Essential role of endocytosis for Interleukin-4 receptor mediated JAK/STAT signalling

Kristina Kurgonaite¹*, Hetvi Gandhi²†*, Thomas Kurth², Sophie Pautot², Petra Schwille¹††, Thomas Weidemann²††**, Christian Bökel¹**

¹Center for Regenerative Therapies Dresden (CRTD), Technische Universität Dresden, Fetscherstr. 105, 01307 Dresden, Germany.
²BIOTEC/Biophysics, Technische Universität Dresden, Tatzberg 47-51, 01307 Dresden, Germany.
†Current address: Institute IMAGINE, 24 Boulevard de Montparnasse, 75015 Paris, France
††Current address: Cellular and Molecular Biophysics, Max Planck Institute of Biochemistry, Am Klopferspitz 18, 82152 Martinsried, Germany

* Co-first authors
** Co-senior authors

For correspondence, contact weidemann@biochem.mpg.de or christian.boekel@crt-dresden.de

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Abstract

Many important signalling cascades operate through specialized signalling endosomes, but a corresponding mechanism has as yet not been described for hematopoietic cytokine receptors. Based on live cell affinity measurements we recently proposed that ligand induced Interleukin-4 receptor (IL-4R) complex formation and thus JAK/STAT pathway activation requires a local, subcellular increase in receptor density. Here we show that this concentration step is provided by the internalization of IL-4R subunits through a constitutive, Rac1/Pak and actin mediated endocytosis route that causes IL-4R subunits to become enriched by about two orders of magnitude within a population of cortical endosomes. Consistently, ligand induced receptor dimers are preferentially detected within these endosomes. IL-4 signalling can be blocked by pharmacological inhibitors targeting the actin polymerization machinery driving receptor internalization, placing endocytosis unambiguously upstream of receptor activation. Together these observations demonstrate a role for endocytosis that is mechanistically distinct from the scaffolding function of signalling endosomes in other pathways.
Introduction

Endocytosis has traditionally been associated with the clearance of active receptors from the plasma membrane and hence signal downregulation. However, around twenty years ago it was shown for different receptor tyrosine kinases (RTKs) that endocytosis of the activated receptor chains controls signal specificity and is required for the full activation of various downstream signal transducers (Di Guglielmo et al., 1994; Grimes et al., 1996; Vieira et al., 1996). Since then, positive contributions of endocytosis to the signal transduction process have also been identified for many other, unrelated signalling pathways (Bökel and Brand, 2014). Despite the molecular differences between these signal transduction cascades, several common themes have emerged at the cell biological level. For example, both for RTKs and TGF-β receptors endosomal signalling is typically driven by clathrin mediated endocytosis, whereas clathrin independent internalization of the receptor chains is associated with degradation and signal downregulation (Di Guglielmo et al., 2003; Sigismund et al., 2008). Both these pathways also involve localized signalling from endosomes that act as platforms where receptors activated at the plasma membrane can interact with downstream pathway components (Miaczynska et al., 2004; Platta and Stenmark, 2011). However, similar endosomal signalling has also been observed in mechanistically unrelated signalling pathways such as the Toll-like receptor cascade (Kagan et al., 2008).

Here we report that endocytosis is also strictly required for signal transduction from specific signalling endosomes in the Interleukin-4 receptor (IL-4R) system. However, the underlying cell biological and biophysical principles differ from all other endocytosis dependent signalling pathways studied to date.

The IL-4R is a typical representative of the class I cytokine receptor (CKR) family that consists of single-pass transmembrane proteins that are non-covalently bound by cytoplasmic Janus kinases (JAKs) contributing the enzymatic activity for signal transduction (Boulay et al., 2003; Nelms et al., 1999). With the exception of a small, homodimerizing group containing e.g. the Erythropoietin receptor (Constantinescu et al., 2001) most class I CKRs form heterodimers (Weidemann et al., 2007). Formation of the active CKR heterodimers occurs in a two step process, whereby the cytokine ligand first binds to one of the subunits that exhibits a higher...
ligand affinity. Subsequently, the second receptor chain is recruited into the complex by the occupied subunit (Whitty and Riera, 2008). Formation of these heterodimeric CKR complexes then triggers cross-activation of the cytoplasmic JAKs that in turn transform the CKR tails into docking sites for various downstream factors (Leonard and O'Shea, 1998). However, the subcellular compartment where these processes occur has as yet not been fully characterized.

