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An arrestin-dependent multi-kinase signaling complex mediates MIP-1β/CCL4 signaling and chemotaxis of primary human macrophages

Ricky Cheung, Mobeen Malik,* Vipa Ravyn,* Brian Tomkowicz,* Andrzej Ptasznik,† and Ronald G. Collman*,1*

*Pulmonary, Allergy and Critical Care and †Hematology and Oncology Divisions, Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA

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ABSTRACT

MIP-1β/CCL4 is a principal regulator of macrophage migration and signals through CCR5. Several protein kinases are linked to CCR5 in macrophages including the src kinase Lyn, PI3K, focal adhesion related kinase Pyk2, and members of the MAPK family, but whether and how these kinases regulate macrophage chemotaxis are not known. To define the role of these signaling molecules, we examined the functions and interactions of endogenous proteins in primary human macrophages. Using siRNA gene silencing and pharmacologic inhibition, we show that chemotaxis in response to CCR5 stimulation by MIP-1β requires activation of Pyk2, PI3K p85, and Lyn, as well as MAPK ERK. MIP-1β activation of CCR5 triggered translocation of Pyk2 and PI3K p85 from the cytoplasm to colocalize with Lyn at the plasma membrane with formation of a multimolecular complex. We show further that arrestins were recruited into the complex, and arrestin down-regulation impaired complex formation and macrophage chemotaxis toward MIP-1β. Together, these results identify a novel mechanism of chemokine receptor regulation of chemotaxis and suggest that arrestins may serve as scaffolding proteins linking CCR5 to multiple downstream signaling molecules in a biologically important primary human cell type. *J. Leukoc. Biol.* 86: 833–845; 2009.

Introduction

Macrophages are recruited to sites of inflammation in response to chemokines, where they play important roles in host defense and aberrant inflammatory diseases. Chemotaxis is mediated by signaling events initiated by binding of chemokines to their cognate receptors. The β-chemokines MIP-1α (CCL3), MIP-1β (CCL4), and RANTES (CCL5) induce migration through the GPCR CCR5, which is highly expressed on macrophages. Our laboratory reported previously that stimulation of CCR5 on primary human MDM activates the SFK Lyn, PI3K, the focal adhesion related kinase Pyk2, and members of the MAPK family, which regulate CCR5-mediated macrophage cytokine and chemokine production [1–4]. Evidence linking CCR5 to each of these kinases in macrophages indicates a complex signaling mechanism and raises the important question of whether they are involved in macrophage chemotaxis.

In this study, we sought to define the roles of these signaling molecules in CCR5 regulation of macrophage chemotaxis and address the interactions between them in mediating this response. We also investigated the role of arrestins, which function traditionally in terminating GPCR signals but have been implicated recently as scaffolding proteins that may link GPCR-activated signaling molecules [5]. For these studies, we examined the functions and interactions of endogenous proteins, rather than overexpression systems, to ensure that the kinases under investigation were present at physiological levels and interacting with native stoichiometry. We also chose to delineate CCR5-mediated signaling pathways in primary human macrophages, instead of monocytoid cell lines, as a more physiologically relevant cellular model. We found that MIP-1β-elicited macrophage chemotaxis mediated by CCR5 is PTX-sensitive and requires concomitant activation of Lyn, class IA PI3K, Pyk2, and the MAPK ERK. MIP-1β activation leads to up-regulation of a physical association among these kinases in a multimeric complex. We showed further that signaling complex formation depends on recruitment of arrestins, which

Abbreviations: Arr2/3=arrestin 2/3, BIO=6-bromoindirubin-3′-oxime, CaMKII=calcium/calmodulin-dependent protein kinase II, C.I.=chemotactic index, FAK=focal adhesion kinase, $G_i\alpha$=inhibitory G-protein α, GPCR=G-protein-coupled receptor, G_s=stimulatory G-protein, GSK3=glycogen synthase kinase 3, MDM=monocyte-derived macrophage(s), PTX=pertussis toxin, Pyk2=proline-rich tyrosine kinase 2, RTK=receptor tyrosine kinase, SDF-1α=stromal cell-derived factor 1α, SFK=Src family kinase, SH2/3=Src homology 2/3, siRNA=small interfering RNA, WASP=*Wiskott-Aldrich syndrome protein,* WCL=whole cell lysate

1. Correspondence: Pulmonary, Allergy and Critical Care Division, Department of Medicine, University of Pennsylvania School of Medicine, 522 Johnson Pavilion, 3610 Hamilton Walk, Philadelphia, PA 19104-6061, USA. E-mail: collmanr@mail.med.upenn.edu

likely serve as scaffolding proteins. Together, these identify a novel mechanism of chemokine receptor signaling and regulation of chemotaxis.

MATERIALS AND METHODS

Reagents

Chemokines were from PeproTech (Rocky Hill, NJ, USA). The CCR5 antagonist M657 [6] was provided by Michael Miller (Merck and Co., Whitehorse Station, NJ, USA). Isoform-specific PI3K inhibitors were from Echelon Biosciences (Salt Lake City, UT, USA). The Lyn peptide inhibitor KRX-123.302 and control peptide KRX-107.110 were synthesized by Bachem (King of Prussia, PA, USA) as described [3, 7]. PTX was from Sigma Chemical Co. (St. Louis, MO, USA). All other inhibitors were from EMD Chemicals (San Diego, CA, USA).

Antibodies

Mouse Pyk2 and Arr2/3 mAb were from BD Bioscience (San Jose, CA, USA). Rabbit polyclonal Lyn, PI3Kp85α, PI3Kp101, and anti-phosphotyrosine antibodies were from Millipore Corp. (Billerica, MA, USA). Antibodies for ERK1/2, phospho-ERK1/2, β-actin, HRP-conjugated anti-rabbit, and anti-mouse IgG were from Cell Signaling Technology (Danvers, MA, USA). Goat polyclonal PI3Kp85, rabbit polyclonal Arr2/3 antibodies, and control mouse, rabbit, and goat IgG were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Alexa Fluor 555-, 488-, and 633-conjugated IgG were from Molecular Probes (Eugene, OR, USA).

Primary human macrophages

Monocytes were isolated by elutriation or selective adherence and allowed to differentiate into macrophages for 7 days as described [3], following which they were used for chemotaxis assays or replated for other studies. Donors were screened for the CCR5Δ32 mutation by PCR [8], and only cells from homozygous wild-type donors were used, except for specified experiments using CCR5-deficient monocytes from CCR5Δ32 homozygotes.

Chemotaxis assay

Macrophages were resuspended in RMPI containing 0.1% BSA and 2×10^5 cells, incubated with inhibitors or vehicle control for 15 min–2 h, and added to 24-well Transwell inserts with 8 μm pore polycarbonate membranes (Corning Costar, Cambridge, MA, USA). Inserts were placed in wells containing 600 μl media plus chemoattractants and incubated at 37°C for 6 h. Cells that migrated through the membrane were stained and counted microscopically in five random high-power fields (400×)/well. Chemotactic responses are expressed as C.I., calculated by dividing the mean number of cells migrating toward chemoattractants by that toward control medium. To distinguish chemotaxis versus chemokinesis, a modified checkerboard analysis was performed. MIP-1β was used for chemotaxis at 1 nM, unless indicated otherwise. Whenever inhibitors or siRNA were used, cell viability was measured in parallel using CellTiter96 AQueous One (Promega, Madison, WI, USA), according to the manufacturer's instructions.

siRNA transfection

Predesigned siRNA pools specific for Lyn, PI3Kp85α, Pyk2, Arr2/3, and nontargeting control siRNA were purchased from Dharmacon Thermo Scientific (Lafayette, CO, USA). siRNA transfection of primary macrophages was performed using the Human Macrophage Nucleofector Kit (Amaxa, Gaithersburg, MD, USA), according to manufacturer's instructions. Week-old macrophages were resuspended (5×10^6 cells/ml) in 100 μl nucleofection solution with 1–3 μg target-specific or nontargeting control siRNA and electroporated using program Y-10. After transfection, macrophages were cultured for 2 days (chosen for maximal protein knockdown) before harvesting to assay in parallel functional responses by chemotaxis assay and protein expression by immunoblot. The sequences of siRNA oligonucleotide pools were as follows: Lyn, AGACUCAACCAGUACGUAA; AGAUUGGAGAAGGCUUGUA; GCGACAUGAUUAAACAUUA; GAUCCAACGUCCAAUAAAC. PI3Kp85 (PIK3R1), GAAGUAAAGCAUUGUGUCA; GACGAGAGACCAAUACUUG; GUUGAAGUCUCGAAUCAGU; AGACCUGGAUUUAGAAUAU. Pyk2 (PTK2B), GAACAUGGCUGACCUCAUA; GGACCACGCUGCUCUAUUU; GGACGAGGACUAUUACAAA; GAGGAAUGCUCGCUACCGA. Arr2 (ARRB1), GAACUGCCCUUCACCCUAA; UGGAUAAGGAGAUCUAUUA; CGAGCACGCUUACCCUUUC; CAAAGGGACGCGAGUGUUC. Arr3 (ARRB2), CGAACAAGAUGACCAGGUA; GAUGAAGGAUGACGACUAU; CGGCGUAGACUUUGAGAUU; CAACCUCAUUGAAUUUGAU. Control, AUGAACGUGAAUUGCUCAA; UAAGGCUAUGAAGAGAUAC; AUGUAUUGGCCUGUAUUAG; UAGCGACUAAACACAUCAA.

Immunoprecipitation and immunoblot analysis

Macrophages in 12-well plates (1×10^6 cells/well) were incubated in serum-free media overnight prior to stimulation and then lysed in radioimmunoprecipitation assay buffer containing protease inhibitors as described previously [3]. Protein concentrations were determined by bicinchoninic acid assay (Pierce, Rockford, IL, USA). For immunoprecipitation, equal

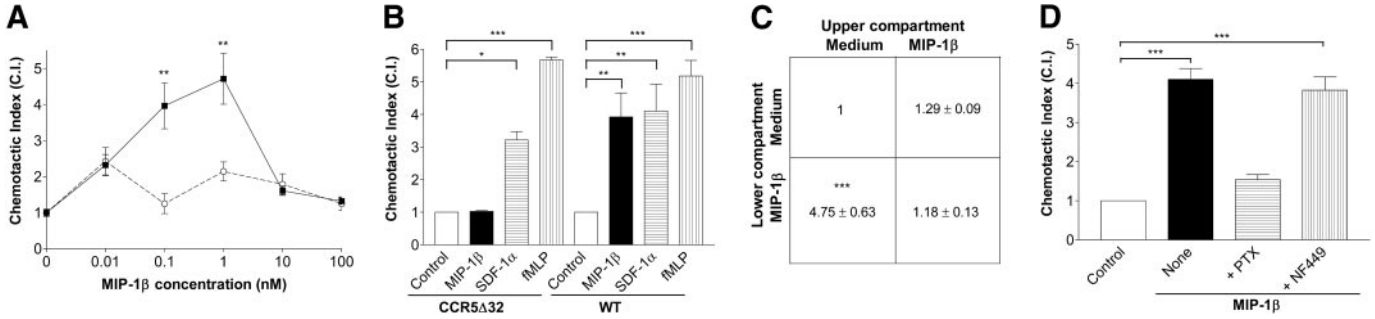

Figure 1. MIP-1β induces CCR5-mediated chemotaxis of primary human macrophages. (A) Macrophages were pretreated for 1 h without (●) or with (□) the CCR5 antagonist M657 (1 μM), placed in Transwell inserts, and stimulated with the indicated concentrations of MIP-1β. Results are expressed as C.I. (B) Migration of macrophages from CCR5Δ32 homozygous donors in response to MIP-1β (1 nM), SDF-1α (1 nM), or fMLP (100 nM). WT, Wild-type. (C) Modified checkerboard analysis of macrophage migration to MIP-1β (1 nM) in the upper and/or lower compartments as indicated. (D) Macrophages were pretreated without or with inhibitors for G_i (PTX, 100 ng/ml) or G_s (NF449, 200 nM) for 2 h prior to measuring migration to MIP-1β. Data shown are means ± SE of three independent experiments using cells from different donors (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

amounts (400 μg) of WCL were incubated at 4°C with antibody (1 μg) for 2 h followed by Pansorbin (50 μl; Calbiochem, San Diego, CA, USA) for 2 h. Immune complexes were washed twice with lysis buffer, eluted by boiling in Laemmli buffer, and subjected to immunoblotting.

