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EPIGENETIC MODIFICATIONS IN *il-10 GENE LOCUS* ARE PIVOTAL FOR THE UNDERSTANDING OF IL-10 COMPETENCE IN B CELLS

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1. ABSTRACT

The homeostasis of the immune system is a finely regulated process, that depends on the equilibrium between the effector and the regulatory compartment, if a misbalance occurs the result could be either excessive inflammation or exaggerate immunosuppression. B cells are traditionally known for their role in production of antibodies and therefore are considered pro-inflammatory cells, however in the last decades, B cells with immune-suppressive functions have been identified, and were termed Regulatory B cells (B_{reg}). Despite great effort of several groups in this field, nowadays, there is no agreement about their origin, nor about their phenotype, and a specific transcription factor, like for example FoxP3 for regulatory T cells, has not been identified yet. B_{reg} mainly exert their immune-suppressive function through the secretion of the anti-inflammatory cytokine Interleukin-10 (IL-10), that is currently used as marker for their detection. A major point in the debate regarding these particular population of B cells is whether the capacity to produce IL-10 is intrinsic property of the cell, or if rather B cells belonging to diverse subset acquire the capacity to produce IL-10 after they are instructed by the surrounding microenvironment.
With the aim to clarify this point, my PhD project focused on IL-10 competent B cells, a particular subset able to produce IL-10 after a short-term stimulation, in the specific, after 5 hours of treatment with LPS, PMA and ionomycin. The initial hypothesis was that IL-10 competent B cells quickly respond to stimulation because they are in a suspended state for its production, and we wanted to clarify which are the possible mechanisms at the basis, with a major focus on epigenetic. The initial observation was that B cells stimulated through CD40, treated with the demethylating agent 5-azacytidine, and then briefly re-stimulated with LPS, present a higher expression of *il-10* mRNA, compared to cells that did not received the chemical compound. That was the starting point that led us to investigate the methylation status of CpGs among the *il-10* gene locus. Starting from a pure and viable population of IL-10 competent B cells, isolated with the combination of an IL-10 secretion assay and cell-sorting, we were able to perform different type of analysis. In first instance, a methylation analysis was performed, taking advantage of a bisulphite-based method, that allows to determine the percentage of methylation at specific sites through sequencing. A specific methylation signature was found, in particular, IL-10 competent B cells are less methylated, compared to the whole population, on two conserved non coding sequence

(CNS) located upstream from the transcription starting site of the gene. Interestingly, we found also that IL-10 competent B cells has an active histones signature, with an enrichment of acetylation in the CNS studied also for DNA methylation. The second step was to test whether B cells that cannot produce immediately IL-10, can do it in a longer time frame, to this purpose the non-competent B cells were re-stimulated, and not surprisingly, they started producing IL-10 after 48 hours, and of note, these cells did not acquire the DNA methylation signature IL-10 competent B cells.

Collectively, the data presented in this thesis support the hypothesis that, among the total B cell population, IL-10 competent B cells are a particular functional subset that, differently from the rest of the population, can respond quickly to a stimulus triggering with the production of IL-10. In the specific, we found that the IL-10 competent B cells can be recognized among the total population as two CNS of their *il-10* gene locus are almost devoid of DNA methylation, contrarily from all other B cells. Finally, IL-10 competent B cells are an effective functional immune-suppressive subset, while all other IL-10 producing B cells acquire this capacity during time and depending on the characteristics of surrounding microenvironment.

2. LIST OF ABBREVIATIONS

5-azacytidine	5-aza
5mC	5-methylcytosine
Ab	Antibody
APC	Antigen Presenting Cells
BCR	B Cell Receptor
BM	Bone Marrow
B_{reg}	Regulatory B cells
CGI	CpG island
CHX	Cycloheximide
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
CNS	Conserved Non coding Sequence
DC	Dendritic Cell
DNMT	DNA methyltransferase
ERK	Extracellular signal-Regulated Kinase
FO	Follicular B cells
gDNA	genomic DNA
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HSC	Hematopoietic stem cell
IL-	Interleukin-
IRF	Interferon Regulatory factors
LMR	Low-Methylated Regions
LPS	Lipopolysaccharide
MCS	Multiple Cloning Site
MD-2	myeloid differentiation factor 2
MMP	Multipotent myeloid/lymphoid Progenitor
MZ	Marginal Zone B cells
NELF	Negative Elongation Factor

NF-κB	Nuclear Factor κB
NK	Natural Killer
PAMP	Pathogen-Associated Molecular Patterns
PMA	Phorbol 12-Myristate 13-Acetate
PRR	Pattern Recognition Receptors
P-TEFb	Positive Transcription Elongation Factor
RISC	RNA-Induced Silencing Complex
SWI/SNF	SWItch/Sucrose Non-Fermentable
TDG	Thymidine DNA-Glycosylase
TET	Ten-Eleven Translocation
TF	Transcription Factor
T_H	T helper
TLR	Toll Like Receptor
T_{reg}	Regulatory T cells
TSS	Transcription Starting Site

3. INTRODUCTION

3.1. The B cell arm of the immune system

The immune system is the main host defence that protects the body from pathogenic microbes and toxins. It is classically divided into the innate and the adaptive compartments. Thus, when a microorganism, like a *bacterium*, disrupts the epithelial barrier, mediators are released and the innate immune system is activated. The process is initiated by resident immune cells such as macrophages, mast cells and dendritic cells (DCs). These cells express cell surface markers called pattern recognition receptors (PRR), which bind molecular patterns associated to pathogens (PAMPs) and not to the host. As a consequence of the recognition, cells release cytokines and chemokines provoking the so-called local inflammation that is characterized by *calor*, *rubor*, *dolor* and *tumor*. These are the macroscopic symptoms of the local blood vessel dilatation and permeabilization. The inflammatory reaction is fundamental for the protection of the organism, but becomes deleterious if it is excessive and uncontrolled since it could also lead to tissue damage (Murphy et al., 2008). In this view, the balance between effector and regulatory cells is essential for a correct resolution of the inflammatory process. A well characterized regulatory subset is represented by regulatory T cells (T_{reg}), instead less is known about their counterpart in the B cell lineage: the regulatory B cell (B_{reg}) population. B cells able to suppress the immune response were first described in the late seventies (Katz, S.I., Parker, D., and Turk, 1974), but only in the last two decades the research on this topic expanded (Wolf et al., 1996). Nowadays we talk about B_{reg} referring to a heterogeneous population of B cells with immunosuppressive properties, but we still do not know which is their precise origin and how they develop (Rosser and Mauri, 2015).

3.1.1. B cells development in the bone marrow

In adult organisms of both human and mouse, B cells origin from the hematopoietic stem cell (HSC) in the bone marrow (BM), where they start their maturation process that continues in secondary lymphoid organs (lymph nodes, spleen and lymphoid tissues). The HSC asymmetrically divides into another stem cell and into the multipotent myeloid/lymphoid progenitor (MPP), that lacks the self-renewal capacity and further develops into the common lymphoid progenitor (CLP) and the common myeloid progenitor

(CMP). The CLP gives rise to B cells, T cells, natural killer (NK) cells and DCs, while the CMP can differentiate into erythrocytes, megakaryocytes, macrophages, neutrophils, eosinophils and basophils (Melchers, 2015). In mice, B lymphopoiesis at early stages is mainly governed by IL-7 and Flt3L. The receptor for Flt3 begins to be expressed in the MPP stage and determines the loss of capacity to differentiate into megakaryocytes and erythrocytes (Adolfsson et al., 2005). Confirming its importance, mice lacking Flt3L have reduced levels of CLP and severely impaired B cell development (Sitnicka et al., 2002). The role of IL-7 is relevant at a later stage, since its receptor is expressed by CLPs. Also in this case mice lacking the receptor (Peschon et al., 1994), or the cytokine itself (von Freeden-Jeffry et al., 1995), do not present a normal B cell population, stating a fundamental role for IL-7 in the progression from the CLP to the pro-/pre- B cell stage.

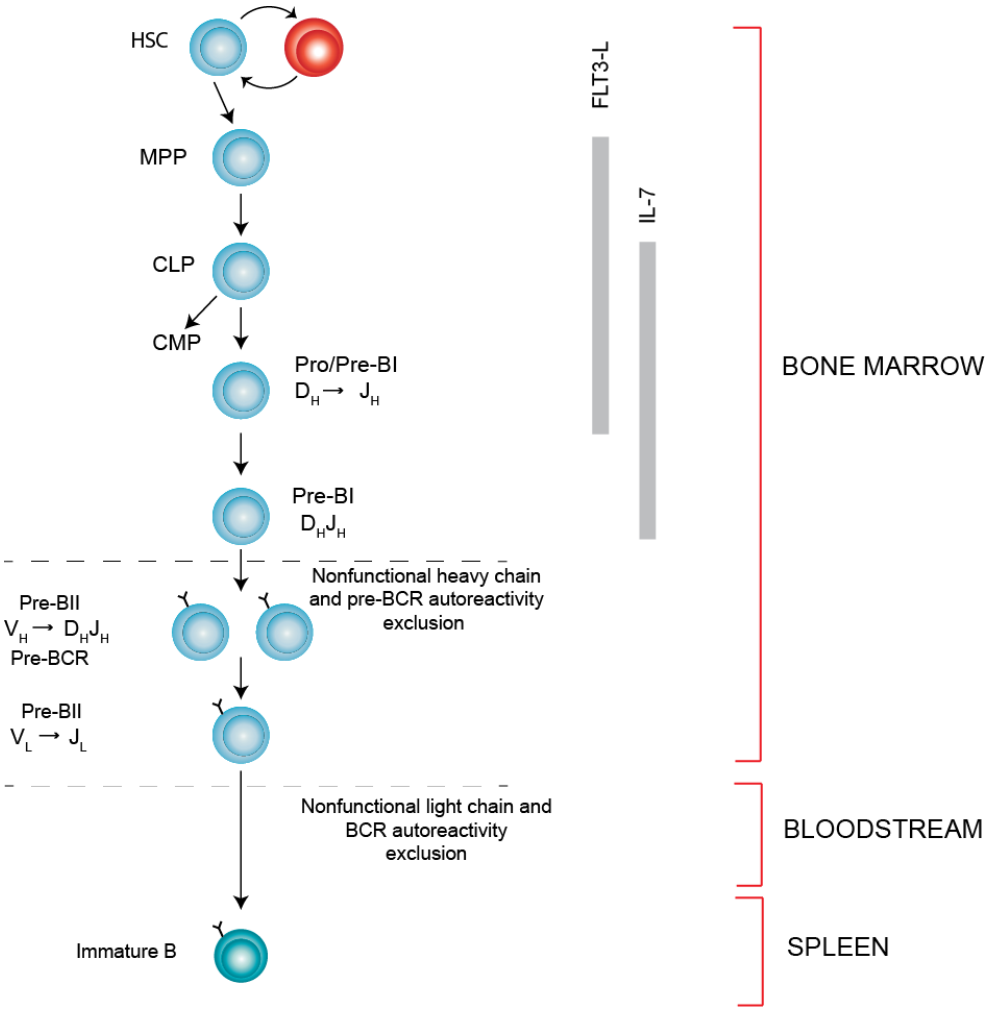


Figure 1. ***Early B cell development.*** *B cells origin from the HSC in the BM and their development is accompanied by the rearrangement of immunoglobulins and the exclusion of B cells expressing auto reactive BCRs.*

After CLP commitment the development of B cells is governed by immunoglobulin formation and expression, that starts in the pro-B cell stage. The formation of the B cell receptor (BCR) is a fundamental process for the maturation of B cells, but also an important feature of the immune system, since it allows the production of millions of antibodies for specific antigens. Basically, as shown in **Figure 2**, an immunoglobulin is made by two heavy chains (IgH) and two light chains (IgL), which are formed thanks to the action of the recombination enzymes (RAG-1 and RAG-2). IgL are composed of a variable (V) and a joining (J) segment coupled by a constant (C) region, while IgH, besides these three domains, contain also the diversity (D) fragment. The rearrangement of the IgL chain starts from the combination of V and J followed by the C segment, on the contrary, the IgH formation starts from the combination of D with J, the V segment joins the DJ sequence and finally the C region is added. The events leading to immunoglobulin formation go together with B cell development, starting from the rearrangement of the D and J (D_HJ_H) in the pro-B cell stage. After the D_HJ_H segment is formed the cell is in the pre-BI stage and undergoes the first checkpoint that excludes the non-functional IgH and the auto reactivity of the pre-BCR formed; survived B cells rearrange the V_H and start the formation of the IgL combining V_L and J_L and, the resulting protein is the final BCR. At this point B cells are tested for non-functional IgL and for BCR reactivity to autoantigens completing the process called central tolerance. As immature B cells they exit from the BM to enter the spleen where the peripheral tolerance and the maturation process starts (Murphy et al., 2008).

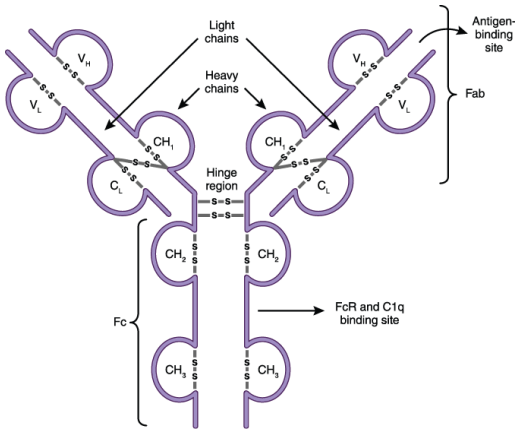


Figure 2. ***Immunoglobulin structure.*** *Each immunoglobulin is composed by two heavy chains (V_H and C_H) and two light chains (V_L and C_L). Adapted from* (Hoffman et al., 2016)

3.1.2. B cell development in the peripheral lymphoid organs

Immature B cells exit from the BM and enter into the spleen where they start the maturation process. From this anatomical compartment B cells can get in the bloodstream and circulate through lymph nodes, gut associated lymphatic tissues, the peritoneal or pleural cavities or again into the spleen. Every day about $2x10^7$ BCR^+ B cells exit from the BM, but only the 10% reaches the periphery and start their maturation process. These immature cells, as they are transitioning from the BM to the peripheral lymphoid organs are called transitional B cells (Carsetti et al., 1995). Transitional B cells are mainly divided into transitional-1 (T1) and transitional-2 (T2), according to their level of maturation, surface markers expression and localization. T1 B cells are the most immature and are characterized by the expression of CD24, but also by a $CD21^{low}CD23^{low}IgM^{high}IgD^{low}$ phenotype, while T2 B cells present $CD21^{high}CD23^{high}IgM^{high}IgD^{high}$ expression. T1 are located in the BM and blood, but the vast majority is found in the outer periarteriolar lymphoid sheath of the spleen, on the contrary T2 are not present outside the spleen. The T2 B cell population matures into other subsets, in the specific they can differentiate into marginal zone (MZ) or follicular (FO) B cells.

3.1.3. B cell activation

The activation of B cells can occur in response to T-independent antigens (TI-antigens), or with the simultaneous activation of T helper (T_H) cells after encountering the so-called T-dependent antigens (Nutt et al., 2015).

The T-dependent activation of B cells is mediated both by soluble factors and by the binding of co-stimulatory molecules on the surface of the two cell types. One of the most relevant mechanism in this context is the CD40-CD154 (or CD40L) axis, CD40 is expressed on the surface of B cells, while CD154 on T cells. The role of this axis has been described in the context of isotype switching, in fact mice lacking CD40 cannot produce IgG, IgA and IgE, preventing an effective immune protection, and thus resulting in immunodeficiency (Kawabe et al., 1994). Interestingly, the signalling through CD40 not only influences the function of B cells, but is also able to induce their proliferation, as reported by several studies (Brines and Klaus, 1993; Nomura et al., 1995).

Another way to activate B cells is through TI-antigens, that are characterized by their intrinsic capacity to generate a response in mice lacking the thymus, and therefore are

deprived of a functional T cell population. TI-antigens are also called PAMPs and are recognized by PRRs that are expressed on the surface of immune cells. One of the most studied class of PRR, are the toll like receptors (TLRs), a specific class able to bind antigens with repeated epitopes like for example lipopolysaccharide (LPS), viral DNA and RNA and other bacterial components like flagellin and lipoproteins. TLRs are expressed on the cell surface or in the endosomal compartment (**Figure 3**), but all share a common structure, with a single-pass through the membrane and an extracellular domain made by 18-25 copies of leucine-rich repeat domains, with a horseshoe-like shape. The activation of TLRs starts when the ligand binds them and induce the formation of dimers or oligomers. The intracellular portion of TLR is a Toll/interleukin-1 receptor (TIR) domain, that is recognized by TIR domain containing adaptors proteins (TIRAP), such as MyD88, TIRAP/Mal, TRIF and TRAM (Murphy et al., 2008). In the context of this thesis the ligand for TLR4 was used, so the attention will be pointed on this particular receptor.

TLR4 is fundamental for the recognition of LPS, a component of Gram-negative bacteria, in fact its absence confers resistance to septic shock in mice (Poltorak, 1998). TLR4 recognizes its ligand with the help of an adaptor protein, the myeloid differentiation factor 2 (MD-2), that localizes into TLR4 pocket and binds LPS. The so-formed activated receptor is made by a dimer of TLR4-MD-2-LPS complex (Park et al., 2009). MD-2 is not the only necessary protein for TLR4 signalling, in fact also the LPS binding protein (LBP) is required, as it ligates LPS in blood and in the extracellular tissue fluid. Moreover, CD14 that is expressed on many immune cells, including lymphocytes (Jersmann, 2005) and augments the sensitivity of cells to the presence of LPS (Murphy et al., 2008). The expression of CD14 on B cells is not well documented, although the murine B cell line CH12, that responds to low doses of LPS, has high levels of CD14 (Kimura et al., 2000). TLR4 is expressed in all murine B cell subpopulations, with little differences in the level; its signalling has a huge impact on the life of these cells, in fact it can induce diverse effects, as proliferation, cytokines release, migration, differentiation and class switch recombination (Gururajan et al., 2007).

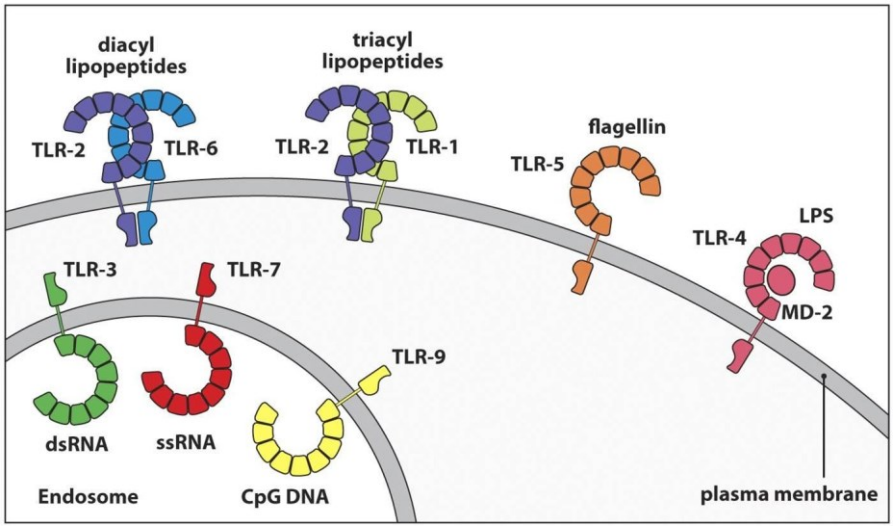


Figure 3. ***Cellular localization of mammalian TLRs****. TLRs are localized both on the surface of the cell and in the endosomal compartment, they all share a common structure. Adapted from* (Murphy et al., 2008)

3.1.4. B cell subsets

B cells can be classified according to several criteria, such as their developmental stage, their localization, their phenotype and functions. In general, B cells are broadly divided into B1 B cells and B2 B cells, that comprises MZ and FO B cells.

Murine B1 cells are mainly localized into the peritoneal and pleural cavity but a small fraction is also present in the spleen. Their main known function is the production of natural IgM Abs, which have several roles such as pathogen neutralization, complement activation, cell-mediated cytotoxicity triggering and prevention of autoimmunity through clearance of dsDNA (Panda et al., 2015). B1 B cells constitute are a self-renewal population, and can be further subdivided into B1a and B1b according to the expression of the CD5 surface marker: B1a are $CD5^+$ while B1b are $CD5^-$. These two subsets have different functions, as B1a B cells produce natural Ab crucial for the protection in the early stage of infections, while antibodies from B1b are necessary in the resolution phase of infections and for long-term protection (Haas et al., 2005).

An interesting aspect of these cells is that, despite the great effort in the study of their functions, their precise ontogeny is still a matter of debate. In particular, there is controversy on two different hypothesis: the “lineage model” states that two different progenitors give rise for the B1 and the B2 population, while the “selection model” proposes that the decision to become a B1 or B2 B cell depends on the type of antigens encountered (Montecino-Rodriguez and Dorshkind, 2006).

MZ and FO B cells are together called B2, but in the spleen are differently localized and have diverse functions. MZ B cells constitute a sessile population that expresses on the surface a $CD19^{+}CD21^{hi}CD23^{-}CD24^{hi}$ phenotype and is the first line of defence against blood-borne pathogens like viruses and bacteria. Differently from MZ B cells, the FO B cell compartment can also recirculate through the blood-stream into lymph nodes, but also again into the spleen. FO B cells are also called conventional B cells, as they can give rise to the formation of germinal centres to start the adaptive response of the immune system through the production of antibodies (Pillai and Cariappa, 2009).

3.1.5. Regulatory B cells

In the last years a new functional subset of B cells with immune-suppressive properties has been recognized and included in the complex network of the immune system and nowadays, B cells belonging to this group are generally called "Regulatory B cells". This expression was first introduced in 2006 by Mizoguchi and Bahn (Mizoguchi and Bhan, 2006), but observation of an immune-suppressive function for B cells goes back to the mid-seventies, when Katz and co-workers discovered that the adoptive transfer of splenocytes depleted from B cells, failed to suppress delayed-type hypersensitivity skin reaction in guinea pigs (Katz, S.I., Parker, D., and Turk, 1974). B_{reg} become a hot topic between the end of nineties and the beginning of the new millennium, starting from a work that described how B-cell deficient mice were unable to recover after the induction of experimental autoimmune encephalomyelitis (EAE), contrarily to the *wt* animals (Wolf et al., 1996). The immune-suppressive function of B_{reg} was attributed to interleukin-10 (IL-10) only in a second moment by other three different studies regarding three distinct pathological settings: colitis (Mizoguchi et al., 2002), EAE (Fillatreau et al., 2002) and arthritis (Mauri et al., 2003). From this moment on, an increasing number of works on this topic have been published, but still there is no agreement about their origins and phenotype and, unlike T_{reg}, that are uniquely identified by the expression of FoxP3 (Rudensky, 2011), an analogous transcription factor (TF) for B_{reg} has not yet been identified.

Although no specific phenotype has been attributed to B_{reg}, several B cells subsets have been reported to have an immune-suppressive activity that can be mediated by different mechanisms such as the release of cytokines, like IL-10, TGF-β or IL-35, or through cell-cell interaction, as summarized in **Table 1**. Several subsets of B_{reg} have been studied among the

murine splenic B cell population, some examples are transitional 2 marginal zone precursor (T2-MZP) (Evans et al., 2007), MZ B cells (Gray et al., 2007), B10 (Yanaba et al., 2008), Tim-1^+ B cells (Ding et al., 2011), $CD9^+$ B cells (Sun et al., 2015), killer B_{reg} cells (Lundy and Boros, 2002), plasma cells (Neves et al., 2010). Other subsets have been identified also in different murine anatomical compartments but also in humans, such as plasmablasts with suppressive function in murine lymph nodes and human blood (Matsumoto et al., 2014), but also a B10 analogue have been identified in human bloodstream (Iwata et al., 2011). These phenotypical heterogeneity suggests, although it cannot exclude, that a lineage specific progenitor for B_{reg} does not exists but, more probably some B cells belonging to different subsets have immune-suppressive capacities (**Figure 4**).

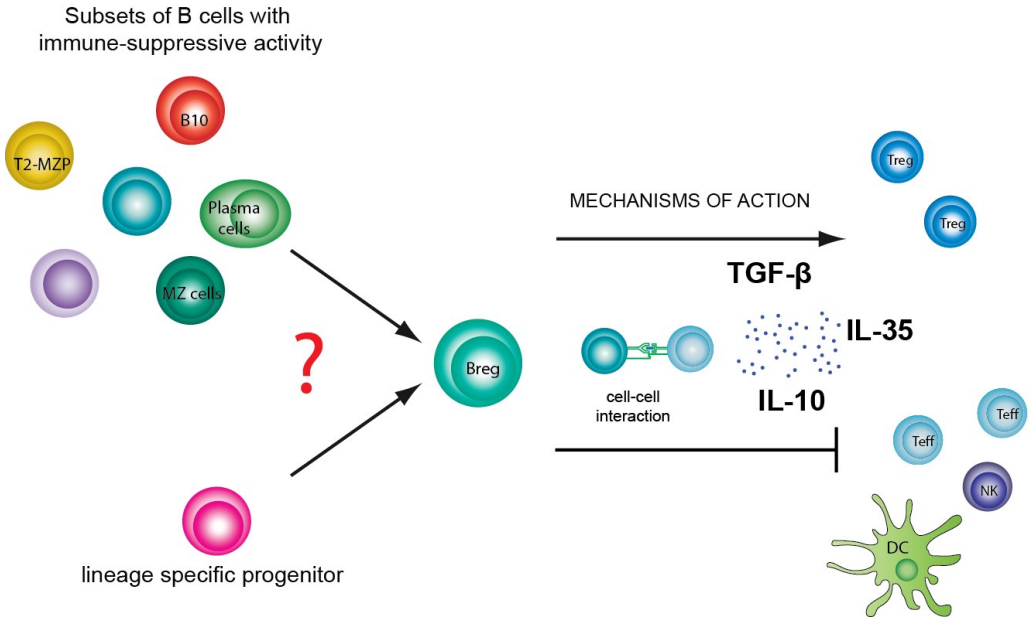


*Figure 4. **Regulatory B cells**. No lineage specific progenitor has been identified for regulatory B cellscells but, on the contrary, several B cell subsets with immune-suppressive functions have been described. Many reports indicate that these cells are able to enhance the number of regulatory T cells and suppress the action of effector T cells and of other immune cells such as natural killer, dendritic cells. both through cell-cell interaction and release of soluble factors.*

The literature is more in agreement regarding the functions of B_{reg}, which can act both through cell to cell interaction and release of soluble factors. B_{reg} influence other cells of the immune system, in particular they are able to induce the T_{reg} population and, at the same time suppress the differentiation of T_H1 and T_H17 by inhibiting the production of inflammatory cytokines by DCs (Carter et al., 2011). The action of B_{reg} is not restricted to the lineage of T cells, but it has been demonstrated that they can target also DCs, limiting their proliferation (Matsushita et al., 2010). The interaction between B_{reg} and other cells

of the immune system is important also for their expansion and/or differentiation: the IL-10 competent B cell population was shown to be impaired in mast cell deficient mice (Mion et al., 2014a), while in the human system the B_{reg} population was expanded in the presence of plasmacytoids dendritic cells (Menon et al., 2016).

Nowadays, the most studied B_{reg} subsets are those that produce IL-10, and this cytokine is used as a marker to identify them among the total B cell pool. In the specific the analysis of these cells and the mechanisms involved in IL-10 production is moving to the ''omics'' and also to the epigenetic field as demonstrated by the increase in number of papers reporting data about transcriptome, miRNome and epigenetic of various B_{reg} subsets (Lin et al., 2014; Sun et al., 2015; Zhang et al., 2013). The lack of these studies is that every group focus on a particular phenotypic B_{reg} subset, or on a particular type of stimulation, making it difficult to shed light on the precise origin of these cells and on the mechanisms involved in IL-10 production.