CKRs can be grouped into families due to the dimerization of different cytokine specific, high affinity receptors with shared, secondarily recruited subunits. When bound by IL-4 the IL-4Rα chain can recruit the IL-2Rγ chain (common gamma chain), which is also used by the high affinity receptors for IL-2, -7, -9, -15, and -21, and is thus classified as a common gamma chain using receptor (Boulay et al., 2003). This IL-4 induced heterodimer of IL-4Rα and IL-2Rγ is referred to as type 1 IL-4R complex. Alternatively, the IL-4 bound IL-4Rα subunit can recruit the IL-13Rα1 chain to form a type 2 complex, which is conversely induced when IL-13Rα1 bound by IL-13 recruits IL-4Rα (Nelms et al., 1999). Unusually amongst class I CKRs, the IL-4Rα chain can thus interact with two different cytokines to form three different, active receptor/ligand complexes (Fig. 1A). Generally, type 1 IL-4R signalling is restricted to cells of hematopoietic origin, while type 2 signalling is more widely distributed (Murata et al., 1998).

Both the interaction of IL-4 with its high affinity receptor IL-4Rα and the subsequent recruitment of the IL-2Rγ chain have been characterized (Zhang et al., 2002a; Zhang et al., 2002b), 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 tightly associated with the cell cortex (hence termed cortical endosomes, CEs) (Gandhi et al., 2014). Trafficking of the IL-4R subunits to the CEs is constitutive and ligand and signalling independent
(Gandhi et al., 2014), consistent with previous observations in murine BaF3 cells (Friedrich et al., 1999). Following ligand stimulation activated receptors are nevertheless preferentially associated with these CEs, suggesting a potential link between endocytosis and IL-4R signal transduction (Gandhi et al., 2014).

In the IL-2R context IL-2Rγ is internalized through a unique, Rac1/Pak regulated, clathrin independent endocytosis route (Grassart et al., 2008; Grassart et al., 2010; Lamaze et al., 2001; Sauvonnet et al., 2005; Subtil et al., 1994) that appears to be highly related to the recently identified fast endophilin mediated endocytosis (FEME) (Boucrot et al., 2014). Receptor internalization also involves the regulatory PI(3)K subunit p85 and the large, modular Guanin exchange factor (GEF) Vav2 (Basquin et al., 2013), although a role for these proteins in pathway activation has not been addressed.

Here we show that the same endocytosis pathway plays a crucial role upstream of IL-4R signalling. We characterize CEs as the target compartment for this internalization step and show that constitutive enrichment of receptor subunits within the CEs constitutes a prerequisite for their efficient, ligand induced dimerization. Consistently, interfering with endocytosis by pharmacologically inhibiting the Rac1/Pak dependent actin polymerization machinery reversibly blocks IL-4R mediated JAK/STAT signalling. Our proposed model of IL-4R pathway activation thus suggests a novel strategy for pharmacological intervention with this important receptor class.
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 type 2 IL-4Rs, but not the type 1 components IL-2Rγ and JAK3 and the transcription factor STAT6 (Gandhi et al., 2014). Since STAT6 activation strictly depends on the IL-4Rα receptor chain (Nelms et al., 1999), we nevertheless chose phosphorylation of transfected STAT6 as a specific readout for JAK/STAT signalling. HEK293T cells expressing STAT6 transduced IL-4 signals through their endogenous type 2 receptors, which was suppressed by RNAi against IL-13Rα1 (Fig. 1B). Signalling was also abolished by overexpression of a truncated, signalling dead IL-13Rα1 chain lacking the intracellular domain (IL-13Rα1-m356) which outcompetes the endogenous receptors and thus prevents signalling in response to either ligand (Gandhi et al., 2014) (Fig. 1C).

Following overexpression of wild type IL-2Rγ and JAK3, STAT6 phosphorylation in response to IL-4 was normal (Fig. 1D). In contrast, IL-4 signalling was blocked by a truncated IL-2Rγ lacking the intracellular domain (IL-2Rγ-m271) (Gandhi et al., 2014), or by a JAK3 version carrying an engineered deletion of the kinase domain (JAK3-ΔJH1) (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). Endogenous and overexpressed receptor chains therefore compete for the limiting pool of occupied IL-4Rα subunits. Accordingly, overexpression of IL-2Rγ/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 have 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 (CEs) (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 CEs (Gandhi et al., 2014). 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 a 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 marks 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 CEs 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 CEs. When quantified using an object based approach, 95.1 ± 3.9% of the IL-2Rg/JAK3-eGFP punctae exhibited an at least partial overlap with the similarly pointlike 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 lysosome marker LAMP1 (12.4 ± 8.4%) (Gandhi et al., 2014).

