Introducción

La endocitosis ha sido tradicionalmente asociada con la clara de receptores desde la membrana plasmática y señales del subcompartimento endocítico. Sin embargo, alrededor de 20 años atrás se demostró que la endocitosis es esencial para la activación del JAK/STAT. Esto se debe a que la endocitosis es un proceso dependiente de clorina que activa el JAK/STAT pathway. Además, los receptores activados en el retículo endoplásmico pueden ser preferentes detectados dentro de estos endomas. IL-4 signalising puede ser bloqueado por inhibidores que actúan en la polymerización de los receptores, incluyendo receptores activados en el retículo endoplásmico, lo que reduce la endocitosis de una manera no retardada en el receptor activado. A pesar de esto, el papel de la endocitosis en la activación del receptor dimerizado es cada vez más evidente. Por lo tanto, la endocitosis es un proceso de señalización endocítica que tiene un papel esencial en la activación del receptor dimerizado.

Bibliografía

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characterized (Zhang et al., 2002a,b), and ectodomain crystal structures are available for all three ligand-induced heterodimers (Hage et al., 1999; LaPorte et al., 2008). Expanding on these in vitro studies, we have recently addressed IL-4R complex formation in the plasma membrane of living HEK293T cells (Gandhi et al., 2014; Weidemann et al., 2011). Surprisingly, we found that the affinities between fully occupied subunits are too low for efficient dimerization at endogenous plasma membrane expression levels by at least two orders of magnitude, implying the existence of a subcellular concentration mechanism that must act during signal transduction (Gandhi et al., 2014). In the course of these studies, we observed that IL-2Rγ-JAK3 complexes concentrate in a specific population of endosomes that are tightly associated with the cell cortex (hence termed cortical endosomes) (Gandhi et al., 2014). Trafficking of the IL-4R subunits to the cortical endosomes is constitutive and independent of ligand binding or pathway activity (Gandhi et al., 2014). IL-4R subunits to the cortical endosomes is constitutive and independent of ligand binding or pathway activity (Gandhi et al., 2014). Endogenous and overexpressed receptor chains therefore compete for the limiting pool of occupied IL-4Rα subunits. Accordingly, overexpression of IL-2Rγ and JAK3 rescued signalling suppressed by the truncated IL-13Rα1-m356 (Fig. 1E).

In summary, expression of different IL-4R subunits in HEK293T cells recapitulates the signalling output expected for both type 1 and type 2 IL-4R complexes, providing a convenient system for studying IL-4R signal transduction by both biochemistry and microscopy.

Endosomal localization of IL-4R subunits

While establishing this model, we previously confirmed that JAK3 and IL-2Rγ are mutually required for their recruitment to the plasma membrane from the cytoplasm and secretory pathway, respectively (Gandhi et al., 2014; Hofmann et al., 2004). However, instead of being smoothly distributed along the cell surface, the two molecules were found concentrated in speckle-like endosomal structures tightly associated with the cell cortex of transfected HEK293T cells (Fig. 1F–H; Fig. S1), which we termed cortical endosomes (Gandhi et al., 2014).

Fluorescence recovery after photobleaching (FRAP) experiments also demonstrated that there is negligible exchange between the cytoplasmic pool of JAK3 and the receptor-bound population associated with the cortical endosomes (Gandhi et al., 2014). Taken together with the absence of visible JAK3 membrane interactions in HEK293T cells (Fig. 1F), this makes the IL-2Rγ–(JAK3–eGFP) complex a suitable marker for the specific subpopulation of endosomes (Fig. 1G) in which we have found all three IL-4R subunits to be enriched at steady state (Gandhi et al., 2014). In contrast, directly labelling the IL-2Rγ chain with eGFP marked additional subcellular compartments, conceivably along the secretory or degradatory pathways (Fig. 1H).

To extend our previous observations using fluorescent fusion proteins (Gandhi et al., 2014), we characterized the endogenous endosomal protein complement of the cortical endosomes by immunofluorescence. The early endosomal proteins Rab5 (Fig. 2A) and EEA1 (Fig. 2B), as well as Rab11, a marker of the recycling compartment (Fig. 2C), were clustered in the vicinity of the cortical endosomes. When quantified using an object-based approach, 95.1±3.9% of the IL-2Rγ–(JAK3–eGFP) punctae exhibited an at least partial overlap with the similarly point-like Rab5 signals. A similar degree of association was observed for Rab11 (94.9±2.0%) and EEA1 (90.3±1.6%) but not for Rab7, a marker of late, degradatory endosomes (13.7±4.7%) (Fig. 2D) or the lysosomal marker LAMP1 (12.4±8.4%) (results are mean±s.d., n=14 cells) (Gandhi et al., 2014).

These cortical endosomes formed without stimulation under conditions where STAT6 phosphorylation was undetectable (Fig. 1D), indicating that receptor subunit internalization does not require pathway activity. Consistent with this, Rab5-positive

RESULTS

HEK293T cells as a model for IL-4R signalling

We recently introduced HEK293T cells as a model system to study the ligand-induced formation of fluorescently tagged IL-4R complexes, as their size and adherent growth makes them amenable to microscopy-based biophysical approaches (Gandhi et al., 2014; Weidemann et al., 2011). HEK293T cells endogenously express IL-2Rα and IL-2Rγ as part of a heterodimeric complex with the gamma chain lacking the intracellular domain (IL-2Rγ-m271) (Gandhi et al., 2014) or by a JAK3 variant carrying an engineered deletion of the kinase domain (JAK3–ΔH1) (Hofmann et al., 2004) (Fig. 1D). Overexpression of IL-2Rγ and JAK3 therefore efficiently reroutes signalling to the type 1 pathway. Importantly, this did not prevent endogenous type 2 signalling in response to IL-13 stimulation (Fig. 1D). Thus, in the absence of ligand, even an excess of defective receptors did not affect the endogenous IL-4Rα chains, consistent with the negligible affinities between unoccupied IL-4R receptor subunits (Gandhi et al., 2014; LaPorte et al., 2008).
Fig. 1. HEK293T cells as a model for IL-4R signalling. (A) Schematic representation of IL-4R complex formation. (B) esiRNA-mediated knockdown of IL-13Rα1 in HEK293T cells inhibits endogenous type 2 IL-4R activity. (C) Overexpression of a truncated signalling-dead IL-13Rα1 receptor (IL-13Rα1-m356) abolishes type 2 IL-4R signalling in response to both IL-4 and IL-13. (D) Overexpression of the type 1 IL-4R components IL-2Rγ and JAK3–eGFP results in normal signal transduction. Signalling in response to IL-4, but not IL-13, is abolished by a truncated IL-2Rγ (IL-2Rγ-m271) or a kinase-dead JAK3 version (JAK3-D949N). (E) The signalling blockade caused by IL-13Rα1-m356 is overcome by an excess of IL-2Rγ and JAK3–eGFP. (F,G) Membrane recruitment of JAK3 by IL-2Rγ–eGFP. (H) JAK3–eGFP transfected into HEK293T cells is fully cytoplasmic (CP) and excluded from the nucleus (N) regardless of expression level. Co-transfection of IL-2Rγ induces recruitment of JAK3–eGFP to the plasma membrane (PM) and speckle-like cortical endosomes (CE). Cells expressing high levels of JAK3–eGFP exhibit both cytoplasmic and membrane-associated pools indicating binding site saturation.
cortical endosomes could also be observed when IL-2Rγ was co-expressed with JAK3-D949N–eGFP, an engineered kinase-dead JAK3 version (Hofmann et al., 2004) (Fig. 2E).

