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SCUOLA DI DOTTORATO DI RICERCA IN
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XXIX CICLO

Immunophenotypic characterization of B-lymphopoiesis in KO mice for oncogenic Ser/Thr kinases by multiparameter flow cytometry

Direttore della Scuola: Ch.ma. Prof. Paola Zanovello

Supervisore: Ch.mo. Dott. Francesco Piazza

Dottorando: Macaccaro Paolo

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ABBREVIATIONS

AA	Aminoacid
Ab	Antibody
AcMo	Monoclonal Antibody
APC	*Antigen Presenting Cells*
ATP	Adenosine triphosphate
BAFF	*B-Cell Activating Factor*
BCR	*B Cell Receptor*
BM	bone marrow
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
BTK	*Bruton's Tyrosine Kinase*
Cdc37	cell division cycle protein 37
CFSE	Carboxyfluorescein succinimidyl ester
CK2	protein kinase CK2
CK2β	β subunit of protein kinase CK2
CK1α	protein kinase CK1
CTRL	control
DL1	delta ligand
dNTPs	deoxyribonucleotide
dpc	*days post coitum*
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
FCS	fetal calf serum
floxed	flanked by loxP sites
FoB	follicular b-cells
FoI	follicular b-cells tipe I
FoII	follicular b-cells tipe II

FSC	*Forward Scatter*
Hsp90	heat shock protein 90
Ig	immunoglobulin
IL4	Interleukin 4
KO	knockout
LPS	Lipopolisaccaride
MAPK	mitogen-activated protein kinase
MDP	Macrophages-dendritic cells progenitor
MUT	Mutant
MZ	marginal zone
MZB	marginal b-cells
MZP	marginal zone precursor
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
N-terminale	amino terminale
PBS	phosphate buffer solution
PI3K	Phosphatidyl-inositol-3-phosphate kinase
PKB/Akt	protein kinase B/Akt
rpm	round per minute
SD	standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Polyacrylamide gel electrophoresis in SDS
SPL	Spleen
TBS	tris buffer solution (buffer solution Tris-HCl)
TCR	T cell receptor
Tris	tris(hydroxymethyl)aminomethane
WT	wild type

AMINO ACID ABBREVIATIONS

A	**Ala**	**Alanine**
C	**Cys**	**Cysteine**
D	**Asp**	**Aspartic acid**
E	**Glu**	**Glutamic acid**
F	**Phe**	**Phenylalanine**
G	**Gly**	**Glycine**
H	**His**	**Histidine**
I	**Ile**	**Isoleucine**
K	**Lys**	**Lysine**
L	**Leu**	**Leucine**
M	**Met**	**Methionine**
N	**Asn**	**Asparagine**
P	**Pro**	**Proline**
Q	**Gln**	**Glutamine**
R	**Arg**	**Arginine**
S	**Ser**	**Serine**
T	**Thr**	**Threonine**
V	**Val**	**Valine**
W	**Trp**	**Tryptophan**
Y	**Tyr**	**Tyrosine**
X	**generic amino acid**	

ABSTRACT

Protein kinase CK2 and CK1 are a pleiotropic and evolutionary conserved serin-threonin kinase that is involved in several cellular processes. A number of studies revealed many mechanisms through which this kinase regulates cell cycle, apoptosis, cell survival and tumorigenesis.

CK2 participates in many developmental pathways, of which particularly relevant for hemo-lymphopoiesis are those dependent on Hedgehog, NF-κB and STAT3, which regulate cell differentiation, proliferation, self-renewal as well as lineage choice commitment.. CK1 regulates also molecular pathways which are important for multiple myeloma plasma cells survival, like WNT/β-catenin pathway and PI3K/AKT pathway.

However, despite all this data, little is known about the role of CK2 and CK1 in B-lymphopoiesis and lymphomagenesis.

To elucidate the physiological and pathogenetic role of CK2 and CK1 in B-lymphocytes, we generated B cell specific conditional KO mice, were we studied the effects of deletion during normal B cell development with multiparameter flow cytometry analysis.

In the bone marrow (BM), CK2β KO mice displayed a reduction of B cells, especially of the recirculating population of transitional and follicular (FO) B-cells. In peripheral blood and spleen the number of B-cells was markedly reduced. In the spleen of CK2β KO we observed an imbalance between the amount of FO and marginal zone (MZ) B-cells was found with an absolute reduction of FO B cells by approximately 2-folds and an increase of MZ B-cells and MZB cell precursors by up to three folds.. *In vitro* class-switch recombination assays demonstrated impairment in IgG_1 and IgG_3 class-switch and a marked reduction of the generation of antibody-producing cells. In CK1α KO mice we observed the totally absence of mature B cells and the presence of early precursors B cells. CK1α HET mice showed a reduction of B cells in bone marrow and an light imbalance of FO B cells an MZB cells in spleen. *In vitro* class-switch recombination assays doesn't showed significant difference between HET and CTRL mice in IgG_1 and IgG_3 class-switch.

Here, we found that the β subunit of protein kinase CK2 is a novel regulator of peripheral B cell differentiation. CK2β has a role in regulation of the GC reaction and in homeostasis of FOB and MZB cells. Furthermore we found that CK1α has a pivotal role in early B cells development. On one side our data enrich the knowledge on the mechanisms regulating B-cell development, on the other side they inform about the potential mechanisms altered by CK2 and CK1 during B-cell tumorigenesis.

1. INTRODUCTION

1.1. General characteristics of B-cells

B lymphocytes are a type of lymphocytes of the humoral immunity of the adaptive immune system. They are specialized in the production of immunoglobulins (Ig) to generate an immune response. The Ig produced can be surface or soluble antibodies and are the specific receptors for antigens. Typically, B-cells express on the plasma membrane two different forms of Ig_s with the same specificity: IgD and IgM ; only a few, express IgG , IgA or IgE.According to the Porter model of 1962 , the antibodies have four chains: two heavy (H, 50-77 kDa) and two light (L , 25 kDa) . The light chains are divided into two types (κ and λ), those heavy in five, and correspond to the respective classes of Ig_s (γ , α , μ , δ and ε). The chains possess both a constant (C) and a variable region (V). Light chains have two disulfide bonds , while, in the heavy, bridges are a total of four . Each of these links leads to the formation of a ring which represents the central portion of a larger region , defined "domain" , consisting of 110 residues . The domain constitutes the aforementioned variable region : VL for the light chains and VH for the heavy. The variable regions of the heavy and light chains associate one another to form the binding site for the antigen , whose specificity depends on the amino acids present in the chains.

Thus, antibodies with different specificities have different amino acid sequences in the V region , the diversity of which are rooted in events of " gene recombination " at the level of the *loci* for both types of chains (**Fig.1**).

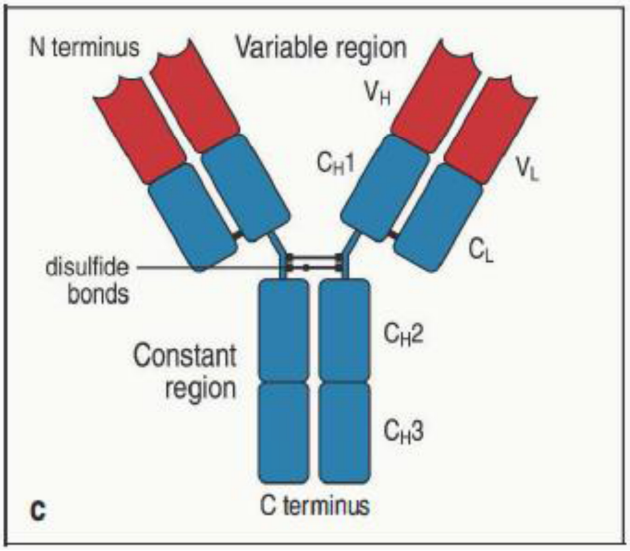

***Fig.1: structure of antibodies** (Modified from Murphy K., Janeway's Immunobiology. 8th. Garland Science. 2012).*
The antibodies have a quaternary structure composed of two light and two heavy chains. The figure shows variable (red) and constant (blue) portions and disulfide bonds.

The B-Cell Receptor (BCR), which plays a key role in the activation of B lymphocytes , is a transmembrane receptor , constituted by an extracellular portion, which binds the antigen and an intracellular region, capable of signal transduction . This region is linked by disulfide bridges with an heterodimer , called Ig - α / Ig – β (or CD79a and CD79b). Each member of the dimer passes through the plasma membrane and has a cytoplasmic tail containing an Immunoreceptor Tyrosine-based Activation Motif (ITAM)[37].

The binding of the antigen with the BCR triggers four main processes :

1) proliferation of B lymphocytes;

2) differentiation into plasma cells (PC)

3) formation of memory cells;

4) antigen presentation to T cells [37]

BCR initiates signal transduction using Src kinases; then in the process is involved a costimulatory complex, CD21 - CD19 - CD81 - Leu13 , which implements the transduction activity. This binding causes a conformational change of the CD19 receptor and a consequent increase in the intracellular signal . Subsequently , the antigen binds the surface Ig_s, the B cell internalizes the Ac- Ag complex, processes and expresses antigenic epitopes in the major histocompatibility complex (MHC) and presents them to T cells (**Fig.2**).

The main function of B-cells is to produce antibodies, but they may perform other functions in the immune response through the expression of Toll Like Receptors (TLR). TLRs are membrane glycoproteins that are able to recognize a large amount of proteins derived from pathogens and the activation of TLRs triggers inflammatory responses [9]. B-cells express high levels of TLR- 9 and TLR-10, which are increased after the activation of the BCR or following the activation of the CD40 molecule.

By means of the TLR-9, B-cells may perform functions typical of the innate immune response , as the receptor recognizes un methylated CpG genomic regions, typical of bacteria with a DNA genome, and is involved in the initial responses to microorganisms. The inducible expression of TLRs in B-cells can , therefore , be a link between the innate and the acquired immune responses [8].

B-cells may also produce inflammatory cytokines (IL-6,IL-10) that induce the differentiation of naïve T cells into T helper 1 (Th1) and T helper 2 (Th2) and secrete factors that may directly mediate the destruction of pathogens . Finally, they can also function as APC , which are capable of internalizing antigens and present them in MHC-II.

B-cell development is a highly regulated process, that occurs in multiple steps during which B-cells undergo cellular and genetic changes. Firstly in the bone marrow from hematopoietic precursors to immature B-cells, then in periphery from transitional to mature B-cells.

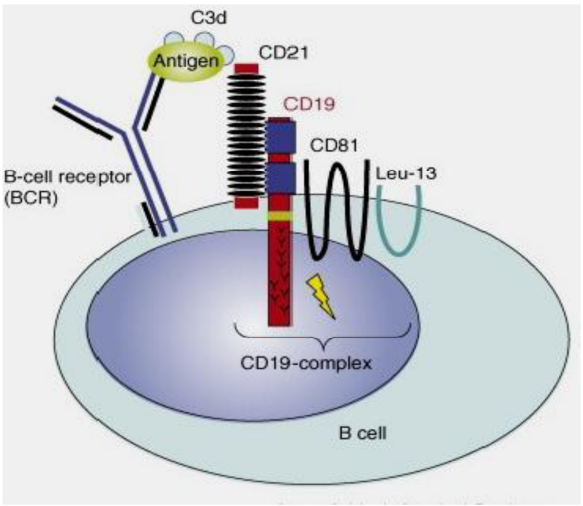

Fig. 2: Activation of the BCR complex and costimulatory molecules CD21 - CD19 - CD81 - Leu13 (Modified from Schäffer. A.A., Current Opinion in Genetics & Development;2007).

1.1.1 The bone marrow

The bone marrow (BM) is the body delegated to haematopoiesis and is crossed by a nutritive artery , which enters the cavity of the bone marrow and here divides into ascending and descending arteries (**Fig.3**). Since these are divided radial arteries, that vascularize endosteum and periosteum through a dense network of capillaries.
The capillaries converge in vascular sinuses , which are sinusoidal vessels [35].
Through the wall of the vascular sinuses mature cells, produced by the BM, go into the blood, while blood cells migrate from the blood in the bone to be removed or to perform specific functions in the processes of defense and immunity (monocytes, granulocytes, lymphocytes). The wall of the vascular sinuses is composed of three layers: endothelium , basal lamina and an incomplete adventitial layer.
The endothelial cells form a continuous foil, not being connected by specialized junctions, are separated by wide gaps , through which the cellular elements can pass in both directions. The endothelium rests on a fenestrated membrane rich in proteoglycans. Outside the basal lamina there is a third layer , which is constituted by fenestrated adventitial cells too. These cells have phagocytic activity that prevents damaged cells to go into the bloodstream.

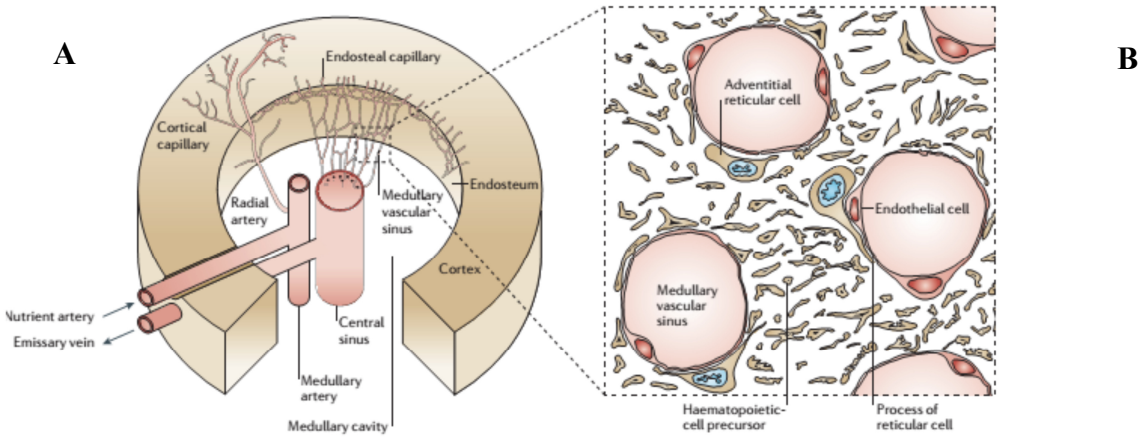

Fig. 3: Bone marrow structure (Modified from Nagasawa T., Nature Reviews Immunology 2006).
A. The main blood source to the bone marrow is provided by the nutrient artery. The nutrient artery crosses the cortex through the nutrient canal into the medullary cavity, where it divides into ascending and descending arteries, from which radial arteries. B. The medullary sinuses in the central cavity of the bone are surrounded by endothelial cells and reticular cells adventitious . The hematopoiesis occurs extravascular spaces between her breasts.

B-cells are generated from hematopoietic stem cells (HSCs) in the liver during the fetal life [20] and the same mechanism occurs in adults BM. The differentiation's pathway from HSCs to mature B-cells can be divided into several processes based on phenotype and functional characteristics that are gradually acquired by cells of the line B [30-46].

Phenotypically, HSCs express high levels of the receptor tyrosine kinase (c-KIT) and the Stem Cell Antigen 1 (SCA-1)[4]. This is a phosphatidyl inositol membrane molecule, which plays a key role in signal transduction during differentiation.

The precursor stem cells closest to the pre–pro B are the *Common Lymphoid Progenitors* (CLP), which have a low level of c-KIT and SCA1 , but a high level of IL 7R [14].

The precursors (pre-proB, proB , preB) are identified by a different expression of CD43 and CD25 (IL2Rα). CD43 is a membrane protein present from the early stages of B lymphocytes differentiation, until the end of the rearrangement of the heavy chains μ. CD43 is , therefore , the cell marker of pre - proB and proB.

In the bone marrow are also present recirculating mature B lymphocytes, and PCs expressing CD138 [28].

Differential expression of heat-stable antigen (HSA) and of the maturation marker BP-1 discriminates four fractions of pro-B cells (A, B, C, and C9). At this stage of development, DNA rearrangement begins in the Ig H chain locus. Most pro-B cells of fraction A carry Ig genes in germline configuration. DH→JH rearrangements are found in almost all cells of fraction B. VH→ DHJH rearrangement occurs in fractions C and C9. Cells in fractions B to C9 are also called pre-B I cells. As soon as mH chain proteins appear in the cytoplasm and can be assembled into a functional precursor B cell receptor (pre-BCR), pre-B I cells develop into large pre-B II cells that are c-KIT and CD43 negative. Successful rearrangement of the H chain and a correctly assembled pre-BCR associate with a functional signaling machinery that allow pre-B II cells to proliferate. [8] The pre-BCR complex plays a critical role in the clonal expansion of μ+ pro-B cells and differentiation to the pre-B cell stage. Pre-B cells undergo Ig L chain rearrangement and the resultant L chain associates with μ heavy chain to form the membrane bound IgM, in addition the signal produced by the pre - BCR receptor activates the trophic factor *Nuclear Factor kappa-light-chain-enhancer of activated B-cells* (NF-kB) [11-35].

After successful rearrangement of both heavy and light chain genes, the BCR, which includes Igα and Igβ, is assembled and expressed on the surface of B-cells. The BCR functions as an antigen-binding and signal transduction molecule during further B cell development.

At the end of this process of development, B-cells leave the bone marrow , coming out from the vascular sinuses, to complete the maturation in secondary lymphoid organs (**Fig.4**).

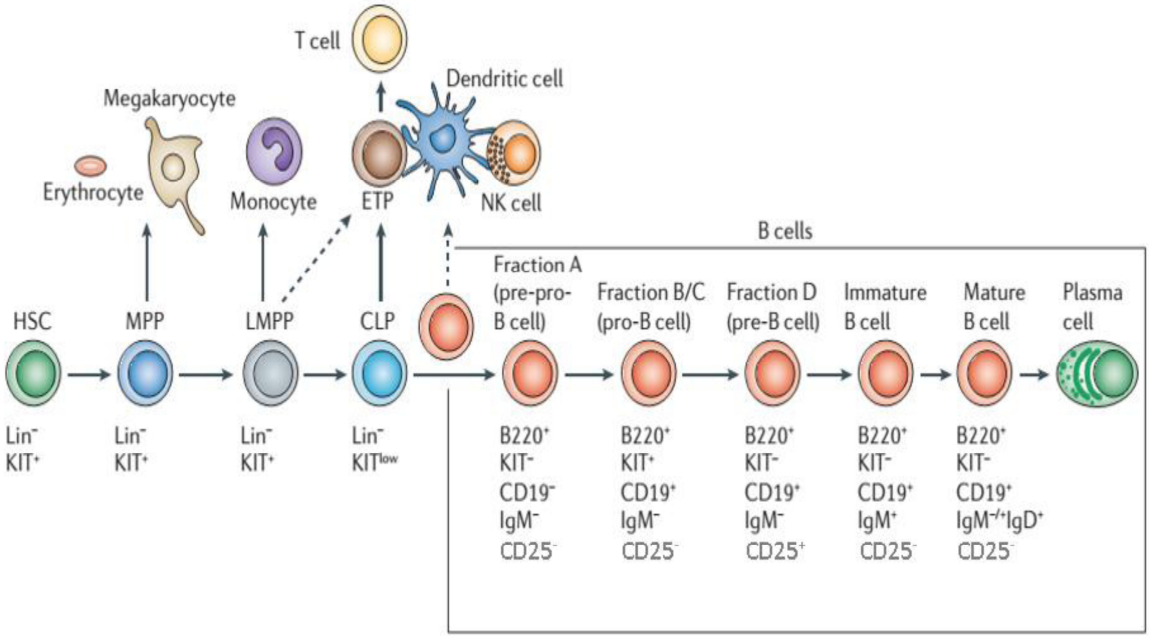

Fig.4: Steps of the B-cell differentiation (Modified from Nagasawa T., Nature Reviews Immunology 2006).

The blood cells originate from a common precursor the HSC. The stem cell with characteristics closer to the precursors of B-cells is the Common Lymphoid Progenitor (CLP). In the box are highlighted the phases of the B-cells development (pre/proB; proB; preB; immature, mature B and plasma cells) and the corresponding surface markers.

1.1.2 Lymph nodes

Lymph nodes (LN) are bean or oval shaped and are highly organized lymphoid structures placed in the sites of convergence of the vessels that are part of the lymphatic circulation. Each LN is surrounded by a fibrous capsule, and inside the LN the fibrous capsule extends to form trabeculae. LNs are divided into the outer cortex and the inner medulla. The cortex is continuous around the medulla except at the hilum, where the medulla comes in direct contact with the hilum [34].

Thin reticular fibers and elastin form a supporting meshwork called a reticular network inside the node. White blood cells (leucocytes), the most prominent ones being lymphocytes, are tightly packed in the follicles (B-cells) and the cortex (T-cells). Elsewhere in the node, there are only occasional leucocytes. As part of the reticular network there are follicular dendritic cells in the B-cell follicle and fibroblastic reticular cells in the T cell cortex. The reticular network not only provides the structural support, but also the surface for the adhesion of dendritic cells, macrophages and lymphocytes. It allows the exchange of material through high endothelial venules and provides the growth and regulatory factors necessary for the activation and maturation of immune cells (**Fig.5**). The number and composition of follicles can change especially when challenged by an antigen, when they develop a germinal center. Lymph enters the convex side of the lymph node through multiple afferent lymphatic vessels, to flow through the sinuses. A lymph sinus, which includes the subcapsular sinus, is a channel within the node, lined by endothelial cells along with fibroblastic reticular cells and this allows for the smooth flow of lymph through them. The endothelium of the subcapsular sinus is continuous with that of the afferent lymph vessel and is also with that of the similar sinuses flanking the trabeculae and within the cortex. All of these sinuses drain the filtered lymphatic fluid into the medullary sinuses, from where the lymph flows into the efferent lymph vessels to exit the node at the hilum on the concave side. These vessels are smaller and don't allow the passage of the macrophages, so that they remain contained to function within the lymph node. In the course of the lymph, lymphocytes may be activated as part of the adaptive immune response.

The LN’s capsule is composed by a dense irregular connective tissue with some plain muscle fibers, and from its internal surface are given off a number of membranous

processes or trabeculae, consisting, in man, of connective tissue, with a small admixture of plain muscle fibers; but in many of the lower animals are composed almost entirely of involuntary muscle. They pass inward, radiating toward the center of the gland, for about one-third or one-fourth of the space between the circumference and the center of the node. In some animals they are sufficiently well-marked to divide the peripheral or cortical portion of the gland into a number of compartments (follicles), but in man this arrangement is not obvious. The larger trabeculae springing from the capsule break up into finer bands, and these interlace to form a mesh-work in the central or medullary portion of the gland. In these spaces formed by the interlacing trabeculae is contained the proper gland substance or lymphoid tissue. The gland pulp does not, however, completely fill the spaces, but leaves, between its outer margin and the enclosing trabeculae, a channel or space of uniform width.

