

Deficiency of β -arrestin1 ameliorates collagen-induced arthritis with impaired T_H17 cell differentiation

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Edited by Robert J. Lefkowitz, Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC, and approved March 18, 2013 (received for review December 12, 2012)

Rheumatoid arthritis (RA) is an inflammatory disease in which interleukin 17 (IL-17)-producing T helper 17 (T_H17) cells have been critically involved. We show that in patients with RA, the expression of a multifunctional regulator β -arrestin1 was significantly up-regulated in peripheral and synovial $CD4^+$ T cells, which correlated well with active phases of RA. In collagen-induced arthritis, deficiency of β -arrestin1 ameliorated disease with decreased T_H17 cell differentiation, proinflammatory cytokine production, synovitis, and cartilage and bone destruction. Further mechanistic study reveals that β -arrestin1 promoted signal transducer and activator of transcription 3 (STAT3) activation required for T_H17 cell differentiation through scaffolding the interaction of Janus kinase 1 and STAT3. These findings indicate a critical role for β -arrestin1 in the pathogenesis of collagen-induced arthritis and T_H17 cell differentiation and suggest β -arrestin1 as a potential diagnostic biomarker and therapeutic target for RA.

Inflammation has been known to be an essential immune response that can be initiated upon infection or injury to maintain tissue homeostasis (1). Rheumatoid arthritis (RA) is a chronic, painful, and disabling disease associated with a typical unrestrained inflammation in diarthrodial joints (2). To elucidate the molecular mechanism of RA development, several animal models of arthritis, in particular collagen-induced arthritis (CIA), have been widely used to explore the key inflammatory inducers and mediators by genetic manipulation of specific genes (3, 4). There is strong evidence that $CD4^+$ T-cell-mediated adaptive immunity is involved in the pathogenesis of RA (5). Interleukin 17 (IL-17)-producing T helper 17 (T_H17) cells represent a distinct subset of $CD4^+$ T cells that are essential in clearing foreign pathogens, but dysregulation of T_H17 cells would induce tissue inflammation in a variety of inflammatory conditions including RA (3, 6). The current understandings of T_H17 cells involvement in RA have been mostly drawn from murine models, whereas less is known about those in human pathologic conditions.

Signal transducer and activator of transcription 3 (STAT3) is one of the nuclear transcription factors from a highly conserved family, which has been involved in various biological processes, including immune response (7). Upon cytokines, such as IL-6, binding to their receptors, STAT3 is associated with and then activated by Janus kinases (JAKs) through phosphorylation at tyrosine residue 705 (Tyr⁷⁰⁵), and activated STAT3 regulates its target genes transcription and $CD4^+$ T-cell lineage commitment (8). Because IL-6, in combination with TGF- β , drives T_H17 cell differentiation, depletion of STAT3 greatly impairs this process (9–11). Several STAT3-interacting proteins, such as PIAS3, GRIM-19, and Rac1, have been reported to regulate STAT3 activation (12–14). However, it remains unknown whether any of those regulators functions in T_H17 cells or is under control by any physiological or pathological signal.

β -Arrestins are multifunctional proteins that play critical roles in G protein-coupled receptor (GPCR) signaling (15). Emerging observations reveal that they could also act as essential adaptors to modulate many other signaling pathways (16). A previous report shows that β -arrestin1 serves as an adaptor to bring the oncoprotein E3 ubiquitin ligase MDM2 to the activated

insulin-like growth factor-1 (IGF1) receptor, thereby promoting receptor ubiquitination and subsequent proteasomal degradation (17). In response to GPCR stimulation, β -arrestin1 regulates histone H4 acetylation and contributes to $CD4^+$ T-cell survival (18). Interestingly, a microRNA, miR-326, encoded by the first intron of β -arrestin1 gene, regulates T_H17 cell differentiation, and its expression level is associated with the pathogenesis of multiple sclerosis (MS) (19). Here, we identify β -arrestin1 as a critical regulator in the pathogenesis of CIA and T_H17 cell differentiation. β -arrestin1 exerts its function through scaffolding the interaction of Janus kinase 1 (JAK1) and STAT3, thereby promoting STAT3 activation. Thus, our findings suggest β -arrestin1 as a potential diagnostic biomarker and therapeutic target for RA.

Results

Expression of β -Arrestin1 Is Up-Regulated in Patients with Active RA and Mice with CIA. In an attempt to identify genes that are differentially expressed in peripheral blood mononuclear cells (PBMC) from patients with active RA and age-matched normal controls, we performed a custom PCR array containing a panel of immune response genes. Among the 93 genes evaluated, the expression of a subgroup of genes was up-regulated in patients with active RA, including *ARRB1* (Fig. 1A). We further confirmed the increased expression of *ARRB1* in PBMC from patients with active RA, compared with normal controls, patients with osteoarthritis (OA), or inactive RA (Fig. 1B). The expression of β -arrestin1 was also up-regulated in patients with active RA at the protein level (Fig. 1C). Next, we examined the expression of *ARRB1* in purified $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, or $CD14^+$ monocytes from patients with active RA and normal controls, respectively. Noticeably, the increased expression of *ARRB1* occurred predominantly in $CD4^+$ T cells, but not in $CD8^+$ T cells, $CD19^+$ B cells, or $CD14^+$ monocytes (Fig. 1D). The expression of *ARRB1* was comparable in $CD4^+$ T cells from normal controls, patients with OA, and inactive RA (Fig. S1). Moreover, the expression of *ARRB1*, in either synovial fluid mononuclear cells (SFMC) or purified $CD4^+$ T cells from SFMC, was even higher than that in PBMC or purified $CD4^+$ T cells from PBMC (Fig. 1E and F).

