Intracellular Dynamics and Fate of a Humanized Anti–Interleukin-6 Receptor Monoclonal Antibody, Tocilizumab

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ABSTRACT

Tocilizumab (TCZ), a humanized anti–interleukin-6 (IL-6) receptor (IL-6R) monoclonal antibody, abrogates signal transducer protein gp130-mediated IL-6 signaling by competitively inhibiting the binding of IL-6 to the receptor, and shows clinical efficacy in autoimmune and inflammatory diseases. Despite accumulating evidence for therapeutic efficacy, the behavior and fate of TCZ at the cellular level remain largely unknown. To address this, we evaluated the endocytosis and intracellular trafficking of IL-6R in HeLa cells. The results of our study provide evidence that IL-6R is constitutively internalized from the cell surface by ligand or TCZ binding and the expression of gp130 in an independent manner and is targeted via endosomes without being significantly directed to the recycling pathway to, and degraded in, lysosomes. Furthermore, the cytoplasmic tail of IL-6R is required for constitutive endocytosis of the receptor, which is mediated by the clathrin and AP-2 complex. We further demonstrate that FcRn, whose function is to regulate the serum persistence of IgG, is confined primarily to early/recycling endosomes and rapidly transits between these compartments and late endosomes/lysosomes without being degraded. Importantly, the expression of FcRn induces the segregation of TCZ from IL-6R, resulting in extensive colocalization of TCZ and FcRn in IL-6R–depleted endosomal compartments. Collectively, our results suggest that FcRn can accelerate the retrieval of the internalized TCZ, not only from endosomes but also from lysosomes. Our findings provide new insight into the mechanism by which the antibody internalized into cells is rescued from lysosomal degradation and into how its serum levels are maintained.

Introduction

Interleukin-6 (IL-6) is a pleiotropic cytokine that is involved in inflammation, acute phase responses, immune responses, and hematopoiesis (Scheller et al., 2011; Ataie-Kachoie et al., 2013). These biologic functions of IL-6 are mediated by binding to the IL-6 receptor (IL-6R) system composed of two type-I membrane glycoproteins: a nonsignaling and ligand-binding α-receptor IL-6R (also known as CD126 or gp80) and a signal transducer protein gp130 (CD130) (Garbers et al., 2012). IL-6R also exists as a soluble form (sIL-6R) that is generated through shedding by proteolytic cleavage of the membrane-bound form (mIL-6R) and mRNA alternative splicing (Chalaris et al., 2011). IL-6 first binds to mIL-6R on the cell surface or sIL-6R circulating in body fluids, and then associates with gp130, inducing homodimerization of gp130 and subsequent activation of the JAK/STAT, ERK, and PI3K signal transduction pathways (Johnson et al., 2012). Therefore, deregulated overproduction of IL-6 is associated with autoimmune and inflammatory diseases including rheumatoid arthritis, juvenile idiopathic arthritis, Crohn’s disease, and Castleman’s disease (Neurath and Finotto, 2011; Spîrchez et al., 2012; Tanaka et al., 2012).

Tocilizumab (TCZ) is a humanized anti-human IL-6R monomeric antibody of the IgG1 subclass that inhibits binding of IL-6 to both sIL-6R and mIL-6R, leading to blockade of many biologic functions of IL-6 (Sato et al., 1993). Accordingly, TCZ administration to patients with rheumatoid arthritis and Castleman’s disease leads to an increase in the serum levels of IL-6 and soluble IL-6R and inhibition of IL-6 signaling (Nishimoto et al., 2008). A growing number of clinical studies have shown the potential uses of TCZ for the treatment of autoimmune and inflammatory diseases (Nishimoto et al., 2003, 2004, 2005; Ito et al., 2004; Yokota et al., 2008; Tanaka et al., 2011). A previous report has shown that the administration of TCZ produces a significant antigen-mediated clearance due to the high turnover of IL-6R (Ohsugi and Kishimoto, 2008). However, the IL-6R–mediated intracellular trafficking of TCZ has not yet been fully elucidated at the cellular level.

The major histocompatibility complex class-I–related receptor, FeRn, which is known to mediate the transfer of IgG from mother to fetus across the placenta (Rodewald and Kraehenbuhl, 1984; Simister and Rees, 1985), also protects IgG from degradation and extends the serum half-life of IgG (Simister et al., 1997; Telleman and Junghans, 2000; Akiles et al., 2011; Spîrchez et al., 2012; Tanaka et al., 2012).

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ABBREVIATIONS: CHX, cycloheximide; E64d, (S)-3-[4-[(3-methylbutyl)amino]carbonyl][butyl]amino]carbonyl]-2-oxiranecarboxylic acid ethyl ester; ER, endoplasmic reticulum; FBS, fetal bovine serum; GFP, green fluorescent protein; GST, glutathione S-transferase; IL-6R, interleukin-6 receptor; ILV, intraluminal vesicle; β2m, β2-microglobulin; PB, Pacific Blue; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; siRNA, small interfering RNA; TGN, trans-Golgi network; TCZ, tocilizumab; WT, wild type.
FcRn binds to IgG at the acidic pH 6.0–6.5 of endosomes, but not at the neutral pH 7.4 of blood (Jones and Waldmann, 1972; Rodewald, 1976; Simister and Rees, 1985; Raghavan et al., 1995). Through such pH-dependent binding of IgG to FcRn, IgG internalized by a nonspecific fluid-phase pinocytosis binds with high affinity to FcRn in acidic endosomes from where the FcRn/IgG complex is recycled back to the cell surface without being delivered to lysosomes. Subsequently, exposure to the neutral pH of the extracellular milieu allows IgG to be released from FcRn (Lobo et al., 2004). In addition, the IgG that does not bind to FcRn is targeted to lysosomes and is eventually degraded (Ward et al., 2003). Therefore, extending the serum half-life of therapeutic monoclonal antibodies could help reduce the dose and/or frequency of administration (Kuo and Aveson, 2011) and enhance in vivo therapeutic efficacy (Zalevsky et al., 2010).

In this study, we examined the internalization mechanism and intracellular dynamics of TCZ in HeLa cells exogenously expressing IL-6R. We further investigated the roles of FcRn in the subsequent cellular fate of TCZ following IL-6R–mediated internalization. Our results revealed that TCZ was internalized intracellularly via constitutive endocytosis taken by IL-6R, for which gp130 was dispensable, and both receptor proteins were targeted to, and degraded in, lysosomes. Moreover, the cytoplasmic domain of IL-6R was required for the endocytosis and lysosomal trafficking of the receptor and TCZ. We showed further that although FcRn was also transported to lysosomes, in contrast to IL-6R it was not significantly degraded. Since the steady-state localization of FcRn was predominantly restricted to early/recycling endosomes, these results suggest that FcRn undergoes rapid retrograde transport to these endosomes immediately after reaching lysosomes. Interestingly, expression of FcRn induced sorting of TCZ to the endosomal compartment from IL-6R–localizing lysosomal compartments. Based on these data, we propose a novel mechanism for the protection of IgG from degradation in which FcRn can retrieve IgG not only from endosomes but also from lysosomes.

