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Regulation of B lymphocyte terminal differentiation and death by the transcription factor Blimp-1

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Dedicated to
my Grandmother, mother,
brothers, sister
and teachers

Contents

Summary

B lymphocyte induced maturation protein-1 (Blimp-1) and X-box-binding protein-1 (XBP-1) are indispensible transcription factors required for B lymphocyte terminal differentiation into Ig secreting plasma cells. Occurrence of an unfolded protein response (UPR) and XBP-1 splicing, due to elevated Ig levels, are critical events during plasma cell generation. However, the upstream molecule sufficient to trigger these events remain elusive. Because ectopic expression of Blimp-1 in B cells is sufficient to generate plasma cells, it is plausible that Blimp-1 might be the upstream molecule, sufficient for the induction of UPR and XBP-1 splicing.

This possibility was investigated by analyzing the function of ectopically expressed Blimp-1 in the mouse B lymphoma line WEHI 231 and in primary mouse splenic B cells. In both cell types, ectopic expression of Blimp-1 led to upregulation of Ig levels, XBP-1 splicing and Ig secretion. Interestigly, the N-terminal part of Blimp-1 comprising the amino acids 1-751 was sufficient to induce the above effects, while the C-terminus comprising amino acids 465-856 had no effect. In addition, in mouse splenic B cells, the UPR target gene BIP was induced in response to either full length or N-terminal domain of Blimp-1. From these results, it is clearly evident that Blimp-1, especially its N-terminal domain, is sufficient to trigger UPR and XBP-1 processing, leading to Ig secretion, in B cells.

Another important aspect of Blimp-1 is its ability to induce apoptosis in B cells. In this context, previous studies identified the down regulation of the anti-apoptotic gene A1, in response to Blimp-1. Results in the current study provide the first evidence that A1 is a direct target of Blimp-1. Putative Blimp-1 binding sites were observed in the A1 promoter and were shown to bind Blimp-1 in vitro. Furthermore, in luciferase reporter assays, Blimp-1 repressed the A1

promoter. Mutation of Blimp-1 binding sites from the A1 promoter led to near abrogation of the repressive effect of Blimp-1, suggesting a direct mode of A1 promoter repression by Blimp-1. In addition, Mad4, a down stream target of Blimp-1, repressed A1 promoter, suggesting an indirect mode of Blimp-1 action..

The repression of A1 gene, by the differentiation inducer Blimp-1, raised the question of involvement of A1 in B cell terminal differentiation. This possibility was tested by analyzing the influence of ectopically expressed A1 on terminal B cell differentiation. Retroviral transduction of A1 into LPS stimulated primary B cells, resulted in reduced Ig secretion and reduction in the plasma cell marker syndecan-1, suggesting a block in terminal differentiation. Furthermore, in A1 expressing WEHI 231 cells, LPS stimulation could not induce down regulation of surface Ig, providing another indication for an inhibitory role for A1 in B cell terminal differentiation. The above findings provide indications for an inhibitory role for A1 in the plasma cell generation. Therefore the repression of A1 gene by Blimp-1 seems to be an important prerequisite for terminal B cell differentiation.

Zusammenfassung

B lymphocyte induced maturation protein-1 (Blimp-1) und X-box-binding protein-1" (XBP-1) sind als Transkriptionsfaktoren unverzichtbar für die terminale Differenzierung von B-Lymphozyten zu Immunglobulin (Ig)-sezernierenden Plasmazellen. Ebenso stellen die unfolded protein response (UPR) und das Spleißen von XBP-1, beides ausgelöst durch erhöhte Ig-Produktion, entscheidende Schritte auf dem Weg zur Plasmazellentstehung dar. Allerdings ist das Molekül/ sind die Moleküle nach wie vor unbekannt, die diesen beiden Ereignissen in der Signalkaskade vorgeschaltet sind. Da die ektope Expression von Blimp-1 in B-Zellen hinreicht, diese zu Plasmazellen zu differenzieren, erscheint es plausibel, dass Blimp-1 das Molekül sein könnte, das die Auslösung einer UPR und das Spleißen von XBP-1 steuert.

Dieser Möglichkeit wurde durch ektope Expression von Blimp-1 in der Maus-B-Zell-Lymphomlinie WEHI 231 und in primären B-Zellen aus der Milz von Mäusen nachgegangen. Die ektope Expression von Blimp-1 führte in beiden Zelltypen zur Erhöhung der Ig Produktion, zum Spleißen von XBP-1 und zur Sekretion von Immunglobulinen. Interessanterweise war der N-terminale Anteil von Blimp-1, bestehend aus den Aminosäuren 1-751, hinreichend, um diese Effekte auszulösen, während der C-Terminus, der die Aminosäuren 465-856 umfaßte, keinen Effekt hatte. Darüberhinaus, wurde die Expression von BIP, dessen Gen ein UPR-Zielgen ist, durch ektope Expression von Blimp-1 bzw. dessen N-Terminus in primären B-Zellen erhöht. Diese Ergebnisse zeigen deutlich, dass Blimp-1, speziell dessen N-terminale Domäne, hinreichend ist, um eine UPR und die Prozessierung von XBP-1 auszulösen, was zur Ig-Sekretion von B-Zellen führt.

Eine weitere wichtige Eigenschaft von Blimp-1 liegt in dessen Fähigkeit, Apoptose in B-Zellen auszulösen. In diesem Zusammenhang konnten frühere

Arbeiten nachweisen, dass die Expression von Blimp-1 zu einer Reduktion der Expression des anti-apoptotischen Proteins A1 führt. Die Ergebnisse dieser Arbeit, wiederum, beinhalten die ersten experimentellen Belege für eine direkte Steuerung der A1 Expression durch Blimp-1. Mögliche Bindungsstellen für Blimp-1 konnten in der Region des A1-Promotors identifiziert werden und die tatsächliche Bindung von Blimp-1 an diese DNA-Abschnitte konnte in vitro nachgewiesen werden. Darüberhinaus zeigten Luziferase-Reporter Assays, dass Blimp-1 den A1-Promotor reprimiert. Mutationen der Blimp-1-Bindungsstellen innerhalb des A1-Promotors führten zur nahezu vollständigen Aufhebung des reprimierenden Effektes von Blimp-1, was eine direkte Repression des A1-Promotors durch Blimp-1 nahelegt. Blimp-1 unterdrückte außerdem durch Induktion der Expression von Mad4 auch auf indirekte Weise den A1-Promotor.
Die Unterdrückung der Transkription des A1 Gens durch das Differenzierungs-induzierende Protein Blimp-1 warf ferner die Frage nach der Rolle von A1 bei der terminalen Differenzierung von B-Zellen auf. Retrovirale Transduktion der A1 Gensequenz in LPS-stimulierte primäre B-Zellen, reduzierte die Ig-Sekretion und die Expression des Plasmazellmarkers Syndekan-1, d.h. verhinderte eine terminale Differenzierung. Desweiteren, führte die Stimulation von A1-exprimierenden WEHI 231 Zellen mit LPS nicht zu einer Verringerung der Expression von Ig an der Zelloberfläche, was einen weiteren, wenn auch indirekten, Hinweis auf eine inhibitorische Rolle von A1 auf die terminale Ausreifung von B-Zellen darstellt. Diese Ergebnisse legen also einen inhibitorischen Effekt von A1 auf die Entstehung von Plasmazellen nahe. Folglich scheint die Repression des A1 Gens durch Blimp-1 eine wichtige Voraussetzung für die terminale Differenzierung von B-Zellen zu sein.

1. Introduction

1.1 Overview of the Immune System

The immune system is a highly versatile defence system that has the remarkable ability to recognize and to mount a protective response against pathogenic organisms or foreign molecules or against abnormal self cells like tumor cells. It consists of a variety of cells and molecules which act in concert to protect the organism.

1.1.1 The Innate and Adaptive components of the Immune System

The immune system broadly consists of two components: the innate immune system and the adaptive or acquired immune system.

1.1.2 Innate Immune System

The innate immune system is comparatively less specific, yet a powerful ancient mode of host defence, present in all multicellular organisms. It provides the first line of host resistance, which is the result of multiple and diverse mechanisms. The innate immune system functions by providing multiple barriers for the microbes like physical, physiological, phagocytic and inflammatory.

Recognition of foreign or altered self molecules by the innate immune system depends on two fundamental aspects : ‘microbial non-self’ and ‘missing self’(1-3). The first, recognition of ‘microbial non-self’ is based on the recognition of conserved molecular structures called pathogen-associated molecular patterns (PAMPs) unique to the pathogen, by the receptors of the innate immune system called pattern-recognition receptors (PRRs) (4-9). The

second, ‘missing self’ is based on the recognition of specific self molecules on normal, uninfected or unaltered cells of the host. Recognition of such a self tag by the inhibitory receptors of the innate immune cells (e.g., NK cells), prevents the activation of effector mechanisms and thereby leaves the normal self cells untouched. Infection or transformation of a cell into abnormality (e.g., tumor cell), results in the loss of such self tags, resulting in the activation of innate immune effector mechanisms which destroy such altered self cells.

In addition, the innate immune system plays an essential role in the induction of adaptive immune responses (5, 10-16). Recognition of PAMPs by PRRs on dendritic cells (DCs) of the innate immune system allows on one hand the maturation of DCs and on the other hand triggers the expression of costimulatory molecules on the DC surface. The costimulatory molecules provide an important second signal for the activation of adaptive immune responses (4, 10-16). Furthermore, triggering PRRs on DCs results in the secretion of cytokines, which play an essential role in T cell differentiation into effector cells.

1.1.3 Adaptive Immune System

The adaptive immune system is a remarkable system capable of specifically recognizing and eliminating any microorganism or foreign molecule. The fundamental characteristics of adaptive immunity are :

1. **Antigenic specificity**

The components of the adaptive immune system exhibit a high degree of specificity, allowing it to distinguish minute differences among antigens. The

recognition is so precise that even protein molecules differing in a single amino acid can be distinguished.

2. **Diversity**

The adaptive immune system evolved as a result of acquisition of RAG genes by vertebrates. Somatic recombination of gene segments encoding antigen receptors facilitated by RAG genes enables the generation of a highly diverse and random repertoire of antigen receptors, which allows the recognition of, in principle, any molecule or structure.

3. **Capacity to display immunologic memory**

Another unique feature of the adaptive immunity is to exhibit immunologic memory. After the initial encounter with the antigen, the immune system acquires a state of immunologic memory. A second encounter with the same antigen therefore elicits a much stronger response, which is more rapid and greater in magnitude. Due to immunologic memory, an individual infected with a microorganism is protected against subsequent infections by the same microorganism and this is the underlying principle of vaccination.

4. **Discrimination between self and non self and tolerance of self antigens**

Even though the adaptive immune system generates a random repertoire of antigen receptors, containing specificities for both foreign and self molecules, the immune system recognizes ignores self molecules. This process of ignorance of self antigens is an active process called self tolerance, which involves elimination or inactivation of cells bearing receptors for self molecules.

1.1.4 Cells of the adaptive immune system

Three main classes of cells, T lymphocytes (T cells), B lymphocytes (B cells) and antigen presenting cells (APCs) constitute the adaptive immune system.

The lymphocytes occupy the central stage in an immune response because they display the hallmark features of the adaptive response, such as specificity, diversity, memory and self tolerance. An effective immune response involves cooperation between APCs and lymphocytes. The APCs trap the antigen and present it to lymphocytes, enabling the initiation of immune responses.

The immune system has evolved different organs, which facilitate the development, activation and differentiation of lymphocytes. The primary lymphoid organs like bone marrow and thymus provide appropriate microenvironments for the development of lymphocytes. The secondary lmphoid organs like spleen, lymph nodes, peyer's patches and tonsils provide the environment where mature lymphocytes get access to the antigen and undergo differentiation into effector cells.

1.1.5 B lymphocytes

B lymphocytes derived their name from the site of their development, the bursa of Fabricius in birds. In mammalian species, bone marrow is the site of B cell maturation. Each mature B cell displays membrane bound immunoglobulin molecules called B cell receptors (BCRs) or antibodies. All of the BCRs on a given B cell have identical specificity for an antigen. The BCR or the antibody is a glycoprotein consisting of two identical heavy chains and two identical light chains. The heavy and light chains are held together by disulfide bonds, with the amino-terminal ends of heavy and light chains forming a molecular fold or cleft with a unique specificity, to which the antigen binds. Encounter of a naive B cell with an antigen triggers proliferation and the progeny generated differentiates into effector B cells called plasma cells or into memory B cells (17-20). Plasma cells generate the

secretory form of antibodies at a rapid rate and hence they secrete huge amounts of antibodies. The secreted antibodies are the fundamental effector molecules of humoral immunity. Most plasma cells have a short life span of only a few days but some plasma cells migrate to the bone marrow where they can survive for longer periods and constitute the pool of long lived plasma cells (18,19). Memory B cells have a longer life span and play a predominant role in secondary humoral immune response, which occurs with greater rapidity and magnitude.

1.1.6 T lymphocytes

The name T lymphocyte is derived from the thymus, the site of T cell development and maturation. Like B cells, T cells express on their surface antigen binding molecules called T cell receptors (TCRs). A given T cell in general expresses on its surface a TCRs with a single specificity for the antigen. However, a fundamental difference exists in the manner in which T cells and B cells recognize the antigen. Whereas a B cell receptor can recognize free antigen, a TCR can not. The TCR on a T cell can recognize antigen only in association with specialized molecules called major histocompatibility complex (MHC) molecules. Two major classes of MHC molecules are expressed in an individuals: Class 1 MHC molecules are expressed on almost all cells of an individual whereas Class 2 MHC molecules are expressed by APCs. Encounter of a naive T cell with antigen-MHC complexes triggers the proliferation and differentiation into various effector cells or into memory cells (21). Furthermore the activation and differentiation is dependant not only on the signal provided by antigen but also needs the acquisition of a second signal called costimulatory signal.

There are two major classes of T cells called T helper (T_H) cells which express a cell surface marker called CD4 and T cytotoxic (T_C) cells with a surface marker CD8. T_H cells recognize antigen in the context of MHC class 2 on APCs, resulting in the activation and secretion of various cytokines. The secreted cytokines play important roles in the activation of other cells of the immune system. T_C cells recognize antigen in a complex with MHC class 1 molecules. Upon recognition of foreign antigen and acquisition of appropriate costimulatory signals, T_C cells differentiate into effector cells called cytotoxic T cells (CTLs). The CTLs exhibit potent cytotoxic activity and play an important role in the elimination of infected self cells or self cells expressing abnormal antigens.

1.1.7 Hematopoiesis and the generation of cells of the immune system

The cells of the immune system, including all other blood cells are derived from pluripotent hematopoietic stem cells (HSCs), by a process called hematopoiesis (22). HSCs have extensive self-renewal capacity and give rise to all blood cells throughout life, by differentiating into progenitor cells with gradually restricted developmental and self-renewal potential(22) During development, hematopoiesis first begins in the embryonic yolk sac, later on in the fetal liver and spleen and from the time of birth, bone marrow is the major site of hematopoiesis. The multipotent HSC first differentiates into common lymphoid progenitors (CLPs) or common myeloid progenitors (CMPs). All lymphoid cells like T cells, B cells, NK cells and NKT cells are derived from lymphoid progenitors. Myeloid progenitors further differentiate into progenitors giving rise to macrophages, neutrophils, eosinophils, basophils, platelets and erythrocytes. Dendritic cells are derived from both myeloid progenitors and lymphoid progenitors (22).

1.1.8 B-lymphocyte development

B cells are continuously produced throughout life, primarily in the fetal liver and spleen before birth and in the bone marrow thereafter. B-lymphocytes, like all other blood cells, are derived from HSC, by a series of developmental stages, each characterized by expression of specific cell surface marker(s) and/or specific transcription factors (22). HSCs are the lineage negative (means they lack the lineage specific differentiation markers) (lin^-), c-kit^+, $sca1^+$, $flt3^-$ subset of bone marrow cells (22). The c-kit^+, lin^-, $sca1^+$, $flt3^-$ HSCs first produce multi potent progenitors (MPPs) with a phenotype c-kit^+, lin^-, $sca1^+$, $flt3^+$, which lose the long term self renewal capacity, marked by the expression of flt3 (22). The MPPs further differentiate into CLPs, identified by the expression of IL-7Rα. The entry of the CLPs into the B lineage restricted population in the mouse is marked by the expression of the surface marker B220, which constitutes a subset of cells called CLP-2 cells (23-26). Further expression of CD19 by CLP-2 cells and D_H-J_H rearrangements at the immunoglobulin heavy chain locus generates the early pro-B cells (23-26). Productive V_H-DJ_H recombination in late pro-B cells results in the cell surface expression of Igμ heavy chain as part of the pre-B cell receptor (pre-BCR). The expression of pre-BCR acts as an important checkpoint, to control the transition from the pro-B to the pre-B cell stage. Pre-BCR signaling triggers proliferative expansion and results in the generation of small pre-B cells. The small pre-B cells generate the immature B cells, upon successful rearrangement of immunoglobulin light chain genes, producing the Ig light chain which is expressed on the cell surface with the already rearranged μ heavy chain, constituting the IgM molecule on the surface. The IgM^+ immature B cells leave the bone marrow to the peripheral

lymphoid organs and undergo further maturation (25). The immature B cell is not fully functional and antigen triggering results in death or unresponsiveness rather than activation and differentiation. Subsequent development in the periphery results in the co-expression of membrane bound IgD, along with IgM. The IgM^+, IgD^+ population of B cells constitutes the mature B cell population which responds to antigenic challenge by eliciting an immune response.

1.1.9 Transcriptional regulation of B cell development

B cell development is regulated by a multitude of transcription factors which are expressed in a stage specific manner, during the developmental progression through different stages. Various kinds of transcription factors act specifically to regulate different steps, starting from the generation of CLPs from HSCs to commitment of CLPS to the B lineage and for the further progression of committed B lineage progenitors to immature, mature and terminally differentiated states (27-37)

1.1.10 Myeloid versus lymphoid lineage choice and PU.1

The Ets family transcription factor PU.1 (Spi.1) is important for the generation of myeloid as well as lymphoid cells as evidenced by studies from PU.1 null embryos (35-38). PU.1 acts at the level of multipotent myeloid-lymphoid progenitors and plays a pivotal role in the regulation of the choice between myeloid and lymphoid lineages of common progenitors (39). Interestingly it was observed that graded expression of PU.1 determines the lymphoid versus myeloid cell fate of early progenitors (39-41). Ectopic expression of low levels PU.1 in $PU.1^{-/-}$ progenitors induced B cell

development while high levels rather suppressed B cell fate and induced macrophage development (40). Recent studies identified IL-7Rα as a direct target of PU.1 and it appears that PU.1 promotes B cell fate by induction of IL-7Rα in progenitor cells (39). In fact it was observed that introduction of the IL-7Rα gene alone into $PU.1^{-/-}$ progenitors was sufficient to induce B cell fate (39).

1.1.11 Pro-B cell development

Generation of pro-B cells and the subsequent development of pro-B cells from the common lymphoid progenitors (CLPs) is dependant on the induction of the transcription factors E2A and EBF (35-37, 42-49). The E2A gene codes for two alternative splice products E12 and E47, which belong to the basic helix-loop-helix family of transcription factors (50). The induction of E2A at the onset of B-lymphopoiesis is very critical for further development of the B-lineage since in E2A-null ($E2A^{-/-}$) mice B cell development was blocked at the very early stage, before the D_H-V_H rearrangement of the Ig_H locus, and all later stages were totally lacking (42, 46, 51).

The early B-cell factor (EBF), like E2A, is required at a similar stage in B cell development, as evidenced by $EBF^{-/-}$ mice, which have a similar developmental block before the D_H-J_H rearrangement of the Ig_H locus, like $E2A^{-/-}$ mice (42, 43, 50, 52-54). The possibility that E2A and EBF act in synergy was raised due to a similar B-cell developmental block in E2A and EBF null mice and was proved in subsequent studies (42, 43, 52, 53).

Pro-B cell development is characterized by successive rearrangements at the IgH locus, the D_H-J_H rearrangement followed by V_H-DJ_H recombination,

which are dependant on the expression of RAG genes. E2A and EBF act in synergy to upregulate RAG1/2 which facilitate genetic rearrangements (42, 43, 52, 53). In addition, these two transcription factors induce other early B lineage specific genes such as Ig-α/ß, VpreB/L5 In the genetic hierarchy, E2A acts upstream of EBF, because EBF null mice have normal levels of E2A but $E2A^{-/-}$ mice have reduced levels of EBF and enforced expression of E2A upregulates EBF transcription (50) .

1.1.12 B-lineage commitment and maintenance by Pax5 (BSAP)

Pax5 also known as B-cell-specific activator protein (BSAP), is expressed throughout B cell development until the plasma cell stage (28). In Pax5 null mice B cell development is arrested at the early pro-B cell stage (27, 31, 55) and pro B cells from these mice exhibit multilineage potential, characteristic of uncommitted progenitors (27, 56). Reconstitution of Pax5 expression in Pax5 null pro-B cells rescues B cell development and suppresses the multilineage potential (27), suggesting a critical role for Pax5 in B lineage commitment and in the restriction of alternate fates of early progenitors. Furthermore, the continued expression of Pax5 throughout B cell development (except the plasma cell stage), is required to maintain B cell identity, as shown by conditional inactivation of Pax5 in mature B cells, where it led to loss of function and identity of mature B cells (57). In addition, conditional inactivation of Pax5 in committed pro-B cells led to the reversal of B lineage commitment and attainment of multilineage potential of early progenitors (56). Therefore, Pax5, at the time of lineage commitment, represses lineage inappropriate genes and induces B lineage specific genes, thereby inducing B lineage commitment. On the other hand, in a committed

B cell, Pax5 activity is continually required to shut off genes corresponding to alternate fates and thereby maintains B cell identity throughout B cell development. The B lineage specific transcriptional targets of Pax5 include the receptor signaling chain Igα, stimulatory coreceptor CD19 and the adaptor protein BLNK, which constitute essential components of pre-BCR signaling pathway (31, 58-61). Therefore, Pax5 by promoting Pre-BCR signaling allows successful development, as the pre-BCR signaling is an important checkpoint during early B cell development.

1.1.13 Peripheral mature B cell subsets and functions

The preimmune mature B cell repertoire is heterogeneous consisting of several B cell compartments or subsets, each with a distinct surface phenotype, functional properties and anatomical distribution (62). These fall into three broad categories, namely the follicular or B2 B cells, marginal zone B cells (MZ B cells) and B1 B cells(17, 62-66). Follicular B cells constitute the recirculating B cells in the spleen in the B cell follicles, whereas MZ B cells and B1 B cells constitute the more static pool, residing in the marginal zone of the spleen (MZ B cells) and pleural and peritoneal cavities (B1 B cells), respectively(17, 62-66). In response to foreign antigens, all these B cell subsets are capable of differentiation into plasma cells. However, they differ in the rapidity of the response, the kind of antigens they recognize and the quality of immune response they generate. B1 and MZ B cells differentiate very rapidly into plasma cells upon antigen encounter and therefore provide the first line of defence against invading pathogens. Their anatomical location and their ability to recognize, in a T independent way, the repetitive antigenic structures like cell wall polysaccharides on pathogen

enables easy and fast access to blood-borne antigens and gut/peritoneum associated infections by MZ and B1 B cells, respectively. The predominant antibodies secreted by the MZ and B1 cells are of low affinity IgM type. Despite the fact that they produce low affinity antibodies, the immune response by MZ and B1 B cells is of immense importance because of its rapidity. In contrast, follicular (B2) B cells, recognize mostly protein antigens in a T dependant manner and in response to foreign antigens do not develop into plasma cells immediately. Instead they enter into a complex set of reactions, collectively called the germinal centre reaction (GC reaction). Here class switch recombination of antigen receptors and somatic hypermutations of the immunoglobulin genes takes place, leading to affinity maturation of antigen receptors. Affinity maturation results in the generation and selection of B cells with very high affinity antigen receptors. Finally, the GC reaction culminates in the generation of both plasma cells and antigen specific memory B cells. The memory B cells have an extensive life time and upon subsequent encounter with the same antigen, they respond very quickly and elicit a very strong response. Therefore memory B cells play a predominant role in the maintenance of immunity for extended periods.

1.1.14 Terminal differentiation of B cells into Plasma cells

The IgM^+ immature B cells leave the bone marrow and enter the peripheral lymphoid organs, where they undergo further maturation, to generate the IgM^+ IgD^+ mature B cell population. Naive mature B cells upon encounter with specific antigen and acquisition of appropriate signals, undergo a complex differentiation programme into effector B cells called plasma cells (Fig. 1).