Pixel-wise colocalization analysis using the Manders correlation coefficients (Manders et al., 1993) confirmed this overall pattern: although the late markers exhibited no sign of colocalization with GFP-labelled cortical endosomes, Manders coefficients for GFP and Rab5, EEA1 and Rab11 indicated moderate colocalization (Table S1), consistent with a partial overlap between the signals. Given that the typical diameter of cortical endosomes labelled by IL-2Rγ and JAK3–eGFP (≈300 nm) (Gandhi et al., 2014) is relatively large for endosomal structures but still near the resolution limit of conventional laser scanning microscopy, we could not unambiguously resolve whether this close, but not fully overlapping, association reflects subdomains within individual cortical endosomes or a tight local clustering of separate endosomes.

Cortical-endosome-like structures partially overlapping with Rab5 (70.1±6.1%) (Fig. 2F) and EEA1 (77.1±6.4%) (Fig. 2G) also formed in HEK293T cells overexpressing the type 2 IL-4R subunit IL-13Rα1–eGFP. (H) EEA1 and JAK3–eGFP colocalize in Jurkat cells. All panels show surface section confocal images except H, which shows a central section.
IL-13Rα1-eGFP, again mirrored by moderate pixel-wise colocalization (Table S1). These colocalization values are lower than for type 1 cortical endosomes, potentially reflecting partial localization of the tagged receptor to non-cortical endosome compartments, as also seen for IL-2Rγ-eGFP (Fig. 1H).

Finally, IL-2Rγ-JAK3-eGFP complexes exhibited a similar subcellular distribution in the T-lymphocyte-derived Jurkat cell line, which endogenously express the type 1 IL-4R (Fig. 2H). The observed partial overlap of 90.7±9.0% between the GFP-positive speckles and the EEA1 antibody signal was corroborated by moderate pixel-wise colocalization (Table S1). Thus, both in a lymphoid and a nonhematopoietic cell line, tagged subunits of the endogenous IL-4R subtype reside in punctate cortical endosomes.

Cortical endosomes are the site of IL-4R-mediated JAK/STAT signalling
We have previously shown that internalization of both IL-2Rγ and IL-4Rα into the IL-2Rγ- and JAK3-positive cortical endosomes is independent of ligand (Gandhi et al., 2014). We extended this here to both IL-4R types by tracking endocytosis of N-terminally His-tagged IL-4Rα by pulse-chase labelling with the hexahistidine-specific dye trisNTA-Alexa Fluor 647 (Gandhi et al., 2014; Lata et al., 2006). At the 0-min time point, no vesicular structures were labelled regardless of ligand presence or IL-4R subtype (Fig. S2A–D). However, following the 20-min chase, most cortical endosomes of either subtype overlapped at least partially with a punctate Alexa Fluor 647 signal (Fig. S2E–H) (for quantification, see Table S2). Thus, for both IL-4R subtypes, IL-4Rα is constitutively internalized into endosomes that already contain its dimerization partner.

Accordingly, GFP-tagged IL-4Rα chains colocalize at steady state with cortical endosomes marked by IL-2Rγ-JAK3-TagRFP in the absence of ligand (Fig. 3A). However, specific immunostaining for activated IL-4Rα (pY497) was only observed following stimulation (Fig. 3B), when 83.3±5.2% (mean±s.d., n=5 cells) of the cortical endosomes overlapped at least partially with the phosphorylated (pY)497 signals, again reflected by moderate pixel-wise colocalization (Table S2). However, IL-4Rα-eGFP is prone to subcellular mislocalization artefacts (Gandhi et al., 2014). We therefore repeated the experiment by co-expressing non-labelled IL-4Rα with IL-2Rγ and JAK3-eGFP. In the absence of ligand, 8.6±4.6% of all cortical endosomes exhibited some overlap with a punctate signal in the pY497-eGFP channel (Fig. 3C), compared with 82.8±4.5% following stimulation with IL-4 (Fig. 3D), again with a concomitant increase in pixel-wise colocalization (Table S2).

The anti-pY497-eGFP antibody used above detects a phosphotyrosine (pY) involved in insulin receptor substrate (IRS)-1/2 signalling (Nelms et al., 1999). Even though activation of the IRS-1/2 and JAK/STAT pathways overall occur in parallel (Nelms et al., 1999; Weidemann et al., 2011), we therefore tested whether activated JAK1 (pY1022 and pY1023) and JAK3 (pY785) similarly colocalized in a ligand-dependent manner with type 1 cortical endosomes (Fig. 3E–H). In nonstimulated HEK293T cells transfected with IL-4Rα and IL-2Rγ-JAK3-eGFP, 14.6±8.2% of the GFP-positive cortical endosomes overlapped at least partially with a phosphorylated JAK1 immunostaining signal (Fig. 3E) and 10.7±2.7% with activated JAK3 (Fig. 3G), potentially reflecting the low-level, ligand-independent activation observed after overexpression of functional IL-4R subunits (Gandhi et al., 2014). Stimulation with IL-4 increased these fractions to 88.1±5.5% (Fig. 3F) and 81.3±5.1%, respectively (Fig. 3H; see Table S2 for pixel-wise analysis). Like at the receptor level, activation of the downstream JAK/STAT branch thus appears biased towards the cortical endosomes.

Ultrastructural analysis of IL-4R subunit localization to cortical endosomes
Immuneelectron microscopy confirmed the tight apposition (average minimum distance 40 nm) of IL-2Rγ- and JAK3-eGFP-containing endosomes with the plasma membrane (Fig. 4A,B). Both in silver-enhanced anti-GFP nanogold stainings (Fig. 4A) and immunostainings with 10-nm gold particles (Fig. 4B), cortical endosomes appeared as multivesicular bodies (MVBs), with receptor subunits present both on the limiting membrane and intraluminal vesicles (Fig. 4A). Consistent with this, 97.9±1.9% of JAK3-eGFP-positive cortical endosomes exhibited partial overlap with the ESCRT-0 component Hrs, as assessed by confocal microscopy (Fig. 4C), an essential protein in MVB biogenesis (Bache et al., 2003), and 96.2±2.4% of all cortical endosomes also contained SARA (Fig. 4D, see Table S3 for pixel-wise colocalization analysis), a marker of signalling endosomes in the TGF-β and BMP pathways (Bökel et al., 2006; Tsukazaki et al., 1998).

These experiments confirmed that IL-2Rγ-JAK3-eGFP subunits are present within multivesicular cortical endosomes. However, the tendency to mislocalize when tagged with GFP (Gandhi et al., 2014) precluded using the same approach for IL-4Rα. Instead, we made use of the high-affinity ligand–receptor interaction with IL-4 (150 pM) (Zhang et al., 2002b) to determine the localization of endogenous IL-4Rα subunits relative to the IL-2Rγ- and JAK3-eGFP-marked cortical endosomes. Following validation by confocal microscopy (Fig. S21J), we therefore stimulated HEK293T cells expressing IL-2Rγ and JAK3-eGFP with biotinlabelled IL-4. After selecting regions of interest by analysing GFP immunofluorescence, we identified cortical endosomes within these areas based on their multivesicular ultrastructure and localization. Even though the endogenous cell surface levels of IL-4Rα are very low in most cells (1–10 receptors/µm²) (Lowenthal et al., 1988; Ohara and Paul, 1987; Park et al., 1987), we could detect a specific localized signal (from 10-nm gold particles conjugated to protein A) for the biotinylated IL-4 within these putative cortical endosomes using an anti-biotin antibody (Fig. 4E).

