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Potential mechanisms of target-independent uptake and toxicity of antibody-drug conjugates

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ABSTRACT

Antibody-drug conjugates (ADCs) are a promising therapeutic modality for oncology indications. The concept of an ADC platform is to increase the therapeutic index (TI) of chemotherapeutics through more selective delivery of cytotoxic agents to tumor cells while limiting exposure to healthy normal cells. Despite the use of antibodies targeting antigens abundantly and/or exclusively expressed on cancer cells (i.e., target cells), dose limiting toxicities (DLTs) in normal cells/tissues are frequently reported even at suboptimal therapeutic doses. Although advancement of ADC technology has helped to optimize all three key components (i.e., mAb, linker, and payload), DLTs remain a key challenge for ADC development. Mechanisms of ADC toxicity in normal cells/tissues are not clearly understood, but the majority of DLTs are considered to be target-independent. In addition to linker-drug instability contributing to the premature release of cytotoxic drug (payload) in circulation, uptake/trafficking of intact ADCs by both receptor-dependent (FcγRs, FcRn and C-type lectin receptors), and-independent (non-specific endocytosis) mechanisms may contribute to off-target toxicity in normal cells. In this article, we review potential mechanisms of target-independent ADC uptake and toxicity in normal cells, as well as discuss components of ADCs which may influence these mechanisms. This information will provide a deeper understanding of the underlying mechanisms of ADC off-target toxicity and prove helpful toward improving the overall TI of the next generation of ADCs.

Contents

Abbreviations: ADC, Antibody Drug Conjugate; CHO, Chinese Hamster Ovary; CLRs, C-type Lectin Receptors; CME, Clathrin-Mediated Endocytosis; DAR, Drug-to-Antibody Ratio; DM1, $N^{2'}$-Deacetyl-$N^{2'}$-(3-mercapto-1-oxopropyl)maytansin; DM4, $N^{2'}$-Deacetyl-$N^{2'}$-(4-mercapto-4-methyl-1-oxopentyl)maytansine; HEK, Human Embryonic Kidney; HER2, Human Epidermal Growth Factor Receptor 2; LSEC, Liver Sinusoidal Endothelial Cells; MMAE, Monomethyl Auristatin E; MMAF, Monomethyl Auristatin F; PBD, Pyrrolobenzodiazepines; PEG, Polyethylene Glycol; VOD, Veno Occlusive Disease; MC, Maleimidocaproyl; MCC, Maleimidomethyl Cyclohexane-1-carboxylate; TI, Therapeutic Index.

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1. Introduction

Antibody-drug conjugates (ADCs) have been pursued for various oncology indications with the hope of increasing efficacy and decreasing the toxicity of small molecule cytotoxic agents by targeted delivery of the payload specifically to tumor cells. To date, four ADCs have been approved by the US Food and Drug Administration (listed in Table 1) (Birrer, Moore, Betella, & Bates, 2019). Mylotarg®, the first ADC to receive marketing approval from the US FDA in 2000, was withdrawn from the market in 2010 based on additional confirmatory trials, which failed to prove clinical benefit and also demonstrated serious treatment-related safety concerns in patients (FDA, 2010). However, Mylotarg® was re-approved in 2017 for newly diagnosed CD33-positive AML (acute myeloid leukemia), with lower recommended dose levels and an altered dosing regimen featuring dose fractionation (Baron & Wang, 2018). The field of ADCs is still expanding with a significant increase in investigational new drug (IND) application submissions to the FDA over recent years. Currently, ≥60 different ADCs are in different stages of clinical development, being evaluated for hematological malignancies and solid tumor indications (Lambert & Morris, 2017; Polakis, 2016) There is also desire to expand ADC technology beyond the current scope of payloads to non-cytotoxic mechanisms of action, and to non-oncology therapeutic areas such as to treat bacterial infections (Lehar et al., 2015), chronic inflammatory conditions (Kern et al., 2016; Wang et al., 2015) and metabolic diseases (Svendsen et al., 2017).

Despite the use of antibodies against tumor-specific and/or tumor-overexpressed antigens and existence of multiple examples of clinically validated ADCs providing benefit to oncology patients, dose limiting toxicities (DLTs) at suboptimal therapeutic doses remain a major challenge for the clinical development of ADCs (de Goeij & Lambert, 2016). DLTs resulting in relatively narrow therapeutic indices (TIs) are also the major cause limiting dose escalation of ADCs to achieve maximum efficacy (Drake, 2018). Details of preclinical toxicities and clinical DLTs of the 4 ADCs approved for marketing by the US FDA are listed in Table 1. Based on the data described in Table 1 and other published literature, the reported ADC toxicities in normal cells/tissues are primarily driven by the payload (Birrer et al., 2019; Donaghy, 2016; Drake & Rabuka, 2017). Details of different classes of payloads used in ADCs and commonly reported primary toxicities in clinical studies are summarized in Table 2.

Even though ADC toxicity is thought to be primarily derived from the payload, the mechanism(s) of ADC uptake for delivery of cytotoxic payload in non-targeted normal cells are not clearly understood. This uptake can occur through variety of mechanisms and considerable efforts are ongoing to understand these mechanisms and to optimize all three key components (i.e., monoclonal antibody, linker and drug) of ADCs. By most estimates, only modest accumulation of ADCs occurs at the target (tumor) site (~0.1% of administered dose per gram of tumor) in humans (Casi & Neri, 2015). Therefore, the major fraction of ADCs remain in circulation or is distributed to normal tissues and may be subject to uptake and catabolism, resulting in toxicity in normal cells. Both target-dependent and-independent uptake mechanisms may potentially contribute to ADC uptake and toxicity in normal cells. Low expression of ADC target antigens in normal tissues may result in target-dependent uptake and subsequent toxicity of ADCs. For example, dose-limiting GI toxicity (hemorrhagic gastritis) of the BR96-doxorubicin (BMS-182248-01) ADC is associated with expression of the Lewis-Y target antigen on normal gastric mucosal cells (Tolcher, 2016). Similarly, skin toxicity (skin rash) from Glembatumumab

Table 1
List of preclinical toxicities and clinical DLTs or SAEs of 4 ADCs approved for marketing by US FDA.

	Gemtuzumab ozogamicin[1] (Mylotarg®)	Brentuximab vedotin[2] (SGN-35)	Trastuzumab emtansine[3] (T-DM1, Kadcyla®)	Inotuzumab ozogamicin[4] (Besponsa®)
Preclinical safety	ADC toxicity profile Monkey and Rat • Bone marrow hypocellularity, ↓ RBC mass and platelets • Renal tubular degeneration/ regeneration • Liver atrophy, ↑ liver enzymes (ALT, AST) Monkey only • Liver sinusoidal dilation • Lymphoid depletion Rat only • Liver karyocytomegaly and oval cell hyperplasia • Testicular degeneration Payload toxicity profile* • Karyocytomegaly in liver and kidney • Lymphoid depletion • Bone marrow hypocellularity • Ulceration of stomach	Monkey and Rat • Bone marrow suppression with neutropenia and ↓ RBC mass • Lymphoid depletion Rat only • ↑ liver enzymes & hepatic focal necrosis • Testicular degeneration • Similar toxicity as seen with ADC	Monkey and Rat • ↑ liver enzymes • Thrombocytopenia • ↑ mitoses in epithelial and phagocytic cells Rat only • Hepatocellular degeneration/necrosis Monkey only • Axonal degeneration (sciatic nerve, spinal cord) • Similar toxicity as seen with ADC	Monkey and Rat • ↑ liver enzymes • Liver sinusoidal dilation, • Hepatocellular hypertrophy • Monkey only • Lymphoid depletion • Ovary atrophy Rat only • Testicular degeneration • Liver adenoma, hepatocellular hyperplasia, karyocytomegaly • Progressive nephropathy • Male mammary gland atrophy • Thrombocytopenia • ↑ liver enzymes (ALT, AST)
Clinical DLT/SAE	• Thrombocytopenia, neutropenia • Liver - increased VOD • Infusion-related AEs	• Peripheral neuropathy • Neutropenia, thrombocytopenia, anemia	• Thrombocytopenia, neutropenia • ↑ liver enzymes • Peripheral neuropathy	• Liver - increased VOD • ↑ Risk of post HSCT non-relapse mortality • Thrombocytopenia, neutropenia

↑, Increased; ↓, Decreased; DLT, Dose limiting toxicity; SAE, Serious adverse events; VOD, Veno-occlusive disease; HSCT, Hematopoietic stem cell transplant; ALT, Alanine transaminase; AST, Aspartate aminotransferase

[1] Anti-CD33 antibody conjugated to calicheamicin via a cleavable hydrazine linker (approved in 2000, Pfizer/Wyeth-Ayerst Laboratories)
[2] Anti-CD30 antibody conjugated to MMAE with a protease cleavable valine-citrulline linker (approved in 2011, Seattle Genetics)
[3] Anti-HER2 antibody conjugated to DM1 via a non-cleavable SMCC linker (approved in 2013, Genentech)
[4] Anti-CD22 antibody conjugated to calicheamicin via an acid-labile butanoic acid linker (approved in 2017, Pfizer)
* Based on results of toxicology studies with payload alone

Table 2
List of different classes of payload used in ADCs and commonly reported clinical toxicity or adverse events

	Tubulin polymerization inhibitors				DNA damaging agents				Topoisomerase inhibitors
	DM1	DM4	MMAF	MMAE	Calicheamicin	PBD	Camptothecin analogues	Duocarmycin	SN-38
Hematotoxicity									
Neutropenia	+	+	−	+	+	+	+	+	+
Thrombocytopenia	+	−	+	+	+	+	−	+	−
Anemia	+	+	−	+	−	+	+	−	−
Neurotoxicity									
Peripheral neuropathy	+	+	−	+	−	−	−	−	−
Ocular toxicity									
Blurry vision, dry eye, corneal deposits and photosensitivity	−	+	+	−	−	−	−	−	−
Liver toxicity									
Increased ALT/AST	+	−	+	−	+	−	−	−	−
Veno-occlusive disease (VOD)	−	−	−	−	+	−	−	−	−
Skin toxicity									
Photosensitivity, dry skin	−	−	−	−	−	+	−	−	−
Serosal effusion									
Pleural	−	−	−	−	−	+	−	+	−
Pericardial	−	−	−	−	−	+	−	+	−
Peripheral edema	−	−	−	−	−	+	−	+	−
Ascites	−	−	−	−	−	+	−	−	−