Materials and Methods

Culture media were purchased from Wako Pure Chemical Industries (Osaka, Japan). Fetal bovine serum (FBS) was obtained from Nichirei Bioscience (Tokyo, Japan). Pacific Blue (PB) Monoclonal Antibody Labeling Kit (P30013), Cy3-conjugated human Tn5, and Alexa488-labeled secondary antibodies were purchased from Molecular Probes (Life Technologies, Carlsbad, CA). Cy5-labeled secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Recombinant human IL-6 was purchased from PeproTech (Rocky Hill, NJ). Cycloheximide (CHX) and 4,4′-diamidino-2-phenylindole were obtained from Sigma-Aldrich (St. Louis, MO). E64d ([25S,3S]-3-[[[[S]-3-methyl-1-[[3-methylbutyl]amino]carbonyl]butyl] amino]carbonyl-2-oxoaracarboxylic acid ethyl ester), leupeptin, and pepstatin A were obtained from Peptide Institute, Inc. (Osaka, Japan).

Cell Culture. HeLa and COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 2 mM glutamine, and 1% penicillin-streptomycin (Sigma-Aldrich) in humidified 95% air and 5% CO2 at 37°C. For immunofluorescence experiments, the cells were plated onto 13-mm coverslips (Matsunami Glass Ind. Ltd., Osaka, Japan) the day before transfection. After 38 hours, the cells were used for immunocytochemical experiments.

Antibodies. A humanized anti–human IL-6R monoclonal antibody of immunoglobulin G1k (IgG1k) subtype, TCZ (Chugai Pharmaceutical Roche Group, Tokyo, Japan), was described previously (Nishimoto et al., 2005). Rabbit polyclonal antibodies to human TGN46 were raised against glutathione S-transferase (GST) fusion protein of the extracellular domain corresponding to amino acid residues 49–162 (K. Fujimoto et al., unpublished data). The antiserum was passed through GST-conjugated CNBr-activated agarose beads (GE Healthcare, Bucks, UK), and then affinity purified using the antigen coupled to CNBr-activated agarose beads according to the manufacturer’s instructions. Mouse monoclonal antibodies to human LAMP-1 and LAMP-2 were obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa (Iowa City, IA). Mouse monoclonal antibodies to β2-sубunit (AP50) and EEA1 were purchased from BD Transduction Laboratories (BD Biosciences, San Jose, CA). Mouse monoclonal antibodies to FLAG-M2 and β-actin were obtained from Sigma-Aldrich. A mouse monoclonal antibody to TfnR was purchased from Life Technologies. A mouse monoclonal antibody to FcRn (B-8) and rabbit polyclonal antibody to gp130 (C-20) were from Santa Cruz Biotechnology (Dallas, TX). A rabbit polyclonal antibody to β2-microglobulin (β2m) (EP2978Y) was purchased from Abcam (Cambridge, MA). Rabbit polyclonal antibodies to green fluorescent protein (GFP) were raised against GST fusion protein of the full length GFP. The antiserum was passed through a GST-conjugated CNBr-activated agarose beads, and then affinity purified using the antigen coupled to CNBr-activated agarose beads.

Plasmids. The cDNAs of human IL-6R, human FcRn, and human β2m were obtained from GE Dharmacon (Lafayette, CO). The IL-6R wild type (WT) and the C-terminal deletion mutants of IL-6R Δ408 were generated by polymerase chain reaction (PCR) amplification. Briefly, the following primer combinations were used: IL-6R WT, using 5′-GACGTCATTACATGCTGGCCCTCGGGCTGC-3′ for the forward primer and 5′-GTGGATCCGCTCTGGGGAAGAAGTACTGCT-3′ for the reverse primer; and IL-6R Δ408, using the IL6R WT for the forward primer and 5′-GTGGATCCGCTCTGGGGAAGAAGTACTGCT-3′ for the reverse primer. The PCR fragments of IL-6R WT and IL6R Δ408 were digested with KpnI and BamHI and cloned into pEGFP-N1 vector (Takara Bio Clontech, Shiga, Japan).

To construct FcRn-ΔS, the FcRn coding sequence was PCR amplified using 5′-GACGTCATTACATGCTGGCCCTCGGGCTGC-3′ for the forward primer and 5′-CGAATTCCGCTCTGGGGAAGAAGTACTGCT-3′ for the reverse primer and cloned into the Xhol/EcoRI sites of the pDsRed-Monomer N1 vector (Clontech Laboratories, Inc., Mountain View, CA).

To coexpress FcRn-ΔS and β2m in the cells, the construct was created by cloning the FcRn-ΔS cDNA and β2m into multiple cloning sites A and B of the pIRE6 vector (Clontech). The β2m cDNA was PCR amplified by using 5′-GAAGGCGGCCGTATCATCTCTCTGTCGGC-3′ for the forward primer and 5′-AAAGGGCCGGCTTTACATGTCTGCTGTCGCCC-3′ for the reverse primer, and the FcRn-ΔS was PCR amplified by using the pDsRed-Monomer N1/FcRn as a template, the FcRn forward primer described previously, and 5′-TCGAGCGCCGCTTTACATGTCTGCTGTCGCCC-3′ for the reverse primer. The β2m gene was then cloned into the SalI/NotI sites of multiple cloning site B of the pIRE6 vector, and FcRn-ΔS and FcRn into Xhol/MluI sites of multiple cloning site A. The human Rab5 (Q79L) cDNA inserted into pCMV5-Flag vector (Koresu and Katada, 2001) was kindly provided by Kota Saito and Toshiaki Katada (Tokyo University, Tokyo, Japan).

The DNA sequence of the construct was always confirmed by dyeodeoxy chain reaction termination sequencing, using the DNA Sequencing Kit (BigDye Termination Cycle Sequencing Ready Reaction, Life Technologies) and ABI PRISM 310 Genetic Analyzer (Life Technologies). Transfections were carried out with FuGENE6 (Promega, Madison, WI) according to the manufacturer’s instructions. After 24 hours, the cells were used for immunocytochemical experiments.

Immunofluorescence Microscopy. Immunofluorescence analysis was performed as described previously (Hirata and Tanaka, 2000). Briefly, cells cultured on coverslips were fixed immediately in 4% paraformaldehyde (Sigma-Aldrich) in phosphate-buffered saline (PBS), pH 7.4, for 30 minutes at room temperature, and permeabilized with 0.05% saponin (Sigma-Aldrich) in PBS for 15 minutes. Cells were quenched with 50 mM NH4Cl (Wako Pure Chemical Industries)
in PBS for 15 minutes and blocked with 1% bovine serum albumin (fraction V; Sigma-Aldrich) or 10% PBS in PBS for 30 minutes. The cells were then incubated for 1 hour in the primary antibody diluted in blocking solution, as described previously (Kuronita et al., 2002). The cells were incubated for 30 minutes with Alexa488-, Cy3-, or Cy5-labeled secondary antibodies, together with 4',6-diamidino-2-phenylindole as a nuclear counterstain. Coverslips were then mounted in Mowiol (Merck Millipore, Billerica, MA) onto glass slides, and the cells were analyzed by laser-scanning confocal microscopy (1A System; Nikon, Tokyo, Japan) equipped with four lasers (405, 488, 561, and 640 nm). 4',6-Diamidino-2-phenylindole and PB were detected with the 405 laser, 450/50 nm filter; Alexa488, and Cy3 were detected with the 488 laser, 525/50 nm filter; Cy3 and DsRed were detected with the 561 laser, 595/50 nm filter; and Cy5 was detected with the 640 laser, 700/75 nm filter. Photographic images were processed using Photoshop (Adobe Systems, San Jose, CA).

