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HYPOTHESIS

SPECIAL ISSUE: CELL BIOLOGY OF THE IMMUNE SYSTEM

Membrane trafficking as an active regulator of constitutively secreted cytokines

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ABSTRACT

Immune-cell activation by inflammatory stimuli triggers the transcription and translation of large amounts of cytokines. The transport of newly synthesized cytokines to the plasma membrane by vesicular trafficking can be rate-limiting for the production of these cytokines, and immune cells upregulate their exocytic machinery concomitantly with increased cytokine expression in order to cope with the increasing demand for trafficking. Whereas it is logical that trafficking is rate-limiting for regulated secretion where an intracellular pool of molecules is waiting to be released, the reason for this is not obvious for constitutively secreted cytokines, such as interleukin-6 (IL-6), interleukin-12 (IL-12) and tumor necrosis factor-α (TNF-α). These constitutively secreted cytokines are primarily regulated at the transcriptional and/or translational level but mounting evidence presented here shows that cells might also increase or decrease the rate of post-Golgi cytokine trafficking to modulate their production. Therefore, in this Hypothesis, we ask the question: why is there a need to limit the trafficking of constitutively secreted cytokines? We propose a model where cells monitor and adjust their production rate of cytokines by sensing the intracellular level of cytokines while they are in transit to the plasma membrane. This self-regulation of cytokine production could prevent an overshooting response of acute-phase cytokines, such as IL-6, IL-12 and TNF-α, upon acute infection.

KEY WORDS: Cytokine secretion, Exocytosis, Interleukin 6, Membrane trafficking

Introduction

Cells release hormones, cytokines, growth factors and neurotransmitters through either regulated or constitutive secretion (Fig. 1; see also Stanley and Lacy, 2010). In regulated secretion, an external stimulus triggers release of molecules from intracellular storage granules, such as insulin-containing pancreatic β-granules or neurotransmitter-containing synaptic vesicles. The final trafficking step of regulated secretion, the fusion of the storage granules with the plasma membrane, is well-understood to be the bottleneck for release (Stanley and Lacy, 2010). In constitutive secretion, there is no long-term intracellular storage of the secreted molecules. Instead, release is primarily regulated at the transcriptional and/or translational level, after a stimulus triggers the biosynthesis of the molecules destined for secretion. These newly formed molecules are then continuously secreted, thus without the need for a second stimulus, by constitutive trafficking through the endoplasmic reticulum (ER), the Golgi complex and the endosomal network to the plasma membrane (Stanley and Lacy, 2010). Nevertheless, evidence shows that trafficking can be rate-limiting for the release of constitutively secreted cytokines from immune cells. In this Hypothesis, we discuss potential mechanisms of how and why this is the case. We propose that newly synthesized interleukin 6 (IL-6) and other acute-phase cytokines encounter their receptors in recycling endosomes while in transit to the plasma membrane, whereas extrinsic foreign IL-6 is endocytosed and signals from the same intracellular compartments. This could enable the cell to monitor and compare its rate of IL-6 secretion and the outside concentration of IL-6, and adapt the transcription of cytokines accordingly. Such negative feedback signaling of newly synthesized cytokines might act to limit the immune response during acute infections in order to prevent sepsis.

Trafficking is rate-limiting for constitutive secretion of cytokines

The best-characterized pathways of constitutive secretion are for the acute-phase inflammatory cytokines interleukins 6 and 12 (IL-6 and IL-12, respectively) and tumor necrosis factor alpha (TNF-α) in macrophages and dendritic cells. For all three cytokines, immune-cell activation by inflammatory stimuli, such as the pathogenic stimulus lipopolysaccharide (LPS), results in a rapid upregulation of transcription and translation (Chiaruttini et al., 2016; Raabe et al., 1998; Tanaka et al., 2016). Following co-translational import in the ER, newly formed IL-6 is trafficked through the Golgi complex to a pool of recycling endosomes that contain the vesicle-associated membrane protein 3 (VAMP3) – a soluble N-ethyl maleimide-sensitive factor (NSF) attachment protein (SNAP) receptor (SNARE) protein – before it is finally secreted at the plasma membrane (Fig. 2; see also Manderson et al., 2007; Murray et al., 2005b; Verboogen et al., 2018a). TNF-α is secreted through the same pathway (Manderson et al., 2007; Murray et al., 2005a,b; Shurety et al., 2000) but is synthesized as a precursor that is membrane-anchored through a transmembrane helix that is cleaved off by metalloproteases at the plasma membrane (Moss et al., 1997). IL-12 is assembled in the ER as a heterodimer of its p35 and p40 subunits, and is trafficked to the plasma membrane via the Golgi complex and late endosomal and/or lysosomal compartments that contain the SNARE protein VAMP7 (Chiaruttini et al., 2016).

