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β-arrestin-1 mediates the TCR-triggered re-routing of distal receptors to the immunological synapse by a PKC-mediated mechanism

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Abstract

T-cell receptors (TCR) recognize their antigen ligand at the interface between T cells and antigen-presenting cells, known as the immunological synapse (IS). The IS provides a means of sustaining the TCR signal which requires the continual supply of new TCRs. These are endocytosed and redirected from distal membrane locations to the IS. In our search for novel cytoplasmic effectors, we have identified β-arrestin-1 as a ligand of non-phosphorylated resting TCRs. Using dominant-negative and knockdown approaches we demonstrate that β-arrestin-1 is required for the internalization and downregulation of non-engaged bystander TCRs. Furthermore, TCR triggering provokes the β-arrestin-1-mediated downregulation of the G-protein coupled chemokine receptor CXCR4, but not of other control receptors. We demonstrate that β-arrestin-1 recruitment to the TCR, and bystander TCR and CXCR4 downregulation, are mechanistically mediated by the TCR-triggered PKC-mediated phosphorylation of β-arrestin-1 at Ser163. This mechanism allows the first triggered TCRs to deliver a stop migration signal, and to promote the internalization of distal TCRs and CXCR4 and their translocation to the IS. This receptor crosstalk mechanism is critical to sustain the TCR signal.

Keywords arrestin; chemokine receptors; GPCR; PKC; receptor crosstalk; signal sustainment

Subject Categories Signal Transduction; Immunology; Membrane & Intracellular Transport

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Introduction

Cells integrate extracellular cues that activate different membrane receptors, which in turn modulate the activity of a panel of transcription factors, and/or mediate the rearrangement of the cytoskeleton and organelles. This signal integration does not always occur in the nucleus or through the cytoplasmic scaffold of signaling proteins. Moreover, some membrane receptors crosscommunicate, whereby one receptor type uses the signaling machinery of another receptor (Natarajan & Berk, 2006). A typical example of this is the crosstalk between the 7-transmembrane G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) initiated upon binding of either GPCR or RTK ligands (Natarajan & Berk, 2006; Shenoy & Lefkowitz, 2011). As such, ligand binding to RTKs can result in the appropriation of several components of the GPCR-mediated signaling machinery, including β-arrestin and G protein-receptor kinases (Shenoy & Lefkowitz, 2011). This crosstalk may be mediated by RTK-triggered activation of GPCR-associated G proteins, or even through direct interactions (Piiper & Zeuzem, 2004; Delcourt *et al*, 2007).

The TCR interacts functionally and physically with the CXCR4 chemokine receptor, a GPCR involved in the migration of T cells into lymphoid organs in response to gradients of the chemokine CXCL12 (Peacock & Jirik, 1999; Molon *et al*, 2005; Kumar *et al*, 2006). TCR triggering provokes the desensitization of T cells to CXCL12, blocking their migratory behavior and facilitating the formation of a stable contact between T cells and antigen presenting cells (APCs) (Bromley *et al*, 2000; Molon *et al*, 2005). Upon contacting an antigen-loaded APC, T cells rearrange their actin cytoskeleton and form a tight apposition with the APC membrane, generating a structure known as the immunological synapse (IS) (Dustin *et al*, 2010; Yokosuka & Saito, 2010). The TCR binds to the peptide-major histocompatibility complex (pMHC) ligand at the periphery of the IS, forming micron-sized clusters termed microclusters. TCRs transmit signals from within these microclusters, which move centripetally to form a

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central aggregation of TCRs, which results in the termination of TCR signaling and their subsequent removal from the center of the IS by a phagocytotic-like mechanism (Alarcon *et al*, 2011; Martinez-Martin *et al*, 2011). For full activation, exhausted TCRs must be replaced by new TCRs to engage pMHC, thereby sustaining TCR signaling for hours (Huppa *et al*, 2003). Thus, while horizontal movements of TCRs initially positioned at the T cell-APC contact site can mediate initial signaling events, distal TCRs must be internalized and rerouted to the IS in order to sustain the TCR signal (Das *et al*, 2004).

At least two independent mechanisms can be used to internalize pMHC-triggered TCRs: a clathrin-coated pit mechanism and a phago cytic-like mechanism. However, in addition to inducing the internalization of pMHC-engaged TCRs, TCR triggering provokes the internalization and downregulation of non-contacted bystander TCRs (Niedergang *et al*, 1998; San Jose *et al*, 2000; Monjas *et al*, 2004). This co-modulation of bystander TCRs occurs via a clathrin-coated pit-dependent mechanism that requires the activation and participation of protein kinase C (PKC) isoforms (Monjas *et al*, 2004; von Essen *et al*, 2006).

TCR engagement by pMHC induces the tyrosine phosphorylation of the so-called immunoreceptor tyrosine-based activation motifs (ITAMs), which consist of a double stretch of tyrosine and leucine (or isoleucine) residues with the consensus sequence YxxL/I(x)6-9YxxL/I. The dual phosphorylated ITAMs serve as high-affinity docking sites for signaling proteins containing tandem SH2 domains, such as the Syk-family tyrosine kinase ZAP-70. However, in their non-phosphorylated form, ITAMs are docking sites for other proteins such as adaptins, which can recognize each of the YxxL/I motifs and participate in endocytosis (Szymczak & Vignali, 2005). Other proteins that bind non-phosphorylated ITAMs include TC21 (also known as RRas2), a member of the Ras-related subfamily of GTPases, and EB1, a plus-end microtubule-tracking protein (Delgado *et al*, 2009; Martin-Cofreces *et al*, 2012).

To identify novel TCR effectors that bind to non-phosphorylated ITAMs, we employed a proteomic approach using synthetic peptides as baits. Among others, we identified β-arrestin-1 (β-Arr1; also known as arrestin-2) as a protein that binds to non-phosphorylated ITAMs in a manner dependent on TCR triggering and mediated by PKC. β-Arr1 binding induces the co-modulation of distal bystander TCRs and their re-routing to the IS, accompanied by CXCR4. We demonstrate that this mechanism is required to supply the IS with new TCRs and hence, to sustain the TCR signal.

Results

β-arrestin-1 is a direct cytoplasmic ligand of the TCR

To characterize new ligands of TCR ITAMs, we employed a proteomic approach using a biotinylated synthetic peptide corresponding to the first ITAM (membrane-proximal, CD3ζa; Fig 1A) of CD3ζ in its unphosphorylated form as bait. For comparison, dual tyrosine phosphorylated peptides corresponding to the first and third ITAMs (CD3ζaP and CD3ζcP) were run in parallel. The three biotinylated peptides were incubated with post-nuclear detergent lysates of the human T cell leukemia line Jurkat, which were incubated in the absence or presence of anti-CD3 to trigger TCRs. Proteins in the cell lysate that co-purified with the three peptides after incubation with streptavidin-agarose beads were characterized by tandem mass spectrometry (MS/MS). In the ITAM-bound fractions we identified 105 proteins from unstimulated cell lysates and 176 proteins from lysates of TCR-triggered cells (Supplementary Fig S1A and Table S1). Some of these ITAM-interacting proteins have been identified previously as phosphorylated substrates downstream of the TCR (Mayya *et al*, 2009). Proteins involved in intracellular/vesicular transport, endocytosis, cytoskeletal function and signal transduction were particularly important for TCR function (Supplementary Fig S1B and Table S1). We selected nine proteins for validation by immunoblotting after pull-down with biotinylated peptides. Four protein interactions were validated (Fig 1B). These proteins are known to participate in endocytosis (McDonald *et al*, 1999; McMahon & Boucrot, 2011; Meyer *et al*, 2012): β-Arr1, vesicle-fusing ATPase (NSF), valosin containing protein (VCP) and clathrin heavy chain (CHC). Interestingly, the four validated proteins only interacted with CD3ζ ITAMs in T cells that had been previously stimulated with anti-CD3, suggesting that their binding to the TCR is regulated by TCR signaling. However, while CHC and VCP showed no preference for tyrosine phosphorylated (CD3ζaP and CD3ζcP) versus unphosphorylated (CD3ζa) ITAMs, NSF and β-Arr1 appeared to bind preferentially the unphosphorylated ITAM (Fig 1B).

While the function of β-Arr1 in GPCRs has been well described (Shukla *et al*, 2011), its role as a TCR effector is unknown. Thus, we investigated the possible function of the interaction between β-Arr1 and the TCR. β-Arr1 was recruited to the TCR of T cells in a TCR triggering-dependent manner (Fig 1C), indicating that the β-Arr1-ITAM interaction (Fig 1B) is not an artifact of peptide overexpression. Using recombinant purified β-Arr1 we found that this protein interacted directly with two unphosphorylated ITAMs (CD3ζa and CD3ζb) and less strongly with two phosphorylated ITAMS (Fig 1D). These results suggest that β-Arr1 is a direct binding partner of the TCR. To characterize the region of β-Arr1 involved in this interaction, we used 2 constructs that express either the first 382 amino acids (β-Arr1 [1-382])(Key *et al*, 2005) or the last 99 amino acids (β-Arr1 [319-418])(Braun *et al*, 2003) of this protein (Fig 2A). The 319-418 fragment contains binding sites for CHC and for the adaptin complex AP2 (Fig 2A), and it therefore plays an important role in β-Arr1-mediated GPCR endocytosis (Krupnick *et al*, 1997; Kern *et al*, 2009). Both fragments were expressed as GFP fusion proteins in HEK-293T cells together with $(His)_6$-tagged CD3ζ. Both truncated proteins co-purified with $(His)_6$-tagged CD3ζ to a similar extent as to the full length wild-type (WT) β-Arr1 ([1-418], Fig 2B). The two truncated proteins share amino acids 319-382 (Fig 2A), indicating that this sequence must mediate the association of β-Arr1 with ITAMs. Indeed, a GST fusion protein with this sequence co-purified with the unphosphorylated ITAMs, confirming that the 319-382 sequence contains the ITAM-binding site (Fig 2C). Notably, despite sharing 76% amino acid identity, β-Arr1 but not β-Arr2 (also known as arrestin-3) interacted with CD3ζa (Fig 1B), suggesting that a non-conserved sequence within amino acids 319-382 mediates this interaction. A comparison of mammalian protein sequences shows different stretches of amino acids that are conserved in β-Arr1 but not in β-Arr2 (Supplementary Fig S2), suggesting that one or several of these sequences could be responsible for the interaction with ITAMs. The recruitment of WT β-Arr1 (1-418) and the truncated β-Arr1 (1-382) and β-Arr1 (319-418) fragments to the TCR was studied in transiently transfected Jurkat T cells stimulated with

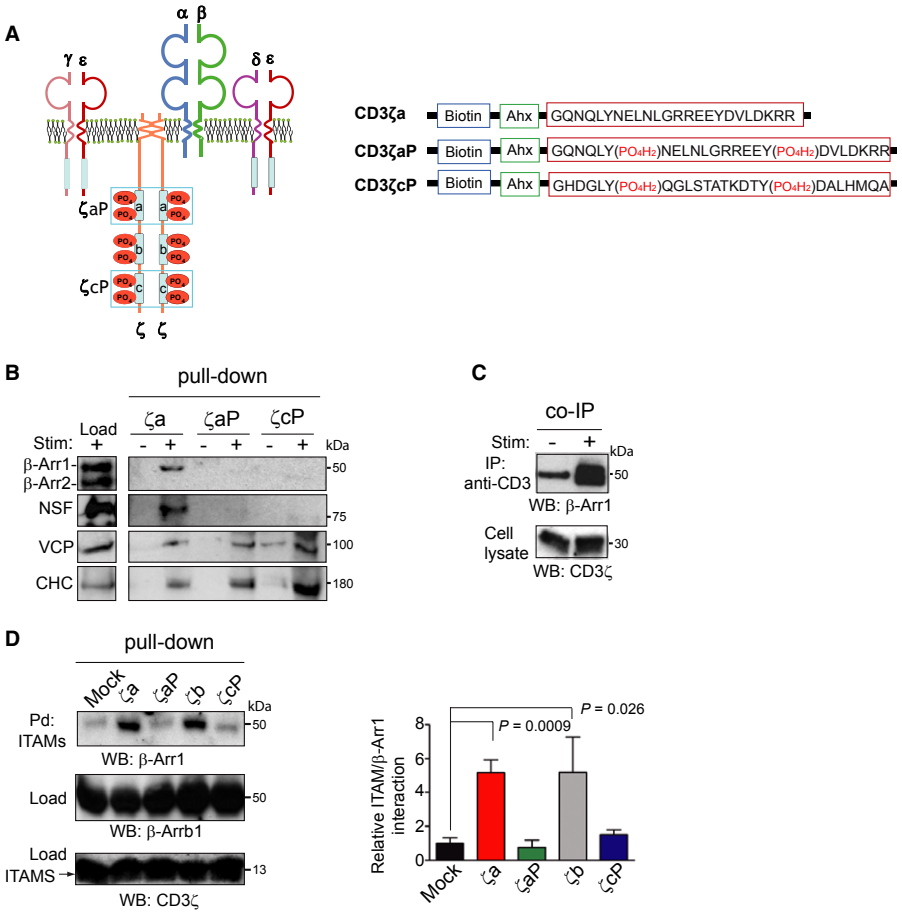


Figure 1. Identification of β-Arr1 as an ITAM-binding protein.