Interestingly, linear correlation analysis between transcripts of β -arrestin1 and the cytokines showed that the expression of *IL17* correlated well with that of *ARRB1* (Fig. 2A and Fig. S2). Furthermore, *ARRB1* was highly expressed in $CD4^+CCR6^+$ T cells (Fig. 2B), a subpopulation enriched for T_H17 cells (20–22). Although CCR6 was also expressed on T_H1 cells (20, 21), the

Author contributions: J.L., B.W., and G.P. designed research; J.L., B.W., A.G., C.L., and S.H. performed research; F.D., W.F., and C.B. contributed new reagents/analytic tools; J.L., B.W., A.G., and G.P. analyzed data; and J.L., B.W., and G.P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1221608110/-DCSupplemental.

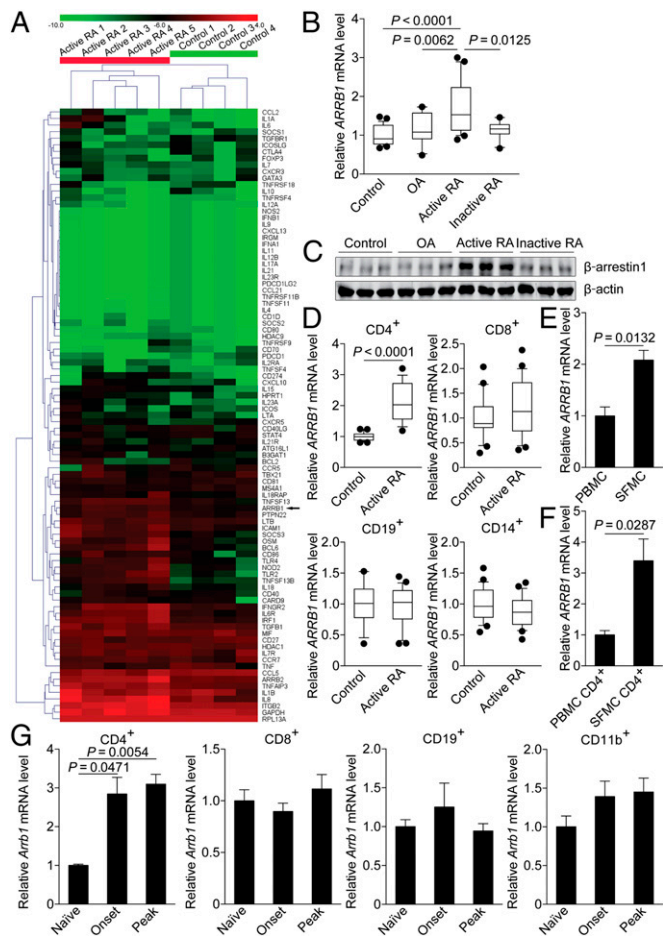


Fig. 1. Expression of β -arrestin1 is up-regulated in $CD4^+$ T cells from patients with active RA and mice with CIA. (A) Heat map of a custom PCR array comparing gene expression in PBMC from normal controls ($n = 4$) or patients with active RA ($n = 5$). (B) Expression of *ARRBT* in PBMC from normal controls ($n = 26$), patients with OA ($n = 16$), active RA ($n = 20$), or inactive RA ($n = 10$). Boxes = 25–75 percentiles. Lines in center of box, median. Whiskers = 10–90 percentiles. Dot, outliers. Results are normalized to expression of the housekeeping gene *RPL13A*. (C) Immunoblot of β -arrestin1 in PBMC from normal controls ($n = 3$), patients with OA ($n = 3$), active RA ($n = 3$), or inactive RA ($n = 3$). (D) Quantitative RT-PCR of *ARRBT* mRNA in $CD4^+$ T cells (Upper Left), $CD8^+$ T cells (Upper Right), $CD19^+$ B cells (Lower Left), or $CD14^+$ monocytes (Lower Right) from normal controls ($n = 17$) or patients with active RA ($n = 18$). Boxes = 25–75 percentiles. Lines in center of box, median. Whiskers = 10–90 percentiles. Dot, outliers. (E and F) Quantitative RT-PCR of *ARRBT* mRNA in PBMC or SFMC (E) and $CD4^+$ T cells from PBMC or SFMC (F) from patients with active RA ($n = 3$). (G) Expression of *Arrb1* in $CD4^+$ T cells (Left), $CD8^+$ T cells (Center Left), $CD19^+$ B cells (Center Right), or $CD11b^+$ myeloid cells (Right) from naive mice or mice with CIA (onset or peak) ($n = 4$ per group). Results are normalized to expression of the housekeeping gene *Hprt*.

expression of *IFNG* was similar in $CD4^+CCR6^-$ and $CD4^+CCR6^+$ T cells (Fig. 2B). Collectively, these data show that the expression of β -arrestin1 is up-regulated in $CD4^+$ T cells, particularly in $CD4^+CCR6^+$ T cells, suggesting it as a potential diagnostic biomarker for RA.

To further explore the expression profile of β -arrestin1 in the development of RA, we induced CIA on C57BL/6 mice as reported (4). Consistent with the data obtained from clinical samples, the expression of *Arrb1* was up-regulated in sorted $CD4^+$ T cells (Fig. 1G and Fig. S3A), especially in $CD4^+CCR6^+$ T cells from mice with CIA at the onset and peak of disease (Fig. 2C). The expression pattern of β -arrestin1 was also confirmed at

the protein level (Fig. S3B). All above results indicate a potential role of β -arrestin1 in the pathogenesis of CIA.

