UNIVERSITY OF UDINE

DEPARTMENT OF MEDICAL AND BIOLOGICAL SCIENCES

PHD COURSE IN BIOMEDICAL AND BIOTECHNOLOGICAL SCIENCES

XIV CICLE

Molecular and cellular environment that leads to the generation of IL-10–producing B cells

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ACADEMIC YEAR 2011-2012

Tesi di dottorato di Francesca Mion discussa presso l'Università degli Studi di Udine

Dedicated to Nonna Rina, who would have
been very proud of this thesis

ABSTRACT

B cells are generally considered as positive regulators of the immune response by producing antigen-specific antibodies; however the regulatory suppressive function of distinct B cell populations has been recently described. Several studies have demonstrated that B cells seem to acquire the ability to suppress immune responses in an IL-10–dependent manner, but it is likely that B cells can exert suppressive functions also by other mechanisms, both contact dependent and independent. The study of B cells suppressive function could lead to the development of novel therapeutic approaches and to the understanding of the mechanism of action of biological drugs in inflammatory and autoimmune diseases and cancer.

The aim of the work described in this thesis was to characterize the microenvironment that induces the expansion and/or differentiation of IL-10–producing B cells, focusing on the contribution of both stimuli activating B cells and immune cell types encountered by B lymphocytes. Specifically, in the first part of the study, the role of endogenous, immune mediated, signals and exogenous, infectious signals on the regulation of several aspects of IL-10–producing B cell biology such as IL-10 production and secretion and expression of specific surface markers, was investigated. Subsequently, the crosstalk between B cells and mast cells (MCs) was studied in order to investigate whether the interaction between these two cell types could influence the development of IL-10–producing regulatory B cells.

The obtained results proved that endogenous and exogenous stimuli can differently affect several properties of IL-10–producing B cells. While B-cell activation through CD40 proved to be the best way to promote IL-10–competent B cell expansion *in vitro*, TLR agonists were the most potent stimuli for inducing IL-10 secretion, at high levels. Moreover, the IL-10–competent B cells induced by LPS or agonistic CD40 mAb displayed different immunophenotypes in regard to CD1d and CD5 expression, suggesting that different activated B-cell subsets with suppressive functions can develop according to the immunological requirements of the specific environment.

Moreover, in this study we observed that bone marrow–derived MCs (BMMCs) were able to increase the percentage of IL-10–producing B cells, regardless of their activation status, while they did not affect IL-10 secretion. MCs-induced IL-10–producing B cells were found at a higher frequency within the $CD19^{+}$ $CD1d^{hi}$ $CD5^{+}$ B cell subset although the majority of these $IL\text{-}10^{+}$ cells displayed a phenotype consistent with the one of transitional 2 and marginal zone B cells.

Among the different mechanisms that could be responsible for the induction of IL-10–producing B cells by MCs the obtained results demonstrated the importance of both the CD40-CD40 ligand (CD40L) interaction and the soluble factors released by MCs. Furthermore, the *in vivo* absence of MCs reduces the percentage of $CD19^+$ $IL\text{-}10^+$ cells in mouse bone marrow, lymph nodes, and peritoneum, suggesting that MCs are key players of IL-10–producing B cell development and/or differentiation in these sites. Altogether these data show that MCs are able to induce the expansion of the IL-10–producing B cell population both *in vitro* and *in vivo* and this can be seen as a very relevant observation in the context of immunologically mediated inflammatory reactions.

Collectively, the data presented in this thesis demonstrate that both the specific microenvironment and mast cells can affect the behavior of B cells in regard to the competence to produce IL-10.

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LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
APCs	Antigen-Presenting Cells
APRIL	A Proliferation-Inducing Ligand
BAFF	B cell activating factor of the TNF family
BCR	B-Cell receptor
BMMCs	Bone Marrow derived Mast Cells
Breg	B regulatory
CD	Cluster of Differentiation
CFSE	Carboxyfluorescein Succinimidyl Ester
CHS	Contact Hypersensitivity
CIA	Collagen-Induced Arthritis
CSR	Class Switch Recombination
DC	Dendritic Cells
DNP	Dinitrophenol
DSS	Dextran Sulfate Sodium
DTH	Delayed-Type Hypersensitivity
EAE	Experimentally induced Autoimmune Encephalomyelitis
FBS	Fetal Bovine Serum
FDCs	Follicular Dendritic Cells
FITC	Fluorescein Isothiocyanate
FO	Follicular
FoxP3	Forkhead box P3
GALT	Gut-Associated Lymphoid Tissue
ICAM	Intercellular Adhesion Molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
mAb	Monoclonal Antibody
MAPK	Mitogen-Activated Protein Kinase

MC	Mast Cell
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MS	Multiple sclerosis
Myd88	Myeloid differentiation primary response gene 88
MZ	Marginal Zone
NK	Natural Killer
NOD	Non-Obese Diabetic
PAMPs	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PE	Phycoeritrin
PE-Cy5	Phycoeritrin-Cyanine 5
PMA	Phorbol 12-Myristate 13-Acetate
RA	Rheumatoid Arthritis
RBCs	Red Blood Cells
SCF	Stem Cell Factor
SCID	Severe Combined ImmunoDeficiency
SHM	Somatic Hypermutation
SLE	Systemic Lupus Erythematosus
STAT3	Signal Transducer and Activator of Transcription 3
TCR	T-Cell Receptor
Teff	T effector
TGF-β	Transforming Growth Factor-β
Th	T helper
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
Treg	T regulatory
UC	Ulcerative Colitis

1. INTRODUCTION

The immune system is one of the most complex biological systems in the human body. Its importance is underlined by the extreme example of people affected by SCID, a severe form of heritable immunodeficiency in which the adaptive immune system is so highly compromised that it is almost considered absent. Such individuals can become infected extremely easily and therefore they are forced to live in a sterile environment, with only limited contact with people.

The strength of the immune system lies on its organization: a network of molecules, cells, tissues and organs work together to ensure protection to the body. The first line of defense against pathogens includes skin and mucosal surfaces, which provide physical and chemical barriers to infection. For example, the normal bacterial flora antagonize the colonization of body surfaces by non-indigenous bacteria. In addition, the internal tissues invariably contain bactericidal substances such as lysozyme, an enzyme that is present in both tissues and secretions. If these barriers are penetrated, the innate immune system provides an immediate response through the recruitment of phagocytic cells. These cells include macrophages and neutrophils that engulf foreign organisms and kill them. Finally, bacterial invasion is also challenged by the activation of the complement system in blood and tissues.

The specificities of the innate immune system are limited and predetermined, therefore we have a second layer of protection, highly specific and extremely diverse, that is given by the adaptive immune system. There are two broad classes of adaptive immune responses, antibody responses and cell-mediated immune responses, which are carried out by B and T lymphocytes, respectively. During its differentiation, each B lymphocyte becomes genetically programmed, through a series of gene-splicing reactions, to produce an antibody molecule with a unique specificity and therefore capable of binding a definite epitope of an antigen (Alberts, 2002).

In antibody responses, B cells are activated to secrete antibodies that circulate in the bloodstream and permeate the other body fluids where they bind specifically to the foreign antigen. For a long time this essential role of B cells in humoral immunity has distracted attention from many other B cell functions, essential for immune homeostasis, which are independent from antibody production. For example, B cells can act as antigen presenting cells (APCs) and release immunomodulatory cytokines that can influence a variety of T cell,

dendritic cells (DCs), and antigen presenting cell functions. They are able to modulate lymphoid tissue organization and neogenesis, regulate wound healing and transplanted tissue rejection and influence tumor development and tumor immunity. In addition, recent findings have revealed the existence of phenotypically diverse B cell subsets with regulatory functions in different models of autoimmune diseases and chronic inflammation (LeBien and Tedder, 2008). Pivotal to the function of these regulatory B cells is interleukin-10 (IL-10), which inhibits proinflammatory cytokines and supports regulatory T (Treg) cell differentiation (Mauri and Blair, 2010). The presence of different subsets of regulatory B cells, potentially distinct for their ontogenesis as well as for the differentiation and activation pathways makes intriguing the study of these cells.

As the experiments performed for this thesis were conducted in order to study different aspects of regulatory B cells, the purpose of this introduction is to present some particular aspects of B cell biology, in particular the development and function of the different subsets of B lymphocytes, and to illustrate the state of the art of these interesting and discussed B cell population with regulatory suppressive functions.

1.1 B cell development

B cells form a diverse and plastic repertoire of immune cells that can be divided into different populations or subsets, characterized by the differential expression of intracellular and cell surface markers and by distinct combination of properties. Mouse B cells have been subdivided into two distinct lineages, B-1 and B-2, which differ for their developmental origin, anatomical localization and functional characteristics (Hardy and Hayakawa, 2001). B-2 B cells, also known as “conventional” B cells, are generally found in spleen and lymph nodes and they act primarily in adaptive immune responses whereas B-1 B cells are predominantly located in body cavities and are considered to be part of the innate immune system. Moreover the development of B-1 and B-2 B cells is also distinct: while the developmental pathway of B-1 cells is still debated, much more is known on B-2 B cells that develop in the bone marrow during postnatal life to then migrate to the spleen where they undergo further maturation into follicular or marginal zone (MZ) B cells (Sagaert and De Wolf-Peeters, 2003).

1.1.1 B-1 B cells

B-1 cells were originally identified as $CD5^+$ B cells that participate in autoimmunity and share similarities with those responsible for human chronic lymphocytic leukaemia (Huang et al., 1999). Later studies showed that B-1 cells constitute a pool of long-lived, self-renewing B cells that produce most of the circulating natural IgM antibodies and that represent about 1 to 5% of total B cells in the mouse (Tung and Herzenberg, 2007). It was also revealed that CD5 was not an identifying marker of these cells: a minor subset of B cells that closely resembled these $CD5^+$ B cells in terms of their tissue distribution, phenotype and development but that did not express CD5 was identified and, therefore, B-1 cells were further subdivided into B-1a ($CD5^+$) and B-1b ($CD5^-$) cells.

B-1 cells are the main B cell population in the peritoneal and pleural cavities but they are also found in other sites such as spleen and various parts of the intestine. However, it must be kept in mind that B-1 B cells display phenotypic differences within different organs: B-1 cells in the peritoneal and pleural cavities can be identified by the $CD11b^+$ $sIgM^{hi}$ $sIgD^{low}$ phenotype while splenic B-1 B cells do not express the surface molecule CD11b (Montecino-Rodriguez and Dorshkind, 2006).

B-1 B cells participate in maintaining tissue homeostasis, as well as in immune defence against mucosal pathogens (Baumgarth, 2011). Despite the fact that this B cell subset constitute only a minor fraction of all B cells in the mouse, it appears to produce most of the natural antibodies that recognize a variety of self-antigens and foreign antigens and appear in the serum and mucosal sites without any known antigenic stimulus (Dono et al., 2004). These polyreactive antibodies have low affinity and broad specificities and function to provide a first line of defense against microbial infection. Furthermore, B-1 B cells express a reduced number of B-cell receptor (BCR) specificities, compared to B-2 follicular B cells, again consistent with their status of innate-like immune cells.

While there is a general consensus about the functional features and the roles of B-1 B cells, the concept that is most at issue is their origin. The first studies supported the idea that B-1 B cells derived from progenitors that were present in fetal omentum and fetal liver but were largely absent from adult bone marrow. As B-1 cells are self-renewing, they are reconstituted by division of fully mature B-1 cells. As described by Baumgarth N., on the basis of recent findings two possible theories on the development of these cells have been proposed: the "induced differentiation hypothesis" states that a common precursor can give rise to either a B-1 or B-2 cell depending on the specific signals received during development and selection while the "lineage hypothesis" proposes that B-1 and B-2 cells develop from distinct B cell

precursors (Baumgarth, 2011). According to the "lineage hypothesis" the progenitor of B-1 cells is present in fetal liver and bone marrow but also in adult bone marrow (Figure 1.1).

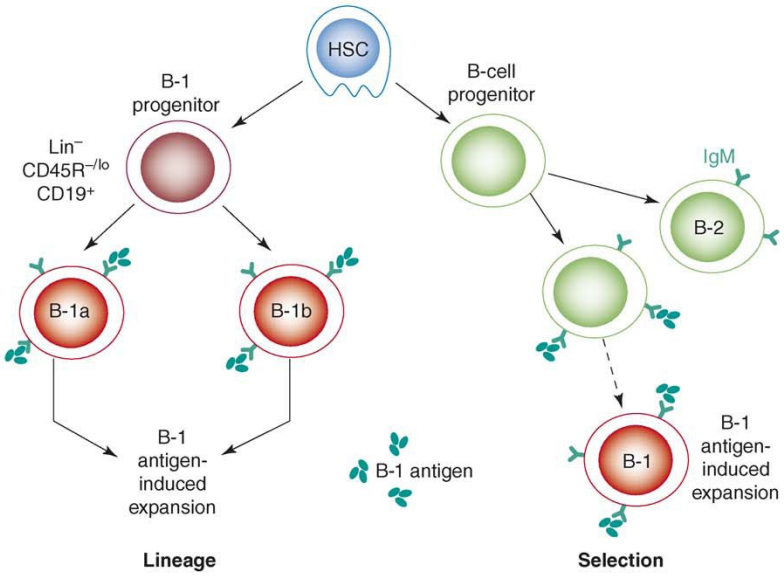


Figure 1.1: Models of B-1 B-cell development. All blood cells, including B-1 B cells, are derived from the hematopoietic stem cell (HSC). The lineage model of B-1 B-cell development proposes the existence of a progenitor with developmental potential restricted to the production of B-1a and B-1b B cells. The induced differentiation model proposes that B-1 and B-2 B cells develop through a single developmental pathway, and that antigen-binding to cells at the sIgM-expressing stage of development determines whether a cell will acquire the characteristics of B-1 or B-2 B cells *(Montecino-Rodriguez and Dorshkind, 2006)*.

This controversy on B-1 cell origin underlies another debate concerning this subset of B cells, which is whether CD5 is a marker of activation or a molecule specific for this B cell lineage. The reason for the expression of CD5 on the B-1a B cells is still unclear even if several studies have demonstrated that CD5 is a negative regulator of BCR signaling that is up-regulated after BCR stimulation and likely contributes to B-cell tolerance *in vivo* (Bikah et al., 1996). In addition it has been shown the existence of a relationship between CD5 expression and IL-10 secretion that promotes B cell survival. In mice, $CD5^+$ B-1 B cells, unlike B-2 cells, are long-lived *in vivo* and *in vitro* and, interestingly, O'garra and coworkers demonstrated that this is due to the ability of B-1 cells to produce IL-10, the most relevant cytokine for B-cell survival in mice and humans (O'Garra et al., 1992). They suggested the existence of a positive autocrine regulatory loop and this theory was actually confirmed by a more recent study showing that, in humans, the CD5 molecule promotes B cell survival through stimulation of autocrine IL-10 production (Gary-Gouy et al., 2002).

1.1.2 B-2 B cells

Murine B-2 cells constitute the predominant population of B lymphocytes present in the spleen and lymph nodes and form the more adaptive part of the B-cell system. In contrast to B-1 cells, which maintain their numbers in adult mice, at least in part, by self-replenishment, B-2 cells are generated throughout life by differentiation of progenitors in the bone marrow. In this regard it is important to keep in mind the significant role of the bone marrow microenvironment for B cell development: progenitor cells receive signals from bone marrow stromal cells both through cell-cell contact and soluble mediators such as IL-7 and Fms-like tyrosine kinase 3 ligand (Flt3L). More specifically, B cells originate from pluripotent hematopoietic stem cells (HSCs) through a highly regulated process that initiates in the bone marrow and is completed in the peripheral lymphoid organs where B lymphocytes are thought to follow either a follicular or a MZ program. The intermediate stages of B-2 B cell developmental pathway have been well characterized and can be easily identified based on the expression of several cell-surface molecules and intracellular transcription factors, the status of immunoglobulin (Ig) heavy and light rearrangement and the expression of the pre-B cell and B cell receptor complex (Samitas et al., 2010).

During the initial phases of lymphocyte development, pluripotent, self-renewing HSCs differentiate into multipotent progenitors, characterized by the loss of self-renewal capacity and the expression of Flt3 which is fundamental for the growth and survival of hematopoietic progenitors (Stirewalt and Radich, 2003). The multipotent progenitor precursor (MPP) is thought to generate two lineage-restricted populations, termed common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs), although recent findings on hematopoietic processes have undermined this classical view of lineage commitment (Kawamoto, 2006). Kawamoto and coworkers observed that the early progenitors in the adult thymus retain myeloid potential and proposed an alternative myeloid-based model of hematopoiesis in which the myeloid potential persists in the T and B cell branches, even after these lineages have diverged (Kawamoto et al., 2010).

The development of lymphoid progenitors is controlled by two transcription factors, PU.1 and Ikaros, which are expressed early in MPPs and act in parallel pathways, in part by regulating the expression of essential signaling receptors (Flt3, c-Kit, and IL-7R). Interestingly, it has been demonstrated that graded expression of PU.1 specifies either the myeloid or lymphoid fate of early hematopoietic progenitors (Busslinger, 2004). Several lines of evidence have proposed that CLP is the common branch point for the generation of the natural killer (NK), T, and B cell precursors and that the entry of CLP into the B cell lineage depends on the

transcription factors E2A, early B cell factor 1 (EBF1), and paired box protein 5 (Pax5), as B cell development is blocked at its earliest stages in the absence of any one of these transcriptional regulators (Figure 1.2). In particular, a huge amount of data have unequivocally identified Pax5 as the critical B cell lineage commitment factor that restricts the developmental options of lymphoid progenitors to the B cell pathway by simultaneously repressing B-lineage-inappropriate genes and activating B-lymphoid-specific genes (Cobaleda et al., 2007). In the absence of Pax5, B cell development is arrested at the early pro-B cell stage of differentiation. Interestingly, these Pax5 -/- pro-B cells are not committed to the B cell lineage but can be cultivated indefinitely *in vitro* in the presence of IL-7 and stroma and are capable of differentiating into a broad spectrum of hematopoietic cell types. The restoration of Pax5 expression in these deficient cells suppresses this multi lineage potential (Rolink et al., 1999; Carotta et al., 2006).

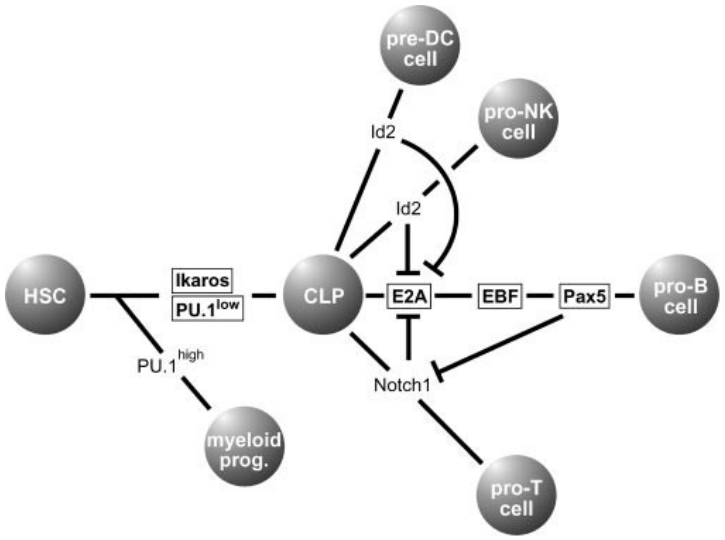
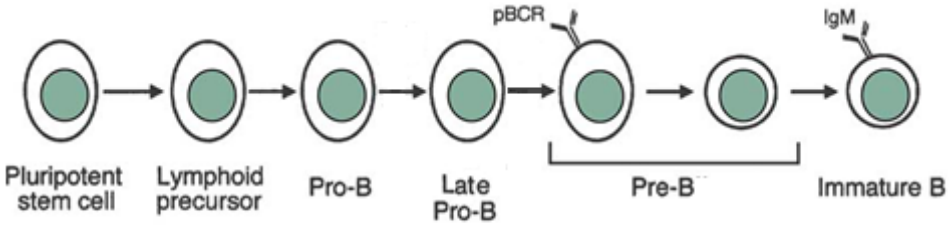


Figure 1.2: Transcriptional control and cross-repression of lymphoid pathways. The primary fate of the CLP is B-cell development, which depends on the transcription factors E2A, EBF and Pax5a. The interference of E2A activity by Id2 blocks B-cell development and promotes commitment to the NK-cell lineage. The exposure of lymphoid progenitors to the thymic microenvironment activates Notch1 signaling, which specifies the T-cell fate and interferes with B-cell development by blocking E2A activity *(Busslinger, 2004)*.

The pre-pro-B stage is the earliest in the B-lineage pathway and is defined by the expression of the B220 surface marker, the lack of CD19, and little or no Ig rearrangement. Going further along the maturation process, we encounter pro-B cells that are characterized by an up-regulation of the recombination-activating gene-1 (RAG-1) and RAG-2, which encode enzymes that play an important role in the rearrangement and recombination of the immunoglobulin genes during the process of V(D)J recombination (De and Rodgers, 2004).

As a matter of fact, during the pro-B stage the rearrangement of the antibody heavy chain takes place: in the early phase we have the D_H-to-J_H gene rearrangement while the joining of a V_H segment to the pre-arranged D_HJ_H complex occurs in the late pro-B stage. Furthermore, the successful rearrangement of the heavy chain gene on one chromosome represses the rearrangement of genetic material from the second chromosome. This not yet well defined mechanism, named allelic exclusion, ensures the monoallelic expression of the IgH gene and, therefore, monospecific Ag recognition (Vettermann and Schlissel, 2010). Once heavy-chain rearrangement is completed, the cell is classified as a pre-B cell and it expresses the pre-BCR in which the rearranged μ heavy chain is combined with the surrogate light chains λ5 and VpreB. In addition, the pre-BCR associates with the Igα/Igβ dimer (even known as CD79a/CD79b dimer) that is needed for the transduction of signals for the survival and development of pro-B cells into pre-B cells (Figure 1.3). The pre-BCR is essential for B cells to develop normally since it has been shown that mutated or altered forms lead to cancer, immunodeficiency and perhaps even autoimmunity (Martensson et al., 2010). The expression of the functional pre-BCR also initiates cell cycling and light chain rearrangement: following proliferation, small pre-B cells, no longer dividing, undergo V-J joining on one light chain chromosome. This rearrangement has to be productive and the resulting light chain must be able to pair with the μ heavy chain in order to form a functional BCR. Once this happens, the cell acquires antigen specificity and is called immature B cell.



IgH	GL	DJ	VDJ	VDJ	VDJ
IgL	GL	GL	GL	GL	VJ
B220	+	+	+	+	+
CD43	+	+	+	-	-
CD25	-	-	+	+	-
λ5/VpreB	-/+	+	+	-	-
CD19	-	+	+	+	+
mb-1/Igα	-/+	+	+	+	+
B29/Igβ	-/+	+	+	+	+

Figure 1.3: B cell development from the pro-B to the immature B cell stage. The stages of B cell development are defined by the rearrangement status of the IgH and IgL chains. The expression of surface markers characterizing the different maturative stages are also indicated. The expression of λ5/VpreB, mb-1, and B29 (−/+) begins late in the pro-B cell stage. *Adapted from (Maier and Hagman, 2002)*.

The antigen receptor gene rearrangement of variable (V), diversity (D) and joining (J) gene segments generates an enormous repertoire of antigen receptors with different antibody specificities, providing the versatility that is essential to normal immune functioning. A logical consequence of the V(D)J random recombination is that a significant fraction of the receptors generated by these process bind to one or more self-antigens. Several studies have shown that B-cell tolerance to the autoreactive B cells is established through four cellular strategies (Figure 1.4). The cell displaying the self-reactive receptor can be triggered to die or can 'edit' the offending receptor by further V(D)J recombination to display a different receptor that is not self-reactive. Then, intrinsic biochemical and gene-expression changes can reduce the ability of the cell to be triggered by self-reactive receptors, a mechanism generally termed clonal anergy. Finally, even if the cells have evaded the three mechanisms above, extrinsic controls can limit the danger of self-reactive receptors limiting the supply of essential growth factors, co-stimuli, pro-inflammatory mediators and other factors (Goodnow et al., 2005).

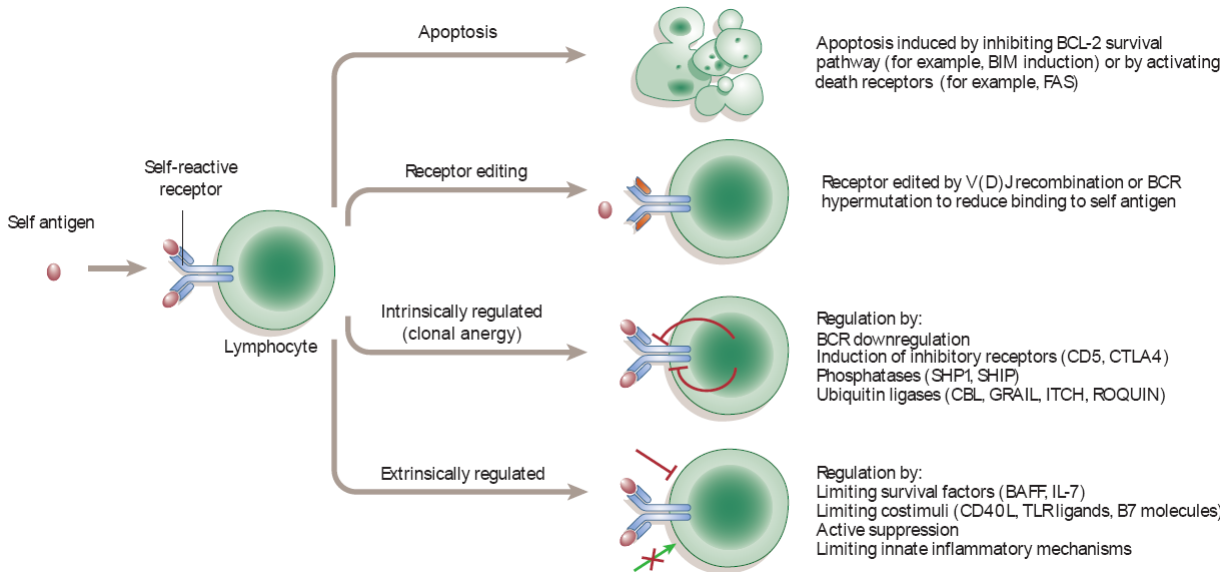


Figure 1.4: Four cellular strategies are used to regulate self-reactive receptors at different points during B cell differentiation. a) The cell is deleted through induction of cell death. b) The receptor is edited to one that is less self-reactive. c) Biochemical or gene-expression changes intrinsically dampen the self-reactive receptor's ability to activate the cell. d) The ability of self-reactive cells or antibody to cause autoimmunity is limited by using extrinsic suppression and by limiting essential growth factors, costimuli and inflammatory mediators. *Adapted from (Goodnow et al., 2005)*.

Most of the immature B cells that survive negative selection are released from the bone marrow and receive signals to home to the spleen where progression through transitional developmental stages is required to form naive mature B cells capable of differentiating into antibody-secreting cells upon encounter with cognate antigen. The transitional B cell

population is heterogeneous and can be divided at least into two subsets, especially on the basis of the variable expression of specific cell surface markers. Transitional 1 (T1) or newly formed (NF) B cells are defined as newly generated B cells that are not able to re-circulate and still don't express follicular markers such as IgD and the low affinity receptor for IgE, CD23. In addition to IgM, they express very low levels of the complement receptor CD21, which is present on the cell surface as part of the CD19-CD21-CD81 co-receptor complex that plays an important role in the integration of innate and humoral immune response (Allman et al., 2001). After emerging from the marginal sinus, T1 B cells further mature into transitional 2 (T2) B cells that are drawn into follicles. T2 B cells still carry markers of immaturity but are able to re-circulate; in addition they acquire cell surface IgD and CD23 and express intermediate levels of CD21 (Carsetti et al., 2004). In the spleen, these T2 B cells can mature into either follicular (FO) or MZ B cells: this cell fate decision depends in part on the strength of BCR signaling but also requires the participation of a distinct member of the Notch family, Notch2 (Figure 1.5). A T2 B cell that receives signals via the BCR makes one of two decisions: if its BCR reacts with intermediate affinity with a self-antigen it is induced to differentiate into a FO B cell while instead if its BCR reacts poorly or not at all to the self-antigen it is receptive to inductive signals that drive it to a MZ B cell fate (Pillai and Cariappa, 2009). Similar to the early stages in the bone marrow, this phase of development in the spleen requires other supporting factors in addition to the BCR signal: the surrounding stromal microenvironment, the presence of appropriate growth factors, as well as the ability of the B cells to respond to them, are all crucial players in the final maturation steps of developing B cells. There is a body of evidence that clearly demonstrates that the B cell activating factor (BAFF), a member of the tumor necrosis factor (TNF) family, plays a fundamental role during the transition from T1 to T2 B cells as mice lacking BAFF or BAFF-receptor (BAFF-R) exhibit a drastic reduction in mature B cell number in the spleen (Shulga-Morskaya et al., 2004). Recently Rauch and coworkers have shown how BAFF-BAFF-R signaling plays a crucial role in the survival and maintenance of mature B cells also *in vivo* (Rauch et al., 2009).

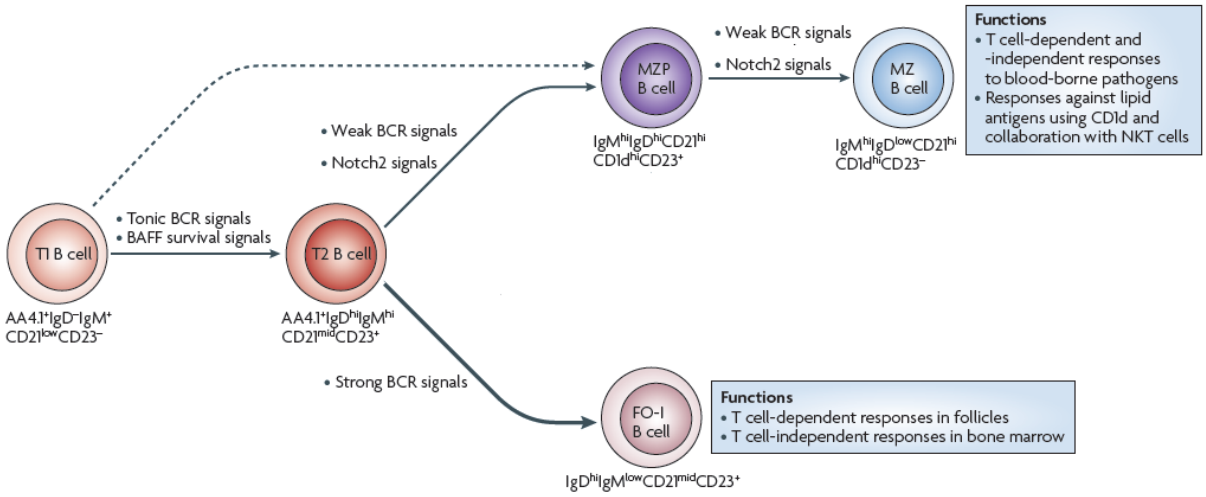


Figure 1.5: The follicular versus marginal zone B lymphocyte cell fate decision. Transitional (T1 and T2) B cells can mature in either follicular (FO) or marginal zone (MZ) B cells and this cell fate decision depends in part on the strength of BCR signalling but also requires the participation of a distinct member of the Notch family, Notch2. It is probable that T2 B cells mature into FO B cells if they recognize self antigen with a high affinity, whereas self-reactive B cells with a lower affinity can mature into MZ B cells. *Adapted from (Pillai and Cariappa, 2009)*.

In the spleen, the FO subset constitutes the main B cell population while MZ B cells represent only about 5% of B lymphocytes. These two subsets of B cells differ first of all for their anatomical localization: if a T2 B cell differentiates into a MZ B cell it migrates to a new location in the spleen where it is physically retained. This new location is the marginal zone, a unique compartment at the interface between the red and the white pulp of the spleen. In this area, the blood circulating through the spleen slows down allowing the efficient encounter of MZ B cells with pathogens present in the bloodstream. MZ B cells are able to initiate a fast and intense antibody response to these blood-borne viral and bacterial agents thereby acting as a bridge between the early innate immune response and the slower adaptive immune response. Therefore, like B-1 cells, MZ B cells appear to function more as an innate-like subset of B cells (Lopes-Carvalho and Kearney, 2004). MZ and B-1 B cells share many other functional characteristics as shown in table 1.1 (Martin and Kearney, 2001).

Table 1.1 – Characteristics of B1 cells, MZ B cells and FO B cells.

Characteristic	B1	MZ	FO
Half-life	Long	Long	Long
Recirculation in lymph	–	–	+
T-independent responses	+++	+++	+
T-dependent responses	+/– ?	+	+
Favorite isotypes	IgM, IgG_3, IgA	IgM, IgG_3	IgG_1
Antigen presentation *in vitro*	+++	+++	+
Time to peak cell cycle	Short	Short	Long
Proliferation to:			
LPS	+++	+++	+
anti-CD40	++	++	+
anti-IgM	–	–	+
anti-IgM + anti-CD40	++	++	++
IgM/co-receptor-dependent selection	++	++	+/–
CD9 expression	+	+	–
Anti-IgM-induced apoptosis	–	+++	+
pyk-2 dependency	–	+	–
Expression of B1 markers (CD5, IL-5R, CD43)	+	–	–
Resistance to Fas-mediated apoptosis	+	–	–

Adapted from (Martin and Kearney, 2001)

Although MZ B cells are defined primarily on the basis of their anatomical localization, the expression of a number of specific surface markers can also be used to identify these cells. Unlike FO B cells, that express high levels of CD23 and IgD but low levels of IgM, MZ B cells can be identified as $CD23^{-}$ IgD^{low} IgM^{hi} and are also characterized by an up-regulation of CD21. Hence, examination of the levels of the surface molecules CD21 and CD23 allows distinguishing MZ B cells from both FO and transitional, newly formed (NF) B cells in the spleen (Figure 1.6). Another marker that can be used to distinguish MZ and FO B cells is CD1d: as reported by Amano and coworkers, MZ B cells are stained brightly for CD1d while instead the remaining subpopulations of B cells in the spleen, lymph node, peritoneal cavity, and bone marrow show either "dull" staining or no staining above background (Amano et al., 1998). CD1d is a glycoprotein with structural homology to the major histocompatibility complex (MHC) class I that binds a variety of self and foreign lipid- and glycolipid-containing antigens and present them mostly to a unique T cell subpopulation, called NKT cells, which shares properties with both T and NK cells (Brigl and Brenner, 2004). Recently, the expression of CD1d on the B cell surface has acquired particular relevance since it has been defined as a marker related to regulatory B cells (Mizoguchi et al., 2002).

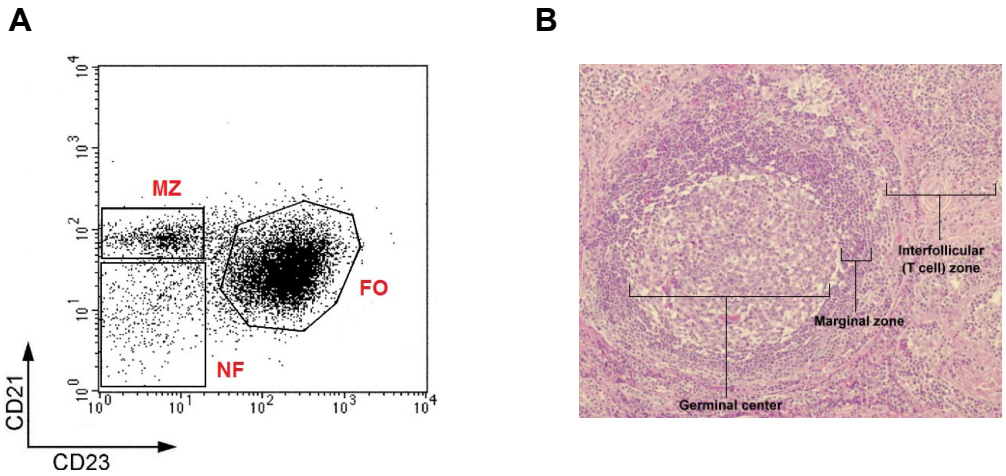


Figure 1.6: Anatomical and immunophenotypic characterization of follicular and marginal zone B cells. (***A***) Mature B cell subpopulations can be identified by anti-CD21 versus anti-CD23 staining. Follicular (FO) B cells express high levels of CD23 and CD21 while marginal zone (MZ) B cells express high levels of CD21, but do not express CD23. (***B***) B cells of the secondary B-cell repertoire can be classified as well according to their homing in the various compartments of the B-follicle, being the germinal centre and the MZ. The latter is well developed in those secondary lymphoid organs where an abundant influx of antigens is known to occur. As such, it is especially well recognizable in the white pulp of the spleen where it was originally described.

Since MZ B cells, compared to other mature naïve B cells, express higher levels of B7 proteins and of the CD38 and CD1d molecules, they are defined as cells that show a pre-activated phenotype. Finally, while MZ B cells are sessile and apparently long-lived cells, FO B cells are freely recirculating lymphocytes and constitute the major B cell subset that, upon activation, undergoes somatic hypermutation and isotype switching and therefore contribute to the production of high affinity antibodies (Pillai et al., 2005).

Whether B cells are FO or MZ, until they do not encounter the antigen for which they are specific they are called mature naïve B cells. They subsequently enter the antigen-dependent phase of their differentiation where these mature but naïve B cells, after encountering their antigen, become activated B cells.

1.2 B cell activation

The antigen-dependent stages of B lymphocyte differentiation occur in the spleen, lymph nodes and other peripheral lymphoid tissues. B cell activation is initiated following the recognition of antigen through the BCR, in the presence of a "second" signal, and results in B cell proliferation and differentiation, rather than cell cycle arrest or apoptosis, as seen with immature cells. Activated B cells can differentiate either into plasma cells, which are capable of antibody secretion, or memory cells, that provide long-lived protection against secondary infection (Harwood and Batista, 2010). The fate of an activated B cell varies depending on the type of antigen and on the anatomical site in which the encounter takes place; moreover, the

different types of mature B cells do not respond equally to the various antigenic stimuli. B cell recognition of antigen is not the only element necessary for B cell activation: naïve B cells require accessory signals that can come either from a helper T cell or, in some cases, directly from microbial constituents (Frauwirth and Thompson, 2002). Depending on the nature of the antigen and of the second signal, B cells can be activated in a T cell-dependent (TD) or T cell-independent manner (TI).

1.2.1 T cell-dependent activation

Protein antigens that are unable to induce antibody responses in animals or humans who lack T cells are known as TD antigens. The second signal required to induce the antibody response to these protein antigens is delivered by a helper T cell that recognizes degraded fragments of the antigen as peptides bound to MHC class II molecules on the B cell surface. The recognition of peptide:MHC class II complexes on B lymphocytes triggers helper T cells to synthesize both cell-bound and secreted effector molecules that synergize in activating the B cell (Janeway, 2005) (Figure 1.7). Some of the most biologically important signals that the B cell receives from the cognate activated T cell are delivered through membrane-bound members of the TNF and TNF receptor (TNF-R) family of proteins such as CD40 and its ligand CD154 (CD40L) or CD134 (OX40) and its ligand OX40L (Bishop and Hostager, 2001).

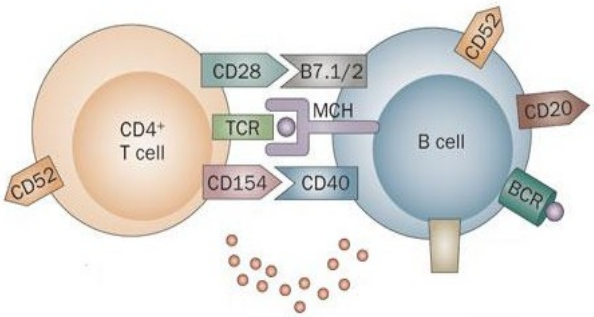


Figure 1.7: The CD40-CD40L co-stimulatory system is required for TD B cell activation. Following Ag presentation we assist to Th cell activation which results in CD40L expression and cytokine secretion. CD40 ligation by CD40L leads to B cell activation. *Adapted from (Kirk et al., 2010)*.