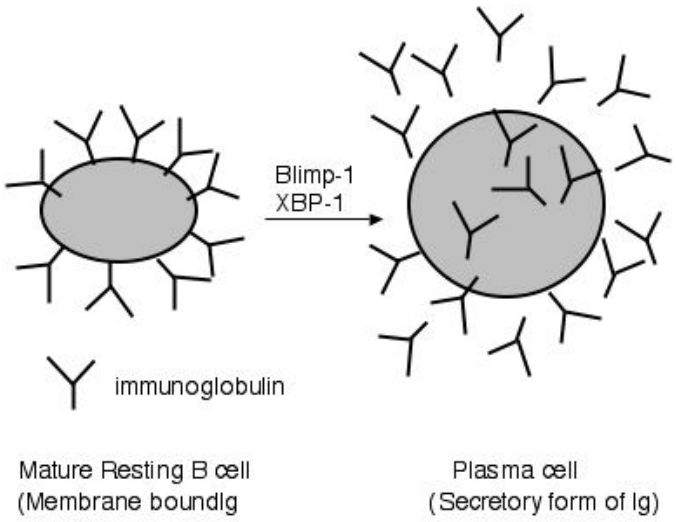


Fig. 1. Fundamental difference between resting B cells and plasma cells.. Resting B cells express membrane bound immunoglobulins (Ig). Where as plasma cells, express secretory form of immunoglobulins and secrete Ig.

Unlike resting mature B cells which express surface immunoglobulins, plasma cells synthesize enormous amounts of secretory form of immunoglobulins which lack the transmembrane domain of the immunoglobulin heavy chains (Fig. 1). Hence plasma cells secrete immunoglobulins at a very high rate, and the secreted immunoglobulins are the ultimate effector molecules involved in providing humoral immunity to the organism. The secreted immmunoglobulins, also called antibodies, act as the effector molecules of the humoral response by several ways. Binding of antibodies to antigen results in the neutralization of the antigen. For example antibodies neutralize toxins or viral particles, by coating them, which blocks them from interacting with host cell receptors, thereby preventing the access to the host. Antibodies also facilitate elimination of foreign antigens. For example antigens coated by antibodies are more readily ingested by phagocytic cells or alternatively, binding of antibodies to antigens on a

microorganism can activate the complement system, resulting in the lysis of the foreign organism.

1.1.15 Transcription factors involved in the regulation of terminal B cell differentiation

The molecular basis of terminal B cell differentiation has started to unravel with the identification of key positive and negative transcription factors involved in this process, such as Blimp-1, XBP-1, IRF-4, BCL-6, Pax-5 and Mitf.

1.1.16 B-lymphocyte induced maturation protein-1 (Blimp-1)

Blimp-1 was originally identified in terminally differentiating B cells (67, 68) Enforced expression of Blimp-1 in B cell lymphoma lines or mature B cells was sufficient to drive terminal differentiation into antibody secreting cells (67-69). Blimp-1 is sufficient to induce, on its own, many of the molecular events associated with plasma cell differentiation (67). It has been shown that Blimp-1 is capable of induction of J chain message, which encodes joining peptide ‘J’ (67). Undifferentiated B cells express monomeric membrane bound Immunoglobulin M (IgM) molecules. Plasma cells secrete IgM as pentameric structures. The organization of monomeric IgM molecules into pentameric structures is facilitated by the joining peptide J, which joins the constant regions of IgM molecules. In addition, Blimp-1 induces immunoglobulin messages corresponding to the secretory form (70). Before the onset of terminal differentiation, B cells express membrane bound Ig molecules, due to the presence of a trans-membrane domain. Ectopic expression of Blimp-1 also leads to upregulation of the plasma cell marker syndecan-1, increased cell size, granularity and eventually secretion of

immunoglobulins. Therefore Blimp-1 was described as a pleiotropic regulator of terminal differentiation (33, 67-69) . Furthermore, Blimp-1 is expressed in all plasma cells and in a small subset of GC B cells that have some plasma cell characteristics, suggesting its involvement in plasma cell differentiation (71). The importance of Blimp-1 in the terminal differentiation programme was further confirmed by B cell specific deletion of Blimp-1 in mice, which resulted in the failure of generation of humoral immune responses to both T dependant and independent antigens (72).

Blimp-1 is a 98KD zinc finger containing transcription factor (67). Blimp-1 contains two acidic domains, one at the N-terminus and the other at the C-terminus (Fig. 5A), (72-74). In addition it contains a PR domain, a proline rich domain and the Zinc finger domain (73-75) (Fig. 5A). The protein contains five consecutive zinc finger motifs of the Drosophila Krueppel C2H2 type, of which the first two confer DNA binding specificity. Blimp-1 is a nuclear protein and functions as a transcriptional repressor by recruiting human groucho, histone deacetylases and histone methyl transferases (73, 75, 76), (Fig. 2). The N-terminal part of Blimp-1 has been shown to be sufficient to mediate transcriptional repression and to induce terminal differentiation while the C-terminus has modest transcriptional activation and had no effect in inducing the secretory phenotype (75, 77, 78). Blimp-1 has been shown to directly repress several target genes e.g., c.myc and Pax5 (34, 75, 79-82). Even though Blimp-1 contains acidic domains characteristic of many transcriptional activators, direct transcriptional activation of target genes has not yet been identified. Recent studies using microarray analyses revealed that Blimp-1 regulates more than 260 genes (82). These genes fall into different functional sets, with each set of genes having a distinct role in plasma cell generation, including regulation of proliferation, cell viability, secretion,

repression of negative regulators of terminal differentiation, blockade of GC reaction and BCR signaling.

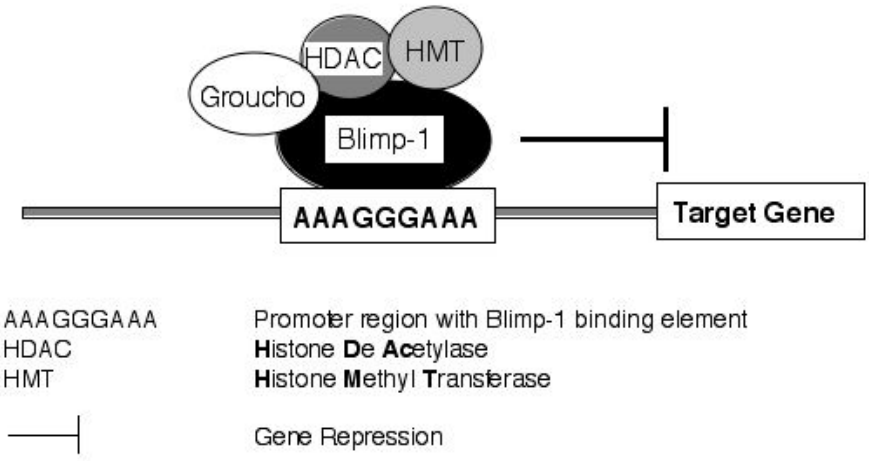


Fig. 2. Mechanism of Blimp-1 mediated gene repression. Blimp-1 binds to specific elements (e.g. AAAGGGAAA) in the promoters of target genes and mediates gene repression by recruiting factors which facilitate transcriptional inactivation.

Of particular importance is first the repression of c-myc, upregulation of MAD-4, upregulation of CDK inhibitors p21, p18, down regulation of E2F, all of which lead to cell cycle arrest, a pre requisite for differentiation (70, 79, 82). Secondly Blimp-1 regulates a set of genes involved in conferring the secretory phenotype to a B cell, e.g., induction of secretory form of immunoglobulin heavy chains, induction of XBP-1 messages (70, 72, 82, 83). In addition, Blimp-1 has been shown to regulate the viability of B cells (70, 84) and the antiapoptotic gene A1 has been identified as a Blimp-1 target (70). Finally Blimp-1 is capable of inducing plasma cell fate by repressing the negative regulators of plasma cell generation such as BCL-6, which in turn is a repressor of Blimp-1, and Pax5 which is a repressor of several important genes like J chain, Ig heavy chain and XBP-1 (32, 82). In addition Blimp-1 represses the induction of the GC reaction, by repressing BCL-6,

which is critically required for the GC reaction, AID and various genes affecting BCR mediated signaling (85-87)((Staudt).

1.1.17 X box binding protein 1 (XBP-1)

XBP-1 is another critical transcription factor for generation of plasma cells (88) Elevated levels of XBP-1 transcripts were found in plasma cells from rheumatoid synovium (88). Furthermore, XBP-1 expression can be upregulated by stimuli that induce plasma cell differentiation, suggesting its association with plasma cell differentiation (88) Consistent with that, XBP-1 null B cells fail to secrete antibodies in response to T dependent or T independent antigens, suggesting the importance of this factor in plasma cell generation (88). XBP-1 is a basic-region leucine zipper protein belonging to the CREB/ATF family of transcription factors (89). It is a ubiquitously expressed protein. However, the 30 KD XBP-1 protein encoded by the ubiquitously expressed XBP-1 mRNA, is unstable and transcriptionally inactive (90). Recent studies unraveled an interesting mechanism of activation of XBP-1, in response to accumulation of unfolded proteins or ER stress, which triggers a signaling pathway called unfolded protein response (UPR) (90-93). The increased protein load is sensed by an ER resident transmembrane protein called IRE1α, which is a serine threonine kinase as well as an endoribonuclease (91, 93). Upon sensing unfolded proteins, IRE1a gets oligomerized and undergoes trans autophosphorylation, leading to the activation of the C-terminal endoribonuclease activity. The active IRE1α then removes 26 nucleotides from the XBP-1 mRNA, resulting in a frameshift in the open reading frame of the mRNA (90, 91, 94). The spliced XBP-1 mRNA now encodes a 54KD transcriptionally active, stable protein

(90). Interestingly, enforced expression in B cells, of a non processible mutant of XBP-1, that can not generate the processed form of XBP-1 protein, blocked immunoglobulin secretion in LPS induced B cell cultures, providing the evidence for a critical role for the processed XBP-1 protein in conferring the secretory phenotype (92). Occurrence of UPR and the generation of the processed form of XBP-1 has been demonstrated during terminal differentiation of B cells (92, 95-97). Furthermore, UPR and XBP-1 processing have been shown to be dependent on immunoglobulins during plasma cell differentiation, as evidenced by lack of XBP-1 processing in immunoglobulin deficient B cells (92).

1.1.18 BCL-6 and Pax5

As described above, MZ and B1 B cells differentiate into plasma cells immediately upon antigen encounter. In contrast, the follicular B cells do not immediately develop into plasma cells. Rather they enter into GC reaction where plasma cell differentiation is actively blocked, to facilitate somatic hypermutations leading to affinity maturation, and to allow class switch recombination. This blockade of plasma cell differentiation is achieved by two transcription factors called BCL-6 and pax5. BCL-6 is highly expressed in GC B cells and pax-5 is expressed throughout B cell development except the plasma cell stage (28, 98-100). BCL-6 mutant mice fail to form GCs but plasma cell differentiation was normal in these mice (100, 101). Pre GC B cells that do not upregulate BCL-6 differentiate into plasma cell. It has been observed that most GC B cells express BCL-6 but a minority do not and these BCL-6 negative cells show plasmacytic morphology. These observations suggest that plasma cell differentiation during pre GC and post GC depends

on the absence of BCL-6 and that BCL-6 is a negative regulator of terminal differentiation.

The more direct evidence that BCL-6 represses plasmacytic diffferentiation came from recent microarray analyses, where it has been shown that BCL-6 represses a large array of genes involved in cell cycle regulation, B cell activation and terminal differentiation, including the most important gene Blimp-1 (87). Independent studies revealed that ectopic expression of BCL-6 in B cells represses plasmacytic differentiation and Blimp-1. In response to BCL-6, down regulation of the CDK inhibitor p27 kip1 was observed (87). This facilitates cell cycle progression and this is one of the requisites for GC reaction because B centroblasts undergo extensive proliferation in the GC. In addition, BCL-6 dependant repression of the master regulator of terminal differentiation, Blimp-1, allows total blockade of plasma cell differentiation in an ongoing GC reaction (87). Repression of Blimp-1 in turn derepresses several genes which play pivotal role in cell cycle progression, with c-Myc and E2F constituting important members of these. In addition, repression of Blimp-1 allows proper expression of pax5 which is another important factor which prevents plasma cell differentiation.

As described above, Pax5 plays a pivotal role in B lineage commitment and maintenance and is expressed throughout B lineage, except at the plasma cell stage (29). This raised the speculation that Pax5 might be inhibitory to plasma cell development and in fact several studies revealed the molecular basis of the negative role of Pax5 in plasma cell development. It has been shown that Pax5 represses J chain, Ig heavy chain transcription and, in addition, it represses the indispensable transcription factor XBP-1 (81, 102). Therefore Pax5 not only blocks plasma cell differentiation by down regulation of (XBP-1) which is required to mediate an important signaling

pathway during terminal differentiation, the unfolded protein response pathway, but also it impairs the very basic transcription of immunoglobulin genes (103). Therefore repression of Pax5 is one of the essential steps required for plasma cell generation but mere lack of Pax5 is not sufficient to drive plasma cell generation (57).

1.1.19 Mitf

Even though the antagonizing mechanisms that prevent plasma cell generation in the GC environment are known, it is not known if plasma cell differentiation is actively blocked in naive B cells. The microphthalamia associated transcription factor (Mitf) has been recently identified as a negative regulator of plasma cell differentiation in naive resting B cells (104). Mitf is highly expressed in naive B cells and rapidly diminishes in activated B cells (104). Transfer of bone marrow (BM) from Mitf null mice into rag deficient recipients, led to increased serum IgM levels and increased numbers of plasma cells, suggesting spontaneous differentiation of B cells into plasma cells. In addition, enforced expression of mitf in B cells under in vitro differentiation conditions, led to impaired development into plasma cells. Furthermore, it was observed that Mitf dependant blockade of spontaneous terminal differentiation is via inhibition of IRF4 expression , a plasma cell specific transcription factor (104).

1.1.20 The role of Blimp-1 in B cell homeostasis

In addition to its functions in the induction of terminal differentiation of B cells, Blimp-1 has been shown to induce reduced viability and cell death in B cells (70, 84, 105). Because Plasma cells are in general short lived effector

cells of the humoral immune response, it seems likely that induction of apoptosis by Blimp-1 might be an essential component of the differentiation programme induced by Blimp-1, which facilitates down regulation of the immune response. Removal of effector cells after execution of their function is very important (1) to provide space for new effector cells, and (2) in terms of biological economy because existence of all the Ig secreting cells even after the successful removal or neutralization of the antigen seems to be against economy principles. Therefore the immune sytem might have evolved in such a way as to accommodate master regulators like Blimp-1, which are solely capable of inducing differentiation and death, ensuring termination of effector phase.

Despite the identification of a role for Blimp-1 in apoptosis, the molecular mechanism of how Blimp-1 affects survival remained largely elusive until recently. Recent observations using truncation mutants of Blimp-1 revealed that a 69 amino acid region within the proline rich region is essential for the apoptotic activity (84). An important finding concerning the molecular targets of Blimp-1 in the context of apoptosis came with the identification of the antiapoptotic BCL2 family member A1 as a repressive target of Blimp-1 (70). This was later on confirmed by other groups by DNA micro array analyses (82). In another study it was observed that ectopic expression of A1 in Blimp-1 transduced B cells, enabled secretion of antibodies for longer periods, confirming that repression of A1 by Blimp-1 indeed plays a role in induction of death (70). Despite the identification of A1 as a target for Blimp-1, the exact mechanism and if it is a direct or indirect target, is not known. Upregulation of Mad4, a transcriptional repressor, was observed in response to Blimp-1 (70). Because the A1 promoter contains some E-box like elements, where Myc & Mad family proteins can bind, it appears possible that Blimp-1 might execute cell death either by direct repression of A1

promoter activity and/or indirectly via upregulation of transcriptional repressors like Mad4 which in turn act on the A1 promoter.

1.1.21 Apoptosis in the development and maintenance of the immune system

Apoptosis is a genetic programme of cellular suicide, which plays a pivotal role in metazoan development and cellular homeostasis (106). This process has an essential role in shaping up the immune repertoire and its maintenance.(106, 107). Abnormalities in genes regulating pro- or anti-apoptotic functions result in disorders like autoimmunity, immunodeficiency or cancer (106). Throughout their life, lymphocytes, the most important components of the immune system, are subject to multiple checkpoints of life and death, from the time of their origin till the effector phase.

Very early in the development, B cells need to express a functional BCR, to receive survival signals. failure to do so results in the elimination of such cells by apoptosis (108-110). After the expression of a functional BCR, those cells expressing autoreactive receptors are eliminated by apoptosis in a process termed negative selection of lymphocytes (111-113). After the negative selection event, the surviving lymphocytes reach the periphery and constitute the naive resting pool. The survival of naive lymphocytes till the encounter of antigen requires the antigen receptor and cytokine dependant signaling and also depends on the homing of lymphocytes to specialized niches within the peripheral lymphoid organs (114, 115). Upon antigen encounter, antigen specific lymphocytes expand rapidly and differentiate into effector cells. Apoptosis plays a key role in bringing back the immune cell numbers after the effector phase, by selectively eliminating the effector cells (106).

1.1.22 The BCL-2 family of proteins : regulators of life and death

The BCL-2 family of proteins comprises both pro- and anti-apoptotic members. They are critical regulators of the mitochondrial pathway of apoptosis (107). The founding member of this family BCL-2 (B-cell lymphoma 2) was identified as a gene that gets linked to immunoglobulin locus after chromosomal translocation in follicular B-cell lymphoma (107). BCL-2 has been shown to protect cells from various death stimuli (116-120). Later on several members of this family were discovered, based on sequence homology in specific domains called BCL-2 homology domains (BH domains). Based on the sequence and structural features, four BH domains called BH1-BH4 were identified in BCL-2 family members (121, 122). The anti-apoptotic members of this family, BCL-2, BCL-X_L , Mcl-1 and A1 contain all four BH domains. Based on the number of BH domains, the pro-apoptotic members of this family were sub divided into two groups, the multidomain pro-apoptotic members and the BH3 only pro-apoptotic members. The multidomain pro-apoptotic members Bax, Bak and Bok possess the BH1, BH2 and the BH3 domains, where as the BH3 only pro-apoptotic members like Bid, Bad, Bik etc., contain homology only within the BH3 domain (123).

The BCL-2 family members are critical regulators mitochondrial membrane permeability and caspase activation (106, 123). Many pro and anti- apoptotic BCL-2 members have a C terminal transmembrane domain, which targets these proteins to the cytoplasmic side of the intracellular membranes of the nucleus, endoplasmic reticulum and mitochondria (123-125). The role of the BCL-2 members at the mitochondria and in the regulation of mitochondrial membrane permeability has been extensively studied (106). The pro-apoptotic members disrupt the mitochondrial membrane integrity leading to

the release of cytochrome C and other apoptogenic factors from mitochondria. This is achieved by the multidomain pro-apoptotic members (e.g., Bax and Bak), which form homo oligomers, resulting in channel formation and efflux of apoptogenic proteins (107). The released Cytochrome C acts as a cofactor in the formation of multimeric complex consisting of Apaf-1 and the initiator caspase, caspase-9. This results in proteolytic cleavage and activation of caspase-9. The active caspase-9 in turn activates effector caspases, which results in the execution of apoptosis (106,107).The anti-apoptotic BCL-2 family members, most of which localized at the mitochondrial membrane, prevent homo oligomerization and channel formation by multidomain pro-apoptotic members and thereby prevent the release of apoptogenic components from mitochondria (106,107).

1.1.23 The anti-apoptotic BCL-2 family member A1

A1 belongs to the anti-apoptotic BCL-2 family. It was originally identified from a cDNA library obtained from mouse bone marrow cultures, as a granulocyte macrophage-colony stimulating factor (GM-CSF) inducible BCL-2 related gene (126). In response to GM-CSF, A1 is induced independent of de novo protein synthesis, suggesting that it is an early response gene in the hematopoietic system (126). Expression of A1 messages was observed in various hematopoietic cell lineages like macrophages, neutrophils, T-cells and B cells (126). In lymphocytes A1 is upregulated in response to antigen receptor stimulation (127, 128). Elevation of A1 levels was observed when immature B cells transit to the long lived pool of resting B cells, suggesting a role for A1 in the maintenance of naive mature peripheral B cell pool (129) Furthermore, CD40 stimulation has been shown to induce A1 in B cell and to confer protection against antigen receptor

induced cell death (130, 131). Interestingly, ectopic expression of A1 can not rescue from the proliferation block induced by antigen receptor triggering, suggesting a specific survival role for A1 in immature B cells (130). The anti-apoptotic functions of A1 were further established in response to diverse apoptotic stimuli like TNFα and p53 induced apoptosis (130-136). In terminally differentiating B cells, A1 has been shown to be down regulated at the RNA level, in response to the master regulator Blimp-1 (70, 82). Ectopic expression of A1 together with Blimp-1 prolonged the lifespan of Ig secreting cells, suggesting a critical role for A1 in determining the effector phase of a terminally differentiated B cell (70). Furthermore, gene expression profiling revealed the down regulation of A1 in immature plasmablasts and mature plasma cells, compared to undifferentiated B cell (137).

Studies addressing the molecular basis of induction of A1 in lymphocytes suggested an NF-kB dependent transcriptional upregulation of A1, in response to various B cell & T cell mitogens like anti-IgM, anti-CD40, anti-CD3/anti-CD28, Concanavalin A, PMA etc., (127, 130, 131). Interestingly it was observed that such induction is dependent in particular on c-rel, as splenocytes from c-rel deficient mice failed to upregulate A1. Such c-rel dependent induction of A1 was attributed to the presence of an NF-kB responsive element in the A1 promoter which has been shown to be indispensable for rel dependent induction of A1 promoter activity (127).

Despite its established role as an anti-apoptotic BCL-2 member, the mechanism of action of A1 is still not clear. Unlike other antiapoptotic BCL-2 members, A1 lacks the membrane anchoring hydrophobic transmembrane domain (138). Furthermore, there are contrasting observations with regard to A1 localization in a cell, with reports of mitochondrial and cytosolic localization (139, 140). Therefore the exact localization and mode of

function of A1 still remains elusive. However, a recent study identified a mechanism of action of A1 (138). In this study it was observed that A1 acts by inhibiting the collaboration between the pro-apoptotic BH3 only protein Bid and the mitochondrial channel forming Bax/Bak proteins, in the induction of cytochrome C release from mitochondria. This is achieved by interaction of A1 with the active truncated form of Bid (tBid), at the mitochondrion, to sequester it from multidomain proapoptotic Bax/Bak proteins (138).

1.1.24 The Myc, Max and Mad family of proteins

The Myc, Max and Mad network consists of a group of transcription factors, comprising both transcriptional activators and repressors (141). They play essential roles in the regulation of cellular proliferation, differentiation and death (142, 143). These proteins belong to the basic helix-loop-helix zipper (bHLHZ) family of proteins. The bHLHZ domain mediates protein protein interactions and DNA binding (141-143). Myc, Mad and other members (e.g., Mga, Mnt) of the family function as transcription factors by forming heterodimers with Max. (141-143). Therefore Max is the common heterodimeric partner for Myc, Mad and other members of the network (144, 145). Only heterodimers comprising Max possess DNA binding ability and transcriptional activation or repressor function. Max can form homodimers possessing the DNA binding capacity but Max homodimers are transcriptionally inactive(141, 142, 146). Other members of the family can form homodimers very poorly and have very weak DNA binding ability, whereas heterodimers with Max are formed more readily and have high affinity DNA binding ability (144, 145). In addition, the Max interacting partners have short half life and their synthesis is highly regulated. So the

activation or repression of specific genes depends on the synthesis of Myc or Mad or other Max interacting partners, in response to a specific stimulus or signal, which upon synthesis associate with Max to form heterodimers. The heterodimers then act as transcription factors on specific target genes. The transcriptional modulatory function of a heterodimer depends on the Max interacting partner in the heterocomplex. For instance a Myc-Max heterodimer functions as a transcriptional activator because Myc has the ability to recruit transcriptional coactivators (histone acetyl transferase), while Mad-Max complexes act as transcriptional repressors due to the recruitment of corepressors by Mad (mSin3 histone deacetylase complex) (147-149). Interestingly, the Max containing heterodimers all recognize the same elements in the DNA called E-boxes with the hexameric sequence 5' CACGTG 3'. The Myc-Max heterodimers recognize the E-box sequence and promote transcriptional activation whereas the Mad-Max complexes recognize the same E-boxes but mediate transcriptional repression (141, 142, 146). The Myc and Mad family of proteins in general have antagonistic functions, with Myc family of proteins being generally pro proliferative and Mad family proteins antagonistic to proliferation and generally linked to terminal differentiation (141, 150).

1.1.25 The Myc family

Myc belongs to a family of proto-oncogenes comprising C-Myc, N-Myc, L-Myc (141, 142). Out of these C-Myc is the most extensively studied gene, in the context of cellular proliferation & tumorigenesis (151). Myc expression is induced in response to diverse growth promoting signals and it induces proliferation (151). Induction of Myc occurs as an immediate early response to most mitogenic signals. Interestingly, growth inhibitory signals like TGF

have been shown to repress c-myc expression (141). Therefore Myc expression strongly correlates with growth and proliferation. Overexpression studies revealed that enforced expression of myc results in growth factor independent proliferation, block in cell cycle exit, accelerated proliferation and increased cell size, confirming its role in proliferation (152). However in the absence of survival factors, c-myc overexpression results in proliferation but concomitant apoptosis (152).

