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Effect of galectin-9 on Immunoglobulin D surface binding and internalization in pre-basophilic and T cell lines

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Effect of galectin-9 on Immunoglobulin D surface binding and internalization in pre-basophilic and T cell lines

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A thesis submitted in fulfilment of the requirements for the degree
Doctor of Philosophy in Immunology at King's College London

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April 2023

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Statement of originality

I hereby declare that the work contained in this thesis has not been previously submitted for a degree or diploma at any other higher education institution. To the best of my knowledge and belief, this work contains no material previously published by another person except where due references are made.

1. Abstract

Immunoglobulin D remains the only antibody class with unknown specific function and structure. It was long thought to simply be expressed as a B cell receptor alongside IgM on B cell surfaces. The discovery of soluble IgD, as well as its elevated presence in certain inflammation-driven human conditions such as asthma and rheumatoid arthritis, has shifted this paradigm and has raised many questions regarding its exact role in health and disease. In recent years, IgD has been ascribed both an innate-like function, being able to bind bacterial superantigens, as well as involvement in hypersensitivity reactions to common antigens, through the action of a yet uncharacterized receptor (IgDR) on effector cell types. Thus, IgD may facilitate the link between the microbiome and asthma development, which has been observed in the past. Recent evidence has suggested the hyaluronan receptor CD44 and its ligand – the immunomodulator galectin-9 – as parts of the putative IgDR, engagement of which leads to immune response enhancement.

In this project I examined the IgD-binding and internalization capabilities of the human pre-basophilic KU812 cell line (CD44⁺) and the human T cell line Jurkat (CD44⁻). A custom IgD fluorescent probe was developed and used in conjunction with flow cytometry and confocal fluorescent microscopy. Flow cytometry showed relatively weak, temperature-dependent binding of IgD to the two cell types, which was markedly enhanced by galectin-9 addition. It is here demonstrated for the first time, via confocal microscopy, that labelled-IgD is internalized and accumulated into early and/or recycling endosomes in the perinuclear region of these cells. This internalization is inhibited by exogenous galectin-9, which retained IgD to the surface. Proteins from cell lysates captured via IgD-pulldown were analyzed by LC/MS for candidate receptor identification. Although speculative, according to the LC/MS data, the glycoproteins CD59 and CD82 seem to be the most promising putative IgD-binding partners in untreated and galectin-9-treated cells, respectively. Taken together, these findings point to a modal function of IgD in effector cells, which is dependent on the presence of inflammation-inducible proteins such as galectin-9.

2. List of commonly used abbreviations

AF – Alexa Fluor

AID – activation-induced deaminase

BAFF – B cell activating factor

BCR – B cell receptor

bp – base pairs

CD – cluster of differentiation

C δ – immunoglobulin heavy chain δ constant domain

CSR – class-switch recombination

δ chain – the heavy chain of IgD

DMSO – dimethyl sulfoxide

Fab – antibody fragment consisting of C_L-V_L and C_{H1}-V_H; antigen-binding region

Fc – fragment crystallizable; tail region of antibody, imparts effector functions

Fc ϵ R1 – high-affinity IgE receptor

Fc ϵ R2 – low-affinity IgE receptor (CD23)

Gal - galectin

GC – germinal centre

HPLC – high performance liquid chromatography

Ig – Immunoglobulin

IgDR – Immunoglobulin D receptor

kDa – kilodalton (1000 atomic mass units)

LC/MS – liquid chromatography and mass spectrometry

MID – *Moraxella Catarrhalis* IgD-binding protein

MID-FP – a protein construct consisting of MID conjugated to a fluorescent protein

MW – molecular weight

N-linked – carbohydrate attached to protein via an asparagine residue

O-linked – carbohydrate attached to protein via threonine or serine residue

PBS – Phosphate-buffered saline

PIPE – polymerase incomplete primer extension

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SPR – surface plasmon resonance

TBS – Tris-buffered saline

TLR – Toll-like receptors

V_H – variable domain of heavy chain

V_L – variable domain of light chain

3. Introduction

3.1 Brief overview of IgD research

Immunoglobulin D was first discovered in 1965 thanks to the study of an irregular multiple myeloma, which was characterized by the presence of a protein different from the known IgA, IgG, or IgM at the time (Rowe and Fahey 1965) . For quite a while after its discovery, IgD was simply considered to shadow the function of IgM as a mature naïve B cell antigen receptor, produced through alternative splicing (Maki et al. 1981), and was not thought to have any additional integral functions in adaptive immunity, mainly because B cells from IgD⁻ transgenic mice were found to retain their humoral immune responses (Nitschke et al. 1993).

In the following couple of decades, IgD's expression was confirmed in several mammalian species such as mice rats, dogs, and primates, but could not be detected in other mammals or birds (Preud'homme et al. 2000). The notable differences in size and sequence between IgD orthologs, as well as its inconsistent presence across species, hinted at a relatively recent evolutionary emergence of this class of immunoglobulins (Chen and Cerutti 2011). The following advances in genetic sequencing and data mining uncovered that IgD's presence was much more ubiquitous than previously thought, and, most interestingly, that its orthologs could be found in species which had diverged several hundred million years ago such as bony and cartilaginous fishes, reptiles, amphibians, and in birds (Han et al. 2016).

The omnipresence of IgD in most vertebrates suggests that, like IgM, it had evolved around the dawn of the adaptive immune system – roughly about 500 million years ago (Ohta and Flajnik 2006).

In more recent years, since around the mid-1990s, an increasing number of clues about the function of IgD have been cropping up. First, T cells were found to bind and respond to IgD (Tamma et al. 2001) via an uncharacterized carbohydrate-binding (lectin) receptor. Notably, increased levels of plasma IgD have been detected in atopic individuals (Peng, Fisher, and Adkinson 1991). Several binding partners of the immunoglobulin have also been documented, including an endogenous lectin involved in inflammation -

galectin-9 (Shan et al. 2018), and several bacterial superantigens (Samuelsson et al. 2006). The specific localization of non-serum IgD to the gut mucosae of fish and in the upper respiratory mucosae of mammals (Chen et al. 2020) have also hinted at IgD's involvement in an innate-like microbial surveillance system (state of "armed peace", discussed later). The source of soluble IgD at mucosal sites was found to be sequestered autoreactive anergic IgD^{high} / IgM^{low} B cells (Pugh-Bernard et al. 2001). Following the study of IgD-class switching (Arpin et al. 1998), and the discovery of allergen-specific IgD in human samples (Zhang et al. 1994), and that certain cell types can act as IgD-effector cells (Sechet et al. 2003), significant leaps in the general understanding of the enigmatic immunoglobulin have been brought about. In the past decade, IgD research has come as far as to propose a specific surface molecule – the hyaluronan receptor CD44 – as the coveted IgD receptor on effector cells (Shan et al. 2018). However, the now many minute pieces of information about IgD are yet to be independently verified and woven together to fully comprehend the function(s) of this mysterious class of immunoglobulins. In the following sections of this introduction, I will go over a detailed overview of the most interesting and relevant pieces of knowledge about our protagonist that have come out thus far, to get us a bit closer to understanding its enigmatic biological role.

3.2 Overview of antibody structure and formation

Since IgD is an antibody, and so are most of the fluorescently labelled probes used in this project, an overview of antibody structure is in order. Antibodies, also known as immunoglobulins, are large Y-shaped glycoproteins secreted by plasma cells, which are derived from B lymphocytes of the adaptive immune system. Antibodies are essentially the secreted form of the variable antigen-specific B cell receptors on their cell surfaces. All antibodies consist of 2 heavy and 2 light chains linked by disulfide bonds. Heavy chains have a varying number of constant domains attached to a single variable domain, while light chains contain a single constant and single variable domain. There are five known classes of antibodies – IgA, IgD,

IgE, IgG, and IgM – their overall structures represented in **Figure 3.1**. Antibody classes differ in the structures of their Fc regions, which impart the ability to bind to different cell surface receptors, from where the different biological functions of immunoglobulins arise. IgG is the most abundant type in plasma and has an opsonizing function – it coats invading pathogens and targets them for phagocytosis via an FcγR. IgM has a similar function to IgG and is also the first class expressed by a B cell as BCR, along with IgD. IgA is secreted by mucosal tissues and prevents colonization by opportunistic bacteria. IgE is thought to have evolved to protect the body from parasitic worms, though it also mediates allergic reactions in atopic individuals by causing basophil and mast cell degranulation (Murphy et al 2017). The known functions of IgD will be discussed later in this chapter.

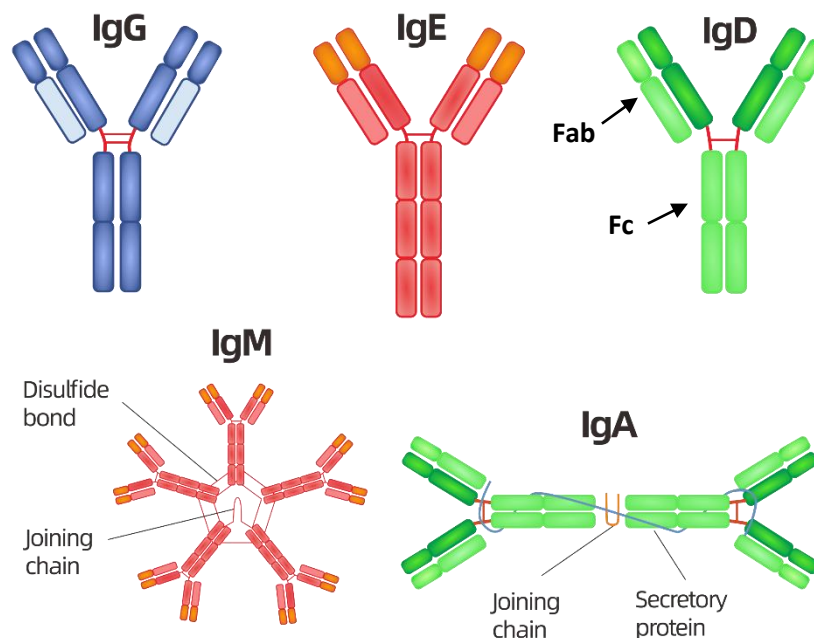


Figure 3.2 Schematic of the structural differences between the different classes of antibodies. IgG, IgE and IgD function as monomers, while IgA and IgM monomers are bound by joining chains into dimers and pentamers, respectively. IgG, IgD, and IgA have 3 constant domains in their heavy chains, while IgM and IgE have 4. Their specificities are imparted by the complementarity determining loops in the variable domains of the light and heavy chains at the tips of the Fab fragments. The effector functions of antibodies are imparted by receptors which recognize their different Fc regions. Fc and Fab regions are separated by hinges of varying length, which impart different mobilities to the Fab fragments. Image adapted [from antibodysystem.com](http://antibodysystem.com).

The wide diversity of antibody specificity is generated by the processes of V(D)J recombination, class switching, and affinity maturation. During B cell maturation in bone marrow, gene recombination occurs between 40-50 variable (V), 6 diversity (D, in heavy chains only), and 27 joining (J) segments, generating a huge number of possible combinations thereof. These segments encode the complementarity determining regions (CDR) of the variable domains for light and heavy chains, situated at the tips of the antigen binding fragments (Fab). These randomly generated combinations of segments are then joined to the constant domain gene segments, which encompass the different heavy chain isotypes. During development, B cells produce IgM and IgD as BCRs by alternative splicing of mRNA transcripts. The many possible VDJ recombinations are then tested for their specificity and affinity as the B cell matures, selecting only viable clones. Upon encounter of the mature B cells with antigen recognized by their BCRs, they can undergo differentiation into antibody-secreting plasma cells, affinity maturation, and class switching. Affinity maturation involves somatic hypermutation through the activation of a cytidine deaminase in order to induce point mutations in the variable (V) regions of the immunoglobulin heavy and light chain genes. Resulting clones are then selected by the immune system depending on responsiveness to antigen and thus tailor-made antibodies for any antigen can be produced (Victora and Nussenzweig 2012). Depending on the immune context defined by what cytokines are present, class switching to different heavy chain constant regions occurs through gene recombination, thus producing antibodies of the different classes.

3.3 Structure and evolution of IgD

IgD is the only remaining immunoglobulin class without a determined atomic structure. It shares the same basic structural characteristics as the other antibody classes, being formed of 2 sets of heavy and light chains made up of Ig domains. The heavy chain has 3 C δ constant domains, but its peculiarity lies in the hinge region between C δ 1 and C δ 2, connecting the Fab and Fc antibody fragments. The unusually long 64 residue hinge is made up of an N-terminal with dense O-linked glycosylations, and a C-terminal highly charged region rich in arginine, lysine, and glutamate residues, separated by a glycine-rich linker (Melliss and Baenziger 1983). The susceptible to proteolysis hinge may be responsible for the relative short half-life for IgD of around three days (Vladutiu 2000). Glycosylations account for around 12% of the 180 kDa molecular weight of IgD, including up to seven O-linked glycosylations at the hinge, as well as three N-linked glycans in C δ 2 and C δ 3 on each chain (ibid). The glycans are necessary for receptor recognition and to protect the highly charged region from enzymatic digestion into Fc and Fab fragments (Griffiths and Gleich 1972), the implication of which has not been studied. Notably, the flexible hinge allows IgD to recognize polyvalent antigens, as when it is replaced by the rigid IgM hinge, only monovalent antigen is bound (Übelhart et al. 2015). Thus, B cells anergic to monomeric antigens can still respond to polymeric ones, such as encountered immune complexes. This fits well with the idea that IgD elicits mucosal surveillance of commensal microbiota, as many bacterial surface antigens are polymeric. It also helps explain the IgD co-expression with IgM as not only its backup (Brink et al. 1992), but also as a recognition tool for separate sets of antigens or antigen concentrations.

The only insight into whole IgD structure comes from small angle X-ray scatter experiments, combined with molecular dynamics modeling, which suggest a semi-extended T-shape for IgD (**Figure 3.2**), restrained by the O-linked glycans (Sun et al. 2005). This sets it apart from the common Y-shape characteristic of other antibody classes and provides more conformational freedom for the Fab arms. However, depending

on the level of vertical restraint imposed by the carbohydrate moiety, the Fab arms may obscure binding sites on the Fc necessary for receptor recognition (ibid).

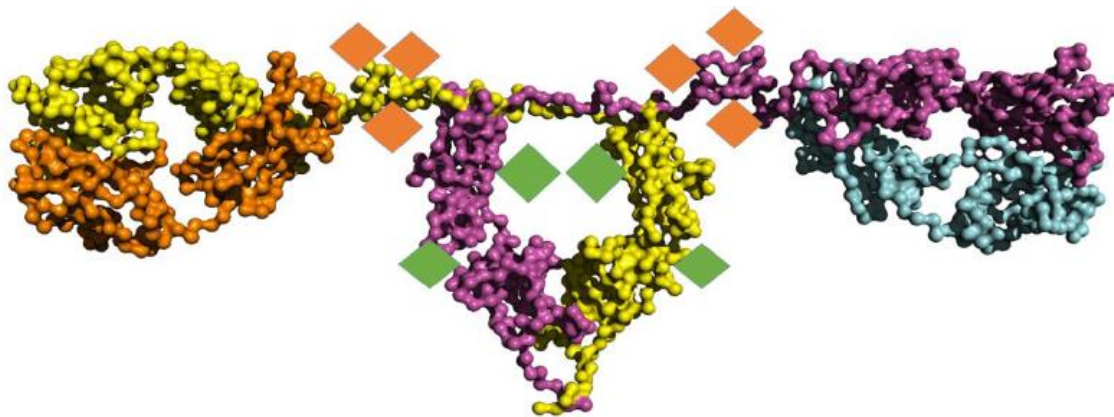


Figure 3.2 PyMol-generated model of whole human IgD, based on alpha-carbon positions, obtained via constrained X-ray scattering (Sun et al. 2005). Heavy chains represented with yellow and magenta, light chains with cyan and orange. Relative positions for O-linked and N-linked glycosylations are represented with orange and green diamonds respectively. The proposed semi-extended structure, imparted by the long hinge region and maintained by O-linked glycosylations, aids the interaction with polyvalent antigens (Übelhart et al. 2015). PDB entry **1zvo**.

The evolutionary antiquity of IgD is evident by the presence of its orthologues and homologues throughout the animal kingdom, going back to cartilaginous fish, evolved over half a billion years ago (Ohta and Flajnik 2006). Unlike the highly preserved IgM, different species have widely different degrees of C δ gene duplications, resulting in large numbers of C δ domains in IgD, with up to 14 C δ domains in bony fish, with yet more diversity generated through alternative splicing of the mRNA transcript, compensating for the lack of CDR in lower vertebrates (Chen and Cerutti 2011). Interestingly, IgD biased for σ light chains (the equivalent of human λ) and lacking V_H regions, produced by IgD-only B cells, has been identified in channel catfish (Edholm et al. 2010), despite their expression of AID, betraying an innate-like pattern recognition role for this immunoglobulin class. The hinge region of IgD is poorly conserved across species, with vastly different glycosylation patterns, for example – the O-linked glycans on the hinge of human IgD are

necessary for its putative receptor binding (Rudd et al. 1994), while the murine IgDR engagement necessitates N-linked glycan presence (Tamma et al. 1991), potentially meaning different surface molecules and effector cells are involved. Overall, the heterogeneity of IgD structures across species, as well as its innate-like pattern recognition properties, signal that since its ancient origin in early jawed vertebrates 500 million years ago, it has been utilized in widely different manners by their evolving immune systems to both complement the activity of IgM and to provide the needed functional flexibility with regards to foreign and self-antigen responses, depending on which surface molecules on effector cells are engaged.

Apart from the transmembrane BCR and soluble antibody forms, IgD has also been shown to also expressed as a GPI-anchored cell surface receptor on B cells, engagement of which triggers cAMP-dependent calcium signaling needed for B cell activation (Chaturvedi et al. 2002), providing another layer of control over the process.

3.4 Source of soluble IgD

Since its discovery, the function of IgD was thought to be limited to that of a redundant B cell membrane receptor, as it is co-expressed with IgM on the surface of naïve B cells by means of alternative splicing of a single heavy chain RNA transcript (Maki et al. 1981), the production of which requires abrogation of transcriptional termination through the action of the zinc-finger protein ZFP318 (Pioli et al. 2014). The fact that IgD-knockout mice have normal immune development and can efficiently mount antibody responses (Nitschke et al. 1993) was interpreted as lack of involvement of IgD in the immune response. IgD could entirely substitute the function of IgM during B cell development in transgenic mice, and vice versa (Iglesias, Lamers, and Köhler 1987). However, the emergence of evidence for allergen-specific IgD in serum (Zhang et al. 1994) and class-switched B-cells to IgD-secreting plasmablasts in the upper respiratory mucosa (Arpin et al. 1998) have since challenged the notion that IgD is simply a backup for IgM, betraying

a more involved role for IgD in immunity. Apart from serum, secreted IgD is mostly detectable in human lacrimal, nasal, salivary, bronchial, and mammary secretions, as well as in the amniotic fluid during pregnancy, with vastly varying concentrations between time points and individuals (Chen and Cerutti 2010). This distribution possibly points to a role similar to that of IgA at mucosal sites, and that it may also be transcytosed across epithelia in a yet unknown manner. Interestingly, soluble IgD levels are elevated in patients with IgA deficiencies, indicating that in some cases IgD could be a substitute for IgA (Brandtzaeg et al. 1999).

To explain the origin of secreted IgD, the recently discovered role of membrane IgD in B cell anergy and peripheral tolerance must be mentioned. Anergy is the process whereby self-reactive B cells acquire tolerance and, rather than being discarded, are maintained in the peripheral lymphoid tissue repertoire, without the ability to proliferate or produce antibody (Goodnow et al. 1988). A common characteristic of anergic B cells is high membrane IgD expression, down-regulation of surface IgM, and the use of a self-reactive set V_H 4-34 heavy chain variable domains (Pugh-Bernard et al. 2001). Interestingly, most of the autoreactive B cell lineages have been shown to be class-switched to IgD (Zheng et al. 2004).

The risk of autoimmunity is outweighed by the masking of tolerance-induced inconsistencies in the B cell repertoire (which could allow for self-like foreign antigens to go unnoticed), to which membrane IgD contributes by dampening the IgM-induced anergy and promoting B cell accumulation in follicles (Sabouri et al. 2016). Conversely, membrane IgD signaling can induce the tolerant state in the first place, by downregulating IgM signaling needed to induce activation or cell death (Duty et al. 2009). Self-reactive B-cells can thus be salvaged through activation of membrane IgD and somatic hypermutation in GCs away from self-reactivity, further aided by N-linked glycosylation of variable domains (Sabouri et al. 2014). Interestingly, over half of antibodies from IgD class-switched B cells are auto-reactive in healthy individuals (Koelsch et al. 2007), part of the repertoire having acquired autoreactivity to dsDNA through somatic hypermutation, while others being naturally self-reactive. This indicates that IgD-only class-switched cells

originate from self-antigen-specific anergic cells in peripheral lymphoid tissues. IgD-producing cells are found in the nasal mucosa, tonsils, adenoids, and lacrimal glands, but not in gut-associated lymphoid tissue (Chen and Cerutti 2010). These cells have been shown to make up to 20% of Ig-secreting cells in human tonsils (Arpin et al. 1998). What causes the homing of these autoreactive B cells to the upper respiratory mucosa and not to the gut mucosa or other lymphoid tissues remains unclear (Brandtzaeg and Johansen 2005).

Class-switching to IgD utilizes a non-canonical $\sigma\delta$ intronic switch region between the $C\mu$ and $C\delta$ genes, is exclusive to GC B cells in the airway mucosa (Liu and de Bouteiller 1996) and has been observed to occur both dependently and independently of T cells (Chen et al. 2009). This unusual class-switching is done through DNA double strand breaks and alternative end-joining, mediated by Rad52 (Xu et al. 2022). IgD derived from these cells is characterized by the highest mutation load of any antibody class, including mutations in the V_L genes, preferential VH 3-30 gene usage (Seifert et al. 2009), and heavy bias towards λ light chain usage (Arpin et al. 1998). Serum IgD also bears these characteristics, indicating its origin from mucosal GC B cells (Seifert et al. 2009). Usage of λ light chain has been shown to be in up to 90% of serum or cell culture secreted soluble IgD (Litwin and Zehr 1987), which is in contrast to the common 2:1 ratio for κ to λ usage by other immunoglobulin classes. Interestingly, the predominant light chain found on transmembrane IgD is κ , the discrepancy being termed “the IgD paradox” (Chen and Cerutti 2010), the implications of which are yet unknown. Furthermore, the IgD-only population in most individuals is dominated by a relatively small number of highly expanded clones (Arpin et al. 1998). These characteristics support the idea that IgD-only B cells emerge from a small subset of cells undergoing GC reactions, driven by superantigens binding to $C\delta$ constant regions of IgD. Of important note is the fact that IgD-CSR has recently been reported to require TLR signaling from the microbial flora (Choi et al. 2017). Other triggers for IgD-CSR remain unknown, however, one of the first instances of its observation was in a murine λ^+

hybridoma line which had spontaneously switched from IgM to IgD production (Neuberger and Rajewsky 1981).

3.5 Innate-like properties of IgD

The role of IgD in innate immunity is not fully understood, but several important observations have been made thus far, most notably the capture of IgD by human myeloid cell types, as detailed in the next section, and the binding to bacterial antigens in a non-immune manner (superantigenic binding). Unlike IgM, IgD lacks the ability to activate the classical complement pathway (Spiegelberg 1989), although there have been some conflicting reports (Konno, Hirai, and Inai 1975), perhaps because of potential autoreactivity towards complement proteins.

IgD has been shown to bind to commensal microbiota of the aerodigestive mucosa in a superantigenic manner (Forsgren and Grubb 1979). By far the best characterized interaction is that of the *Moraxella Catarrhalis* IgD-binding protein (MID) (Nordström, Forsgren, and Riesbeck 2002). *M. Catarrhalis* is a gram-negative diplococcus and is among the most common inhabitants of the nasopharynx in healthy children (Karalus and Campagnari 2000). However, it often exhibits pathogenicity, being a common cause of otitis media and, more importantly, its overabundance is associated with treatment-resistant severe asthma (Green et al. 2014), and the exacerbation of allergic airway inflammation (Alnahas et al. 2017). Moreover, children with lower microbial diversity and overabundance of *M. Catarrhalis* in the nasal mucosa (Depner et al. 2017) and children with aberrant immune responses towards the bacterium (Larsen et al. 2014) have a higher chance to go on to develop asthma. It is of interest that children growing up on farms, who have a richer microbiome diversity, are protected from the detrimental effects of *Moraxella* (Depner et al. 2017), which is somewhat reminiscent of the hygiene hypothesis of asthma and allergies (A. H. Liu 2015), according to which disrupted health or diversity of the airway microbiome, due to the increased sterility of modern urban life, can be a major driving factor for the development of these conditions.

The most solid evidence that MID binding to IgD is of biological relevance is that the minimal IgD-binding MID fragment (res 962-1200) alone can stimulate B cells to proliferate and secrete IL-6, but only in the presence of IL-4 and IL-2 – cytokines, derived from T cells (Nordström et al. 2006). Since the nasal and oropharyngeal mucosa are sites where both IgD-only B cells and *M. Catarrhalis* reside, it is logical to assume, taken together with previously stated findings, that IgD class-switching and production may, at least in part, result from germinal centre reactions, triggered by the bacterium. It is possible that the MID: IgD interaction evolved as an evasion mechanism whereby engaging the IgD BCR in a non-immune manner results in production of polyclonal and potentially harmless to the pathogen antibodies (ibid).

Full-length MID, also known as adhesin or hemagglutinin, is a 200 kDa outer membrane trimeric autotransporter, i.e. several monomers insert pore-forming subunits into the outer bacterial membrane, with the rest of the protein transported through the resulting pore (Hallström et al. 2008), and serves as an adhesion molecule to host cells. We are only concerned with the 238 amino acid soluble IgD-binding portion of MID, spanning residues 962-1200, which forms tetramers in solution, facilitating enhanced IgD binding (Nordström, Forsgren, and Riesbeck 2002) . The MID binding site on IgD has been mapped to the Cδ1 constant region, with amino acid residues 198-206 being crucial for the interaction (Samuelsson et al. 2006).

3.6 IgD effector cells

IgD has been documented to be captured by several types of myeloid cells, as well as by T cells, by means of a yet uncharacterized receptor termed IgDR. However, due to the vastly different cell phenotypes and responses observed, it is more likely that several distinct surface molecules have the property to respond to IgD, eliciting different effects under varying conditions.

Murine and human T cells were the first type shown to bind IgD over 35 years ago (Coico et al. 1985). The murine receptor was hypothesized to be a lectin, with specificity for N-linked glycans, as it failed to bind

deglycosylated IgD, while binding was also inhibited by monosaccharides (Tamma et al. 1991). Human IgDR was later found to bind O-linked glycans of both IgD and IgA1 on CD4 and CD8 T cells (Swenson et al. 1998). Engaging of the receptor on T cells by IgD results in robust proliferation (Coico et al. 1985). IgDR was later found to be upregulated by exposure to oligomeric IgD, as well as T cell-derived cytokines IL-2 and IL-4 (Tamma and Coico 1992). IgDR upregulation has been shown to promote the humoral immune response by facilitating B-T cell interactions and expansion of antigen-specific T cells (Yang and Coico 1996). IgD-mediated IgDR expression and upregulation have also been observed in the Burkitt's B cell lymphoma Daudi line, which are also stimulated to proliferate upon engagement with IgD (Dai et al. 2016).

The binding of IgD to several myeloid cell types has also been discovered. Eosinophils and neutrophils bind IgD in the context of skin allergy (Hunyadi, Hamerlinck, and Cormane 1976), it being the most abundant Ig type bound by the latter cell type. Monocytes have been shown to produce TNF- α and IL-1 β in response to IgD stimulation (Drenth et al. 1996). Elevated levels of IgD in rheumatoid arthritis patients were shown to result in activation of peripheral blood mononuclear cells (PBMCs), whereby they proliferate and secrete pro-inflammatory cytokines such as IL-1 β , IL-6, and TNF- α (Y. Wu et al. 2016). Mastocytoid cell lines have also exhibited IgD-binding capabilities (Chen et al. 2009).

Oligomeric IgD can trigger IL-6 production in KU812 pre-basophilic cell line, without inducing degranulation (Sechet et al. 2003), while circulating basophils have been shown to be 'armed' with IgD on their surface, much like IgE on Fc ϵ RI, which upon crosslinking elicits anti-microbial and B cell stimulating programs through the secretion of antimicrobials such as cathelicidin, as well as cytokines enhancing the humoral immune response - IL-1, IL-4, IL-13 and BAFF (Chen et al. 2009). Recently it was shown that this effect is imparted by the binding of soluble IgD to galectin-9, the latter reportedly directing the former to the hyaluronan receptor and adhesion molecule CD44 on the basophil surface (Shan et al. 2018).

Galectin-9 is a β -galactoside-binding tandem repeat type lectin with two carbohydrate recognition domains, allowing it to bind to two separate glycoproteins at the same time, and has diverse, if somewhat controversial, immunoregulatory actions. It has been shown to suppress basophil (Hayen et al. 2018), as well as mast cell line degranulation (Kojima et al. 2014), while at the same time activating IL-6 release and limiting survival of the latter. Gal-9, like Gal-3, can bind to IgE, but, unlike Gal-3, can disrupt the formation of IgE-antigen complexes (Niki et al. 2009). Gal-9 has been implicated in the skewing of the immune response to Th2 cytokine-driven immunity in several ways - firstly, by downregulating Th1 CD4 T cells, which express a cell surface receptor for it named Tim3 (Zhu et al. 2005); secondly, by triggering the differentiation of Th2 CD4 T cells and M2 macrophages (Enninga et al. 2016); and finally, through the recruitment of eosinophils (Rao and Sriramarao 2017). However, binding to its partner CD44 on Th2 T cells, which displaces its hyaluronan ligand, prevents the cells from infiltrating into the airways (Katoh et al. 2012). Other observed effects of gal-9 engagement of CD44 are the induction of differentiation in osteoblasts (Tanikawa et al. 2010) and the differentiation and maintenance of induced regulatory T cells (C. Wu et al. 2014), both processes propagated via the SMAD signaling pathway. Treatment with galectin-9 is beneficial in sublingual allergen-specific immunotherapy, most likely by inducing TGF- β production (Ikeda et al. 2017). Gal-9 expression has been shown to positively correlate with rheumatoid arthritis severity (J. Sun et al. 2021). Interestingly, gal-9 expression can be induced by gut microbiota and is correlated with reduced acute allergic symptoms (de Kivit et al. 2012). Thus, galectin-9 has somewhat of a conflicting role regarding atopy, with most data suggesting it enhances Th2 differentiation, while others point to a protective anti-inflammatory role. Importantly, gal-9 can have anti-proliferative functions by inducing apoptosis in T cell lymphomas (Fujita et al. 2017), and is necessary for LAMP2-mediated autophagosome formation (Sudhakar et al. 2020).

Galectins are known for their fluctuating expression during immune responses and for their modulatory role, both from the inside and at the surface of cells (Liu and Rabinovich 2010), and, along with other

lectins, are involved in the orchestration of some antibody responses (Bambouskova et al. 2016). Specific interactions with the carbohydrate moiety of IgD may impart an extra level of control over its functions, directing it to different cell types or receptors in different inflammatory conditions, but these conjectures remain to be further investigated. That sort of control could explain, for instance, why neutrophils can only bind IgD in an inflamed milieu and not in homeostasis (Hunyadi, Hamerlinck, and Cormane 1976).

Of note is that gal-9 itself can specifically bind to bacteria expressing blood group-like antigens and induce their death on its own (Blenda et al. 2022), which is apparently a non-immune arm of the epitope masking prevention system. The microbial surveillance, along with the Th2-enhancing capabilities of both, makes it plausible that IgD and galectin-9 are part of the same regulatory machinery. The dual carbohydrate binding capabilities of galectin-9 render it an excellent adaptor molecule, which can facilitate interaction of glycoproteins such as immunoglobulins and cell surface molecules, imparting an IgDR status to the latter.

3.7 IgD in the middle

Despite making up only around 0.25% of serum immunoglobulins (Vladutiu 2000), soluble IgD apparently has important immunomodulatory functions confined to aerodigestive mucosal surfaces. Taken together, the available evidence points to a regulatory role for IgD in the mucosal immune system and the enhancement of the humoral response, at a crossroads between activation and tolerance. A picture for the possible involvement of IgD in asthma and allergies emerges when we consider the following known information:

IgD-only cells in the mucosa differentiate from peripheral tolerant IgD⁺ IgM^{low} B cells, following their encounter with overabundant (due to microbiome imbalance) *Moraxella Catarrhalis* or other IgD-binding bacteria in the airway microbiome, which activate the B cells through superantigens on their surfaces. Since those cells were likely induced to be tolerant due to their reactivity to environmental or self-antigens,

so as to prevent epitope masking of potential pathogens, their specificity could be towards any common allergen. In a less-likely scenario reactivity toward environmental antigens could be acquired through somatic hypermutation after bacteria-induced IgD-CSR. The result would be generation of IgD with allergen specificity, which either came about randomly through mutation, or, more likely, was produced by a B cell tolerant to it. Through capture on IgDR, this allergen-specific antibody can arm basophils and other cells, either locally or in circulation. Upon ligation of the IgD: IgDR complex by cognate antigen, effector cells secrete the known cytokine inducers of IgE-CSR – IL-4 and IL-13 – as well as other B cell-stimulating factors. The coating of human primary basophils with anti-Phl p 7 IgD, followed by Phl p 7 stimulation was confirmed to trigger IL-4 and IL-13 secretion in experiments done by Balraj Sandhar (Gould group, data not yet published). From there, either a population of the original allergen-tolerant/reactive B cells, or B cells tolerant/reactive to another antigen, could be induced to switch to and produce IgE, thus propagating the ‘allergic march’, which defines atopic conditions (Gabryszewski and Hill 2021). This progression is also known to be triggered by *S. aureus* superantigens in a yet unknown manner (Gould et al. 2007).

This emerging model (**Figure 3.3**) for IgD’s potential involvement in allergen-specific IgE generation in the upper respiratory mucosa is also in agreement with the observation that levels of allergen-specific IgE and IgD are correlated in atopic individuals (Zhang et al. 1994). However, the direct progression from elevated IgD to elevated IgE remains to be concretely proven.

IgD – linking the microbiome and atopy

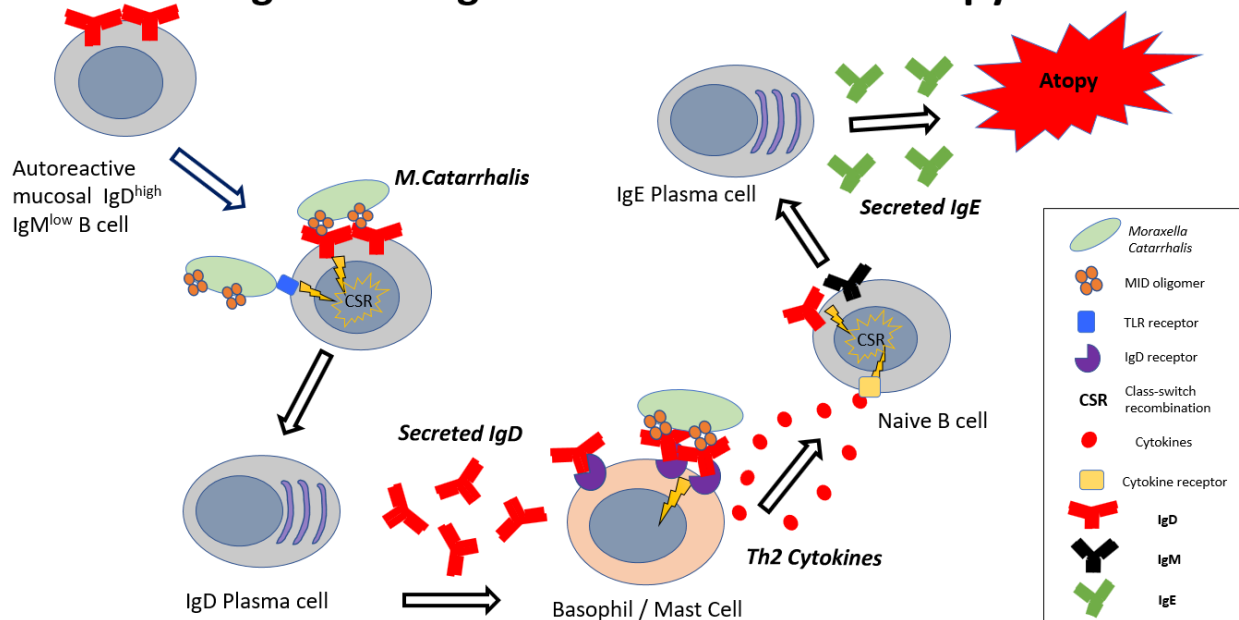


Figure 3.3 Proposed model for the involvement of IgD in the progression from microbiome disruption due to overabundance of some bacterial species such as *M. catarrhalis*, to generation of allergen-specific IgE, based on review of available literature. B cells induced to be tolerant to environmental or self-antigens are triggered by IgD-binding proteins on bacterial surfaces, causing the B cells to undergo IgD-CSR and secrete IgD, which is then captured by IgDRs on effector cells in the mucosa. Exposure to cognate antigen or bacterial superantigen can then trigger the release of cytokines enhancing the Th2 humoral response, including IgE generation by CSR in other local B cells of the same or different specificity as the original IgD-switched clone, leading to the establishment and propagation of hypersensitivity.

3.8 Structural characteristics of proteins used in this project

Phl p 7

Phl p 7 is a small 8.5 kDa member of the polcalcin family of allergens and is the major allergen of *Phleum pratense* (common timothy grass). Being a polcalcin, it requires the binding of calcium ions for its antigenic function and stability (Mitropoulou et al 2018). The Phl p 7 protein has a single-domain globular fold consisting of 4 α -helices and 2 short anti-parallel β -strands (**Figure 3.4**). The overall structure of Phl p 7 is stabilized by the two bound calcium ions, which help to maintain the protein's stability and prevent its degradation by proteases.

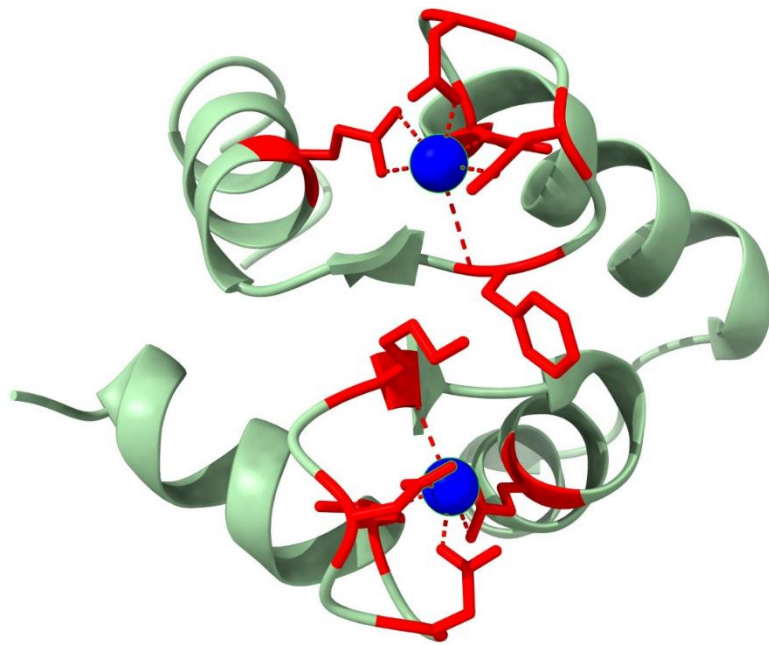


Figure 3.4 Structure model for Phl p 7 obtained by X-ray crystallography. The calcium ions are chelated by electrostatic interactions with the oxygen atoms of three glutamate residues and one backbone carbonyl oxygen. PDB: **5OTJ**. Model generated with Yasara.

MID

The *Moraxella Catarrhalis* IgD binding protein is a large 200 kDa trimeric autotransporter and serves as a cell surface adhesion molecule to host cells. The N-terminal region of MID contains a signal peptide for secretion, the IgD-binding domain in its central region, which is essential for the bacterium's survival in the mucosa. The central region also contains several repeats of a conserved motif called the MCE (Moraxella catarrhalis element), which is believed to play a role in protein folding and stability. The C-terminal region of MID contains several repeats of a conserved motif known as the RTX (repeat in toxins) domain, which is found in many bacterial toxins and is involved in protein secretion and translocation across bacterial membranes. This domain has been shown to be essential for the ability of MID to be expressed on the surface of the bacterial cell and to interact with host cells. In addition to its structural domains, MID also contains several post-translational modifications, including glycosylation and phosphorylation, which can modulate its activity and function (Hallström et al 2008). No solved structures have yet been published for MID. **Figure 3.5** represents a model of the full-length MID trimer generated by the AlphaFold algorithm (EBI).

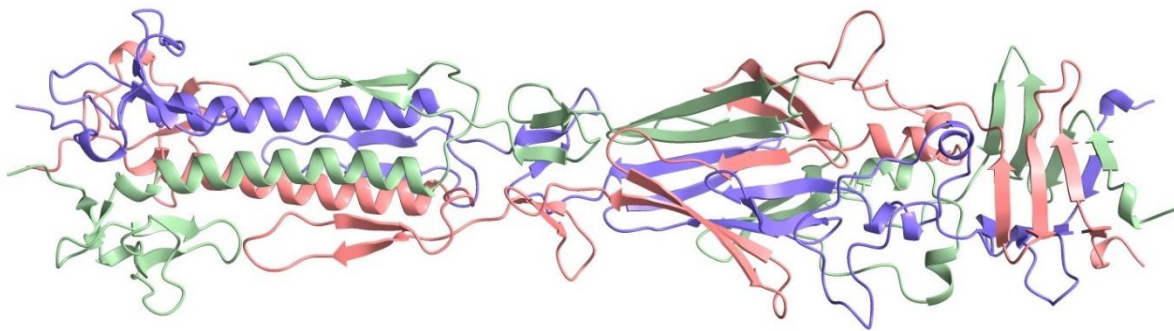


Figure 3.5 AlphaFold-generated model of a full-length MID trimer, coloured by monomer. The IgD-binding domain is towards the middle of the protein and the pore-forming RTX C-terminal domain is to the left in the model. Image generated with Yasara.

