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B-cell receptor-dependent antigen phagocytosis drives the germinal center response *in vivo* and *in vitro*

Ana Martínez Riaño

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B-cell receptor-dependent antigen phagocytosis drives the germinal center response *in vivo* and *in vitro*

This thesis is submitted by Ana Martínez Riaño in fulfillment of the requirements for the degree of Doctor in Molecular Biology

Thesis director: **Dr. Balbino Alarcón Sánchez**

Research professor in Consejo Superior de Investigaciones Científicas

Centro de Biología Molecular Severo Ochoa

Universidad Autónoma de Madrid

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CONTENTS

FIGURES CONTENT

FIGURES CONTENT

ABBREVIATIONS

ACK	Erythrocytes lysis buffer
BCR	B cell receptor
BSA	Bovine Serum Albumin
B2	B cell subtype
CD	Cluster of differentiation
cDNA	complementary DNA
CSR	Class-switch recombination
CXCR4	C-X-C chemokine receptor type 4
CXCR5	C-X-C chemokine receptor type 5
DC	Dendritic cells
DMEM	Dulbecco's Modified Eagle's medium
DNA	Deoxyribonucleic acid
DZ	Dark zone
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immuno-absorbent Assay
ERK	Extracellular signal–regulated kinase
FACS	Flow Cytometry
FBS	Fetal Bovine Serum
FO	Follicular B cells
GAP	GTP-ase activating protein
GC	Germinal center
GDP	Guanosine diphosphate
GEF	Guanine exchange factor
GTP	Guanosine triphosphate
SHM	Somatic hypermutation
HRP	Horseradish peroxidase
ICOS	Inducible T-cell costimulatory
IC	Immuno-complexes
IF	Immunofluorescence
I.p	Intraperitoneal

ABBREVIATIONS

IS	Immunological synapse
I.v	Intravenous
LZ	Light zone
MFI	Mean Fluorescence Intensity
MHC	Molecular Histocompatibility complex
MZ	Marginal zone B cells
NIP-OVA	4-hydroxy-5-iodo-3-nitrophenyl acetyl hapten bound to Ovalbumin
NP-PE	4-hydroxy-3-nitrophenyl acetyl hapten bound to phycoerythrin
NP-CGG	4-hydroxy-3-nitrophenyl acetyl hapten bound to chicken gamma globulin
PBS	Phosphate buffered saline
PC	Plasma cell
PD1	Programmed cell death protein 1
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase enzyme
PIP2	Phosphatidylinositol (4,5)biphosphate
PIP3	Phosphatidylinositol (3,4,5)biphosphate
PFA	Paraformaldehyde
RNA	Ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RRas	Ras related protein
RT-qPCR	real time quantitative-polymerase chain reaction
SCS	Subcapsular sinus
SV40	Simian virus 40
SYK	Spleen tyrosine kinase
TCR	T cell receptor
TF	Transcription factor
TFH	T follicular helper cells
WB	Western Blot
WT	Wild-type

SUMMARY

B cells have two main roles in the adaptive immune response: the generation of antibodies that help neutralizing pathogens and the antigen presentation to T cells. It is believed that B cells mainly acquire antigens by BCR-driven endocytosis, and that phagocytosis of antigens is a process exclusive of professional phagocytic cells, such as macrophages, neutrophils and DCs.

However, in this work we show that splenic Follicular naïve B cells are in fact able to phagocytose latex 1-3μm size beads coated with anti-IgM by a BCR-driven mechanism mediated by two GTPases of the Ras superfamily: RRas2 and RhoG. We show that phagocytosed antigens are processed and presented to naïve CD4 T cells, inducing their activation. *Rras2*-/- and *Rhog*-/- B cells have less capacity to phagocytose antigen, and this effect is more prominent for bigger particles. In consequence, RRas2- and RhoG-deficient B cells induce less CD4 T-cell activation *in vitro*.

We further show that a simple 2-cell culture can mimic an *in vivo* germinal center reaction. Naïve CD4 T cells stimulated by B cells presenting peptides derived from phagocytic antigens can differentiate into TFH cells. Those *in vitro*-derived TFH cells express CXCR5 and PD1 and secrete TFH cytokines (IL-4 and low levels of IL-21). In turn, cultured FO B cells with phagocytic abilities differentiate into germinal center B cells. The *in vitro* derived GC B cells produce class-switched antigen-specific antibodies of high affinity and can differentiate into plasma cells. Importantly, we show that the terminal differentiation depends on how B cells acquire antigen, since it is negligible when it is given in solution or in RhoG-deficient B cells.

We further explore the *in vivo* role of B cell phagocytosis. The success of vaccines commonly rely on the generation of high-affinity class-switched antibodies able to neutralize pathogens. We show that *in vivo* B cell phagocytosis is important for germinal center generation and antibody production, not only upon immunization with latex beads, but also using a common vaccination adjuvants that generates large immuno-complexes.

We propose that antigen phagocytosis by B cells is a mechanism for BCR affinity-discrimination in the GC. Only those B cells carrying high-affinity BCRs are able to phagocytose antigens and receive the TFH costimulus to survive.

RESUMEN

Las células B tienen dos funciones principales durante la respuesta inmune adaptativa: la producción de anticuerpos capaces de neutralizar patógenos y la activación de linfocitos T mediante la presentación de antígeno. Se cree que la adquisición de antígenos se produce mayoritariamente a través de procesos endocíticos mediados por el BCR, considerándose otros mecanismos alternativos como la fagocitosis exclusiva de células fagocíticas profesionales tales como macrófagos, neutrófilos y células dendríticas.

A lo largo de este trabajo mostramos que las células B foliculares también son capaces de fagocitar antígenos a través del BCR en un proceso dependiente de dos GTPasas de las superfamilia Ras: RRas2 y RhoG. Los linfocitos B de ratones *Rras2*-/- y *Rhog*-/- son deficientes en dicho proceso fagocítico, siendo su efecto mayor cuanto más grande es la partícula a fagocitar.

También mostramos que con un simple cultivo celular de linfocitos T y B naif podemos mimetizar una reacción de centro germinal. Células CD4 naif en cultivo se diferencian a TFH cuando son estimuladas por células B que presentan péptidos derivados de antígenos fagocitados. Estas TFH generadas in-vitro expresan CXCR5 y PD1 además de secretar citoquinas características de las TFH (IL-4 y IL-21). A su vez, estas células B foliculares cultivadas se diferencian a células B de centro germinal. Dichas células B de CG producen anticuerpos antígeno-específicos de alta afinidad con cambio de clase y se diferencian a células de plasma. Curiosamente, la diferenciación completa a centro germinal depende de cómo la célula B adquiere el antígeno, viéndose así inhibida tanto cuando se estimulan con antígeno soluble como cuando las células B son deficientes en RhoG.

Asimismo, hemos estudiado la importancia *in vivo* de la fagocitosis por linfocitos B. Ésta no solamente es importante durante la inmunización con micro-esferas recubiertas de antígeno, sino también cuando se utiliza un adyuvante comúnmente utilizado en vacunas que produce la agregación del antígeno en grandes inmuno-complejos.

Consecuentemente, proponemos que el proceso de adquisición de antígeno por fagocitosis puede ser utilizado durante la discriminación entre células B de diferente afinidad dentro del CG dado que solamente aquellas con suficiente afinidad por el antígeno serán capaces de fagocitarlo y así recibir el coestimulo de los linfocitos TFH.

INTRODUCTION

INTRODUCTION

1. Brief view into the adaptive immune response

Our body develop two important types of immunological responses to fight against pathogenic infections. The first one, called **innate immune response**, is found both in vertebrates and invertebrates and is characterized by the fast and generic response to pathogens without inducing long-lasting protection. The second one, called **adaptive immune response**, is a more specialized system found only in vertebrates. The adaptive immune response is characterized by their ability to respond to small amounts of pathogens in a specific way, leading to a more efficient and fast reply in subsequent encounters with the same pathogen. It is the basis of vacuums and it is founded in the existence of immunological memory. This adaptive immune response is carried out by lymphocytes and can be divided in two different types: humoral response, which is B-cell mediated; and cell response, T-cell mediated.

Both, T and B lymphocytes, are capable to recognize pathogens in a specific way through their antigen receptors: T-cell receptor (TCR) and B-cell receptor (BCR) respectively. B cells recognize native or denatured pathogen proteins in a soluble, particulated or membrane-bound manner. However, T cells only recognize proteolytically processed peptides (8-15 aa) presented by major histocompatibility complex (MHC) molecules on antigen-presenting cells. Both types of lymphocytes can be subdivided in specific B or T-subsets according to their phenotype and function (Cantrell 2015).

B cells have two major roles: as antibody producers and as antigen presenting cells. In adult mammals, they develop in the bone marrow and once they mature, colonize peripheral lymphoid organs, where they get fully differentiated into different B cell subsets; being the Follicular (FO) and Marginal zone (MZ), comprised in the B2 subtype, and B-1 B cells the most abundant. When pathogen-derived antigens gain access to the peripheral lymphoid organs, B cells develop a germinal center response in order to fight and eliminate the infection. B cell responses can be subclassified according to the requirement for T cell help as T-independent and T-dependent responses. T-independent responses induce a limited germinal center reaction, being aborted after 5 days, with low class-switch and affinity maturation. T-Independent antigens are multivalent molecules, usually polysaccharides, which promotes B cell response by inducing BCR crosslinking and activation of secondary receptors such as Toll-like receptors (TLRs) (de Vinuesa et al. 2000, Obukhanych and Nussenzweig 2006). T-dependent response is characterized by the stimulation and acquisition of antigen by B cells

for T cell presentation and coestimulation. This response, which induces the expansion of B cells with high affinity BCR for the pathogen, is going to be studied more in detail throughout this thesis.

T cells, the other lymphocyte population implicated in the responses against foreign antigens, also begin to develop in the bone marrow, but they migrate to the thymus as early progenitors to finalize their development. Once they mature and express correctly the TCR, exit the thymus to colonize the peripheral lymphoid organs. The predominant T cells are α/β TCR T cells, which can be classified in two main subsets: the CD8 cytotoxic (CTL) which kill infected cells; and the CD4 helper cells, which produce chemokines and cytokines to modulate the activity of other lymphocytes or cells (Cantrell 2015).

2. B cell signaling

B lymphocytes need to get stimulated in order to develop and perform their functions during the humoral response. The first step in their stimulation is the recognition of specific antigens though their B-cell receptor.

2.1 B-cell receptor

The BCR is a transmembrane protein composed by a membrane-bound immunoglobulin (mIg), the antigen binding subunit, coupled non-covalently to a signal transduction module consisting of a heterodimer of CD79α (Igα) and CD79β (Igβ). The antigen binding complex consists on an Immunoglobulin heavy chain (IgHC) bound to an immunoglobulin light chain (IgLC) by disulfide bonds (Packard and Cambier 2013). The amino-terminal regions of the immunoglobulin chains, called variable regions, participate in the antigen recognition trough specific domains termed complementary-determining regions (CDRs). The carboxy-terminal region of the IgHC defines the different immunoglobulin isotypes (IgM, IgG, IgA, IgE) that will determine their functionality (Cantrell 2015). The high BCR diversity found in our bodies is produced by two important mechanisms occurring during B cell development and humoral response: gene rearrangement and somatic hypermutation. First of all, Tonegawa in 1976 described the rearrangement of immunoglobulin genes during the formation of the mature BCR (Hozumi and Tonegawa 1976). At the pro-pre B cell stage of development the V(D)J rearrangement of IgH gene is produced, and subsequently, at the pre-B cell stage the VJ IgL rearrangement for the

generation of the mature BCR. In addition, during the humoral response to specific pathogens, stochastic mutations are introduced in the immunoglobulin genes changing their affinity against the pathogen (Kim et al. 1981). These two mechanisms produce the generation of a high degree of Ig diversity (>10^{11}) (Schatz and Ji 2011). Throughout this thesis we will focus more deeply in the processes that occur during the humoral response.

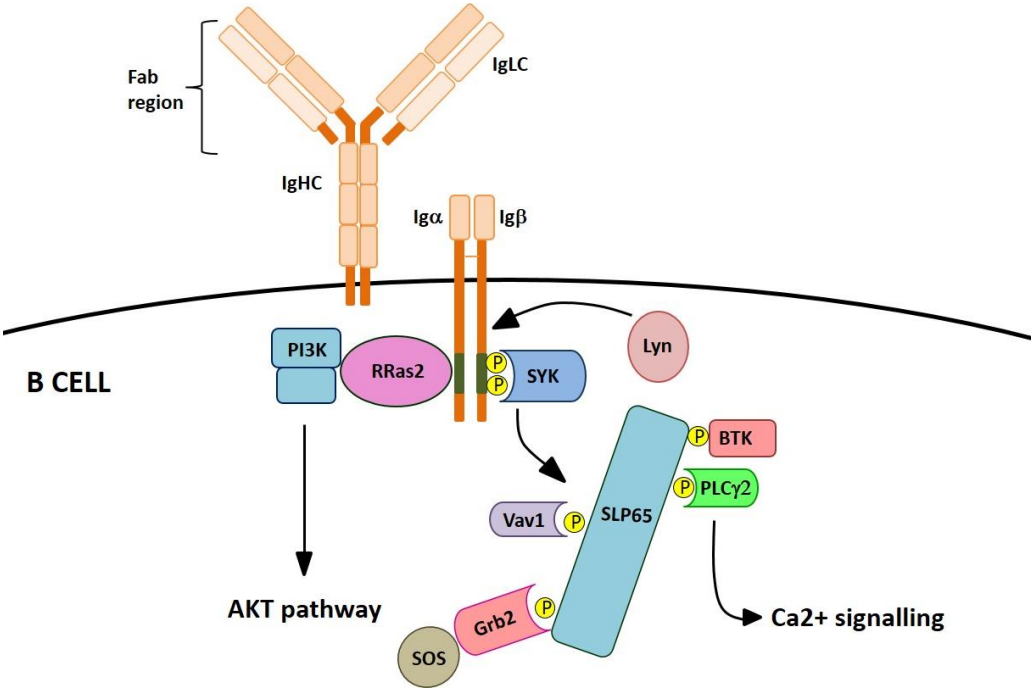

Illustration 1. **BCR signalosome**. The illustration shows the composition of the BCR structure and the early signalosome. The antigen binding unit, composed by an immunoglobulin light chain (IgLC) bound to an immunoglobulin heavy chain (IgHC), interacts non-covalently with the signaling subunits Igα/β. Phosphorylation of the ITAM motifs of the Igα/β dimer fires the BCR signaling cascade that finishes with the activation of many downstream pathways for the survival, proliferation and differentiation of the B cell.

Since mIg has a small intracellular tail that cannot transmit signals, it is necessary their binding to Igα/β complex. Both Igα and Igβ contain an Immunoreceptor Tyrosine-based Activation Motif (ITAM) characterized by the consensus sequence [YxxL/I $(x)_{6-9}$ YxxL/I]. Upon antigen binding to mIg, signal is transmitted across the plasma membrane leading to the phosphorylation of ITAM motifs by kinases of the Src-family, especially by Lyn. This phosphorylation induces generation of SH2 docking sites where Syk kinase binds, allowing its phosphorylation and activation, which leads to signal propagation via the recruitment of scaffold proteins such as SLP-65 (also called BLNK). SLP-65 binds to Igα via non-ITAM tyrosines and gets phosphorylated by Syk allowing the recruitment of downstream effectors as PLCγ2,

Vav1 and Btk (Brezski and Monroe 2008). These proteins have pleckstrin domains (PH) that bind lipid phosphoinositoides important for the propagation of the signal. PLCγ2 cleaves PIP2 in IP3 and DAG which induce the release of intracellular Ca^{2+} stores and the activation of PKCβ respectively (Packard and Cambier 2013). Moreover, the phosphorylation of BCAP by Syk creates docking sites for PI3K recruitment and activation. Recently, it has been described by our laboratory that the PI3K pathway can also be activated through a small Ras GTPase called RRas2 that also interacts with the Igα/β complex (Delgado et al. 2009). A more extensive description of this GTPase is given below.

CD19 is a membrane glycoprotein that functions as a BCR correceptor. It has a dual function, amplifying Src-family kinase (SFK) signals and activating PI3K. It has recently been shown that CD19 is required for BCR stimulation by membrane-bound antigens but not by soluble ones (Depoil et al. 2008). This requirement could be due to the function of CD19 amplifying SFK signaling, which is required for BCR activation upon low-avidity ligand interaction (Fujimoto et al. 2000, Mukherjee et al. 2013). BCR stimulation activates different downstream pathways such as PI3K-Akt, Ca2+, MAPK-ERK and NFKB, which promote the B cell proliferation, survival and differentiation.

3. Antigen presentation to B cells

The BCR fulfills two important roles: a signaling role that takes place upon antigen binding and an antigen internalization role that is followed by antigen processing and presentation to helper T cells. The secondary lymphoid organs are specialized structures that increase the likelihood that lymphocytes find their cognate antigens since the lymphatic fluid containing samples of soluble and particulate antigens moves through these organs (Harwood and Batista 2010). Transport of antigens through the follicle depends on their characteristics since they can reach the B cells in a soluble form or directly presented by other cells:

- Soluble antigens, smaller than 70KDa, can diffuse into the draining lymph nodes where FO B cells can acquire them and migrate to the T-B border. It was thought earlier that antigen gains access into the lymph nodes thanks to small pores found in the subcapsular sinus (SCS), although, later, the existence of a conduit network has been described which allows the faster diffusion of antigen into the follicles (Farr et al. 1980, Pape et al. 2007, Roozendaal et al. 2009).

- Large antigens, bigger than 70KDa, are supposed not to be able to diffuse into the follicle and they need cell-mediated transport. Usually antigens reach the follicles in an opsonized form coated with soluble molecules such as IgGs (immuno-complexes), lectins or complement mediators. These opsonized antigens are then recognized by different membrane receptors of the antigen-presenting cells in order to be presented to the B cells. There are different cell types implicated in this acquisition and presentation into the follicle (Batista and Harwood 2009):
    - Subcapsular macrophages: type of macrophages that form a layer at the subcapsular sinus (SCS) and that differ from medullar macrophages by their low phagocytic ability (Szakal et al. 1983). These cells participate in the acquisition of virus, particles and immuno-complexes (IC) through three different receptors: FCγRIIB, Mac1 and SIGN-R1. It has been shown that after immunization, FO B cells stop near them and acquire and accumulate antigen before moving to the T-B zone (Carrasco and Batista 2007, Junt et al. 2007, Phan et al. 2007).
    - Dendritic cells: can present antigens at the follicle or in an extrafollicular location to newly arriving B cells from high endothelium venules. These cells use two kinds of receptor to acquire and present antigen: FCγRIIB and DC-SIGN (Kwon et al. 2002, Itano et al. 2003).
    - Follicular Dendritic cells (FDCs): are mainly found inside the follicle. FDCs retain antigens in an IC-form trough Complement receptors CR1 and CR2 and the FCγRIIB receptor, although this last one is less expressed. Their antigen-depots remain bound to the membrane for long-times, which facilitates a long-lasting response (Yoshida et al. 1993, Fang et al. 1998).
    - B cells: it has been shown that marginal zone B cells can transport IC from the SCS to the follicle to load FDCs located there (Carrasco and Batista 2007).

Although B cells can acquire and be activated by soluble antigens, it has been reported that membrane-bound antigens are more efficiently recognized *in vivo* (Carrasco and Batista 2006, Depoil et al. 2008). Moreover, it has been shown that while for soluble antigen it is necessary the crosslinking of 2 or more BCRs for signaling (Schamel and Reth 2000), monovalent membrane-bound antigen is sufficient to induce BCR conformational change that stimulates their association with neighboring BCRs to propagate the signal (Tolar et al. 2009). The interaction between the B cells and the APC generates a specialized cell-cell contact structure

called **'Immunological Synapse'**. These structure was first reported in T cells when their TCRs interact with peptide-MHC molecules (Dustin and Shaw 1999). Later, it was shown that B cells also form this characteristic structure after binding to membrane-attached antigens (Batista et al. 2001). The immunological synapse mediates cell activation, antigen acquisition and internalization requiring extensive remodeling of the cell cytoskeleton. The actin cytoskeleton has been reported not only to be essential for immunological synapse formation (Depoil et al. 2009) but also to start and sustain BCR signaling facilitating membrane organization (Treanor et al. 2010).

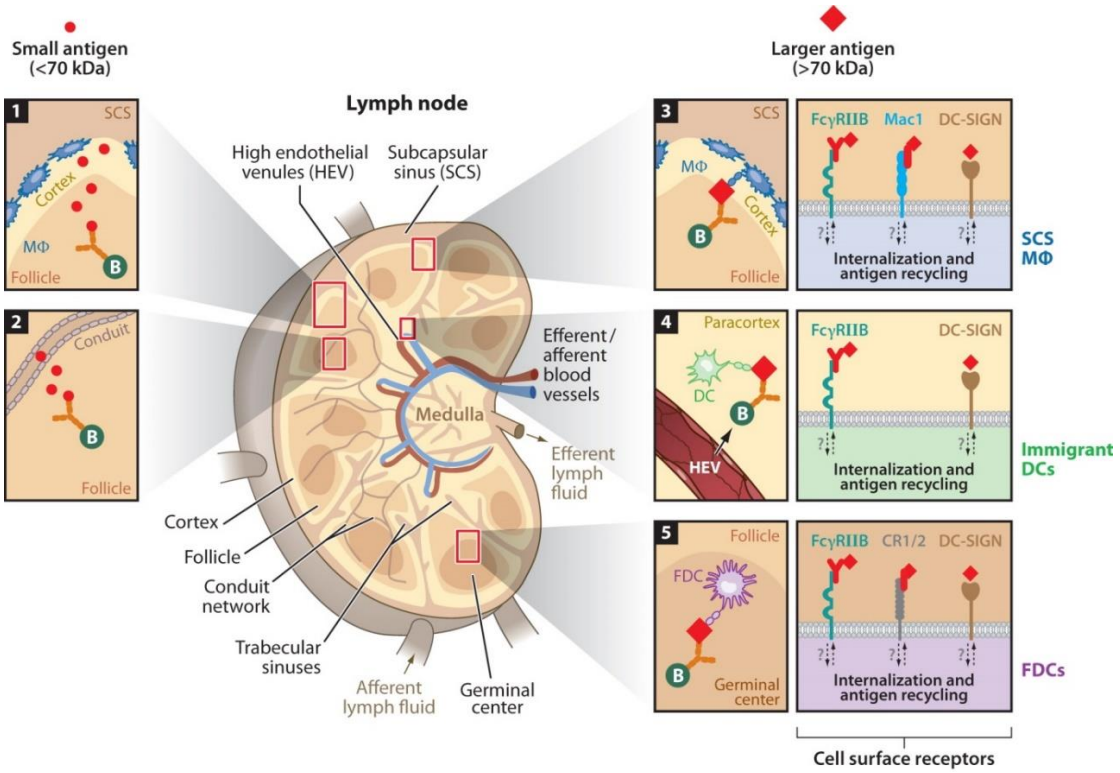

Illustration 2. **Antigen presentation to B cells.** Visualization of the internal structure of a lymph node. Small (< 70 kDa) antigen (red circle) can enter into the follicle trough small pores generated between the macrophages of the subcapsular sinus (1) or through a conduit network coming from the afferent vessels (2). Larger (> 70 kDa) antigens (red square) can be presented to B cells by macrophages of the SCS (3), immigrant dendritic cells that extravasate from high endothelium venules (HEV) (4) and by follicular dendritic cells (5) having antigen bound to membrane specific receptors. Adapted from (Harwood and Batista 2010).

4. Antigen uptake and presentation

B lymphocytes are considered professional presenting cells although they are less efficient than DC in their capacity to take antigen in a non-specific manner (Avalos and Ploegh 2014).

Nevertheless, it is essential that B cells acquire antigens through their BCR to present them to CD4 T cells and generate an efficient T-dependent humoral response.

4.1 Antigen uptake: phagocytosis

There are three major pathways for antigen internalization. All three exist both in DC and B cells but their regulation and efficiency differs (Roche and Furuta 2015):

- **Endocytosis**: this process involves the internalization of soluble molecules as well as small receptor-ligand complexes and other membrane proteins into intracellular compartments. B cells can use this antigen acquisition mechanism thanks to different membrane receptors such as BCR, FCγR or Complement Receptor. This process can be independent or dependent of clathrin, and although the latter is considered the main pathway for antigen internalization in B cells (Natkanski et al. 2013), there are other mechanisms relevant for endocytosis of antigen that are clathrin-independent (Stoddart et al. 2005, Mayor and Pagano 2007).
- **Macropinocytosis**: it is a non-selective process that consists in the capture and internalization of large quantities of extracellular material into vesicles called pinosomes. This process involves the formation of membrane ruffles to acquire the material. Although B cells can acquire antigens using this process, the efficiency is very low compared to other receptor-dependent mechanisms.
- **Phagocytosis:** it is the process by which cells recognize and engulf particles bigger than 0.5μm in an actin-dependent mechanism that drives the generation of a phagocytic cup. The rearrangement of the plasma membrane around the particles proceeds in a zipper-like manner as receptors in the membrane progressively bind more antigen molecules.

Phagocytosis is an ancient and evolutionary conserved mechanism that has two main roles: feeding in unicellular organisms and clearance of pathogens. There are three main groups of receptors involved in the detection and clearance of foreign bodies: pattern-recognition receptors, opsonic receptors and apoptotic receptors (Flannagan et al. 2012). During phagocytosis, a characteristic structure called **'phagocytic cup'** is generated which shares several functional and structural similarities with the immunological synapse: receptor clustering, actin reorganization, microtubule dynamics, intracellular trafficking and internalization of the triggered receptor (Niedergang et al. 2016).

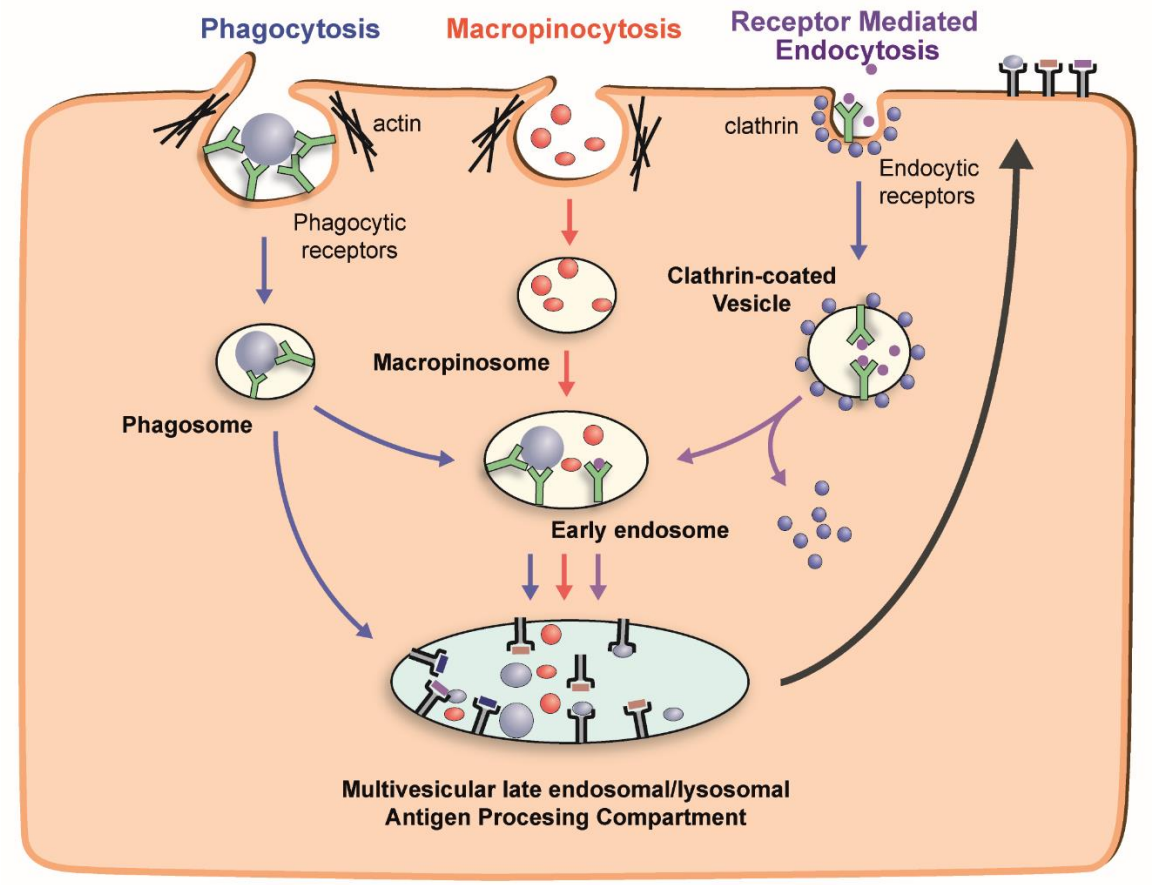

Illustration 3. Pathways of exogenous antigen uptake by antigen-presenting cells. The antigen internalized enters to organelles that favor its denaturalization and degradation to be loaded onto MHC II molecules. Phagocytosis process (blue) engulfs particulate antigens bigger than 0.5 μm through its binding to membrane-receptors. Phagocytosed antigen (found in phagosome vesicles) can fuse to early and late endosomal/lysosomal compartments where peptide-MHCII complexes are generated. Macropinocytosis process (red) uptakes extracellular antigens in a non-specific manner. Vesicles generated are called macropinosomes that enter to the early endocytic pathway of antigen processing. Receptor-mediated endocytosis (purple) uptakes soluble particles using clathrin-coated vesicles. Adapted from (Liu and Roche 2015).

In mammalian macrophages the most studied phagocytic pathways are the two which imply the activation of the opsonic receptors FcγR, which interact with IgG and is dependent of CDC42 and Rac GTPases, and CR3, that interacts with the complement component iC3b and is dependent of RhoA GTPase (Groves et al. 2008). They were classified as type I and type II phagocytosis respectively (Caron and Hall 1998).

FcγR-mediated phagocytosis takes place after ligand binding, which distribute the receptors in closer proximity, generating their aggregation and ITAM phosphorylation. This phosphorylation is produced by Src-family kinases that in turn promote the recruitment and

activation of the Syk kinase, whose function is essential (Crowley et al. 1997), and adaptor proteins. In addition, PLCγ and PI3K are important in order to eliminate PIP2 lipids found in the membrane and allow particle internalization (Flannagan et al. 2012).

Particle uptake needs remodeling of the actin cytoskeleton, which requires the activity of nucleators such as the Arp2/3 complex and nucleation-promoting factors (WASP and Scar/WAVE family of proteins) (May et al. 2000). GTPases of the Rho family control phagocytosis at different steps: Cdc42 during the early phagocytosis, Rac1 in the nascent phagosome and Rac2 at the base of the phagocytic cup (Flannagan et al. 2012). RhoG must also participate since cells deficient in this GTPase are defective in phagocytosis mediated by FcγR and CR3 receptors (Tzircotis et al. 2011). The internalization of large particles requires extensive remodeling of the plasma membrane that requires the delivery of new plasma membrane from intracellular compartments by focal exocytosis.

B cells were thought not to be able to phagocytose antigens, but few year ago, three different groups demonstrated that a specific subtype of B cells (B1 B cells) phagocytose latex beads and bacteria *in vivo* (Gao et al. 2012, Nakashima et al. 2012, Parra et al. 2012). These papers go along with data published several years ago showing that lymphoblastic B cell lines acquire antigen bound to latex beads and present it to T cells with 10^4-fold higher efficiency than soluble antigen. Nevertheless, Vidard and colleagues could not demonstrate the phagocytosis of latex beads by naïve B cells (Vidard et al. 1996).

4.2 Peptide loading

MHC class II is assembled in the endoplasmic reticulum (ER) but functional maturation is achieved in the endosomal compartment, rich in antigenic peptides. In the ER, MHC-II associates with a protein called Invariant chain (Ii) or CD74. This association promotes MHC-II folding and, thanks to specific motifs present in the Ii sequence, MHC-II can traffic to the endosomal compartment (Roche and Furuta 2015). The interaction with Ii has also a role preventing MHC-II early loading with antigen peptides. In acidic endosomes, the Ii protein is proteolysed and released, only leaving an Ii-derivate peptide, called CLIP, associated with the MHC II binding groove. Afterwards, in late endosomes, H2-DM protein (HLA-DM in humans) promotes the dissociation of CLIP, facilitating MHC II-loading with internalized antigen peptides (Blum et al. 2013). After antigen peptide binding, MHC II-antigen traffics to the plasma membrane where it will be able to present the peptide to T cells.

4.3 CD40/CD40L signaling

Presentation of MHC-II antigen complexes by B cells to cognate CD4 T cells results in the activation of the latter that subsequently express CD40L (CD154). CD40L expressed by activated T cells can then interact with CD40 in B cells. CD40 is a transmembrane protein of Tumor Necrosis factor family (TNF) expressed on B cells, DC, monocytes, platelets, macrophages and other non-hematopoietic cells as fibroblasts. CD40L is primarily expressed by activated T cells, as well as B cells and other cell subtypes under inflammatory conditions (Schonbeck and Libby 2001). CD40/CD40L interaction plays an important function during T-cell dependent humoral response because CD40 signaling is essential for germinal center formation, immunoglobulin class-switch, somatic hypermutation and generation of memory and long-lived plasma cells. The engagement of CD40 by its ligand promotes the recruitment of TNFR-associated factors (TRAFs) to its cytoplasmic domain. TRAF signaling cascade activates different pathways including canonical and non-canonical NF-Kβ, PI3K, MAPK and PLCγ. However, it has been shown that CD40 can also signal through the recruitment of Jak3 to its cytoplasmic domain, than in turn activates the STAT5 cascade (Elgueta et al. 2009).

5. Germinal center

5.1 Germinal center reaction

The germinal center is an anatomical, functional and transient structure that is produced in response to antigens in secondary lymphoid organs. In germinal centers antigen-specific B cells proliferate as well as experiment immunoglobulin class-switch and BCR affinity maturation due to somatic hypermutation. It was first described in 1884 by Walter Flemming as distinct anatomical regions with dividing cells that appear after immunization. Much later, in 1957 Burnet postulated the clonal selection theory about the expansion of the B cell clones with high BCR affinity for the immunized antigen (Burnet 1976). Previous experiments had already shown that immunoglobulin affinity in serum of immunized animals increased over time in concordance with Neil Jerne's postulation of a Darwinian-like process among the antigen-reactive B cells (Jerne 1951, Eisen and Siskind 1964). The germinal center reaction peaks after 6-7 days of immunization under experimental conditions, and after that period, it starts to decrease. Nevertheless, in response to pathogens, GC reaction can be more sustained, as for example upon *Plasmodium* infection (Robbiani et al. 2015).

In non-immunized animals the spleen is divided in follicles composed of naïve IgM^+ IgD^+ B lymphocytes. Once pathogenic antigens reach the follicles, activated antigen-specific T and B cells migrate towards the interfollicular T:B zone, where B cells present antigen to T cells, generating long-lasting interactions and the first wave of B cell proliferation. At this stage, B cells can either differentiate towards **short-lived extrafollicular plasma cells**, or plasmablast, or migrate deep into the follicle to generate a germinal center response. This extrafollicular B cell response produces antigen-specific antibodies of low affinity responsible for the first rapid reaction (Paus et al. 2006). Recently it has also been described the generation of **early-memory B cells**, independently of germinal center reaction, which can have isotype switched BCRs but do not undergo hypermutation (Inamine et al. 2005, Kaji et al. 2012, Weisel et al. 2016).

Precursors of T Follicular Helper (TFH) and pre-GC B cells migrate into the follicle three days after antigen administration. Therefore, the commitment of T and B cells to GC takes places outside the follicle, but after that, they need to migrate inside the follicle to generate the GC. During these first steps of GC formation, proliferating antigen-specific B cell blasts displace IgM+ IgD+ naïve B cells generating a mantle zone around the GC. This massive proliferation takes place after 5-6 days postimmunization, inducing a vast increase of GC size, which is fully mature after day 7 of immunization. Mature germinal center is subdivided in two compartments which were originally separated according to their appearance in histochemical sections: the Dark Zone (DZ) and the Light Zone (LZ). The DZ is of high cell density due to packed lymphoblasts, while the LZ is more sparsely populated. There is a continuous trafficking of B cells between both areas (Allen et al. 2007, Hauser et al. 2007, Schwickert et al. 2007).

The **DZ** is mainly formed by highly proliferating B cells called *centroblasts* intermingled in a network of *reticular cells* and *tangible-body macrophages*. During this massive proliferation, B cells undergo somatic hypermutation in the variable regions of their Ig receptor in order to enhance their affinity for the antigen proteins (Victora et al. 2010). This process is carried out by AID, an enzyme that deaminates cytosine residues in ssDNA to uracil, generating point mutations. It is also responsible for Ig class-switch recombination during B cell maturation due to the generation of double strand breaks caused by DNA mismatched that induce a deletional recombination reaction (Pavri and Nussenzweig 2011). Class-switch recombination can also occur earlier than germinal center (Pape et al. 2003).

The proliferated B cells carrying mutated BCRs migrate to the **Light Zone**, where the BCRs are interrogated for their affinity resulting in the selection of higher-affinity clones. In this GC

compartment we can find germinal center B cells called *centrocytes*, *T follicular helper cells*, *Follicular Dendritic cells* and *macrophages* (Victora and Nussenzweig 2012). Newly arrived B cells need to acquire more antigen through their mutated BCR to present it to TFH cells in order to obtain their co-estimulus and survive (Allen et al. 2007). The limiting numbers of TFH cells allows the competition among antigen-specific B cells to acquire antigen and load new MHCII molecules (Gitlin et al. 2014). Regulation of MHC expression and degradation between centrocytes and centroblasts prevents the accumulation of old MHC-peptide complexes and favors the competition for T cell help (Bannard et al. 2016). The iterative cycles between DZ and LZ promote the selection of B cells with high affinity BCRs. The LZ is constantly repopulated with 50% of centroblasts, but only 10-30% of centrocytes returns to the DZ suggesting that clonal selection takes place in the LZ (Victora et al. 2010).

5.2 Germinal center populations

Germinal center B cells

GC B cells can be distinguished from naïve B cells according their size, as they have a more blastic and polarized structure with a leading edge and an uropod. They are the fastest dividing mammalian cells, dividing every 6 to 12 hours (Allen et al. 2007, Hauser et al. 2007). GC B cells can be identified by high expression of CD95 and n-glycolylneuraminic acid (recognized by GL7 antibody clone), together with high binding to peanut agglutinin (PNA), loss of surface IgD and modulation of CD38 expression (in mice is downregulated while in human is upregulated) (Victora and Nussenzweig 2012). Bcl6 is the master regulator of germinal center B cell differentiation, since it has been shown that *Bcl6*-/- mice are unable to generate germinal centers and high-affinity antigen specific antibodies (Dent et al. 1997, Ye et al. 1997). Bcl-6 functions as a transcriptional repressor and has four major roles controlling germinal center reaction (Basso and Dalla-Favera 2010):

- Silencing antiapoptotic molecules such as Bcl2. The fate of germinal center B cells is to die unless they receive prosurvival signals. The maintenance of a pro-apoptotic genetic fingerprint favors the survival of clones with the highest BCR affinity and prevents to some degree the appearance of autoreactive clones that emerge during the SHM process (Saito et al. 2009).

- Repressing the expression of p53 and ATR, which increases the ability of B cells to tolerate DNA damage induced by rapid proliferation and AID function (Phan and Dalla-Favera 2004, Ranuncolo et al. 2007).
- Silencing Blimp1 gene, the master regulator of plasma cell differentiation, therefore preserving the germinal center identity (Tunyaplin et al. 2004). In this regard, Bcl6 also repress some BCR and CD40 downstream effectors to allow a correct responsiveness of GC cells to selective signals in order to maintain GC characteristics and prevent their differentiation (Shaffer et al. 2000, Basso et al. 2010).