References: (Nagayama, Ellisen, Chabner, & Bardia, 2017; Robak & Robak, 2016; Yan, Yu, Zhang, Liu, & Li, 2017; Masters et al., 2018; Eaton et al., 2015; Oak & Bartlett, 2016; Yardley et al., 2015; Advani et al., 2010; Lambert et al., 2019; Rudin et al., 2017; Stein et al., 2017; Bardia et al., 2017; Dotan et al., 2017; Owonikoko et al., 2016; Aftimos CvH et al., 2016; Starodub et al., 2014)

vedotin and Bivatuzumab mertansine is considered to be mediated by low expression of target antigens gpNMB (glycoprotein non-metastatic melanoma protein B) and CD44v6 respectively in healthy keratinocytes (Maric, Rose, Annis, & Siegel, 2013; Tijink et al., 2006; Yardley et al., 2015). Despite such examples, the majority of DLTs of ADCs under clinical development are considered to be mediated by target-independent uptake into normal cells (de Goeij & Lambert, 2016; Hinrichs & Dixit, 2015; Masters, Nickens, Xuan, Shazer, & Amantea, 2018). It is also important to note that target antigen expression in normal cells is often not predictive of ADC toxicity. A prime example is the clinical toxicity profile of Trastuzumab emtansine (T-DM1, Kadcyla®), an ADC targeting human epidermal growth factor receptor 2 (HER2). Even though vital organs such as heart and kidney express significant levels of HER2 (Fuchs et al., 2003; Press, Cordon-Cardo, & Slamon, 1990), no evidence of T-DM1 clinical toxicity related to these organs was reported (Dieras et al., 2014). However, a severe decrease in platelets (thrombocytopenia) is the common DLT of T-DM1. Due to the absence of HER2 expression on circulating platelets or platelet-producing megakaryocytes, this toxicity is largely considered to be a target-independent effect (de Goeij & Lambert, 2016; Krop et al., 2010). Hence, in addition to target-antigen expression, factors such as the rate of internalization, recycling/trafficking kinetics of target antigen, intrinsic sensitivity to the payload and in vivo distribution of ADCs to normal cells/tissue may potentially determine ADC toxicity (Junttila et al., 2015). In general, tissues with high perfusion and relatively leaky vasculature (with incomplete basement membrane and/or fenestrations) such as liver, bone marrow and spleen are expected to have higher distribution and exposure to IgG/ADCs compared to other normal tissues (Liu, 2018; Poisson et al., 2017).

Several ADCs sharing the same payload-linker composition, but targeting divergent antigens, have similar maximum tolerated doses (MTDs) and also exhibit overlapping toxicities in normal cells/tissues. This toxicity exists even though the endogenous target antigen expression is significantly different across normal cells/tissues (de Goeij & Lambert, 2016). The most common ADC toxicities, unrelated to target antigen expression, are observed in bone marrow/hematology, eye, liver, peripheral nerve, kidney, GI and serosal effusions (Donaghy, 2016; Masters et al., 2018) For example, neutropenia is commonly observed as a DLT with the majority of MMAE-based ADCs (with cleavable linkers), despite targeting a wide range of antigens including CD30, MUC16 (Mucin 16), CD22 and PSMA (prostate-specific membrane antigen) (Advani et al., 2017; Liu et al., 2013; Petrylak et al., 2013; Younes et al., 2010). Similarly, ocular (corneal) toxicity is reported as a DLT for multiple DM4-containing ADCs targeting unrelated antigens not expressed in the eye (CD19, CanAg, folate receptor alpha, and mesothelin) (de Goeij & Lambert, 2016). In addition, ocular toxicity is observed in multiple MMAF-based ADCs for diverse targets with non-cleavable linkers (Thompson et al., 2015; Younes et al., 2012). All this evidence suggests that ADC toxicity is largely target-independent and also further suggests a potential role of drug-linker combinations (ADC platform) to specific off-target toxicities. This is further supported by recent reports on the role that each component of the ADCs has on the toxicities exhibited by 35 active and discontinued compounds, concluding that all three components of ADCs (i.e., mAb, drug and linker) contribute to toxicity in normal cells/tissues (Donaghy, 2016).

ADC technology has modestly improved across generations of biologic and chemical advancements in optimizing all three key components of ADCs (Beck, Goetsch, Dumontet, & Corvaia, 2017); however, achieving a higher clinical TI for ADCs still remains elusive. This review will focus on the current knowledge regarding the mechanistic understanding of target-independent uptake (also referred to as non-specific uptake) of ADCs in normal cells which would potentially contribute to off-target toxicity.

ADC uptake into normal cells independent of target-antigen may occur via multiple potential mechanisms. As shown in Fig. 1, different receptor-dependent and receptor-independent (non-specific endocytosis) mechanisms may contribute to uptake of intact ADC and/or released free payload in normal cells. In addition, linker-drug instability with premature release of cytotoxic payload in circulation may also contribute to target-independent ADC toxicities. Understanding mechanisms of target-independent ADC uptake and toxicity to healthy normal cells is critical to improve ADC technology. Although the challenges are substantial, by developing approaches to circumvent these mechanisms, it may be possible to improve the overall TI of ADCs. In the following sections, different mechanisms for target-independent uptake of ADCs are described in detail.

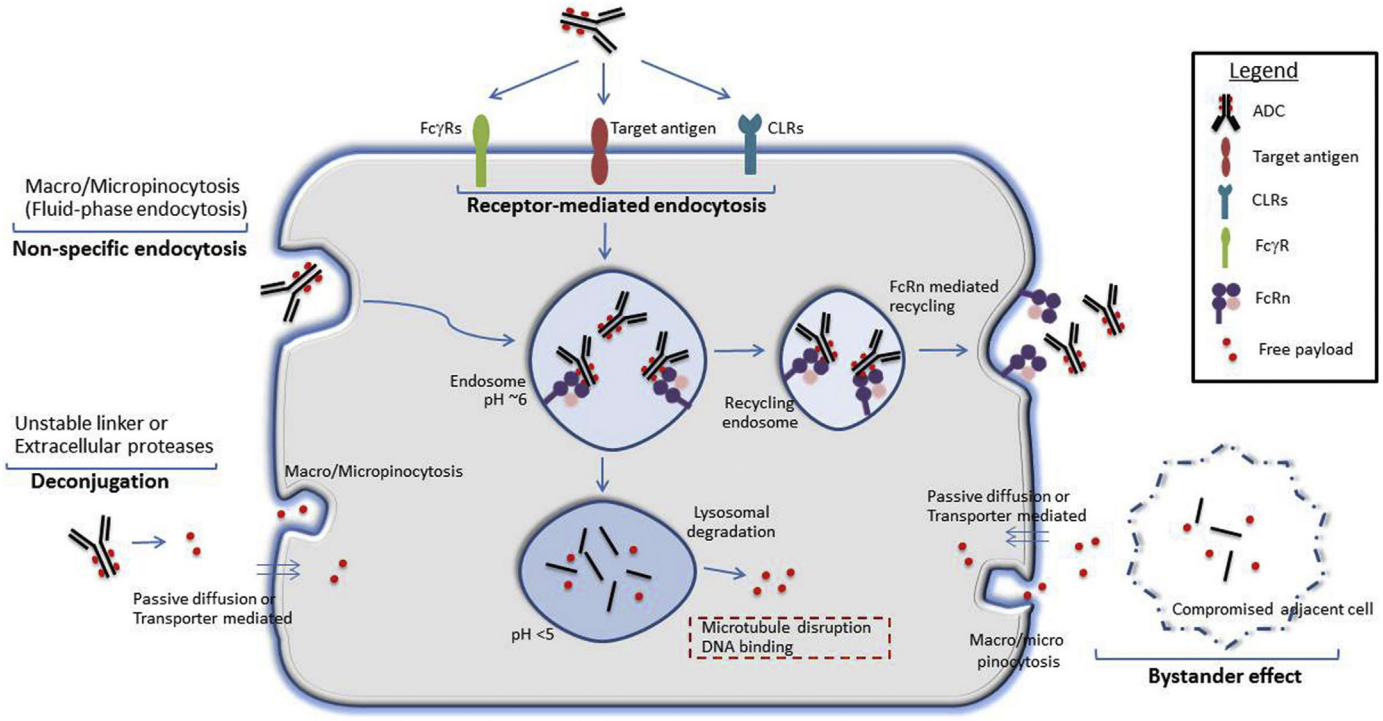

Fig. 1. Potential mechanisms for ADC or free payload uptake in normal cells. Target antigen may be expressed on normal cells and contribute to target-dependent uptake of ADCs. In addition, other receptors which bind conserved Fc regions of IgG antibodies such as Fc gamma receptors (FcγRs), neonatal Fc receptor (FcRn) and C-type lectin receptors (CLRs) may also contribute to target-independent internalization/trafficking of ADCs in normal cells. Non-specific endocytic mechanisms such as macropinocytosis or micropinocytosis could also contribute to internalization of intact ADC or free payload (released extracellularly due to linker-drug instability or extracellular protease activity). Free payload may also enter normal cells by other mechanisms such as passive diffusion (if membrane permeable), non-specific endocytosis or specific transporters (if substrate for membrane transporters)-mediated uptake. In addition, antigen-positive target cells are also able to mediate toxicity by releasing payload into the local environment that is subsequently taken up by antigen-negative normal cells (bystander effect) by either passive diffusion, transporter-mediated uptake or by other non-specific endocytosis mechanisms.

2. Potential mechanisms for target-independent ADC uptake and toxicity

2.1. Linker-drug instability

Linker-drug instability can result in premature release of payload into blood and lead to off-target toxicity of ADCs (Beck et al., 2017). Linker selection is one of the primary drivers behind ADC stability. First-generation ADCs had acid-cleavable linkages (e.g., hydrazone) that were designed to be stable in the neutral pH of plasma with release occurring in the lower pH of lysosomes following ADC internalization. However, these early ADCs were often plagued by poor plasma stability, which lead to measurable free payload in the circulation (Doronina et al., 2003) (Fig. 1). Factors contributing to plasma instability of ADCs include susceptibility to serum proteases, particularly for peptide-based linkers (Dokter et al., 2014) and spontaneous de-conjugation, such as retro-Michael maleimide transfer onto free thiols present on plasma proteins (Alley et al., 2008). In addition to safety issues, the efficacy of unstable ADCs can also be diminished as the ADCs arriving at the tumor are no longer fully loaded with payload. The introduction of non-cleavable linkers has in some cases alleviated linker cleavage issues (Lu, Jiang, Lu, & Zhang, 2016). Comparison of ADCs with cleavable (SPP-DM1 and mc-vc-PAB-MMAE) and non-cleavable linker (mcc-DM1 and mc-MMAF) types has shown reduced toxicity (hepatic and hematological toxicity) with non-cleavable linkers for specific targets (CD22) (Polson et al., 2009). Similarly, a non-cleavable thioether MCC linker improved preclinical safety profile over a cleavable disulfide linker with maytansinoid ADCs (Lewis Phillips et al., 2008). Reduced toxicity with non-cleavable linker types is proposed to be due to reduced release of free cytotoxic payload into the systemic circulation. However, not all targets are suitable for treatment by non-cleavable ADCs since full mAb catabolism is needed to release the linker-drug. ADCs with cleavable linkers may also provide increased efficacy through the bystander effect (discussed in next section) and are a top choice for antigens with a lower copy number, heterogeneous tumor expression or low internalization rates (Polson et al., 2009). Therefore, linker-drug stability remains a key area for optimization to maximize the probability of having a successful ADC (Durbin, Nottoli, Catron, Richwine, & Jenkins, 2017). Such stability considerations are especially important with exquisitely potent and membrane permeable toxins such as pyrrolobenzodiazepines (PBDs) (Sutherland et al., 2013). It is also important to note that, in addition to cleavability of linkers, membrane permeability of released payload may influence potential off-target cytotoxicity in normal cells, which can be leveraged to influence TI.