Galectin-9

Galectin-9 is a member of the galectin family, a group of proteins that bind to beta-galactoside sugars in a calcium-dependent manner. It is a widely expressed glycan-binding protein with a molecular weight of approximately 40 kDa. It is a tandem-repeat galectin, meaning it contains two structurally similar domains connected by a linker peptide. Each domain contains a carbohydrate recognition domain (CRD) that can bind to beta-galactoside sugars. The two domains can impart adapter-like functions of galectin-9. The protein also contains a C-terminal tail that can mediate oligomerization. Studies have shown that galectin-9 can form dimers, tetramers, and higher-order oligomers through interactions between its C-terminal tail and the CRD of other galectin-9 molecules. The formation of these oligomers can modulate galectin-9's activity and affect its interactions with other proteins and cells (Hayen et al. 2018). Structure of gal-9 has not yet been solved and **Figure 3.6** represents an AlphaFold (EBI) model.

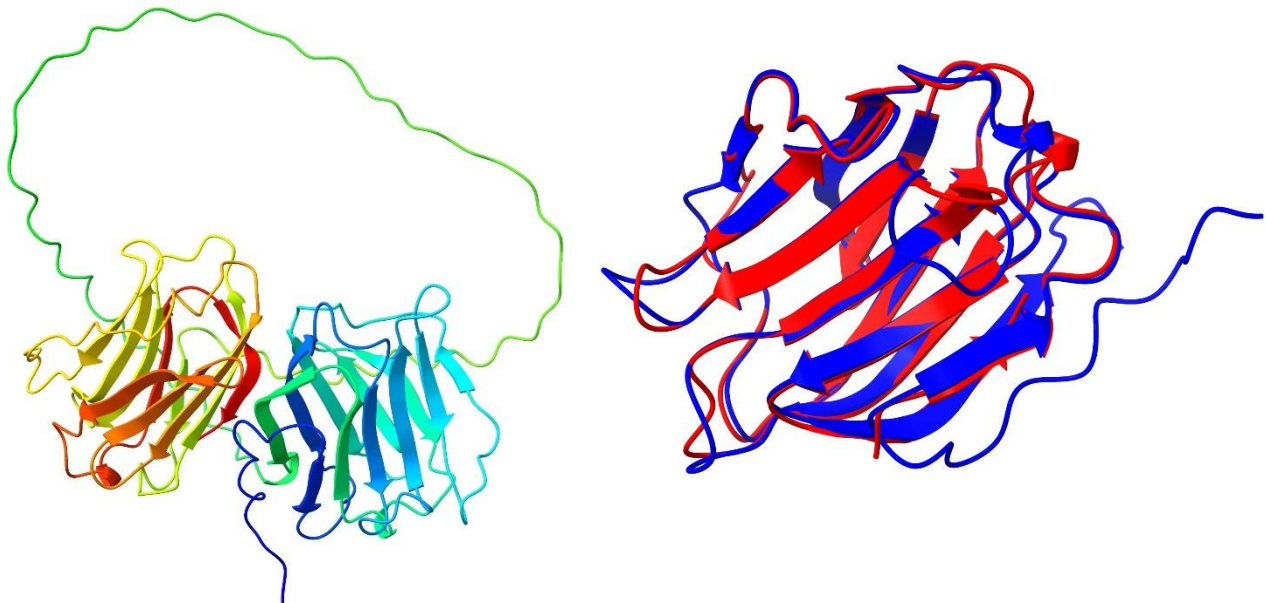


Figure 3.6 Left – AlphaFold model of full-length galectin-9 consisting of two globular carbohydrate recognition domains, connected by a long unstructured linker. **Right** – Superimposition of the N- (blue) and C-terminal (red) binding domains shows they are nearly identical with an RMSD of 0.6 Å. Image generated with Yasara.

CD44

CD44 is a type I transmembrane glycoprotein with molecular weight of around 85 kDa and serves as a receptor for hyaluronan. It consists of a signal sequence, the N-terminal hyaluronan-binding domain (HABD), a very long and flexible unstructured linker, a 25-residue transmembrane helix, and a C-terminal cytoplasmic tail. HABD is composed of approximately 100 amino acid residues and has a globular fold (**Figure 3.7**) with a characteristic "link module" structure. The link module consists of two β -sheets that are connected by a conserved disulfide bond and a calcium ion coordinated by several acidic residues. The hyaluronan-binding site is composed of a cleft formed by several conserved amino acid residues (Teriete et al. 2004).

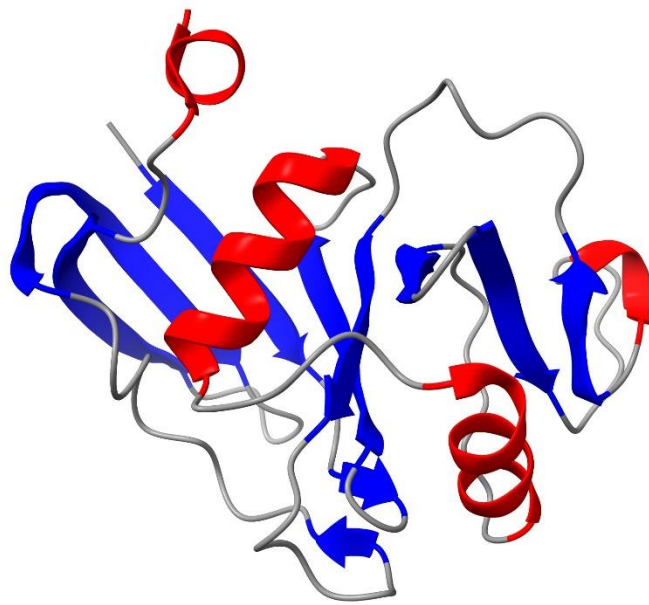


Figure 3.7 X-ray Crystallography structure of the hyaluronan binding domain of human CD44. The globular domain is connected to the cell surface via an at least 500 residue long sequence, predicted to be unstructured by AlphaFold (EBI). The predicted linker flexibility allows for extension of up to 75 nm away from the surface in search of its hyaluronan ligand. PDB: **1UUH**. Image generated with Yasara.

4. Aims and Objectives

Since the start of the project, our main aim has been to identify and characterize the elusive IgD receptor on effector cells, however, the individual objectives changed dynamically throughout its duration due to various unforeseen circumstances or discoveries. The establishment and optimization of protein production of the titular constructs used in the project, as well as the optimization of the major techniques employed, took a considerably longer time than initially expected, spanning the first two years of the project. By the time everything was set up, the Coronavirus pandemic in 2020 occurred, which severely disrupted workflow and access to necessary equipment. This resulted in significant time limitations for experimental data acquisition, thus excluding a big part of the initial objectives we had planned, which included protein crystallization trials, binding experiments, examining responses of various cell types to IgD & MID, and so forth. Additionally, the 2018 publication by Shan and colleagues, heavily cited in this thesis, set further boundaries for my research, shifting the focus to galectin-9 and CD44 as potential participants in the IgD receptor complex. The unexpected discovery that IgD is internalized by both KU812 and Jurkat cells also prompted further examination of the method of entry and its subcellular localization.

Thus, the concrete objectives followed through, as parts of the aim of identifying IgDR, were the following:

- To establish production of the recombinant monoclonal IgD used throughout the project, a directly labelled fluorescent IgD probe, as well as its cognate antigen (Phl p 7) for IgD purification.
- To examine the ability of KU812 (CD44⁺) and Jurkat (CD44⁻) cell lines to bind soluble IgD and to observe the effect of exogenous galectin-9 addition on binding by fluorescence flow cytometry.
- To confirm IgD binding and to observe its potential internalization by those cell lines, its subcellular localization, as well as the effect of galectin-9 addition, via confocal fluorescence microscopy.
- To identify potential IgD-receptor complex candidate proteins through IgD-mediated pulldown of cell lysate samples, followed by LC/MS and proteomics analysis.

5. Principles of experimental techniques

5.0 Brief overview of fluorescence

The following two techniques - flow cytometry and confocal microscopy, among many others – are reliant on the phenomenon of fluorescence, so at least a very short summary of its physical principles is in order.

Fluorescence is a type of photoluminescence that occurs when electrons of a molecule absorb photons of light with a specific wavelength, move to a higher energy state, and then emit photons with a longer wavelength, thus returning to the ground state, after a brief time delay (Lakowicz, 2006). Fluorescence refers to this emitted longer-wavelength, lower-energy light signal. The energy of the absorbed photons must match the energy difference between the ground state and the excited state of the electrons, so certain molecules with their electron configurations can only be excited by specific photon wavelengths. The excited state lifetime is a measure of the length of time in which the electrons are in the unstable higher energy state. As they return to the ground state, they emit photons of lower energy than the ones absorbed, which therefore have longer wavelength. The difference in absorbed and emitted wavelength is due to dissipation of some of the energy absorbed as heat or non-radiative decay and is called the Stokes shift. Different fluorophores have different Stokes shifts that depend on their specific electron configurations, enabling effective discrimination between their emitted fluorescence in the same biological sample through the use of dichroic mirror filters, which reflect photons of a specific wavelength towards a detector, while letting the remaining photons through.

The custom design of fluorophores with specific excitation and emission spectra, as well as the development of fluorescent proteins, photoswitchable dyes, and so on, has kickstarted a revolution in cell imaging methods and has led to the establishment of more complex techniques such as fluorescence resonance energy transfer (FRET), the various super-resolution microscopy methods, and many more.

5.1 Flow cytometry

Flow cytometry is a powerful technique for the swift analysis of single cells, which is routinely used in molecular biology, immunology, microbiology, and so on. In this fluorescence-based method, cells are either stained directly with fluorescent dyes (e.g DNA-staining dyes for viability analysis), stained with fluorescent antibody conjugates specific to certain cell surface molecules (e.g an IgG1 antibody specific to CD44, conjugated to Alexa Fluor 488), or transfected with fluorescent protein constructs, expressed by the cells (e.g. Green Fluorescent Protein attached to protein of interest). Cells are then suspended in a focusing buffer solution and channeled through the multiple lasers of a flow cytometer in single file, yielding visible light scatter and fluorescence signals for each passing object (McKinnon, 2018). Detectors read the light signals and convert them into digital form, which is stored in a standard data file format (.fcs) for analysis (**Figure 5.1**). This method can be used not only for the detection of cell surface molecules, but also for the sorting and purification of different cell types from a heterogeneous mixture, based on their size, shape, and profile of bound fluorescent antibody probes – an extension of flow cytometry called fluorescence-activated cell sorting (FACS). Thanks to advances in the generation and optimization of available small dye molecules and fluorescent proteins, flow cytometry has rapidly developed over the past couple of decades and multidimensional analyses of multiple parameters are now a possibility, especially in very advanced cytometers that combine this technique with fluorescent microscopy or mass spectrometry.

In this project, basic flow cytometry was utilized to determine and compare IgD binding to pre-basophilic (KU812) and T (Jurkat) cell lines, previously shown to interact with IgD, and whether that is affected by incubation temperature and galectin-9 addition. The exact experimental procedures applied are described in the Methods section.

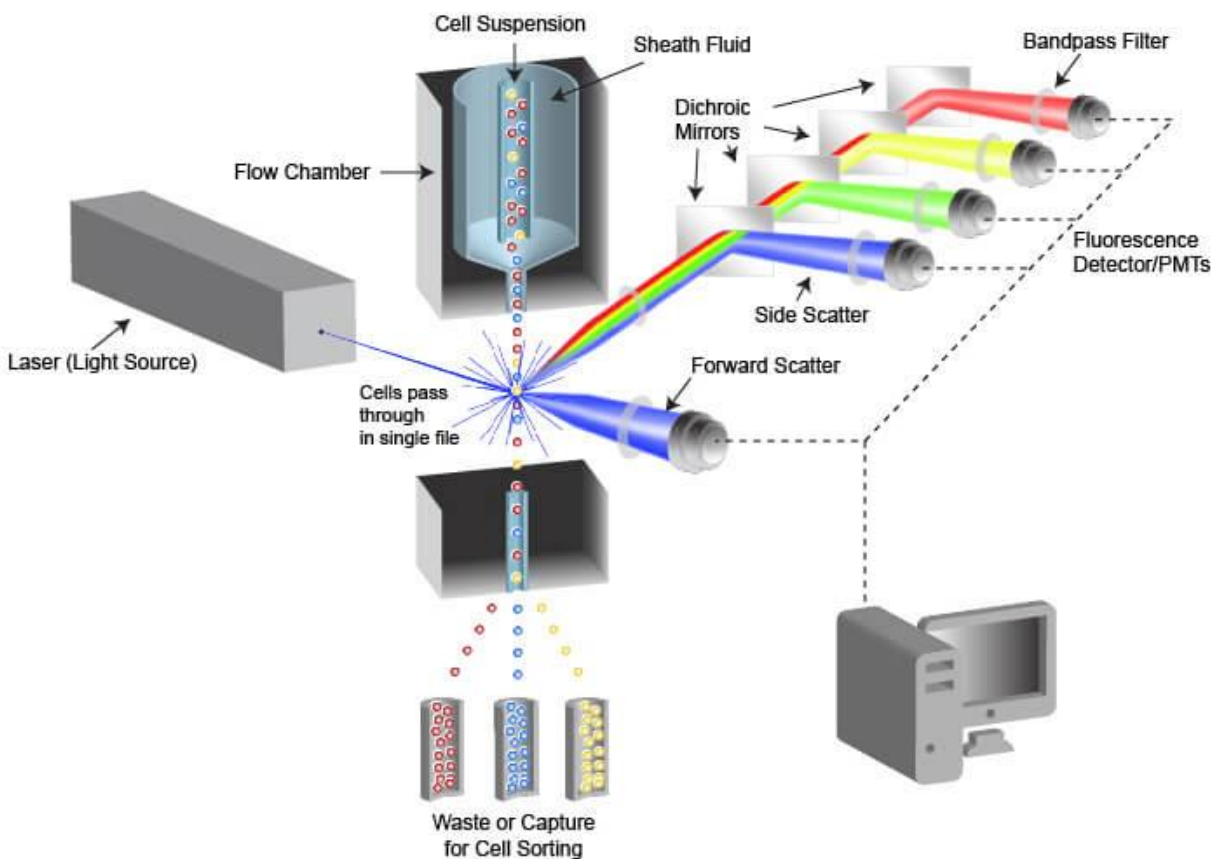


Figure 5.1 Overview of a common flow cytometer setup – Cell sample is injected at high pressure into the centre of a laminar stream of liquid known as sheath or focusing fluid. Thanks to the hydrodynamic focusing effect of the fluid (or through acoustic vibrations), cells are organized into a single file line and each cell passes separately through one or more laser beams of different wavelengths. Scattered laser light and fluorescence are detected by photomultiplier tubes (PMTs) or photodiodes (PDs). Forward scatter is used to measure the relative cell size and side scatter is used to determine cell granularity and density. The light emitted by fluorescent probes (or from the cells' own autofluorescence) passes through dichroic mirrors, which act as filters, separating the light signal into its constituent wavelengths, allowing the use of multiple fluorescent probes of different colours. The detectors digitalize the photon counts and feed them to a computer for analysis and data storage. Passing cells are either automatically sorted according to differences in light scattering and fluorescence, or simply discarded as waste. Figure taken from 'Overview of Flow Cytometry' at cellsignal.com

The sample preparation for flow cytometry starts with the harvesting of an appropriate amount of cells grown to certain density. Common applications would require roughly between 50 000 and 200 000 cells per sample condition. The cells are washed of their nutritional medium via centrifugation and resuspended in a saline solution (most commonly PBS), which is supplemented with bovine serum albumin (BSA) to maintain their survival and membrane integrity, and with ethylenediaminetetraacetic acid (EDTA) – a calcium chelating agent, which prevents the calcium-mediated attachment of cells to each other. The combination of PBS, 2% BSA, and 2 mM EDTA will henceforth be referred to as ‘FACS buffer’. The samples are then usually incubated with a reagent that blocks the cell surface receptors for the Fc regions of IgG antibodies (FcγR blocker), since most of the antibody-fluorophore conjugates used for staining are of the IgG class. This step is necessary to prevent any non-specific binding and internalization of the fluorescent antibody probes, which must only bind to their cognate cell surface antigens. After a wash step, the next stage is to incubate the samples with the desired fluorescent probes for the appropriate amount of time and at the correct temperature to allow optimal antibody binding to the surface molecules of interest – usually 1 hour at 4°C. This can be done with either directly labelled antibodies specific to cell surface molecules, or through the incubation with non-labelled primary antibodies, followed by staining with secondary labelled antibodies that recognize the primary ones. A membrane-impermeable DNA-binding viability dye, such as propidium iodide, is also added to the staining cocktail, so that cells with lysed membranes, which are non-viable, become dyed and will be excluded from the flow cytometry analysis. After a couple of wash steps to remove unbound antibody, the samples are resuspended once again in FACS buffer and are ready for analysis in the flow cytometer, as described above. However, before data acquisition can begin, the laser voltages need to be adjusted in order to improve signal-to-noise ratio and any potential spectral overlaps between the fluorescence channels in multicolour experiments (colour spill when the emission spectra of two fluorophores are close to each other) need to be accounted for. This is achieved by using compensation beads – small polystyrene particles, coated with capture antibodies that

recognize and bind the fluorescently labelled antibodies from the staining cocktail. Each set of beads is exposed to only one fluorophore and thus it can be calculated how much of its signal spills over to other channels, and the signal values for the samples of interest can be automatically corrected.

Once the calibration has been completed, the cells, focused in the sheath fluid into single file line, either by pressure or through acoustic vibrations, pass through the laser beams of the cytometer, one of which is a lower power HeNe or diode laser in the visible light range. As the cells pass through it, light from the beam is scattered in a way determined by their size and morphology. The forward scattered photons (FSC) are recorded by a detector parallel to the beam and the scatter intensity depends on a cell's size. The side scattered photons (SSC) are recorded by a detector at a 90° angle to the beam and the scatter intensity depends on a cell's granularity and shape (Ibrahim and van den Engh 2007). Simultaneously, fluorophores in the samples are excited by the respective higher power lasers. The resulting fluorescence signal passes through or is reflected by dichroic mirrors towards the detectors for the respective emitted light bandwidths. The recorded fluorescence intensity reflects the amount of molecule of interest tagged by the fluorophore present on or inside a passing cell. The detectors convert signal from light to digital and send it to a computer. Thus, the intensity of scatter and fluorescence in different wavelengths is recorded for each individual cell as a separate event and plotted by a computer software like FlowJo™ (BD Life Sciences) as histograms.

The next step is the data analysis, via the same software, which begins with setting up gates to define cell populations. Usually, an unstained cell sample is used for the initial FSC/SSC gating, which is used to exclude small particles, cell debris, and cell aggregates, which have different sizes from the main cell populations. Next, the defined cell populations are divided into living and dead cells based on the fluorescent signal from the viability dye added during staining. Then, the fluorescence signals from the dyes tagging the molecules of interest are examined in the live cell populations, which can be subdivided into positive and negative for the fluorophore. Mean fluorescence intensity (MFI) is recorded for the gated

populations, compared across the samples, and can be used to further differentiate cells based on the expression levels of the molecule of interest. Thus, gating is an essential step in the elimination of background and irrelevant signals to maintain the quality and accuracy of collected data. Gating can be set up manually or via algorithms and is usually saved for future data acquisition. MFI of live cells from the main population are compared to an isotype control or to unstained cells. The isotype control is an antibody of the same class and subtype and conjugated to the same fluorophore as the probe used to detect the molecule of interest, which is added to the staining cocktail instead of the probe in a control sample. This is done as reference to exclude any signal that could arise from non-specific binding of the antibody's Fc region to cell components.

In summary, flow cytometry is a very useful commonly employed technique in various areas of biomedical research. The proper execution of each step from sample preparation to gating and analysis is vital for the quality of obtained data. In this project, I have used flow cytometry in a relatively straightforward way – to confirm the differences in expression of CD44 for my two cell types and to study their ability to bind IgD with and without galectin-9 - but the technique is powerful enough for a much wider range of applications – from cell sorting to biophysical characterization of molecular interactions.

5.2 Confocal microscopy

Confocal microscopy is a technique used to create high-resolution, three-dimensional images of biological samples, which has revolutionized the field of cellular imaging. It is based on the principle of optical sectioning, which allows the imaging of thin sections of a sample while rejecting out-of-focus light from above and below the focal plane, which makes it possible to obtain images with great depth and detail. The basic setup of a confocal microscope includes a light source, a scanning system, and a detector. The light source is typically a laser that emits light of a specific wavelength. The scanning system consists of a series of mirrors that reflect the laser beam to scan the sample in a two-dimensional plane. The detector,

which is commonly a photomultiplier tube, collects the emitted fluorescence signal from the sample, digitalizes the photon count, and feeds it to a computer. In order to achieve optical sectioning, confocal microscopy uses pinhole apertures in front of the laser and detectors. The pinhole in front of the laser allows for the illumination of a specific two-dimensional focal plane, while the one in front of the detector blocks any out-of-focus fluorescent signal, which results in a sharp image of the sample in that plane (Elliott 2020). A completed two-dimensional confocal image is achieved by scanning multiple points in the same plane. By scanning the sample in the z-axis, multiple focal planes can be imaged, which can then be reconstructed into a three-dimensional image, however that technique is mostly limited to fixed, i.e dead cells, due to the dynamics of molecules and organelles inside a living cell.

Confocal microscopy provides significant advantages over conventional widefield microscopy for the high-resolution imaging of biological samples by excluding out-of-focus light thanks to light source and detector pinholes, which allow for the focusing of both exciting and emitted light (**Figure 5.2**). This is even more relevant in fluorescence microscopy, where excitation of out-of-focus dye molecules would significantly blur the image, while also causing them to photobleach (expend their fluorophore ability). In the case of point-scanning confocal microscopy, used to obtain the images presented in this thesis, laser illumination and light detection are focused on the same diffraction-limited spot, which is moved across the sample to construct an image. Lasers, rather than conventional light sources, must be used to generate photons of specific energy for the excitation of distinct fluorophores, while dichroic mirrors are used to filter and reflect emitted light into detection channels to distinguish signals from the separate dyes, similar to the principle described earlier for flow cytometry. Data collection may vary from the common point-scanning method, depending on the sample and desired resolution – spinning disc confocal microscopy can produce higher-resolution images, but with weaker optical sectioning, light-sheet fluorescence microscopy is used to illuminate thin sections of thick specimens, while two-photon microscopy uses two-photon excitation with long-wavelength lasers to excite fluorophores in a small area deep within thick samples.

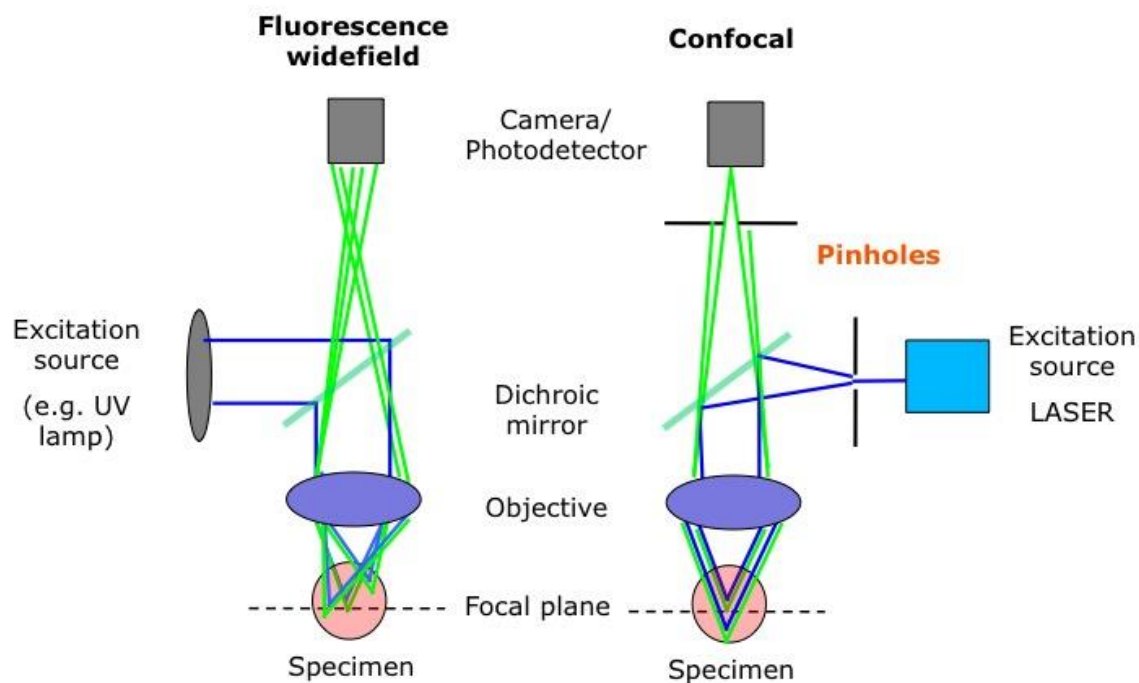


Figure 5.2 The main difference between conventional widefield and confocal microscopy setups is the addition of pinholes. One at the light source limits illumination and fluorophore excitation to one focal point, while another pinhole in front of the detector excludes emitted light from outside the focal point. The light source for fluorescent confocal imaging must be a laser, as it needs to provide a constant directed stream of photons of specific wavelengths needed to excite the fluorophores in the specimen. Resulting image is generated by scanning multiple points in the sample. Image from Cellularimaging.nl.

Confocal microscopy is not without its disadvantages over conventional widefield microscopy, other than the much higher costs of equipment and maintenance. The speed of scanning makes signal acquisition much slower and is not suitable for time-sensitive experiments. The slower acquisition means longer exposure of samples to the laser light, which can cause photodamage to the specimens, while also resulting in photobleaching of fluorophores, causing them to irreversibly lose their fluorescence, which may necessitate the use of high probe concentrations to compensate for this loss, which makes experiments more costly. The field of view in confocal microscopy is much smaller, which limits the ability to image large specimens or multiple cells simultaneously. Sample preparation also more time-consuming.

Sample preparation for confocal microscopy can vary significantly depending on whether the imaged cells or tissues must be alive or not and whether the cells are adherent – i.e. attach to their specially coated containers and form a monolayer – or cells that thrive in suspension. Most common imaging applications use fixed adherent cells – cells are grown and adhere onto the imaging slide or plate, after which they are exposed to a powerful cross-linking agent such as 4% paraformaldehyde, which instantly kills the cells but retains the conformation and state of their extra- and intracellular structures, thus creating a sort of cell snapshot. Cell membranes can then be permeabilized with a detergent such as Triton X-100 to allow intracellular staining. The following staining with fluorescent probes follows a similar protocol for the staining of samples for flow cytometry – first a FcγR blocker is added to prevent the binding of labelled antibodies to any Fc receptors in the sample, then, after washes, the staining cocktail is added. Once again, either labelled antibodies are used directly, or non-labelled primary antibody incubation is done first, followed by addition of the labelled secondary antibodies specific for the primary ones. DNA-binding dyes such as DAPI or Hoechst are also commonly added to the staining cocktail to distinguish the cell nuclei. After the staining and wash steps are completed, the specimen is covered with a cover slip, with or without the addition of mounting medium and sealant, depending on whether the sample must be kept wet or dried respectively, and is ready for imaging. The fixation, permeabilization, and staining of non-adherent cells must be done in suspension (in test tubes) prior to their transfer onto imaging slides or plates. Fixed cell staining offers the advantages of imaging in the z-axis to construct a three-dimensional image of an immobile sample, and the ability to image intracellular stained structures.

On the other hand, the imaging of live cells permits neither fixation nor membrane permeabilization. For obvious reasons, live cells must be used to follow processes such as probe internalization, cell movement, division, and so on. Therefore, different methods for sample preparation must be used, which again depend on whether the imaged cells are adherent or in suspension. Staining and imaging of live adherent cells are more straightforward and are done in a similar way as for fixed adherent cells, of course excluding

the fixation and permeabilization steps. However, for the imaging of suspension cells there must be a way to limit their movement in solution in order to obtain accurate high-resolution images. This is commonly performed by either centrifugation of the cell suspension onto defined areas of a glass slide through a small opening, or by immobilization through the application of a pad made from agarose gel, or other transparent non-toxic materials, on top of the suspension. For the live cell imaging described in this thesis, the latter method was performed, as described in the next chapter. As part of the confocal microscope setup, a special chamber for the sample stage maintains humidity, temperature, and CO₂ concentration during imaging, to maintain cell viability. Imaging in the z-axis is not desirable, as the live cells' internal structures exhibit constant movement, but time-lapse acquisition is applicable for tracking of changes within and outside the cells. The use of high-power laser voltages and long exposure times can cause phototoxicity and damage to live cells and must therefore be avoided.

After staining and mounting, samples are imaged according to the specific experimental requirements. The microscope setup includes focus adjustment, setting optimal laser voltages to achieve signal of the required intensity without excess photobleaching, selecting fluorescence filters, choosing the appropriate lens magnification, and selecting an area of the sample to be viewed.

During the better part of this project, confocal microscopy was performed exclusively on live suspension cells for the purpose of tracking the cell surface binding, internalization, and subcellular localization of our fluorescently labelled IgD. Cell fixation was only used towards the end of the project as a way of verification that labelled IgD does not bind non-specifically to intracellular structures within permeabilized cells.

Fluorescence confocal microscopy has led to a plethora of innovations in cell imaging thanks to its advantages over widefield microscopy. Its significantly improved spatial resolution with optical sectioning and improved signal-to-noise ratio render it a very useful technique for the observation of fine intracellular structures and processes, despite limitations such as slow imaging speeds and high cost of maintenance.

5.3 Mass spectrometry

Mass spectrometry (MS) is an indispensable analytical technique in modern proteomics used to determine the mass and other structural characteristics of molecules. It is commonly used in tandem with liquid chromatography (LC), which is employed to separate the constituents of complex mixtures, such as the proteins in cell lysates, based on their physical and chemical properties. The combination of these two techniques, LC/MS, is a highly sensitive and selective method for the identification and quantification of proteins in biological samples.

The principles of MS involve ionization of separated peptides derived from enzymatic digestion of proteins, followed by their discrimination based on mass-to-charge ratios (m/z) within a mass analyzer. The resulting mass spectra provide information on peptide molecular weights, while tandem mass spectrometry (MS/MS) allows for peptide sequence determination through fragmentation and subsequent proteomics analysis of fragment ions. Comparing experimental mass spectra to theoretical spectra generated from protein sequence databases enables identification and quantification of proteins present in complex biological samples (Domon and Aebersold 2006). The individual components and methods of the technique may vary depending on the experimental requirements. **Figure 5.3** outlines the mass spectrometry workflow used for this project.

The first step of analysis is the preparation of the biological sample, which in our case is proteins from cell lysates. Microsomal enrichment through ultracentrifugation was performed on the lysates, followed by incubation with recombinant IgD, pulled down by incubation with Phl p 7-conjugated Sepharose, and captured proteins were separated by SDS-PAGE, as described in detail in the next chapter. Protein bands excised from the polyacrylamide gel are treated with reducing agent and cut into smaller peptides by treatment with a protease such as trypsin.

Electrophoresis is mentioned often throughout this thesis, so it would be relevant to briefly mention how it works in principle. Proteins are first boiled, and their disulfide bonds reduced by β -mercaptoethanol, if necessary, which leaves all polypeptide chains separated and unfolded. Dodecyl sulfate (DS) anions from SDS in the protein sample coat the denatured chains, giving them a net negative charge proportional to their size and maintaining their unfolded state. Samples are applied to the wells of a gel made of varying concentrations cross-linked polyacrylamide, determined by the desired resolution for particular ranges of molecular weight. Electrical current is run through the gel and the voltage difference between the top and the bottom of the gel causes the proteins coated in DS to migrate in lanes. The speed at which they move through the polyacrylamide matrix depends on their net charge, which in turn depends on their size. Thus, protein bands of different sizes are separated and then stained for visualization.

The peptide mixture is then separated by size via high-performance liquid chromatography (HPLC) and by hydrophobicity via reversed-phase liquid chromatography (RPLC). In HPLC, the mobile phase containing the analytes is filtered through the stationary phase, which is a gel of spherical porous beads. Smaller molecules are retained by the pores and take longer to elute, which results in their separation by size. In RPLC, the stationary phase (column medium) is a hydrophobic material, such as C18 (an 18-carbon hydrophobic compound, bonded to the silica support), and the mobile phase is typically a gradient of water and organic solvent, such as acetonitrile. As the mobile phase passes through the column, the peptides or proteins in the sample interact differently with the stationary phase and the mobile phase. More hydrophobic molecules interact more strongly with the stationary phase, while more hydrophilic molecules are more soluble in the mobile phase. As the proportion of organic solvent in the mobile phase is increased, the strength of the interaction between the molecules and the stationary phase decreases, allowing the molecules to elute from the column. Less hydrophobic molecules are the least retained by the medium and elute first, while more hydrophobic molecules elute when the organic solvent concentration increases. Separation based on hydrophobicity provides a much higher resolution between

molecules, as some of the many peptides in the sample will have similar size and charge, thus reducing sample complexity and signal suppression, which improves the overall quality and precision of protein identification. It also improves the following process of electrospray ionization (ESI).

ESI is a soft ionization technique used to convert peptides or other analytes from the liquid phase into gas-phase ions for direct mass spectrometry analysis. Peptides with different hydrophobicities exhibit varying ionization efficiencies, therefore the separation by hydrophobicity allows for a more uniform ionization process, ensuring that peptides with different ionization properties can be effectively analyzed. The solvents used in the previous step (water and acetonitrile) are volatile and are easily evaporated in this process. In ESI, high voltage is applied to the tip of a capillary through which the sample is running at a very slow flow rate, which induces a charge on the surface of the liquid. As the liquid exits the capillary tip, the electric field causes the liquid surface to become unstable, forming a Taylor cone. At the tip of the cone, the electrostatic repulsion overcomes the surface tension of the liquid, resulting in the ejection of highly charged droplets into the surrounding environment, which undergo desolvation as the solution evaporates. The electrostatic repulsion between charges on the droplets' surface causes them to undergo Coulombic fission, whereby the droplets break into smaller droplets with a higher m/z until the droplets become sufficiently small that the electrostatic repulsion results in the ejection of a jet of gas-phase ions directly from the droplet surface, a process known as ion evaporation (Fenn et al. 1989).

Matrix-assisted laser desorption/ionization (MALDI) is another soft ionization method of converting peptides from the liquid to the gas-ion phase, which instead relies on the use of a laser to desorb and ionize the analytes which are co-crystallized with a solid matrix. MALDI is not amenable to direct coupling with liquid chromatography techniques compared to ESI, as it uses dried sample mixed with the matrix, therefore the latter is preferred for the analysis of complex biological samples.

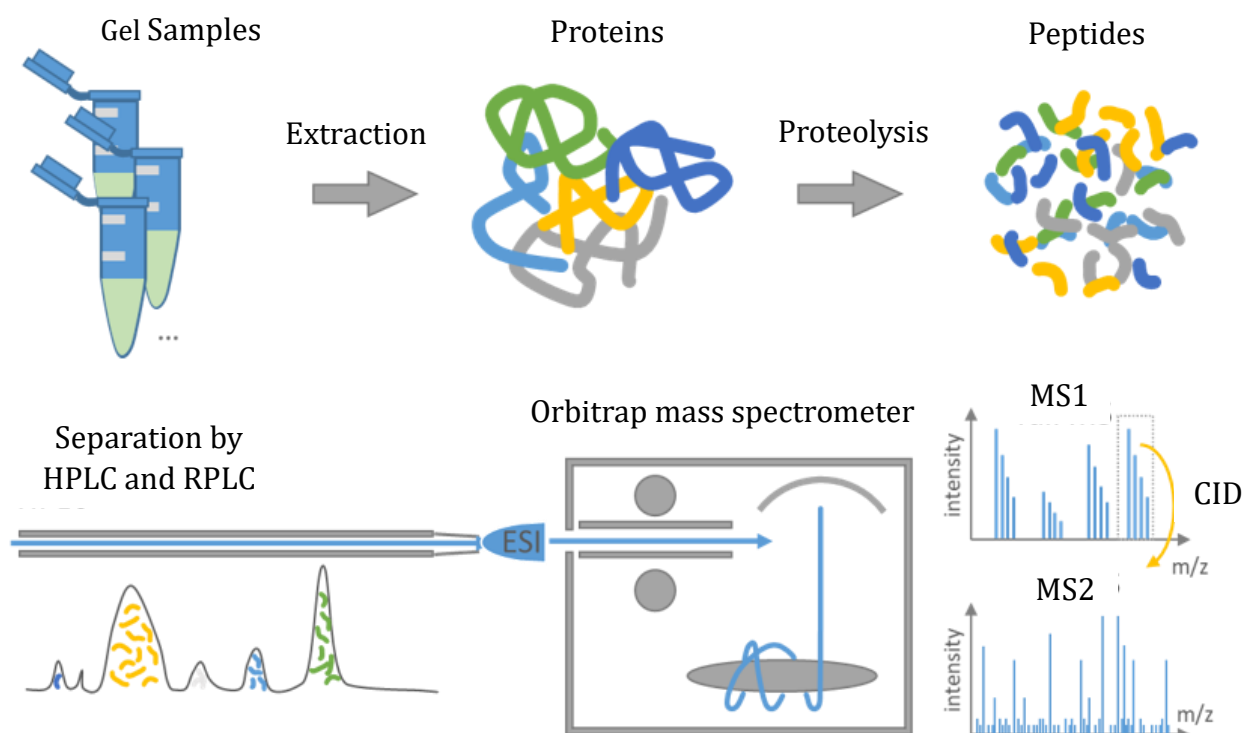


Figure 5.3 Overview of the LC/MS workflow used in the project – Enriched cell lysates are incubated with recombinant IgD, followed by a pull-down with PhI p 7-conjugated Sepharose. Captured proteins are by size and treated with proteases. Peptides are then separated by size and hydrophobicity (HPLC and RPLC respectively) and ionized through electrospray ionization. Ions are guided to an Orbitrap mass analyzer and released towards the electric field of the central spindle electrode, causing them to oscillate. The frequency of their oscillations induces current in the detector electrode proportional to their abundance and mass-to-charge ratios. The induced signals are transformed to mass spectra (MS1) which show the peptides' mass (m/z) and presence (intensity) in the sample. Individual peptide ions are selected for tandem mass spectrometry (MS/MS) and fragmented by acceleration and collision into inert gas molecules (CID), which breaks their atomic bonds. The resulting fragment ions can be run through the Orbitrap again. Their mass spectra (MS2) can be matched with theoretical spectra from proteomics databases via software to determine the amino acid sequence of the chosen peptide, as well as its parent protein. Image adapted from [CSBiology](https://www.csbiology.com/).

The generated gas-phase ions are transferred to the mass analyzer of the spectrometer for separation and detection, which differentiates the ions based on their mass-to-charge ratio (m/z). Several types of mass analyzers exist, each with their specific advantages and limitations. The type used to analyze samples from this project is an Orbitrap mass analyzer. Other spectrometers include time-of-flight (TOF), ion cyclotron resonance (ICR), quadrupole: quadrupole mass analyzers, and so on.

The Orbitrap is a high-resolution and high-mass accuracy analyzer that employs an electrostatic field to confine ions in an orbital motion around a central spindle electrode. The gas-phase ions from ESI are directed via an ion funnel to an electrodynamic ion guide called the “C-trap” released into the center of the Orbitrap where they are forced into an orbital motion around a spindle electrode by its electrostatic field, while also oscillating around the central axis. Their oscillation frequencies are determined by their m/z values with lighter ions oscillating at higher frequencies. The moving ions create an alternating electric field, which in turn induces an electrical signal in the detector electrode, known as image current, proportional to the ions’ oscillation frequencies. The time-domain signal from the current is Fourier-transformed into a frequency-domain signal to determine ion oscillation frequency, and thus, m/z (Makarov et al. 2006). The obtained m/z values are used to generate full mass spectra for the analytes (MS1), enabling their quantification - their presence is proportional to image current intensity - and their identification via the following step of tandem mass spectrometry (MS/MS).

In MS/MS, specific ions from MS1 are selected for further fragmentation and analysis to determine their amino acid sequences. The fragmentation can be induced by different methods – in our case it is collision-induced dissociation (CID), where the ions are accelerated to high kinetic energy using an electric field and directed to collide with an inert gas (e.g., argon or nitrogen), converting some of the ions’ kinetic energy into internal energy, which causes them to become unstable and break apart at specific bonds like the peptide bond. Various types of fragment ions, primarily b-ions and y-ions are produced - b-ions result from the cleavage of the peptide bond with the charge retained on the N-terminal side, while y-ions result from the cleavage of the peptide bond with the charge retained on the C-terminal side. The fragment ions are separated by m/z and their spectra (MS2) detected in the Orbitrap in the same way as for MS1. The mass differences between adjacent fragment ions in the MS2 spectrum correspond to the masses of the amino acid residues in the peptide sequence (Aebersold & Mann 2003). By analyzing the mass differences across all MS2 spectra of fragment ions derived from one selected MS1 peptide, its entire amino acid sequence

can be reconstructed. This process is performed by software like Proteome Discoverer (Thermo) that compares and matches the experimental MS2 spectra to theoretical spectra generated from protein sequence databases using algorithms such as SEQUEST, which can determine the amino acid sequences of peptides and identify their parent proteins. The method described as such is referred to as “shotgun proteomics”, or “bottom-up proteomics”.

Thus, mass spectrometry employs the ionization of chromatography-separated peptides derived from complex biological samples to determine their mass-to-charge ratios and relative abundance, or MS1 spectra. Through subsequent fragmentation of the peptides and database matching of the fragment ions’ MS2 spectra in tandem mass spectrometry, information about the identity of the peptides’ parent proteins in the samples can be acquired. In this project, we used mass spectrometry in conjunction with cell lysate pull-downs in the search of proteins associated with IgD which could be candidates for cell surface IgDR.

5.4 Surface plasmon resonance

Surface plasmon resonance (SPR) is a label-free, real-time optical technique widely used to study the binding affinities and kinetics of biomolecular interactions. In this method, a ligand (in our case, a protein) is immobilized onto a thin gold layer within the flow cell of an SPR chip. An analyte (also a protein) in an aqueous solution is then flowed over the immobilized ligand, allowing it to bind. As the analyte binds to the ligand, its accumulation on the flow cell surface causes a change in the local refractive index. This change affects the angle at which surface plasmons are resonantly excited (the SPR angle). The shift in the SPR angle is measured as response units (RU) over time, generating a sensorgram and is compared to a control flow cell without ligand. From the sensorgram, association and dissociation rates (on- and off-rates) can be determined, and the equilibrium dissociation constant for the interaction (K_D) can be calculated. **Figure 5.4** represents the common setup of an SPR experiment.