A Cartoon of a TCR and the sequence of the ITAM-encoding biotin-labelled synthetic peptides used in this paper.

B Validation of a set of ITAM-interacting proteins in a pull-down and immunoblot assay. ITAM-interacting proteins from lysates of control (–) and anti-CD3-stimulated (+) Jurkat T cells were pulled-down with the indicated biotinylated ITAMs and identified in Western blots (WB) probed with the specific antibodies indicated. To demonstrate the reactivity of the antibodies, 5% of the pull-down input was analyzed directly. β-Arr1, β-arrestin 1; β-Arr2, β-arrestin 2; NSF, vesicle-fusing ATPase; VCP, valosin containing protein; CHC, clathrin heavy chain (representative of three experiments).

C T-cell stimulation induces the recruitment of β-Arr1 to the TCR. Murine T-cell lymphoblasts were stimulated with anti-CD3 for 20 min. β-Arr1 co-purified with the TCR in anti-CD3 immunoprecipitates. Whole cell lysates were used as controls of expression (representative of three experiments).

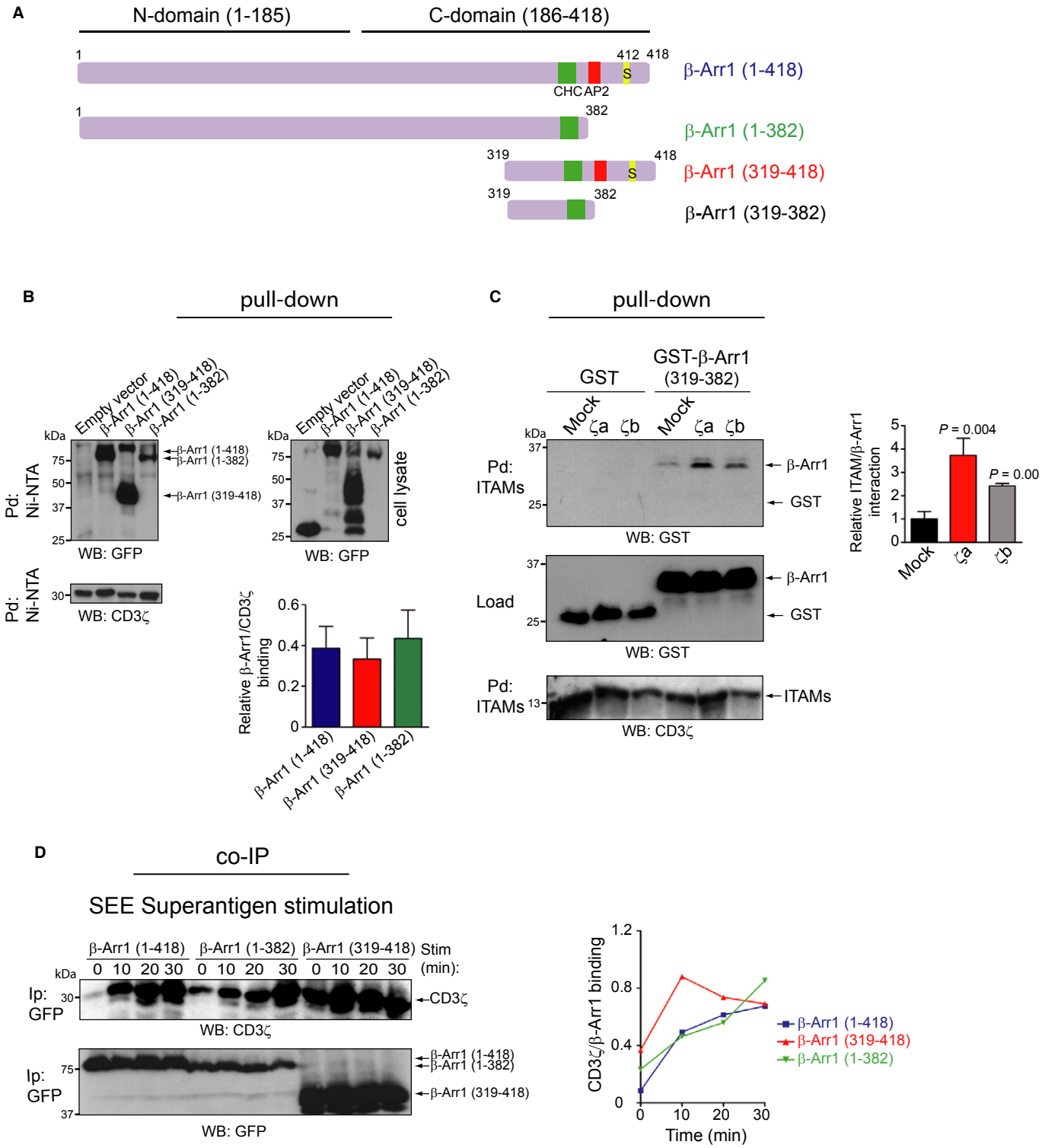
D β-Arr1 interacts directly with the unphosphorylated ζa and ζb ITAMs. Recombinant β-Arr1 was incubated with an irrelevant biotinylated peptide (mock) or with the indicated biotinylated ITAMs and pulled-down with streptavidin-sepharose beads. The initial input of recombinant β-Arr1 and biotinylated peptides was estimated by immunoblotting with anti-β-Arr1 and anti-CD3ζ antibodies. The densitometric intensity of the co-purified β-Arr1 bands was compared to that of the initial input and all values are expressed relative to the control condition (mock), considered as 1. The data represent the mean ± s.d. of three independent experiments.

Source data are available online for this figure.

superantigen-loaded APCs. Whereas binding to the TCR by the WT and the β-Arr1 (1-382) constructs was clearly induced by TCR triggering, β-Arr1 (319-418) bound strongly even in basal conditions (Fig 2D), suggesting that the ITAM-binding motif is constitutively exposed in the β-Arr1 (319-418) fragment. These results suggest important differences in the manner in which β-Arr1 binds to TCRs and GPCRs, as the β-Arr1 (319-418) construct alone does not bind to GPCRs (Krupnick *et al*, 1997).

β-arrestin-1 mediates the downregulation of bystander TCRs

Given that the β-Arr1 (319-418) contains CHC and AP2 binding sites, and that it binds constitutively to the TCR, we investigated the effect of over-expressing this construct on TCR plasma membrane expression. TCR expression was constitutively reduced 2–3 fold in Jurkat cells transfected with the β-Arr1 (319-418) construct as compared with control cells (pEGFP vector) or cells transfected with the WT or β-Arr1 (1-382) constructs (Fig 3A), suggesting that the β-Arr1 (319-418) construct downregulates TCR expression in resting conditions. Interestingly however, over-expression of β-Arr1 (319-418), and to a lesser extent β-Arr1 (1-382), delayed ligand (superantigen)-induced downregulation of the TCR (Fig 3B), an inhibitory effect that was particularly notable during the first 20 min of stimulation. Of note, cells transfected with β-Arr1 (1-382) had basal TCR levels similar to those transfected with the WT construct, and therefore the inhibition of



ligand-triggered TCR downregulation could not be attributed to reduced initial TCR expression.

There was significant co-localization of internal TCR and CHC in Jurkat T cells expressing either WT β-Arr1 or β-Arr1 (319-418) when incubated for 5 min in the presence (Fig 3C, left panel) or absence of anti-CD3 (Supplementary Fig S3). However, the co-localization coefficient between the transfected β-Arr1 constructs and TCR or CHC was much higher for β-Arr1 (319-418) (Fig 3C, middle panel and plot). Hence, β-Arr1 (319-418) over-expression would appear to induce the accumulation of the TCR in CHC-positive endocytotic vesicles, thereby accounting for the observed reduction in TCR surface expression (Fig 3A and B). The effect of this construct in

Figure 2. β-Arr1 is a direct cytoplasmic ligand of the TCR.

A Schematic representation of β-Arr1 showing the regions of β-Arr1 interacting with various partners and the various deletion mutants. CHC, clathrin heavy chain, AP2, adaptor protein complex 2.

B CD3ζ interacts with both the β-Arr1 (1-382) and β-Arr1 (319-418) constructs. HEK-293T cells were co-transfected with His_6-tagged CD3ζ and WT β-Arr1 (1-418) (full length protein), or either of the 2 truncated mutants [β-Arr1 (1-382) and β-Arr1 (319-418)], all fused to GFP, or with empty vector (expressing non-fused GFP). Ni-NTA beads were used to pull-down CD3ζ and co-purify the β-Arr1 constructs. Data were normalized to the total expression of GFP-fused proteins and expressed as the mean ± s.d. of three independent experiments.

C Amino acids 319-382 of β-Arr1 mediate its binding to the ITAMS. Unphosphorylated biotinylated ITAMS (ζa and ζb) were incubated with purified GST or purified GST-β-Arr1 (318-382) fusion protein before pull-down with streptavidin-sepharose beads. Co-purified proteins were detected after immunoblotting with anti-GST. Data were normalized to the total expression of GST proteins and expressed as the mean ± s.d. of two independent experiments.

D High basal levels of β-Arr1 (319-418) mutant binding to the TCR in non-stimulated cells. Jurkat cells were transiently transfected with vectors encoding the indicated GFP fusion proteins and stimulated with unloaded (0 min) or SEE superantigen-loaded Raji APCs for the times indicated (10, 20 or 30 min). Immunoprecipitation was carried out using an anti-GFP antibody and the co-purified TCR was detected in Western blots with anti-CD3ζ. Data were normalized to the total expression of the GFP-fusion proteins. Results shown are representative of three independent experiments.

Source data are available online for this figure.

promoting constitutive TCR internalization while inhibiting ligand-induced TCR down-regulation was also evident when the inside/outside ratio of TCR fluorescence intensity was evaluated by confocal microscopy. In cells transfected with WT β-Arr1, increased inside/outside ratios were observed 5 and 10 min after stimulation. By contrast, the inside/outside ratio was higher at time 0 in cells transfected with the β-Arr1 (319-418) construct than in cells transfected with the WT construct, although it did not increase further upon stimulation (Fig 3C, right panel). These results suggest that β-Arr1 (319-418) promotes the internalization and sequestering of TCRs in endocytotic vesicles, and blocks any increase in TCR down-regulation provoked by the ligand.