Table 1. ***Main B cell subsets with immune-suppressive functions and their characteristics****.*

B_{reg}	Surface markers	Mechanism of suppression	Notes	Reference
T2-MZP	$CD19^+CD21^{hi}CD23^{hi}CD24^{hi}$	IL-10	Induction of T_{reg}, suppression of effector CD4+ and CD8+ T cells	(Evans et al., 2007)
MZ B cells	$CD19^+CD21^{hi}CD23^-$	IL-10	Induction of T_{reg}, suppression of effector CD4+ and CD8+ T cells	(Gray et al., 2007)
B10	$CD19^+CD5^+CD1d^{hi}$	IL-10	suppression of effector CD4+ and CD8+ T cells, monocytes and DCs	(Yanaba et al., 2008)
Plasma cells	$CD138^+$MHC-$11^{lo}B220^+$	IL-35, IL-10	Suppression of NK, neutrophils and effector CD4+ T cells	(Shen et al., 2014)
	$CD73^{hi}$ B-1	IL-10, adenosine	Suppression of T cell activation	(Kaku et al., 2015)
Tumor evoked B_{reg}	$CD19^+B220^+CD25^+$	TGF-b	Induction of T_{reg}, metastasis promotion	(Olkhanud et al., 2011)
Tim-1+ B cells	$CD19^+$Tim-1^+	IL-10	Suppression of effector CD4+ T cells	(Ding et al., 2011)
Killer B_{reg} cells	$CD19^+$ $FasL^+$	FasL, IL-10	Promotion of T cell death	(Lundy and Boros, 2002)

3.2. Epigenetics

Epigenetics, literally “above genetics”, resemble all changes resulting in alterations of the phenotype without directly involving the DNA sequence (Arrowsmith et al., 2012). These peculiar modifications are heritable and can alter gene expression acting at various levels: from modifications at the level of DNA residues and histone tails, to the action of miRNA and other non-coding RNA.

The DNA in the nucleus of cells is not present as single filament but complexed with proteins, that together form a structure called chromatin. Two forms of chromatin exist: euchromatin that has a loose composition and is highly accessible to the transcription machinery, and heterochromatin, that instead is dense and transcriptionally silent. The basic unit of chromatin are nucleosomes, that are composed by eight different proteins called histones, on which the DNA is wound, each nucleosome has 147 bp of DNA wrapped on it. Chromatin is further organized in a cell-type specific manner, for example nucleosomes located on the same genomic region can carry different modifications depending on the cell type, thus determining changes in the regulation of the genes located in that DNA section. The degree of difference in the chromatin structure among different cells is mainly concentrated within particular regions, that thanks to their capacity of regulating gene expression, are able for example to influence the differentiating fate of a cell, and also its behaviour. These peculiar regions are represented by promoters and enhancers, that collectively can be called *cis*-regulatory regions (CREs). These DNA regions are relatively short (up to 1500 bp), and differently localized: promoters are proximal to the transcription starting site (TSS), instead enhancers are positioned also several kb upstream or downstream from the TSS. Enhancers can recruit general coactivators, such as p300/CBP, and serve as binding sites for TFs. They loop on the TSS where the promoter is located and enhance the transcription of their target gene.

Chromatin is highly dynamic and its structure can be modified both by remodelling complexes and modifications directed on nucleosomes. In the context of the immune system, it is becoming clear that there is a connection between the chromatin structure near some genes and the specificity of the immune response (Smale et al., 2014). In particular, chromatin remodelers can act on the chromatin structure in order to generate a stimulus- and tissue-specific response, in a way that makes it possible, for example, to

maintain genes involved in inflammation in a repressed state under normal conditions, but also to rapidly induce them after receiving the right trigger (Smale et al., 2014).

3.2.1. Transcriptional control concepts

Transcription can be divided in four different steps: initiation, promoter proximal pausing/release, elongation and termination. The critical phase is the transition of the RNApol II from the binding on the promoter to the entering into productive elongation, which is made possible both by the 5' capping of nascent RNA and the phosphorylation of the Pol II by the protein kinase positive transcription elongation factor (P-TEFb). Before starting transcribing the gene, RNA pol II is stabilized on the proximal promoter thanks to the negative elongation factor (NELF) and the DRB sensitivity-inducing factor (DSIF), as shown in **Figure 5A**. The binding is disrupted after the recruitment of P-TEFb that is mediated by activator proteins like c-Myc or NF-kb, or by coactivators such as BRD4 (**Figure 5B**). The Pol II is then released when NELF is phosphorylated and, as shown in **Figure 5C,** at this point the elongation starts (Kwak and Lis, 2013).

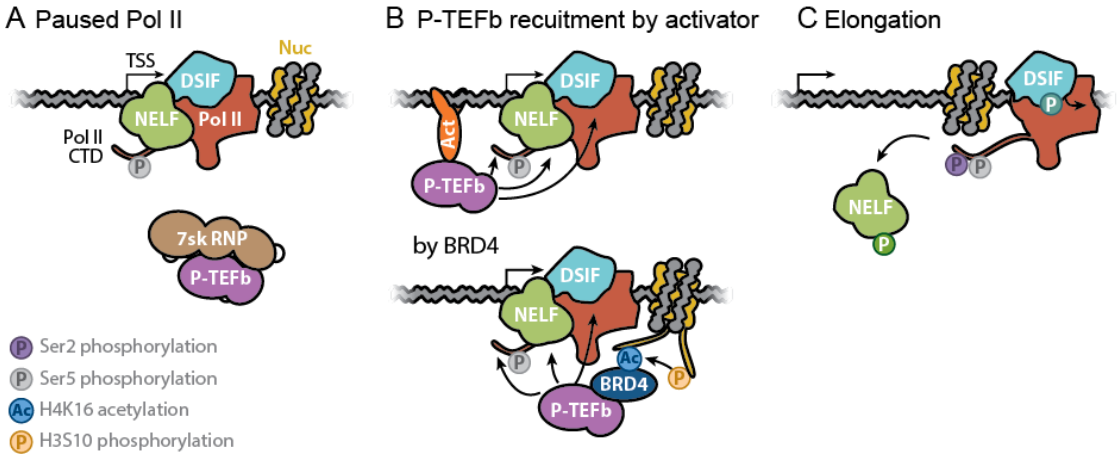


Figure 5. ***Transcriptional elongation control. (A)*** *Paused Pol II is bound by DSIF and NELF on the promoter.* ***(B)*** *Pol II is released after P-TEFb recruitment by activator or BRD4.* ***(C)*** *Finally NELF is dissociated and the elongation starts. Adapted from* (Kwak and Lis, 2013).

A fundamental aspect of transcription is the time frame occurring between cell triggering and the start of transcription. In this view, genes can be classified into primary-response genes (PRGs) and secondary-response genes (SRGs) if, after stimulation, they are transcribed in the first two hours or later, respectively (Medzhitov and Horng, 2009).

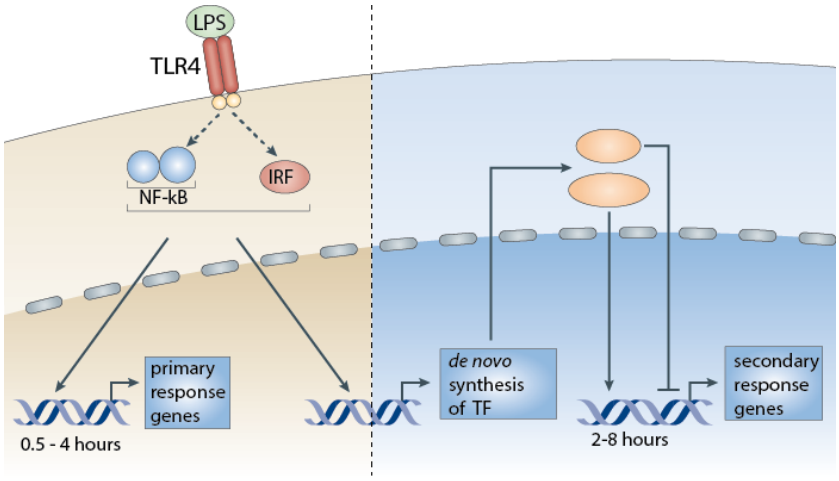


Figure 6. ***Primary (PRGs) and secondary (SRGs) response genes****. PRGs are transcribed within 4h from stimulation by TF already present in the cell, while SRGs require new protein synthesis in order to be transcribed. Adapted from* (Medzhitov and Horng, 2009).

Expression of PRGs depends on the action of TFs constitutively expressed, such as NF-κB that, following cell stimulation, translocates into the nucleus and activates transcription (Saccani et al., 2002). SRGs require instead *de novo* synthesis of TF induced by stimulation (Smale et al., 2014) (**Figure 6**).

In the context of the immune system, PRGs and SRGs have been well characterized in LPS-stimulated macrophages, where the differential timing in the regulation of expression has been linked not only to the action of different type of TF, but also to chromatin structure. In particular, it has been discovered that the majority, but not all, of PRGs are independent from the action of the SWItch/Sucrose Non-Fermentable (SWI/SNF) complex. The SWI/SNF complex can remodel nucleosomes weakening the interaction between DNA and histones, both by sliding on the DNA or directly removing them (**Figure 7**). The fact that PRGs do not need the action of the SWI/SNF complex, suggests that their promoters are almost devoid of assembled nucleosomes (Ramirez-Carrozzi et al., 2009). These characteristics suggest a model in which the SWI/SNF independent genes are rapidly induced by general stimuli (as their promoter are free of nucleosomes), while SWI/SNF dependent genes (the majority of SRGs) need a selected set of stimuli as they require the action of remodelling complex to be activated.

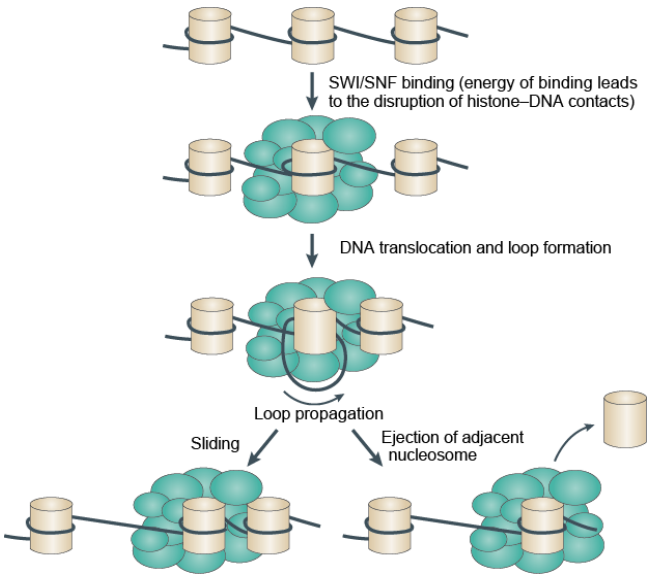


Figure 7. ***SWI/SNF chromatin remodelling complex.*** *SWI/SNF chromatin remodelling complex is able to weak the interaction between DNA and protein, both by sliding nucleosomes or directly removing them. Adapted from* (Wilson and Roberts, 2011).

Interestingly, a gene can be either primary or secondary depending on the specific cell type. An example is made by the *il-6* gene, that is a primary SWI/SNF-independent gene in fibroblasts, while it is a secondary SWI/SNF-dependent gene in macrophages. This can be partially explained by the function of this cytokine in the two different cell types: the IL-6 released by fibroblast functions just as a general systemic mediator of the acute phase of inflammation, on the contrary the same cytokine produced by macrophages is necessary to modulate the T cell differentiation and so is part of a more specific and tightly regulated process (Smale et al., 2014).

Overall, these evidences lead to the idea that chromatin structure can influence the expression and the specificity of a response.

3.2.2. DNA methylation

About the 70-80% of CpGs in the genome are methylated and can be found either in CpGs island (CGIs), which are DNA segments > 200bp with > 50% of CG content, or as isolated dinucleotides. Methylation of cytosine residues located in CpGs dinucleotides is the most common DNA covalent modification in mammals. The methyl group is added at the 5' position of cytosine, forming 5-methylcytosine (5mC), by three DNA methyltransferase (DNMT) enzymes. DNMTs can be divided into *de novo* and maintenance methyltransferase. In particular, Dnmt1 recognizes hemimethylated filaments during DNA replication and

maintains the methylation pattern on the new strand. Dnmt3a and Dnmt3b are instead two *de novo* methyltransferases, and are more active during embryo development (Rose and Klose, 2014).

The percentage of methylation among the mouse genome of embryonic stem cells varies depending on the genomic localization, as demonstrated in a paper published by Stadler and co-workers, in which it was shown that CpGs methylation is lower at promoter regions, but higher on genic and intergenic portions (Stadler et al., 2011a), as shown in **Figure 8**. They further classified genomic regions on their percentage of methylation (not on their localization), identifying three different classes: fully methylated regions (most of the genome), unmethylated regions (mostly promoters) and low-methylated regions (LMRs). They pointed their attention on LMRs that are characterized by evolutionary conservation, low CpG content and have chromatin features resembling enhancers, suggesting for them a regulatory function. Interestingly, they also found an enrichment of 5-hydroxymethylcytosine (5hmC) on LMR, a modification correlated to enhancers (Pastor et al., 2011) and to a dynamic change in DNA methylation, since it is an intermediate state that occurs before cytosine demethylation.

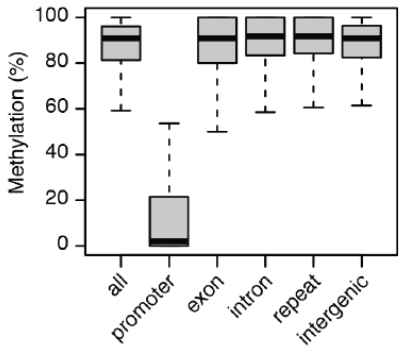


Figure 8. ***Methylation distribution in mouse methylome.*** *Across the genome the promoters are almost unmethylated, while the majority of methylation is located in intra and intergenic regions. Adapted from* (Stadler et al., 2011a).

DNA demethylation can occur as a passive process, when the methylation pattern is not maintained in DNA duplication, or as an active mechanism thanks to particular enzymes. As summarized in **Figure 9**, two pathways are involved in cytosine demethylation: oxidation of 5mC by Ten-Eleven Translocation (TET) protein and DNA repair-based mechanisms. The TET enzyme catalyses the oxidation of 5mC into 5hmC, that is further transformed into 5-formylcytosine (5fC) and 5-carboxylcysteine (5caC); the modified bases are then recognized

by the thymidine DNA-glycosylase (TDG) and excised. In the formed abasic site is finally restored the unmodified C (Kohli and Zhang, 2013).

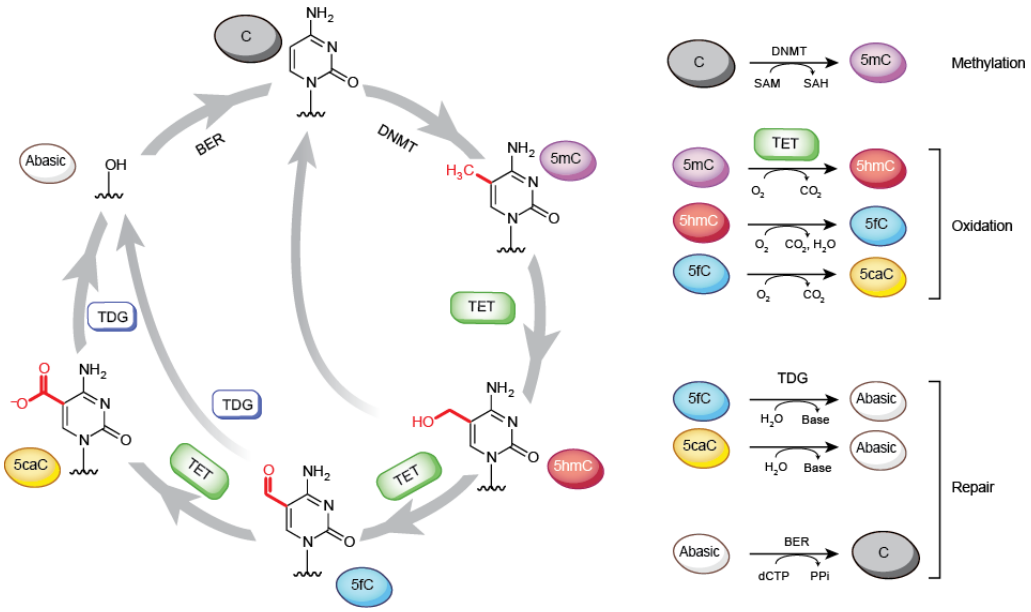


*Figure 9**. Dinamic DNA methylation and demethylation**. DNA methylation is a dynamic process, active demethylation depends on the action of TET and TDG enzymes. Adapted from* (Kohli and Zhang, 2013).

Since the seventies when it was discovered, DNA methylation was usually correlated to gene silencing (Holliday and Pugh, 1975; Riggs, 1975), but new approaches such as genome-wide studies of the methylome shed light on its more variable function. It is known that methylation localized near TSS prevents transcription initiation, instead it appears that methylation along the gene body can enhance transcription elongation. Moreover, methylation localized at enhancer and insulator can alter their regulatory function (Jones, 2012). In general, the mammalian genome tends to be unmethylated at CGIs and highly methylated on other regions. Demethylated CGIs are often located at promoters of fully or potentially active genes, whereas methylated CGI are linked to stable silencing, like for genes located on the inactive X chromosome. An exception to this rule is represented by intragenic CGIs: these genomic portions can be fully methylated, but yet they do not alter the transcription elongation of the gene (Maunakea et al., 2010). Conversely, methylation at regulatory regions such as enhancers is still a challenge. As already mentioned, they are usually CpG-poor, and present a variable percentage of methylation suggesting that CpG sites are in a dynamic state (Stadler et al., 2011b). There are evidences that differential methylation at these regions can influence gene transcription, and an example is represented by a study that compares T_{reg} and conventional T cells. The authors found that

the main differences are located at regions distal from promoters, but also that a correlation between the methylation level of some enhancer with the regulatory activity of T_{reg} exists (Schmidl et al., 2009).

3.2.3. Histones modifications and their functions

Each nucleosome contains histone octamer composed by two copies of histone H2A, H2B, H3 and H4, their involvement into gene transcriptional control depends on modifications of their tails. Nowadays, up to 60 modifications have been described, the most well-known and characterized are methylation, acetylation, phosphorylation, ubiquitination and sumoilation. Depending on the modified tails, each alteration has different effects on chromatin structure and gene expression (Bannister and Kouzarides, 2011). For the purposes of this thesis, only acetylation and methylation signatures and their functions will be described, as these are the most studied and more abundant modifications among the genome.

Histone modifications are not only static signatures, but in some ways they actively contribute to transcriptional regulation interacting, for example, with TFs and the assembly of complexes. Proteins and complexes involved in epigenetic regulation can be divided in three main classes (**Figure 10**): *epigenetic writers*, that directly modify histones adding chemical groups to them, *epigenetic erasers*, that remove modifications, and *epigenetic readers* which have the function to recognize all these changes (Arrowsmith et al., 2012).

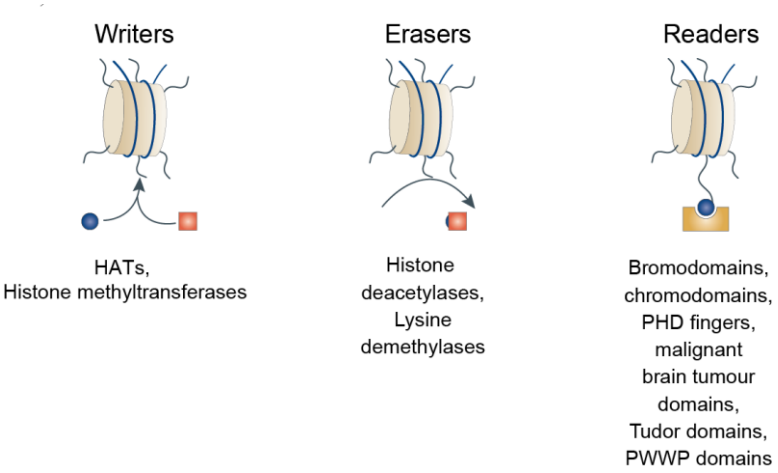


Figure 10. ***Classes of proteins involved in epigenetic regulation of gene transcription.*** *Proteins that can covalently add methyl or acetyl groups to histones tails are called ''writers'', while proteins that can recognize these modifications are generally called ''readers'' and finally enzymes that can remove histones marks are called ''erasers''. Adapted from* (Arrowsmith et al., 2012)

Epigenetic writers

Acetylation occurs on lysine residues located at the N-terminus of histones and depends on the balance between the action of two different classes of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). Basically, by adding an acetyl group, HATs neutralize the positive charge of the lysine, diminishing in this way the binding between DNA and histones, leading to a more open chromatin: for this reason acetylation associates with expressed genes (Bannister and Kouzarides, 2011). HATs are classified into three main classes on the structure of their catalytic domain: the Gcn5 N-acetyltransferases (GNAT) family, the MYST-like HAT domain (MYST) family and the p300/CPB (CREB binding protein) family (Lee and Workman, 2007). Histone proteins can be methylated both on arginine and lysine residues and, compared to acetylation, the situation is more complex. In fact, lysine may be mono-methylated (me1), di-methylated (me2) or tri-methylated (me3) and, depending on the position of the residue modified, methylation can have both an activating or repressing effect on gene transcription. For example, tri-methylation of lysine 4 (K4) of histone H3 (H3K4me3) located near TSS is correlated with gene expression, on the other hand, the same modification on lysine 27 (H3K27me3) is associated with repressed genes. Methyl marks are added by specific histone methyltransferase: every modification has its specific modifier. In mammals H3K4 is the substrate for the MLL/Set1 family, which comprises six different proteins with a SET catalytic domain, capable to methylate the H3K4 transferring the methyl group from S-adenosylmethionine (Calo and Wysocka, 2013); the H3K27me3 modification, instead, is catalysed by a different protein called Enhancer of zeste homolog2 (EZH2) (Kuzmichev et al., 2002).

Epigenetic erasers

Among the *epigenetic erasers* we find lysine demethylases: each modification is targeted by a specific protein or complex. For example, Lysine-specific demethylase 1 (LSD1) removes the methyl group from H3K4me1/me2 (Shi et al., 2004), H3K4me3 is instead demethylated by the JARID1 subfamily, proteins belonging to the bigger Jumonji C domain protein family (JmjC) (Christensen et al., 2007). On the other hand, acetylation is removed from histones by HDACs, their action is however less specific from demethylase, so in general more HDACs can have the same target (Bannister and Kouzarides, 2011).

Epigenetic readers

This category comprises a broad variety of protein families spanning from TFs, epigenetics writers themselves (e.g. p300) and transcriptional regulators (e.g. BRD4). A great number of interaction between *epigenetic readers* and histone modifications have been described (Musselman et al., 2012), so just a few examples will be reported. At the promoter level, the tri-methylation at H3K4 is recognized by TAF3, a PHD (Plant Homodomain) protein that is part of a complex implicated in transcription (Vermeulen et al., 2007). The already mentioned transcriptional controller BRD4, a bromodomain protein, specifically recognizes acetylated histone tails and binds a subunit of P-TEFb, allowing the transcriptional pause release (Moon et al., 2005). Regarding enhancers, that are enriched in H3K4me1 (as it will be explained later), it has been reported that the acetyltransferase TIP60 (belonging to the MYST family) recognizes them and transfers acetyl groups allowing the formation of a more relaxed chromatin (Jeong et al., 2011).

3.2.4. Specific epigenetic signature of promoters and enhancers

In the last years, advances in techniques for chromatin study, like ChIP-sequencing (ChIP-seq), allowed to define precise signatures for regulatory regions at a genome-wide level. In general, promoters are characterized by H3K4me3, enhancers by H3K4me1, while both are enriched in H3K27Ac upon activation; moreover, when these regions are silenced, H3K27me3, a marker of closed chromatin, is found. Epigenetics marks create a “snapshot” of the activation status of the whole genome of a cell, allowing the recognition not only of active and repressed genes, but also of genes which are ready to be rapidly transcribed when there is the need, these genes are called poised, or primed, genes.

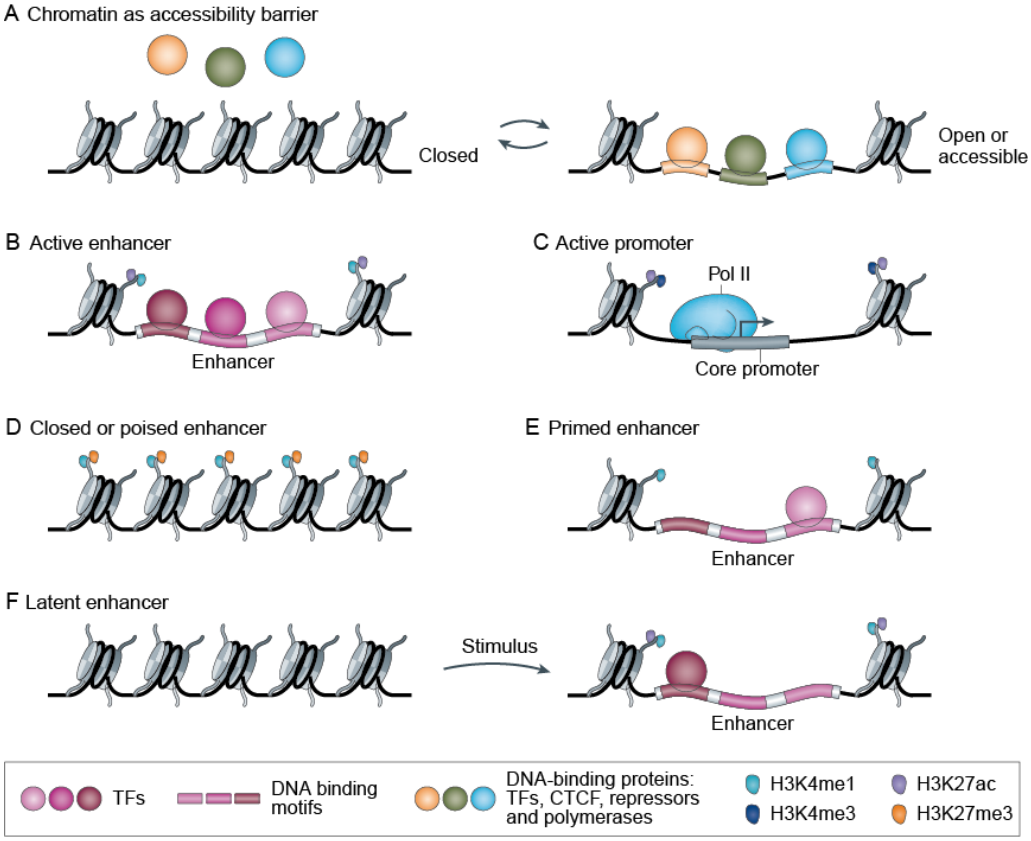


Figure 11. ***Chromatin accessibility and histone marks at enhancers and promoter****.* ***(A)*** *Chromatin accessibility to TFs, DNA-binding proteins and other proteins depends on nucleosomes density.* ***(B)*** *Histones flanking active enhancers are characterized by H3K4me1 and H3K27ac, while at active promoters* ***(C)*** *H3K4me3 and H3K27ac are found.* ***(D)*** *At closed or poised enhancers are present both H3K4me1 and the repressive mark H3K27me3, primed enhancers instead are identified by the sole presence of H3K4me1* ***(E)****. Latent enhancers* ***(F)*** *are located in regions with compact chromatin, but after stimulation can acquire the signature of active enhancers. Adapted from* (Shlyueva et al., 2014)

Promoters

Promoters are located at the 5' of genes and have a fundamental role in transcription initiation. From an epigenetic point of view, they are generally characterized by the co-presence of H3K4me3, H3K27Ac and RNA pol II when located near active genes, as shown in **Figure 11C**. At promoters of poised gene instead H3K4me3 and RNA pol II (without mRNA presence) are found, in this way transcription can be activated immediately after the stimulus. This situation is of a particular interest for cells of the immune system that are required to respond immediately after antigen triggering (Lenhard et al., 2012; Lim et al., 2009).

The activity of a promoter depends also on its intrinsic structure, according to their CpGs percentage, these genomic regions can be classified in high (CGIs promoters) and low CpG-content promoters. High CpG-content promoters, located near constitutively active genes,

tend to have an accessible chromatin, since the presence of CGIs leads to unstable nucleosome assembly; therefore, these genes are also SWI/SNF-independent genes and with a primary-response kinetics (Ramirez-Carrozzi et al., 2009). CGIs promoter at active genes are almost unmethylated, since the presence of H3K4me3 impedes the interaction of DNMT3L with the DNA strand (Ooi et al., 2007). On the other hand, genes coupled with CpG-poor promoters are subjected to a tighter regulation and at basal state are inactive. Contrarily from CGIs promoter, they are enriched in assembled nucleosomes and the expression of their associated genes depends on the action of SWI/SNF complex (Ramirez-Carrozzi et al., 2009). Interestingly, they are often located near genes encoding proteins specific for terminally differentiated cells (Zhou et al., 2011).