This is termed the subcapsular sinus (lymph path or lymph sinus). Running across it are a number of finer trabeculæ of reticular connective tissue, the fibers of which are, for the most part, covered by ramifying cells [8].

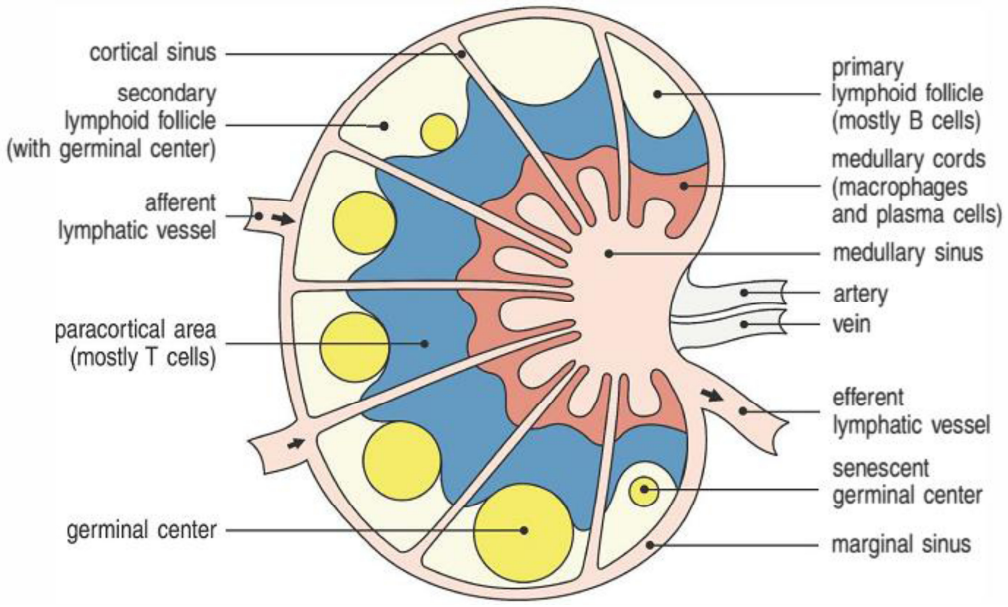

Fig.5: ***lymph node structure (Modified from Murphy K., Janeway's Immunobiology. 8th. Garland Science. 2012).***

A lymph node consists of a cortex and an inner medulla. The cortex is composed of a cuter cortex of B-cells organized into lymphoid follicles, and deep, or paracortical, are smade up mainly of T-cells and dendritic cells. The medulla consists of strings of macrophages and antibody secreting plasma cells.

1.1.3 The spleen

The spleen (SPL) is a lymphoid organ that is located in the abdominal cavity and is delegated to the removal of senescent erythrocytes , cell debris and micro-organisms present in the blood. It is covered by a connective tissued capsule from which originate highly vascularized trabeculae of connective tissue, which gives rigidity to the body [29]. By trabecular vessels entering the parenchyma of the organ , consisting of red pulp and white pulp (**Fig.6**).

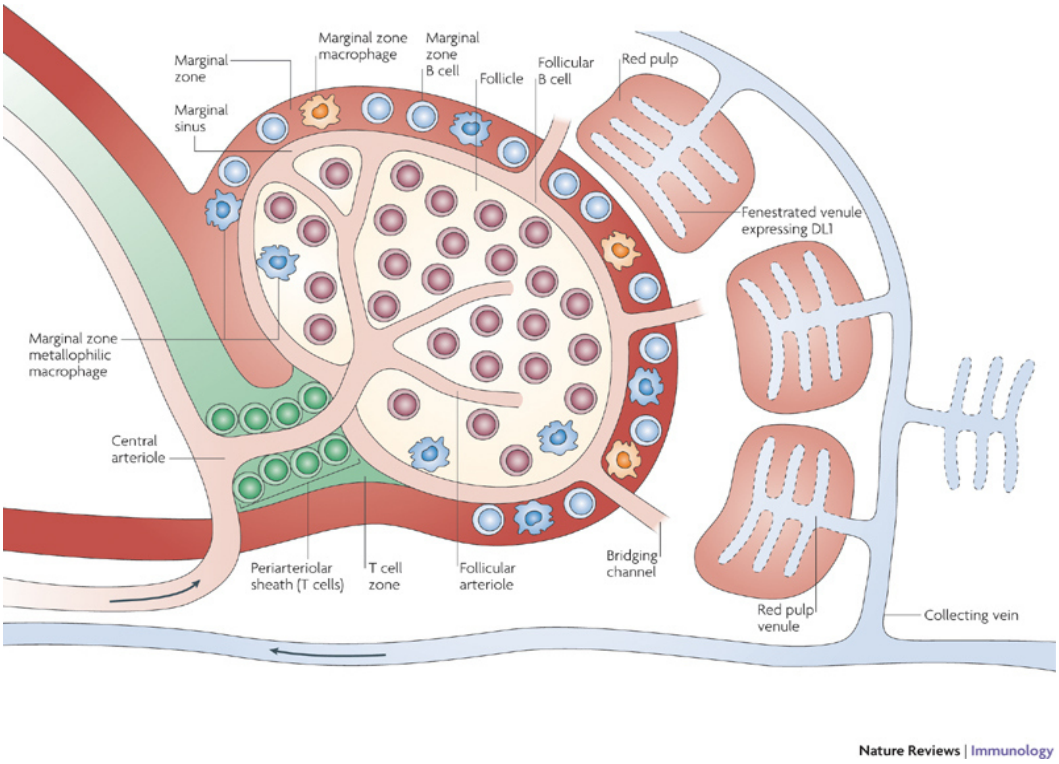

Fig.6: mouse spleen structure (Modified from Pillai and Cariappa; Nature Reviews Immunology 2009).

The spleen is composed of red pulp and white pulp, the latter plays an immunologic function and is the site where development and differentiation of lymphocytes occurs.
In the white pulp stands a marginal zone, which separates white blood cells from the red pulp, a T-zone and a follicular zone where takes place B-cell differentiation.

Red pulp

In the red pulp of the spleen can be identified two systems : filtering the blood and recycling the iron [29].

Filtering the blood: The specialized structure of the venous system of the red pulp gives this area its unique capacity to filter the blood and remove old erythrocytes.

Arterial blood arrives into cords in the red pulp, which consists of fibroblasts and reticular fibres and forms an open blood system without an endothelial lining.

The contractility of the stress fibres might also aid in the retention of erythrocytes in the spleen thereby forming a reservoir of erythrocytes and reducing stress on the heart by reducing the viscosity of the blood during rest.

Recycling of iron: Erythrophagocytosis is important for the turnover of erythrocytes, and recycling of iron is an important task of splenic macrophages, in conjunction with those of the liver. Erythrocytes are hydrolysed in
the phagolysosome of macrophages, from which haem is released after the proteolytic degradation of haemoglobin.

In addition to such phagocytosis of erythrocytes, a considerable portion of erythrocytes are also destroyed intravascularly throughout the body, as a result of the continuous damage to their plasma membrane. This leads to the release of haemoglobin, which is bound rapidly by haptoglobin. Receptor-mediated endocytosis of CD163, an haemoglobin-specific receptor at the cell surface of macrophages, leads to scavenging of haemoglobin from the circulation in the spleen. Iron is important for survival of both the host and the bacterium.

The red pulp is also known to be the site where plasmablasts and PCs lodge. After antigen-specific differentiation in the follicles of the white pulp, plasmablasts migrate into the red pulp, initially just outside the marginal zone.

The position of plasmablasts in the red pulp resembles the localization of plasmablasts in the medullary cords of lymph nodes, and this extrafollicular antibody production leads to rapid entry of antibody into the bloodstream. The plasmablasts are attracted to the red pulp after upregulating their expression of the CXC-chemokine receptor 4 (CXCR4), which binds the CXC-chemokine ligand 12 (CXCL12), which is expressed in the red pulp. This coincides with downregulation of expression of the chemokine receptors CXCR5 and CC-chemokine receptor 7 (CCR7), which binds the homeostatic

chemokines that are present in B-cell follicles and the T-cell zone of the white pulp [32].

White pulp

The white pulp is organized as lymphoid sheaths, with T- and B-cell compartments, around the branching arterial vessels, so it closely resembles the structure of a lymph node. The correct organization and maintenance of the white pulp is controlled by specific chemokines that attract T and B-cells to their respective domains, thereby establishing specific zones within the white pulp [9].

T cells interact with dendritic cells (DCs) and passing B-cells, whereas in the B-cell follicles (also known as the B-cell zones), clonal expansion of activated B-cells, which leads to isotype switching and somatic hypermutation, can take place. CXCL13 is required by B-cells to migrate to B-cell follicles, whereas CC-chemokine ligand 19 (CCL19) and CCL21 are involved in attracting T cells and DCs to T-cell zones of the white pulp. Expression of these chemokines is controlled by lymphotoxin-α1β2 (LT-α1β2) and tumour necrosis factor (TNF). On the inside of the white pulp is an area in which are located activated follicular and follicular dendritic cells. In the follicles, during the humoral response , these cells generate germinal centers (GCs), which take part in the process of maturation of B lymphocytes , which ends with the formation of PCs and memory cells [28]. In GCs it is possible to distinguish a darker area, where there are proliferating B lymphocytes (centroblasts) and a lighter area , in which there are dendritic cells and where there is the selection and differentiation of the progeny (centrocytes). The outest part of the GC is the mantle zone, where there are no proliferating B-cells, which divide the inner zone of the GC from the T-zone of the white pulp (**Fig.7**) [9-29].

The marginal zone is an important transit area for cells that leave the bloodstream and enter the white pulp. It is constituted by two specific populations of macrophages (marginal zone macrophages and marginal zone metallophilic macrophages) and no recirculating B lymphocytes [28].

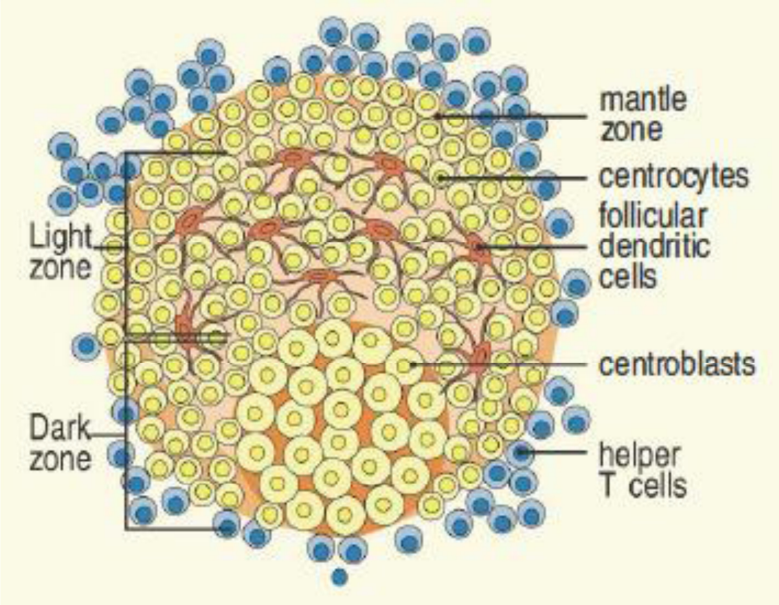

Fig.7: The germinal center (Modified from Murphy K., Janeway's Immunobiology. 8th. Garland Science. 2012)

The germinal center is divided into : mantle zone , light area and dark area . The mantle zone divides the the germinal center from the T-zone and presents especially centrocytes . The light area is composed of centrocytes and dendritic cells , while the dark area is composed of centroblasts.

1.1.4 Splenic B lymphocytes

Peripheral B-cells are lymphocytes that leave the bone marrow after the formation and expression of the BCR.

Mature B lymphocytes, newly formed, that express surface IgM but have not yet come into contact with an antigen, are defined naive B-cells. The differentiation of B lymphocytes, which leads to the formation of PCs, capable of secreting Igs, and memory cells, is in secondary lymphoid organs.

In these organs can be distinguished three main populations of B lymphocytes: transitional, follicular and marginal. Transitional B-cells are the most immature, they express markers of bone marrow precursors and may differentiate in both follicular and marginal B. Follicular B cells reside in the follicle and are responsible for the humoral response with high affinity. MZB are present in the marginal zone and are involved in innate (as APCs) and adaptive (low affinity antibody response) immunity. Based on the differential expression of a series of surface markers it is possible to distinguish different B lymphocytes' subtypes (**Table 1**) [1].

Marker/Cell	AA4.1	IgM	IgD	CD23	CD21	CD1d
T1	+	High	-	-	Low	-
T2	+	High	High	+	Low	-
Fo I	-	Low	High	+	Med	-
Fo II	Low	High	High	+	Med	-
MZP	Low	High	High	+	High	+
MZB	-	High	Low	-	High	+

Table 1: markers of peripheral B-cells (Modified from Allman D. et al., Current Opinion in Immunology. 2008)[2].

The different classes of peripheral B-cells have different patterns of markers that define the state of maturation. AA4.1 is a midollar precursor marker and, in peripheral organs, identifies transitional B-cells. CD23 and IgD are markers of follicular B-cells, while CD21 high and CD1 are markers of marginal B-cells. IgM identifies mature B-cells.

Marginal B-cells(MZB)

In mice, MZB cells are ontogenetically distinct from follicular B cells and B-1 cells, selectively occupy the MZ, have an $IgM^{hi}IgD^{low}CD21^{hi}CD23^{-}$ $CD1d^{hi}$ phenotype and primarily express non-mutated immunoglobulin variable (IgV) genes, some of which encode polyreactive BCRs [9]. These BCRs recognize conserved molecular signatures that are often shared by foreign and autologous antigens. As a result of this promiscuous reactivity, antibodies from mouse MZB can recognize and clear bacteria from the external environment and also damaged cells from the host [28].

Mouse MZB also express high levels of TLRs, similarly to myeloid cells of the innate immune system. This dual expression pattern permits them to uniquely integrate signals from both clonally distributed and germline-encoded microorganism-recognition

receptors. Signals generated via dual BCR and TLR engagement induce the extensive production of low-affinity antibodies by MZB, which bridges the temporal gap required for the production of high-affinity antibodies by FoB cells. In mice, similar signals regulate B cell tolerance, indeed, dysregulated co-engagement of BCR and TLR molecules by self antigens contributes to the onset of autoimmunity through the pathogenic activation of autoreactive B-cells (including MZB) [9]. MZB cells are also specialized in the recognition of complement opsonins via the complement receptors CD21 and CD35 and in the recognition of microbial lipids via the MHC class I-like molecule CD1d. After recognizing a microorganism, these B-cells rapidly give rise to non-mutated antibody-secreting plasmablasts via extrafollicular TI or TD pathways. However, mouse MZ B cells can also generate long-lived PCs that secrete high-affinity antibodies via a canonical follicular TD pathway that involves the presentation of peptide–MHC class II complexes to $CD4^+$T helper (T_H) cells, including T follicular helper cells (T_{FH} cells). Indeed, mouse MZ B cells have a high expression level of MHC class II, CD80 and CD86 molecules, which are required for the activation of T_{FH} cells.

Mouse MZ B cells are functionally versatile, as they respond to multiple types of microbial challenge, including TI carbohydrate antigens and TD protein antigens.

The development of MZB cells requires the migration of these cells to the region of the marginal sinus and their retention there until they are activated. Clearly, signals for commitment to a MZ B cell fate (through the BCR, Notch2 and the canonical NF-κB pathway) dictate the acquisition of the ability of the committed cell to migrate, differentiate further, self-renew and be retained in the marginal zone. However, a consideration of the anatomy of the spleen and the location of the ligands for Notch2 supports the notion that 'retention' is an equilibrium state [37].

The sphingosine 1-phosphate receptor (S1PR1) participates in marginal zone B-cell migration and retention *in vivo* and is crucial to overcome CXC-chemokine ligand 13 (CXCL13) mediated attraction of B-cells to the follicles. The lymphocyte function-associated antigen 1 (LFA1) and α4β1 integrins on MZ B cells bind to intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1), respectively, and contribute to the retention of MZ B cells in the marginal zone [37].

The Wiskott-Aldrich syndrome protein (WASP) is a cytoskeletal protein that connects haematopoietic cell surface receptors to the regulation of actin polymerization through the activation of the ARP2–ARP3 complex. The lymphocyte function-associated

antigen 1 (LFA1) and α4β1 integrins on MZ B-cells bind to intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1), respectively, and contribute to the retention of MZ B cells in the marginal zone [19].

Follicular B-cells (FoB)

FoB cells are the population of B lymphocytes more represented in secondary lymphoid organs. This type of recirculating lymphocytes are responsible for the high affinity antibody response.

The follicles are adjacent to the T-zone, inside of which are T helper (Th), which regulate the activation of B-cells [26]. The entry of B-cells in the follicle is regulated by a CXCL13 gradient produced by follicular dendritic cells and stromal cells. CXCL13 interacts with the receptor CXCR5 expressed by B-cells [9].

The activation of FoB lymphocytes is divided into several phases:

1. Th cells are activated in the T zone by dendritic, and after activation, T cells express CD40L [26].
2. B-cells are activated by soluble antigens or membrane antigens presented by follicular dendritic. The antigen binds to BCR and is processed and displayed on the membrane via MHC II;
3. Activated B and T cells migrate to the perifollicular level, where takes place the immunological synapse. This migration is regulated by gradients of CXCL13 and CCL19 / CCL21, which bind to CXCR5 and CCR7 respectively [28]
4. interaction CD40 / CD40L and antigen presentation results in the production by the Th lymphocytes of chemokines (IL-2, IL-4, IL21) that promote the proliferation and differentiation of B-cells and the production of transcription factors CD40 dependent, essential for the formation of GCs [26]
5. within the GC occur two processes: isotype switching and somatic hypermutation;
6. at the end of the maturation process are generated PCs and memory cells.

Fo B cells are characterized by high intensity of expression of IgD and CD23 markers, that identify recirculating B-cells. FoB cells, infact, when activated and become PCs, migrate to the the red pulp to produce the antibodies directly into the bloodstream.

Recirculating B-cells are also present in the bone marrow where they form the niches around the medullary sinusoidal venules [37].

There are two distinct populations of follicular cells:

Follicular type I (FoI) and Follicular type II (FoII). The difference between these two populations is the signal cascade leading to their formation. The follicular type I depend on a strong signal of BCR that activates the Bruton's Tyrosine Kinase (BTK) and inhibits the pathway of Notch2, while the follicular type II do not depend on the antigen and, therefore, the activation of the BCR . The FoII shall nevertheless be considered follicular cells, because they are independent from the activation of the Notch2 pathway (essential for the formation of MZB) and are, therefore, considered an intermediate population that can, with suitable signals, differentiate into marginal or follicular B-cells [7-17].

Transitionl B-cells

Transitional B-cells are immature B-cells that originate from the bone marrow [32].

These cells are identified through the high expression of the marker AA4.1 (CD93) [1], which regulates cell adhesion and identifies the precursors. There are three distinct populations of transitional B-cells: T1 (AA4.1 $^{+}$, CD23^{-}, IgM^{high}), T2 (AA4.1 $^{+}$, CD23 $^{+}$, IgM^{high}) and T3 (AA4.1 $^{+}$, CD23 $^{+}$, IgM^{low}) [1].

Transitional cells are characterized by a low ability to proliferate in the presence of stimuli(for example the anti- IgM), and their survival depends on the signal of the BCR and the trophic factor BAFF [17].

T1 cells do not express CD23 and IgD, and are considered the earliest stage in peripheral B cell maturation.

While T2 cells have follicular markers and have the capacity to recirculate, as mature cells, and, according to the stimulus, can differentiate, both in mature follicular, or in marginal cells [7-17].

T3 cells are an anergic population that expresses the same markers of T2, however, with respect to T2, have a low expression of IgM [7-17].

In fact, it was observed that the response of the BCR to self antigens in immature B lymphocytes IgM^{high}, can lead to a reduction of surface IgM, as in the case of T3[31].

Marginal B-cell precursors (MZP)

In the literature, various methods have been used to define the different populations of mature B lymphocytes, and several studies are in agreement in defining, considering the surface markers, FoB ($IgM^{med/low}$, IgD^{high}, $CD23^{+}$, $CD21^{med}$, $AA4.1^{-}$) and MZB (IgM^{high}, IgD^{low}, $CD23^{-}$, $CD21^{high}$, $AA4.1^{-}$) [7-32].

Instead, it is controversial the characterization of immature cells.

The main nomenclatures are those of Loder and Allmann [1-25].

Loder defines two populations of immature B-cells, T1 and T2, distinguishing these cells by the expression of CD21, CD23, IgD and a marker of differentiation, CD24, which is lost in mature cells.

Allmann, however, distinguishes three populations of transitional cells, T1, T2 and T3, basing the analysis on the expression of AA4.1 (CD93).

The existence of a population of precursor marginal cells (MZP) was proposed by Allmann in 2005. In this study MZP were characterized by cytometry, as $IgM^{high}CD21^{high}$ and $CD23^{+}$, and the same area subpopulation of T2 according to the classification of Loder. MZP differ from T2 for a more intense expression of CD21 and a lower expression of AA4.1.

This result shows that MZP are immature cells, but still in a stage of maturity with respect to the following populations of T1 and T2. AA4.1, in fact, is lost with cell maturation. Moreover, it was observed in knockout mice for Notch2, a reduction of MZB and of MZP, but not a change in T2 [29-39].

From a functional point of view, recirculating MZP are cells that originate in the red pulp, where they receive the stimulation of Notch2, necessary to differentiate into marginal cells. When the marginal zone is not able to receive more cells, MZP go back in the follicle where they accumulate [7].

1.1.5 Splenic B-cell development

Newly formed or T1 B-cells arrive through the central arterioles and the marginal sinus in the white pulp of the spleen [9]. They exit the circulation through fenestrations in the marginal sinus and, in response to a CXCL13 gradient, move into splenic B-cell follicles. These transitional B-cells initially (as T1 B-cells) only require tonic BCR signals for survival but subsequently receive and require both tonic BCR signals and trophic signals from BAFF as they enter the follicle (produced by more than one cell type but mainly by follicular dendritic cells in follicles) and begin to differentiate into recirculating follicular B cells. The B-cells in the follicular zone express high levels of IgD and CD23 and intermediate levels of CD21 and acquire the ability to recirculate. Soon after their generation, these cells still express the AA4.1 marker of immaturity and are therefore defined as T2 cells [39].