The localization of B cells in and around the germinal center is due to the expression and downregulation of different chemokines and G-protein coupled membrane receptors (GPCRs). Naïve B cells express high amounts of the GPCR Ebi2, which maintain the cells in the outer part of the follicle and allows to perform an extrafollicular B cell response. Ebi2 has to be repressed in GC B cells to allow their localization in the center of the follicle and the formation of the GC (Gatto et al. 2009, Gatto et al. 2011). Likewise, CCR7 expression in cognate-activated B cells allows their localization in the T:B border to interact with T cells since the CCR7 ligands CCL19 and CCL21 are produced in the T cell areas (Forster et al. 1999, Gatto et al. 2011). Homing to the follicles is directed by CXCR5 given that its ligand CXCL13 is produced by stromal cells located inside the follicle, while the induction of S1P2 by GC B cells and TFH cells prevents their migration towards the outer follicle (Green et al. 2011). The later trafficking inside the germinal center between the DZ and LZ is regulated by the expression of CXCR4 (Allen et al. 2004), since its ligand (CXCL12) is produced by stromal cells located in the DZ (Bannard et al. 2013). GC B cells found in the DZ are characterized by the expression of $CXCR4^{hi}$ $CD86^{lo}$ / $CD83^{lo}$ and downregulation of their BCR in the membrane, while GC B cells found in the LZ, centrocytes, express lower levels of CXCR4 and higher of CD86 and CD83 correceptors (Victora et al. 2010), indicating an activated-like phenotype.

Follicular dendritic cells

FDCs form a reticular network in the follicle and germinal center, mainly localizing in the Light Zone. They have two major roles:

- Working as a reservoir of antigen in the germinal center retaining antigens for long periods. FDCs, as it has been mentioned before, can present and retain the antigen in immune complexes (IC) through their Complement receptor 1 and 2 (CR1 and

CR2) and the FCγRIIB receptor (Barrington et al. 2002). The FDCs are found in both GC areas (DZ/LZ), although FDCs presenting immuno-complexes are only localized in the LZ.

- Secreting chemokines and cytokines to attract and sustain GC B cells. They express CXCL13, which is the ligand of CXCR5 that promotes the location of B and follicular T cells in the germinal center and is supposed to play a role in DZ/LZ localization (Allen et al. 2004, Haynes et al. 2007). In addition, FDCs express cytokines such as BAFF and IL-6 which favor germinal center reaction and the survival of B cells (Kosco-Vilbois and Scheidegger 1995).

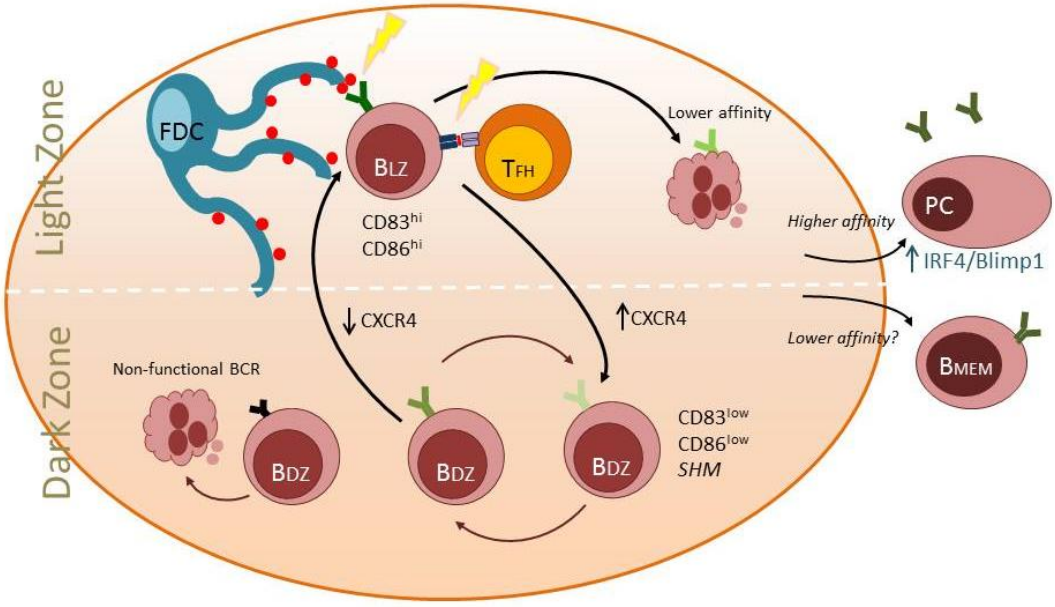

Illustration 4. Germinal center generation. The cartoon shows a mature germinal center subdivided in the Dark zone (DZ) and Light Zone (LZ). Germinal center B cells cycle between these two areas in order to proliferate, perform class-switch and increase their BCR affinity thanks to the differential expression of chemokine receptors. In the Light Zone, B cells with high BCR affinity acquire antigen from FDCs to present it to the TFHs and obtain their help. In the dark zone, GC B cells proliferate and perform CSR and SHM. B cells with lower BCR affinities or non-functional receptor enter in an apoptotic process. B cells that express a high affinity BCR exit GC program towards memory cell or plasma cell fate. Adapted from (Mesin et al. 2016).

T follicular helper cells

Despite being a smaller population, T cells play an essential role in the generation, maintenance and clonal selection of germinal center B cells. After the first CD4 T cell priming

event by DC, the CD4 cell undertakes a cell-fate decision in which if CXCR5 expression is upregulated, the cell migrates to the T-B border where, if it correctly interacts with a B cell presenting antigen, it differentiates to TFH cells (Ramiscal and Vinuesa 2013, De Silva and Klein 2015). This differentiation is directed by the Bcl6 transcription factor, as described for GC B cells (Johnston et al. 2009, Yu et al. 2009). **GC TFH** are characterized by the expression of $CXCR5^+$ $PD1^+$ $Bcl6^+$ $CD84^+$ $ICOS^+$ (Hutloff et al. 1999, Haynes et al. 2007), and the production of high concentrations of IL21 and variable levels of IL4, IFNγ, and IL2. These GC TFH cells play a fundamental role providing survival and selection signals to centrocytes (Ramiscal and Vinuesa 2013). Their number is limited during germinal center reaction, for that reason GC B cells compete for obtaining their help since more antigen B cells present, increased probability to interact and obtain TFH co-stimulus and prosurvival signals (Schwickert et al. 2011). Moreover, it has been shown that increasing numbers of TFH cells impair somatic hypermutations and affinity maturation of GC B cells since they do not need to compete for their help (Preite et al. 2015).

In addition to CD4 TFH cells, there are other germinal center T cell populations: **NK TFH** that are found after immunization with CD1d-a-Galceramide and also express CXCR5 and PD1 under the control of Bcl6 and CD28 signaling. They induce early antibody responses which promote the appearance of early extrafollicular plasma cells, but not memory B cells nor bone-marrow plasma cells (Detre et al. 2012, King et al. 2012, Tonti et al. 2012); **T follicle regulatory cells** that are characterized as $CXCR5^{hi}$ $PD1^{hi}$ $Foxp3^+$ cells but also express GITR, CTLA-4 and IL-10. Their roles are still discussed but it seems that they repress self-reactive germinal center B cells and diminish germinal center reactions (Chung et al. 2011, Wollenberg et al. 2011); **CD8+ T regulatory cells,** which also play a suppressing role on GC TFH cells that express MHC class I Qa-1. They are identified by the expression of CD44, ICOSL and CXCR5, as well as CD8 (Kim et al. 2010).

Other cell populations

Tingible body-macrophages (TBM) whose function is to engulf and eliminate apoptotic B cells residual from the selection process carried out during the GC process (Aguzzi et al. 2014).

Germinal center-resident DCs have also been found in human tonsils and mouse spleen but their function is not well understood (Grouard et al. 1996, Lindquist et al. 2004).

5.3 Germinal center B cells fate: Memory and Plasma cells

The humoral response generates two important barriers to a secondary infection: pathogen-experienced memory B cells and plasma cells secreting protective-antibodies.

Memory B cells are long-lived quiescent cells that upon reestimulation secrete antigen-specific antibodies and can reenter into the germinal center or differentiate into plasma cells. They are found in the spleen and in circulating blood and in humans can be distinguished by the expression of CD27. There is not, however, a specific marker for mouse cells, although there have been different suggestions (Weisel et al. 2016). What promotes the fate decision towards memory B cell generation is still unknown since class-switched memory B cells arising from GC are phenotypically indistinguishable from GC B cells in terms of number of mutations and affinity of their BCR. Indeed, defects in germinal center formation and development have an effect on the generation of the memory B cell population coming from GC, but not always in plasma cell formation (Good-Jacobson and Shlomchik 2010).

The other important cell population that comes up from GC are the **long-lived plasma cells**, or antibody secreting cells. These cells remain in non-dividing state at the bone marrow in specialized niches. They differentiate from GC and memory B cells as they secrete immunoglobulins bearing a wide range of selecting mutations (Phan et al. 2006), which function as a barrier for secondary re-infections. They do not express Ig receptor at the membrane and in human can be differentiated by the expression of CD38, CD27 and $CD20^{low}$, while in mouse by CD138 and $B220^{low}$. Blimp1 is the master regulator which directs GC B cells towards PC fate. Blimp1 expression is upregulated upon CD40 ligation, which induces the expression of Irf4 TF through the activation of NFkB. Irf4 repress Bcl6 (Klein et al. 2006, Sciammas et al. 2006, Bolduc et al. 2010, Kishi et al. 2010).

Although the precise mechanism that induce germinal center B cell differentiation towards memory B cells or plasma cells is still not known, it has been hypothesized that the BCR affinity and the Ig isotype can direct them into one or the other fate (Phan et al. 2006, Gitlin et al. 2016). The existence of several gaps about how the germinal center response occurs and how memory and plasma cells differentiate is in part due to the absence of an *in vitro* system to study these processes in detail. This thesis tries to develop a new *in vitro* system culture that recreates a germinal center response.

6. Ras superfamily

Ras superfamily consist of a big family of proteins of low molecular weight (20-29 KDa) formed by more than 150 members. They are much conserved across species, having a 30-55% homology between sequences (Goitre et al. 2014). All of them share structural and mechanistic common features since they switch between an active GTP-bound and an inactive GDP-bound state, which induces a conformational change in the protein. Ras proteins have in general low intrinsic GTPase activity, so their activity is regulated by guanine nucleotide exchange factors (GEF) and GTPase-activating proteins (GAPs). The first ones promote the transition from the inactive GDP-bound state to an active-GTP bound conformation, while GAP proteins accelerate GTP hydrolysis promoting the switch of Ras proteins to an inactive GDP-bound state (Quilliam et al. 2002, Johnson and Chen 2012). Ras proteins are posttranscriptionally modified, mainly in their C-terminal region, which allows its specific localization and anchoring to the plasma membrane. Moreover, these specific modifications determine the effector proteins of each member. In addition to GEFs and GAPs regulators, the activity of some Ras proteins can be modulated by a third group of regulators called guanine nucleotide dissociation inhibitors (GDIs) that interact with the C-terminal regions of Ras proteins, avoiding their correct localization in the plasma membrane next to their effectors.

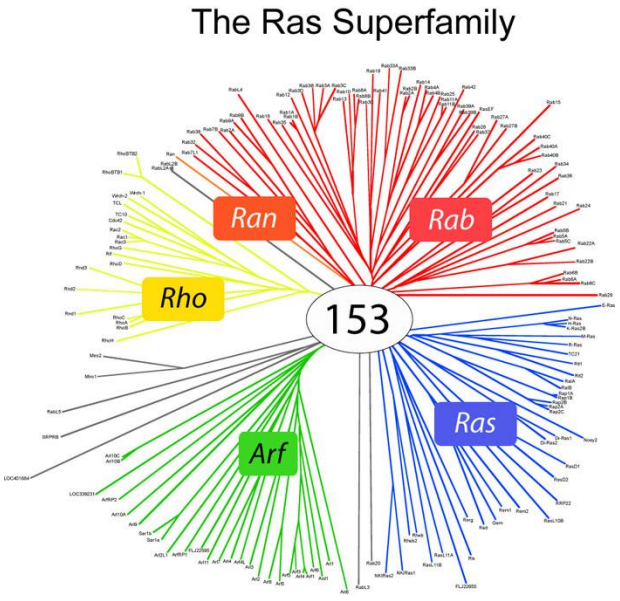

Illustration 5. Ras superfamily members. The illustration shows the distribution of the members of the five different families of Ras superfamily: Ras, Rab, Ran, Rho and Arf. Modified from (Wennerberg et al. 2005).

Ras superfamily is subdivided in 5 different subfamilies: Ras, Rac/Rho, Rab, Arf and Ran which exert different functions (Wennerberg et al. 2005). This thesis is going to be focus in two proteins belonging to the **Rho** and **Ras** families.

6.1 Ras family: RRas2

The Ras family was found in the 70s as a consequence of studying transforming retroviruses isolated from **ra**t **s**arcomes, but it was in 1982 when mutational activated forms of Ras protein were found in human cancer cell lines (Goitre et al. 2014). Nowadays, in humans there are 40 Ras proteins subdivided in 6 different subfamilies: Ras, RRas, Rap, Ral, Rheb and Rin. They share high sequence homology mainly in their N-terminal region, which includes the effector binding domains (Switch I and II), and the GTP/GDP interaction regions. They intervene in a wide range of functions: cell proliferation and differentiation, survival, GTP-dependent exocytosis, cell adhesion, morphology, migration and polarization. The classical Ras family is composed of KRas, NRas and HRas and has been extensively studied because they are found mutated in 15-20% of human tumors, reaching 90% in some tumors as pancreatic (Prior et al. 2012). Ras family expression is ubiquitous, although some Ras proteins have a cellular-restricted expression. Most of the Ras proteins are localized in the plasma membrane through posttranslational modifications of their CAAX C-terminal domain (Ahearn et al. 2012).

RRas2

The subfamily of RRas is composed of three members: RRas1 and RRas2, which are ubiquitously expressed, and MRas (or RRas3) whose expression is restricted to the brain. RRas2 was found in 1990 in a cDNA library of teratocarcinomas (Drivas et al. 1990). It is the only Ras superfamily protein with transforming abilities similar to the oncogenic classic Ras proteins (Graham et al. 1994). It shares a 55-60% homology with classical rases, and the effector loop is basically indistinguishable, it is probably for that reason that it has not been studied in depth. Although RRas2 has a high intrinsic GTPase activity (Movilla et al. 1999), it has some described GEFs as SOS1, RasGRF and RasGRPs; and GAPs as p120-GAP and NF1, which are also active on classical Rases (Graham et al. 1996, Ohba et al. 2000). RRas2 has been found mutated in their active form in several types of cancer at low frecuency rates (0,6-2%) such as uterine and kidney carcinoma, large B-cell lymphoma, lung, stomach and colorectal adenocarcinomas, skin melanoma and ovarian carcinoma (Cerami et al. 2012) (Gao et al. 2013), although it is mainly found overexpressed in its wild-type version in tumors of the oral cavity, esophagus, skin carcinomas, breast cancer and lymphomas (Clark et al. 1996, Sharma et al. 2005, Delgado et al. 2009, Macha et al. 2010, Larive et al. 2012). Moreover, it has been found that specific RRas2 polymorphisms are linked to unfavorable Tamoxifen treatment of breast cancer (Rokavec et al. 2008). In spite of its important role in tumorigenesis and its high

homology with classical Rases, RRas2 has been poorly studied in many other biological processes, and in most cases the studies have been generalized to Ras proteins without specification of which type of Ras protein.

Several years ago, it was described the existence of a GTPase activity coupled to CD3γ and CD3ζ chains of the TCR, but it was not specified what protein was responsible (Peter et al. 1993). In 2009, our laboratory, in search for TCR direct effectors using a yeast two-hybrid approach, found that RRas2 interacts in its inactive form with all CD3 chains through their ITAM motifs, 'preferentially' when they are non-phosphorylated. This interaction was also found in B cells where RRas2 binds to Igα chain of the BCR. RRas2 plays an important role during survival and homeostatic proliferation of T and B cells by recruiting p110δ subunit of PI3K to the antigen receptors (both TCR and BCR) and activating the PI3K-AKT pathway. RRas2-deficient mice have lower numbers of marginal zone and follicular B cells and a deficient germinal center response. The effect on T cells was not so apparent but they also show a decrease in age-developed memory T cells and in total numbers (Delgado et al. 2009). Moreover, in our laboratory it was described that RRas2 colocalize with the TCR at the immunological synapse and through PI3K directs the activation of RhoG, participating in the uptake of antigen from antigen-presenting cells trough a trogocytic process (Martinez-Martin et al. 2011).

6.2 Rho family: RhoG

In mammals, this family is composed of 23 members subdivided in 6 different families: Rho, Rac, CDC42, Rnd, RhoBTB and MIRO (Wennerberg and Der 2004). These GTPases are activated by 79 different GEFs, which belong to two different families, being the major one the Dbl family, with at least 66 members. Downregulation of their activity is promoted by 70 different known GAPs and 3 mammal GDIs. Their strong and stringent regulation suggest their important role in cellular processes such as actin cytoskeleton organization, cell adhesion, polarity, motility, cell-cycle progression and gene regulation (Tybulewicz and Henderson 2009). The importance of this family of proteins was manifested when in 1992 the laboratory of Allan Hall implicated the Rac and Rho families in cytoskeleton reorganization and stablished a crosstalk between Rac/Rho and Ras/Rho families (Ridley and Hall 1992, Ridley et al. 1992). Some of the most studied Rho GTPases are: Rac1, involved in the generation of lamellipodia and membrane ruffles; RhoA, involved in the formation of stress fibers and microspikes; and Cdc42, important for filopodia formation. This family of GTPases has been long studied but

mainly using dominant negative versions of the proteins or using *Clostridium botulinum* C3 toxin, which ADP-rybosylates and inactivated the GTPases. It is for that reason that it is still not known if many processes are carried out by one or more specific Rho GTPases (Tybulewicz and Henderson 2009).

RhoG

RhoG is a member of the Rac subfamily composed by Rac1, Rac2, Rac3 and RhoG. Rac1 and RhoG have a widespread expression while Rac2 and Rac3 are more restricted to hematopoietic and neural tissues respectively (Burridge and Wennerberg 2004). RhoG was discovered in 1992 analyzing genes induced by different extracellular stimuli such as growth factors, thrombin and serum (Vincent et al. 1992). It has a structure similar to Rac1 and CDC42, with a 72% and 62% homology respectively in their general sequence, but 92% in their effector domains. There has been much speculation about the effectors shared between Rac1 and RhoG, as it was shown that they have some in common such as JNK, but differ in others such as PAK1 kinase (Prieto-Sanchez and Bustelo 2003). Nevertheless, it was shown that RhoG can activate Rac1, since RhoG activation by the GEF Trio promotes its binding to ELMO effector, which generates a trimeric complex with Dock180 that functions as a GEF for Rac1 (Katoh et al. 2006). RhoG participates in multiple processes: engulfment of apoptotic bodies (deBakker et al. 2004); transendothelial migration by leucocytes (van Buul et al. 2007), micropinocytosis (Ellerbroek et al. 2004), neurite outgrowth (Katoh et al. 2000, Estrach et al. 2002), fibroblast migration (Katoh et al. 2006), macrophage FcγR and CR3-dependent phagocytosis (Tzircotis et al. 2011), gene expression in T and B lymphocytes (Vigorito et al. 2003), as well as activation of proapoptotic and antiapoptotic pathways trough JNK and PI3K activation respectively (Murga et al. 2002). Nevertheless, although the inhibition of thymic Rho GTPases in mouse by C3 toxin cause severe problem in lymphocyte development, the mouse deficient for RhoG did not show any impairment in T and B cell development (Galandrini et al. 1997, Henning et al. 1997, Vigorito et al. 2004). The only difference found in those mice was a slightly upregulated humoral response in the absence of RhoG with an increase of serum IgG1 and IgG2b immunoglobulins after immunization with soluble antigen. In our laboratory it was described that RhoG participates in the trogocytosis and phagocytic TCR-driven antigen uptake in mature T lymphocytes. The activation of RRas2 through the TCR induces the activation of PI3K and subsequently RhoG activity (Martinez-Martin et al. 2011).

In this thesis, we study if B cells, like T cells, can also uptake phagocytic antigens in a RhoG- and RRas2- driven mechanism. In addition, we study the importance of this mechanism during the generation of the humoral response both *in vitro* and *in vivo*.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. MATERIALS

1.1 Cell lines

MOLT-4 is a T cell line originated from a patient with acute lymphoblastic leukemia. These cells are cultured in RPMI with 5% fetal bovine serum (FBS) supplemented with 2mM L-Glutamine, 100U/ml penicillin and 100U/ml streptomycin.

HEK293T is a human embryonic kidney cell line transformed with adenovirus (Graham et al. 1977). These cells contain the SV40 large T-antigen allowing the episomal replication of plasmids containing the SV40 origin and the early promotor region. This cell line is cultured in DMEM with 5% fetal bovine serum (FBS) supplemented with 2mM L-Glutamine, 100U/ml penicillin and 100U/ml streptomycin.

Naïve T and B lymphocytes isolated from mouse peripheral lymphoid organs were maintained in RMPI with 10% FBS supplemented with 2mM L-Glutamine, 100U/ml penicillin and 100U/ml streptomycin, 10mM Sodium pyruvate and 20 μM β–Mercaptoethanol. When indicated the medium is supplemented with 1ng/ml IL-21 and 10ng/ml IL-4.

Table 1. Material used for culture cells

PRODUCT	COMERCIAL BRAND
RPMI 1640	CBMSO culture service
DMEM	CBMSO culture service
Culture plates	BD-Falcon
FBS	Sigma
L-Glutamine	CBMSO culture service
Penicillin	CBMSO culture service
Streptomycin	CBMSO culture service

β-Mercaptoethanol	Sigma

1.2 Mice

Throughout this thesis we have used different mice models characterized by RRas2 and RhoG GTPases deficiency and the expression of transgenic BCR and TCR:

Non-transgenic C57BL/6: express the allele CD45.2 of the *Ptprc* gene.

Congenic C57BL/6 CD45.1: express the other allele CD45.1 of the *Ptprc* gene. These animals are used to carry out adoptive transfer experiments in order to differentiate the donor form the receptor cells. Those mice were kindly provided by Dr. Carlos Ardavín (CNB, Madrid).

Rag1^{tm1Mom}: do not have neither mature T nor B cells when are in homozygosis (Mombaerts et al. 1992). These mice were kindly provided by Dr. Cesar Cobaleda (CBMSO, Madrid).

Rras2-/-: these mice are found in C57BL/6 background and were generated as it is described in (Delgado et al. 2009).

Rhog-/-: these mice are found in C57BL/6 background and were generated as it is described in (Vigorito et al. 2004).

B1-8^{hi} knock-in: these mice bear a pre-arranged VDJ region of the Igh chain (Vh186.2) which combined with a specific λ1 IgL recognize 4-Hydroxy-3-nitrophenylacetyl hapten (NP) or its derivate 4-Hydroxy-3-iodo-5-nitrophenylacetyl (NIP) (Shih et al. 2002). These mice were kindly provided by Prof. Tomohiro Kurosaki (RIKEN, Japan). *Rras2*-/- and *Rhog*-/- mice were crossed with B1-8^{hi} in order to obtain ***Rras2-/-*** **B1-8^{hi}** and ***Rhog-/-*** **B1-8^{hi}** mice. These mice are found in C57BL/6 background.

OT-2: these transgenic mice express the OT-2 TCR (Vα2/Vβ5) specific for a peptide 323-339 of chicken ovalbumin presented by I-A^{b} (Barnden et al. 1998).

All the experiments were performed using mice homozygotes for the WT version or the mutated gene with similar age (6-12 weeks). Mice were maintained under SPF conditions in the animal facility of the Centro de Biología Molecular Severo Ochoa in accordance with applicable national and European guidelines. All animal procedures were approved by the ethical committee of the Centro de Biología Molecular Severo Ochoa.

In order to genotype the deficient mice, PCR from genomic DNA was carried out using primers of **Table 2.** In the case of OT-2^{tg} and CD45.1 mice, their genotyping was performed by Flow Cytometry using peripheral blood stained for CD4/Vα2 and CD45.1/CD45.2 respectively.

Table 2. Oligonucleotide sequences for mice genotyping

ALLELE	OLIGO 5'	OLIGO 3'
Rras2+/+	GGATCATGTTGTGGAGTTGTGGC	GTCCAAGAAGACATGGATGGGGG
Rras2-/-	GGATCATGTTGTGGAGTTGTGGC	AAACCCTCTTGCAGTTGCATCCG
Rhog+/+	GGCACAAATGGCACCCAGAGG	GAGTTTCCAGGCAAGGGGTGC
Rhog-/-	CCTCGTCTTGGAGTTCATTC	GAGTTTCCAGGCAAGGGGTGC
B1-8^{hi} KI	CAGGTCCAACTGCAGCAGCCT	CAACTATCCCTCCAGCCATAGGAT
B1-8^{hi} WT	TCAGGTCATGAAGGACTAGG	GACCCTGAAATTGTCACCAT
Rag1^{tm1Mom}	TGGATGTGGAATGTGTGCGAG	GAGGTTCCGCTACGACTCTG
Rag1+/+	CCGGACAAGTTTTTCATCGT	GAGGTTCCGCTACGACTCTG

1.3 Reagents

Table 3. Reagents, source and application

REAGENTS	SOURCE	APPLICATION
Organic salts, acids, bases and solvents	MERCK, PANREAC, SIGMA	Diverse
Cell strainer 40μm	BD Pharmigen	Tissue disruption
0.45μm filters	Millipore	Supernatant filtration
P96 plates	Nunc	Flow cytometry staining
P100, p6, p24 and p96 plates	Falcon	Cell culture
TEMED	BioRad	Western Blot

Nitrocellulose membranes 0.2 μm	BioRad	Western Blot
APS	BioRad	Western Blot
Pierce ECL Western Blotting Substrate	Thermo Fisher	Western Blot
Dynabeads M-280 Streptavidin	Invitrogen	B and T cell purification
Sodium heparin	Chiesi	Peripheral blood collection
Polybrene	Sigma	Lentiviral transduction
JetPEI	PolyPlus Transfection	Hek293T cell transfection
SBA Clonotyping system-HRP	Southern Biotech	ELISA
Poly-L-Lysine	SIGMA	Immunofluorescence
Foxp3 / Transcription Factor Staining Buffer Set	eBiosciences	Flow Cytometry
Imject Alum	Sigma	Mice immunization
RNeasy Plus Mini Kit	QIAGEN	RNA extraction
GoTaq qPCR Master Mix	PROMEGA	qPCR
QIAamp DNA kit	QIAGEN	DNA extraction
Mouse IL-2 Flex Set	BD Biosciences	FACS IL-2 quantification
MS/Rat Soluble Protein master Buffer Kit	BD Biosciences	FACS IL quantification
Mouse IL-21 Flex Set	BD Biosciences	FACS IL-21 quantification
IL-10 CBA Flex Set	BD Biosciences	FACS IL-10 quantification
IL-6 CBA Flex Set	BD Biosciences	FACS IL-6 quantification
IL-4 CBA Flex Set	BD Biosciences	FACS IL-4 quantification

Ovalbumin	SIGMA	Cell stimulation (bead-bound)
NIP(15)-Fluorescein-BSA	Biosearch Technology	B cell staining
NP(7)-BSA	Biosearch Technology	ELISA
NP(41)-BSA	Biosearch Technology	ELISA
NIP(15)-Ovalbumin	Biosearch Technology	Cell stimulation / Mice immunization
NP(25)-CGG	Biosearch Technology	Cell stimulation
NP(36)-PE	Biosearch Technology	B cell staining
HIV-1 p17/p24/gp120	Jena Biosciences	Cell stimulation/ELISA
IL-4	Peprotech	Cell culture
IL-21	Peprotech	Cell culture
Polybead Carboxylate 1 □m	Polysciences	Cell stimulation
Polybead Carboxylate 3 □m	Polysciences	Cell stimulation
FluoSpheres Carboxylate 1 □m Crimson (625/645)	ThermoFischer	Phagocytosis assays
Fluoresbrite Carboxylate 3 □m Y/O (529/546)	Polysciences	Phagocytosis assays
Fluoresbrite Carboxylate 1 □m Y/G (441/486)	Polysciences	Phagocytosis assays
Fluoresbrite Carboxylate 3 □m Y/G (441/486)	Polysciences	Phagocytosis assays
Fluoresbrite Carboxylate 10 □m Y/G (441/486)	Polysciences	Phagocytosis assays
Carbodiiamide Kit	Polysciences Inc.	Covalent binding of proteins to latex beads
Streptavidin-PercP	BD Pharmigen	FACS
Streptavidin-APC	BD Pharmigen	FACS
Streptavidin-APC-Cy7	BD Pharmigen	FACS

Table 4. Buffers

BUFFERS	COMPOSITION	APPLICATION
PBS	137 mM NaCl, 2,7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH2PO4 pH 7,4	Diverse
TBS	50 mM TrisHCl, 276 mM NaCl, 54 mM KCl pH 8	Western Blot
PBS-BSA 1% Azide	PBS 1x, Azide 0,02%, BSA 1%.	FACS
PBS-BSA 1%	PBS 1x, BSA 1%.	ELISA
TBS-TWEEN	TBS 1x, 0,1% Tween	WB
PBS-TWEEN	PBS 1X, 0,05% Tween	ELISA
TNB	100mM Tris-HCl pH 7,4, 150mM NaCl, 2% BSA	Immunofluorescence
ACK lysis buffer	0,15 M NH_4Cl, 10 mM $KHCO_3$, 0,1 mM EDTA, pH 7,2-7,4	Erythrocytes lysis
BRIJ96	20 mM Tris-HCl pH 7.8, 150 mM NaCl, 10mM IOAA, 1 mM PMSF, 1 µg/ml aprotinina, 1µg/ml leupeptina, 0.33% Brij96, phasphatase and proteases inhibitors	Cellular lysis for protein extraction
Tissue digestion	100mM Tris-HCl pH 8,5, 0,2% SDS, 200 mM NaCl, 5 mM EDTA	Genomic DNA extraction
Loading buffer 1X	Glicerol 10%, βmercaptoethanol 5%, SDS 3%, blue bromphenol, 1/8 v upper buffer	SDS-PAGE
Upper buffer	0,4 M Tris pH 6,8, 0,4% SDS	SDS-PAGE
Lower buffer	1,5 M Tris pH 8,8, 0,4% SDS	SDS-PAGE
Polyacrylamide	Acrilamida 30%, Bisacrilamida 0,8%	SDS-PAGE

1.4 Antibodies and fluorescent probes

Table 5. Antibodies and fluorescent probes

ANTIBODIES/ FLUORESCENT PROBES	CLON/ CODE	DESCRIPTION	APLICATION	SOURCE	USE
ANTIBODIES					
Anti-Actin	AC-74	Mouse mAb	WB	Sigma	1:4000
Anti-AKT	9272	Rabbit mAb	WB	Cell Signaling	1:100
Anti-CD45R- APC, –Biotin, –V450, -FITC	RA3-6B2	Rat mAb	FACS/Purification/ IF	BD Pharmigen	1:200
Anti-Bcl6-647, -PE	K112-91	Mouse mAb	FACS	BD Pharmigen	1:75
Anti-Blimp1-PE	5E7	Rat mAb	FACS	BD Pharmigen	1:75
Anti-CD4 -647, -PerCP -Biotin	RM4-5	Rat mAb	FACS /IF	BD Pharmigen	1:200
Anti-CD8-Biotin	53-6.7	Rat mAb	Purification	BD Pharmigen	1:200
Anti-CD11c-Biotin	HL3	Rat mAb	Purification	BD Pharmigen	1:200
Anti-CD16/32 purified	2.4G2	Rat mAb	FACS	BD Pharmigen	1:250
Anti-CD19 –PE, –APC, –PE-cy7	1D3	Rat mAb	FACS/ IF	eBiosciences	1:200
Anti-CD21-PE	7G6	Rat mAb	FACS	BD Pharmigen	1:200
Anti-CD23-V450	B3B4	Rat mAb	FACS	BD Pharmigen	1:200
Anti-CD25-APC	3C7	Rat mAb	FACS	BD Pharmigen	1:200
Anti-CD38-A488	90	Rat mAb	FACS	Biolegend	1:200
Anti-CD40-PE-Cy5	3/23	Rat mAb	FACS	Biolegend	1:200
Anti-CD43-Biotin	S7	Rat mAb	Purification	BD Pharmigen	1:150

Anti-CD45.1-APC-Cy7	A20	Rat mAb	FACS	BD Pharmigen	1:200
Anti-CD45.2-APC	104	Rat mAb	FACS	BD Pharmigen	1:200
Anti-CD86-PE-Cy5	GL1	Rat mAb	FACS	eBiosciences	1:200
Anti-CD95 –FITC, -PE-Cy7	Jo2	Hamster Ab	FACS	BD Pharmigen	1:200
Anti-CD138-APC	281-2	Rat mAb	FACS	BD Pharmigen	1:200
Anti-CD154-PE	MR1	Hamster Ab	FACS	BD Pharmigen	1:200
Anti-CD275-Biotin	HK5.3	Rat mAb	FACS	eBiosciences	1:200
Anti-CD278-PE	7E.17G9	Rat mAb	FACS	Biolegend	1:200
Anti-CD279 (PD1)-FITC	J43	Rat mAb	FACS	eBiosciences	1:100
Anti-CXCR4-Biotin	2B11	Rat mAb	FACS	eBiosciences	1:100
Anti-CXCR5-Biotin	2G8	Rat mAb	FACS	BD Pharmigen	1:100
Anti-F4/80-Biotin	BM8	Rat mAb	FACS/ Purification	Biolegend	1:200
Anti-Gr1-Biotin	RB6-8C5	Rat mAb	Purification	BD Pharmigen	1:200
Anti-GL7- 647, -FITC	GL7	Rat mAb	FACS/IF	BD Pharmigen	1:200
Anti-IgD-Biotin, -FITC, -V450	11-26c	Rat mAb	FACS	eBiosciences	1:200
Anti-Ovalbumin-FITC	Ab85584	RbAb	FACS	Abcam	1:100
Anti-kappa-Biotin	RMK-12	Rat mAb	Purification	Biolegend	1:200
Anti-IgM-PE, -APC	II/41	Rat mAb	FACS	eBiosciences	1:200
Anti-IgM F(ab')2	Polyclonal	Goat pAb	Beads	Jackson Immunoresearch	40μg/ml
Anti-NK1.1-Biotin	PK136	Rat mAb	Purification	BD Pharmigen	1:200

Anti-pAkt (S473)	D9E	Rabbit mAb	WB	Cell Signaling	1:1000
Anti-pCD79A(Y182)	5173	Rabbit pAb	WB/IF	Cell Signaling	1:1000/ 1:100
Anti-pErk (T202/Y204)	9101	Rabbit pAb	WB	Cell Signaling	1:1000
Anti-pS6 (S240/244)	D68F8	Rabbit pAb	WB	Cell Signaling	1:1000
Anti-pSYK (Y525/Y526)	2711	Rabbit pAb	WB/IF	Cell Signaling	1:1000/ 1:200
Anti-Vα2-PercP, -Cy5.5	B20.1	Rat mAb	FACS	Biolegend	1:200
SECONDARY ANTIBODIES					
Anti-rabbit-647	A-31573	Donkey anti-rb	IF	Invitrogen	1:300
Anti-rabbit F(ab')2 Peroxidase (HRP)		Sheep anti-rb	WB	Amersham	1:5000
Anti-mouse Peroxidase (HRP)		Sheep anti-m	WB	Amersham	1:5000
Anti-goat IgGs-FITC	705-096-147	Donkey pAb	FACS / IF	Jackson Immunoresearch	1:150
FLUORESCENT PROBES					
Phalloïdin-TRICT	P-1951		IF	SIGMA	1:200
Phalloïdin- 488	A-12379		FACS	ThermoFisher	1:100
DAPI	268298		IF	Merck	1:500
Cell Trace Violet	C34557		FACS/IF	Life Technology	5 μg/mL
CFSE	C34554		FACS/IF	Thermo Fisher	5 □g/mL

IF: Immunofluorescence FACS: Flow Cytometry Beads: bead-bound WB: Wester Blotting

1.5 Vectors

Table 6. Vectors

NAME	ORIGIN	DESCRIPTION
pLKO.1-GFP	Addgene (David Sabatini)	pLKO.1 vector backbone containing GFP protein
pSRα-NL4.3	(Zaldivar et al. 2009)	pSRα plasmid containing HIV envelope protein (gp120)
psPAX2	Addgene (Didier Trono)	Plasmid expressing the viral GAG and POL proteins
pMD2.G	Addgene (Didier Trono)	Plasmid expressing the VSV-G envelope proteins

1.6 Oligonucleotides

Table 7. Oligonucleotides used for quantitative PCR

ALLELE	5' SEQUENCE	3' SEQUENCE
Bcl6	GGAAGTTCATCAAGGCCAGT	GACCTCGGTAGGCCATGA
Bcl2	GTACCTGAACCGGCATCTG	GGGGCCATATAGTTCCACAA
Blimp1	GGCTCCACTACCCTTATCCTG	TTTGGGTTGCTTTCCGTTT
Aid	AGGGAGTCAAGAAAGTCACG	CAGGAGGTGGCACTATCTCT
HPRT	TCCTCCTCAGCAAGCTTTT	CCTGGTTCATCATCGCTAATC
GAPDH	CTCCCACTCTTCCACCTTCG	CATACCAGGAAATGAGCTTGACAA

Table 8. Oligonucleotides used for sequencing

ALLELE	5' SEQUENCE	3' SEQUENCE
B1-8 IgH (Vh186.2)	CCATGGGATGGAGCTGTATCATCC	GAGGAGACTGTGAGAGTGGTGCC

2. METHODS

2.1 Isolation and purification of mouse T and B cells

In order to obtain naïve T and B cells, lymph nodes and spleen were removed from non-immunized SPF mice and cell suspensions in PBS+2%FBS were obtained using 40μm filters.

The cellular suspensions were centrifuged for 5 minutes at 1500 rpm. Cells isolated from lymph nodes were resuspended again in PBS+2% FBS, while splenocytes needed to be treated with the ACK lysis buffer for 3 minutes in order to lyse the erythrocytes. Lysis was stopped by washing the cells once with PBS+2% FBS. Cells that did not require to be purified were resuspended in PRMI+10% FBS (detailed composition in Reagents 1.1) to be used for functional experiments.

For T:B cultures, purified B and T cells were used. Purification was carried out by negative selection using a cocktail of biotinylated antibodies. The antibody cocktails used were:

- **Follicular B cell purification**: anti-CD43 and anti-CD11b. CD43 is expressed in T lymphocytes, granulocytes, monocytes and in certain B cells populations. CD11b is expressed by macrophages, monocytes, granulocytes, DC and some subsets of T and B cells, such as B1 B cells. This type of purification did not exclude MZ B cells, nevertheless the majority of cells obtained belong to the Follicular B cell subset.
- **Follicular B1-8^{hi} B cell purification**: anti-CD43, anti-CD11b and anti-kappa. B1-8 B cells express the Vh186.2 IgH and a lambda IgL chain. This restricted purification is only used for B cells signalling experiments (Methods 2.10, 2.11, 2.12 and 2.14).
- **OT2 T cell purification**: anti-B220, anti-CD11b, anti-CD8, anti-NK1.1, anti-Gr1, anti-F4/80. This cocktail of biotinylated antibodies allows the removal of B cells, CD8 T cells, NK cells, granulocytes, monocytes, DC, macrophages and activated CD4 T cells.

Incubation with the purification cocktail was performed at 0ºC in PBS+2%FBS for 20 minutes at $70x10^6$ cells/ml concentration. Subsequently, cells were washed and a small aliquot was taken in order to test the purification efficiency.

Once cells were stained and washed, they were incubated with magnetic beads covered with streptavidin in a 1,5:1 beads:cells ratio in the same volume as previously used for the staining ($70x10^6$ cells/ml). Those beads that were going to be used were previously washed in PBS+2%FBS. The cell suspensions were incubated with the beads at 4ºC for 45 minutes under rotation. After that, beads were removed using a magnet and only the supernatant was

recovered. This step was repeated three times in order to eliminate all the beads, and in consequence all the biotinylated stained cells.

The final cellular suspension was washed and resuspended in RPMI+10% FBS to be counted and resuspended at the concentration of interest.

Purification success was tested staining cells taken before and after incubation with the streptavidin beads:

- B cell purification: streptavidin (to detect the biotinylated antibodies) and anti-B220.
- OT2 purification: streptavidin (to detect the biotinylated antibodies) and anti-CD4.

2.2 Mouse peripheral blood collection for genotyping

A small aliquot of peripheral blood (100μl) was taken from each mouse in an Eppendorf containing heparin. Blood was centrifuged 5 minutes at 7000rpm and treated for 5 minutes at 0ºC with Ack lysis buffer to remove the erythrocytes. Samples were centrifuged once more and washed in PBS+1%BSA to be stained with anti-Vα2/anti-CD4 or anti-CD45.1/anti-CD45.2 antibodies for 20 minutes at 0ºC. After that, cells were washed and analysed by flow cytometry.

