**Hck Tyrosine Kinase Activity Modulates Tumor Necrosis Factor Production by Murine Macrophages**

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**Summary**

The hematopoietic cell kinase (hck) is a member of the src family of tyrosine kinases, and is primarily expressed in myeloid cells. Hck expression increases with terminal differentiation in both monocyte/macrophages and granulocytes and is further augmented during macrophage activation. Recent evidence has implicated src-related tyrosine kinases in critical signaling pathways in other hematopoietic lineages. Herein we demonstrate that manipulation of the level of hck expression in the murine macrophage cell line BAC1.2F5 alters the responsiveness of these cells to activation by bacterial lipopolysaccharide (LPS) but does not affect survival or proliferation. Overexpression of an activated mutant of hck in BAC1.2F5 cells augments tumor necrosis factor (TNF) production in response to LPS, whereas inhibition of endogenous hck expression, by antisense oligonucleotides, interferes with LPS-mediated TNF synthesis. Together, these observations suggest that hck is an important component of the signal transduction pathways in activated macrophages.

**Materials and Methods**

**Materials.** DMEM, t-glutamine, penicillin, streptomycin, G418 (Geneticin), and herbimycin A were purchased from GIBCO (Grand Island, NY). FCS was obtained from HyClone Laboratories, Inc. (Logan, UT). LPS purified from *Escherichia coli* strain 0111:B4 was purchased from Sigma Chemical Co. (St. Louis, MO). L cell con-

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1 Abbreviations used in this paper: hck, hematopoietic cell kinase; LCM, L cell conditioned medium.
ditioned medium (LCM) was prepared as described by Stanley and Heard (27) and was used as the source of CSF-1 for routine growth and maintenance of the BAC1.2F5 cell line and subclones. Recombinant human CSF-1 was obtained from the Genetics Institute (Cambridge, MA) and recombinant murine GM-CSF was obtained from Amgen, Inc. (Thousand Oaks, CA).

Cells and Cell Culture. BAC1.2F5 cells were obtained from Charles Sherr (St. Jude Children’s Research Hospital) and were routinely cultured in DMEM supplemented with 15% FCS, 25% LCM as a source of CSF-1, 2 mM L-glutamine, 20 mM Hepes (pH 7.3), 50 μM penicillin, and 50 μg ml⁻¹ streptomycin.

Retrovirus Production and Infection of BAC1.2F5 Cells. LNSL7-based vectors (28) expressing cDNAs encoding wild-type, activated (p59hck and kinase-negative (p59kE269) forms of hck have been previously described (29). These vectors were introduced into the ecotropic retroviral packaging cell line Psi-2 (30) by calcium chloride transfection. Psi-2 cells expressing the constructs were selected by growth in G418 400 μg ml⁻¹ (active drug), and supernatants from LNSL7-expressing Psi-2 cells were used to infect BAC1.2F5 cells. BAC1.2F5 clones expressing LNSL7-based constructs were isolated by the use of cloning cylinders in the presence of G418 400 μg ml⁻¹.

Antisense Oligonucleotides. Phosphorothioated 21-mer oligonucleotides corresponding to the seven codons immediately downstream from the AUG translational initiation site of the murine hck gene (8) were purchased from Synthecell Corp. (Rockville, MD). The antisense oligonucleotide sequence was 5'-GAACCTGGA-CTTCCAGCAGATCC-3'. The sense (control) oligonucleotide sequence was 5'-GGATGCGTGAAGTCCAGGTTC-Y).

Results and Discussion

BAC1.2F5 cells express hck mRNA (Fig. 1 a), immunoreactive p59hck protein (Fig. 2 a), and hck kinase activity (Fig. 2, b and c). Hck mRNA levels increase three- to sixfold within 1-4 h of exposure of quiescent BAC1.2F5 cells to growth factors (CSF-1 and GM-CSF) and activating stimuli including LPS (Fig. 1 a); hck protein expression parallels the accumulation of hck mRNA (Fig. 2 a). The upregulation of hck expression by growth factors and LPS in BAC1.2F5 cells is similar to that reported in human monocyte-derived macrophages (9) and murine bone marrow-derived monocytes and macrophages (10, 12).