Immunoblotting was done on immunoprecipitates generated as described above or directly on WCL (20 μg). Samples were denatured by boiling in Laemmli buffer, resolved by 8% SDS-PAGE, and transferred to nitrocellulose membranes. Conditions for blocking, washing, and dilution were based on instructions from antibody providers. Proteins were visualized by ECL (GE Healthcare, Waukesha, WI, USA) on autoradiographic films. Multiple exposures were used to ensure that quantitations were conducted within the linear range of the images. Films were digitally scanned, and relative intensities of the protein bands were quantitated by Image J (National Institutes of Health, Bethesda, MD, USA) software. In some experiments, membranes were stripped with Restore Stripping Buffer (Pierce) and reprobed with different antibodies.

Lyn kinase activity

Lyn activation was measured by in vitro kinase assay as described previously [3]. Lyn was immunoprecipitated as above, and half of the immunoprecipitate was incubated for 10 min at room temperature with kinase buffer containing 10 μCi [γ-^{32}P]ATP, boiled in Laemmli buffer, resolved by SDS-PAGE, and visualized by autoradiography. The remaining immunoprecipitate was eluted by boiling in sample buffer and immunoblotted with anti-Lyn antibody to assess total Lyn content.

Immunofluorescence confocal microscopy

Macrophages were replated onto glass coverslips, cultured overnight in serum-free media, exposed to MIP-1β, then fixed in 4% paraformaldehyde for 20 min, and permeabilized with 0.2% Triton X-100 for 10 min. Fixed cells were blocked with donkey serum for 20 min and incubated for 1 h with primary antibodies (1:100 dilution). Controls included replacing primary antibodies with species-specific IgG and omitting primary antibodies. Cells were washed, incubated for 30 min with fluorophore-conjugated secondary antibodies (1:100 dilution), washed again, and mounted using VectorShield (Vector Laboratories, Burlingame, CA, USA). Confocal images were captured with a Zeiss LSM510 Meta laser-scanning confocal device attached to an Axioplan2 microscope (63× Plan-Apochromat oil objective). To avoid bleed-through, fluorophores were scanned independently using the multitracking function of the LSM510 device. Images were merged electronically using LSM Image Browser software and saved as Tagged Image File Format files.

Statistical analysis

Quantitative data show means ± SE of at least three independent experiments using cells from different donors. Multiple group comparisons were analyzed by one-way ANOVA, followed by the Bonferroni correction for comparison of means. All immunoblot and immunofluorescence images are representative of experiments carried out with macrophages from at least three different donors.

RESULTS

MIP-1β induces macrophage chemotaxis through binding to CCR5 and coupling of $G_i\alpha$ protein

We generated a concentration-response curve in primary human macrophages for MIP-1β, the most specific ligand for CCR5. Macrophage migration showed a bell-shaped concentration dependence typical of chemokines, with maximal response at 1 nM and less migration at higher concentrations (**Fig. 1A**). Migration toward MIP-1β was mediated through CCR5, as it was abrogated by the specific CCR5 antagonist

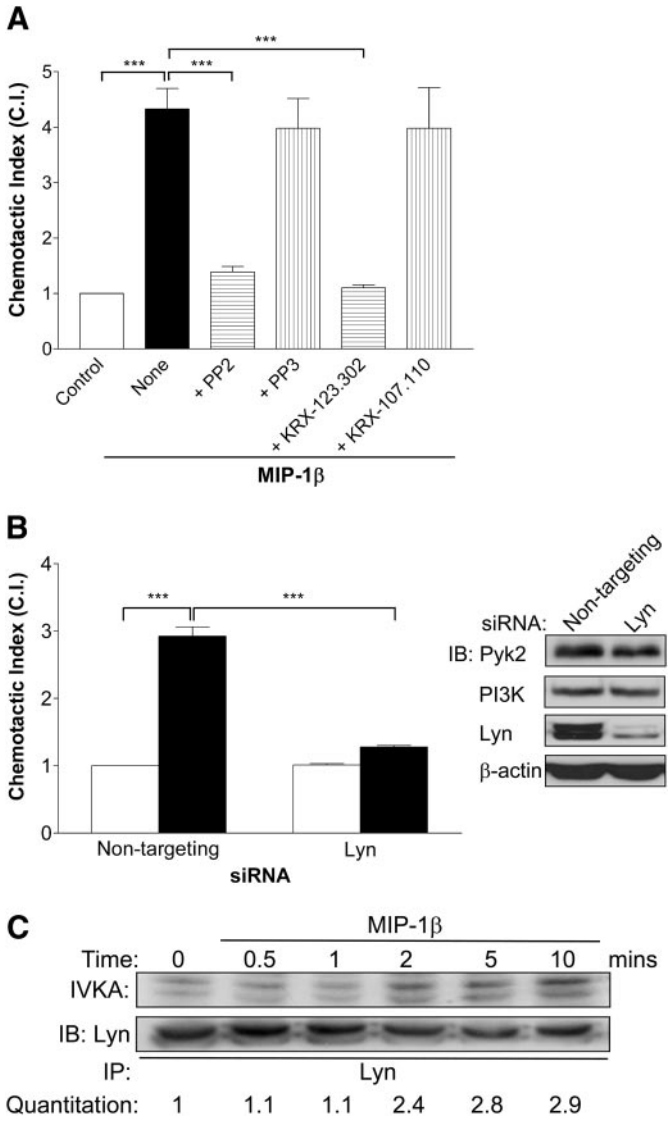

Figure 2. Macrophage chemotaxis in response to MIP-1β requires activation of Src family kinase Lyn. (A) Macrophages were pretreated for 1–2 h with the broad-spectrum SFK inhibitor PP2 or its inactive analog PP3 (each at 10 μM), a Lyn-specific peptide inhibitor KRX-123.302 or its negative control peptide KRX-107.110 (each at 10 μM), or control vehicle alone (0.1% DMSO) and then stimulated with MIP-1β. (B) Macrophages were transfected with nontargeting control or Lyn-specific siRNA prior to stimulation without (open bars) or with (solid bars) MIP-1β to induce chemotaxis. To monitor protein expression, immunoblots (IB) were performed in parallel to the chemotaxis assay on the same batch of transfected macrophages. Inset, Immunoblots showing Pyk2, PI3K, Lyn, and β-actin protein levels in siRNA-transfected macrophages from representative parallel transfection. (C) Macrophages were stimulated with MIP-1β for indicated times prior to cell lysis. Cell lysates were immunoprecipitated (IP) with anti-Lyn antibody for 4 h at 4°C. Half of the immunoprecipitate was subjected to in vitro kinase assay (IVKA; upper panel), and the other half was subjected to immunoblot with anti-Lyn antibody to determine total protein (lower panel). Data shown are means ± SE or representative blots of three independent experiments with cells from different blood donors (***, $P<0.001$).

M657. Furthermore, CCR5-deficient macrophages from CCR5Δ32 homozygous donors failed to migrate toward MIP-1β but responded normally to SDF-1α and fMLP (Fig. 1B), which mediate cell movement through CXCR4 and fMLP receptors, respectively. Heat denaturation eliminated the MIP-1β response (data not shown). A modified checkerboard analysis (Fig. 1C) showed that macrophages migrated only when a concentration gradient existed between upper and lower compartments, confirming the response was chemotaxis rather than chemokinesis.

Although $G_i\alpha$ is the primary G-protein coupled to CCR5, other G-proteins as well as G-protein-independent pathways have also been implicated [1, 9–11]. Therefore, we determined whether CCR5-mediated macrophage chemotaxis toward MIP-1β requires $G_i\alpha$ coupling. Pretreatment with the $G_{i/o}$ inhibitor PTX markedly attenuated MIP-1β-induced chemotaxis, but the G_s-specific inhibitor NF449 [12] had no effect (Fig. 1D). Together, these results indicate that MIP-1β induces macrophage chemotaxis through binding to CCR5 and coupling of $G_i\alpha$.

Activation of Lyn is required for MIP-1β-induced macrophage chemotaxis

We reported previously that CCR5-elicited macrophage TNF-α and IL-1β production were mediated through the SFK Lyn [3, 4]. To determine if Lyn is required for chemotaxis, we tested the broad SFK inhibitor PP2 and a Lyn-specific pseudosubstrate peptide inhibitor KRX-123.302 [7]. As shown in **Figure 2A**, PP2 and KRX-123.302 blocked chemotaxis toward MIP-1β, whereas the inactive analog PP3 and control peptide KRX-107.110 had no effect. In parallel, we monitored the effects on macrophage viability, which excluded inhibitor cytotoxicity as a cause of decreased migration (data not shown).

We then confirmed Lyn involvement using siRNA gene knockdown. As shown in Figure 2B, siRNA silencing reduced Lyn protein expression in primary macrophages by ~88%, whereas other proteins, including Pyk2, PI3K, and β-actin, were unaffected. Lyn knockdown impaired MIP-1β-induced macrophage chemotaxis significantly (Fig. 2B). Inhibition of migration was not a result of cytotoxicity, as there was no difference in viability between control and Lyn siRNA-transfected macrophages (data not shown). In accord with this functional data implicating Lyn in macrophage migration, an in vitro kinase assay confirmed that MIP-1β increased Lyn kinase activity (Fig. 2C).

PI3Kp85α is involved in MIP-1β-induced macrophage chemotaxis

In macrophages, CCR5 activation of PI3K regulates cytokine production and survival [2, 4, 13], but it is unknown whether this pathway is involved in chemotaxis. As shown in **Figure 3A**, migration toward MIP-1β was markedly inhibited by LY294002 and wortmannin, two potent PI3K inhibitors with different mechanisms of action.

GPCRs, including chemokine receptors, are classically linked to class IB PI3K (p101–p110γ) isoforms, and class IA (p85-p110α,β,δ) are typically activated by RTKs [14]. However, class