Enhancers

The fundamental importance of enhancers in lineage determination and maintenance of cell identity is intrinsic in their capacity to drive specific gene expression induced by environmental stimuli. These genomic DNA portions are classically identified by a higher ratio of H3K4me1 compared to H3K4me3, the binding of co-activators and HATs (e.g. CBP/p300) and the enrichment for TF binding site (Heintzman et al., 2007). They can be classified into poised enhancers, which are characterized by the sole presence of H3K4me1 or by the co-presence of H3K4me1 and H3K27me3 (**Figure 11D**), and active enhancers, which are also enriched in acetylation (**Figure 11B**) (Jeong et al., 2011). A third class of enhancers, named latent enhancers (**Figure 11F**), has been recently discovered in LPS-stimulated macrophages (Ostuni et al., 2013). In resting cells these regions completely lack any known enhancer-specific marker but, after the appropriate triggering, they acquire a mono-methylated signature.

The functionality of enhancers, or the indication of their activation, is influenced also by the methylation status of CpGs sites. Keeping in mind that enhancers localize with LMR, recent studies at a genome-wide level detected a correlation between their activity and low methylation levels. It has been demonstrated that the presence of CpG methylation correlates with the absence of the binding of TF and coactivators (Stadler et al., 2011b). However, the most interesting features, is the discovery that methylation at enhancers is a very dynamic process. A hint is the co-presence of 5hmC, on intermediate of the active demethylation pathway, and H3K4me1 and H3K27Ac (Sérandour et al., 2011). These data

leads to the hypothesis that, if a cell expresses demethylases proteins (e.g. TET), the activation of an enhancer could involve active DNA demethylation (Yu et al., 2012).

All the cells belonging to the immune system originate from the HSC; however, they are able to act in a cell- and stimulus-specific manner building a very intricate and finely regulated response. Moreover, immune cells have also the ability to remember their experience, allowing them to refine an eventual second response (Smale et al., 2014). These mechanisms depend, at least in part, from the action of enhancers. These peculiar genomic regions are fundamental for the cell-fate determination: in the early stages of the development from the HSC, the so-called pioneer factors, such as PU.1 for macrophages or Pax5 for B cells, are able to shape the genomic environment, establishing a permissive-region at the level of cell-type specific enhancers (Winter and Amit, 2014). Once cell-identity is established, enhancers, and more in general, the chromatin landscape surrounding a gene, are able to influence the response of a cell to a specific stimulus. An example comes from the human setting, where neutrophils are not able to produce IL-10 contrarily from monocytes and, in this case the explanation of this difference is found in the chromatin status of the *il-10* gene. While monocytes are characterized by the presence of H3K27Ac along the locus, neutrophils are completely devoid, linking their inability to produce this cytokine to the non-permissive status of chromatin (Tamassia et al., 2013).

An interesting aspect of many cell types of the immune system, such as macrophages, DC or neutrophils, is their plasticity: they are able to change their transcriptional output without affecting cell-identity. In other words, after the exposure to different stimuli, a "new" cellular subtype with specific functions is generated (Biswas and Mantovani, 2010). A major role in the determination of cell plasticity is played by enhancers and, in particular, by the recently described latent enhancers discovered in murine macrophages after a short-term stimulation with LPS. The most interesting feature of these latent enhancers is that, once cells are deprived of the source of stimulation, the classical H3K4me1 marker of enhancers is retained, allowing a faster and stronger induction of the related gene. This confers to the cell an epigenetic memory for the specific stimulation and allows the establishment of different functions that distinguish them from all the other cells of the population (Ostuni et al., 2013).

3.2.5. MicroRNA

Another mechanism by which gene expression can be controlled is through the action of non-coding RNA (ncRNA), that can be divided into long ncRNA and small ncRNA. Among the family of small RNA, the most represented are the micro RNA (miRNA), which function is to bind the 3'UTR of target mRNA genes, driving a post-transcriptional negative regulation through the induction of mRNA degradation or the block of translation.

miRNA biogenesis

MicroRNAs are single stranded RNA made by 20-23 nucleotides, which biogenesis starts when they are initially transcribed into primary miRNA (pri-miRNA) by RNA Pol II (**Figure 12**). The shape of pri-miRNA is quite peculiar, as it is made by a double-stranded hairpin stem, a terminal loop and two single-stranded regions at the ends. The pri-miRNAs are then cleaved at 5' and 3' ends by the endonucleases Drosha and DGCR8 (DiGeorge critical region 8) into pre-miRNA. At this step, pre-miRNAs are exported from the nucleus, by Exportin-5 complexed with Ran-GTP, to the cytoplasm, where they can finally mature. Once in the cytoplasm, the hairpin is cleaved by DICER, that cuts the loop generating a 22nt miRNA duplex, with two nucleotides protruding at each 3' end. After the cleaving, Dicer dissociates from the miRNA duplex, that can be bound by the effector RNA-induced silencing complex (RISC). RISC is composed by one helicase that disrupts the duplex separating the guide strand from the one complementary to the target gene, by Argonaute2 (Ago2), that mediates the effector function of the complex, and by the miRNA itself. After the binding to the 3'UTR of its target, RISC can silence gene expression following three main pathways: inducing the cleavage of the deadenylation of the target mRNA or through the inhibition of translation (Winter et al., 2009).

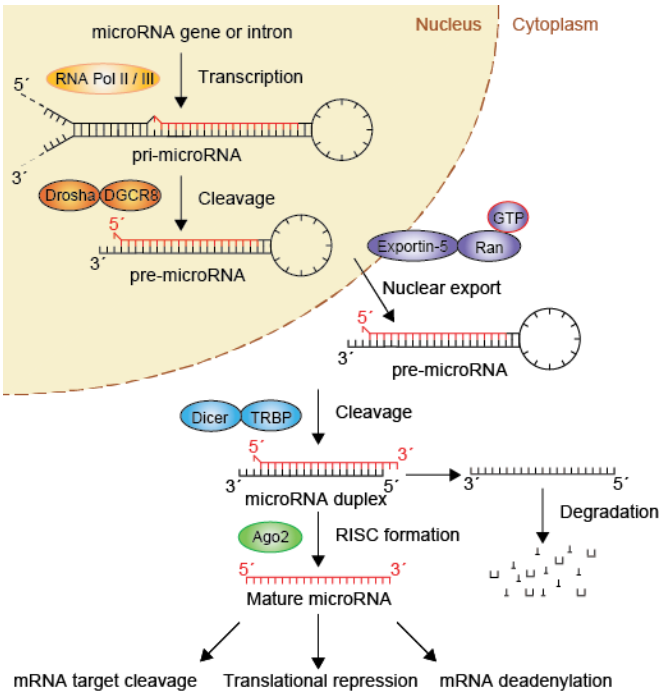


Figure 12. ***miRNA biogenesis****. The pri-miRNA is synthetized in the nucleus, and initially cleaved by Drosha to form the pre-miRNA that is exported in the cytoplasm. The pre- miRNA is then processed by Dicer and the final miRNA is loaded to the RISC complex that mediates the cleavage of the mRNA target, the translational repression or the mRNA deadenylation. Adapted from* (Winter et al., 2009).

miRNA and TLR signalling

The role of miRNAs was initially studied in the regulation of the development and differentiation of cells, for example miR-223 expression is peculiar for myeloid cells, while miR-181 is specific for the B cell lineage (Chen et al., 2004). However, the action of miRNAs is not limited to lineage determination, in fact they are also involved in the regulation of the immune response. The first profiling of miRNAs induced by TLR signalling was performed in 2006 on human macrophages, when the authors of the study described an induction of miR-155, miR-146 and miR-132 after LPS triggering (Taganov et al., 2006). Of note, bioinformatics analysis and experimental evidences show that binding sites for miRNAs are located in the 3'UTR of cytokines and chemokines, for example the *il-6* gene is targeted by the let-7 family of miRNAs, suggesting a direct action of miRNAs on its expression (Iliopoulos et al., 2009). Targeting different cytokines and chemokines confers to miRNAs potential anti or pro-inflammatory properties. An anti-inflammatory miRNA is miR-146a since a consequence of its increase, in monocytes, is the down-regulation of several pro-inflammatory chemokines and cytokines such as TNF, IL-1β and IL-6 (Nahid et al., 2009). An opposite example is represented by miR-155 that is able to promote a pro-

inflammatory response as it can suppress the expression of negative regulators of TLR-signalling such as SOCS1 (Wang et al., 2010). Interestingly, after TLR induction, the immune-regulatory cytokine IL-10, is able to inhibit miR-155, amplifying the anti-inflammatory effect (McCoy et al., 2010). In light of the fact that miRNAs can have both pro- and anti-inflammatory properties and can act at different levels of the immune response, O'Neill and co-workers hypothesized their role in the resolution of inflammation (O'Neill et al., 2011). In particular, TLR triggering induces the upregulation of miR-155 that limits the expression of TLR-pathway repressors and promotes the expression of pro-inflammatory cytokines. Upregulation of pro-inflammatory mechanisms is a consequence of the expression of miR-146 that can negatively regulate the immune response turning off TLR-signalling. Its action is facilitated by miR-21 that enhances the expression of IL-10 (Sheedy et al., 2010), starting in this way the negative feed backing that downregulates miR-155 and switch off the TLR pathway.

3.3. Interleukin-10

IL-10 belongs to the IL-10 family of cytokines that comprises also IL-19, IL-20, IL-22, IL-24 and IL-26, and was originally described by Mosmann *et al.* as a factor able to inhibit the production of other cytokine (Fiorentino et al., 1989). Specifically, it was shown that, when secreted by T_H2, it diminished the synthesis of IL-2 and IFN-γ by T_H1 cells. Over years, it has been discovered that IL-10 has pleiotropic functions in the regulation of the immune response and moreover that is produced by many type of cells belonging both to the adaptive and innate immune system (Saraiva and O'Garra, 2010). Interestingly, IL-10 is a very conserved cytokine, in fact a viral form of IL-10 has been described in Epstein-Barr and other viruses. Its main function is to down modulate the production of IFN-γ and other inflammatory cytokines by peripheral blood cells after infection (Ouyang et al., 2014).

3.3.1. Locus and protein structure

The *il-10* gene is located both in mouse and human on chromosome 1, on the qE4 and q32.1 segments, respectively. The gene is near other members of the IL-10 family, indeed *il-19*, *il-20* and *il-24* are located upstream of *il-10*. Downstream, the coding sequence for the *Mapkapk2* (Mitogen-activated protein (MAP) kinase-activated protein kinase 2) is found. The *il-10* gene is highly conserved and, in both species, it comprises 5 exons and 4 introns, moreover, a discrete number of conserved non coding sequences (CNS) with human are found among the *locus* (Gabryšová et al., 2014). The TATA box in mice is located among 98 and 95 bp upstream from the TSS (the first methionine), while in human it is between 91 and 88 bp (Mosser and Zhang, 2008). Both in human and mouse, the protein is made by 178 aa and is secreted as a homodimer composed by two monomers non-covalently bound, as shown in **Figure 13**.

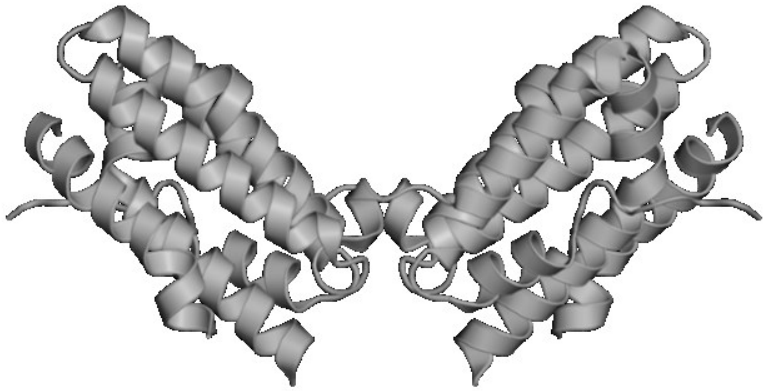


Figure 13. ***Human IL-10****. IL-10 is a very conserved protein and both in mouse and human, is a homodimer composed by two monomers non-covalently bound. Image from UniprotPDB*

3.3.2. IL-10 functions

IL-10 has a wide range of functions, both pro- and anti-inflammatory, and for this reason it is considered an immune-regulatory cytokine. Its main anti-inflammatory characteristics are the capacity to inhibit the activity of macrophages and DCs through the blocking of inflammatory mediators such as TNF-a, IL-1b, IL-6, but also cytokines required for T cell differentiation and proliferation (Hedrich and Bream, 2010). On the other hand, IL-10 has been described as a differentiation/growth factor for B cells as well as for mast cells, NK and other cells (Burdin et al., 1997).

IL-10 has been described to have functions in many pathological settings, for example it has a fundamental role in gut homeostasis, as IL-10 deficient mice spontaneously develop severe inflammatory bowel disease (Moore et al., 2001). Moreover, IL-10 loss of function, described in five patients, determines a non-treatable infantile Crohn's-like disease (Glocker et al., 2011; Kotlarz et al., 2012). Unfortunately, the use of recombinant IL-10 in clinical trials for Crohn's disease did not lead to the expected results, in fact there were no significant benefits in patients after the treatment (Buruiana et al., 2010). The involvement of IL-10 has been described also in autoimmune diseases, in particular, high levels of IL-10 in the serum have been reported for patients with systemic lupus erythematosus (Mongan et al., 1997), although its precise role still remains unclear. The cytokine is involved also in the control of inflammatory response after an infection, in fact it can dampen the action of T_H1, NK cells and macrophages. The crucial point in this setting is the timing of the release, indeed, if IL-10 is produced too early it will switch off the response allowing the persistency

of the infection; on the other hand, the absence of IL-10 will result in tissue damages caused by overwhelming infections (Couper et al., 2008).

3.3.3. IL-10 transcriptional regulation

IL-10 is produced by cells belonging to both the innate and adaptive immune system. However, the pathways controlling this mechanism are quite well characterized in macrophages, DCs and T cells, while for B cells, mast cells and T_{reg} less is known. The regulation of *il-10* gene expression in all these subsets does not depend on a single TF or a unique pathway, but rather, several different proteins have been described to activate the gene, depending both on the cell type and stimulation (Saraiva and O'Garra, 2010).

In macrophages and DCs, IL-10 can be induced by the stimulation through several TLRs that mediate the activation, through adaptor proteins, of several signalling pathways such as MAPK pathway, the phosphatidylinositol 3 kinase (PI(3)K)- AKT pathway, the nuclear factor (NF)-κB pathway and interferon regulatory factors (IRF) (Kawai and Akira, 2010). Many studies in macrophages demonstrate that, upon stimulation through TLR, a fundamental role is played by the MAPK extracellular signal-regulated kinase (ERK) and moreover, the absence of the upstream kinase tumour progression locus 2 (TPL-2) implies a reduction of IL-10 production compared to wild-type cells (McNab et al., 2013). Downstream to the ERK-dependent pathway, the activator protein -1 (AP-1) can be activated and is associated with positive regulation of IL-10. Another MAPK described in the regulation of IL-10 is p38: in human macrophages it has been demonstrated that the binding of the TF Sp-1 to the promoter, depends on the activation of this protein (Ma et al., 2001).

Another important pathway for IL-10 induction is the one dependent on NF-κB activation, noteworthy is the fact that macrophages with impaired NF-κB activation present a reduction in IL-10 production (Kanters et al., 2003). Moreover, in the context of this thesis, it is of primary interest the finding from Saraiva and colleagues (Saraiva et al., 2005) that the p65 subunit of NF-κB is recruited to a region 4.5 kb upstream from *il-10* TSS following the stimulation of macrophages through TLR4.

Unlike macrophages and DC, the T cell population is not able to produce IL-10 immediately upon microbial stimulation, but rather needs to differentiate in T_H1, T_H2 or T_H17 and, of note, the cytokine can be produced by all three subsets (Saraiva and O'Garra, 2010). As for macrophages, the activation of MAPK has a great importance in the induction of IL-10 also

in T cells, but in this context a great effort has been made to uncover which are the specific factors that directly bind the *il-10* gene to regulate the expression of this cytokine. The TF IRF4 has been shown to bind at a region 9kb from the TSS and, further, in *irf4*$^{-/-}$ T cells *il-10* mRNA is not detected upon stimulation (Li et al., 2012). Another TF, NFAT1 has been described to bind both the promoter and an intronic region of *il-10* gene (Im et al., 2004), but when in conjunction with IRF4 it binds the region 9 kb upstream from the TSS and together the two factors are able to enhance *il-10* transcription (Lee et al., 2009).

As mentioned above, little is known regarding *il-10* transcriptional regulation in B cells. Taking advantage of specific chemical inhibitors, we demonstrated an important role of the MAPK p38 upon TLR triggering (Mion et al., 2014b). Of note, a recently published work found an implication of p38 also in a human B cell line: cells were treated with bedamustine and an increase of IL-10 production dependent on p38-Sp1 was observed (Lu et al., 2016). A role for NFAT has been described also in B cells, as the deletion of the calcium sensors STIM1 and STIM2 leads to a defective activation of this factor and, as a consequence a lack of IL-10 production (Matsumoto et al., 2011). A role for STAT3 has been documented in human B cells from peripheral blood, as B cells from SLE patients have defects in IL-10 production that can be correlated with impaired STAT3 phosphorylation (Blair et al., 2010). It has also been discovered that the B-cell linker protein (BLNK) is a critical component for IL-10 production, as BLNK deficiency significantly reduces the *il-10* transcription level (Jin et al., 2013). Very recently, a suppressive role in the induction of B_{reg} has been described for the TF Foxd3, that directly binds the *il-10* gene promoter and inhibits the transcription of the gene (Zhang et al., 2016).

3.3.4. IL-10 epigenetic regulation and more: what do we know?

Basically, epigenetic regulation lays on the chromatin structure: if the DNA is open and accessible it can be bound by TF, otherwise it cannot. A simple method to determine the chromatin status is through the identification of sites hypersensitive to DNase I digestion (HSS). Over years, these HSSs have been characterized mainly in macrophages and T cells (Saraiva and O'Garra, 2010). Upon TLR stimulation, macrophages present five different HSS located at -4.5, -2, -0.12, +1.65 and +2.98 kb from the TSS, but, very interestingly, these regions have a degree of sensitivity to digestion also in unstimulated cells, and this is consistent with their capacity to produce IL-10 immediately after stimulation (Saraiva et

al., 2005). In the same work, on the other hand, it was demonstrated that in DC the only HSS, after stimulation, is the one located at -4.5 kb. Chromatin accessibility in T cells is much more different, in fact the locus of naïve T cells is almost closed, and HSS start their formation upon differentiation. Of note differently from macrophages and DC, the region at -4.5kb does not seem to have any particular role for T cells (Saraiva et al., 2005).

There are several reports concerning the epigenetic regulation of *il-10* gene transcription in T cells. In the murine system a direct role for GATA-3 in chromatin remodelling in the *il-10 locus* has been proved. In particular, inducing the acetylation of histones H3 and H4, the TF is able to switch to a transcriptionally active status in $CD4^+$ T cells (Shoemaker et al., 2006). Dong *et al* studied CpGs methylation on *il-10* locus in human T_H cells. This group did not find a clear pattern, but only a slight demethylation in two not conserved CpGs proximal to the promoter in IL-10^+ cells compared to IL-10^-, so they hypothesized a more intricate regulation involving also histones modifications. In fact, in this region they found histones modifications correlated with transcriptional activity (Dong et al., 2007). However, the same study showed that the treatment of T_H with DNMT inhibitors induced the expression of IL-10, demonstrating that, at least in principle, its expression can be regulated by methylation. Another study instead found a region in the fourth intron that binds STAT5 and have enhancer activity and appears to be methylation-dependent (Tsuji-Takayama et al., 2008).

Few data exist in literature on the epigenetic regulation of IL-10 in B cells. A study on peripheral blood human B cells demonstrated that treatment with histone deacetylase and DNMT inhibitors enhance *il-10* mRNA level (Larsson et al., 2012). It was also demonstrated that, in murine B cells, the treatment with the HDAC inhibitor SAHA enhances IL-10 secretion (Zhang et al., 2013). Regarding innate immune cells, a correlation between the phosphorylation of histone H3 in the *il-10* promoter and the binding with TF and mRNA expression has been described (Zhang et al., 2006). Also NK cells are able to produce this cytokine, in particular its regulation is regulated by STAT4 after IL-12 stimulation, through the binding of a conserved region located in the fourth intron (Grant et al., 2008).

The production of IL-10 can also be regulated at a post-transcriptional level, in fact the 3'UTR of *il-10* mRNA is enriched in AU rich elements (AREs). AREs identify short-lived mRNAs, as they are binding factors for destabilizing proteins such as tristetraprolin (TTP) (Gabryšová et al., 2014). In macrophages, the absence of TTP leads to an increased

expression of *il-10* after TLR4 triggering, but the same result was not obtained in DCs (Gaba et al., 2012). The 3'UTR of *il-10* gene was also shown to be bound by the ARE/poly(U) binding degradation factor1 (AUF1): its absence in a melanoma cell line again led to an increase of the half-life of *il-10* mRNA (Brewer et al., 2003).

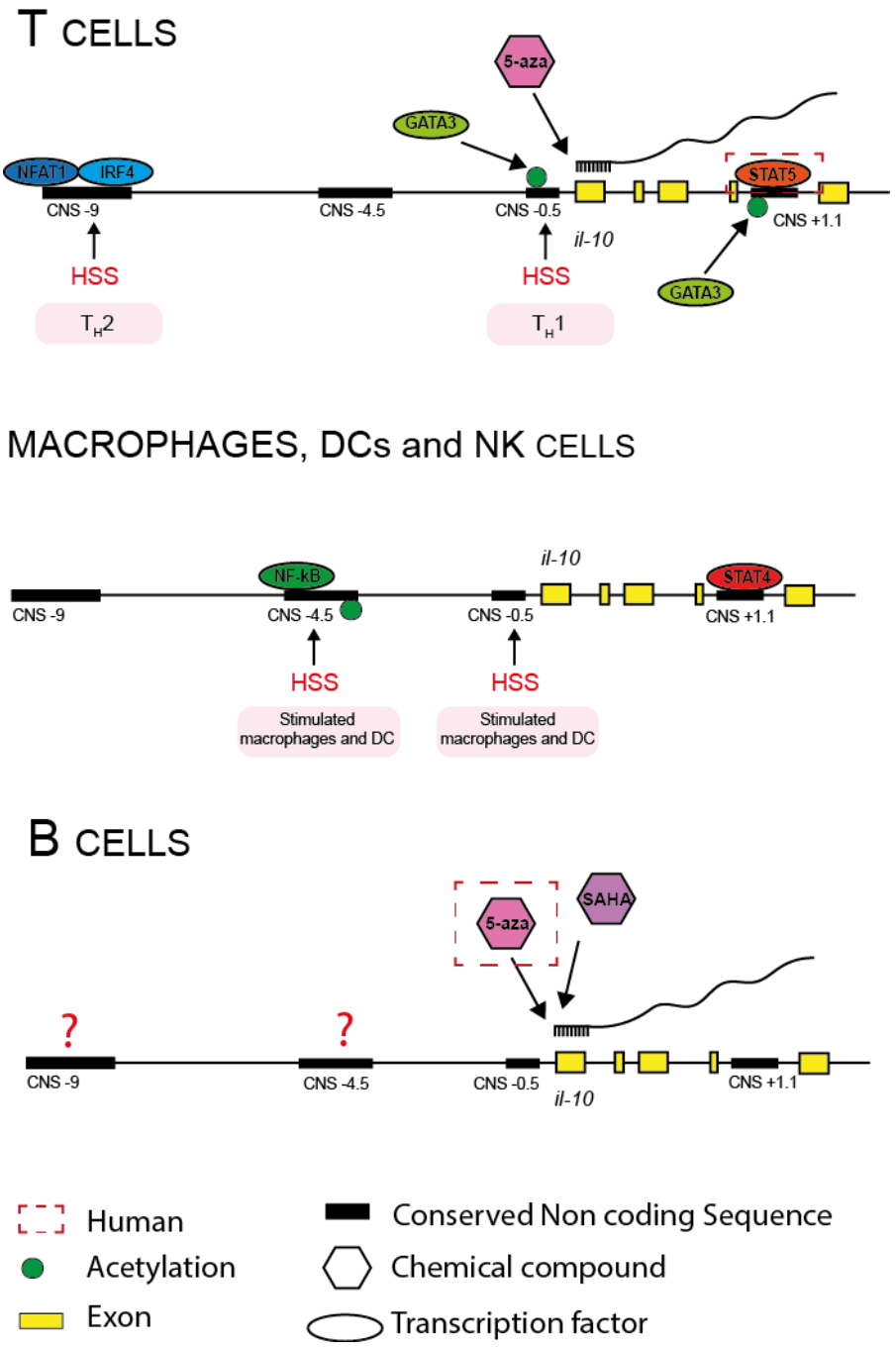
Among the post-transcriptional regulation of *il-10* mRNA, we find also miRNA-mediated mechanisms, that turned out to have both silencing and inducing functions. If on one hand, in a human cell line, miRNA-106a have been described to bind the UTR of *il-10* gene downregulating its expression (Sharma et al., 2009), on the other miRNA-466L, in macrophages, is able to increase IL-10 expression by antagonizing TTP-mediated mRNA degradation (Ma et al., 2010).

Overall, these studies shed light on the different pathways and mechanisms involved in the regulation of IL-10 production among diverse cell lineages, stating the importance of investigating regulatory networks in the specific subset analysed.

4. AIMS OF THE THESIS

B cells are usually considered positive regulators of the immune response, but in the last decades they have been also described as immune regulatory cells. The precise origin and development of B cells with immunosuppressive feature are still under debate, on the contrary, their mechanisms of action are quite well characterized. In particular, it is documented that their regulatory capacity mainly depends on the production of the cytokine IL-10. This work of thesis focuses on a particular subset of immune-suppressive B cells, named IL-10 competent B cells, that are defined as cells in a suspended and preparatory state that, after encountering the appropriate stimulus, immediately start producing IL-10.

The precise objective of my PhD project was to uncover which are the possible mechanisms at the basis of IL-10 competence in B cells, with a major focus on epigenetics. In first instance, a method to isolate this rare population was developed. Taking advantage of a bisulphite-based method the methylation of DNA was analysed to uncover its possible role in the competence for IL-10. Four regions of the *il-10* gene locus were analysed both in IL-10 competent and non-competent B cells isolated from different starting materials. In order to further characterize the epigenetic status of the selected regions, an analysis of histone modifications was performed, with a major focus on active markers. The final task was the study of the possible involvement of epigenetic in the production of IL-10 by IL-10 non-competent B cells population.



Regulation of il-10 gene expression by immune cells: a focus on epigenetic.
The knowledge about how il-10 gene expression is regulated on the epigenetic level is extended in T cells, macrophages and DCs while for B cells a little is known. In particular, a major role is played by CNS -9 in T cells while CNS -4.5 is specific for DCs and macrophages. In T cells il-10 gene expression is upregulated by the binding of NFAT1-IRF4 to the CNS -9, and also by the chemical compound 5-azacytidine. It has also been demonstrated that GATA3 remodels the chromatin of the locus inducing histone acetylation near the promoter and in the fourth intron. In the human setting instead STAT5 binds a region of the fourth intron that appears to have an enhancer-like activity. Regarding macrophages and DCs, a major role in the regulation of il-10 gene is played by CNS-4.5, which is bound by NF-kB, and CNS +1.1 that in NK is a binding site for STAT4. In the human setting, 5-azacytidine is able to induce il-10 expression in B cells, while in the murine context an effect for SAHA has been proved. However, it remains to be determined which is, if present, the role of CNSs in the regulation of il-10 gene expression in B cells.

5. RESULTS

5.1. IL-10 competence and production

In the last years, the laboratory where I did this work of thesis has focused its attention on a particular subset of B_{reg}, that is **IL-10 competent B cells**. This particular population has been studied in the context of the interaction with mast cells (Mion et al., 2014a), but also at a more molecular level, pointing the attention on the effects of different types of stimuli on the regulation of two distinct processes: IL-10 competence and IL-10 production (Mion et al., 2014b). In general, cells competent for a cytokine, or a protein, are defined as the ones that have the capacity to quickly produce it after stimulation, in other words they are ready for its synthesis, compared to the rest of the cell pool. According to the literature, competence can be acquired in a two stepwise model (Mohrs et al., 2005). The process starts with the development of the cell, where the gene that will confer the competence undergoes chromatin alterations which renders it more accessible. The open state of chromatin usually correlates with the basal presence of the transcript, and makes cells poised for the production of the corresponding protein. The important feature is that only competent cells start producing the protein immediately upon specific activation, while all the others require a longer timeframe or different signals (Mohrs et al., 2005).