A colocalization module of Nikon software was used to measure the colocalization of the internalized PB-labeled TCZ (PB-TCZ) with IL-6R-GFP or FcRn-DsRed. PB labeling of TCZ was performed according to the manufacturer’s instructions.

**Western Blotting.** Cells cultured on 35-mm dishes were washed with PBS, scraped, and homogenized in 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 0.5% Triton X-100, 0.1% SDS, 1 mM EDTA, 10 mM NaF, and 1 mM Na3VO4 containing protease inhibitor cocktail (Nacalai Tesque, Inc., Kyoto, Japan). Then, 10 μg protein was subjected to SDS-PAGE (10% polyacrylamide) under reducing conditions. Proteins were transferred to a polyvinylidene difluoride membrane (Merck Millipore), which was subsequently blocked with 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Triton X-100 (TBS-T), and 5% milk powder for 1 hour at room temperature. The blot was incubated overnight at 4°C with the primary antibody, followed by washing with TBS-T. Subsequently, incubation with horseradish peroxidase–coupled sheep anti-rabbit or donkey anti-mouse antibody (GE Healthcare) was performed for 1 hour at room temperature followed by washing with TBS-T. Blots were finally analyzed using the ECL Detection System (GE Healthcare). Immunoblots were quantified using an LAS1000 Imaging System (Fuji Film, Tokyo, Japan) or Molecular Image ChemiDoc XRS+ with Image Laboratory Software (BioRad, Hercules, CA).

**Antibody Uptake and Endocytosis Assays.** HeLa or COS-1 cells transfected with or without IL-6R-GFP were incubated with the antibody to PB-TCZ (2 μg/ml) for 15 minutes at 4°C. For Tfn internalization, cells cultured on coverslips were incubated in serum-free Dulbecco’s modified Eagle’s medium with 1 mg/ml bovine serum albumin for 30 minutes and then with Cy3-labeled Tfn (25 μg/ml) for 15 minutes at 4°C. Cells were washed twice with PBS at 4°C and further incubated in Dulbecco’s modified Eagle’s medium containing 10% FBS at 37°C for the indicated time periods. After internalization, cells were fixed in 4% paraformaldehyde in PBS. K+-depletion and sucrose loading were performed as described previously by Di Guglielmo et al. (2003) and Heuser and Anderson (1989), respectively.

**RNA-Mediated Interference.** The following small interfering RNA (siRNA) oligonucleotides synthesized by B-bridge International (Cupertino, CA) were used: AUCCGCGGAUAAGAGUATT for the negative control, GUGGAUCCUUCGGGUCATT for the μ2-subunit (Masuyama et al., 2009). Also, gpi30 siRNA was purchased from Sigma-Aldrich. HeLa cells were transfected with the indicated siRNAs using Lipofectamine RNAiMAX Reagent (Life Technologies) according to the manufacturer’s protocol. All experiments were performed 48–72 hours after siRNA transfection.

**Statistical Analysis.** Data were expressed as mean ± S.E.M. Samples were analyzed using Student’s t test. A value of P < 0.05 was considered to be statistically significant.

**Results**

**Intracellular Localization and Stability of IL-6R-GFP.** We first examined the localization of human IL-6R in HeLa cells. For this, we generated a construct encoding fusion protein that fused GFP to the COOH terminus of IL-6R (IL-6R-GFP), which was transiently expressed in HeLa or COS-1 cells. As shown in Fig. 1A, IL-6R-GFP was distributed primarily both on the cell surface and in reticular structures around the perinuclear region, and was also occasionally visible in small punctate structures scattered throughout the cytoplasm. Then, to investigate the intracellular localization of IL-6R-GFP in more detail, we performed colocalization experiments of IL-6R-GFP with several organelle-specific marker proteins. IL-6R-GFP partially colocalized with the trans-Golgi network (TGN) marker TGN46 in the perinuclear reticular structures (Fig. 1A). In addition, IL-6R-GFP–positive small puncta were labeled with antibodies for an early endosome marker EEA1 or a late endosome/lysosome marker LAMP-1 (Fig. 1A). However, IL-6R-GFP did not significantly colocalize with recycling endosome marker TfnR (Fig. 1A). Nearly the same results were obtained in COS-1 cells expressing IL-6R-GFP (Supplemental Fig. 1). To further examine whether the localization of IL-6R-GFP in LAMP-1–positive late endosomes/lysosomes reflects the site where the receptor is degraded, HeLa cells transiently expressing IL-6R-GFP were incubated with medium containing lysosomal protease inhibitors, E64d, leupeptin, and pepstatin A (E64d/Leup/Pep) for 6 or 24 hours. E64d/Leup/Pep treatment largely shifted the steady-state localization of IL-6R-GFP to LAMP-1-positive vesicles, in which most of the IL-6R-GFP was primarily enclosed inside the vesicles (Fig. 1B), reflecting the lysosomal degradation of IL-6R-GFP. In addition, western blot analysis showed that although treatment of cells with CHX, a protein synthesis inhibitor, for 6 hours reduced expression levels of IL-6R-GFP to ∼30% of those in untreated cells, this was restored almost to basal levels by supplementing E64d/Leup/Pep (Fig. 1C). However, under the same conditions the level of expression of TfnR was not significantly affected (Fig. 1C). Collectively, these results suggest that IL-6R-GFP expressed in HeLa cells is constitutively delivered to, and is subsequently degraded in, late endosomes/lysosomes without being recycled to the cell surface. Accordingly, the localization of IL-6R-GFP in compartments along the endocytic pathway—including the cell surface, early endosomes, and late endosomes/lysosomes observed in immunofluorescence experiments—appears to reflect a constitutive trafficking pathway of the receptor. This hypothesis was further supported by the demonstration that the half-life of IL-6R-GFP was not significantly affected following treatment of cells with a ligand IL-6 (t1/2 = 2.0 hours) compared with that in control cells (t1/2 = 2.8 hours) (Fig. 1D), which is in agreement with a previous report (Gerhartz et al., 1994).