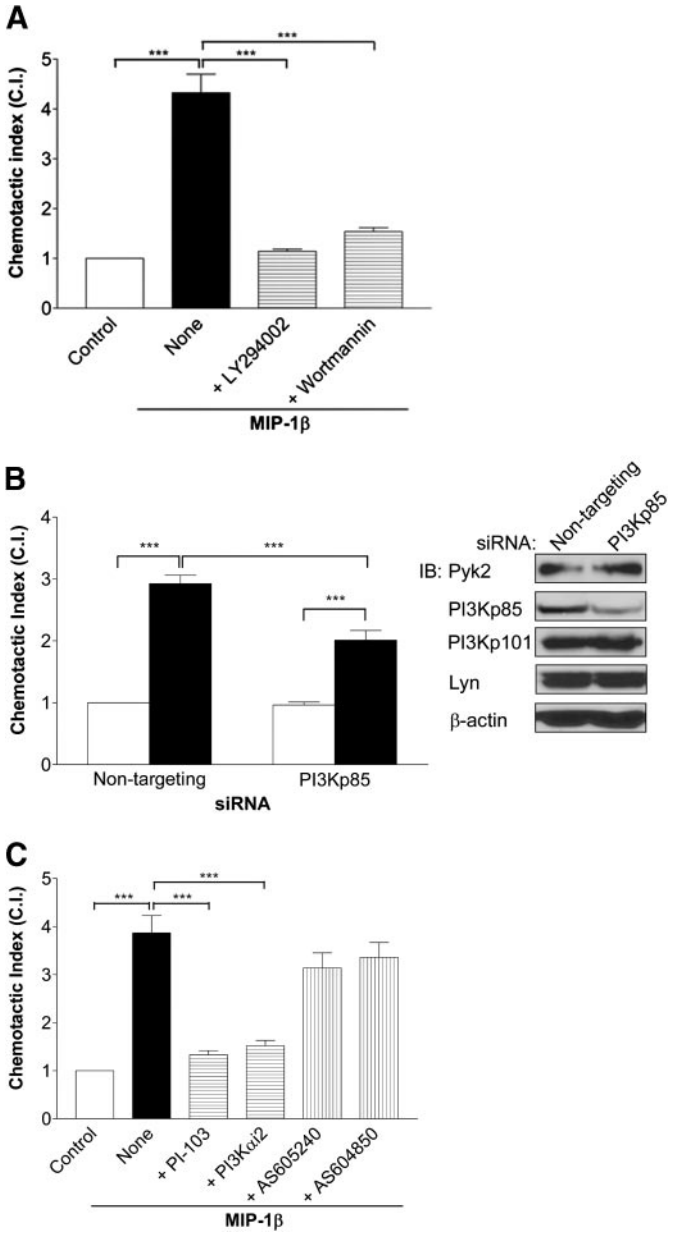

Figure 3. MIP-1β-induced chemotaxis of macrophages requires class IA PI3K activation. (A) Macrophages were pretreated for 1–2 h with the PI3K inhibitor LY294002 (10 μM), wortmannin (100 nM), or control vehicle alone (0.1% DMSO) prior to exposure to MIP-1β. (B) Chemotaxis of unstimulated (open bars) or MIP-1β-stimulated (solid bars) macrophages that had been transfected with control or PI3Kp85-specific siRNA. Inset, Immunoblots for Pyk2, PI3Kp85, PI3Kp101, Lyn, and β-actin protein were performed in parallel on the same batch of siRNA-transfected macrophages. (C) Macrophages were pretreated for 30–60 min with the isoform-specific class IA PI3K inhibitors (horizontal striped bars) PI-103 (40 nM) and PI3Kαi2 (10 nM), class IB PI3K inhibitors (vertical striped bars) AS605240 (10 nM) and AS604850 (250 nM), or control vehicle alone (open bar; 0.1% DMSO), followed by stimulation with MIP-1β. Data shown are means ± SE of three independent experiments with cells from different donors, along with representative immunoblots (***, $P<0.001$).

IA PI3K may also be activated by GPCRs and in some models, are involved in chemokine receptor-mediated leukocyte migration [15–19]. We therefore used siRNA and specifically reduced PI3Kp85α protein expression by ~68%, whereas Pyk2, PI3Kp101, Lyn, and β-actin were unaffected (Fig. 3B). PI3Kp85α knockdown suppressed macrophage chemotaxis (Fig. 3B), implicating the p85α isoform in the response to MIP-1β. We attempted to suppress class IB PI3K by silencing p101 and p110γ genes in primary human MDM but could not achieve efficient knockdown of either protein (data not shown). Therefore, we used recently available isoform-specific pharmacological PI3K inhibitors. As shown in Figure 3C, MIP-1β-stimulated chemotaxis was blocked significantly by PI-103 and PI3Kαi2, two agents that inhibit class IA but not IB PI3Ks [20]. In contrast, class IB-specific inhibitors AS605240 and AS604850 [21] had minimal, nonsignificant effects. Thus, CCR5-mediated macrophage chemotaxis depends largely on class IA PI3Kp85α, and involvement of class IB PI3K, if any, is likely minor. This result contrasts with the classical involvement of class IB PI3K in GPCR-mediated signaling and some studies of murine macrophage chemotaxis [21, 22] but consistent with other reports of class IA involvement in chemotaxis and chemokine receptor-mediated activation of monocytes, macrophages, and other cell types [17–19, 23].

MIP-1β-induced chemotaxis requires Pyk2

Studies from our laboratory and others have shown that CCR5 activates Pyk2 in several cell types and regulates cell movement in some models [1, 4, 24, 25], but whether it is involved in MIP-1β-induced macrophage chemotaxis is unknown. As specific Pyk2 inhibitors are not available, we first used agents targeting upstream activators of Pyk2 that are often used as indicators of Pyk2 involvement (**Fig. 4A**). Although not completely specific, dantrolene blocks the ryanodine receptor-mediated intracellular calcium release important for Pyk2 activation [26], and AG17 blocks calcium release-activated calcium channel-mediated intracellular calcium elevation [27]. Dantrolene and AG17 abrogated chemotaxis toward MIP-1β, whereas the inactive compound AG43 had no effect.

To assess Pyk2 dependence more specifically, we then transfected primary macrophages with Pyk2-specific siRNA. Pyk2 knockdown suppressed protein expression by ~72% in a gene-specific manner and attenuated MIP-1β-induced migration significantly (Fig. 4B). Consistent with these findings, phosphotyrosine immunoblotting confirmed Pyk2 activation following

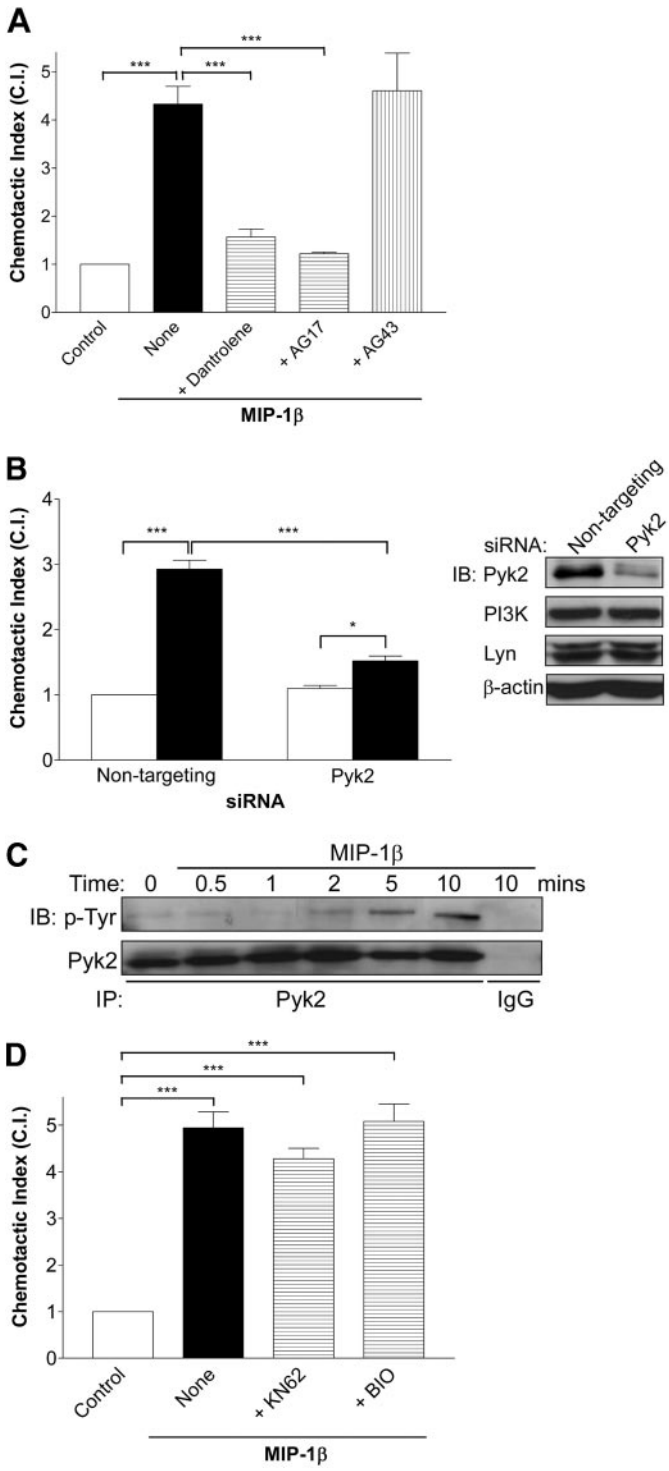

Figure 4. Chemotaxis of macrophages triggered by MIP-1β requires proline-rich tyrosine kinase Pyk2. (A) Macrophages were pretreated for 15–60 min with the upstream Pyk2 inhibitors dantrolene (10 μM) and AG17 (20 μM), inactive analog AG43 (20 μM), or control vehicle alone (0.1% DMSO) prior to stimulation with MIP-1β. (B) Macrophages were transfected with nontargeting control or Pyk2-specific siRNA followed by stimulation without (open bars) or with (solid bars) MIP-1β to induce chemotaxis. Immunoblots were performed in parallel on the same batch of transfected macrophages. (C) Macrophages were stimulated with MIP-1β for indicated times prior to cell lysis. Cell lysates were immunoprecipitated with anti-Pyk2 antibody or control IgG and immunoblotted with anti-phosphotyrosine (p-Tyr) antibody (upper panel) to assess Pyk2 activation. The blots were stripped and reprobed with anti-Pyk2 antibody to determine total Pyk2 protein (lower panel). (D) Macrophages were pretreated with the CaMKII inhibitor KN62 (1 μM), GSK3 inhibitor BIO (10 nM), or control vehicle alone (0.1% DMSO) for 1 h prior to stimulation with MIP-1β. Data shown are the means ± SE of three independent experiments with cells from different donors, along with representative blots (***, $P<0.001$).

MIP-1β stimulation (Fig. 4C) and showed a time course similar to that observed for Lyn activation (Fig. 2C).

Given the calcium dependence of macrophage Pyk2 activation, we asked if there was a role for the CaMKII, which regulates multiple macrophage functions, lymphocyte chemotaxis, and Pyk2 activation in some cell model [28, 29]. We also tested GSK3, a downstream target of PI3K implicated in macrophage cytoskeletal rearrangement and chemokine receptor signaling [30]. Neither the CaMKII inhibitor KN62 nor GSK3 inhibitor BIO affected MIP-1β-triggered macrophage chemotaxis (Fig. 4D). In addition to excluding roles for CaMKII and GSK3, these results highlight the specificity of blocking effects seen with Lyn, PI3K, and Pyk2 inhibitors.

MIP-1β induces physical association among Lyn, PI3Kp85, and Pyk2 in macrophages

These data indicated that Lyn, PI3K, and Pyk2 are all critical for MIP-β-induced macrophage chemotaxis, so we next determined if a physical association existed among these kinases in primary human macrophages. We focused on endogenous proteins rather than overexpression systems to ensure that they were being tested under physiologically relevant conditions. WCLs of macrophages were subjected to immunoprecipitation followed by immunoblot analysis (**Fig. 5**).

When endogenous Lyn protein was immunoprecipitated in unstimulated macrophages, modest amounts of PI3Kp85 and Pyk2 were sometimes pulled down as well (Fig. 5A, first lane, and data not shown). In MIP-1β-stimulated macrophages, however, markedly increased amounts of PI3Kp85 and Pyk2 were coprecipitated with Lyn (second lane). In contrast, immunoprecipitation with nonspecific IgG yielded no signals (third lane, all panels). We then carried out reciprocal experiments using PI3Kp85 or Pyk2 antibody for immunoprecipitation, and immunoprecipitation of PI3Kp85 revealed stimulation-enhanced Pyk2 and Lyn coprecipitation (Fig. 5B), and concordant results were seen following Pyk2 immunoprecipitation (Fig. 5C).