Our assumption is that the principle of competence can be applied to B cells in the context of IL-10 production. Indeed, just a little fraction of B cells is able to immediately produce this specific cytokine under certain stimulation circumstances, while all the others necessitate an extended timing. The conditions necessary to follow IL-10 competent B cells were already described by work performed in this and in other groups (Mion et al., 2014b)(Yanaba et al., 2009). Specifically, the detection of IL-10 competent B cells can be accomplished by flow cytometry through an intracellular cytokine staining (ICS) in which cells receive, in combination with the stimulation, a secretion inhibitor, in this case monensin, to retain IL-10 in the cytoplasm. The best stimulation condition to induce *ex vivo* IL-10 competent B cells in the murine setting, is by stimulating B cells for 5h with LPS, phorbol 12-myristate 13-acetate (PMA) and ionomycin. Also other combinations of stimuli are able to induce IL-10 competence, such as PMA and ionomycin alone, but at a lower percentage compared to LPIM, as shown in **Figure 14**.

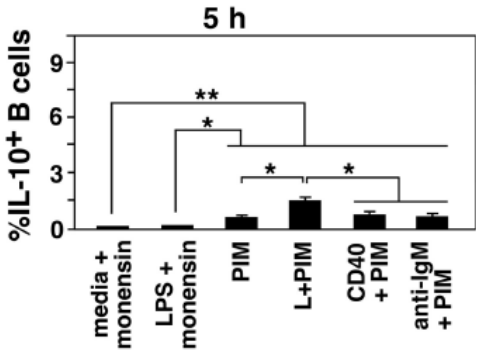


Figure 14 . ***Different stimulation for the induction of IL-10 competent B cells****. Mean values of IL-10+ B cells after 5 hours of different treatment. Adapted from* (Yanaba et al., 2009)

Under homeostatic conditions, the IL-10 competent B cell population can be detected in several murine organs at basal conditions, such as spleen, BM, peritoneal cavity, lymph nodes. Except for the peritoneal context, IL-10 competent B cells are very rare and in the spleen they represent the 2-3% of the total B cell population (Yanaba et al., 2008), as shown in **Figure 15**.

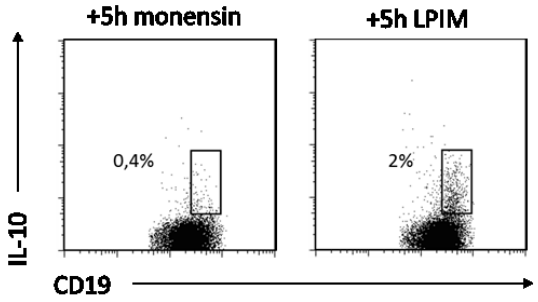


Figure 15. ***Splenic B cells positive for intracellular IL-10.*** *The frequency of B cells competent to express cytoplasmic IL-10 following a 5 h stimulation with LPS, PMA, ionomycin and monensin (LPIM) was analyzed in freshly isolated B cells. As a control, the condition in which cells received only monensin during the last 5 h of culture is also shown. Dot plots for one representative experiment are reported with indicated the frequencies of IL-10⁺ cells among total CD19⁺ lymphocytes. Adapted from* (Mion et al., 2014a) .

To investigate which are the mechanisms at the basis of IL-10 competence, B cells were treated with different type of stimuli. In particular, we compared the effects of immune-mediated, or endogenous, (BAFF and a-CD40 mAb) and infective, or exogenous (LPS and CpG) signals. The graph in **Figure 16A** clearly shows that IL-10 competent B cells are expanded by immune-mediated stimulation and LPS, but not by CpG. However, looking for IL-10 secretion (**Figure 16B**), CpG is the major inducer together with LPS, while the stimulation through CD40 or with BAFF does not induce IL-10 production. Therefore, IL-10 competence and IL-10 production are two different mechanisms and are induced by

different stimuli. It was hypothesized that endogenous and exogenous signals act at different time points: immune-mediated signals expand the IL-10 competent B cell population, that start to produce IL-10 after encountering the appropriate signals. An increase of *il-10* mRNA transcription was observed after a short term-stimulation of 2h with LPS, CpG or anti-IgM Ab both on B cells left unstimulated or treated for 48h with anti-CD40 mAb. Interestingly, the expression of the gene is at least doubled in B cells activated through CD40 compared to the unstimulated samples **(Figure 16C)**, indicating that the population competent for IL-10 production was expanded. The initial hypothesis was further confirmed by the detection of the secreted cytokine **(Figure 16D)**, only B cells stimulated through CD40 for 48h are able to quickly secrete and release IL-10 (Mion et al., 2014b).

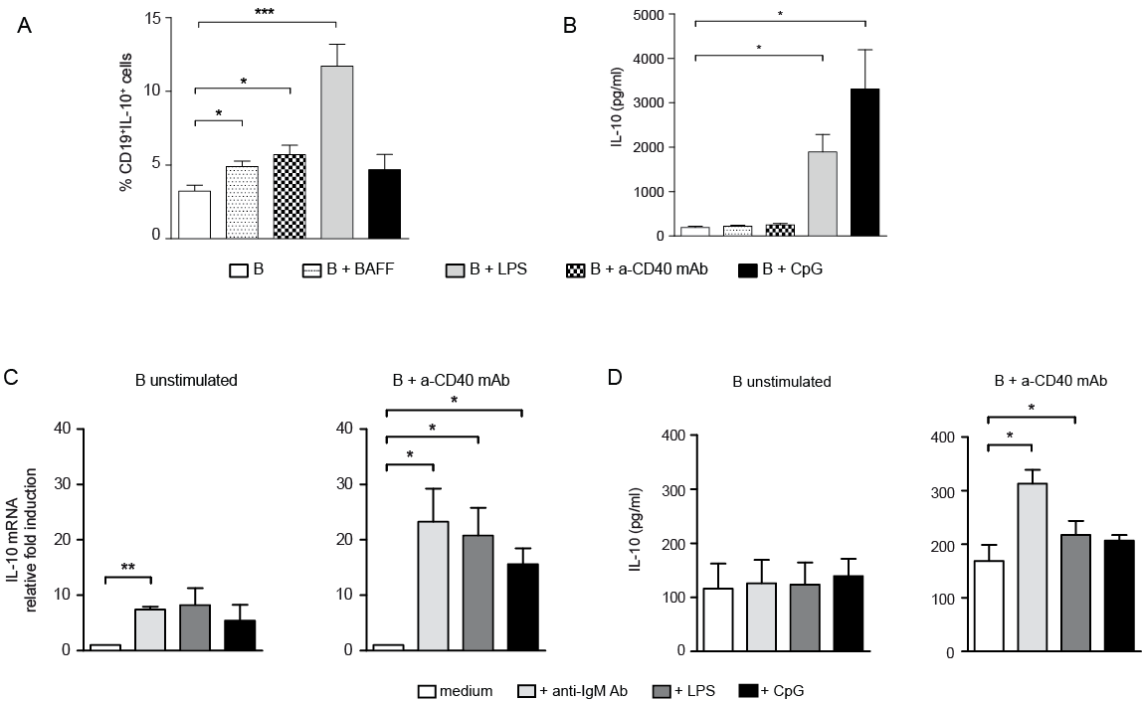


Figure 16. ***IL-10 competence and IL-10 production are two distinct mechanisms****. **(A)** IL-10 secretion after 48h of stimulation. B cells were cultured either alone (NST) or in the presence of BAFF, anti-CD40 mAb, LPS or CpG. Cell supernatants were collected and IL-10 levels detected by ELISA. **(B)** B cells positive for intracellular IL-10 after 48h of stimulation with BAFF, LPS, CpG or through CD40, and re-stimulated for 5 hours with LPIM. **(C-D)** Short term stimulation through TLR4, TLR9 or BCR after 48h of culture with a-CD40 mAb. B cells were incubated either alone (B unstimulated) or in the presence of a-CD40 mAb for 48 h. During the last 2 h of culture anti-IgM Ab, LPS, CpG or medium were added to both conditions. **(C)** Supernatant were harvested from cultured cells and IL-10 concentrations determined by ELISA. **(D)** mRNA from all conditions were extracted and tested for il-10 gene expression. Adapted from* (Mion et al., 2014b)

On the basis of the above presented data, we hypothesized that, among the total splenic B cell population, both IL-10 competent and non-competent B cell are present: once cells are stimulated, *il-10* gene transcription takes place with a different kinetics in the two functional subsets. In particular, our hypothesis was that in IL-10 competent B cells the gene acts as a primary-response gene (PRG) while in IL-10 non-competent B cells *il-10* could behave as a secondary-response gene (SRG). Based on the literature, the main difference among these classes of genes is that transcription of PRG is rapid since it depends on TF already present inside the cell, while SRGs need *de novo* protein synthesis, and more time, in order to be transcribed (Burger et al., 1994). Interestingly, PRGs can be further divided into early and late PRGs depending on the time frame in which their transcription starts: early PRGs are transcribed in the first 2h after stimulation, while late PRGs after 3-4 hours (Tullai et al., 2007). To determine to which class a gene belongs, cells must be treated with cycloheximide (CHX), a chemical compound that inhibits the protein synthesis by blocking the mRNA translation elongation (Tullai et al., 2007).

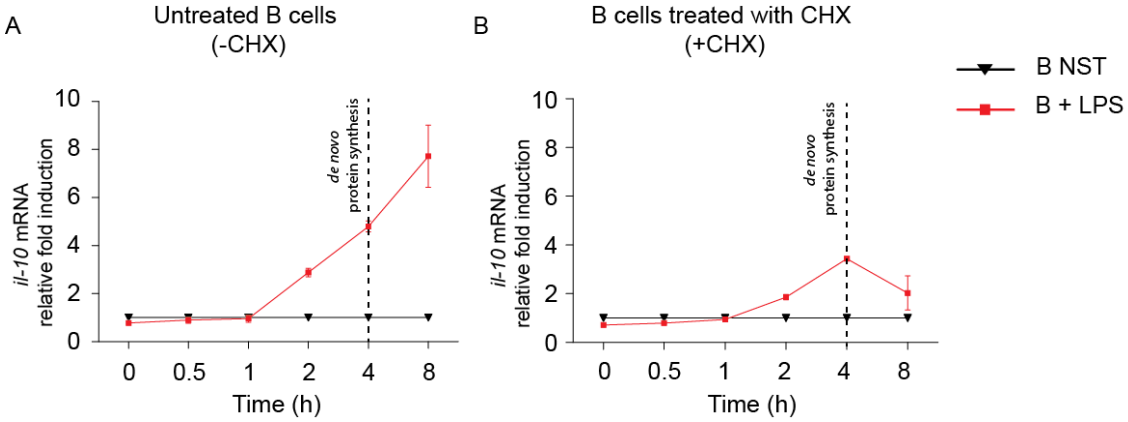


Figure 17. ***il-10 gene expression in B cells after LPS stimulation and treatment with CHX.*** *B cells were pre-treated with CHX* ***(B)*** *or not* ***(A)*** *for 30 minutes and then stimulated with LPS (B+LPS) or left unstimulated (NST). At different time-point cells were collected and lysed, qPCR was performed to compare the mRNA level of il-10 gene. In the two conditions (-CHX and +CHX) the expression level shown is calculated as fold induction over unstimulated B cells (B NST). Mean values ± SEM from two independent experiments are shown.*

To define the class to which *il-10* gene belongs, purified splenic B cells were pre-treated with 10 µM CHX and then stimulated or not with LPS. The expression of *il-10* gene was tested by qPCR at different time-points, and was calculated as fold induction of LPS-treated B cells over the unstimulated samples. After LPS stimulation and without the presence of the inhibitor, the transcription of *il-10* gene starts between the first and the second hour, and continues to increase during all the tested time frame (**Figure 17A**). When B cells are

pre-treated with CHX, the transcription starts at the same time point, but decreases between 4 and 8 hours **(Figure 17B)**. This is the "breaking point" that defines the moment in which B cells require *de novo* protein synthesis to transcribe *il-10* gene. The initial hypothesis was confirmed, as in the absence of CHX (**Figure 17A**) the level of the transcript continues to augment: IL-10 competent B cells, that are poised for IL-10 production, do not require *de novo* protein synthesis, while IL-10 non-competent B cells, that respond later to the stimulation, necessitate the production of other factors.

On the basis of these results, the main question to be answered was: why IL-10 competent B cells are able to immediately produce the cytokine, while all the other require more time? Moreover, if the same gene in the same cell lineage has this intricate regulation and kinetics, which are the mechanisms underlie?

Answering to these questions was the aim of my thesis. Therefore, I decided to first analyse among the *il-10* gene *locus* the possible involvement of epigenetic, taking into consideration modifications on DNA methylation and histone modifications in IL-10 competent B cells. In a second moment the characteristics of the non-competent subset were investigated. Given that these cells have a different regulation of IL-10 production, the last task was to perform a more genome-wide analysis, focusing on their transcriptome and miRNome.

5.2. IL-10 competence in B cells is associated to DNA methylation

Gene expression is tightly regulated by many different mechanisms, among them several belong to the epigenetics world. DNA methylation and histones modifications are just examples (Smale and Natoli, 2014). In first instance, DNA methylation was taken into consideration as a possible mechanism regulating *il-10* gene expression in B cells. DNA methylation it has already been described to be involved in the regulation of other cytokines; such as IFN-γ, the expression of which is correlated with the level of DNA methylation at its promoter (Yano et al., 2003). In mammals, DNA methylation occurs on CpGs dinucleotides and is maintained throughout cell division by Dnmt1, an enzyme that recognizes the 5meC on the hemimethylated strand and preserves the modification also on the new filament (Bogdanović and Veenstra, 2009).

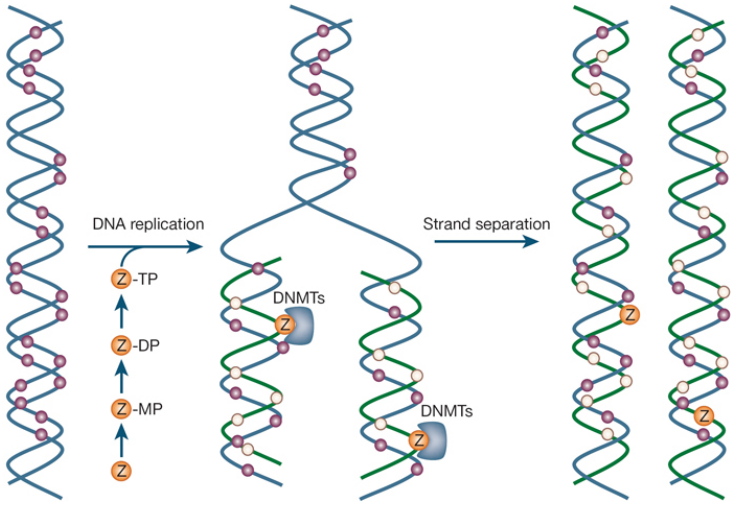


Figure 18. ***The mechanism of action of 5-azacytidine****. 5-aza acts in a cycle-dependent manner as, after its reduction, it can substitute cytosine in actively replicating DNA. Once incorporated in the DNA strand, the compound covalently binds DNMTs, leading to their degradation and preventing them to propagate the methylation signature on the new synthesized strand. Adapted from* (Egger et al., 2004)

To investigate whether *il-10* gene expression depends on CpGs methylation, B cells were treated with 5-azacytidine (5-aza), a compound that inhibits Dnmt1. Basically, 5-aza is a nucleoside analogue that, after reduction to 5-aza-2-deoxycytidine, substitutes cytosines into the replicating DNA and covalently binds Dnmt1 enzymes. In this way, 5-aza prevents Dnmt1 from exerting their function, as they are degraded. As a consequence, the methylation is lost (**Figure 18**). Since 5-aza functions in a cell-cycle dependent way, it was necessary to determine at which concentration B cells still proliferate, given that this compound has toxic effects. To test cell proliferation rate, a CFSE-based assay was

performed: this fluorescent dye binds all free amines on the surface and inside the cells, so if cells divide the compound is diluted among daughter cells, resulting in a decrease of fluorescence in younger generations.

B cells labelled with CFSE and pre-activated with a-CD40 mAb for 24h, were treated with increasing doses of 5-aza (1, 2.5, 5 and 10 µM) and the intensity of CFSE was tested after 48h. **Figure 19** shows that the increase of 5-aza concentration causes a progressive decrease of the percentage of proliferating cells. Specifically, B cells still proliferate when treated with 5-aza at the 2.5 µM dose, while at higher doses cell-division is almost completely abolished. Therefore, this concentration proved to be the most suitable for the following experiments.

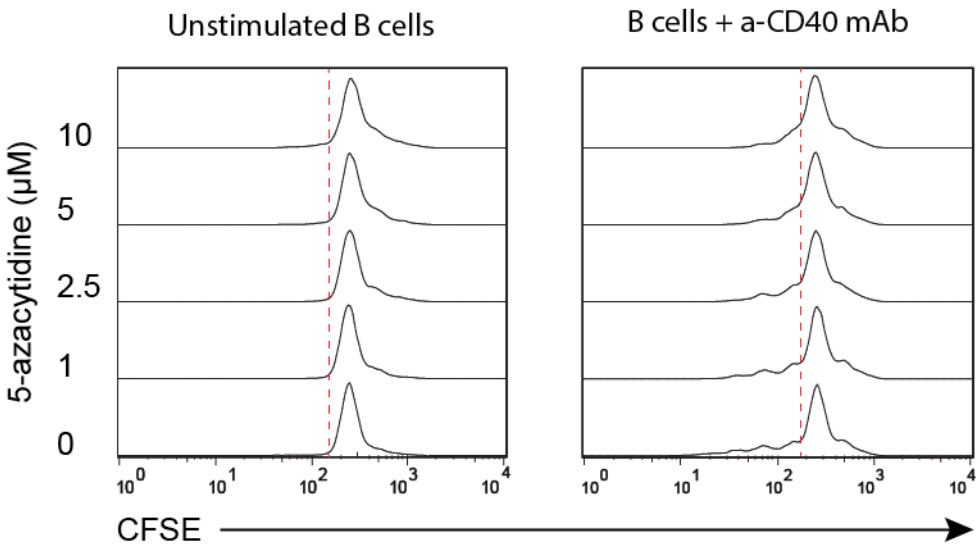


*Figure 19. **B cell proliferation after 5-azacytidine treatment**. Cells were marked with CFSE and left in culture without stimulation or with a-CD40 mAb and with increasing doses of 5-aza. Representative data from one experiment are shown.*

The effects of 5-aza on *il-10* gene expression were tested in the setting of the short-term restimulation described in the first paragraph (**Figure 16c**). B cells were pre-activated through CD40 for 24h, treated with 5-aza for other 48h and, during the last 2h, medium alone or containing LPS was added to the culture. The data in **Figure 20** are reported as fold induction of cells treated with 5-aza and stimulated for 2h with LPS, on the condition that received medium alone. A significant increase of *il-10* gene expression was detected between the untreated and the 2.5 µM dose condition.

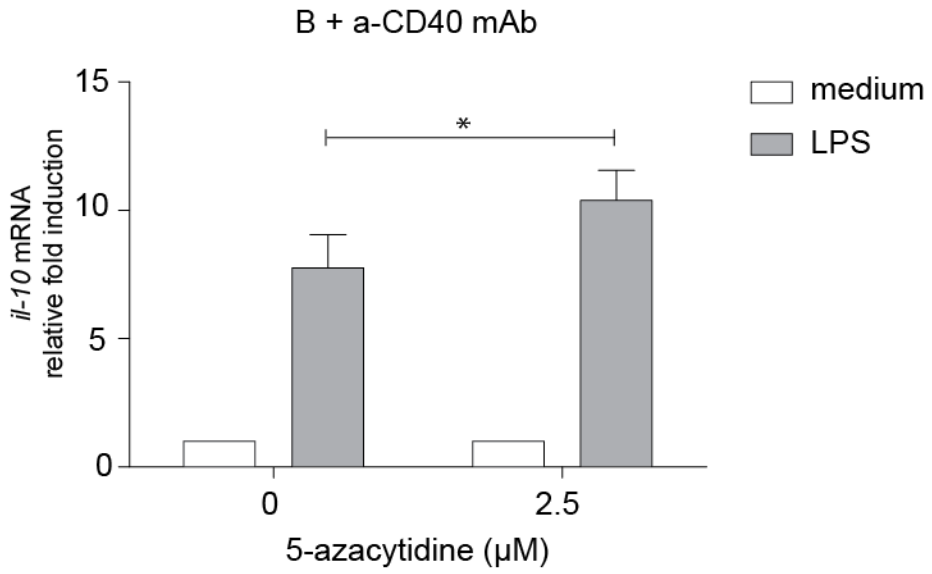


Figure 20. ***Effects of 5-azacytidine on short term-stimulation of a-CD40 mAb treated B cells****. Il-10 mRNA levels were tested by qPCR and expressed as a fold induction of samples re-stimulated with LPS over conditions that received only medium. Mean values ± SEM are shown from three different experiments, * p<0.05.*

These results hint that the expansion of the IL-10 competent B cell pool is dependent on DNA methylation. Since the level of *il-10* gene expression increases after re-stimulation with LPS in B cells that received the 5-aza, compared to the condition without the inhibitor. So, in light of this result it was decided to study more in depth the methylation status of the *il-10* gene locus, comparing IL-10 competent and non-competent B cells.

5.3. Set up of the methodology to purify splenic IL-10 competent B cells

To date, two methods are available for the detection of IL-10 competent B cells: the intracellular cytokine staining (ICS) for IL-10 coupled with cell-sorting, and the secretion assay for IL-10, available in commercial kits (Regulatory B cell Isolation Kit, Miltenyi Biotec). In first instance, the two methods were compared in terms of yield and purity, in order to choose the more appropriate to isolate the population of interest.

The main difference between the two methods relies on the system used for the detection of IL-10: while ICS needs cells to be fixed and permeabilized for the staining of the cytokine present in the cytoplasm, the secretion assay allows to obtain viable B cells that are actively secreting IL-10, capturing the cytokine on the surface of the cell. **Figure 21** shows the purity of the two methods: the dot plots in panel **A** demonstrate that with ICS coupled with cell-

sorting, it is possible to reach a 90% of purity for both fractions, while with the secretion assay alone the two populations present high background (**Figure 21B**).

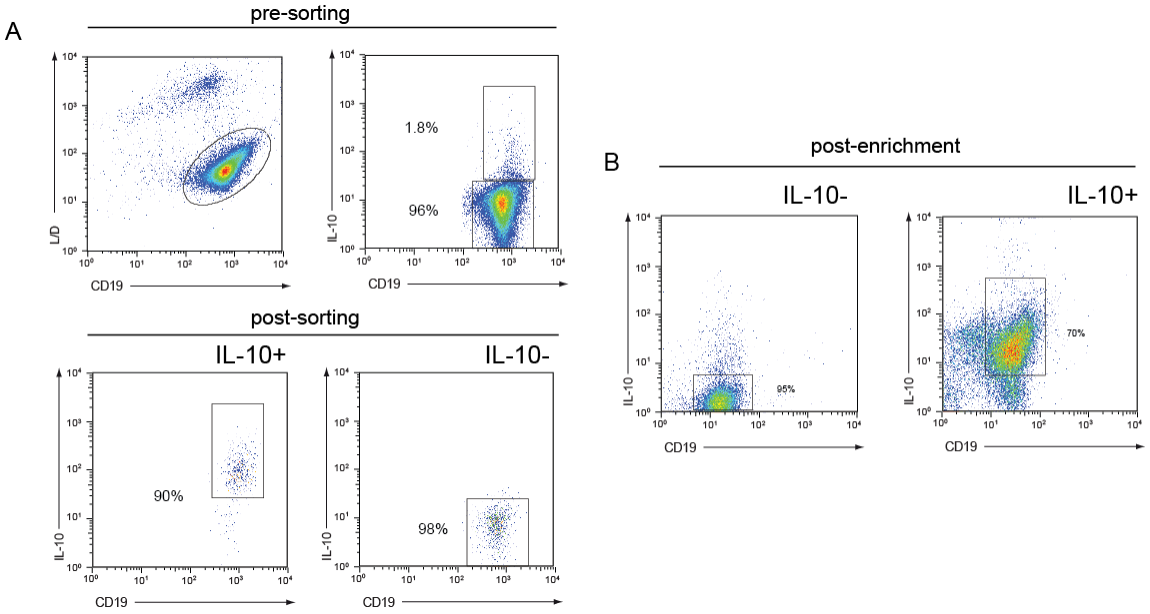


Figure 21. ***Comparison of methods for the isolation of IL-10 competent and non-competent B cells.*** ***(A)*** *IL-10 competent (IL-10$^+$) and non-competent (IL-10$^-$) B cells where isolated after ICS for IL-10. In the pre-sorting phase, viable B cells were selected for their positivity to CD19 and negativity for Live and dead (L/D). The purity of the two fractions after cell-sorting are reported in the lower part of the panel.* ***(B)*** *IL-10 competent (IL-10$^+$) and non-competent (IL-10$^-$) B cells where isolated with the Regulatory B cell Isolation kit and the purity after the enrichment is reported.*

The first method allows to obtain IL-10$^+$ B cells with high purity but not more viable, while the strength of the second is represented by the viability of the isolated cells, which allows to perform studies on RNA or *in vivo*. In light of these results, we tried to set up a combination of the two methods. After purification, B cells were stimulated for 5h with LPI, and underwent a single column passage for IL-10$^+$ cell enrichment; the pre-enriched fraction was then sorted. The gating strategy for sorting is shown in **Figure 22A**: viable cells are selected from the morphological dot plot, then doublets are excluded and finally the IL-10$^+$ and IL-10$^-$ gates are set. The pre-enriched fractions together with the purity of the populations after sorting are showed in **Figure 22B**. For both populations the percentage of purity is 98-99%. In conclusion, with this third method, we were able to obtain viable IL-10 competent B cells (IL-10$^+$) and IL-10 non-competent B cells (IL-10$^-$) at high purity and suitable for *in vivo* experiments and for several analyses of molecular biology.

