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The Src Family Kinases Hck and Fgr Are Dispensable for Inside-Out, Chemoattractant-Induced Signaling Regulating β_2 Integrin Affinity and Valency in Neutrophils, but Are Required for β_2 Integrin-Mediated Outside-In Signaling Involved in Sustained Adhesion[1]

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Neutrophil β_2 integrins are activated by inside-out signaling regulating integrin affinity and valency; following ligand binding, β_2 integrins trigger outside-in signals regulating cell functions. Addressing inside-out and outside-in signaling in $hck^{-/-}fgr^{-/-}$ neutrophils, we found that Hck and Fgr do not regulate chemoattractant-induced activation of β_2 integrin affinity. In fact, β_2 integrin-mediated rapid adhesion, in static condition assays, and neutrophil adhesion to glass capillary tubes cocoated with ICAM-1, P-selectin, and a chemoattractant, under flow, were unaffected in $hck^{-/-}fgr^{-/-}$ neutrophils. Additionally, examination of integrin affinity by soluble ICAM-1 binding assays and of β_2 integrin clustering on the cell surface, showed that integrin activation did not require Hck and Fgr expression. However, after binding, $hck^{-/-}fgr^{-/-}$ neutrophil spreading over β_2 integrin ligands was reduced and they rapidly detached from the adhesive surface. Whether alterations in outside-in signaling affect sustained adhesion to the vascular endothelium in vivo was addressed by examining neutrophil adhesiveness to inflamed muscle venules. Intravital microscopy analysis allowed us to conclude that Hck and Fgr regulate neither the number of rolling cells nor rolling velocity in neutrophils. However, arrest of $hck^{-/-}fgr^{-/-}$ neutrophils to >60 μm in diameter venules was reduced. Thus, Hck and Fgr play no role in chemoattractant-induced inside-out β_2 integrin activation but regulate outside-in signaling-dependent sustained adhesion. *The Journal of Immunology*, 2006, 177: 604–611.

L eukocyte recruitment into inflamed tissues is viewed as a multistep cascade of adhesive interactions between leukocytes and endothelial cell adhesion molecules. These involve selectin-mediated leukocyte tethering and rolling, integrin-dependent firm adhesion, and, ultimately, transmigration across the endothelial layer (reviewed in Refs. 1 and 2).

As demonstrated by studies (3–6) on human and cattle leukocyte adhesion deficiency type I, β_2 integrin-mediated stable adhesion represents an essential step for leukocyte transmigration. Interaction of β_2 integrins with endothelial counterreceptors depends on their capability to undergo dynamic functional changes commonly referred to as activation and occurring as a consequence of inside-out signaling triggered by a variety of leukocyte agonists collectively named chemoattractants (1, 7).

Integrin activation by chemoattractants is very rapid and is believed to involve both a conformational change, resulting in an increase of integrin affinity for the ligand, and clustering of integrin molecules in discrete areas of the plasma membrane that would increase strength of binding (increased valency) (see Refs. 7–9) and references contained therein). In addition, integrins may act as signal transduction devices in leukocytes (10–12). Despite reorganization of the actin cytoskeleton via signals triggered by integrin ligation is believed to strengthen adhesive interactions in mesenchymal and epithelial cells (reviewed in Refs. 13 and 14), it is still unclear whether signals delivered by leukocyte integrins and leading to cytoskeleton rearrangements contribute to leukocyte firm adhesion to the endothelium (see Ref. 2).

Accumulating evidence suggests that the hemopoietic cell lineage-specific Src family kinases Fgr and Hck are implicated in integrin signaling in myeloid cells (reviewed in Refs. 10–12). Noteworthy, deficiency of Fgr and Hck renders neutrophils and macrophages nonresponsive to adhesion-mediated activation following engagement of different integrin subfamilies (15–19). The defective response of $hck^{-/-}fgr^{-/-}$ neutrophils and macrophages includes marked alteration in cytoskeleton rearrangement and cell spreading.

Although neutrophils from $hck^{-/-}fgr^{-/-}$ animals do not exhibit alteration in their migratory ability, both in Transwell assays in vitro and in a chemical peritonitis model in vivo (19–21), evidence that such alterations may manifest in certain inflammatory conditions has been also presented. For example, in the LPS-induced systemic inflammatory reaction, $hck^{-/-}fgr^{-/-}$ neutrophils accumulate in the blood and are impaired in their capability to migrate into the liver (22). In addition, Fgr deficiency results in a marked

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reduction in the accumulation of eosinophils in the lung in a murine model of allergic inflammation (23). Noteworthy, mice expressing a constitutively active form of Hck or with the selective granulocyte inactivation of the Src family kinase inhibitor C-terminal Src kinase develop an exaggerated pulmonary inflammation and their granulocytes are more responsive to integrin-dependent stimulation (24, 25).

To address the role of Fgr and Hck in integrin-dependent adhesion of neutrophils to vascular endothelial ligands, we investigated whether deficiency of these kinases results in alterations of integrin affinity and/or valency mediating firm adhesion to the endothelium. In this study, we show that β_2 integrin affinity and valency are not positively regulated by Fgr and Hck. However, $hck^{-/-}fgr^{-/-}$ neutrophils displayed the property to adhere for a much shorter time to β_2 integrin ligands and were inhibited in their capability to arrest to inflamed striate muscle venules under flow. These findings suggest that whereas inside-out, chemoattractant-induced, integrin activation does not require Fgr and Hck, outside-in integrin signaling regulated by these kinases critically contributes to strengthening neutrophil adhesion to inflamed endothelium.

