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.

Exposure of monocytes and macrophages to bacterial LPS triggers a series of biochemical and functional changes including the activation of protein kinase C (1), tyrosine phosphorylation of several proteins (2), production of reactive oxygen metabolites (3), and production and secretion of cytokines with critical roles in host defense (1, 4, 5). TNF is an important monokine with pleiotropic effects on the host inflammatory response, including the induction of fever, direct antiviral and antitumor activity, and augmentation of monocyte, granulocyte, and lymphocyte function (for a review see reference 5).

Expression of the hematopoietic cell kinase (*hck*)[1] tyrosine kinase is essentially limited to cells of monocyte/macrophage and granulocyte lineages (6–9), increases with differentiation along either the macrophage or granulocyte pathways (7, 10, 11), and is further augmented by activation stimuli including LPS in mature monocytes and macrophages (9, 10, 12). This pattern of expression suggested a potential role for *hck* in the terminal differentiation of macrophages and/or in the activation pathways of mature monocytes and macrophages. Several lines of evidence support a role for other members of the *src* family of kinases in signal transduction pathways of lymphocytes, mast cells, and basophils (13–23).

To study the possible role of *hck* in macrophage activation and cytokine gene expression, we have manipulated the level of expression of *hck* in the murine macrophage cell line, BAC1.2F5. BAC1.2F5 cells require CSF-1 or GM-CSF for maintenance of viability and proliferation, grow as an adherent monolayer, and morphologically resemble normal macrophages (24–26). These cells express Ia antigen, possess Fc receptors, engage in Fc receptor–mediated phagocytosis, and produce cytokines in response to LPS (24–26). In this report, we show that BAC1.2F5 cells express *hck* mRNA, protein and kinase activity, and that *hck* expression and kinase activity are augmented by activation stimuli (e.g., LPS) and by growth factors (e.g., CSF-1 and GM-CSF). Furthermore, we demonstrate that manipulation of the level of *hck* kinase activity in BAC1.2F5 cells alters the response of these cells to LPS but does not affect viability or proliferation. Inhibition of endogenous *hck* expression, with antisense oligonucleotides, leads to diminished LPS-mediated TNF production in BAC1.2F5 cells, whereas constitutive expression of an activated mutant of *hck* ($p59^{hckF501}$) in BAC1.2F5 cells augments TNF production in response to LPS.

Materials and Methods

Materials. DMEM, L-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-

[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 U ml^{-1} penicillin G, and 50 μg ml^{-1} streptomycin.

Retrovirus Production and Infection of BAC1.2F5 Cells. LNSL7-based vectors (28) expressing cDNAs encoding wild-type, activated ($p59^{hckF501}$) and kinase-negative ($p59^{hckE269}$) 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'-GAACCTGGACTTCACGCATCC-3'). The sense (control) oligonucleotide sequence was (5'-GGATGCGTGAAGTCCAGGTTC-3').

RNA Isolation and Northern Blot Analysis. Total cellular RNA was isolated from BAC1.2F5 cells by the guanidinium isothiocyanate/cesium chloride method and quantitated spectrophotometrically as described (31). For blots, 20 μg RNA was electrophoresed on 2.2 M formaldehyde, 1% agarose gels, transferred to Nytran membranes, UV irradiated, and baked, as described (32). Blots were hybridized overnight with 10^6 dpm ml^{-1} of ^{32}P-labeled murine or human *hck* cDNA probes or a murine TNF cDNA probe, washed twice with 3× SSC, 0.1% SDS, and twice with 0.2× SSC, 0.1% SDS at 42°C, then autoradiographed at −70°C in the presence of two enhancing screens.