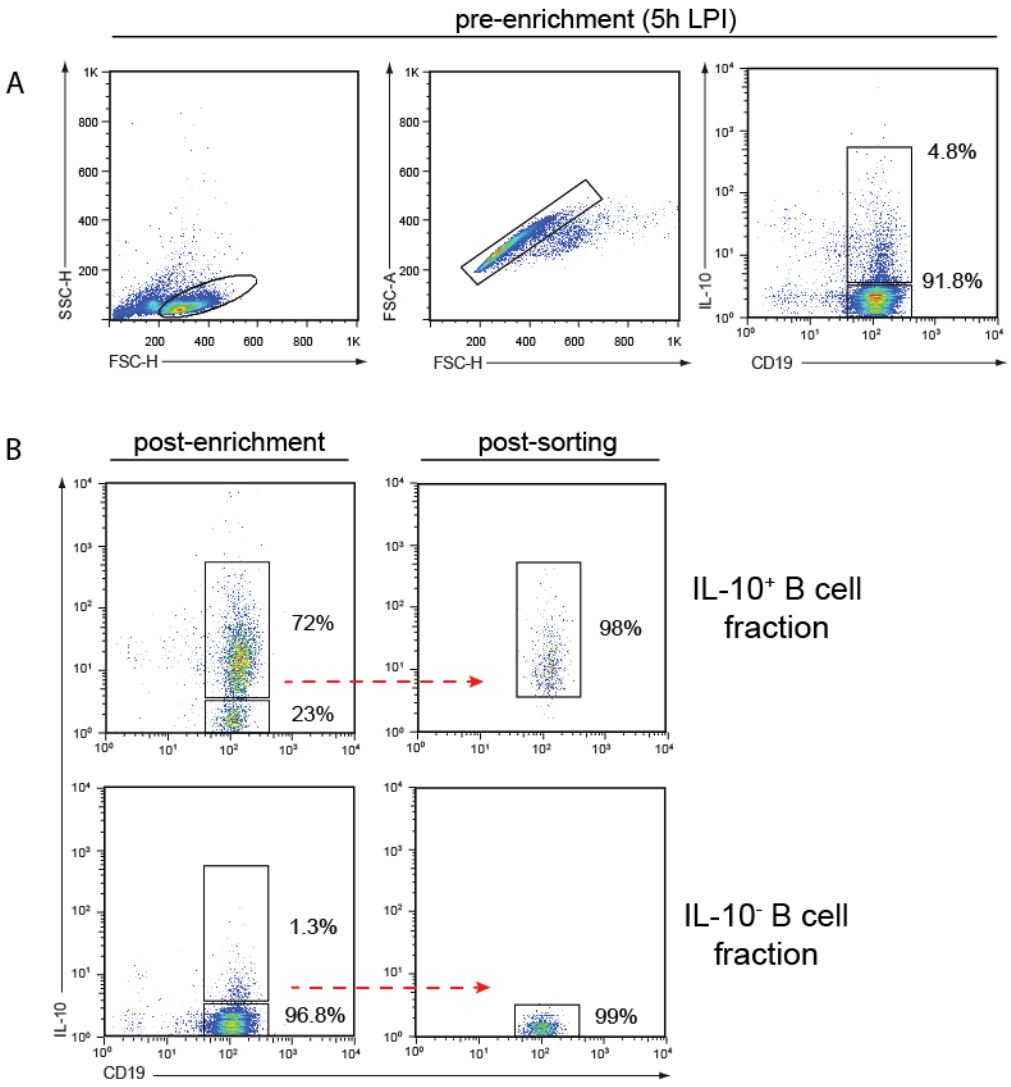


Figure 22. ***Method for the isolation of IL-10 competent B cells based on the combination of the regulatory B cell isolation kit and cell-sorting. (A)*** *The dot plot shows the percentage of IL-10 competent B cells after 5h of LPI (CD19 vs IL-10) before the enrichment and the gating strategy used for the sorting, viable cells were selected on the base of their morphology, then doublets were excluded and gates are set for IL-10+ and IL-10- B cells.* ***(B)*** *the purity of IL-10 competent and non-competent B cells is shown after the magnetic enrichment and post cell-sorting.*

5.4. Study of CpGs methylation in IL-10 competent B cells

Although several methods allow the study of CpG methylation, the gold standard is the technique based on the treatment of DNA with bisulphite, a chemical compound that is able to convert unmethylated cytosines into uracils. The methylation of short regions of interest can be studied through bisulphite methylation PCR (BSP). Basically, using specific primers, the selected regions are amplified trough a PCR step, the 5meC will remain C while the unmethylated residues will turn into T. The PCR products are then sequenced and the methylation status is determined by analysing the percentages of C or T in the CpGs sites located in the original genomic sequence. In order to study small regions of the genome,

and not to have a genome-wide resolution, two different approaches are possible: to directly sequence the PCR products (direct BSP) or to clone them into vectors and then proceed with the sequencing (cloning-based BSP). Direct BSP with Sanger sequencing is the fastest technique, but inefficient when analysing regions presenting a variable rate of methylation, as the result will not be a clear peak in the electropherogram (**Figure 23 left**). This problem is overcome if pyrosequencing is available, or with cloning-based BSP (Tost and Gut, 2007). In this thesis cloning-based BSP was chosen to analyse the methylation status of IL-10 competent B cells. Theoretically, this method gives a single-cell resolution analysis, as each vector is cloned with a single PCR product and, after sequencing it gives clear peaks (**Figure 23 right**), allowing the calculation of CpG methylation very easily if a consistent number (usually 10 to 15) of vectors are sequenced (Hernández et al., 2013).

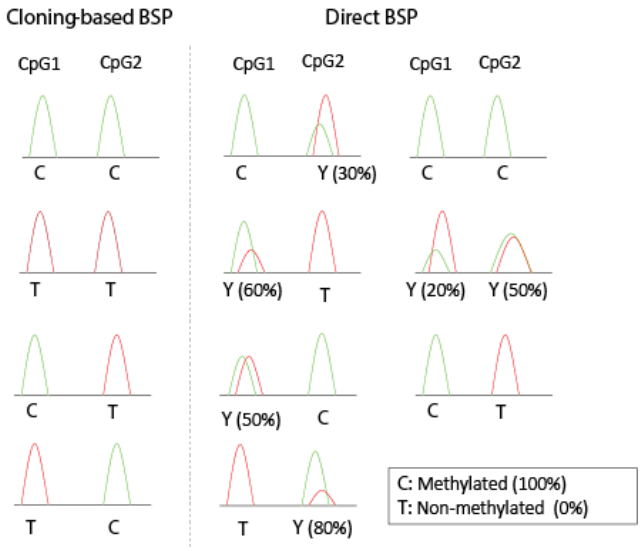


Figure 23. ***Electropherogram from cloning-based BSP and direct BSP****. Cloning-based BSP allows to have a single cell resolution of DNA methylation (left panel), while direct BSP gives an average methylation percentage of the PCR product sequenced. Adapted from* (Hernández et al., 2013)

5.4.1. Identification of regions of interest

The analysis of the *il-10* gene locus was done taking advantage of the USCS and VISTA databases. All regions were selected on the basis of their level of conservation between mouse and human (>70%) and their CG content. Murine regions were selected on GRCm38/mm10 assembly (chromosome 1: 131,010,441-131,021,799) and human regions on GRCh38/hg38 assembly (chr1: 206,784,453-206,772,920). In the murine setting, a total of four regions encompassing 30 CpGs were analysed. Instead, in the human setting 22 CpG

sites were selected among three fragments. All selected conserved noncoding sequence (CNS) are shown in **Figure 24A** for the murine system and **24B** for the human setting. In particular, mCNS -9 is evolutionary conserved with hCNS -12.5 and mCNS -4.5 with hCNS -6. In both species the region near the TSS is conserved and finally, mCNS +1.1 was not analysed in human because there were no conserved CpGs.

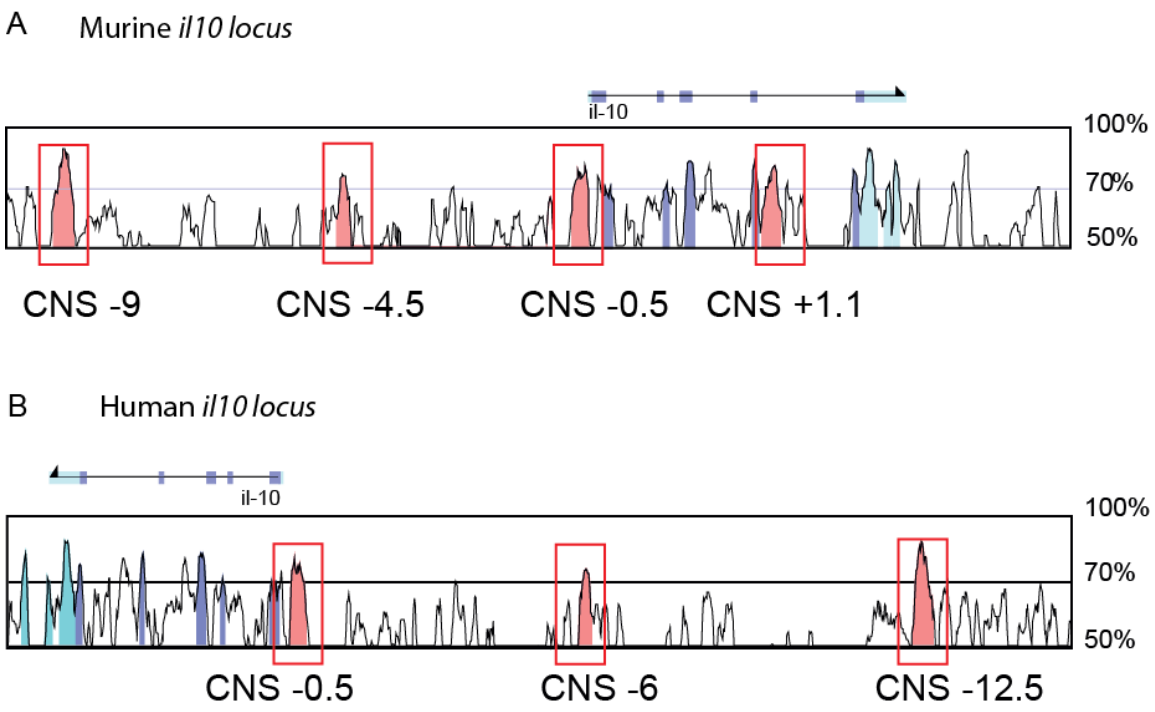


*Figure 24. **Analysis of il-10 gene locus in mouse and human**. The representative figure of il-10 murine **(A)** and human **(B)** locus were generated taking advantage of VISTA browser. In particular regions highlithed in pink have a high degree of conservation between mouse and human (>70%).*

5.4.2. Methylation pattern of murine IL-10 competent B cells

In first instance, the methylation level of IL-10 competent B cells isolated from the spleen was analysed. Cells were isolated as described in 4.4.1 and for methylation analysis $0.1x10^6$ cells for each condition were lysed and their genomic DNA (gDNA) was bisulphite-converted as described in methods. All four regions highlighted in **Figure 24A** were tested. The mean of methylation percentage ± SEM from four independent experiments is shown in **Figure 25A**, while **Figure 25B** reports the heatmap together with the statistics. From this data it is clear that splenic IL-10 competent and non-competent B cells have a different methylation profile: B cells able to produce IL-10 after a short stimulation *ex vivo* (5h LPI),

have a lower methylation percentage on mCNS-9 and mCNS-4.5. In mCNS -9 the overall methylation is about 40% for IL-10 competent B cells and 70% for non-competent, while in mCNS -4.5 methylation is 40% and 90% respectively. The intronic region and the one proximal to the TSS are instead almost fully unmethylated in both fractions.

To confirm these data, it was decided to determine the methylation pattern of IL-10 competent B cells in the *tiger* transgenic mouse model. The peculiarity of this transgenic model is that cells simultaneously produce IL-10 and GFP as two separate proteins, since the coding sequence for the reporter protein is cloned after the 3'-UTR of the *il-10* gene (Kamanaka et al., 2006). This allows to follow IL-10 production without the need of ICS, as the production of IL-10 goes together with GFP synthesis, but while IL-10 is secreted, the reporter protein is retained in the cells, enabling the detection of B cells that produced IL-10 by flow cytometry. The gating strategy and sorting results are shown in **Figure 37**, in MATERIALS AND METHODS section.

The found methylation signature has the same trend of the *wt* counterpart, in fact the major differences among the two cellular fractions were found in mCNS-9 and mCNS-4.5, as shown in **Figure 26**. In mCNS-9 the overall methylation is collectively lower, with a 20% for IL-10 competent B cells and 35% for non-competent, while in mCSN-4.5 is more similar as it was found a 70% and 95% of methylation rate, respectively. Also in this case, mCNS-0.5 and mCNS+1.1 are almost unmethylated in both fractions.

To test whether the found methylation pattern was common to all IL-10 competent B cells, we decided to analyse another anatomical compartment: the peritoneum. This context was chosen because the peritoneal cavity is enriched in B1 cells, a subset of B cells that naturally produce huge amounts of IL-10 (O'Garra et al., 1992). **Figure 27** reports the data for peritoneal cells and shows that both fractions are substantially fully demethylated among all the *il-10* gene locus, with the exception of mCNS-4.5, that resulted again the region with the most significant difference. In fact, the overall methylation in this region is around 10% in IL-10 competent B cells while it is about 50% in non-competent B cells.

Collectively, these data highlight the role of DNA methylation in the regulation of IL-10 expression. In particular, significant differences were detected in both mCNS-9 and mCNS-4.5 for the spleen compartment, while for peritoneal cells mCNS -4.5 is the only region with statistically significant differences.

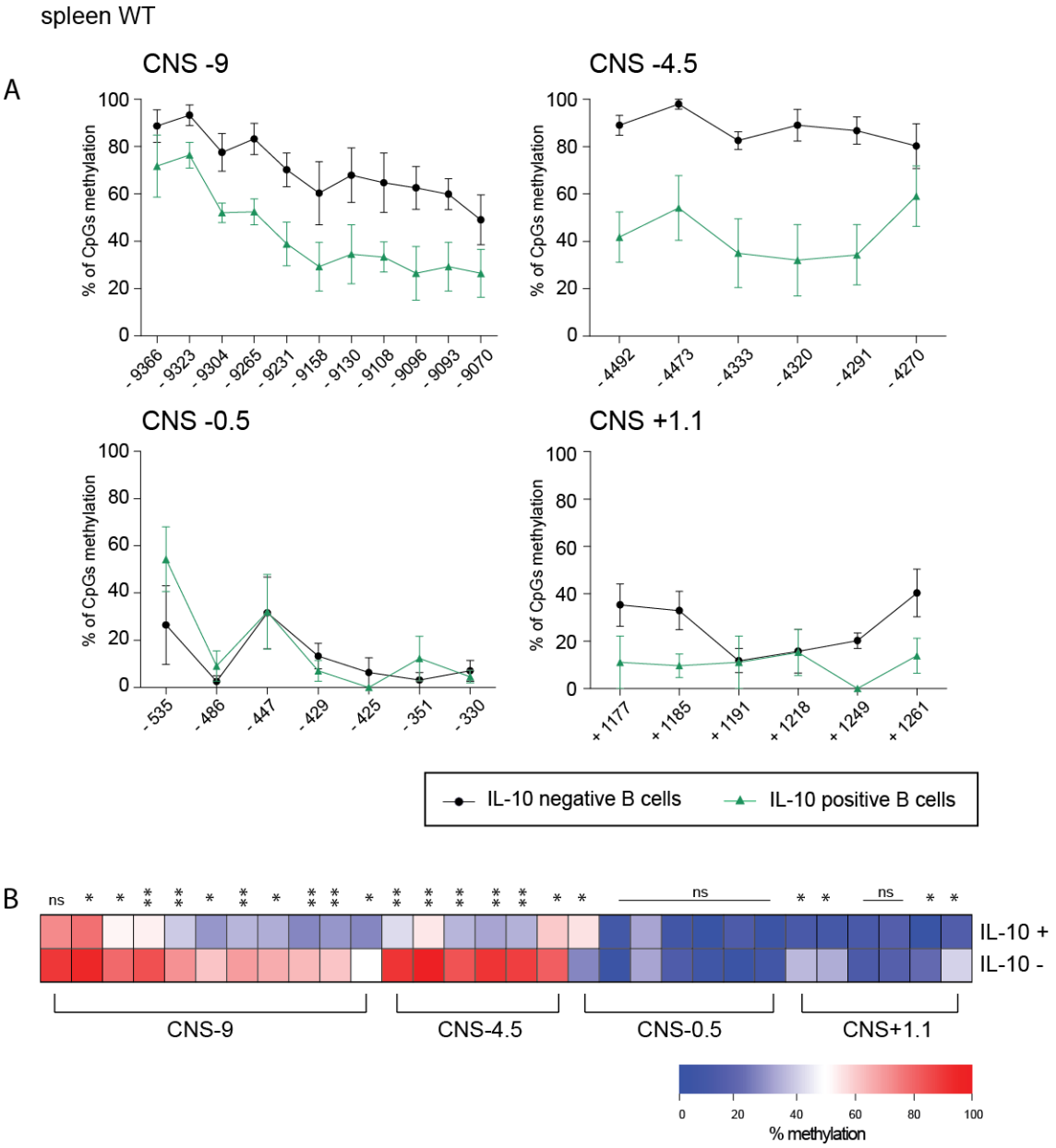


Figure 25. ***CpGs methylation pattern of IL-10 competent non-competent murine splenic Wt B cells****. A total of 30 CpGs across four different regions were analyzed for their level of methylation.* ***(A)*** *graphs show the percentage of methylation ±SEM at single CpGs nucleotides.* ***(B)*** *the heatmap reports the percentage of methylation at single CpG sites and the statistics comparing the IL-10^{+} vs IL-10^{-} B cells. Statistical analysis was conducted with Mann-Whitney U-test; n=4*

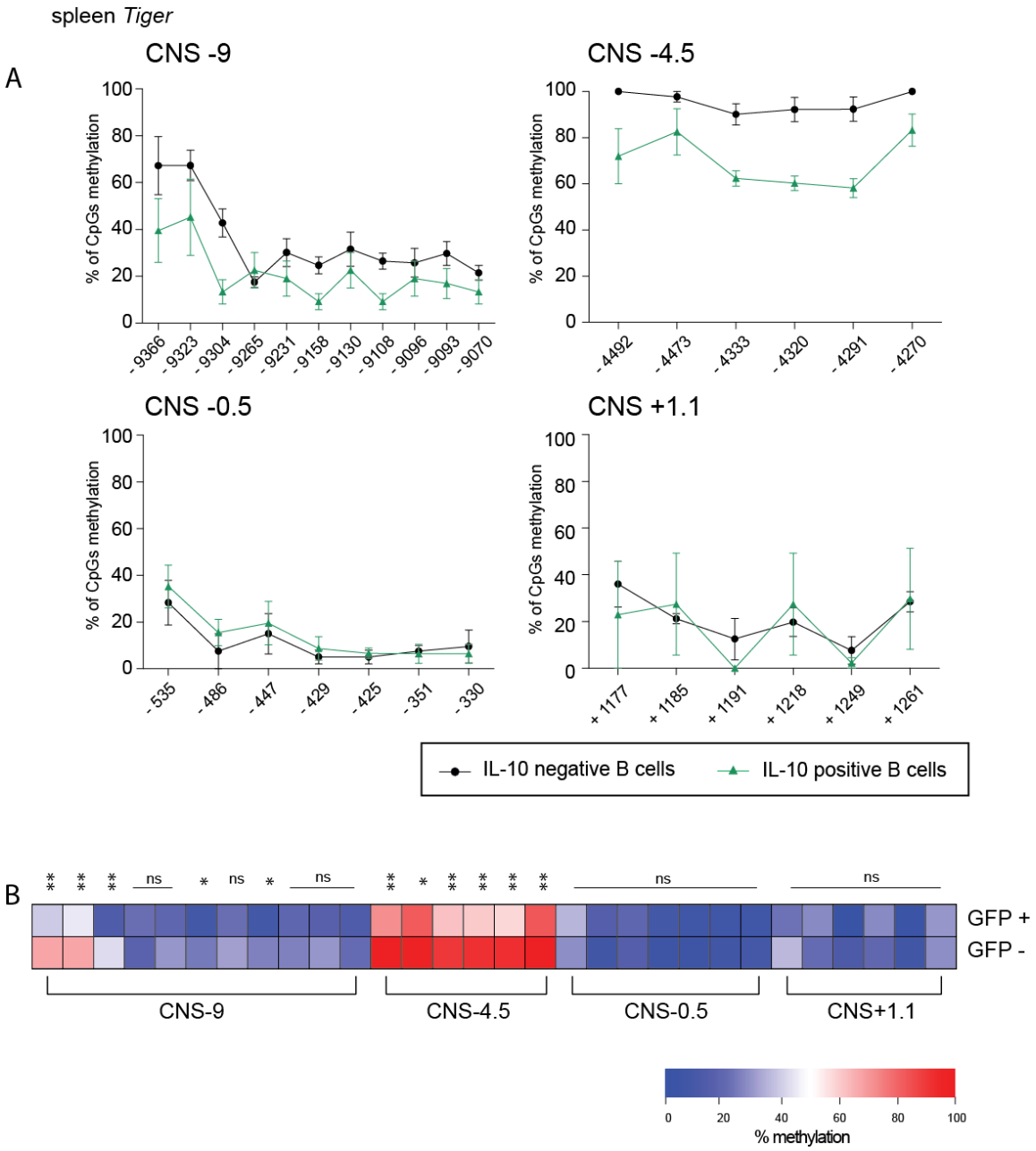


Figure 26. ***CpGs methylation pattern of IL-10 competent and non-competent murine splenic B cells of tiger mice****. A total of 30 CpGs across four different regions were analyzed for their level of methylation.* ***(A)*** *graphs show the percentage of methylation ±SEM at single CpGs nucleotides.* ***(B)*** *The heatmap reports the percentage of methylation at single CpG sites and the statistics comparing the GFP⁺ vs GFP⁻ B cells. Statistical analysis was conducted with Mann-Whitney U-test; n=3*

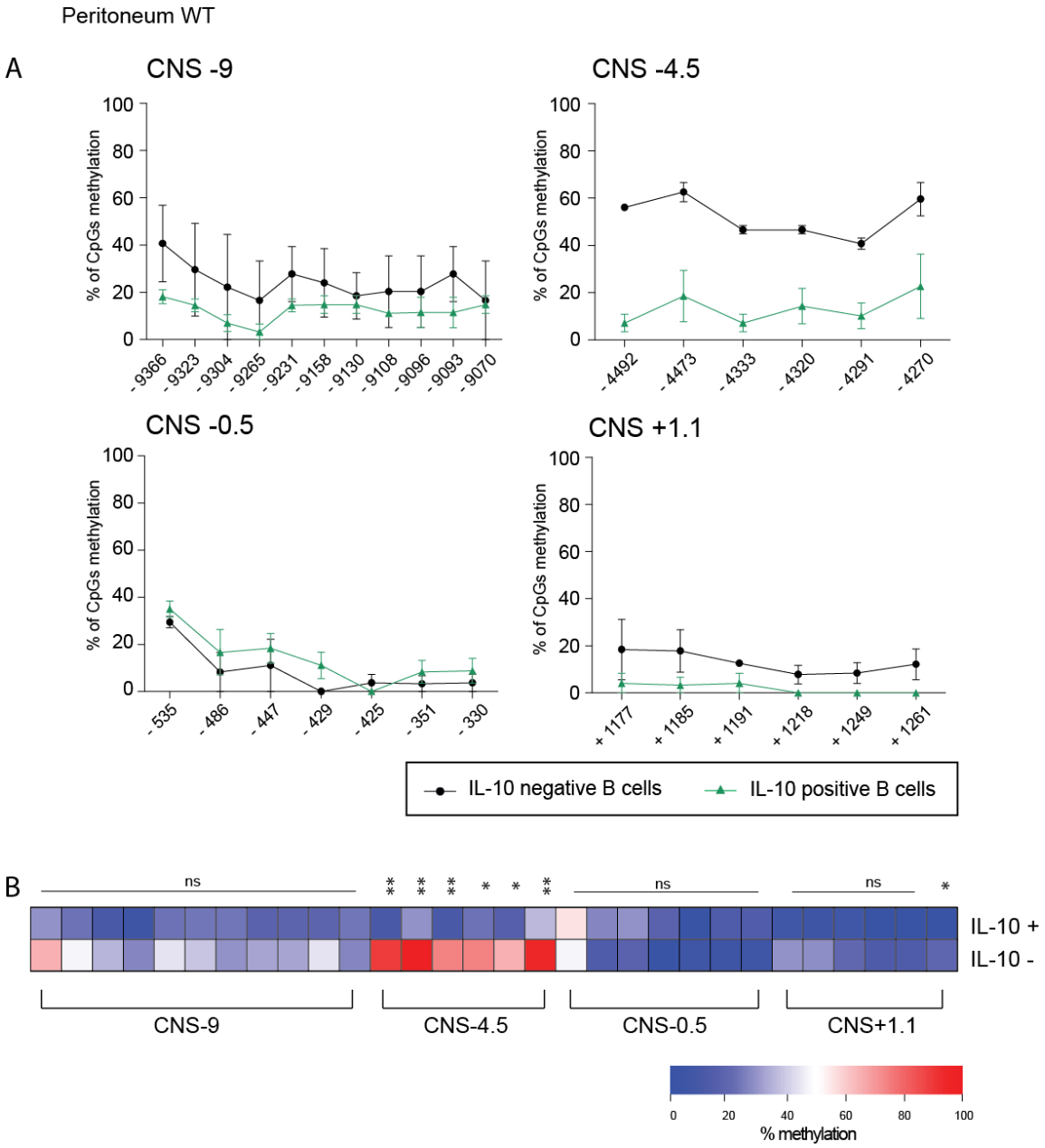


Figure 27. ***CpGs methylation pattern of IL-10 competent and non-competent murine peritoneal B cells****. A total of 30 CpGs across four different regions were analyzed for their level of methylation.* ***(A)*** *graphs show the percentage of methylation ±SEM at single CpGs nucleotides.* ***(B)*** *The heatmap reports the percentage of methylation at single CpG sites and the statistics comparing the IL-10^{+} vs IL-10^{-} B cells. Statistical analysis was conducted with Mann-Whitney U-test; n=3*

5.4.3. Methylation pattern of human IL-10 competent B cells

Since the *il-10* gene and its locus are very conserved among species, the methylation pattern of human IL-10 competent B cells was determined. Cells were isolated from peripheral blood of healthy donors taking advantage of the ICS for IL-10 followed by cell-sorting. The IL-10 secretion assay strategy was not used as, for the methylation analysis alone, also fixed cells are suitable. Differently from the murine setting, the best conditions to follow IL-10 competence in human B cells is by stimulating cells for 5h with CpG, instead of LPS, plus PMA and ionomycin (CPI). Moreover, the secretion must be blocked adding brefeldin A (the complete cocktail acronym is CPIB) and not monensin (Iwata et al., 2011). The gating strategy is depicted in **Figure 28** and, similarly to the murine condition, two populations were isolated with a purity around 95-98%.

The methylation pattern is reported in **Figure 29**, and it can be noticed how the two populations mainly differ on hCNS-12.5 and hCNS -6. hCNS -0.5 is fully unmethylated both in IL-10^{+} and IL-10^{-} B cells. Interestingly, the identified signature is conserved with the murine counterpart, in fact hCNS -12.5 is conserved with mCNS-9 and hCNS-6 with hCNS-4.5, reinforcing the possible regulatory role for methylation at these regions, since the pattern is maintained across species.

