Septin Cytoskeleton Dynamics on B cell Activation and Antibody Responses

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Abstract

B cells perform a vital role in keeping the host organism safe against pathogens by recognizing exogenous and endogenous antigens via their B cell receptors (BCRs) and producing antibodies against said antigens. After an antigen binds to a BCR, the BCR-antigen complex is internalized and processed further. Several known mechanisms and players such as the actin cytoskeleton and endocytosis help explain how the BCR-antigen complex is internalized and processed; however, the whole picture remains unclear. In this study we focused on the septin cytoskeleton, particularly on the role of septin-7, and how its function affects B cells' activation and antibody responses.

To infer the possible role of septin cytoskeleton in B cell activation, forchlorfenuron (FCF) was used to inhibit the dynamics of septin filaments in antigen activated mouse lymphoma cells. These cells were then probed for phosphorylated proteins to examine whether the septin cytoskeleton plays a role in cell signalling. To examine organelle dynamics septin cytoskeleton dynamics were again inhibited with FCF after antigen activation to examine how the septin filaments associate with other cytoskeletal components, notably the microtubules. To look at humoral responses, mice that were either septin-7 wild type (WT) or conditional septin-7 knock-out in B cells (cKO) were immunized with an immunogen that would elicit T cell help. After immunization, the changes in the serum antibody levels were tracked.

This study contains preliminary results showing that inhibition of septin cytoskeleton dynamics leads to a decrease on BCR activation upon antigen stimulation, which is reversible upon washing off the inhibitor FCF. At another level, we confirm that septin inhibition impairs B cells' ability to spread over an antigen- coated surface, thus making it difficult for the B cells to form immune synapses. Furthermore, we can tentatively verify that in *in vivo* septin-7 conditional knockout mouse model the T-dependent response in B cells is diminished.

Key words: septins, cytoskeleton, B cells, immunology, antibodies.

List of abbreviations

AIS – adaptive immune system

AID - activation-induced cytidine deaminase

APC – antigen presenting cell

BCR – B cell receptor

BSA – bovine serum albumin

cKO – condition knockout

cRPMI – complete RPMI

cSMAC – central supramolecular activation cluster

DBB – donkey blocking buffer

DMSO – dimethyl sulfoxide

dSMAC – distal SMAC

ECD – extracellular domain

ELISA - enzyme-linked immunosorbent assay

Erk ½ - extracellular signal-regulated kinases 1 and 2

FCF – forchlorfenuron

FCS – fetal calf serum

FOXO – forkhead box, subgroup O

GC – germinal center

HRP – horseradish peroxidase

Ig – Immunoglobulin

IIS – innate immune system

IL - interleukin

IS – immune synapse

ITAM – immunoreceptor tyrosine activated motif

KO – knockout

MHC – major histocompatibility complex

MTOC – microtubule organizing center

NP-KLH – Nitrophenyl-Keyhole Limpet Hemocyanin

NF-κB - nuclear factor kappa B

PBS – Phosphate-buffered saline

PFA – paraformaldehyde

pSMAC – peripheral SMAC

PVDF – polyvinylidene fluoride

RAG1 – recombination activation gene 1

RAG2 – recombination activation gene 2

SHM – somatic hypermutation

SMAC – supramolecular activation cluster

TBS – Tris-buffered saline

TBST – Tris-buffered saline with 0,05 % Tween

Tfr – T follicular regulatory cell

Tfh – T follicular helper cell

TMD – transmembrane domain

WT – wild type

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1 Introduction

1.1. The immune system - origins

The immune system is complex in how each individual factor is organized to protect the host organism from pathogens. One factor in such complexity is the large and varied group of cells that together form the immune system. Most vertebrate animals have cells of both innate and adaptive immunity, the former being the first line of defense, and the latter a slower, more sophisticated system.

1.1.1. Innate immunity system – the faithful and fast first line

The innate immune system (IIS) is not only involved in keeping pathogens out of the host organism, but it also plays an important role in the regular upkeep of tissues. All eukaryotes, even protozoans, have an innate immune system in some form. However, the latter group may at most have receptor-like peptides that recognize, bind, and eliminate bacteria. Considering protozoans in this case akin to more derived animals, such as mammals, only demonstrates how conserved this pathogen recognition system is. It is even suggested that the white blood cells' ability to phagocytose was originally derived from amoebas (Dzik., 2010; Martins et al., 2023).

The innate immune system is called such because it exists remains unchanged throughout the life of the host organism, meaning that it reacts to a predetermined, and relatively limited, set of stimuli. This feature may seem like it would debilitate the IIS and prevent it from working efficiently against fast-mutating pathogens. Yet when a pathogen evolves, not every part of it changes, as some structures are far more prone to mutations than others. The innate immunity system targets various structures that remain unchanged, thus making up a line of defense that is reliable against a myriad of threats. The IIS also detects whether the host's tissues are damaged or not, as healthy cells express antigens that do not activate the IIS, while the opposite is true in damaged cells. This gives the IIS the role of waste control and repair in tissues (Kaur and Secord., 2021). This truly makes the IIS an invaluable asset in the host organism's arsenal in defence against pathogens from the outside and harm done on the inside.

Simple protozoans have simple IISs, and more derived organisms have more derived IISs, and not all of them are the same. These more derived organisms possess specialized cells, such as macrophages and neutrophils, that hunt down and eliminate the threats to the host. Humans

have, in addition to the two aforementioned types of leukocytes, natural killer cells, eosinophils, basophils, mast cells, and dendritic cells. However, these patrolling and phagocytizing cells are only a fraction of the entire IIS. Most of the IIS is made up of passive tissue, such as skin, and mucous membranes lining all the various inner surfaces of the body that are still in contact with the outside, be it with air, urine, or food passing through. Keratinized skin is a wall pathogens can scarcely get through, and mucous membranes, while permeable, contain receptors that recognize pathogens. These endothelium-bound receptors are called pattern recognition receptors, and their ligands are the common and conserved molecules on pathogens, much like on the leukocytes of the IIS and their receptors (Li and Wu., 2021).

Other immediate protections are the blood-brain barrier for the central nervous system, and some chemical obstructions, such as the low pH of the stomach in some organisms. However, when these protective barriers are breached by pathogens, the mobile cells of the IIS are the first to arrive on site and create an inflammatory response. The IIS also plays a role in mobilizing the adaptive immune system (Sonnenberg and Hepworth., 2019).

1.1.2. Adaptive immune system – the specialized and slow second line

The adaptive immune system (AIS) first began to evolve in the last common ancestor of jawed vertebrates around 500 million years ago, meaning that almost all jawed vertebrates share a similar immune system. However, while jawless vertebrates have an adaptive immune system of their own, it bears some similarities to the AIS of jawed vertebrates, including lymphoid cells, which raises questions about whether the basics for an AIS existed even earlier than was thought before. It has been noted that even pre-vertebrates have lymphoid cells, yet the number of these cells in each such individual is not great enough to establish a robust AIS like in larger organisms (Flajnik and Kasahara., 2010).

As to how the evolution of the AIS in jawed vertebrates could have happened, besides the increased size of the organisms, several changes in the genome had to take place for lymphocytes to achieve the level of variety in their receptors as is observed today. One such change is the insertion of a transposon near or within an immunoglobulin superfamily-encoding gene in the genome. This transposon is recognized as recombination-activation genes 1 and 2 (RAG1 and RAG2) which mediate recombination of the heavy and light chain genes of the immunoglobulins (Igs), creating a variety of structures with affinities for different antigens. The

RAGs code for proteins that, together in a complex, cleave the DNA and create double strand breaks around certain gene segments. After this cleaving action, the gene segments are joined together in a new order, and thus the DNA sequence is repaired. This event is called a V(D)J recombination event, where the name stems from the three most important kinds of gene segments involved: variable (V), diversity (D), and joining (J). Out of these three, however, the D segment is not always present, so the three segments thusly marked as V(D)J when discussed. This recombination event may happen multiple times per lymphocyte, especially if later the resulting immunoglobulin reacts to self-antigens. This mechanism of repeatedly breaking and repairing DNA is understandably dangerous for an organism, and thus there are certain regulating factors for the RAG proteins not to wreak havoc on the genome. Two of these factors pertain to how the chromatin is stored: the areas of the DNA that the RAGs bind to are more easily accessible for the proteins than segments that the RAGs should not bind to. The RAGs also bind only to certain sequences that are unlikely to be found elsewhere in the genome. Lastly, for the RAGs to be truly effective, they must appear in high amounts in a cell. This high expression occurs only early in the development of lymphocytes, thus ensuring that the proteins do not affect other kinds of cells (Schatz and Ji., 2011; Nemazee., 2017). Of the two transposon genes, RAG1 is the major factor in creating the diversity seen in Igs, while RAG2 interacts with histones at the active transcription sites. Another vital change in the genome to accommodate the emergence of AIS was a twofold whole-genome duplication. This would have made the organism more resistant to new mutations and given the materials for a wider range of variability in the immune system (Flajnik and Kasahara., 2010).

Two major lineages of cells make up the AIS: T cells and B cells. Both originate from hematopoietic stem cells in the bone marrow. However, they undergo distinct differentiation pathways: B cells develop and mature in the bone marrow, while T cells migrate to and mature in the thymus.

1.2. The components of the adaptive immune system

1.2.1. T cells

T cells are generated from hematopoietic stem cells which, after maturation, go through a negative selection for self-antigens. Immature hematopoietic precursor cells migrate from the bone marrow to the thymus where they develop into their mature variants through three distinct steps. This sequence is quite an intricate process and happens entirely in the thymus. During

the first step, the hematopoietic stem cell may commit to becoming a T cell, or alternatively a B cell, a myeloid cell, or an innate T cell. Once the stem cell has committed to the non-innate T cell lineage, it may become an αβ or a γδ T cell. The two T cell lines are different, and only the αβ T cell line moves from the cortex of the thymus to the medulla, where the final step of the maturation process occurs. T cells can be divided into two groups according to the presence of a certain co-receptor: CD4+ cells, or CD8+ cells. Both receptors are expressed on the surface of a premature T cell, only right before maturation, in the medulla of the thymus, do the cells commit to one or the other. The presence of either of these receptors defines whether the T cell is a helper, or a killer T cell respectively (Sun et al., 2023).

The CD4+, or the helper T cells, can be further divided into two gross subcategories, these being the conventional helper T cells, and regulatory T cells, the latter of which can be distinguished by the CD25 co-receptor they have on their membranes. In short, they have the opposite roles when it comes to modulating the immune system's activity. Helper T cells increase the activity, while regulatory T cells suppress it through multiple separate pathways, most of which are shared by the immune system (Dikiy et al., 2023). Of course, there are multiple other types of helper T cells, though their differences are too minute to detail in this paper. Killer T cells are also not an entirely homogenous class of cells, as they can be largely sorted into similar subcategories as helper T cells, differentiated from each other by minor details. In addition to the conventional killer T cells, however, there are regulatory T cells that express the CD8 co-receptor that defines killer T cells (Koh et al., 2023).

The T cell-defining co-receptors are of particular importance, as they respond to major histocompatibility complexes (MHC). The CD4 co-receptor responds to the ligand bound to MHC class II, while the CD8 co-receptor responds to the MHC class I-bound ligand (Germain., 2002). The MHC class I structures are found on the surface of virtually all cells, where they present self-antigens. These can be normal, non-pathogenic particles that belong to the presenting cell's regular function, or pathogenic particles from a viral infection. If the ligand particle on the MHC class I structure is of pathogenic origin, the binding CD8+ T cell is prompted to kill the presenting cell. Class II MHC structures are found almost exclusively on the surface of APCs, and they are vital for the recognition of exogenous, non-self-antigens. If the ligand bound to the MHC class II structure is of pathogenic origin, the CD4+ cell that recognizes and binds to it is prompted to recruit B cells for an immune response. The same antigens the T cells react to could be found on the surface of a pathogen, for example, but this

would not cause a similar reaction as when bound to an MHC structure. Thus, the MHC I and II are truly important in inducing an immune reaction. However, this is only the case in αβ T cells, as γδ cells do not require binding to an MHC-ligand complex to generate an immune response (Harryvan et al., 2021; Sun et al., 2023).