As the name suggests, SPR relies on the excitation of surface plasmons, which are electromagnetic waves that propagate along the interface between a metal (such as gold) and a dielectric material (such as glass) in response to incident light. When light passes through an interface from a high refractive index material, which in our case is a prism mounted in the instrument, to a low refractive index material - glass on the SPR chip - some light is reflected off the interface. At an angle greater than the critical angle, total internal reflection occurs. However, when the glass is coated with a thin gold film, such as in an SPR chip, the reflection is not complete, and some light is absorbed by the film. The angle of light at which the most significant loss of total internal reflection occurs, resulting in the minimum reflected light intensity, is called the SPR angle (Homola 2008). This reduction in reflected light intensity (SPR dip) occurs due to the oscillations of mobile electrons (plasma) at the metallic film surface, induced by the incident light coming through the prism. These oscillations form an electromagnetic wave, called the surface plasmon wave or surface plasmon polariton, that propagates along the glass-metal interface. At the SPR angle, the wave vector (describing the wavelength and direction) of the incident polarized light matches the surface plasmon wave vector, leading to resonance. This coupling causes a loss of light energy and, consequently, a reduction in the reflected light intensity. A decaying evanescent electric field generated by the resonance penetrates a short distance into the dielectric medium from the metal film and is sensitive to small changes in the refractive index at the interface. These changes occur due to the presence of an analyte bound to a ligand immobilized on the metal surface. The increase in the refractive index when the protein binds to the ligand results in a small shift in the SPR angle. This shift is recorded by the SPR instrument's photodetector array and plotted via software as response units (RU) over time - a sensogram - with 1 unit equivalent to a shift in the SPR angle by 0.001 degrees (Damborský et al. 2016).

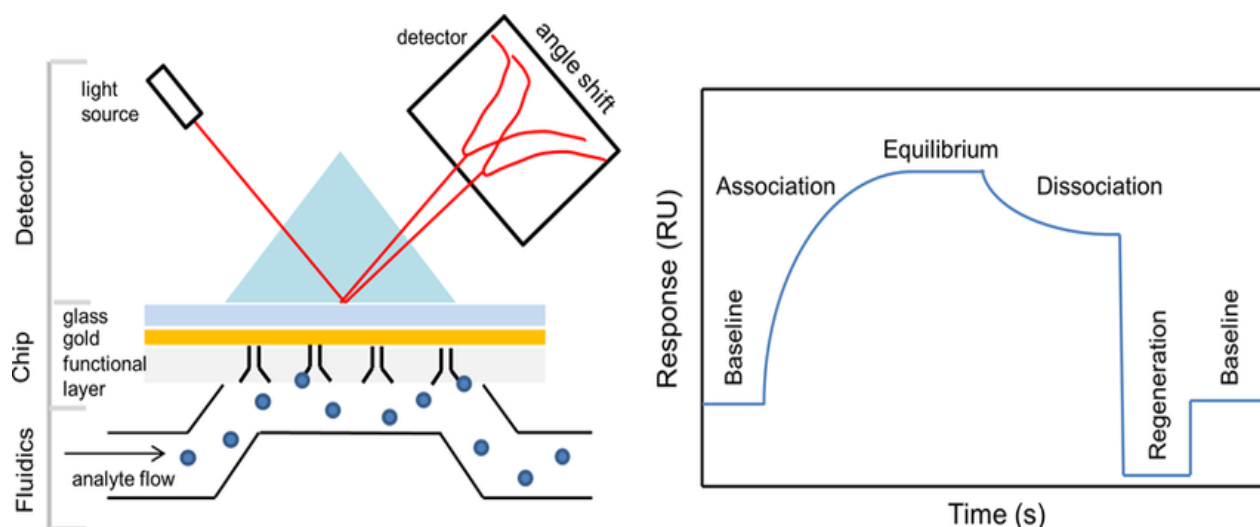


Figure 5.4 Setup of an SPR experiment – Ligand is captured on a gold film covering a glass chip in a flow cell and analyte is flown through it. Polarized light passing through the interface of a prism and the glass causes surface plasmons on the gold layer to resonate at a specific incident angle (SPR angle). The binding of analyte to the ligand causes a local change in refractive index at the interface, which slightly shifts the SPR angle. The SPR angle change is proportional to the amount of analyte bound and is recorded in real-time as a sensorgram. Sensorgrams are fitted to a binding model – the association phase yields rate of analyte binding (k_{on}), while the dissociation phase yields rate of analyte dissociation (k_{off}), which are then used to calculate the dissociation constant for the interaction (K_D), and thus the affinity of analyte for ligand. The chip can then be regenerated. Figure from Damborský et al. 2016.

Data analysis in SPR involves fitting the obtained sensorgrams to mathematical models to extract the kinetic and affinity parameters for the interaction, such as the association rate constant (k_{on}), dissociation rate constant (k_{off}), and the equilibrium dissociation constant (K_D). Before analyzing the data, it is essential to correct the baseline drift, if any. The baseline represents the SPR signal in the absence of binding events and is acquired from a reference flow cell with passing analyte without any captured ligand. By subtracting the baseline signal from the entire sensorgram, the data is corrected, and the focus is solely on the binding event. Sensorgrams obtained from different analyte concentrations are fitted to a binding model, typically the 1:1 Langmuir binding model or other more complex models if needed (e.g., two-state reaction models, heterogeneous ligand models). The choice of the model depends on the characteristics of the interacting molecules and the experimental conditions. A sensorgram curve consists of baseline, association phase,

equilibrium phase, and dissociation phase. The association phase of the sensorgram shows the binding of the analyte to the immobilized ligand on the sensor chip. The association rate constant can be derived by fitting the association phase to an exponential association equation. The fitted curve represents the theoretical binding response based on the chosen model. Similarly, the dissociation phase of the sensorgram represents the release of the analyte from the immobilized ligand and the dissociation rate constant can be derived by fitting the dissociation phase to an exponential dissociation equation. The equilibrium phase represents the point at which the binding and dissociation rates of the analyte and the immobilized ligand have reached a balance, resulting in a steady-state condition. During this phase, the rate at which the analyte molecules bind to the ligand molecules is equal to the rate at which the bound analyte molecules dissociate from the ligand, thus the SPR signal remains constant. The equilibrium dissociation constant represents the affinity between the ligand and the analyte, and it is calculated from the derived rate constants using the equation $K_D = k_{off} / k_{on}$. A lower K_D value indicates a higher affinity between the interacting molecules (rate of binding must be higher than the rate of dissociation). To ensure the validity and reliability of the extracted parameters, it is crucial to evaluate the goodness-of-fit of the model to the experimental data. This can be done by visually inspecting the residuals (the difference between experimental data and fitted curves) and calculating the Chi-square (χ^2) value. A good fit should show random residuals and a low χ^2 value.

Sample preparation for SPR may vary depending on the type and number of analytes and the method for ligand capture on the SPR chip. In our SPR experiments we used immobilized anti-His-tag antibody on the surface of the chip to capture His-tagged Phl p 7. Recombinant monoclonal anti-Phl p 7 IgD antibody was then captured and used as ligand, after which a serial dilution of the recombinant Moraxella IgD-binding protein (MID) was introduced as analyte in order to confirm that the two constructs bind each other, and to obtain the binding kinetics for the IgD: MID interaction. The SPR instrument we used in this project is an in-house Biacore T100 system (Biacore AB).

There are different ways to couple the ligand to the chip surface, however the most common type used for protein ligands is amine coupling - same chemistry used for the fluorescent probe labelling of IgD. The gold surface of the chip is covered with a self-assembled thin monolayer of organic material such as alkanethiols with terminal carboxyl groups (-COOH). These groups are activated by addition of N-hydroxysuccinimide (NHS) and a carbodiimide compound, resulting in NHS-ester formation, which can react with primary amino groups (-NH₂) on proteins such as their N-terminal amines and those of lysine sidechains. The reaction forms a stable amide bond and thus the protein ligand is immobilized. Other methods for ligand coupling include the streptavidin-mediated capture of biotinylated proteins, direct binding of free thiol groups to the gold surface, His-tag capture via nickel ions, and so on.

Both ligand and analyte proteins must be previously purified and dialysed into SPR buffer by HPLC so that only monomers are used, as protein oligomers can significantly alter the experimental results. During the HPLC step, their buffers are also exchanged with SPR running buffer such as HEPES buffer (minimal light absorbance). The purified analyte must be concentrated to a desired molarity and then serially diluted to a range of concentrations for analysis. After analyte is bound and dissociated, the chip can be reused for another binding cycle, following a regeneration step. Regeneration is necessary to ensure all analyte has come off the ligand and is usually done by adding very high or low pH buffers to the flow cell to disrupt the interaction, followed by washes with running buffer, which removes traces of regeneration buffer and returns the SPR signal to baseline.

SPR provides significant advantages over other techniques used to study biomolecular interactions such as isothermal titration calorimetry (ITC) or enzyme-linked immunosorbent assays (ELISA). It requires only small amounts of label-free sample, which is not only more financially feasible, but also bypasses the use of fluorophores and tags, which may alter the biomolecules' properties. SPR also provides real-time monitoring and quantification of kinetic parameters and can be applied to any type of biomolecules – proteins, lipids, nucleic acids, etc. However, SPR is not without its drawbacks and common pitfalls of SPR

are associated with achieving the correct ligand density during coupling to the chip, the proper regeneration of the chip without disruption of the ligand, and the technique's sensitivity to buffer conditions such as pH, temperature, and ionic strength. However, with proper optimization and careful selection of the immobilization chemistry and buffer compositions, these pitfalls can be easily avoided. The only real drawback is the insensitivity of SPR to analytes smaller than 100 daltons, as the tiny change in refractive index associated with their binding results in a negligible shift in SPR angle and cannot be measured accurately. Thus, SPR is mostly limited to larger molecules.

6. Materials and Methods

6.1 Overview of protein constructs

6.1.1 Titular protein constructs summary

The bulk of the laboratory work done in the first two years of the project consisted of optimizing, troubleshooting, and experimenting with methods for the expression, production, and purification of various protein constructs used for flow cytometry, microscopy, pull-downs, and binding experiments. All recombinant proteins used throughout the project, unless explicitly stated, were produced in-house. Expression and purification of the main protein constructs utilized in the project are described in detail later in the chapter. Most proteins batches used were produced and purified immediately before application. Results of protein expression and purification are presented in the next chapter. In-house expression and purifications of protein constructs followed the same general workflow – introduction of plasmid bearing gene of interest into the relevant organism – competent bacteria or mammalian cells - expanding them, collecting protein from the cell lysate or nutritional medium, respectively, purifying the protein through affinity chromatography, separating protein monomers through size exclusion chromatography (HPLC), while also exchanging their buffers, inferring their purity and molecular weight by SDS-PAGE, and storing them at appropriate concentrations in a -80°C freezer for long-term storage, or in a 4°C refrigerator for immediate use. Protein preparations were always handled on ice for all experimental procedures.

Recombinant Phl p 7

Phl p 7 is the major antigen in the pollen of *Phleum Pratense* (common timothy grass) and is a member of the polcalcin family of allergens and so it requires the binding of calcium ions for stability and for its antigenic function (Mitropolou et al. 2018). Being a small soluble protein with monomers at around 8.5

kDa in molecular weight, it is easily expressed in *E. coli*. The Phl p 7 gene insert was cloned into a pET151 TOPO bacterial plasmid expression vector (Thermo) (**Figure 6.1**) and the resulting plasmid was kindly provided by Gintare Bucaite. The *lac* operator sequence upstream of the gene insert facilitates inducible protein expression through IPTG addition to growth medium at a set time point, or through the gradual depletion of glucose and subsequent lactose utilization in autoinduction medium. Protein expression and purification methods are detailed later in this section.

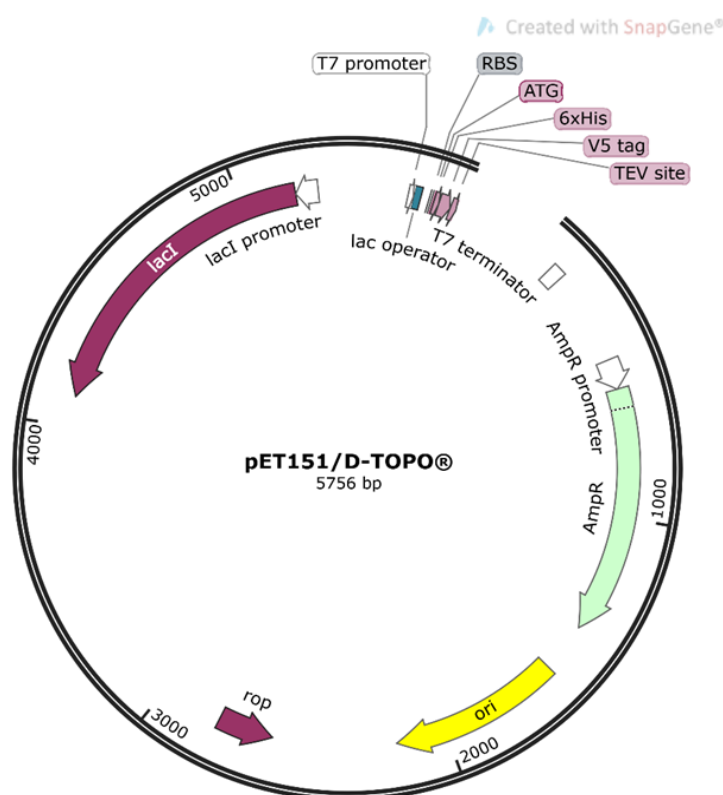


Figure 6.1 Map of the pET151/D-TOPO vector (Thermo) used for Phl p 7 expression in *E. coli*. Topoisomerase-I facilitates the addition of the gene insert of interest to the linearized vector by means of single-stranded overhangs annealing to the CACC 5'-sequence of the insert. Gene of interest expression is under the control of a promoter and terminator for the T7 RNA-polymerase, expressed by expression-competent bacterial strain. T7 promoter is in turn regulated by the *lac* operator sequence, resulting in protein production when lactose is the available food source (Studier and Moffatt, 1986). The resulting protein has a hexahistidine for purification, as well as a V5 tag for detection, linked to the N-terminus of the protein of interest via a TEV protease cleavage site. Plasmid confers β -lactamase expression (AmpR) to transformants, rendering them resistant to ampicillin. Map generated with SnapGene (GSL Biotech).

Recombinant human monoclonal IgD antibody with specificity to Phl p 7

The **pVitro1-D-102.1F10** IgD plasmid construct (**Figure 6.2**) was engineered by Balraj Sandhar via an established PIPE-cloning protocol for transferring antibody isotype or specificity (Dodev et al. 2014) from a pre-existing human anti-Phl p 7 IgE construct (Mitropoulou et al. 2018). Due to the structure's complexity and lack of the necessary folding and glycosylation machinery in bacteria, it was expressed in transfected human embryonic kidney cell line (HEK293). The mammalian pVitro1 expression vector allows for the simultaneous co-expression of two protein constructs of interest, which in this case are the λ light chain and δ heavy chain, thanks to the two separate sets of promoters and enhancer flanking those gene inserts. Secreted IgD in the nutritional medium was then purified by binding to a custom in-house built Phl p 7-conjugated Sepharose column, detailed below.

For flow cytometry and confocal microscopy experiments the recombinant IgD was labelled with Alexa Fluor 546 dye (Thermo), as described later.

Recombinant *Moraxella* IgD-binding protein (MID) (residues 962-1200)

Fortunately, the soluble minimal IgD-binding fragment of the giant 200 kDa MID membrane protein encompasses just 238 amino acid residues (Nordström, Forsgren, and Riesbeck 2002), with molecular weight of about 27 kDa and is amenable to expression in *E. coli*. The pET26b plasmid construct bearing the MID (962-1200) insert was kindly provided by prof. Kristian Riesbeck (Lund University, Malmö), after my initial failure to produce a working stable MID construct (901-1250) due to inconsistencies in literature, as described in detail below. In its native form, the construct forms tetramers of about 100 kDa (ibid). MID was expressed the same way as Phl p 7 – bacteria transformed with the plasmid were also grown in autoinduction medium.

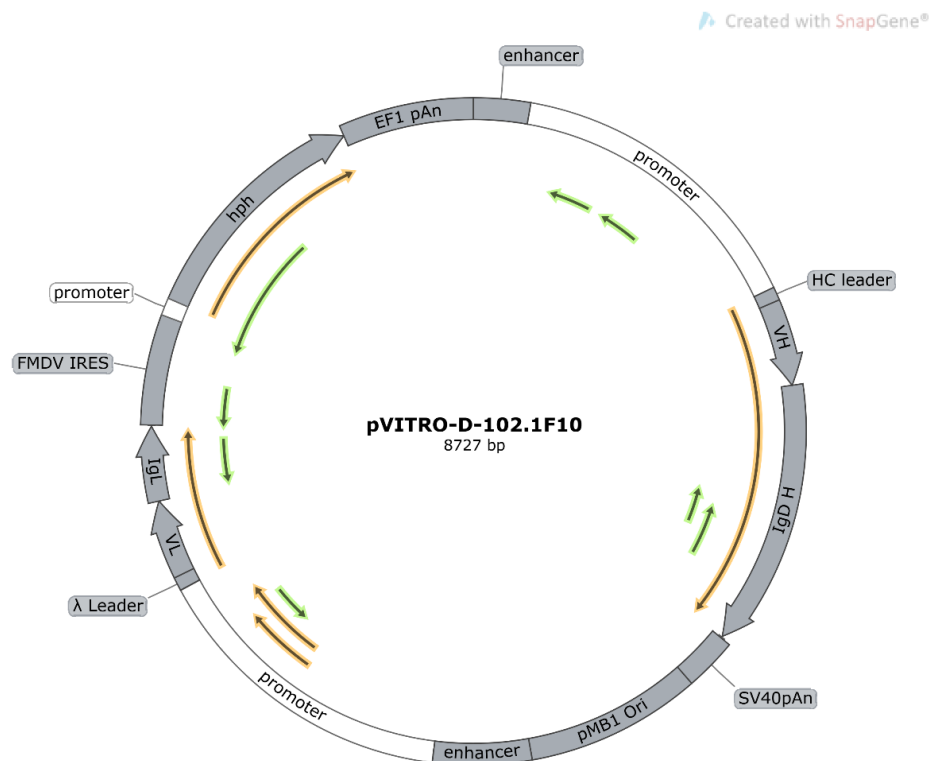


Figure 6.2 Map of the vector used for monoclonal IgD expression. The pVITRO vectors are widely used for antibody production due to their ability to facilitate the simultaneous expression of two proteins of interest from the same vector – in this case the light and heavy chains making up the antibody. The vector has two separate cloning sites and protein expression is under the control of two separate sets of promoters and viral enhancers, as well as a Foot and Mouth Disease Virus (FMDV) internal ribosome entry site (IRES), which allows translation of two open reading frames from the same mRNA. hPh gene encodes a hygromycin phosphotransferase enzyme, which confers hygromycin B resistance as a selection marker. Map generated with SnapGene (GSL Biotech).

6.1.2 Other protein constructs produced

As before the disruption of the project due to the coronavirus pandemic in 2020 we aspired to do a range of structural, binding, and cell studies involving IgD, MID, Phl p 7, as well as different constructs and complexes of these proteins, several other protein constructs were created that were either under-utilized or under-optimized due to the resulting time restraints.

Generation of IgD-Fab fragments by site-directed mutagenesis

Early in the project it was decided to employ the use of IgD-Fab fragments (C δ 1 and V_H domains of the heavy chain joined to the light chain) as controls, and later for crystallization trials, but were unfortunately not experimentally utilized in this project due to time limitations. To generate the Fab fragments a novel and simplistic strategy was developed – the introduction of a point mutation in the original pVtro1-D-102.1F10 IgD DNA construct by site-directed mutagenesis (SDM), whereby a single cytidine nucleotide, in the region corresponding to the hinge of the δ heavy chain, was mutated to thymidine, rendering a CAG codon, which encodes a glutamine residue, into a TAG stop codon, which results in terminating the amino acid chain during protein translation. The assembly of Fab fragments within the HEK293 cells should follow the same principle as for whole antibody expression. The benefit of this strategy is its simplicity, as conventional Fab fragment production requires papain digestion of whole purified antibody, or *de novo* cloning (Zhao et al. 2009), the employment and optimization of which would be very time consuming.

Initially, I attempted to carry out the mutagenesis through PCR using primers to introduce the mutation, without using a commercially available kit. The primers for SDM must be 25-45 nucleotides long, with the mutated base in the middle of the sequence, with temperatures of above 78°C. Unfortunately, subsequent sequencing via Mix2Seq Kit (Eurofins Genomics) of plasmids purified from pelleted transformants via Monarch Plasmid MiniPrep kit (NEB), according to the manufacturer's instructions, revealed no induced mutations in the original IgD template. Therefore, I employed the use of QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent), which induced the mutation on the first attempt, as confirmed by sequencing. PCR reaction was carried out according to the manufacturer's protocol. DNA polymerization was done by the QuickChange Lightning *Pfu*-based polymerase. Methylated parental template was digested with DpnI. No gel extraction was performed. Reaction was directly transformed into XL10-Gold Ultracompetent *E. coli*, supplied with the kit, according to manufacturer's instructions. Cells were plated onto LB-agar plates with 50-150 μ g/ml hygromycin B as selection marker. Expanded XL10-Gold bacteria

were used for plasmid purification via GenElute HP Maxiprep Kit (Sigma), according to the manufacturer's manual. SDM on the plasmid was confirmed by sequencing via Mix2Seq Kit (Eurofins genomics). HEK293 cells were transfected with the plasmid in the same way as for the full-IgD bearing pViro construct and Fab fragments were expressed and purified the same way as for whole IgD, as described later. Yield was somewhat higher than for whole IgD and averaged around 2.5 mg per litre of cell culture. Fab fragments were concentrated to 0.22 mg/ml in TBS-Ca buffer after HPLC purification, concentration was measured via NanoDrop spectrophotometer (Thermo) as absorbance at 280 nm, size and purity checked by SDS-PAGE, and stored at -80°C.

Initial attempt at MID IgD-binding fragment (901-1250) cloning and production

In the early days of the project, prior to receiving the proper MID-pET26b construct from prof. Riesbeck, I attempted to clone it *de novo*. The full protein sequence for MID was copied from NCBI (RefSeq: WP_041786631.1). Due to uncertainties in the literature as to the MID residue numbering, differing with around 50 amino acid residues, depending on what strain of *M. Catarrhalis* was described, a sequence corresponding to the minimal IgD-binding fragment (res. 962-1200), with additional 50 residues or so on either terminus, was chosen for cloning - MID (res 901-1250). The protein sequence was reverse-translated to RNA and then into DNA sequence, which was codon optimized for *E. Coli* expression, using the online tool Sequence Manipulation Suite (bioinformatics.org). Optimised DNA sequence was sent to Integral DNA Technologies (IDT) for DNA gblock fragment synthesis. IDT gene fragments were resuspended in RNase-free deionized water (Sigma) to a concentration of 5 ng/μl and were stored at -20°C upon arrival. Gene fragments were amplified via 2-step PCR program, using Phusion Flash High-Fidelity polymerase (Thermo) and primers to introduce a 5' CACC overhang, required for the following TOPO cloning, as well as a 3' TAA stop codon. The 2-step PCR program omits the primer annealing step due to the higher melting temperature of the primers used and the high efficiency of the Phusion polymerase. When a plasmid

template was used for amplification, methylated parental DNA was digested through the addition of 1 µl FastDigest DpnI restriction enzyme and 2 µl FD Buffer (Thermo) to PCR reaction, following completion. Digestion reaction was incubated for 15 min at 37°C.

PCR reaction was then transferred to a 1% agarose-TBE gel, with GelRed (Biotium) UV stain, for electrophoresis in TBE buffer to confirm the size of the amplified product. Gel was viewed in a Gel Doc XR+ gel documentation system (Bio-Rad) under UV light and DNA bands of the appropriate size were excised and purified via Monarch DNA Gel Extraction Kit (NEB) as per the manufacturer's instructions. DNA concentration was determined on a NanoDrop spectrophotometer (Thermo) by measuring light absorption at 230 nm.

The gel-purified PCR products were then cloned into a pET151 TOPO expression vector by means of Champion pET151 directional TOPO expression kit (Thermo). A molar ratio of 2:1 insert: vector was used for the cloning reaction (**Table 5.1**). Expression of this plasmid should yield the protein of interest, with a hexahistidine tag, for subsequent purification nickel-affinity chromatography, attached to the protein's N-terminus via a V5-epitope sequence and TEV-protease cleavage site for tag removal.

Table 5.1 TOPO cloning reaction

Salt Solution	0.5 µl
Purified PCR product	0.2-2 µl
H2O	Up to 2.5 µl
pET151 vector	0.5 µl (10 ng)
Total	3 µl

Cloning reaction was incubated for 5 min at room temperature and was transformed into 10 µl NEB 10β chemically competent cells (NEB). Cells were kept on ice for 30 min, heat shocked at 42°C for 30 sec, and put back on ice for another 5 min. 100 µl of super optimal broth with catabolite repression (SOC medium, Thermo) were added to the bacteria, and incubated for 1 hour in a 37°C shaking incubator. Cells were then plated onto LB-agar with 100 µg/ml ampicillin as a selection marker, as the pET151 vector bears the AmpR

gene encoding the β -lactamase ampicillin degrading enzyme, and incubated overnight. Colonies were then screened for the presence of insert in the pET plasmid via colony PCR with T7 promoter/terminator-specific primers and REDTaq DNA polymerase (Sigma), followed by 1% agarose-TBE gel electrophoresis. Successful transformants were inoculated into 5 ml LB medium with 100 μ g/ml ampicillin and grown overnight shaking at 37°C for plasmid extraction with the Monarch Plasmid Miniprep Kit (NEB) as per the manufacturer's instructions. Plasmid DNA concentration was measured via NanoDrop spectrophotometer (Thermo) by registered absorbance at 230 nm, and samples were sent for sequencing via a Mix2Seq Kit (Eurofins Genomics). MID (901-1250) was expressed and purified the same way as Phl p 7, detailed later in the chapter. HPLC fractions were concentrated by centrifugation in 50 kDa cutoff filters (Milipore) and were stored in TBS buffer at 5 mg/ml concentration at -80°C. MID (901-1250) was initially stable, with yields in BL21 (DE3) *E. coli* (NEB) of up to 20 mg per litre of bacterial culture. The molecular weight of MID (901-1250) was estimated to be 40.6 kDa and, with the addition of the N-terminal tags, as well as the protein being unfolded by SDS, the apparent molecular weight should be slightly larger (around 46 kDa). This construct was used for binding experiments with IgD until certain problems arised with mutations in the plasmid template, described in the Results section. Despite managing to rescue the original MID (901-1250) plasmid construct, we had come into contact with prof. Kristian Riesbeck, who provided the correct MID (962-1200) sequence, cloned into a pET26b vector, which was the construct used for the rest of the project.

Fluorescent MID conjugates

Four MID (901-1250) constructs conjugated to fluorescent proteins (FPs) via 16-residue flexible linkers (MID-eGFP, eGFP-MID, mRFP1-MID and mCherry-MID) were created by Rebecca Beavil, by means of Polymerase Incomplete Primer Extension (PIPE) cloning. The MID insert was cloned out of the previously generated pET151 plasmid, and into sites of linearized pET151 mCherry-mNAMPT, RFP-GFP, or GFP-GFP

plasmid constructs, also made by Rebecca Beavil. PIPE cloning utilizes incomplete extension and compatible primer overhangs to join two DNA sequences. The Phusion Flash 2-step PCR program with no final extension was used to amplify the MID fragment, while vector amplification was done with the slower Pfu polymerase (Promega). The resulting reaction with linearized amplification product was digested with DpnI for parental strand removal and the product was circularized via covalent end ligation with Rapid DNA Ligation Kit (Thermo), according to its manual. Ligation reaction was loaded onto 1% agarose-TBE gel, stained with GelRed (Biotium) for electrophoresis. Gel bands of appropriate sizes were excised under UV illumination, and DNA was extracted using Monarch DNA Gel Extraction Kit (NEB). Plasmid transformation, expansion, sequencing, as well as protein expression and purification, were dealt with in the same manner as described in detail for Phl p 7 in section 6.2.

Dot blots with MID or MID-FP were used to test whether the preparations bind IgD, as well as to test the fluorophore efficiency of the MID-FP constructs. MID-FP constructs were tested after being stored at 4° for about a month, to simulate conditions for storage of labelled secondary antibodies. 0.5 µg of 102.1F10 anti-Phl p 7 IgD were dotted onto all membranes, which were later incubated with 1 µg/ml of MID-FP conjugates in 5% milk powder. Blots were viewed under 3 different light filters – AF-488, AF-546 and AF-647 (green, red, and far-red, respectively) in a Gel Doc XR+ (Bio-Rad).

Attempts at cloning and expressing galectin-3 and galectin-9

Due to budgetary reasons, because of the high usage of galectin-9 after the Shan et al. paper was published in 2018, it was decided to attempt to clone and produce recombinant human gal-9, as well as gal-3 as an IgE-binding control, in-house. Protein sequences for Gal-3 (NP_002297.2) and Gal-9 (NP_033665.1) were dealt with in the same manner as previously described for the initial MID (901-1250) construct. IDT gene fragments for the two sequences were obtained and cloned into pET151 expression vectors conferring

ampicillin resistance to transformants. Due to low protein yield and degradation, the attempts at producing recombinant human gal-3 and gal-9 were abandoned in the interest of time.

After my initial attempts to clone and express gal-9 (as well as gal-3) in *E. coli* were unsuccessful, it was decided to use commercially available gal-9 due to time limitations. Recombinant human galectin-9 (40 kDa), produced in *E. coli*, was purchased from R&D systems (2045-GA-01M). Some of the gal-9 used in this project was kindly provided by prof. Fu-Tong Liu (University of California-Davis), also recombinant human variant, produced in *E. coli* at their facility.

Primer Specificity	Sequence
MID_F TOPO	CACCGCTGGGGAGATTTCGCACA
MID_R_STOP	TTAGTCCGACGCCGCCTTCTCC
MID-FP_F	AGCGGTGGCGGTGGTTCCGCTGGGGAGATTTCGCAC
MID-FP_R	TCTGAGCTCGCCCTTTTATTAGTCCGACGCCGCCTTCTCC
Open_vector_FPMID_F	TAAAAGGGCGAGCTCAGATCCGG
Open_vector_FPMID_R	GGAACCACCGCCACCGCTGCC
MID-GFP_F	CTCCTCGGTCTCGATTCTACGAAAACCTGT
MID-GFP_R	TACCGCCACCGCCCGAGTCCGACGCCGCCTTCTCCA GTCCC
Open_vector_MID-GFP_F	TCGGGCGGTGGCGGTAGCG
Open_vector_MID-GFP_R	ATCGAGACCGAGGAGAGGGTTAGGG
IgD_Fab_STOP_F (SDM)	GAGTCTCCAAAGGCATAGGCCTCCTCAGTGC
IgD_Fab_STOP_R (SDM)	GCACTGAGGAGGCCTATGCCTTTGGAGACTCTG
Gal_3_TOPO_F	CACCATGGCTGATAATTTCAGC
Gal_3_TOPO_R	TTAAATCATTGTGTAGCTGGCG
Gal_9_TOPO_F	CACCATGGCATTCTCTGGTTCAC
Gal_9_TOPO_R	TTACGTCTGAACATGCGTCAATTGG

6.2 Phl p 7 expression and purification

The pET151-Phl p 7 plasmid construct (Figure 6.1), kindly provided by Gintare Bucaite, was first transformed in NEB 10 β *E. coli* (NEB) for plasmid expansion. All production steps were according to the protocol described in Mitropoulou et al. 2018. Cells were kept on ice for 30 min, heat shocked at 42°C for 30s and put back on ice for another 5 min. 100 μ l of super optimal broth with catabolite repression (SOC medium, Thermo) were added to the competent cells, which were incubated for 1 hour in a 37°C shaking incubator. Cells were then plated onto LB-agar plates with 100 μ g/ml of ampicillin as a selection marker and incubated overnight.

For generation of large quantities of plasmid DNA, colonies from the LB-agar plates were expanded in 50 ml of LB broth with 100 μ g/ml ampicillin (**Table 5.3**) overnight at 37°C. Bacteria were then pelleted by centrifugation and plasmid DNA was purified using the Monarch Plasmid MiniPrep kit (NEB), according to manufacturer's protocol. Purified plasmid was sent to EuroFins genomics for sequencing prior to the following step.

Table 5.3 Luria-Bertani (LB) broth recipe (per 1L)

Component	Amount
Tryptone (Sigma)	10 g
Yeast Extract (Sigma)	5 g
NaCl	10 g
Ampicillin (100 mg/ml)	1 ml
Deionized water	to 1000 ml

Plasmid was then transformed into BL21 DE3 expression-competent *E. coli* (NEB) in the same manner as for the NEB 10 β . This bacterial strain expresses the T7 RNA-polymerase needed for transcribing inserts in the pET151 plasmid. Colonies from the LB-agar plate were then grown in LB starter cultures with 100 μ g/ml ampicillin in a shaking incubator at 37°C to sufficient density before being transferred to ZYP-5052 rich auto-induction medium (Studier 2014), the recipe for which is described in **Table 5.4**, and were incubated

shaking at 30°C (to reduce further bacterial growth) for 72 hours. The benefit of the autoinduction medium is that it does not require manual induction of expression via the addition of IPTG as a stimulant of the lac operon on the vector. Instead, the composition is such that once the initial preferred glucose carbon source is diminished, only lactose remains, which triggers the lac operon on the vector and results in the induction of robust recombinant protein expression.

Table 5.4 ZYP-5052 rich auto-induction medium recipe (per 1L)

Component	Amount
N-Z amine AS (Sigma)	10 g
Yeast Extract (Sigma)	5 g
1M MgSO ₄	1 ml
1000x metals mix (Sigma)	1 ml
50x 5052 (0.5% glycerol; 0.05% glucose; 0.2% lactose)	20 ml
20x NPS (0.5M (NH ₄) ₂ SO ₄ ; 1M KH ₂ PO ₄ ; 1M Na ₂ HPO ₄)	50 ml
Ampicillin (100 mg/ml)	1 ml
Deionized water	to 1000 ml

Phl p 7 was grown in vented-cap flasks and all open handling was done in a biological safety cabinet, as it can be a very potent allergen. Bacterial cultures were centrifuged, and pellets stored overnight at -80°C, which causes ice crystals to crack the frozen cell membranes. Complete lysis buffer (**Table 5.5**) was added to pellets (roughly 5 ml per g of pellet). Cell lysate was left to stir for 30 min with frequent mixing before spinning down. Supernatant was then run through a HisTrap FF crude 1ml column with Ni²⁺ ions (GE Healthcare) on an AKTA Prime pump system, at a flow rate of 1 ml/min, at 4°C. Recombinant protein binds to the column resin by virtue of its N-terminal hexahistidine tag. Column is then washed with binding buffer A (**Table 5.6**), with low imidazole concentration to outcompete non-specific binding. The tight interaction of the histidine tag and the nickel ions on the NTA resin is outcompeted by the high imidazole concentration in the elution buffer B. A step gradient between binding buffer A and elution buffer B was set. UV elution profile was viewed with PrimeView software. Due to the lack of tryptophans in the amino

acid sequence of Phl p 7, very low UV absorption at 280 nm, typically used to quantify proteins, is observed, and the UV trace is not an accurate representation of protein presence.

Table 5.5 Composition of lysis buffer (pH 7.4)

Component	Final concentration
Na ₂ PO ₄	20 mM
NaCl	500 mM
Imidazole	20 mM
Lysonase Bioprocessing Reagent (Novagen)	1:2500
IGEPAL CA-630 (Sigma)	1 %
cOmplete EDTA-free protease inhibitors (Sigma)	1 tablet per 10 ml

Table 5.6 Composition of Ni-NTA buffers

Component	Buffer A (binding)	Buffer B (elution)
Na ₂ PO ₄	20 mM	20 mM
NaCl	500 mM	500 mM
Imidazole	20 mM	500 mM
NaN ₃	0.05%	0.05%

Protein fractions were sampled via SDS-PAGE, concentrated to >1 mg/ml via centrifugation in 15 kDa cutoff filters (Milipore) in 5 min steps at 2 500 g and further purified and examined by HPLC (custom Gilson system) on a Superdex 75 Increase 10/300 GL gel filtration column (GE Healthcare), while also having its buffer changed to TBS. 4 mM calcium chloride was added to the purified Phl p 7 subsequently, as to avoid formation of calcium phosphate salt in the HPLC system's fluidics, and stored for subsequent use. All following buffer exchanges were done through HPLC or buffer dialysis.

6.3 Phl p 7-conjugated column

For more efficient purification of large quantities of recombinant monoclonal anti-Phl p 7 IgD, purification via antigen binding was employed, as opposed to previously used methods based on lectin-binding (Andrew et al. 2001). For this purpose, recombinant Phl p 7, expressed and purified as described above, was conjugated to NHS-activated Sepharose 4 Fast Flow resin (GE Healthcare), which was packed into a chromatography column (GE Healthcare). The resin itself is an agarose matrix with pre-activated NHS (N-hydroxysuccinimide) spacer arm groups available to form covalent bonds with free primary amino groups on proteins, like the N-terminal amino groups and lysine sidechains.

Two iterations of a Phl p 7-conjugated Sepharose resin were made with different bed volumes and amounts of conjugated antigen before the final third column was produced, which was used throughout the latter half of the project. The first column was used for testing and it had a less than 5 ml bed volume, while the second one had about 15 ml bed volume, but Phl p 7 ligand density was too high and resulted in the aggregation of IgD during the purification process.

Average ligand density of the Sepharose 4 Fast Flow matrix is about 20 μ mol of activated ester groups with a total dried medium volume of 20 ml. Assuming 50% average coupling rate, 800 μ moles of purified Phl p 7 (9.97) mg, dialyzed beforehand into coupling buffer (0.2 M NaHCO_3 , pH 8 and 0.5 M NaCl), were added to HCl-washed Sepharose matrix medium and mixed at 4°C overnight. It is important to avoid using high protein concentrations in low volume, as that could lead to spontaneous oligomer and multimer formation, which upon conjugation could obscure the epitopes recognized by the anti-Phl p 7 IgD, which was a problem in the second iteration of the Phl p 7 column. Non-reacted groups were then blocked by addition of blocking buffer (0.5 M ethanolamine, pH 8.0, 0.5 NaCl). Medium was then washed several times with two alternating alkaline (0.1 M Tris-HCl, pH 8) and acidic (0.1 M acetate, pH 4, 0.5 M NaCl) buffers, before being stored in 20% ethanol to prevent microbial contamination. The final optimized Phl p 7 column had a bed volume of 15 ml and was connected to the AKTA Prime pump system. If we assume 50% coupling

efficiency leading to 10 μ moles Phl p 7 per ml of resin, of which half could bind IgD, then the column should theoretically be able to bind roughly 0.1 mg of IgD per ml bed volume, or in this case – up to 1.5 mg of antibody, which were indeed roughly the amounts obtained in each purification batch. Flow rates of 1-2 ml/min were used for full IgD and IgD-Fab purification via the column.

6.4 MID (962-1200) production

Production and purification of recombinant minimal IgD-binding fragment of MID (res 962-1200) was done the same way as for Phl p 7. The pET26b plasmid bearing the MID gene insert, kindly provided by prof. Kristian Riesbeck, bears much the same features as the pET151 vector, except that the antibiotic used for selection in this case is 50 μ g/ml kanamycin. Plasmid was amplified in NEB 10 β *E. coli*, purified, sequenced and then transformed into expression competent BL21 (DE3) *E. coli*, which were grown in autoinduction medium for 72 hours at 30°C in a shaking incubator. Cell pellets from cultures were frozen, then lysed, after which lysate was run through a Ni-NTA column. Protein fractions were concentrated to >1 mg/ml in 50 kDA cutoff filters (Milipore) by centrifugation, after which HPLC was performed to separate monomers and exchange buffer to SPR buffer. Fractions were reconcentrated to 2 mg/ml to avoid aggregation and stored at 4°C for immediate use.

6.5 Attempt at creating MID-conjugated column

To facilitate potential future production of IgD of different specificities, I attempted to create a MID-conjugated Sepharose column, which has previously been described (Samuelsson, Forsgren, and Riesbeck 2006). This was done at the stage prior to producing the correct (962-1200) MID construct, which may explain the lack of success with it. Since the initial MID (901-1250) was problematic due to issues with template plasmid mutation described in the Results chapter, the best-expressing and most stable FP-MID conjugate, produced and purified by Rebecca Beavil, was used for the column – mCherry-MID. Protocol

for the conjugation of mCherry-MID in coupling buffer to Sepharose 4 Fast Flow (GE Healthcare) was the same as for the Phl p 7 column, detailed above, with the appropriate recalculation of available primary amines on this construct. Unfortunately, SDS-PAGE post-purification of IgD with this mCherry-MID column revealed a lot of unspecific proteins in elute, as well as significant amounts of IgD left in the flowthrough, therefore the antigen-mediated purification of IgD via the Phl p 7-conjugated columns was relied on for most of the project instead.

6.6 HEK293 cell transfection with IgD- and IgD-Fab-encoding plasmids

pVitro1-D-102.1.F10 IgD and IgD-Fab plasmid constructs were first expanded in NEB 10 β *E. coli*, purified with GenElute HP Maxiprep Kit (Sigma), according to manufacturer's instructions, checked by sequencing (Mix2Seq - Eurofins Genomics), and finally transfected into HEK293 (human embryonic kidney) cells with the use of the FuGENE transfection reagent (Promega). The reagent consists of cationic lipids in 80% ethanol, which form liposome complexes with negatively charged DNA (Jacobsen et al. 2004). HEK293 cells were previously used for the generation of pVitro1-D-102.1.F10 anti-Phl p 7 IgD and were obtained from Balraj Sandhar. Cells were grown in high-glucose Dulbecco's modified eagle medium (DMEM, Gibco), supplemented with 10% fetal calf serum (Sigma), 2 mM glutamine (Sigma), 100 μ g/ml penicillin and 100 U/ml streptomycin (Sigma), henceforth referred to as full DMEM. 24 hours prior to transfection, cells were plated into 24-well plates at a density of 4×10^5 cells per 500 μ l full DMEM medium in each well. Plasmid was diluted to 20 ng/ μ l in DEPC-treated water. For a FuGENE: DNA ratio of 3:1, 3.3 μ l of reagent was added to 52 μ l diluted plasmid solution, mixed and incubated for 10 min at room temperature. 25 μ l of complex was added per well (2 wells transfected). A hygromycin B kill-curve was set up in the said 24-well plate – a range from 50-1000 μ g/ml of antibiotic was added in duplicates, to select the lowest hygromycin concentration, at which most of the untransfected cells perish. 72 hours later, cells were observed and the appropriate concentration of hygromycin B was selected to be 50 μ g/ml. Cells from the 2 transfected wells and 4 other control wells were digested with cold Trypsin-EDTA to detach, then were split 1:4 with warm

full DMEM, and transferred into 6-well plates, with the addition of 50 µg/ml hygromycin B. Upon reaching confluency, transfectants were sequentially transferred into T25 -> T75 -> T175 flasks, and then into 1L of full DMEM in magnetic spinner flasks. Cells were left in the flasks at 37°C and 5% CO₂ for 4 weeks before harvesting. The gentle stirring stops cell attachment and provides equally rich oxygen and nutrient distribution for all cells, thus optimizing protein expression.