The CD40-CD40L co-stimulatory system plays a fundamental role in B cell activation. As a matter of fact, several *in vitro* and *in vivo* studies have implicated direct signaling of B cells by CD40 as a critical step at numerous stages of the B cell response including proliferation and clonal expansion, Ig production, germinal center formation, isotype switching, affinity maturation, and induction of B cell memory (Chirmule et al., 2000). In addition, it has been shown that CD40 can induce re-expression of telomerase activity in memory B cells, thereby contributing to an expanded lifespan of these cells (Hu et al., 1997). However, if on the one

hand CD40 activation guides the B cells through their differentiation program, on the other hand it prevents the terminal differentiation of activated mature B cells into plasma cells (Randall et al., 1998). The importance of the CD40-CD40L axis during TD humoral responses is best illustrated by its genetic deficiency that, in humans, causes the X-linked hyper-IgM syndrome (HIGM). Patients affected by this disorder develop recurrent infections due to abnormal humoral immunity, which is the consequence of the failure to form germinal centers in lymph nodes and isotype switch to IgG, IgA, and IgE (Callard et al., 1993). A similar phenotype was seen in mice rendered CD40L-deficient by germline knockout (Xu et al., 1994) or treated with a CD40L blocking Ab (Foy et al., 1993). Following immunization with a TD Ag these mice were unable to mount a primary or a secondary antibody response against the antigen, did not form germinal centers, and did not generate antigen-specific memory B cells.

In a T cell-dependent immune response mainly FO B cells are involved even if MZ and B-1 B cells can also respond. Following interaction with the cognate T cell, antigen-activated B cells can either differentiate into short-lived plasma cells or enter B cell follicles in secondary lymphoid organs and establish histological structures called germinal centers (GCs) (Figure 1.8). MZ B cells responding to a TD antigen presumably differentiate directly into extrafollicular plasma cells and do not participate to the GC reaction (Shapiro-Shelef and Calame, 2005). In the germinal centers, B cells undergo massive clonal expansion: this proliferation takes place in the GC dark zone and is accompanied by somatic hypermutation (SHM), a process which introduces mutations at a very high rate in the immunoglobulin variable region genes. The mutated GC B cells then migrate to the GC light zone, which is rich in T helper (Th) cells and follicular dendritic cells (FDCs). By interacting with these cells, GC B cells that have acquired high-affinity variants of the original antigen-specific BCR are selected while GC B cells bearing unfavorable mutations undergo apoptosis (Zenz et al., 2010). In the GC light zone, many GC B cells also undergo class switch recombination (CSR). This biological process changes the immunoglobulin heavy chain constant region (C_H) gene from C_μ to one of the other C_H genes and this results in switch of the immunoglobulin isotype from IgM or IgD to either IgG, IgE, or IgA. Each isotype determines the manner by which captured antigens are eliminated and the location where the immunoglobulin is delivered and accumulated (Muramatsu et al., 2000).

Positively selected GC B cells finally differentiate either into memory B cells or plasma cells and leave the GC. These two quiescent and long-lived B cell types persist within local environmental survival niches that afford cellular longevity. However, the factors supporting

memory B cell survival within the secondary lymphoid organs and allowing plasma cell persistence in the bone marrow remain poorly characterized. Interestingly, Noelle and coworkers showed that the members of the TNF family of ligands, BAFF and a proliferation-inducing ligand (APRIL), support the survival of plasma cells but not of memory B cells *in vivo*, and this suggests that memory B cells are the first identified B-2 lineage subset that survives independently of BAFF and APRIL (Benson et al., 2008).

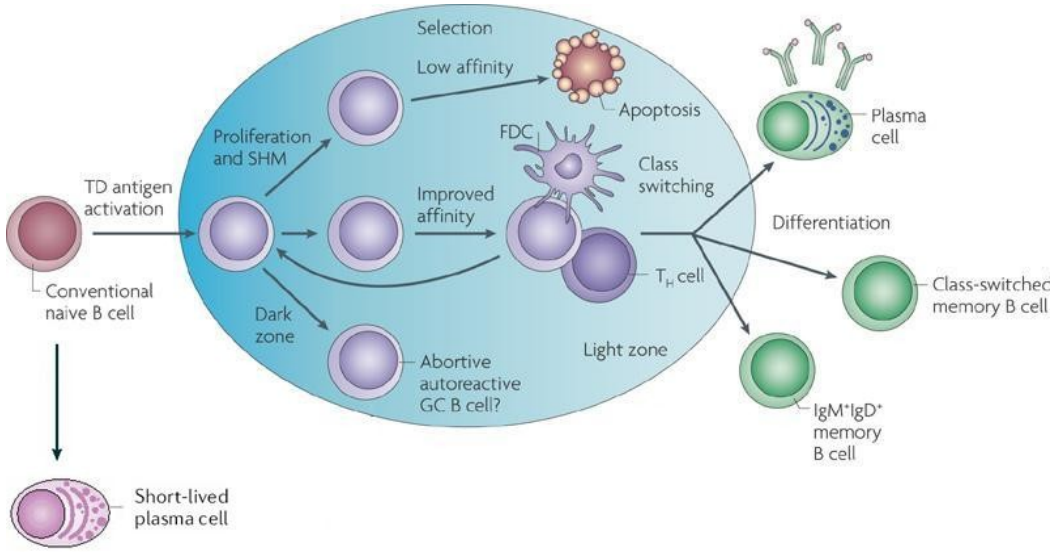


Figure 1.8: Germinal center reaction. After activation by antigen, mature naïve B cells migrate to the edge of the follicles, where they receive help from cognate T cells. Following interaction with the T cell, Ag-activated B cells can either differentiate into short-lived plasma cells or enter B cell follicles and establish histological structures called germinal centers (GCs). In the dark zone of the GC the B cells proliferate and undergo somatic hypermutation (SHM). At this point, cells that introduce mutations that impair BCR expression die by lack of a BCR signal. After one (or potentially more) division/mutation cycles, surviving B cells migrate towards the light zone of the GC where they interact with Ag in immune complexes on follicular dendritic cells (FDCs). Antigen signals are not limiting at this point, and all B cells transiting to the light zone upregulate CD83 and CD86. B cells present Ag to T helper cells (Th) and the B lymphocytes that successfully interact with Th can then follow two potential fates: exit from the GC into the plasma cell or the memory B cell fate. *Adapted from (Zenz et al., 2010)*.

1.2.2 T cell-independent activation

Some microbial products can activate B cells directly, in the absence of T cell help: these microbial antigens are known as TI antigens because they induce antibody responses in individuals who have no T lymphocytes. The second signal required to activate antibody production to these TI antigens is either provided directly by recognition of a common microbial constituent or by a nonthymus-derived accessory cell. This is of fundamental importance since the ability of B cells to respond directly to these antigens provides a rapid response to many important bacterial pathogens.

TI responses are subdivided into two categories referred to as type 1 (TI-1) and type 2 (TI-2). TI-1 antigens are defined as polyclonal B cell activators, since, at high concentrations, they are able to induce proliferation and antibody secretion by B cells in the absence of specific antigen binding to surface immunoglobulin. The most classical example of a TI-1 antigen is lipopolysaccharide (LPS), a component of gram negative bacterial cell walls that activates B cells via Toll-like receptor (TLR) 4, regardless of their BCR specificity. LPS induces B cell proliferation and differentiation into Ab secreting cells and it stimulates IL-6 secretion in mature B cells through phosphatidylinositol 3-kinase (PI 3-kinase) signaling pathways (Venkataraman et al., 1999). Human B cells lack TLR4, but express TLR9, and undergo proliferation and IgM production in response to CpG DNA (Krieg et al., 1995).

TI-2 antigens, such as capsular polysaccharides and polymeric proteins, are molecules with multiple, repeating subunits. These highly repetitive molecules activate B lymphocytes by engagement and crosslinking of their BCR (Janeway, 2005).

In a TI immune response, mainly MZ and B-1 B cells are involved: in rodents, these B cell subsets exhibit an activated phenotype that allows their rapid proliferation and differentiation into short-lived Ab-secreting cells upon stimulation with TI antigens (McHeyzer-Williams, 2003). Unlike FO B cells, MZ and B-1 B cells express polyspecific antibodies that recognize TI antigens with low affinity. This induces weak, predominantly IgM-mediated responses, with an inefficient induction of isotype switching, affinity maturation, and little or no booster effect after the second exposure to the antigen.

1.2.3 Role of other immune cell types in B cell activation

For a long time the two Ig gene-diversifying processes CSR and SHM were thought to be almost exclusively dependent on CD40L and on T cells. However, towards the end of the 90, several papers reported that viral glycoproteins and bacterial polysaccharides could stimulate IgG and IgA production in the absence of CD40L-expressing $CD4^+$ T cells (Cyster, 2000; Fagarasan and Honjo, 2000). These observations implicated the existence of SHM and CSR-inducing molecules different from CD40L.

As already mentioned above, the TD pathway provides immunological memory but is relatively slow to occur. To compensate for this limitation, MZ B cells and mucosal B-1 cells rapidly undergo TI Ab production in response to highly conserved microbial Ags with repetitive structures. Both TI-1 and TI-2 Ags encompass pathogen-associated molecular patterns (PAMPs), which stimulate MZ and B-1 cells by cross-linking poorly diversified surface BCRs encoded by a restricted set of V(D)J genes carrying no or few mutations

(Bendelac et al., 2001). During a TI response the interaction of B cells with DCs and activated macrophages is also required (Balazs et al., 2002). These innate immune cells recognize PAMPs through pattern recognition receptors, including TLR family members. In addition to triggering immediate innate responses, PAMP-TLR interaction favors TI B cell Ab production. Following the observation that APCs interact with B cells to enhance IgG and IgA production, the group of Cerutti investigated the possibility that APCs could play a key role in the initiation of CD40-indipendent CSR. They demonstrated that human DCs and monocytes up-regulate BAFF and APRIL upon stimulation with interferon (IFN)-α, IFN-γ, LPS or CD40L and that, in the presence of appropriate cytokines, BAFF and APRIL induced CD40-independent CSR to C_γ, C_α or C_ε in B cells (Litinskiy et al., 2002). More recently, Puga and coworkers demonstrated that splenic neutrophils, which have a phenotype distinct from that of circulating neutrophils, can form MZ B cell–interacting NET-like structures, and elicit Ig class switching, somatic hypermutation and antibody production by activating MZ B cells via a mechanism that involved BAFF, APRIL and the cytokine IL-21 (Puga et al., 2012).
These findings suggest that BAFF, APRIL and the immune cell type that produce these cytokines belong to a pathway that, by mediating TI class switching, critically links the innate and adaptive immune responses (Cerutti et al., 2011).
Interestingly, another cell type of the innate immune system was shown to regulate B-cell activation and differentiation. In a recent study published on *Blood*, Merluzzi and coworkers reported that mast cells (MCs) were able to promote both survival and activation of näive B cells, as well as proliferation and further plasma cell differentiation of activated B cells. B-cell proliferation was reduced when MCs and B cells were separated by a transwell membrane or when MCs were IL-6 deficient, suggesting that both soluble factors and cell-cell contact are important in this event. Moreover, they demonstrated that activated MCs were able to induce IgA surface expression on activated B cells and IgA secretion, suggesting a new role of MCs in the induction of humoral immune responses (Merluzzi et al., 2010).

All together these data suggest that antigen-sampling DCs, MCs and other cells of the innate immune response are able to promote and enhance B cell responses at mucosal surfaces inhabited by commensal bacteria and at the interfaces between the circulation and the immune system, through a TI pathway (Figure 1.9).

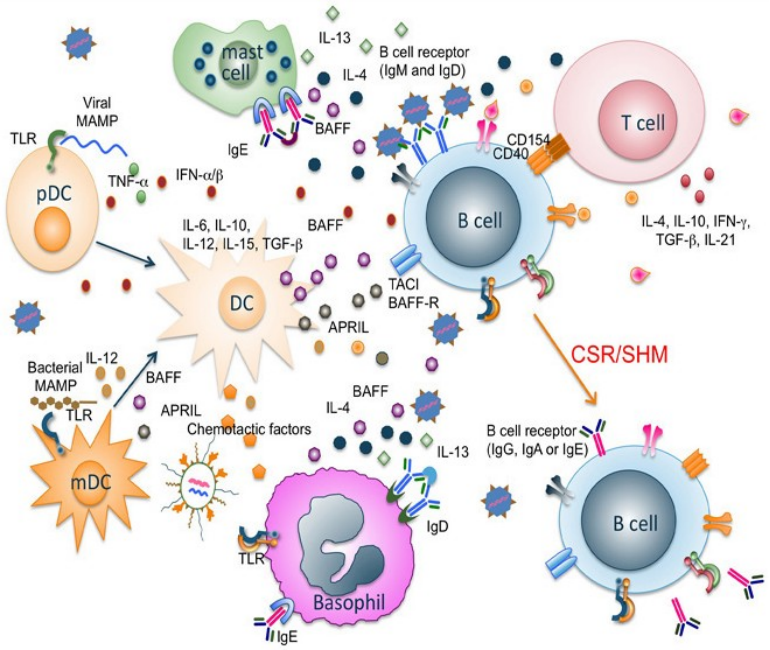


Figure 1.9: Role of different immune cell types in B-cell activation. *In vivo*, the stimuli that affect B-cell survival, proliferation and differentiation derive from the crosstalk between B lymphocytes and other cells of the immune system. Cells of both the innate and adaptive immune system can interact with B cells through cell to cell contact or through the release of specific soluble factors. *Adapted from Casali and coworkers, University of California, Irvine.*

1.3 Regulatory B cells

B cells are classically associated to their capacity to produce antibodies: these cells have been classified as positive regulators of humoral immune responses and are distinguished by their ability to terminally differentiate into antibody-secreting plasma cells. However, over the last two decades, a growing body of evidence has demonstrated that B lymphocytes have many other functions, unrelated to antibody production (Figure 1.10). B cells can act as APCs, they produce numerous cytokines and they express B cell-specific co-stimulatory molecules, such as CD80, CD86, CD40 and OX40L, that mediate their interaction with different immune cell types (LeBien and Tedder, 2008). Several papers have shown that B cells are not simply the passive recipients of T cell help, but actively participate in cellular immune responses by directing the magnitude and quality of the T cell response to foreign and self antigens (Lund and Randall, 2010).

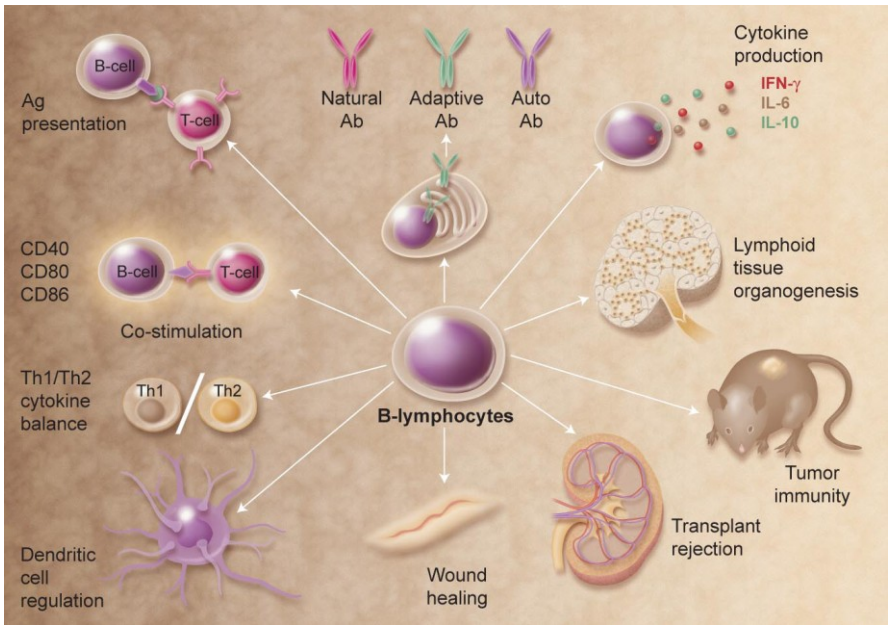


Figure 1.10: Multifunctional attributes of B lymphocytes. Selected examples of how B cells regulate immune homeostasis are shown. Many of these functions are independent of antibody production. *(LeBien and Tedder, 2008)*.

In addition to the aforementioned B cell functions, recent findings have revealed a regulatory role of B cells that place them again under the spotlight as important immune regulators.

In 2002, Bhan and collaborators termed these cells "regulatory B cells" (Bregs) (Mizoguchi et al., 2002) and, since then, many papers have shown the role of these cells in the maintenance of the fine equilibrium required for tolerance. Bregs restrain the excessive inflammatory responses that occur during autoimmune diseases or that can be caused by unresolved infections and they exert this function mainly through the production of the regulatory cytokine IL-10. B cells from several different subsets seem to acquire the ability to suppress immune responses in an IL-10-dependent manner even if it is likely that these Breg cells may exert suppressive functions also by other mechanisms, both contact dependent and independent. While some of these Breg cells belong to truly distinct and specialized subsets, some are likely activated B cells that acquired a suppressive function (Vitale et al., 2010).

Since Bregs are rare, lack a specific marker, and express detectable IL-10 only upon *ex vivo* stimulation, the definitive identification of this functional subset of B cells still remains challenging.

1.3.1 Identification of B cells with suppressive functions in distinct immunological settings

The first evidence of the existence of a B cell population with a negative regulatory function dates back to 1974 when Katz and coworkers reported that spleen B cells were found to impair delayed type hypersensitivity (DTH) responses in guinea pigs (Katz et al., 1974). Specifically, they showed that adoptively transferred B cell–depleted splenocytes were unable to inhibit delayed-onset skin reactions. Although this finding led to conclude that DTH responses and T-cell function can be regulated by suppressor B cells, the potential role of B cells in the regulation of immune response was mostly overlooked for at least another decade.
In recent years, regulatory B cells were identified in both mouse and humans and are now recognized as an important new component of the immune system.

- ***Bregs and autoimmunity***

In the majority of autoimmune diseases, B cells are generally considered to be pathogenic because of their capacity to secrete autoantibodies. For this reason, highly targeted B-cell depleting therapies, focused on restoring normal B cell function and eliminating pathogenic autoantibodies, have been developed (Martin and Chan, 2004). Paradoxically, studies in the mouse have shown that, in certain models of autoimmune disease and chronic inflammation, B cell elimination leads to the exacerbation of the disease. This suggests that, in addition to pathogenic B cells, we have the coexistence of distinct protective B cell subsets that suppress the progression of and/or enhance the recovery from acquired immune mediated inflammation. Janeway and coworkers were the first to provide direct evidence for a role of B cells in the suppression of a T cell-mediated autoimmune reaction (Wolf et al., 1996). Using a mouse model of experimentally induced autoimmune encephalomyelitis (EAE), they demonstrated that disease onset, severity and recovery were extremely impaired in μMT mice (a strain genetically deficient of B cells as a consequence of the disruption of the membrane exon of the immunoglobulin μ heavy chain gene (Kitamura et al., 1991)) compared to controls. Later the group of Anderton established that autoantigen-reactive splenic B cells were responsible for the suppression of EAE and uncovered the key suppressive role of IL-10 produced by these B cells upon their activation with the autoantigen and through CD40 ligation (Fillatreau et al., 2002). At around the same time, similar observations underlining the importance of B cell-derived IL-10 in immunoregulation were made in other mouse models of autoimmune conditions, including chronic intestinal inflammation and collagen-induced arthritis (CIA). In 2002, Mizoguchi and coworkers, while studying the spontaneous colitis that develops in T-cell

receptor α (TCRα) knockout mice, defined a gut-associated lymphoid tissue (GALT)-associated B cell subset characterized by high expression of the MHC Class I-like surface molecule CD1d and enhanced production of IL-10. This B cell subset appeared after the development of chronic intestinal inflammation and suppressed the progression of intestinal inflammation by downregulating inflammatory cascades associated with IL-1 upregulation and signal transducer and activator of transcription 3 (STAT3) activation, rather than by altering polarized Th responses (Mizoguchi et al., 2002). A year later, work done by Mauri and coworkers revealed that activation of arthritogenic splenocytes with antigen and an agonistic anti-CD40 Ab gives rise to a B cell population that produces high levels of IL-10. The transfer of these cells into mice affected by CIA suppressed the induction of arthritogenic Th1 response and restrained the severity of inflammation in the synovia (Mauri et al., 2003).

Over the years, Breg cells were shown to be able to down-regulate immune responses in several other murine models of autoimmunity, such as type 1 diabetes (Hussain and Delovitch, 2007) and systemic lupus erythematosus (SLE) (Haas et al., 2010; Watanabe et al., 2010), and, therefore, it seems probable that the induction of Bregs or the identification of pathways leading to their expansion will become the preferred option for treating autoimmune conditions, as an alternative to the present B-cell depletion therapy (Mauri and Ehrenstein, 2008).

- ***Bregs and immunity against pathogens***

Although the number of studies describing Bregs during infections is increasing, conclusive interpretation of the current data has been hampered by conflicting results.

B cells from mice infected with the filarial nematode *Brugia pahangi* produce IL-10 that down-regulates the expression of B7 molecules on the B cell surface, attenuating their efficiency as APC to $CD4^+$ T cells and restricting their expansion. Thus, IL-10–producing B cells have an important role in filarial infection via their ability to modulate T cell responses (Gillan et al., 2005). Recently, the group of Launois demonstrated that in BALB/c mice $CD1d^{hi}$ $CD5^+$ IL-10–producing B cells are required for susceptibility to infection with the protozoan parasite *Leishmania major*, suggesting that parasites can exploit and take advantage of this immune regulatory mechanism (Ronet et al., 2010).

Several studies in both human and animal models have demonstrated that helminths are powerful modulators of their host's immune response and that they are prone to evoke a regulatory environment (Jankovic et al., 2006; Fallon and Mangan, 2007). Mangan and coworkers demonstrated that *Schistosoma mansoni* infection protects mice from an

experimental model of systemic fatal anaphylaxis, an extreme allergic response. Interestingly, the *in vivo* depletion experiments performed in this study demonstrated that the Breg population induced during helminthic infection and the IL-10 produced by these B cells played a fundamental role in the worm-mediated protection to anaphylaxis (Mangan et al., 2004). Moreover, the group of Razzitte assessed the role of B cell control during helminthic infections in multiple sclerosis (MS) patients and showed how these parasites are able to induced regulatory B cells capable of dampening the immune response through production of IL-10. In MS patients, helminths may lead to increased IL-10–secreting B cells numbers or activity, either by generating new cells or by activating/expanding existing cells (Correale et al., 2008).

- ***Bregs and tumor immunity***

The role of B lymphocytes in tumor immunity has primarily been studied in the context of their role as positive regulators of the immune response through antibody production and T-cell activation (LeBien and Tedder, 2008). With the increase of evidences demonstrating the immunosuppressive function of specific B cell subsets, the hypothesis that Bregs could also downregulate the protective cytotoxic T lymphocyte responses directed against tumor cells began to take hold.

In 2005 Shah and coworkers examined the role of B cells in antitumor immunity by comparing tumor resistance of wild type mice and mice genetically lacking B cells. They observed that B-cell-deficient mice showed higher resistance to several histologically diverse primary syngeneic tumors. The enhanced antitumor immunity was associated with an increased activity of T and NK cells, both of which are important for the promotion of natural tumor surveillance (Shah et al., 2005). One year later, Inoue and coworkers demonstrated that B cells can function as regulatory cells in some specific tumor settings and provided a mechanistic explanation for why B cells can limit effective immune responses against certain tumor cells. CD40L-expressing tumor cells activate B cells and these CD40 activated B cells release IL-10 which, in turn, diminishes CD8 and NK cell IFN-γ secretion, antitumor activity and CD8 T-cell memory development (Inoue et al., 2006).

More recently it has been shown that during 7,12-dimethylbenz[α]anthracene/12-O-tetradecanoylphorbol 13-acetate (DMBA/TPA)-induced skin carcinogenesis TNF-α mediates tumor-promoting activity via IL-10–producing regulatory B cells, that repress antitumor immunity (Schioppa et al., 2011).

Moreover, the group of Tedder demonstrated that IL-10^{+} B cells are potent negative regulators of innate immune responses and that their removal is essential for optimal CD20 mAb clearance of malignant B cells *in vivo* (Horikawa et al., 2011).

All together, these data suggest that IL-10–producing regulatory B cells are likely to be involved in regulating antitumor immunity and, therefore, the study of these cells and of their role in tumor immunity could pave the way for the development of novel therapies for tumor treatment.

- ***Bregs in humans***

Following the identification of IL-10–producing regulatory B cells in mice, researchers started looking for Bregs in humans. Although indirect evidence indicates the presence of these cells in humans, their isolation and characterization has been limited by experimental restrictions, mainly due to the difficulties in obtaining sufficient human samples.

The existence of Bregs in humans was first hypothesized by the group of Bar-Or. In a first report, they demonstrated that effector cytokine production by normal human B cells is context-dependent and that bystander B cells activated through CD40-mediated T cell stimulation, without preceding Ag/BCR engagement, produce negligible amounts of proinflammatory cytokines but secrete significant levels of the immune regulatory cytokine IL-10 (Duddy et al., 2004). Moreover, they observed that IL-10 was produced almost exclusively by näive B cells and that, compared with healthy individuals, patients with MS had a reduced frequency of IL-10-producing näive CD27^{-} B cells, in response to CD40 stimulation (Duddy et al., 2007).

More recently a regulatory B cell subset with a CD19^{+} CD24^{hi} CD38^{hi} CD5^{+} CD1d^{hi} phenotype has been described (Blair et al., 2010). In an Immunity paper, Blair and coworkers demonstrated that, upon CD40 engagement, IL-10-producing B cells were enriched within the CD19^{+} CD24^{hi} CD38^{hi} subset of circulating B cells and that these activated CD19^{+} CD24^{hi} CD38^{hi} cells were able to suppress the differentiation of Th1 cells in an IL-10, CD80 and CD86–dependent manner. Interestingly, they showed that, although numerically increased compared to healthy individuals, CD19^{+} CD24^{hi} CD38^{hi} B cells isolated from SLE patients failed to produce IL-10 in response to CD40 stimulation.

Intriguingly, stimulation of healthy B cells with CpG coupled to anti-Ig induce both the memory (CD27^{+}) and transitional (CD24^{hi} CD38^{hi}) B cell subsets to release IL-10 (Bouaziz et al., 2010), thus indicating the compelling need for more extensive characterization of human Bregs to better understand their immune regulatory function (Mauri and Bosma, 2011).

Given that human B cells can produce functional levels of IL-10 and that IL-10–producing Bregs play a intriguing role in the context of autoimmune diseases, the manipulation of IL-10 production by human B cells might represent a potential target for modulating the immune response in autoimmunity and cancer.

1.3.2 Immunosuppressive mechanisms of Bregs

All the aforementioned studies demonstrated, both in humans and mice, that B cells are critical for the effective regulation of the immune system, in different immunological settings. Although the immunosuppressive properties of B cells have for long received very little attention, it is now well established that regulatory B cells exist and are commonly defined as a functional B cell subset that suppresses the progression of and/or enhances the recovery from acquired immune mediated inflammation (Mizoguchi and Bhan, 2006).

Most of the immune suppressive mechanisms previously attributed to regulatory T cells have also been demonstrated in B cells. Among the regulatory T cell population, two different subsets have been described on the basis of their distinct suppressive mechanisms: naturally occurring $CD4^+$ $CD25^+$ Tregs exert their suppressive effects via cell contact by membrane-bound molecules while transforming growth factor-β (TGF-β)–producing Th3 cells and IL-10-producing T regulatory 1 (Tr1) cells act by releasing immunosuppressive cytokines (Jonuleit and Schmitt, 2003). At the same way, B cells employ a wide array of immunosuppressive mechanisms which include the production of IL-10 and TGF-β, secondary Ag presentation, and interaction with other immune cells.

The hallmark of Bregs suppressive function is their capacity to secrete IL-10, a multifunctional cytokine with diverse effects on most hematopoietic cell types and best known for its anti-inflammatory properties (Moore et al., 2001). Many of the immuno-inhibitory characteristics of IL-10 can be traced to the capacity of this cytokine to prevent the production of the Th1-associated cytokines IL-2 and IFN-γ and the Th2-associated cytokines IL-4 and IL-5 from APCs. The other profound effect of IL-10 is to inhibit the production of proinflammatory cytokines and mediators from macrophages and DCs: the major inflammatory cytokines, IL-1, IL-6, IL-12, and TNFα, are all dramatically repressed following exposure to IL-10 (Mosser and Zhang, 2008).

As already described above, the activity of IL-10-producing Bregs has been widely demonstrated in experimental models of autoimmunity, infectious disease and tumor rejection. Moreover, the role of IL-10 secretion by B cells was also emphasized in a murine model of

oral tolerance where B cell-derived IL-10 enhanced the tolerogenic capacity of DCs (Gonnella et al., 2001).

Bregs suppressive function has also been correlated with the production of the anti-inflammatory cytokine TGF-β. Tian and coworkers showed that LPS-activated B cells, but not control B cells, secrete TGF-β and that the transfusion of these LPS-treated B cells into prediabetic non-obese diabetic (NOD) mice inhibited the spontaneous Th1 immunity to B cell Ags and disease progression (Tian et al., 2001). The importance of TGF-β for Breg effector functions was also shown in SAMP1/Yit mice, which are widely recognized as a murine model of Crohn's disease. Mishima and coworkers reported that TLR-activated intestinal B cells from SAMP1/Yit mice produced significantly less IL-10 and TGF-β compared to control mice and correlated this observation to the intestinal inflammation spontaneously developed by these mice (Mishima et al., 2010).

In addition to the production of soluble factors such as IL-10 and TGF-β, regulatory B cells exert their immunosuppressive functions also by interacting with other cells. The group of Bhan investigated the role of B cell co-stimulatory molecules in the development of chronic colitis in $TCR\alpha^{-/-}$ mice and reported that direct contact between CD40 and CD40L was critical for the B cell-mediated T cell regulation in the pathogenesis of colitis in these mice. As a matter of fact, B cells isolated from $CD40^{-/-}$ mice as well as B cells treated with anti-CD40 mAb lost their ability to regulate the number of pathogenic $TCR\alpha^{-}\beta^{low}$ T cells (Mizoguchi et al., 2000). The expression of CD80 and CD86 by regulatory B cells might also be important in contact-dependent suppression of immune cells by Bregs, via their ligation of cytotoxic T-lymphocyte protein 4 (CTLA4) or CD28 (Mauri and Blair, 2010). Evidence supporting this hypothesis were reported in a study conducted by Mann and coworkers in which the authors investigated the mechanism whereby B cells regulate EAE and found that the expression of B7 by B lymphocytes was essential for clinical recovery as well as for the up-regulation of IL-10 and forkhead box P3 (FoxP3) in the central nervous system (Mann et al., 2007).

Finally, several studies indicate that Abs may also play a critical role in the suppression of immune responses depending on the nature of host environment. These Abs can neutralize harmful soluble factors, dampen DC/macrophage activation through the IgG/FcγRIIB interaction or enhance the clearance of apoptotic cells that constitute a potential source of self-Ags for activating self-reactive T cells (Mizoguchi and Bhan, 2006).

Altogether the above-mentioned evidence indicates that B cells can exert their regulatory function both by contact-independent and contact-dependent interactions and highlights the

potential diversity of mechanisms used by B cells for their regulatory suppressive functions in different immunological settings. An overview of Breg cells suppressive functions is shown in figure 1.11.

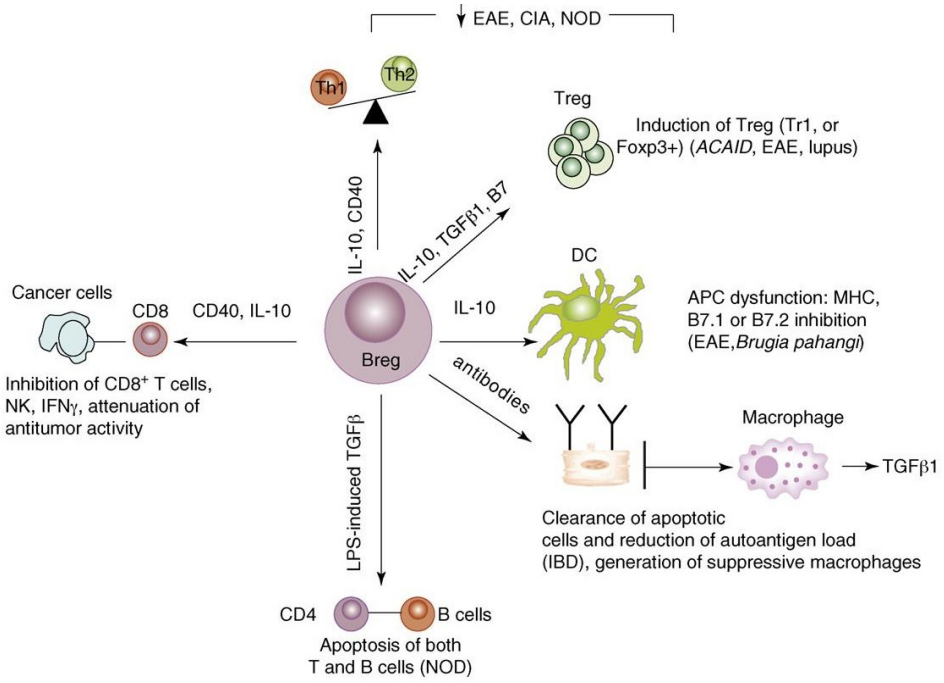


Figure 1.11: Bregs defining features and suppressive functions. Bregs suppress activation and differentiation of $CD4^+$, $CD8^+$, and NK T cells primarily via the release of the anti-inflammatory cytokines IL-10 and TGF-β. Engagement of costimulatory pathways including CD40-CD40L and CD28-B7 are required for the release of IL-10 by Bregs. A Breg-T cell dialogue results in an inhibition of T cell activation and Th1 differentiation, induction of Tregs (Tr1), inhibition of DC activation and clearance of apoptotic cells mediated via the release of autoreactive antibodies. The suppressive effect that Bregs exert on T cells might also provoke immune responses against autologous tumor cells. *(Mauri and Ehrenstein, 2008)*.

1.3.3 Targets of Bregs suppressive action

The effect of Breg cells suppressive activity has been demonstrated on many cell types of the innate and adaptive immune system. Bregs can suppress the activation of dendritic cells and macrophages, as well as both Th1 and Th2 responses (Figure 1.12). An example of Breg-mediated suppression of DCs activation comes from a study conducted by Tedder's group. In the mouse EAE model of human MS they show that IL-10–producing B cells downregulated the ability of DCs to act as APCs and thereby indirectly modulated T cell proliferation (Matsushita et al., 2010). Furthermore, Lampropoulou and coworkers reported that IL-10 from TLR-activated B cells does not suppress T cells directly but rather through the inhibition of antigen-presenting cells used in the culture assay (Lampropoulou et al., 2008).

Moreover, a role for Bregs in controlling Treg cells was proposed. Several disease models demonstrate that IL-10 produced by Bregs is important for the generation and/or maintenance of the pool of Tregs although a cognate interaction mediated by CD80 and CD86 is also required (Carter et al., 2011).

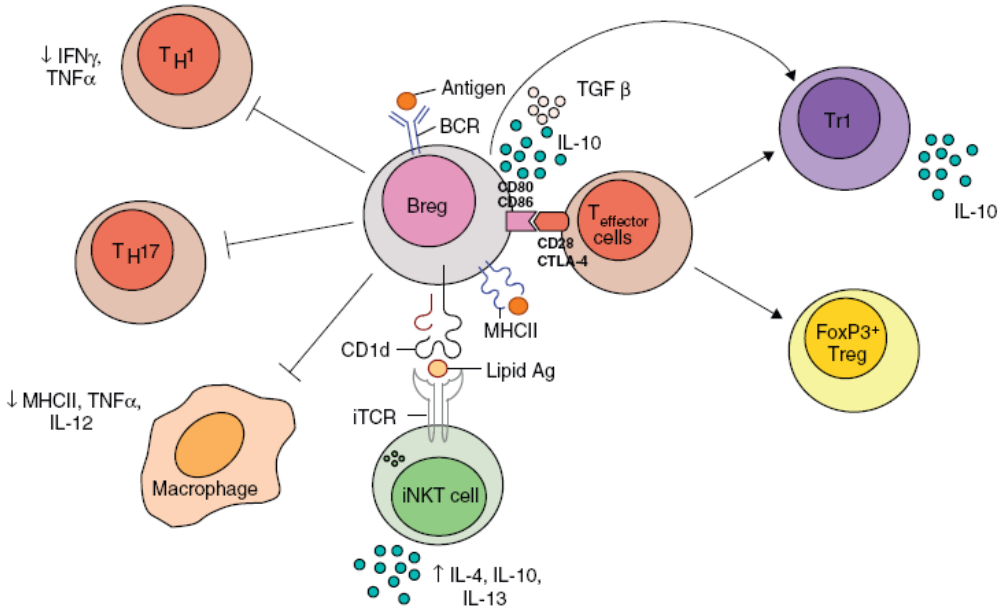


Figure 1.12: Targets of Bregs suppressive action. Regulatory B cells inhibit Th1 cells differentiation and activation of macrophages. Inhibition is mediated via the release of soluble factors (IL-10 and TGF-β), but also requires antigen recognition and engagement of co-stimulatory molecules including CD80/CD86. Interactions between Bregs and Teff cells can result in the induction of both FoxP3$^+$ Tregs and regulatory CD4$^+$ T cells producing IL-10 (Tr1). Lipid-antigen presentation via CD1d on Bregs may lead to activation of invariant NK T cells with regulatory function (not yet investigated). *(Mauri, 2010)*.

1.3.4 Regulatory B cells: different immunophenotypes, same effector functions

Besides the general agreement on the existence of a B cell population that exhibit immunomodulatory functions, an accurate identification of these regulatory B cells is challenging since these cells are rare, express detectable IL-10 only upon *ex vivo* stimulation and, above all, lack a specific marker (analogous to the expression of FoxP3 by Treg cells). Recently, the T cell Ig domain and mucin domain protein-1 (TIM-1) was reported to identify a large majority (70%) of B cells capable of producing IL-10, making this marker the most specific yet identified for IL-10–producing B cells (Ding et al., 2011).

The fact that more than one phenotype has been reported for Breg cells further complicates the whole issue. At least two phenotypically distinct subsets of B cells, transitional 2-marginal zone precursor (T2-MZP) Breg cells and B10 cells, have so far been demonstrated to exert immunosuppressive functions *in vivo* as well as *in vitro*. B10 and T2-MZP B cells are

phenotypically similar in terms of their surface markers (Figure 1.13), but important differences exist between these two B cell subsets (Mauri and Blair, 2010).

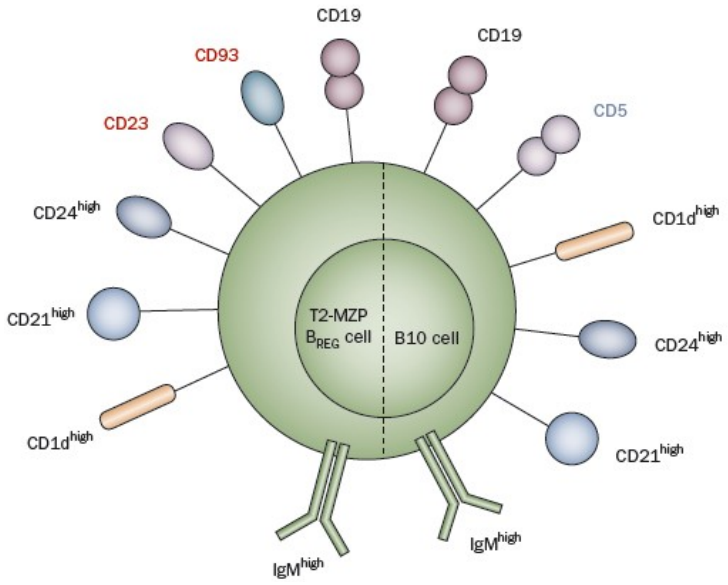


Figure 1.13: Surface molecules that characterize B10 and T2-MZP Breg cells. B10 cells and T2-MZP B cells are phenotypically similar in terms of their surface markers but the expression of some specific surface molecules distinguish these two B cell subsets. Both these two subsets express high levels of CD1d, CD21, CD24, and IgM and moderate levels of CD19 (text in black). T2-MZP Breg cells are also positive for CD93 and CD23 (text in red). By contrast, B10 cells do not express CD23 or CD93 but do express CD5 (text in blue). *(Mauri and Blair, 2010)*.

