

Deficiency of Src Family Kinases p59/61^{hck} and p58^{c-fgr} Results in Defective Adhesion-dependent Neutrophil Functions

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Abstract. Cross-linking of the neutrophil β_2 - or β_3 -related leukocyte response integrins by extracellular matrix (ECM) proteins or monoclonal antibodies (mAb) stimulates cytoskeletal rearrangement leading to cell spreading and respiratory burst. Tyrosine phosphorylation of a variety of proteins and activation of the Src family kinases within polymorphonuclear leukocytes (PMN) have recently been implicated in the intracellular signaling pathways generated by leukocyte integrins (Yan, S.R., L. Fumagalli, and G. Berton. 1995. *J. Inflammation*. 45:217–311.) To directly test whether these functional responses are dependent on the Src family kinases p59/61^{hck} and p58^{c-fgr}, we examined adhesion-dependent respiratory burst in PMNs isolated from *hck*^{-/-}, *fgr*^{-/-}, and *hck*^{-/-}*fgr*^{-/-} knockout mice. Purified bone marrow PMNs from wild-type mice released significant amounts of O₂⁻ when adherent to fibrinogen-, fibronectin-, or collagen-coated surfaces, in the presence of activating agents such as tumor necrosis factor (TNF) or formyl-methionyl-leucyl-phenylalanine, as described for human PMNs. PMNs from *hck*^{-/-}*fgr*^{-/-} double-mutant mice, however, failed to respond. This defect was specific for integrin signaling, since respiratory burst was normal in *hck*^{-/-}*fgr*^{-/-}

PMNs stimulated by immune complexes or PMA. Stimulation of respiratory burst was observed in TNF-primed wild-type PMNs plated on surfaces coated with murine intracellular adhesion molecule-1 (ICAM-1), while *hck*^{-/-}*fgr*^{-/-} PMNs, failed to respond. Direct cross-linking of the subunits of β_2 and β_3 integrins by surface-bound mAbs also elicited O₂⁻ production by wild-type PMNs, while the double-mutant *hck*^{-/-}*fgr*^{-/-} cells failed to respond. Photomicroscopy and cell adhesion assays revealed that the impaired functional responses of *hck*^{-/-}*fgr*^{-/-} PMNs were caused by defective spreading and tight adhesion on either ECM protein- or mAb-coated surfaces. In contrast, *hck*^{-/-} or *fgr*^{-/-} single mutant cells produced O₂⁻ at levels equivalent to wild-type cells on ECM protein, murine ICAM-1, and antiintegrin mAb-coated surfaces. Hence, either p59/61^{hck} and p58^{c-fgr} is required for signaling through leukocyte β_2 and β_3 integrins leading to PMN spreading and respiratory burst. This is the first direct genetic evidence of the importance of Src family kinases in integrin signaling within leukocytes, and it is also the best example of overlapping function between members of this gene family within a defined signal transduction pathway.

DURING acute inflammatory responses, the interaction of leukocyte integrins with counterreceptors on the vascular endothelium and ECM proteins plays a major role in localizing neutrophils to the inflammatory site (reviewed in Springer, 1994). The recognition of the appropriate ligands by leukocyte integrins also serves to regulate many activation functions of inflammatory neutrophils (Kownatzki et al., 1988; Nathan, 1987; Shappell et al., 1990). In the presence of inflammatory mediators such as TNF or FMLP, cross-linking of leukocyte integrins by ECM proteins induces rearrangement of the actin cytoskeleton, resulting in the formation of focal ad-

hesion-like structures and cell spreading (Fuortes et al., 1993; Nathan and Sanchez, 1990). Coincident with these morphological changes, activated PMNs release proteases from specific granules and mount a sustained respiratory burst resulting in the release of superoxide (Nathan and Sanchez, 1990; Nathan et al., 1989; Richter et al., 1990; Sutherland and Boxer, 1994). In monocytes, binding of fibronectin by surface β_1 integrins elicits cytokine gene expression (Lin et al., 1994; Yurochko et al., 1992). The dependence of these functional responses on leukocyte integrins has been shown using PMNs from leukocyte adhesion deficiency patients, which lack β_2 integrins and fail to respond to TNF or FMLP when adherent to ECM proteins (Nathan and Sanchez, 1990; Nathan et al., 1989; Shappell et al., 1990). Moreover, either in monocytes or PMNs cross-linking of β_1 -(CD29), β_2 -(CD18), or β_3 -related

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leukocyte response integrin (CD69) by mAbs elicits the same functional responses (Berton et al., 1992; Lin et al., 1994; Zhou and Brown, 1993).

The intracellular signaling pathways elicited by integrin receptor cross-linking that result in cytoskeletal rearrangement and functional responses are currently being defined (Clark and Brugge, 1995; Hynes, 1992; Rosales et al., 1995). Signaling pathways involving changes in intracellular pH, caused by activation of the Na^+/H^+ antiporter, and increases in intracytoplasmic Ca^{2+} have been observed after ECM protein cross-linking of surface integrins (Jaconi et al., 1991; Schwartz et al., 1991). Likewise, in fibroblasts and tumor cells spreading on ECM protein-coated surfaces, activation of the Ras-MAPK pathway, resulting in pPLA_2 activation, as well as activation of PI-3K, have been observed; inhibition of either pPLA_2 or PI-3K blocks actin cytoskeletal rearrangements, impairing cell spreading (Chen et al., 1994; Peppelenbosch et al., 1993; Wymann and Arcaro, 1994). Proximal to these intracellular signaling events, however, integrin receptor-mediated activation of tyrosine phosphorylation has been observed in both nonhematopoietic cells as well as in leukocytes. The importance of tyrosine kinase activation in leukocyte integrin signaling is demonstrated by the fact that tyrosine kinase inhibitors block spreading and respiratory burst in both TNF-stimulated human PMNs plated on ECM proteins or resting cells plated on anti-CD18-mAb coated surfaces (Fuortes et al., 1993; Laudanna et al., 1993a). Many of these newly tyrosine-phosphorylated proteins appear to localize to focal adhesion structures at the cell membrane (Fuortes et al., 1993). Tyrosine phosphorylation of one of these focal adhesion proteins, paxillin, has been shown to be dependent on signals transduced by β_2 integrins (Fuortes et al., 1994; Graham et al., 1994).

Several protein tyrosine kinases have been implicated in mediating integrin-dependent signals. In carcinoma cells, fibroblasts, and platelets, pp125^{F_{AK}} has been implicated as playing a central role in integrin-signaling events because it is localized to focal adhesion structures, it is activated in response to cell adhesion, and it phosphorylates several focal adhesion proteins such as paxillin and tensin (Rosales et al., 1995). pp125^{F_{AK}}, however, is weakly expressed in PMNs and not activated during adhesion, while monocytes do not express pp125^{F_{AK}} at all (Fuortes et al., 1994; Lin et al., 1994). In these cell types, therefore, other kinases must function in integrin-signaling pathways. In platelets and fibroblasts pp60^{c-src} has been shown to become activated and associate with Triton X-100-insoluble cytoskeletal structures after integrin cross-linking (Eide et al., 1995;

Huang et al., 1993). Indeed, a critical role for pp60^{c-src} in integrin signaling is suggested by the observation that *src*^{-/-} fibroblasts are defective in spreading on fibronectin-coated surfaces, and that this phenotype can be rescued by transfection with wild-type pp60^{c-src} (Kaplan et al., 1995). The major Src family kinases expressed in PMNs are p59/61^{hck}, p58^{c-fgr} and p53/56^{lyn} (Tsygankov and Bolen, 1993). Cross-linking of PMN β_2 integrins has been shown to cause activation of p58^{c-fgr} and p53/56^{lyn} and translocation of these kinases to a Triton X-100 cytoskeletal fraction, suggesting that they play a central role in integrin signaling (Berton et al., 1994; Yan et al., 1995). Indeed, a number of newly tyrosine-phosphorylated proteins, as well as components of the NADPH oxidase, are redistributed to a Triton X-100-insoluble fraction after β_2 integrin cross-linking in human PMNs (Yan et al., 1995).