6.7 IgD production and purification

After transfected HEK293 were grown in 1L full DMEM in spinner flasks for 4 weeks, cell cultures were centrifuged at 3 000 g for 20 min at 4°C, cells and debris were discarded, while the supernatant was filtered and stored with 0.05% NaN₃ at 4°C until purification. Antigen affinity chromatography was then performed with the supernatant, which was run through the Phl p 7-conjugated Sepharose column, described in section 6.3, attached to an AKTA Prime pump system (GE Healthcare). In a similar manner to the production of the other protein constructs, after running the supernatant through the column, it was washed with TBS-Ca binding buffer (0.1 M Tris-HCl, pH 8, 0.15 M NaCl, 4 mM CaCl₂, 0.05% NaN₃) (calcium is necessary for Phl p 7 binding) to remove unspecifically weakly bound proteins, after which the elution buffer (0.2 M glycine, pH 2.5, 50 mM NaCl, 0.05% NaN₃) disrupts the antibody: antigen interaction, thus eluting the antibody. 1 M Tris solution was added 1:1 to the protein fractions to raise the pH back to about 7. Flowthrough of unbound proteins was run through the column again, as unbound IgD was still present in flowthrough due to saturation of Phl p 7 immobilized on the column medium. Phl p 7-Sepharose resin was then restored by washing with binding buffer. Diluted protein fractions were then pooled concentrated with a 150 kDa cutoff centrifugal filtering unit (Milipore) and run through a Superdex 200 Increase 10/300 GL gel filtration column (GE Healthcare) via custom Gilson HPLC system. Protein was concentrated to 0.5 mg/ml aliquots to ensure lack of aggregation and stored at -80°C until further use. Protein yields were on average in the order of 2.2 mg of IgD per litre of HEK293 cell culture.

6.8 Surface Plasmon Resonance

To biophysically confirm the interaction between the in-house produced anti-Phl p 7 IgD and MID, SPR was carried out on a Biacore T-100 system (Biacore AB). In this technique, the refractive index near a sensor surface is measured, as described in the previous chapter. His-tagged Phl p 7 at 20 nM concentration, previously dialysed into SPR buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% NaN₃, 0.001% P-20 surfactant) via HPLC, was immobilized on the surface of the target flow cell of a CM5 chip (GE Healthcare) by means of binding to an anti-His antibody, prebound to the monolayer coating of the chip through NHS-mediated amine coupling. Meanwhile, no Phl p 7 is flown through the reference flow cell, as its signal will be used for baseline correction. Anti-Phl p 7 IgD at 20 nM concentration, also dialysed in SPR buffer via HPLC, was then allowed to bind to Phl p 7, thus becoming itself the immobilized ligand thanks to the very high affinity of the antigen: antibody interaction. Then, after flowing some SPR buffer to remove unbound proteins, MID, also dialysed by HPLC, was flown through the flow cells at a serial dilution down from 500 nM to 8 nM MID in SPR buffer. As the analyte of interest accumulates onto the immobilized ligand, the refractive index near the chip surface is increased and recorded as response versus time, or a sensogram, which is compared to a reference flow cell without ligand (Nguyen et al. 2015). The ligand: analyte interaction is then disrupted through addition of 0.2 M glycine solution with pH 3 to regenerate the chip for the next cycle after a wash with SPR buffer.

Data from the Biacore instrument were exported as tab-separated value (tsv) files denoting resonance units (RU) at 0.1 sec intervals, after subtraction from reference cell values, as well as subtraction of the previously acquired signal for IgD binding to Phl p 7. The following steps of data analysis were performed using a python script, which is contained in this GitHub repository: <https://github.com/soclemys/spr-analysis>. Python script was developed with the invaluable help of Danail Stoilov. The raw data were first cleaned by dropping null values, outlier interpolation, and smoothing. Data were roughly split to association data up to the highest response, indicating maximum binding of analyte to ligand, and

dissociation data following the maximum value. The next step is to fit the association data for each analyte concentration to the curve of a 1:1 Langmuir binding model, represented by the following equation: $R(t) = R_{\max} \times (1 - \exp(-k_{\text{obs}} \times t))$, where $R_{\max}$ is the maximum response value, t is time, and k_{obs} is the observed association rate. The Langmuir model describes the binding kinetics between single ligand and receptor. Association phase data for the different concentrations were fitted using the model and values for $R_{\max}$ (maximum response) and the observed rate of association k_{obs} were obtained. The fitting process involved minimizing the residuals, which represent the differences between the observed and expected response. Values for k_{obs} for each concentration of analyte were plotted and linear regression is performed on the resulting curves, represented by the following equation: $k_{\text{obs}} = k_{\text{on}} \times [C] + k_{\text{off}}$, where k_{on} is the rate of binding (on-rate) k_{off} is the rate of dissociation (off-rate) and $[C]$ is analyte concentration. k_{on} (in $\text{M}^{-1} \text{s}^{-1}$) is derived from the slope, and k_{off} (in s^{-1}) from the y-intercept. The dissociation phase binding data was then analyzed using a hyperbolic binding model, which assumes that the response (R) is related to the concentration ($[C]$) of the analyte through the equation: $R = R_{\max} \times [C] / (K_D + [C])$, where K_D is the equilibrium dissociation constant in molar units, which can also be derived by the equation: $K_D = k_{\text{off}} / k_{\text{on}}$. To determine the values of $R_{\max}$ and K_D , the experimental data was fitted to the hyperbolic binding equation using a nonlinear least squares method. The fitting was performed by minimizing the sum of squared residuals between the observed response values and the values predicted by the model equation. The fitting process involved optimizing the parameters $R_{\max}$ and K_D to minimize the residuals. The initial guesses for the parameters were set to the maximum observed response ($R_{\max}$) and a value of 1 for K_D . The optimization algorithm iteratively adjusted the parameters until a satisfactory fit was obtained. The quality of the fit was assessed using the χ^2 statistic, which measures the discrepancy between the observed and predicted response values. The χ^2 value was calculated by summing the squared differences between the observed and expected responses, normalized by the expected values.

6.9 Cell culture

All cell cultures were grown in a 37°C incubator, maintaining 5% CO₂. Cells were counted before regular splitting. Suspension cells were directly added to equal volume of Trypan Blue (Sigma) and counted manually under a microscope, using a hemocytometer (Neubauer). The mean value of 4 counted areas was multiplied by 2×10^4 to account for dilution. Adherent HEK293 cells were first treated with cold porcine Trypsin-EDTA (Sigma), which after 10 min was inactivated by 1:1 addition of warm full DMEM, at which point a sample of cells is added to equivalent volume of Trypan Blue (Sigma) and counted. Suspension cells were always split and seeded 48 hours prior to staining for flow cytometry and confocal microscopy experiments.

HEK293 cell line for protein production was grown in high-glucose DMEM (Gibco) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 µg/ml penicillin, 100 U/ml streptomycin, as well as 50 µg/ml hygromycin B after transfection.

KU812 and Jurkat suspension cell lines were both grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco), containing sodium bicarbonate, supplemented with 10% fetal calf serum, 2 mM glutamine, 100 µg/ml penicillin, 100 U/ml streptomycin. KU812 is a human pre-basophilic cell line expressing the CD44 hyaluronan receptor and has been previously used in IgD-related research (Sechet et al. 2003), acquired from ATCC. Jurkat is a human cell line obtained from acute T cell leukemia, not expressing CD44, used in this project both due to availability, and because of previous IgD-related research done on T cells (Tamma et al. 1991). Jurkat cell line was kindly provided by Paul Lavender.

The LAD2 human mature mast cell line (Kirshenbaum et al. 2003), kindly provided by Grzegorz Woszczek, was originally used for flow cytometry experiments, as mast cells, along with basophils, have also been shown to be IgD-binding effector cells (Chen et al. 2009). However, due to the high cost of the Stem Cell factor supplementation to its StemPro-34 growth medium (Gibco), as well as slow and inconsistent

doubling rate, the cell line was used for a brief period prior to setting on microscopy experiments, which require a high amount of cells over a long period of time.

For long-term storage all cells were frozen in 90% fetal calf serum with 10% DMSO at density of 1×10^6 /ml - first in a container for slow freezing with isopropanol at -80°C overnight, and then in a liquid nitrogen tank. When thawing the cells, they were first put on ice, after which they were added to warm medium and centrifuged to remove any DMSO.

6.10 Fluorescent labeling of IgD

To detect IgD on the surface or inside tested cells, and to avoid the use of anti-IgD-fluorescent conjugates, which could potentially trigger IgDR cross-linking in live cells, a directly labelled IgD probe was produced using Alexa Fluor 546 NHS ester conjugation (Thermo, labeling kit A20183) to the anti-Phl p 7 IgD. The chemical principal of the reaction is the same as for the Phl p 7 conjugation to NHS-activated Sepharose through primary amine coupling, described earlier. All labeling and purification steps were done according to the manufacturer's manual. Recombinant Anti-Phl p 7 IgD was concentrated to 2 mg/ml in 0.5 ml after having its buffer exchanged to 0.1 M sodium bicarbonate (without azide), pH 8.3 via HPLC. About 50 mg of the succinimidyl dye ester were dissolved in 0.5 ml sterile anhydrous DMSO, aiming for 5:1 dye to protein molar ratio, and was added to the antibody solution. Reaction was kept overnight at 4°C and was then blocked with 0.5 M ethanolamine. Resulting product was put through a PD10 desalting column to separate unbound dye from labelled protein by gravity. Labelled IgD was then run through HPLC for buffer exchange to TBS, concentrated to 0.5 mg/ml, and stored at -4°C until the following experiments. Recombinant SPE7 IgE, provided by Rebecca Beavil, was also fluorescently labelled the same way to be used as a control in later confocal microscopy experiments.

6.11 Flow cytometry

Initial flow cytometry experiments testing temperature-dependency of IgD binding were done with the LAD2 cell line and non-labelled IgD (followed by anti-IgD staining), while later experiments with the KU812 and Jurkat cell lines were done with the directly labelled IgD-AF546. For all experiments, 2×10^5 cells per sample were used. All wash steps were done by addition of FACS buffer (PBS with 2% BSA and 2mM EDTA), 10 min centrifugation, and resuspension in the same buffer. All samples were analysed with an Attune NxT Flow Cytometer (Thermo), which uses acoustic focusing of the cells in the sheath fluid.

Before staining, cells were washed and blocked with 10% Fc γ R blocker (Miltenyi) in FACS buffer for 10 min on ice, to prevent binding of the IgG probes to IgG-receptors, and then washed. Next, for indirect IgD labelling, unlabelled anti-Phl p 7 IgD (with or without 50 μ g/ml galectin-9) was added to cells at 50 μ g/ml concentration (as per the staining described in Shan et al. 2018) and incubated either on ice or at 37°C for 40 min. After washing, the full staining antibody cocktail was added to cells (antibody conjugates diluted according to manufacturer instructions), including an anti-IgD-PE conjugate (SouthernBiotech), anti-CD44-AF488 (Thermo), along with the 10 μ l FVD viability dye (Thermo) per sample, and the appropriate isotype controls (IgG2b-AF488 for the anti-CD44 and IgG1-PE for the anti-IgD), then incubated on ice for 1 hour. For direct labelling, IgD-AF546 and galectin-9 were added (at 50 μ g/ml final concentrations) along with the rest of the staining cocktail. Each cell sample was then washed and resuspended in 1 ml FACS buffer before analysis. Obtained data were analysed with the FlowJo software (BD Life Sciences). Main cell populations were defined through gating of scatter signal from unstained cells and applied uniformly for all samples. Compensations for signal leakage between channels and the cytometer's laser voltages were set up using UltraComp eBeads (Thermo), previously incubated with the fluorescent probes used in the experiment.

6.12 Fluorescence confocal microscopy

To visually confirm the binding of IgD to cell lines and to inspect its potential subcellular internalization, point scanning confocal microscopy was performed on KU812 and Jurkat live and fixed cell samples. Phl p 7-specific IgD was labelled in-house with AlexaFluor 546 fluorophore, as described earlier. Initial experiments were done with non-labelled IgD incubation, followed by staining with anti-IgD-AF647 (Biolegend). KU812 (human pre-basophilic cell line) and Jurkat (human T cell line) were selected for these experiments due to their reported (and confirmed by flow cytometry) expression and lack of expression, respectively, of the surface molecule CD44, which has been proposed as a potential receptor for IgD (Shan et al. 2018). For each experiment, unless indicated, approximately 2×10^5 live cells of either cell line were stained with 50 $\mu\text{g/ml}$ anti-Phl p 7 IgD-AF546, with or without 50 $\mu\text{g/ml}$ galectin-9, and the appropriate concentration of commercial reagents, as advised by the manufacturers. One of the aims of the project was to track potential endocytosis of IgD in cells, which is an active process, requiring cells to be alive, ruling out the conventionally used cell fixing methods for microscopy, which permeabilize and thus kill the cells. Since both cell lines used for these experiments are non-adherent suspension cells, they had to be somehow immobilized, which was done by a non-conventional method previously used by Ivanusic, Madela, and Denner 2017. The method is to essentially squish the cells under transparent pads made of 2% agarose to prevent their free movement in solution (**Figure 6.3**). One caveat is that cells can appear to have different sizes because of different degrees of pressure applied to them by the agarose pad, due to imperfections in the optical dish surface, but on the other hand, certain cell features, such as nuclei and intracellular vesicles can become more readily apparent in brightfield view at high magnification if cells are more flattened.

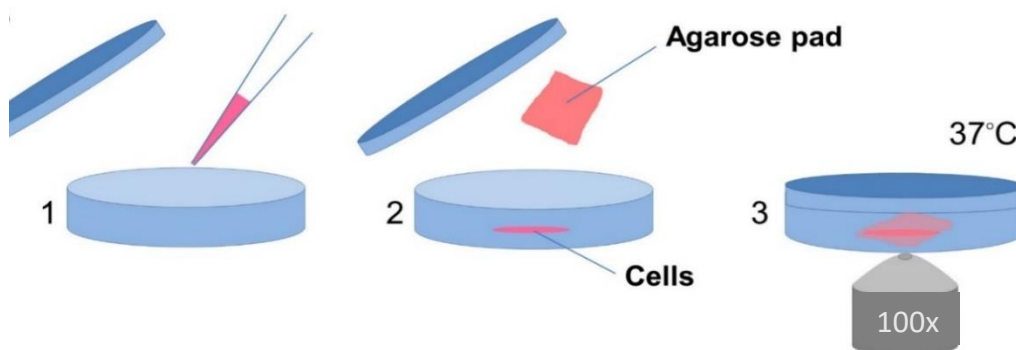


Figure 5.18 Method for suspension cell immobilization – Agarose was added to phenol red-free, FBS-free RPMI 1640 medium at 2% w/v. Solution was boiled until agarose was fully dissolved and then poured into square moulds. Set pads were then excised and placed onto drops of cells in imaging well plates, restraining the movement of cells trapped under them. Image adapted from Ivanusic et al 2017.

Staining was performed in one step with a pre-made staining cocktail, at a cell density of 2×10^6 per ml (2×10^5 in 100 μ l total volume per sample), at 37°C, for 40 min, in phenol red-free, FCS-free RPMI medium (Gibco). Following the staining step, cells were washed with said medium, after which they were resuspended in 100 μ l medium and placed as a single drop onto the inner surface of Ibidi 35 mm μ -dishes. Over each drop, an approximately 5 mm thick agarose pad was placed to limit the movement of the cells in suspension. The agarose pads were made by pouring 2% agarose molten agarose solution, dissolved in the same phenol red-free, FBS-free RPMI, into plastic molds of appropriate sizes (app. 20x20 mm), followed by cooling. Pads were transferred gently onto the drops of cells, protected from light during transportation, to avoid photobleaching, and imaged on an enclosed stage at constant temperature of 37°C, with 5% CO₂ supply.

Later in the project staining was also performed on fixated suspension cell lines as controls to infer whether IgD would bind anywhere inside the permeabilized cells. 2×10^5 cells per sample were washed and resuspended in 4% paraformaldehyde, which is a potent cross-linker, and incubated for 10 min at room temperature. After further 3 washes with PBS, cells were resuspended in 1% Triton X-100 detergent to permeabilize them, and were incubated for another 10 min at room temperature. After another 3 PBS

washes, 3% BSA was added as a blocking reagent for non-specific binding and cells were washed again prior to staining with the titular fluorescent conjugates, along with 5 μ L DAPI per sample, which is normally used to dye the nuclei of permeabilized cells blue. Stained samples were then mounted onto glass coverslips, left to dry, and sealed onto glass slides with nail polish. Imaging was done the same way as for cells in suspension, excluding the temperature and CO₂ control normally applied to living cells.

All microscopy experiments were performed using a Nikon A1R inverted confocal microscope setup with 25mm field of view, housed at the Nikon Imaging Centre at King's College London. Cells were imaged using a 60x or 100x magnification oil-immersion lens. Oil immersion allows for a higher lens numerical aperture (allows for sampled light of wider angles to enter the lens) thanks to the oil's higher refractive index, which results in improved resolution (shorter distance between two objects at which they appear separate) at greater than 40x magnification. Samples were first viewed in brightfield at low magnification to locate cell clusters. Laser voltages were set up depending on observed signal from the samples. Time-lapses of varying length (5-15 min) were recorded via Nikon's NIS-Elements software using the Galvano scan feature. Individual frames were then analysed and edited for image presentation via the same software.

Since the antibody of interest was labelled with Alexa Fluor (AF) 546, emitting yellow light, the remaining reagents were chosen as to have absorption/emission spectra at either shorter or longer wavelengths, in order to minimize any potential overlap. The numbers in these fluorophore names denote the light wavelength at which they exhibit maximum absorption (e.g – AF546 has maximum absorption at 546nm). Thus AF488, emitting green light, and AF647, emitting red light, were utilized (**Figure 6.4**). Fluorophores with shorter absorption wavelengths than 488nm were also considered, as there is some amount of overlap between their emission spectrum and the absorption spectrum of the AF546 fluorophore, but were disregarded, as they require the absorption of UV light, which damages living cells and may disrupt intracellular processes, as well as membrane integrity. Through application of the appropriate dichroic mirror filters for the fluorescence signals, the small overlap between AF488 emission and AF546

absorption is further minimized and has therefore not been problematic. Brightfield view (without laser light) was used for contrast and to find appropriate fields of cells and to discern their shape, size, and any large intracellular compartments (e.g. nuclei borders can usually be easily noticed).

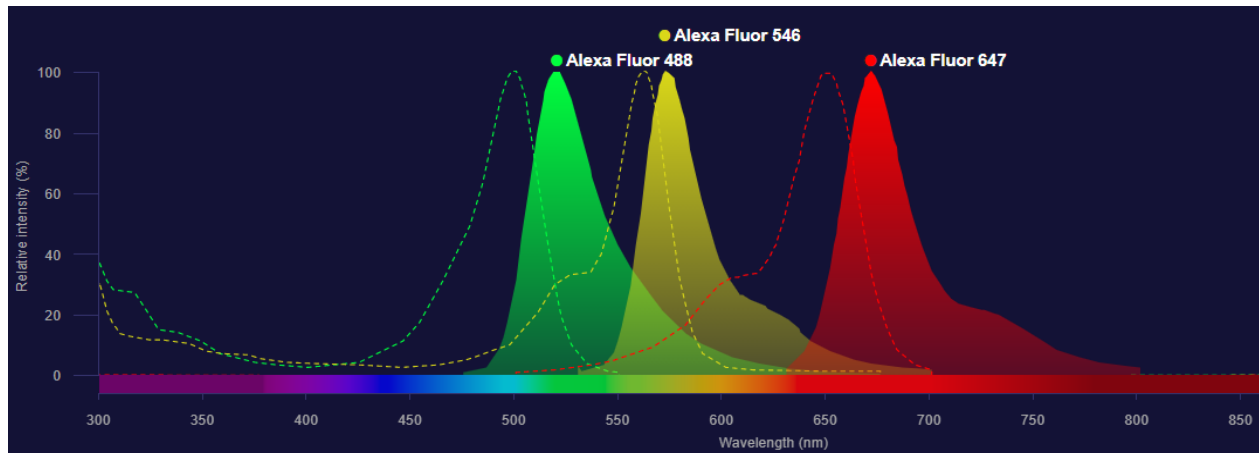


Figure 6.4. Absorption (dotted lines) and emission (solid lines) spectra for fluorophores used in this project. AF488 (green), AF546 (yellow), and AF647 (red) dyes conjugated to antibodies are used in tandem for cell imaging. Thanks to the distinct absorption/emission peaks and the use of filters, no signal overlap was observed in experiments. Image produced with SpectraViewer at ThermoFisher.com

For detection of CD44, AF488-labelled anti-CD44 (Thermo) was used. For detection of early endosomes AF647-labelled anti-EEA1 (Abcam) was used. Initial experiments were also performed with AF647-labelled 10 000 MW dextran (Thermo) to detect pinocytosis, but results were inconclusive, as no signal was recorded above background. Anti-Rab11-AF488 (Thermo) was also briefly used to label recycling endosomes, but was not efficient in non-permeabilized cells, as prohibitively high concentrations were needed to achieve slight signal above background.

Anti-CD44-AF488 was added to the staining cocktail for both KU812 and Jurkat cells, as initial negative control for the latter. Since Jurkat cells express no CD44, as evident by the lack of any green signal in their images, supported by the earlier flow cytometry experiments with those cells, the reagent was not used on Jurkat cells further than initial control experiments for budget reasons.

6.13 IgD pull-downs and sample preparation for mass spectrometry

To attempt to infer any candidate proteins associated with the IgD: IgDR complex, bound anti-Phl p 7 IgD on cell surfaces, or within cell lysates, was pulled-down with Phl p 7-conjugated Sepharose, proteins attached to it were run on SDS-PAGE, and consistent protein bands were analysed with LC/MS.

Two pull-down protocols were tested, differing in whether IgD binding was done before or after cell lysis. 1×10^7 KU812 or Jurkat cells were harvested per sample and washed with DPBS (PBS, 0.5 mM EDTA). For binding before lysis, cells were washed and then resuspended with 5 ml binding buffer (HEPES, 2% BSA), to which $1 \mu\text{M}$ (0.18 mg/ml) recombinant anti-Phl p 7 IgD was added, with or without $1 \mu\text{M}$ galectin-9 (0.04 mg/ml). After 2 hours spinning on a wheel at room temperature, samples were washed twice in binding buffer and cell pellets were resuspended in 2 ml lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 5% glycerol, with protease inhibitor). Samples were then incubated spinning at 4°C for 30 min before centrifugation at 20 000 g for 10 min to remove cellular debris. In earlier experiments, BS3 crosslinker (Thermo) was added after the binding step to lock any IgD binding partners in place, but it produced no significant differences.

For the alternative method, which proved to be more successful, the harvested and washed cells were lysed with the lysis buffer first, after which IgD and gal-9 were added to the lysate supernatant. As later in the project it was observed that IgD colocalizes with endosomes, differential centrifugation and ultracentrifugation were applied to the cell lysates for microsomal enrichment (Pérez-Rodríguez et al. 2020) prior to IgD and gal-9 incubation. Differential centrifugation makes use of the different weight and density, and thus, sedimentation rate, of different subcellular structures. Cell lysate samples were first centrifuged at 2 000 g for 10 min at 4°C to pellet nuclei, debris, and other large aggregates. Resulting supernatant was further centrifuged at 20 000 g for 30 min at 4°C to pellet other large organelles such as mitochondria and peroxisomes. The supernatant from the previous step was then spun in an

ultracentrifuge (Beckman) at 120 000 g for 1 hour at 4°C, which results in the separation of the microsomal fraction, containing small intracellular membrane-bound vesicles such as Golgi and ER remnants, as well as endo-lysosomal vesicles, as hard pellets sticking to the bottom of the ultracentrifugation tubes. Microsomal pellets were then resuspended in 1 ml binding buffer and incubated with IgD with or without galectin-9 addition, as described above.

Next, Phl p 7-conjugated Sepharose (same as the column) was washed several times in lysis buffer and incubated with the enriched lysate samples, gently spinning at 4°C overnight. Resin was then washed several times in PBS and samples were centrifuged at 2 500 g for 5 min. All supernatant was removed and the Sepharose, bearing Phl p 7 bound to IgD: protein complexes, was added to 2x Laemmli loading buffer (125 mM Tris-HCl, pH 7, 4% SDS, 20% glycerol, bromophenol blue, and 10% β -mercaptoethanol for reduced samples) and boiled at 95°C for 10 min to remove bound proteins from the resin. Samples were centrifuged for 5 min at 2 500 g to remove Sepharose remnants. Supernatant was then carefully collected without disturbing the resin sediment and loaded onto mini-PROTEAN TGX 4-20% gradient acrylamide gels for SDS-PAGE (Bio-Rad). Individual bands were excised from the protein gels and collected in distilled water before being sent to the Centre of Excellence for Mass Spectrometry at the James Black Centre, King's College London for analysis.

Liquid chromatography followed by mass spectrometry (LC/MS) is routinely used for protein identification in samples by observing the mass-to-charge ratios of fragmented peptides, which helps determine their amino acid sequence (Domon and Aebersold 2006). The principles of the type of mass spectrometry used in this project are outlined in Chapter 5.

The following steps were performed at the facility and methods report was provided by Xiaoping Yang. Protein samples were first reduced with dithiothreitol, treated with iodoacetamide to form carbamidomethyl derivatives, and proteolyzed with trypsin into a mixture of small peptides. Samples were

resuspended in 30 µl resuspension buffer (2% acetonitrile in 0.1% formic acid), 10 µl of which was injected to be analysed by liquid chromatography. Chromatographic separation was performed using a U3000 UHPLC NanoLC system (Thermo). Peptides were resolved by reversed-phase chromatography on a 75 µm C18 column (50 cm length) using a three-step linear gradient of 80% acetonitrile in 0.1% formic acid. The gradient was delivered to elute the peptides at a flow rate of 250 nl/min over 60 min. The eluate was ionized by electrospray ionization using an Orbitrap Fusion Lumos (Thermo) operating under Xcalibur v4.1.5. The instrument was first programmed to acquire using a “Universal CID” method by defining a 3s cycle time between a full MS scan and MS/MS fragmentation (tandem MS). This method takes advantage of multiple analysers on Orbitrap-Fusion-Lumos and drives the system to use all available time in parallel, resulting in decreasing the dependence on method parameters. The instrument was programmed to acquire in automated data-dependent switching mode, selecting precursor ions based on their intensity for sequencing by collision-induced fragmentation using a TopN CID method. The MS/MS analyses were conducted using collision energy profiles that were chosen based on the mass-to-charge ratio (m/z) and the charge state of the peptide.

Raw mass spectrometry data were processed into peak list files using Proteome Discoverer v2.2 (Thermo). Processed data were then searched using Sequest search engine embedded in PD 2.2, against the current version of the reviewed Swissprot Homo Sapiens database downloaded from Uniprot.org. Scaffold 5 (by Proteome Software) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.9% probability and contained at least 3 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al. 2003). Proteins sharing significant peptide presence and sequence similarity were grouped by the algorithm into clusters.

7. Production and purification of protein constructs

Since the experimental techniques in this project all require a large amount of IgD over long time periods (especially for confocal microscopy), it was deemed best to establish its in-house production and purification via protocols previously used in the group (Dodev, 2014). For that purpose, we also needed to produce and purify recombinant Phl p 7, used for the capture and purification of the recombinant monoclonal anti-Phl p 7, also via protocol previously established in the group (Mitropolou, 2018). IgD-Fab constructs were also produced for the first time via SDM and were supposed to be used for structural and binding studies with Phl p 7 and/or MID but were also not experimentally utilized due to time limitations. Two MID constructs were also produced – MID res. 901-1250, and MID res. 962-1200 – which were supposed to be used for cytokine secretion assays for MID binding to IgD captured by KU812 or Jurkat cells, and for co-crystallization studies, however, due to time limitations, they were only used for SPR to confirm their interaction with IgD and to estimate binding affinity. Several MID-FP conjugates were also produced by Rebecca Beavil, to be potentially used as fluorescent probes for IgD, but were not utilized, as directly labelled IgD-AF546 was produced and used for most experiments instead. Galectin-9 expression and purification was also attempted due to the requirements for high amounts for experiments, but proved difficult and time-consuming to optimize, so commercially available or gifted (by prof. Fu-Tong Liu) gal-9 was used instead. Despite not being used as much as the titular protein constructs – 102.1F10 anti-Phl p 7 IgD and Phl p 7 – a fair amount of time was spent for the optimization and production of the under-utilized constructs, and it will also be briefly described in this section, as they could still be utilized in the future. As explained previously, the same general workflow was followed for all protein production: introduction of plasmid with gene of interest and antibiotic resistance into the relevant organism, growth, harvesting, purification by affinity chromatography, separation by HPLC, concentration, and storage.

7.1 Phl p 7 production and purification

Recombinant Phl p 7 was expressed with the pET151 expression vector, described in section 6.1.1, which was kindly provided by Gintare Bucaite. All steps were performed according to the established Phl p 7-production protocol used in our group (Mitropolou, 2018) and are described in detail in section 6.2.

Nickel-NTA affinity chromatography was used for purification by virtue of the protein's N-terminal hexahistidine tag, introduced to the protein as part of the pET151 vector sequence. Column used was a HisTrap FF crude 1 ml column (GE Healthcare) with a flow rate of 1 ml/min. Affinity chromatography traces (AKTA, GE) do not show discernable UV absorbance peaks for the protein, since the construct lacks any tryptophan residues, which normally confer UV absorbance at 280 nm, while the detector is also not very sensitive (**Figure 7.1**). About 9 ml of bacterial lysate were run per cycle as to not exceed the binding capacity of the small Ni-NTA column.

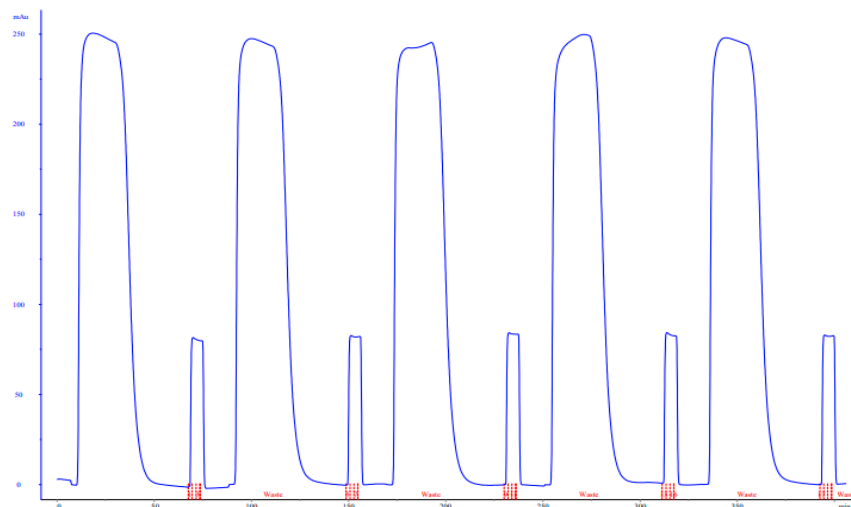


Figure 7.1 Example AKTA Nickel-NTA affinity chromatography trace for Phl p 7 purification from bacterial cell lysate – UV absorbance of solution (Y axis) after it has passed through the Ni-NTA column is plotted against time (X axis). Large UV waves indicate the unspecific cell lysate proteins going through the column during the binding step. The smaller rise during the elution steps (fraction collection depicted in red) is due to the high imidazole concentration of the elution buffer, used to outcompete the histidine attachment to the nickel ions. No discernable peak for Phl p 7 elution is present, due to its lack of tryptophan residues and therefore low UV absorbance. Chromatogram generated with PriveView 5 software.

Next, following concentration in 15 kDa filters by centrifugation, size exclusion chromatography (SEC) via HPLC was performed on the eluted Phl p 7, following concentration, in order to assess purity and oligomeric state, and to exchange its buffer to TBS. Phl p 7 was run through a Superdex 75 size exclusion column (GE Healthcare), suitable for smaller proteins, and an example elution detector voltage trace from a Phl p 7 preparation used for the Phl p 7-conjugated Sepharose column for IgD purification is shown in **Figure 7.2**. The UV detector on the custom Gilson HPLC system is much more sensitive, therefore the sample elution produces noticeable peaks, represented in millivolts of voltage produced by the detector due to UV absorbance. In SEC large proteins pass through the column matrix faster and are eluted first in the early fractions, while small proteins, such as Phl p 7, are eluted at later time points.

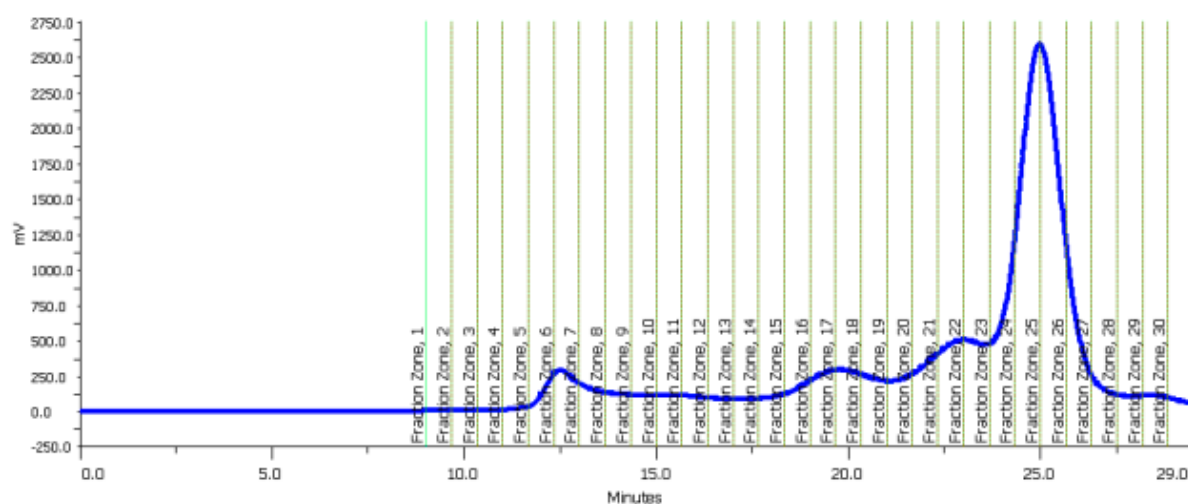


Figure 7.2 Example size exclusion HPLC UV trace for a Phl p 7 purification from the concentrated eluted fractions from the Ni-NTA affinity chromatography. Detector voltage due to UV absorbance of eluant (Y axis) is plotted versus time (X axis). SEC column used: Sepharose 75 (GE healthcare) Large peak to the right indicates the smaller Phl p 7 monomers, as inferred by the later time point at which they elute. Smaller bumps on the trace represent different oligomeric states or high MW aggregate (left-most bump). HPLC also exchanged the sample buffer from Ni-NTA elution buffer to TBS. Fractions under the large monomer peak were collected, run on SDS-PAGE, concentrated, and stored at 4°C for immediate use, or at -80°C for long-term storage. Chromatogram generated with the TRILUTION LC 2.1 software (Gilson).

HPLC revealed that, while mostly monomeric, different oligomers and Phl p 7 aggregates were present in the eluent after Ni-affinity chromatography. Fractions under the large monomeric peak of the HPLC trace were collected, concentrated to 5 mg/ml aliquots and investigated via SDS-PAGE (**Figure 7.3**) prior to storage at 4°C (immediate use) or at -80°C (long-term). 4 mM calcium chloride was added to the Phl p 7 samples for stability and to improve binding to the IgD antibody. Calcium addition is done post-HPLC, as it could form salts with the phosphates of the Ni-NTA elution buffer, in which the protein is dissolved prior to HPLC purification, forming calcium phosphate depositions in the system's fluidics. For SDS-PAGE Phl p 7 samples were run either unboiled or boiled for 10 min at 95°C in order to infer any oligomer presence in the unboiled samples.

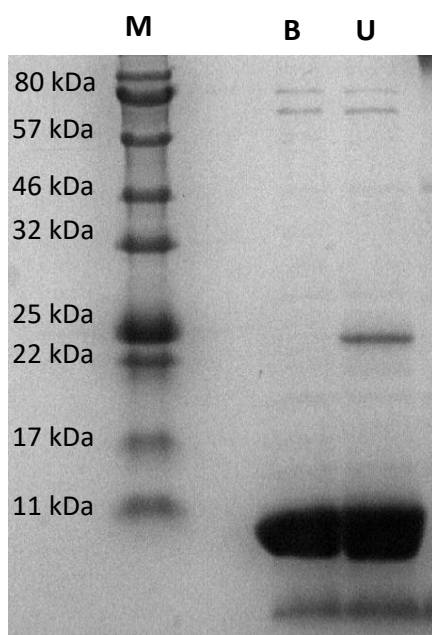


Figure 7.3 Example SDS-PAGE of HPLC-purified and concentrated recombinant Phl p 7 in TBS-Ca buffer, run on 15% acrylamide gel. Monomer bands appear at about 10 kDa due to the addition of ca. 1.5 kDa tags to the 8.5 kDa polcalcin. Despite sampling of only monomers post-HPLC, some oligomers have subsequently formed, as evident by the unboiled (U) sample, compared to monomer only in the boiled sample (B). Complex is most likely dimers, judging by the size difference. Bands at furthestmost top and bottom are likely due to the sample buffer dye. Marker – color prestained protein standard, broad range (11-245 kDa) (NEB). Gel imaged with the Gel Doc XR+ system (Bio-Rad).

SDS-PAGE revealed that the protein was of the correct size and purity after the purification steps, and that some oligomer formation occurs, as inferred by the presence of a 23 kD band in the unboiled sample lane. Boiling would disrupt any oligomer formation. Complexes are most likely dimers, judging by their size difference, forming spontaneously due to the 5 mg/ml protein concentration, despite collecting just the fractions under the monomeric peak of the chromatogram. Overall Phl p 7 expressed very well, as expected, with average final protein monomer yields of over 30 mg per litre of cell culture. Purified Phl p 7 was then mostly used for IgD purification and IgD pull-downs for mass spectrometry sample preparation.

7.2 Human monoclonal 102.1F10 anti-Phl p 7 IgD production and purification

As described in the Methods section, HEK293 cells were transfected with the pVito1 vector, bearing the inserts for the δ -heavy chain and λ light chain, cloned by Balraj Sandhar from an anti-Phl p 7 IgE plasmid construct as per the established PIPE-cloning protocol for production of antibodies with different isotypes (Dodev et al. 2014). Transfectants were cultured in full DMEM with 50 μ g/ml hygromycin B, which kills any non-transfected cells (**Figure 7.4**).

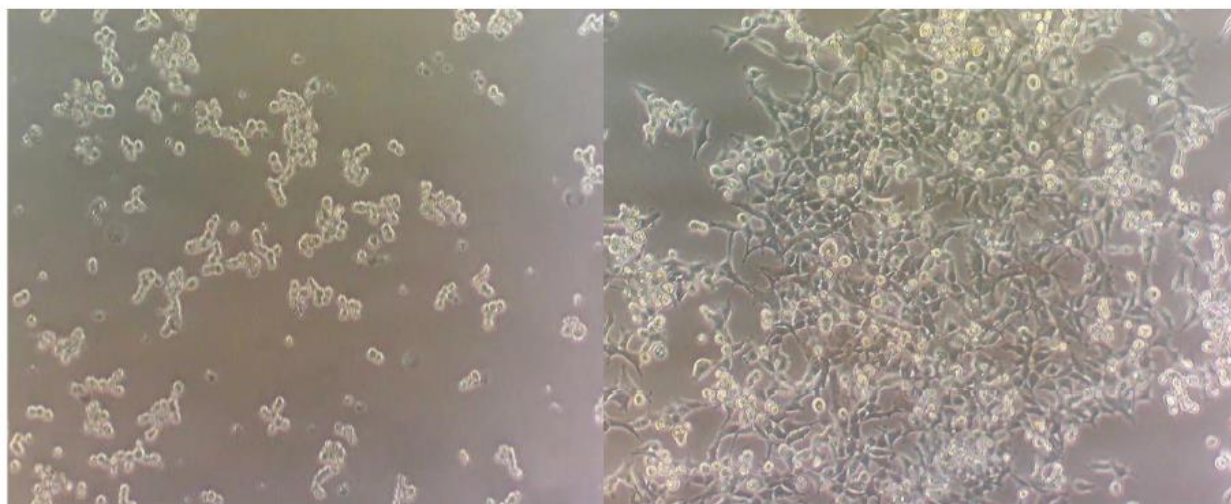


Figure 7.4 Untransfected (left) and transfected (right) HEK293 cells under selection with 50 μ g/ml hygromycin B. Majority of untransfected cells seem to be killed, judging by their rounded shape and lack of attachments. While there is some cell death evident in the transfected well, most of the cells are attached and dividing, indicating the uptake of the pVito1 plasmid and expression of hygromycin B phosphotransferase, conferring antibiotic resistance. 40x magnification, image taken with MotiCam.

After HEK293 transfected cell expansion into 1L full DMEM for 4 weeks, IgD-containing medium was collected and centrifuged for the removal of cell pellets and debris, and sodium azide added to prevent microbial growth. Supernatant was then run through the Phl p 7-conjugated Sepharose column, the production of which is described in section 6.3, at a flowrate of 1 ml/min. Bound IgD to the Phl p 7-conjugated resin was eluted with the low pH elution buffer, as indicated by the sharp peak in the UV chromatogram (**Figure 7.5**), after which 1M Tris was added to the protein fractions for pH normalization. Flowthrough of unbound proteins contained some unbound IgD due to saturation of the Phl p 7 immobilized on the column resin and was run for a second time in order to extract higher amounts of IgD. UV traces for the flowthrough runs were the same but with smaller UV peaks due to less material eluting.

