DECODING THE IMMUNE PUZZLE: THE ROLE OF ANTIGEN PRESENTING CELLS IN FOOD ALLERGIES

The development of a methodology to analyse HLA-DR presented peptides derived from the glycan-modified allergen BLG in B-lymphocytes by Mass Spectrometry

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DECODING THE IMMUNE PUZZLE: THE ROLE OF ANTIGEN PRESENTING CELLS IN ALLERGIC REACTIONS

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ABSTRACT

Food allergies (FAs) involve abnormal immune responses triggered by specific food antigens. Individuals with FAs can manifest a spectrum of symptoms, spanning from mild to life-threatening, and the absence of curative therapies significantly diminishes their quality of life. A potential innovative therapy includes the targeting of inhibitory Siglec receptors expressed by immune cells (e.g., antigen presenting cells) with glycan-modified allergens to skew the allergic response towards a tolerogenic response. It remains unknown whether glycan-modified allergens are processed and presented differently within Major Histocompatibility Complex II by antigen-presenting cells. This study focused on establishing a mass spectrometry-based methodology for analysing HLA-DR presented peptides derived from glycan-modified beta-lactoglobulin (BLG) in B-lymphocytes. The Epstein–Barr virus (EBV)-immortalized B cell line JY was cultured under varying conditions and HLA-DR-peptide complexes were isolated using immunoprecipitation. These peptides were then separated by Solid Phase Extraction (SPE) and identified using Liquid Chromatography with tandem Mass Spectrometry (LC-MS/MS) and Mascot analysis. Serum-Free Medium (SFM) appeared as an alternative to Fetal Calf Serum (FCS)-supplemented media to eliminate potential interference with bovine proteins during the identification of allergenic bovine protein-derived peptides. Efficient DMP cross-linking of the L243 antibody with Protein A/G magnetic beads enhanced immunoprecipitation efficiency and reduced experimental preparation time. Overall, more than 500 human self-presented peptides were identified, with approximately half matching known sequences in literature, along with the detection of 29 unique bovine peptides, including the BLG-derived peptide VEELKPTPE. While validating the immunoprecipitation protocol for HLA-DR complexes, the study emphasized the need for further optimization in contamination control and sensitivity to advance our understanding of this vital immunological pathway.

Keywords: food allergy, cow's milk allergy, B-lymphocytes, immunopeptidomics, immunoaffinity purification, HLA-DR peptide presentation, mass spectrometry

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LAYMAN'S SUMMARY

A food allergy (FA) is an unusual response of the immune system that happens when certain foods are consumed. Instead of recognizing these foods as harmless, the immune system treats them as threats and reacts to protect the body. Most of the time the component of a certain food that causes an allergic reaction is a protein. For example, in Cow's Milk Allergy (CMA), a common food allergy in children, one of the allergenic proteins is β-lactoglobulin (BLG). Food allergies can cause a range of symptoms, from mild and localized issues like oral and skin symptoms to life-threatening anaphylaxis.

Anaphylaxis is a severe reaction characterized by symptoms like throat and tongue swelling, breathing difficulty, loss of consciousness, low blood pressure, and shock. Immediate treatment, often with an epinephrine auto-injector (EpiPen), is crucial to manage anaphylaxis. Epinephrine helps by opening airways, increasing blood pressure, and countering the effects of the allergic reaction. Unfortunately, there are no curative therapies for food allergies, and people with allergies are typically advised to avoid foods that trigger their allergies. This significantly impacts their quality of life, leading to constant caution, limited dietary choices, emotional stress, and social exclusion.

The immune response in food allergies involves two phases: sensitization and elicitation. In the sensitization phase, allergenic proteins are processed by immune cells called Antigen Presenting Cells (APCs). One of the most important molecules in this pathway is the Major Histocompatibility Complex class II molecule (MHC-II). This molecule is able to present fragments of proteins, called peptides, to other immune cells, eventually leading to the production of allergen-specific antibodies called immunoglobulin E (IgE). In the elicitation phase, re-exposure to the allergen causes the allergen to bind to those specific IgE antibodies and subsequently triggers the release of inflammatory mediators such as histamine, leading to allergic symptoms.

One potential therapy for food allergies is allergen immunotherapy, which gradually exposes patients to increasing doses of allergens. However, this treatment has limitations, including the risk of adverse reactions like anaphylaxis. Another approach involves modifying allergenic proteins with complex sugar molecules called glycans. Some glycans can induce immune tolerance, suppressing allergic reactions. Suppressing Siglecs receptors, which recognize specific glycan patterns, can be targeted to reduce allergies.

This study aims to improve our understanding of the MHC-II pathway and how it presents therapeutic glycopeptides derived from glycan-modified allergens, using BLG as a

model allergen. The research used different cell culture conditions and a technique called immunoprecipitation, to fish out MHC-II molecules with the bound peptides from other cell components by using antibody cross-linked to beads as a bait. Then, mass spectrometry supported the detection of these peptides, and software such as Mascot identified whether they were human or cow-related proteins. This research leads to a better understanding of the allergenic pathway, eventually leading to the development of potential treatments for FAs, and ultimately improving the lives of those who have them.

LIST OF ABBREVIATIONS

Abbreviation	Definition
APC	Antigen-presenting cell
BLG	β-lactoglobulin
BSA	Bovine Serum Albumin
CMA	Cow's Milk Allergy
DBPCFC	Double-blind placebo-controlled food challenge
DCs	Dendritic cells
DMP	Dimethyl-pimelimidate-dihydrochloride
EBV	Epstein-Barr virus
ER	Endoplasmic reticulum
ESI	Electrospray ionization
FA	Food allergy
FCS	Fetal calf serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HLA	Human Leukocyte Antigen
HRP	Horseradish peroxidase
HSPA8	Heat shock cognate 71 kDa protein
IgE	Immunoglobulin E
ILC	Innate lymphoid cell
LC-MS/MS	Liquid Chromatography with tandem Mass Spectrometry
MFI	Mean fluorescence intensity
MHC	Major Histocompatibility Complex
PBS	Phosphate Buffered Saline
PEG	PolyEthylene Glycol
PGK1	Phosphoglycerate kinase 1
Score_BA	Log-scale predicted binding affinity
Score_EL	Eluted ligand prediction score
SCX	Strong cation exchange
SFM	Serum-free medium
SPE	Solid phase extraction

TBS-T	Tris-buffered saline with Tween 20
TCR	T-cell receptor
Th2	T helper 2

INTRODUCTION

What is a food allergy?

Food allergy (FA) is an atypical immune response induced by the ingestion of specific food antigens. The immune system of food allergic individuals mistakenly identifies otherwise harmless food antigens as potential threats resulting in an immune response aimed at protecting the body (1,2).

Accurately, estimating the prevalence of FA in Europe presents a considerable challenge due to the reliance on self-reported data in most studies (3-5). In a 2013 systematic review spanning from 2000 to 2012, Nwaru et al. estimated FA at 3% in adults and 6% in children using double-blind placebo-controlled food challenge (DBPCFC), the gold-standard in diagnosing FAs (3). However, a more recent review spanning from 2012 to 2023 reveals a rise in self-reported FA cases up to 36% in adults and nearly 50% in children) without a corresponding increase in confirmed cases through DBPCFC (4). Even though conclusive data are lacking, the increase in self-reported and confirmed cases underscores the need for further research to improve the quality of life for those who suffer from FA (3-5).

This immune reaction can manifest in a range of symptoms, varying from mild and localized manifestations, such as oral and skin symptoms, to even life-threatening conditions termed anaphylaxis (1,6). Anaphylaxis is characterized by the simultaneous occurrence of various, critical symptoms such as swelling of the throat and tongue, which can impair breathing, loss of consciousness, low blood pressure, and shock. Given the rapid progression of anaphylaxis, immediate management is essential, typically involving the intramuscular administration of epinephrine via an auto-injector (i.e., EpiPen). Upon administration, epinephrine acts on adrenergic receptors, leading to bronchodilation to facilitate improved breathing. Additionally, it induces vasoconstriction and thereby increasing the blood pressure (6).

Despite the life-threatening situations that can arise from a FA, no curative therapies without risk of (severe) adverse effects are currently available. Besides emergency medication, allergic individuals are mostly advised to avoid causative foods resulting in a reduced quality of life compared to non-allergic individuals. The reduced quality of life for individuals with food allergies is a result of the constant need for caution, the limitations on their dietary choices, the emotional impact of living with the risk of severe reactions, and social exclusion (1,7).

Pathophysiology

The atypical immune response triggered by an allergenic protein can be divided into a sensitization and an elicitation phase (Figure 1). The sensitization phase is marked by the initial allergen exposure to antigen-presenting cells (APCs) such as dendritic cells (DCs), macrophages, and B-cells (8,9). These APCs possess the capability to engulf the allergen by endocytosis, subjecting it to enzymatic cleavage by lysosomal proteases, resulting in the generation of allergen-derived peptides.

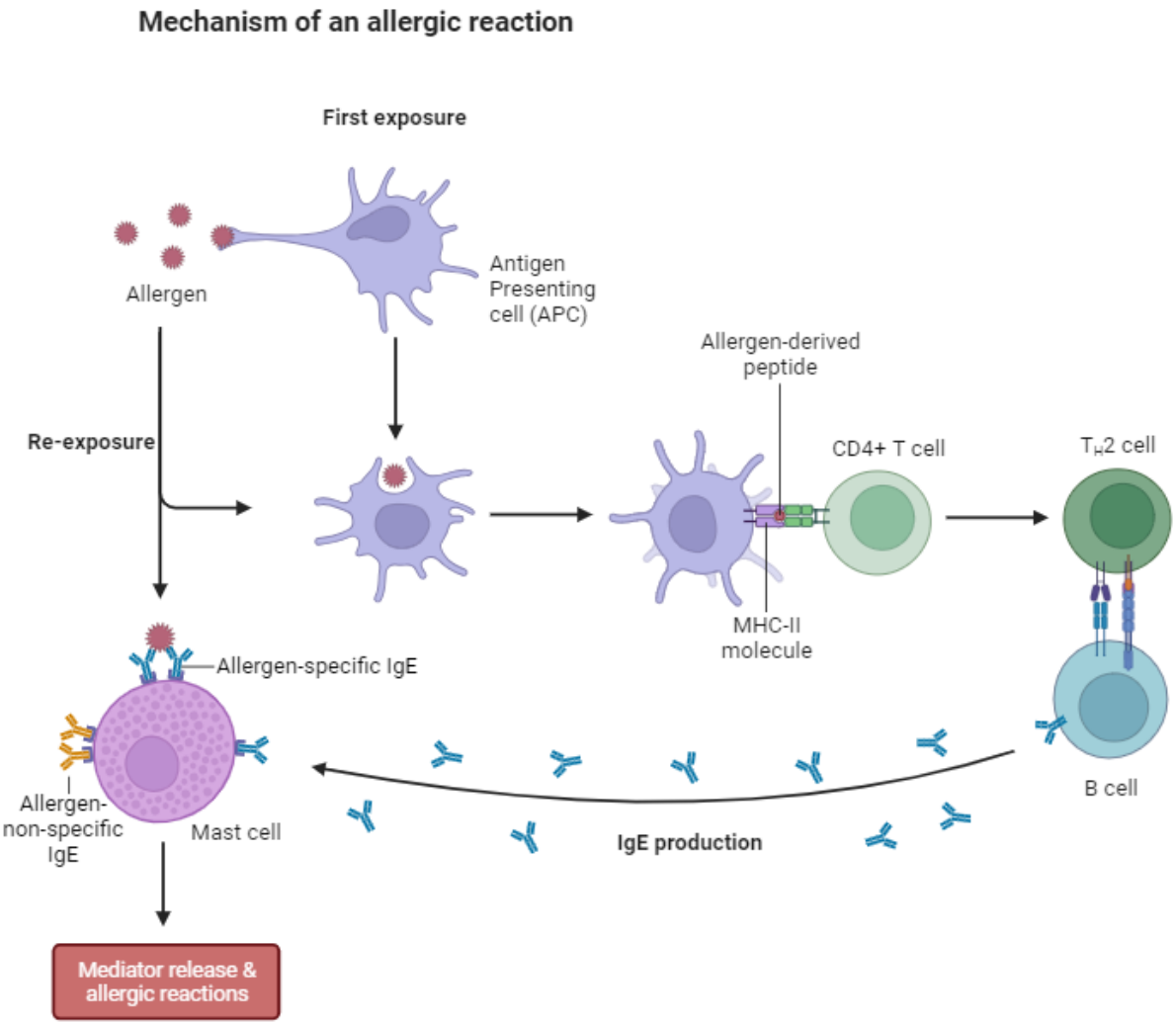


Figure 1 - An illustration of the mechanism of an allergic reaction showing the first exposure (sensitization) phase and a re-exposure (elicitation) phase prior release of mediators causing allergic symptoms.

Subsequently, these peptide-containing endosomes fuse with Major Histocompatibility Complex class II (MHC-II)-containing endosomes assembled in the endoplasmic reticulum (ER), leading to the replacement of the invariant chain (CD74) with allergenic peptides (Figure 2). This results in the presentation of MHC-II-peptide complexes on the cell surface for

recognition by an allergen-specific T-cell receptor (TCR) of CD4+ T helper cells (10,11). These CD4+ T helper cells differentiate into T helper 2 (Th2) cells, a process regulated by the cytokine interleukin-4 (IL-4) or, in the case of DCs, by neighbouring innate lymphoid cells (ILCs) that are not completely knwon. Th2 cells upregulate IL-4 production and, when the allergen is presented by B cells, initiate CD40 (B cell)-CD40L (T cell) communication. This recognition of the allergen by B cells, along with engagement of the B cell receptor, triggers the activation and differentiation of B cells, leading to the synthesis and secretion of allergen-specific immunoglobulin E (IgE) antibodies (9,13). These IgE antibodies, when bound to high-affinity FcεRI receptors on mast cells, prime these cells for rapid responses upon subsequent allergen exposures (elicitation phase). Upon re-exposure to the allergen, the cross-linking of at least two IgE antibodies bound to the FceRI receptor on basophils and mast cells initiates degranulation, causing the release of inflammatory mediators including histamine (8,9,13). The crucial distinction between allergic and non-allergic individuals lies in the immune response balance, characterized by Th2 dominance and heightened IgE production, ultimately leading to increased sensitivity to allergens and the distinctive allergic symptoms caused by the release of mediators (1).

