

**IDENTIFICATION OF DOWNSTREAM TARGETS  
OF THE LYMPHOID-SPECIFIC  
TRANSCRIPTIONAL COACTIVATOR OBF-1**

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# 1 Introduction

## 1.1 The murine immune system

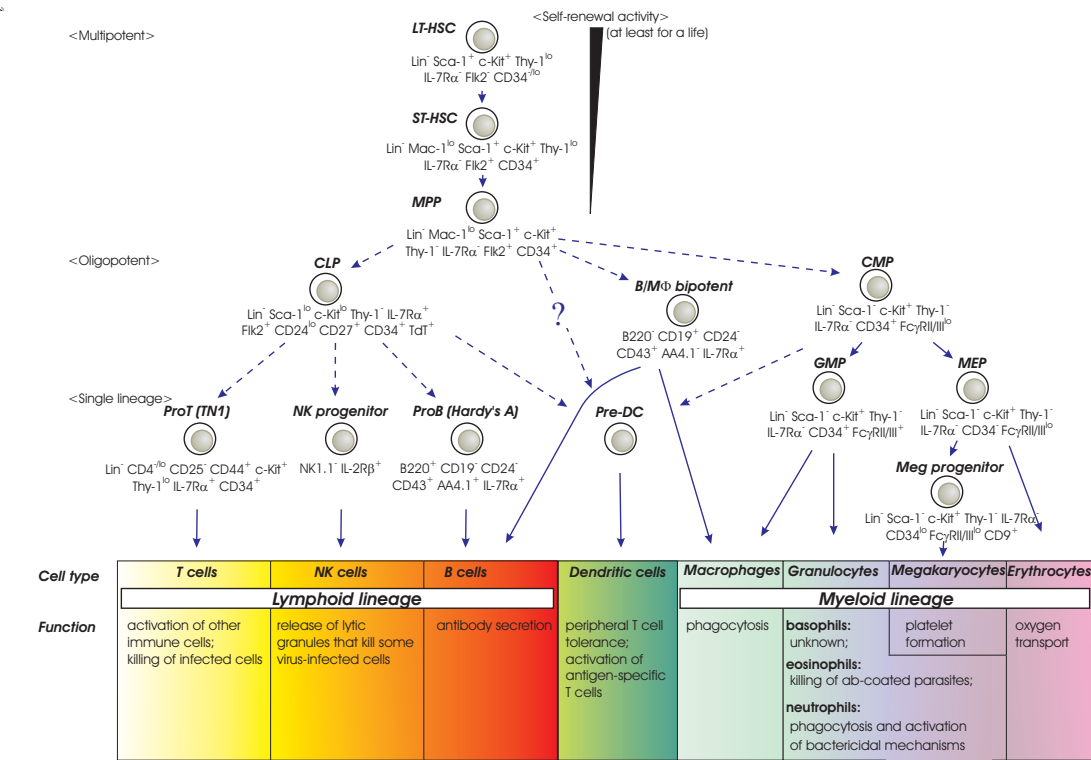
The first chapter serves as a general introduction into the constituents of the immune system of the mouse, whose physiological function is the defense against pathogens (antigens) invading the body. It bears high resemblance to other mammalian immune systems, such as its human equivalent. All cell types making up the immune system are continuously regenerated from multipotential precursor cells, the hematopoietic stem cells (HSCs) that reside mainly in the bone marrow of adult mammals. Commitment to the different hematopoietic lineages and differentiation of HSCs into the different effector types of cells of the immune response proceeds in a cascade of irreversible steps, as depicted in Figure 1. The HSCs differentiate in the hematopoietic organs, including bone marrow, spleen, thymus and lymph nodes, and migrate and patrol through the body in the bloodstream and in the lymphatic system.

One can distinguish two types of immune responses: **innate** and **adaptive immune responses**. The adaptive immune response is a specific reaction of an individual to infection with a pathogen, while the innate immune response does not rely on previous exposure to the pathogen and acts in the same way in different individuals. Both responses are, however, intricately linked: the specific immune

responses often rely on components of the innate immune system. We will focus here on the mechanisms and effector cells of adaptive immunity.

The major cellular components of the adaptive immune responses are the B and T lymphocytes that arise from a common lymphoid progenitor (CLP) detectable early in embryonic development in the fetal liver and, during adulthood, in the bone marrow. Both B and T cells bear highly diverse receptors on their cell surface that are able to recognize a vast diversity of antigens. Both share an ordered rearrangement of their antigen receptor genes, obligatory expression of a surrogate, invariant component of their pre-receptors, and ligand-dependent positive and negative selection of their mature antigen receptor repertoires. Each cell is genetically programmed to encode unique cell surface receptors specific for a particular antigen. The B cell antigen receptor (BCR) is the membrane-bound form of an antibody (immunoglobulin) that would be secreted upon activation of the cell. Each B cell presents only one type of BCR on its surface. The T cell antigen receptor (TCR) is a membrane-bound molecule related to immunoglobulin, and recognizes a complex of a peptide fragment bound to a molecule specialized in antigen presentation, an MHC molecule (Janeway et al., 1999).

We will focus first on the development of T cells and then dedicate a chapter to B cell development in the mouse.



**Figure 1** Differentiation of hematopoietic stem cells (HSCs) to the different lymphoid and myeloid lineages. The different progenitor cells can be distinguished by the expression of different cell surface molecules (as indicated). (Figure modified from (Kondo et al., 2003).)

### 1.1.1 Murine T cell development

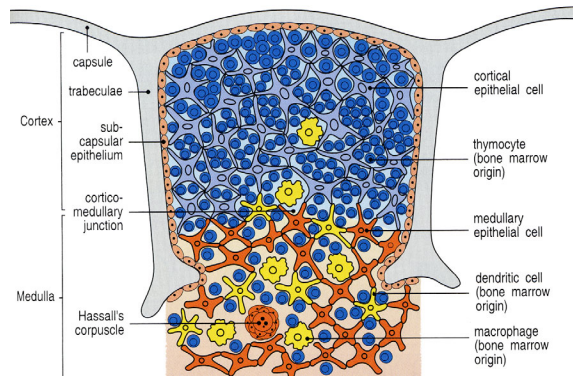
#### 1.1.1.1 Main stages of T cell development in the thymus

As mentioned before, T cells originate from pluripotent hematopoietic stem cells (HSCs) present in the fetal liver and, during life, in the bone marrow of mammals. These HSCs can give rise to proT cells that are the earliest committed T cell progenitors that migrate to the thymus, the main site of T cell development. Here, the immature T cells undergo distinct stages of proliferation and differentiation, which can be identified by discrete cell surface expression of a number of protein markers. T cell development is accompanied by gene rearrangements that produce the T cell receptor and is guided by positive and negative selection processes that determine the mature TCR repertoire and that are the cause of extensive cell death in the thymus. The main stages of thymic T cell development are depicted in Figure 2 and reviewed extensively in (Gill et al., 2003). The thymus itself is structured in several lobules, each containing discrete cortical (outer) and medullary (central) regions. As shown in Figure 3, the cortex consists mainly of immature thymocytes embedded in a network of branched cortical epithelial cells and intermittent macrophages. The outer cortical area contains mostly proliferating immature cells, while the deeper layers of cortical thymocytes are mostly in the process of undergoing thymic selection. The medulla consists of mature thymocytes, surrounded by medullary epithelial cells, macrophages and dendritic cells.

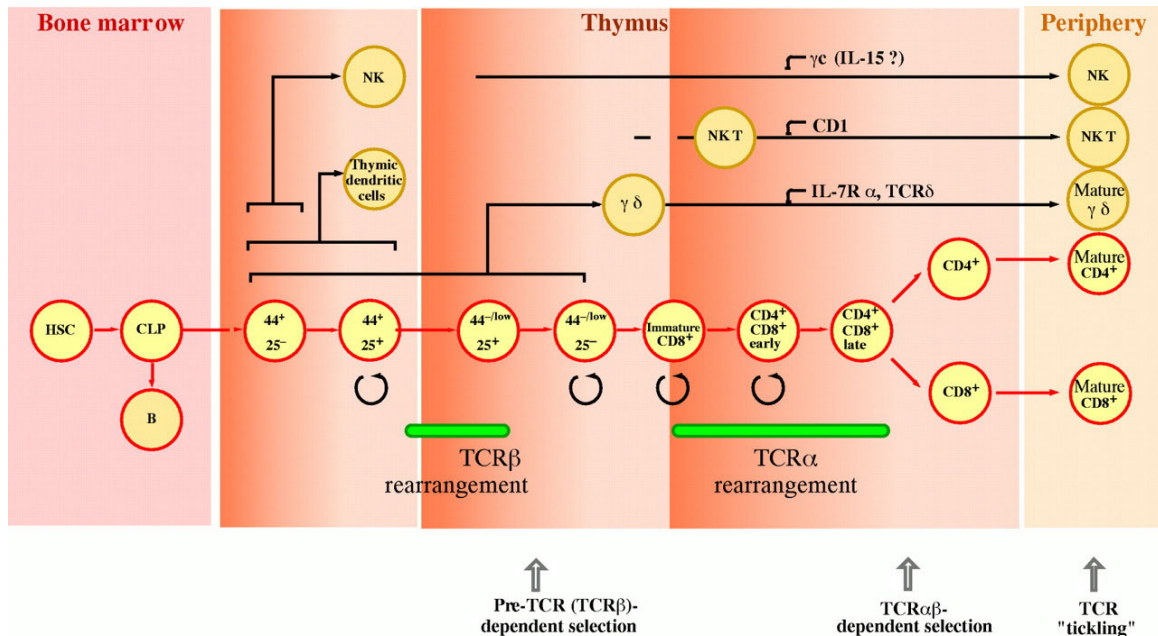
#### 1.1.1.1.1 The classical pathway of $\alpha\beta$ T cell development

The majority of lymphoid progenitor cells present in the thymus follow the main pathway of T cell development (Figure 2), resulting in the generation of a repertoire of T cells that carry a unique T cell receptor (TCR), consisting of an alpha and a beta chain, on their surface, and a co-receptor molecule that can be either CD4 or CD8. The functional consequences of this will be discussed later.

However, there are several branch points during early thymocyte development that can divert the cells into other lineages.



**Figure 3** Thymic architecture. (Taken from (Janeway et al., 1999).) The thymus lies above the heart and is composed of several lobules with cortical (outer) and medullary (inner) regions. The architecture and the cells making up the thymus are indicated.



**Figure 2** T cell development. The developmental stages can be distinguished by the expression of cell surface molecules and the TCR rearrangement status. Sub-lineage branch points and selection checkpoints are indicated.

#### 1.1.1.1.2 $\gamma\delta$ T cells

A second T cell lineage, the  $\gamma\delta$  T cells, express  $\gamma\delta$  T cell receptors instead of  $\alpha\beta$  TCRs, and make up 1-5% of the T cells in the thymus.  $\gamma\delta$  T cells are also the first T cells that appear during embryonic mouse development as bursts of cells that populate distinct sites in the adult animal, starting with the epidermis, leading to the establishment of so-called dendritic epidermal T cells (DETCs), and proceeding with the epithelial layers of the reproductive tract (Havran and Allison, 1988; Hayday, 2000).  $\alpha\beta$  T cells appear only a few days after the earliest  $\gamma\delta$  T cells and rapidly become the predominant thymocyte population. There is still some controversy about how the decision between  $\alpha\beta$  and  $\gamma\delta$  T cell fate is made. What is clear is that following the definitive commitment to the T cell lineage in the thymus, T cell precursors express the IL-2 receptor  $\alpha$  chain (CD25) and begin to rearrange and express their TCR  $\beta$ ,  $\gamma$  and  $\delta$  genes. Cells that successfully rearrange TCR  $\gamma$  and TCR  $\delta$  express a  $\gamma\delta$  TCR and can proceed along the  $\gamma\delta$  lineage pathway. Similarly, cells that successfully rearrange their TCR  $\beta$  gene express a pre-TCR (formed by association of TCR  $\beta$  with the invariant pT $\alpha$  chain) and are able to differentiate along the  $\alpha\beta$  lineage. However, to which extent the  $\gamma\delta$  TCR and pre-TCR play an instructive - rather than a selective - role in the  $\alpha\beta/\gamma\delta$  lineage decision remains controversial (Fehling et al., 1999; Kang and Raulet, 1997; MacDonald and Wilson, 1998; Robey and Fowlkes, 1998). The instructive model postulates that expression of a  $\gamma\delta$  TCR or pre-TCR *per se* is sufficient to direct a bipotential precursor cell to adopt a  $\gamma\delta$  or  $\alpha\beta$  T cell fate. In contrast, the selective model assumes that the  $\alpha\beta/\gamma\delta$  cell fate is determined independently of the TCR, but that appropriate TCR (or pre-TCR) signaling is required for survival and subsequent differentiation along the predetermined lineage pathway (MacDonald et al., 2001).

The biological function of this T cell subset is still poorly understood.  $\gamma\delta$  T cells are often considered to be a more primitive cell type, since they are emerging earlier than  $\alpha\beta$  T cells in phylogeny and in ontogeny, and might be defining an interface between the adaptive and innate immune systems (reviewed in (Hayday and Tigelaar, 2003)). In addition,  $\gamma\delta$  T cell deficits are more often associated with defective immunoregulation than with a failure of immunity (Girardi et al., 2002; Roberts et al., 1996). These immunoregulatory  $\gamma\delta$  T cell subsets have several effector potentials, including the potential for cytolysis and chemokine secretion.

#### 1.1.1.1.3 NK cells

Second, natural killer (NK) cells can develop from a common T/NK cell precursor. While NK

cells arise mostly from a bone marrow precursor, a minor subset (approximately 0.1%) of cells with NK phenotype can be detected in the adult thymus, arising from bipotential T/NK precursors. A working model for the NK cell development is presented in a recent review (Colucci et al., 2003)

NK cells are lymphocytes which are able to recognize and kill a limited range of abnormal cells by a cytotoxic attack highly similar to that of cytotoxic T cells, even though they do not bear any known antigen-specific receptors on their cell surface. This cytotoxic attack is triggered when an antibody of the IgG1 or IgG3 subclass bound to a cell makes contact with the Fc $\gamma$ RIII receptor (CD16) on the NK cell surface.

#### 1.1.1.1.4 NKT cells

Yet another cell type, the NKT cells, constitute a lymphocyte subpopulation that is abundant in the thymus, spleen, liver and bone marrow and is also present in the lung. NKT cells express surface markers that are characteristic of both natural killer cells (such as NK1.1 and CD122) and conventional T cells (such as TCRs). They have been implicated in the regulation of immune and autoimmune responses, particularly in the development of type 1 diabetes. The majority of NKT cells utilizes a restricted TCR repertoire that recognizes glycolipids in association with the non-polymorphic MHC-like molecule CD1d. These cells preferentially use a single invariant V alpha 14 antigen receptor (Taniguchi et al., 2003). A natural ligand for V14 NKT cells has been identified, namely  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer), a glycolipid which is presented by CD1d (Kawano et al., 1997). It is generally assumed that a very small population of conventional CD4<sup>+</sup>CD8<sup>+</sup> cells randomly express the NKT semi-invariant TCR, and these are selected by CD1d to develop into NKT cells (MacDonald, 2002). NKT cells are extensively reviewed in (Sharif et al., 2002).

#### 1.1.1.2 T cell function

T cells are able to distinguish cells that are harboring pathogens by recognizing peptide fragments of pathogen-derived proteins displayed by the cell in a complex with the Major Histocompatibility Complex molecule (MHC). There are two different classes of MHC, class I and class II, that are specialized in the presentation of peptides derived from different cellular compartments. MHC class I molecules present peptides from the cytosol and are recognized by CD8<sup>+</sup> killer T cells, while MHC class II molecules display peptides generated in vesicles and are recognized by CD4<sup>+</sup> helper T cells. Thus, the two different functional subsets of T cells can become activated and contribute to the host defense against different types of pathogens. Proteins from viruses or other intracellular parasites are displayed on



MHC class I molecules and the infected cell can be killed by a killer T cell ( $CD8^+$  T cell) that recognizes the antigen as non-self. On the other hand, extracellular pathogens and toxins taken up in vesicles, usually by phagocytic cells such as macrophages, are presented in MHC class II complexes to helper T cells ( $CD4^+$  T cells) that activate B cells to secrete specific antibodies that help to eliminate the pathogens (Janeway et al., 1999). The MHC class I and class II molecules are depicted in Figure 4.

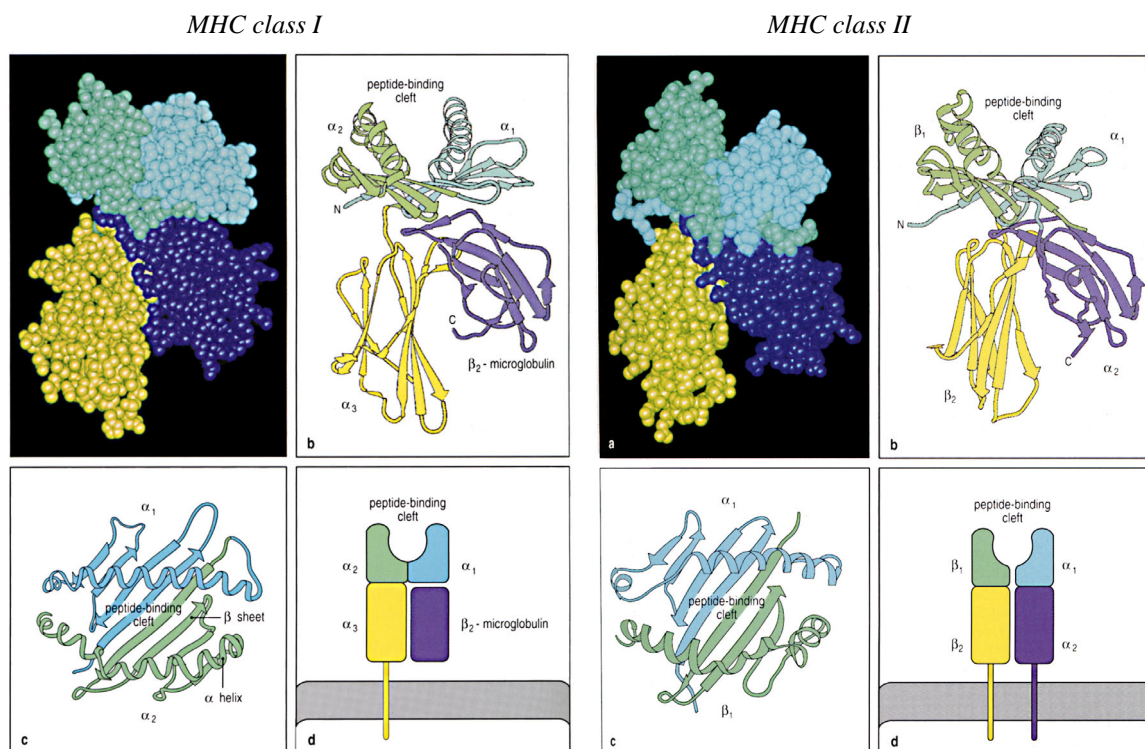
Each individual TCR is specific for a particular MHC-peptide complex or, in other words, MHC-restricted for antigen recognition. Thymocytes with TCRs that are able to recognize self-MHC molecules and function in self-MHC-restricted responses to foreign antigens are positively selected for survival in the thymus. The positive selection relies critically on thymic cortical epithelial cells that make close contacts with the T cells which express CD4 and CD8 co-receptors on their cell surface during the process. The positive selection depends on the engagement of both the antigen receptor and co-receptor with an MHC molecule, and determines the survival of single-positive cells that express only the appropriate co-receptor. Having accomplished positive selection, these single-positive thymocytes are ready for export to the periphery. The function of the CD4 or CD8 co-receptors of the mature T cells lies in their distinct abilities to bind invariant sites on MHC class II or class I molecules, respectively.

Negative selection in the thymus occurs to cells that encounter their corresponding antigen (a self-peptide) on a self-MHC of an antigen presenting cell (APC). The most important types of APCs are the

bone marrow-derived dendritic cells and macrophages. These are professional antigen-presenting cell types that also activate mature T cells in peripheral lymphoid tissues. Negative selection is controlled by the transcription factor AIRE (autoimmune regulator), which promotes ectopic expression of peripheral tissue-restricted antigens in thymic medullary epithelial cells. This “immunological self shadow” is an important mechanism in central tolerance to autoantigens and thus a key factor in controlling autoimmunity (Anderson et al., 2002; Liston et al., 2003).

Once they have completed their development in the thymus, T cells enter the bloodstream and are carried by the circulation. On reaching a peripheral lymphoid organ they leave the blood again to migrate through the lymphoid tissue, returning to the bloodstream to recirculate between blood and peripheral lymphoid tissue until they encounter their specific antigen.

The activation of naive T cells in response to antigen, and their subsequent proliferation and differentiation, constitutes a primary immune response. At the same time as providing armed effector T cells, this response generates immunological memory, which gives protection from subsequent challenge by the same pathogen. The generation of memory T cells, long-lived cells that give an accelerated response to antigen, is much less well understood than the generation of effector T cells. Memory T cells differ in several ways from naive T cells, but like naive T cells they are quiescent and require activation by antigen-presenting cells with co-stimulatory activity in order to regenerate effector T cells. This cell type is reviewed in (Sad and Krishnan, 2003).



**Figure 4** Molecular structure of MHC class I (left panels (a) to (d)) and MHC class II molecules (right panels (a) to (d)) (from (Janeway et al., 1999)).

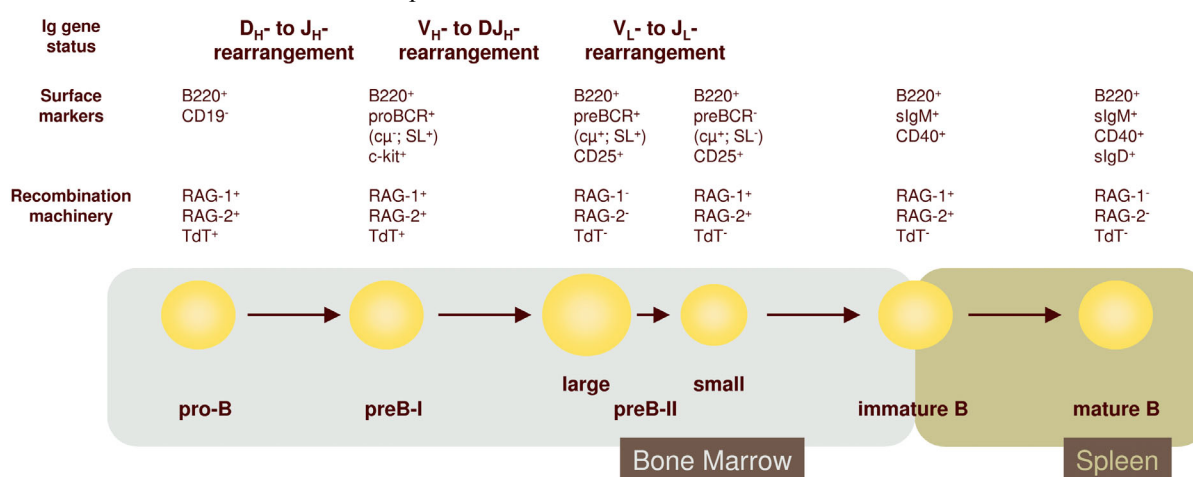


## 1.1.2 Murine B cell development

### 1.1.2.1 Development of immature B cells

Just like the aforementioned T lymphocytes, B cells arise from pluripotent progenitors in the fetal liver or in the bone marrow of adult mice. They start their maturation in these tissues and complete it in the secondary lymphoid organs, such as the spleen and the lymph nodes. Commitment to the B cell lineage is followed by rearrangement and expression of the B cell receptor genes, the immunoglobulin heavy and light chain genes. First, rearrangement of the immunoglobulin heavy chain gene is observed, followed by expression of the pre-B cell receptor (pre-BCR), composed of the heavy chain and the surrogate light chains  $\lambda 5$  and V-preB. Signaling through this pre-BCR leads to a transient phase of cellular proliferation, and triggers the transition from early pre-B cells to late small pre-B cells. Recombination of the  $\kappa$  and  $\lambda$  light chains loci is initiated at the pre-B cell stage. Successful pairing of one of the light chains with the heavy chain results in surface expression of Immunoglobulin M (IgM), the hallmark of immature B cells. Upon encounter

with autoantigens that are recognized by this sIgM, the cells can downregulate the IgM surface expression and induce a further rearrangement of the Ig light chain gene by a process termed **receptor editing**. Cells that remain autoreactive undergo programmed cell death (apoptosis), but those that lose the autoreactivity through receptor editing can proceed normally in their development. IgM-expressing cells that were not negatively selected for recognition of self molecules can subsequently exit the bone marrow and complete their maturation in the spleen. Discrete stages of B cell development have been defined based on (i) cell cycle status and cell size, (ii) the status of Ig heavy (H) and light (L) chain rearrangement and, (iii) the expression of several genes on the cell membrane or in the cytoplasm (especially those of the rearrangement machinery (Rag-1, Rag-2, TdT) and the pre-BCR and BCR complex (IgH chain, (surrogate) IgL chain, Ig $\alpha$  and Ig $\beta$  chains). **Figure 5**, in combination with **Table I**, depicts these successive stages of antigen independent B cell development. In addition, it indicates the stages at which various transcription factors have been found to be important, usually on the basis of knockout studies.



**Figure 5** Murine B cell development. Discrete stages of B cell development, based on cell size, the rearrangement status of the immunoglobulin genes and the expression of genes on the cell membrane or in the cytoplasm, are shown. Some of the markers are only indicated where their expression/inexpression is required to identify a specific B cell subset. Immature B cells are depicted overlapping the bone marrow and the spleen because these cells are present in both organs.

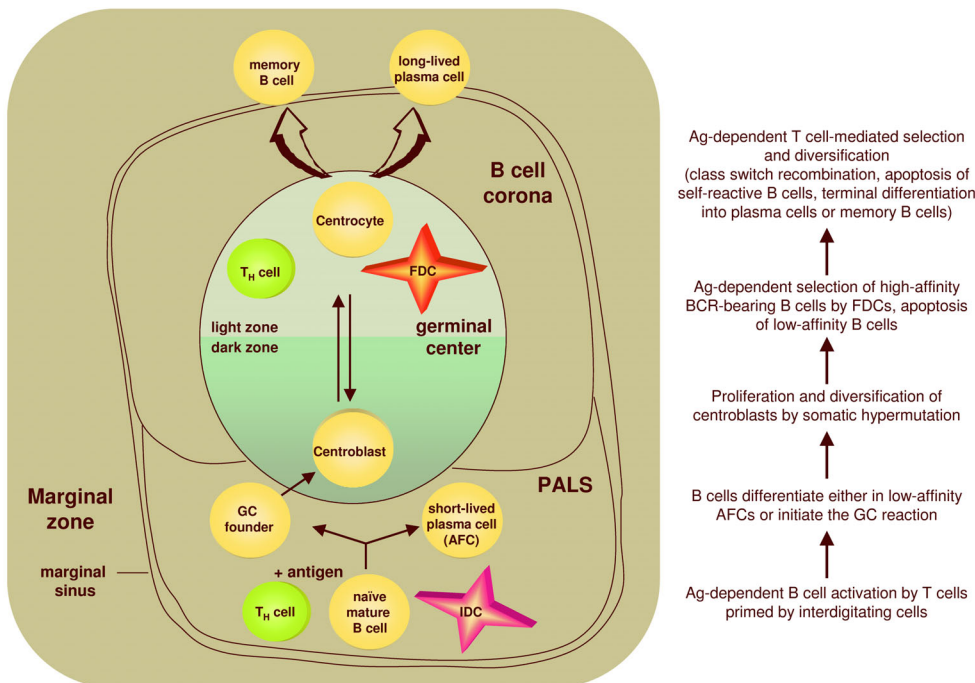
**Table I** Genes controlling murine early B cell development

Developmental transitions	pro-B to preB-I	preB-I to large preB-II	large preB-II to small preB-II	small preB-II to immature B	immature B to mature B	centrocyte to plasma cell
Hallmark events	D <sub>H</sub> - to J <sub>H</sub> - rearrangement	V <sub>H</sub> - to DJ <sub>H</sub> - rearrangement	V <sub>L</sub> - to J <sub>L</sub> - rearrangement	IgM surface expression	IgD surface expression	
Important transcription factors	<i>Sox-4</i> <i>E2A</i> <i>EBF</i> <i>Pax-5</i>	<i>Pax5</i>	<i>IRF-4</i> / <i>IRF-8</i>	<i>OBF-1</i> / <i>Aiolos</i>	<i>OBF-1</i> <i>Oct-2</i> <i>NF-<math>\kappa</math>B</i>	<i>Blimp-1</i> <i>XBP-1</i> <i>Aiolos</i> (?)
Important other genes	<i>RAG-1</i> , -2 <i>SCID</i> <i>BLNK</i> <i>JH</i> <i><math>\mu</math>mb</i> <i>Ig<math>\beta</math></i> <i>IL-7</i> ; <i>IL-2R<math>\beta</math></i>	<i>Syk</i> <i>PI3K</i> <i><math>\lambda 5</math></i> <i>VpreB1</i> / <i>VpreB2</i>	<i>DNA Polymerase Mu</i> <i>Btk</i> / <i>Tec</i>	<i><math>\kappa</math> / <math>\lambda</math> L chain</i>	<i>Btk</i>	

1.1.2.2 T cell dependent immune responses

Immature B cells enter the spleen from the blood at the level of the marginal zone (MZ) where they can be selected for self-reactivity in the periphery and consequently enter an anergic state, or they can mature further. B cells that have freshly entered the spleen can be identified by their cell surface reaction with the monoclonal antibody 493 (Rolink et al., 1998), which presumably recognizes the cell surface marker AA4.1, which is the complement component C1q like receptor C1qRp (Rolink et al., 2002). Subsequently, the (naïve) B cells cross the marginal sinus and migrate via the periarteriolar lymphoid sheath (PALS) to the lymphoid follicles, which are areas specialized in antigen retention and presentation. Upon encounter with T cell dependent (TD) antigen such mature  $IgM^+ IgD^+$  B cells become activated and proliferate with T cell help. The T helper cells ( $T_H$ ) required for this activation are  $CD4^+ CD8^-$  cells that can be divided into two subpopulations according to their cytokine profile: Activated  $T_H1$  cells secrete mainly IL-2 and IFN- $\gamma$ , while activated  $T_H2$  cells secrete IL-4 and IL-5.  $T_H$  cells get activated by binding of antigen processed and displayed by antigen presenting cells (APCs). Upon activation,  $T_H$  cells upregulate the expression of a number of accessory receptors and ligands (e.g. CD40L and CD28) that are essential for B cell activation in TD immune responses. The activated

mature B cells then differentiate predominantly into low affinity antibody-forming cells (AFCs), which are short-lived plasmacytes that initially secrete IgM, but subsequently switch to secondary isotypes and die by apoptosis within two weeks of immunization. Some cells from the expanded B cell pool do not become AFCs but migrate into the adjacent follicles, which are subsequently transformed into germinal centers (GCs). GC B cells undergo iterative cycles of proliferation, somatic hypermutation and apoptosis in cooperation with antigen-primed T cells and follicular dendritic cells (FDCs), a process resulting in the selection of B cell clones that make an antibody with high affinity for a cognate antigen. Additionally, Ig class switching occurs during the GC reaction. These steps expand, refine and diversify the repertoire of the early immune response to assure the long-term maintenance of protective immunity. Two types of terminally differentiated B cells eventually emerge from the GC reaction: nonsecreting, Ig surface-positive memory B cells and high affinity antibody-secreting plasmablasts that carry no immunoglobulins at their surface and that are the final mediators of the humoral immune response. Figure 6 gives an overview over the B cell fate in the spleen.



**Figure 6** B cell maturation in the spleen. Immature B cells enter the spleen in the marginal zone (MZ) where they are selected for self-reactivity. Positively selected (naïve) B cells migrate via the periarteriolar lymphoid sheath (PALS) to the lymphoid follicles, where they can encounter TD antigens, become activated, proliferate and differentiate into short-lived low affinity antibody-forming cells (AFCs). Some cells do not become AFCs but migrate into the adjacent follicles, which are then transformed into germinal centers (GCs). GC B cells undergo iterative cycles of proliferation, somatic hypermutation and apoptosis in cooperation with antigen-primed T cells and follicular dendritic cells (FDCs), a process resulting in the selection of B cell clones that make a high affinity antibody for the specific antigen. Two types of terminally differentiated B cells eventually emerge from the GC reaction: non-secreting memory B cells and high affinity antibody-secreting plasmablasts.  $T_H$ , T helper; IDC, interdigitating cell.

### 1.1.2.3 T cell independent immune responses

As we have seen in the previous section, antigens recognized by the BCR usually require costimulatory signals from helper T cells to elicit a specific (TD) immune response. Some antigens however, including mostly bacterial cell wall components, such as LPS and certain sugars (e.g. Ficoll), can stimulate the B cells by themselves to proliferate and to secrete antibodies independently of T cell help. These T cell independent (TI) antigens do not lead to the formation of immunological memory, and the antibodies that arise from a TI immune response usually do not undergo affinity maturation or class switching. The B cell subsets that are required for TI immune responses are preactivated splenic marginal zone (MZ) B cells, as well as B1 B cells which provide a bridge between the very early innate and the later appearing adaptive immune response by generating an initial wave of IgM producing plasmablasts during the first three days of a primary response to particulate bacterial antigens (Martin and Kearney, 2000a; Martin et al., 2001).

#### 1.1.2.3.1 B-1 B cells

B-1 cells constitute a subset of B cells that are localized predominantly in the peritoneal and pleural cavities and can be distinguished from the conventional (B-2) B cells by their self-renewal capacity and unique cell surface proteins. In contrast to other B cell populations, they are CD45 (B220<sup>lo</sup>), IgM<sup>hi</sup>, CD23<sup>-</sup>, CD43<sup>+</sup>, and IgD<sup>lo</sup>. While the origins of B-1 cells remain controversial, it is well established that they secrete natural antibodies important for innate immunity – preferentially low-affinity, poly-reactive, mostly self-reactive antibodies (Forster and Rajewsky, 1987; Hayakawa et al., 1990; Hayakawa et al., 1984; Mayer and Zaghoulani, 1991; Shirai et al., 1991). B-1 cells make antibody responses mainly to polysaccharide antigens in a T cell independent manner. It appears that they arise either as a distinct lineage from committed fetal/neonatal precursors (Hayakawa et al., 1985; Lam and Stall, 1994), or from follicular B-2 cells in response to BCR ligation (Clarke and Arnold, 1998; Haughton et al., 1993). For a detailed review of B1 cells see (Berland and Wortis, 2002). In either case it appears that BCR specificity and surface density together are also decisive factors in the development of B-1 versus B-2 cells (Lam and Rajewsky, 1999).

The B-1a cells subset expresses CD5, but is otherwise almost indistinguishable from the B-1b cells (Kantor et al., 1992; Stall et al., 1992).

#### 1.1.2.3.2 Marginal zone (MZ) B cells

MZ B cells consist mainly of a large, mostly non-circulating subset of mature B cells that are

localized in the marginal zone of the spleen. As mentioned before, they have an important function at the early stages of the immune response. This could be attributed to their observed lower activation threshold that triggers them more easily into proliferation or differentiation than immature or recirculating mature B cells. Several recent reviews discuss these properties in more detail (Bendelac et al., 2001; Martin and Kearney, 1999; Martin and Kearney, 2000a; Martin and Kearney, 2002).

A number of genes that are crucial for the generation or maintenance of the MZ B cell compartment have been identified by targeted disruption or overexpression studies.

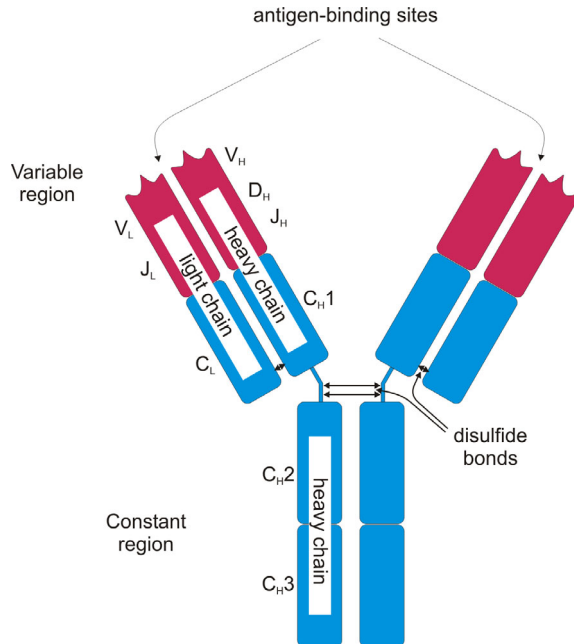
A first group of mutations that specifically affect MZ B cells is modulating the BCR signal strength. For example, mice with a targeted disruption of the Aiolos transcription factor have a strongly reduced number of MZ B cells, most likely due to an increase in BCR signal strength in the absence of Aiolos (Cariappa et al., 2001). In *Lyn*<sup>-/-</sup> mice and in mice with a mutated Igα ITAM motif, MZ B cell numbers are also strongly decreased, and both *Lyn* and Igα are known to negatively regulate BCR signaling. Conversely, MZ B cell numbers are enhanced in mice lacking the coreceptor CD21, and this enhancement correlates with an expected decrease in BCR signal strength (Cariappa et al., 2001). In the absence of the BCR costimulatory molecule CD19, MZ B cells are lacking completely (Hiemstra et al., 1999; Martin and Kearney, 2000b).

A second group of genes is presumably required for the migration of precursors to the MZ, such as NFκB p50, Pyk2 and Lsc, or for the development and maintenance of the microarchitecture and microenvironment of the spleen (such as *Lta*, *Ltb*, *LtβR*, NFκB p52, RelB, and Dock2) (reviewed in (Cariappa and Pillai, 2002)). Recently, the transcriptional coactivator OBF-1 has also been suggested to be important for MZ B cell development, probably due to altered homing properties of OBF-1 deficient cells (Samardzic et al., 2002b).

Another recent report investigating the effect of conditional mutagenesis of the transcription factor RBP-J (recombination signal binding protein-J) in B cells demonstrates that Notch-RBP-J signaling is involved in cell fate determination of marginal zone B cells (Tanigaki et al., 2002). It has been suggested that Notch-RBP-J signaling regulates the lineage commitment of mature B cells into follicular versus MZ B cells.

### 1.1.3 B cell function / Immunoglobulins

The expression of immunoglobulins, either as cell surface receptors, or in secreted form as antibody molecules, is a unique feature of B cells. An immunoglobulin (Ig) molecule consists of two identical heavy (H) and two identical light (L) polypeptide chains held together by a combination of noncovalent bonds and covalent disulfide bonds (Figure 7). Each polypeptide chain features one variable (V) and one constant (C) domain. The antigen is bound by both the  $V_H$  and  $V_L$  domains. The variable region can be further divided into hypervariable regions, also called complementarity determining regions (CDRs), and more conserved framework regions (FR). The C region is characteristic for each distinct isotype and is non-variable within the same isotype group. There are five main heavy-chain classes (isotypes), some of which have several subtypes: IgM, IgD, IgG, IgA, and IgE. Antibodies of different isotypes operate in distinct places in the body and have distinct functional activities. The different functions for human isotypes are listed in Table II. The corresponding heavy-chains are denoted  $\mu$ ,  $\delta$ ,  $\epsilon$ ,  $\gamma$  and  $\alpha$ , respectively. Initially, all immature B cells express the IgM isotype and may switch to a different isotype at later stages of B cell maturation.



**Figure 7** Structure of a prototypic immunoglobulin molecule. The constant regions of the heavy and light chains are depicted in blue, the variable regions are shown in red. (Figure modified after (Janeway et al., 1999), Fig. 3.1 to 3.3)

**Table II** Effector functions and distribution of human immunoglobulin isotypes (from (Janeway et al., 1999), Fig. 8.16)

Functional activity	IgM	IgD	IgG1	IgG2	IgG3	IgG4	IgA	IgE
Neutralization	+	-	++	++	++	++	++	-
Opsonization	-	-	+++	-	++	+	+	-
Sensitization for killing by NK cells	-	-	++	-	++	-	-	-
Sensitization of mast cells	-	-	+	-	+	-	-	+++
Activation of complement system	+++	-	++	+	+++	-	+	-
Distribution	IgM	IgD	IgG1	IgG2	IgG3	IgG4	IgA	IgE
Transport across epithelium	+	-	-	-	-	-	+++ (dimer)	-
Transport across placenta	-	-	+++	+	++	+/-	-	-
Diffusion into extravascular sites	+/-	-	+++	+++	+++	+++	++ (monomer)	+
Mean serum level (mg/ml)	1.5	0.04	9	3	1	0.5	2.1	$3 \times 10^{-3}$

#### 1.1.3.1 Organization and transcription of the Ig loci

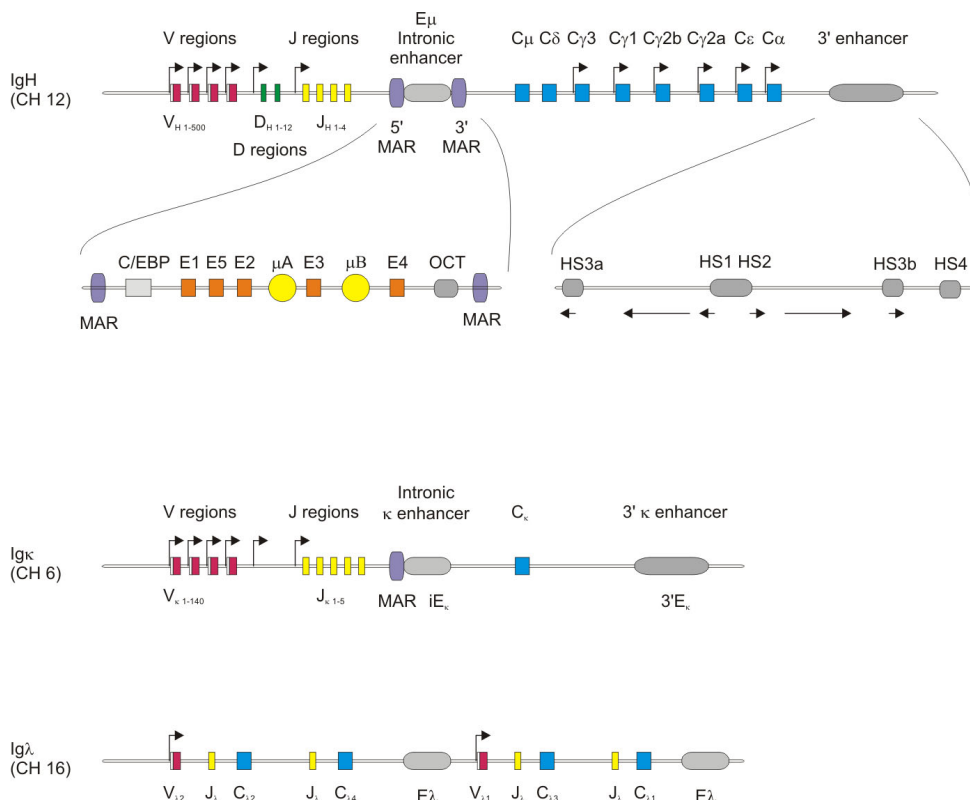
The functional gene segments that make up the Ig L and H chains are organized into three clusters, located on different chromosomes: the  $\kappa$  and  $\lambda$  light-chain genes, and the heavy-chain genes. Multiple copies of all of these gene segments exist in germline DNA (see Figure 8). The two loci of light-chain genes,  $\kappa$  and  $\lambda$ , are alternatively used, with species-specific preferences ( $\kappa$  to  $\lambda$  ratios of 2:1 in man and 20:1 in mouse), but apparently without functional differences.

In order to form the functional Ig molecules, the gene segments have to be assembled by somatic recombination. Two types of recombinatorial events take place at the Ig locus during B cell development: **V(D)J recombination** at both H and L loci, and **class switch recombination (CSR)** at the H locus.

##### 1.1.3.1.1 V(D)J recombination

During the process of **V(D)J recombination**, the H chain rearrangement occurs first, starting with a rearrangement joining one of the  $D_H$  gene segments with one of the  $J_H$  genes. Subsequently, one  $V_H$  gene segments is joined to shape a  $VDJ-C_H$  gene. Rearrangement of the L chain follows, where one of the  $V_L$  gene segments is directly joined to a  $J_L$  gene segment, leading to a  $VJ-C_L$   $\kappa$  or  $\lambda$  gene. In both cases, RNA splicing after transcription joins the  $V(D)J$  segments to the closest downstream C region coding sequence. In immature B cells, that is  $C_\mu$  or, if alternative splicing occurs during late phases of B cell development,  $C_\delta$ . The C genes are organized in the following order in the mouse genome: 5'- $V(D)J-C_\mu-C_\delta-C_\gamma3-C_\gamma1-C_\gamma2b-C_\gamma2a-C_\epsilon-C_\alpha-3'$  (D'Eustachio et al., 1980).