An affinity-defined subset of T1 or T2 cells that recognize self antigens with high affinity, but apparently below a threshold that would induce anergy, would presumably activate BTK downstream of the BCR and differentiate into follicular type I B-cells. Another subset of T1 or T2 B-cells that express BCRs that do not recognize self antigens with sufficient affinity to activate BTK signalling, instead, if the marginal zone compartment is not 'full', when these cells encounter DL1 in the splenic red pulp they will be induced to differentiate into marginal zone precursor and MZ B-cells and migrate to and be retained in the marginal zone. The exact mechanism of sensing the pool size of the marginal zone B-cell compartment remains to be determined. The marginal zone does not contain significant numbers of IgD^+ or $CD23^+$cells and it therefore seems less likely that marginal zone precursor B-cells actually reside in the marginal zone; these precursor cells have never been precisely localized.

Recent study demonstrated that marginal zone precursor B-cells respond to CXCL13 and accumulate in the follicle or that they transiently occupy the red pulp as they differentiate into MZ B cells. In either case, some chemotactic signals, probably S1P, contributes in a Gα12 or Gα

13 and LSC-dependent manner to promote the differentiation of marginal zone precursor B-cells and their migration towards the marginal zone. Migration requires WASP to mediate actin reorganization in conjunction with RAC1 and RAC2 and this in

turn contributes to integrin–ligand interactions and the retention of MZB cells in the vicinity of the marginal sinus. Interactions between myeloid cells and marginal zone precursor B-cells induce canonical NF-κB signalling that collaborates with Notch2 derived signals to mediate the differentiation and maintenance of MZB cells during or after completion of the process of migration.

Perhaps, if the marginal zone B-cell compartment is 'full', a T2 B-cell that does not have sufficient avidity for self antigen to differentiate into the BTK-dependent follicular type I B-cell compartment will mature by default into a long-lived follicular type II B-cell. Different studies suggest that follicular type I B-cells are instructed not to respond to DL1, perhaps in response to BTK-derived BCR signals, as they pass through red-pulp venules, and this ensures the existence of a stable recirculating follicular B-cell compartment. As MZ B-cells might need to be regularly stimulated through Notch2, and as it is known that murine red pulp venules are fenestrated, it is probable that MZ B cells slip in and out of venules in the marginal zone to receive signals from DL1. In this model, the decision to become a marginal zone B-cell might be initiated at the T1, T2 or follicular type II B-cell stage. We assume that only self antigens available through the circulation in the bone marrow or the spleen can contribute to the decision to drive a developing B-cell into a follicular type I-B-cell fate. However, if the developing B-cell lacks the ability to become a follicular type I B cell, it might then be induced to assume a MZB cell fate in the red pulp or the marginal zone through weak BCR signals, Notch ligands and BAFF. Based on anatomical considerations we assume that the temporal order of events that drive marginal zone B-cell development is as follows: weak BCR signalling, followed by inductive signals from DL1 and BAFF, followed then by chemotactic signals by either a poorly understood mechanism including S1PR1 or an unknown Gα12- or Gα13-linked receptor, and followed finally by a migration and retention event that involves the activation of integrins. By comparing BCR repertoires of transitional, Fo and MZ B cells, it has been argued that marginal zone B-cells could be derived from T1 B cells (**Fig.8**).

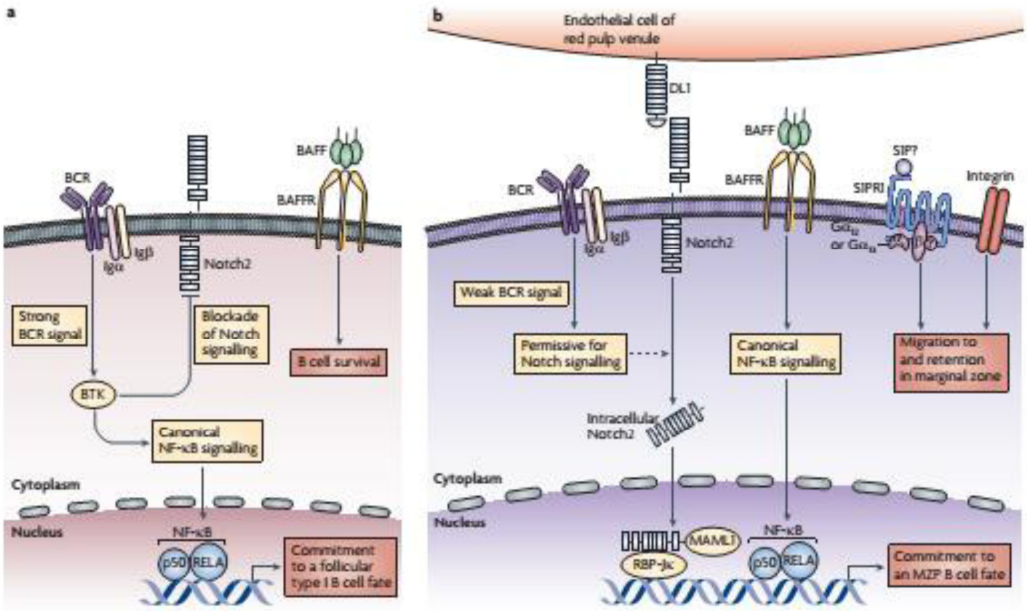

Fig. 8: signals required for the B-cell commitment ***(Modified from Pillai and Cariappa Nature Reviews Immunology 2009).***

a. Strong signalling through the B cell receptor (BCR) activates Bruton's tyrosine kinase (BTK), which in turn activates the canonical nuclear factor-κB (NF-κB) signalling pathway and might instruct B-cells not to respond to signals through Notch2 receptor. Signals through the B cell-activating factor receptor (BAFFR) are required for B cell survival but not for the commitment to follicular type I B cell fate.

b. Although weak BCR signalling is the initial event driving commitment, acquisition of the marginal zone B cell phenotype also requires further signalling pathways. Signals resulting from the interaction of delta-like 1 (DL1) expressed by endothelial cells of red pulp venules with Notch2 on developing B-cells leads to activation of the transcription factors Mastermind-like 1 (MAML1) and RBP-Jκ. Signals resulting from the interaction of BAFF with BAFFR activate NF-κB through the canonical pathway.

1.2 Protein kinase CK2

CK2 is a highly conserved and expressed serine/threonine kinase. It is now abundantly clear that it is a promiscuous enzyme as a diverse and somewhat bewildering array of more than 300 potential substrates have been identified. CK2 partecipates in many cellular process including cell proliferation, survival and differentiation [38]. There is an increasing body of evidence indicating that CK2 is involved in protein kinase networks controlling cell cycle progression and cellular responses to stress including ultraviolet light, heat schock, TNFα. Furthermore, abnormally high levels of CK2 have been observed in various types of cancer both solid tumours (breast, prostate, lung, kidney, neck and head) and hematological malignancies (Multiple Myeloma, Burkitt Lymphoma, Mantel cell Lymphoma and Diffuse Large B-Cell Lymphoma). Based on this involvement in trasformation and tumorigenesis, CK2 has recently attracted attention as a potential therapeutic target.

1.2.1 CK2 structure

CK2 has typically been viewed as tetrameric complex consisting of two catalytic subunits (38-42 kDa) and two regulatory subunits (27 kDa) (**Fig.9**). However the catalytic subunits can perform its activity also as monomer in the absence of the regulatory counterpart. CK2 was distinguished among other protein kinase for its ability to phosporylate serine or threonine residues that are proximal to acidic amino acids. Pinna defined a minimal consensus sequence for phosphorylation by CK2, however there are sites that are efficiently phosphorylated by CK2 despite the absence of this consensus sequence. CK2 has also the ability to phosphorylate tyrosine residues, although the kinetic parameters for this phosphorylation are much less favourable than those in ser/thr residues [24].

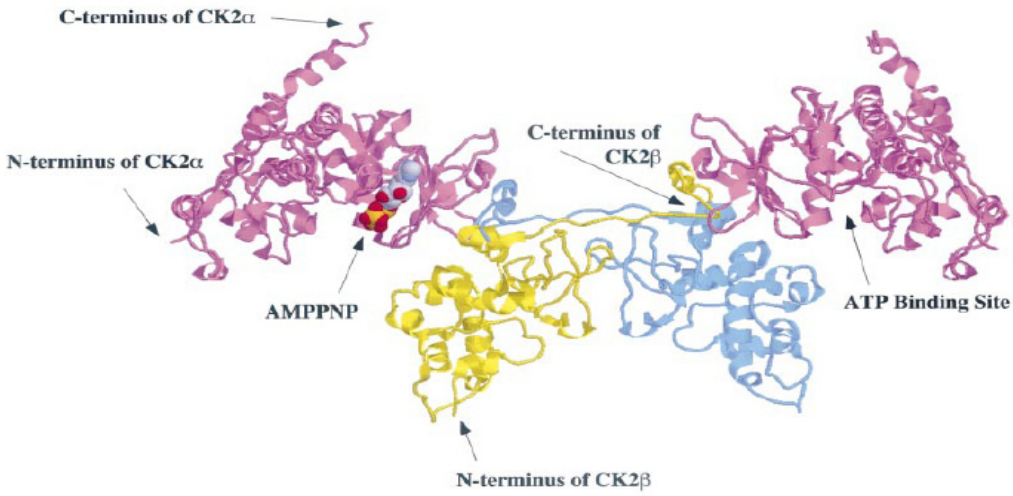

Fig.9: Ribbon diagram illustrating the high-resolution structure of tetrameric CK2.

While consideration of CK2 as a tetrameric complex remains relevant, significant evidence has emerged to challenge the view that its individual subunits exist exclusively within these complexes. Indeed, a lot of data indicate that the regulatory β subunit exists and performs functions independently of tetramers. In particular:

-X-ray crystallography revealed that the CK2α and CK2β interface is relatively small and flexible; this result raises the possibility that CK2 tetramers are subjected to disassembly and reassembly.

-Expression of CK2. Relatively little is known about how either CK2α or CK2β expression is regulated. Earlier studies had shown that CK2β protein was synthesized in excess of the catalytic subunit, underling a lack of coordinated expression. Several reports have also revealed an unbalanced expression of the two subunits in different tissues. For example the level of CK2β in testis was significantly higher in comparison to the level of CK2α.

The intriguing demonstration that aberrantly high levels of CK2β have also been observed in tumors, highlights the importance of understanding the dynamic role of CK2β both within the context of the holoenzyme and as an independent protein.

-Localization of CK2 subunits.

Immunofluorescence studies confirmed that catalytic and regulatory subunits of CK2 are not exclusively co-localized. While the majority of both subunits are localized to nuclear fractions, a major proportion of CK2α was tightly bound to nuclear components whereas CK2β was only loosely associated with other nuclear components. In addition, it was demonstrated in mammalian cells that all the three subunits of CK2 were localized to the smooth endoplasmic reticulum and the Golgi complex, instead only CK2α and CK2α' could be detected in the rough endoplasmic reticulum. In addition to confirm the predominantly nuclear and moderately cytoplasmic localization of both CK2α and CK2β, these studies showed that nuclear import and export of CK2 subunits are regulated independently and can result in rapid changes of their steady-state distribution. However, when associated in a stable holoenzyme complex, the two subunits are dynamically retargeted in the cytoplasm. Moreover, they demonstrated that the binding of fibroblast growth factor 2 (FGF-2) to the holoenzyme provokes its nuclear accumulation, supporting the concept of a signal-mediated localization, which may result in a sophisticated regulation of the kinase**.**

CK2α

In humans, two different forms of the catalytic subunits (designated CK2α and CK2α') which are encoded by distinct genes, were initially characterized. With the exception of their unrelated C-terminal domains, these two isoforms are very similar to one another, exhibiting approximately 90% identity within their catalytic domain. Recently, a third isoform, CK2α", almost completely identical to CK2α, was identified; the only distinguishing feature lies in the completely distinct C-terminal domain. It is known that the different CK2 isoforms are closely related and show considerable functional overlap; indeed, knocking out the gene encoding CK2α' in mice results in variable offspring when heterozigous mice are bred to homozygosity, suggesting that CK2α has the capacity to compensate for CK2α' in the context of viability. However the male are sterile and display defects in speramatogenesis, demonstrating that the functional compensation is not absolute. There is also evidence for functional specialization of the individual CK2 isoforms in yeast, mice and mammals and there may also be differences in the subcellular localization of CK2α andCK2α' [24].

CK2β

In contrast to the catalytic isoforms of CK2, only one known form of the regulatory subunit β has been identified in mammals, but multiple forms have been identified in other organisms, such as *Saccharomyces cerevisiae*. CK2β is remarkably conserved among species and x-ray crystallography studies have determined that a dimer of CK2β subunits forms the core of the CK2 tetramer [24].

A large portion of CK2β has been shown to be phosphorylated at an autophosphorylation site consisting of serine 2, 3 and 4 at its N-terminus.

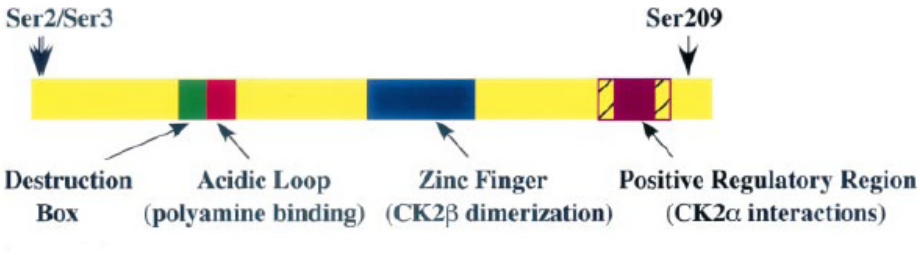

Fig.10: the regulatory CK2β subunit.
Linear representation of CK2β, illustrating the main elements within its amino acid sequence.

It was hypothesized that this autophosphorylation could be mediated by an intermolecular reaction through the formation of higher order CK2 structures and it could enhance CK2β stability. CK2β is also phosphorylated at S^{209} near its C-terminus, a residue which is phosphorylated in a cell-cycle dependent manner by $p34^{cdc2}$**(Fig.10)** .

-It is particularly intriguing that CK2β has motifs that have been previously characterized as motifs that regulate cyclin degradation. Indeed, this sequence is similar to the amino acid motif called destruction box that plays a key role in the specific degradation of cyclin B at the end of mitosis [24].

X-ray crystallography revealed the importance of the zinc-finger region: this sequence is characterized by four cysteine residues which mediate the interaction allowing the CK2β dimer to form the core of the holoenzyme. CK2β dimerization precedes catalytic subunits binding and it is a prerequisite for CK2 tetramer formation.

-C-terminal region is responsible for the ability of CK2β to enhance and stabilize CK2 activity.

-One additional important sequence is the acidic loop: it has been identified as the site on CK2 that binds polyamines which are known to stimulate CK2 activity *in vitro* [24].

Over the last decade a plethora of CK2β-specific interaction partners have been identified through studies performed *in vitro* and *in vivo.* Some of these proteins have undergone more extensive validation allowing for their classification as either CK2 dependent or CK2 independent partners of CK2β.

-CK2-dependent binding partners are proteins that interact with tetrameric CK2 through binding sites on CK2β. Within CK2 complex a major role for CK2β appears to be substrate docking or recruitment where it brings the substrate protein and the catalytic subunit close enough to facilitate the phosphorylation reaction. A second function of CK2β appears to involve transmission of regulatory signals provided by other proteins in manner that could be analogous to that seen with polyamines. FGF-2 exemplifies this, as binding of FGF-2 to CK2β stimulates CK2 activity.

Thus, these two functions of CK2β modulate the ability of CK2 to phosphorylate specific cellular targets**.**

- CK2β independent binding partners are proteins that interact with CK2β in the absence of catalytic subunits. These proteins include A-Raf, c-Mos, and Chk1, that are ser/thr protein kinases containing sequences reminiscent of the CK2β binding region present in the CK2 catalytic subunit. In the case of A-Raf-CK2β interaction it was demonstrated that the presence of CK2α abolishes the activation observed with CK2β, suggesting that CK2α was competing with A-Raf for binding to CK2β. Interestingly, while in the case of c-Mos, the interaction with CK2β leads to down-regulation of the latter, inducing mitotic arrest in rapidly dividing embryonic cells; in the case of A-Raf and Chk1, the kinase activity is enhanced upon interaction with CK2β [24].

1.2.2 Regulation of CK2 in cells

The traditional view of CK2 looks at this protein as a constitutive active [84] and unregulated kinase, nevertheless, several studies support the idea that there are distinct mechanisms contributing to the physiological regulation of CK2:

- the first one is represented by the CK2β subunit that influences CK2 recruitment of the substrate and CK2 localization; moreover, it was demonstrated that the presence of the destruction box in CK2β, and consequently its degradation through proteasome, determines the oscillation of CK2 activity during cell cycle [24].
- Phosphorylation of CK2: several works indicate that phosphorylation is not absolutely required to activate CK2 in a manner analogous to that seen with MAP kinases. However they do not exclude the possibility that phosphorylation partecipates to some degree in aspects of CK2 regulation. Examination of CK2, isolated from mammal cells, has led to the identification of a number of physiological phosphorylation sites on both CK2α and CK2β. Indeed CK2β is phosphorylated at its autophosphorylation site and at Ser^{209}, a residue that is phosphorylated in a cell-cycle dependent manner. Autophosphorylation of CK2β could indirectly regulate CK2 activity. CK2α is phosphorylated in a cell-cycle dependent manner at four sites within its unique c-terminal domain even if these sites do not appear to effect a dramatic change in the catalytic activity of the kinase. CK2 can also be phosphorylated by the Src tyrosine kinases, by c-Abl tyrosine kinase and by the pathological counterpart Bcr-Abl fusion protein (typical of chronic myeloid leukemia). In this last context CK2 activity is inhibited by phosphorylation. [16].
- Protein-protein interaction: it has been shown that CK2 interacts with proteins such as FGF-1, FGF-2, HSP90 (heat shock protein 90) and the cochaperonine cdc37 that may directly alter or stabilize its catalytic activity. CK2 also interacts with tubulin, FAF-1 and cKIP-1, that could be involved in the targeting of CK2 to specific sites or structures within cells. There are three tumor suppressors that bind and inhibit CK2 activity: p53 interacts with the β subunit affecting its

function; in a similar way also p21WAF1 binds to CK2 β; adenomatous polyposis coli protein (APC) inhibits CK2 through the interaction with α subunits [18].

- Role of small molecules in CK2 regulation: CK2 is inhibited by negatively charged compounds such as heparin and activated by positively charged compounds, including polyamine [24].

1.2.3 CK2 functions

CK2 always behaves as an antiapoptotic agent implying on different cellular functions, signalling pathways and biochemical reactions which ultimately cooperate to promote cell survival **(Fig.11).**

- CK2 is a multisite regulator of different signalling pathways that are potentiated by phosphorylation:
  -NFkB: this transcription factor is normally sequestered in the cytosol by the binding to its inhibitor IkB. CK2 acts at different levels: it phosphorylates IkB promoting its degradation through proteasome, increases the expression of IKK kinases, phosphorylates p65 subunit of NF-kB increasing its transcriptional capability.

  -Wnt pathway: in the presence of Wnt, the destruction complex which targets β-catenin to the proteasome is inhibited by the stabilizing protein dishevelled (Dvl). CK2 phosphorylates Dvl and β-catenin promoting their stabilization, and TCF/LEF, facilitating its association to partner molecules;

  -PI3K/Akt: here again CK2 operates as a multisite regulator: a first level is represented by the tumor suppressor PTEN, the phosphatase which dephosphorylates PIP3 (phosphatidylinositol 3, 4, 5 triphosphate), thus maintaining the PI3K/Akt signal down, under resting conditions; it has been demonstrated that the constitutive phosphorylation of PTEN by CK2, while regulating the PTEN protein stability, has an inhibitory effect on its phosphatase activity as well, with the final effect of stimulating Akt-dependent signalling. A second level of CK2 intervention on this pathway is represented by Akt itself:

beside a physical interaction between the two kinases, a direct phosphorylation of Akt on Ser 129 by CK2 has been found, which promotes an hyper-activated state of Akt. [10] There is moreover an indirect effect of this CK2-mediated phosphorylation, since it contributes to maintain an high level of phospho Thr-308, by ensuring a stable association with the chaperone protein Hsp90, known to protect Thr308 from dephosphorylation.

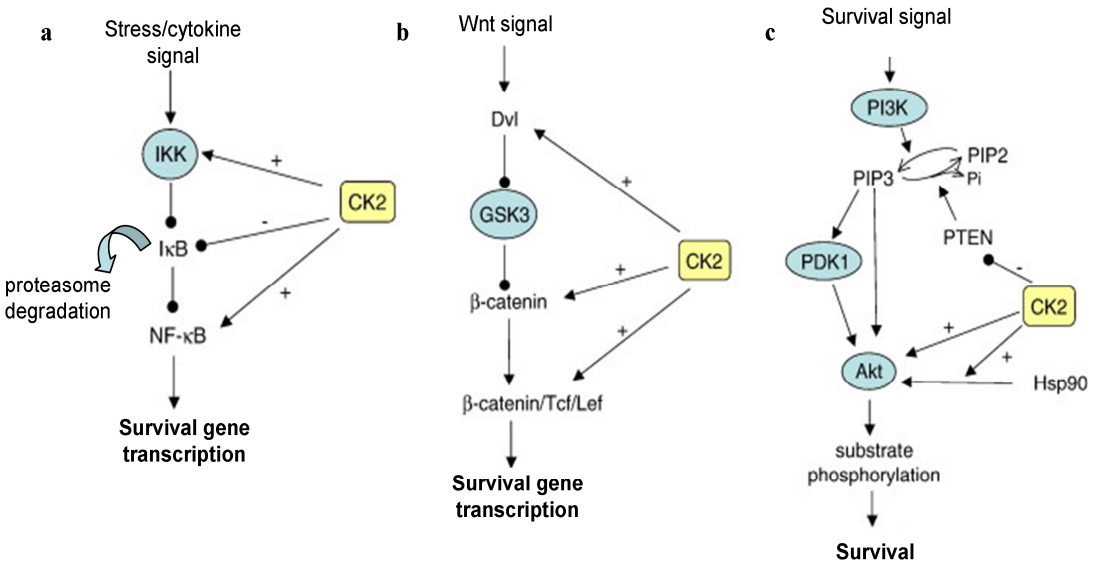

Fig.11: CK2 –dependent multisite regulation of NF-kB (a), β-catenin (b), and Akt (c) signaling.

- Jak-Stat pathway: Zheng and co-workers provided the first evidence that ser/thr kinase CK2 binds and phosphorylates Jak2 and these events are critical for the activation of Jak2-Stat signalling pathway [50].
- CK2 and apoptotic signalling: the caspase inhibitor ARC is phosphorylated and activated by CK2 while survivin, a member of the inhibitor of apoptosis protein (IAP) family, is upregulated whenever CK2 expression is increased. Other CK2 targets are Bid, Max, HS1, presenilin, connexin, whose previous phosphorylation generates caspase resistant sites. Caspase 9 itself falls in this category, since its phosphorylation by CK2 protects caspase 9 from caspase 8 cleavage [27].

