Function and therapeutic potential of intracellular antibodies

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Abstract

Therapeutic antibodies targeting disease associated antigens are key tools in the treatment of cancer and autoimmunity. So far, therapeutic antibodies have targeted antigens which are, or are presumed to be, extracellular. A largely overlooked property of antibodies is their functional activity inside cells. The diverse literature dealing with intracellular antibodies emerged historically from studying the properties of some autoantibodies. The identification of TRIM21 as an intracellular Fc receptor, which links cytosolic antibody recognition to the ubiquitin proteasome system, brings this research into sharper focus. We review critically the research related to intracellular antibodies, link this to the TRIM21 effector mechanism and highlight how this work is exposing the previously restricted intracellular space to the potential of therapeutic antibodies.

Autoantibodies: friend or foe?

Antibodies targeting self-cellular components, autoantibodies, are a defining feature particularly of the autoimmune rheumatic conditions systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS). Tissue specific autoimmune diseases, exemplified by multiple sclerosis (MS) and type I diabetes (T1D), also present with prominent and disease specific autoantibody profiles to self-antigens, many of which are intracellular. Autoantibodies have been studied extensively as a route to understanding the selective breakdown of self-tolerance which this phenomenon clearly illuminates. Generally considered to be a secondary manifestation of underlying regulatory defects in antigen presentation, perhaps linked to a dysregulated immune response to apoptotic and/or necrotic cell death, autoantibodies historically were considered to be contributors rather than primary mediators in disease initiation. However, some autoantibodies have been linked definitively with pathology, for instance in myasthenia gravis and Graves' disease.

In contrast to the perceived limited functional role of autoantibodies, they may in fact be revealing unrecognised capabilities of humoral immunity. The properties of disease-ameliorating anti-IFNα autoantibodies detected in AIRE-deficient patients is consistent with this more nuanced assessment [1]. Natural antibodies, where antigen binding CDR3 regions are germ-line encoded un-mutated V(D)J gene segments, are often directed to self-antigens and are prevalent in healthy individuals [2]. Autoantibodies are associated with many diseases, including cancer and Alzheimer's disease [3]. Their presence in serum often predates overt symptoms by many years, adding the need for vigilance to their long standing use in disease definition/classification [4-6]. The reduced rates of some cancers observed in autoimmune disease patients has led to speculation of possible beneficial effects of autoantibodies with precise specificity [7].

Identification of cell penetrating autoantibodies

The idea that autoantibodies binding nuclear antigen (ANA), notably DNA or the so-called ribonucleoprotein particles, could penetrate inside cells, enter the cell cytoplasm and modulate function, was first reported by Alarcon-Segovia et al in a series of papers beginning in

the 1970s [8-10]. Variable effects on cell viability by cell penetrating autoantibodies were claimed subsequently by a number of laboratories, using different *in vivo* cell culture systems [11-13]. Cell penetration was a property of some mouse and human monoclonal autoantibodies and was not isotype restricted [14]. Uptake of IgG autoantibodies into various cell types, including mononuclear cells, lymphocytes, hepatocytes, epithelial cells and neurons [10, 12, 15, 16] was proposed initially to be via Fc gamma receptor mediated endocytosis [17]. Other mechanisms of uptake have been described [18-20]. However, it is clear that in much of these early data, there is insufficient resolution to determine whether antibodies are located in the cytosol and therefore whether antibodies have actually traversed the cell membrane.