Although primarily being regulated at the biosynthesis level (Chiaruttini et al., 2016; Raabe et al., 1998; Tanaka et al., 2016), mounting evidence suggests that post-Golgi trafficking can be rate-limiting for the secretion of IL-6, IL-12 and TNF-α. Thus, in order to cope with the increasing demand for cytokine production in response to pathogenic stimuli, immune cells upregulate their trafficking machinery at both the transcriptional and translational level, as well as at the post-translational level. As a consequence, activated dendritic cells increase the delivery rate of IL-6-containing

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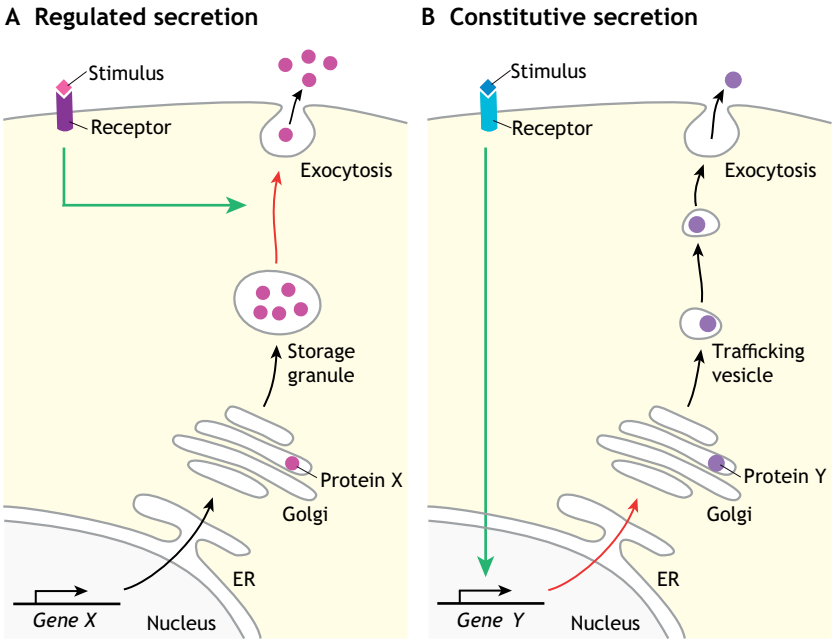

Fig. 1. Regulated versus constitutive secretion. (A) Regulated secretion. After its transcription and translation, a protein accumulates in storage granules. Upon arrival of a stimulus, these storage granules fuse with the plasma membrane resulting in exocytosis. Exocytosis is, thus, rate-limiting for secretion (red arrow). (B) Constitutive secretion. A stimulus upregulates transcription and/or translation of a factor that, after its *de novo* synthesis and trafficking to the plasma membrane, is continuously secreted. Here, transcription and/or translation are rate-limiting for secretion (red arrow).

vesicles at the plasma membrane about two-fold, as shown by quantitative imaging (Verboogen et al., 2018b). At the post-translational level, the SNARE protein syntaxin-3, which is involved in IL-6 release, moves from intracellular compartments to the plasma membrane in LPS-activated macrophages (Collins et al., 2014). Moreover, Förster resonance energy transfer (FRET) coupled to fluorescence lifetime imaging microscopy (FLIM) revealed that LPS activation of dendritic cells results in increased formation of the complex between the SNARE protein VAMP3 – which is also involved in IL-6 release – and syntaxin-4 at the plasma membrane (Verboogen et al., 2017). At transcriptional and translational levels, the expression of SNAREs and many other proteins involved in trafficking of cytokines is upregulated in activated macrophages and dendritic cells (Chiaruttini et al., 2016; Collins et al., 2014; Manderson et al., 2007; Mori et al., 2011; Murray et al., 2005a; Pagan et al., 2003). Although not directly proven, this is likely to result in an increased rate of exocytic trafficking that is required to secrete all the newly synthesized cytokines.