The site of conjugation may also modulate stability and pharmacokinetics of ADCs. The conventional non-specific conjugation methods using surface exposed amino acids such as lysines or cysteines generate highly heterogeneous ADCs (drug to antibody ratio [DAR], 0 to 8) with increased chances for aggregation and decreased plasma stability (Zhou, 2017). Hence, these non-specifically conjugated ADCs may also contribute to safety challenges with increased target-independent uptake and toxicity in normal cells. In recent years, advanced linker technologies and improved knowledge of the role of conjugation sites have produced more homogenous ADCs with improved overall linker-drug stability and reduced premature cytotoxic payload loss in plasma (Lyon et al., 2014; Strop et al., 2013b). These ADCs with higher linker-drug stability can also undergo more target-driven release of payload resulting in improved efficacy (Donaghy, 2016). Hence, variety of conjugation site engineering strategies including several site-specific conjugations using specific amino acids (natural or engineered), Fc glycans and short peptide tags have been used to generate homogenous DAR ADCs with improved safety profiles (Strop et al., 2013a; Zhou, 2017). For example, ADCs conjugated with first site-specific conjugation technology such as THIOMAB (unpaired cysteine-mediated conjugation),

are shown to be relatively better tolerated pre-clinically than a conventional ADC having same payload with non-specific conjugation (Junutula et al., 2010; Thompson et al., 2016). In addition, ADCs composed of a cysteine-engineered antibody conjugated with a PBD using a novel self-immolative disulfide linker is shown to improve safety profile (decrease toxicity) with equivalent efficacy in preclinical studies, demonstrating potential to increase the TI of ADCs (Pillow et al., 2017).

2.1.1. ADC toxicities associated with linker-drug instability

Neutropenia (decreased neutrophil count) is an important target-independent DLT of ADCs associated with systemic release of membrane permeable free payload due to instability of cleavable linker in the plasma. Neutropenia is a common toxicity for many ADCs conjugated to MMAE via protease cleavable valine-citrulline linkers such as Brentuximab vedotin (Adcetris, Seattle Genetics), ASG-5ME (Agensys), Glembatumumab vedotin (Celldex Therapeutics), Indusatumab vedotin (Millennium Pharmaceuticals), Polatuzumab vedotin (Genentech) and PSMA ADC (Progenics Pharmaceuticals) (Beck et al., 2017; Coveler et al., 2016; de Goeij & Lambert, 2016; Donaghy, 2016; Hinrichs & Dixit, 2015; Younes et al., 2012). A recent in vitro mechanistic study by Zhao et al described that vc-MMAE based ADC-induced neutropenia is due to a direct cytotoxic effect of released payload on differentiating neutrophils in the bone marrow (Zhao et al., 2017). As per the linker chemistry, valine-citrulline linkers are expected to undergo intracellular cysteine protease-mediated cleavage in lysosomes (Sutherland et al., 2006). The results of Zhao et al revealed that serine proteases secreted by differentiating neutrophils locally in the bone marrow microenvironment contribute to the cleavage of the vc linker extracellularly and release membrane permeable MMAE resulting in cytotoxicity to differentiating neutrophils in the bone marrow. However, results of this study open another question as to why membrane permeable MMAE payload released extracellularly in the bone marrow is toxic to only differentiating neutrophils (myeloid lineage cells), but not to other multiple sensitive cell types in bone marrow such as hematopoietic stem/progenitor cells, erythroid and megakaryocytic lineage cells. Additional exploratory studies, particularly using in vivo models comparing hematotoxicity (specifically toxicity to differentiating bone marrow cells) of cleavable and non-cleavable MMAE based ADCs, are needed to definitively understand and confirm the role of extracellular serine proteases in neutropenia.

Similarly peripheral neuropathy (a result of damage to peripheral nerves) is another important target-independent clinical toxicity associated with microtubule inhibitor ADCs (irrespective of target antigen) leading to treatment discontinuation and/or dose reduction (Litvak-Greenfeld & Benhar, 2012). PN is thought to be driven by linker-drug instability with premature release of membrane permeable free payload (microtubule inhibitor) in systemic circulation. Microtubule inhibitors disrupt interphase microtubule function critical for active transport of key essential proteins from the neuron cell body to distal synapses ultimately resulting in peripheral neuropathy (Starobova & Vetter, 2017). PN is characterized by numbness and tingling in the extremities that may eventually worsen to cause extreme pain and weakness and is commonly noted in the clinic after repeated dosing of ADCs containing membrane permeable MMAE (attached with protease cleavable linkers, e.g., valine citrulline), but not with ADCs containing membrane impermeable MMAF (Polakis, 2016). In addition, PN is a frequent adverse event for almost all ADCs with membrane permeable maytansinoid (DM-1 and DM-4) conjugated with cleavable linkers (Galsky et al., 2008; Stagg et al., 2016).

In addition to passive diffusion of unconjugated membrane permeable payload in systemic circulation, distribution/uptake of conjugated ADCs either by receptor-dependent or-independent mechanisms (discussed in later sections) may also occur in peripheral nerves. Hence, it requires additional in vivo investigative studies for definitive understanding of mechanisms of payload/ADC exposure to peripheral nerves. It is important to note that, PN observed in the clinic is not always predicted in preclinical animal models. For example, standard pre-clinical toxicology studies conducted for vcMMAE based ADCs have not predicted the PN observed in the clinic (Saber & Leighton, 2015). However, for other non-MMAE ADC containing microtubule inhibitors such as DM1 or DM4, PN is observed in preclinical species with good translatability to the clinic (Stagg et al., 2016).

2.1.2. Bystander effect

In addition to direct cytotoxicity following uptake of ADCs by target antigen-positive cells, free payload from ADCs may also be cytotoxic to adjacent target antigen-negative cells through a phenomenon referred to as bystander effect (Singh, Sharma, & Shah, 2016). In antigen-expressing target cells, ADC uptake and catabolism in lysosomes releases free payload in the cytoplasm. The free payload can then either passively enter the extracellular space (membrane permeable, highly lipophilic payloads) or be released due to loss of membrane integrity (after target cell death). Released free payload may move into target antigen-negative cells by passive diffusion, transporter-mediated uptake, or by other non-specific endocytosis mechanisms to cause cytotoxicity (Staudacher & Brown, 2017) (Fig. 1).

The bystander effect in ADCs is often associated with increased tumor killing (efficacy), especially for tumors with heterogeneous antigen expression (Okeley et al., 2010). Its effect on potency and efficacy of ADCs with membrane permeable payloads has been demonstrated using in vitro colony spheroid assays and co-culture systems (Kovtun et al., 2006; Okeley et al., 2010) as well as in vivo xenograft models (Breij et al., 2014; Golfier et al., 2014; Li et al., 2016). However, the increased cellular permeability needed to achieve the bystander effect can also contribute to off-target toxicity. Released payload can permeate into normal tissues and lead to increased toxicity when compared to non-cleavable, impermeable payload (Doronina et al., 2006). For example, Cantuzumab mertansine (Can-M) and Cantuzumab ravtansine (Can-R) are two ADCs that target CanAg (tumor-associated carbohydrate antigen, a novel glycoform of MUC1), but have a linker/payload combination of relatively labile SPP (N-succinimidyl 4-(2-pyridyldithio) pentanoate) linker/DM1 and stable SPDB (N-succinimidyl-4-(2-pyridyldithio)butanoate) linker/DM4 respectively. Hepatic toxicity (elevated transaminases) was observed only with Can-M in patients with hepatic metastases of solid malignancies and is suggested to be due to bystander effect on adjacent normal hepatocytes (Tolcher et al., 2003). Recent advances in ADC technology have brought about cytotoxic payloads able to be metabolized in cancer cells to membrane-impermeable metabolites (e.g., Dolaflexin) (Hofland, 2016). This approach may control the bystander effect, retaining beneficial chemical properties for killing cancer cells while also significantly minimizing systemic toxicity to normal cells.

2.2. Non-specific endocytosis

Endocytosis is an essential process in cells for the uptake of nutrients, regulation of transmembrane dynamics, and synaptic vesicle recycling etc. Endocytosis can also play an important role in the uptake and distribution of macromolecules including IgG/ADCs into normal cells (Xiao & Gan, 2013). Endocytosis is broadly categorized as phagocytosis (internalization of particulates) and pinocytosis (internalization of soluble molecules, also called as fluid-phase endocytosis). In addition, based on the size of endocytic vesicle formation, endocytosis has been classified as macroscale and microscale endocytic processes. It is not uncommon, however to see some overlap in different nomenclature for endocytic mechanisms used in different published references. Key characteristics of major endocytic mechanisms potentially contributing to non-specific uptake of IgG/ADCs are listed in Table 3.

Macroscale endocytosis includes phagocytosis and macropinocytosis, which involve the internalization of large particles or large volume fractions, respectively. Phagocytosis involves uptake of large-scale particles into deformations of cell membranes (localized

rearrangement of actin) that encircle the particle. It is possible that immune-complexes containing ADCs or aggregates of ADCs could also be taken up by this process. Similar to phagocytosis, macropinocytosis is also an actin-dependent process and it involves formation of ruffled extensions of the plasma membrane around a region of relatively large amounts of extracellular fluid (rather than particles) to mediate endocytosis. Microscale endocytic processes involve the engulfment of smaller volumes with microsomes less than 200 nm in size. These processes often require special coat proteins like clathrin or caveolin (Fig. 2). Clathrin-mediated endocytosis (CME) is the predominant form of receptor-mediated microscale endocytosis in most cell types (Muro, Koval, & Muzykantov, 2004) and is also a primary mechanism for target-dependent internalization of ADCs into target antigen/receptor expressing cells (Kovtun & Goldmacher, 2007; Schrama, Reisfeld, & Becker, 2006). Binding of ligands to specific membrane receptors initiates a cascade of signaling events leading to recruitment of specific adaptor proteins for clathrin-coated vesicle formation (Popova, Deyev, & Petrenko, 2013) (Fig. 2). These newly formed vesicles are severed by dynamin (GTPase enzyme) and released for further intracellular transport (Cao, Chen, Awoniyi, Henley, & McNiven, 2007). Caveolin-mediated endocytosis involves flask-shaped structures (caveolae) formed by the membrane coating protein caveolin and it also depends on dynamin for vesicle scission (Palade, 1953). Caveolin-mediated uptake plays a major trafficking role in many cell types (Doherty & McMahon, 2009) and especially predominates in endothelial cells (Muro et al., 2004). It is important to recognize that in addition to clathrin and caveolin-mediated endocytosis, several other micropinocytic processes including CLIC/GEEC, ARF6, Flotillin and Tetraspanin associated processes exist (Doherty & McMahon, 2009; Kumari, Mg, & Mayor, 2010), which are not discussed in this review.