Because of the generally variable and confusing reports and notwithstanding the possibility of cell culture or fixation artefacts, much of this work was overlooked [21]. An intracellular function for antibodies was also counter to the prevailing view of humoral immunity focusing exclusively on extracellular antigens. Critically, being able to distinguish between true intracellular localisation for antibody in the cytosol and merely compartmentalisation within endosomes/lysosomes turned out to be difficult to achieve experimentally [22]. The action of conventional Fc receptors, particularly FcRn for example, could account for localisation and retention within the endolysosomal compartment. Antibody engagement by other signalling Fc receptors, expressed on a variety of lymphoid and myeloid cells (but not generally by epithelial or endothelial cells) could account for additional functional consequences in some experiments. Instead, it was considered that cell penetration by autoantibodies was likely to be a rare or aberrant phenomenon, which nevertheless could have important consequences for autoimmune disease, by influencing tolerance to intracellular self-antigen and by initiating apoptotic cell death [9, 11, 23]. Cell penetration has also been proposed as an intrinsic property of some germ-line encoded natural autoantibodies, which, if correct, has led to speculation of additional immunological functions for these molecules [2, 24, 25]. Importantly, it was also recognised that delivery of antibodies into the cell could open up new therapeutic areas [23].

Characteristics of intracellular and cell penetrating antibodies

Several strands of research address the functional capabilities of antibodies inside cells and the properties which contribute to stability and cell penetration. Much of this effort has been driven by their therapeutic potential.

Intrabodies are antibody fragments composed of linked variable region heavy and light chains used to target intracellular proteins [26]. Targeting of disease specific protein variants using intrabodies for therapeutic purposes has been effective in cell culture [27-29]. Screening of recombinant antibody libraries for optimisation of intrabody structure with enhanced binding characteristics has been used [30-32]. The reducing environment inside cells negatively impacting disulphide bridge formation, as well as improper glycosylation, have been seen as a key factors affecting IgG structure and function in the cytosol [33]. Introduction of intrabodies usually requires expression of DNA plasmids encoding antibody sequences within the target cell, either by transfection or by gene therapy approaches using adeno-associated virus in the whole animal [34]. Chemical modification to aid delivery or covalent fusion of antibody molecules with cell-penetrating peptides, such as HIV-1 TAT, have also been used, as well as modification with amino acid motifs which support localisation to distinct subcellular compartments, for example endoplasmic reticulum retention using the KDEL motif [35-37]. The efficiency of some of these mechanisms of introduction has been questioned [22]. Therefore, while intrabodies demonstrate the effectiveness of antibodies inside cells and offer a means for optimisation of stability and binding characteristics, the technology is at present limited by the difficulties of first delivering them into the cytosol.

Cell-penetrating autoantibodies have been identified, with many of the reports focusing particularly on the properties of anti-DNA monoclonal autoantibodies. Sequencing studies of V_H regions of cell-penetrating anti-DNA autoantibodies showed a preference for charged amino acids in the antigen binding (CDR3) region [38, 39]. Defined motifs were not identified, admittedly from comparison of a relatively small number of anti-DNA monoclonal antibodies (n=6), although it was proposed that the position of a number of arginine residues could resemble nuclear localisation signals and thereby influence trafficking [40]. A single human IgG monoclonal anti-DNA autoantibody, clone 3E10, has been studied in detail [39, 41-43].

Antibody Fab and single chain Fv fragments (scFv), a construct similar to intrabodies composed of only the variable heavy and light, V_H and V_L, segments from clone 3E10, efficiently penetrated into cells and in addition localised to the nucleus. Localization therefore was a property of the antigen binding region of 3E10 and independent of Fc receptors [38, 44]. Mutations which enhanced the ability of 3E10 scFv fragments to penetrate cell nuclei and precipitate DNA damage have been identified and 3E10 appeared to be particularly toxic to cancer cells with dysregulated DNA repair pathways, for instance with BRCA2 mutations [45, 46]. Cellular uptake into tumour cells of 3E10 scFv was enhanced by the presence of extracellular DNA [39], although 3E10 also selectively immunoprecipitated heavy chain of myosin IIb on the cell surface of muscle cells, implying both a role for this protein in cell uptake but also a degree of cross- or poly- reactivity for the antibody [47]. Purified 3E10 IgG has been investigated as a potential therapeutic agent in its own right and as a delivery vehicle for covalently attached moieties [48]. Bivalent or bispecific antibody derivatives which take advantage of the cell-penetrating properties of 3E10, but which target, in addition, disease associated protein variants, have been shown to be effective [49, 50]. A large literature therefore reports the investigation of cell penetration and intracellular localisation, particularly of anti-DNA autoantibodies. However, the requirements for cell penetration have not been formally defined and the caveats we have outlined of proving true cytosolic delivery in most cases remain. Also, it is not clear whether the characteristics of these antibodies are rare or unique and therefore not representative of normal antibody function.