IL-2Rγ-JAK3 complexes are enriched at cortical endosomes
Nanogold-tagged secondary antibodies can partially penetrate into sections, resulting in variable silver enhancement that precludes reliable quantification. We therefore quantified images obtained with protein A conjugated to 10-nm gold particles (Fig. 4B), which exclusively label epitopes exposed on the section surface. We focused on the cortical endosome limiting membrane, given that receptors on lumenal vesicles are isolated from the cytoplasm and thus, at least temporarily, unavailable for signalling. Quantification yielded on average 22.0±11.8 gold particles per endosome, 18.0±9.4 of which were associated with the limiting membrane (83.1±12.6%, mean±s.d., n=20 cells) (Table S4), translating into a linear density of 10.7±4.1 gold particles per µm of limiting membrane. In parallel, we separately quantified the density of anti-GFP immunogold particles at the plasma membrane directly overlying the cortical endosomes (3.98±1.57 gold particles per µm) and at the remainder of the plasma membrane elsewhere in the field of view (0.92±0.45 gold particles per µm). The density of goldlabelled anti-GFP particles per unit length in the cortical endosome limiting membrane was thus increased 14.0±7.6-fold relative to the
distant plasma membrane and 3.3±1.8-fold relative to the directly adjacent plasma membrane (Fig. 4F; Table S4). Given that receptor densities per area scale with the square of the densities per unit length, the local two-dimensional receptor concentrations in the limiting membrane of the cortical endosomes are increased by about 200-fold relative to the general plasma membrane. The order of magnitude of this concentration step is potentially sufficient to compensate for the inherently low affinities between the receptor subunits.

Fig. 3. Cortical endosomes are the site of IL-4R mediated JAK/STAT signalling. (A,B) HEK293T cells transfected with IL-2Rγ, JAK3–TagRFP and IL-4Rα–eGFP. The two type 1 receptor subunits colocalize in the absence of ligand (A). Following ligand stimulation (B), phosphorylated IL-4Rα (pIL-4Rα) becomes detectable at the cortical endosomes. (C–H) HEK293T cells transfected with IL-2Rγ and JAK3–TagRFP and unlabelled IL-4Rα. Markers for pIL-4R pathway components are largely absent from the cortical endosomes of unstimulated cells (C,E,G). pIL-4Rα (D), phosphorylated JAK1 (pJAK1) (F), and phosphorylated JAK3 (pJAK3) (H) become detectable at the cortical endosomes following IL-4 stimulation. All panels show surface confocal sections.
Ligand-induced IL-4R heterodimerization preferentially occurs at the cortical endosomes

To visualize IL-4R complex formation directly, we used fluorescence lifetime imaging of Förster resonance energy transfer (FLIM–FRET) microscopy to track the subcellular distribution of IL-4R heterodimers carrying the CyPet fluorescence donor (IL-4Rα) or the YPet acceptor (IL-2Rγ or IL-13Rα1) within their cytoplasmic tails (Nguyen and Daugherty, 2005) (Fig. 5A).

In the absence of ligand, the IL-4Rα–CyPet donor-only lifetime was slightly higher at the plasma membrane (2.42±0.07 ns) in comparison with endosomes (2.32±0.07 ns; P<0.05) (Fig. 5B,C). Both values were weakly reduced by the presence of IL-4 (P<0.05). Introduction of either YPet-tagged acceptor subunit further reduced donor lifetime, potentially due to FRET caused by protein crowding and stochastic clustering. Consistently, this ligand-independent effect was especially noticeable in the cortical endosomes, where according to our confocal and electron microscopy data both subunits are constitutively enriched. Addition of IL-4 caused a significant shift towards even shorter lifetimes relative to each of these four different baselines, reflecting the ligand-induced formation of receptor heterodimers. For the low-affinity type 1 IL-4R complexes, this reduction was much more pronounced in endosomes (from 2.06±0.05 ns down to 1.57±0.06 ns in the presence of ligand, P<0.01) than at the plasma membrane (2.29±0.05 ns versus 2.18±0.07 ns, P<0.01). In contrast, under the same conditions...
conditions the high-affinity type 2 IL-4R complexes were readily detected both within endosomes (2.16±0.06 ns versus 1.78±0.04 ns; *P*<0.01) and at the plasma membrane (2.34±0.04 ns versus 1.85±0.04 ns, **P**<0.01) (Fig. 5B,C).

These FLIM results confirm our previous fluorescence cross-correlation spectroscopy (FCCS) observations: at comparable expression levels (Gandhi et al., 2014), ligand-induced dimerization at the plasma membrane was readily detectable by FCCS for the higher affinity type 2 IL-4R, whereas dimers of the lower affinity type 1 IL-4R were just above detection threshold (Gandhi et al., 2014). The IL-4-induced lifetime shifts at the membrane thus correlate qualitatively with both the affinities governing formation of the respective receptor complexes (Gandhi et al., 2014) and their relative signalling strength (Gandhi et al., 2014; LaPorte et al., 2008).

In contrast, within the cortical endosomes the FLIM results indicate pronounced ligand-dependent heterodimerization for both receptor types. Thus, endocytic trafficking to the cortical endosomes facilitates efficient dimerization also for the low-affinity type 1 IL-4R complexes, potentially reflecting the increased local receptor concentrations detected by independent means.

**IL-4R ligand and receptor subunits are internalized by a specific, Rac1-, Pak- and actin-dependent endocytosis route**

The type 1 IL-4R and IL-2R complexes share IL-2Rγ, which in the IL-2R context is internalized by a specific actin-mediated and dynamin-dependent endocytosis route (Lamaze et al., 2001; Subtil et al., 1994). This pathway is regulated by the small GTPase Rac1 and its downstream kinases Pak1 and Pak2 that trigger local actin branching and polymerization (Sauvonnet et al., 2005). In agreement with this pathway also acting in our HEK293T model, most cortical endosomes overlapped with immunostaining against the activated phosphorylated form of the RacGEF Vav2 (Vav2-pY172, 95.7±3.9%, mean±s.d., *n*=5 cells) (Fig. 6A; Table S3), which has previously been implicated in IL-2R endocytosis (Basquin et al., 2013).

Consistently, 93.0±2.7% of all cortical endosomes colocalized with Arp2, a nucleation factor for actin branching (Fig. 6B), and cortical endosomes were surrounded by basket-like actin structures, potentially explaining their stable cortical localization (Fig. 6C). We confirmed this actin association by correlative light electron microscopy (CLEM) (Fig. S2K). Arp2 also colocalized with cortical endosomes containing the type 2 receptor subunit IL-13Rα1-eGFP (90.2±4.0%) (Fig. 6D, Table S3).

Even though the transferrin receptor is exclusively internalized by standard clathrin-mediated endocytosis, cortical endosomes could also be loaded with transferrin after a 5-min chase (Fig. S3A) as expected for regular Rab5-positive early endosomes.