**Figure 8** Organization and control elements of the murine immunoglobulin heavy (*IgH*) and light chain (*Igκ*, *Igλ*) gene loci. The variable (V), joining (J) and constant (C) gene segments are represented as boxes. The enhancers are shown as grey boxes, and the detailed view of the *IgH* enhancer shows important regulatory elements and transcription factor binding sites. The intronic enhancer of the *IgH* locus is flanked by matrix attachment regions (MARs). The *IgH* 3' enhancer contains four DNase hypersensitive sites (HS1 to HS4) essential for its activity; the arrows below indicate three inverted repeats that illustrate the palindromic structure of the 3' enhancer.

#### 1.1.3.1.2 Class switch recombination

The second type of rearrangement occurring at the Ig locus during B cell development is **class switch recombination** (CSR) at the *IgH* locus. CSR is a deletional process that places a constant region (C) gene other than  $C_{\mu}$  or  $C_{\delta}$  directly downstream of the rearranged VDJ segments, leading to the expression of other, secondary Ig isotypes encoded by downstream C genes, such as *IgG*, *IgE* and *IgA* (Stavnezer, 1996). CSR occurs between highly repetitive G-rich switch (S) sequences with abundant palindromes that precede every C region, except for  $C_{\delta}$ . This process is controlled by a series of signals involving BCR and cytokine signaling, and in which B cell - T cell interactions play a critical role (Stavnezer, 1996). The activation and targeting of CSR correlates with the capability of certain mitogens and cytokines to either induce or suppress germline transcription of specific C genes (Manis et al., 2002; Snapper et al., 1997). Both recombinatorial events are thus intimately linked to transcription of the locus.

#### 1.1.3.1.3 The recombination machinery

In both V(D)J rearrangement and CSR, double-strand breaks in the DNA are generated and differentially re-ligated, thereby deleting the interjacent genomic sequence. In the case of V(D)J rearrangement, this relies on highly conserved **recombination signal sequences (RSS)**, short stretches of DNA present 3' of each V region, 5' and 3' of D regions and 5' of each J region. RSS always consist of a conserved heptamer and nonamer separated by 12 or 23 base pairs. V(D)J rearrangement occurs only between gene segments located on the same chromosome and follows the **12/23 rule**: Recombination can link only a gene segment flanked by a 12mer-spaced RSS to one with a 23mer-spaced RSS. This has the effect that  $D_H$  to  $J_H$  segment joining and  $V_H$  to  $D_H$  segment joining is possible, but  $V_H$  to  $J_H$  segment joining is not possible, as both  $V_H$  and  $J_H$  segments are flanked by 23bp spacers and the  $D_H$  segments are flanked by 12bp spacers (Eastman et al., 1996; Tonegawa, 1983; van Gent et al., 1996).

The recombination activating enzymes RAG-1 and RAG-2 catalyze the double strand break and re-

ligation reactions, assisted by components of the DNA double-strand break repair machinery, including Ku proteins and the DNA-dependent protein kinase (DNA-PK).

Isotype switch recombination also requires DNA-PK and Ku proteins (Ku70 and Ku80), but can occur in the absence of RAG-1 and RAG-2 gene expression; the enzymes that perform the recombination reactions are yet unknown.

#### 1.1.3.1.4 Sterile and productive transcripts

Two types of Ig transcripts are expressed during B cell development: sterile germline transcripts and productive transcripts. Sterile transcripts result from Ig gene transcription preceding gene rearrangement and class switching and are not transcribed. It is generally believed that sterile transcripts alter the accessibility of the Ig loci and thereby influence the regulation of gene rearrangements. Sterile transcripts have been characterized from most of the CH genes, as well as for C $\kappa$  and C $\lambda$  light chain genes.

Productive transcripts originate from the transcription of rearranged Ig genes and are translated into functional proteins.

#### 1.1.3.1.5 Affinity maturation

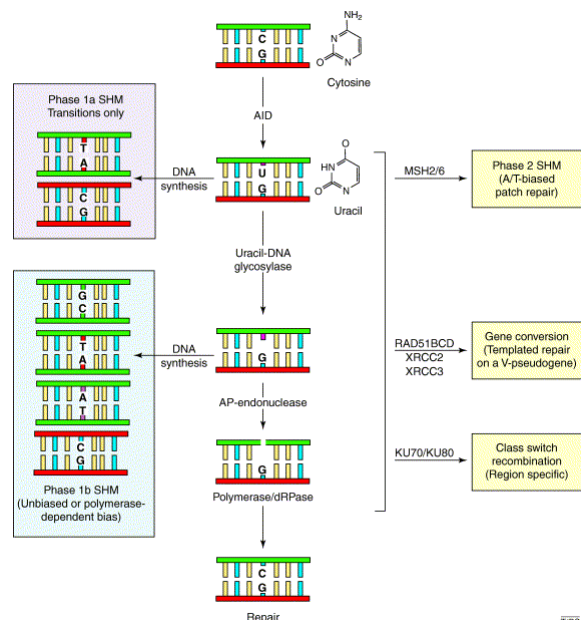
The efficiency of antigen elimination is also enhanced by **affinity maturation**, which is accomplished by excessive point mutations in the V-region gene, coupled with selection of high-affinity antibody-producing cells by limited amounts of antigen. Point mutations are introduced by two types of molecular mechanisms: non-templated **somatic hypermutation (SHM)**, and **gene conversion**, which takes place in chickens and rabbits, with pseudogenes serving as template (Reynaud et al., 2003).

The process of somatic hypermutation can be divided into three phases: targeting, DNA recognition and cleavage, and repair. The first phase of targeting a nuclease to the Ig locus necessitates transcription (reviewed in (Jacobs and Bross, 2001)). While some sequence specificity was reported, it remains unclear what is recognized by this nuclease (Michael et al., 2002). The cleavage mediated by the nuclease leads to a DNA double strand break (DSB) (Bross et al., 2000; Papavasiliou and Schatz, 2000), which might be preceded by a single strand lesion (Kong and Maizels, 2001). In the final phase, the DSB is probably repaired into a mutation by a subset of error-prone polymerases ((Gearhart and Wood, 2001), reviewed in (Papavasiliou and Schatz, 2002)).

#### 1.1.3.1.6 AID

The activation-induced deaminase (AID) is an enzyme specifically expressed in germinal center B

cells (Muramatsu et al., 1999) which is required for CSR, and also for the processes of somatic hypermutation (SHM) (Muramatsu et al., 2000; Nagaoka et al., 2002) and gene conversion (Arakawa et al., 2002; Harris et al., 2002). The analyses of several AID mutants indicate a requirement for class-switch-specific cofactors (Ta et al., 2003). Still, the mechanism of action of AID remains uncertain. Since AID displays a sequence similarity with the RNA-editing enzyme APOBEC-1, it is believed by some that AID may also act as an RNA-editing enzyme. Alternatively, it has been shown in *E. coli* and in *in vitro* assays to directly edit DNA (Petersen-Mahrt et al., 2002) by catalyzing deamination of deoxycytidine (dC) on single-strand DNA in a transcription-dependent manner, but not on double-strand DNA, RNA-DNA hybrids or RNA (Bransteitter et al., 2003; Chaudhuri et al., 2003). According to the DNA deamination model (Neuberger et al., 2003), AID deaminates dC on one strand of DNA to generate dU residues. Following the deglycosylation and removal of the misintegrated dU residues by UNG, the apyrimidic endonuclease (APE) could then generate DNA breaks, a necessary step common to both CSR and SHM. In agreement with this model, a partial CSR defect and a skewed pattern of SHM in uracil N-glycosylase (UNG)-deficient mice was described recently (Rada et al., 2002), providing further evidence that AID acts directly on DNA. A model integrating AID in CSR, SHM and gene conversion pathways is shown below (Figure 9).



**Figure 9** DNA deamination model for antibody diversification according to the Neuberger group, taken from (Neuberger et al., 2003). This model shows how different mechanisms resolving the initiating dU-dG lesion could lead to various patterns of antibody diversification. Phase 1A: somatic hypermutation (SHM) with activation-induced deaminase (AID) triggers dC → dU deamination. DNA synthesis occurring opposite this dU residue leads to dC → dT (and dG → dA) transitions. Phase 1B: the action of a uracil-DNA glycosylase (UNG) that excises a base before DNA synthesis can occur leads to both transitions



and transversions subsequent to DNA synthesis opposite the apyrimidinic site. Phase 2: Alternatively, recognition of the dU–dG mismatch by components of the mismatch repair pathway could trigger a mutagenic DNA repair synthesis leading to mutations at dA and dT that also occur during SHM. Alternatively, the lesion could be repaired by the mismatch repair system. In the case of chicken or rabbit, IgV gene conversion results when the initiating dU–dG lesion (or an intermediate in its repair) is resolved by a recombinational process templated on one of the proximal IgV pseudogenes. Switch recombination is thought to be triggered when AID-mediated deamination is targeted to dC residues in the vicinity of the m switch region with the resolution involving a partner switch-region from the downstream immunoglobulin (Ig) isotype. The major pathway of switch recombination might occur by a form of non-homologous end-joining involving Ku70 or Ku80 (Casellas *et al.*, 1998; Manis *et al.*, 1998). Abbreviations: AP-endonuclease, apyrimidinic endonuclease; dRPase, 5'-deoxyribosephosphodiesterase.

#### 1.1.3.1.7 Allelic exclusion, receptor editing

For each Ig locus, there are two alleles that can undergo gene rearrangement, and this increases the chance of a successful rearrangement. After successful rearrangement of the first allele, the recombination machinery is quickly downregulated, so that generally only one successfully rearranged IgH and IgL locus is found in any one B cell. This phenomenon is called **allelic exclusion**. The other allele remains in germline configuration, is partially rearranged or has completed an unproductive rearrangement.

Another rescue mechanism that prevents excessive unproductive rearrangements is the process of **receptor editing**. Unlike the heavy-chain genes, repeated light-chain gene rearrangements of unused V and J gene segments can occur, thereby increasing the chance of the B cell precursors to generate progeny that bears intact IgM molecules: the immature B cells.

**Note:** Diversity of the antibody repertoire is achieved by a number of mechanisms:

- **many V-region gene segments** exist in the genome of an individual, providing a heritable source of diversity,
- **random recombination** of separate V, D, and J gene segments during **V(D)J recombination** provide additional diversity,
- **variability of the junctions** between the gene segments is increased by the **random insertion** of P- and N-nucleotides and by **deletion** of nucleotides at the ends of some coding sequences,
- the **association of different light- and heavy-chain V regions** adds diversity,
- the **modification of V regions** of expressed immunoglobulins in the process of **somatic hypermutation** upon antigen stimulation of mature B cells further increases diversity of the antigen-binding site

## 1.2 Transcription

At this point, it is useful to give a brief overview over the general mechanisms of eukaryotic gene transcription, which constitute the basis of the most common and most immediate point of regulation of gene expression in a cell.

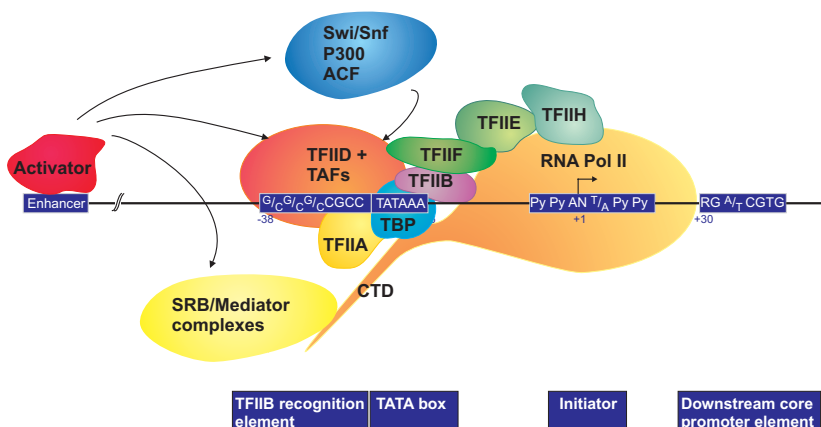
Transcription of the eukaryotic genes is performed by three RNA polymerases: RNA polymerase I (Pol I) synthesizes the large ribosomal RNA (rRNA), Pol II synthesizes mRNA and Pol III synthesizes tRNA and 5S rRNA. We will focus here on Pol II transcription; a review on Pol I and Pol III transcription can be found in (Paule and White, 2000).

### 1.2.1 Pol II transcription

#### 1.2.1.1 The basal transcription machinery

Synthesis of mRNA by RNA polymerase II (Pol II) is governed by two distinct DNA elements: a **core promoter** and upstream – or downstream – **enhancer** sequences. The core promoter determines the transcription start site and directs the assembly of the pre-initiation complex (PIC), which consists of Pol II and general transcription factors (GTFs), namely TFIIA, TFIIB, TFIID, TFII E, TFII F and TFII H. TFIID itself is a multi-protein complex containing the TATA-binding protein (TBP) and a set of TBP-associated factors (TAF<sub>II</sub>s), which have sequence-specific DNA-binding activity and are involved in promoter selectivity (Burley and Roeder, 1996).

The core promoter often contains a TATA box, an AT-rich motif located 25-30 bp upstream of the transcription start site which is bound by TBP. Another common element is the initiator (Inr), which encompasses the transcription start site and which serves as a binding site for additional regulatory factors. The sequence elements of a typical core promoter and a model of the Pol II transcription initiation machinery are depicted in Figure 10.



**Figure 10** Components of the DNA polymerase II transcription initiation machinery.

The largest subunit of Pol II contains a highly conserved C-terminal domain (CTD) consisting of tandem repeats of the heptapeptide YSPTSPS, which can be differentially phosphorylated during the different stages of transcription. During the initiation of transcription, the CTD is unphosphorylated and binds to the large SRB/Mediator complex. At the transition to transcription elongation, the CTD becomes phosphorylated and Pol II dissociates from the SRB/Mediator complex and recruits the elongator complex as well as several pre-mRNA processing factors. The cell cycle-specific regulation of Pol II activity also depends on the phosphorylation of its CTD.

#### 1.2.1.2 Transcription factors

To achieve a specific and controlled regulation of gene transcription, the basal transcription machinery interacts with additional proteins that bind to the promoter or enhancer region, so-called **transcription factors**. Transcription factors contain at least two essential features: a sequence-specific **DNA-binding domain** and an independent **regulatory domain** that can be either transactivating or inhibitory. Factors that do not feature a DNA-binding domain but are recruited by transcription factors to the active site of transcription and thereby modulate transcriptional activity are termed **transcriptional coactivators** or **corepressors**.

There are various ways in which transcription factors regulate transcription. First, they can interact with components of the basal transcription machinery and thereby modulate the assembly of the preinitiation complex (Ptashne and Gann, 1997). Other processes that can be targeted by transcription factors are transcription elongation and re-initiation of transcription. Transcription factors can also recruit chromatin modifying activities that modulate the acetylation state of the local chromatin and thereby can facilitate or inhibit transcription. For example, the coactivators p300 and CBP contain histone acetyltransferase activity that activates transcription by altering or disrupting the repressive chromatin structure (Ogryzko et al., 1996).

Moreover, transcription factors could be involved in targeting promoters to regions in the nucleus with high transcriptional activities (Brown et al., 1997).

## 1.2.2 Transcriptional regulation of B cell development

The development, maturation and selection of mammalian B cells is a complex and strictly regulated process. As we have seen previously, the successive stages of B cell differentiation can be characterized by sequential rearrangement of the immunoglobulin (Ig) genes, and by the ordered appearance or disappearance of proteins at the cell surface, or within the cell (Rolink and Melchers, 1993). This entire program of differentiation involves the selective expression of genes that are characteristic of the B cell lineage. The correct temporal expression of these cell type-specific genes is determined by the action of lineage-restricted transcriptional regulators. Experiments using targeted gene disruptions have shown that the loss of some transcription factors, such as PU.1, affects multiple hematopoietic lineages, whereas the loss of other transcriptional regulators specifically affects early B cell development (Scott et al., 1994). These transcription factors include, for example, EBF (early B cell factor), E2A, and Pax5 (Bain et al., 1994). Since I have published an extensive review on the transcriptional control of B cell development, which can be found in Section 2.5 of this thesis, I will focus at this point specifically on the transcription of the immunoglobulin genes.

## 1.2.3 Immunoglobulin gene transcription

The Ig genes were among the first genes studied in depth in mammalian cells. For that reason, they are some of the best-studied transcriptional units in mammalian cells today, and thus constitute a very useful model to analyze the regulation of gene transcription in general. Ig gene transcription is a highly regulated process driven by a number of ubiquitous and tissue-specific transcription factors that bind to regulatory regions in the Ig locus. These control regions include the V gene proximal promoters and promoters used for sterile transcription of the H and L chain loci, the intronic enhancers in the J-C region of the H and  $\kappa$  chain loci ( $E_{\mu}$ ,  $iE_{\kappa}$ ), the silencers located near the intronic enhancers, and the 3' enhancers of the  $\kappa$ ,  $\lambda$  and  $C_{\alpha}$  C region genes ( $3'E_{\kappa}$ ,  $E_{\lambda}$ , and  $3'EH$ ). Additional regulatory elements are the matrix attachment regions (MARs), flanking the  $E_{\mu}$  and  $iE_{\kappa}$  intronic enhancers. The various control elements are depicted in Figure 8.

### 1.2.3.1 V region promoters

The V region promoters contain several control elements, the most highly conserved elements being a TATA box about 25 bp upstream of the initiation site and an octamer element (ATGCAAT) or its reverse complement. This element is also conserved in some Ig enhancers and in the promoters of other B cell specific genes, including  $Ig\beta$ , CD20 and CD21, and will be discussed more in detail later. In the  $V_H$  promoters, but not in the  $V_L$  promoters, the octamer site is often found in conjunction with a heptamer sequence (CTCATGA) 2 to 22 bp upstream. Other conserved elements in many V promoters and enhancers include C/EBP (CAAT enhancer binding protein) binding sites and  $\mu E3$  elements, which are binding sites for E box proteins, such as TFEB, TFE3 and USF (Beckmann et al., 1990).

### 1.2.3.2 IgH intronic enhancer ( $E_{\mu}$ Enhancer)

The intronic enhancer is located between the J regions and  $C_{\mu}$  and spans approximately 220 bp sequence featuring a number of E box motifs (CANNTG), such as  $\mu E1$ ,  $\mu E2$ ,  $\mu E3$ ,  $\mu E4$ ,  $\mu E5$ ,  $\mu A$ , as well as C/EBP and octamer binding sites (Arulampalam et al., 1997; Engel and Murre, 2001; Ernst and Smale, 1995). The E box binding proteins (E-proteins) E2A, E2-2 and HEB belong to a subclass of bHLH factors that act as transcriptional activators and can homo- or heterodimerize on the E box motifs E2, E4 and E5 (Hu et al., 1992; Murre et al., 1989). Another subclass of bHLH factors possesses an additional leucine zipper motif, allowing them to form higher order multimers. Members of this subclass, including USF, TFE3 and TFEB, bind to the E3 box (Beckmann et al., 1990). Additional motifs termed  $\mu A$  and  $\mu B$  were also implicated in the activation of IgH gene expression. These binding sites are recognized by the transcription factors of the Ets family, including Ets-1, Ets-2, Elf-1, Erp, and NERF, which can bind to the  $\mu A$  motif, and PU.1, which binds to the  $\mu B$  site (Nelsen et al., 1993; Rivera et al., 1993). The Ets transcription factors are characterized by a conserved DNA-binding domain (the ETS domain) that forms a helix-loop-helix structure that binds specific purine-rich DNA sequences with a GGA core (Crepieux et al., 1994; Sharrocks, 2001). They are generally weak activators of transcription by themselves and act mainly through cooperative binding with other transcription factors.

### *Matrix attachment regions*

The intronic enhancer is flanked by two matrix attachment regions (MARs), consisting of A/T-rich sequences that associate with the nuclear matrix (Cockerill et al., 1987). They have been associated with the control of chromatin structure of entire gene loci and thus overlap functionally with locus control

regions (LCRs). The MARs can contribute positively to E $\mu$  function by enhancing transcription from a transgene promoter (Forrester et al., 1994; Jenuwein et al., 1997). In addition, MARs have been implicated in constituting physical boundaries between genes by forming chromosome loops (Cockerill and Garrard, 1986; Cockerill et al., 1987).

### 1.2.3.3 The 3' IgH enhancer

The 3' IgH enhancer is located downstream of the IgH locus, 3' of the C $\alpha$  region. It spans over 30 kb and encompasses four DNaseI hypersensitive sites with enhancer activity, termed HS1 to HS4. The initially identified major enhancer contains HS1 and HS2. This enhancer region is not active in resting B cells, but its activity is inducible by mitogens and by BCR and CD40 crosslinking in activated B cells and plasma cells. It was discovered early that an octamer site present in several of the 3' enhancer elements is crucial for its activity during B cell activation (Yuan et al., 1995) and that interaction of Oct-2 and its coactivator OBF-1 with the 3' IgH during B cell activation are essential (Tang and Sharp, 1999). HS3a and HS3b respond less strongly to mitogenic signals and appear to be influenced, among others, by Bach2 binding, together with one of the small Maf proteins, to Maf recognition elements (MAREs) in HS3a and HS3b (Muto et al., 1998). Binding of Pax-5 and NF- $\kappa$ B to their cognate DNA recognition sequences seems to be important as well.

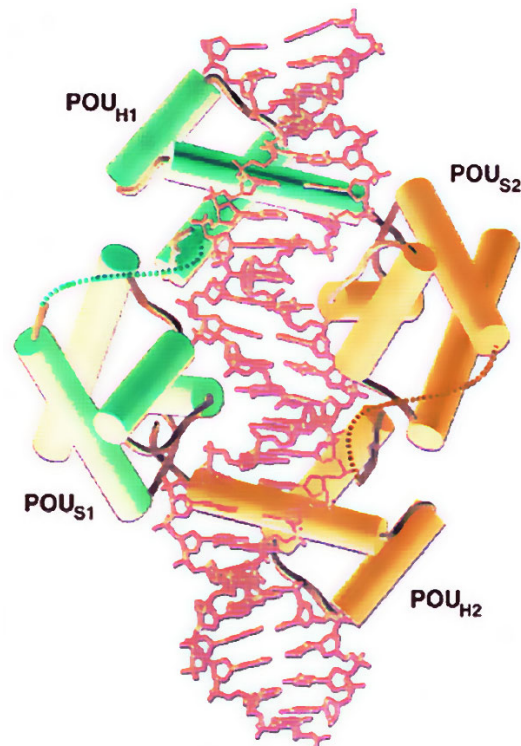
It is generally believed that the 3' IgH enhancer plays an important role in the high expression of switched Ig loci in plasma cells compared to unswitched loci, presumably because it is then brought into the vicinity of the V<sub>H</sub> promoter and interacts with it (Arulampalam et al., 1997).

## 1.2.4 Specific transcription factors regulating Ig gene transcription

### 1.2.4.1 Oct factors

As we have seen in the previous section, the V region promoters appear to be quite simple and contain a highly conserved octamer element (5'-ATGCAAAT-3', or its reverse complement), located upstream of a TATA box. This octamer element is found in the promoters of a variety of eukaryotic genes, including ubiquitously active genes, such as H2B (LaBella et al., 1988) and most U snRNA genes (Herr, 1992; Janson and Pettersson, 1991; Mittal et al., 1996; Murphy et al., 1992), but also genes that are restricted to distinct cell types or tissues (Christensen et al., 1992; Hermanson et al., 1989; Thevenin et al., 1993). Importantly, the octamer element is present in all immunoglobulin heavy and light chain gene promoters as well as in the heavy

chain intronic enhancer (Staudt and Lenardo, 1991) and appears to be critical for Ig gene transcription, as shown by a variety of *in vitro* and *in vivo* experiments (Bergman et al., 1984; Dreyfus et al., 1987; Jenuwein and Grosschedl, 1991; Mason et al., 1985; Matthias, 1998; Wirth et al., 1987). The best studied transcription factors that interact specifically with the octamer motif in B cells are the POU homeodomain proteins Oct-1 and Oct-2. While Oct-1 is ubiquitous, Oct-2 is predominantly expressed in B cells, as well as in activated T cells and in the nervous system. Oct-1 and Oct-2 are members of the POU family of transcription factors (POU stands for Pit-1, Oct-1 and nematode Unc-86; for review see (Herr and Cleary, 1995; Latchman, 1999; Phillips and Luisi, 2000)). The common characteristic of the POU family is the 150-160 amino acid bipartite DNA-binding domain (POU domain), consisting of two structurally independent subdomains: the homeodomain POU<sub>H</sub> (which is related to the DNA binding domain of homeobox proteins), and the specific domain POU<sub>S</sub> (which is unique to the POU factors). These two subdomains are connected through a short flexible linker, conferring conformational flexibility and thereby functional diversity to this class of transcription factors. The linker spans 14-26 amino acids and allows the protein to contact the DNA on both sides of the double helix ((Phillips and Luisi, 2000); Figure 11).



**Figure 11** Three-dimensional structure of the Oct-1 POU domain bound to an octamer site. The POU homeodomain (POU<sub>H</sub>) and POU specific domain (POU<sub>S</sub>) bind to opposite sites of the DNA. Figure adapted from (Andersen and Rosenfeld, 2001).

Oct-1 and Oct-2 show identical DNA binding activities and share 90% sequence identity in their DNA binding POU domain, but differ in other



protein regions, mostly in the C-terminal activation domain (Tanaka et al., 1992).

Due to its B cell-restricted expression pattern, Oct-2 was considered as one prime candidate for the activation of immunoglobulin transcription in B cells. Yet, targeted disruption of the Oct-2 gene in mice failed to demonstrate an essential role of Oct-2 in Ig gene transcription (Corcoran et al., 1993; Schubart et al., 2001). The analysis of Oct-2 deficient mice showed that this factor is required for B cell maturation and also, surprisingly, for postnatal survival (Corcoran and Karvelas, 1994; Corcoran et al., 1993). In addition, Oct-2 was found to be essential for cell proliferation following LPS stimulation and also for development of B1 B cells (Humbert and Corcoran, 1997).

Oct-1 appears to rely on cooperative binding with other factors to achieve its full activation potential. It was shown that Oct-1 activates snRNA promoters by binding to octamer sites in the core promoter in cooperation with SNAPc. Ternary complex formation with tissue-restricted co-activators, such as OBF-1 and SNAPc (Ford et al., 1998), can increase the specificity of the transcriptional activation mediated by Oct factors.

While it was known early that Oct-1 and Oct-2 can bind to DNA elements as monomers (Staudt et al., 1986), it was discovered only recently that they can also form homo- and heterodimers on specific octamer-related DNA motifs: the PORE (Palindromic Oct-factor Recognition Element: ATTTGAAATGCAAAT) and the MORE (More PORE: ATGCATATGCAT) (Remenyi et al., 2001; Tomilin et al., 2000). This can confer additional selectivity, as discussed below.

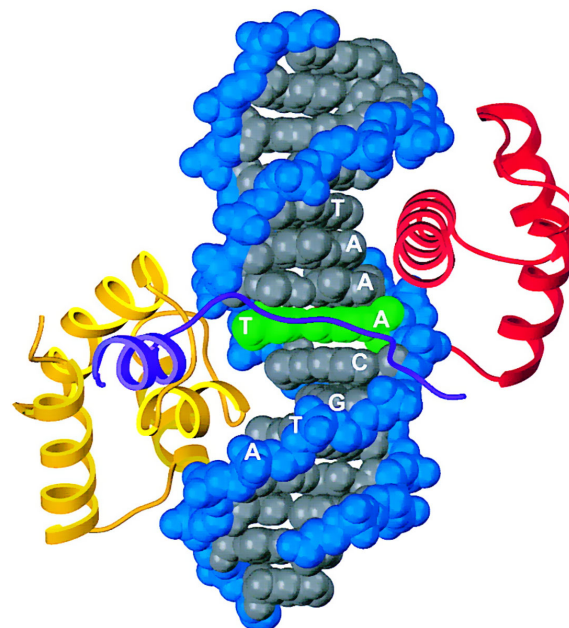
#### 1.2.4.2 OBF-1

OBF-1 (OCA-B, Bob-1), is a proline-rich lymphoid-specific transcriptional coactivator protein that interacts with the POU domains of Oct-1 or Oct-2 and with the conserved octamer element on the DNA (Gstaiger et al., 1996; Luo and Roeder, 1995; Strubin et al., 1995) to form a ternary complex on a subset of octamer sites. The structure of this complex was solved by co-crystallization, showing that OBF-1 makes contacts to both the POU<sub>H</sub> and POU<sub>S</sub> domains, as well as to the major groove of the DNA (Chang et al., 1999; Chasman et al., 1999; Sauter and Matthias, 1998) (Figure 12).

OBF-1 strongly potentiates transcription from octamer-containing promoters such as the immunoglobulin  $\kappa$  light chain promoter in transfection assays and *in vitro* (Luo and Roeder, 1995; Schubart et al., 1996b; Strubin et al., 1995). In B cells derived from OBF-1 knockout mice containing an inducible OBF-1 allele it was found that activity of strictly octamer-dependent reporters was also dependent on OBF-1 (Laumen et al., 2000).

However, *in vivo* data from OBF-1 deficient mice indicated that OBF-1 is dispensable for early antigen-independent B cell development and that the

level of unswitched Ig gene transcription is unaffected in the absence of the co-activator (Kim et al., 1996; Nielsen et al., 1996; Schubart et al., 1996a; Schubart et al., 2001).



**Figure 12** Ternary complex of OBF-1 (purple) and Oct-1 POU<sub>H</sub> (red) and POU<sub>S</sub> (yellow) domains on an octamer site in the DNA. OBF-1 traverses the major groove at the A:T base pair at position 5 of the octamer motif. Figure taken from (Chasman et al., 1999).

However OBF-1 seems to play a role for cell survival at earlier B cell stages, since a significant reduction of transitional (immature 493<sup>+</sup>) B cells in the spleen was observed by FACS analyses, indicating a malfunction in the B cell homing from bone marrow to the spleen in the absence of OBF-1 (Schubart et al., 2000) or an impaired production of transitional B cells in the bone marrow of OBF-1 deficient mice (Hess et al., 2001).

Importantly, histochemical studies have shown that OBF-1 function is crucial for the formation of germinal centers in secondary lymphoid organs, which are absent in OBF-1 deficient mice. As a consequence, OBF-1 deficient mice have defects in the Ag-dependent B cell development and show a strongly impaired immune response to T cell-dependent antigens (Kim et al., 1996; Schubart et al., 1996a).

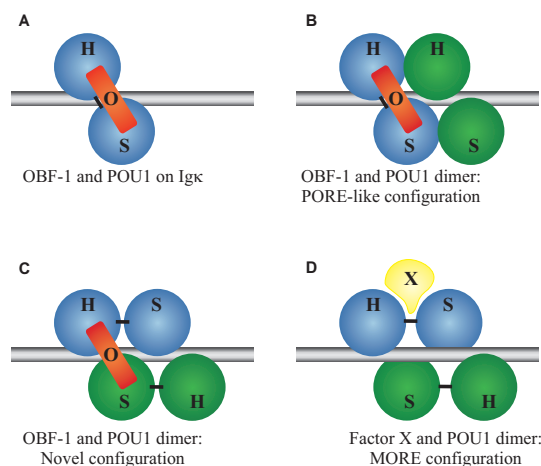
OBF-1 expression is largely restricted to B cells (Schubart et al., 1996b), but it can also be induced in T lymphocytes by *in vitro* costimulation (Sauter and Matthias, 1997; Zwilling et al., 1997). The phenotype observed in OBF-1 deficient mice clearly coincides with the expression pattern of the OBF-1 gene in B cells, which peaks at two distinct time points in B cell development: at the immature B cell stage in the bone marrow, and with highest expression levels in germinal centers and germinal center-derived B cell lymphomas (Greiner et al., 2000; Qin et al., 1998). The increased expression levels in germinal center B cells are partly due to

increased transcription (Qin et al., 1998) and partly due to increased protein stability (Boehm et al., 2001; Tiedt et al., 2001).

In addition, OBF-1 expression is strongly upregulated in murine activated splenic B cells after immunization (Qin et al., 1998). Following stimulation of the BCR, CD40 or IL-4 signaling pathways OBF-1 is upregulated specifically in GC B cells where it acts as an integrator essential for full differentiation and function of these cells (Qin et al., 1998).

OBF-1 expression can also be specifically upregulated *in vitro* at the level of mRNA and protein in resting B cells by stimulation with a combination of IL-4 and CD40 ligand or LPS alone (Greiner et al., 2000; Qin et al., 1998).

Interestingly, recent structural and functional data have demonstrated that, at least on some of the Ig heavy chain promoters, Oct-1 binds to the octamer site in a dimeric conformation (MORE) that precludes recruitment of OBF-1 to the DNA (Tomilin et al., 2000). Conversely, the osteopontin gene contains a permissive dimeric binding site (PORE) for Oct factors that specifically recruits OBF-1; on this site the binding of the Oct dimer is dramatically stabilized by OBF-1 (Lins et al., 2003). These different dimer configurations are depicted in Figure 13.



**Figure 13** Models of Oct-1 binding to specific octamer sites on the DNA. **A.** “Archetypical” binding of an Oct-1 monomer, together with OBF-1 on a canonical octamer element (as described in (Chasman et al., 1999)). **B.** Proposed POU1 dimer configuration (Botquin et al., 1998) with OBF1 binding to the POU1 molecule at the octamer half-site (Remenyi et al., 2001) on a PORE-type of octamer site. **C.** POU1 dimer as in (B), but with a different linker connectivity causing OBF-1 to bind to the POU<sub>S</sub> and POU<sub>H</sub> domains of two different POU1 molecules on the octamer half-site. Configuration based on the crystal structure described previously (Remenyi et al., 2001), proposed in (Lins et al., 2003). **D.** POU1 dimer precluding OBF-1 binding (as described in (Tomilin et al., 2000)), binding on a MORE-type of octamer site, probably in conjunction with an unknown cofactor X. The 3D crystal structure clearly differs from (C). Both the POU homeodomain (H) and POU specific domain (S) of individual Oct-1 molecules are represented in blue and green; OBF-1 (O) is represented in red.

To date, known direct target genes depending on OBF-1 include the BLR1 gene (which is cooperatively regulated by NF- $\kappa$ B, OBF-1 and Oct-2 in B cells (Wolf et al., 1998)), the CCR-5 gene (regulated by OBF-1 and Oct-1) in T cells (Moriuchi and Moriuchi, 2001), as well as the B cell specific B29 and mb1 promoters (Malone and Wall, 2002). Recently, the Kcnn4 promoter, the Lck distal promoter (Kim et al., 2003b) and the Adh2-like promoter (Brunner et al., 2003b) have been identified to be directly bound and activated by OBF-1. However, the physiological relevance of these target genes for GC formation and TD immune responses remains unclear. I have recently identified the Ets factor Spi-B as another, crucial target of OBF-1 coactivation during the germinal center reaction (see Section 2.1).

In addition, a myristoylated isoform of OBF-1 has been identified that is membrane-bound and could play a role as a signal transducer itself (Yu et al., 2001).

OBF-1 deficient mice also show a severe reduction of the number of immature B cells in the spleen; this reduction is even more dramatic in OBF-1/Oct-2 (Schubart et al., 2001) and OBF-1/btk (Bruton’s tyrosine kinase) (Schubart et al., 2000) compound mutant mice. The molecular mechanisms underlying these defects are still unclear. However, a bcl-2 transgene expressed in the B lineage rescues, to a large extent, the defect in OBF/btk double-mutant mice, suggesting that lifespans of immature B cells may play a role in this transition (Rolink et al., unpublished data), an effect that might again be related to BCR signaling strength. In line with this, it was very recently reported that Bcl-2 is downregulated in the bone marrow of OBF-1 deficient mice (Brunner et al., 2003b). Finally, mice lacking simultaneously OBF-1 and the chromatin regulator Aiolos show a severe developmental block in the bone marrow, at the transition between pre-B and immature B cells (Sugai et al., 2003; Sun et al., 2003). This phenotype is all the more surprising given the fact that the individual knockouts of these two factors show defects only in late stages of B cell development, as mentioned above. This observation underscores the concept that the same factors may perform different functions (and therefore may have different targets) at different developmental stages.

In addition, recent studies have shown that even in the absence of both Oct-2 and OBF-1, B cells were able to develop normally to the IgM<sup>+</sup> stage and immunoglobulin gene transcription was still largely unaffected (Schubart et al., 2001). These unexpected observations imply that the ubiquitous factor Oct-1 may play a previously unrecognized role in the control of immunoglobulin gene transcription and suggest the possible existence of another, as yet unidentified, cofactor. In line with this is the binding of Oct dimers on several IgH promoters in the MORE configuration which precludes OBF-1 recruitment (Tomilin et al., 2000). On the other hand, it was very recently found that OBF-1 is critical for the activity of a subset of IgL promoters



(Casellas et al., 2002) and also strongly enhances the expression of the osteopontin gene in B cells (Lins et al., 2003).

#### 1.2.4.3 Spi-B

Spi-B is a member of the Ets family of transcription factors that encompasses over 30 members characterized by the presence of a conserved DNA-binding domain (the ETS domain, Figure 14), a 85 amino acid motif which forms a unique helix-loop-helix structure that binds purine-specific DNA sequences (Crepieux et al., 1994; Sharrocks, 2001). Spi-B is closely related to another Ets factor crucial for hematopoiesis, PU.1/Spi-1, through high structural homology and by its ability to transactivate identical target genes as PU.1 *in vitro* (Ray et al., 1992; Ray-Gallet et al., 1995). Spi-B is also able to interact with the coactivator PU.1 interacting protein (Pip/IRF-4) (Eisenbeis et al., 1995). *In vivo* data shows however, that the target genes for PU.1 and Spi-B overlap only partially (Rao et al., 1999). Both PU.1 and Spi-B are required for normal BCR signaling (Garrett-Sinha et al., 1999), but while Spi-B can functionally replace PU.1 in myeloid development, it cannot replace PU.1 in lymphoid development (Dahl et al., 2002). Spi-B deficient mice exhibit severe abnormalities in B cell function and selective T cell-dependent humoral immune responses accompanied by a dramatic defect in germinal center formation and maintenance (Su et al., 1997).

Spi-B<sup>-/-</sup> mice have a BCR signaling defect and appear to initiate the production of germinal centers within splenic primary B cell follicles, but these structures decay prematurely due to BCR-mediated apoptosis. The Peyer's patches in Spi-B knock-out mice, just as in OBF-1 deficient mice, are normal in appearance and cell numbers. However, the Peyer's patch is under constant stimulation by antigens from

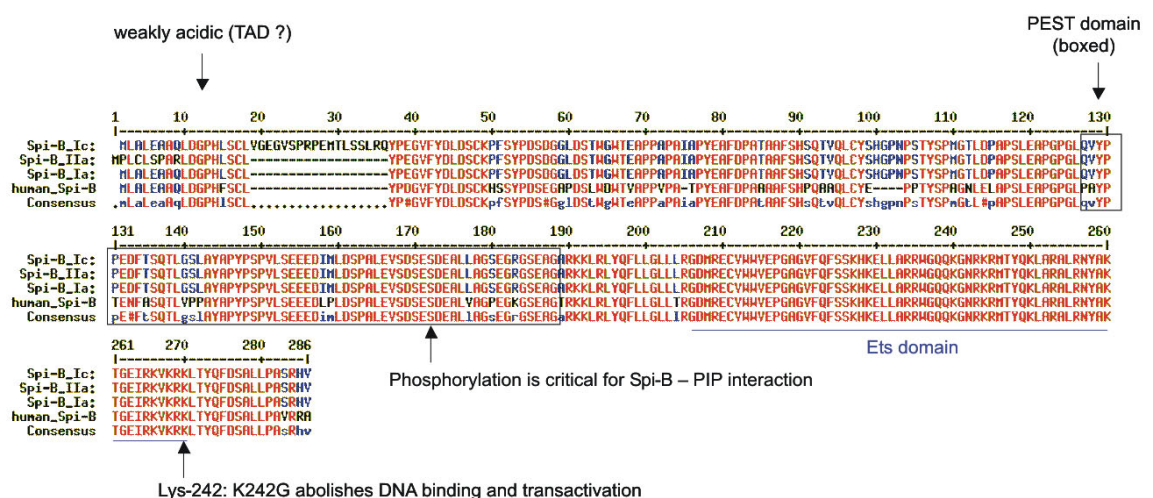
the intestine, while the splenic germinal centers arise after immunization and vanish after a few weeks.

The effect of Spi-B deficiency in gene targeted mice manifests itself in later stages of B cell development and involves mainly B cell activation through T cell dependent antigen stimulation, including germinal center formation (Su et al., 1997). Since T cell dependent antigenic stimulation and germinal centers in the secondary lymphoid organs are important factors in the activation of Ig somatic hypermutation (Kelsoe, 1996) and since Spi-B can bind to several of the Ig enhancers, it was postulated that Spi-B deficiency may lead to diminished activation of Ig somatic mutation. In a recent study, however, somatic hypermutation was not diminished in Peyer's patches of Spi-B deficient mice. In contrast, the B cell clonal selection and affinity maturation processes seemed to be affected in Spi-B deficient animals (Kim et al., 2003a).

Recently, Spi-B was implicated in the regulation of Igλ transcription, but not of Igκ or IgH transcription at the pro-B to pre-B cell transition in IL-7 dependent PU.1<sup>-/-</sup> Spi-B<sup>-/-</sup> pro-B cells (Schweitzer and DeKoter, 2004).

#### 1.2.4.4 Other key transcription factors in B cell development

Many other transcription factors that are essential for B cell development, including other Ets factors, GATA factors, Ikaros family members, E2A, EBF, Pax-5, Sox-4, Lef-1, BCL-6, STATs, IRFs, the NF-κB family, Blimp-1 and XBP-1, are discussed in detail in the recently published review (Bartholdy and Matthias, 2004) in Section 2.5. To avoid redundancy, they are not discussed again in the present context.



**Figure 14** Alignment of the protein sequence of different Spi-B isoforms of murine and human origin. The figure shows differential splicing and different N-termini caused by the use of two different promoters. It also illustrates the high conservation among the two species and the protein domains that have been identified in (Rao et al., 1999).

## 1.3 Objective of research projects

Gene targeting of OBF-1 revealed that this transcriptional coactivator is not required for B cell genesis or for the initial immunoglobulin gene transcription, despite the well documented importance of the conserved octamer motif in many B cell-specific promoters and enhancers. OBF-1 can be transiently induced in T cells, but does not seem to be required there, as OBF-1 deficient T cells appeared to be functionally normal. Thus, the possible function of OBF-1 in T cells has remained unclear. In contrast, OBF-1 is absolutely necessary for later stages of B cell development, most obviously for the formation of germinal centers and the subsequent production of secondary immunoglobulin isotypes following a T cell dependent antigen challenge. However, the target genes or mechanisms that could explain this phenotype on a molecular basis were still missing.

We approached this problem by following two complementary strategies: First, we generated transgenic mice overexpressing OBF-1 in a constitutive manner in T cells to analyze its function in this cell type. This type of analysis proved to be a valid approach for the identification of novel OBF-1 target genes, and we identified one important OBF-1 target, Spi-B. These findings will be discussed in greater detail the first part of the following section.

Secondly, we generated and analyzed transgenic mice overexpressing OBF-1 in a B cell-specific manner throughout B cell development, thereby overriding the tight transcriptional control imposed on the endogenous OBF-1 gene. For this, *OBF-1* was driven by a  $\mu$  enhancer-V region promoter construct that allowed us to study the effect of *OBF-1* expression starting at early stages of B cell development where it is normally not yet expressed. The data obtained from this – still ongoing – analysis underlines the importance of the tight control of OBF-1 expression at these early stages, as B cell-specific overexpression was shown to lead to a partial block of B cell development. This work is presented in [Section 2.1](#).