1.2.2. B cells

B lymphocytes emerge from the pluripotent hematopoietic stem cells in the bone marrow. This maturation can be defined by three different stages; the pro-B cell, the pre-B cell, and lastly the mature B cell which will further differentiate in a response for a certain antigen in the peripheral lymphoid tissues. This progression of the cells' maturation is influenced by the cytokines present in the microenvironment of the marrow, released by mature B cells. Of these, interleukin 7 (IL-7) is especially necessary to develop a pre-B cell into maturity, as it has been shown that the absence of IL-7 or the receptor for IL-7 arrests B cell maturation partially or entirely to the pre-B cell stage. This is seen in drastic reduction of the number of the immature, and thus mature, B cells as the cells fail to proliferate (Carsetti., 2000).

After reaching their mature state, B cells can be divided into three different kinds, these being naïve B cells, plasma cells, and memory B cells. Of these, naïve B cells have not been exposed to any antigens yet and are circulating in the host's body, waiting for an antigen exposure that will activate them. When they encounter an antigen, they differentiate into plasma cells and later memory B cells, which are the hallmarks of B cell biology. Plasma cells can secrete high-affinity antibodies against a specific antigen, which bind to the antigen and opsonize it, this process marks the antigen to be destroyed by phagocytes. Such an antigen may be a surface protein of a pathogen, in which case the entire pathogen becomes marked. Memory B cells, in turn, provide the immunological memory for a quicker response against the same antigen in a re-infection (Abbas, A., et al., 2024, p. 144-166).

Mature B cells may also be differentiated by their location in the body. Follicular B cells reside in the secondary lymphatic tissues. Marginal zone B cells are located in the marginal sinus of the spleen, affording them the capability to rapidly react to antigens in blood. B1 cells are found in mucosal tissues that, like marginal zone B cells, are close to possible pathogens and their antigens. The B cells at these sites are slightly different, for example marginal zone B cells and B1 cells can effectively respond to T cell-independent antigens, an ability suitable to them

regarding their location at the perimeters of the protected tissues. Follicular B cells, in turn, are not as effective when it comes to responding to T cell-independent antigens (Nutt et al., 2015).

Antigens come in many of forms, they can be proteins secreted by other cells of the body, surface receptors on pathogens, or chemicals found in cosmetics, or even metals. Indeed, there are various antigens even in the host organism the B cells could react to in the case of autoimmune disorders, ranging from type 1 diabetes to rheumatoid arthritis to multiple sclerosis. B cells recognize antigens through their transmembrane B cell receptor (BCR). The BCR has two major structures: the antigen-recognizing homodimeric immunoglobulin that may be any of the five major Ig classes (A, D, E, G, or M), and the signal-transducing heterodimeric Igα/β immunoglobulin. The two structures are non-covalently bound and work intrinsically with each other (Dong et al., 2022).

The BCR can be divided into three domains: the extracellular domain (ECD) which recognizes antigens, the transmembrane domain (TMD) which anchors the receptor to the cell membrane, and the intracellular domain which transduces the signal from an activated BCR inside the cell. The intracellular domain contains the Igα/β co-receptor's immunoreceptor tyrosine-based activation motifs (ITAMs) that become phosphorylated and kickstart the intracellular signaling cascade after BCR activation (Dong et al., 2022).

Although all specialized BCRs are unique in the way that they bind to a single kind of antigen, each one of them follows the same basic blueprint of a heavy chain and a light chain that are joined together by disulfide bridges. The heavy chain is embedded into the cell membrane, while the light chain is the extracellular domain. The heavy-light chain monomers are joined together via disulfide bridges, thus forming the homodimer (Dong et al., 2022).

To achieve the specificity for a new kind of antigen, the B cell goes through somatic hypermutation (SHM) in its variable region that is made up of the V(D)J gene segments. SHM happens by the cell deliberately causing itself to repair its DNA via activation-induced cytidine deaminase (AID) causing a cytosine-to-uracil conversion. Since uracils are not compatible with the DNA structure, the cell's DNA repair mechanisms activate. However, the change in the bases leads to double strand breaks, which leads to complete change in the affected segments, instead of returning the strands to their original state. The V(D)J segments are present in both the heavy and light chains in dispersed sections, meaning that the hypervariable region still has

some non-variable sections throughout it. The variable-invariable structure is important for preserving the integrity of the receptor (Abbas, A., et al., 2025, p. 76-99; Stavnezer et al., 2008).

As mentioned, the heavy chain of the BCR can be of any immunoglobulin class, but as to which class the cell expresses is not arbitrary. All B cells are naïve after maturation, and these B cells express IgM and IgD BCRs. Plasma and memory B cells express all types of BCRs. To achieve this necessary change to the Ig class to better combat infections, B cells must undergo a process called class switching. This is a cellular event where the heavy chain of the BCR changes, leaving the variable region largely intact. The variable region of the BCR is important, but so is the invariable heavy chain, as it determines many of the receptor's functions and affinities in relation to other white blood cells and proteins, for example how soluble the antibody is, and if it successfully prompts other cells. The Ig heavy chain is expressed by a continuous series of genes, this gene series, when unaltered, expresses the IgM isotype. The class switch happens by intrachromosomal deletional recombination event triggered by AID, causing parts of this gene series to be removed entirely. This leads to the expression of the other Ig isotypes, depending on how much of the series is deleted. However, the mechanism which causes the IgD to be expressed also requires alternative splicing of the genes (Stavnezer et al., 2008).

Although producing antibodies against pathogens is the primary function of B cells, it is not all they do. B cells also prompt T cells' function by presenting antigens, develop and repair secondary lymphatic tissues, inhibit the function of the immune system when its activity is unnecessary, and prompt immature B cells to mature (Abbas, A., et al., 2024, p. 144-166; Shen et al., 2015, Shahaf et al., 2016).

1.3. The immune synapse – formation and significance

The immune synapse is a unique and transient structure between a lymphatic cell and an APC. While T cells also form synapses with APCs, they are slightly different than B cell – APC synapses due to the differences between BCRs and T cell receptors. The IS' formation is a complicated event which includes distinct steps. B cells can recognize antigens with their BCRs in two forms: soluble or tethered to the surface of antigen presenting cells (APCs). Even though B cells are able to capture antigens circulating freely in a liquid medium, such as the interstitial fluid or blood plasma, the most prominent form of antigen recognition *in vivo* is the latter one. Binding to antigens tethered to an APCs surface lowers the threshold for the B cell's activation,

leading to higher activation with lower amounts or affinity of antigen, compared to soluble recognition (Batista et al., 2001). B cells interact with the presented antigens by stretching their cell membrane over the APC's surface to capture as many antigens with their BCRs as possible. Soon after, the B cell's membrane contracts, causing the BCR-antigen complexes to gather in one central structure that is called an immune synapse (IS). The IS is an intricately coordinated structure that can be divided into three main sections: the central supramolecular activation cluster (cSMAC), the peripheral SMAC (pSMAC), and the distal SMAC (dSMAC). The pSMAC, which is rich in actin and adhesion molecules, surrounds the cSMAC to which all BCR-antigen complexes are gathered. The dSMAC encircles both the cSMAC and pSMAC, containing only actin filaments (Kuokkanen et al., 2015). Of course, the IS is not merely a superficial structure, but one that is anchored to actin filaments within the cell.

1.4. The cytoskeleton

The cytoskeleton is largely known to consist of three major components: actins, intermediate filaments, and microtubules. These are highly dynamic and constantly changing structures that do far more than give the cell shape. Actins, for example, are responsible for the movement of cells, pushing against the cell membrane on the inside and thus creating filopodia and lamellipodia with which a cell drags itself along a substrate. On the other hand, microtubules are largely responsible for determining cell polarity, intracellular movements of vesicles and cell organelles, even separating duplicated chromosomes from each other during mitosis. Intermediate filaments anchor cell organelles to the outer membrane of the cell, along with stabilizing actins and microtubules (Goldmann, 2018). In this study, I will mainly focus on the dynamics of the actin cytoskeleton and microtubules.

1.4.1. Actin cytoskeleton – movement and shape

The actin cytoskeleton is one of the main elements of cell morphology, intracellular trafficking, and cell migration. The filaments are helical in structure, and capable of forming branches. The basic building block of the actin cytoskeleton is called G-actin, whereas the filaments themselves are called F-actin. The F-actin has a fast-growing barbed end, and a slow-growing pointed end. Although both the filament ends are named as growing, G-actins at the pointed end may come apart, thus shortening the F-actin. The growth of F-actin may also be stopped by capping proteins that attach to the barbed end. There are other structures that are associated with the actin filaments, such as the Arp2/3 complex, which is made up of seven different

subunits, and together they act as a nucleation site for F-actin, and cap the pointed end of the filament. The Arp2/3 complex is also what allows F-actin to form branches, as it connects to the filaments in near-perpendicular angles and nucleates new F-actin from those positions (Mullins and Pollard., 1999; Bolger-Munro et al., 2019).

The barbed end of the filament is the one growing towards the direction of the cell's movement, pushing against the cell membrane and thus creating protrusions that may attach to an extracellular surface. However, there are two major types of protrusion cells may utilize to generate movement: lamellipodia and filopodia. Lamellipodia are wide structures, in which the actin cytoskeleton is branched to generate force on as much of the cell's surface area as possible. Cells utilizing lamellipodia for movement are fast but imprecise in their direction. On the other hand, filopodia are relatively thin and sometimes present as very long protrusions that are used for slow but exact movements. A cell may also use filopodia to sense targets that are not in its immediate vicinity. Filopodia often emerge from lamellipodia, meaning that these two structures are not necessarily separate from each other (Svitkina., 2018).

Not only are actin filaments important for cell migration and morphology, but they are also involved in phagocytosis and intake of proteins from the cell membrane. In the case of B cells, the actin cytoskeleton is vital for immune synapse formation. The actin cytoskeleton causes the B cell to stretch over the APC, after which it constricts to bring the BCR-antigen complexes into a cluster. The actins' work does not end there, however. The BCR-antigen complexes need to be internalized and transported deeper into the cell for processing.

It has been noted that actins form concentrated spots, or foci, in cells. These formations are important in that they aid in BCR-antigen complex internalization by generating forces that pull the complexes inward into the cell. These foci are generated by the Arp2/3, a complex formed of seven different proteins (Roper et al., 2019). This suggests that the heptameric complex has a major role in internalizing the antigens on the B cell's surface. Moreover, as a nucleation site for actin branches, Arp2/3 is largely responsible for the formation of lamellipodia. This bears importance to the case of the IS, as the actin structures associated with the synapse's formation resemble lamellipodia. Similarly, Arp2/3 has a responsibility in bringing the newly formed BCR-antigen complexes together into the cSMAC of the IS, as inhibiting the protein heptamer prevents full IS formation. Instead, without Arp2/3, the BCR-antigen complexes gather into small clusters but do not form an IS (Bolger-Munro et al., 2019). This would mean that Arp2/3

is deeply involved in the generation of the IS, a notion which only underscores the significance of the actin cytoskeleton in said process.

1.4.2. Microtubules – organization and structure

Consisting of repeating αβ subunits, microtubules are wide compared to the other cytoskeletal filaments, approximately 25 nanometers in width. They are hollow and rigid filaments that have quite a few purposes in a cell's function, such as internal organelle trafficking and aiding in mitosis. Microtubules are in a near constant, dynamic flux where they grow from the plus end. The filaments can disassemble in a process called *microtubule catastrophe*, where the growing filament rapidly shortens (Gardner et al., 2013). At their minus ends, microtubules are connected to a structure called microtubule organizing center (MTOC). MTOCs act as nucleation sites for microtubules, since spontaneous formation of these filaments is exceedingly rare. Their organization and polarization are crucial for directing microtubules towards the B cell immune synapse, enabling proper antigen extraction and signaling. In addition, microtubules serve as highways for vesicle trafficking (Goodson and Jonasson., 2018). A proof of this concept is seen in cells that lack the ability of microtubule nucleation, as these cells appear unpolarized or move without clear direction (de Forges et al., 2012). Moreover, microtubules have very specific roles depending on the cell utilizing them. These filaments form the backbone of certain cellular features, such as the cilia and flagella, in which they are relatively stable. Microtubules are also vital in neurons, as they are at the core of axons. This is important not only in a structural sense, but as mentioned, microtubules also act as highways for transporting organelles and compounds inside the cell. This enables an elongated cell, like a neuron, to quickly transport matter inside itself (Goodson and Jonasson., 2018).