Regardless of the mechanisms involved, endocytosed cargo is subjected to intracellular processing, including trafficking through the endo-lysosomal pathway. Cargo marked for degradation progress from the early endosome to the late endosome and then to their final destination in the lysosome. Alternatively, endocytosed cargo can be re-routed back to the surface by recycling endosomes. Other trafficking outcomes are transcytosis to the adluminal space or sorting into different subcellular compartments such as the Golgi complex and endoplasmic reticulum. Rates of intracellular trafficking may also vary depending on endocytic mechanism. For example, the existence of a rapid recycling route back to the cell surface from the early endosome has been documented for clathrin-mediated endocytosis (Grant & Donaldson, 2009). Similarly, contents internalized via macropinocytosis and phagocytosis processes may also get recycled, but are more typically processed for lysosomal degradation. Interestingly in endothelial cells, ligands internalized by caveolar-mediated endocytosis are preferentially sorted into sub-cellular compartments avoiding the lysosomal degradation pathway (Muro et al., 2004).

Overall, both macro and microscale endocytic processes described above may contribute to uptake of ADCs into normal cells. With respect to target-independent ADC toxicity to normal cells, non-specific endocytic mechanisms such as caveolar-dependent endocytosis, macropinocytosis and phagocytosis are potentially important mechanisms. It is also clear that endocytic mechanisms and overall rates of endocytosis vary across normal tissues and cell types (Adler et al., 2018). Many specialized immune cells (including macrophages and dendritic cells) specifically do this as a primary function, and have a higher rate of endocytosis. For example, Kupffer cells (resident macrophages in the liver) are known to play a major role in non-specific uptake and clearance of immuno-conjugates including ADCs (Kraynov et al., 2016). Endothelial cells also have a relatively high rate of endocytosis of macromolecules due to their strategic positioning at the interface between blood vessels and the interstitial compartment. However to our knowledge, no full description of which normal cell types and tissues are the most endocytically active has been published to date. Therefore, understanding the rate of endocytosis in different normal cells/tissues would be valuable towards understanding the role of non-specific endocytosis as a potential mechanism for ADC uptake and toxicity.

2.2.1. Factors influencing non-specific endocytosis of IgG/ADCs

The physicochemical properties of macromolecules may influence the likelihood of being endocytosed in normal cells/tissues. Molecular charge on surface of IgG/ADC is an important one among the many parameters that collectively influence antibody tissue distribution and PK (Iznaga-Escobar, Mishra, & Perez-Rodriguez, 2004). Positively-charged molecules are attracted to the negatively-charged groups in the cell membrane of most mammalian cells as well as within the extracellular matrix (heparin sulfate proteoglycans) (Varki, 2008; Wiig, Gyenge, Iversen, Gullberg, & Tenstad, 2008). The close proximity increases the local concentration, leading to more non-specific endocytic uptake in normal tissues/cells. Detailed summary of reported in vivo preclinical studies evaluating PK and tissue disposition of charge-modified IgG

Table 3
Key characteristics of major endocytic mechanisms

Major classes	Major sub types	Key characteristics	Pharmacological Inhibitors
Microscale endocytosis (< 200 nm)	Clathrin-mediated endocytosis	• Clathrin coat-dependent microscale pinocytosis • Well characterized among all other endocytosis mechanisms • Predominant form of receptor-mediated endocytosis. • Involves wide variety of transmembrane receptors (including ADC targets)-mediated endocytosis in most cell types • Facilitated by accessory/adaptor proteins and requires dynamin (GTPase) for vesicle scission	Dynasore (Dyngo), NSC23766, Phenylarsine oxide, Chlorpromazine, K^+ depletion
	Caveolin-mediated endocytosis	• Clathrin coat-independent, caveolae (flask-shaped 60–80 nm size invaginations)-mediated microscale endocytosis • Receptor-independent non-specific endocytosis • Important role in endocytosis and transcytosis across endothelial barriers • Dynamin (GTPse) is required for vesicle scission	Dynasore (Dyngo), NSC23766, Indomethacin, Filipin, Genistein
Macroscale endocytosis(0.2 to 10μM)	Macropinocytosis	• Receptor-independent, non-specific macroscale pinocytosis • Direct contact with the internalized material is not required. Ruffled extensions of the plasma membranes around a region of extracellular fluid mediate endocytosis • Actin (actin polymerization)-dependent process. Also require Rac1, cholesterol- and Na+/H+ exchanger activity	EIPA, EDTA, Cytochalasin B, Cytochalasin D, Latrunculin A, Rottlerin, Rapamycin
	Phagocytosis	• Receptor-dependent (Fc-mediated cross-linking) non-specific macroscale endocytosis. • Internalization of larger opsonized particulate matter • Actin-dependent (actin polymerization) endocytic process and PI3Ks play an important role • Well characterized in specialized immune cells such as macrophages	Wortmannin, LY294002

References: (Doherty & McMahon, 2009; Grant & Donaldson, 2009; Hackstein, Taner, Logar, & Thomson, 2002; Kumari et al., 2010; Mulcahy, Pink, & Carter, 2014; Sato, Nagai, Mitsui, Ryoko, & Takano, 2009; Vercauteren et al., 2010)

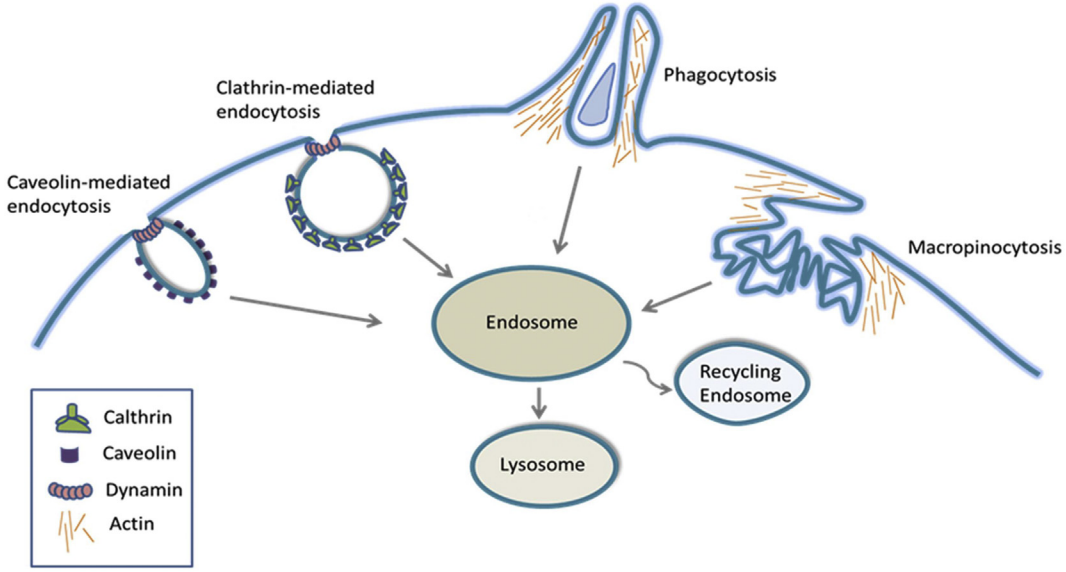

Fig. 2. Schematic representation of key structural characteristics of macroscale and microscale endocytic processes. Phagocytosis involves FcγR binding with localized activation of Cdc42 (cell division control protein 42) and Rac1(Ras-related C3 botulinum toxin substrate) leading to actin polymerization. The process of Macropinocytosis involves the uptake of large fluid volumes and is morphologically characterized by ruffled macropinosomes. Caveolar endocytosis is characterized by a spike-like coating of caveoli associated with cholesterol that is visible by electron microscopy (EM). The best defined microscale endocytic processes include the clathrin-mediated endocytosis, which involves the formation of a complex lattice composed of clathrin trimers forming bristled appearing endosomes that can be identified morphologically by EM.

antibodies are described in an excellent review by Boswell et al., (Boswell et al., 2010). In general, these investigative studies revealed that increases in net positive-charge of IgG antibodies result in increased plasma clearance with increased tissue distribution, whereas decreases in net positive charge result in decreased tissue distribution. Importantly shifts in isoelectric point (pI) of at least one unit or more is sufficient to produce measurable changes in tissue distribution and PK (Boswell et al., 2010). These conclusions may also be applicable to ADCs, supporting the hypothesis that ADC charge may influence non-specific endocytosis in normal cells. Therefore, charge modification, either by reduction of positive charge or by balancing the overall surface charge distribution, is an approach to consider while designing future ADCs. But it is important to note that similar to normal tissues, charge modifications may also influence target antigen-dependent ADC uptake required for efficacy in tumor cells. Optimization of ADC surface charge to decrease target-independent uptake in normal cells while retaining target-mediated uptake in tumor cells could favorably improve TI.

Hydrophobicity of ADCs may also play a role in their non-specific uptake by normal cells. Many drug-linker combinations used in ADCs are hydrophobic; imparting significant hydrophobicity onto antibodies, particularly for ADCs with high DAR. Increased hydrophobicity of high DAR species can contribute to ADC aggregation and accelerated non-specific clearance mainly by Kupffer cells and hepatic sinusoidal endothelial cells (Lyon et al., 2015) (Drake & Rabuka, 2017). Similar to hepatic cells, ADCs with high DAR may get rapidly cleared by other normal cells with high non-specific endocytic capacity contributing for target independent (off-target) toxicity. For example, for MMAE based ADCs, DAR4 purified ADCs have a two-fold higher tolerability than DAR8 in an in vivo mouse model (Hamblett et al., 2014). This conclusion may also be applicable in general for other ADCs, hence DAR optimization is a key design parameter to improve TI of ADCs. Recent advancement in conjugation and linker technologies may overcome these unwanted PK issues of high DAR ADCs. For example, a reduction in ADC hydrophobicity by engineering hydrophobicity out of a drug-linker or masking their inherent hydrophobicity with polyethylene glycol (PEG) are proposed to decrease non-specific clearance of high DAR species, improving both PK profile and TI of ADCs (Burke et al., 2017; Lyon et al., 2015; Viricel et al., 2019).