## 2 Results

### 2.1 Research Publication (in press)

# The Ets factor Spi-B is a direct critical target of the coactivator OBF-1

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**OBF-1 (Bob.1, OCA-B) is a lymphoid-specific transcriptional coactivator that associates with the transcription factors Oct-1 or Oct-2 on the conserved octamer element present in the promoters of immunoglobulin genes and other lymphoid-specific or ubiquitous genes. OBF-1 deficient mice have B cell-intrinsic defects, completely lack germinal centers and have severely impaired immune responses to T cell dependent antigens. Although several genes have been shown to be regulated by OBF-1, crucial genes that might explain the observed phenotype of OBF-1 deficiency have remained elusive to date. Here we have generated transgenic mice expressing OBF-1 specifically in T cells and examined these together with mice lacking OBF-1 to discover novel transcriptional targets of this coactivator. Using microarray analysis, we have identified the Ets transcription factor Spi-B as a direct target gene critically regulated by OBF-1, that can help explain the phenotype of OBF-1 deficient mice. Spi-B has been implicated in signaling pathways downstream of the BCR and is essential for germinal center formation and maintenance. The present findings establish a hierarchy between these two factors and provide a molecular link between OBF-1 and BCR signaling.**

Running title: Spi-B is a direct critical OBF-1 target gene

Supplemental material will be available at the journal's website

[*Keywords*: Spi-B; OBF-1; germinal center; transcription; BCR signaling]

#### Introduction

The proline-rich lymphoid specific protein OBF-1 (Bob.1, OCA-B (Luo and Roeder 1995; Strubin et al. 1995)) forms a ternary complex with the POU proteins Oct-1 or Oct-2 and DNA on the conserved octamer element (5'-ATGCAAAT-3'). This motif is present in the promoters of a number of eukaryotic genes, including ubiquitously active genes, such as H2B and U snRNA genes, but also genes that are restricted to distinct cell types or tissues (reviewed in (Matthias 1998)). Notably, the octamer element is present in all immunoglobulin (Ig) heavy and light chain gene variable (V) region promoters as well as in the heavy chain intronic enhancer (Staudt and Lenardo 1991) and is critical for Ig gene transcription (Bergman et al. 1984; Mason et al. 1985; Dreyfus et al. 1987; Wirth et al. 1987; Jenuwein and Grosschedl 1991; Matthias 1998). OBF-1 strongly potentiates transcription from octamer-

containing promoters such as the immunoglobulin  $\kappa$  light chain promoter in transfection assays and *in vitro* (Luo and Roeder 1995; Strubin et al. 1995; Schubart et al. 1996a). In B cells containing an inducible OBF-1 allele it was found that the activity of octamer-dependent reporters was dependent on OBF-1 (Laumen et al. 2000). Yet, *in vivo* data from OBF-1 deficient mice indicated that OBF-1 is dispensable for early antigen-independent B cell development and that the level of unswitched Ig  $\mu$  gene transcription in mature B cells is unaffected in the absence of this coactivator (Kim et al. 1996; Nielsen et al. 1996; Schubart et al. 1996a; Schubart et al. 2001). OBF-1 appears to play a role for cell survival at earlier B cell stages, and in absence of OBF-1 a significant reduction of transitional B cells in the spleen was observed by FACS analysis. This suggests that OBF-1 is important for production of transitional B cells in the bone marrow (Hess et al. 2001) or B cell homing from the bone marrow to the spleen (Schubart et al. 2000).

Importantly, histochemical studies have shown that OBF-1 function is essential for the forma-

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tion of germinal centers (GC) in secondary lymphoid organs. As a consequence, OBF-1 deficient mice have defects in the antigen-dependent B cell development and show a dramatically impaired immune response to T cell-dependent (TD) antigens (Kim et al. 1996; Schubart et al. 1996a).

OBF-1 expression is largely restricted to B cells (Schubart et al. 1996a), but it can also be induced in T lymphocytes by costimulation *in vitro* (Sauter and Matthias 1997; Zwilling et al. 1997). The phenotype observed in OBF-1 deficient mice clearly coincides with the expression pattern of the OBF-1 gene in B cells, which peaks at two distinct time points in B cell development: at the immature B cell stage in the bone marrow, and with highest expression levels in germinal centers and germinal center-derived B cell lymphomas (Qin et al. 1998; Greiner et al. 2000). The increased expression levels in germinal center B cells are partly due to increased transcription (Qin et al. 1998) and partly due to increased protein stability (Boehm et al. 2001; Tiedt et al. 2001).

Interestingly, recent structural and functional data have demonstrated that, at least on some of the Ig heavy chain promoters, Oct-1 binds to the octamer site in a dimeric conformation (MORE) that precludes recruitment of OBF-1 to the DNA (Tomilin et al. 2000). Conversely, the osteopontin gene contains a permissive dimeric binding site (PORE) for Oct factors that specifically recruits OBF-1; on this site the binding of the Oct dimer is dramatically stabilized by OBF-1 (Lins et al. 2003).

To date, known direct target genes depending on OBF-1 are the BLR1 gene (which is cooperatively regulated by NF- $\kappa$ B, OBF-1 and Oct-2 in B cells (Wolf et al. 1998)), the CCR-5 gene (regulated by OBF-1 and Oct-1) in T cells (Moriuchi and Moriuchi 2001), as well as the B cell specific B29 and mb1 promoters (Malone and Wall 2002). Recently, the Kcnn4 promoter, the Lck distal promoter (Kim et al. 2003) and the Adh2-like promoter (Brunner et al. 2003) have been identified to be directly bound and activated by OBF-1. However, the physiological relevance of these target genes for GC formation and TD immune responses remains unclear. Here we present evidence that the Ets factor Spi-B is a novel, crucial target of OBF-1 coactivation during the germinal center reaction.

Spi-B is a member of the Ets family of transcription factors that encompasses over 30 members characterized by the presence of a conserved DNA-binding domain (the ETS domain), a motif of 85 amino acids which forms a unique helix-loop-helix structure binding purine-rich DNA sequences (Crepieux et al.

1994; Sharrocks 2001). Spi-B is closely related to another Ets factor crucial for hematopoiesis, PU.1/Spi-1, through high structural homology and by its ability to transactivate identical target genes as PU.1 *in vitro* (Ray et al. 1992; Ray-Gallet et al. 1995). Spi-B is also able to interact with the coactivator PU.1 interacting protein (Pip/IRF-4) (Eisenbeis et al. 1995). *In vivo* data show however, that the target genes for PU.1 and Spi-B overlap only partially (Garrett-Sinha et al. 1999). Both PU.1 and Spi-B are required for normal BCR signaling (Garrett-Sinha et al. 1999), but while Spi-B can functionally replace PU.1 in myeloid development, it cannot replace PU.1 in lymphoid development (Dahl et al. 2002). Spi-B deficient mice exhibit severe abnormalities in B cell function and selective T cell-dependent humoral immune responses accompanied by a defect in germinal center formation and maintenance (Babb et al. 1997). Spi-B deficient mice have a BCR signaling defect and appear to initiate the production of germinal centers within splenic primary B cell follicles, but these structures decay prematurely due to BCR-mediated apoptosis. The Peyer's patches in Spi-B deficient mice, just as in OBF-1 deficient mice, are normal in appearance and cell numbers. However, the Peyer's patch is under constant stimulation by antigens from the intestine, while the splenic germinal centers arise after immunization and vanish after a few weeks.

Here we show conclusively that OBF-1 deficient mice have strongly reduced levels of Spi-B and that OBF-1 acts directly on the Spi-B promoter to enhance transcription of this Ets factor. These results establish a molecular link between these two transcription factors which are both crucial for the TD immune response and GC formation.

## Results

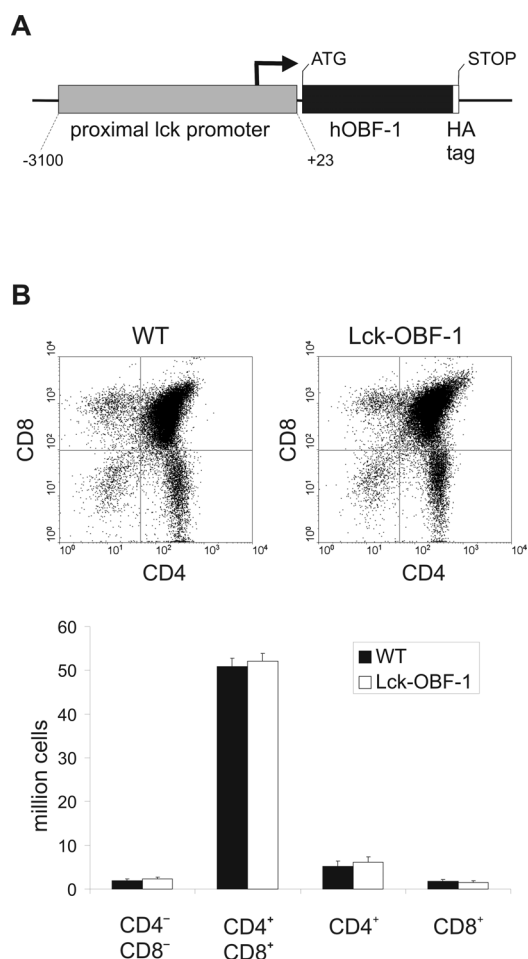
### *Generation and phenotype of lck-OBF-1 mice*

The coactivator OBF-1 was previously found to be expressed in a mostly B cell restricted manner, but expression was also induced in T cell lines and primary murine thymocytes following *in vitro* stimulation with PMA and ionomycin (Sauter and Matthias 1997; Zwilling et al. 1997). However, the physiological role of OBF-1 in T cells has remained unclear and in OBF-1 deficient mice T cell development and function were found to be normal (Schubart et al. 1996a; Qin et al. 1998). To further define the potential function of OBF-1 in T cells we generated transgenic mice expressing an HA epitope-

tagged OBF-1 cDNA under the control of the murine proximal lck promoter (Fig. 1A).

This T cell specific promoter is highly active in thymocytes throughout their development and then shows low activity in peripheral T cells (Shimizu et al. 2001). In agreement with the expected activity pattern of the lck promoter high levels of transgenic OBF-1 mRNA and protein were found in the thymus and lower levels were found in the spleen (see Supplementary material); by contrast, transgenic OBF-1 mRNA or protein were not detectable in non-lymphoid organs of these transgenic lines (not shown). Several independent transgenic mouse lines were generated, which all showed the same phenotype described below.

We first examined overall T cell development by flow cytometry, using antibodies against



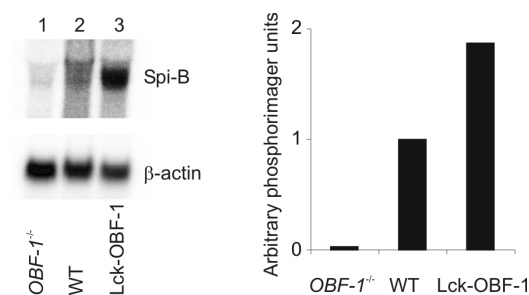
**Figure 1.** Normal T cell development in lck-OBF-1 transgenic mice. (A) Structure of the construct used for generation of transgenic mice. (B) FACS analysis of WT or lck-OBF-1 transgenic mice. Thymocytes were analyzed by flow cytometry with FITC-conjugated anti-CD4 and PE-conjugated anti-CD8 antibodies. The dot plot shows data from one representative animal of each genotype. The histogram in the lower part shows the mean  $\pm$  SD of data from 5 mice of each genotype.

the T lineage surface markers CD4 and CD8. As shown in Figure 1b total thymocyte numbers, as well as the numbers of double positive, double negative or single positive CD4 or CD8 T cells were all found to be normal in these transgenic mice. Upon further characterization by FACS analysis, some specific alterations in distinct T cell populations were observed in the thymus or in the periphery. For example, a subset of CD4<sup>+</sup>CD8<sup>+</sup> thymocytes also co-expressed the IL-2 receptor alpha chain (CD25). These CD4<sup>+</sup>CD8<sup>+</sup>CD25<sup>+</sup> cells were found to express a higher level of OBF-1 than the CD25<sup>-</sup> cells, thus establishing a potential link between CD25 expression and OBF-1 levels (see Supplementary material). These cells, however, do not appear to correspond to CD25<sup>+</sup>CD4<sup>+</sup> regulatory helper T cells, and no elevated number of CD25<sup>+</sup> cells was observed in the spleen (data not shown). In addition, other specific T cell populations such as  $\gamma\delta$  and dendritic epidermal T cells were also influenced by OBF-1 expression (see Supplementary material). Together, these results indicate that OBF-1 overexpression in the T cell compartment leads to very specific effects on a number of minor T cell subsets, but does not overtly perturb normal T cell development.

Because overall T cell development and total T cell numbers were largely unchanged by the expression of OBF-1 we decided to use thymocytes RNA for microarray experiments, in order to identify potential OBF-1 target genes. To this end two independent comparisons were performed: (i) transgenic vs WT total thymocytes, (ii) transgenic thymocytes, CD4<sup>+</sup>CD8<sup>+</sup>CD25<sup>+</sup> vs CD4<sup>+</sup>CD8<sup>+</sup>CD25<sup>-</sup> (see Supplementary material). The results of these independent microarray experiments were combined and allowed to identify overlapping subsets of genes that are modulated by OBF-1 expression.

*The expression of Spi-B is proportional to OBF-1 levels in mouse thymocytes and reflects the activity of the octamer-containing promoter 2*

The lymphoid-specific ETS family transcription factor Spi-B was identified as one interesting potential OBF-1 target gene in the microarray analysis. Its elevated expression in the transgenic mice was confirmed by northern blot analysis and was compared to the expression in thymocytes of *OBF-1*<sup>-/-</sup> mice: While the wild type mice express low levels of Spi-B mRNA, the expression of this gene is severely reduced in OBF-1 deficient thymocytes (Fig. 2). Thus, expression of Spi-B and OBF-1 mRNA are directly correlated in murine thymocytes *in vivo*.

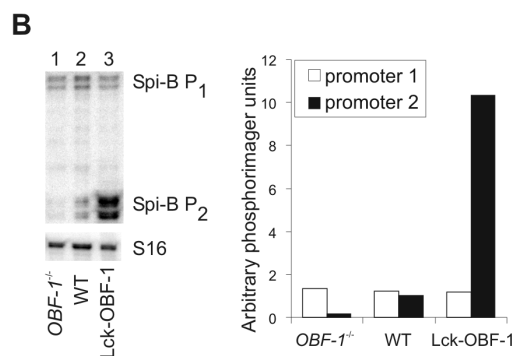
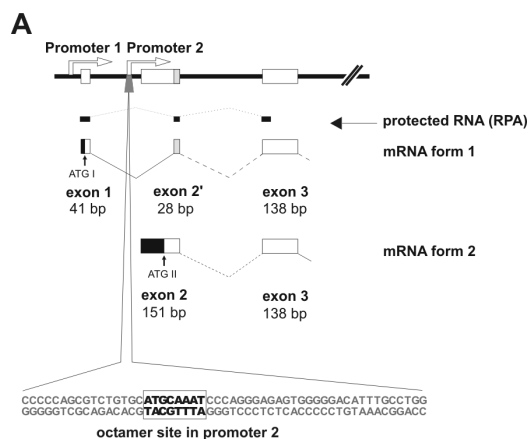


**Figure 2.** Spi-B mRNA level is proportional to the amount of OBF-1 expression in thymocytes. Thymocytes were isolated from OBF-1 deficient, WT or lck-OBF-1 transgenic mice and total RNA was analyzed by Northern blotting with a Spi-B specific probe (left). The same membrane was rehybridized with a probe for beta-actin as a control for loading. Quantification by Phosphorimaging and normalization to the expression level of beta-actin are shown on the right.

Interestingly, the Spi-B gene is transcribed from two distinct promoters (Chen et al. 1998); the downstream promoter (P<sub>2</sub>) contains a perfect octamer site and could therefore represent a good potential target of coactivation by OBF-1. The Spi-B promoters and mRNA isoforms are depicted in Figure 3A. The use of an RNase protection probe that distinguishes between the mRNA isoforms transcribed from the two different promoters revealed that promoter 1 is equally active in cells of the different OBF-1 genotypes, while promoter 2 activity is strongly modulated in thymocytes lacking or overexpressing OBF-1 (Fig. 3B).

#### *Transcription from Spi-B promoter 2 is controlled by OBF-1*

To verify the importance of the octamer site in promoter 2, we performed transient transfection assays using luciferase reporter genes driven by the Spi-B gene P<sub>1</sub> (P<sub>1-luc</sub>) or P<sub>2</sub> (P<sub>2-luc</sub>); we also used variants of P<sub>2</sub> with different mutations in the octamer site. As shown in Figure 4A, upon cotransfection of an OBF-1 expression vector in 293T cells activity of P<sub>1</sub> remained unaffected, but activity of P<sub>2</sub> was strongly enhanced. In addition, a point mutation in the octamer site that specifically abolishes binding of OBF-1 to the Oct-1/2 - DNA complex (P<sub>2-S-luc</sub>, (Babb et al. 1997)) strongly reduced promoter activation, to the same extent as a multiple mutation of the octamer site (P<sub>2-M-luc</sub>) which completely abrogates binding of Oct factors (Fig. 4A). Electrophoretic mobility shift assays (EMSA, Fig. 4B) provided additional *in vitro* evidence for the formation of a ternary complex between Oct-1 - or the Oct-1 POU domain - and OBF-1 on the octamer site of promoter 2 (lanes 9 and



**Figure 3.** Spi-B promoter 2 is under the control of OBF-1. (A) Schematic representation of the mouse Spi-B gene in the promoter region. The structure of the mRNAs originating from promoter 1 (upstream) or 2 (downstream) is indicated. Exon sequences are depicted as boxes. The sequence surrounding the octamer site in promoter 2 is shown below. (B) RNase protection assay with RNA from thymocytes of the indicated genotypes. A probe for S16 was included as a control in the hybridization reaction and its signal was used for the normalized expression presented in the right part of the Figure.

15), visible as a supershifted complex, compared to the complex obtained with Oct-1 or POU-1 alone (lanes 6 and 12). These results demonstrate that the octamer site in the Spi-B promoter 2 is necessary for allowing OBF-1 binding in a ternary complex with Oct-1 (or possibly Oct-2) and that it mediates coactivation by OBF-1.

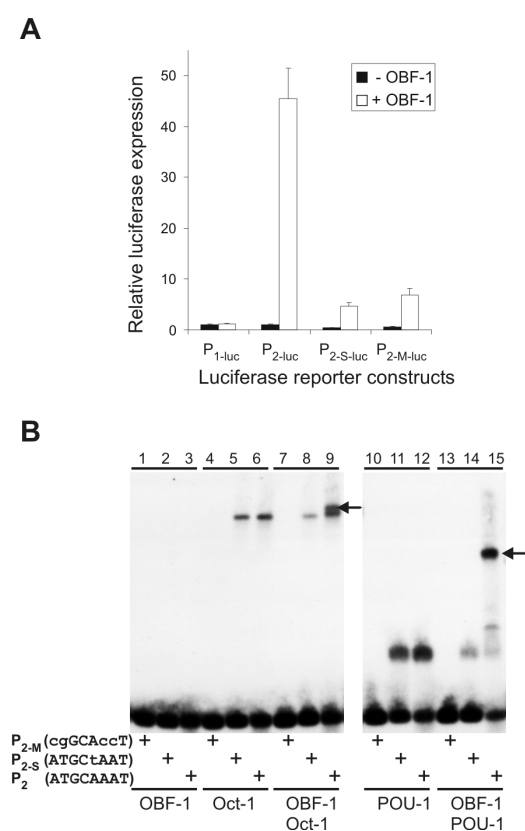
#### *OBF-1 binds in vivo on Spi-B promoter 2*

To determine whether OBF-1 is indeed bound on the Spi-B promoter 2 *in vivo* we performed chromatin immunoprecipitation (ChIP) experiments. For this, primary thymocytes from WT or transgenic mice were isolated, chromatin bound proteins were crosslinked with glutaraldehyde, and OBF-1 was immunoprecipitated with an anti-HA antibody. As shown in Figure 5, the Spi-B promoter 2

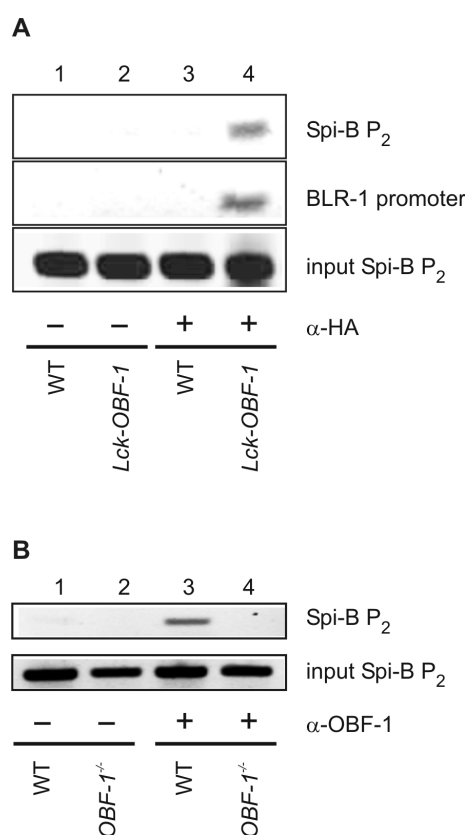


could be amplified by PCR in the precipitate from transgenic thymocytes, but not from WT samples. The BLR-1 promoter, which has been shown earlier to be regulated by OBF-1 (Wolf et al. 1998), was used as a positive control for these ChIP experiments and was also robustly amplified in the immunoprecipitated transgenic sample. Previous work had established that the primary function of OBF-1 is in B cells (Schubart et al. 1996a; Qin et al. 1998). Therefore we attempted to establish a link between expression of OBF-1 and Spi-B in B cells. For this, ChIP experiments were first repeated with primary splenic B cells isolated from WT mice. However, because OBF-1 is expressed only at low levels in these cells (Qin et al. 1998) and the available anti OBF-1 antibodies are not

sensitive enough, no conclusive results were obtained (data not shown). We next used Abelson virus transformed proB cell lines established from WT or *OBF-1*<sup>-/-</sup> mice. Unlike primary mouse splenic B cells, v-Abl transformed proB cells are homogeneous and express high levels of OBF-1 mRNA and protein (our unpublished data). In this case, ChIP analysis with an anti-OBF-1 antibody showed that the Spi-B promoter 2 could be amplified in immunoprecipitates from WT, but not from *OBF-1*<sup>-/-</sup> cells. In contrast, control primers in the coding region of the Spi-B gene failed to amplify a fragment (data not shown). These results thus demonstrate unambiguously the presence of OBF-1 on the Spi-B promoter 2 *in vivo*.



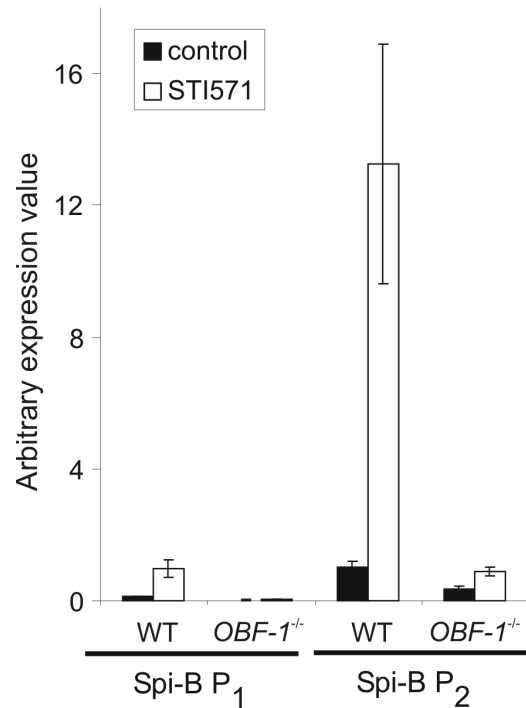
**Figure 4.** Critical role of the octamer motif for activation of the Spi-B promoter 2. (A) Luciferase reporter constructs under the control of the Spi-B promoter 1 or 2 were transfected into 293T cells together with an empty expression vector (black bars) or an OBF-1 expression vector (white bars). The histograms represent the mean  $\pm$  SD of three independent experiments. (B) Electrophoretic mobility shift assay with a WT ( $P_2$ ) or mutated ( $P_{2,S}$ ,  $P_{2,M}$ ) octamer site DNA probe derived from the Spi-B promoter and *in vitro* translated proteins. Either full-length Oct-1 (lanes 4 to 9) or its isolated POU domain (lanes 10 to 15) was used for complex formation.



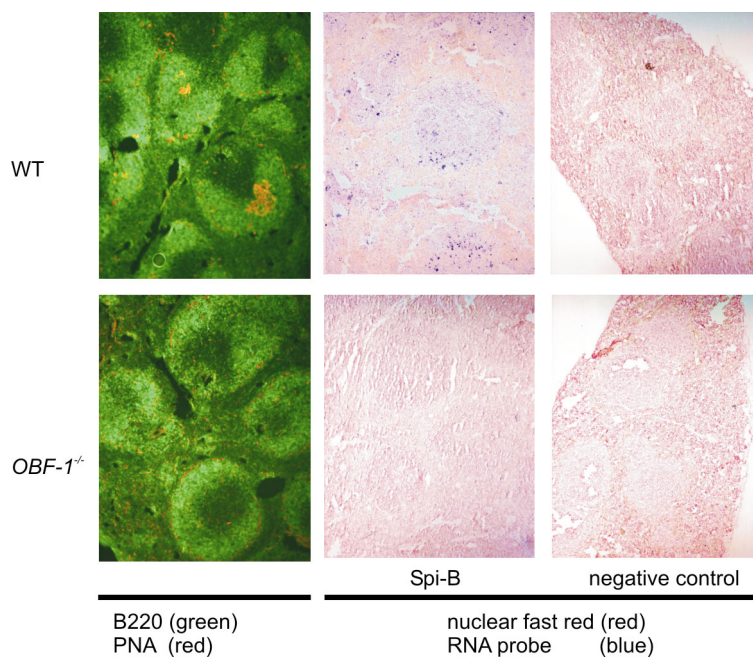
**Figure 5.** *In vivo* OBF-1 is bound on the Spi-B promoter 2. (A) Crosslinked chromatin was isolated from WT or lck-OBF-1 thymocytes and immunoprecipitated with an anti-HA antibody. The precipitated material was used as template for a PCR reaction with primers derived from Spi-B  $P_2$ , or from the BLR1 promoter. The lower panel (input) shows PCR amplification with the chromatin before immunoprecipitation. (B) Crosslinked chromatin was isolated from WT or *OBF-1*<sup>-/-</sup> Abelson virus transformed proB cells and immunoprecipitated with antibodies against OBF-1. A PCR reaction with primers derived from Spi-B  $P_2$  is shown for the precipitate (upper panel) and for the input (lower panel).

*OBF-1 is essential for Spi-B transcription in v-Abl transformed proB cell lines*

It was shown recently that transcription of Spi-B (and of its coactivator IRF-4) is induced upon treatment of v-Abl transformed proB cells with the Abelson kinase inhibitor STI571 (Gleevec); this treatment elicits further differentiation of the cells, as characterized by increased transcription of Rag-1 and Rag-2, Ig  $\kappa$  light chain gene rearrangement, and enhanced expression of CD25 at the cell surface (Muljo and Schlissel 2003). We therefore used v-Abl transformed proB cells to test whether OBF-1 is essential for STI571-induced expression of the Spi-B gene. For this, WT or *OBF-1*<sup>-/-</sup> proB cells were treated for 10 hours with STI571 and expression of the Spi-B gene was measured by real-time PCR with primer pairs that discriminate mRNA originating from P<sub>1</sub> or P<sub>2</sub>. As shown in Figure 6, Spi-B transcription from promoter 2 was efficiently induced in WT cells, but not in cells lacking OBF-1. In addition, promoter 1 was marginally induced by STI571 in WT cells, but not in mutant cells, suggesting that OBF-1 may also influence moderately the activity of promoter 1. This is not unreasonable, as the start sites of the two transcripts are only about 600 bp apart.



**Figure 6.** In Abelson proB cells OBF-1 is necessary for STI571-induced expression of Spi-B. WT or *OBF-1*<sup>-/-</sup> Abl proB cells were cultured in the presence (open bars) or absence (solid bars) of STI571 (Gleevec, 10  $\mu$ M) for 10 hrs and total RNA was extracted. After cDNA synthesis, expression of the Spi-B gene was measured by real-time PCR with primers specific for RNA originating from P<sub>1</sub> or P<sub>2</sub>. The histograms show the mean  $\pm$  SD of 3 independent experiments.



**Figure 7.** Absence of Spi-B expression in *OBF-1*<sup>-/-</sup> follicular B cells. Ten days after immunization with DNP-KLH splenic cryosections were prepared. Formation of GCs was monitored by staining of B cells with an anti-B220 antibody (green) together with Peanut Agglutinin (PNA, red). Spi-B expression was detected by immunohistochemistry with a specific riboprobe (antisense, blue staining) and nuclei were counterstained with Nuclear Fast Red (red staining). A control with a sense Spi-B probe is shown.

*Impaired in vivo Spi-B expression in splenic follicles of immunized mice in the absence of OBF-1*

We and others have shown previously that OBF-1 deficient mice completely fail to form germinal centers upon challenge with a T cell dependent antigen such as DNP-KLH (Schubart et al. 1996a) and this arguably represents the most conspicuous phenotype of these mice. We therefore examined whether absence of OBF-1 influences expression of Spi-B in splenic follicles.

As shown in Fig. 7, in immunized WT mice peanut agglutinin (PNA) positive GC B cells can be detected in the secondary follicles, and these cells also distinctly express Spi-B mRNA. By contrast, in immunized OBF-1 deficient mice no GC B cells can be evidenced and Spi-B expression is completely absent from the follicles.

### Discussion

Here, we present compelling evidence that, in the mouse, the coactivator OBF-1 is necessary for efficient transcription of the Spi-B gene in T and B cells. Overexpression of OBF-1 from the Ick promoter in transgenic mice did not lead to major changes in T cell development or function but modulated expression of specific genes (data not shown). One of them, encoding the gene for the transcription factor Spi-B, was identified as a physiologically relevant candidate.

While ectopic overexpression might represent an artificial situation, the comparison with OBF-1 deficient thymocytes strongly enhances the relevance of our findings. In the absence of OBF-1, a general reduction of Spi-B mRNA levels was observed in thymocytes, mainly reflecting a reduction in the use of promoter 2 (Figs 2 and 3). The normal physiological significance of these findings still remains to be elucidated, since OBF-1 protein is undetectable in total thymic extracts and the absence of OBF-1 (and of Spi-B) does not notably affect T cell development. It is possible, however, that low levels of endogenous OBF-1 protein exist in a subset of thymocytes, be it T or thymic B cells.

A number of *in vitro* experiments showed that OBF-1 can bind to Spi-B P<sub>2</sub> and transactivate transcription from that promoter (Fig. 4). The importance of the perfect octamer site present in Spi-B P<sub>2</sub> is clearly visible in EMSA and transient transfection experiments, strongly suggesting a direct binding of OBF-1 on the Spi-B promoter. This was confirmed *in vivo* by ChIP experiments. A weak effect of OBF-1 expression was noticed on P<sub>1</sub> as well (Fig. 6), which seemed to be independent of the P<sub>2</sub> octamer site, but could reflect activation of the nearby P<sub>2</sub> promoter. Furthermore, chromatin immunoprecipitation assays clearly showed a specific association of the transgenic OBF-1 with the Spi-B promoter P<sub>2</sub> *in vivo* (Fig. 5).

While it is still possible that OBF-1 also regulates additional gene(s) required for the proper expression of Spi-B, our data clearly illustrate that OBF-1 directly controls Spi-B promoter activity via a consensus octamer site important for its expression. Other possible low affinity OBF-1 sites in the Spi-B promoter may explain why the octamer mutations created in our reporter constructs (P<sub>2-S-luc</sub> and P<sub>2-M-luc</sub>) do not completely abolish transcription activation in transient assays.

Given the fact that OBF-1 expression is mainly required in B lymphocytes, we directed our focus to the more physiologically relevant B cell compartment. It was recently shown that Spi-B is induced upon treatment of v-Abl transformed proB cells with the v-Abl kinase inhibitor STI571 (Muljo and Schlissel 2003). We show here that OBF-1 is absolutely crucial for this induction – which is caused mainly by strongly increased transcription from Spi-B P<sub>2</sub> (Fig. 6). Although necessary, the presence of OBF-1 is in itself not sufficient for induction of Spi-B transcription in these cells, and the OBF-1 protein or RNA levels in v-Abl transformed B cells are not noticeably altered by STI571 treatment (data not shown). This indicates that inhibition of the v-Abl kinase does not influence OBF-1 expression, but rather prevents Spi-B transcription through a different mechanism. For example, v-Abl activity could repress Spi-B transcription by directly phosphorylating OBF-1 thereby leading to inhibition of ternary complex formation or coactivation, or by phosphorylating other targets, such as Oct-1 or Oct-2. In the case of Oct-2 it has been proposed that phosphorylation of a residue in the POU domain interferes with binding of Oct-2 to the non-canonical octamer sequence in the murine BLR-1 promoter (Pevzner et al. 2000). While v-Abl transformed proB cells are useful to establish the requirement for OBF-1 in Spi-B transcription in B cells, these cells do not correspond to the developmental stage at which the main phenotype has been described in mice lacking either Spi-B or OBF-1. Neither of these two factors appears to be essential at this early stage of B cell development, at which they might be redundant: In the absence of Spi-B other related Ets factors, such as PU.1 or Spi-C could compensate for Spi-B function.

It is interesting to note that Spi-B levels are also reduced in the bone marrow of OBF-1 knockout mice (data not shown), and that loss of OBF-1 expression has effects on early B lymphopoiesis that have been recognized earlier. There is a reduction of transitional B cells in the spleen of OBF-1 deficient mice (Schubart et al. 2000; Hess et al. 2001), possibly caused by apoptosis during the negative selection of self-reactive B cells occurring at this stage (Carsetti et al. 1995; Sater et al. 1998). Interestingly, OBF-1 deficiency also has an impact on the Ig κ repertoire (Jankovic and Nussenzweig 2003) and leads to a reduction of a subset of immunoglobulin kappa genes (Casellas et al. 2002), suggesting the involvement of OBF-1 in the

selection of transitional B cells. This effect of OBF-1 deficiency might be independent of Spi-B and could be caused by direct binding of OBF-1/Oct-1 or-2 complexes to specific octamer sites in some of the Ig  $\kappa$  promoters. In contrast, constitutive overexpression of OBF-1 in B cells from a  $\mu$  enhancer-V region promoter results in a severe block of B cell development in the bone marrow of transgenic mice (our unpublished data), further supporting the notion that tight control of OBF-1 level is critical for early B cell development.

There are several lines of evidence for the involvement of OBF-1 in modulating BCR signaling strength. Firstly, *OBF-1*<sup>-/-</sup> B cells are unable to induce BCR-triggered Ca<sup>2+</sup> mobilization; this defect was restored in *CD22*<sup>-/-</sup> *OBF-1*<sup>-/-</sup> double deficient animals (Samardzic et al. 2002a). CD22 is a negative regulator of BCR signaling, and CD22 deficient mice display a lowered activation threshold for BCR crosslinking and increased Ca<sup>2+</sup> mobilization upon BCR stimulation (Nitschke et al. 1997). Secondly, *OBF-1*<sup>-/-</sup> B cells also showed a reduced proliferation following anti-IgM stimulation (Sun et al. 2003). It is thus very tempting to assume that the B cell differentiation defect in the bone marrow of *OBF-1*<sup>-/-</sup> mice is BCR-signal dependent. Second, Spi-B itself, together with PU.1, was implicated in signaling downstream of the BCR, probably by controlling the transcription of a component coupling Syk to downstream targets, such as PLC $\gamma$  and BLNK (Garrett-Sinha et al. 1999). In addition, increased apoptosis in the bone marrow, possibly caused by the increased sensitivity of *OBF-1*<sup>-/-</sup> transitional B cells to BCR cross-linking observed *in vitro* (Jankovic and Nussenzweig 2003) could explain the receptor specific block at the T1 stage of B cell development in *OBF-1* mutant mice. These effects, but also the reduced numbers of MZ B cells found in one specific *OBF-1* deficient mouse strain (Samardzic et al. 2002b) can be explained by a model in which OBF-1 is required for setting the normal threshold for immature B cell development and selection in the bone marrow.

A second function of OBF-1, its crucial role for the development of GCs in the spleen, reflects a different mechanism, since *OBF-1*<sup>-/-</sup> *CD22*<sup>-/-</sup> double deficient mice were still unable to mount humoral immune responses and to form germinal centers, although their BCR activation threshold was similar to the WT situation (Samardzic et al. 2002a). The data presented here indicate that the expression of Spi-B, controlled by the upregulation of OBF-1 protein in the GC B cells may be a key element in the GC formation. To conclude, we have identified Spi-B as the most physiologically relevant *in vivo* target of OBF-1 to date. These findings greatly help to extend our understanding of how OBF-1 is involved in B cell activation and GC formation and place it directly upstream of Spi-B in a transcriptional cascade.

## Materials and Methods

### Mouse strains and cell lines

The Lck-OBF-1 construct used to generate transgenic mice contains an N-terminally HA epitope-tagged human OBF-1 cDNA under the control of the murine proximal lck promoter (-3100 to +23 relative to the transcription start site). Transgenic mouse lines were obtained and bred in B6CF1 x C57BL/6 background and were bred to a wild type partner of the same genetic background. *OBF-1* deficient mice were in 129SV-C57BL/6 background. All animal work and experiments were done in compliance with local and institutional regulations. Mice were sacrificed, the thymi were taken, rinsed in PBS and immediately homogenized in TRIZOL reagent (Life Technologies). Total RNA was prepared using TRI-ZOL reagent and purified on RNeasy Miniprep columns (Qiagen) according to the manufacturers' instructions. Sequence data is available upon request.

Cell lines: Abelson pro-B cell lines were derived from wild type and *OBF-1*<sup>-/-</sup> mice and were described previously (Schubart et al. 1996b)

### PCR

To determine the *OBF-1* genotype, genomic DNA was isolated from tail biopsies and used as a template for polymerase chain reaction (PCR). The PCR assay for the *OBF-1* wild type and *OBF-1* mutant loci was carried out with the following primer combinations, respectively: 5'-GCT CCC TGA CCA TTG AC-3', 5'-TCC TGT CCC ATC CCC CTG TAA-3' and 5'-GGC TTA GAT AAC AAA GCG TGT GCT C-3', 5'-GCG TGC CCA TCT TGT TCA ATG G-3'. The size of the PCR products for the WT allele was 560bp and for the mutant allele 830bp.

Transgenic *OBF-1*-HA was detected using the following primer combinations: 5'-GGT TGA GTG GTG GGG GTA GGG-3' and 5'-CAC ACG GAC GCC CTG GTA TGG-3' (for genotyping), 5'-CAC TCT CTC TGT GGA AGG CTT TG-3' and 5'-TTC TCA GCT CTA GAC GGC GTA GT-3' (for QPCR).

Spi-B was detected with the PCR primers 5'-CAG TTC TCC TCC AAG CAC AAG-3', 5'-CCA GCC ATT CAT CTC GCA TAG-3' (standard RT-PCR for all isoforms and splice variants, also used to generate a probe for Northern blotting), 5'-CTC TGA ACC ACC ATG CTT GCT-3', 5'-TCC TTC TGG GTA CAA ACA GCT TAA-3' (QPCR for form 1), 5'-AGG GCG GCC CTG ACA T-3', 5'-TCC TTC TGG GTA CAA ACA GCT TAA-3' (QPCR for form 2). To amplify Spi-B promoter 2 in chromatin immunoprecipitations (ChIP) samples, the primers 5'-GCG CCC AGC CTT CTC CTC CA-3' and 5'-AAA TGT CCC CCA CTC TCC CTG-3' were used. To amplify the BLR-1 promoter in ChIP samples, the following primers were used: 5'-TGT AGA GGA GGC TGG GAG TA-3' and 5'-CGA GAA GGT TTG TGC TGG TG-3'.

GAPDH (QPCR): 5'-ACC TGC CAA GTA TGA TGA CAT CA-3', 5'-GTA GCC CAA GAT GCC CTT CAG T-3'.  $\beta$ -actin (for Northern blot probe):

5'-ATG GAT GAC GAT ATC GCT GCG CTG-3',  
5'-CTA GAA GCA CTT GCG GTG CAC GAT-3'.

Quantitative real-time PCR (QPCR) was performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using a SybrGreen-based kit from Applied Biosystems.

#### *Cell culture and protein expression in eukaryotic cells*

Adherent cells were maintained in DMEM plus 10% fetal calf serum. All B cell lines were grown at 37°C and 10% CO<sub>2</sub> in RPMI supplemented with 10 % FCS (Life Technologies, Inc.), penicillin/streptomycin and 50 mM β-mercaptoethanol (Fluka).

HEK293T cells were transfected in 6-well-plates using FuGENE6 (Roche, Basel, Switzerland) according to the manufacturers instructions.

#### *Protein extract, western blotting and luciferase assay*

For western blotting, B and T cells were lysed by repeated cycles of freeze and thaw in 20 mM HEPES pH7.9, 20 % glycerol, 400 mM NaCl, 0.5 mM EDTA, 0.25 mM EGTA, 0.025 % NP-40, 1 mM DTT, 0.5 mM NaF and 'complete' protease inhibitor cocktail (Roche). Protein concentrations were determined with Bradford reagent (BioRad) and 30 mg of protein for each sample was resolved by SDS-PAGE with subsequent transfer onto Immobilon membrane (Millipore). HA-tagged proteins were detected with the monoclonal antibody 12CA5, and endogenous OBF-1 was detected using a polyclonal antibody that was raised in rabbits against the C-terminal 154 amino acids of murine OBF-1. Bands were visualized with the ECL system (Amersham).

To measure luciferase activity, reporter plasmids were cotransfected and cells were lysed in 0.1 M potassium phosphate buffer, pH7.8 containing 1 mM DTT by repeated cycles of freeze and thaw. The cell lysates were measured in a Bertold Luminometer according to standard protocols. The reporter plasmids P<sub>1-luc</sub> (D2393) and P<sub>2-luc</sub> (D2397) were kindly provided by D. Tenen and are described elsewhere (Chen et al. 1998). In brief, P<sub>1-luc</sub> consists of the 170 bp Spi-B promoter 1 driving the firefly luciferase gene in the pXP2 expression vector. P<sub>2-luc</sub> is based on the same vector, but contains the 800 bp Spi-B promoter 2 instead of the Spi-B promoter 1. P<sub>2-S-luc</sub> and P<sub>2-M-luc</sub> are identical to P<sub>2-luc</sub>, but contain specific mutations in the octamer site (Figure 4b shows the exact mutations, which are identical to the mutations in the oligonucleotides P<sub>2-M</sub> and P<sub>2-S</sub>). For transient transfections, 600 ng of OBF-1 expression vector (Strubin et al. 1995) or empty expression vector and equimolar amounts of luciferase reporter vectors were used. Samples were filled up with empty vector to a total amount of 1200 ng DNA per transfection.

#### *Electrophoretic mobility shift assay (EMSA)*

*In vitro* translated proteins were prepared as described previously (Sauter and Matthias 1998).

Binding reactions contained equimolar amounts of proteins, 1 mM EDTA, 250 µg/ml BSA, 20 mM HEPES (pH 7.9), 50 mM KCl, 4% Ficoll, 6.25 x 10<sup>5</sup> cpm/ml of <sup>32</sup>P-labeled double-stranded oligonucleotide probe, 25 µg/ml single stranded DNA, and either 25 µg/ml poly(dI-dC) (Amersham Pharmacia Biotech). Protein-DNA complexes were resolved on a 4% (30:1) polyacrylamide:bisacrylamide, 0.25 x TBE gel at 200 V for 1 h, dried, and subjected to autoradiography.

The following double-stranded synthetic oligonucleotides were used:

P<sub>2</sub> probe: 5'-CCC CCA GCG TCT GTG CAT GCA AAT CCC AGG GAG AGT GGG GG-3', 5'-CCC CCA CTC TCC CTG GGA TTT GCA TGC ACA GAC GCT GGG GG-3'

P<sub>2-S</sub> probe: 5'-CCC CCA GCG TCT GTG CAT GCT AAT CCC AGG GAG AGT GGG GG-3', 5'-CCC CCA CTC TCC CTG GGA TTA GCA TGC ACA GAC GCT GGG GG-3'

P<sub>2-M</sub> probe: 5'-CCC CCA GCG TCT GTG CCG GCA CCT CCC AGG GAG AGT GGG GG-3', 5'-CCC CCA CTC TCC CTG GGA GGT GCC GGC ACA GAC GCT GGG GG-3'

#### *Northern blotting*

RNA was prepared by Trizol (Life Technologies, Inc.) according to the manufacturer's instructions. 15 mg of total RNA was analyzed by Northern blotting using Hybond-XL membranes (Amersham Pharmacia Biotech). OBF-1 was visualized with a radiolabelled full length probe. Quantification of signal intensity was performed on a Molecular Dynamics PhosphorImager using Image Quant software.

#### *RNase protection assay*

RNase protection was performed as described earlier (Schubart et al. 1996b). In brief, a probe was *in vitro* transcribed from a vector containing the murine Spi-B promoter and 5' coding region (kindly provided by D. Tenen, described in (Chen et al. 1998)). The gel-purified radiolabeled probe was afterwards hybridized with 20 mg of total RNA at 50 °C over night. After RNase digestion, RNA was resolved on a 5% polyacrylamide gel and autoradiographed. The protected part of the murine Spi-B RNA is shown in Figure 3A. In the same assay, a control probe of S16 mRNA was used for normalization of loaded RNA quantity. Signal intensity was quantified on a Molecular Dynamics PhosphorImager using Image Quant software.