Immunoblotting and Immunoprecipitations. For Western blotting and immunoprecipitations, cells were lysed in extraction buffer (20 mM Tris, 100 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM Na_3VO_4, 0.2 mM PMSF, 10 μg ml^{-1} leupeptin, and 10 μg ml^{-1} aprotinin). For immunoblotting, lysates were electrophoresed on 7.5% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and reacted with rabbit polyclonal antisera raised against an *hck*-TrpE fusion protein (9). Blots were reacted with a donkey anti-rabbit IgG horseradish peroxidase conjugate (Amersham Corp., Arlington Heights, IL), and proteins were detected by enhanced chemiluminescence (Amersham Corp.). For immunoprecipitations, lysates were incubated with anti-*hck* rabbit polyclonal antiserum, and the immunocomplexes were collected with protein A–agarose (Bethesda Research Laboratories, Gaithersburg, MD) and analyzed by SDS-PAGE.

In Vitro Kinase Assays. For in vitro kinase assays, PBS-washed cells were lysed in 2% NP-40 and TEN (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.15 M NaCl). Lysates were incubated with the rabbit polyclonal anti-*hck* antiserum and immunocomplexes were collected with protein A–agarose. Immunoprecipitated proteins were suspended in 10 μl kinase buffer (containing 50 mM Pipes, pH 7.0, 1 mM MnCl, 1 mM Na_3VO_4, and 10 μCi of γ-[^{32}P]ATP) and incubated at room temperature for 20 min as described (33). In some experiments, denatured rabbit muscle enolase was added as an exogenous substrate, as described (34). Products of the kinase reactions were analyzed by SDS-PAGE.

Determination of TNF Concentrations. TNF protein levels were measured with a solid-phase sandwich ELISA specific for murine TNF-α, as specified by the manufacturer (Genzyme Corp., Cambridge, MA).

Phosphoamino Acid Analysis. ^{32}P-labeled protein bands were excised from polyacrylamide gels, rehydrated, digested with trypsin, lyophilized, hydrolyzed in 0.5 ml 6N HCl for 1 h at 110°C, and subjected to two-dimensional thin-layer chromatography in the presence of cold phosphoserine, phosphothreonine, and phosphotyrosine in pH 1.9 buffer, then autoradiographed at −70°C, as described (35, 36). Markers were visualized with ninhydrin after autoradiography.

Results and Discussion

BAC1.2F5 cells express *hck* mRNA (Fig. 1 *a*), immunoreactive $p59^{hck}$ 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

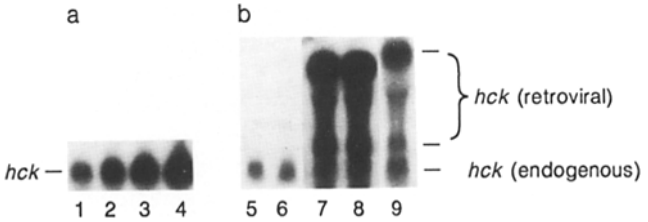

Figure 1. Northern blot analysis of *hck* mRNA expression in BAC1.2F5 parental cells and transfected cell lines. (*a*) BAC1.2F5 cells were incubated for 16 h in the absence of growth factors, then exposed to medium alone (lane *1*) or medium plus rHuCSF-1 100 pg ml^{-1} (lane *2*), rMuGM-CSF 100 pg ml^{-1} (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-Huhck^{E269} (lane *7*), LNSL7-Huhck^{F501} (lane *8*), and LNSL7-Muhck^{F501} (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.