Materials and Methods
Cell preparation

Male and female C57BL/6J, 6- to 8-wk-old mice were used as wild-type (WT)[4] control. Generation of $hck^{-/-}fgr^{-/-}$ double knockout (KO) and $hck^{-/-}$ and $fgr^{-/-}$ single knockout mice in this same background has been previously described (20). Mouse bone marrow neutrophils were isolated from femurs and tibias as previously described (15). Briefly marrow cells were flushed from the bones using HBSS (137 mM NaCl, 0.53 mM KCl, 0.033 mM Na_2HPO_4, 0.4 mM $NaHCO_3$, 0.044 mM KH_2PO_4, and 2 mM HEPES, pH 7.4) without Ca^{2+} and Mg^{2+}, and containing 0.1% BSA. Cells were centrifuged and, after hypotonic lysis of erythrocytes, resuspended in 3 ml of 45% Percoll (Amersham Biosciences) in Ca^{2+}/Mg^{2+}-free HBSS supplemented with 0.1% BSA. Bone marrow cells were then loaded on top of a Percoll discontinuous density gradient (26) and, after centrifugation at $1600 \times g$ for 30 min at room temperature, cells at the interface between 81 and 62% and 62 and 55% Percoll layer were harvested and diluted in Ca^{2+}/Mg^{2+}-free HBSS supplemented with 0.1% BSA. After a further wash, neutrophils were resuspended at 10×10^6 ml in modified HBSS with a total osmolarity of 308 mOsmol/L (27) and supplemented with 0.5 mM $CaCl_2$ and 5 mM D-glucose. Routinely, cell suspensions were left at room temperature for 1 h before assay.

Adhesion assays

Eighteen-well glass slides were coated for 120 min at 37°C with human fibrinogen (Sigma-Aldrich) (20 μg per well in endotoxin-free PBS). Neutrophils (5×10^4 per well; 2.5×10^6 per ml in PBS, containing 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10% heat-inactivated FCS, pH 7.3) were added, incubated for 10 min at 37°C, and then stimulated by the addition of the agonist. After three washings in cold PBS, cells were fixed on ice in 1.5% glutaraldehyde for 60 min; computer-assisted enumeration of cells bound in 0.2 mm^2 was performed as described previously (28). As stimuli, leukotriene B_4 (LTB_4; Sigma-Aldrich) or the peptide Trp-Lys-Tyr-Met-Val-D-Met (WKYMVm; Sigma-Genosys) were used. Under-flow adhesion assay was performed as previously described (8).

Measurement of LFA-1 affinity

Induction of LFA-1 high-affinity state by chemoattractants was evaluated by measuring binding of soluble ^{125}I-ICAM-1, as previously described (8). Briefly, native ICAM-1 isolated from spleens was iodinated with ^{125}NaI by the Chizzonite method. The binding assay was performed at 37°C in a 500-μl Eppendorf tube. Neutrophil suspensions (40 μl of 5×10^7 ml in PBS containing 1 mg/ml BSA, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM D-glucose, pH 7.2) were directly layered on a 100-μl oil cushion of 2:1 dibutyl:dioctyl phthalates. Neutrophils were stimulated with 10 μl of PBS containing WKYMVm (0.5 μM) and ^{125}I-ICAM-1 (5×10^5 cpm corresponding to ~2 μg of ^{125}I-ICAM-1). The binding reaction was stopped by

rapid centrifugation in a microfuge (Biofuge, Heraeus). Radioactivity bound to neutrophils was counted with a gamma counter.

Immunofluorescence microscopy

To study the surface distribution of LFA-1, a confocal imaging system was used. Neutrophils were stimulated in suspension under stirring and then immediately fixed in 1% ice-cold paraformaldehyde in PBS (pH 7.4) for 10 min. Cells were washed and incubated with 10 μg/ml TIB213, a rat anti-mouse LFA-1 Ab (American Type Culture Collection) for 30 min on ice, washed three times, and then incubated for 30 min with Texas Red-conjugated goat anti-rat secondary Ab. The washed cells adhered for 30 min at 4°C on 0.1% poly-L-lysine-coated, round 13-mm glass coverslips. The cells were washed, rinsed in absolute ethanol and once in PBS, and then mounted in 30% glycerol. Fluorescent specimens were analyzed with a Zeiss LSM 510 confocal imaging system, with a ×63 C-Apochromat objective (aperture 1.2). The cells were serially scanned in horizontal sections 0.6 μm apart.

Preparation of mice for intravital microscopy

C57BL/6 young females were purchased from Harlan-Nossan and were housed and used according to current European community rules for the usage of laboratory animals. Mice were injected i.p. with 12 μg LPS (E. coli 026:B6, Sigma-Aldrich) 5–6 h before starting the intravital experiment. Animals were anesthetized by i.p. injection (10 ml/kg) of physiologic saline containing ketamine (5 mg/ml) and xylazine (1 mg/ml). The recipient was maintained at 37°C by a Linkam CO102 (Olympus) stage-mounted strip heater. A heparinized PE-10 polyethylene catheter was inserted into the right common carotid artery toward the aortic arch. The skin was removed and the pectoral muscle was bathed with sterile saline, and a 24×24-mm coverslip was applied and fixed with silicon grease. A round chamber with 11-mm internal diameter was attached on the coverslip and filled with water (29).

Intravital videomicroscopy

The muscle preparation was placed on an Olympus BX50WI microscope and a water immersion objective with long focal distance (focal distance 3.3 mm, aperture 0.5 ∞; Olympus Achroplan) was used. Blood vessels were visualized by using fluorescent dextrans: 3 mg of FITC-dextran (148 kDa; Sigma-Aldrich) and/or 6 mg of TRITC-dextran (155 kDa; Sigma-Aldrich) was diluted in 0.3 ml of sterile physiologic saline and centrifuged for 5 min at $14,000 \times g$ (each mouse received 0.05 ml of supernatant). Neutrophils were labeled with either green 5-chloromethylfluorescein diacetate or orange 5-(and-6)-(((chloromethyl)benzoyl)amino)tetramethylrhodamine) (Molecular Probes). Fluorescent-labeled cells per condition (2.5×10^6) were injected into the common carotid toward the aortic arch by a digital pump. The images were visualized by using a silicon-intensified target videocamera (VE-1000 SIT; Dage-MTI) and a Sony SSM-125CE monitor. Recordings were digitalized and stored on videotapes using a digital VCR (Panasonic NV-DV10000).