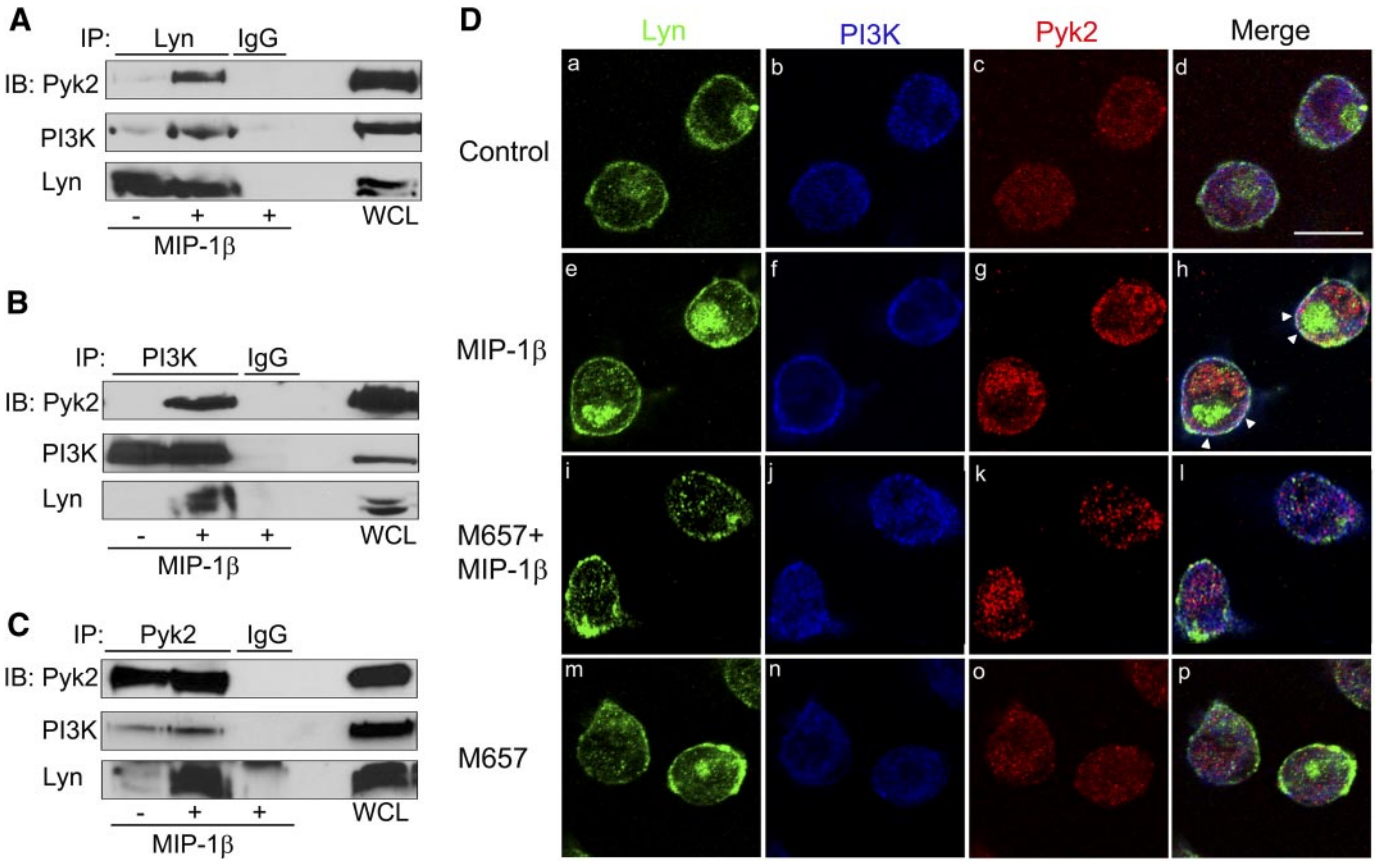

Figure 5. MIP-1β induces Lyn, PI3K, and Pyk2 association and colocalization to form a multi-kinase signaling complex. Macrophages were treated without or with MIP-1β for 5 min prior to cell lysis. Cell lysates were immunoprecipitated with antibody specific for (A) Lyn, (B) PI3K, (C) Pyk2, or with control IgG. Immune complexes were resolved on SDS-PAGE and subjected to immunoblot with antibodies specific for Pyk2, PI3K, and Lyn. WCL of unstimulated cells served as a positive control for immunoblotting. (D) Macrophages were pretreated without or with the CCR5 antagonist M657 (1 μM) for 1 h prior to stimulation with MIP-1β for 5 min. Macrophages were fixed, permeabilized, and triple-labeled with antibodies to Pyk2, PI3K, and Lyn before examination by confocal microscopy. Subcellular distribution of Lyn (green; a, e, i, m), PI3K (blue; b, f, j, n), and Pyk2 (red; c, g, k, o) is shown in the single-channel images. Colocalization of Lyn, PI3K, and Pyk2 is indicated by arrowheads shown in the Merge images (white; d, h, l, p). Original scale bar = 10 μm (shown in d; applies to a–p).

In contrast to Lyn, immunoprecipitation with antibody to Hck, another SFK abundantly expressed in macrophages, showed no constitutive or stimulation-induced association with PI3Kp85 or Pyk2, nor did probing the Lyn, PI3K, or Pyk2 immunoprecipitates with anti-Hck antibody yield any signal (data not shown). Of note, the degree of baseline association varied among cells from different donors, which may be a result of variable levels of basal activation and is typical of studies using primary human macrophages from different individuals [2, 31, 32]. Together, these results demonstrate that Lyn, PI3Kp85, and Pyk2 associate to form a complex that is up-regulated by MIP-1β stimulation.

MIP-1β induces subcellular colocalization of Lyn, PI3Kp85, and Pyk2

We next carried out triple-labeling immunofluorescence confocal microscopy to determine the subcellular distribution of these kinases and the putative signaling complex (Fig. 5D). In unstimulated macrophages (Fig. 5D, a–d), Lyn was found mainly at the plasma membrane, consistent with its typical subcellular distribution as a result of N-terminal myristoylation and palmitoylation. In contrast, PI3Kp85 was distributed at the plasma membrane and in the cytoplasm, and Pyk2 was localized primarily in the cytoplasm. Colocalization of the three kinases was minimal in unstimulated macrophages. Control experiments in which primary antibodies were omitted or substituted with isotype IgG of the same species showed no labeling (data not shown).

When macrophages were stimulated with MIP-1β (Fig. 5D, e–h), Lyn remained primarily at the plasma membrane, but the majority of PI3Kp85 and Pyk2 also became membrane-associated. MIP-1β-induced PI3K/Pyk2 translocation thus resulted in colocalization of the three kinases at the plasma membrane. Relocalization of PI3K and Pyk2 to the plasma membrane in response to MIP-1β was blocked by the CCR5 antagonist M657 (Fig. 5D, i–l). These results are in accord with the immunoprecipitation data and further support the notion that CCR5 stimulation induces a physical association among Lyn, PI3Kp85, and Pyk2.

Activation of ERK is important for MIP-1β-induced macrophage chemotaxis

Previous data about macrophages indicate that CCR5 activates MAPKs, which are important for cytokine and chemokine production [1–3]. ERK, p38, and JNK have each been implicated in regulating cell migration, but the roles of individual MAPKs in macrophage chemotaxis are defined incompletely. Therefore, we examined the involvement of ERK, p38, and JNK in MIP-1β-induced chemotaxis using specific pharmacological inhibitors. As shown in **Figure 6A**, two ERK inhibitors (U0126 and PD98059) with distinct modes of action abolished MIP-1β-triggered chemotaxis. In contrast, neither the p38 inhibitors (SB202190 and PD169316) nor JNK inhibitor (SP600125) affected chemotaxis. None of the MAPK inhibitors affected macrophage viability (data not shown). Immunoblotting confirmed that MIP-1β induced ERK phosphorylation in a manner that was

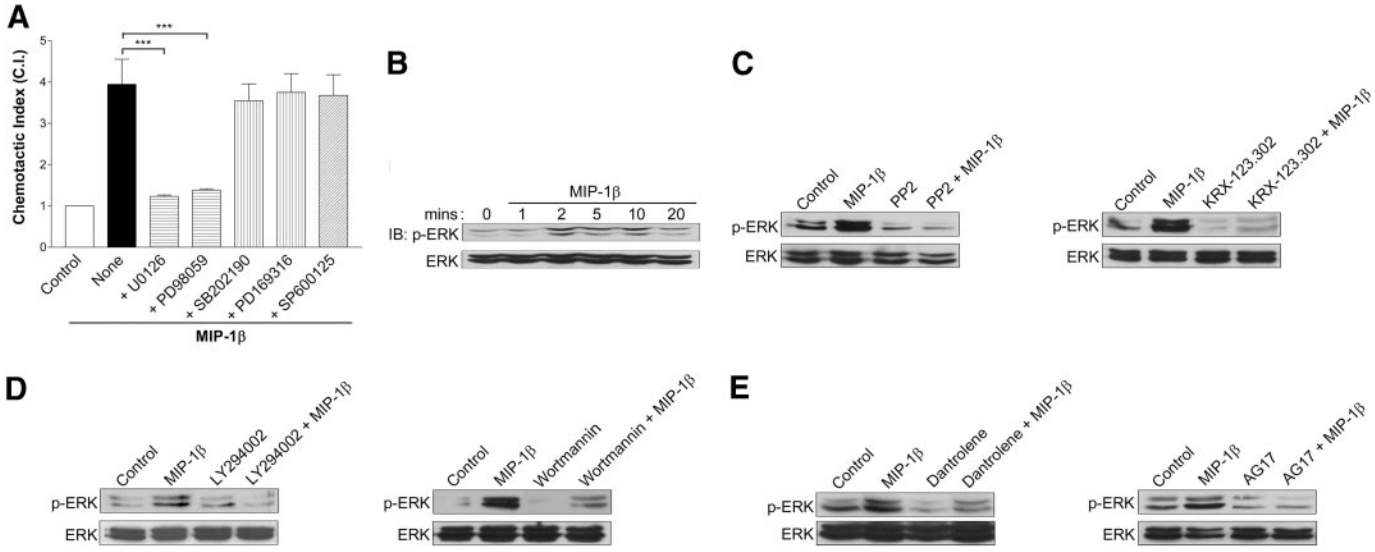

Figure 6. Macrophage chemotaxis in response to MIP-1β involves ERK, which is downstream of Lyn and PI3K. (A) Macrophages were pretreated with inhibitors of ERK (horizontal striped bars) U0126 (1 μM, 1 h) and PD98059 (10 μM, 2 h); p38 (vertical striped bars) SB202190 and PD169316 (both 10 μM for 1 h); JNK (hatched bar) SP600125 (100 nM, 1 h); or vehicle control (open bar; 0.1% DMSO) prior to stimulation with MIP-1β. (B) Macrophages were stimulated with MIP-1β for indicated times prior to cell lysis. Activation of ERK was determined by immunoblot of cell lysates with an antibody specific for the phosphorylation sites of ERK (p-ERK; upper panel). The blots were stripped and reprobed with anti-ERK antibody to determine total ERK protein (lower panel). (C–E) Macrophages were pretreated with inhibitors for (C) Lyn [KRX-123.302 (10 μM, 2 h)] or Src family kinase [PP2 (10 μM, 1 h)]; (D) PI3K [LY294002 (10 μM, 2 h) or wortmannin (100 nM, 1 h)]; or (E) Pyk2 [dantrolene (10 μM, 15 min) or AG17 (20 μM, 1 h)]. Cells were stimulated with MIP-1β (1 nM, 5 min), and WCLs were resolved by SDS-PAGE and immunoblotted with anti-phosphorylated ERK (upper panels) or anti-ERK (lower panels) antibody. Data reflect means ± SE of three independent experiments with cells from different donors, along with representative blots (***, $P<0.001$).

time-dependent (Fig. 6B) and similar to kinetics shown previously for Lyn and Pyk2 (Figs. 2C and 4C). These data indicated that ERK activation is involved in MIP-1β-induced chemotaxis of primary macrophages, and p38 and JNK are not.