Despite being hollow, no trafficking of compounds happens inside the filaments. However, the lumen of microtubules is not entirely useless, as the acetylation of the microtubule subunits appears to take place in it. The significance of acetylation in microtubule function is still somewhat contested, but it is believed to stabilize the tubulin filaments, thus preventing them from degenerating. It may also have a role in the microtubule-associated motor protein attachment and detachment from the filaments (Soppina et al., 2012). The transportation of cellular structures takes place on the outer surface of the filaments via motor proteins such as dynein and kinesin (Goodson and Jonasson., 2018).

1.4.3. Septin cytoskeleton – modulation and synergy

In recent years, a fourth less-known family of proteins belonging to the cytoskeleton, septins, has risen into prominence. These are highly conserved GTP-proteins. Septins were first discovered in 1970 in *Saccharomyces cerevisiae,* where they were identified as a part of the yeast's budding process (Hartwell et al., 1970). Since then, they have been discovered in all eukaryotic kingdoms of life except for plants, where they are entirely absent (Spiliotis and Nakos., 2021).

Although septins are not a new discovery by any means, for a long time they were not paid much attention. Their later discovery in mammalian cells made them more significant in the eyes of researchers, and now they are known to be a source of many pathological conditions when functioning improperly. For example, absence of septin-2 in epithelial cells prevents the formation of columnar epithelium due to insufficient guidance of microtubule growth, it has also been observed to upkeep epithelial integrity through interactions with F-actin (Spiliotis et al., 2008; Dolat et al., 2014). Septin-4 has been isolated from colorectal cancer tissue samples, while the protein is absent in healthy tissue (Zieger et al., 2000; Tanaka et al., 2001). Homozygous septin-4, and heterozygous septin-12 absence in male mice have been linked to infertility due to immotile sperm and deformed sperm, respectively (Kissel et al., 2005; Lin et al., 2009). Elevated septin-5 expression is associated with platelet dysfunction leading to excessive bleeding (Kato et al., 2004). Gradual septin-9 depletion has been observed in different kinds of tumors through their progression, and its increased methylation is used as a biomarker for colon cancer in its early stages (Tóth et al., 2011; Dolat et al., 2014).

Septins are incredibly well conserved across species, but they do have differences in the structures and number of septins. To date, 13 distinct septin monomers have been identified in humans (septins 1-12 and 14), and they are sorted into four family groups (SEPT2, SEPT3, SEPT6 and SEPT7) according to their sequences' similarity to each other. Together, the septin monomers form either heterohexameric complexes or hetero-octameric complexes. These hexamers and octamers then form even larger hetero-oligomeric structures. The basic building blocks are not formed arbitrarily, however, as different monomers have different affinities for each other. The septin arrangements 2/6/7/9, 2/6/7, 2/7/9, 5/3/7, and 5/7/11 are dubbed the canonical conformations, although other non-canonical ones have been discovered (Kim et al., 2011; Dolat et al., 2014).

Interestingly, all the canonical oligomer conformations include septin-7, which has been discovered to be one of the few whose absence in mouse knockout models leads to an embryonically lethal condition. Septin-7 has been noted to be unique, as it differs from other septin monomers and forms its own family group SEPT7, in which it is the only member. It has been suggested that septin-7 is necessary for septin cytoskeletal filaments' formation, as without it septins cease to form oligomers (Wang et al., 2018). Two other septins whose absence leads to embryonic lethality are monomers 9 and 11. Of the three embryonically lethal knockout models, the one without septin-7 has been noted to stop developing in utero the earliest, marking the absolute importance of said monomer for basic cell function, most likely pertaining to mitosis (Mostowy and Cossart., 2012; Dolat et al., 2014). If the fact that septin-7 is always present in the canonical heterohexamers is considered, perhaps septin-7's importance lies in its ability to aid in septin oligomerization.

Since not all septin knockouts lead to equally drastic outcomes, it is fair to conclude that not all of the monomers are equally as important. Other monomers of a family group can fulfil the same roles as another monomer in the same family could, for example, knocking out septin-3 entirely does not affect nerve function in mice, despite septin-3 being specific to neurons. This is perhaps explained by septin-9, which is able to effectively replace septin-3, and coincidentally they are both in the SEPT2 family group. Septin-11 has also been observed to replace septin-6, at least in the 2/6/7 hexameric configuration, both septins 6 and 11 belong to the SEPT6 family group. This ability to replace other monomers of the same family group has been termed as "Kinoshita's rule" after Makoto Kinoshita, who discovered this septin capability (Kinoshita., 2003; Güler and Mostowy., 2023).

Not all 13 different septin monomers are expressed in every cell, either. Septins 2, 7, and 9 are expressed in nearly all tissues, septins 4, 5, 8, and 10 are not quite as ubiquitous as the previous three, and lastly septins 1, 3, 12, and 14 are expressed only in very limited tissues. Septin-1 is expressed in lymphoid and epidermal tissues, septin-3 is found in neuronal tissues, septin-12 is exclusive to testes, and septin-14 is expressed both in neuronal and testicular tissues (Mostowy and Cossart., 2012; Dolat et al., 2014).

Septins have been described to perform many roles, such as acting as scaffolding for a cell's internal protein and endosome distribution, as diffusion barriers, and they frequently interact

with both microtubules and F-actin. In fact, it has been noted that when septin octamers specifically are absent, cells lose much of their stiffness, a characteristic that actin filaments are responsible of. Furthermore, septins have been observed to link actin filaments to the plasma membrane. The absence of septins also disrupts vesicle transport, a cellular function largely maintained by microtubules (Mostowy and Cossart., 2012; Güler and Mostowy., 2023). This further proves that septins and the actin cytoskeleton are interlinked and cooperative in upholding the internal structure of cells. Septins are distinct from the other cytoskeletal components in that in addition to simple filaments, they may also form rings and cage-like structures. These cages are rather curious, as they have been observed to trap bacteria that have invaded the host cell, with the impairment of septins leading to increased cell infection rate. In this way, septins may directly take part in defense against pathogens (Mostowy et al., 2010; Mostowy and Cossart., 2012).

As far as the immune synapse formation and importance is understood, several questions regarding the spatio-temporal activation and antigen processing remain unanswered. The actin cytoskeleton and other endolysosomal machinery alone have not explained the process, and thus it is speculated that perhaps septins play a role in this important step of immune system activation.

1.5. B cell's internal signaling cascades after activation

The ITAMs at the intracellular tails of the homodimers that make up the BCR become phosphorylated upon BCR activation. This is the beginning of a vast signaling cascade within the B cell that will eventually lead to cell differentiation, antibody secretion, and memory formation.

1.5.1. Syk

Syk is an intracellular protein tyrosine kinase, which is almost immediately downstream from BCR. It is vital in B cell maturation, as totally Syk-deficient cells never reach the fully matured state. The kinase has a major role in many functions of the mature B cell, these ranging from antibody responses to differentiation of the B cells into plasma cells, and in the case of memory B cells, secondary antibody response and survival. *In vitro* it has been observed that when the ability to express Syk is removed from naïve, mature B cells, their number in their host drops rapidly, and up to 80% of these cells are lost within 21 days. In the surviving B cells, after the

loss of ability to express Syk, the cells may still activate and proliferate following stimulation from an antigen (Ackermann et al., 2015).

Syk was chosen as a protein to probe for in this study's immunoblot experiment due to its multifaceted and vital role in B cell function. If disrupting septin dynamics negatively affects Syk's activity in the cell, it would highlight the importance of a functioning septin cytoskeleton.

1.5.2. HS1

HS1, or hematopoietic lineage cell-specific protein 1, is immediately downstream from Syk, and it is phosphorylated by the tyrosine kinase upon BCR activation. The relationship between Syk and HS1 is tight, as has been deduced from B cells without Syk, where HS1 does not phosphorylate upon BCR activation. After its phosphorylation, HS1 promotes rearrangements in the actin cytoskeleton (Hao et al., 2004). In addition, HS1 may dictate if a B cell proliferates or apoptoses (Yamanashi et al., 1997).

HS1 was chosen as a protein to probe in this study's immunoblot experiment due to its importance in actin cytoskeleton modulation. If septin inhibition also inhibits HS1 phosphorylation, it could be evidence of a less active actin cytoskeleton, which could lead to impaired antigen processing and antibody secretion.

1.5.3. Akt

Akt, also known as Protein Kinase B (PKB), is a group of three highly similar protein kinases (Akt1, Akt2, and Akt3). Due to their similarities in structure and function, the three kinases will be referred to as a singular Akt. In the BCR activation cascade it is rather close downstream, between the receptor and Akt being the major signaling molecules PI3K and PIP_3. Akt's activation leads to many different functions of the cell, such as ATP generation and gene transcription, kicking in. Akt is an important part of many different signaling cascades further downstream (Manning and Toker., 2017). By the virtue of Akt's involvement in multiple cellular functions, it is understandably vital in B cells' lifecycle from development to activation and differentiation. However, there is a mechanism Akt is involved in, which is unique to B cells. When the BCR is not activated, a transcription factor called Forkhead Box, Subgroup O (FOXO) remains in the B cell nucleus and prevents the cell from proceeding further into its cell cycle. However, when the BCR becomes activated, Akt moves into the nucleus where it

phosphorylates FOXO, which causes FOXO to move out of the nucleus into the cytoplasm. This leads to the B cell resuming its cell cycle, differentiating into a plasma cell (Limon and Fruman., 2012).

Akt was chosen as a protein to probe in this study exactly for its multifaceted role in B cell function. Although it is involved in specifically B cell cycle, it also sustains other types of cells' functions. If septin inhibition leads to less phosphorylated Akt, it could mean B cells are less likely to differentiate after BCR activation.

1.5.4. Erk ½

The Extracellular Signal-Regulated Kinases 1 and 2 (Erk 1 and Erk 2, also known as Erk ½) are very far downstream from the starting point of a given signaling cascade. The kinases are isoforms of each other, and of them the absence of Erk 2 causes embryonically lethal defects in mice (Lucas et al., 2022). Although the kinases are separate, they are usually not distinguished from each other in text, and thus they will be referred to as a singular molecule, Erk ½ here.

Erk ½ is a protein which, upon phosphorylation, translocates into the nucleus, where it exerts various effects through multiple different transcription factors (Wortzel and Seger., 2011). The role of Erk ½ in B cells specifically has not been investigated much, although some studies have been performed. For example, in one study, up to 4 hours after BCR activation the Erk ½ was phosphorylated, but this phosphorylation became inhibited after 8 hours. This means that in B cells Erk ½ is most likely responsible for activating the fast-acting genes (Adem et al., 2015).

Erk ½ was chosen as a protein to be probed for in this study due to its involvement in nuclear processes. If septin inhibition leads to less phosphorylated Erk ½, it could mean that B cells proliferate less after BCR activation. This would result in a less effective immune response due to the lower number of differentiated B cells.

1.5.5. NF-κB

The Nuclear Factor kappa B (NF-κB) is a family of five proteins which are directly responsible for regulating immune and inflammatory responses by activating genes. The proteins belonging to this family are NF-κB1, NF-κB2, ReIA, ReIB, and c-ReI. They are usually bound to specific

inhibitory proteins in the cytoplasm, but upon phosphorylation and subsequent uncoupling from the inhibitory proteins they translocate into the nucleus. Being nuclear factors, the NF-κB proteins are at the end of the signaling cascades they are a part of.

The NF-κB pathway can activate due to many different stimuli, and these mechanisms of activation can be divided into two types: canonical, and non-canonical. In canonical activation, the cell receives stimuli via cytokines, growth factors, stress agents, ionizing radiation, microbial antigens, and more, whereas in non-canonical activation the stimuli come from ligands that bind to Tumor Necrosis Factor Receptors. In canonical activation, the inhibited NF-κB proteins become unbound from their inhibitory proteins, while in non-canonical activation the NF-κB2's precursor protein is processed into NF-κB2. In most cases of activation, the canonical path is employed (Liu et al., 2017). However, the non-canonical pathway is not without merit, as it leads to increased development, maturation, and survival of B cells, in addition to being involved in antibody class switching by inducing the expression of AID (Stavnezer et al., 2008; Guo et al., 2024).