Exposure of quiescent BAC1.2F5 cells to CSF-1, LPS (Fig. 2, b and c), and GM-CSF (data not shown) also promptly augments hck kinase activity, as measured both by autophosphorylation kinase reactions (Fig. 2 b) and trans-phosphorylation reactions with denatured rabbit muscle enolase serving as a substrate (Fig. 2 c). At optimal concentrations of CSF-1 and LPS, the kinetics of induction of augmented hck kinase activity by these stimuli are very similar (Fig. 3), with some increase in kinase activity observed as early as 5 min after stimulation and maximal kinase activity detected by 10-20

Figure 1. Northern blot analysis of hck mRNA expression in BAC1.2F5 parental cells and transfected cell lines. (a) BAC1.2F5 cells were incubated for 16 h in the absence of growth factors, then exposed to medium alone (lane 1) or medium plus rHuCSF-1 100 pg ml⁻¹ (lane 2), rMuGM-CSF 100 pg ml⁻¹ (lane 3), or LPS 1 μg ml⁻¹ (lane 4) for 4 h. (b) BAC1.2F5 cells (lane 5) and subclones expressing the LNSL7 vector alone (lane 6), LNSL7-Huhck296 (lane 7), LNSL7-Huhck301 (lane 8), and LNSL7-Mukck296 (lane 9) were grown to confluence in medium containing LCM (27) as a source of CSF-1. Total RNA was isolated from the indicated cells and analyzed by Northern blotting with human (lanes 7 and 8) and murine (lanes 5, 6, and 9) hck cDNA probes.
muscle enolase as described in Materials and Methods.

were lysed and kinase assays performed in the presence of denatured rabbit kinase activity of Figure 2. Expression of immunoblotting with a rabbit polyclonal anti-hck antiserum. (b) In vitro BAC1.2F5 cells and subclones. BAC1.2F5 parental cells were incubated from BAC1.2F5 subclones expressing vector alone (lane 4), human kinase-negative p59hck 269 (lane 5), human activated p59hck 269 (lane 6), or murine activated p59hck 269 (lane 7) were cultured in medium containing LCM (27) as a source of CSF-1. Lysates were prepared and analyzed by immunoblotting with a rabbit polyclonal anti-hck antisera. (b) In vitro kinase activity of hck proteins in BAC1.2F5 cells and subclones: autophosphorylation reactions. Kinase activities of hck proteins immunoprecipitated with Anti-hck antisera from BAC1.2F5 cells incubated overnight in the absence of growth factors, then exposed to medium alone (lane 1), rCSF-1 100 pg ml\(^{-1}\) (lane 2), or LPS 1 \(\mu\)g ml\(^{-1}\) (lane 3) for 16 h. BAC1.2F5 subclones expressing LNSL7 vector alone (lane 4), human kinase-negative p59hck 269 (lane 5), human activated p59hck 269 (lane 6), or murine activated p59hck 269 (lane 7) were cultured in medium containing LCM (27) as a source of CSF-1. Lysates were prepared and analyzed by immunoblotting with a rabbit polyclonal anti-hck antisera. (b) In vitro kinase activity of hck proteins in BAC1.2F5 cells and subclones: autophosphorylation reactions. Kinase activities of hck proteins immunoprecipitated with Anti-hck antisera from BAC1.2F5 cells incubated overnight in the absence of growth factors, then exposed to medium alone (lane 1), rCSF-1 100 pg ml\(^{-1}\) (lane 2), or LPS 1 \(\mu\)g ml\(^{-1}\) (lane 3) for 10 min, and from BAC1.2F5 subclones expressing vector alone (lane 4), human p59hck 269 (lane 5), human p59hck 269 (lane 6), and murine p59hck 269 (lane 7). Cells were lysed and in vitro kinase assays performed as described in Materials and Methods. (c) Trans-phosphorylation kinase activity of hck proteins in BAC1.2F5 cells and subclones. Kinase activities of hck proteins immunoprecipitated from BAC1.2F5 cells incubated overnight in the absence of growth factors, then exposed to medium alone (lane 1), rCSF-1 100 pg ml\(^{-1}\) (lane 2), or LPS 1 \(\mu\)g ml\(^{-1}\) (lane 3) for 10 min, and from BAC1.2F5 subclones expressing vector alone (lane 4), human p59hck 269 (lane 5), human p59hck 269 (lane 6), and murine p59hck 269 (lane 7). Cells were lysed and kinase assays performed in the presence of denatured rabbit muscle enolase as described in Materials and Methods.