- CK2 participates in the regulation of proteins that have important functions associated with cell cycle progression: topoisomerase II, p34, cdc34, $p27^{kip}$**,** MDM2, p21WAF/CIP and p53 [27].
- CK2 cooperates also with proto-oncogenes such as c-Myc, c-Myb, c-Jun, Ha-Ras and A-Raf [38].
- The RNA polymerase I and RNA polymerase II complexes were among the first substrates to be discovered. Then RNA polymerase III was also shown to be target of CK2. Phosphorylation by CK2 of the TATA-binding protein (TBP), a subunit of TFIIIB (the core component of the Pol III transcriptional machinery), promotes a remarkable increase in Pol III activity, favouring the synthesis of tRNA and 5SrRNA. Thus CK2 enhances rRNA and tRNA biogenesis [38]**.**
- Y. Miyata and colleagues demonstrated that the cochaperone cdc37 is a CK2 target. Cdc37 is involved in the folding process of several protein kinases in tight collaboration with Hsp90; however, cdc37 shows molecular chaperone activity *per se*. Phosphorylation of cdc37 by CK2 is essential for the proper function of the chaperone, moreover, CK2 itself operates in a cdc37-dependent manner being directly associated with the latter one. Thus CK2 may control many growth-related protein kinases simultaneously via cdc37 phosphorylation (**Fig.12**) [27].

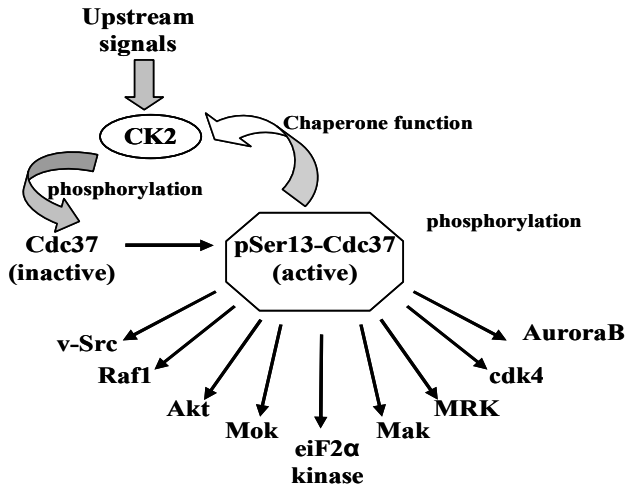

Fig. 12: Cdc37 as substrate of CK2.
CK2 and Cdc37 cooperate to create a positive feedback to control a number of important kinases.

1.2.4 CK2 in lymphoid tumors

The first evidence of a pro-survival role for CK2 in lymphocytes came from *in vivo* mouse models of lymphomagenesis that were prompted by previous demonstration of CK2 involvement in lymphomas in the cattle. CK2α-overexpression favored T-cell lymphomagenesis in T-ALL-transgenic mice. MRL-lpr/lpr mice bearing a Fas receptor mutation develop a lymphoproliferative disease and autoimmuity resembling systemic erythematous lupus. Double MRL-lpr/lpr CK2-transgenic mice displayed an accelerated tumor phenotype characterized by massive enlargement of lymphoid organs due to proliferation of aberrant, though polyclonal, T-lymphocytes. Parallel and subsequent work demonstrated in this model that CK2 cooperates both with loss of the tumor suppressor p53 and with overexpression of the oncogene c-myc in causing lymphocyte proliferation and clonal expansion. These initial studies established a proliferative and apoptosis-protective role for CK2 in T lymphocyte in the mouse. However, a more straightforward role of this kinase in lymphocyte oncogenesis has been recently suggested by studies in human lymphoid tumors, both arising from precursor cells and from mature lymphocytes [36].

CK2 in LLC

In addition to having a role in immature lymphoid precursor derived blood tumors, CK2 has recently been implicated also in the pathogenesis of B-chronic lymphocytic leukemia (B-CLL).

The group of Jaeger et al. found elevated CK2β phospho-Ser209 levels in primary samples from 44 B-CLL patients.

Remarkably, CK2 inhibition with apigenin or TBB caused a reduction in the phosphorylation of PTEN at Ser380 and of AKT at Ser473 and was associated to B-CLL cell apoptosis. Remarkably, the combination of CK2 and

PI3K inhibitors (LY294002) was shown to produce a synergistic cytotoxic effect on B-CLL cells. Importantly, parallel work confirmed the pro-survival function of CK2 in the growth of B-CLL cells. Martins et al. showed that both CK2α and CK2β subunits are elevated in primary B-CLL from patients and CK2 inhibition in B-CLL cells is coupled with inactivation of protein kinase C, increased PTEN activity and apoptosis.

Interestingly, the cytotoxic effect of CK2 inhibition was more pronounced on B-CLL cells isolated from advanced stage (Binet B or C) patients. Normal
T and B lymphocytes were slightly affected by the treatment with CK2 inhibitors.
Of note, in this work the authors used the novel compound CX-4945 as a CK2 inhibitor, which is currently under investigation in phase-I clinical trials in cancer patients [36].

CK2 in MM

CK2 has also been implicated in the pathogenesis of MM. Our group described that CK2 is crucial for malignant plasma cell survival demonstrating that this protein kinase positively regulates STAT3 and NF-kB-dependent signalling (**Fig.13**). CK2 activity and CK2a and b protein levels were found upregulated in MM cell lines and primary cells. CK2a knockdown or CK2 inhibition with TBB and TBB-derived agents caused MM cell apoptosis, which was not counteracted by the addition of growth factors, such as interleukin-6 and insulin-like growth factor-I. We also found that CK2 inhibitors made MM cells more sensitive to the cytotoxic effect of melphalan. Remarkably, CK2 silencing or inhibition was associated to IkBa stabilization and decreased NF-kB transcriptional activity. Similarly, our group demonstrated that CK2 inhibition caused a reduction in STAT3 phospho-Tyr705 and phospho-Ser727 levels, implying this kinase as a positive regulator of two critical pro-survival pathways in MM. Moreover, CK2 could be instrumental for the modulation of MM cell sensitivity to novel therapeutic agents. A recent study has reported that CK2 could represent a central kinase downstream inhibition of the proteasome with bortezomib, thus influencing
the cell phosphoproteome and many signaling pathways upon treatment with this central, widely employed therapeutic agent [36].

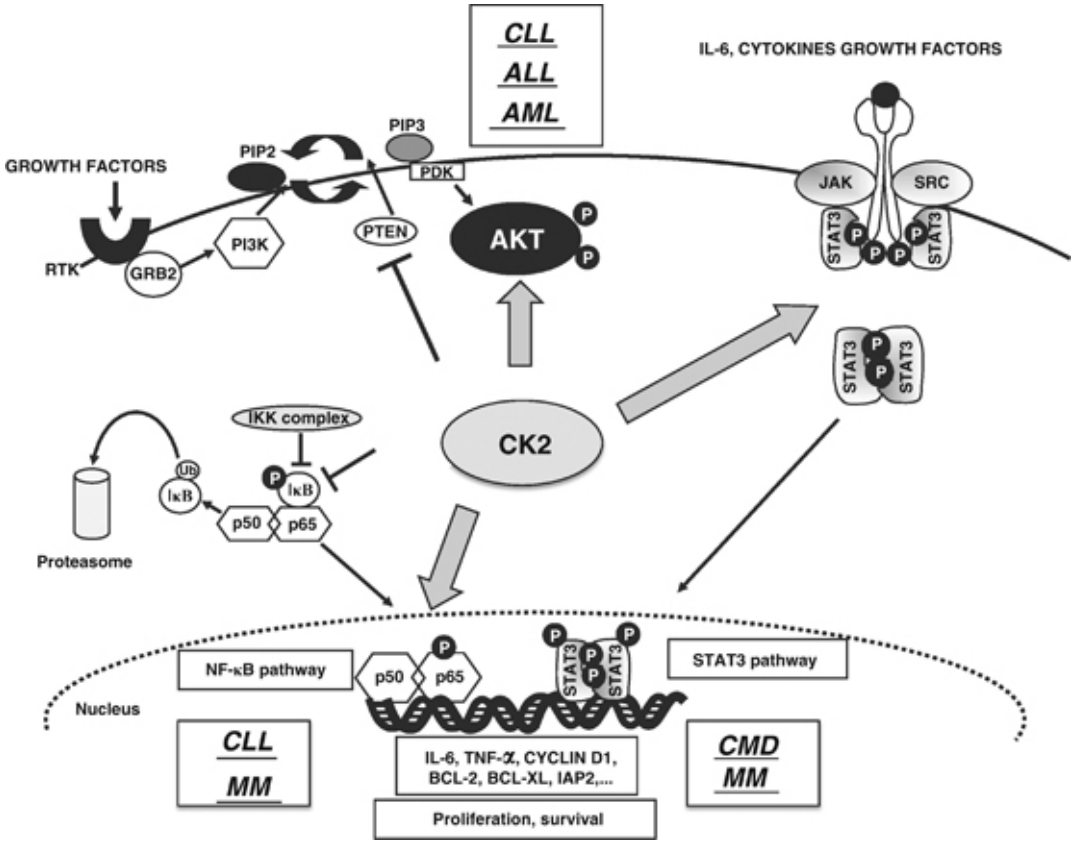

Fig.13:involvement of protein kinase CK2 in blood tumor-associated signaling pathways(Piazza F.,Leukemia 2012).

Among the several signaling pathways regulated by CK2, the modulation of the AKT/PKB/PI3K/PTEN cascade may be critical for the survival and proliferation of ALL, CLL and AML tumor cells; in CMD and in MM. CK2 regulates the extent of JAK/STAT activation downstream of cytokine/growth factor signaling; CK2-mediated phosphorylation of IκBα in its PEST domain leads to IκBα inhibition through proteasome degradation and CK2 phosphorylation of NF-κB p65 on Ser529 causes the activation of the NF-κB pathway and this could be important for CLL and MM cell survival and resistance to cytotoxic therapies

1.3 PROTEIN KINASE CK1

1.3.1 CK1 family and activity

Protein kinase CK1 (formerly called "casein kinase 1") belongs to the superfamily of serine-threonine kinase (STK), proteins constitutively active and localized in many cellular region (nucleus, cytoplasm, cellular membrane and vescicles) [43] [44].

In mammalians, the family of CK1 is composed by seven members encoded by distinct genes (α, β, γ1, γ2, γ3, δ and ε) which display the highest homology in their kinase domain (50-90% identical) and a lower degree of homology in the

N-term and C-term of the protein [45]. The molecular weight vary between 37 kDa of CK1α and 51 kDa of CK1γ3.

CK1 functions as a monomeric kinase, it uses only ATP as phosphate donors and the family members show similar specificity in substrates which are enzymes, transcription factors, cytoskeletal protein and membrane proteins [46].

Canonical CK1 consensus site is S/T(p)-X-X-S/T, where S/T(p) refers to a phospho-serine or a phospho-threonine, X refers to any amino acid and S/T to the target serine/threonine. This consensus site requires priming by another kinase. However, CK1 can also phosphorylate other consensus sites containing a cluster of acidic aminoacids N-terminal to S/T target [45].

Cellular localization of the kinase in the cell is fundamental; infact the phosphorylation event occurs only if the substrate is near to the kinase. CK1 modulates its own activity through auto-phosphorylation in the C-term domain, but some studies demonstrate that it can inhibit itself through dimerization [43] [44].

1.3.2 Role of CK1 in the cell

CK1 plays many different roles in the cell modulating several cellular processes.

- *Functions of CK1 in membrane transport processes*

In eukaryotic cells the subcellular localization of the isoforms CK1α and δ is well characterized. Both isoforms interact with membrane structures of the ER, Golgi and/or Trans Golgi Network (TGN) and various transport vesicles, but their functions in membrane transport have not been elucidated in detail. Furthermore, CK1 isoforms have been found in association with the SNARE complex and phosphorylation of

SNARE-associated proteins is important for the regulation of protein–protein interaction and the synaptic vesicle exocytosis [47].

- Molecular regulation of the circadian rhythm

CK1 is one of the "clock genes", as CK1ε phosphorylates the circadian rhythm gene PERIOD, promoting its ubiquitination and degradation [47].

- Regulation of cell proliferation, division and apoptosis

Several CK1 isoforms have been shown to phosphorylate the tumor suppressor p53. CK1 α, δ and ε are able to phosphorylate S4, S6, S9 and T18 in the p53 molecule, weakening p53 binding to the p53-inhibitor Mdm2 and stabilizing the molecule [45].

However other evidences suggest that CK1α physically interacts with Mdm2 promoting p53 inhibition and degradation as observed in human amelanotic malignant melanoma cell line [48]. Therefore, depending on cell type and/or cellular conditions CK1a could inhibit or activate p53 [49].

CK1α, δ and ε have been implicated in the regulation of cell division by modulating the activity of centrosomal proteins and CK1δ, in response to genotoxyc stress, prevents genomic instability through phosphorylation of tubulin and microtubules associated proteins like MAP4, MAP1A, tau and stathmin [47].

At present, increasing evidence shows that CK1 isoforms are involved in blocking apoptosis through different pathways. Recently, it has been shown that these isoforms, especially CK1α, are involved in mediating tumor cell resistance to TRAIL (Tumor necrosis-factor-Related Apoptosis-Inducing Ligand) induced apoptosis. It is also thought that CK1 phosphorylates death-inducing signalling complex (DISC) providing the cells with resistance against caspases action, inhibiting apoptosis [46].

Furthermore CK1 (isoforms α, δ, and ε) is involved in the regulation of Fas-mediated apoptosis which leads to the activation of caspase 8 [46].

Finally CK1α phosphorylates the RXR class of retinoic acid receptor, inhibiting their ability to induce apoptosis, in a mechanism not clarified in details [47].

1.3.3 CK1 in normal and malignant hematopoiesis

Expression of CK1 in hematopoietic cells has been reported in few studies and its role in normal hematopoiesis is still largely unidentified. However recently new evidence for a potential involvement of CK1 in malignant hematopoiesis are emerging and different members of the CK1 family do not seem to display the
same functions [45].

For example CK1ε up-regulates PTEN activity upon genotoxic stress in myeloid cells, suggesting a "tumor suppressor" function of CK1ε in myeloid malignancies [45].

Another study described the critical involvement of CK1α in the pathogenesis of Activated B-cell-like Diffuse Large B-cell (DLBC) and Non-Hodgkin Lymphomas (NHL) by regulating NF-kB signaling intensity through the control of CARMA1. CARMA1 was identified as a CK1α binding partner and CK1α was shown to be required for NF-kB activation and proliferation in lymphocytes [45].

Thus, these initial reports suggest a potential critical involvement of CK1 members in the pathogenesis of blood tumors and indicate that CK1 is endowed with oncogenic functions in the hematopoietic compartment [45].

1.3.4 CK1 in important pathways of MM

CK1 regulates also molecular pathways which are important for multiple myeloma plasma cells survival, like WNT/β-catenin pathway and PI3K/AKT pathway.

1.3.4.a CK1 and the WNT/β-catenin pathway

All CK1 family members participate in the regulation of canonical WNT/β-catenin signaling pathway are involved. Positive as well as negative regulatory functions for CK1 have been described.

In absence of the WNT ligand CK1α interacts with and phosphorylates Axin, APC and β-catenin (at Ser-45), thereby priming β-catenin for further phosphorylation by GSK3β and subsequent degradation.

In contrast, CK1α phospholylates LRP6 triggering WNT-mediated intracellular signaling [49].

1.3.4.b CK1 and the PI3K/AKT pathway

As mentioned above, CK1 seems to activate PTEN by physical interaction, down-regulating PI3K/AKT signaling [42].

In addiction, CK1α phosphorylates DEPTOR (mTOR inhibitor) which is then targeted to the proteasome, activating mTOR-mediated survival pathways [49].

1.4 GENERATION OF A CONDITIONAL KO MICE MODEL

Conditional mutagenesis is a critical tool to study pleiotropic genes, that exert their functions in several organs and tissues during embryogenesis and adult life.

For this reason, several mouse models have been generated in order to obtain the conditional activation of gene expression in one or more cell types (spatial control) or in specific developmental stages (temporal control). One of the principal strategies used for these purposes, combines the homologous recombination with the properties of the Cre recombinase.

Cre Recombinase is a tyrosine recombinase enzyme derived from the P1 Bacteriophage. The enzyme uses a topoisomerase I like mechanism to carry out site specific recombination events. The enzyme (38kDa) is a member of the Integrase family of site specific recombinase and it is known to catalyse site specific recombination between two DNA recognition sites (loxP sites). This 34 base pair (bp) loxP recognition site consists of two 13 bp palindromic sequences which flank an 8bp spacer region. The products of Cre-mediated recombination at loxP sites are dependent upon the location and relative orientation of the loxP sites. Two separate DNA species both containing loxP sites can undergo fusion as the result of Cre mediated recombination. DNA sequences found between two loxP sites are said to be "floxed". In this case the products of Cre mediated recombination depends upon the orientation of the loxP sites. DNA found between two loxP sites oriented in the same direction will be excised as a circular loop of DNA whilst intervening DNA between two loxP sites that are opposingly orientated will be inverted. The enzyme requires no additional cofactors (such as ATP) or accessory proteins for its function (**FIG.14**).

The enzyme plays important roles in the life cycle of the P1 Bacteriophage such as cyclization of the linear genome and resolution of dimeric chromosomes that form after DNA replication [21-3].

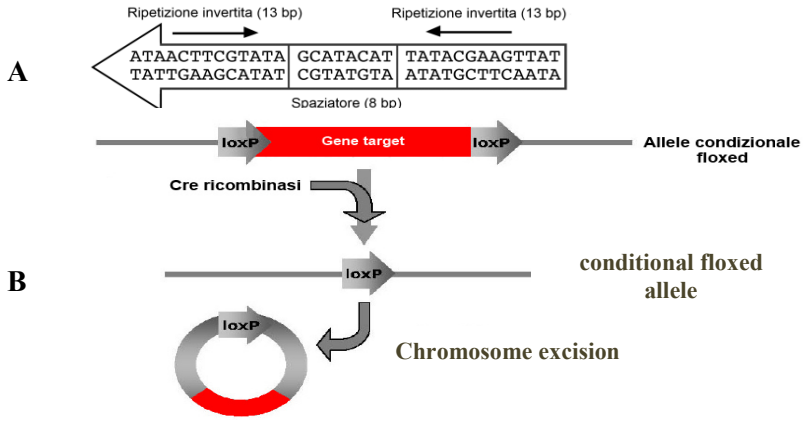

Fig.14: A. ***Canonic loxP sequence (Modified from Araki K et al., BMC Biotechnology 2010); B. Conditional genetic inactivation through the loxP system (Modified from Kim JE, Journal of Korean Endocrine Society 2006).***

Using the combination of gene targeting and site-specific recombination techniques, it is possible to generate *KO* mice in a desired specific tissue or cell line. The rationale consists in using a target vector to insert two loxP sites flanking the gene of interest or a sequence included within the open reading frame (ORF), maintaining its correct expression [6]. The embryo or the homozygous mice that possess both alleles flanked by loxP sites, and transgenic for the expression of Cre recombinase will be characterized by a normal gene function for the tissue or cell type of interest. With the term gene targeting we identify the manipulation of the murine genome based on the homologous recombination. Linear DNA molecules represent the preferred substrate for the homologous recombination, which is more frequent during the S phase of cell cycle. The targeting vector is studied to introduce loxP sites in the desired genomic site in embryonic stem cells (ES). The selection of the recombined ES cells is carried out through a marker gene (generally a gene that confers a drug resistance) inserted in the targeting vector. For example, the gene that codifies the neomycin phosphotransferase (*neo*): only cells that have correctly made the recombination are able to survive when in

their culture medium is present G418, as this drug is lethal for cells that don't bear the neo cassette. The negative selection eliminates ES cells that have incorporated the vector through a mechanism of non-homologous recombination. A marker gene is usually insert externally to the vector region included in the genome. The timidine kinase, isolated from the herpes virus (HSV-tk) constitute an example: if the vector incorporation happens through the linearized far ends, HSV-tk gene is inserted together with *neo* gene. Adding the 2'-fluoro-2'-deoxy-1beta-D-arabinofuranosyl-5-iodouracil (*FIAU*) occurs the negative selection of HSV-tk^{+} cells. After the enrichment of ES cells that bear the correct modified genetic locus, the next step is to generate chimeric mice, that are able to pass on the offspring the mutant gene. For this reason, ES mutant cells are injected in a host blastocyst that originates the chimeric mice after being transplanted in a foster mother. In order to make easier the isolation of mice bearing the mutant gene, ES cells and the host blastocyst derive from mice that express distinguishable pigmentation alleles [6].

Finally, to obtain mice with conditional deletion of the target gene, it is necessary to cross mice that bear the floxed gene with mice that express the Cre recombinase under the control of a tissue or cell type specific promoter (**Fig.15**) [21-3].

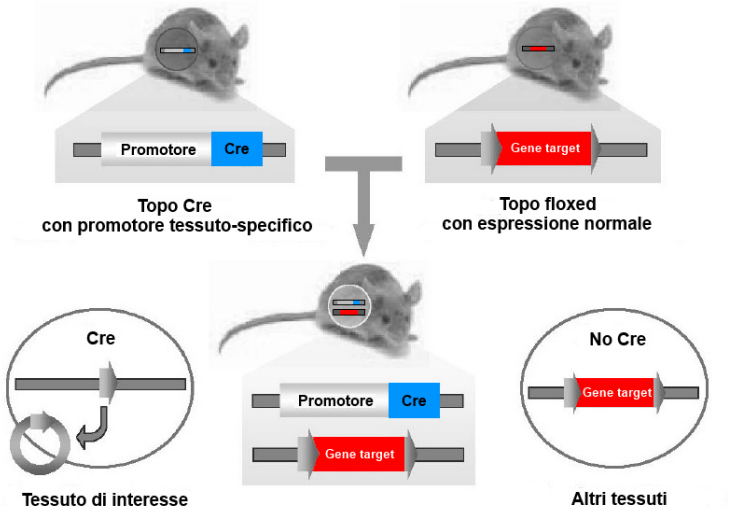

Fig. 15: Tissue specific conditional mutagenesis (Modified from Kim JE, Journal of Korean Endocrine Society 2006).

Target gene is excised by Cre recombinase, selectively expressed in floxed mice. Arrows indicate the orientation of the loxP sites.