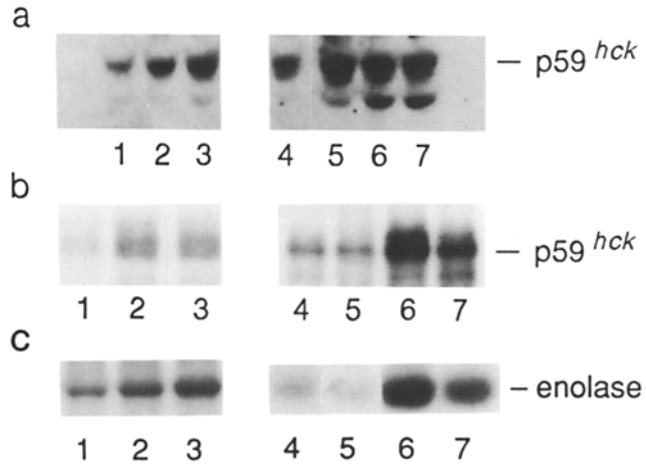

Figure 2. Expression of *hck* proteins and kinase activities by BAC1.2F5 cells and transfected cell lines. (*a*) Immunoblot of whole cell lysates from BAC1.2F5 cells and subclones. BAC1.2F5 parental cells were incubated overnight in the absence of growth factors, then exposed to medium alone (lane *1*), rCSF-1 100 pg ml^{-1} (lane *2*), or LPS 1 μg ml^{-1} (lane *3*) for 16 h. BAC1.2F5 subclones expressing LNSL7 vector alone (lane *4*), human kinase-negative $p59^{hckE269}$ (lane *5*), human activated $p59^{hckF501}$ (lane *6*), or murine activated $p59^{hckF501}$ (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* antiserum. (*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 μg ml^{-1} (lane *3*) for 10 min, and from BAC1.2F5 subclones expressing vector alone (lane *4*), human $p59^{hckE269}$ (lane *5*), human $p59^{hckF501}$ (lane *6*), and murine $p59^{hckF501}$ (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 μg ml^{-1} (lane *3*) for 10 min, and from BAC1.2F5 subclones expressing vector alone (lane *4*), human $p59^{hckE269}$ (lane *5*), human $p59^{hckF501}$ (lane *6*), and murine $p59^{hckF501}$ (lane *7*). Cells were lysed and kinase assays performed in the presence of denatured rabbit muscle enolase as described in Materials and Methods.

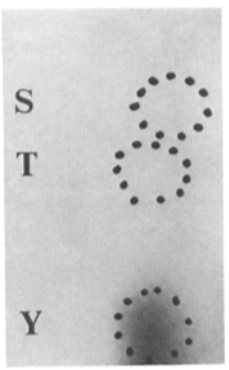

Figure 4. Phosphoamino acid analysis of rabbit muscle enolase after incubation with anti-*hck* immunoprecipitate from BAC1.2F5 cells. BAC1.2F5 cells were incubated overnight in the absence of growth factors, then stimulated for 10 min with LPS 1 μg ml^{-1}. Cells were lysed, lysates subjected to immunoprecipitation with a rabbit polyclonal antiserum specific for *hck*, immunoprecipitates collected with protein A–agarose, and a trans-phosphorylation in vitro kinase assay was performed in the presence of exogenous denatured rabbit muscle enolase, as described in Materials and Methods. The band corresponding to rabbit muscle enolase (~45 kD) was cut out of the gel, solubilized, reacted with trypsin, and analyzed by thin-layer chromatography to determine the content of phosphotyrosine (*Y*), phosphoserine (*S*), and phosphothreonine (*T*), as described in Materials and Methods.

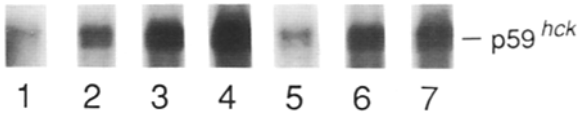

Figure 3. Augmented *hck* kinase activity in BAC1.2F5 cells stimulated with rCSF-1 or LPS. BAC1.F5 cells were incubated overnight in the absence of growth factors, then exposed to medium alone (lane *1*), rCSF-1 100 pg ml^{-1} for 5 min (lane *2*), 10 min (lane *3*), or 20 min (lane *4*); or LPS 1 μg 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* antiserum. 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-γ have also 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 $p59^{hck}$ protein, rather than augmented kinase activity per se.

