ORIGINAL RESEARCH

β-Arrestin-2–Dependent Signaling Promotes CCR4–mediated Chemotaxis of Murine T-Helper Type 2 Cells

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Abstract

Allergic asthma is a complex inflammatory disease that leads to significant healthcare costs and reduction in quality of life. Although many cell types are implicated in the pathogenesis of asthma, $CD4^+$ T-helper cell type 2 (Th2) cells are centrally involved. We previously reported that the asthma phenotype is virtually absent in ovalbumin-sensitized and -challenged mice that lack global expression of β-arrestin (β-arr)-2 and that $CD4^+$ T cells from these mice displayed significantly reduced CCL22–mediated chemotaxis. Because CCL22-mediated activation of CCR4 plays a role in Th2 cell regulation in asthmatic inflammation, we hypothesized that CCR4-mediated migration of $CD4^+$ Th2 cells to the lung in asthma may use β-arr–dependent signaling. To test this hypothesis, we assessed the effect of various signaling inhibitors on CCL22-induced chemotaxis using *in vitro*–polarized primary $CD4^+$ Th2 cells from β-arr2–knockout and wild-type mice. Our results show, for the first time, that CCL22-induced, CCR4-mediated Th2 cell chemotaxis is dependent, in part, on a β-arr2–dependent signaling pathway. In addition, we show that this chemotactic signaling mechanism involves activation of P-p38 and Rho-associated protein kinase. These findings point to a proinflammatory role for β-arr2–dependent signaling and support β-arr2 as a novel therapeutic target in asthma.

Keywords: CCR4; T-helper type 2 cell; chemotaxis; β-arrestin-2–dependent signaling; asthma

Clinical Relevance

Pharmacological modulation of G protein-coupled receptor activity is a well-established treatment approach that is pervasive in clinical medicine. The results reported herein emphasize the importance of considering the effect of potential pharmacologic agents on dual signaling pathways. Our study points to a proinflammatory role for β-arrestin-2–dependent signaling in T-helper type 2 cells. If, like the murine cells, human T-helper type 2 cells use a β-arrestin-2–dependent chemotactic signaling pathway, then this pathway could be selectively targeted in patients with asthma.

Allergic asthma is a chronic inflammatory syndrome characterized by airway inflammation, hyperresponsiveness, remodeling, and reversible airflow obstruction that leads to significant healthcare costs and reduction in quality of life (1). In a sensitized individual, antigen exposure triggers a coordinated interaction among several immune (2) and lung structural cell types (3), resulting in lung inflammation. Allergen-specific T lymphocytes differentiate into various T-helper (Th) cell subtypes that migrate to the lung, where they participate in the allergic inflammatory response (2). Studies

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using RAG (recombinase-activating gene) 1 knockout (KO) mice show that T lymphocytes are both necessary and sufficient for the development of allergic airway inflammation (4, 5). Similarly, activated $CD4^+$ T cells are localized in the bronchial mucosa of patients with asthma, and allergen challenge of patients with asthma induces a selective recruitment of $CD4^+$ T cells into the airways (6, 7). Although many Th cell subsets (including Th1 and Th17) are implicated in the pathogenesis of asthma, $CD4^+$ T-helper type 2 (Th2) cells are centrally involved (reviewed in References 8–12).

Directed migration of immune cells from secondary lymphoid tissue to the site of inflammation, is initiated by chemokine receptor activation. One outcome of allergen-induced Th2 polarization is elevated cell surface expression of CCR4, which enables Th2 cells to preferentially respond to inflammatory, rather than homeostatic, chemokines (13, 14). Evidence that CCR4 is implicated in asthma pathogenesis comes from human studies, where increased lung production of C-C motif chemokine (CCL) 17 and CCL22, chemokines that selectively activate CCR4, occurs after segmental allergen challenge (15–17). Despite an early study using CCR4-KO mice that showed no change in ovalbumin-induced airway inflammation (18), the importance of the CCL17/22–CCR4 axis to murine asthma models has been demonstrated in several subsequent studies, where genetic, pharmacologic, and immunologic inhibition of CCR4 activation significantly impairs both Th2 cell extravasation into the lung and asthma phenotype development (19–22).

We previously showed that mice genetically devoid of β-arrestin (β-arr)-2 are significantly protected from developing the asthma phenotype (23). In addition, $CD4^+$ T cells harvested from the lungs of these mice displayed a significant impairment in CCL22-mediated chemotaxis *in vitro*. We concluded that β-arr2 regulates CCR4-mediated chemotaxis in Th2 cells; however, the mechanism by which β-arr2 exerts this regulation is unknown.

β-arr2, a ubiquitously expressed scaffold protein, regulates and transduces 7 transmembrane receptor (7TMR) signaling, including chemokine receptors (24). β-arr2 was first shown to play a role in chemokine receptor–mediated chemotaxis in a 2002 report where naive T lymphocytes derived from β-arr2–knockout (β-arr2–KO) mice demonstrated significantly impaired CXCR4-mediated migration (25). Although canonical signaling pathways associated with CCR4-mediated chemotaxis in human Th2 cells have been examined (26), contribution by β-arr–dependent signaling was not considered.

For many reasons, delineation of the mechanistic role for β-arrs in chemotactic signaling is difficult to unravel. First, β-arrs are known to both arrest G protein–mediated, and transduce β-arr–dependent, signaling at a single chemokine receptor, and each of these dual signaling pathways may contribute to migration (reviewed in References 27 and 28). Second, ligand activation of chemokine receptors induces cell chemotaxis as well as several other cellular functions, including adhesion, proliferation, and gene expression (16). Thus, cellular levels of signaling intermediates must be proven to be associated with chemotaxis. Third, signaling intermediates, such as second messengers or mitogen-activated protein kinases (MAPKs), associated with G protein–mediated signaling may be identical to those activated by the β-arr–dependent pathway. Currently, the signaling source of these intermediates is only distinguishable by their spatiotemporal localization (29–32). Finally, chemotactic signaling pathways are cell type– and context-specific and, thus, must be individually examined.

The goal of the current study was to carefully dissect the role for β-arr2 in regulating CCR4-mediated chemotaxis in primary murine $CD4^+$ Th2 cells.

Methods

Animals

Animal care protocols were approved by Duke University Institutional Animal Care and Use Committee and performed in accordance with U.S. Animal Welfare Act standards and NIH guidelines. Male β-arr2–KO (23) and littermate wild-type (WT) mice were used for all experiments at 8–16 weeks of age.

Chemicals

SB203580, Y27632, LY294002, and 227013 (CCR4 antagonist) were purchased from Calbiochem Corp. MK2206 was purchased from SelleckChem. Pertussis toxin (PTX) was purchased from Sigma Aldrich. Murine CCL22 and CCL17 were purchased from R&D system.

Polarization of $CD4^+$ T Cells to Th2

Naive $CD4^+$ T cells, purified and enriched (EasySep; StemCell Technologies) from spleens, were cultured in RPMI 1640 and polarized to Th2 using standard methods (*see* the data supplement). Th2 skewing was assessed by GATA-3 or IL-4 measurement using anti–GATA3–Phycoerythrin (PE) antibody (no. 12-9966-42; Ebioscience) or anti–IL-4–PE antibody (no. 554436; BD Pharmingen). Cells that underwent IL-4 staining were restimulated before surface staining. FACSVantage (Becton Dickinson) and FlowJo software (Tree Star Inc.) were used for flow cytometric analysis.

Chemotaxis Assays

Chemotaxis assays were performed in 24-well transwells as described previously (23). The bottom chamber was filled with 0.6 ml of RPMI 10% (vol/vol) FBS media with or without various concentrations of murine CCL17 or CCL22 (R&D Systems). Murine Th2 cells (1×10^6) in 0.1 ml of media were added to the top chamber. After 90 minutes of incubation at 37°C, the transmigrated cells in the bottom chamber were stained and counted by flow cytometry. The number of cells that transmigrated to media alone (chemokinesis) was subtracted from the number of cells found in chemokine-containing lower chambers. The difference represents the number of cells that underwent agonist-induced chemotaxis. This value was divided by the total number of cells placed on the upper transwells, and expressed as "percentage directed migration." Enumeration of cells using flow cytometry was based on sample collection time, not percentage of cells.