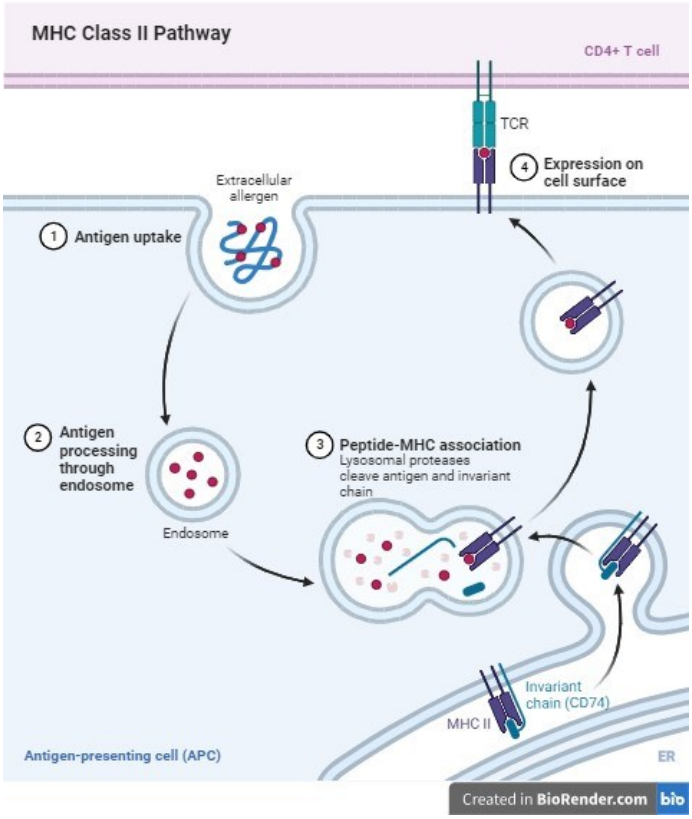


Figure 2 - An illustration of the MHC-II pathyway including antigen uptake, processing, peptide MHC association, cell surface expression and finally peptide presentation to CD 4+ T cells

Potential therapies

In the quest for sustained allergen tolerance, allergen immunotherapy remains the primary approach, involving a gradual increase in allergen doses administered over months to years. However, its efficacy is often compromised by patient discontinuation due to the extended treatment duration and the risk of adverse reactions, including anaphylaxis. A notable breakthrough in allergen immunotherapy would include the safe delivery of therapeutic allergen quantities to sensitized individuals without the risk of anaphylaxis (14,15).

One innovative approach in targeting the allergic pathway involves the modification of allergenic proteins with glycans. Glycans, intricate carbohydrate structures composed of linked monosaccharides, become covalently attached to proteins through enzymatic glycosylation. Glycoproteins play crucial roles in various biological processes, including protein folding, immune responses, cell signalling, and cell proliferation. The presence of glycans on allergens may impact protein antigen uptake, proteolytic processing, MHC presentation, and subsequent immune responses. Some glycans enhance immune activation by improving peptide binding to MHC-II, while others induce tolerogenic responses, suppressing immune reactions and contributing to immune regulation to prevent excessive allergies (16,17). Sialic Acid-Binding Immunoglobulin-like Lectins, also known as Siglecs, form a family of lectin receptors capable of recognizing various sialic acid patterns on glycan-conjugated proteins. Siglecs are expressed primarily on leukocytes and possess inhibitory signalling capabilities through their cytoplasmic domains (16,18). Exploiting their potential to modulate immune responses and mitigate allergic reactions, particularly in IgE-mediated allergies, holds promise for innovative immunotherapies involving glycan-modified allergens. By engaging specific glycans, certain Siglecs can transmit inhibitory signals, reducing immune cell activation in allergic responses (16-19).

Two primary strategies are currently under exploration: the use of antibodies to diminish Siglec-expressing cells and induce inhibition, and the deployment of liposomal nanoparticles displaying allergens and high-affinity Siglec ligands to co-crosslink Siglecs with the respective activating receptors (20-23). In 2019, Duan et al. described an approach employing liposomal nanoparticles with both allergen and Siglec ligands, effectively preventing IgE-sensitized mast cell activation and desensitizing them to subsequent triggers. Additionally, the liposomes accelerated the clearance of circulating anti-allergen IgE and thus preventing re-sensitization (23). The area of liposomal nanoparticles displaying allergens to include the safe delivery of therapeutic quantities and glycan ligands targeting high-affinity

Siglec receptors holds potential in the field of FA to provide patients with a safer and more efficient route toward allergen tolerance, ultimately enhancing their quality of life.

Although conventional wisdom held that T-cells are not able to recognize carbohydrates as T-cell independent antigens, recent research has shown that glycopeptides can undergo processing in antigen-presenting cell endosomes. If these glycopeptides bind to MHC-II, they can be presented to and recognized by T cells (18, 24). Research in mouse models has shown that glycan-modified allergens engaging Siglec-9 have the capacity to redirect APCs, tilting the balance in favor of Treg responses as opposed to Th2 responses (Figure 3). It remains uncertain, whether this redirection arises from the presentation of glyco-peptides or from the engagement of Siglec on DCs, which can alter their cytokine secretion and other processes. Tregs serve as key players in immune regulation, mitigating excessive immune reactions and fostering tolerance (25). Sustaining glycan modifications on allergenic peptides throughout the antigen processing cascade might hold the promise of consistently augmenting Treg responses, thereby carrying substantial therapeutic potential.

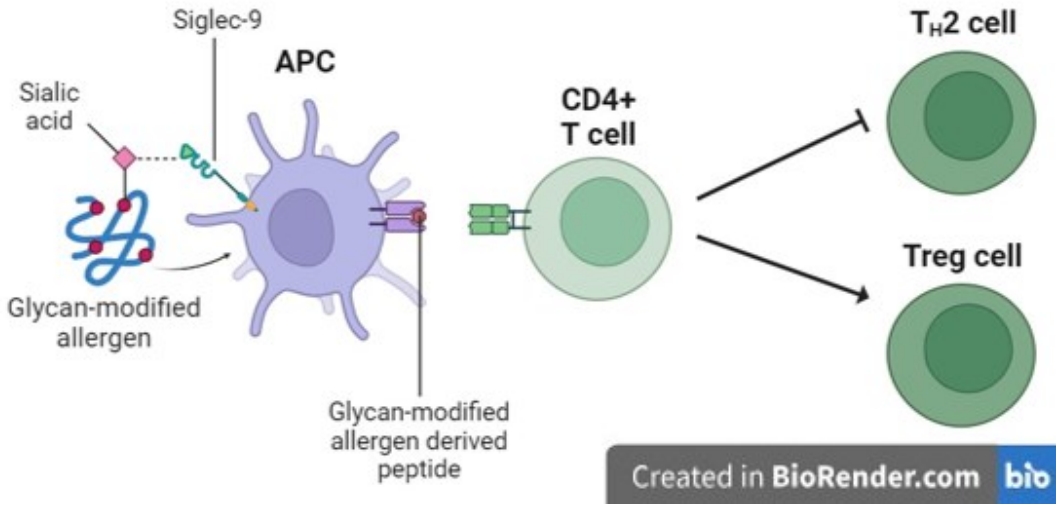


Figure 3 - An illustration of the potential pathway of a sialic acid-modified allergen binding to the Siglec-9 receptor and after antigen uptake, processing and presentattion of glycopeptides to CD4+ cells consistently favor Treg responses over Th2 responses

Nevertheless, the question remains whether allergen-derived glycopeptides will be presented, or if other allergen-derived peptides lacking the sialic acid glycan take precedence. Alternatively, the presentation of distinct self-presenting antigens could occur due to the possible obstruction of digestion sites during the endosomal processing. A comprehensive understanding of glycan-modified allergen processing by MHC and the potential downstream effects of glycopeptides offers unique prospects for developing glycopeptide-specific immunotherapies in the context of treating allergies.

Cow's milk allergy

Cow's Milk Allergy (CMA) is a prevalent food allergy commonly affecting infants and young children, with prevalence estimates ranging from 0.5% to 3% in developed countries by the age of one (26,27). CMA can result in severe anaphylactic reactions and has significant nutritional implications, impacting growth not only in childhood but also persisting into adulthood (26,28). Cow's milk primarily contains two major proteins: casein, constituting approximately 80% of the total milk protein content, and whey, making up around 20%, with β-lactoglobulin (BLG) being a key allergen within the whey fraction (26).

Efforts to improve the lives of individuals suffering from CMA involve both academic and industrial research, particularly focusing on therapeutic peptides associated with oral tolerance. Academic studies, such as those involving nanoparticles co-encapsulating immune-activating DNA sequences and BLG-derived peptides, have shown promise in preventing whey-induced CMA in mice, especially when maintaining strict hygiene conditions in their environment, mimicking real-world scenarios (29).

In the industrial sector, research has contributed by developing and analysing hydrolysed infant formulas, which have revealed the presence of functional MHC-II-restricted T cell epitopes. These findings offer potential support for the development of oral tolerance to whey allergens (30). However, there remains an unexplored realm in CMA research concerning liposomal nanoparticles that can deliver allergens safely in therapeutic quantities and glycan ligands targeting high-affinity Siglec receptors resulting in the consistent reduction of Th2 cell development using glycopeptides. To further explore this area, a comprehensive understanding of the fundamental MHC-II pathway, encompassing antigen uptake, processing, and the potential presentation of therapeutic glycopeptides, is essential.

Aim of the study

In order to contribute effectively to ongoing research in potential FA immunotherapy involving glycan-modified allergens, it is crucial to enhance the fundamental understanding of the MHC-II pathway, which is referred to as Human Leukocyte Antigen class II (HLA-II) in humans. This will facilitate a deeper insight into the mechanisms of antigen uptake, proteolytic processing, and, ultimately, the presentation of potential therapeutic glycopeptides derived from glycan-modified allergens. Within this study, a methodology has been developed to analyse the presentation of the peptide cargo embedded in HLA-DR, an HLA-II molecule. These peptides may originate from either self-presenting antigens or derived from the allergenic protein BLG, which acts as a model allergen in this research.

EXPERIMENTAL PROCEDURES

In this study, a more practical methodology to isolate and analyse HLA-II presented peptides was developed building upon the protocol established by Purcell et al. (31). The Epstein–Barr virus (EBV)-immortalized B cell lymphoblastoid line, JY, has been utilized as a model cell line and cultured under varying conditions. Immunoaffinity techniques, such as immunoprecipitation, have been employed to isolate HLA-DR-peptide complexes from lysed cells. Subsequently, the peptides have been separated through Solid Phase Extraction (SPE), followed by their analysis and identification using Liquid Chromatography with tandem Mass Spectrometry (LC-MS/MS) and Mascot (Figure 4).

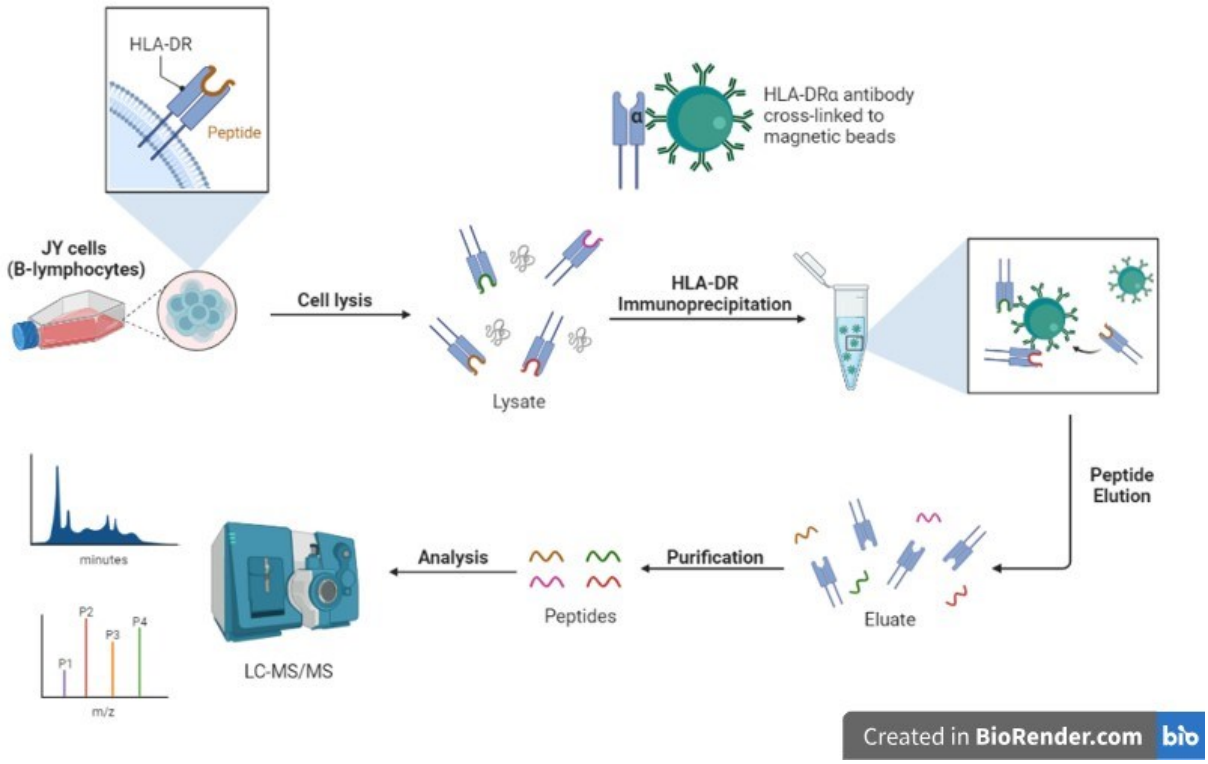


Figure 4 - Experimental workflow applied for HLA-DR peptide ligand analysis. Cells were cultivated under various conditions and harvested and lysed to prepare lysate for HLA-DR immuno-affinity purification. HLA-DR complexes were immuno-affinity purified using L243 antibodies coupled to protein A/G magnetic beads. Subsequent to elution, purification and identification were performed.