Image analysis

Video analysis was performed by playback of digital videotapes in real time or at reduced speed and frame-by-frame. Vessel diameter (D), hemodynamic parameters, and the velocities of rolling were determined by using a PC-based system and the NIH Image 1.61 software. The velocities of ≥ 20 consecutive freely flowing cells/venule were calculated, and from the velocity of the fastest cell in each venule (V_{fast}), we calculated the mean blood flow velocities (V_m): $V_m = V_{fast}/(2 - \varepsilon^2)$, where ε is the ratio of the neutrophil diameter to vessel diameter (30). The wall shear rate (WSR) was calculated from WSR $= 8 \times V_m/D$ (s^{-1}), and the shear stress acting on rolling cells was approximated by WSR $\times$ 0.025 (dyne/cm^2), assuming a blood viscosity of 0.025 Poise. Neutrophils were considered as rolling whether they traveled at velocities below V_{crit} ($V_{crit} = V_m \times \varepsilon \times (2 - \varepsilon)$) (30). Neutrophils that remained stationary on venular wall for ≥ 30 s were considered adherent. At least 140 consecutive cells/venule were examined. Rolling and firm arrest fractions were determined as the percentage of cells that rolled or firmly arrested within a given venule in the total number of cells that enter that venule during the same period (31).

Statistics

A two-tailed Student's t test was used for statistical comparison of two samples. Velocity histograms were compared using the Mann-Whitney U test (32) and Kolmogorov-Smirnov test. Differences were regarded as significant with a value of $p < 0.05$.

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[4] Abbreviations used in this paper: WT, wild type; KO, knockout; LTB_4, leukotriene B_4; WSR, wall shear rate.
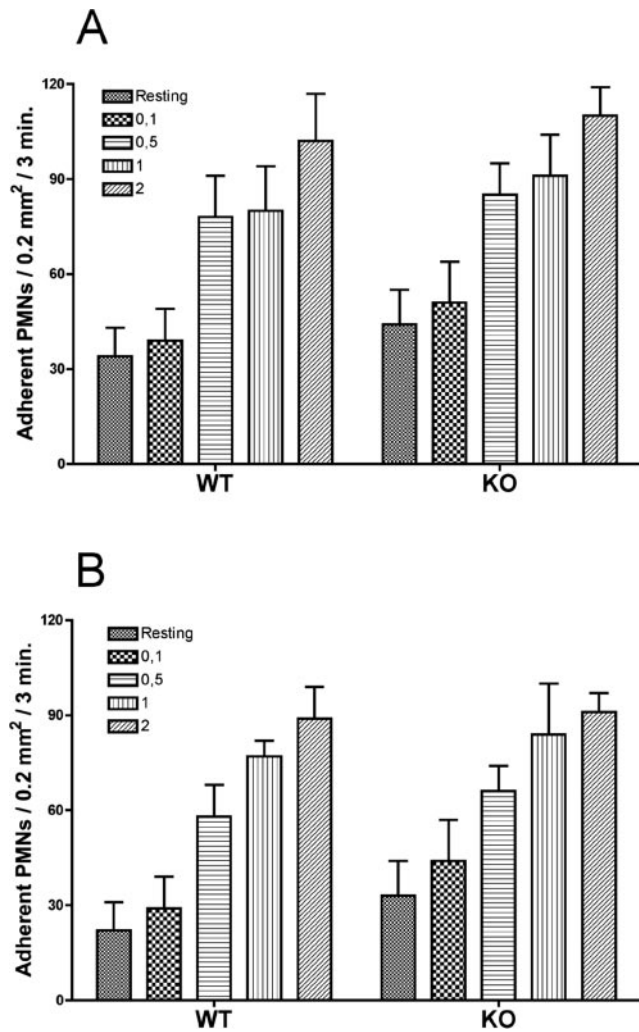
FIGURE 1. Neutrophils from $hck^{-/-}fgr^{-/-}$ mice display normal β_2 integrin-dependent rapid adhesion to fibrinogen. Eighteen glass wells were coated with 1 mg/ml purified fibrinogen. Rapid adhesion was triggered with DMSO (resting) or with the indicated nanomolar concentrations of WKYMVm (*A*) or micromolar concentrations of LTB$_4$ (*B*). The number of adherent cells in 0.2 mm^2 in 3 min is shown. In these assay conditions, percent adherent cells at optimal doses of the chemoattractant was ~12–15% of input cells. Mean results of triplicate assays of two experiments are reported.

FIGURE 2. Neutrophils from $hck^{-/-}fgr^{-/-}$ mice display normal LFA-1-dependent rapid adhesion to ICAM-1. Eighteen glass wells were coated with the indicated number of molecules per square micrometer of mouse native-purified ICAM-1. Rapid adhesion was triggered with DMSO (resting) or with 100 nM WKYMVm. The number of adherent cells in 0.2 mm^2 in 3 min is shown. Mean results of triplicate assays ($\pm$SD) of one representative experiment of the three performed are reported.