Pharmacologic Cell Signaling Inhibitors

The effect of various signaling inhibitors on CCL22-induced chemotaxis was assessed in Th2 cells replete with, and devoid of, β-arr2. Th2 cells were preincubated with inhibitors for 30 minutes before chemotaxis stimulation with 10 nM CCL22, except for PTX, which required 16-hour incubation.

CCR4 Expression

Radioligand binding and RT-PCR were used to assess CCR4 expression in Th2 cells.

Saturation binding technique was employed where Th2 cells were preincubated (5 min) with 1-μM cold murine CCL17 followed by 0.3 nM [^{125}I]-labeled CCL17 (Perkin Elmer) for 60 minutes on ice. Maximal CCR4 binding (Bmax) was calculated and normalized to WT maximal binding (*see* the data supplement). Real-time PCR was performed to measure the expression of CCR4 relative to the housekeeping gene, GAPDH. The fold change in CCR4 expression over the labortory standard is presented. cDNA was synthesized using total cellular RNA isolated from Th2 cells.

Western Blot Analysis

CD4$^+$ Th2 cells were serum starved for 18–24 hours before stimulation with 10-nM CCL22. Cells were harvested at different time points after stimulation and resuspended in RIPA buffer containing protease inhibitors. Lysates were mixed with sample buffer and proteins were separated on 10% Tris-glycine gels, transferred to polyvinylidene difluoride (PVDF) membranes and probed using antibodies against p38, P-p38, extracellular signal–regulated kinase (ERK), and P-ERK (*see* the data supplement). Using chemiluminescent detection (Pierce), each band in the immunoblots was quantified by densitometry using the GeneTools program (SynGene).

Statistical Analysis

Data are expressed as mean (±SEM). Statistical calculations were performed using GraphPad Prism (GraphPad Software, Inc.) or SPSS software. A two-tailed Student's *t* test was applied as appropriate, and repeated measures analysis was used for dose–response comparisons. A *P* value of less than or, where noted, equal to, 0.05 was considered statistically significant.

Results

Effect of β-arr2 on Th2 Cell Migration

The β-arr–dependent and -independent signaling pathways can be distinguished by distinct temporal signatures, where signaling via the former pathway is delayed and prolonged compared with the latter (29–32). Thus, we chose to examine chemotaxis at 90 minutes. Chemokinesis of WT and β-arr2–KO Th2 cells at 90 minutes was not different (16.5 ± 1.6% and 18.7 ± 2.4%, respectively), indicating that β-arr2 does not regulate baseline Th2 cell migration. CCL22-stimulated Th2 cell chemotaxis occurs in a dose-dependent manner, but the magnitude of the chemotactic response was significantly impaired in cells lacking β-arr2 (β-arr2–KO) (Figure 1A). Given that CCL17 is a CCR4-specific ligand (33) and has been shown to have different signaling effects than CCL22 (34, 35), we tested its chemotactic effect on Th2 cells. WT Th2 cells migrated less well to CCL17 than to CCL22 (Figure 1A versus 1B), and the chemotactic response to CCL17 was not different between WT and β-arr2–KO Th2 cells (Figure 1B). Directed migration in response to CCL17 was dose dependent, but even at the highest CCL17 concentration, the directed migration was only around twice that observed in the absence of chemokine. Taken together, these results suggest that CCL17 does not promote β-arr2–dependent chemotaxis, in contrast to CCL22. Therefore, to study the role for β-arr2–dependent signaling in CCR4-mediated chemotaxis, we chose to use CCL22 in our experiments. Antagonists of CCR4 blocked CCL22-induced chemotaxis, suggesting that CCR4 is the operative chemokine receptor for CCL22-induced responses in Th2 cells (Figure 1C).

Effect of β-arr2 on CCR4 Expression

Because β-arr can regulate internalization of several chemokine receptors, we measured surface expression of CCR4 in WT and β-arr2–KO Th2 cells. CCR4 binding efficiency and expression levels in β-arr2–KO Th2 cells were similar to that in WT Th2 cells, as shown by measurement of radioligand binding (Figure 2A) and receptor mRNA levels using the RT-PCR method (Figure 2B). The fact that chemotaxis to CCL17 was the same in WT and β-arr2–KO cells also supports the notion that the two cell types have equal levels of CCR4 expression.

β-arr2 Does Not Regulate CD4$^+$ T Cell Differentiation

GATA-3 expression and IL-4 secretion are distinguishing features of Th2 cells (36, 37). As shown in Figure 3, naive WT and β-arr2–KO T cells similarly polarized to the Th2 phenotype as assessed by IL-4 (Figures 3A and 3B) and GATA-3 expression (Figures 3C–3E). These results demonstrate that Th2 cell expression of GATA-3 and production of IL-4 are independent of β-arr2.

Effect of Signaling Inhibitors on CCL22-induced Th2 Cell Chemotaxis

To determine the signaling molecules that mediate chemotaxis, Th2 cells were preincubated with inhibitors of p38 MAPK (SB203580) and Rho-associated protein kinase (ROCK; Y27632) before stimulation with CCL22. Inhibition of either p38 or ROCK significantly reduced chemotaxis in WT Th2 cells, but had no significant effect on that of β-arr2–KO Th2 cells (Figures 4A and 4B), suggesting that a significant proportion of Th2 cell chemotaxis is mediated by a β-arr2–dependent signaling pathway involving these signaling intermediates. In contrast, inhibitors of PI3K (LY294002) or Akt (MK2206) similarly reduced CCL22-induced chemotaxis in WT and β-arr2–KO Th2 cells (Figures 4C and 4D, respectively), suggesting that the PI3K/Akt signaling pathway to chemotaxis is operative even in the absence of β-arr2. Interestingly, the heterotrimeric Gi protein inhibitor, PTX, completely abolished CCL22-induced chemotaxis in WT and β-arr2–KO Th2 cells (Figure 4E) without affecting chemokinesis (data not shown). These data suggest that the CCR4 dual signaling pathways (β-arr2–dependent and -independent) are both downstream of Gi protein.

To further verify that p38 MAPK is involved in β-arr2–dependent signaling downstream of CCR4 activation, we measured cellular levels of P-p38. Because β-arr–dependent MAPK signaling is characteristically delayed and prolonged relative to that of β-arr–independent signaling (29), we measured MAPKs at early and late time points after CCL22 stimulation. CCL2-induced activation of P-p38 was similar in WT and β-arr2–KO Th2 cells at 5 minutes. In contrast, at the 15- and 30-minute time points, the P-p38 signal was significantly less in the β-arr2–KO Th2 cells compared with the WT cells in which the P-p38 signal remained elevated. These results suggest that the MAPK signal at later time points (15 and 30 min) results from β-arr2–dependent signaling. Because ERK1/2 signaling is known to be involved in β-arr–dependent signaling in other cellular contexts (38), we measured CCR4-mediated activation of P-ERK1/2 (Figures 5D and 5E). P-ERK1/2 was induced weakly, but similarly, in WT and β-arr2–KO Th2 cells at the early time points and absent in both genotypes at the late time point