#### *Immunofluorescent staining and flow cytometry (FACS) analysis*

Single cell suspensions of freshly prepared thymocytes were prepared, stained and analyzed according to standard procedures (Rolink et al. 1994). FACS analysis was performed on a FACSCalibur (BD Biosciences, San Jose, CA), and fluorescence data were collected on 30,000 events by gating on living cells and typical forward-side scatter appearance of lymphocytes. CellQuest software was used for collecting and analyzing data. Cell sorting was per-

formed on a MoFlo (DakoCytomation) with a purity of at least 94 %.

The mAbs 7D4 (anti-CD25; biotinylated), 53-6.7 (anti-CD8a; PE-labeled) and GL3 (anti- $\gamma\delta$ -TCR; PE-labeled) were purchased from Pharmingen (BD Biosciences), San Diego, CA.; RA3-6B2 (anti-B220; FITC labeled (IHC)), and GK1.5 (anti-CD4; FITC-labeled), anti-CD122-FITC (#1905-02), and streptavidin-RPE were purchased from Southern Biotechnology Associates (Birmingham, USA). Biotinylated peanut agglutinin (PNA-biotin, Vector Lab) was used for immunohistochemistry

#### *Immunohistochemistry and in situ hybridization*

Young adult mice were sacrificed, the thymi were taken, rinsed in PBS and fixed for 16 hours in 4% paraformaldehyde. After a series of washes in PBS, 0.85% NaCl, 50% ethanol in PBS and 70% ethanol in PBS, the thymi were paraffin embedded according to standard procedures and 6  $\mu$ m sections were prepared for histochemistry.

For in situ hybridization, a 450 bp probe from the 3' UTR of the murine Spi-B cDNA and the corresponding antisense probe were DIG-labeled with the DIG RNA labeling kit (Roche) according to the manufacturer's instructions.

#### *Chromatin immunoprecipitation (ChIP)*

ChIP was performed as described previously (Masternak et al. 2003). In brief,  $4 \times 10^7$  thymocytes (or  $5 \times 10^6$  STI571-treated Abelson B cells) were harvested, suspended in 40 ml (4 ml) RPMI 1640 and crosslinked for 10 minutes at room temperature with 4 ml (400  $\mu$ l) 11% formaldehyde / 0.1 M NaCl / 50 mM Hepes pH 7.9. Crosslinking was stopped by addition of 6.5 ml (650  $\mu$ l) 1 M glycine pH 8 and incubation for 5 minutes at room temperature. Cells were washed with 50 ml (5 ml) cold PBS containing protease inhibitors (Complete, Roche, Basel, Switzerland), resuspended in 40 ml (4 ml) ice cold cell lysis buffer (5 mM Pipes, pH8.0 / 85 mM KCl / 0.5% NP40) + Complete (Roche) and vortexed. Nuclei were spun down immediately for 7 minutes at 2,500 rpm, then lysed for 10 minutes at room temperature in 2.2 ml Nuclei lysis buffer (50 mM Tris, pH8.0 / 10 mM EDTA / 1% SDS). Chromatin was sonicated with a Branson 250 sonicator:  $3 \times 20''$  pulses constant power output setting 7, precleared by centrifugation at 12000 rpm for 10 minutes at 4°C. Chromatin was transferred to fresh vials and stored at -80°C.

To immunoprecipitate chromatin with specific antibodies, 10  $\mu$ g (3  $\mu$ g) of chromatin were diluted with immunoprecipitation (IP) buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 2 mM EDTA, 0.1% sodium deoxycholate, 0.1% SDS and the protease inhibitor complex Complete) containing 50 g/ml salmon sperm DNA, 100 g/ml Escherichia coli tRNA and 1 mg/ml bovine serum albumin. Immunoprecipitation was performed with an anti-HA antibody (12CA5) in case of the thymocytes and with the monoclonal OBF-1 antibody C-20 (SC-955

X, Santa Cruz Biotechnology, CA) in case of the Abelson cell lines. Immune complexes were captured at room temperature with protein A-Sepharose beads. Beads were washed twice with IP buffer, twice with IP buffer containing 500 mM NaCl, twice with 20 mM Tris-HCl, pH 8.0, 0.25 M LiCl, 2 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate and once in TE containing 0.1% NP-40. Immunoprecipitated chromatin fragments were eluted with 100 mM Tris-HCl, pH 8.0, 1% SDS for 10 min at 65 °C, diluted twofold with 400 mM NaCl, 200 g/ml proteinase K, digested for 1 h at 37 °C and incubated overnight at 65 °C to reverse the crosslinks. After extraction with phenol-chloroform and chloroform, DNA was precipitated with isopropanol in the presence of glycogen, washed in 80% ethanol and resuspended in TE. Immunoprecipitated DNA derived from 100 ng of input chromatin DNA and a standards containing 10 ng of total input chromatin DNA were analyzed by PCR.

#### *Affymetrix Microarrays MG-U74 and MG-U74v2*

*RNA preparation and microarray hybridization:* Cells were harvested. Total RNA from thymus was immediately isolated with the Trizol method (Life Technologies, Inc.), followed by a purification using the RNeasy kit from Qiagen according to the manufacturer's instructions. Microarray analysis was performed using MG\_U74A version 2 GeneChips™ (Affymetrix). 10  $\mu$ g of total RNA was reverse transcribed using the SuperScript Choice system for cDNA synthesis (Life Technologies) according to the protocol recommended by Affymetrix.

*Data processing:* Chip analysis was performed using the Affymetrix Microarray Suite v5 and GeneSpring 5.1 (Silicon Genetics). Changes in gene expression were assessed by looking for concordant changes between replicates using a signed Wilcoxon rank test. The "change" p-value threshold was < 0.003. Genes whose detection p-value was > 0.05 (absent) in all conditions were also discarded. Detailed information conforming to MIAME standards is provided upon request.

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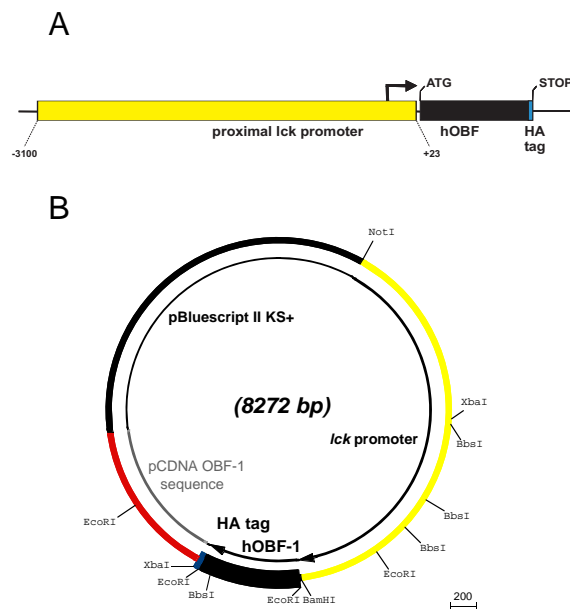
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## 2.2 *Lck-OBF-1* transgenic mice

### 2.2.1 Generation of *lck-OBF-1* transgenic mice

*In vivo*, OBF-1 was found to be expressed mostly in a B cell-restricted manner, but its expression was also induced in T cell lines and primary murine thymocytes by *in vitro* stimulation with PMA and ionomycin. However, the physiological role of OBF-1 in T cells has remained unclear and in OBF-1 deficient mice T cell development and function were found to be normal (Qin et al., 1998; Schubart et al., 1996a). To further define the potential function of OBF-1 in T cells, we generated transgenic mice expressing an HA epitope-tagged OBF-1 cDNA under the control of the murine proximal *lck* promoter. This T cell-specific promoter is highly active in thymocytes throughout their development and then has low activity in peripheral T cells (Shimizu et al., 2001). The expression cassette was cloned into pBluescript II KS+ from previously described plasmids containing the proximal *lck* promoter (p1017, kindly provided by R. Perlmutter), and human OBF-1 cDNA with an additional C-terminal HA tag (pCDNA-OBF-1, pEV OBF-1/9HA). The DNA sequence of this T cell-specific construct is available online in digital form at <http://www.fmi.ch/groups/matthias.p/Bartholdy.html>. A plasmid map is shown in Figure 15.

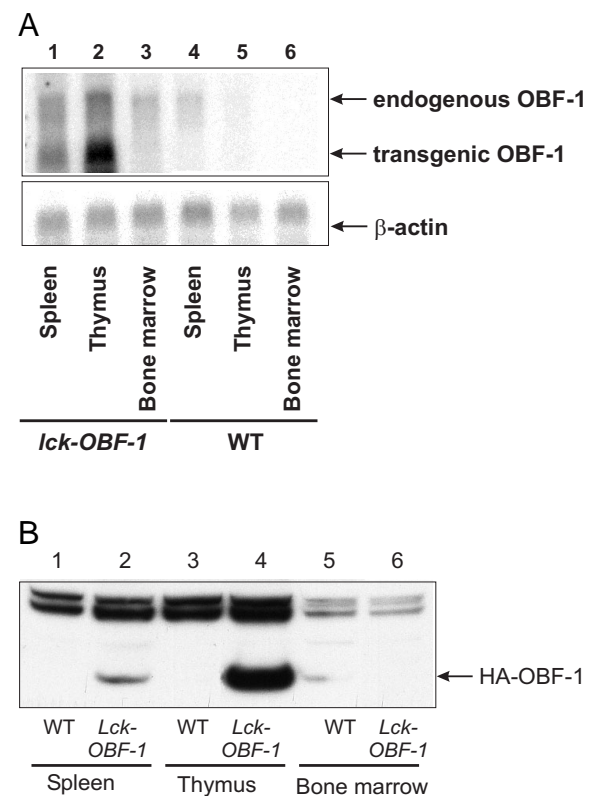


**Figure 15** *Lck-OBF-1* construct used to generate transgenic mice constitutively expressing HA-tagged hOBF-1 in a T cell-specific manner. (A) Construct, (B) in the context of the expression vector (important restriction sites are indicated).

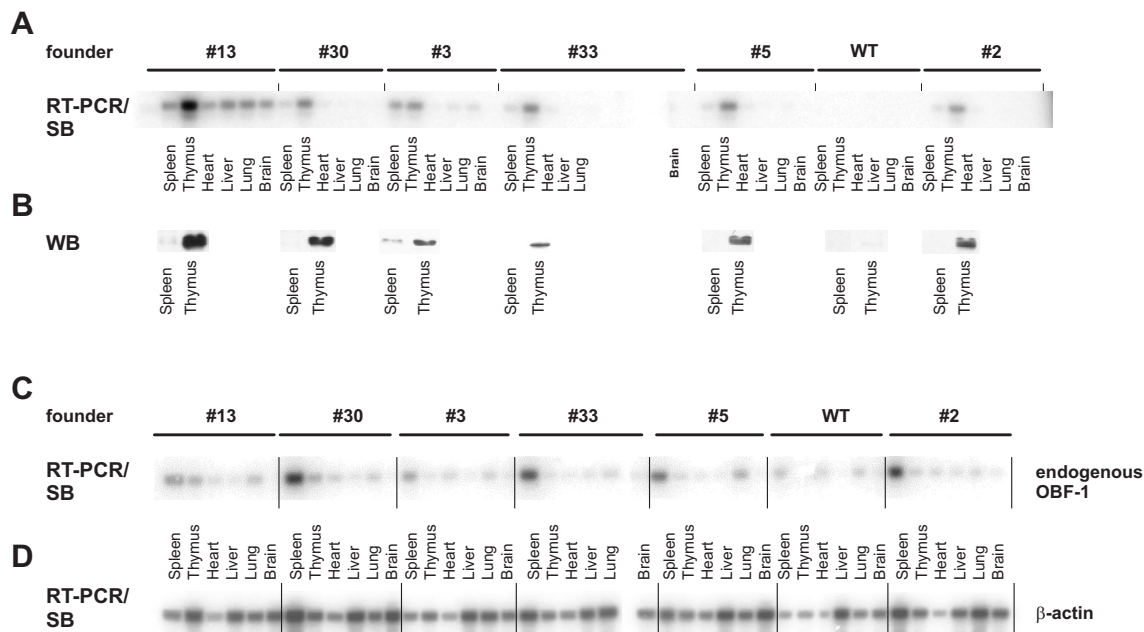
### 2.2.2 Genotyping of transgenic mouse lines and expression analysis

At first, potential founder mice were screened by PCR analysis and by Southern blotting. The positive founders were subsequently crossed to WT mice of the same background to establish distinct transgenic lines. Transgenic offspring from these lines was used to determine the mRNA and protein expression pattern of the transgene.

In agreement with the expected activity pattern of the *lck* promoter, high levels of transgenic OBF-1 mRNA and protein were found in the thymus and lower levels were found in the spleen (Fig. 16). By contrast, transgenic OBF-1 mRNA and protein were barely detectable in non-lymphoid organs of these transgenic lines (Fig. 17). Several independent transgenic mouse lines were generated, which showed the same phenotype described below. We focused on the lines #13 and, later, #466, which had the highest transgene expression and the most pronounced phenotype.



**Figure 16** (A) mRNA expression of *lck-OBF-1* and endogenous OBF-1 in lymphoid tissue (northern blot).  $\beta$ -actin was used as a loading control. (B) Expression of transgenic OBF-1 protein in the lymphoid compartment of 6-8 weeks old mice. Western blot analysis was performed with the monoclonal anti-HA antibody 12CA5. The upper band is unspecific, but shows that equal amounts of WT and transgenic protein were loaded for each tissue.



**Figure 17** Analysis of the expression pattern of *lck-OBF-1* in transgenic mouse lines. **A.** RT-PCR/Southern blot analysis of the *lck-OBF-1* transgene in several transgenic mouse lines. **B.** Protein expression measured by western blot analysis of spleen and thymus extracts of the same mouse lines. The (HA-tagged) transgenic *OBF-1* protein was detected with a monoclonal anti-HA antibody (12CA5). **C.** and **D.** Control reactions detecting the expression levels of endogenous *OBF-1* mRNA (**C**) and  $\beta$ -actin mRNA (**D**).

### 2.2.3 FACS analysis

To study the effect of *OBF-1* overexpression in T cells, flow cytometric analyses were performed of cells from several lymphoid organs, such as the bone marrow, spleen, thymus and lymph nodes. For that,

the expression of a number of cell surface markers characteristic of discrete stages of B and T cell development was monitored by FACS. A list of the surface markers that were used for this study is presented in [Table III](#).

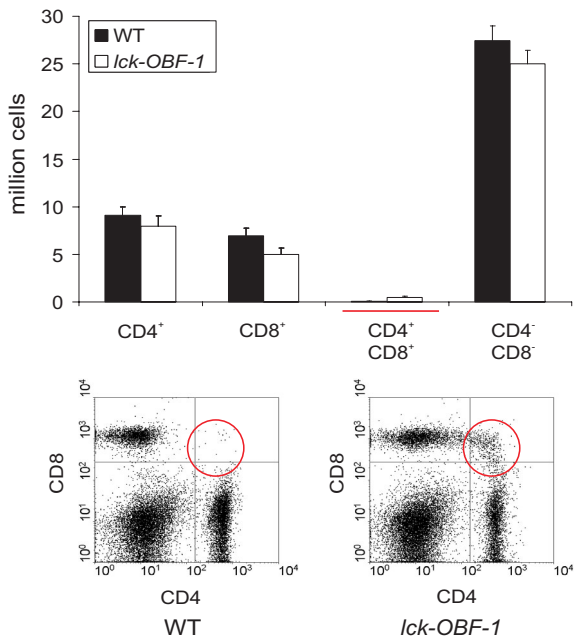
**Table III** Cell surface markers used to study B and T cell development.

marker	description	marker	description
c-kit (CD117)	Stem cell factor receptor, 145 kDa transmembrane tyrosine kinase encoded by the c Kit proto-oncogene. c Kit acts to regulate a variety of biological responses including cell proliferation, apoptosis, chemotaxis and adhesion. Expressed on some multipotent hematopoietic cells.	CD25 (IL-2-R $\alpha$ ; TAC)	IL-2 receptor $\alpha$ chain, expressed on activated T and B cells. Resting T and B cells and NK cells do not express CD25. Transiently expressed during B-cell development in the bone marrow (during pre-B/preB-II stage). Expressed at higher levels during very early stage of T-cell development in fetal and adult thymus.
493 antigen (C1qRp, AA4.1)	Complement component C1q like receptor C1qRp; expressed on immature B cells in the bone marrow and in the spleen.	CD90 (Thy-1)	Surface glycoprotein of the Ig superfamily, in all mammalian brain and HSCs. Pan-T cell marker, found on thymocytes, peripheral T cells, and some intraepithelial T lymphocytes.
B220	Isoform of CD45, a major cell-membrane glycoprotein expressed on all hematopoietic cells except mature erythrocytes. B220 primarily recognizes only cells of B-lineage from pro-B cell through mature B lymphocytes; pan-B cell marker.	CD122 (IL-2R $\beta$ )	Expressed by early T cell progenitors, some peripheral T cells, and NK cells. Upregulated by activation. Associates with CD25 and CD132 ( $\gamma_c$ ) to form the high affinity IL-2 receptor.
CD3	TCR-associated CD3 complex expressed on all T cells; pan-T cell marker.	$\gamma\delta$ -TCR	Specific TCR expressed by a subset of T cells found mainly in gut epithelium and skin.
CD4	Transmembrane glycoprotein expressed on most thymocytes and on mature T helper cells.	IgD	Immunoglobulin D (isoform found on mature B cells).
CD5 (Ly-1)	Major ligand of CD72, found on thymocytes, T cells, and a subset of B cells (the B-1 B cells), but not NK cells.	IgM	Immunoglobulin M (isoform that is found bound to the membrane of immature and mature B cells).
CD8	Differentiation antigen found on most thymocytes and the mature cytotoxic T cells.	NK1.1	Expressed on all NK cells and it initiates transmembrane signals that activate cytotoxicity.

As expected, B cell development in the spleen and bone marrow was unaffected by expression of the transgene in T cells (data not shown), and normal numbers of B lymphocytes of all developmental stages were present in the lymphoid system of the transgenic animals.

### 2.2.3.1 Normal development of conventional T cells

After observing that the B cell compartment appeared to be unaltered, we turned our attention to the T cell compartment of the *lck-OBF-1* transgenic mice. The main pathway of T cell development, as judged by the cell surface expression of the CD4 and CD8 coreceptors on the maturing thymocytes, appeared to be normal (see Section 2.1, Figure 1), and normal numbers of mature cells were measured in the peripheral lymphoid organs. However, a small subpopulation of CD4<sup>+</sup> CD8<sup>+</sup> cells appeared in the spleen of the transgenic mice that was undetectable in WT littermates (Fig. 18).



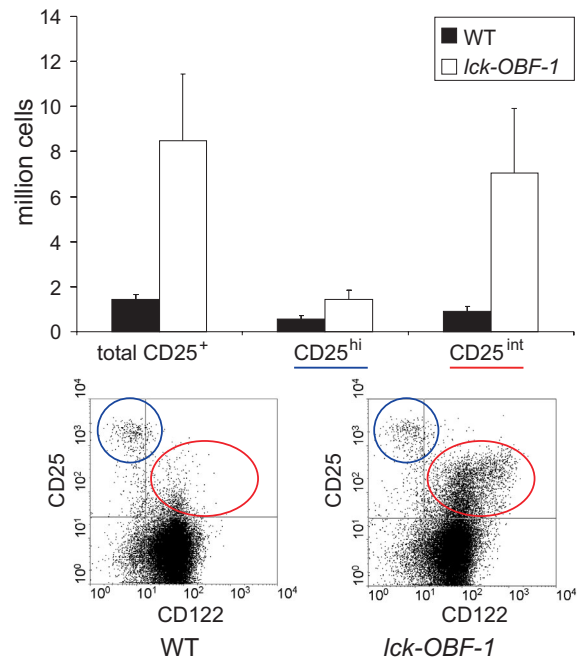
**Figure 18** Presence of CD4<sup>+</sup> CD8<sup>+</sup> splenocytes in transgenic mice (encircled in red). Splenocytes from 11-12 week old mice were isolated and analyzed by FACS with FITC-labelled anti-CD4 and PE-conjugated anti-CD8 antibodies. The dot plot of one representative mouse of each genotype is shown. In the upper part of the Figure the results from 6 animals of each genotype have been pooled and the data are presented  $\pm$  SD (average of 3 independent experiments, each time in duplicate).

### 2.2.3.2 Massive increase in CD25<sup>+</sup> thymocytes

Prompted by the appearance of this abnormal T cell subset, we analyzed the T cell compartment of the *lck-OBF-1* transgenic mice in greater detail and found a number of alterations in its composition. The most obvious change was the appearance of a population of thymocytes expressing CD25, the IL-2 receptor alpha chain, on the cell surface (Fig. 19). Usually, immature thymocytes express CD25 at the

CD4<sup>-</sup> CD8<sup>-</sup> (double negative, DN) stages, which correspond to the CD122<sup>-</sup> CD25<sup>hi</sup> population in Figure 19. While this population is only slightly upregulated in the transgenic thymus, most of the ~7-fold increase in CD25<sup>+</sup> cells is caused by the appearance of CD4<sup>+</sup> CD8<sup>+</sup> (double positive, DP) cells that express CD25. Usually, these DP cells have downregulated CD25 expression which gets only reinitiated in activated peripheral T cells.

Interestingly, the expression of CD25 on mature T cells in the spleen and in the lymph nodes was not increased in the transgenic animals. This is in concordance with the finding that other T cell activation markers, such as CD69 and HSA were also normal (not shown).



**Figure 19** Increase in CD25<sup>+</sup> thymocytes in *lck-OBF-1* transgenic mice. Thymocytes from 11-12 weeks old mice were prepared and analyzed by FACS with FITC-conjugated anti-CD122 and PE-conjugated anti-CD25 antibodies. The dot plot of one representative mouse of each genotype is shown. In the upper part the results from 6 mice of each genotype have been pooled and the data are presented  $\pm$  SD. The CD25<sup>high</sup> or CD25<sup>intermediate</sup> cells correspond to the dot plot areas circled in blue or red, respectively (average of 3 independent experiments with 11-12 week old mice (each time in duplicate)).

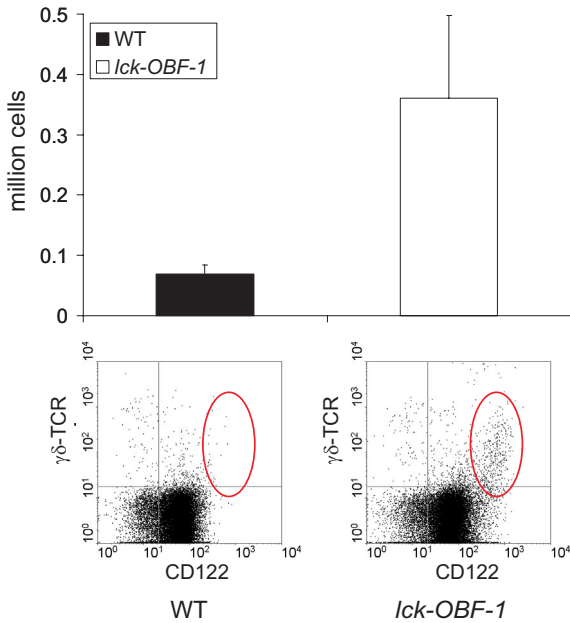
### 2.2.3.3 Alterations in gammadelta T cell development

Another T cell subpopulation that was upregulated in *lck-OBF-1* transgenic thymus were the gammadelta T cells. These cells do not express a conventional T cell receptor composed of an alpha and a beta chain, but use the gamma and delta chain instead. As shown in the introduction (Fig. 2), these cells branch off from the main T cell pathway early in thymic T lymphopoiesis. In the transgenic mice, a 4- to 5-fold increase in  $\gamma\delta$ -TCR<sup>+</sup> T cells was found in the thymus (Fig. 20). Additional FACS analyses

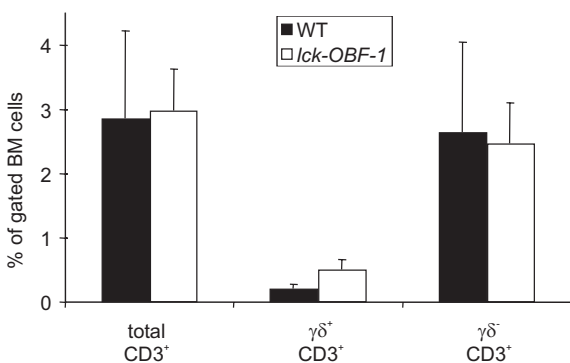


showed that these cells were CD4<sup>+</sup> CD8<sup>+</sup>, and mostly CD25<sup>+</sup> as well.

It is of interest to note that the number of  $\gamma\delta$  T cells is normal in the spleen and is only slightly increased in the bone marrow of *lck-OBF-1* mice (Fig. 21). Another site where  $\gamma\delta$  T cells are found frequently is the gut epithelium as part of the intestinal intraepithelial lymphocytes (iIEL). Murine iIEL contain predominantly CD8<sup>+</sup> T cells, but also some CD4<sup>+</sup> CD8<sup>-</sup> DN T cells. About 50% of the T cells express the  $\gamma\delta$  TCR. In the *lck-OBF-1* transgenic mice there was only a marginal increase in these  $\gamma\delta$  T cells in the gut (not shown).



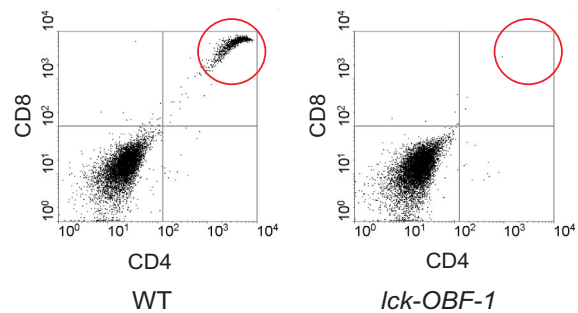
**Figure 20** Increase in  $\gamma\delta$ -TCR<sup>+</sup> thymocytes in *lck-OBF-1* transgenic mice. Thymocytes from 6-9 weeks-old mice were isolated and analyzed by FACS with FITC-labelled anti-CD122 and PE-conjugated anti- $\gamma\delta$ -TCR antibodies. The dot plot of one representative mouse of each genotype is shown. In the upper part of the Figure the results from 6 mice of each genotype have been pooled and the data are presented  $\pm$  SD. The gated area for the quantification is indicated in red (average of 3 independent experiments, each time in duplicate).



**Figure 21** Moderately increased levels of  $\gamma\delta$ -TCR<sup>+</sup> T cells in the bone marrow of transgenic mice. Isolated bone marrow cells were stained with FITC-labelled anti-CD3 and PE-conjugated anti- $\gamma\delta$ -TCR antibodies. While the

total T cell number (CD3 total) and the number of “conventional”  $\gamma\delta$ -TCR<sup>+</sup> T cells (right column) were normal in the *lck-OBF-1* transgenic bone marrow population, the  $\gamma\delta$ -TCR<sup>+</sup> T cells (middle column) were moderately increased in the transgenic animals. The results from 3 mice of each genotype have been pooled and the data are presented  $\pm$  SD.

In contrast, dendritic epidermal T cells (DETC), which are a specific subset of  $\gamma\delta$  TCR<sup>+</sup> T cells (that use the V $\gamma$ 3 gene segment), were lacking completely in the skin of the transgenic mice (Fig. 22). V $\gamma$ 3 cells are making up the first wave of TCR<sup>+</sup> cells that can be found in the fetal thymus around day 14-16 of gestation. In adult mice, they are found exclusively in the epidermis, where they represent the main T cell population.

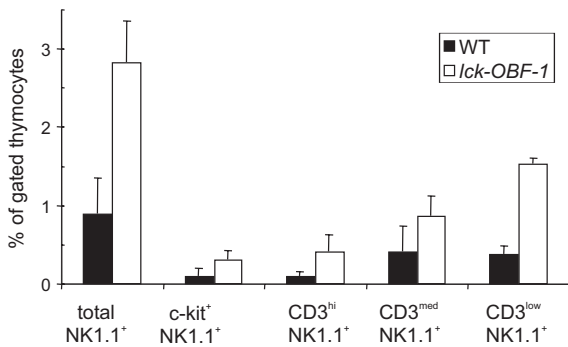


**Figure 22** Absence of dendritic epidermal T cells in the skin of transgenic mice (encircled in red). Isolated dendritic epidermal T cells (DETCs) were stained with FITC-labelled anti-CD3 and PE-conjugated anti- $\gamma\delta$ -TCR antibodies. The dot plot of one representative mouse of each genotype is shown. As one can clearly see, CD3<sup>+</sup>  $\gamma\delta$ -TCR<sup>+</sup> DETCs are virtually absent in the skin of the transgenic animals but can be readily detected in WT mice (and also in *OBF-1* deficient mice, data not shown).

### 2.2.3.4 NK cells

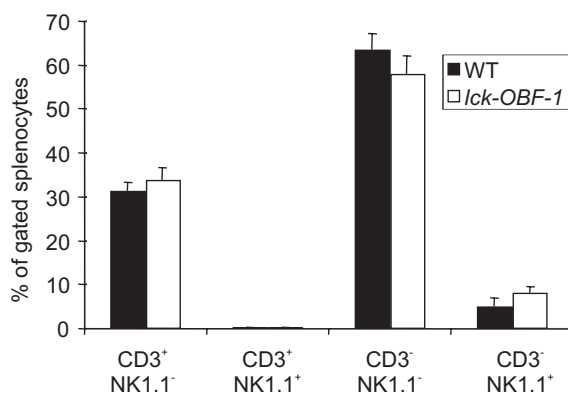
Other cell populations that arise from a common lymphoid progenitor are the natural killer (NK) and NKT cells. Similarly to B cells and T cells, this cell population critically depends on interleukin signaling through receptors that share the common  $\gamma_c$  chain ( $\gamma_c$ ), such as IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Consequently,  $\gamma_c$  deficient mice have a marked impairment of B, T, and NK cell development. The defective B and T cell development observed in  $\gamma_c$  deficient mice seems to reflect the lack of IL-7R signaling, while the defect in NK cell development is due to the absence of IL-15R signaling. Mice lacking IL-7 or its receptor IL-7R have major defects in B and T cell development, but an uncompromised NK cell development (see also the review in Section 2.5). Studies in mice deficient in IL-15 or IL-15R $\alpha$  have confirmed a critical role for IL-15 in regulating the development and/or expansion of NK cells, NK-T cells, and distinct intestinal intraepithelial lymphocyte populations (Kennedy et al., 2000; Lodolce et al., 1998).

It appeared thus possible that the NK development in the transgenic mice could also be altered, since a dramatic increase in IL-2R $\alpha$  (CD25) in the thymus of these animals might interfere with signaling through the IL-15R, which also uses the IL-2-R $\beta$  chain (CD122). The number of NK cells in the thymus of *lck-OBF-1* mice was indeed ~3-fold higher, constituting approximately 2.8% of the thymic lymphocytes, compared to 0.9% in the WT animals. This increase does not seem to be caused by one specific subset of NK or NKT cells, since all NK1.1<sup>+</sup> cell populations differing in the expression of the pan-T cell marker CD3 were affected similarly (Fig. 23).



**Figure 23** Increased number of NK1.1<sup>+</sup> cells in the thymus of *lck-OBF-1* transgenic mice. Thymocytes from 6-9 weeks-old mice were isolated and analyzed by FACS with FITC-labelled anti-NK1.1 and PE-conjugated anti-CD3 or anti-c-kit antibodies. The total number of NK1.1<sup>+</sup> cells was increased ~3-fold among the *lck-OBF-1* transgenic thymocytes. This increase was due to an increase in both NKT cells expressing high or intermediate levels of CD3, and in NK cells that do not express CD3 (CD3<sup>low</sup> cells). A part of these NK1.1<sup>+</sup> cells was also c-kit<sup>+</sup>. The Figure shows the results from cells of 3 mice of each genotype and the data are presented  $\pm$  SD.

In contrast to the thymus, the numbers of NK and NKT cells in the spleen were normal (Fig. 24).



**Figure 24** Normal ratio of T cells (first column), NKT cells (second column) and NK cells (last column) in the spleen of *lck-OBF-1* transgenic mice. The Figure shows the results from cells of 3 mice of each genotype and the data are presented  $\pm$  SD.

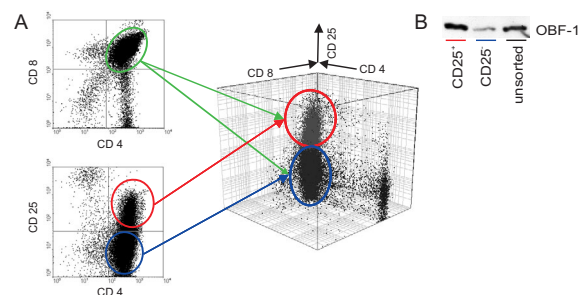
## 2.2.4 Microarray experiments

We have seen previously that OBF-1 overexpression in the T cell compartment leads to very specific effects on a number of minor T cell subsets, but does not overtly perturb normal T cell development. Because overall T cell development and total T cell numbers were largely unchanged by the expression of OBF-1 we decided to use thymocytes RNA for microarray experiments, in order to identify potential OBF-1 target genes. To this end two independent comparisons were performed: (i) transgenic vs WT total thymocytes, and (ii) transgenic thymocytes, CD4<sup>+</sup>CD8<sup>+</sup>CD25<sup>+</sup> vs CD4<sup>+</sup>CD8<sup>+</sup>CD25<sup>-</sup> cells (see Figure 18).

Microarray data complying with MIAME standards can be found in electronic form on the web at the public ArrayExpress database at <http://www.ebi.ac.uk/arrayexpress>. A table listing the genes that were differentially regulated in the same manner in all microarray experiments is listed in Appendix (Section 6.1).

The most interesting candidates that we identified by the microarray experiments comparing WT to *lck-OBF-1* total thymic RNA are listed in Table IV. In the present context, “interesting” genes are preferentially fulfilling the following criteria: (i) they are upregulated (or downregulated) in the transgene tissue, and, respectively, downregulated (or upregulated) in *OBF-1* deficient tissue, (ii) they are usually expressed in lymphocytes, just like OBF-1, and (iii) they contain an octamer site in their gene regulatory regions to which OBF-1 can bind.

The genes listed in Table IV were those genes that were upregulated and that contained certain common motifs in their promoter region. Besides the octamer motif that was identified in 18 of 36 genes (50%), other motifs appeared several times in this analysis. One such motif is a PU.1 binding site, thus also a putative Spi-B binding site. Another recurring motif is the combination of binding sites for ICSBP (IRF-8) and AR (androgen receptor), and another is the site for MLTF (Adenovirus Major Late Transcription Factor, USF1).



**Figure 25** FACS sorting of CD25<sup>high</sup> vs CD25<sup>low</sup> CD4/CD8 DP thymocytes. (A) Thymocytes from 15 *lck-OBF-1* transgenic mice were pooled and stained with FITC-labelled anti-CD4, PE-labelled anti-CD8 and APC-conjugated anti-CD25 antibodies. Subsequently, cells double-positive for CD4 and CD8 (green) were separated

## 2.2 *Lck-OBF-1* transgenic mice

in  $CD25^{high}$  (red) or  $CD25^{low}$  (blue) subsets using a MoFlo FACS sorter. RNA from the different fractions was purified and used for an Affymetrix GeneChip analysis. (B) Total protein was isolated in parallel from the corresponding cell populations and western blot analysis using a polyclonal antibody against *OBF-1* were used to measure the *OBF-1* protein levels in the sorted T cell subsets. Unsorted thymocytes were included as a control.

**Table IV** Genes upregulated in *lck-OBF-1* transgenic mice and recurring transcription factor binding sites in the gene promoter region. The table refers to motifs found in the promoters of human orthologs.

n.d.: not determined; no data for the promoter was available for the analysis.

/: not found; a sequence resembling the consensus binding sequence of the respective transcription factor was not detected in the gene promoter region.

Gene	Oct-1	PU.1	AR/ ICSBP	MLTF
Spi-B	perfect	no	yes	no
CD25	tTGCAAAT	yes	yes	yes
RW1	/	no	no	yes
FCRIIb	ATGCAAT	yes	yes	no
AW548096 (C/EBP-induced)	n.d.	n.d.	n.d.	n.d.
TCR gamma 2/4	/	yes	yes	no
Hcph	perfect	yes	no	yes
STAT4	perfect	no	yes	yes
CD44	perfect	yes	no	yes
S100A8	/	yes	no	yes
Syp/PLCgamma2	/	no	yes	yes
Atp2a3 (Serca)	n.d.	n.d.	n.d.	n.d.
AA409502	n.d.	n.d.	n.d.	n.d.
DKFZP5643116	n.d.	n.d.	n.d.	n.d.
ODC	ATGCAATa	yes	yes	yes
160122_at	ATGC AAAA A	yes	yes	yes
Tcf4	n.d.	n.d.	n.d.	n.d.
Myla	/	yes	yes	yes
Ly6D	perfect	no	no	yes
DC12 ortholog	n.d.	n.d.		
160820_at	ATGCAAAG	yes	yes	yes
Gadd45beta	ATGC AAAa T	yes	yes	yes
Mef2b	/	yes	yes	no
Crip	n.d.	n.d.	n.d.	n.d.
XBP-1	/	no	yes	yes
Akap12	ATGCAAgc	no	no	no
96158_at	ATGCAAt, ATGCAtt,AT GtAAAT, ATGaAAAT	yes	yes	no
CIKS (96221_at)	ATGC AAAc, cTGCAAAT	yes	yes	yes
Egln2	perfect	no	yes	yes
Notch1	perfect	yes	no	no
Hip1R (97551_at)	/	yes	yes	yes
97853_at	ATGCAAt	yes	yes	yes
Zfpn/FOG	/	yes	yes	yes
Casp7	perfect	yes	yes	yes
99366_at	n.d.	n.d.	n.d.	n.d.
lbp2	/	yes	no	yes

## 2.3 E $\mu$ -V<sub>H</sub>-OBF-1 transgenic mice

### 2.3.1 Generation of E $\mu$ -V<sub>H</sub>-OBF-1 transgenic mice

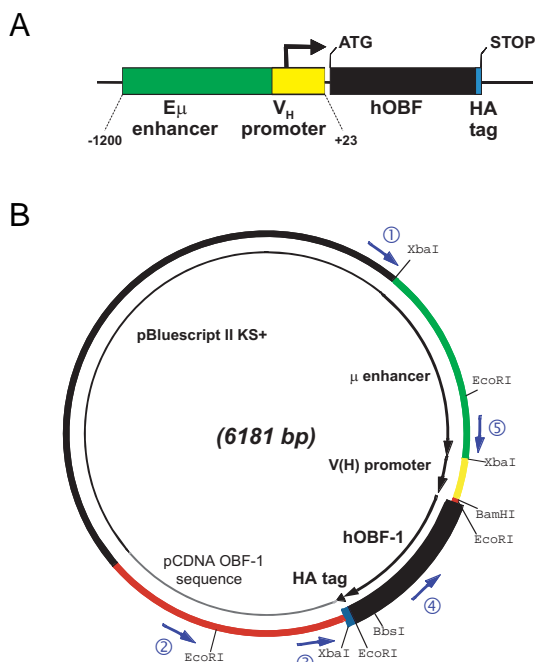
In order to learn more about the function of OBF-1 in B cells, we generated another line of transgenic mice overexpressing human HA-tagged OBF-1. We made use of promoter/enhancer elements that were characterized previously to confer B cell-specific expression (Banerji et al., 1983; Dreyfus et al., 1987; Gillies et al., 1983; Grosschedl and Baltimore, 1985; Mason et al., 1985), starting early in B cell ontogenesis: the V heavy-chain promoter and the  $\mu$  enhancer.

The expression cassette (depicted in Figure 26) was cloned into pBluescript II KS+ from previously described plasmids containing the V<sub>H</sub> promoter (plasmid S-19), the  $\mu$  Enhancer (plasmid 1-27), and the human OBF-1 cDNA with an additional C-terminal HA tag (plasmids pCDNA OBF-1, pEV OBF-1/9HA). The sequence of the B cell-specific construct (E $\mu$ -V<sub>H</sub>-OBF-1 vector) is available online in digital form at <http://www.fmi.ch/groups/matthias.p/Bartholdy.html>. A plasmid map featuring essential restriction sites is shown in Figure 26 B.

### 2.3.2 Genotyping of transgenic mouse lines and expression analysis

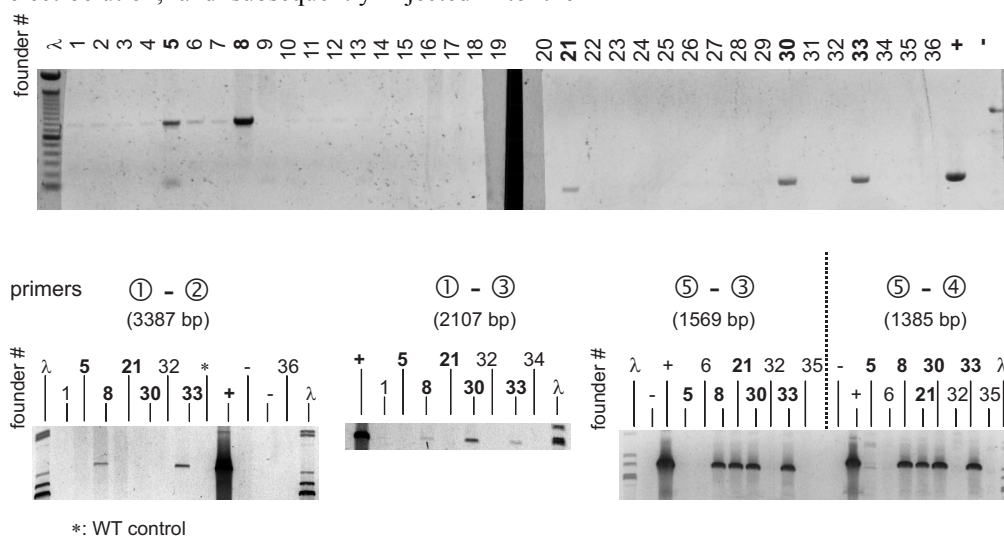
#### 2.3.2.1 Identification of transgenic founders

The construct was cloned, excised from pBluescript II KS+ by SfiI digestion, separated by agarose gel electrophoresis followed by electroelution, and subsequently injected into the



**Figure 26** E $\mu$ -V<sub>H</sub>-OBF-1 construct used to generate transgenic mice constitutively expressing HA-tagged hOBF-1 in a B cell-specific manner. **A.** Structure of the construct used for generation of transgenic mice. **B.** Plasmid map of the vector containing the E $\mu$ -V<sub>H</sub>-OBF-1 construct. The location of primers (① through ⑤) used for PCR analysis is indicated. The sequence of these primers can be found in Section 4.

male pronuclei of mice. Transgenic founders were screened by PCR and Southern blot analysis. Figure 27 shows the results of this screening.

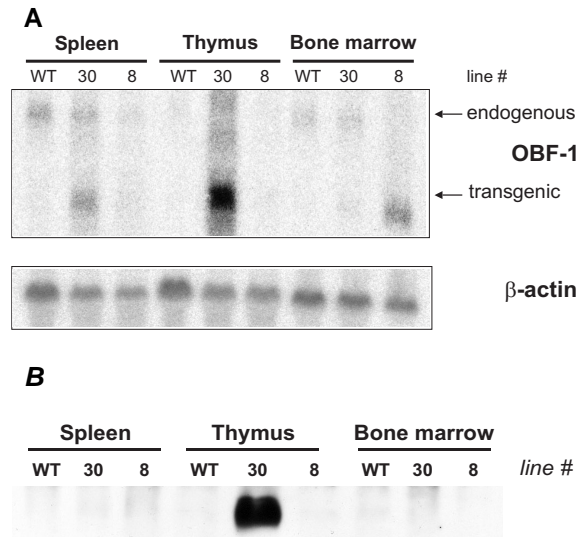


**Figure 27** PCR analysis of tail DNA from E $\mu$ -V<sub>H</sub>-OBF-1 transgenic founders. Founders that gave positive PCR reactions indicating the presence of the transgene are marked in bold. Additional PCR reactions with the primers ① through ⑤ were performed in the indicated combinations (below) to verify full-length integration of the transgene.