Deficiency of β -Arrestin1 Ameliorates CIA. We next examined the clinical and histopathological features of β -arrestin1-deficient (*Arrb1*^{-/-}) mice in CIA. Although most of the wild-type (WT) littermates developed arthritis, *Arrb1*^{-/-} mice exhibited significantly reduced incidence of disease (Fig. 3A). The severity of arthritis was also markedly attenuated in *Arrb1*^{-/-} mice throughout the chronic phase of disease (Fig. 3B). Compared with WT littermates, *Arrb1*^{-/-} mice displayed much milder joint swelling (Fig. 3E). Histopathological analysis of ankle joints showed a remarkable decrease in synovial inflammation, pannus formation, and cartilage and bone destruction in *Arrb1*^{-/-} mice (Fig. 3C). The loss of matrix proteoglycan content in the articular cartilage, another hallmark of chronic destructive arthritis, was also decreased in *Arrb1*^{-/-} mice (Fig. 3D). Further assessment by microscopic computed tomography (micro-CT) showed that *Arrb1*^{-/-} mice exhibited less severe bone destruction (Fig. 3E). Additionally, the expression of proinflammatory cytokines including *Il17*, *Il17f*, and *Il22* in the joints was down-regulated in *Arrb1*^{-/-} mice (Fig. 3F). These observations demonstrate that deficiency of β -arrestin1 ameliorates CIA, further suggesting a proinflammatory role of β -arrestin1.

Knockout or Knockdown of β -Arrestin1 Reduces the Production of T_H17 -Associated Cytokines. Because both T-cell-mediated and humoral immune responses have been described to be essential in the pathogenesis of CIA, we first examined the serum levels of collagen II (CII)-specific antibodies, which have been suggested to correlate well with the severity of CIA (23, 24). Circulating levels of CII-specific IgG1, IgG2b, and IgG3 were comparably elevated in *Arrb1*^{-/-} mice and WT littermates at day 35 after initial immunization (Fig. 4A). We next performed multilineage analysis to evaluate the infiltration of immune cells in the spleen and draining lymph node (DLN). Although the frequency and absolute number of T_H1 cells or regulatory T (T_{reg}) cells were similar in both groups of mice, those of T_H17 cells were markedly reduced in *Arrb1*^{-/-} mice (Fig. 4B and Fig. S4A). Concurrently, the absolute number of $B220^+$ cells, $CD11b^+$ cells, or $CD11c^+$ cells was not different between *Arrb1*^{-/-} mice and WT littermates (Fig. S4B). Furthermore, the production of IL-17, but not of IFN- γ , in

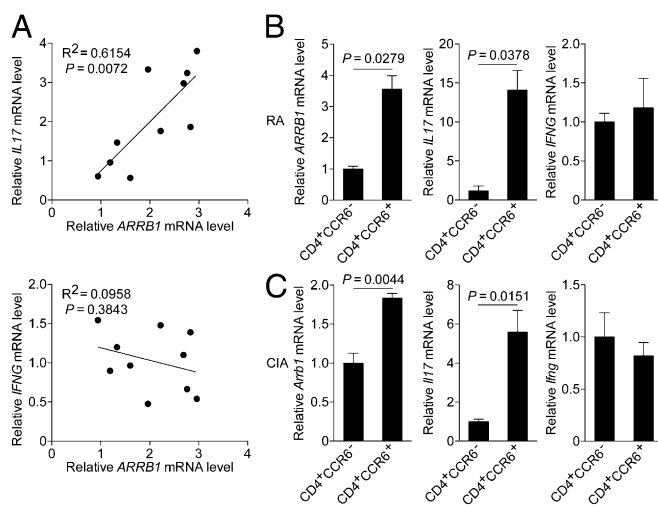


Fig. 2. Expression of β -arrestin1 correlates with that of IL-17 in $CD4^+$ T cells. (A) Linear correlation analysis between transcripts of β -arrestin1 and IL-17 (Upper) and β -arrestin1 and IFN- γ (Lower) in $CD4^+$ T cells from patients with active RA ($n = 10$). (B) Expression of *ARRBT*, *IL17*, or *IFNG* in $CD4^+CCR6^-$ or $CD4^+CCR6^+$ T cells from patients with active RA ($n = 5$). (C) Expression of *Arrb1*, *Il17*, or *Ifng* in $CD4^+CCR6^-$ or $CD4^+CCR6^+$ T cells from mice with CIA ($n = 4$).