Results

Deficiency of Fgr and Hck does not affect chemoattractant-stimulated rapid adhesion of neutrophils to β_2 integrin ligands

Because signal transduction by chemoattractants in granulocytes implicates Src family kinases and deficiency of Fgr and Hck results in a defect in some neutrophil responses to chemoattractants (33–37), we asked whether β_2 integrin-dependent adhesion also requires Fgr and Hck. To this purpose, we exploited assays of rapid adhesion to immobilized β_2 integrin ligands that have been validated to reflect integrin affinity changes in studies with lymphocytes (8, 38). As shown in Fig. 1*A*, adhesion to immobilized fibrinogen could be easily detected within 3 min from the stimulation with different doses of the synthetic peptide WKYMVm, a potent agonist of the mouse receptor for formylated peptides (39). Comparable results were obtained with LTB4 (Fig. 1*B*) and fMLP (data not shown). Notably, $hck^{-/-}fgr^{-/-}$ neutrophils behaved as WT cells in this type of assay and rapidly adhered in response to both WKYMVm and LTB4 (Fig. 1).
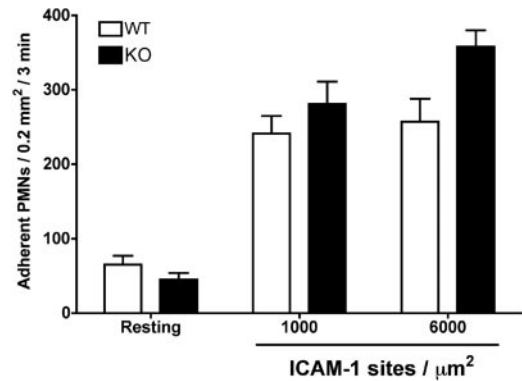
To strengthen these findings we examined rapid adhesion to ICAM-1, the major β_2 integrin ligand expressed by endothelial cells (Fig. 2). As shown in Fig. 2, rapid adhesion of WT and $hck^{-/-}fgr^{-/-}$ neutrophils to immobilized ICAM-1 in response to WKYMVm was equal. It is important to note that rapid adhesion assays were performed on both low- and high-density ICAM-1, i.e., in conditions that with lymphocytic cells have been shown (8) to require both affinity and mobility/clustering changes or affinity changes alone, respectively. Thus, Fgr and Hck seem to be dispensable for induction of both affinity and valency (clustering) modifications in response to chemoattractants in murine neutrophils.

The conclusion that Fgr and Hck do not regulate binding of neutrophil β_2 integrins to ICAM-1 also derived from studies of under-flow adhesion (Fig. 3). In fact, under a shear stress of 2 dyne/cm^2, adhesion of $hck^{-/-}fgr^{-/-}$ or WT neutrophils to glass capillary tubes coated with ICAM-1 and WKYMVm, in combination with P-selectin (Fig. 3), was comparable. Notably, this experimental approach allowed us to exclude that deficiency of these kinases results in a decrease in neutrophil rolling. In fact, $hck^{-/-}fgr^{-/-}$ neutrophils rolled as well as WT cells on P-selectin.

Deficiency of Fgr and Hck does not affect chemoattractant-stimulated activation of β_2 integrin affinity or lateral mobility

To validate the conclusion suggested by the results of the experiments reported in Figs. 1 and 2, i.e., that Fgr and Hck do not regulate β_2 integrin affinity or lateral mobility in murine neutrophils, we addressed this issue by alternative and more direct assays (Figs. 4 and 5).

Changes in β_2 integrin affinity, as a function of time, were examined by binding of soluble ICAM-1 following stimulation with WKYMVm (Fig. 4). As previously described with lymphocytes (8), chemoattractant stimulation induced a rapid increase of ICAM-1 binding to murine neutrophils that reached a plateau at ~3 min and then declined to the level of unstimulated cells within 10 min. Interestingly, ICAM-1 binding to WKYMVm-stimulated $hck^{-/-}fgr^{-/-}$ neutrophils was even higher than to WT cells. Because β_2 integrin expression by the two mouse neutrophil strains is absolutely comparable (15, 18), and, therefore, changes in integrin expression cannot affect ICAM-1 binding, we conclude that β_2 integrin affinity changes are independent of, or perhaps even inhibited by, Fgr and Hck (see *Discussion*).
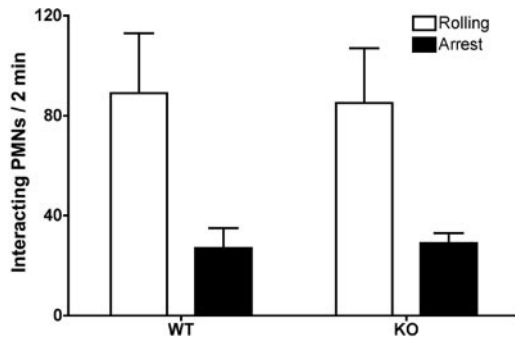
FIGURE 3. Neutrophils from $hck^{-/-}fgr^{-/-}$ mice display normal rolling and integrin-dependent rapid adhesion under-flow. Glass capillary tubes were cocoated with P-selectin, ICAM-1, and WKYMVm. Rolling and adhesion were analyzed under a shear stress of 2 dyne/cm². The percentage of total cells interacting with capillary wall undergoing rolling and/or adhesion is shown. One representative experiment of the three performed is reported.

FIGURE 4. Triggering of LFA-1 high-affinity state is increased in neutrophils from $hck^{-/-}fgr^{-/-}$ mice. Neutrophils were stimulated with DMSO (resting) or with 100 nM WKYMVm for the indicated time. The cpm of bound ^{125}I-ICAM-1 for 2×10^6 neutrophils are shown. Mean results of triplicate assays ($\pm$SD) of one representative experiment of the three performed is reported.

Changes in β_2 integrin clustering were examined by fluorescence confocal microscopy (Fig. 5). Notably, WKYMVm (or fMLP, not shown) induced a rapid and consistent redistribution of β_2 integrins on the neutrophil surface. However, no difference in the lateral mobility of β_2 integrins was detected in WT vs $hck^{-/-}fgr^{-/-}$ neutrophils both in response to WKYMVm or PMA. We conclude that Fgr and Hck have no role in the regulation of the two modalities of β_2 integrin activation induced in neutrophils by chemoattractants.