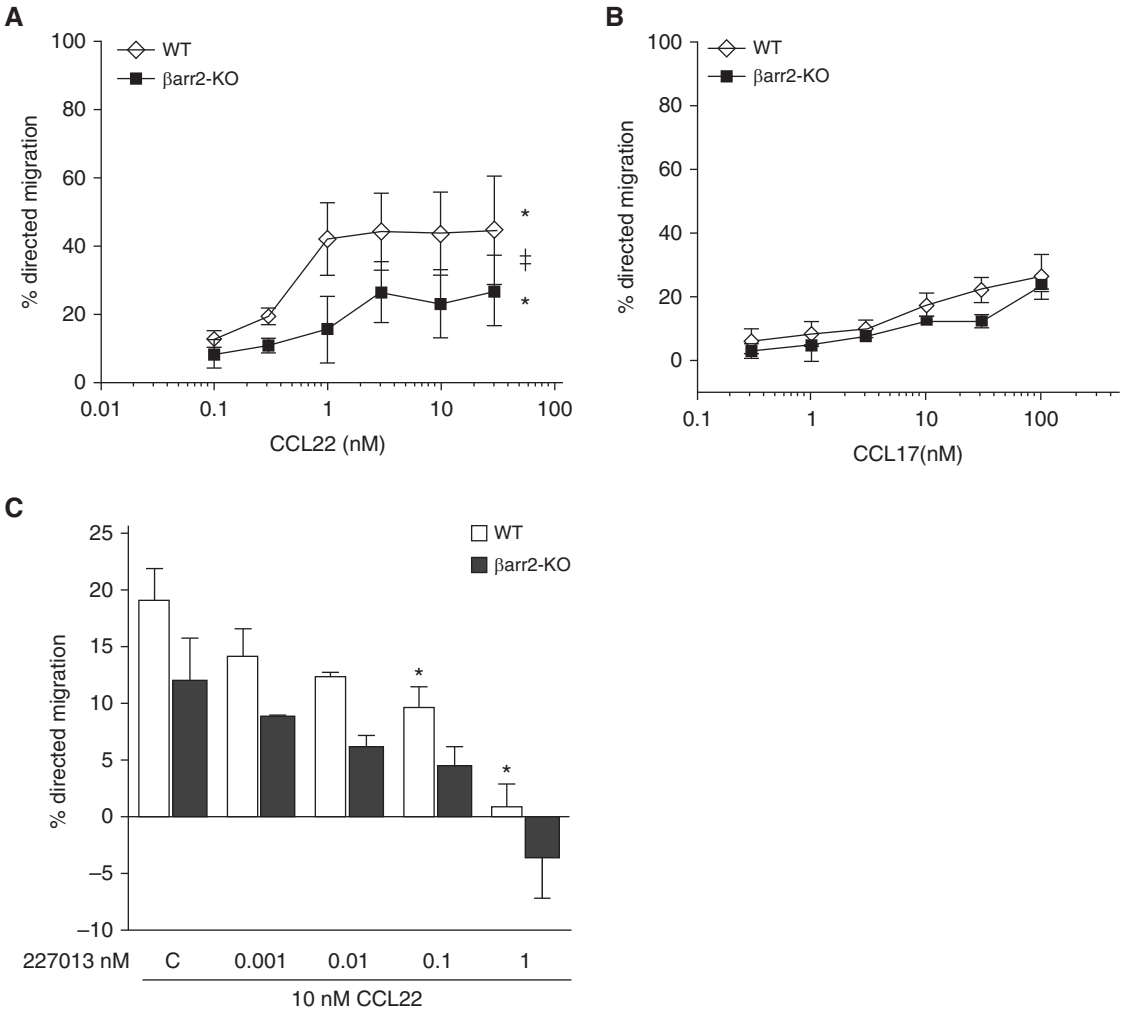

Figure 1. Effect of β-arrestin (β-arr)-2 on CCL22– and CCL17-induced T-helper cell type 2 (Th2) migration. Migration of *in vitro* polarized wild-type (WT) and β-arr2–knockout (KO) Th2 cells was measured in response to a 90-minute exposure to increasing doses of (*A*) CCL22 or (*B*) CCL17. (*C*) Before stimulation with 10 nM CCL22, Th2 cells underwent a 30-minute pretreatment with the CCR4 antagonist, 227013. Data are presented as the mean (±SEM) of four independent experiments. $^{‡}P < 0.05$ effect of genotype and $^{*}P < 0.05$ effect of dose according to repeated measures ANOVA for WT versus β-arr2–KO.

(30 min), indicating that, although there is precedence for β-arr–dependent P-ERK1/2 signaling to chemotaxis, this pathway/effector combination is not used by Th2 cells in response to CCR4 activation. Taken together, our observations are consistent with the inhibitor data, which suggest that the MAPK effector molecule associated with CCR4-mediated β-arr–dependent signaling to chemotaxis is P-p38, not P-ERK1/2.

Discussion

In this study, we reveal that, in addition to the classical G protein–dependent/β-arr–independent signaling pathway, CCR4-mediated Th2 cell chemotaxis is also transduced by a β-arr–dependent mechanism. Furthermore, we delineated that β-arr–dependent signaling uses P-p38 and ROCK effector molecules, whereas PI3K/Akt are associated with the β-arr–independent pathway (Figure 6).

To characterize the CCR4-mediated chemotactic signaling pathways in Th2 cells, we reasoned that inhibition of signaling intermediates downstream of the β-arr–dependent chemotactic signaling pathway would reduce chemotaxis only in WT cells, because the β-arr–dependent pathway is absent in β-arr2–KO cells. Our results show that the β-arr–dependent signaling pathway uses both P-p38 and ROCK to initiate cell migration.

ROCK is a downstream effector of small, GTP-binding proteins, or GTPases, of the Rho family, such as rho, Rac, and Cdc42. These GTPases are central regulators of actin-based cytoskeletal reorganization (39). Our finding that T lymphocytes use ROCK signaling to chemotax is consistent with that of others. For example, using human peripheral blood mononuclear cells (PBMCs) and a lymphoblastoid $CD4^{+}$ T cell line (CEM cells), Bardi and colleagues (40) showed that lymphocyte migration to homing chemokines CCL19 and CCL21 is dependent upon the activation of ROCK. Similarly, the ROCK pathway has been implicated in promoting lymphocyte chemotaxis to CXCL12 (41). Precedence for β-arr involvement in ROCK activation and

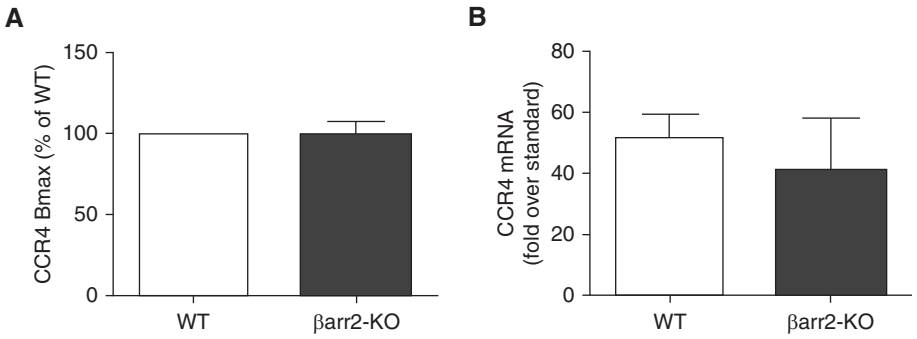

Figure 2. CCR4 expression in Th2 cells. Expression of CCR4, as assessed by (*A*) radioligand binding and (*B*) RT-PCR, was the same in WT and β-arr2–KO Th2 cells. Data are presented as the mean (±SEM) of three independent experiments. Bmax = maximum specific binding.

stress fiber formation came from a study by Barnes and colleagues (42) who used angiotensin II type 1A receptor (AT1aR) stably expressing HEK293 cells. A report that linked ROCK-mediated chemotaxis to asthma came from Cronshaw and colleagues (43), who showed that migration of human $CD4^+$ Th2 cells to CCL22 was reduced by the ROCK inhibitor, Y-27632. Their finding is consistent with the murine results presented herein. Moreover, in the current study, we have further delineated that ROCK-mediated chemotaxis is β-arr dependent. Although there was a trend for a small inhibitory effect of Y27632 on chemotaxis of β-arr2–KO Th2 cells, statistical significance was not reached even with a sample size of five experiments. However, it is possible that the β-arr2–independent, or an as-yet undiscovered, signaling pathway downstream of CCR4 and independent of β-arr2 uses ROCK to contribute, modestly, to Th2 cell chemotaxis.

In addition to ROCK, we show that the MAPK, P-p38, is also involved in CCR4-mediated, β-arr–dependent signaling to chemotaxis. Several previous studies using immortalized cell lines have demonstrated a role for P-p38 in mediating 7TMR-induced chemotaxis (33, 38, 44). For example, in HEK 293 cells, knockdown of β-arr2 blocked CXCR4- or AT1aR-mediated chemotaxis by a mechanism involving the activation of p38 MAPK (38, 44). In a separate study, where a role for β-arr was not examined, Lee and colleagues (45) showed that activation of the lysophosphatidic acid receptor, a 7TMR, by lysophosphatidylserine causes chemotaxis of a human glioma cell line (U87) via a P-p38–dependent mechanism. The work presented herein is the first to demonstrate that a primary immune cell uses a β-arr–dependent P-p38 signaling cascade to migrate toward an inflammatory chemokine. Whether or not P-p38 and ROCK directly interact to facilitate β-arr–dependent chemotaxis is unknown and was not tested in this study.