A murine anti-DNA autoantibody with unique characteristics, clone 3D8, has been used to develop whole IgG antibodies reportedly capable of cell penetration, called cytotransmabs [51]. In common with clone 3E10, 3D8 scFv internalized into living cells, but was capable of hydrolyzing DNA and RNA and of localization to the cell cytoplasm [52]. Cell penetration by 3D8 was reportedly mediated by the single V_L segment [53]. The 3D8 V_L segment was used as a targeting adaptor by engineering it into the human therapeutic antibodies adalimumab (Humira®) and bevacizumab (Avastin®). The resulting humanized recombinant chimaeric antibodies were efficiently internalized into the cytoplasm in live cells, while maintaining binding affinity for their target antigen, tumour necrosis factor alpha (TNF-α) and vascular

endothelial growth factor A (VEGF-A), respectively [51]. Cytotransmabs were reported to be internalized by endocytosis through interactions with cell surface heparin sulfate proteoglycan and to escape from early endosomes into the cytosol where they were stable for more than 6 h. Degradation of internalized cytotransmabs was inhibited by drug MG132, indicating the involvement of proteasomes in their turnover [51]. Again, however, the data presented in these reports do not provide sufficient resolution to prove definitively cytosolic antibody delivery and the underlying molecular mechanism by which cytotransmabs escape from early endosomes into the cytosol is not addressed.

In vitro screening systems have been developed to optimize cytotransmabs for stability and cell-penetration, as well as to identify the cellular factors involved. A method that enables direct assay of the efficiency of antibody penetration into HeLa cells based on split GFP complementation has been developed for these purposes [54]. Such systems show cytosolic antibody delivery, but at low frequency [22, 51]. A cell-free protein synthesis system was also developed in order to screen large numbers of antibodies for enhanced cytosol-penetrating characteristics [55]. Clearly, if the claims presented in these reports can be verified, then this technology will have important practical applicability for therapeutic antibody production, by the direct targeting of disease associated protein variants in the cytosol [51, 55].

TRIM21 is a cytosolic Fc receptor required for antibody-dependent pathogen restriction

During infection, antibodies are delivered efficiently to the cytosol when bound to obligate intracellular pathogens, such as viruses and bacteria [56, 57]. Once in the cytosol, antibodies contribute to innate immune signalling through NFkB and IRF3 pathway activation. They also neutralise pathogens via proteasome-dependent degradation. These sensor and effector functions are dependent on recruitment of the cytosolic antibody receptor TRIM21 [58]. The terms antibody dependent intracellular neutralisation (ADIN) and intracellular antibody-mediated degradation (IAMD) have been coined in order to describe the TRIM21 antibody effector mechanism [59].

TRIM21 is a member of the tripartite motif (TRIM) protein family of RING E3 ubiquitin ligases. TRIM21 was first identified as an antibody binding protein of unusual structure by yeast

two-hybrid analysis and by investigation of its auto-antigenic characteristics compared to other related TRIM proteins [60, 61]. The TRIM21 interaction with immunoglobulins was found to occur with specificity and extremely high affinity, requiring a complex tertiary fold of the carboxyl-terminal B30.2 domain of TRIM21, binding to residues on Fc from both C_{H2} and C_{H3} [62, 63]. The TRIM21/Fc interaction exemplifies a general mechanism of B30.2 domain binding to complex conformational epitopes inside cells. Other examples identified so far, are retroviral restriction factor TRIM5α targeting the HIV capsid and the $\gamma\delta$ T cell receptor BTN3A1 binding of bacterial phosphoantigen [64-66]. These molecules link specific recognition of unusual intracellular antigenic epitopes to potent effector functions.