Figure 3. Augmented hck kinase activity in BAC1.2F5 cells stimulated with rCSF-1 or LPS. BAC1.2F5 cells were incubated overnight in the absence of growth factors, then exposed to medium alone (lane 1), rCSF-1 100 pg ml\(^{-1}\) for 5 min (lane 2), 10 min (lane 3), or 20 min (lane 4), or LPS 1 \(\mu\)g ml\(^{-1}\) for 5 min (lane 5), 10 min (lane 6), or 20 min (lane 7). Lysates were prepared and subjected to immunoprecipitation with a rabbit polyclonal anti-hck antisera. Autophosphorylation in vitro kinase assays were performed on the immunoprecipitates, and reaction products were analyzed by SDS-PAGE, as described in Materials and Methods.

min after stimulation. Phosphoamino acid analysis confirmed that this in vitro kinase activity resulted in phosphorylation of enolase exclusively on tyrosine residues (Fig. 4). LPS and IFN-\(\gamma\) also have been reported to provoke increased hck kinase activity in murine bone marrow–derived macrophages (12), but the effect was noted only after more than 6 h of stimulation and may have primarily reflected increased steady-state levels of p59hck protein, rather than augmented kinase activity per se.

Unstimulated BAC1.2F5 cells contain little or no TNF mRNA (Fig. 5a) and do not secrete detectable amounts of TNF protein (data not shown). In response to LPS (but not CSF-1 or GM-CSF; data not shown), BAC1.2F5 cells accumulate large amounts of TNF mRNA and secrete TNF protein (Fig. 5).

To determine if p59hck was an important signaling molecule in activated macrophages, we employed complementary strategies to modulate hck kinase activity in BAC1.2F5 cells. First, we used retroviral constructs to overexpress a mutant form of human and murine hck with increased kinase activity (p59hck 4E259, Y501-F501) (29) in BAC1.2F5 cells. Cells expressing vector alone (LNSL7) or a kinase-defective mutant of hck protein (p59hck 4E259, K269-E269) were used as controls. Second, we used antisense oligonucleotides specific for murine hck to inhibit endogenous p59hck expression in BAC1.2F5 cells. We also examined the effect of an inhibitor of tyrosine kinase activity, herbimycin A, on the activation requirements of these cells.