We next asked if ERK activation was linked to Lyn, PI3K, and Pyk2. Macrophages were pretreated with Lyn, PI3K, or Pyk2 inhibitors, and ERK phosphorylation was detected by immunoblotting. As shown in Figure 6C, treatment with the broad-spectrum SFK inhibitor PP2 or the Lyn-specific peptide inhibitor KRX-123.302 inhibited MIP-1β-stimulated ERK phosphorylation, whereas control compound and peptide did not. Similarly, PI3K inhibitors LY294002 and wortmannin markedly attenuated ERK phosphorylation triggered by MIP-1β (Fig. 6D). The two upstream inhibitors of Pyk2, dantrolene and AG17, also blocked ERK phosphorylation (Fig. 6E). Together, these results suggested that MIP-1β-induced ERK activation is regulated by Lyn, PI3K, and Pyk2 in this CCR5-mediated macrophage signaling cascade.

Arrestins physically associate with Lyn, PI3Kp85, Pyk2, and ERK in response to MIP-1β

Arrestins classically play a role in terminating GPCR-mediated signals but more recently, have been found to participate also in signal activation and in particular, can act as scaffolds to link GPCR-activated signaling molecules [33, 34]. Arrestins have been linked to CCR5 desensitization and internalization following chemokine stimulation in overexpression systems [35, 36], but a role in positive regulation of CCR5 signals has not been described. As our results demonstrated physical asso-

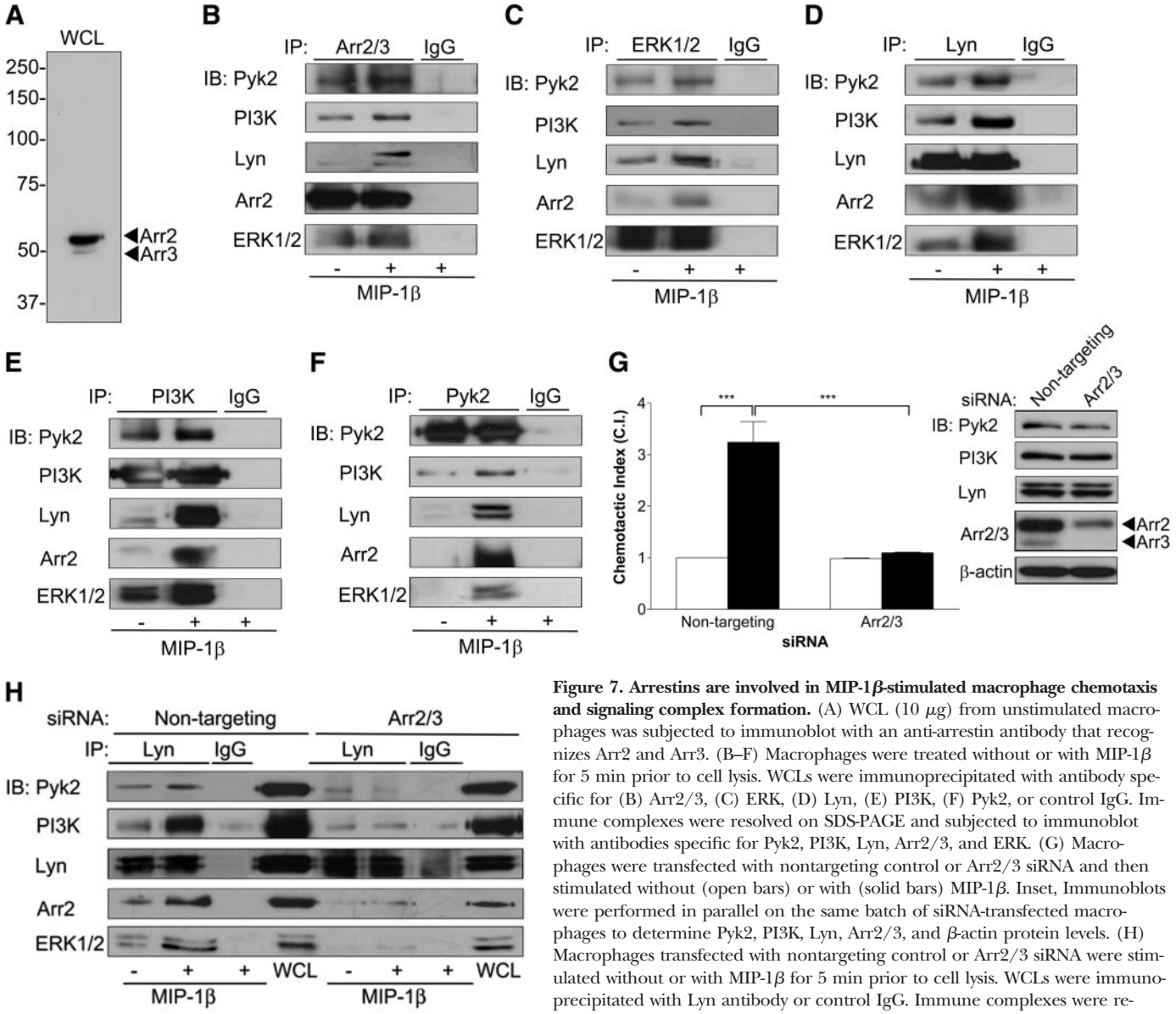

Figure 7. Arrestins are involved in MIP-1β-stimulated macrophage chemotaxis and signaling complex formation. (A) WCL (10 μg) from unstimulated macrophages was subjected to immunoblot with an anti-arrestin antibody that recognizes Arr2 and Arr3. (B–F) Macrophages were treated without or with MIP-1β for 5 min prior to cell lysis. WCLs were immunoprecipitated with antibody specific for (B) Arr2/3, (C) ERK, (D) Lyn, (E) PI3K, (F) Pyk2, or control IgG. Immune complexes were resolved on SDS-PAGE and subjected to immunoblot with antibodies specific for Pyk2, PI3K, Lyn, Arr2/3, and ERK. (G) Macrophages were transfected with nontargeting control or Arr2/3 siRNA and then stimulated without (open bars) or with (solid bars) MIP-1β. Inset, Immunoblots were performed in parallel on the same batch of siRNA-transfected macrophages to determine Pyk2, PI3K, Lyn, Arr2/3, and β-actin protein levels. (H) Macrophages transfected with nontargeting control or Arr2/3 siRNA were stimulated without or with MIP-1β for 5 min prior to cell lysis. WCLs were immunoprecipitated with Lyn antibody or control IgG. Immune complexes were resolved on SDS-PAGE and immunoblotted with antibodies specific for Pyk2, PI3K, Lyn, Arr2, and ERK. Data shown are means ± SE of three independent experiments with cells from different donors, along with representative blots (***, $P<0.001$).

ciation among Lyn, PI3Kp85, and Pyk2, we asked if arrestins were involved in this complex.

Arr2 and Arr3 (β-arrestins 1 and 2) are expressed in murine macrophage-like cell lines [37], but the arrestin subtypes expressed in primary human macrophages have not been reported. Therefore, we carried out immunoblot analysis of unstimulated macrophages using an antibody recognizing Arr2 and Arr3 (**Fig. 7A**). Immunoblotting detected a strong band migrating at an apparent molecular mass of 54 kDa and a fainter band at ~48 kDa, which are consistent with Arr2 and Arr3, respectively [38], suggesting that primary human macrophages express Arr2 and Arr3, with Arr2 as the predominant subtype.

Next, we asked if endogenous arrestins associate with the Lyn/PI3Kp85/Pyk2 complex in primary macrophages, as well as with ERK. WCLs of unstimulated and MIP-1β-stimulated macrophages were subjected to immunoprecipitation and immunoblot analysis using the Arr2/3 antibody, as well as antibodies to each of the implicated signaling molecules.

As shown in Figure 7B, immunoprecipitation with Arr2/3 antibody pulled down a 54 kDa protein consistent with Arr2. In unstimulated macrophages (first lane), modest amounts of Pyk2, PI3Kp85, and ERK (and to a lesser extent, Lyn) were coprecipitated, levels of which varied somewhat among donors, suggesting a low but variable level of constitutive association among Arr2/3 and these kinases. This association was markedly up-regulated by MIP-1β stimulation (second lane), indicating that complex assembly was triggered by CCR5 activation. Reciprocal experiments were then performed in which ERK, Lyn, PI3Kp85, and Pyk2 were immunoprecipitated, followed by immunoblotting for other members of this signaling complex (Fig. 7, C–F). Although the absolute levels of baseline association and the magnitude of up-regulation triggered by MIP-1β varied somewhat among donors, these results confirmed that endogenous Arr2/3 associates with ERK as well as Lyn, PI3Kp85, and Pyk2 in a complex that is augmented by CCR5 activation.

MIP-1β-stimulated chemotaxis and signaling complex formation are arrestin-dependent

We next tested if arrestins play a role in MIP-1β-induced macrophage chemotaxis using a combination of siRNAs that target Arr2 and Arr3, along with a control siRNA. As shown in Figure 7G, Arr2/3 siRNA suppressed endogenous Arr2 and Arr3 expression by ~81% and ~93%, respectively, with no effect on other proteins including Pyk2, PI3K, Lyn, and β-actin. Arr2/3 silencing abolished migration toward MIP-1β completely, and macrophages transfected with control siRNA exhibited normal MIP-1β-induced chemotaxis (Fig. 7G). Inhibition of chemotaxis was not a result of decreased cell viability, as there was no difference in viability of macrophages transfected with nontargeting control and Arr2/3 siRNA (data not shown).

To ascertain whether signaling complex assembly depended on arrestins, we then examined complex formation in Arr2/3 siRNA-transfected macrophages by coimmunoprecipitation (Fig. 7H). In macrophages transfected with control siRNA (left lanes), MIP-1β stimulation up-regulated Pyk2/PI3Kp85/Lyn/Arr2/ERK complex formation (Fig. 7H, left lanes), similar to untransfected macrophages (shown in Fig. 7D). In contrast, arrestin depletion abrogated the ability of MIP-1β to promote interaction of these signaling molecules (Fig. 7H, right lanes). This result indicates that arrestins are required for the MIP-1β-stimulated physical association among Pyk2, PI3K, Lyn, and ERK. Together, these data indicate that Arr2/3 play a crucial role in CCR5-mediated chemotaxis of primary human macrophages, likely by acting as scaffolds to assemble a multi-kinase signaling complex involving of Lyn, PI3K, Pyk2, and ERK.

MIP-1β-induced multi-kinase complex is not blocked by PTX

We show here that chemotaxis in response to MIP-1β is blocked by PTX, indicating involvement of $G_i\alpha$ proteins (Fig. 1D) and that Pyk2 is involved in the arrestin-dependent signaling complex required for chemotaxis (Fig. 4). In earlier studies, however, we reported that CCR5-mediated Pyk2 phosphorylation in macrophages was independent of $G_i\alpha$ [1]. Therefore, we aimed to determine whether formation of the MIP-1β-elicited multi-kinase signaling complex was dependent on $G_i\alpha$ signaling. Macrophages were stimulated by MIP-1β, with or without PTX pretreatment, and evaluated for complex formation by coimmunoprecipitation.