In this study, NF-κB2 was chosen as a protein to be probed for due to its immediate involvement in the immune response. If inhibiting septins leads to less phosphorylated NF-κB, it could mean that the affected B cells have lower rates of survival, and they cannot perform the antibody class switch.

1.6. Antibody generation and long-lasting immunity

The germinal center (GC) is an important hub for B cell activity, present in secondary lymphoid tissues such as the lymph nodes and the spleen. The GC response is relatively slow, taking multiple days to play out in full effect. Although B cells are at the forefront of this process, many other cells work in the background, tightly controlling the microenvironment of the germinal center. This process will, in the end, lead to reliable and robust high-affinity antibody generation against foreign pathogens.

The GC is a transient structure in secondary lymphoid tissues. In the pre-GC state, when the germinal center is still forming, these tissues can be divided into two distinct sections: the T cell zone and the mantle. The T cell zone hosts helper T cells which interact with the naïve B cells of the mantle at the border of the two sections. In this pre-GC state, the activated B cells develop into GC-independent, low-affinity memory B cells or extrafollicular plasmablasts.

These cells are distinct from the ones that develop in the active germinal center in numbers, gene expression, and antigen-binding affinity (Young and Brink, 2021; Viant et al., 2021). Once the germinal center has developed, it can be split into the light zone (LZ) and dark zone (DZ). The DZ is the area where the B cells both divide rapidly and experience several rounds of SMH in an attempt to achieve a higher specificity to the challenged antigen. In the LZ the freshly mutated B cells are positively selected before being released from the lymphoid tissue or targeted for a new round of SMH in the DZ if their BCRs are not specific enough (Stebegg et al., 2018). It has been noted that the GC response begins on the first day of immunization as the migration and interaction of B cells and T cells within the lymph nodes (De Silva and Klein., 2015). This means that the GC response itself is fast, the only hurdle in the AIS deployment being reaching the required BCR affinity for the antigen.

The germinal center is composed of other cells participating in the maturation and differentiation of B cells into effector and memory responders. Some of these important cells are follicular dendritic cells, T follicular helper (Tfh) cells, T follicular regulatory (Tfr) cells, and killer T cells within the GC. The FDCs are professional antigen presenting cells like the non-follicular dendritic cells. However, residing in the GC, they have an important role in guiding the B cells' position through the light and dark zones of the structure. They also aid in the B cells' positive selection alongside with Tfh cells. The Tfh cells are derived from naïve helper T cells by dendritic cells, and their main role is to select the B cells which have undergone SHM and generated the BCRs with the highest affinities. The Tfr cells have long been enigmatic in their origin and purpose, but in recent years their roles in the GC have been elucidated. They take part in the upkeep of the GC response via Tfh cells, and they select antigen specific Tfh and B cells. They also have a role in the B cell class switching. Having been derived from regulatory T cells, Tfr cells also possess immune suppressive abilities, for example limiting the size of the germinal center and guiding the function of Tfh cells. The handful of killer T cells in the light zone of the germinal center are responsible of guarding the GC from external pathogens by eliminating infected cells that may infiltrate it. All the four aforementioned cell types reside in the light zone of the GC (De Silva and Klein., 2015; Stebegg et al., 2018; Young and Brink., 2021).

There are two types of antibody-secreting cells: plasmablasts and plasma cells. Plasmablasts are short-lived (up to days), dividing cells whose antibody secretion capacity is somewhat limited. Plasma cells, on the other hand, are nondividing, long-lived (up to months or years),

and able to secrete large amounts of antibodies to counteract specific antigens. Plasmablasts emerge from activated follicular B cells or B1 cells. They are the fast AIS responders while the GC is still forming, and they can terminally differentiate into plasma cells. Although plasma cells can differentiate from plasmablasts, they most often originate from GC B cells (Nutt et al., 2015; Khodadadi et al., 2019).

Memory B cells do not secrete antibodies, but they can activate by BCR-antigen binding and differentiate into antibody-secreting plasma cells, or alternatively they can home in on GCs and aid in their formation. Since memory B cells are pre-existing and primed for re-activation from a specific antigen, they are the first line of defense of the AIS, whereas the newly divided B cells in the GCs are the second line. It must be pointed out that memory B cells are not the same as the long-lasting plasma cells whose life span may reach even decades. These two cell categories have their differences that are most likely rooted in the selection mechanism during the GC response. Some memory B cells have been noted to cross-react to pathogens that are similar to the ones they were generated against, such as new variants of an influenza virus, while long-lasting plasma cells do not appear to do the same. This makes the memory B cells as a whole have wider coverage for different pathogens, compared to long-lasting plasma cells. Like with other B cell types, memory B cells are also not homogenous, as they can be sorted into two classes according to the dependency or independency from the GC, denoting if a GC is necessary for the memory B cell's generation (Inoue and Kurosaki., 2024).

2 Aims, hypotheses and goals

The aim of this study is to determine if the septin cytoskeleton is essential for immune synapse formation, BCR activation, and, ultimately, antibody production. We hypothesize that if the septin cytoskeleton is disrupted, B cells are less capable in forming an IS and processing the BCR-antigen complexes. In addition, changes in organelle dynamics, and the potential role of septin filaments in the spatial orientation of microtubules after BCR activation was studied through immunofluorescence after the use of the same pharmacological inhibitor. Finally, to study the importance of the septin cytoskeleton in vivo, a conditional knockout of septin-7 in B cells was used to measure antibody responses with an immunogen eliciting T-dependent responses.

3 Results

3.1. B cell activation

BCR activation can be initiated through specific antigen when this is recognized through the antigen-binding site of the BCR, or through surrogate antigen that crosslinks the BCRs and triggers the downstream signaling cascade. As such, we take advantage of the surrogate antigen properties to obtain the same pattern of activation throughout our assays. To ascertain the possible effect of septins in BCR signaling and the extent of that effect, we used a reversible pharmacological pan-septin inhibitor, forchlorfenuron (FCF). Mouse primary B cells were incubated with a low serum buffer for 15 minutes ("starvation") and acutely treated for 5 minutes with different concentrations of FCF, or with DMSO as a control treatment. Afterwards, FCF-treated cells were washed to reverse the inhibitor effect, where septin cytoskeleton dynamics are able to proceed as normal. The cells were then activated with surrogate antigen $F(ab')_2$ IgM and probed for the phosphorylation of two key proximal (HS1, and Syk) and three distal effector proteins (Akt, NF-κB, and Erk1/2). This means that each DMSO, FCF, and wash-off treatment has two conditions: the nonactivated and activated. For normalizing the amount of protein loaded, β-actin was probed afterwards for every blot.

Syk is a tyrosine kinase known to be the first key player directly phosphorylated upon the BCR becoming antigen-activated, and it initializes the BCR signaling cascade. HS1 is tightly linked to actin cytoskeleton remodeling upon activation. Akt has been known to play a vital role in inducing the cell cycle to proceed to the proliferative state, it is a distal protein in the signaling cascade. Erk ½ directly phosphorylates the transcription factors that lead to B cell proliferation. NF-κB is a nuclear factor, directly having a role in gene activation and transcription. Together these chosen proteins represent the entire intracellular BCR signaling cascade from the first phosphorylation events to further gene activation and cell development.

As B cells have different activation kinetics depending on how antigen is delivered, to study BCR signaling in greater detail, we stimulated splenic B cells with either soluble or surface antigen. Unfortunately, due to time constraints and technical difficulties with the reagents at the time, the necessary replicates could not be performed to infer statistical significance. In Figure 1 available blots for analysis are shown, they have been probed for the housekeeping protein β-actin. Even though significant differences cannot be shown and discussed, some notable

differences can be perceived and quantified, as shown in Figure 2. To compare between conditions, the intensities of the imaged blots were quantified and normalized to β-actin. The β-actin blots are not shown due to them being used only for normalizing the data acquired from the blots shown.

As expected upon activation, with both soluble and surface stimulatory conditions, there is an increase of phosphorylated proteins (DMSO 15 min), pSyk being the clearest example of this as it is directly downstream from the BCR. Notably, when acutely treating the splenic B cells with FCF the intensity of said phosphorylation decreases to less than 50% of most proteins, the only exception to this is in surface-activated pHS1, as is seen in Figure 2. Looking at both proximal and distal signaling proteins, the same effect of FCF is evident. Moreover, this effect is highlighted even more when the FCF concentration is increased. Nonetheless, upon washing the FCF away, the B cells recover the signaling phosphorylation patterns seen without the drug treatment.

After quantifying the phosphorylated proteins in the blots, no statistical significances were observed due to the lack of replicates. However, there is a trend where the FCF treatment inhibits protein phosphorylation, suggesting that septin dynamics play a part in the cell signaling cascade. This is especially seen in the downstream proteins (Fig. 2). Upon FCF treatment, phosphorylation of Syk remains largely unchanged, meaning that the septin cytoskeleton's dynamics do not seem to affect early BCR signaling. As the cascade is observed further downstream, the effect of the FCF treatment becomes more pronounced, even overriding the effects of BCR activation. This may be explained with the septin cytoskeleton's ability to act as a diffusion barrier that may disrupt the flow of the cascade as FCF impairs its dynamics, an effect that may be compounded along the signaling cascade. In Figure 2 it is seen that FCF treatment and its intensity strongly correlate with lowered protein phosphorylation, meaning that disrupted septin cytoskeleton dynamics have most likely affected the cascade, ending up in lower NF-κB phosphorylation.

Curiously, when the soluble-activated wash-off conditions of pNF-κB are observed, the protein levels do not return to the activated or even non-activated control levels, as is seen in Figure 2, meaning that the phosphorylation of NF-κB is prevented even after washing off the FCF. This may simply be variability or perhaps not all of the FCF was washed off, since in the same surface-activated pNF-κB conditions the lowest data points are at the same levels by a rough

estimate, except for the activated wash-off 100 µM FCF-treated column, where even the lowest data point rises to the non-activated control levels.

Taking everything into account, despite the lack of statistical significance, Figure 2 shows a trend where disrupting the septin cytoskeleton dynamics with FCF leads to lower phosphorylated protein levels. This means that although activated via BCR, the B cell's ability to react accordingly to its changing environment becomes impaired due to disrupted septin cytoskeleton dynamics.