The over-expression of the β-Arr1 (319-418) construct in Jurkat cells suggests that β-Arr1 mediates the ligand-induced down-regulation of TCRs. To corroborate this finding using an alternative method, we used a panel of 3 shRNA constructs to silence β-Arr1 mRNA expression. When transfected into the 2B4 murine T cell hybridoma, the 3 constructs produced a variable reduction in β-Arr1 mRNA expression: transfection of shRNA construct 1 reduced β-Arr1 mRNA expression by up to 80% of that with a non-target shRNA control construct (Fig 3D, left panel);. shRNA construct 3 was slightly less effective than construct 1; while construct 2 had no significant effect on mRNA expression. The effect of these 3 shRNA constructs on β-Arr1 mRNA expression correlated with their effect on antigen-induced TCR down-regulation in 2B4 cells (Fig 3D, middle panel). Thus, shRNA construct 1 was used to knockdown β-Arr1 expression in all subsequent experiments. It induced a decrease in β-Arr1 protein expression of over 50% in 2B4 cells (Fig 3D, right panel). Unlike expression of the β-Arr1 (319-418) construct, the knockdown approach did not affect the levels of basal TCR expression (data not shown).

We and others have previously reported that TCR triggering induces the down-regulation of non-triggered bystander TCRs via a TCR signaling-dependent mechanism (Niedergang *et al*, 1998; San Jose *et al*, 2000; Bonefeld *et al*, 2003; Geisler, 2004; Monjas *et al*, 2004). To determine whether β-Arr1 mediates this trans-acting effect, we used $CD8^+$ T cells from double transgenic mice expressing two TCRs with different antigen specificities: the OT-I TCR and the HY TCR (Fig 4A). While the OT-I TCR responds to OVAp, the HY TCR responds to an antigen (SMCY) expressed only in male cells. We studied in $CD8^+$ T cells from double transgenic female mice (Fig 4B) if stimulation of 1 of the TCRs (e.g., OT-I) down-regulated the engaged OT-I TCR complexes and induced the co-modulation of non-engaged HY and OT-I TCR complexes (Fig 4A). As expected, stimulation with OVAp induced the down-regulation of both TCRs (Fig 4C). Of note, while some of the down-regulated OT-I TCRs may have been directly engaged by ligand, all the down-regulated HY TCRs were bystander. Down-regulation of bystander HY TCRs was not due to unforeseen cross-reactivity with OVAp as stimulation of $CD8^+$ T cells from single HY TCR transgenic mice with OVAp did not induce the down-regulation of HY TCRs (Fig 4D). By contrast, stimulation of single HY TCR transgenic or OT-I transgenic T cells with their cognate antigens (SMCYp and OVAp, respectively) resulted in HY and OT-I TCR down-regulation, respectively (Fig 4D).

The role of β-Arr1 in the down-regulation of bystander TCRs was assessed in double transgenic $CD8^+$ T cells transduced with the shRNA construct 1 or a control construct. This shRNA reduced β-Arr1 protein expression by ~50%, compared with a control scrambled shRNA construct, but it did not affect β-Arr2 protein expression (Fig 4E). β-Arr1 knockdown inhibited the OVAp-triggered down-regulation of the stimulated (OT-I), but also of the non-stimulated (HY) TCRs (Fig 4F), suggesting that β-Arr1 mediates the down-regulation in *trans* of unbound TCRs. We next investigated the mechanism underlying β-Arr1 recruitment to non-engaged receptors in double TCR transgenic T cells using specific inhibitors. As expected (San Jose *et al*, 2000; Monjas *et al*, 2004), PP2 inhibition of Src kinases, immediate transducers of TCR signals, blocked the down-regulation of bystander HY TCRs (Fig 4G), indicating that activation of TCR-triggered signaling is required to co-modulate non-engaged TCRs. Moreover, the PKC inhibitor Gö6976 (Martiny-Baron *et al*, 1993) also blocked down-regulation of bystander TCRs (Fig 4G), further demonstrating that this effect is mediated by PKC, as reported previously (von Essen *et al*, 2006).

TCR-triggering induces the phosphorylation of β-arrestin-1 at Ser163 by PKC, required for TCR binding

We hypothesized that regulation of β-Arr1 recruitment to the TCR occurs through the phosphorylation of β-Arr1 by PKC. We transfected Jurkat T cells with the WT β-Arr1 (1-418)-GFP construct and stimulated them with superantigen. Phosphorylation of transfected β-Arr1 by PKC was assessed in anti-GFP immunoprecipitates by immunoblotting with an anti-phosphoserine antibody specific

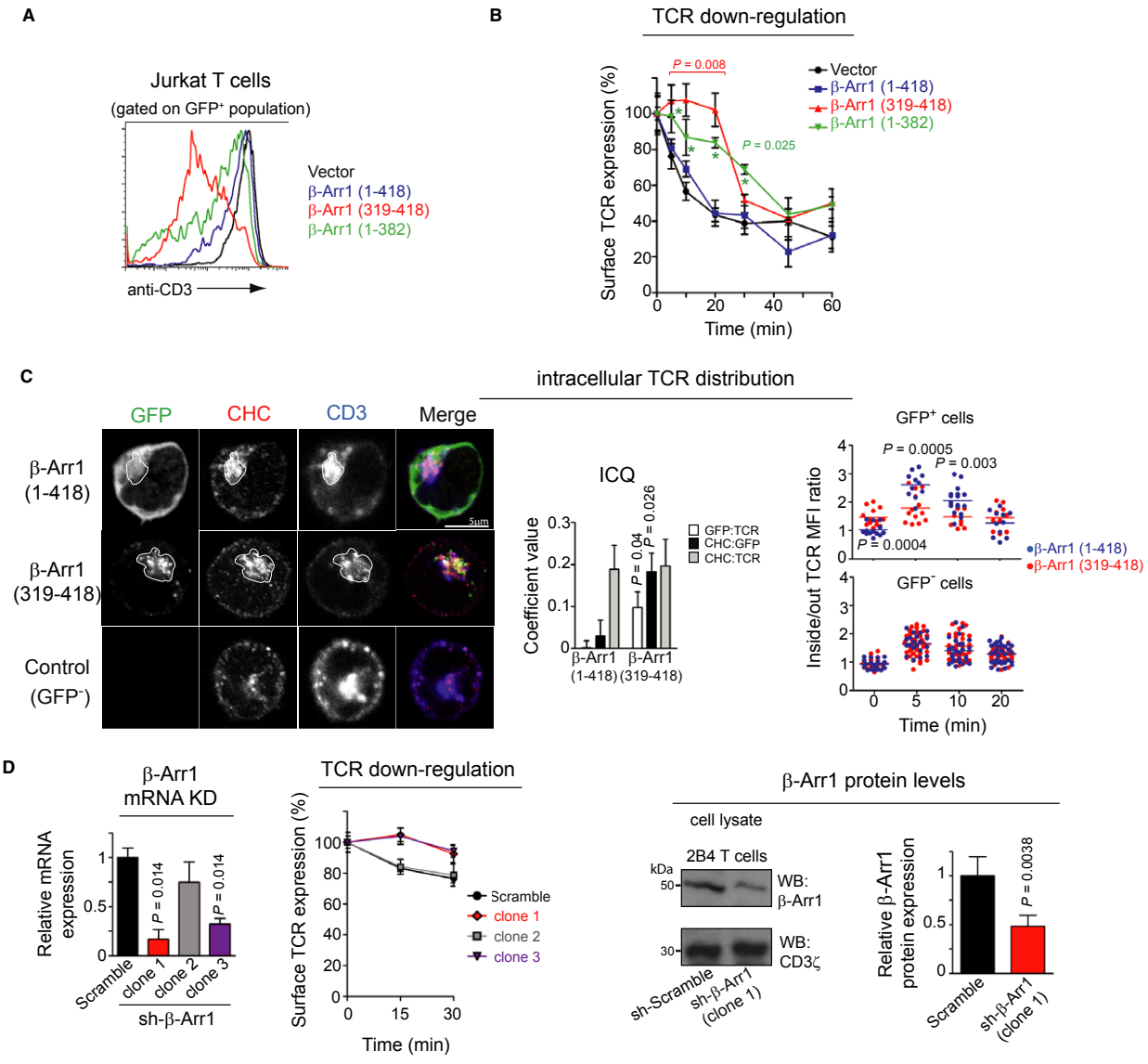


Figure 3. β-Arr1 mediates TCR down-regulation.

A Expression of β-Arr1 (319-418) reduces surface TCR expression. TCR expression at the surface of Jurkat cells transiently transfected with an empty GFP vector or GFP fused to WT or β-Arr1 mutant constructs was analyzed by flow cytometry after staining with anti-CD3 and gating on the GFP$^+$ populations.

B The β-Arr1 (319-418) construct, and to a lesser extent the β-Arr1 (1-382) construct, reduced the ligand (SEE)-induced down-regulation of the TCR. Jurkat cells transiently transfected with the indicated GFP-constructs were stimulated with SEE-loaded Raji APCs and TCR down-regulation was determined according to the mean fluorescence intensity (MFI) after staining with anti-CD3, and by flow cytometry. Values represent the mean ± s.d. of four data sets (representative of four experiments).

C The β-Arr1 (319-418) fragment induces the accumulation of the TCR in clathrin-positive vesicles but prevents TCR triggering-induced endocytosis. Jurkat cells transiently transfected with the indicated GFP-constructs were incubated with fluorochrome-conjugated anti-CD3 for 30 min on ice, and after extensive washing they were incubated for varying durations at 37°C to allow TCR internalization (a medial confocal optical section is shown after a 5 min incubation, left). After fixation, the cells were stained with anti-CHC. Intensity correlation analysis (ICA) was performed to compare the β-Arr1, CD3ζ and CHC signals (middle) and an intensity correlation coefficient (ICQ) was obtained for the regions of interest (selected areas in the Figure), combining the values from different time points (5, 10 and 20 min). TCR endocytosis was quantified by calculating the inside/outside ratio of CD3 MFI in both GFP-positive and GFP-negative (control) cells (right). A total of 11–30 cells per condition and time point were studied. Each dot represents a T cell and the bars represent the mean value (representative of two experiments).

D Quantitative RT-PCR analysis of β-Arr1 mRNA expression in sh-β-Arr1 transduced 2B4 T cells. Three different shRNA constructs were tested (left) and the values expressed relative to those of controls (sh-Scrambled construct, considered to be 1) (representative of two experiments). Kinetics of TCR down-regulation in sh-β-Arr1 transduced murine 2B4 T cells after stimulation with MCC antigen-loaded DCEKs. TCR down-regulation was calculated based on the MFI of the Vβ3-stained TCR. Each value represents the mean ± s.d. of three data points (middle, representative of two experiments). Immunoblot analysis of β-Arr1 in lysates of sh-β-Arr1 (clone 1)-transduced 2B4 T cells (right). The membranes were re-probed with anti-CD3ζ as a loading control (right). Data were normalized to the total expression of CD3ζ and expressed as the mean ± s.d. of two independent experiments.

Source data are available online for this figure.

for PKC-phosphorylated substrates. Phosphorylation of β-Arr1 increased above basal levels 5 min after stimulation with PKC, decaying after 30 min (Fig 5A). This phosphorylation was inhibited by the PKC inhibitor Gö6976, especially during the first 15 min, further suggesting that PKC could directly phosphorylate β-Arr1. In addition, we observed that PKC inhibition greatly reduced the β-Arr1/TCR association induced upon stimulation (Fig 5A), suggesting that TCR-triggered PKC activation is required for β-Arr1 recruitment to the TCR.