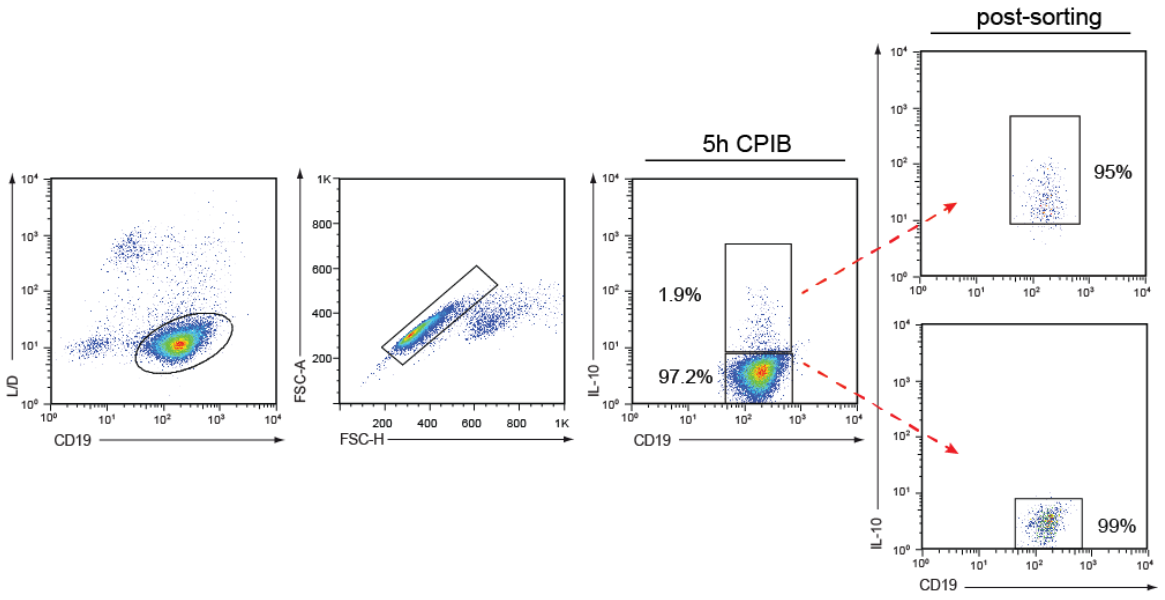


Figure 28. ***Isolation of IL-10 competent and non-competent B cells from human peripheral blood****. In the first dot plot, viable B cells were selected for their positivity for CD19 and negativity for the fluorescent dye Live and Dead (L/D). Doublets were excluded from the sorting.*

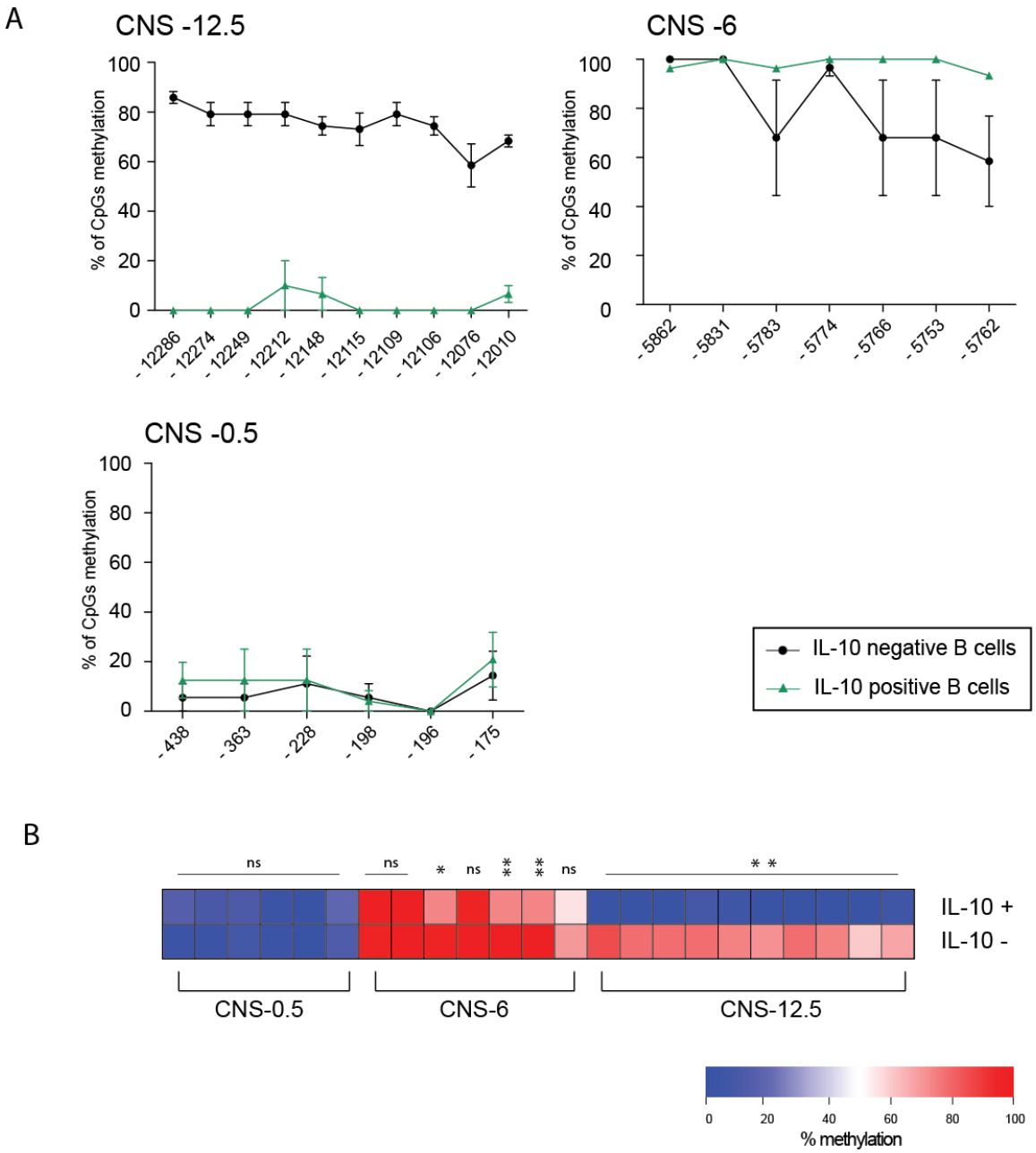


Figure 29. ***CpGs methylation pattern of IL-10 competent and non-competent human peripheral blood B cells****. A total of 23 CpGs across four different regions were analyzed for their level of methylation.* ***(A)*** *graphs show the percentage of methylation ±SEM at single CpGs nucleotides.* ***(B)*** *The heatmap reports the percentage of methylation at single CpG sites and the statistics comparing the IL-10+ vs IL-10 B cells. Statistical analysis was conducted with Mann-Whitney U-test; n=3*

5.5. Histone modification signature of IL-10 competent B cells

DNA regulatory regions can be identified on the basis of their chromatin signature by analysing the modifications located at histone tails. Enhancers are a class of regulatory regions located also several kb upstream from the TSS and are able to enhance the transcription of the target gene. Promoters instead are usually located near the TSS and serve as binding site for the transcriptional machinery. These two regions have different histone modification signatures, in particular enhancers are identified by the presence of H3K4me1, while promoters are enriched in H3K4me3. Interestingly, when active, both enhancers and promoters are characterized by the presence of acetylation (Zhou et al., 2011).

In order to define if IL-10 competent and non-competent B cells differ also at the level of histone modification, the chromatin immune precipitation (ChIP) technique was used to identify possible differences in mCNS-9, mCNS-4.5 and mCNS-0.5. The enrichment for histones modification is expressed as percentage of input and was determined by qPCR. Prolactin was used as negative control, while the housekeeping gene GAPDH and the B cell specific marker CD19 as positive controls.

Active promoters are characterized by the presence of H3K4me3. As shown is **Figure 30A**, at the level of mCNS-0.5, IL-10 competent B cells, that are actively producing the cytokine, have an 8-times higher enrichment, expressed as percentage of input, for this modification compared to the IL-10 negative fraction. As expected, at the level of mCNS-9 and mCNS-4.5, that are located several kb upstream from the TSS, H3K4me3 is not present in both fractions. A general marker of activation that accompanies transcription is the acetylation occurring on K27 of histone H4. **Figure 30B** shows the enrichment over the input for H3K27Ac in all the studied regions. Interestingly, although not significantly different, all CNSs were characterized by a higher presence of this modification in the IL-10 competent B cells population compared to the IL-10 negative fraction. In particular, the fold increase is 2.5 for mCNS-0.5, 1.8 for mCNS -4.5 and 2 for mCNS-9.

The data obtained from ChIP experiments suggest that mCNS-9 and mCNS -4.5 can be putative enhancers, as they are characterized by H4K27Ac but not by H3K4me3 (Ostuni et al., 2013). On the other hand, mCNS-0.5 that is proximal to the TSS has the characteristic signature of an active promoter only in IL-10 competent B cells. Collectively, IL-10 competent and non-competent B cells have a different histone modification signature.

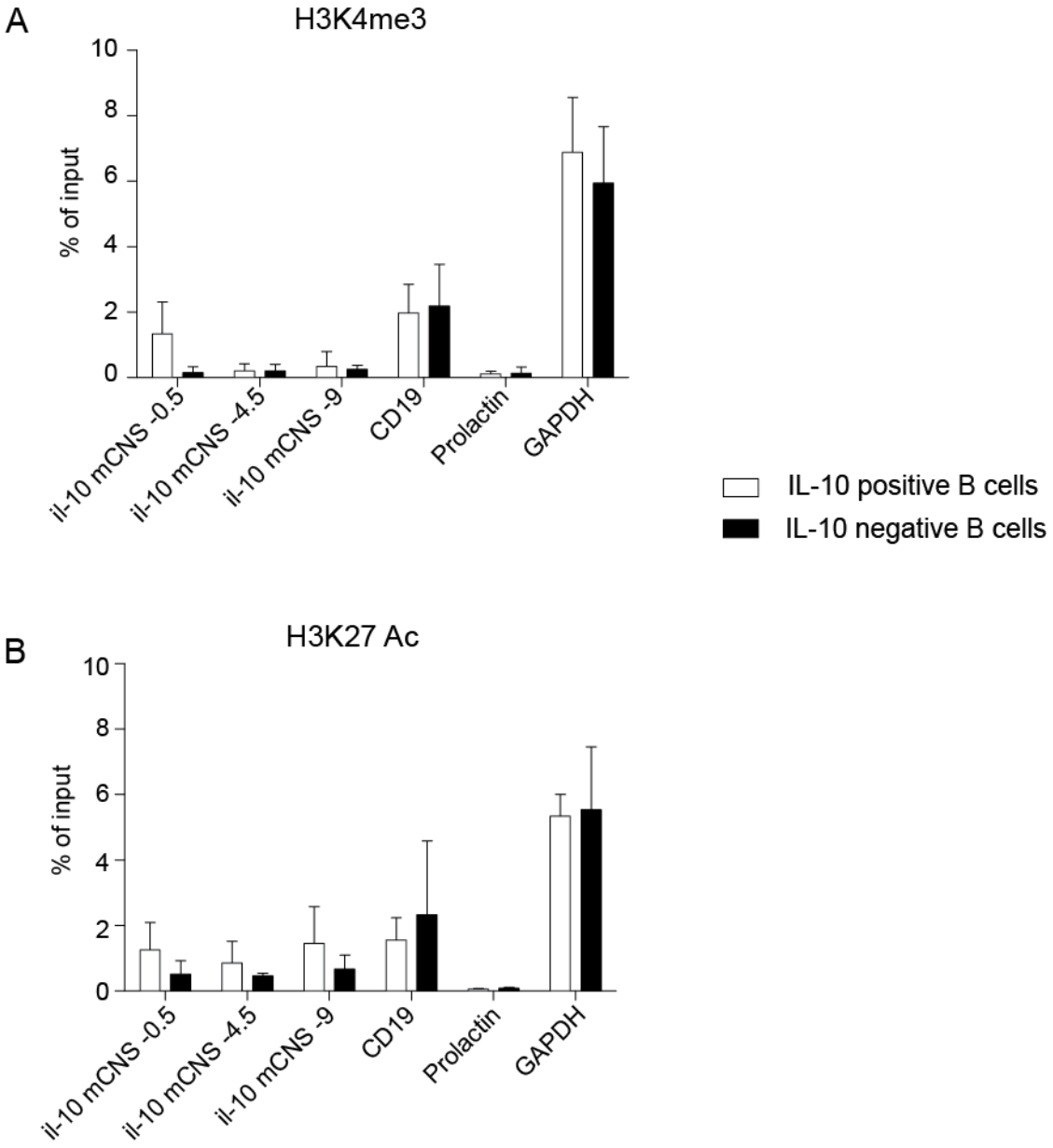


Figure 30. ***ChIP for H3K4me3 and H3K27Ac****. The immunoprecipitation was performed for H3K4me3* ***(A)*** *and H3K27Ac* ***(B).*** *The fold enrichment over the percentage of input for each modification was tested through qPCR, using specific primers for each region. Mean values ± SEM from three independent experiments are shown.*

5.6. IL-10 production by IL-10 non-competent B cells

The epigenetic data reported in the previous paragraphs state that IL-10 competent and non-competent B cells have different DNA methylation and histone modification profiles, but also gave us an important information: there are no epigenetic obstacles for IL-10 production, as for example methylation on the region near the TSS, in IL-10 non-competent B cells. This finding supports our initial hypothesis that these two populations can respond

to stimulation in a time-dependent manner, with IL-10 competent B cells acting as first-responder and IL-10 non-competent B cells as second-responders.

To further demonstrate that IL-10 competent B cells are not responsible for total IL-10 production found in the supernatants after 48h of stimulation (**Figure 16B**), but that is in part due to the capacity of IL-10 non-competent B cells to secrete the cytokine in a second moment, IL-10$^-$ B cells were cultured in the presence or absence of LPS for 24h or 48h. It is important to underline that these cells already received a stimulation, as they were separated from IL-10 competent B cells after 5 h of LPI. The supernatants were then tested through ELISA assay, and, as shown **in Figure 31A**, IL-10 non-competent B cell re-stimulated with LPS are able to secrete the cytokine after 48h but not 24h. To further confirm this data, at 48h IL-10 was detected also through ICS and the results confirmed that, after LPS stimulation, B cells are able to produce the cytokine. Indeed, the percentage of IL-10$^+$ B cells obtained after LPS treatment was significantly higher compared to the unstimulated sample (**Figure 31B**).

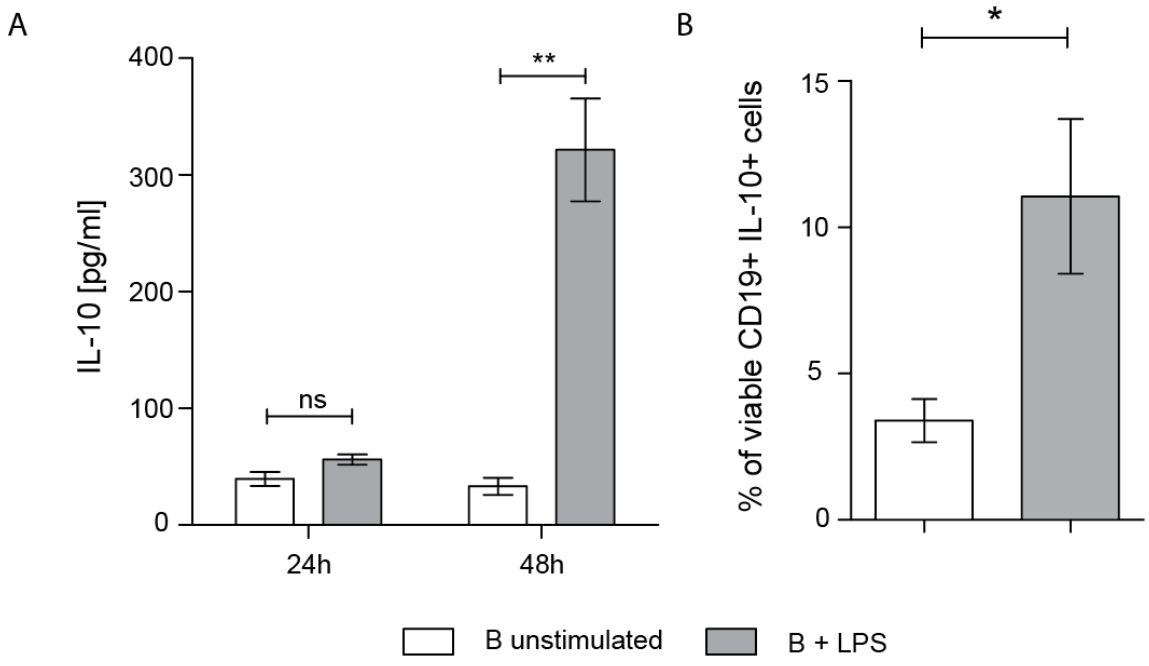


Figure 31. ***IL-10 production by IL-10 non-competent B cells.*** ***(A)*** *IL-10 non-competent B cells secrete IL-10 after 48h of stimulation with LPS.* ***(B)*** *ICS for IL-10 on IL-10 non-competent B cells. n=4, paired t-student test * p<0.05, **p<0.01.*

In light of the result that also IL-10 non-competent B cells are able to produce IL-10, we asked whether the capacity to produce IL-10 was the consequence of the acquisition of an IL-10 competent methylation profile. For this purpose, IL-10 non-competent B cells were

seeded in the presence of LPS, left in culture for 48h, subjected to a second IL-10 secretion assay and then re-sorted for positivity/negativity to IL-10. The gating strategy and the purity of the population obtained are reported in **Figure 32**. As already described, gDNA was bisulphite-converted and the methylation analysis for mCNS-9 and mCNS-4.5 was performed. The choice of analysing these two regions was due to the results of the previous experiments, which showed that they were the most differently methylated in IL-10 competent and non-competent B cells.

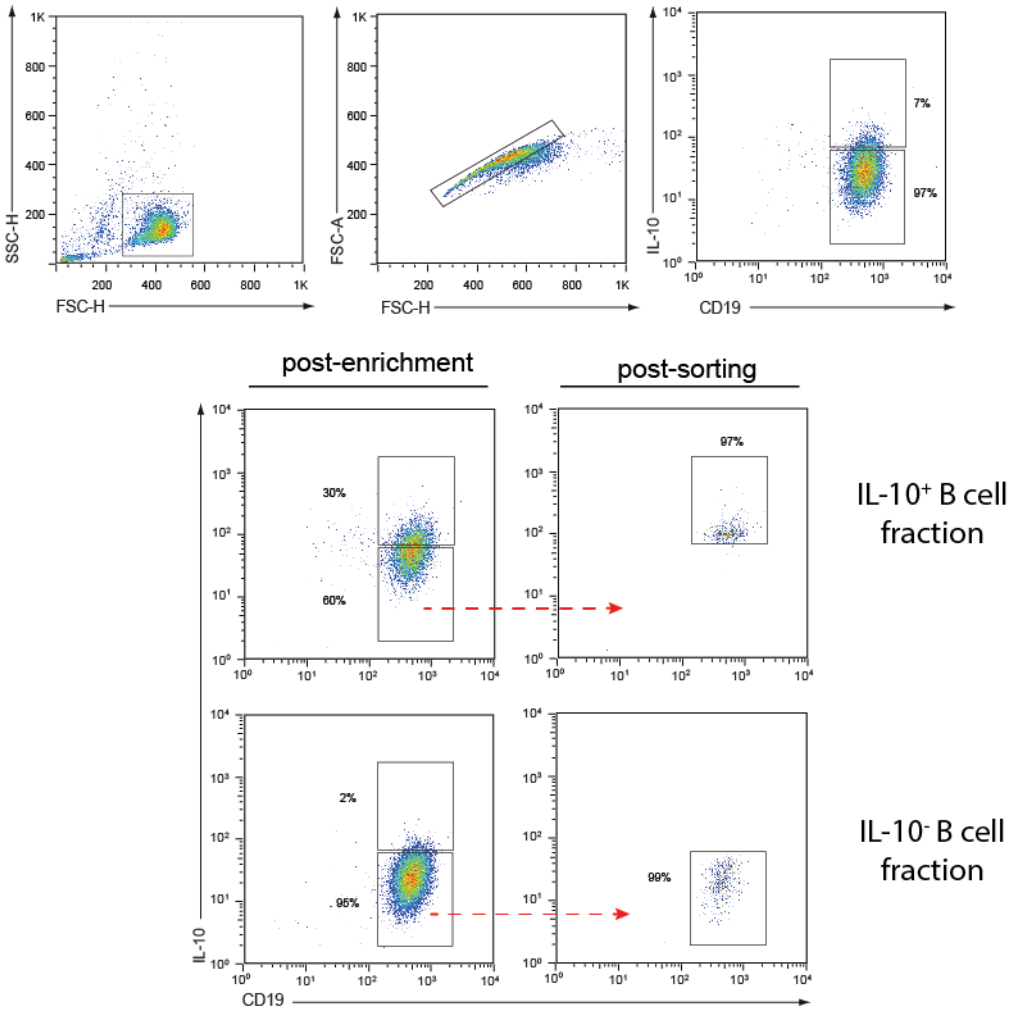


Figure 32. ***Re-sorting of IL-10 non-competent B cells****. IL-10 non-competent B cells were stimulated with LPS for 48h hours. Viable cells were selected on the morphologic plot and doublets were excluded. Gate for sorting were set on the IL-10 $^{-}$ fraction post-enrichment.*

Results from a single experiment are reported in **Figure 33**. Contrarily from the data reported in paragraph 5.4.2, the two populations do not have distinct patterns. In fact, the percentage of the overall methylation are comparable with 60% for IL-10^{+} and 55% for IL-10^{-} B cells in mCNS-9, and 95% and 88% in mCNS-4.5.

The initial hypothesis was that IL-10 competence and IL-10 production are two different mechanisms and that only a small portion of the B cell population is competent for IL-10 production. Accordingly to that assertion, this experiments demonstrate that IL-10 production at late timing by the non-competent B cells population does not depend on the acquisition of an IL-10 competent signature, but rather by other mechanisms that should be further characterized.

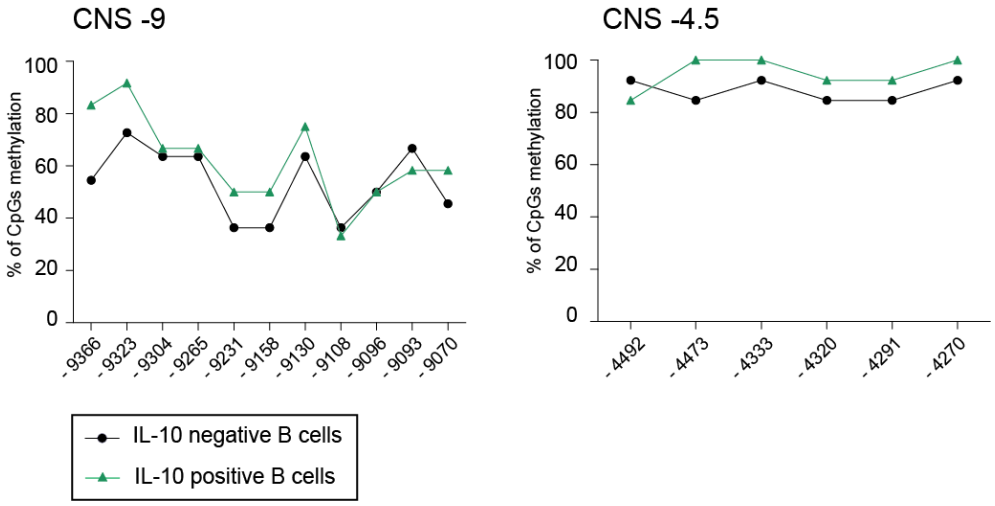


Figure 33. ***Methylation pattern of re-stimulated IL-10 negative B cells.*** *Values of methylation percentage at single CpG sites. n=1*

5.7. Pathways network in IL-10 competent B cells: a future study

In light of the results presented in the previous paragraph, it is clear that IL-10 competent and non-competent B cells take advantage of different pathways for the regulation of IL-10 production. The method used in this thesis to isolate the two populations include the stimulation through TLR4, therefore all B cells are activated and start the downstream signalling. In order to investigate if different pathways are activated in the IL-10 competent and non-competent B cells, it was decided to conduct the analysis both of the transcriptome and miRNome, with the aim, in future studies, to cross the results to identify possible regulatory networks. In literature there no data about transcriptome and

miRNome regarding IL-10 competent B cells, only one work separated B cell on their capacity to produce IL-10 in the human context (Lin et al., 2014), while in the murine setting analysis were conducted on subset only enriched in IL-10 producing B cells, or on B cells that received long stimulation (e.g. 48h of a-CD40 mAb) (Sun et al., 2015).

The results that will be presented below are just preliminary, and do not report a precise list of genes and miRNAs that are differently regulated in the two populations.

For transcriptome and miRNome analysis, IL-10 competent and non-competent B cells were separated as described in **Figure 22** of **paragraph 5.3**. The analysis of the miRNome was performed in Berlin by the group of Prof. Radbruch at the DRFZ, but the analysis is still ongoing.

The processing of transcriptome data was conducted instead by the bioinformatics facility of "Fondazione IRCCS Istituto Nazionale Tumori". The experiment was done in quadruplicate and, after normalization, the analysis of significantly different regulated genes showed that in the IL-10^{+} population there are 95 up-regulated and 47 down-regulated genes compared to the IL-10^{-} population, as reported in the plot in **Figure 34.**

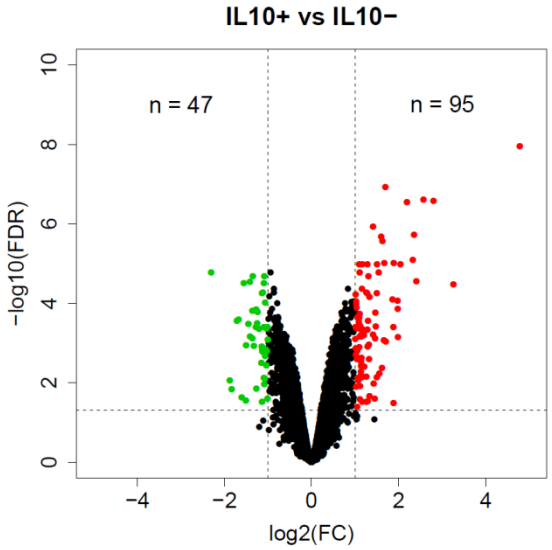


Figure 34. ***Number of significantly up and down-regulated genes between IL-10 competent (IL-10^{+}) and non-competent (IL-10^{-}) B cells****. Significantly* up-regulated genes are highlighted in red. Significantly* down-regulated genes are highlighted in green. *Significance threshold: fold change ≥ 2 (or ≤ -2) and false discovery rate < 0.05.*

A first analysis of these data was generated taking advantage of The Immunological Genome Project (https:// www.immgen.org/), a database that contains the information of

many murine immune cell subsets and allows the comparison with user-inserted datasets. The two lists of genes characterizing IL-10^{+} and IL-10^{-} B cells were compared with the main mature and immature murine population from splenic and peritoneal compartment, in particular, transitional, follicular, marginal zone and B1a B cells from the spleen and B1a and B1b from the peritoneal cavity. The data shown in **Figure 35** are automatically generated from the online software uploading a list of gene, and allows to visualize the expression of the dataset uploaded across the ImmGen populations. Interestingly, IL-10 negative B cells resemble the transitional and FO B cell population, that are respectively an immature and a specialized subset, so it is realistic that these cells do not need the capacity to quickly produce IL-10 after encountering an exogenous stimulation. The analysis for IL-10 positive population has instead revealed a similar transcriptional profile to MZ and B1 B cells from the spleen and the peritoneal cavity. This result is very peculiar, as B1 and MZ B cell are considered innate-like of B cells, that are able to respond quickly to external triggering from blood-borne pathogens (Zhang, 2013), a behaviour that resembles the properties of IL-10 competent B cells to immediately produce the cytokine after the trigger of the stimulus.

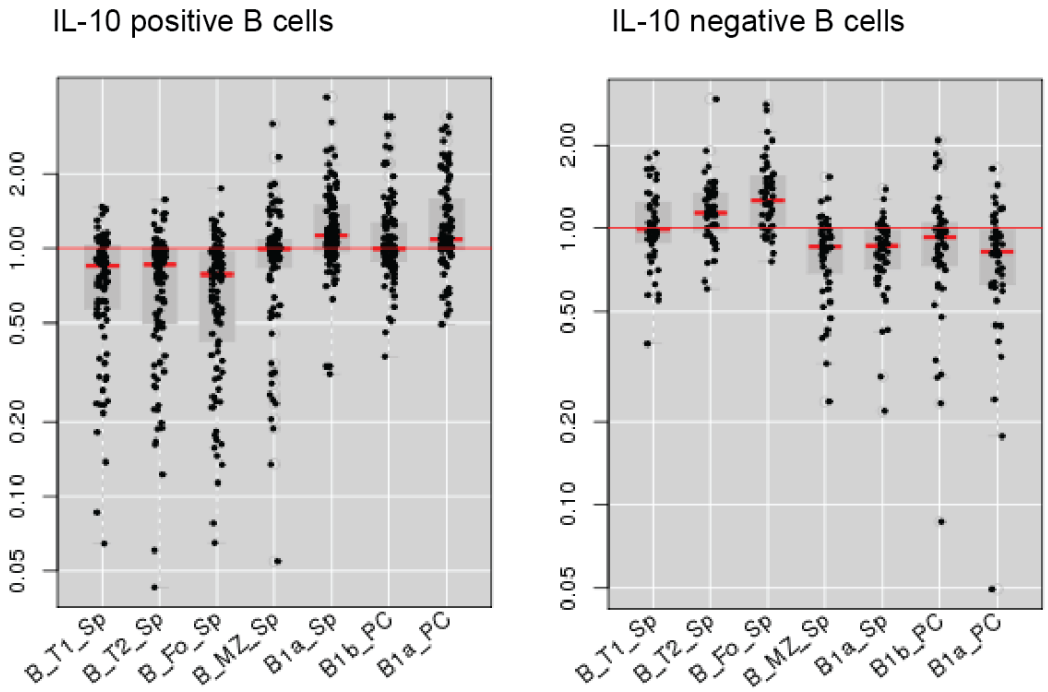


Figure 35. ***Comparison of transcriptome signature of IL-10 competent and non-competent B cells with the major murine B cell subsets****. The plot represent the similirity of the dataset uploaded with the main murine B cell population, where Sp=spleen and PC=peritoneal cavity.*

6. DISCUSSION

In the last twenty years, a growing body of evidences highlighted the role of B cells in the context of immunosuppression. Despite B cells with the capacity to downregulate the immune response were described both in healthy and pathological settings, their precise origin and phenotype are still a matter of debate (Rosser and Mauri, 2015). These cells are collectively called B_{reg} and act mainly through the secretion of the regulatory cytokine IL-10 (Fillatreau et al., 2002; Mauri et al., 2003; Mizoguchi et al., 2002). IL-10 can be produced by several immune cell types and is fundamental in the control of inflammation, as it is able to limit the damage that an uncontrolled response can have (Couper et al., 2008). Although the regulation of IL-10 expression has been extensively studied in many immune cell-types, for B cells little is known (Saraiva and O'Garra, 2010). B cells with the capacity to produce IL-10 were shown to be enriched in several subsets, for example B10 (Yanaba et al., 2008), T2-MZP (Evans et al., 2007) or MZ B cells (Gray et al., 2007), but none of these populations have been described to be a "fully committed" B_{reg} subset, as the percentage of IL-10 producing B cells does not correspond to the entire pool of cells. In our opinion, the literature concerning B_{reg} was rendered confusing as a consequence of the misleading interpretation of two different concepts: IL-10 production and IL-10 competence. In a previous work (Mion et al., 2014b), we reported evidences of the fact that these are two distinct mechanisms that co-exists among the splenic B cell population, as IL-10 competent B cells are able to produce and secrete IL-10 immediately after a short stimulation, while IL-10 non-competent B cells need a longer timeframe to produce the cytokine. The concept of competence is not limited to IL-10 in B cells but rather it has been described also for other cytokines in other cell lineages. An example is represented by the production of IL-4 by T cells, where the competence is acquired in a two stepwise model. Only cells that will become competent, during their development, generate an accessible chromatin conformation at the level of the gene that will confer the competence, allowing the production of the cytokine directly after stimulation. On the contrary, all the other cells that are also able to produce IL-4, need other signals and more time to produce the cytokine (Mohrs et al., 2005).