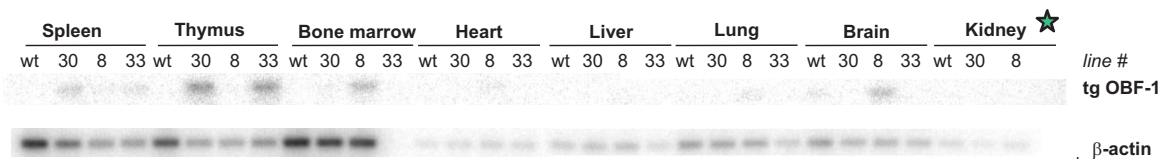
2.3.2.2  $E\mu-V_H$ -*OBF-1* expression in the transgenic mouse lines

Expression of the transgenic OBF-1 was determined by western blot, northern blot and RT-PCR/SB techniques (Figures 28 and 29). Three mouse lines had integrated the full length transgene into their genome and were further characterized for OBF-1 expression: the lines #8, #30 and #33. The transgenic mice were viable and had a normal appearance, although line #30 showed at first a reduced rate of transgenic founders compared to the expected mendelian ratio. After embryo transfer, however, this changed to a normal distribution.

Transgene mRNA was detected in the spleen, in the bone marrow, but – at highest levels – in the thymus of the  $E\mu-V_H$ -*OBF-1* mice of the lines #8, #30 and #33. The high expression in the thymus and relatively low expression in the B cell compartment was unexpected, although activation of similar V heavy-chain promoter- $\mu$  enhancer constructs was reported previously (Wasylyk and Wasylyk, 1986). However, the protein expression pattern reflected the mRNA expression; HA-tagged OBF-1 protein was only detected in the thymus of the  $E\mu-V_H$ -*OBF-1* mice (Fig. 29). The construct is thus expressed specifically in lymphoid cells, not only in B cells, but to an even greater extent in T cells.



**Figure 29** mRNA and protein expression of two  $E\mu-V_H$ -*OBF-1* transgenic mouse lines. (A) mRNA expression of *Ick-OBF-1* and endogenous *OBF-1* in lymphoid tissue (northern blot). A full length *OBF-1* probe was used, which detects both endogenous (upper band) and transgenic (tg) *OBF-1* mRNA (lower band).  $\beta$ -actin was used as a loading control. (B) Expression of the western blot analysis was performed using the monoclonal HA-antibody 12CA5.



★: Kidney RNA was taken from a mouse of line #8 that had one inflamed kidney (first RNA on the blot) and one normal looking one (second RNA on the blot). Thus, no *OBF-1* expression was observed in either of the two kidneys.

**Figure 28** RT-PCR-Southern Blot analysis of  $E\mu-V_H$ -*OBF-1* mRNA expression. A probe specific for the transgenic *OBF-1* (tg *OBF-1*) was used to monitor the expression of the transgene in the indicated mouse tissues.  $\beta$ -actin was used as loading control.



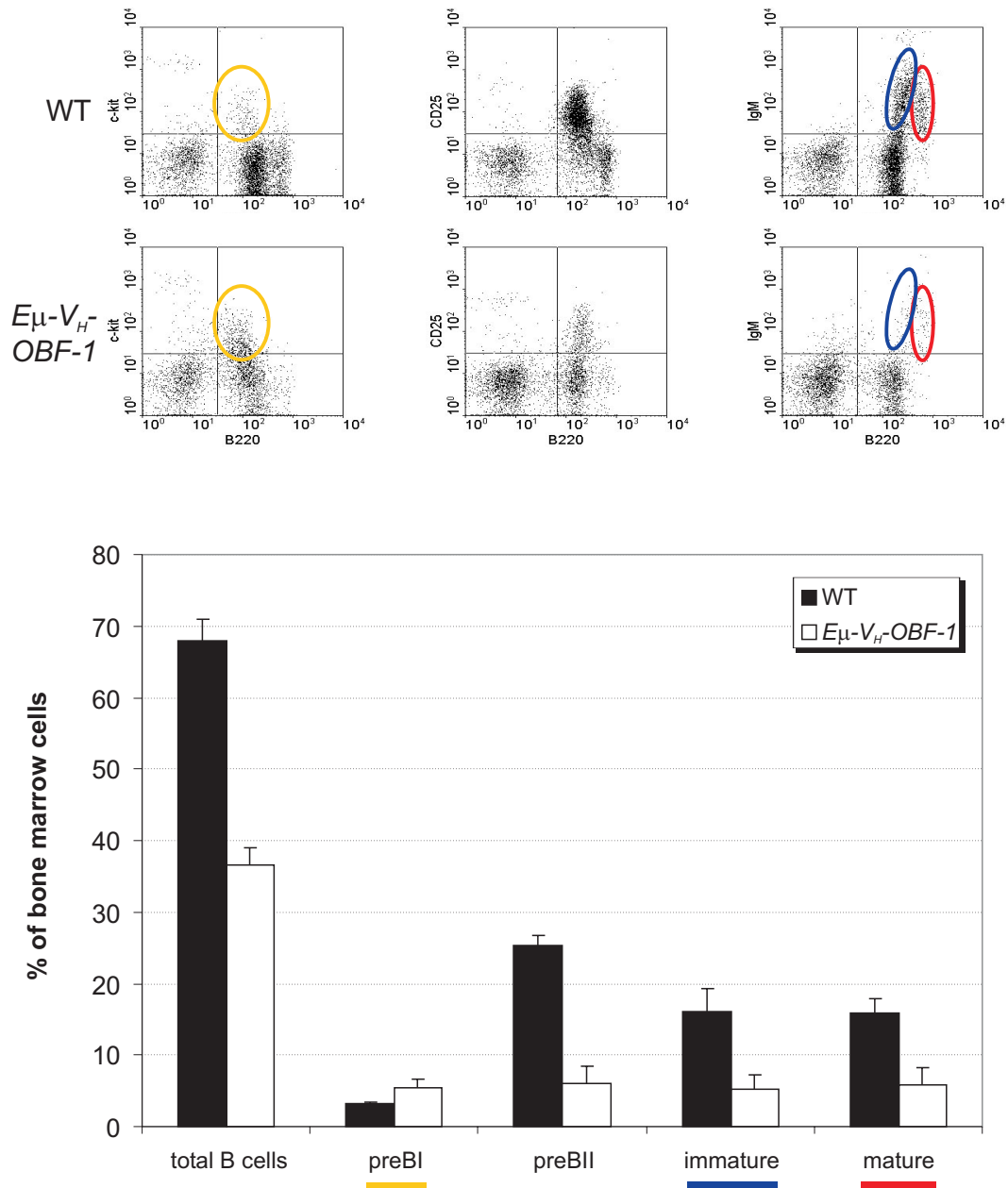
### 2.3.3 FACS analysis

#### 2.3.3.1 Early block in B cell development in the bone marrow

The cellular composition of the B cell compartment of each mouse line was determined by flow cytometry and using monoclonal antibodies directed against various B and T cell surface molecules. These include anti-B220, anti-c-Kit, anti-CD19, anti-CD25, anti-IgM, anti-IgD, 493, anti-CD90, anti-CD4, and anti-CD8. For a description of the antigens recognized by these antibodies, see Table V. In brief, the expression of specific

combinations of cell surface molecules can be monitored by fluorescence-labeled antibodies and allows the distinction of cells from different developmental stages or different cell lineages.

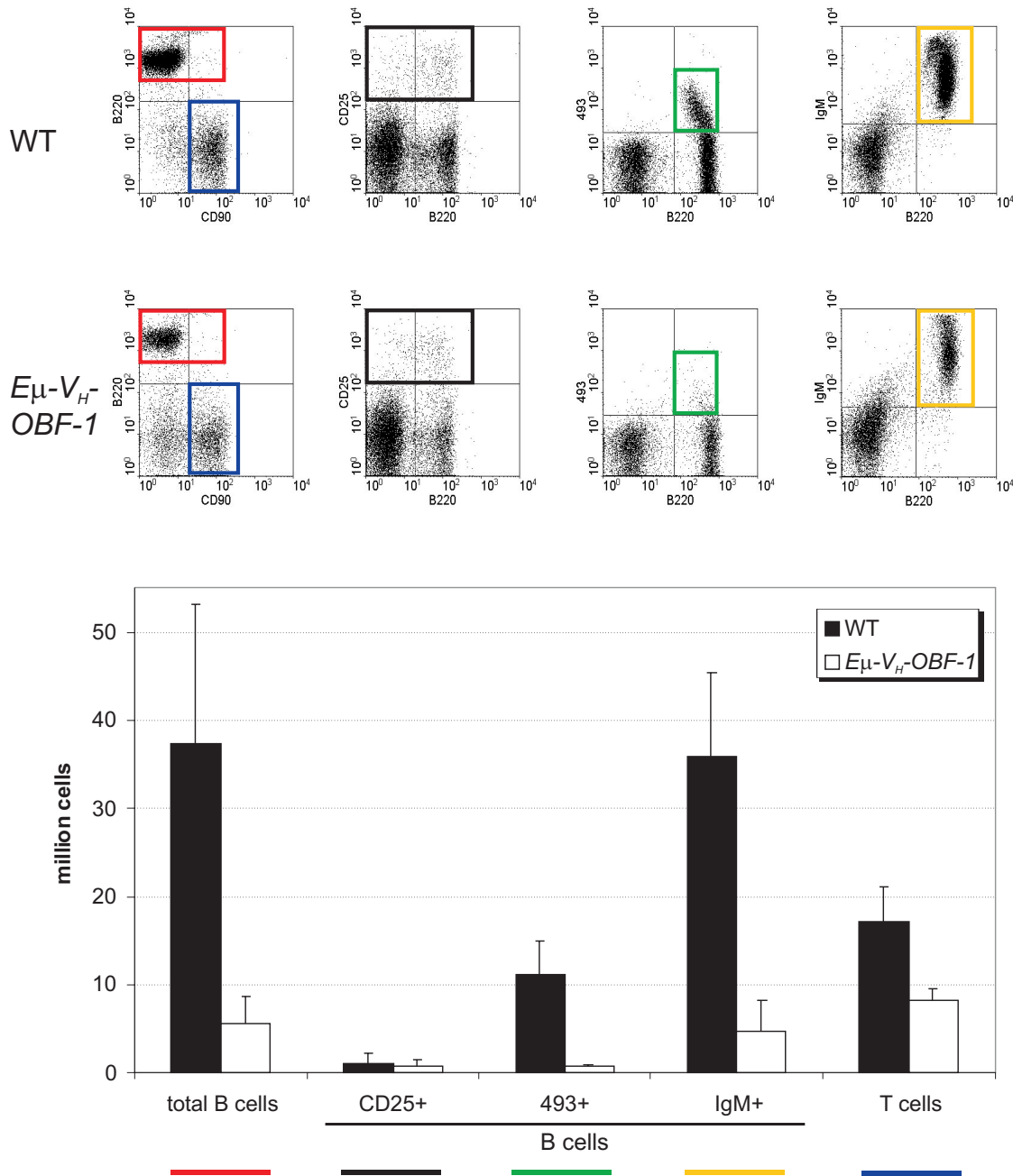
$E\mu-V_H\text{-}OBF\text{-}1$  mice of the lines #8 and #33 showed no gross perturbations of their lymphoid compartment, but line #30 had a clearly reduced number of B cells in the lymphoid organs. Figure 30 shows the strongly impaired B cell development in the bone marrow of the transgenic mice from line #30. We focused on line #30 for further analysis of the B cell compartment, since we observed the most pronounced effect on B cell development in this line.



**Figure 30** FACS analysis of the lymphoid compartment of the bone marrow of  $E\mu-V_H\text{-}OBF\text{-}1$  mice (line #30). Distinct developmental stages of B cell development are distinguished by specific cell surface staining with fluorescent antibodies shown in the upper part of the figure. A quantification of these cell populations is presented in the graph below. The histograms represent the mean  $\pm$  SD of four mice from two independent experiments. Cells from distinct B cell stages are encircled in colors in the upper part of the figure, and the same colors were used to highlight these populations in the quantification. PreBII cells consist of the subgroup of  $B220^+ CD25^+$  cells that do not express IgM; they cannot be directly highlighted as one population in the upper graphs, therefore there is no color code attributed to this population.

## 2.3.3.2 Strongly reduced splenic B cell compartment

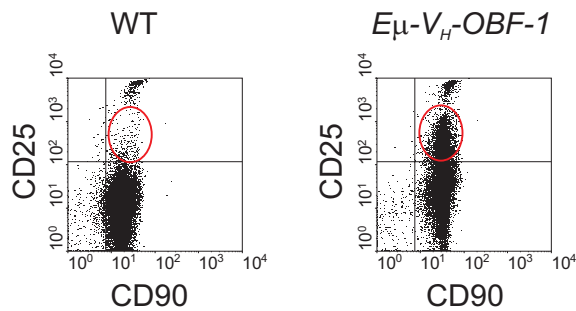
As shown in Figure 31, the reduction of B cells in  $E\mu-V_H$ -*OBF-1* mice of line #30, which first occurs in the bone marrow, is propagated to the spleen, where it is even more pronounced with a remaining amount of approximately 12-13% of the normal B cell numbers. The T cell numbers are also slightly reduced.



**Figure 31** FACS analysis of  $E\mu-V_H$ -*OBF-1* mice (line #30). Cellular composition of the spleen. Distinct developmental stages of B cell development are distinguished by specific cell surface staining with fluorescent antibodies shown in the upper part of the figure. A quantification of these cell populations (color coded) is presented in the graph below. The histograms represent the mean  $\pm$  SD of two independent experiments (five mice per genotype).

### 2.3.3.3 Abnormal CD25<sup>+</sup> cells in the thymus of *E $\mu$ -V<sub>H</sub>-OBF-1* mice

The western blot expression analysis (Fig. 29) already showed a strong expression of the transgenic OBF-1 in the thymus of the mice from line #30, similar to the *lck-OBF-1* mice. To find out whether this expression has similar effects in both transgenic mouse lines, we also performed FACS analysis of the thymus of *E $\mu$ -V<sub>H</sub>-OBF-1* mice. As for the *lck-OBF-1* mice, there was the appearance of CD25<sup>+</sup> CD4<sup>+</sup> CD8<sup>+</sup> cells in the thymus (Fig. 32). However, the other abnormal cell populations, such as  $\gamma\delta$  TCR<sup>+</sup> and NK1.1<sup>+</sup> cells, but also CD4<sup>+</sup> CD8<sup>+</sup> cells in the spleen did not appear in the *E $\mu$ -V<sub>H</sub>-OBF-1* mice.



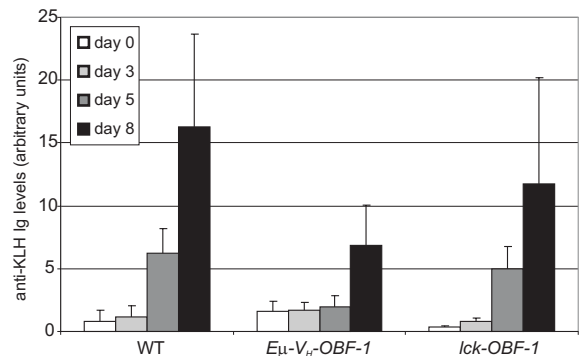
**Figure 32** Appearance of a CD25<sup>+</sup> CD4<sup>+</sup> CD8<sup>+</sup> T cell population in the thymus of *E $\mu$ -V<sub>H</sub>-OBF-1* transgenic mice (circled in red).

It has to be stressed at this point that this project is still at a preliminary state, given that most results were obtained exclusively the line #30 of *E $\mu$ -V<sub>H</sub>-OBF-1* mice. Therefore, one has to be careful about the interpretation of the results. We observed some variation of the phenotype, which could be due to different copy numbers and sites of integration, ultimately leading to variations in the transgene expression level. For instance, mice from lines #30 and #33, showed increased numbers of CD25-expressing thymocytes, similar to the *lck-OBF-1* mice. Other lines, such as the lines #8, #21, #487 and #543 did not show such an obvious increase in this cell type. However, mice from the lines #543 and #487 also have a reduced number of B cells in the spleen and in the bone marrow, but the effect is less severe than in mice from line #30. We still need to carefully analyze the transgene expression in other mouse lines to assure that the observed phenotype in the line #30 is fully attributable to OBF-1 expression in B cells.

## 2.4 Functional assays of both transgenic mouse lines

### 2.4.1 Immunization with a TD antigen

To assess the status of the immune system of both *lck-OBF-1* and *E $\mu$ -V<sub>H</sub>-OBF-1* transgenic mice, these animals were immunized intraperitoneally with a T cell dependent antigen (DNP-KLH) and the immunoglobulin levels in the blood were measured at day 0, 3, 5 and 8 of immunization by ELISA. The results are depicted in Figure 33. The *lck-OBF-1* mice show a normal response to DNP-KLH, whereas the *E $\mu$ -V<sub>H</sub>-OBF-1* mice only respond weakly to the antigen stimulation. This is most probably due to the reduced number of B cells in the spleen of these mice.

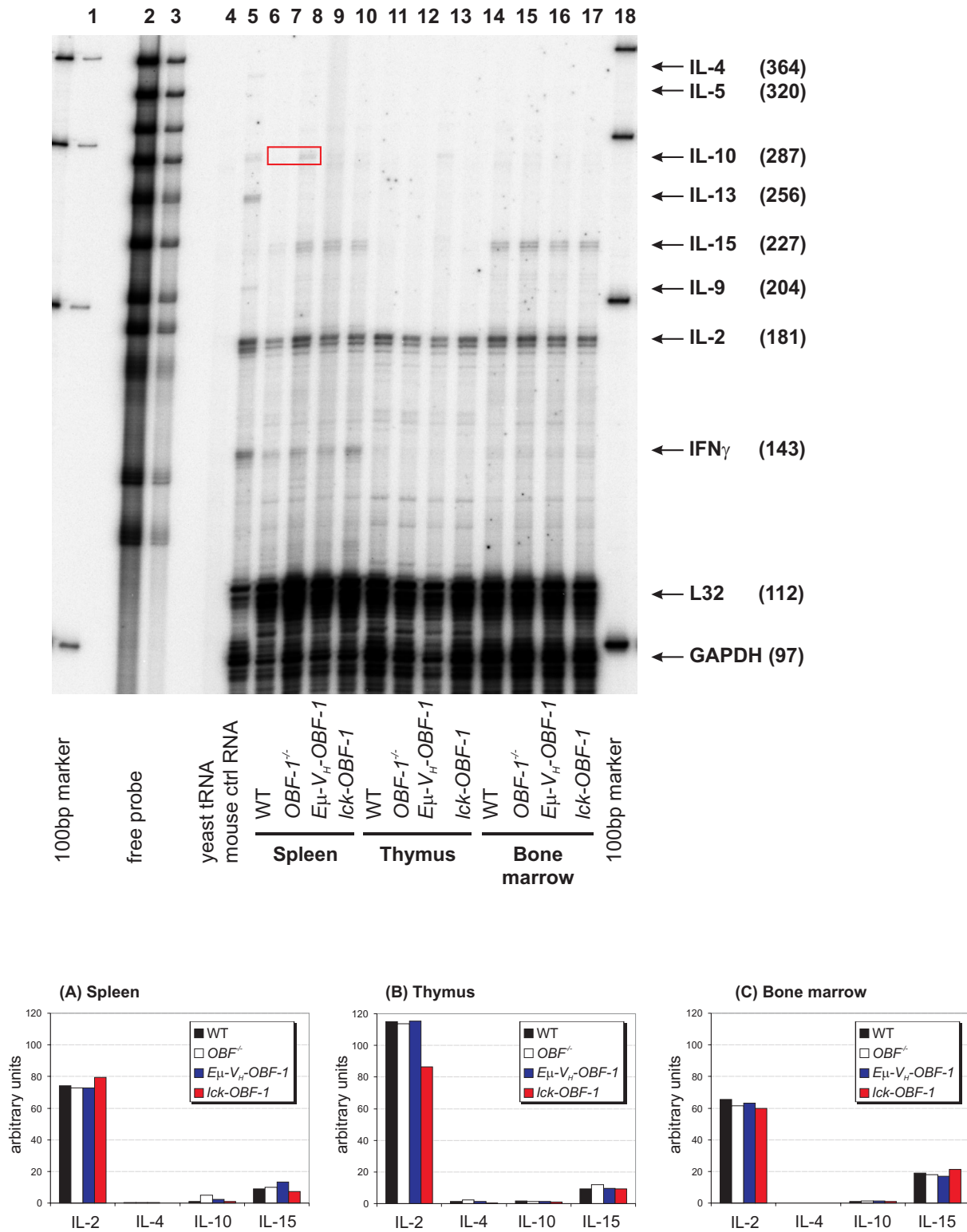


**Figure 33** Immune response against a TD antigen (DNP-KLH). WT and both *E $\mu$ -V<sub>H</sub>-OBF-1* and *lck-OBF-1* transgenic mice were immunized intraperitoneally and total Ig levels in the blood were measured at day 0, 3, 5 and 8 of immunization. The histograms represent the mean  $\pm$  SD of three mice per genotype.

### 2.4.2 Cytokine expression analysis

In addition, the cytokine profile of the transgenic animals was investigated by a multiprobe RNase protection assay. This assay helps to see differences in the level of a number of cytokines, including the interleukins IL-2, IL-4, IL-5, IL-9, IL-10, IL-13, IL-15, and interferon gamma (IFN $\gamma$ ), in the lymphoid organs. We also included RNA from WT and OBF-1 deficient mice in our assay for comparison. As shown in Figure 34, IL-2 levels were normal in all mice, showing that the upregulation of CD25 is not brought about by increased levels of IL-2.

The most striking difference in chemokine levels was the increased level of IL-10 mRNA in OBF-1 deficient spleen. This result was confirmed in a second RNase protection experiment (not shown).



**Figure 34** Cytokine profile of the lymphoid organs of WT, *OBF-1* deficient and *OBF-1* transgenic ( $E\mu-V_H$ -*OBF-1* and *lck-OBF-1*) mice. The raw data from the RNase protection is shown in the upper part of the figure. The graphs below show a quantification by phosphorimaging and normalization to the expression level of GAPDH. The only clear difference of cytokine levels observed was an increase in IL-10 levels in *OBF-1*<sup>-/-</sup> compared to WT splenic RNA (highlighted in red in the upper part of the figure).

## 2.5 Review

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Review

## Transcriptional control of B cell development and function

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**Abstract**

The generation, development, maturation and selection of mammalian B lymphocytes is a complex process that is initiated in the embryo and proceeds throughout life to provide the organism an essential part of the immune system it requires to cope with pathogens. Transcriptional regulation of this highly complex series of events is a major control mechanism, although control is also exerted on all other layers, including splicing, translation and protein stability. This review summarizes our current understanding of transcriptional control of the well-studied murine B cell development, which bears strong similarity to its human counterpart. Animal and cell models with loss of function (gene “knock outs”) or gain of function (often transgenes) have significantly contributed to our knowledge about the role of specific transcription factors during B lymphopoiesis. In particular, a large number of different transcriptional regulators have been linked to distinct stages of the life of B lymphocytes such as: differentiation in the bone marrow, migration to the peripheral organs and antigen-induced activation.

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*Keywords:* Transcriptional; B cell; Development**1. Introduction**

The development of B lymphocytes from pluripotent progenitors is a tightly regulated process that originates in the fetal liver and in the adult bone marrow and further proceeds in the secondary lymphoid organs. Commitment to the B cell lineage and subsequent rearrangement of the immunoglobulin (Ig) heavy chain gene is followed by expression of the pre-B cell receptor (pre-BCR), which is composed of the heavy chain and the surrogate light chains

$\lambda 5$  and VpreB. Signaling through the pre-BCR leads to a transient phase of cellular proliferation, and triggers the transition from early pre-B cells to late small pre-B cells. Recombination of the  $\kappa$  and  $\lambda$  light chain loci is initiated at the pre-B cell stage. Successful pairing of one of the light chains with the heavy chain results in surface expression of IgM, the hallmark of immature B cells. IgM-expressing cells then exit the bone marrow and complete their maturation in the spleen. Antigen encounter then leads to activation of mature B cells and their differentiation into memory B cells or antibody-secreting plasma cells (PCs), the final mediators of the humoral immune response.

The entire developmental program of B lymphopoiesis is tightly regulated, and one of the most important regulatory mechanisms is the transcriptional control executed by a large number of cell-specific or ubiquitous factors that drive cells into the B lineage, lead to their selection and differentiation or to cell death, or maintain them in a defined differentiation stage. Gene targeting studies revealed that several transcription factors are essential for early B cell development: Their absence leads to a block in differentiation primarily at either of two stages: (i) at the initiation of B lineage commitment and (ii) at the onset of  $V_H D J_H$  recombination (see Fig. 1 and Table 1). Successful initiation of immunoglobulin gene rearrangement is thus a critical checkpoint in B lymphopoi-

*Abbreviations:* Ig, immunoglobulin; BCR, B cell receptor; TCR, T cell receptor; SC, stem cell; HSC, hematopoietic stem cell; CLP, common lymphoid progenitor; DC, dendritic cell; FDC, follicular dendritic cell; NK cell, natural killer cell; AFC, antibody-forming cell; IDC, interdigitating cell; PC, plasma cell; TdT, terminal deoxynucleotidyl transferase; bHLH, (basic) helix–loop–helix; CSR, class switch recombination; SHM, somatic hypermutation; M-CSF, macrophage colony-stimulating factor; IFN- $\gamma$ , interferon- $\gamma$ ; LPS, lipopolysaccharide; PALS, periarteriolar lymphoid sheath; MZ, marginal zone; GC, germinal center; TD, T cell-dependent; TI, T cell-independent; HDAC, histone deacetylase; HAT, histone acetyl transferase; LCR, locus control region; MHC, major histocompatibility class; MAR, matrix attachment region; UPR, unfolded protein response; ER, endoplasmic reticulum.

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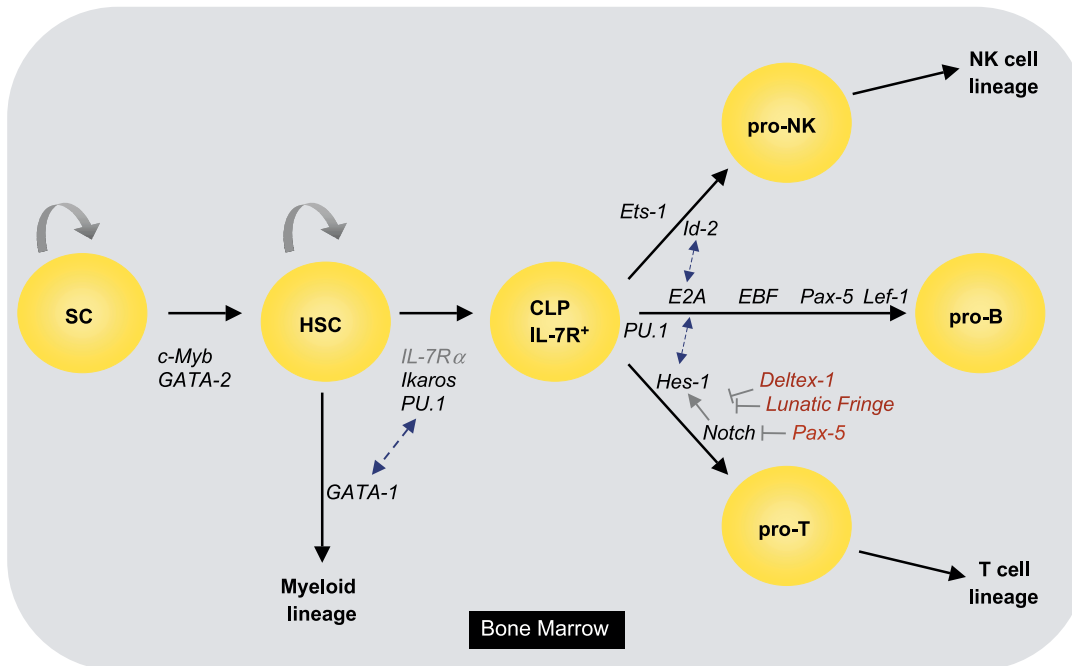


Fig. 1. Lineage commitment in the bone marrow. The position of the transcription factors on the scheme represents the stage at which their absence (based usually on knockout studies) leads to a developmental block. Proteins normally acting as inhibitors of the differentiation pathways are depicted in red. Physical interactions between transcription factors are indicated by double-headed blue arrows. SC, stem cell; HSC, hematopoietic stem cell; CLP, common lymphoid progenitor; NK, natural killer cell.

Table 1  
Genes controlling murine early B cell development

Developmental transitions	Pro-B to pre-B-I	Pre-B-I to large pre-B-II	Large pre-B-II to small pre-B-II	Small pre-B-II to immature B	Immature to mature B	Centrocyte to plasma cell
Hallmark events	D <sub>H</sub> - to J <sub>H</sub> -rearrangement	V <sub>H</sub> - to DJ <sub>H</sub> -rearrangement	V <sub>L</sub> - to J <sub>L</sub> -rearrangement	IgM surface expression	IgD surface expression	
Important transcription factors	<i>Sox-4, E2A, EBF, Pax-5</i>	<i>Pax5</i>	<i>IRF-4/IRF-8</i>	<i>OBF-1/Aiolos</i>	<i>OBF-1, Oct-2, NF-κB</i>	<i>Blimp-1, XBP-1, Aiolos (?)</i>
Other important genes	<i>RAG-1, -2, SCID, BLNK, J<sub>H</sub>, μ<sub>mb</sub>, Igβ, IL-7; IL-2Rβ</i>	<i>Syk, PI3K, λ5, VpreB1/VpreB2</i>	<i>DNA Polymerase Mu, Btk/Tec</i>	<i>κ/λ L chain</i>	<i>Btk</i>	

esis. Other factors have been found to be dispensable for early B cell development but to affect function of mature peripheral cells. In addition, sophisticated conditional gene targeting strategies have revealed that some factors execute their function at several discrete stages of B cell development and it remains to be seen how general this may be.

There is little doubt that the highly ordered combinatorial interplay of DNA-binding transcription factors controls the stepwise development of B cells that is accompanied by their sequential acquisition of novel properties (e.g., DNA rearrangement, BCR signaling, etc. . .). In many cases, however, the molecular explanations are still lacking, and the critically affected target genes that may ultimately explain the phenotypes of the mutant mice have not yet been identified unambiguously. Furthermore, at a mechanistic level, the mode of operation of the critical transcription factors has often not been dissected in their natural B cell context and there are certainly multiple ways how one and the same

transcription factor can regulate gene expression, depending on the cellular and/or developmental context.

Evidently, the regulation of gene expression is a multi-level process requiring at the minimum opening of the locus, binding of transcription factors to their target sequences in promoter and enhancers and recruitment of the basal transcription machinery. Each one of these steps can be rate-limiting and offers a possibility for regulation. In addition, superimposed, epigenetic mechanisms that regulate the accessibility of a locus and lead to local or global changes in chromatin structure are being increasingly examined and will be addressed briefly in the last section of this review. For the sake of clarity, this review is organized chronologically, following the principal stages of B cell differentiation: commitment to the B lineage, early B lymphocyte development in the bone marrow, homing to the secondary lymphoid organs, T cell-dependent (TD) responses (B cell activation and germinal center (GC) formation), T cell-

independent (TI) responses (marginal zone (MZ) and B1 B lymphocytes) and plasma cell differentiation.

## 2. B lineage commitment

An initial step in lymphopoiesis is the commitment of pluripotent hematopoietic progenitors to the lymphoid lineage, in other words the generation of common lymphoid progenitors (CLPs) from hematopoietic stem cells (HSCs). The IL-7R<sup>+</sup> common lymphoid progenitor (Kondo et al., 1997b) gives rise exclusively to lymphocytes (T, B and NK cells), although it can still be impelled to differentiate into the myeloid lineage by signaling through ectopically expressed IL-2 or GM-colony-stimulating factor (CSF) receptors. Transcriptional control of such cytokine receptors may therefore be an important step in the cell lineage decision, as pointed out by Kondo et al. (2000). Targeted mutation of the IL-7R $\alpha$  gene (Peschon et al., 1994; Maki et al., 1996), which is primarily expressed in the common lymphoid progenitors in fetal liver and bone marrow, in T lineage cells and in developing B cells (Sudo et al., 1993; Kondo et al., 1997a; Mebius et al., 2001), results in a severe early block in B and T cell development. In this context, ectopic expression of the anti-apoptotic gene bcl-2 partially restores  $\alpha\beta^+$  T cell, but not B cell development (Akashi et al., 1997; Kondo et al., 1997a; Maraskovsky et al., 1997). These findings suggest that IL-7 signaling is required not only for survival and proliferation of B lineage progenitors but also for their differentiation into B cells.

Recent reports revealed that lymphocytes and subsets of dendritic cells (DCs) originate from a common myelo-lymphoid progenitor (Lu et al., 2002); the loss of developmental potential to the DC lineage appears to be the final step in B-lineage commitment of the precursor cells (Izon et al., 2001). It is therefore tempting to suggest that expression of IL-7 could act as a regulator of cell fate decisions in multipotent B/dendritic cell precursors by driving these cells to differentiate into B lineage-committed cells (reviewed in Prohaska et al., 2002).

Given the fact that IL-7 signaling is so crucial for B cell development in mice, it is hardly surprising to note that this cytokine exhibits similar positive effects on proliferation and development of human B lineage cells in vitro (Uckun et al., 1991; Dadi et al., 1993). Yet, in vivo, the situation appears to be more complex, as evidenced from the impaired T cell development—but normal B cell development—observed in individuals with a defective IL-7 receptor gene (Fugmann et al., 1998; Puel et al., 1998; Puel and Leonard, 2000).

### 2.1. PU.1

Recently, the Ets family transcription factor PU.1 has been shown to directly regulate the expression of IL-7R $\alpha$  in lymphoid progenitors and thereby to play a pivotal role in the myeloid versus lymphoid lineage decision (DeKoter et

al., 2002). Low PU.1 expression levels promote the lymphoid versus myeloid cell development, whereas high levels suppress it (DeKoter and Singh, 2000). PU.1 null mice die during fetal development at day 18.5 of gestation and lack B, T, and myeloid progenitors. (Scott et al., 1994, 1997). *PU.1*<sup>-/-</sup> fetal livers also contain reduced numbers of multipotential lymphoid-myeloid progenitors (AA4.1<sup>+</sup>, Lin<sup>-</sup>). Furthermore, these mutant progenitors fail to proliferate and differentiate into pro-B cells in response to stromal contact and IL-7 (Scott et al., 1997). Retroviral transduction of IL-7R $\alpha$  into *PU.1*<sup>-/-</sup> progenitors restores their IL-7-dependent proliferation and, at low frequency, their differentiation into pro-B cells that express the mb-1, B29, VpreB,  $\lambda$ 5, RAG-1 and RAG-2 genes and undergo IgH D-J rearrangement. In addition to this critical role in early lymphoid development, PU.1, as well as other Ets family members, has also been reported to be crucial at later stages of B cell development; this will be discussed within the context of these stages.

### 2.2. GATA factors

The transcriptional activity of PU.1 can be modulated by its interaction with other hematopoietic regulators. Such an interaction was reported between GATA proteins and PU.1 (Zhang et al., 1999). GATA family members are transcription factors that contain a two-zinc finger domain and share a consensus binding sequence-WGATAR-. Interaction of GATA-1 with PU.1 leads to repression of PU.1-dependent transcription (Nerlov et al., 2000).

PU.1 in turn inhibits the in vitro or in vivo acetylation of GATA-1 by CREB-binding protein (CBP) (Hong et al., 2002) and can negatively regulate expression of the GATA-2 gene, thereby promoting distinct myeloid cell fates (Walsh et al., 2002). A similar mechanism could operate in the lymphoid compartment, where PU.1 and GATA-3 are exclusively expressed and where they are required for the development of B cells and T cells (Scott et al., 1994; McKercher et al., 1996; Ting et al., 1996). Both factors might thus play a pivotal role in the B- versus T-lymphoid decision (Nerlov et al., 2000). Another GATA family member, GATA-2, is essential at the earliest stages of hematopoiesis, since mice with a targeted deletion of this gene fail to generate a sufficient number of primitive erythroid cells and die at day 10–11 of gestation (Tsai et al., 1994).

The PU.1–GATA interactions can serve as examples of negative regulatory interactions between hematopoietic transcription factors. Other examples include the inhibition of Ets-1 on erythroid promoters by MafB5 and the inhibition of GATA-1 transactivation by Friend of GATA-1 (FOG)-mediated recruitment of the CtBP corepressor (Fox et al., 1999).

### 2.3. Ikaros

The Ikaros gene encodes a number of Krüppel-type zinc finger proteins, generated by alternative splicing, which act as lymphoid-specific transcription factors required at an

early stage of lymphopoiesis. In the absence of Ikaros, lineage restriction of multipotent hematopoietic progenitors towards the lymphoid pathways is severely affected. Ikaros-deficient mice lack all B, natural killer (NK) and fetal T cells as well as the earliest described lymphoid progenitors (Wang et al., 1996). Like several other transcription factors, Ikaros was shown to act either as a repressor or a potentiator of gene transcription depending on the context.

In lymphocytes, Ikaros exists as an integral component of chromatin-remodeling complexes, including the Mi-2 $\beta$ /nucleosome remodeling and deacetylation (NuRD) complex. In line with this, Ikaros, together with these associated activities, can cause repression of target genes. Recent reports showed that Ikaros is involved in repression of the  $\lambda 5$  gene in mature B cells and of the terminal deoxynucleotidyl transferase (TdT) gene in CD4<sup>+</sup> CD8<sup>+</sup> thymocytes (Koipally et al., 1999; Sabbattini et al., 2001; Trinh et al., 2001). In addition, several Ikaros family proteins, including Ikaros (Koipally and Georgopoulos, 2000), Eos and TRPS1 (Perdomo and Crossley, 2002), also utilize CtBP corepressors to inhibit gene expression. Conversely, Ikaros-containing chromatin remodeling complexes may also increase gene expression in cycling cells (Koipally et al., 2002). It has been shown that Ikaros is capable of enhancing gene expression as a potentiator of bona fide transcriptional activators rather than by functioning itself as a classical activator. Potentiation by Ikaros requires its intact DNA binding and dimerization domains, both of which are also necessary for its recruitment into a pericentromeric-heterochromatin-associated nuclear compartment in cycling cells (Cobb et al., 2000).

#### 2.4. *c-Myb*

Another transcription factor involved in very early cell fate decisions is *c-Myb*. Foetuses lacking *c-Myb* die at E15 as they fail to switch from embryonic yolk sac erythropoiesis (during which *c-Myb* is not expressed; Sitzmann et al., 1995) to adult hepatic erythropoiesis; a generalized defect in haemopoiesis is also apparent (Mucenski et al., 1991).

*c-Myb* cooperates with several other transcription factors to achieve transactivation of its target promoters. For example, it associates with the histone acetyl transferase (HAT) CBP/p300, which interacts with  $\nu$ -, *c*- and *A-Myb* via sequences encompassing the conserved central activation domain and acts as transcriptional coactivator (Dai et al., 1996; Oelgeschlager et al., 1996a; Facchinetti et al., 1997; Yamamoto et al., 2002). Other interaction partners include the CCAAT binding proteins (C/EBP) alpha and beta (Burk et al., 1993; Mink et al., 1996; Klempt et al., 1998), the Ets factors Ets-2 (Dudek et al., 1992) and PU.1 together with C/EBP $\alpha$  (Oelgeschlager et al., 1996b), the runt homology domain factor AML-1/Runx-1 (Zaiman and Lenz, 1996; Britos-Bray and Friedman, 1997), as well as GABP, a heteromeric transcription factor complex of the Ets family (Nuchprayoon et al., 1997). Interestingly, interaction between the hematopoietic Ets transcription factor Spi-B and

the coactivator CBP is associated with negative cross-talk with *c-Myb* (Yamamoto et al., 2002), revealing another part of this intricate network of transcriptional regulation of lymphopoiesis.

### 3. Antigen independent stages of B cell development

#### 3.1. Initiation of B lymphopoiesis in the bone marrow

Ig heavy chain gene rearrangement occurs at the pro-B cell stage where D<sub>H</sub> to J<sub>H</sub> joining in the early pro-B cells precedes V<sub>H</sub> to DJ<sub>H</sub> joining in the late pro-B cells. A productive VDJ<sub>H</sub> joining leads to the expression of the  $\mu$  chain, which is the hallmark of the pre-B cell stage. A fraction of the  $\mu$ H chain is expressed at the cell surface of large pre-B cells where it forms the pre-BCR by pairing with a surrogate light chain consisting of  $\lambda 5$  and VpreB, and with the signaling molecules Ig $\alpha$  (mb-1) and Ig $\beta$  (B29) (Fig. 2 and Table 1).

##### 3.1.1. *E2A*, *EBF* and *Pax5*

Three transcription factors have been identified by gene targeting to play crucial roles at the onset of B cell development: the basic helix-loop-helix (bHLH) proteins early B cell factor (EBF), E2A, and the paired domain factor B cell-specific activator protein (BSAP), also known as Pax5. Both E2A and EBF act upstream of Pax5 in the genetic hierarchy of B cell development (Bain et al., 1994; Lin and Grosschedl, 1995) and are equally expressed in Pax5<sup>-/-</sup> and wild-type pro-B cells (Nutt et al., 1997). Mutation of Pax5 is considered to dissociate the initial activation of B-lineage-specific gene expression by E2A and EBF from the Pax5-dependent control of B lineage commitment (Nutt et al., 1999). Inactivation of any of these three genes leads to a block in B lymphopoiesis before complete rearrangement of the IgH gene and only DJ segments are found to be recombined (Bain et al., 1994; Urbanek et al., 1994; Zhuang et al., 1994; Lin and Grosschedl, 1995; Nutt et al., 1997). Ectopic expression of E2A proteins in lymphoid cell lines leads to the upregulation of EBF, Pax5, VpreB and RAG1 (Kee and Murre, 1998). Forced expression of both E2A and EBF in hematopoietic precursor cells leads to the upregulation of an entire B cell differentiation program (O'Riordan and Grosschedl, 1999) including Pax5, the surrogate light chain genes  $\lambda 5$  and VpreB (Sigvardsson et al., 1997; Persson et al., 1998; Sigvardsson, 2000), the signaling molecules Ig $\alpha$  and Ig $\beta$  and the recombination-activating RAG1 and RAG2 genes (Fuller and Storb, 1997; Sigvardsson et al., 1997; Kee and Murre, 1998; Kishi et al., 2002; Maier et al., 2003). Remarkably, ectopic expression of E2A or EBF in concert with RAG1 or RAG2 in nonlymphoid cells is sufficient to promote Ig gene rearrangement and leads to the generation of a diverse repertoire of D<sub>H</sub>-J<sub>H</sub> recombinations (Romanow et al., 2000). However, as in Pax5-deficient B cells, no V-D<sub>H</sub>J<sub>H</sub> rearrangement takes place; thus, other lymphoid

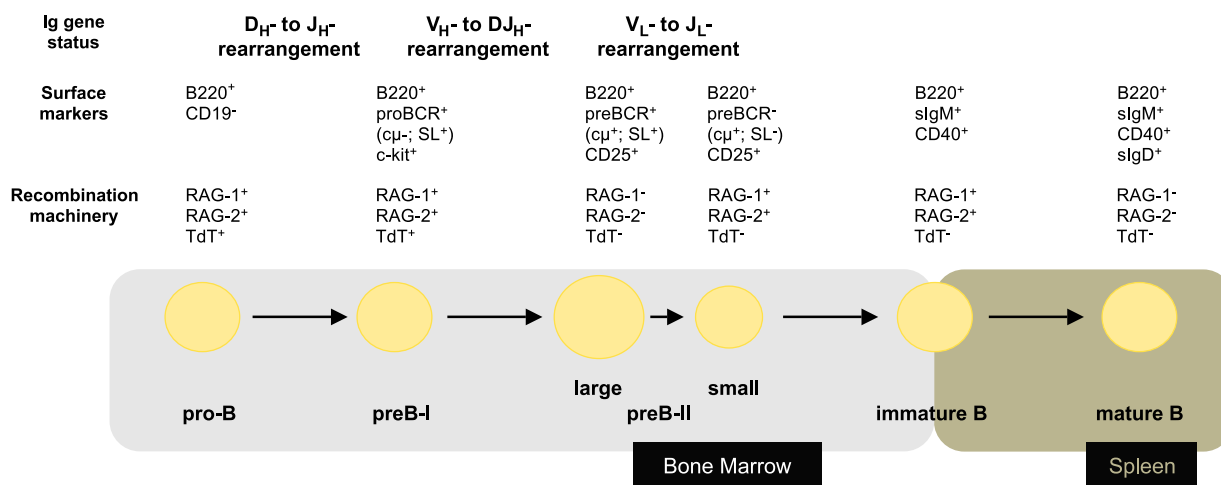


Fig. 2. Murine B cell development. Discrete stages of B cell development have been defined based on (a) cell cycle status and cell size, (b) the status of Ig heavy (H) and light (L) chain rearrangement and (c) the expression of specific molecules on the cell membrane or in the cytoplasm (especially factors of the rearrangement machinery (Rag-1,-2, TdT) and of the pre-BCR and BCR complex (IgH chain, (surrogate) IgL chain, Igα and Igβ chains). Some of the markers are only indicated where their expression/inexpression is required to identify a specific B cell subset. Immature B cells are depicted overlapping the bone marrow and the spleen because these cells are present in both organs.

factors are necessary to obtain a true immunoglobulin repertoire. In addition, other transcription factors involved in the regulation of the RAG gene expression are NF- $\kappa$ B, Ikaros, and c-Myb (Brown et al., 1997; Kishi et al., 2000, 2002).