Cell culture

The B-lymphoblastoid cell line JY (HLA-DRA∗01, HLA-DRB1∗04:04/13:01, HLA-DRB4∗04, HLA-DRB5∗02) was cultured under three distinct conditions to examine their influence on the results (32). JY cells were either cultured in the serum-free medium (SFM) ExCellerate B Cell Media, Xeno-Free (Bio-Techne) or in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (FCS). Purified BLG was added to JY cells cultured in FCS-supplemented RPMI medium at a concentration of 12.5 µg/mL, 24 hours before

harvesting. All media were supplemented with 10 µL/ml (1%) penicillin-streptomycin (Sigma Aldrich) and incubated in a humidified incubator at 37 °C with 5% CO_2.

Flow cytometric measurement of HLA-DR and CD19 expression

Flow cytometry was used to measure the expression of HLA-DR and CD19 on JY cells to elucidate the potential differential impact of SFM, RPMI medium supplemented with FCS and the application of BLG. Cells were harvested by centrifugation at 300 x g, 4 °C for 5 minutes and the cell pellet was resuspended in FACS buffer (PBS, 0.5% BSA, 2mM EDTA) to a concentration of 1 x 10^6 cells/mL. Resuspended cells were transferred to a 96-well plate and stained with anti-HLA-DR-PE (fluorophore: phycoerythrin, clone: L243, dilution: 1:50) and anti-CD19-APC (fluorophore: allophycocyanin, clone: HIB19, dilution: 1:50) for 20 minutes at 4 °C in the dark. An isotype control, IgG2b Rat PE, was included to confirm specific binding. Fixation of cells was performed by incubation with 0.5% paraformaldehyde for 30 minutes. Acquisition was performed on a Canto II (BD Bioscience) and the data were analyzed using FlowJo (V10, Becton Dickinson).

Generation of cell lysate

Cell lysis disrupts the cell membrane, causing the release of cellular contents. This allows the access, analysis, and isolation of both intracellular and extracellular HLA-DR complexes. The cells were harvested by centrifugation and washed three times with an excess of ice-cold Phosphate Buffered Saline (PBS) after removal of the media. Subsequently, the cells were disrupted with 1 mL freshly prepared lysis buffer (0.5% NP-40, 50 mM Tris pH 8.0, 150 mM NaCl and protease inhibitor cocktail (10 µL per 1 mL buffer) in Milli-Q water) per 1 x 10^7 pelleted cells for 1 hour at 4 °C, on gentle end-to-end rotation. The lysate was then cleared by centrifugation for 15 minutes at 11,000 x g at 4 °C, snap frozen in liquid nitrogen and stored at -80 °C. Table 1 shows the number of harvested cells and an aliquot of each lysate was kept a side to determine the total protein concentration by using the Bradford method.

Table 1 - The three different cell cultures showing the condition they are grown in and the amount of harvested cells

Cell culture	***Conditions***	***Amount of harvested cells***
1	ExCellerate B Cell Media, Xeno-Free	4 x 10^7
2	RPMI 1640 medium with 10% FCS	2.0 x 10^8
3	RPMI 1640 medium with 10% FCS and 12.5 µg/mL BLG	1.25 x 10^8

Cross-linking anti-human HLA-DR antibody to Protein A/G Magnetic Beads

To enhance the reusability of the Pierce™ Protein A/G Magnetic Beads (ThermoFischer) and prevent co-elution of antibodies, a cross-linking reaction was performed using the anti-human HLA-DRa specific antibody (L243, Biolegend). Initially, the bead slurry was thoroughly washed with binding buffer (0.1 M NaPhosphate Buffer, pH 8.0), a magnetic separator was employed to efficiently separate the magnetic beads, along with the bound antibody, from the binding buffer. Subsequently, the beads were incubated with 80 µg of antibody per 100 µL of bead slurry, ensuring coverage of the manufacturer's specified maximum binding capacity. This incubation took place in binding buffer at a 4:1 ratio of binding buffer to antibody, with agitation at 4 °C for 30 minutes. Following antibody incubation, the beads were resuspended in Cross-linking Buffer (0.2 M triethanolamine, pH 8.2, J.T. Baker Chemicals B.V.) and vortexed to ensure proper mixing. Subsequent steps included incubation with Cross-linking Buffer containing 25 mM dimethyl-pimelimidate-dihydrochloride (DMP, Merck) at room temperature for 45 minutes, with agitation. Further processing of the beads involved resuspending in Blocking Buffer (0.1 M ethanolamine, pH 8.2, Medchem) followed by vortexing and incubation. After blocking the beads were washed with PBS and to elute any bound antibody that was not cross-linked with DMP, 1 mL Elution Buffer (0.1 M glycine-HCl, pH 2.5) was added to the beads. Finally, the beads were resuspended and stored in PBS containing 0.1% Tween 20 and 0.02% sodium azide.

HLA-DR immunoaffinity purification with cross-linked anti-human HLA-DR antibody to Protein A/G Magnetic Beads

In order to isolate the HLA-DR-peptide complexes from the lysate, HLA-DR immunoaffinity purification was performed by immunoprecipitation HLA-DRa specific antibody (L243, Biolegend) cross-linked to Pierce™ Protein A/G Magnetic Bead slurry (ThermoFischer). Beads were washed three times with a volume of 4 mL PBS per 100 µL beads prior loading the lysate, subsequently a volume of 4 mL lysate was loaded per 100 µL cross-linked beads and incubated overnight. After incubation, the flow-through was kept aside to determine successful isolation of HLA-DR-peptide complexes by western blotting. Prior elution, beads were washed three times with 4 mL PBS per 100 µL beads. Elution was done with 250 µL 10% acetic acid (Sigma-Aldrich) per 100 µL beads and 100 µL 1 M Tris-base was added as neutralization buffer. After elution, beads were washed in PBS three times with a volume of 4 mL per 100 µL beads and stored in 100 µl PBS supplemented with 0.02% sodium azide.

SDS-PAGE and Western Blotting

To confirm the presence of HLA-DR in the lysate, flowthrough, and eluates, SDS-PAGE and Western blotting were performed. Gel preparation followed the CBDD lab standard protocol, and the gel was cast and allowed to polymerize. Samples, including lysate, flowthrough, and eluates, were diluted at a 1:1 ratio with 2x Laemmli sample buffer (Biorad). The sample mixtures were then heated for 5 minutes at 95°C to denature the proteins. The denatured protein samples were loaded onto the gel. Electrophoresis was performed at 200 V for 32 minutes to separate the proteins according to their molecular weights. After electrophoresis, the gel was stained with Coomassie brilliant blue to visualize the separated proteins.

Following SDS-PAGE, the proteins from the gel were transferred to a pre-activated PVDF membrane (Biorad) using a standard transfer protocol. After protein transfer, a membrane-blocking step was carried out by incubating the membrane in 5% Bovine Serum Albumin (BSA) in tricine-buffered saline (TBS) for one hour at 4 °C. This step helps to prevent non-specific binding of antibodies. Post-blocking, the PVDF membrane was incubated overnight with the primary antibody L243, which was diluted at a 1:4000 ratio in a TBS containing 1% BSA. This primary antibody specifically targets HLA-DR. Following the primary antibody incubation, the membrane was washed three times with TBS-T (Tris-buffered saline with Tween 20, Sigma Aldrich) to remove unbound primary antibody. The membrane then underwent a one-hour incubation with a secondary antibody, which was a rabbit anti-mouse IgG (NB7544, Novus Biologicals) conjugated with horseradish peroxidase (HRP). To visualize the isolated HLA-DR complexes, Clarity™ Western ECL Substrate (Biorad) was applied to the membrane.

Separation of HLA-DR associated peptides by Solid Phase Extraction and nano-LC–HRMS/MS analysis

A solid phase extraction (SPE) was performed in order to prepare the eluate, containing HLA-DR complexes and their associated peptides, for LC-MS/MS analysis. A Sep-Pak C18 1 cc Vac Cartridge, 50 mg Sorbent per Cartridge, 55 - 105 µm, 100/pk (Waters) was used for SPE. Conditioning of the column was achieved with MS grade Acetonitrile (VWR International) and 0.1% formic acid in Milli-Q water. Subsequently, the eluate was loaded on the SPE column diluted in 0.1% formic acid in Milli-Q water. Peptides were eluted from the column with 0.1% formic acid in 70% acetonitrile. The total volume was put into a vacuum

concentrator at 45 °C and evaporated until almost dry. A volume of 10 µL was injected into the LC-MS/MS.

The LC-MS/MS procedure was done as described by Meiring et al. The nanoflow liquid chromatography using an UltiMate™ 300 RSLCnano System (Thermo Fischer Scientific, Waltham, MA, United States) was connected online to a Q-Exactive Plus Mass spectrometer (Thermo Fischer Scientific, Waltham, MA, United States) and used to analyse all samples (33). Peptides were trapped on a Acclaim™PepMap™ 100 C18 HPLC trap column (1 mm Trap Cartridge, 5 µm, 1mm inner diameter, 5mm length (Thermo Fischer Scientific, Waltham, MA, United States) at 5 µL/minute in 100% solvent A (0.1 mol/L acetic acid in water). Afterwards the peptides were eluted at ~100nL/minute in a 90 minutes gradient from 10% - 40% solvent B (0.1 mol/L acetic acid in 95:5 (v/v) acetonitrile/water) to a 15 cm bioZen 2.6 µm Peptide XC-C18, nano Column, 140 x 0.075 mm (00f-4782-AW-21, Phenomenex, Utrecht, The Netherlands) Via liquid junction attached to the analytical column the eluent was sprayed by electrospray ionization (ESI). The mass spectrometer operated in data dependent mode, repeatedly switching between MS and MS/MS. At a resolution of 70,000 (FWHM) full scan mass spectra(from m/z 375 to 1,500) were obtained after accumulation to a target value of 3 x 10^6 or 100 ms (whichever was reached first). The 15 most abundant ions at a threshold above 3E4 were picked for collision-induced dissociation (HCD) at normalized collision energy of 28% after accumulation to a target value of 1 x 10^5 or 64 ms at a resolution of 17,500 (FWHM).

All MS data were processed by Proteome Discoverer (version 2.1,Thermo Scientific). Peak lists were generated using a standard work-flow. Peptide identification was performed by searching individual peak lists of CID fragmentation spectra against a database containing human proteins in the case of self-presenting peptides and bovine whey proteins in the case of BLG presented peptides using Mascot (version 2.4.1, Matrix Science Ltd, London, United Kingdom).

RESULTS

Morphological changes in EBV-transformed B-lymphocytes induced by serum-free medium

To ascertain whether SFM can replace culture medium supplemented with 10% FCS, the morphology was assessed by microscopy using Trypan Blue as a live/dead marker (Figure 5). JY cells cultured in either SFM or FCS-supplemented RPMI medium showed morphological differences. Besides the increase in debris and the lower concentration of cells for the SFM condition, there were indications for differential physiological properties. A subpopulation of JY cells grown in SFM have a smaller circumference compared to the FCS-supplemented culture. These differences indicate that the choice of culture medium can exert differential effects on surface antigen expression and potentially influencing B cell biology.

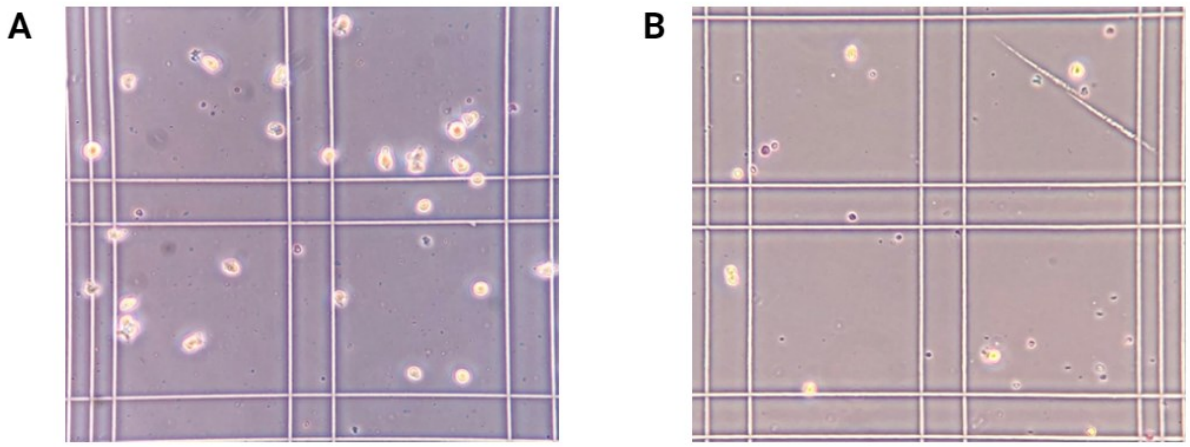


Figure 5 - Microscopic Visualization of the cell Culture by 1:1 dilution in Trypan Blue. (A) Visualization of cells grown in SFM. (B) Visualization of cells grown in 1640 RPMI supplemented with 10% FCS.

Reduced HLA-DR and CD19 expression by cultivating JY cells in serum-free culture medium

In order to verify the assumption that SFM may influence surface antigen expression and potentially B cell biology, the surface expression of HLA-DR, the peptide-presenting molecule of interest, and CD19, a B cell marker, were evaluated by flow cytometry in comparison to an IgG2b Rat PE isotype control (Figure 6).

A rightward shift in the fluorescence signal indicates increased fluorescence intensity and upregulation of the target surface protein. Both SFM and FCS-supplemented medium demonstrated nearly identical rightward shifts in comparison to the isotype control. These results strongly suggest HLA-DR expression on the cell surface of EBV-transformed B-lymphocytes (Figure 6A). Nonetheless, the culture supplemented with 10% FCS yielded a

slight greater mean fluorescence intensity (MFI), 50078 versus 32160 respectively, indicative of elevated HLA-DR expression by JY cells cultured in 10% FCS compared with those cultivated in SFM.