Unstimulated BAC1.2F5 cells contain little or no TNF mRNA (Fig. 5 *a*) 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 $p59^{hck}$ 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 ($p59^{hckF501}$, Y501-F501) (29) in BAC1.2F5 cells. Cells expressing vector alone (LNSL7) or a kinase-defective mutant of human *hck* ($p59^{hckE269}$, K269-E269) were used as controls. Second, we used antisense oligonucleotides specific for murine *hck* to inhibit endogenous $p59^{hck}$ 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 $p59^{hckF501}$ and kinase-negative $p59^{hckE269}$ 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. 1 *b*), protein (Fig. 2 *a*), and kinase activity (Fig. 2, *b* and *c*). BAC1.2F5 subclones expressing either human or murine $p59^{hckF501}$ or human $p59^{hckE269}$ 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

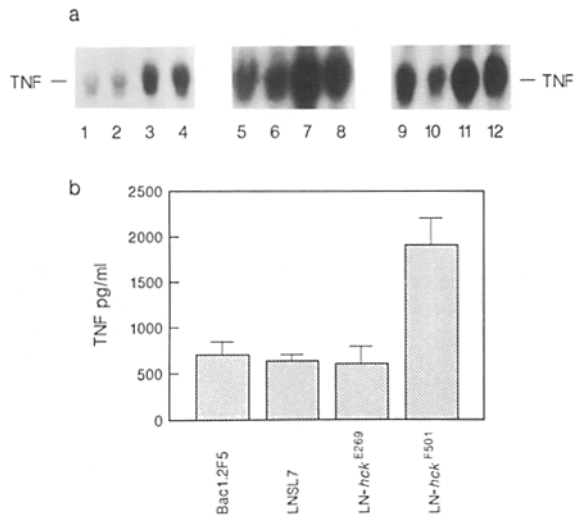

Figure 5. Augmented production of TNF mRNA and protein by BAC1.2F5 cells and subclones expressing p59^{hckF501}. (*a*) Northern blot analysis of TNF mRNA accumulation by BAC1.2F5 cells and subclones: unstimulated, lanes *1–4*; after exposure to LPS 100 ng ml^{-1} (lanes *5–8*) or LPS 500 ng ml^{-1} (lanes *9–12*) for 4 h. RNA was isolated from unstimulated BAC1.2F5 cells (lane *1*) and subclones expressing human kinase–defective p59^{hckE269} (lane *2*), human activated p59^{hckF501} (lane *3*), and murine activated p59^{hckF501} (lane *4*); and after LPS stimulation of BAC1.2F5 cells (lane *5*) and subclones expressing vector alone (lane *6*), human kinase–defective p59^{hckE269} (lanes *9* and *10*), human activated p59^{hckF501} (lanes *7*, *11*, and *12*), and murine activated p59^{hckF501} (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 p59^{hckE269}, and human activated kinase, p59^{hckF501}. 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.