Activated p59hck 4E259 and kinase-negative p59hck 4E259 mutants (29) of hck were expressed in BAC1.2F5 cells by infecting the cells with retroviral stocks produced by introduction of LNSL7-hck constructs (28, 29) into the Psi-2 retroviral packaging cell line (30). Clones were selected in G418 and screened for expression of hck mRNA (Fig. 1b), protein (Fig. 2a), and kinase activity (Fig. 2b). BAC1.2F5 subclones expressing either human or murine p59hck 4E259 or human p59hck 4E259 were morphologically similar to parental cells and cells expressing vector alone and continued to require CSF-1 for proliferation and survival. BAC1.2F5 parental cells and hck-expressing subclones also exhibited similar surface marker phenotypes. All expressed Ly1, Mac1, and Mac2 surface antigens and were negative for Ly2 and Mac3 expression (data not shown). However, in response to LPS, BAC1.2F5 clones
Figure 5. Augmented production of TNF mRNA and protein by BAC1.2F5 cells and subclones expressing p59k>F501. (a) Northern blot analysis of TNF mRNA accumulation by BAC1.2F5 cells and subclones: unstimulated, lanes 1-4; after exposure to LPS 100 ng ml⁻¹ (lanes 5-8) or LPS 500 ng ml⁻¹ (lanes 9-12) for 4 h. RNA was isolated from unstimulated BAC1.2F5 cells (lane 1) and subclones expressing human kinase-defective p59k>F501 (lane 2), human activated p59k>F501 (lane 3), and murine activated p59k>F501 (lane 4); and after LPS stimulation of BAC1.2F5 cells (lane 5) and subclones expressing vector alone (lane 6), human kinase-defective p59k>F269 (lanes 9 and 10), human activated p59k>F501 (lanes 7, 11, and 12), and murine activated p59k>F501 (lane 8). Total RNA was isolated from the indicated cells and analyzed by Northern blotting with a murine TNF cDNA probe. (b) TNF secretion by BAC1.2F5 cells and subclones expressing vector alone (LNSL7), human kinase-negative p59k>E269, and human activated kinase, p59k>F501. Cells were cultured in 6-well tissue culture plates in medium containing LCM as a source of CSF-1, and were exposed to LPS 1 μg ml⁻¹ for 16 h. Supernatants were collected and stored at -70°C until analysis. TNF protein levels were determined by use of a solid-phase sandwich ELISA (Genzyme Corp.). Data represent means of three experiments ±SD.

expressing either human or murine p59k>F501 accumulated 5-20-fold more TNF mRNA (Fig. 5 a) and secreted two-to-four-fold more TNF protein (Fig. 5 b) than did parental cells, clones expressing vector alone (LNSL7), or clones expressing human kinase-defective p59k>F269. In the absence of LPS, BAC1.2F5 clones expressing p59k>F501 accumulated appreciable TNF mRNA (Fig. 5 a) but produced little or no immunoreactive TNF (data not shown).

In parallel experiments, we employed antisense oligonucleotides to study the effects of inhibiting endogenous p59k expression in BAC1.2F5 cells. BAC1.2F5 cells were exposed to either sense or antisense hck oligonucleotides at concentrations varying from 1-30 μM for intervals ranging from 24 h to 4 wk. Exposure of BAC1.2F5 cells to hck antisense oligonucleotides for 72 h resulted in a significant reduction (two-to-five-fold) in the expression of p59k protein (Fig. 6 a) and kinase activity, as measured by autophosphorylation (Fig. 6 b) and trans-phosphorylation (Fig. 6 c) kinase assays, whereas exposure of the cells to the sense control oligonucleotide had no effect. BAC1.2F5 cells exposed to hck antisense oligonucleotides accumulated significantly less TNF mRNA (25-50% of control, data not shown) and produced significantly less TNF protein (30-35% of control, Fig. 6 d) in response to LPS than did untreated cells or cells exposed to an equimolar concentration of the sense oligonucleotide control. In parallel experiments, exposure of BAC1.2F5 cells to sense or antisense
oligonucleotides corresponding to the analogous seven codons of murine lyn kinase (33) had no effect on TNF production by BAC1.2F5 cells, which also express p56lck (data not shown). Proliferation of BAC1.2F5 cells in response to CSF-1 was not affected by exposure to lck sense or antisense oligonucleotides (data not shown).

The effect of lck antisense oligonucleotides on TNF production by BAC1.2F5 cells was comparable with that observed with preincubation of these cells with the tyrosine kinase inhibitor herbimycin A. Pretreatment of BAC1.2F5 parental cells and subclones overexpressing p59hck with herbimycin A inhibited LPS-stimulated TNF production by these cells in a dose-dependent manner, leading to a maximal four- to five-fold reduction in TNF secretion at optimal concentrations (Fig. 7). Weinstein, et al. (2), have reported that herbimycin A blocks the LPS-stimulated release of arachidonic acid metabolites in the murine RAW 264.7 macrophage cell line, and herbimycin A also inhibits LPS-induced TNF production by human alveolar macrophages (Beatty, C., and C. B. Wilson, personal communication).