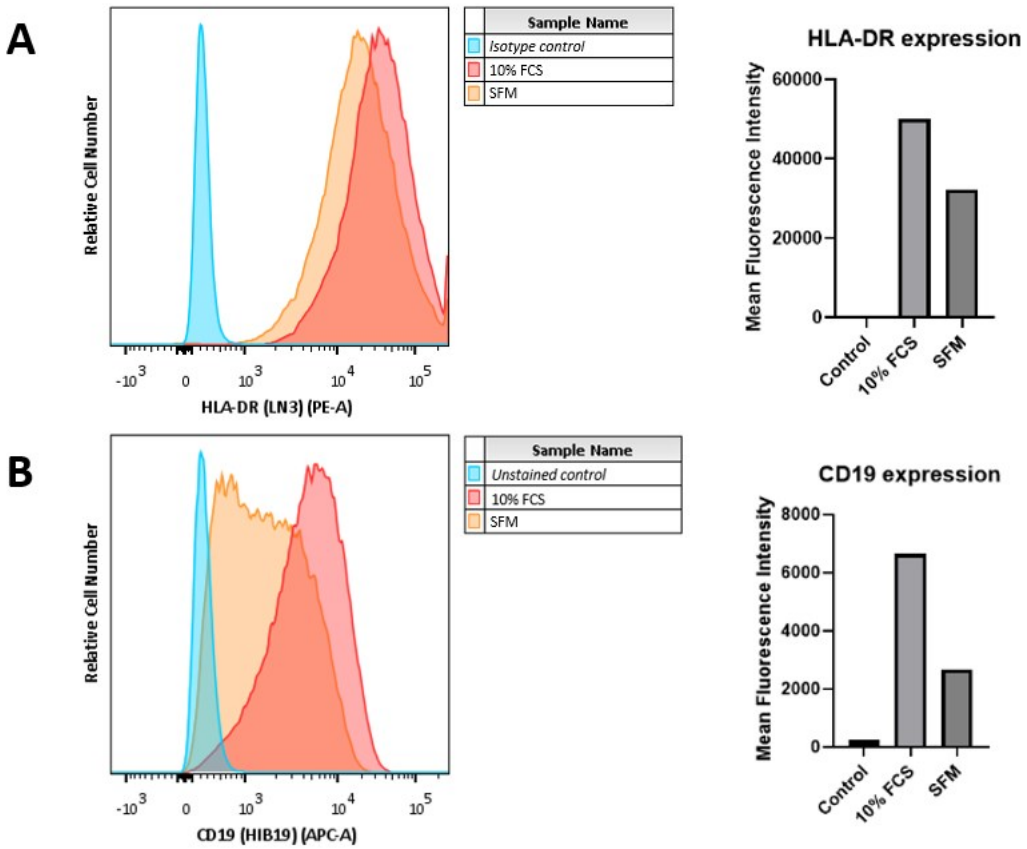


Figure 6 - Flow cytometry analysis of the expression of HLA-DR and CD19 on the cell surface of JY cells grown in SFM (Serum-Free Medium) and RPMI 1640 supplemented with 10% FCS. (A) A PE-A histogram and a MFI (Mean Fluorescence Intensity) graph showing the expression of HLA-DR in the two different media including an IgG2b Rat PE isotype control. (B): An APC-A histogram and a MFI (Mean Fluorescence Intensity) graph showing the expression of CD19 in the two different media including an unstained sample as a control.

Conversely, the CD19 histograms revealed intriguing distinctions (Figure 6B). The histogram for JY cells cultured in SFM exhibited a more pronounced disparity compared to those grown in FCS. The distribution pattern of JY cells cultured in SFM manifested a broader peak that partially encompassed the unstained control's histogram. The broader peak in the SFM condition implies a wider range of fluorescence intensity values, which could be attributed to factors such as variations in cellular activation or altered cellular physiology induced by the absence of serum components. The data presented in figures 5 and 6 suggest that the choice of culture medium can exert differential effects on surface antigen expression and potentially influencing B cell biology.

Unanticipated HLA-DR expression by cultivating JY cells in FCS supplemented medium incubated with BLG

To evaluate the influence of antigen exposure (BLG, 12.5 µg/ml, 24 hours) on HLA-DR and CD19 surface expression, another flow cytometry experiment was performed with identical labelled antibodies and dilutions. JY cells showed an increase in the expression of HLA-DR on the cell surface compared with the isotype control in presence and absence of BLG (Figure 7A). Whilst the cell culture that has not been exposed to BLG displayed a narrow and defined peak, the HLA-DR expression histogram of JY cells exposed to BLG exhibited a peak with two broad shoulders (Figure 7B). This indicates a more heterogeneous distribution of HLA-DR expression levels upon BLG application. The width of the peak may be influenced by factors such as changes in cell size, granularity, or fluorescence intensity due to the exposure itself. BLG treatment may induce cell activation or differentiation, leading to more diverse cell populations with different characteristics.

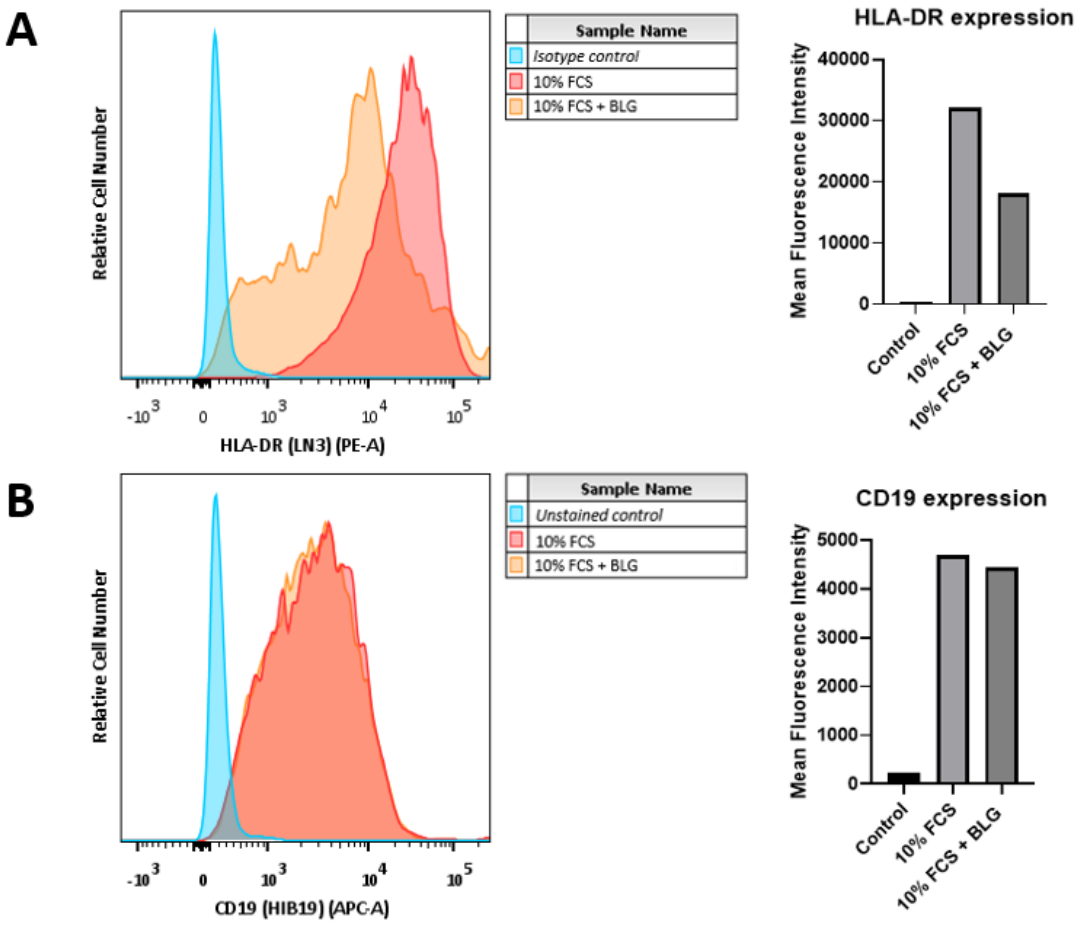


Figure 7 - Flow cytometry analysis of the expression of HLA-DR and CD19 on the cell surface of JY cells grown in RPMI 1640 supplemented with 10% FCS with and without exposure to 12.5 ug/mL BLG. (A) PE-A histogram and MFI (Mean Fluorescence Intensity) graph showing the expression of HLA-DR in the two different conditions including an IgG2b Rat PE isotype control. (**B**): APC-A histogram and MFI (Mean Fluorescence Intensity) graph showing the expression of CD19 in the two different conditions including an unstained sample as a control.

Immunoprecipitation of HLA-DR peptide complexes requires cross-linking of the L243 antibody with Protein A/G magnetic beads

The flow cytometry examination of JY cells cultured under distinct growth conditions confirmed the expression of HLA-DR on the cell surface. In order to identify the peptides presented by the HLA-DR molecule, immunoprecipitation was utilized to separate these complexes from the lysate. In the initial endeavour to isolate HLA-DR from the lysate, the HLA-DR-specific antibody L243 was non-covalently linked to Protein A/G magnetic beads using a sodium phosphate binding buffer. SDS-PAGE analysis of the eluate revealed the antibody's tendency to elute from the Protein A/G magnetic beads (Figure 8, lane F). In light of this finding, a strategic decision was made to proceed with bead cross-linking, effectively addressing this challenge and enhancing subsequent isolation procedures.

Cross-linking L243 to Protein A/G magnetic beads with DMP

To enhance the reusability of magnetic beads and mitigate antibody co-elution, the anti-human HLA-DR antibody (clone: L243) was cross-linked to Protein A/G magnetic beads utilizing the cross-linker DMP. In figure 9, successful cross-linking of the antibody to Protein A/G magnetic beads using DMP is demonstrated using the validation strategy of Purcell et al. The first lane loaded with pure antibody displays distinct bands corresponding to the antibody's light (25 kD) and heavy chain (50 kD). In the lane C, a sample of magnetic beads with non-covalently bound antibody is shown prior to the introduction of the cross-linking buffer containing DMP. In accordance with Purcell et al. cross-linking efficiency test, this lane revealed a subtle presence of antibody.

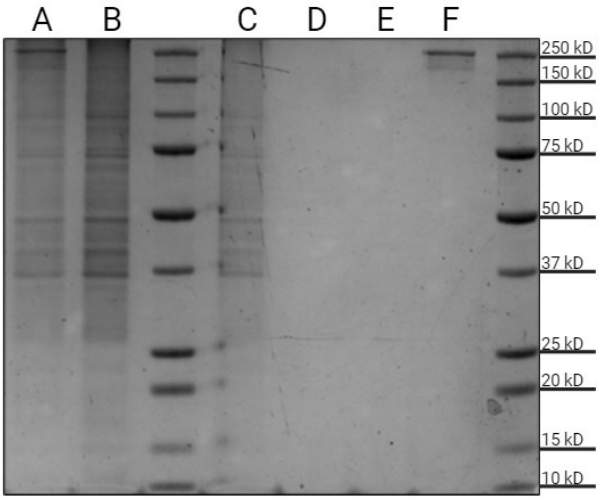


Figure 8 - A SDS-PAGE analysis of the first immunoprecipitation experiment using the HLA-DR-specific antibody L243 non-covalently linked to Protein A/G magnetic beads. (A) non-covalently linked beads incubated with the lysate of JY cells grown in FCS-supplemented RPMI 1640 medium. (B) The raw lysate (C) Flowthrough of lysate after incubating with non-covalently linked beads. (D and E) PBS washes. (F) Obtained eluate after eluting with glycine-HCl

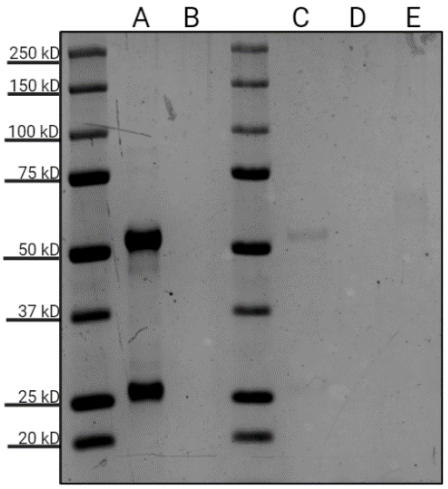


Figure 9 - The validation of cross-linking efficiency was conducted by following the procedures outlined by Purcell et al. (A) 1.25 µg pure antibody loaded in a volume of 5 µL. (B) Flowthrough after incubation of magnetic beads with the antibody in. (C) Beads in triethanolamine before adding the DMP cross-linker. (D) Beads after incubation with 25 mM DMP in triethanolamine. (E) Eluate, that elutes bound antibody that is not cross-linked with DMP

Successful isolation of HLA-DR complexes by immunoprecipitation

To validate the immunoprecipitation procedure, 4 x 10^7 cells cultured in SFM were lysed and subjected to L243 antibody cross-linked protein A/G magnetic beads to isolate the HLA-DR peptide cargos. In view of SDS-PAGE results not yielding observable bands indicative of an adequate HLA-DR concentration in the eluate, a subsequent western blot analysis was conducted on the eluate to ascertain the presence of HLA-DR molecules. The resultant blot of the first half exhibited a distinct band within the lysate, flowthrough, and eluate, all around 60-67 kDa, signifying the presence of the HLA-DR heterodimer (Figure 10). Furthermore, the eluate displayed a signal approximately at 33-35 kDa, consistent with the HLA-DR alpha subunit. This observation aligns with the specificity of L243, the employed antibody, known to target the alpha subunit specifically. In contrast, the unincubated half of the membrane exclusively exhibited the signal corresponding to the co-eluted antibody. These results confirm successful immune precipitation of the HLA-DR alpha subunit and most probably presented peptides.

