a pathway involving the Hck-mediated activation of PKCδ leading to the modulation of AP-1 activity may offer an explanation for the ability of Hck to effect cytokine transcription in the absence of changes in MAPK activity. Clearly, further work is needed to confirm this hypothesis.

In summary, this study has shown that of the SFK family members expressed in primary human macrophages, it is Hck that plays the dominant role in LPS-induced expression of TNF and IL-6 production, two important cytokines not only in the inflammatory response but also in disorders such as rheumatoid arthritis. The study of SFK in TLR signaling has been beset by contradictory results and problems of redundancy between the kinases that could be a particular problem in knockout mice. However, this study, by using siRNA in primary human macrophages, shows that one particular SFK is required for signaling by a TLR. Moreover, the data show that this family of tyrosine kinases controls a distinct pathway leading to AP-1, and that the SFK-mediated regulation of gene transcription does not require NF-κB or MAPK pathways. Thus, the SFKs appear to represent another distinct facet of the signaling by TLR4.

Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure S1. LPS driven TNF production in human macrophages after infection with adenoviral constructs: maximal effect at 100 moi. Primary human macrophages were infected with adenoviral constructs encoding wild type (wt) or kinase dead (kd) versions of the Src kinases and Pyk2 kinase at various multiplicities of infection (moi) (virus particle:cell ratio). 48 hours after infection, cells were stimulated with LPS and supernatants analysed for TNF content by ELISA. Results are expressed as percentage increase compared to Pyk2 infected control cells and are combined mean data (±SEM from at least 3 separate donors.)