These experiments provide the first direct evidence for a functional role for the lck tyrosine kinase and suggest that p59hck is an integral component of the signaling pathways involved in macrophage activation and TNF production. Whereas other stimuli (e.g., CSF-1 and GM-CSF) also upregulate lck expression and kinase activity, the results of our experimental manipulation of lck expression in BAC1.2F5 cells suggest that p59hck does not play a critical role in the mitogenic responses to those stimuli. An improved understanding of the role of p59hck in macrophage signaling pathways will require identification of associated cell surface molecules and substrates of the lck kinase.

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References

1. Adams, D.O., and T.A. Hamilton. 1987. Molecular transductional mechanisms by which IFNγ and other signals regulate macrophage development. Immunol. Rev. 97:5.

2. Weinstein, S.L., M.R. Gold, and A.L. DeFranco. 1991. Bacterial lipopolysaccharide stimulates protein tyrosine phosphorylation in macrophages. Proc. Natl. Acad. Sci. USA. 88:4148.

3. Adams, D.O., and T.A. Hamilton. 1984. The cell biology of macrophage activation. Annu. Rev. Immunol. 2:283.

4. Burchett, S.K., W.M. Weaver, J.W. Westall, A. Larsen, S. Kronheim, and C.B. Wilson. 1988. Regulation of tumor necrosis factor/cachectin and IL-1 secretion in human mononuclear phagocytes. J. Immunol. 140:3473.

5. Beutler, B., and A. Cerami. 1989. The biology of Cachectin/TNF—a primary mediator of the host response. Annu. Rev. Immunol. 7:625.

6. Ziegler, S.F., J.D. Marth, D.B. Lewis, and R.M. Perlmutter. 1987. Novel protein-tyrosine kinase gene (lck) preferentially expressed in cells of hematopoietic origin. Mol. Cell. Biol. 7:2276.

7. Quintrell, N., R. Lebo, H. Varmus, J.M. Bishop, M.J. Pette- tenati, M.M. Le Beau, M.O. Diaz, and J.D. Rowley. 1987. Identification of a human gene (HCK) that encodes a protein-tyrosine kinase and is expressed in hematopoietic cells. Mol. Cell. Biol. 7:2267.

8. Holtzman, D.A., W.D. Cook, and A.R. Dunn. 1987. Isolation and sequence of a cDNA corresponding to an src-related
gene expressed in murine hematopoietic cells. *Proc. Natl. Acad. Sci. USA.* 84:8325.

9. Ziegler, S.F., C.B. Wilson, and R.M. Perlmutter. 1988. Augmented expression of a myeloid-specific protein tyrosine kinase gene (*hck*) after macrophage activation. *J. Exp. Med.* 168: 1801.

10. Yi, T., and C.L. Willman. 1989. Cloning of the murine c-scr proto-oncogene cDNA and induction of c-scr expression by proliferation and activation factors in normal bone marrow-derived monocytic cells. *Oncogene.* 4:1081.

11. Lichtenberg, U., N. Quintrell, and J.M. Bishop. 1992. Human protein-tyrosine kinase gene HCK: expression and structural analysis of the promoter region. *Oncogene.* 7:849.

12. Boulet, I., S. Ralph, E. Stanley, P. Lock, A.R. Dunn, S.P. Pawar, M.P. Cooke, and K.M. Abraham. 1988. Specialized protein-tyrosine kinase proto-oncogenes in hematopoietic cells. *Biochim. Biophys. Acta.* 948:245.

13. Eiseman, E., and J.B. Bolen. 1990. Src-related tyrosine protein kinases as signaling components in hematopoietic cells. *Cell.* 2:303.

14. Veillette, A., M.A. Bookman, E.M. Horak, L.E. Samelson, and J.B. Bolen. 1988. The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-kinase p56Lck. *Cell.* 55:301.

15. Veillette, A., M.A. Bookman, E.M. Horak, L.E. Samelson, and J.B. Bolen. 1989. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase p56Lck. *Nature (Lond.)* 338:257.