- ***T2-MZP Breg cells***

Mauri's group was the first to investigate in detail the phenotype of splenic IL-10–producing B cells. In 2007 they reported that the majority of splenic $B220^+$ IL-10^+ cells of both normal and autoimmune mice expressed high levels of CD21, CD24, IgM and CD1d and were $CD23^+$, a phenotype consistent with the one of a splenic subpopulation that has been alternatively designated as either T2 (Loder et al., 1999; Hardy and Hayakawa, 2001) or MZP B cells (Cariappa et al., 2000). Moreover, they reported that the transfer of these T2-MZP B cells reduced the incidence and severity of CIA in recipients, to a greater extent compared to other mature B cell populations, suggesting the presence of regulatory B cells within this subset (Evans et al., 2007).

The same research group has also investigated the existence and function of T2-MZP Breg cells in MRL/lpr mice, which spontaneously develop a lethal SLE-like disease. Blair and coworkers reported that anti-CD40 stimulation enriches this set of Bregs, contained within the T2-MZP B cell subset, and they showed that, *in vitro*, these IL-10–producing T2-like B cells suppress Th1 differentiation, convey suppressive capacity to $CD4^+$ T cells, and induce the

differentiation of $CD4^+$ $FoxP3^-$ IL-10^+ T cells. *In vivo*, these IL-10^+ Bregs reverse lupus-like disease and induce long-term tolerance via the inhibition of Th1 responses and T cell proliferation (Blair et al., 2009).

- ***B10 Breg cells***

Among the best characterized Breg subsets, a numerically rare (~1-3%) and phenotypically unique $CD19^{hi}$ $CD1d^{hi}$ $CD5^+$ subset of regulatory B cells has been identified by Tedder and colleagues in the spleens of both normal and autoimmune mice. This regulatory B cell subset was shown to be Ag-specific and to significantly influence T cell activation and inflammatory responses through IL-10 production. These $CD1d^{hi}$ $CD5^+$ B cells were called B10 cells since they are more enriched for IL-10–producing cells (9%–15%) than other B cell subsets and because other B cell populations with their own unique regulatory properties may also exist (DiLillo et al., 2011). B10 cells were further defined as B cells that are competent to express detectable IL-10 after 5 h of *in vitro* stimulation with a cocktail of LPS, phorbol 12-myristate 13-acetate (PMA), ionomycin, and monensin (LPIM). B10 cells were shown to represent around 2% of splenocytes and 25% of peritoneal B cells in wild type adult mice while they do not seem to circulate around the body since these cells were found at low frequencies in the peripheral blood and in lymph nodes (Table 1.2). B10 progenitor (B10pro) cells have also been identified within the spleen $CD19^{hi}$ $CD1d^{hi}$ $CD5^+$ B cell subset. B10pro cells are defined as those B cells that are not induced to express cytoplasmic IL-10 after LPIM stimulation for 5 h but that can be induced to mature into IL-10–competent B10 cells by *in vitro* culture with agonistic CD40 mAb for 48 h. B10pro cells that do not express either CD5 or CD1d are also found in other lymphoid tissues such as the peritoneal cavity, lymph nodes, and blood. However, since IL-10 expression is the defining marker for B10 cells, it is not possible to differentiate between B10 cells and B10pro cells after 48 h *in vitro* cultures (Matsushita and Tedder, 2011).

Table 1.2 – B10 and B10pro cell distributions within tissues of C57BL/6 mice.

	B10 cells		B10pro + B10 cells	
Tissue	Percentage	Number ($\times 10^{-6}$)	Percentage	Number ($\times 10^{-6}$)
Blood	1.5 ± 0.1	0.029 ± 0.01	3.5 ± 0.5	0.07 ± 0.02
Bone marrow	2.8 ± 0.5	0.20 ± 0.03	3.5 ± 0.2	0.25 ± 0.03
Lymph nodes	0.7 ± 0.1	0.01 ± 0.01	1.5 ± 0.2	0.03 ± 0.01
Spleen	2.0 ± 0.3	1.18 ± 0.1	6.2 ± 0.3	3.7 ± 0.25
Peritoneal cavity	25.8 ± 3.0	0.51 ± 0.03	30.2 ± 2.0	0.62 ± 0.12

(Matsushita and Tedder, 2011)

Regulatory B10 cells share surface markers with several other B cell subsets, including $CD5^+$ B-1a cells, $CD21^{hi}$ $CD23^-$ IgM^{hi} $CD1d^{hi}$ MZ and $CD21^{hi}$ $CD23^+$ IgM^{hi} $CD1d^{hi}$ T2-MZP B cell subsets. Nevertheless the relationship between B10 cells and other B cell subsets remains unresolved (DiLillo et al., 2010).

B10 cell regulatory function was first defined in the contact hypersensitivity (CHS) mouse model of T cell-mediated inflammation, when functional comparisons were made between $CD19^{-/-}$, wild type, and human CD19 transgenic (hCD19Tg) mice which have hyper-responsive B-cells due to CD19 overexpression. Yanaba and coworkers reported that oxazolone-induced inflammation was dramatically reduced in hCD19Tg mice and this reduction in inflammation correlated with an increased number of $CD1d^{hi}$ $CD5^+$ IL-10-producing B cells compared to wild type mice. Adoptive transfer of $CD1^{hi}$ $CD5^+$ B cells to $CD19^{-/-}$ mice, which develop an exacerbated inflammatory response compared to that of wild type mice, led to markedly reduced inflammation via the production of IL-10 (Yanaba et al., 2008). Since their characterization in 2008, B10 cells were found to inhibit inflammation, autoimmunity, and innate and adaptive immune responses through the production of IL-10. Spleen B10 cell numbers were shown to significantly increase in diabetes- and lupus-prone mice (Yanaba et al., 2009; Haas et al., 2010), and the adoptive transfer of antigen-primed $CD1d^{hi}$ $CD5^+$ B cells reduced inflammation during CHS and EAE (Matsushita et al., 2008; Matsushita et al., 2010). Moreover, Yanaba and coworkers reported that this B10 cell subset plays a critical regulatory role also in dextran sulfate sodium (DSS)-induced intestinal injury, which is a model for human ulcerative colitis (UC) (Yanaba et al., 2011). Finally, human regulatory B10 cells that parallel their mouse counterparts have also been described (Iwata et al., 2011).

- ***Other B cell subsets with regulatory properties***

Although T2-MZP and B10 B cells are the major candidates to exert the regulatory role assigned to B cells, other IL-10–producing B cell subsets have also been described, such as $CD21^{hi}$ $CD23^{-}$ IgM^{hi} $CD1d^{hi}$ MZ B cells and $CD5^{+}$ B-1a cells (Lampropoulou et al., 2010).
B-1 cells were shown to regulate immunity through the production of IL-10 both in neonatal and adult mice. In neonatal mice, the large amounts of IL-10 produced by B cells upon activation by TLR agonists contributes to the potent inhibition of Th1 responses characteristic of newborn mice (Sun et al., 2005). For what concern the immune regulating functions of B-1 cells in adult mice, Shimomura and coworkers reported that TCR deficient mice showed reduced incidence and severity of UC after transfer from a specific pathogen-free animal unit to a less hygienic facility, suggesting the existence of a protective component in the new microbial environment. The mice kept in less hygienic conditions had more B-1 cells in the peritoneal cavity and B-1 cells isolated from these "dirty" mice could adoptively transfer the protection to "clean" recipient mice. They conclude that regulatory B-1 B cells prevent the development of colitis and are induced without intestinal inflammation under non-hygienic conditions (Shimomura et al., 2008).
The regulatory properties of MZ B cells were demonstrated in a study conducted by Gray and coworkers. The authors showed that regulatory B cells can be induced during the priming and activation of ovalbumin specific $CD4^{+}$ T cells if cognate antigen is administered together with apoptotic cell extracts and complete Freund's adjuvant. These cells were shown to localize predominantly in the spleen marginal zone and had the ability to suppress T cell-dependent CIA in an IL-10 dependent manner (Gray et al., 2007).

1.3.5 Activation and differentiation pathways of Bregs

B cells with regulatory suppressive activity have been discovered in autoimmunity, inflammation, infection and tolerance settings, so it is likely that different stimuli can prime their activation. On the converse, Breg cells generated in different settings might display different activation requirements. As a matter of fact, a number of signals, both endogenous and exogenous, have been reported to prime the regulatory suppressive function of B cells and induce the secretion of IL-10 (Vitale et al., 2010).
Among the different molecules associated to regulatory B cell activation and differentiation, TLRs were shown to play a fundamental role. The requirement for TLR signaling has been described in several models of B cell mediated suppression of autoimmunity and of other inflammatory conditions. *Ex vivo* LPS stimulation has been shown to induce the capacity of

total splenic B cells, adoptively transferred in pre-diabetic NOD mice, to suppress Th1 responses and spontaneous autoimmune diabetes (Tian et al., 2001). TLR9 agonists were shown to induce *ex vivo* the ability of MZ B cells to suppress the production of effector cytokines by splenic non-B cells (Lenert et al., 2005). Murine neonatal $CD5^+$ B cells were induced by several TLR agonists in their ability to inhibit inflammation and suppress both plasmacytoid and conventional dendritic cells in an IL-10-mediated manner (Zhang et al., 2007). The role of TLR signaling in both B cell suppression of T cell mediated autoimmunity and in the induction of IL-10 secretion by B cells has been thoroughly investigated by the group of Fillatreau. They induced EAE in mice by immunization with a myelin oligodendrocyte glycoprotein-derived peptide emulsified in complete Freund adjuvant and demonstrated that recovery from EAE is both B cell and myeloid differentiation primary response gene 88 (MyD88) dependent, indicating that TLR signaling plays an essential role in this mouse model of T cell mediated autoimmunity. The authors also showed that MyD88 dependent TLR signaling is both necessary and sufficient to stimulate IL-10 production by splenic mouse B cells (Lampropoulou et al., 2008).

Moreover, multiple studies have identified the CD40-CD40L interaction as an essential pathway for the activation of Bregs. In 2000 Mizoguchi and coworkers reported that B cells isolated from CD40-deficient mice, as well as B cells pretreated with blocking anti-CD40 mAb, failed to suppress the pathogenic $TCR\alpha^-\beta^{low}$ T cell responses and inflammation observed in the colon of αμ-deficient mice (Mizoguchi et al., 2000). A couple of years later, the group of Anderton showed that, when stimulated with autoantigen, B cells from normal mice that have recovered from EAE produced IL-10 and that this was dependent on concurrent ligation of CD40. Bone marrow chimeric mice in which either IL-10 or CD40-deficiency was restricted to the B cell compartment failed to recover from EAE (Fillatreau et al., 2002). The importance of the CD40-CD40L axis in the activation and differentiation of IL-10–producing regulatory B cells has been demonstrated in several other studies and in many other immunological settings (Mauri et al., 2003; Blair et al., 2009; Blair et al., 2010).

Recently it has been shown that BAFF, a key regulator of B cell maturation and function, increased the frequency of IL-10–producing B cells, *in vitro* and *in vivo* (Yang et al., 2010a). Although BAFF has been generally considered as a driving factor for its proinflammatory function, Yang and coworkers reported that BAFF, at low concentrations, induced MZ B cell differentiation into IL-10–producing B cells and that the *in vivo* transfer of BAFF-induced $IL\text{-}10^+$ B cells significantly inhibited the arthritis development in CIA mice.

Antigenic activation appears a crucial but likely late signal in the induction of B cells with regulatory suppressive functions. For instance, the generation of Breg cells depends on the stimulation with cognate antigen and efficient BCR signaling in several autoimmune settings. BCR requirement is clearly demonstrated in mouse models of autoimmunity suppression, where regulatory B cells are autoantigen reactive (Fillatreau et al., 2002; Mauri et al., 2003). Moreover, mice deficient for the CD19 co-receptor fail to efficiently induce Breg cells (Yanaba et al., 2008), further demonstrating the importance of BCR signaling. Interestingly, BCR-mediated Ca^{2+} flux appears to be required for IL-10 production in B cells, as B cells deficient in the calcium sensors stromal interaction molecule (STIM) 1 and STIM2 have a profound defect in IL-10 secretion and abrogated suppression abilities *in vivo* (Matsumoto et al., 2011). Remarkably, antibody responses and B cell development are largely intact in these animals, suggesting that BCR-mediated calcium flux is crucially important for B cell-mediated immune suppression but is dispensable for effector functions (Klinker and Lundy, 2011).

In conclusion, although the nature of signals that drive the maturation and activation of Breg cells have not been univocally characterized, what seems clear is that the expansion and differentiation of regulatory B cells depends on both the activation context and the specific B cell subset involved.

1.3.6 Current hypothesis on Bregs origin

The relationship between the recently identified IL-10–producing regulatory B cell population and the B-1 or B-2 cells is currently unclear (Figure 1.14). These regulatory B cells share some functional (IL-10 production) and phenotypical ($CD19^{hi}$ $CD5^{+}$) characteristics with B-1a cells but they also have similar characteristics to T2-MZP and MZ B cells (high-level expression of CD1d and CD21). In the opinion of Baumgarth, the regulatory B cell subset might therefore be a new member of an emerging group of 'innate-like' B cell populations, which in mice include B-1a, B-1b and MZ B cells (Baumgarth, 2011).

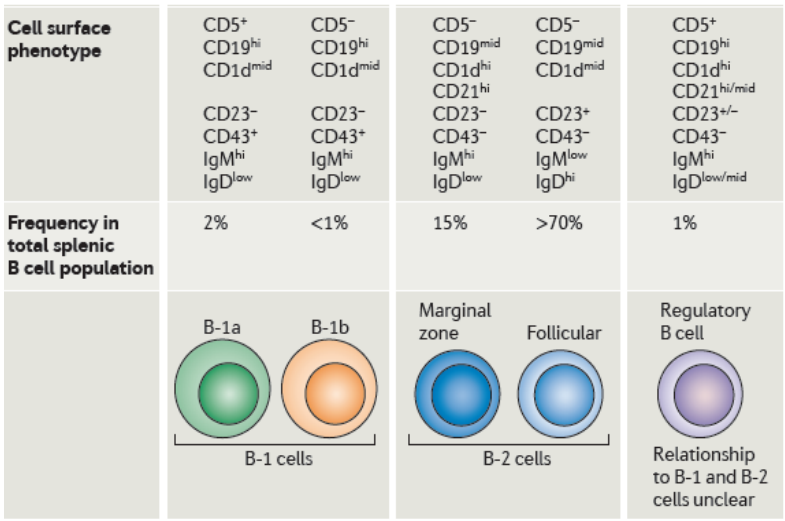


Figure 1.14: Relationship between Bregs and other mature splenic B cell subsets. Five subsets of mature B cells are present in the mouse spleen. FO and MZ B cells, which form the B-2 cell population, constitute the majority of splenic B cells. B-1a and B-1b cells are minor subsets in terms of their frequency in the spleen. A regulatory B cell subset that expresses high levels of CD1d was recently identified among $CD23^{+}$ B cells. These cells have also been described as $CD1d^{hi}CD5^{+}CD23^{-}$ B cells. Therefore, regulatory B cells have phenotypical markers of both B-1 and B-2 cells. *(Baumgarth, 2011)*.

Much controversy still exists concerning the origin and development of regulatory B cells and different hypothesis have been proposed in regard to their ontogenesis.

The first was formulated by Mizoguchi and Bhan, who argue that IL-10–producing Bregs appear under inflammatory conditions and that are not detected in normal states. On this basis, they suggest that, depending on the type of inflammation present, there are at least two possible pathways by which splenic B cells may differentiate into Bregs. "Innate type regulatory B cells" develop from MZ B cells (and possibly B-1 cells), primed by infective inflammatory signals (TLR4/LPS, TLR9/CpG). On the contrary, "acquired type regulatory B cells" develop in acquired immune-mediated diseases, such as EAE and rheumatoid arthritis (RA), from activated FO B cells, through BCR ligation with self-Ag and/or CD40-CD40L interaction (Mizoguchi and Bhan, 2006). According to this model, different Breg cell populations are generated from existing B cell subsets under distinct activation processes (Figure 1.15A).

Fillatreau and collaborators have proposed a different model on the hypothesis that B cell suppressive functions are acquired during the process of B cell activation in a stepwise fashion "through a hierarchical process initiated by TLRs and later reinforced via BCR and CD40" (Lampropoulou et al., 2010). According to this model all activated B cells can become "suppressive B cells" (Vitale et al., 2010). This two-step pathway for Breg development was reproposed in a modified version by Mauri and Bosma, which suggest that Bregs may arise from a common progenitor, namely T2-MZP B cells (Mauri and Bosma, 2011). In their view, it's a potential autoreactive T2-MZP B cells that, during a response to pathogens, becomes activated and release a first wave of IL-10. As inflammation goes on, activation of

autoreactive T cells occurs, and Bregs receive full activating signals (CD40, BCR, CD80-CD86), which enhance and stabilize the production of IL-10 (Figure 1.15B).

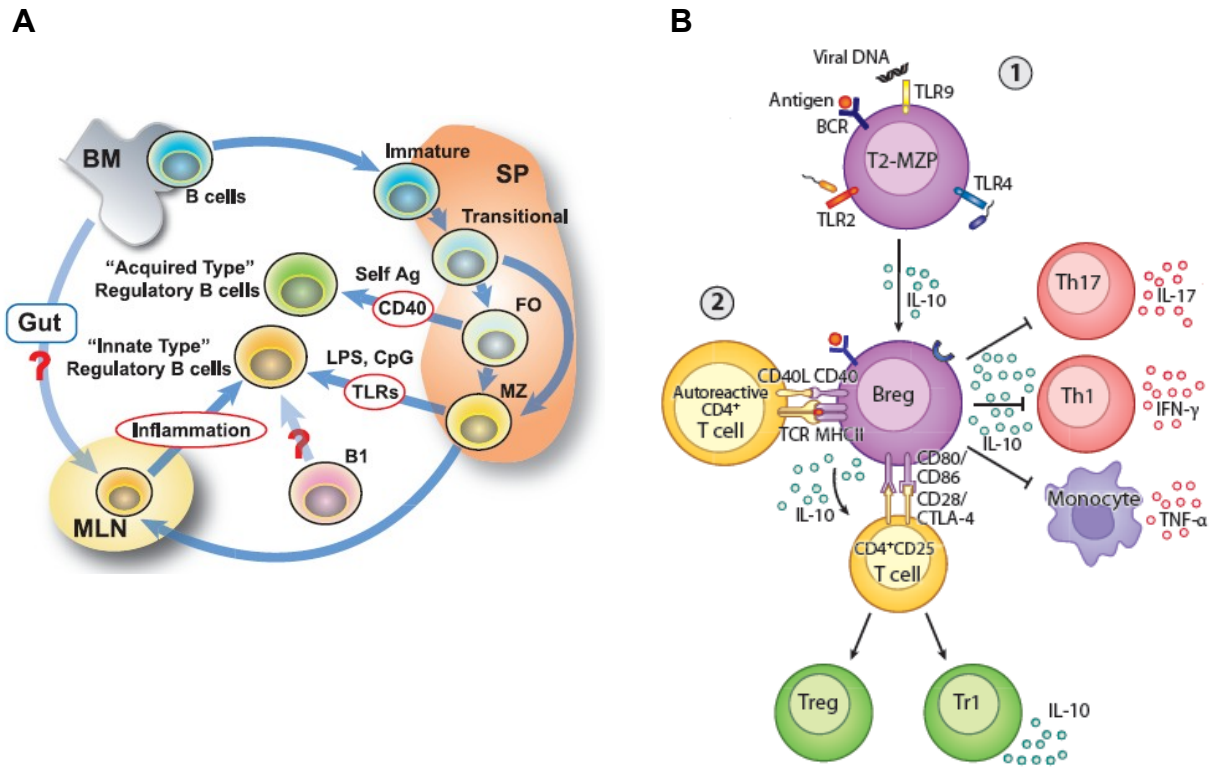


Figure 1.15: Two possible models proposed for Bregs origin and development. (***A***) In the model proposed by Mizoguchi and Bhan there are two possible pathways that may lead to the development of Bregs. Bregs may develop from activated FO B cells following further activation through CD40 pathway or BCR ligation with self-Ags ("acquired type" regulatory B cells). When pathogenesis involves exposure to bacterial products, an "innate type" Breg subset may develop from splenic MZ B cells, following activation through TLR pathways. It is also possible that the "innate type" Breg subset origins from B-1 cells. *(Mizoguchi and Bhan, 2006)*. (***B***) In Mauri's opinion, autoreactive T2-MZP B cells are the common progenitors of Bregs. After TLR stimulation, T2-MZP Bregs produce IL-10 (step 1) but Bregs release an optimal amount of IL-10 only in response to further activation through the BCR and CD40 engagement (step 2). *Adapted from (Mauri and Bosma, 2011)*.

Finally, Tedder's group suggested that CD1d^{hi} CD5^{+} IL-10–producing B cells represent a distinct lineage of B cells which they termed "B10" cells. The unique B10 cell phenotype has led them to propose that B10 cells derive from B10 progenitor (B10pro) cells that may not fit into any preconceived subset (DiLillo et al., 2010). IL-10–competent B cells normally represent only 1–2% of spleen B cells but prolonged (48 h) culture of adult spleen B cells with mitogenic LPS or agonistic CD40 mAb induced a significant increase in the numbers of IL-10-competent B cells, suggesting that a subset of CD1d^{hi} CD5^{+} B cells can be induced to mature further during culture with prolonged stimulation (Yanaba et al., 2009). How or whether this is recapitulated *in vivo* is currently unknown, but, on the basis of these results, DiLillo and coauthors proposed a linear differentiation scheme (Figure 1.16), whereby B10pro

cells may become IL-10–competent as they progress to become functionally mature CD1d^{hi} CD5^{+} B10 cells within the spleen (DiLillo et al., 2010).

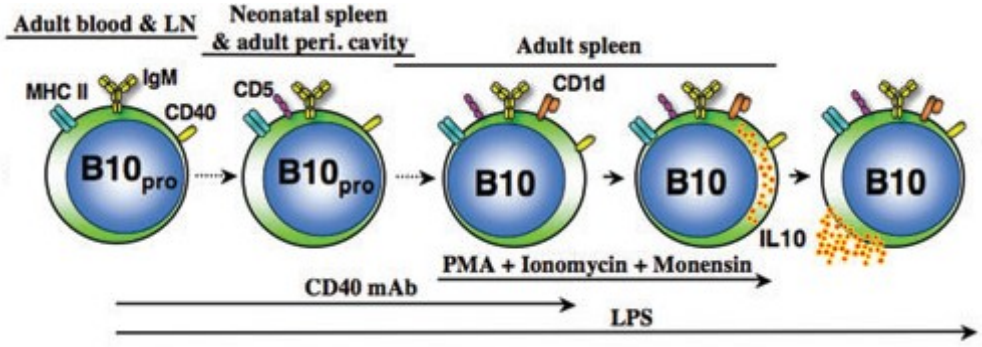


Figure 1.16: Model proposed for B10 cell development. CD1d^{hi}CD5^{+} IL-10-competent B10 cells in the adult spleen are induced to express cytoplasmic IL-10 following stimulation with PMA, ionomycin, and monensin (PIM) or LPS plus PIM (L+PIM) for 5 h. B10pro cells are found among the CD1d^{-}CD5^{-} adult blood and lymph node B-cell subsets and within the CD1d^{-}CD5^{+} neonatal spleen and adult peritoneal cavity B-cell subsets. CD40 stimulation induces B10pro cells to become competent for PIM-induced cytoplasmic IL-10 expression. LPS not only induces B10pro cells to become competent for PIM-induced cytoplasmic IL-10 expression but also promote both IL-10 production and secretion by B10 cells. *Adapted from (DiLillo et al., 2010)*.

1.3.7 Outstanding questions

Regulatory B cells constitute a recently described population and, therefore, there are still several unresolved issues in this field. Among the many outstanding questions regarding this B cell subset, it remains to be understood whether Bregs constitute a truly distinct and specialized subset or, more likely, are activated B cells that acquired a suppressive function (Vitale et al., 2010). Both experimental and literature data support the idea that regulatory and effector B cells arise from the same progenitors, which are capable of being reprogrammed according to the immunological requirements of the inflammatory environment (Mauri and Bosma, 2011). Of great help in solving this issue would be the identification of specific surface markers that, coupled with transcriptional factors, would provide a unique signature. To date, no transcription factor specifically responsible for Breg differentiation or development has yet been identified while several surface molecules (CD1d, CD5, CD23 and CD21) have been associated to the B-cell regulatory phenotype. Moreover, the possible role of a specific cell type in the generation of IL-10–producing regulatory B cells has never been investigated so far and, therefore, the immune cell types involved in the development and differentiation of Bregs are currently unknown.

A great deal of attention has been paid to the factors that generate or expand IL-10–producing B cells. Stimuli known to increase the number of IL-10–producing B cells *in vitro* include

signaling through TLRs, the BCR, CD40 or combinations thereof (Klinker and Lundy, 2011). Since the study of Breg cells could lead to the development of novel therapeutic approaches and to the understanding of the mechanism of action of biological drugs in inflammatory and autoimmune disease and cancer, the methods that could be used to expand them *in vivo* for therapy need to be researched further (Vitale et al., 2010).

1.4 Mast Cells

Mast cells represent a specific type of immune cells characterized by the expression of high affinity plasma membrane receptors (FcεRI) that bind to IgE antibodies, and by their content of numerous large cytoplasmic granules, storing biogenic amines, proteoglycans, cytokines and neutral serine proteases. These cells are best known for their role in the defense against parasites as well as in pathologic conditions such as allergy and asthma, where they release large amounts of histamine and leukotrienes following IgE cross linking. However, in recent years, MCs have been identified as being responsible for a far more complex range of functions (Metz and Maurer, 2007). Mast cells are now known to participate in several normal physiologic processes, including wound healing (Weller et al., 2006), angiogenesis (Soucek et al., 2007) and tissue remodeling (Galli and Tsai, 2008) as well as to play a central role in the pathogenesis of autoimmune disease, cardiovascular disorders, and cancer (Rao and Brown, 2008). Very interestingly, MCs were shown to act as regulatory linkers between innate and acquired immunity: through the expression of various co-stimulatory molecules and the release of specific soluble factors, these cells can influence many aspects of the biology of different immune cells, including granulocytes, monocytes/macrophages, DCs, T cells, B cells and NK cells (Galli et al., 2008; Frossi et al., 2010).

1.4.1 Mast cell development and tissue distribution

Mast cells originate in the bone marrow from pluripotent $CD34^+CD13^+CD117^+$ hematopoietic stem cells. In contrast to most other myeloid cells, such as monocytes and neutrophils, MCs do not terminally differentiate in the bone marrow, but rather circulate as immature mast cell precursors (MCPs) that enter peripheral sites and then mature locally (Figure 1.17). Since mast cells undergo terminal differentiation in the target tissues, their phenotype is influenced by factors present in the local microenvironment (Rao and Brown, 2008). Cytokines important in the development and maturation of mast cells include IL-3, IL-6 and IL-4 (Chen et al., 2005; Kulka and Metcalfe, 2005). One of the most important signals from tissue to local MCs is

stem cell factor (SCF). The receptor for SCF, c-kit (or CD117), is widely expressed on hematopoietic lineages early in differentiation, but mast cells are the only terminally differentiated hematopoietic cells that still express c-Kit at high levels. In mice and humans, SCF is an irreplaceable survival signal for tissue MCs. The W/Wv, Sl/Sld, and Wsash mouse strains are perfect examples of how SCF or c-Kit mutations leads to severe defects in the production of mast cells (Jarboe and Huff, 1989). Similarly, MCs obtained from patients with systemic mastocytosis commonly exhibit activating mutations in c-kit (Akin et al., 2004; Akin and Metcalfe, 2004).

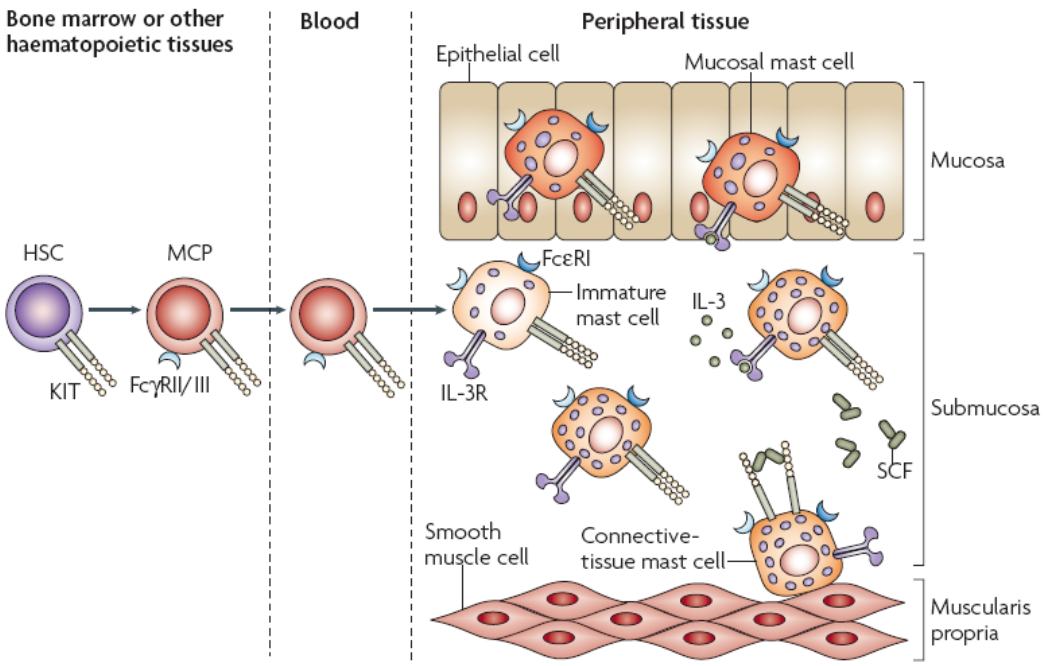


Figure 1.17: Mast cell development and tissue distribution. Tissue mast cells are derived from hematopoietic stem cells (HSCs), which ultimately give rise to mast cell progenitors (MCPs). MCPs circulate in the blood and enter the tissues, where they undergo differentiation and maturation to become mature mast cells. Stem cell factor is normally required to ensure mast cell survival in the tissues, but the phenotype of mature mast cells can vary depending on the growth factor and on other microenvironmental factors *(Galli et al., 2008)*.

In mice, two mast cell subsets have been described. Connective tissue mast cells are predominantly found in the skin and peritoneal cavity, and their granules contain the proteoglycan heparin and large amounts of histamine and carboxypeptidase A. On the other hand, mucosal mast cells are instead found mainly in the mucosal layer of the gut and lungs, and their granules contain chondroitin sulfate as the major proteoglycan and relatively less histamine and carboxypeptidase A (Figure 1.18A). In humans, two analogous subsets of mast cells have been described that differ based on whether their granules contain the protease

tryptase alone (MC_T) or tryptase along with chymase (MC_{TC}). MC_T are predominantly found at mucosal sites while MC_{TC} within connective tissue (Rao and Brown, 2008).

1.4.2 Mast cell activation

As defined by Frossi and collaborators, the mast cell is an antenna of the microenvironment that sense a great variety of signals and rapidly and selectively directs the immune response (Frossi et al., 2004). MCs are located at strategic sites, such as skin and vascular or mucosal barriers, where they can respond to pathogens, through the multiple TLRs expressed on their cell surface, or to allergens, through surface-bound IgE (Rivera and Gilfillan, 2006; Bischoff, 2007). Mast cells also express receptors for complement components, particularly C3a and C5a (Marshall and Jawdat, 2004). The type of stimulus responsible for the activation of MCs modulates the quality and magnitude of the mast cell response (Figure 1.18B).

The canonical pathway that leads to MC activation is via IgE and its receptor FcεRI. The FcεRI is a tetrameric receptor with high affinity for IgE and it is constitutively expressed on the surface of mast cells. The number of surface FcεRI is up-regulated by increased concentrations of IgE and recent work indicates that monomeric IgE (in the absence of crosslinking) can render mast cells resistant to apoptosis and can induce the release of cytokines (Kawakami and Galli, 2002). The interaction of antigen with IgE bound to FcεRI, on the cell surface, leads to the aggregation of FcεRI and initiates a series of biochemical events resulting in MC-degranulation and subsequent release of biologically active mediators. This IgE–dependent mast cell activation response results in rapid release (in minutes) of preformed inflammatory mediators (such as histamine, heparin and other proteoglycans, proteases) and of certain cytokines (TNF-α), the secretion of *de novo*–synthesized lipid mediators (including cysteinyl leukotrienes and prostaglandins) and the production, with a prolonged kinetics, of many cytokines, chemokines and growth factors (Siraganian, 2003).

Among the diverse IgE-indipendent triggers that regulate mast cell activation, much interest has been devoted to innate signals since these cells have a unique 'armamentarium' of receptor systems and mediators for responding to pathogen-associated signals. MCs express many TLRs and mast cell responses to TLR agonists can vary among different populations of mast cells (Marshall, 2004). As a matter of fact, Supajatura and coworkers observed quantitative and qualitative differences in the activation of mast cells via TLR2 and TLR4 stimulation. TLR2-mediated mast cell activation by peptidoglycan leads to degranulation and production of IL-4 and IL-5, in contrast to TLR4-mediated mast cell activation, in which TNF-α, IL-1β, IL-6, and IL-13 are the major cytokines produced (Supajatura et al., 2002).

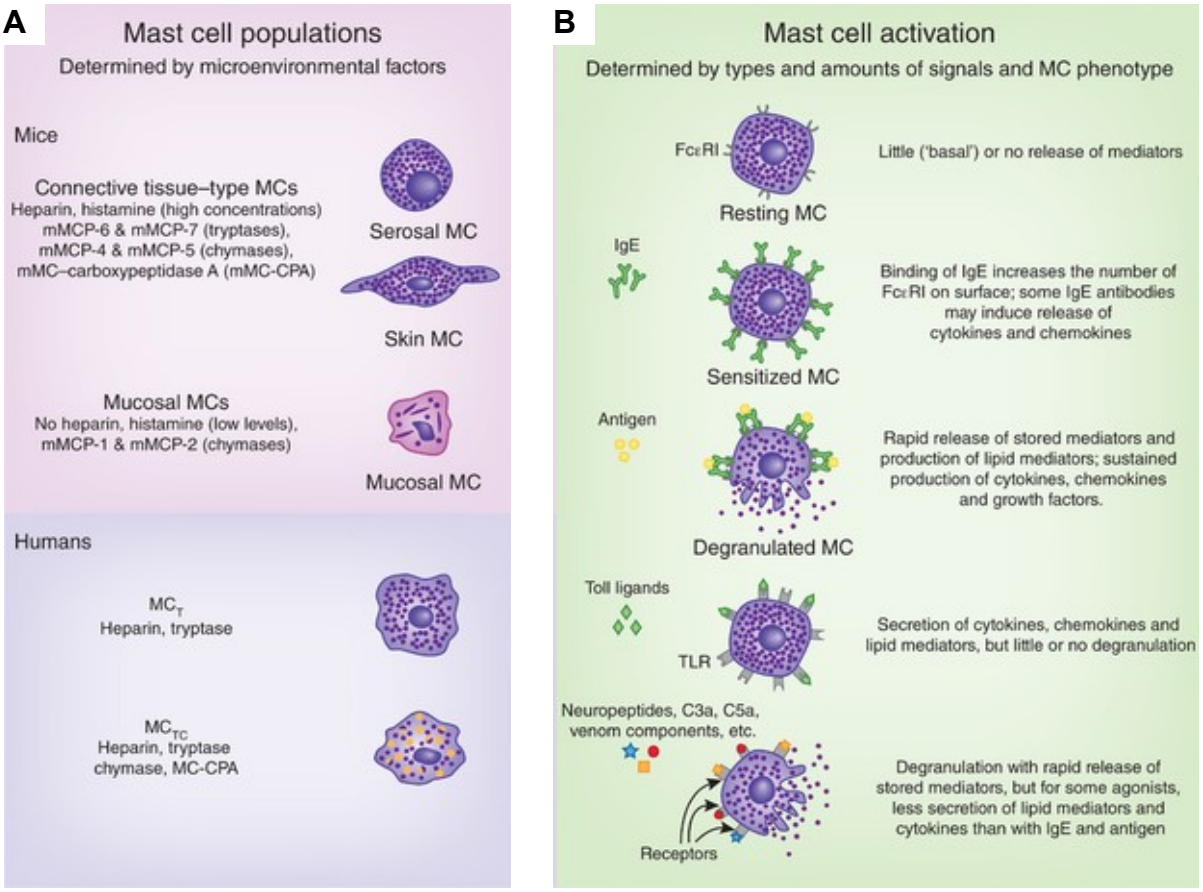


Figure 1.18: Mast cell populations and their activation. (***A***) Mast cells in mice or humans can be subcategorized into populations defined by anatomical location and/or mediator content, such as proteoglycans or proteases. (***B***) MCs respond to pathogens, through the multiple TLRs expressed on their cell surface, or to allergens, through surface-bound IgE. MCs also express receptors for complement components, particularly C3a and C5a. *Adapted from (Galli et al., 2011)*.

1.4.3 Crosstalk between mast cells and other immune cell types

Mast cells have for long been mainly associated to immediate-type hypersensitivity responses (Christy and Brown, 2007). It has now been widely demonstrated that mast cells may influence the development, intensity and duration of adaptive immune responses that contribute to host defense, allergy and autoimmunity, rather than simply functioning as effector cells in these settings (Galli et al., 2005b). In addition to the expression of the surface receptors MHC I and II, MCs have been reported to express other important co-stimulatory molecules, such as intercellular adhesion molecule (ICAM)-1, ICAM-3, β2-integrins, CD43, CD40L, CD80 and CD86 (Henz et al., 2001), which allow them to interact with several cell types among which DCs, lymphocytes, endothelial cells, and fibroblasts (Figure 1.19). Moreover, as already mentioned above, a variety of stimuli can activate mast cells to release a

diverse array of biologically active products, many of which can mediate potential proinflammatory, anti-inflammatory and/or immunoregulatory effects (Galli et al., 2005b).