The model proposed in this thesis is that IL-10 competent B cells are a genuine B_{reg} subset, as they are able to respond immediately after 5 h of stimulation with LPS, PMA and

ionomycin, in terms of IL-10 production, while IL-10 non-competent B cells acquire an immune-suppressive capacity after being instructed by the surrounding environment.

In this study, we demonstrated that, among the total splenic B cell population, the *il-10* gene has a double transcriptional kinetic, since cells that are treated with CHX and then stimulated with LPS are not able to transcribe the gene after the fourth hour while, in the absence of the inhibitor, the transcription continues. The CHX treatment completely blocks protein synthesis, meaning that if a gene is transcribed in its presence, it needs only factors already present in the cell and can be rapidly transcribed. This process resembles the behaviour of B cells for IL-10 production. In particular, the *il-10* gene acts as a late PRG in IL-10 competent B cells as it is transcribed only after 2h of stimulation in a protein synthesis independent-manner. In all other B cells, *il-10* gene can be considered a SRG, as it needs *de novo* protein synthesis and a longer timeframe to be transcribed. Ramirez-Carozzi and colleagues demonstrated that, in LPS-stimulated macrophages, there is a connection between chromatin accessibility and the regulation of PRGs and SRGs. More specifically, PRGs do not require nucleosomes remodelling, while SRGs depend on the action of the SWI/SNF complex (Ramirez-Carrozzi et al., 2009). Also in light of these observations, we decided to test whether at the basis of IL-10 competence in B cells there are epigenetic modifications involved in chromatin or DNA accessibility.

The most common DNA modification in mammals is the methylation of C located in CpG sites (Deaton and Bird, 2011). In the past, this modification has been extensively studied at the level of gene promoters and has usually been associated with transcriptional silencing. Advances in technologies allowed genome-wide studies of the methylome, that located the majority of methylation not at the level of promoters, but rather in intra and intergenic regions. Moreover, these studies revealed that there is a correlation between the level of methylation on certain areas and the regulation of gene transcription (Stadler et al., 2011b). We first demonstrated that by interfering with methylation, through the use of the demethylating agent 5-aza, the pool of IL-10 competent B cells expands (**Figure 20**), so we decided to test whether between IL-10 competent and non-competent B cells there are differences at the level of methylation among the *il-10* gene locus. We selected four different regions on the basis of their level of conservation and CG content: two are located several kb from the TSS (mCNS-9 and mCNS-4.5), one is near it (mCNS-0.5) and the last one is located in the fourth intron (mCNS +1.1). We discovered that the level of methylation is

different between IL-10 competent and non-competent B cells, and is mainly located on mCNS-9 and mCNS-4.5 in the splenic compartment (**Figure 25 and 26**) and only on mCNS-4.5 in peritoneal cells (**Figure 27**). Interestingly, the same methylation signature of splenic B cells was conserved also in human IL-10 competent B cells (**Figure 29**). Noteworthy, a study that compared the methylome of effector T cells and T_{reg}, found that the majority of differences, in term of methylation, are located at promoters distal regions, rather than on promoters themselves (Schmidl et al., 2009). This observation resembles our findings, as a different methylation level was found on regions located several kb from the TSS, but no differences between IL-10 competent and non-competent B cells were detected in close proximity of the TSS. Interestingly, the analysed regions located upstream from the TSS, were already described to have a role in the regulation of *il-10* gene expression in macrophages and T cells. In T cells, mCNS-9 has been described as a binding site for NFAT1 and IRF4 that, together, are able to enhance *il-10* expression (Lee et al., 2009). A regulatory role for mCNS-4.5 has been described instead only in macrophages, where this region is bound by NF-κb following TLR4 stimulation (Saraiva et al., 2005). Collectively, our observation, together with the literature data, suggest that mCNS-9 and mCNS-4.5 may have a role in the regulation of *il-10* gene expression in B cells. Interestingly, these two regions were also tested in T cells for enhancer activity and it was discovered that mCNS-9 has enhancer properties in T cells (Jones et al., 2005; Lee et al., 2009). What still remains to be determined is whether if mCNS-9 or mCNS-4.5 have an enhancer-like activity in B cells. In order to clarify this point, a luciferase-assay must be performed and, to this aim, we have already cloned the CNSs of *il-10* gene locus into the pGL3 vector. In the near future, the generated constructs will be transiently transfected in the CH12.F3 B cell line and tested for luciferase activity (see APPENDIX).

Enhancers can be identified by their chromatin signature, in particular these regions are characterized by the presence of H3K4me1 and the absence of H3K4me3, and by the presence of active acetylation (Zhou et al., 2011). Taking advantage of ChIP, we were able to identify the specific histone signature of IL-10 competent and non-competent B cells with a particular focus on H3K4me3 and H3K27Ac. mCNS-9 and mCNS-4.5 are almost devoid of H3K4me3, while the level of H3K27Ac is higher in IL-10 competent B cells compared to the IL-10 non-competent population. On the basis of these data we can speculate that mCNS-4.5 and mCNS-9 are enhancers, and they are more active only in IL-

10 competent B cells, as characterized by higher acetylation. Moreover, although mCNS-0.5 does not present differences in DNA methylation, it has a specific histone signature, as the level of H3K4me3 and H3K27Ac are higher in IL-10 competent B cells compared to the IL-10 non-competent B cells.

Interestingly, although more methylated at mCNS-9 and mCNS-4.5, IL-10 non-competent B cells were fully demethylated at mCNS-0.5, similarly to the IL-10 competent B cell population. Although repressive histone modifications were not tested, the evidence that mCNS-0.5 DNA is not methylated leads to hypothesize that no major epigenetic obstacle to *il-10* transcription at a later time-point is present. It should be kept in mind that both IL-10 competent and non-competent B cells received the same LPI stimulation, so also the IL-10 non-competent fraction underwent to activation as TLR4 is widely expressed in B cells (Bekeredjian-Ding and Jego, 2009). To test whether also IL-10 non-competent B cells are able to produce IL-10, these cells were re-stimulated with LPS and, not surprisingly, IL-10 was detected in supernatants after 48h in the presence of the stimulus, confirming the initial hypothesis that the *il-10* gene can act as a SRG in IL-10 non-competent B cells, as it is transcribed in a second moment. A delayed induction of IL-10 has been reported also for human B cells. Indeed, Heine and colleagues performed an experiment quite similar to the one set up by us in the murine system: they first stimulated B cells for two days through CD40 and with IL-4, and CpG, then sorted them for their capacity to secrete IL-10 and finally re-stimulated the IL-10$^-$ fraction with the same stimuli. The authors showed that after the second three-days stimulation, also the B cells that were not able of producing IL-10 at day two started to secrete this cytokine (Heine et al., 2014), resembling the result that we obtained for IL-10 non-competent B cells re-stimulation in the murine system. To further investigate the mechanisms of IL-10 production in IL-10 non-competent B cells, as a next step of our analysis we decided to characterize the methylation signature at mCNS-9 and mCNS-4.5 of the IL-10 negative fraction. B cells that were initially IL-10 negative, were then re-sorted and separated for their positivity/negativity for IL-10, and their methylation signature at mCNS-9 and mCNS-4.5 was determined. Interestingly, in these settings there were no differences between IL-10 positive and IL-10 negative B cells, but rather, both populations presented the same methylation profile of the starting IL-10 non-competent B cell population.

Collectively, we demonstrated that after a short stimulation (5h LPI), B cells behave in two different ways: only IL-10 competent B cells are able to rapidly produce the cytokine without the need to synthetize TF, while IL-10 non-competent B cells necessitate more time as new protein synthesis is required. Moreover, as shown in **Figure 36,** IL-10 competent and non-competent B cells can be clearly distinguished by their specific methylation signature. Our hypothesis is that mCNS-9 and mCNS-4.5 can serve as binding sites for TF already present in IL-10 competent B cells, while non-competent cells have these regions masked by CpG methylation, that prevents the binding of TF. Moreover, IL-10 non-competent B cells need also the synthesis of new TF, as demonstrated by the CHX experiment. On the basis of our findings, the research of a specific TF that can uniquely identify IL-10 competent B cells is pointless, as these cells are already poised for IL-10 production and take advantage of already available factors. Nevertheless, we started the characterization of these two populations from the point of view of the global gene and miRNA expression, as to our knowledge in literature there are no data relative to the transcriptome and miRNome of IL-10 competent and non-competent B cells. Our purpose is to determine whether different pathways are activated in the two fractions and, moreover, if it is possible to identify common general networks between miRNAs and genes involved in the regulation of IL-10 expression. In order to contextualize this phenomenon into the *in vivo* setting, it will also be fundamental to determine which are the possible contributing external factors. For example, it has been shown that for human B cells, the secretion of IL-10 is strictly dependent on the action of autocrine calcitriol (Heine et al., 2008). Indeed, if cells are stimulated and treated with an antagonist of calcitriol, the levels of secreted IL-10 are lower compared to the untreated condition.

In conclusion, in the future this work will focus on the identification of the pathways that are activated in the IL-10 non-competent population after the first triggering, and that lead to IL-10 synthesis after 48h, but also on the TF that allows the rapid production of IL-10 by IL-10 competent B cells.

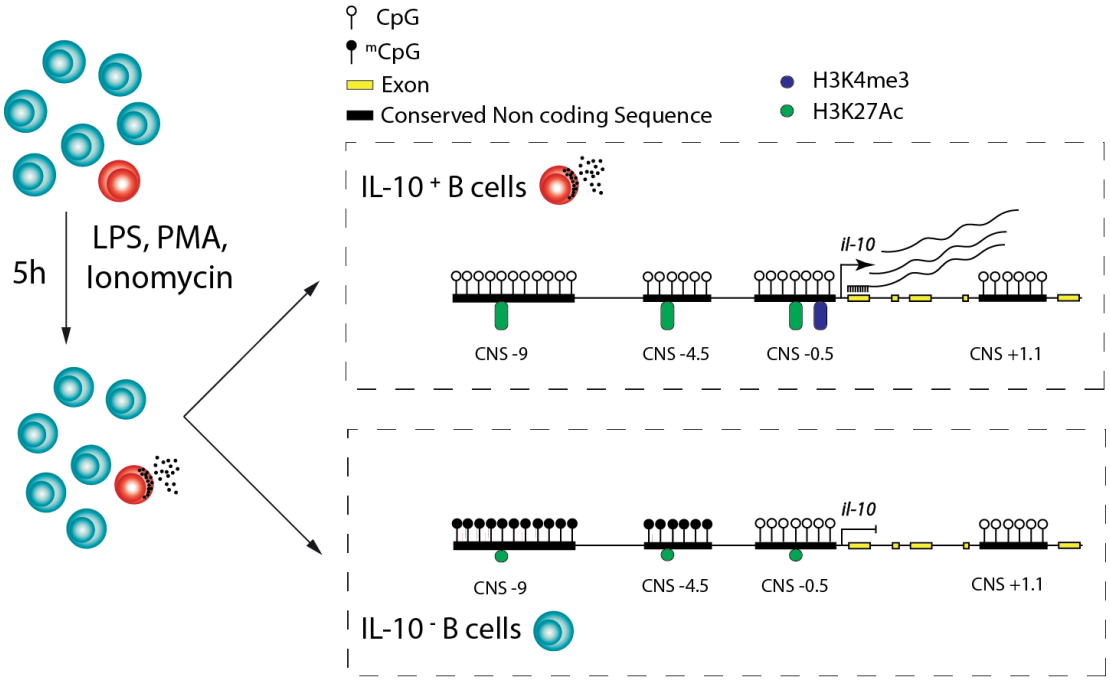


Figure 36. ***The epigenetic signature of murine splenic IL-10 competent (IL-10^{+}) and non-competent (IL-10^{-}) B cells.*** *IL-10 competent B cells have a fully demethylated locus, while IL-10 non-competent B cells are methylated at CNS-9 and CNS-4.5. Tri-methylation at H3K4 is found only at CNS-0.5 of IL-10 competent B cells. Acetylation is present in all regions analyzed, but is higher in IL-10 competent B cells compared to IL-10 non-competent fraction.*

7. MATERIALS AND METHODS

7.1. Murine B cell isolation

7.1.1. Animals

C57BL/6 mice were purchased from Envigo+++, spleens from IL-10 reporter GFP knock-in *tiger* mice were kindly provided by P.Scapini (University of Verona, Division of Medicine). All animal experiments were performed in accordance with the animal care and use committees of the respective institutes.

7.1.2. Splenic B cells

- ***Purification of Total B cells population***

B cells were isolated from spleen of 6-12 week old female mice (Harlan Laboratories, Indianapolis, IN, USA). Briefly, spleens were disrupted and the erythrocytes fraction was depleted by hypotonic lysis with ACK lysing buffer (SIGMA-Aldrich). B cells were isolated with *B cell isolation kit* (Miltenyi, 130-090-862) following the manufacturer's instructions, the obtained purity is around 95-99%.

- ***Purification of splenic IL-10 competent B cells***

B cells were isolated from spleens of 6-12 week old female C57BL/6 mice (Harlan Laboratories, Indianapolis, IN, USA). Briefly, spleens were disrupted and the erythrocytes fraction was depleted by hypotonic lysis with ACK lysing buffer (SIGMA-Aldrich). Using the *Regulatory B cell isolation kit* (Milteny 130-095-873), IL-10 secreting B cells were isolated from the splenocytes suspension following manufacturer instruction after 5h of stimulation with LPS, PMA and ionomycin (SIGMA-Aldrich) (LPI).

The isolation of IL-10 competent B cells in *tiger* mice was performed by isolation of total splenic B cells coupled by cell-sorting for positivity/negativity for GFP after 5h of stimulation with LPI. The purity and the gating strategy for sorting are shown in **Figure 37**.

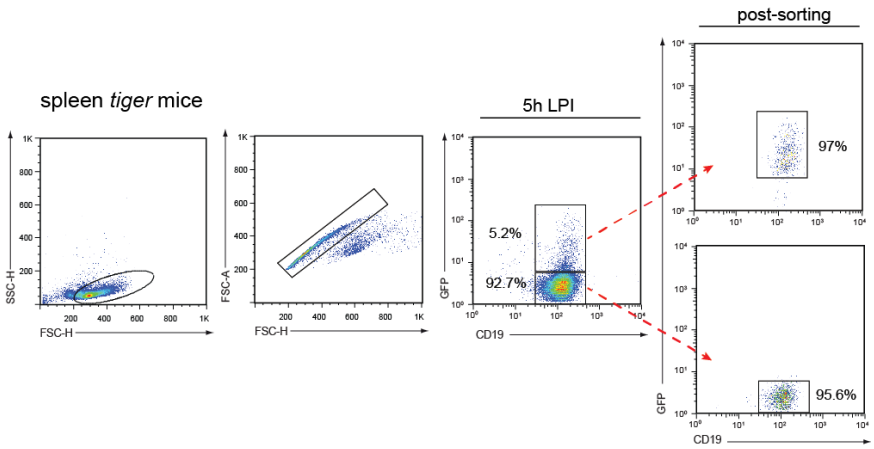


*Figure 37. **Isolation of IL-10 competent B cells from the spleen of tiger mice**. Viable B cells were selected on their morphology, then doublets were excluded from the sorting, and finally gates for positivity/negativity to GFP were set. The purity of the two fractions after sorting are shown.*

7.1.3. Peritoneal B cells

B cells from the peritoneal cavity were obtained after peritoneal lavage performed with cold PBS supplemented with 3% of fetal bovine serum (FBS) (SIGMA-Aldrich). Briefly, after cutting the skin above the abdomen, 5-10 ml of the solution are injected with a syringe using a 27G needle in the peritoneal cavity and cells are push into suspension through a gentle massage. Peritoneal IL-10 competent B cells were FACS-sorted after an intracellular staining for IL-10 (see paragraph 7.5.1) performed as described in shown in **Figure 38.**

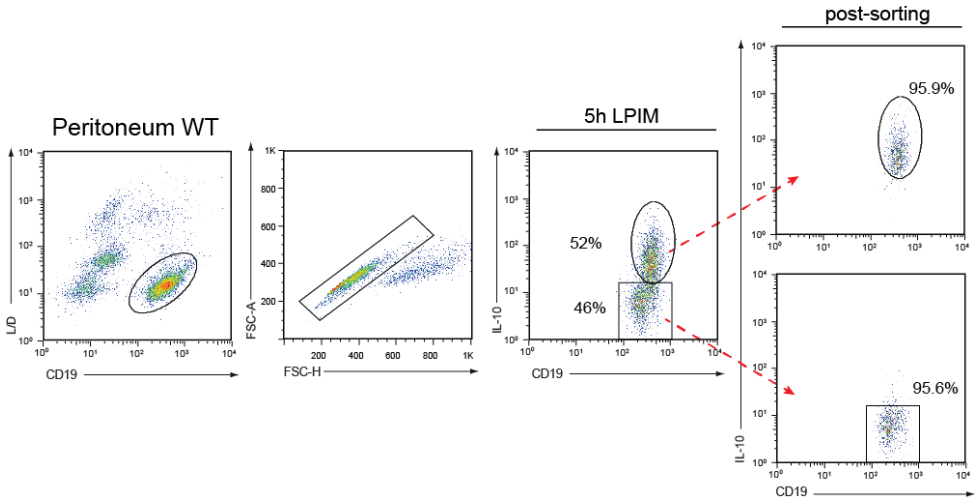


*Figure 38. **Isolation of IL-10 competent B cells from peritoneal cavity**. Viable B cells were selected for their positivity for CD19 and negativity for the fluorescent dye Live and Dead (L/D). Doublets were excluded from the sorting. The purity of the two fractions after sorting are shown.*

7.2. Human B cells isolation

Human IL-10 competent B cells were isolated from peripheral blood of healthy donors, according to the ethic committee of Azienda Ospedaliero-Universitaria di Udine. The population of B cells were enriched using *RosetteSep™ Human B cell Enrichment Cocktail* (STEMCELL) and IL-10 competent B cells were separated with FACS-cell sorting after 5 hours of stimulation with CpG (MWG), PMA (SIGMA-Aldrich) and ionomycin (SIGMA-Aldrich) plus Brefeldin A (SIGMA-Aldrich) and the intracellular staining for IL-10 (see paragraph 7.5.1).

7.3. Proliferation assay

B cells were marked with 5 µM carboxyfluorescein succinimidyl ester (CFSE; Invitrogen-Molecular Probes) by incubating them for 15 minutes at 37°C, the reaction is stopped with FBS (SIGMA-Aldrich) and cells are seeded at 10^6 cell/well. The proliferation rate was evaluated by citofluorimetry after 72 hours.

7.4. Inhibitor assay

7.4.1. Cycloheximide treatment

B cells are pre-treated with 10 µM CHX (SIGMA-Aldrich) for 30 minutes before stimulation with LPS (SIGMA-Aldrich) 10 µg/ml. B cells at 10^6 cell/ml are treated with CHX 2x, seeded and then the medium alone or with LPS (SIGMA-Aldrich) (2x) is added.

7.4.2. 5-azacytidine treatment

The protocol used for 5-aza treatment is summarized in **Figure 39**; basically, B cells are pre-stimulated with a-CD40 mAb (Becton Dickinson) at 1µg/ml for 24h, then the inhibitor is added to the culture at various concentration in 70µL of medium. The cells are then left in the incubator and in the last 2h of culture LPS (SIGMA-Aldrich) at a final concentration of 10 µg/ml in 70 µL, or medium alone are added to each well.

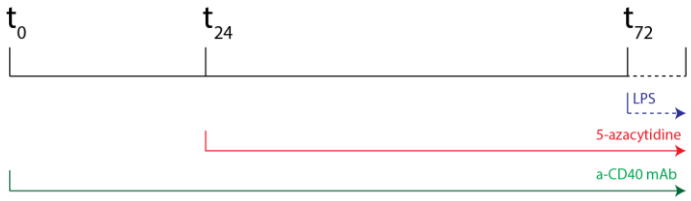


*Figure 39. **Timeline of 5-azacytidine treatment**. B cells are pre-stimulated with a-CD40 mAb for 24h, then treated with 5-aza for additional 48h; the medium alone or with LPS is added to the culture in the last 2h.*

7.5. IL-10 detection

7.5.1. Intracellular staining for IL-10

For immunofluorescent staining of intracellular IL-10, B cells are resuspended at $1x10^6$ cells/ml in culture medium containing 50 ng/ml PMA (SIGMA-Aldrich), 500 ng/ml ionomycin (SIGMA-Aldrich), 10 µg/ml LPS (SIGMA-Aldrich) and 2 µM monensin (eBiosciences) and cultured for 5 h at 37°C and 5% CO_2. At the end of the 5 h of stimulation cells are collected and washed with PBS before being stained with the green fluorescent probe LIVE/DEAD Fixable Green Dead Cell Stain Probe (Molecular Probes, Life Technologies) in order to discriminate viable cells from dead ones. Subsequently, the anti CD16/CD32 mAb is added to avoid non-antigen-specific binding of Fc portion of antibodies. The two reagents are incubated together for 15 min after which cells are washed and incubated for 30 min with the CD19 mAb (BioLegend). After surface staining, cells are fixed with 250 µl of Cytofix/Cytoperm cell fixation buffer (Becton Dickinson) for 20 min and then washed two times with Perm/Wash buffer (Becton Dickinson) for cell permeabilization. Hereafter Perm/Wash buffer is used for staining and washes in order to maintain cells in a permeabilized state. Cells are resuspended in Perm/Wash buffer containing PE-labeled anti-IL-10 mAb for 30 min. Finally, cells are washed twice with Perm/Wash buffer and resuspended in 250 µl of 1.5% formaldehyde fixative. Cells were kept at 4°C until analyzed by flow cytometry, or in some experiments sorted.

7.5.2. ELISA assay

The levels of IL-10 in cell supernatants were quantified by ELISA. $0.6x10^6$ B cells were cultured in the presence or absence of stimulation for the time requested by the experiment, in 24-well flat bottom plate (Corning costar, Tewksbury, MA, USA). The mouse IL-10 ELISA Ready-SET-Go kit (eBioscience) was used following manufacturer's instructions.

7.5.3. qPCR for *il-10* mRNA expression

IL-10 mRNA levels were determined by real-time PCR, total cellular RNA was isolated from $1x10^6$ B cells with Eurogold TriFast reagent (Euroclone) following manufacturer's instructions. RNA (1 µg) was reverse transcribed to cDNA using the SensiFast cDNA synthesis kit (Bioline). The generated cDNA was amplified by quantitative real-time PCR with the BioRad iQ5 device and using SYBR green as detection agent (iQTM SYBR Green Super Mix, BioRad). Data were collected and analyzed by the complementary computer software (iQ5 software, Bio-Rad). Results were expressed as fold induction compared to the control condition. The G3PDH transcript levels were used to normalize samples. The primers used for the real-time PCR reactions were synthesized and purified by Sigma-Aldrich and are listed in the **paragraph 7.9**.

7.6. CpGs methylation analysis

7.6.1. Bisulphite treatment and PCR amplification

Using the EpiTect Fast Bisulfite Conversion kit (Qiagen), genomic DNA was extracted from 100.000 cells for each condition and bisulphite-converted following manufacturer instructions. For PFA-fixed samples 1 hour at 65°C of decross-linking preceded the standard protocol of bisulphite-converted DNA extraction. Quantification was done as ssDNA with NanoDrop. Subsequently, PCR products were obtained from bisulphite-converted DNA using specific primers designed with the online software tool BiSearch and listed in **Paragraph 7.9**.

For all primer the following thermic protocol was used:

Step	**T [°]**	**Time**	
Initial denaturation	95	5 minutes	
Denaturation	95	1 minute	
Annealing	50	45 seconds	40 cycles
Extension	72	1 minute	
Final extension	72	10 minutes	

7.6.2. Cloning, transformation and sequencing

Fresh PCR were cloned using the pCR4-TOPO TA cloning kit for sequencing (Invitrogen) and TOP 10 one shot competent cells (Invitrogen) were transformed. Plasmids from single clones were extracted and sequenced using Sanger method (**Figure 40**). The percentage of methylation was determined using both QUMA and BISMA online software.

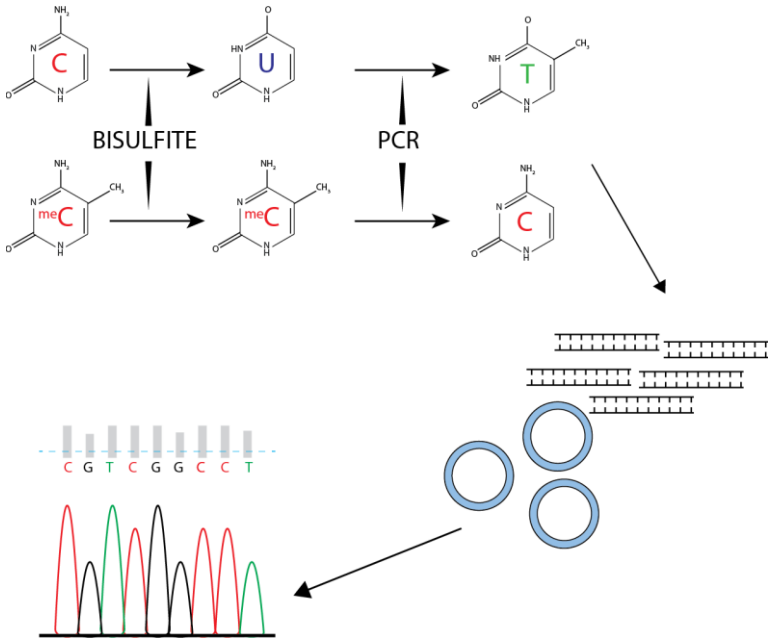


Figure 40. ***Cloning-based BSP.*** *After bisulphite conversion, specific regions were amplified by PCR, and the products were cloned into vector which were used to transform Top10 E.Coli were then transformed. For each clone the plasmid was sequenced (Sanger sequencing) allowing the calculation of the percentage of methylation of the basis of the number of C or T in the resulting sequence.*

7.7. Histones modification analysis

7.7.1. Chromatin immune precipitation

Up to 0.5 x 10^6 B cells for each condition are processed, and fixed in media added with formaldehyde (SIGMA-Aldrich) to a final concentration of 1% for 5 minutes in constant slow rotation. The fixation is then stopped by quenching with glycine (SIGMA-Aldrich) 0.125 M for 5 minutes and washed twice with cold PBS supplemented with protease inhibitor (Roche). Cells pellet are then lysed with L2 buffer, considering 150µl for each subsequent immunoprecipitation (example: 300 µl of L2 to study H3K27Ac and H3K4me3). Samples are then sonicated (Diagenode) to obtain DNA fragments between 200bp and 1kb as shown in **Figure 41**. After sonication the samples are diluted ten times with Dilution Buffer and the specific antibodies added: for these experiment H3K27Ac and H3K4me3 (all antibodies where purchased from Abcam) were used. The immunoprecipitation is performed overnight at 4°C on slow rotation. In order to purify the DNA-protein complexes bounded

to the antibodies, magnetic beads coupled with protein A (Dynabeads, Invitrogen) must be added to samples and incubated for 20 minutes at 4°C under rotation. After this passage the Input, so all the unbound fraction, is collect from each condition when put on the magnet, in the experiment for this thesis the 10% of the entire sample was taken. At this point, the remaining supernatant is through away and the beads-DNA-Protein complexes are washed three times with Wash Buffer (supplemented with PMSF) and one time with TE buffer, all the washing steps are performed keeping samples on the magnet. In order to separate the DNA-protein complexes from the beads, an incubation with Extraction Buffer must be performed for 25 minutes at 37°C on a thermomixer, setting 5 seconds at 1200rpm and 5 seconds steady. Finally, DNA-protein complexes are released through the reversion of the initial cross-linking leaving the samples overnight at 65°C. DNA is then purified using the QIAquick Gel Extraction Kit (QIagen), following the manufacturer's protocol.