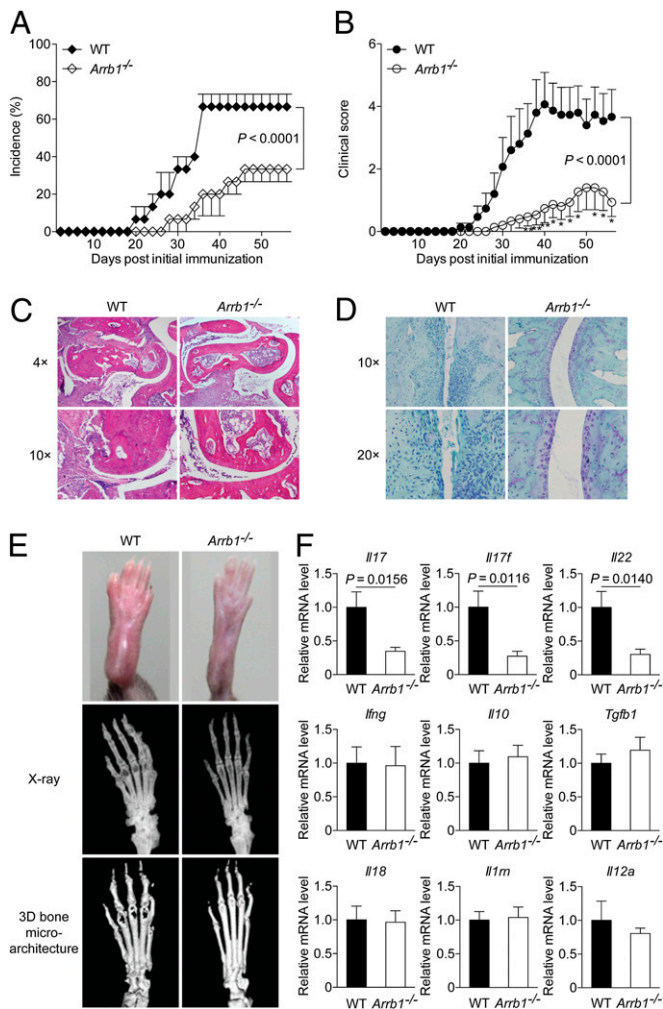


Fig. 3. Deficiency of β -arrestin1 ameliorates CIA. (A and B) Incidence (A) and severity (B) of CIA in $Arrb1^{-/-}$ mice and WT littermates ($n = 15$ per group). $*P < 0.05$ and $**P < 0.01$, versus WT littermates. (C and D) Hematoxylin and eosin (H&E) staining (C) and safranin O staining (D) of paraffin sections of ankle joints at day 56 after initial immunization ($n = 8$ per group). (E) Representative photograph and radiography of the hind paws ($n = 8$ per group). (F) Expression of various cytokines in the joints of collagen II (CII)-immunized $Arrb1^{-/-}$ mice and WT littermates ($n = 8$ per group).

the supernatants of cell cultures after in vitro CII challenge, was profoundly decreased in $Arrb1^{-/-}$ mice (Fig. 4C). In addition, chemotaxis assay showed no significant difference in CCL20-mediated migration of DLN cells or $CD4^{+}$ T cells between $Arrb1^{-/-}$ mice and WT littermates (Fig. 4D and Fig. S5).

To further confirm the potential role of β -arrestin1 in vivo, we used lentiviral vectors expressing shRNA to silence β -arrestin1 gene for long-lasting suppressive capacity. Local administration of lentivirus expressing GFP and shRNA for $Arrb1$ (Lenti-sh $Arrb1$) into inflamed joints of mice with CIA could effectively reduce the protein level of β -arrestin1, compared with lentivirus expressing GFP and control shRNA (Lenti-control) (Fig. 4E). We then intraarticularly injected Lenti-sh $Arrb1$ or Lenti-control into the joints of CII-immunized mice at the onset of disease. Consistent with the data obtained from $Arrb1^{-/-}$ mice and WT littermates, lentivirus-mediated silencing of β -arrestin1 in vivo in the joints led to significant decreased expression of proinflammatory cytokines, including $Il17$, $Il17f$, and $Il22$ (Fig. 4F). Based on the increased expression of $Arrb1$ in $CD4^{+}CCR6^{+}$ T cells from mice with CIA, knockdown of β -arrestin1 ex vivo in these cells remarkably reduced the expression of several T_H17 -

associated genes, such as $Il17$ and $Il22$ (Fig. 4G–I), which have been critically involved in CIA (3, 25). Together, these results indicate that knockout or knockdown of β -arrestin1 reduces the production of T_H17 -associated cytokines.

Depletion of β -Arrestin1 Impairs T_H17 Cell Differentiation in Vitro. To determine whether β -arrestin1 directly regulated T_H17 cell differentiation, we performed an in vitro T-cell differentiation assay. We first examined the expression of $Arrb1$ in $CD4^{+}$ T-cell subsets and found that $Arrb1$ was most highly expressed in T_H17 cells (Fig. S4). Bioinformatic analysis revealed a putative binding site for ROR γ t, the T_H17 lineage-specific transcription factor, in the 2-kb region of mouse $Arrb1$ promoter. In cells expressing a reporter containing the $Arrb1$ promoter, ROR γ t substantially enhanced luciferase activity in a dose-dependent manner (Fig. S6), indicating that ROR γ t may regulate β -arrestin1 expression in T_H17 cells. In addition, the up-regulation of $Arrb1$ expression