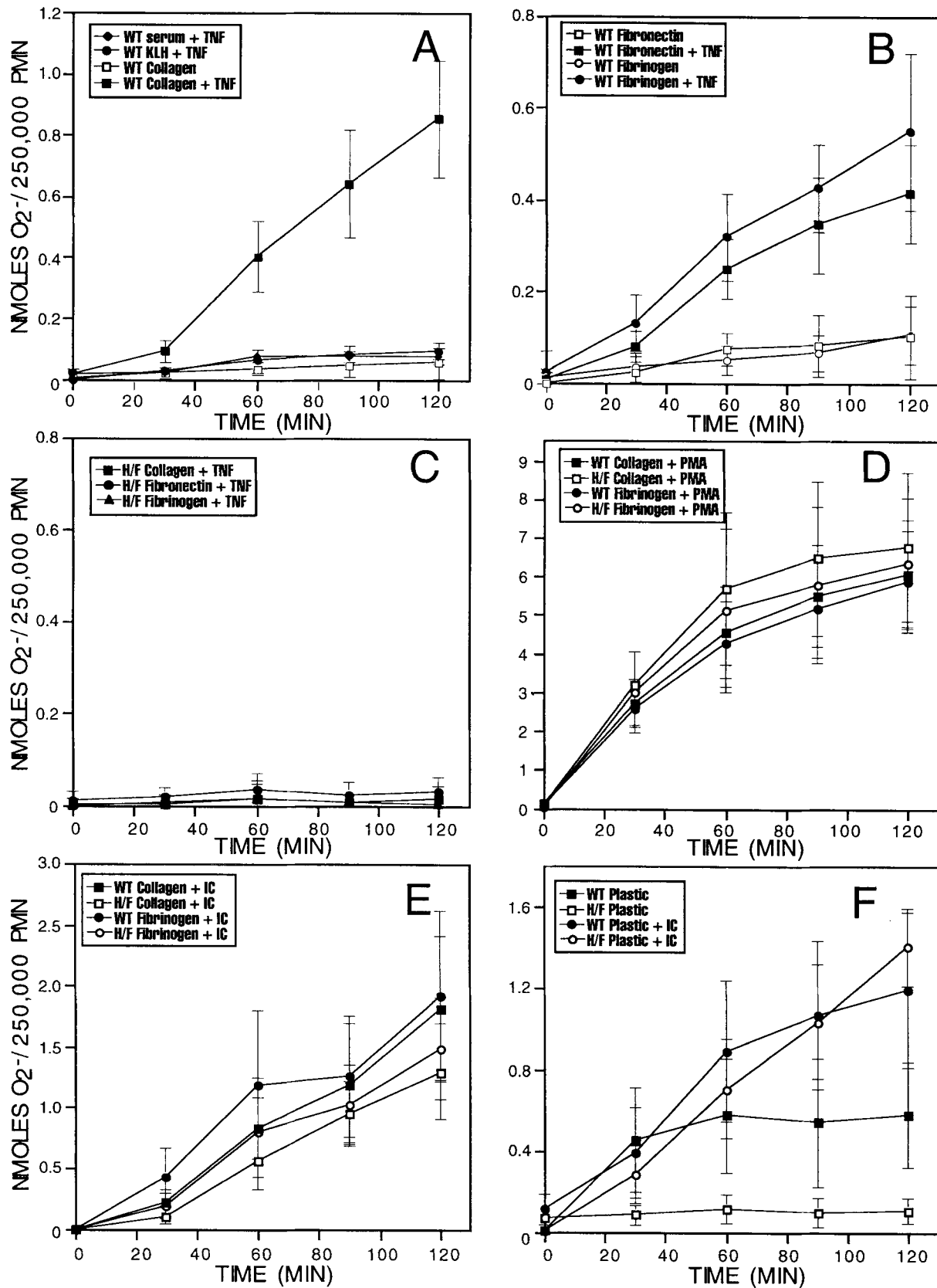
To test directly whether the Src family kinases p59/61^{hck} and p58^{c-fgr} play functional roles in integrin signaling in PMNs, we tested neutrophil functions in *hck*^{-/-}, *fgr*^{-/-}, and *hck*^{-/-}*fgr*^{-/-} double-mutant mice. Previous analysis of these mice revealed that the double-mutant animals were susceptible to *Listeria monocytogenes* infection, while single-mutant mice were not, indicating that both kinases serve at least partially redundant functions in the innate immune system (Lowell et al., 1994). In this work, we report that *hck*^{-/-}*fgr*^{-/-} PMNs are defective in adhesion-dependent respiratory burst mediated by cross-linking of integrin receptors with either ECM proteins or surface-bound anti-integrin mAbs. This functional defect is the result of defective cell spreading. Single-mutant *hck*^{-/-} or *fgr*^{-/-} PMNs, however, are completely normal. These results strongly argue that the p59/61^{hck} and p58^{c-fgr} kinases are critically involved in transducing signals through neutrophil integrins.

Materials and Methods

Isolation of Bone Marrow PMNs

Mouse bone marrow PMNs were isolated from femurs and tibiae as described (Hart et al., 1986). Marrow cells were flushed from the bones using HBSS (without $\text{Ca}^{2+}/\text{Mg}^{2+}$; GIBCO BRL, Gaithersburg, MD) + 0.1% BSA, RBCs were lysed with 0.15 M NH_4Cl , 1 mM KHCO_3 , 0.1 mM Na_2EDTA (4 min at room temperature), and remaining leukocytes were washed twice in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS + 0.1% BSA, then resuspended in 3 ml of a 45% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) solution in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS. The leukocytes were then loaded on top of a Percoll density gradient prepared in a 15-ml polystyrene tube (Nalgene) by layering successively 2 ml each of 62, 55, and 50% Percoll solutions on top of 3 ml of an 81% Percoll solution. Cells were then centrifuged at 1,200 g for 30 min at room temperature in a swinging bucket rotor. The cell band formed between the 81 and 62% layer was harvested using a pas-

Figure 1. Generation of O_2^- by PMNs from wild-type and *hck*^{-/-}*fgr*^{-/-} double-homozygous mice adherent to different surfaces. (A) O_2^- produced from wild-type PMNs stimulated with TNF (20 ng/ml) plated in wells coated with collagen, mouse serum, and KLH. O_2^- production by wild-type PMNs plated on serum or KLH in the presence or absence of TNF (not shown) was equal. (B) O_2^- produced by wild-type PMNs plated in wells coated with fibrinogen or fibronectin in either the presence or absence of TNF. (C) O_2^- produced by *hck*^{-/-}*fgr*^{-/-} double-mutant PMNs plated in wells coated with collagen, fibronectin, or fibrinogen, in the presence of TNF. O_2^- production by double-mutant PMNs plated in the absence of TNF was 0 (not shown). (D) O_2^- produced by wild-type and *hck*^{-/-}*fgr*^{-/-} PMNs plated in collagen or fibrinogen-coated wells in the presence of PMA (2.5 ng/ml). (E) O_2^- produced by wild-type and *hck*^{-/-}*fgr*^{-/-} PMNs plated in collagen or fibrinogen-coated wells in the presence of immune complexes (3 $\mu\text{g}/\text{ml}$ total protein). (F) O_2^- produced by wild-type and *hck*^{-/-}*fgr*^{-/-} PMNs plated in uncoated tissue-culture plastic wells in the presence or absence of immune complexes. Purified bone marrow PMNs were plated in wells coated with the different proteins, and O_2^- production was monitored as described in Materials and Methods. O_2^- production by PMNs from wild-type or *hck*^{-/-}*fgr*^{-/-} mutant mice in response to TNF was compared in nine independent experiments on collagen, in seven independent experiments on fibrinogen, and in five independent experiments on fibronectin. In each



experiment, a control stimulus (PMA in six experiments, immune complexes in three experiments) was included. O₂⁻ production by wild-type and *hck*^{-/-}*fgr*^{-/-} PMNs plated on plastic was compared in nine independent experiments (with PMA stimulus in six experiments (not shown; values equivalent to *D*) and immune complexes in three experiments (*F*). Mean results ± SD are reported. Experiments were performed using bone marrow PMNs pooled from two to three mice per experiment or from individual mice. Defective adhesion-dependent O₂⁻ production by PMNs from *hck*^{-/-}*fgr*^{-/-} double-mutant mice was found in a total number of 24 animals.

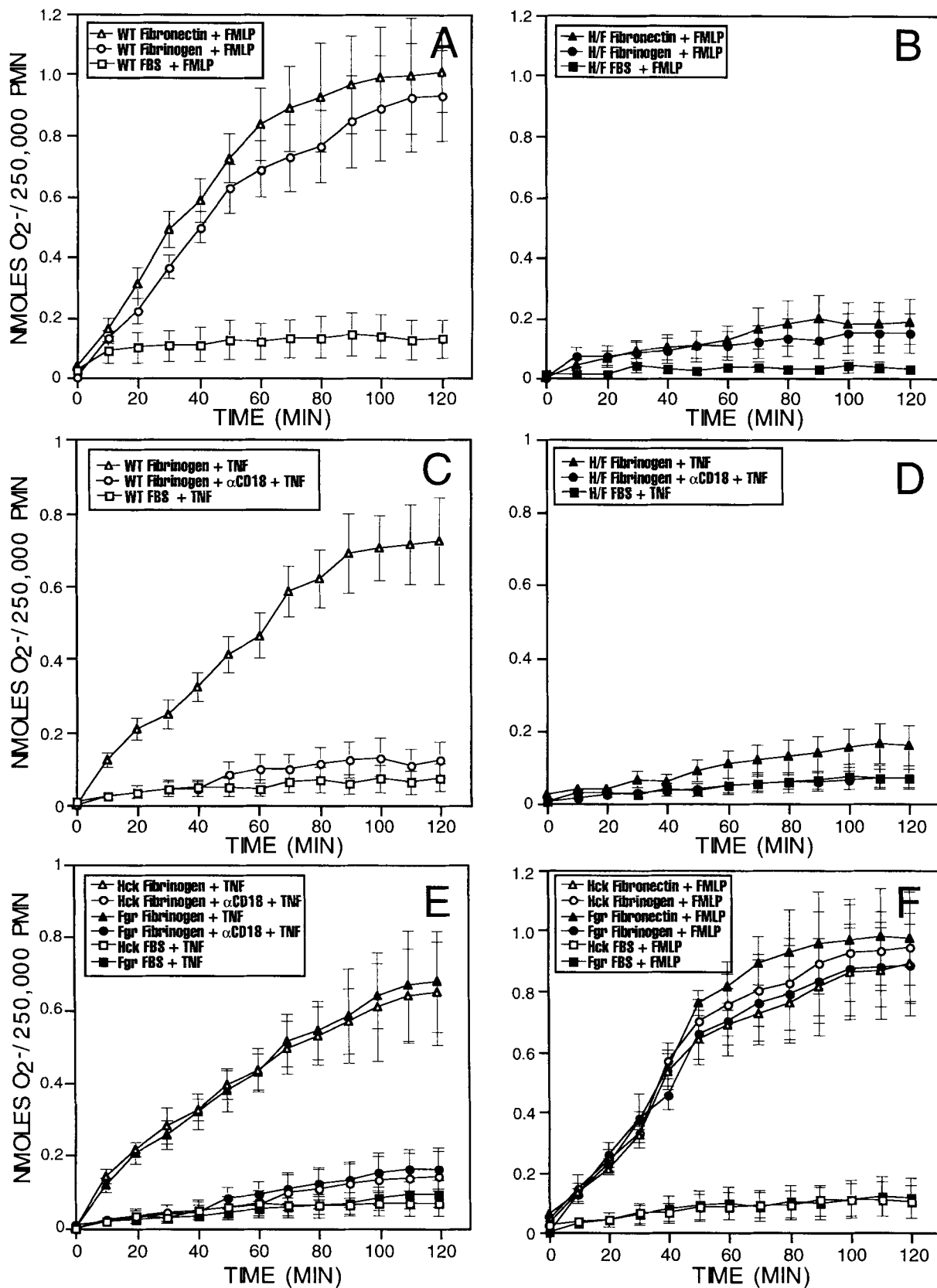


Figure 2. *hck*⁻¹*fgr*⁻¹ double-mutant PMNs are defective in adhesion-dependent O₂⁻ production, while *hck*⁻¹ and *fgr*⁻¹ single-mutants are not. (A) O₂⁻ produced from wild-type PMNs, stimulated with FMLP (200 nM), and plated in wells coated with fibronectin, fibrinogen, or FBS. (B) O₂⁻ produced from *hck*⁻¹*fgr*⁻¹ double-mutant PMNs, stimulated with FMLP, plated in wells coated with fibronectin, fibrinogen or FBS. (C) O₂⁻ produced from wild-type PMNs, stimulated with TNF (20 ng/ml), plated on fibrinogen-coated wells, with/without addition of anti-CD18. (D) O₂⁻ produced from *hck*⁻¹*fgr*⁻¹ PMNs, stimulated with TNF, plated on fibrinogen-coated wells,