The offspring will have the gene deleted only in the cells that express the integrase. Another accurate control of recombination is possible if these mice are transgenic also for a reporter gene, the expression of which is activated by Cre recombinase. A common example is the β galactosidase gene (LacZ), included in ROSA26 locus, that is active only after the excision of a floxed STOP codon. The efficiency of recombination can be site-dependent, for this reason the recombination pattern obtained is not necessary comparable for different genes. Another important parameter is the control of potential phenotypes generated by the only presence of the Cre transgene.

2. AIM OF THE STUDY

Protein kinase CK2 and CK1 are a pleiotropic and evolutionary conserved serin-threonin kinase that is involved in several cellular processes. A number of studies revealed many mechanisms through which this kinase regulates cell cycle, apoptosis, cell survival and tumorigenesis.

CK2 participates in many developmental pathways, of which particularly relevant for hemo-lymphopoiesis are those dependent on Hedgehog, NF-κB and STAT3, which regulate cell differentiation, proliferation, self-renewal as well as lineage choice commitment.. CK1 regulates also molecular pathways which are important for multiple myeloma plasma cells survival, like WNT/β-catenin pathway and PI3K/AKT pathway.

However, despite all this data, little is known about the role of CK2 and CK1 in B-lymphopoiesis and lymphomagenesis.

To elucidate the physiological and pathogenetic role of CK2 and CK1 in B-lymphocytes, we generated B-cell specific conditional KO mice, were we studied the effects of deletion during normal B-cell development. Aim of this study is optimize a analysis strategy using a multiparameter flow cytometry.

3. MATERIALS AND METHODS

3.1 Generation of conditional CK2β KO mice in the hematopoietic compartment

CK2β KO mice in hematopoietic cells were generated through several sequential crossings.

The first cross was made between C57BL6 mice homozygous for the *Csnk2b floxed* ($CK2\beta^{Fl/Fl}$) allele (C. Cochet, Grenoble), with C57BL6 mice hemizygous for the transgene *Cd19-Cre* ($Cd19^{+/Cre}$) (Jackson laboratories). This cross originates mice with the genotype $CK2\beta^{+/Fl}$ $Cd19^{+/Cre}$ (heterozygous mice). To obtain KO mice, heterozygous mice were then crossed with $CK2\beta^{Fl/Fl}$ mice. The genotype of our KO mouse model possesses the following genotype $CK2\beta^{Fl/Flx\backslash}$ $Cd19^{+/Cre}$.

Figure 16 shows $CK2\beta^{Fl}$ and $Cd19^{Cre}$ alleles.

A

Fig.16: A. $CK2\beta^{Fl}$ allele.

Rectangles represent the exons of the *Csnk2b* gene. The first exon is indicated by the white rectangle, with an arrow that indicates the transcription initiation site. LoxP sites are indicated by black arrowheads. The promoter (not highlighted in the picture) is located between the first loxP site and the first exon.

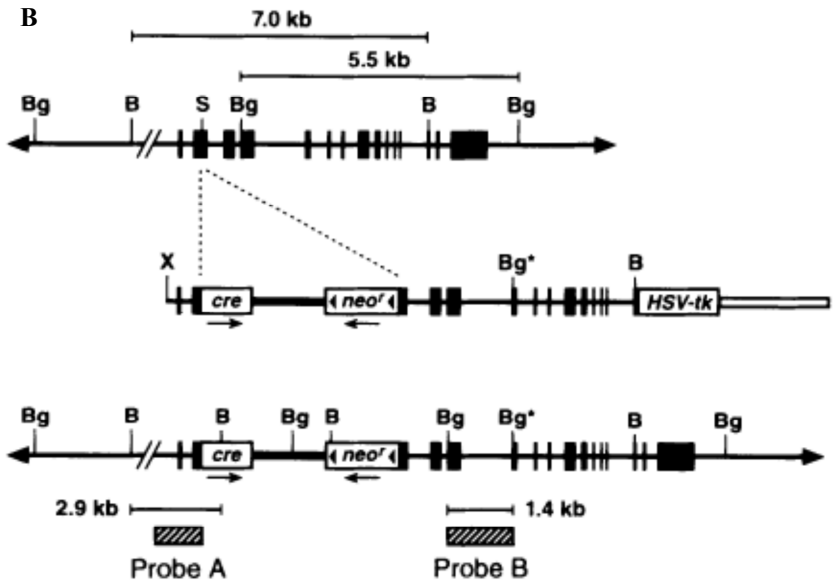

Fig 16: B) Cd19-Cre transgene.

A targeting vector containing Cre recombinase, the rabbit beta-globin intron /poly A signal sequence, and an FRT-flanked neomycin resistance gene, was used to disrupt exon 2 of the Cd19 gene and to express cre under the regulation of the endogenous promoter. Herpes simplex virus thymidine kinase gene was placed at 3' of the Cd19 sequence to allow for the selection against random integration. The construct was transfected into 129P2/OlaHsd-derived E14-1 embryonic stem cells. Correctly targeted ES cells were injected into 129/Sv blastocysts. The resulting chimeric animals were backcrossed to BALB/c IgHb congenics for a number of generations and then backcrossed to C57BL/6 for 10 generations.

3.2 Generation of conditional CK1α KO mice in the early B cell lineage

CK1α KO mice in the early B cell lineage were generated through several sequential crossings.

The first cross was made between C57BL6 mice homozygous for the *Csnk1a1 floxed* (CK1 $\alpha^{Fl/Fl}$) allele (genOway), with C57BL6 mice hemizygous for the transgene *mb1-Cre* ($Mb1^{+/Cre}$) (E. Hobeika, Freiburg). This cross originates mice with the genotype CK1 $\alpha^{+/Fl}$ $Mb1^{+/Cre}$ (heterozygous mice). To obtain KO mice, heterozygous mice were then crossed with $CK1\alpha^{Fl/Fl}$ mice. The genotype of our KO mouse model possesses the following genotype $CK1\alpha^{Fl/Fl}$ $Mb1^{+/Cre}$.

Figure17 shows $Mb1^{Cre}$ alleles and $CK1\alpha^{Fl}$.

A

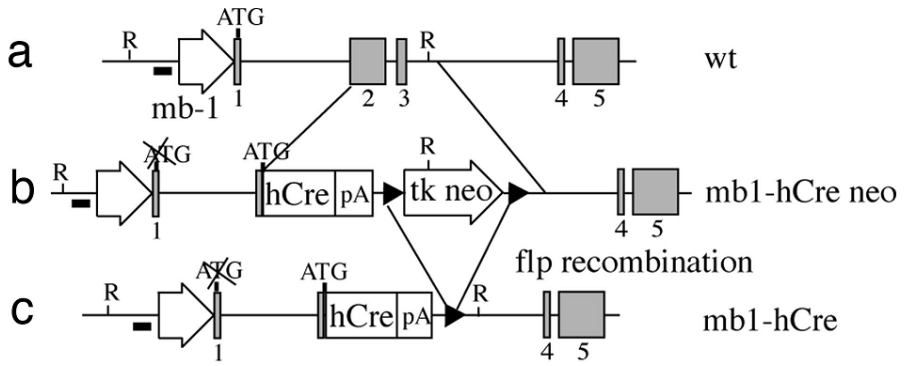

Fig. 17A: Targeting construct for Mb1 locus with hCre recombinase.
Mb1 WT locus (a) and Mb1 locus (b-c) targeted by the Mb1-cre and the delation of the neo cassette by flp recombinase. The endogenous Mb1 ATG in exon 1 was deleted. The FRT sites are rapresented by filled blackwheads, and the tk neo cassette is rapresented by an open arrow. The Mb1 exson are shown as grey boxes numbered 1-5.

B

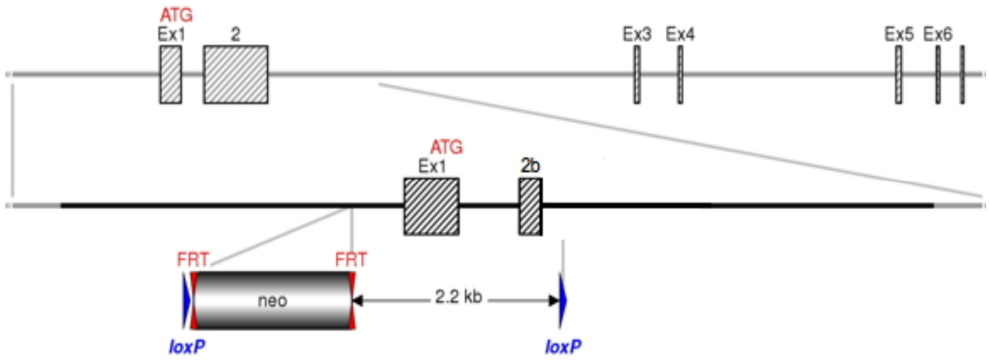

Fig. 17B: Insertion of loxP site in Csnk1a1 endogenus locus
Hatched rectangles represent Csnk1a1 coding sequences, grey rectangles indicate non-coding exon portions, solid lines rapresent chromosome sequences. The neomycine positive selection cassette is indicated. LoxP sites are rapresented by triangles and FRT sites by double triangles.

3.3 Isolation of genomic DNA from mice tales

The far ends of tails of adult mice were digested for 3 to 4 hours at 55°C, after having been resuspended in 500µl of a suitable lysis buffer, the composition of which is described below:

- 250 µL of *mouse tail* 2X lysis buffer,
- 25 µL di Proteinase K 10 mg/mL (Ambion, Life Technologies, Carlsbad, USA),
- 25 µL of SDS 10% w/v,
- H_2O Milli-Q to 500 µL.

mouse tail 2X lysis buffer contains:

- Urea 8M,
- EDTA (pH 8.0) 20 mM,
- SDS 1% w/v,
- Tris-HCl (pH 8.0) 1M,
- NaCl 5M.

Each sample was subsequently centrifuged at 13,000 rpm for 10 minutes at 4°C and the supernatant was collected and transferred to a new eppendorf containing 1ml of absolute ethanol and mixed vigorously.
This leads to the formation of a white precipitate that corresponds to genomic DNA.
Samples were centrifuged at 13,000 rpm for 30 minutes at 4°C, supernatant was discarded and pellet dryed up at room temperature for 10 minutes.
DNA was then rehydrated with 500µl of H_2O and stored at 4°C till usage.

3.4 Protocol for mouse genotyping

Amplification of the CK2β^{Fl} allele

The presence of the CK2β^{Fl} allele was determined through PCR using the following primers (F = Forward; R = Reverse):

- F (BB3): 5′-CTAGCTCGAGATGAGTAGCTCTGAGGAGGTG-3′
- R (BB4): 5′-GGATAGCAAACTCTCTGAG-3’

The reaction mix for one sample is composed by:

- 12.5 µL ofREDTaq® ReadyMix™ (Sigma-Aldrich, Steinheim, Germania), containing Tris-HCl (20mM, pH 8.3), KCl (100 mM), $MgCl_2$ (3 mM), gelatine (0.002%), dNTPs and DNA *Taq* polimerase (0.006 Units/µL),
- 1.0 µL of each primer (20 pmol/µL),
- 8.5 µL of H_2O, included in the commercial kit,
- 2.0 µL of purified genomic DNA.

The thermal protocol of the PCR reaction is listed below

Amplification protocol		
94°C	5min	Initial denaturation
94°C	30sec	Denaturation
55°C	30sec	annealing of primers to genomic DNA
72°C	2min	Extension
72°C	7min	Final extention
4°C	Endless	Hold
denaturation, annealing and extension: 40 repeats		

The amplification products were separated by gel electrophoresis on a 1.5% agarose gel. The size expected for the amplification products are between 400
and 600 bp.

Detection of the *Cd19-Cre* transgene

The CD19-Cre knock-in/knock-out allele has the Cre recombinase gene inserted into the first coding exon of the CD19 gene; abolishing endogenous Cd19-gene function and

placing cre expression under the control of endogenous Cd19 promoter/enhancer elements. Cre recombinase expression is directed at the earliest stages and throughout B-lymphocytes development and differentiation.
PCR reaction is carried out using the following primers (F = Forward; R= Reverse):

- Primer for transgene allele:
  - F: 5'-GCG GTC TGG CAG TAA AAA CTA TC-3'
  - R: 5'-GTG AAA CAG CAT TGC TGT CAC TT-3'
- Primer for wild type allele:
  - F: 5'- CCT CTC CCT GTC TCC TTC CT-3'
  - R: 5'-TGG TCT GAG ACA TTG ACA ATC A-3'

The reaction mix for one sample is composed by:

- 12.5 µL of REDTaq® ReadyMix™ (Sigma-Aldrich, Steinheim, Germany), comtaining Tris-HCl (20mM, pH 8.3), KCl (100 mM), $MgCl_2$ (3 mM), gelatine (0.002%), dNTPs and DNA *Taq* polimerase (0.006 Units/µL),
- 1.0 µL of each primer (20 pmol/µL),
- 6.5 µL di H_2O, included in the commercial kit,
- 2.0 µL of genomic DNA.

The thermal protocol of the PCR reaction is listed below:

Amplification Protocol		
94°C	3min	Initial denaturation
94°C	30sec	Denaturation
62°C	1min	annealing primers-genomic DNA
72°C	1min	Etesio
72°C	2min	Final extension
10°C	endless	Hold
denaturation, annealing and extension: 35 repeats		

The size of the expected amplification products are: 477 bp for the *wild type* allele and 100 bp for the *transgene*. In heterozygous mice are present both amplification products. The amplification products were separated by gel electrophoresis on a 1.5% agarose gel.

3.5 Isolation of hematopoietic organs

Spleen, thymus, lymph nodes were isolated from mice 8-12 weeks old. Organs were extracted and put in a dish containing PBS.

They were disrupted through pipetting and the solution obtained was filtered with a cell strainer of 70 μm (Becton Dickinson, Milan, Italy) posed on the top of a 50ml Falcon tube.

Cell strainers were washed several times with RPMI-1640 supplemented with 1% v/v (100 U/μl) antibiotics (penicillin/streptomycin, Euroclone, Italy) and 10% v/v of fetal bovine serum (FBS, Euroclone, Italy).

After this passage, tubes were centrifuged at 1200 rpm for 5 minutes at 4°C. The supernatant is discarded and cells resuspended in PBS 1X without Calcium and Magnesium.

To analyze bone marrow cells, femurs and tibiae were carefully cleaned from adherent soft tissue. The tip of each bone was removed with a rongeur and the marrow was harvested by inserting a syringe needle into one end of the bone and flushing with RPMI. Bone marrow cells were filtered with a cell strainer of 40 μm (Becton Dickinson, Milan, Italy) on the top of a 50ml Falcon tube. Cells were counted after staining with trypan-blue dye.

3.6 Splenic B-cells purification

Splenic B-cells from mice were purified using EasySep™ Mouse B Cell Isolation Kit (Stemcell).

EasySep™ Mouse B Cell Isolation Kit is designed to isolate B-cells from single cell suspensions of splenocytes by negative selection. Unwanted cells are targeted for removal with biotinylated antibodies directed against non-B-cells (CD4, CD8, CD11b, CD43, CD49b, CD90.2, Ly-6C/G (Gr-1), TER119) and streptavidin-coated magnetic

particles (RapidSpheres). Labeled cells are separated using an EasySep magnet without the use of columns. Desired cells are poured off into a new tube.
This procedure is used to process 0.25 - 2 mL of sample (up to 2 x 10^8 cells).

1. Cell suspension was prepared at a concentration of 1 x 10^8 cells/mL in recommended medium (PBS + 2% FBS with 1 mM EDTA) in a 5 mL polystyrene tube to properly fit into the EasySep Magnet. Normal Rat Serum was then added (50 µL/mL) to cells.
2. EasySep Mouse B Cell Isolation Cocktail was then added (50 µL/mL) and cells were incubated at room temperature for 10 minutes.
3. EasySep Streptavidin RapidSpheres (75 µL/mL), previously vortexed for 30s'', were added to cells and incubated at room temperature for 2.5 minutes.
4. Cell suspension was brought up to a total volume of 2.5 mL by adding recommended medium. The tube was inserted into the magnet at room temperature for 2.5 minutes.
5. In one continuous motion magnet and tube were inverted, pouring off the desired fraction into a new 5 mL polystyrene tube. The magnetically labeled unwanted cells will remain bound inside the original tube, held by the magnetic field of the EasySep Magnet (**Fig.18**).

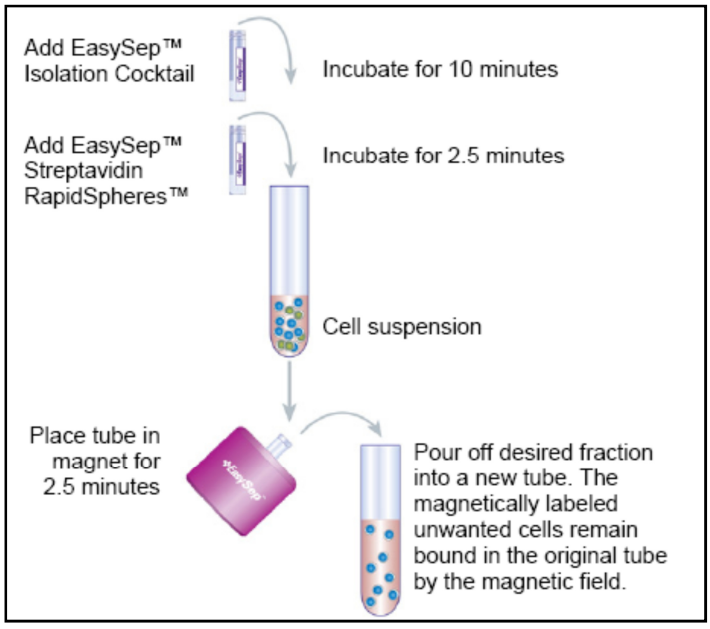

Fig.18 Manual Easysep protocol diagram

3.7 B-cells culture

B-cells purified from the spleen of mice were coltured at a density of 10^6 cells/ml in RPMI medium plus 10% FBS and 2-mercaptoethanol for 36 h with 1 μg/ml anti–mouse CD40 (clone HM40-3; BD) +/- 15 μg/ml goat anti–mouse IgM F(ab′)2 fragments (Jackson ImmunoResearch Laboratories, Inc.) +/- 10ng/ml murine IL-4 (Sigma).

3.8 Splenic B-cells stimulation

For *in vitro* class switch assays, B-cells were cultured at 10^6 cells/ml with 20ug of Lipopolysaccharide (LPS; Sigma-Aldrich) and recombinant murine IL-4 at 10ng/ml to induce switching to IgG1 or with LPS alone to induce switching to IgG3.
The percentage of IgG1 or IgG3 expression was measured by flow cytometric analysis after 3 days of stimulation and using anti-mouse IgG1, IgG3 and anti-B220.

3.9 Flow citometry

To investigate the role of CK2β and CK1α during lymphopoiesis, 500,000 cells from organs of mice, sacrificed between 8 and 12 weeks, were stained.
The staining was performed in 5 ml tubes in a maximum volume of 100 ul of Phosphate Buffered Saline (PBS) without Calcium and Magnesium. Prior to the staining, cells were incubated in the dark for 5 minutes, at 4°C, with CD16/CD32 Antibodies (FcBlock, BD Bioscences).
Then cells were stained with the following antibodies:

ANTIBODY	FLUOROCHROME	COMPANY
CD21/CD35	FITC	BD Biosciences
CD43	FITC	BD Biosciences
CD3	FITC	BD Biosciences
IgD	FITC	BD Biosciences
Gr-1 (Ly6GELY-6C)	FITC	BD Biosciences
B220/CD45R	FITC	BD Biosciences
CD19	PE	BD Biosciences

Mac-1(CD11B)	PE	BD Biosciences
IgM	PE	BD Biosciences
CD23	PE	BD Biosciences
CD8	PerCP-Cy5.5	BD Biosciences
CD19	PerCP-Cy5.5	BD Biosciences
Streptavidin	PerCP-Cy5.5	BD Biosciences
CD25	APC	BD Biosciences
B220/CD45R	APC	BD Biosciences
CD21/CD35	APC	BD Biosciences
IgG1	APC	BD Biosciences
IgM	PE-Cy7	BD Biosciences
cKit(CD117)	PE-Cy7	BD Biosciences
B220/CD45R	APC-Cy7	BD Biosciences
IgD	V450	BD Biosciences
biotin IgG3		BD Biosciences
CD138	PE	BD Biosciences
CD38	PE	BD Biosciences
CD95	PE-CY7	BD Biosciences
CD24	PerCP-Cy5.5	BD Biosciences
CD127	BV421	BD Biosciences
AA4.1/CD93	PE-594	BD Biosciences

After the staining, cells were acquired through FACSCanto I and FACSAria III (BD Biosciences, NY, USA). FACSCanto I is a cell analyser with two laser 488(Blue) and 633(Red) which can acquire up to six fluorescence signal. FACSAria III is a cell sorter with four laser 405(Violet), 488(Blue), 561(Yellow-Green) and 633(Red) which can acquire up to fifteen fluorescence signal.

The analysis of dot plots was carried out with FacsDiva (BD) and FlowJo software (TreeStar).

Flow Cytometry Protocol Summary:

BrDU staining:

1. Harvest BM from BrdU-treated mice and collect 1-1.5x106 cells/tube
2. Add Fc blocking (4uL dil 1:10)
3. Add the following surface antibodies in staining buffer*:
    -B220 APC-Cy7
    -CD25 PE
    -CD24 Percp Cy5.5
    -CD127 BV421
4. Mix well and incubate on ice for 20 min.
5. Wash with staining buffer once. Centrifuge for 4 min at 1200 rpm at 4°C.
6. Aspirate of the supernatant.
7. Resuspend cells in 100 uL of Cytofix/Cytoperm Buffer and incubate on ice for 20 min.
8. Wash the cells with 1 mL of 1X Perm/Wash Buffer. Centrifuge for 4 min at 1200 rpm at 4°C. Discard the supernatant. [Washing fixed cells and store at 4°C overnight in staining buffer to proceed the day after with intracellular staining].
9. Resuspend the cells in 80 uL of Cytoperm Permeabilization Buffer Plus.
10. Incubate the cells for 10 min on ice.
11. Wash the cells with 1 mL of 1X Perm/Wash Buffer. Centrifuge for 4 min at 1200 rpm at 4°C. Discard the supernatant.
12. Re-fix cells: Resuspend cells in 100 uL of Cytofix/Cytoperm Buffer and incubate on ice for 5 min.
13. Wash the cells with 1 mL of 1X Perm/Wash Buffer. Centrifuge for 4 min at 1200 rpm at 4°C. Discard the supernatant.
14. Treat cells with DNaseI to expose incorporated BrdU: resuspend the cells in 100 ul of diluted DNase (diluted to 300 ug/ml in DPBS with Ca2+ and Mg2+) per tube (ie, 30 ug of DNase/106 cells).
15. Incubate at 37°C for 1h.
16. Wash the cells with 1 mL of 1X Perm/Wash Buffer. Centrifuge for 4 min at 1200 rpm at 4°C. Discard the supernatant.