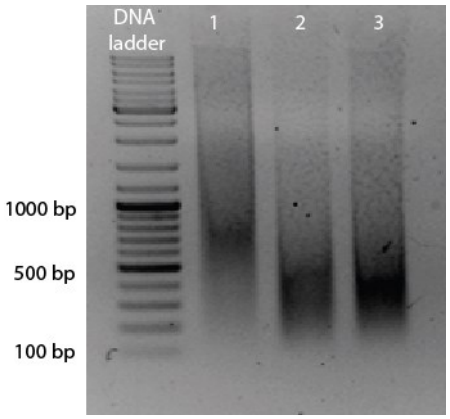


Figure 41. ***Genomic DNA after sonication****. Lane 2 is the condition chosen for experiments; 8 cycles at high power set on Bioruptor (Diagenode)*

Buffer used for ChIP

L2 Buffer: 50 mM Tris pH 8.0, 5 mM EDTA, 1% SDS

Dilution Buffer (DB): 50 mM Tris pH 8.0, 5 mM EDTA, 200 mM NaCl, 0.5% NP-40

Washing Buffer (WB): 20 mM Tris pH 8.0, 2 mM EDTA, 0.1% SDS, 1%NP-40, 500 mM NaCl

TE buffer: 10 mM Tris pH 7.5, 1mM EDTA

Extraction Buffer (EB): TE buffer 1X containing 1% SDS

7.7.2. ChIP qPCR conditions

DNA from ChIP, obtained as described in paragraph 7.7.1, are tested in real-time PCR using 3 µl for each replicate. The reaction was performed taking advantage of the intercalant dye SYBR green (BioRad) on the CFX96 machine (BioRad). A total of six regions were tested, the primers are listed in **paragraph 7.9**. The data from ChIP experiments are calculated as the percentage of input.

7.8. RNA preparation for transcriptome and miRNome

Both for transcriptome and miRNome cells fractions were isolated as described in paragraph 5.3. For both experiments up to 10^6 cells for each condition were lysed. Cells for transcriptome were lysed in Qiazol (Qiagen), while RNA from cells for miRNA analysis was extracted taking advantage of the RNeasy Kit (Qiagen) with minor modification to include the smallRNA fraction, as described by the manufacture's protocol.

7.9. Primer list

target	Technique	Chromosomic localization	N° CpGs	Forward primer	Reverse primer
mil-10	qPCR			TGTGAAAATAAGAGCAAGGCAGTG	CATTCATGGCCTTGTAGACACC
mG3PDH	qPCR			TCAACAGCAACTCCCACTCTTCCA	ACCCTGTTGCTGTAGCCGTATTCA
mCNS -9	Bisulphite	chr1:131,010,441-131,010,839	11	AGTGTAAAGATTATAGGAAGGTT	CTACCCAACTCTAAAAATAC
mCNS –4.5	Bisulphite	chr1:131,015,283-131,015,667	6	TAGAGATGTTATTGGTGTTT	CCACTTATACACAATATAAC
mCNS -0.5	Bisulphite	chr1:131,019,234-131,019,658	7	GTAGAGGAGGTATTAGAATTTTT	ACTTCTACATTACAACTATTTTT
mCNS +1.1	Bisulphite	chr1:131,021,587-131,021,799	6	AGATTAGATAGGAGATTAGGTAAA	AAAAAAACCATAACTAAAACCCC
hCNS -12.5	Bisulphite	chr1:206,784,418-206,784,795	10	AAGTTTTTTTTGAAAGAGATG	AACCTCTTCTAACTCATAACTT
hCNS – 6	Bisulphite	chr1:206,777,989-206,778,360	7	GGTGGTTAAGGTTTAAATAT	CTATATTCCTCTAAACTTTC
hCNS -0.5	Bisulphite	chr1:206,772,534-206,772,918	6	TGGGTATTTATTTTAGGTTG	AAAAAAACCTCTTCATTCAT
mCNS -9	ChIP	chr1:131,010,617-131,010,776		ACCCTGGTCATGCTCTTGAG	GCGTGTTCACCTGTGTTTCC
mCNS -4.5	ChIP	chr1:131,015,466-131,015,648		TGGCACCACAGTTACACAAAG	CTGGGGTCATAGGTTTAGAAGG
mCNS -0.5	ChIP	chr1:131,019,508-131,019,671		GGAGTGCGTGAATGGAATC	CTGGTCGGAATGAACTTCTG

mCD19 promoter	ChIP	chr7:126,414,184-126,414,281		CTGCCAGACCAAAGAACTTC	CAGTGAACGTGGAGGATAGTG
mProlactin promoter	ChIP	chr13:27,057,392-27,057,591		CCTTCATTTCTGGCCAATGT	GCCTGAGAGAACCACAGCTT
mGAPDH promoter	ChIP	chr6:125,165,082-125,165,205		GTACTGTGGGGAGGTGGATG	CAAAGGCGGAGTTACCAGAG

7.10. Statistical analysis

Experimental data are shown as mean ± standard error of mean (SEM). The unpaired or paired Student's t-tests (Prism, GraphPad Software, La Jolla, CA, USA) were used to analyse the results for statistical significance. P values below 0.05 were considered as significant. To test if the percentage of CpGs methylation were statistically significant, the online tool QUMA (http://quma.cdb.riken.jp/) was used (Kumaki et al., 2008).

8. APPENDIX

It has become progressively clear that appropriate expression of IL-10 is a precise key modulator in the balance between inflammation and immunoregulation when accurately spatial/temporal regulated by discrete IL10-producing cells. The mechanisms that govern the cell type- and receptor-specific induction of IL-10, however, remain unclear, but a fundamental role is played by changes in CpG DNA methylation status in gene regulatory regions. In this work of thesis, I focused my attention on four CNSs located in the *il-10* gene locus, and I analysed the epigenetic modifications which occur in IL-10 competent B cells. I found that IL-10 competent B cells show open chromatin and active transcription at two CNSs, which are located 9kb and 4.5kb upstream from the TSS and an additional mCNS-0.5 that is the more proximal to *il-10* gene. These experimental evidences, together with literature data (Jones et al., 2005; Saraiva et al., 2005), led us to hypothesize an enhancer-like activity for mCNS-9 and mCNS-4.5.

Experimental strategy to identify enhancers use transient transfection assays. DNA segment containing putative enhancers are cloned into a luciferase reporter vector usually under the control of a, CMV or SV40 minimal promoter. In my effort to define physiological conditions I decided to clone the identified CNS in reporter gene containing the of the *il-10* gene TSS. To this purpose, I cloned the entire region (containing mCNS-9, mCNS-4.5 and mCNS-0.5) and the single or combinations of the CNS to identify the contribution of each DNA-segments in the enhancement of the. All the clonings were made in pGL3-Basic Vector (Promega) (**Figure 42**), which lacks eukaryotic promoters and enhancers, and has luciferase gene, which expression exclusively depends on the correct insertion of promoter and enhancers in the vector. Moreover, the plasmid contains an ampicillin-resistance gene for selection, and multiple restriction sites (MCS) for insertion of DNA fragments are located upstream and downstream of the luciferase gene.

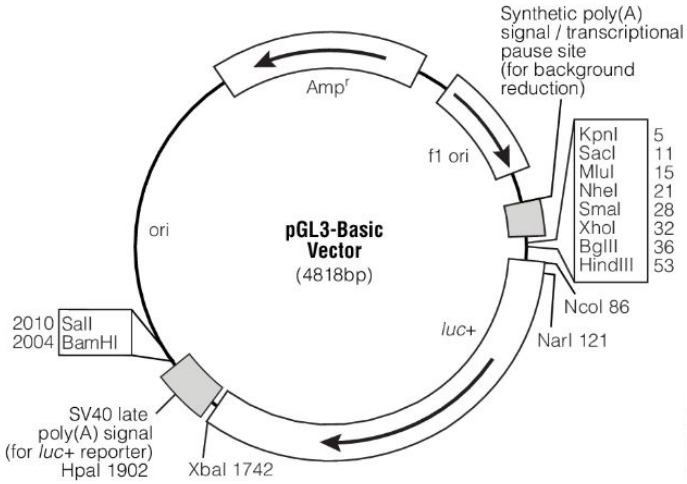


Figure 42. ***pGL3-Basic Vector****. The pGL3-Basic vector is 4818bp long and contains the coding sequence for the luciferase (luc+) and the gene for ampicillin resistance (Amp^r). (Adapted from Promega).*

Since no natural restriction site compatible with the MCS of the pGL3-Basic Vector are present in the studied region of the *il-10* gene locus, it was decided to insert them through the PCR reaction, adding a poly-AAA and the sequence for the enzyme at the 5' of each primer. For each cloning, different enzymes and primer pairs were used. In all cases, the digestion of the PCR product and of the plasmid were conducted separately and the ligation was done after a purification step. The single cloning strategies are described below.

In order to effectively test the enhancer-like activity of these regions, the subsequent step will be to transiently transfect the B cell line CH12.F3 with these plasmids and, after stimulation with PMA, ionomycin and LPS, to measure the activity of the synthetized luciferase. To date we managed to perform all the cloning and we are setting the conditions for the transfection on the Neon Transfection System (Life Technologies).

Cloning strategy for the entire region of the il-10 locus region studied

The PCR amplicon containing mCNS-9, mCNS-4.5 and mCNS-0.5 is 9716 bp long. At the 5' and 3' of the PCR product the restriction sites for MluI and SalI were synthetized (**Figure 43A**), while the plasmid was digested with MluI and XhoI, as this last enzyme creates compatible ends for SalI ligation (**Figure 43B**). The resulting plasmid is shown in **Figure 43C** and is 14523 bp long.

Cloning strategy for the exclusion of mCNS-9

The exclusion of mCNS-9 was made by the production of a PCR product that amplifies the region downstream mCNS-9 to the beginning of the gene (**Figure 44A**). The enzymes used are MluI and XhoI both for the PCR product and the plasmid (**Figure 44A-B**) and the resulting plasmid is 9495 bp long (**Figure 44C**)

Cloning strategy for the exclusion of mCNS-4.5

The cloning strategy for the exclusion of mCNS-4.5 comprises two different steps and two distinct PCR products. The first PCR amplifies the segment located upstream from mCNS-4.5, with the addition of the restriction site for KpnI and MluI. The insertion in the pGL3-Basic vector is made through digestion with the same enzymes (**Figure 45A-B, red primer and enzymes**) and, after ligation the plasmid is 9852 bp long (**Figure 45C**). The second PCR product comprises the downstream region from mCNS-4.5 to the beginning of *il-10* gene, this last cloning is made possible through the cut with MluI and BamHI on the PCR product (**Figure 45A, green primer**) and with MluI and BglII on the plasmid showed in **Figure 45C**. The insertion is possible as BamHI and BglII create compatible ends. The resulting plasmid without mCNS-4.5 in showed in **Figure 45D.**

Cloning strategy for the exclusion of mCNS-0.5

The exclusion of the region proximal to the promoter should comprise the segment of DNA before the ATG of the gene and, for this reason also in this case two sequential cloning are necessary. The first cloning is made into the basic plasmid by cutting both the PCR product and the plasmid with the enzymes XhoI and HindIII (**Figure 46A-B, red primers**). The resulting vector, shown in **Figure 46C**, is cut with MluI and XhoI for the second cloning, while the PCR product including mCNS-9 and mCNS-4.5 is processed with MluI and SalI. The final vector without mCNS-0.5 is shown in **Figure 46D**.

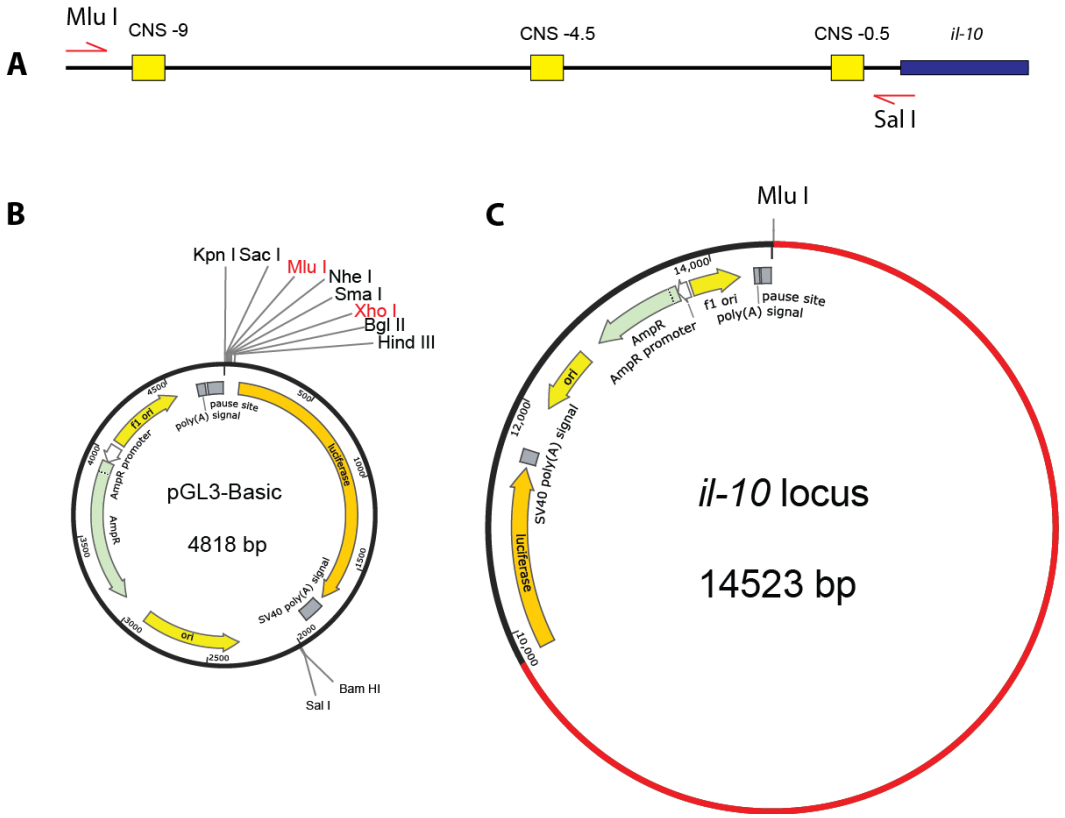


Figure 43. ***Cloning strategy for all studied regions of the il-10 gene locus****.* ***(A)*** *The region with mCNS-9, mCNS-4.5 and mCNS-0.5 was amplified with the insertion of MluI and SalI restriction site at the extremities.* ***(B)*** *pGL3-Basic vector was digested with MluI and XhoI.* ***(C)*** *Map of the resulting plasmid, the red segment represents the PCR product inserted.*

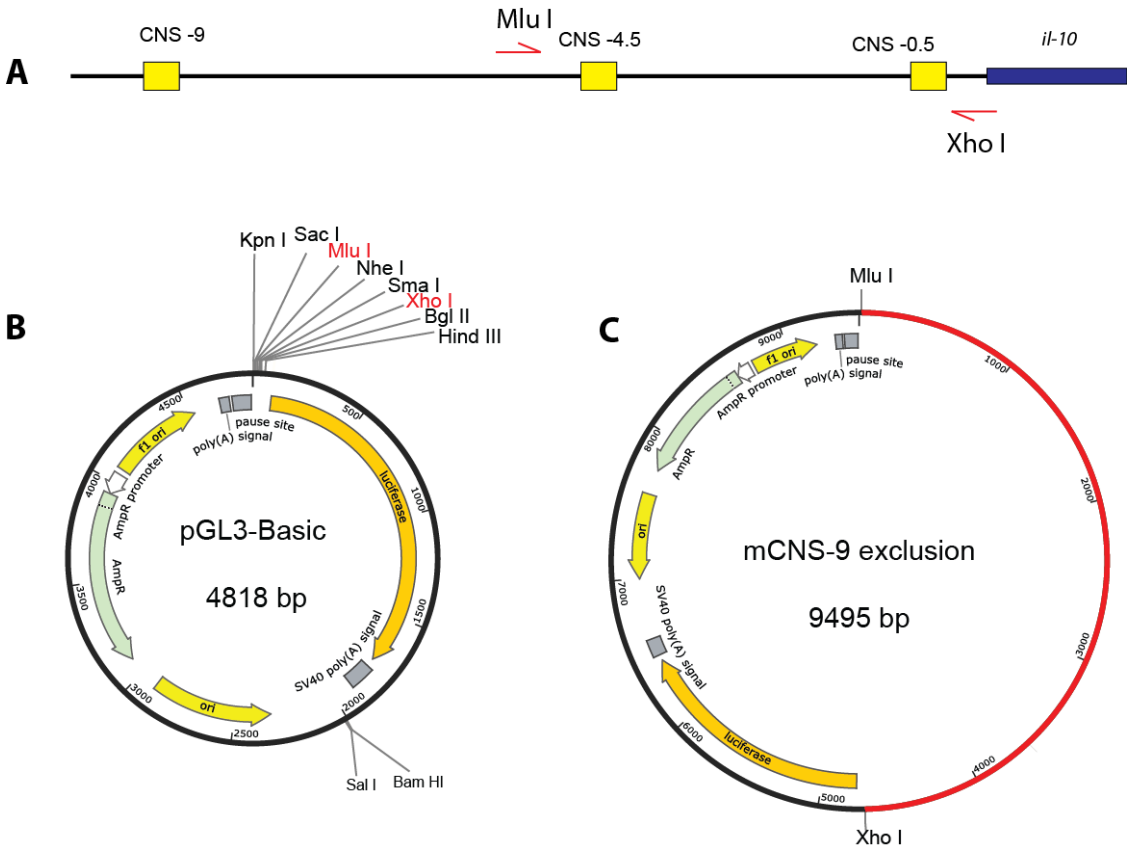


Figure 44. ***Cloning strategy for the exclusion of mCNS-9****.* ***(A)*** *The region without mCNS-9 was amplified with the insertion of MluI and XhoI restriction site at the extremities.* ***(B)*** *pGL3-Basic vector was digested with MluI and XhoI.* ***(C)*** *Map of the resulting plasmid, the red segment represents the PCR product inserted.*

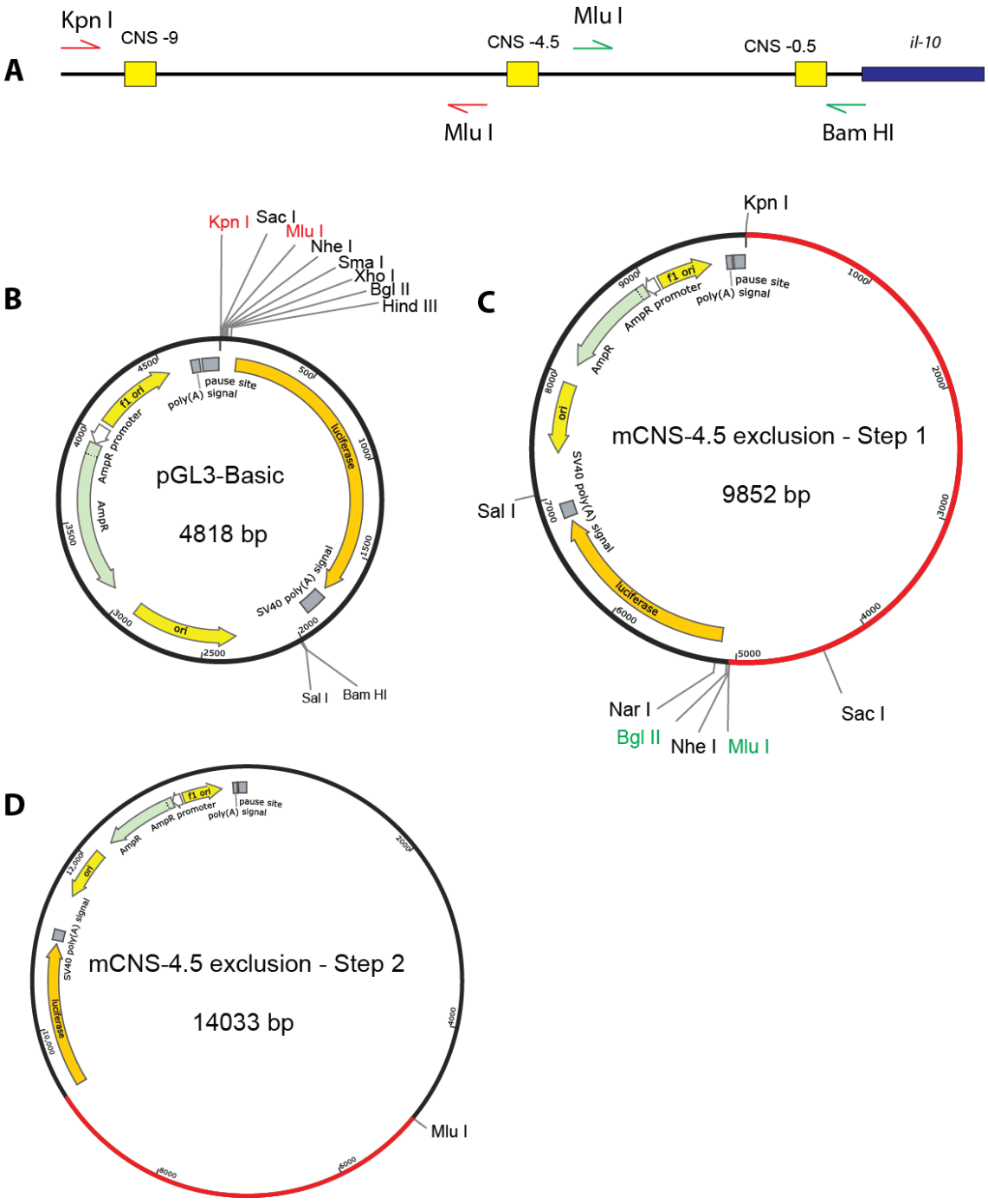


Figure 45. ***Cloning strategy for the exclusion of mCNS-4.5. (A)*** *The PCR for the first cloning is amplified with insertion of KpnI and MluI sites (red primers), while the second fragment has MluI and BamHI (green primers).* ***(B)*** *pGL3-Basic vector is digested with KpnI and MluII.* ***(C)*** *the resulting vector from the first cloning is digested with MluI and BglII.* ***(D)*** *Final vector with the insertion of the second PCR product.*

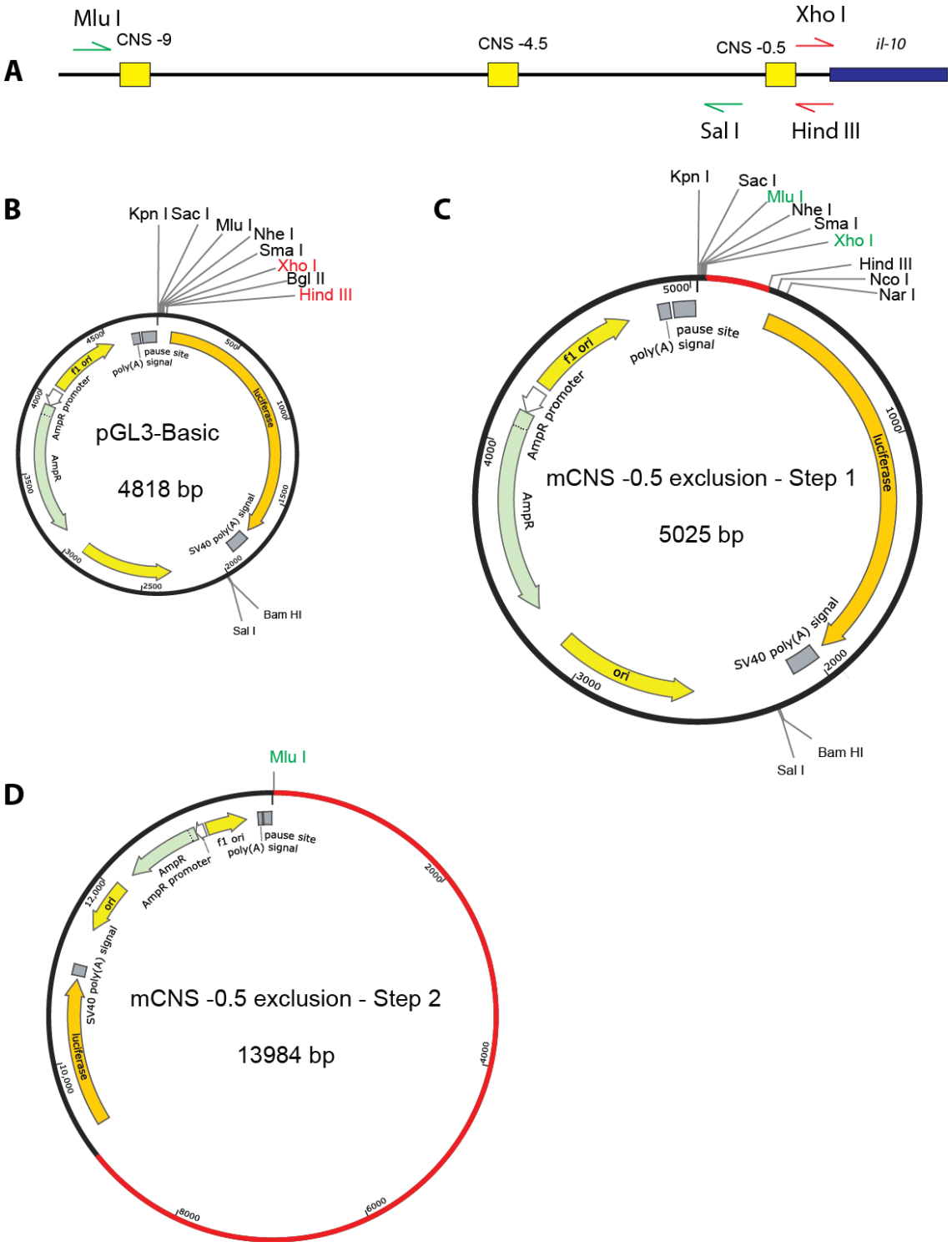


Figure 46. ***Cloning strategy for the exclusion of mCNS-0.5. (A)*** *The PCR for the first cloning is amplified with insertion of XhoI and HindIII sites (red primers), while the second fragment has MluI and SalI (green primers).* ***(B)*** *pGL3-Basic vector was digested with XhoI and MluII.* ***(C)*** *Vector resulting from the first cloning is digested with MluI and XhoI.* ***(D)*** *The final vector is formed insertion of the second PCR product.*

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10. LIST OF PUBLICATIONS

Full papers

- Francesca Mion, **Silvia Tonon**, Barbara Toffoletto, Daniela Cesselli, Carlo E. M.Pucillo, Gaetano Vitale

  IL-10 production by B cells is differentially regulated by immune-mediated and infectious stimuli and requires p38 activation.

  Mol. Immunol. doi:10.1016/j.molimm.2014.05.018

- Elizabeth C Rosser, Kristine Oleinika, **Silvia Tonon**, Ronan Doyle, Anneleen Bosma, Natalie A Carter, Kathryn A Harris, Simon A Jones, Nigel Klein & Claudia Mauri

  Regulatory B cells are induced by gut microbiota–driven interleukin-1β and interleukin-6 production

  Nature Medicine 20,1334–1339 (2014) doi:10.1038/nm.3680

Conference proceedings

- *IL-10-competent B cell expansion and IL-10 production are differently regulated by immune-mediated and infectious stimuli.*

  Poster presented at "IX national congress of SIICA", Firenze 28-31 May 2014

- *Regulation of IL-10 production in B cells*

  Oral presentation at "International Retreat of PhD students in immunology", Catanzaro 19-20 June 2015

- *Is epigenetics controlling IL-10 production in B cells?*

  Poster presented at SIBBM, Torino 1-3 July 2015

- *Does Epigenetics play a role in IL-10 production by B cells?*

  Oral presentation at "X national congress of SIICA", Abano Terme 25-28 May 2016