### 3.1.2. Pax5

Pax5 directly acts as a transcriptional activator of several lymphoid genes such as CD19, N-myc, Igα and LEF-1 (Nutt et al., 1998). It has been shown to bind to the VpreB and lambda5 promoters as well as to the KI and KII sites upstream of the J kappa genes (Tian et al., 1997) and possibly contribute to their activation. In addition, Pax5 acts as a transcriptional repressor of lineage-inappropriate genes, including the M-CSF receptor gene, and thus commits the progenitor cells to the B-lymphoid lineage and maintains that commitment by suppressing alternative cell fates (Kee and Murre, 1998; Nutt et al., 1999; O’Riordan and Grosschedl, 1999; Busslinger et al., 2000; Mikkola et al., 2002). In line with this, Pax5-deficient pro-B cells maintain a broader developmental potential than wild type pro-B cells and can still differentiate in vitro into natural killer cells, dendritic cells, macrophages, osteoclasts and granulocytes (Nutt et al., 1999). In addition, in these Pax5-deficient pro-B cells—but not in wt pro-B cells—ectopic expression of C/EBPα and GATA factors induces a switch to the myeloid lineage (Heavey et al., 2003). Interaction with distinct proteins that act as corepressors (Groucho and perhaps also TBP, Rb and PTIP) or coactivators (Daxx; Emelyanov et al., 2002) is regarded as the key to the differential transcriptional activity of Pax5.

Intriguingly, the transcription factor PU.1 is a direct target of the Pax5-mediated repression and, inversely, PU.1 can inhibit Pax5 transactivation (Maitra and Atchison, 2000).

In transgenic mice, pan-hematopoietic Pax5 expression under control of the Ikaros locus strongly promotes B cell development at the expense of T lymphopoiesis. This is achieved by interfering with T lineage commitment and early thymocyte development through repression of the transcription of Notch1, a key regulator of T cell specification (Souabni et al., 2002).

### 3.1.3. Notch1 versus E2A

A regulatory network of repressors has emerged, which has a strong influence on cell lineage decision and might also be implicated in regulating “trans-differentiation”.

Notch signaling has first been identified as the crucial component of lateral inhibition in the nervous system and was implicated in the regulation of cell fate determination in various organisms ranging from nematode to man (reviewed in Guidos, 2002; Portin, 2002). Notch is a family of large (ca. 3000 aa) transmembrane proteins expressed at the surface of cells in the nervous and lymphoid systems. Activation of Notch by interaction with one of its ligands leads to cleavage (by  $\gamma$ -secretase) of the intracellular domain, which can then enter the cell nucleus where it binds to DNA in conjunction with CBF1/RBP-Jk and act as a bona fide transcription factor. Recently, Notch signaling was described as an early event in T cell commitment from a common lymphoid progenitor at the expense of B cell development: Overexpression of active Notch1 directs bone marrow stem cells to differentiate into immature T cells in the bone marrow and inhibits B cell development (Pui et al., 1999), whereas, inversely, deletion of Notch1 promotes B lymphopoiesis in the thymus (Wilson et al., 2001). These differential effects of Notch signaling appear to be mediated by distinct Notch ligands. Inhibition of Notch signaling by the Notch modifier



Lunatic Fringe leads to a phenotype similar to the inactivation of Notch1 (Koch et al., 2001). Similarly, enforced expression of Deltex1, another Notch modifier, in hematopoietic progenitors results in B cell development at the expense of T cell development in fetal thymic organ culture and in vivo, most likely by antagonizing Notch1 signaling via inhibition of coactivator recruitment (Izon et al., 2002). At the molecular level, Notch signaling induces expression of Hes1, a transcriptional repressor, which can heterodimerize with E47, a bHLH protein encoded by the E2A gene and antagonize its function in early B cell development (Sasai et al., 1992; Jarriault et al., 1995).

### 3.1.4. *Id* genes

While E2A proteins act as transcriptional activators, the *Id* transcription factors form another class of HLH proteins which lack the basic region essential for DNA binding and operate as inhibitory molecules. All known mammalian *Id* proteins, *Id*1 through *Id*4, can heterodimerize with E2A and other bHLH proteins, thereby inhibiting their DNA binding and consequently functioning as dominant negative antagonists of bHLH transcription factors (Benezra et al., 1990; Ellis et al., 1990; Christy et al., 1991; Sun et al., 1991; Riechmann et al., 1994). *Id* proteins are expressed in lymphoid tissues including fetal liver, bone marrow, spleen and thymus (Christy et al., 1991; Riechmann et al., 1994; Rivera et al., 2000; Ikawa et al., 2001). In vivo interactions between *Id* and E2A proteins (Deed et al., 1998), and also Pax5 have been described (Roberts et al., 2001).

While no obvious defect in the B cell differentiation pathway was noted in *Id*1-deficient mice (Yan et al., 1997), probably due to compensation by *Id*3, overexpression of *Id*1 in tissue culture models was found to antagonize E-protein-mediated activation of Ig enhancers (Pongubala and Atchison, 1991; Wilson et al., 1991). In addition, overexpression of *Id*1 in the B cells of transgenic mice was found to block B cell differentiation at the pro-B cell stage, a phenotype similar to that caused by E2A deficiency (Bain et al., 1994; Zhuang et al., 1994; Sun, 1994). Since *Id*1 expression in B cells ceases after the pro-B cell stage, whereas E2A gene products are present at all stages, it is tempting to speculate that *Id* proteins keep the existing E proteins inactive at the pro-B cell stage, while in subsequent stages when *Id* expression is turned off, E proteins are allowed to enhance the expression of their downstream targets required for further B cell differentiation (Jacobs et al., 1993; Pongubala and Atchison, 1991; Sun et al., 1991; Wilson et al., 1991). In agreement with this model, transgenic mice expressing *Id*1 or *Id*2 in T cells under the control of the *lck* proximal promoter show developmental defects similar to those described for E2A- and HEB-deficient mice (Kim et al., 1999; Morrow et al., 1999), while *Id*2-deficient mice have a severely impaired NK development (Yokota et al., 1999) and an increased E2A activity accompanied by an

abnormally high IgE class switch recombination (CSR) frequency in B cells (Sugai et al., 2003).

### 3.1.5. *Ets* factors

The *Ets* family of transcription factors encompasses over 30 members that are characterized by the presence of a conserved DNA-binding domain (the ETS-domain), a motif of 85 amino acids which forms a unique helix-loop-helix structure that binds purine-specific DNA sequences (Crepieux et al., 1994; Sharrocks, 2001). Tissue-specific knockouts have emphasized the key role of *Ets*-1 in T cell survival and for the production of NK cells (Bories et al., 1995; Muthusamy et al., 1995). Other *Ets* family members important for hematopoiesis include the aforementioned PU.1, and its closest relatives Spi-B and Spi-C, which will be discussed later in this review. *Ets* factors act as nuclear targets of signal transduction pathways and thereby usually collaborate with other proteins. For example, it was recently found that concomitant overexpression of Spi-B and its interaction partner PIP/IRF-4 in Abelson pre-B cells leads to DNA rearrangement and induction of I $\kappa$ B transcription (Muljo and Schlissel, 2002), thereby overcoming the differentiation block elicited by the Abl kinase. In addition, IRF-4, together with the related factor IRF-8, is required for Ig light-chain gene transcription and rearrangement. In mice deficient for both transcription factors, B cell development is arrested at the pre-B to B cell transition and the mutant cells fail to down regulate the pre-BCR (Lu et al., 2003).

### 3.1.6. *LEF-1* and *Wnt* signaling

The *Wnt* signal transduction pathway induces the nuclear translocation of membrane-bound beta-catenin and plays a key role in cell-fate determination. Recently, a regulatory function of *Wnt* signaling in B cell proliferation through lymphoid enhancer-binding factor-1 (*LEF-1*) has been identified (Reya et al., 2000). *LEF-1* is a member of a family of proteins containing an HMG-box as their DNA-binding domain. These include *LEF-1*, TCF-3, TCF-4 and the Sry-like transcription factor Sox-4, which is important for very early B cell differentiation. It was known previously that *LEF1*<sup>-/-</sup> mice have several developmental defects, including a defect in pro-B cell differentiation (van Genderen et al., 1994). Recently, it was shown that *LEF-1* binds and activates the RAG-2 promoter together with c-Myb and Pax-5 in immature B cells (Jin et al., 2002). Signaling through the BCR can regulate beta-catenin levels via a phospholipase C/protein kinase C/GSK-3 pathway (Christian et al., 2002), and *Wnt*/ $\beta$ -catenin signaling has been shown to be essential for the self-renewal of hematopoietic stem cells in vivo (Reya et al., 2003).

Sox-4 is highly expressed in the central nervous system, and to a lesser extent in heart, lung and thymus throughout embryogenesis. Mice deficient for Sox-4 die at embryonic day E14 due to cardiac malformation. In adult mice, Sox-4 acts as a crucial transcriptional activator in lymphocytes and



is expressed in bone marrow and thymus, but also in the gonads. In transfer experiments using *Sox-4*<sup>-/-</sup> fetal liver cells to reconstitute the lymphoid compartment of lethally irradiated recipients, B cell development was shown to be blocked almost completely at the pro-B cell stage (Schilham et al., 1996).

### 3.2. Homing to secondary lymphoid organs and cell adhesion

Immature IgM<sup>+</sup> IgD<sup>-</sup> B cells that previously have made successful in-frame rearrangements at the heavy and light chain locus and were positively selected, acquire the ability to exit the bone marrow and to home to the red pulp of the spleen. It has been estimated that only about 10% of the 15–20 million immature B cells generated each day in the mouse bone marrow emerge as mature B cells in the periphery. A considerable part of this loss is probably caused by the deletion of autoreactive B cells in the bone marrow. Additional loss could either be caused by an emigration defect from the bone marrow or a failure to properly home to the secondary lymphoid organs.

While this review focuses primarily on transcriptional control of B cell development, it is worth mentioning here the importance of several chemokines, cytokines and their receptors, the expression of which in turn is often controlled by lineage-specific transcription factors. A number of genes have been implicated in B cell motility and homing. Essentially, the cytokines lymphotoxins and tumor necrosis factor (Fu and Chaplin, 1999), interferon- $\gamma$  (IFN- $\gamma$ ) (Flaishon et al., 2000), as well as homeostatic chemokines, such as Exodus-2 (Hromas et al., 1997; Gunn et al., 1998), BCA-1 (Legler et al., 1998), SLC (Campbell et al., 1998; Nagira et al., 1998; Ngo et al., 1998; Gunn et al., 1999), MIP-3 $\beta$  (Kim et al., 1998), LARC (Tanaka et al., 1999) and chemokine receptors (Forster et al., 1996, 1999) were all shown to regulate lymphocyte trafficking and positioning in the subcompartments of lymphoid organs.

Most importantly, CCR7 and CXCR5 (BLR1) and their respective ligands, SLC or MIP-3 $\beta$  and BCA-1, are known to be essential for B cell homing (Forster et al., 1996, 1999; Cyster, 1999; Melchers et al., 1999; Ansel et al., 2000; Bowman et al., 2000). It appears that balanced responsiveness to chemoattractants from adjacent zones determines the B cell position in the follicle (Reif et al., 2002): Increased expression of CCR7 is sufficient to direct B cells to the T zone while overexpression of CXCR5 overcomes the antigen-induced B cell movement to the T zone. BCA-1 is also required for B1 cell homing to peritoneal and pleural cavities, natural antibody production, and body cavity immunity by the B1 lymphocytes (Ansel et al., 2002).

While the transcription factors regulating CCR7 expression are largely unknown, CXCR5 expression is controlled, at least in part, by Oct-2, OBF-1 and NF- $\kappa$ B (Wolf et al., 1998).

Additionally, the levels of the cytokine interferon- $\gamma$  secreted in autocrine fashion by B cells also modulate their homing and adhesion properties. IFN- $\gamma$  is transcribed and secreted by immature B cells in which it negatively regulates homing, but its transcription is drastically downregulated in mature B cells (Flaishon et al., 2000). Low levels of IFN- $\gamma$  down regulate the integrin-dependent adhesion of B cells by activating a pathway that interferes with cytoskeleton rearrangement (Flaishon et al., 2001). Thus, immature B cells could prevent premature encounter with antigen on their way to the spleen by downregulating their own migration to lymph nodes or inflammatory sites. Transcription factors regulating IFN- $\gamma$  transcription in T cells include CREB/ATF1, Jun/ATF2, NFAT, YY1, AP-1, NF- $\kappa$ B and GATA-3 (Campbell et al., 1996; Sica et al., 1997; Sweetser et al., 1998; Zhang et al., 1998; Ferber et al., 1999; Nakahira et al., 2002).

## 4. Antigen-dependent stages of B cell development

A number of transcription factors play important roles in late-stage B cell differentiation. The fact that the same factors can be important at several different developmental stages and can interact in various combinations on a particular gene promoter reflects a high regulatory complexity that is currently only partly understood. In fact, several different approaches using various techniques, including single or multiple gene knockouts, conditional knockouts, knock-ins, reconstitution studies and specific overexpression mediated by various promoters, have helped to clarify functions of factors such as Pax-5, E2A or NF- $\kappa$ B that would not have been revealed using one single approach. For example, B cells that lack E2A activity (which is normally induced during B cell activation and has been mentioned earlier) are blocked in their ability to express secondary Ig isotypes. This defect lies at the level of DNA rearrangements between the Ig switch regions suggesting that E2A is an essential target during B cell activation and its induction is required to promote Ig class switch recombination (Quong et al., 1999).

### 4.1. T cell-dependent antigen responses

Most antigens can only activate B cells to proliferate and differentiate into antibody-secreting cells in the presence of an additional signal from CD4<sup>+</sup> helper T (T<sub>H</sub>) cells. These T cell-dependent immune responses take place in a specialized microenvironment, the GCs (Fig. 3).

#### 4.1.1. B cell activation and germinal center formation

4.1.1.1. *Oct-1, Oct-2 and OBF-1.* B cell-specific expression and rearrangement of immunoglobulin genes is controlled by variable region promoters (V<sub>H</sub> and V<sub>L</sub>), intronic enhancers (E $\mu$  or E $\kappa$ ) and 3' enhancers, all of which are selectively active in B cells. The V region promoters appear

to be quite simple and contain a highly conserved octamer element (5' -ATGCAAAT-3', or its reverse complement), located upstream of a TATA box. Several *in vitro* and *in vivo* studies have demonstrated the crucial importance of the octamer site for the activity and B cell specificity of V region Ig promoters (Dreyfus *et al.*, 1987; Wirth *et al.*, 1987; Jenuwein and Grosschedl, 1991). The best studied transcription factors that interact specifically with the octamer motif in B cells are the POU homeodomain proteins Oct-1 and Oct-2. While Oct-1 is ubiquitous, Oct-2 is predominantly expressed in B cells, as well as in activated T cells and in the nervous system. Another factor, OBF-1 (also called OCA-B or Bob-1), is a proline-rich B cell-specific transcriptional coactivator that interacts with the POU domains of Oct-1 or Oct-2 and with DNA to form a ternary complex on a

subset of octamer sites. Because of their B cell-restricted expression pattern, Oct-2 and OBF-1 have been considered to be the principal candidates for mediating the activity of immunoglobulin promoters in B cells and initial cell transfection experiments have supported this postulate. Yet, individual inactivation of the Oct-2 or OBF-1 gene in mice failed to demonstrate an essential role for these factors in Ig transcription (Corcoran *et al.*, 1993; Kim *et al.*, 1996; Nielsen *et al.*, 1996; Schubart *et al.*, 1996). The analysis of Oct-2-deficient mice showed that this factor is required for B cell maturation and also, surprisingly, for postnatal survival (Corcoran *et al.*, 1993; Corcoran and Karvelas, 1994). In addition, Oct-2 was found to be essential for cell proliferation following lipopolysaccharide (LPS) stimulation and also for development of B1 B cells (Humbert and Corcoran,

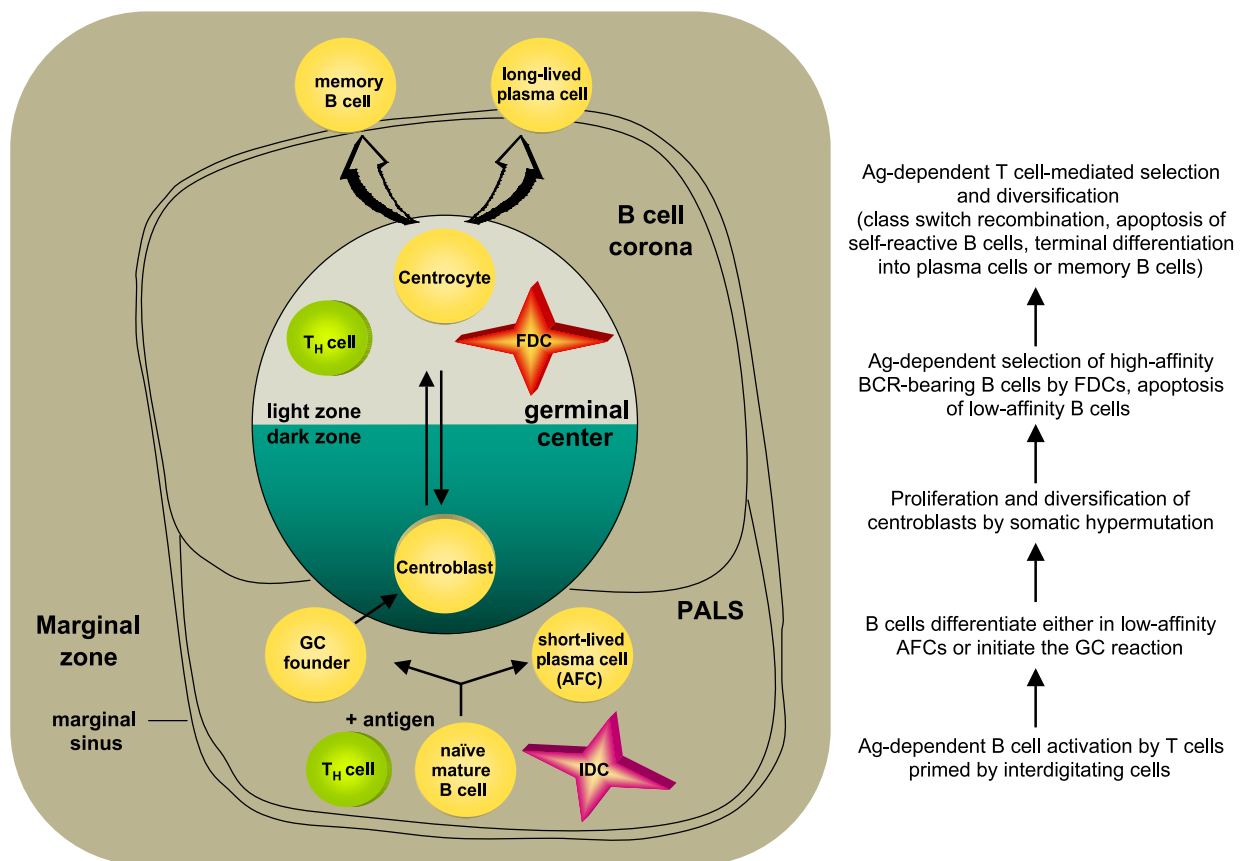


Fig. 3. B cell maturation in the spleen. Immature B cells enter the spleen from the blood at the level of the MZ where they are selected for self-reactivity in the periphery (and enter an anergic state), or they mature further. Subsequently, the (naïve) B cells cross the marginal sinus and migrate via the periarteriolar lymphoid sheath (PALS) to the lymphoid follicles, which are areas specialized in antigen retention and presentation. Upon encounter with TD antigens such mature  $IgM^+ IgD^+$  B cells become activated and proliferate with T cell help and predominantly differentiate into low affinity antibody-forming cells (AFCs). AFCs are short-lived plasmacytes that initially secrete IgM, but subsequently switch to secondary isotypes and die by apoptosis within 2 weeks of antigen encounter. Some cells from the expanded B cell pool do not become AFCs but migrate into the adjacent follicles, which are subsequently transformed into germinal centers (GCs). GC B cells undergo iterative cycles of proliferation, somatic hypermutation and apoptosis in cooperation with antigen-primed T cells and follicular dendritic cells (FDCs), a process resulting in the selection of B cell clones that make an antibody with high affinity for a cognate antigen. Additionally, Ig class switching occurs during the GC reaction. These steps expand, refine and diversify the repertoire of the early immune response to assure the long-term maintenance of protective immunity. Two types of terminally differentiated B cells eventually emerge from the GC reaction: non-secreting, Ig surface-positive memory B cells and high affinity antibody-secreting plasmablasts that no longer carry immunoglobulins at their surface.  $T_H$ , T helper; IDC, interdigitating cell.

1997). Similarly, mice deficient for the coactivator OBF-1 show defects in late stage B cell differentiation resulting in the disruption of antigen-dependent immune responses and in complete absence of germinal center formation (Kim et al., 1996; Nielsen et al., 1996; Schubart et al., 1996). Following stimulation of the BCR, CD40 or IL-4 signaling pathways OBF-1 is upregulated specifically in GC B cells where it acts as an integrator essential for full differentiation and function of these cells (Qin et al., 1998). In vivo, accumulation of OBF-1 protein in GC B cells is controlled largely at the post-transcriptional level and the Ring finger protein Siah, which is the homolog of *Drosophila* Sina and interacts with OBF-1, may be part of the regulatory mechanism (Boehm et al., 2001; Tiedt et al., 2001). In addition, a myristoylated isoform of OBF-1 has been identified that is membrane-bound and could play a role as a signal transducer itself (Yu et al., 2001).

OBF-1-deficient mice also show a severe reduction of the number of immature B cells in the spleen; this reduction is even more dramatic in OBF-1/Oct-2 (Schubart et al., 2001) and OBF-1/btk (Bruton's tyrosine kinase) (Schubart et al., 2000) compound mutant mice. The molecular mechanisms underlying these defects are still unclear. However, a bcl-2 transgene expressed in the B lineage rescues, to a large extent, the defect in OBF/btk double-mutant mice, suggesting that lifespans of immature B cells may play a role in this transition (Rolink et al., unpublished data), an effect that might again be related to BCR signaling strength. In line with this, it was very recently reported that Bcl-2 is down-regulated in the bone marrow of OBF-1-deficient mice (Brunner et al., 2003). Finally, mice lacking simultaneously OBF-1 and the chromatin regulator Aiolos show a severe developmental block in the bone marrow, at the transition between pre-B and immature B cells (Sugai et al., 2003; Sun et al., 2003). This phenotype is all the more surprising given the fact the individual knockouts of these two factors show defects only in late stages of B cell development, as mentioned above. This observation underscores the concept that the same factors may perform different functions (and have therefore different targets?) at different developmental stages.

In addition, recent studies have shown that even in the absence of both Oct-2 and OBF-1, B cells were able to develop normally to the IgM<sup>+</sup> stage and immunoglobulin gene transcription was still largely unaffected (Schubart et al., 2001). These unexpected observations imply that the ubiquitous factor Oct-1 may play a previously unrecognized role in the control of immunoglobulin gene transcription and suggest the possible existence of another, as yet unidentified, cofactor. In line with this, it was found that on many Ig<sub>H</sub> promoters Oct factors bind as dimers in a way precluding recruitment of OBF-1 (Tomilin et al., 2000). On the other hand, it was very recently found that OBF-1 is critical for the activity of a subset of Ig<sub>L</sub> promoters (Casellas et al., 2002) and also strongly enhances the expression of the osteopontin gene in B cells (Lins et al., 2003).

**4.1.1.2. CCAAT/enhancer binding protein (C/EBP).** C/EBPs are a family of transcription factors containing a highly conserved C-terminal basic-leucine zipper domain, which is involved in dimerization and DNA binding. Six family members, C/EBP $\alpha$  to  $\zeta$  have been isolated and characterized. Additional diversity is brought about by differential use of translation initiation sites and protein interactions among the family members and with other transcription factors, which may allow for differential regulation of target genes in a tissue-specific and cell-stage-specific manner. Gene targeting of several family members has identified essential roles for C/EBPs in the control of proliferation and differentiation, metabolism and inflammation, particularly in hepatocytes, adipocytes and hematopoietic cells. C/EBPs are critical components of the transcriptional regulation of hematopoiesis (for review, see Yamanaka et al., 1998; Hu et al., 2000; Ramji and Foka, 2002). For instance, C/EBP $\alpha$  was described as a master regulator of myeloid progenitors, C/EBP $\beta$  plays an important role in macrophage and B cell development, C/EBP $\gamma$  is involved in B cell development, and C/EBP $\delta$  is upregulated during myelopoiesis. Furthermore, C/EBP $\epsilon$  is a regulator of terminal differentiation of eosinophils and functional maturation of neutrophils.

In the case of B cells, the C/EBP family of transcriptional activators is functionally important at many stages of B lymphopoiesis, as witnessed by: (i) the impaired generation of bone marrow B lymphocytes in mice deficient in C/EBP $\beta$  (Chen et al., 1997), (ii) the requirement for C/EBP proteins for the regulation of the pro-B cell-specific enhancer of the Id1 gene (Saisanit and Sun, 1997), (iii) the requirement for C/EBP $\alpha$ , together with PU.1, for the regulation of the granulocyte-macrophage colony-stimulating factor receptor alpha gene (Hohaus et al., 1995) and (iv) the requirement for C/EBP proteins for Ig V<sub>H</sub> promoter activity in vivo and in vitro (Cooper et al., 1992).

**4.1.1.3. NF- $\kappa$ B/Rel family members.** NF- $\kappa$ B was originally described as a nuclear transactivator of the immunoglobulin  $\kappa$ -light chain enhancer (the  $\kappa$ B enhancer), and is known today to be a cytokine-inducible transcription factor governing the expression of an important set of genes involved in inflammation and cell survival (Siebenlist et al., 1994; Beg and Baltimore, 1996; Barnes and Karin, 1997; Karin, 1999). Members of the NF- $\kappa$ B/Rel family of transcription factors share a highly conserved 300 amino acid domain, the rel domain, which is essential for DNA binding, dimerization and interaction with other transcription factors. At least five members of this family of ubiquitously expressed proteins were shown to be involved in B cell development. These include NF- $\kappa$ B1 (p105), NF- $\kappa$ B2 (p100), RelA (p65), RelB, and c-Rel. NF- $\kappa$ B1 and NF- $\kappa$ B2 generate precursor proteins that are proteolytically cleaved to give rise to the p50 and p52 NF- $\kappa$ B subunits, respectively. These can homo- or heterodimerize and bind their cognate DNA recognition sequences.

NF- $\kappa$ B2-deficient mice have defects in the microarchitecture of secondary lymphoid organs and show impaired humoral immune responses. The formation of B cell follicles is impaired as well as the formation of follicular dendritic cell (FDC) networks upon antigenic challenge of the mutant mice. The defects in formation of B cell follicles may be attributed, at least in part, to impaired production of the B lymphocyte chemoattractant chemokine BCA-1, possibly a result of defective FDCs (Caamano et al., 1998; Franzoso et al., 1998; Poljak et al., 1999). RelB-deficient mice, in contrast to *nfkb1*<sup>-/-</sup>, but similar to *nfkb2*<sup>-/-</sup> mice, are unable to form GCs and FDC networks upon antigen challenge in the spleen. RelB is also required for normal organization of the marginal zone and its population by macrophages and B cells. The expression of homing chemokines, particularly of BCA-1, is strongly reduced in *relB*<sup>-/-</sup> spleen (Weih et al., 2001). Similarly to c-Rel and p50/NF- $\kappa$ B, RelB plays a role in B cell proliferation, but in contrast to these two factors, it is not critically involved in maturation to Ig secretion or expression of Ig isotypes, although retroviral expression of RelB in activated B cells inhibits switching to IgG1, but not to IgE (Snapper et al., 1996; Bhattacharya et al., 2002). The transcription of the RelB gene itself is regulated by NF- $\kappa$ B family members, in particular by NF- $\kappa$ B p50, RelA and RelB (Bren et al., 2001). B cells lacking the c-Rel transactivation domain have selective defects in germline C<sub>H</sub> transcription and Ig class switching, featuring isotype induction profiles that differ from those of mice deficient in p50/NF- $\kappa$ B (Zelazowski et al., 1997). In addition, in cellular model systems, the NF- $\kappa$ B family was found to critically regulate rearrangement of the  $\lambda$  light chain gene (Bendall et al., 2001) and of the Ig  $\kappa$  locus (O'Brien et al., 1997), in the latter case partly by inducing a B cell-specific demethylation of the Ig  $\kappa$  locus (Kirillov et al., 1996).

**4.1.1.4. I $\kappa$ B family.** I $\kappa$ B proteins associate with the NF- $\kappa$ B transcription factors via their ankyrin repeat domain and are involved in an important regulatory mechanism of NF- $\kappa$ B activity by controlling the nuclear abundance of the various NF- $\kappa$ B subunits, most likely in an independent manner. In case of the prototypical I $\kappa$ B proteins I $\kappa$ B $\alpha$ , - $\beta$  and - $\epsilon$ , this leads to the retention of NF- $\kappa$ B in the cytoplasm, and consequently to the inhibition of NF- $\kappa$ B activity. Cellular activation can lead to I $\kappa$ B phosphorylation and its subsequent degradation via the ubiquitin-proteasome pathway. NF- $\kappa$ B is released in this process and can translocate to the nucleus where it acts as a transcriptional regulator. Among its target genes are several I $\kappa$ B family members, whose expression level is thus regulated, at least in part, by an autoregulatory feedback loop (Sun et al., 1993; Chiao et al., 1994; Brasier et al., 2001). Another rather unusual I $\kappa$ B family member, the proto-oncogene Bcl-3, is primarily nucleoplasmic and can lead to enhanced NF- $\kappa$ B-dependent transcription. Bcl-3 interacts with NF- $\kappa$ B1, NF- $\kappa$ B2, Jab1, Pirin, Tip60 and Bard1. The latter are nuclear proteins which also bind to other transcription factors including c-Jun, nuclear

factor I (NFI), HIV-1 Tat or the tumor suppressor and PolII holoenzyme component Brca1, respectively. It is hypothesized that Bcl-3 might act as an adaptor between NF- $\kappa$ B p50/p52 and other transcription regulators and its gene activation function may at least in part be due to recruitment of the Tip60 histone acetylase (Dechend et al., 1999). Deletion of Bcl-3 leads to a defect in GC formation (Schwarz et al., 1997) (Table 2).

**4.1.1.5. BCL-6.** The proto-oncogene and transcriptional repressor BCL-6 was characterized as a multifunctional regulator of lymphocyte differentiation and immune responses (reviewed in Dalla-Favera et al., 1999 and Staudt et al., 1999). BCL-6 contains an N-terminal BTB/POZ domain and Krüppel-type zinc finger motifs in the COOH-terminal region (Kerckaert et al., 1993; Ye et al., 1993; Fukuda et al., 1995) and can function as a sequence-specific transcriptional repressor (Chang et al., 1996a; Dewindt et al., 1995; Seyfert et al., 1996; Baron et al., 1995; Kawamata et al., 1994). Postulated BCL-6 target genes include Blimp-1, CD69, CD44, EB12, Id2, STAT1, MIP-1 $\alpha$ , IP-10, p27kip1 and cyclin D2 (Shaffer et al., 2000). BCL-6-deficient mice fail to form germinal centers during a T cell-dependent immune response and exhibit a fatal inflammatory disease distinguished by the presence of T helper type 2 (T<sub>H</sub>2) cells (Dent et al., 1997; Fukuda et al., 1997; Ye et al., 1997). Studies of BCL-6-deficient B cells recently showed that BCL-6 and germinal center formation are essential for somatic hypermutation (SHM), while generation of memory B cells can occur independently of germinal center formation, somatic hypermutation and Ig class switching (Toyama et al., 2002).

BCL-6 protein expression in B cells is exquisitely regulated following antigen encounter. Nascent pre-GC B cells upregulate BCL-6 protein, migrate to the follicular area and initiate GC formation. Antigen-specific B cells that do not upregulate BCL-6 protein expression differentiate in the PALS into plasmablasts and provide an initial burst of low-affinity antibody. While plasmacytic differentiation within the PALS, which allegedly takes place only in the absence of BCL-6 expression (Falini et al., 2000), occurs normally in BCL-6-deficient mice, GC differentiation is blocked (Fukuda

Table 2  
Genes essential for GC reactions

Function	Gene
GC B cell activation	<b>Bright, BCL-6, Spi-B, Oct-2, OBF-1, STAT6, CD40, CD40L</b>
FDC maturation	<b>BCL3, NF-<math>\kappa</math>B p52, LT-<math>\alpha</math>, LT-<math>\beta</math>, LT-<math>\beta</math>R, TNFR-1, TNF-<math>\alpha</math></b>
T cell activation	<b>CD24, CD28, B7, TCR, MHC class II</b>
B cell homing to follicle	<b>BLR1, BCA-1</b>
Class switch recombination	<b>NF-<math>\kappa</math>B p52, c-Rel, Bcl-3; PU.1; BCL-6; STAT6; T-bet; Pax-5; E2A; LRI</b>
Somatic hypermutation	<b>BCL-6, LT-<math>\alpha</math>, TNF-<math>\alpha</math></b>

Transcription factors are listed in **bold** type, other proteins in normal type.



et al., 1997). These observations suggest that BCL-6 controls a cell fate decision made by antigen-specific B cells, and thereby acts as a key regulator of terminal B cell differentiation.

Mechanistically, BCL-6 binds to a number of STAT6 binding sites in vitro and regulates the transcription of a subset of IL-4-inducible genes in transiently transfected cells (e.g.,  $I\epsilon$ , but not CD23b). Furthermore, lack of BCL-6 deregulates IgE class switching in vivo. Thus, BCL-6 can modulate the transcription of selective STAT6-dependent IL-4 responses, including IgE class switching in B cells (Harris et al., 1999). BCL-6 function is inhibited by p300-mediated acetylation which disrupts the ability of BCL-6 to recruit class II histone deacetylases (HDACs) (Lemercier et al., 2002), thereby preventing it from repressing transcription and inducing cell transformation (Bereshchenko et al., 2002).

**4.1.1.6. STATs.** As a competitor of BCL-6, STAT6 is a signal transducer and activator of transcription (STAT) critically involved in regulating IL-4-responsive genes (Kohler and Rieber, 1993; Kotanides and Reich, 1993; Hou et al., 1994; Schindler et al., 1994; Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996; Davey et al., 2000; Zhu et al., 2001). Notably, it plays an essential role in the IL-4-mediated activation of germline  $\gamma 1$  and  $\epsilon$  Ig gene transcription and class switch recombination in B cells (Linehan et al., 1998). Synergistic activation of Ig gene transcription was observed between STAT6 and either NF- $\kappa$ B or PU.1 (Schaffer et al., 1999; Stutz and Woisetschlager, 1999). Similar suppressive effects of BCL-6 were observed for another STAT transcription factor, STAT3 (Reljic et al., 2000).

Yet another member of the STAT family involved in class switch recombination is STAT5, which has an essential role in IL-5-dependent IgH switch recombination in mouse (Horikawa et al., 2001), probably via NF- $\kappa$ B. It appears that both Stat5a and Stat5b are essential for IL-5-dependent  $\mu$  to  $\gamma 1$  CSR and Ig secretion (Nakamura et al., 2002).

**4.1.1.7. T-bet.** T-bet, a recently discovered member of the T-box transcription factor family, which is rapidly induced by IFN- $\gamma$  in lymphoid and myeloid cells (Lighvani et al., 2001), was shown to directly regulate Th1 lineage commitment by CD4 T cells (Szabo et al., 2000). In addition, it acts as a selective transducer of IFN- $\gamma$ -mediated T-independent IgG2a class switching in B cells (Peng et al., 2002; Gerth et al., 2003).

**4.1.1.8. Spi-B.** The Ets family member Spi-B is closely related to PU.1 through structural homology and by its ability to transactivate identical target genes as PU.1 in vitro (Ray et al., 1992; Ray-Gallet et al., 1995). In vivo, however, the target genes of Spi-B and PU.1 overlap only partially (Rao et al., 1999). Both PU.1 and Spi-B are required for normal BCR signaling (Garrett-Sinha et al., 1999), but while Spi-B can functionally replace PU.1 in myeloid development, it cannot replace PU.1 in lymphoid development (Dahl et al., 2002).

Spi-B-deficient mice exhibit severe abnormalities in B cell function and selective T cell-dependent humoral immune responses accompanied by a dramatic defect in germinal center formation and maintenance. *Spi-B*<sup>-/-</sup> mice have a BCR signaling defect and appear to initiate the production of germinal centers within splenic primary B cell follicles, but these structures decay prematurely due to BCR-mediated apoptosis (Su et al., 1997). Recent data from our laboratory showed that Spi-B expression in GC B cells is under the control of OBF-1, and this observation may explain a crucial part of the phenotype of OBF-1-deficient mice (Bartholdy and Matthias, manuscript submitted).

**4.1.1.9. CIITA.** Class II transactivator (CIITA) activates the expression of multiple genes involved in antigen presentation and is believed to be required for both constitutive and IFN- $\gamma$ -inducible expression of these genes. Importantly, the expression of major histocompatibility complex (MHC) class II, which is required for antigen presentation to T cells and GC formation, is regulated by CIITA (Silacci et al., 1994). CIITA-deficient mice are impaired in T-dependent antigen responses and MHC class II-mediated allogeneic responses (Chang et al., 1996b). Mechanistically, CIITA, in conjunction with the regulatory factor X (RFX), binds to the promoter-proximal S-Y motifs of MHC class II genes and activates transcription by inducing local histone acetylation and recruitment of the transcription machinery, as well as long-range histone acetylation and chromatin remodeling over a large domain by binding to the distal MHC class II locus control region (LCR) (Masternak et al., 2003). CIITA arguably has a role not only in class II, but also in class I MHC gene induction (Martin et al., 1997).

**4.1.1.10. Bright.** Bright, the B cell regulator of Ig heavy chain transcription, represents a new family of DNA-binding proteins that shares regions of high homology with the yeast SWI1 and *Drosophila* dri proteins (Herrscher et al., 1995; Gregory et al., 1996) that are part of chromatin remodeling complexes (Peterson and Herskowitz, 1992). Bright has been found to interact with regions of the murine Ig heavy chain locus that contain matrix attachment regions (MARs) (Webb et al., 1991b) and may be yet another important regulator of Ig expression at late stages of differentiation (Webb et al., 1998). MARs are A + T-rich DNA sequences that have also been implicated in organizing chromatin into transcriptionally active domains (Fey et al., 1991; Getzenberg, 1994). Bright binding is associated with increases in Ig transcription (Webb et al., 1991a; Herrscher et al., 1995), and one study suggested that the MARs flanking the intronic heavy chain enhancer are indeed critical for Ig expression (Oancea et al., 1997).

#### 4.1.2. Plasma cell differentiation.

Centrocytes that receive survival signals during affinity maturation exit the GC and continue to mature as either long-lived plasma cells (PCs) or memory B cells (reviewed



in Gray et al., 1996; O'Connor et al., 2003). PC precursors complete their terminal differentiation either within the GCs, or after having reached the bone marrow (O'Connor et al., 2002). Plasma cells are terminally differentiated, post-mitotic, antibody-producing cells. A small number of these cells remain in the bone marrow where they can survive and secrete antibodies for many months.

The regulation of several transcription factors is crucial for lymphocyte differentiation into plasma cells: The expression of a number of B cell-specific transcription factors required in earlier stages of B cell development is decreased or extinguished in plasma cells. These include EBF, Pax5, A-Myb, BCL-6 and CIITA. Concomitantly, a large set of B cell-specific surface proteins is downregulated at the plasma cell stage, including MHC class II, B220, CD19, CD21, CD22 and the chemokine receptors BLR1 and CCR7. In contrast, a smaller number of transcription factors are upregulated in plasma cells, including the bona fide key regulators of plasma cell differentiation, XBP-1, Blimp-1 and IRF-4.

**4.1.2.1. Blimp-1.** Blimp-1 (B lymphocyte-induced maturation protein-1) is a zinc finger protein that was first identified as a plasmacytoma-specific repressor that binds to a negative element in the murine *c-myc* promoter and was subsequently found to repress *c-myc* in B cells (Kakkis et al., 1989; Lin et al., 1997). Transcriptional repression mediated by Blimp-1 involves the recruitment of histone deacetylases (Yu et al., 2000). Blimp-1 expression is sufficient to drive terminal differentiation of BCL-1 cells into plasma-like cells, making it a good candidate for being a master regulator of plasma cell development (Turner et al., 1994). However, repression of *c-myc* by Blimp-1 is necessary, but not sufficient, for differen-

tiation of mature B cells into plasma cells (Lin et al., 2000), hinting at the importance of other Blimp-1 target genes. Blimp-1 determines plasma cell fate by triggering both the inhibition of earlier GC B cell activities and the activation of plasma cell functions. After downregulation of BCL-6, which directly represses Blimp-1 at the GC B cell stage (Reljic et al., 2000; Shaffer et al., 2000; Vasanwala et al., 2002), Blimp-1 is induced and directly represses several genes required for B cell receptor signaling and GC reactions [such as Id3, *c-Myc* (Lin et al., 1997, 2000), Spi-B, CIITA and Pax5 (Shaffer et al., 2002)]. In a feedback loop, Blimp-1 also represses BCL-6 transcription, and this leads to terminal plasmacytic differentiation (Fig. 4; for a review, see Calame, 2001).

**4.1.2.2. XBP-1.** XBP-1 (X-box-binding protein-1) is a ubiquitously expressed basic-region leucine zipper protein of the CREB/ATF (cyclic AMP response element binding protein/activating transcription factor) family of transcription factors, which binds to a specific DNA sequence called an X-box, first identified in the regulatory region of an MHC class II gene (Liou et al., 1990). Mice lacking XBP-1 die as embryos as a result of liver abnormalities and severe anaemia (Reimold et al., 2000). In a complementation experiment using RAG-2/XBP-1-deficient chimaeric mice, it was shown that XBP-1 is required specifically for plasma cell development (Reimold et al., 2001). XBP-1 gene expression is repressed by Pax5, which is essential for early B cell development but downregulated in plasma cells (Barberis et al., 1990; Reimold et al., 1996).

Importantly, XBP-1 appears to function as a transcriptional activator that amplifies the unfolded protein response (UPR) during differentiation of antibody-secreting B cells

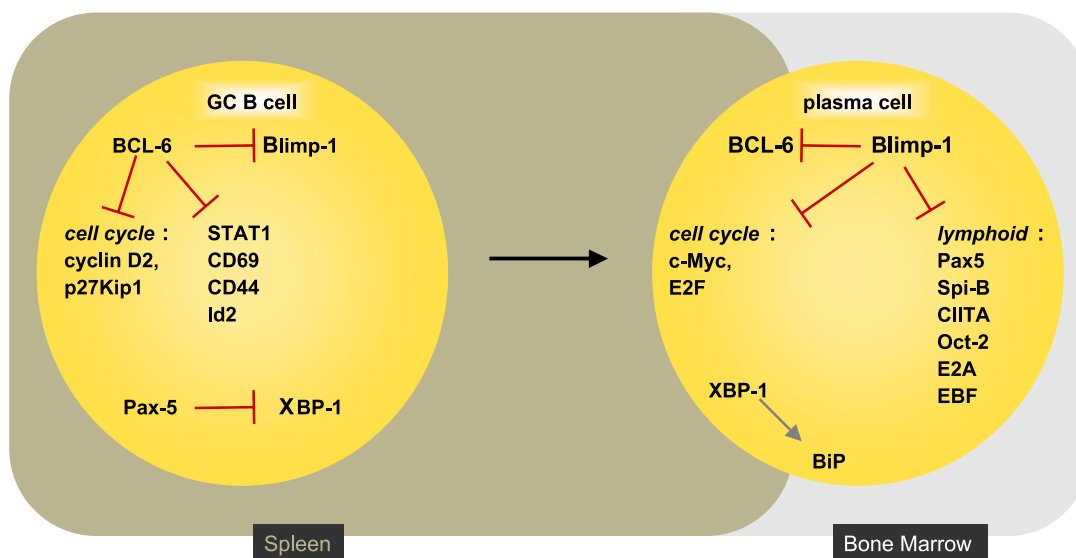


Fig. 4. Transcription factors in plasma cell differentiation. At the GC B cell stage, BCL-6 represses Blimp-1, a key regulator of plasma cell differentiation. Another transcription factor crucial in plasma cells, XBP-1 is repressed by Pax-5. Upon transition to the plasma cell stage, Blimp-1 expression leads to the repression of BCL-6 transcription, and thereby to the inhibition of earlier GC B cell activities, including the transcription of a number of B cell specific transcription factors, such as Pax-5. As a consequence of Pax-5 downregulation, XBP-1, specifically required for plasma cell development, is expressed.