To the best of our knowledge, phosphorylation of β-Arr1 by PKC has not been described. A motif mining study combining different kinase-specific phosphorylation site prediction tools (see Supplementary Materials and Methods) revealed the presence of 6 putative PKC phosphorylation sites in β-Arr1 (Fig 5B). To identify the specific sites of phosphorylation of β-Arr1 by PKCα, we next performed an *in-vitro* kinase assay with the constitutively active form of PKCα in the presence of recombinant β-Arr1 and we carried out phosphopeptide analysis by tandem mass spectrometry after enrichment by immobilized metal affinity chromatography (IMAC) (Supplementary Fig S4). We detected a single β-Arr1-derived phosphopeptide that corresponded to amino acids 161-170 phosphorylated on Ser163 (Fig 5C). This phosphorylation was mediated by PKCα since it was not detected in the control condition without added kinase (Supplementary Fig S4). Therefore, the *in vitro* phosphorylation assay suggested that β-Arr1 is a potential substrate of PKCα. Noteworthy, the fact that phosphorylation was limited to a single residue, Ser163, argues against a non-specific effect derived from widespread phosphorylation by PKCα in this 2-protein system. Nonetheless, to determine if β-Arr1 becomes phosphorylated at Ser163 *in vivo*, a flag-tagged β-Arr1 construct was transiently expressed in Jurkat T cells and immunoprecipitated from both anti-CD3-stimulated cells and non-stimulated controls. The 161-170 peptide phosphorylated on Ser163 was again found in the IMAC-bound fraction from CD3-stimulated samples (Fig 5D) but not from the control unstimulated samples (Supplementary Fig S5). These results demonstrate that β-Arr1 is phosphorylated on Ser163 in response to TCR activation *in vivo*. Together with the *in vitro* phosphorylation (Fig 5C) and the immunoblotting data (Fig 5A), these results strongly indicate that the activation of PKC upon TCR triggering is responsible for the phosphorylation of β-Arr1 at Ser163.

To determine if phosphorylation of Ser163 is required for the association of β-Arr1 to the TCR, we generated a S163A mutant of β-Arr1 and studied its recruitment to the TCR upon TCR triggering. The GFP-tagged WT and mutant form of β-Arr1 were transiently transfected in Jurkat T cells and TCR-bound β-Arrb1 was monitored by WB (Fig 5E). Ser163 mutation strongly reduced the recruitment of β-Arr1 to the TCR (Fig 5E), thus demonstrating that phosphorylation of this residue is required for this interaction. In addition, immunoblotting with the pan-PKC substrate-specific antibody showed a strong reduction of β-Arr1 phosphorylation, further confirming the identity of PKC as the kinase that phosphorylates β-Arr1 in TCR-stimulated T cells. Overall, our data indicate that TCR triggering leads to the phosphorylation of β-Arr1 by PKC at Ser163, enabling β-Arr1 to bind and down-regulate bystander TCRs. Interestingly, Ser163 is within a polybasic amino acid sequence, the phosphate sensor (Fig 5B and F), a site that upon binding to phosphorylated GPCRs contributes to make β-Arr1 shift to its active conformation (Gurevich & Gurevich, 2006).

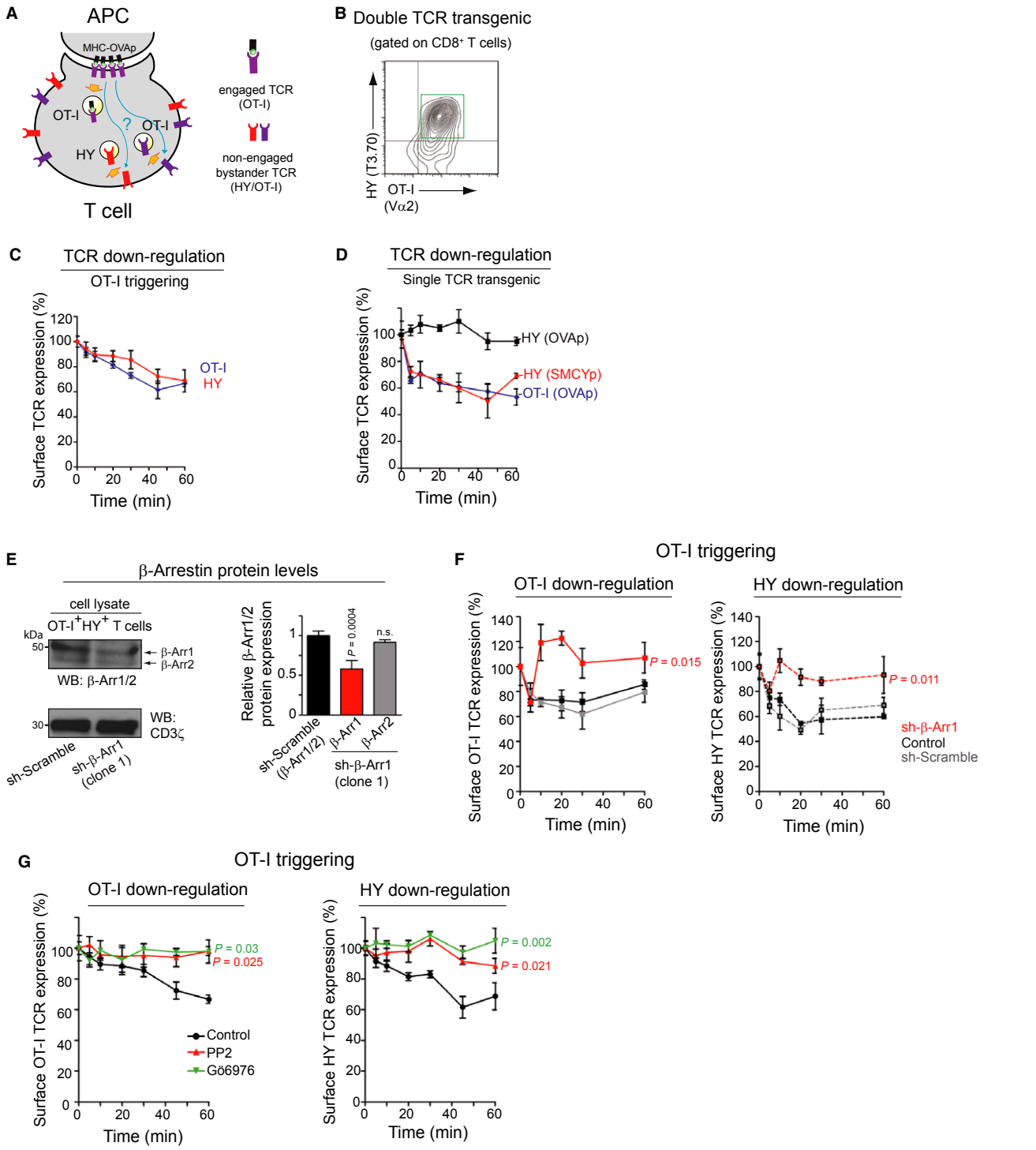
β-arrestin-1 is required for the trafficking and concentration of the TCR at the immunological synapse

The concentration of the TCR at the IS is made possible by lateral movements of unbound TCRs to the APC-T cell contact site, as well as by intracellular trafficking mechanisms that facilitate the endocytosis of TCRs distal to the IS, to where they are redirected (Das *et al*, 2004). Given that β-Arr1 was required to down-regulate unengaged TCRs upon TCR-triggering, we proposed that the modulation of bystander TCRs may be the first step in the redirection of distal TCRs to the IS. We investigated how expression of the β-Arr1 (319-418) construct affected the accumulation of TCRs at the IS in Jurkat cells stimulated with superantigen-loaded APCs. In cells transfected with WT β-Arr1, TCR accumulation was observed at the IS by confocal microscopy (Fig 6A). By contrast, a substantial proportion of TCRs accumulated in intracellular vesicles distal to the IS in β-Arr1 (319-418)-transfected cells. TCR accumulation at the IS was quantified in relation to that of the total plasma membrane. This showed that β-Arr1 (319-418) expression abrogated the accumulation of the TCR at the IS induced by antigen (Fig 6A, bottom). Likewise, β-Arr1 knockdown in OT-I cells abrogated the antigen-induced accumulation of TCRs at the IS (Fig 6B).

We examined whether the decrease in the accumulation of TCRs at the IS in β-Arr1-inactivated cells was due to defective rerouting of TCRs endocytosed at sites distal to the IS. To this end, Jurkat cells double transfected with CD3ζ-Cherry and either the WT β-Arr1-GFP or the β-Arr1 (319-418)-GFP constructs were analyzed by live time-lapse video microscopy. These cells were pre-incubated at 0°C with Alexa647-labeled anti-TCR β antibody before incubation at 37°C with superantigen-loaded APCs to follow the fate of TCRs endocytosed from sites distal to the IS. In cells transfected with WT β-Arr1 the internalized anti-TCR β was translocated to the IS, evident through the accumulation of CD3ζ-Cherry (Fig 6C and Supplementary Movie S1). By contrast, in cells transfected with β-Arr1 (319-418), endocytosed anti-TCR β remained sequestered in β-Arr1 (319-418)-positive vesicles that were also positive for CD3ζ-Cherry (Fig 6C, Supplementary Movie S2, and Fig S6). These vesicles were not rerouted to the IS. Thus, together these results suggest that β-Arr1 is required for the TCR-induced down-regulation of distal bystander TCRs and their translocation to the IS.

Crosstalk of TCR and CXCR4 mediated by β-arrestin-1

Based on the trans-modulation of distal TCRs mediated by β-Arr1 following TCR triggering, we investigated whether this co-modulation was restricted to the TCR or if it affected membrane receptors in general. To determine the specificity of the trans-acting effect, Jurkat T cells transfected with WT, β-Arr1 (1-382) or β-Arr1 (319-418) constructs were stimulated with superantigen and the effect on transferrin receptor (TR) internalization was measured by the incorporation of labeled transferrin within the cells. Expression of the β-Arr1 (319-418) construct had no effect on the rate of transferrin internalization (Fig 7A, left panel) and likewise, knockdown of β-Arr1 in OT-I T cells had no effect on the rates of transferrin internalization (Fig 7A, central panel). Moreover, TCR triggering with OVAp antigen failed to stimulate MHC-I down-regulation (Fig 7A, right panel). These results indicate that the effect in *trans* on distal TCRs induced by TCR triggering is not a generalized phenomenon



affecting all membrane receptors, even if these receptors (TR) are endocytosed by a clathrin-dependent mechanism. As β-Arr1 is best known as a mediator of GPCR internalization, we investigated the effect of TCR triggering on the internalization of the chemokine receptor CXCR4. Superantigen-mediated TCR triggering in Jurkat T cells and antigen-mediated TCR triggering in OT-I T cells resulted in the down-regulation of CXCR4 (Fig 7B). This effect was mediated by β-Arr1, as it was inhibited by both β-Arr1 (319-418) over-expression and β-Arr1 knockdown. Interestingly, TCR stimulation did not induce the down-regulation of the chemokine receptor CCR7 (Fig 7C), which does not recruit β-Arr1 in basal conditions (Byers *et al*, 2008). These findings suggest therefore a correlation between the potential of the TCR to provoke the co-modulation of chemokine receptors and the ability of these receptors to recruit β-Arr1.

Figure 4. β-Arr1 mediates the down-regulation of bystander TCRs.

A Dual TCR transgenic model of bystander TCR down-regulation. $CD8^+$ T cells from OT-I TCR and HY TCR double transgenic female mice were stimulated with OVAp-loaded APCs. This stimulus triggers the OT-I but not the HY TCR. Accordingly, all HY TCRs and the non-engaged OT-I TCRs are bystanders.

B $CD8^+$ T cells from double transgenic mice expressing the OT-I TCR (anti-Vα2 staining) and the HY TCR (T3.70 antibody staining) were analyzed by flow cytometry in all subsequent experiments.