**Internalization and Endocytic Trafficking Pathways of TCZ.** We examined whether localization of IL-6R-GFP in the endocytic compartments is associated with the fate of TCZ after binding with the receptor. HeLa cells transiently expressing IL-6R-GFP were incubated with PB-labeled TCZ (PB-TCZ) or PB-labeled nonimmune human IgG (PB-IgG) at 4°C for 30 minutes to avoid nonselective internalization of the antibody by fluid-phase endocytosis. Cells were immediately fixed or subsequently chased at 37°C for up to 3 hours, and the internalization of PB-TCZ and pathway taken by PB-TCZ were analyzed by confocal laser microscopy. Both cell surface binding and internalization of PB-TCZ, but not PB-IgG, were visible only in cells expressing IL-6R-GFP (Fig. 2A), indicating the absence of expression of endogenous IL-6R in
Fig. 1. Intracellular localization of IL-6R-GFP. (A) HeLa cells were transiently transfected with IL-6R-GFP, fixed, and then incubated with primary antibodies to TGN46, TfnR, EEA1, or LAMP-1. The primary antibodies were revealed by incubation with Cy3-labeled secondary antibodies. Nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI) (cyan). Cells were visualized by confocal microscopy. Right columns show the merged images for triple staining of IL-6R-GFP (green), each organelle marker (red), and DAPI (cyan). Bars, 20 μm. (B) HeLa cells were transiently transfected with IL-6R-GFP. After 24 hours of transfection, cells were further cultured with fresh medium containing E64d/Leup/Pep for 6 or 24 hours. Cells were then fixed and incubated with primary antibodies to LAMP-1 and Cy3-labeled secondary antibodies. Nuclei were labeled with DAPI (cyan). Intracellular localization of IL-6R-GFP and LAMP-1 was observed by confocal microscopy. Right columns show the merged images for triple staining of IL-6R-GFP (green), LAMP-1 (red), and DAPI (cyan). Squares indicate magnified regions. Bars, 20 μm. Graph represents IL-6R-GFP colocalization with LAMP-1 at each time point. Error bars indicate S.E.M. of five independent experiments (n = 20 cells for each condition). **p < 0.01; ***p < 0.001. (C) HeLa cells were transiently transfected with IL-6R-GFP. After 24 hours of transfection, cells were treated with media alone, containing 10 μg/ml CHX or a combination of CHX and E64d/Leup/Pep at 37°C for 6 hours and harvested. Equal amounts of cell lysates (20 μg) were subjected to SDS-PAGE and immunoblotting using antibodies to GFP, TfnR, or β-actin. (D) HeLa cells transiently transfected with IL-6R-GFP were incubated in the absence or presence of IL-6 (100 ng/ml) or TCZ (2 μg/ml) with medium containing CHX (10 μg/ml) for the indicated time periods and harvested. Equal amounts of cell lysates were subjected to
HeLa cells and the specificity of IL-6R-GFP expression–dependent internalization of PB-TCZ. The same results were observed in COS-1 cells (unpublished data). After an internalization of 30 minutes, PB-TCZ was significantly reduced from the cell surface and was simultaneously visible in small punctate structures dispersed throughout the cytoplasm (Fig. 2B). These vesicles exhibited a tendency to accumulate around the perinuclear region with increasing chase periods (60 and 180 minutes) (see Fig. 2B) and the rate of colocalization between IL-6R-GFP and PB-TCZ was hardly altered during chase periods up to 3 hours (Fig. 2B). Furthermore, treatment of cells with TCZ did not affect the half-life of IL-6R-GFP (Fig. 1D), suggesting that PB-TCZ is internalized by the constitutive endocytosis of IL-6R-GFP.

We further examined the compartments where the internalized PB-TCZ was localized. As shown in Fig. 2C, the internalized PB-TCZ was hardly detected in TGN46-positive structures during the chase for up to 3 hours, suggesting that IL-6R-GFP does not recycle between the cell surface and the TGN as does TGN46 (Maxfield and McGraw, 2004). This result also implies that IL-6R-GFP partially colocalizing with TGN46 (Fig. 1A) might reflect a population of the newly synthesized receptor en route to the cell surface and late endosomes/lysosomes. The internalized PB-TCZ was already extensively colocalized with EEA1 and TfnR after a 30-minute chase (Fig. 2C). Although the colocalization of PB-TCZ with EEA1 was constantly visible for up to 3 hours of chase, colocalization with TfnR decreased time dependently. In addition, the rate of colocalization of PB-TCZ with LAMP-1 increased over time (Fig. 2C), and a significant colocalization between PB-TCZ and LAMP-1 was observed in cells incubated with the chase medium containing E64d/Leupeptin/Pep compared with control cells (Fig. 2D). Collectively, these results suggest that part of the internalized PB-TCZ is either delivered from early endosomes to, and is degraded in, late endosomes/lysosomes in cells expressing IL-6R-GFP, without being significantly directed to the recycling pathway, while the remainder is retained in EEA1-positive early endosomes with IL-6R-GFP.

The Cytoplasmic Domain of IL-6R Is Necessary for Its Internalization. It has been shown that effective internalization of IL-6 and down regulation of IL-6R are mediated by association with gp130 (Zollnhoffer et al., 1992; Graeve et al., 1996). However, it remains unclear whether gp130 is involved in the internalization of PB-TCZ mediated by IL-6R. To this end, we knocked down the expression of gp130 in HeLa cells by RNA interference. Western blot analyses revealed that transfection of gp130-specific siRNA already effectively reduced the expression level of gp130 by ∼95% of control cells after 12 hours of siRNA transfection (Fig. 3A). This is very consistent with, and was accounted for by, the short half-life of gp130 (∼1 hour) estimated in HeLa cells treated with CHX (Fig. 3B). Furthermore, the depletion of gp130 did not significantly influence the expression level and stability of IL-6R-GFP (Fig. 3C). As shown in Fig. 3D, knockdown of gp130 did not significantly affect the internalization of PB-TCZ, or the localization of IL-6R-GFP. Therefore, these data apparently indicate that gp130 is not involved in the constitutive trafficking of IL-6R and concomitant internalization of PB-TCZ.

These results prompted us to further examine whether IL-6R itself has a signal responsible for endocytosis from the cell surface and following trafficking to late endosomes/lysosomes without intervention of gp130. Membrane proteins endocytosed from the cell surface contain the information necessary for internalization in their cytoplasmic domain such as a tyrosine-based motif (YXXΦ, where X is any amino acid and Φ is a bulky hydrophobic residue) and a dileucine-based motif ([DE]XxxL[Ll]) (Bonifacino and Traub, 2003), both of which are also known to function as a lysosomal targeting signal (Eskelinen et al., 2003). Indeed, in the COOH-terminal cytoplasmic tail of IL-6R, consisting of 79 amino acids, there are two endocytosis motifs: a typical tyrosine-based type motif, 408-YSLG, and a dileucine-like motif, 427-LI (Fig. 4A). Also, it has been shown that both motifs are necessary for post-Golgi biosynthetic trafficking to the basolateral plasma membrane in polarized epithelial cells (Martens et al., 2000). However, previous reports have demonstrated that the dileucine-like motif within the cytoplasmic tail of gp130 is essential for IL-6–induced internalization and downregulation of IL-6R, while the cytoplasmic domain of IL-6R is dispensable (Dittrich et al., 1994, 1996). Then, to examine whether the cytoplasmic tail of IL-6R including both tyrosine- and dileucine-based motifs is required for receptor internalization and trafficking, we created a mutant (IL-6R Δ408-GFP) that deleted the COOH-terminal 60 amino acids including both motifs (Fig. 4A). As shown in Fig. 4B, the molecular mass of IL-6R Δ408-GFP (∼100 kDa) estimated from the mobility on SDS-PAGE was ∼10 kDa smaller than that of IL-6R WT-GFP (∼110 kDa). This difference was quite consistent with the difference in the molecular mass between the full-length and deletion mutants of IL-6R predicted from the amino acid sequence.