Consistent with its role in cellular proliferation and growth, Myc has been shown to modulate important genes involved in cell cycle, growth adhesion and apoptosis (141). Of these, some targets are directly activated due to the transcriptional activation function of c-myc while some are repressed due to Myc activity. For example, the important components of cell cycle progression like D type cyclins and CDK4 are transcriptional induced by c-myc while the genes inhibitory to cell cycle like p21 ^{CIP1} are repressed. Even though, as described above, the mechanism of transcriptional activation by Myc has been established, the mechanism of transcriptional repression was solved only very recently (153, 154). In these studies, it was shown that the transcriptional repression is mediated by interaction of Myc with another protein called Miz-1 at specific DNA elements called 'initiator' elements, in certain target genes. Through such interaction, Myc opposes the transcriptional activation function of Miz-1. This kind of mechanism has been shown for the Myc mediated repression of cell cycle inhibitor p15^{INK4b} and for the Myc antagonist Mad4 (155).

1.1.26 The Mad family

The Mad family of transcription factors is composed of Mad1, mxi1, Mad 3

and Mad4 proteins. The Mad family proteins resemble the Myc family in their association with Max to become a functional transcriptional regulator and in their ability to recognize the same DNA binding elements (E-boxes) (141, 150). However, in contrast to Myc, Mad-Max complexes act to repress E-box containing promoters (146, 150, 156). Also in contrast to Myc, Mad protein expression is correlated with terminal differentiation in many cell types (146, 150, 156). Furthermore, under differentiation conditions, a switch from Myc-Max to Mad-Max complexes occurs, suggesting an important function for Mad proteins in cellular differentiation. In support of this, ectopic expression of Mad has been shown to interfere with proliferation and to block cooperative transformation by Myc and Ras (141). However, till date, there is no evidence if ectopic expression of Mad proteins is sufficient to induce terminal differentiation.

1.1.27 Myc, Mad, Blimp-1 and B cell terminal differentiation

Myc levels rapidly diminish during terminal differentiation of many cell types including B cells (70, 141). Possibly Myc down regulation results in the cell cycle exit that is required for terminal differentiation. In the context of B cell terminal differentiation, c-Myc has been shown to be repressed by Blimp-1 (70, 79). The down regulation of Myc is generally associated with upregulation of Mad factors, under differentiation conditions. In fact, in terminally differentiating B cells Mad4 was found to be upreguated in response to Blimp-1 (70). However, the mechanism of Mad4 upregulation in response to Blimp-1 is not known. Because c-myc is a repressor of Mad4 , upregulation of Mad4 in response to Blimp-1 could atleast in part is due to repression of c-myc (155). Therefore, Blimp-1 dependant terminal differentiation seems to occur, at least, via the modulation of Myc-Max to

Mad-Max ratios (70). Perturbation of Myc, Mad network by Blimp-1 might also have some influence in the regulation of B cell homeostasis, because the the promoter of anti-apoptotic gene A1 contains E-box like elements, where Myc/Mad can bind.

2. Aims of the project

Terminal differentiation of B cells into antibody secreting plasma cells is an essential step for eliciting a successful humoral immune response. The molecular basis of this differentiation programme has started to unravel with the identification of B lymphocyte induced maturation protein-1 (Blimp-1) and X-box-binding protein-1 (XBP-1) as indispensable transcription factors required for this process (67, 88).

Despite the requirement of both Blimp-1 & the processed form of XBP-1 in plasma cell generation, it was not yet clear how these two transcription factors collaborate during plasma cell generation. Especially, it was not evident if these two transcription factors act in the same pathway or in alternate pathways of plasma cell generation. Because ectopic expression of Blimp-1 in B cells is sufficient to generate plasma cells, which synthesize and secrete large amounts of immunoglobulins, it appears possible that the induction of immunoglobulins in response to Blimp-1 might be the trigger for UPR which results in XBP-1 processing. Therefore one of the major aims of this project was to understand (1) if Blimp-1 is the upstream molecule providing the signal for UPR resulting in the splicing of XBP-1 and (2) to study if Blimp-1 alone is sufficient to trigger UPR, in B cells. To achieve these goals, the effect of ectopic expression of Blimp-1 on the induction of

UPR and XBP-1 processing was studied in primary splenic B cells and in a mouse B lymphoma line, WEHI 231.

Another important feature of Blimp-1 is its ability to induce apoptosis in B cells. The identification of Bcl-2 family anti-apoptotic gene A1 as a Blimp-1 target, opened up the molecular basis of Blimp-1 induced apoptosis. However it still remains to be understood if A1 is a direct or indirect target of Blimp-1. So another focus of this study was to identify the mechanism of Blimp-1 dependant repression of A1. Because Blimp-1 has been described as transcriptional repressor capable of binding to specific elements in promoter elements of target genes like c-myc, pax5, etc., the possibility of direct repression of A1 promoter by Blimp-1 was analyzed. To achieve this, truncated and site specific mutants of A1 promoter coupled to luciferase reporters were used, to study the effect of Blimp-1 on the A1 promoter.

The repression of A1 gene by Blimp-1, might have important implications in the induction of terminal differentiation. Because Blimp-1 is an inducer of terminal differentiation, possibly repression of A1 might be one of the pre requisites for terminal B cell differentiation. Therefore another focus of this study is to understand the significance of Blimp-1 mediated repression of A1, in the context of B cell terminal differentiation

3. Materials

3.1 Antibodies:

anti-XBP-1 (Rabbit polyclonal)	Santa Cruz
anti-ERK (Rabbit polyclonal)	Santa Cruz
anti-Flag (mouse monoclonal)	Sigma
anti-GFP (Rabbit polyclonal	Clontech
anti-μchain F(ab')$_2$ (Goat monoclonal)	Jackson labs
PE-anti-mouse IgM^b	Pharmingen
PE-anti-mouse IgM^a	Pharmingen3
PE-Rat IgG$_{2a}$	Pharmingen
Streptavidin-PE	Pharmingen
PE-anti-mouse syndecan-1	Pharmingen
PE-anti-mouse kappa	Pharmingen

3.2 Antibiotics

Ampicilin	Sigma
G418	Calbiochem
Penicilin	Medical stores
Streptomycin	Medical stores
Zeocin	Invitrogen

3.3 Chemicals

Agarose	Roth
EDTA	Roth
Tris	Roth
$ZnCl_2$	Sigma
DMSO	Sigma

DTT	Sigma
Trypanblue	Sigma
SDS	AppliChemGMBH
Saponin	Sigma
Glycine	Roth
Boric acid	Roth
Magnesium chloride	Roth
Sodium chloride	Roth
Sodium azide	Sigma
Bovine serum albumin (BSA)	Sigma
Polyacrylamide	Applichem
APS	Sigma
Ethanol	Roth
TEMED	AppliChemGMBH
Tween20	AppliChemGMBH
Ethydium bromide	Roth
Potassium chloride	Sigma
NP-40	Sigma
Trizol	GIBCO/Invitrogen
Nitrocellulose membrane	Amersham/ Bioscience

3.4 Radioactive material

Alpha-P32 dATP	Amersham/Hartman analytik

3.5 Enzymes

BamH1	NEB
Bgl11	MBI

EcoR1	NEB
Ssp1	MBI
Pst1	MBI
Pfu DNA Polymerase	Promega
T4 DNA ligase	NEB
SAP	MBI
Klenow fragment	NEB
Superscript reverse transcriptase	Invitrogen

3.6 Media

RPMI	Institute facility
Glutamine (0.07%)	GibcoBRL
Non-essential aminoacids	GibcoBRL
Sodium pyruvate	GibcoBRL
FCS	GibcoBRL
β mercaptoethanol	GibcoBRL
DMEM	Institute facility

4. Methods

4.1 Cell Culture

WEHI 231 cells (murine B cell lymphoma line) and 293T cells (human embryonic kidney cell line 293 expressing SV40 T antigen) were obtained from American Type Culture Collection. The B cell line was maintained in RPMI 1640 medium supplemented with 4.8 mM L-glutamine, 1 mM sodium pyruvate, 1x non essential amino acids (Life Technologies), 5% (v/v) FCS, streptomycin (0.2 mg/ml), penicillin (400U/ml) and ß-mercaptoethanol (5×10^{-5} M). Cells were maintained at 1×10^5 cells/ml and replenished with fresh medium every two days. The 293T cells were propagated in DMEM supplemented with 10% (v/v) FCS and streptomycin/penicillin as mentioned above. 293T cells were always plated at a density of 2×10^5 cells/ml and replenished with fresh medium every two 3 days.

Murine splenic B cells were cultured at 1×10^6 cells/ml in RPMI 1640 (X vivo-15) supplemented as described (Mishell RJ 1967 Jexmed). Cells were stimulated with LPS 20 µg/ml (Pseudomonas aeruginosa, Sigma, St. Louis, MO) or with LPS 10 µg/ml plus anti-µ F (ab'_2) 5 µg/ml (goat anti-mouse IgM, µ chain specific from Jackson Immuno research laboratories). Stimulated B cells were replenished with fresh medium every 24 hrs.

4.2 Long Term storage of WEHI 231 cells

WEHI 231 cells were pelleted and washed once with PBS**.** Cells were then resuspended in freezing buffer (90% FCS, 10% DMSO), at a density of 2-3 million cells/ml and aliquoted into cryovials. The cryovials were frozen at -80^{o}C. Repropagation was performed by placing the vial with frozen cells

briefly at 37^{o}C and gradually thawing the cells by the addition of complete medium. The cell suspension was transferred to a Falcon tube and cells were pelleted by centrifugation. The cell pellet was once washed with complete medium, to remove the DMSO and cells were pelleted. The cell pellet was suspended in complete medium and plated in tissue culture flasks.

4.3 Preparation of murine splenic B cells

High density mouse splenic B cells were prepared as described (Chen-Bettecken U., PNAS 1985), from spleens of 6-8 week old C57BL6 mice bred at the institute from breeding couples obtained from Iffa Credo (Saint Germain sur L'Arbelse,Lyon, France). B cells with a density of =1.087 g/cc were isolated from a step wise Percoll Gradient (Pharmacia, Freiburg,Germany) and further enriched by positive selection using CD19 magnetic beads (Miltenyi Biotech). Approximately 1x10 ^{7} B cells were incubated with anti-CD19 mAb in 1 ml of FACS buffer on ice for 25 min and then washed with 10 times excess of MACS buffer. After centrifuging at 1300 rpm for 5 min at 4^{o}C, cells were resuspended in 600 µl of MACS buffer and mixed with 100 µl of anti-rat.IgG Microbeads (Miltenyi Biotech) and incubated for 15 min on ice. After incubation, excess magnetic beads were washed away by using 10-121 ml of cold FACS buffer and centrifuged at 1300 rpm for 5 min at 4^{o}C. Cell pellet was resuspended in 1 ml of cold MACS buffer and loaded on to the MACS positive selection column (LS ; Miltenyi Biotech). B cells bound to the column were eluted from the column by removing the magnetic field.

MACS Buffer : 0.5% BSA in PBS without Ca^{2+} and Mg^{2+}, with 2 mM EDTA.

4.4 Transfection of 293 T cells

293T cells were transfected by the calcium phosphate method. The day before transfection, 293 T cells were plated at a density of 2 x 10^5 cells/m, with a total of 5 ml in 6 cm petri dishes. Next day, cells were replenished with fresh medium. Precipitation of DNA to be transfected was done by using $CaCl_2$ in HBS buffer. 6-8µg of DNA to be transfected was taken into an eppendorf tube with 60 µl of 2 M $CaCl_2$, and the volume is made to 500 µl with autoclaved water. Now 500 µl of 2x HBS buffer was added into the eppendorf tube containing DNA, with vigorous aeration. The DNA precipitates were added on to the cells and dispersed all over the cells evenly, by gentle shaking of the petri plate. Next day, medium was removed from petri plates and fresh medium was added.

4.5 Luciferase Assays

293 T cells were plated at a density of 2x10^5 cells per well in 12 well plates, the day prior to transfection. Next day, cells were fed with fresh medium, 3 hours prior to transfection. Transfections were carried out as described above. Cells were co-transfected with pGL3 luciferase reporter under the regulation of either wild type, truncated or mutant versions of A1 promoter, together with pEYZ7MCS, pEGN/MCS vector control plasmids or pEYZ/FBlimp-1, pEGN/FMAD4 respectively. 36 hours after transfection, the medium was aspirated and cells were washed once with cold PBS. PBS was aspirated and cells were lysed using 250µl lysis buffer. The lysates were taken into eppendorf tubes kept on ice. Wherever indicated, an aliquot 50µl of each lysate was used for SDS-PAGE and western blotting, to detect GFP by using anti-GFP antibody, as a transfection efficiency control. For the

analyses of Luciferase activity, 50 µl of lysate was mixed with 50 ml of assay buffer in 96 well plates. The luciferase activity was measured in a luminometer, using luciferin substrate solution. Before measuring the luciferase activity, the luminometer was once washed with Luciferin substrate solution. After measurement, the luminometer was once washed with water and then with air.

Lysis Buffer: 50mM Tris, 50mM MES, 1mM DTT, 0.1% TritonX-100 pH7.8

Assay Buffer: 125mM MES, 125mM Tris, 25 mM Mg_2oAc, 5mM ATP pH7.8

Luciferin substrate solution: 250µM Luciferin in 5mM Khpo4 pH7.8

4.6 Bicistronic retroviral vectors

The bicistronic retroviral vector pEYZ/MCS **is** based on the pcz CFG2 hCD8/EYZ retroviral vector (kuss). In this vector, the CMV enhancer replaces the U3 region of the 5' long terminal repeat (LTR) of the murine leukemia virus (MuLV). Using this vector, co-ordinate expression of the chimeric marker gene and the gene of interest is possible via an internal ribosome entry site (IRES), derived from encephalo -myocardis virus . The pEYZ/MCS consists of a multiple cloning site encompassing the restriction sites for Eco R 1, Smi 1 and BamH 1. In the pEGN/MCS, GFP/neomycin resistant protein serves as a chimeric selection marker. In order to generate flag-tagged versions of proteins, an oligonucleotides encoding the flag-peptide was inserted into the MCS, to yield pEYZ/MCS-F or pEGN/MCS-F.. In this study, the DNA sequences encoding Blimp-1, N-Blimp-1, C-Blimp-1, A1 and Mad4 were introduced into the multiple cloning site of pEYZ/MCS or pEGN/MCS respectively. The retroviral vector pEYZ/F-Blimp-1 was

generated by cloning Eco R 1/Bam H 1 fragment of pEGN/Blimp-1 into pEYZ/MCS-F. cDNAs corresponding to truncated mutants of Blimp-1 encoding N-Blimp-1 (aa 1-751) and C-Blimp-1 (aa 465-856) were generated by PCR amplification of the respective regions from full length Blimp-1 and were inserted into the MCS of pEYZ/MCS-F. The generation of pEYZ/F-A1 was previously described (Knodel). The Mad4 cDNA was obtained by RT-PCR from RNA isolated from murine splenic B cells stimulated with LPS and infected with Blimp-1. The Mad4 cDNA was inserted into pEGN/MCS-F by using Eco R 1 and Bam H 1 sites.

4.7 Generation of recombinant retroviruses

Recombinant revtroviruses (rRV), harbouring the gene of interest were generated by using the pHIT packaging system (Soneka et al). 293T cells were transiently transfected with the expression construct for murine leukemia virus Gag/Pol (pHIT60) and Env (pHIT123), as well as the respective expression constructs pEYZ/MCS, pEGN/MCS, pEYZ/FBlimp-1F, pEYZ/N-Blimp-1F, pEYZ/C-Blimp-1F, pEYZ/FA1 or pEGN/MAD4F. Next day, cells were replenished with fresh medium. Viral supernatants were harvested every 24 hrs for 3 days. The supernatants were filtered through 0.45 μm filters. Polybrene was added to a final concentration of 10 μg/ml and the supernatants were used either freshly or stored at -70^{o}C for later use.

4.8 Infection of cells using recombinant retroviruses

1 x 10^5 WEHI 231 cells were suspended in 5 ml of recombinant retroviral supernatant, in 15 ml tubes. Cells were incubated for 10 min at RT with gentle shaking and then infection was carried out with spin centrifugation.

Spin centrifugation was carried out at 2200 rpm, at 32^{o}C for 3 hrs. After 3 hrs, the viral supernatant was removed and cells were resuspended in fresh medium in a 37^{o}C incubator. After one day, the infected cells were further selected incubating with either 250 µg/ml of Zeocin or 50 mg/ml of Neomycin, based on the antibiotic resistance marker present in the expression construct, for 1 or 2 days. With this method in general 80% infected cells were obtained. The positivity or percent infected cells was measured by FACS analysis of EYFP (Enhanced Yellow Fluorescent protein) or EGFP (Enhanced Green Fluorescent protein).

Primary murine B cells were infected, essentially, in the same way as described above with some modifications. Splenic B cells were stimulated with either LPS or with LPS plus anti-µ as described above and kept in culture for 1 day. After 1 day, 1.5 - 2 x 10^{6} of stimulated cells were used per 5 ml of retroviral supernatant. After infection, the cells were again kept in the same stimulatory conditions as before like LPS or LPS plus anti-.µ, till further analysis. Antibiotic mediated selection of the infected cells was started one day after infection, with 100 µg/ml of Zeocin.

4.9 FACS analyses

All the FACS analyses were carried out on Beckton Dickinson FACScan, using cellQuest software. For the quantification of infected cells, expression of EYFP or EGFP was analyzed.

Analyses of Immunoglobulin expression levels by flow cytometry

2 x 10^{5} primary B cells or WEHI 231 cells, either infected or variously stimulated, were washed once with cold FACS buffer (1xPBS/1%BSA). FC

receptors were blocked by incubating with Fc-specific non-fluorescent antibodies for 15 min on ice. Surface kappa chains were stained with non-fluorescent anti-kappa antibodies for 20 min on ice. Thereafter, cells were washed once with cold FACS buffer, followed by cold PBS alone. Cells were then fixed by using formaldehyde at a final concentration of 2%, for 20 min at room temperature. Cells were washed once with FACS buffer. Fixed cells were either used for FACS staining or stored at this stage at 4^{o} C, in dark up to 3 days for further analyses. Cells were permeablized by incubation with saponin buffer with 0.5% saponin in FACS buffer., for 10 min at room temerature.. Cells were washed once in saponin buffer and intracellular Fc receptors were blocked by using Fc block. Then cells were incubated with phycoerethrin (PE) labeled isotype control antibodies or with PE labeled anti- kappa chain or anti-μ chain antibodies for 30 min at room temperature, in saponin buffer. Cells were washed twice with saponin buffer and once with FACS buffer and used for analysis.

4.10 Immunoglobulin secretion assay

Immunoglobulin secretion assay was carried out for LPS stimulated or rRV infected WEHI 231. Unstimulated, LPS stimulated, pEYZ/MCS or pEYZ/F-Blimp-1 infected cells were cultured for 2 days at a density of 1 x 10^5 / ml in 12 well plates. In case of pEYZ/MCS or pEYZ/Blimp-1 infections, infected cells were selected with antibiotic 250 μg/ml Zeocin for 1 day. Culture supernatants were taken after 2 days and centrifuged at 1400 rpm for 10 min at 4^{o}C. The supernatants were either used immediately or frozen at -70^{o}C for later use. Secretory form of IgM in the supernatants was detected by subjecting the supernatants for SDS PAGE on a 10% polyacrylamide gel,

followed by western blot analyses. μ chain specific antibody was used to detect the IgM molecules in the supernatants.

For detection of secreted IgM, murine splenic B cells were cultured at a density of 1 x 10^6 / ml in 12 well plates, stimulated with either LPS or with LPS + anti-μ. Alternatively, splenic B cells were stimulated with LPS + anti-μ and infected with either pEYZ/MCS or with pEYZ/FBlimp-1, pEYZ/FN-Blimp-1, pEYZ/FC-Blimp-1 constructs. In another set up, LPS stimulated splenic B cells were infected with pEYZ/MCS or pEYZ/FA1. Two days after infection, culture supernatants were processed as described above and used in western blot analysis to detect secreted IgM, by using μ chain specific antibody.

4.11 Preparation of whole cell extracts

WEHI 231 cells and murine splenic B cells were taken from culture flasks/plates into falcon tubes and cells were pelleted. In case of 293 T cells, cells were harvested by either trypsinization or scrapping. Cell pellets were washed once with PBS. The cell pellets were resuspended in 2 fold pellet volume of ice cold suspension buffer. Then an equal volume (2 fold pellet volume) of 2X SDS loading buffer was added, so that the final concentration of the loading buffer becomes 1x. The sample was boiled at 95^{o}C for 10 min and then kept on ice. The chromosomal DNA was sheared by sonication. The sonicated samples were then centrifuged at 14000 rpm for 10 min at RT and the supernatants were transferred to a new tube and used for analyses by SDS-PAGE.

Suspension buffer: 0.01 M Tris.Cl pH 7.6, 0.1M NaCl, 0.001M EDTA.

2x SDS gel loading buffer: 100 mM Tris-Cl pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue and 20% glycerol.

4.12 Preparation of Nuclear / Cytoplasmic fractions

1 x 10^6 Cells were pelleted in an eppendorf tube. Cells were washed once with cold PBS and the cell pellet was suspended in 400 µl ice cold buffer A. The sample was incubated on ice for 15 min and then 25 µl of 10% NP-40 was added, vortexed vigorously for 10 seconds and centrifuged for 1 min at 14000 rpm, at 4^{o}C. The supernatant contains the cytoplasmic fraction and was taken into a new eppendorf tube. The pellet contains the nuclear fraction and was washed once with 200µl of cold buffer A and nuclei were pelleted by centrifugation at 14000 rpm, for 1 min at 4^{o}C. The supernatant was removed and 100 µl of buffer C was added. The sample was placed on an eppendorf shaker for 15 min at 4^{o}Cwith vigorous shaking. The sample was centrifuged at 13000 rpm for 20 min at 4^{o}C. the supernatant containing the nuclear proteins was aliquoted into new eppendorf tubes. Cytoplasmic and nuclear fractions were stored at -70^{o}C till further use. In order to use these fractions for SDS-PAGE , appropriate amounts of protein extracts were mixed with SDS gel loading buffer to a final concentration of 1x. Samples were boiled for 10 min at 95^{o}C and, centrifuged at 14000 rpm for 10 min and supernatants were loaded on to the gel.

Buffer A: 10mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1mM EGTA, 2.5 mM DTT and proteinase inhibitor cocktail .

Buffer C: 20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 25% glycerol, 2.5 mM DTT and proteinase inhibitor cocktail.

Both buffers were stored at 4^{o}C, without DTT and proteinase inhibitors. DTT and proteinase inhibitors are always added freshly, just before use.

4.14 Western Blotting

4.14.1 SDS-PAGE

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed, by using 10 to 12.5 % polyacrylamide resolving gels. Resolving and stacking gels were made as described below.

Resolving gel	10%	12.5%
dd H_2O	6.25 ml	5.00 ml
acryl : bisacryl 30%	5.00 ml	6.25 ml
lower Tris ph 8.8	3.75 ml	3.75 ml
10% APS	150 µl	150 µl
TEMED	25 µl	25 µl

Stacking gel	
dd H_2O	3.00 ml
acryl : bisacryl 30%	0.65 ml
lower Tris ph 8.8	1.25 ml
10% APS	100 µl
TEMED	10 µl

Resolving gel solution was made as described above and poured in between glass plates. After polymerization was complete, stacking gel solution was poured on top of polymerized resolving gel and an appropriate comb was inserted. Following polymerization, comb was removed and the gel was used for electrophoresis. Proteins samples were boiled for 10 minutes at 90^{o}C, in 1x SDS gel loading buffer and loaded on to the wells. Electrophoresis was carried out in 1x Tris-glycine running buffer.

1X Tris-Glycine buffer: 25 mM Tris, 250 mM Glycine, 0.1% SDS

Upper Tris: 30.3 g Tris, 10 ml 20% SDS, H_2O to a final volume of 500 ml, pH adjusted to 6.8

Lower Tris: 90.85 g Tris, 10 ml 20% SDS, H_2O to a final volume of 500 ml, pH adjusted to 8.8

10% APS: 1 g Ammoniumpersulfate dissolved in ddH_2O and brought to a final volume of 10 ml.

20% SDS: 20 g SDS dissolved in 100ml of ddH_2O.

2x SDS gel loading buffer: 100 mM Tris-Cl pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue and 20% glycerol.

4.14.2 Transfer of Proteins from gel to membrane

According to the dimensions of the gel., Whatman filter papers and PVDF membrane (Immobilon^{TM}, Millipore) were cut. The PVDF membrane was activated for 15 seconds in methanol. Whatman filters were soaked in transfer buffer. Gel was placed on whatman filters presoaked in transfer buffer and the activated PVDF membrane was placed on the gel. The PVDF membrane was in turn covered with 3 more presoaked whatman filters. Air bubbles were removed by rolling over a glass pipette gently. The sandwich containing the gel and membrane was kept in a semidry transfer system and the transfer was carried out at 70 ma for 2 hours.