The precise molecular mechanisms involved in antibody bound pathogen restriction by TRIM21 are complex and involve a number of essential cofactors [67]. The first step in TRIM21 activation is recruitment of the E2 enzyme Ube2W and catalysis of auto-mono-ubiquitination [68]. The E2 heterodimer Ube2N/2V2 is then recruited, which uses the mono-ubiquitination to prime anchored lysine-63 poly-ubiquitin chain extension. Ubiquitinated TRIM21, like TRIM5, is a substrate for the proteasome and both proteins depend on proteasomes to degrade targeted viral complexes. Lysine-63 poly-ubiquitin chains are subsequently liberated, a critical step linked to proteasome recruitment, which simultaneously contributes to the initiation of innate immune signalling [68]. TRIM21-mediated degradation of virus also requires the unfoldase and segregase enzyme p97/VCP [69].

TRIM21 exhibits broad antibody class specificity, binding with high affinity to IgG, IgM and IgA, a characteristic unique to this Fc receptor [70]. By targeting structural elements of non-enveloped viruses for degradation, TRIM21 allows for recognition of viral nucleic acid by host cytosolic DNA sensors such as cGAS and the RNA sensor RIG-I, to contribute to the triggering of innate immune signalling [71]. Biophysical characteristics of an anti-adenovirus capsid monoclonal antibody, 9c12, has allowed the kinetic and thermodynamic requirements for efficient TRIM21 recruitment and effector function to be determined [72]. The physiological relevance of these processes has been demonstrated using TRIM21 -/- knockout mice, which are more susceptible to lethal adenovirus infection [73].

TRIM21 and regulation of autophagy

The elegant molecular and structural studies of TRIM21 function show, that upon infection, TRIM21 links immunoglobulin dependent recognition of virus in the cytosol to innate immune signalling and virus degradation contingent on proteasomes. In a separate line of research, a number of TRIM molecules, including TRIM21, have been linked to autophagy, a system for controlled degradation of cell components and of invading microorganisms, distinct from proteasome function [74]. TRIM21 has been shown to interact with ULK1, ATG16L1 and BECLIN1 (BECN1), key components for autophagosome assembly, and target cytosolic IRF3 for degradation by a process termed precision autophagy [75, 76]. So far, no mechanistic link to autophagy has been made for cytosolic immunoglobulins, although IRF3 is shown to bind to the TRIM21 B30.2 domain. It will be important to resolve these two functions for TRIM21 and to assess whether cytosolic immunoglobulin can influence cellular degradation mechanisms through these two mutually exclusive systems [75].

Therapeutic implications of intracellular antibodies

There are obvious intracellular protein variants, associated particularly with cancer and with neurodegenerative disease, that make attractive therapeutic targets for antibodies [33, 77, 78]. A bispecific scFv antibody linking the cell-penetrating anti-DNA antibody 3E10 to target MDM2 protein in the nucleus has been produced [50] and intrabodies expressed in cells have been used to disrupt the function of p53, p21Ras and BCR-ABL oncoproteins [30, 32, 35]. Cytotransmab functionality has been engineered into the commonly used human therapeutic antibodies adalimumab (Humira®) and bevacizumab (Avastin®), targeting TNF-α and VEGF-A, a strategy which offers the potential to block these proteins before their extracellular release [51]. Intracellular antibodies optimised for binding to the microtubule-associated protein TAU, found in neurofibrillary lesions of the brains of Alzheimer's disease sufferers have been investigated [79]. Recent data show that incoming TAU seeds are subject to degradation by antibody dependent recruitment of TRIM21, preventing them from triggering aggregation of soluble TAU protein [80].