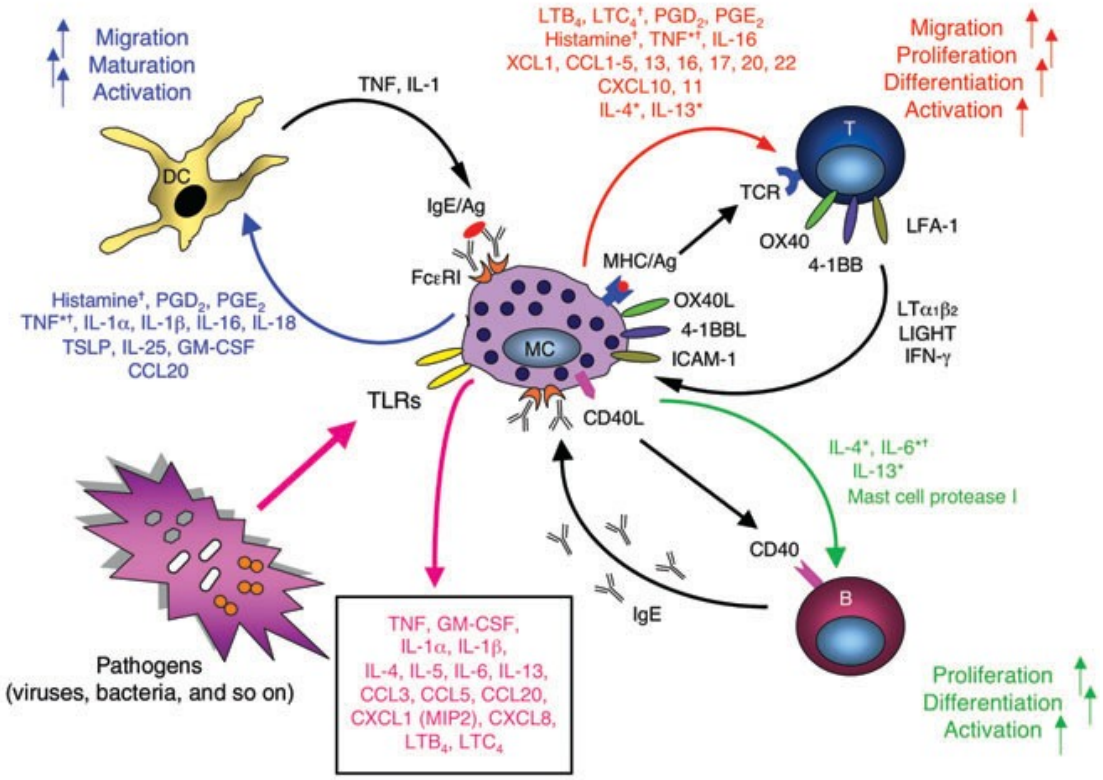


Figure 1.19: Mechanisms by which mast cells may influence DCs, T cells and B cells. The mast cell surface structures and secreted products that can influence various aspects of the biology of DCs, T cells and/or B cells are presented. Mast cell activation with IgE and specific antigen (Ag) results in the secretion of a diverse range of products. *(Galli et al., 2005b)*.

Both *in vitro* and *in vivo* evidence indicate that mast cells have the potential to influence DC migration, maturation and function (Galli et al., 2005b; Galli et al., 2008). For example, Mazzoni and coworkers, following the observation that DC and mast cells are located in close proximity in peripheral compartments, demonstrated that mast cell products could influence the maturation of DCs at sites of inflammation and hence play a critical role in determining the polarity of Ag-specific T cell responses *in vivo* (Mazzoni et al., 2006).

Evidence for the migration of mast cells to draining lymph nodes during immune responses and the close proximity of mast cells and lymphocytes at sites of tissue inflammation (Mekori and Metcalfe, 1999; Merluzzi et al., 2010), suggest that mast cells and lymphocytes may influence each other's functions by bidirectional cell–cell interactions (Galli et al., 2005a).
Mast cells were shown to modulate T cell functions; in particular antigen processing and presentation has been proposed as a mechanism by which MCs might regulate T cell responses

(Galli et al., 2005b). For example, in a very recent paper the group of Bulfone-Paus demonstrated that MCs induced antigen specific $CD8^+$ T cell activation and proliferation. This process required direct cell contact and MHC class I-dependent antigen cross-presentation by MCs and induced the secretion of IL-2, IFN-γ, and macrophage inflammatory protein-1a by $CD8^+$ T cells (Stelekati et al., 2009).

As mentioned above, most of the cell-cell interactions of which we have discussed so far, have been shown to be bi-directional and, therefore, even T cells can modulate mast cell function. Interestingly, Gri and coworkers, following the observation that Treg cells and MCs could be found in close proximity *in vivo*, investigated the consequence of the interaction of these two cell types and reported that Treg cells can inhibit MC degranulation through cell-cell contact. Specifically, they found that the interaction of OX40-expressing Treg cells with OX40L-expressing MCs inhibited the extent of MC degranulation *in vitro* and of the immediate hypersensitivity response *in vivo* (Gri et al., 2008).

Finally, a functional connection between MCs and B cells has also been proven to exist. Mast cells produce several cytokines, such as IL-4, IL-5, IL-6 and IL-13, that are known to regulate, directly or in combination with other factors, B cell development and function. Moreover, the CD40L co-stimulatory molecule is constitutively expressed on mast cell surface and this further supports the existence of a functional crosstalk between these two cell types. The first evidence of an effect of MCs on B cells, mediated by the physical interaction through the CD40-CD40L axis, was reported by Gauchat and coworkers. They showed that CD40L was expressed on both freshly purified human lung mast cells and on the human cell line HMC-1 and further demonstrated that these MCs can interact with B cells to induce the production of IgE, in the presence of IL-4 and in absence of T cells (Gauchat et al., 1993). Furthermore, the role of the CD40-CD40L axis in the induction of IgE production by B cells was also observed in perennial allergic rhinitis (PAR), an IgE-mediated atopic disease. Nasal mast cells (NMCs) from patients with PAR displayed significantly higher expression levels of FcεRI, CD40L, IL-4, and IL-13 compared to NMCs from patients with chronic infective rhinitis (CIR). The essential role of CD40L in this allergic disease context was further substantiated by the finding that the IgE production was inhibited by anti-CD40L mAb (Pawankar et al., 1997).

The group of Mécheri was the first to show that unstimulated mouse bone marrow-derived mast cells (BMMCs) were able to induce resting B cells to proliferate and to become IgM-producing cells. In this case, B cell activation was mediated by mast cell-derived factors and contact between these two cell types seemed not to be required (Tkaczyk et al., 1996). Some

years later the same research group reported that membrane vesicles, released by the mast cell cytoplasmic granules and termed exosomes, were responsible of MC-driven B cell proliferation and activation. Interestingly, they showed that important co-stimulatory molecules, such as MHC II, CD86, CD40, CD40L, LFA-1, and ICAM-1, were associated with exosomes (Skokos et al., 2002).

Only recently the study of the specific role of MCs in B-cell growth and differentiation has been investigated more in detail. Merluzzi and coworkers proved that both nonsensitized and activated MCs were able to induce a significant inhibition of cell death and an increase in proliferation of naïve B cells. Such proliferation was further enhanced in activated B cells. This effect required cell-cell contact and MC-derived IL-6. Activated MCs were shown to regulate CD40 surface expression on unstimulated B cells and the interaction between CD40 and CD40L on MCs, together with MC-derived cytokines, were involved in the differentiation of B cells into $CD138^{+}$ plasma cells and in selective IgA secretion. These data were corroborated by *in vivo* evidence of infiltrating MCs in close contact with IgA-expressing plasma cells within inflamed tissues (Merluzzi et al., 2010).

2. AIMS OF THE THESIS

B cells are traditionally known for their essential role in humoral immunity as they produce specific antibodies against antigens. Nevertheless, an emerging, significant body of evidence indicates that B lymphocytes are much more than antibody-producing cells since they mediate and regulate many other functions, essential for immune homeostasis, through the secretion of cytokines or the interaction with other immune cell types (LeBien and Tedder, 2008).
Interestingly, over the last decade, several papers have reported the presence of an IL-10–producing B cell population that negatively regulates immune response in different murine models of autoimmune disease and chronic inflammation (Mauri and Blair, 2010). The idea of the existence of "regulatory B cells" has originated from these observations and great effort has been directed to shed light on their development and expansion. However, a conclusive identification of these cells is challenging since they are rare, lack a specific marker and express detectable IL-10 only upon *ex vivo* stimulation. Moreover, based on shared phenotypic markers, several studies have assigned these cells to distinct B cell subsets, including MZ, T2-MZP and B10 cells, therefore raising the question of whether the ability to suppress immune responses through the production of IL-10 is a general property of all B cells or a specific characteristic of a defined lineage. Considering the role of regulatory B cells in autoimmunity and chronic inflammation, the study of their origin and developmental pathways is of considerable importance in the view of the development of novel and more effective therapeutic approaches for these pathological conditions.

The aim of the present thesis was to characterize the microenvironment that induces the expansion and/or differentiation of IL-10–producing B cells, focusing on the contribution of both stimuli activating B cells and immune cell types encountered by B lymphocytes.
It was investigated whether endogenous signals, produced by other cells of the immune system, and exogenous signals, resulting from foreign substances and infectious agents, could trigger the expansion and/or differentiation of IL-10–producing B cells. Moreover, other aspects of the induced IL-10–producing B cells, such as their immunophenotype and cytokine release, were studied in order to understand whether these stimuli acted according to common or different mechanisms.

Moreover, the attention was focused on the role of mast cells (MCs) in the expansion and/or differentiation of IL-10–producing B cells, both *in vitro* and *in vivo*. The evidence of a direct B-MC interaction, together with data demonstrating the role of MCs as important regulators of physiological and pathological immune responses, leads to hypothesize that MCs could regulate different aspects of IL-10–producing B cell biology such as their proliferation, survival, homing or differentiation.

3. RESULTS

3.1 Role of stimuli activating B cells on the induction of IL-10–producing B cells

B cells with regulatory suppressive activity have been discovered in autoimmunity, inflammation, infection and tolerance settings, so it is likely that different stimuli can prime their expansion and/or differentiation, in a context-dependent manner (Vitale et al., 2010). Although B cells can exert their suppressive function through both soluble factors and cell-to-cell interaction, the most widely studied and best understood immunosuppressive mechanism of B lymphocytes is the secretion of IL-10 (Klinker and Lundy, 2011).
The B-cell capacity to produce this anti-inflammatory cytokine was described many years ago but only recently the role of stimuli involved in B-cell maturation and activation was taken in account in relation to the production and secretion of IL-10. Increasing attention is being paid to those factors that generate or expand IL-10–producing B cells *in vitro*; despite this, no study has yet been able to clarify whether the ability to produce IL-10 and act as a regulatory B cell is a general property of all B lymphocytes or a specific characteristic of a defined lineage. It is also still unclear whether all the cells that produce IL-10 are the same: while some IL-10^{+} B cells may truly belong to distinct and specialized subsets of regulatory B cells, other are more likely to be activated B cells, that acquired a suppressive function.

The experiments performed in this first part of the thesis were aimed to investigate these open questions related to IL-10–producing B cells. For this purpose, B cells were cultured with four different molecules, known to promote B cell maturation and/or activation: LPS and CpG were chosen as they mimic exogenous, infectious signals, whereas BAFF and the agonistic CD40 mAb resembled endogenous, “immune mediated” signals. In particular, BAFF, LPS and CD40 have already been shown to be related to the acquisition of the regulatory phenotype by B cells (Lampropoulou et al., 2008; Yanaba et al., 2009; Yang et al., 2010a), while specific studies regarding the role of CpG are lacking. Following stimulation of splenic B cells with these factors, differences in the frequency of IL-10–producing B cells, IL-10 secretion and expression of surface molecules, related to the regulatory phenotype, were assessed and further analyzed.

3.1.1 Identification of IL-10–producing B cells: gating strategy

Since most of the work done for this thesis was focused on the study of IL-10–producing B cells, a great amount of effort was dedicated to the development of the experimental system for their detection. In normal conditions, cells that are "competent" to express *Il-10* are found among the total B lymphocytes purified from the spleen of wild type mice, although at very low frequencies. These IL-10–competent B cells do not produce detectable intracellular IL-10 and, therefore, an *ex vivo* stimulation is required to detect and quantify these cells.

In the experiments performed for this thesis the stimulation protocol designed by Yanaba and coworkers was used: B cells were stimulated *in vitro* for 5 h with a cocktail of LPS, PMA and ionomycin (LPI) to enhance *Il-10* expression in IL-10–competent B cells. For optimal detection, monensin was also added to the stimulation cocktail (LPIM) to block intracellular protein transport and thereby retain newly generated IL-10 within the cell cytoplasm (Yanaba et al., 2008).

Following the 5 h LPIM stimulation, the IL-10–producing B cells were directly detected by intracellular staining using CD19 and IL-10 mAbs. As reported in literature (Matsushita and Tedder, 2011), IL-10–competent B cells are rare and therefore even low-level background staining can lead to an incorrect analysis and quantification of these cells. Dead cells are the main cause of artifacts in immunophenotyping analysis as they nonspecifically bind monoclonal antibody conjugates (Perfetto et al., 2006). For this reason, a live/dead dye that brightly stained dead cells was used, allowing their exclusion from the analysis. Figure 3.1 shows the analysis of the flow cytometry data and the gating strategy performed to identify the IL-10–producing B cells among the total B lymphocytes, freshly purified from mouse spleen. Gate G1 defines the viable B cells while it excludes the dead cells (G2), which are positive for live/dead staining. A representative cell surface CD19 and cytoplasmic IL-10 staining of viable B cells deriving from gate G1 is shown in the upper middle panel of the figure: the reported percentage indicates the frequency of IL-10–producing B cells within the selected gate, among total $CD19^+$ B cells. To increase the specificity of $IL\text{-}10^+$ and $IL\text{-}10^-$ B cell discrimination, the described strategy employed a perfectly matched isotype control (Rat IgG2b) in addition to the use of B cells not stimulated with LPIM, as a negative control.

This approach was also used to identify IL-10–producing B cells among total B cells that were cultured for 48 h in the presence of an activatory stimulus or of MCs, before LPIM stimulation.

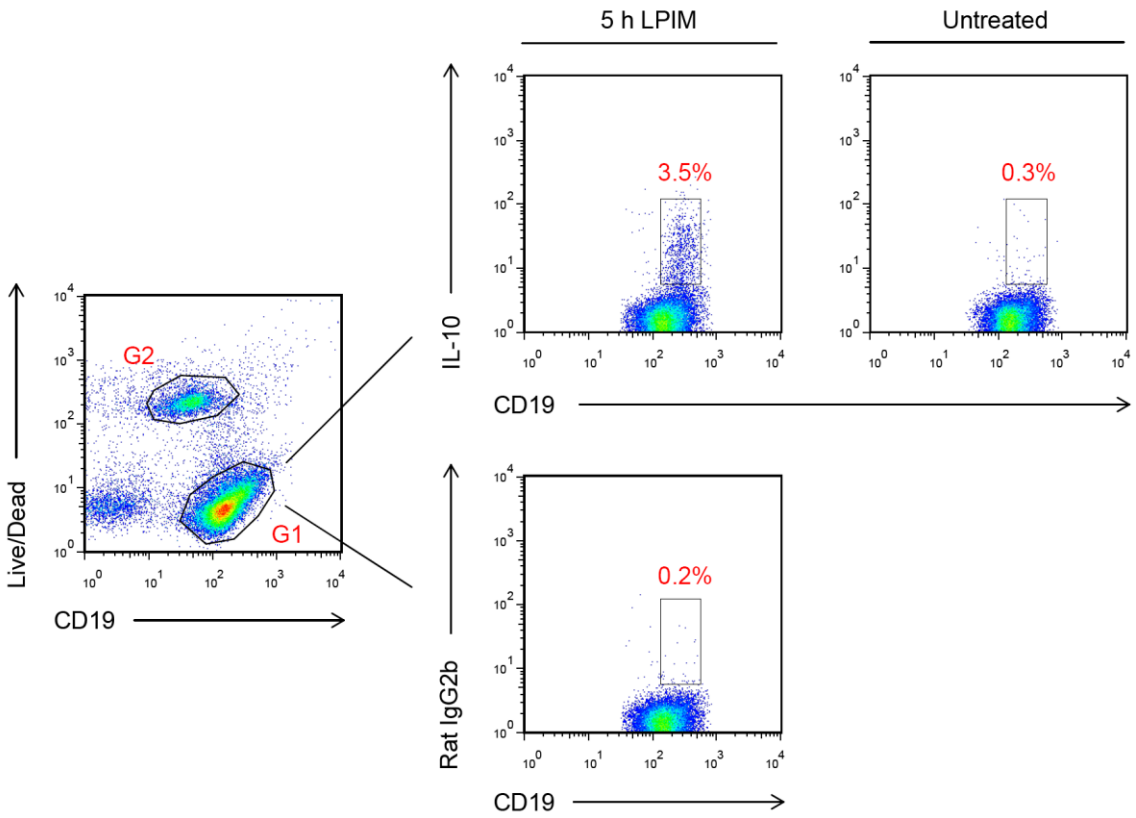


Figure 3.1: Gating strategy and flow cytometric analysis for identifying IL-10$^+$ B cells among total B cells. Using the CD19 *vs* live/dead plot (left panel) it is possible to identify viable B cells (G1) that are positive for CD19 staining but negative for live/dead staining. This allows to exclude dead cells (G2) from the analysis. The panels on the right show a representative cell surface CD19 and cytoplasmic IL-10 staining by viable B cells (upper middle dot plot) and the negative controls used for the correct identification of CD19$^+$ IL-10$^+$ cells. As negative controls, the strategy employed a perfectly matched isotype control (Rat IgG2b) in addition to the use of B cells not stimulated with LPIM.

3.1.2 Role of stimuli activating B cells on the expansion of IL-10–competent B cells *in vitro*

Primary B cells can undergo maturation and/or activation by means of different signals, including mediators of both innate and adaptive immunity (Zarnegar et al., 2004). Upon ligand binding, receptors such as CD40, BCR, BAFF-R, TLR4, and TLR9, promote survival, proliferation and differentiation responses, which vary depending on the B cell subset under analysis. For example, MZ B cells proliferate and differentiate into plasma cells more rapidly than FO B cells in response to LPS; in addition they expand preferentially compared to FO B cells in BAFF transgenic mice (Kaminski and Stavnezer, 2006). It is therefore possible that, given the different effects on B-cell survival, proliferation and maturation, these stimuli may also differently regulate the expansion and/or differentiation of IL-10–competent B cells.

With the purpose of clarifying this aspect, wild type spleen B cells were cultured for 48 h with LPS, CpG, BAFF or agonistic CD40 mAb, at predetermined optimal concentrations; LPIM was added to the media during the last 5 h of culture, in order to enhance *Il-10* expression in IL-10–competent B cells. Then, following intracellular staining, the percentages of IL-10–producing B cells, obtained after culture with these stimuli, were compared to the ones normally found within the spleen of adult mice. As shown in figure 3.2A, all the tested stimuli were able to expand the IL-10–competent B cell population of the spleen, since the percentages of IL-10–producing B cells, obtained after stimulation, were significantly different from the ones found among freshly isolated B lymphocytes. However, the histogram clearly shows that the stimuli did not have the same inductive capacity. CD40 ligation, with subsequent LPIM stimulation, induced cytoplasmic IL-10 production by 15,7% (± 0.5) of B cells, which was 5.4-fold higher than for unstimulated B cells (2.9% ± 0.2) and 1.8, 2.0 and 2.6-fold higher than for B cells cultured respectively with LPS (8.5% ± 1.0), BAFF (7.6% ± 0.6) and CpG (5.9% ± 1.0). Therefore, in our system, B cell stimulation through CD40 proved to be the best way to promote IL-10–competent B cell expansion and/or differentiation. This result is in agreement with the *in vitro* experiments performed by Yanaba and coworkers (Yanaba et al., 2009) and with several papers that demonstrated that CD40 signaling is pivotal for the generation and function of regulatory B cells in experimental autoimmune encephalomyelitis, collagen-induced arthritis and inflammatory bowel disease models (Mizoguchi et al., 2000; Fillatreau et al., 2002; Mauri et al., 2003).

Regardless of the observed differences in the frequency of IL-10^+ B cells after stimulation with either agonistic CD40 mAb, BAFF, LPS or CpG, the percentages of IL-10–producing B cells were in any case higher than the ones found among freshly isolated B lymphocytes. This increase could be explained either by the differentiation of non IL-10–competent B cells in IL-10–competent B cells or by the preferential proliferation and/or survival of IL-10–competent B cells in respect to other B cell subsets. In both cases, the result is an increased percentage of IL-10–producing B cells (Figure 3.2B). Understanding the biological process (proliferation, survival and/or differentiation) responsible for the expansion of IL-10–competent B cells that occurs after activation with these stimuli remains a goal of future work.

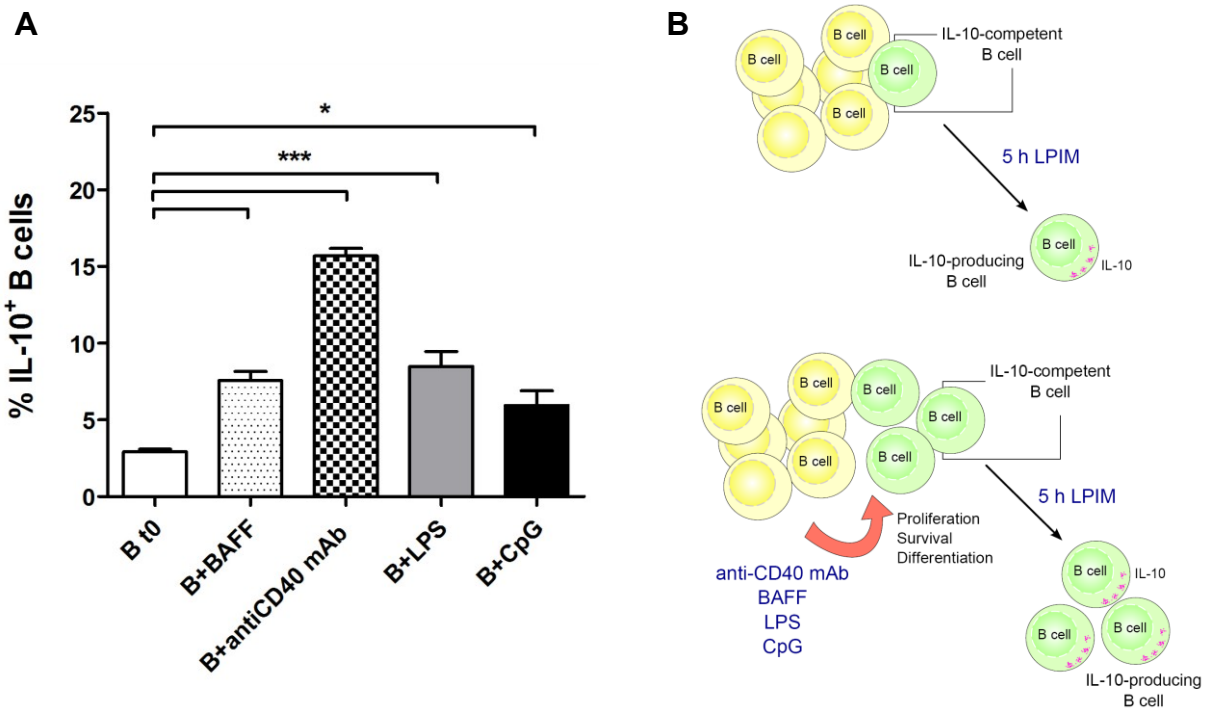


Figure 3.2: ***In vitro*** **B cell stimulation induces the expansion of IL-10–competent B cells.** (***A***) The frequency of IL-10–producing B cells was analyzed in freshly isolated B lymphocytes (indicated as t0) or in purified B cells cultured for 48 h with agonistic CD40 mAb, BAFF, LPS or CpG. LPIM stimulation was used to induce IL-10 expression in IL-10–competent B cells. Bar graphs indicate mean (± SEM) percentages of IL-10^+ B cells from five independent experiments. Significant differences between sample means are indicated, as follows: *, $p< 0.05$; ***, $p< 0.001$. The statistical analysis was performed using an unpaired *t* test. (***B***) Schematic representation of the expansion of the IL-10 competent B cell population after treatment with the four stimuli.

3.1.3 Diverse B-cell stimuli induce different levels of cytoplasmic IL-10

From the analysis of the flow cytometry data relative to IL-10 intracellular staining it is possible not only to know the percentage of cells that express this cytokine but also to have an idea of the levels of cytoplasmic IL-10, present in the cells. While the histogram reported in figure 3.2A shows very clearly that all four stimuli tested are effective in expanding the IL-10–competent B cell population, the plots reported in figure 3.3 demonstrate that also the levels of cytoplasmic IL-10 are differently affected by LPS, CpG, BAFF and CD40 mAb. Figure 3.3 shows the results of the flow cytometric analysis of one representative experiment out of three: both the CD19 *versus* IL-10 dot plots (left part of the picture) and the single-color histograms, relative to IL-10 fluorescence intensity (right part of the picture), are reported. The latter representation shows that, when the B cells were cultured with LPS or CpG, the fluorescence intensity relative to IL-10 expression (solid line) appears uniformly higher, compared to the isotype control (filled histogram), even if the cell population seems to produce IL-10 at medium-low levels. On the contrary, when the B cells were stimulated either with the CD40 mAb or with BAFF, the histogram relative to B cell IL-10 expression perfectly

overlaps the one of the isotype control, with the only exception of a minor and clearly distinct part of the population, expressing medium-high levels of IL-10. Therefore this analysis suggests that IL-10–competent B cells respond differently to the tested B-cell stimuli.

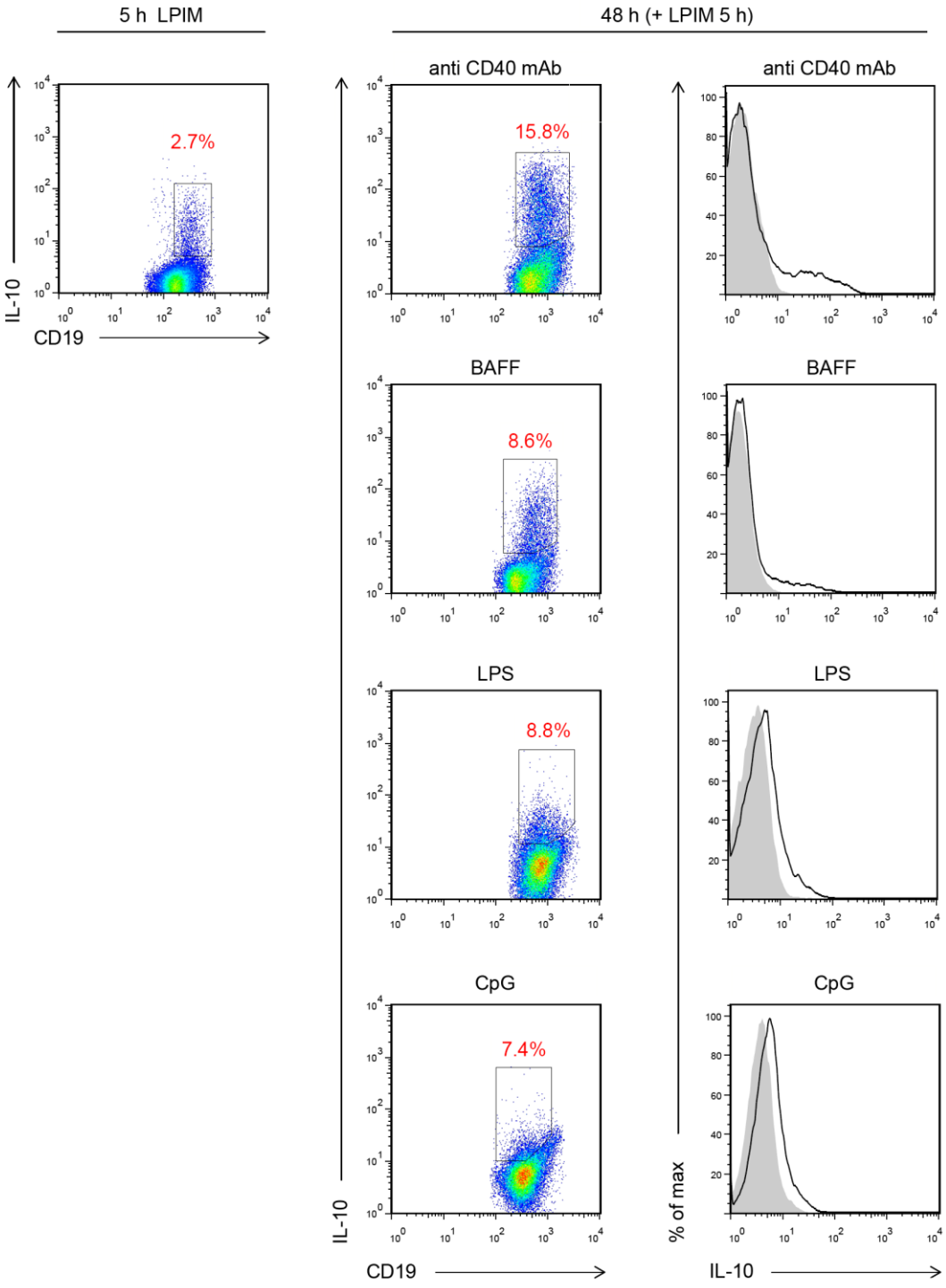


Figure 3.3: The fluorescence intensity relative to IL-10 expression differs when B cells are stimulated by exogenous, infectious signals, or endogenous, immune mediated signals. Spleen B cells were freshly isolated or cultured for 48 h with agonistic CD40 mAb, BAFF, LPS or CpG. Values indicate the percentage of $CD19^+$ $IL\text{-}10^+$ cells among total $CD19^+$ cells, within the indicated gates. Single-color histograms in which cell numbers (y-axis) are plotted against log fluorescence intensity of IL-10 (x-axis) are also reported. Filled histograms (gray) indicate isotype control antibody.

3.1.4 Role of stimuli activating B cells on the secretion of IL-10

IL-10 is an anti-inflammatory cytokine with pleiotropic effects in immunoregulation and inflammation. It is capable of inhibiting the synthesis of pro-inflammatory cytokines such as IFN-γ, IL-2, IL-3, TNF-α and GM-CSF in cells such as macrophages and Tregs. It also displays a potent ability to suppress the antigen-presentation capacity of antigen presenting cells. However, it is also stimulatory towards certain T cell subsets and mast cells and stimulates B-cell maturation and antibody production (Sabat et al., 2010). Moreover, the secretion of IL-10 was shown to be critical for regulatory B cell effector functions (Mauri et al., 2003). In order to assess whether signals provided by agonistic CD40 mAb, BAFF, LPS or CpG could differentially regulate B-cell IL-10 secretion, wild type spleen B cells were cultured for 48 h either alone or in the presence of one of the aforementioned stimuli. Then, culture supernatants were collected and the levels of IL-10 were determined by ELISA.

As shown in figure 3.4A, no significant differences in IL-10 secretion were found between unstimulated B cells (0.20 ng/mL ± 0.05) and B cells cultured either with agonistic CD40 mAb (0.25 ng/mL ± 0.03) or BAFF (0.21 ng/mL ± 0.03). On the contrary, LPS and CpG stimulation induced significant B-cell IL-10 secretion; in particular, the concentrations of this cytokine in the supernatants of B cells stimulated through TLR4 or TLR9 were respectively 9.8 and 17-fold higher than for unstimulated B cells.

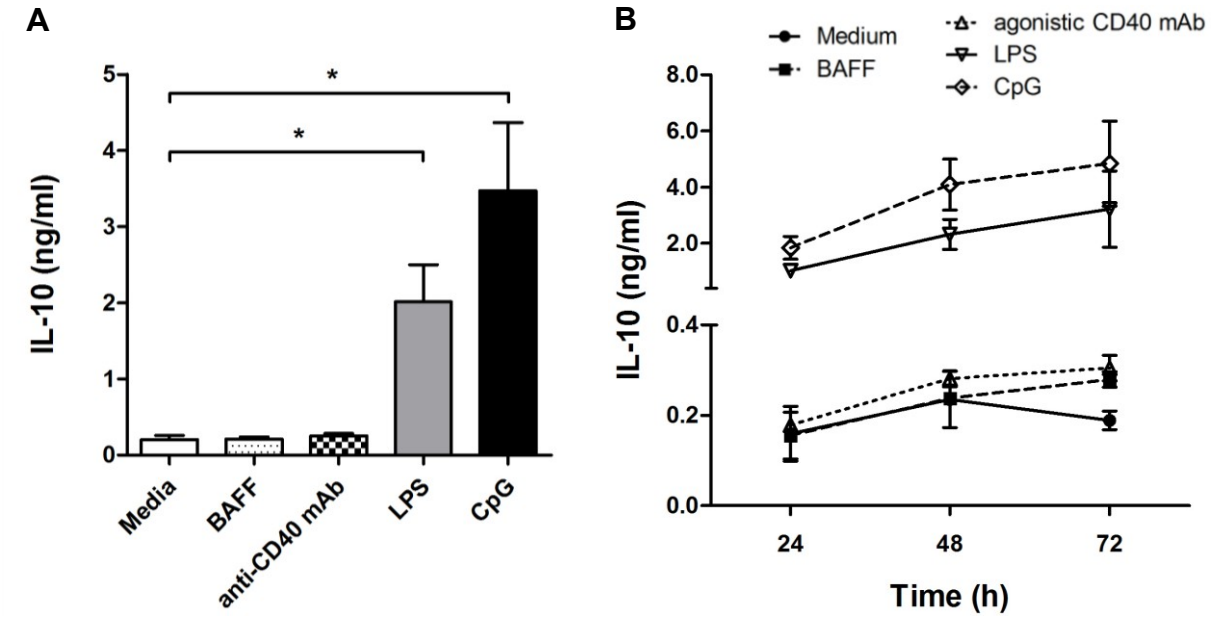


Figure 3.4: LPS and CpG stimulation promotes IL-10 secretion by B cells. (***A***) For the quantification of secreted IL-10, culture supernatants were harvested from cells at 48 h from the initial exposure to the stimuli and tested by ELISA. Bar graphs indicate mean (± SEM) concentrations of four independent experiments. Significant differences between sample means are indicated, as follows: *, $p < 0.05$. The statistical analysis was performed using a paired *t* test (***B***). In order to study the kinetics of IL-10 secretion in unstimulated and stimulated B cells, the supernatants were collected after 24, 48 and 72 h of culture and analyzed by ELISA.

Further on, the kinetics of IL-10 secretion in unstimulated and stimulated B cells were studied (Figure 3.4B). The supernatants were collected at 24, 48 and 72 h from the initial exposure to the stimulus and stored at -20 C until analyzed by ELISA. At 24h from the start of stimulation, IL-10 was detected in the culture supernatants and, already at this time point, it was evident that stimulation with LPS and CpG led to increased secretion of IL-10 compared to all the other conditions. In the stimulated B cells, IL-10 continued to be produced throughout the whole culture period; on the contrary, a decrease in IL-10 concentration was observed in unstimulated B cells after 48 h of culture. IL-10 is the most relevant cytokine for B-cell survival in mice and humans (Gary-Gouy et al., 2002), therefore the observed decrease in IL-10 concentrations in the supernatants of unstimulated B cells could be due to the fact that, in the absence of stimulation, IL-10 acts as an autocrine growth factor, used by B cells for their survival.

All together these results show, once again, that stimulation of B cells with innate or immune mediated signals leads to different outcomes: while LPS and CpG promoted the secretion of IL-10 at high levels, the agonistic CD40 mAb and BAFF did not affect the basal release of this cytokine by B cells. The IL-10 secreted by B cells after stimulation with LPS or CpG is likely due to the fact that signaling via TLRs provides an important sensory mechanism for the detection of infectious threats (Takeda et al., 2003). When TLRs recognize conserved microbial structures, they initiate immune responses and produce proinflammatory mediators; however, all the immune responses, including the one against infection, at some point in time, must be turned off. In the infectious scenario, IL-10 is a rather “late” cytokine, produced after the proinflammatory mediators in order to limit the immune response to pathogens and therefore preventing damage to the host (Saraiva and O'Garra, 2010).

3.1.5 Short-term stimulation via TLR4 or BCR induces the secretion of IL-10 by CD40-induced IL-10–producing B cells

Although CD40-generated signals were shown to induce the expansion of IL-10–competent B cells, they do not induce the release of this cytokine. The reason for this outcome of B cell stimulation through CD40 could be that a second signal is required to promote IL-10 secretion by CD40-induced IL-10–producing B cells. In order to investigate this hypothesis, B cells were cultured for 48 h with agonistic CD40 mAb and either LPS or anti–mouse IgM Ab were added to the medium during the last two hours of culture. Then, culture supernatants were collected and the levels of IL-10 were determined by ELISA. The representative experiment reported in figure 3.5A clearly shows that a short-term stimulation via TLR4 or BCR was able

to induce the secretion of the IL-10 produced by B cells during stimulation with agonistic CD40 mAb. As a matter of fact, the concentrations of this cytokine in the supernatants of B cells stimulated for the last 2 h of culture with LPS or anti–IgM Ab were respectively 3.6 and 2.1-fold higher than for the same B cells that didn't undergo the short-term stimulation.

To exclude the possibility that the observed increase in IL-10 concentration was due to a *de novo* production of IL-10 rather than to the secretion of the IL-10 produced during the previous 48 h of stimulation with agonistic CD40 mAb, freshly isolated splenic B cells were cultured for 2 h either alone or in the presence of LPS or anti–IgM Ab. As reported in the representative experiment shown in figure 3.5B, the short-term stimulation alone was not able to induce IL-10 secretion by B cells.

Thereby, LPS, anti–IgM Ab or other signals may optimally induce IL10–competent B cell effector functions in IL-10^{+} B cells induced *via* activation through CD40.

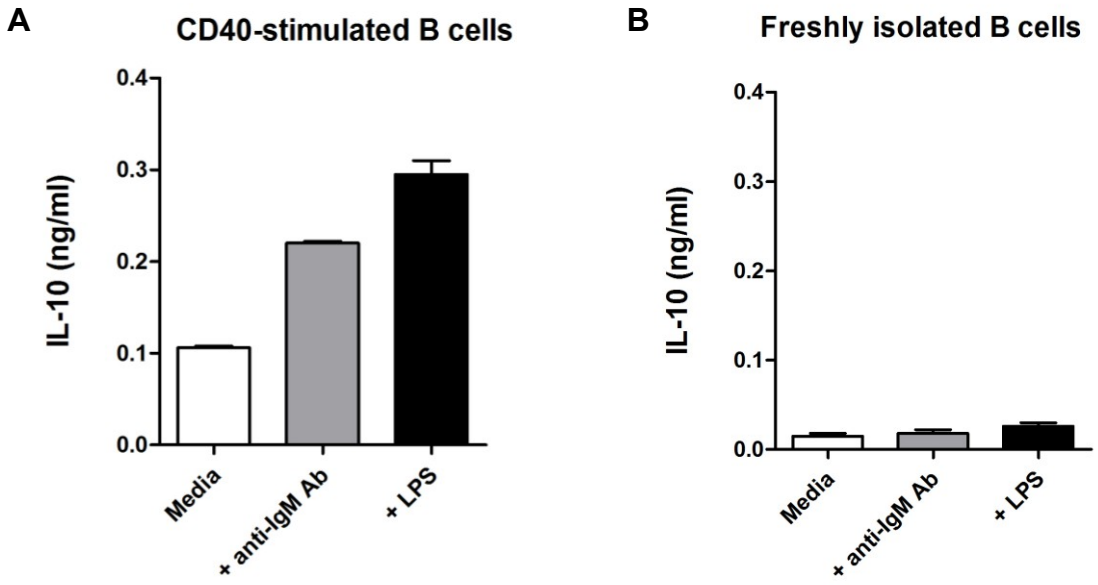


Figure 3.5: Short-term stimulation via TLR4 or BCR induces the secretion of IL-10 by CD40-induced IL-10 competent B cells. (***A***) Purified B cells were cultured at 10^{6}/mL in the presence of agonistic CD40 mAb for 48 h. During the last 2 h either LPS or anti-IgM Ab were added to the culture medium at predetermined optimal concentrations. At the end of stimulation, supernatant fluids were harvested from cultured cells and IL-10 concentrations were determined by ELISA. (***B***) In parallel, as a control, freshly purified naïve B cells were cultured for 2 h in the presence of either LPS or anti-IgM Ab. Bar graphs indicate mean (± SEM) concentrations from one representative experiment in which each supernatant fluid was tested in duplicate.