2.3 Genomic DNA extraction for genotyping

For genotyping, genomic DNA was extracted from a small fragment of the tail cut when the animal was 3-4 weeks old. Tails tissue was digested overnight at 55ºC using a specific buffer detailed in Table 4 and Proteinase K (10μg per sample). Once digested, 1 volume of phenol:clorophorm:isoamilic alcohol (25:24:1) was added, vortexed and centrifuged 10 minutes at 10000 rpm at RT. The aqueous phase (were the DNA is retained) was laid on 1 volume of phenol:clorophorm (24:1), vortexed and centrifuged for 10 minutes at 10000 rpm. Finally, the aqueous phase was mixed with 2 volumes of ethanol, allowing the DNA precipitation. After another centrifugation, the DNA was washed another time with 500μl of 70% ethanol. After a last centrifugation, the liquid was decanted for the removal of ethanol residues allowing the DNA to dry. DNA was resuspended in 50μl of sterile water for its further usage in a PCR reaction.

2.4 Antigen-coated bead preparation

To prepare beads with adsorbed antigen, a total of $130x10^6$ carboxylated latex beads of 1 μm diameter were incubated overnight at 4ºC under continuous rotation with a concentration of 40 μg/ml of protein in 1 ml of PBS. When 3 and 10 μm diameter beads are used, the number of beads to be incubated was reduced 3-fold and 30-fold, respectively. Beads were subsequently washed twice with PBS+1% BSA and resuspended in complete RPMI medium in order to be used.

To prepare beads with covalently bound antigen, the PolyLink Protein Coupling Kit (Polysciences) was used as indicated by the manufacturer. An equivalent to 12.5 mg of beads were washed in Coupling Buffer (50 mM MES, pH 5.2, 0.05% Proclin 300), centrifuged 10 minutes at 1000g and resuspended in 170 μL Coupling Buffer. A 20 μl volume of Carbodiimide solution (freshly prepared at 200 mg/ml) was added to the bead suspension and incubated for 15 minutes. After that, a total of 400 μg of NIP-OVA were added at 5 mg/ml final concentration. To prepare beads coupled to two different proteins we follow a sequential procedure: the first protein was added at sub-saturating conditions (100 μg p17/p24/gp120 HIV-1 fusion protein) for one hour, and after that, the second one was added to reach saturation (400 μg NIP-OVA) and incubated for an additional hour. Incubations were carried out at room temperature with gentle mixing. Beads were centrifuged and washed twice in Wash/Storage buffer (10 mM Tris, pH 8.0, 0.05% BSA, 0.05% Proclin 300). To remove non-covalently bound protein, beads were washed once with 0.1% SDS followed by two washes with PBS + 1% BSA to remove the SDS. Beads were resuspended finally in the Wash/Storage buffer to be stored, or in PBS or complete medium if they were going to be used immediately.

2.5 Phagocytosis assay

In-vitro phagocytosis assay

Purified naïve Follicular B cells from WT, *Rras2-/-*and *Rhog-/-* C57BL/6 mice were resuspended in RPMI+0.2%BSA and plated in 96-well V-bottom plates at a concentration of $1x10^6$ cells per well in 50μl. 1 or 3 μm fluorescent beads coated with anti-IgM antibody were added at a 1:10 and 1.3 ratio B cell:beads respectively in 30μl per well. The plate was briefly centrifuged at 1500 rpm for 10 seconds and incubated at 37ºC for 1 or 2 hours. Subsequently, all the remaining procedures were performed at 0ºC in order to avoid actin remodelling and

phagocytosis. When phagocytosis was analysed by flow cytometry, cells were washed once with cold PBS+1%BSA and stained with anti-B220 and with a fluorescent isotype-specific antibody against anti-IgM antibody in order to distinguish external beads from those beads already internalized. After 30 minutes of staining, cells were washed in PBS+1%BSA and resuspended in 200µl of PB1+1%BSA in order to be analysed by flow cytometry. B cells (B220+) positive for fluorescent beads have either attached or internalised beads. The staining with the isotype-specific Ig allows discrimination between these last two conditions: B cells with attached beads in the membrane are B220+ Beads+ isotype Ig+; B cells with only phagocytosed beads are B220+ Beads+ isotype Ig-. The increase in bead fluorescence correlates with the number of beads either attached or internalized, allowing us to calculate a phagocytic index.

Phagocytic index = (%cells with 1 internal bead*1) + (%cells with 2 internal beads*2) + …

When cells were going to be analysed by confocal microscopy, the subsequent stainings are detailed in Methods 2.9.

In-vivo phagocytosis assay

In order to study whether B cell phagocytosis takes place *in vivo*, WT and *Rhog-/-* B1-8^{hi} mice were immunized intraperitoneally with 2x10^{7} Crimsom fluorescent 1µm beads covalently bound to NIP-OVA. After 5 hours, spleens were harvested and disrupted in PB2+2% FBS on ice as it is explained in Methods 2.1. All the subsequent ex-vivo procedures were performed at 0ºC. To identify those beads phagcytosed from those ones still attached to the plasma membrane, cells were stained with an anti-Ovalbumin FITC antibody for 30 minutes together with the antibodies required to distinguish cell populations: macrophages (anti-CD11b and anti-F4/80), B cells (anti-CD19 and anti-B220), antigen-specific B cells (NP-PE), marginal zone and follicular B cells (anti-CD21 and anti-CD23). Afterwards, cells were washed once in PBS+1%BSA and analysed by flow cytometry.

2.6 Germinal center differentiation culture

Cultures of non-tg B cells

When non-tg B cells were used, naïve Follicular B cells were purified from C57BL/6 WT, *Rhog-/-* and *Rras2-/-* mice and naïve CD4 T cells from OT-2 mice (Methods 2.1). B cells were seeded in

a p96 round-bottom plate at a concentration of $0.2x10^6$ cells/well in 75µl. 1µm beads coated with anti-IgM plus Ovalbumin or anti-IgM and Ovalbumin separately were resuspended in 50µl and added to the B cells at different ratios (1:0.3; 1:1; 1:3; 1:10; and 1:20 B cells:beads) according to the kind of experiment. The plate was spun at 1500 rpm for 15 seconds and incubated 1 hour at 37ºC. After this hour, CD4 T cells were added to the B cells ($0.2x10^6$ CD4 per well) and co-cultured for 3 days. After 72 hours, the activation levels and TFH markers were analysed on CD4 T cells by flow cytometry using anti-CD25, anti-CXCR5 and anti-PD1 antibodies. Simultaneously, expression of GC markers by B cells was also tracked using anti-GL7, anti-CD95 and anti-CD40 antibodies. When proliferation was also measured, cells were previously stained with Cell-Trace Violet or CFSE as explained in Methods 2.8.

When non-tg B cell cultures were used to study the formation large T:B clusters, cells were cultured in 6 flat-bottom well plates and $5x10^6$ of B and T cells were cultured with 1:10 B cell:beads ratio.

B1-8^{hi} B cell cultures

When B cells from B1-8^{hi} mice were used, naïve Follicular B cells from WT, *Rras2-/-* and *Rhog-/-* B1-8^{hi} and CD4 T cells from OT-2 mice were purified. $0.2x10^6$ B cells were incubated at 37ºC for 1 hour in 96-well round-bottom plate with 1µm beads coated with NIP-OVA or NP-CGG (1:3 B cells:beads ratio) or with soluble NIP-OVA (100ng/ml). After 60 minutes, purified CD4 T cells were added to the plate ($0.2x10^6$ cells per well) and co-cultured for 4 days. After 96 hours, GC and TFH cell markers were analysed by flow cytometry. In some experiments, cells were removed daily for flow cytometry staining in order to study the kinetics of GC generation. When cultures were extended for 7 days, 50µl extra RMPI+10%FBS medium were added at day 5 of culture.

When formation of large T:B clusters by confocal microscopy or antibody production was measured, cultures were carried out in 6-well flat-bottom plates by incubating $4.5x10^6$ B and T cells in 3ml and using a 1:3 Bcell:beads ratio or 100ng/ml soluble NIP-OVA.

2.7 Flow cytometry

Flow cytometry stainings were performed using p96 V-bottom. Each well contained 2-5x10^5 cells and all procedures were carried out at 0ºC using a staining buffer of PBS+1%BSA+0.02%azide.

For all flow cytometry stainings cells were incubated with anti-CD16/32 together with the primary antibodies diluted in the staining buffer at the specific dilution (listed in Material and Methods 1.4) in a final volume of 50 µl per well for 20 minutes. After the first incubation, the plate was centrifuged for 2 minutes at 1500 rpm, the supernatant was discarded and cells were washed with the PBS+1%BSA buffer. If some of the primary antibodies were not directly labelled with a fluorophore, staining with a secondary antibody or with fluorescent streptavidin during 15 minutes at the dilution required was carried out (listed in Material and Methods 1.4). Cells were washed once and resuspended in a final volume of 200µl to analyse the samples by flow cytometry.

For intracellular stainings the Foxp3/Transcription Factor Staining Buffer Set was used. Cells were fixed for 30 minutes using fixation buffer (4.2% formaldehyde), washed twice in the permeabilization buffer and incubated for 30-60 minutes with the corresponding antibody diluted in the permeabilizaton buffer. After two subsequent washes, cells were resuspended in 200µl of PBS+1%BSA in order to be analysed by flow cytometry.

In the long-term cultures (longer than four days), cells were stained with a cell-dye probe in order to study more accurately the live population. The LIVE/DEATH Fixable Violet Dead Cell Stain kit, which reacts with free amines, was used. After extracellular staining, cells were washed twice in PBS and incubated in 50µl of the LIVE/DEATH probe (1:1000 dilution in PBS) for 30 minutes. After that, cells were washed once in PBS alone and once again with PBS+1%BSA.

Cells were analysed using the Flow Cytometers FACSCanto II and FACSCantoA and the FACSDiva software. In both cases data was analysed with the FlowJo software program.

2.8 Proliferation assay

In order to measure the proliferation of B and T cells in our GC cultures after 72-96 hours of stimulation, cells were previously stained with Cell Trace Violet (CTV) or Carboxifluoresceinsuccinimidil ester (CFSE). To do that, after cellular purification cells were

counted and respuspended in a 50ml-Falcon tube in RPMI 20mM Hepes at 6-10x10^6 cells/ml. Cells were incubated 10 minutes at 37ºC in order to get tempered. Subsequently, cells were vortexed while CTV or CFSE was being slowly added at a 5μM final concentration (1:1000 dilution) and incubated for 5-10 minutes at 37ºC protected from light. To stop the reaction RPMI 20%FBS was added and cells were washed twice using complete RPMI medium. After that, cells were counted and cultured as explained above (Methods 2.6). This kind of staining allows to monitor the number of cell divisions, and in consequence to calculate the proliferation index (PI) which takes into account the percentage of cells that have proliferated and the number of divisions that have undergone.

$$\text{Proliferation index} = \left[\frac{(\%p1*1) + (\%p2*2) + (\%p3*3) + (\%p4*4) + \ldots}{\%\ p0} \right] *100$$

2.9 Confocal microscopy

Phagocytosis of 1 and 3μm beads

Purified naïve Follicular B cells from C57BL/6 mice were resuspended in RPMI+0.2% BSA + 20mM Hepes and plated in a p96 V-bottom plate at 1x10^6 cell/well in 30μl at 37ºC. 1μm or 3μm fluorescent beads coated with anti-IgM ($F(ab')_2$ fragment) were resuspended in 30 μl of RPMI and added to the cells (1:10 and 1:3 B cell:beads ratio respectively). Cells were spun after bead addition and incubated for 1 or 2 hours at 37ºC. All the remaining procedures were performed at 0ºC. Cells were transferred to coverslips previously treated for 2 hours at RT or overnight at 4ºC with 50μg/ml Poly-L-lysine. After 20 minutes, attached cells were extracellularly stained with anti-goat-488 diluted in TNB buffer for 30 minutes. Coverslips were washed in TNB, fixed using 4% paraformaldehyde for 15 minutes and washed again to be stained with Phalloïdin-TRICT diluted in 0.1% Triton X-100 in TNB. After 30 minutes of intracellular staining, coverslips were washed twice and fixed onto glass slides with Mowiol. The samples were left dried at RT for 24 hours and stored at 4ºC afterwards.

Culture clusters of T:B lymphocytes

Purified naïve FO B cells were cultured with purified OT2 T cells for 4 and 7 days in a 6 well flat-bottom plates with either 1μm beads coated with anti-IgM+Ovalbumin or NIP-OVA or

100ng/ml soluble NIP-OVA as it is explained in Methods 2.6. Afterwards, 4% paraformaldehyde was added to the p6 well plate to get a final concentration of 2%. Clusters were collected using a trimmed tip and incubated onto Poly-L-lysine treated coverslips. After 30 minutes at RT, coverslips with attached cells were washed in TNB buffer and stained with anti-CD4, anti-B220 and anti-GL7 primary antibodies diluted in TNB for 1 hour at RT. After washing in TNB buffer, cells were stained with DAPI diluted in TNB for 2 minutes. After the last two washes, coverslips were transferred to glass slides containing Mowiol.

In order to monitor the degree of cell proliferation inside the cluster, cells were stained with Cell Trace Marker and CFSE as it is described in Methods 2.8 before culturing.

For cell number calculation, a specific algorithm to quantify the DAPI dots in a cluster image was used. For quantification of the distribution of the proliferated B and T cells, and the GL7 expression, another algorithm was used. This one allows drawing concentric circles equidistant between them (radius, radius*2, radius*3, radius*4…) and to calculate the mean intensity of the selected staining in those drawn areas. The data obtained was normalized to the higher value for each cluster (considered as 100) in order to compare different clusters and represent the data.

BCR stimulation with 1μm latex beads

Naïve purified FO B1-8^{hi} B cells were starved for 1 hour in RPMI plus 20mM Hepes pH 7.4 and plated in a p96 well V-bottom plate in 30 μl ($5x10^5$ cells per well). Subsequently, cells were stimulated with 1μm Y/G fluorescent beads coated with NIP-OVA for 5 or 30 minutes at 37ºC. Once beads were added, the plate was spun at 1500 rpm for 10 seconds. After incubation, cells were fixed in 4% paraformaldehyde for 15 minutes at 0ºC to stop stimulation. Afterwards, cells were transferred to Poly-L-lysine treated coverslips and after 30 minutes washed in TNB and stained with the extracellular antibody (anti-B220) diluted in TNB for 1 hour at 0ºC. After that, cells were washed and permeabilized with 0.3% Triton X-100 in PBS for 30 minutes. pSyk and pCD79a antibodies were diluted in 0.1% triton X-100 and cells were incubated with 50μl of the dilution for 1 hour. After washing the coverslips, a secondary staining with anti-rabbit-647 antibody was added for 30 minutes. Subsequently, coverslips were washed and transferred to glass slides.

Confocal images were acquired with Zeiss LSM710 system and a Zeiss AxioObserver LSM710 Confocal microscopes.

2.10 BCR downmodulation

In order to study if soluble or bead-bound antigen induces similar BCR downmodulation, FO B1-8 B cells from B1-8^{hi} mice were purified and resuspended in complete RPMI medium. Cells were plated in a 96-well V-bottom plate at a density of $2.5x10^5$ cells per well in 50μl. 1μm NIP-OVA bead-bound (1:3 B cell:beads ratio) or 100ng/ml soluble NIP-OVA were added to the cells in a volume of 30μl. After spinning the cells (1500 rpm for 10 seconds), they were incubated at 37ºC for different time-points. Subsequently, cells were washed in cold PBS+1%BSA and stained for IgM, CD19 and B220 at 0ºC for 20 minutes. After a last wash, cells were resuspended in 200μl of cold PBS+1%BSA to be analysed by flow cytometry.

2.11 Measurement of actin polymerization

To measure the actin polymerization induced by BCR stimulation with soluble or bead-bound antigen, B1-8 B cells were purified from B1-8^{hi} mice (Methods 2.1) and starved for 1 hour in RPMI+20mM Hepes at 37ºC. Afterwards, cells were stimulated either with NIP-OVA bound to beads (1:3 B cell:beads ratio) or 100ng/ml of soluble NIP-OVA at 37ºC for different time points. Subsequently, cells were washed once in PBS+1%BSA at 37ºC and fixed with 4% paraformaldehyde for 15 minutes at room temperature. After washing with PBS+1%BSA, cells were extracellularly stained with anti-B220 antibody diluted in PBS+1%BSA for 20 minutes, washed and permeabilized in 4% paraformaldehyde plus 0.1% Triton X-100 for 2 minutes at room temperature. Phalloïdin-Alexa 488 dye was diluted 1:200 in PBS+1%BSA and incubated for 1 hour with the cells. After washing, cells were resuspended in 200μl of PBS+1%BSA to be analysed by flow cytometry.

2.12 NP saturation assay

In order to test if determined concentrations of soluble or bead-bound antigen were able to contact with similar amounts of BCRs. $1x10^5$/well of purified B1-8 B cells from B1-8^{hi} mice were plated in a p96 well V-bottom plate and incubated for 1 hour at 0ºC with different doses of soluble NIP-OVA or NIP-OVA bead-bound (1μm). After that, cells were washed once in cold PBS+1%BSA and stained in 50μl with 2.5 μg/ml NP(36)-PE at 0ºC for 30 minutes. After another wash, cells were resuspended in PBS+1%BSA to be analysed by flow cytometry. The mean

fluorescence intensity (MFI) of NP-PE in CD19+ B220+ cells reflected the levels of antigen-specific BCR saturation.

2.13 Interleukin measurement

In order to study if in the *in vitro* GC culture cells secrete interleukins required for GC reaction, the levels of IL-2, IL-4, IL-6, IL-10 and IL-21 were measured in the supernatants using the BD Cytometric Bead Assay (CBA) Kit.

When interleukin production was measured comparing 1 and 3μm beads size, purified Follicular B cells from WT B1-8^{hi} mouse were co-cultured with purified naïve CD4 T cells from OT2 mouse and stimulated with bead-bound NIP-OVA (1μm or 3μm beads size) at different ratios. After 4 days, the supernatant obtained was used to measure the cytokines release at the culture medium following the manufacturer instructions.

When RhoG deficiency was studied, FO B cells were purified from WT or *Rhog*-/- B1-8^{hi} mice and cultured for 4 and 7 days with purified naïve CD4 T cells from OT2 mice and 1μm beads coated with NIP-OVA (1:3 B cell:beads ratio). Supernatants obtained after 4 and 7 days of culture were used for interleukin measurement.

2.14 Immunoblot analysis of B cell activation

In order to compare the stimulatory effects between soluble and bead-bound stimulus, purified B1-8^{hi} B cells were resuspended in RPMI+20mM Hepes and incubated under starving conditions for 1 hour. After that, cells were stimulated at different time-points with NIP-OVA bead-bound (1:3 B cell:beads ratio) or 100ng/ml of soluble NIP-OVA. After the last time-point, stimulation was stopped. Cells were spun at 10000 rpm for the removal of the supernatant, and subsequently, lysed in Brij96 lysis buffer containing protease and phosphatase inhibitors (1% Brij96, 140mM NaCl, 10mM Tris-HCl (pH 7,8), 10mM iodoacetamide, 1mM PMSF, 1μg/ml leupeptin, 1 μg/ml aprotinin, 1mM sodium orthovanadate, 20mM sodium fluoride and 5mM of MgCl2). The lysis was performed at 0ºC for 15 minutes and afterwards, the lysate was centrifuged for 15 minutes at 13000 rpm at 4ºC. Supernatant was recovered and diluted in 3X sample buffer, boiled for 5 minutes at 95ºC and ran in 10% polyacrylamide gels in order to separate the proteins by SDS-PAGE. Gel system from BIO-RAD was used for electrophoresis. Subsequently, proteins were transferred by dry blotting to nitrocellulose membranes (Bio

Rad), which were incubated in 10% skimmed milk in TBS-0.1%Tween for 1 hour at RT. Membranes were washed using TBS-0.1%Tween and incubated overnight at 4ºC with the primary antibody solution. After three sequential washes, membranes were incubated with HRPO coupled to a secondary antibody for 1 hour of at RT. Detection was performed using the enhanced chemiluminescence detection system (ECL, Bio Rad).

2.15 Real-time qPCR

A total of $5x10^6$ purified FO B cells (Methods 2.1) from WT or *Rhog*$^{-/-}$ B1-8^{hi} mice were cultured with purified OT2 (ratio 1:1) and different BCR stimuli (NIP-OVA bound to 1 μm beads or 100 ng/mL soluble NIP-OVA) in a 6 well flat-bottom plate. After 7 days, cells were stained with anti-CD19 antibody in order to sort the B cells (FACSAria Fusion (BSC II). RNA from sorted B cells was isolated using the RNAeasy Plus Mini Kit (QIAGEN) and cDNA was synthetized with the SuperScript III (Invitrogen) using Oligo-dT primers.

The cDNA obtained by retrotransciption from 50ng of mRNA was amplified using specific primers (Table 7). These primers were designed to amplify regions comprised between two consecutive exons in order to avoid genomic DNA amplification. Quantitative real-time PCR was performed in triplicate using the SYBR Green PCR Master Mix. *Gapdh* and *Hprt* expression were used as normalizers. The qPCR reaction was performed using the ABI 7300 Real Time PCR System and the results were analysed using the SDS2.4 software. Obtained cycle threshold (Ct) values were used to calculate mRNA levels relative to HPRT and GAPDH expression using the $2^{-\Delta\Delta Ct}$ method.

2.16 Measurement of antigen-specific antibodies

In vitro GC cultures

To measure the release of NP-specific Igs in B1-8^{hi} B cell cultures, naïve WT, *Rras2*-/- and *Rhog*-/- FO B cells were purified and cultured with OT2 T cells and either NIP-OVA bound to 1μm beads (1:3 B cell:beads ratio) or soluble (100ng/ml) for 7 days (Methods 2.1 and 2.6). The supernatant recovered after seven days of culture was used to measure the secretion of antigen-specific antibodies.

To measure NP-specific Igs in cultures of non-trangenic B cells, purified naïve C57BL/6 FO B cells were preincubated with a mixture of NIP-OVA and HIV-1 p17/p24/gp120 fusion protein (Jena Biosciences) covalently bound to 1 μm beads (1:1 bead:Bcell ratio) and cultured with OT-2 T cells. After 5 days of culture, some cultures were supplemented with 1 ng/mL IL-4 and 10 ng/mL IL-21 (Methods 2.6). Supernatants recovered at day 7th and 10th were used to measure the secreted Igs.

To perform the ELISA, Costar p96 flat-bottom plates were incubated overnight at 4ºC with 100 μl of NP(7)-BSA or NP(41)-BSA at 5μg/ml, or in the case of non-tg B cells, with p17/p24/gp120 fusion protein in PBS at 10μg/ml. Afterwards, plates were washed twice with 100μl of PBS+0.05%Tween and incubated for 1 hour with PBS+1%BSA at RT. After two sequential washes with PBS+0.05%Tween, plates were incubated with the culture supernatants diluted ½ in PBS for 1-2 hours at room temperature. After three sequential washes the SBA Clonotyping System-HRP (Southern Biotech) was used to detect the presence of class-switched antigen-specific Igs. Antibodies were diluted 1/350 in PBS+1%BSA and incubated for 1 hour at RT at the plates, after that, plates were washed three times and developed using the ABTS Single Solution (Life Technology). The reaction was stopped using 0.1M Citric acid and the absorbance was measured with a Microplate reader (iMARK, BioRad).

In vivo immunized mice

WT or *Rhog-/-* C57BL/6 mice were immunized intraperitoneally with 200 μg of NIP-OVA embedded with Alum or PBS, and 14 days after this first immunization, mice were reimmunizated in the same conditions. 6 days after the second immunization, mice were bleed, obtaining around 500μl of blood per animal. Blood was incubated for 2 hours at 37 ºC and centrifuged 5 minutes at 8000 rpm, inducing the sera separation. In the case of *Rag1-/-* mice, serum was obtained two days after the second immunization. NP-antigen specific antibodies were detected by ELISA as it was performed for culture supernatants. In this case, the mice sera were diluted 1/175 in PBS.

2.17 Cell line transduction and neutralization assay

MOLT-4 transduction

In order to infect MOLT-4 cell line with lentiviral vectors, viral particles were first produced in HEK293T cells using the JetPei transfection system. HEK293T cells were transiently transfected with a vector including the sequence of interest (GFP to monitor the efficiency of infection: pLKO.1-GFP vector) and vectors containing viral genes necessary for the envelope (VSV-G envelope: pMD2.G vector; HIV envelope: pSRα-NL4.3 vector) and for the viral packaging (psPAX2 vector). The viral particles were produced and concentrated in the supernatant medium of the HEK293T cells. The protocol followed was:

-**DAY 1**: $4x10^6$ HEK293T cells were plated in a p100 with 10 ml of DMEM 10% FBS to obtain a 70-80% confluence the day after.

-**DAY 2**: HEK293T cells were transfected with the three viral plasmids explained above using two mixes: MIX A: 250μl of 150mM NaCl in PBS + 26μl 225 μM JetPei

MIX B: 250μl of 150mM NaCl in PBS + 1μg envelope vector (pSRα-NL4.3 or pMD2.G) + 4,8μg psPAX2 + 6,6μg pLKO.1-GFP

Mix A was added over Mix B, incubated for 15-30 minutes at room temperature and dropped into HEK293T cells medium.

-**DAY 4**: supernatant of HEK293T cells was removed and filtered (0.45μm filter) to eliminate cellular debris. Supernatant was supplemented with 8μg/ml Polybrene, which is a cationic polymer that avoids the repulsion between the virions and the sialic acid from the plasmatic membrane, increasing the efficiency of the infection. Subsequently, MOLT-4 cells were plated in a p24 well plate at $0.3x10^6$ cells/well concentration with 350μl of DMEM+10%FBS and 350μl of viral supernatant. Cells were centrifuged at 2200 rpm for 90 minutes at 32ºC and cultured at 37ºC for 24 hours.

-**DAY 5**: MOLT-4 cells were analysed by flow cytometry to track the levels of infection (GFP expression).

Viral titration

In order to use MOLT-4 cells with comparable levels of infection using VSV-G or HIV virions, HEK293T supernatants were titrated. After 24 hours, the infectivity was analysed by Flow

cytometry tracking GFP expression. VSV-G and HIV supernatant dilution inducing similar GFP expression was the one selected to carry out the neutralization experiments.

HIV neutralization assay

$5x10^6$ naïve FO B cells from non-tg C57BL/6 were cultured with OT-2 T cells (1:1 B:T cells ratio) previously purified by negative selection as explained in Methods 2.1. Cells were cultured in a p6 well plate with 1μm beads bound covalently with NIP-OVA and HIV-1 p17/p24/gp120 fusion protein (1:1 beads:B cell ratio) for 7 days. Culture supernatant was used to test if B cells have secreted antigen-specific immunoglobulins against HIV proteins. In order to test that, the viral supernatants previously obtained from HEK293T cells using virions containing either VSV-G or HIV envelope were incubated for 1 hour at 37ºC with two dilutions (1:2 and 1:4) of the T:B cell culture medium. These mixed mediums were used to infect MOLT-4 cells plated in a p24 plate ($0.3x10^6$ MOLT-4 cells/350μl DMEM per well) as it was explained before, at the end having different conditions:

- MOLT-4 cells infected with VSV-G viral supernatant
- MOLT-4 cells infected with HIV viral supernatant
- MOLT-4 cells infected with VSV-G viral supernatant preincubated for 1 hour with the T:B cell culture supernatant (1:2 and 1:4 dilution)
- MOLT-4 cells infected with HIV viral supernatant preincubated for 1 hour with the T:B cell culture supernatant (1:2 and 1:4 dilution)
- MOLT-4 cells cultured in DMEM+10%FBS medium (negative control)

After 24 hours, MOLT-4 cells were centrifuged at 1500 rpm for 5 minutes, washed in PBS+1%BSA+0.02%azide and analysed by Flow cytometry. The Mean Florescence Intensity of GFP reflected the levels of MOLT-4 infection after the different treatments. VSV-G viral supernatant was used as control for immunoglobulin antigen-specificity neutralization.

2.18 Somatic hypermutation

In order to study if the GC B cells obtained in culture performed somatic hypermutation at their variable Ig region, FO B cells were purified from WT or *Rhog*-/- B1-8^{hi} mice and cultured with purified CD4 T cells from OT2 mice in a 6-well flat-bottom plate (Methods 2.1 and 2.6). B cells were stimulated either with 1μm NIP-OVA bead-bound (1:3 B cell:beads ratio) or soluble

NIP-OVA (100ng/ml) and cultured for 7 days. After that, cells were washed in PBS+1%BSA and stained with anti-CD19 for 20 minutes at 4ºC. Genomic DNA was extracted from CD19+ sorted cells using the QIAamp DNA kit (QIAGEN). These genomic DNA was used to amplify by PCR the specific Vh rearrangement that NP-reactive B cells carry (Vh186.2). PCR was performed using the Expand High Fidelity (Roche) polymerase in order to avoid extra mutations during the PCR reaction. The specific primers (Table 8) and the used program had been described previously (Shih et al. 2002). PCR products were subcloned into PCR2.1 vector using the TA cloning PCR2.1 kit (QIAGEN). DH5α bacteria were transformed with the subcloned products and plated in Ampicillin+LB plates. Individual clones grown in the presence of Ampicillin conditions were selected, analysed by PCR to test if they carried the subcloned product (it was used the same PCR conditions as explained before), and sent for sequencing using the SUPREMERUN 96 system of GATC. Obtained sequences were analysed looking for mutations comparing with the standard sequence. Genomic DNA extracted from fresh sorted naïve CD19+ cells from B1-8^{hi} mouse was used as a control for putative point mutations introduced during DNA amplification.

2.19 Adoptive transfer and immunizations

In order to assess the *in vivo* GC formation, 6- to 12- weeks-old WT and *Rhog-/-* mice were immunized intraperitoneally with 200μg of NIP-OVA diluted in 200μl of PBS or complexed with 100μl of Alum diluted 1:1 in PBS. After 7 days spleens were harvested and homogenized (Methods 2.1) to be analysed by flow cytometry. When the generation of anti-NP antibodies was studied, mice were reimmunized after 14 days in the same conditions.

For immunization with NIP-OVA bound to beads, WT and *Rhog*-/- mice were injected intraperitoneally with 2x10^7 1μm beads covalently bound to NIP-OVA diluted in 200μl of PBS.

For adoptive transfer into CD45.1 mice, naïve FO B cells from WT and *Rhog-/-* B1-8^{hi} mice were purified. 1x10^7 purified B cells diluted in 100μl of PBS were injected intravenously in the recipient mice, and at the day after they were immunized with 200μg of NIP-OVA in PBS or embedded in Alum. Alternatively, those mice were immunized with 2x10^7 1μm beads covalently bound to NIP-OVA diluted in 200μl of PBS.

For adoptive transfer into *Rag1*-/- mice, 1x10^5 purified CD4 T cells from OT2 mice and 1x10^6 naïve FO B cells from WT or *Rhog*-/- B1-8^{hi} mice resuspended in 100μl of PBS were injected intravenously to recipient *Rag1-/-* mice. Afterwards, mice were immunized with 200μg of NIP-

OVA either mixed with Alum or in PBS. 14 days later, animals were reimmunized in order to obtain their sera two days later.

2.20 Statistical analysis

To perform the statistical analysis it was used the non-parametric two-tailed unpaired t-test in order to assess the confidence intervals. Statistical parameters including the exact value of n, the means +/- s.d. are reported at the Figure and Figure legends.

OBJECTIVES

OBJECTIVES

The main objective of this thesis is to evaluate the possible role of phagocytosis as a mechanism for antigen uptake by B lymphocytes during the humoral response. In order to develop this main objective, we undertook the subsequent specific objectives:

1. **To determine the phagocytic ability of follicular B cells *in vitro*.**
   - 1.1 To study the ability of B cells to phagocytose antigen in a BCR-driven process.
   - 1.2 To determine the role of RRas2 and RhoG GTPases during BCR-driven phagocytosis.
2. **To describe the effect of B-cell antigen phagocytosis during *in vitro* antigen presentation.**
   - 2.1 Analysis of CD4 T cell activation and TFH differentiation.
   - 2.2 Analysis of germinal center B cell generation.
3. **To study the generation of a humoral response *in vitro*.**
   - 3.1 To determine the role of phagocytic antigen uptake by B cells for *in vitro* plasma cell differentiation.
   - 3.2 To study the role of antigen phagocytosis for antibody production *in vitro*.
4. **To study BCR signalling induced by phagocytic stimulus.**
5. **To determine the relevance of B cell phagocytosis during an *in vivo* humoral response.**
   - 5.1 To analyse the effect of RhoG deficiency during the germinal center response *in vivo*.
   - 5.2 To study the consequences of the defective phagocytosis by B cells on the humoral response *in vivo*.

OBJETIVOS

El principal objetivo de esta tesis es el estudio de la importancia del mecanismo de fagocitosis durante la adquisición de antígenos por parte de las células B durante el desarrollo de la respuesta humoral. En base a este objetivo principal se enumeran los siguientes objetivos concretos:

1. **Determinar la capacidad fagocítica de los linfocitos B foliculares en cultivo.**
   - 1.1 Determinar la capacidad fagocítica de antígenos por parte de linfocitos B mediada por el BCR.
   - 1.2 Estudiar el papel que desarrollan las GTPasas RRas y RhoG durante este proceso.
2. **Describir el efecto de la adquisición de antígenos mediante fagocitosis por parte de los linfocitos B durante la presentación de antígenos en cultivo.**
   - 2.1 Analizar la activación de los linfocitos T CD4 y su diferenciación a TFH.
   - 2.2 Analizar la generación de células B de centro germinal.
3. **Estudiar la generación de respuesta humoral en cultivo.**
   - 3.1 Determinar el papel de la adquisición de antígenos por fagocitosis en la diferenciación de las células B a células de plasma.
   - 3.2 Estudiar el efecto de la fagocitosis de antígeno en la generación de anticuerpos *in vitro*.
4. **Estudiar la señalización a través del BCR inducida por el estímulo fagocítico.**
5. **Determinar la relevancia de la función de la fagocitosis de antígenos llevada a cabo por los linfocitos B durante la respuesta humoral 'in-vivo'.**
   - 5.1 Analizar el defecto de RhoG en linfocitos B durante la respuesta de centro germinal.
   - 5.2 Estudiar las consecuencias del defecto en la fagocitosis por parte de linfocitos B en la respuesta humoral '*in vivo*'.

RESULTS

RESULTS

1. B lymphocytes phagocytose and present antigens through their BCR by a RRas2- and RhoG-dependent mechanism.

It was described in our laboratory that T cells acquire membrane-bound antigens from APC through a process called trogocytosis. It was shown that naïve T cells have the ability to phagocytose latex beads of up to 6 μm size by a TCR-driven mechanism. This process is dependent of two small GTPases from the Ras superfamily: RRas2 and RhoG (Martinez-Martin et al. 2011). RRas2 interacts with the TCR, but also with the BCR, and has a fundamental role in the homeostatic proliferation and survival of mature T and B lymphocytes (Delgado et al. 2009). Moreover, RRas2-deficient mice have a deficient humoral response, developing smaller germinal centers. In spite of the participation of RRas2 and RhoG in the same pathway downstream the TCR during trogocytosis, RhoG-deficient mice do not show the same phenotype as *Rras2-/-*. Turner and collegues reported that *Rhog-/-* mice do not present any anomaly during T and B cell development or in the germinal center response to immunization with soluble antigen (Vigorito et al. 2004). Nonetheless, in a screening of more than 20 Rho proteins, RhoG was found to be involved in FcγR- and CR3- mediated phagocytosis by macrophages (Tzircotis et al. 2011), which implies active acquisition of particles bigger than 0.5 μm through a receptor and an actin-driven process.

1.1 B cells can phagocytose antigens through a BCR-driven mechanism

First of all, we wondered if follicular B cells have also the ability to acquire phagocytic antigens in a BCR dependent manner since phagocytosis ability has already been reported for B1 B cells (Gao et al. 2012, Nakashima et al. 2012, Parra et al. 2012). To test our hypothesis, we purified naïve FO B cells from C57BL/6 mouse by negative selection using anti-CD43 and anti-CD11b antibodies to exclude all non-B cells and B1 B cell subsets. Although MZ B cells were not excluded with this cocktail of antibodies, the majority of B cells obtained belong to the FO B cell subset. Afterwards, we incubated those purified B cells for 1 hour at 37ºC with fluorescent latex beads coated with a $F(ab')_2$ fragment of a goat anti-IgM antibody. In order to distinguish totally phagocytosed beads from those just adhered to the membrane, cell cultures were incubated with fluorescent anti-goat IgGs antibody able to recognize the anti-IgM used to cover the beads. Cells with beads attached to the membrane could be identify by their

positivity for the secondary antibody staining, while cells with beads only internalized would be negative (**Illustration1**).

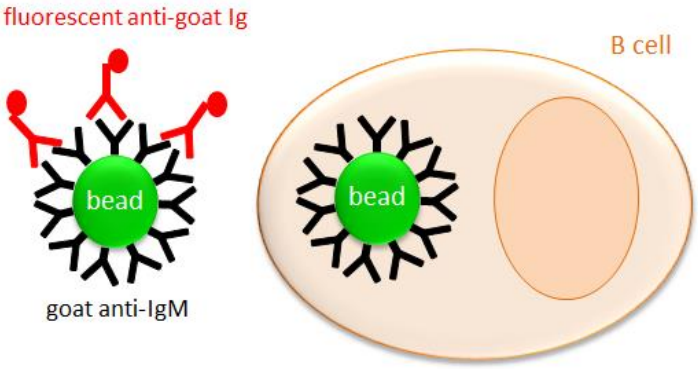

Illustration 1. Experimental set-up to distinguish B cells with exclusively internal beads from B cells that have adhered beads. Extracellular fluorescent beads coated with a goat F(ab')$_2$ fragment of anti-IgM antibody can be recognized by a fluorescent secondary anti-goat antibody, while beads already internalized are inaccessible to the secondary antibody. Therefore, B cells with membrane-bound beads are positive for the bead and secondary antibody fluorophores, while B cells with phagocytosed beads are positive only for the bead fluorophore.

Purified B cells incubated with 1 μm and 3 μm latex beads coated with anti-IgM F(ab')$_2$ were prepared for immunofluorescence. Confocal images show that naïve FO B cell are able to acquire particulate antigens of up to 3 μm of size through a BCR-driven process (**Figure 1**).

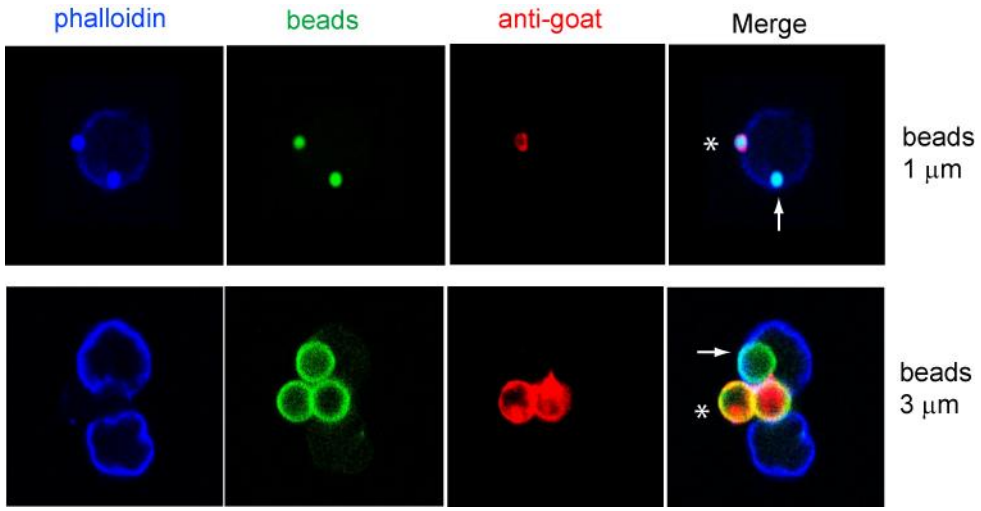

Figure 1. B cells can phagocytose 1 and 3 μm latex beads coated with anti-IgM antibody. Purified naïve FO B cells were incubated for 1 hour with fluorescent 1 μm (upper panel) or 3 μm (lower panel) beads (green) bound to a goat anti-IgM. Anti-goat 488 staining (red) shows those beads still attached to the membrane and not internalized. Phalloïdin staining (blue) allows visualization of F-actin. Asterisks indicate beads not fully internalized since they are positive for the secondary anti-goat antibody; arrows point at beads already phagocytosed, negative for the secondary antibody.