Deficiency of Fgr and Hck renders β_2 integrin-dependent adhesion a rapidly reversible event

Besides changes in affinity and avidity, signals ensuing from the integrin itself (outside-in signaling) upon its ligation, and leading to rearrangement of the cell cytoskeleton and stabilization of cell-substrate interaction, are believed to regulate integrin-mediated adhesiveness in mesenchymal and epithelial cells (13, 14). Because we previously (see Introduction) implicated Fgr and Hck outside-in integrin signaling in granulocytes, we asked whether the stability of adhesion was affected by Fgr and Hck deficiency. As shown in Fig. 6, we found that this was indeed the case. In fact, whereas WKYMVm-stimulated adhesion on fibrinogen in static conditions lasted up to 6 min in WT neutrophils, $hck^{-/-}fgr^{-/-}$ cells adhered as rapidly as WT cells, but after 4 min started to detach from the adherence surface and, at 6 min, very few of them were still adherent. We conclude that when integrin affinity starts to regain its resting levels (see Fig. 4), persistence of an adherent state is maintained as a consequence of Src family kinase-dependent signaling from ligand-engaged integrins. To further investigate the difference in persistence of adhesion of WT vs $hck^{-/-}fgr^{-/-}$ neutrophils, we addressed whether both Hck and Fgr are concurrently required to regulate the stability of cell-substrate interaction. To this purpose, we performed assays with single knockout $hck^{-/-}$ or $fgr^{-/-}$ neutrophils. As reported in Fig. 6, we did not detect any difference in adherence persistence between WT, Hck-, and Fgr-deficient cells. We conclude that, as it emerged in previous studies (15–17, 20, 22, 35), these kinases play a concurrent and/or redundant regulatory function. Confirming previous studies (15), stabilization of adhesion by Fgr and Hck requires cytoskeleton rearrangement and cell spreading. In fact, as reported in Table I, a reduced number of $hck^{-/-}fgr^{-/-}$ neutrophils spread over fibrinogen (or ICAM-1; data not shown) in response to WKYMVm. Additionally, the mean surface area of adherent/spread $hck^{-/-}fgr^{-/-}$ neutrophils was significantly lower than that of WT cells.
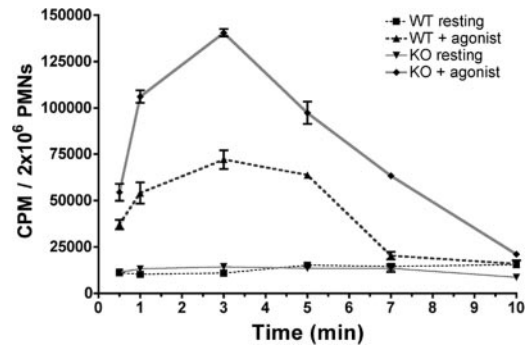
Behavior of Fgr- and Hck-deficient neutrophils in inflamed venules in vivo

To understand whether our findings on the role of Fgr and Hck in regulating integrin-mediated adhesion in static conditions had a possible significance in vivo under flow, we examined neutrophil adhesiveness in inflamed venules. It was previously shown (40, 41) that treatment with LPS or TNF is able to up-regulate adhesion molecules on endothelium in vivo, inducing a subacute inflammation. Therefore, mice were treated with LPS and after 5–6 h we performed intravital microscopy in subacutely inflamed venules of the striate muscle. After this time of LPS treatment, we confirmed previous findings (40–42) that the endothelium expresses E-, P-selectin, ICAM-1, and VCAM-1 (data not shown).

Examining rolling and arrest of fluorescently labeled neutrophils (see *Materials and Methods*) in striate muscle venules with a diameter <60 μm, we found that cells derived from control and $hck^{-/-}fgr^{-/-}$ mice were able to roll in a comparable manner (Fig. 7A). We also analyzed the quality and strength of rolling interactions by measuring neutrophil rolling velocities (V_{rol}). V_{roll} calculated from different experiments were pooled in velocity classes to better analyze the rolling differences between control and $hck^{-/-}fgr^{-/-}$ cells. As shown in Fig. 7B, no significant differences were observed between the two neutrophil populations. Examining venules with a diameter <60 μm also, the capability to arrest on the inflamed endothelium was not different in WT and $hck^{-/-}fgr^{-/-}$ neutrophils (Fig. 7A).

When we extended examination of neutrophil behavior to inflamed striate muscle venules with a diameter >60 μm (Fig. 7C), we found that, in agreement with previous data (41), the percentage of interacting cells was lower than in smaller venules (cf. with Fig. 7A), but $hck^{-/-}fgr^{-/-}$ neutrophils still did not display any alteration in the percentage of rolling cells and rolling velocity (Fig. 7C and data not shown). In marked contrast, arrest (sticking) of $hck^{-/-}fgr^{-/-}$ neutrophils to >60-μm diameter venules was significantly inhibited (62% inhibition, $p < 0.002$; Fig. 7C). It is important to note that the comparison between cells derived from WT and mutant animals was performed in the same venules, i.e., in similar hemodynamic conditions and in the presence of equivalent expression of endothelial ligands. As fluctuations in blood flow occurred, hemodynamic parameters were measured during the injection of cells derived from WT and $hck^{-/-}fgr^{-/-}$ mice. Notably, microvascular hemodynamics were similar during the injection of control or $hck^{-/-}fgr^{-/-}$ cells, supporting the accuracy of the results reported in Fig. 7 (data not shown).
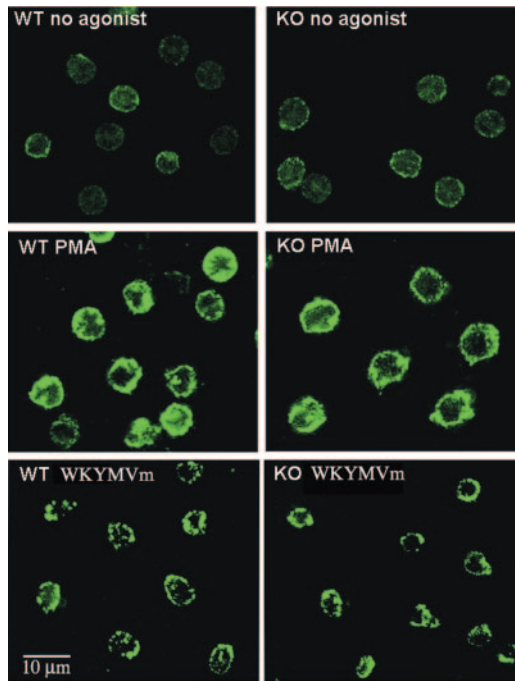
FIGURE 5. Agonist-induced LFA-1 plasma membrane redistribution is not altered in $hck^{-/-}fgr^{-/-}$ neutrophils. Cells were triggered in suspension with DMSO (no agonist) or with 100 ng/ml PMA for 10 min. or 100 nM WKYMVm for 1 min. Shown are confocal microscopy images of cells stained with anti-mouse LFA-1 mAb TIB-213. Images are representative of three distinct experiments.
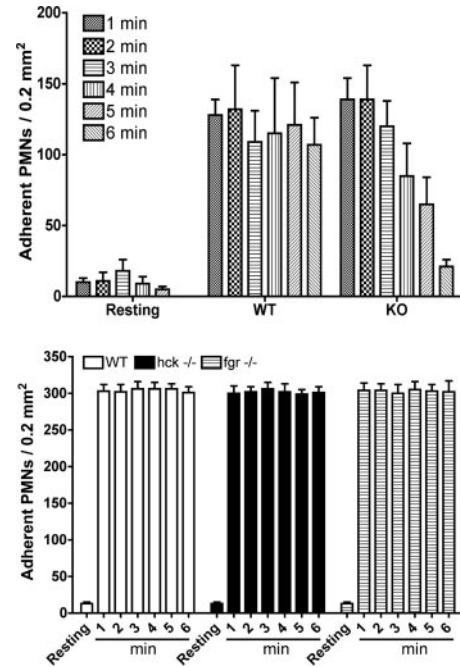