2.2.2. ADC toxicities associated with non-specific endocytosis

Non-specific endocytosis (macropinocytosis in particular) has been suggested as the route of ADC uptake in normal corneal epithelial cells and megakaryocytes contributing to ocular toxicity and thrombocytopenia respectively (Zhao et al., 2018; Zhao et al., 2017). Evidence for macropinocytosis-mediated uptake in corneal epithelial cells was mainly due to decreased cytotoxicity in human primary corneal epithelial cells (in vitro) to AGS-16C3F (ADC targeting ENPP3 antigen with mcMMAF linker-payload) based tool ADCs with decreasing positive charge and/or hydrophobicity and inhibition of ADC uptake with co-incubation with EIPA (5-(N-ethyl-N-isopropyl)-amiloride, macropinocytosis inhibitor) (Zhao et al., 2018). In this study, the authors altered ADC charge or hydrophobicity by 3 different mechanisms, including the addition of attachment of poly-glutamate peptides, mutational changes of certain charged amino acids, and attachment of PEG moieties to antibodies. The results of these experiments in corneal epithelial cells were generally consistent with the hypothesis that charge modification (to increase net positive charge) alters uptake and toxicity of ADCs in normal cells. Of the 9 mutant antibody ADCs (with increase in the number of negatively charged amino acids) produced, 3 showed improved TI *in vitro* in human corneal epithelial cells (Zhao et al., 2018). The authors further reported lower uptake and toxicity of charge modified tool ADCs (with decreased positive charge) in some other normal cell types (HUVECs and fibroblasts); however, no consistent improvement was seen in megakaryocytes, which were previously reported to be sensitive to the same non-specific uptake mechanism (Zhao, Gulesserian, Ganesan, et al., 2017). In addition, it is difficult to relate the *in vitro* findings of normal corneal epithelial cells in this study to actual *in vivo* improvement since ocular findings in the rabbit model used did not have any lesion/defect (cytotoxicity) primarily in corneal epithelial cells as has been reported for other ADCs clinically or in other animal models (Eaton, Miller, Mannis, & Murphy, 2015; Poon et al., 2013). The results of this work overall provide strong evidence of the importance of charge in non-target related cellular uptake in human corneal epithelial cells, but conclusions about the mechanism of uptake mainly being macropinocytosis are still somewhat unresolved. ADC charge modifications may also influence other potential receptor (FcγRs, CLRs)-mediated uptake mechanisms (discussed in later sections) in corneal epithelial cells, which are not completely ruled out by this study.

Similarly, modulation of macropinocytosis-mediated internalization was shown to decrease ADC (AGS-16C3F) toxicity to megakaryocytes (thrombocytopenia) (Zhao, Gulesserian, Ganesan, et al., 2017). However, there is some inconsistency in the literature regarding macropinocytosis as the mechanism of target-intendent ADC uptake in differentiating megakaryocytes (Uppal et al., 2015)as is discussed in a later section. Macropinocytosis can be conclusively identified by electron microscopy (EM) evaluation, where "ruffled" macropinosome morphology is observed (Grimmer, van Deurs, & Sandvig, 2002). EM evaluations combined with labeled ADCs could be helpful for further confirming involvement of macropinocytosis-mediated uptake and subcellular localization of ADC in normal cells.

2.3. Receptor-mediated uptake mechanisms

Target-independent uptake and toxicity of ADCs could also be mediated by different candidate receptors which recognize the Fc (fragment crystallizable) region in the IgG backbone in ADCs (Fig. 3). IgG constant domains are highly conserved in structure allowing interaction with other components of the immune system through Fc receptors to initiate effector immune functions (Ravetch, 1994). Although Fc-mediated effector functions are not typically required for achieving ADC efficacy, recognition and binding of Fc receptors to the antibody (IgG) component of ADCs could mediate target-independent internalization to normal cells (Uppal et al., 2015). In this section, the potential role of different Fc binding receptors including Fc gamma receptors (FcγRs), neonatal Fc receptor (FcRn) and C-type lectin receptors (CLRs) in mediating IgG/ADC internalization/trafficking and toxicity to normal cells is discussed. In addition, physicochemical properties of IgG/ADC which contribute to binding of these receptors are also included.

2.3.1. Fc gamma receptors (FcγRs)

FcγRs play a pivotal role in linking the cellular and humoral immune responses through antibody-mediated effector functions such as antibody dependent cellular cytotoxicity (ADCC), complement dependent cytotoxicity (CDC), phagocytosis of IgG-opsonized particles/immune complexes and release of cytokines (IFN-γ and TNF-α) and other inflammatory mediators (Chan, Ong, Mok, & Ooi, 2015; Nimmerjahn & Ravetch, 2008). These effector functions play an important role in modulating the efficacy of several therapeutic IgG antibodies (Stewart, Hammond, Oberst, & Wilkinson, 2014). For ADCs, FcγR-mediated effector functions are not typically critical for target-related efficacy, but may potentially contribute to target-independent uptake and toxicity in normal cells. Therefore, knowledge of FcγR biology, expression patterns in normal cells/tissues, and physiochemical factors of ADCs which contribute to FcγR binding is of great importance for understanding potential target-independent toxicity mechanisms. Based on the type of signaling pathway initiated following receptor cross linking, FcγRs are mainly divided into activating (FcγRI, FcγRIIa, FcγRIIc, FcγRIIIa and FcγIIIb) and inhibitory type (FcγRIIb1/b2) receptors (Bruhns, 2012). Interactions of activating FcγRs with immunoreceptor tyrosine-based activation motif (ITAM) positively regulate effector functions, whereas interactions of inhibitory type receptors with immuno-receptor tyrosine-based inhibition motif (ITIM), negatively regulate IgG-mediated effector functions including endocytosis/phagocytosis (Gerber & Mosser, 2001; Ravetch, 1994). In addition to immune cells, expression of different FcγRs was shown in multiple other normal cell types including epidermal keratinocytes (Cowan, Broomfield, & Smith, 1998), sensory neurons (Andoh & Kuraishi, 2004), mesangial cells, osteoclasts (Daeron, 1997), endothelial cells (Lyden et al., 2001), fibroblasts (Antonsson & Johansson, 2001), salivary gland epithelial cells (Lisi et al., 2007), various cell types in kidney (Aarli, Matre, & Thunold, 1991) and eye (Niu et al., 2011; Tripathi, Borisuth, & Tripathi, 1991; Wang, Jeng, & Kaplan, 1989), megakaryocytes, platelets (Wu, Markovic, Chesterman, & Chong, 1996), and differentiating bone marrow derived immature cells including hematopoietic stem/progenitor cells (Mahalingaiah et al., 2017).

2.3.2. FcγR-mediated internalization of IgG/ADCs

FcγRs are important molecules not only for mediating the effector functions of IgG antibodies, but are also among the most characterized endocytic cell surface receptors playing a role in internalizing/clearing IgG-opsonized antigens from the systemic circulation (Zhu et al., 2016). Binding of FcγRs to the Fc region induces clustering/crosslinking of IgGs on cells and initiates downstream signaling events that result in phosphorylation and activation of kinases such as PI3K, p70S6K and Akt (Ganesan et al., 2004). These are directly involved in reorganization of the actin cytoskeleton and membrane remodeling in formation of pseudopods and phagosomes (Chan et al., 2015; Maxeiner et al., 2015).

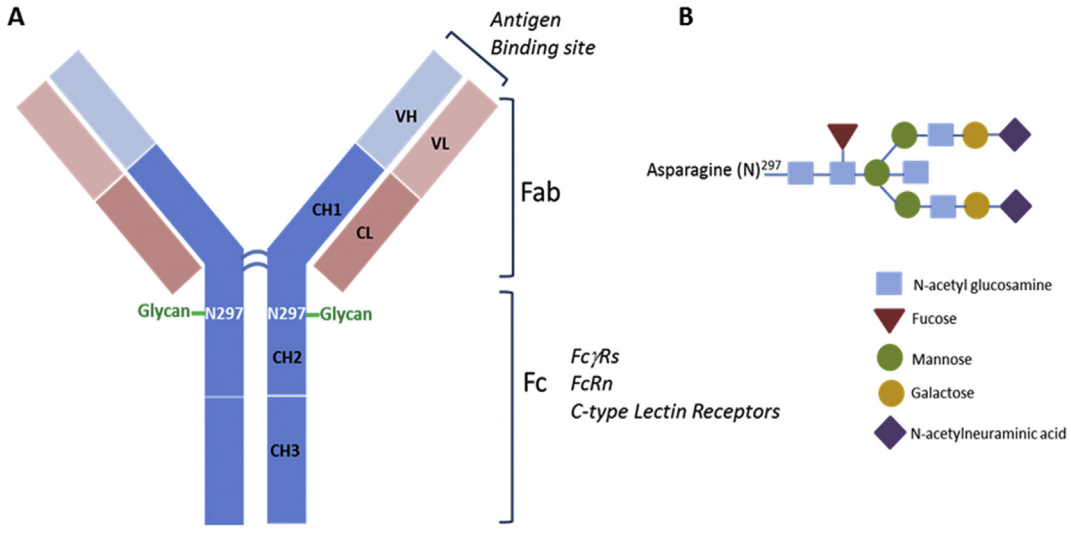

Fig. 3. A. Schematic representation of basic structure of IgG antibody showing Fab (fragment, antigen binding) region composed of one constant (C) and one variable (V) domain from each heavy (H) and light (L) chain and the variable domain in Fab (antigen binding fragment) region also referred to as the Fv region (region for binding to antigens). The Fc (fragment, crystallizable) region composed of two heavy chains which bind to various cell receptors, such as Fc receptors (FcγRs, FcRn), C-type lectin receptors (CLRs) and other general scavenger receptors. B. Typical structure of a fully processed *N*-glycan at Asparagine (N)-297 sites within CH2 domain of Fc region. The N297-linked complex-type glycan is located within the each heavy chain and consists of a bi-antennary complex structure composed of N-acetylglucosmaine and mannose, and followed by variable extensions of galactose, sialic acid (N-acetylneuraminic acid), fucose and bisecting residues of N-acetylglucosmaine.

Similar mechanisms may also hold true for FcγR-mediated internalization of ADCs contributing to target-independent toxicity in normal cells.