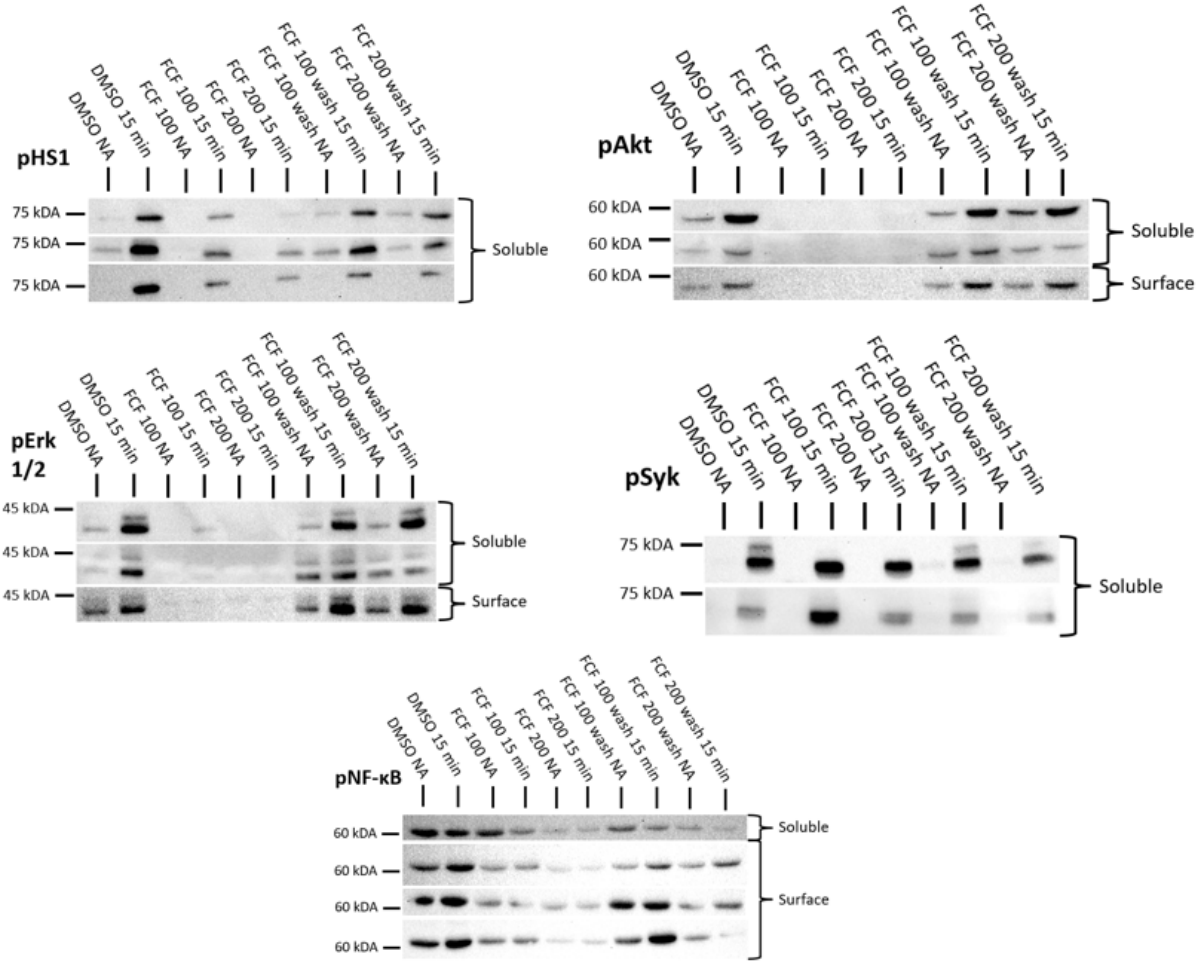

Figure 1. Immunoblots used for protein quantifications. The kDa size markers correspond to the protein ladder used to discern the protein sizes. NA means not activated with antibodies, 15 min means 15 minutes of antibody activation, DMSO means treated with DMSO, 100 means treated with 100 µM of FCF, 200 means treated with 120 µM of FCF, wash means FCF treatment was washed off after application. The soluble and surface markers explain how the cells the blots were acquired from were activated.

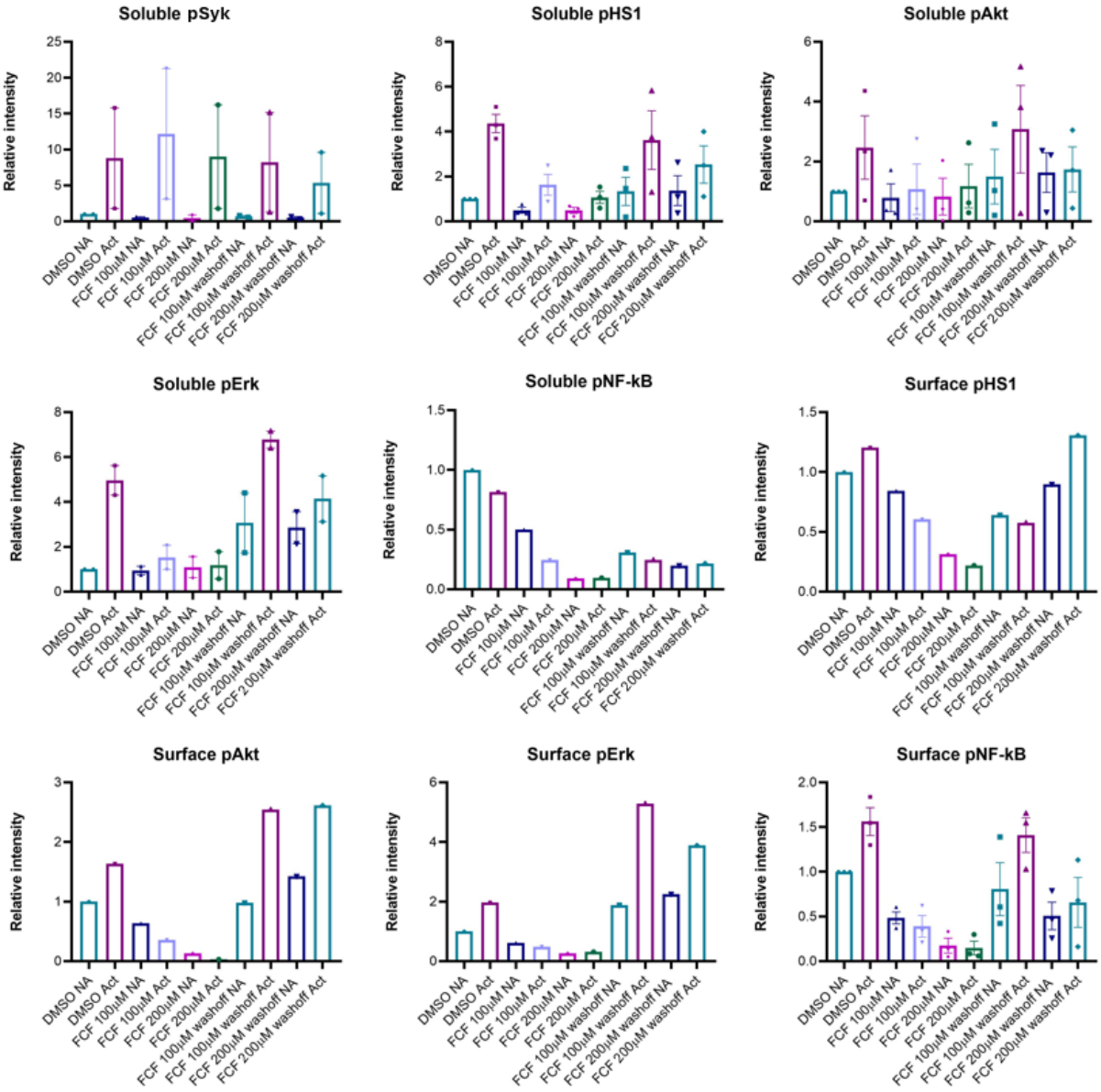

Figure 2. The measured intensities of the imaged protein bands normalized to β-actin. Each column corresponds to the calculated mean intensity of imaged blots of same mode of activation, probed protein, and treatment. The X axes show which treatment corresponds which column. The Y axes show the relative intensity of the blots per treatment, with the DMSO NA treatment always being 1.0. NA means not activated with antibodies, 15 min means 15 minutes of antibody activation, DMSO means treated with DMSO, 100 means treated with 100 µM of FCF, 200 means treated with 120 µM of FCF, wash means FCF treatment was washed off after application. There is a trend in almost all figures where the protein levels are lower when the cells were treated with FCF, but the drug was not washed off. In addition, in all cases, especially in Surface pHS1, pAkt, and pErk 1/2, the sample sizes are too small for definitive conclusions to be drawn.

3.2. Microtubule dynamics upon septin cytoskeleton inhibition

Based on the previous results where BCR signaling becomes defective under the effect of the pan-septin inhibitor, we aimed to visualize if the immune synapse formation would also be affected by targeting septin dynamics. For this, we used a mouse lymphoma cell line A20 D1.3, a common cell line used for B cell assays. This cell line is different from mouse primary B cells in their behavior, as they exhibit high proliferation and an increased basal level of activation characteristic of a cancer cell line. Nonetheless, it is a useful tool for assays regarding activation, and organelle-actin dynamics due to the increased size. Therefore, after treating the cells with increasing concentrations of the inhibitor and DMSO as a control, we plated the cells in coverslips coated with surrogate antigen $F(ab')_2$ IgM where B cells became activated. This activation leads to actin remodeling and changes in organelle position, as each B cell spreads to gather the maximum amount of antigen possible. This is what also happens in an *in vivo* setting.

At 60 minutes of activation B cells are mostly fully spread with a clear polarization of the BCR-antigen complexes concentrated in the central region of the synapse. At this time, B cells were fixed and stained with fluorescent anti-septin-7, anti-α-tubulin, and anti-actin antibodies. The confocal microscopy was done to visually infer the association of septin filaments with microtubules upon stabilization with FCF. All figures shown are composites of the cells at their cell-surface junction where the BCR-antigen complexes form. Microtubules are largely responsible for intracellular trafficking of compounds and organelles, vesicles belonging to the latter group. Normally, when a B cell activates via an antigen binding to a BCR, the cell internalizes this BCR-antigen complex. This occurs by wrapping the plasma membrane around the engaged receptors, which are then taken into the cytoplasm as vesicles. However, when a surrogate antigen is used, no true BCR-antigen complex is formed. Instead, the BCR is engaged and internalized through the vesicular uptake on its own. These vesicles are subsequently trafficked along microtubules to their appropriate intracellular destinations.

In Figure 5, the activated B cells have spread wide, with their edges relatively smooth and even, without sharp and long protrusions. In both cells the MTOC along with microtubules, colored green, are clearly visible. Notable is that in both cells presented, the MTOC has migrated to the center of the cell, this is perhaps to aid in antigen extraction and internal vesicle transport via the microtubules.

Septins have a role in organizing microtubules (Mostowy and Cossart., 2012; Spiliotis and Nakos., 2021). This means that septins physically associate with microtubules, which should be seen as septin filaments along microtubules if the septin cytoskeleton's dynamics are inhibited. However, under normal conditions the septin cytoskeleton is in a constant flux, which causes the septins to appear rather evenly spread within the cell. This normal condition is indeed seen in Figure 5, where the septin cytoskeleton, colored yellow, appears as fine granules spread across the cytoplasm.

The septin cytoskeleton is deeply involved in the dynamics of both the actin cytoskeleton and microtubules (Spiliotis and Nakos., 2021). If its dynamics are inhibited, we should be able to see aberrations in the other cytoskeletal components' behaviors as well. Figure 6 showcases this, the cells in it were treated with 100 µM FCF which inhibits septin cytoskeleton dynamics. It is apparent that the cells' ability to spread is severely disrupted, since in Figure 6 the cells' cytoplasm is constricted close around the cells' nuclei with only a few long and branching filopodia. In these cells the microtubules are also rather disordered, and the MTOC is not clearly visible. The septins do not appear as filaments aligned with the microtubules as hoped, instead they are densely packed into one spot or multiple large aggregates within the cell. This bundling of septins is in fact an expected outcome. Overall, it is clear from Figure 6 that inhibiting septin cytoskeleton dynamics causes severe disruptions both in actin cytoskeleton and microtubule dynamics.

Figure 7 shows the result of treating B lymphocytes with 200 µM FCF. The results of this experiment were not quantified, so it is unclear whether increasing the FCF concentration has any difference in the disruption of the cytoskeleton dynamics. However, it is perhaps worth noting that many of the cells treated with this higher concentration detached from the glass' surface and were washed off, suggesting that FCF might affect antigen binding. Septin clusters also appear outside the cells, this is seen already in Figure 6 with the 100 µM FCF treatment, and it is a sometimes-observed occurrence.