FIGURE 6. Neutrophils from $hck^{-/-}fgr^{-/-}$ mice display accelerated down-modulation of adhesion. Eighteen glass wells were coated with 1 mg/ml purified fibrinogen. Rapid adhesion was triggered for the indicated time with DMSO (resting) or with 100 nM of WKYMVm. The number of adherent cells in 0.2 mm^2 after different times of incubation is shown. Mean results of triplicate assays ($\pm$SD) of one representative experiment of the five performed is reported. *Lower panel,* The results obtained with single KO $hck^{-/-}$ or $fgr^{-/-}$ neutrophils.

Discussion

The current general view of activation of the integrin-binding capacity to appropriate ligands conceives three steps, which likely includes additional transitional states: from a bent, inactive conformation integrins switch to an extended state in which the integrin displays an intermediate affinity for the ligand, and finally to a fully active, high-affinity state; concurrently with affinity changes, clustering of integrins in discrete sites strengthens the extent of binding (increased valency) (43, 44). In the context of the leukocyte integrin LFA-1, this finely regulated process dictates firm adhesion to ICAM-1 and the leukocyte arrest to the inflamed endothelium (2, 7, 44). The final step of integrin activation, i.e., resulting in ligand binding with high affinity and valency, has been traditionally viewed as that switching the integrin to a canonical signal-transducing receptor (see Introduction and below).

The current paradigm of signal transduction mechanisms implicated in integrin activation and function relies on the concepts of inside-out (agonist- or, in the case of leukocyte integrin, chemoattractant-induced) and outside-in (ligand-induced) signals. Inside-out signaling regulating integrin activation in response to chemoattractants in leukocytes involves small GTPases of the Rho and Rap families, lipid and protein kinases such as PI3-kinase and protein kinase Cζ, and cytoskeletal proteins such as talin and α-actinin (see Refs. 7, 8, 45, and 46 and references therein). As pioneered by studies in mesenchymal cells (13, 14), cytoplasmic tyrosine kinases of the Src (Hck and Fgr) and focal adhesion kinase (Pyk2) families, as well as Syk, play a major role in outside-in integrin signaling in leukocytes (reviewed in Ref. 12).

In this study, we demonstrate that the Src family kinases Hck and Fgr are dispensable for up-modulation of β_2 integrin affinity and valency in neutrophils in response to chemoattractants. However, lack of expression of these kinases is accompanied by a reduced capability to maintain a prolonged adhesion to β_2 integrin

ligands in vitro. Furthermore, this defect manifests in vivo as a consistent reduction of the capability of neutrophils to arrest to larger inflamed venules. Additionally, we demonstrate that Hck and Fgr do not regulate neutrophil rolling.

Gαi-linked chemoattractant and chemokine receptors inducing integrin affinity activation in neutrophils trigger different signal transduction pathways (47). In the last few years, accumulating evidence implicated Src family kinases in the regulation of neutrophil degranulation in response to both fMLP and chemokines (34–37). However, neutrophil chemotaxis in response to these ligands does not appear to be defective in Src kinase-deficient neutrophils (19) and $hck^{-/-}fgr^{-/-}$ neutrophils were recently reported (21) to be even more responsive to a number of different chemokines. These findings suggest that more than being implicated in upstream Gαi signaling, these kinases might be required for more distal signaling events, a possibility also consistent with the physical association of Hck and Fgr with primary and secondary granules (48, 49). In this study, we provide evidence suggesting that Hck and Fgr are not implicated in activation of neutrophil β_2 integrin affinity in response to chemoattractants. This relies on three different experimental approaches. First, rapid adhesion to two different β_2 integrin ligands, in static condition assays, which were validated as reflecting LFA-1 affinity changes in lymphocytes (8), was equal in WT and $hck^{-/-}fgr^{-/-}$ neutrophils (Figs. 1 and 2). Second, neutrophil adhesion to glass capillary tubes coated with adhesion molecules and a chemoattractant under a shear stress of 2 dyne/cm^2 was not regulated by expression of Hck and Fgr (Fig. 3). Finally, direct examination of β_2 integrin affinity by assaying binding of soluble ICAM-1 showed that $hck^{-/-}fgr^{-/-}$ neutrophils bound even higher amounts of ICAM-1 following stimulation with a chemoattractant (Fig. 4). It is worth noting that the rho small GTPase RhoA has been demonstrated to regulate LFA-1 affinity

TABLE I. *Neutrophils from hck$^{-/-}$fgr$^{-/-}$ mice display defect in spreading*[a]

	Spread cells on total adherent Polymorphonuclear neutrophils (%)	Mean area (μm^2)[b]
WT	71	192 ± 28
KO	39	98 ± 14

[a] Mean values ($\pm$SD) from $\geq$100 analyzed cells in two separate experiments are reported.