These various reports show the potential for intracellular antibodies to disrupt function inside cells and could be used to identify and optimise relevant cellular machinery. Importantly, some reports now show therapeutic effectiveness in the whole animal. Antibodies to intracellular proteins delivered systemically have been shown to target intracellular antigen and prevent tumour growth in an *in vivo* system [81]. Three different immunogenic targets were used to assess the effectiveness of systemic antibody delivery. The three intracellular proteins used in the tumour vaccine studies were cancer-associated protein tyrosine phosphatase of regenerating liver 3 (PRL3), the polyomavirus middle T oncoprotein (mT) and the general reporter green fluorescent protein (EGFP). Tumour cells expressing these intracellular proteins, either endogenously or by over-expression, were inhibited by their respective exogenous antibodies [81]. Remarkably, tumour growth was also inhibited by host antibodies induced by vaccination with the appropriate antigen [81].

Further evidence of specificity in this therapeutic approach has been presented. Two mouse melanoma cell lines with different PRL3 expression levels, B16F0 and B16F10, were used to induce tumours in mice. Tumour metastasis was inhibited by a humanized recombinant anti-PRL3 antibody, with the level of inhibition correlating with PRL3 protein expression in the two cell lines [82]. Inhibitory effects of anti-PRL3 antibody on tumour metastasis induced by human cell lines with high endogenous PRL3 expression, including colorectal cancer cell line HCT116 and ovarian cancer A2780, was also shown [82]. Inhibitory effects of the antibody were not seen using lung cancer line NCI-H460 which does not express PRL3, consistent with the notion that the efficiency of PRL3 antibody treatment correlated with PRL3 protein expression in the tumour. Similar experiments were used to show the potency of specific antibodies directed to other members of the PRL family of proteins, with PRL1 antibody specifically blocking metastatic tumour formation by PRL1, but not PRL3, expressing cells, and *vice versa* [83].

Concluding Remarks and Future Perspectives

During infection antibodies access the cytosol when bound to viral or bacterial pathogens. In the absence of infection, cells are either intrinsically or transiently permissive for

antibody penetration, or alternatively, cell penetration is a property of (presumably) a minor fraction of antibodies.

The identification of intracellular TRIM21-dependent antibody recognition necessitates a re-assessment of the literature regarding the function of antibodies inside cells. It is possible that the potent effects of these antibodies have been overlooked because only a few antibody molecules are required to elicit TRIM21 effector function and cell penetration by antibody is normally infrequent [72]. Historically, there has been speculation of a mechanism of intracellular pathogen restriction by antibodies [84]. For example, anti-Sendai virus IgA monoclonal antibody neutralized Sendai virus in the cytoplasm [85]. The identification of the TRIM21 mechanism provides a molecular explanation for some of these observations. The confusing and often contradictory effects of intracellular antibodies on cell function and viability, particularly the work on autoantibodies, could be interpreted as variation in efficiency of TRIM21 recruitment. Different antibody molecules, antibody derivatives and cells have been used. Effects of antibody isotype and allotype on TRIM21 binding were detected using defined variants of humanized recombinant IgG CAMPATH-1H (anti-CD52) in initial experiments [61]. It is likely that polymorphisms within TRIM21 will also affect its function.

Theoretically, all antibodies capable of penetrating into the cell cytoplasm will engage TRIM21 and target intracellular antigens for degradation. However, the antibody effector mechanism is not fully understood and it is not yet clear what structural requirements allow for TRIM21 activation. Although TRIM21 appears to be expressed ubiquitously in cells and tissues, immune signalling will be induced dependent only on the availability of accessory factors, which may not be present [58, 68]. The role of TRIM21 in selective autophagy of activated IRF3, acting as a negative regulator of innate immune signalling, also requires further investigation in terms of antibody binding [75, 76].