These CEs formed without stimulation under conditions where STAT6 phosphorylation was undetectable (Fig. 1D), indicating that receptor subunit internalization does not require pathway activity. Consistently, Rab5 positive CEs
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).

Pixelwise co-localization analysis using the Manders correlation coefficients (Manders et al., 1993) confirmed this overall pattern: While the late markers exhibited no sign of colocalization with GFP labelled CEs, Manders coefficients for GFP and Rab5, EEA1, and Rab11 indicated moderate co-localization (Table S1), consistent with a partial overlap between the signals. Since the typical diameter of CEs labelled by IL-2Rγ/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 CEs or a tight, local clustering of separate endosomes.

CE-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 subunit IL-13Rα1-eGFP, again mirrored by moderate, pixelwise colocalization (Table S1). These colocalization values are lower than for type 1 CEs, potentially reflecting partial localization of the tagged receptor to non-CE 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 that endogenously expresses 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, pixelwise 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 had previously shown that internalization of both IL-2Rγ and IL-4Rα into the IL-2Rγ/JAK3-positive CEs is independent of ligand (Gandhi et al., 2014). We extended this here to both IL-4R types by tracking endocytosis of N-terminally Histagged IL-4Rα pulse/chase labelled with the hexahistidine-specific dye trisNTA-Alexa647 (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 CEs of either subtype overlapped at least partially with a punctate Alexa647 signal (Fig. S2E-H) (for quantification see Table S2). Thus, for either IL-4R subtype, IL-4Rα is constitutively internalized into endosomes already containing its dimerization partner.

Accordingly, GFP tagged IL-4Rα chains colocalize at steady state with CEs 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% of the CEs overlapped at least partially with the phospho-IL-4Rα signals, again reflected by moderate pixelwise 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/JAK3-eGFP. In the absence of ligand, 8.6 ± 4.6% of all CEs exhibited some overlap with a punctate signal in the phospho-IL4Rα channel (Fig. 3C), compared with 82.8 ± 4.5% following stimulation with IL-4 (Fig. 3D), again with a concomitant increase in pixelwise colocalization (Table S2).

The phospho-IL4Rα antibody used above detects a phosphotyrosine 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 (pY1022/23) and JAK3 (pY785) similarly colocalized in a ligand dependent manner with type 1 CEs (Fig. 3E-H). In nonstimulated HEK293T cells transfected with IL-4Rα and IL-2Rγ/JAK3-eGFP 14.6 ± 8.2% of the GFP positive CEs overlapped at least partially with a phospho-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 co-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 pixelwise analysis). Like at receptor level, activation of the downstream JAK/STAT branch thus appears biased towards the CEs.

**Ultrastructural analysis of IL-4R subunit localization to cortical endosomes**

Immmuno-electron microscopy confirmed the tight apposition (average minimum distance 40 nm) of IL-2Rγ/JAK3-eGFP containing endosomes with the plasma
membrane (Fig. 4A,B). Both in silver-enhanced anti-GFP nanogold stainings (Fig. 4A) and 10 nm immunogold stainings (Fig. 4B) CEs appeared as multivesicular bodies (MVBs), with receptor subunits present both on the limiting membrane and intraluminal vesicles (Fig. 4A). Consistently, by confocal microscopy 97.9 ± 1.9% of JAK3-eGFP positive CEs exhibited partial overlap with the ESCRT-0 component Hrs (Fig. 4C), an essential protein in MVB biogenesis (Bache et al., 2003), and 96.2 ± 2.4% of all CEs also contained SARA (Fig. 4D, see Table S3 for pixelwise 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 CEs. However, the tendency to mislocalize when GFP-tagged (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/JAK3-eGFP marked CEs. Following validation by confocal microscopy (Fig. S2I,J) we therefore stimulated HEK293T cells expressing IL2Rγ/JAK3-eGFP with biotin-labelled IL-4. After selecting regions of interest by anti-GFP immunofluorescence we identified CEs within these areas based on their multivesicular ultrastructure and localization. Even though the endogenous cell surface levels of IL-4Rα are in most cells very low (1-10 receptors / µm²) (Lowenthal et al., 1988; Ohara and Paul, 1987; Park et al., 1987), we could detect a specific, localized 10 nm protein A gold signal for the biotinylated IL-4 within these putative CEs using an anti-biotin antibody and (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 10 nm immunogold (Fig. 4B) which exclusively labels epitopes exposed on the section surface. We focused on the CE limiting membrane, since receptors on luminal 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%, 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 CEs (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 anti-GFP gold particles per unit length in the CE limiting membrane is 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). Since the ratio of receptor densities per area scales with the square of that value, the local, two dimensional receptor concentrations in the limiting membrane of the CEs are increased by about 200-fold relative to the general plasma membrane, an order of magnitude potentially sufficient to compensate for the inherently low affinities between the receptor subunits.