C Stimulation of double transgenic $CD8^+$ T cells with OVAp-loaded APCs induced the down-regulation of both the triggered (OT-I) and bystander (HY) TCRs. Each value represents the mean ± s.d. of three data sets (representative of three experiments).

D Down-regulation of the bystander HY TCR is not due to ligand cross-reactivity. $CD8^+$ T cells from single OT-I TCR transgenic (blue) or HY TCR transgenic mice (red) were stimulated with APCs pulsed with their cognate antigen (1 μM OVAp and 100 μM SMCYp, respectively) for the times indicated. In parallel, single HY TCR transgenic mice (black) were stimulated with OVAp-pulsed APCs under the same conditions. Each value represents the mean ± s.d. of three data points (representative of three experiments).

E Immunoblot analysis of β-Arr1/2 levels in lysates of sh-β-Arr1 (clone 1)-transduced naïve OT-I^+ HY^+ T cells. The membranes were re-probed with anti-CD3ζ as a loading control. Data were normalized to the total expression of CD3ζ and expressed as the mean ± s.d. of three independent experiments.

F β-Arr1 mediates the TCR signal-mediated down-regulation of bystander TCRs. Double TCR transgenic $CD8^+$ T cells were mock-transduced (black) or transduced with sh-Scrambled (gray) or sh-β-Arr1 (clone 1, red), and stimulated with OVAp-loaded APCs. Each value represents the mean ± s.d. of six data points (representative of four experiments). Solid lines represent directly-engaged OT-I TCRs, while dashed lines represent bystander HY TCRs.

G The Src kinase inhibitor PP2 and the PKC α/β inhibitor Gö6976 block the down-regulation of bystander TCRs. Double transgenic $CD8^+$ T cells were pre-incubated with the inhibitors indicated (20 μM PP2 or 1 μM Gö6976) for 30 min prior to stimulation with unloaded (0 time point) and OVAp-loaded APCs for the times indicated. TCR (engaged OT-I and bystander HY) down-regulation was quantified as described above. Each value represents the mean ± s.d. of three data sets (representative of three experiments).

Source data are available online for this figure.

While co-localization of CXCR4 and TCR at the IS has been described previously (Molon *et al*, 2005), the molecular elements participating in the crosstalk between these receptors are yet to be identified. We assessed the role of β-Arr1 in mediating CXCR4 translocation to the IS by analyzing Jurkat cells stimulated with superantigen-loaded APCs. In the presence of WT β-Arr1, CXCR4 localized with the TCR at the IS (Fig 7D), whereas expression of β-Arr1 (319-418) induced the accumulation of both CXCR4 and the TCR in intracellular vesicles, preventing the accumulation of CXCR4 at the IS (Fig 7D). Based on these findings, we propose that the recruitment of β-Arr1 to distal TCRs and other β-Arr1-binding receptors, such as CXCR4, promotes their internalization and redirection to the IS.

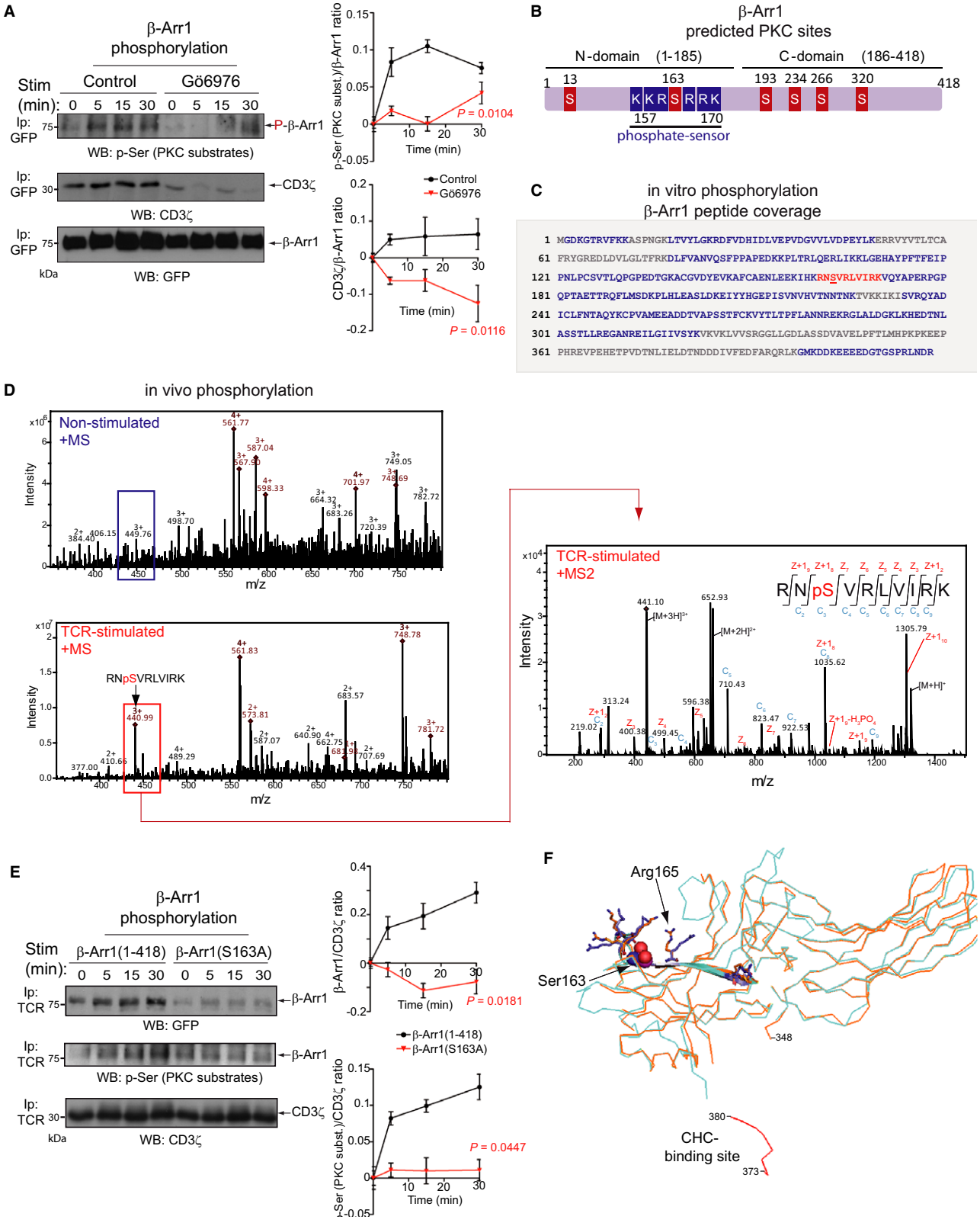
β-arrestin-1 knockdown shortens the duration of the TCR signal and abrogates the TCR-mediated stop signal on CXCR4-driven T-cell migration

The formation of an IS is thought to sustain TCR signaling, whereby TCR molecules in nanoclusters are engaged by pMHC at the edges of the IS and form bigger signaling microclusters (Schamel & Alarcon, 2013). These microclusters move centripetally, gradually losing their capacity to recruit signaling proteins (Lee *et al*, 2002, 2003; Dustin *et al*, 2010; Yokosuka & Saito, 2010; Dustin & Depoil, 2011), and eventually, the exhausted TCRs are removed from the center of the IS (Alarcon *et al*, 2011). Thus, new TCRs must be supplied to the IS to sustain TCR signaling and fully activate T cells. Given that β-Arr1 is required for distal TCRs to reach the IS (Fig 6), we hypothesized that it may also be required for T-cell activation. To investigate this possibility, OT-I T cells transduced with shRNA constructs were stimulated with OVAp antigen-loaded APCs and T-cell activation was measured based on the expression of activation markers (CD69, CD25) and T-cell proliferation. Compared with control cells, β-Arr1-deficient OT-I cells responded poorly to TCR triggering (Fig 8A). Interestingly, rather than decreasing the sensitivity of OT-I T cells to low doses of antigen, β-Arr1 knockdown reduced the amplitude of the activation response, suggesting a defect in sustaining TCR signaling. This effect was more clearly observed when tyrosine phosphorylation of the CD3ζ subunit was analyzed by immunoblotting (Fig 8B) and flow cytometry (Fig 8C). These results demonstrated that β-Arr1 is required to sustain TCR signaling, probably due to its involvement in the continuous supply of new TCRs to the IS.

T cells can migrate to and from lymphoid organs in response to chemotactic gradients. This migratory activity stops when the T cell encounters an APC displaying an appropriate antigen, whereby the *stop* signal emitted by the TCR opposes the *go* signal of chemokine receptors (Dustin, 2004). To investigate the role of β-Arr1 in TCR-triggered *stop* signals, we analyzed shRNA-transduced OT-I T-cell migration towards CXCL12 in transwell plates. While control cells migrated from the upper to the lower chamber in response to a CXCL12 gradient (Fig 8D), this migratory activity was attenuated in the presence of antigen, consistent with the down-regulation of surface CXCR4 observed in TCR-triggered T cells (Fig 7B) and with previous data (Peacock & Jirik, 1999). Compared with control cells, knockdown of β-Arr1 increased T-cell migration and abrogated the stop signal generated by TCR triggering (Fig 8D). These results suggest that β-Arr1 mediates the endocytosis of distal TCRs and CXCR4 induced by TCR triggering, and their redirection to the IS (Fig 8E).

Discussion

In this study, we have adopted a proteomic approach to characterize new ligands of the TCR-signaling ITAM motifs. In this way, we identified the GPCR-interacting protein β-Arr1 as a novel direct ligand of the TCR that is recruited to unphosphorylated ITAMs in a manner dependent on TCR triggering. While β-Arr1 was previously described as a cytoplasmic effector of both GPCRs and RTKs (Hupfeld & Olefsky, 2007), this is the first demonstration of β-Arr1 binding to the TCR, a multi-subunit receptor without intrinsic enzymatic activity. We found that β-Arr1 recruitment to the TCR was induced by TCR triggering. However, in contrast to the recruitment of other TCR signaling effectors, such as the tyrosine kinase ZAP-70 or the adaptor protein Nck (Gil *et al*, 2002), that is mediated



by changes in the TCR itself, recruitment of β-Arr1 to the TCR was mediated by modifying β-Arr1. The mechanism by which TCR triggering induces the recruitment of β-Arr1 to non-triggered TCRs involves the activation of PKC, in line with the proposed role of PKC in the down-regulation of bystander TCRs (Geisler, 2004). We demonstrate that PKC phosphorylates β-Arr1 at a single residue, Ser163,

Figure 5. Identification of a PKC phosphorylation site in β-Arr1.

A PKC inhibition impairs the phosphorylation of β-Arr1 and its recruitment to the TCR. Jurkat cells transiently transfected with WT β-Arr1 (1-418)-GFP construct were stimulated with unloaded (0 time point) or SEE-loaded Raji APCs at the time points indicated. Cell lysates were immunoprecipitated with an anti-GFP antibody and co-precipitated TCR complexes or β-Arr1 phosphorylation by PKC was detected in immunoblots probed with anti-CD3ζ and anti-phospho-(Ser) PKC substrates, respectively. The membrane was re-probed with anti-GFP as a loading control. Quantification was carried out by densitometry and by dividing the intensity of the bands detected with both anti-CD3ζ and anti-phospho-(Ser) PKC substrates in the immunoprecipitated cell lysates by that of the bands detected with anti-GFP (representative of three experiments).

B Schematic representation of β-Arr1 showing predicted PKC phosphorylation sites by the combination of different kinase-specific phosphorylation site prediction tools (see Materials and Methods). One of the predicted sites, Ser163, is included in the phosphate-sensor region.

C *In vitro* phosphorylation of recombinant β-Arr1 on residue Ser163 by constitutively active PKCα. Combined Mascot result of the IMAC-bound and flow-through fractions showing identified sequence coverage (72%) of recombinant bovine β-Arr1 protein phosphorylated by PKCα *in vitro*. The only detected phosphorylated peptide is shown in red (Ser163 is underlined); detected non-phosphorylated peptides in blue.