1.2 B cell phagocytosis is dependent of RRas2 and RhoG GTPases

In order to study if BCR-driven phagocytosis in B cells was dependent of RRas2 and RhoG, purified FO B cells from Wild Type, *Rras2-/-* and *Rhog-/-* mice were incubated for 1 or 2 hours with fluorescent 1 μm or 3 μm beads coated with anti-IgM. Cells were stained for the secondary antibody and analyzed by FACS in order to differentiate internal from external beads (**Figure 2**).

The technique was set up using two different controls: performing the incubation at 0ºC to avoid bead internalization and incubating at 37ºC beads not coated with anti-IgM, to exclude possible unspecific binding of the secondary antibody (**Figure 2a**). The stepwise increase in fluorescence intensity allowed to calculate the phagocytic index, which reflects the number of phagocytosed beads (**Figure 2b**). FO B cells, as previously seen by microscopy, are able to phagocytose 1 and 3 μm beads in a process dependent of RRas2 and RhoG GTPases since deficient cells have lower phagocytic index. Moreover, the effect of RRas2 and RhoG is even more remarkable when bead size is increased to 3 μm, suggesting that their requirement grows with particle size (**Figure 2c**). Indeed, B cell phagocytic capacity has a size limitation since B cell phagocytosis of 10 μm beads coated with anti-IgM was almost negligible compared with phagocytosis of 1 and 3 μm beads (**Figure 2d**).

The data show that splenic FO cells are able to phagocytose beads in a BCR-driven process dependent of RRas2 and RhoG GTPases. The ability to phagocytose beads decreases with the size of the particle while becoming more dependent of RRas2 and RhoG activity.

1.3 B cells can process and present phagocytic antigens to CD4 T cells

B lymphocytes are considered professional antigen presenting cells since they express MHC class II and are able to activate T cells (Roche and Furuta 2015). B lymphocytes acquire antigen by a BCR-dependent mechanism or by a receptor independent process, such as pinocytosis. Although both types of antigen acquisition can induce T cell responses, the BCR-dependent process is more efficient. BCR-antigen uptake has always been linked to molecules and particles smaller than 200 nm, and therefore to endocytic processes rather than phagocytic. Recently, it was seen that peritoneal (PerC) B1 B cells acquire phagocytic antigens and present them to CD4 T cells in a more efficient way than macrophages (Parra et al. 2012).

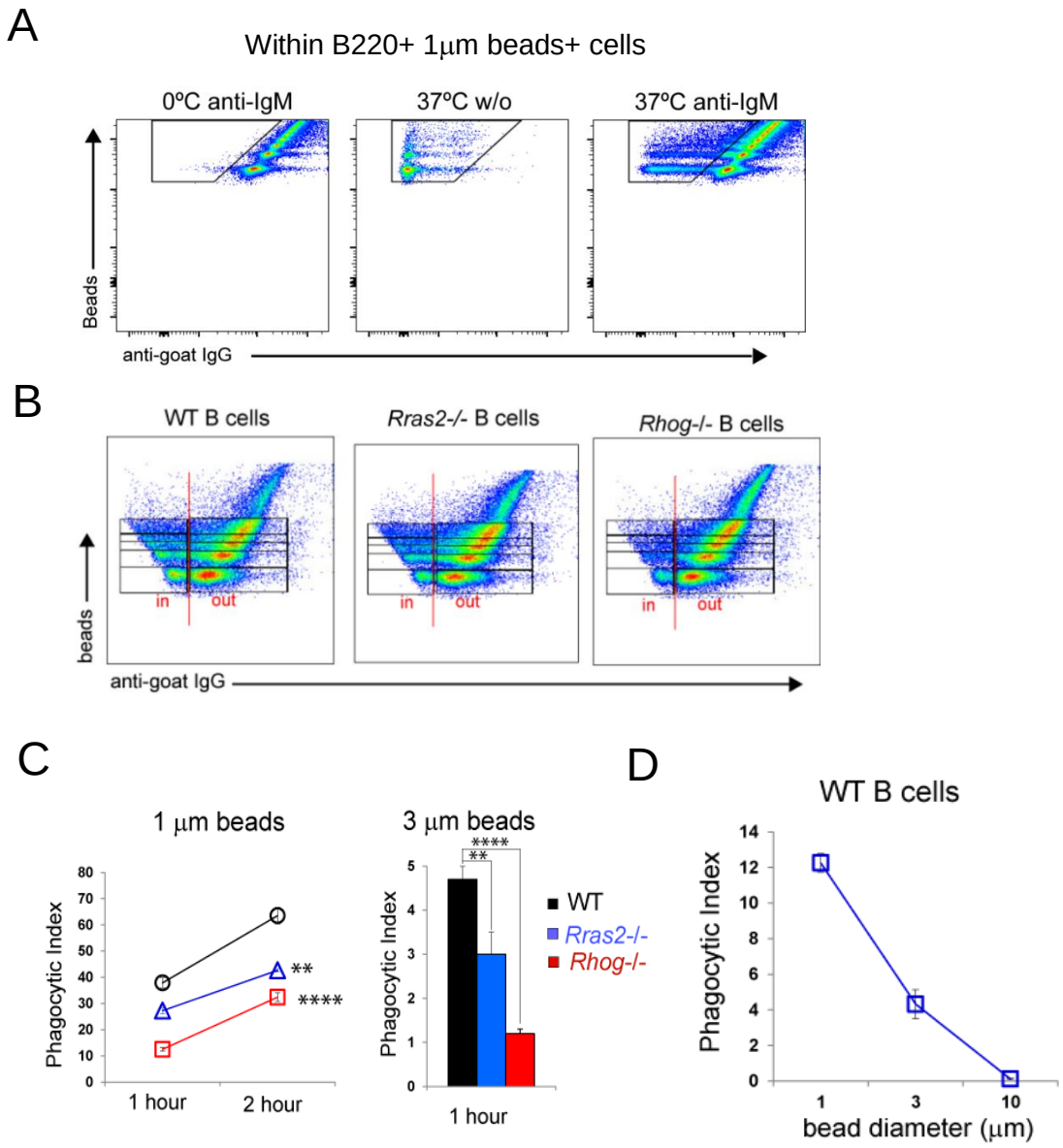

Figure 2. BCR-driven phagocytosis in FO B cells depends on RRas2 and RhoG GTPases. (A) Flow cytometry plots show B cells with 1μm fluorescent beads bound to anti-IgM positive (external) or not (internal) for the secondary anti-goat IgG. B cells were incubated for 1 hour with anti-IgM beads at 0ºC or at 37ºC or with beads without BCR stimulus at 37ºC. Subsequently cells were stained with the secondary antibody at 0ºC. (B) Flow cytometry plots show WT, *Rras2-/-* and *Rhog-/-* FO B cells (B220+ CD43- CD11b-) incubated for 1 hour with 1μm fluorescent beads bound to anti-IgM and stained for a secondary antibody anti-goat IgG. The stepwise fluorescent intensity in the beads wavelength indicates the number of beads, while the positivity (out) or negatively (in) for the secondary antibody discriminates cells with membrane-attached beads from cells with only internalized beads respectively. (C) Graphs show the phagocytic index quantification of WT, *Rras2-/-* and *Rhog-/-* B cells incubated with 1 or 3 μm beads. Data represent the mean +/- S.D. (n=3) **p< 0.005; ***p < 0.0005 (Unpaired Student's t-Test). (D) Graph shows the phagocytic index of WT B cells incubated for 1 hour with 1, 3 or 10 μm size fluorescent beads bound to anti-IgM. Data represent the mean +/- S.D. (n=3)

Once we knew that splenic FO B cells can phagocytose antigen, it was evaluated if those B cells could present phagocytic antigens to CD4 T cells. To test this idea, purified FO B cells from WT, *Rras2-/-* and *Rhog-/-* mice were incubated with different ratios of 1μm beads bound to a mixture of anti-IgM antibody and ovalbumin. Subsequently, those cells were co-cultured for 3 days with purified naïve CD4 T cells from OT-2^{tg} mice labeled with Cell Trace Violet (CTV) (**Figure 3a**). OT-2 transgenic mice express a clonotypic TCR composed by Vα2 and Vβ5 TCR chains that specifically recognize an ovalbumin-derived peptide presented by I-A^b (Barnden et al. 1998). Consequently, OT-2 T cells could only be activated if B cells phagocytose anti-IgM+Ova beads, process the ovalbumin protein and load the fragments peptides onto I-A^b MHC. The labeling of OT-2 T cells with CTV allowed us to study the percentage of proliferating cells and calculate the Proliferation Index (PI) taking into account the number of cell divisions, since CTV intensity is diluted by half after each cell division. Incubation of B cells with anti-IgM+Ova beads induced OT-2 T cells proliferation in a dose-dependent manner according to the bead/B cell ratio whereas preincubation with beads coated with either anti-IgM or Ovalbumin alone did not. This data show that B cell activation is not enough to induce OT-2 T cell proliferation, indicating that this process is not caused by B cell cytokine release. Moreover, in *Rras2-/-* and *Rhog-/-* B cell cultures, OT-2 T cells proliferated less than in WT conditions, suggesting that the deficiency in phagocytosis showed by *Rras2-/-* and *Rhog-/-* FO B cells is transduced in a lower presentation of antigen to T cells.

In addition, expression of CD25 (IL-2Rα) was also monitored in those cultures. OT-2 T cells upregulated CD25 in a bead dose-dependent manner. This increased expression was also antigen- and BCR-dependent and mediated in part by RRas2 and RhoG GTPases (**Figure 3b**). These results suggest that the acquisition of phagocytic antigens by FO B cells induced their processing, loading onto MHC-II molecules and presentation to T cells, which in consequence get activated.

1.4 CD4 T cells differentiate *in vitro* into follicular helper T cells upon their activation by processed phagocytosed antigens presented by B cells

The main function of B cells as antigen presenting cells takes place in the secondary lymphoid organs when antigen arrives and germinal centers are generated. B cells need to present antigens to CD4 T cells to receive costimulatory signals from a T-cell subset (T follicular helper T cells) to survive (Allen et al. 2007). Moreover, B cell stimulation to T cells is important for TFH differentiation, although it is still not clear if it is necessary for the differentiation or only for its

maintenance (Crawford et al. 2006, Kerfoot et al. 2011, Barnett et al. 2014). TFH cells express characteristics extracellular markers such as CXCR5 and PD1, and their differentiation is mediated by the master regulator *Bcl6*.

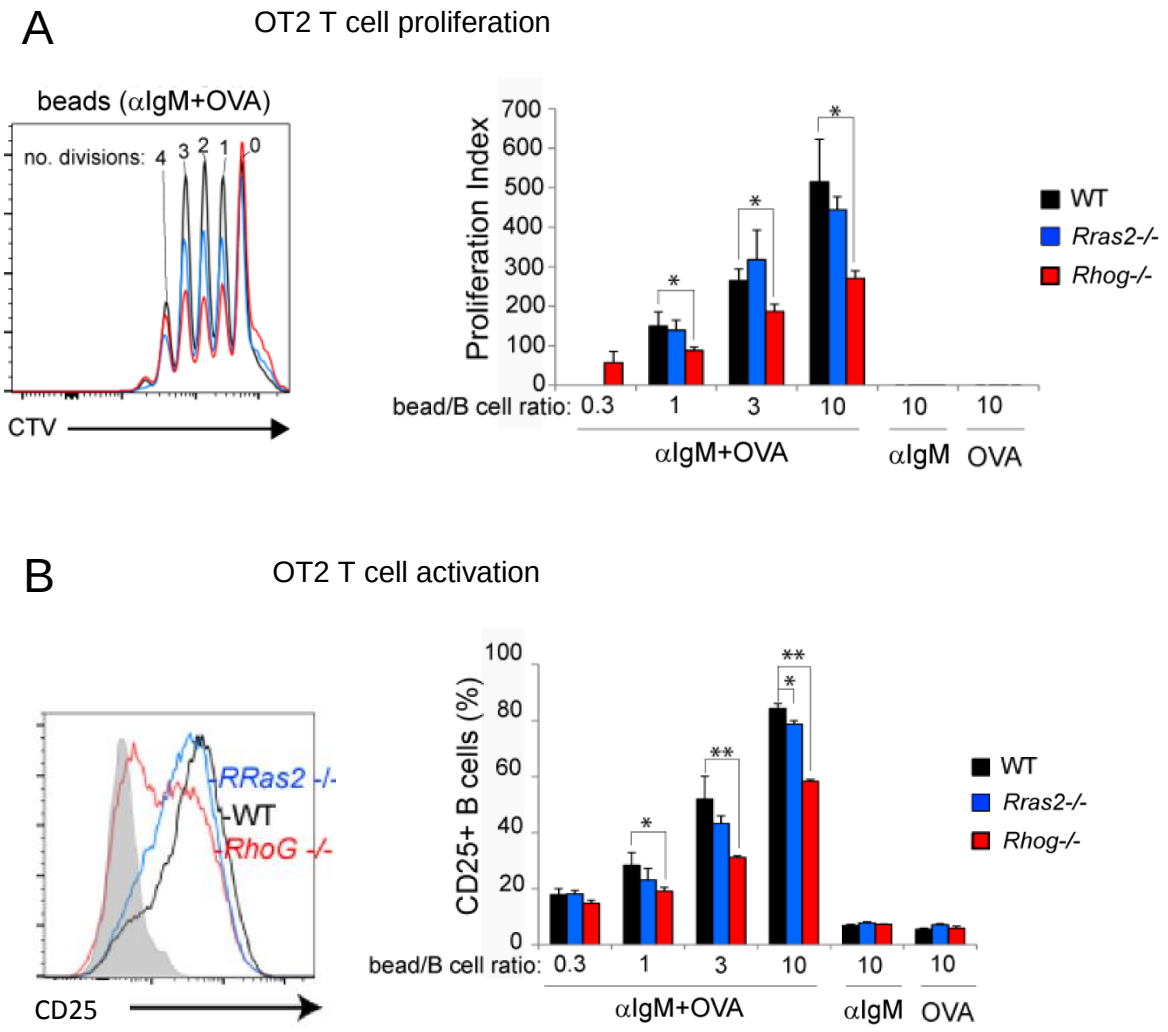

Figure 3. B cells activate CD4 T cells trough the presentation of phagocytosed antigens. (A) Histogram plot shows CTV staining of OT2 T cells after three days of stimulation with WT (black), *Rras2-/-* (blue) and *Rhog-/-* (red) purified FO B cells preincubated with anti-IgM+OVA bound to 1 μm beads in a 10:1 bead:B cell ratio. Plot to the right shows the proliferation index of OT-2 T cells after three days of culture with B cells and different bead:B cell ratios. Beads bound only to anti-IgM or ovalbumin were used as controls. Data represent the mean +/- S.D. (n=3) *p < 0.05 (Unpaired Student's t-Test). (B) Histogram plot shows CD25 expression by OT2 T cells stimulated with WT (black), *Rras2-/-* (blue) or *Rhog-/-* (red) B cells for three days as in (A) . Grey histogram represents CD25 expression in the control condition consisting of B cells stimulated with beads coated with anti-IgM but not ovalbumin. The bar plot shows the percentage of CD25+ OT2 T cells after three days of culture in the conditions indicated in the graph. Data represent the mean +/- S.D. (n=3) *p < 0.05; **p < 0.005 (Unpaired Student's t-Test)

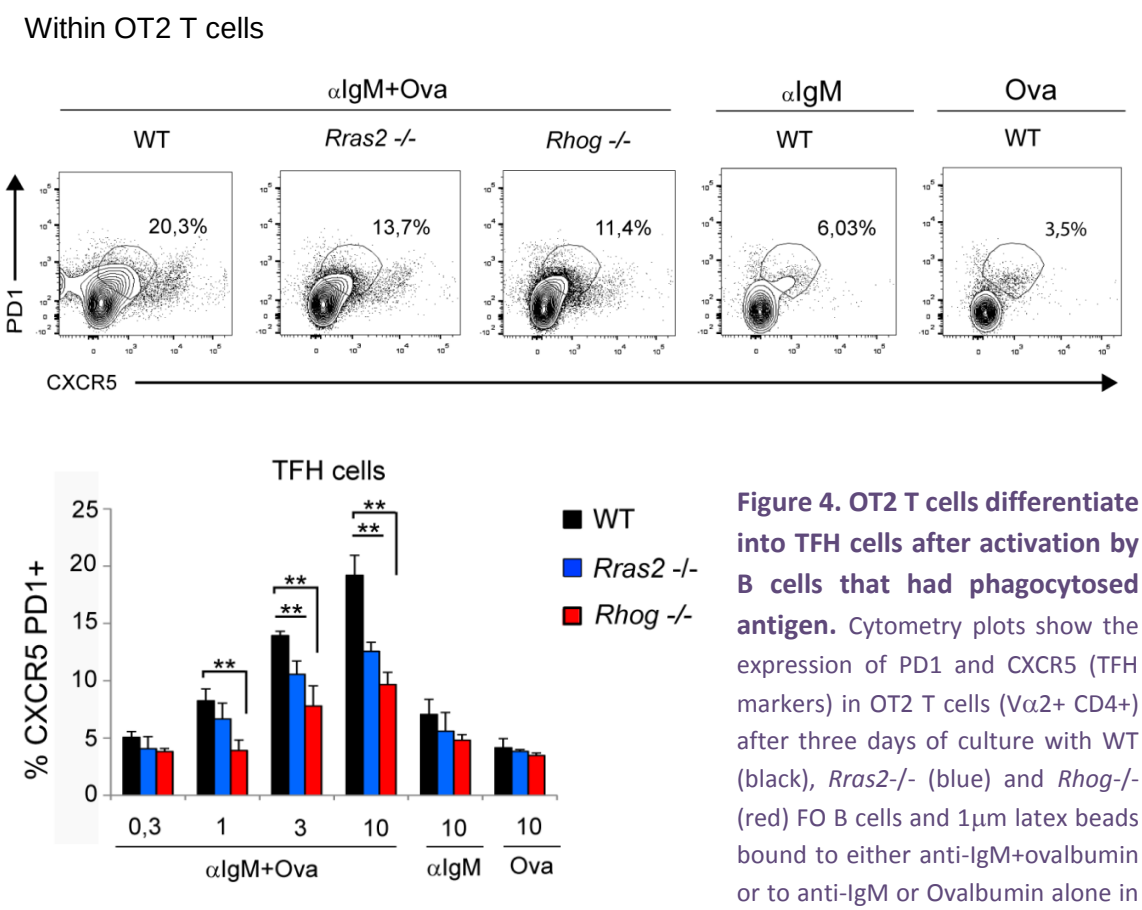

Figure 4. OT2 T cells differentiate into TFH cells after activation by B cells that had phagocytosed antigen. Cytometry plots show the expression of PD1 and CXCR5 (TFH markers) in OT2 T cells (Vα2+ CD4+) after three days of culture with WT (black), *Rras2-/-* (blue) and *Rhog-/-* (red) FO B cells and 1μm latex beads bound to either anti-IgM+ovalbumin or to anti-IgM or Ovalbumin alone in a 10:1 bead:B cells ratio. Gates indicate the percentage of positive cells for CXCR5 and PD1. Lower bar graph shows the percentage of TFH (CXCR5+PD1+ in CD4 T cells) after three days of culture in the conditions indicated in the graph. Data represent the mean +/- S.D. (n=3) **p < 0.005 (Unpaired Student's t-Test)

In order to test if FO B cell presentation of phagocytic antigens to OT2 T cells could be inducing their differentiation to TFH, purified naïve OT2 T cells were cultured together with splenic naïve B cells and 1 μm latex beads coated with anti-IgM+ovalbumin at different doses (**Figure 4**). We found that OT2 T cells express surface TFH markers after three days of culture in an antigen- and BCR-driven manner, reaching 20% of positive cells with the highest dose of beads. The importance of RRas2 and RhoG during phagocytic acquisition of antigen is also translated into TFH differentiation since OT2 T cells cultured with *Rras2-/-* and *Rhog-/-* B cells have lower percentages of TFH. These data suggest that presentation of phagocytic antigens by B cells induces TFH differentiation of OT2 T cells.

2. Naïve B cells differentiate into Germinal Center B cells *in vitro* by a phagocytic-dependent mechanism

Germinal center formation requires the activation of antigen-specific Follicular B cells when pathogen-derived antigens access to the secondary lymph nodes. These activated B cells need the costimulation by CD40L and ICOS induced by TFH to survive and be selected (Kawabe et al. 1994, Dong et al. 2001, McAdam et al. 2001). Therefore, two kinds of signals are necessary for germinal center B cells formation: a BCR stimulus provided by the proper antigen and a costimulatory signal provided by the TFH cells.

2.1 B cells differentiate to GC *in vitro* by a RRas2 and RhoG dependent process

In order to test if GC B cells were generated *in vitro*, naïve non-transgenic FO B cells were cultured with OT2 T cells and 1μm latex beads coated with anti-IgM+ovalbumin. B cells acquired GC markers CD95 and GL7 after three days in culture (**Figure 5a**), upregulated CD40 expression (**Figure 5b**) and proliferated extensively (**Figure 5c**). GC B cell markers expression required T cell help since cells only stimulated with anti-IgM (i.e. without OVA) did not show comparable expression. Furthermore, antigen has to be phagocytosed because RRas2- and RhoG-deficient B cells were also deficient in GC differentiation and proliferation. These data suggest that antibody-induced antigen phagocytosis by FO B cells promote T and B cell differentiation to TFH and GC cells respectively.

In order to demonstrate that B cell phagocytosis was BCR antigen-driven, the B1-8^{hi} knock-in mice were used. These mice have a specific VDJ rearrangement of IgH locus, which in combination with a specific λ IgL chain confers B cells high specificity for 4-hydroxy-3-nitrophenylacetyl (NP) hapten and its derivate 4-hydroxy-3-iodo-nitrophenylacetyl (NIP). The light chain is not fixed, for that reason in non-immunized mice only 3-5% of B cells express the specific λ light chain and are able to interact with NP (Shih et al. 2002, Shih et al. 2002). NP and NIP haptens bound to carrier proteins induce T-cell dependent GC responses.

Purified naïve FO B cells from WT, *Rras2-/-* and *Rhog-/-* B1-8^{hi} mice were cultured with naïve OT2 T cells and 1μm latex beads coated with either NIP(15)-ovalbumin or, as a control, with NP(26)-CGG (chicken gamma globulin) which activates B1-8 B cells but not OT2 T cells (**Figure 6**).

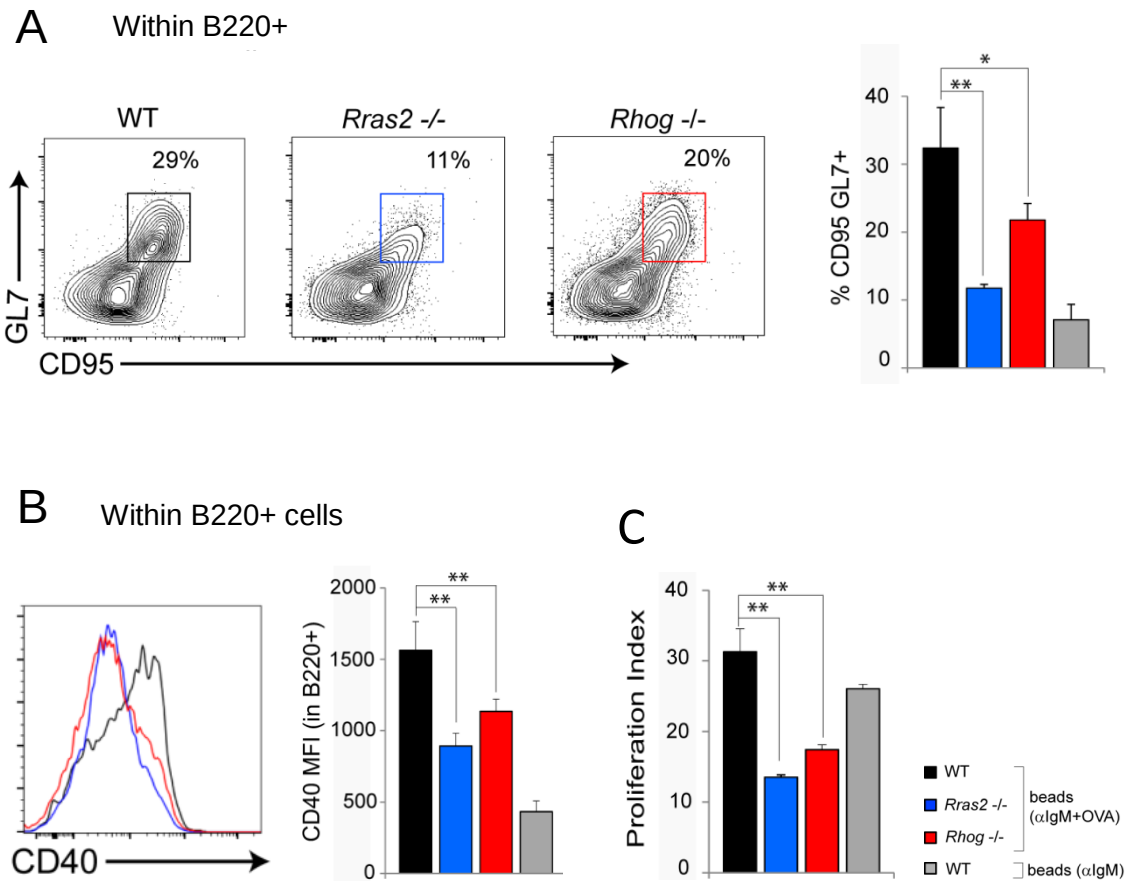

Figure 5. Non-transgenic naïve B cells differentiate into germinal center B cells *in vitro* by a RRas2- and RhoG-dependent process. (A) Cytometry plots show CD95 and GL7 expression in WT (black), *Rras2-/-* (blue) and *Rhog-/-* (red) FO B cells after three days in culture with OT2 CD4 T cells and 1μm latex beads coated with anti-IgM+ovalbumin (20:1 beads:Bcell ratio) or only anti-IgM (grey). Gates indicate the Germinal Center population (CD95+ GL7+ in B220+) which is quantified in the bar plots. Data represent the mean +/- S.D. (n=3) *p < 0.05; **p < 0.005 (Unpaired Student's t-Test). (B) Histogram plot of CD40 expression of B cells cultured for three days as in (A). Quantification of Mean Fluorescence Intensity (MFI) of CD40 in the cultured B cells is shown in the graph. Data represent the mean +/- S.D. (n=3) **p < 0.005 (Unpaired Student's t-Test). (C) Proliferation Index quantification of CTV-labelled B cells cultured for 3 days as in (A). Data represent the mean +/- S.D. (n=3) **p < 0.005 (Unpaired Student's t-Test)

Four days of culture led to the emergence of a large population of CD95 GL7 double-positive cells. RRas2 (blue) and RhoG (red) deficient B cells showed again a defect in the generation of GC B cells, indicating the importance of antigen phagocytosis and T-cell help for this *in vitro* differentiation (**Figure 6a**).

Bcl6 is a master regulator of GC B and T cell differentiation whose expression is rapidly upregulated after B cell encounter with its cognate peptide. Its expression was found upregulated in GL7hi cells when B cells were stimulated with NIP-OVA beads, while *Rras2-/-* and *Rhog-/-* B cells showed lower expression (**Figure 6b**).

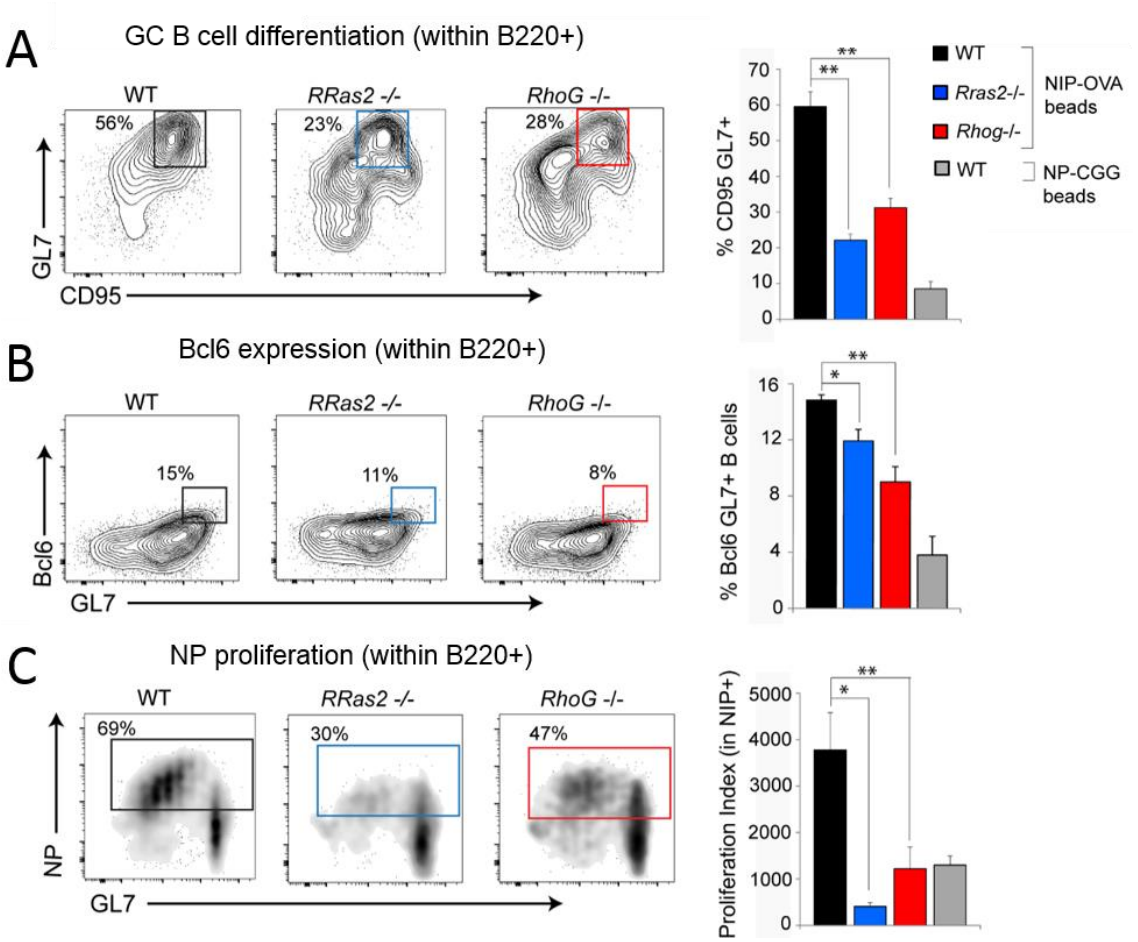

Figure 6. B cells from B1-8^{hi} mice acquire GC phenotype *in vitro* upon phagocytic antigen presentation in a RRas2- and RhoG-dependent process. (A) Flow cytometry plots show the expression of CD95 and GL7 in B220+ cells from WT (black), *Rras2-/-* (blue) and *Rhog-/-* (red) B1-8^{hi} mice cultured for 4 days with purified OT2 T cells and 1µm NIPOVA beads (3:1 beads:Bcell ratio). Gates indicate the considered germinal center B cell population. The bar graph quantifies the percentage of CD95+GL7+ cells in B cells stimulated with NIP-OVA or control stimulus NP-CGG (grey). Data represent the mean +/- S.D. (n=3) **p < 0.005 (Unpaired Student's t-Test). (B) Cytometry plot shows the expression of Bcl6 and GL7 in B cells cultured for 4 days as in (A). Gates indicate the Bcl6+GL7+ population within B220+ cells. Bar graph quantifies the percentage of Bcl6+GL7+ B cells. Data represent the mean +/- S.D. (n=3) *p < 0.05; **p < 0.005 (Unpaired Student's t-Test). (C) Cytometry plots show the expression of NP and Cell Trace Violet dilution in B cells after being cultured as in (A). Bar graph quantifies the proliferation index in NP+ B220+ B cells. Data represent the mean +/- S.D. (n=3) *p < 0.05; **p < 0.005 (Unpaired Student's t-Test).

Afterwards, to monitor antigen-driven proliferation in those cultured cells, FO B cells from WT, *Rras2-/-* or *Rhog-/-* B1-8^{hi} mice were stained with Cell Trace Violet and put in culture with OT2 T cells and NIP-OVA or NP-CGG beads as previously done (**Figure 6c**). Four days later, the proliferation index in NP-reactive B cells was calculated. B cells proliferate upon phagocytic antigen acquisition in an RRas2- and RhoG-dependent mechanism. This proliferation is not only due to BCR stimulation but to CD4 T cell costimulus as well, since NP-CGG condition was less stimulatory.

These data suggest that antigen-driven phagocytosis induces the *in vitro* differentiation of B cells into GC cells. RRas2 and RhoG GTPases are required during this process as well as CD4 T cell help.

Surprisingly, the defect in expression of GC markers in *Rras2-/-* B cells was stronger than in *Rhog-/-* although phagocytic antigen uptake was more impaired in the latter. *Rras2-/-* mice have been shown previously to be deficient in homeostatic B cell proliferation and in the formation of GCs due to a B cell-intrinsic defect in tonic and antigen-triggered BCR signaling (Delgado et al. 2009) (Mendoza 2017). In order to avoid possible side-effects, it was decided to only use *Rhog* knock out B cells as control of the relevance of phagocytosis in the processes studied.

With the aim to monitor GC B cell formation in culture and to study the kinetics of acquisition of GC markers, WT and *Rhog-/-* FO B cells from B1-8^{hi} mice were cultured with 1μm NIPOVA or NP-CGG beads and OT2 T cells for 7 days. Every day, cultured cells were analysed for the expression of GC markers: CD95+GL7+ and CD38lo BCl6+ (**Figure 7**).

In order to exclude dead cells, a Cell-Viability marker was used. After three days of culture, a double-positive population (CD95+GL7+) appeared in B220+ B cells, which peaked at day 4 and decreased afterwards (**Figure 7a**). The GC was also followed as a CD38low Bcl6+ population. This population emerged after 3 days of culture and followed a similar kinetics as for the CD95+GL7+ population (**Figure 7b**). *Rhog-/-* cultured B cells were found to be defective in the generation of GC from the beginning of the stimulation. Likewise, B cells cultured with NP-CGG beads, without OT2 T cell costimulus, did not differentiate to GC cells.

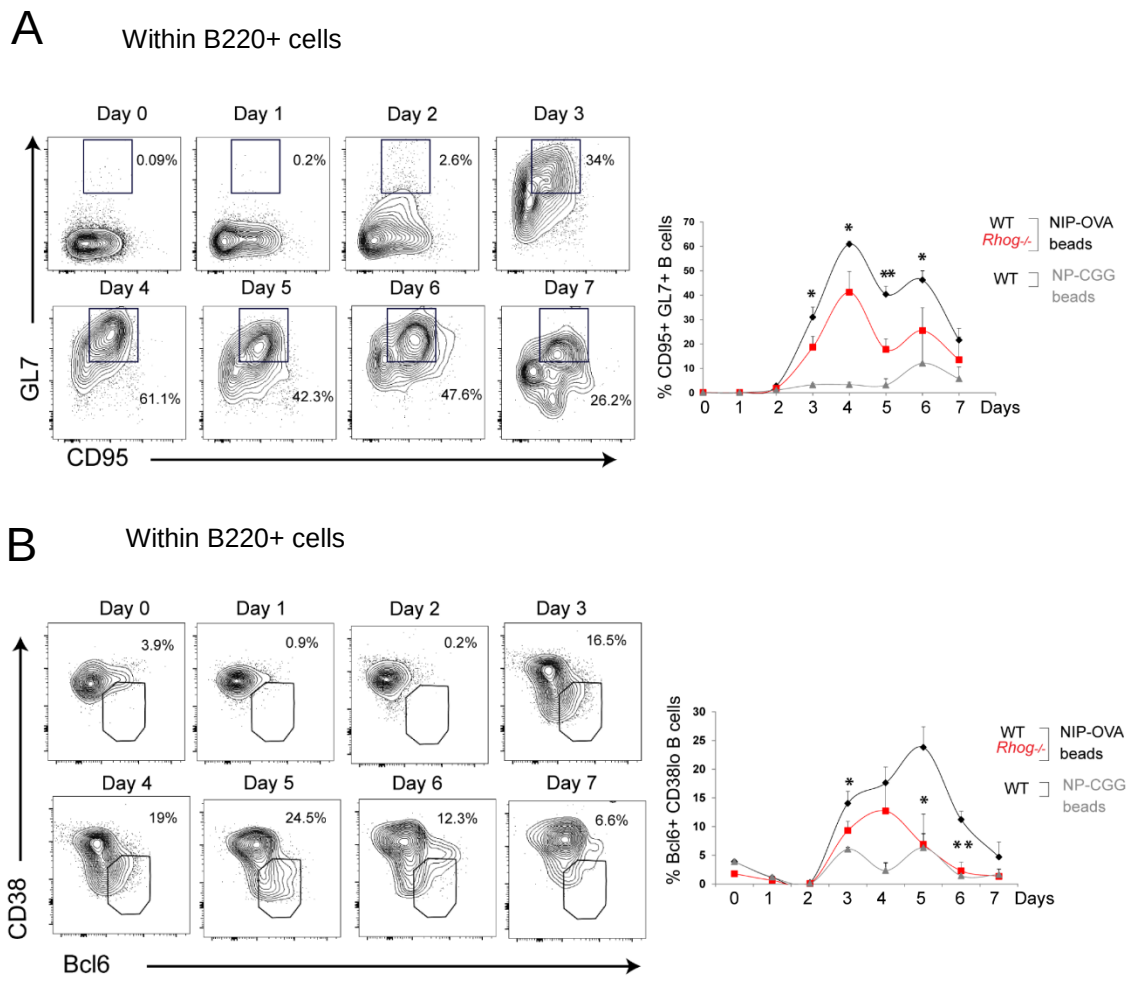

Figure 7. Expression kinetics of GC B cell markers in in-vitro cultures. (A) Flow cytometry panel illustrates the daily expression of CD95 and GL7 GC markers in purified naïve WT FO B cells from B1-8^{hi} mice cultured for 7 days with 1μm NIP-OVA beads (3:1 Beads:Bcell ratio) and OT2 T cells. Gates indicate the double-positive population. Line graph quantifies the time-dependent emergence of GC B cells (CD95+GL7+ in B220+) in cultured WT (black) or *Rhog*-/- (red) B cells stimulated with NIP-OVA beads. In parallel, WT cells were also stimulated with NP-CGG bead-bound (grey) as explained before. Data represent the mean +/- S.D. (n=3) *p < 0.05; **p < 0.005 (Unpaired Student's t-Test). (B) Cytometry panel shows the daily expression of CD38 and Bcl6 in WT B cells from B1-8^{hi} mice cultured as in (A). Line graph quantifies the daily generation of CD38low Bcl6+ cells in WT (black) and *Rhog*-/- (red) B cells cultured with NIP-OVA beads or with NP-CGG (grey). Data represent the mean +/- S.D. (n=3) *p < 0.05; **p < 0.005 (Unpaired Student's t-Test)

In order to monitor the expression of GC B cell markers along the proliferation of cultured cells, CTV-labelled purified WT FO B cells from B1-8^{hi} mice were cultured with purified OT2 T cells and stimulated with 1μm beads-bound to either NIP-OVA or NP-CGG (**Figure 8**). After four days of stimulation with NIP-OVA beads, Bcl6 expression peaked in cells that had divided 3 times and decreased in more divided cells. The NP-CGG stimulus was unable to induce Bcl6

expression. CD38 downregulation needed also T cell help and occurred after the second cell division. In contrast, IgD downregulation and GL7 upregulation did not require T cell help since NP-CGG coated beads also induced both phenomena. In addition to the differential effect of CD38 downregulation and Bcl6 expression, B cell stimulated with NP-CGG coated beads did not proliferate extensively as those stimulated with NIP-OVA.