The molecular mechanism by which β-arr mediates chemotaxis is poorly understood; in part owing to the complexity of β-arr–mediated regulation of 7TMR signaling. In general, a cell is able to directionally migrate to an area of high chemokine concentration due, in part, to a greater ligand occupancy of chemokine receptors on the leading edge of the cell than on the trailing edge (27, 46). This differential activation of chemokine receptors leads to gross reorganization of the actin cytoskeleton, cell shape change, and cell chemotaxis (27, 39). Acting in a classical role (24, 47), β-arr may regulate chemotaxis by promoting chemokine receptor desensitization and recycling; a collective mechanism by which cells maintain their ability to sense a chemoattractant gradient (27). On the other hand, acting in a signal transduction role (24, 47), β-arr may scaffold multiple kinases that ultimately regulate actin cytoskeletal rearrangement (28). Our P-p38 data suggest that the latter role for β-arr is at work in CCR4-mediated migration of Th2 cells. Progress in delineation of the chemotactic cytoskeletal substrates activated by β-arr–dependent signaling complexes is an area of active investigation, and the reader is directed to reports from DeFea's group (28, 48) for a thorough review.

The absence of β-arr2 does not completely impair the CCR4-induced chemotaxis of $CD4^+$ Th2 cells, illustrating the existence of a β-arr–independent signaling pathway to chemotaxis. PI3K, and its substrate, Akt, are well known as chemotactic signaling intermediates in many cell types (49–51). Thus, as anticipated, inhibition of PI3K or Akt resulted in a significant decline in WT Th2 cell chemotaxis. The observation that β-arr2–KO Th2 cell chemotaxis was also significantly impaired in the presence of LY294002 or MK2206 places PI3K and Akt on the β-arr–independent arm of the chemotactic signaling pathways (Figure 6). Our results are in agreement with the finding by Xue and colleagues (52) who used human $CD4^+$ Th2 cells to show that chemotaxis induced by agonist activation of the 7TMR CRTH2 (chemoattractant receptor-homologous molecule expressed on Th2 cells) was significantly inhibited by 100 μM LY294002 as were actin polymerization and Akt phosphorylation. Interestingly, although in their study LY294002 completely blocked Akt phosphorylation, it only partially inhibited chemotaxis. Thus, we speculate that a β-arr–dependent signaling pathway may also contribute to chemotaxis in human $CD4^+$ Th2 cells. Although we did not measure intracellular Ca^{2+} concentration in the present studies, Cronshaw and colleagues (26) showed that CCR4-mediated Th2 cell chemotaxis was not impaired in the presence of 2-aminoethoxydiphenyl borane, which blocks Ca^{2+} release from intracellular stores (53).

Interestingly, the β-arr2–dependent, CCR4-mediated chemotactic signaling pathway is downstream of Gi family heterotrimeric proteins (Figure 6), as PTX completely abrogated CCL22-induced migration in WT (β-arr–replete) Th2 cells. This finding is consistent with that of Cronshaw and colleagues (43) who showed that human Th2 cell migration to CCL22 was abolished by prior treatment with PTX. To date, β-arr–dependent 7TMR signaling has been shown to be completely independent of Gs or Gq protein family member–mediated signaling (32, 44, 47, 54, 55), and Cheung and colleagues (56) showed that CCR5-mediated, β-arr–dependent chemotaxis is independent of Gi. Thus, it may seem curious that our study demonstrates that β-arr–dependent signaling to chemotaxis is downstream of Gi protein. However, Kohout and colleagues (57) showed that CCR7-mediated, β-arr–dependent chemotaxis is PTX sensitive, and speculated

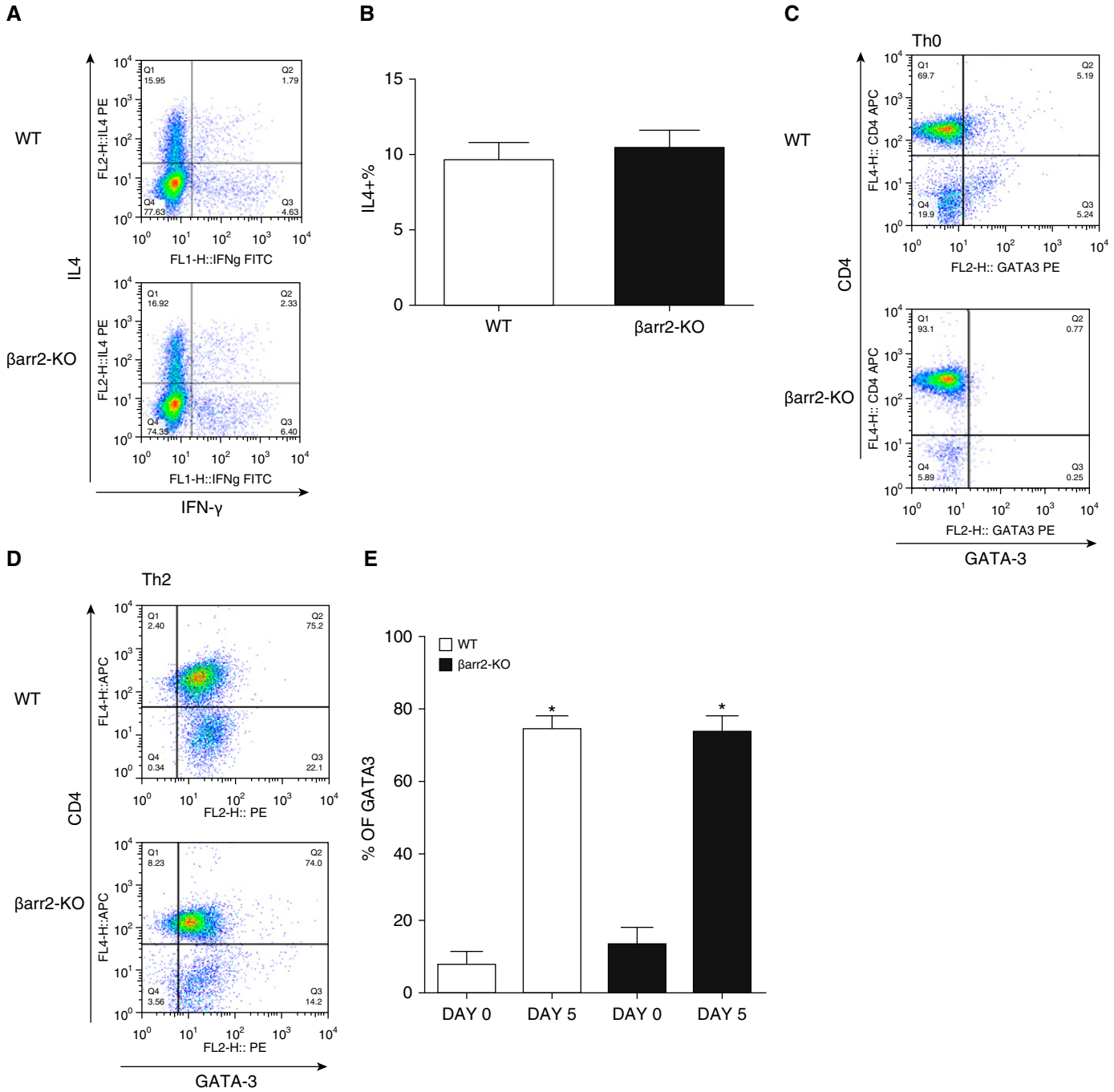

Figure 3. Effect of β-arr2 deficiency on $CD4^+$ Th2 cell polarization. (*A*) Dot displays and (*B*) summary bar graphs show the proportion of $CD4^+$ cells that express IL-4 (representative of 10 separate experiments). (*C* and *D*) Dot displays show the proportion of total WT (top) and β-arr2–KO (bottom) $CD4^+$ cells that are GATA-binding protein 3 (GATA3) positive before (*C*) and after (*D*) Th2 cell polarization. (*E*) Summary bar graphs show the percent of $CD4^+$ $GATA3^+$ cells. Data are presented as the mean (±SEM) of 3–10 experiments. $^*P < 0.05$ according to Student's *t* test versus Day 0.