Ligand induced IL-4R heterodimerization preferentially occurs at the cortical endosomes

To test this directly we used 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 ± 0.06 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 CEs, where according to our confocal and EM 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 vs. 2.18 ± 0.07 ns, p < 0.01). In contrast, under the same conditions the high affinity type 2 IL-4R complexes were readily detected both within
endosomes (2.16 ± 0.06 ns vs. 1.78 ± 0.04 ns; p < 0.01) and at the plasma membrane (2.34 ± 0.04 ns vs. 1.85 ± 0.04 ns, p < 0.01) (Fig. 5B,C).

These FLIM results confirm our previous 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 CEs the FLIM results indicate pronounced, ligand dependent heterodimerization for both receptor types. Thus, endocytic trafficking to the CEs 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/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 kinase Pak1/2 that trigger local actin branching and polymerization (Sauvonnet et al., 2005). In agreement with this pathway also acting in our HEK293T model, most CEs overlapped with immunostaining against the activated, phosphorylated form of the RacGEF Vav2 (Vav2-pY172: 95.7 ± 3.9%) (Fig. 6A, Table S3), which has previously been implicated in IL-2R endocytosis (Basquin et al., 2013).

Consistently, 93.0 ± 2.7% of all CEs colocalized with Arp2, a nucleation factor for actin branching (Fig. 6B), and CEs 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 CEs 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, CEs 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, while EHT-1864 (Shutes et al., 2007) and IPA-3 (Deacon et al., 2008) were used to block Rac1 and Pak1/2 dependent pathways, respectively. Chlorpromazine (Wang et al., 1993) served as a control reagent targeting clathrin-mediated endocytosis that 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/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, while chlorpromazine had no effect. Signalling via 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/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 IC\textsubscript{50} for STAT6 phosphorylation of 29 ± 4 µM for EHT-1864 and 4.2 ± 0.3 µM for IPA-3 (Fig. 6H). Both values are roughly consistent with published IC\textsubscript{50} 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 branching complex (Hetrick et al., 2013) (Fig. 6J), demonstrating that it is the actin organizing role rather than another output of the potentially pleiotropic Rac1/Pak cassette that is required for IL-4R pathway activation.

**Inhibition of the actin dependent endocytosis pathway reduces ligand and receptor trafficking to the CEs**

We finally quantified the effect of drug treatment on the uptake of ligand and receptor subunits to the CEs. HEK293T cells transfected with IL-4Rα1, IL-2Rγ, and JAK3-eGFP were pretreated with compounds and loaded at 4°C with a fully active, Alexa647 labelled IL-4 (Fig. S4A) (Duppatla et al., 2014). Localization of the labelled IL-4 to the CEs 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, due to a ubiquitous fluorescent background and the appearance of vesicular inclusions in the cytoplasm that precluded the measurement of ligand uptake relative to individual CEs. Since 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 via clathrin-independent, Rac1/PAK/actin mediated endocytosis, endosomal ligand levels were similar for a DMSO control and Chlorpromazine treatment (Fig. 7A,B). In contrast CK-666, EHT-1864, and IPA-3 (Fig. 7C-E) all caused a significant reduction in the in the average, total amount of ligand fluorescence internalized into all CEs of a given cell (Fig. 7F) at concentrations validated for inhibition of signalling (Fig. S4I). Due to the close apposition of CEs 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 CEs 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-Alexa647 (Gandhi et al., 2014; Lata et al., 2006). While nonspecific internalization of the trisNTA dye contributes around 10% to the endosomal signals (Gandhi et al., 2014), uptake of H6-IL-4Rα and H6-IL-2Rγ into
CEs 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 assay 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 with in 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 μm$^2$ (Gandhi et al., 2014). At endogenous IL-4R cell surface densities of 1-10 receptors per μm$^2$ (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 signalling endosomes (CEs) 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 CEs relative to the plasma membrane. This subcellular concentration step compensates for the low affinities between ligand occupied and secondarily recruited IL-4R subunits. In the presence of ligand endocytosis thus permits the local formation of receptor heterodimers at the endosomes, 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:

i) EM quantification revealed an increase in subunit density of around two orders of magnitude from the plasma membrane to the limiting membrane of the
multivesicular CEs. Increasing endogenous receptor densities by the same factor would suffice to bring the resulting endosomal receptor densities into the range of the $K_{d,2D}$ values of the respective complexes, which is a prerequisite for efficient dimerization in response to ligand. Although our quantifications were done on CEs formed by 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.