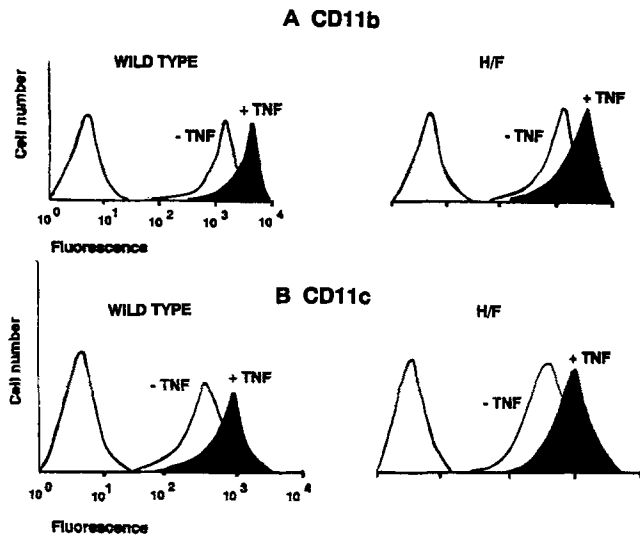


Figure 3. *hck*^{-/-}*fgr*^{-/-} double-mutant PMNs upregulate CD11b and CD11c normally in response to TNF. Wild-type and double-mutant PMNs treated without or with TNF (20 ng/ml for 30 min at 37°C) were stained with biotin-labeled anti-CD11b (A) or anti-CD11c (B) followed by streptavidin-phycoerythrin to detect antibody binding and analysis by FACS. Profiles to the left show binding of streptavidin-PE alone. In the experiment reported, (A) mean fluorescence index (MFI) of wild-type without TNF was 1,484 and with TNF was 3,288; *hck*^{-/-}*fgr*^{-/-} without TNF was 1,139 and with TNF was 2,044; (B) MFI of wild-type without TNF was 316 and with TNF was 562; *hck*^{-/-}*fgr*^{-/-} without TNF was 395 and with TNF was 651. In another experiment, TNF increased expression of CD11b 1.7- and 2.9-fold in wild-type and *hck*^{-/-}*fgr*^{-/-} PMNs, respectively, while expression of CD11c was increased 3.4- and 2.5-fold in wild-type and *hck*^{-/-}*fgr*^{-/-} cells, respectively.

tear pipette, diluted into fresh Ca²⁺/Mg²⁺-free HBSS + 0.1% BSA, washed twice, and resuspended in HBSS containing Ca²⁺/Mg²⁺ (GIBCO BRL). Total cell number was quantitated using a hemacytometer. FACS analysis (Becton Dickinson & Co., Mountain View, CA) of cell preparations revealed high expression of the murine granulocyte marker Gr-1 (Pharmingen, San Diego, CA) on >95% of the cells isolated from the 62–81% interface and successively less expression on less dense cells, indicating that these were less differentiated PMN precursors (Hestdal et al., 1991). Lymphocytes were found at the 45–50% interface.

For FACS analysis ~10⁶ bone marrow cells or PMNs were stained with mAbs listed in Table I for 60 min at 4°C, washed once, stained with secondary reagents (FITC- or PE-conjugated streptavidin or goat anti-rat; Boehringer Mannheim Biochemicals, Indianapolis, IN), washed twice, and resuspended in 1 ml PBS/0.2% FCS + 1 μg/ml propidium iodide. Viable cells were analyzed with FACScan (Becton Dickinson).

Preparation of ECM-, Intracellular Adhesion Molecule-1 (ICAM-1)¹- and mAb-coated Microtiter Plates and Coverslips

Immulon 4 (Dynatech Laboratories, Chantilly, VA) flat-bottom polystyrene microtiter plates were used for adhesion of ECM proteins, recombinant murine (rm) ICAM-1, and mAbs. ECM proteins were purchased from Sigma Immunochemicals (St. Louis, MO) or Collaborative Biomed-

1. *Abbreviations used in this paper:* ICAM-1, intracellular adhesion molecule-1; MAPK, mitogen-activated protein kinase; rmICAM-1; recombinant murine intercellular adhesion molecule; FBS, fetal bovine serum.

with/without addition of anti-CD18. (E) O₂⁻ produced from *hck*^{-/-} or *fgr*^{-/-} single-mutant PMNs, stimulated with TNF, plated in wells coated with fibrinogen, or FBS, with/without addition of anti-CD18. (F) O₂⁻ produced from *hck*^{-/-} or *fgr*^{-/-} single-mutant PMNs, stimulated with FMLP, plated in wells coated with fibronectin, fibrinogen, or FBS. Mean results ± SD of three independent experiments, using bone marrow PMNs pooled from three mice per experiment, are reported.

cal Products (Bedford, MA). 200 μl of fibrinogen, fibronectin, collagen, and KLH (200 μg/ml) were added to each well, and plates were incubated overnight at 4°C or for 1–2 h at 37°C. Protein samples were removed and wells were washed twice with PBS. For coating with rmICAM-1 (kindly provided by Dr. F. Takei, Terry Fox Laboratory, Vancouver, Canada), 50 μl of rmICAM-1 in 0.1 M sodium bicarbonate buffer, pH8.0, were added to each well, and plates were incubated as above. Unbound rmICAM-1 was removed, plates were blocked with 200 μl of 20% FBS in PBS for 1 h at 37°C, and then washed twice with PBS.

Antibodies used in this study are shown in Table I. The FD441.8 anti-CD11a mAb was provided by Dr. F. Takei (Terry Fox Laboratory), and the Ha2/11 anti-CD29 mAb was from C. Damsky (University of California, San Francisco). Antibodies were either purchased with biotin conjugation or were biotinylated using a commercial kit (Pierce Chemical Co., Rockford, IL). Solutions were made in PBS. For coating microtiter wells with mAbs, plates were incubated with 100 μl of streptavidin (25 μg/ml) for 1 h at 37°C, washed, then incubated with biotinylated mAbs (10 μg/ml) for 37°C for 1 h, washed, then blocked with 20% FBS + 25 μg/ml protein A (Sigma) for 1–2 h at 37°C for 1 h, and washed. Plates were not stored. ELISA assays were used to determine maximal concentrations for binding of each mAb, and no significant difference was found using 10, 20, or 50 μg/ml mAbs; however, higher concentrations of streptavidin paradoxically reduced the amount of bound mAb. The final blocking step using 20% FBS + 25 μg/ml of protein A was required to reduce O₂⁻ release elicited by irrelevant mAbs, most likely caused by the interaction of PMNs with polystyrene plastic, as previously reported with human PMNs (Berton et al., 1992; Nathan, 1987).

For photomicroscopy, plastic Thermanox coverslips (Nunc, Roskilde, Denmark) were coated with fibrinogen or antiintegrin mAbs as described above. Coverslips were placed in 24-well plates, then ~10⁵ bone marrow PMNs were plated in the wells, and cells were incubated for 30 min at 37°C in 95% air/5% CO₂ and then fixed by replacing the media with 4% paraformaldehyde (in PBS) for 30 min at room temperature. Coverslips were washed with PBS and water, then mounted in glycerol and photographed under phase contrast on an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY).

Measurement of O₂⁻ Release

96-well microtiter plates, coated with the appropriate protein or mAb, were filled with 50 μl of 200 mM ferricytochrome C (Sigma C-7752) in HBSS + 2 mM NaN₃ and warmed to 37°C in an automated microtiter plate reader (vMax, attached to Macintosh IIsi running Softmax[®] software; Molecular Devices, Sunnyvale, CA). 50 μl of PMN suspension in HBSS (5 × 10⁵ cells), with/without additional stimuli, was added to each well, and plates were immediately inserted into the plate reader. Absorbance at 550 and 490 nm (or 468 nm for Fig. 1) were recorded every 10 min, and differences were used to calculate nanomoles of O₂⁻ produced (see Berton et al., 1992). In some experiments, 30 U/well of superoxide dismutase (Sigma) was added to confirm that reduction of cytochrome C was caused by O₂⁻ production. rmTNF-α (Genzyme, Cambridge, MA), FMLP (Sigma), and PMA (Sigma) were used as stimulants. Immune complexes were prepared as described (Dusi et al., 1994). All time points were performed in triplicate.

Neutrophil Adhesion Assays

200 μl of bone marrow PMNs suspended in HBSS (at 2 × 10⁶/ml) were plated into microtiter wells precoated with fibrinogen and incubated at 37°C in 95% air/5% CO₂. At varying times of incubation, 100 μl of media was removed from the top of the wells and the plates were centrifuged upside down on 3MM paper (Whatman Chemical Co., Clifton, NJ) in a swinging bucket rotor for 5 min at 60 g. Plates were then washed once with 100 μl of prewarmed (37°C) PBS and centrifuged again as described above. The percentage of PMNs remaining adherent to the bottom of the wells was measured by assaying membrane enzyme acid phosphatase exactly as described (Bellavite et al., 1992). Enzyme activity was assayed in parallel on 200 μl of the original cell suspension and was taken as the reference value of 100% to calculate the percentage of adhesion. Assays were performed in triplicate.