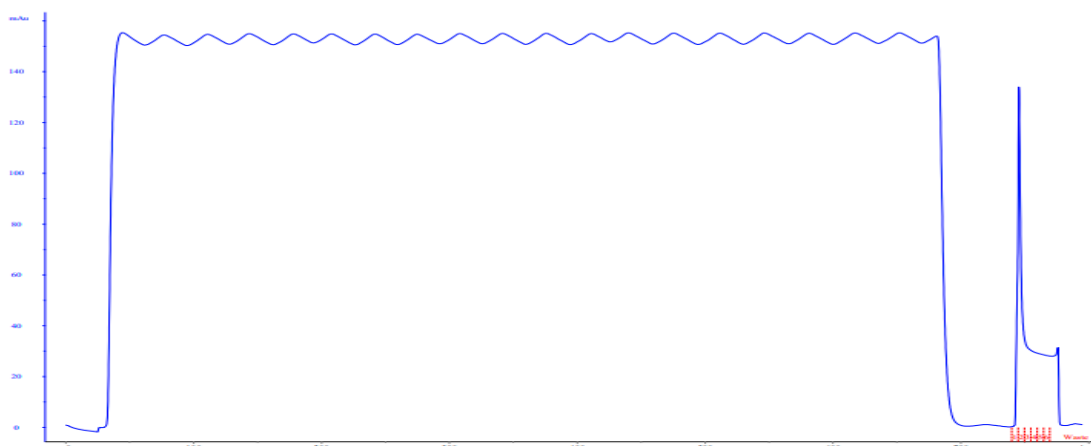


Figure 7.5 Example Phl p 7 affinity chromatography trace for recombinant anti-Phl p 7 IgD purification from HEK293 cell supernatant. Column used was the third and final high-binding capacity column (section 6.3). Large UV signal until fraction collection represents all the unbound proteins from the supernatant that are passing through the column. After washing with binding buffer to remove unbound proteins, the low pH elution buffer causes IgD separation from Phl p 7, thus causing it to elute, as seen by the sharp peak over the fraction collection (red lines) towards the right of the figure (end of the run). Flowthrough can be run on the column again in case of unbound IgD presence due to Phl p 7 saturation during the initial run. Chromatogram generated with PriveView 5 software.

Eluted fractions of the initial and flowthrough runs were pooled and concentrated by centrifugation in 150 kDa cutoff filters (Milipore) prior to size-exclusion by HPLC. IgD concentration after each run was measured and I estimated that the final Phl p 7-Sepharose column had a maximum binding capacity of about 1.5 mg IgD, while a further 0.5-0.7 mg of unbound IgD on average were repurified from the flowthrough, resulting in average total yield for IgD production of just above 2 mg per litre of HEK293 cell culture.

Concentrated IgD was then subjected to size exclusion via HPLC through a Superdex 200 Increase column (GE Healthcare), suitable for larger proteins, for additional purification, to infer its state and potential presence of aggregate, as well as to exchange its buffer to TBS-Ca (same buffer for Phl p 7). No discernable aggregates or degradation products were observed for IgD purified with the final third generation Phl p 7-conjugated Sepharose column (**Figure 7.6**).

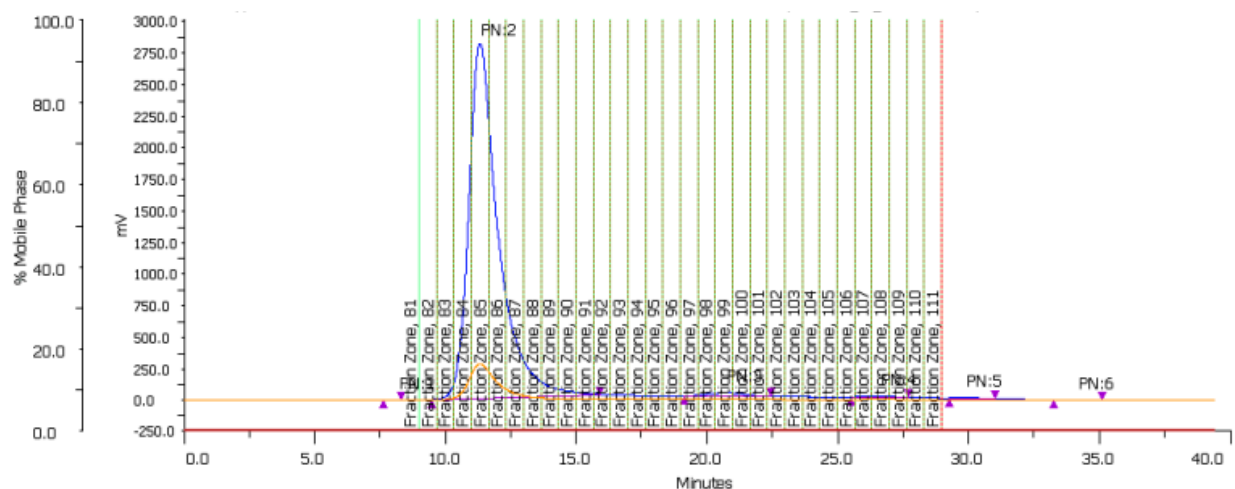


Figure 7.6 Example size exclusion HPLC trace for IgD purification from the eluted samples post affinity chromatography using the third generation Phl p 7-Sepharose column and 150 kDa cutoff filter concentration by centrifugation. SEC column used – Superdex 200 Increase. Large peak represents monomeric anti-Phl p 7 IgD and no aggregates or degradation products to the left or to the right of the peak, respectively, are observable. Fractions under the large monomer peak were collected, run on SDS-PAGE, concentrated, and stored at 4°C for immediate use, or at -80°C for long-term storage. Chromatogram generated with the TRILUTION LC 2.1 software (Gilson).

SDS-PAGE was performed for each of the purification steps, as this is a novel protocol employed in our group. Samples for electrophoresis were treated with β -mercaptoethanol to reduce the disulfide bonds covalently holding together the light and heavy chains of the antibody, thus separating them. Electrophoresis revealed that a noticeable amount of protein contaminants was present following affinity chromatography, indicating significant non-specific binding of proteins from the cell culture supernatant to the Phl p 7-Sepharose column medium. However, those contaminants seem to have been removed by centrifugation in the large MW cutoff filters during concentration and the subsequent size-exclusion HPLC (**Figure 7.7**). The IgD present in the AKTA flowthrough samples indicates that the binding capacity of the Phl p 7-Sepharose has been exceeded.

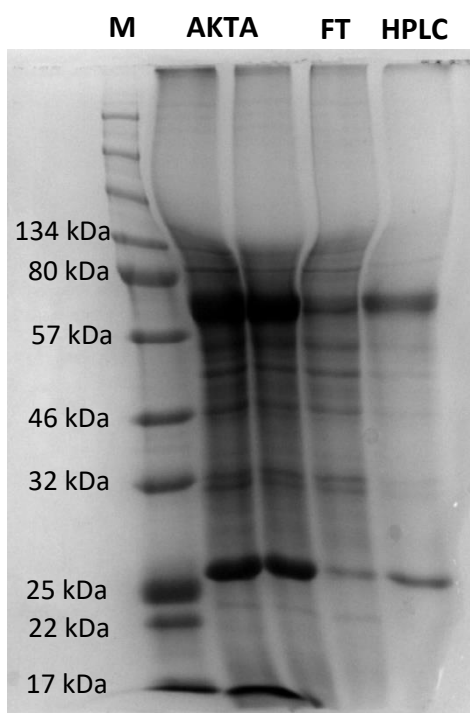


Figure 7.7 Example SDS-PAGE of anti-Phl p 7 IgD samples at different stages of preparation, under reducing conditions. Following Phl p 7 column purification (AKTA, GE) there are still many protein contaminants apparent in the samples. Some IgD is also present in the column flowthrough (FT), indicating that the loaded IgD must have exceeded the binding capacity of the Phl p 7-conjugated Sepharose. After concentration in the high MW cutoff filters, followed by HPLC, many of the protein contaminants have been removed, leaving just the 25 kDa light chain and 65 kDa heavy chain of IgD (and contamination traces from the other sample lanes due to loading). 7% acrylamide gel, marker – color prestained protein standard, broad range (NEB). Gel imaged with Gel Doc XR+ (Bio Rad).

SDS-PAGE of the IgD samples purified by HPLC showed the clear presence of protein bands at 25 kDa and 65 kDa, corresponding to the light and heavy chains of the antibody. The low acrylamide percentage in the gel allowed for better resolution of the protein bands in this range of molecular weight. Anti-Phl p 7 IgD was concentrated to 0.5 mg/ml aliquots which were kept in a -80°C freezer for long-term storage, or in a 4°C refrigerator for immediate use.

The recombinant IgD used for labelling with AF546, as described in 6.10, was re-purified by HPLC and concentrated to 2 mg/ml in coupling buffer. After coupling and removal of unbound fluorophore via PD10 desalting column, the IgD-AF546 was concentrated, purified by HPLC to remove any free fluorophore traces and buffer was exchanged to TBS. The HPLC traces for the conjugate (**Figure 7.8**) were nearly identical to the traces for free IgD, indicating no aggregation or degradation products. The probe was stored at 4°C and its effectiveness was confirmed via the later described fluorescence-based techniques.

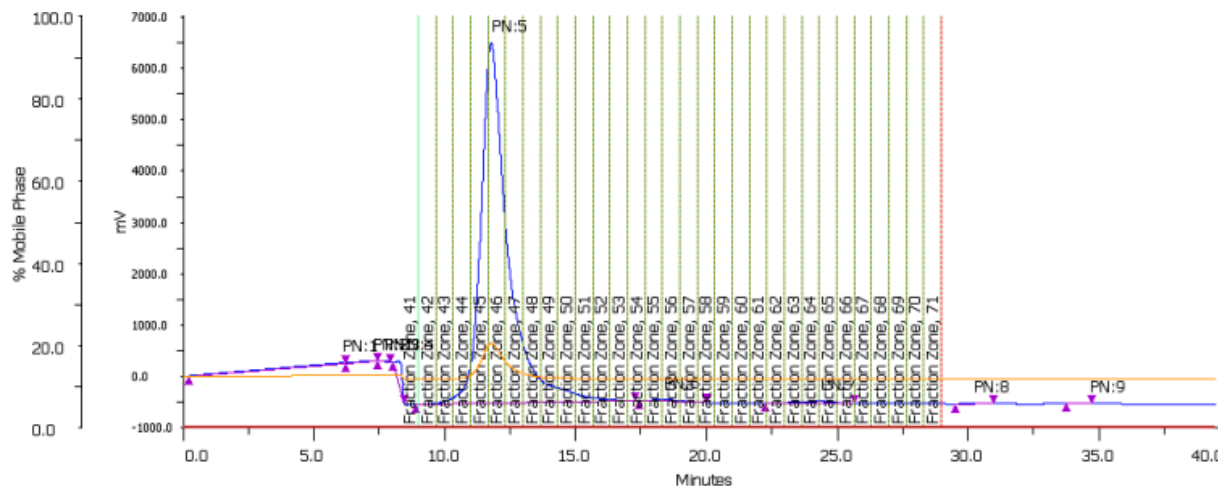


Figure 7.8 Size exclusion HPLC trace for concentrated IgD-AF546 following removal of unbound fluorophore by desalting. SEC column used – Superdex 200 Increase. No aggregation or degradation products are observed, although there seems to be a small rise in UV2 signal (orange trace), which could be due to elution of unbound fluorophore left after the passing IgD-AF546. Fractions under the peak were collected and stored at 4°C. Chromatogram generated with the TRILUTION LC 2.1 software (Gilson).

7.3 Production and purification of IgD-Fab fragments

Fab fragments were generated via SDM, as explained in 6.1.2, for use in binding experiments and crystallization trials, the plans for which were unfortunately scrapped due to time restraints. The PCR reaction of SMD (**Figure 7.9**) was digested with DpnI and run on a 1% TBE-agarose gel for separation. Electrophoresis revealed no partial PCR products or contaminants. DNA band at approximately 12 kbp, same size as the plasmid for whole IgD, was excised, purified, and sent for sequencing. Sizes of the plasmids encoding full IgD and IgD-Fab are the same, as no changes to length were introduced, only the mutation of a single nucleotide.

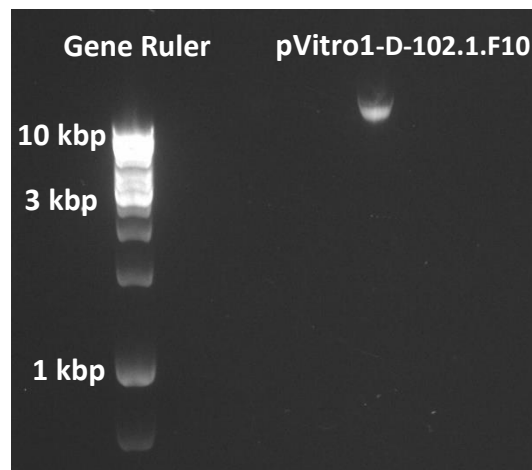


Figure 7.9 1% TBE agarose gel electrophoresis of PCR of amplified pVitro1-D-102.1.F10 plasmid with primers for SDM. 12 kbp band is the same size as the full IgD plasmid, since no changes to its length were imparted. Marker used is 1kb GeneRuler (Thermo). Image generated with Gel Doc XR+ (Bio Rad).

After purification of the excised band, the desired mutation of a cytidine to thymidine in order to introduce a TAG stop codon in the heavy chain, generated by the QuickChange lightning kit and appropriate primers, was confirmed as successful by DNA sequencing at Eurofins Genomics (**Figure 7.10**). Sequenced SDM product was aligned with template sequence using Clustal Omega (www.ebi.ac.uk).

pVitro_IgD_sequencing_F	AGATACAAAGACGGGACAGCTACTACATGACAAGCAGCCAGCTCTCCACCCCTCCAGC	194
pVitro_IgD_template	AGATACAAAGACGGGACAGCTACTACATGACAAGCAGCCAGCTCTCCACCCCTCCAGC	240

pVitro_IgD_sequencing_F	AGTGGCGCCAAGGCGAGTACAAATGCGTGGTCCAGCACACCGCCAGCAAGAGTAAGAAGG	254
pVitro_IgD_template	AGTGGCGCCAAGGCGAGTACAAATGCGTGGTCCAGCACACCGCCAGCAAGAGTAAGAAGG	300

pVitro_IgD_sequencing_F	AGATCTTCCGCTGGCCAGAGTCTCCAAAGGCATAGGCCTCCTCAGTGCCCACTGCACAAC	314
pVitro_IgD_template	AGATCTTCCGCTGGCCAGAGTCTCCAAAGGCACAGGCCTCCTCAGTGCCCACTGCACAAC	360

pVitro_IgD_sequencing_F	CCCAAGCAGAGGGCAGCCTCGCCAAGGCCAACCACAGCCCCAGCCACCACCGTAACACAG	374
pVitro_IgD_template	CCCAAGCAGAGGGCAGCCTCGCCAAGGCCAACCACAGCCCCAGCCACCACCGTAACACAG	420

pVitro_IgD_sequencing_F	GAAGAGGAGGAGAAGAGAAGAAGGAGAAGGAGAAAGAGGAACAAGAAGAGAGAGAGA	434
pVitro_IgD_template	GAAGAGGAGGAGAAGAGAAGAAGGAGAAGGAGAAAGAGGAACAAGAAGAGAGAGAGA	480

Figure 7.10 Eurofins Genomics sequencing of mutated pVitro1-D-102.1.F10 α -Phl P 7 IgD heavy chain aligned with the original template sequence. The C->T point mutation is marked, along with the mutated CAG->TAG stop codon. Reverse primer sequencing also confirms the introduced mutation (not shown). No other changes within the template were inferred. Sequencing reads viewed with the Clustal Omega Multiple Sequence Alignment tool at www.ebi.ac.uk

The same steps for the preparation of whole anti-Phl p 7 IgD were performed for the Fab fragments, as they contain the antigen binding site specific to Phl p 7. Affinity chromatography was run on the third generation Phl p 7-Sepharose column and UV traces (**Figure 7.11**) were identical to the whole IgD traces, indicating robust elution of protein bound to the Phl p 7-Sepharose after lowering the pH with elution buffer, albeit the UV absorbance was weaker due to less eluting material, which could be both due to the smaller size of the construct.

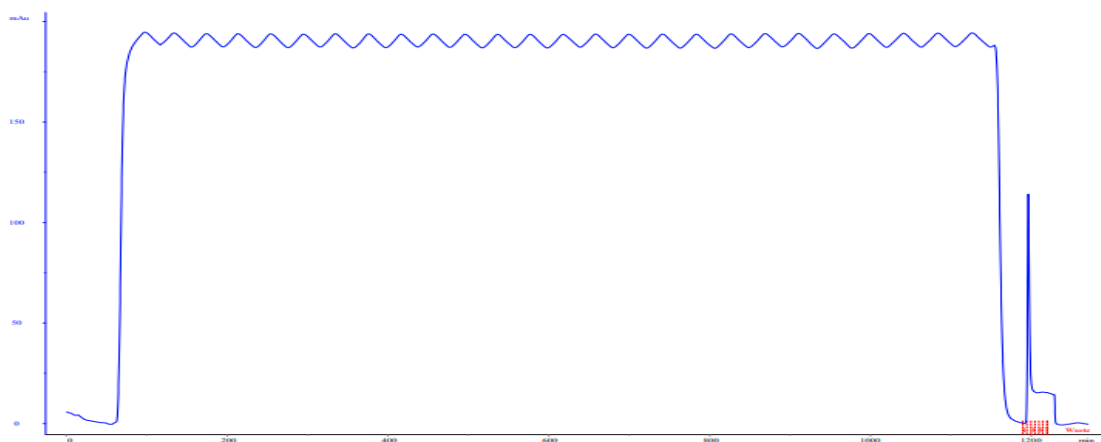


Figure 7.11 Example Phl p 7 affinity chromatography UV trace for recombinant anti-Phl p 7 IgD-Fab fragment purification from HEK293 cell supernatant via the third generation Phl p 7-conjugated Sepharose column (section 6.3). Overall similar trace as for whole IgD, including the sharp peak over the fraction collection (red lines) towards the right of the figure (end of the run). Lower UV absorbance due to lower expression and smaller size of the fragment. Chromatogram generated with PriveView 5 software.

Further purification and buffer exchange to TBS-Ca by size exclusion HPLC (**Figure 7.12**) of concentrated eluent showed later elution through the Superdex 200 Increase column (GE Healthcare), as expected due to the smaller size of the fragment. No noticeable aggregation or degradation products were observed.

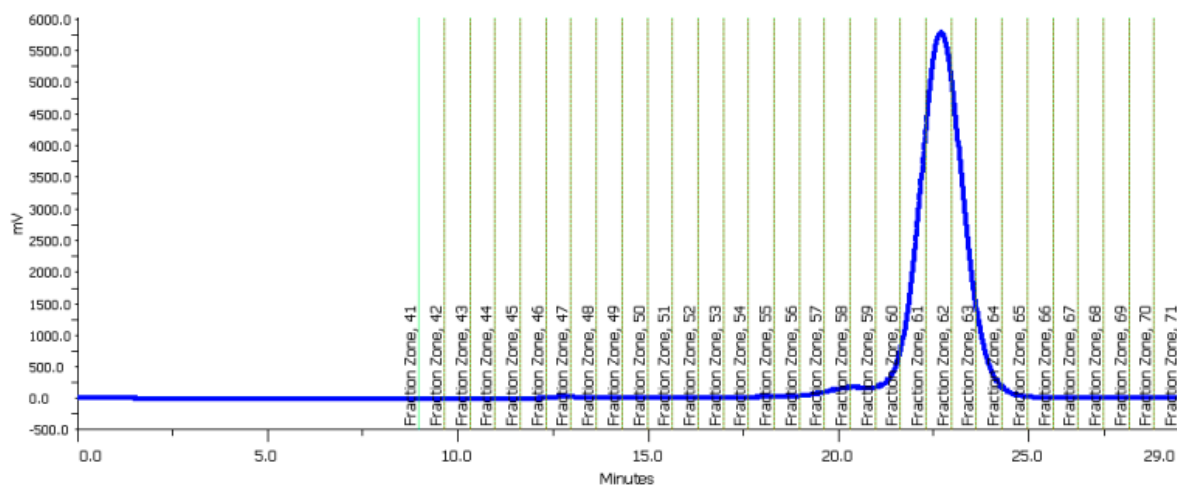


Figure 7.12 Example size exclusion HPLC UV trace for IgD-Fab purification from samples post Phl p 7-Sepharose affinity chromatography and 150 kDa cutoff filter concentration by centrifugation. SEC column used – Superdex 200 Increase. No aggregation or degradation products present. Fractions under the peak were collected, aliquoted and stored at -80°C at 0.22 mg/ml concentration. Chromatogram generated with the TRILUTION LC 2.1 software (Gilson).

Fractions corresponding to the HPLC trace peak were collected, concentrated to 0.22 mg/ml for storage, and examined by SDS-PAGE under reducing conditions to disrupt the disulfide bonds between the light and heavy chains. 10% acrylamide gel was used for better resolution of the appropriate molecular weight range. SDS-PAGE revealed high purity of the λ light chain and terminated δ heavy chain at 25 kDa and 28 kDa respectively, as expected. No contaminants, aggregates or degradation products were observed (Figure 7.13).

Despite not using this construct in the project, it is currently being studied by other members of the group and its structure has been solved and is pending publication as of April 2023 (Davies et al).

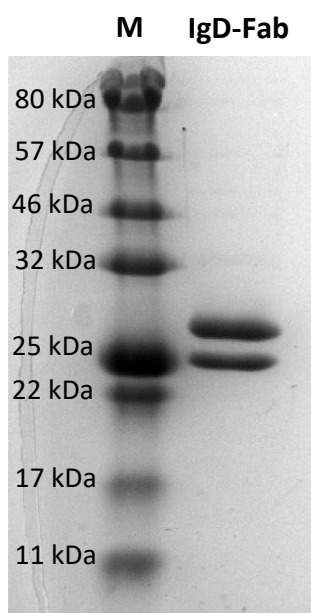


Figure 7.13 SDS-PAGE of purified pVtro1-D-102.1F10 IgD-Fab fragment in reducing conditions. 10 μ g of protein were run on a 10% acrylamide gel. The λ light chain has a molecular weight of about 25 kDa, while the δ heavy chain, terminating just before the hinge region, is about 28 kDa, as expected. No contaminants, aggregates or degradation products present, and the protein preparation is of high purity. Marker – Color prestained protein standard, broad range (11-245 kDa) (NEB). Imaged with Gel Doc XR+ (Bio-rad).

7.4 Production and purification of MID constructs

7.4.1 MID (901-1250)

The *Moraxella Catarrhalis* IgD binding protein was to be used for various cell activation, binding, and structural studies, but due to time limitations and problems with the production on the initial 901-1250 construct, we were only able to perform SPR binding experiments with the recombinant anti-Phl p 7 IgD. The initial MID (901-1250) construct sequence was selected and sent to IDT for synthesis as described in 6.1.2. The received gBlock was amplified by PCR, resulting product was run on a 1% TBE-agarose gel for size determination (**Figure 7.14**), and the DNA band was excised and purified.

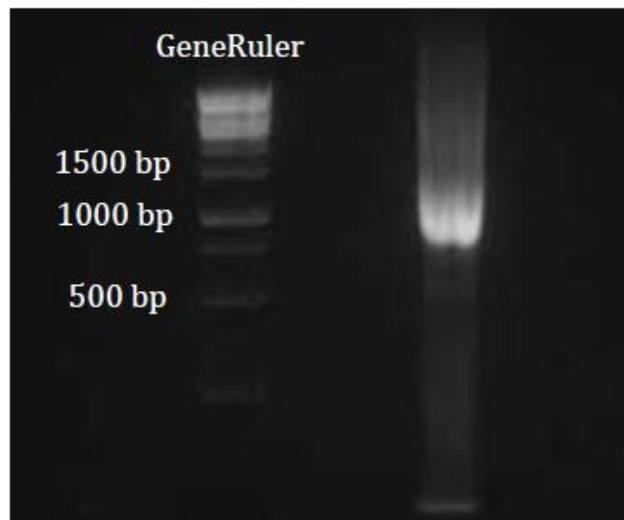


Figure 7.14 1% TBE agarose gel electrophoresis of PCR product of amplified 1050 bp MID (901-1250) gene fragment from the gblock generated by IDT. For all cloning, DNA was extracted from agarose gel, as described. Marker used is 1kb GeneRuler (Thermo). Image generated with Gel Doc XR+ (Bio Rad).

Same steps for plasmid amplification and subsequent protein expression and purification of the MID (901-1250) constructs were followed as for Phl p 7, as both methods rely on the expression of the hexahistidine tag-generating and ampicillin resistance-conferring pET151 vector in BL 21 *E. coli*. Ni-NTA affinity chromatography traces revealed the expected elution of MID (901-1250) due to the high imidazole

concentration of the elution buffer outcompeting it for nickel ion binding. UV traces were indiscernible from the IgD construct traces and are not shown.

Following fraction collection and concentration in 50 kDa cutoff centrifugal filters (Milipore), eluted fractions from the Ni-NTA chromatography were subjected to size exclusion HPLC for separation and buffer exchange to TBS. Chromatograms (**Figure 7.15**) revealed some amount of aggregation and degradation products, represented by small bumps to the left and right of the peak respectively. It was not yet clear whether the large peak represents monomers or oligomers, as MID is known to oligomerize in solution (Nordström et al 2002). In the latter case, the bump to the right of the peak could be due to monomers, although when compared to the Phl p 7 trace (**Figure 7.2**) from the same Superdex 75 column, the eluent causing this signal seems to be too small to be monomeric MID.

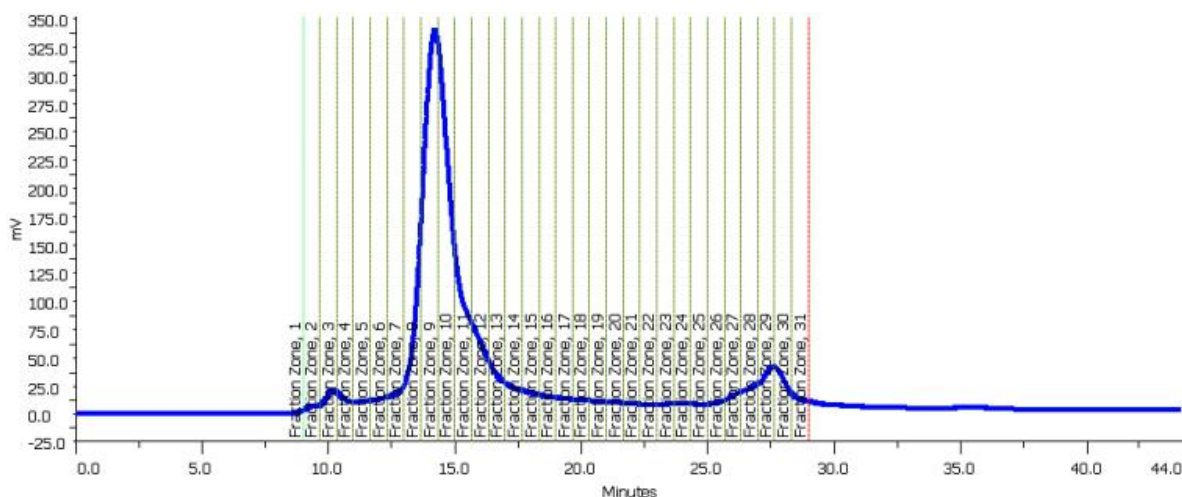


Figure 7.15 Example size exclusion HPLC UV trace for MID (901-1250) samples following Ni-NTA affinity chromatography. SEC column used – Superdex 75. It is unclear whether the large peak represents monomers or oligomers. The bump in absorbance to the left of the peak is indicative of aggregate, while the bump to the right is likely due to MID degradation. Fractions under the peak were collected, concentrated, and stored at -80°C. Chromatogram generated with the TRILUTION LC 2.1 software (Gilson).

SDS-PAGE of concentrated MID (901-1250) samples following HPLC (**Figure 7.16**) clearly showed that this construct exists in solution predominantly as oligomers, as evident by the unboiled samples. In the boiled samples, MID was entirely monomeric, as shown by the apparent 46 kDa protein band. The discrepancy between the estimated 40.6 kDa size of the MID (901-1250) construct is due to the introduction of the purification tags encoded in the pET151 vector, as well as due to the shape and DS-applied charge of the unfolded protein resulting in slower migration through the polyacrylamide gel. No protein contaminants, degradation products, or high MW aggregates were observed in both boiled and unboiled samples. Flowthrough from affinity chromatography contained some unbound MID indicating that the binding capacity of the Ni-NTA column was exceeded. The 8% acrylamide gel allowed for better clear band separation between monomer and oligomer.

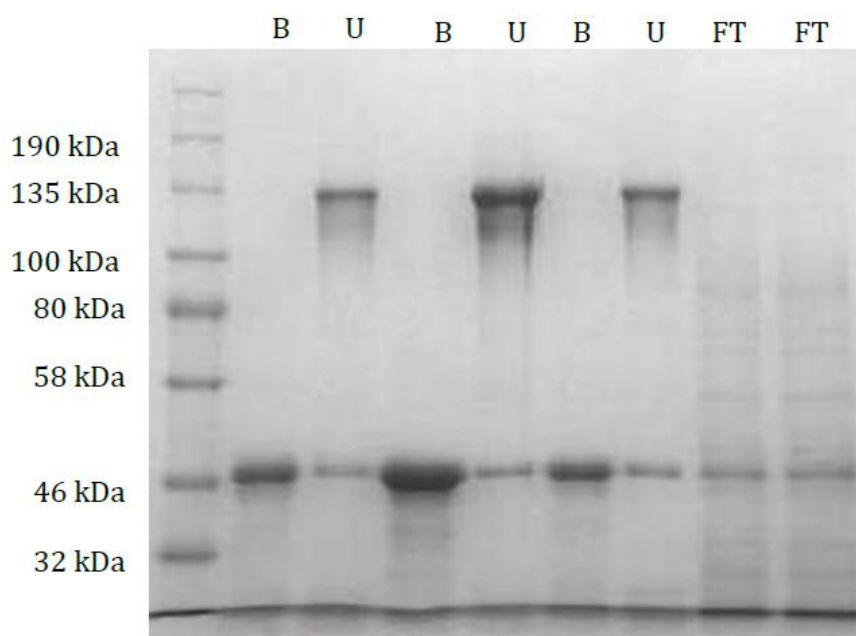


Figure 7.16 SDS-PAGE of boiled (B) and unboiled (U) concentrated MID (901-1250) protein fractions following Ni-NTA column affinity purification and HPLC, run on 8% acrylamide gel. The estimated size of MID monomers should be 40.6 kDa and the apparent larger molecular weight is due to the presence of tags, and due to unfolding of the protein by SDS. No oligomers are present in boiled samples, whereas MID exists predominantly as trimers in unboiled samples, which is inconsistent with the tetrameric form of MID (962-1200) reported in literature, but consistent with the full-length protein's oligomeric state. Presence of MID in the flowthrough (FT) of the affinity chromatography step indicates that the amount of loaded protein exceeds binding capacity of the Ni-NTA column. Marker – Color prestained protein standard, broad range (11-245 kDa) (NEB). Gel imaged with Gel Doc XR+ (Bio-Rad).

The observed protein bands at approximately 135 kDa in the unboiled samples seem to represent trimers, since that value is roughly 3 times the size of the 46 kDa monomers in the boiled samples. This is inconsistent with the tetrameric structure of the MID (962-1200) that has been reported in literature (Nordström et al 2002), but consistent with the trimer formation of full-length bacterial membrane MID (Hallström et al 2008). Trimer instead of tetramer formation could be due to the additional sequence present in the MID (901-1250) construct, which may result in a different monomer association mode compared to MID (962-1200).

The extra sequence in the (901-1250) construct proved to be more problematic than we expected. It was unclear whether due to aberrant oligomerization and aggregation or due to exposure of hydrophobic residues, but the longer this protein was stored, the “stickier” it got – preliminary SPR experiments showed that it stucked tightly to the proteins on the SPR chip even without the presence of the IgD ligand in the reference cell, and it was impossible to “unstick” it with even the lowest buffer pH. Later protein preparations showed significant aggregation and degradation occurring in the MID (901-1250) samples, as seen on SEC chromatograms (**Figure 7.17**). These MID preparations were therefore disposed of.

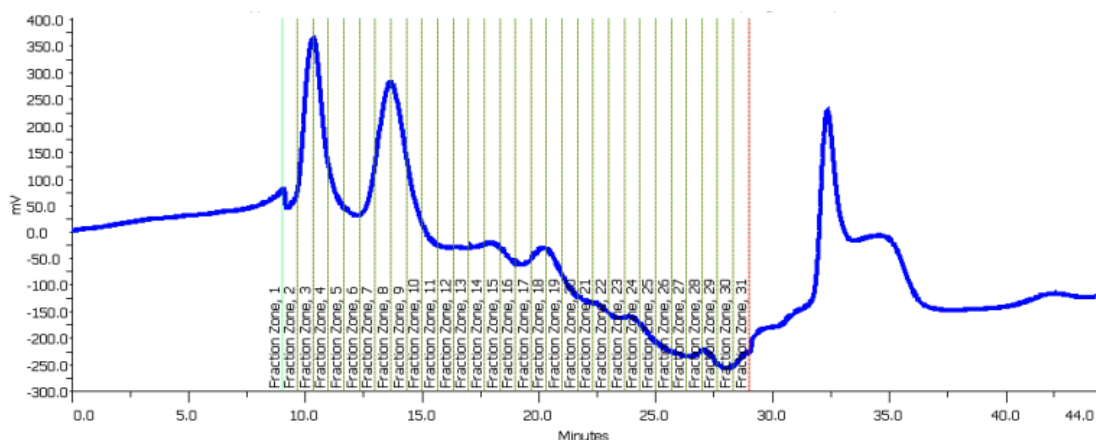


Figure 7.17 HPLC UV trace for MID (901-1250) samples after a month of storage. SEC column used – Superdex 75. The peak from Figure 7.9 has shifted to the left, and peaks to the left and right of it appear showing significant aggregation and degradation, respectively. Therefore, these preparations were scrapped. Chromatogram generated with the TRILUTION LC 2.1 software (Gilson).

To find out whether the problem with instability and aggregation of the MID (901-1250) construct was due to aberrant pET151 plasmid sequence, sequencing with T7 promoter (F) and T7 terminator (R) was performed again on the template (Eurofins Genomics) (**Figure 7.18**). The forward and reverse sequencing reads confirmed a point mutation in the protein coding region of template – a guanosine was mutated to thymidine, which converts a UCC codon in the mRNA transcript, encoding a serine residue, to a UAC codon, which encodes tyrosine. The mutation could be due to spontaneous cytidine deamination on the opposite strand, which converts it to uridine, which pairs with adenosine instead of guanosine. The larger aromatic tyrosine residue in the protein construct could disrupt folding and lead to exposure of hydrophobic patches, which would explain the apparent stickiness, aggregation, and instability of the preparation. Later in the project, the original MID template was rescued by re-cloning it from the GFP-MID plasmid back into the pET151 vector, but soon after we got a hold of the correct MID (962-1200) sequence.

T7_F_MID_20.06_col4	TACCTCCAAAACGTTTGAGACTGTAACGTTTGC	314
Target_MID	TACCTCCAAAACGTTTGAGACTGTAACGTTTGC	360
T7_F_MID_20.06_col4	TGATAAAACAAAAGGGAAAGTTAAGGTGGGCATAGACCAACAAATG	374
Target_MID	TGATAAAACAAAAGGGAAAGTTAAGGTGGGCATAGACCAACAAAGG	420
T7_F_MID_20.06_col4	TAAGCTGACCGTAGGCAATAACAATGGAAGGAATAGTAATTGATTCCAAGACGGTCA	434
Target_MID	TAAGCTGACCGTAGGCAATAACAATGGAAGGAATAGTAATTGATTCCAAGACGGTCA	480
T7_R_MID_20.06_col4	TAACATTTGCAAGTGTGTTACTAAGGCCTGTGATGGTGTGTTTGACCGTCTTTGGAATCAA	827
Target_MID	TAACATTTGCAAGTGTGTTACTAAGGCCTGTGATGGTGTGTTTGACCGTCTTTGGAATCAA	900
T7_R_MID_20.06_col4	TTACTATTCCCTTTTCCATTGTTATTGCCTACGGTCAGCTTAGGGGTCGTAAGGCCATTG	887
Target_MID	TTACTATTCCCTTTTCCATTGTTATTGCCTACGGTCAGCTTAGGGGTCGTAAGGCCCTTTG	960
T7_R_MID_20.06_col4	TTTGGTCTATGCCACCTTAACCTTCCCTTTTGTTTATCATTTCGAGATTGTTATCCCAT	947
Target_MID	TTTGGTCTATGCCACCTTAACCTTCCCTTTTGTTTATCATTTCGAGATTGTTATCCCAT	1020

Figure 7.18 Sequencing by Eurofins Genomics of the MID-pET151 expression plasmid, obtained from a transformed colony, using T7 promoter (Forward, F) and T7 terminator (Reverse, R) primers. A point mutation in the protein coding region, marked here by the black ovals, was discovered in all sequenced colonies transformed with the plasmid. Mutation theoretically results in the change of a UCC serine codon in the mRNA transcript to a UAC phenylalanine codon. The mutation could be due to spontaneous deamination of a cytidine on the reverse strand, which turns it into a uridine (U), which caused an adenosine (bottom image) to be incorporated in future replication cycles, which pairs with thymidine (top image). Sequencing alignment done with Clustal Omega (www.ebi.ac.uk).

7.4.2 MID-FP conjugates

The MID (901-1250) was only used for preliminary SPR experiments and for the generation of fluorescent MID-conjugates. All cloning and purification steps for the MID-FPs were done by Rebecca Beavil. Overall, the fluorescent protein conjugates expressed much more robustly than MID alone, with average yields of above 30 mg per litre of bacterial culture, perhaps due to the stabilizing effect of the fluorescent protein construct conjugates since their expression and stability have previously been optimized.

The binding of the MID-FP conjugates to IgD, as well as their fluorescent signal, were assessed via dot blot binding assays (**Figure 7.19**). 0.5 µg of Anti-Phl p 7 IgD, as well as a TBS negative control, were dotted onto nitrocellulose membranes, which were blocked with 2% BSA and then incubated with the respective MID-FP conjugates – MID-GFP, GFP-MID, mCherry-MID, and RFP-MID, as well as with anti-IgD-FITC and anti-IgD-AF647 as positive fluorescence controls.

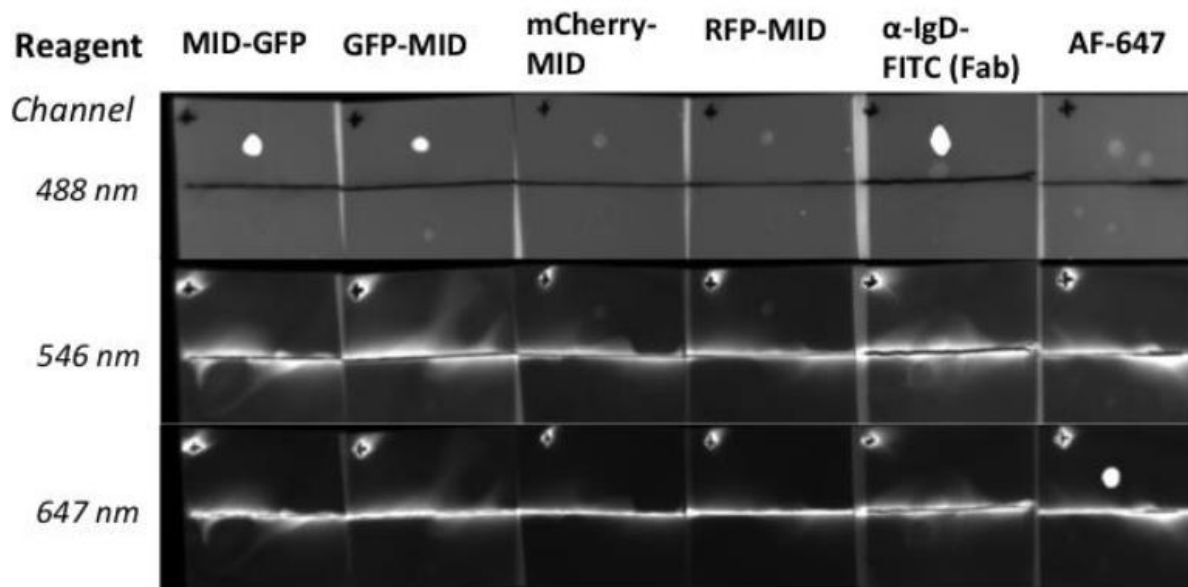


Figure 7.19 Dot blots of the four different FP-MID constructs, created by Rebecca Beavil, along with two IgD-binding fluorophore controls, viewed under three light filters. 0.5 µg of 102.1F10 α-Phl p 7 IgD was dotted onto each nitrocellulose square (top half) along with a TBS negative control (bottom half). MID-FP, or controls, at equivalent concentrations of 1 µg/ml in 5% milk powder were used as secondary antibodies. It is evident that MID-GFP and GFP-MID have retained IgD binding and fluorophore activity, following a month of storage at 4°. mCherry-MID and RFP-MID have perhaps lost either fluorophore activity or their ability to bind IgD. Viewed with Gel Doc XR+ (Bio-rad).

Overall, both the N- and C-terminally attached GFP conjugates showed good binding and fluorescence signal, with some amount of GFP-MID sticking to the negative TBS control. The mCherry-MID and RFP-MID seem to have bound to IgD, but do not exhibit fluorescence in the red 647 nm channel.

The most highly expressed and stable MID-FP construct was mCherry-MID and it was decided to attempt to build a mCherry-MID-Sepharose column for the purification of IgD of various specificities, which has been done previously (Samuelsson et al. 2006). Coupling of the mCherry-MID to the Sepharose resin was also done by Rebecca Beavil. Unfortunately, despite the nice looking red-dyed Sepharose column, lots of unspecific binding to the mCherry-MID-Sepharose during affinity purification occurred, as evident by the presence of various protein contaminants in SDS-PAGE (not shown). The non-specific binding could be due to the presence of the fluorescence protein or misfolding of the MID (901-1250) part of the construct.

7.4.3 MID (962-1200)

Towards the later stages of the project, the original correct MID (962-1200) sequence cloned into a pET26b expression plasmid vector was kindly provided by prof. Kristian Riesbeck. The construct was only used for SPR binding experiments to anti-Phl p 7 IgD due to time limitations. MID (962-1200) was expressed and purified following the same steps as for the previous MID construct, except for the antibiotic selection, which was done with 50 µg/ml kanamycin instead of ampicillin. Size exclusion traces (**Figure 7.20**) showed no aggregation or degradation of the construct. The peaks for MID (962-1200) were further to the right compared to MID (901-1250), since the protein is 88 amino acids shorter and about 8 kDa smaller in MW. Following HPLC, samples were concentrated to 2 mg/ml in SPR buffer to avoid aggregation, sampled by SDS-PAGE (**Figure 7.21**) and stored at 4°C for immediate use. Overall, this construct showed more robust expression than the MID (901-1250) with average yields of above 30 mg per litre bacterial culture, perhaps due to lack of destabilization and aggregation imparted by the extra sequence in the previous construct.