17. Stain for BrdU: resuspend the cells with anti-BrdU FITC antibody (15 uL/tube) diluted in Perm/Wash Buffer and incubate at room temperature for 30 min.
18. Wash the cells 1 mL of 1X Perm/Wash Buffer. Remove the supernatant.

Stain total DNA for cell cycle analysis. Resuspend the cells in 10 ul of the 7-AAD in PBS for 20 min on ice.
Acquire stained cells on a flow cytometer.

*Staining Buffer: Dulbecco's PBS without Mg2+ or Ca2+, 1% inactivated FCS (or BSA 3%), 0.09% NaN3; adjust pH 7.4-7.6 and filter. Store at 4°C.

CFSE stainig:

1. Resuspend B cells in PBS at concentration of 20x10^6 cell/ml
2. Add CFSE at final concentration of 5uM
3. Incubate 5 min at 37°C
4. Block staining with 1/5 of FCS on total volume (400ul/2mL)
5. Wash cell with PBS+FCS 2%
6. Plate cells in 6 well plate
7. Add stimulus LPS , LPS+IL4
8. Harvest B cells from well and collect 1-1.5x106 cells/tube
9. Add Fc blocking (4uL dil 1:10)
10. Add the following surface antibodies in staining buffer:
    -B220 APC-Cy7
    -CD138 PE
    -IgG3 biotin-streptavidin Percp Cy5.5
    -IgM PEcy7
    -IgG1 APC

3.10 Statistical analysis

Experiments were performed at least three times. We used t-test to analyze data as appropriate. We considered p values < 0,05 as significant. Analyses were performed using Excel (Microsoft Office) or Origene 7.0 software.

3.11 Software

Sono stati utilizzati per l'acquisizione e l'analisi dei dati citoflorimetrici *BD Cell Quest Pro*, *BD FACSDiva*, *FlowJo* v.X.
I grafici di dispersione e gli istogrammi sono stati realizzati con *GraphPad Prism* v.VI e con *Microsoft Office Excel*.

4. RESULTS

4.1 Generation of CD19 conditional CK2β KO mice

In order to obtain a conditional knockout mice for CK2β, we performed crossings of CK2β Flox / Flox mice (generated by Dr. B.Boldyreff , Grenoble) with CD19Cre / Cre mice (Jackson Lab) .

The first generation of mice obtained were heterozygous for the two loci.

Heterozygotes mice were crossed with each other , generating Flox / Flox , Cre / + (KO) and + / + , Cre / + (CTRL) mice.

For the experiments we only used CD19 Cre/+ mice , because CD19 Cre/Cre mice have an abnormal phenotype in the development of B lymphocytes. Mice obtained were viable, fertile and had a normal phenotype. Mice genotype was verified by a PCR (**FIG.19**).

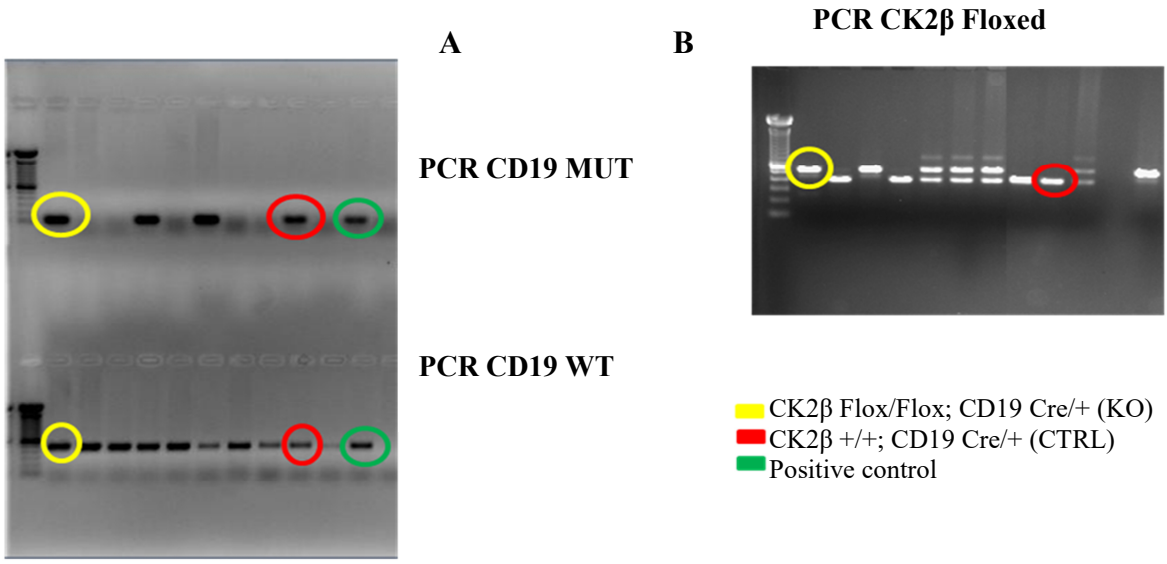

Fig.19: mice genotyping.

A. PCR for CD19 mutated and CD19 wt
B. PCR for the site CK2β. The positive control (C +) is a CK2βFlox / Flox mouse.

The PCR to determine the presence of the WT CD19 gene, provides a 477 bp fragment, while the mutant CD19 provides a 100 bp fragment. In the PCR for CK2β gene, product of 550 bp and 400 bp are expected for Flox and WT, respectively.

In Figure 19 are shown the PCR products of 10 mice, generated with the previously described crossings.

4.2 CK2β KO mice present an evident splenomegaly

CK2β KO and CTRL mice were sacrified between 8 and 12 weeks.

During the isolation of organs, the spleen of CK2β KO mice showed an increase in size and weight (splenomegaly) (**Fig.20**), while the remaining hematopoietic organs did not show any phenotypic alteration.

In addition, the staining with the trypan-blue dye, showed, an increase in cell count in CK2β KO mice (**Fig.20**).

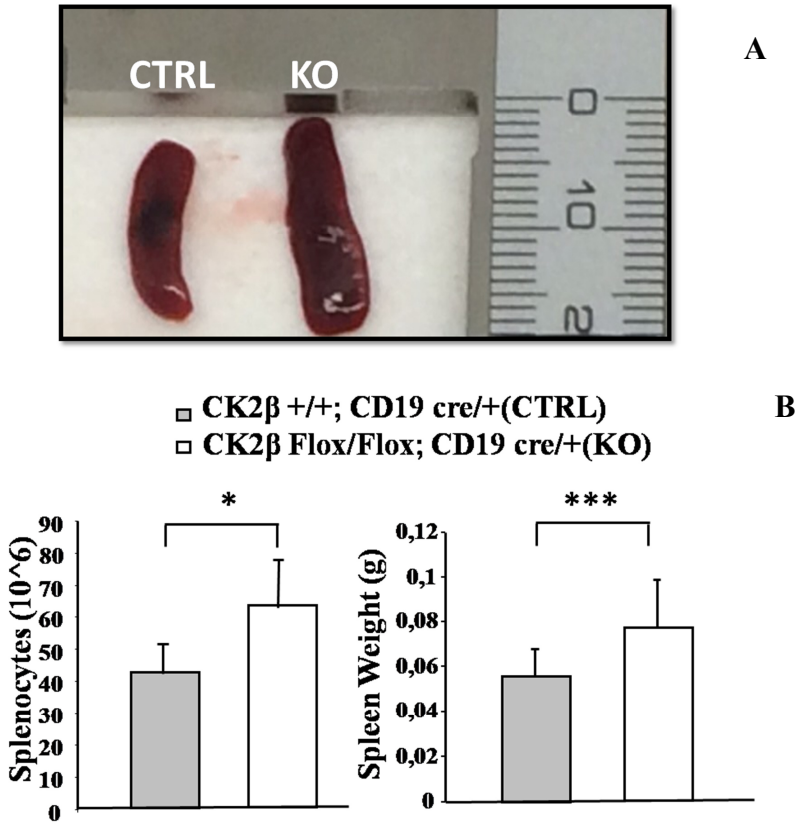

Fig.20: CK2β KO mice are characterized by a marked splenomegaly.
A. CK2β KO mice showed a phenotypic alteration (splenomegaly) when compared to CTRL mice.
B. Histograms summarizing the increase in cellularity and weight of spleens in CK2β KO mice compared to CTRL mice.

4.3 Quantification of total B-cells

In order to assess potential differences in the percentage of B-cells present in the hematopoietic organs of CK2β KO and CTRL mice, flow cytometryc analysis was performed using fluorescent antibodies that recognize specific markers for these cells: CD19 and B220.

We analyzed bone marrow, spleen, and peripheral blood (**Fig.22**).

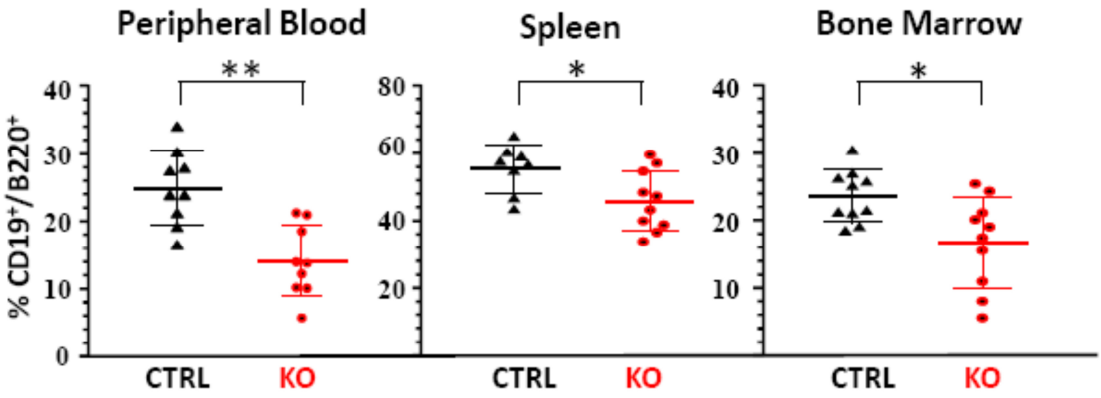

Fig. 22: percentage of B-cells in CK2β KO and CTRL mice.
The analysis was performed with FACSCalibur, using CD19 and B220 markers in peripheral blood, spleens and bone marrow. Graphs show mean and standard deviation (SD). Numerosity: peripheral blood (CTRL = 8, KO = 9); spleen (CTRL = 8, KO = 10); bone marrow (CTRL = 10, KO = 10). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

As observed in figure 22, in peripheral blood, spleen and BM, there is a significant reduction in B-cells ($p < 0.05$) in CK2β KO when compared to CTRL mice.

There is no variation of B lymphocytes in the lymph nodes (data not shown).

The most significant varation was observed in peripheral blood ($p = 0.00155$), indicating a possible reduction of the recirculating B-cell population.

4.4 Analysis of recirculating B lymphocytes

After quantification of total B-cells, we analyzed the populations of naive B-cells ($CD19^{+}/B220^{+}$), newly formed lymphocytes, and recirculating B lymphocytes ($CD19^{high}/B220^{high}$), the most mature component of B lymphocytes. Flow cytometric

analysis showed a reduction of the ($CD19^{high}$/$B220^{high}$) in KO mice, in particular in the bone marrow (**Fig.23**).

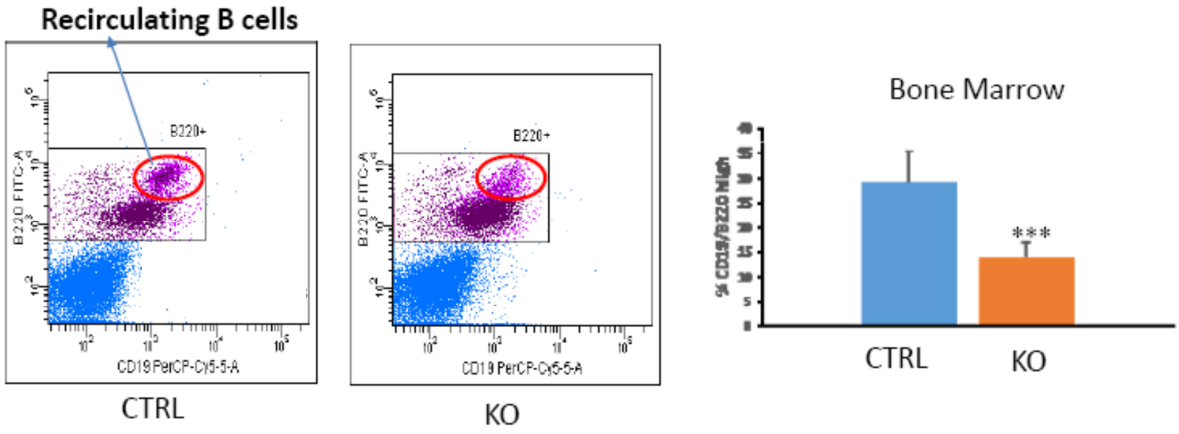

Figure 23: reduction of recirculating B lymphocytes in KO mice.
The analysi was performed with FACSCalibur flow cytometric for the markers CD19 and B220 in the peripheral blood, spleen, bone marrow. The graphs show the mean and standard deviation (SD). Numerosity: peripheral blood (CTRL = 8, KO = 9); spleen (CTRL = 8, KO = 10); bone marrow (CTRL = 10, KO = 10). * P <0.05, ** p <0.01, *** p <0.001

To confirm the reduction of recirculating B lymphocytes, we used two specific markers IgM and IgD, which allow to discriminate *naïve* B-cells (IgM^+/IgD^-) and recirculating B lymphocytes (IgM^+/IgD^+) (**Fig.24**).

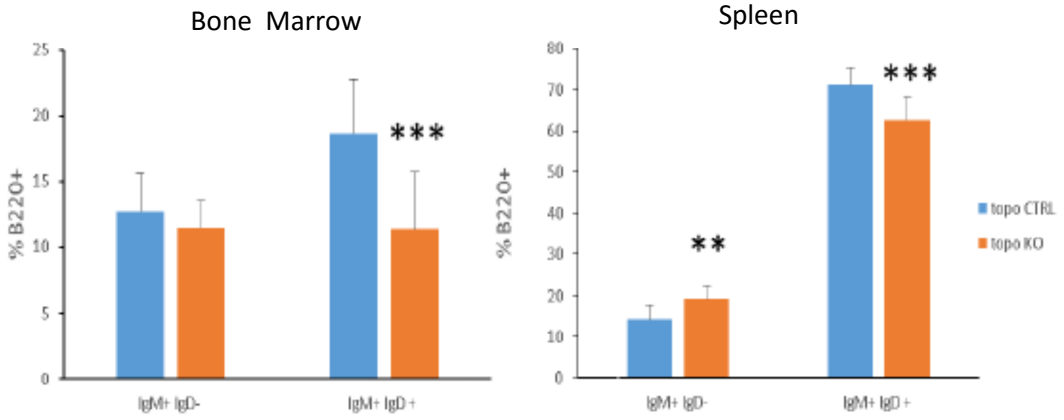

Fig.24: analysis of IgM and IgD markers in spleen and bone marrow.
Analysis B220/IgM /IgD on B-cells of BM and SP. Graphs show means and SD. Numerosity: BM (CTRL = 10, KO = 11); SP (CTRL = 11, KO = 11). *** p <0.001; ** p <0.01.

Figure 24 showed no significant variation of naive B-cells (IgM^+/IgD^-) in the bone marrow, while these increase significantly ($p<0.01$) in the spleen of KO when compared to CTRL mice.

In addition there is a reduction of recirculating B-cells (IgM^+/IgD^+), both in the bone marrow and spleen of KO, compared to CTRL mice. Confirming previous result obtained with ($CD19^{high}/B220^{high}$) staining.

4.5 Analysis of BM precursors

To evaluate a possible reduction of B-cells during their development , we analyzed the bone marrow precursors proB ($CD19^+$, $B220^+$, IgM^-, $CD25^-$, $CKIT^+$) and preB ($CD19^+$, $B220^+$, IgM^-, $CD25^+$, $cKit^-$) .

For this analysis we stained cells of bone marrow with the following fluorochromes: B220-FITC, IgM-PE, CD19-PerCPCy5.5, CD25-APC, CKIT-PECy7. During this analysis were considered B220 and CD19 markers of B cell line, and in particular we analyzed the population $B220^+$, because it is expressed earlier than CD19 (**Fig.25**).

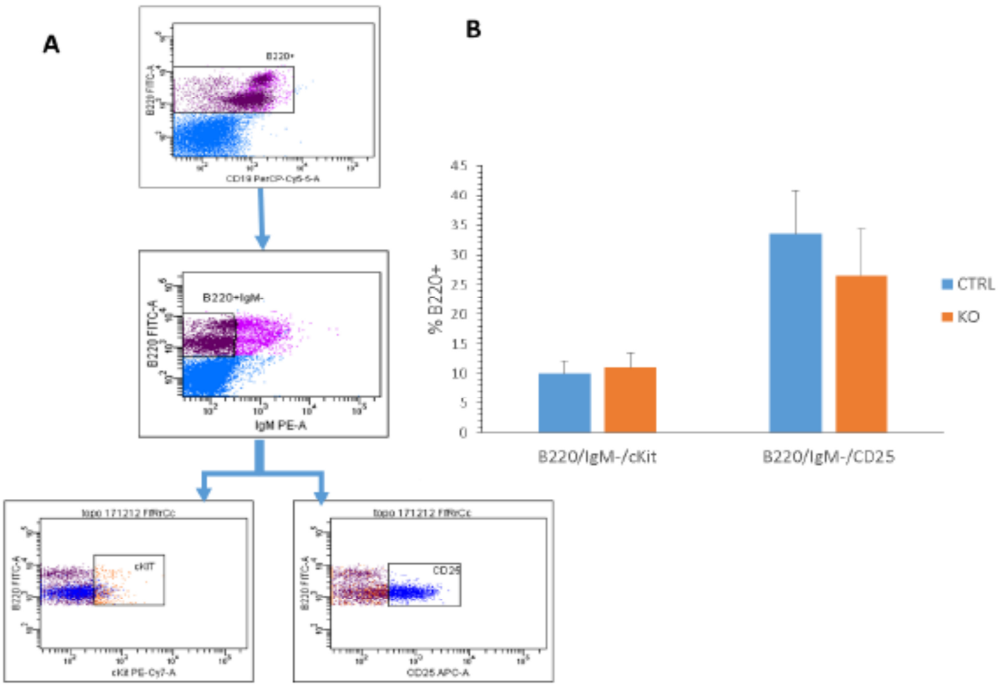

Fig. 25: analysis of preB and proB in BM.
A.summary cytograms of flow cytometric analysis. only the cells B220 were selected. Among the $B220^+$ IgM were excluded. Finally among the $B220^+/IgM^-$ we selected $CKIT^+$ (PROB) and $CD25^+$ (preb). B. histogram of populations $B220^+/IgM^-/CKIT^+$ (PROB) and $B220^+/IgM^-/CD25^+$ (preb). Numerosity: BM (CTRL = 7, KO = 10). The graphs show the mean ± SD.

Statistical analysis shows that the proB lymphocytes do not have a significant variation between KO and CTRL mice, while preB lymphocytes have a tendency of reduction in KO mice (p 0.08).

4.6 Characterization of peripheral B lymphocytes

After analyzing in detail the B-cells of the bone marrow and their precursors, we analyzed peripheral B lymphocytes. We stained the spleen cells with specific markers of follicular and marginal B lymphocytes, which are the most represented classes of splenic B-cells. Over the two line markers CD19 and B220, we added CD21 and CD23. CD23 is expressed in lymphocytes that enter in the follicle, and is a marker of recirculating B-cells. CD21 is expressed at high intensity in MZB is a marker of proteins complement (C3b and C3d) and is part of the BCR coreceptor.

B-cells were divided into FoB ($CD19^+$, $B220^+$, $CD21^-$, $CD23^+$) and MZB ($CD19^+$, $B220^+$, $CD21^+$, $CD23^-$). The plots of the acquisition are described in detail in Figure 26.

The results of this analysis show that, in the spleen of KO mice, there is a marked reduction of follicular B-cells when compared to CTRL mice ($p < 0.001$). This reduction was offset by an accumulation of MZB in KO mice.

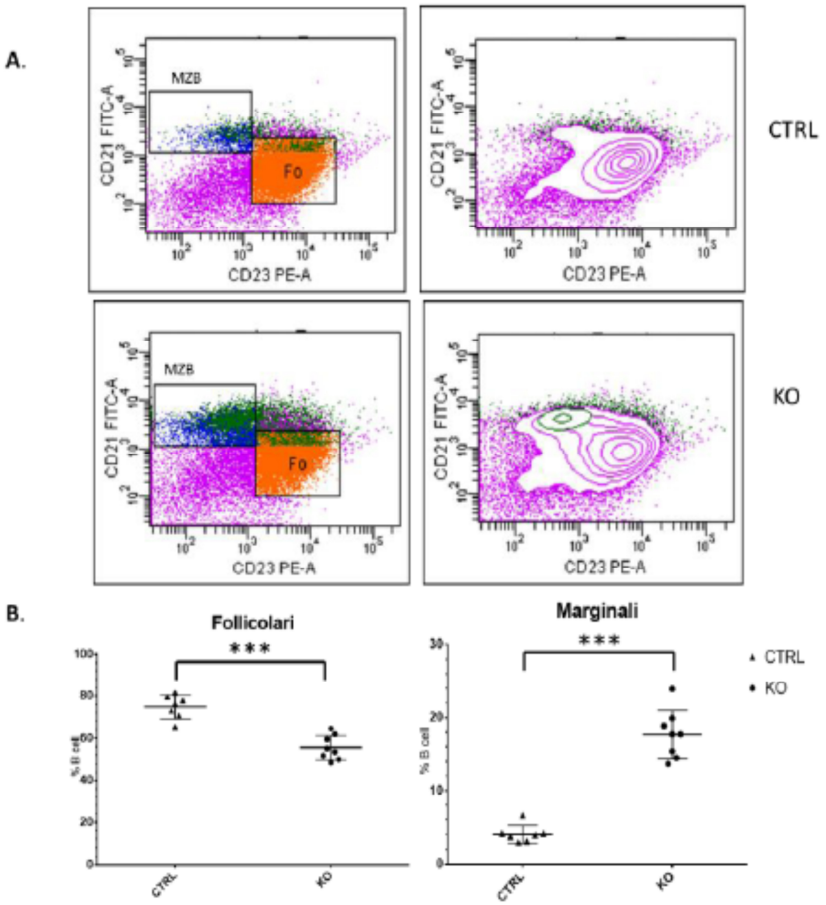

Fig.26:reduction of FOB and increase of MZB lymphocyte number in CK2β KO mice.
(A) Representative dot plot cytogram showing the stain for CD21 and CD23 used to characterize B lymphocyte populations. (B) Graphs summarizing FACS analysis of MZB and FOB in CTRL and KO mice. CK2β KO mice showed a marked reduction of FOB and a significant increase of MZB in the spleen. . Data representing mean +/- SD 8 CTRL and 8KO mice. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

After finding these differences, we performed a more accurate analysis to define all the population residing in the follicle ($CD23^{+}$, $CD21^{-}$) and analyze the precursors of MZB (MZP).