TRIM21 (Ro52) is a prominent autoantigen in systemic lupus erythematosus, suggesting a link between autoantibody production and TRIM21 restriction. TRIM21 autoantibodies (anti-Ro52) could be an attempt by humoral immunity to enhance TRIM21 function, perhaps precipitated by cryptic viral infection for which the TRIM21 mechanism has presumably evolved to combat. It is notable that TRIM21 binds Fc in a region at the C_{H2}-C_{H3} interface that overlaps a

number of bacterial and virus encoded Fc receptors [86]. Convergent binding by molecules which are structurally distinct could imply pathogen interference with TRIM21 recruitment. The same argument goes for all autoantibodies which target intracellular components, suggesting more specific roles in autoimmune pathology than previously thought. The notion that some natural antibodies could be engaging TRIM21 suggests additional potential in pathogen restriction and immune regulation for the antigen inexperienced humoral immune response [87].

Reports of the targeting of tumours with antibodies to intracellular antigens also necessitates a re-appraisal of intracellular oncoproteins as targets for anticancer therapy, although a number of questions remain [81]. The effects documented did not appear to be restricted by the antigen, as different molecules were targeted, even the cell marker EGFP which is not functionally related to tumorigenesis. It is unclear what properties of the antibodies allowed for intracellular delivery in these experiments, nor which effector mechanisms are involved. There are reports of broader protective effects of naturally occurring autoantibodies in disease, for example in HER2/neu positive breast cancer [88] and the possibility exists that diseased cells become transiently permissive for antibody uptake. If they can be verified, the data suggest that focusing on extracellular molecules for therapeutic antibodies, is over-restrictive. It will also be important to discover whether the TRIM21 effector mechanism is involved and if so whether it can be optimized for therapeutic benefit. Finally, these new data may help us to understand why the prevalence of certain cancers, notably breast, ovarian and endometrial, is reduced in patients with SLE [89, 90].

Figure 1 IgG structure

Diagram of antibody IgG structure highlighting antibody fragments and the site of TRIM21 binding. TRIM21 binds via its carboxyl terminal B30.2 (also termed PRYSPRY) domain to the Fc portion of IgG at residues overlapping the C_H2 and C_H3 domains. TRIM21 is believed to form a head-to-tail dimer which binds both immunoglobulin G heavy chains simultaneously. Presence/absence of N-linked glycosylation did not affect TRIM21 interaction [61].

Figure 2 Intracellular antibodies recruit TRIM21

Schematic representation of the possible effects of intracellular antibodies and TRIM21 recruitment. Antibodies are delivered efficiently to the cytoplasm when bound to infectious micro-organisms. Inside cells, antibodies recruit the cytosolic Fc receptor TRIM21. A potent restriction mechanism is activated, resulting in proteasome dependent degradation of the antigenic target. Innate immune signalling via transcription factors NFkB and IRF3, induced in part, by free lysine-63 linked polyubiquitin chains (modified from [67]). In addition to proteasome activation and innate signalling, TRIM21 has been linked to the initiation of autophagy, resulting in the compartmentalisation and degradation of activated IRF3, thereby limiting immune signalling (modified from [76]. So far, cytosolic immunoglobulins have not been linked to TRIM21 dependent autophagy. Some antibodies, particularly autoantibodies, penetrate into the cell cytoplasm in the absence of infection. It is not known by what mechanism this is achieved nor whether cell penetrating (auto)-antibodies recruit TRIM21 and activate similar molecular programs.

Trends

Monoclonal antibodies targeting extracellular antigens are well established as key therapeutic tools in cancer and autoimmunity. Prominent examples include anti-HER2/neu (Herceptin®) in the treatment of breast cancer, anti-TNFα (Humira®) used in rheumatoid and psoriatic arthritis and anti-VEGF-A (Avastin®) used for various types of cancer and (off-label) in age-related macular degeneration (wet-AMD).