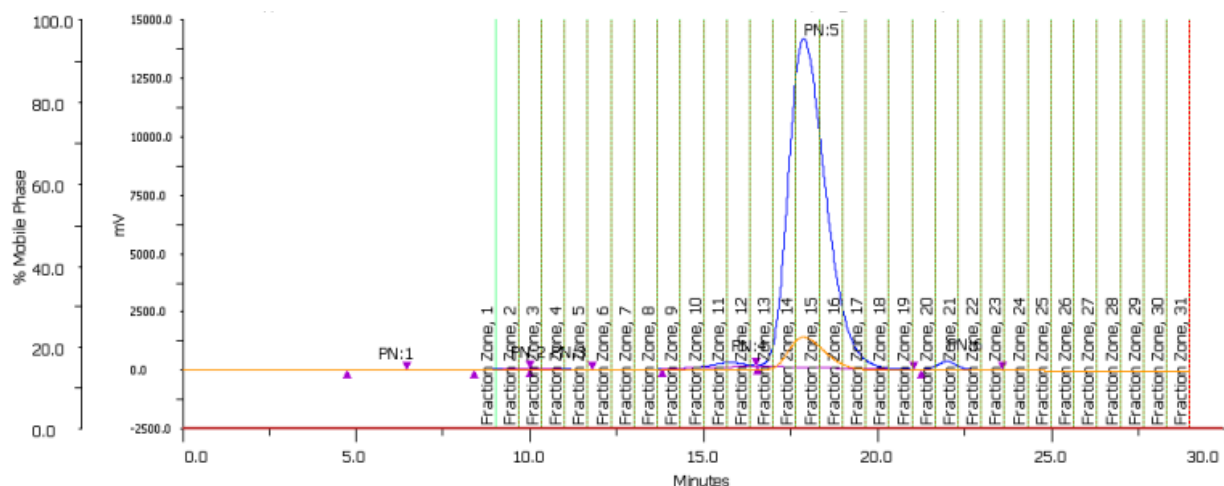


Figure 7.20 Example HPLC UV trace for MID (962-1200) samples following Ni-NTA affinity purification. SEC column used – Superdex 75. The peak here is further to the right compared with the peak for MID (901-1250) on **Figure 7.15**, indicating the smaller size of the construct, as it is 88 amino acids shorter. No aggregation and minimal degradation products were observed. Fractions under peak were collected and stored at 4°C in SPR buffer. Chromatogram generated with the TRILUTION LC 2.1 software (Gilson).

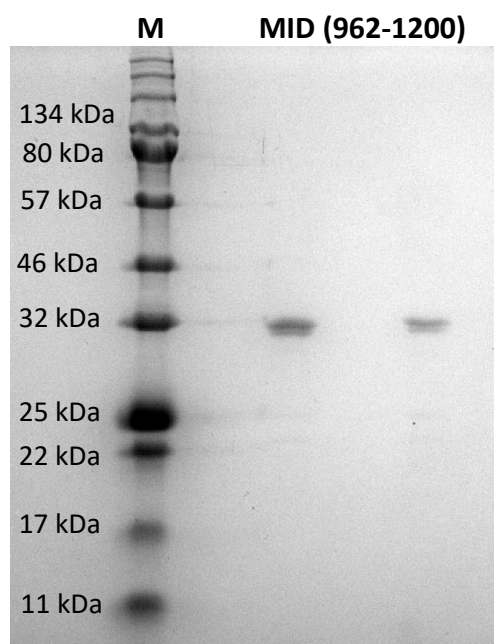


Figure 7.21 SDS-PAGE of HPLC-purified and concentrated recombinant MID (962-1200). Samples were boiled beforehand and only monomers are present with size of about 30 kDa due to the 1.5 kDa tags added. Protein preparations were pure with a small amount of degradation barely visible as faint bands right under the main bands. 10% acrylamide gel, marker – color prestained protein standard, broad range (11-245 kDa) (NEB). Imaged with Gel Doc XR+ (Bio-Rad).

7.5 Surface plasmon resonance with MID (962-1200) and anti-Phl p 7 IgD

Preliminary SPR experiments were carried out with MID (901-1250), as described in the Methods section. However, due to the instability and aggregation of the construct, the data obtained were inaccurate. The aggregation also caused extensive non-specific binding to the surface of the SPR chip in both reference and target flow cells, which made it impossible to regenerate the chip.

The newer MID (962-1200) construct was much more stable and well-behaved and produced interpretable data. Serial dilution from 500 nM to 8 nM of MID in SPR buffer was run through the flow cells of the SPR chip. Data from the BIAcore T100 instrument were exported as tsv files and were analyzed using the python script linked in the Methods section and all figures were produced using it. Sensograms were plotted with cleaned data for each concentration (**Figure 7.22**). Lower concentrations were omitted.

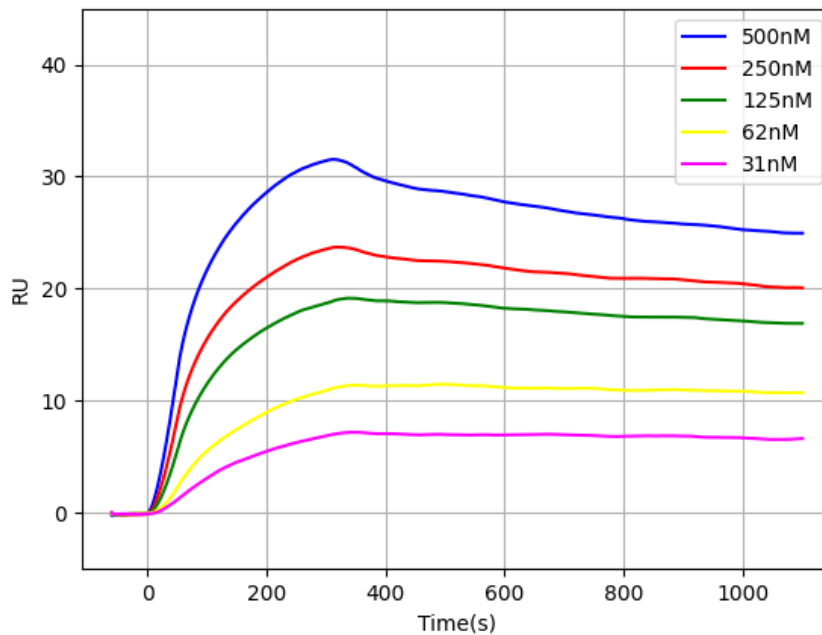


Figure 7.22 Sensograms of a serial dilution of MID (962-1200) binding to anti-Phl p 7 IgD immobilized on the SPR chip. Data was previously subtracted from reference cell and cleaned using the scripts in Methods. Adequate association and dissociation phases can be discerned.

Association phases of the generated cleaned sensograms were fit to the 1:1 Langmuir binding model, as described (**Figure 7.23**). The values for k_{obs} were obtained from the data fitting and used for linear regression analysis. (**Figure 7.24**) in order to obtain K_{on} and K_{off} values. Linear regression fit was not ideal, indicating a non-linear component to the analyte binding at higher concentrations, possibly due to an avidity effect.

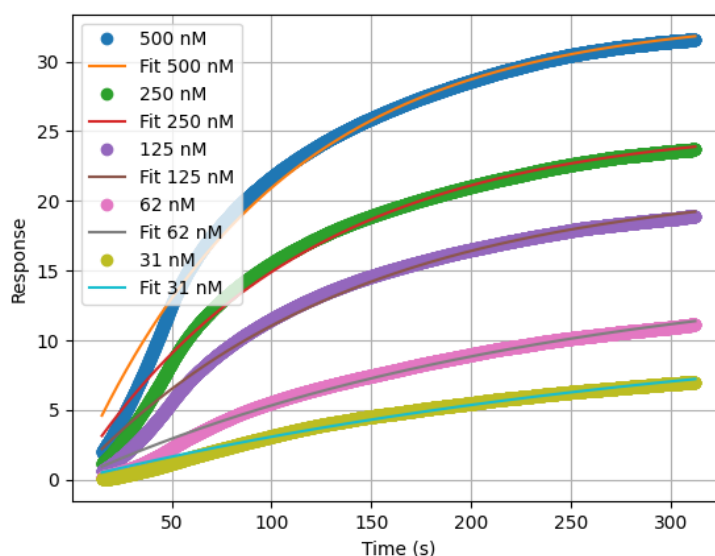


Figure 7.22 Curve fittings of association data to the Langmuir binding model. Streaks represent experimental data and lines represent the fit to the model curves.

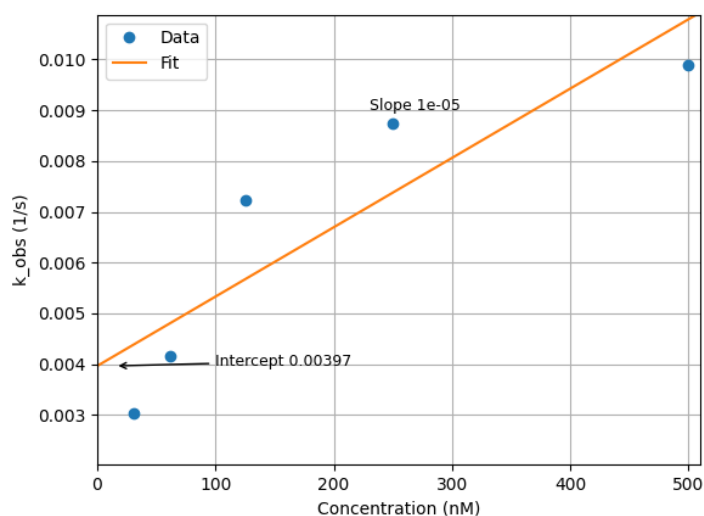


Figure 7.23 Values for k_{obs} from the Langmuir binding model were plotted for each analyte concentration and linear regression was performed, as described in Methods. The slope of the resulting line represents $k_{on} = 0.00001364 \text{ M}^{-1}\text{s}^{-1}$ and the y-intercept represents $k_{off} = 0.00397 \text{ s}^{-1}$. The estimated reduced χ^2 value is 0.7862, which represents an imperfect fit due to overfitting or a non-linear element in the binding events. Estimated K_D was calculated to be 291.05 nM.

The observed $R_{\max}$ values were then analyzed with a hyperbolic binding model (Figure 7.24) by plotting $R_{\max}$ for each analyte concentration, assuming that the response is related to the concentration via the equation described in Methods. The data fitting and χ^2 statistics produced an estimated $R_{\max} = 38.004 \pm 2.04$ RU and $K_D = 136.879 \pm 18.847$ nM. The reduced χ^2 value, calculated by dividing the χ^2 value by the degrees of freedom, was found to be 0.051. This indicates a good fit between the model and the data. The R-squared value, a measure of the proportion of response variation explained by the model, was determined to be 0.991, indicating a high degree of fit.

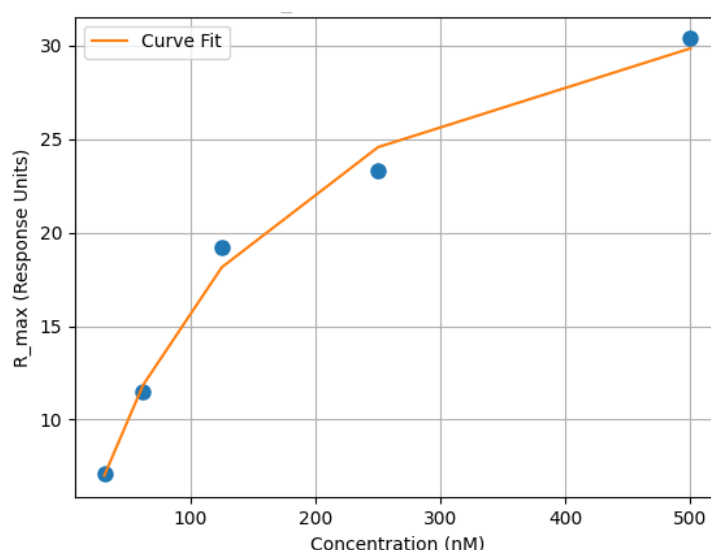


Figure 7.23 Fitted curve with a hyperbolic binding model, as described in Methods. The data (blue points) provided a good fit to model. Estimated $K_D = 136.879 \pm 18.847$ nM.

Overall, the SPR data confirmed the binding of the two in-house produced protein constructs MID (962-1200) and anti-Phl p 7 IgD. Estimated K_D for the interaction was roughly 136 nM, indicating the concentration at which half $B_{\max}$ is achieved. Nanomolar affinity indicates that the strength of the interaction is relatively high. Poorer fits to linear regression indicate that there may be an avidity effect involved, especially at higher concentrations. This would be expected, as MID is known to form oligomers in solution.

8. Flow cytometry results

In the very early stages of the project, two other cell lines not described in the methods section, were used – the B cell leukaemia Daudi cell line (Dai et al. 2016) and the U937 immature monocyte cell line (Y. Wu et al. 2016), due to their supposed IgD-responding capabilities cited previously in literature. However, due to inconsistencies in results, as well as malfunction of the Guava EasyCyte flow cytometer (Luminex) used in-house initially, experiments with those cell lines were discontinued early on and data from them will not be presented here. Experiments with the LAD2 cell line yielded results similar to the data from KU812 cells with regards to IgD binding and will not be discussed due to the short use of that cell line. The focus instead will be on just the KU812 and Jurkat cell lines, which were the only cell lines used consistently for all three major techniques throughout the project.

Before gaining insight to the potential involvement of galectin-9 thanks to the Shan et al. publication in 2018, staining for flow cytometry was done with non-labelled IgD and without the use of galectin-9. IgD bound to cells was detected with anti-IgD-PE (Biolegend).

Initially, following standard staining protocol in which all cell incubations were done on ice, almost no anti-IgD-PE signal was detected, compared to cells stained with isotype control alone. Cell samples were then split into three conditions for IgD incubation – on ice, at room temperature (25 °C, and at 37°C. Almost no change was observed between the ice and room temperature conditions, but **in the 37°C samples an increase in IgD detection was observed (Figure 8.1)**. Therefore, all following IgD incubations were done at that temperature. **The discovery that IgD-binding is temperature dependent hints at the involvement of an active cellular mechanism taking part, which would otherwise not occur at lower temperatures.** It is also possible that temperature sensitivity is due to increased rotational freedom of Fab fragments, thanks to IgD's lengthy hinge, which could improve receptor accessibility to the Fc fragment.

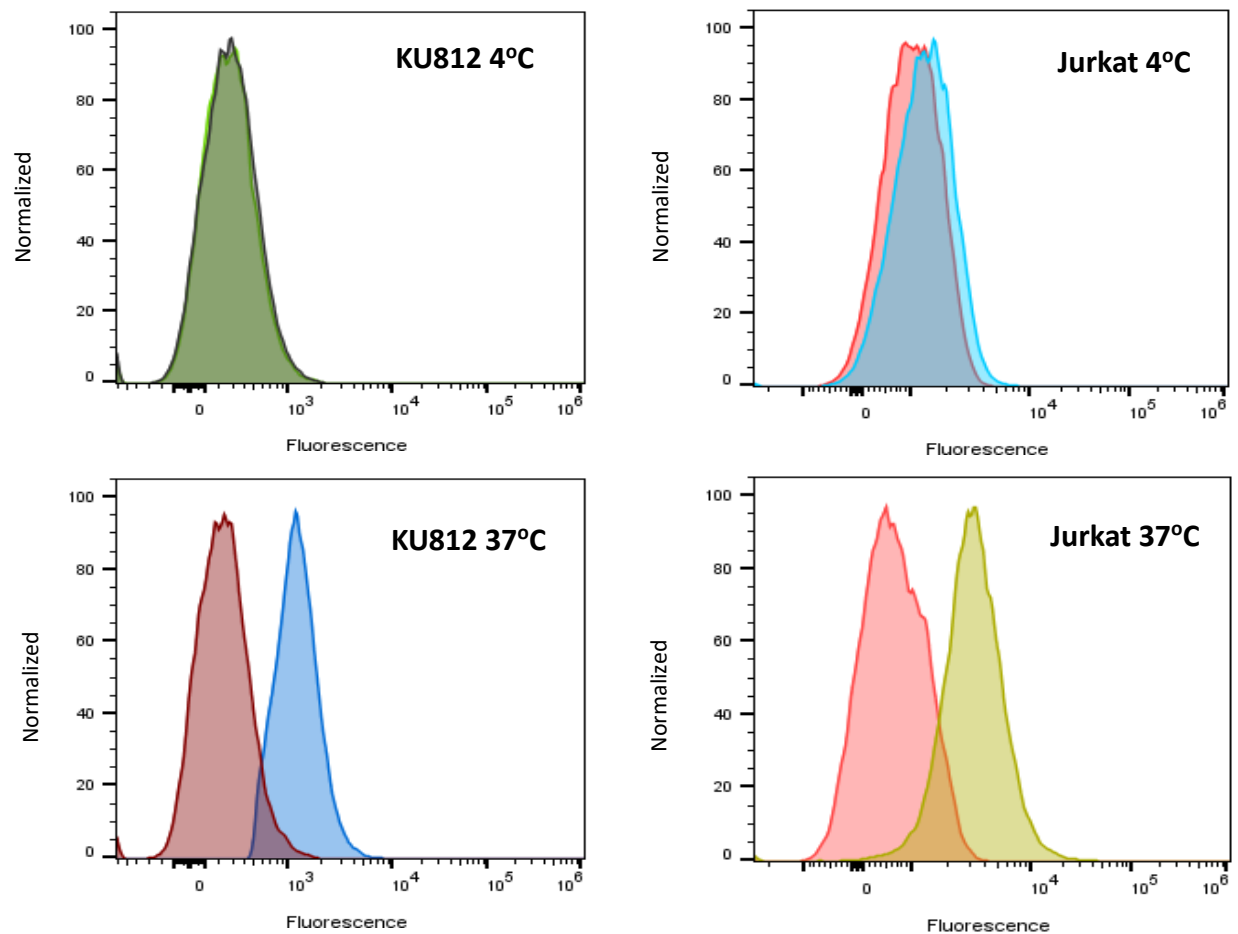


Figure 8.1 Normalized mean fluorescence intensity obtained following staining of KU812 (left) and Jurkat (right) cell lines with non-labelled IgD. The signal detected is that of the phycoerythrin (PE) fluorophore conjugated to the anti-IgD detection IgG antibody, which is compared to the basal IgG1-PE isotype control signal (pink or dark pink), used as a blank for the IgG-PE probe. Cell populations were gated according to forward and side scatter profiles, live cells were gated by viability dye exclusion. For both cell lines almost no binding of IgD was observed when incubation was done at 4°C (top), while a significant increase is visible for the 37°C incubations (bottom), indicating the potential involvement of an active cellular process. Figure generated with FlowJo (BD Biosciences).

After the forementioned Shan et al. paper was published in 2018, my focus was turned to the proposed involvement of CD44 and galectin-9. First, to confirm whether Jurkat cells in fact exhibit no CD44 expression, I compared its recorded expression, along with the expression of other relevant genes, via available information deposited in Expression Atlas (ebi.ac.uk), represented in **Figure 8.2**. Jurkat cells should express no CD44, nor any of the IgE receptors. Both cell types should have endogenous gal-9 production but is unknown how much of it they would secrete.

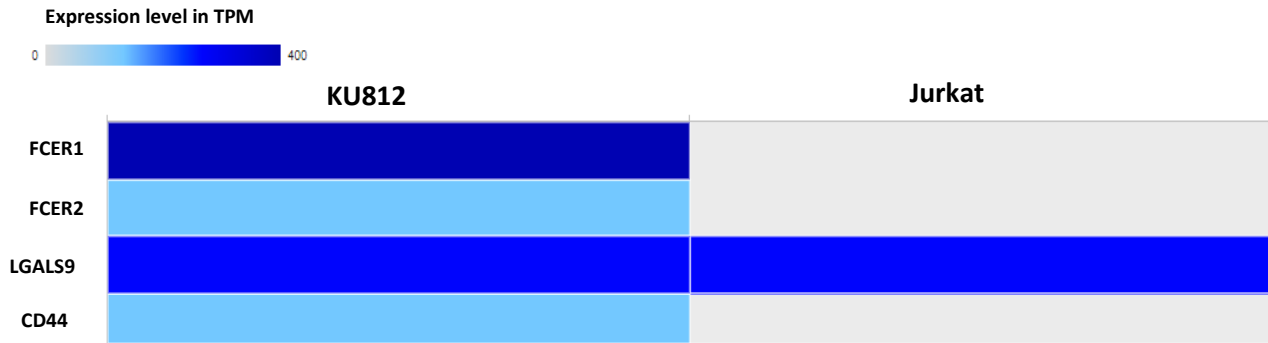


Figure 8.2 Expression profile of KU812 and Jurkat cell lines with regards to some molecules of interest, available from literature, represented as transcript per million (TPM). Notably, Jurkat cells do not express any of the two IgE receptors (FcεR1 and CD23) or CD44, unlike KU812. Both cell lines show similar expression of endogenous galectin-9, which should therefore not interfere with data obtained after exogenous addition. Source: Expression Atlas (ebi.ac.uk).

KU812 and Jurkat cells were stained with the same anti-CD44-AF488 (Biolegend) used for confocal microscopy, at 4°C, to confirm the lack of cell surface CD44 in the Jurkat cells. Unsurprisingly, Jurkat cell samples had almost no AF488 signal, unlike the robust signal evident in KU812 samples (**Figure 8.3**). The confirmation of this key difference between the two cell lines is pivotal for any conclusions drawn later about the involvement of CD44 in IgD binding to effector cells.

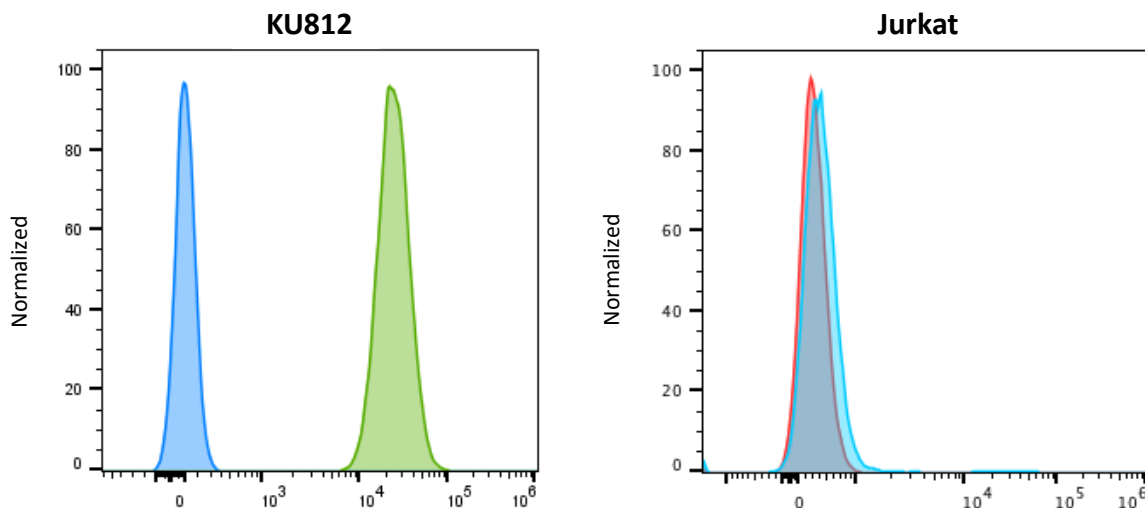


Figure 8.3 Normalized mean fluorescence intensity obtained following staining of KU812 (left) and Jurkat (right) with anti-CD44-AF488. Sample signal was compared to IgG1-AF488 isotype control signal (blue) as a blank. No signal above blank was observed for Jurkat cells, while KU812 cells exhibited quite strong binding of the anti-CD44 probe. This data confirms that KU812 have a fair amount of CD44 on their surfaces, while Jurkat cells have none. Figure generated with FlowJo (BD Biosciences).

Experiments with galectin-9 addition coincided with the production of the earlier described recombinant anti-Phl p 7 IgD-AF546 fluorescent conjugate, and were done using that probe, to exclude the effects of any potential cross-linking of captured IgD on cell surfaces by the anti-IgD antibody used previously. 50 µg/ml galectin-9 was added to samples with the rest of the staining cocktail (IgD-AF546 included) and cells were incubated at 37 °C for 40 min. **Incubations with galectin-9 for longer than an hour resulted in significantly more cell death**, discussed later. No isotype control (IgD) could be used in this case, so stained cell signal was compared to unstained cells instead.

Overall, a noticeable **improvement of IgD binding was observed for both cell lines upon exogenous galectin-9 addition (Figure 8.4)**. Gal-9 is a known ligand for CD44 (Tanikawa et al. 2010), and increased binding to KU812 CD44⁺ cells was expected, as per the recent findings (Shan et al. 2018). However, the interesting thing in this case is that galectin-9 addition produced an even more robust improvement of IgD **binding in the CD44⁻ Jurkat cells, indicating that the gal-9-mediated effect on cell surface binding is facilitated through an unknown mechanism independent of CD44**, at least in the Jurkat T cell line.

The minimal but non-zero presence of IgD without galectin-9 on the surface of cells only when they were stained at 37°C posed the question what cellular process necessitating this temperature could be responsible for the small IgD signal. **We hypothesized that the weak IgD surface signal could be due to endocytosis of the probe**, which only occurs at or around that temperature, and could also explain the lower cell surface presence of the fluorescent probe. Endocytosis of the IgD probe was indeed discovered to occur in the latter fluorescence confocal microscopy experiments.

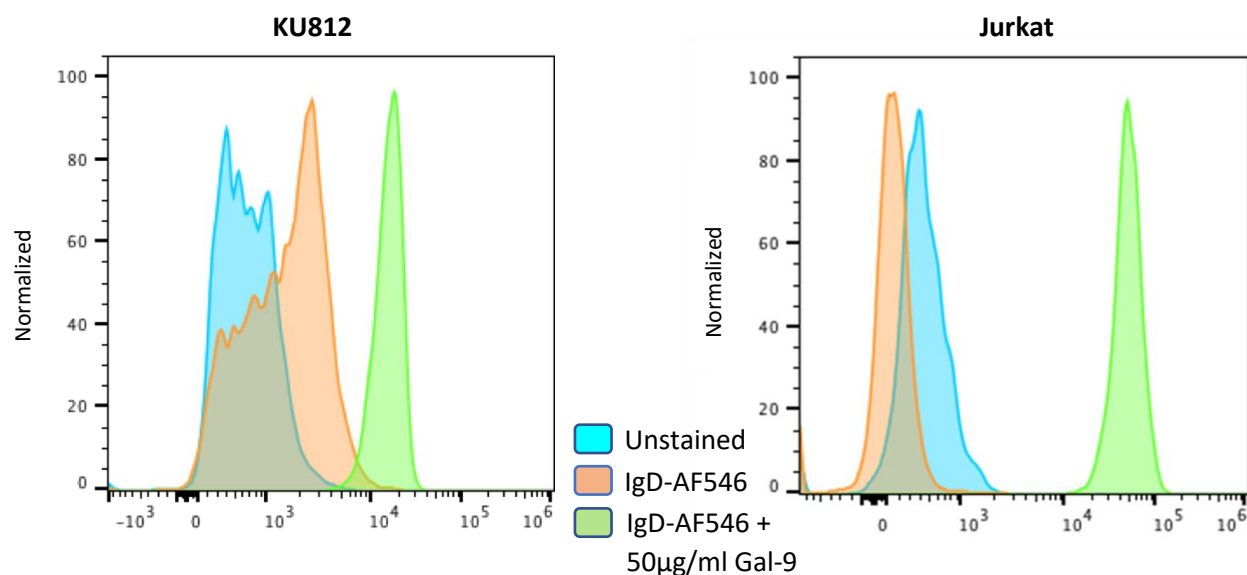


Figure 8.4 Normalized fluorescence data obtained following staining of KU812 (left) and Jurkat (right) with anti-Phl p 7 IgD-AF546 with (green) and without (red) exogenous galectin-9 addition. Sample signal was compared to unstained cells (blue) as blank control. Gal-9 significantly improved binding of IgD to cell surfaces, compared to IgD-stained samples without it. For KU812 cells this would confirm the gal-9 effect on IgD binding through CD44 described by Shan et al. Jurkat cells however bear no CD44, yet are even more so influenced by gal-9, indicating a CD44-independent mechanism for the improved binding. Figure generated with FlowJo (BD Bioscience).

Despite the expected result for the CD44⁺ KU812 cells, confirming somewhat the statement by Shan et al. that gal-9 targets IgD to CD44, thus improving IgD surface retention, these data raised a very important question with regards to the CD44⁻ Jurkat cells – **how is gal-9 enhancing IgD presence on the Jurkat T cell surface and what is IgD (or IgD: gal-9 complex) binding to?** We attempted to answer these questions through mass spectrometry analysis of cell lysates incubated with IgD, as described in Chapter 10.

An important observation was made in the early days of staining experiments with galectin-9, which resulted in method optimization. It was noticeable that incubations with exogenous gal-9 for longer than 1 hour at 37°C led to a significant increase in observed cell death, as evident by the greater amount of cells taking up FVD in all samples and the depletion of the cell populations gated by forward and side scatter (**Figure 8.5**). This effect was also observed by confocal microscopy of samples stained over 4 hours prior to experiments. Evidence in literature was then found stating that sustained increased exogenous galectin-

9 presence can have pro-apoptotic effects on cells (Saita et al. 2002), which is why it has been tested in cancer therapy through cell apoptosis (Fujita et al. 2017). For a brief period, this effect caused us to believe IgD was binding to mostly dead or dying cells, but that was quickly dismissed, as it is normal for ruptured dying cells to non-specifically intake the probe. Therefore, cell incubations with galectin-9 for maximum of 40 min were employed for all experiments, which did increase cell viability in later experiments.

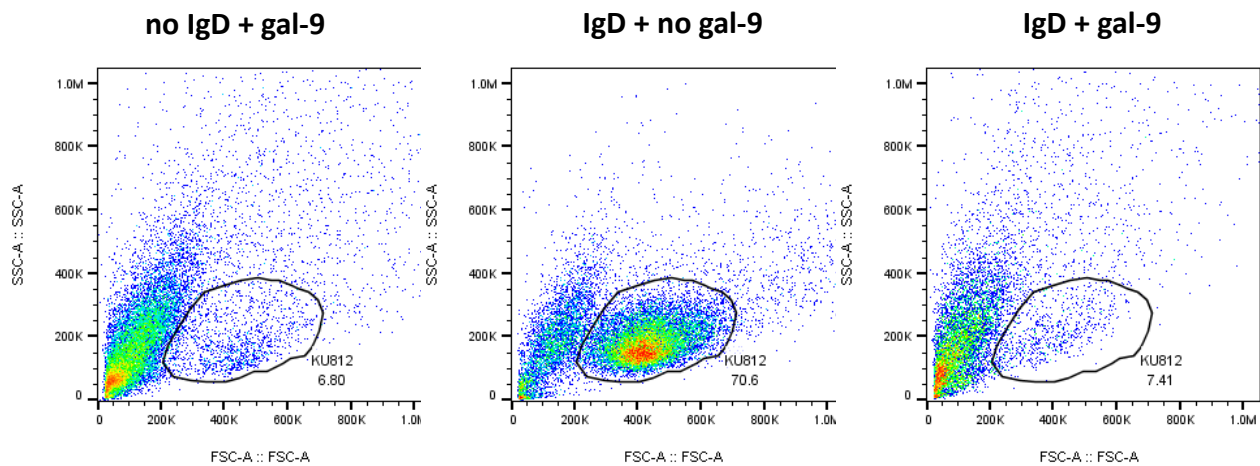


Figure 8.5 Forward and side scatter collected from KU812 cells stained with or without 50 $\mu\text{g}/\text{ml}$ IgD-AF546 and 50 $\mu\text{g}/\text{ml}$ galectin-9, incubated with the reagents for over 2 hours at 37°C. Gating was placed according to unstained cell populations. In the samples with gal-9 (left, right) there is an obvious absence of the healthy cell population normally observed (middle). Most cells seem to have died off, apparent by the majority of events recorded having very low forward (FSC) and side scatter (SSC) due to the loss of the cells' membrane integrity. IgD presence had no apparent influence on the gal-9 induced effect (right). Figure generated with FlowJo (BD Bioscience).

9. Confocal microscopy results

Between the temperature and galectin-9 dependence for IgD binding to cell surfaces, the simple flow cytometry experiments hinted at a more complex picture for IgD's cellular interactions than initially anticipated, which prompted us to dig deeper. And so, we turned to fluorescence confocal microscopy to directly view what is happening with IgD on (or inside) the KU812 and Jurkat cells.

The initial fluorescent probe used for IgD detection for confocal microscopy was a commercially available anti-IgD-AF647 (Biolegend, same clone as the anti-IgD-PE for cytometry). Initial experiments with the probe were successful (**Figure 9.1**), but after the generation of the anti-Phl p 7-IgD-AF546, the former became obsolete due to high cost and much weaker signal compared to the latter, which also liberated the red fluorescence channel for other reagents such as anti-EEA1-AF647 and anti-LAMP1-647 (also red-wavelength fluorescent probes are somewhat more widely used and therefore more easily available commercially), as described below. Cells stained with IgD-probes were also stained with anti-CD44-AF488 (KU812 cells only) and later with other markers for different stages of endocytosis.

Overall, every microscopy experiment betrayed the same features across numerous samples of both Jurkat and KU812 cells – **IgD seemed to be taken up within cells, not at all colocalizing with the CD44 probe on the cell surface when no exogenous galectin-9 was present. Conversely, when gal-9 was added during IgD incubation, IgD signal localized almost exclusively to the cell surface, along with CD44.** This discovery could somewhat explain the weak IgD-signal from cell surfaces without gal-9 addition at 37°C only, as observed by flow cytometry earlier – the IgD was in fact being taken up within cells, limiting the detected surface signal, while with gal-9 it was kept locked to the cell surface. Following those observations, probes for early endosomes, recycling endosomes, late endosomes, and lysosomes were utilized to track their potential colocalization with the IgD probe and its trafficking within cells. All following images were captured and viewed with the NIS-elements software (Nikon). Figures generated with ImageJ.

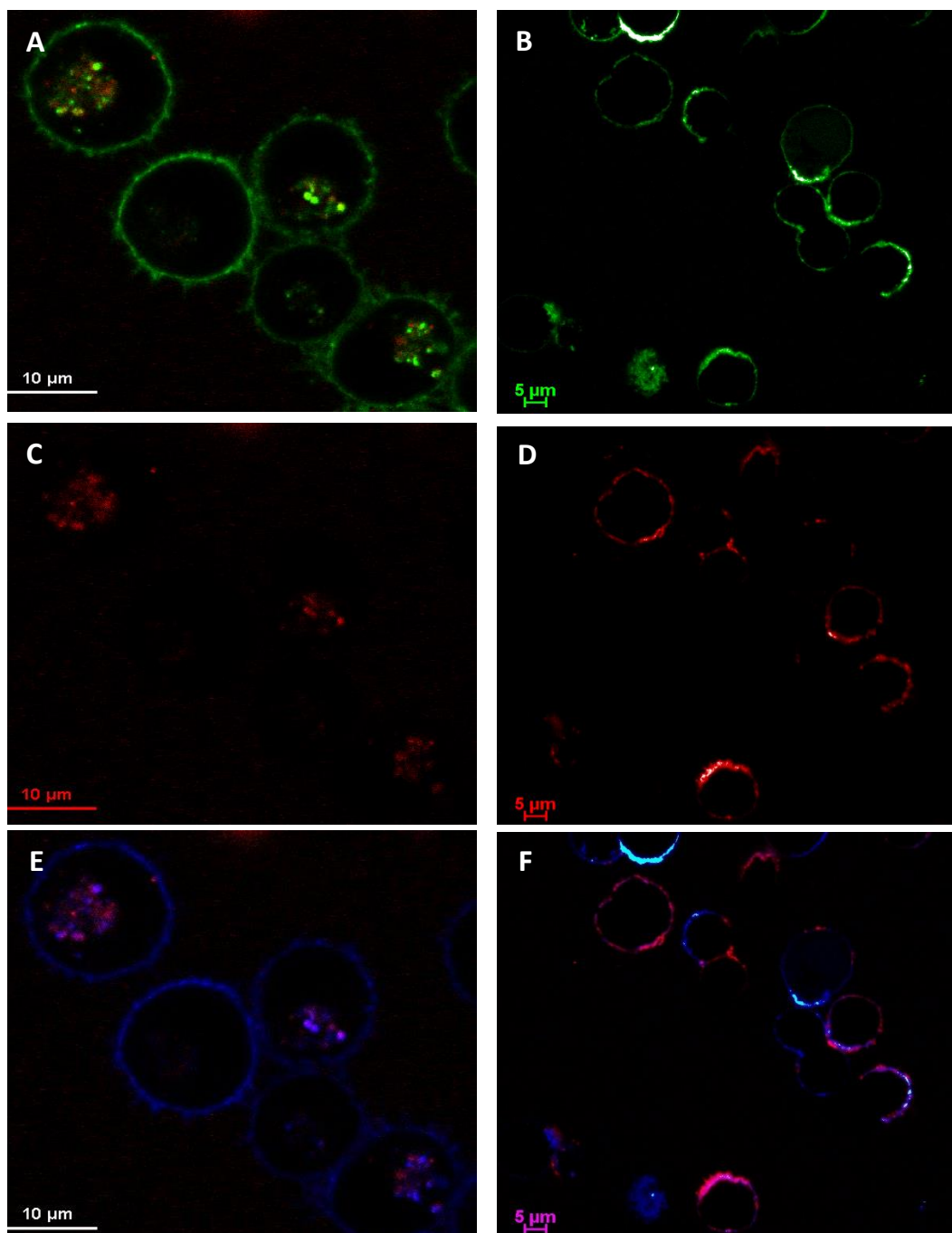


Figure 9.1 60x magnification with 2x digital zoom of **KU812** cells, stained with anti-CD44-AF488 (green) and anti-IgD-AF647 (red). The Anti-CD44 signal clearly demarks the cell membranes. **Left** – all channels (**A**), red channel (**C**), and false green to blue merged channel (**E**) of cell sample without added gal-9. IgD is seen entirely taken up within cells, along with some CD44, with no AF647 signal present on the cell membranes. Intracellular CD44 may be colocalizing with IgD (**E**, purple) either due to binding or because it was taken up in endocytic vesicles. **Right** – green (**B**), red (**D**), and false green to blue merged (**F**) channels of samples with 50 µg/ml gal-9. Neither IgD nor CD44 signals are visible intracellularly and all the probes signal is coming from cell surfaces, where the two probes colocalize, evident by the blue false coloring of the green channel overlapping with the red to form purple in **F**.

Ever since the trial experiments with just KU812 cells (**Figure 9.1**), it was confirmed that IgD **did in fact colocalize with CD44 on the cell surface, as was claimed, but only if exogenous gal-9 was present.** Without it, most of the IgD signal detected was intracellular. Following experiments done with Jurkat cells (**Figure 9.2**) showed **the same behavior of the IgD probe as it was observed in KU812 cells, despite Jurkat not expressing CD44.** Therefore, other surface molecules must be binding to IgD – potentially wholly different, depending on the presence of galectin-9.

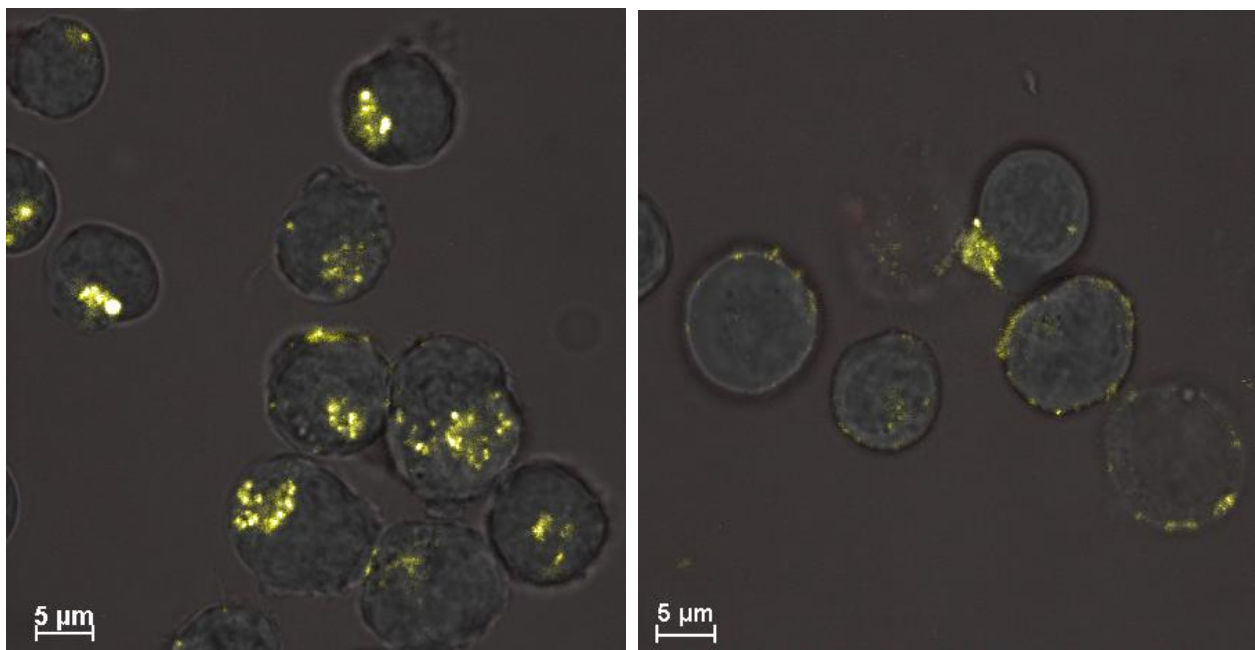


Figure 9.2 60x magnification with 2x digital zoom of **Jurkat cells**, stained with anti-Phl p 7 IgD-AF546, with (**left**), or without (**right**) exogenous galectin-9; brightfield and yellow channels. The IgD signal (yellow) is seen only inside of cells on the left, and almost entirely on the cell surface on the right. Lack of CD44 expression in these cells implies other IgD binder(s), which may differ depending on gal-9.

Next, to determine how IgD is being taken up within non-gal-9-incubated cells, I employed the use of labelled dextran (for pinocytosis) and endocytosis markers, all labelled with the red dye AF647, as that detection channel was free once I switched to the IgD-AF546 probe. 10 000 MW Dextran-AF647 addition yielded no signal above background in all experiments with it, excluding any involvement of pinocytosis, and will therefore be not discussed further.