2.3.2.1. ADC toxicities associated with FcγRs-mediated uptake in normal cells. Even though the expression pattern of FcγRs in normal healthy cells/tissues corresponds with several reported target-independent toxicities of ADCs (Hall, Diaconu, & Pearlman, 2001; Lovdal, Andersen, Brech, & Berg, 2000; Mahalingaiah et al., 2017; Wang et al., 1989), the role of FcγRs in mediating ADC off-target toxicity are mainly proposed as a potential mechanism for hematotoxicity (toxicity to blood cells). Hematotoxicity is the most common target-independent DLT for ADCs containing auristatin (MAME, MMAF), calicheamicin, and maytansinoid (DM-1) (Hinrichs & Dixit, 2015) (de Goeij & Lambert, 2016). Key data to support the role of FcγRs in ADC-induced hematotoxicity are provided by Uppal et al. (2015), who investigated target-independent uptake as a potential mechanism for the development of thrombocytopenia with Trastuzumab-emtansine (T-DM1) (Uppal et al., 2015). T-DM1-induced thrombocytopenia was observed as a DLT in clinical studies. HER2 expression is not reported on circulating platelets or differentiating megakaryocytes (MKs) in the bone marrow (Buhring et al., 1995; Press et al., 1990), hence target-dependent uptake in these cells is not expected. Using in vitro experiments and a novel human bone marrow hematopoietic stem/progenitor cell differentiating model, Uppal et al., not only ruled out a direct effect of the ADC on mature platelets, but also revealed FcγRIIa-mediated internalization and cytotoxicity (cytoskeletal disruption) of T-DM1 in differentiating MKs (in the bone marrow) as a mechanism for decreased platelet production. T-DM1 internalization in differentiating MKs was significantly inhibited by using FcγRII blocking antibodies or by using the Fc mutant version of T-DM1 that shows decreased FcγR binding (T-DM1-DANA carrying D265A and N297A mutations). Blocking T-DM1 interaction with FcγRIIa receptors significantly decreased T-DM1 internalization, but did not completely block either uptake or cytotoxicity to MKs suggesting that additional mechanisms, such as non-specific endocytic uptake, could also contribute to platelet loss. This is supported by a recent report published by Zhao, Gulesserian, Ganesan, et al., 2017 (Zhao, Gulesserian, Ganesan, et al., 2017), who investigated mechanisms of both AGS-16C3F (ADC targeted against ENPP3 (Ectonucleotide pyrophosphatase/phosphodiesterase family member 3), conjugated via a non-cleavable linker to MMAF) and T-DM1 induced thrombocytopenia using an in vitro MK cell differentiation platform similar to Uppal et al (Uppal et al., 2015). There was no direct effect of AGS-16C3F on mature platelets similar to T-DM1, and also ENPP3 (target antigen) expression was not detected on circulating platelets or its precursor megakaryocytes. In contrast to Uppal et al, Zhao et al (Zhao, Gulesserian, Ganesan, et al., 2017) concluded that target-independent macropinocytosis of ADCs in MKs and inhibition of their differentiation plays a role in ADC-induced thrombocytopenia.

In addition to FcγR-mediated and/or macropinocytosis-mediated uptake of ADCs into differentiating MKs in bone marrow, other mechanisms such as peripheral destruction or increased clearance/sequestration of circulating platelets could also contribute to ADC-induced thrombocytopenia. Human platelets survive (life span) for 9 to 11 days (Rowley, Schwertz, & Weyrich, 2012) and then get cleared by different mechanisms including aging-induced surface glycan modification (desialylation), exposing an underlying galactose moiety (which is recognized by the Ashwell-Morrell receptor) for clearance in hepatocytes and immune-mediated (antibody or T-cell dependent) mechanisms (Hoffmeister & Falet, 2016). Understanding the change in platelet kinetics following ADC treatment could be helpful for differentiating thrombocytopenia due to decreased production vs. peripheral destruction or clearance. A rapid (acute) drop in platelet counts (in <5 to 7 days) indicates accelerated platelet destruction in peripheral blood or sequestration at distant sites of damage, rather than decreased production in the bone marrow. As an example, ADC induced thrombocytopenia, independent of FcγR-mediated or macropinocytosis-mediated uptake into MKs, is demonstrated for calicheamicin based ADCs in Cynomolgus monkeys (Guffroy et al., 2017). In this study, hepatic toxicity of a calicheamicin ADC primarily in liver sinusoidal endothelial cells (LSECs) is associated with platelet sequestration in liver sinusoidal spaces contributing to thrombocytopenia. Hepatic toxicity characterized by sinusoidal obstruction syndrome (SOS) (previously known as veno occlusive disease [VOD]) is a serious complication reported in patients treated with calicheamicin based ADCs. The exact mechanisms for uptake of these ADCs in LSECs contributing to initial insult to hepatic sinusoids are unknown. Hepatic toxicity is associated with ADCs targeting different antigens including one which is known to be expressed in liver cells such as CD33 (Gemtuzumab ozogamicin, GO), as well as antigens not expressed in liver cells such as CD22 (Inotuzumab ozogamicin, IO), suggesting potential target-independent uptake mechanisms (Godwin, McDonald, & Walter, 2017). This is further supported by a recent Cynomolgus monkey study demonstrating hepatic toxicity consistent with early SOS using a non-binding (IgG1 mAb targeted against non-mammalian protein) ADC containing the same linker-payload as GO and IO (Guffroy et al., 2017). Based on these results and the reported expression pattern of key candidate receptors, target-independent uptake mechanisms mediated by FcγRs (Godwin et al., 2017) and mannose receptor (Gorovits & Krinos-Fiorotti, 2013) in hepatic sinusoidal endothelial cells have been proposed as potential mechanisms. A role of un-conjugated calicheamicin circulating in the blood stream as well as other potential receptor-independent endocytic mechanisms contributing for ADC uptake cannot be ruled out as endothelial cells also retain relatively higher levels of non-specific macropinocytosis (fluid-phase endocytosis) (Goebl et al., 2008).

2.3.2.2. Factors influencing IgG/ADC binding to FcγRs. The IgG subclass used in ADCs and other physicochemical properties (such as amino acid sequence in the Fc region and the glycosylation profile), may influence FcγR binding affinity of ADCs. Each of the IgG subclasses has a unique binding profile to different FcγRs (reviewed in (Vidarsson, Dekkers, & Rispens, 2014)). A major distinction is that IgG1 and IgG3 subtypes interact efficiently, whereas IgG2 and IgG4 show reduced affinity to the majority of FcγRs (Vidarsson et al., 2014). If the presence of FcγR-mediated effector functions would be deleterious, then use of human IgG2 or IgG4 rather than commonly used human IgG1 may represent a better choice (Liu, 2015; Presta, 2005). This may also hold true for ADCs, wherein using mAb with an IgG2 or IgG4 backbone would potentially decrease FcγR-mediated internalization and therefore toxicity in normal cells. Several ADCs with an IgG2 backbone are currently in clinical development by Agensys/Astellas (AGS-16C3F, ASG-15ME and AGS67E) (Beck et al., 2017). Hence comparison of clinical safety data from these ADCs when available with other IgG1 based ADCs with similar linker-drug profiles might inform as to whether IgG2 offers a path forward to overcome or decrease FcγR-mediated toxicities.

In addition, several amino acids comprising the N-terminus of the CH2 domains and strands adjacent in the three dimensional immunoglobulin fold of human IgG play a critical role in FcγR binding (Siberil et al., 2006; Vidarsson et al., 2014). Engineering the Fc portion of the IgG to change amino acids in these regions may either decrease or enhance the FcγR-mediated effector functions. For therapeutic antibodies, most of the Fc engineering strategies are centered on increasing the binding affinity to FcγRs to improve the effector functions (Kellner, Derer, Valerius, & Peipp, 2014; Presta, 2008). For ADCs, antibody engineering to decrease or remove the binding affinity to FcγRs (Fc silencing) is a potential approach to overcome FcγR-mediated off-target toxicities (Beck et al., 2017; Mimoto, Kuramochi, Katada, Igawa, & Hattori, 2016). Common approaches for Fc silencing include introducing mutations into the FcγR binding regions, such as L234A/L235A (LALA mutants), V234A/G237A, and G236R/L328R (Mimoto et al., 2016). Even though these Fc engineering approaches are well characterized and tested to modulate FcγR binding, Fc engineered IgG or ADCs present

other concerns which need to be considered. Undesirable effects of PK profile, impaired stability, and increased risk of immunogenicity with ADA (anti-drug antibody) development are all potential concerns related to this approach.

Another factor that can potentially influence antibody binding to FcγRs and antibody-mediated effector functions is the sugar moiety (N-linked glycosylation) attached to all IgG subclasses at the asparagine residue 297 (N297) in the CH2 region of the antibody (Arnold, Wormald, Sim, Rudd, & Dwek, 2007; Siberil et al., 2006). Fc glycans consist of a branched (bi-antennary) heptameric core sugar structure containing N-acetylglucosamine (GlcNac) and mannose with variable amounts of branching and terminal sugar residues such as sialic acid, galactose, fucose, and GlcNac (Subedi & Barb, 2016) (Fig. 3). ADC-FcγR interactions can be significantly influenced by the presence or absence of these terminal or branching sugar residues in IgG (Liu, 2015). Glycosylation is the most complex post-translational modification and the composition of IgG glycoforms are known to vary greatly with the expression host cell line (i.e., CHO or HEK or mouse myeloma cells) and culture conditions (Goh & Ng, 2018) used to produce mAbs. Therefore, selection of specific mammalian cells that selectively produce desired IgG glycoforms or glyco-engineering to alter glycosylation profiles (aglycosylation/deglycosylation) could favorably alter FcγR binding profiles. A common approach for removal of the N-glycan (aglyscosylation) is mutating the Fc region (N297 mutant) and is shown to decrease IgG binding to FcγRs (Schroeder Jr. & Cavacini, 2010). Antibodies with glycan modifications have been evaluated in rodent models (Presta, 2005), but there are no reports of complete evaluations of these in humans. Similar to Fc engineering, the majority of the information available for IgG glyco-engineering is to improve antibody-mediated effector functions by increasing FcγR binding (Wang, Mathieu, & Brezski, 2018). Better understanding of the impact of glycosylation modifications designed to decrease FcγR binding and how such modifications impact IgG stability and overall PK are required to facilitate the development of the next-generation of ADCs with more optimized efficacy and safety profiles. Different tools to investigate potential role of FcγRs in mediating ADC uptake and toxicity to normal cells are listed in supplementary Table 1.