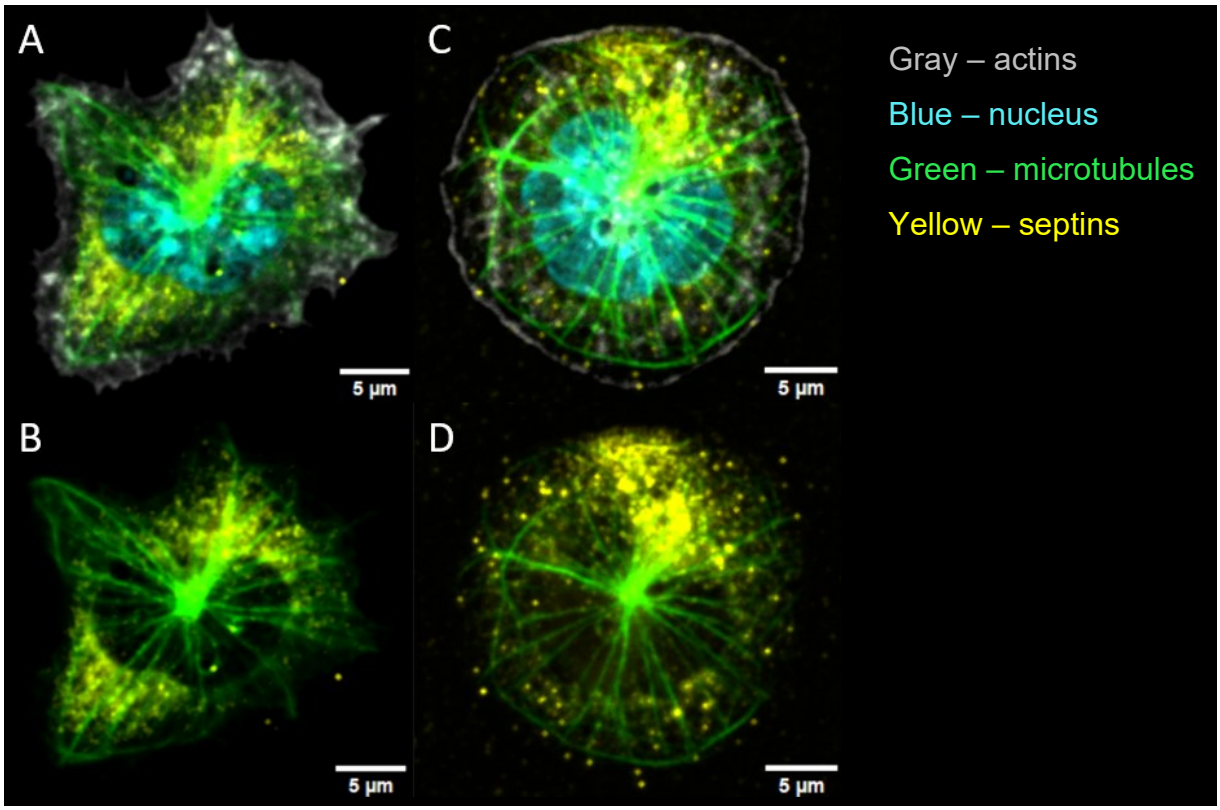

Figure 5. Confocal microscopy of DMSO-treated and antibody-activated A20 D1.3 B cells at 60 minutes activation. A and C sections show four different cell structures in different colors: blue corresponds to the nucleus, green to microtubules, yellow to septins, and gray to actins. The scale bar of 5 µm is shown in the bottom right corner of each image. Sections B and D only show the green microtubules and the yellow septins for easier comparison. The cells are relatively round and smooth at the edges, exhibiting proper spreading over an antibody-coated surface. Notables are the septin structures diffused relatively evenly within the cell, appearing as small granules in the images.

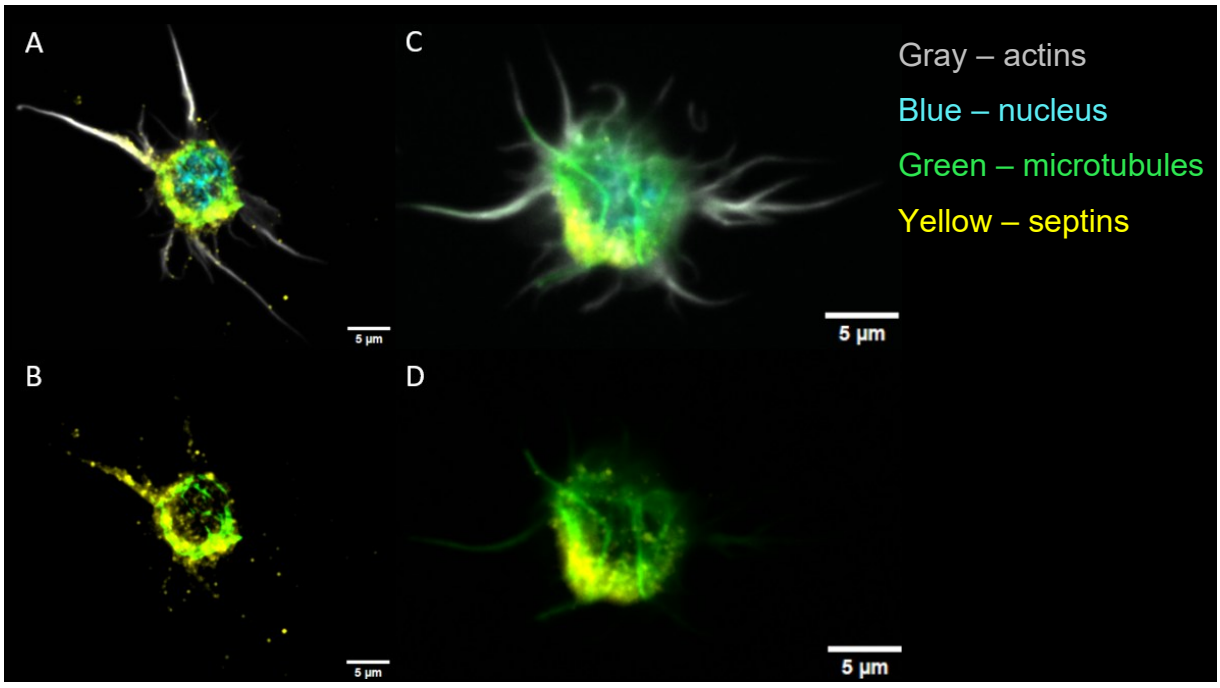

Figure 6. Confocal microscopy of 100 µM FCF-treated and antibody-activated A20 D1.3 B cells at 60 minutes activation. A and C sections show four different cell structures in different colors: blue corresponds to the nucleus, green to microtubules, yellow to septins, and gray to actins. Sections B and D only show the green microtubules and the yellow septins for easier comparison. The cells are constrained due to the FCF treatment, spreading only long filopodia out of the central mass. The septins are concentrated into multiple large blots as is seen in images A and B, or into one bigger area as seen in images C and D.

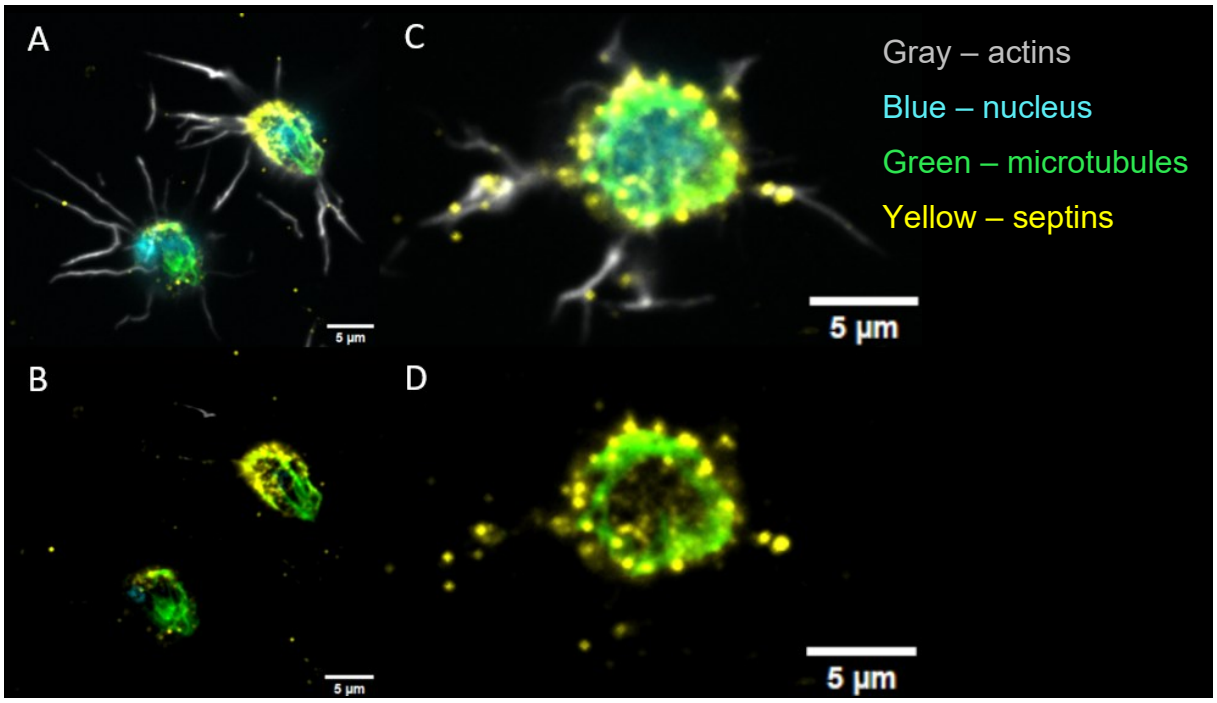

Figure 7. Confocal microscopy of 200 µM FCF-treated and antibody-activated A20 D1.3 B cells at 60 minutes activation. A and C sections show four different cell structures in different colors: blue corresponds to the nucleus, green to microtubules, yellow to septins, and gray to actins. Sections B and D only show the green microtubules and the yellow septins for easier comparison. The cells are heavily constrained due to the FCF treatment, spreading only long and thin filopodia out of the central mass. Many of the cells died and became washed off the microscopy slide during the 200 µM FCF treatment. The septins are concentrated into one bigger area as is seen in images A and B, or multiple large blots as seen in images C and D.

3.3. Antibody responses

To assess if the septin cytoskeleton is, in fact, essential for B cell functions such as activation, proliferation, and class switching, we took advantage of our septin-7 cKO mouse model to evaluate the antibody responses as a final readout of humoral immunity. Mice were immunized with NP-KLH, an immunogen eliciting T cell help, GC formation, and consequently class switching. We directly compare the antibodies' titers amount and affinity produced in septin-7 cKO mice versus WT mice by using enzyme-linked immunosorbent assay (ELISA). In short,

an ELISA measures the concentration of a substrate in solution, in this case the antibodies secreted by T cell activated B cells in the serum of mice.

Mice were immunized intraperitoneally with 50 µg of NP-KLH (Nitrophenyl – keyhole limpet hemocyanin) with 2% alhydrogel at day 0. The cohort in this study had a matching number of septin-7 WT and cKO mice (4 WT and 4 cKO mice). The mice were followed for the next 28 days, and blood was collected from the saphenous leg vein every 7 days (Days 7, 14, 21, and 28). In addition, blood was also collected on the day before the immunization (Day -1) to evaluate the basal levels of antibody concentration. Blood was let to coagulate and was centrifuged to extract the serum, the fraction rich in antibodies.

According to literature, when immunizing mice with NP-KLH in 2% alhydrogel/alum intraperitoneally, the B and T cells around the spleen will be under prolonged exposure to the antigen (NP-KLH). These cells can then present the processed antigen in germinal centers giving origin to higher affinity, class-switched antibodies (IgG1, IgG2b, IgG2c). However, in the first wave (up to 14 days) most antibodies produced will be of the IgM and IgG3 types. These originate from low affinity plasma cells in the extrafollicular area and are not required to undergo class-switching in the GC follicles. After day 14, when germinal centers hit maturity, plasma cells producing high affinity antibodies take charge and dominate the immune response.

NP-KLH, also known as (4-hydroxy-3-nitrophenyl) acetyl -keyhole limpet hemocyanin, consists of a carrier molecule, the KLH, and an effective hapten antigen, the NP. The NP-KLH is a widely used combination of molecules that reliably causes an immune response. In these cases, B cells develop NP-specific antibodies. (Wirguin et al., 1995; Erkes and Selvan., 2014). The NP-KLH was used in this experiment due to its reliability and the strong reaction it causes, making it easier to measure NP-specific antibody levels.

As seen in Figure 3, at the basal level, the septin-7 cKO mice had lower total antibody concentration in serum compared to the septin-7 WT mice, and overall, the IgG1 antibody levels were the highest while the IgG3 antibody levels were the lowest. No statistical significance was observed in any antibody concentrations between the septin-7 WT and cKO conditions, although within the IgG3 antibodies the difference approached significance ($P = 0.09$). The observation of this baseline trend shows that knocking out septin-7 from B cells already disrupts their ability to secrete antibodies, even in the absence of concrete statistical significance.

IgM, IgG1, IgG2b, IgG2c, and IgG3 antibody isotypes were measured throughout the experiment. Due to naïve B cells already expressing IgM, its levels were expected to increase before the others and then drop steadily as the B cells go through class switching in the newly-formed germinal centers. This is seen in Figure 4, where the NP-specific IgM antibodies reach their concentration peak between days 7 and 14, after which they begin to decrease. Simultaneously, the other Ig isotypes increase in concentration through the duration experiment, effectively reaching their peak on day 21.