Despite this upregulation of trafficking, evidence indicates that there is no or only little surplus capacity of trafficking, and the transport of newly synthesized cytokines to the plasma membrane can still be an apparent bottleneck for cytokine production in activated immune cells. First, RNA interference (RNAi) to partially knock down golgin p230 (officially known as Golga4), a long coiled-coil protein residing at the trans-Golgi network and required for export of TNF-α from the Golgi, leads to reduced TNF-α secretion (Lieu et al., 2008). Similarly, TNF-α release from recycling endosomes at the plasma membrane is sensitive to the levels of proteins orchestrating this process, as shown for the SNARE proteins VAMP3, syntaxin-4, syntaxin-6 and Vti1b, and the small GTPase Rab37 (Mori et al., 2011; Murray et al., 2005a,b; Pagan et al., 2003). Interestingly, not only does their downregulation by RNAi or the overexpression of dominant-negative mutants lead to reduced secretion of TNF-α – but their overexpression has the opposite effect (Mori et al., 2011; Murray et al., 2005a,b; Pagan et al., 2003), indicating that the levels of these trafficking proteins can still be rate-limiting for secretion. Second, post-Golgi trafficking of IL-6 is mediated by the SNARE proteins VAMP3, syntaxin-3, syntaxin-6 and Vti1b, and their overexpression increases IL-6 production, whereas its knockdown following RNAi results in a reduction of IL-6 release (Collins et al., 2014; Manderson et al., 2007; Verboogen et al., 2017). Finally, for IL-12, its secretion from late endosomal and/or lysosomal compartments is sensitive to the levels of VAMP7, because knockout or knockdown of VAMP7 leads to reduced IL-12 secretion (Chiaruttini et al., 2016). These data show that, although the exocytic pathway is upregulated upon immune-cell activation, trafficking can still be rate-limiting for the release of IL-6, IL-12 and TNF-α, even though these cytokines are constitutively secreted and are primarily regulated at the transcriptional and/or translational level (Chiaruttini et al., 2016; Raabe et al., 1998; Tanaka et al., 2016). These findings point to a conundrum: why is there a need for this apparent bottleneck?

Mechanisms underlying rate-limiting trafficking of constitutively secreted cytokines

There are two possible mechanisms that explain how trafficking can be rate-limiting for constitutive cytokine secretion. First, it is possible that the trafficking machinery cannot cope with high levels of protein transcription and/or translation. Indeed, after LPS stimulation of macrophages and dendritic cells, newly synthesized intracellular IL-6, IL-12 and TNF-α become visible at the perinuclear Golgi area where they reach maximum concentrations ∼2–4 h after stimulation (Chiaruttini et al., 2016; Manderson et al., 2007; Murray et al., 2005b; Shurety et al., 2000; Verboogen et al., 2018b), suggesting a possible accumulation of these cytokines in the Golgi complex at early time points after stimulation. It is also possible that newly synthesized cytokines are degraded in lysosomes and/or through the ER-associated degradation (ERAD). Support that post-Golgi trafficking might be rate-limiting for secretion of TNF-α (and other highly produced secretory proteins) comes from experiments with the retention using selective hooks (RUSH) system (Boncompain et al., 2012). The RUSH system is based on the reversible retention of recombinantly expressed GFP-tagged TNF-α in the ER through a streptavidin-binding protein that anchors to a stable ER ‘hook protein’ that is tagged with streptavidin. The addition of excess biotin to the cells results in