2.3.3. Neonatal Fc receptor (FcRn)

FcRn, also called the Brambell receptor, is a member of MHC class I glycoproteins, which specifically binds to the Fc domain and plays a critical role in the characteristically long half-lives of IgGs (~21 days) relative to other plasma proteins by protecting them from lysosomal degradation and recycling them back to extracellular space (Akilesh, Christianson, Roopenian, & Shaw, 2007). Unlike other Fc receptors, FcRn interacts with ligands in a strictly pH dependent manner with high affinity binding at slightly acidic pH (<6.5), but with very low affinity binding at physiological neutral pH (~7.4) (Rodewald, 1976). This pH dependence is considered key to the mechanism by which FcRn extends IgG/ADC half-life. FcRn is broadly expressed in many normal adult tissues/cell types (Table 4). Especially vascular endothelial cells and myeloid derived hematopoietic cells (antigen presenting cells) play a predominant role in FcRn-mediated salvage and thus, the catabolism and PK of IgG/ADCs (Sand et al., 2014). FcRn expressed in polarized epithelial cells (e.g., intestinal epithelial cells, renal proximal tubular epithelial cells) also contributes to bidirectional transcytosis of IgG or immune complexes (Rojas & Apodaca, 2002). It is also important to note that, although in general, the FcRn expression pattern is comparable there is a major difference in IgG binding affinity between human and other preclinical species (Latvala, Jacobsen, Otteneder, Herrmann, & Kronenberg, 2017). While human FcRn binds only human IgG, murine FcRn is highly promiscuous and binds to IgGs from several species (including human) with higher (~10 fold) affinity than mouse IgG (Ober, Radu, Ghetie, & Ward, 2001). Interestingly binding affinity of human IgG to Cynomolgus monkey FcRn is also ~2 folds higher than to human FcRn (Abdiche et al., 2015).

Table 4
Expression of FcRn in different normal cells/tissues

Organ system	Cell type	Reference(s)
Vascular system	Endothelial cells	(Latvala et al., 2017)
Reproductive system	Placenta (syncytiotrophoblasts), mammary gland endothelial cells	(Kuo et al., 2010)
GI tract	Intestinal epithelial cells	(Yoshida et al., 2004)
Hematopoietic cells	Macrophages, monocytes, dendritic cells, and differentiating hematopoietic (myeloid, erythroid and megakaryocytic) lineages	(Mahalingaiah et al., 2017; Zhu et al., 2001)
Respiratory system	Nasal epithelium, bronchial epithelial cells, tracheal epithelial cells and alveolar macrophages	(Heidl, Ellinger, Niederberger, Waltl, & Fuchs, 2016; Kuo et al., 2010; Spiekermann et al., 2002)
Kidney	Podocytes in glomerulus, brush border of proximal tubular epithelial cells and vascular endothelium	(Haymann et al., 2000)
Liver	Hepatocytes, Kupffer cells and sinusoidal endothelial cells	(Blumberg et al., 1995; Borvak et al., 1998; Telleman & Junghans, 2000)
Brain	Capillary endothelium, choroid plexus epithelium and blood brain barrier (BBB)	(Kuo et al., 2010)
Eye	Corneal epithelium, lens epithelium, conjunctiva lymphatic vessel, non-pigmented ciliary epithelium and blood vessels	(Kim et al., 2008)
Skin	Vascular endothelium, keratinocytes, melanocytes, hair follicles, and sebaceous glands	(Cauza et al., 2005; Cianga, Cianga, Plamadeala, Branisteanu, & Carasevici, 2007)
Muscle	Endothelial cells of small arterioles and capillaries	(Borvak et al., 1998)

2.3.3.1. FcRn binding and potential role in ADC toxicity. ADCs internalized by FcRn expressing cells, primarily via non-specific fluid phase endocytosis (macro or micropinocytosis), bind to FcRn in acidic early endosomes and then FcRn-ADC complexes are sorted into recycling endosomes to divert away from lysosomal degradation and recycled back to the cell surface for release of ADC at neutral pH into the extracellular space or systemic circulation (Pyzik, Rath, Lencer, Baker, & Blumberg, 2015). In each cycle of endocytosis, only the ADCs not bound to FcRn are trafficked to lysosomes for catabolism and release of payload (Fig. 4). FcRn binding is also important from a safety perspective for reducing ADC accumulation and catabolism to release cytotoxic payload in normal cells (Akilesh et al., 2007; Telleman & Junghans, 2000). Hence, modification of FcRn binding of ADCs is potentially a useful approach to overcome undesired toxicity/adverse events in normal cells with significant FcRn expression. Different tools/models to investigate the role of FcRn mediated trafficking in ADC accumulation and toxicity to normal cells is listed in Table 2.

The impact of modulation of FcRn binding on efficacy and tolerability of anti-CD70 maytansine (DM1)-based ADCs with non-cleavable linkers was investigated by Hamblett et al (Hamblett et al., 2016). These authors compared ADCs with wild type human IgG1 and a mutant version (H435A) with attenuated FcRn binding. Mutation of H435 to alanine (A) attenuated IgG binding to FcRn only at pH 6.0 without any impact on FcRn binding at physiological pH 7. Results of this study revealed that loss of FcRn binding impacted both efficacy (decreased) and tolerability (increased toxicity, more severe thrombocytopenia) of ADCs in SCID mice. Decreased efficacy is associated with rapid systemic clearance and decreased (~ 3fold) exposure (AUC) and shorter half-life of H435A mutant ADCs than their wild-type counterparts. The specific mechanism through which loss of FcRn binding increased

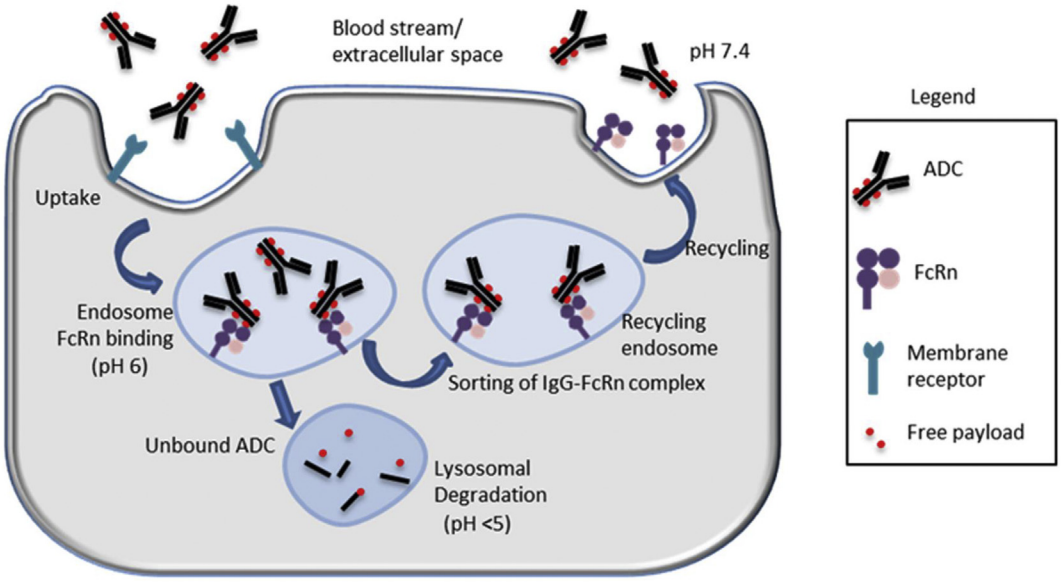

Fig. 4. Role of FcRn in ADC recycling

thrombocytopenia was not investigated, but considering the previously reported mechanism demonstrating differentiating megakaryocytes (MKs) as the primary targets for DM1-based ADCs (Uppal et al., 2015), it has been suggested that MKs express FcRn and are able to recycle wild-type ADCs to protect from lysosomal degradation and payload release. Therefore, loss of FcRn binding in experimental ADCs might have resulted in decreased recycling and increased lysosomal trafficking for degradation and cytotoxic payload (DM1) release in MKs contributing to a severe drop in platelets. Surprisingly, in this study the authors didn't report any effects of H435A mutation on other potential target organs (eg., Liver) for DM1 based ADCs.

Based on the results of Hamblett et al experiments using FcRn mutant tool ADCs and the known function of FcRn in normal cells, modulation of the IgG framework of ADCs to increase FcRn binding (specifically at acidic pH) appears to be a viable approach for decreasing target-independent toxicity in FcRn expressing normal cells, while retaining desired therapeutic efficacy in target cells. Fc engineering (Fc mutants) within the CH2-CH3 domains of IgG spanning the FcRn binding regions (H410, H435 and L253 residues) increases FcRn binding affinity and has been explored with relative success in extending serum half-life of IgG therapeutics reducing the needed frequency of administration and/or increasing efficacy. Although such engineered IgGs were shown to have extended half-life and increased efficacy in preclinical studies, they have not yet tested clinically (Pyzik et al., 2015). Recent studies demonstrated that the IgG charge distribution in the Fab region (distal variable fragment, Fv) is also involved in FcRn interaction (Schoch et al., 2015), which provides a potential non-Fc modifying approach to optimize FcRn affinity. It is noteworthy that cancer cells (solid tumor samples, cell lines and xenograft models) also express functional FcRn, which may regulate IgG tumor distribution, metabolism, and efficacy (Cianga, Cianga, Cozma, Ward, & Carasevici, 2003; Palma et al., 2011). Therefore the expression level of FcRn in target cancer cells (especially tumors derived from FcRn expressing cells) and its role in recycling of ADCs needs to be investigated further to clearly understand how ADCs with increased FcRn binding affect overall efficacy in target cells.

2.3.4. C type lectin receptors (CLRs)

It has been proposed that N-linked Fc glycosylation sites on IgG antibodies or the antibody component of ADCs can serve as recognition/binding ligands for CLRs (Fig. 3), providing a target-independent route for their internalization/uptake in normal cells (Fig. 1) (Boesch et al., 2014; Gorovits & Krinos-Fiorotti, 2013). Therefore, understanding CLR biology and expression patterns in normal cells/tissues may offer some insight into the mechanisms of commonly observed off–target ADC toxicities. CLRs comprise a large and diverse, yet highly conserved family of well characterized endocytic receptors. Type I CLRs are calcium-dependent, have multiple (6 to 8) carbohydrate recognition domains (CRDs) and may also contain cysteine-rich and fibronectin domains. Members include macrophage mannose receptor (MR, MRC1, CD206), Endo180 (CD280, MRC2, uPAR associated protein, uPARAP), DEC-205, PLA2R and DCL-1. Type II CLRs contain a single CRD and can be either calcium-dependent like Dectin 2, Mincle, CLECSF8, DCIR, DCAR, BDCA-2, DC-SIGN, MGL or calcium-independent like Dectin 1, CLEC5A, DNGR-1 (CLEC9A) (Chiffoleau, 2018).