that β-arr translocation to a chemokine receptor at the cell surface, which is required for β-arr–dependent signaling, is triggered by free β-γ Gi protein subunits. Using human Th2 cells, Cronshaw and colleagues (26, 43) showed that CCR4-mediated Th2 cell chemotaxis requires Gi signaling, and uses both a Rho-dependent signaling pathway, as well as a phospholipase C(PLC)/PKC-δ signaling cascade that is calcium independent and diacylglycerol (DAG) dependent. Although at the time of the Cronshaw studies it was known that β-arr signaling is significantly involved in naive and allergen-activated $CD4^+$ T cell migration (23, 25), as well as the migration of multiple transformed cell lines (24, 38, 57), tools for examining β-arr–dependent signaling in primary human cells with endogenous levels of receptor expression were lacking. Thus, future

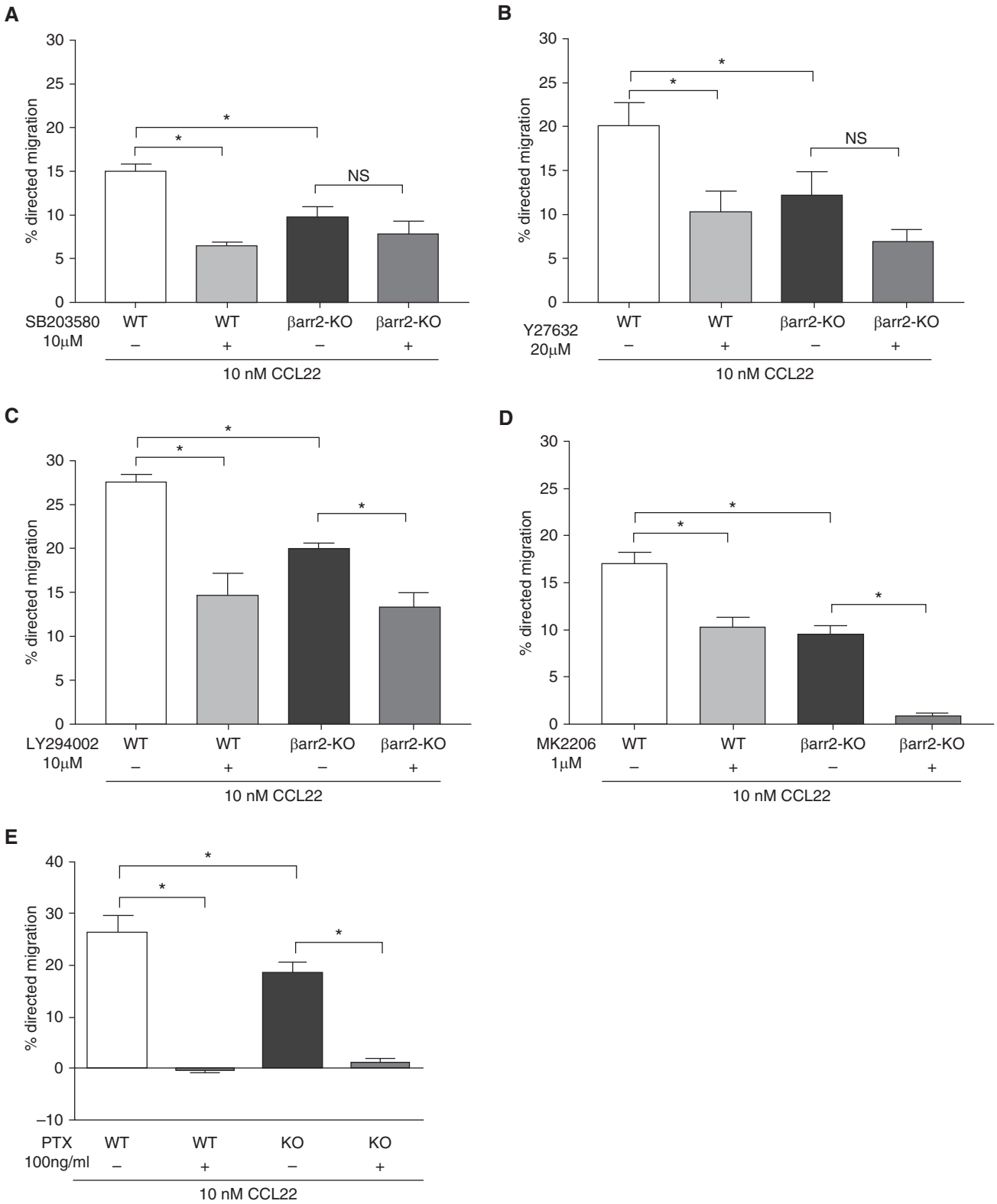

Figure 4. Effect of cell signaling inhibitors on CCL22–induced Th2 cell chemotaxis. (*A–E*) Shown is the percent directed migration of WT and β-arr2–KO $CD4^+$/Th2 cells that were pretreated, or not, with indicated concentrations of cell signaling inhibitors (*A*) SB203580, (*B*) Y27632, (*C*) LY294002, (*D*) MK2206, and (*E*) pertussis toxin (PTX) before incubation with 10-nM CCL22. All inhibitor incubations were for 30 minutes, except PTX, which was 16 hours. Data are presented as the mean (±SEM) of three to five experiments. $^*P \leq 0.05$ according to paired *t* test for within-subject effect of inhibitor or Student's *t* test for WT versus β-arr2–KO. NS = not significant.