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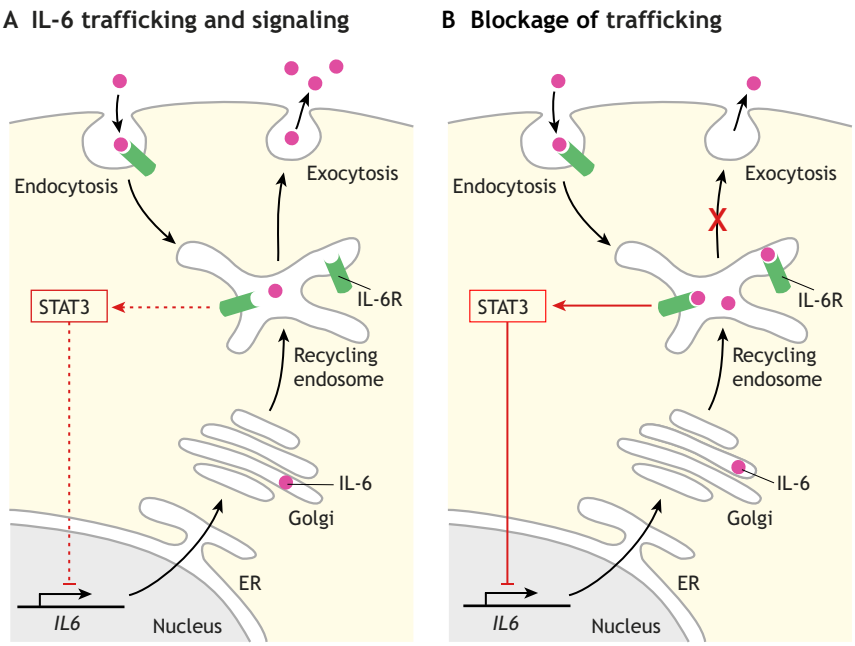

Fig. 2. Trafficking regulates the production of the constitutively secreted cytokine IL-6. (A) Proposed mechanism for the self-limiting production of IL-6. Both endocytosed and newly synthesized IL-6 molecules bind to the IL-6 receptor that resides in recycling endosomes. This, in turn, leads to signaling and activation of the transcription factor STAT3, its translocation to the nucleus and the downregulation of *IL6* transcription. Through this mechanism, immune cells might monitor their rate of IL-6 production, compare it to the outside concentration of IL-6 to adjust their rate of IL-6 synthesis and prevent an overshooting cytokine response. (B) Potential mechanism explaining how trafficking can be a bottleneck for IL-6 production. Upon a perturbation of trafficking (e.g. by using RNAi to knock down an exocytic SNARE protein) IL-6 accumulates in recycling endosomes where it binds to the IL-6 receptor, resulting in increased activation of STAT3. This, in turn, leads to stronger inhibition of *IL6* transcription, thus, eventually shutting down IL-6 synthesis and, therefore, secretion.

the rapid release of the GFP-tagged TNF-α from this hook protein and triggers the synchronous transport of TNF-α molecules via the secretory pathway, which can be visualized by microscopy (Boncompain et al., 2012). After biotin addition, TNF-α (and other secretory cargoes) rapidly leaves the ER and accumulates at the Golgi region where it reaches peak levels ∼10 min after biotin addition. Although the first TNF-α already reaches the plasma membrane within minutes after ER release, it takes a further 20–30 min until all TNF-α has left the Golgi complex and reached the plasma membrane (Boncompain et al., 2012). Thus, trafficking from the Golgi to the plasma membrane is slower than trafficking from the ER to the Golgi. Accordingly, when the rate of cytokine synthesis is faster than post-Golgi trafficking, cytokines accumulate in the Golgi region, just as observed for IL-6, IL-12 and TNF-α 2–4 h after immune-cell activation (Chiaruttini et al., 2016; Manderson et al., 2007; Murray et al., 2005b; Shurety et al., 2000; Verboogen et al., 2018b). However, at later timepoints, the immune cells might have sufficiently upregulated their trafficking machinery because accumulation in the Golgi complex is no longer observed (Chiaruttini et al., 2016; Manderson et al., 2007; Murray et al., 2005b; Shurety et al., 2000; Verboogen et al., 2018b). Therefore, the accumulation of cytokines at the Golgi cannot explain why overexpression of trafficked proteins results in increased cytokine secretion at these later timepoints of immune-cell activation (Manderson et al., 2007; Mori et al., 2011; Murray et al., 2005a, b; Pagan et al., 2003). An open question is whether this transient accumulation of cytokines at the Golgi is just a non-functional consequence because the trafficking pathway is unable to cope with the high rate of cytokine synthesis or whether it has any functional role in cytokine signaling.