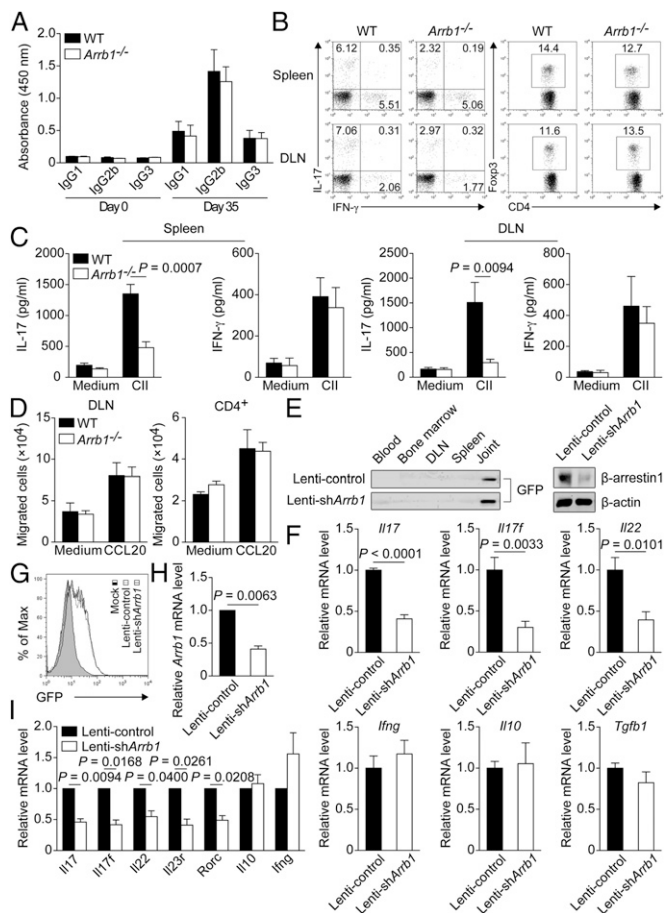


Fig. 4. Knockout or knockdown of β -arrestin1 reduces the production of T_H17 -associated cytokines. (A) ELISA of CII-specific IgG1, IgG2b, or IgG3 in the serum of $Arrb1^{-/-}$ mice and WT littermates at day 0 and day 35 after initial immunization ($n = 7$ per group). (B) Flow cytometry of intracellular IL-17, IFN- γ , or Foxp3 in gated $CD4^{+}$ T cells from the spleen and draining lymph node (DLN) of CII-immunized $Arrb1^{-/-}$ mice and WT littermates ($n = 8$ per group). (C) Concentration of IL-17 or IFN- γ in the supernatants of cell cultures challenged with CII (100 μ g/mL) for 4 d ($n = 6$ per group). (D) Chemotaxis assay of DLN cells or $CD4^{+}$ T cells from CII-immunized $Arrb1^{-/-}$ mice and WT littermates ($n = 5$ per group). (E and F) Immunoblot of GFP or β -arrestin1 (E) and expression of various cytokines (F) in the joints of CII-immunized mice intraarticularly injected with lentivirus expressing GFP and shRNA for $Arrb1$ (Lenti-sh $Arrb1$) or GFP and control shRNA (Lenti-control) ($n = 5$ per group). (G–I) Flow cytometry of GFP (G) and expression of various genes (H and I) in $CD4^{+}CCR6^{+}$ T cells from mice with CIA transduced with Lenti-sh $Arrb1$ or Lenti-control.

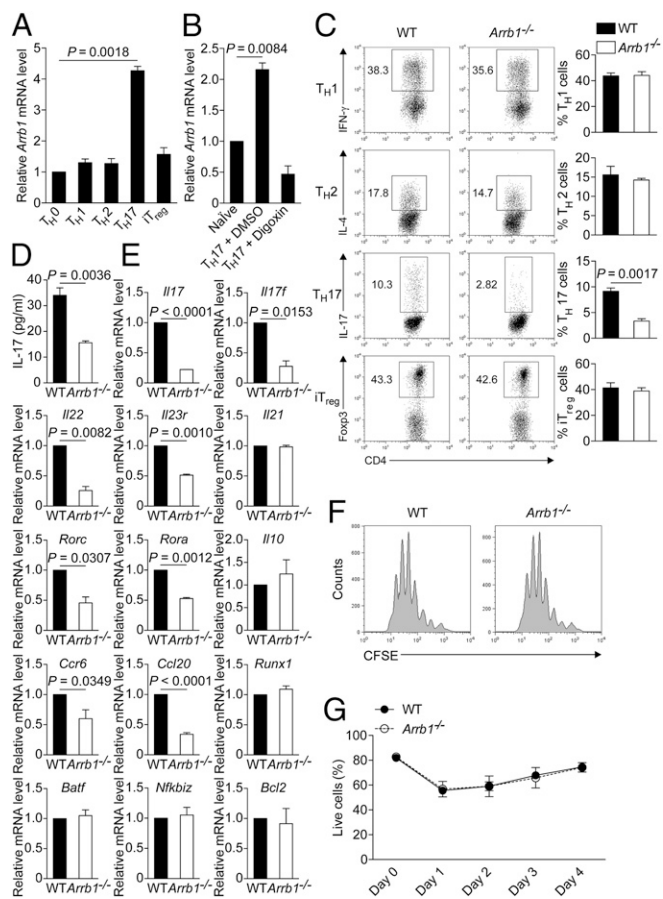


Fig. 5. Depletion of β -arrestin1 impairs T_H17 cell differentiation in vitro. (A) Expression of *Arrb1* in T_H0 , T_H1 , T_H2 , T_H17 , or inducible T_{reg} (iT_{reg})-polarized cells from C57BL/6 mice. (B) Quantitative RT-PCR of *Arrb1* mRNA in naive $CD4^+$ T cells or T_H17 -polarized cells in the presence of DMSO or Digoxin (10 μ M). (C) Flow cytometry of naive $CD4^+$ T cells from *Arrb1*^{-/-} mice and WT littermates cultured for 4 d under T_H1 , T_H2 , T_H17 , or iT_{reg} -polarizing conditions. Percentage of each subset is shown. (D) Production of IL-17 in the supernatants of T_H17 -polarized cells. (E) Expression of T_H17 -associated genes in naive $CD4^+$ T cells from *Arrb1*^{-/-} mice and WT littermates cultured for 2 d under T_H17 -polarizing conditions. (F) Flow cytometry of naive $CD4^+$ T cells labeled with carboxyfluorescein succinimidyl ester (CFSE) from *Arrb1*^{-/-} mice and WT littermates cultured for 4 d under T_H17 -polarizing conditions. (G) Naive $CD4^+$ T cells were cultured under T_H17 -polarizing conditions, and live cells were identified by propidium iodide exclusion.

in T_H17 cell differentiation was inhibited by Digoxin (Fig. 5B), a ROR γ t-specific inhibitor in T_H17 cells (26, 27). Further investigation is needed for clarification of β -arrestin1 expression regulated by ROR γ t.