The markers used in this analysis were: CD19, B220, CD21, CD23 and IgM (**Fig.27**).

- CD19, B220 to identify B-cells;
- IgM to distinguish FoI (IgM^{low}) from other follicular populations expressing IgM^{high} (T2, FoII, MZP);
- CD23 to distinguish follicular populations ($CD23^{+}$) from the extrafollicolar populations $CD23^{-}$ (T1 and MZB);
- CD21 to discriminate, based on the intensity of the marker, between T1, T2 ($CD21^{low}$), FoB, FoI, FoII ($CD21^{med}$) and MZB, MZP ($CD21^{high}$).

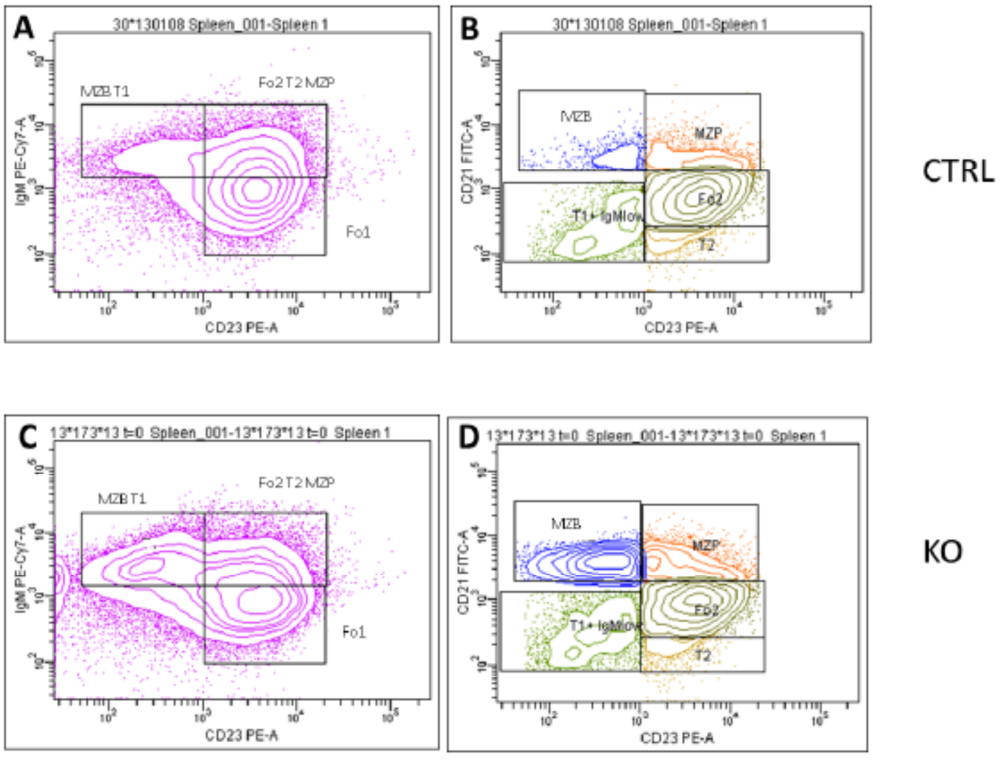

Fig. 27: cytograms of splenic B-cells population.
The analysis was performed on the gate of B-cells (CD19/B220) and, were identified by the expression of CD23 and IgM, the FoI and a large population that contains FoBII, T2, MZP (plot A-C). From this population, based on the expression of CD21, were distinguished the MZP, FoII and T2 (plot B-D). While T1 cells were identified as IgM^{+} / $CD21^{-}$ / $CD23^{+}$.

Analysis of the results show a several differences between KO and CTRL mice, indeed in KO mice there is an increase in MZB ($CD19^{+}$, $B220^{+}$, IgM^{+}, $CD21^{high}$, $CD23^{-}$), and an in precursors MZP ($CD19^{+}$, $B220^{+}$, IgM^{+}, $CD21^{high}$, $CD23^{+}$) (**Fig.27**).
Statistical analysis shows that in KO mice there is a significant increase of MZB and MZP, and a reduction of a follicular populations (FoI, FoII, T2) (**Fig.28**).

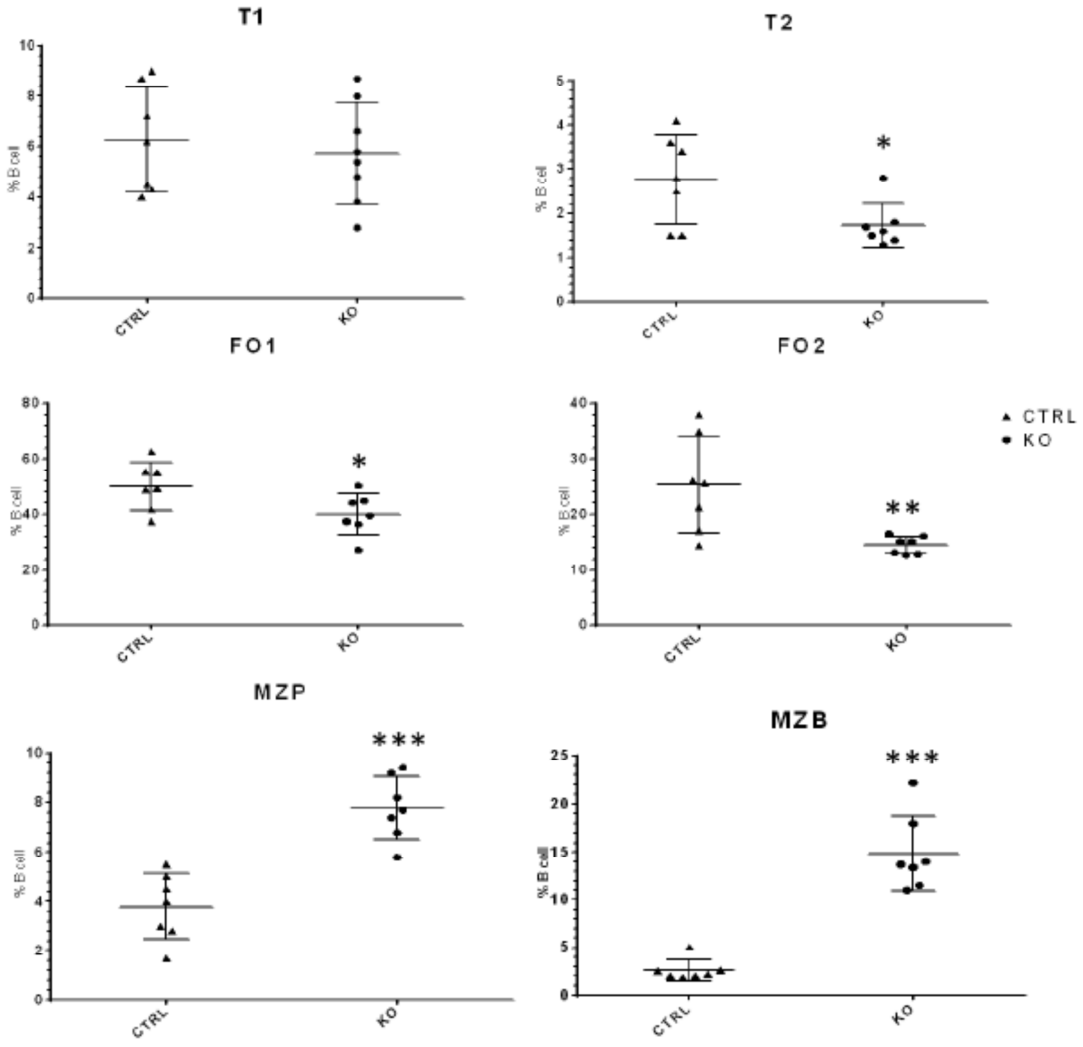

Fig.28:analysis of splenic B- cells population.
Graphs scatter representative cytometric analysis. Have compared the distributions of B subpopulations in KO and CTRL mice.. The graphs shows the mean ± SD. Mice analyzed: CTRL = 7, KO = 7. *** p <0.001, ** p <0.01, * p <0.05.

4.7 Functional analysis of GC

Having observed a clear reduction of FoB in KO mice, a study was performed to evaluate if these cells react to stimulus that induce isotype switching.

Splenocytes were treated with LPS and LPS + IL4. LPS induces the proliferation and differentiation of B-cells into plasma cells (PC) secreting IgG3 (T-independent response). The stimulus LPS + IL4 induces differentiation in PC secreting IgG1 (T-dependent response). Splenocytes obtained from CTRL and KO mice were stimulated for 72 hours and analyzed by flow cytometry. Splenocytes were stained with anti-B220 and anti-IgG1 and IgG3. Results obtained demonstrate that in KO mice there is a reduction in the number of IgG produced in presence of both stimuli, demonstrating the existence of anomalies that compromise isotype switching (**Fig.29**).

In addition, to assess the formation of PC, splenocytes were stained with anti-B220 and anti-CD138. Results showed that in KO mice there is a reduction of PC when compared to CTRL mice, after stimulation with LPS and LPS + IL4 (**Fig.30**).

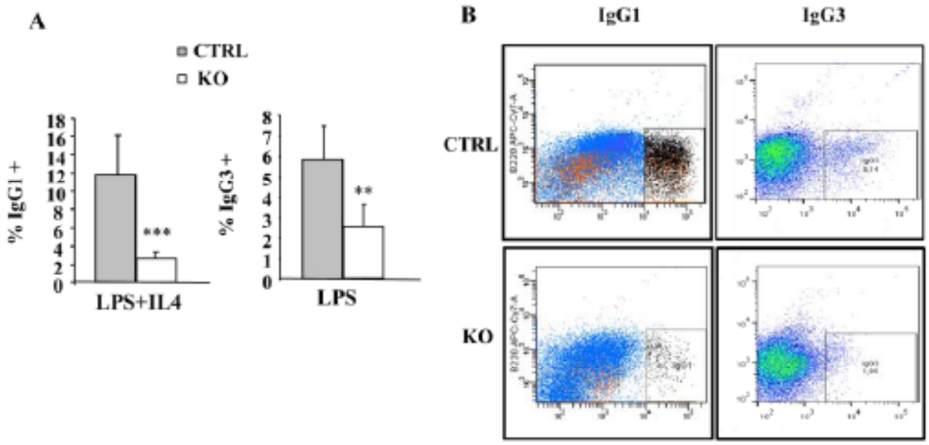

Fig.29: functional analysis of the GC reaction.
A.Histograms summarizing the FACS analysis of IgG1 and IgG3 expression of $CD19^{+}$ splenocytes from CTRL and KO mice treated for 72 hours with LPS (for IgG3) or LPS + IL-4 (for IgG1). Numerosity: IgG1 (CTRL = 8, KO = 6); IgG3 (CTRL = 5, KO = 4) *** $p < 0.001$, ** $p < 0.01$. Graphs show mean and SD. B. Representative dot plots are shown.

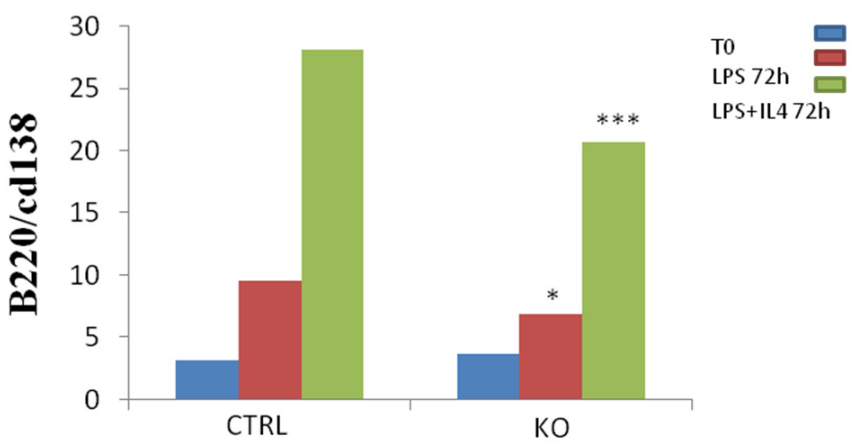

Fig.30: reduced production of PC in KO mice.
Histograms summarizing the FACS analysis of CD138 expression in $CD19^{+}$ splenocytes from CTRL and KO mice treated for 72 hours with LPS and LPS + IL-4 .Numerosity: (CTRL = 4, KO = 4); *** $p < 0.001$ * $p < 0.05$. Mean and SD are shown.

4.8 Generation of Mb-1 conditional CK1α KO mice

In order to obtain a conditional knockout mice for CK1α, we performed crossings of CK1α Flox / Flox mice (generated by Genoway) with Mb1Cre / Cre mice (E. Hobeika, Freiburg).

The first generation of mice obtained were heterozygous for the two loci.

Heterozygotes mice were crossed with each other , generating Flox / Flox , Cre / + (KO) and + / + , Cre / + (CTRL) mice.

For the experiments we only used Mb1 Cre/+ mice , because Mb1 Cre/Cre mice have an abnormal phenotype in the development of B lymphocytes. Mice obtained were viable, fertile and had a normal phenotype. Mice genotype was verified by a PCR (**FIG.31**).

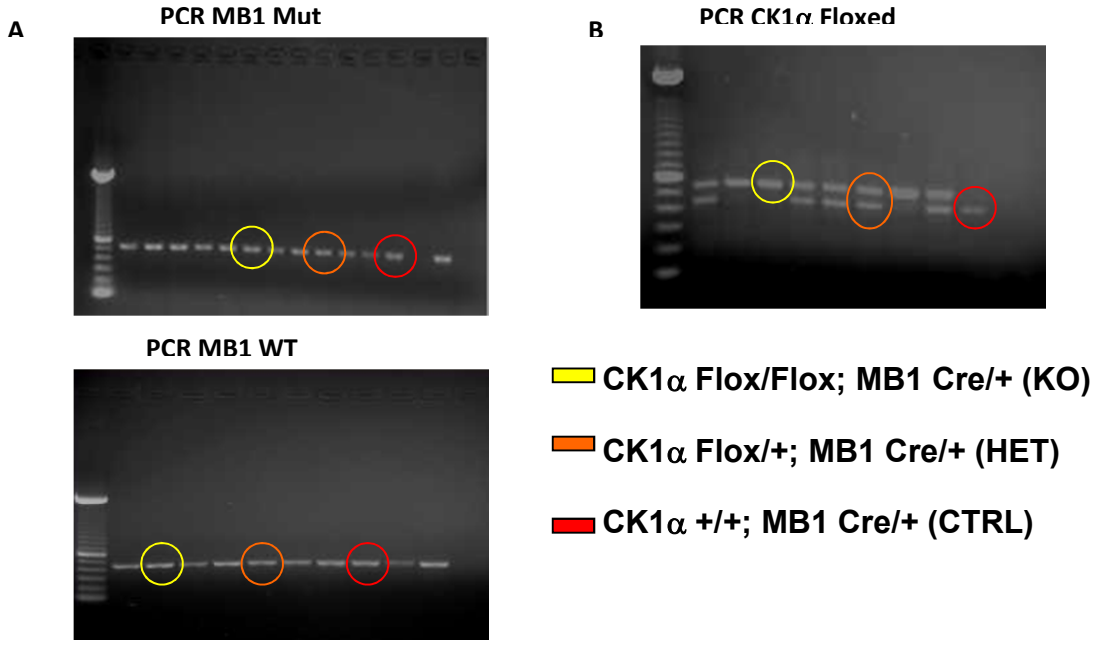

Fig.31: mice genotyping.

A. PCR for MB1 mutated and MB1 wt
B. PCR for the site CK1α.

The PCR to determine the presence of the WT MB1 gene, provides a 400 bp fragment, while the mutant Mb1 provides a 500 bp fragment. In the PCR for CK1α gene, product of 545 bp and 435 bp are expected for Flox and WT, respectively.

4.9 Quantification of total B-cells in CK1 model

In order to assess potential differences in the percentage of B-cells present in the hematopoietic organs of CK1α KO, HET and CTRL mice, flow cytometryc analysis was performed using fluorescent antibodies that recognize specific markers for these cells: CD19 and B220.

We analyzed bone marrow, spleen, and peripheral blood (**Fig.32**).

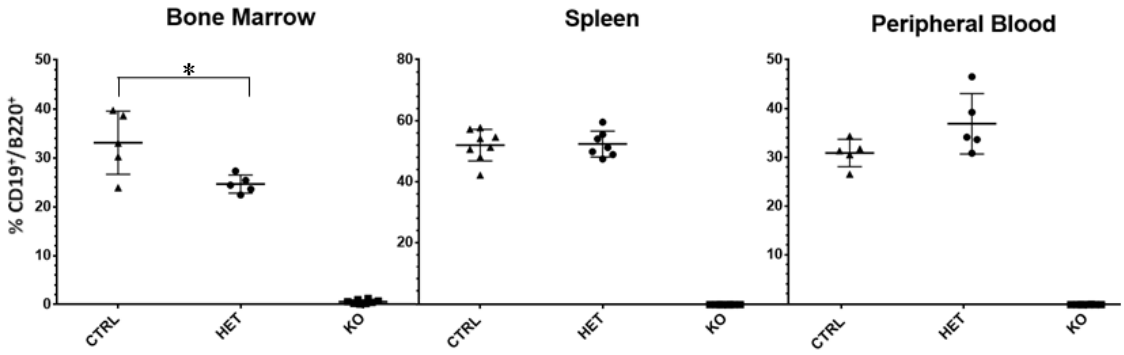

Fig. 32: percentage of B-cells in CK1α KO and CTRL mice.
The analysis was performed with FACSCalibur, using CD19 and B220 markers in peripheral blood, spleens and bone marrow. Graphs show mean and standard deviation (SD). Numerosity: peripheral blood (CTRL = 5, HET=5, KO = 8); spleen (CTRL = 8, HET=7 KO = 8); bone marrow (CTRL = 5, HET=5 KO = 8). *p<0,05

As shown in figure we observed the absence of mature B cells in all organs in KO mice while there was a significant differences (p<0,05) only in bone marrow B cells between control mice and heterozygous.

4.10 Analysis of BM precursors

Unlike the CK2 KO mice the model CK1 does not have mature B cells, so we went to assess the population of bone marrow precursors with Hardy's stain. We have identified all three B cells precursor PreproB(B220,IgM+,CD127+;CD24-;CD25-),ProB(B220,IgM+,CD127+;CD24-;CD25-)andPreB(B220,IgM+,CD127+;CD24-;CD25-). **Fig.33**

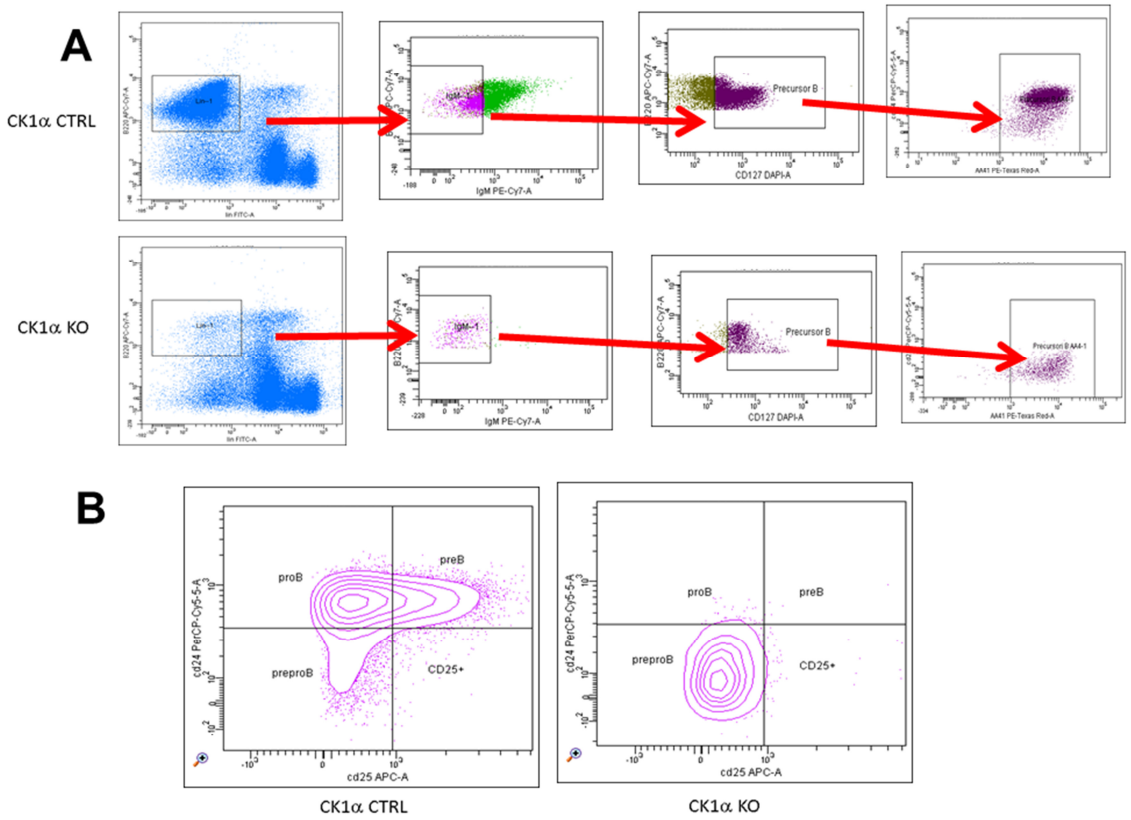

Fig. 33: comparison between CTRL and KO precursors .

A. Summary of cytometric analysis gate strategy to identified the precursors B cells in bone marrow. Firstly we excluded non-B cells with line markers (Lin). then we selected the precursors (IgM-, CD127, AA4.1).
B. Is shown the identification of three B cell precursors population in the gate of precursors with markers CD25 and CD25.