CLRs can be both membrane-bound (primarily) or soluble /secreted and are often found mainly on myeloid lineage cells including tissue resident macrophages, monocytes, dendritic cells, and antigen-presenting cells where they have been most extensively studied (Chiffoleau, 2018; Lech et al., 2012). Increasing evidence also points to their functional expression in multiple normal epithelial and endothelial cells, including dermal microvascular endothelial cells (DMECs), liver sinusoidal endothelial cells (LSECs), perivascular microglial cells, glomerular mesangial cells, and corneal epithelial cells (Cummings & McEver, 2015; Linehan, Martinez-Pomares, Stahl, & Gordon, 1999). It is worth noting that, even though CLRs are constitutively expressed in various normal tissues, certain pathophysiological events such as inflammation and infection (fungal, microbial) have been shown to significantly modulate expression of these receptors (Chiffoleau, 2018; Lech et al., 2012; Wang et al., 2016). Important endocytic functions and constitutive expression of CLRs in several normal tissues, including commonly reported ADC target organs (liver, skin and cornea), suggests their potential role in mediating target-independent ADC internalization and toxicity in these tissues.

2.3.4.1. ADC toxicities associated with CLRs binding. Even though there is no definitive evidence to demonstrate a direct role of CLRs in off-target toxicity of ADCs, mannose receptor (MR)-mediated uptake has been proposed as a potential mechanism of hepatic toxicity of ADCs (Gorovits & Krinos-Fiorotti, 2013). Hepatic toxicity independent of target antigen expression has been reported for several ADCs (Masters et al., 2018) and these toxicities are consistently associated with initial insult to liver sinusoidal endothelial cells (LSECs), where MR expression might play a role (Gillespie et al., 2000; Guffroy et al., 2017; Tolcher et al., 2003). LSECs are highly specialized endothelial cells with a

known scavenger function to endocytose and remove soluble macromolecular tissue waste products via several high affinity scavenger receptors including MR (Elvevold et al., 2008). Significantly higher levels of lysosomal enzyme activities in LSECs compared to other hepatic cells, such as Kupffer cells and hepatocytes, may also further contribute to their high capacity degradation of endocytosed ligands (including ADCs) and release of degraded material/cytotoxic payload to the surrounding compartment (Elvevold, Nedredal, Revhaug, & Smedsrod, 2004; Knook & Sleyster, 1980). Interestingly, LSECs depend on MR-mediated uptake of lysosomal enzymes (glycoproteins) to maintain their high degradation capacity (Elvevold et al., 2008). Rapid internalization of MR-ligand complexes and quick recycling of MRs back to the cell surface after releasing ligands in the early endosome (Stahl, Schlesinger, Sigardson, Rodman, & Lee, 1980) may further contribute to the high continuous endocytic capacity of MR expressing cells. MR-mediated uptake is also an important mechanism for the clearance of endogenous and therapeutic glycoproteins and immunoglobulins in LSECs. For example, clearance of mannosylated antibody-enzyme fusion proteins is predominantly by endocytic MR in LSECs, suggesting an important role of MR in the clearance of bio-therapeutic compounds (Kogelberg et al., 2007). Therefore, based on the known MR-mediated scavenger function of LSECs and reported location of initial insult of hepatic toxicities of ADCs, MR-mediated uptake could potentially contribute to off-target hepatic toxicity. In addition, due to the presence of large fenestrae (~50 to 150 nm) surrounded by microtubule (actin) filaments, absence of diaphragm and lack of basement membrane, LSECs are the most permeable endothelial cell type in the body (Poisson et al., 2017). Therefore, scavenger receptor-independent diffusion of macromolecules (including ADCs) through membrane pores into LSECs may also potentially contribute to hepatic toxicity. Hence, additional in vivo investigative experiments are required to definitively identify MR-dependent or -independent uptake mechanisms contributing for hepatic toxicity of ADCs.

It is important to note that Kupffer cells also express MR (Schlesinger et al., 1978) and play an important role in non-specific uptake and processing of ADCs (Kraynov et al., 2016). Hence MR-mediated uptake of ADCs in Kupffer cells and release of cytotoxic payload into surrounding cells contributing to hepatic toxicity cannot be ruled out. In addition, certain CLRs and other endocytic scavenger receptors are constitutively expressed on the surface of several other normal cell types including corneal epithelial cells (Wang et al., 2016) potentially contributing to target-independent corneal (ocular) toxicity observed with several ADCs, but has not been shown to be causative.

2.3.4.2. Factors influencing IgG/ADC binding to CLRs. The glycosylation profile of IgGs (and potentially ADCs) is the key determinant for MR binding affinity. Studies have shown that modification of the glycosylation patterns of IgGs can affect the degree of their MR-mediated uptake in macrophages and dendritic cells (Dong, Storkus, & Salter, 1999). MR recognizes and binds N-glycosylation residues on the asparagine (Asn-297) amino acid within the CH2 domain of the Fc region of IgG/ADCs (Fig. 3) and its binding affinity is determined by the nature of the terminal sugar residue found on the Fc glycan (Allavena, Chieppa, Monti, & Piemonti, 2004). Binding affinity is highest for terminal mannose and N-acetylglucoseamine residues, but lowest for galactose residues (Arnold et al., 2007). Therefore, MR binding affinity is high for IgGs/ADCs, which lack terminal galactose (agalactosylated, G0F IgG) and expose high affinity sugar residues. Hence, understanding and regulating glycosylation profiles (either by modifying expression host system or by glyco-engineering) of IgGs may reduce MR binding affinity and off-target toxicity of ADCs in normal cells. It is also important that in addition to the IgG framework, the presence of any carbohydrate moieties on ADC payloads may also contribute to mannose receptor-mediated uptake of an ADC. Investigative in vitro and in vivo studies using ADCs with modified N-glycosylation profiles, CLRs blockers (blocking antibodies or pharmacological inhibitors e.g., mannan), or testing in specific knockout models could add value in confirming a potential role of CLRs in off-target toxicity from ADCs. Different tools to investigate the role of C-type lectin receptors (CLRs) in mediating ADC uptake and toxicity to normal cells are listed in supplementary Table 3. Limited availability of functional blocking antibodies, specific ligands or pharmacological inhibitors as well as potential compensation/redundancy by other receptor(s) both known and unknown in the same family may complicate the interrogation of all CLRs as mediators of non-target-mediated ADC uptake.

3. Summary

Despite considerable past efforts towards development of safer and more efficacious ADCs through selective targeting of a cytotoxic payload to antigens highly expressed on tumor cells, off-target dose-limiting toxicities (DLTs) continue to represent a significant challenge in the development of ADCs to treat cancer. These toxicities mainly include effects on different organs/tissues such as bone marrow/hematology, liver, eye, peripheral nerve, kidney, and serosal effusion (vascular leak syndrome) (Donaghy, 2016; Masters et al., 2018). As discussed throughout this manuscript, both receptor-dependent and -independent mechanisms may contribute to off-target ADC toxicities. These mechanisms may significantly vary from one cell/tissue type to another depending on expression of key candidate receptors and functional specialization (e.g., rate of non-specific endocytosis). Even though common DLTs including thrombocytopenia (FcγRIIa-mediated or macropinocytosis-mediated), ocular toxicity (macropinocytosis-mediated), neutropenia (extracellular protease-mediated), liver injury (mannose receptor-mediated) and peripheral neuropathy (membrane permeable free payload in circulation) have been associated with potential mechanisms of ADC/payload uptake, these associations are either not always supported by strong experimental data or not consistent between reports (Gorovits & Krinos-Fiorotti, 2013; Polakis, 2016; Uppal et al., 2015; Zhao et al., 2018; Zhao, Gulesserian, Ganesan, et al., 2017; Zhao, Gulesserian, Malinao, et al., 2017). It is also possible that more than one mechanism of target-independent ADC uptake or a combination of both intact ADC and free payload uptake contribute to specific DLTs. Additionally, mechanisms of uptake contributing to other delayed clinical toxicities involving multiple organs/tissues, such as capillary leak syndrome or serosal effusions (pleural and pericardial) and peripheral edema with PBD and duocarmycin based ADCs (Owonikoko et al., 2016; Rudin et al., 2017; Stein et al., 2017), are still not understood. These multi-organ toxicities may be consequent to conjugated ADC or free payload uptake in organ specific vascular endothelial cells or secondary to toxicity in normal tissues/organs elsewhere in the body.

ADC technology has improved modestly across multiple generations and different approaches have been considered to address associated toxicities including optimization of the IgG framework, linker chemistry, and DAR thereby decreasing non-specific uptake into normal cells/tissues (Beck et al., 2017). Other potential approaches being investigated to improve therapeutic value includes assessing alternative formats/platforms of IgG and technologies such as Fc engineering, masking peptide technology (e.g., pro-bodies), single-chain variable fragments (scFvs) or dual variable domains (DVDs), which may further provide new opportunities to improve the TI of ADCs (Beck et al., 2017; Hendriks, Choi, de Bruyn, Wiersma, & Bremer, 2017). In addition, modification of physiochemical properties of ADCs is also a useful approach to favorably alter the PK/distribution of ADCs and create improved safety profiles. Specifically, overall surface charge of ADC may influence multiple potential target-independent uptake mechanisms discussed in this paper; hence charge modifications through antibody engineering or chemical modifications to decrease ADC uptake into normal cells, although incompletely understood at this time, is another exciting strategy to consider. Some of the ADC toxicities may also be driven by Cmax-dependent tissue disposition of ADCs. Hence, dose modification/

fractionation to optimize peak (Cmax) versus sustained systemic (AUC) exposures has been employed, with variable success, in an attempt to prolong the dosing period to decrease off-target toxicity while maintaining efficacy (Hinrichs et al., 2017). This avenue of research may yet bear beneficial fruit as well as for improving ADC selectivity. Ultimately, a better understanding of the potential mechanisms of target-independent ADC toxicities discussed in this paper and factors influencing those mechanisms may improve optimization of all three components of the ADC (mAb, payload and linker) to achieve better safety profiles.

Overall, it has become clear that selectively delivering payloads to target expressing tumor cells with ADCs is much more complicated than originally anticipated. Simply selecting antibody targets that are highly expressed in tumors with little/no expression in normal tissues has not proven sufficient to provide selective in vivo delivery of ADC payloads to tumor cells while minimizing toxicity to normal cells. Multiple avenues for target-independent (non-specific) ADC entry into normal cells have been described, which likely vary across cell types and depend on individual characteristics of the ADCs themselves. It also appears increasingly less likely that a single universal uptake mechanism is responsible for target-independent ADC uptake in normal cells. Not all parameters influencing non-specific ADC uptake have been identified to date and it appears that the complexity will continue to expand the more we know. However, there have been significant successes despite the challenges and interest in ADCs as potential therapeutic agents remains high.

Conflict of interest statement

All authors are or were former employees of AbbVie and may own AbbVie stock. Kenneth Durbin is currently an employee of Proteinaceous Inc. AbbVie sponsored and funded the study; contributed to the design; participated in the collection, analysis, and interpretation of data, and in writing, reviewing, and approval of the final publication.

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Appendix A. Supplementary data

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