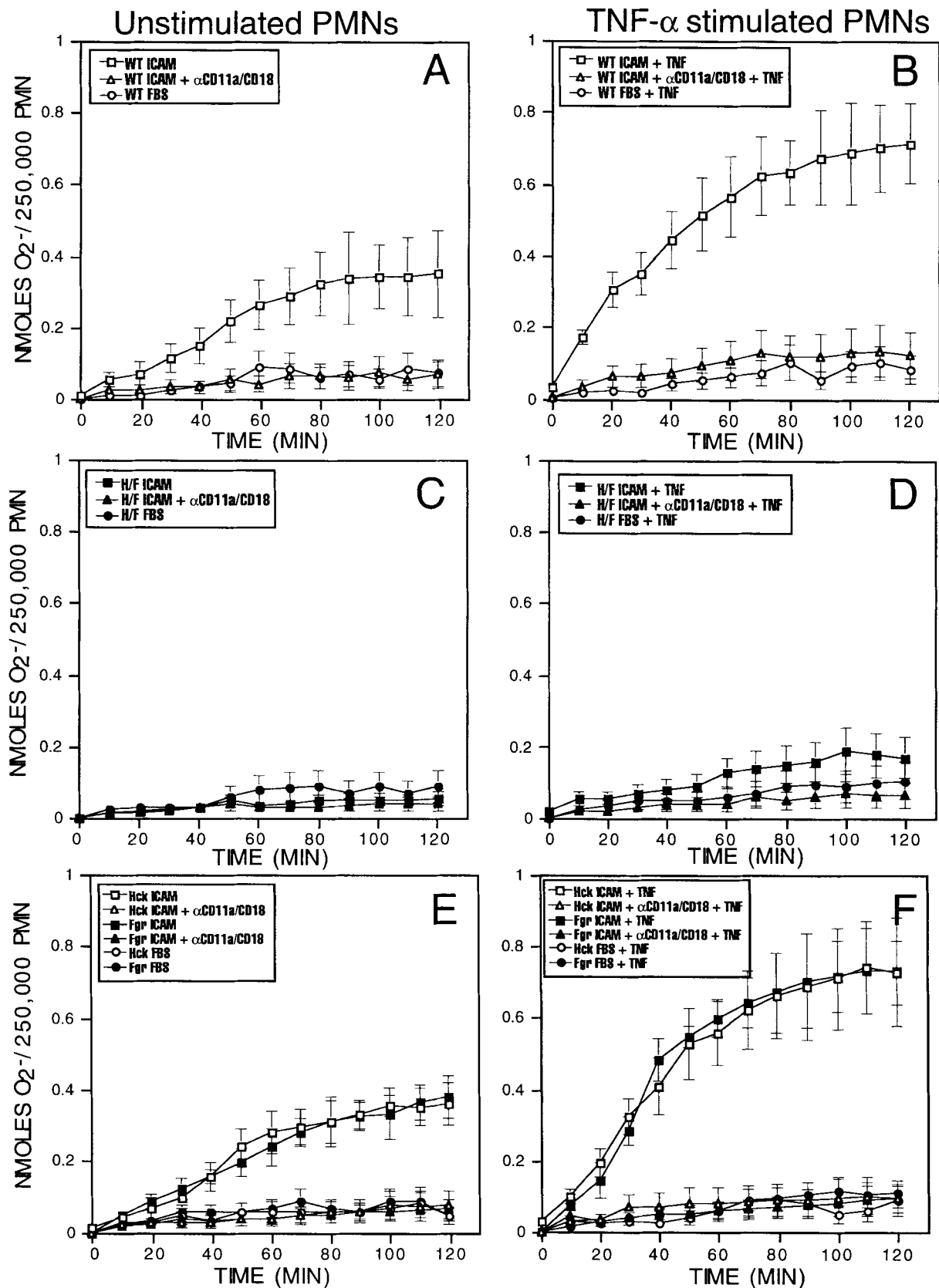


Figure 4. *hck*^{-/-}*fgr*^{-/-} double-mutant PMNs are defective in adhesion-dependent O_2^- production elicited by plating on murine ICAM-1-coated surfaces, while *hck*^{-/-} and *fgr*^{-/-} single mutants are not. (A) O_2^- produced from wild-type PMNs plated in wells coated with murine ICAM-1 or fetal bovine serum (FBS). To inhibit ICAM-1 binding, cells were preincubated for 5 min with anti-CD11a and -CD18 mAbs at 5 μ g/ml each. (B) Same as A, with addition of TNF stimulus (20 ng/ml). (C) O_2^- produced from *hck*^{-/-}*fgr*^{-/-} double-mutant PMNs plated in wells coated with murine ICAM-1 or FBS, with/without addition of anti-CD11a/CD18 mAbs. (D) Same as C, with addi-

Results

hck^{-/-}*fgr*^{-/-} Neutrophils Are Defective in Adhesion-dependent Superoxide Production

To determine if murine neutrophils demonstrated adhesion-dependent O₂⁻ production in response to murine TNF, as seen in human cells, bone marrow PMNs were prepared from wild-type mice and plated on microtiter wells precoated with mouse serum, KLH, or ECM proteins in the presence or absence of TNF. As shown in Fig. 1 (A and B), O₂⁻ production was observed in TNF-stimulated wild-type murine PMNs adherent to rat tail collagen type I, human fibrinogen, or human fibronectin, but not to serum- or KLH-coated plates. Superoxide production occurred with the characteristic lag period of 20–30 min described for human PMNs (Nathan, 1987) and was completely dependent on TNF stimulation. No differences were observed if plates were precoated with murine vs human fibrinogen (not shown).

In contrast, bone marrow PMNs isolated from *hck*^{-/-}*fgr*^{-/-} double-mutant mice failed to produce O₂⁻ when plated on collagen-, fibrinogen- or fibronectin-coated surfaces in the presence of TNF (Fig. 1 C). To confirm that the double-mutant PMNs had the capability to undergo respiratory burst, cells were plated on ECM-coated wells in the presence of PMA. Both wild-type and double-mutant PMNs produced abundant amounts of O₂⁻ in response to PMA (Fig. 1 D). As described for human PMNs (Fuortes et al., 1994), PMA-stimulated O₂⁻ production from murine PMNs is not dependent on adhesion to ECM proteins, since both wild-type and mutant cells released large amounts of O₂⁻ when plated on serum- or BSA-coated plates (not shown). The above data demonstrate that TNF-primed *hck*^{-/-}*fgr*^{-/-} PMNs are defective in integrin-mediated stimulation of respiratory burst.

Immune complex phagocytosis is also able to stimulate production of toxic oxygen metabolites by PMNs. Immune complex-stimulated respiratory burst mediated by cross-linking of the FcγRIIIa receptor is not dependent on adhesion to ECM proteins (Zhou and Brown, 1994). We investigated whether stimulation of PMNs from wild-type and *hck*^{-/-}*fgr*^{-/-} mutant mice via Fc receptors was able to stimulate O₂⁻ release by plating cells in either ECM-coated or uncoated wells in the presence of immune complexes. As shown in Fig. 1 (E and F), both wild-type and *hck*^{-/-}*fgr*^{-/-} mutant PMNs released O₂⁻ in the presence of immune complexes, confirming that signaling through Fc receptors is intact in these mice. Interestingly, plating of wild-type PMNs on noncoated (plastic) wells induced moderate O₂⁻ production, while *hck*^{-/-}*fgr*^{-/-} PMNs failed to respond to plastic surfaces (Fig. 1 F). This confirms observations with human PMNs that plain polystyrene is a potent activator of PMN respiratory burst presumably through cross-linking of surface integrins.

To confirm that the defective O₂⁻ production observed in *hck*^{-/-}*fgr*^{-/-} mutants was not caused by impaired TNF

responses, PMNs adherent to ECM proteins were stimulated with FMLP. As observed with human PMNs (de la Harpe and Nathan, 1989; Shappell et al., 1990), wild-type murine PMNs released large amounts of O₂⁻ when adherent to fibronectin or fibrinogen in the presence of FMLP (Fig. 2 A). However, *hck*^{-/-}*fgr*^{-/-} plated on either fibronectin or fibrinogen failed to produce O₂⁻ in the presence of FMLP (Fig. 2 B). To further examine TNF responses, we examined upregulation of surface integrin expression in PMNs after TNF treatment. Both wild-type and *hck*^{-/-}*fgr*^{-/-} PMNs showed an approximately twofold increase in CD11b and CD11c expression, assayed by FACS, within 30 min of TNF stimulation (Fig. 3), approximating the response seen with human PMNs (Naccache et al., 1994). FACS analysis of surface integrin α-chain (CD11a, CD11b, and CD11c) or β chain (CD18, CD29, and CD61) expression levels showed no difference between wild-type and *hck*^{-/-}*fgr*^{-/-} PMNs (not shown). Therefore, the defective responses in *hck*^{-/-}*fgr*^{-/-} PMNs were most likely caused by impaired signals generated from integrin-ECM protein interactions rather than from impaired TNF responses or integrin expression.

Adhesion-dependent Superoxide Production on Fibrinogen-coated Surfaces Is Mediated by β₂ Integrins

To demonstrate that TNF-stimulated O₂⁻ production in PMNs adhering to fibrinogen-coated surfaces was dependent on binding by β₂ integrins, wild-type PMNs were plated on serum- or fibrinogen-coated plates, in the presence of TNF, with and without previous incubation with anti-CD18 mAb. As demonstrated with human PMNs (Nathan et al., 1989; Richter et al., 1990), blocking of β₂-integrin binding inhibited O₂⁻ production of TNF-stimulated wild-type PMNs plated on fibrinogen-coated wells (Fig. 2 C). *hck*^{-/-}*fgr*^{-/-} PMNs failed to respond, irrespective of previous incubation with anti-CD18 (Fig. 2 D). Preincubation of PMNs with either anti-CD29 or -CD61 partially reduced the production of O₂⁻ by TNF-stimulated wild-type PMNs adherent to fibronectin-coated wells (not shown), demonstrating the role of β₁- and β₃-integrin binding to this ECM protein. *hck*^{-/-}*fgr*^{-/-} PMNs were unaffected by anti-CD29 treatment.

Fibrinogen-adherent *hck*^{-/-} and *fgr*^{-/-} Single-mutant PMNs Produce Normal Levels of Superoxide in Response to TNF and FMLP

To determine if *hck*^{-/-} and/or *fgr*^{-/-} single-mutant PMNs manifested the same phenotype as the *hck*^{-/-}*fgr*^{-/-} double-mutant cells, bone marrow PMNs were isolated from single-mutant animals and plated on fibrinogen-coated wells, in the presence or absence of TNF, with and without previous incubation with anti-CD18 mAb. Like wild-type cells, both *hck*^{-/-} and *fgr*^{-/-} single-mutant PMNs produced abundant amounts of O₂⁻ in the presence of TNF, which was inhibited by previous incubation with anti-CD18 mAb

tion of TNF stimulus. (E) O₂⁻ produced from *hck*^{-/-} or *fgr*^{-/-} single-mutant PMNs plated in wells coated with murine ICAM-1 or FBS, with/without addition of anti-CD11a/CD18 mAbs. (F) Same as E, with addition of TNF stimulus. Mean results ± SD of three independent experiments, using bone marrow PMNs pooled from three mice per experiment, are reported.