We further assessed the internalization of the mutant by monitoring the uptake of PB-TCZ. In contrast to IL-6R WT-GFP expressing cells, most of the PB-TCZ stayed bound to the cell surface, and was scarcely internalized into cells expressing IL-6R Δ408-GFP (Fig. 4C). These results are also supported by the elevated cell surface expression level of IL-6R Δ408-GFP compared with IL-6R WT-GFP (Fig. 4C). Thus, these data suggest that the cytoplasmic domain including tyrosine- and/or dileucine-like motifs is essential for effective internalization of IL-6R.

Internalization of IL-6R Is Mediated by a Clathrin/ AP-2–Dependent Mechanism. We further investigated whether the internalization of IL-6R-GFP is mediated in a clathrin-dependent manner. The clathrin-mediated endocytosis motifs present in cargo molecules are specifically recognized by the μ2-subunit of the AP-2 complex (Bonifacino and Traub, 2003). Therefore, the requirement of a μ2-subunit in IL-6R-GFP endocytosis was investigated by transfection of HeLa cells with siRNA directed against the μ2-subunit in direct comparison with Cy3-labeled Tfn, a ligand that is well

SDS-PAGE and immunoblotting using antibodies for GFP. The amount of IL-6R-GFP was quantified by densitometry and expressed as the percentage of initial IL-6R-GFP remaining at each time point. Half-lives were determined by curve fitting using an exponential equation. Error bars represent S.E.M. of three independent experiments.
documented to be internalized by TfnR in a manner that is dependent on clathrin/AP-2 (Motley et al., 2003; Masuyama et al., 2009).

The knockdown of the \( \mu \)-2-subunit substantially reduced the internalization of PB-TCZ relative to control cells (Fig. 5, A–C), which was correlated with, and accounted for, the increase in both cell surface-bound PB-TCZ and the cell surface expression level of IL-6R-GFP (Fig. 5, A and B) Similarly, inhibition of Cy3-Tfn internalization and increased cell surface expression of TfnR were also observed in cells transfected with siRNA directed against the \( \mu \)-2-subunit (Fig. 5, A and B). We further investigated the internalization...
Fig. 3. Involvement of gp130 in endocytosis of IL-6R. (A) HeLa cells were transfected with siRNA directed to scramble negative control (NC) or to gp130. At the indicated time periods after transfection, cells were harvested and equal amounts of cell lysates of siRNA-treated cells were subjected to SDS-PAGE and immunoblotting using antibodies to gp130, GFP, or β-actin. (B) HeLa cells were incubated in the presence of CHX (10 μg/ml) for the indicated time periods and harvested. Equal amounts of cell lysates were subjected to SDS-PAGE and immunoblotting using antibodies for gp130. The amount of gp130 was quantified by densitometry and expressed as the percentage of initial gp130 remaining at each time point. Half-lives were determined by curve fitting using an exponential equation. Error bars represent S.E.M. of four independent experiments. (C) HeLa cells expressing IL-6R-GFP were transfected with siRNA directed to NC or gp130. After 12 hours, cells were treated with or without 10 μg/ml CHX at 37°C for 6 hours and harvested. Equal amounts of cell lysates (20 μg) were subjected to SDS-PAGE and immunoblotting using antibodies to gp130, GFP, TfnR, or β-actin. (D) Twelve hours after transfection of siRNA directed to NC or gp130, HeLa cells were transiently transfected with an expression plasmid encoding IL-6R-GFP. After 24 hours, cells were incubated with PB-TCZ (2 μg/ml) at 4°C for 30 minutes. Cells were then internalized with PB-TCZ for 3 hours at 37°C, fixed, and visualized by confocal microscopy. Right columns show the merged images for double staining of IL-6R-GFP (green) and PB-TCZ (cyan). Bars, 20 μm.
of PB-TCZ using pharmacological inhibitors, such as potassium depletion (Di Guglielmo et al., 2003) or sucrose loading (Heuser and Anderson, 1989), for clathrin-dependent endocytosis, and found that the uptake of PB-TCZ was strongly inhibited by either potassium depletion or sucrose loading (unpublished data). Altogether, these data suggest that IL-6R is internalized by clathrin/AP-2-dependent endocytosis. Furthermore, the depletion of the μ2-subunit not only increased the expression levels of IL-6R-GFP, but also significantly abrogated degradation of the receptor (Fig. 5D), suggesting that IL-6R-GFP is directly delivered from the TGN to the cell surface and from where it is transported to lysosomes for degradation via a clathrin/AP-2-dependent endocytic pathway. Again, this result confirms that IL-6R-GFP is constitutively targeted to the lysosomal degradation pathway and is not significantly recycled to the cell surface. It is also noteworthy that, in addition to increased expression of IL-6R-GFP on the cell surface by the μ2-knockdown observed in confocal laser microscopy (Fig. 5, A and B), western blot analysis revealed that IL-6R-GFP expression levels were markedly elevated in μ2-knockdown cells relative to control cells (Fig. 5D). This suggests that in our experimental system ectodomain shedding of the surface IL-6R caused by ADAM10 and ADAM17, members of the ADAM family of metalloproteases (Garbers et al., 2011), is not significantly responsible for the turnover of the receptor. Interestingly, the effect of μ2-knockdown on the turnover rate of gp130 was less sensitive than that on IL-6R-GFP (Fig. 5D), suggesting that substantial amounts of gp130 may be internalized in a μ2-independent manner or may be targeted directly from the

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**Graph**

Graph represents the relative amount of internalized PB-TCZ in control (NC) or gp130 knockdown (KD) cells (normalized to 1 for the NC). Error bars indicate S.E.M. of five independent experiments ($n=20$ cells for each condition); NS, not significant.
TGN to late endosomes/lysosomes without passing through the cell surface and being degraded.

**Effect of FcRn-DsRed Expression on the Intracellular Localization of IL-6R-GFP.** The results shown in Fig. 2C suggested that part of the internalized PB-TCZ was segregated from IL-6R-GFP in early endosomes, and was retained there without being subsequently transported together with the receptor to late endosomes/lysosomes. This may be accounted for by the acidic pH-dependent dissociation of TCZ from IL-6R (Igawa et al., 2010), which helps the antibody avoid lysosomal degradation and subsequent exocytosis. In this regard, FcRn is known to play a central role in the maintenance of serum IgG levels by the acidic pH dependence of the interaction with the Fc domain of IgG and recycling of IgG away from the lysosomal degradation pathway toward the cell surface, leading to release of IgG from FcRn owing to the low affinity at a neutral extracellular milieu pH (Lobo et al., 2004). Thus, FcRn functions as a receptor protecting IgG from degradation, thereby extending the antibody half-life (Simister et al., 1997; Telleman and Junghans, 2000; Akilesh et al., 2007; Montoyo et al., 2009).