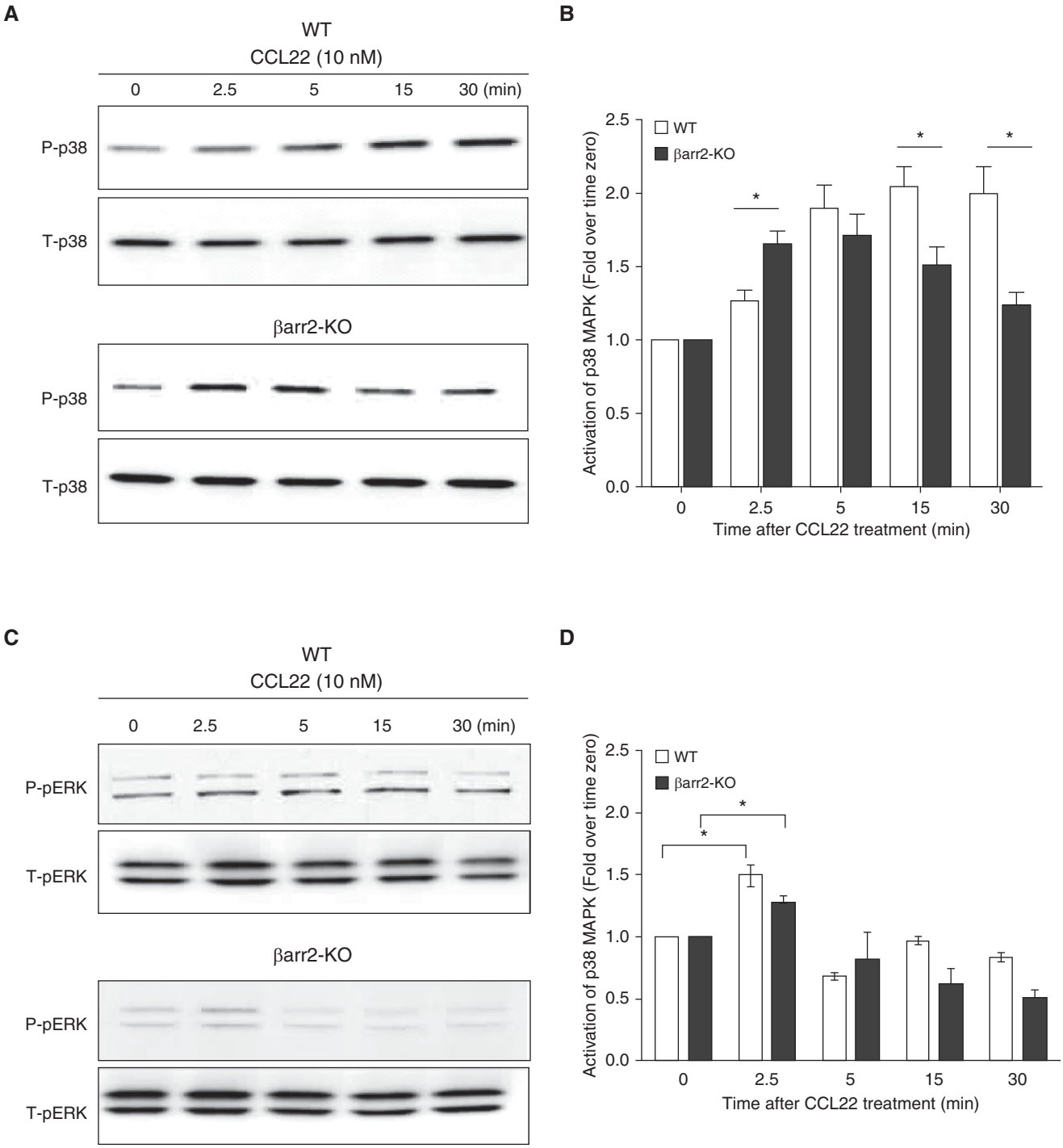

Figure 5. Effect of β-arr2 on CCL22–induced activation of mitogen-activated protein kinases (MAPKs) in Th2 cells. The time course of CCL22-induced Th2 cell production of (*A* and *B*) P-p38 and (*C* and *D*) P-ERK1/2 was assessed by immunoblot and quantified by densitometry. Open bars, WT Th2 cells; solid bars, β-arr2–KO Th2 cells. Western blot is representative of three separate experiments. Data are expressed as mean (±SEM). $^*P < 0.05$ significant difference between WT and β-arr2–KO Th2 cells at the indicated time point according to Student's *t* test. Readers may view the uncut gels for *C* in the data supplement.

studies may show that CCR4-activated, Rho-dependent signaling to chemotaxis in human Th2 cells is, like murine cells, mediated by β-arr–dependent signaling.

Several lines of evidence suggest that CCL17 does not trigger β-arr–dependent signaling and is, therefore, a biased CCR4 ligand (8, 35, 58). Using human immortalized cells (HUT78 cells) transfected with CCR4, Ajram and colleagues (34) showed that CCL22, but not CCL17, triggered β-arr recruitment to the CCR4

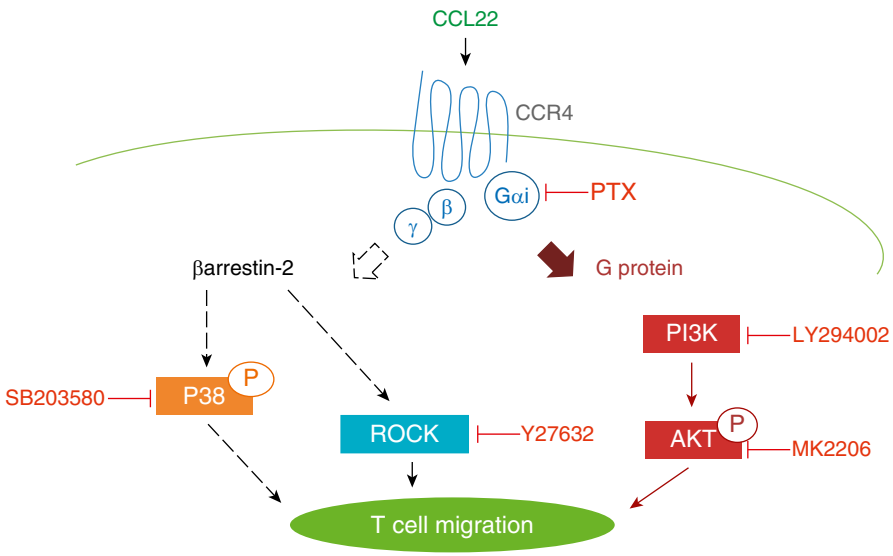

Figure 6. Role for β-arr2–dependent signaling in Th2 cell chemotaxis. CCL22–induced Th2 cell chemotaxis is promoted by both β-arr2–dependent and β-arr2–independent signaling pathways, both of which are downstream of Gαi activation. The β-arr2–dependent signaling arm uses P-p38 and Rho-associated protein kinase (ROCK), whereas the β-arr2–independent arm uses PI3K and Akt.

receptor as assessed by DiscoveRx. Current knowledge indicates that translocation of β-arr to the cell surface receptor is required for β-arr–dependent signaling. The data presented herein are consistent with the notion that CCL17 is biased toward G protein signaling. Our observation, that WT Th2 cells migrate less well to CCL17 relative to CCL22 and about the same as cells lacking β-arr2, suggests that the β-arr–dependent signaling is not activated by CCL17 in WT Th2 cells.

β-arr2–mediated regulation of cell chemotaxis has gained prominence in the study of cancer (38, 59, 60; reviewed in References 28 and 39), atherosclerosis (61), and inflammatory disorders (reviewed in Reference 62), where it appears that β-arr is a proinflammatory/prometastatic protein (reviewed in Reference 27). Collectively, our prior work shows that β-arr2 plays a similar proinflammatory role in controlling cell migration crucial to asthma pathogenesis (23, 63). An example of the immunopathologic relevance of β-arr–dependent chemokine receptor chemotactic signaling is highlighted in WHIM (warts, hypogammaglobulinemia, infection, and myelokathexis) syndrome (64). Genetic mutations that cause truncation of the CXCR4 cytoplasmic tail underlie the highly varied disease characteristics observed in patients afflicted with WHIM syndrome. The truncation of CXCR4 augments and prolongs β-arr–dependent, CXCL12-induced signal transduction leading to enhanced leukocyte chemotaxis. Similarly, truncation of the CCR4 carboxy terminal cytoplasmic domain observed in adult T cell leukemia/lymphoma cells results in enhanced CCR4-mediated chemotaxis (65). If the parallel to WHIM syndrome holds true, where the CCR4 truncation enhances β-arr–dependent chemotaxis, then β-arr2 regulation of CCR4 may play a role in the pathogenesis of adult T cell leukemia/lymphoma.

Despite the importance of CCR4-mediated, β-arr–dependent Th2 cell migration to the development of asthma, it is doubtful that the absence of the β-arr–dependent arm of the chemotactic signaling cascade in Th2 cells is solely responsible for the striking lack of asthma phenotype observed in β-arr2–KO mice (23). In fact, β-arr–dependent regulation of nonchemokine 7TMRs plays a role in asthma pathogenesis. For example, protease-activated receptor-2 stimulation has been shown to stimulate β-arr2–dependent actin cytoskeletal rearrangement (reviewed in References 27 and 28) and $CD4^+$ T cell migration, which may underlie much of the protection from asthma phenotype development observed in protease-activated receptor-2–KO mice (66). Another mechanism by which β-arr–mediated regulation of 7TMRs, other than chemokine receptors, may influence the asthma phenotype is through regulation of β2-adrenoceptor (β2AR) signaling. Endogenous activation of β2ARs is required for full development (67–69) and exacerbation (70) of the asthma phenotype, and β-arrs are well known to regulate β2AR signaling and function (32, 53).

Although *in vitro* models of chemotaxis may not always predict the *in vivo* migration of cells, our prior work (23, 63) suggests that β-arr–dependent signaling downstream of CCR4 in Th2 cells is indeed pathophysiologically relevant. We previously showed that WT mice that received hematopoietic cells from β-arr2–KO donors were significantly protected from development of the inflammatory characteristics of the asthma phenotype (63). Although transplanted marrow contains dendritic cell and B lymphocyte precursors that could impact asthma phenotype development, it is unlikely that function of these cell types in asthma is mediated by β-arr2, as our original study (in β-arr2–KO mice) showed that responses to allergen sensitization and challenge mediated by these cells were normal (23). Transplant of β-arr2–null eosinophils could potentially confound the interpretation of the role for Th2 cells in promoting asthma inflammation in chimeric mice, as eosinophil migration to the lung in allergic inflammatory airway disease is β-arr2 dependent; however, eosinophil activation is secondary to chemokine release by Th2 cells (71), supporting the importance of β-arr2 regulation of Th2 cell migration in allergic inflammation.