4.14.3 Immunoblotting

Following transfer, membrane was placed in methanol for few seconds and then allowed to air dry. The membrane was kept with protein side up in blocking solution and blocked for at least 1 hour at RT or O/N at 4^{o}C, on a shaker with gentle shaking. Following blocking, the membrane was incubated

with primary antibody solution,used at a desired dilution and for a specified time, based on the type of antibody used, with gentle shaking . After incubation with primary antibody, the membrane was washed 3 times, 10 min each with TBS-T, on a shaker. After washings, the membrane was incubated with horseradish peroxidase (HRP) conjugated secondary antibody for 1 h at RT. Again the membrane was washed 3 times with TBS-T, as described above. Following washes, chemiluminescence detection was performed to detect the proteins, by using ECL western blotting system (Amersham), according to the manufacturer's instructions.

The membrane was used several times to detect different proteins either by stripping the membrane of the bound antibodies or without stripping. Stripping was carried out by incubating the membrane in stripping buffer at 50^{o}C, for 30 min, with occasional gentle shaking. Following stripping, the membrane was washed twice with large volume of TBS-T at RT on a shaker. The membrane was then blocked with blocking solution for 1 h at RT, on a shaker. The membrane is now ready for further probing.

10x TBS: 250 mM Tris, 1.5M NaCl. pH8.0

TBS-T: 1x TBS with Tween-20 added to 0.1%

Blocking solution: 5% non-fat dried milk in TBS-T

Stripping buffer: 100 mM 2-Mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7

Antibody solutions: Desired amount of primary or secondary antibody was made in blocking solution. Primary antibody solutions were frozen at -20^{o}C for repeated use.

4.15 Preparation of oligonucleotide probes

Complementary single stranded oligonucleotides with 5' overhangs, were annealed in the following reaction mix.

Reaction volume	50.0 µl
5' oligo (100 µM)	10.0 µl
3' oligo (100µM)	10.0 µl
NaCl (3M)	1.5 µl
Tris (1M pH 7.5)	5.0 µl
$MgCl_2$ (1M)	5.0 µl
TE	18.5 µl

The reaction mix was heated for 2 min at 85 ^{o}C, on a heat block and allowed to cool down to 25^{o}C O/N, by setting the heat block to 25^{o}C.

4.16 Radioactive labeling of the annealed oligonucleotides

All the oligonucleotides used were with 5' T overhangs. Hence Radiolabelling of the annealed oligonucleotides was achieved by using α ^{32}P-dATP, in a klenow reaction.

Klenow reaction was performed by using the following reaction mix:

10 µl ddH_2O

2.0 µl annealed oligo

2.0 µl 10x reaction buffer

2.0 µl dNTPs (0.5 mM each without dATP)

3.0 µl α ^{32}P-dATP

1.0 µl klenow fragment

The 20 µl reaction mix was incubated for 20 min at RT.

4.16.1 Purification of the probe from free nucleotides

10 µl of blue dextran solution (0.7% (W/V) in water) was added to the 20 µl reaction mix. Sephadex G-50 in autoclaved water was used to prepare 1ml column in a 1ml syringe, the bottom of which was plugged with glass wool. The reaction mix with blue dextran was loaded on to the column and separation of oligonucleotides from free nucleotides was carried out under gravity. Elution was carried out with TE buffer. Under these conditions, the oligonucleotides move along with blue dextran solution where as the free nucleotides migrate slowly. Therefore, 200-300µl of blue solution containing the oligonucleotides was eluted and unincorporated radioactivity was left in the column. One µl of the eluate was measured for incorporated radioactivity. A total of 50000 cpm were used per reaction in EMSA.

4.17 Electrophoretic Mobility Shift Assay (EMSA)

Mobility-shift assays were carried out by incubating 5 µg of nuclear extract with 50000 cpm of α ^{32}P-dATP labeled oligonucleotides. Oligonucleotides representing putative Blimp-1 binding sites 1,2,3 and 4 from A1 promoter were used in the EMSA, with the Blimp-1 binding site from the c-myc promoter used as a positive control. The oligonucleotides were incubated in a reaction mix containing 1 µg of poly d(I-C) (Roche) for 25 min on ice, in a buffer containing 10mM Tris-HCl pH 7.5, 5mM $MgCl_2$, 1 mM EDTA, 1 mM $ZnCl_2$, 1% glycerol and 0.5 mM DTT. For supershift assays, nuclear extracts were incubated first with 1µg of monoclonal anti-Flag antibody (Sigma) or with control antibody (anti-pTyr (1G2), SC-020, Santa Cruz Biotechnology) for 20 min on ice and for a further 20 min after the addition

of the radiolabelled probe. For competition assays, the nuclear extracts were first incubated with non-labelled oligonucleotides for 20 min and then radiolabelled probes were added and incubated for further 20 min on ice. The reaction mix was loaded on to a 5 % polyacrylamide gel and DNA-protein complexes were resolved at 4^0C. After the run, the gel was dried at 80^oC for 90 min and subjected to autoradiography.

5% poly acrylamide gel: 8.3 ml acrylamide:bisacrylamide (30%), 2.5 ml 5x TBE, 39.2 ml ddH_2O.

5x TBE: Tris base 54.0 g , Boric acid 27.5 g, EDTA 3.75 g, dissolved in 1L ddH_2O.

The following oligonucleotides were used for EMSAs:

A1 promoterBlimp-1 binding sites:

Site-1:	AACGAAAAAAGTAAATG
	TTCATTTACTTTTTTCGTT
Site-2	AAACATTTTCCTCCTTCACAGTCTG
	TTCAGACTGTGAAGGAGGAAAATGTT
Site-3	CGGTGTGAAAGCCAAAGTTC
	TGAACTTTGGCTTTCACAGACCG
Site-4	ACATAAGTGACGAGAAGGAAGGAC
	TGTCCTTCCTTCTCGTCACTTATGT
E-Box	TCAGACCACGTGGTCGGG
	CCCGACCACGTGGTCTG
	TTTCCAGGCACGTGATACATATACATACTC
	TGAGAGTATGTATATGTATCACGTGCCTGG

4.18 Oligonucleotide precipitation assay

Nuclear extracts containing Flag tagged cRel, flag tagged Blimp-1 and nuclear extracts containing GFP as a control, were made from 293T cells, transfected with pEYZ/FcRel, pEYZ/Fblimp-1 and pEYZ respectively, as described above. The nuclear extracts were then precleared for 2 hours at 4^0C, by incubating with streptavidin-agarose beads, in binding buffer, to remove proteins non-specifically binding to beads. Biotinylated olionucleotides representing the putative Blimp-1 binding site (Site-1) from A1 promoter were coupled to streptavidin-magnetic beads, by incubation at 4^0C, for 1 hour. Oligonucleotide coupled magnetic beads were then separated from freeoligonucleotides by magnetic separation. !0µl of the oligonucleotide coupled magnetic beads were used in a binding reaction with 5µl of the precleared nuclear extracts in binding buffer, in a total reaction volume of 100µl. The reaction mix was incubated for 2hours at 4^0C. Following incubation, magnetic beads isolated by magnetic separation. The beads were removed from the magnetic field and the bound proteins were eluted from the oligo coupled magnetic beads by using elution buffer. The eluates were separated by SDS-PAGE followed by western blotting and probed with anti-flag antibody.

Binding Buffer: 10mM Tris-HCL pH7.5 1mM DTT, 1mM EDTA, 5% Glycerol, 1mM $ZnCl_2$.
Elution Buffer: Tris-HCL pH 7.5, NaCl 1M
Biotinylated oligonucleotides: c
site-1 of A1 promoter: AAGTATTCATTTACTTTTTTCGTTGG
CCAACGAAAAAAGTAAATGAATACTT

4.19 PGL3-A1 promoter Luciferase constructs

A1 promoter regulated Luciferase reporters were made in pGL3 vector back ground. A putative Blimp-1 binding site present in the pGL3 vector sequence was removed by Ssp1 cleavage. The Blimp-1 site deleted pGL3 vector background was used to construct all the A1 promoter-luciferase reporters used in this study. The previously cloned pGL3-2010 A1 Luc was used for the subsequent construction pGL3-luc reporter plasmids regulated by truncated and site specific mutants of A1 promoter. The pGL3-500 A1-luc was constructed by amplifying 500bp A1 promoter region from 2010 A1-luc. The PCR product was digested with Bgl11 and Nco1 and inserted into pGL3 vector digested with the same enzymes. PGL3-81 A1-Luc was constructed by the PCR amplification of 81 bp region from 2010-A1 luc and subsequent digestion of PCR product and vector with Bgl11 and Nco1 and subsequent ligation. PGL3 81 A1 triple-mut luc, where all the three Blimp-1 binding sites (sites 2,3 &4) were mutated was generated by site specific mutagenesis, by using wild type 81 A1 luc as a template. Each of the Blimp-1 binding sites was replaced with a previously reported mutant blimp-1 site CTAACCAGCT (Calame), by site specific mutagenesis.

4.20 RT-PCR

Total RNA was isolated from WEHI 231 cells and primary B cells using Trizol reagent. Approximately 5x10 ^{cells} were suspended in Trizol reagent, 0.5ml Trizol reagent, incubated at RT for 5 min. 100µl chloroform was added and incubated for 5 min at RT with vigorous shaking. Following incubation, the samples were centrifuged at 14,000 rpm for 15 min at 4□C. The aqueous phase taken into a fresh eppendorf tube and 250 □l isopropanol

reagent was added, incubated at RT for 10 min. Samples were centrifuged at 14,000 rpm for 10 min at 4□C and the RNA pellets were washed once with 75% ethanol. Pellets were air dried and dissolved in DEPC water. RNA concentration was determined with a spectrophotometer.

cDNA was synthesized with SuperScript reverse transcriptase from 1 μg of RNA. The cDNAs were used for RT-PCR analysis, to detect Blimp-1expression, XBP-1, Bip, Ire1α, BIP, and β-actin, using the following primer pairs:

Blimp-1	GAAGAAACAGAATGGCAAGA
	CTGACTGGATCCCGCAAAGGCAGCCCAGCAGCTG
BIP	TCTACTCGAATTCCAAAGATTCAG
	GGTCGTTCACCTTCATAGAC
Ire1α	GATCCAGTCCTGCAGGTCCC
	GATGGGCAGAGACTATCAGCA
β-actin	CCAGGTCATCACTATTGGCAACGA
	GAGCAGTAATCTCCTTCTGCATC

PCRs with the above primers were performed in 2x mastermix (Fermentas), according to the manufacturer's instructions, with the following conditions: denaturation 1min, 94^0C,

Annealing 1min, 62^0C

Elongation 2min, 72^0C

For the specific amplification of processed XBP-1 (XBP-1p) and unprocessed XBP-1 (XBP-1u), the f following primer pairs were used.

XBP-1p AAGCGGCAGCGGCTCACGCAC
GGGCCTGCACCTGCTGCGGAC
XBP-1u AAGCGGCAGCGGCTCACGCAC
GGTGCACATAGTCTGAGTGTGCTGC

XBP-1 processing was analyzed using an alternate set of primers which amplify both processed and unprocessed forms of XBP-1. The primer pairs were: CAGGAGTTAAGAACACGCTTGG
TTGACACTAATCAGCTGGGGG
The PCR conditions were same as mentioned above.
These PCR primers yielded amplification products of 773 and 747 bp for the XBP-1 unprocessed (XBP-1u) and processed (XBP-1p) mRNA, respectively. Because the separation of these PCR products is difficult on a gel, PCR products were digested with Pst1 enzyme, which only cuts within the 26bp region that is removed by the Ire1α. After the digestion, the expected lengths in the agarose gel for XBP-1u and XBP-1p were 636 and 747bp respectively.

5. Results

Part I

5.1 Blimp-1 is sufficient to induce UPR and XBP-1 splicing in B cells

5.1.1 Correlation between Blimp-1 expression & XBP-1 processing during LPS induced differentiation of WEHI 231 cells

Lipopolysaccharide (LPS) stimulation of murine B cell lymphoma line WEHI 231 results in Ig secretion (157), suggesting that these cells have the intrinsic molecular machinery required for terminal differentiaion. Therefore, WEHI 231 cells were used to study the molecular events involved in terminal differentiation of B cells. Because one of the main objectives of this study is to understand the role of Blimp-1 in XBP-1 processing, in the first step, expression of endogenous Blimp-1 and XBP-1 processing was analyzed in LPS stimulated WEHI 231 cells. Expression of Blimp-1 was analyzed by RT PCR analysis. As shown in Fig. 3A, Blimp-1 mRNA was detected only in LPS stimulated cells but not in unstimulated control cells. Because the induction of an unfolded protein response (UPR) resulting in XBP-1 splicing had been shown during terminal differentiation of primary splenic B cells (92), the occurrence of these events in LPS stimulated WEHI 231 cells was analyzed. To investigate UPR, XBP-1 splicing was analysed by RT PCR, using primers which specifically amplify either the spliced or unspliced XBP-1. In LPS stimulated WEHI 231 cells, an increase in spliced form of XBP-1 was observed compared to unstimulated cells (Fig. 3A, middle row). Specificity of the primers used to detect spliced and unspliced forms of XBP-

1 was verified in a PCR reaction, using control vectors encoding spliced, unspliced or empty vectors (Fig. 3C). Furthermore, Western blot analysis of the whole cell extracts showed an overall increase in IgM levels, with a specific increase in secretory form of μ chain, in LPS stimulated cells, compared to unstimulated cells (Fig. 3B upper panel). As expected, upregulation of Blimp-1 and processing of XBP-1 correlated with Ig secretion, the hall mark feature of the terminally differentiated B cells (Fig. 3B bottom row). These results indicate that a correlation exists between Blimp-1 expression & XBP-1 processing in immunoglobulin secreting WEHI 231 cells.

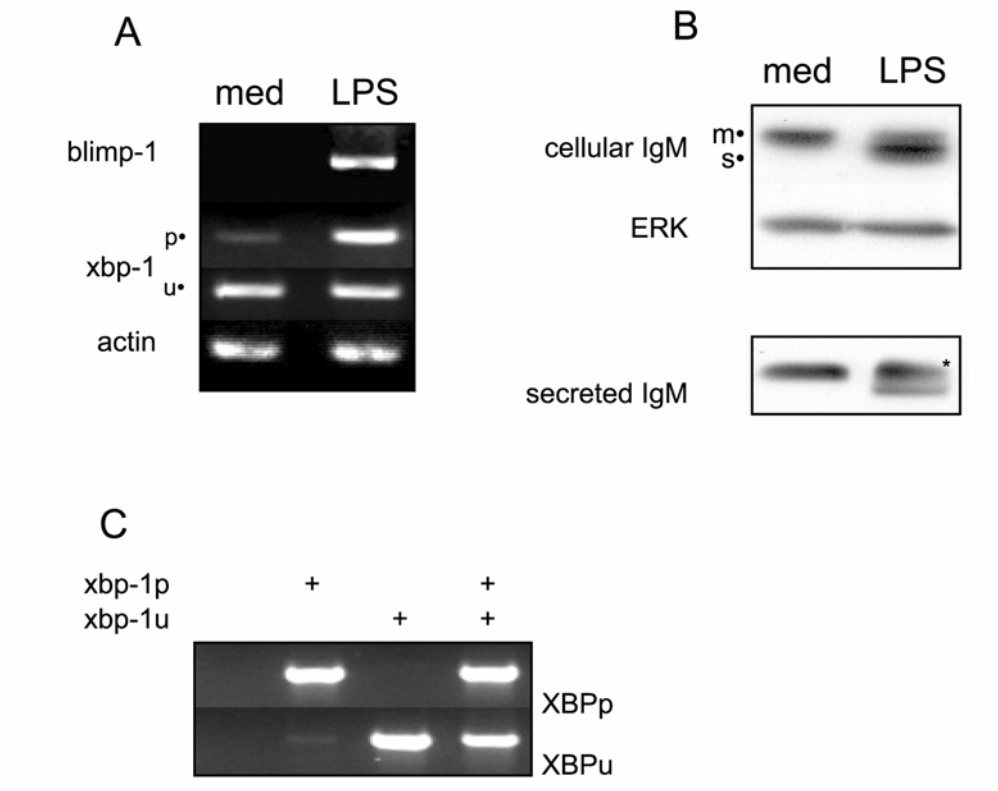


Fig. 3. Endogenous Blimp-1 expression correlates with XBP-1 processing in WEHI 231 cells. A. RT-PCR analysis of total RNA from unstimulated or LPS stimulated (3 days) WEHI 231 cells. Upper panel: Blimp-1.Midddle panel: P: processed XBP-1 and u: unprocessed XBP-1. Lower panel: Actin detection was used as a normalization control.

B. Upper panel: Whole cell lysates of unstimulated or LPS stimulated (3 days) WEHI 231 cells were analyzed by Western blot. Detection with a μ chain specific antibody recognized two bands, with the upper band representing membrane bound μ chain and the lower band corresponding to the secretory form of μ chain. ERK detection was used as a loading control. Lower panel: Culture supernatants from unstimulated or LPS stimulated (3 days) WEHI 231 cells were used to detect secreted IgM by western blotting. The star mark represents a non-specific band. C. To check the specificity of the XBP-1 primers, PCR reactions containing the following plasmids was carried out.: Lane 1: empty vector, lane 2: vector encoding processed form of XBP-1, lane 3: vector encoding unprocessed form of XBP-1, and lane 4: both vectors from lanes 2 and 3. The samples of the upper panel were amplified with primer pair specific for processed form of XBP-1 and the sample of the lower panel with the primer pair specific for the unprocessed form of XBP-1

5.1.2 Ectopic expression of Blimp-1 is sufficient to induce UPR in WEHI 231 cells.

To investigate whether Blimp-1 is the causal factor for the induction of XBP-1 processing, WEHI 231 cells were infected with bicistronic recombinant retroviruses (rRV) encoding Blimp-1 plus Enhanced Yellow Fluorescent Protein(EYFP) or with control rRV encoding only EYFP. As shown in the RT-PCR analyses, introduction of Blimp-1 led to enhanced splicing of XBP-1 mRNA (Fig. 4A), in comparison to control infected cells. Western blot analysis of whole cell extracts showed that Blimp-1 expression led to the generation of a 54 KD processed version of XBP-1 protein(Fig. 4B). In addition to the 54KD band, a 37KD band was observed which represents the exact molecular weight of the protein corresponding to the 371 amino acids, encoded by the spliced version of XBP-1. The 54KD processed form of XBP-1 might be the result of post-translational modification(s). The molecular nature of modification(s) resulting in the generation of 54KD band is currently unknown. Even the 54 KD band seems to be subject to further modifications because in pulse chase experiments it was observed that, with time, an upper band slightly above 54 KD accumulates (90).

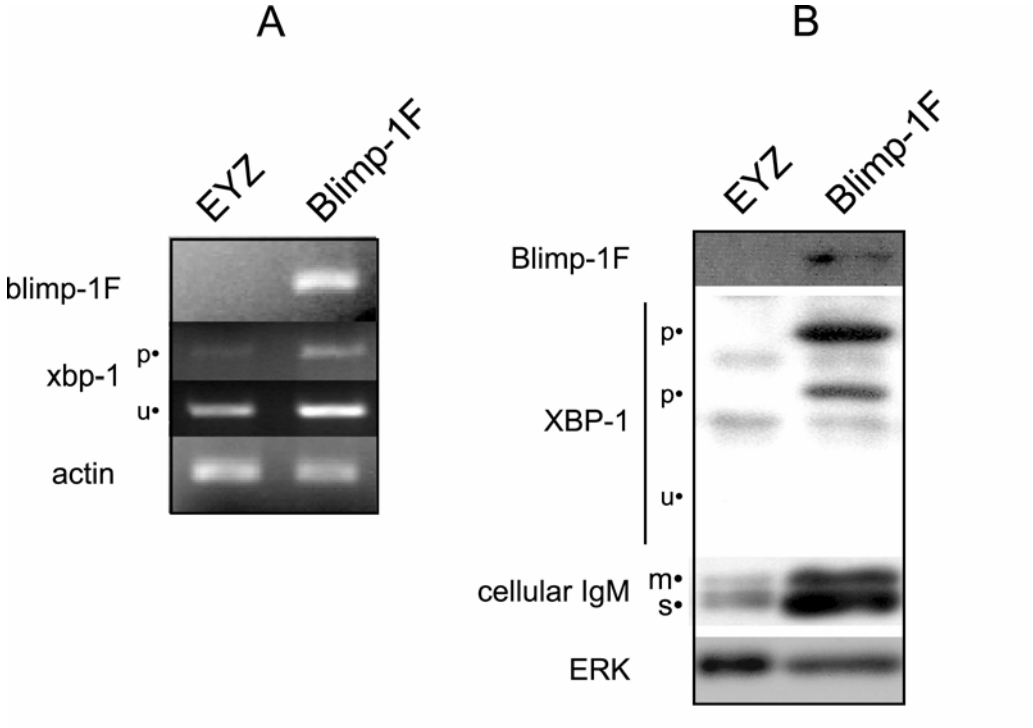


Fig 4. Influence of ectopic expression of Blimp-1 in WEHI 231 cells. A. RT-PCR analysis of total RNA from WEHI 231 cells infected with either control virus encoding EYFP (EYZ) or EYFP plus flag-tagged Blimp-1 (Blimp-1F). Upper panel: Blimp-1.Midddle panel: P: processed XBP-1 and u: unprocessed XBP-1. Lower panel: Actin detection was used as a normalization control. B. Western blot analysis of whole cell lysates from EYFP or Blimp-1F infected WEHI 231 cells. Upper panel: Blimp-1.Midddle panel: P: processed XBP-1 and u: unprocessed XBP-1. Unprocessed XBP-1 was below the level of detection, most likely due to its known instability. Lower panel: Cellular IgM detected by μ chain specific antibody. m: membrane bound μ chain and s: secretory form of μ chain. ERK detection was used as a loading control.

In addition to these events, Blimp-1 introduction led to an overall increase in the immunoglobulin μ heavy chain proteins, in accordance with the previously published results, which showed upregulation of IgM mRNA representing both secretory and membrane bound forms (Fig. 4B) (69). Therefore, from these results it is evident that introduction of Blimp-1 by itself is sufficient to induce intracellular immunoglobulins, UPR and XBP-1 processing in WEHI 231 cells.

5.1.3 The N-terminal part of Blimp-1 is sufficient to induce UPR & XBP-1 processing in WEHI 231 cells

Specific domains of Blimp-1 have been shown to be required for transcriptional repression of target genes and the transcriptional repressive function of Blimp-1 has been shown in the context of several genes (73, 75, 77, 82). Broadly, the N-terminal part of the Blimp-1 is sufficient to mediate transcriptional repression while the C-terminal part has been shown to exert moderate activation of transcription. As detailed in the introduction, different domains of Blimp-1 have different biological functions.

To determine which domain(s) of Blimp-1 is necessary and sufficient to mediate the essential functions like induction of UPR, XBP-1 splicing and Ig secretion, rRV were generated encoding the aminotermial part of the Blimp-1 comprising amino acids 1-751 (N-Blimp-1F) and carboxy terminal domain comprising amino acids 465-851 (C-Blimp-1F)(Fig. 5A). Both the N and C terminal Blimp-1 constructs contain the zinc finger domain, which facilitates DNA binding. The expression levels of the Blimp-1 proteins corresponding to different constructs was analyzed by western blotting (Fig. 5B).

The DNA binding ability of the truncated mutants of Blimp-1 was verified by EMSA, using an oligonucleotide representing the previously described Blimp-1 binding site from the c-myc promoter (79). As shown in Fig. 5C, the full length, N-terminal & C-terminal Blimp-1 proteins were capable of binding to the Blimp-1 binding site corresponding to the c-Myc promoter. The potential of Blimp-1 truncated mutants in the context of B cell terminal

differentiation was analyzed in WEHI 231 cells. Retroviral transduction of WEHI 231 cells with the N-terminal domain led to the induction of the same effects as did the full length Blimp-1, in terms of induction of XBP-1 processing, as shown by the generation of the spliced version of XBP-1 in RT PCR analysis (Fig. 6).