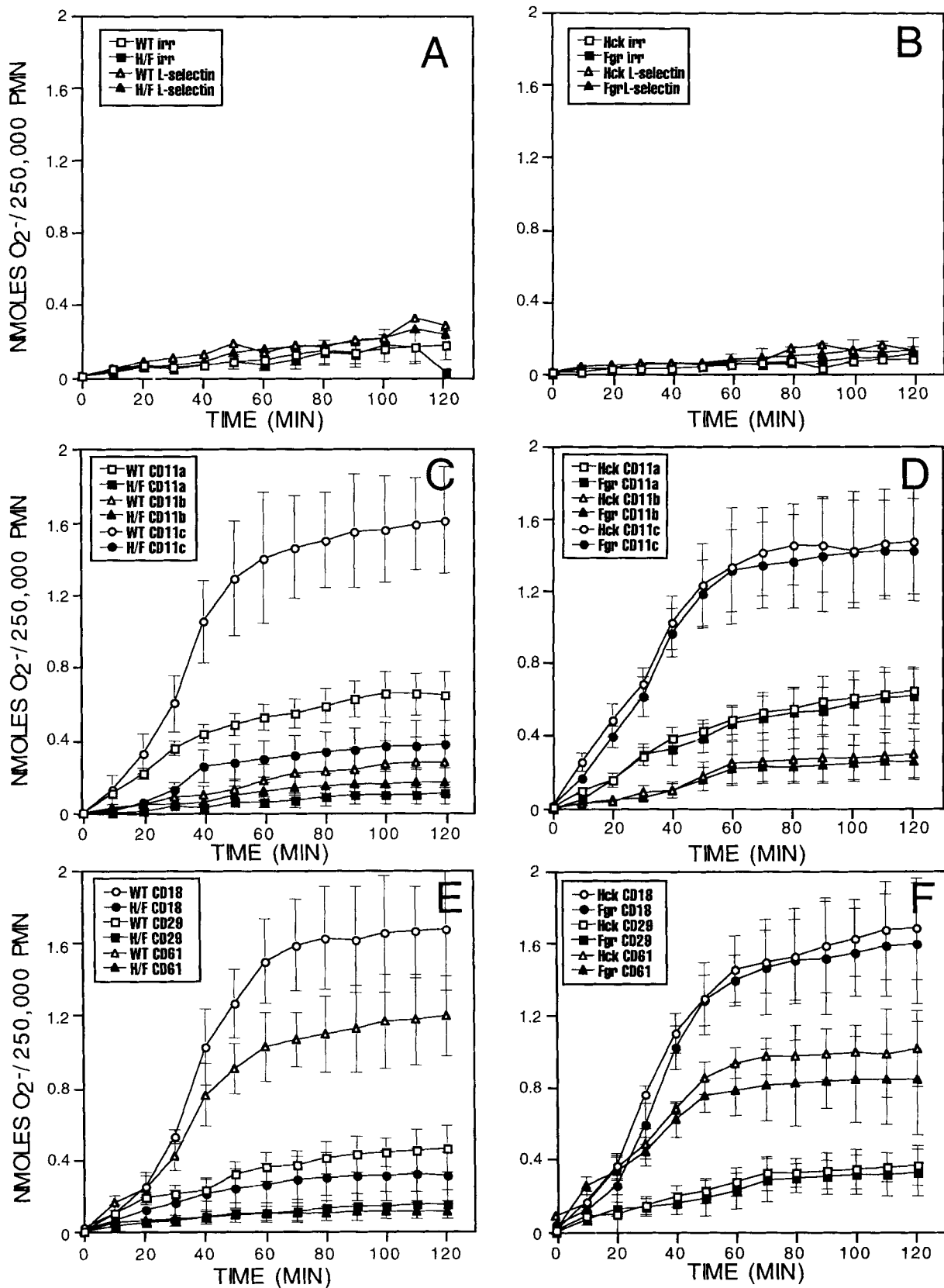


Figure 5. *hck*^{-/-}*fgr*^{-/-} double-mutant PMNs are defective in O₂⁻ production elicited by plating on antiintegrin mAb-coated surfaces, while *hck*^{-/-} and *fgr*^{-/-} single mutants are not. (A) O₂⁻ produced from wild-type or *hck*^{-/-}*fgr*^{-/-} PMNs plated in wells coated with an irrelevant mAb or anti-L-selectin mAb. mAbs were bound to streptavidin-coated polystyrene plates as described in Materials and Methods. Source of mAbs is shown in Table 1. (B) Same as A, using PMNs from either *hck*^{-/-} or *fgr*^{-/-} single-mutant mice. (C) O₂⁻ produced from wild-type or *hck*^{-/-}*fgr*^{-/-} PMNs plated in wells coated with mAbs to α chains of leukocyte integrins: anti-CD11a, -CD11b, and

(Fig. 2 E). Additionally, *hck*^{-/-} and *fgr*^{-/-} single-mutant PMNs adherent to fibronectin or fibrinogen produced O₂⁻ in response to FMLP stimulation at levels equivalent to wild-type cells (Fig. 2 F). These results demonstrate that defective expression of both p59/61^{hck} and p58^{c-fgr} are required to abrogate the O₂⁻ response in adhering PMNs.

Adhesion to ICAM-1-coated Surfaces Stimulates Superoxide Production in Wild-type and Single-mutant PMNs, but Not in *hck*^{-/-}*fgr*^{-/-} Double Mutants

Adhesion of human PMNs to endothelial cells induces cell spreading and respiratory burst (Nathan, 1987; Shappell et al., 1990). Since the major counterreceptor for PMN integrins α_Lβ₂ (LFA-1; CD11a/CD18) and α_mβ₂ (CR3; CD11b/CD18) expressed by endothelial cells is ICAM-1, we addressed whether PMN interaction with ICAM-1 induces respiratory burst. Soluble rICAM-1 was used to coat microtiter wells and bone marrow PMNs from wild-type, *hck*^{-/-}, *fgr*^{-/-}, or *hck*^{-/-}*fgr*^{-/-} mice were plated in the absence or presence of TNF, with and without previous incubation with anti-CD11a and -CD18 mAbs. Wild-type PMNs produced modest amounts of O₂⁻ when plated on rICAM-1 in the absence of TNF, and significantly more O₂⁻ in the presence of TNF (Fig. 4, A and B). This response was completely inhibited by previous incubation with both anti-CD11a and -CD18 mAbs (Fig. 4 B; only partial inhibition of O₂⁻ production was seen with anti-CD11a alone as previously reported [Shappell, et al., 1990]). Both resting and TNF-stimulated *hck*^{-/-}*fgr*^{-/-} PMNs failed to produce significant amounts of O₂⁻ when plated on rICAM-1 (Fig. 4, C and D). Single-mutant PMNs, however, responded normally (Fig. 4, E and F). These results demonstrate that adhesion to surface-bound ICAM-1 induces respiratory burst in wild-type, *hck*^{-/-}, and *fgr*^{-/-} PMNs, but not in double-mutant *hck*^{-/-}*fgr*^{-/-} cells.

Adhesion to Surface-bound mAbs against the α Chains (CD11a and CD11c), β₂ Chain (CD18), and β₃ Chain (CD61) of Leukocyte Integrins Triggers Superoxide Release in Wild-type and Single-mutant PMNs, but not Double-mutant Cells

To directly test whether impairments in signaling through leukocyte integrins explains the defective induction of respiratory burst in adherent *hck*^{-/-}*fgr*^{-/-} PMNs, bone marrow neutrophils from wild-type, single- and double-mutant animals were plated in microtiter wells coated with various mAbs. Streptavidin-coated microtiter wells were treated with biotinylated mAbs, then the plates were blocked with FBS and soluble protein A to inhibit binding of antibodies to cellular Fc receptors (see Materials and Methods and Table I). Plating of PMNs on irrelevant mAb or anti-L-selectin mAb (CD62L)-coated surfaces did not trigger O₂⁻ release (Fig. 5, A and B; FACS analysis indicated equivalent high expression of L-selectin on wild-type and mutant PMNs, not shown). Wild-type, *hck*^{-/-} and *fgr*^{-/-} PMNs

produced large amounts of O₂⁻ when plated on anti-CD11c mAb-coated wells, and less on anti-CD11a coated wells, while *hck*^{-/-}*fgr*^{-/-} double-mutant cells failed to respond (Fig. 5, C and D). Wild-type and single-mutant PMNs responded poorly to surface-bound anti-CD11b, as described with human PMNs (Berton et al., 1992). Stimulation with TNF resulted in a minor enhancement of O₂⁻ production from PMNs plated on anti-CD11a mAb- and anti-CD11b mAb-coated surfaces but did not alter O₂⁻ production from cells plated on other mAbs (data not shown). Using mAbs directed to the β chains of leukocyte integrins, we observed abundant stimulation of respiratory burst in wild-type and single-mutant cells plated on anti-CD18 (β₂ chain)- or anti-CD61 (β₃ chain)-coated wells, and weak stimulation of O₂⁻ release on anti-CD29 (β₁ chain) wells (Fig. 5, E and F). As seen with the α chain mAbs, cross-linking of the β chains failed to induce O₂⁻ release in *hck*^{-/-}*fgr*^{-/-} double-mutant cells. These results demonstrate that either kinase was required to generate intracellular signals produced from a variety of leukocyte integrins.

***hck*^{-/-}*fgr*^{-/-} PMNs Fail to Spread on Fibrinogen-coated or Antiintegrin mAb-Coated Surfaces**

To determine if the defective production of O₂⁻ by *hck*^{-/-}*fgr*^{-/-} PMNs was a result of impaired cell spreading, purified bone marrow PMNs were plated on tissue culture plastic coverslips coated with either serum, fibrinogen, or antiintegrin mAbs, then processed for photomicroscopy. As shown in Fig. 6, TNF-stimulated wild-type PMNs do not spread on serum-coated coverslips and spread moderately on fibrinogen (Fig. 6, A and C), while *hck*^{-/-}*fgr*^{-/-} PMNs fail to spread on either surface (Fig. 6, B and D). Both cell types spread extensively after treatment with PMA (Fig. 6, E and F). No spreading on fibrinogen was observed in the absence of TNF (not shown).