We then sought to evaluate the effect of FcRn expression on intracellular trafficking of the internalized PB-TCZ. We first examined the localization of FcRn in cells expressing FcRn tagged with DsRed at its COOH terminus (FcRn-DsRed) alone by comparison with that of several organelle-specific marker proteins. As shown in Fig. 6A, FcRn-DsRed was extensively colocalized with TfnR in HeLa cells, which agrees with a previous report (Tzaban et al., 2009). Although only a very small fraction of FcRn-DsRed was found in EEA1- or LAMP-1–positive vesicles, little if any FcRn-DsRed coincided with TGN46 (Fig. 6A). By contrast, FcRn-DsRed expressed in COS-1 cells showed an endoplasmic reticulum (ER)–like reticular staining pattern (Supplemental Fig. 2A). Since the exit of FcRn from the ER is required for the association of FcRn with β2m (Praetor and Hunziker, 2002), the ER-like localization of FcRn-DsRed we observed in COS-1 cells may be due to failure of β2m endogenously expressed in COS-1 cells to interact with FcRn-DsRed. Indeed, immunofluorescence and western blot analyses revealed that the expression level of β2m in COS-1 cells was substantially lower than that in...
HeLa cells (Supplemental Fig. 2B). Furthermore, the ER-like localization of FcRn-DsRed seen in single transfected COS-1 cells shifted to TfnR-positive structures assembled in the perinuclear region by coexpression with human β2m and FcRn-DsRed colocalized with β2m in scattered small vesicles (Supplemental Fig. 2A, bottom columns). However, such a localization change of FcRn-DsRed by exogenous expression of β2m was not significantly observed with HeLa cells (unpublished data). Furthermore, the perinuclear localization of endogenous β2m was markedly altered by the expression of FcRn-DsRed, but not DsRed, where most of the β2m was colocalized with FcRn-DsRed (Fig. 6B), which is consistent with previous reports (Praetor and Hunziker, 2002; Tesar et al., 2006). However, the expression of IL-6R-GFP did not cause such an alteration in β2m localization (Fig. 6B).}

**Fig. 6.** Intracellular localization of FcRn-DsRed. (A) HeLa cells were transiently transfected with an expression plasmid encoding FcRn-DsRed, fixed, and then incubated with primary antibodies to TGN46, TfnR, EEA1, or LAMP-1. The primary antibodies were revealed by incubation with Cy3-labeled secondary antibodies. Nuclei were labeled with 4',6-diamidino-2-phenylindole (DAPI). Cells were visualized by confocal microscopy. Right columns show the merged images for triple staining of FcRn-DsRed (red), each organelle marker (green), and DAPI (cyan). Bars, 20 μm. (B) HeLa cells were transiently transfected with either an expression plasmid encoding DsRed, FcRn-DsRed, IL-6R-GFP, or FcRn-DsRed and IL-6R-GFP. After 24 hours, cells were treated with or without 10 μg/ml CHX at 37°C for 6 hours, fixed, and then incubated with primary antibodies to β2m. The primary antibodies were revealed by incubation with Alexa488-, Cy3-, or Cy5-labeled secondary antibodies. Nuclei were labeled with DAPI. Cells were visualized by confocal microscopy. In cells transfected with DsRed, FcRn-DsRed, or IL-6R-GFP alone, right columns show the merged images for triple staining of DsRed, FcRn-DsRed, or IL-6R-GFP (red), β2m (green), and DAPI (cyan). In cells cotransfected with IL-6R-GFP and FcRn-DsRed, right columns show the merged images for triple staining of FcRn-DsRed (red), β2m (green), and DAPI (cyan). IL-6R-GFP is indicated in white. Asterisks show the cells expressing FcRn-DsRed. Bars, 20 μm.
Fig. 7. Effect of FcRn-DsRed on intracellular trafficking of the internalized PB-TCZ. (A) HeLa cells were transiently cotransfected with an expression plasmid encoding IL-6R-GFP and FcRn-DsRed. After 24 hours, cells were incubated with 2 μg/ml PB-TCZ at 4°C for 30 minutes and subsequently...
secretion extracellularly, thereby suggesting the proper association of exogenously expressed FcRn-DsRed and endogenous β2m in HeLa cells. In addition, although CHX treatment also resulted in a striking loss of fluorescence intensity of IL-6R-GFP, which is attributed to rapid degradation of the receptor, as shown in Fig. 1C, it did not significantly affect the expression level of FcRn-DsRed, as shown from both immunofluorescence and western blot analyses of CHX-treated cells (Figs. 6B and 8B, respectively). Collectively, we concluded that the level of β2m expressed endogenously in HeLa cells is sufficient at least for normal intracellular trafficking of FcRn. Therefore, we performed the following experiments using HeLa cells.

Effect of FcRn-DsRed on the Intracellular Sorting of Internalized PB-TCZ. As shown in Supplemental Fig. 3, there was no detectable cell surface binding of PB-TCZ and PB-IgG in cells expressing FcRn-DsRed alone. This result is consistent with the proposed characteristic for very low binding affinity between FcRn and IgG at a neutral pH (Jones and Waldmann, 1972; Rodewald, 1976; Simister and Rees, 1985; Raghaban et al., 1995). Furthermore, coexpression of IL-6R-GFP and FcRn-DsRed did not influence the cell surface binding of PB-TCZ at 4°C (unpublished data), or also the steady-state localization of each (Supplemental Fig. 4), compared with cells expressing FcRn-DsRed alone (Fig. 6A).

We next examined the effect of FcRn-DsRed expression on intracellular trafficking of the internalized PB-TCZ in HeLa cells. It was noteworthy that the majority of PB-TCZ–positive punctate structures significantly colocalized with FcRn-DsRed during a 3 hour chase (Fig. 7A). However, the rate of colocalization between PB-TCZ and IL-6R-GFP in cells coexpressing FcRn-DsRed decreased with an increase in the chase periods (Fig. 7A), which was different from that in cells expressing IL-6R-GFP alone (Fig. 2B), thereby suggesting that the expression of FcRn-DsRed may accelerate a divergence of the internalized PB-TCZ from IL-6R-GFP. As shown in Fig. 7B, during chase periods up to 3 hours, localization of the internalized PB-TCZ coincided extensively with EEA1 in cells expressing both IL-6R-GFP and FcRn-DsRed, as observed in cells expressing IL-6R-GFP alone (Fig. 2C). Furthermore, the time-dependent decrease in the rate of colocalization between PB-TCZ and TfnR in cells expressing IL-6R-GFP alone (Fig. 2C) was significantly abrogated by coexpression of FcRn-DsRed (Fig. 7B). However, a time-dependent increase in colocalization between PB-TCZ and LAMP-1, as was seen in cells expressing IL-6R-GFP alone (Fig. 2C), was not observed in cells expressing both IL-6R-GFP and FcRn-DsRed (Fig. 7B). Nevertheless, similar to cells transfected with IL-6R-GFP alone (Fig. 2D), treatment of cells coexpressing IL-6R-GFP and FcRn-DsRed with E64d/Leup/Pep resulted in extensive colocalization of PB-TCZ with IL-6R-GFP, most of which were detected in LAMP-1–positive compartments and where FcRn-DsRed was also colocalized (Supplemental Fig. 5). Collectively, these results suggest that FcRn-DsRed may increase not only retention of PB-TCZ dissociated from IL-6R-GFP in early endosomes but also retrieval of PB-TCZ from late endosomes/lysosomes to early endosomes.