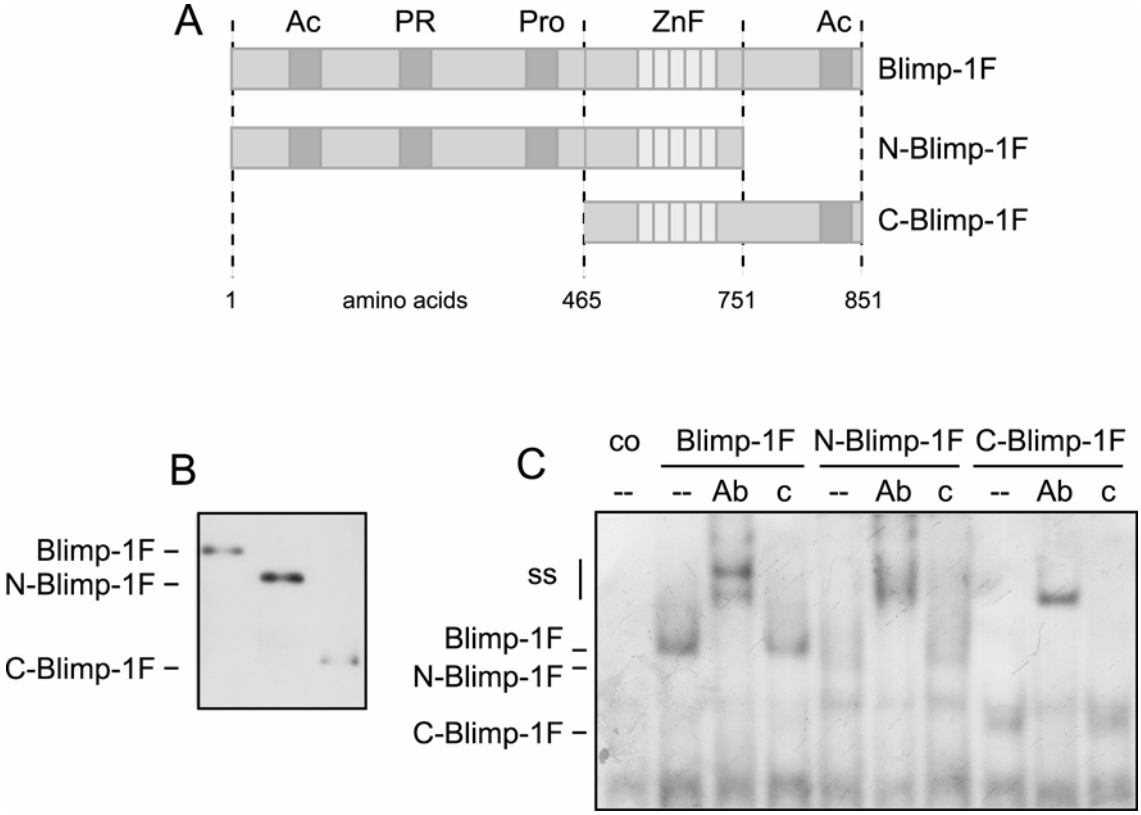


Fig. 5. Analysis of expression and DNA binding ability of full length Blimp-1 (Blimp-1F), N (N-Blimp-1F) and C-terminal C-Blimp-1F). A. Full length Blimp-1 and the N-terminal and C-truncated versions of Blimp-1. PR: PRD1-BF1-RIZ homology domain, Pro: proline rich domain, ZnF: zinc finger domain, Ac: acidic rich region. B. Nuclear extracts from 293 T cells transfected with either full length Blimp-1 or N or C-terminal Blimp-1 were analyzed by western blotting to detect expression of the relevant proteins. C. The same nuclear extracts were used in electrophoretic mobility shift assay, to confirm the DNA binding ability , using an oligonucleotide representing the Blimp-1 binding site from c-MYC promoter.

The C terminal domain, had no effect on induction of XBP-1 processing and was comparable to the control virus infected population (Fig 6). RT-PCR analysis of a serial dilution of cDNA sample indicated an approximately 5-fold increase in the amount of the processed form of XBP-1, in response to Blimp-1 (Fig. 6). These observations suggest that the N-terminal part of Blimp-1, is sufficient to mediate the major functions of Blimp-1, which are required for plasma cell generation namely : induction of UPR and XBP-1 processing.

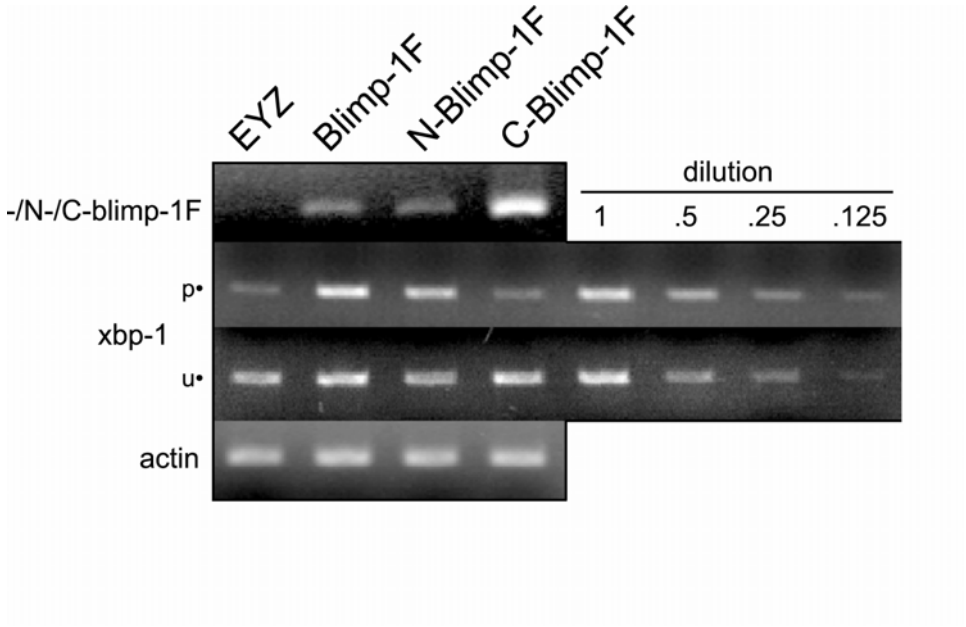


Fig. 6. Full length Blimp-1 or its N-terminus are sufficient to induce XBP-1 processing in WEHI 231 cells. Total RNA from WEHI 231 cells infected with control (EYZ) recombinant retroviruses (rRV) or with rRV encoding Blimp-1-F, N-Blimp-1F or C-Blimp-1F, was analyzed by RT-PCR to detect Blimp-1 expression and XBP-1 processing. Upper panel: Blimp-1 expression .Middle panel: P: processed XBP-1 and u: unprocessed XBP-1. In order to assess the Blimp-1 dependant change in the processed and unprocessed XBP-1 mRNA, two fold serial dilution of Blimp-1F samples were run in parallel in the same gel. Lower panel: Actin detection was used as a normalization control.

5.1.4 Correlation between Blimp-1 expression and UPR induction in mouse primary splenic B cells

Because primary B cells are physiologically more relevant, the role of Blimp-1 in the induction of UPR and XBP-1 splicing was analyzed in primary murine splenic B cells. Primary B cells were analyzed under endogenous Blimp-1 expressing and non expressing conditions, to observe if a correlation exists between Blimp-1 expression & XBP-1 processing. LPS stimulation of primary mouse B cells leads to induction of Blimp-1 & Ig secretion while stimulation with LPS plus anti-μ fails to induce endogenous Blimp-1 expression & Ig secretion (68).

Accordingly, stimulation of primary splenic B cells for 3 days with LPS led to induction Blimp-1 compared to LPS & anti-μ stimulated cells, as shown by RT PCR analysis (Fig. 7B). Furthermore compared to LPS plus anti-μ stimulation, LPS stimulation led to the generation of spliced form of XBP-1 and upregulation of UPR target gene BIP (Fig. 7B). Interestingly, LPS treatment led to massive increase in cellular imunoglobulins of both IgM heavy & Kappa light chains, compared to LPS plus anti μ stimulation, as shown by FACS analysis (Fig 7A). In accordance with previous studies, LPS stimulation resulted in Ig secretion (Fig. 7C). From these observations it is clearly evident that a strong correlation exists between endogenous Blimp-1 expression, induction of intracellular immunoglobulins, UPR and XBP-1 processing in primary splenic B cells.

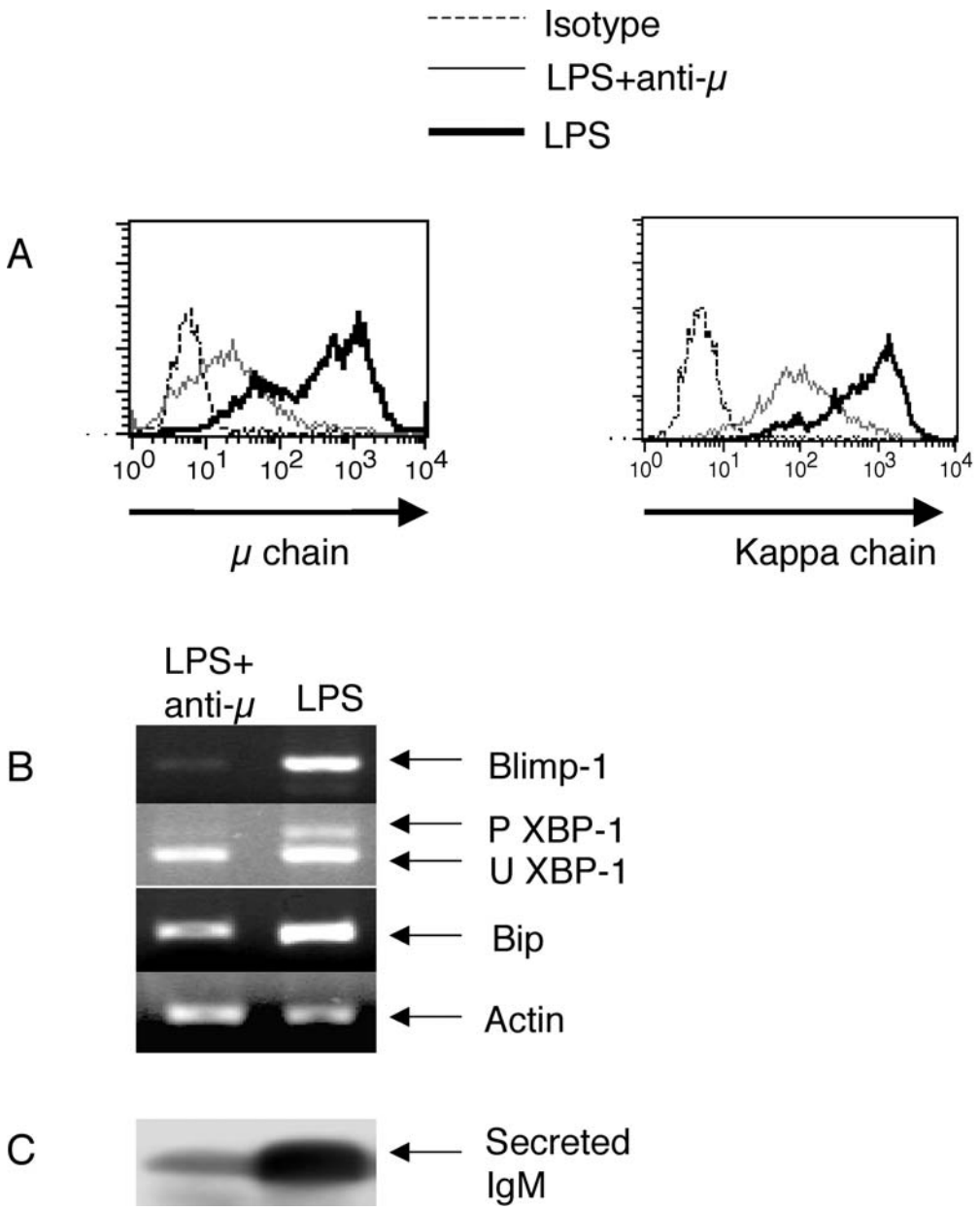


Fig. 7. Endogenous Blimp-1 expression in primary mouse B cells correlates with cellular immunoglobulin (Ig) induction and XBP-1 processing. A. Primary splenic B cells were stimulated with either LPS+anti-μ or LPS for 3 days. Cells were stained for μ chains (Left panel and kappa chains (right panel) and analyzed by FACS. Shown was the histogram overlay, representing control isotype stained cells (grey dotted line), LPS+anti-μ stimulated cells (grey line) and LPS stimulated cells (thick black line). B. RT-PCR analysis of the total RNA isolated from LPS or LPS+antiμ stimulated primary B cells. Upper row: Blimp-1 expression .Middle row: P: processed XBP-1 and u: unprocessed XBP-1 and BIP expression. Lower row: Actin detection used as a normalization control. C. Western blot analysis of the 3 day culture supernatants from LPS+anti-μ or LPS stimulated primary B cells. Secreted IgM was detected using μ chain specific antibody.

5.1.5 Introduction of Blimp-1 or its N-terminal domain is sufficient to induce intracellular Ig levels & UPR in mouse splenic B cells

Ectopic expression of Blimp-1 or N-terminal Blimp-1 was found to be sufficient for induction UPR and XBP-1 splicing in WEHI 231 cells. To confirm whether this holds true in primary B cells, the influence of full length Blimp-1, N and C terminal Blimp-1 was analyzed in these cells. Mouse splenic B cells were stimulated with LPS plus anti-μ, and infected with rRV encoding either full length Blimp-1F, the N-Blimp-1F comprising or C-Blimp-1F plus EYFP or with control vectors encoding only EYFP. Whole cell extracts of infected populations were first analyzed by western blotting, to verify the expression of proteins corresponding to different constructs (Fig. 8A). Introduction of full length Blimp-1F or N-Blimp-1F led to a massive increase in cellular levels of both IgM heavy chains & Kappa light chains, revealed by FACS staining (Fig. 8C, D). On the other hand, C-Blimp-1F had no effect, as shown by the fact that the levels of IgM heavy & Kappa light chains remained the same as that of vector control infections (Fig. 8C, D). Furthermore, full length Blimp-1F and N- Blimp-1F induced XBP-1 splicing, as shown by the generation of the processed form of XBP-1 (Fig. 9). XBP-1 processing was further analyzed by an alternate method using primers which amplify both processed and unprocessed forms of XBP-11, in an RT-PCR reaction. After the PCR, Pst1 resistance of the processed form of XBP-1 was used to distinguish processed from unprocessed XBP-1 (Fig. 9 bottom panel).

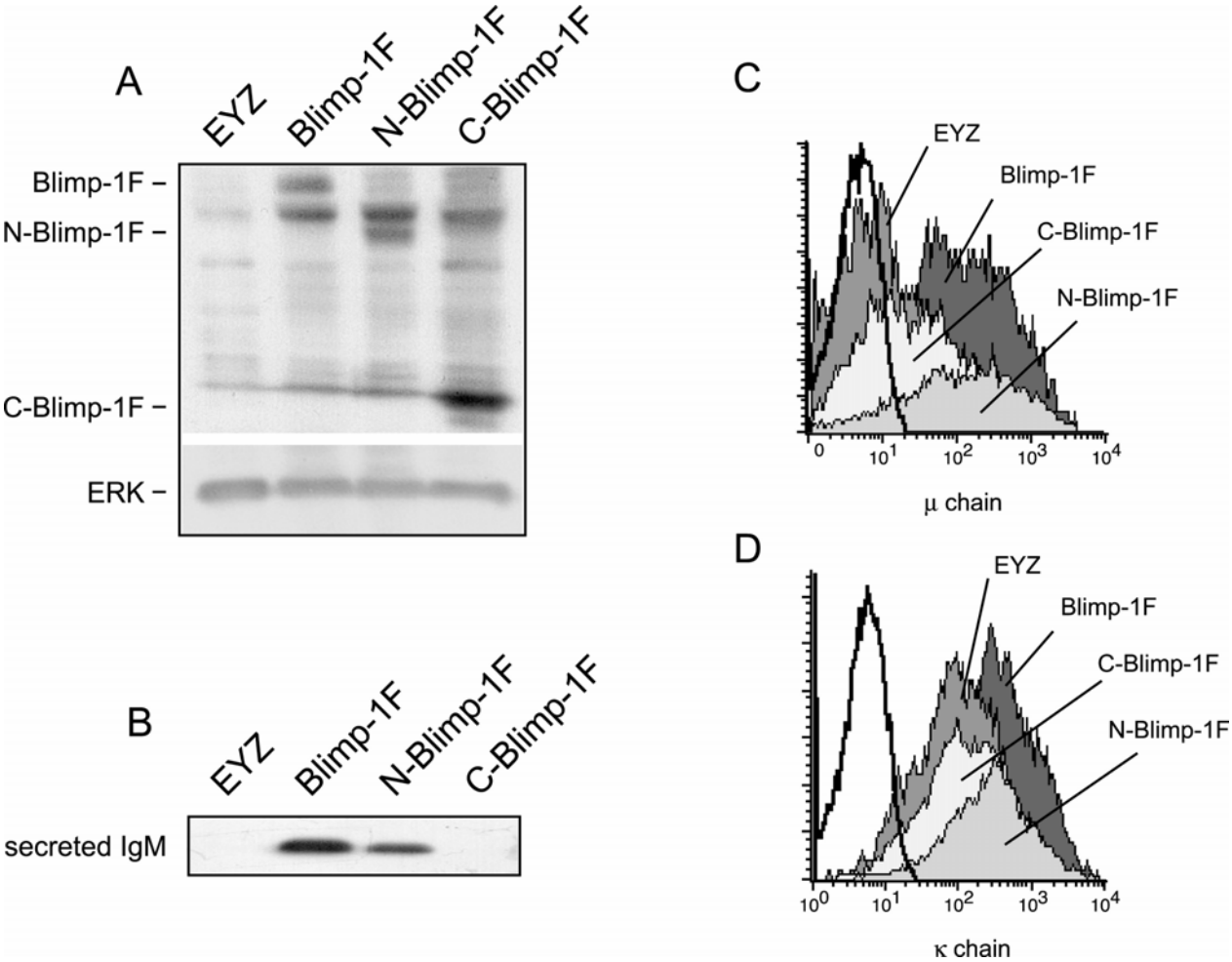


Fig. 8. Ectopic expression of Blimp-1 or its N-terminus in primary B cells leads to upregulation of cellular Ig levels and Ig secretion. A. Western blot anaysis of whole cell lysates from primary B cells, infected with rRV coding for various flag-tagged Blimp-1 proteins. C & D. FACS analysis: shown were the histogram overlays representing the cellular Ig levels corresponding to μ chains (C), and kappa chains (D). B. western blotting to detect secreted IgM in the culture supernatants of primary B cells transduced with the indicated Blimp-1 proteins.

From both the methods, it is clearly evident that Blimp-1 or its N-terminus were sufficient to induce XBP-1 processing (Fig. 9). In addition, the levels BIP, a classical UPR target gene were upregulated in response to either full length or the N- Blimp-1F (Fig. 9), suggesting the induction of UPR. The levels of IRE1α, an enzyme involved in XBP-1 processing remained un altered in response to Blimp-1F or N-Blimp-1F (Fig. 9).

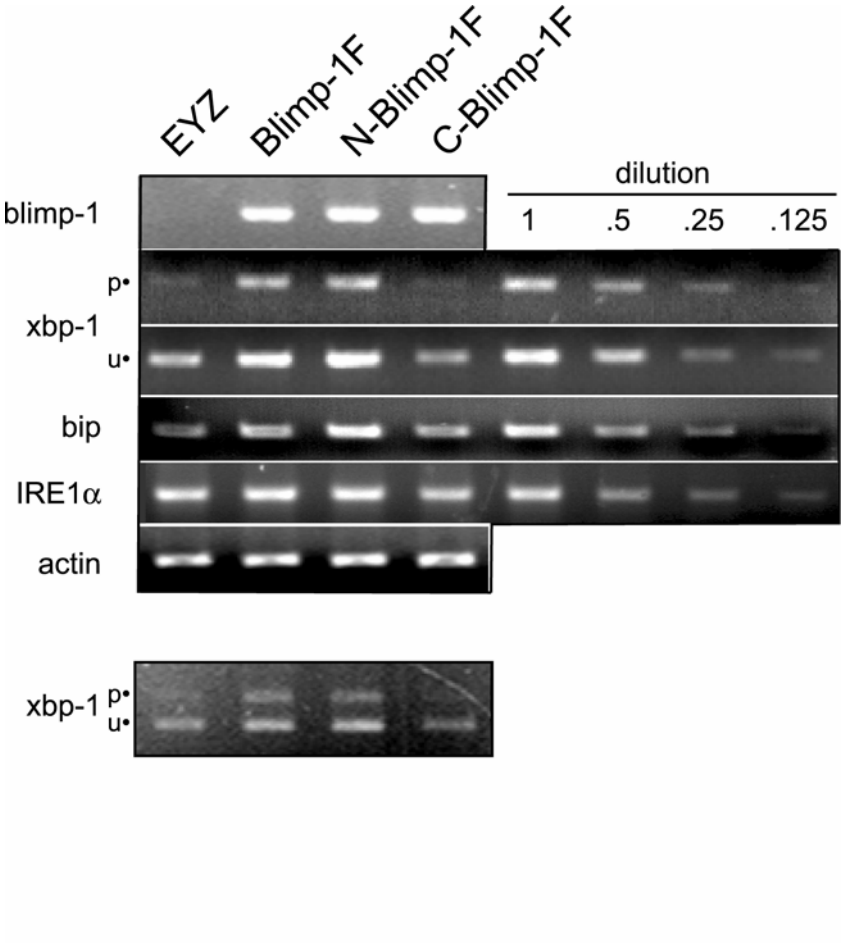


Fig. 9. Induction of XBP-1 processing and UPR target gene BIP, in response to Blimp-1F or N-Blimp1F. RT-PCR analysis of total RNA obtained from primary B cells infected with control rRV encoding EYFP or various versions of Blimp-1, to detect expression of Blimp-1, processed (P) XBP-1, unprocessed (u) XBP-1, BIP, and IRE1α. The change in expression levels of XBP-1, BIP and IRE1α, was assessed by two fold serial dilutions of the Blimp-1F sample. Lower panel: XBP-1 processing was analyzed by RT-PCR with primers which amplify both processed and unprocessed forms. After the PCR, the cDNA from processed form was distinguished from the cDNA of the unprocessed form by its resistance to the enzyme Pst1.

Most importantly, compared to C-Blimp-1F transduced cells or control populations expressing only EYFP, introduction of either full length Blimp-1 or its N terminal part led to secretion of immunoglobulins, as it is the case with LPS stimulated B cells (Fig. 8B). These results clearly indicate that in primary B cells ectopically expressed Blimp-1 is sufficient, on its own, to

induce intracellular Ig levels, UPR, XBP-1 splicing and Ig secretion. Importantly, the N-Blimp-1F with previously attributed transcriptional repressive function was found to be sufficient to induce the critical events UPR and XBP-1 splicing.

5.2 Part II

5.2.1 The mechanism of Blimp-1 dependant repression of the anti-apoptotic gene A1

5.2.1.1 Plasmacytic differentiation conditions or ectopic expression of Blimp-1 results in down regulation of A1 messenger RNAs

The anti-apoptotic Bcl-2 family member A1 has been shown to be down regulated in response to Blimp-1,at the RNA level, in WEHI 231 cells (70). To study the influence of Blimp-1 on the A1 gene, in primary B cells, A1 expression was analyzed during LPS induced differentiation of primary murine splenic B cells. Compared to LPS plus anti-μ stimulation

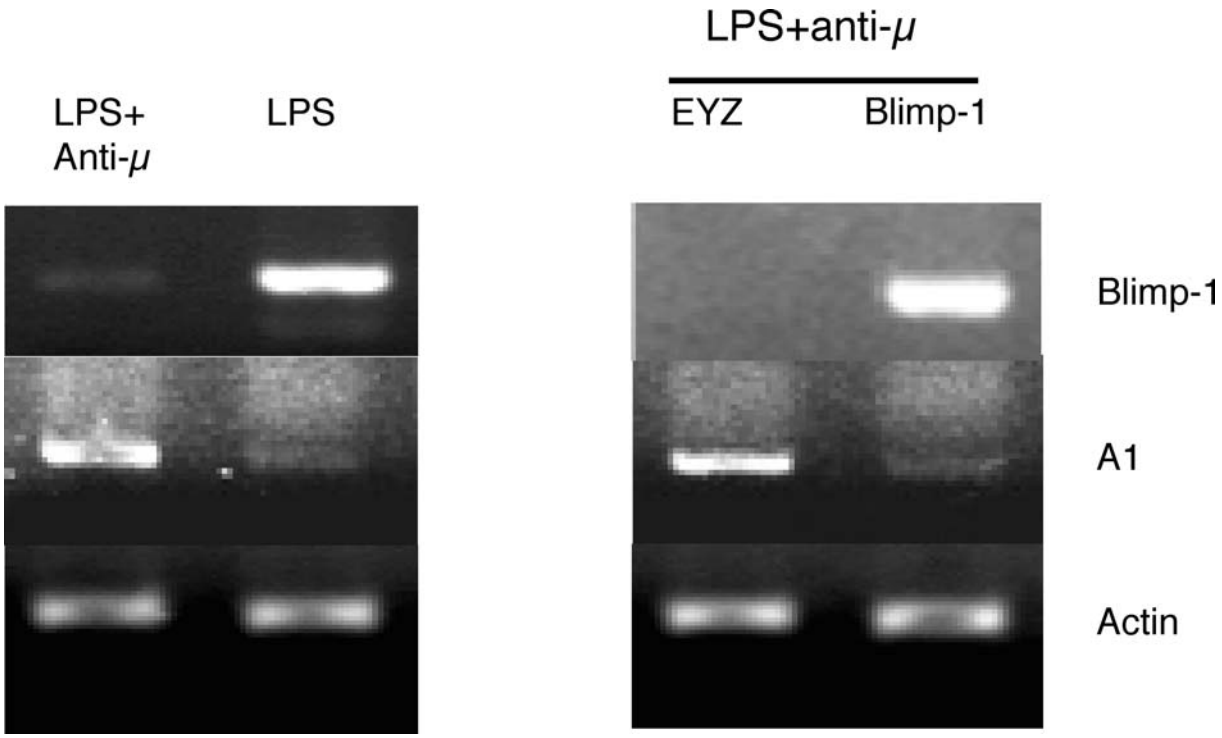


Fig. 10. Blimp-1 dependant down regulation of A1 in primary mouse B cells.. Left panel: Total RNA isolated from LPS or LPS+anti-μ stimulated primary B cells was

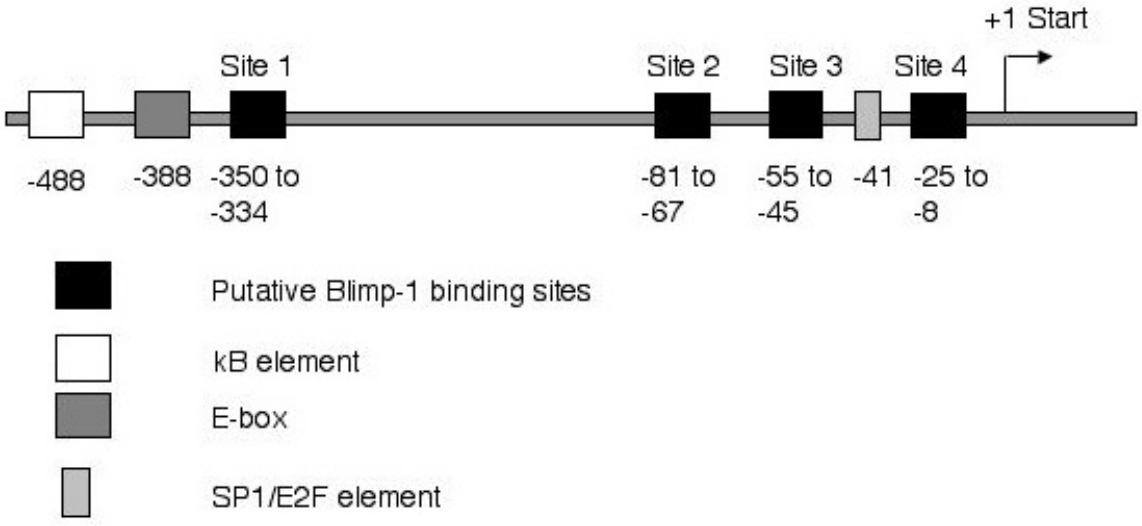
analyzed by RT-PCR analysis. Right panel: RT-PCR analysis of primary B cells infected with rRV encoding EYFP or Blimp-1 under LPS+anti-μ condition. Upper row: Blimp-1 expression, middle row: A1, bottom row: Actin expression as a normalization control.

of B cells, which blocks endogenous Blimp-1 expression, LPS stimulation alone which promotes endogenous Blimp-1 expression, led to down regulation of A1 (Fig. 10 left panel). This result indicates a correlation between endogenous Blimp-1 expression and A1 repression in primary mouse B cells. To confirm that, in primary B cells, A1 repression is a Blimp-1 dependant event, LPS and anti-μ stimulated murine splenic B cells were retrovirally transduced with rRV encoding Blimp-1 or with rRV expressing only EYFP. Three days after infection, Blimp-1 infected cells and control vector infected cells were analyzed by RT-PCR analysis for the expression of A1. As shown in Fig. 10 (right panel), ectopic expression of Blimp-1 led to a reduction in the A1 messages, suggesting that in primary B cells A1 expression is down regulated by Blimp-1.