We then assessed T_H17 cell differentiation in *Arrb1*^{-/-} mice and WT littermates. Obviously, the frequency of IL-17-positive cells, *Il17* mRNA expression, and IL-17 production were markedly decreased in *Arrb1*^{-/-} mice (Fig. 5C–E). However, the frequency of IFN- γ -positive cells, IL-4-positive cells, or Foxp3-positive cells was comparable in *Arrb1*^{-/-} mice and WT littermates (Fig. 5C), suggesting no significant impact of β -arrestin1 on the differentiation of T_H1 , T_H2 , or inducible T_{reg} (iT_{reg}). Besides *Il17*, the expression of other T_H17 -associated genes including *Rorc*, *Rora*, *Il17f*, *Il22*, *Il23r*, *Ccr6*, and *Ccl20*, was also remarkably down-regulated in *Arrb1*^{-/-} T cells (Fig. 5E and Fig. S7). Our previous report showed that β -arrestin1 regulates $CD4^+$ T-cell survival via *Bcl2* (18). Under T_H17 -polarizing conditions, we observed no apparent difference in proliferation or viability between WT and *Arrb1*^{-/-} T cells (Fig. 5F and G and Fig. S8). The expression of *Bcl2* was also unchanged in the same

conditions (Fig. 5E). These data clearly demonstrate the pivotal role of β -arrestin1 in T_H17 cell differentiation.

Interaction of JAK1/ β -Arrestin1/STAT3 Regulates STAT3 Activation. Given the essential role of STAT3 in T_H17 cell differentiation (9–11, 28), we next investigated whether loss of β -arrestin1 affected STAT3 Tyr⁷⁰⁵ phosphorylation, which is required for its activation. Under T_H17 -polarizing conditions, STAT3 Tyr⁷⁰⁵ phosphorylation in *Arrb1*^{-/-} T cells was significantly attenuated (Fig. 6A), resulting in decreased occupancy of STAT3 on the promoter of *Il17*, *Il17f*, and *Rorc* (Fig. 6B). However, STAT1 Tyr⁷⁰¹ phosphorylation or STAT5 Tyr⁶⁹⁴ phosphorylation was not altered under these conditions (Fig. 6A). The effect of β -arrestin1 on STAT3 phosphorylation was further confirmed by overexpression or knockdown of β -arrestin1 (Fig. 6C and D). In addition, the expression of other transcription factors involved in T_H17 cell differentiation, such as *Runx1*, *Batf*, and *Nfkbiz*, was comparable in WT and *Arrb1*^{-/-} T cells (Fig. 5E).

Because β -arrestins have been reported to function as adaptors in various signaling pathways (29, 30), we examined whether a similar scaffolding mechanism was involved in STAT3 activation. In $CD4^+$ T cells from C57BL/6 mice, endogenous β -arrestin1, JAK1, and STAT3 associated with each other (Fig. 7A). Interestingly, administration of IL-6 triggered a remarkable increase in JAK1/STAT3 interaction as well as β -arrestin1/STAT3 interaction (Fig. 7A), and this interaction was further confirmed in T_H17 -polarized cells (Fig. S9). These results implicate that IL-6 stimulation promotes the JAK1/ β -arrestin1/STAT3 interaction. However, the interaction of JAK1 and STAT3 was markedly reduced in β -arrestin1-knockout or -knockdown cells (Fig. 7B and C) and, conversely, promoted by β -arrestin1 overexpression (Fig. 7D), which suggest that β -arrestin1 is indispensable for promoting the IL-6-induced interaction of JAK1 and STAT3. More importantly, neither β -arrestin1 1–180 (a C-terminal deletion mutant consisting of β -arrestin1 amino acids 1–180) nor β -arrestin1 181–418 (a N-terminal deletion mutant consisting of β -arrestin1 amino acids 181–418) possessed the potential to promote the JAK1/STAT3 interaction (Fig. 7E and F). Therefore, STAT3 activation and T_H17 cell differentiation were unchanged in the presence of both β -arrestin1 mutants (Fig. 7G and H). Taken together, these findings indicate that β -arrestin1 positively regulates STAT3 activation through scaffolding the JAK1/STAT3 interaction.

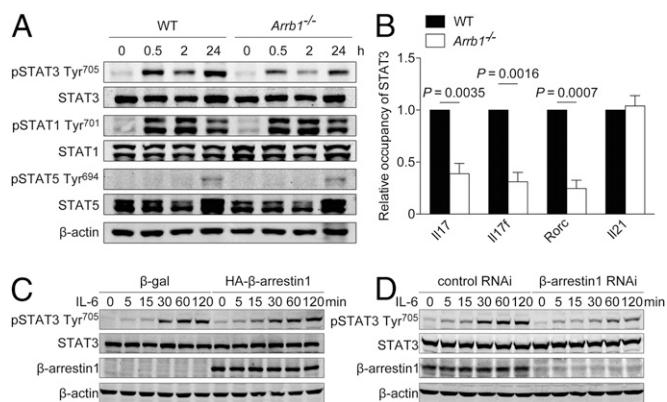


Fig. 6. Regulation of STAT3 activation by β -arrestin1. (A) Immunoblot of phosphorylation and expression of STAT3, STAT1, or STAT5 in naive $CD4^+$ T cells under T_H17 -polarizing conditions for the indicated times. (B) ChIP assay of the binding of STAT3 to the *Il17*, *Il17f*, *Rorc*, or *Il21* promoter in *Arrb1*^{-/-} mice and WT littermates. (C and D) Immunoblot of phosphorylation and expression of STAT3 in HEK293 cells expressing β -galactosidase (β -gal) or HA- β -arrestin1 (C) and control RNAi or β -arrestin1 RNAi (D).