In summary, the *in vitro* work presented herein demonstrates, for the first time, that β-arr2–dependent chemotactic signaling occurs in Th2 cells, and that this signaling pathway is downstream of CCL22-mediated activation of CCR4. Although *in vivo* experiments will be required to unravel exactly where, or at what time point in Th2 cell migration, β-arr2–dependent chemotaxis is important for asthma pathogenesis, this study provides a better understanding of the nature of signaling pathways that underlie cell chemotaxis. Such information will help generate more specific pharmacological

tools with which to treat pathologies characterized by aberrant cell migration. In fact, CCR4 is currently being targeted for antiasthma therapy (57, 72, 73). An advantage of antimigration-based treatment is reduced risk of toxicity compared with immunosuppressive therapy. Because CCR4-mediated signaling pathways can now be additionally distinguished by dependence, or not, on β-arr2, the potential exists to further reduce therapeutic side effects by searching for and testing orthosteric ligands or allosteric modulators of CCR4 that selectively modulate one signaling pathway over the other. Selective manipulation of CCR4-mediated chemotactic signaling pathways is highly feasible given that CCL17 is an endogenous biased ligand, and that the receptor has distinct conformational binding sites for CCL17 and CCL22 (35), which can be selectively inhibited by existing monoclonal antibodies and allosteric antagonists. ■

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βarrestin-2-dependent signaling promotes CCR4-mediated chemotaxis of murine T helper type 2 cells

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Online Supplemental Materials and Methods

Animals

Male β-arrestin-2–deficient (βarr2-KO) and littermate WT mice were backcrossed for at least 8 generations onto the C57BL/6 background Mice were euthanized by CO_2 inhalation followed by bilateral thoracotomy according to animal welfare guidelines.

Polarization of $CD4^+$ T cells to Th2

Spleens were harvested and ground with the flat end of a syringe in 5 ml of RPMI 1640 medium (Invitrogen Corp., Maryland, USA) containing 10 % FBS on a 60 mm culture dish. Cells were filtered through a 70 µm BD Falcon strainer (Becton, Dickinson and Co. USA) and washed with RPMI before centrifugation at 300 x *g* for 10 min at 4 °C. The pelleted cells were re-suspended in 10 ml of red blood cell lysis buffer (0.14 M NH_4Cl and 0.017 M Tris, pH 7.4) followed by incubation on ice for 10 min. After centrifugation, cells were resuspended in RPMI / 10 % FBS, and counted. Naïve $CD4^+$ T cells were purified and enriched from total splenocytes using EasySep negative mouse T cell selection kit (StemCell Technologies, BC, Canada) as described in procedures from the manufacturer. Th2 cell lines were generated using two different methods which produced similar Th2 skewing as assessed by GATA-3 or IL-4 measurement (see results). In the first method 2.5 X 10^6 cells/well on a 24 well plate were stimulated with plate-bound anti-CD3 (145-2C11) (1 µg/mL) and anti-CD28 (37.51) (5 µg/mL) and skewed to Th2 using polarization media that contained anti-IFNɣ (20 µg/mL), anti-IL-12 (10 µg/mL) and 10 ng/mL IL-4 in RPMI 1640 containing 10 % FBS, 2 mM L-glutamine, 50 µM β-mercaptoethanol, 100 U / mL penicillin, 100 µg / mL streptomycin sulfate for 3 days at 37 °C with 5 % CO_2. After 3 days, polarized T cells were re-stimulated with polarization media plus interleukin (IL)-2 (5 ng/mL). The second Th2-skewing method was the same as described above except that in place of plate-bound anti-CD28, irradiated APCs (2 million irradiated

(3000 rads) mixed splenocytes) were used instead. For FACS analysis, the cells were stained with APC-CD4 and FITC-CD3 ligand (CD3) Abs (BD Pharmingen™, San Diego, CA, USA), and then fixed in 4 % paraformaldehyde and analyzed using a FACSVantage (Becton Dickinson) at the Duke University Comprehensive Cancer Center Flow Cytometry Shared Resource and data were analyzed using FlowJo software (Tree Star Inc).

Intracellular Cytokine Staining (ICS) assay

To assess intracellular cytokine staining for GATA-3 and IL-4 on day 5 we washed and resuspended 1 X 10^6 $CD4^+$ Th2 cells in a 100 µl volume of eFluor® NC Flow Cytometry Staining Buffer (eBioscience, Inc. San Diego, CA, USA). Samples of 1 × 10^6 cells from each animal were placed into fresh tubes containing 0.5 µg of anti-mouse CD16 / 32 F_c block (BD Pharmingen™, San Diego, CA, USA) in RPMI 1640 and incubated for 20 min at 4 °C to reduce nonspecific binding. Following the blocking step, cells were stained with combinations of antibodies for 30 min at 4 °C. After washing with Flow Cytometry Staining Buffer, cells were surface labeled and treated with the Fixation / Permeabilization Solution (eBioscience Inc. San Diego, CA, USA), according to the manufacturer's suggested protocol. Anti-GATA3-PE antibody (Ebioscience, San Diego, CA, USA #12-9966-42) or anti-IL-4-PE antibody (BD Pharmingen™, San Diego, CA, USA #554436) was added to cells and stained for 60 min at 4 °C. After the incubation period, cells were washed and fixed in 4 % paraformaldehyde solution. Cells that underwent IL-4 staining were re-stimulated prior to surface staining with 50ng/ml PMA and 500ng/ml ionomycin for 5 hours in the presence of 2µM monensin. All flow cytometric analysis was performed with the FACSVantage (Becton Dickinson) at the Duke University Comprehensive Cancer Center Flow Cytometry Shared Resource and data were analyzed using FlowJo software (Tree Star Inc).

Chemotaxis assays

Th2 cells (1 × 10^5) were placed in 100 µl RPMI / 10 % FBS in the upper chamber of a Trans-well insert (6.5 mm diameter, 5 µm pore size; Corning Costar Corp., Acton, Massachusetts, USA). The lower chamber contained 0.1 to 30 nM murine CCL22 (R&D system, MN, USA) or 0.3 to 100 nM murine CCL17 in 600 µl RPMI prepared with 10 % FBS. After incubation at 37°C for 90 min, the migrated cells were collected from the lower chamber. The cells were stained with the following antibodies: APC anti-CD4 and APC anti-IgG control (BD Pharmingen™, San Diego, CA, USA). Analysis and cell sorting were done on a FACSVantage (Becton Dickinson)

at the Duke University Comprehensive Cancer Center Flow Cytometry Shared Resource. The number of CD4+ cells found in the lower chamber that contained no chemokine (neither CCL22 nor CCL17) represented cell chemokinesis which was subtracted from the number of cells found in the lower chamber containing chemokine. All assays were performed in triplicate.

Pharmacologic Cell Signaling Inhibitors

Inhibitors used were as follows: 20 μM SB203580, an inhibitor of p38 MAPKs; 20 μM Y27632, an inhibitor of Rho kinase; 10 μM LY294002, an inhibitor of PI3 Kinase; and 0.01 μM MK2260, an inhibitor of AKT. After 90 min cells found in the lower chamber were collected and processed by Flow Cytometry according to the methods above. Although not subtype specific, we used the pan-PI3K inhibitor Ly294002 to ensure inhibition of PI3K-mediated signaling.