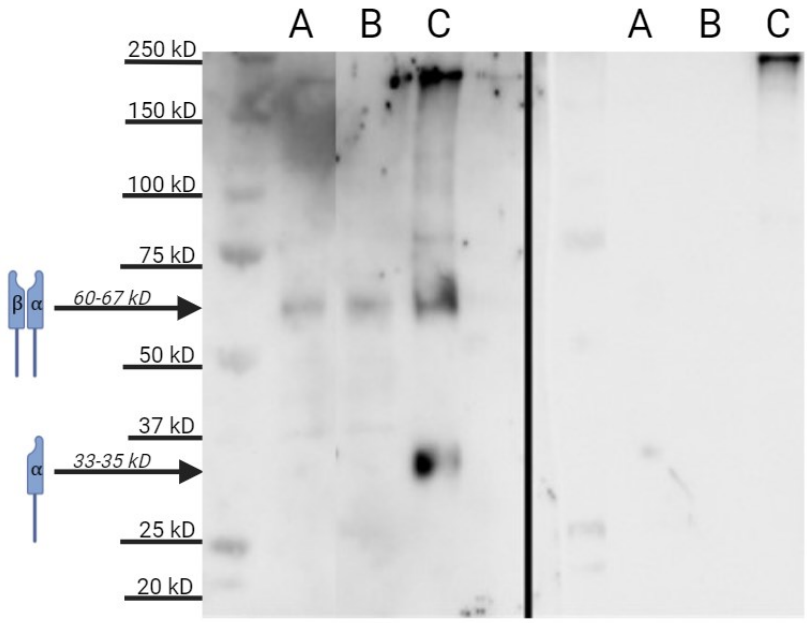


Figure 10 - A Western Blot analysis of the immunoprecipitation experiment using the anti-human HLA-DR antibody (clone: L243) cross-linked to Protein A/G magnetic beads in order to isolate HLA-DR peptide complexes from JY lysate cultivated in SFM. On the left half of the membrane (A) Lysate, (B) Flowthrough and (C) Eluate are exposed to primary antibody (L243) and a secondary antibody, a rabbit anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP). The right half of the membrane was solely exposed to a rabbit anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP)

Identification of self-presenting and BLG derived peptides

Subsequent to the immunoprecipitation of the HLA-DR peptide complexes under various conditions, the eluates were separated via SPE to isolate them from HLA-DR molecules, potential leached antibodies, non-specific binding molecules, as well as certain detergents and salts. Three distinct eluates were analysed in order to ascertain the presence of peptides that can be identified based on their mass-to-charge ratio (m/z) and characteristic fragmentation patterns.

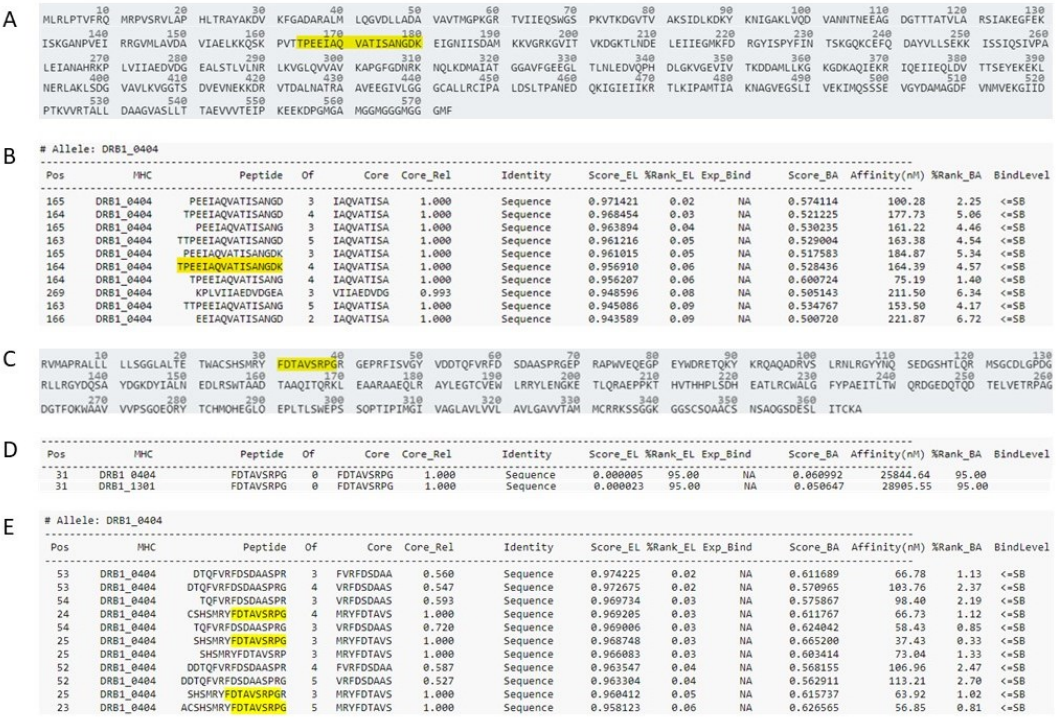
Serum-Free Medium and self-presenting peptides

Due to the limited access to ExCellerate B Cell Media, a high cell count could not be grown up. Therefore, an analysis of self-presenting peptides was chosen instead of exposing the culture to BLG. After collecting the eluate, a sample preparation process involving solid-phase extraction (SPE) was conducted. The isolated peptides were eluted and then concentrated under vacuum. Following this, a 10 µL volume of the prepared sample was introduced into the LC-MS/MS system for analysis. Subsequently, the sequence was determined with proteome discoverer and two unique self-presenting peptides could be identified that could be linked to two human proteins using Mascot.

One of the identified peptide sequence, TPEEIAQVATISANGDK, corresponds to HSPD1, a 60 kDa heat shock protein (Figure 11A). Using NetMHCIIpan-4.0, a predictive tool,

peptides derived from the HSPD1 protein that could conceivably bind to the HLA-DR beta chain produced by the alleles DRB1∗04:04/13:01 were predicted (Figure 11B). The outcome of this prediction, performed within the context of peptide lengths spanning 13 to 18 amino acids, unveiled that the peptide TPEEIAQVATISANGDK could be considered a strong binder to the HLA-DR beta chain produced by the allele DRB1∗04:04. The peptide scored among the highest of on both the eluted ligand prediction score (Score_EL) and the log-scale predicted binding affinity (Score_BA). Intriguingly, according to literature, this peptide has been found to be presented in a JY cell culture grown in RPMI 1640 supplemented with 10% FCS.

The second peptide sequence, FDTAVSRPG, aligns with HLA-C, a HLA class I protein (Figure 11C). Existing literature shows that peptides stemming from HLA class I proteins are more frequently associated with HLA-DR molecule presentation. NetMHCIIpan-4.0 was used to predict potential presented peptides derived from HLA-C, utilizing a sequence length of 9 amino acids, the peptide scored relatively low on both Score_EL and Score_BA (Figure 11E). The prediction results indicate a low likelihood of this peptide being presented in this manner. Considering the identification of only two unique peptides, it is anticipated that the peptide would exhibit elevated scores in terms of eluted ligand and binding affinity. Interestingly, when changing the peptide length into 13 to 18 amino acids in the prediction of peptides derived from HLA-C, the sequence FDTAVSRPG emerges within the peptide sequences associated with the most robust binding affinities (Figure 11D). However, in light of the eluted ligand and binding affinity scores, this outcome hints at the possibility of incomplete peptide fragmentation or peptide degradation prior to analysis. This observation prompts a deeper exploration into the experimental conditions that might have contributed to these results.



A

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          10        20        30        40        50        60        70        80        90       100       110       120       130
MLRLPTVFRQ MRPVSRVLAP HLTRAYAKDV KFGADARALM LQGVDLLADA VAVTMGPKGR TVIIEQSWGS PKVTKDGVTV AKSIDLKDKY KNIGAKLVQD VANNTNEEAG DGTTTATVLA RSIAKEGFEK
      140       150       160       170       180       190       200       210       220       230       240       250       260
ISKGANPVEI RRGVMLAVDA VIAELKKQSK PVTTPEEIAQ VATISANGDK EIGNIISDAM KKVGRKGVIT VKDGKTLNDE LEIIEGMKFD RGYISPYFIN TSKGQKCEFQ DAYVLLSEKK ISSIQSIVPA
      270       280       290       300       310       320       330       340       350       360       370       380       390
LEIANAHRKP LVIIAEDVDG EALSTLVLNR LKVGLQVVAV KAPGFGDNRK NQLKDMAIAT GGAVFGEEGL TLNLEDVQPH DLGKVGEVIV TKDDAMLLKG KGDKAQIEKR IQEIIEQLDV TTSEYEKEKL
      400       410       420       430       440       450       460       470       480       490       500       510       520
NERLAKLSDG VAVLKVGGTS DVEVNEKKDR VTDALNATRA AVEEGIVLGG GCALLRCIPA LDSLTPANED QKIGIEIIKR TLKIPAMTIA KNAGVEGSLI VEKIMQSSSE VGYDAMAGDF VNMVEKGIID
      530       540       550       560       570
PTKVVRTALL DAAGVASLLT TAEVVVTEIP KEEKDPGMGA MGGMGGGMGG GMF
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B

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# Allele: DRB1_0404
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Pos          MHC          Peptide   Of       Core  Core_Rel       Identity      Score_EL %Rank_EL Exp_Bind      Score_BA  Affinity(nM) %Rank_BA  BindLevel
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165    DRB1_0404      PEEIAQVATISANGD   3   IAQVATISA     1.000       Sequence      0.971421     0.02       NA      0.574114        100.28     2.25      <=SB
164    DRB1_0404     TPEEIAQVATISANGD   4   IAQVATISA     1.000       Sequence      0.968454     0.03       NA      0.521225        177.73     5.06      <=SB
165    DRB1_0404      PEEIAQVATISANG    3   IAQVATISA     1.000       Sequence      0.963894     0.04       NA      0.530235        161.22     4.46      <=SB
163    DRB1_0404    TTPEEIAQVATISANGD   5   IAQVATISA     1.000       Sequence      0.961216     0.05       NA      0.529004        163.38     4.54      <=SB
165    DRB1_0404     PEEIAQVATISANGDK   3   IAQVATISA     1.000       Sequence      0.961015     0.05       NA      0.517583        184.87     5.34      <=SB
164    DRB1_0404    TPEEIAQVATISANGDK   4   IAQVATISA     1.000       Sequence      0.956910     0.06       NA      0.528436        164.39     4.57      <=SB
164    DRB1_0404      TPEEIAQVATISANG   4   IAQVATISA     1.000       Sequence      0.956207     0.06       NA      0.600724         75.19     1.40      <=SB
269    DRB1_0404      KPLVIIAEDVDGEA    3   VIIAEDVDG     0.993       Sequence      0.948596     0.08       NA      0.505143        211.50     6.34      <=SB
163    DRB1_0404    TTPEEIAQVATISANG    5   IAQVATISA     1.000       Sequence      0.945086     0.09       NA      0.534767        153.50     4.17      <=SB
166    DRB1_0404      EEIAQVATISANGD    2   IAQVATISA     1.000       Sequence      0.943589     0.09       NA      0.500720        221.87     6.72      <=SB
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C

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          10        20        30        40        50        60        70        80        90       100       110       120       130
RVMAPRALLL LLSGGLALTE TWACSHSMRY FDTAVSRPGR GEPRFISVGY VDDTQFVRFD SDAASPRGEP RAPWVEQEGP EYWDRETQKY KRQAQADRVS LRNLRGYYNQ SEDGSHTLQR MSGCDLGPDG
      140       150       160       170       180       190       200       210       220       230       240       250       260
RLLRGYDQSA YDGKDYIALN EDLRSWTAAD TAAQITQRKL EAARAAEQLR AYLEGTCVEW LRRYLENGKE TLQRAEPPKT HVTHHPLSDH EATLRCWALG FYPAEITLTW QRDGEDQTQD TELVETRPAG
      270       280       290       300       310       320       330       340       350       360
DGTFQKWAAV VVPSGQEQRY TCHMQHEGLQ EPLTLSWEPS SQPTIPIMGI VAGLAVLVVL AVLGAVVTAM MCRRKSSGGK GGSCSQAACS NSAQGSDESL ITCKA
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D

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Pos          MHC          Peptide   Of       Core  Core_Rel       Identity      Score_EL %Rank_EL Exp_Bind      Score_BA  Affinity(nM) %Rank_BA  BindLevel
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 31    DRB1_0404         FDTAVSRPG    0   FDTAVSRPG     1.000       Sequence      0.000005    95.00       NA      0.060992      25844.64    95.00
 31    DRB1_1301         FDTAVSRPG    0   FDTAVSRPG     1.000       Sequence      0.000023    95.00       NA      0.050647      28905.55    95.00
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E

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# Allele: DRB1_0404
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Pos          MHC          Peptide   Of       Core  Core_Rel       Identity      Score_EL %Rank_EL Exp_Bind      Score_BA  Affinity(nM) %Rank_BA  BindLevel
-----------------------------------------------------------------------------------------------------------------------------------------------
 53    DRB1_0404     DTQFVRFDSDAASPR   3   FVRFDSDAA     0.560       Sequence      0.974225     0.02       NA      0.611689         66.78     1.13      <=SB
 53    DRB1_0404     DTQFVRFDSDAASPRG  4   VRFDSDAAS     0.547       Sequence      0.972675     0.02       NA      0.570965        103.76     2.37      <=SB
 54    DRB1_0404      TQFVRFDSDAASPR   3   VRFDSDAAS     0.593       Sequence      0.969734     0.03       NA      0.575867         98.40     2.19      <=SB
 24    DRB1_0404     CSHSMRYFDTAVSRPG  4   MRYFDTAVS     1.000       Sequence      0.969205     0.03       NA      0.611767         66.73     1.12      <=SB
 54    DRB1_0404      TQFVRFDSDAASPRG  3   VRFDSDAAS     0.720       Sequence      0.969006     0.03       NA      0.624042         58.43     0.85      <=SB
 25    DRB1_0404     SHSMRYFDTAVSRPG   3   MRYFDTAVS     1.000       Sequence      0.968748     0.03       NA      0.665200         37.43     0.33      <=SB
 25    DRB1_0404      SHSMRYFDTAVSRP   3   MRYFDTAVS     1.000       Sequence      0.966083     0.03       NA      0.603414         73.04     1.33      <=SB
 52    DRB1_0404     DDTQFVRFDSDAASPR  4   FVRFDSDAA     0.587       Sequence      0.963547     0.04       NA      0.568155        106.96     2.47      <=SB
 52    DRB1_0404     DDTQFVRFDSDAASPRG 5   VRFDSDAAS     0.527       Sequence      0.963304     0.04       NA      0.562911        113.21     2.70      <=SB
 25    DRB1_0404     SHSMRYFDTAVSRPGR  3   MRYFDTAVS     1.000       Sequence      0.960412     0.05       NA      0.615737         63.92     1.02      <=SB
 23    DRB1_0404     ACSHSMRYFDTAVSRPG 5   MRYFDTAVS     1.000       Sequence      0.958123     0.06       NA      0.626565         56.85     0.81      <=SB
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Figure 11 - An overview of derived peptides of the human proteins and the NetMHCIIpan-4.0 prediction results. (**A**) One of the identified peptides highlighted in the sequence of HSPD1, a 60 kDa heat shock protein. (**B**) Prediction results of presented peptides by allele DRB1∗04:04 derived from HSPD1 using NetMHCIIpan-4.0 spanning 13 to 18 amino acids. (**C**) One of the identified peptides highlighted in the sequence of HLA-C, a HLA class I protein. (**D**) Prediction results of FDTAVSRPG by alleles DRB1∗04:04 and DRB1∗13:01 with a length of 9 amino acids derived from HLA-C. (**E**) Prediction results of presented peptides by DRB1∗04:04 derived from HLA-C spanning 13 to 18 amino acids.