5.2.1.2 The A1 promoter contains multiple elements with sequence homology to the Blimp-1 binding elements

Despite the establishment of A1 as a repressive target of Blimp-1, the mechanism of A1 repression is not known. The direct transcriptional repressive activity of Blimp-1 has been shown in the context of genes like c-Myc, Pax-5, IFN-ß etc (75, 79, 81). Because one possibility of repression is by direct binding of Blimp-1 to specific Blimp-1 binding elements in the promoter regions of target genes (e.g., c-Myc, Pax-5 etc.,), the A1 promoter region upstream to the transcription start site was analyzed for putative Blimp-1 binding sites.

Sequence alignment studies of Blimp-1 binding sites from known Blimp-1 target genes and a recent study (CalA) directed towards identifying a probable Blimp-1 binding consensus sequence enabled the construction of ($^{A}/_{C}$) AG ($^{T}/_{C}$) GAAAG ($^{T}/_{C}$) ($^{G}/_{T}$) as the Blimp-1 binding consensus sequence (CalA). Comparison of the A1 promoter with the known Blimp-1 binding sites from various target genes revealed the presence of putative



Known Blimp-1 binding sites

Myc PRF site	AAAGGGAAA
Pax5	AAAGTGAACT

Putative Blimp-1 binding sites from the A1 promoter

A1 Site 1	AAAAAGTAAA
A1 Site 2	AAGGAGGAAAA
A1 Site 3	AAAGCCAAA
A1 Site 4	AAGTGACGAGAAGGAA

Fig. 11. Putative Blimp-1 binding sites in the A1 promoter, within the 500 base region upstream to the transcription start site. Black boxes: putative Blimp-1 binding sites. White box: previously described kB element. Dark Grey box: E-box like element .

Light grey box: SP1/E2F like element. Lower panel Comparison of the sequences of known Blimp-1 binding elemetns and putative Blimp-1 binding elements from the A1 promoter.

Blimp-1 binding sites with varying homology to the known Blimp-1 binding elements (Fig. 11).

An upstream site with weak homology to the known Blimp-1 binding sites or consensus sequence was found to be located between -350 and -334 (here after called as site-1). Quite surprisingly three more Blimp-1 binding sites were observed quite close to the transcriptional start site. Of these three, two sites, the first one located between -81 and -67 (site-2) and the second located between -55 and -45 (site-3) show a reverse orientation relative to the transcriptional start site. The third sequence was located between -25 and -8 (site-4) (Fig. 11). So the presence of putative Blimp-1 binding elements suggests the possibility of a direct repression of the A1 promoter by Blimp-1.

5.2.1.3 The putative Blimp-1 binding sites from the A1 promoter bind Blimp-1 in vitro

Before studying the functional relevance of putative Blimp-1 binding sites of the A1 promoter in mediating the repressive effect of Blimp-1, it is important to establish their ability to bind Blimp-1. This was achieved by oligonucleotide pull-down assay and electrophoretic mobility shift assays (EMSAs). Essentially, oligonucleotides representing the putative Blimp-1 binding sites of the A1 promoter were tested for their Blimp-1 binding ability, using Flag tagged Blimp-1 containing nuclear extracts, with Flag tagged c-Rel used as a negative control.

First the expression & nuclear localization of Blimp-1 (Blimp-1F) and c-Rel (c-RelF) was analyzed by western blot analysis, using an ant-Flag antibody..

Nuclear & cytosolic extracts were made from 293Tcells transfected with either Blimp-1F or c-RelF. As shown in Fig. 12 (A), both Blimp-1F & Fc-Rel were predominantly localized in the nucleus.

To study the Blimp-1 binding ability of the upstream Blimp-1 binding site (Site-1) of the A1 promoter, oligonucleotide pull-down assay was performed. A biotinylated double stranded oligonucleotide probe spanning site-1 was used to precipitate Blimp-1F from nuclear extract. Nuclear

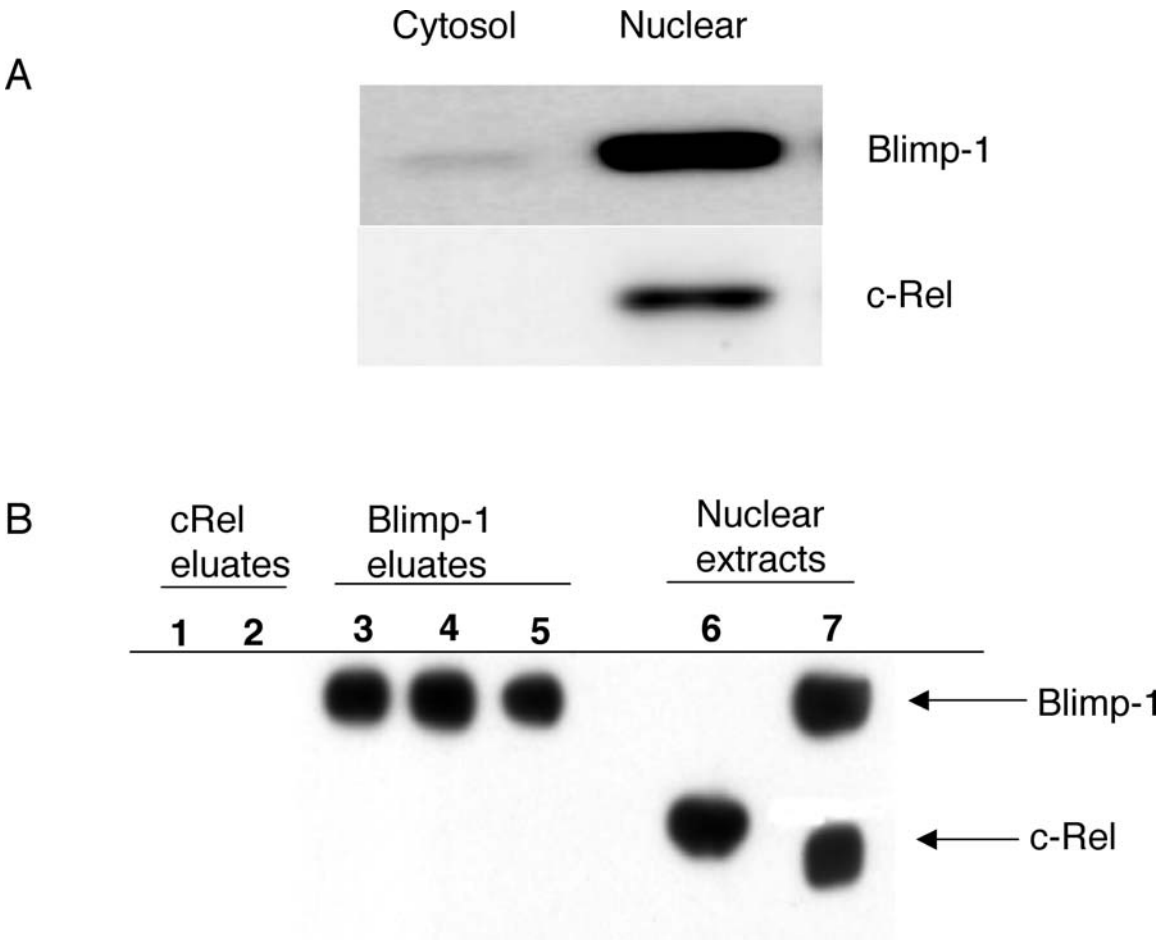


Fig. 12. Putative Blimp-1 binding element (site-1) from A1 promoter binds Blimp-1 in vitro. A. Western blot analysis of nuclear and cytosolic extracts from Blimp-1F and Fc-Rel transfected 293 T cells confirms nuclear localization of Blimp-1F and Fc-Rel in 293 T cells. B. Oligonucleotide precipitation assay showing the binding of Blimp-1 to an oligo corresponding to site-1 of A1 poromoter. Biotinylated site-1 oligo was incubated with either flag-tagged c-Rel or Blimp-1 containg nuclear extracts,DNA-protein coplexes were
precipitated by incubation with streptavidin coupled magnetic beads. Bound proteins were eluted by high salt and analyzed by western blot analysis, using anti-flag antibodies. As shown in lanes 1 & 2, c-Rel was not detected in the eluates whereas

binding of Blimp-1 was clearly evident (lanes 3-5). Lanes 6 & 7 represent nuclear extracts containing flag tagged cRel & Blimp-1,respectively.

extract containing c-RelF was used as a control. Eluates from site-1 oligo incubated with Blimp-1F nuclear extracts contained Blimp-1, as detected by anti-Flag antibody, indicating the binding of Blimp-1 (Fig. 12B). In contrast, c-RelF was not detected in the eluates from the site-1 oligo incubated with c-RelF nuclear extracts (Fig. 12B). This experiment indicates that Blimp-1 binds specifically to site-1 and further excludes the possibility that the observed binding was due to the Flag tag because c-RelF, which also contains a Flag tag failed to bind site-1.

The Blimp-1 binding ability of sites 1-4 from the A1 promoter was further analyzed by EMSAs and competition assays, by using oligonucleotide probes representing the corresponding sites from the A1 promoter. Oligonucleotides representing each site were radiolabelled and incubated with nuclear extracts made from 293T cells transfected with either Blimp-1F or empty vectors. As shown in Fig. 13, specific DNA-protein complexes were observed in case of sites 1 and 3 incubated with Blimp-1F containing nuclear extracts, compared to vector control nuclear extracts. The presence of Blimp-1 in these specific DNA-protein complexes was confirmed by super shift assays by using anti-Flag antibody which resulted in the formation of retarded slowly migrating complexes. Interestingly, with both site-1 and site-3 a specific band was observed but much stronger band was observed in case of site-3 compared to site-1 (Fig.13). This finding indicates that both sites 1 and 3 corresponding to

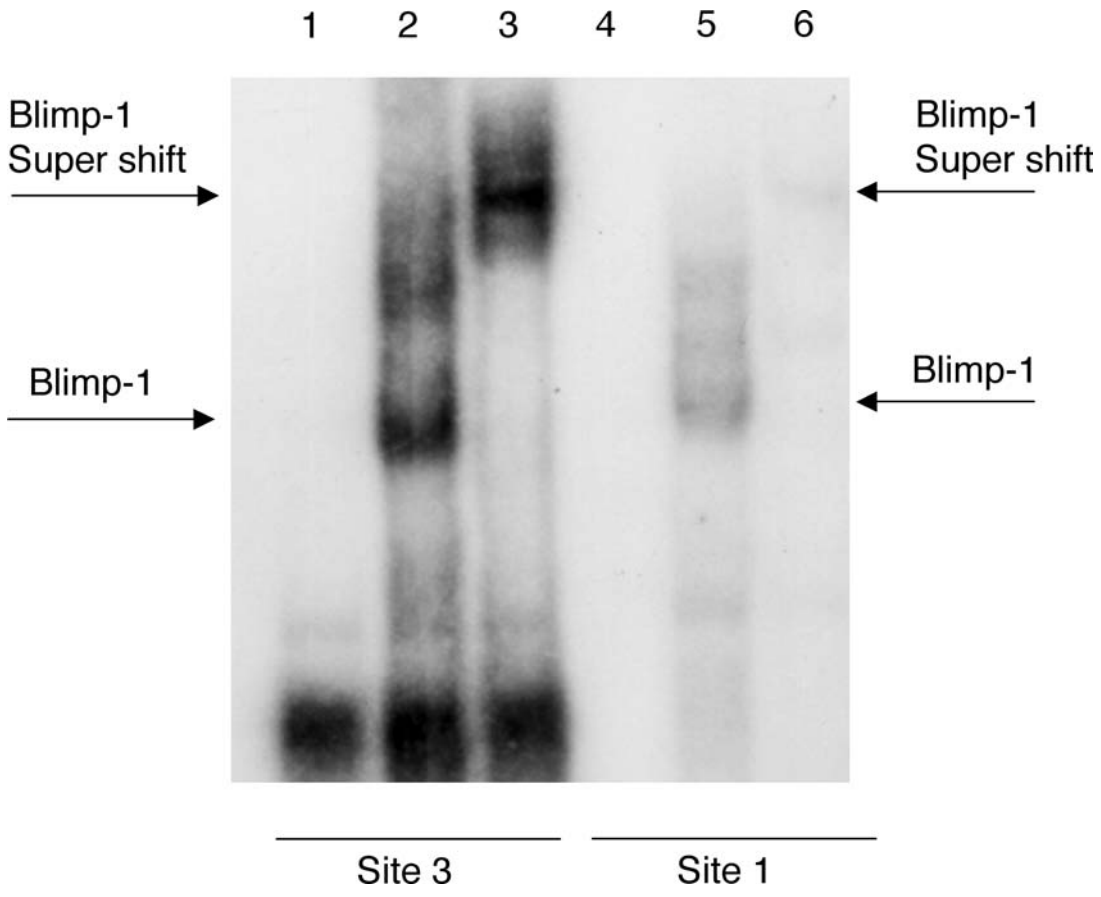


Fig. 13. Electrophoretic Mobility Shift Assay (EMSA) reveals binding of Blimp-1 to putative Blimp-1binding sites 1 & 3 corresponding to A1 promoter. Lanes 1-3: site-3 EYZ, site-3 Blimp-1, site-3 Blimp-1+anti-flag antibody, respectively. Lanes 4-6: site-1 EYZ, site-1 Blimp-1, site-31Blimp-1+anti-flag antibody, respectively

the A1 promoter can bind to Blimp-1, with site 3 having higher affinity than site-1.In contrast to sites 1 and 3, sites 2 and 4 failed to form specific DNA-protein complexes upon incubation with Blimp-1F nuclear extracts. This can be due to the fact that they lack the minimal sequence requirements for Blimp-1 binding and hence do not bind Blimp-1 at all, or, alternatively, they form weaker complexes with Blimp-1 due to lesser affinity and hence can not be resolved on a gel in an EMSA. To test the latter possibility, a competition

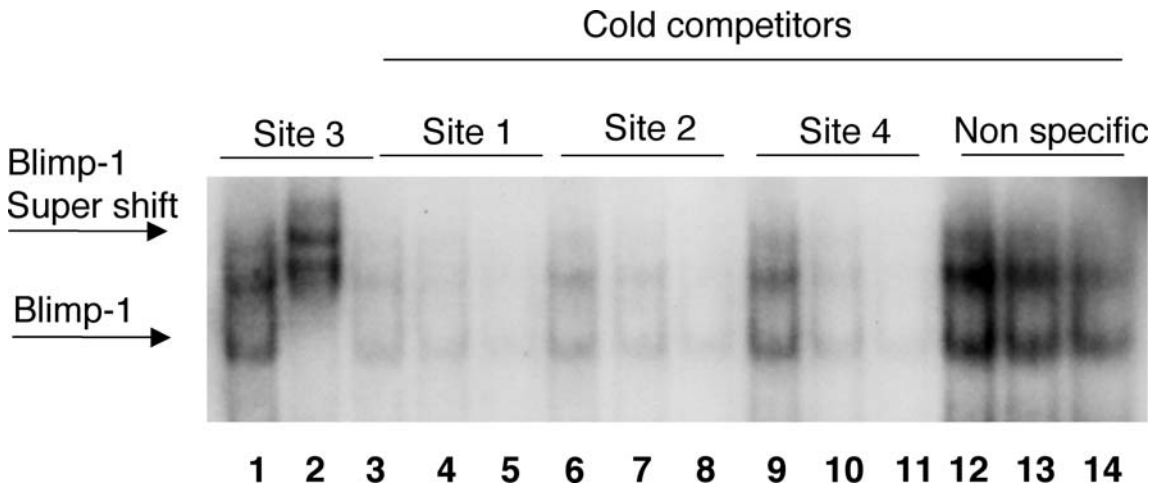


Fig. 14. Competition analysis to show the blimp-1 binding ability of sites 2 & 4 of the A1 promoter. Lane 1: Radiolabelled Blimp-1 binding oligo corresponding to site-3 of A1 promoter shows a specific band upon incubation with flag-tagged Blimp-1 containing nuclear extract. Lane 2: The identity of Blimp-1 was confirmed by using an antibody against flag- tag, as revealed by the super shift.Lanes 3-14: Radiolabelled Site-3 oligo of A1 promoter was incubated with increasing amounts of cold competitor oligos corresponding to either site-1 (lanes 3-5) , site-2 (lanes 6-8), site-4 (lanes 9-11) or with a non specific oligo representing the E-box (lanes 12-14).

assay was performed in which site-3 oligo was incubated with Blimp-1F nuclear extracts, with increasing excess of oligos representing sites 2 and 4 respectively and with an E-Box oligo, which binds to Myc and Mad family proteins, as a control non specific oligo.

As shown in fig. 14, the use of increasing amounts of sites 2 and 4 abolished the binding of Blimp-1 to site 3, where as the control non specific oligo, the E-box, did not. From this result it is evident that sites 2 and 4 of the A1 promoter also have the inherent capacity to bind Blimp-1 but with a lower affinity. Taken together the in vitro binding assays reveal that among the putative Blimp-1 binding sites from the A1 promoter, site-3 has a stronger affinity for Blimp-1, compared to site-1, and sites 2 and 4 are even much weaker binding sites.

5.2.1.4 Blimp-1 dependant A1 repression occurs at the transcriptional level due to an effect on the A1 promoter

The reduction of the A1 mRNA level (Fig. 10) seen in the above experiment can be due to repression of the A1 promoter by Blimp-1 or, alternatively, it can be due to induction of post transcriptional decay of A1 mRNA by Blimp-1. However, the identification of putative Blimp-1 binding elements in the A1 promoter, and the confirmation that these sites indeed bind Blimp-1, suggest the possibility of a direct repressive role for Blimp-1 on the A1 promoter.

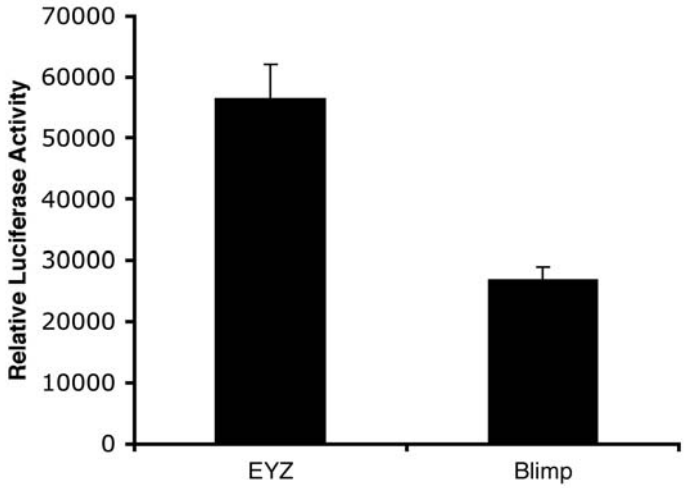


Fig. 15. Repressive effect of Blimp-1 on a luciferase reporter regulatable by 2010 base region (2010A1Luc) upstream to thetranscription start site of murine A1 gene. 100ng of 2010A1 Luc luciferase reporter with 500 ng of either empty vector expressing EYZ or 500 ng of Blimp-1 was cotransfected into 293 T cells and luciferase activity was assayed after 36 hours.

Therefore, luciferase reporter assays (Fig. 15) were performed using a luciferase reporter regulated by an A1 promoter region, consisting of 2010 base pairs corresponding to the 5' flanking region of the murine A1 gene (here after called 2010 A1-luc). Luciferase assays were carried out in 293T

cells by transient cotransfections of 2010 A1-luc together with expression vectors encoding FBlimp-1 or empty control vectors. As shown in figure 15, transient transfection of 293T cells with 2010 A1-luc plus Blimp-1F led to marked reduction in the A1 promoter activity, as indicated by reduction in the luciferase activity, compared to cotransfections with control vectors. This result indicates that Blimp-1 mediated reduction in the A1 messages is due to a repressive effect of Blimp-1 on the A1 promoter, suggesting a transcriptional repressive effect.

5.2.1.5 Truncation mutants of the A1 promoter identify the minimal Blimp-1 responsive region within the A1 promoter

To study the relevance of Blimp-1 binding elements and the repression of the A1 promoter by Blimp-1, A1 promoter regulated luciferase reporters were generated, with successive deletions of the A1 promoter region 5′ to the transcriptional start site (Fig. 16). The truncated versions of A1 promoter-luciferase reporters include -500 to +1 of the A1 promoter (500 A1 Luc) and -81 to +1 (81 A1 Luc). The 500 A1 Luc contains all the four Blimp-1 binding sites, the sites 1-4 (Fig. 16). The 81 A1 Luc lacks the upstream Blimp-1 binding element, the site-1, but contains all the remaining sites 2,3 and 4 (Fig. 16). Luciferase reporter assays using the reporter plasmid 500 A1-luc in cotransfections with either Blimp-1F expression vectors or empty vectors revealed the repressive effect of Blimp-1 on the -500 to +1 region of the A1 promoter (Fig. 17). To study the influence of down stream Blimp-1 binding sites in the regulation of A1 promoter activity, the luciferase reporter regulated by the shorter region of the A1 promoter -81 to +1 (81 A1 Luc)

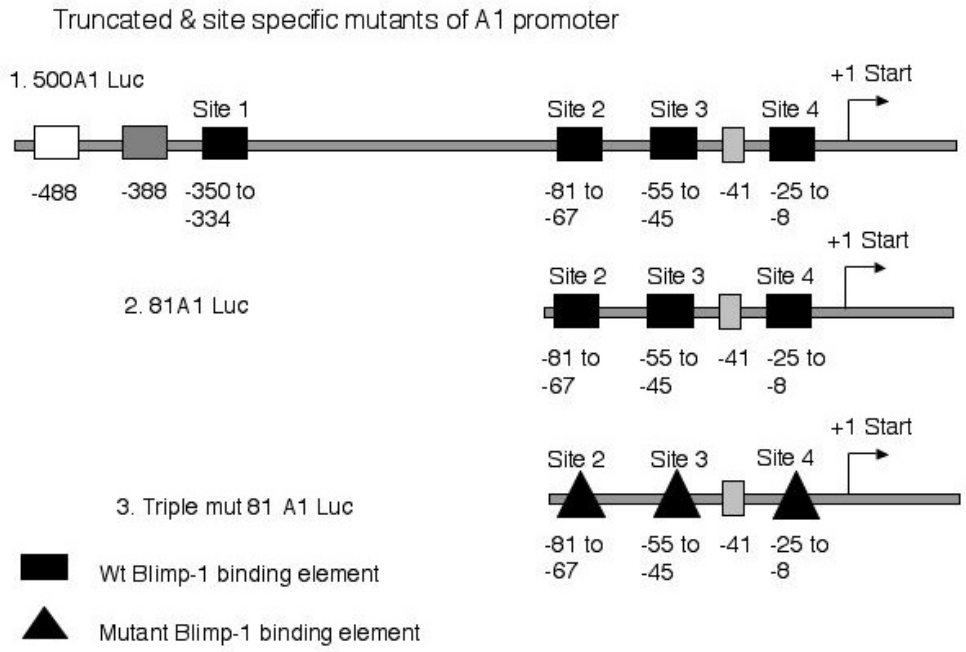


Fig. 16. Schematic figure shows the truncated and mutant versions of A1 ptomoter –luciferase reporters. 1: 500A1-luc represents the luciferase reporter regulated by 500 base pair, upstream to the transcription start site of A1 promoter. 2: 81A1-luc represents short version of luciferase reporter, regulated by 81 bp A1 promoter region. 3: Triple mut 81A1-luc represents the 81A1-luc with all the three Blimp-1 binding elements mutated. Black squares represent wild type Blimp-1 binding elements. Black triangles represent mutant Blimp-1 sites.

was used in cotransfection assays. Cotransfection of 81 A1 Luc with increasing amounts of Blimp-1F expression vectors led to progressive reduction in luciferase activity, compared to vector control, suggesting a repressive effect of Blimp-1 on this shortest region of A1 promoter (Fig.18). This is in agreement with the presence of specific Blimp-1 binding elements 2,3 and 4 within this region. As an internal control for transfection efficiency, aliquots of the same lysates used for measuring Luciferase activity were analyzed by Western blotting to detect the expression of EYFP (Fig. 18).