ii) 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 $\mu m^2$) (Gandhi et al., 2014) are still below the two-dimensional dissociation constant for the low affinity type 1 receptor complexes ($k_{d,2D} \approx 1000$ receptors per $\mu m^2$) (Gandhi et al., 2014). Consistently, 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 CEs. In contrast, for the type 2 IL-4R these plasma membrane expression levels lie at or above the dissociation constant of the IL-4 induced heterodimers ($k_{d,2D} \approx 180$ receptors per $\mu m^2$). Correspondingly, we could readily detect type 2 complex formation by FLIM also at the plasma membrane.

iii) Phosphorylated, active IL-4R pathway components (IL-4Rα, JAK1, and JAK3) are enriched at the CEs following ligand exposure, suggesting that the CEs are the site of IL-4R mediated JAK/STAT signal transduction. CEs 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) CEs are Rab5 and EEA1 positive, multivesicular endosomes. In addition, BMP signalling endosomes and CEs 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 CEs and internalization of IL-4Rα to CEs containing its heterodimerizing partners IL-2Rγ or IL-13Rα1 occur constitutively in unstimulated cells, even with kinase dead JAK3 versions.

iv) Inhibition of Rac/Pak/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/Pak1 cassette in this process. We also tested several acting stabilizing and disassembling compounds (Phalloidin, LatrunculinA, CytochalasinD) 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/Actin mediated endocytosis reduced the internalization of both ligand and receptors to the CEs, consistent with the notion that the block in signalling is caused by reducing the endosomal density of occupied and recruited subunits.

Since the IL-2Rγ subunit is known to interact with regulators of the endocytosis machinery (Basquin et al., 2013) it is likely that CEs 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 pVav and Arp2/3, may cause extended retention of the CEs 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/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 CEs 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 the IL-4R may extend at least to the other common gamma chain using CKRs: Conditional knockouts of Rac1/2 (Dumont et al., 2009; Guo et al., 2008), PAK2 (Phee et al., 2014), and Vav1/2/3 (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, AKT) or defects in cytoskeletal organization and cell migration. However, this phenotype is surprisingly similar to the SCID caused by loss of IL-2Rγ, JAK3 or the IL-7R (O'Shea et al., 2004). A general requirement for Rac/Pak/actin mediated endocytosis in IL-2Rγ associated CKR signalling would thus provide a more parsimonious explanation for this shared hematopoietic phenotype.

Combining low affinity interactions between receptor subunits with constitutive, endocytic enrichment in long lived signalling endosomes allows cells to integrate weak extracellular signals over time and buffers these signals against temporal fluctuations. This may 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 weight 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). pCMV6M-PAK1, pCMV6M-PAK1-K299R (Sells et al., 1997), pcDNA3-Rac1 and pCDNA3-Rac1T17N (Kraynov et al., 2000) were obtained from Addgene. pH6-IL4Rα-CyPet, pIL2RγYPet, and pIL13Rα1-YPet were generated by inserting the CyPet and YPet ORFs (Nguyen and Daugherty, 2005) into the respective receptor expression plasmids (Weidemann et al., 2011; Worch et al., 2010). All sequences available upon request.

Cell culture
HEK293T cells were cultured in DMEM (Gibco / Life Technologies, Darmstadt, Germany) with 10% FCS and transfected with plasmids or esiRNA (Eupheria Biotech, Dresden, Germany) using Lipofectamine 2000 (Sigma-Aldrich, Taufkirchen, Germany) or Turbofect (Thermo Scientific, Bonn, Germany) in OptiMEM medium (Gibco). Jurkat cells were cultured in RPMI-1640 (Gibco) with 10% FCS and transfected using Turbofect. Cells were stimulated with 10 ng/ml IL-4 (Invitrogen / Life Technologies, Darmstadt, Germany) or 20 ng/ml IL-13 (Invitrogen).