**Inhibition of IL-4R endocytosis blocks JAK/STAT pathway activation**

To functionally link this endocytosis route with IL-4R signalling, we used pharmacological inhibitors for different endocytic pathways. Dynasore (Macia et al., 2006) was used to interfere with all dynamin-dependent endocytosis pathways, whereas EHT-
Fig. 6. Inhibition of actin-mediated endocytosis reversibly blocks IL-4R-mediated STAT6 phosphorylation. (A–C) Cortical endosomes marked by IL-2Rγ/JAK3–eGFP complexes colocalize with activated Vav2 phosphorylated at Y172 (pVav2) (A), Arp2 (B) and basket-like actin accumulations (C). Arp2 also decorates endosomes marked by IL-13Rα1–eGFP (D). (E–G) Inhibition of dynamin (dynasore, 320 µM), Rac1 (EHT-1864, 50 µM), Pak1 and Pak2 (IPA-3, 10 µM), and clathrin-mediated endocytosis (chlorpromazine, 7 µM). Dynasore, EHT-1864 and IPA-3 reversibly block type 1 IL-4R signalling in HEK293T cells overexpressing IL-2Rγ and JAK3 (E), endogenous type 1 signalling in Jurkat cells (F) and endogenous type 2 signalling in HEK293T cells in response to both IL-4 and IL-13 (G), whereas chlorpromazine has no effect (E–G). (H) Dose–response curves of IL-4R pathway inhibition by EHT-1864 and IPA-3 (four independent experiments per compound). Error bars denote s.d. (I,J) Inhibition of type 1 IL-4R signalling in HEK293T cells overexpressing IL-2Rγ and JAK3 by dominant-negative (DN) Rac1 (T17N) and Pak1 (K299R) (I) or the Arp2/3 inhibitor CK-666 (400 µM) (J). A–D show surface confocal sections; in E–J, phosphorylated STAT6 (pSTAT6) is the signalling readout and GAPDH is a loading control; w, compound washout; WT, wild type.
1864 (Shutes et al., 2007) and IPA-3 (Deacon et al., 2008) were used to block Rac1- and Pak-dependent pathways, respectively. Chlorpromazine (Wang et al., 1993) served as a control reagent targeting clathrin-mediated endocytosis, which was not predicted to affect CKR internalization (Subtil et al., 1994). As expected, dynasore and chlorpromazine, but not EHT-1864 and IPA-3, were able to block clathrin-mediated transferrin uptake (Fig. S3B) without affecting STAT6 expression (Fig. S3C).

Consistent with a role of Rac1- and Pak-mediated endocytosis in signalling, EHT-1864 and IPA-3 caused a strong but reversible reduction of ligand-dependent STAT6 phosphorylation in HEK293T cells with a reconstituted type 1 IL-4R (Fig. 6E). The influence of dynasore was consistent but somewhat weaker, whereas chlorpromazine had no effect. Signalling through the endogenous type 1 IL-4R of Jurkat cells (Fig. 6F) and endogenous type 2 signalling in HEK293T cells in response to both IL-4 and IL-13 (Fig. 6G) were equally affected. Thus, all three ternary IL-4R–ligand complexes require Rac1- and Pak-mediated endocytosis for productive JAK/STAT signalling. To further support this link we determined dose–response curves for the inhibition of endogenous type 2 IL-4R activity by EHT-1864 and IPA-3, allowing us to estimate an IC50 for STAT6 phosphorylation of 29±4 µM for EHT-1864 and 4.2±0.3 µM for IPA-3 (mean±s.d.) (Fig. 6H). Both values are roughly consistent with published IC50 values for these drugs in different systems [e.g. 12.5 µM for EHT-1864-induced cancer cell apoptosis (Hinterleitner et al., 2013) and 2.5 µM for inhibition of Pak1 kinase activity by IPA-3 (Deacon et al., 2008)].

Signalling through the reconstituted type 1 pathway was also reduced by dominant-negative Rac1 (D17N) (Kraynov et al., 2000) and Pak1 (K299R) (Sells et al., 1997) (Fig. 6I), confirming the pharmacological inhibition data. Finally, signalling was also inhibited by CK-666, a specific inhibitor of the Arp2/3 actin-organizing role rather than another output of Rac1- and Pak-dependent pathways, respectively.

**Inhibition of the actin-dependent endocytosis pathway reduces ligand and receptor trafficking to the cortical endosomes**

We finally quantified the effect of drug treatment on the uptake of ligand and receptor subunits to the cortical endosomes. HEK293T cells transfected with IL-4Rα1, IL-4Rγ and JAK3-eGFP were pretreated with compounds, and loaded at 4°C with a fully active Alexa-Fluor-647-labelled IL-4 (Fig. S4A) (Duppata et al., 2014). Localization of the labelled IL-4 to the cortical endosomes was then monitored by quantitative confocal microscopy (Fig. S4B–D), either immediately (Fig. S4E,F) or after a chase of 20 min at 37°C in the presence of inhibitors (Fig. S4G,H). Cells treated with dynasore had to be excluded from this analysis, owing to a ubiquitous fluorescent background and the appearance of vesicular inclusions in the cytoplasm that precluded the measurement of ligand uptake relative to individual cortical endosomes. Given that we used unlabelled IL-4Rα, we limited our analysis to cells exhibiting robust surface labelling by the fluorescent ligand, which confirmed the presence of its high-affinity receptor (Fig. 7A–E). Consistent with internalization occurring through clathrin-independent and Rac1-, Pak- and actin-mediated endocytosis, endosomal ligand levels were similar for the DMSO control and after chlorpromazine treatment (Fig. 7A,B). In contrast CK-666, EHT-1864, and IPA-3 (Fig. 7C–E) all caused a significant reduction in the average total amount of ligand fluorescence internalized into all cortical endosomes of a given cell (Fig. 7F) at concentrations validated for inhibition of signalling (Fig. S4I). Owing to the close apposition of cortical endosomes and plasma membrane, restricting the analysis to cells exhibiting visible surface labelling invariably lead to overestimation of endosomal ligand levels by projection effects, which was reduced by focussing on cortical endosomes near the bottom membrane (Fig. 7G).

Receptor internalization in the absence of ligand was tracked by a similar loading assay using pulse labelling of cells expressing N-terminally His-tagged receptor subunits with trisNTA–Alexa-Fluor-647 (Gandhi et al., 2014; Lata et al., 2006). Although nonspecific internalization of the trisNTA dye contributes ~10% to the endosomal signals (Gandhi et al., 2014), uptake of H6-IL-4Rα and H6-IL-2Rγ into cortical endosomes following a chase of 10 min at 37°C was reduced to about 50% of control levels in the presence of EHT-1864 (Fig. 7H), again taking projection effects into account. Both assays thus provide conservative estimates of the true reduction in ligand and receptor uptake caused by the inhibitor treatment.