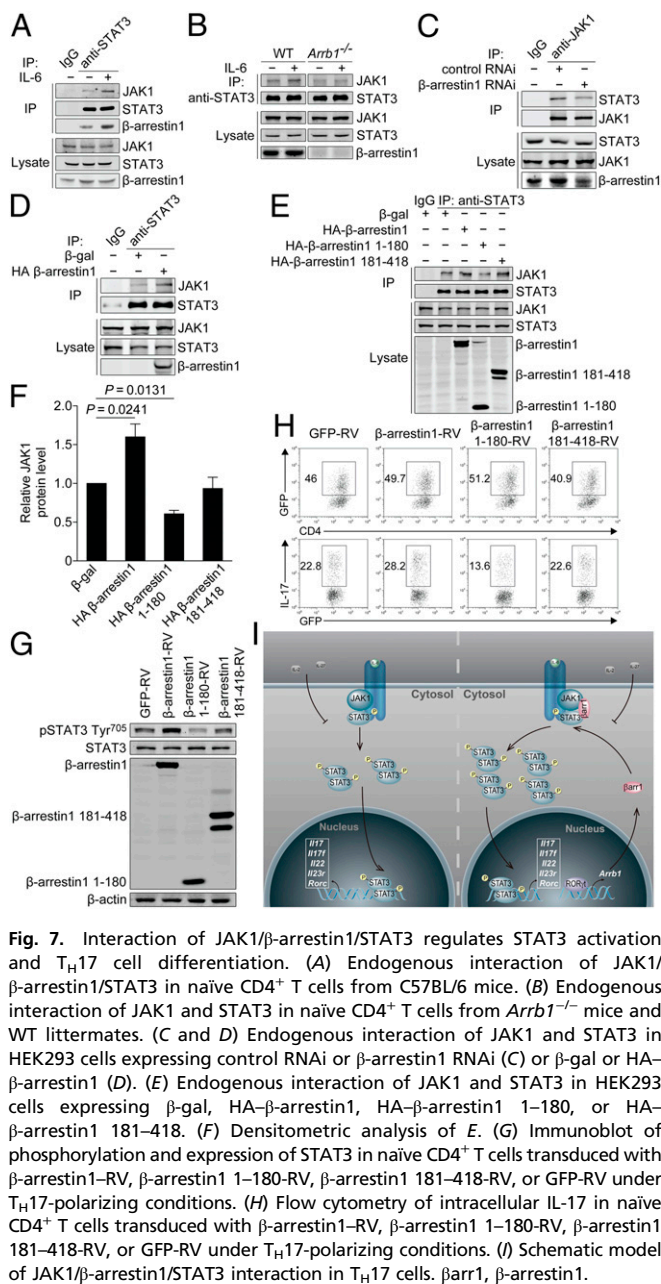


Fig. 7. Interaction of JAK1/β-arrestin1/STAT3 regulates STAT3 activation and T_H17 cell differentiation. (A) Endogenous interaction of JAK1/β-arrestin1/STAT3 in naive CD4⁺ T cells from C57BL/6 mice. (B) Endogenous interaction of JAK1 and STAT3 in naive CD4⁺ T cells from *Arbb1*^{-/-} mice and WT littermates. (C and D) Endogenous interaction of JAK1 and STAT3 in HEK293 cells expressing control RNAi or β-arrestin1 RNAi (C) or β-gal or HA-β-arrestin1 (D). (E) Endogenous interaction of JAK1 and STAT3 in HEK293 cells expressing β-gal, HA-β-arrestin1, HA-β-arrestin1 1–180, or HA-β-arrestin1 181–418. (F) Densitometric analysis of E. (G) Immunoblot of phosphorylation and expression of STAT3 in naive CD4⁺ T cells transduced with β-arrestin1-RV, β-arrestin1 1–180-RV, β-arrestin1 181–418-RV, or GFP-RV under T_H17-polarizing conditions. (H) Flow cytometry of intracellular IL-17 in naive CD4⁺ T cells transduced with β-arrestin1-RV, β-arrestin1 1–180-RV, β-arrestin1 181–418-RV, or GFP-RV under T_H17-polarizing conditions. (I) Schematic model of JAK1/β-arrestin1/STAT3 interaction in T_H17 cells. βarr1, β-arrestin1.

Discussion

The essential role of STAT3 in T_H17 cell differentiation has been clearly described (9–11, 28). However, the understanding of STAT3 activation remains limited. Our observations have demonstrated that in response to IL-6, β-arrestin1 may function as a scaffold protein to promote the JAK1/STAT3 interaction (Fig. 7). Subsequently, the phosphorylation of STAT3 by JAK1 is increased, and the expression of STAT3 target genes, particularly a group of T_H17-associated genes including *Il17*, *Il17f*, *Il22*, and *Rorc*, is up-regulated. Although the phosphorylation of STAT3 by JAK1 has been well documented (7, 8), our data indicate that promotion of the JAK1/STAT3 interaction by β-arrestin1 is also critical for STAT3 activation, which provide insights into regulation of STAT3 activation. Collectively, our findings suggest β-arrestin1 as an important regulator of STAT3 activation in T_H17 cell differentiation. Interestingly, our preliminary results reveal that RORγt, the T_H17 lineage-specific transcription factor, transactivates *Arbb1* promoter. This

molecular mechanism might account for the selective higher expression of β-arrestin1 in T_H17 cells. Therefore, β-arrestin1 possesses the potential to specifically regulate T_H17 cell differentiation, but not the differentiation of T_H1, T_H2, or T_{reg} cells. Because STAT3 regulates the expression of RORγt through binding to its promoter (28), based on our data presented, we propose a feed-forward loop of β-arrestin1–STAT3–RORγt to regulate T_H17 cell differentiation. Furthermore, whether any extracellular signal could terminate this loop is of interest. It is noteworthy that IL-2, a growth factor for most T cells, has been reported to inhibit T_H17 cell differentiation via STAT5 activation (9). A recent study has confirmed this finding and further proposed the reciprocal actions of STAT3 and STAT5 on the genetic locus encoding IL-17 (31). Likewise, IL-27 has been shown to have antiinflammatory activity through its restraint of T_H17 cell differentiation, in a STAT1-dependent manner (32, 33). Thus, IL-2/STAT5 or IL-27/STAT1 axis might restrain this β-arrestin1–STAT3–RORγt loop under certain physiological or pathological conditions.