3.1.6 Role stimuli activating B cells on the secretion of other anti-inflammatory cytokines

Although IL-10 is an important anti-inflammatory cytokine both in human and mouse immune response, the immune system has provided several other immunomodulatory components that limit the potentially injurious effects of sustained or excessive immune and inflammatory reactions. These immunomodulatory components can be either cells or soluble mediators (Opal and DePalo, 2000). The hallmark of regulatory B cells is their capacity to secrete the regulatory cytokine IL-10; however, the mechanisms by which this subset of B cells suppress inflammation are likely to vary depending on the type of inflammatory response. In addition to IL-10-producing B cells, a subset of regulatory B cells that is capable of producing TGF-β after *in vitro* stimulation with LPS has also been identified (Tian et al., 2001). For these reasons, the ability of signals provided by agonistic CD40 mAb, BAFF, LPS or CpG to promote the secretion of other anti-inflammatory cytokines was assessed. Wild type spleen B cells were cultured either alone or in the presence of one of the aforementioned stimuli and, after 48 h, the cell supernatants were harvested and tested by ELISA to determine the levels of TGF-β and IL-13.

Figure 3.6 shows the results from one representative experiment, out of three, for both TGF-β (3.6A) and IL-13 (3.6B). For what concerns TGF-β, no differences in cytokine secretion were found between unstimulated (37.3 pg/mL ± 16.3) and CD40-stimulated B cells (51.3 pg/mL ± 7.0) whereas a 2.5-3-fold increase in TGF-β concentrations was observed when B cells were cultured with BAFF, LPS or CpG. Taking into account B cell activation through LPS or CpG and comparing the result obtained for TGF-β to the one previously observed for IL-10, it can be noticed how the levels of secreted TGF-β were lower than the ones observed for IL-10, which was secreted in high amounts already after 24 h of culture. A similar result was observed in TLR-activated intestinal B cells (Mishima et al., 2010).

Concerning IL-13, both unstimulated and stimulated B cells produced very low levels of this cytokine and this result is in accordance with data reported in literature relatively to IL-13 secretion by human B lymphocytes (Hajoui et al., 2004). Unfortunately, any observed difference between samples were not considered reliable since the measured concentrations were very close to the lower limit of detection of the ELISA kit used to perform these experiments.

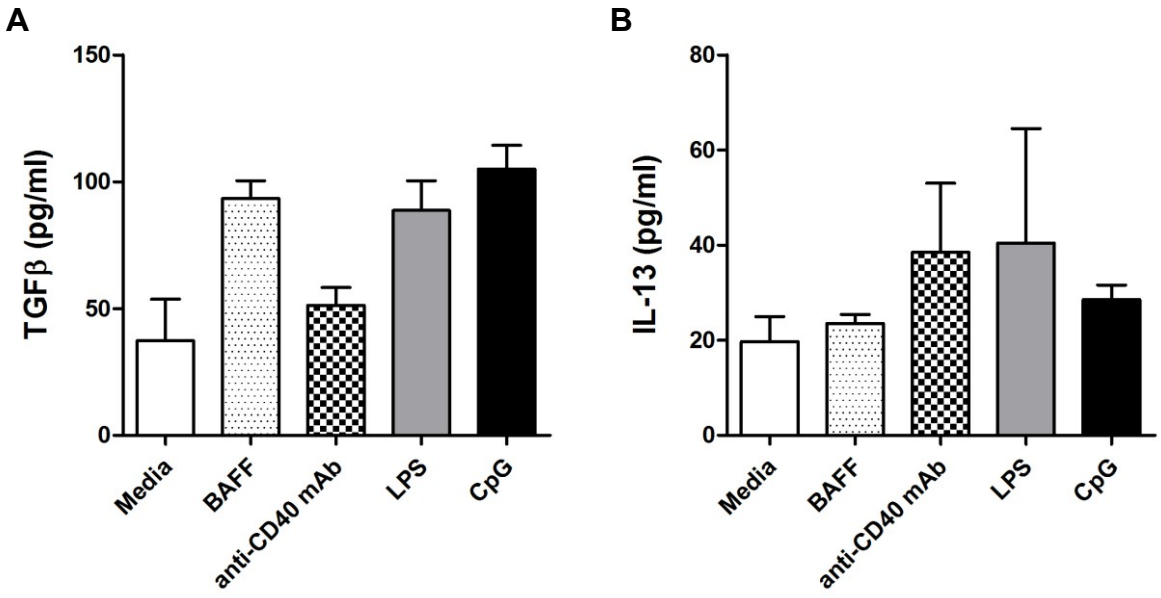


Figure 3.6: B cells produce other anti-inflammatory cytokines, in addition to IL-10. For measuring secreted TGF-β (***A***) and IL-13 (***B***), purified B cells were cultured at 10^6/mL in the absence of stimulation or in the presence of agonistic CD40 mAb, BAFF, LPS or CpG. Supernatant fluids were harvested from cultured cells at 48 h from the initial exposure to the stimuli and protein levels of TGF-β and IL-13 were determined by ELISA. Bar graphs indicate mean (± SEM) concentrations from one representative experiment in which each supernatant fluid was tested in duplicate.

3.1.7 Supernatants derived from LPS or CpG–stimulated B cells suppress effector T cell proliferation *in vitro*

As an increasing body of evidence showed that B cells had many other functions, unrelated to Ab production, it became clear that B cells were not simply the passive recipients of T cell help, but actively participated in cellular immune responses by directing the magnitude and quality of the T cell response to foreign and self-antigens. In particular, it has been shown that both effector and regulatory B cell subsets modulate the function of T cells by presenting antigen, providing co-stimulation and producing cytokines that direct the proliferation and the effector functions of responding T cells (Lund and Randall, 2010).

Since B cell cultured in the presence of agonistic CD40 mAb, BAFF, LPS or CpG led to different outcomes in cytokine production and secretion, the conditioned media of unstimulated and stimulated B cells were tested in order to determine whether the substances produced by the treated cells were able to suppress T cell proliferation. With this purpose, conditioned media from B-cell cultures were prepared by harvesting cell-supernatants 48 h after the addition of the stimulus. Furthermore, the effector ($CD4^+$ $CD25^-$) T (Teff) cells were purified from mouse spleen, labeled with carboxyfluorescein succinimidyl ester (CFSE) and cultured for 72 h in the presence of both T-cell mitogens and B-cell conditioned media.

Figure 3.7A shows the flow cytometric analysis of one representative experiment, out of four, from which it can be noticed that, although the proliferation of $CD4^+$ $CD25^-$ cells occurred in the presence of all the tested conditioned media, T cells cultured with supernatants from LPS or CpG-activated B cells progressed through generations slower than the others. This result was further confirmed by analyzing the proliferation index, which is the average number of divisions of just the responding cells (cells that underwent at least one division). As shown in figure 3.7B, a significant decrease in the T-cell proliferation index was observed between $CD4^+$ $CD25^-$ cells cultured with the supernatants from LPS or CpG-activated B cells (respectively 1.7 ± 0.2 and 1.5 ± 0.2) and the positive control (2.3 ± 0.1), represented by the same T cells cultured only in the presence of T cell mitogens. Instead, no significant reduction was observed when the Teff cells were cultured with the conditioned medium of unstimulated B cells (2.4 ± 0.1) or of B cells activated either with agonistic CD40 mAb (2.2 ± 0.1) or BAFF (2.3 ± 0.1). Therefore, these results clearly demonstrate that the supernatants of B cells stimulated through TLR4 or TLR9 were able to suppress T cell proliferation, indicating the presence of suppressive secreted molecules, produced by the activated B cells. These findings are consistent with the result of the ELISA experiment performed in order to investigate IL-10 secretion by activated B cells and suggest that IL-10 is responsible for the suppression of T-cell proliferation observed with the conditioned media of LPS and CpG-activated B cells.

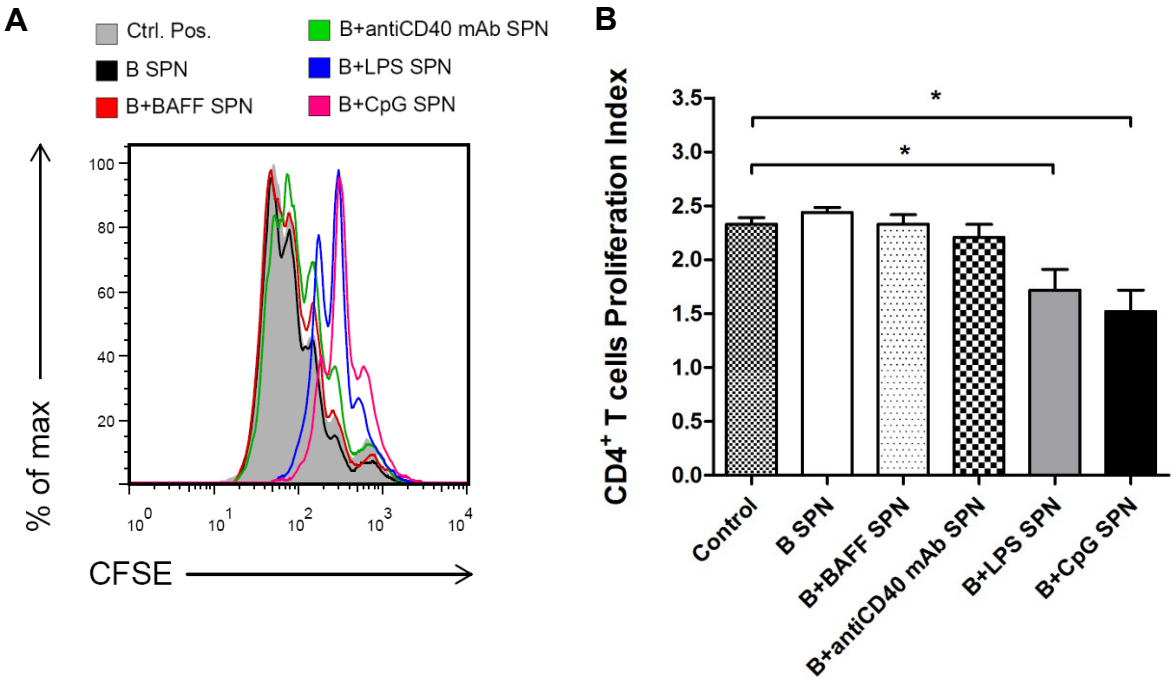


Figure 3.7: Supernatants derived from LPS or CpG–stimulated B cells suppress effector T cell proliferation. (***A***) CFSE-labeled $CD4^+CD25^-$ purified T cells were incubated for 72 h with anti-CD3 and anti-CD28 mAbs, in the presence of allogeneic irradiated APCs. Cultures additionally received medium (control), supernatants from unstimulated B cells (B SPN) or supernatants from B cells activated for 48 h with agonistic CD40 mAb (B+agonistic CD40 mAb SPN), BAFF (B+BAFF SPN), LPS (B+LPS SPN) or CpG (B+CpG SPN). Diminished CFSE intensity, indicative of proliferation, was detected in $CD4^+$ T cells by flow cytometry. CFSE profiles of effector T cell proliferation in the different conditions are shown for one representative experiment, out of four. (***B***) Bar graphs indicate mean (± SEM) of the T cell proliferation index from four independent experiments. Significant differences between sample means are indicated, as follows: *, $p< 0.05$. The statistical analysis was performed using a paired *t* test.

3.1.8 B cell stimulation through CD40 and TLR4 differently affect the immunophenotype of IL-10–competent B cells

The results presented so far clearly show that exogenous, infectious signals and endogenous, immune mediated signals differently affect IL-10 production and secretion by B cells. This led to investigate whether the tested stimuli could also have a different effect on the immunophenotype of the induced $IL\text{-}10^+$ B cells. In particular, the expression of the CD1d and CD5 molecules on $IL\text{-}10^+$ B cells obtained after stimulation with agonistic CD40 mAb or LPS was analyzed. The choice of studying the immunophenotype of IL-10–competent B cells in relation to the CD1d and CD5 molecules was due to the fact that, on the basis of the differential expression of these two markers on the B cell surface, it is possible to identify four distinct subsets ($CD1d^-$ $CD5^-$, $CD1d^+$ $CD5^-$, $CD1d^+$ $CD5^+$, $CD1d^-$ $CD5^+$) that resemble the various phenotypes of regulatory B cells described in literature (Figure 3.8). IL-10–producing

B cells have been detected within the splenic marginal zone population (CD21^{hi} CD23^{-} IgM^{hi} CD1d^{hi}) or the less-mature transitional 2–MZ precursor population (CD21^{hi} CD23^{+} IgM^{hi} CD1d^{hi}) (Evans et al., 2007; Gray et al., 2007). In addition, some studies suggest that regulatory B cells may also reside within the much larger follicular B cell subset (Miyazaki et al., 2009). Finally, Yanaba et al. identified B10 cells, which constitute a small subset of splenic B cells that express a CD1d^{hi} CD5^{+} phenotype and partially overlaps with that of MZ, T2-MZ, and B-1a cells. It is important to specify that although CD1d^{hi} CD5^{+} B cells are more enriched for IL-10–producing cells (they represent between 9 and 15% of the cells of CD1d^{hi} CD5^{+} B cells) than other B cell subsets, most IL-10^{+} B cells fall outside of the B10 population (Ding et al., 2011).

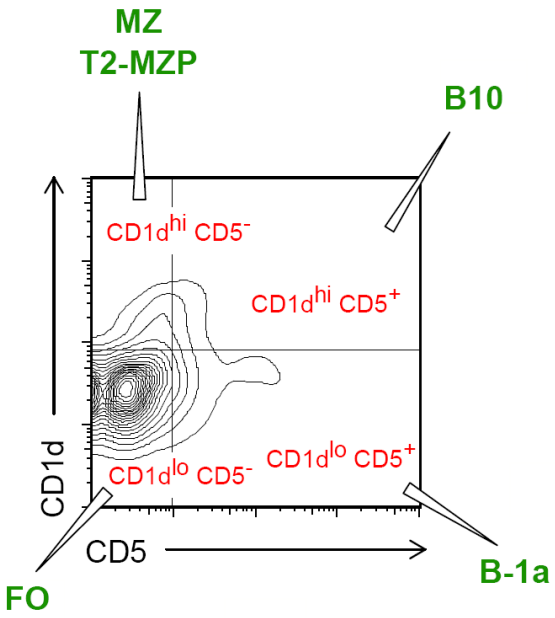


Figure 3.8: B cell populations identified on the basis of the differential expression of the CD1d and CD5 markers. The differential expression of CD1d and CD5 on the B cell surface leads to the definition of four distinct subsets: CD1d^{-} CD5^{-}, CD1d^{+} CD5^{-}, CD1d^{+} CD5^{+} and CD1d^{-} CD5^{+}. This B cell compartments resemble respectively the follicular (FO), marginal zone (MZ) or transitional 2–MZ precursor (T2-MZP), B10 and B-1a subsets.

In order to characterize the immunophenotype of the IL-10^{+} B cells obtained after activation through CD40 or TLR4, purified CD19^{+} splenocytes were cultured for 48 h either with agonistic CD40 mAb or LPS and stimulated with LPIM for 5 h, before permeabilization and staining with CD19, CD1d, CD5 and IL-10 mAbs. Finally, flow cytometric analysis was performed and B cells within both the IL-10^{+} and IL-10^{-} gates were assessed for CD1d and CD5 expression. The results of this analysis show the distribution of both IL-10^{+} and IL10^{-} cells among the four subsets defined by the differential expression of the two markers. As a consequence, the sum of the percentages of IL-10^{+} or IL-10^{-} cells within the four subsets is equal to 100%.

Figure 3.9A shows the result obtained when the B cells were stimulated with the agonistic CD40 mAb: while the IL-10^{-} cells were found almost exclusively (85.7% ± 3.9) in the CD1d^{-} CD5^{-} follicular B cell compartment, the IL-10^{+} cells were distributed mainly in the CD1d^{-}

$CD5^-$ (44.5% ± 5.1) and in the $CD1d^+$ $CD5^-$ (47.4% ± 4.0) subsets. Only a minor part of the CD40-induced IL-10^+ B cells presented a $CD1d^+$ $CD5^+$ (4.8% ± 1.4) or a $CD1d^-$ $CD5^+$ (3.3% ± 1.5) phenotype. On the contrary, the IL-10^+ B cells induced by LPS presented an immunophenotype very similar to the IL-10^- cells since 80.9% (± 4.6) of the IL-10–producing cells were found within the follicular B cell compartment and only 14.5% (± 4.0) within the $CD1d^+$ $CD5^-$ subset (Figure 3.9B).

Therefore, the performed phenotypical analysis revealed that the IL-10–producing B cells resulting from B cell activation through CD40 or TLR4 present a different immunophenotype. The vast majority of LPS-induced IL-10^+ B cells were negative for CD5 and expressed low levels of CD1d, thus resembling the phenotype of FO B cells. On the contrary, CD40-induced IL-10^+ B do not belong exclusively to the FO compartment since 50% of the IL-10^+ population expressed high levels of CD1d, a defining marker of MZ or T2-MZP B cells.

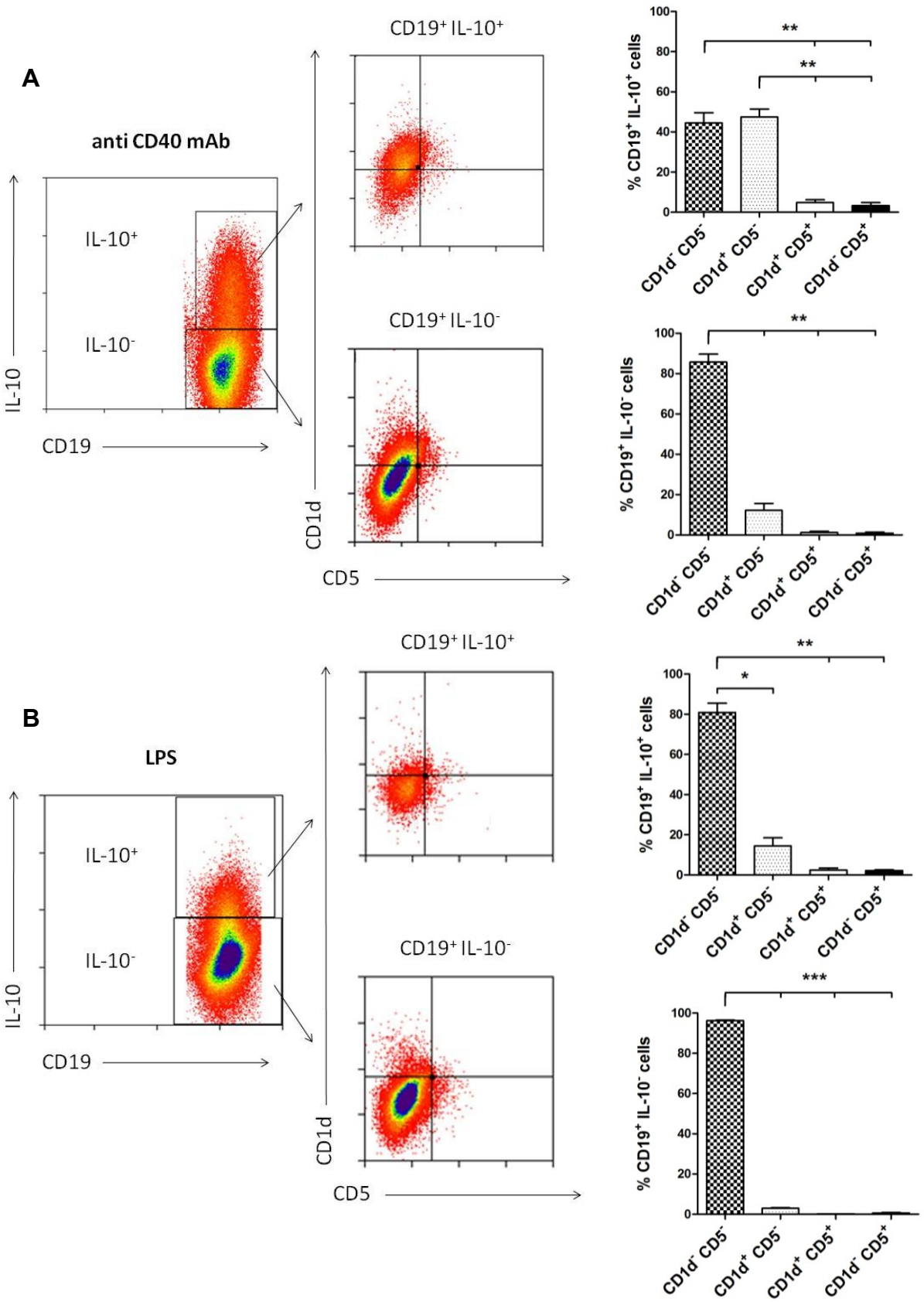


Figure 3.9: Immunophenotype of IL-10$^+$ B cells induced by agonistic CD40 mAb or LPS. Spleen B cells were cultured for 48 h with agonistic CD40 mAb (***A***) or LPS (***B***) and stained using CD19, CD1d, CD5 and IL-10 mAbs. In both cases, the left panels of the figure show the flow cytometric analysis performed to study the expression of the CD1d and CD5 surface molecules. The histograms reported on the right show the average percentages (± SEM) of IL-10$^+$ or IL-10$^-$ cells that display a CD1d$^-$ CD5$^-$, CD1d$^+$ CD5$^-$, CD1d$^+$ CD5$^+$ or CD1d$^-$ CD5$^+$ phenotype, from four independent experiments. Significant differences between sample means are indicated, as follows: *, p< 0.05; **, p< 0.01; ***, p< 0.001. The statistical analysis was performed using a paired *t* test.

In order to further investigate the reasons of the observed differences between the two conditions, the immunophenotype of the IL-10$^+$ B cells obtained after culture with agonistic CD40 mAb or LPS was compared to the one of the IL-10$^+$ B cells normally found within the spleen of adult mice. Freshly isolated B lymphocytes were cultured for 5 h with LPIM in order to enhance *Il-10* expression in IL-10–competent B cells, then the expression of the surface molecules CD1d and CD5 on the IL-10$^+$ B cells was investigated. As shown in figure 3.10A, these IL-10–producing B cells were found within all the four subsets defined by the differential expression of CD1d and CD5, although the highest percentage (46.6% ± 3.9) of these IL-10$^+$ cells expressed high levels of CD1d and low levels of CD5, thus resembling the phenotype of MZ or T2-MZP B cells. On average, IL-10$^+$ B cells with a CD1d$^+$ CD5$^+$ or CD1d$^-$ CD5$^+$ phenotype represented respectively 5.1% (± 2.1) and 9.4% (± 1.8) of total IL-10$^+$ B cells. The remaining fraction of IL-10$^+$ B cells (39% ± 0.1) was found in the FO compartment.

The comparison of the IL-10$^+$ B cell distribution in the four subsets, before and after treatment with agonistic CD40 mAb or LPS, showed an interesting result: while activation through CD40 did not affect the immunophenotype of IL-10–producing B cells, the B cell treatment with LPS favored the acquisition of a FO-like phenotype (Figure 3.10B,C).

As a matter of fact, while no significant differences in the distribution of the IL-10$^+$ B cells were observed between freshly isolated B lymphocytes and CD40-activated B cells, the percentage of IL-10$^+$ cells with a CD1d$^-$ CD5$^-$ phenotype was 2.1-fold higher in LPS-stimulated B cells than in naïve B lymphocytes. With regard to this last comparison, the observed differences within the CD1d$^-$ CD5$^-$, the CD1d$^+$ CD5$^-$ and the CD1d$^-$ CD5$^+$ subsets were all statistically significant.

To conclude, while activation through CD40 promoted the expansion of IL-10–competent B cells without altering the overall distribution of these cells among the different B cell compartments, LPS stimulation seemed to favor the expansion of IL-10$^+$ B cells through a mechanism that selectively affects the CD1d$^-$ CD5$^-$ subset.

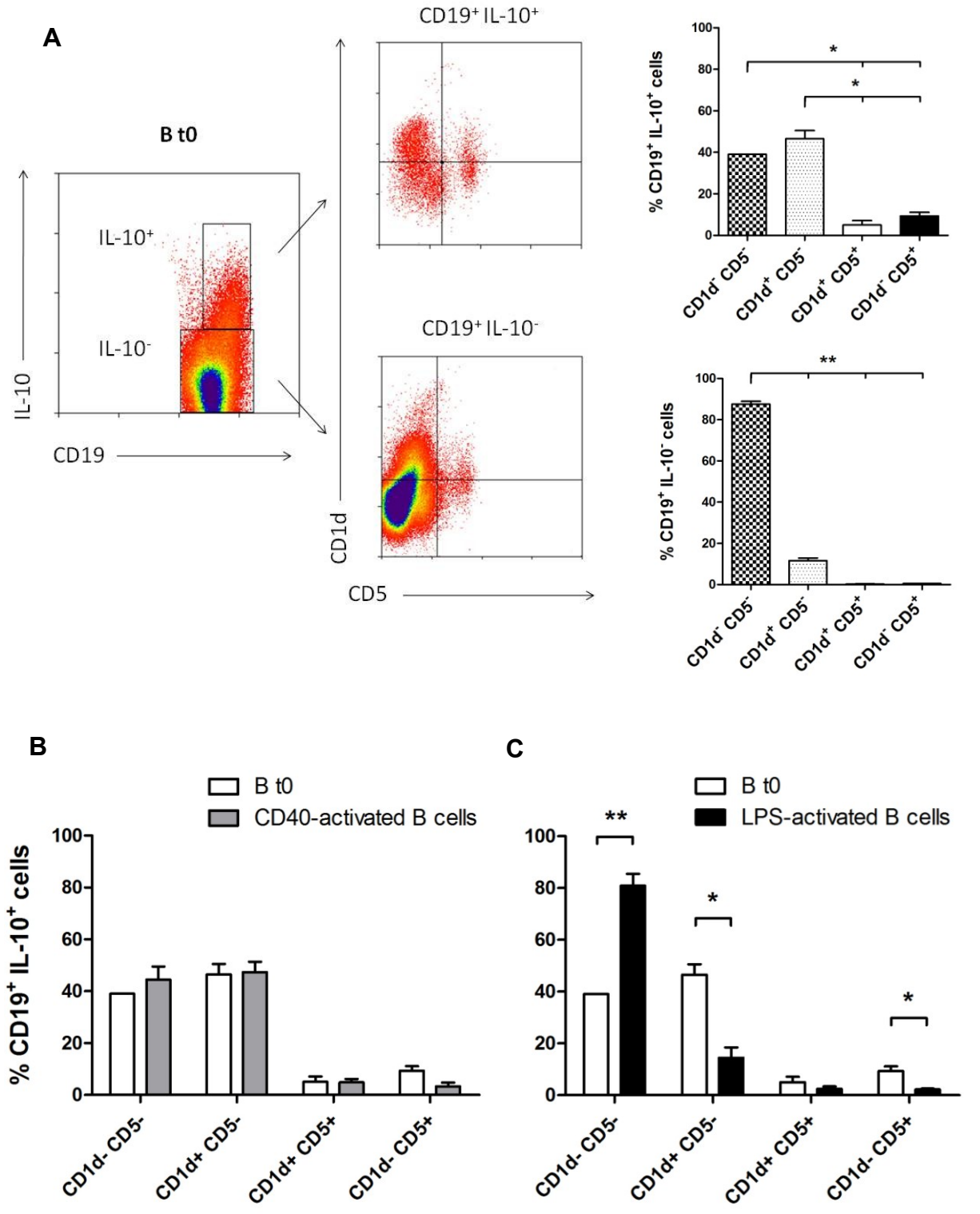


Figure 3.10: Immunophenotype of IL-10–producing B cells before and after activation through CD40 or TLR4. (***A***) B cells were freshly isolated from mouse spleen and stimulated for 5 h with LPIM in order to induce IL-10 expression in IL-10–competent B cells. The cells were therefore stained using CD19, CD1d, CD5 and IL-10 mAbs and the immunophenotype of both IL-10$^+$ and IL-10$^-$ cells was evaluated. The histograms reported on the right side of the picture show the average percentages (± SEM) of IL-10$^+$ or IL-10$^-$ cells that display a CD1d$^-$ CD5$^-$, CD1d$^+$ CD5$^-$, CD1d$^+$ CD5$^+$ or CD1d$^-$ CD5$^+$ phenotype. Moreover, the distribution of total IL-10$^+$ B cells among the four subsets defined by CD1d and CD5 was assessed before and after stimulation of B cells with agonistic CD40 mAb (***B***) and before and after stimulation with LPS (***C***). Significant differences between sample means are indicated, as follows: *, $p< 0.05$; **, $p< 0.01$. The statistical analysis was performed using a paired *t* test.

3.1.9 Both CD40- and LPS- induced IL-10–competent B cells are found to be enriched in the CD1d$^+$ CD5$^-$ subset

Several papers have reported that, although the majority of IL-10–competent B cells found in mouse spleen did not present a CD1d$^+$ CD5$^+$ phenotype, the subset characterized by the expression of both these two markers was the one that displayed the highest frequency of IL-10$^+$ B cells (Ding et al., 2011; Klinker and Lundy, 2011). Therefore, when analyzing IL-10$^+$ B cells in relation to their phenotype, it is important to clearly identify the question that one wants answered: the percentage of IL-10$^+$ cells expressing the CD1d$^+$ CD5$^+$ phenotype or the percentage of CD1d$^+$ CD5$^+$ cells that produces IL-10.

The experiments presented in the previous paragraph of this thesis were performed in order to investigate whether B-cell activation with agonistic CD40 mAb or LPS differently affected the immunophenotype of IL-10–producing B cells and, therefore, their distribution among the four subsets identified by the differential expression of the CD1d and CD5 markers. The next step was to assess whether these two stimuli differently affected the frequency of IL-10$^+$ B cells within each subset. For this purpose, freshly purified or activated B cells were stained with CD19, CD1d, CD5 and IL-10 mAbs, before performing flow cytometric analysis. In this case, B cells within the CD1d$^-$ CD5$^-$, CD1d$^+$ CD5$^-$, CD1d$^+$ CD5$^+$ and CD1d$^-$ CD5$^+$ populations were assessed for IL-10 expression.

Figure 3.11A shows the gating strategy and the flow cytometric analysis performed to identify the frequency of IL-10–producing B cells among the four subsets. Firstly, the CD1d$^-$ CD5$^-$, CD1d$^+$ CD5$^-$, CD1d$^+$ CD5$^+$ and CD1d$^-$ CD5$^+$ populations were identified on the basis of the differential expression of the CD1d and CD5 molecules. Then, for each subset, the percentage of IL-10$^+$ B cells was calculated using a perfectly matched isotype control.

As shown in figure 3.11B, spleen B cells that are competent to express cytoplasmic IL-10 following a 5 h LPIM stimulation were found to be particularly abundant within the CD1d$^+$ CD5$^-$, CD1d$^+$ CD5$^+$ and CD1d$^-$ CD5$^+$ subsets in wild type B6 mice. Among these three populations, the CD1d$^+$ CD5$^+$ was the one most enriched in IL-10$^+$ B cells since 17.1% (± 0.9) of the cells found within this subset produced IL-10. By contrast, IL-10–expressing B cells were significantly less common within the CD1d$^-$ CD5$^-$ B cell population. Actually, the percentage of CD1d$^-$ CD5$^-$ B cells producing IL-10 was 7.5-fold lower than the one of CD1d$^+$ CD5$^+$ B cells expressing this cytokine.

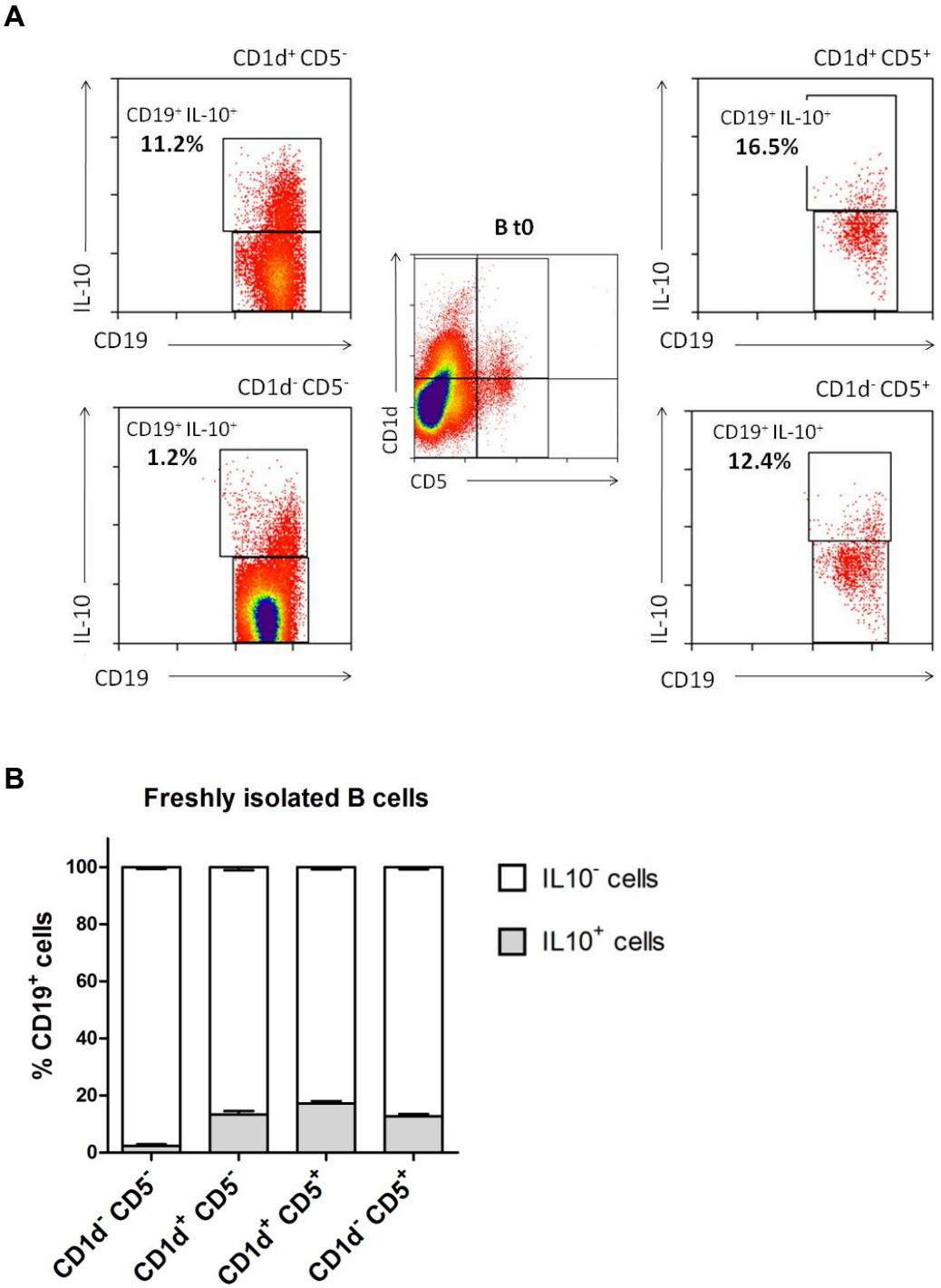


Figure 3.11: Frequencies of IL-10–producing B cells among the CD1d$^-$ CD5$^-$, CD1d$^+$ CD5$^-$, CD1d$^+$ CD5$^+$ and CD1d$^-$ CD5$^+$ subsets of naïve B cells. (***A***) B cells were freshly isolated from mouse spleen and stimulated for 5 h with LPIM in order to induce IL-10 expression in IL-10–competent B cells. The cells were therefore stained using CD19, CD1d, CD5 and IL-10 mAbs and the frequencies of IL-10–producing B cells among the CD1d$^-$ CD5$^-$, CD1d$^+$ CD5$^-$, CD1d$^+$ CD5$^+$ and CD1d$^-$ CD5$^+$ populations were evaluated. The reported dot plots result from the flow cytometric analysis of one representative experiment. (***B***) Bar graphs indicate the average percentages (± SEM) of IL-10$^+$ (gray) and IL-10$^-$ (white) cells in each of the four subsets defined by the differential expression of the CD1d and CD5 molecules. For each subset, the sum of the IL-10$^+$ and IL-10$^-$ cells is equal to 100%. The results are from two independent experiments.

After 48 h of *in vitro* stimulation with either agonistic CD40 mAb or LPS, the CD1d$^-$ CD5$^-$ subset still remained the one with the lowest frequency of IL-10–producing cells whereas, in both cases, the CD1d$^+$ CD5$^-$ was the one most enriched in IL-10$^+$ B cells (Figure 3.12A,B).

For what concerns the CD40–activated B cells, an average of 7.2% (± 0.5) of total CD1d$^-$ CD5$^-$ cells expressed IL-10, which was significantly lower than the 29.4% (± 2.4), 23.1% (± 4.5) and 18.9% (± 4.0) detected respectively for the CD1d$^+$ CD5$^-$, CD1d$^+$ CD5$^+$ and CD1d$^-$ CD5$^+$ populations (Figure 3.12A). Similarly, even in the case of LPS–stimulated B cells, CD1d$^+$ CD5$^-$, CD1d$^+$ CD5$^+$ and CD1d$^-$ CD5$^+$ B cells were more enriched for IL-10–producing cells (respectively 18.5% ± 1.8, 13.9% ± 3.6 and 9.6% ± 3.5) than the CD1d$^-$ CD5$^-$ B cell subset, where only 5.8% (± 0.7) of the total cells produces IL-10 (Figure 3.12B). Figure 3.13 shows the flow cytometric analysis for one representative experiment out of three.

In conclusion, although signals provided through CD40 and TLR4 differentially affected the cell surface phenotype of IL-10$^+$ B cells, in both cases the CD1d$^+$ CD5$^-$, CD1d$^+$ CD5$^+$ and CD1d$^-$ CD5$^+$ populations remained the subsets in which it is possible to observe the higher frequencies of IL-10–expressing B cells.

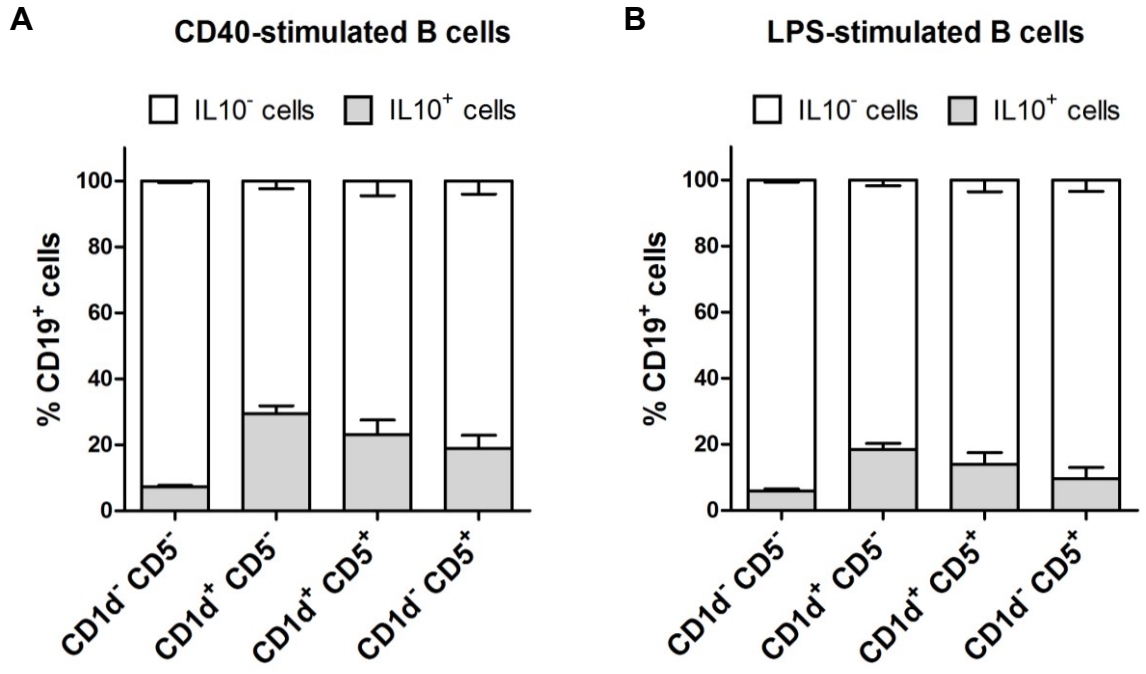


Figure 3.12: Frequencies of IL-10–producing B cells among the CD1d$^-$ CD5$^-$, CD1d$^+$ CD5$^-$, CD1d$^+$ CD5$^+$ and CD1d$^-$ CD5$^+$ subsets of CD40– or LPS–activated B cells. Spleen B cells were cultured for 48h in the presence of either agonistic CD40 mAb (***A***) or LPS (***B***) and stimulated with LPIM for the last 5 h of culture in order to induce IL-10 expression in IL-10–competent B cells. The cells were therefore stained using CD19, CD1d, CD5 and IL-10 mAbs and the frequencies of IL-10–producing B cells among the CD1d$^-$ CD5$^-$, CD1d$^+$ CD5$^-$, CD1d$^+$ CD5$^+$ and CD1d$^-$ CD5$^+$ populations were evaluated. Bar graphs indicate the average percentages (± SEM) of IL-10$^+$ (gray) and IL-10$^-$ (white) cells in each of the four subsets defined by the differential expression of the CD1d and CD5 molecules. For each subset, the sum of the IL-10$^+$ and IL-10$^-$ cells is equal to 100%. The results are from three independent experiments.