Internalized PB-TCZ Is Sorted from IL-6R-GFP and Escapes Lysosomal Degradation by the Expression of FcRn-DsRed. Overexpression of a GTPase-deficient Rab5 (Q79L) mutant can stimulate homotypic fusion of early endosomes or heterotypic fusion of early endosomes and late endosomes/lysosomes, leading to formation of enlarged early endosomes or hybrid organelles with features of early endosomes and late endosomes/lysosomes, respectively (Kuronita et al., 2002; Hirota et al., 2007). Thus, taking advantage of the enlargement of endocytic compartments by expression of Rab5(Q79L), may enable us to analyze increased sorting of PB-TCZ away from IL-6R-GFP (i.e., the lysosomal degradation pathway) by the expression of FcRn-DsRed. In addition, the large vacuolar size of Rab5(Q79L)-induced compartments (average diameter ~5 μm) may also make it possible to easily see whether PB-TCZ is bound to the receptor or not; that is, localization of PB-TCZ to the limiting membrane or lumen, respectively.

We first analyzed the effect of 3xFLAG-Rab5(Q79L)-expression on the distribution of IL-6R-GFP or FcRn-DsRed. As shown in Fig. 8A, FcRn-DsRed was highly enriched on the limiting membrane of Rab5(Q79L)-induced enlarged vacuoles (referred to as QL compartments), which is consistent with a previous report (Gan et al., 2009). By contrast, IL-6R-GFP was predominantly detected in the lumen of QL compartments, which were occasionally visible as small vesicle structures (Figs. 8, A and C), presumably corresponding to the intraluminal vesicles (ILVs) of multivesicular bodies. The same results were also observed in cells triple transfected with IL-6R-GFP, FcRn-DsRed, and FLAG-Rab5(Q79L) (Fig. 8A, bottom columns; and Fig. 8D). In support of the hypothesis that the sorting of membrane proteins into the ILVs of multivesicular bodies is associated with their degradation following fusion with lysosomes (Raiborg and Stenmark, 2009; Shields and Piper, 2011), IL-6R-GFP was degraded relatively rapidly, with a half-life of ~2 hours (Fig. 1D), while FcRn-DsRed was not significantly degraded as assessed by western blotting of CHX-treated cells (Fig. 8B), confirming the immunofluorescence data obtained using CHX-treated cells (Fig. 6B).

When PB-TCZ was internalized to cells expressing both 3xFLAG-Rab5(Q79L) and IL-6R-GFP, it constantly showed
Fig. 8. Effect of FcRn-DsRed expression on the escape of internalized PB-TCZ from lysosomal degradation. (A) HeLa cells were transiently cotransfected with an expression plasmid encoding 3xFLAG-tagged Rab5(Q79L) and IL-6R-GFP, FcRn-DsRed, or IL-6R-GFP and FcRn-DsRed. After 24 hours, cells were fixed, permeabilized, and incubated with a mouse monoclonal antibody to FLAG for labeling of Rab5(Q79L). The primary antibodies were revealed by incubation with Alexa488-, Cy3-, or Cy5-labeled secondary antibodies. Cells were visualized by confocal microscopy. Right columns show the merged images for double or triple staining of 3xFLAG-Rab5(Q79L) (red, green, and cyan in the top, middle, and bottom columns, respectively) and IL-6R-GFP (green), FcRn-DsRed (red), or IL-6R-GFP (green) and FcRn-DsRed (red). Boxed regions indicate the area used for the zoomed insets. The white lines in the zoomed insets were analyzed by line scanning and the fluorescence intensity profiles are shown in histograms. Bars, 20 μm. (B) HeLa cells transiently transfected with an expression plasmid encoding IL-6R-GFP or FcRn-DsRed and IL-6R-GFP were treated with media alone, containing
extensive colocalization with IL-6R-GFP in the QL compartments during chase periods up to 3 hours (Fig. 8C). Moreover, luminal or membrane localization of PB-TCZ in the QL compartments was closely correlated with that of IL-6R-GFP. Thus, the colocalization of PB-TCZ and IL-6R-GFP in the lumen of the QL compartments may reflect the IL-6R-GFP–mediated lysosomal degradation of PB-TCZ. However, in cells expressing FcRn-DsRed together with 3xFLAG-Rab5(Q79L) and IL-6R-GFP, at 30 minutes after internalization PB-TCZ was detected in nearly all of both IL-6R-GFP– and FcRn-DsRed–positive QL compartments, where it colocalized with IL-6R-GFP in the lumen or the membranes, although FcRn-DsRed was localized only in the membranes (30 minutes) (see Fig. 8D). Interestingly, with increasing chase times, the internalized PB-TCZ became colocalized more extensively with FcRn-DsRed than IL-6R-GFP in the QL compartments; that is, it was visible in the QL compartments containing FcRn-DsRed but little or no IL-6R-GFP (60 and 180 minutes) (see Fig. 8D). Moreover, the expression of FcRn-DsRed markedly shifted the localization of the PB-TCZ from the lumen to the limiting membrane in the QL compartments, whereas it had no effect on the sorting of IL-6R-GFP into the ILVs of the QL compartments (Fig. 8D). These results may reflect an association of PB-TCZ with FcRn-DsRed in endosomal compartments, although direct evidence is lacking. Collectively, these results suggest the potentially useful role of FcRn in helping internalized PB-TCZ avoid lysosomal degradation.

**Discussion**

Available evidence suggests that unlike LDL and Tfn receptors, which escape lysosomal degradation and recycle to the cell surface via recycling endosomes (Maxfield and McGraw, 2004), IL-6R is delivered to, and is degraded in, lysosomes without recycling back to the cell surface, and also that gp130 plays a crucial role in this process. This hypothesis came from the results showing that coexpression of the cytoplasmic tail–deleted IL-6R with gp130 caused IL-6 internalization as efficiently as the WT protein (Dittrich et al., 1994), and that the complex of sIL-6R (which lacked the transmembrane and cytoplasmic domains) and IL-6 was internalized by gp130 and degraded within lysosomes (Graeve et al., 1996). Therefore, these lines of evidence led to the conclusion that there are no available signals responsible for the internalization of IL-6R within the cytoplasmic tail.

Here, we offer evidence that the internalization of PB-TCZ, which is specific for the expression of IL-6R-GFP as well as the half-life of IL-6R-GFP, is not significantly affected by the presence or absence of the expression of gp130. Moreover, the basal half-life of IL-6R-GFP (2.8 hours) was much shorter than that of TfnR (~19 hours), which continuously recycles to the cell surface (Rutledge et al., 1991), and that of the epidermal growth factor receptor (~20 hours), which undergoes ligand-inducible downregulation (Ware et al., 1997). These results, together with the absence of any effect of IL-6 and TCZ on the half-life of IL-6R-GFP (Fig. 1D), suggest that IL-6R-GFP is constitutively internalized and is directed to the lysosomal degradation pathway without being recycled back to the cell surface. Thus, it is conceivable that gp130 is largely unnecessary for the endocytic pathway taken by IL-6R-GFP. Our results further demonstrate that the cytoplasmic tail of IL-6R, including the tyrosine- and dileucine-like motifs, is necessary for at least the internalization of the receptor itself, which is mediated by clathrin in an AP-2–dependent manner. Thus far, all studies of signals involved in trafficking of IL-6R have been analyzed in complex with IL-6 and gp130. Therefore, one possible explanation for the disagreement between our results and previous reports is that the cytoplasmic tail of gp130 might preferentially function as the internalization signal only in the IL-6 signaling pathway via soluble IL-6R rather than the membrane-bound receptor. However, we believe this possibility to be unlikely because the turnover rate of IL-6R-GFP was not affected by addition of IL-6, regardless of the expression of gp130 (unpublished data). Thus, our results give new insight into the mechanisms of trafficking of IL-6R, which is involved in the antigen-mediated clearance of antibodies. We cannot yet completely exclude the presence of some internalization signal(s), other than the tyrosine- and dileucine-based motifs, because internalization of a small amount of IL-6R 4G8-GFP was occasionally visible after prolonged chase periods of up to 6 hours (unpublished data). Therefore, future experiments are needed to identify in more detail the signal(s) responsible for endocytosis and intracellular trafficking of IL-6R.