The second explanation of how trafficking can be rate-limiting for protein secretion is that intracellular trafficking might somehow affect the biosynthesis of cytokines, so that cells sense their rate of cytokine production; this might provide a negative feedback mechanism for their biosynthesis (Fig. 2). At least for IL-6, current data provide evidence for this second mechanism. First, quantitative imaging performed by our group has shown that single secretory vesicles contain only a few IL-6 molecules in LPS-activated dendritic cells (Verboogen et al., 2018b); moreover, given the thousands of insulin molecules observed in pancreatic β-vesicles (Michael et al., 2007), it is unlikely that these few molecules reflect capacity limits of the secretory vesicles. Second, macrophages and dendritic cells contain the receptors for IL-6, IL-12 and TNF-α, which makes them prone to so-called autocrine signaling, whereby cells respond to the same cytokine they produce (Fukao et al., 2001; Parameswaran and Patial, 2010; Verboogen et al., 2018a). For the IL-6 receptor, we have recently shown that more than half of its signaling occurs from within recycling endosomes because only ∼40% of IL-6 receptors reside at the plasma membrane, with the remainder being located within endosomes and the Golgi complex (Verboogen et al., 2018a). Furthermore, blockage of IL-6 uptake either by using the dynamin inhibitor dynasore or by overexpression of a dominant-negative mutant of the endocytic cargo adapter epsin 2, significantly inhibited activation of its downstream factor signal transducer and activator of transcription 3 (STAT3) by ∼60% (Verboogen et al., 2018a). To enable endosomal signaling, IL-6 has to be endocytosed prior to activating the IL-6 receptor from within the lumen of endosomal compartments. Thus, these recycling endosomes have to contain the IL-6 receptor (Verboogen et al., 2018a) but – similar to the recycling endosomes encountered by newly synthesized IL-6 during its trafficking from the Golgi to the plasma membrane – they also contain VAMP3 (Manderson et al., 2007; Murray et al., 2005b; Verboogen et al., 2018a). In fact, we have shown that both newly synthesized IL-6 in transit to the plasma membrane and endocytosed exogenous IL-6 signal from within recycling endosomes of the same signature, and are able to activate STAT3 downstream of the IL-6 receptor (Verboogen et al., 2018a). Although STAT3 has pro-inflammatory effects, it inhibits activation of dendritic cells and its signaling results in a reduced transcription of the *IL6* gene (Fig. 2) (Bode et al., 2012; Melillo et al., 2010). This negative feedback inhibition of IL-6 synthesis is present for the first hours after LPS stimulation (Verboogen et al., 2018a). At sustained periods of LPS exposure, the increased expression of suppressor of cytokine signaling 3 (SOCS3) switches off IL-6 signaling (Bode et al., 2012; Croker et al., 2012), thereby inhibiting this negative feedback loop and allowing for an increase in IL-6 transcription (Bode et al., 2012; Verboogen et al., 2018a). SOCS3 is a well-known STAT3-inducible negative regulator of STAT3 (Bode et al.,

2012; Croker et al., 2012), and mice with impaired SOCS3 signaling develop IL-6-related inflammatory diseases (Croker et al., 2004).