CCR4 Expression

Radioligand binding and RT-PCR were used to assess CCR4 expression in Th2 cells. Th2 cells (4×10^6) in 125 μl buffer (RPMI 1640 with 1% BSA, 10 mM HEPES, pH7.4) were pre-incubated with or without 1 μM cold murine CCL17 for 5 min and were followed by incubation with 0.3nM [^{125}I]-labeled CCL17 (Perkin Elmer) for 60 min on ice. To terminate the binding reaction, the cells were pelleted and the free ligand in the supernatant was aspirated. The specific binding was calculated by subtracting the binding in the presence of cold CCL17 from the binding in the absence of cold CCL17. The maximal binding (Bmax) of cell surface CCR4 was calculated by dividing the specific binding by the specific activity of the free ligand and the amount of protein. This value was normalized to the WT CCR4 expression and graphed as percent of WT Bmax. Total cellular RNA was isolated from Th2 cells (2×10^6) using a Trizol reagent kit (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. cDNA was synthesized using high capacity cDNA reverse transcription kit (Applied Biosystems, USA) following the manufacturer's protocol. Real-time PCR was performed with the TaqMan Universal PCR Master Mix (Applied Biosystems) using 25 ng cDNA. The expression of CCR4 relative to the housekeeping gene GAPDH was measured using a Statgene Max300x sequence detector. Total RNA from control mouse lung was used as a lab standard. The fold change in CCR4 expression over the lab standard is presented.

Western blot analysis

To analyze the expression of the P-p38 protein (Cell Signaling; 1: 1,000, Beverly, USA), and pERK MAPK (Cell Signaling; 1: 1,000, Beverly, USA), $CD4^+$ Th2 cells were grown to an optimal density on 24 well plates. $CD4^+$ Th2 cells were serum-starved for 18 – 24 hrs in cytokine supplemented polarization medium prior to stimulation with chemokine. Cells were harvested at different time points after 10 nM CCL22 stimulation. After stimulation, media was removed and 50 µl of radioimmunoprecipitation assay (RIPA) lysis buffer (50mM Tris-HCl, 150mM NaCl, 5mM EDTA, 10mM NaF, 0.5% Na Deoxycholate, 1% Triton X-100, 0.1% SDS) containing protease inhibitor cocktail (Roche, IN, USA) was added to each tube. Whole cell lysates were sonicated, and same amounts of total cellular protein (25 µg, as measured by a Bradford assay read at 570 nm ; Bio-Rad, Hercules, CA, USA) were subjected to 10 % Novex® Tris-Glycine gels (Life Technologies, NY, USA), and transferred to PVDF membranes for immunoblotting (Millipore, MA, USA). The PVDF membranes were blocked for 1 hr in TBST (137 mM NaCl, 20 mM Tris–HCl, pH 7.6, 0.1 % Tween 20) containing 5 % nonfat dry milk and were incubated with primary antibodies overnight at 4 °C. After three washes in TBST, membranes were incubated with HRP-conjugated anti-rabbit IgG (ECL anti-rabbit IgG, Horserahish Peroxidase-Linked Species-Specific Whole antibody, GE Healthcare Life Sciences, NJ, USA) for 2 hrs at room temperature. The membranes were washed, and the proteins were detected by a Chemiluminescent detection system (SuperSignal West Femto reagent, Pierce, Rockford, IL, USA). Each band in the immunoblots was quantified by densitometry using the GeneTools program (SynGene, Frederick, MD, USA); representative images are shown as inverted grayscale.

Figure 5C Uncut Gels

Fig5C P-ERK 1/2 (black and white tones inverted)

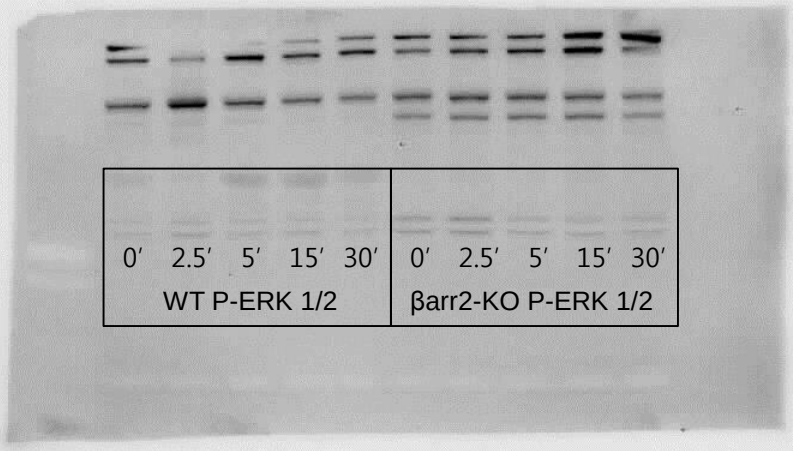

Fig5C P-ERK 1/2 inverted

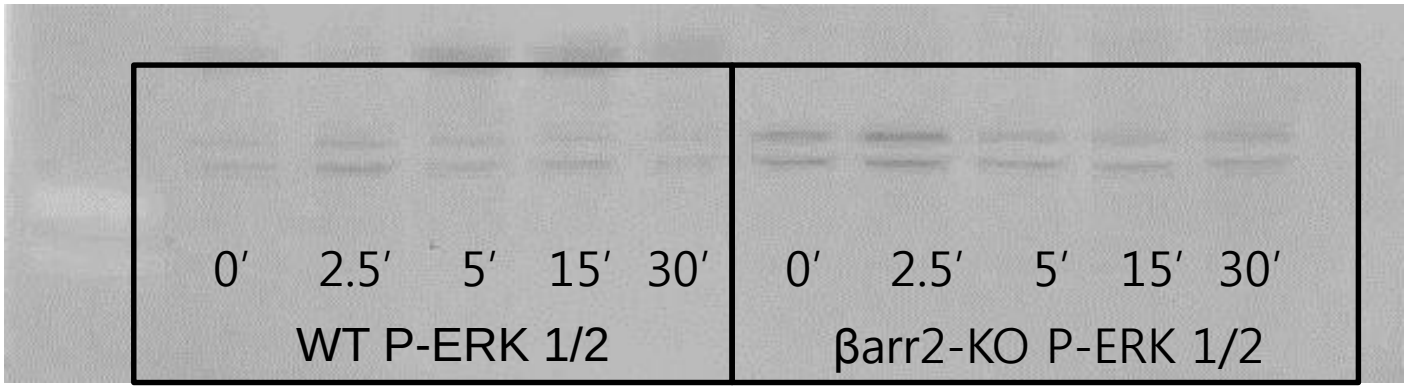

Fig5C Total-ERK 1/2 inverted

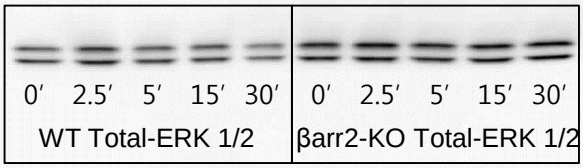
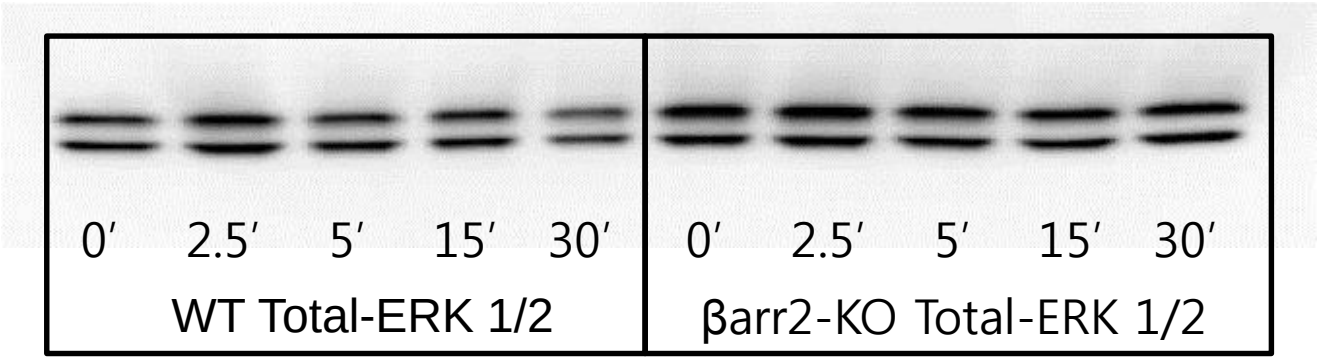
Fig5C Total-ERK 1/2 inverted