First of the endocytosis markers used was anti-EEA1-AF647 (Abcam). EEA1 (Early Endosomal Autoantigen-1) is a 162 kDa protein, functioning as homodimers, which mediates the sorting and fusion of nascent endocytic vesicles into the early endosome compartment by interacting with GTP-bound Rab5a and SNARE proteins on their membranes, and is exclusively localized to these compartments. Its structure is formed of a long coiled-coil motif, making up the dimer interface, and a C-terminal zinc finger-like FYVE domain, which docks to phosphatidylinositol-3-phosphate (PIP₃) on the vesicle surfaces (Wilson et al. 2000).

Overall, the **EEA1-AF647 probe colocalized almost entirely with IgD** when added to the staining cocktail in live cells, irrespective of gal-9 addition. With gal-9, it appeared to be locked to the cell surface, along with IgD, while without it, both probes were seen within cells, in some cases clearly seen in discernible enclosed endocytic vesicles. This discovery, along with the previously discussed temperature dependence of IgD binding, **cements the notion that IgD is endocytosed by living cells when no exogenous galectin-9 is present (Figure 9.3)**. Conversely, with gal-9, the anti-EEA1 probe localized almost entirely on the cell surface and no internalization was visible (**Figure 9.4**), meaning that **gal-9 either locks the probe to the surface, or halts endocytosis entirely**. It is also possible that gal-9 may nonspecifically bind the anti-EEA1-AF647 IgG probe as well and locking it to CD44 or an unknown cell surface protein in Jurkat. Interestingly, **internalized IgD was confined to discrete punctae**, which could be endocytic vesicles. These punctae of signal were observed to be trafficked in tight channels within cells in time lapse recordings, circumventing nuclei and **accumulating in what seem to be perinuclear folds**. The implications of this observation are discussed later. Another interesting feature observed is that, when galectin-9 is present, the distribution pattern of probes on the cell surface mostly overlaps. This could be explained for the proposed binding partners IgD and CD44, but why the EEA1 probe colocalizes strictly with them, with almost no intracellular endocytic vesicle staining, is another question, which is tackled later.

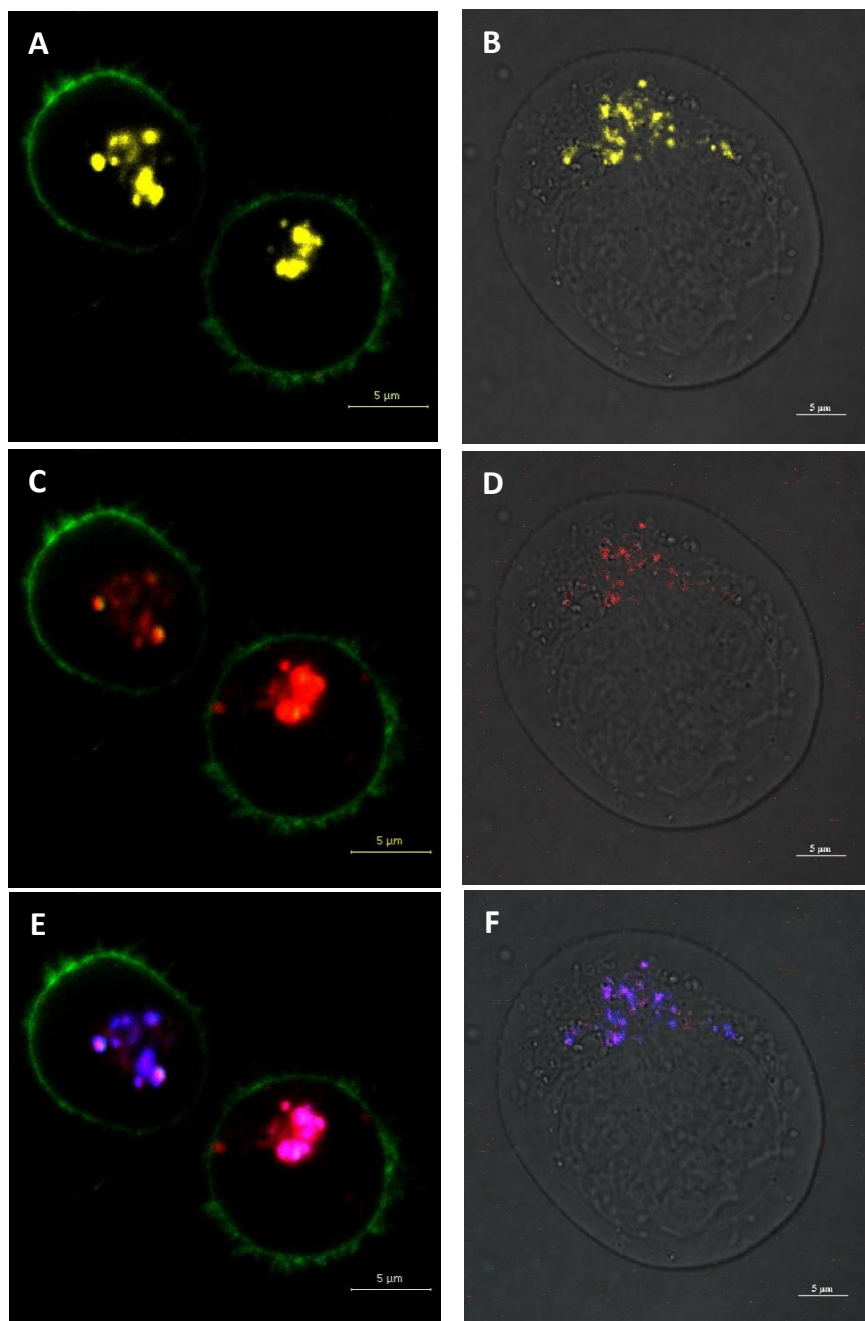


Figure 9.3 100x magnification with 2x digital zoom of **KU812 (Left)** and **Jurkat cells (R)**, stained with IgD-AF546 (yellow) and anti-EEA1-AF647 (red), **without exogenous galectin-9**. **A, C** – yellow or red channel overlaid with green channel to show CD44 demarking the cell membrane of KU812. No IgD or EEA1 signal is visible on the cell surface, all of it being present intracellularly and mostly colocalizing, as evident by the false color merging in **E**. **B, D** – yellow or red channel overlaid with brightfield view for contrast of cell borders and features of a single Jurkat cell. It appears more flattened and therefore larger due to the agarose pad's pressure (Methods). Again, IgD and EEA1 probes colocalize intracellularly in distinct loci, with no signal from cell membrane, as seen in the false colour merging in **F**. Additionally, thanks to the flattened shape of the Jurkat cell, accumulation of the probes near the lightly discernible nucleus can be seen.

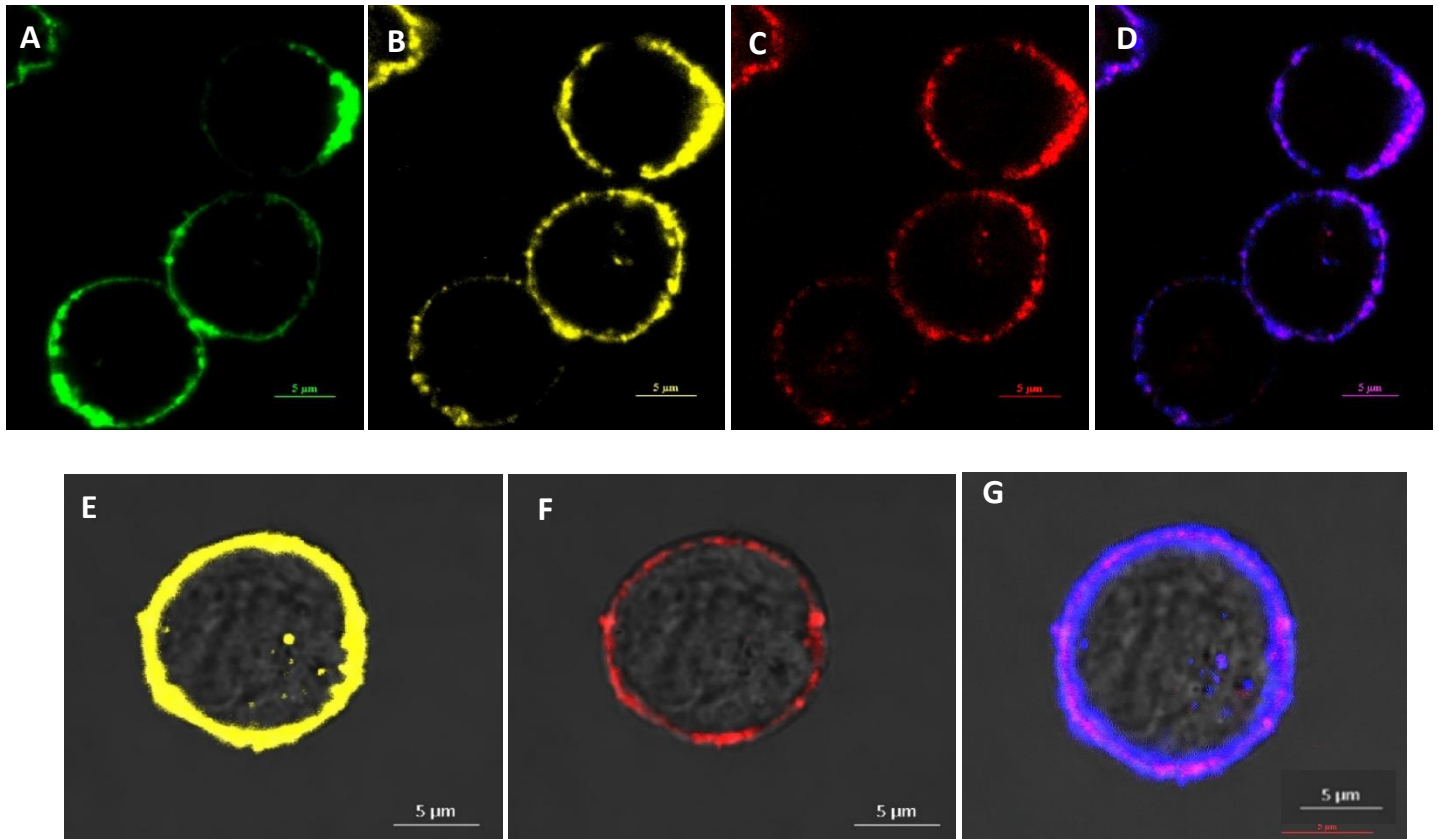


Figure 9.4 100x magnification with 2x digital zoom of **KU812 (Top)** and **Jurkat cells (Bottom)**, stained with anti-CD44-AF488 (green), IgD-AF546 (yellow) and anti-EEA1-AF647 (red), **with 50 μg/ml galectin-9**. **A, B, C** – green, yellow, and red channels of KU812. Little to no signal coming from inside the cells, instead all three probes colocalize on the cell surfaces, with almost the same overlapping distribution patterns, as evident by the yellow to blue false colour merging with the red channel in **D** – weaker/stronger signal for one probe corresponds to the same for the. **E, F** – Yellow and red channel overlapped with brightfield view of a Jurkat cell. Very strong uniform IgD signal is present on the surface, with a small amount of intracellular signal (possibly overstained). EEA1 probe is seen entirely on the cell surface, colocalizing with IgD, as seen in the false colour merged channels in **G**.

It is interesting to note that when I switched to direct labelling with IgD, much less CD44 signal was visible intracellularly, perhaps because cross-linking of captured unlabelled IgD by the anti-IgD-AF647 probe may trigger CD44 internalization as a potential part of the IgD receptor complex.

The fact that IgD is internalized without galectin-9 and retained on the surface when the latter is added exogenously, on both CD44-positive and CD44-negative cells, suggests differential modes of effector cell function, depending on the presence of gal-9, the levels of which fluctuate during inflammation (Liu and Rabinovich 2010). This effect is independent of CD44, as demonstrated by the Jurkat cell staining. On the other hand, it is unclear why the endocytic marker is kept at the surface by galectin-9. A possible explanation could be that gal-9 is either wholly halting endocytosis or is simply preventing IgD from being endocytosed (with nothing else to be endocytosed in the samples). It is also possible that gal-9 targets the anti-EEA1 IgG probe to an unknown cell surface molecule. To gain a better understanding, the effect of gal-9 was compared to that of Dynasore (Abcam) (**Figure 9.5**) – a cell-permeable reversible inhibitor of dynamin (Macia et al. 2006). Dynamin is a 100 kDa GTPase which normally facilitates the fission of clathrin-coated endocytic vesicles from the intracellular side of the membrane, by means of self-polymerization into rings along the budding invagination's cervix, followed by GTP hydrolysis, thus pinching off a vesicle (Schmid and Frolov 2011). 25 μ M Dynasore in DMSO was added to the cell staining cocktail.

Notably, the **Dynasore-mediated prevention of IgD internalization confirms that IgD is otherwise taken up via clathrin-mediated endocytosis**. Overall, the addition of endocytosis inhibitor to both cell lines mimicked the effect of galectin-9 with regards to EEA1 and IgD probe accumulation on the cell surfaces. However, some notable differences can be observed from these images. First, in KU812 cells, the identical surface signal pattern of IgD and anti-EEA1, showing that IgD is recruited for endocytosis, does not match that of anti-CD44 (**Figure 9.5**), unlike when gal-9 is added (**Figure 9.4**). Second, as opposed to having relatively uniform signal on cell surfaces with gal-9 on Jurkat cells, the probes in the Dynasore-inhibited condition seemed to co-accumulate in specific locations, again indicating primed endocytosis. Thus, it can be inferred that IgD is normally recruited for endocytosis, but gal-9 is preventing internalization by forcing IgD to co-localize with CD44, at least in KU812 cells. Questions remain with regards to Jurkat, in which another surface molecule must be engaged via gal-9 addition.

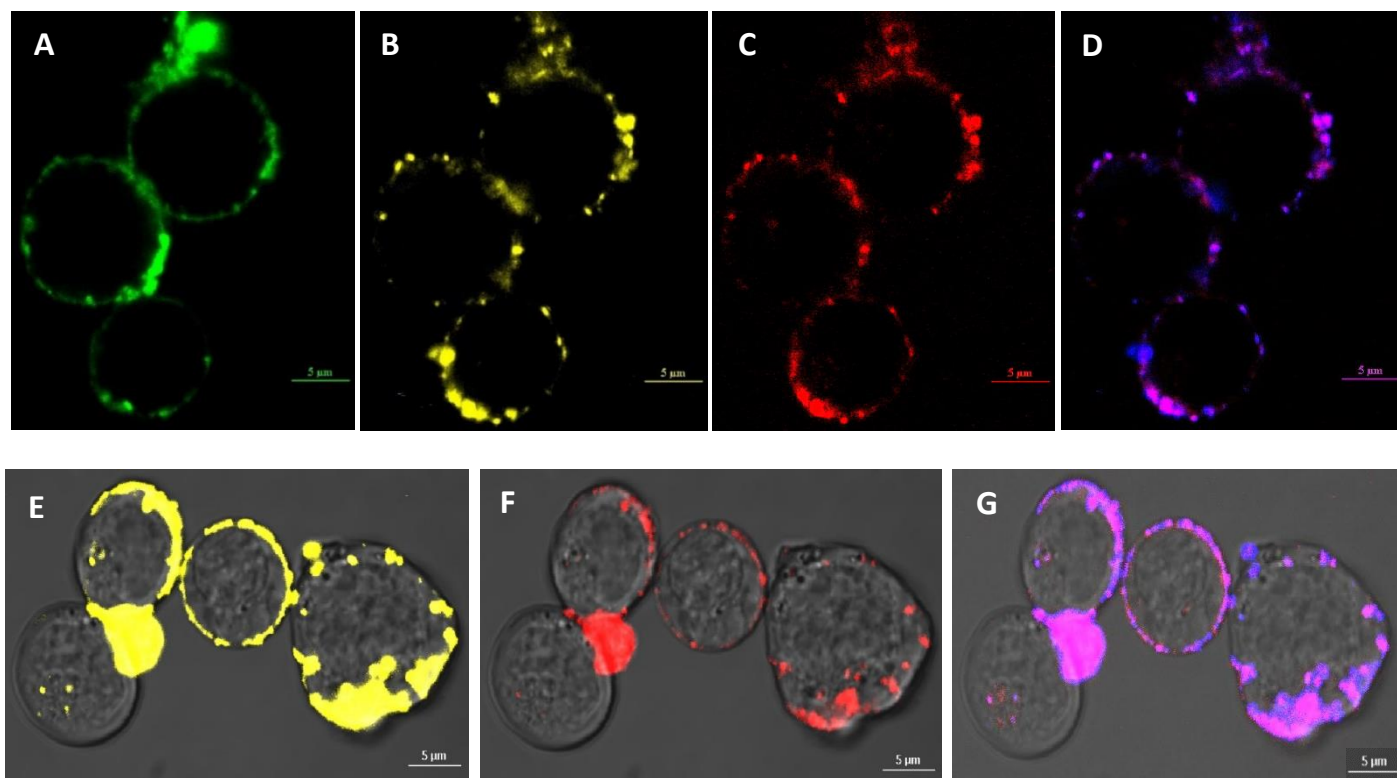


Figure 9.5 100x magnification with 2x digital zoom of **KU812 (Top)** and **Jurkat cells (Bottom)**, stained with anti-CD44-AF488 (green), IgD-AF546 (yellow) and anti-EEA1-AF647 (red), **with 25 µM Dynasore** addition. **A, B, C** – green, yellow, and red channels of KU812. Note the difference in CD44 and IgD distribution pattern, compared to Figure 9.4. Once again IgD colocalizes with the EEA1 probe, as observed by the intense purple in the false colour merged channel in **D**. **E, F** – Yellow and red channel overlapped with brightfield view of Jurkat. For both cell lines Dynasore has halted endocytosis, leading to pronounced probe accumulation in distinct areas of the cell surface. IgD and EEA1 probe colocalize in distinct surface loci, as seen by the yellow to blue false colouring and merger in **G**. Effect of Dynasore seems very similar to that of gal-9.

After discovering that IgD is found in early endosomes, taken up via clathrin-mediated endocytosis, questions remained regarding how it is trafficked further. Early endosomes form a network of incoming vesicles, in which cargo is sorted to recycling endosomes for return to the surface via several routes, or for degradation through the late endosome-lysosome pathway (Huotari and Helenius 2011). The different trafficking compartments differ in their associated surface proteins, which is a useful way of telling them apart in fluorescence microscopy. Anti-Rab11-AF488 (Thermo) was tested as a marker for recycling endosomes, while anti-LAMP1-AF647 (Abcam) was used to mark late endosomes/lysosomes.

Rab11 is a member of the Rab family of small GTPases, which mostly serve as regulators of membrane trafficking processes. Rab11 is necessary for lipid domain organization which is integral for cargo sorting from early to recycling endosomes (Sönnichsen et al. 2000). Unfortunately, only a couple of successful microscopy sessions with Rab11 staining were done (**Figure 9.6**), as the reagent required very high concentrations to achieve signal above background, which proved to be too costly.

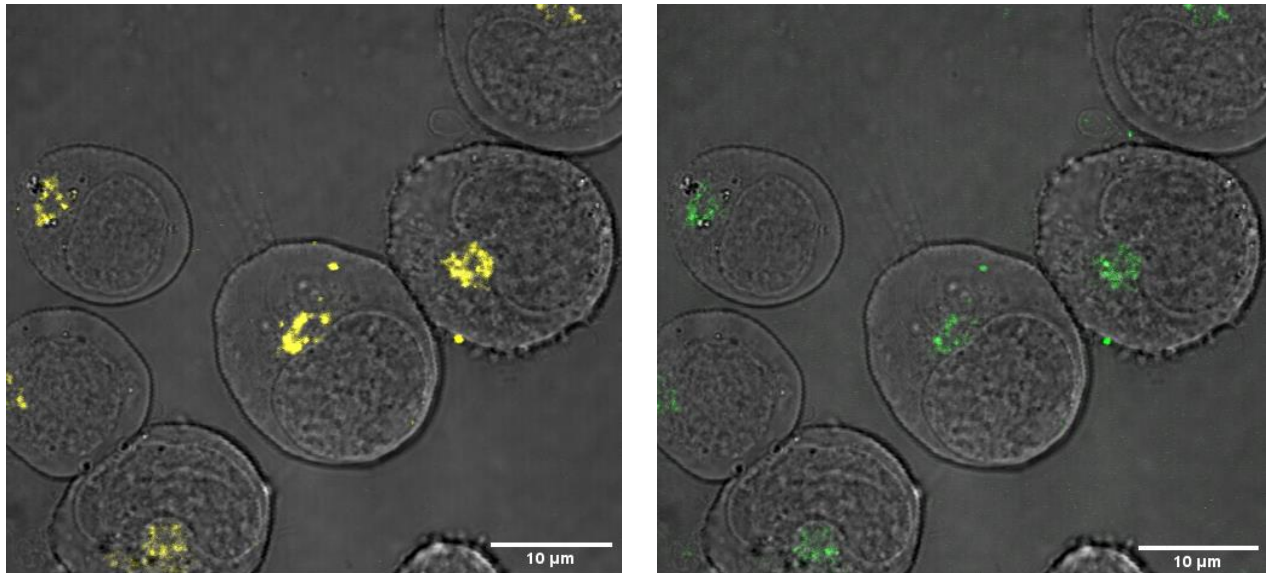


Figure 9.6 100x magnification with 2x digital zoom of **Jurkat** cells, stained with anti-Phl p 7 IgD-AF546 (yellow channel, left) and anti-Rab11-AF488 (green channel, right), in brightfield overlay. Weak signal above background is noticeable for the Rab11 probe, which seems to be mostly colocalizing with the IgD probe, accumulating in regions next to the nuclear membrane. Nuclei are well distinguishable, due to the flattening of the cells, as denser regions surrounded by membrane.

Rab11 probe colocalized with internalized IgD and drew my attention to the predominant location of the signal, which in all samples was very close to the nucleus, in sort of indentations in the nuclear membrane, which is made obvious when cells are properly flattened by the agarose pad. It cannot be yet concluded for certain that IgD is being sorted into recycling endosomes due to the very few successful tries with the Rab11 probe (KU812 images were blurry, with weak signal and therefore inconclusive). However, there is ample evidence in literature of a perinuclear-localized endocytic recycling compartment (ERC), situated

next to the microtubule-organising centre (minus ends of all microtubules in a cell). This compartment is a sort of hub for recycling endosomes, carried to and from it by motor proteins along microtubules (Grant and Donaldson 2009). The implications of IgD being seemingly found in that compartment will be elaborated on in the discussion section.

The canonical alternative to cargo being recycled is for it to be shipped through late endosomes, which fuse with lysosomes. The marker used to locate this compartment is anti-LAMP1-AF647. LAMP1 is a heavily glycosylated transmembrane protein making up a large part of the lysosome's protein component, with a role in membrane integrity maintenance and regulation of autophagosome formation (Eskelinen 2006).

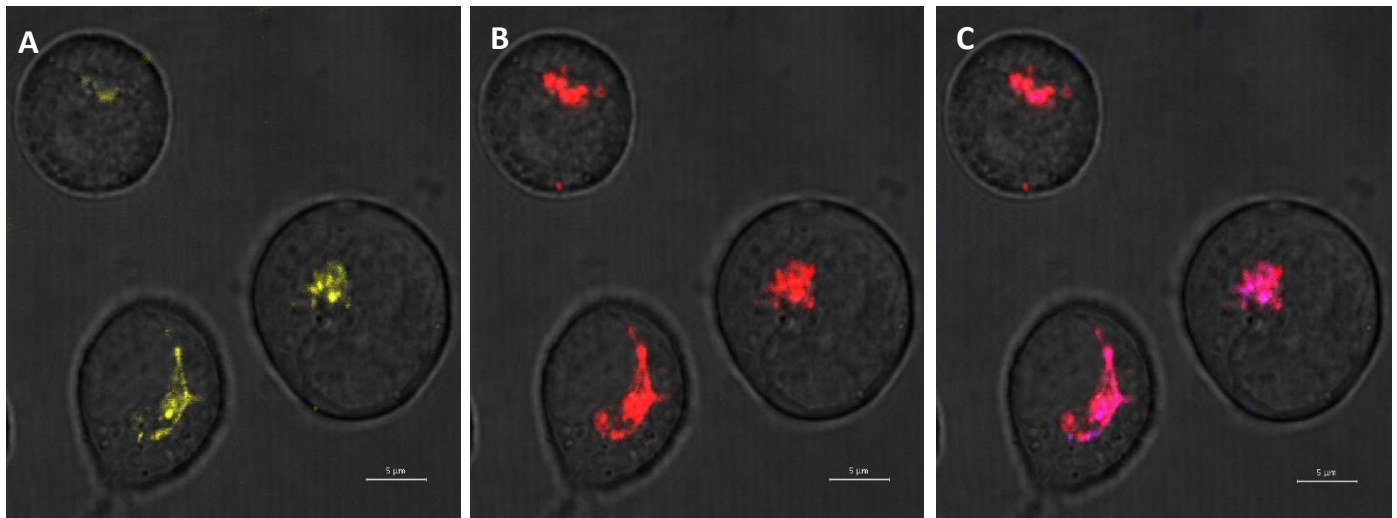


Figure 9.7 100x magnification with 2x digital zoom of **Jurkat** cells, stained with IgD-AF546 (yellow channel, **A**) and anti-LAMP1-AF647 (red channel, **B**), **without galectin-9**. Interestingly, IgD and the lysosomal marker have almost matching (false colour merged channel, **C**), entirely intracellular distribution, which, at least in the Jurkat cells, also matches the localization of the Rab11 and EEA1 probes to the perinuclear region, as seen in **Figure 9.6**.

LAMP1 staining in KU812 did not yield publication quality images, as they were quite blurry, and their data is therefore omitted. However, same patterns of colocalization were discernable, despite high noise.

IgD colocalized with anti-LAMP1 in both cell types (Figure 9.7), which was not expected after having previously confirmed its colocalization with Rab11, leading to seemingly conflicting conclusions – IgD was observed to have been taken up by both recycling endosomes and lysosomes, the pathways in which they are involved should be mutually exclusive. However, it could be that LAMP1 is not strictly limited to the lysosomal compartment (Cheng et al. 2018), and the discoveries may not be as conflicting as initially thought. More on the implications of these findings will be discussed later.

Labelled murine SPE7 IgE-AF546 was also briefly used on Jurkat cells as a proposed control, as Jurkat cells do not express any of the canonical FcεR1 or FcεR2 (CD23) IgE receptors (**Figure 8.2**). SPE7 IgE is a highly cytokinergic murine DNP-specific antibody, able to trigger responses in human mast cell lines without the presence of antigen (Bax et al. 2015). Interestingly however, **the IgE probe seemed to be internalized in much the same manner as IgD within those cells, colocalizing with the early endosomal marker EEA-1, with little to no effect of galectin-9 addition (Figure 9.8)**, despite its reported ability to bind IgE (Niki et al. 2009). This surprising discovery raises several questions with regards to the specificity of the IgD internalization observed across all samples without galectin-9, while also indicating that the effect of surface retention by galectin-9 may be IgD-specific. Notably, once again the probe accumulates in the same perinuclear region as IgD, and the process in this case is not as affected by gal-9 addition, as for IgD. These implications will be discussed further in the next chapter. Unfortunately, due to time limitations, and limited access to the Nikon facility during 2020, experiments with labelled anti-Phl p 7 IgE and with KU812 cells could not be performed, so no concrete conclusions can be drawn as of yet, and future work is needed to confirm whether both IgD and IgE are indeed internalized via the same pathway.

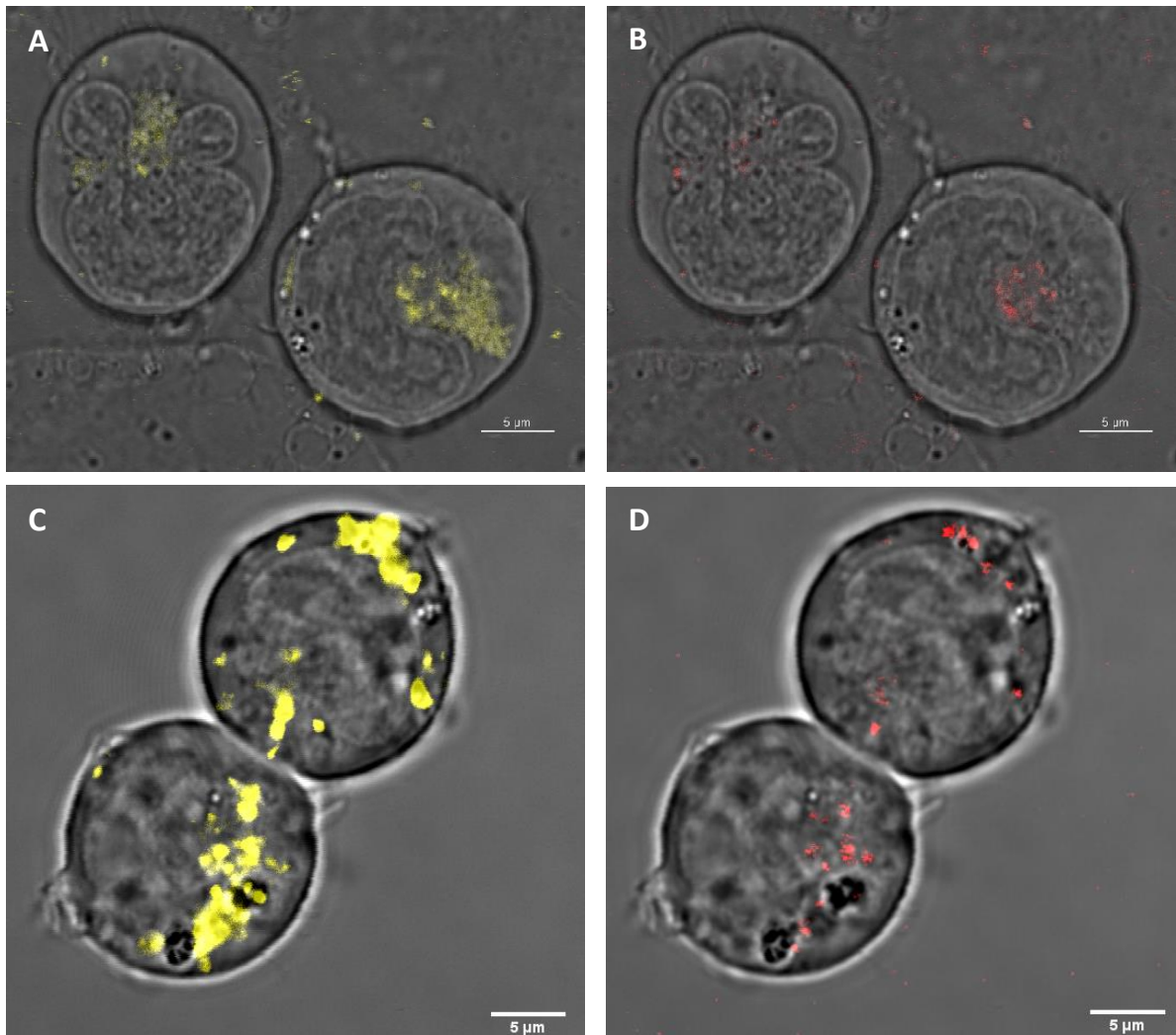


Figure 9.8 100x magnification of **Jurkat** cells, stained with SPE7 IgE-AF546 (yellow channel, left) and anti-EEA1-AF647 (red channel, right), **without (A, B), or with (C, D) galectin-9 addition**. Signal from red probe is only slightly discernible due to photobleaching of these samples. **A, B** - Without gal-9 IgE seems to be localizing to the same perinuclear region as IgD. It colocalizes with EEA1, indicating that IgE may be going through the same internalization pathway as IgD. **C, D** – gal-9 addition has little effect on IgE internalization, although it seemingly causes more IgE to be accumulated, judging by the stronger signal. This effect could be due to gal-9 binding to IgE and its aggregation.

Finally, a couple of experiments right at the end of the project were done with **fixed Jurkat (Figure 9.9)**, to observe potential IgD binding to dead and permeabilized cells, and whether that would be affected by exogenous galectin-9 addition. In this case, all staining steps were done on ice, and the DAPI dye was added to staining cocktail to mark cell nuclei in the blue fluorescence channel. Gal-9 addition seemed to have an impact by concentrating the IgD-AF547 signal to distinct punctae.

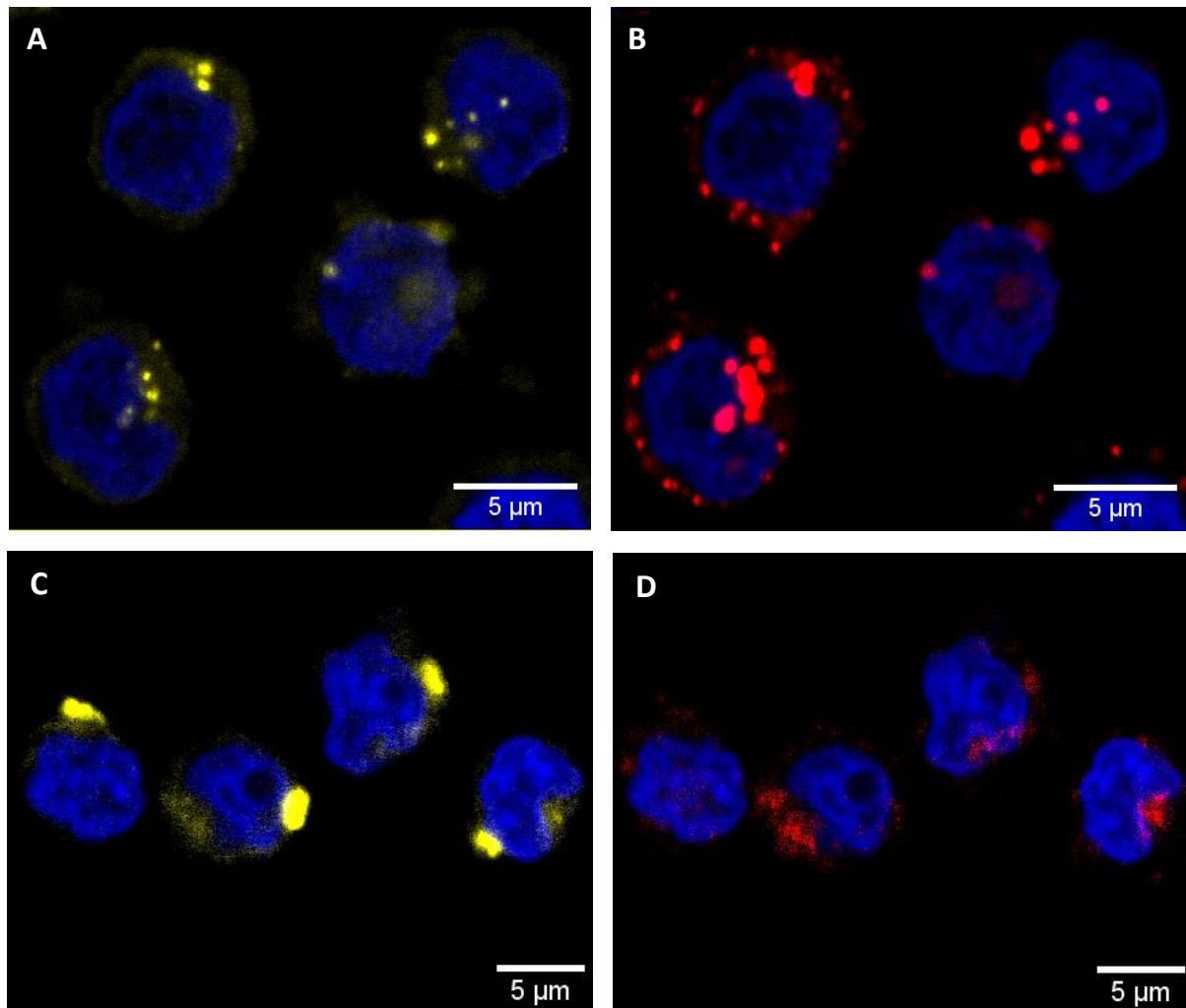


Figure 9.9 100x magnification and 2x digital zoom of **fixed Jurkat** cells, stained with DAPI (blue channel, all), IgD-AF546 (yellow channel, left), and anti-LAMP1-AF647 (red channel, right), **without (A, B), or with (C, D) exogenous galectin-9 addition**. Note how the nuclei take up a large portion of intracellular space in this cell type. **A, B** – most of the IgD probe seems to be uniformly dispersed around the cell membrane, indicated by the yellow halos around the large nuclei, while some small punctae of IgD accumulation are also visible. These once again localize to where concentrated LAMP1 signal is visible, however perinuclear accumulation, as seen before, is not observed here, and instead IgD-LAMP1 punctae are closer to cell surfaces (signal seen at the same place as DAPI most likely coming from membrane above nuclei). **C, D** – with galectin-9 addition, the IgD probe seems to be grouped into specific loci on the cell surface, with some of it also dispersed around the cell membrane. Notably, these punctae of IgD accumulations in this case do not colocalize with the dispersed LAMP1 signal.

Fixed cell staining of KU812 yielded no conclusive results, as recorded images were of very low quality and staining protocol could not be further optimized due to time limitations at the end of the project.

Overall, fluorescence microscopy qualitatively confirmed IgD internalization in the pre-basophilic KU812 and Jurkat T cell lines, via clathrin-mediated endocytosis, as evident by the dynamin inhibitor experiments. This process is CD44-independent, at least in Jurkat cells. Conversely, with exogenous galectin-9 addition, IgD stuck to the surface of both cell lines, with no observable internalization, which is once again CD44-independent, with regards to Jurkat cells. The intracellular fate of internalized IgD still remains unclear, as following endocytosis and accumulation into early endosomes, as evidenced by the anti-EEA1 probe, IgD seems to show up in both the recycling endosomal (Rab11 probe) and the endo-lysosomal (LAMP1 probe) compartments. This may not be as conflicting as it sounds, as LAMP1-labelled organelles have been shown to not necessarily exhibit lysosomal characteristics (Cheng et al. 2018), and that LAMP1 has previously been found in other cellular locations, such as the perinuclear space (Baba et al. 2020), raising questions regarding the validity of LAMP1 as an exclusively lysosomal marker. Either way, IgD signal, after internalization, was seen to mostly accumulate in the perinuclear space.

The few experiments with labelled murine IgE staining of Jurkat cells showed that it also accumulates in the same perinuclear location after being endocytosed, as evident by the EEA1 probe colocalization. This could mean that IgD endocytosis in this cell line is not specific and applies to at least one other class of antibody, however work on the matter remains inconclusive. Interestingly, the effect of antibody retention to the cell surface by gal-9 addition was not observed for the IgE probe, despite IgE and gal-9 being known binding partners.

Fixed cell staining showed IgD still colocalizing with LAMP1 signal punctae, despite the dispersed cell surface presence of both, which could mean there is an IgD binding partner within LAMP1-positive membrane compartments (not necessarily lysosomes). With gal-9 addition, accumulation of IgD probe signal were evident on cell surfaces, which does not colocalize with LAMP1, indicating that gal-9 potentially targets IgD to other surface proteins. The accumulation of signal in such a manner is somewhat peculiar, since all proteins on the membrane should be cross-linked, limiting their lateral movement.

10. Pulldowns and mass spectrometry results

Following gel electrophoresis of the microsome-enriched cell lysate samples, as described in the methods section, the most prominent protein bands were excised, presuming they would contain the best/most IgD binders, and sent for LC/MS analysis. The first attempt was to lyse cells after IgD incubation and employed no microsomal enrichment. The resulting SDS-PAGE showed that very little protein was pulled down with this method (**Figure 10.1**), so I switched to the alternative method of lysing cells prior to IgD incubation, followed by microsomal enrichment, which yielded many distinct protein bands pulled down by IgD (**Figure 10.2**). The resulting protein bands were the same after 0.2 mM BS3 crosslinker (Thermo) in DMSO addition to cell lysates incubated with IgD. Lack of difference between these samples could indicate that the cross-linker concentration used was too low, but due to time restrictions it was not further optimized. Lysates with added gal-9 were not subjected to microsomal enrichment, as IgD in those samples localized to outer cell membrane, as shown previously.

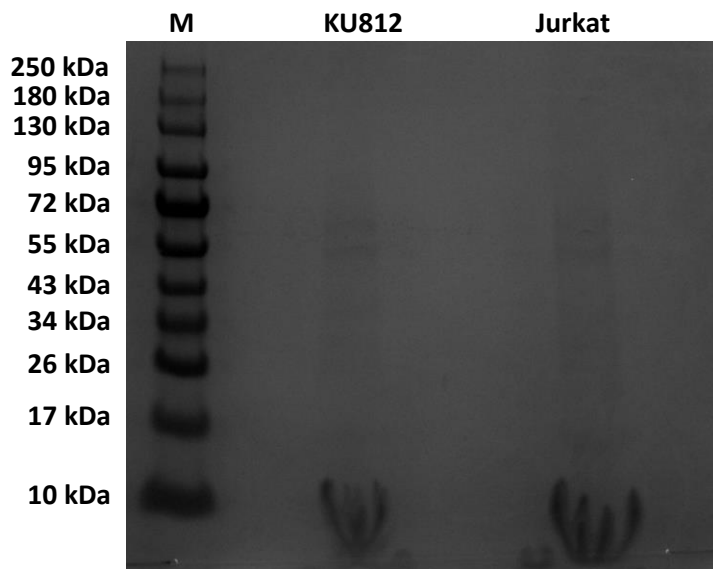


Figure 10.1 SDS-PAGE of Phl p 7-Sepharose-mediated pulldown of cell lysate from KU812 and Jurkat cells, which were previously incubated with IgD, without microsomal enrichment. Very faint bands above the 55 kDa mark are visible, most likely corresponding to the 62 kDa IgD heavy chain. Large swathes at the bottom of the gel possibly from Phl p 7 broken off the Sepharose. 4-20% gradient gel, marker – color prestained protein standard, broad range (10-250 kDa) (NEB). Viewed with Gel Doc XR+ (Bio-Rad).