As shown in Fig.33 in KO mice is observed a drastic reduction of B cells in particular the mature B cells populations (IgM+) is absent. The analysis shown that in KO are conserved only PreproB cells population while ProB cell are a rare population that

express CD24 at low level respect the normal intensity in HET and CTRL. Furthermore in HET mice there are a reduction of all precursors.(**Fig. 34)** .

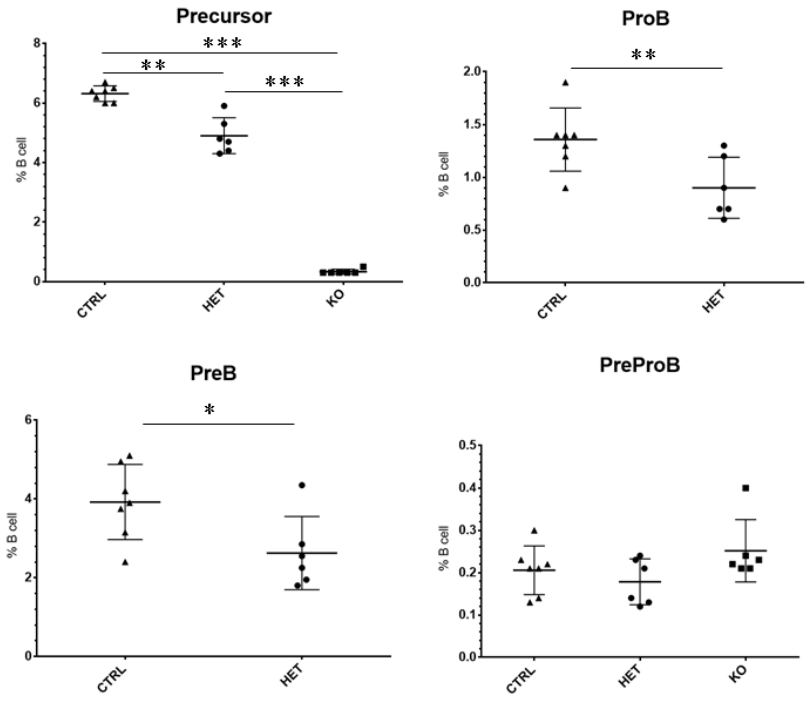

Fig.34: Analysis of precursors in bone marrow

The analysis of all precursors B cells in bone marrow. KO mice conserved only preproB cells(CD24-; CD25-) that is equal to HET and CTRL. Graphs show mean and standard deviation (SD). Numerosity: CTRL = 7, HET=6, KO = 6. *p<0,05, **p<0,01, *** p<0,001

4.11 BrdU staining in precursors B cells

To evaluate the proliferation of purified B cell precursor B cells were labeled with the line markers CD127 AA4.1 (Precursors), CD24 and CD25 (PreproB, ProB, PreB) and 7AAD and BrdU.

The staining with BrdU and 7AAD helps to identify proliferating cells and the various phases of the cell cycle: Go ($BrdU^-$; $7AAD^-$) ; G1($BrdU^+$; $7AAD^-$) ; S/G2($BrdU^+$; $7AAD^+$) G2/M($BrdU^-$; $7AAD^+$). Plot of result of CTRL HET and KO mice in all populations are reported in fig.35

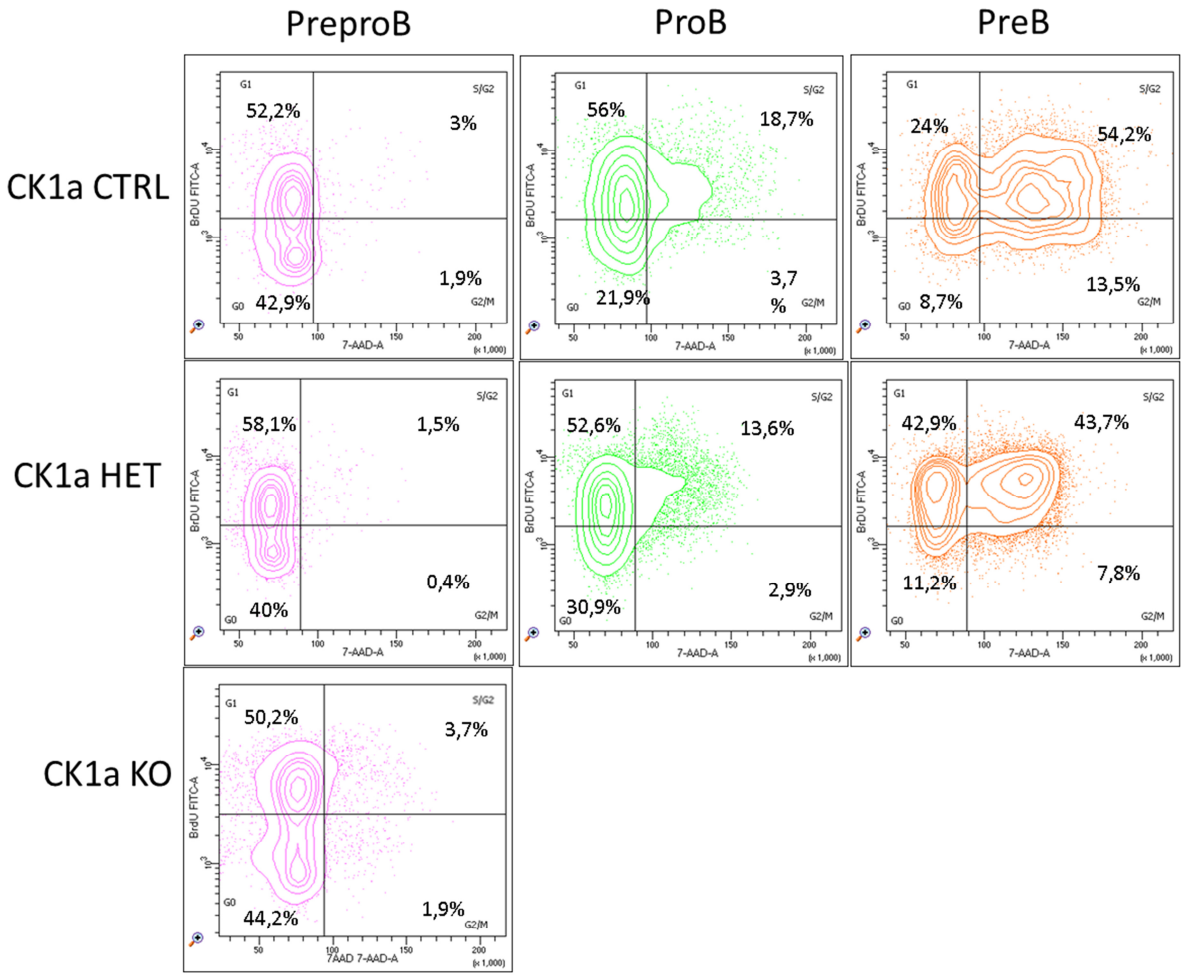

Fig. 35: BrdU 7aad in precursors B cells .
The contour plot showed the expression of 7AAD/BrdU in PreproB, ProB and PreB. In the graph was reported the mean of each phase of cell cycle.

The figure shows how each population has a different proliferation. Among HET and CTRL aren't observed differences, PreproB are mainly in G1 while the ProB have a S/ G2 phase and the most proliferating population are preB.

In KO there aren't a PreB and ProB population and the PreproB have the same distribution of CTRL and HET.

This data could indicate a cell cycle block, because the mice were treated with BrdU for one week. During this period all cells could be cycling then the presence of cells in G2/M phase that have not incorporated BrdU is not possible in a normal cell cycle.

4.12 Characterization of peripheral B lymphocytes

The bone marrow of the data showed a complete absence of mature B, then it is continued the analysis of B lymphocytes evaluating if there are differences between CTRL and HET.

The analysis strategy is the same as that described for the model CK2

B-cells were divided in T1($CD19^{+}$, $B220^{+}$, IgM^{high}, $CD21^{low}$, $CD23^{-}$); T2($CD19^{+}$, $B220^{+}$, IgM^{high}, $CD21^{low}$, $CD23^{-}$); FO1($CD19^{+}$, $B220^{+}$, IgM^{med}, $CD21^{med}$, $CD23^{+}$); FO2($CD19^{+}$, $B220^{+}$, IgM^{high}, $CD21^{med}$, $CD23^{+}$); MZB($CD19^{+}$, $B220^{+}$, IgM^{high}, $CD21^{high}$, $CD23^{-}$); MZP($CD19^{+}$, $B220^{+}$, IgM^{high}, $CD21^{high}$, $CD23^{+}$).

Statistical analysis shows that in HET mice there is a significant increase of MZB and MZP, and a reduction of a follicular populations Fo1 and transitional B cells T1 and T2 (**Fig.36**).

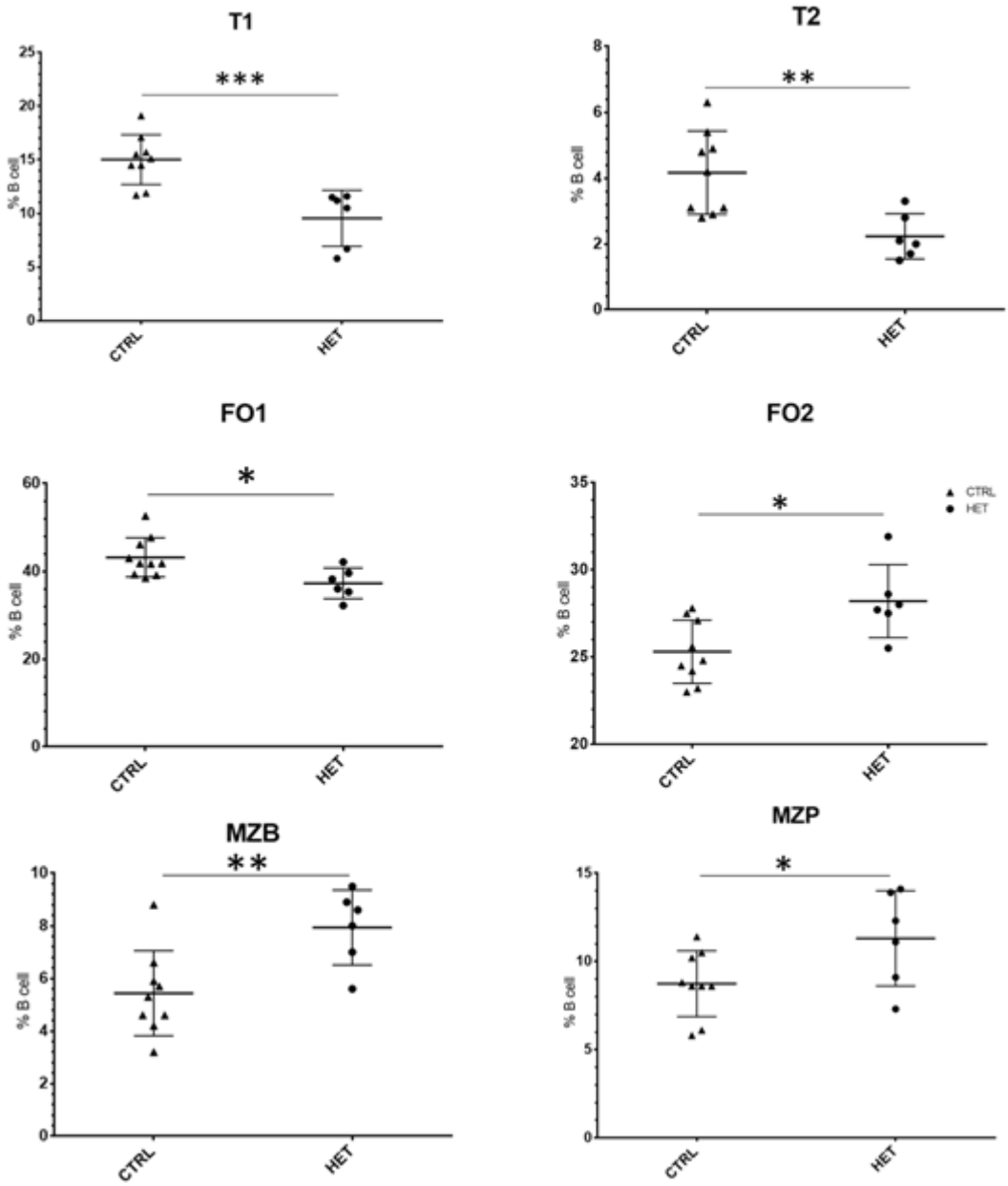

Fig.36:analysis of splenic B- cells population.
Graphs scatter representative cytometric analysis. Have compared the distributions of B subpopulations in HET and CTRL mice.. The graphs shows the mean ± SD. Mice analyzed: CTRL = 9, HET = 6. *** p <0.001, ** p <0.01, * p <0.05**.**

4.13 Functional analysis and CFSE staining

Splenocytes of CK1 CTRL and HET were treated with LPS and LPS + IL4 for 72h like described for CK2 model. In addition, in this model were tested the cell proliferation with CFSE (Carboxyfluorescein succinimidyl ester) a cells markers that was diluted during the cell divisions. Results showed that in the HET and CTRL mice there weren't a significant differences in IgG1 and IgG3 expressions (**Fig.37)**.

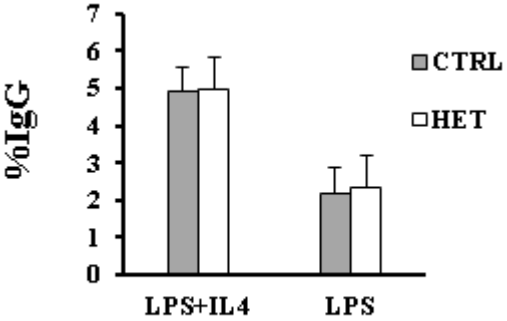

Fig.37: functional analysis of the isotype swithing
A.Histograms summarizing the FACS analysis of IgG1 and IgG3 expression of $CD19^{+}$ splenocytes from CTRL and HET mice treated for 72 hours with LPS (for IgG3) or LPS + IL-4 (for IgG1). Numerosity: IgG1 (CTRL = 3, HET = 3); IgG3 (CTRL = 3, KO = 3) Graphs show mean and SD. B. Representative dot plots are shown.

The analysed mice didn't show a difference in proliferation in both treatment

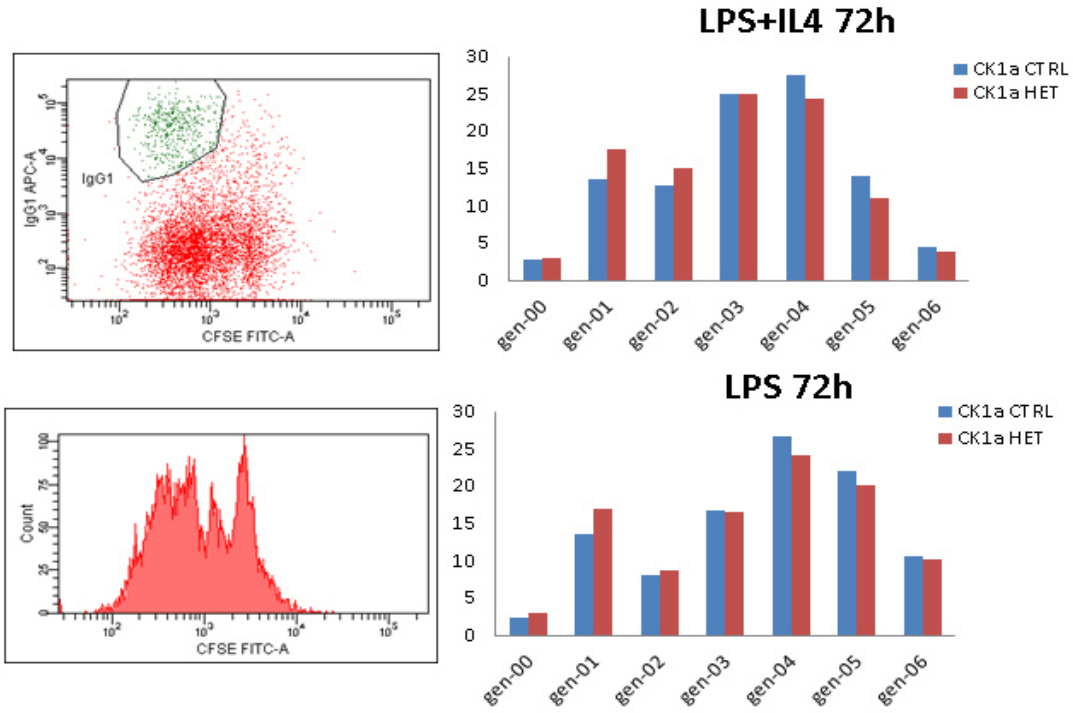

Fig.38: CFSE proliferation analysis.
A.Histograms compared the seven generation analysed in HET and CTRL after 72h of treatment with LPS and LPS+IL4. A dotplot exemples show how the IgG are expressed only in the last generation where the CFSE is weaker.

5. DISCUSSION

CK2 is a pleiotropic and evolutionary conserved serin-threonin kinase that is involved in several cellular processes. A number of studies revealed many mechanisms through which this kinase regulates cell cycle, apoptosis, cell survival and tumorigenesis.

However, despite all this data, little is known about the role of CK2 in B-lymphopoiesis and lymphomagenesis.

Considering this knowledge, we decided to investigate the role of CK2 using a conditional CK2β KO mouse model in B-cells.

To make this, we crossed homozygous mice for the *floxed Csnkb* allele with hemizygous mice for the *Cd19-Cre* transgene, in order to obtain the deletion of the *Csnk2b gene* only in B-cells.

The first thing we observed a marked splenomegaly, derived by an increase in cellularity and weight of spleens in CK2β KO compared to CTRL mice..

Flow cytometry analysis of BM, SP and PB-cells, demonstrated a remarkable reduction of total B-cells in CK2β KO compared to CTRL mice.

In bone marrow, B-lymphocyte reduction interested, in particular, recirculating B-cells, which reside only temporarily in the bone marrow, but, as their name suggests, they normally recirculate.

These results propose, for the first time, that CK2β subunit is necessary for the maintenance of the correct abundance of peripheral B lymphocytes.

Furthermore, these data were confirmed by the analysis of IgM and IgD immunoglobulins in naive B- (IgM^{+}/IgD^{-}) and recirculating B-cells (IgM^{+}/IgD^{+}) of bone marrow and spleen.

Analysis showed that there are no differences in the naive B-cell population, while there is a marked reduction of recirculating B-cells in bone marrow and spleen of KO compared to CTRL mice. These data support the idea that CK2 has a crucial role in determining the survival of peripheral B-cells, but not in the ontogeny of B-cells in the bone marrow.

Furthermore, the analysis of bone marrow precursors proB ($B220^{+}/IgM^{-}/CKIT^{+}/CD25^{-}$) and preB ($B220^{+}/IgM^{-}/cKit^{-}/CD25^{+}$) showed no differences between KO and CTRL mice, reinforcing the idea that CK2 appears not to be essential for B-cell

maturation in the bone marrow, but is critical for correct subsequent stages in B-cell development.

Since we observed a marked reduction of peripheral B-cells, we characterized this population, in particular we analyzed splenic B-cell subpopulations.

Flow cytometry analysis showed that spleens of CK2β KO mice are characterized by a reduction in FOB and an increase in MZB. Specifically, follicular populations are decreased (FoI, FoII, T2), while the marginal populations are increased (MZB, MZP).

This results suggest that CK2 may be involved in addressing immature cells to marginal or follicular differentiation. It is important to notice that there are no differences in T1 B-cells, which represent the peripheral B-cells more similar to the naive. These findings confirm the hypothesis that CK2 is principally involved in peripheral B-cell differentiation. In addition, functional analysis of GC demonstrated an hypogammaglobulinemia, and, after stimulation, revealed that in KO mice there is a reduction in the number of IgG, demonstrating the existence of anomalies that compromise isotype switching.

CK1α like CK2 is a pleiotropic conserved serin-threonin kinase constitutively active that is involved in several cellular processes. Expression of CK1 in hematopoietic cells has been reported in few studies and its role in normal hematopoiesis in still largely unidentified. We decided to investigate the role of CK1α using a conditional CK1α KO mouse model in B-cells.

To make this, we crossed homozygous mice for the *floxed Csnk1a1* allele with hemizygous mice for the *Mb1-Cre* transgene, in order to obtain the deletion of the *Csnk1a1 gene* only in B-cells.

From the analysis with the line of markers for B cells CD19 B220 it was observed that in the KO mice the mature population is absent in all the analyzed organs. These results propose, that CK1α is necessary for early B cells development, so unlike CK2 CK1 could have a central role in bone marrow B cells maturation. Conseguently we went to characterize all bone marrow B cell precursors PreproB ProB and PreB with a hardy's staining in CTRL HET and KO mice. The results showed that in Ck1a KO mice the PreB and ProB cells are absent while the PreproB population are conserved. There are significant differences between CTRL and HET that present a reduction in ProB and PreB cells population. Knowing that the promoter Mb1 start expression at proB level

the results suggest that there is a differentiation block or cell death when ko of ck1 occurs. In hemizygous there is a intermediate phenotype with a significant reduction of ProB and PreB cells.

To deepen the phenotype was performed a proliferation analysis by administering the Brud diluted in drinking water for a week. In the analysis were identified the phases of the cell cycle by evaluating the BrdU in combination with 7AAD and the data showed that between CTRL and HET there isn't differences and in KO the distribution of Go and G1 population in PreproB are conserved. Furthermore appears to be a rise of sub-G1 but it's a preliminary data.

The analysis on mature B populations was carried out by comparing HET and KO to investigate whether partial absence of CK1 can lead to an peripheral phenotype seen the dramatic results of the KO in the bone marrow. took place in spleen the same analysis performed in the model CK2 characterizing transitional B cells(T1,T2), follicular B cells(FoI, FoII) and marginal B cells (MZB, MZP).

Flow cytometry analysis showed that there is a significant increase of FOB and a reduction of MZB. Furthermore there is a reduction of T1 B cells that confirm the phenotype in bone marrow. The functional analysis of GC after stimulation with LPS and LPS+IL4 revealed that in HET mice the percentage of IgG is comparable to CTRL than the analysis excludes functional problem in isotype switching. Furthermore

Furthermore also the analysis of proliferation of B cells with CFSE does not reveal differences between CTRL and HET.

In conclusion these project shows that the β subunit of protein kinase CK2 is a novel regulator of peripheral B-cell differentiation. CK2β controls the GC reaction, class-switch recombination and somatic hypermutation.

CK1a instead has a key role in the differentiation of bone marrow progenitors and cell survival.

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CK2 regulates B cell commitment and the germinal center reaction counteracting NOTCH2 and sustaining the BCR signaling
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