Taken together, these data, thus, show how endosomal signaling (Irannejad et al., 2015; Lobingier and von Zastrow, 2019; Murphy et al., 2009; Platta and Stenmark, 2011) allows dendritic cells to sense both their production rate of IL-6 and the extracellular concentration of IL-6. This mechanism makes it possible for cells to self-limit their production of IL-6 during the first hours after encountering a pathogenic stimulus (Verboogen et al., 2018a). Such a regulation of IL-6 production explains how trafficking can be an apparent bottleneck for the production of the constitutively expressed cytokine IL-6. If the rate of IL-6 release is reduced (for instance, due to knockdown of a SNARE), IL-6 spends more time within recycling endosomes, enabling prolonged activation of STAT3 and reduced transcription of *IL6*. By contrast, when the trafficking rate is increased (for instance, due to overexpression of a SNARE), IL-6 spends less time in recycling endosomes, leading to reduced activation of STAT3 and increased *IL6* expression. This model, thus, can explain why, in cell culture, increasing or reducing IL-6 trafficking by overexpression or knockdown of the SNARE proteins VAMP3, syntaxin-3, syntaxin-6 and Vti1b results in increased or reduced production of IL-6, respectively (Collins et al., 2014; Manderson et al., 2007; Verboogen et al., 2017). Importantly, there is *in vivo* evidence supporting such a negative feedback for IL-6 production. Mice with conditional knockout of the IL-6 receptor in liver cells show increased IL-6 secretion and higher blood levels of IL-6 (Klein et al., 2005; Wunderlich et al., 2010). In addition, increased serum levels of IL-6 have been observed upon antibody therapy against the IL-6 receptor in patients with rheumatoid arthritis or other auto-immune diseases (Nishimoto et al., 2008; Shimamoto et al., 2013); although, in both cases, these findings might also be explained by reduced removal of IL-6 from the circulation by the liver. The negative feedback loop of IL-6 signaling potentially also limits the production of other cytokines, including TNF-α and IL-12 (Bode et al., 2012; Xing et al., 1998). Moreover, unpublished observations obtained in our laboratory show that production of IL-12 might be self-limited in a manner similar to that described for IL-6 here (our unpublished observations). We thus propose that immune cells monitor the release rate and extracellular concentration of cytokines, and use this information to adjust their *de novo* synthesis, thereby enabling a self-limitation of their cytokine production. This, however, raises the question why cells would need to transiently self-limit their production of cytokines.

Why is there a need to limit the trafficking rate for constitutive secretion?

A balanced production of acute-phase cytokines is important for the induction and propagation of the immune response to acute infections, as well as for the control of the resolution phase. The acute systemic infection triggered by the presence of viable bacteria, fungi or microbial products in the blood can elicit sepsis and septic shock (Chaudhry et al., 2013; Hotchkiss et al., 2016). During sepsis, vascular endothelial cells and immune cells produce high amounts of acute-phase cytokines – including IL-6, IL-12 and TNF-α – in a process called the cytokine storm (Chaudhry et al., 2013; Hotchkiss et al., 2016; Paardekooper et al., 2017). The cytokine storm then triggers the inflammatory immune responses that are required to resolve infection, including activation of B and T lymphocytes, modulation of haematopoiesis, release of hepatic acute-phase proteins – such as C-reactive protein – and induction of fever (Chaudhry et al., 2013; Hotchkiss et al., 2016). However, an excessive inflammatory response can lead to tissue damage and organ failure because of insufficient blood flow to one or more organs caused by low blood pressure, to lactic acidosis or to reduced urine production (Chaudhry et al., 2013; Hotchkiss et al., 2016). The most-severe form of sepsis is septic shock – the main cause of death in intensive care units (Schulte et al., 2013) – worldwide leading to ∼5.3 million deaths annually (Hotchkiss et al., 2016).

Low serum levels of IL-6 are associated with a better prognosis for sepsis as IL-6 levels in patients with septic shock are higher than in patients without shock (Damas et al., 1992; Hack et al., 1989; Martí Arjona and Moreno Camacho, 2017; Pathan et al., 2004; Spittler et al., 2000). Indeed, plasma IL-6 shows the best correlation with mortality rate in patients with sepsis (Hack et al., 1989; Kumar et al., 2009; Martí Arjona and Moreno Camacho, 2017). This is also true for animal models. Compared to wild-type mice, LPS administration in IL-6-knockout mice led to reduced inflammatory responses and protection against mortality and organ failure (Cuzzocrea et al., 1999; Remick et al., 2005). Therefore, it might be beneficial to limit the levels of acute-phase cytokines upon acute infection, particularly those of IL-6. The production of appropriate levels of IL-6 and other acute-phase cytokines is very important for triggering an adequate immune response while preventing septic shock; however, their expression is strictly controlled at different levels, including access to their promoter region, regulation of transcription and posttranscriptional modifications (Tanaka et al., 2016). We now propose that these mechanisms of regulation of IL-6 production are, in turn, controlled by negative feedback signaling from the pool of IL-6 residing within recycling endosomes, which consists of a combination of newly synthesized IL-6 in transit to the plasma membrane and endocytosed IL-6 from the outside of the cell. This negative feedback control could prevent an overshooting response that could have devastating consequences upon acute systemic infection. As the rate of exocytosis determines the time IL-6 spends within recycling endosomes, the trafficking rate of IL-6 might directly correlate with the extent of this negative feedback signaling, which can explain how trafficking is an apparent bottleneck for cytokine secretion. However, the existence of this self-limiting mechanism of IL-6 production and its potential contribution in preventing septic shock still needs to be formally proven, for instance by using LPS injections in mice with conditional knockout for the IL-6 receptor in those cells that produce IL-6.