However, the essential role of these Blimp-1 binding elements in mediating the repressive effect of Blimp-1 can only be understood by mutagenesis of

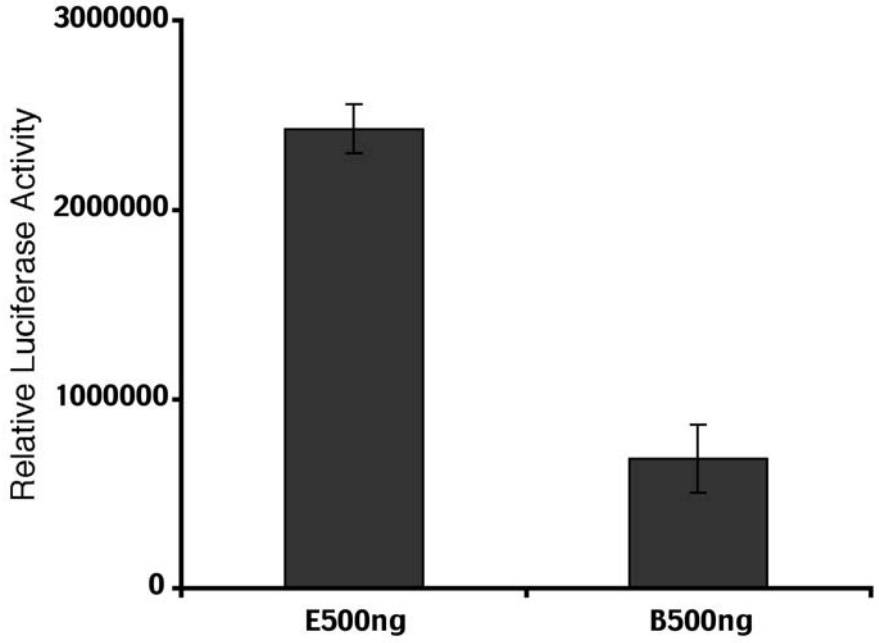


Fig. 17. Repression of truncated A1 promoter representing the -500 to+1 of A1 promoter (500 A1 Luc)By Blimp-1. 100ng of 500 A1-Luc luciferase reporter with 500 ng of either empty vector expressing EYZ or 500 ng of Blimp-1 was cotransfected into 293 T cells and luciferase activity was assayed after 36 hours.

these sites. Therefore site specific mutagenesis was carried out to generate a triple mutant, in which all the three Blimp-1 binding elements 2,3 and 4 were mutated, within the 81 A1 Luc (fig. 13). The triple mutant was tested in luciferase assays for the repressive effect of Blimp-1. Wild type 81 A1 Luc (WT 81 A1 Luc) or the triple mutant (triple mut 81 A1 Luc) in which all the three Blimp-1 binding sites were mutated was used in cotransfections together with Blimp-1F and the repressive effect of Blimp-1 on the triple mutant was compared to that of WT 81 A1 Luc. The WT 81 A1 Luc was repressed by Blimp-1 but with the use of triple mut 81 A1 Luc, the repressive effect of Blimp-1 was nearly abolished (Fig.19A & B). Again aliquots of the 293T

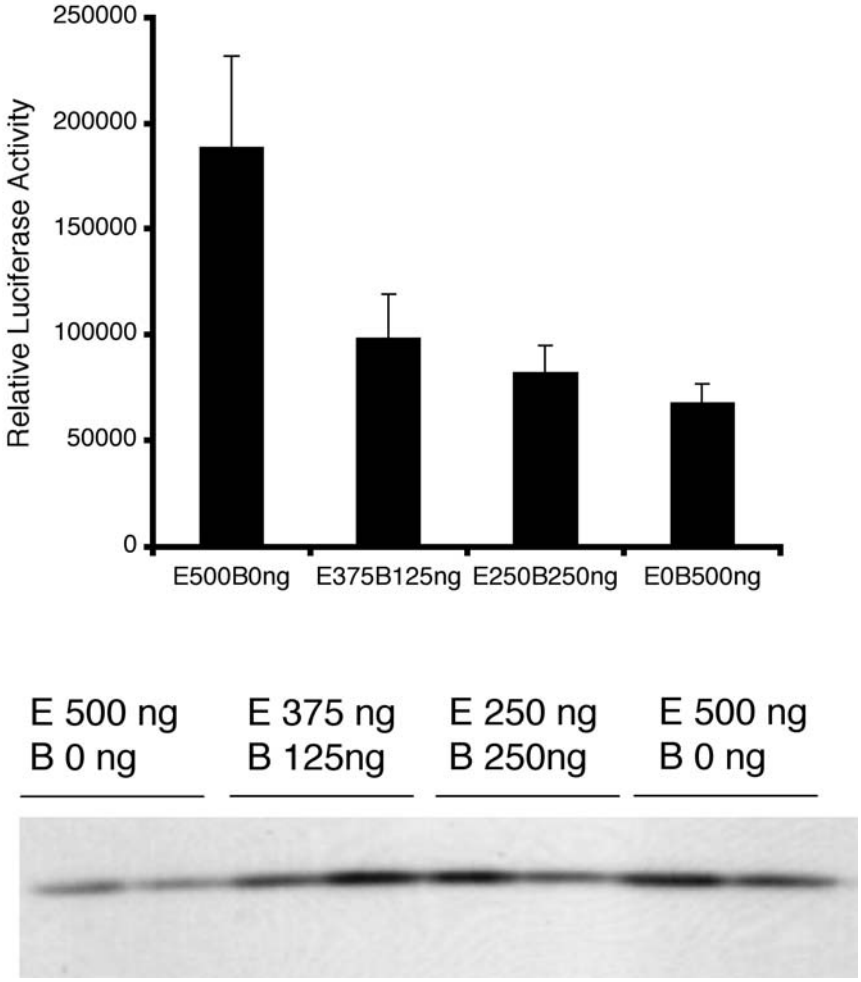


Fig. 18. Luciferase reporter assay revealing the dose dependant repressive effect of Blimp-1 on the unmutated short A1 Promoter (81 A1-Luc). Upper panel: 100ng of WT 81 A1-Luc luciferase reporter with 0-500 ng of of Blimp-1 was cotransfected into 293 T cells and luciferase activity was assayed after 36 hours. The total amount of transfected DNA was kept constant to 500 ng by using empty vector expressing EYFP. Lower panel: The same duplicate lysates of 293 T cells used in the luciferase assay were assayed by Western blot analysis to confirm the transfection efficiency. Shown was the expression of EYFP protein encoded by the bicistronic retroviral vectors which encode EYFP. From the figure it is clearly evident that all the lysates expressing Blimp-1 infact have higher EYFP but still the Luciferase activity was low, suggesting that the observed reduction in luciferase activity was not due to lower transfection efficiency but rather due to repressive effect of Blimp-1.

cell lysates used for measuring luciferase activity were analyzed by western blotting for EYFP expression, as a transfection efficiency control (Fig. 19C). From these results it is clearly

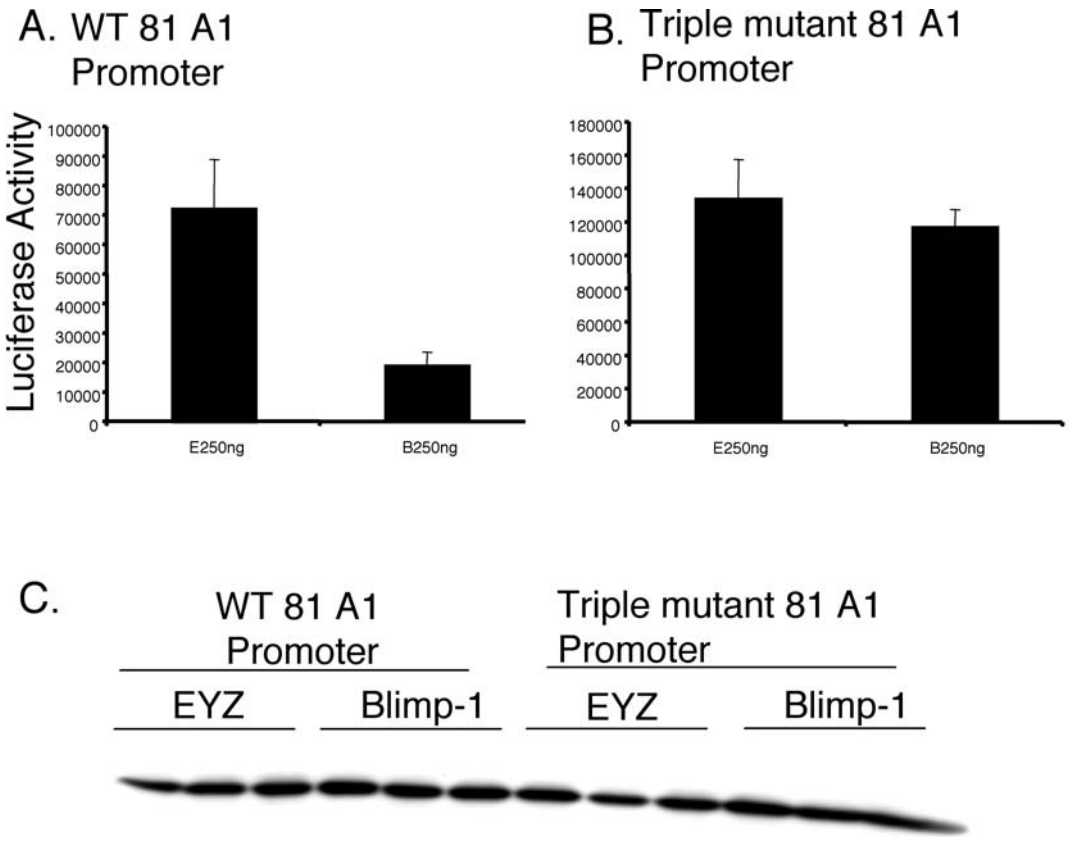


Fig. 19. Loss of the repressive effect of Blimp-1 on the triple mutant short version of A1 promoter (81A1Luc) in 293T cells. A. Luciferase reporter assay revealing the repressive effect of Blimp-1 on the unmutated short version of A1 Promoter (81 A1-Luc). 100ng of WT 81 A1 Luc luciferase reporter with 250 ng of either empty vector expressing EYZ or 250 ng of Blimp-1 was cotransfected into 293 T cells and luciferase activity was assayed after 36 hours. B. Luciferase reporter assay revealing the loss of repressive effect of Blimp-1 on the triple mutant 81A1 Luc Luciferase reporter. 100ng of triple mutant 81 A1-Luc luciferase reporter with 250 ng of either empty vector expressing EYZ or 250 ng of Blimp-1 was cotransfected into 293 T cells and luciferase activity was assayed after 36 hours. B. The same triplicate lysates of 293 T cells used in the luciferase assay were assayed by western blot analysis to confirm the transfection efficiency. Shown was the expression of EYFP protein encoded by the bicistronic retroviral vectors which encode EYFP. Equal expression of EYFP suggests that the observed effects were not due to variations in transfection efficiency.

evident that Blimp-1 exerts a direct repressive effect on the A1 promoter and that such a repressive effect is dependant on specific Blimp-1 binding elements present within the promoter.

5.2.1.6 Blimp-1 dependent indirect mode of A1 promoter repression

In addition to the Blimp-1 binding sites, the A1 promoter region contains an E-box element CACGTG, located between -389 and -386 and an SP1/E2F binding element located between -43 and –30 (Fig. 11). As described in the introduction, E-box elements bind the Myc/Mad family proteins and play a pivotal role in transcriptional regulation, with Myc involved in the transcriptional activation and Mad proteins involved in transcriptional repression, through the same E-boxes (141). In response to Blimp-1, a shuffle in the balance between Myc and Mad proteins was observed with down regulation of c-Myc and upregulation of Mad4 (70). Because Mad4 is inducible by Blimp-1 and because the A1 promoter contains a Mad responsive E-box element, Blimp-1 might exert an indirect mode of A1 promoter repression via Mad4 through the E-boxes. Alternatively the upregulated Mad4 might exert A1 promoter repression through the SP1 / E2F like element, via the repression of E2F transcription factors.

5.2.1.7 Cloning and characterization of Mad4

To study the influence of Mad4 on the A1 promoter regulation, recombinant retroviruses expressing the mouse Mad4 were constructed. Because Blimp-1 induces Mad4 upregulation, first strand cDNAs were synthesized from

Blimp-1 infected primary mouse splenic B cells, followed by PCR amplification of Mad4, by using Mad 4 specific primers. PCR amplified Mad4 cDNA was inserted into the MCS of the bicistronic retroviral vector pEGN/MCS-F, to generate pEGN/ Mad44F.

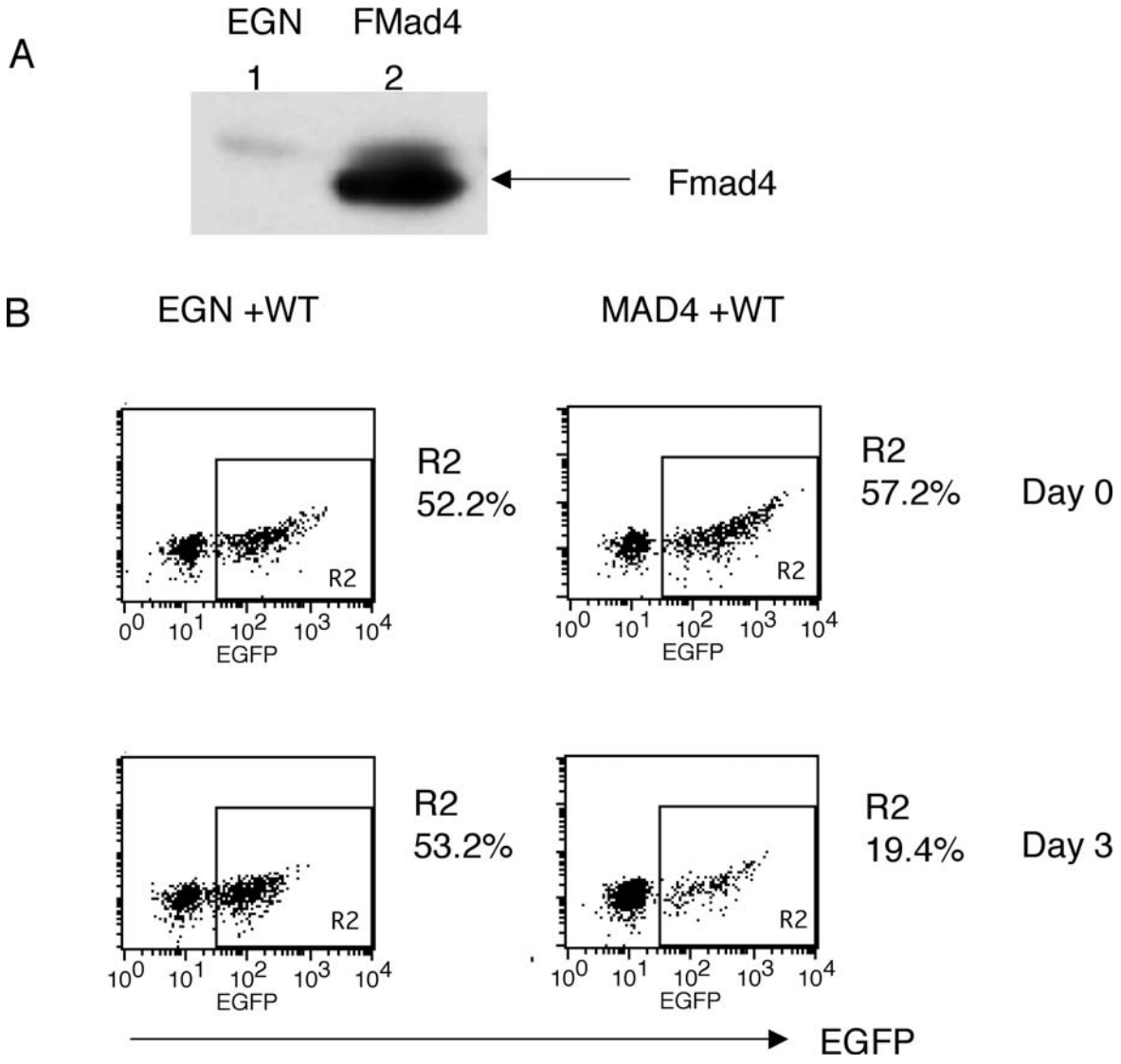


Fig. 20. Expression and functional analysis of cloned mouse Mad4. A. Western blot analysis of 293T cell lysates transfected with either control vector (pEGN) encoding EGFP or pEGN/ Mad4F encoding flag-tagged Mad4. Mad4 was detected by anti-flag antibody. B. Functionality of Mad4 was tested by analyzing the previously described growth inhibitory function of Mad4, by FACS analysis of mixed cultures, where either pEGN or pEGN/ Mad4F infected M12 cells were mixed with wild type M12 cells. In the

Mad4 containing mixed cultures, the EGFP+ cells representing the Mad4 population declained drastically by day 3, indicating growth disadvantage for Mad4 expressing cells. Where as in the mixed cultures containing the pEGN vector control infected cells, the EGFP+ population remained unaltered.

The Mad4 expression vector generated was further verified for the expression of the protein by transient transfections into 293 T cells. As shown in the western blot (fig. 20A), 293T cells transfected with Mad4 show a specific band corresponding to Mad4, detected by an antibody against Flag.. Because Mad4 has been described to function as an anti-proliferative molecule, the functionality of cloned Mad4 was analyzed by mixed culture experiments in B cell line M12. pEGN or pEGN/ Mad44F infected M12 cells were mixed with wild type M12 cells. The mixed cultures were followed over a period of time and the EGFP positive cells were measured by FACS analysis. In the mixed cultures, Mad4 expressing cells gradually declined while vector control infected cells remained constant, suggesting the growth disadvantage of Mad4 infected cells (Fig. 20B). This result confirms the functionality of the cloned Mad4 expression vector pEGN/Mad4F.

5.2.1.8 Mad 4 exerts a repressive effect on the A1 promoter

As described above, Mad4 can be the linking molecule to mediate Blimp-1 dependant indirect mode of A1 promoter repression. To test this possibility, luciferase assays were carried out in 293T cells by cotransfecting with the 500 A1 Luc together with Mad4 expression vector pEGN-Mad4 or control pEGN vectors. Luciferase activity was measured as a read out for promoter function. Cotransfection of 500A1 Luc together with Mad4 led to siginificant reduction in the luciferase activity, indicating a repressive effect of Mad4 on the A1 promoter (Fig. 21 left panel).

Because the Mad4 responsive E-box element was located between -389--386 of the A1 promoter, the shorter version of the A1 promoter 81 A1 Luc which lacks the E-box was used in luciferase assays to study whether Mad4 dependant A1 promoter repression is E-box dependant or independent. Surprisingly, as shown in figure 21 (right panel), Mad4 was able to repress even the shorter form A1 promoter which lacks the E-box. This can

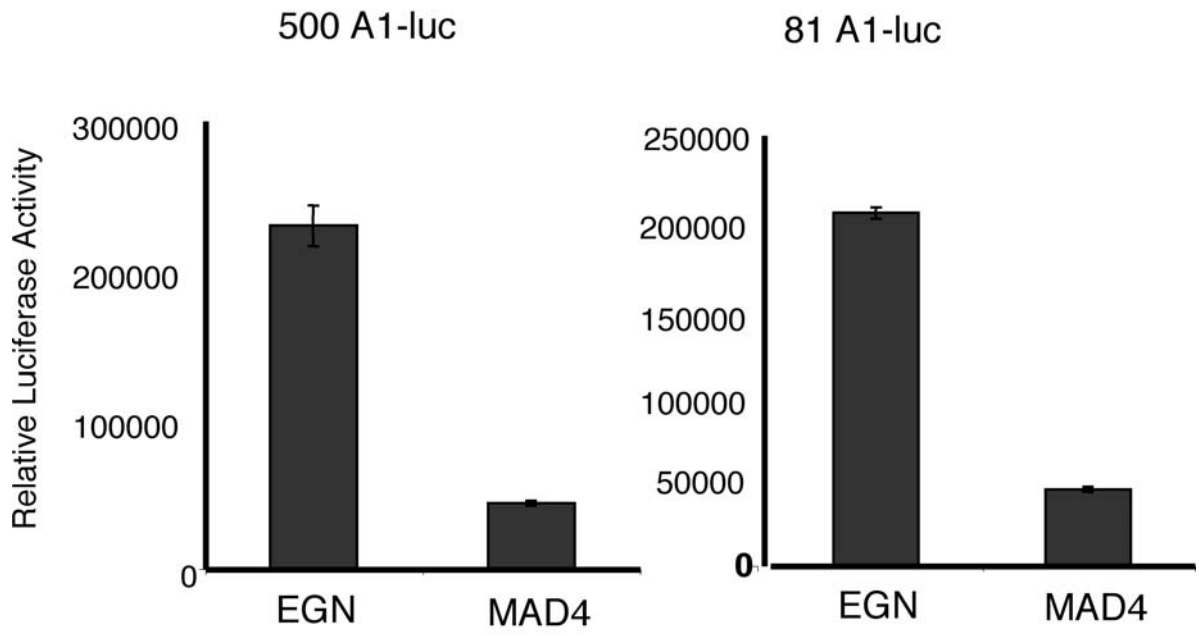


Fig. Repression of the A1 promoter by Mad4, a down stream target of Blimp-1. Luciferase reporter assay revealing the repressive effect of Mad4 on the 500 A1-Luc reporter (left panel) and 81A1-Luc reporter (right panel). 100ng of 500 A1 Luc luciferase or 81A!-luc reporter with 1µg of either empty vector expressing EYZ or Blimp-1 was cotransfected into 293 T cells and luciferase activity was assayed after 36 hours.

possibly, be due to novel unidentified Mad4 responsive elements within the shorter version of A1 promoter or alternatively via the modulation of other factors by Mad4, which in turn exert a repressive effect on the A1 promoter. The repression of A1 promoter by Mad4, a down stream target of Blimp-1

implies that Blimp-1 in addition to its direct repressive effect on the A1 promoter, also exerts an indirect mode of repression via Mad4.

5.3 Part III

5.3.1 The functional significance of Blimp-1 dependant repression of A1 : implications in B cell terminal differentiation

The results presented above indicate that Blimp-1 exerts direct and indirect modes of A1 promoter repression. As described previously (70), repression of A1 is a potential mechanism to induce apoptosis of terminally differentiated B cells because ectopic expression of A1 rescues Blimp-1 infected B cells from cell death and enables prolonged survival. From this study it appears that induction of B cell death by Blimp-1 via repression of A1 is a mechanism to down modulate the immune effector phase. However, the down modulation of A1 promoter activity by the differentiation inducing factor Blimp-1 and its down stream target Mad4, which is generally expressed in cells during terminal differentiation, raises the possibility of involvement of A1 in the interference of B cell terminal differentiation.