Immunocytochemistry
Cells were processed as described (Kupinski et al., 2013). Primary antibodies: EEA1 (Abcam, Cambridge, UK, ab70521; mouse, 1:500), Arp2 H-84 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA; sc-15389R; rabbit, 1:1000), phospho-Y497-IL-4Rα (Abcam; ab61099; rabbit; 1:1000), phospho-JAK1 (Santa Cruz; sc-16773R; rabbit; 1:1000), phospho-JAK3 (Santa Cruz sc-16567R; rabbit; 1:1000), Rab5 (BD, Heidelberg, Germany; 610725; mouse; 1:1000), Rab11 (BD, 610657; mouse; 1:1000), Hrs (Acris, Herford, Germany; GTX101718; rabbit; 1:1000); phospho-Vav2 (Santa Cruz; sc-16409-R; rabbit; 1:1000), SARA (Abcam ab124875; rabbit; 1:1000); Alexa568-Phalloidin (Invitrogen; 1:1000).

His-tags were detected using trisNTA-Alexa647 (Lata et al., 2006) as described (Gandhi et al., 2014).
Imaging was performed on a Zeiss 780 confocal LSM. For object based colocalization analysis colour channels were separately thresholded and regions of overlap between the resulting bitmap images quantified using Fiji/ImageJ (Schindelin et al., 2012). Pixelwise colocalization analysis was performed by Manders analysis using the automated thresholding functionality of the JaCoP Fiji/ImageJ plugin (Bolte and Cordelieres, 2006).

**Immunoblots, inhibitor treatments**

Cells pre-treated for 20 min in medium containing DMSO (up to 2%) or endocytosis inhibitors (Chlorpromazine, up to 10 µM; Dynasore, up to 400 µM; EHT-1864, up to 60 µM; IPA-3, up to 20 µM; CK-666, up to 400 µM; concentrations retested batchwise and used as indicated), were stimulated with IL-4 or IL-13 (Gibco) at 37 °C for 30 min in presence of compound. To test reversibility, cells were allowed to recover for 30 min in medium under continuous stimulation. Immunoblots were performed as described (Gandhi et al., 2014) with phospho-STAT6 (Santa Cruz; sc-101808; rabbit; 1:200), STAT6 (Santa Cruz; sc-981; rabbit or mouse; 1:400), and GAPDH (Abcam; ab8245; mouse; 1:4000) antisera.

STAT6 phosphorylation and protein levels were quantified from band intensities (ImageQuant, GE Healthcare) and IC$_{50}$ values calculated by fitting with a dose response function

$$y = A_1 + \frac{A_2 - A_1}{1 + 10^{(IC_{50} - c)/p}}$$

where A1, A2 and p are descriptive parameters and c the varied inhibitor concentration (Origin 9.1, OriginLab).

**Loading assays**

Transferrin uptake was assayed by incubating cells with 20 µg/ml rhodamin- or Alexa647-transferrin (Life Technologies) for 10 min at 4 °C. After washing, cells were transferred to 37 °C under continued presence of inhibitors (30 min) before acid stripping (150 mM NaCl, 100 mM Glycin, pH 2.5), fixation, and imaging.

IL-4-biotin was produced with the EZ-Link Sulfo-NHS-Biotinylation Kit (Thermo Scientific) according to instructions and cells loaded on ice (10 min) and incubated at 37°C (30 min).
IL-4 loading was quantified using a site specifically labelled IL-4-Alexa647 (Duppatla et al., 2014) (gift from Walter Sebald, Würzburg, Germany). HEK293T cells expressing IL-4Rα and H6-IL-2Rγ/JAK3-eGFP were pre-incubated with inhibitors in culture medium at 37°C (15 min), and labelled on ice with 1 nM IL-4-Alexa647 (10 min). After washing, endocytosis was released in the presence of inhibitor at 37°C (20 min). For quantification cells were imaged (LSM780, Zeiss) at three different planes using the GaAsP detector in integration mode. For image analysis (ImageJ), CEs were selected in the GFP channel by thresholding and the ROI measured in the Alexa647 channel to determine area (Aₖ) and mean intensity (Iₖ) for a single cell in each frame (k). The total intensity per endosomes (I_{endo}) was calculated by

\begin{equation}
\langle I_{endo} \rangle = \frac{\sum_i \langle I_\xi \rangle A_k}{\sum_i A_k} = \frac{\sum_i \langle I_\xi \rangle \langle A_{vads} \rangle N_{endo}}{\sum_i A_k}
\end{equation}

where the average area per endosome A_{endo} = 0.67 µm² was determined independently in the DMSO control to derive the number of endosomes in each frame (N_{endo}). For statistics we calculated the variance of the weighted sample mean

\begin{equation}
\sigma = \left( \sum_k \left( \langle I_\xi \rangle - \langle I_{endo} \rangle \right)^2 w_k^2 \right)^{1/2} \text{ with } w_k = A_k / \sum_i A_k
\end{equation}