More complete spreading of wild-type PMNs was observed on coverslips coated with antiintegrin mAbs. As shown in Fig. 7, wild-type PMNs remain rounded after settling on irrelevant mAb-coated coverslips (Fig. 7 A), but spread well over surfaces coated with anti-CD11c (Fig. 7 C) and anti-CD18 (Fig. 7 E). Similar results were seen with anti-CD61-coated coverslips (not shown). In contrast, cross-linking of these integrins on *hck*^{-/-}*fgr*^{-/-} PMNs failed to trigger cell spreading (Fig. 7, D and F). We conclude, therefore, that the defective production of O₂⁻ in adherent *hck*^{-/-}*fgr*^{-/-} was caused by a failure of integrin-mediated signaling of cell spreading.

TNF-stimulated *hck*^{-/-}*fgr*^{-/-} PMNs Are Defective in Tight Adhesion to Fibrinogen-coated Surfaces

As a more quantitative assessment of cell spreading, we conducted static adhesion assays of wild-type, *hck*^{-/-}, *fgr*^{-/-} and *hck*^{-/-}*fgr*^{-/-} PMNs on fibrinogen-coated microtiter

-CD11c. (D) Same as C, using PMNs from either *hck*^{-/-} or *fgr*^{-/-} single-mutant mice. (E) O₂⁻ produced from wild-type or *hck*^{-/-}*fgr*^{-/-} PMNs plated in wells coated with mAbs to β chains of leukocyte integrins: anti-CD18, -CD29, and -CD61. (F) Same as E, using PMNs from either *hck*^{-/-} or *fgr*^{-/-} single-mutant mice. Mean results ± SD of three independent experiments, using bone marrow PMNs pooled from three mice per experiment, are reported.

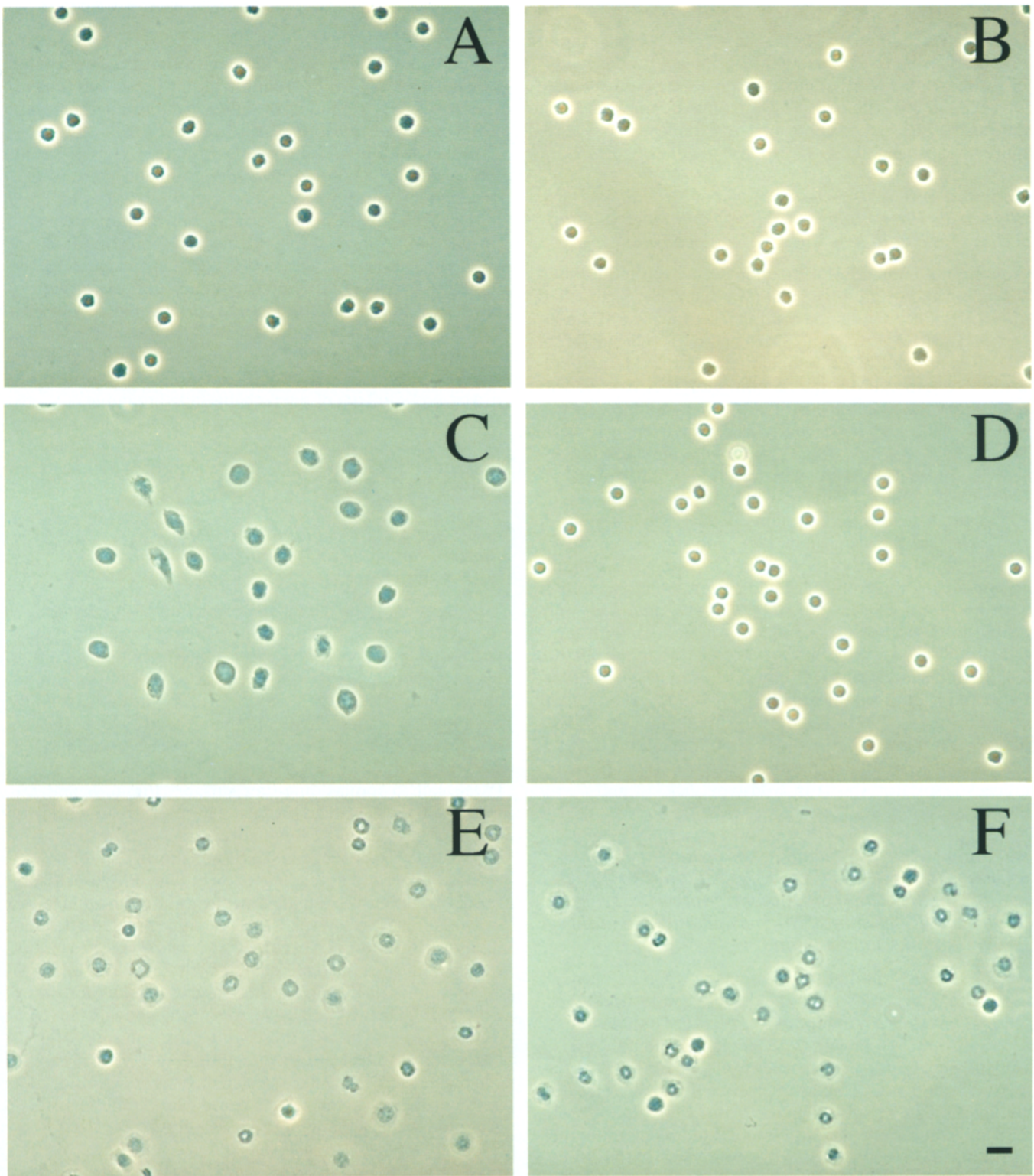


Figure 6. *hck*^{-/-}*fgr*^{-/-} mutant PMNs are defective in spreading on fibrinogen-coated surfaces. Plastic coverslips were coated with human fibrinogen at 100 μ g/ml or FBS and used to bind wild-type or *hck*^{-/-}*fgr*^{-/-} PMNs stimulated with either TNF (20 ng/ml) or PMA (2.5 ng/ml). After a 30-min incubation at 37°C, coverslips were prepared for photomicroscopy as described in Materials and Methods. (A) Wild-type PMNs stimulated with TNF plated on FBS-coated coverslips. (B) *hck*^{-/-}*fgr*^{-/-} PMNs stimulated with TNF plated on FBS-coated coverslips. (C) Wild-type PMNs stimulated with TNF plated on fibrinogen-coated coverslips. (D) *hck*^{-/-}*fgr*^{-/-} PMNs stimulated with TNF plated on fibrinogen-coated coverslips. (E) Wild-type PMNs stimulated with PMA plated on fibrinogen-coated coverslips. (F) *hck*^{-/-}*fgr*^{-/-} PMNs stimulated with PMA plated on fibrinogen-coated coverslips. Bar, 10 μ m.

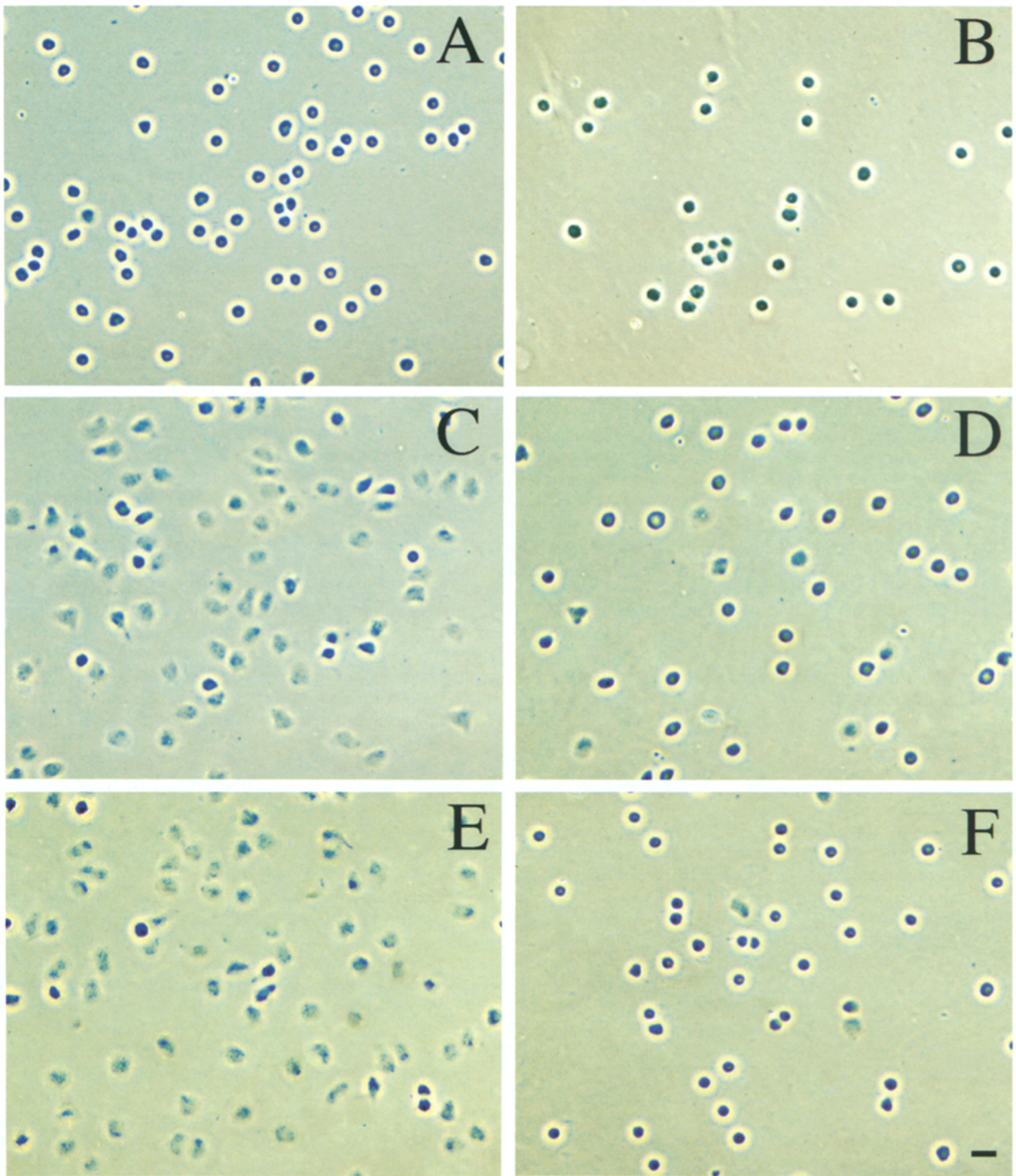


Figure 7. mAbs to CD11c and CD18 fail to trigger spreading of *hck*^{-/-}*fgr*^{-/-} PMNs. Plastic coverslips were coated with streptavidin and used to bind biotinylated irrelevant mAb, anti-CD11c, and anti-CD18. Wild-type and *hck*^{-/-}*fgr*^{-/-} mutant PMNs were plated on the coverslips, incubated at 37°C for 30 min, and coverslips were processed for photomicroscopy as described in Materials and Methods. (A) Wild-type PMNs plated on irrelevant mAb. (B) *hck*^{-/-}*fgr*^{-/-} PMNs plated on irrelevant mAb. (C) Wild-type PMNs plated on anti-CD11c. (D) *hck*^{-/-}*fgr*^{-/-} PMNs plated on anti-CD11c. (E) Wild-type PMNs plated on anti-CD18. (F) *hck*^{-/-}*fgr*^{-/-} PMNs plated on anti-CD18. Bar, 10 μ m.