However, what is obvious in Figure 4 is the lack of difference in antibody levels between the septin-7 WT and cKO conditions. Although there was no statistical significance between the conditions at base level, the trend was observable. In Figure 4, the IgM graph shows the clearest difference between the WT and cKO conditions, with the latter condition consistently having lower levels of NP-specific antibodies. In the IgG1 graph the conditions are indistinguishable from each other on day 7, after which the septin-7 cKO condition begins to exhibit lower antibody levels when compared to the WT condition. However, in the IgG2b and IgG2c isotypes this difference between the conditions is observed only on day 28, until which the conditions have been indistinguishable. Statistical significance between septin-7 WT and cKO conditions was observed only in the IgM isotype, and only on the day 14 mark ($P < 0.05$).

The failure in the immunization experiment can be attributed to technical difficulties during the experiment. In addition, the results for the NP-specific IgG3 measurements were discarded due to entirely failed experiments and thus are not presented. It must also be noted that one of the septin-7 WT mice had to be sacrificed before the end of the experiment due to a serious injury.

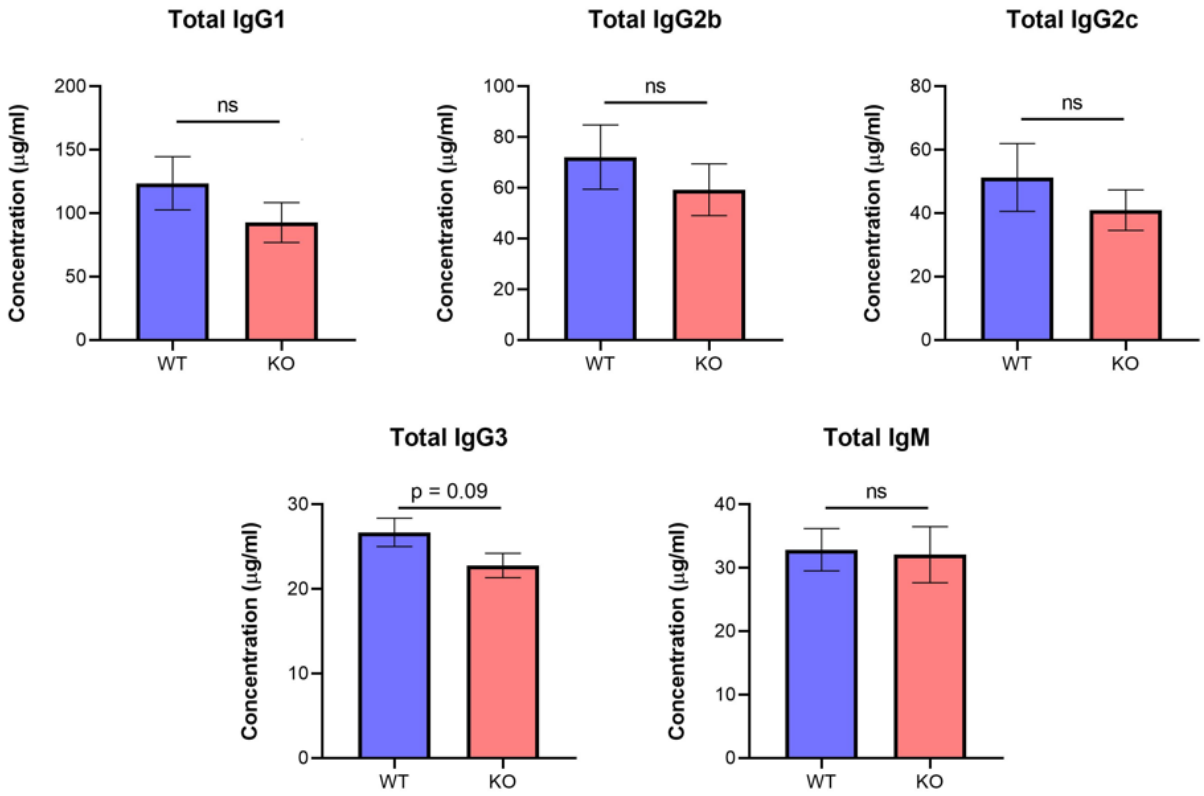

Figure 3. Comparison of the basal IgM, IgG1, IgG2b, IgG2cm, and IgG3 antibody levels between septin-7 WT and KO mice before immunization. Comparatively, the IgG1 levels are the highest, and the IgG3 levels are the lowest. The Y axes show the absolute concentration of antibodies in serum at the basal level. Note that the values of the Y axis are not the same between figures. No statistical significance was observed between the septin-7 WT and cKO conditions in any antibodies.

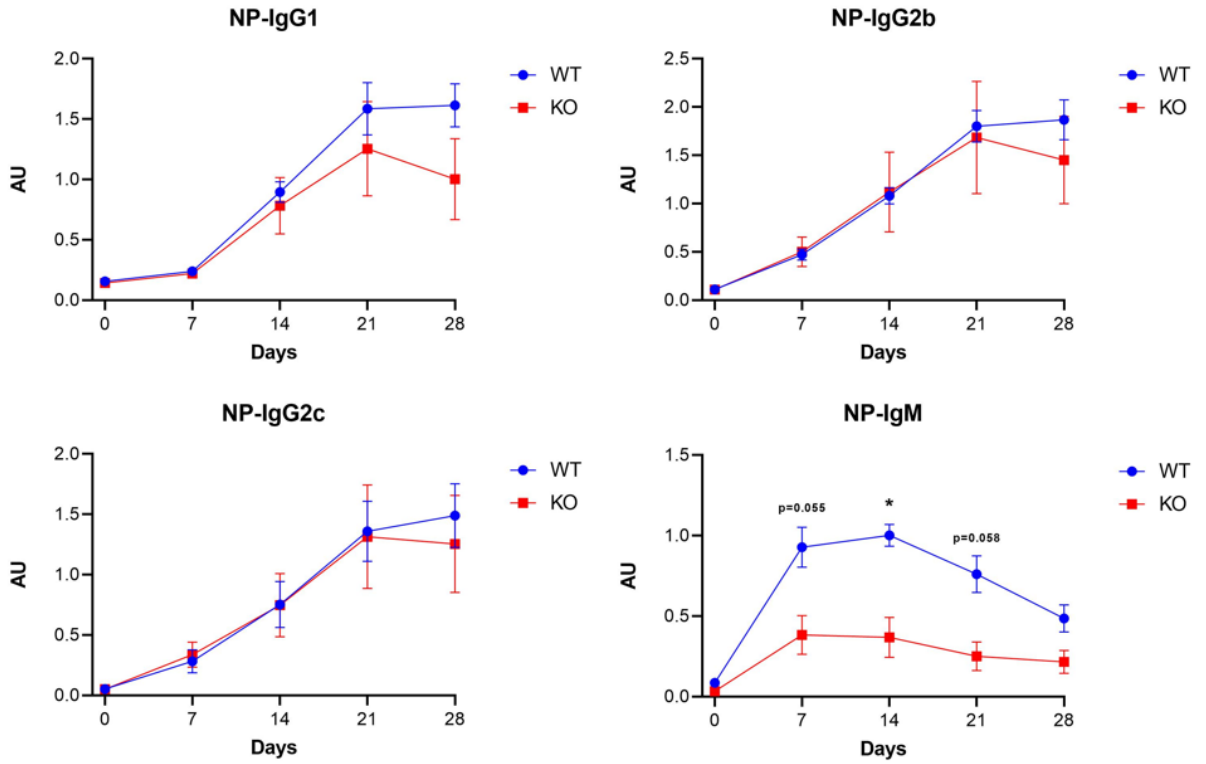

Figure 4. Comparison of the NP-specific IgM, IgG1, IgG2b, and IgG2c antibody levels over 28 days between septin-7 WT and KO mice. Arbitrary unit is used; the measurement values are compared to a mixture of all mice's serum samples at the 14-day mark. No statistical significance was found except in NP-IgM antibodies on day 14 ($P < 0.05$). There is a trend in the figures where the antibody concentration in septin-7 cKO mice falls slightly between the days 21 and 28. This trend of lower antibody levels is also seen in the IgM figure throughout the time measured.

4 Discussion

This study met with multiple issues at nearly every step of the way. Completing the immunoblots was particularly difficult, and the process was subject to continuous scrutiny. For most of the time, 1.5 mm thick gels were used, only later was it realized that 1.0 mm thick gels were ideal for running samples. The technique for doing and imaging the immunoblots was also refined on multiple separate occasions. As for microscopy, much of the time the A20 D1.3 cells used for it were not thriving and thus refused to spread on the antibody-coated slide appropriately. The ELISAs were not without issues, either. The ELISAs were done with two cohorts of mice, with approximately 2 months between the experiments. The results acquired from the first cohort did not match what has been seen in literature, as immunizing mice with NP-KLH should lead to a fast and strong reaction (Wu et al., 1998), in our study only the changes in the IgM antibody levels followed the expected course. Our results thus led to initial

confusion. It was soon determined that the reason for the lackluster effects was due to the immunization reagents being contaminated. The analysis of the second cohort's blood samples and resulting data was started but not finished in time for this study due to missing mouse cohorts.

Although no concrete conclusions can be drawn from the results, weak due to the many difficulties encountered during the study, the trends in the data suggest that septins indeed do have a vital role in B cells' immunity acquisition. The immunoblots suggest that when treated with FCF and thus impairing the septin cytoskeleton, the cells' internal signaling pathways become defective downstream despite the cells being presented with either soluble or surface-bound antibodies. Defects in the cascade are not similarly observed upstream, which appears unaffected by the same septin cytoskeleton dynamics impairment.

When the signaling cascade becomes weak downstream, as is seen in the reduction of pErk ½ and pNF-kB levels in the cells, the cells fail to produce new proteins in sufficient quantities to respond to the presented stimulus. These two signaling agents directly regulate transcription factors inside the cell nucleus, and if the amount of these proteins is lowered, it would lead to reduced transcription of DNA and thus an impaired immune response in the form of lowered antibody levels. The septin cytoskeleton has been observed to act as a diffusion barrier inside the cells, separating compounds dissolved in the cytoplasm from one another. Perhaps this ability affects the BCR signaling cascade as well, and disrupting the septin cytoskeleton dynamics causes rigidity of the septin filaments, and this way the components of the signaling cascade may become permanently sequestered from one another. Such an effect on the signaling cascade may be compounding, as the previous step in the cascade would be unable to become phosphorylated in effective quantities and subsequently move the signal to the next step because of the impaired septin cytoskeleton dynamics, leading to a gradually diminishing signal. However, this is mere speculation, and more detailed studies must be conducted to determine the mechanics of the septin cytoskeleton and how it affects the intracellular signaling cascade.

The confocal microscopy images show that after a B cell is activated with antibodies and then the septin filaments are inhibited with FCF, the septins bundle together. At the same time, disrupted microtubule and actin cytoskeleton dynamics are observed, leading to the cells developing long and sometimes branching filopodia instead of spreading evenly. Since the B cell cannot spread to gather antigens presented to it, its ability to become BCR-activated is

prevented to begin with. Although it is not visible in the figures presented in this study, septins have been observed to associate with microtubules by forming filaments when treated with FCF. This enforces the idea that the septin cytoskeleton does play a role in intracellular vesicle transport, a function that is vital in antibody internalization and transport.

While my part of the larger study gave inconclusive results, the part conducted by a more experienced researcher has already yielded results which indicate that septin-7's role in B cell specialization and immune response is undeniable. Therefore, this experiment should not be taken as evidence for the opposite. In fact, it should encourage another look to be taken into the matter.

5 Experimental design

5.1. Mice and Ethical Issues

The Mb1-cre mouse colony (carrying an allele in which exons 2 and 3 of the Cd79a gene have been replaced with a codon optimized Cre recombinase gene and the ATG codon of exon 1 is deleted) were shared by Dr. M. Reth (University of Freiburg and Max-Planck Institute for Immunobiology, Freiburg, Germany) and the Septin7flox/flox mouse colony was a kind gift from Prof. Dr. Matthias Gaestel, Institute of Physiological Chemistry Hannover Medical School, and it is previously described elsewhere (Menon et al., 2014). Wildtype (WT) C57BL/6NCrl mice were purchased from the University of Turku Central Animal Laboratory. All strains were on a C57BL/6 background and maintained under specific-pathogen-free conditions. All experiments were done with age- (8-12 weeks), and sex-matched animals, and with littermate controls (Mb1 WT/WT, Sept7flox/flox) were used whenever possible. All animal experiments were approved by the Ethical Committee for Animal Experimentation in Finland and adhered to the Finnish Act on Animal Experimentation (62/2006; animal license numbers: 7574/04.10.07/2014 KEK/2014-1407-Mattila, 10727/2018).