(Gass et al., 2002); in addition, the UPR and plasma cell differentiation appear intricately linked via XBP-1 (Iwakoshi et al., 2003). Upon UPR activation, XBP-1 mRNA becomes cleaved in a site-specific manner to remove a 26-nucleotide intron. This generates a spliced XBP-1 mRNA with an altered reading frame encoding a 54-kDa bZIP transcription factor, p54XBP-1, which is a more potent and more stable transcriptional activator than the 30-kDa protein translated from unprocessed XBP-1 mRNA (Shen et al., 2001; Yoshida et al., 2001; Calfon et al., 2002). p54XBP-1 can bind endoplasmic reticulum stress response elements (ERSEs) and may also act through a second *cis*-acting element to regulate promoter activity (Yoshida et al., 2001).

**4.1.2.3. IRF-4/MUM-1/Pip.** Another protein that is very important for late B cell functions, Pip [Pu.1 interacting protein, also termed IRF-4 (interferon regulatory factor 4), LSIRF, NF-EM5 or ICSAT], is a lymphoid-specific transcriptional activator (Eisenbeis et al., 1995). It is recruited to DNA by interaction with the Ets factors PU.1 or Spi-B through a regulatory domain that is important for formation of a ternary complex, and for autoinhibition (Brass et al., 1996). Recently, the crystal structure of the ternary PU.1/IRF-4/DNA complex has been solved and shows that the phosphorylated PEST region of PU.1 interacts with the autoinhibitory domain of IRF-4 and thereby stabilizes the complex (Escalante et al., 2002). IRF-4 is expressed in GC centrocytes and plasmablasts/plasma cells and is also involved in the late stages of B cell differentiation as well as in T-cell activation (Falini et al., 2000). Deletion of this transcription factor in mice revealed that it is essential for the function and homeostasis of both mature B and mature T lymphocytes (Mittrucker et al., 1997). Interestingly, Pip also cooperatively binds to DNA in association with the E2A protein E47. This cooperative interaction leads to a very strong transcriptional synergy between Pip and E47 (Nagulapalli and Atchison, 1998).

#### 4.2. T cell-independent antigen responses, MZ B and B1 cells

Both the preactivated splenic marginal zone B cells and B1 B cells are part of a “natural memory response” and provide a bridge between the very early innate and the later appearing adaptive immune response by generating an initial wave of IgM producing plasmablasts during the first 3 days of a primary response to particulate bacterial antigens (Martin and Kearney, 2000a; Martin et al., 2001).

##### 4.2.1. B1 cells

B-1 cells are found predominantly in the peritoneal and pleural cavities and can be distinguished from the conventional (B-2) B cells by their self-renewal capacity and unique surface proteins. In contrast to other B cell populations, they are CD45<sup>B220<sup>lo</sup></sup>, IgM<sup>hi</sup>, CD23<sup>-</sup>, CD43<sup>+</sup> and IgD<sup>lo</sup>. While the origins of B-1 cells remain controversial, it is well established that they secrete natural antibodies important

for innate immunity—preferentially low-affinity, poly-reactive, mostly self-reactive antibodies (Hayakawa et al., 1984, 1990; Forster and Rajewsky, 1987; Mayer and Zaghouni, 1991; Shirai et al., 1991). It appears that they arise either as a distinct lineage from committed fetal/neonatal precursors (Hayakawa et al., 1985; Lam and Stall, 1994), or from follicular B-2 cells in response to BCR ligation (Haughton et al., 1993; Clarke and Arnold, 1998; for a detailed review of B1 cells, see Berland and Wortis, 2002). In either case, it appears that BCR specificity and surface density together are also decisive factors in the development of B-1 versus B-2 cells (Lam and Rajewsky, 1999).

The B-1a cells subset expresses CD5, but is otherwise almost indistinguishable from the B-1b cells (Kantor et al., 1992; Stall et al., 1992). An NFAT-dependent enhancer has been found to be necessary for anti-IgM-mediated induction of murine CD5 expression in primary splenic B cells (Berland and Wortis, 1998).

##### 4.2.2. Marginal zone B cells

MZ B cells consist mainly of a large, mostly non-circulating subset of mature B cells that are localized in the marginal zone of the spleen. As mentioned above, they have an important function in the early stages of the immune response. This could be due to the fact that MZ B cells appear to have a lower activation threshold and are therefore more easily triggered into proliferation or differentiation than immature or recirculating mature B cells. Several recent reviews discuss these properties in more detail (Martin and Kearney, 1999, 2000a, 2002; Bendelac et al., 2001).

A number of genes that are crucial for the generation or maintenance of the MZ B cell compartment have been identified by targeted disruption or overexpression studies.

One group of mutations that specifically affect MZ B cells is modulating the BCR signal strength. For example, mice with a targeted disruption of the Aiolos transcription factor have a strongly reduced number of MZ B cells, most likely due to an increase in BCR signal strength in the absence of Aiolos (Cariappa et al., 2001). In *Lyn*<sup>-/-</sup> mice and in mice with a mutated Ig $\alpha$  ITAM motif, MZ B cell numbers are also strongly decreased, and both *Lyn* and Ig $\alpha$  are known to negatively regulate BCR signaling. Conversely, MZ B cell numbers are enhanced in mice lacking the CD21 coreceptor, and this enhancement correlates with an expected decrease in BCR signal strength (Cariappa et al., 2001). In the absence of the BCR costimulatory molecule CD19, MZ B cells are lacking completely (Hiemstra et al., 1999; Martin and Kearney, 2000b).

Apart from altered BCR signal strength, several other genes are presumably required for the migration of precursors to the MZ, such as NF $\kappa$ B p50, Pyk2 and Lsc, or for the development and maintenance of the splenic microarchitecture and microenvironment (such as Lt $\alpha$ , Lt $\beta$ , Lt $\beta$ R, NF $\kappa$ B p52, RelB and Dock2) (reviewed in Cariappa and Pillai, 2002). Another recent finding using conditional mutagenesis of the transcription factor RBP-J (recombination signal

Table 3  
Genes implicated in marginal zone development or maintenance

Model	MZ phenotype	Reference
<i>Aiolos</i> <sup>-/-</sup>	Decreased MZ and B1 B cells; hyperactive B cells	(Cariappa et al., 2001)
<b><i>RelB</i></b> <sup>-/-</sup>	Decreased MZ B cells	(Weih et al., 2001)
<b><i>RelA</i></b> <sup>-/-</sup> / <i>c-rel</i> <sup>-/-</sup>	Decreased MZ B cells	(Grossmann et al., 2000)
<b><i>NFκB p50</i></b> <sup>-/-</sup>	Lack of MZ B cells	(Cariappa et al., 2000)
<b><i>RBP-J</i></b> <sup>-/-</sup>	Lack of MZ B cells	(Tanigaki et al., 2002)
<i>CD19</i> <sup>-/-</sup>	Lack of MZ B cells; defects in TD and TI responses	(Martin and Kearney, 2000b)
<i>CD21</i> <sup>-/-</sup> , <i>Igα</i> <sup>-/-</sup>	Increased MZ B cells	(Cariappa et al., 2001)
<i>Lt α</i> <sup>-/-</sup> , <i>Lt α</i> <sup>-/-</sup> , <i>Lt β R</i> <sup>-/-</sup>	Reduced MZ B cells; disorganized B/T areas	(Matsumoto et al., 1996, 1997; Ngo et al., 1999; Ansel et al., 2000; Korner et al., 2001)
<i>Dock2</i> <sup>-/-</sup>	Low MZ and B1 B cells; reduced chemokine migration	(Fukui et al., 2001)
<i>Pyk2</i> <sup>-/-</sup>	Lack of MZ B cells; normal B1 cells; hyporesponsivity to chemokines	(Guinamard et al., 2000)
<i>Lyn</i> <sup>-/-</sup>	Lack of MZ B cells	(Seo et al., 2001)
<i>Lsc</i> <sup>-/-</sup>	Low MZ B cells; normal B1 cells	(Girkontaite et al., 2001)
<i>XID/Btk</i> <sup>-/-</sup>	Decreased MZ B cells	(Khan et al., 1995)

Transcription factors are listed in **bold** type, other proteins in normal type.

binding protein-J) in B cells demonstrates that Notch-RBP-J signaling is involved in cell fate determination of marginal zone B cells (Tanigaki et al., 2002). It has been suggested that Notch-RBP-J signaling regulates the lineage commitment of mature B cells into follicular versus MZ B cells (Table 3).

## 5. Conclusions and perspectives

As described here, the control of gene expression is a multi-level process that offers multiple possibilities for regulation. Apart from the competition of transcription factors for binding sites and interacting proteins, and the regulation of protein and mRNA stability, superimposed mechanisms that regulate the accessibility of a locus, such as local changes in chromatin structure through modifications in histone acetylation or demethylation of CpG dinucleotides—or even global changes in the condensation of chromatin, silencers and locus control regions (Ernst and Smale, 1995)—are absolutely essential for the initiation of transcription.

Several transcription factors, coactivators and corepressors have been shown to directly interact with HDACs, or HATs and thereby modulate chromatin remodeling and promoter activity of B cell-specific genes. Some examples were already mentioned in previous chapters: Repression via association with histone deacetylases was observed with Ikaros, PU.1, BCL-6, Blimp-1 and Notch (Kao et al., 1998). Activation via complex formation with the HAT CBP/p300 was described for Pax5–Daxx complexes, c-Myb, CIITA and several bHLH proteins, including E2A (Eckner et al., 1996; Qiu et al., 1998; Fontes et al., 1999; Romanow et al., 2000; Emelyanov et al., 2002; Yamamoto et al., 2002). In line with this, V(D)J recombination, which partly depends on E2A activity, is also stimulated by histone acetylation (Kwon et al., 2000; McBlane and Boyes, 2000). While effects of chromatin structure and remodeling activ-

ities on V(D)J recombination are hardly surprising, many questions remain about how the remodeling activities are recruited to the right recombination sites, in the right cells, at the right times.

Fascinating recent findings about the regulation of the immunoglobulin kappa locus underline the importance of nuclear architecture for cell stage and cell type-specific transcription. They encompass a role of DNA demethylation and increased chromatin accessibility in the process of allelic exclusion at the kappa chain locus (Goldmit et al., 2002), which seems to be also regulated by a differential subnuclear localization (Skok et al., 2001; Kosak et al., 2002). Interestingly, all of the B cell receptor loci ( $\mu$ ,  $\kappa$  and  $\lambda$ ) and also the T cell receptor (TCR)  $\beta$  locus replicate asynchronously, a phenomenon that can be considered an epigenetic mark for allelic exclusion, since the early-replicating allele is almost always selected to initially undergo rearrangement in B cells (Mostoslavsky et al., 2001). The identification of factors that target these remodeling activities will no doubt be a focus of intense research in the near future, as will be the kinetics of chromatin opening relative to the initiation of recombination. Transcription itself might very well participate in the initiation of chromatin opening for recombination. The subsequent “closing” of chromatin at nonproductive sites once a productive recombination event has been achieved raises additional questions that are critical to our overall understanding of the regulation and coordination of chromatin remodeling events in B cell development.

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## 3 Discussion

### 3.1 Overexpression of OBF-1 in T cells

We have generated transgenic mice specifically overexpressing OBF-1 in T cells. The observed expression pattern (Figs. 16 and 17) corresponds to the published pattern of a GFP transgene driven by the same *lck* proximal promoter construct as the one that was used in the present study (Shimizu et al., 2001). Expression was highest in the thymus, but also present in the spleen of young adult mice. Due to the method used to generate transgenic animals, the gene expression can differ among different mouse lines. This can be caused by different numbers of gene copies that have integrated in the genome, or by the accessibility and intrinsic activity of the site of integration. The phenotype that we observed was most pronounced in the lines that had the highest transgene expression, and we carried out our experiments with two independent lines with high transgene expression that showed a very similar phenotype.

The transgenic mice were viable, healthy, and did not show any obvious signs of immune problems. They had normal serum Ig levels, and a normal induction of an immune response to DNP-KLH, a T cell dependent antigen, was witnessed by enzyme-linked immunosorbent serologic assay (ELISA) (Fig. 32). The numbers of B and T cells in the lymphoid organs were found to be normal and no obvious signs of autoimmunity, decreased lifespan or similar immune-dependent diseases were discernible. The cytokine expression profile in the lymphoid organs was normal, as far as it was investigated (Fig. 33). Thus, the ectopic expression of OBF-1 in T cells of transgenic mice did not lead to major alterations of T cell development or function. In a similar manner, it was found earlier in mice deficient for OBF-1 that the observed defects were B cell-intrinsic, as OBF-1<sup>-/-</sup> T cells behaved normally in WT background (REF.). To conclude, neither the presence, nor the absence of OBF-1 in T cells appears to have a fundamental impact on the immunocompetence in mice.

#### 3.1.1 Phenotypic abnormalities

However, some phenotypic abnormalities and a skewed repertoire and distribution of specific lymphocyte subpopulations, most prominently of  $\gamma\delta$  T cells and NK cells, have been discovered and are described in Section 2.2.3.2. These abnormalities did not visibly affect the well-being and immune status of the mice, probably because those more specialized immune cells that were affected were not required in the environment of the mouse facility.

However, specifically challenging these cells might reveal abnormalities in the transgenic mice that could be caused by the skewed repertoire of these minor cell populations.

##### 3.1.1.1 CD4<sup>+</sup> CD8<sup>+</sup> CD25<sup>+</sup> thymocytes

The most striking phenotype observed in *lck-OBF-1* mice was the appearance of T cell populations featuring an abnormal cell surface marker expression pattern. Most obvious was the appearance of CD4<sup>+</sup> CD8<sup>+</sup> double positive (DP) thymocytes expressing CD25, the IL-2 receptor alpha chain (Fig. 19). This receptor component is usually expressed only in early CD4<sup>-</sup> CD8<sup>-</sup> double-negative (DN) thymocytes where commitment to the T cell lineage has been established, but subsequently it is dramatically downregulated for the remainder of T cell development. It was shown recently that the downregulation of CD25 transcription correlated with (i) loss of the basal transcriptional machinery from the core promoter; (ii) dissociation of histone acetylase p300 and BRG1, a member of the ATP-dependent SWI/SNF chromatin remodeling complex; and (iii) the dephosphorylation and deacetylation of histone tails (Yeh et al., 2002). The CD25 expression at the DP stage in the transgenic animals might therefore be caused rather by the sustained cell surface expression of this protein, than by its reexpression. This phenomenon might directly depend on *lck-OBF-1* expression, which starts early in T cell development and might assist in keeping the CD25 locus in an active conformation. The early CD4<sup>-</sup> CD8<sup>-</sup> (DN) thymocytes expressing CD25 at high levels were slightly increased in numbers in the transgenic animals, but the bulk of CD25 surface expression occurred on DP thymocytes. Considering the relatively mild differences in gene expression patterns between these DP CD25<sup>+</sup> and conventional DP (CD25<sup>-</sup>) thymocytes in a microarray experiment using *lck-OBF-1* transgenic mice (discussed later), this population is probably not reflecting a novel population, but rather normal thymocytes that have specifically upregulated the expression of the CD25 gene.

We speculated that these CD25<sup>+</sup> thymocytes might be more easily activated and more prone to apoptosis, but we could not find any indication for this hypothesis. Preliminary experiments using total thymocytes of *lck-OBF-1* and of WT mice in a proliferation assay did not reveal any significant differences in proliferation (not shown). Given the fact that DP thymocytes are undergoing massive apoptosis, we cannot exclude the possibility that a slightly increased rate of apoptosis in transgenic mice could have remained unnoticed. Therefore, it would be of interest to specifically monitor the apoptosis of CD25-expressing cells *in vivo*, or to compare sorted CD25<sup>+</sup> vs. CD25<sup>-</sup> thymocytes in a proliferation assay.



3.1.1.2 CD4<sup>+</sup> CD8<sup>+</sup> DP splenocytes

Another T cell population with an abnormal cell surface marker expression pattern that was found in the transgenic animals were CD4<sup>+</sup> CD8<sup>+</sup> DP splenocytes (Fig. 18). T cells that leave the thymus to circulate in the body or to migrate to secondary lymphoid organs, such as the spleen, are usually committed either to the T helper cell fate and express only CD4 (not CD8), or to the cytotoxic T cell fate and express only CD8 (not CD4). A small population of peripheral CD4<sup>+</sup> CD8<sup>+</sup> T lymphocytes was described in mice and in rats, where they represent a minor thymus-derived subpopulation in the adult animals. These peripheral DP T cells in rats appear among the first T cells that colonize the peripheral lymphoid organs during fetal life, and represent about 40% of peripheral T cells around birth. Their proportion then decreases to reach the low values seen in adulthood. These cells are resistant to undergo apoptosis in response to glucocorticoids, but show poor proliferative responses upon CD3/TCR stimulation due to their inability to produce IL-2 (Jimenez et al., 2002). For mice, it was hypothesized before that this DP population consists of prematurely exported immature thymocytes that could potentially cause autoimmunity (Bonomo et al., 1994).

The splenic DP T cells observed in our study behave similarly to the DP T cells described in rats. They do not seem to proliferate much and do not feature an activated phenotype characterized by CD69 or CD25 expression. Autoimmune responses were not tested, but the average lifespan of the transgenic animals was not shorter than the lifespan of their WT littermates.

It has to be pointed out that the majority of the peripheral T cells had a normal phenotype and that the cytokine profile of the spleen was normal in the transgenic animals. The total numbers of T cells as well as the percentage of CD4 single positive (SP) and CD8 SP T cells were also normal (Section 2.1, Fig. 1).

3.1.1.3  $\gamma\delta$  T cells

Another cell population that is generally established very early during embryogenesis was strongly affected in the *lck-OBF-1* mice: The V $\gamma$ 3 subset of  $\gamma\delta$  T cells, constituting the vast majority of dendritic epidermal T cells (DETC), was missing completely in the skin of young adult transgenic animals (Fig. 22). It was recently postulated that DETCs play a role in tissue repair (Jameson et al., 2002), since they produce keratinocyte growth factors (KGFs) and chemokines upon activation. DETCs that bear  $\gamma\delta$  TCRs on the cell surface are found frequently in murine skin and they produce cytokines and proliferate in response to damaged or stressed keratinocytes, indicating a functional interaction between these two neighboring cell types *in vivo*. The complete lack of DETCs in the transgenic mice could therefore lead to defects in tissue

repair and wound healing. This hypothesis has not been deliberately tested. However, it was not obvious, e.g. in injuries caused by fights among male littermates.

Aside from the DETC population, other  $\gamma\delta$  T cells were affected as well by OBF-1 overexpression, as shown in Figures 20 and 21. In the thymus of *lck-OBF-1* mice, the  $\gamma\delta$  T cells were approximately fourfold increased. This T cell lineage might be favored by the upregulated CD25 expression in the transgenic thymus (see Table V and Section 6.1).

Interestingly, the deregulated expression of CD25, the IL-2R alpha chain, in the transgenic mice, could be explaining part of the observed abnormalities of the  $\gamma\delta$  T cell and NK cell lineages. It is possible that the inappropriate expression of CD25 influences the lineage commitment to NK cells, which rely on IL-15 signaling. As mentioned in the results section (Section 2.2.3.4), the IL-15 receptor also uses CD122, the IL-2 receptor beta chain as one of its components. It is thus conceivable that inappropriately expressed IL-2R $\alpha$  competes with IL-15R $\alpha$  for their common beta chain.

## 3.1.2 Possible target genes identified by microarray analysis

In order to identify genes directly regulated by OBF-1, we first performed microarray analysis comparing WT to transgenic thymocytes. Given the fact that the overall T cell development was normal and the major T cell populations were present at normal ratios in the transgenic animals, we assumed that the comparison of RNA from the entire organ should reflect mostly the main differences in gene expression within the T cells expressing the transgene, rather than reflecting merely a change in cellular composition of the thymus. The analysis revealed a rather defined set of differentially regulated genes in the total thymocyte populations of transgenic animals compared to WT mice. In a second microarray experiment we used fluorescence-sorted thymocytes from *lck-OBF-1* transgenic mice and compared CD25<sup>+</sup> CD4<sup>+</sup> CD8<sup>+</sup> cells to CD25<sup>-</sup> CD4<sup>+</sup> CD8<sup>+</sup> cells. The gene expression analysis of these sorted subpopulations of transgenic T cells showed an expression profile largely overlapping with the profile found in the first microarray experiment, thus demonstrating that the observed changes in global gene expression are rather T cell-intrinsic, and not caused by other (non-T) cells that contribute to the gene expression pattern of total thymocyte RNA.

It should be pointed out at this point that the overexpression of OBF-1 in T cells can be considered as a useful model, especially when compared with cells from OBF-1 deficient mice. In some cases, overexpression can yield clearer effects; the findings can then be studied in WT and OBF-1 deficient background, thus bringing into focus the genes that are the most closely co-regulated with OBF-1.



Using a genome-wide expression analysis to identify potential target genes can be a very successful approach, provided that the experimental setup is suitable. Given the fact that OBF-1 expression is mainly required in B cells, while we study its overexpression in T cells, the most interesting potential target genes should have a defined function in B cells, and the effect that OBF-1 deficiency has on their expression level should ideally be the opposite.

### 3.1.2.1 Spi-B

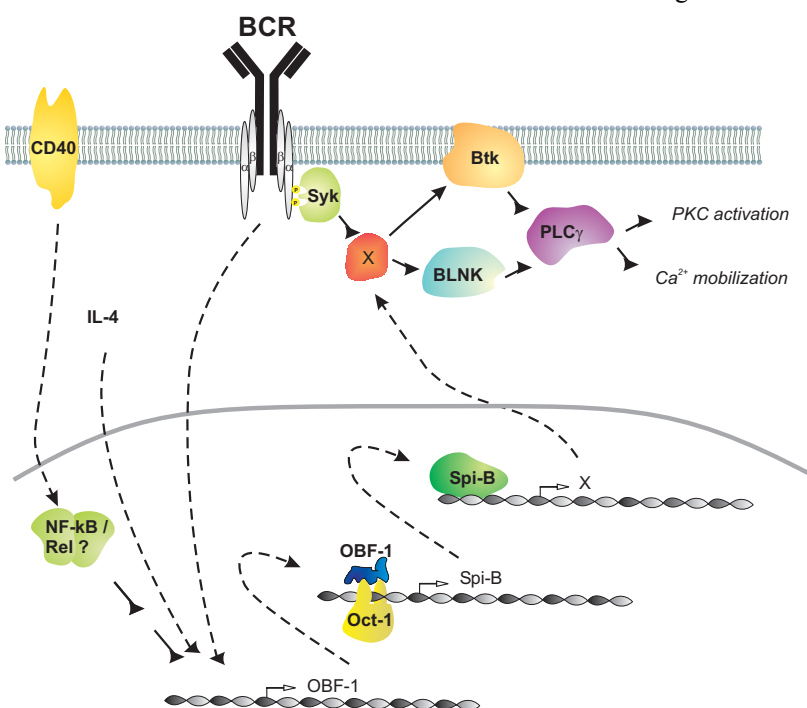
We have indeed successfully applied the microarray analysis technique to compare the global gene expression of the *lck-OBF-1* transgenic thymus to WT thymus. As discussed earlier, OBF-1 overexpression lead to the modulated expression of a small number of specific genes (see Table V and Appendix (Section 6.1)). Without setting any threshold for differentially expressed genes, other than eliminating background noise by replicate experiments, we found about 69 genes upregulated and 123 genes downregulated (out of approximately 12,000 genes and ESTs) in the transgenic thymocytes, compared to WT littermates. One of these genes, encoding the transcription factor Spi-B, was identified as a physiologically relevant candidate. The comparison with OBF-1 deficient thymocytes, which have markedly decreased Spi-B mRNA levels (Section 2.1, Fig.2), strongly enhances the relevance of our findings. The results are presented and discussed in greater detail in the publication in Section 2.1 of the thesis. They constitute the main findings of this work and present compelling evidence that OBF-1 is necessary for efficient transcription of the Spi-B gene in T and B cells of the mouse. We show that OBF-1 exerts its effect by directly binding to the Spi-B promoter 2, thereby enhancing its transcription.

Spi-B was upregulated almost 9-fold in

transgenic thymus – that was the most highly upregulated gene on the array. Our data suggest that the expression of Spi-B, directly controlled by the upregulation of OBF-1 protein in germinal center (GC) B cells, may be a key element in the formation of GCs. The target gene Spi-B could in fact explain a substantial part of the observed phenotype of OBF-1 deficient mice, since the lack of Spi-B leads to significant defects in GC formation and maintenance (Su et al., 1997). OBF-1 acting directly upstream of Spi-B transcription might thus serve as an important regulator of this transcriptional cascade. Spi-B can therefore be considered as the most physiologically relevant *in vivo* target of OBF-1 identified to date.

Figure 35 presents a model that places OBF-1 at the crossroads between being an integrator of CD40, interleukin and BCR signaling and being an effector molecule controlling, *via* Spi-B, the coupling of BCR signaling events to downstream pathways.

Interesting new findings have shown that in Spi-B<sup>-/-</sup> PU.1<sup>-/-</sup> IL-7 and stromal cell dependent proB cell lines, transcription of the Ig lambda light chain is reduced, but transcription of other putative Spi-B targets, such as Ig kappa light chain and Ig heavy chain (IgH) genes, is not reduced, or even increased (Schweitzer and DeKoter, 2004). The OBF-1 levels were not investigated in this work, but it is tempting to speculate that OBF-1 levels might not be affected by the lack of PU.1 and Spi-B, or might even be upregulated in a compensatory manner. This could explain the higher levels of CD25 and the increase in IgH and Igκ transcription, which appear to be independent of Spi-B/PU.1, but perhaps dependent on OBF-1. In contrast, transcription of Igλ, which takes place at the preB cell stage after successful rearrangement of IgH, is impaired in PU.1/Spi-B double deficient B cell lines. The authors hypothesize that interference with PU.1 and/or Spi-B activity in proB cells promotes differentiation to a stage intermediate between late proB cells and preBI



**Figure 35** Model integrating OBF-1 in a transcriptional cascade leading to activation of downstream effectors of BCR signaling in activated B cells. OBF-1 transcription is induced upon signals coming from CD40, IL-4 and the BCR, and OBF-1 protein expression modulates Spi-B transcription which, in turn, acts upon an unknown target X that couples BCR activation and Syk phosphorylation to downstream phosphorylation events, such as BLNK, Btk and PLCγ phosphorylation.

cells. In this context, Spi-C (Prf), a close relative of Spi-B and PU.1, might serve as a negative regulator of Spi-B/PU.1 activity. In fact, Spi-C is specifically expressed in B cells between the preB cell and the mature B cell stages, but not in plasma cells. It is a very weak transcriptional activator that can bind to PU.1 DNA binding sites, but unlike PU.1 and Spi-B, it cannot interact with IRF-4 (Pip, LSIRF, NF-EM5) to synergistically activate gene expression on Ets-IRF composite elements (EICE) (Bemark et al., 1999; Carlsson et al., 2003; Hashimoto et al., 1999). Perhaps, Spi-C can normally interfere with PU.1/Spi-B activity starting at the preBI stage of B lymphopoiesis, and this negative regulation might be required for normal B cell development.

This hypothesis is suitable to explain defects that we observed in the  $E\mu$ -V<sub>H</sub>-OBF-1 transgenic mice that overexpress OBF-1 in B cells. In these animals, B cell development is blocked at the preB cell stage. This block might be directly due to higher expression levels of Spi-B. To investigate this, RNA expression analysis of sorted proB cells of WT and  $E\mu$ -V<sub>H</sub>-OBF-1 transgenic mice would be a suitable approach. It would also be useful to stain the B220<sup>+</sup> c-kit<sup>+</sup> proB cells with a marker for apoptosis, such as Merocyanin-540.

The related interferon regulatory factors IRF-4 and IRF-8 (ICSBP) were identified as additional important players besides Spi-B/PU.1 in the preB to B cell transition (Lu et al., 2003; Muljo and Schlissel, 2003). IRF-4 and IRF-8 are selectively expressed in the lymphoid and myeloid cells of the immune system. Both can be recruited to IRF DNA binding sites by interaction with other transcription factors that bind close to the IRF site. In particular, it was shown that Spi-B and PU.1 can recruit IRF-4 or IRF-8 to Ets-IRF composite elements (EICE) present in the Ig  $\kappa$  3' and  $\lambda$  enhancers, where they play a role in the control of Ig light chain gene transcription (Brass et al., 1999; Eisenbeis et al., 1995; Escalante et al., 2002; Pongubala et al., 1992).

In the microarray experiments, IRF-4 and IRF-8 were not affected by OBF-1 expression in transgenic thymocytes. On the other hand, the promoter analysis of the genes that were upregulated in the T cells overexpressing OBF-1 revealed a high incidence of IRF-8 binding sites (Table IV). It is possible that the overexpression of Spi-B that we observed contributed to an increased stabilization of IRF-8 on EICEs.

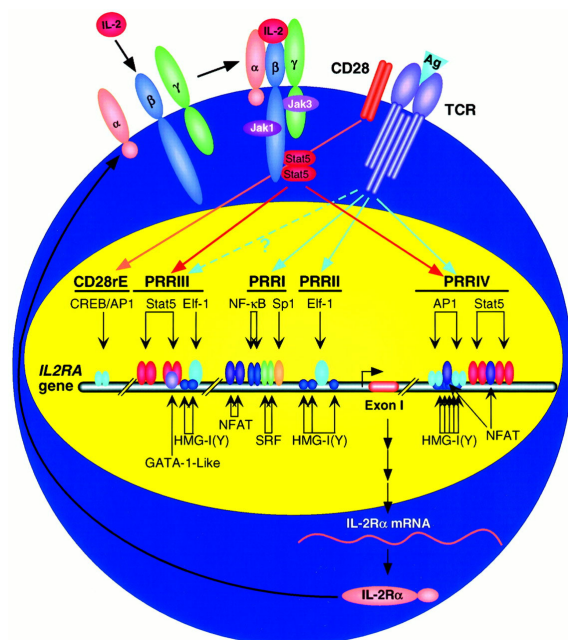
In proB cell lines transformed with the Abelson virus, enforced expression of Spi-B and IRF-4 was shown to be sufficient to induce Ig $\kappa$  germline transcription and to promote B cell differentiation (Muljo and Schlissel, 2003). In this experimental system, transcription of Spi-B and of IRF-4 is upregulated upon treatment with the Abl kinase inhibitor STI571 (Gleevec). We show that the upregulation of Spi-B transcription critically depends on OBF-1 (Section 2.1, Fig. 6). We observe that inhibition of the v-Abl kinase does not influence OBF-1 expression, but rather prevents Spi-B transcription through a different mechanism, since

the presence of OBF-1 is not sufficient in itself for induction of Spi-B transcription in these cells, and the OBF-1 protein or RNA levels in v-Abl transformed B cells are not noticeably altered by STI571 treatment (not shown). Neither Spi-B, nor OBF-1 appear to be essential at this early stage of B cell development, at which they might be redundant: Other related Ets factors, such as PU.1 or Spi-C could compensate for Spi-B function in the absence of Spi-B.

### 3.1.2.2 CD25

As mentioned previously, the cell surface expression of CD25 was greatly increased on a subpopulation of thymic T cells, thus it was hardly surprising to observe a 2.4-fold upregulation of CD25 mRNA levels on the microarray. The upregulation of this gene also provided an internal control to the microarray experiment. Interestingly, a high expression of transgenic OBF-1 was detected in sorted CD25<sup>+</sup> DP thymocytes, showing a direct correlation between these two molecules (Fig. 25). In addition, endogenous OBF-1 mRNA was expressed at detectable levels in the CD25<sup>+</sup> population, but not in the CD25<sup>-</sup> population. This finding raises the possibility of a positive feedback loop in which the overexpression of OBF-1 protein would promote its own gene transcription.

The CD25 promoter is well-studied, and there is no evidence that OBF-1 might directly lead to its activation. The regulatory elements of the CD25 promoter are discussed in (Kim and Leonard, 2002).



**Figure 36** Regulatory elements of the *IL-2R $\alpha$*  gene (CD25). The scheme depicts the transcription factor binding sites in the five positive regulatory regions (PRRI to PRRV (CD28-responsive enhancer, CD28rE). It shows how PRRI, PRRII and PRRIV are regulated for optimal TCR activation whereas PRRIII and PRRIV are regulated for IL-2 inducibility. Figure from (Kim and Leonard, 2002). No octamer or PU.1 binding sites were identified.

They are summarized in Figure 36. It is generally believed that a number of transcription factors belonging to unrelated families work in cooperation to activate the CD25 gene by interacting with the five positive regulatory regions (PRRI to V). However, octamer sites to which OBF-1 could bind were not identified in the studies of the CD25 gene regulatory regions. OBF-1 could have an influence on CD25 expression *via* an effect on IL-2 expression, but we found IL-2 levels to be unaltered in the transgenic thymocytes (Fig. 35). It is possible that binding of other transcription factors, such as AP-1, NF- $\kappa$ B, NFAT, CREB, Stat5, SRF, HMG-I(Y), GATA-1-Like, or Elf-1 to their recognition sites in the CD25 promoter could be modulated by OBF-1, but we have no evidence for that.

### 3.1.2.3 PLC $\gamma$ 2

Yet another interesting candidate target gene that has been identified in the microarray analysis is the phospholipase C gamma 2 (PLC $\gamma$ 2), whose transcription was 7.2-fold upregulated in transgenic thymocytes. This enzyme catalyzes the hydrolysis of phosphatidylinositols to generate diacylglycerol (DAG) and inositol phosphates, including inositol 1,4,5-trisphosphate (IP<sub>3</sub>). DAG activates protein kinase C (PKC), while IP<sub>3</sub> mediates the mobilization of Ca<sup>2+</sup> from internal stores, resulting in a transient intracellular Ca<sup>2+</sup> flux (Rhee and Bae, 1997). Expression of PLC $\gamma$ 2 was more than 7-fold upregulated in *lck-OBF-1* transgenic thymus compared to WT thymus. We could not identify a perfect octamer site in the putative promoter sequence, therefore it still needs to be tested whether PLC $\gamma$ 2 is a direct OBF-1 target, or whether the effect is indirect.

PLC $\gamma$ 2 is a very interesting molecule that is required for B cell development and survival. It was shown recently that coupling between the BCR and PLC $\gamma$ 2 is essential for mature B cell development. (Hikida et al., 2003). The ablation of PLC $\gamma$ 2 leads to a block in proB cell differentiation, reduced numbers of follicular mature B cells, B1 B cell deficiency, and to poor responses to T-independent type 2 (TI-2) antigens. PLC $\gamma$ 2<sup>-/-</sup> mice have defects in the IgM-induced Ca<sup>2+</sup> flux and in the mitogen-induced proliferation. Fc $\gamma$ R and Fc $\epsilon$ R signaling are also defective, resulting in a loss of collagen-induced platelet aggregation, mast cell Fc $\epsilon$ R function, and NK cell Fc $\gamma$ RIII and 2B4 function, as well as to resistance to Fc $\epsilon$ R-mediated, as well as to Fc $\gamma$ R-mediated, cutaneous inflammatory skin reaction (Hashimoto et al., 2000; Wang et al., 2000; Wen et al., 2002).

### 3.1.2.4 Fc $\gamma$ RII $\beta$

In this context, it is interesting to note that one downstream target of PLC $\gamma$ 2, Fc $\gamma$ RII $\beta$ , is also upregulated (2.2-fold) in the thymocytes overexpressing OBF-1. Fc receptors (FcR) belong to the Ig receptor superfamily and recognize the Fc portion of immunoglobulins. They couple humoral

and cellular immunity by directing the interaction of antibodies with effector cells. Distinct FcRs exist for each Ig isotype: Fc $\gamma$ R binds IgG; Fc $\alpha$ R binds IgA, and Fc $\epsilon$ R binds IgE. In addition, the FcRs are classified by their binding affinities: High affinity FcRs are referred to as FcRI, and these are able to bind noncomplexed, monomeric immunoglobulins. Low affinity FcRs bind only aggregated immunoglobulins or antibodies complexed to multivalent antigens, and they are referred to as FcRII/III (Ravetch, 1997; Ravetch and Clynes, 1998). Fc $\gamma$ Rs are widely expressed in most hematopoietic cells, and are capable of triggering several cell signaling pathways via activation of cytoplasmic protein tyrosine kinases, such as the Src, Syk and Tec family kinases (Durden et al., 1995; Eiseman and Bolen, 1992; Hutchcroft et al., 1992; Kawakami et al., 1994; Pignata et al., 1993). The PLC $\gamma$ /Ca<sup>2+</sup>/PKC pathway has been shown to be involved in the activation of all types of mitogen-activated protein kinases (MAPK; ERKs, c-Jun N-terminal kinases (JNKs), and p38 MAPKs) (Hashimoto et al., 1998; Jiang et al., 1998; Ueda et al., 1996). The PLC $\gamma$ /Ca<sup>2+</sup>/PKC pathway leads to the activation of transcription factors, including NFAT, CREB, and Ets transcription factors, such as Elk-1 (Jain et al., 1993; Vaishnav et al., 2003; Wasylyk et al., 1998). Ultimately, activation of the FcR can lead to phagocytosis, Ab-dependent cell cytotoxicity, respiratory burst, degranulation, production of inflammatory cytokines, and enhanced Ag presentation (Ravetch, 1997).

Other potential target genes that were identified in the microarray experiments include muscle-specific genes, such as the MADS box transcription factor myocyte enhancer factor 2B (MEF2B), and the myosin alkali light chain gene 4 (Myl4), which is expressed in the cardiac atria and in the embryonic muscle.

### 3.1.2.5 MEF2B

The members of the MEF2 family of transcription factors (MEF2A to MEF2D) bind to a conserved A/T-rich sequence in the control regions of many skeletal and cardiac muscle genes. MEF2B transcripts are expressed during mouse embryogenesis in the developing cardiac and skeletal muscle cells, but not in the adult mouse, in a pattern distinct from but overlapping with those of the other Mef2 genes (Edmondson et al., 1994). It was shown that MEF2B, unlike the other Mef2 family members, is part of a smooth muscle-specific complex that binds to an A/T-rich element important for smooth muscle myosin heavy chain gene expression (Katoh et al., 1998). Both the *mef2B* and *mef2C* genes are expressed in the B cells of the peripheral white pulp of the postnatal spleen, but are not detected above background in the plasma cells present in the red pulp (Swanson et al., 1998). MEF2 proteins have also been shown to interact with other transcription factors, including myogenic bHLH factors and the ETS transcription factor PEA3 to express muscle-



specific genes in muscle cell culture (Molkentin et al., 1995; Taylor et al., 1997). MEF2 proteins could possibly interact with E2A bHLH and other ETS transcription factors, such as Spi-B, in a synergistic manner.

In our microarray experiments, MEF2B mRNA was 6.7-fold upregulated in *lck-OBF-1* thymocytes compared to WT samples. We could not identify an octamer site in its promoter, but PU.1 and IRF-8 binding sites were present. The increased expression of Spi-B as a direct result of *lck-OBF-1* expression in the transgenic thymus might be the cause of enhanced expression of MEF2B.

#### 3.1.2.6 Myosin alkali light chain gene 4 (Myl4)

MEF2B was identified as a component of a smooth muscle-specific complex important for smooth muscle myosin heavy chain gene expression. Myosin is the major component of the thick filament of striated muscle, and it is formed by two myosin heavy chain molecules and four myosin light chains (MLC). There are two types of light chains, the regulatory (MLC2-type, or phosphorylatable) light chains, and the alkali (MLC1- and MLC3-type, or essential) light chains. Different isoforms of both heavy and light chains exist in different striated muscle types. The alkali light chains are associated with the head region of the myosin molecule (Waller and Lowey, 1985) and are in close contact with actin (Henry et al., 1985; Labbe et al., 1986). They appear to play a role in the actomyosin interaction (Barton et al., 1988) and to be involved in the stabilization and regulation of the myosin structure.

The 7.6-fold upregulation of Myl4 that we observed in transgenic thymocytes is at the same highly elevated level as the upregulation of MEF2B. It is possible that their gene expression is linked, controlled by the same signaling events that trigger the expression of a muscle-specific gene program. One important factor known to modulate gene expression in muscle-specific genes is intracellular calcium signaling. Indeed, an increased activity of PLC $\gamma$ 2 might lead to increased Ca<sup>2+</sup> release from internal stores. This might be one way how the expression of Myl4 can be activated in the transgenic thymocytes, which expressed higher levels of PLC $\gamma$ 2 than WT thymocytes.

#### 3.1.2.7 Gadd45 $\beta$

Gadd45 beta is a growth-arrest and DNA-damage-inducible gene that gets activated in response to various cellular stress signals, and also by inflammatory cytokines, such as IL-6, IL-18 and IL-12, TNF and TGF- $\beta$ 1. It can also be induced by treatment with lipopolysaccharide (LPS). Gadd45 $\beta$  is involved in cell cycle arrest and apoptosis and is required for the generation of T<sub>H</sub>1 cells *in vivo* (Lu et al., 2001). Gadd45 is transcriptionally upregulated by p53 *via* an intronic p53 consensus-binding site in the Gadd45 gene (Kastan et al., 1992), but it also can

be regulated in a p53-independent manner (Zhan et al., 1996). It was shown recently that the Gadd45 promoter contains binding sites for Oct-1 and NF-Y which are required for and concertedly contribute to the p53-independent activation of Gadd45 following binding of BRCA1 (Fan et al., 2002) and following treatment with the HDAC inhibitor TSA (Hirose et al., 2003). Moreover, Gadd45 $\beta$  expression is regulated by NF- $\kappa$ B (Jin et al., 2002), resulting in the suppression of JNK signaling by targeting MKK7/JNKK2 and inhibiting its enzymatic activity. This inhibition is crucial for the suppression of TNF- $\alpha$  induced apoptosis and links the NF- $\kappa$ B signals to the MAPK pathway (De Smaele et al., 2001; Papa et al., 2004; Tang et al., 2001).

In our microarray analyses, Gadd45 $\beta$  was mildly, but consistently upregulated (1.4-fold) in the transgenic thymocytes. This could be explained by the direct binding of OBF-1 together with Oct-1 on the perfect octamer site present in the Gadd45 $\beta$  promoter. As we have seen previously, Oct-1 and NF-Y act in concert on the Gadd45 $\beta$  promoter. Binding of OBF-1 could interfere with binding of either BRCA1 or other ternary factors and thereby modulate the transcriptional activity of the Gadd45 $\beta$  promoter. Thus, Gadd45 $\beta$  might be a direct target gene, although the physiological importance of OBF-1 under these conditions remains unclear. It might be a purely artificial transcriptional increase resulting from ectopic overexpression of this coactivator, but it might just as well be a mechanism that operates under certain physiological conditions in cells in which stress or DNA-damage signals, such as single or double strand breaks occur – as it happens in T and B cells undergoing Ig rearrangement.

#### 3.1.2.8 Other upregulated genes

Another expected result was the increased expression of TCR gamma (we detected a 1.8-fold increase in V $\gamma$ 2 transcription), a component of the TCR of  $\gamma\delta$  T cells. As shown in Figure 20, the  $\gamma\delta$  T cell population was increased in the thymus of transgenic animals. We assume that the enhanced V $\gamma$ 2 expression is caused solely by the altered thymic composition, and not by a direct activation of  $\gamma\delta$  TCR transcription by OBF-1.

Similarly, the hyaluronate binding protein CD44 was 2.8-fold upregulated in transgenic thymocytes. This increase might be due to the increased number of early T cells from the CD44<sup>+</sup> CD25<sup>-</sup> or the CD44<sup>+</sup> CD25<sup>+</sup> CD4<sup>-</sup> CD8<sup>-</sup> (DN) populations that we observed by FACS analysis (Fig. 19).

#### 3.1.2.9 Downregulated genes

Aside from genes that were upregulated in the thymus of *lck-OBF-1* transgenic mice, we found an even larger portion of genes that were downregulated compared to WT thymus. So far, OBF-1 has always been characterized as an activator of transcription, and not as a repressor. The manner in which

OBF-1 may achieve downregulation of genes can be indirect *via* the induction of a repressor protein, or direct, e.g. by competing with another Oct-binding factor, such as the small nuclear RNA-activating protein complex (SNAPc), or by stabilizing Oct-1/Oct-2 on promoters, and thereby allowing or precluding the binding of another transcription factor on an adjacent or overlapping binding site. One example for such an interaction of OBF-1/Oct-1 complexes with another transcription factor is the association of the CCAAT/enhancer-binding protein (C/EBP) with octamer factors on Ig promoters. This association presumably contributes to the repressory action of the inhibitory isoform C/EBP $\beta$ -3 on Ig promoters, which occurs mainly through the octamer elements (Hatada et al., 2000).