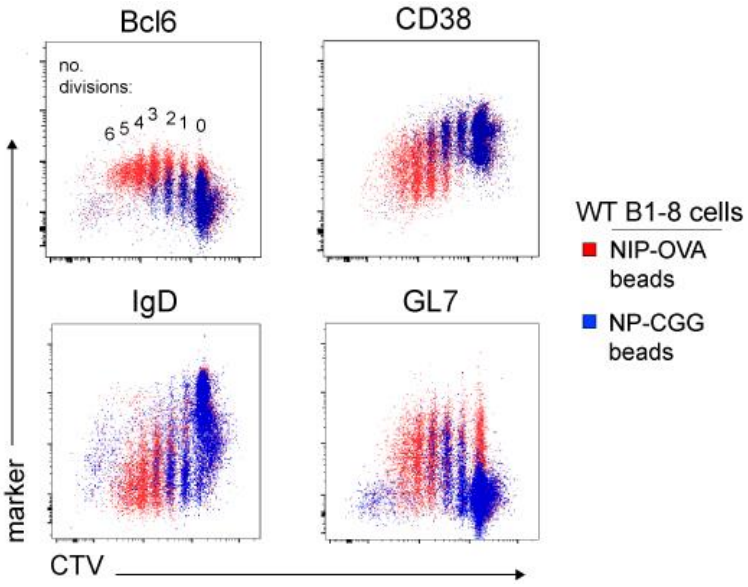

Figure 8. Expression of GC markers with the number of divisions. Dot plots of Bcl6, CD38, IgD and GL7 expression according to Cell Trace Violet (CTV) dilution in gated B cells from B1-8^{hi} mice cultured for four days with OT2 T cells and 1μm NIP-OVA (red) or NP-CGG (blue) bead-bound (3:1 ratio beads:Bcell).

B cells do not only need CD40:CD40L and ICOSL:ICOS costimulus produced by CD4 T cells, it is also necessary a specific microenvironment created by some cytokines release for GC and plasmablast differentiation,. To monitor if those cytokines were also secreted in the *in vitro* B:T cultures, WT FO B cells from B1-8^{hi} mice were cultured for four days with CTV-labelled OT2 T cells and different doses of 1μm and 3μm NIP-OVA beads. Proliferation, TFH differentiation and cytokine release (IL-2, IL-4, IL-6, IL-10 and IL-21) were monitored in those cultures (**Figure 9**). TFH are the main producers of IL-4 and IL-21, necessary for IgG1 class-switch and GC development respectively. IL-6 is produced by B cells, as well as DC, and plays an important role in TFH differentiation; while IL-2 and IL-10 are described as inhibitors of early GC but important players during plasmablast differentiation and maintenance of plasma cells (Wali et al. 2016). Cultured cells showed that OT2 T cells proliferate in a bead dose-dependent manner, but surprisingly, TFH differentiation and cytokine release have an optimal bead-dose related with the size of the bead since it was different for 1 and 3μm beads. This optimum dose coincides for TFH differentiation and cytokines release, although IL-21 was poorly secreted.

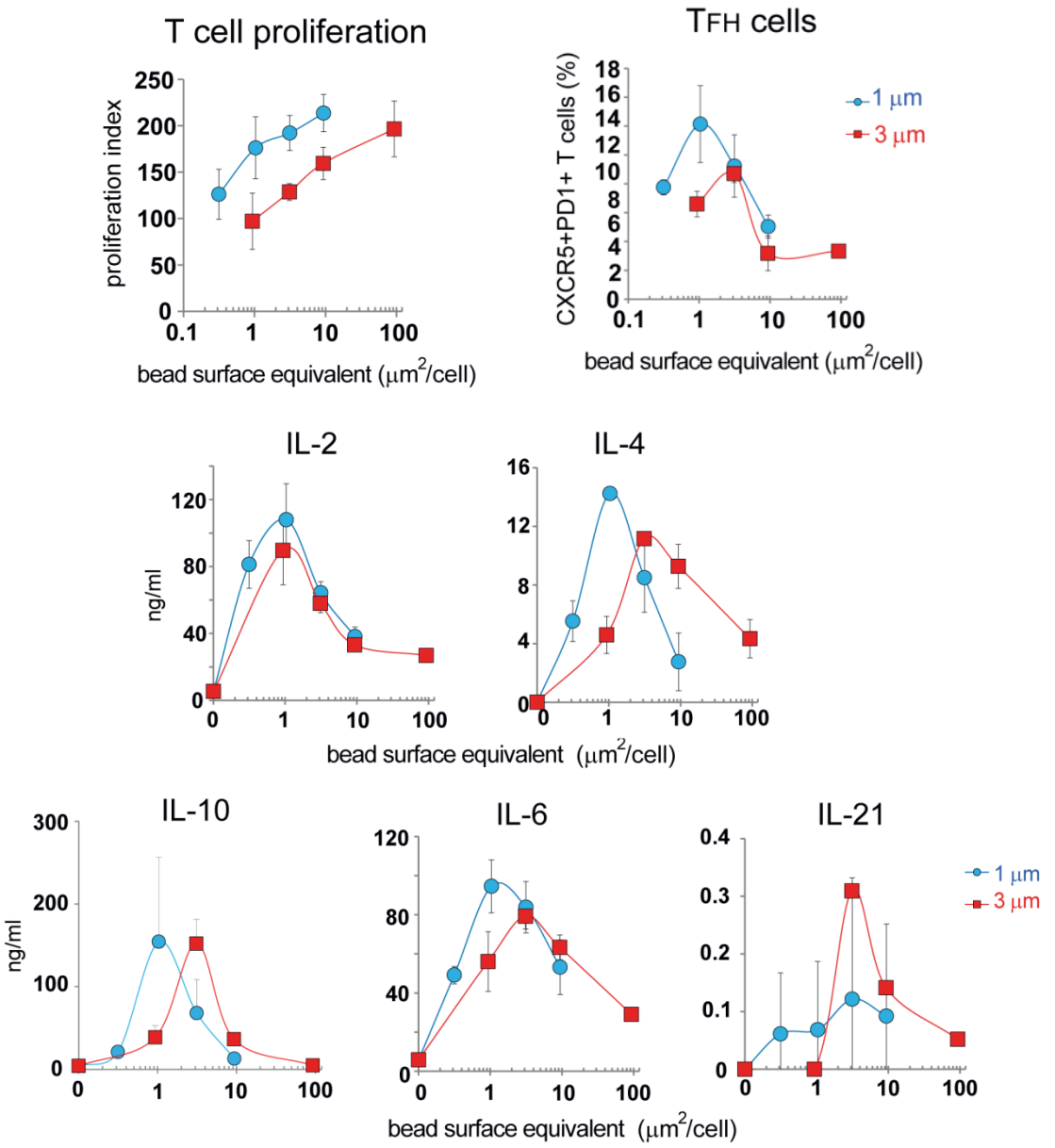

Figure 9. *In vitro* T:B cell cultures produce typical GC cytokines with an optimum bead-dose response. Graphs show OT2 T cell proliferation, TFH (CXCR5+PD1+ in CD4+) differentiation and cytokine release in the supernatant of naïve FO B cells from B1-8^{hi} mice cultured for four days with OT2 T cells and different doses of 1μm (blue) and 3μm (red) bead-bound NIP-OVA. The beads:B cell ratio for 1μm beads used were: 0,1:1 to 3:1; for 3μm beads were: 0,033:1 to 3:1. The dose of beads is represented in the graphs normalized according to their theoretical surface considering them as spheres. Data represent the mean +/- S.D. (n=3)

These data shows that cytokines important for germinal center reaction are secreted in the supernatant of the T:B cultures.

In order to know if cytokine release in T:B cell cultures was also phagocytosis-dependent, WT and *Rhog*-/- B cells from B1-8^{hi} mice were cultured with 1µm NIP-OVA bead-bound for four and seven days and cytokines released to the culture supernatant were measured (**Figure 10**). *Rhog*-/- B cell cultures produced lower amounts of cytokines, indicating that antigen phagocytosis was required for their generation. Moreover, the concentration of IL-2 increases substantially between days 4 and 7, suggesting the existence of conditions that favour plasmablast differentiation at late stages.

These data suggest that B cell antigen phagocytosis and presentation to CD4 T cells is important for the secretion of GC-type interleukins.

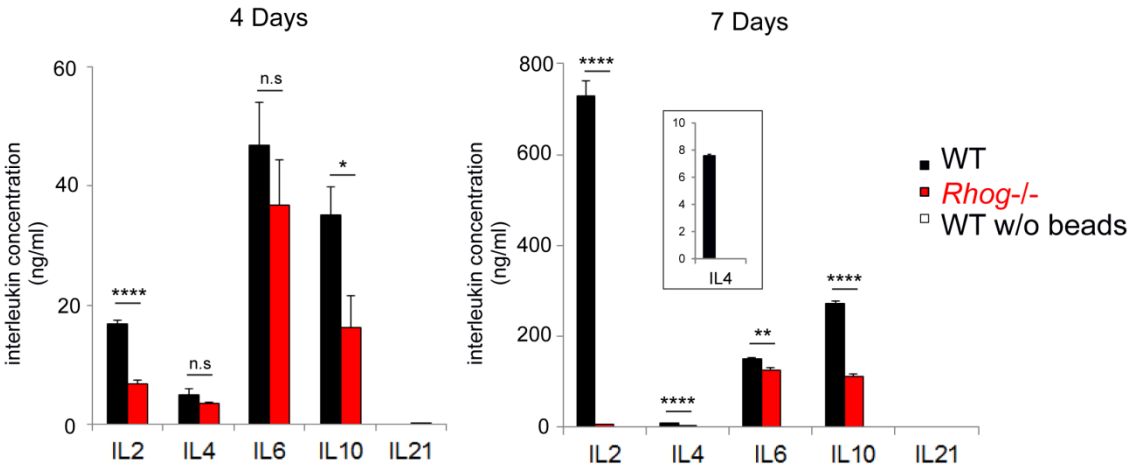

Figure 10. Interleukin secretion in cultured germinal centers is RhoG-dependent. Graphs show the concentration of interleukin IL-2, IL-4, IL-6, IL-10 and IL-21 in the supernatants of cultured WT (black) or *Rhog*-/- (red) naïve FO B cells from B1-8^{hi} mice together with OT2 T cells and NIP-OVA bead-bound (3:1 beads:Bcell ratio) for 4 and 7 days. Supernatant of cultured cells for 7 days without stimulus was used as control. Data represent the mean +/- S.D. (n=3) n.s $p > 0.05$; **$p < 0.005$; ****$p < 0.00005$ (Unpaired Student's t-Test)

2.2 Cultured germinal center B cells differentiate to plasma cells *in vitro*

B cell differentiation to plasma cell is directed by the expression of the transcription factors Blimp1 and Irf4 (Shapiro-Shelef et al. 2003). Blimp1 is involved in the B cell exit from the GC program by promoting their terminal differentiation to plasma cells. Bcl6 and Blimp1 expression are involved in a mutually regulatory loop in a way that Bcl6 repress Blimp1, and the latter repress Bcl6.

In order to study if the observed downregulation of Bcl6 in the last B cell divisions was accompanied by an upregulation of plasma cell differentiation, expression of Bcl6 and Blimp-1 was monitored in CTV-labelled B1-8^{hi} B cells cultures stimulated with bead-bound NIP-OVA and cultured with purified OT2 T cells. As expected, Bcl6 had a bell-shaped expression with cell division while Blimp1 expression increased. Therefore, downregulation of Bcl6 in the last divisions could be originated by the growing repression caused by Blimp1 (**Figure 11a**).

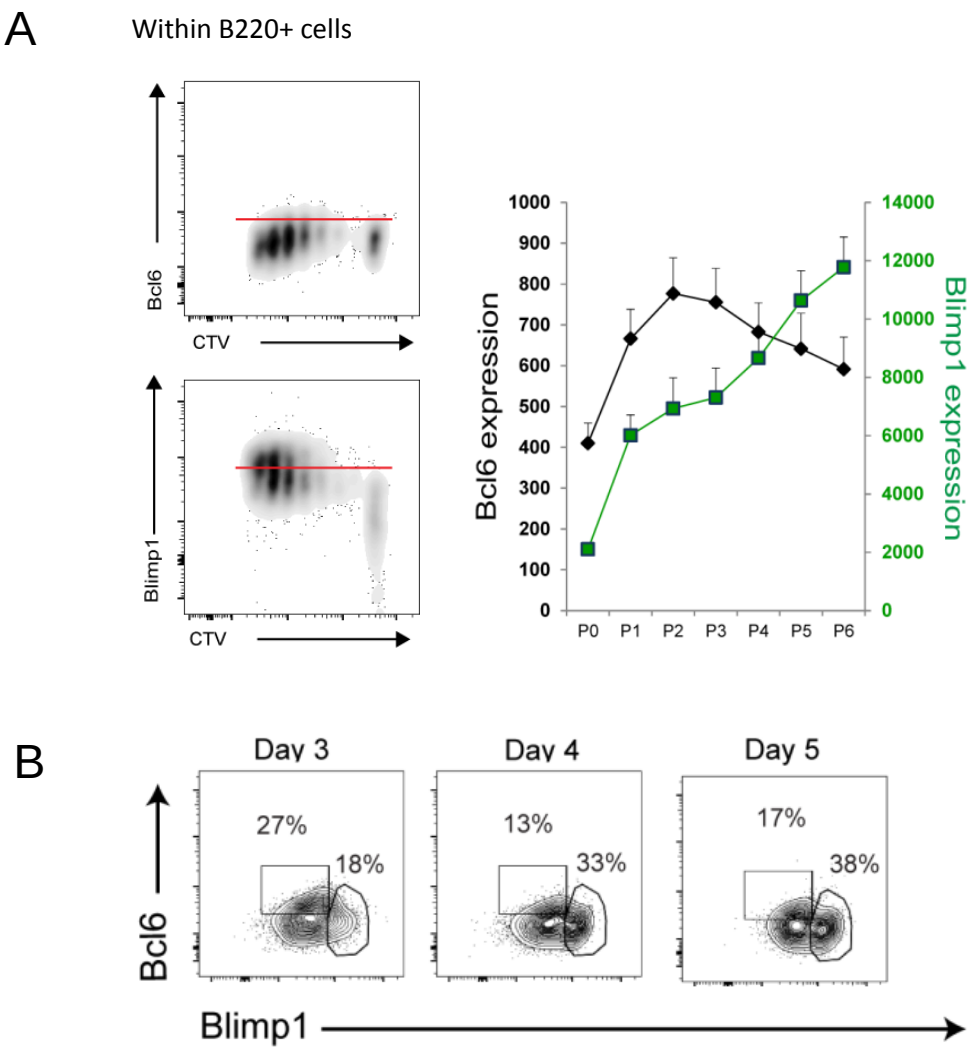

Figure 11. Cultured GC B cells express Blimp1 and downregulate Bcl6 in the last cell divisions. (A) Cytometry plots show expression of Bcl6 (upper plot) and Blimp1 (lower plot) according to CTV dilution in FO B cells from B1-8^{hi} mice cultured for three days with OT2 cells and stimulated with NIP-OVA beads (3:1 beads:Bcell ratio). Red line indicates the transcriptional factor expression over that of non-divided cells. Right graph shows the mean fluorescence expression (MFI) of Bcl6 (black) and Blimp1 (green) according to B cell division. (B) Contour-plots illustrate the expression of Bcl6 and Blimp1 in B cells cultured as in (A) for 3, 4 or 5 days. Gates indicate Bcl6^{hi} Blimp1^{low} and Blimp1^{hi} Bcl6^{low} populations.

Moreover, when the expression of Blimp1 and Bcl6 was tracked simultaneously in B cells between days 3, 4 and 5 of culture, a $Bcl6^{low}Blimp1^{hi}$ population increased over days at expenses of a $Bcl6^{hi}$ $Blimp1^{low}$ population (**Figure 11b**). These data suggest that *in vitro* GC B cells have similar molecular regulation as *in vivo*.

With the idea of monitoring the formation of plasma cells in the cultures, B cells from WT and *Rhog-/-* $B1\text{-}8^{hi}$ mice were followed over seven days of culture with OT2 T cells and 1µm bead-bound NIP-OVA or NP-CCG. Daily, cells were analysed for the expression of Blimp1 and CD138, which expression is upregulated in plasma cells (**Figure 12a**).

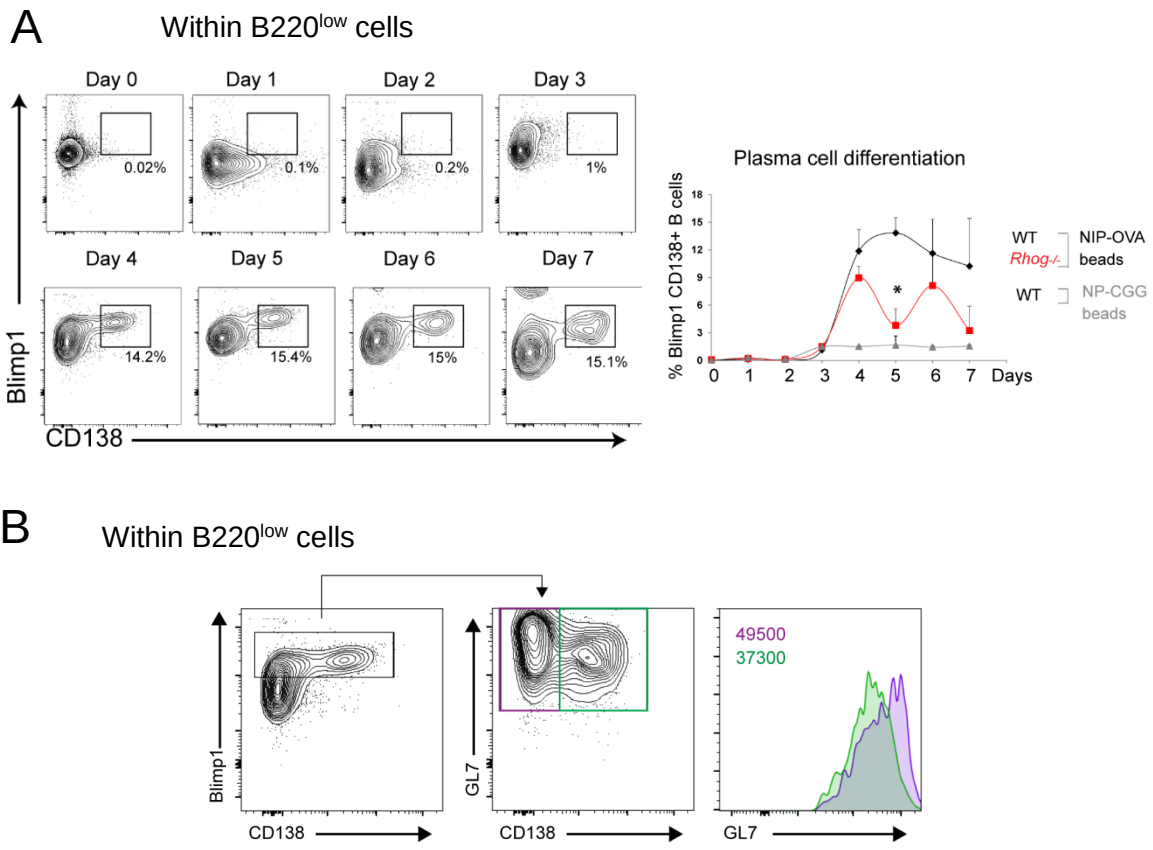

Figure 12. Germinal center B cells differentiate to plasma cells after four days of culture in a RhoG-dependent process. (A) Upper panel shows the expression of CD138 and Blimp1 inside $B220^{low}$ B cells along seven days of culture of WT (black) or *Rhog-/-* (red) FO B cells from $B1\text{-}8^{hi}$ mice with OT2 T cells and 1µm beads coated with NIP-OVA or NP-CGG (grey) (3:1 beads:Bcell ratio). Quantification of plasma cells (CD138+ Blimp1+) generation with time of culture is shown in the lower graph. Data represent the mean +/- S.D. (n=3) *p < 0.05 (Unpaired Student's t-Test). (B) FACS plots show the expression of CD138 and Blimp1 in B220low B cells after 4 days of culture as in (A). Blimp1+ B cells were analysed for the expression of GL7 and CD138, showing two different populations (CD138- vs CD138+) with different expression of GL7. Histogram shows the expression and mean fluorescence intensity (MFI) value of GL7 within the CD138- (purple) and CD138+ (green) cells.

A double-positive population appeared at day 4 that was slightly increased at day 5 and maintained after that. This plasma cell population was completely abolished in B cells stimulated with NP-CGG beads, indicating its dependency on T cell help and the correct B cell acquisition and presentation of phagocytic antigens. Moreover, we could identify after four days of culture a plasmablast population, precursor of plasma cells, derived from germinal center B cells as they were positive for Blimp1 and GL7 but still negative for CD138 (**Figure 12b**). These data suggest that *in vitro* generated GC B cells can differentiate into plasma cells in a RhoG-dependent manner.

2.3 Soluble antigen is not able to induce a complete GC differentiation of B cells *in vitro*

The B cell receptor has a dual role in GC formation: B cell stimulation and acquisition of antigens for presentation to CD4 T cell. We wonder if the way how the antigen is given to the cells was important for B cell differentiation to GC, independently of its role in antigen presentation to CD4 T cells. For that reason, it was decided to compare soluble versus phagocytic BCR stimuli.

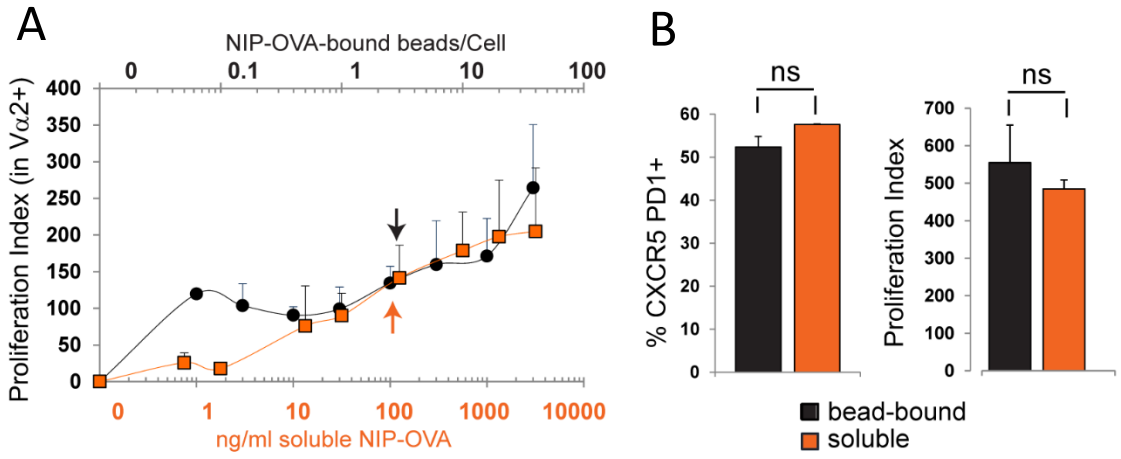

Figure 13. B cells cultured with 3 NIP-OVA coated beads per B cell induce equivalent OT2 activation to 100ng/mL soluble antigen. (A) Proliferation Index quantification of CTV-labelled OT2 T cells cultured for four days with FO B cells from B1-8^{hi} mice stimulated with a wide range of soluble NIP-OVA concentrations (orange) or different doses of NIP-OVA beads-bound (black). Data represent the mean +/- S.D. (n=3). (B) Percentage of TFH cells (CXCR5+PD1+ in Vα2+ OT2 T cells) and Proliferation Index of OT2 T cells after four days of culture with B cells from B1-8^{hi} mice stimulated with 100ng/mL soluble NIP-OVA (orange) or a 3:1 NIP-OVA beads:Bcell ratio (black). Data represent the mean +/- S.D. (n=3) n.s $p > 0.05$ (Unpaired Student's t-Test)

In order to find a soluble concentration and a bead dose that exerts similar CD4 T cell activation, a titration experiment of both stimuli was performed (**Figure 13**). Purified FO B cells from B1-8^{hi} mice were stimulated with different ratios of beads coated with NIP-OVA and in parallel with a broad range of soluble NIP-OVA concentrations in presence of CTV-labelled OT2 T cells for 4 days (**Figure 13a**). We found that 100 ng/ml soluble NIP-OVA induces similar OT2 T cell proliferation and TFH differentiation than a 3:1 ratio of beads coated with NIP-OVA (**Figure 13a and 13b**). These doses of both stimuli were selected to study B cell activation and differentiation.

In such conditions the proliferation and expression of GC markers was compared in CTV-labelled B cells from B1-8^{hi} mouse cultured with OT-2 T cells for 4 days and stimulated with soluble or bead-bound antigen (**Figure 14**).

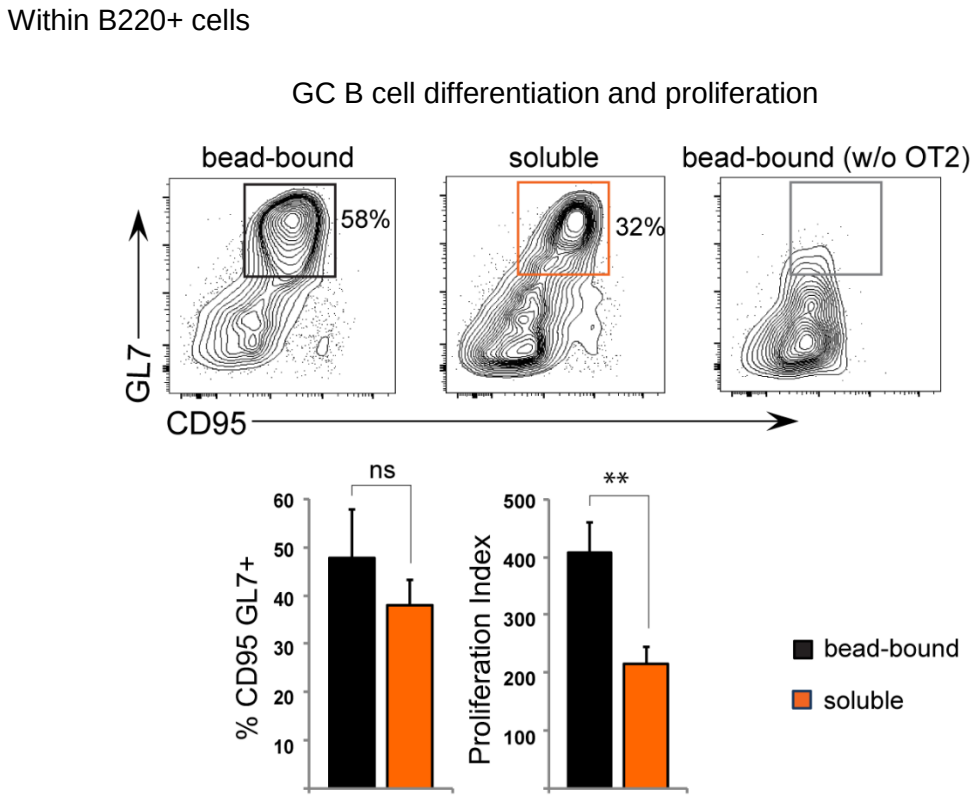

Figure 14. Soluble antigen induces GC markers *in vitro* but stimulates less B cell proliferation. Upper cytometry plots show the expression of CD95 and GL7 by CTV-labelled FO B cells from B1-8^{hi} mouse stimulated with bead-bound NIP-OVA (3:1 beads:B cell ratio) (black) or 100ng/mL soluble NIP-OVA (orange) and cultured with or without (grey) OT2 T cells for four days. Gates indicate the considered double-positive population. Plots at bottom show the percentage of GC B cells (CD95+ GL7+ within B220+) and the Proliferation Index of those B cells. Data represent the mean +/- S.D. (n=3) n.s $p > 0.05$; *$p < 0.05$; **$p < 0.005$ (Unpaired Student's T-Test).

Both types of stimulus induced similar levels of GC surface marker expression. Nevertheless, the way of B cell antigen delivery affected B cell proliferation since B cells stimulated with phagocytic antigen proliferated more than with the soluble one. The costimulus received by OT2 T cells was necessary for GC differentiation, since B1-8 B cells stimulated with NIP-OVA bead-bound but cultured without OT2 T cells did not differentiate towards GC.

It has been shown above (**Figure 2**) that *Rhog-/-* B cells are deficient in the uptake of phagocytic antigens and in consequence, in the CD4 T cell activation and differentiation to GC B cells and TFH. To test if the *in vitro* phenotype of *Rhog-/-* B cells was only due to their defect in phagocytosis, purified FO B cells from WT or *Rhog-/-* B1-8^{hi} mice were cultured for four days with OT2 T cells and stimulated with bead-bound or soluble NIP-OVA (**Figure 15**).

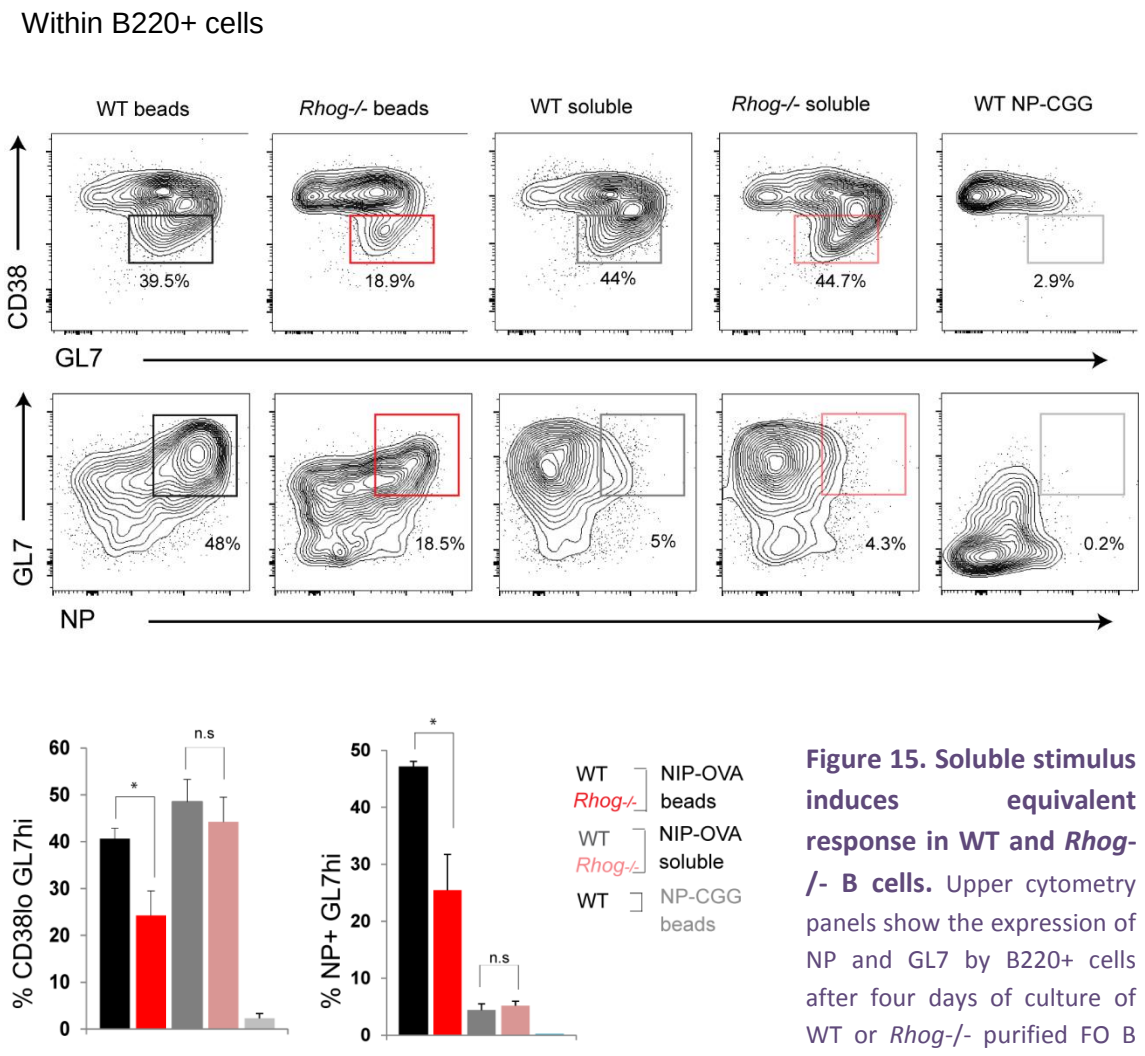

Figure 15. Soluble stimulus induces equivalent response in WT and *Rhog-/-* B cells. Upper cytometry panels show the expression of NP and GL7 by B220+ cells after four days of culture of WT or *Rhog-/-* purified FO B cells from B1-8^{hi} mice with OT2 T cells and soluble (100ng/mL) or bead-bound NIP-OVA (3:1 beads:Bcell ratio). As a control, cells were stimulated with NP-CGG bead-bound (light grey). Lower panel shows CD38 and GL7 expression in those cultured B220+ cells. Bar graphs indicate the quantification of gated populations: CD38^{lo} GL7^{hi} and NP+ GL7^{hi}. Data represent the mean +/- S.D. (n=3) n.s $p > 0.05$; *$p < 0.05$; (Unpaired Student's T-Test).

As it was expected, soluble stimulus induced equivalent response in WT and *Rhog-/-* B cells regarding the downmodulation and expression of CD38 and GL7 respectively, as well as the expansion of high-affinity NP reactive B cells. In contrast, *Rhog-/-* B cells stimulated with bead-bound NIP-OVA generated a lower percentage of GC B cells ($CD38^{low}$ $GL7^{hi}$) and less high-affinity NP+ cells than their WT counterparts, corroborating that RhoG deficiency is restricted to phagocytosis of antigen. Of note, percentages of high-affinity WT NP+ B cells were very different between soluble and bead-bound stimuli, suggesting that high-affinity B cells get more expanded when are exposed to a phagocytic stimulus.

The genetic footprint of GC and plasma B cells have long been studied (Recaldin and Fear 2016). While germinal center B cells have *Bcl6* as master regulator gene, plasma cells have *Blimp1*. Ig class-switch and somatic hypermutations, key points during GC reactions, are regulated by Aid (Activation-induced cytidine deaminase) enzyme, whose activity induces dC:dU mismatches that are repaired by the mismatch or base excision repair machinery. Although its important role during affinity maturation of GC B cells, its expression is not exclusive of this process and can be induced by different stimulus independently of GC reaction, as for example by TLR agonists (Pone et al. 2012). To study this genetic footprint in our GC cultures, qPCR analysis of sorted WT or *Rhog-/-* B cells from $B1\text{-}8^{hi}$ mice was performed after 7 days of culture with OT2 T cells and 1µm bead-bound or soluble NIP-OVA stimuli (**Figure 16**).

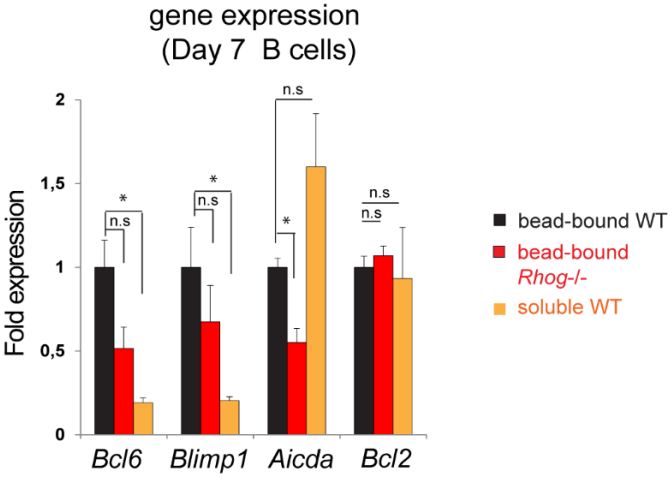

Figure 16. Soluble stimulus induces a different mRNA expression of genes involved in GC than phagocytic antigen. Graph quantification of fold change mRNA expression of *Bcl6, Blimp1, Aicda* and *Bcl2* genes relative to the WT bead-bound condition. Purified FO B cells from WT (black) or *Rhog-/-* (red) $B1\text{-}8^{hi}$ mice were cultured for seven days with OT2 T cells and NIP-OVA beads, or stimulated with soluble NIP-OVA (orange). B cell sorted cells were used for qPCR analysis. *Hprt* and *Gapdh* genes were used as normalizer genes. (n=3) n.s $p > 0.05$; *$p < 0.05$ (Unpaired Student's T-Test).

Phagocytic antigen uptake is a good inducer of *Bcl6* and *Blimp1* expression since soluble antigen resulted in poorer expression. *Rhog-/-* B cells also have lower expression, although not

significantly different, which could be due because RhoG-deficiency does not block completely the phagocytosis. *Aicda* induction was also defective in *Rhog-/-* B cells. However, the soluble stimulus strongly induced its expression. It could be explained by new data that indicates that upregulation of AID expression is not enough for its activation, it also need to be phosphorylated to be functional (Basu et al. 2005, Pasqualucci et al. 2006). *Bcl2*, a pro-survival factor which needs to be downregulated during GC reaction to favour the selection of high affinity clones (Smith et al. 2000), was equally maintained, suggesting similar pro-apoptotic environment in all culture conditions independently of the stimulus.

Another GC hallmark is the somatic hypermutation that takes places in the immunoglobulin genes. Spontaneous somatic mutations occur at a very low rate in dividing normal cells (around 10^{-9} mutations per base par), but this mutational rate increase up to 10^{-3} to 10^{-4} during the GC reaction in a process called somatic hypermutation that introduce point mutations in specific regions of the Ig loci (McKean et al. 1984, Rajewsky et al. 1987, Odegard and Schatz 2006). To test if somatic hypermutation occurred *in vitro*, genomic DNA was extracted from sorted B cells of WT or *Rhog-/-* B1-8^{hi} mice after being stimulated with 1μm bead-bound or soluble NIP-OVA and cultured for seven days with OT2 T cells. B1-8V_H specific Ig sequences were amplified, subcloned in a PCR2.1 vector and introduced into DH5α strain of E.Coli to obtain individual clones. Each clone was sequenced and analysed to search for point mutations (**Figure 17**). B1-8^{hi} B cells have already introduced a point mutation (W33L) that confer them high affinity for NP, for that reason, their mutational rate *in vivo* is lower than in their counterpart B1-8^{lo} animals or non-transgenic B cells, which do not carry this mutation (Shih et al. 2002). Analysing the data obtained by clone sequencing, it was observed that WT B1-8 B cells cultured with phagocytic antigen had low nucleotide mutation rate ($3.17x10^{-4}$) but higher than non-stimulated cells ($4x10^{-9}$). Moreover, *Rhog-/-* B1-8 B cells stimulated with phagocytic antigen showed a strong defect in somatic hypermutation, in concordance with its poor ability to form GCs. Surprisingly, stimulation with soluble antigen also increased the mutational rate compared to non-stimulated cells although it did not reach the numbers obtained with phagocytic antigen, which were 1.5-fold higher, meaning that, indeed, soluble stimulus is also activating Aid.