It has been well documented that β-arrestin1 functions as multiprotein scaffolds to coordinate complex signal transduction networks in diverse cellular processes, including differentiation, cytokine production, proliferation, cell viability, and migration (15, 34). It could be of interest to examine whether β-arrestin1 regulates the above events in T_H17 cells. Our observations show that (i) depletion of β-arrestin1 impairs in vitro T_H17 cell differentiation; (ii) knockout or knockdown of β-arrestin1 reduces the production of T_H17-associated cytokines in vivo; (iii) proliferation of CFSE-labeled cells is not altered by loss of β-arrestin1; (iv) viability of T_H17 cells is unchanged in the absence of β-arrestin1; and (v) deletion of β-arrestin1 does not affect CCL20-mediated migration. Together, these observations suggest that β-arrestin1 could selectively be involved in T_H17 cell differentiation and production of T_H17-associated cytokines.

Besides T_H17 cells, fibroblast-like synoviocytes have also been reported to be involved in the pathogenesis of CIA (2). Recent reports show that β-arrestin1 is up-regulated in fibroblast-like synoviocytes, the primary effector of cartilage destruction and inflammatory pathogenesis in CIA (35, 36). Because β-arrestin1 is reported to play an important role in fibroblast invasion and pulmonary fibrosis (34), further investigation is needed to clarify the role of β-arrestin1 in fibroblast-like synoviocytes.

Our investigation on clinical samples demonstrates that the expression of β-arrestin1 is significantly up-regulated in peripheral or synovial CD4⁺ T cells from patients with active RA, and it correlates well with the active phases of RA. These data are consistent with our previous finding that β-arrestin1 expression is remarkably up-regulated in peripheral CD4⁺ T cells from patients with relapsing-remitting MS (18). Our preliminary results indicate that β-arrestin1 expression seems to be up-regulated also in CD4⁺ T cells from patients with inflammatory bowel disease (IBD). Taken together, all of the evidence accumulated so far indicate the functional involvement of β-arrestin1 in many inflammatory and autoimmune diseases. Although it is not exactly known at this point how β-arrestin1 is extensively involved, it could be further explored as a potential diagnostic biomarker or even a prognostic indicator for those diseases. Furthermore, silencing of β-arrestin1 either in joints or in CD4⁺CCR6⁺ T cells from mice with CIA results in decreased production of proinflammatory cytokines. These results suggest that β-arrestin1 might serve as a potential therapeutic target for RA. It is noticeable that the expression of β-arrestin1 in SFMC is markedly higher than that in PBMC. This increased expression of β-arrestin1 in specific lesions may also occur in other inflammatory and autoimmune diseases, such as MS and IBD. Given that MS has been characterized as a central nervous system (CNS) disorder and IBD has been classified as an intestinal disease, tissue-specific targeting of β-arrestin1 in individual disease is necessary, although β-arrestin1 expression in those lesions remains to be further explored.

Materials and Methods

Study Subjects. Volunteer subjects included patients from Renji Hospital of Shanghai Jiaotong University, who fulfilled the American College of Rheumatology (ACR) classification criteria for RA, and age-matched healthy volunteers from personnel of Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences). Blood and synovial fluid samples were obtained from subjects after informed-consent procedures, and the sampling was completed in accordance with the guidelines of the Ethics Committee of Renji Hospital, Shanghai Jiao Tong University School of Medicine.

Mice. C57BL/6 mice were obtained from Shanghai Laboratory Animal Center (Chinese Academy of Sciences). *Arrb1*^{-/-} mice on a C57BL/6 background were provided by Robert J. Lefkowitz (Duke University Medical Center, Durham, NC). All mice were maintained in pathogen-free conditions. Animal care and use were in accordance with the guidelines of the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences.

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Statistics. Data are presented as mean ± SEM. A two-tailed Student t test was used to compare two groups, or where appropriate, the two-way analysis of variance followed by Bonferroni post hoc test for multiple comparisons and a Mann-Whitney test for nonparametric data (CIA scoring). For all tests, a P value of 0.05 or less was considered statistically significant.

Details of T-cell differentiation, induction of CIA, histopathology, ELISA, flow cytometry, immunoprecipitation and immunoblot, and ChIP are provided in *SI Materials and Methods* and *Tables S1* and *S2*.

ACKNOWLEDGMENTS. We thank Robert J. Lefkowitz (Duke University Medical Center) for *Arrb1*^{-/-} mice; Biao Zheng (GlaxoSmithKline Research and Development Center) for technical assistance on CIA induction; Dan R. Littman (New York University) for plasmid FLAG-RORγt; Dangsheng Li (Shanghai Institutes for Biological Sciences), Jiahong Kang (Tongji University), and Jian Zhao for helpful discussions; and Wenjing Li, Shunmei Xin, Hui Xu, and Xianglu Zeng for technical assistance. We also thank all laboratory members for sharing reagents and advice. This research was supported by Chinese Academy of Sciences (XDA01010302), Ministry of Science and Technology (2011CB946102), and Ministry of Health (2012BA110B03).

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