The majority of disease associated protein variants are arguably found inside cells. The function of antibodies inside cells is being investigated in order to target intracellular components for therapeutic benefit.

To be therapeutically effective, the mechanism(s) by which antibodies cross the cell membrane and penetrate the cell cytoplasm need to be identified and optimized. Some autoantibodies which target intracellular components can penetrate inside cells and are being optimised for efficient intracellular delivery.

Antibodies are carried efficiently inside the cell when bound to infectious micro-organisms, where they recruit the cytosolic Fc receptor TRIM21.

TRIM21 links Fc mediated antibody recognition to the ubiquitin proteasome system, a general mechanism of immune-surveillance coupled to innate immune signaling and antigen degradation. TRIM21 has also been linked to initiation of autophagy.

Antibodies to some intracellular proteins delivered systemically *in vivo* are reported to be therapeutically effective.

Outstanding Questions

How may antibodies, particularly autoantibodies, penetrate inside cells?

Does cell penetration represent a normal function of some antibodies, or is this an aberrant characteristic of rare autoantibodies only?

What characteristics of either cells or antibody allow for cell penetration?

Can cell penetration be optimised to allow for efficient antibody delivery to the cytosol?

Cytosolic Fc receptor TRIM21 has been linked functionally to proteasome dependent degradation of virus and to autophagy.

Do all cytosolic antibodies engage TRIM21? Are intracellular antigens bound by antibody degraded by the proteasome or by autophagy?

Do all intracellular antibodies induce innate immune signalling?

The reported therapeutic benefit in tumour metastasis for antibodies targeting intracellular antigens needs to be confirmed.