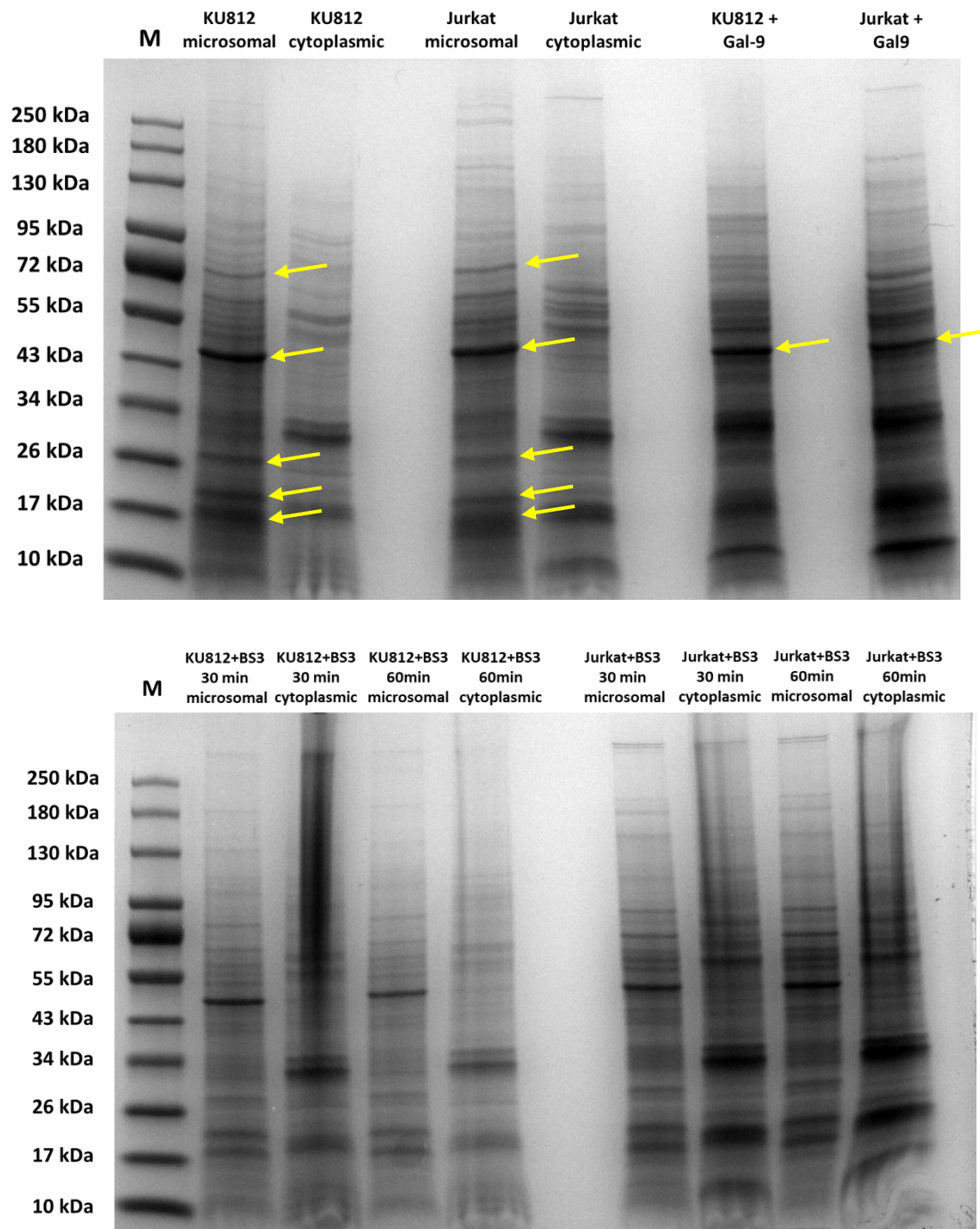


Figure 10.2 SDS-PAGE of microsomal and cytoplasmic fractions of proteins pulled down by Phl p 7—Sepharose from IgD-treated cell lysate from KU812 and Jurkat cells. Lysates with added gal-9 were not subjected to microsomal enrichment. Prominent bands present across microsomal fraction samples, marked with yellow arrows, were excised by scalpel and sent for LC/MS analysis. Incubation with 0.2 mM BS3 crosslinker for 30 min or 1 hour (**bottom**) yielded no change in corresponding protein bands. 4-20% gradient gel, marker – color prestained protein standard, broad range (10-250 kDa) (NEB). Gel imaged with Gel Doc XR+ (Bio Rad).

A total of 12 protein bands were sent for mass spectrometry analysis – the prominent 17, 20, 27, 45, and 72 kDa bands of the microsomal samples of KU812 and Jurkat lysates, as well as the 45 kDa bands from the gal-9-treated lysates. These bands were chosen with the presumption that they carry the most amount of IgD-pulled down proteins. **A total of 2562 proteins were identified** from peptide sequencing of the twelve excised gel bands following database searching and were aligned in the Scaffold data file. Inspection of the data in this format identifies proteins in gel bands that are outside of the expected molecular weight for that protein. Manual inspection shows several reasons why this occurs including truncated protein sequences and proteins identified that have been retained at a higher weight due to inefficient resolution or trapping within the gel matrix. Thresholds were set at 99% protein identification and 95% peptide identification probability and a minimum of 3 peptides present across samples, which resulted in 1248 individual proteins. They were then ranked according to most amount of exclusive unique spectral matches present across samples. Comparing corresponding bands from the two cell type lysates reveals a large degree of overlap, but also many unique proteins present in the samples (**Figure 8.3**).

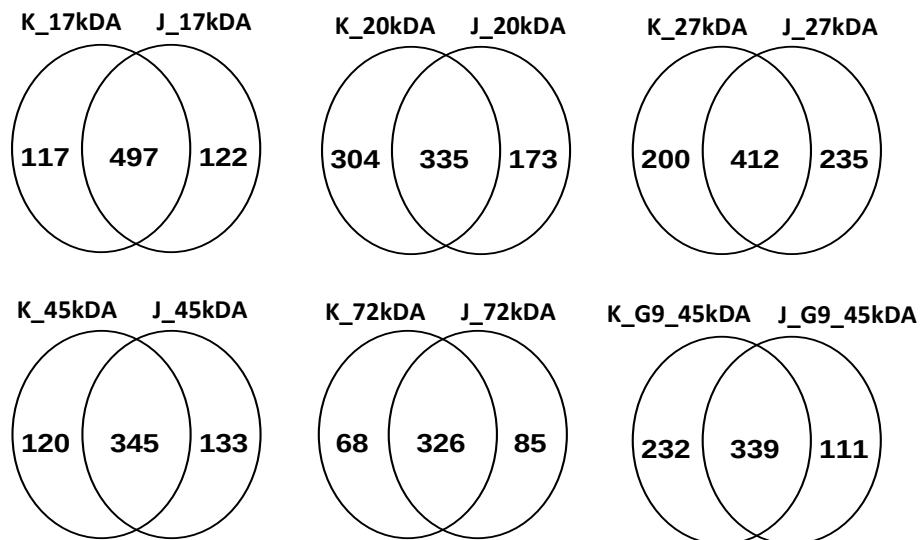


Figure 10.3 Venn diagrams generated by Scaffold 5 (Proteome Software), showing the degree of overlap between proteins of corresponding MW bands of lysates from the KU812 (K) and Jurkat (J) cells. Overall, the shared proteins between the samples were more than the unique proteins for each cell type, as would be expected for human leukocytes.

When proteins are ranked by number of exclusive unique spectrum count, it is immediately evident that the samples are unfortunately dominated by the presence of human actin, keratin, and tubulin clusters (**Figure 10.4**), which are most likely signs of contamination with skin proteins from dust, which must have happened during gel band excision and handling. Many spectra of other intracellular proteins were also present in all samples, such as heat shock proteins, ribosomal proteins, and metabolic enzymes, indicating that the IgD-pulldowns were not at all as specific as I had hoped and that the method needs further optimization, or at least more wash steps for the Phl p 7-Sepharose resin.

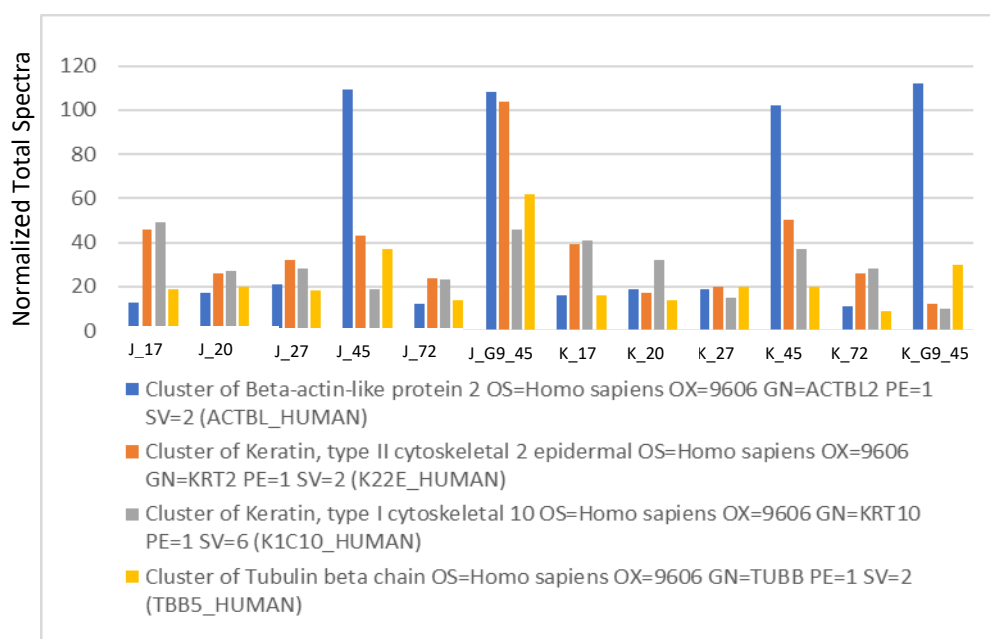


Figure 10.4 Bar chart of Scaffold 5 data for the four most represented protein clusters by spectral count across all samples. The massive presence of keratin clusters most likely indicates contamination with skin particles from dust. J – Jurkat; K – KU812; G9 – Galectin-9. Figure generated with Microsoft Excel.

Despite the discouragingly large amount of non-relevant proteins and the keratin contamination present in the samples, some sense could still be made from the dataset. First, I examined IgD spectra to confirm its presence in the samples. As expected, IgD heavy chain was present in all samples, while the lambda light chain was present in the lower MW bands (**Figure 10.5**), confirming the IgD pulldown.

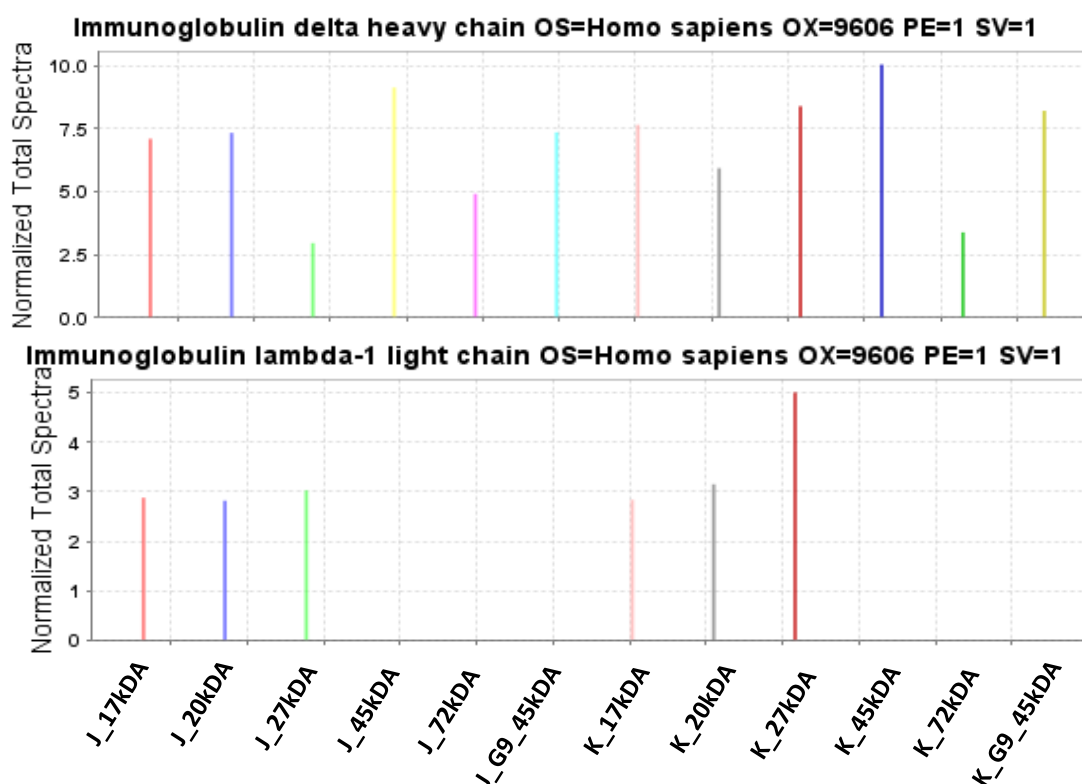


Figure 10.5 Normalized total peptide mass spectra for human IgD δ heavy chain (top) and Ig λ light chain (bottom). IgD is present in all samples, confirming that it was captured by the PhI p 7 resin. Figures generated with Scaffold 5 (Proteome Software). J – Jurkat; K – KU812; G9 – Galectin-9.

Next, I examined the presence of proteins suggested by the microscopy experiments to colocalize with IgD. Even after considerably lowering the stringency for peptide presence and certainty, only Rab11 convincingly appeared to be in the samples (**Figure 10.6**). EEA1 and LAMP1 were not present even at lower protein and peptide identification stringency (although LAMP2 was barely detectable in only the gal-9 samples). Other proteins associated with recycling endosomes were also searched, of which the transferrin receptor-1, or CD71, occurred in samples. Proteins associated with endocytosis also appear at lower stringency, such as clathrin light chain, myosin light chain cluster, and dynein heavy and light chains (not shown). CD44 was also checked, but only appeared in KU812 samples at very low stringency with a very low signal, potentially indicating lack of involvement (**Figure 10.6**). When proteins only come up at lower stringency settings, the quality of their peptide spectra was manually checked.

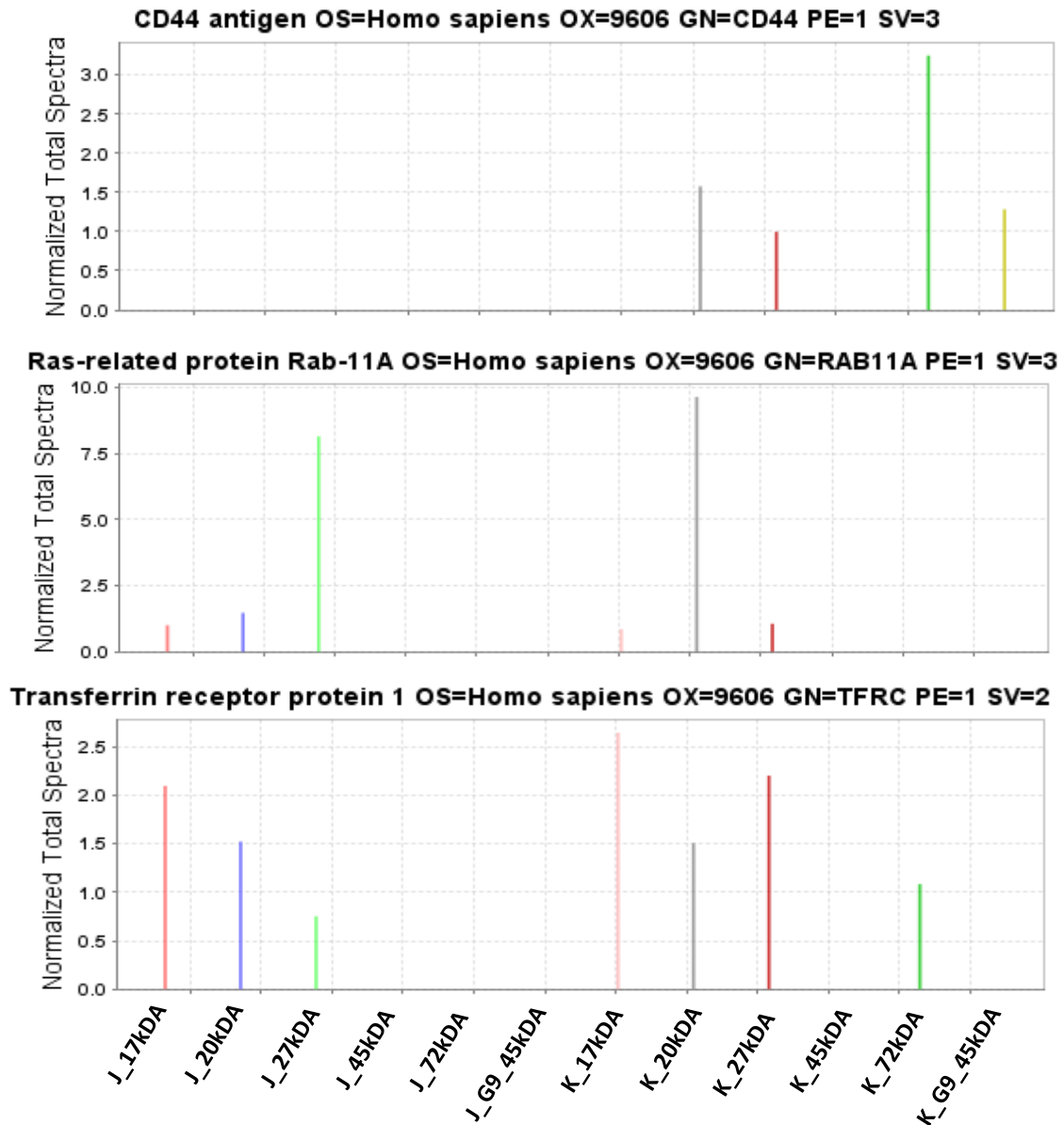


Figure 10.6 Normalized total peptide spectra corresponding to CD44 (top), Rab11a (mid), and CD71 (bottom). As expected, CD44 only comes up in KU812 samples, albeit in small quantities at the lowest stringency criteria, perhaps indicating lack of direct involvement with IgD. Peptides from the recycling endosomal proteins Rab11a and CD71 show up in much greater quantities across samples (especially Rab11a), possibly confirming the association of IgD with this cellular compartment. Figures generated with Scaffold 5 (Proteome Software). J – Jurkat; K – KU812; G9 – Galectin-9.

The reported molecular weight of CD44, Rab11a and CD71 is 82 kDa, 24 kDa, and 90 kDa respectively, which is close to the MW of samples they were identified in, bar CD71.

The next step of my analysis was to cross-reference samples of the same cell origin and molecular weight, differing in galectin-9 addition, specifically looking for any lectins or cell surface glycoproteins with carbohydrate binding capabilities, as the oligosaccharide component of IgD is necessary for its receptor binding (Rudd et al. 1994). For this search the highest stringency criteria were applied. The cross-reference and targeted glycoprotein search yielded just two proteins with relatively convincing differential presence across samples untreated or treated with galectin-9 – Clec11a and CD82, as well as another glycoprotein present only in untreated samples - CD59 (**Figure 10.7**). Clec11a (C-type lectin domain family 11 member A), also known as osteolactin, is a 40 or 47 kDa (depending on isoform) secreted glycoprotein involved in osteoblast growth and differentiation (Wang et al. 2020). CD82, is a 30-34 kDa member of the tetraspanin family of transmembrane glycoproteins and has been ascribed tumour suppressor functions. It is expressed on both intracellular membranes and on the cell surface and has roles in cell adhesion, motility, and protein trafficking (Yan et al. 2021). CD59, or protectin, is a 14-20 kDa (depending on isoform) secreted, or cell-surface GPI-linked glycoprotein which is essential for negative control over the membrane attack complex formation during complement activation (Davies and Lachmann 1993), known to be internalized following engagement with bacterial inhibitor toxins (Cai et al. 2014). Generally, their reported MW roughly matched the estimated MW of the protein bands, bar CD59. Clec11a is an extracellular secreted protein, so it is ruled out as a receptor candidate, although small lectins are generally known for their antibody-binding capabilities and it may have a role similar to gal-9. Interestingly, CD82 was found only in the gal-9-treated samples and in much more convincing quantity and quality than CD44. CD59 is a surface protein with a role in native immunity, is inhibited by bacteria in order for them to evade complement attack and is known to be endocytosed when engaged by ligand, which fits both the internalization and microbial surveillance themes for IgD. However, due to the relatively poor overall quality of the samples due to lack of optimization and contamination, the putative involvement of these proteins remains an informed proposal, rather than a concrete finding and will be further discussed later.

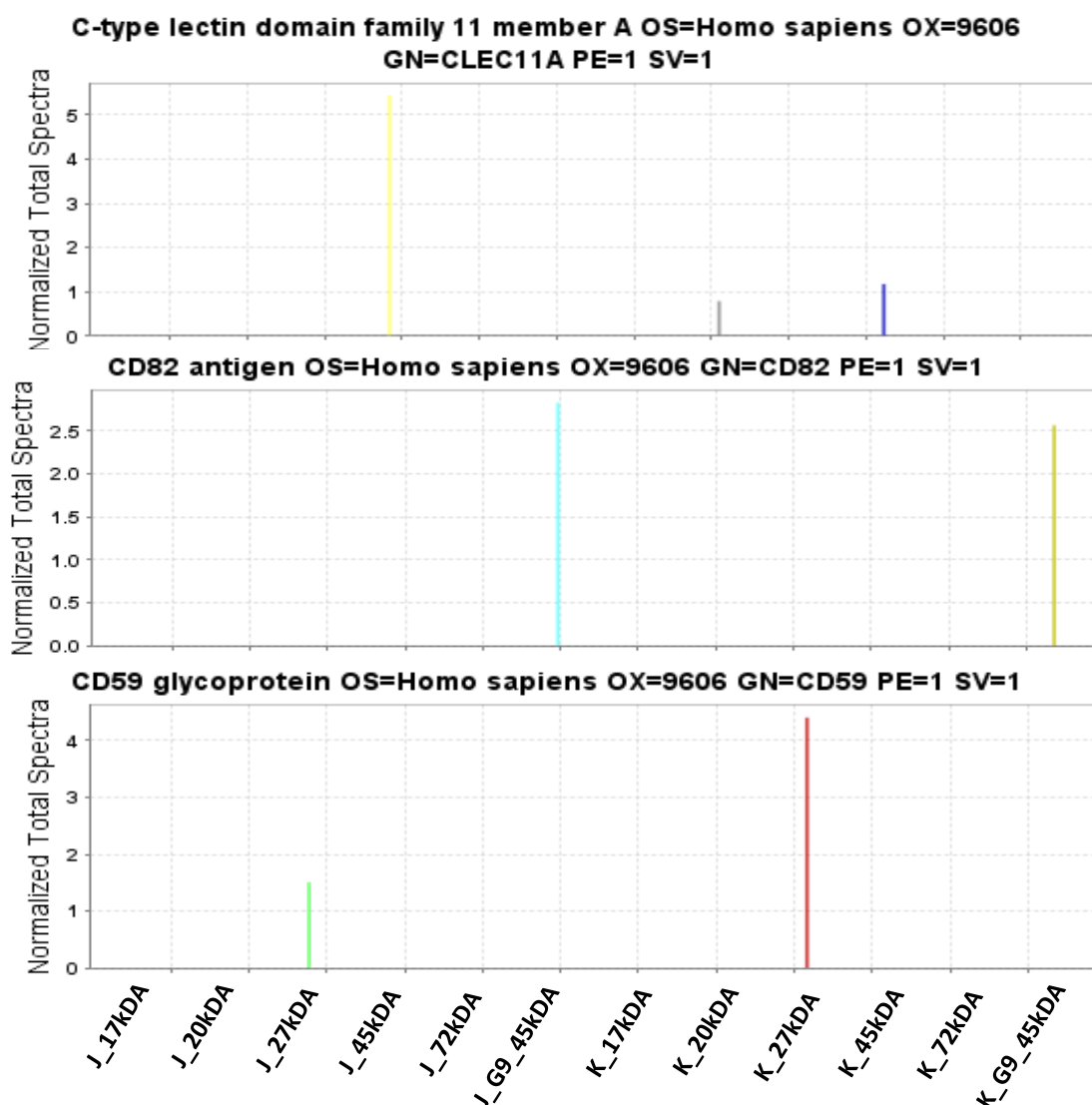


Figure 10.7 Normalized total peptide spectra corresponding to the three glycoproteins with differential presence in samples, remaining in the highest stringency settings – Clec11a, CD82 and CD59. Interestingly, CD82 is present in the gal-9-treated samples from both cell lines and missing from the corresponding MW sample without gal-9, making it a viable gal-9-binding candidate. Conversely, Clec11a shows up in the Jurkat gal-untreated sample and not in the corresponding gal-9 sample. CD59 is present in the 27 kDa bands of both cell lines. Figures generated with Scaffold 5 (Proteome Software). J – Jurkat; K – KU812; G9 – Galectin-9.

Overall, the analysis identified both heavy and light chains of IgD across samples, the presence of recycling endosome markers Rab11a and CD71 in samples without gal-9, and **two putative IgD-binding cell surface glycoproteins with scores higher than CD44 – CD59 in untreated, and CD82 in gal-9 treated samples.**

Three titular protein constructs, three orthogonal experimental setups, and over three years later, I have managed to prove with certainty that IgD is internalized within pre-basophilic and T cell lines via clathrin-mediated endocytosis, and that this process is inhibited by galectin-9, which locks IgD to the surface in a CD44-independent manner, contradictory to some of the findings of Shan and colleagues. What is hinted at by my data, but remains to be concretely confirmed, is that IgD, when endocytosed, localizes to the perinuclear region in what is most likely recycling endosomes. IgD pulldowns and mass spectroscopy analysis were on the one hand suboptimal, incomplete due to time restrictions, samples were overcrowded and contaminated with intracellular and *ex vitro* proteins, but on the other hand still useful and informative with regards to the putative IgD interactome. In the end, albeit with some reserves, I am inclined by the data to propose two different candidate proteins for IgDR, which from my analysis seem to be more plausible than CD44 – CD59 when no galectin-9 is present, and CD82 when galectin-9 is present - because of their presence in samples, cell surface localization, and thematic relations, which will be discussed in the next section, as well as the implications of my findings.

11. Discussion

Despite the relative lack of experimental optimization and late streamlining of my project, I have managed to discover several previously unknown facts about IgD, which uncover new avenues for further research into IgD effector functions. First, I have shown in this thesis, with a fair amount of certainty, that IgD is endocytosed by pre-basophils and T cells in homeostasis, and that this process is abrogated by elevated amounts of galectin-9, which are known to occur in an inflammatory context. Galectin-9 does indeed interact with IgD, as shown by others, by retaining it on the cell surface in a CD44-independent manner, which may be necessary for its biological function in Th2-driven inflammation. To the best of my knowledge, IgD's internalization by cells has been proven here for the first time. The CD44-independency seems in conflict with the findings of Shan et al. about its involvement in IgD-mediated responses, but it is very plausible that galectin-9 interacts with different cell surface receptors depending on cell type, due to its carbohydrate binding promiscuity, and also given the vastly different functions of basophils and T cells. It is of note that Shan et al. used CD44 knockdown via siRNA in KU812 cells, leading to about a 2-fold decrease in IgD binding (Shan et al. 2018), but these findings need to be revisited and corroborated. It is still very much possible that CD44 is the surface molecule targeted by gal-9 in KU812 cells, as the two proteins are known binding partners, but CD44 is definitely not expressed by Jurkat cells, yet they display the same effect of IgD surface retention by galectin-9. One could argue that if CD44 was in fact a receptor for IgD, a prominent band at above 70 kDa would have been present in the KU812 gal-9 treated cell lysates and not in other samples, which was not the case (Figure 6.13). Traces of CD44 peptides were spotted by mass spectrometry, but were of negligible quantity and quality, compared to the other observed glycoproteins. If we accept that CD44 is a receptor for the IgD-gal-9 complex on KU812 cells, then that raises the pivotal question of what the receptor for the IgD-gal-9 complex on Jurkat T cells would be. It is important to note that CD44 engagement by galectin-9 has previously been shown to activate cell signaling

through the SMAD family of transcription factors (Tanikawa et al. 2010), which normally trigger proliferation or apoptosis in response to stimuli such as TGF- β (Walton, Johnson, and Harrison 2017). Conversely, production of the Th2 cytokines IL-4 and IL-13 is under the control of the STAT6 and GATA3 transcription factors (Stark, Tibbitt, and Coquet 2019), which questions the direct involvement of CD44 in Th2 cytokine production. It is of course plausible that CD44 may be linked to different signalling systems depending on cell type, and its study needs further elucidation.

Since one of the first discovered pieces of information on IgDR showed that carbohydrate binding is necessary for the capture of IgD by T cells (Swenson et al. 1998), the targets of my proteomics search following mass spectrometry data analysis were lectins and glycoproteins. Analysis of those data does need to be taken with a lot of caution due to the contamination of samples and low optimization of sample preparation, but still yields some potential targets for IgD on the examined cell lines. Cross-referencing proteins present in different samples showed only three glycoproteins differing in expression at the most stringent selection criteria. Notably, CD44 spectra were not highlighted at all with this stringency. Of the three, two are cell surface proteins – CD59 and CD82, while Clec11a is a secreted lectin with similar size to galectin-9. Knowledge of the functions of these proteins, however, is quite limited and no literary evidence suggests any direct involvement in antibody responses. The least studied of these proteins is Clec11a, which has only been shown to function as a differentiation stimulator for bone cells (Wang et al. 2020). The property of CD59 to downregulate complement-induced membrane attack complex formation on host cell surfaces (Davies and Lachmann 1993), which is a humoral immune process normally leading to bacterial permeabilization and death, as well as the observation that this receptor is internalized and degraded upon ligation by bacterial toxins (Cai et al. 2014), make it a viable IgD-binding target in conditions without galectin-9 elevation. Quite intriguingly, one study has shown that knocking down galectin-3 expression in HeLa cells resulted in increased specific uptake of cell surface CD59 (Mathew and Donaldson 2018), which is reminiscent of the galectin-9-mediated effect on IgD cell surface retention exhibited by my results,

although in this case the researchers were examining clathrin-independent endocytosis. This modulatory role for CD59 with regards to host-microbiome interactions and its ability to be endocytosed fit well with IgD's role in bacterial surveillance and the observations derived from my microscopy data. CD59 tissue distribution seems to be across most cell types, but among the highest-expressing CD59 tissues are tongue and salivary glands (protein atlas), which is also where soluble IgD can be found. The other surface glycoprotein – CD82 – interestingly shows up in just the gal-9 treated samples, with convincing presence. So far, CD82 has mostly been studied in the context of cancer metastasis by virtue of its role in cell adhesion (Yan et al. 2021), although some evidence suggests it has at least a minor role in immunity. The tetraspanin superfamily of transmembrane proteins, of which CD82 is a member, have been described as regulators of membrane microdomain organization in the immunological synapse formation between T cells and antigen-presenting cells by directing recruitment of MHC class II molecules, integrins, and other co-receptor molecules (Rocha-Perugini, Sánchez-Madrid, and del Hoyo 2015). CD82 in particular has been shown to be recruited to phagosomes with internalized microbes, prior to their lysosomal fusion in dendritic cells (Artavanis-Tsakonas et al. 2011), and to also be associated with TLR9 receptor complex modulation in macrophages (Khan et al. 2019). Levels of CD82 are upregulated in fibroblasts in the context of rheumatoid arthritis, which decreases cell migration, invasion, and adhesion (Neumann et al. 2018). Most importantly, ligation of CD82, along with two other tetraspanins, by the CD28 co-stimulatory molecule on T cells leads to their activation and differentiation into Th2-type IL-4, IL-5, and IL-13-secreting cells through the STAT6/GATA3 signalling pathway (Serra et al. 2008). Thus, CD82 has roles in both adaptive immunity as an antigen presentation and Th2 differentiation regulator, and in native immunity, as a TLR-signalling regulator. No direct binding of CD82 to lectins or other glycoproteins has been proven, however it has been shown to be necessary for the microdomain clustering of the Dectin-1 protein, which is a receptor for fungal cell wall carbohydrates (Tam et al. 2019), and that its expression correlates with that of galectin-3 (Wu et al. 2013). The cell surface microdomain organizational properties of CD82 may

bode well for its potential interaction with, and cell surface retention of, the IgD-gal-9 complex. CD82 also has above normal tissues expression in salivary glands and lungs (protein atlas). Particularly, it could explain the signal bunching of the IgD fluorescent probe in the gal-9 treated Jurkat cells (Figure 6.11).

If we suppose that IgD binds to CD59 in the absence of galectin-9, and to CD82 in its presence, that could have some very interesting potential implications. If IgD is bound by cells in homeostasis, aberrant activation of effector function can be prevented through its endocytosis via CD59 engagement. Conversely, inferring from the individual proteins' known utilization by microbial evasion strategies, IgD-CD59 complex engagement, and its resulting internalization, could be a way for pore-forming bacteria to enter cells unnoticed. On the other hand, in the context of inflammation and resulting galectin-9 elevation, CD82 may organize cell-surface IgD, captured via gal-9 or other carbohydrate-binding adaptors, into distinct microdomains, which would improve CD82-mediated signalling into the STAT6/GATA3 pathway, resulting in Th2 cytokine secretion by the effector cell (Serra et al. 2008). So far it is yet to be proven whether the internalization of IgD is specific, as the few experiments with the fluorescent IgE probe exhibited similar intracellular localization (Figure 6.11). However, the galectin-9 effect of surface retention was only observable with IgD and not IgE.

Another interesting finding in my research, which warrants further exploration, is the localization and accumulation of IgD-bearing endocytic vesicles to the perinuclear regions of cells. This process may not be specific to IgD, as shown by the fluorescent IgE probe localizing to the same areas. IgD seemed to colocalize both with the Rab11 recycling endosome marker and the LAMP1 lysosomal marker, although the latter has been shown to also stain endocytic vesicles other than lysosomes (Cheng et al. 2018). The perinuclear and relatively central location of IgD probe accumulation is reminiscent of the endocytic recycling compartment (ERC) – a system of perinuclear tubulovesicular membranes, situated next to the microtubule organization centre for ease of vesicular transport along microtubules (Xie et al. 2016). ERC is a sorting station for the 'slow' recycling of cargo, past the sorting endosome level normally leading to

immediate recycling (Naslavsky and Caplan 2018). Surface receptors often need to be recycled back to the cell surface in outbound exosomes following dissociation with their ligands in endosomes (Huotari and Helenius 2011). The functional significance of slower recycling through the ERC, as opposed to faster recycling occurring closer to the cell surface, remains to be fully elucidated. In general, the involvement of the endosomal recycling system in inflammation has not been properly investigated thus far. Appearance of the recycling endosome markers Rab11a and CD71 (transferrin receptor) mass spectra from the gal-9-untreated cell lysates complements the observed colocalization of IgD with Rab11 to perinuclear regions, observed with confocal microscopy. Therefore, the assumption that IgD is going through the ‘long’ recycling endocytic pathway in homeostasis in certain cell types would not be so far-fetched. However, this process may not be specific to IgD. It remains to be seen what happens to the IgD probe after longer than 1 hour incubation times – whether it is released back to the surface, with or without external stimuli, or whether it ends up being degraded, which also needs to be tested for other antibody isotypes. The implications for such immunoglobulin retention and potential recycling to the cell surface remain unclear. A bold conjecture would be that there may exist a process whereby immunoglobulins of certain classes can be stored short-term intracellularly in primed, but not activated cells, to be exported and utilized when given the proper inflammatory signaling milieu. This would aid in preventing proteolytic digestion of IgD by extracellular proteases, enhancing its otherwise short half-life, ensuring that locally produced IgD would not be wasted. Another explanation would be that there may be a system in place for recycling of antibodies following antibody: antigen complex internalization and separation, instead of the common route of degradation known for IgG-Fc receptors (Bournazos, Gupta, and Ravetch 2020). The existence of intracellular IgG-Fc receptors (Foss et al. 2015) also point to a plausible way of utilizing IgD internalization. Alternatively, this internalization could be a remnant of the existing transcytosis systems for IgA and IgM via the polymeric Fc receptor (Wei and Wang 2021), or for IgG via the neonatal Fc receptor (Roopenian and

Akilesh 2007), potentially explaining the presence of IgD in fetal amniotic fluid and abundance at mucosal sites.

The completely different localization of the IgD probe when cells were exposed to galectin-9 not only betrays the notion that different cell surface molecules are involved in IgD binding in different contexts, but also further cements galectin-9 as a regulator of inflammation. Carbohydrate recognition may have evolved early in the evolution of the eukaryotic immune system, as it is necessary for recognition of pathogen-associated surface molecules in most multicellular animals. This ancient but proven defence mechanism may have been merged and revamped by the adaptive immune system in the form of antibody glycosylations being recognized by various host adaptor lectins with differential expression depending on inflammatory context. The glycations on cell surface receptors and antibodies, as well as their associated carbohydrate-binding proteins, collectively referred to as the glycome, may differ vastly depending on environmental stimuli (Reily et al. 2019). Thus, modulation of the glycome is a significant, but rather underexplored level of inflammation control.

Much work remains to be added on by future IgD researchers in order to help ground such conjectures with further evidence, especially with regard to underexplored due to time limitations avenues of this project. The immediate miss currently is experimental setups with cognate IgD antigen to determine whether internalization would still occur. Interaction of anti-Phl p 7 IgD with its cognate antigen may drastically change IgD's cell-binding capabilities, as Fab engagement may either further obscure or unmask the Fc for receptor binding. The opposite could also be true – binding of IgD to a cell surface receptor may make it more readily able to bind antigen. This would have to be tested with both incubation with antibody: antigen complexes, and addition of antigen following incubation with antibody alone, with or without galectin-9. The same would have to also be done with the MID superantigen and it would be interesting to see whether its effects would differ from those of Phl p 7. Through SPR I have confirmed that MID superantigen is able to bind Phl p 7-immobilized IgD, but not whether Phl p 7 can bind MID-

immobilized IgD, and whether affinities for one or the other would differ. This could have potential implications in mucosal immunity, as it would mean that the presence of *Moraxella Catarrhalis* may either enhance or decrease IgD's affinity for antigen, or vice versa. To add another layer of complexity, presence of either superantigen or allergen could impact IgD affinity for IgDR. Extensive research needs to be done to corroborate such logical proposals, which needs to be backed up not only by SPR, but also with crystal structures of different IgD complexes to infer directly any structural flexibility exhibited between them.

Confocal microscopy with more endosomal probes is probably warranted, especially for other recycling endosome markers. Tests need to be done with other classes of fluorescently labelled immunoglobulins, such as IgA and IgM. Longer timelapses with weaker laser strength or more bleaching-resistant dyes could answer the question whether IgD eventually becomes recycled to the cell surface or instead becomes degraded, how long is it being stored for, and whether stimulation of intracellular IgD-laden cells would lead to an observable change in the antibody's localization.

Further optimized IgD pulldowns need to be performed, more gel bands analyzed, and higher cross-linker concentrations tested. With proper handling and more Sepharose resin washes, results will most likely look cleaner with a higher signal-to-noise ratio. Incubation with Phl p 7: IgD and MID: IgD complexes prior to pulldown should also be examined for differences to control samples.

Preliminary research done by Balraj Sandhar has shown that primary basophils are able to respond to Phl p 7 stimulation following anti-Phl p 7 IgD incubation, resulting in the release of IL-4 and IL-13. These results need to also be confirmed in the cell lines used in this project and corroborated through other orthogonal experiments such as qPCR to examine changes in cytokine gene expression. The same experimental setup needs to also be done with the MID superantigen and potential differences from the Phl p 7 response investigated.

Another further step would be to validate the candidacy of CD59 and CD82 as surface receptor molecules for IgD by targeted si-RNA knockdown in the KU812 and Jurkat cell lines, which would be confirmed with qPCR and western blotting. It would be interesting to see whether downregulating these molecules would ablate IgD internalization or surface retention by gal-9 respectively.

The importance of IgD glycosylations and interactions with other carbohydrate-binding proteins also warrants years of work. There could potentially be other small lectin adaptors for IgD which could target it to different receptors and concomitant fate, depending on inflammatory context, not to mention other cell surface and extracellular large polysaccharides, known to be involved in cytokine and chemokine sequestration (Zhang 2010). Additionally, the glycosylations on IgD itself may change depending on external factors, as is the case for other glycoproteins during inflammation (Reily et al. 2019).

Interestingly, despite mast cells being said to be IgD effector cells (Gutzeit, Chen, and Cerutti 2018), there is only a single piece of evidence for the mastocytoid cell lines HMC-1 and LAD2 binding to IgD (Chen et al. 2009). These cell lines and their responses to IgD and antigen/superantigen would be a next logical step in IgD research. Another good candidate cell type for future research would be neutrophils.

More complex experimental setups need to be employed in order to produce a clearer image of IgD's overall function in the development of atopy, as per the previously described model (Figure 3.2). A good first step towards that would be experiments with co-cultures of B cells and basophils or mast cells, in which one could try and recreate the putative progression from bacterial superantigen or allergen stimulation of membrane IgD to soluble IgD-mediated responses in effector cells. The reported IgD-mediated IgE-response attenuation (Shan et al. 2018) should be further investigated, as it could have great implications for the maintenance of responsiveness without activation (degranulation) of basophils. The potential intracellular sequestration of IgD reported here could add to the notion of cells being kept primed to an antigen, without producing a full-on response.

If said future experiments are successful in confirming such a pivotal immunoregulatory role for IgD, very real pharmaceutical implications would ensue. For one, interrupting the interaction of superantigens with IgD through small molecule inhibitors could lead to attenuation of the 'allergic march' and thus ameliorating the severity of atopic diseases. Elevated soluble IgD is present in other autoimmune conditions such as rheumatoid arthritis and blocking the interaction with its effector cells by inhibiting IgDR could prove beneficial in terms of disease severity. Much remains to be learned about IgD and hopefully its potential use as a drug target in autoinflammatory and atopic conditions will boost research efforts in the next few decades.

The findings presented in this thesis, however minute in the grand scheme of past and future IgD research, have nevertheless produced some key knowledge with regards to how the effector functions of IgD may be varied in different inflammatory contexts. In summary, I would say that my research has raised a lot more questions than it has answered, which would hopefully in the future open new avenues for research into the ever-increasing complexity of Immunoglobulin D.

Acknowledgements

I would like to first and foremost thank my supervisors Andrew Beavil and James McDonnell for their guidance, support, and patience throughout the project. The same extends to the rest of my thesis committee – Brian Sutton, Paul Lavender, and James Pease. I am thankful for the funding by the Asthma UK Centre in Allergic Mechanisms of Asthma and the enormous opportunity I was provided with. Many thanks to Rebecca Beavil, Balraj Sandhar, and Gintare Bucaite for providing the vector constructs and for invaluable advice in protein production methods. Thanks to Kristian Riesbeck for providing the correct MID plasmid construct, and to Fu-Tong Liu for providing some of the recombinant galectin-9. Analysis of microscopy and mass spectrometry data wouldn't have been possible without the help and advice from Isma Ali and Steven Lynham respectively. Thanks to Grzegorz Woszczek for the LAD2 cell line and Paul

Lavender for Jurkat cell line, and for the access to their flow cytometer. Special thanks to Danail Stoilov for working on the Python scripts for SPR data analysis. Last, but not least, I would like to thank my partner, my friends, and my family for their unending support, and finally, thank you, the reader, for your attention.

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