While a number of genes were less expressed in *lck-OBF-1* transgenic thymus than in WT thymus, the relative decrease was generally less pronounced than the increase in several putative target genes, such as Spi-B, MEF2b, Myl4, and PLC $\gamma$ 2. Most strikingly, several genes encoding lymphoid-specific cell adhesion molecules were downregulated, including CD6, CD5, lymphotoxin beta (LT $\beta$ ) and LFA-1.

#### 3.1.2.9.1 CD6

The CD6 cell surface glycoprotein is preferentially expressed by T lymphocytes and B1a cells, and functions both as a co-stimulatory and adhesion receptor on T cells. It interacts with CD166 (ALCAM) on thymic epithelial cells and keratinocytes, and probably other ligands expressed on such antigen presenting cells. It was shown that CD6 expression increases as double-positive thymocytes are selected to a single-positive stage and that it correlates with expression of the selection marker CD69 (Singer et al., 2002). In addition, CD6 mediates binding of a transformed CD4<sup>+</sup> T cell line to gamma-interferon activated keratinocytes (KCs) (Singer et al., 1997), and could therefore have a function in the immune responses in the skin.

The expression of CD6 was downregulated approximately 2.2-fold in the transgenic thymocytes.

#### 3.1.2.9.2 CD5

CD5, a lymphocyte surface protein of the scavenger receptor cysteine-rich superfamily, closely related to CD6 and highly homologous in the extracellular region, was also downregulated to a similar extent (1.8-fold) as CD6. Both can physically interact on the cell surface of lymphocytes and colocalize at the immunological synapse upon lymphocyte activation (Gimferfer et al., 2003).

#### 3.1.2.9.3 Lt $\beta$

Yet another cell adhesion molecule, Lt $\beta$  (lymphotoxin beta), which is important for B cell homing to the germinal center, was found to be downregulated (2.3-fold) in the transgenic thymo-

cytes. Lt $\beta$ -deficient mice have a disrupted marginal zone, lack splenic germinal centers and follicular dendritic cells, as well as Peyer's patches and peripheral lymph nodes (Koni et al., 1997). Mice with a B cell-specific deletion of Lt $\beta$  retained the morphological defects in marginal zone and germinal center formation (Tumanov et al., 2002). Similar defects, notably in the GC formation, but in one specific mouse strain also in the MZ formation (Samardzic et al., 2002b), were also observed in mice deficient for OBF-1. It would therefore be interesting to study the expression of Lt $\beta$  in the spleen OBF-1 deficient mice.

#### 3.1.2.9.4 Integrin alpha L (LFA-1)

LFA-1 is exclusively expressed on leukocytes and interacts with its ligands ICAM-1, -2, and -3 to promote a variety of homotypic and heterotypic cell adhesion events required for normal and pathological functions of the immune systems. The integrin LFA-1, together with alpha 4 beta 1, has an important function in promoting lymphocyte entry into the splenic white pulp and the retention of B cells in the marginal zone (Lo et al., 2003; Lu and Cyster, 2002). It is downregulated 1.7-fold in transgenic thymocytes.

In this context, it is interesting to note that PGRL, a CD81-associated protein on lymphocytes is upregulated (2.3-fold) in the transgenic thymocytes. CD81 is a costimulatory molecule that is reported to influence adhesion, morphology, activation, proliferation, and differentiation of B, T, and other cells. Anti-CD81 antibody was shown to activate LFA-1 on human thymocytes and can also affect B-T cell interactions through an increase in IL-4 synthesis by T cells responding to antigen presented by B cells (Levy et al., 1998). In view of the reported function of CD81, it appears possible that the increased expression of the CD81-associated molecule PGRL leads to the reduction of LFA-1, and possibly also other cell adhesion molecules.

#### 3.1.2.10 Previously identified putative OBF-1 target genes

Direct OBF-1 target genes known to date include the BLR1 gene (Wolf et al., 1998), the CCR-5 gene (regulated by OBF-1 and Oct-1) in T cells (Moriuchi and Moriuchi, 2001), the B cell-specific B29 and mb1 genes (Malone and Wall, 2002). Recently, the *Kcnn4* promoter, the *Lck* distal promoter (Kim et al., 2003b) and the *Adh2*-like promoter (Brunner et al., 2003a) have been identified to be directly bound and activated by OBF-1 in early B cell cultures. In addition, the osteopontin gene was characterized as being an OBF-1 target gene (Lins et al., 2003). In the microarray experiments of *lck-OBF-1* transgenic thymocytes, the expression of these genes was unchanged. It is possible that these genes are not OBF-1 target genes in early T cells, and that the presence of other, B cell lineage-specific and B cell stage-specific factors is required for coactivation by OBF-



1. It is also possible that additional signaling events are necessary to change the phosphorylation status of either OBF-1 directly, or of other targets, such as Oct-1 or Oct-2. In the case of Oct-2 it has been proposed that phosphorylation of a residue in the POU domain interferes with binding of Oct-2 to the non-canonical octamer sequence in the murine BLR-1 promoter (Pevzner et al., 2000).

## 3.2 Overexpression of OBF-1 in B cells

OBF-1 expression is mainly required in B cells, and therefore we generated transgenic mice that express OBF-1 under the control of the  $\mu$  enhancer and the VDJ promoter, which were characterized previously to confer B cell-specific expression. In contrast to the endogenous OBF-1 promoter, however, the transgenic promoter/enhancer construct should activate transcription from early stages of B cell development on, where OBF-1 is normally not yet expressed. Upon generating B cell-specific OBF-1 transgenic mice, we hypothesized that expression of this transcriptional coactivator might lead to an activated phenotype, increased B cell responses, or similar signs of hyperreactivity. We were surprised to find rather the opposite phenotype and a strongly reduced B cell compartment in these  $E\mu-V_H-OBF-1$  mice.

The analysis of  $E\mu-V_H-OBF-1$  mice of the mouse line #30 suggests that enforced expression of OBF-1 in early B cell development can be deleterious for these cells. The block in B lymphopoiesis appeared at the preBI to preBII transition. At that stage in B cell development, the V to DJ<sub>H</sub> and V-J<sub>L</sub> rearrangements occur. Involvements of OBF-1 in the expression of the Ig light chain genes have been reported, and they could be one reason for the developmental block. In addition, overexpression of Spi-B in human hematopoietic progenitor cells can block T, B and NK cell development *in vitro* (Schotte et al., 2003).

B cells that have differentiated beyond the preBII cell stage do not express noticeable levels of transgenic OBF-1 protein, as judged by western blot analysis of total bone marrow and spleen extracts (Fig. 29). mRNA, however, can be detected by northern blot and RT-PCR/southern blot in the bone marrow and in the spleen of the  $E\mu-V_H-OBF-1$  mice of line #30 and, at lower levels, line #8 (Figures 28 and 29).

We plan to analyze the early B cells in this transgenic mouse model in greater detail to address the question whether the block of B cell development and the lack of OBF-1 protein expression in B cells beyond the preBII cell stage are caused, as we hypothesize, by an increase of apoptosis of B cells that highly express OBF-1. We also plan to analyze sorted c-kit<sup>+</sup> B220<sup>+</sup> and CD25<sup>+</sup> B220<sup>+</sup> bone marrow

B cells for expression of OBF-1, Spi-B and markers of apoptosis.

As mentioned earlier, our analysis relies mostly on experiments conducted in this one mouse line (line #30), which shows the most severe and most obvious phenotype in B cell development. A reduction of mature B cells was also observed in mice from other mouse lines, but it was clearly less severe than in line #30. We need to analyze at least another line to study its transgene expression and phenotype more in detail in order to assure that what we observe in line #30 is not an artifact specific to this line, but directly due to transgenic OBF-1 expression in bone marrow B cells of these animals. Of course, the generation of transgenic animals ectopically overexpressing the transgene always creates an artificial situation that will easily lead to artifacts that do not reflect the natural *in vivo* function of the protein being studied. Therefore, one has to be cautious with the interpretation of the results. We specifically used this method to identify candidate target genes that can be verified in the wild type situation, and that also clearly depend on OBF-1, based on the comparison of OBF-1 deficient mice to wild type mice. Thus, the analysis of line #30 alone is already useful, but should be backed up by data from at least one additional transgenic line. We are currently planning to investigate line #543 more in detail and to deepen our analysis of ectopic OBF-1 expression in B cells.

## 3.3 Summary

There is growing evidence that OBF-1 has an important function in modulating BCR signaling strength, most likely by acting downstream of the BCR in the PLC $\gamma$ 2/PKC/Ca<sup>2+</sup> pathway. Firstly, *OBF-1* deficient B cells have a defect in BCR-triggered Ca<sup>2+</sup> mobilization, which was restored in *CD22*<sup>-/-</sup> *OBF-1*<sup>-/-</sup> compound mutant mice (Samardzic et al., 2002a). CD22 is a negative regulator of BCR signaling, and CD22-deficient mice have a lowered activation threshold for BCR crosslinking and increased Ca<sup>2+</sup> mobilization upon BCR stimulation (Nitschke et al., 1997).

Additional evidence for this is offered by the work comprised in the present thesis. We show that OBF-1 transcriptionally activates the Spi-B transcription factor, which was shown previously to be involved in this pathway. It was assumed that this involvement of Spi-B (together with PU.1) is mediated by an as yet unidentified protein X coupling Syk phosphorylation to the phosphorylation of downstream targets, such as PLC $\gamma$  and BLNK (Garrett-Sinha et al., 1999). In this model, the level of Spi-B protein would directly control the transcription of this factor X (see also Fig. 35).

Second, we observed a transcriptional upregulation of PLC $\gamma$ 2 in T cells overexpressing OBF-1. We plan to investigate PLC $\gamma$ 2 expression in OBF-1 deficient B cells, especially of splenic follicular B cells.

If the lack of OBF-1 lead to reduced PLC $\gamma$ 2 expression, this would represent a second manner how OBF-1 activity could interfere with parts of the BCR signaling cascade essential for B cell maturation. Several downstream targets of the PLC $\gamma$ /Ca<sup>2+</sup>/PKC pathway were also affected by the overexpression of OBF-1 in T cells, including Fc $\gamma$ RII $\beta$ , and, possibly, Myl4.

A third line of evidence for the involvement of OBF-1 in BCR signaling was the finding that B cells lacking OBF-1 also show a reduced proliferation following anti-IgM stimulation *in vitro* (Sun et al., 2003). *In vivo*, it has been recognized earlier that OBF-1 deficiency affects early B lymphopoiesis, leading to a reduction of transitional splenic B cells (Hess et al., 2001; Schubart et al., 2000), possibly caused by apoptosis during the negative selection of self-reactive B cells occurring at this stage (Carsetti et al., 1995; Sater et al., 1998). Therefore, OBF-1 might well be directly involved in the selection of transitional B cells. Lack of OBF-1 has an impact on the Ig  $\kappa$  repertoire (Jankovic and Nussenzweig, 2003), leading to a reduction of a subset of Ig  $\kappa$  genes (Casellas et al., 2002). On the other hand, we observe that constitutive overexpression of OBF-1 in B cells of *E $\mu$ -V $H$ -OBF-1* transgenic mice results in a severe block of B cell development at the preB1 to preBII transition in the bone marrow (Fig. 29), further supporting the concept that a tight control of OBF-1 expression is critical for early B cell development. In addition, the reduced numbers of marginal zone B cells found in one specific OBF-1 deficient mouse strain (Samardzic et al., 2002b) could be explained by a model in which OBF-1 is required for setting the normal threshold for immature B cell development and selection in the bone marrow. It is currently unclear whether this effect of OBF-1 deficiency is independent of Spi-B, whose expression levels were also reduced in the bone marrow of OBF-1 deficient mice (data not shown). It could be caused by direct binding of OBF-1/Oct-1 or OBF-1/Oct-2 complexes to specific octamer sites in some of the Ig  $\kappa$  promoters.

While the modulation of BCR signaling strength could be one important physiological function of OBF-1, the crucial function of OBF-1 in the development of GCs in the spleen reflects a different mechanism, since OBF-1<sup>-/-</sup> CD22<sup>-/-</sup> double deficient mice were still unable to form GCs and to mount humoral immune responses, although their BCR activation threshold was similar to the WT situation (Samardzic et al., 2002a). Our data presented here indicate that the expression of Spi-B, controlled by the upregulation of OBF-1 protein in the GC B cells may be a key element in the GC formation that does not rely on an altered BCR signaling strength, but rather on the activation of one specific downstream pathway following BCR activation.

In brief, we have generated transgenic mice overexpressing OBF-1 and have identified Spi-B as the most physiologically relevant *in vivo* target of OBF-1 to date. These findings greatly help to extend

our understanding of how OBF-1 is involved in B cell activation and GC formation and place it directly upstream of Spi-B in a transcriptional cascade that appears to be intimately linked to B cell receptor signaling.

## 4 Materials and Methods

### Mouse strains and cell lines

The *lck*-OBF-1 construct used to generate transgenic mice contains an N-terminally HA epitope-tagged human OBF-1 cDNA under the control of the murine proximal *lck* promoter (-3100 to +23 relative to the transcription start site).

The  $E\mu$ - $V_H$ -OBF-1 construct used to generate the second line of transgenic mice contains the same N-terminally HA epitope-tagged human OBF-1 cDNA, this time under the control of the murine VDJ enhancer ( $E\mu$ ) coupled to the  $V_H$  promoter. Sequence data is available at <http://www.fmi.ch/groups/matthias.p/Bartholdy.html>.

Both transgenic mouse lines were obtained and bred in B6CF1 x C57BL/6 background and were bred to a wild type partner of the same genetic background. OBF-1 deficient mice were in 129SV-C57BL/6 background. All animal work and experiments were done in compliance with local and institutional regulations.

For RNA expression analysis, mice were sacrificed, the organs were taken, rinsed in PBS and immediately homogenized in TRIzol reagent (Life Technologies). Total RNA was prepared using TRIzol reagent and purified on RNeasy Miniprep columns (Qiagen) according to the manufacturers' instructions.

Cell lines: Abelson pro-B cell lines were derived from wild type and *OBF-1*<sup>-/-</sup> mice and were described previously (Schubart et al. 1996b)

### PCR

To determine the OBF-1 genotype, genomic DNA was isolated from tail biopsies and used as a template for polymerase chain reaction (PCR). The PCR assay for the OBF-1 wild type and OBF-1 mutant loci was carried out with the following primer combinations, respectively:

5'-GCT CCC TGA CCA TTG AC-3',

5'-TCC TGT CCC ATC CCC CTG TAA-3'

and

5'-GGC TTA GAT AAC AAA GCG TGT GCT C-3',

5'-GCG TGC CCA TCT TGT TCA ATG G-3'.

The size of the PCR products for the WT allele was 560bp and for the mutant allele 830bp.

Transgenic OBF-1-HA was detected using the following primer combinations:

5'-GGT TGA GTG GTG GGG GTA GGG-3' and

5'-CAC ACG GAC GCC CTG GTA TGG-3' (for genotyping),

5'-CAC TCT CTC TGT GGA AGG CTT TG-3' and

5'-TTC TCA GCT CTA GAC GGC GTA GT-3' (for QPCR).

Spi-B was detected with the PCR primers 5'-CAG TTC TCC TCC AAG CAC AAG-3', 5'-CCA GCC ATT CAT CTC GCA TAG-3' (standard RT-PCR for all isoforms and splice variants, also used to generate a probe for Northern blotting), 5'-CTC TGA ACC ACC ATG CTT GCT-3', 5'-TCC TTC TGG GTA CAA ACA GCT TAA-3' (QPCR for form 1), 5'-AGG GCG GCC CTG ACA T-3', 5'-TCC TTC TGG GTA CAA ACA GCT TAA-3' (QPCR for form 2).

To amplify Spi-B promoter 2 in chromatin immunoprecipitations (ChIP) samples, the primers 5'-GCG CCC AGC CTT CTC CTC CA-3' and 5'-AAA TGT CCC CCA CTC TCC CTG-3' were used.

To amplify the BLR-1 promoter in ChIP samples, the following primers were used:

5'-TGT AGA GGA GGC TGG GAG TA-3' and

5'-CGA GAA GGT TTG TGC TGG TG-3'.

GAPDH (QPCR):

5'-ACC TGC CAA GTA TGA TGA CAT CA-3',

5'-GTA GCC CAA GAT GCC CTT CAG T-3'.

$\beta$ -actin (for Northern blot probe):

5'-ATG GAT GAC GAT ATC GCT GCG CTG-3',

5'-CTA GAA GCA CTT GCG GTG CAC GAT-3'.

Primers used to amplify parts of the  $E\mu$ - $V_H$ -OBF-1 expression cassette in pBluescript II KS+:

① T7 (primer in pBluescript II KS+):

5'-TAA TAC GAC TCA CTA TAG GG-3'

② T3 (primer in pBluescript II KS+):

5'-ATT AAC CCT CAC TAA AGG GA-3'

③ pEV< (primer in pCDNA3):

5'-CCC CAA ACT CAC CCT GAA GTT C-3'

④ #12542 (primer in hOBF-1):

5'-AAG GAC TGG CTT GGG ATA G-3'

⑤ #30015 (primer in VDJ promoter):

5'-CTG TCC AGC CCC ACC AAA CCG-3'

Quantitative real-time PCR (QPCR) was performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using a SybrGreen-based kit from Applied Biosystems.

### Plasmids

The expression cassettes used to generate *lck*-OBF-1 and  $E\mu$ - $V_H$ -OBF-1 transgenic mice are shown in Section 2.2 and Section 2.3, respectively. The HA-tagged hOBF-1 sequence was derived in both cases from two plasmids available in our lab: The N-terminal part was obtained as a 0.7 kb BamHI-BbsI fragment from the plasmid pCDNA OBF-1, while the C-terminal part was obtained as a 1.3 kb BbsI-SfiI fragment from pEV OBF-1/9HA. The *lck* proximal promoter/enhancer region was obtained as a 3.3 kb

NotI-BamHI fragment from the plasmid p1017 kindly provided by Roger Perlmutter. The VDJ enhancer ( $E\mu$ ) was obtained as a 1 kb XbaI fragment from the plasmid 1-27 and the VH promoter was obtained as a 0.2 kb fragment from the plasmid S-19. Both plasmids 1-27 and S-19 were kindly provided by Thomas Jenuwein. The cloning strategy is depicted in Figure 34.

### Cell culture and protein expression in eukaryotic cells

Adherent cells were maintained in DMEM plus 10% fetal calf serum. All B cell lines were grown at 37°C and 10% CO<sub>2</sub> in RPMI supplemented with 10% FCS (Life Technologies, Inc.), penicillin/streptomycin and 50 mM  $\beta$ -mercaptoethanol (Fluka).

HEK293T cells were transfected in 6-well-plates using FuGENE6 (Roche, Basel, Switzerland) according to the manufacturers instructions.

### Protein extract, western blotting and luciferase assay

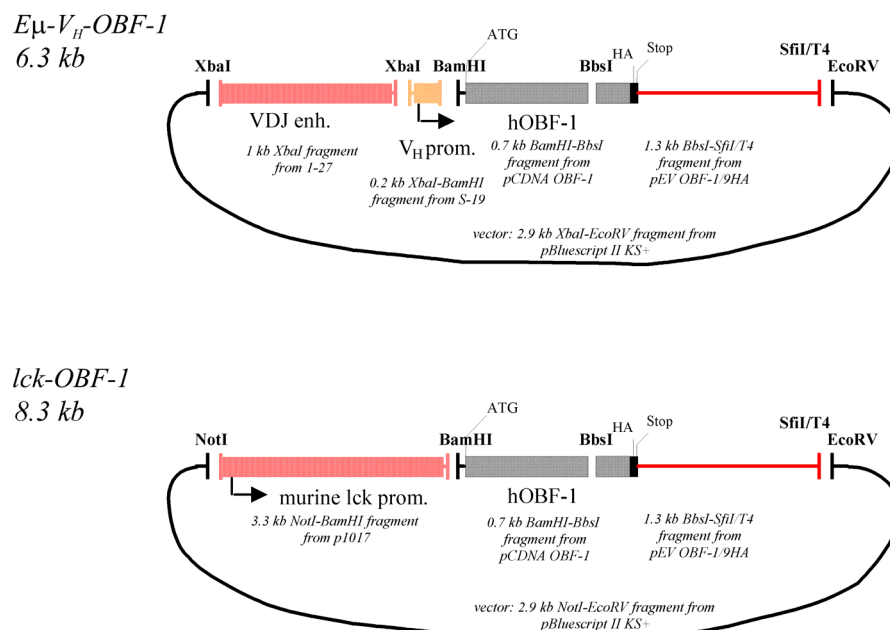
For western blotting, B and T cells were lysed by repeated cycles of freeze and thaw in 20 mM HEPES pH7.9, 20% glycerol, 400 mM NaCl, 0.5 mM EDTA, 0.25 mM EGTA, 0.025% NP-40, 1 mM DTT, 0.5 mM NaF and 'complete' protease inhibitor cocktail (Roche). Protein concentrations were determined with Bradford reagent (BioRad) and 30  $\mu$ g of protein for each sample was resolved by SDS-PAGE with subsequent transfer onto Immobilon membrane (Millipore). HA-tagged proteins were detected with

the monoclonal antibody 12CA5, and endogenous OBF-1 was detected using a polyclonal antibody that was raised in rabbits against the C-terminal 154 amino acids of murine OBF-1. Bands were visualized with the ECL system (Amersham).

To measure luciferase activity, reporter plasmids were cotransfected and cells were lysed in 0.1 M potassium phosphate buffer, pH7.8 containing 1 mM DTT by repeated cycles of freeze and thaw. The cell lysates were measured in a Bertold Luminometer according to standard protocols. The reporter plasmids P<sub>1-luc</sub> (D2393) and P<sub>2-luc</sub> (D2397) were kindly provided by D. Tenen and are described elsewhere (Chen et al. 1998). In brief, P<sub>1-luc</sub> consists of the 170 bp Spi-B promoter 1 driving the firefly luciferase gene in the pXP2 expression vector. P<sub>2-luc</sub> is based on the same vector, but contains the 800 bp Spi-B promoter 2 instead of the Spi-B promoter 1. P<sub>2-S-luc</sub> and P<sub>2-M-luc</sub> are identical to P<sub>2-luc</sub>, but contain specific mutations in the octamer site (Figure 4b shows the exact mutations, which are identical to the mutations in the oligonucleotides P<sub>2-M</sub> and P<sub>2-S</sub>). For transient transfections, 600 ng of OBF-1 expression vector (Strubin et al. 1995) or empty expression vector and equimolar amounts of luciferase reporter vectors were used. Samples were filled up with empty vector to a total amount of 1200 ng DNA per transfection.

### Electrophoretic mobility shift assay (EMSA)

*In vitro* translated proteins were prepared as described previously (Sauter and Matthias 1998). Binding reactions contained equimolar amounts of proteins, 1 mM EDTA, 250  $\mu$ g/ml BSA, 20 mM HEPES (pH 7.9), 50 mM KCl, 4% Ficoll, 6.25 x 10<sup>5</sup> cpm/ml of <sup>32</sup>P-labeled double-stranded oligonucleotide probe, 25  $\mu$ g/ml single stranded DNA, and ei-



**Figure 34** Cloning strategy applied to obtain the  $E\mu$ -V<sub>H</sub>-OBF-1 (upper figure) and lck-OBF-1 expression cassettes (lower figure) that were used to generate transgenic mice. The name of the plasmid from which the DNA fragments were derived are indicated in the figure. The restriction sites that were used for cloning and the approximate size of the DNA fragments are also indicated.

ther 25 µg/ml poly(dI·dC) (Amersham Pharmacia Biotech). Protein-DNA complexes were resolved on a 4% (30:1) polyacrylamide:bisacrylamide, 0.25 x TBE gel at 200 V for 1 h, dried, and subjected to autoradiography.

The following double-stranded synthetic oligonucleotides were used:

P<sub>2</sub> probe: 5'-CCC CCA GCG TCT GTG CAT GCA AAT CCC AGG GAG AGT GGG GG-3', 5'-CCC CCA CTC TCC CTG GGA TTT GCA TGC ACA GAC GCT GGG GG-3'

P<sub>2-S</sub> probe: 5'-CCC CCA GCG TCT GTG CAT GCT AAT CCC AGG GAG AGT GGG GG-3', 5'-CCC CCA CTC TCC CTG GGA TTA GCA TGC ACA GAC GCT GGG GG-3'

P<sub>2-M</sub> probe: 5'-CCC CCA GCG TCT GTG CCG GCA CCT CCC AGG GAG AGT GGG GG-3', 5'-CCC CCA CTC TCC CTG GGA GGT GCC GGC ACA GAC GCT GGG GG-3'

### Northern blotting

RNA was prepared by Trizol (Life Technologies, Inc.) according to the manufacturer's instructions. 15 mg of total RNA was analyzed by Northern blotting using Hybond-XL membranes (Amersham Pharmacia Biotech). OBF-1 was visualized with a radio-labelled full length probe. Quantification of signal intensity was performed on a Molecular Dynamics PhosphorImager using Image Quant software.

### RNase protection assay

RNase protection was performed as described earlier (Schubart et al. 1996b). In brief, a probe was *in vitro* transcribed from a vector containing the murine Spi-B promoter and 5' coding region (kindly provided by D. Tenen, described in (Chen et al. 1998)). The gel-purified radiolabeled probe was afterwards hybridized with 20 mg of total RNA at 50 °C over night. After RNase digestion, RNA was resolved on a 5% polyacrylamide gel and autoradiographed. The protected part of the murine Spi-B RNA is shown in Figure 3A. In the same assay, a control probe of S16 mRNA was used for normalization of loaded RNA quantity. Signal intensity was quantified on a Molecular Dynamics PhosphorImager using Image Quant software.

### Immunofluorescent staining and flow cytometry (FACS) analysis

Single cell suspensions of freshly prepared thymocytes were prepared, stained and analyzed according to standard procedures (Rolink et al. 1994). FACS analysis was performed on a FACSCalibur (BD Biosciences, San Jose, CA), and fluorescence data were collected on 30,000 events by gating on living cells and typical forward-side scatter appearance of

lymphocytes. CellQuest software was used for collecting and analyzing data. Cell sorting was performed on a MoFlo (DakoCytomation) with a purity of at least 94 %.

The mAbs 7D4 (anti-CD25; biotinylated), 53-6.7 (anti-CD8a; PE-labeled) and GL3 (anti-γδ-TCR; PE-labeled) were purchased from Pharmingen (BD Biosciences), San Diego, CA.; RA3-6B2 (anti-B220; FITC labeled (IHC)), and GK1.5 (anti-CD4; FITC-labeled), anti-CD122-FITC (#1905-02), and streptavidin-RPE were purchased from Southern Biotechnology Associates (Birmingham, USA). Biotinylated peanut agglutinin (PNA-biotin, Vector Lab) was used for immunohistochemistry

### Immunohistochemistry and *in situ* hybridization

Young adult mice were sacrificed, the thymi were taken, rinsed in PBS and fixed for 16 hours in 4% paraformaldehyde. After a series of washes in PBS, 0.85% NaCl, 50% ethanol in PBS and 70% ethanol in PBS, the thymi were paraffin embedded according to standard procedures and 6 µm sections were prepared for histochemistry.

For *in situ* hybridization, a 450 bp probe from the 3' UTR of the murine Spi-B cDNA and the corresponding antisense probe were DIG-labeled with the DIG RNA labeling kit (Roche) according to the manufacturer's instructions.

### Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously (Masternak et al. 2003). In brief, 4 x 10<sup>7</sup> thymocytes (or 5 x 10<sup>6</sup> ST1571-treated Abelson B cells) were harvested, suspended in 40 ml (4 ml) RPMI 1640 and crosslinked for 10 minutes at room temperature with 4 ml (400 µl) 11% formaldehyde / 0.1 M NaCl / 50 mM Hepes pH 7.9. Crosslinking was stopped by addition of 6.5 ml (650 µl) 1 M glycine pH 8 and incubation for 5 minutes at room temperature. Cells were washed with 50 ml (5 ml) cold PBS containing protease inhibitors (Complete, Roche, Basel, Switzerland), resuspended in 40 ml (4 ml) ice cold cell lysis buffer (5 mM Pipes, pH8.0 / 85 mM KCl / 0.5% NP40) + Complete (Roche) and vortexed. Nuclei were spun down immediately for 7 minutes at 2,500 rpm, then lysed for 10 minutes at room temperature in 2.2 ml Nuclei lysis buffer (50 mM Tris, pH8.0 / 10 mM EDTA / 1% SDS). Chromatin was sonicated with a Branson 250 sonicator: 3 X 20" pulses constant power output setting 7, precleared by centrifugation at 12000 rpm for 10 minutes at 4°C. Chromatin was transferred to fresh vials and stored at -80°C.

To immunoprecipitate chromatin with specific antibodies, 10 µg (3 µg) of chromatin were diluted with immunoprecipitation (IP) buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 2 mM EDTA, 0.1%



sodium deoxycholate, 0.1% SDS and the protease inhibitor complex Complete) containing 50 g/ml salmon sperm DNA, 100 g/ml Escherichia coli tRNA and 1 mg/ml bovine serum albumin. Immunoprecipitation was performed with an anti-HA antibody (12CA5) in case of the thymocytes and with the monoclonal OBF-1 antibody C-20 (SC-955 X, Santa Cruz Biotechnology, CA) in case of the Abelson cell lines. Immune complexes were captured at room temperature with protein A-Sepharose beads. Beads were washed twice with IP buffer, twice with IP buffer containing 500 mM NaCl, twice with 20 mM Tris-HCl, pH 8.0, 0.25 M LiCl, 2 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate and once in TE containing 0.1% NP-40. Immunoprecipitated chromatin fragments were eluted with 100 mM Tris-HCl, pH 8.0, 1% SDS for 10 min at 65 °C, diluted twofold with 400 mM NaCl, 200 g/ml proteinase K, digested for 1 h at 37 °C and incubated overnight at 65 °C to reverse the crosslinks. After extraction with phenol-chloroform and chloroform, DNA was precipitated with isopropanol in the presence of glycogen, washed in 80% ethanol and resuspended in TE. Immunoprecipitated DNA derived from 100 ng of input chromatin DNA and standards containing 10 ng of total input chromatin DNA were analyzed by PCR.

#### *RNA preparation and hybridization to Affymetrix Microarrays MG-U74 and MG-U74v2*

Sorted thymocytes were collected, or, alternatively, total thymi were isolated from mice, briefly rinsed with PBS and sonicated in Trizol. Total RNA from these sorted cells or thymi was immediately isolated with the Trizol method (Life Technologies, Inc.), followed by a purification using the RNeasy kit from Qiagen according to the manufacturer's instructions. Microarray analysis was performed using MG\_U74A version 2 GeneChips™ (Affymetrix). 10 µg of total RNA was reverse transcribed using the SuperScript Choice system for cDNA synthesis (Life Technologies) according to the protocol recommended by Affymetrix.

#### *Data processing*

Chip analysis was performed using the Affymetrix Microarray Suite v5 and GeneSpring 5.1 (Silicon Genetics). Changes in gene expression were assessed by looking for concordant changes between replicates using a signed Wilcoxon rank test. The "change" p-value threshold was < 0.003. Genes whose detection p-value was > 0.05 (absent) in all conditions were also discarded. Detailed information complying with MIAME standards can be found in electronic form on the web at the public ArrayExpress database at <http://www.ebi.ac.uk/arrayexpress>.

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## 6 Appendix

### 6.1 Genes differentially regulated similarly in all microarray experiments

#### 6.1.1 Genes upregulated in total thymocytes of lck-OBF-1 mice and upregulated in the subfraction of CD25<sup>+</sup> CD4<sup>+</sup> CD8<sup>+</sup> thymocytes of lck-OBF-1 mice

Reference	Name	Gene Symbol	Description	Fold up-regulation
<b>B or T cell surface molecules / Cell adhesion</b>				
101917_at	interleukin 2 receptor, alpha chain	Il2ra	activation marker; receptor component B, T cells	2.45
102337_s_at	Fc receptor, IgG, low affinity IIb	Fcgr2b	IgG binding receptor	2.16
102695_at, 102744_at	T-cell receptor gamma, variable 2	Tcrg-V2	T cell receptor component of $\gamma\delta$ -T cells	1.77
160553_at	lymphocyte antigen 6 complex, locus D, ThB	Ly6d	cell surface antigen expressed by most CD4+CD8+ (DP) thymocytes at high levels; downregulated in mature CD4+ and CD8+ SP T cells. Interaction of TCR/coreceptor with the self-MHC-peptide contributes to the downregulation of ThB expression on developing thymocytes.	1.99
160820_at	immunoglobulin superfamily, member 8; PGRL	Igsf8	Ig superfamily; most prominent CD81-associated cell surface protein on thymocytes as well as a number of T cell and B cell lines; probably modulates T cell motility	2.3
103005_s_at	CD44 antigen	Cd44	hyaluronate binding protein; homing/cell adhesion; results indicate that CD44 expression is part of a genetic program controlled by the beta-catenin/Tcf-4 signaling pathway and suggest a role for CD44 in the generation and turnover of epithelial cells	2.8
<b>Transcription factors / cofactors</b>				
102994_at	signal transducer and activator of transcription 4	Stat4	IFN- $\gamma$ activation site-binding protein expressed in early myeloid differentiation. Can be activated by specific cytokines, such as IL-12, IFN- $\alpha$ , and IL-2. Requirement for STAT4 and a role for IFN-g as an amplifying factor in IL-12 induction of the functional IL-18 receptor complex.	1.57
160483_at	transcription factor 4	Tcf4	antiapoptotic; target genes: c-myc and cyclin D1; in Wnt signaling pathway; binds beta-catenin. expressed in embryo, intestine...	2.03
93417_at, 93418_g_at	myocyte enhancer factor 2B	Mef2b	MADS box TF; early embryo heart/skeletal muscle; myocyte differentiation	6.73
93657_at	Spi-B transcription factor (Spi-1/PU.1 related)	Spib	Ets TF; T, B cells; immune response, GC formation, secondary Ig isotypes	8.84
94821_at	X-box binding protein 1	Xbp1	bZIP TF; essential for Ig secretion and plasma cell development; high XBP-1 mRNA levels are found in specialized secretory cells; stimulated by LPS	1.78
97497_at	Notch gene homolog 1 (Drosophila)	Notch1	Notch 1-deficient CLPs adopt a B cell fate in the thymus; antiapoptotic	1.93
97974_at	zinc finger protein, multitype 1 / FOG-1	Zfpm1	GATA-1 cofactor; can repress GATA-3-dependent activation of the IL-5 promoter in T cells and inhibit GATA-3 dependent Th2 development in CD4+ T cells when overexpressed during primary activation of naive T cells. FOG -/- mice die during mid-embryonic development with severe anemia. Partial block of erythroid differentiation and lack of megakaryocytes in the absence of FOG.	1.99
97853_at	PC4 and SFRS1 interacting protein 2 (Psp2, LEDGF)	Psp2	survival factor in stress reaction; transcriptional activator of heat shock and stress-related genes	1.61

## 6.1 Genes differentially regulated ...

Intracellular Signaling				
102851_s_at	hemopoietic cell phosphatase	Hcph	protein Tyr phosphatase; myeloid; motheaten	1.54
95022_at	A kinase (PRKA) anchor protein (gravin) 12	Akap12	kinase (PRKA) anchor protein	1.86
99372_at	endothelial differentiation, sphingolipid G-protein-coupled receptor, 5	Edg5	endothelial differentiation, sphingolipid G-protein-coupled receptor	4.27
Calcium signaling				
103448_at	S100 calcium binding protein A8 (calgranulin A)	S100a8	expression is negatively regulated by c-Fos/AP-1	2.28
103503_at	phospholipase C, gamma 2	Plcg2	essential for specific functions of Fc epsilon R and Fc gamma R	7.15
103935_at	ATPase, Ca <sup>++</sup> transporting, ubiquitous (SERCA3a)	Atp2a3	calcium ion transport	1.77
160487_at	myosin, light polypeptide 4, alkali; atrial, embryonic	My14	calcium ion binding;	7.61
Proapoptotic Genes				
96338_at	EGL nine homolog 2 (C. elegans)	Egln2	putative regulator of growth and apoptosis	2.5
98498_at	caspase 7	Casp7		1.43
97551_at	huntingtin interacting protein 1 related	Hip1r	proapoptotic; Overexpression resulted in rapid caspase 3-dependent cell death; component of the endocytic machinery; neurons	2.37
Other Genes				
94061_at	cysteine-rich protein 1 (intestinal)	Crip1	LIM domain; mainly expressed in intestine, thymus, spleen, and lung. Transgenic mice overexpressing Crip1 had only about 50% of WT WBC count - proportionately fewer lymphocytes and more monocytes, eosinophils, neutrophils. FACS: CRIP tg have more CD4 <sup>+</sup> /CD8 <sup>+</sup> thymic lymphocytes.	1.78
161666_f_at	growth arrest and DNA-damage-inducible 45 beta	Gadd45b	essential for promoting TNF-alpha-mediated cell survival. However, neither TNF-a signalling nor cell survival is affected in mice lacking <i>gadd45b</i>	1.37
160084_at	ornithine decarboxylase, structural	Odc	degraded by the 26 S proteasome via a ubiquitin-independent pathway; degradation is greatly accelerated by association with the polyamine-induced regulatory protein antizyme 1 (AZ1)	1.56
102336_at	RW1 protein	Rw1-pending	integral to membrane	2.14
As Yet Uncharacterized Genes				
102383_at	RIKEN cDNA 5730593F17 gene	5730593F17Rik	C/EBP-induced protein (ortholog of human and rat)	1.43
104294_at	RIKEN cDNA 5430411K16 gene	5430411K16Rik	DKFZP564B116 protein Homolog; Putative ortholog of inhibitor of BTK (IBTK)	1.62
160122_at	RIKEN cDNA 2410004D18 gene	2410004D18Rik	asparaginase 2 superfamily	2.27
160684_at	RIKEN cDNA 8430410A17 gene	8430410A17Rik	DC12 protein Putative Ortholog	1.2
96158_at	cDNA sequence BC017133	BC017133		1.61
96221_at	expressed sequence AI429613	AI429613	CIKS / ACT1 homolog (NFkappaB-activating protein, connection to I kappa B-kinase)	2.37
99366_at	RIKEN cDNA E030024M05 gene	E030024M05Rik		1.88

### 6.1.2 Genes downregulated in total thymocytes of lck-OBF-1 mice and downregulated in the subfraction of CD25<sup>+</sup> CD4<sup>+</sup> CD8<sup>+</sup> thymocytes of lck-OBF-1 mice

Reference	Name	Gene Symbol	Description	Fold down-regulation
<b>B or T cell surface molecules / Cell adhesion</b>				
101311_at	T-cell receptor beta, variable 13	Tcrb-V13		-1.53
102237_at	CD28 antigen	Cd28		-1.79
104121_at	junction plakoglobin	Jup		-1.11
104606_at	CD52 antigen / B7	Cd52		-1.66
92203_s_at	CD6 antigen	Cd6	binds activated leukocyte cell adhesion molecule (ALCAM, CD166), a member of the immunoglobulin (Ig) superfamily (IgSF)	-2.17
92204_at	CD6 antigen	Cd6		
94726_at	T-cell receptor beta, variable 13	Tcrb-V13		-1.76
94764_at	integrin alpha L	Itgal		-1.69
98323_at	chemokine (C-C motif) receptor 9	Ccr9		-1.87
99053_at	intercellular adhesion molecule 2	Icam2		-1.55
102940_at	lymphotoxin B	Ltb		-2.28
<b>Transcription factors / cofactors</b>				
160617_at	Kruppel-like factor 13	Klf13		-1.44
99602_at	TGFB inducible early growth response 1	Tieg1		-1.31
<b>Intracellular Signaling</b>				
100511_at	RIKEN cDNA 6330406L22 gene	6330406L22Rik		-1.4
103279_at	SH2 domain protein 1A	Sh2d1a		-1.56
160545_at	cyclin D3	Ccnd3		-1.57
92185_at	RIKEN cDNA A630084M22 gene	A630084M22Rik		-1.5
<b>Calcium Signaling</b>				
103596_at	diacylglycerol kinase, alpha	Dgka		-1.5
<b>Thymocyte-specific genes</b>				
102203_at	placental protein 11 related	Pp11r / Tcl-30		-1.99
92471_i_at	schlafen 2	Slfn2		-1.81
93637_at	CD5 antigen	Cd5		-1.76
<b>Other Genes</b>				
94835_f_at	tubulin, beta 2	Tubb2		-2.15
92642_at	carbonic anhydrase 2	Car2		-1.77
93652_i_at	vesicle-associated membrane protein 1	Vamp1		-1.63
93861_f_at	Mouse endogenous murine leukemia virus modified polytropic provirus DNA, complete			-1.62
96172_at	immune associated nucleotide 1	Ian1		-1.69
102850_at	tyrosine kinase, non-receptor, 2	Tnk2		-1.66
104598_at	dual specificity phosphatase 1	Dusp1		-1.76
97384_at	glia maturation factor, gamma	Gmfg		-1.62

## 6.1 Genes differentially regulated ...

### As Yet Uncharacterized Genes

160379_at	erythrocyte protein band 4.1	Epb4.1	-1.76
93195_at	malignant fibrous histiocytoma amplified sequence 1	Mfhas1	-1.85
94428_at	ilvB (bacterial acetolactate synthase)-like	Ilvbl	-1.61
96640_at	RIKEN cDNA 3110001A13 gene	3110001A1	-1.46
97967_at	Mus musculus 11 days embryo head cDNA, RIKEN full-length enriched library, clone:6230425C21 product:hypothetical protein, full insert sequence.	3Rik	-2.12



## 7 Abbreviations

AFCs	antibody-forming cells	Ig	Immunoglobulin
APCs	antigen presenting cells	iIEL	intestinal intraepithelial lymphocytes
APC	allophycocyanin	IP	immunoprecipitation
BCR	B cell receptor	ITAM	immunoreceptor tyrosine-based activation motif
bHLH	(basic) helix-loop-helix	kb	1000 base pairs
bp	base pairs	KCs	keratinocytes
CDR	complementarity determining region	KGF	keratinocyte growth factor
ChIP	chromatin immunoprecipitation	LCR	locus control region
CLP	common lymphoid progenitor	LPS	lipopolysaccharide
CSR	class switch recombination	Lt	lymphotoxin
CTD	C-terminal domain	MAR	matrix attachment region
DC	dendritic cell	M-CSF	macrophage colony-stimulating factor
DETC	dendritic epidermal T cell	MHC	Major histocompatibility complex
DIG	digoxigenin	MIAME	minimum information about a microarray experiment
DMEM	Dulbecco's modified Eagle medium	MZ	marginal zone
DN	double negative	NK cell	natural killer cell
DNP-KLH	dinitrophenyl-keyhole limpet hemocyanin	nt	nucleotides
DP	double positive	PALS	periarteriolar lymphoid sheath
DSB	(DNA) double strand break	PBS	phosphate buffered saline
EICE	Ets-IRF composite elements	PC	plasma cell
ELISA	enzyme-linked immunosorbent serologic assay	PCR	polymerase chain reaction
ER	endoplasmatic reticulum	PIC	pre-initiation complex
EST	expressed sequence tag	PE	phycoerythrin
FACS	fluorescence activated cell sorting	PMA	phorbol 12-myristate 13-acetate (also known as TPA)
FCS	fetal calf serum	PNA	peanut agglutinin
FDC	follicular dendritic cell	Pol	RNA polymerase
FITC	fluorescein (mistakenly abbreviated by its commonly-used reactive isothiocyanate form)	QPCR	quantitative PCR
FR	framework region	RPA	RNase protection assay
GC	germinal center	rpm	revolutions per minute
GFP	green fluorescent protein	RT-PCR	reverse transcription PCR
GTFs	general transcription factors	RSS	recombination signal sequence
HA	hemagglutinin	SC	stem cell
HAT	histone acetyl transferase	SD	standard deviation
HDAC	histone deacetylase	SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
HSC	hemotopoietic stem cell	SHM	somatic hypermutation
IDC	interdigitating cell	SP	single positive
IFN- $\gamma$	interferon- $\gamma$	TCR	T cell receptor
		TD	T cell dependent
		TdT	terminal deoxynucleotidyl transferase
		tg	transgenic
		TGF	transforming growth factor
		TI	T cell independent
		TNF	tumor necrosis factor
		UPR	unfolded protein response
		UTR	untranslated region
		WT	wild type

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