5.3.1.1 Ectopic expression of A1 in WEHI 231 cells interferes with terminal differentiation

To test the possible influence of A1 on B cell terminal differentiation, WEHI 231 cells were used in an in vitro differentiation assay. As described in the first part, LPS triggers the terminal differentiation of WEHI 231 cells and Blimp-1 is induced under these conditions. If Blimp-1 induced repression of A1 were required for terminal differentiation, then ectopic expression of A1 from a heterologous promoter should counter differentiation.

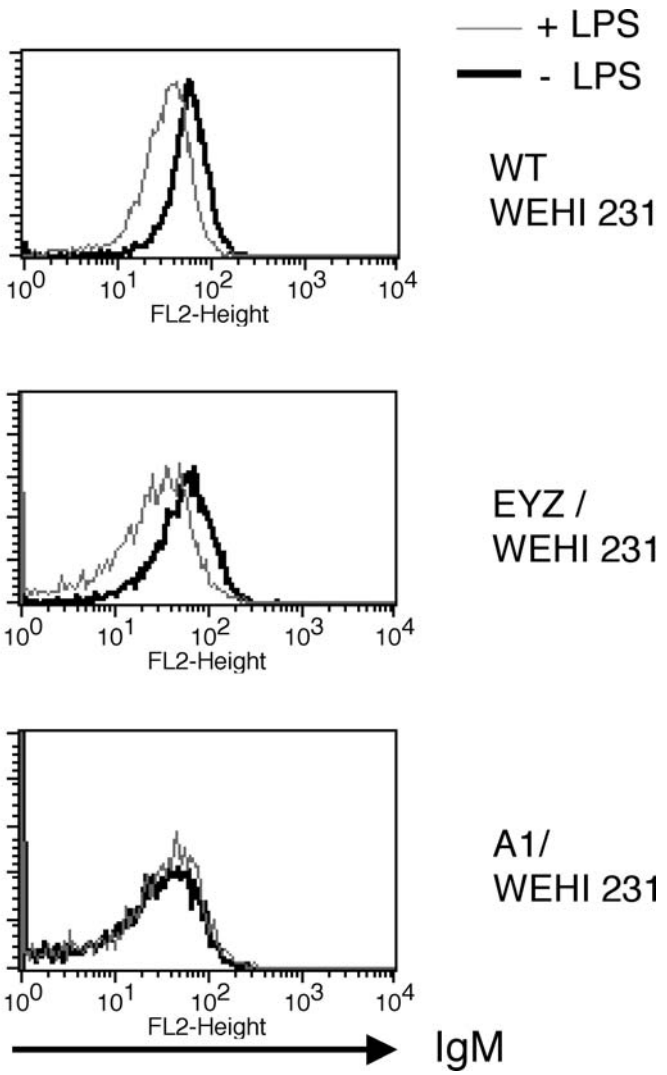


Fig. 22. Ectopic expression of A1 blocks LPS induced surface immunoglobulin (Ig) down regulation in WEHI 231 cells. FACS analysis shows down regulation of surface Ig in control vector EYZ infected or WT WEHI 231 cells, upon LPS stimulation (Upper and middle panels). Where as the A1 infected WEHI 231 cells do not respond to LPS stimulation, as the surface IG levels of unstimulated and LPS stimulated cells show the same profile of surface Ig (Lower panel). Shown were the FACS histogram overlays.

To test this possibility, WEHI 231 cells were transduced with rRV encoding A1 and EGFP or control rRV encoding only EGFP and stimulated with LPS for 3days, to induce terminal differentiation. The surface expression of IgM was used as a marker for differentiation, because, upon differentiation,

WEHI 231 cells lose the surface expression of IgM, due to the generation of secretory form of IgM. As shown in the FACS analysis (fig.22), control vector infected cells and WT WEHI 231 cells show a reduction in the surface expression of IgM upon LPS stimulation, while such a reduction was not evident in A1 infected cells. This result provides the first indication that A1 might interfere with the terminal differentiation programme of B cells.

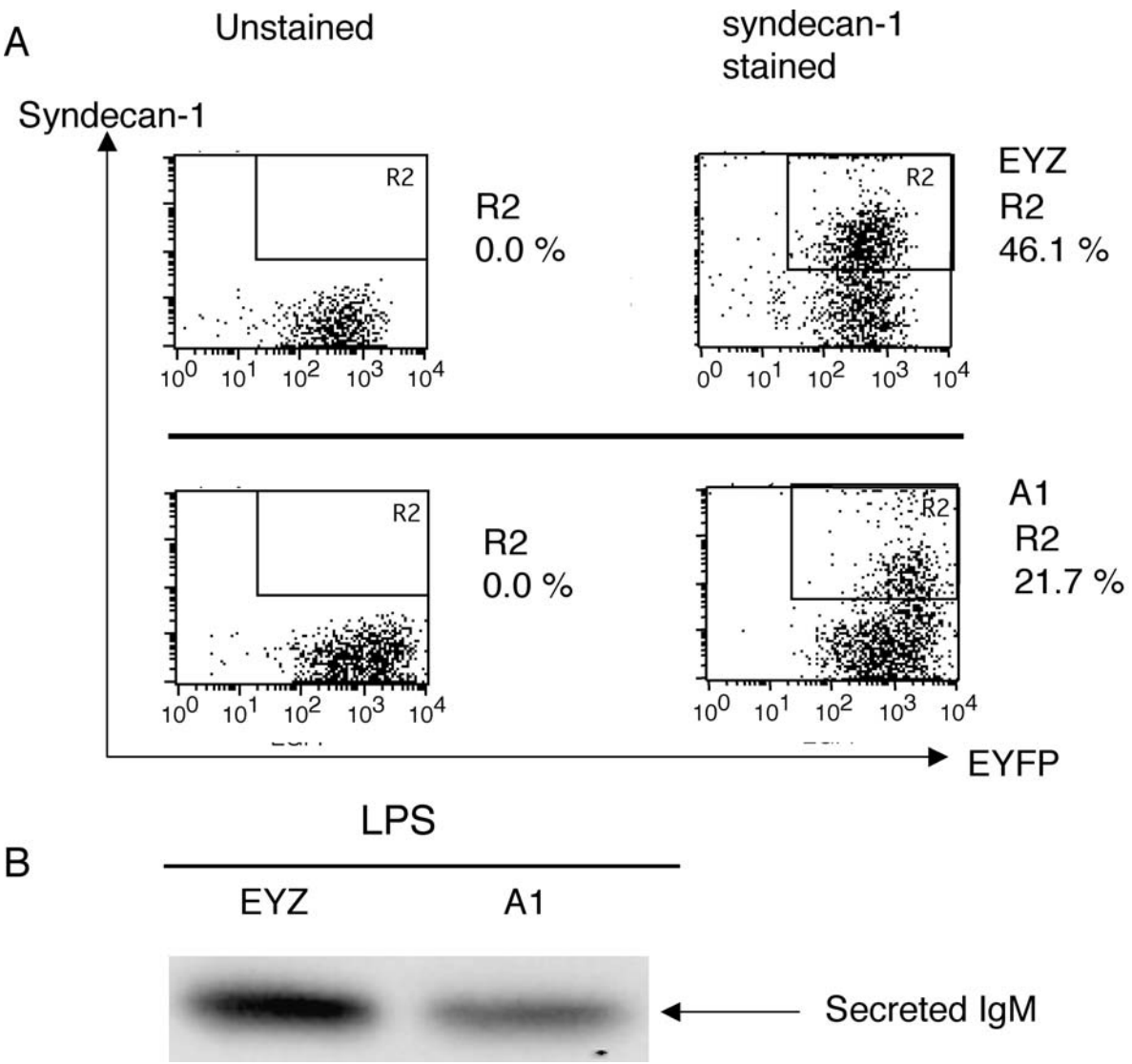


Fig. 23. Ectopic A1 expression interferes with terminal differentiation of of mouse splenic B cells. A: Primary B cells were infeted with A1 or control vector EYZ under LPS stimulation and further cultured for 3 days with LPS stimulation. Facs analysis reveals the

reduced expression of plasma cell marker syndecan-1, in A1 infected cells, compared to control vector infected cells. B. Culture supernatants of the same cells used above were analyzed by Western blotting to detect the secreted IgM. A1 infected cells secreted reduced amounts of Ig molecules, in comparison to EYZ infected cells.

5.3.1.2 Ectopic expression of A1 in primary mouse splenic B cells results in reduced expression of plasma cell marker syndecan-1 and reduced Ig secretion.

To further study the role of A1 in terminal B cell differentiation, the influence of A1 on Ig secretion in mouse splenic B cells was analyzed. LPS stimulated splenic B cells undergo differentiation and secrete Ig, which is a hall mark feature of terminally differentiated B cells. Furthermore LPS stimulation induces Blimp-1 and down regulates A1. Again to study the influence of A1, primary splenic B cells were stimulated with LPS and transduced with rRV encoding A1 or empty vector controls. Infected cells were further maintained for 3 days in culture and culture supernatants were analyzed for secretory IgM by Western blot analysis. Splenic B cells infected with A1 encoding rRV secreted reduced amounts of IgM compared to vector control infected cells (Fig. 23B). Furthermore, A1 infected splenic B cells showed reduced expression of plasma cell marker syndecan-1 (Fig. 23A).The reduction in secreted IgM and reduction in syndecan-1 in A1 infected cells indicate an inhibitory role for A1 in B cell terminal differentiation. Therefore repression of A1 by Blimp-1 might be an essential step not only to ensure shut down of immune effector phase but also to ensure the initiation of proper effector phase.

6. Discussion

Induction of UPR to generate a transcriptionally active form of XBP-1 via XBP-1 mRNA splicing, has been shown to be an essential step during plasma cell differentiation (90, 92). Furthermore, enforced Blimp-1 expression in B cell lines or in primary B cells is sufficient to induce terminal differentiation, resulting in the generation of Ig secreting plasma cells. Plasma cells secrete huge amounts of immunoglobulins (Ig). Secretion of immunoglobulins requires continuous synthesis of Ig molecules as well. The increased Ig synthesis might be the trigger for UPR and XBP-1 splicing. Based on these possibilities it is assumed that Blimp-1 might be the upstream molecule sufficient to induce all these events in a B cell. This possibility was tested by ectopic expression of Blimp-1 in WEHI 231 cells and in primary mouse B cells.

The findings in the current study revealed that introduction of Blimp-1 into WEHI 231 cells or into primary splenic B cells, is sufficient to induce UPR and XBP-1 splicing. Furthermore, studies with the truncated mutants of Blimp-1 identified the N-terminal part of Blimp-1 as the important region pocessing the capacity to mediate the important functions Blimp-1 required for terminal B cell differetion..

Before studying the effects of ectopically introduced Blimp-1 in the context of induction of UPR, it was first verified whether a correlation existed between endogenous Blimp-1 expression and XBP-1 processing in B cells. The B cell lymphoma line WEHI 231 is known to secrete immunoglobulins upon LPS stimulation indicating that these cells have the inherent capacity to undergo terminal differentiation. Analysis of unstimulated & LPS stimulated WEHI 231 cells revealed a correlation between endogenous Blimp-1

expression, generation of processed form of XBP-1 and Ig secretion (Fig). A similar correlation was observed in primary splenic B cells. LPS & anti-μ stimulated primary B cells proliferate but fail to differentiate. Under these conditions Blimp-1 was not expressed and UPR was not observed. Whereas LPS stimulation led to induction of Blimp-1, Ig heavy & light chains & UPR as shown by XBP-1processing & upregulation of UPR target BIP. These results show a strong correlation between endogenous Blimp-1 expression & XBP-1 processing in B cells.

Blimp-1 is sufficient and the sole molecule capable of triggering UPR & XBP-1 processing in B cells

Even though a correlation was observed between Blimp-1 expression and XBP-1 processing, it is not evident if it is a mere correlation or strictly a Blimp-1 induced function. This question was analyzed by ectopic expression of Blimp-1 in WEHI 231 cells. Because WEHI 231 cells do not express endogenous Blimp-1 under normal culture conditions, ectopic expression of Blimp-1 in these cells facilitates identification of functions soley derived from Blimp-1. Accordingly, transduction of WEHI 231 cells with Blimp-1 encoding retroviruses resulted in the generation of spliced version of XBP-1, in comparison to control vector transduced cells. This result indicates the sufficiency of Blimp-1 in the induction of UPR. Furthermore, Blimp-1 function was analyzed in mouse primary B cells, under LPS & anti-μ conditions. Under these conditions endogenous Blimp-1 was not expressed and therefore provides a possibility to study the functions derived exclusively from ectopically introduced Blimp-1. Again, Blimp-1 introduction resulted in the induction of UPR and XBP-1 splicing, confirming the sufficiency of

Blimp-1 in inducing these events. In both WEHI 231 cells and mouse primary B cells, enforced expression of Blimp-1 led to elevation of of immunoglobulin proteins and UPR, as revealed by the splicing of XBP-1. Furthermore in primary B cells Blimp-1 upregulated the UPR target gene BIP. These results clearly demonstrate that Blimp-1 is the upstream molecule sufficient to trigger UPR and XBP-1 splicing in B cells.

Domains of Blimp-1 associated with transcriptional repressive function induce UPR while the region of Blimp-1 associated with transcriptional activation is dispensable

Previous studies using serial truncated mutations of Blimp-1 defined the domains of Blimp-1 needed for transcriptional repression (75) and the repressive functions of blimp-1 were established in the context of several genes (83). The N-terminal region of Blimp-1 consists these established domains and has been shown to mediate transcriptional repression whereas the C-terminal region of Blimp-1 shows modest transcriptional activation. The transcriptional repressive function of Blimp-1 is associated with the repression of key transcription factors (e.g. c-Myc, Pax5, Bcl6 etc.) which antagonize plasma cell differentiation (82). Therefore it appears plausible that the Blimp-1 domain(s) comprising the transcriptional repressive function might be sufficient to trigger important events needed for plasma cell differentiation like UPR and XBP-1 splicing. In this context, it was interesting to understand whether the region of Blimp-1 important for transcriptional repression was sufficient to induce UPR and XBP-1 processing or if they are separable processes, meaning that induction of XBP-1 processing originated from domains or regions of Blimp-1 independently of

transcriptional repressive domains. The fact that Blimp-1 represses Pax5 which in turn leads to derepression of XBP-1 and Ig transcription, provides interesting clues about the possible mechanism of UPR induction and XBP-1 processing (81). Blimp-1 dependant repression of Pax5 might lead to upregulation of Ig levels and derepression of XBP-1 messages. The elevated Ig levels activate IRE1α, which acts on XBP-1 RNA and removes an intron to generate a functional XBP-1 message (92). Because Pax5 repression as described above seems to unfold a series of events necessary for UPR and XBP-1 processing, it is logical to speculate that the transcriptional repressive function of Blimp-1 is important in the induction of UPR. Therefore, it is conceivable that the N-terminal region of Blimp-1 associated with transcriptional repressive function of Blimp-1 might play a role in the induction of UPR and XBP-1 processing.

In the current study, these possibilities were analyzed by using truncated mutants of Blimp-1 encoding the N-terminal or the C-terminus of Blimp-1. As speculated, ectopic expression of N-terminal Blimp-1 (amino acids 1-751) in B cells was sufficient to induce the same effects as the full length Blimp-1 in terms of Ig heavy & light chain upregulation, UPR & XBP-1 processing. Whereas the C-terminal part of Blimp-1 comprising the amino acids 465-856 with previously described transcriptional activation function is not capable of inducing Ig synthesis and UPR.

In summary the current study confirms the view that Blimp-1 is a master regulator of terminal differentiation. The evidence showing the sufficiency of Blimp-1 in triggering an UPR and XBP-1 processing, in B cells, in addition to previously ascribed functions for Blimp-1 such as a block in proliferation

and generation of the secretory form of immunoglobulins (69, 79), confirms Blimp-1 as the single gene capable of regulating every essential step involved in terminal B cell differentiation. Furthermore, the N-terminal region of Blimp-1 associated with the transcriptional repressive function was identified as a region sufficient to mediate UPR induction & XBP-1 processing and Ig secretion, whereas the C-terminal region appears to be dispensable. Such a predominant effect of Blimp-1 containing the repressive domains might be initiated by direct repression of plasma cell antagonists like c-Myc and Pax5. Loss of c-Myc results in a block in proliferation, a prerequisite for differentiation. Down regulation of pax-5 by Blimp-1, on the other hand, possibly regulates the set of events involved in induction of an physiological UPR via upregulation of XBP-1 mRNA and IG levels, with the elevated Ig levels in turn providing the signal for UPR & XBP-1 processing.

In addition to the undisputed role for Blimp-1 in triggering the plasma cell differentiation, induction of apoptosis is another important aspect of Blimp-1 function. However, the exact mechanism and significance of Blimp-1 mediated apoptosis is unknown. Possibly, induction of apoptosis by Blimp-1 might be essential to regulate effector cell homeostasis, providing space for new immune effector cells. Alternatively, Blimp-1 induced B cell death is needed to define the effector phase of a differentiated B cell, or it is a safety mechanism, which kills any terminally differentiated B cell, until and unless the cell receives specific survival signals, obtained only during proper differentiating conditions. With regard to the mechanism of Blimp-1 induced apoptosis, despite the identification anti apoptotic gene A1 as a Blimp-1 target, the molecular basis of A1 repression is unknown (70).

Results in the current study provide the first evidence that A1 is a direct target of Blimp-1. The presence of multiple Blimp-1 binding sites in the A1 promoter, and the ability of these sites to bind Blimp-1 raised the possibility of direct repression of the A1 promoter by Blimp-1. In vitro binding analyses (oligonucleotide precipitation assays and EMSAs) clearly showed that Blimp-1 binds to these elements with varying affinities, in accordance with the variation in the homology of these sites to the consensus sequence. The repression by Blimp-1 of luciferase reporter gene under the regulation of A1 promoter clearly indicates that Blimp-1 exerts its repressive effect on the A1 promoter. Such a repression of A1 promoter by Blimp-1 seems to be due to binding of Blimp-1 to specific Blimp-1 binding elements in the A1 promoter because mutation of all the Blimp-1 binding elements led to significant reversal of the repressive effect of Blimp-1. From these results it is clearly indicative that at least one mode of A1 downregulation by Blimp-1 is due to direct repression of A1 promoter mediated by specific Blimp-1 binding elements. The presence of multiple Blimp-1 binding elements with weak homology to the consensus sequence suggests that possibly all these sites have a cumulative effect in mediating the repressive effect of Blimp-1 on the A1 promoter. Furthermore, as shown in the EMSAs, except site3 of A1 promoter, the other sites 1,2 and 4 are weak blimp-1 binding sites. The presence of weak Blimp-1 binding sites in the A1 promoter has possibly important functional significance, in terms of viability of effector plasma cells. Possibly due to the weak affinity of Blimp-1 to the Blimp-1 sites in the A1 promoter, Blimp-1 might target A1 only when its concentration becomes high. Due to this reason, during earliest phases of plasma cell differentiation, when Blimp-1 levels just start to upregulate, the A1 promoter would not be repressed by Blimp-1 but on the other hand A1 induction might be blocked allowing only basal level expression of A1. This is because B cell terminal

differentiation inducing signals like LPS induce activation of NF-kB. which is a known inducer of A1 promoter (127). Subsequent induction of Blimp-1 might start to counter the positive effect of NF-kB on the A1 promoter, through site3, the strong Blimp-1 binding site, leading to a block in A1 induction but allowing basal expression. With time, i.e., with accumulation of more Blimp-1, the protein might occupy all the sites in the A1 promoter, exerting repression. This sort of a mechanism where Blimp-1 targets A1 as a late repressive target will explain how Blimp-1 despite being a pro-apoptotic molecule will allow proper differentiation of B cells into plasma cells. Therefore, B cells might have evolved such a pleotropic factor Blimp-1 which induces differentiation into effector cells and which also brings on death of effector cells as a late effect, providing time for effector phase.

Additionally, an indirect mode of repression of A1 promoter is possible via other factors which are in turn Blimp-1 modulated factors. An interesting candidate in this context is the Myc/Mad family protein Mad4 (70). This is because it was previously observed that Myc levels go down and Mad4 levels go up in response to Blimp-1. Interestingly, the A1 promoter contains E-box where Myc/Mad family proteins can bind. Therefore, it appears that, Mad4, a transcriptional repressor, induced by Blimp-1 might repress A1 promoter. This possibility was tested by studying the effect of ectopically expressed Mad4 on the A1 promoter activity in luciferase reporter assays and indeed Mad4 repressed A1 promoter. However, this repression of the A1 promoter by Mad4 seems to be independent of E-boxes because Mad4 repressed a truncated mutant of A1 promoter lacking E-box. Possibly Mad4 mediated repression of the A1 promoter occurs via novel Mad4 binding sites specific for Mad4. Alternatively Mad4 might exert its repressive effect by interaction with other factors through other DNA binding elements. Possibly full shut

down of A1 promoter resulting in plasma cell death is a combined effect of direct and indirect modes of promoter repression by Blimp-1.

Blimp-1 mediated repression of A1 as a pre requisite for differentiation:

The presence of multiple Blimp-1 binding sites in the A1 promoter and its repression by Blimp-1 raises an important biological question, with regard to the function of A1 during terminal B cell differentiation. An interesting possibility is the involvement of A1 in the interference of plasma cell generation. This possibility was tested by ectopically expressing the A1 in B cells. Indeed ectopic expression of A1 interfered with terminal differentiation, as shown by reduced Ig secretion and reduction in the plasma cell marker syndecan-1, in splenic B cells. In the WEHI 231 cells, overexpression of A1 led to a slight decrease in surface Ig levels. This could be due to a negative influence of A1 on the Ig synthesis itself. Furthermore, the surface Ig levels were unaltered in A1 transduced WEHI 231 cells upon LPS stimulation, whereas in control vector transduced cells loss os surface Ig was evident. This could possibly be due to impairment of generation of secretory form of Ig or due to perturbance of secretory apparatus, in the prisence of A1. The above findings indicate interesting clues about an inhibitory role for A1 in the plasma cell generation and therefore repression of A1 by Blimp-1 seems to be an important pre requisite for plasma cell generation.

The immune system might have evolved such a mechanism with an anti-apoptotic molecule like A1 blocking the differentiation of B cells into immune effector cells. Such a mechanism where repression of anti-apoptotic molecule is required for effector cell generation, possibly ensures that the differentiated cell is destined for death. This kind of mechanism meets

important requisites of the immune system like, defined timing of the effector cell viability, prevention of prolonged survival of effector cells, which allows space for new effector cells.

7. Literature

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8 Abreviations

Ab	antibody
Ag	antigen
APC	antigen presenting cell
BCR	B cell receptor
Blimp-1	B lymphocyte induced maturation protein-1
bHLH	basic helix-loop-helix
BSS	balanced salt solution
BSA	bovine serum albumin
cRPMI-1640	complete RPMI-1640 medium
CREB	cAMP response element binding protein
CTL	cytotoxic T-lymphocyte
DC	dendritic cell
DEPC	diethylpyrocarbonate
DMSO	dimethylsulfoxide
DTT	dithiothreitol
ERK	extracellular signal regulated kinase
EGN	enhanced green fluorescent/neomycin protein
EYZ	enhanced yellow fluorescent / zeocin protein
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
HDAC	histonedeacetylase
HMT	histone methyl transferase
IFN-□	interferon gamma

Ig	immunoglobulin
IRES	internal ribosome entry site
LPS	lipopolysaccharide
LTR	long terminal repeat
MCS	multiple cloning site
MHC	major histocompatibility complex
NF-□B	nuclear factor □B
NK cell	natural killer cell
NLS	nuclear localization signal
PBS	phosphate buffered saline
PRR	pattern recognition receptor
RAG1	recombinase activating gene-1
RT	room temperature
SDS	sodium dodecyl sulfate
TCR	T-cell receptor
TGF□	transforming growth factor□
Th1	T helper type 1
Th2	Thelper type 2
UPR	unfolded protein response
WT	wild type
XBP-1	x box binding protein-1

Publications

1. Kushwaha A, Rao PP, **Duttu VS**, Malhotra P, Chauhan VS. Expression and characterisation of Plasmodium falciparum acidic basic repeat antigen expressed in Escherichia coli. *Mol Biochem Parasitol*. 2000 Mar 5;106(2):213-24.

2. **Duttu S. Vallabhapurapu**, Anneliese Schimpl, and Ingolf Berberich B lymphocyte induced maturation protein-1 (blimp-1) is sufficient to trigger an unfolded protein response and XBP-1 processing in B cells. *Signal Transduction Receptors, Mediators and Genes* (**in press**).

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Publications

1. Kushwaha A, Rao PP, **Duttu VS**, Malhotra P, Chauhan VS. Expression and characterisation of Plasmodium falciparum acidic basic repeat antigen expressed in Escherichia coli.
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Oral Presentation

34th Annual meeting of the German Scociety of Immunology
Berlin September 24-27, 2003
Title: Blimp-1 acts upstream to XBP-1 and induces ist processing during Plasma cell generation.

Poster presentations

1. 33rd Annual meeting of the German Scociety of Immunology
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Title: Direct and indirect modes of repressive effect of Blimp-1 on the A1 promoter

2. 32nd Annual meeting of the German Scociety of Immunology
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Title: Analysis of Blimp-1 responsive elements in the A1 promoter

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Declaration

I here by declare that all the information furnished above is true to the best of my knowledge.

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