These data indicate that *in vitro* cultures of naïve FO B and T cells develop somatic mutations although the rates do not reach the levels of hypermutation that occurs in immunized mice. Even though, phagocytic stimulus seems to be a better inducer of germinal center hallmarks than soluble antigen.

	no. sequences	no. bases	no. mutations	mutation rate (nucleotide) x10E-5	mutation rate (protein)
non-stim WT	116	50209	2	0.0004	0.000000006
beads WT	360	154607	49	31.7	0.0006
soluble WT	232	98725	21	21.3	0.0004
beads RhoG KO	47	20194	1	0.0049	0.0

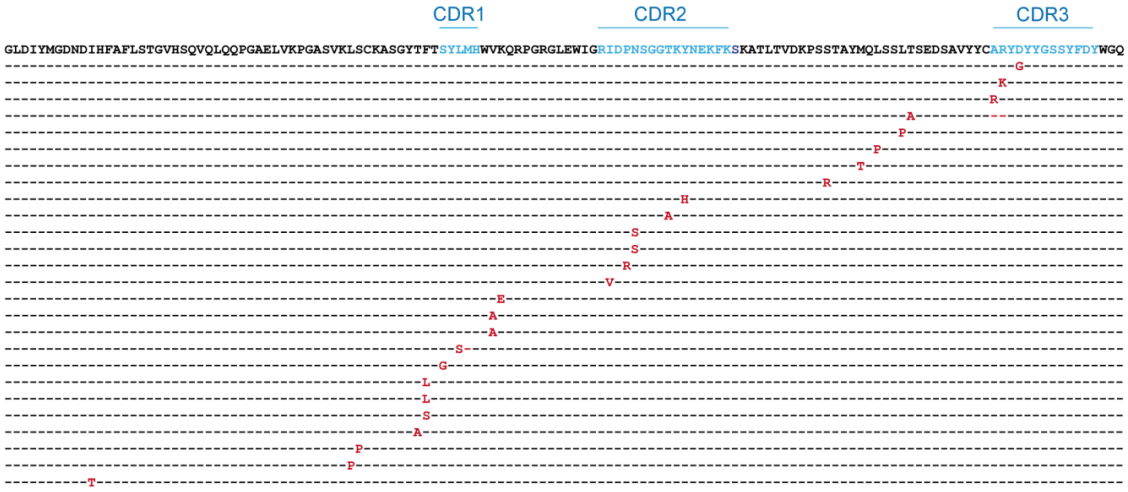

Figure 17. *In vitro* germinal center induces somatic mutations in the IgH locus in a phagocytic-dependent process. Upper table indicates the number of sequences and nucleotide bases studied in the four different conditions analysed: Naïve non-stimulated B cells from B1-8^{hi} mouse, WT B cells cultured for seven days with OT2 T cells and stimulated either with beads (beads WT) or soluble NIP-OVA (soluble WT), and *Rhog*-/- B cells cultured for seven days as WT cells and stimulated with NIP-OVA beads (beads *Rhog* KO). The number of mutations found in the analysed sequences allows us to calculate the mutational rate of nucleotides and amino acids. The amino acid mutations found in WT B cells stimulated with beads are represented in the lower graph that also shows the original sequence of Vh B1-8^{hi} B cells with the CDRs highlighted in blue.

2.4 Soluble antigen does not promote the *in vitro* differentiation of germinal center B cells to plasma cells

Germinal center B cells with higher BCR affinities differentiate into plasma cells, which are responsible for high-affinity antibody secretion. In order to study the importance of the form in which the antigen is given to cultured B cells for plasma cell differentiation, purified FO B cells from B1-8^{hi} mice were put in culture with OT2 T cells and stimulated with bead-bound (3:1 beads: B cell ratio) or soluble NIP-OVA (100ng/ml). Appearance of class-switched B cells was monitored at three different time-points by studying the expression of IgG1 in the membrane: IgG1+ IgD- population (**Figure 18a**). Phagocytic stimulus was necessary to induce class-switching. Moreover, emergence of IgG1+ B cells peaks after four days and decays

afterwards, which could be due to their differentiation to plasma cells. Using CD138 staining, it was noticed that B cells stimulated with bead-bound antigen begin to differentiate to plasma cells after four days of culture, in concordance with previous data, while soluble antigen was unable to induce this differentiation (**Figure 18b**).

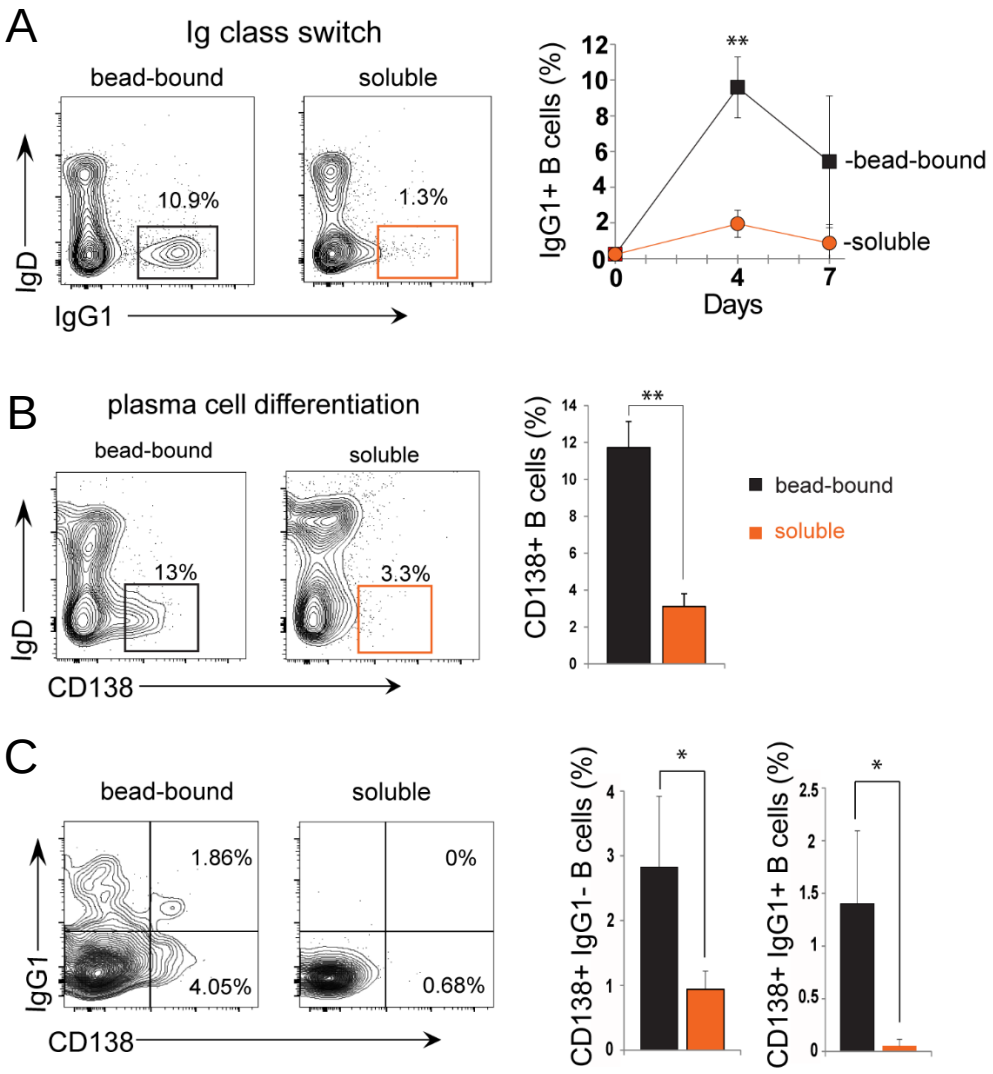

Figure 18. Soluble BCR stimulus does not promote GC B cells differentiation into plasma cells. (A) Cytometry plots show the expression of IgD and IgG1 in B220+ B cells from B1-8^{hi} mice cultured for four days with OT2 cells and bead-bound (3:1 beads:B cell ratio) (black) or soluble (100ng/ml) NIP-OVA (orange). Graph quantifies the percentage of class-switched IgG1 B cells at three time-points of incubation. Data represent the mean +/- S.D. (n=3) **p < 0.005 (Unpaired Student's t-Test). (B) Expression of CD138 and IgD in B cells (B220^{+}) after four days of culture as in (A). Graphs indicate the percentage of CD138+IgD- B cells. Data represent the mean +/- S.D. (n=3) **p < 0.005 (Unpaired Student's t-Test). (C) Expression of IgG1 and CD138 in B cells (B220^{+}) after seven days of culture as in (A). Graphs indicate the percentages of plasma cells (CD138+ IgG1-) and plasmablasts (CD138+IgG1+). Data represent the mean +/- S.D. (n=3) *p < 0.05 (Unpaired Student's t-Test).

After seven days of culture with OT2 T cells, B cells stimulated with bead-bound NIP-OVA show three different populations according to the expression of CD138 and IgG1: class-switched germinal center B cells (IgG1+ CD138-), plasmablast (IgG1+ CD138+) and plasma cells (IgG1- CD138+), while soluble-stimulated B cells do not perform either class-switch or differentiate to plasma cells (**Figure 18c**).

These data indicate the importance of the way how the BCR gets stimulated. Although the soluble stimulus is a good inducer of B cell activation, it is unable to induce Ig class-switch and terminal differentiation of GC B cells into plasma cells.

2.5 Generation *in vitro* of germinal center-like structures upon B cell stimulation with phagocytic antigen

Cultures of purified B cells and CD4 T cells stimulated with phagocytic antigen recapitulates most of the characteristics of *in vivo* germinal center: expression of surface markers distinctive of GC B cells and TFH cells, upregulation of transcriptions factors essential for the process, class-switch recombination, some levels of somatic hypermutation and finally, terminal differentiation of germinal center B cells into plasma cells.

Upon *in vivo* immunization, proliferating antigen-specific B cells form clusters that segregate from the non-responding B cells. After 3-4 days of naïve B and CD4 T cells co-culturing in the presence of phagocytic antigens, the generation of large cellular aggregates that could be seen even without microscope magnification was detected. Naïve FO B cells from non-tg mice and OT2 T cells stimulated with 1μm beads coated with anti-IgM+ovalbumin generate large clusters containing as many as 8000 cells (**Figure 19a**). The broad range of cluster cellularity could be in part produced by hand-manipulation during the transfer to coverslips for immunostaining. B and T cells distribution analysed by CD4 and B220 staining is not compartmentalized although it seems not to be completely random.

In order to study clustering differences between the phagocytic and soluble stimuli, FO B cells from B1-8^{hi} mice were stimulated with bead-bound (3:1 beads:B cell ratio) or soluble NIP-OVA (100ng/mL) and cultured with OT-2 T cell. Bead-bound antigen also induced the formation of large clusters of intermingled B and CD4 T cells (**Figure 19b**), while soluble antigen resulted in the formation of smaller clusters.

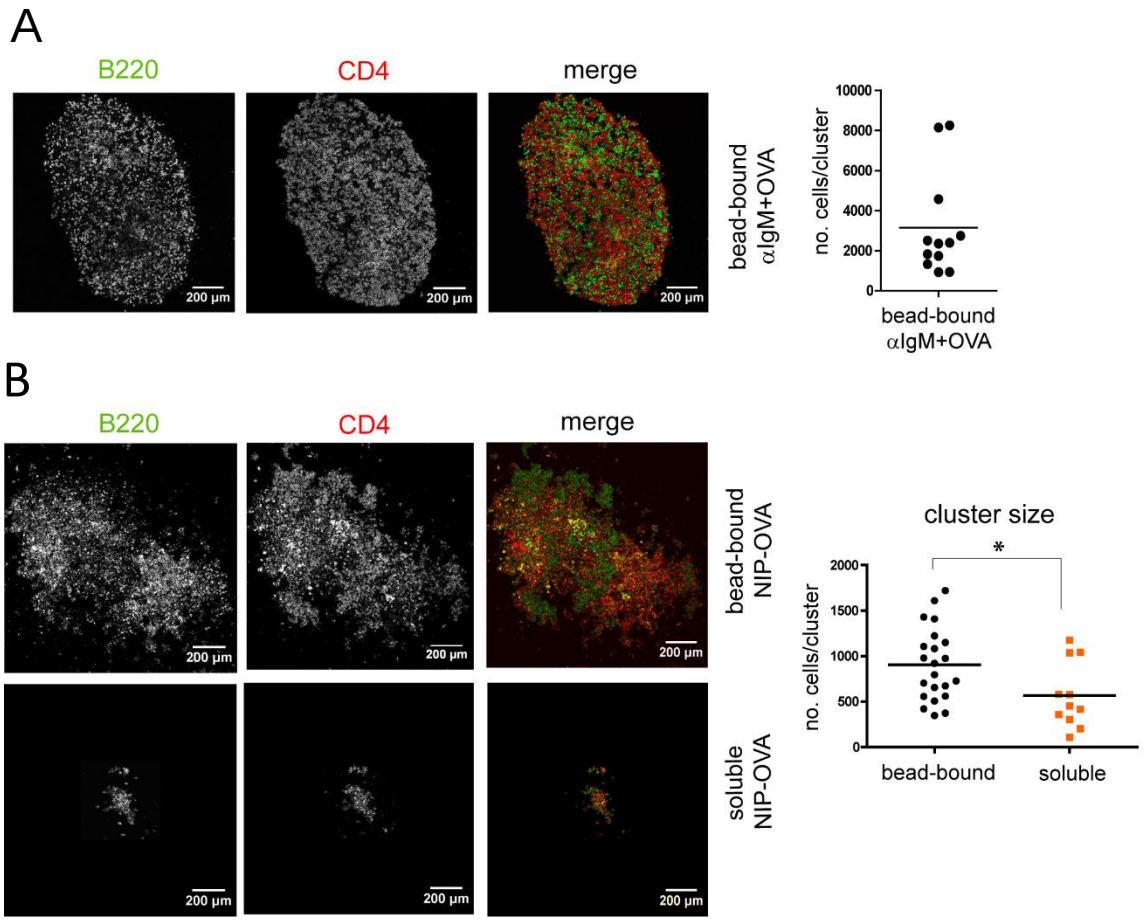

Figure 19. Generation of large cell clusters *in vitro* in the presence of bead-bound antigen. (A) Immunofluorescence section of a cell cluster after four days of culture of non-tg FO B cells with OT2 T cells and 1µm beads bound to anti-IgM+Ovalbumin. B cells were identified using B220 staining (green), OT2 T cells with CD4 (red), and total cellularity was calculated using DAPI staining. Graph shows the quantification of number of cells per cluster (n=12). (B) Immunofluorescence sections of cell clusters of FO B cells from B1-8^{hi} mice cultured for seven days with OT2 T cells and stimulated either with bead-bound NIP-OVA (upper panel) or 100ng/mL soluble NIP-OVA (lower panel). Staining and cellular quantification per cluster was performed as in (A). *$p < 0.05$ (Unpaired Student's T-Test).

Using mixtures of CTV-labelled B cells from B1-8^{hi} mouse and CFSE-labelled OT-2 T cells stimulated for seven days with bead-bound NIP-OVA, allowed to study the spatial distribution of proliferated cells (**Figure 20a**). Analysis of CTV and CFSE dilution in concentric regions from the center of the cluster determined that cultured cells proliferate and migrate towards the edges of the cluster. In the same way, expression of GL7 is also increased in those cells located more in the periphery, in agreement with the idea that the most proliferated B cells are those ones that have entered into the germinal center reaction and express GL7 (**Figure 20b**).

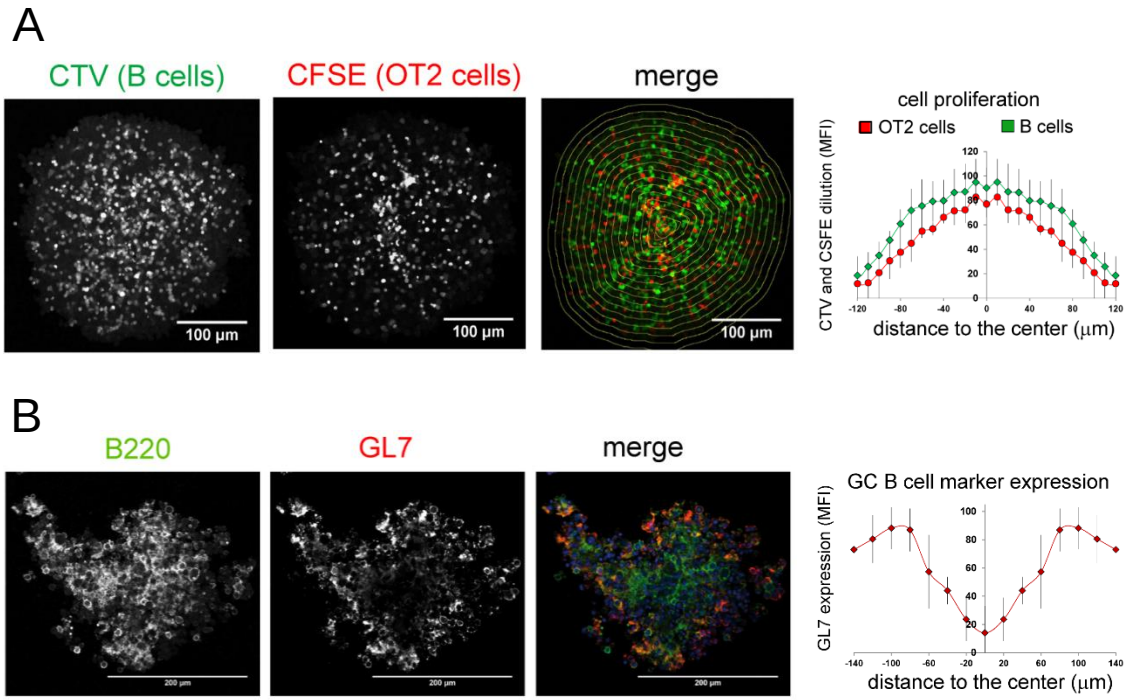

Figure 20. *In vitro* cellular clusters show huge proliferation in concordance with expression of germinal center markers. (A) Immunofluorescence of cellular cluster of CTV-labelled B cells (green) from B1-8^{hi} mouse and CFSE-OT2 T cells (red) cultured for seven days with bead-bound NIP-OVA. Yellow concentric circles show the delimitations analysed for CTV and CFSE expression normalized for the higher value, considered as 100. Graph indicates the fluorescence intensity of CFSE and CTV in those delimited areas. Data represent the mean +/- S.D. (n=5). (B) Immunofluorescence of a cellular cluster of B1-8^{hi} B cells and OT2 T cells culture as in (A) showing expression of GL7 as germinal center marker (red), distribution of B cells (green) and nucleus (DAPI in blue). Graph indicates the expression of GL7 along the cluster in delimited concentric regions Expression is normalized for the higher value (considered as 100). Data represent the mean +/- S.D. (n=5).

These data illustrate that B:T cell cultures of B cells stimulated with phagocytic antigen induce the generation of structures that could mimic *in vivo* GC.

2.6 Phagocytic antigens induce in-vitro Ig class-switch and the secretion of antigen-specific high affinity immunoglobulins

Key in GC reaction is the generation of high-affinity class-switched Igs against pathogens. Class-switched Igs have specific roles during pathogen removal such as neutralization, opsonization or complement's activation. There exist several protocols to perform B cell class-switch *in*

vitro, but none of them allow the production of antigen-specific immunoglobulins in a process that could mimic an *in vivo* GC (Nojima et al. 2011, Kato et al. 2012, Inagaki et al. 2013).

In order to assess if in-vitro germinal center B cells produce antigen-specific antibodies in a phagocytic-dependent manner, purified FO B cells from B1-8^{hi} WT and *Rhog-/-* mice were cultured for seven days with purified OT2 T cells and 1 µm beads bound to NIP-OVA. Supernatants were used to evaluate the production of low and high-affinity NP-reactive immunoglobulins by ELISA (**Figure 21**).

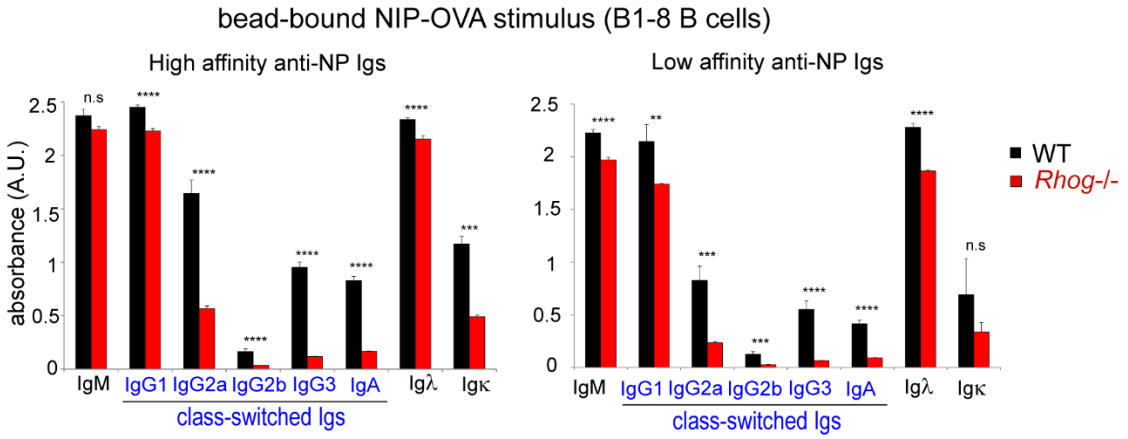

Figure 21. *In vitro* GC B cells secrete high-affinity antigen-specific immunoglobulins in a RhoG-dependent manner. ELISA quantification of high-affinity (NP(7)-BSA plate bound) or low affinity (NP(41)-BSA plate bound) Igs in supernatants obtained after seven days of culture of purified FO B cells from WT (black) or *Rhog-/-* (red) B1-8^{hi} mice with OT2 T cells and bead-bound NIP-OVA. Data represent the mean +/- S.D. (n=3) n.s $p > 0.05$; **$p < 0.005$; ***$p < 0.0005$; ****$p < 0.00005$ (Unpaired Student's t-Test).

A high production of anti-NP immunoglobulins was detected in those supernatants, which were comprised of IgMs but also of class-switched Igs: IgG1, IgG2a, IgG3 and IgA, and to a lesser extent, IgG2b. *Rhog*-deficient B cells show an important impairment in the generation of class-switched immunoglobulins suggesting that phagocytosis of antigen was required.

Likewise, when antigen-specific Ig secretion was compared between cultured FO B cells from B1-8^{hi} mouse stimulated with bead-bound and soluble NIP-OVA, it became clear that the soluble antigen is much less competent to induce the production of antigen-specific Igs than the phagocytic stimulus (**Figure 22**). Moreover, class-switched Igs were absent in the supernatants of soluble stimulated cells, in concordance with previous results of flow cytometry that show very few B cells class-switched to IgG1. Interestingly, the presence of

mature Igs was more evident in supernatants of B1-8^{hi} B cells stimulated for 7 days than for 4 days, indicating that the production of high-affinity class-switched Igs needs a temporal B cell maturation, coinciding with the expression of plasma cell markers (**Figure 12**) and with the pace of the GC response *in vivo*.

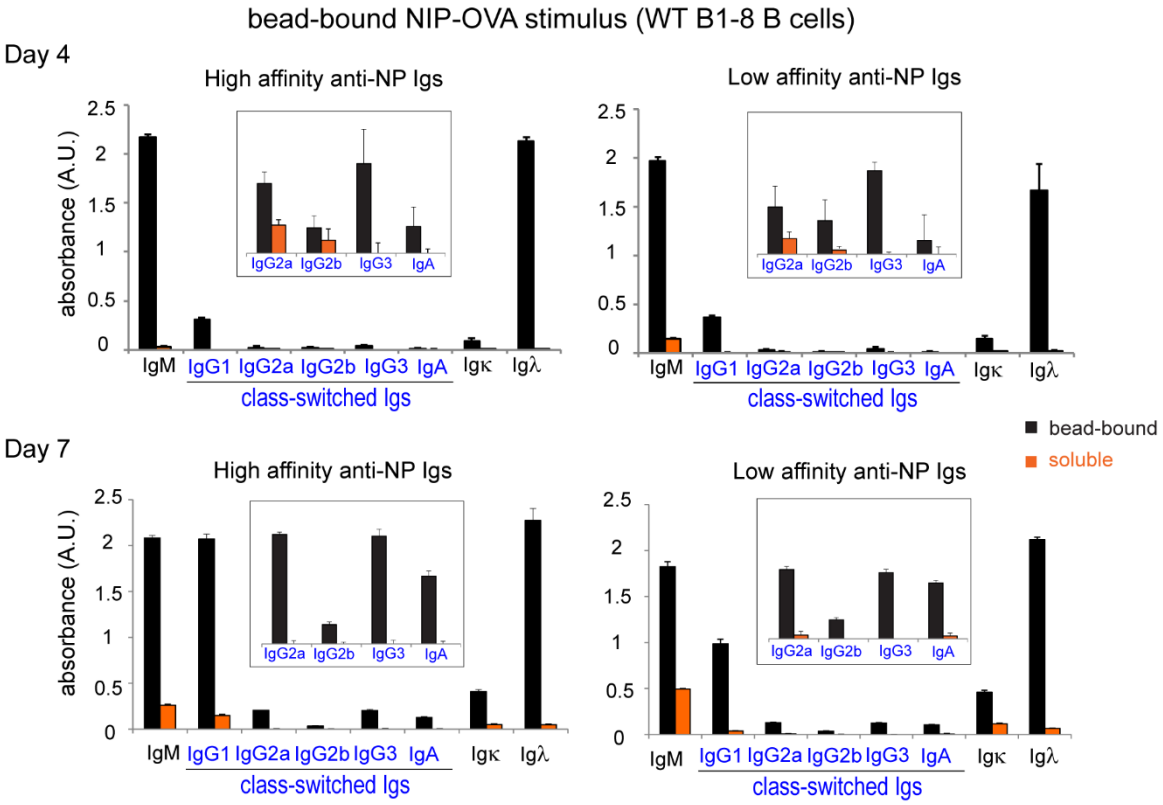

Figure 22. Antigen-specific Igs production *in vitro* is phagocytosis-dependent. Quantification by ELISA of high-affinity and low-affinity anti-NP immunoglobulins in the supernatants obtained after 4 (upper graphs) or seven days (lower graphs) of culture of FO B cells from B1-8^{hi} mice with OT2 T cells and bead-bound (black columns) or soluble NIP-OVA (orange). Data represent the mean +/- S.D. (n=3).

These data indicate that high-affinity antigen-specific immunoglobulin secretion by B cells in culture is due to specific BCR signalling produced during phagocytic antigen intake.

2.7 Naïve T and B lymphocytes are sufficient to generate GC *in vitro*

All in-vitro experiments had been performed purifying cells by negative selection, which allowed reaching 90% of cell purity. In order to exclude the participation of a third cell type that could be contaminating the cell populations, and to determine that FO B cells were the B

cells responsible for carrying out the GC reaction *in vitro*, cell culture experiment with sorted naïve FO B cells and CD4 T cells stimulated with bead-bound or soluble NIP-OVA stimulus was carried out (**Figure 23**).

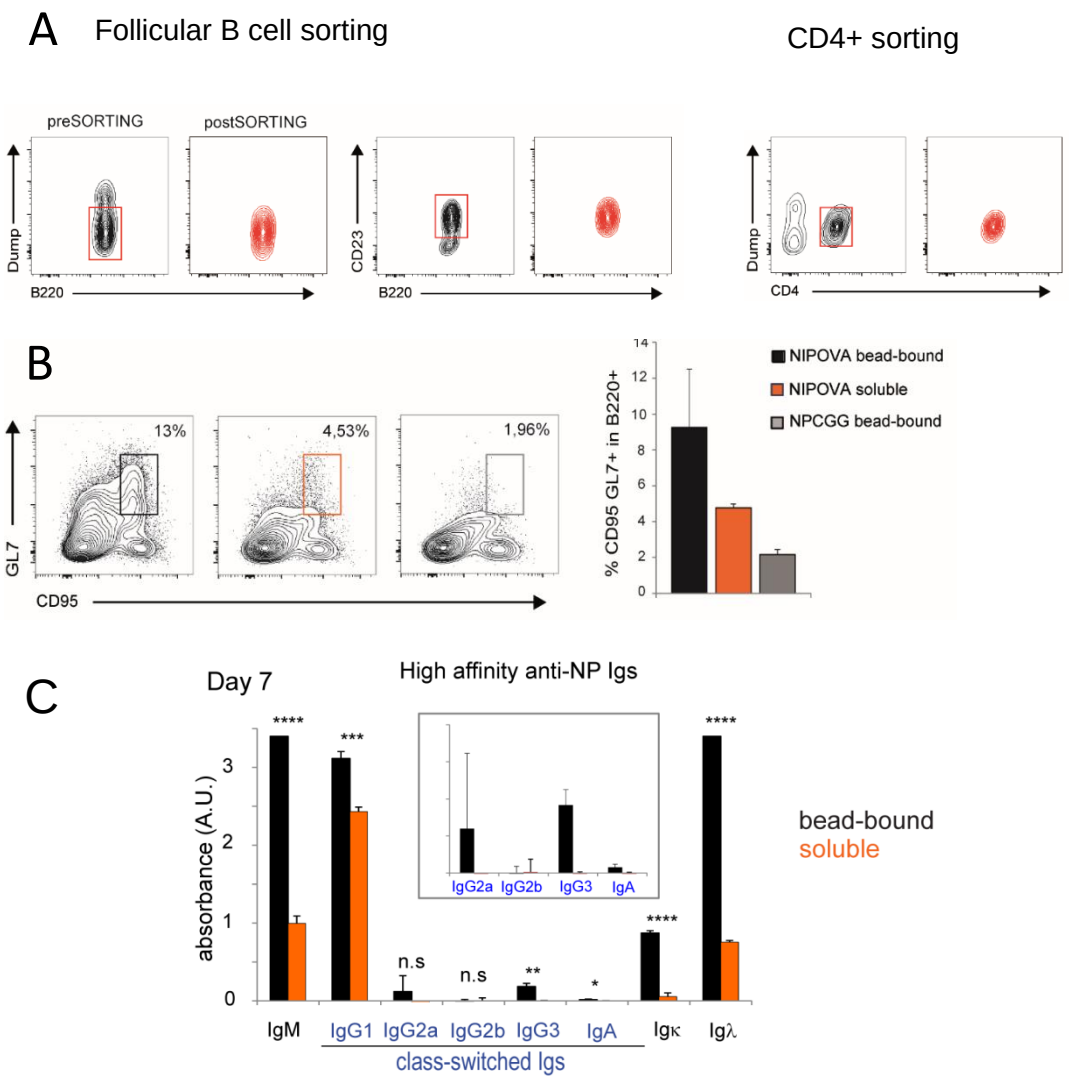

Figure 23. T and B cells are sufficient to induce *in vitro* germinal center cells and high-affinity Igs. (A) Cytometry plots indicate the gating strategy for sorting FO naïve B cells from B1-8^{hi} mouse and CD4 T cells from OT2 mouse. Left panels show the expression of Dump channel (CD43 and CD11b) and B220 in CD43-CD11b- splenocytes from B1-8^{hi} mouse generated by negative selection as well as the expression of CD23 within the gated B220+ B cells. Right panel shows expression of Dump channel (B220, CD11b, NK1.1, CD8, Ly6.6 and F4/80) and CD4 expression in all cells from OT2 lymph nodes negatively selected previously. Gates indicate the selected populations. (B) Cytometry plots show CD95 and GL7 expression after four days of culture of sorted FO B cells from B1-8^{hi} mice and CD4 T cells from OT2 mouse stimulated with bead-bound (black) or soluble (orange) NIP-OVA, or bead-bound NP-CGG (grey). Data represent the mean +/- S.D. (n=3). (C) ELISA quantification of high-affinity anti-NP immunoglobulins in supernatants of cultured cells (as in B) for seven days. Data represent the mean +/- S.D. (n=3) *p < 0.05; **p < 0.005; ***p < 0.0005; ****p < 0.00005 (Unpaired Student's t-Test).

Cells were first stained and negatively purificated using a cocktail of biotinylated antibodies: CD11b and CD43 to negatively select B cells from B1-8^{hi} mice; and CD11b, B220, CD8, NK1.1, F4/80 and Ly6.6 to negatively select CD4 T cells from OT2 mice. Afterwards, remaining cells were stained to be positively selected in the FACS sorter: B220+ CD23+ to purify follicular B cells and CD4+ to purify CD4 T cells, reaching a 99% of culture purity (**Figure 23a**). After four days of culture, cells were analysed for germinal center markers (**Figure 23b**). These data corroborate that only B and T cells are necessary to recreate a germinal center *in vitro* and that the phagocytic stimulus induces better differentiation than the soluble one. Supernatants of those sorted cells cultured for seven days confirm that antigen-specific high-affinity Igs are produced in the B:T cell culture with phagocytic stimulus since B cells stimulated with soluble antigen produce much less Igs (**Figure 23c**). Overall, these data indicate that B and T cells cultures stimulated with bead-bound antigen produce high-affinity antigen-specific class-switched immunoglobulins.

2.8 Immunoglobulins generated *in vitro* are functional

To interrogate if antigen-specific Ig could also be found in supernatants of non-transgenic B cell stimulated with therapeutic relevant antigens, purified FO B cells from non-tg C57BL/6 mice were stimulated with NIP-OVA+HIV fusion protein (p17/p24/gp120) covalently bound to 1 μm beads and cultured with purified OT2 T cells for 7 days. While NIP-OVA was used as a carrier to obtain OT2 help, HIV protein was used to produce antibodies against Env glycoproteins and test their neutralization effect during infection. Both proteins were bound covalently to the beads to increase the amount of protein per bead available. Since the initial number of antigen-specific B cells in non-immunized and non-tg mouse is predictably very low, we tried to extend the life of T+B cell cultures by supplementing them with IL-4 and IL21 after 5 days of culture and cultured them over 10 days as it has been described (Nojima et al. 2011). Supernatants were tested for antigen-specific Ig against NP and HIV protein using as control supernatants of cultured cells without bead-stimulus (**Figure 24**).

Antigen-specific IgMs against NP and HIV fusion protein were produced both in cultured cells for 7 days only stimulated with NIP-OVA+HIV beads and in cells cultured for 10 days and supplemented with Interleukins (**Figure 24a**). Nevertheless, class-switched Igs reactive against NP were only found in supernatants of cells supplemented with IL4 and IL21. These data show that cultures *in vitro* of non-tg B and OT2 cells can generate antigen-specific class-switched Igs.

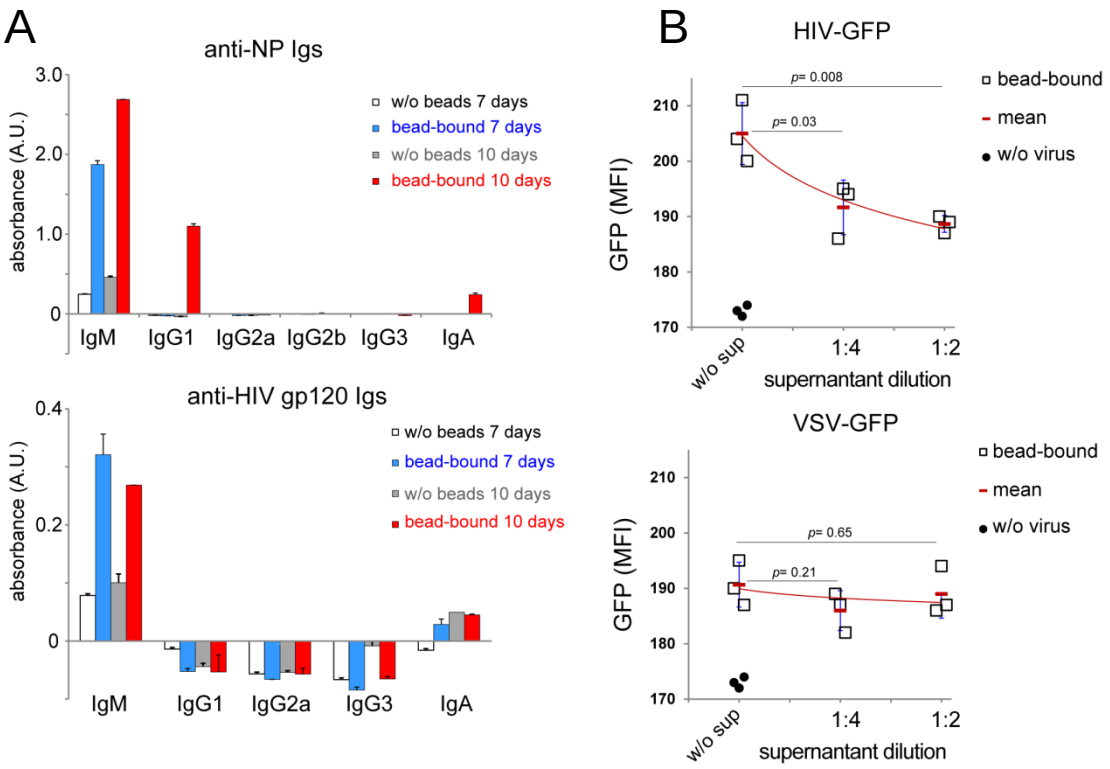

Figure 24. anti-Env HIV protein Igs generated *in vitro* have viral neutralizing effect. (A) Quantification of anti-NP and anti-HIV (p17/p24/gp120) fusion protein antibodies obtained from supernatants of cultured non-tg B cells and OT2 T cells stimulated or not with NIP-OVA+HIV fusion protein covalently bound to 1µm beads for seven or ten days. Cultured cells for ten days were previously supplemented with IL-4 and IL-21 at day 5. Data represent the mean +/- S.D. (n=3). (B) Quantification of GFP expression in MOLT-4 cells after 24 hours of infection with HIV Env-coated or VSV G protein-pseudotyped GFP-expressing virions incubated with two different dilutions of supernatants obtained after seven days of culture as in (A). Data represent the mean +/- S.D. (n=3) (Unpaired Student's T-Test).

In order to determine the functional relevance of the antibodies produced in culture, neutralizing experiments of HIV infection were conducted (**Figure 24b**). The MOLT-4 leukemic T cell line was infected either with GFP-expressing viral particles coated with either HIV or VSV (Vesicular stomatitis virus) envelopes. Supernatant obtained after seven days of culture and previously tested for ELISA was used for the neutralization assay. Culture supernatant was incubated at two different dilutions with the viral supernatants for 60 minutes at 37º C prior to the infection of MOLT-4 cells. Viral infection was analysed after 24 hours by flow cytometry tracking GFP expression in those cells. The supernatant inhibited in a dose-dependent manner the entry of HIV Env-coated virus but not the VSV G-coated one, demonstrating the neutralization effect and specificity of the antibodies produced in the *in vitro* cultures.

3. Phagocytic antigen stimulation induces a stronger and more sustained BCR signal than soluble stimulus

B cell stimulation with soluble antigen has long been used to study signalling pathways downstream the BCR. However, it has been proposed that the predominant form of antigen encounter by B cells *in vivo* is associated to membranes and in a particulate form (Carrasco and Batista 2006, Depoil et al. 2008). Moreover, the activation threshold necessary for BCR stimulation is lower when the antigen is associated to membrane than in a soluble manner (Batista et al. 2001, Tolar et al. 2009). To provide a mechanistically explanation to the different B cell response produced by soluble or bead-bound stimuli in *in vitro* germinal center recreation, it was decided to study the early BCR events that take place after antigen-mediated stimulation.

3.1 Bead-bound antigen provokes stronger actin remodeling at lower BCR occupancy than soluble stimulus

First of all, it was determined the grade of BCR occupancy using a fluorescent NP-derivate in FO B1-8^{hi} B cells (negatively selected: CD43- CD11b- IgL kappa-) incubated for one hour on ice with either different doses of bead-bound NIP-OVA or its soluble form (**Figure 25a**). The soluble concentration used for in-vitro GC cultures (100ng/ml) reached almost 100% BCR occupancy, while the bead-bound stimulus (3:1 beads: B cell ratio) only 66%, indicating that the soluble antigen interacts with more BCRs than the bead-bound dose. Moreover, both stimuli produce similar BCR downregulation when B1-8^{hi} B cells were stimulated at 37ºC for different times (**Figure 25b**). These data indicate that the bead-bound antigen is not more effective in BCR occupancy and BCR downregulation than the soluble one, so the better production of high-affinity class-switched immunoglobulins could not be justified by a better BCR occupancy or higher dose of antigen.

Phagocytosis requires the rearrangement of actin cytoskeleton around the particle in the phagocytic cup (Yuseff et al. 2013). Consequently, it was studied the intensity of actin polymerization upon stimulation with soluble and phagocytic antigen. B1-8^{hi} B cells were stimulated with both types of antigen at different time-points and stained with phalloïdin, which binds to polymerized actin (**Figure 25c**). Both kinds of stimuli induced an early actin polymerization, but bead-bound antigen induces a more sustained polymerization than the soluble one. Indeed, stimulation with soluble antigen lead to an early polymerization phase

followed by a rapid depolymerization. These data suggest that phagocytic antigen elicits an extended actin polymerization.