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

**FLIM / FRET**

FLIM measurements were performed on a Nikon TE-2000 microscope (Nikon, Tokyo, Japan) equipped with a 60x, NA 1.49 TIRF lens, a Becker and Hickl (Berlin, Germany) FLIM setup (DCS-120 scanner, PMC-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 x 128 pixel images were scanned with a pixel dwell time of 11.55 µs and a pixel distance of 0.33 µm. Photon arrival times for individual pixel were fitted offline with a single exponential (binning 3, threshold 20 photons, fit range 1.5-13 ns). For each cell, donor lifetimes were separately averaged for five positions at the plasma membrane and five CEs 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 labeling as described (Slot and Geuze, 2007). 70 nm sections were cut on a Leica UC6+FC6 cryo-ultramicrotome and picked up in methyl cellulose / sucrose (2% methyl cellulose, Sigma M-6385, and 2.3 M sucrose, 1:1). The grids were incubated with primary antibodies (rabbit anti-GFP: TP 401, Torrey Pines, 1:100; rabbit anti-biotin, Enzo # 43881, 1:100) followed by protein A 10 nm gold, post-fixed in 1% glutaraldehyde, and contrasted by incubation in methyl cellulose containing 0.4% uranylacetate. Alternatively, the primary anti-GFP was detected with secondary antibodies coupled to ultra small gold (Aurion, ~ 1 nm) followed by silver enhancement using the R-Gent SE/Kit (Aurion) before contrasting. For CLEM, ultra thin cryosections were labelled with chicken-anti-GFP (Abcam; ab13970), mouse-anti-actin (Cedarlane CLT 9001), rabbit-anti-mouse, protein A 10 nm gold, and goat-anti-chicken Alexa488 as described (Fabig et al., 2012). Sections were analyzed on a Philips Morgagni 268 TEM (FEI) at 80 kV.

**Statistics**

Data were plotted using box-and-whisker plots or as mean ± s.d. (standard deviation) column charts. Differences were tested for significance using Student’s t-test for pairwise and ANOVA followed by Tukey’s HSD post-hoc test for multiple comparisons. All data sets measured at least in triplicate.
Supplementary information
Tables S1 - S4
Supplementary Figures S1-S4

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Conflict of interest
The authors declare no competing interest.

Author contributions
T.W. discovered CEs and initiated the experiments, T.W. and C.B. devised the experimental strategy, K.K. performed pharmacological studies, H.G. and K.K. characterized the endosomes, T.K. & H.G. performed the EM analysis, S.P. & C.B. performed FLIM/FRET experiments, P.S., T.W. and C.B. were responsible for data interpretation, T.W. & C.B. wrote the manuscript.

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References


Figures

**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 and 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 complexes 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)
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. JAK3-eGFP transfected into HEK293T cells is fully cytoplasmic (CP) and excluded from the nucleus (N) regardless of expression level. (G) Cotransfection of IL-2Rγ induces recruitment of JAK3-eGFP to the plasma membrane (PM, open arrowhead) and speckle-like cortical endosomes (CEs). Cells expressing high levels of JAK3-eGFP exhibit both cytoplasmic and membrane associated pools indicating binding site saturation. (H) IL-2Rγ-eGFP and JAK3-TagRFP colocalize at the plasma membrane and at CEs. Note structures exclusively labeled by IL-2Rγ-eGFP. (B-E) Phosphorylation of transfected STAT6, signalling readout; GAPDH, loading control; (F-H) central confocal sections.
Fig. 2.

**Endosomal localization of IL-4R subunits.** (A-E) CEs marked by IL-2Rγ/JAK3-eGFP associate with the early endosomal markers Rab5 (A) and EEA1 (B), and the recycling endosomal marker Rab11 (C), but not with the late endosomal marker Rab7 (D). Rab5 also marks CEs formed by IL-2Rγ and the kinase dead JAK3-D949N-eGFP (E). (F, G) Colocalization with Rab5 (F) and EEA1 (G) is also seen for the type 2 IL-4R subunit IL-13Rα1-eGFP. (H) EEA1 and JAK3-eGFP colocalize in Jurkat cells. All panels surface section confocal images except (H), central section.
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 becomes detectable at the CEs. (C-H) HEK293T cells transfected with IL-2Rγ/JAK3-TagRFP and unlabelled IL-4Rα. Markers for phosphorylated IL-4R pathway components are largely absent from the CEs of unstimulated cells (C,E,G). Phosphorylated IL-4Rα (D), phospho-JAK1 IL-4Rα (F), and phospho-JAK3 (H) become detectable at the CEs following IL-4 stimulation. All panels surface confocal sections.
Fig. 4.