D Identification of Ser163 as a TCR-induced phosphorylation site in β-Arr1 *in vivo*. Flag-tagged β-Arr1 (1-418) was purified from Jurkat cells stimulated or not with anti-CD3 (10 min), digested with LysC and the resulting phospho-peptides were enriched in IMAC columns and analyzed by tandem MS. The two left spectra illustrate the precursor mass scan of zoomed region (*m/z* 400–750) at 33.0–33.3 min of non-stimulated (up) and CD3-stimulated (bottom) samples. Red inset highlights the 161–170 peptide phosphorylated on Ser163 found only in IMAC-eluates from CD3-stimulated samples and not in control un-stimulated samples (blue inset). The right spectrum illustrates the ETD MS2 scan of the *m/z* 440.99 ion as the phosphorylated β-Arr1 peptide (aminoacids 161-170; sequence RNpSVRLVIRK where pS (red) indicates phosphorylated serine); *c* and *z* ion series are shown.

E Ser163 is required for inducible β-Arr1 binding to the TCR. Jurkat cells transiently transfected with WT (1-418) β-Arr1-GFP or β-Arr1 (S163A)-GFP constructs were stimulated with unloaded (0 time point) or SEE-loaded Raji APCs for the time points indicated. Cell lysates were immunoprecipitated with an anti-CD3 antibody and co-precipitated β-Arr1 was detected by immunoblotting with anti-GFP. The membrane was sequentially re-probed with anti-phospho-(Ser) PKC substrates to monitor the PKC-dependent phosphorylation of β-Arr1 WT and (S163A). Anti-CD3ζ blotting was used as loading control. Quantification was carried out by densitometry as previously described (representative of three experiments is shown).

F Alignment of active and inactive β-Arr1 showing the position of Ser163 within the phosphate sensor region. Backbone representation of active (orange; PDB 3GC3) aligned with inactive (cyan; PDB 1JSY) bovine β-Arr1. The backbone of amino acids 373-380 of active β-Arr1 that interact with CHC is shown in red. Basic amino acids from the phosphate sensor are shown with sticks (orange for active; blue for inactive) while the lateral chain of Ser163 in both β-Arr1 crystals is represented with spheres. Residues 349-372 are not resolved in any of the structures.

Source data are available online for this figure.

both *in vitro* and *in vivo*; thus being this, to our knowledge, the first report showing that β-Arr1 is a substrate of PKC. Interestingly, Ser163 is placed within the previously characterized "phosphate sensor" of β-Arr1, which upon interaction with phosphorylated GPCRs provokes the opening of β-Arr1 into an active conformation enabled to bind GPCRs through C-terminal sequences (Gurevich & Gurevich, 2006). Thus, the finding that Ser163 becomes phosphorylates by PKC and that this phosphorylation is required to bind the TCR, suggests a new mechanism to change the charge of the "phosphate sensor" by direct phosphorylation and resulting in β-Arr1 activation.

While pMHC ligand binding to the TCR induces tyrosine phosphorylation of ITAMs within seconds and the activation of most of the TCR downstream pathways occurs within minutes, full T-cell activation requires contact between T cells and 1 or more antigen-loaded APCs for several hours (Grakoui *et al*, 1999). Thus, T cells require a sustained TCR signal over hours to proliferate or fully differentiate into effector cells. However, TCRs are rapidly internalized from the IS, the site of contact between the pMHC and the APC, and down-regulated (Lee *et al*, 2003; Alarcon *et al*, 2011). Down-regulation of TCRs at the IS involves their replacement by other unengaged TCRs. It has been recently demonstrated that engagement of a single TCR is sufficient to promote its translocation to the center of the IS (Xie *et al*, 2012). However, once all available TCRs at the T cell:APC contact surface have been engaged and down-regulated, other non-engaged TCRs must repopulate the IS, a process that involves the internalization of TCRs from sites distal to the IS and their relocation via endocytotic and exocytotic routes (Das *et al*, 2004; Soares *et al*, 2013). Our finding that β-Arr1 is required to down-regulate TCR-triggered bystander TCRs provides a mechanism for the crosstalk between pMHC-engaged TCRs at the IS and distal TCRs. In addition to its role in down-regulating bystander TCRs, β-Arr1 is required for TCR accumulation at the IS, which decreases in T cells either expressing the β-Arr1 (319-418) construct or depleted of β-Arr1. Taken together, inhibiting the TCR-induced down-regulation of distal TCRs and TCR accumulation at the IS by strategies that interfere with β-Arr1 function suggests that this protein mediates the TCR signal-mediated internalization of distal TCRs and their rerouting to the IS. Indeed, in cells expressing the β-Arr1 (319-418) construct, TCRs are trapped in clathrin-positive endocytotic vesicles, suggesting that this construct not only acts as a dominant-negative mutant for TCR internalization but that it also prevents the rerouting of endocytotic vesicles to the IS.

Interfering with β-Arr1 function inhibits late T-cell activation events such as T-cell proliferation and expression of activation markers but also, β-Arr1-deficient T cells exhibit less sustained phosphorylation of the CD3ζ subunit of the TCR, indicating that β-Arr1 is required to sustain the TCR signal at the TCR level. This is in line with β-Arr1 participating in the rerouting of distal TCRs to the IS and sustaining the TCR signal. Interestingly, β-Arr1 is overexpressed in patients with multiple sclerosis. In addition, neurological symptoms in a mouse model of multiple sclerosis are attenuated and exacerbated by decreases and increases in β-Arr1 expression, respectively (Shi *et al*, 2007). These findings were interpreted in the context of the multiple roles of β-Arr1 as a scaffolding protein and activator, promoting the expression of pro-survival factors such as Bcl-2. Thus, the role of β-Arr1 in multiple sclerosis could be explained as a pro-survival effect on non-activated and activated $CD4^+$ T cells. However, these results could also be interpreted in light of the present findings demonstrating a role of β-Arr1 in sustaining the TCR signal at the IS, suggesting that a more sustained TCR signal enhances autoimmunity. It would be of interest to

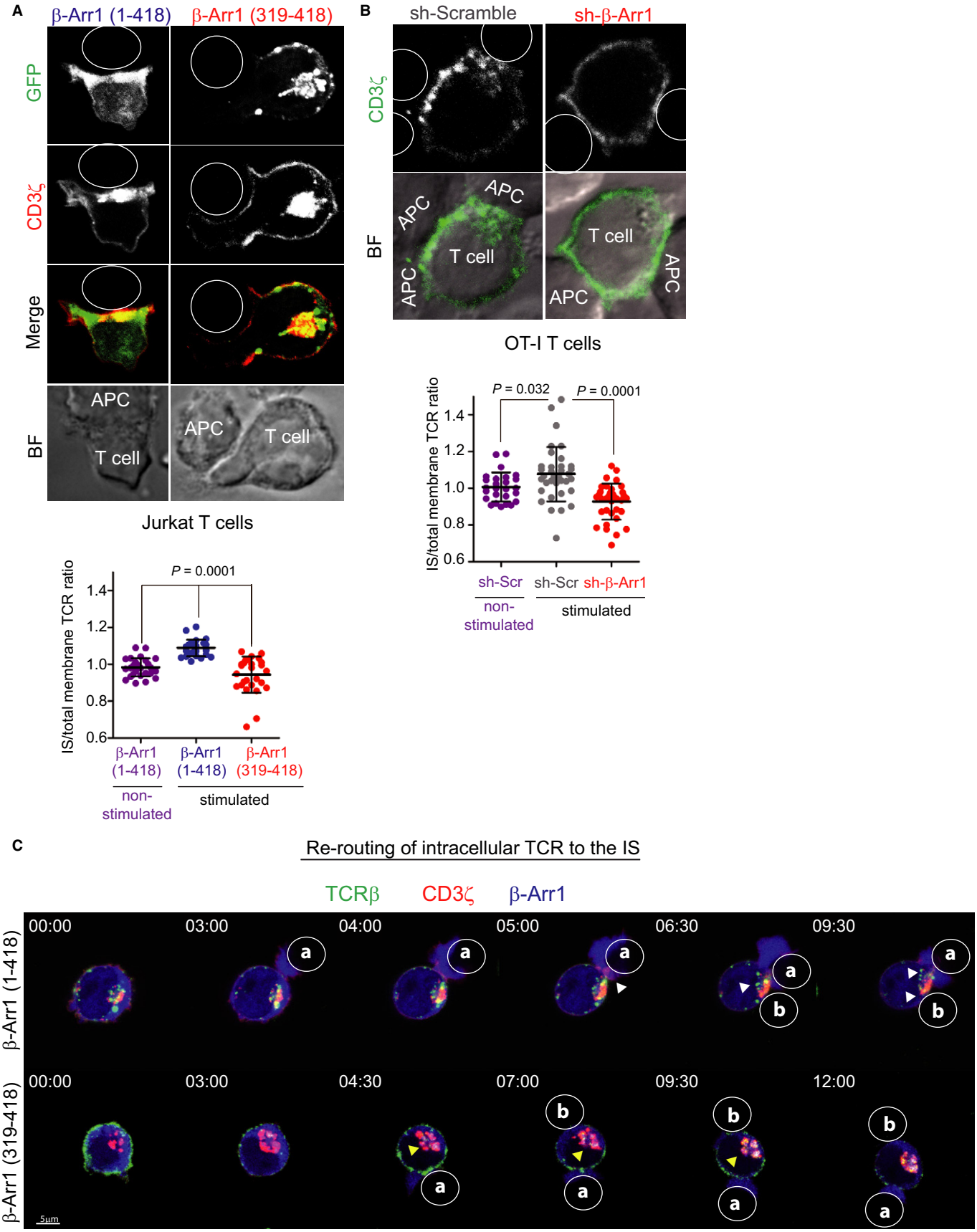


Figure 6. β-Arr1 is required for the trafficking and accumulation of the TCR at the immunological synapse (IS) .

A Quantification of TCR accumulation at the IS. Jurkat cells were co-transfected with CD3ζ-Cherry and either β-Arr1 WT (1-418) or β-Arr1 (319-418) constructs, and stimulated either with SEE-loaded APCs or unloaded APCs (condition of non-stimulation). TCR accumulation at the IS was quantified based on the MFI ratio of CD3ζ-Cherry at the IS vs. that in the total plasma membrane of GFP-positive cells. A total of 25–30 single T cell:APC conjugates were analyzed per condition and time point. A mid-plane confocal optical section from representative T cell:APC conjugates is shown for each condition (10 min time point) (representative of two experiments). Each dot represents a T cell:APC conjugate and the bar represents the mean value ± s.d.

B β-Arr1 knockdown reduces TCR accumulation at the IS. OT-I $CD8^+$ lymph node T cells were co-transduced with a lentivirus encoding CD3ζ-GFP and with either the sh-Scrambled or sh-β-Arr1 clone 1 constructs before stimulation with OVAp-loaded APCs or with unloaded APCs (condition of non-stimulation). Accumulation of the TCR at the IS was quantified as described above. A mid-plane confocal optical section from representative T cell:APC conjugates is shown for each condition (10 min time point).