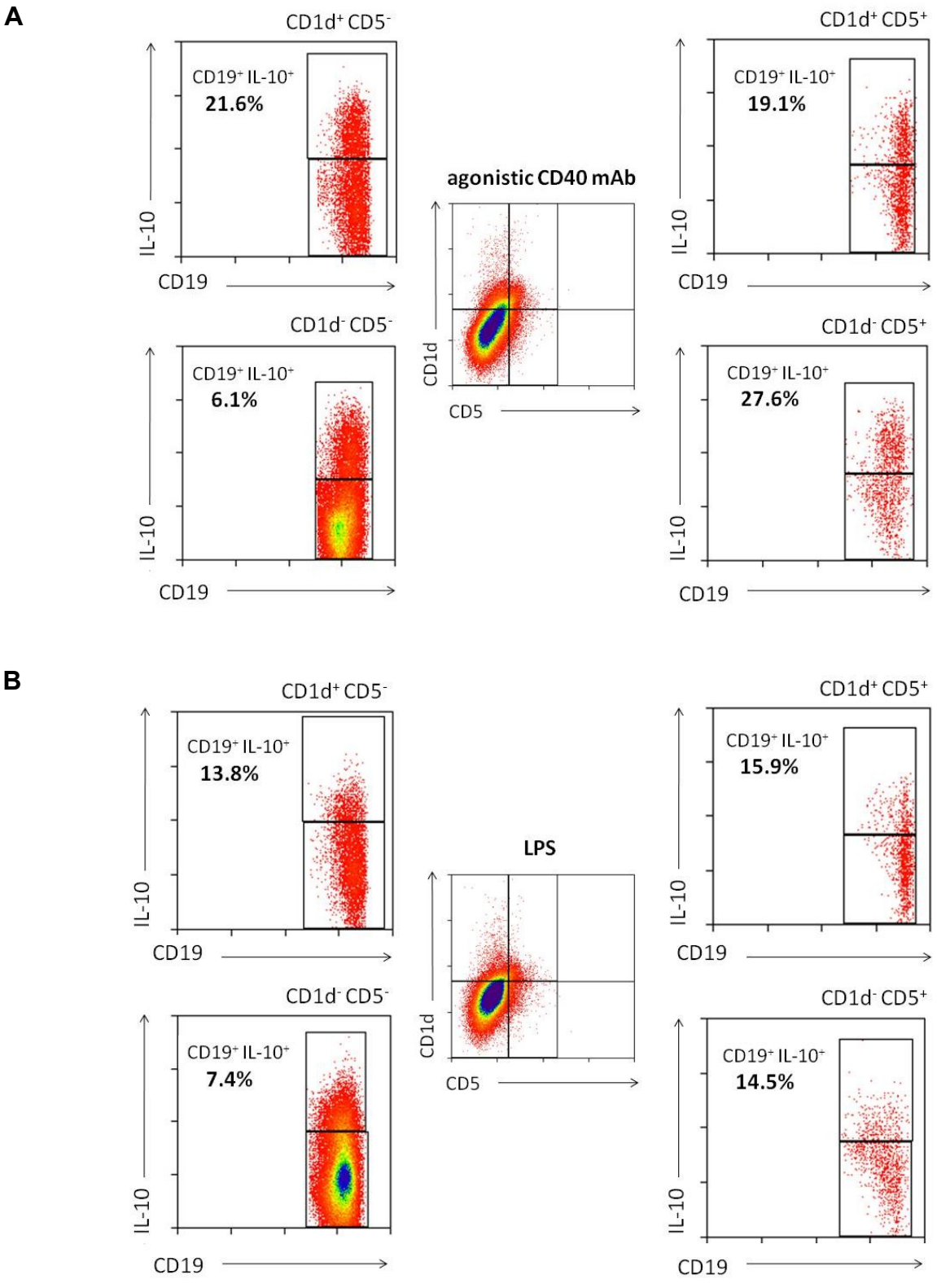


Figure 3.13: Gating strategy and the flow cytometric analysis performed to identify the frequency of IL-10–producing B cells among the CD1d$^-$ CD5$^-$, CD1d$^+$ CD5$^-$, CD1d$^+$ CD5$^+$ and CD1d$^-$ CD5$^+$ subsets of CD40– or LPS–activated B cells. Spleen B cells were cultured for 48h in the presence of either agonistic CD40 mAb (***A***) or LPS (***B***) and stimulated with LPIM for the last 5 h of culture in order to induce IL-10 expression in IL-10–competent B cells. The cells were stained using CD19, CD1d, CD5 and IL-10 mAbs before undergoing flow cytometric analysis. Firstly, the CD1d$^-$ CD5$^-$, CD1d$^+$ CD5$^-$, CD1d$^+$ CD5$^+$ and CD1d$^-$ CD5$^+$ populations were identified on the basis of the differential expression of the CD1d and CD5 molecules. Then, for each subset, the percentage of IL-10$^+$ B cells was calculated using a perfectly matched isotype control.

3.2 Induced regulatory B cells: possible role of mast cells

Most of the studies investigating the origin and differentiation of regulatory B cells are based on the use of mouse models of autoimmune disease and chronic inflammation and on the comparison of the effects of various B-cell stimuli on the *in vitro* expansion of the $CD19^+$ $IL\text{-}10^+$ population (Klinker and Lundy, 2011).
Both in a physiological and pathological context, the stimuli that can affect the development, differentiation and/or expansion of regulatory B cells likely derive from the crosstalk between B lymphocytes and other cells of the immune system. However the possible role of a specific cell type in the generation or expansion of IL-10–producing B cells has never been investigated so far and, therefore, the immune cell types involved in the development and differentiation of regulatory B cells are currently unknown.

Mast cells reside predominantly in tissue exposed to the external environment, including skin, intestinal tract and trachea, where they exert an important function as "sentinels" of tissue and immune homeostasis (Frossi et al., 2004). Furthermore, a growing body of evidence indicates that MCs occupy a critical niche at the interface of innate and acquired immunity and that they can function as both effector and immunoregulatory cells, depending on the immunological context (Galli and Tsai, 2010). For what concerns the immunomodulatory properties of MCs, it has been reported that, through the direct or indirect action of a wide variety of products and surface receptors, these cells are able to affect the recruitment, survival, development, phenotype or function of several immune cells, including B lymphocytes (Galli et al., 2008).

The evidence of a direct B/mast cell interaction (Merluzzi et al., 2010), together with data demonstrating the role of MCs as important regulators of physiological and pathological immune responses, led to the investigation of whether MCs could drive B cell differentiation towards regulatory B cells. Considering that the production of IL-10 is the hallmark of various B cell subsets with regulatory properties, the first point was to address whether MCs were able to promote the expansion of IL-10–competent B cells and/or to induce B-cell IL-10 secretion *in vitro*. Then, a series of experiments were performed in order to phenotypically characterize the IL-10–competent B cells derived from the co-culture with MCs. Finally the role of mast cells in the expansion and/or differentiation of IL-10–competent B cells was evaluated *in vivo*.

3.2.1 B cells can physically interact with MCs

Although the direct interaction between B lymphocytes and mast cells has for long been considered highly likely and important to study (Pawankar et al., 1997; Skokos et al., 2002; Sayed et al., 2008), only few papers have investigated the crosstalk between these two immune cell types. Work done in the laboratory where this thesis was performed has shown that MCs promote both survival and proliferation of naïve B cells, as well as activation and further plasma cell differentiation of activated B cells. B-cell proliferation was reduced when MCs and B cells were separated by a transwell membrane or when MCs were IL-6 deficient, suggesting that both cell-cell contact and soluble factors are necessary for the B-cell proliferation enhancement by MCs (Merluzzi et al., 2010).

To further confirm the occurrence of cellular interactions between MCs and B cells, live cell imaging of the forming contacts between MCs and B cells was carried out by time-lapse bright-field video microscopy. Equal numbers of bone marrow-derived MCs and splenic B cells were plated onto glass bottom Petri dishes and cells behavior was observed every minute for a total of 30 minutes. Under resting conditions the two cell types were typically rounded and easily distinguishable by their distinct morphological features: MCs were large (about 15-20 μm) whereas B cells were smaller (8-10 μm), due to their tiny cytoplasm. As shown in figure 3.14, numerous cell conjugates were effectively observed after only few minutes of co-culture: following cell-cell contact formation, the shape of both cells changed from round to an elongated and/or flattened one, in a very dynamic manner. Individual conjugates showed sequential phases of adhesion, slow lateral movement and stable interaction in different proportions and of different duration. Furthermore, similar observations were recorded in IgE–sensitized MCs-B cells co-cultures, after Ag addition: the time series started after Ag addition and cell behavior was observed every minute for a total of 30 minutes (data not shown).

These results illustrate the formation of cognate interactions between MCs and B cells and, therefore, further confirms the existence of a direct interplay between these two cell types.

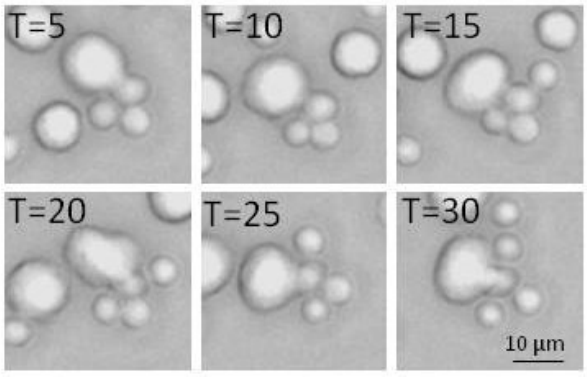


Figure 3.14: **B cells physically interact with MCs**. Representative images of conjugate formation between MCs and B cells are shown. Equal numbers of nonsensitized MCs and B cells were seeded onto glass bottom Petri dishes and conjugate formation was monitored by timelapse video microscopy for 30 min.

3.2.2 IL-10 detection in B cells co-cultured with MCs: gating strategy

The precise identification of IL-10–producing B cells through intracellular staining and consequent flow cytometric analysis is a fundamental requirement for the study of the possible role of MCs in the development of IL-10–competent B cells. Therefore, the same approach used to identify the IL-10$^+$ B cells induced by B-cell stimuli (described in paragraph 3.1.1 and summarized in figure 3.15) was applied also to the B cell-MC co-culture system.

An important aspect to emphasize is that, in the context of the B cell-MC co-culture, this gating strategy allows to simultaneously exclude from the analysis both dead cells and MCs, without the need to add a marker for MCs. The left panel of figure 3.15 shows that, by analyzing the CD19-live/dead double staining it is possible to identify three distinct populations: gate G1 defines the viable B cells and excludes both the dead cells (G2), that are positive for live/dead staining, and viable MCs (G3), that are negative for live/dead and CD19 staining. A representative cell surface CD19 and cytoplasmic IL-10 staining of viable B cells deriving from gate G1 is shown in the upper middle panel of the figure: the reported percentage indicates the frequency of IL-10–producing B cells within the selected gate among total CD19$^+$ B cells.

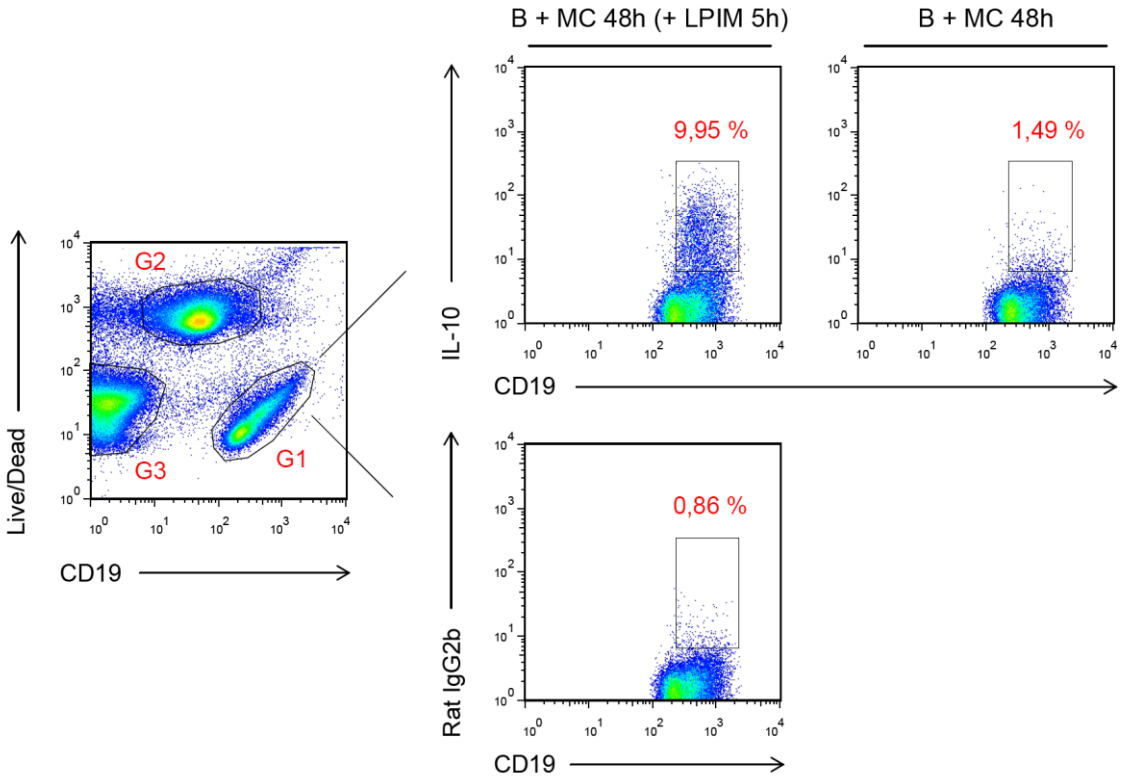


Figure 3.15: Gating strategy and flow cytometric analysis for identifying IL-10$^+$ B cells in the B-MC co-culture. Using the CD19 vs live/dead plot (left panel) it is possible to identify viable B cells (G1) that are positive for CD19 staining but negative for live/dead staining. The cells outside the G1 gate are either dead cells (G2) or viable MCs (G3). The panels on the right show a representative cell surface CD19 and cytoplasmic IL-10 staining by viable B cells (upper middle dot plot). As negative controls, the strategy employed a perfectly matched isotype control (Rat IgG2b) in addition to the use of B cells not stimulated with LPIM.

3.2.3 MCs increase the frequency of IL-10–competent B cells *in vitro*

The interplay between MCs and B cells can affect several aspects of both MC and B cell biology and, therefore, it could also regulate the expansion and/or differentiation of IL-10–competent B cells. In both cases, this would turn into an increase in the percentage of $CD19^+$ $IL\text{-}10^+$ cells. In order to assess whether MCs were able to influence the expansion and/or differentiation of IL-10–competent B cells *in vitro*, a 48 h co-culture assay using murine bone marrow-derived MCs and splenic B cells was performed, in a 1:1 ratio. The $CD19^+$ $IL\text{-}10^+$ population was analyzed in freshly isolated B lymphocytes and in the same B cells after co-culture with either non-sensitized or IgE-Ag–stimulated MCs. In both cases, LPIM was added to the culture medium to enhance *Il-10* expression in IL-10–competent B cells and to block intracellular protein transport and secretion.

As shown in figure 3.16A, following co-culture with MCs, the percentages of $IL\text{-}10^+$ B cells were significantly increased. Furthermore, similarly to what was seen for B cells stimulated with agonistic CD40 mAb (see paragraph 3.1.2), the flow cytometric analysis revealed that the overall intensity of cytoplasmic IL-10 staining in the MC–induced $IL\text{-}10^+$ B cells was high (figure 3.16A, right panels). The co-culture with non-sensitized and IgE-Ag–stimulated MCs induced cytoplasmic IL-10 expression by respectively 8.4% (± 0.9) and 7.1% (± 0.8) of B cells, which was 2.5-3-fold higher than for B lymphocytes that were competent to express cytoplasmic IL-10 following a 5 h LPIM stimulation (2.9% ± 0.2). In addition, although the frequency of $CD19^+$ $IL\text{-}10^+$ cells was always slightly higher when the B cells were co-cultured with non-sensitized MCs rather than with IgE-Ag–stimulated MCs, the observed difference was not significant (Figure 3.16B). These results suggest that MCs are able to increase the frequency of $CD19^+$ $IL\text{-}10^+$ cells, either by promoting the proliferation and/or the survival of existing IL-10–competent B cells or by inducing the differentiation of B lymphocytes into IL-10–competent cells. Moreover, MC-activation by anaphylactic stimuli was not necessary to observe this novel aspect of B/MC crosstalk, supporting the idea that the potential to carry out immunomodulatory functions is a basic property of the mast cell-lineage (Galli et al., 2008).

In a physiological context, it is likely that a single mast cell can interact with more than one B lymphocyte or *vice versa*. For this reason, the co-culture was performed using two different B:MC ratio (3:1 and 1:3) and the frequency of $IL\text{-}10^+$ B cells was analyzed in these different condition. Figure 3.16C reports the result obtained with the 3:1 B:MC ratio and shows that, compared to the IL-10–competent cells found within freshly isolated B lymphocytes, a 2.4-fold increase in the percentage of $CD19^+$ $IL\text{-}10^+$ cells was observed when the co-culture was performed using this different culture condition. A similar result was obtained with the 3:1

B:MC ratio (data not shown). Considering these results together with the one obtained with the 1:1 ratio, it is possible to conclude that MCs promote the expansion and/or differentiation of IL-10–competent B cells, independently from the co-culture ratio.

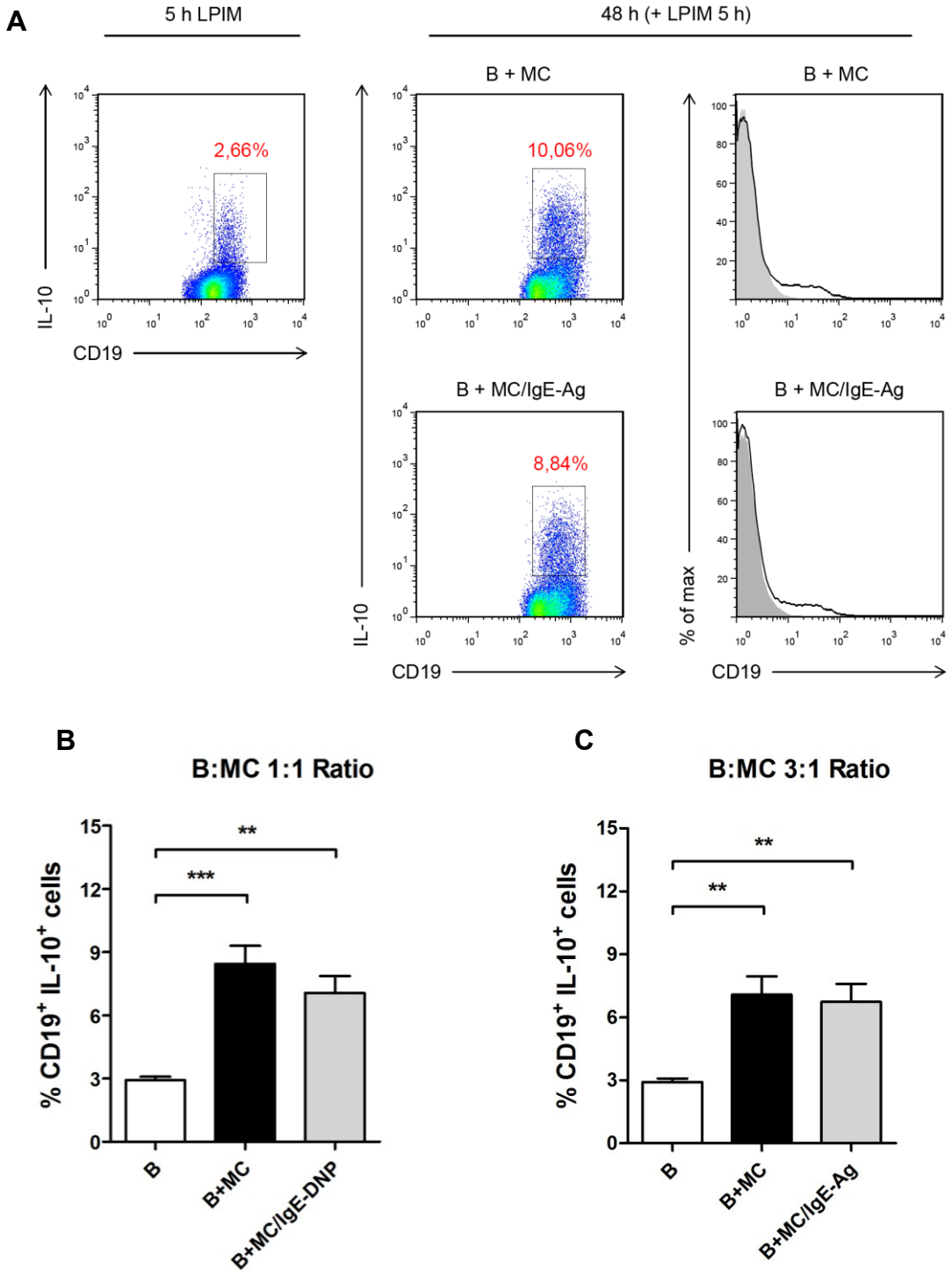


Figure 3.16: MCs promote the expansion of IL-10–competent B cells *in vitro*. (***A)*** B cells were cultured either alone for 5 h with LPIM or for with MCs for 48 h, with LPIM added during the last 5 h of culture. In both cases, cells were permeabilized and stained for IL-10. Dot plots for one representative experiment are shown with indicated the percentages of IL-10^+ cells among total B cells. Bar graphs indicate mean (±SEM) percentages from eight independent experiments, in the case of the 1:1 B:MC co-culture (***B***), and from four experiments, for the 3:1 B:MC co-culture (***C***). The statistical analysis was performed using an unpaired *t* test. Significant differences between sample means are indicated as follows: **, $p< 0.01$; ***, $p< 0.001$.

3.2.4 The CD40-CD40L axis is involved in the induction of IL-10$^+$ B cells by MCs

Following the observation that MCs were able to increase the frequency of IL-10–competent B cells, the mechanism responsible for this phenomenon at the single-cell level was investigated. Besides the capacity to produce soluble factors, MCs can express several co-stimulatory molecules, including members of both the B7 and the TNF/TNFR families (Nakae et al., 2006). Among these, there are several B cell–modulating molecules such as CD153 (CD30L) and CD154 (CD40L). CD154 is the natural ligand of CD40, it is constitutively expressed on the surface of MCs (Figure 3.17A) and its expression is increased by IgE-Ag stimulation (Pawankar et al., 1997; Merluzzi et al., 2010).

Since CD40 ligation is the most potent strategy for inducing *in vitro* B-cell maturation into IL-10–competent cells (Yanaba et al., 2009), the role of the CD40-CD40L axis in the MC–driven expansion and/or differentiation of IL-10$^+$ B cells was investigated. For this purpose, the MC/B cell co-culture experiment was performed in the presence of an anti-CD40L blocking Ab or of its isotype control and the result was that the CD40-CD40L axis played a key role in the MC–induced increase of IL-10$^+$ B cells. As shown in figure 3.17B, the blockade of CD40L on MCs produced a significant decrease in the percentage of the IL-10$^+$ B cells and this reduction was observed both with non-sensitized and IgE-Ag–stimulated MCs (respectively a 1.5 and 1.4 fold decrease). The observed result was exclusively due to the interference with the CD40-CD40L pathway as the same effect was not observed when the co-culture was performed in the presence of the CD40L isotype control Ab.

This result was further validated by a transwell experiment that showed the requirement of cell-cell contact for the increased frequency of IL-10$^+$ B cells after co-culture with MCs. As shown in figure 3.17C, when the two cell populations were physically separated by a membrane filter there was a partial, although significant, decrease in the percentage of IL-10–producing B cells.

These findings reveal that the CD40-CD40L axis played an important role in the MC–driven expansion and/or maturation of IL-10–competent B cells. These do not exclude the possibility that other interactions or soluble factors might significantly contribute to the increase in the frequency of IL-10$^+$ cells induced by MCs.

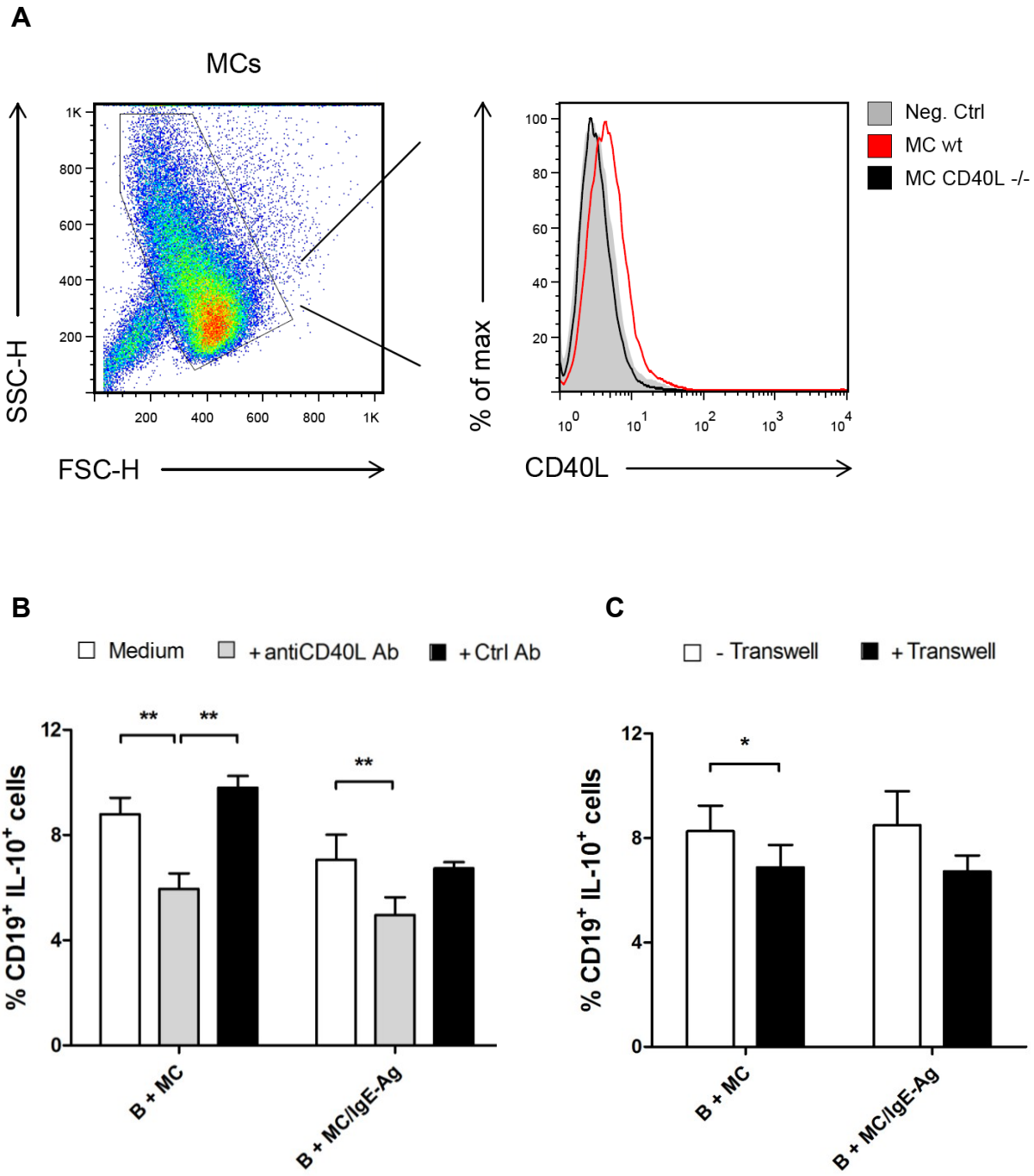


Figure 3.17: The CD40-CD40L axis is involved in the induction of IL-10$^+$ B cells by MCs. (***A***) Surface expression of CD40L was analyzed by flow cytometry on wild type (red histogram) and CD40L-/- (black histogram) bone marrow MCs. A representative histogram is shown here for three independent experiments. The filled histogram (gray) indicate the negative control that is the wild type MCs not stained with the anti-CD40L Ab. (***B***) B cells were cultured for 48 h with either IgE-sensitized or non-sensitized MCs in the presence (gray bar) or absence (white bar) of an anti-CD40L blocking Ab. As a control, the same co-culture experiment was performed in the presence of the CD40L isotype control antibody mouse IgG (black bar). (***C***) The same co-culture experiment was performed in the presence (black bar) or absence (white bar) of a transwell polycarbonate membrane with pores of 0,4-µm that allow the passage of soluble factors, but not of cells. (***B, C***) Bar graphs indicate mean (±SEM) percentages from four independent experiments. The statistical analysis was performed using a paired *t* test. Significant differences between sample means are indicated as follows: *, p< 0.05; **, p< 0.01.

3.2.5 MCs generated signals do not promote IL-10 secretion

The hallmark of regulatory B cell suppressive function is their capacity to secrete the regulatory cytokine IL-10. Therefore, since MCs were shown to increase the frequency of IL-10–competent B cells, either by acting on their expansion or differentiation, the possibility that they could also regulate the process of IL-10 secretion was evaluated.

In order to investigate this aspect, MCs and B cells were cultured either alone or together for 48 h; then culture supernatants were collected and IL-10 concentration was measured by ELISA assay. In this experimental setting, unstimulated B cells released low, although detectable, levels of IL-10 (113.2 ± 22.6 pg/ml). This basal secretion of IL-10 was not affected by the presence of non-sensitized MCs (Figure 3.18A) as similar concentrations of the cytokine were measured in the co-culture supernatant (109.9 ± 30.0 pg/ml). In the presence of IgE-Ag–activated MCs, IL-10 levels were slightly higher (165.8 ± 49.7) but the observed difference was not statistically significant (Figure 3.18B). These results lead to the conclusion that in the B/MC co-culture system IL-10 competence and secretion are independently regulated.

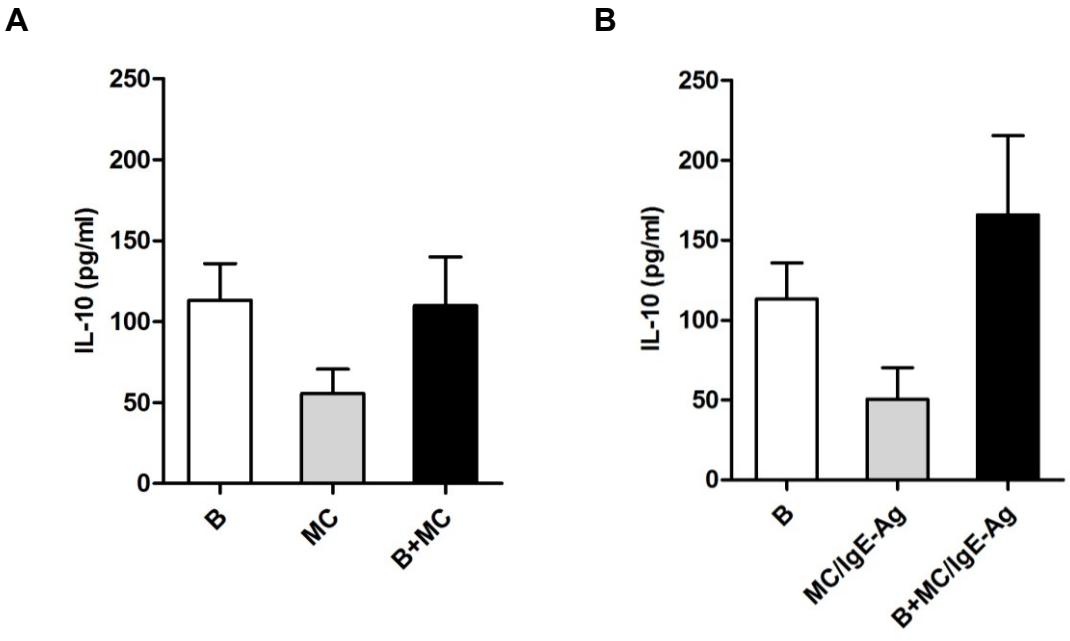


Figure 3.18: MCs do not promote IL-10 secretion by B cells. (***A***) For measuring secreted IL-10, culture supernatant fluids from B cells, MCs or B+MCs were harvested after 48 h of culture and IL-10 concentrations were determined by ELISA. (***B***) The same experiment was performed using IgE-Ag–stimulated MCs. Bar graphs indicate mean (± SEM) concentrations of four independent experiments. The statistical analysis was performed using a paired *t* test.

3.2.6 Supernatants of the B/MC co-cultures do not suppress effector T cell proliferation

Although IL-10 was not detected at increased levels in the supernatants of the B/MC co-cultures, the conditioned media was tested in order to determine whether other substances produced by the crosstalk between these two cell types were able to suppress T cell proliferation. With this purpose, B cells were cultured for 48 h either alone or with non-sensitized or IgE-Ag–stimulated MCs and conditioned media were prepared by harvesting cell-supernatants. Furthermore, the Teff cells were purified from mouse spleen, labeled with CFSE and cultured for 72 h in the presence of both T-cell mitogens and conditioned media from B-cell, MC or B/MC cultures. Figure 3.19 reports the results relative to the co-culture between B cell and non-sensitized MCs. The single-color histograms, relative to CFSE fluorescence intensity, show how T cells activated with anti-CD3 and anti-CD28 mAb progressed through generations while the same T cells did not proliferate when unstimulated (Figure 3.19A). Activated $CD4^+$ $CD25^-$ cells cultured with the supernatants of the B/MC co-culture proliferated as well as the same T cells cultured in the presence of only T-cell mitogens (positive control) or with control supernatants (from B cells or MC cells alone), indicating the absence of a suppressive cytokine in the co-culture system. This result was further confirmed by analyzing the T-cell proliferation index. As shown in figure 3.19B, no significant differences in the proliferation index were observed between the positive control (2.3 ± 0.1) and the $CD4^+$ $CD25^-$ cells cultured with the supernatants of the B/MC co-culture (2.2 ± 0.1). Similar results were obtained using the B/MC IgE-Ag conditioned media.

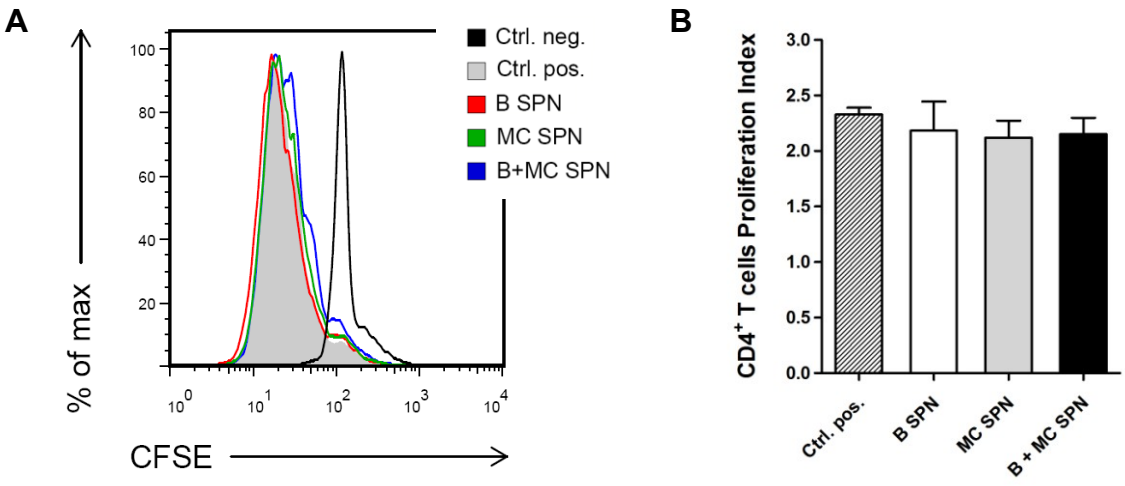


Figure 3.19: Supernatants derived from B/MC co-cultures do not suppress effector T cell proliferation. (***A***) CFSE-labeled $CD4^+CD25^-$ T cells were incubated for 72 h either alone (Ctrl. neg.) or with anti-CD3 and anti-CD28 mAbs, in the presence of allogeneic irradiated APCs (Ctrl. pos.). Cultures additionally received medium (controls), supernatants from unstimulated B cells (B SPN), unstimulated MCs (MC SPN) or B/MC co-cultures (B+MC SPN). CFSE profiles of effector T cell proliferation in the different conditions are shown for one representative experiment. (***B***) Bar graphs indicate mean (± SEM) of the T cell proliferation index from four independent experiments. The statistical analysis was performed using a paired *t* test.

3.2.7 MC–generated IL-10$^+$ B cells: importance of the CD1d and CD5 markers

As previously indicated, MC–generated signals were able to induce the expansion of the spleen IL-10$^+$ B-cell population. MCs could either promote the proliferation and/or survival of existing IL-10–competent B cells or drive the differentiation of B lymphocytes towards IL-10–competent cells. Either way, it still needs to be understood if, in this context, the signals provided by MCs could act indiscriminately on all the B cells of the spleen or preferentially affect a specific B-cell subset. For this reason, an extensive study focused on the MC–induced IL-10–competent B cells and on the expression of the CD1d and CD5 surface molecules was undertaken. As already explained in detail in paragraph 3.1.7, the choice of studying the IL-10$^+$ B-cell population in relation to the CD1d and CD5 markers was due to the fact that these markers are common to all the regulatory B-cell subsets described in literature.

- ***Role of MC–generated signals on the expression of the CD1d and CD5 molecules on the B-cell surface***

First of all, the possibility that MC–generated signals could affect the expression of the CD1d and CD5 molecules on the B cell surface was assessed. Splenic B lymphocytes were cultured for 48 h with either nonsensitized or IgE-Ag–stimulated MCs and the expression of these molecules on B cells was determined, before and after co-culture with MCs, by flow cytometry.

Total B cells obtained from mouse spleen expressed high amounts of cell-surface CD1d while CD5 was present at low levels. In addition, the overall frequency of the CD1d^{hi} CD5$^+$ population represented 1.5-3% of the purified spleen B cells. As shown in figure 3.20A, the CD1d^{hi} CD5$^+$ subset was not affected by the presence of MCs as the percentage of this population remained almost the same. Nevertheless, the counter plots revealed a change in the expression of the two surface markers that was best appreciated analyzing the fluorescence intensity data. After co-culture with both nonsensitized and IgE-Ag–stimulated MCs a small number of B cells showed a markedly enhanced expression of CD5 on their surface (Figure 3.20B); by contrast, B-cell CD1d expression was slightly reduced (Figure 3.20C). This result was confirmed by the analysis of the mean fluorescence intensity (MFI) of the two molecules (Figure 3.20D,E). Compared to freshly isolated B lymphocytes, CD5 expression was significantly increased in B cells co-cultured with MCs, independently from the MC-activation status. On the contrary, CD1d expression was significantly reduced. These results prompt the suggestion that MCs could regulate the expansion and/or differentiation of IL-10–competent B cells acting on CD5$^+$ cells or inducing the expression of this marker on CD5$^-$ B cells.

