Intracellular dynamics and fate of a humanized anti-interleukin-6 receptor monoclonal antibody,Tocilizumab (TCZ)

藤本, 景子

Intracellular dynamics and fate of a humanized anti-interleukin-6 receptor monoclonal antibody, Tocilizumab (TCZ) ヒト化抗インターロイキン-6 受容体モノクローナル抗体（トシリズマブ）の細胞内動態

Pharmaceutical Cell Biology　3PS09015K　Keiko Fujimoto

[INTRODUCTION]

Interleukine-6 (IL-6) is a pleiotropic cytokine that is involved in inflammation, acute phase responses, immune responses, and hematopoiesis (1). These biological functions of IL-6 are mediated by binding to the IL-6 receptor (IL-6R) system composed of two type-I membrane glycoproteins: a non-signaling and ligand-binding α-receptor IL-6R, which also exists as a soluble form (sIL-6R), and a signal transducer protein gp130. 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 (1, 2). Deregulated overproduction of IL-6, therefore, is associated with autoimmune and inflammatory diseases including rheumatoid arthritis, juvenile idiopathic arthritis, Crohn's disease, and Castleman's disease.

Tocilizumab (TCZ) is a humanized anti-human IL-6R monoclonal antibody of the IgG1 subclass that inhibits binding of IL-6 to both sIL-6R and mIL-6R, leading to blockade of many biological functions of IL-6. 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 (2, 3).

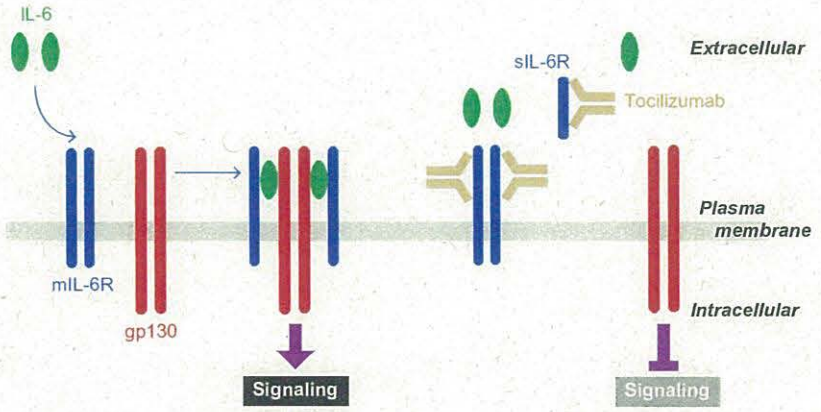


Figure 1. Tocilizumab blocks IL-6-mediated signaling pathway by inhibiting IL-6 binding to mIL-6R and sIL-6R.

Despite accumulating evidence for the therapeutic efficacy, the behavior and fate of TCZ at the cellular level remain largely unknown. To address this, I evaluated the endocytosis and intracellular trafficking of the TCZ antigen IL-6R in HeLa cells.

[RESULTS AND DISCUSSION]

1. Internalization and endocytic trafficking pathways of TCZ

I first examined the localization of human IL-6R in HeLa cells. For this, I generated a construct encoding fusion protein that fused GFP to the COOH-terminus of IL-6R (IL-6R-GFP) and which was transiently expressed in HeLa cells. IL-6R-GFP was distributed primarily both on the cell surface and in the *trans*-Golgi network-like reticular structures around the perinuclear region, and was also occasionally visible in endosomes/lysosomes-like small punctate structures scattered throughout the cytoplasm. I examined whether localization of IL-6R-GFP in the endocytic endosomes/lysosomes-like compartments is associated with the fate of TCZ after binding with the receptor. HeLa cells transiently expressing IL-6R-GFP were incubated with Pacific Blue (PB)-labeled TCZ (PB-TCZ) at 4 °C for 30 min to avoid nonselective internalization of the antibody by fluid phase endocytosis. Cells were immediately fixed or subsequently

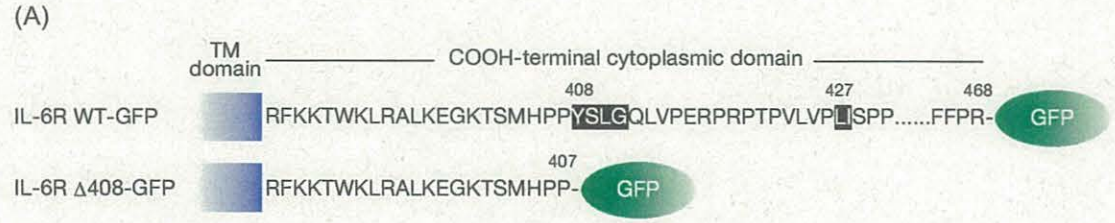
chased at 37 °C for up to 3 h and the internalization of, and the pathway taken by, PB-TCZ were analyzed by confocal laser microscopy. Both cell surface binding and internalization of PB-TCZ were visible only in cells expressing IL-6R-GFP, indicating the absence of expression of endogenous IL-6R in HeLa cells and the specificity of IL-6R-GFP expression-dependent internalization of PB-TCZ. After an internalization of 30 min, PB-TCZ was significantly reduced from the cell surface and was simultaneously visible in small punctate structures dispersed throughout the cytoplasm. These vesicles exhibited a tendency to accumulate around the perinuclear region with increasing chase periods and the rate of colocalization between IL-6R-GFP and PB-TCZ hardly altered during chase periods up to 3 h. Furthermore, treatment of cells with TCZ did not affect the half-life of IL-6R-GFP, suggesting that PB-TCZ is internalized by the constitutive endocytosis of IL-6R-GFP.