Table 1. mAbs Used in This Study

mAb	Subclass	Specificity	References/sources
R35-95	Rat IgG2a	Irrelevant control	Pharmingen
RB6-8C5	Rat IgG2b	Gr-1/myeloid differentiation antigen	Pharmingen
MEL-14	Rat IgG2a	L-selectin	Endogen
M17/4	Rat IgG2a	CD11a	Pharmingen TIB 213 (American Type Culture Collection, Rockville, MD)
FD441.8	Rat IgG2b	CD11a	(Sanchez-Madrid et al., 1983)
M1/70	Rat IgG2b	CD11b	Pharmingen
N418	Hamster IgG1	CD11c	Endogen
M18/2	Rat IgG2a	CD18	Pharmingen
2E6	Hamster IgG1	CD18	Endogen
Ha2/11	Hamster IgG1	CD29	(Stephens et al., 1995)
2C9.G2	Hamster IgG1	CD61	Pharmingen

wells, using unstimulated cells or after treatment with TNF or PMA. In this assay, cells were plated on fibrinogen-coated microtiter wells, allowed to adhere tightly, then non-bound cells were centrifuged off the dish and the remaining cells were quantitated (see Materials and Methods). As shown in Fig. 8, ~15% of wild-type PMNs adhere tightly to the fibrinogen in the presence of TNF, while <5% of *hck*^{-/-}*fgr*^{-/-} remain bound (Fig. 8A). In contrast, pretreatment of PMNs with PMA stimulated tight adhesion of 45% of both wild-type and double-mutant cells (Fig. 8B). As observed with superoxide production, single-mutant *hck*^{-/-} and *fgr*^{-/-} PMNs behaved the same as wild-type PMNs, demonstrating ~15% tight adhesion to fibrinogen-coated plates (Fig. 8C).

Discussion

The integrin family of leukocyte adhesion molecules are responsible, not only for cell-cell and cell-ECM adhesion events, but also for transmitting signals leading to specific functional responses. In this work, we demonstrate that adhesion-dependent stimulation of PMN respiratory burst is dependent on the expression of the Src family kinases p59/61^{hck} and p58^{c-fgr}. In the absence of these kinases, PMNs fail to produce O₂⁻, despite treatment with TNF or FMLP, when plated on ECM protein-, ICAM-1-, or anti-integrin mAb-coated surfaces. The defective adhesion-dependent respiratory burst is caused by the inability of the *hck*^{-/-}*fgr*^{-/-} PMNs to spread appropriately over these surfaces. In contrast, single-mutant *hck*^{-/-} or *fgr*^{-/-} PMNs respond normally, indicating that these kinases serve a partially overlapping function in transducing integrin signals.

TNF-, FMLP-, or cytokine-stimulated human PMNs undergo sustained respiratory burst when adherent to fibrinogen-, collagen-, fibronectin-, vitronectin-, or laminin-coated surfaces (Nathan et al., 1989). Binding to fibrinogen and laminin was demonstrated to be mediated by β₂ integrins by blocking with soluble anti-CD18 mAb. In contrast, activation on fibronectin-coated surfaces is likely caused by binding via β₃ or β₁ integrins, since it is not inhibitable with anti-CD18 mAb. Murine PMNs respond in an equivalent fashion: in the presence of TNF or FMLP, wild-type PMNs produced abundant amounts of O₂⁻ when plated on surfaces coated by ECM proteins that are recognized by either β₂ integrins (fibrinogen) or β₁/β₃ integrins

(fibronectin and collagen, Fig. 1). Moreover, respiratory burst was inhibited in murine PMNs plated on fibrinogen by pretreatment with soluble anti-CD18 mAb (Fig. 2), and was not observed on surfaces coated with FBS or KLH, as previously reported with human cells (Dri et al., 1991). Murine PMNs, like human PMNs, can be directly activated to undergo a respiratory burst when plated on anti-integrin mAb-coated surfaces, independent of TNF treatment (Fig. 5). Hence, the role of TNF or FMLP appears to be the activation of high affinity binding capacity by the integrin receptors for ECM proteins (inside-out signaling), rather than direct involvement in integrin intracellular signaling pathways (outside-in signaling, see Hynes, 1992). Therefore, we found no difference in the integrin-dependent activation of neutrophil functions between murine and human cells.

The adhesion-mediated activation of neutrophil functions in TNF-primed cells is dependent on tyrosine phosphorylation responses elicited after the cross-linking of surface β₂- or β₃-related integrins (Berton et al., 1994; Fuortes et al., 1993; Laudanna et al., 1993a; Zhou and Brown, 1993). These signals initiate a pathway leading to cytoskeletal rearrangement, cell spreading, assembly of the subunits of the NADPH oxidase complex at the plasma membrane, and production of O₂⁻ (Fuortes et al., 1993; Yan et al., 1995). The identity of the tyrosine kinases responsible for mediating integrin signaling in leukocytes and other cell types has been suggested by a variety of biochemical studies. In neutrophils, the Src family kinases p58^{c-fgr} and p53/56^{lyn} are activated and translocated to a Triton X-100-insoluble fraction after plating of TNF-treated PMNs on fibrinogen-coated surfaces (Berton et al., 1994; Yan et al., 1995). The other major src family kinase present in PMNs, p59/61^{hck}, is also activated by β₂-integrin cross-linking (Fumagalli, L., unpublished observation). In platelets and fibroblasts, pp125^{FAK} and pp60^{c-src} have been implicated as the major tyrosine kinases mediating signals through β₃ or β₁ integrins that result in cytoskeletal rearrangements (Burrige et al., 1992; Clark et al., 1994a; Lipfert et al., 1992; Schlaepfer et al., 1994). The role pp125^{FAK} in signaling through leukocyte integrins in PMNs is unclear, however, since neutrophils express very low amounts of pp125^{FAK} and there is no apparent activation of pp125^{FAK} kinase activity after cross-linking of β₂ integrins (Fuortes et al., 1994). In human monocytes, where

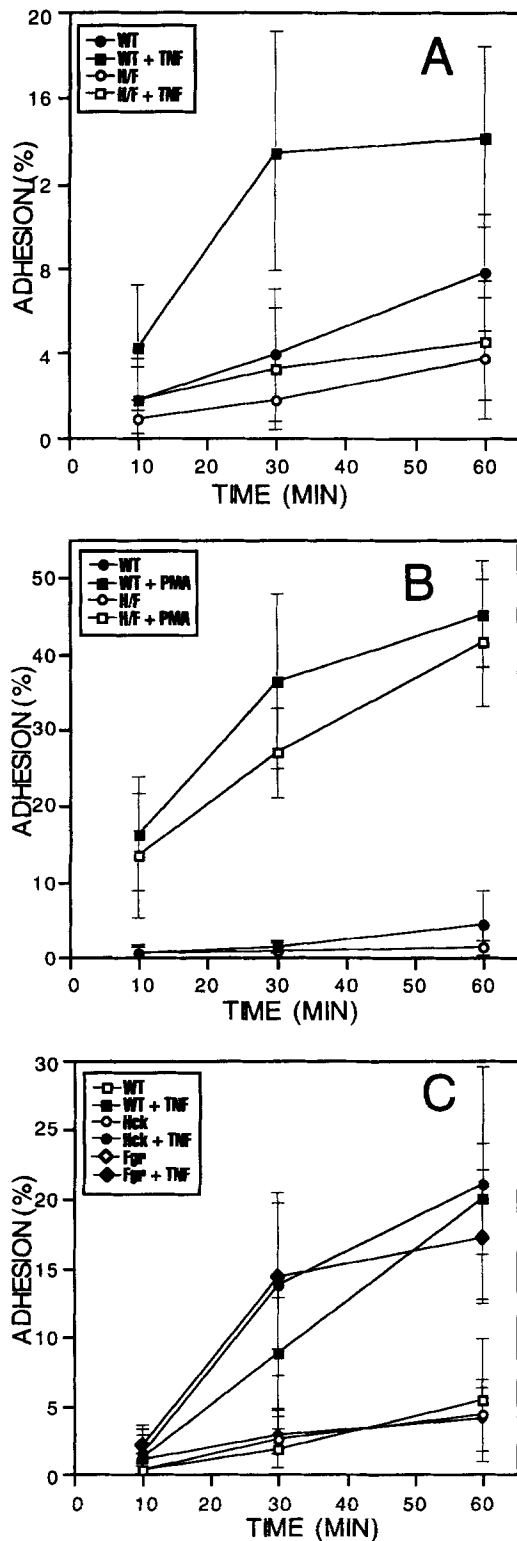


Figure 8. *hck*^{-/-}*fgr*^{-/-} mutant PMNs are defective in TNF-stimulated adhesion to fibrinogen-coated surfaces, while *hck*^{-/-} and *fgr*^{-/-} single mutants are not. The time course of neutrophil adhesion to fibrinogen-coated microtiter wells was assayed as described in Materials and Methods. (A) Wild-type or *hck*^{-/-}*fgr*^{-/-} mutant PMNs were plated in the absence or presence of TNF (20 ng/ml). (B) Same as A, except that PMNs were plated in the absence or presence of PMA (2.5 ng/ml). (C) Wild-type, *hck*^{-/-} or *fgr*^{-/-} PMNs were plated in absence or presence of TNF. Results of 10 (A), 7 (B), and 5 (C) independent experiments \pm SD are reported.

pp125^{FAK} is not expressed at all, p72^{syk} has been found to be a major integrin-responsive tyrosine kinase (Lin et al., 1995). p72^{syk} is also activated in platelets after cross-linking of the α IIb/ β ₃ by fibrinogen (Clark et al., 1994b). Therefore, a number of different tyrosine kinases have been suggested to be responsible for integrin signaling.