**DISCUSSION**

We have previously shown that individual IL-4R subunits diffuse freely within the plasma membrane (Gandhi et al., 2014; Weidemann et al., 2011). Following ligand binding to one subunit, we observed recruitment of a second subunit into heterodimers, consistent with the canonical view of IL-4R activation (LaPorte et al., 2008; Zhang et al., 2002a). However, our FCCS experiments demonstrated that the two-dimensional dissociation constants of the IL-4R complexes are of the order of several hundred to a thousand receptors per µm2 (Gandhi et al., 2014). At endogenous IL-4R cell surface densities of 1–10 receptors per µm2 (Lowenthal et al., 1988; Ohara and Paul, 1987; Park et al., 1987), the fraction of subunits entering active heterodimers would thus be negligible even with saturating ligand levels. Productive signalling therefore demands a subcellular concentration step that is missing from current models of CKR activation.

Based on the experiments described here, we therefore propose a revised model of IL-4R pathway activation, whereby endocytosis is an essential process upstream of receptor dimerization. According to this model, receptor subunits are continuously and constitutively endocytosed to a population of cortical endosomes (cortical endosomes) and recycled to the plasma membrane, thus sampling the environment for presence of ligand (Fig. 8). Fine tuning of endocytosis and recycling rates establishes a steady state increase in receptor density in the limiting membrane of the cortical endosomes relative to the plasma membrane. This subcellular concentration step compensates for the low affinities between ligand occupied and secondarily recruited IL-4R subunits. Thus, in the presence of ligand endocytosis, the local formation of receptor heterodimers at the endosomes can occur, which would, according to the law of mass action, be disfavoured at endogenous plasma membrane densities. This unusual role of endocytosis for JAK/STAT pathway activation is supported by several lines of evidence as described below.

First, electron microscopy quantification revealed an increase in subunit density of around two orders of magnitude from the plasma membrane to the limiting membrane of the multivesicular cortical endosomes. Increasing endogenous receptor densities by the same factor would suffice to bring the resulting endosomal receptor densities into the range of the Kd,JAK values of the respective complexes, which is a prerequisite for efficient dimerization in response to ligand. Although our quantifications were performed on
cortical endosomes formed upon overexpression of IL-2Rγ and JAK3–eGFP, our estimate of the concentration factor is conservative; in cells with exceptionally high expression levels, visible levels of receptor subunits tend to uniformly accumulate at the plasma membrane, indicating saturation of the endocytic machinery. Thus, the concentration factor we derived will, if anything, underestimate the true concentration step achieved by the cells at the lower endogenous receptor densities.

Fig. 7. Inhibition of actin-mediated endocytosis blocks ligand and receptor trafficking to cortical endosomes. (A–G) IL-4 loading assay. HEK293T cells expressing IL-2Rγ and JAK3–eGFP and preincubated with compounds at indicated concentrations were pulse-labelled on ice with IL-4–Alexa-Fluor-647. Following a 20-min chase at 37°C internalization of ligand into cortical endosomes was detectable in cells treated with 2% DMSO (vehicle control) (A) or chlorpromazin (B). Internalization was reduced in cells treated with CK-666 (C), EHT-1864 (D) or IPA-3 (E). (F,G) Quantification of results shown in A–E, evaluating all cortical endosomes (F) or focussing on cortical endosomes adjacent to the bottom membrane (G). (H) EHT-1864 treatment reduces the uptake of His-tagged IL-4Rα or IL-2Rγ by cells subjected to a pulse–chase labelling experiment with trisNTA–Alexa-Fluor-647 into cortical endosomes. Arrowheads indicate position of selected cortical endosomes. Error bars indicate s.d.
Second, the dimerization behaviour of the different receptor complexes as observed by FLIM–FRET microscopy is consistent with the corresponding FCCS affinity measurements. Overexpression levels in our HEK293T model (200–300 receptors per µm²) (Gandhi et al., 2014) are still below the two-dimensional dissociation constant for the low-affinity type 1 receptor complexes (Kₐ,2D ≈ 1000 receptors per µm²) (Gandhi et al., 2014). Consistent with this, in our FLIM experiments, these receptors exhibit a comparatively weak life time shift at the plasma membrane, and effective IL-4-induced formation of type 1 receptor complexes is largely confined to the cortical endosomes. In contrast, for type 2 IL-4R, these plasma membrane expression levels lie at or above the dissociation constant of the IL-4-induced heterodimers (Kₐ,2D ≈ 180 receptors per µm²). Correspondingly, we could also readily detect type 2 complex formation by FLIM at the plasma membrane.

Third, phosphorylated active IL-4R pathway components (IL-4Rα, JAK1 and JAK3) are enriched at the cortical endosomes following ligand exposure, suggesting that the cortical endosomes are the site of IL-4R-mediated JAK/STAT signal transduction. Cortical endosomes share certain properties with signalling endosomes of other pathways. Like the signalling compartments described for the Trk, neurotrophin (Valdez et al., 2007) and bone morphogenic protein (BMP) pathways (Bökel et al., 2006), cortical endosomes are Rab5- and EEA1-positive multivesicular endosomes. In addition, BMP signalling endosomes and cortical endosomes share the endosomal adaptor protein SARA (Bökel et al., 2006).

However, there are also clear differences: in most pathways, receptor uptake into signalling endosomes is a consequence of pathway activation, as exemplified by the TGF-β and RTK pathways (Bökel and Brand, 2014; Platta and Stenmark, 2011). Clearly this would not work if endocytosis were required upstream of receptor dimerization. Consistently, both steady state enrichment of the IL-2Rγ–JAK3 complexes at the cortical endosomes and internalization of IL-4Rα to cortical endosomes containing its heterodimerizing partners, IL-2Rγ or IL-13Rα1, occur constitutively in unstimulated cells, even with kinase-dead JAK3 versions.

Finally, inhibition of Rac-, Pak- and actin-mediated endocytosis reversibly blocks JAK/STAT signalling through all three IL-4R–ligand complexes, both at endogenous expression levels and under the overexpression conditions of our reconstitution system. This places endocytosis unambiguously upstream of signalling. Inhibition of signalling by CK-666 (Hetrick et al., 2013), which specifically affects the actin-branching factor Arp2, implicates the actin-organizing role of the Rac1 and Pak1 cassette in this process. We also tested several acting-stabilizing and -disassembling compounds (Phalloidin, Latrunculin A, cytochalasin D) for effects on STAT6 phosphorylation, but were unable to identify dosage windows where the observed effects were robust and reversible. Nevertheless, in pulse–chase experiments, inhibition of the Rac1-, Pak- and actin-mediated endocytosis reduced the internalization of both ligand and receptors to the cortical endosomes, consistent with the notion that the block in signalling is caused by reducing the endosomal density of occupied and recruited subunits.

Given that the IL-2Rγ subunit is known to interact with regulators of the endocytosis machinery (Basquin et al., 2013), it is likely that cortical endosomes are increased in size relative to the endogenous situation. In addition, local re-recruitment of the actin-branching machinery to the overexpressed IL-2Rγ chains, as reflected by the presence of phosphorylated Vav and Arp2/3, might cause extended retention of the cortical endosomes in the actin cortex, which could account for the presence of Rab11 in the periphery. The low physiological receptor levels prevented the characterization of the corresponding endogenous signalling compartment. Nevertheless, our inhibitor experiments unambiguously demonstrate that signalling through the endogenous type 1 and type 2 IL-4R complexes also requires Rac- and Pak-mediated endocytosis.