Conclusions

In this Hypothesis, we propose a mechanism of how trafficking can be both an apparent bottleneck and a regulator of the production of the constitutively secreted cytokines IL-6, IL-12 and TNF-α. We suggest that newly synthesized cytokines signal from within recycling endosomes while they are in transit to the plasma membrane. Extracellular IL-6 is also endocytosed by the cell and signals from within the same recycling endosomes. The signals from newly synthesized cytokines are, thereby, likely to be combined with the signals from extracellular cytokines (autocrine signaling), allowing the cell to adapt its IL-6 production rate depending on the extracellular concentration of IL-6. As the exocytosis rate directly influences the level of cytokines residing within intracellular compartments, our model explains why trafficking can be rate-limiting for the release of IL-6 and other cytokines, even though they are primarily regulated at the transcriptional and/or translational level. The self-limiting production of IL-6 is transient and is switched off at longer activation times through IL-6-induced expression of SOCS3. With regard to a possible reason for why cells would transiently self-limit their production of IL-6, IL-12 and TNF-α, we suggest that this prevents an overshooting response of these acute-phase cytokines

upon acute infection, which would lower the risk of sepsis and septic shock.

The monitoring of production rates might not only be limited to the examples we discussed here, but could also occur for other constitutively secreted molecules. This seems possible because many (if not all) cells that produce a particular cytokine, hormone or growth factor also express the receptor for this molecule, which is a prerequisite for self-signaling. Moreover, signaling within intracellular compartments has now been observed for many different molecules, including epidermal growth factor (EGF), Wnt signals and ligands of Toll-like receptors (Irannejad et al., 2015; Lobingier and von Zastrow, 2019; Murphy et al., 2009; Platta and Stenmark, 2011), raising the possibility that their production rates are monitored in a way similar to that we propose here. Intracellular signaling of newly synthesized cytokines might not be restricted to cytokines that are released by canonical ER–Golgi vesicular trafficking but might also occur for non-conventionally released cytokines, such as interleukin-1 beta (IL-1β). IL-1β is a cytosolic protein that is released from immune cells through an incompletely understood pathway (Sitia and Rubartelli, 2018). Moreover, cells that produce IL-1β also express both activating and inhibitory receptors for IL-1β, including a cytosolic isoform of the inhibitory type 2 IL-1β receptor (Peters et al., 2013), which might enable signaling of IL-1β from the cytosol prior to release. Another open, but intriguing, possibility is that the receptors that mediate self-signaling are also responsible for the differential intracellular sorting of cargo molecules to different compartments of the trafficking pathway as, for instance, observed for IL-12 that is sorted to late endosomes (Chiaruttini et al., 2016), and IL-6 and TNF-α that accumulate in different sub-compartments of recycling endosomes (Manderson et al., 2007; Murray et al., 2005b). Future studies investigating the trafficking of constitutively secreted molecules, thus, should not only measure the final secretion rate but, also, address their transcription and translation levels as well as the intracellular fate of non-secreted molecules.

Taken together, we, therefore, argue that constitutive secretion is likely to be not just the mere flux of material from the ER and Golgi to the plasma membrane but that this trafficking route is used by the cell to monitor protein synthesis rates and to compare this to uptake from outside of the cell, in order to regulate and adapt protein production.

Competing interests

The authors declare no competing or financial interests.

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