A major problem with the studies cited above, however, has been the inability to demonstrate that any individual kinase is essential for signaling through a given cell surface integrin; rather, kinase activation may only accompany signaling through more critical pathways. The fact that multiple kinases are activated after integrin cross-linking, e.g., in PMNs, suggests that more than one kinase may play a functional role. We therefore examined PMNs from *hck*^{-/-}, *fgr*^{-/-}, and *hck*^{-/-}*fgr*^{-/-} double-mutant mice for defects in adhesion-dependent neutrophil functions to assess whether these kinases played a critical role in these integrin-dependent signaling pathways. In contrast to wild-type PMNs, *hck*^{-/-}*fgr*^{-/-} failed to undergo respiratory burst when stimulated with either TNF or FMLP and plated on a fibrinogen-, fibronectin-, or collagen-coated surface (Figs. 1 and 2). The defective superoxide response was not caused by defects in NADPH oxidase activity, since treatment with PMA resulted in robust O₂⁻ release from *hck*^{-/-}*fgr*^{-/-} PMNs. Moreover, initiation of respiratory burst by signals through Fc γ receptors was also normal in *hck*^{-/-}*fgr*^{-/-} mutant PMNs (Fig. 1). This is supported by previous observations that Fc γ receptor mediated-induction of tyrosine phosphorylation and phagocytosis were normal in macrophages and PMNs from *hck*^{-/-}*fgr*^{-/-} mice (Lowell et al., 1994). The defective adhesion-dependent responses of *hck*^{-/-}*fgr*^{-/-} PMNs were not the result of defects in TNF signaling, since TNF induced normal upregulation of surface CD11b and CD11c (Fig. 3).

Cytoskeletal rearrangement and cell spreading are required for respiratory burst in human PMNs (Nathan and Sanchez, 1990). To determine if the impaired adhesion-dependent respiratory burst in *hck*^{-/-}*fgr*^{-/-} PMNs was caused by defective cell spreading, we performed photomicroscopy and cell adhesion assays on TNF-stimulated cells adhering to fibrinogen-coated surfaces. TNF-primed *hck*^{-/-}*fgr*^{-/-} PMNs failed to spread on fibrinogen, resulting in defective tight adhesion, while wild-type cells underwent shape changes and displayed tight adhesion (Figs. 6 and 8). Stimulation of *hck*^{-/-}*fgr*^{-/-} PMNs with PMA produced extensive spreading and adhesion, leading to respiratory burst. Therefore, it is likely that the defective functional responses of TNF-treated *hck*^{-/-}*fgr*^{-/-} PMNs was caused by the inability of these cells to rearrange the actin cytoskeleton after cross-linking of integrins by ECM proteins.

TNF-primed *hck*^{-/-}*fgr*^{-/-} PMNs also failed to respond to surface-bound ICAM-1, the ligand for α _L β ₂ (LFA-1, CD11a/CD18) and α _m β ₂ (CR3, CD11b/CD18) (Marlin and Springer, 1987), while wild-type cells produced moderate amounts of O₂⁻ (Fig. 4). ICAM-1-mediated cross-linking of LFA-1 has been shown to deliver signals in lymphocytes leading to enhanced proliferative responses, better antigen presentation, and increased secretion of IL-2 (Chong et al., 1994; Moy and Brian, 1992; Van Seventer et al., 1991). Purified surface-immobilized ICAM-1 has also been shown to elicit PMN spreading and tight adhesion (Pysznik et al., 1994). Therefore, *hck*^{-/-}*fgr*^{-/-} mice were defective in

neutrophil responses normally elicited by a variety of integrin ligands.

Direct demonstration that cross-linking of integrin receptors leads to intracellular signals resulting in neutrophil responses has been shown using surface-bound mAbs to the various subunits of β_2 - and β_3 -related integrins (Berton et al., 1992; Zhou and Brown, 1993). Similar results have been reported in eosinophils using surface-bound anti- β_1 and anti- β_2 mAbs and in monocytes using anti- β_1 mAbs (Laudanna et al., 1993b; Lin et al., 1994). In each case, cross-linking of the appropriate integrin subunits triggered cell spreading coincident with production of O_2^- (in PMNs and eosinophils) or cytokine expression (monocytes). In human PMNs, the best responses were seen when cross-linking the β_2 integrins $\alpha_L\beta_2$ (CD11a/CD18) and $\alpha_X\beta_2$ (p150/95, CD11c/CD18), using mAbs to the α chains (Berton et al., 1992), or the β_3 -related leukocyte response integrin (Zhou and Brown, 1993). Cross-linking of $\alpha_M\beta_2$ (Mac-1, CD11b/CD18) with an α chain mAb failed to induce O_2^- production (Berton et al., 1992; Zhou and Brown, 1994). Therefore, a direct test of defective integrin signaling in *hck*^{-/-}*fgr*^{-/-} PMNs was obtained by plating cells on anti-integrin mAb surfaces and assessing respiratory burst and cell spreading. As shown in Figs. 5 and 7, surface-bound mAbs against CD11a and CD11c, as well as anti- β_2 and - β_3 mAbs, triggered abundant O_2^- release and extensive cell spreading in wild-type PMNs, but not in *hck*^{-/-}*fgr*^{-/-} cells, despite the fact that mutant PMNs express normal levels of integrin subunits on the cell surface.

In contrast, *hck*^{-/-}*fgr*^{-/-} double-mutant PMNs, *hck*^{-/-}, or *fgr*^{-/-} single-mutant cells displayed no defects in adhesion-dependent respiratory burst (on any of the three types of surfaces used), cell spreading, or tight adhesion. This observation demonstrates that these kinases must serve overlapping roles in integrin-signaling pathways in PMNs. Redundant functions between Src family kinases have been postulated as an explanation for the limited phenotypes of single mutant animals (Lowell et al., 1994; Soriano et al., 1991). More severe phenotypes present in double-mutant animals provides genetic evidence that such redundancy is functionally significant (Lowell et al., 1996; Stein et al., 1994); however, this is the first direct demonstration of overlapping function in a specific signaling pathway within a single cell type. Functional overlap between p59/61^{hck} and p58^{c-fgr} is also indicated by the observation that *hck*^{-/-}*fgr*^{-/-} double mutants are more susceptible to *L. monocytogenes* infection while wild-type and single-mutant mice are resistant (Lowell et al., 1994). It is unclear whether the defective PMNs responses of these mice are responsible for the defective innate immunity to *Listeria*. Immune responses to *Listeria* involve production of IL-12 by infected macrophages causing a Th1 response that leads to production of high levels of IFN- γ resulting in full activation of macrophages to kill the organism (Scott, 1993). It is possible that defective mobilization of PMNs to the site of initial *Listeria* inoculation may hinder the early response to this infection in double-mutant mice. Alternatively, since macrophages express p59/61^{hck} and p58^{c-fgr}, it is possible that defective integrin signaling in these cells may lead to impaired anti-*Listeria* responses. Indeed, initial experiments have indicated defective tyrosine phosphorylation responses in thioglycollate-elicited

peritoneal macrophages from *hck*^{-/-}*fgr*^{-/-} double mutants (Lowell, C., unpublished observation). If the defective PMN responses are physiologically significant, this would predict that *hck*^{-/-}*fgr*^{-/-} mice would manifest a variety of inflammatory defects, in the same fashion as observed for CD18^{-/-} or ICAM-1-deficient animals (Sligh et al., 1993; Xu et al., 1994). These defects would include impaired migration of PMNs to inflammatory sites and impaired margination of PMNs during endotoxic shock; these models are currently being tested.

A second major conclusion of this work is that both p59/61^{hck} and p58^{c-fgr} participate in β_2 - and β_3 -integrin-mediated signaling in PMNs. We found no specificity for one kinase signaling preferentially for one type of integrin. While it is clear that the cytoplasmic domain of the β chain of $\alpha_L\beta_2$ is required for signaling, the molecular organization of the integrin signaling complex is unknown (Hibbs et al., 1991; Peter and O'Toole, 1995). There has been no direct demonstration of association of either p59/61^{hck} or p58^{c-fgr} with cytoplasmic portions of β_2 or β_3 integrins, although the SH3 domain of pp60^{c-src} has been found to associate with paxillin (Weng et al., 1993). This suggests a model by which p59/61^{hck} and p58^{c-fgr} may interact indirectly with integrins within focal adhesion structures in a large multicomponent complex. The domain structures within the kinase that are responsible for this interaction (SH2, SH3, or unique regions) are unknown; however, given the result that defective spreading of *src*^{-/-} fibroblasts can be rescued by transfection with kinase inactive versions of pp60^{c-src} (Kaplan et al., 1995), it is interesting to speculate that p59/61^{hck} or p58^{c-fgr} kinase activity may not be completely required for leukocyte integrin signaling.

Since signaling through both β_2 and β_3 integrins is defective in *hck*^{-/-}*fgr*^{-/-} PMNs, this implies that signaling through different integrins proceeds through the same pathway. This would predict that common downstream substrates, such as MAP kinases, would be activated after either β_2 - or β_3 -integrin cross-linking, and that these substrates would not be activated in *hck*^{-/-}*fgr*^{-/-} PMNs. Indeed, there is no evidence for differential functional responses after cross-linking of β_2 versus β_3 integrins in PMNs. Therefore, the specific functional response of any given cell type to ECM proteins (or counterreceptors) is most likely the result of differential cell-surface integrin expression, rather than different intracellular signals generated by different integrin subunits.

The Hck and Fgr kinases have been implicated in a variety of signaling pathways in PMNs and macrophages, including Fc γ R, LPS, and MHC class II signaling pathways (Boulet et al., 1992; Morio et al., 1994; Zhou et al., 1995). However, functional responses elicited by signaling through these receptors are normal in *hck*^{-/-}*fgr*^{-/-} PMNs and macrophages. Rather, it appears that these kinases serve a physiologically significant role in adhesion-dependent PMN functions. If the defective PMN functions we have observed in vitro result in impaired inflammatory responses in *hck*^{-/-}*fgr*^{-/-} mice, this would indicate that therapeutics targeted at Src family kinases in PMNs could provide novel approaches to control inflammatory diseases.

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