I further examined the compartments where the internalized PB-TCZ was localized. The results suggest that a 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.

2. 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 (4). However, it remains unclear whether gp130 is involved in the internalization of PB-TCZ mediated by IL-6R. To this end, I knocked down the expression of gp130 in HeLa cells by RNA interference. The depletion of gp130 did not significantly influence the expression level and stability of IL-6R-GFP. Moreover, knock down 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.

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 (Figure 2A). Then, to examine whether the cytoplasmic tail of IL-6R including both tyrosine- and dileucine-based motifs is required for receptor internalization and trafficking, I created a mutant (IL-6R Δ408-GFP) that deleted the COOH-terminal 60 amino acids including both motifs (Figure 2A). 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 (Figure 2B). Thus, these data suggest that the cytoplasmic domain including tyrosine- and/or dileucine-like motifs is essential for effective internalization of IL-6R.



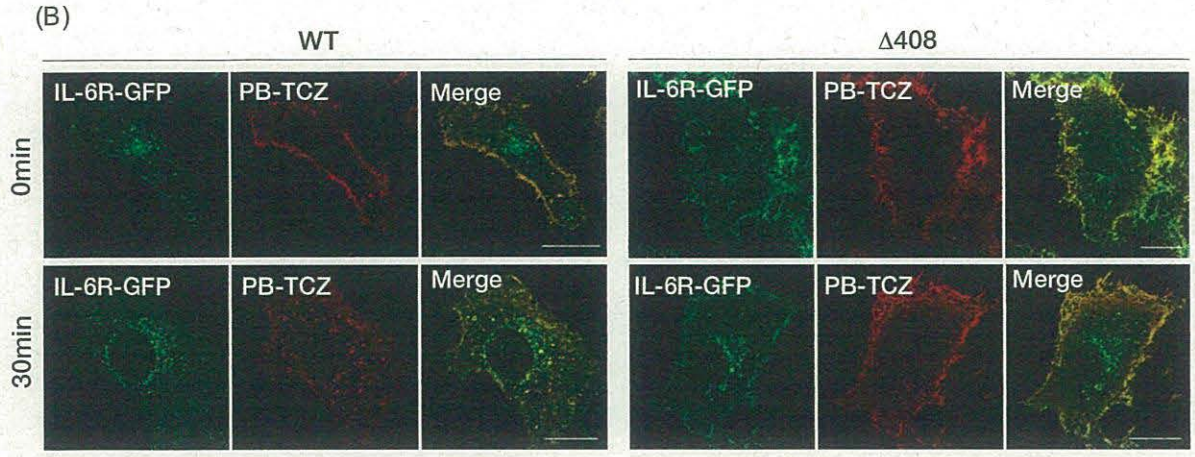


Figure 2. Analysis of endocytosis signal of IL-6R.
(A), Schematic representation of wild-type (WT) IL-6R-GFP and IL-6R Δ408-GFP. IL-6R Δ408-GFP lacking 60 amino acids from the COOH-terminus of the cytoplasmic domain consisting of 79 amino acids. Numbers correspond to amino acids. TM; transmembrane. Potential tyrosine and dileucine-like motifs are highlighted as white on a black background.
(B), HeLa cells expressing IL-6R WT-GFP or IL-6R Δ408-GFP were preincubated with 2 μg/ml PB-TCZ at 4 °C for 30 min and immediately fixed or subsequently allowed to internalize the antibody for 30 min at 37°C. Cells were then visualized by confocal microscopy. Right columns show the merged images for double staining of IL-6R WT-GFP or IL-6R Δ408-GFP (green) and PB-TCZ (red). Bars, 20 μm.