16. Shaw, A.S., K.E. Amrein, C. Hammond, D.F. Stern, B.M. Sefton, and J.K. Rose. 1989. The Lck tyrosine protein kinase interacts with the cytoplasmic tail of the CD4 glycoprotein through its unique amino-terminal domain. *Cell.* 59:627.

17. Turner, J.M., M.H. Brodsky, B.A. Irving, S.D. Levin, R.M. Perlmutter, and D.R. Litman. 1990. Interaction of the unique N-terminal region of the tyrosine kinase p56Lck with the cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. *Cell.* 60:755.

18. Samelson, L.E., A.F. Philips, E.T. Loung, and R.D. Klausner. 1990. Association of the lyn protein tyrosine kinase with the T cell antigen receptor. *Proc. Natl. Acad. Sci. USA.* 87:4358.

19. Cooke, M.P., K.M. Abraham, K.A. Forbush, and R.M. Perlmutter. 1991. Regulation of T cell receptor signaling by a src family protein-tyrosine kinase (*p59^m^*). *Cell.* 65:281.

20. Davidson, D., L.M.L. Chow, M. Fournel, and A. Veillette. 1992. Differential regulation of T cell antigen responsiveness by isoforms of the src-related tyrosine protein kinase p59^m^. *J. Exp. Med.* 175:1483.

21. Yamanashi, Y., T. Kakiuchi, J. Mizuguchi, T. Yamamoto, and K. Toyoshima. 1990. Association of B cell antigen receptor with the protein tyrosine kinase Lyn. *Science (Wash. DC).* 251:192.

22. Eiseman, E., and J.B. Bolen. 1992. Engagement of the high-affinity IgE receptor activates src protein-related tyrosine kinases. *Nature (Lond.)* 355:78.

23. Schwarzbaum, S., R. Halpern, and B. Diamond. 1984. The generation of macrophage-like cell lines by transfection with SV40 origin defective DNA. *J. Immunol.* 132:1158.

24. Morgan, C., J.W. Pollard, and E.R. Stanley. 1987. Isolation and characterization of a cloned growth factor dependent macrophage cell line, BAC1.2F5. *J. Cell. Physiol.* 130:420.

25. Wheeler, E.F., C.W. Rettenmier, A.T. Look, and C.J. Sherr. 1986. The v-fms oncogene induces factor independence and tumorigenicity in CSF-1 dependent macrophage cell line. *Nature (Lond.)* 324:377.

26. Stanley, E.R., and P.M. Heard. 1977. Factors regulating macrophage production and growth. *J. Biol. Chem.* 252:4305.

27. Marth, J.D., D.B. Lewis, M.P. Cooke, E.D. Mellins, M.E. Gearn, L.E. Samelson, C.B. Wilson, A.D. Miller, and R.M. Perlmutter. 1989. Lymphocyte activation provokes modification of a lymphocyte-specific protein tyrosine kinase (*p56Lck*). *J. Immunol.* 142:2430.

28. Ziegler, S.F., S.D. Levin, and R.M. Perlmutter. 1989. Transformation of NIH 3T3 fibroblasts by an activated form of p^59^Lck. *Mol. Cell. Biol.* 9:2724.

29. Mann, R., R.C. Mulligan, and D. Baltimore. 1983. Construction of a retrovirus packaging mutant and its use to provide helper-free defective retrovirus. *Cell.* 33:153.

30. Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

31. Yu, T., J.B. Bolen, and J.N. Ihle. 1991. Hematopoietic cells express two forms of lyn kinase differing by 21 amino acids in the amino terminus. *Mol. Cell. Biol.* 11:2391.

32. Cooper, J.A., F.S. Esch, S.S. Taylor, and T. Hunter. 1984. Phosphorylation sites in enolase and lactate dehydrogenase utilized by tyrosine protein kinases in vivo and in vitro. *J. Biol. Chem.* 259:7835.

33. Kmiecik, T.E., and D. Shalloway. 1987. Activation and suppression of pp60^src^ transforming ability by mutation of its primary sites of tyrosine phosphorylation. *Cell.* 49:65.

34. Boyle, W.J., P. Van Der Geer, and T. Hunter. 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymol.* 210:110-149.