The combined abundance of the two identified peptides in the purified peptide eluate from SFM resulted in 6.2 x 10^5. However, the supplementary materials include an LC/MS-MS chromatogram displaying numerous peaks, exhibiting, a consistent recurring pattern with intensities reaching up to 10^8 (Figure S1 and S2). Upon closer examination of the highest relative abundance m/z value for each peak, it was determined that the consistent difference of 44 between peaks indicates their origin as PolyEthylene Glycol (PEG), a polymer compound composed of repetitive units of ethylene glycol. This PEG contamination might suppress the intensity of low-abundant peptides resulting in a lower number of unique peptides.

RPMI 1640 supplemented with 10% FCS and self-presenting peptides

Before subjecting the eluate from a notably larger cell count grown in the FCS-supplemented culture (Table 1) to peptide purification and LC/MS-MS analysis, a Western blot analysis was conducted to validate the presence of HLA-DR molecules (Figure 12). Lane E, corresponding to the eluate, exhibited a signal at the expected mass of the HLA-DR molecule, confirming the presence of HLA-DR molecules within the eluate. Subsequently, a MS analysis,

which resulted in the identification of 225 unique peptides derived from human proteins, was performed.

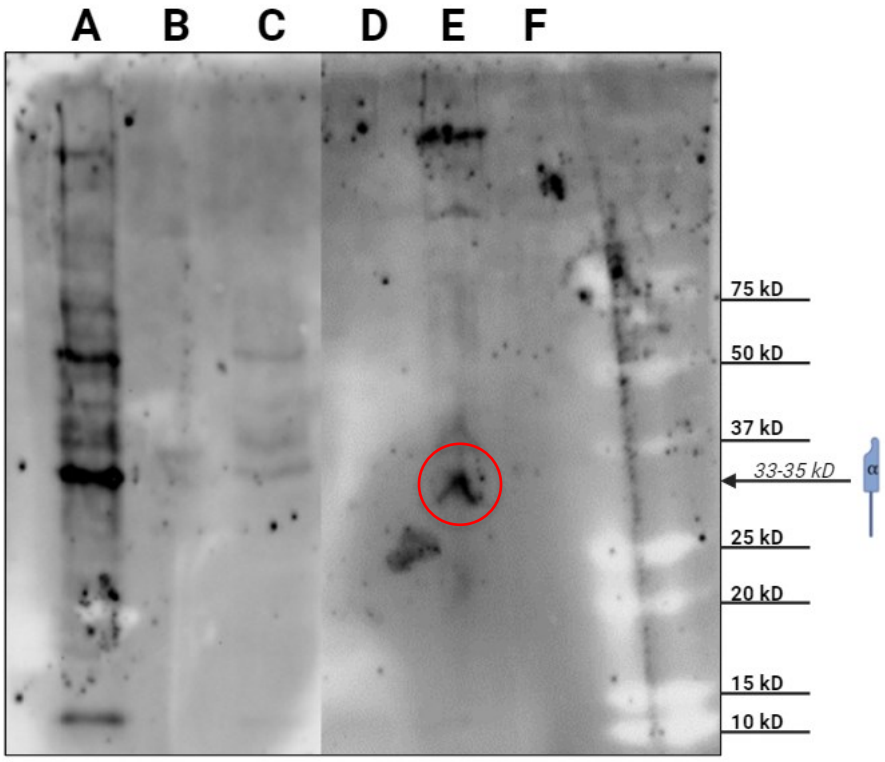

Figure 12 **-** A Western Blot analysis of the immunoprecipitation experiment using the anti-human HLA-DR antibody (clone: L243) cross-linked to Protein A/G magnetic beads in order to isolate HLA-DR peptide complexes from JY lysate cultivated in FCS supplemented medium. Lanes: (A) Lysate, (B) Wash beads prior loading the lysate, (C) Flowthrough, (D) Wash after removing flowthrough, (E) Eluate and (F) Wash after elution.

Remarkably, HLA class I proteins emerged as the predominant class, yielding the highest number of unique human protein-derived peptides in this sample (figure 13A). HLA-A and HLA-C are normally involved in the presentation of peptides derived from cytosolic proteins, this observation suggests a potential interplay wherein HLA-I molecules on the cell surface are internalized and processed into peptides for subsequent presentation by HLA-II molecules. Additionally, among the top five human proteins contributing to the most unique peptides, cytoskeletal keratins were notable. Keratin, primarily associated with palmar skin tissue, is not naturally presented by B cells. The other proteins in the top five included Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Heat shock cognate 71 kDa protein (HSPA8), and Phosphoglycerate kinase 1 (PGK1) respectively. Collectively, these top five human proteins accounted for approximately 35% of all identified peptides and contributed to over half of the total intensity (figure 13B). Particularly, GAPDH, a key enzyme in the glycolytic pathway, made the most substantial contribution to the overall intensity among the top five proteins from which the peptides were derived.

To gain deeper insights into the peptide characteristics of HLA-DR presented peptides, a frequency distribution analysis of peptide lengths with the identified peptides, was conducted (Figure 13C). This analysis revealed a normal distribution, with the majority of peptides falling within the range of 13 to 18 amino acids. These findings underscore the success in isolating HLA-DR-presented peptides derived from human proteins. Furthermore, when analysing the MS data against a bovine protein library, two alpha-S1-casein derived peptides, RPKHPIKHQ and RPKHPIKHQGLPQ, were identified.

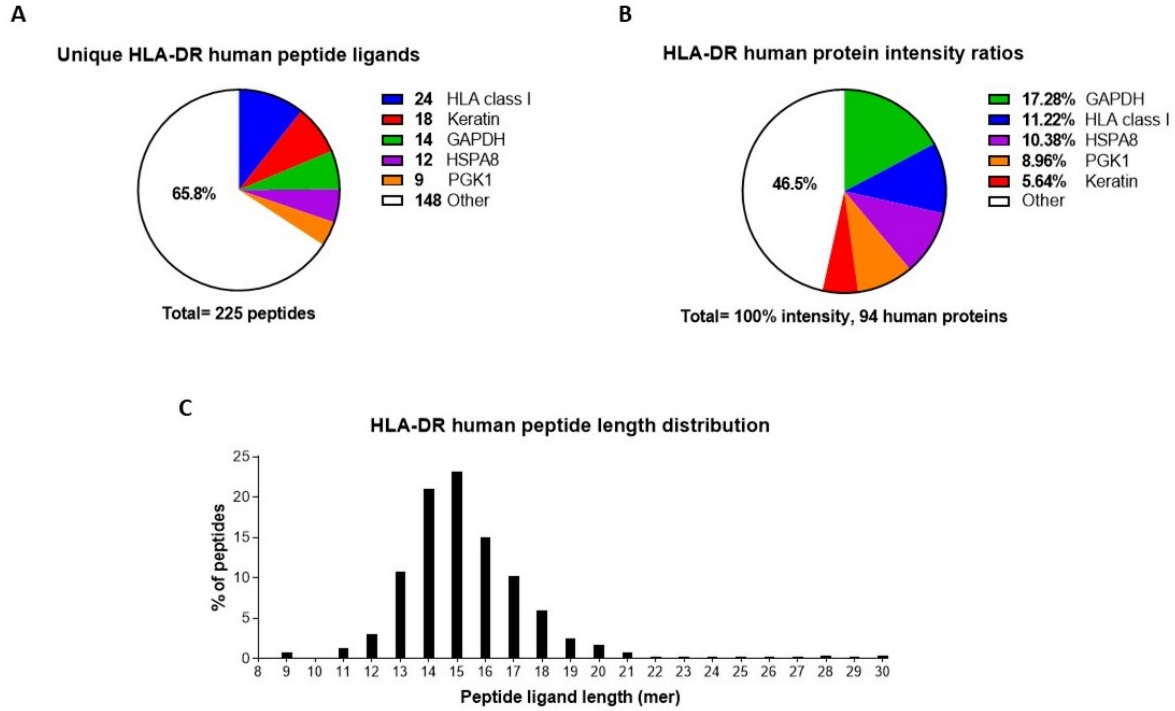

Figure 13 - An overview of the identified human protein derived self-presenting peptides presented by HLA-DR on the cell-surface of cells grown in FCS-supplemented medium and their characteristics. (A) A pie chart showing the top 5 most abundant human proteins. (B) A pie chart showing the intensity distribution in the top 5 human proteins. (C) A frequency distribution of the peptide length.

RPMI 1640 supplemented with 10% FCS and BLG derived peptides

The research findings provide valuable insights into the immunoprecipitation and identification of peptides associated with HLA-DR complexes in a BLG-incubated FCS-supplemented cell culture. The study aimed to enhance the yield of unique peptides derived from human- and bovine proteins in BLG-incubated culture and explore the diversity of peptides presented by HLA-DR molecules on JY cells. The previous Western blot analyses (Figure 10 and 12) revealed that HLA-DR complexes were still present in the flowthrough of the lysate, indicating the potential for further peptide isolation. To improve the immunoprecipitation yield, the lysate was incubated with cross-linked beads for three times. Figure 14 demonstrates that increasing the number of lysate loading cycles led to a decrease

in the presence of HLA-DR molecules in the subsequent flowthroughs, indicating successful enrichment of HLA-DR-associated peptides.

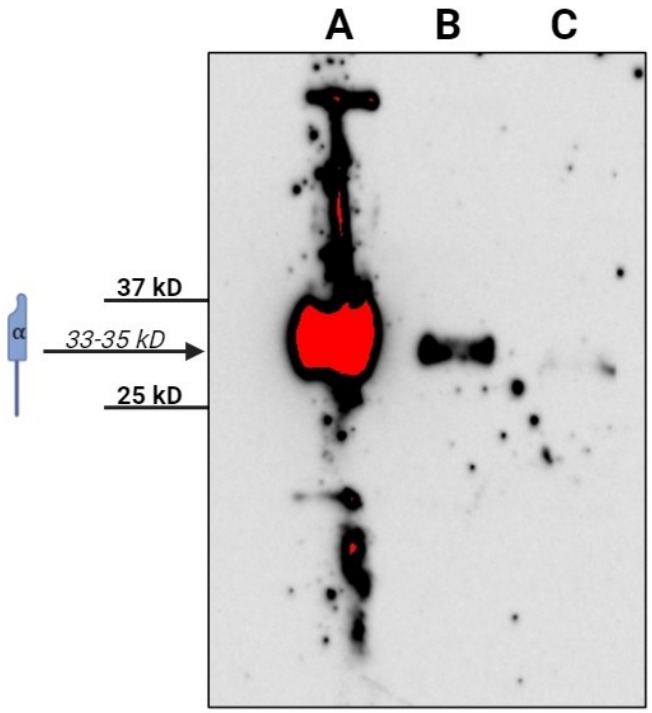

Figure 14 - A Western Blot analysis of the lysate and flowthroughs after loading the flowthrough twice on the beads. The anti-human HLA-DR antibody (clone: L243) cross-linked to Protein A/G magnetic beads was used in order to isolate HLA-DR peptide complexes from JY lysate cultivated in FCS supplemented medium in presence of BLG. Lanes: (A) Lysate, (B) First flowthrough, and (C) Second flowthrough.

After pooling all the eluates, which contained HLA-DR peptide complexes from the lysate and subsequent flowthroughs, a total of 483 unique human protein-derived peptides was identified using MS (Figure 15A). This analysis was conducted with a cell count of 1.25 x 108 cells. Interestingly, the top five human proteins contributing to the most peptides in the BLG-incubated sample were the same as those in the FCS-supplemented culture in the absence of BLG. However, the BLG-incubated sample exhibited a much more diverse portfolio of unique peptides, including two unique CD74-derived peptides not found in the FCS-supplemented culture. Remarkably, while the top five human proteins accounted for only 25% of the total number of unique peptides, they contributed to nearly half of the total intensity (Figure 15B). This suggests that despite the increased diversity of human proteins, a select few play a significant role in peptide presentation via HLA-DR molecules. Moreover, the length distribution of peptides originating from human proteins exhibited a pattern closely resembling a normal distribution, with the majority falling within the range of 13 to 18 amino acids, consistent with the prior findings of self-presenting peptides (Figure 15C).