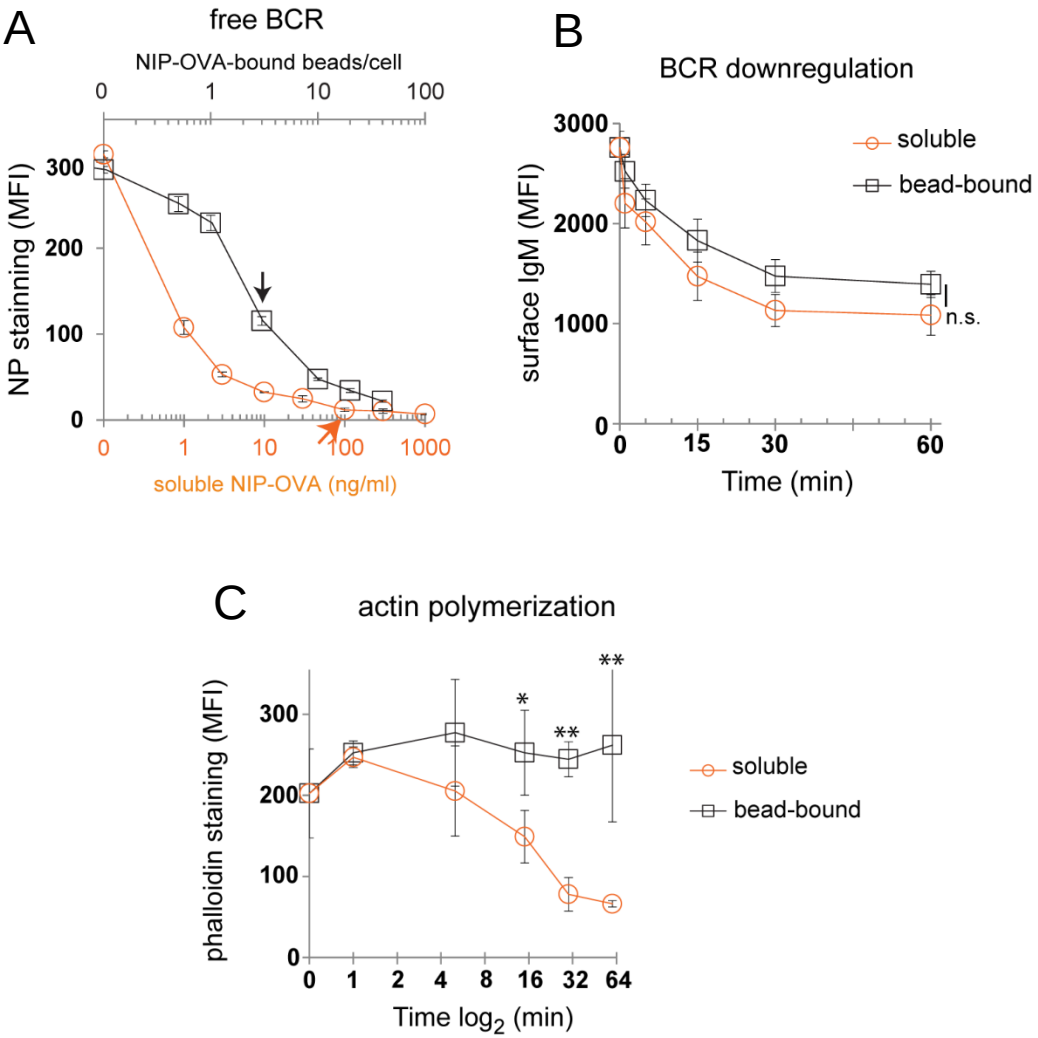

Figure 25. Bead-bound antigen elicits a stronger actin polymerization than soluble antigen but equal BCR downmodulation at lower BCR occupancy. (A) Measurement of BCR occupancy staining with fluorescent NP-reagent FO B1-8^{hi} B cells previously incubated on ice with different concentrations and doses of soluble (orange) and bead-bound NIP-OVA (black) for one hour. Arrows indicate the doses of soluble (100ng/ml) and bead-bound (3:1 ratio) NIP-OVA used in culture. Data represent the mean +/- S.D. (n=3). (B) Graph shows BCR downregulation in FO B1-8^{hi} B cells stimulated with soluble (100ng/ml) and bead-bound (3:1 ratio) NIP-OVA at different time-points at 37ºC. Cells were stained late with a surface anti-IgM antibody. Data represent the mean +/- S.D. (n=3) (Unpaired Student's t-Test). (C) Graph indicates phalloïdin mean fluorescence intensity (MFI) in B1-8^{hi} B cell stimulated as in (B). Data represent the mean +/- S.D. (n=3) *p < 0.05; **p < 0.005 (Unpaired Student's t-Test).

3.2 Bead-bound antigen induces stronger and more sustained BCR signaling due to a phagocytic-dependent process

In order to study early BCR activation events, purified B1-8^{hi} B cells were stimulated with soluble (100ng/ml) or NIP-OVA bead-bound (3:1 ratio beads:B cell) for different early time-points. Immunoblot of cell lysates allowed us to analyse the activation of Akt, Erk and Syk molecules downstream of the BCR (**Figure 26**). Bead-bound antigen induces a stronger and more sustained phosphorylation of Erk and Akt, being the latter linked to PI3K pathway which has also been related with RRas2 and RhoG activation in T cells (Martinez-Martin et al. 2011). Moreover, Syk phosphorylation, which has been shown to mediate FCγR-dependent phagocytosis (Crowley et al. 1997), is also more intense and sustained in B cells stimulated with bead-bound antigen. These data suggest that the bead-bound stimulus induces a stronger and more sustained BCR activation than the soluble antigen.

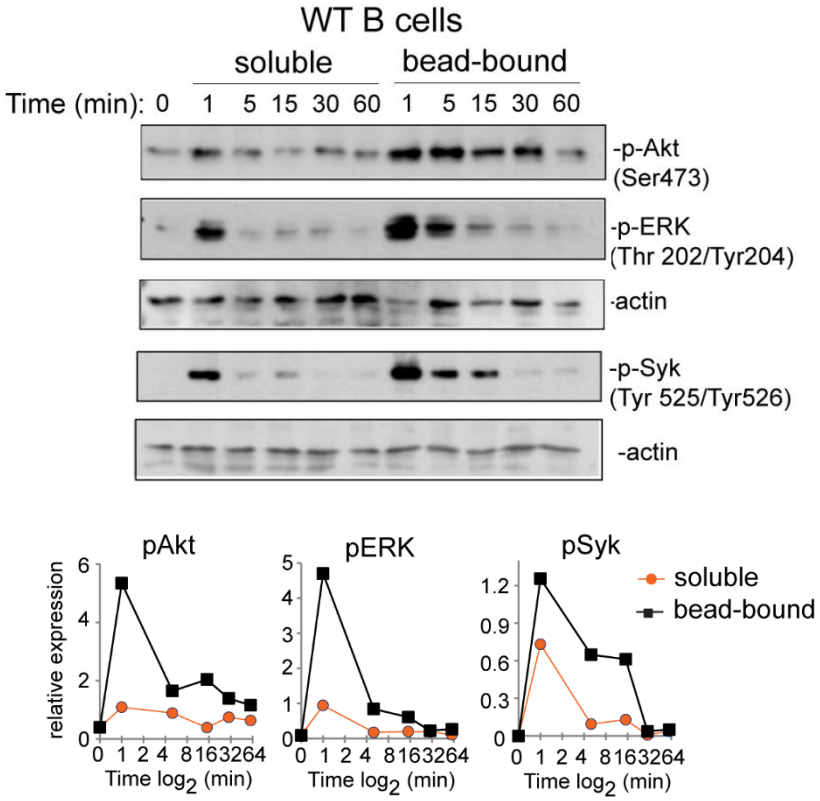

Figure 26. Bead-bound antigen induces a stronger and more sustained BCR signal than soluble antigen. Immunoblots show the phosphorylation of Akt, Erk and Syk in purified B1-8^{hi} B cells stimulated with soluble (100ng/ml) or beads-bound (3:1 beads:B cell ratio) NIP-OVA at different time-points. Actin was used as a loading control. Quantifications of phosphorylated proteins are represented in the graphs.

In order to test if this stronger BCR signalling observed in bead-bound stimulated B cells was related to a phagocytic process, it was also analysed in *Rhog*-deficient B cells (**Figure 27**). WT and *Rhog-/-* B1-8^{hi} B cells were stimulated at different time points with bead-bound NIP-OVA (3:1 beads:B cell ratio). Activation of PI3K kinase was also studied in this case analysing the phosphorylation of Akt and S6 downstream effectors. It was found more activated in stimulated WT than in *Rhog*-deficient B cells, as well as Erk activation. Phosphorylation of Syk was also RhoG-dependent since its induction was lower in those cells. This data suggests that phagocytic stimulus elicits a longer and more sustained BCR signal.

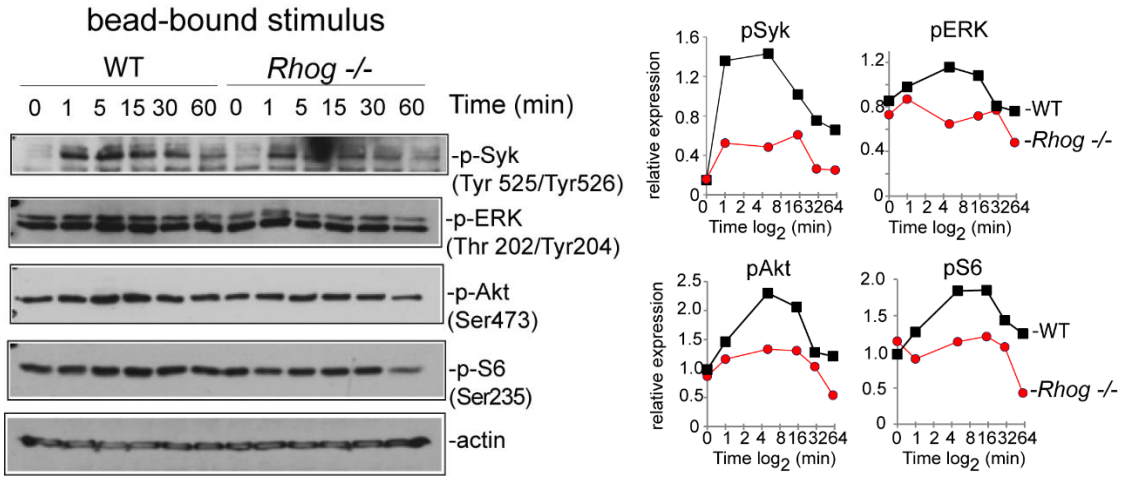

Figure 27. Bead-bound antigen induces a strong BCR signal by a RhoG-dependent mechanism. Immunoblots show phosphorylation of Syk, Erk, Akt and S6 in WT and *Rhog-/-* B1-8^{hi} B cells stimulated with bead-bound NIP-OVA for different time-points. Phosphorylation levels normalized for actin are represented in the graphs.

Confocal microscopy was used to assess the cellular localization of the active BCR during bead-bound stimulation. B1-8^{hi} B cells were stimulated with NIP-OVA covalently bound to fluorescent 1µm beads for 5 and 30 minutes in order to analyse BCR-early activation events during cup formation and after beads intake (**Figure 28**). After 5 minutes of stimulation, phosphorylation of Igα and Syk took place in the membrane cup where the beads were bound. Interestingly, BCR phosphorylation still persisted after 30 minutes and both proteins were found phosphorylated all around the phagocytosed beads. This result indicates that BCR signalling persist in intracellular phagosomes which could be responsible for the sustained signalling.

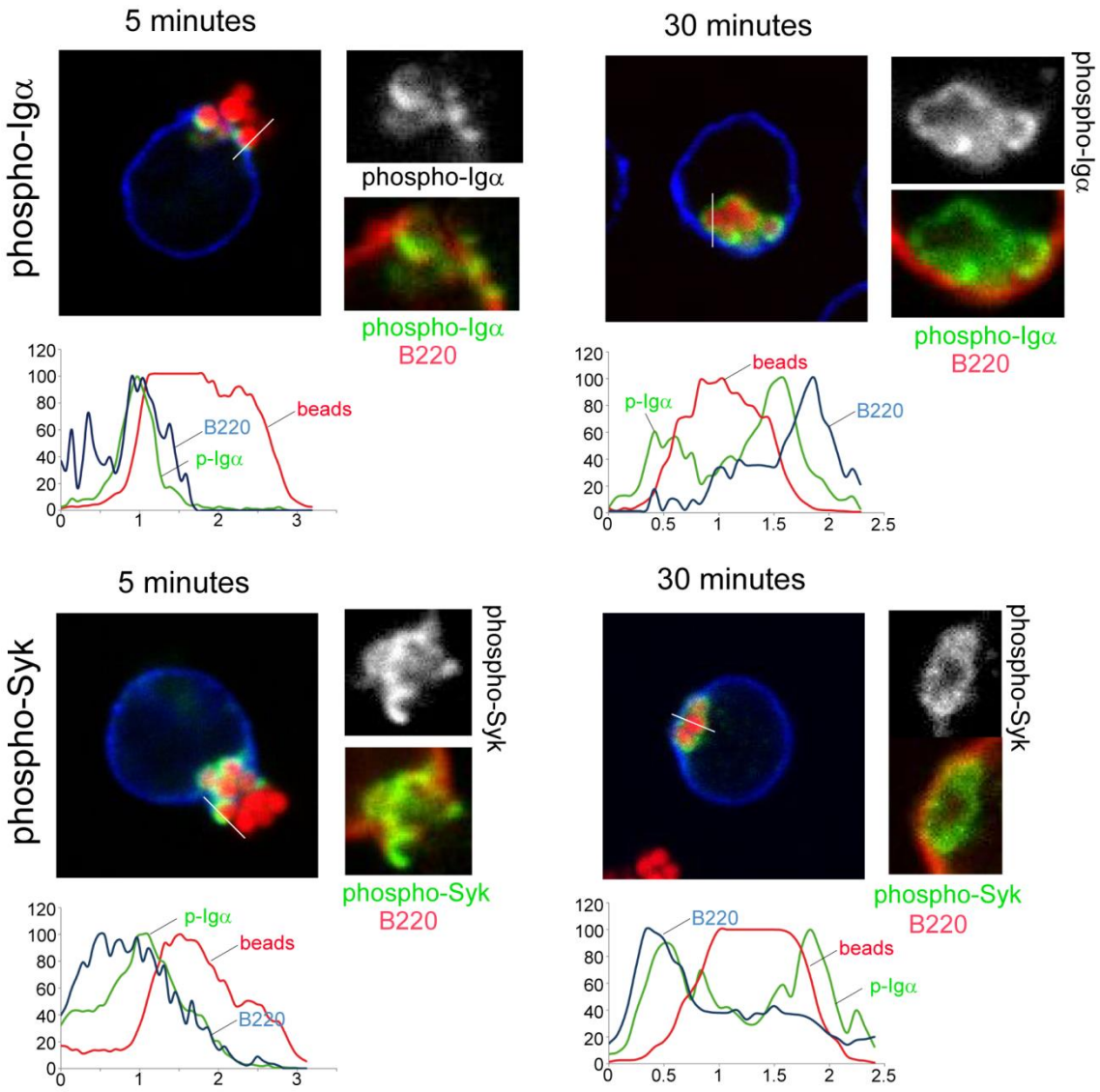

Figure 28. BCR signaling takes place during the phagocytic cup formation and persists in intracellular phagosomes. Confocal microscopy images show phosphorylation of Igα chain and Syk upon bead-bound NIP-OVA stimulation of purified B1-8^{hi} B cells for 5 and 30 minutes. The B220 B cell marker is shown in blue, the beads in red and phosphorylation of Igα and Syk in green. Histograms overlays show the expression of the three colours along the white lines drown in the main images. Details of bead-contact area are shown in the enlarged pictures where phosphorylation of Igα is shown in green and B220 in red.

4. B cell phagocytosis of antigens stimulates the humoral response in-vivo

In vivo germinal centers are generated when antigen gets access to the secondary lymphoid organs. The current paradigm of how antigen is sensed by follicular B cells is that it has to be presented on the membrane of APCs such as follicular dendritic cells (FDCs) from where the antigen-specific B cells acquire, degrade and present it to T cells. Although several years ago it was thought that soluble antigen could gain access to the center of the follicle to be directly acquired by FO B cells, a growing number of reports show that at most times the antigen arrives presented by other cell populations located between the afferent lymphatic and blood vessels and the inner follicle. Nonetheless, the presence of micron-sized intracellular bacteria has been found in B cells, which are processed and can be presented to T cells (Gao et al. 2012).

4.1 Splenic B lymphocytes phagocytose bead-bound antigens in a BCR- and RhoG-dependent manner

Once it was shown that follicular B cells can phagocytose 1 and 3 μm particles inducing the generation of germinal center structures *in vitro*, we wonder if that process could also be produced *in vivo* and its possible function and relevance. First of all, in order to study if B cells could phagocytose beads *in vivo* in a BCR-dependent manner, NIP-OVA covalently bound to 1 μm fluorescent beads was injected intraperitoneally into WT and *Rhog-/-* B1-8^{hi} mice. After five hours spleens were harvested for flow cytometry analysis. To distinguish cells that have phagocytosed beads from others with beads still attached to the membrane, splenocytes were stained extracellularly with a fluorescent antibody against ovalbumin. Using as a control B cells incubated on ice with beads (**Figure 29a**), it was possible to designate those B cells that have beads attached to the plasma membrane. B1-8^{hi} mice have only a small percentage of NP-reactive B cells, taking advantage of that, we could compare within the same animal the BCR-dependency of bead uptake. B cells with beads inside (negative for fluorescent anti-Ovalbumin) were NP+, while almost no B cells with a non-reactive BCR for NP (non-NP+) have phagocytosed beads, indicating that this acquisition is BCR-mediated (**Figure 29b**). *Rhog-/-* B cells phagocytose 3 folds fewer beads than WT cells, corroborating the data obtained *in vitro* and suggesting that B cells can also phagocytose beads *in vivo* through their BCR in a RhoG-dependent mechanism.

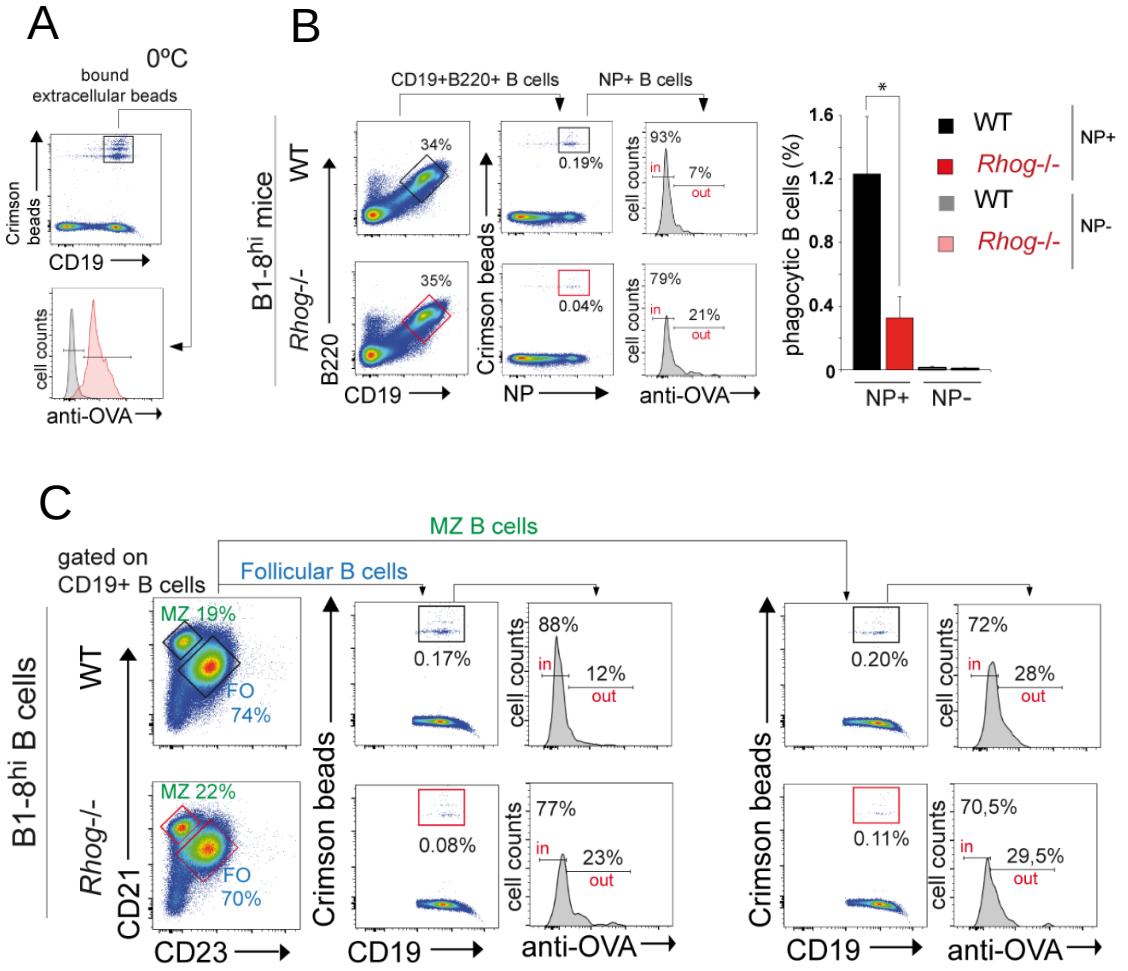

Figure 29. B cells phagocytose bead-bound antigens *in vivo*. (A) Cytrometry plots show WT splenic cells from B1-8^{hi} mice incubated at 0ºC for 1 hour with fluorescent (Crimssom) 1µm NIP-OVA beads and subsequently stained with (red histogram) or without (grey histogram) a fluorescent anti-ovalbumin antibody. (B) Cytometry panel shows the *in vivo* internalization of Crissom 1µm NIP-OVA beads in WT (black;grey) and *Rhog-/-* (red;pink) B1-8^{hi} mice. After five hours of intraperitoneal injection of NIP-OVA beads, splenocytes of these two groups of animals were analysed. Within total B cells (CD19+ B220+), NP-reactive B cells positive for NIP-OVA beads were analysed for the presence of internalised beads (in) or still attached to the membrane (out), using anti-ovalbumin staining. Bar graph shows the quantification of phagocytosed beads in the NP+ B cells and in the non-specific B cells (NP-). Data represent the mean +/- S.D. (n=3) *p < 0.05 (Unpaired Student's t-Test). (C) Cytometry plots show the NIP-OVA bead-bound acquisition by MZ (CD21hi CD23-) and FO (CD21med CD23+) CD19+ B cells from WT and *Rhog-/-* B1-8^{hi} mice immunized as in (B). B cells with Crissom beads were analysed for the phagocytosis of this beads (in and out histograms). Quantification of the phagocytosis is shown in the lower bar graph. Non-tg WT mouse was used as a control. Data represent the mean +/- S.D. (n=3) *p < 0.05 (Unpaired Student's t-Test).

With the purpose of distinguish which subtypes of splenic B cells were able to phagocytose beads, marginal zone (MZ) and follicular (FO) cells were distinguished according to the expression of CD21 and CD23 (**Figure 29c**) and a non-tg mouse was used as a control for BCR specificity. Both subtypes of B cells phagocytosed beads with a similar efficiency. Moreover, *Rhog-/-* MZ and FO cells showed also a defect in the acquisition of beads, while non-tg B cell subtypes did not almost have intracellular beads, indicating that in both populations the phagocytosis is BCR-driven.

The percentage of B cells that had phagocytosed beads was comparable to populations of 'professional' phagocytes such as macrophages and dendritic cells. These subpopulations were analysed in WT and *Rhog-/-* B1-8^{hi} animals and in non-tg C57BL/6 (**Figure 30**). While CD11b^{low} F4/80+ dendritic cells are very inefficient in the acquisition process, splenic CD11b+ F4/80+ macrophages have better phagocytic activity but still lower than BCR-reactive B cells (1.5 vs 0.05%), suggesting that antigen-specific B cells have a comparable phagocytosis efficiency than professional phagocytes.

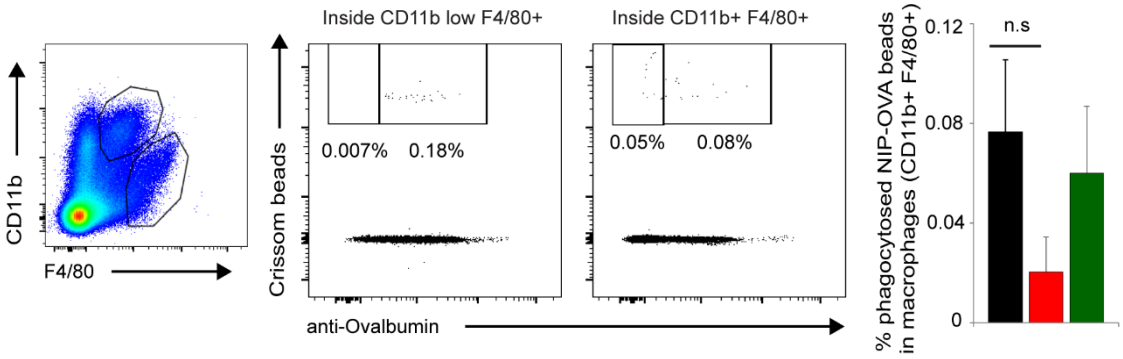

Figure 30. Beads phagocytosis by spleen macrophages is lower than that of antigen-specific B cells. Cytometry plots show fluorescent NIP-OVA bead-bound acquisition in DC (CD11b^{low} F4/80^{+}) and macrophages (CD11b^{+} F4/80^{+}). WT (black) and *Rhog-/-* (red) B1-8^{hi} mice and non-tg C57BL/6 mouse (green) were immunized with fluorescent beads covalently bound to NIP-OVA as in Figure 29. Dendritic cells and macrophages were analysed for the presence of internalized (negative for anti-Ovalbumin) or still external (positive for anti-Ovalbumin) beads. Quantification of beads phagocytosis in macrophages is shown in the graph. Data represent the mean +/- S.D. (n=3) n.s $p > 0.05$ (Unpaired Student's t-Test).

4.2 Antigen phagocytosis by B cells plays an important role during humoral response *in vivo*

Once it was verified that B cells phagocytose antigen-coated beads *in vivo*, we aimed to test its relevance during the humoral response. First, WT and *Rhog-/-* mice were immunized intraperitoneally with NIP-OVA covalently bound to 1µm beads in order to study germinal

center generation (**Figure 31**). WT mice developed GC B cells after seven days of immunization with beads while this B cell response was less effective in *Rhog-/-* mice (3-fold less), suggesting the relevance of B cell phagocytosis during germinal center reaction.

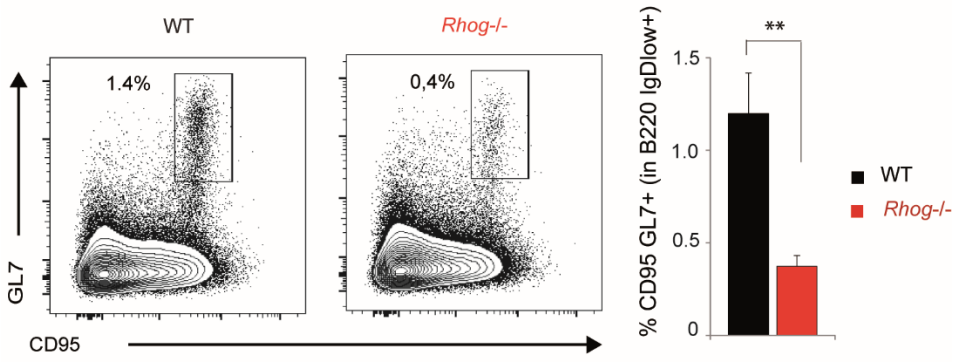

Figure 31. *Rhog*-knock out mice have defective GC B cell differentiation upon immunization with bead-bound antigen. Cytometry plots show the expression of GC markers (CD95 and GL7) in CD19+ B cells from WT (black) and *Rhog-/-* (red) mice immunized intraperitoneally with 1μm beads covalently bound to NIP-OVA. Gates show the considered GC positive population which is quantified in the right bar graph. Data represent the mean +/- S.D. (n=3) **p < 0.005 (Unpaired Student's t-Test).

The implication of RhoG in phagocytic processes had already been described in other cell subtypes as macrophages and T cells (Martinez-Martin et al. 2011, Tzircotis et al. 2011) . Since *Rhog-/-* mice are full knock-out animals, to verify that the defect in germinal center formation after 1μm beads immunization was B-cell intrinsic and not caused by deficiencies in other cell types, a B cell transfer experiment was carried out (**Figure 32**). Purified WT or *Rhog-/-* B cells from B1-8^{hi} mice, that express the CD45.2 allele, were injected intravenously into acceptor CD45.1+ C57BL/6 mice. Mice were immunized with NIP-OVA covalently bound to 1μm beads and their spleens were analysed 7 days later. Congenic markers CD45.2 and CD45.1 allowed to distinguish donor from acceptor B cells. The percentages of transferred B cells recovered after 7 days were similar for the WT and *Rhog-/-* genotypes. However, *Rhog*-deficient B cells showed a strong defect in GC marker expression. Possible differences during the immunization could not be responsible for the different response between WT and *Rhog-/-* B cells because endogenous CD45.1+ B cells from the two animal groups developed a similar germinal center response (**Figure 32** green bar graph). These data illustrate that B cell phagocytosis of antigens induces a potent humoral response in a process dependent of RhoG.

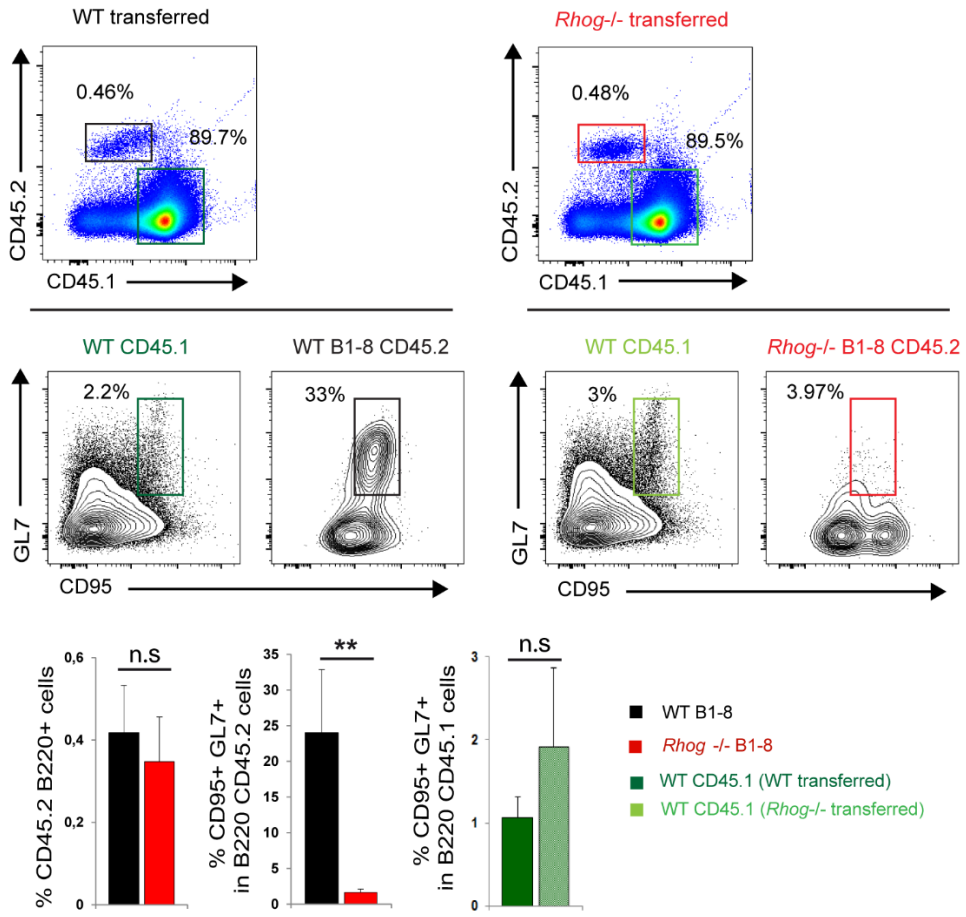

Figure 32. The *in vivo* defect in GC formation after administration of bead-bound NIP-OVA is B cell intrinsic. Cytomery plots show the presence of endogenous (CD45.1+) and transferred (CD45.2+) B cells in CD45.1+ mice inoculated with WT (black) or *Rhog-/-* (red) B cells from B1-8^{hi} mice and subsequently immunized with NIP-OVA covalently bound to 1μm beads. After 7 days the presence of GC markers (CD95+GL7+) inside both B cell populations in each group of mice was analyzed. Quantifications of gated populations are shown in the lower bar graphs. Data represent the mean +/- S.D. (n=3) **p < 0.005 (Unpaired Student's t-Test).

4.3 Alum-based vaccinations are potent inducers of humoral response due to elicit B cell antigen phagocytosis

At present, vaccination protocols do not include the use of antigens bound to spheres, for that reason we aimed to test the *in vivo* relevance of B phagocytosis in a more common system of immunization and vaccination. Aluminium-based adjuvants are commonly used in human vaccines to elicit a potent humoral response (Ghimire 2015). As a first approach to test if phagocytosis by B cells of alum-antigen complexes is behind the efficacy of alum adjuvant, WT and *Rhog-/-* mice were immunized with NIP-OVA protein complexed with Alum. There were

not statistically significant differences in terms of GC marker expression (**Figure 33a**). Nonetheless, when the production of high-affinity immunoglobulins against NP was analysed in sera of WT and *Rhog-/-* mice immunized with NIP-OVA embedded in Alum (particulate) or in PBS (soluble), it was noticed that *Rhog-/-* mice have a defective production of some class-switched high-affinity immunoglobulins in response to antigen+Alum immunization (**Figure 33b**). *Rhog-/-* mice produce less high-affinity IgG2b and IgG3 than WT animals when were immunized with Alum, but not with soluble antigen, in concordance with the data published by Vigorito and colleagues (Vigorito et al. 2004). These data suggest that RhoG-dependent phagocytosis mechanism is involved in the humoral response to antigen+Alum immunization.

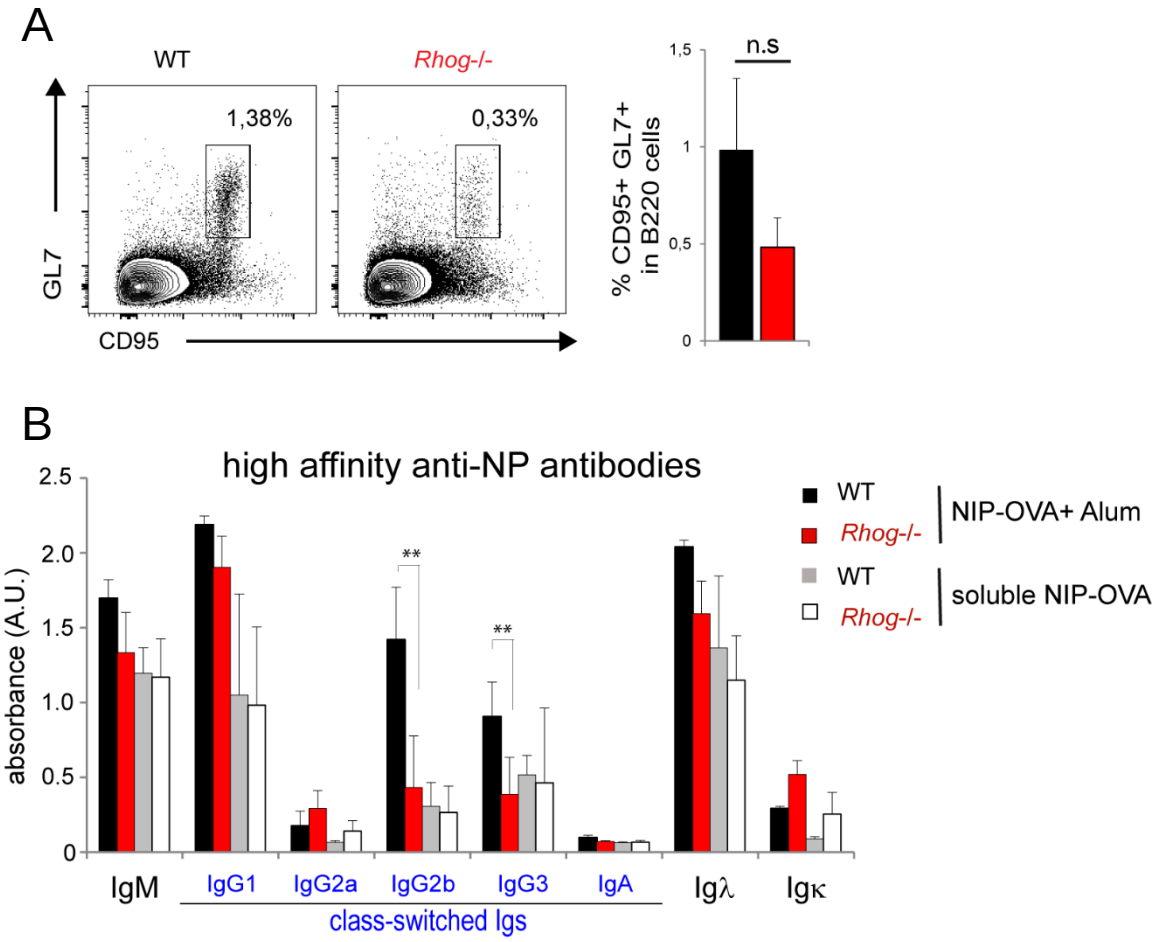

Figure 33. RhoG-deficient mice show impaired humoral response to NIP-OVA Alum-immunization. (A) Cytometry plots show the expression of CD95 and GL7 germinal center markers in splenic B cells (B220+) from WT (black) and *Rhog-/-* (red) mice 7 days after immunization with NIP-OVA plus Alum complexes. Gated populations (CD95+GL7+) were quantified in the bar graph. Data represent the mean +/- S.D. (n=3) n.s $p > 0.05$ (Unpaired Student's T-Test). (B) ELISA quantification of high-affinity anti-NP immunoglobulins in sera of WT (black;grey) and *Rhog-/-* (red;white) mice 20 days after the first immunization with NIP-OVA complexed with Alum (particulate) or alone (soluble). Mice were reimmunized at day 14. Data represent the mean +/- S.D. (n=3) ** $p < 0.005$ (Unpaired Student's T-Test).

To determine if the defective humoral response to Alum plus antigen immunization was B cell intrinsic, an adoptive transfer experiment was carried out. Purified WT and *Rhog-/-* B cells from B1-8^{hi} mice expressing the CD45.2 allele were inoculated into acceptor C57BL/6 CD45.1+ mice and subsequently immunized with NIP-OVA complexed with Alum. One week later it was noticed that transferred *Rhog-/-* B cells had an impaired GC marker expression response compared with their WT counterpart cells, while endogenous CD45.1+ B cells from both groups of mice responded similarly (**Figure 34a**).