As yet, it remains unclear how widely this mechanism applies to other CKRs. Indeed, different processes may be used to subcellularly concentrate the various receptors, including clustering into lipid rafts or membrane associated signalosomes as reported for the IL-7R (Rose et al., 2010; Tamarit et al., 2013) and the IL-2R (Pillet et al., 2010) systems. However, membrane
partitioning and endocytosis need not be exclusive, since at least for the IL-2R subunits sorting into lipid ordered domains is thought to precede internalization (Lamaze et al., 2001). The increased local density of IL-2Rγ/JAK3 complexes observed in the plasma membrane immediately adjacent to the cortical endosomes relative to more distant regions may reflect the presence of such a mechanism also in the IL-4 system.

Importantly, the link between receptor endocytosis and pathway activation we describe for IL-4R might extend at least to the other common γ-chain-using CKRs; conditional knockouts of Rac1 and Rac2 (Dumont et al., 2009; Guo et al., 2008), Pak2 (Phee et al., 2014), and Vav1, Vav2 and Vav3 (Dumont et al., 2009; Fujikawa et al., 2003) exhibit a block in T-cell development at the CD4+CD8− stage that is currently attributed to various signalling pathways (e.g. Notch, Ras–MAPK and AKT) or defects in cytoskeletal organization and cell migration. However, this phenotype is surprisingly similar to the severe combined excessive IL-4R activation, such as allergy and asthma.

Our inhibitor studies demonstrate that CKR signalling can in must respond to potentially distant and fluctuating cues. In addition, with hematopoiesis and the regulation of immunity, where cells might be especially useful for a receptor family largely associated with constitutive endocytic enrichment in long-lived signalling with temporal and buffers these signals against temporal fluctuations. This endosomes allows cells to integrate weak extracellular signals over time and buffers these signals against temporal fluctuations. This might be especially useful for a receptor family largely associated with hematopoiesis and the regulation of immunity, where cells must respond to potentially distant and fluctuating cues. In addition, our inhibitor studies demonstrate that CKR signalling can in principle be targeted by interfering with receptor endocytosis through low molecular mass compounds. Our model therefore offers a new perspective for tackling diseases associated with excessive IL-4R activation, such as allergy and asthma.

**MATERIALS AND METHODS**

**Molecular biology**

Expression plasmids for pathway components have been described (Gandhi et al., 2014; Weidemann et al., 2011; Worch et al., 2010). pJAK3-R402H-eGFP and pJAK3-D949N-eGFP (Hofmann et al., 2004) were a gift from Sigrun Hofmann (Dresden University Children’s Hospital, Dresden, Germany). pCMV6M-Pak1, pCMV6M-Pak1-K299R (Sells et al., 1997), pCDNA3-Rac1 and pCDNA3-Rac1T17N (Kraynov et al., 2000) were obtained from Addgene. pHu-IL4Rα-CyPet, pIL2Rγ-YPet, and pIL13Rα1-YPet were generated by inserting the CyPet and YPet open reading frames (Nguyen and Daugherty, 2005) into the respective receptor expression plasmids (Weidemann et al., 2011; Worch et al., 2010). All sequences are available upon request.

**Cell culture**

HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco/Life Technologies, Darmstadt, Germany) with 10% calf serum (FCS) and transfected with plasmids or esiRNA (Eupheria Biotech, Dresden, Germany) using Lipofectamine 2000 (Sigma-Aldrich, Germany). HEK293T cells expressing IL-4Rα and JAK3–eGFP were pre-incubated with inhibitors in culture medium at 37°C (15 min) and labelled on ice with 1 nM IL-4 (10 min). After washing, endocytosis was released in the presence of inhibitor at 37°C (20 min). For quantification, cells were imaged at three different planes using the GaAsP detector in integration mode. For image analysis (ImageJ), cortical endosomes were selected in the GFP channel by thresholding and the region of interest (ROI) measured in the Alexa Fluor 647 channel to determine area (AEnd) and mean intensity (INEnd) for a single cell in each frame (k). The total intensity per endosome (IEnd) was calculated by:

\[
I_{\text{End}} = \sum_k \frac{\langle I_k \rangle A_k}{A_{\text{End}}} = \frac{\sum_k \langle I_k \rangle A_k}{\sum_k A_k} N_{\text{Endo}},
\]

where the average area per endosome \( A_{\text{End}} = 0.67 \mu m^2 \) was determined independently in the DMSO control to derive the number of endosomes in...
each frame ($N_{\text{frame}}$). For statistics, we calculated the variance of the weighted sample mean:

$$\sigma = \left( \sum_k (q_k - \mu_{\text{frame}})^2 w_k^2 \right)^{1/2}$$

with $w_k = A_k / \sum A_k$.

where $w_k$ represent the weights accounting for the different number of endosomes per cell.

**FLIM and FRET**

FLIM measurements were performed on a Nikon TE-2000 microscope (Nikon, Tokyo, Japan) equipped with a 60×, NA 1.49 TIRF lens, a Becker and Hickl (Berlin, Germany) FLIM setup (DLY-120 scanner, PM-100 high-speed PMT detector, Simple-Tau-152-DX time-correlated single photon counter), and a cooled laser diode module (445±5 nm, full width at half maximum 60–90 ps for 0.5 mW, 50 MHz).

Measurements were performed in air buffer at 22°C. 128×128 pixel images were scanned with a pixel dwell time of 1.55 ps and a pixel distance of 0.33 μm. Photon arrival times for individual pixels were fitted offline with a single exponential (binning 3, threshold 20 photons and fit range 1.5–13 ns). For each cell, donor lifetimes were separately averaged for five positions at the plasma membrane and five cortical endosomes identified by comparing the donor lifetime image with a fluorescence image in the acceptor channel.

**Electron microscopy**

Cells were processed for Tokuyasu cryosectioning and immunogold labelling as described previously (Slot and Geuze, 2007). 70-nm sections were cut on a Leica UC6+FC6 cryo-ultramicrotome and picked up in methyl cellulose with sucrose (2% methyl cellulose, Sigma M-6385, and 2.3 M sucrose, 1:1). The grids were incubated with primary antibodies (rabbit anti-GFP antibody, TP 401, Torrey Pines, 1:100; rabbit anti-biotin antibody, sucrose, 1:1). The grids were incubated with primary antibodies (rabbit anti-

**Supplementary information**

Supplementary information available online at http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.170969/-/DC1

**References**


Supplementary information

Figure S1:

A. JAK3-TagRFP + H6-IL-2Rγ

B. IL-13Ra1-eGFP + JAK3-TagRFP

C. IL-2Rγ-eGFP

D. H6-IL-2Rγ-eGFP

E. H6-IL-2Rγ-eGFP + JAK3-TagRFP

F. Side view
**Figure S1: Characterization of cortical endosomes I.** Live confocal images of non-stimulated, transiently transfected HEK293T cells. (A) **Recruitment of JAK3-TagRFP by the IL-2Rγ subunit.** Like the eGFP-tagged JAK3 construct (Fig. 1F,G), JAK3-TagRFP alone (inset) is homogeneously distributed in the cytoplasm (CP) and excluded from the nuclear compartment (N). Co-expression of a non-fluorescent common gamma chain construct (H6-IL-2Rγ) efficiently recruits JAK3-TagRFP to the plasma membrane (PM) and into cortical endosomes (CE). (B) **JAK3-eGFP distribution in the presence of the IL-13Rα1.** In the presence of the type 2 subunit IL-13Rα1-eGFP, JAK3-TagRFP remains homogeneously distributed in the cytoplasm. None of the eGFP-labelled structures, the plasma membrane (PM), endogenous membranes (EM), or CEs show visible enrichment of JAK3. (C,D) **IL-2Rγ requires JAK3 for accurate surface expression.** (C) A C-terminally tagged IL-2Rγ chain construct is largely retained in endogenous membranes (EM) like the ER, the nuclear envelope, and perinuclear vesicles. In many cells, the receptors do not reach the plasma membrane (PM, upper right inset). Occasionally, in some highly expressing cells, a small fraction of IL-2Rγ appears evenly at the PM (lower left inset). (D) Fusion of a His-tag at the N-terminus does not change this pattern. (E,F) **Cellular localization of cortical endosomes.** (E) Confocal live images of a non-stimulated, transiently transfected HEK293T cell co-expressing H6-IL-2Rγ and JAK3-TagRFP. Example frames from a z-stack showing the bottom membrane and the centre plane of the cell. (F) 3D reconstruction (ZEN software, Zeiss) showing a side view from the direction indicated in (E) (large grey arrow). Most of the CEs intersect the bottom or top planes, only a few CEs intersect the centre plane. The particular CE from the centre plane image is indicated. Note that this algorithm is based on maximum intensity and does not indicate colocalization by composite colours.
Figure S2:

A. IL-2Rα / JAK3-eGFP, trisNTA-A647, Merge
B. IL-2Rα / JAK3-eGFP, trisNTA-A647, Merge
C. IL-13Rα-eGFP, trisNTA-A647, Merge
D. IL-13Rα-eGFP, trisNTA-A647, Merge
E. IL-2Rα / JAK3-eGFP, trisNTA-A647, Merge
F. IL-2Rα / JAK3-eGFP, trisNTA-A647, Merge
G. IL-13Rα-eGFP, trisNTA-A647, Merge
H. IL-13Rα-eGFP, trisNTA-A647, Merge
I. HIV-2Rα + JAK3-eGFP
J. HIV-2Rα + JAK3-eGFP
K. IL-2Rα / JAK3-eGFP

Acin: 10 nm gold
PM
CE
Figure S2: Characterization of cortical endosomes II. (A-H) Constitutive trafficking of IL-4Rα into CEs. Confocal images of transiently transfected HEK293T cells co-expressing the His-tagged construct H6-IL-4Rα with either the type 1 marker IL-2Rγ/JAK3-eGFP. (A,B,E,F) or the type 2 marker IL-13Rα1-eGFP (C,D,G,H). To assay the background staining (A-D), cells were cooled on ice (10 min), stained with 150 nM trisNTA-Alexa647 (15 min), washed and immediately fixed ("0 min loading"). The bottom plane images show that surface expressed His-tagged IL-4Rα chains have not yet accumulated within CEs. (E-H) Following a 20 min chase, HEK293T cells accumulate trisNTA-Alexa647 under non-stimulated and stimulated conditions in both types of CEs. (I,J) Validation of IL-4-biotin. Confocal images of transiently transfected HEK293T cells co-expressing H6-IL-2Rγ and JAK3-eGFP. (I) Live images containing streptavidin-Cy3 showing the absence of non-specific binding to the cell surface. (J) IL-4-biotin loading. To avoid fluid phase uptake of inactivated IL-4, cells were were cooled on ice (10 min), stained with IL-4-biotin/streptavidin (10 min), and washed. Endocytosis was released by a temperature shift to RT during imaging. Since both IL-4-biotin and streptavidin-Cy3 were randomly labeled, the procedure associated multiple fluorescent Cy3 molecules with each IL-4Rα chain. (K) Actin association of CEs visualized by CLEM. HEK293T cells co-expressing IL-2Rγ and JAK3-eGFP were stained with anti-GFP and anti-actin antibodies, nuclei counterstained with DAPI. (a,b) Overview of a section of fluorescently stained cells (arrowheads) in a region of interest (ROI, rectangle) imaged by fluorescence (a) and TEM mode (b) indicating the field of view as displayed in (c). (c) Fluorescence image in which two cells were selected for CLEM (rectangle). (d, e) Correlative imaging of two labeled cells in fluorescence mode (d) and TEM mode (e). (f-g) TEM images at increasing magnifications reveal actin accumulation (gold particles) in the vicinity of a plasma membrane associated multivesicular CE (g). The arrowheads indicates an electron dense structure underneath the plasma membrane.
Figure S3:

A.

IL-2Rγ / JAK3-eGFP

Transferrin-A555

Merge

5 µm

B.

DMSO

Chlorpromazine

CK-666

EHT-1864

IPA-3

Dynasore

Bright field

0.2%

7 µM

400 µM

50 µM

10 µM

320 µM

Transferrin

Merge

C.

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<th>IPA-3</th>
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Figure S3: Transferrin uptake. (A) Transferrin loading of CEs. Confocal images of transiently transfected, non-stimulated HEK293T cells co-expressing IL-2Rγ and JAK3-eGFP. For staining, cells were cooled on ice (10 min), and labelled with 20 µg/ml Transferrin-Alexa555 (15 min). After washing, endocytosis was released at 37°C (5 min). Transferrin uptake in CEs indicates that these compartments are rapidly accessible for cargo that enters the cell via other endocytosis pathways, as expected for Rab5 positive endosomes. (B) Specificity control for pharmacological small molecule endocytosis inhibitors. Confocal images of non-transfected, non-stimulated HEK293T cells. The cells were pre-incubated with endocytosis inhibitors in culture medium at 37°C (15 min), cooled on ice (10 min), and labelled with 20 µg/ml Transferrin-Alexa647 (15 min). After washing, endocytosis was released in the presence of compounds at 37°C (15 min). Receptor mediated, dynamin and clathrin dependent transferrin uptake was blocked by chlorpromazin and dynasore, but not by EHT-1864 or IPA-3, or CK-666. (C) STAT6 expression in the presence of inhibitors. Non-stimulated HEK293T cells transiently transfected with STAT6 were pre-treated with endocytosis inhibitors at 37°C (30 min), lysed, and assayed by immunoblotting. STAT6 protein levels are not affected by drug treatment.
Figure S4:

A. Comparison of IL-4 and IL-4-Alexa647 effects on pY-STAT6 and STAT6.

B. Graph showing frequency [kcpm] of different concentrations of Alexa647.

C. Line graph depicting mean counts (0-255) vs. Alexa647 [nM].

D. Images showing GFP-channel, Mask, and Alexa647-channel.

E. Images demonstrating IL-4Ra + H6-IL2Rg + JAK3-eGFP, showing JAK3-eGFP, IL-4-Alexa647, and Merge at different time points.