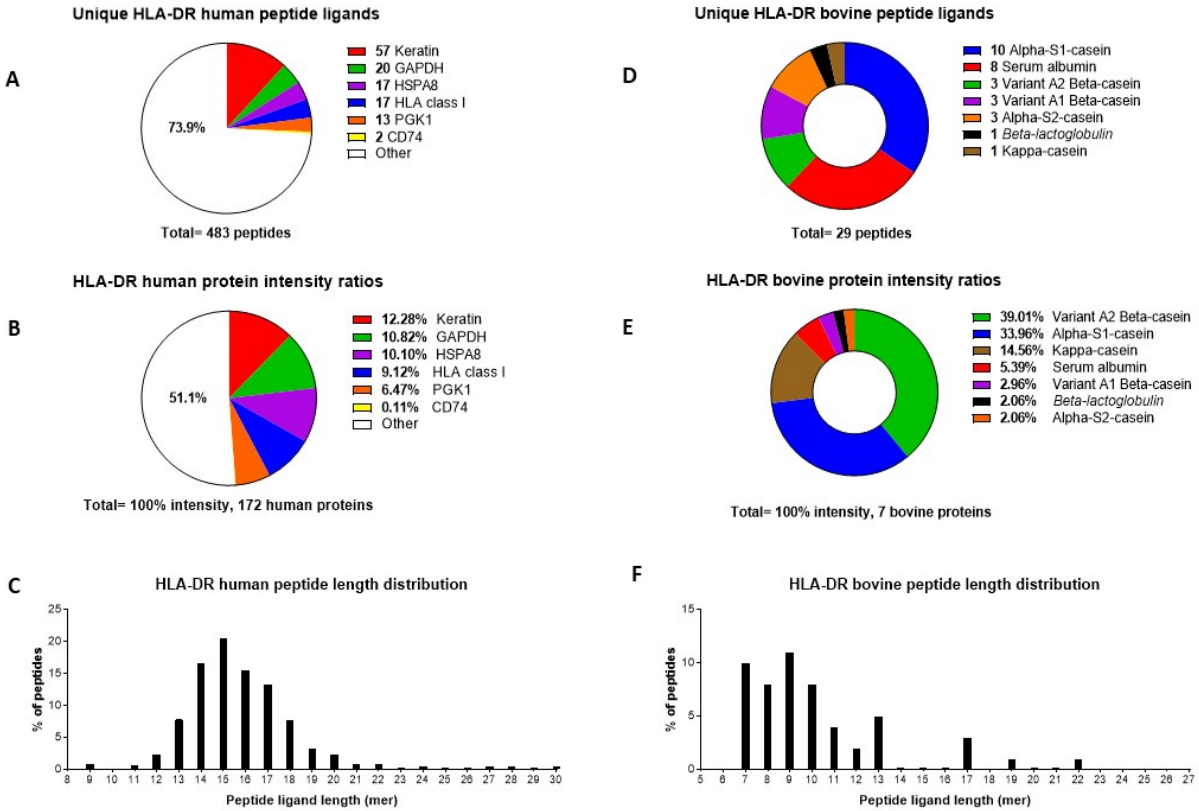

Figure 15 - An overview of the identified human protein derived self-presenting peptides and whey bovine protein derived peptides presented by HLA-DR on the cell-surface of cells grown in FCS-supplemented medium and their characteristics. (A) A pie chart showing the top 5 most abundant human proteins. (B) A pie chart showing the intensity distribution in the top 5 human proteins. (C) A frequency distribution of the human protein derived peptide length. (D) A pie chart showing the identified whey bovine proteins. (E) A pie chart showing the intensity distribution in the whey bovine proteins. (F) A frequency distribution of the whey bovine derived peptide length.

Subsequently, when analysing the MS data against a bovine protein library, a total of 29 unique peptides was identified (Figure 15D). Most of these peptides were derived from casein, with varying numbers associated with 10 Alpha-S1-casein derived peptides and 1 Kappa-casein derived peptide. Additionally, eight serum albumin-derived peptides and one BLG-derived peptide were identified. The A2 beta-casein variant exhibited the highest intensity, despite only three unique peptides being identified compared to Alpha-S1-casein, that also contributed substantially to the total bovine protein intensity, with its 10 unique peptides accounting for almost 34% of the intensity (Figure 15E). In contrast, the BLG-derived peptide, a 9-mer (VEELKPTPE), contributed to only 2% of the total intensity. Notably, the distribution of peptide lengths for bovine protein-derived peptides differed from human protein derived-peptides as the majority of these peptides were found to be between 7 and 13 amino acids in length (Figure 15F). This observation underscores the distinct characteristics of bovine protein processing and presentation.

In summary, this study demonstrates the successful isolation and identification of self-presenting peptides (Figure 16) as well as peptides from bovine proteins in BLG-incubated cell cultures. These peptides are processed and presented on the cell surface through HLA-DR molecules by JY cells. These findings provide insights into immune responses, potentially impacting our understanding of CMA and dietary antigen-related immunology.

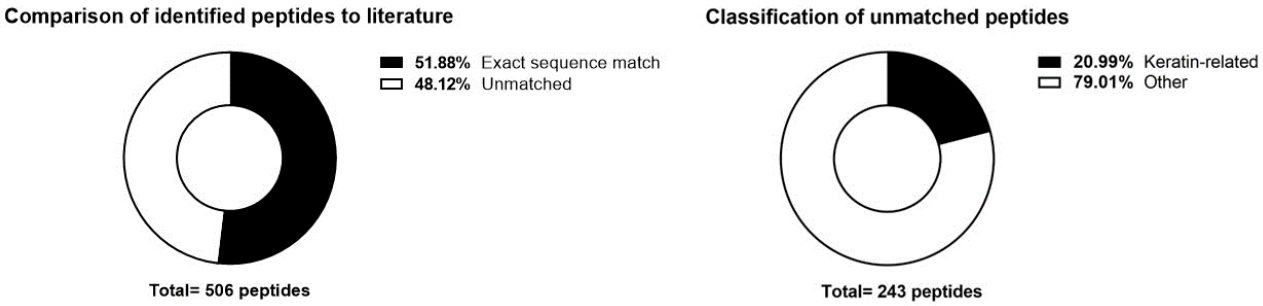

Figure 16 - A comparison between the self-presenting peptides identified in this study and those found in the work of Demmers et al. The graph reveals that over 50% of the identified peptides exhibited an exact match with those documented in the literature. Among the remaining 50%, approximately 20% were related to keratin but did not correspond to previously reported sequences.

DISCUSSION

Understanding the HLA-II pathway plays a crucial role in the pursuit of more effective immunotherapies for FAs involving glycan-modified allergens. This knowledge enhances the comprehension of critical aspects such as antigen uptake, processing, and the potential presentation of therapeutic glycopeptides. These glycopeptides, characterized by specific carbohydrate structures, have the potential to selectively target Siglec receptors on antigen-presenting cells, thereby mitigating immune cell activation in FA responses (16-24). The central objective of this study was to establish a robust methodology for the analysis of HLA-DR-presented peptides derived from glycan-modified beta-lactoglobulin (BLG) in B-lymphocytes. A robust methodology to identify HLA-DR presented peptides was developed. It resulted in the identification of more than 500 unique self-presenting peptides, of which 50% matched exactly with peptides described in literature for the JY B cell line (32). Additionally, a peptide derived from BLG was identified upon applying unmodified BLG to the cell culture. This peptide matched with a peptide identified in the infant formula of Danone using fragment ion-match analysis

To prevent potential interference from bovine proteins with the identification of BLG-derived peptides, we explored the use of SFM as an alternative to FCS-supplemented media in cell culture. FCS contains a complex mixture, including bovine-related proteins, which can lead to false positives in peptide identification when doing research into allergenic bovine proteins. Microscopic analysis revealed significant differences in cell morphology depending on the growth medium employed, with cells cultured in SFM displaying reduced cellular size compared with those in FCS-supplemented medium. Furthermore, HLA-DR expression was higher for JY cells cultured in FCS-supplemented medium compared with JY cells cultured in SFM. The lack of growth factors in the SFM medium caused the changes in morphology and HLA-DR expression required for the cell culture of JY cells. Notably, SFM culture was exclusively maintained in ExCellerate B Cell Media, devoid of additional cytokines or growth factor supplements. The manufacturer recommends the CellXVivo Human B Cell Expansion Kit, emphasizing the critical role of cytokines or growth factor supplements such as IL-4 in cell expansion. In this study, EBV-immortalized B lymphocytes were used, and the requirement for a cytokine mix was not as crucial as it would be when using primary B cells. Surprisingly, the FCS-supplemented culture with BLG displayed a lower mean fluorescence intensity (MFI) and a heterogeneous distribution of HLA-DR expression, contrary to expectations of upregulation upon antigen exposure (34). To conclusively validate the flow cytometry data,

replication of experiments and the inclusion of a viability stain, to eliminate any influence from dead cells, are essential. Although dead cells mostly result in a stronger background.

DMP has been successfully employed as a cross-linker for the anti-human-HLA-DR antibody L243 to Protein A/G magnetic beads. Immunoprecipitation without cross-linked antibody to the beads resulted in excessive antibody elution from the beads. Cross-linking the anti-human-HLA-DR antibody L243 with Protein A/G magnetic beads using DMP aimed to enhance the reusability of the beads and reduce antibody co-elution. Although the suitability of DMP as a cross-linker for L243 antibody and both Protein A Sepharose and Protein A/G beads has been well-documented in the literature, this study represents the first successful demonstration of DMP cross-linking of an IgG antibody to Protein A/G magnetic beads (31,32). The optimized immunoprecipitation protocol not only increases practicality but also significantly reduces experiment preparation time as it eliminates the need for column preparation as outlined by Purcell et al. (31).

Over 500 unique self-presented peptides were identified in this study, with at least 50% matching the exact sequence of peptides described in the literature for JY cells (32)Both the FCS-supplemented culture, in the presence and absence of BLG, showed presentation of HLA-I derived peptides, suggesting a potential interplay wherein HLA-I molecules on the cell surface are internalized and processed into peptides for subsequent presentation by HLA-DR molecules(32,36,37). However, there were some inconsistencies with existing literature. For instance, while a previous study by Demmers et al. found that CD74-derived peptides contributed significantly to the intensity, this study identified only 2 unique CD74-derived peptides, contributing to 0.10% of the total intensity (32).

Unique peptides primarily originating from different casein proteins were identified in the FCS-supplemented cultures, both in the presence and absence of BLG. It has to be confirmed if these peptides derived from FCS or is related to a cross-contamination by re-using the magnetic beads. While the frequency distribution of self-presenting peptide length was normally distributed, with the majority of peptides falling into the expected peptide length for HLA-II presented peptides (13 to 18 amino acids)(32,38), bovine-related peptides were ranging between 7 and 13 amino acids. This discrepancy may be related to the digestion sites recognized by the endosomal proteases involved in the HLA-II pathway. A better understanding of the involved enzymes and their digestion sites is necessary to comprehend the potential peptides that can be presented.

Moreover, one BLG-derived peptide (VEELKPTPE) was identified that partially overlaps with a highly allergenic peptide (LLDAQSAPLRVYVEELKP) described by

Meulenbroek et al. (39). Although, proteins with only one unique peptide match are typically not considered reliable, a fragment ion match spectrum analysis resembles the identical peptide in the Optimix control sample from Nutricia and indicates that the identified BLG peptide is not a false positive. The choice to incubate the culture with a 12.5 ug/ml concentration for 24 hours was based on a publication by Bourdeau et al. (40). Nevertheless, variations in incubation time and concentration could potentially increase the number of peptides and should be explored.

Despite the success of the developed method in identifying self-presenting peptides and a BLG-related peptide, several areas of optimization are warranted. The most critical limitation to address is contaminations that may suppress the intensity of low-abundant peptides. Keratin-derived peptides, considered contaminants as B-cells do not naturally present them, can be minimized by using gloves to avoid skin contact with materials (41). Overcoming PEG contamination, which was consistently observed in all chromatograms of the analysed samples, is particularly challenging due to its primary source, NP-40 in the lysis buffer. To reduce PEG contamination, alternative detergent-free cell lysis methods should be explored, such as sonification or freeze-thaw cycles (42,43). However, it is essential to ensure that these methods do not compromise the integrity of HLA-DR-peptide complexes and ensure their preservation throughout the process. Another approach to reduce PEG contamination is to introduce strong cation exchange (SCX) as a purification step before sample analysis, as C18-based SPE is not able to remove PEG from the sample (44).

Another limitation is the high cell count required for peptide identification. Sensitive measurements have improved our understanding of peptide presentation, but further advancements in sensitivity are necessary due to the high cell numbers currently needed (45). The results in this study indicate that cell count matters, as higher cell counts in the FCS-supplemented cultures resulted in the identification of more peptides. Loading the lysate multiple times on the beads increased the number of peptides even with a lower cell count (1.25×10^8 vs 2×10^8). However, this approach may become less practical when applied to primary cells. Adding cytokines such as IL-4 and interferon gamma could potentially increase the expression of HLA-DR molecules and their corresponding peptides, offering a solution to identify more peptides of interest. Additionally, The NetMHCIIpan 4.0 prediction tool proved to be a reliable tool for confirming the reliability of identified peptides in the SFM culture. This tool can predict strong binders of BLG-derived peptides and even if they are present in very low concentrations, they still can be manually searched for in the raw data (46).

In conclusion, this study provides valuable insights into the HLA-II pathway and the presentation of allergen-derived peptides. While the methodology has shown promise in identifying self-presenting and a BLG-related peptide, optimization is essential to overcome contamination issues and the need for a high cell count. Further exploration of SFM in combination with cytokines and growth factors, varying incubation times and concentrations for recombinant BLG, the use of detergent-free cell lysis methods, repeated loading of lysates on re-usable beads, and the implementation of SCX as a purification method are recommended for follow-up research. These enhancements will significantly contribute to higher yield of BLG-derived (glyco)peptides resulting in an enhanced comprehension of the immunological mechanisms implicated in FA and hopefully facilitate the development of more efficacious immunotherapies in the future.

SUPPLEMENTARY MATERIALS

This report includes additional materials provided in separate appendices.