As shown in **Figure 8A**, PTX did not prevent MIP-1β-induced association of Pyk2, Lyn, and PI3K. Similarly, activation

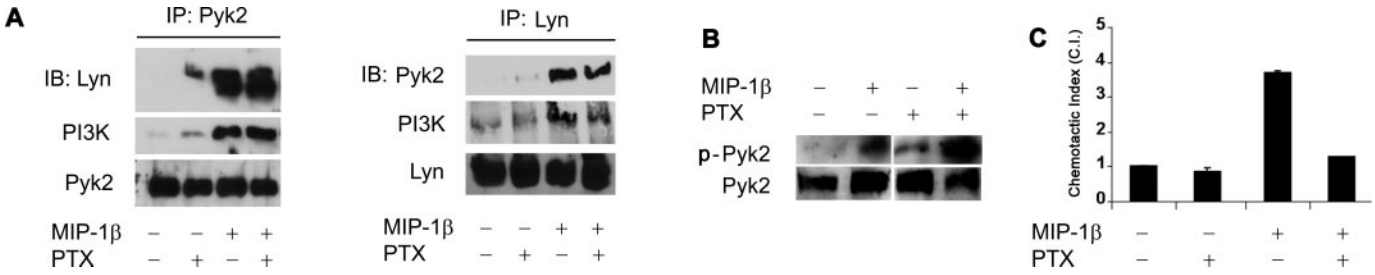

Figure 8. PTX does not block MIP-1β-induced formation of the Lyn/Pyk2/PI3K multi-kinase complex. (A) Macrophages were incubated for 2 h in the presence or absence of PTX (100 ng/ml) followed by stimulation with MIP-1β for 5 min. Cell lysates were immunoprecipitated with the indicated antibodies, resolved on SDS-PAGE, and subjected to immunoblot with antibodies specific to Pyk2, PI3K, and Lyn. (B) Macrophages were stimulated with MIP-1β, with or without PTX pretreatment, and WCLs were analyzed by immunoblotting with an antibody specific for the phosphorylated form of Pyk2 (p-Pyk2) to assess Pyk2 activation or anti-Pyk2 antibody to determine total Pyk2 protein. (C) Macrophages were pretreated with or without PTX for 2 h prior to measuring chemotaxis in response to MIP-1β.

of Pyk2 by MIP-1β was insensitive to PTX (Fig. 8B), which is consistent with our data published previously [1]. Thus, taken together with the PTX sensitivity of macrophage migration in response to MIP-1β (Figs. 1D and 8C), these data indicate that MIP-1β/CCR5-elicited macrophage chemotaxis requires both $G_i\alpha$-dependent signaling, and a $G_i\alpha$-independent pathway that involves Pyk2 activation and arrestin-mediated Lyn/PI3K/Pyk2 complex formation (see Fig. 10).

RANTES-induced chemotaxis is also associated with the Lyn/PI3K/Pyk2 complex formation

Finally, we asked whether another CCR5 ligand, RANTES, also up-regulated this multi-kinase signaling complex in macrophages. A concentration-response analysis showed a bell-shaped curve typical of chemokines with maximal migration at 100 ng/ml (**Fig. 9A**). Like MIP-1β, RANTES-induced migration was inhibited by PTX (Fig. 9B). Similar to our finding with MIP-1β, RANTES stimulation also up-regulated Lyn, Pyk2, and PI3K coassociation (Fig. 9C). Thus, MIP-1β and RANTES induce formation of a complex involving Lyn, PI3K, and Pyk2, suggesting that this is a consistent mechanism of CCR5 signaling in response to multiple ligands.

DISCUSSION

Macrophage chemotaxis plays a critical role in host defense and many pathological inflammatory responses, but the signaling pathways regulating macrophage migration are incompletely defined. Here, we showed that macrophage chemotaxis mediated through CCR5 requires concomitant activation of protein kinases Lyn, PI3K, Pyk2, and ERK; that these kinases act coordinately with formation of a multimeric signaling complex; and that arrestins play a role structurally in complex assembly and functionally in linking CCR5 to chemotaxis (**Fig. 10**).

SFKs, PI3K, and Pyk2 have each individually been implicated in modulating migration in many cell types [39–41], including mouse macrophages [23, 42–44]. Our study is the first to demonstrate in a primary human cell system that concomitant activation of all three kinases is necessary for chemokine receptor-mediated macrophage migration. Our results are in agreement with some cell line models showing coordinated activation of SFK, PI3K, and Pyk2 (or FAK) in response to GPCR and RTK stimulation and that migration may be abrogated by blocking any single pathway [45, 46]. These results are also consistent with our recent report that release of IL-1β by macrophages exposed to HIV-1 virions or gp120 envelope glycoprotein that interact with CCR5 involves activation and association of Lyn, PI3K, and Pyk2 [4], and it is likely that the complex induced following HIV-1 exposure is also arrestin-dependent. As RANTES-induced chemotaxis is also associated with Lyn/Pyk2/PI3K complex formation (Fig. 9), these results suggest that this interaction is an important general mechanism by which CCR5 signals in macrophages. It will be important to determine if formation of this complex is seen in other cell types that express CCR5 and whether other chemokine receptors signal through a similar mechanism.

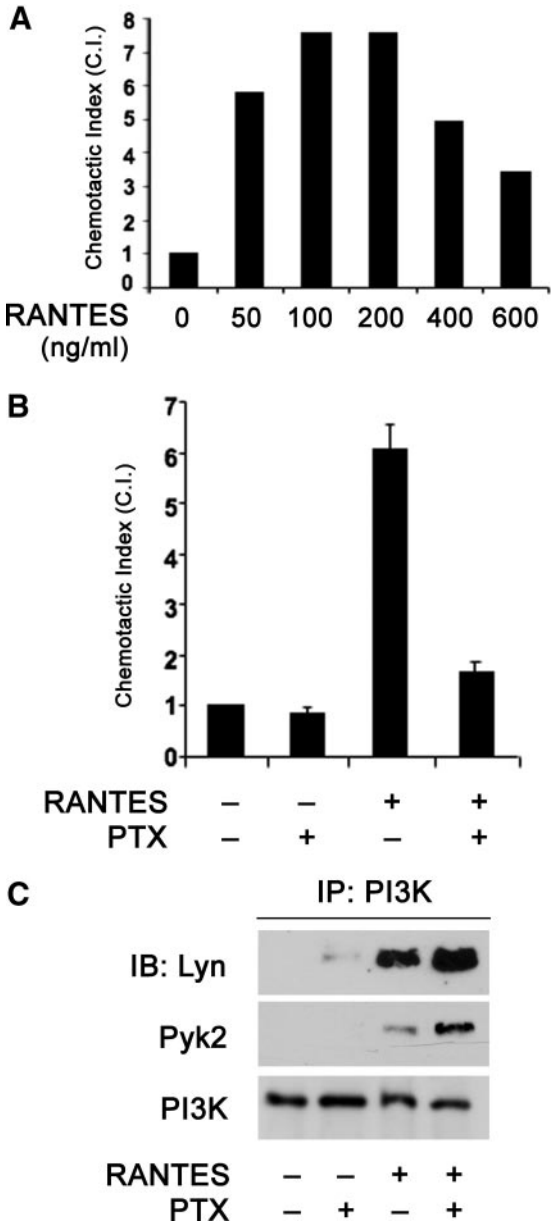

Figure 9. Lyn/PI3K/Pyk2 complex up-regulation in response to RANTES in primary human macrophages. (A) Dose-dependent migration of primary macrophages in response to RANTES. (B) Migration in response to RANTES (100 ng/ml) was measured following pretreatment for 2 h with or without PTX (100 ng/ml). (C) Macrophages with or without PTX treatment were stimulated for 5 min with RANTES, and WCLs were immunoprecipitated with PI3K antibody. Immune complexes were resolved on SDS-PAGE and immunoblotted with antibodies specific for Lyn, Pyk2, or PI3K.

Several possibilities could explain why inhibiting a single kinase would result in near-complete rather than partial attenuation of chemotaxis. One mechanism is that Lyn, PI3K, and Pyk2 could act in sequence such that activation of one kinase leads to activation of a downstream partner. Another nonmutually exclusive possibility is that Lyn, PI3K, and Pyk2 act in parallel to control different crucial downstream targets. Lyn

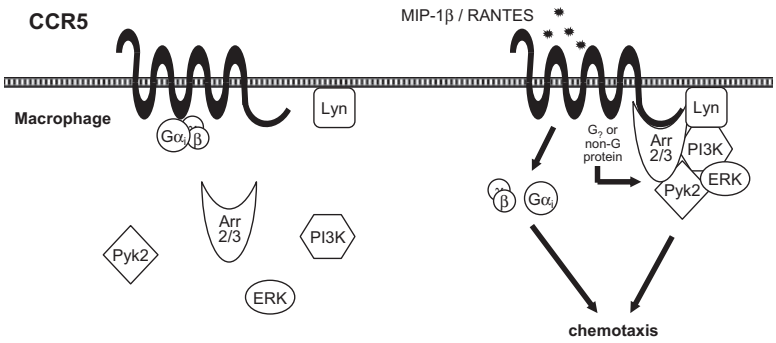

Figure 10. A proposed model for signaling mechanisms of CCR5-induced chemotaxis in primary human macrophages. Binding of MIP-1β or RANTES to macrophage CCR5 triggers $G_i\alpha$-dependent signaling and $G_i\alpha$-independent mechanisms that induce translocation of arrestins, PI3K and Pyk2 to the membrane to coassociate with Lyn and form a multi-kinase signaling complex. CCR5 stimulation also triggers the activation of downstream MAPK ERK and association with the arrestin-mediated complex, which together, result in macrophage chemotaxis.

and other SFKs can phosphorylate actin-binding proteins directly, including vinculin, talin, and tensin, to modulate cytoskeletal rearrangement and cell migration [47–49]. PI3K can trigger actin reorganization and cell migration through multiple downstream mediators including Rac, WASP/WASP family verprolin-homologous protein, and other proteins [50]. Pyk2 can bind and phosphorylate paxillin, which is involved in macrophage migration [51]. Defining how each of these kinases links to cell migration machinery in macrophages will require further studies.

We found that CCR5 activation triggers PI3Kp85α and Pyk2 translocation from the macrophage cytoplasm to the plasma membrane, where they colocalize with Lyn and form a multimolecular complex. Complex formation is recognized increasingly as a mechanism of integrating signals and modulating specificity. Lyn, PI3K, and Pyk2 possess structural motifs known to facilitate protein–protein interactions. Lyn and PI3K each contain SH2 domains that can interact with phosphorylated tyrosine residues and SH3 domains that bind to proline-rich motifs [52, 53]. PI3K and Pyk2 possess proline-rich motifs that can interact with SH3 domains [54, 55]. Furthermore, all three kinases contain tyrosine residues that undergo phosphorylation upon activation [56–58]. Additional studies will be needed to define how these motifs are involved in interactions among these kinases and with arrestins. In a murine cell lines overexpressing CCR5, MIP-1β-induced chemotaxis was associated with a complex involving Pyk2, Syk, SH2-containing tyrosine phosphatase 1, and growth factor receptor-bound granulocyte chemotactic protein 2 [24]. Whether these additional molecules participate in the signaling complex identified here in primary human macrophages remains to be determined. Interestingly, non-GPCR-mediated migration of different cell lines has also been associated with Src, PI3K, and Pyk2 (or FAK) complex formation [45, 59].

In several cell types, arrestins link GPCRs to MAPK activation and downstream functions [34, 60], including p38 MAPK-mediated chemotaxis in CXCR4-stimulated human embryo kidney 293 cells [61]. Consistent with this, we demonstrated here in primary macrophages that arrestins mediate ERK incorporation into this CCR5-induced complex. Furthermore, formation of the Lyn/PI3K/Pyk2 signaling complex itself is dependent on arrestins. Arrestin-dependent Src/ERK complex formation has been shown previously in GPCR-stimulated rat kidney cells [33], and our results extend the members of the signaling complex incorporated through the arrestin mechanism. Indeed, this is the first evidence to our knowledge for Pyk2 recruitment into an arrestin-dependent signaling complex. On the other hand, direct interactions between Arr2/3 and SFK and PI3Kp85 are, like ERK, well-established [46, 62]. Additional molecules are recruited by arrestins into chemokine receptor-triggered signaling complexes in other cell types, such as the small GTPase Rho, Rac, and Cdc42 following CXCR4 stimulation of T cells [63, 64], and further studies will be important to determine if these or other molecules are also recruited by arrestins in CCR5-stimulated primary human macrophages.

Arrestins are known to mediate CCR5 desensitization and internalization [35] but have not been identified previously as positive mediators of CCR5 function. These results, involving at least four kinases, suggest that arrestins likely act as scaffolds upon which multiple signaling molecules are recruited in response to CCR5 activation and that formation of this complex is required for chemotaxis. Chemotaxis has been shown to be arrestin-dependent in several cell lines [61, 65], as well as in response to CXCR2 and CXCR4 stimulation in lymphocytes and neutrophils from Arr3 knockout mouse [66, 67] but has not been examined previously in macrophages. Finally, our finding that chemotaxis triggered by MIP-1β through CCR5 requires arrestin-dependent/$G_i\alpha$-independent scaffolding and $G_i\alpha$-dependent signaling (Fig. 10) suggests a synergistic mechanism involving these two pathways of GPCR coupling [5]. Responses requiring cooperative signaling through G protein and arrestin pathways have been described for a few GPCRs such as angiotensin II type 1A, delta opioid, and growth hormone secretagogue type 1a receptors [5, 68–70] but have not been identified previously for chemokine receptors. The mechanisms involved in $G_i\alpha$-independent arrestin activation, and the nature of the $G_i\alpha$-dependent responses will require further investigation.