[b] Mean area of unstimulated cells settled on fibrinogen-coated plastic is about 42 μm for both WT and KO cells.

changes (38, 45, 46). Because the Rho inhibitor p190RhoGAP is a Src substrate (50, 51) and p190RhoGAP tyrosine phosphorylation is reduced in $hck^{-/-}fgr^{-/-}$ macrophages (52), it is tempting to speculate that this may account for enhanced binding of ICAM-1 to mutant neutrophils.

On a whole, the evidence that Hck and Fgr do not regulate affinity changes of β_2 integrins in neutrophils is in line with the very recent demonstration that inside-out and outside-in signalings regulating the $aII_b\beta_3$ integrin are biochemically distinct events (53); whereas talin binding to the β_3 integrin cytoplasmic tail regulates affinity changes, the Src kinase binding to a distinct site regulates outside-in signaling. According to this model, chemoattractants and chemokines would trigger Hck/Fgr-independent signals leading to β_2 integrin activation and cell migration (see Refs. 19 and 21); ligand binding would then result in activation of Hck and Fgr and the integrin-dependent pathway of activation of neutrophil effector functions (12). We believe this view will have to stand the challenge of further investigation to be definitively validated. In fact, a very recent study (44) proposed that immobilized chemokine-induced intermediate-affinity binding of LFA-1 to ICAM-1 in lymphocytes is followed by rapid transition to a high-affinity state possibly induced by outside-in signals. Additionally, clustering of activated β_2 integrins at sites of contact between Chinese hamster ovary cells expressing E-selectin and human neutrophils is inhibited by Src family kinase inhibitors (54). Therefore, we cannot exclude that chemoattractant signaling triggers a transient shift to an intermediate-affinity state of β_2 integrins in neutrophils, but, following ligand binding, outside-in, possibly Hck/Fgr dependent, signals induce full integrin activation. If true, this hypothesis could imply that transition from intermediate- to high-affinity state is important not for leukocyte arrest under flow (which was unaffected in $hck^{-/-}fgr^{-/-}$ neutrophils) but in maintaining a prolonged adhesion. Unfortunately, because suspension assays of ligand binding in response to soluble activators mainly detect integrin triggering to high-affinity state (see Ref. 44) and anti-mouse β_2 integrin Abs against activation epitopes specific for the two states are not available, we cannot, at present, address this relevant issue.

However, it is also possible that Hck and Fgr are not at all involved in the modulation of transitions between LFA-1 conformers in neutrophils. Indeed, we found that after adhesion, $hck^{-/-}fgr^{-/-}$ neutrophils displayed a reduced capability to spread over β_2 integrin ligands (Table I). Notably, after an initial, likely affinity-mediated, binding to fibrinogen, $hck^{-/-}fgr^{-/-}$ neutrophils rapidly detached from, whereas WT cells remained adherent to the adhesive surface (Fig. 6). Data from two different experimental approaches allowed us to conclude that the role played by Hck and Fgr in stabilization of adhesion is unlikely dependent on modulation of integrin valency via clustering of the molecule on the cell surface. First, adhesion assays to low-density surface-bound ICAM-1 (Fig. 3), that with lymphocytic cells have been shown to
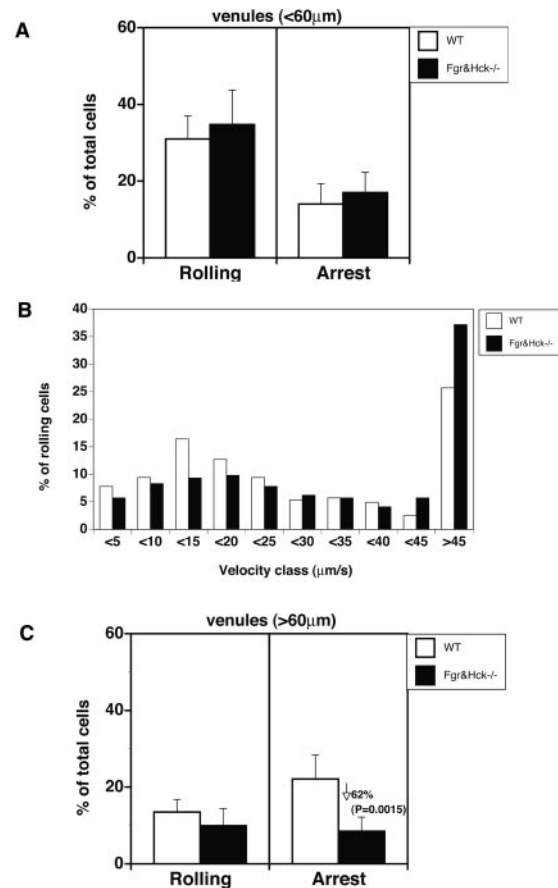