APPENDIX A: Supplementary figures
APPENDIX B: Identified peptides derived from human proteins
APPENDIX C: Identified peptides derived from whey bovine proteins
APPENDIX D: Revised Protocol for Isolating HLA-DR Presented Peptides from B-Lymphocytes for Mass Spectrometry Analysis

REFERENCES

1. Renz, H., Allen, K. J., Sicherer, S. H., Sampson, H. A., Lack, G., Beyer, K., & Oettgen, H. C. (2018). Food allergy. Nature Reviews Disease Primers, 4(1). https://doi.org/10.1038/nrdp.2017.98

2. De Martinis, M., Sirufo, M. M., Suppa, M., & Ginaldi, L. (2020). New perspectives in food allergy. International Journal of Molecular Sciences, 21(4), 1474. https://doi.org/10.3390/ijms21041474

3. Tang, M. L., & Mullins, R. J. (2017). Food allergy: is prevalence increasing? Internal Medicine Journal, 47(3), 256–261. https://doi.org/10.1111/imj.13362

4. Nwaru, B. I., Hickstein, L., Panesar, S. S., Muraro, A., Werfel, T., Cardona, V., Dubois, A., Halken, S., Hoffmann-Sommergruber, K., Poulsen, L. K., Roberts, G., Van Ree, R., Vlieg-Boerstra, B., & Sheikh, A. (2013). The epidemiology of food allergy in Europe: a systematic review and meta-analysis. Allergy, 69(1), 62–75. https://doi.org/10.1111/all.12305

5. Spolidoro, G. C. I., Amera, Y. T., Ali, M. M., Nyassi, S., Lisik, D., Ioannidou, A., Rovner, G., Khaleva, E., Venter, C., Van Ree, R., Worm, M., Vlieg-Boerstra, B., Sheikh, A., Muraro, A., Roberts, G., & Nwaru, B. I. (2022). Frequency of food allergy in Europe: An updated systematic review and meta-analysis. Allergy, 78(2), 351–368. https://doi.org/10.1111/all.15560

6. Yue, D. T., Ciccolini, A., Avilla, E., & Waserman, S. (2018). Food allergy and anaphylaxis. Journal of Asthma and Allergy, Volume 11, 111–120. https://doi.org/10.2147/jaa.s162456

7. Herbert, L., Shemesh, E., & Bender, B. G. (2016). Clinical management of psychosocial concerns related to food allergy. The Journal of Allergy and Clinical Immunology: In Practice, 4(2), 205–213. https://doi.org/10.1016/j.jaip.2015.10.016

8. Poulsen, L. K. (2005). In search of a new paradigm: mechanisms of sensitization and elicitation of food allergy*. Allergy, 60(5), 549–558. https://doi.org/10.1111/j.1398-9995.2005.00821.x

9. Vickery, B. P., Chin, S., & Burks, A. W. (2011). Pathophysiology of food allergy. Pediatric Clinics of North America, 58(2), 363–376. https://doi.org/10.1016/j.pcl.2011.02.012

10. Roche, P. A., & Furuta, K. (2015). The ins and outs of MHC class II-mediated antigen processing and presentation. Nature Reviews Immunology, 15(4), 203–216. https://doi.org/10.1038/nri3818

11. Cresswell, P. (1996). Invariant chain structure and MHC Class II function. Cell, 84(4), 505–507. https://doi.org/10.1016/s0092-8674(00)81025-9

12. Satitsuksanoa, P., Daanje, M., Akdis, M., Boyd, S. D., & Van De Veen, W. (2020). Biology and dynamics of B cells in the context of IgE-mediated food allergy. Allergy, 76(6), 1707–1717. https://doi.org/10.1111/all.14684

13. Iweala, O. I., & Burks, A. W. (2016). Food allergy: our evolving understanding of its pathogenesis, prevention, and treatment. Current Allergy and Asthma Reports, 16(5). https://doi.org/10.1007/s11882-016-0616-7

14. Duan, S., Koziol-White, C., Jester, W. F., Nycholat, C. M., Macauley, M., Panettieri, R. A., & Paulson, J. C. (2019). CD33 recruitment inhibits IgE-mediated anaphylaxis and desensitizes mast cells to allergen. Journal of Clinical Investigation, 129(3), 1387–1401. https://doi.org/10.1172/jci125456

15. Wood, R. A. (2016). Food allergen immunotherapy: Current status and prospects for the future. The Journal of Allergy and Clinical Immunology, 137(4), 973–982. https://doi.org/10.1016/j.jaci.2016.01.001

16. Wolfert, M. A., & Boons, G. (2013). Adaptive immune activation: glycosylation does matter. Nature Chemical Biology, 9(12), 776–784. https://doi.org/10.1038/nchembio.1403

17. Rudd, P. M., Elliott, T., Cresswell, P., Wilson, I. A., & Dwek, R. A. (2001). Glycosylation and the immune system. Science, 291(5512), 2370–2376. https://doi.org/10.1126/science.291.5512.2370

18. Avci, F. Y., Li, X., Tsuji, M., & Kasper, D. L. (2013). Carbohydrates and T cells: A sweet twosome. Seminars in Immunology, 25(2), 146–151. https://doi.org/10.1016/j.smim.2013.05.005

19. Doungstop, B. K., Van Vliet, S. J., Van Ree, R., De Jong, E. C., & Van Kooyk, Y. (2021). Carbohydrates in allergy: from disease to novel immunotherapies. Trends in Immunology, 42(7), 635–648. https://doi.org/10.1016/j.it.2021.05.002

20. Smith, B., & Bertozzi, C. R. (2021). The clinical impact of glycobiology: targeting selectins, Siglecs and mammalian glycans. Nature Reviews Drug Discovery, 20(3), 217–243. https://doi.org/10.1038/s41573-020-00093-1

21. Bénac, O., Gaudin, M., Ors, M., Roy, A. L., Blanc, H. R., & Soulas, C. (2018). Preclinical development of first-in-class antibodies targeting Siglec-9 immune checkpoint for cancer immunotherapy. Cancer Res., 78(13 Suppl.), 2713.

22. Bochner, B. S., O'Sullivan, J. A., Chang, A., & Youngblood, B. (2023). Siglecs in allergy and asthma. Molecular Aspects of Medicine, 90, 101104. https://doi.org/10.1016/j.mam.2022.101104

23. Duan, S., Arlian, B. M., Nycholat, C. M., Wei, Y., Tateno, H., Smith, S. A., Macauley, M., Zhu, Z., Bochner, B. S., & Paulson, J. C. (2021). Nanoparticles displaying allergen and SIGLEC-8 ligands suppress IGE-FCERI–Mediated anaphylaxis and desensitize mast cells to subsequent antigen challenge. Journal of Immunology, 206(10), 2290–2300. https://doi.org/10.4049/jimmunol.1901212

24. Avci, F. Y., Li, X., Tsuji, M., & Kasper, D. L. (2011). A mechanism for glycoconjugate vaccine activation of the adaptive immune system and its implications for vaccine design. Nature Medicine, 17(12), 1602–1609. https://doi.org/10.1038/nm.2535

25. Perdicchio, M., Ilarregui, J. M., Verstege, M. I., Cornelissen, L. a. M., Schetters, S., Engels, S., Ambrosini, M., Kalay, H., Veninga, H., Haan, J. M. M. D., Van Berkel, L. A., Samsom, J. N., Crocker, P. R., Sparwasser, T., Berod, L., Garcia-Vallejo, J. J., Van Kooyk, Y., & Unger, W. W. J. (2016). Sialic acid-modified antigens impose tolerance via inhibition of T-cell proliferation and de novo induction of regulatory T cells. Proceedings of the National Academy of Sciences of the United States of America, 113(12), 3329–3334. https://doi.org/10.1073/pnas.1507706113

26. Flom, J. D., & Sicherer, S. H. (2019). Epidemiology of Cow's milk allergy. Nutrients, 11(5), 1051. https://doi.org/10.3390/nu11051051

27. Høst, A. (2002). Frequency of cow's milk allergy in childhood. Annals of Allergy Asthma & Immunology, 89(6), 33–37. https://doi.org/10.1016/s1081-1206(10)62120-5

28. Sinai, T., Goldberg, M. R., Nachshon, L., Amitzur-Levy, R., Yichie, T., Katz, Y., Monsonego-Ornan, E., & Elizur, A. (2019). Reduced Final Height and Inadequate Nutritional Intake in Cow's Milk-Allergic Young Adults. The Journal of Allergy and Clinical Immunology: In Practice, 7(2), 509–515. https://doi.org/10.1016/j.jaip.2018.11.038

29. Liu, M., Thijssen, S., Hennink, W. E., Garssen, J., Van Nostrum, C. F., & Willemsen, L. E. M. (2023). Oral pretreatment with β-lactoglobulin derived peptide and CpG co-encapsulated in PLGA nanoparticles prior to sensitizations attenuates cow's milk allergy development in mice. Frontiers in Immunology, 13. https://doi.org/10.3389/fimmu.2022.1053107

30. Gouw, J. W., Jo, J., Meulenbroek, L. A., Heijjer, T. S., Kremer, E., Sandalova, E., Knulst, A. C., Jeurink, P. V., Garssen, J., Rijnierse, A., & Knippels, L. (2018). Identification of peptides with tolerogenic potential in a hydrolysed whey-based infant formula. Clinical & Experimental Allergy, 48(10), 1345–1353. https://doi.org/10.1111/cea.13223

31. Purcell, A. W., Ramarathinam, S. H., & Ternette, N. (2019). Mass spectrometry–based identification of MHC-bound peptides for immunopeptidomics. Nature Protocols, 14(6), 1687–1707. https://doi.org/10.1038/s41596-019-0133-y

32. Demmers, L. C., Wu, W., & Heck, A. J. R. (2021). HLA Class II presentation is specifically altered at elevated temperatures in the B-Lymphoblastic cell line JY. Molecular & Cellular Proteomics, 20, 100089. https://doi.org/10.1016/j.mcpro.2021.100089

33. Meiring, H. D. (2002). Nanoscale LC–MS(n): technical design and applications to peptide and protein analysis. Analytical Science Journals. https://doi.org/10.1002/1615-9314(20020601)25:9

34. Mommaas, A. M., Wijsman, M. C., Mulder, A. A., Van Praag, M. C. G., Vermeer, B., & Koning, F. (1992). HLA class II expression on human epidermal langerhans cells in situ: Upregulation during the elicitation of allergic contact dermatitis. Human Immunology, 34(2), 99–106. https://doi.org/10.1016/0198-8859(92)90035-l

35. Stricher, F., Macri, C., Ruff, M., & Muller, S. (2013). HSPA8/HSC70 chaperone protein. Autophagy, 9(12), 1937–1954. https://doi.org/10.4161/auto.26448

36. Grommé, M., Uytdehaag, F. G. C. M., Janssen, H., Calafat, J., Van Binnendijk, R. S., Kenter, M., Tulp, A., Verwoerd, D., & Neefjes, J. (1999). Recycling MHC class I molecules and endosomal peptide loading. Proceedings of the National Academy of Sciences of the United States of America, 96(18), 10326–10331. https://doi.org/10.1073/pnas.96.18.10326

37. Montealegre, S., & Van Endert, P. M. (2019). Endocytic recycling of MHC Class I molecules in non-professional antigen presenting and dendritic cells. Frontiers in Immunology, 9. https://doi.org/10.3389/fimmu.2018.03098

38. Montealegre, S., & Van Endert, P. M. (2019). Endocytic recycling of MHC Class I molecules in non-professional antigen presenting and dendritic cells. Frontiers in Immunology, 9. https://doi.org/10.3389/fimmu.2018.03098

39. Meulenbroek, L. A., Van Esch, B. C., Hofman, G. A., Jäger, C., Nauta, A. J., Willemsen, L. E. M., Bruijnzeel-Koomen, C. A., Garssen, J., Van Hoffen, E., & Knippels, L. (2013). Oral treatment with β-lactoglobulin peptides prevents clinical symptoms in a mouse model for cow's milk allergy. Pediatric Allergy and Immunology, 24(7), 656–664. https://doi.org/10.1111/pai.12120

40. Bourdeau, T., Affolter, M., Dupuis, L., Panchaud, A., Lahrichi, S., Merminod, L., Martin-Paschoud, C., Adams, R. L., Nutten, S., & Blanchard, C. (2021). Peptide Characterization and Functional Stability of a Partially Hydrolyzed Whey-Based Formula over Time. Nutrients, 13(9), 3011. https://doi.org/10.3390/nu13093011

41. Keller, B. O., Sui, J., Young, A. L., & Whittal, R. M. (2008). Interferences and contaminants encountered in modern mass spectrometry. Analytica Chimica Acta, 627(1), 71–81. https://doi.org/10.1016/j.aca.2008.04.043

42. Hopper, J. T. S., Yu, Y. T., Li, D., Raymond, A., Bostock, M. J., Liko, I., Mikhailov, V. A., Laganowsky, A., Benesch, J. L. P., Caffrey, M., Nietlispach, D., & Robinson, C. V. (2013). Detergent-free mass spectrometry of membrane protein complexes. Nature Methods, 10(12), 1206–1208. https://doi.org/10.1038/nmeth.2691

43. Shen, W., Berning, K., Tang, S. W., & Lam, Y. W. (2020). Rapid and Detergent-Free decellularization of cartilage. Tissue Engineering Part C-methods, 26(4), 201–206. https://doi.org/10.1089/ten.tec.2020.0008

44. Kanaujia, P. K., Tak, V., Pardasani, D., Gupta, A., & Dubey, D. K. (2008). Application of cation-exchange solid-phase extraction for the analysis of amino alcohols from water and human plasma for verification of Chemical Weapons Convention. Journal of Chromatography A, 1185(2), 167–177. https://doi.org/10.1016/j.chroma.2008.01.080

45. Leichtle, A. B., Dufour, J., & Fiedler, G. M. (2013). Potentials and pitfalls of clinical peptidomics and metabolomics. Schweizerische Medizinische Wochenschrift. https://doi.org/10.4414/smw.2013.13801

46. Karosiene, E., Rasmussen, M., Blicher, T., Lund, O., Buus, S., & Nielsen, M. (2013). NetMHCIIpan-3.0, a common pan-specific MHC class II prediction method including all three human MHC class II isotypes, HLA-DR, HLA-DP and HLA-DQ. Immunogenetics, 65(10), 711–724. https://doi.org/10.1007/s00251-013-0720-y