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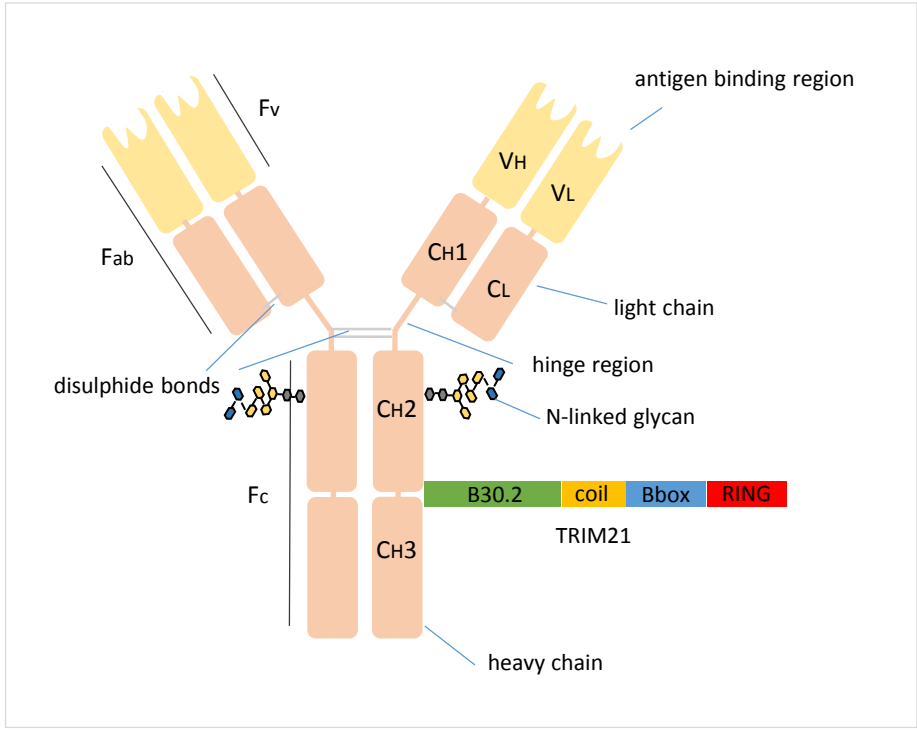
Figures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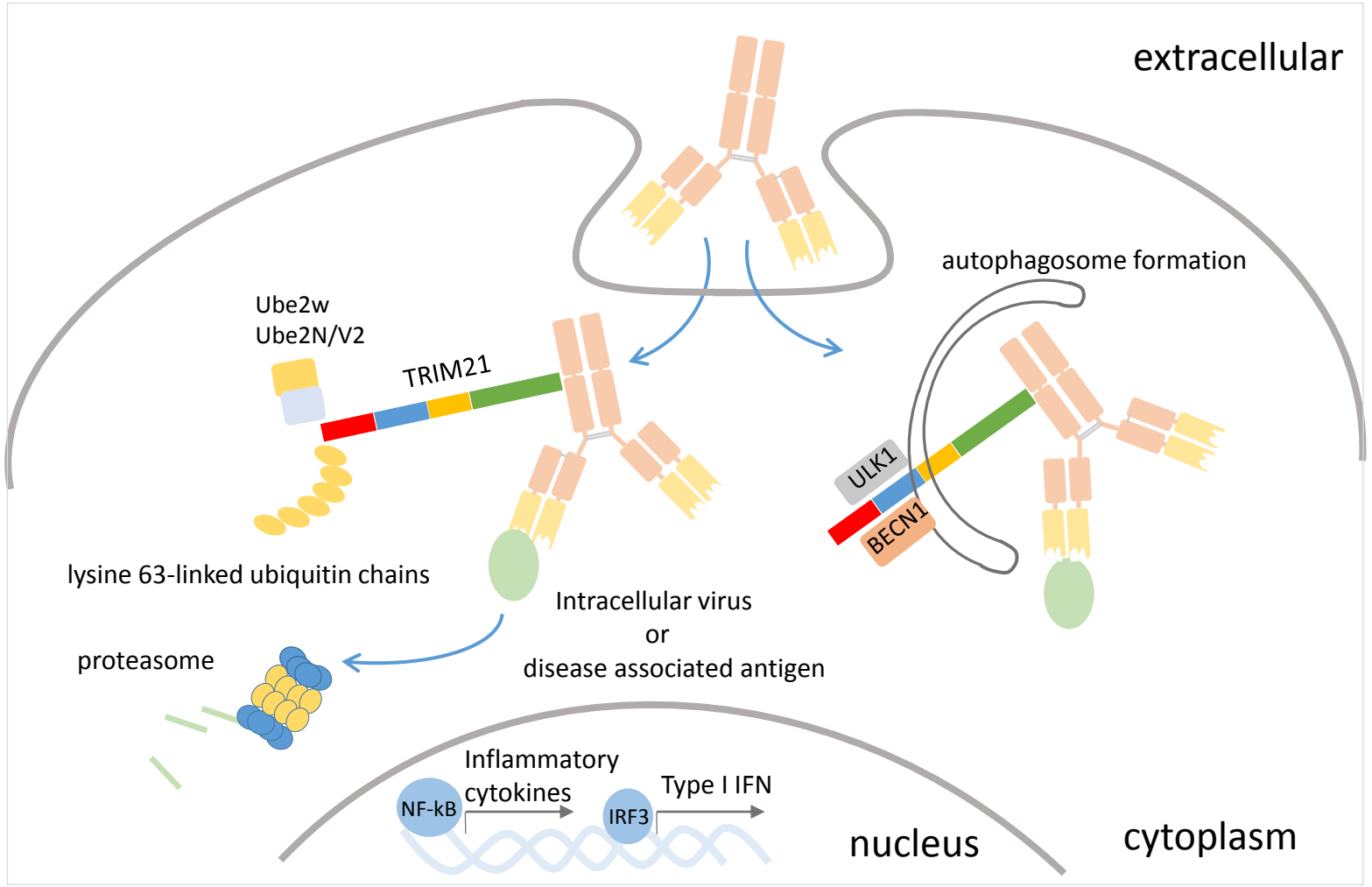
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