C β-Arr1 (319-418) expression results in TCR retention in intracellular vesicles that are not redirected to the IS. Jurkat cells co-transfected with CD3ζ-Cherry and either β-Arr1 WT (1-418) or β-Arr1 (319-418) constructs were stimulated with SEE-loaded Raji APCs. An Alexa647-labeled anti-TCRβ(clone JOVI.1) was added several seconds previously to track endocytosis and intracellular trafficking of surface TCRs. This process was followed by time-lapse video-microscopy and selected snapshots are shown: white arrowheads indicate the accumulation of CD3ζ-Cherry and TCRβ-Alexa647 at the IS; yellow arrowheads indicate the accumulation of CD3ζ-Cherry and TCRβ-Alexa647 in β-Arr1 (319-418)-GFP^+ intracellular vesicles. Both the WT- and β-Arr1 (319-418)-transfected cells formed 2 sequential ISs, first with APC labelled a and later with APC labelled b. The full sequence is shown in Supplementary Movies 1 and 2. Results shown are representative of four independent experiments.

explore if β-Arr1 plays a similar role in other autoimmune diseases. In fact, a recent report has shown that β-Arr1 levels are increased in T lymphocytes from patients with primary biliary cirrhosis, an autoimmune disease characterized by T cell-mediated destruction of bile ducts, and that β-Arr1 overexpression increased T-cell proliferation and cytokine production (Hu *et al*, 2011).

The effect of TCR signaling on the internalization and down-regulation of distal membrane receptors was not generalized, as it failed to affect transferrin internalization or MHC-I expression. Nevertheless, TCR signaling did affect receptors other than the TCR itself, as TCR triggering provoked the down-regulation of the chemokine receptor CXCR4 via a mechanism mediated by β-Arr1. TCR signaling could therefore potentially mediate the down-regulation of all membrane receptors that recruit β-Arr1. The effect on CXCR4 is clearly relevant, as CXCR4 and the TCR associate at the IS (Molon *et al*, 2005). The activation of β-Arr1 by the TCR and its effect on CXCR4 down-regulation is also in line with the *stop* signal emitted by the TCR that terminates chemokine-driven T-cell migration when T cells begin to form an IS. Therefore, the modification of β-Arr1 induced by TCR signals appears to be instrumental in the transition from a migratory to a static behavior, whereby T cells must remain associated to an APC long enough to activate as many TCRs at the IS as are required for full T-cell activation. Indeed, it has been reported that unlike CXCR4, the chemokine receptor CCR7 is not recruited to the IS (Molon *et al*, 2005). Since CCR7 ligation inhibits T-cell activation, it is tempting to speculate that this is so because it does not receive the *stop* signal generated by TCR signaling (Bromley *et al*, 2000). We demonstrate that this lack of response correlates with an unresponsiveness to TCR-induced receptor down-regulation, suggesting that this effect is due to the inability of CCR7 to recruit β-Arr1 (Byers *et al*, 2008).

Although TCR triggering induces the down-regulation of distal TCRs and CXCR4, β-Arr1 appears to interact differently with each receptor. It was previously reported that the β-Arr1 (319-418) fragment exerts a dominant negative effect on GPCR down-regulation but at it does not bind to GPCRs (Krupnick *et al*, 1997; Gurevich & Gurevich, 2006), while the present findings demonstrate that this construct binds the TCR. Furthermore, we delimit the ITAM-binding domain of β-Arr1 to residues 319-382, which differ from the residues involved in GPCR-binding that appear to be scattered over the sequence of β-Arr1 (Moore *et al*, 2007), and which contains conserved sequences not present in β-Arr2 that might be responsible for ITAM binding. Regardless of which domains are responsible for the binding of β-Arr1 to TCRs and GPCRs, we describe a mechanism of membrane receptor cross-communication mediated by β-Arr1 that promotes the internalization and rerouting of distal receptors to a location in the membrane where another receptor is signaling. It remains to be determined whether this crosstalk also occurs in membrane receptors functionally linked to β-Arr1 other than the TCR.

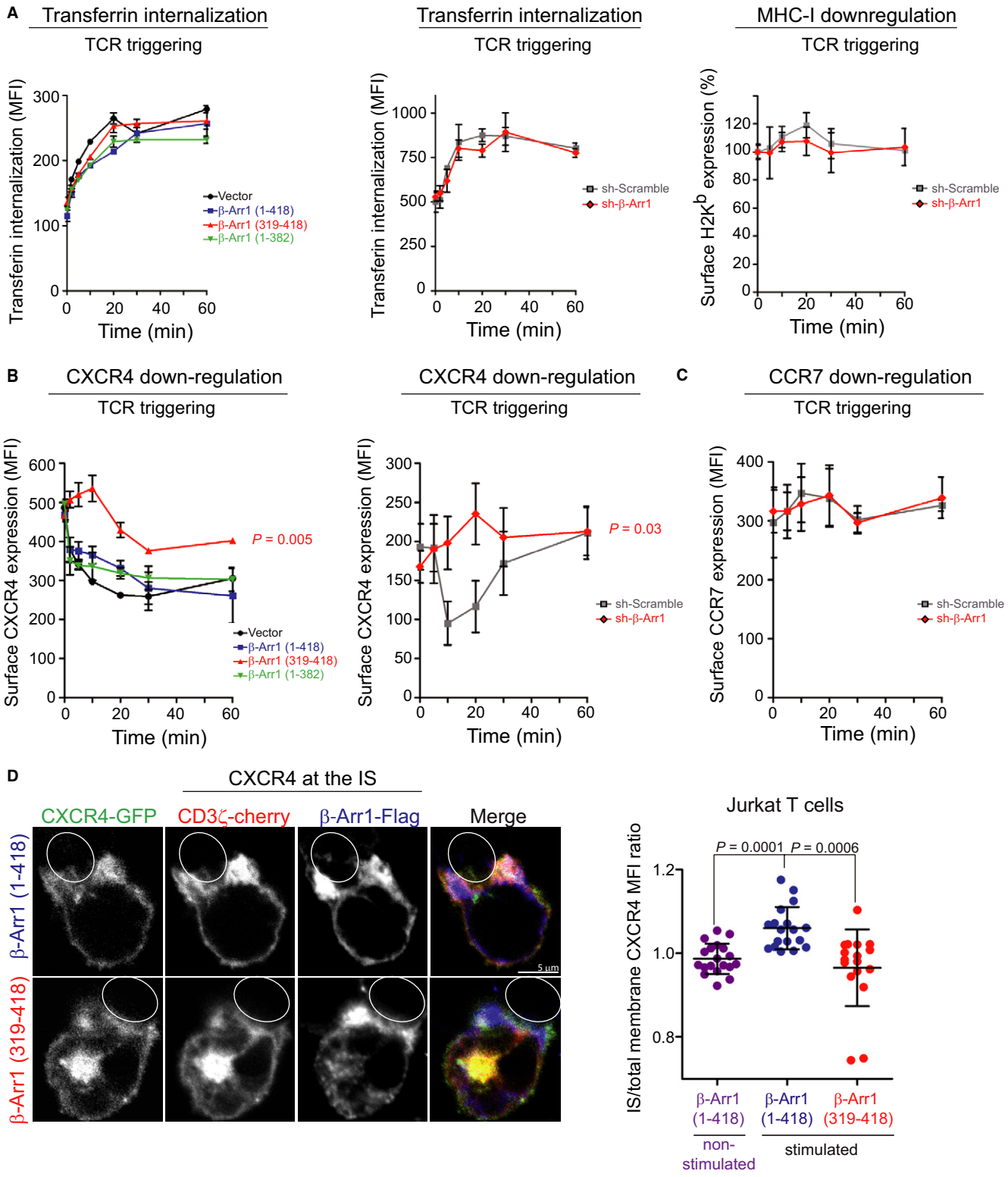
Materials and Methods

Mice, cells and treatments

TCR single and double transgenic mice expressing the OT-I-TCR (H-$2K^b$–restricted, specific for ovalbumin OVAp, peptide SIINFEKL) (Hogquist *et al*, 1994), and/or the HY-TCR (H-$2D^b$ restricted, specific for mouse male antigen SMCY peptide KCSRNRQYL) (Kisielow *et al*, 1988) were used where indicated. Mice were maintained under SPF conditions in the animal facility of the Centro de Biología Molecular Severo Ochoa in accordance with national and European guidelines. All animal procedures were approved by the ethical committee of the Centro de Biología Molecular Severo Ochoa. The human Jurkat T cell lymphoma, human Raji lymphoblastoid B cell, human embryonic kidney HEK-293T, and murine 2B4 hybridoma cell lines, as well as DCEK fibroblast cells and lymph node T cells, were grown as described previously (Delgado *et al*, 2009; Martinez-Martin *et al*, 2011). Where indicated, double transgenic mouse lymphoblasts were pre-treated 30 min before stimulation with the Src tyrosine kinase inhibitor PP2 (20 μM) and the PKC inhibitor Gö6976 (1 μM), both obtained from Sigma.

Cell line transfection and naïve cell transductions

Jurkat cells were transiently transfected by electroporation with the indicated constructs (see Supplementary Table S2), as described previously (Borroto *et al*, 1999). For lentiviral transductions, transducing supernatants were produced from transfected packaging HEK-293T cells as described previously (Martinez-Martin *et al*, 2009). Bacterial glycerol stocks containing lentivirus plasmid vector pLKO.1-puro with shRNAs specific for murine β-Arr1



(3 different clones; Supplementary Table S2), and the pLKO.1-puro non-mammalian shRNA control (targets no known mammalian genes) were purchased from Mission shRNA (Sigma). Lentiviruses were produced in HEK-293T cells after co-transfection of lentivirus plasmid vector with shRNA or control vector with vectors expressing HIV gag/pol genes and the VSV G protein using the JetPEI transfection reagent (Polyplus Transfection). Naïve T cell transductions were carried out as described previously (Martinez-Martin *et al*, 2011), and transduced T cells were treated for 4–6 days with puromycin (1–2 μg/ml) before use. For specific IF experiments, the

Figure 7. TCR triggering promotes the down-regulation of CXCR4 via a β-Arr1-dependent mechanism.

A β-Arr1 does not affect transferrin internalization or MHC-I down-regulation. Jurkat cells were transfected with the indicated β-Arr1-GFP constructs and stimulated with SEE-loaded APCs in the presence of Alexa633-labeled transferrin. Bound but not internalized transferrin was removed after an acidic wash and transferrin internalization was determined by flow cytometry (left). OT-I T cells transduced with sh-Scrambled or sh-β-Arr1 clone 1 were stimulated with OVAp-loaded APCs (middle and right). Transferrin internalization and MHC-I down-regulation was quantified as described above.

B Antigen-mediated TCR triggering induces CXCR4 down-regulation. Jurkat cells transfected with the indicated β-Arr1 GFP constructs (left) were stimulated with SEE-loaded APCs, while OT-I T cells transduced with the indicated shRNA constructs were stimulated with OVAp-loaded APCs (right). CXCR4 down-regulation was quantified as described in Fig 3B and E, respectively.

C Antigen-mediated TCR triggering does not induce CCR7 down-regulation. OT-I T cells transduced with the indicated shRNA constructs were stimulated with OVAp-loaded APCs. CCR7 down-regulation was quantified as described in Fig 3E.

D Expression of β-Arr1 (319-418) prevents the translocation of TCR and CXCR4 to the IS. Jurkat cells were co-transfected with CXCR4-GFP and CD3ζ-Cherry constructs and either Flag-tagged β-Arr1 (1-418) or β-Arr1 (319-418) constructs before stimulation with SEE-loaded Raji APCs (stimulated) or unloaded APCs (non-stimulated). After fixation, cells were intracellularly stained with anti-Flag. The accumulation of CXCR4 at the IS was quantified as described in Fig 5A and a total of 18 single T cell:APC conjugates were counted per condition. A mid-plane confocal optical section is shown (10 min time point, representative of two experiments).

Data information: In (A–C), each value represents the mean ± s.d. of 3 data points (representative of three experiments).

pHR-CD3ζGFP construct expressing CD3ζ fused at the C-terminus to GFP was co-transfected in HEK-293T cells together with the aforementioned PLKO.1 vectors (sh- β-Arr1 or sh-Scrambled) plus packaging plasmids.