5.2. Cell culture

A20 mouse lymphoma cells stably expressing a hen egg lysozyme (HEL)-specific IgM BCR (D1.3) were a kind gift from Prof Facundo Batista (the Ragon Institute of MGH, MIT and

Harvard, USA). Cells were maintained in complete RPMI [cRPMI; RPMI 1640 with 2.05mML-glutamine supplemented with 10% fetal calf serum (FCS (Fisher Scientific #10270-106)), 50 µM β-mercaptoethanol, 4 mM L-glutamine, 10 mM HEPES and 100 U/ml penicillin/ streptomycin].

At the beginning of the experiment, 10 million A20 D1.3 cells frozen in Freezing Medium (FCS (Fisher Scientific #10270-106) supplemented with 10% DMSO) were thawed. The cells were resuspended in 10 ml of fresh cRPMI, to a final concentration of 1 million/ml. Cells were split every other day when optimal confluency was achieved. Fresh cRPMI was added so the cells stayed in the growing phase.

5.3. B cell isolation from mice

WT C57BL/6NCrl mice were sacrificed by gassing them with CO_2 until no movement could be observed, and then breaking the neck, after which the spleen was extracted. The spleen was mechanically disintegrated in B cell isolation buffer (Phosphate-buffered saline without Ca^{+2} and Mg^{+2} (PBS) (Sigma-Aldrich P4417-50TAB) 2 % FCS (Fisher Scientific #10270-106), 2 mM EDTA (Invitrogen AM9260G)), and pipetted through a 70 µm cell strainer (Fisherbrand Sterile Cell Strainer #22363548) to obtain single cells in solution. The B cells were isolated by using a negative isolation kit (EasySep Mouse B cell Isolation Kit, #19854, StemCell Technologies) according to the manufacturer's instructions. The isolated B cells were centrifuged (Eppendorf Centrifuge 5810R) (450 G, 5 min, +4 °C), the supernatant was discarded, and the cells were resuspended in fresh cRPMI and allowed to rest for 1 hour at 37ºC, 5% CO2.

5.4. Sample lysate preparation

Primary B cells isolated from WT C57BL/6NCrl mice spleens were treated for desired conditions. These conditions were either treatment with dimethyl sulfoxide (DMSO, 1:200 in PBS) or forchlorfenuron (FCF), the latter in concentrations of 100 µM or 200 µM. The cells were activated with surface-bound or soluble antigens for 15 minutes or not activated at all. The cells were lysed with 5x SDS (62.5 mM TrisHCL pH 6.8, 2 % SDS, 10 % glycerol, 100 mM 2-ME, bromophenol blue) lysis buffer. The samples were collected and sonicated (Diagenode Bioruptor Plus) for 5 minutes on high program, on/off in 30 second intervals. The

samples were then boiled in a block heater (Biosan Thermo-Shaker TS-100) at +90 °C for 5 minutes before storing them in -20 °C for further experiment.

5.5. Western blot

Lysates were thawed and heated to + 70 °C for each run. 20 µl of each sample was run through 10 % acrylamide gels of 1,0 mm or 1,5 mm in thickness and transferred onto polyvinylidene fluoride (PVDF, BioRad Trans-Blot Turbo Transfer Pack #1704157) membranes (Trans-Blot Turbo Transfer System, BioRad). The membranes were blocked for 1 hour in room temperature (RT) in rotation with 5 % bovine serum albumin (BSA) (Biowest P6154-500GR) in Tris-buffered saline supplemented with 0,05 % Tween (TBST). The membranes were incubated overnight (O/N) at +4 °C with primary antibodies in 5 % BSA in TBST. The membranes were washed 5x5 minutes with TBST. The membranes were then incubated for 1 hour RT with secondary horseradish peroxidase (HRP)-conjugated antibodies (Jackson ImmunoResearch #111035144) (1: 10 000) in 5 % milk in TBST. The membranes were then washed 5x5 minutes with TBST. The membranes were incubated for 5 minutes with Immobilon Western Chemiluminescent HRP Substrate (WBKLS0500, Millipore) (1:1). The membranes were scanned with ChemiDoc MP Imaging System (BioRad). The raw integrated densities of the protein bands were background subtracted and measured in ImageJ.

To probe for the housekeeping protein (β-actin) in a sample, the imaged membranes were stripped in 10 ml of stripping buffer (glycine-HCL, 2,4 pH, 1 % SDS) for approximately 12 minutes at 37 °C in rotation. The membranes were washed 5x5 minutes with TBST. The membranes were incubated with anti-β-actin (1:50 000 in 5 % milk in TBST) for 1 hour at RT. The membranes were then washed 5x5 minutes with TBST. The membranes were incubated for 5 minutes with Immobilon Western Chemiluminescent HRP Substrate (WBKLS0500, Millipore) (1:1). The membranes were scanned with ChemiDoc MP Imaging System (BioRad). The raw integrated densities of the protein bands were background subtracted and measured in ImageJ.

5.6. Immunofluorescence staining

Cells (mouse B cells or A20 D1,3) were taken and resuspended in imaging buffer (PBS supplemented with 0,1% glucose, 0,5% FCS, 0,5 mM $CaCl_2$, and 2 mM $MgCl_2$) and allowed to rest for 15 minutes at 37 °C. The cells were split evenly into different conditions. The cells were

either treated with DMSO (1:200 in PBS) for control, or with 100 µM or 200 µM final concentration of FCF.

Twelve-well PTFE diagnostic slides (Thermo Fisher, #10028210) were coated with 4 µg/ml fibronectin in PBS and F(ab')$_2$ Fragment goat-anti-mouse IgM (Jackson ImmunoResearch #115007020) 10 µg/ml, for 30 minutes at RT. Approximately 20 000 treated cells were pipetted into each well and incubated at 37 °C, 5 % CO_2 for the desired timepoint of 1 hour. The slide was placed on ice to stop the activation, and the supernatant was removed. Afterwards, the cells were fixed with 1 % paraformaldehyde (PFA) (Sigma-Aldrich 16005-1KG-R) (diluted in PBS) at RT for 20 minutes. The cells were then blocked with donkey blocking buffer (DBB) (5 % donkey serum buffer in PBS) to prevent nonspecific binding of primary antibodies for 20 minutes. The samples were washed 3 times with PBS and incubated with primary antibodies (Anti-α-Tubulin Antibody, clone DM1A, Alexa Fluor 488 conjugate (16-232, Merck Millipore); Acti-Stain Fluorescent Phalloidin 670, #PHDH3, Cytoskeleton; Anti-Septin-7 Rabbit Polyclonal Antibody IgG (13818-1-AP, Protein Tech)) each diluted to 1:200 in saponin permeabilization buffer (0,2 % BSA; 0,05 % saponin in PBS) O/N at +4 °C. The next day, the cells were washed 3 times with PBS and incubated with secondary antibody (Alexa Fluor 555 Donkey Anti-Rabbit IgG (H+L) (A31572, Thermo Fisher) diluted to 1:500 in PBS for 30 minutes in RT. Finally, the samples were washed 3 times with PBS and mounted using Fluoromount-G containing DAPI (Thermo Fisher, #00495952), and allowed to dry for 30 minutes at +37 °C.

5.7. Spinning disk confocal microscopy

Images were acquired using a 3i CSU-W1 spinning disk confocal microscope equipped with 405, 488, 561 and 640 nm laser lines, 510 – 540, 580 – 654 and 672 – 712 nm filters, and a 63x Zeiss Plan-Apochromat objective.

5.8. Immunization

T-dependent immunization was performed on 18 septin-7 WT and 18 septin-7 cKO mice, in addition to 4 Mb1 Cre mice. For both the septin-7 WT and cKO mouse groups, the sex ratio was 10:8 males to females. All Mb1 Cre mice used were female. All mice were 6-8 weeks old at the time of immunization.

T-dependent and T-independent immunizations were performed on separate mouse cohorts. On day -1 approximately 50-100 µl of blood was collected from each mouse. The samples were incubated in RT for coagulation before spinning them at 3000 rpm for 8 minutes at +4 °C, followed by a 1-minute spin at 15 000 rpm. The serum was then collected and transferred to separate tubes and stored at -20 °C. On day 0 the T-dependent mice were immunized with Nitrophenyl-Keyhole Limpet Hemocyanin (NP-KLH) 0,33 µg/µl in Imject Alum. The T-independent mice were immunized with NP_{40}-Ficoll (1:2) in PBS. The immunization was done intraperitoneally. Approximately 50-100 µl of blood was collected from each mouse on days 7, 14, 21 and 28. The samples were incubated in RT for coagulation before spinning them at 3000 rpm for 8 minutes at +4 °C, followed by a 1-minute spin at 15 000 rpm. The serum was then collected and transferred to separate tubes and stored at -20 °C.

5.9. ELISA

Serum samples were collected from 8 immunized mice (4 septin-7 WT, 4 septin-7 cKO) at different timepoints (d0, d7, d14, d21, d28). The samples were collected from the saphenous vein. The blood was coagulated at RT for approximately 1 hour, after which it was spun down, and the serum was collected and stored at -20 °C. The serum samples and standards for antibodies were prepared in a 96-well plate, 60 µl/well. All serum sample dilutions were made with blocking buffer (1% BSA in PBS), these final dilutions used in the experiment are shown in Table 1. The standards were made from the d14 samples combined from the different mice and diluted from 1:10 000 to 1:640 000 in halving concentrations.

Table 1. The serum sample dilutions for the ELISA protocol. These dilutions were used for all samples collected at different timepoints.

IgM	1:25 000	**IgG2b**	1:50 000	**IgG3**	1:25 000
IgG1	1:75 000	**IgG2c**	1:25 000		

5 ELISA plates, one for each probed antibody (IgM, IgG1, IgG2b, IgG2c, IgG3), were coated with capture protein (NP-BSA (Ratio 31), Biosearch Technologies, N-5050H-10; 1:20; 50 µg/ml in PBS) 50 µl/well, and left to incubate O/N at +4 °C under cling film cover. The plates were then washed 3 times with 150 µl of washing buffer (0,05 % Tween-20 in PBS) and blocked with 150 µl of blocking buffer (1 % BSA in PBS) for 1-2 hours at RT. The plates were washed 3 times with 150 µl of washing buffer.

The serum dilutions shown in Table 1, and standard dilutions made of d14 serum samples were prepared in a volume of 60 µl/well. The samples and standards were pipetted in duplicates onto the blocked ELISA plates by pipetting 50 µl of each solution in their designated wells. The ELISA plates were wrapped with cling film and incubated O/N at +4 °C. The plates were then washed 6 times with 150 µl of washing buffer. 50 µl of biotinylated detection antibody (Southern Biotech, GαM IgM-Biotin, 1021-08; GαM IgG1-Biotin, 1071-08; GαM IgG2b-Biotin, 1091-08; GαM IgG2c-Biotin, 1078-08; GαM IgG3-Biotin, 1103-08) in blocking buffer (1:250) was then added to each well, according to the antibody probed for, and incubated for 1 hour at RT. The plates were then washed 6 times with 150 µl of washing buffer. 50 µl of Extravidin Alkaline Phosphatase in blocking buffer (1:5000) was pipetted into the wells and incubated for 1 hour at RT. The plates were then washed 6 times with 150 µl of washing buffer and 2 times with 150 µl of mQ-H_2O. 50 µl of 1 mg/ml pNPP substrate in 0,2 M Tris was added to the wells. The plates were covered from light in RT, and the absorbance was recorded at different time points with BioTek Cytation 5 Cell Imaging MultiMode Microplate Reader.

5.10. Illustrations and statistics

Statistical significance for immunoblotting was calculated using ordinary one-way ANOVA with Holm-Sidak's multiple comparisons test. Statistical values are denoted as: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Graphs were created in GraphPad Prism 8.

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