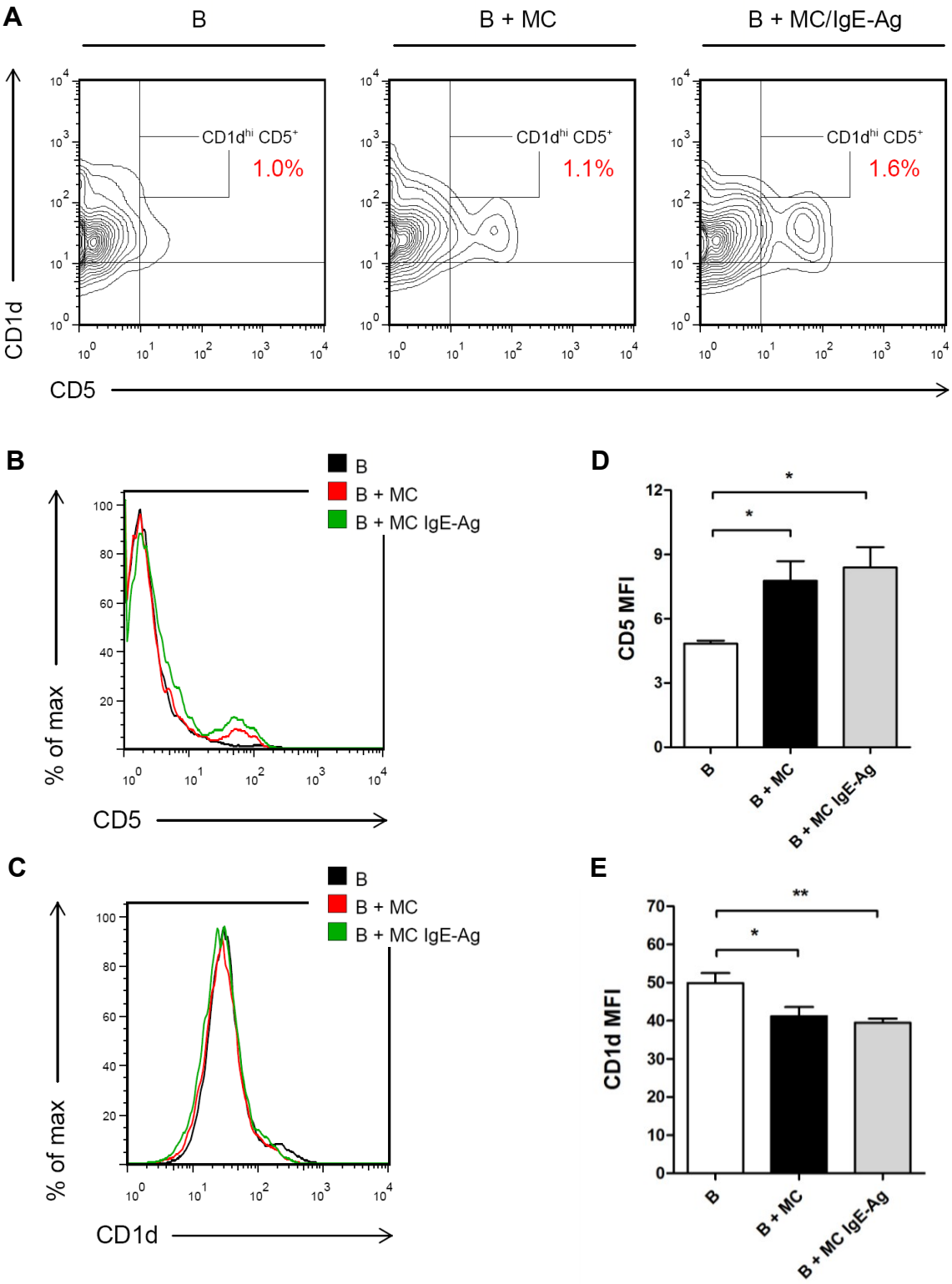


Figure 3.20: Role of MC–generated signals on the expression of the CD1d and CD5 molecules on the B-cell surface. (***A***) Spleen B cells were freshly isolated or cultured for 48 h with either nonsensitized or IgE-Ag–stimulated MCs. Subsequently, the cells were stained using CD19, CD1d, CD5 mAbs and cell surface CD1d and CD5 expression by $CD19^+$ cells was determined by immunofluorescence staining. The counter plots are representative of results obtained from five independent experiments. Values indicate the percentage of $CD1d^{high}$ $CD5^+$ B cells among total B cells, within the indicated gates. Single-color histograms in which cell numbers (y-axis) are plotted against log fluorescence intensity of CD5 (x-axis) (***B***) or CD1d (***C***) are also reported. The bar plots report the mean fluorescence intensity (MFI) of CD5 (***D***) and CD1d (***E***) expression and the reported results show means (± SEM) from four independent experiments. Significant differences between sample means are indicated, as follows: *, $p< 0.05$; **, $p< 0.01$. The statistical analysis was performed using an unpaired *t* test.

- ***Phenotypic characterization of the MC–induced IL-10$^+$ B cells***

The next step in order to shed light on the origin of MC–induced IL-10$^+$ B cells was the phenotypic characterization of these cells.

Purified spleen B cells were cultured for 48 h with either non-sensitized or IgE-Ag–activated MCs and stimulated for 5 h with LPIM, in order to enhance *Il-10* expression in IL-10–competent B cells. Following permeabilization and staining with CD19, CD1d, CD5 and IL-10 mAbs, flow cytometric analysis was performed and MCs were excluded from data analysis by gating on CD19$^+$ cells. Finally, B cells within both the IL-10$^+$ and IL-10$^-$ gates were analyzed for the expression of the CD1d and CD5 molecules (Figure 3.21A, dot plots).

The IL-10–competent B cells generated from the co-culture with nonsensitized MCs were found within all the four subsets defined by the differential expression of these two markers, although the highest percentage (51.1% ± 3.1) of these IL-10$^+$ expressed high levels of CD1d and low levels of CD5, thus resembling the phenotype of MZ or T2-MZP B cells. As expected, only a minor part of total IL-10$^+$ B cells presented a CD1d$^+$ CD5$^+$ (8.6% ± 1.0) or a CD1d$^-$ CD5$^+$ (4.8% ± 1.1) phenotype. On the contrary, the majority of IL-10$^-$ cells (83.1% ± 2.6) expressed low levels of both CD1d and CD5 and therefore displayed a follicular-like phenotype (Figure 3.21A, histograms). The reported data refer to the B/MC co-culture but the same experimental procedure was also followed to characterize the immunophenotype of the IL-10–competent B cells found after co-culture with IgE-Ag–stimulated MCs. Even in this case the majority of IL-10$^+$ expressed a surface phenotype that could be attributed to MZ or T2-MZP B cells, since high levels of CD1d and low levels of CD5 were detected (data not shown). Therefore, the majority of IL-10–competent B cells obtained after co-culture with MCs displayed a CD1d$^+$ CD5$^-$ phenotype, independently from the MC-activation status.

In order to investigate whether the specific immunophenotype observed for MC–induced IL-10–competent B cells was due to a change in the expression of the two surface molecules, the immunophenotype of the IL-10$^+$ B cells obtained after culture with non-sensitized MCs was compared to the one of the IL-10$^+$ B cells normally found within the spleen of adult mice (Figure 3.21B). As already reported in paragraph 3.1.7, the CD1d$^+$ CD5$^-$ and the CD1d$^-$ CD5$^-$ phenotypes were the more represented among total IL-10$^+$ cells identified in the freshly purified B cell population. The IL-10$^+$ B cells resulting from the B/MC co-culture presented a similar phenotype since the majority of these cells displayed either a FO– or MZ/T2-MZP–like phenotype. Although none of the observed differences were statistically significant, a higher percentage of MC–induced IL-10$^+$ B cells expressed the CD1d$^+$ CD5$^-$ and CD1d$^+$ CD5$^+$ phenotypes. An analogous result was obtained also from the comparison of the

immunophenotypes of the IL-10$^-$ B-cell population in freshly purified B cells or in B cells co-cultured with MCs.

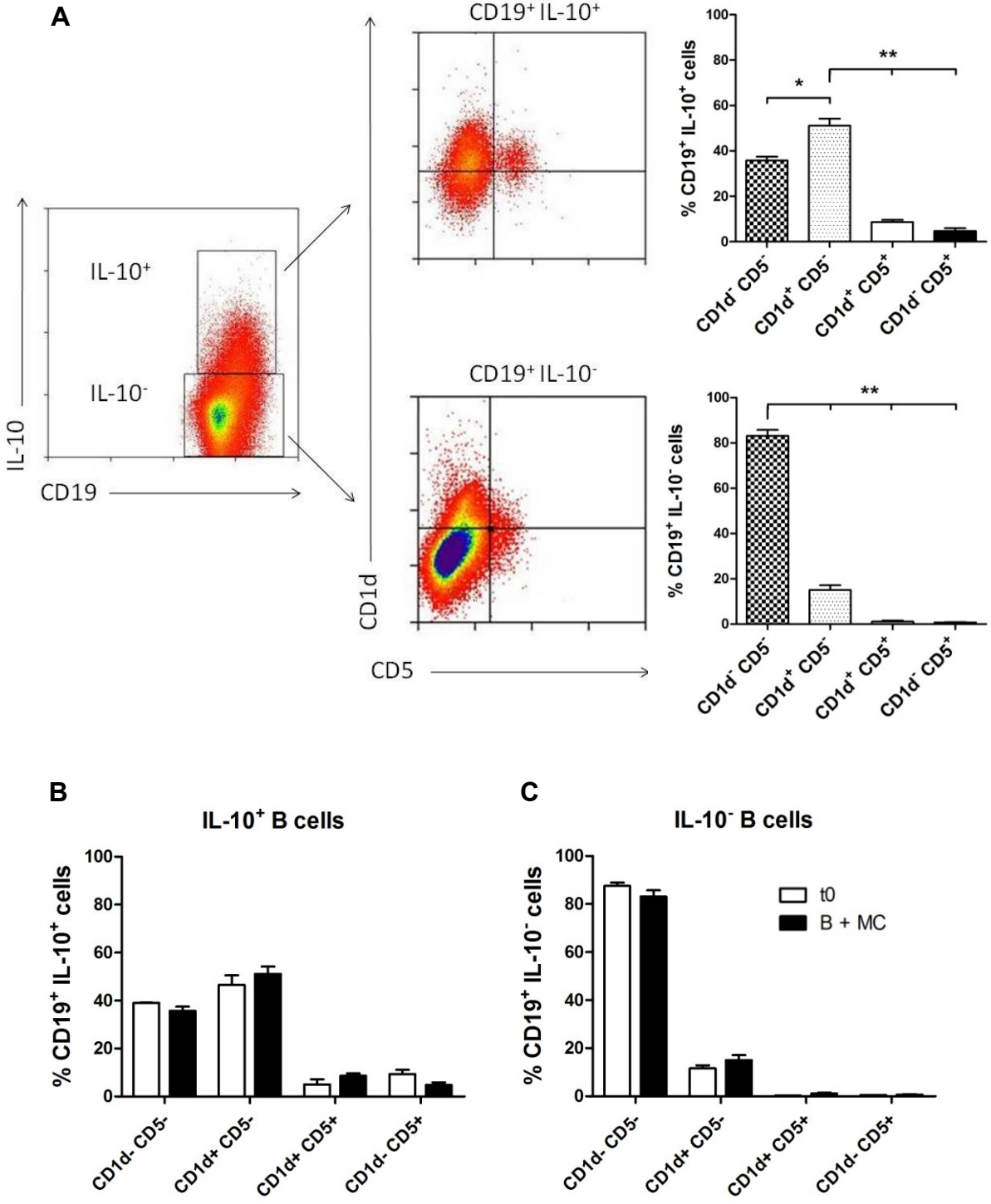


Figure 3.21: Immunophenotype of IL-10$^+$ B cells induced by MC–generated signals. (***A***) Spleen B cells were cultured for 48 h with nonsensitized MCs and stained using CD19, CD1d, CD5 and IL-10 mAbs. The left panels of the figure show the flow cytometric analysis performed to study the expression of the CD1d and CD5 surface molecules on the IL-10$^+$ and IL-10$^-$ cells. The histograms reported on the right side of the figure show the average percentages (± SEM) of IL-10$^+$ or IL-10$^-$ cells that display a CD1d$^-$ CD5$^-$, CD1d$^+$ CD5$^-$, CD1d$^+$ CD5$^+$ or CD1d$^-$ CD5$^+$ phenotype, from four independent experiments. The distribution of total IL-10$^+$ (***B***) or IL-10$^-$ (***C***) B cells among the four subsets defined by CD1d and CD5 was assessed before and after culture of B cells with nonsensitized MCs. Significant differences between sample means are indicated, as follows: *, $p< 0.05$; **, $p< 0.01$. The statistical analysis was performed using an unpaired *t* test.

- ***Frequency of MC–induced IL-10$^+$ B cells among the CD1d$^-$ CD5$^-$, CD1d$^+$ CD5$^-$, CD1d$^+$ CD5$^+$ and CD1d$^-$ CD5$^+$ subsets***

Finally, the frequency of the MC–induced IL-10$^+$ B cells within the CD1d$^-$ CD5$^-$, CD1d$^+$ CD5$^-$ CD1d$^+$ CD5$^+$ and CD1d$^-$ CD5$^+$ was evaluated, in order to identify which are the subsets more enriched for IL-10–producing cells. Figure 3.22A shows the gating strategy and the flow cytometric analysis performed to identify the frequency of IL-10–producing B cells among the four subsets. Firstly, MCs were excluded from data analysis by gating on CD19$^+$ cells and the CD1d$^-$ CD5$^-$, CD1d$^+$ CD5$^-$, CD1d$^+$ CD5$^+$ and CD1d$^-$ CD5$^+$ B-cell populations were identified on the basis of the differential expression of the CD1d and CD5 molecules. Then, for each subset, the percentage of IL-10$^+$ B cells was calculated using a perfectly matched isotype control.

As shown in figure 3.22B, MC–induced IL-10$^+$ B cells were found to be particularly abundant within the CD1d$^+$ CD5$^-$, CD1d$^+$ CD5$^+$ and CD1d$^-$ CD5$^+$ subsets while they were significantly less common within the CD1d$^-$ CD5$^-$ B cell population. As a matter of fact, the percentage of CD1d$^-$ CD5$^-$ B cells producing IL-10 was from 4 to 5-fold lower than the percentage of CD1d$^+$ CD5$^-$, CD1d$^+$ CD5$^+$ and CD1d$^-$ CD5$^+$ expressing this cytokine.

The CD1d$^+$ CD5$^+$ population was the one most enriched in IL-10$^+$ B cells since 13.2% (± 1.2) of the cells found within this subset produced IL-10. It is important to emphasize that, although the double positive population was the one that presented the highest frequency of IL-10$^+$ cells, only 8.6% (± 1.0) of the MC–induced CD19$^+$ IL-10$^+$ cells presented a CD1d$^+$ CD5$^+$ phenotype. This result is in accordance with several recent papers showing that, although strongly enriched for IL-10 expression, the CD1d$^+$ CD5$^+$ population is very small and actually accounts for less than 25% of all IL-10$^+$ B cells in spleen (Ding et al., 2011; Klinker and Lundy, 2011).

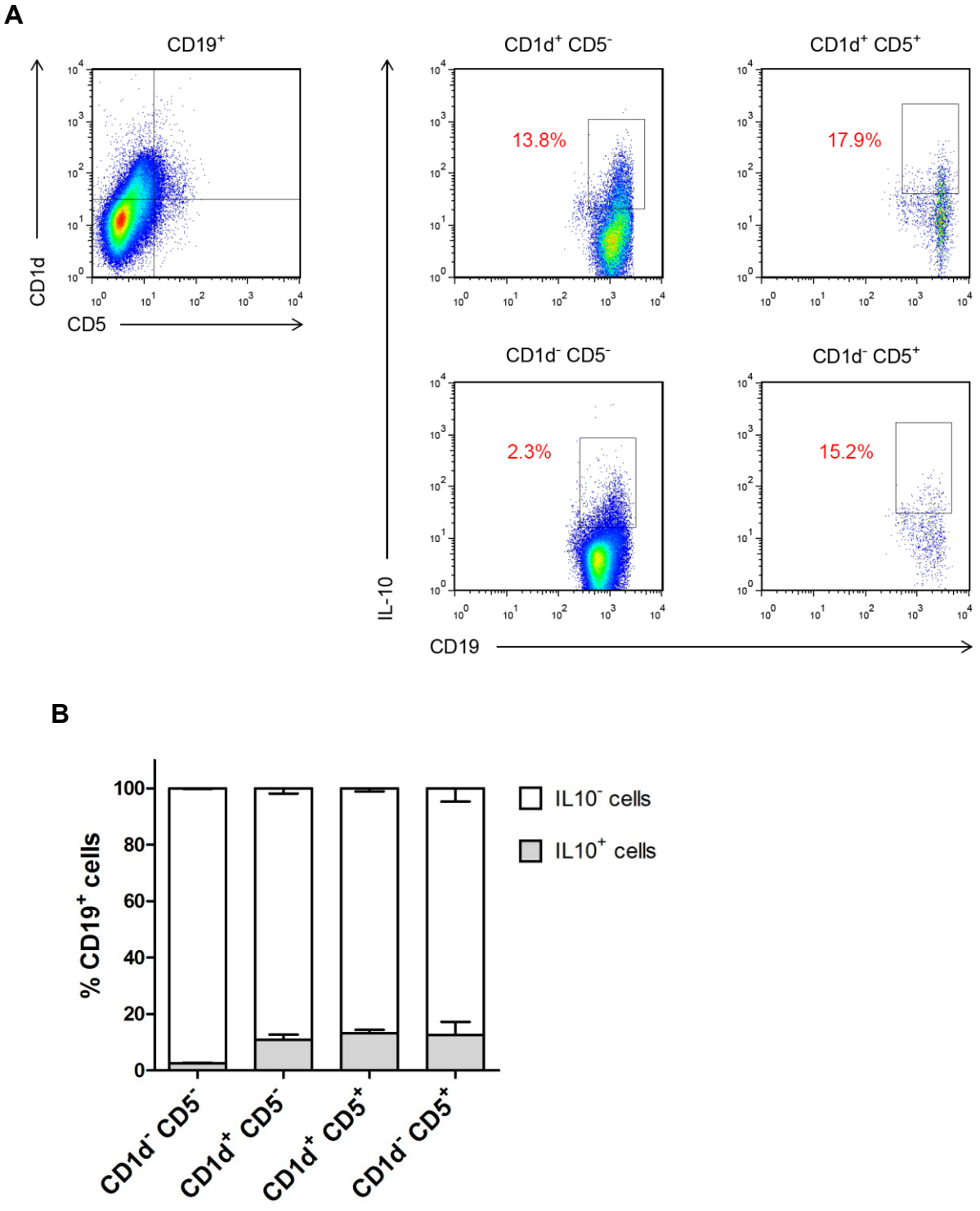


Figure 3.22: The CD1d$^+$ CD5$^+$ subset is the most enriched in MC–induced CD19$^+$ IL-10$^+$ cells. (***A***) Spleen B cells were cultured for 48h, in the presence of nonsensitized MCs, and stimulated with LPIM for the last 5 h of culture in order to induce IL-10 expression in IL-10–competent B cells. The cells were stained using CD19, CD1d, CD5 and IL-10 mAbs before undergoing flow cytometric analysis. MCs were excluded from data analysis by gating on CD19$^+$ cells and the CD1d$^-$ CD5$^-$, CD1d$^+$ CD5$^-$, CD1d$^+$ CD5$^+$ and CD1d$^-$ CD5$^+$ populations were identified on the basis of the differential expression of the CD1d and CD5 molecules. Then, for each subset, the percentage of IL-10$^+$ B cells was calculated using a perfectly matched isotype control. (***B***) Bar graphs indicate the average percentages (± SEM) of IL-10$^+$ (gray) and IL-10$^-$ (white) cells in each of the four subsets defined by the differential expression of the CD1d and CD5 molecules. For each subset, the sum of the IL-10$^+$ and IL-10$^-$ cells is equal to 100%. The results are from four independent experiments.

3.2.8 MCs induce IL-10–competent B cell differentiation *in vivo*

The above-presented results revealed that MCs were able to induce the expansion and/or differentiation of IL-10–competent B cells *in vitro*. In order to assess whether MCs were able to exert this function also *in vivo*, the frequency of $CD19^+$ $IL\text{-}10^+$ cells was evaluated in a recently characterized murine model of MCs deficiency, the *C57BL/6-Kit*$^{W\text{-}sh/W\text{-}sh}$ (*Kit*$^{W\text{-}sh}$) mouse.

Kit$^{W\text{-}sh}$ mice carry an inversion mutation upstream of the *c-Kit* promoter region which results in a reduced c-kit receptor function (Nautiyal et al., 2009). The reduction in c-kit expression on stem cells in the bone marrow inhibits the differentiation of myeloid progenitors into mast cell precursors and this results in MC-deficiency in multiple anatomical sites of adult *Kit*$^{W\text{-}sh}$ mice (Wolters et al., 2005). Therefore, the *Kit*$^{W\text{-}sh}$ mouse was considered an interesting model to study the direct or indirect role of MCs in IL-10–competent B cell differentiation *in vivo*.

The IL-10–competent B-cell population was analyzed in the spleen, lymph nodes, peritoneal cavity and bone marrow of C57BL/6 (B6) and *Kit*$^{W\text{-}sh}$ mice directly by intracellular cytokine staining after LPIM stimulation for 5 h. In both mice, IL-10–producing B cells were identified in all the four organs: $IL\text{-}10^+$ B cells were predominantly located within the spleen and peritoneal cavity while bone marrow and lymph nodes presented lower frequencies of these cells. Interestingly, in three of the four organs considered, the *Kit*$^{W\text{-}sh}$ mice had a lower percentage of IL-10–producing B cells than the B6 mice (Figure 3.23). In particular, the observed differences were more pronounced and statistically significant in peritoneal cavity and lymph nodes, where the frequencies of IL-10–producing B cells were respectively 1.7 and 1.6-fold higher in the wt mice than in the MC–deficient mice. In the bone marrow, the percentage of $IL\text{-}10^+$ B cells was 1.5-fold higher in B6 mice than in *Kit*$^{W\text{-}sh}$ mice, however this difference was not statistically significant. In the spleen compartment, the differentiation of the IL-10–competent B cell population seemed not to be affected by the absence of MCs as no differences were observed in the percentages of $IL\text{-}10^+$ B cells of B6 and *Kit*$^{W\text{-}sh}$ mice. This result could be explained by the fact that the role of MCs in IL-10–competent B cell differentiation is contextual to the considered organ as it could depend both on the particular function that these cells play in that tissue and on the specific resident and recirculating populations found within it.

Compared to other available strains of mast cell–deficient mice, the *Kit*$^{W\text{-}sh}$ model is experimentally advantageous because of its background strain and fewer developmental abnormalities; moreover, these animals are fertile and not anemic (Grimbaldeston et al., 2005). Nevertheless, even the *Kit*$^{W\text{-}sh}$ mice bear abnormalities beyond the mast cell lineage: they

exhibit a marked expansion of immature myeloid cells in spleen and marrow as well as an elevated number of circulating neutrophils and platelets (Nigrovic et al., 2008). To test whether the observed differences in the frequency of $CD19^+$ IL-10^+ cells between B6 and $Kit^{W\text{-}sh}$ mice were truly due to the absence of MCs rather than to other *c-Kit* related alterations, the same experiment was performed in $Kit^{W\text{-}sh}$ mice in which the MC population was reconstituted by injecting *in vitro* differentiated BMMCs, obtained from B6 mice. BMMCs were intraperitoneally (i.p.) transferred into 4-6-weeks-old female $Kit^{W\text{-}sh}$ and the experiments with these mice were initiated 6–8 weeks after the adoptive transfer of the cells. As shown in the bar plots reported in figure 3.23, the decrease in the frequency of IL-10^+ B cells was corrected in all the analyzed tissues of BMMC-reconstituted $Kit^{W\text{-}sh}$ mice except in the peritoneal cavity where the percentage of IL-10^+ B cells is significantly lower than in B6 mice. This result might be explained by the fact that the peritoneal cavity, unlike spleen and lymph nodes, is mainly populated by B-1 B cells which are self-renewing and, therefore, reconstituted by division of fully mature B-1 cells. Therefore, in the peritoneum of $Kit^{W\text{-}sh}$ mice, MC reconstitution might not be sufficient to restore the frequency of IL-10^+ B cells observed in wild type mice as the B cells may already have been committed towards a particular phenotype.

All together these results strengthened the hypothesis, resulting from the *in vitro* experiments, that MCs are important regulators of IL-10–competent B cell differentiation. However, the *in vivo* experiments showed that the role of MCs on IL-10–competent B cell differentiation may vary depending on the analyzed tissue and on the type of B cells that are present in that specific context.

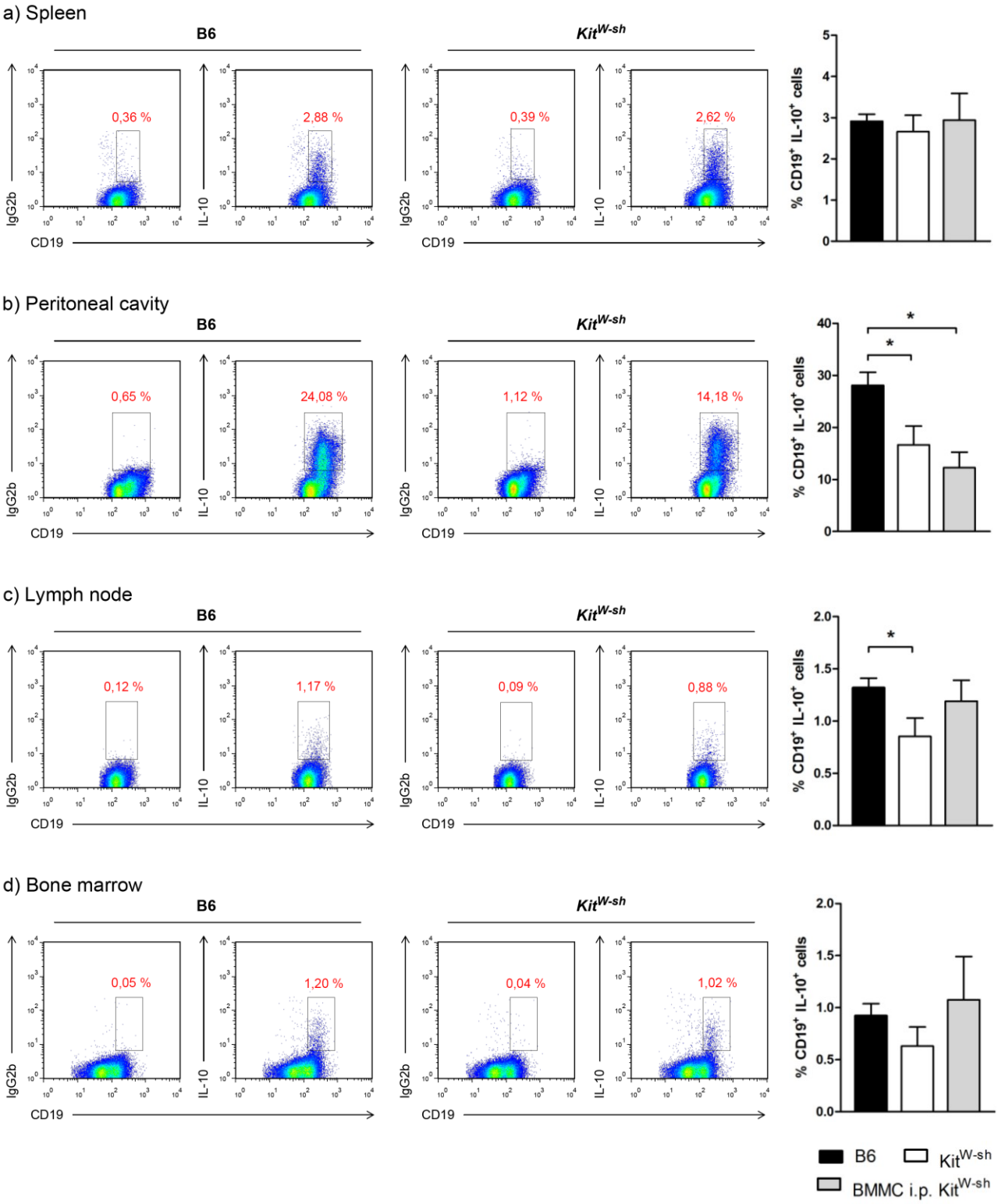


Figure 3.23: **IL-10 production by B cells from B6 and *Kit*$^{W\text{-}sh}$ mice.** Cells derived from spleen (a), peritoneal cavity (b), lymph nodes (c) and bone marrow (d) were cultured with LPS, PMA, ionomycin and monensin for 5 h. The isolated cells were first stained with the Live/Dead green fluorescent probe and with CD19 mAb and, after permeabilization, with IL-10 mAb. CD19 and Live/Dead staining were used to draw the initial gate for identifying viable B cells. The dot plots from one representative experiment show frequencies of IL-10-producing cells among total B cells within the indicated gates. Bar graphs indicate mean (± SEM) percentages of IL-10^{+} B cells from five independent experiments. In this case, the data derived from the intraperitoneally reconstituted *Kit*$^{W\text{-}sh}$ mice, was also plotted. Significant differences between sample means are indicated as follows: *, $p < 0.05$.

3.2.9 The role of MCs in IL-10–competent B cell differentiation is maintained in a pathological context

The above described findings reveal a novel and intriguing role of MCs in the regulation of IL-10–competent B-cell differentiation *in vivo*. Several papers have reported that the frequency of the IL-10–producing B-cell population undergoes variations in the context of chronic inflammation that takes place in autoimmune disease or in tumor (DiLillo et al., 2010). This outcome could be due to a modification of the specific inductive functions of the immune cell types that, directly or indirectly, regulate IL-10–competent B-cell differentiation.

In order to investigate whether the role of MCs in IL-10–competent B-cell differentiation changed in a pathological setting, the frequency of IL-10–poducing B cells was analyzed in B6 and $Kit^{W\text{-}sh}$ tumor-bearing mice. The choice of the tumor context was due to several reasons. Firstly, it is now becoming increasingly evident that, as important immune regulators, these cells are likely to be involved also in regulating anti-tumor immunity. Regulatory B cells were shown to repress anti-tumor immunity during DMBA/TPA-induced squamous carcinogenesis (Schioppa et al., 2011) and to be potent negative regulators of lymphoma depletion during CD20 immunotherapy (Horikawa et al., 2011). Moreover, there are several evidences demonstrating MC–contribution to tumor development (Huang et al., 2008; Heneberg, 2011). Increased number of MCs have been reported, among the others, in B-cell neoplasms including Hodgkin's lymphoma, diffuse large B-cell lymphoma, lymphoplasmacytic lymphoma, and chronic lymphocytic leukemia (Fukushima et al., 2006; Hedstrom et al., 2007; Galinsky and Nechushtan, 2008).

For these experiments, an inflammation–related mouse colon carcinogenesis model induced by azoxymethane (AOM) and DSS was chosen. The experimental procedure adopted is a modification of a protocol already described in literature (Suzuki et al., 2004). Mice were given a single intraperitoneal administration (10 mg/kg body weight) of the genotoxic colonic carcinogen AOM and further underwent 3 cycles of a 1-week oral exposure (2% in drinking water) to the non-genotoxic carcinogen DSS plus 2 weeks of normal drinking water. Mice were sacrificed 90 days after the administration of AOM since, at this time point, both B6 and $Kit^{W\text{-}sh}$ mice developed detectable tumors. Spleen, lymph nodes and peritoneal fluid were collected and IL-10 production by isolated B cells was verified directly by intracellular cytokine staining after LPIM stimulation for 5 h. In tumor–bearing mice, the absence of MCs significantly affected the differentiation of IL-10–competent B cells in the peritoneal cavity and lymph nodes, as the percentages of IL-10^{+} B cells were respectively 1.9 and 1.6-fold higher in the wild type than in the MC–deficient mice (Figure 3.24). It is worth noting the

result obtained in the spleen since, unlike to what observed in the other organs and in the spleen of healthy mice, the *Kit*$^{W\text{-}sh}$ tumor–bearing mice had a higher frequency of IL-10^{+} B cells (4.1% ± 0.6) than the B6 mice (3.3% ± 0.4). A possible explanation of this result could be related to the MC-ability to mobilize immunosuppressive cells to the tumor site. Yang and coworkers have recently demonstrated that mast cells mobilize myeloid-derived suppressor cells and Treg cells in tumor microenvironment in a murine hepatocarcinoma model (Yang et al., 2010b). In the wild type mice, MCs might contribute to the recruitment of IL-10–competent B cells with suppressive capability in the tumor microenvironment where they might sustain tumor tolerance. In the *Kit*$^{W\text{-}sh}$ mice, the absence of MCs could affect the homing of IL-10–producing B cells to the tumor site and this could explain the higher frequency of these cells in the spleen of the MC-deficient mice.

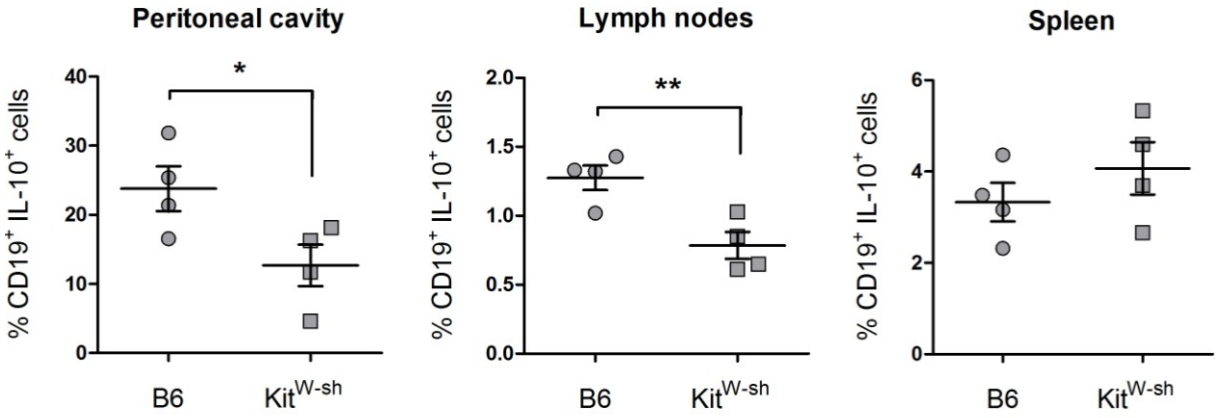


Figure 3.24: **IL-10 production by B cells from B6 and *Kit*$^{W\text{-}sh}$ tumor–bearing mice.** Cells derived from peritoneal cavity, lymph nodes and spleen of B6 and *Kit*$^{W\text{-}sh}$ tumor–bearing mice were cultured with LPS, PMA, ionomycin and monensin for 5 h. The isolated cells were first stained with the Live/Dead green fluorescent probe and with CD19 mAb. After permeabilization, the cells were stained with IL-10 mAb. Each symbol reported in the plots indicate the percentage of IL-10–producing cells among total B cells from the isolated cells. The mean (± SEM) percentages of IL-10^{+} B cells from the four independent experiments is indicated on the graphs. Significant differences between sample means are indicated as follows: *, $p< 0.05$; **, $p< 0.01$.

4. DISCUSSION

During the last decade, a functional plasticity was ascribed to immune cells, which assume different behavior depending on the stage of immune response. In this context, it is well established that B cells, behind their well-known function as effector cells of humoral immune response, are also important in the suppression of immune responses. One of the earliest observations concerning the anti-inflammatory role of B cells was achieved by Flood and colleagues, who showed that B-cell depletion might interfere with the function of suppressor T cells (Flood et al., 1984). Moreover, a regulatory B-cell population that suppressed the progression of intestinal inflammation through the production of IL-10 was described (Mizoguchi et al., 2002). Subsequently, the transfer of B cells that produce IL-10 was correlated with the modulation and prevention of arthritis (Mauri et al., 2003), while an interesting study by the group of Anderton showed that B cells that produce IL-10 were necessary for the recovery from EAE. These B cells produced antigen-specific IL-10 and, remarkably, were able to transfer suppression to EAE induced in bone marrow chimeric mice, in which IL-10 deficiency was restricted to B cells (Fillatreau et al., 2002). More recently, a subset of B suppressor cells was characterized as transitional 2-marginal zone precursor B cells and this phenotype was associated with remission of arthritis and IL-10 secretion (Evans et al., 2007). Finally, the group of Tedder, identified a numerically rare (~1-3%) and phenotypically unique $CD19^{hi}$ $CD1d^{hi}$ $CD5^{+}$ subset of regulatory B cells in the spleens of both normal and autoimmune mice (Yanaba et al., 2008).

Although an increasing body of evidence strongly supports the existence of regulatory B cells, there are still many open questions. Depending on the specific immunological setting under investigation, these cells have been described as either $CD23^{-}$ $CD21^{hi}$ $CD1d^{hi}$ IgM^{hi} MZ B cells, $CD23^{+}$ $CD21^{hi}$ IgM^{hi} $CD1d^{hi}$ T2-MZP B cells, $CD1d^{hi}$ $CD5^{+}$ B10 cells and $CD5^{+}$ B-1a cells, making it difficult to understand whether the ability to produce IL-10 and to contribute to the maintenance of the fine equilibrium required for tolerance is a general property of all B cells or a specific characteristic of a defined lineage. Although different mechanisms, both contact–dependent and –independent, were shown to be responsible for the regulatory properties exerted by these B cells, to date, the production and secretion of IL-10 remains the unifying hallmark of the regulatory, suppressive properties of this functional subset of B cells (Vitale et al., 2010).

B-cell maturation, activation and effector functions are strictly related to the specific immunological context under analysis and are influenced by mediators of both innate and adaptive immunity. Therefore, a study aimed to characterize the microenvironment that induces the expansion and/or differentiation of IL-10–producing B cells was undertaken.

Initially, the contribution of stimuli activating B cells was investigated. B cells were cultured with four different molecules, known to promote B-cell activation through different signaling pathways: LPS and CpG were chosen as they mimic exogenous, infectious signals, and bind membrane or endosomal TLRs, whereas BAFF and the agonistic CD40 mAb resembled endogenous, "immune-mediated" signals. The results obtained showed that the subset of B cells that was competent to produce IL-10 after a 5 h *in vitro* stimulation with LPIM expanded significantly in response to all the tested stimuli, although activation through CD40 proved to be the best way to promote IL-10–competent B cell expansion *in vitro*. On the contrary, when the ability to secrete IL-10 was assessed, it was observed that cytokine release occurred only when the B cells were cultured with TLR agonists. Interestingly, an increase in the levels of IL-10 secreted by CD40-activated B cells was observed only following a short-term stimulation of 2 h with either LPS or anti-IgM Ab. These results lead to hypothesize that while infectious signals induce the production and release of IL-10 in order to regulate and, eventually, switch off the inflammatory response triggered by danger signals, immune-mediated stimuli promote the expansion of the IL-10–competent B cell population normally found in different anatomical sites. In the infectious scenario, the production and secretion of IL-10 are necessary to limit and turn off the immune response initiated by microbial components and, therefore, prevent damage to the host. On the contrary, the signals provided by BAFF or through CD40 are preparatory, since they instruct the B cell and render it competent to exert its suppressive function as a result of the encounter of an appropriate and context-specific second signal (Figure 4.1).