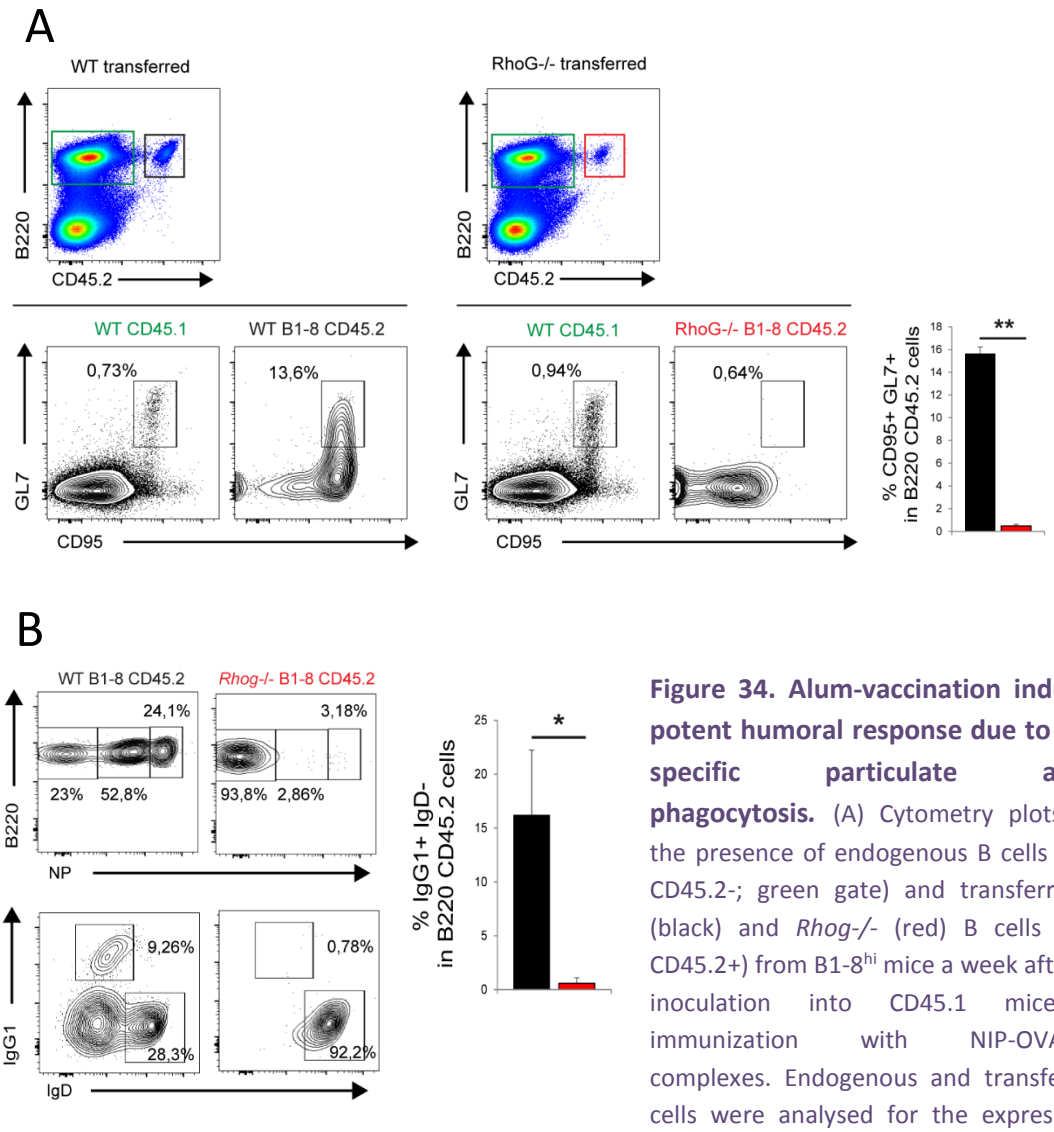

Figure 34. Alum-vaccination induces a potent humoral response due to B cell-specific particulate antigen phagocytosis. (A) Cytometry plots show the presence of endogenous B cells (B220+ CD45.2-; green gate) and transferred WT (black) and *Rhog-/-* (red) B cells (B220+ CD45.2+) from B1-8^{hi} mice a week after their inoculation into CD45.1 mice and immunization with NIP-OVA+Alum complexes. Endogenous and transferred B cells were analysed for the expression of germinal center markers (CD95+GL7+). Quantification of CD95+GL7+ population in transferred B cells is shown in the bar graph. Data represent the mean +/- S.D. (n=3) ** $p < 0.005$ (Unpaired Student's T-Test). (B) Cytometry plots show the presence of NP-reactive (upper panels) and class-switched IgG1+ IgD- (lower panels) by transferred WT and *Rhog-/-* B220+ B cells in response to NIP-OVA+Alum immunization (as in A). Bar graph shows the quantification of IgG1+ class-switched cells in transferred B cells. Data represent the mean +/- S.D. (n=3) * $p < 0.05$ (Unpaired Student's t-Test).

Moreover, expansion of NP-binding B cells was also affected by the absence of RhoG, as well as the expression of class-switched IgG1 cells (**Figure 34b**). These results indicate that Alum-immunization boost is produced by B cell specific antigen phagocytosis in a RhoG-dependent process.

In order to assess the B cell-specific defect in antibody production previously detected in WT and *Rhog-/-* mice, an adoptive cell transfer experiment in *Rag1-/-* recipient mice, which lack T and B cells, was performed. *Rag1-/-* mice were inoculated with purified B cells from WT or *Rhog-/-* B1-8^{hi} mice and purified OT-2 T cells and subsequently immunized twice with NIP-OVA in PBS (soluble) or complexed with Alum (particulate) at day 0 and 14 (**Figure 35**).

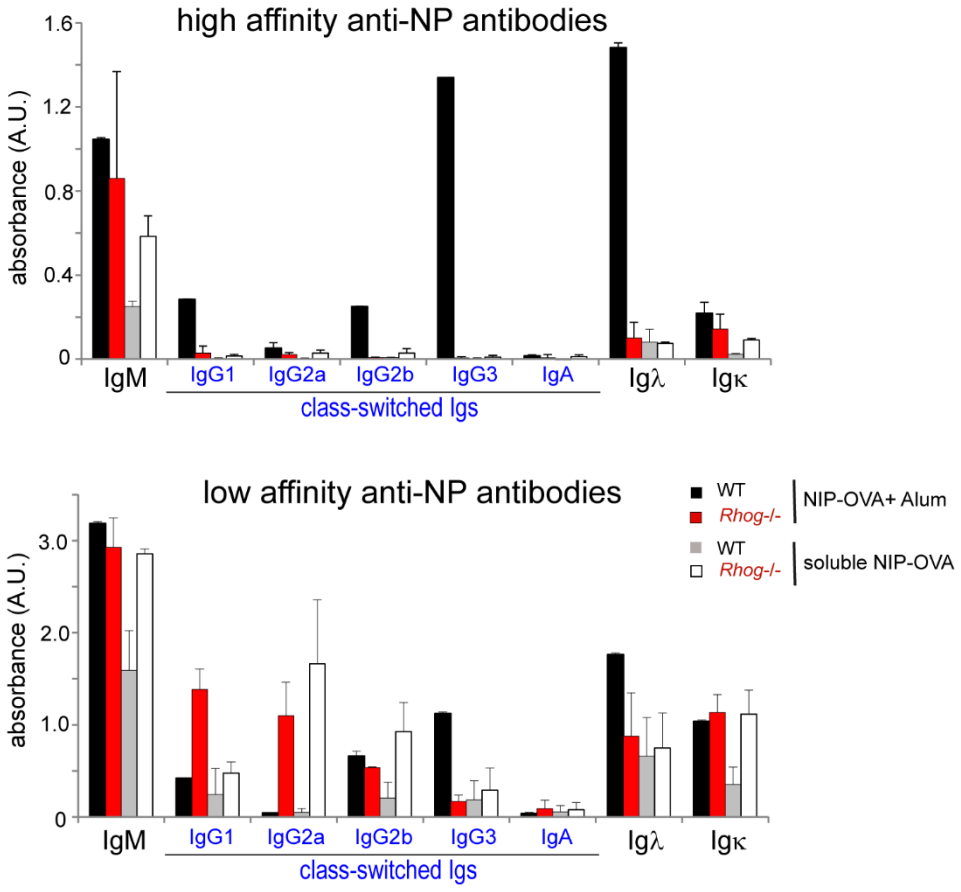

Figure 35. Production of high-affinity antibodies in response to antigen plus alum is partially due to B cell antigen phagocytosis. ELISA quantification of high-affinity (upper graph) and low-affinity (lower graph) anti-NP immunoglobulins in sera of *Rag1-/-* mice reconstituted with WT (black;grey) and *Rhog-/-* (red;white) B cells from B1-8^{hi} mice and OT2 T cells and subsequently immunized with soluble (in PBS) or particulate (embedded in Alum) NIP-OVA. Sera were obtained 16 days after the first immunization. (n=1-3).

Sera from those mice were extracted to determine the presence of high and low affinity immunoglobulins specific for NP. *Rag1-/-* immunized with NIP-OVA+Alum and reconstituted with WT B cells produce high and low-affinity IgMs, as well as class-switched immunoglobulins, whereas mice reconstituted with *Rhog-/-* B cells show a very strong impairment in the production of high affinity anti-NP antibodies. These differences in high-affinity antibodies were not observed in mice immunized with soluble NIP-OVA, suggesting that RhoG is only required for the response to particulate antigen. In contrast, low-affinity antibodies were produced by transferred *Rhog-/-* B cells at WT levels or even more independently of the type of immunization. This could be caused because *Rhog-/-* mice produce more immunoglobulins in sera than WT mice, but these immunoglobulins are of lower affinity.

These data suggest that the potent humoral boost generated by Alum-based vaccines, which induce the production of high affinity immunoglobulins, is due in part to direct B cell phagocytosis of antigen aggregates.

DISCUSSION

DISCUSSION

Throughout this thesis, we have shown a novel mechanism both *in vivo* and *in vitro* for antigen uptake by Follicular B cells. B cell phagocytosis seems key not only to induce a potent humoral response *in vivo* upon antigen immunization in the presence of alum adjuvant, but also to recreate a germinal center *in vitro*. This mechanism is BCR-driven and dependent of two small GTPases: RRas2 and RhoG. The B cell forces necessary to phagocytose a particulate antigen could drive the affinity-discrimination mechanism necessary to allow the survival and affinity maturation during the GC reaction. We describe a simple 2-cell type culture system in which we can detect Ig class-switch, BCR affinity maturation, B cell differentiation to plasma cells and antigen-specific Ig secretion.

1. Phagocytosis as a new mechanism for antigen uptake by Follicular B cell

Within this work we have studied the ability of mouse B cells, in particular Follicular B cells to phagocytose antigens through their BCR. We show that naïve B cells can acquire latex beads coated with antigen up to 3μm size both *in vitro* and *in vivo*. This antigen acquisition mechanism has been controverted within the B cell field, having detractors and supporters. In 1996, Vidard and colleagues studied the ability of different antigen presenting cells to acquire particulate antigens and present them to T cells. They already demonstrated that lymphoblastoid B cells were able to phagocytose latex beads coated with ovalbumin, process the antigen and present it to T cells. However, they could not demonstrate this capacity for circulating naïve B cells (Vidard et al. 1996). This paper contradicted previous data supporting the inability of B cells to acquire antigens through phagocytosis (Galelli et al. 1993). In the last years several different papers came out that corroborated the phagocytic ability of B cells in different organisms: early vertebrates (Li et al. 2006, Zimmerman et al. 2010), mouse (Gao et al. 2012, Nakashima et al. 2012, Parra et al. 2012), and human (Souwer et al. 2009, Zhu et al. 2016) and its importance during clearance of bacteria. In the three sequential studies that appeared in 2012, it was shown that peritoneal cavity and liver B1 B cells were able to acquire latex beads and bacteria (Gao et al. 2012, Nakashima et al. 2012, Parra et al. 2012), in a process that could be performed in a BCR-independent manner. Nevertheless, these studies did not focus on FO B cells, as these cells did not undergo BCR-independent phagocytosis.

Our data demonstrate that in fact FO B cells also have phagocytic capacity driven by BCR engagement. 1μm latex beads coated with anti-IgM were found inside naïve FO B cells after 1 hour of incubation in a process that is RRas2- and RhoG- dependent. In addition, we have shown that the proteins bound to the beads are degraded and can be presented trough MHC class II to CD4 naïve T cells, inducing its activation. This process is dose-dependent, meaning that by increasing the beads:B cell ratio, more antigen is acquired and presented to T cells, which in turn get more activated.

Small antigens are thought to be acquired by B cells by a clathrin-dependent endocytosis and pinocytosis mechanism, while larger antigens are acquired by direct uptake from antigen presenting cells. Upon B cell contact with the APC, BCR triggering induces membrane spreading on the APC and the polarization of BCRs and downstream signalling molecules towards the contact area with the APC, generating a well-structured synapse (Batista et al. 2001). Batista and colleagues proposed that B cells acquire tethered antigens from APC using considerable mechanical forces (Batista and Neuberger 2000), a fact that has been confirmed by Tolar and colleagues using membrane bilayers with bound antigens (Natkanski et al. 2013). This group showed that B cells pull on the antigen during synapse formation and provoke the deformation of the APC's membrane. This mechanism can be reminiscent of a frustrated phagocytosis and similar to what it is described in T cells as trogocytosis, mechanism in which a T cell can extract MHC presenting antigens and other membrane proteins from the APC at the immunological synapse contact (Martinez-Martin et al. 2011).

In 2011, another mechanism was put forward to explain how B cells extract antigen from the APC's membrane. The group of Ana Marie-Lennon described the recruitment of lysosomes to the synaptic interface, inducing the release of proteases to the contact Bcell-APC interface. These proteases digest antigens bound to the APC, facilitating its uptake by B cells (Yuseff et al. 2011). Recently, these two mechanisms have been integrated in a model that proposes that a B cell uses one or the other depending on the stiffness of the APC's membrane (Spillane and Tolar 2017). Although this model is based on the use of artificial membrane bilayers, our data do not oppose it, and in fact, all models could be integrated. Phagocytosis could be the mechanism used by B cells to engulf bacteria, yeast or even large immuno-complexed antigens. It is not likely that our results are explained by antigen extraction from the latex beads through a protease-dependent degradation since: 1) we find beads inside B cells 2) it has been shown previously that incubation with larger beads (25μm) decrease the ability of B cells to present antigen to T cells (Batista and Neuberger 2000).

2. Role of RRas2 and RhoG in BCR-driven phagocytosis

Phagocytosis is a widespread mechanism that can be utilized by many different cell types under specific conditions such as pathogen or apoptotic bodies removal (Underhill and Goodridge 2012, Arandjelovic and Ravichandran 2015). Phagocytosis requires the interaction of membrane receptors with their ligands, which are covering the particle. This mechanism is tightly controlled by pathways that promote actin remodelling, microtubule relocalization, intravesicular trafficking and generation of membrane protrusions, which fuse for the engulfment of the particle. Phagocytosis is classified in two types according to the Rho GTPase family members and the opsonized receptors involved (FcγRII or CR3) (Caron and Hall 1998, Groves et al. 2008). Tzircortis and colleagues described that both types of phagocytosis are dependent of RhoG in macrophages. The importance of this GTPase in phagocytic processes was first detected during the engulfment of apoptotic bodies (deBakker et al. 2004), and later during the trogocytic uptake of membrane antigens by T cells (Martinez-Martin et al. 2011). Our group described the involvement of RRas2 and RhoG trogocytosis and phagocytosis of large particles triggered by TCR engagement. Here we demonstrate that these two GTPases are also involved in BCR-driven phagocytosis by Follicular B cells. Nevertheless, RRas2- and RhoG- deficient B cells do not present a complete blockage of antigen-driven phagocytosis, suggesting a possible redundancy with other GTPases, or even between them. Although it was described that RRas2 activates RhoG through the recruitment and activation of PI3K in T cells (Martinez-Martin et al. 2011), it is not known if this mechanism is operating in B cells or if alternative pathways control RhoG activation. It would be unlikely that RhoG deficiency induces a stronger phagocytic defect than RRas2 if RRas2 activation were exactly upstream of RhoG activation. Thus, we suggest that additional alternative pathways are able to activate RhoG independently of RRas2.

Taking into account the increasing evidence of RhoG importance during phagocytosis, and the fact that until a few years ago most studies addressing the participation of Rho GTPases were carried out using not totally specific inhibitors, it could be interesting to determine in which other phagocytic pathways is RhoG implicated. Indeed, expression of this GTPase across the immunological system is high, especially in neutrophils and some populations of dendritic cells (https://www.immgen.org/ database), which are considered as professional phagocytes (van Kessel et al. 2014).

3. *In vitro* germinal center recreation: can we mimic the *in vivo* process?

Although germinal centers were discovered more than 100 years ago and in spite of the extensive work performed, there are still much unknown about how the affinity maturation process takes place or how cells differentiate towards specific cellular fates. Recent use of live imaging techniques has implied a step-forward in the study of germinal center generation; nevertheless, the inability to study the reaction *in vitro* has been one of the main obstacles to dissect the process in a detailed timely manner.

In this thesis, we show that presentation of phagocytosed antigens by B cells to CD4 T cells induces their differentiation to T Follicular cells, expressing CXCR5 and PD1, as well as producing typical TFH cytokines as IL-4 and IL-21 at low levels. Interestingly, even though their activation, measured as T cell proliferation, is dose-dependent, which means more antigen-presentation more CD4 proliferation, their differentiation to TFH has an optimal bead-dose. Although this data apparently contradicts a recent paper published by Sallusto and colleagues, in which it is described that increasing amounts of antigen induce increasing numbers of TFH and the maintenance of their phenotype (Baumjohann et al. 2013), could be that in our in-vitro system, excessive T cell stimulation induce the differentiation of CD4 T cells towards other T cell subsets.

Our data also show that in parallel to naïve CD4 T cell differentiation to TFH, naïve Follicular B cells differentiate into GC B cells. This differentiation is induced by BCR triggering during phagocytosis of bead-bound antigens with the help of costimulatory signals received from T cells. Neither one of the two stimuli is sufficient to induce survival and GC differentiation in culture, since B cells from B1-8^{hi} mice stimulated with bead-bound NP-CGG or without a BCR-specific antigen, do not differentiate. These results are in concordance with published data *in vivo* where it is shown that B cell presentation to T cells is essential for germinal center formation (Gitlin et al. 2014). Although there exist much controversy about the importance of the BCR as a signalling module or just as a mere mechanism to acquire antigens (Victora et al. 2010, Khalil et al. 2012, Mueller et al. 2015), it seems that both roles are essential for germinal center development.

Interestingly, our data also point to a role for antigen phagocytosis for the GC response *in vivo*. This evidence derives from the use of RhoG-deficient B cells. Although we found that both RRas2-deficient and RhoG-deficient B cells have impaired BCR-driven phagocytosis, we have preferred using the latter because *Rras2*-/- B cells have additional intrinsic proliferative and BCR signalling defects (Delgado et al. 2009) (Mendoza 2017). RhoG-deficient mice were

however shown not to have any apparent deficiency in lymphocyte development and RhoG has been involved in phagocytosis by different receptors but not in any other cell process.

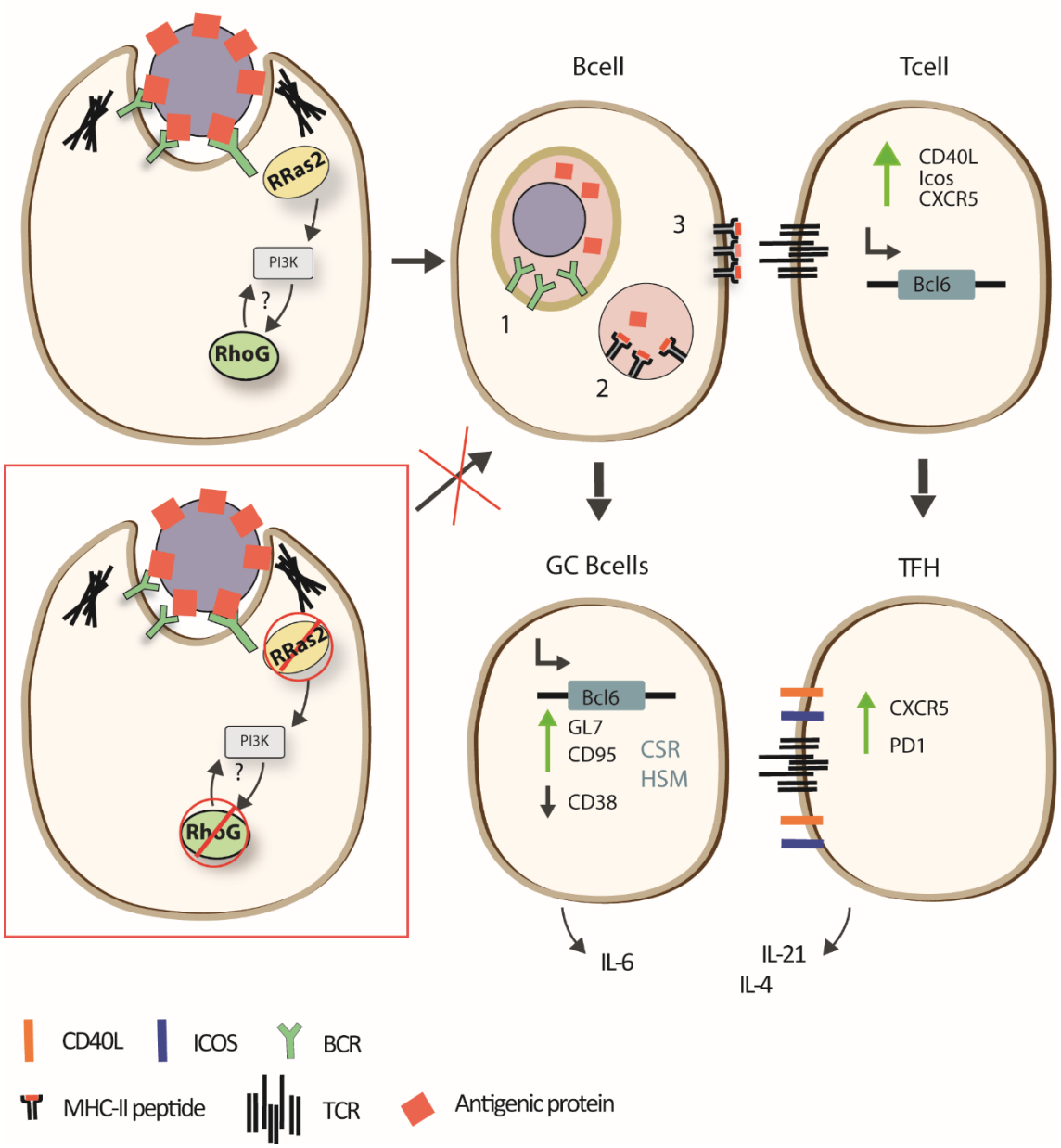

Figure 1. Dynamic of Tcell-Bcell cultures in the presence of antigen that is phagocytosed. Naïve cultured B cells phagocytose 1-3µm beads coated with antigen by a BCR-driven process. This mechanism is RRas2- and RhoG-dependent. Once antigen is phagocytosed, it traffics trough endosomal and lysosomal compartments (1), where the antigen is processed and MHC II molecules are loaded with the processed peptides (2) and expressed at the plasma membrane (3). Naïve cognate T cells can recognize the B cell-presented peptides, to become activated. In turn, T cell co-stimulation together with the BCR stimulus pushes the GC reaction.

Our data demonstrate that *in vitro* generated GC B cells secrete antigen-specific class-switched immunoglobulins to the medium. Indeed, those secreted antibodies are of high affinity and their generation increase with time of culture, in line with an affinity-maturation process (Mesin et al. 2016). Although naïve B1-8^{hi} B cells express a BCR which already carries the mutation W33L that confers high affinity for NIP (Shih et al. 2002), these cells undergo somatic hypermutation in the in-vitro GC recreation, although with a lower degree, as it has been described *in vivo* (Shih et al. 2002).

In summary, the three major hallmarks of germinal center reactions seem to be present in the in-vitro 2-cell culture upon phagocytic antigen uptake: expression of TFH and GC markers, class-switching and Ig affinity-maturation. Furthermore, our culture system allows to study the differentiation of GC cells to plasma cells (PC), since we can detect their generation after 5 days of culture. Interestingly, the appearance of CD138+ Blimp1+ PC population occurs when the numbers of Bcl6+ GC B cells begin to decline. Bcl6 and Blimp1 TF are mutual repressors and for that reason, it is necessary the downregulation of Bcl6 and the gradual upregulation of Blimp1 to exit the GC program and acquire a PC fate (Tunyaplin et al. 2004, Crotty et al. 2010).

Several protocols to achieve GC *in vitro* have been described (Nojima et al. 2011, Kato et al. 2012, Inagaki et al. 2013, Purwada et al. 2015, Fang et al. 2017). However, in none of them it has been achieved the generation of antigen-specific GCs. The protocol described by Nojima et al. induces the production of class-switched immunoglobulins and the differentiation of B cells towards memory B cells thanks to the use of a fibroblast-feeder cell line that expresses CD40L and BAFF. However, they were not able to activate sufficiently AID expression to induce SHM in the cultured B cells, making impossible affinity-maturation in those cell cultures (Nojima et al. 2011). In other cases, the production of antigen-specific Ig has been achieved but provoking a massive B cell stimulation which impedes to use this technique as a translation of the *in vivo* process (Kato et al. 2012, Inagaki et al. 2013).

Although most of the experiments have been carried out using naïve B cells from B1-8^{hi} tg mice, we show that this 2-cell culture assay can also be applied to the generation of antigen-specific immunoglobulins with putative therapeutic functions using non-tg B cells. A possible function for the 2-cell culture could be the production of human antibodies of therapeutic interest.

4. The importance of how antigen is given: soluble vs phagocytic

It was previously shown that B cells can acquire soluble antigen, although the efficiency of its presentation to T cells is several fold lower than when particulate antigens are acquired (Vidard et al. 1996). We have shown in this thesis that the way how the antigen is acquired is key to generate a GC *in vitro*. In our cultures, soluble antigen is able to stimulate B cells and induce their proliferation and expression of germinal center markers. Nevertheless, this GC reaction gets abrogated after 4 days, inducing a scarce production of class-switched antigen-specific Igs.

There has been much controversy about how GC B cells signal through their BCR. In 2012 it was published a paper that described the unresponsiveness of GC B cells after soluble stimulation. They justify this absence of BCR signalling by the increased expression of some phosphatases such as SHP-1 found in those cells (Khalil et al. 2012). However, more recently it has been shown that when antigen is displayed embedded in membrane bilayers, GC B cells are indeed activated. This could indicate that the physical format of the antigen is important for the interaction with B cells and for GC development: a soluble stimulus is not able to induce B cell signalling to overpass the threshold imposed by high levels of phosphatases, whereas particulate antigens are able to do that stimulating those B cells with high-affinity BCRs.

We show that phagocytosed antigens continue triggering BCR signalling after their complete internalization in phagosomes inducing a more sustained BCR and Syk phosphorylation. Pierce and colleagues described that Akt signalling was regulated during BCR internalization upon stimulation since this pathway was found decreased when BCR endocytosis was blocked (Chaturvedi et al. 2011). Their data fits with our results that show a more sustained Akt phosphorylation upon particulate B cell stimulation, indicating that Akt signalling can persist in the internalised phagocytic vesicles. Moreover, phagocytic particle uptake is linked to the PI3K pathway, indicating that bigger particles need more AKT signalling (Cox et al. 1999, Araki et al. 2003). This data is in concordance with the proportional requirement for RRas2 and RhoG with particle size, since these two GTPase are upstream or downstream the PI3K-Akt pathway (Rosario et al. 2001, Murga et al. 2002, Delgado et al. 2009, Martinez-Martin et al. 2011). Moreover, Akt activation is related with CSR and plasma cell differentiation, the balance between the activation and downregulation of this pathway controls the first wave of plasmablast formation and CSR (Omori et al. 2006).

In addition to a stronger and more sustained stimulation of the PI3K-AKT pathways, we have detected a stronger Erk and Syk activation. Interestingly, Syk signalling has been related to membrane spreading necessary to contact the APC-associated antigen (Flannagan et al. 2012). This membrane spreading allows extraction of higher amounts of antigen and the discrimination between a wide range of affinities (Fleire et al. 2006). In addition, Erk pathway has been related to plasma cell differentiation by inducing the expression of Blimp1 (Yasuda et al. 2011). B cells deficient in RhoG show defective Syk, Akt and Erk phosphorylations, comparable to stimulation of WT cells with soluble antigen, suggesting that both the membrane spreading around the particle and the persistent activation found in phagosomes sum up to induce a stronger BCR signalling activation. The persistence of signalling in early phagosome stand against the idea that BCR internalization extinguishes active signalling (Stoddart et al. 2005). It would be interesting to track the intracellular vesicle trafficking followed by a particulate antigen after its BCR-dependent phagocytosis to compare it with trafficking of FCγR-phagocytosed antigens and with endocytosed ones.

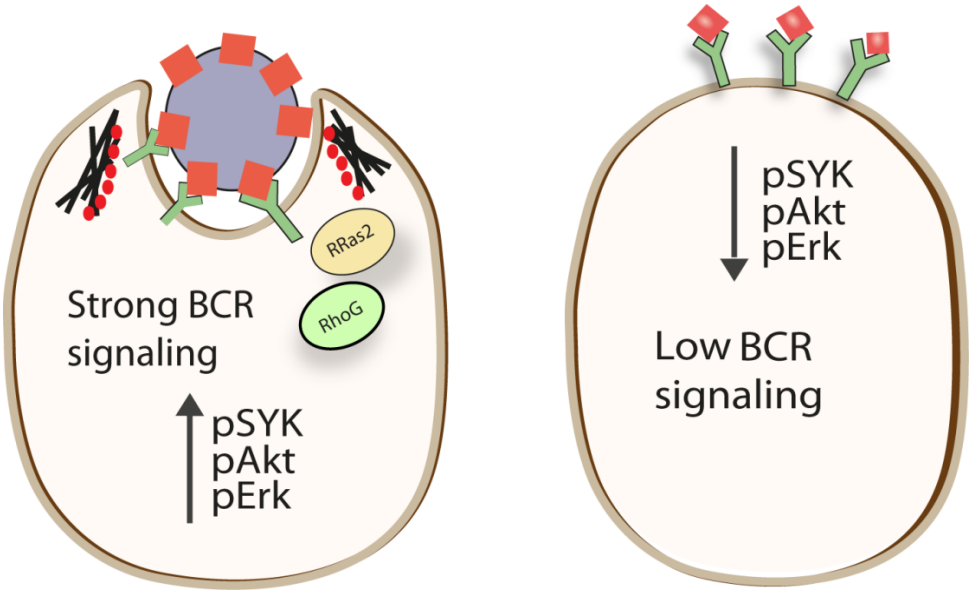

Figure 2. Phagocytosis induces stronger BCR signalling than soluble stimulus. Phagocytic stimulus induces more robust activation of Syk, Akt and Erk pathways than soluble antigen. To be able to acquire particulate antigens, B cells generate a phagocytic cup in the contact area where thanks to myosin activity and actin remodelling, plasma membrane extends around the particle to engulf it.

Recently it has put forward the idea that affinity discrimination could be due to the different physical forces used by B cells to extract the antigen depending of their BCR affinity. In that way, high-affinity B cells induce spreading, membrane invagination and pulling out the antigen

through a mechanism directed by myosin II (Natkanski et al. 2013). Phagocytosis also induces this massive spreading where myosins play a fundamental role (Flannagan et al. 2012), indicating that phagocytic antigen uptake could be also a driving mechanism for affinity discrimination during the generation of the humoral response. Therefore, we hypothesize that those B cells with high affinity BCRs can acquire antigens by phagocytosis resulting in stronger and more sustained BCR signalling.

Indeed, we show that stimulation with soluble antigen is not able to promote plasma cell differentiation in the B:T cultures, while phagocytic antigen does. Although the mechanism of PC differentiation is not yet fully understood, it is thought that B cells carrying high-affinity BCRs are more prone to differentiate into PC during the GC response (Phan et al. 2006, Gitlin et al. 2016), indicating that phagocytic stimulus could induce the expansion of higher affinity clones that differentiate to PCs compared to soluble one. Nevertheless, it was surprising when analysing somatic hypermutation and expression of AID transcript in the *in vitro* cultures, very few differences appear between B cells stimulated with a phagocytic and soluble antigen. One possible explanation is that somatic hypermutation takes place in B cells stimulated with soluble antigen but they do not survive enough to perform affinity maturation and differentiate into PC.

5. Is B cell phagocytosis important for *in vivo* humoral response?

We show in this thesis that the phagocytic BCR-driven role on the humoral response is not only important *in vitro*; it also takes place *in vivo*. Indeed, we show that phagocytosis of particulate antigens by B cells is a key process to develop a potent humoral response since RhoG-deficient mice develop smaller GCs after immunization with bead-bound antigen. Phagocytic uptake of big antigens, as bacteria, by B cells has been previously linked to removal of infection through their microbicidal abilities (Nakashima et al. 2012, Parra et al. 2012). Here, we go one step further, by proposing that acquiring phagocytic antigens by B cells respond to a mechanism for affinity discrimination between B cell clones during GC response.

It is accepted that B cells acquire antigens in immuno-complexes retained at the surface of APCs such as FDCs and macrophages, but several groups have shown that mice lacking either FDCs (Matsumoto et al. 1996), or *Cr2-/-* (Chen et al. 2000), or transgenic mice with blocked Ig secretion that impedes the formation of IC (Hannum et al. 2000), can generate a humoral response in specific conditions. The germinal center reaction is blocked in those animals when

they are immunized with soluble antigen, but upon immunization with alum embedded antigens their B cells undergo SHM and secrete high-affinity Ig. Interestingly, this effect in humoral response is not due to a compensatory mechanism by FCγR presentation (Wu et al. 2000), and in fact, in some cases, the Igs found in the sera of these animals were of higher affinity than in their WT counterparts (Chen et al. 2000). One hypothesis would be that the embedded antigens in fact generate particulate antigens big enough to be able to be phagocytosed directly by B cells, allowing the existence of affinity discrimination. Following that idea, only B cells with enough BCR affinity to phagocytose antigens would be able to enter into GC reaction. Indeed, aluminium-based immunogens generate antigen aggregates from 1-10 μm size (Ghimire 2015). In that way, one explanation to the strong humoral boost that this kind of adjuvants induce would be its effect of promotion of particulate antigen formation that enables their phagocytosis by B cells. Our data point out in that direction since *Rhog-/-* B cells show a strong impairment in the GC response in mice immunized with NIPOVA+Alum, but not with soluble NIPOVA. This impairment is also showed in the production of high-affinity Igs.

However, if we think that in WT animals particulate antigens can also be presented by other APCs, is this B cell phagocytic ability essential? One of the important points is that B cell phagocytosis is not a reminiscent mechanism since we show that antigen-specific B cells have even higher phagocytic ability than cells considered phagocytes, as macrophages. Two years ago, it was published that upon specific viral infections, the organization of subcapsular macrophages (SCS) get disrupted being displayed towards inner follicular areas. This organization is not recovered until 4 weeks after infection. Batista and colleagues showed that during these time-lapse, small antigens were not able to be retained as well in the SCS, being the GC response and PC differentiation diminished upon secondary infection (Gaya et al. 2015). Moreover, it has also been shown that Follicular B cells *in vivo* can transport antigen from SCS towards GC LZ, being their transport important for Ig affinity maturation (Phan et al. 2007, Suzuki et al. 2009). Considering those, we propose that Follicular B cells could be playing a role trapping and phagocytosing antigens in the SCS in normal conditions, but more importantly after primary infections, where the macrophage barrier function is disrupted, in order to bring the antigens to the LZ. In the paper of Batista and colleagues they show a diminished GC response upon secondary immunization, but it could be produced by using small beads (0.2 μm beads), which are not uptaken by phagocytosis. It would be interesting to test if upon secondary infection with bacteria or particulate antigens (>0.5μm size) this strong defect in humoral response is still found, or B cell phagocytosis process compensate at some degree this disruption of the macrophage barrier. Indeed, it would also be interesting to test if

B cell phagocytosis of bacteria, such Salmonella, could start a GC reaction without the necessity of other helper cells as FDCs.

The migration of B cells between the DZ and LZ has been shown to be important during GC reaction in order to select higher affinity B cell clones (Victora et al. 2010) since FDCs presenting immune-complexes are found in the LZ and it is the place where B cells acquire new antigens to present them to TFH. However, some years before stablishing that CXCR4 was one of the chemokines which drives the GC localization between the two areas, it was shown that CXCR4-deficient B cells where still capable to produce antigen-specific antibodies and PC (Nie et al. 2004). This last article indeed suggested that the humoral response upon immunization with particulate antigens (antigen embedded in alum) could not be affected by the misslocalization of GC B cells in the GC. Altogether, those data prompt us to hypothesize the existence of a parallel role in the acquisition and presentation of particulate antigens between the FDCs and Follicular B cells, in line with the data shown in this thesis.

6. Function of Rho GTPases during humoral response: RhoG asks for a place

The *in vivo* function of RhoG GTPase during the humoral response was previously unnoticed due to the kind of immunization used during the experimental procedures. Since immunization with soluble or particulate antigens do not activate the same *in vivo*, or *in vitro*, processes in B cells, it could be that during B cell studies some protein functions have been overlooked. Indeed, RhoG-deficiency does not block completely the process of phagocytosis neither the humoral response induced upon immunization with particulate antigens. This indicates that there must be some redundancy with other GTPases and emphasizes the importance of B cell phagocytosis, which need to be controlled by more than one protein. Indeed, the function of different GTPases of the Rho family, as well as GEF proteins for the Rho family, has been studied during B cell development and humoral response (Doody et al. 2001, Randall et al. 2009, Jabara et al. 2012, Zhang et al. 2012, Burbage et al. 2015, Gerasimcik et al. 2015). Nevertheless, although some reports identify defects in the humoral response to T-dependent antigens, they do not discriminate between particulate or soluble antigens. Importantly, the members of the Rho family play an important role in the control of cytoskeleton reorganization, so it would not be surprising if B lymphocytes lacking some of these proteins are also defective in BCR-driven phagocytosis. Interestingly, Cdc42-deficient B cells are

defective in the antibody-response to Alum-embedded antigen immunization with scarce differentiation to PC, in part produced by deficiency in antigen presentation to CD4 T cell (Burbage et al. 2015, Gerasimcik et al. 2015). Moreover, these B cells show impaired activation of PI3K and Erk pathways, similarly to the defects that we find in *Rhog*-/- animals. In addition, mice deficient in Dock8, a GEF of Cdc42 and Rac1, show also low antibodies titers after immunization with particulate antigens and in the formation of the IS (Randall et al. 2009), similar to what it has been described in CD19-deficient B cells (Wang and Carter 2005). Interestingly, CD19 function has been linked to the acquisition of low-avidity and membrane-absorbed antigens via the activation of PI3K, so it would be interesting to study if its function is also important for BCR-driven phagocytosis. Moreover, although it has not been studied during this thesis, it would be interesting to analyse the paper of RhoG and B cell phagocytosis during the humoral response to bacterial infection, since these pathogens are big enough to be phagocytosed by B cells.

Overall, the data obtained throughout this thesis show a new role for FO B cells in the development of a potent humoral response upon phagocytosing particulate antigen in a BCR-driven process. This mechanism, which promotes a more sustained BCR signalling, is dependent on actin remodelling and the function of two small GTPases: RhoG and RRas2. This new mechanism of antigen acquisition allowed us to mimic a germinal center process in in-vitro cultures, which opens a broad range of possibilities for basic molecular investigation and for the generation of therapeutical antibodies.

CONCLUSIONS

CONCLUSIONS

1. Follicular B cells phagocytose particulate antigens *in vitro* in a BCR-driven process dependent on RRas2 and RhoG GTPase function.
2. B cells present phagocytosed antigens to CD4 T cells, inducing their activation and TFH differentiation *in vitro*.
3. *In vitro* B cell antigen phagocytosis, and subsequent CD4 T cell stimulation, induces B cell differentiation towards GC B cells.
4. B cell antigen phagocytosis is required for complete *in vitro* GC differentiation, antigen-specific high-affinity antibody production, BCR affinity maturation and plasma cell generation.
5. Neither soluble antigen stimulation of B lymphocytes nor RhoG- RRas2-deficient lymphocytes completely differentiate into GC cells.
6. Follicular and Marginal zone B cells phagocytose particulate antigens *in vivo* by a BCR-driven process dependent of the GTPase RhoG.
7. B cell antigen phagocytosis is required to develop a potent humoral reaction in response to particulate antigens *in vivo*.
8. RhoG is required for the humoral response to particulate antigens but not to soluble ones.
9. Phagocytosed antigens induce a stronger and more sustained BCR signalling than soluble antigens.

CONCLUSIONES

1. Los linfocitos B foliculares fagocitan antígenos particulados *in vitro* a través del BCR en un proceso dependiente de las GTPasas RRas2 y RhoG.
2. Los linfocitos B en cultivo presentan péptidos derivados de antígenos fagocitados a células T CD4 promoviendo su activación y diferenciación a TFH.
3. El mecanismo de fagocitosis de antígenos por células B y su presentación a linfocitos CD4 induce la diferenciación de linfocitos B naif a linfocitos B de centro germinal.
4. La adquisición de antígenos mediante fagocitosis es necesaria para la completa diferenciación en cultivo de los linfocitos B a células de centro germinal, la producción de anticuerpos antígeno-específicos de alta afinidad y la posterior diferenciación a células de plasma.
5. Linfocitos B estimulados con antígeno soluble o deficientes para RRas2 o RhoG GTPasas no realizan centros germinales completamente diferenciados en cultivo.
6. Los linfocitos B foliculares y de la zona marginal son capaces de fagocitar antígenos particulados *in vivo* a través del BCR en un proceso dependiente de la GTPasa RhoG.
7. El proceso fagocítico de antígenos mediado por los linfocitos B es necesario para el desarrollo de una respuesta humoral potente frente a antígenos particulados.
8. RhoG media la respuesta humoral de los linfocitos B a antígenos particulados pero no a antígenos solubles.
9. Los antígenos fagocíticos inducen una señalización a través del BCR más fuerte y sostenida que los antígenos solubles.

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ADDENDUM