Time-lapse fluorescence confocal microscopy and immunofluorescence

Jurkat cells and preactivated transduced T cells from OT-I mice were adhered to fibronectin-coated plates in 1 ml of HBSS containing 2% or 10% FBS (for Jurkat and primary cells, respectively), and they were placed on a microscope stage and maintained at 37°C. Antigen-presenting cells (Raji, T2-K^b, or APCs from CD3ε knockout mice (DeJarnette *et al*, 1998) preloaded with antigen (SEE and OVAp) were added, and a series of fluorescence and bright-field frames was captured sequentially every 30 s using a Zeiss LSM710 Laser scanning microscope coupled to an AxioObserver inverted microscope with a 100× PlanApo oil immersion objective (1.4 numerical aperture). The images were processed and videos were generated using Metamorph software 6.2r6 (Universal Imaging).

For confocal microscopy, cells were first adhered to poly-L-lysine-coated coverslips, and they were then fixed and permeabilized as described previously (Delgado *et al*, 2009), prior to staining with the appropriate antibodies. Cells were viewed on a Zeiss Confocal LSM710 system coupled to a vertical AxioImager.M2 microscopy system with 63× and 100× PlanApo oil immersion objective lens (1.4 numerical aperture). For quantification, 12-bit images were subjected to background correction and threshold-based analysis with Fiji software(Schindelin *et al*, 2012). Briefly, TCR intensity in the plasma membrane or cytoplasm was quantified in cells segmented using the Analyze Particles tool in combination with different contour/shape filters. After generating the regions of interest (ROIs), the mean fluorescence intensity (MFI) of each region was measured for each channel. In other experiments ROIs were manually selected. Where indicated, quantitative co-localization was carried out using the intensity correlation analysis (ICA) imaging method as described previously (Li *et al*, 2004; Martinez-Martin *et al*, 2011).

T-cell stimulation and receptor downregulation

T-cell stimulation was carried out as described previously (Martinez-Martin *et al*, 2011). For receptor down-regulation studies, Raji cells preloaded with 1 μg/ml SEE and T2-k^b cells preloaded with 1 μM OVAp were used as APCs, while in some experiments we also used spleen cells from T cell-deficient CD3$\varepsilon^{-/-}$ mice (DeJarnette *et al*, 1998). After 2 washes, the APCs were seeded onto a flat bottom 96-well plates (10^5 cells) and at the time points indicated, transiently transfected Jurkat cells or transduced naïve T cells were added and maintained at 37°C (ratio 1:1). At the end of the incubation surface expressed TCRs and chemokine receptors were stained with the antibodies indicated (Supplementary Table S2) and analyzed in a FACSCanto II flow cytometer (BD).

Immunoprecipitation and Western blot analysis of T-cell activation

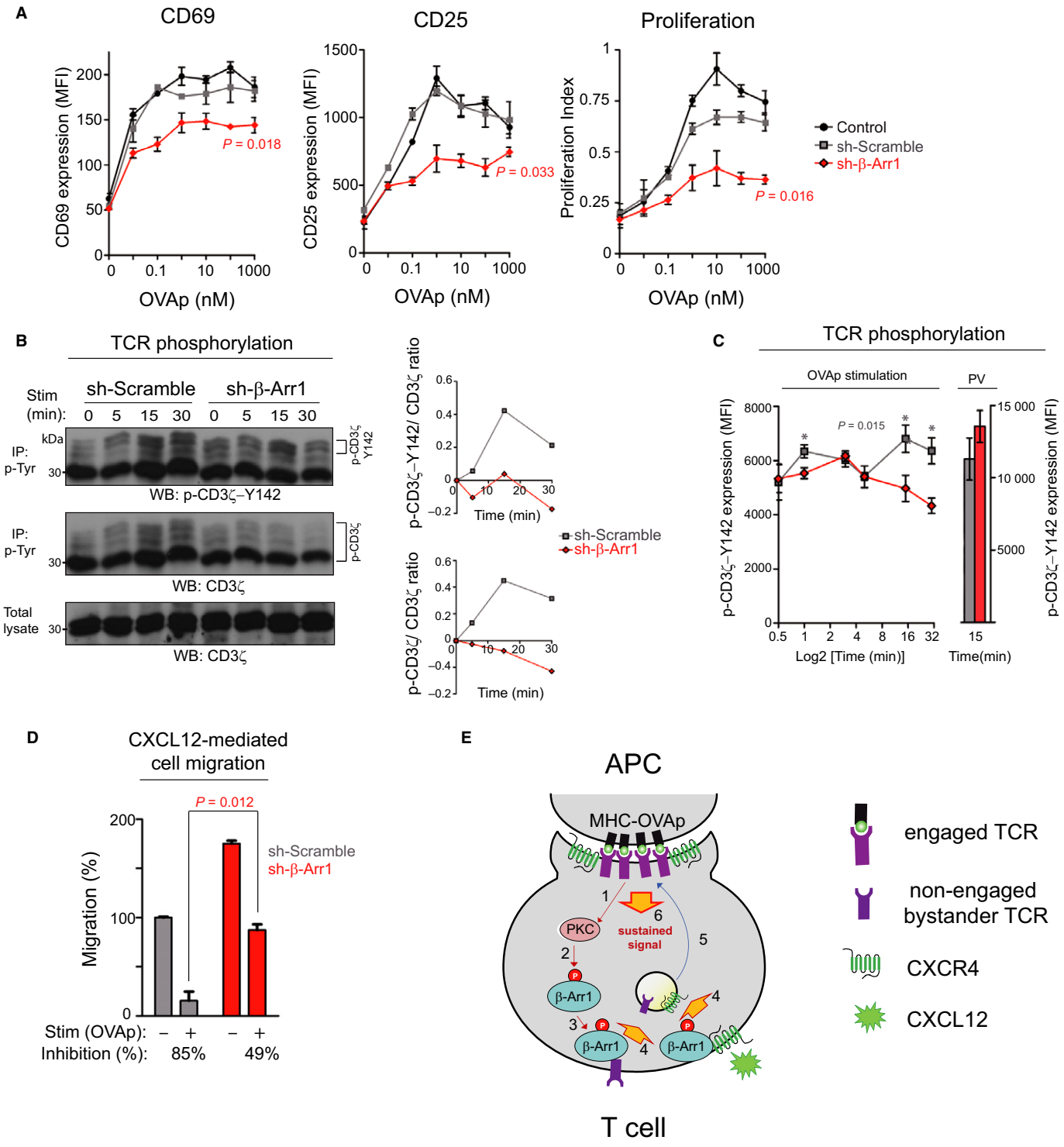
Transiently transfected Jurkat cells (3×10^7 per time point) or transduced naïve $CD8^+$ T cells (1×10^7 per time point) were activated for different periods with a soluble antibody (anti-CD3ε) or with APCs preloaded with 1 μM OVAp or 1 μg/ml SEE, depending on the experiment. Immunoprecipitation and immunoblotting were performed as described previously (Martinez-Martin *et al*, 2009, 2011). Band density was quantified by laser densitometry using Fiji Software (Schindelin *et al*, 2012). In co-immunoprecipitation experiments, the amount of co-precipitated protein was normalized to that of the immunoprecipitated protein, as assessed using specific antibodies.

Protein purification and pull-down assays

For *in vitro* association, 50 ng of recombinant β-arrestin-1 (kindly provided by Dr. V.V. Gurevich, Vandervilt University, USA) was incubated with 50 ng of an irrelevant biotinylated peptide (mock) or with the indicated biotinylated ITAMs (phosphorylated and unphosphorylated) in 250 μl of binding buffer, as described previously (Nogues *et al*, 2011). GST pull-down assays were carried out as described elsewhere (Gil *et al*, 2002).

Chemotaxis assays

Chemotaxis was assayed in 96-well ChemoTx System plates using filters with a pore size of 5 μm (Neuro Probe). CXCL12 (PeproTech) was added to the lower compartment in RPMI 1640 containing 1% FBS. Double transgenic TCR (OT-I and HY) transduced cells



(sh-Scrambled and sh-β-Arr1) were stimulated with OVAp (1 μM) or mock-treated for 30 min at 37°C, washed and then resuspended in RPMI 1640 1% FBS. Cells (2.5×10^5) were placed on the upper compartment, and the plate was incubated at 37°C for 1 h. The total number of cells that migrated to the lower compartment of each well was determined under a light microscope. Assays were performed in triplicate.

Statistical analysis

Quantitative data are represented as the means ± s.d., except for the chemotaxis assay where data represent the means ± s.e.m. A non-parametric 2-tailed unpaired *t*-test was used to assess the confidence intervals using GraphPad Prism software (http://www.graphpad.com/).

Figure 8. β-Arr1 knockdown shortens the duration of TCR signaling and abrogates the TCR-mediated stop signal for CXCR4-driven T-cell migration.

A β-Arr1 silencing impairs T-cell activation. OT-I cells were mock-transduced (black) or transduced with sh-Scrambled (gray) or sh-β-Arr1 clone 1 (red) and stimulated with OVAp loaded APCs for 24 h (for CD69), 48 h (for CD25) or 72 h (for proliferation). Each value represents the mean ± s.d. of three data points (representative of three experiments).

B β-Arr1 depletion reduces the duration of TCR signaling. OT-I cells transduced with either sh-Scrambled or sh-β-Arr1 clone 1 constructs were incubated with OVAp-loaded APCs. Phospho-tyrosine immunoprecipitates and the total cell lysates were resolved by SDS-PAGE, and examined in Western blots probed with anti-phospho-CD3ζ-Y142 and anti-total CD3ζ (left). Quantification was carried out by densitometry and by dividing the intensity of the bands detected with both phospho-CD3ζ-Y142 and CD3ζ in the immunoprecipitated cell lysates by that of the bands detected with total CD3ζ in the whole cell lysates (right; representative of three experiments).

C β-Arr1-deficient T cells exhibit reduced phosphorylation of the CD3ζ subunit. CD3ζ-Y142 phosphorylation was measured by flow cytometry in OT-I transduced cells (sh-Scrambled, gray; shArr1, red) incubated with OVAp-loaded APCs at the time points indicated (left). In parallel, cells were stimulated with pervanadate (PV, 5 mM) for 15 min as a control of CD3f-Y142 phosphorylation (right). Each value represents the mean ± s.d. of three data points (representative of three experiments).

D β-Arr1 depletion abrogates the stop migration signal generated by TCR triggering, as evident when migration in response to CXCL12 was assayed in OT-I cells transduced with sh-Scrambled or sh-β-Arr1 constructs. Bars represent the mean percentage of migrated cells (± s.e.m.) over a 1.5 h exposure to CXCL12 alone (-) or a combination of CXCL12 plus 1 μM OVAp (+). Values are expressed as a percentage of those from the controls (sh-Scrambled construct in the presence of CXCL12). Experiments were performed three times in quadruplicate.

E Model of receptor crosstalk mediated by the TCR-induced phosphorylation of β-Arr1 by PKC. Initial TCR engagement by its pMHC cognate ligand activates PKC (1) which subsequently phosphorylates β-Arr1 on Ser163 (2). This phosphorylation opens β-Arr1 which can then bind distal TCRs and CXCR4 (3), promoting their internalization (4) and redirection to the IS (5). This allows more TCR to be allocated to the IS and sustainment of the TCR signal (6).

Source data are available online for this figure.

Antibodies and other reagents

A full list of antibodies and other reagents used is provided in Supplementary Table S2.

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions

EF-A performed most of the experiments. BA, PP, JPA and FM designed and supervised the research. EC generated β-Arr1 constructs and mutants. NM-M and AA performed the migration assays. EF-A, SIG and RN generated the phosphoproteomics data. EF-A, NM-M, NG-M, SIG, RN and BA analyzed the data. BA prepared the manuscript, which was revised by EF-A, AA, PP, JPA and FM.

Conflict of interest

The authors declare that they have no conflict of interest.

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