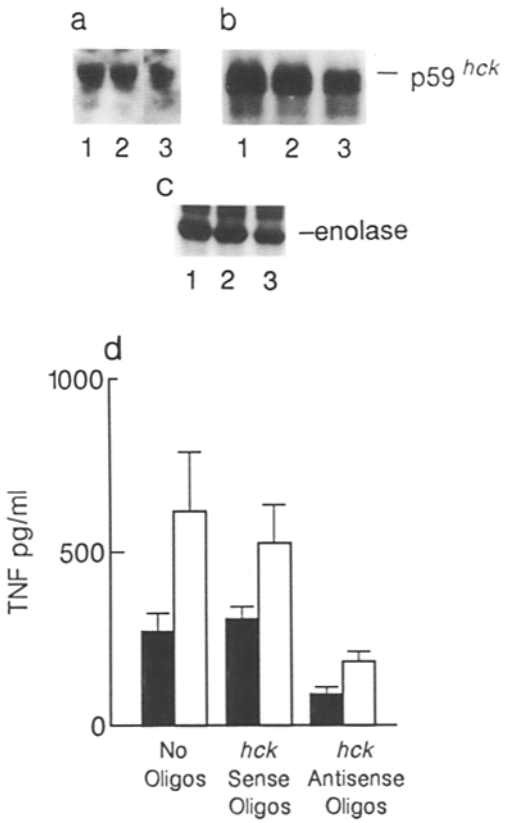

Figure 6. *Hck* antisense oligonucleotides reduce *hck* protein expression and inhibit TNF production by BAC1.2F5 cells. (*a*) Immunoblot of lysates from BAC1.2F5 cells exposed to medium alone (lane *1*), medium with 10 μM *hck* sense oligonucleotide control (lane *2*), or 10 μM *hck* antisense oligonucleotide (lane *3*) for 72 h. Lysates were prepared, subjected to SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting with rabbit polyclonal anti-*hck* antisera. (*b*) Determination of *hck* autophosphorylation kinase activity in BAC1.2F5 cells incubated in medium alone (lane *1*), medium with 10 μM *hck* sense oligonucleotide (lane *2*), or 10 μM *hck* antisense oligonucleotide (lane *3*) for 72 h, then exposed to LPS 100 ng ml^{-1} for 10 min. Lysates were prepared and in vitro kinase assays were performed as described in Materials and Methods. (*c*) Determination of *hck* trans-phosphorylation kinase activity in BAC1.2F5 cells incubated in medium alone (lane *1*), medium with 10 μM *hck* sense oligonucleotide (lane *2*), or 10 μM *hck* antisense oligonucleotide (lane *3*) for 72 h, then exposed to LPS 100 ng ml^{-1} for 10 min. Lysates were prepared and trans-phosphorylation kinase assays performed with denatured rabbit muscle enolase as a substrate, as described in Materials and Methods. (*d*) TNF secretion by Bac1.2F5 cells exposed to *hck*-specific oligonucleotides. Bac1.2F5 cells were cultured in 6-well plates for 72 h in the presence of medium alone (with LCM as a source of CSF-1) or in the presence of 10 μM concentrations of sense or antisense *hck* oligonucleotides, then exposed to LPS 250 ng ml^{-1} (*solid bars*) or LPS 1 μg ml^{-1} (*open bars*) for 16 h. Supernatants were collected and frozen at −70°C until analyzed. TNF concentrations were quantitated by use of a solid-phase antibody sandwich ELISA. Data represent means of three experiments ±SD.

expressing either human or murine p59^{hckF501} 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 p59^{hckE269}. In the absence of LPS, BAC1.2F5 clones expressing p59^{hckF501} 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 p59^{hck} 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 p59^{hck} 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

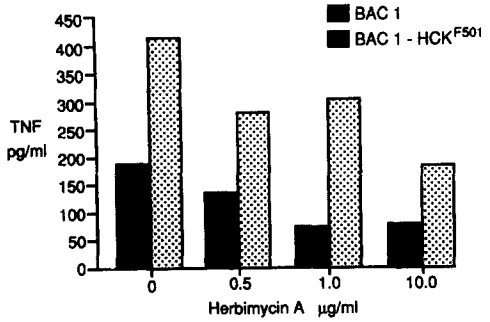

Figure 7. Herbimycin A inhibits TNF production by BAC1.2F5 cells and subclones expressing p59^{hckF501}. BAC1.2F5 cells and subclones expressing human p59^{hckF501} were exposed to herbimycin A at the indicated concentrations for 4 h, then stimulated with LPS 250 ng ml^{-1} for 16 h. Supernatants were collected and stored at −70°C until analyzed. TNF concentrations were quantitated by use of a solid-phase antibody sandwich ELISA.

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 p56^{lyn} (data not shown). Proliferation of BAC1.2F5 cells in response to CSF-1 was not affected by exposure to *hck* sense or antisense oligonucleotides (data not shown).

The effect of *hck* 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 p59^{hckF501} 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 *hck* tyrosine kinase and suggest that p59^{hck} 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 *hck* expression and kinase activity, the results of our experimental manipulation of *hck* expression in BAC1.2F5 cells suggest that p59^{hck} does not play a critical role in the mitogenic responses to those stimuli. An improved understanding of the role of p59^{hck} in macrophage signaling pathways will require identification of associated cell surface molecules and substrates of the *hck* kinase.

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