FIGURE 7. Mice were treated with 12 μg LPS 5–6 h before starting the intravital microscopy experiments. *A*, Rolling and arrest fractions of WT and $hck^{-/-}fgr^{-/-}$ neutrophils were calculated. Eleven venules with diameter <60 μm were examined in five animals. Mean $\pm$ SD of *D* was 38.6 $\pm$ 17.7. Groups were compared using Student's *t* test. Data shown are mean $\pm$ SEM. *B*, Velocity histograms were generated by measuring rolling velocities in venules with *D* <60 μm. Frequency distributions were calculated after cells were assigned to velocity classes from >0 to 5 μm/s, 5 to 10 μm/s, 10 to 15 μm/s, and so on. One hundred eighty-eight rolling cells were examined for WT neutrophils and 183 rolling cells were considered for $hck^{-/-}fgr^{-/-}$ neutrophils. *C*, Rolling and arrest fractions were calculated. Ten venules with *D* >60 μm were examined in five animals. Mean $\pm$ SD of *D* was 99.2 $\pm$ 44.3. Groups were compared using Student's *t* test. *p* = 0.0015 for the arrest fraction of $hck^{-/-}fgr^{-/-}$ neutrophils when compared with WT cells. Data shown are mean $\pm$ SEM.

reflect both LFA-1 affinity and mobility/clustering changes, gave similar results using WT or mutant neutrophils. Second, LFA-1 clustering induced by a chemoattractant or PMA was not impaired in $hck^{-/-}fgr^{-/-}$ neutrophils (Fig. 5). The most conservative interpretation of these findings is that Src family kinase-dependent formation of actin-based multimolecular complexes linked to integrin cytoplasmic tails firmly anchor the cell to the adhesive surface. Indeed, β_2 integrin-mediated neutrophil responses are strictly dependent on an intact, actin-based cytoskeleton (reviewed in Ref. 10). Additionally, the ICAM-1-induced LFA-1-dependent lymphocyte arrest under flow requires integrin association with the actin cytoskeleton (44).

What is the possible meaning of a reduction in Src family kinase-dependent signals downstream of integrin ligation in the context of neutrophil recruitment into the inflammatory site? As outlined in the Introduction, in vitro assays and in vivo recruitment in some model of experimental inflammation excluded that $hck^{-/-}fgr^{-/-}$ neutrophils display a reduced migratory ability (19,

21). However, mice expressing a constitutively active form of Hck or with the selective granulocyte inactivation of the Src family kinase inhibitor C-terminal Src kinase develop an exaggerated pulmonary inflammation (24, 25). Additionally, in the endotoxin-induced systemic inflammation model, deficiency of Hck and Fgr results in a paradoxical, marked neutrophilia and a reduced accumulation of neutrophils into the liver (22). Thus, decreased or increased neutrophil integrin signaling results in reduced or enhanced neutrophil recruitment, respectively, at least in the lungs and the liver. One possible reason for these findings is that changes in the robustness of firm adhesion to the endothelium result in a lower or higher percentage of neutrophils remaining attached to the inflamed endothelium; by default, a lower or higher cell number, respectively, would then be able to transmigrate. We addressed this issue examining neutrophil adhesiveness to inflamed muscle venules in vivo by intravital microscopy. Notably, this type of analysis excluded that Hck and Fgr regulate neither rolling nor rolling velocity in neutrophils (Fig. 7), a conclusion also supported by the examination of rolling in glass capillary tubes coated with P-selectin (Fig. 3). Hence, Hck and Fgr play no role in regulation of selectin-mediated functions. Examining neutrophil arrest in vivo, we could not detect any alteration in the behavior of $hck^{-/-}fgr^{-/-}$ neutrophils when this analysis was performed in venules with a diameter <60 μm (Fig. 7). This concords with the results derived from the examination of neutrophil arrest to glass capillary tubes coated with ICAM-1 under a shear stress of 2 dyne/cm^2 (Fig. 3). However, the arrest of $hck^{-/-}fgr^{-/-}$ neutrophils to >60-μm diameter venules was significantly inhibited compared with WT cells. We do not know the reason for these findings. However, it is worth noting that recent studies showed that there is a direct relationship between shear stress and ICAM-1 expression in endothelial cells challenged with proinflammatory cytokines (55). Because larger venules have a lower shear stress and, consequently, a lower expression of ICAM-I, it is tempting to speculate that Hck and Fgr may regulate firm adhesion to the endothelium in a manner dependent on β_2 integrin ligand expression on the endothelial cells. Thus, the role played by these kinases in neutrophil recruitment in different tissues could indeed vary on the basis of different factors, including tissue vascularization.

It is of interest that the phenotype of $hck^{-/-}fgr^{-/-}$ neutrophils described in this study is strictly similar to that of Vav-1 and -3-deficient neutrophils (56). In fact, the double inactivation of Vav-1 and -3 results in a severe defect in spreading over β_2 integrin ligands, normal inside-out activation of β_2 integrin affinity, and reduced stable adhesion to cremaster muscle venules. Notably, Vav proteins are substrates of Src kinases and Syk (19, 57), a Src family kinase downstream substrate in the context of neutrophil integrin signaling (12). Indeed, Src family kinase inhibition (58) or deficiency (L. Fumagalli and G. Berton, unpublished observations) results in reduced Vav tyrosine phosphorylation; additionally, Vav is a major substrate of Fgr in Fgr-expressing COS cells (52). Hence, whereas trimeric G protein-coupled receptors activate neutrophil β_2 integrin affinity via signals largely independent of Hck and Fgr, upon ligand binding, these kinases, along with Syk, trigger a Vav-dependent signaling pathway, leading to adhesion strengthening. Whereas a large body of evidence already implicated this pathway in neutrophil activation in the inflammatory site, this study suggests that adhesion strengthening by Src family kinases may also play a role in certain vascular districts. Together with the very recent evidence that the interaction of the α_4 integrin with paxillin, an event that follows activation of $\alpha_4\beta_1$ (59), is required for the recruitment of mononuclear leukocytes into inflamed tissue (60), and the data obtained with Vav-1 and -3-deficient neutrophils (Ref. 56 and see above), our findings highlight a possible role of outside-in integrin signaling in leukocyte recruitment.

Disclosures

The authors have no financial conflict of interest.

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