In addition to normal host-defense function, dysregulated chemokines and aberrant macrophage migration have been implicated in the pathogenesis of many inflammatory diseases including sarcoidosis, rheumatoid arthritis, multiple sclerosis, atherosclerosis, inflammatory bowel disease, and HIV encephalopathy. Indeed, the MIP-1β concentration used here (1 nM) is comparable with levels observed in synovial fluid and serum of subjects with rheumatoid arthritis and sepsis, respectively [71, 72]. Results from our study help elucidate the signaling pathways involved in chemokine-triggered chemotaxis of primary human macrophages and provide insight into the mecha-

nism of interaction, which are important in understanding the pathogenesis of diseases involving dysregulation of macrophage migratory function.

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REFERENCES

1. Del Corno, M., Liu, Q. H., Schols, D., de Clercq, E., Gessani, S., Freedman, B. D., Collman, R. G. (2001) HIV-1 gp120 and chemokine activation of Pyk2 and mitogen-activated protein kinases in primary macrophages mediated by calcium-dependent, pertussis toxin-insensitive chemokine receptor signaling. *Blood* **98,** 2909–2916.
2. Lee, C., Tomkowicz, B., Freedman, B. D., Collman, R. G. (2005) HIV-1 gp120-induced TNF-{α} production by primary human macrophages is mediated by phosphatidylinositol-3 (PI-3) kinase and mitogen-activated protein (MAP) kinase pathways. *J. Leukoc. Biol.* **78,** 1016–1023.
3. Tomkowicz, B., Lee, C., Ravyn, V., Cheung, R., Ptasznik, A., Collman, R. G. (2006) The Src kinase Lyn is required for CCR5 signaling in response to MIP-1β and R5 HIV-1 gp120 in human macrophages. *Blood* **108,** 1145–1150.
4. Cheung, R., Ravyn, V., Wang, L., Ptasznik, A., Collman, R. G. (2008) Signaling mechanism of HIV-1 gp120 and virion-induced IL-1β release in primary human macrophages. *J. Immunol.* **180,** 6675–6684.
5. Defea, K. (2008) β-Arrestins and heterotrimeric G-proteins: collaborators and competitors in signal transduction. *Br. J. Pharmacol.* **153** (Suppl. 1), S298–S309.
6. Finke, P. E., Meurer, L. C., Oates, B., Mills, S. G., MacCoss, M., Malkowitz, L., Springer, M. S., Daugherty, B. L., Gould, S. L., DeMartino, J. A., Siciliano, S. J., Carella, A., Carver, G., Holmes, K., Danzeisen, R., Hazuda, D., Kessler, J., Lineberger, J., Miller, M., Schleif, W. A., Emini, E. A. (2001) Antagonists of the human CCR5 receptor as anti-HIV-1 agents. Part 2: structure-activity relationships for substituted 2-Aryl-1-[N-(methyl)-N-(phenylsulfonyl)amino]-4-(piperidin-1-yl)butanes. *Bioorg. Med. Chem. Lett.* **11,** 265–270.
7. Goldenberg-Furmanov, M., Stein, I., Pikarsky, E., Rubin, H., Kasem, S., Wygoda, M., Weinstein, I., Reuveni, H., Ben-Sasson, S. A. (2004) Lyn is a target gene for prostate cancer: sequence-based inhibition induces regression of human tumor xenografts. *Cancer Res.* **64,** 1058–1066.
8. Rana, S., Besson, G., Cook, D. G., Rucker, J., Smyth, R. J., Yi, Y., Turner, J. D., Guo, H. H., Du, J. G., Peiper, S. C., Lavi, E., Samson, M., Libert, F., Liesnard, C., Vassart, G., Doms, R. W., Parmentier, M., Collman, R. G. (1997) Role of CCR5 in infection of primary macrophages and lymphocytes by macrophage-tropic strains of human immunodeficiency virus: resistance to patient-derived and prototype isolates resulting from the Δ CCR5 mutation. *J. Virol.* **71,** 3219–3227.
9. Zhao, J., Ma, L., Wu, Y. L., Wang, P., Hu, W., Pei, G. (1998) Chemokine receptor CCR5 functionally couples to inhibitory G proteins and undergoes desensitization. *J. Cell. Biochem.* **71,** 36–45.
10. Gosling, J., Monteclaro, F. S., Atchison, R. E., Arai, H., Tsou, C. L., Goldsmith, M. A., Charo, I. F. (1997) Molecular uncoupling of C-C chemokine receptor 5-induced chemotaxis and signal transduction from HIV-1 coreceptor activity. *Proc. Natl. Acad. Sci. USA* **94,** 5061–5066.
11. Leach, K., Charlton, S. J., Strange, P. G. (2007) Analysis of second messenger pathways stimulated by different chemokines acting at the chemokine receptor CCR5. *Biochem. Pharmacol.* **74,** 881–890.
12. Hohenegger, M., Waldhoer, M., Beindl, W., Boing, B., Kreimeyer, A., Nickel, P., Nanoff, C., Freissmuth, M. (1998) Gsα-selective G protein antagonists. *Proc. Natl. Acad. Sci. USA* **95,** 346–351.
13. Tyner, J. W., Uchida, O., Kajiwara, N., Kim, E. Y., Patel, A. C., O'Sullivan, M. P., Walter, M. J., Schwendener, R. A., Cook, D. N., Danoff, T. M., Holtzman, M. J. (2005) CCL5-CCR5 interaction provides antiapoptotic signals for macrophage survival during viral infection. *Nat. Med.* **11,** 1180–1187.
14. Li, Z., Jiang, H., Xie, W., Zhang, Z., Smrcka, A. V., Wu, D. (2000) Roles of PLC-β2 and -β3 and PI3Kγ in chemoattractant-mediated signal transduction. *Science* **287,** 1046–1049.
15. Turner, L., Ward, S. G., Westwick, J. (1995) RANTES-activated human T lymphocytes. A role for phosphoinositide 3-kinase. *J. Immunol.* **155,** 2437–2444.
16. Vicente-Manzanares, M., Rey, M., Jones, D. R., Sancho, D., Mellado, M., Rodriguez-Frade, J. M., del Pozo, M. A., Yanez-Mo, M., de Ana, A. M., Martinez-A, C., Merida, I., Sanchez-Madrid, F. (1999) Involvement of phosphatidylinositol 3-kinase in stromal cell-derived factor-1 α-induced lymphocyte polarization and chemotaxis. *J. Immunol.* **163,** 4001–4012.
17. Boulven, I., Levasseur, S., Marois, S., Pare, G., Rollet-Labelle, E., Naccache, P. H. (2006) Class IA phosphatidylinositide 3-kinases, rather than p110 γ, regulate formyl-methionyl-leucyl-phenylalanine-stimulated chemotaxis and superoxide production in differentiated neutrophil-like PLB-985 cells. *J. Immunol.* **176,** 7621–7627.
18. Schymeinsky, J., Then, C., Sindrilaru, A., Gerstl, R., Jakus, Z., Tybulewicz, V. L., Scharffetter-Kochanek, K., Walzog, B. (2007) Syk-mediated translocation of PI3Kδ to the leading edge controls lamellipodium formation and migration of leukocytes. *PLoS ONE* **2,** e1132.
19. Selvaraj, S. K., Giri, R. K., Perelman, N., Johnson, C., Malik, P., Kalra, V. K. (2003) Mechanism of monocyte activation and expression of proinflammatory cytochemokines by placenta growth factor. *Blood* **102,** 1515–1524.
20. Knight, Z. A., Gonzalez, B., Feldman, M. E., Zunder, E. R., Goldenberg, D. D., Williams, O., Loewith, R., Stokoe, D., Balla, A., Toth, B., Balla, T., Weiss, W. A., Williams, R. L., Shokat, K. M. (2006) A pharmacological map of the PI3-K family defines a role for p110α in insulin signaling. *Cell* **125,** 733–747.
21. Camps, M., Ruckle, T., Ji, H., Ardissone, V., Rintelen, F., Shaw, J., Ferrandi, C., Chabert, C., Gillieron, C., Francon, B., Martin, T., Gretener, D., Perrin, D., Leroy, D., Vitte, P. A., Hirsch, E., Wymann, M. P., Cirillo, R., Schwarz, M. K., Rommel, C. (2005) Blockade of PI3Kγ suppresses joint inflammation and damage in mouse models of rheumatoid arthritis. *Nat. Med.* **11,** 936–943.
22. Lopez-Ilasaca, M., Crespo, P., Pellici, P. G., Gutkind, J. S., Wetzker, R. (1997) Linkage of G protein-coupled receptors to the MAPK signaling pathway through PI 3-kinase γ. *Science* **275,** 394–397.
23. Vanhaesebroeck, B., Jones, G. E., Allen, W. E., Zicha, D., Hooshmand-Rad, R., Sawyer, C., Wells, C., Waterfield, M. D., Ridley, A. J. (1999) Distinct PI(3)Ks mediate mitogenic signaling and cell migration in macrophages. *Nat. Cell Biol.* **1,** 69–71.
24. Ganju, R. K., Brubaker, S. A., Chernock, R. D., Avraham, S., Groopman, J. E. (2000) β-Chemokine receptor CCR5 signals through SHP1, SHP2, and Syk. *J. Biol. Chem.* **275,** 17263–17268.
25. Park, S. Y., Avraham, H., Avraham, S. (2000) Characterization of the tyrosine kinases RAFTK/Pyk2 and FAK in nerve growth factor-induced neuronal differentiation. *J. Biol. Chem.* **275,** 19768–19777.
26. Sajan, M. P., Bandyopadhyay, G., Kanoh, Y., Standaert, M. L., Quon, M. J., Reed, B. C., Dikic, I., Farese, R. V. (2002) Sorbitol activates atypical protein kinase C and GLUT4 glucose transporter translocation/glucose transport through proline-rich tyrosine kinase-2, the extracellular signal-regulated kinase pathway and phospholipase D. *Biochem. J.* **362,** 665–674.
27. Fuortes, M., Melchior, M., Han, H., Lyon, G. J., Nathan, C. (1999) Role of the tyrosine kinase Pyk2 in the integrin-dependent activation of human neutrophils by TNF. *J. Clin. Invest.* **104,** 327–335.
28. Ginnan, R., Singer, H. A. (2002) CaM kinase II-dependent activation of tyrosine kinases and ERK1/2 in vascular smooth muscle. *Am. J. Physiol. Cell Physiol.* **282,** C754–C761.
29. Poggi, A., Zocchi, M. R., Carosio, R., Ferrero, E., Angelini, D. F., Galgani, S., Caramia, M. D., Bernardi, G., Borsellino, G., Battistini, L. (2002) Transendothelial migratory pathways of V δ 1+TCR γ δ+ and V δ 2+TCR γ δ+ T lymphocytes from healthy donors and multiple sclerosis patients: involvement of phosphatidylinositol 3 kinase and calcium calmodulin-dependent kinase II. *J. Immunol.* **168,** 6071–6077.
30. Cai, X., Li, M., Vrana, J., Schaller, M. D. (2006) Glycogen synthase kinase 3- and extracellular signal-regulated kinase-dependent phosphorylation of paxillin regulates cytoskeletal rearrangement. *Mol. Cell. Biol.* **26,** 2857–2868.
31. Reiling, N., Blumenthal, A., Flad, H. D., Ernst, M., Ehlers, S. (2001) Mycobacteria-induced TNF-α and IL-10 formation by human macrophages is differentially regulated at the level of mitogen-activated protein kinase activity. *J. Immunol.* **167,** 3339–3345.
32. Bosco, M. C., Puppo, M., Santangelo, C., Anfosso, L., Pfeffer, U., Fardin, P., Battaglia, F., Varesio, L. (2006) Hypoxia modifies the transcriptome of primary human monocytes: modulation of novel immune-related genes and identification of CC-chemokine ligand 20 as a new hypoxia-inducible gene. *J. Immunol.* **177,** 1941–1955.
33. DeFea, K. A., Vaughn, Z. D., O'Bryan, E. M., Nishijima, D., Dery, O., Bunnett, N. W. (2000) The proliferative and antiapoptotic effects of substance P are facilitated by formation of a β-arrestin-dependent scaffolding complex. *Proc. Natl. Acad. Sci. USA* **97,** 11086–11091.
34. Luttrell, L. M., Roudabush, F. L., Choy, E. W., Miller, W. E., Field, M. E., Pierce, K. L., Lefkowitz, R. J. (2001) Activation and targeting of extracellular signal-regulated kinases by β-arrestin scaffolds. *Proc. Natl. Acad. Sci. USA* **98,** 2449–2454.
35. Aramori, I., Zhang, J., Ferguson, S. S. G., Bieniasz, P. D., Cullen, B. R., Caron, M. G. (1997) Molecular mechanism of desensitization of the chemokine receptor CCR-5: receptor signaling and internalization are dissociable from its role as an HIV-1 co-receptor. *EMBO J.* **16,** 4606–4616.
36. Huttenrauch, F., Pollok-Kopp, B., Oppermann, M. (2005) G protein-coupled receptor kinases promote phosphorylation and β-arrestin-mediated