One of the clinical problems associated with therapeutic monoclonal antibodies targeting particular antigens including TCZ is antigen-mediated clearance of antibodies from plasma (Kelley et al., 2006; Ng et al., 2006; Tabrizi et al., 2006, 2010; Ohsugi and Kishimoto, 2008). Therefore, reducing the elimination of IgG antibodies is expected to lead to sustained and increased long-term clinical efficacy. A potential strategy to overcome antigen-mediated clearance of antibodies is to increase the efficiency of FcRn-mediated recycling of the IgG antibody. One successful approach to reduce the elimination of IgG antibodies has been described by Igawa et al. (2010), who engineered TCZ by introducing histidine residues into the antigen-binding site to dissociate from IL-6R at the acidic pH of endosomes, without affecting the antigen-binding affinity at a neutral pH in plasma. This enables the antibody to recycle back to the plasma and to avoid lysosomal degradation through binding with FcRn in acidic endosomes.

10 μg/ml CHX, or a combination of CHX and E64d/Leup/Pep at 37°C for 6 hours and then harvested. Equal amounts of cell lysates (20 μg) were subjected to SDS-PAGE and immunoblotting using antibodies to GFP, FcRn, or β-actin. (C) and (D) HeLa cells were transiently cotransfected with an expression plasmid encoding 3xFLAG-tagged Rab5(Q79L) and IL-6R-GFP (C) or IL-6R-GFP and FcRn-DsRed (D). After 24 hours, cells were incubated with 2 μg/ml of PB-TCZ at 4°C for 30 minutes and allowed to internalize the antibody for the indicated time periods at 37°C. After internalization, cells were fixed, permeabilized, and incubated with a mouse monoclonal antibody to FLAG for labeling of Rab5(Q79L). The primary antibodies were revealed by incubation with Cy3- or Cy5-labeled secondary antibodies. Cells were visualized by confocal microscopy. In (C), right columns show the merged images for triple staining of IL-6R-GFP (green), PB-TCZ (cyan), and 3xFLAG-Rab5(Q79L) (red). In (D), right columns show the merged images for triple staining of IL-6R-GFP (green), PB-TCZ (cyan), and FcRn-DsRed (red). 3xFLAG-Rab5(Q79L) is indicated in white. Arrowheads show the QL compartments containing both PB-TCZ and FcRn-DsRed, but lacking or containing little IL-6R-GFP. Boxed regions indicate the area used for the zoomed insets. The white lines in the zoomed insets were analyzed by line scanning; the fluorescence intensity profiles are shown in histograms. Bars, 20 μm.
In vitro binding affinity assays between TCZ and sIL-6R using surface plasmon resonance have revealed that the unmodified TCZ we used in this study has a much lower dissociation rate from sIL-6R at pH 6.0 than modified TCZ (Igawa et al., 2010), thereby leading to IL-6R-mediated lysosomal degradation of TCZ. Although we do not directly analyze this difference at a cellular level between modified and unmodified TCZ, our results demonstrate that the expression of FcRn-DsRed caused a divergence of the internalized PB-TCZ from IL-6R-GFP (Fig. 7, A and B), and shifted the localization of PB-TCZ from the lumen to the limiting membrane of Rab5(Q79L)-induced enlarged endosomal compartments (Fig. 8, C and D). However, such phenomena were never seen in cells double-transfected with IL-6R-GFP and Rab5(Q79L). Accordingly, these data suggest that the expression of FcRn-DsRed contributes to the recovery of the internalized PB-TCZ from the lysosomal degradation pathway.

A previous study reported that monomeric red fluorescence protein-tagged FcRn expressed in the human endothelial cell line HMEC-1 is localized to both early endosomes and late endosomes/lysosomes, representing bidirectional transport of FcRn for recycling and constitutive degradation, respectively (Weßen et al., 2013). In this study, we also observed that a small fraction of FcRn-DsRed colocalizes with LAMP-1-positive late endosomes/lysosomes, and such colocalization significantly increases upon treatment of cells with lysosomal protease inhibitors, E64d/Leupeptin. However, in contrast to IL-6R-GFP, the very low degradation and the lack of sorting into endosomal ILVs of FcRn-DsRed (Fig. 8, B and A, respectively) suggest that colocalization of FcRn-DsRed with LAMP-1 seems unlikely to involve constitutive degradation of the receptor. Rather, these results could be accounted for by continuous shuffling of FcRn-DsRed between endosomes and lysosomes via kiss-and-run and/or kiss-and-linger processes, as described previously (Gan et al., 2009), although the underlying molecular mechanism remains to be elucidated. Consistent with previous suggestions (Gan et al., 2009), our results also indicate that transfer of membrane proteins from endosomes to lysosomes by kiss-and-run and/or kiss-and-linger processes between endosomes and lysosomes is selective because TfnR, which is exclusively colocalized with FcRn-DsRed (Figs. 6A and 7B), never shifted its localization to LAMP-1-positive compartments upon treatment of cells with E64d/Leupeptin (unpublished data). However, two targeting signals, tryptophan- and dileucine-based motifs present in the cytoplasmic tail of FcRn, have been postulated to mediate internalization of FcRn from the plasma membrane or transport of FcRn from the TGN to the endocytic pathway (Wu and Simister, 2001; Wernick et al., 2005). The tryptophan-based endocytosis signal resembles a tyrosine-based motif and has the capability to interact with the μ2-subunit (Wernick et al., 2005). However, our results suggest that the cell surface expression level of FcRn-DsRed, unlike TfnR, is not significantly increased in cells depleted of the μ2-subunit (unpublished data) and suggest that most, if not all, of the FcRn unoccupied with IgG does not continuously cycle between recycling endosomes and the cell surface. Accordingly, the retention of PB-TCZ in FcRn-DsRed-positive endosomes we observed here (Fig. 7A) may be attributed to incomplete saturation of FcRn with PB-TCZ or reflect the slow dissociation rate of FcRn complexes at the cell surface after recycling and subsequent reinternalization as described previously (Ober et al., 2004).

Furthermore, since FcRn can also bind IgG at a pH of 5.0 (Tesar et al., 2006), it is conceivable that FcRn may be able to trap TCZ within not only endosomes, but also lysosomes. On the basis of these lines of evidence, we conclude that PB-TCZ, once delivered to, and released in, lysosomes, might again be recovered from lysosomes for sorting and/or recycling endosomes by FcRn-mediated kiss-and-run and/or kiss-and-linger processes. This hypothesis also implies that the delivery of IgG to lysosomes does not necessarily reflect degradation, at least in cells expressing FcRn. Collectively our data provide new insights into the role of FcRn in the rescue of internalized IgG from lysosomal degradation.

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