**Ultrastructural analysis and quantification.** (A-E) HEK293T cells expressing IL-2Rγ/JAK3-eGFP. (A-B) Anti-GFP immuno EM enhanced anti-GFP detected by Ag-enhanced nanogold (A) and 10 nm immunogold (B) reveals accumulation of JAK3-eGFP at multivesicular CEs. (C,D) CEs are marked by the MVB markers Hrs (C) and SARA (D). (E) anti-biotin immuno-EM reveals the presence of biotinylated IL-4 within CEs selected by GFP fluorescence. (F) Quantification of 10 nm anti-GFP immunogold stainings. At the CE limiting membrane gold particles are enriched 3.3 fold relative to the adjacent plasma membrane (PM) and 14-fold relative to more distant membrane regions. (C,D) Surface confocal sections.
**Fig. 5.**

**Detection of receptor dimerization by FLIM.** (A) Experimental strategy: FRET caused by ligand induced heterodimerization of CyPet-tagged IL-4Rα and YPet-tagged IL-2Rγ or IL-13Rα1 reduces donor fluorescence lifetime. (B) FLIM images of HEK293T cells expressing IL-4Rα-CyPet in combination with nonfluorescent IL-2Rγ (donor only), IL-2Rγ-YPet or IL-13Rα1-YPet (acceptors). Homogeneous membrane area (open arrows) and CEs (arrows) as used for quantification. Color indicates fluorescence lifetime (sidebar). (C) Quantification of FLIM measurements. IL-4 exposure shifts the donor lifetimes to lower values (red arrows). Type 1 IL-4R complexes preferentially form within CEs whereas type 2 IL-4 receptors also form at the plasma membrane. Error bars denote s.d., box-and-whisker plots indicate 2nd and 3rd quartile (box), median (horizontal line), and 1.5x interquartile range (whiskers). ***, p < 0.01.
Fig. 6.

Inhibition of actin mediated endocytosis reversibly blocks IL-4R mediated STAT6 phosphorylation. (A-C) CEs marked by IL-2Rγ/JAK3-eGFP colocalize with activated Vav2 (pY172) (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), Pak1/2 (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-4R.
IL-2Rγ/ 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), while Chlorpromazine has no effect (E-G). (H) Dose response of IL-4R pathway inhibition by EHT-1864 and IPA-3 (four independent experiments / compound). (I,J) Inhibition of type 1 IL-4R signalling in HEK293T cells overexpressing IL-2Rγ/JAK3 by dominant negative Rac1 (T17N) and Pak1 (K299R) (I) or the Arp2/3 inhibitor CK-666 (400 µM) (J). (A-D) surface confocal sections, (E,J) pSTAT6, signalling readout; GAPDH, loading control; w, compound washout. Error bars denote s.d.
Fig. 7.

Inhibition of actin mediated endocytosis blocks ligand and receptor trafficking to CEs. (A-G) IL-4 loading assay. HEK293T cells expressing IL-2Rγ/JAK3-eGFP and preincubated with compounds at indicated concentrations pulse labelled on ice with IL-4-Alexa-647. Following a 20 min chase at 37°C internalization of ligand into CEs 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 (A-G), evaluating all CEs (F) or focussing on CEs adjacent to the bottom membrane (G). (H) EHT-1864 treatment reduces the uptake of His-tagged IL4Ra or IL-2Rγ by cells pulse/chase labelled with trisNTA-Alexa647 into CEs. Error bars indicate s.d..
The role of endocytosis in IL-4R signal transduction. (A) Low lateral affinities between IL-4R subunits prevent ligand induced dimerization at the plasma membrane. (B) Receptors are continuously internalized by constitutive, actin and dynamin mediated endocytosis. (C) The relative rates of endocytosis and recycling lead to an increased receptor density at the cortical endosome limiting membrane, which locally shifts the equilibrium towards receptor heterodimerization. Endocytosis is thus required for downstream signal transduction to compensate for the low, intrinsic affinities governing receptor complex formation.