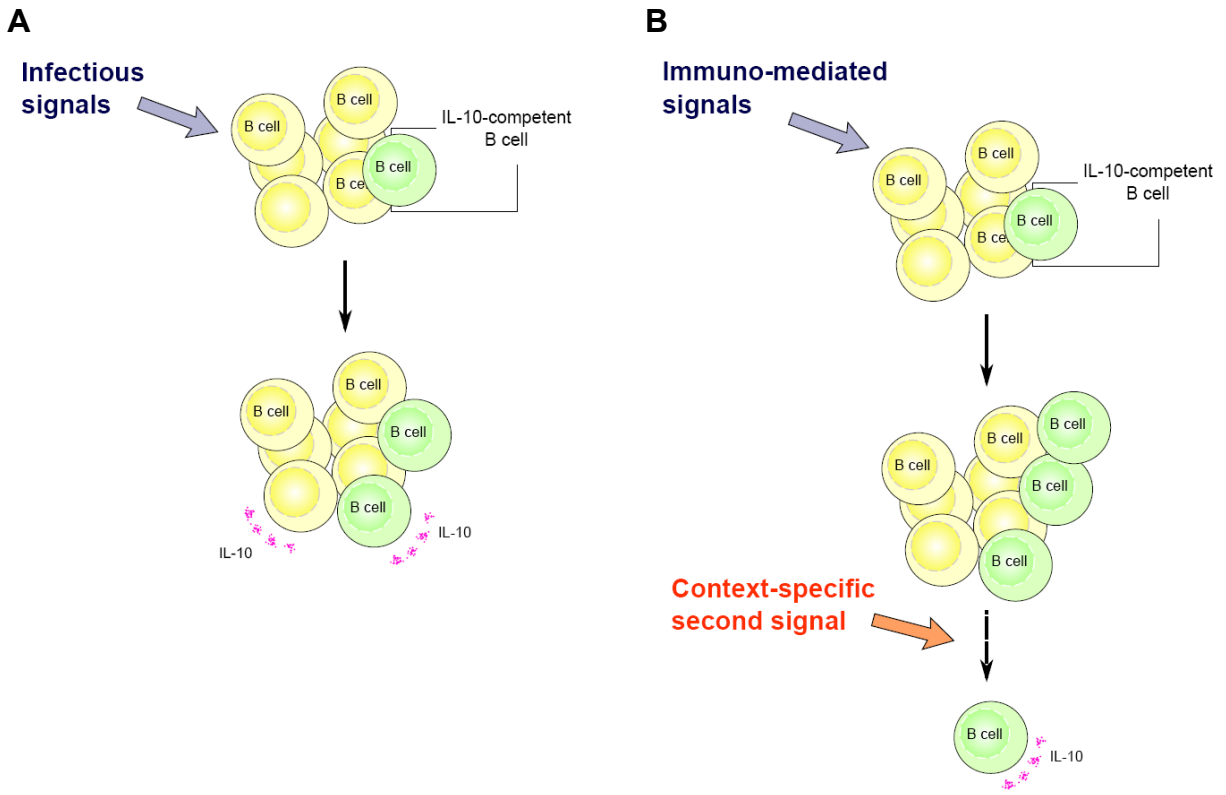


Figure 4.1: IL-10 competence and secretion are differently regulated in different activation contexts. ***(A)*** When the B-cell population encounter an infectious signal, both IL-10–competent and non IL-10–competent B cells are induced to produce and release high amounts of IL-10. ***(B)*** Immune mediated signals promote the expansion of the IL-10–competent B cell population but do not induce IL-10 secretion. A specific second signal is required to promote IL-10 secretion by these IL-10–competent B cells.

The model proposed in figure 4.1 is further supported by the experiments performed to characterize the immunophenotype of the induced IL-10–producing B cells. Among freshly isolated B lymphocytes, the $CD19^+$ $IL\text{-}10^+$ cells were found within all the four subsets defined by the differential expression of CD1d and CD5, although the highest percentage of these $IL\text{-}10^+$ cells expressed high levels of CD1d and low levels of CD5, thus resembling the phenotype of MZ or T2-MZP B cells. On this basis, it can be suggested that all the known B-cell populations contribute to the generation of the functional subset of $IL\text{-}10^+$ B cells. After stimulation through CD40, no changes in the overall distribution of these cells among the different B cell compartments were observed, indicating that the signals provided by this type of activation induce the expansion of IL-10–competent B cells without favoring a subset rather than another. On the contrary, the B-cell treatment with LPS results in a selective increase in the frequency of $IL\text{-}10^+$ cells with a $CD1d^-$ $CD5^-$ FO-like phenotype. This finding could be explained by the fact that LPS differently affects the proliferation and differentiation of the diverse B-cell populations, possibly resulting in a different distribution of the IL-10–producing B cells. The interpretation of the result relative to LPS is further complicated by the fact that,

during the 48 h of stimulation, these B cells are producing and secreting great amounts of IL-10. Hence, it is possible that cells that had already released the cytokine were not detected by intracellular staining. Incidentally, the analysis of the immunophenotype revealed that the IL-10–producing B cell population missing in LPS-activated B cells, compared to freshly isolated B cells, was the one that displayed a MZ-like CD1d^{hi} subset. Compared to FO B cells, MZ B cells are known to be more sensitive and to proliferate in response to bacterial components like LPS (Su et al., 2004; Srivastava et al., 2005). Moreover, they were shown to possess an enhanced secretory apparatus and to exhibit superior secretory activity (Gunn and Brewer, 2006).

As already pointed out in other studies (Mauri and Bosma, 2011; Scapini et al., 2011), these results reveal that the production and secretion of IL-10 and the expansion of the IL-10–competent B-cell population depend on both the activation context and the specific B-cell subset involved. This concept should be kept in mind in the view of the development of novel and more effective therapeutic approaches for pathological conditions such as autoimmunity and chronic inflammation, based on the use of regulatory B cells.

In vivo, the stimuli that affect B-cell survival, proliferation and differentiation derive from the crosstalk between B lymphocytes and other cells of the immune system. Cells of both the innate and adaptive immune system can interact with B cells via cell to cell contact or through the release of specific soluble factors and, therefore, could drive B-cell differentiation towards regulatory B cells. The existing evidence of a direct B-mast cell interaction (Gauchat et al., 1993; Tkaczyk et al., 1996), together with data demonstrating the role of MCs as important regulators of physiological and pathological immune responses (Frossi et al., 2010), led to the investigation of whether MCs could play a role in the expansion and/or differentiation of IL-10–competent B cells, both *in vitro* and *in vivo*.

Work done previously in the laboratory where this thesis was performed showed that MCs promote both survival and proliferation of naïve B cells, as well as activation and further plasma cell differentiation of activated B cells (Merluzzi et al., 2010). The existence of a crosstalk between these two cell types was further confirmed in the present study. First, the formation of cognate interactions between MCs and B cells was investigated *in vitro*: after only few minutes of co-culture, numerous cell conjugates were observed, demonstrating that these two cell types are able to physically interact.

In order to study the nature of this interaction and the possible effects on the differentiation of IL-10–competent B cells, MCs and B lymphocytes were co-cultured and analyzed in different

experimental settings. Similarly to what happened when the B lymphocytes were activated through CD40, MCs were able to increase the frequency of IL-10–competent B cells, *in vitro*, without affecting the overall distribution of the IL-10^+ cells among the four subsets defined by the differential expression of the CD1d and CD5 surface molecules. MCs could induce the expansion of the IL-10^+ B-cell population either by promoting the proliferation and/or survival of existing IL-10–competent B cells or by inducing the differentiation of B lymphocytes into IL-10–competent cells. Understanding the biological process responsible for the increased frequency of $CD19^+$ IL-10^+ cells that occurs as a result of the crosstalk between these two cell types remains a goal of future work.

Taking advantage of a recently characterized murine model of MC-deficiency, the role of signals provided by MCs in IL-10–competent B-cell expansion was also investigated *in vivo*. The results obtained showed that MCs are important regulators of IL-10–competent B-cell differentiation since the MC-deficient mice had lower basal levels of IL-10–producing B cells in the peritoneum, bone marrow and lymph nodes compared to the wild type mice. No differences were observed in the spleen. This last result can be explained by the consideration that although MCs play an important role in the differentiation of IL-10–competent B cells, other factors may also regulate this process *in vivo*. Therefore, it is possible that, in the spleen, other immune cell types or soluble mediators play a specific inductive function and/or could functionally compensate the role of MCs in this organ.

As a next step, the possibility that mast cells could also regulate the process of IL-10 secretion by B cells was evaluated. The results obtained revealed that in the B/MC co-culture system, IL-10 competence and secretion are independently regulated since MCs did not affect IL-10 secretion.

Concerning IL-10 production and secretion, many similarities were observed between the B cells activated through CD40 and those co-cultured with MCs. Since CD40L is constitutively expressed on the surface of mast cells, the role of the CD40-CD40L axis in the MC–driven expansion and/or differentiation of IL-10^+ B cells was assessed. Both co-culture experiments performed in the presence of an anti-CD40L blocking Ab and transwell assays revealed the importance of this co-stimulatory interaction, since a significant reduction of the induced IL-10^+ B cells was observed in these conditions. Nevertheless, other interactions or soluble factors might significantly contribute to the MC-driven expansion of $CD19^+$ IL-10^+ cells. Mast cells are known to release cytokines, such as IL-5, IL-6 and TGF-β, that were shown to affect several aspects of B cell biology (Takatsu, 1998; Lebman and Edmiston, 1999). Moreover, histamine released by MCs can bind the histamine receptors on B cells, and increase the

proliferative response to B-cell receptor cross-linking (Banu and Watanabe, 1999). These factors all constitute very interesting points for further investigation.
Altogether these data show that MCs are able to induce the expansion of the IL-10–competent B-cell population both *in vitro* and *in vivo* and this can be seen as a very relevant observation in the context of immunologically mediated inflammatory reactions.

Collectively, the data presented in this thesis demonstrate that both the molecular cues present in the specific microenvironment and mast cells can affect the behavior of B cells in regard to the competence to produce IL-10.

5. MATERIALS AND METHODS

5.1 Solutions and culture media

- **Wash medium**

  RPMI 1640 (Euroclone)
  10% FBS (Sigma Aldrich)
  20 mM Hepes (Euroclone)
  2 mM L-glutamine (Euroclone)
  antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) (Euroclone)

- **Complete culture medium for splenocytes**

  RPMI 1640 (Euroclone)
  10% FBS (Sigma Aldrich)
  20 mM Hepes (Euroclone)
  2 mM L-glutamine (Euroclone)
  1 mM sodium pyruvate (Euroclone)
  1X non-essential amino acids (from 100X mix, Euroclone)
  antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) (Euroclone)
  50 mM β-mercaptoethanol (Sigma Aldrich)

- **Complete culture medium for BMMCs**

  RPMI 1640 (Euroclone)
  20% FBS (Sigma Aldrich)
  20 mM Hepes (Euroclone)
  2 mM L-glutamine (Euroclone)
  1 mM sodium pyruvate (Euroclone)
  1X non-essential amino acids (from 100X mix, Euroclone)
  antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) (Euroclone)
  50 mM β-mercaptoethanol (Sigma Aldrich)
  20 ng/ml IL-3 (PeproTech)
  20 ng/ml SCF (PeproTech)

- **Medium for rabbit complement reconstitution**

  RPMI 1640 (Euroclone)

  25 mM Hepes (Euroclone)

  3% BSA (Sigma Aldrich)

- **Phosphate Buffered Saline (PBS)**

  137 mM NaCl (Sigma Aldrich)

  27 mM KCl (Sigma Aldrich)

  4.3 mM Na_2HPO_4 (Sigma Aldrich)

  1.4 mM KH_2PO_4 (Sigma Aldrich)

  pH 7.4

- **MIN buffer**

  PBS pH 7.4

  2 mM EDTA (Sigma Aldrich)

  0.5% BSA (Sigma Aldrich)

- **FACS Buffer**

  PBS

  0,5% Sodium azide (Sigma Aldrich)

  0,1% BSA (Sigma Aldrich)

5.2 Cell preparation and culture conditions

5.2.1 Animals

Female C57BL/6 (B6) mice (Harlan Laboratories) were used for almost all the experiments performed in this thesis. Mast cell-deficient *c-Kit* mutant *C57BL/6-Kit$^{W\text{-}sh/W\text{-}sh}$* (*Kit$^{W\text{-}sh}$*) mice were purchased from The Jackson Laboratories and, in some cases, the MC population was reconstituted in these mutant mice by injecting *in vitro* differentiated BMMCs derived from B6 mice. The cells ($5*10^6$ cells in 0.2 ml PBS) were intraperitoneally (i.p.) transfered into 4-6-weeks-old female *Kit$^{W\text{-}sh}$* and the experiments with these mice were initiated 6–8 weeks after adoptive transfer of the BMMCs. The *Kit$^{W\text{-}sh}$* were maintained under pathogen-free conditions at the animal facility of Fondazione IRCCS "Istituto Nazionale dei Tumori" (Milan, Italy), in collaboration with the group of M. Colombo.

5.2.2 Purification of mice spleen B cells

Purified splenic B cells were obtained from 6-12-week-old mice by a negative depletion method that removes all unwanted cells without manipulating the B cells themselves. Briefly, spleens were removed and dissociated in PBS supplemented with 0.0192 M sodium citrate. Splenocyte cell suspension was depleted of red blood cells (RBCs) by hypotonic lysis with ACK lysing buffer (Sigma Aldrich) and of T cells by complement-mediated cytotoxic lysis using an anti-Thy 1.2 monoclonal antibody (a gift from K. Hathcock, Experimental Immunology Branch, NIC/NIH, Bethesda, MD USA) in conjunction with rabbit complement (Low-Tox M; Cedar Lane). The cell suspension was then cultured for at least one hour at 37°C and 5% CO_2 to allow the adhesion of mononuclear cells at the bottom of the flask. At the end of incubation, the cells in suspension were collected, washed and resuspended at a final concentration of 106 cell/ml in complete culture medium for splenocytes. To test the purity of the isolated B cells, a small aliquot of cells was checked by cytofluorimetric analysis for the expression of CD19; the percentage of $CD19^+$ cells was between 85 and 95% (Figure 5.1).

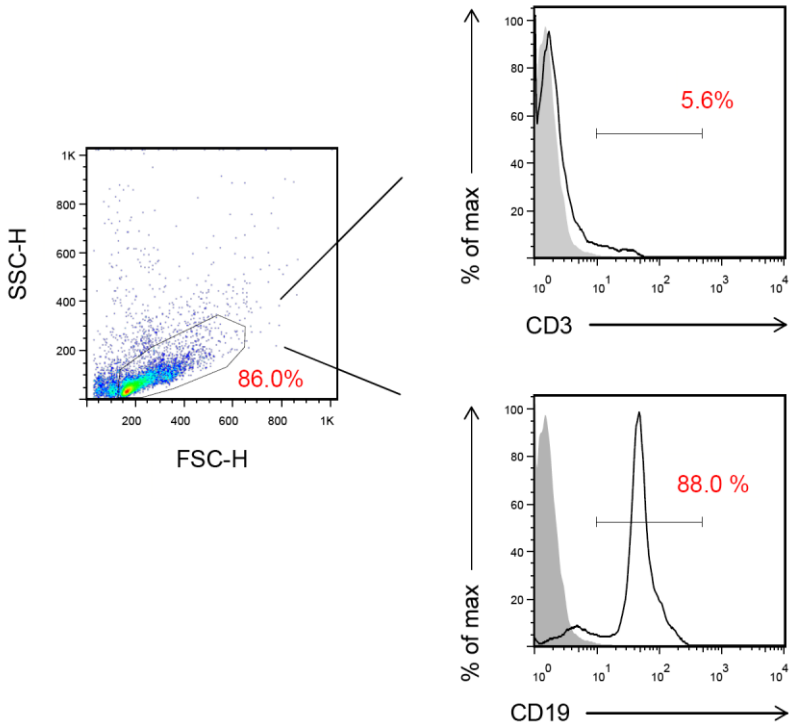


Figure 5.1: Purity of the B cell population isolated from mouse spleen. In the scatter plot of forward scatter (FSC) vs side scatter (SSC) the cells are distributed based on their size and granularity. The lymphocytes (gated) form a tight population, clearly distinguishable from dead cells (on the left of the gated region) and from granulocytes (above the gated region). The hystograms reported on the right side of the picture show how the great majority of the isolated lymphocytes (87.9%) are B cells and that the contamination from T cells accounts for only the 5.6% of total cells.

5.2.3 Isolation of myeloid precursors from mouse bone marrow and mast cell *in vitro* differentiation

Mast cells are widely distributed in most tissues but in low numbers. Therefore, the isolation of these cells is not easy and it leads to low yields. Generating murine mast cells from bone marrow precursor cells is a useful tool to study the biological functions of these cells.

Myeloid precursors were isolated from the femurs of 5-6-week-old mice: these bones were dissected using scissors, cutting through the tibia below the knee joints, as well as through the pelvic bone close to the hip joint. Muscles connected to the bone were removed and the femurs were kept into a polypropylene tube containing sterile PBS, until ready to process them. Under the hood, the epiphyses were removed from the collected femurs and wash medium was injected inside femur diaphisis using a syringe (BD Plastipak) with a 30 gauge needle (Microlance). The cell suspension was collected in a conical polypropylene tube (Sarstedt).

In vivo, these stem cells can become committed to differentiation along a number of distinct haemopoietic lineages, e.g. the erythroid, megakaryocytic, mast cell, granulocytic and macrophage and lymphocytic lineages. The committed progenitor cell then undergoes

proliferation accompanied by increasing maturation which eventually gives rise to the mature blood cell (Whetton et al., 1986). This process is governed, at least in part, by a number of haemopoietic growth factors and it has been shown that, depending on the specific growth factors added to the culture media, these bone marrow-derived cells can differentiate, *in vitro*, into various lineages, such as macrophages and dendritic cells (Zanoni et al., 2009).

In order to obtain mast cells, the extracted myeloid precursors were centrifugated at 300 g and resuspended in complete culture medium for BMMCs which contains IL-3 and SCF, the ligand of the c-Kit receptor (CD117). The standard procedure for BMMCs generation takes 4-6 weeks. After this period, mast cells constitute between 85 and 95% of the whole population, as assessed by flow cytometry for positive expression of FcεRI and c-Kit (Figure 5.2).

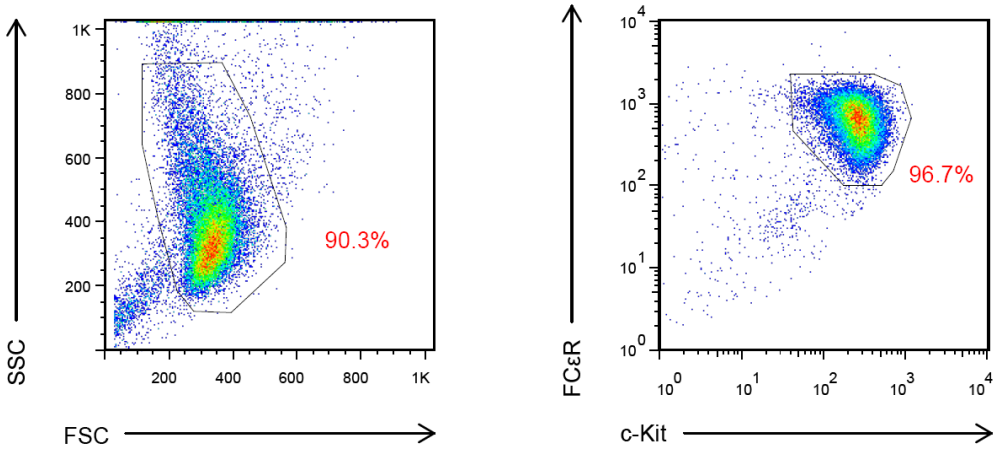


Figure 5.2: Purity of the bone marrow-derived mast cell population. In the scatter plot of forward scatter (FSC) vs side scatter (SSC) the cells are distributed based on their size and granularity. The BMMCs (gated) are large in size and are also granular. The dot plot reported on the right side of the picture shows how mature BMMCs are positively stained for both FcεRI and c-Kit.

In the experiments of co-culture with B cells (paragraph 5.2.7), BMMCs were activated through the receptor for IgE. MCs were resuspended at 10^6 cell/ml in wash medium (that does not contain IL-3) and then incubated for two hours at 37°C and 5% CO_2. Then MCs were sensitized for four hours with 1µg/ml IgE, directed against the antigen dinitrophenol (DNP). Finally, the cells were washed with PBS before being resuspended in complete culture medium for splenocytes, supplemented with 100 ng/ml DNP.

5.2.4 Isolation of Mouse Peritoneal Cavity Cells

The peritoneal cavity is a membrane-bound and fluid-filled abdominal cavity of mammals, which contains the liver, spleen, most of the gastro-intestinal tract and other viscera. It harbors a number of immune cells including macrophages, B cells and T cells. In particular, the

peritoneal cavity is important for the study of B cells because of the presence of a unique peritoneal cavity-resident B cell subset known as B1 cells, in addition to conventional B2 cells (Ray and Dittel, 2010).

Peritoneal cavity cells were obtained from 6-12-week-old mice. Using scissors and forceps the outer skin of the peritoneum was cut and gently pulled back to expose the inner skin lining the peritoneal cavity. Then 5 ml of ice cold PBS, supplemented with 3% fetal bovine serum (FBS), were injected into the peritoneal cavity using a 27 gauge needle (Microlance) being careful not to puncture any organs with the needle. After injection, the peritoneum was gently massaged to dislodge any attached cells into the PBS solution and as much fluid as possible was collected and deposited in tubes kept on ice. The collected cell suspension was centrifuged (450 g for 8 minutes) and the pellet was then resuspended in complete culture medium for splenocytes, at a final concentration of 10^6 cell/ml. Cells were kept at 37°C and 5% CO_2.

5.2.5 Isolation of Lymph Nodes Cells

Lymph nodes function as filters of tissues and tissue fluids and are sites of origin and production of lymphocytes for normal physiological functions. Lymph nodes are somewhat difficult to find: they are generally “pearly white” and/or translucent in color and can blend in with surrounding fat.

Lymph node cells were obtained from either axillary, inguinal or mesenteric lymph nodes of 6-12-week-old mice. The excised lymph nodes were placed in a 24-well flat bottom plate (1 lymph node per well) in 1 ml of PBS and a single-cell suspension was obtained by gently disrupting the lymph node using the sterile plunger of a 1-ml syringe. The lymph node cells were then transferred into a plastic centrifuge tube making them pass through a cell strainer (BD Biosciences) and then washed once in 10 ml of fresh PBS by centrifuging 5 minutes at 580 g. After the wash, the cells were resuspended at 10^6 cell/ml in complete culture medium for splenocytes and cultured at 37°C and 5% CO_2.

5.2.6 *In vitro* stimulation of spleen B cells

Purified B cells were plated at $0{,}6*10^6$ cell/well in a 24-well flat bottom plate (Corning Costar), at the final concentration of 10^6 cell/ml, and cultured for 48 h in the presence or absence of substances known for their ability to induce B cell maturation and/or activation. Specifically, we stimulated the cells with either purified anti-mouse CD40 monoclonal

antibody HM40-3 (BD Pharmingen) at 1.0 μg/ml, BAFF (PeproTech) at 20 ng/ml, LPS (Sigma Aldrich) at 10 μg/ml or CpG (Dynavax) at 5 μg/ml.

5.2.7 B lymphocyte-mast cell co-colture

When B cells were co-cultured with MCs, $0.6*10^6$ B cells were added to an equal number of IgE-sensitized or non-sensitized MCs, in the presence or absence of DNP, and cultured for 48 hours in a 24-well flat bottom plate (Corning Costar).

In some co-culture experiments, B cells and MCs were separated by a transwell polycarbonate membrane (Corning Costar) with pores of 0,4-μm that allow the passage of soluble factors, but not of cells. The B cells ($0.6*10^6$ cells) were cultured in the bottom chamber in a final volume of 600 μl while instead the MCs ($0.2*10^6$ cells) were placed in the top chamber, in a final volume of 100 μl.

To evaluate the role of CD40/CD40L signaling pathway in the B lymphocyte-MC co-culture, the anti-CD40L blocking Ab (clone MR1) was added to the culture medium at the final concentration of 50 μg/mL and maintained for all the 48 hours of culture. As a control, the same co-culture experiment was performed in the presence of the CD40L isotype control antibody mouse IgG (Sigma Aldrich). The anti-CD40L Ab was purified from hybridoma and was a kind gift from M. Colombo, Molecular Immunology Unit, Department of Experimental Oncology and Molecular Medicine, Fondazione IRCCS Istituto Nazionale Tumori).

5.2.8 Effector T cell purification

$CD4^+$ $CD25^-$ T cells constitute the effector T cell population and were purified from mouse spleen using the $CD4^+$ $CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec). In fact, in isolating $CD4^+$ $CD25^+$ Treg cells through the immunoaffinity protocol on which is based this kit, purified $CD4^+$ $CD25^-$ Teff cells are obtained as a side product. The purification of Teff cells from the splenocyte suspension is performed in a two-step procedure:

- flow in a nylon column to eliminate the great majority of B cells that electrostatic interact with nylon polymers;
- double immuno-affinity purification with magnetic beads.

In order to obtain an homogeneous single-cell suspension, mouse spleens were perfused with culture media using a 27 gauge needle (Microlance). After centrifugation for 5 minutes at 625

g, 1 ml/spleen of cold ACK lysis buffer (Sigma Aldrich) was added to the cell pellet and the suspension was incubated at 4°C for 5 minutes to allow osmotic lysis of RBCs. After the 5 minutes incubation, culture medium was added to stop the lysis. The cells were therefore centrifuged as before, resuspended in culture medium and then loaded on the nylon column, where they were left to incubate for 1 hour at 37°C. At the end of the incubation the negatively selected cell fraction was collected, centrifuged and, immediately after, incubated with anti-CD8 Ab (80 µl antibody/100 million cells) and anti-CD45R/B220 Ab (60 µl antibody/100 million cells), both conjugated with magnetic beads. After an incubation step of 20 minutes at 4°C, the cells were washed with MIN buffer and loaded on a LS magnetic column (Miltenyi Biotec). The eluted $CD4^+$ fraction was subsequently incubated first for 20 minutes at 4°C with anti-CD25 PE Ab and, then, for additional 20 minutes with anti-PE Ab, conjugated to magnetic beads. At the end of incubation, cells were washed, resuspended in culture medium and then loaded on a LS magnetic column. The magnetically labeled $CD4^+$ $CD25^+$ Treg cells were retained in the column while, instead, the unlabeled $CD4^+$ $CD25^-$ Teff cells ran through and were collected in a conical polypropylene tube (Sarstedt).

5.2.9 Effector T cell proliferation assay

Freshly isolated Teff cells were CFSE-labeled (paragraph 5.2.3) and seeded at $5*10^5$ cell/ml in a 96-well round bottom plate (Corning Costar) in complete culture medium for splenocytes. The T cells were stimulated with 5 µg/ml anti-CD3 mAbs (eBioscience) and 2.5 µg/ml anti-CD28 mAbs (eBioscience). In many cases the induction of T-cell proliferation is dependent on the presence of non-T cells, that function as accessory cells providing additional co-stimulatory signals for T-cell proliferation. For this reason, $7.5*10^4$ accessory cells were added to each well. The accessory cells used for these experiments derived from the unfractionated mouse spleen cell suspension, treated with mitomycin C (Sigma Aldrich).
Thus, the activated Teff cells were cultured for 72h and cell proliferation was evaluated by flow cytometry by measuring the decrease in the MFI of CFSE in the activated and comparing it to the one observed for the unstimulated cells.

In the experiments performed for this thesis, supernatants from purified B cells activated for 48h with either anti-mouse CD40 mAb, BAFF, LPS or CpG or through interaction with MCs were compared with supernatants from unstimulated B cells for their effects on T-cell proliferation. In all the cases, B cell conditioned media was added to fresh culture media in a 1:1 ratio.

5.3 Flow cytometric analysis

5.3.1 Staining for surface markers

A vast array of different cell surface molecules are involved in mediating immune responses. Methods that determine the types and levels of such surface markers provide important information regarding cell lineage, activation status, adhesion, migration and homing capacity, and ability to respond to stimuli and to interact with other cells.

To assess cell-surface expression of different co-stimulatory molecules and/or activation markers, $0.5*10^6$ cells were collected into polystyrene tubes (Sarstedt), washed with PBS, resuspended in FACS buffer and then incubated in the dark for 30 minutes at 4°C with a fluorescent mAb specific for a cell surface marker or with an Ig isotype-matched control. In case of multicolor staining, other fluorescent antibodies directed at various cell surface antigens were added at the same time. After the incubation, the cells were washed with FACS buffer, resuspended in 0.5 ml of PBS and kept at 4°C until flow cytometric analysis. The Abs used in the experiments performed for this thesis were conjugated either with fluorescein isothiocyanate (FITC), phycoeritrin (PE) or PE-cyanine 5 (PE-Cy5) flurochromes. A complete list of the antibodies used in this work are shown in the table here below:

Table 5.1 – List of antibodies

Specificity of Antibody	Reactivity	Isotype	Clone	Conjugated	Manufacturer	Work diluition
CD3	Mouse	Hamster IgG	145-2C11	FITC	ImmunoTools	1:100
CD19	Mouse	Rat IgG2a	1D3	FITC	eBioscience	1:100
CD19	Mouse	Rat IgG2a	6D5	PE-Cy5	BioLegend	1:125
CD5	Mouse	Rat IgG2a	53-7.3	FITC	BioLegend	1:50
CD1d	Mouse	Rat IgG2b	1B1	PE	eBioscience	1:100
CD154 (CD40L)	Mouse	Hamster IgG	MR1	PE	BioLegend	1:50
FcεR	Mouse	Hamster IgG	MAR-1	PE	BioLegend	1:100
CD117 (c-Kit)	Mouse	Rat IgG2b	2B8	PE-Cy5	eBioscience	1:100
Isotype controls			**Clone**	**Conjugated**	**Manufacturer**	**Work diluition**
Rat IgG2a				FITC	BioLegend	1:100
Rat IgG2a				PE	BioLegend	1:100
Rat IgG2a				PE-Cy5	BioLegend	1:100

5.3.2 Intracellular staining

A method widely used in this work is the intracellular staining of the cytokine IL-10.
A modification of the basic immunofluorescent staining protocol described above was used for the simultaneous analysis of surface molecules and intracellular antigens at the single-cell level. The protocol used is a slightly modified version of the method described by Tedder and Matsushita (Matsushita and Tedder, 2011).
In vitro stimulation of cells is usually required for detection of cytokines by flow cytometry since cytokine levels are typically too low in resting cells. In our case 1-2*10^6 cells were resuspended (1*10^6 cells/ml) in culture medium alone (as a control) or containing 50 ng/ml PMA (Sigma Aldrich), 500 ng/ml ionomycin (Sigma Aldrich) and 10 µg/ml LPS (Sigma Aldrich) and cultured for 5 hours at 37°C and 5% CO_2. The protein transport inhibitor monensin (Sigma Aldrich) was added at the final concentration of 2 µM together with PMA, ionomycin and LPS to block the secretion of IL-10 during the five hours of stimulation.
The intracellular staining protocol starts at the end of the 5 hours of stimulation: cells were kept at 4°C, with minimal exposure to light, and, after each wash, were pelleted by centrifugation at 300 g for 5 minutes. Briefly, the cells were collected in propylene tubes and washed with PBS. In order to discriminate viable cells from dead ones, B cells were stained with a green fluorescent probe LIVE/DEAD Fixable Green Dead Cell Stain Probe, that binds to the cell membrane and that can permeate the necrotic cells. Sequentially the purified anti-mouse CD16/CD32 mAb was added to avoid non-antigen-specific binding of Fc portion of antibodies used hereafter with Fc receptors on B cell surface. After 15 minutes of incubation, cells were washed with cold PBS and incubated for 30 minutes with 100 µl of ice-cold PBS containing PE-Cy5 CD19 mAb. In certain cases, cells were also stained with PE-Cy7 CD5 mAb and Pacific Blue CD1d mAb. Then cells were washed and fixed with 250 µl of Cytofix/Cytoperm cell fixation buffer (BD Biosciences) for 20 minutes and, at the end of the incubation, the cells were washed two times with the Perm/Wash buffer for cell permeabilization (BD Biosciences). Hereafter Perm/Wash buffer was used for staining and washes in order to maintain the cells in a permeabilized state. The cells were resuspended in 100 µl of Perm/Wash buffer containing PE-labeled anti-IL-10 mAb or PE-labeled Isotype control Ab and incubated for 30 minutes. Finally, cells were washed twice with Perm/Wash buffer and resuspended in 250 µl of 1.5% formaldehyde fixative. The cells were kept at 4°C until they were analyzed by flow cytometry. The working dilutions of reagents and antibodies are reported in table 5.2.

5.2 – List of antibodies and fluorescent dyes

Reagent	Clone	Manufacturer	Work diluition
Live/Dead Fixable Green Dead Cell Stain Probe		Molecular Probes, Invitrogen	1:750
CD16/32	2.4G2	BD Biosciences	1:100
CD19 PECy5	6D5	BioLegend	1:125
CD5 PECy7	53-7.3	eBioscience	1:50
CD1d PacificBlue	1B1	BioLegend	1:100
IL-10 PE	JES5-16E3	BioLegend	1:100
Rat IgG 2b PE		eBioscience	1:100

5.3.3 CFSE staining of cell division

In order to study lymphocyte proliferation, cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) (Molecular Probes, Invitrogen), a novel cell-tracing fluorescent dye used to examine the proliferative activity of cells by the labeling of a parent generation and the inheritance of the label by daughter generations. CFDA-SE diffuses into cells where the acetate groups on the molecule are cleaved to yield a highly fluorescent derivative (CFSE) that is retained in the cell and can be detected by flow cytometry. Cell division results in sequential halving of fluorescence and up to eight cell divisions can be monitored before the fluorescence is decreased to the background fluorescence of unstimulated cells (Parish et al., 2009).

Briefly, 10^5-10^7 T lymphocytes were washed and resuspended in 1 ml of complete culture medium for splenocytes. CFDA-SE was then added to a final concentration of 5 μM and incubated for 15 minutes at 37°C, in the dark. The reaction was blocked by adding 1 ml of cold FBS and cells were then centrifuged (300 g for 5 minutes) and washed two times with wash medium. Finally, Teff cells were resuspended at the final concentration of $5*10^5$ cell/ml in complete culture medium for splenocytes.

5.3.4 Flow cytometry data acquisition and analysis

Depending on the number of channels needed to detect the fluorescence signals of the sample in analysis, the fluorescence analyses were performed either on the FACScan (Becton Dickinson) or on the Cyan ADP 9-color cytometer (DakoCytomation).

The flow cytometry data were analyzed with FlowJo software (Tree Star) which allows to import and analyze cytometry data regardless of which FACS (Fluorescence Activated Cell Sorting) machine is used to collect the data.

5.4 Quantification of secreted proteins

5.4.1 Enzyme Linked Immunosorbent Assay

Cytokine quantification in cell supernatants was performed using the Enzyme Linked Immunosorbent Assay (ELISA), a highly sensitive and easy to use biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample.
The ELISA experiments performed for this thesis are "sandwich ELISAs" in which the plate is coated with a capture antibody and, after an o/n incubation and the block of nonspecific binding sites on the surface, sample is added and any antigen present binds to the capture antibody. Then enzyme-linked detecting antibody is added and binds to antigen and, at last, a substrate is applied and is converted by the enzyme to detectable form.
All ELISA kits were purchased from eBioscience, except for the one for IL-13 (PeproTech), and were all used according to the manufacturer's instructions.

5.5 Microscopy

5.5.1 Real-time video microscopy and conjugate formation evaluation

The formation of MC-B cell conjugates in real time was analyzed by time-lapse epiluminescent microscopy using the Leica AF6000LX system (microscope, DMI6000 B; camera, DFC350FX; software: LAS AF). In total, $0.5*10^6$ pre-sensitized MCs and $0.5*10^6$ B cells were plated on glass bottom Petri dishes (Nunc). The chamber was placed on heating plate prewarmed at 37°C and DNP was added. Phase-contrast images were recorded at indicated time points and resulting video-recorded movies were processed with the Photoshop Cs3 software.

5.6 Statistical analysis

Experimental data are shown as means ± standard error of mean (SEM). The unpaired or paired Student's t-tests (Prism GraphPad Software, San Diego, CA, USA) were used to analyze the results for statistical significance. P values below 0.05 were considered as significant.

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7. LIST OF PUBLICATIONS

Full Papers

1. Vitale G., **Mion F.**, Pucillo C.

   Regulatory B cells: evidence, developmental origin and population diversity.

   Mol Immunol. 2010 Nov-Dec;48(1-3):1-8. Epub 2010 Oct 14.

2. Boscolo S., **Mion F.**, Licciulli M., Macor P., De Maso L., Brce M., Antoniou M., Marzari R., Santoro C., Sblattero D.

   Simple scale-up of recombinant antibody production using an UCOE containing vector.

   N Biotechnol. Epub 2011 Dec 28.

3. **Mion F.**, et al.

   Mast cells promote the expansion of the IL-10–competent B-cell population through the CD40-CD40L axis.

   Manuscript in preparation.

Conference proceedings

1. **Mion F.**, Cattaruzzi G., Pucillo C., Vitale G.

   Biochemical and Functional aspects of Pax5 SUMOylation.

   Poster presented at "1st IRB Barcelona PhD Student Symposium" (November 2009; Barcelona, Spain).

2. **Mion F.**, D'Orlando O., Gri G., Cesselli D., Toffoletto B., Frossi B., Beltrami A.P., Beltrami C.A., Pucillo C.

   Human MSCs affect the B cell survival and differentiation in CD19 dim and CD19 bright B cell subpopulations.

   Poster presented at "SIICA 7th National Conference" (May 2010; Bari, Italy).

3. **Mion F.**, D'incà F., Frossi B., Toffoletto B., Danelli L., Pucillo C. Vitale G.

   B lymphocytes: more than antibody-producing cells.

   Oral presentation at "ESF-EMBO Symposium on B Cells and Protection: Back to Basics" (June 2011; Sant Feliu de Guixols, Spain).

4. **Mion F.**, D'incà F., Frossi B., Toffoletto B., Danelli L., Pucillo C. Vitale G.

   B lymphocytes: more than antibody-producing cells.

   Poster presented at "2011 Joint annual meeting SIICA–DGfI" (September–October 2011; Riccione, Italy).

8. ACKNOWLEDGMENTS

It's amazing how fast time passes: it seems like yesterday that I joined the group of Professor Pucillo and now the PhD course is at the end. I'd be lying if I said that during these three years, everything was great. Trying to do research is not an easy thing and sometimes I felt like giving up. Today I would like to thank all the people that, with their actions and their words, helped me to not let go.

First of all I would like to thank Professor Carlo Pucillo which, more than any other person, was able to transmit me the enthusiasm necessary to do this job. Thanks for having always found a solution and for having believed in me.

Another person that has been very patient with me was my tutor, Dott. Gaetano Vitale. I can imagine that for him it must not have been easy to stand to hear me talk for hours and hours of my experiments. I'm happy that we managed to work together, in spite of our opposite characters.

A very special thank goes to Dr. Barbara Frossi, Dr. Elena Betto and Dr. Giorgia Gri. They have taught me many things and have always been very helpful and kind. Most importantly, the three of them always encouraged me and I'm grateful to them for this.

And now, it is the turn of Federica, Luca and Riccardo who were the people, the *Johnnys*, with whom I spent most of my time in the lab.

Luca doesn't appreciate kind and sweet words but I still want to thank him because he was the one from which I learnt most of the things that I know.

Thank you Federica for having been a friend and not only a colleague. Together we had fun, we got angry and we got to know each other.

And then there's Riccardo.
At the beginning I would have never thought to become so close to him but life is very strange some times. I will miss him a lot now that he is going to work for *"Mr.Galli"*.
Tortel, *Nonna Papera* wishes you all the best!

I want to thank the new entries of the Pucillo's lab, Alice, Raffaella and Silvia, and all the members of the CB lab with whom I spent funny moments, inside and outside Piazzale Kolbe.

I would like to acknowledge Dr. Barbara Toffoletto and Dr. Daniela Cesselli that were very helpful with the experiments of flow cytometry.

A special thank goes to my parents, to Giuliana and Flavio and to Marina, Paolo and Stefano. And finally to Marco, the most important person in my life.