internalization of CCR5 homo- and hetero-oligomers. *J. Biol. Chem.* **280,** 37503–37515.
37. Parameswaran, N., Pao, C. S., Leonhard, K. S., Kang, D. S., Kratz, M., Ley, S. C., Benovic, J. L. (2006) Arrestin-2 and G protein-coupled receptor kinase 5 interact with NFκB1 p105 and negatively regulate lipopolysaccharide-stimulated ERK1/2 activation in macrophages. *J. Biol. Chem.* **281,** 34159–34170.
38. Huttenrauch, F., Nitzki, A., Lin, F. T., Honing, S., Oppermann, M. (2002) β-Arrestin binding to CC chemokine receptor 5 requires multiple C-terminal receptor phosphorylation sites and involves a conserved Asp-Arg-Tyr sequence motif. *J. Biol. Chem.* **277,** 30769–30777.
39. Nakata, Y., Tomkowicz, B., Gewirtz, A. M., Ptasznik, A. (2006) Integrin inhibition through Lyn-dependent cross talk from CXCR4 chemokine receptors in normal human CD34+ marrow cells. *Blood* **107,** 4234–4239.
40. Benard, V., Bohl, B. P., Bokoch, G. M. (1999) Characterization of rac and cdc42 activation in chemoattractant-stimulated human neutrophils using a novel assay for active GTPases. *J. Biol. Chem.* **274,** 13198–13204.
41. Di Cioccio, V., Strippoli, R., Bizzarri, C., Troiani, G., Cervellera, M. N., Gloaguen, I., Colagrande, A., Cattozzo, E. M., Pagliei, S., Santoni, A., Colotta, F., Mainiero, F., Bertini, R. (2004) Key role of proline-rich tyrosine kinase 2 in interleukin-8 (CXCL8/IL-8)-mediated human neutrophil chemotaxis. *Immunology* **111,** 407–415.
42. Munugalavadla, V., Borneo, J., Ingram, D. A., Kapur, R. (2005) p85α subunit of class IA PI-3 kinase is crucial for macrophage growth and migration. *Blood* **106,** 103–109.
43. Meng, F., Lowell, C. A. (1998) A β 1 integrin signaling pathway involving Src-family kinases, Cbl and PI-3 kinase is required for macrophage spreading and migration. *EMBO J.* **17,** 4391–4403.
44. Okigaki, M., Davis, C., Falasca, M., Harroch, S., Felsenfeld, D. P., Sheetz, M. P., Schlessinger, J. (2003) Pyk2 regulates multiple signaling events crucial for macrophage morphology and migration. *Proc. Natl. Acad. Sci. USA* **100,** 10740–10745.
45. Reiske, H. R., Kao, S. C., Cary, L. A., Guan, J. L., Lai, J. F., Chen, H. C. (1999) Requirement of phosphatidylinositol 3-kinase in focal adhesion kinase-promoted cell migration. *J. Biol. Chem.* **274,** 12361–12366.
46. Wang, P., DeFea, K. A. (2006) Protease-activated receptor-2 simultaneously directs β-arrestin-1-dependent inhibition and Gαq-dependent activation of phosphatidylinositol 3-kinase. *Biochemistry* **45,** 9374–9385.
47. Zhang, Z., Izaguirre, G., Lin, S. Y., Lee, H. Y., Schaefer, E., Haimovich, B. (2004) The phosphorylation of vinculin on tyrosine residues 100 and 1065, mediated by SRC kinases, affects cell spreading. *Mol. Biol. Cell* **15,** 4234–4247.
48. Pasquale, E. B., Maher, P. A., Singer, S. J. (1986) Talin is phosphorylated on tyrosine in chicken embryo fibroblasts transformed by Rous sarcoma virus. *Proc. Natl. Acad. Sci. USA* **83,** 5507–5511.
49. Davis, S., Lu, M. L., Lo, S. H., Lin, S., Butler, J. A., Druker, B. J., Roberts, T. M., An, Q., Chen, L. B. (1991) Presence of an SH2 domain in the actin-binding protein tensin. *Science* **252,** 712–715.
50. Kolsch, V., Charest, P. G., Firtel, R. A. (2008) The regulation of cell motility and chemotaxis by phospholipid signaling. *J. Cell Sci.* **121,** 551–559.
51. Duong, L. T., Rodan, G. A. (2000) PYK2 is an adhesion kinase in macrophages, localized in podosomes and activated by β(2)-integrin ligation. *Cell Motil. Cytoskeleton* **47,** 174–188.
52. Hibbs, M. L., Dunn, A. R. (1997) Lyn, a src-like tyrosine kinase. *Int. J. Biochem. Cell Biol.* **29,** 397–400.
53. Funaki, M., Katagiri, H., Inukai, K., Kikuchi, M., Asano, T. (2000) Structure and function of phosphatidylinositol-3,4 kinase. *Cell. Signal.* **12,** 135–142.
54. Pleiman, C. M., Hertz, W. M., Cambier, J. C. (1994) Activation of phosphatidylinositol-3′ kinase by Src-family kinase SH3 binding to the p85 subunit. *Science* **263,** 1609–1612.
55. Seabold, G. K., Burette, A., Lim, I. A., Weinberg, R. J., Hell, J. W. (2003) Interaction of the tyrosine kinase Pyk2 with the N-methyl-D-aspartate receptor complex via the Src homology 3 domains of PSD-95 and SAP102. *J. Biol. Chem.* **278,** 15040–15048.
56. Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Plowman, G. D., Rudy, B., Schlessinger, J. (1995) Protein tyrosine kinase PYK2 involved in Ca(2+)-induced regulation of ion channel and MAP kinase functions. *Nature* **376,** 737–745.
57. Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S. A., Schlessinger, J. (1996) A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. *Nature* **383,** 547–550.
58. Felsch, J. S., Cachero, T. G., Peralta, E. G. (1998) Activation of protein tyrosine kinase PYK2 by the m1 muscarinic acetylcholine receptor. *Proc. Natl. Acad. Sci. USA* **95,** 5051–5056.
59. Basile, J. R., Afkhami, T., Gutkind, J. S. (2005) Semaphorin 4D/plexin-B1 induces endothelial cell migration through the activation of PYK2, Src, and the phosphatidylinositol 3-kinase-Akt pathway. *Mol. Cell. Biol.* **25,** 6889–6898.
60. DeFea, K. A., Zalevsky, J., Thoma, M. S., Dery, O., Mullins, R. D., Bunnett, N. W. (2000) β-Arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *J. Cell Biol.* **148,** 1267–1281.
61. Sun, Y., Cheng, Z., Ma, L., Pei, G. (2002) β-Arrestin2 is critically involved in CXCR4-mediated chemotaxis, and this is mediated by its enhancement of p38 MAPK activation. *J. Biol. Chem.* **277,** 49212–49219.
62. Luttrell, L. M., Ferguson, S. S., Daaka, Y., Miller, W. E., Maudsley, S., Della Rocca, G. J., Lin, F., Kawakatsu, H., Owada, K., Luttrell, D. K., Caron, M. G., Lefkowitz, R. J. (1999) β-Arrestin-dependent formation of β2 adrenergic receptor-Src protein kinase complexes. *Science* **283,** 655–661.
63. Del Pozo, M. A., Vicente-Manzanares, M., Tejedor, R., Serrador, J. M., Sanchez-Madrid, F. (1999) Rho GTPases control migration and polarization of adhesion molecules and cytoskeletal ERM components in T lymphocytes. *Eur. J. Immunol.* **29,** 3609–3620.
64. Haddad, E., Zugaza, J. L., Louache, F., Debili, N., Crouin, C., Schwarz, K., Fischer, A., Vainchenker, W., Bertoglio, J. (2001) The interaction between Cdc42 and WASP is required for SDF-1-induced T-lymphocyte chemotaxis. *Blood* **97,** 33–38.
65. Ge, L., Ly, Y., Hollenberg, M., DeFea, K. (2003) A β-arrestin-dependent scaffold is associated with prolonged MAPK activation in pseudopodia during protease-activated receptor-2-induced chemotaxis. *J. Biol. Chem.* **278,** 34418–34426.
66. Su, Y., Raghuwanshi, S. K., Yu, Y., Nanney, L. B., Richardson, R. M., Richmond, A. (2005) Altered CXCR2 signaling in β-arrestin-2-deficient mouse models. *J. Immunol.* **175,** 5396–5402.
67. Fong, A. M., Premont, R. T., Richardson, R. M., Yu, Y. R., Lefkowitz, R. J., Patel, D. D. (2002) Defective lymphocyte chemotaxis in β-arrestin2- and GRK6-deficient mice. *Proc. Natl. Acad. Sci. USA* **99,** 7478–7483.
68. Barnes, W. G., Reiter, E., Violin, J. D., Ren, X. R., Milligan, G., Lefkowitz, R. J. (2005) β-Arrestin 1 and Gαq/11 coordinately activate RhoA and stress fiber formation following receptor stimulation. *J. Biol. Chem.* **280,** 8041–8050.
69. Camina, J. P., Lodeiro, M., Ischenko, O., Martini, A. C., Casanueva, F. F. (2007) Stimulation by ghrelin of p42/p44 mitogen-activated protein kinase through the GHS-R1a receptor: role of G-proteins and β-arrestins. *J. Cell. Physiol.* **213,** 187–200.
70. Bradbury, F. A., Zelnik, J. C., Traynor, J. R. (2009) G Protein independent phosphorylation and internalization of the delta opioid receptor. *J. Neurochem.* **109,** 1526–1535.
71. Koch, A. E., Kunkel, S. L., Harlow, L. A., Mazarakis, D. D., Haines, G. K., Burdick, M. D., Pope, R. M., Strieter, R. M. (1994) Macrophage inflammatory protein-1 α. A novel chemotactic cytokine for macrophages in rheumatoid arthritis. *J. Clin. Invest.* **93,** 921–928.
72. O'Grady, N. P., Tropea, M., Preas II, H. L., Reda, D., Vandivier, R. W., Banks, S. M., Suffredini, A. F. (1999) Detection of macrophage inflammatory protein (MIP)-1α and MIP-1β during experimental endotoxemia and human sepsis. *J. Infect. Dis.* **179,** 136–141.

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