F. Images showing H6-IL2Rg + JAK3-eGFP, with images at different time points.

G. Images showing IL-4Ra + H6-IL2Rg + JAK3-eGFP, with images at different time points.

H. Images showing H6-IL2Rg + JAK3-eGFP, with images at different time points.

I. Comparison of IL-4 inhibition and washout effects on pY-STAT6 and STAT6.

J. Legend for inhibitors: EH1-1864, IPA-2, Choxpromazine, and Dynasore.
Figure S4: IL-4-Alexa647 loading. (A-D) Quantification procedure (A) Immunoblot showing successive STAT6 phosphorylation upon stimulation of IL-4 and IL-4-Alexa647 (dilution series in factors of 5). The indicated 1 nM IL-4-Alexa647 solution was used for the quantitative image analysis (Figure 7). (B) Detector response for imaging settings used to quantify endosome loading. Histograms showing the frequency of counts per pixel as stored in 512 x 512 images recorded in solutions of Alexa647 in PBS. Background noise was measured for PBS only (red). (C) Linearity of the mean values from (B) for the dilution series of Alexa647 in PBS. (D) Example images of a HEK293T cell expressing IL-2Rγ/Jak3-eGFP as a marker for CEs after loading with IL-4-Alexa647. The ROI containing CEs (arrow head) was selected in the eGFP-channel and transferred to the Alexa647 channel for measuring the area and mean intensity. (E-H) Specificity control for IL-4-Alexa647 binding and internalization. Confocal images of transiently transfected HEK293T cells expressing H6-IL-2Rγ, and JAK3-eGFP as a CE marker with (E,G) or without (F,H) IL-4Rα chain. (E,F) 0 min loading. Cells were were cooled on ice (10 min), stained with 1 nM IL-4-Alexa647 (10 min), and immediately fixed. (E) The centre plane images show that IL-4-Alexa647 is bound to surface expressed IL-4Rα chains at the plasma membrane (PM). Accumulation in cortical endosomes (CE) has not yet occurred. (F) By the same procedure, endogenous levels of IL-4Rα, produce no signals in the Alexa647 channel for identical contrast settings. (G,H) 20 min loading. Endocytosis was released by a temperature shift to 37°C in culture medium and stopped by fixation following a 20 min chase. (G) Residual IL-4-Alexa647 could still be found at the plasma membrane (PM), while most fluorescence accumulated in cortical endosomes (CE). (H) Under the same conditions, fluorescence uptake is not detectable in cells lacking overexpressed IL-4Rα. (I) Validation of endocytosis inhibitors. HEK293T cells transiently transfected with STAT6 showing specific and reversible inhibition of IL-4 induced STAT6 phosphorylation for the same batch and concentrations of compounds as used for the quantitative IL-4-Alexa647 loading assay (Figure 7).
Table S1: Colocalization of CEs with endosomal markers:

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<td>HEK293T</td>
<td>JAK3-eGFP IL-2Rγ</td>
<td>eGFP</td>
<td>Anti-Rab5</td>
<td>95.1 ± 3.9</td>
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<td>Jurkat</td>
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<td>90.7 ± 9.0</td>
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Table S2: Internalization and activation of IL-4R components in HEK293T cells:

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<td>97.7 ± 1.3</td>
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<td>0.186 ± 0.125</td>
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<tr>
<td>IL-4Rα JAK3-eGFP IL-2Rγ</td>
<td>no</td>
<td>eGFP</td>
<td>Anti-pJAK1 (pY1022/1023)</td>
<td>14.6 ± 8.2</td>
<td>0.381 ± 0.281</td>
<td>0.198 ± 0.171</td>
</tr>
<tr>
<td>IL-4Rα JAK3-eGFP IL-2Rγ</td>
<td>yes</td>
<td>eGFP</td>
<td>Anti-pJAK1 (pY1022/1023)</td>
<td>88.1 ± 5.5</td>
<td>0.829 ± 0.018</td>
<td>0.226 ± 0.057</td>
</tr>
<tr>
<td>IL-4Rα JAK3-eGFP IL-2Rγ</td>
<td>no</td>
<td>eGFP</td>
<td>Anti-pJAK3 (pY785)</td>
<td>10.7 ± 2.7</td>
<td>0.212 ± 0.161</td>
<td>0.016 ± 0.011</td>
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<tr>
<td>IL-4Rα JAK3-eGFP IL-2Rγ</td>
<td>yes</td>
<td>eGFP</td>
<td>Anti-pJAK3 (pY785)</td>
<td>81.3 ± 5.1</td>
<td>0.703 ± 0.149</td>
<td>0.163 ± 0.037</td>
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Table S3: Markers of the Rac1/Pak dependent endocytosis and signalling endosomes

<table>
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<tr>
<th>cell type</th>
<th>transfected plasmids</th>
<th>imaging channel 1</th>
<th>imaging channel 2</th>
<th>object based coloc. [%]</th>
<th>Manders coefficient M1</th>
<th>Manders coefficient M2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK293T JAK3-eGFP IL-2Rγ</td>
<td>eGFP</td>
<td>Anti-Hrs</td>
<td>97.9 ± 1.9</td>
<td>0.663 ± 0.062</td>
<td>0.271 ± 0.039</td>
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</tr>
<tr>
<td>HEK293T JAK3-eGFP IL-2Rγ</td>
<td>eGFP</td>
<td>Anti-Sara</td>
<td>96.2 ± 2.4</td>
<td>0.624 ± 0.067</td>
<td>0.111 ± 0.059</td>
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<tr>
<td>HEK293T JAK3-eGFP IL-2Rγ</td>
<td>eGFP</td>
<td>Anti-pVav2</td>
<td>95.7 ± 3.9</td>
<td>0.643 ± 0.070</td>
<td>0.159 ± 0.054</td>
<td></td>
</tr>
<tr>
<td>HEK293T JAK3-eGFP IL-2Rγ</td>
<td>eGFP</td>
<td>Anti-Arp2</td>
<td>93.0 ± 2.7</td>
<td>0.665 ± 0.022</td>
<td>0.271 ± 0.039</td>
<td></td>
</tr>
<tr>
<td>HEK293T IL-13Rα1-eGFP</td>
<td>eGFP</td>
<td>Anti-Arp2</td>
<td>90.2 ± 4.0</td>
<td>0.613 ± 0.061</td>
<td>0.196 ± 0.137</td>
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Table S4: Quantification of immunogold labeled IL-2Rγ/JAK3-eGFP complexes in the membrane by TEM

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<tr>
<th>Image</th>
<th>PM adjacent</th>
<th>PM distant</th>
<th>CE</th>
<th>Ratio</th>
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<tr>
<td></td>
<td>Contour length [µm] (# gold)</td>
<td>gold density per µm</td>
<td>Contour length [µm] (# gold)</td>
<td>gold density per µm</td>
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<td>1</td>
<td>1.18 (3)</td>
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<td>2.37 (1)</td>
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<td>2</td>
<td>3.38 (12)</td>
<td>3.55</td>
<td>8.33 (4)</td>
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<tr>
<td>3</td>
<td>0.7 (4)</td>
<td>5.71</td>
<td>1.63 (1)</td>
<td>0.61</td>
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<tr>
<td>4</td>
<td>1.16 (3)</td>
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<tr>
<td>5</td>
<td>1.1 (4)</td>
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<tr>
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<td>1.54</td>
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<tr>
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<td>13.8 (13)</td>
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<td>5.24</td>
<td>6.3 (5)</td>
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<tr>
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<tr>
<td>16</td>
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<td>17</td>
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<td>1.34 (8)</td>
<td>5.97</td>
<td>2.1 (1)</td>
<td>0.48</td>
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<tr>
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<td>3.0 (3)</td>
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<td>7.02</td>
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<tr>
<td>Average</td>
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<td>0.92</td>
<td>22.0</td>
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<tr>
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