3. Internalized PB-TCZ is sorted from IL-6R-GFP and escapes lysosomal degradation by the expression of FcRn-DsRed

One of the clinical problems associated with therapeutic monoclonal antibodies targeting particular antigens including TCZ is antigen-mediated clearance of antibodies from plasma (5). Therefore, reducing the elimination of IgG antibodies is expected to lead to sustained and increased long-term clinical efficacy. The MHC class-I-related receptor, neonatal Fc receptor (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. I 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/Leup/Pep. However, in contrast to IL-6R-GFP, the very low degradation and the lack of sorting into endosomal intraluminal vesicles of FcRn-DsRed suggest that colocalization of FcRn-DsRed with LAMP-1 seems unlikely to involve constitutive degradation of the receptor. Moreover, the expression of FcRn-DsRed markedly shifted the localization of the PB-TCZ from the lumen to the limiting membrane in the enlarged endosomes/lysosomes caused by overexpression of a GTPase-deficient Rab5(Q79L) mutant, whereas it had no affect on the sorting of IL-6R-GFP into the lumen of the enlarged endosomes/lysosomes (Figure 3), suggesting the potentially useful role of FcRn in helping internalized PB-TCZ avoid lysosomal degradation. Rather, these results could be accounted for by continuous shuttling of FcRn-DsRed between endosomes and lysosomes via kiss-and-run and/or kiss-and-linger processes. Interestingly, my 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 transferrin receptor, which is a marker of the recycling endosome and exclusively colocalized with FcRn-DsRed, never shifted its localization to LAMP-1-positive compartments upon treatment of cells with E64d/Leup/Pep.

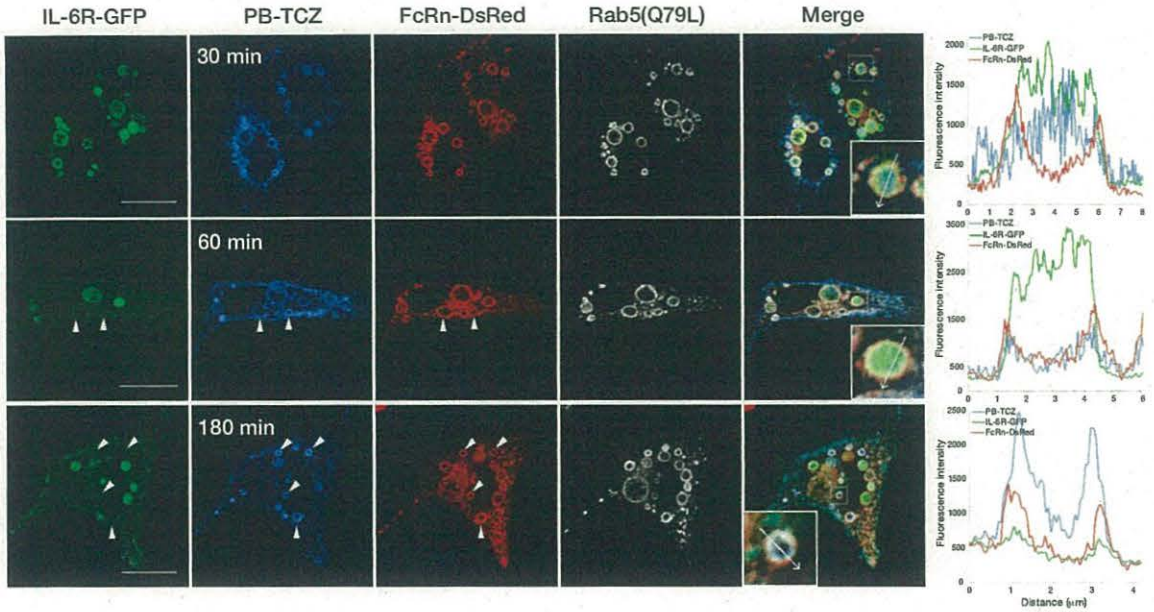


Figure 3. Effect of FcRn-DsRed expression on the escape of internalized PB-TCZ from lysosomal degradation. HeLa cells were transiently co-transfected with an expression plasmid encoding 3xFLAG-tagged Rab5(Q79L), IL-6R-GFP, and FcRn-DsRed. After 24 h, cells were incubated with 2 μg/ml of PB-TCZ at 4 °C for 30 min 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 Cy5-labeled secondary antibodies. Cells were visualized by confocal microscopy. Right columns show the merged images for triple staining of IL-6R-GFP (green), PB-TCZ (cyan), and FcRn-DsRed (red). 3xFLAG-Rab5Q79L 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 and the fluorescence intensity profiles are shown in histograms. Bars, 20 μm.

[CONCLUSION]

My 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. I 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, I 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.

[REFERENCES]

1. Ataie-Kachoie P, Pourgholami MH, and Morris DL (2013) *Cytokine Growth Factor Rev* **24:**163-73.
2. Tanaka T, Narazaki M, and Kishimoto T (2014) *Cold Spring Harb Perspect Biol* **6:**a016295.
3. Nishimoto N, Terao K, Mima T, et al., (2008) *Blood* **112:**3959-3964.
4. Graeve L, Korolenko TA, Hemmann U, et al., (1996) *FEBS Lett* **399:**131-134.
5. Amano J, Masuyama N, Hirota Y, Tanaka Y, et al., (2010) *Drug Metab Dispos* **38:**2339-2346.