Molecular physiology of plasma cell differentiation

Thesis

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Molecular physiology of plasma cell differentiation

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Abstract

Plasma cells are central to an effective immune response, being the sole producers of the antibodies, yet they can cause severe disease in autoimmunity and multiple myeloma. Therefore their differentiation and survival must be tightly regulated. In this work I investigated some of the mechanisms regulating plasma cell differentiation and life span. The differentiation of a long lived B cell to a short lived plasma cell entails a profound structural and functional metamorphosis finalized to the massive production of immunoglobulins (Ig). Exuberant Ig synthesis causes several types of stress in differentiating plasma cells. My work deals with the characterization of the C/EBP transcription factor CHOP in plasma cell differentiation. Comparing differentiation of B cells harvested from *chop*$^{-/-}$ mice to wt cells I found a mild phenotype, consisting in an increased accumulation of intracellular IgM aggregates and a decreased secretion of this antibody class, *in vitro* and *in vivo*. These findings reveal a novel role for CHOP in ensuring optimal functionality of the secretory pathway in the course of plasma cell differentiation. CHOP is involved in the differentiation of various cell types, where it interacts with other members of the C/EBP family favoring or impeding differentiation and it is an important factor in the ER stress response named unfolded protein response (UPR), in which it plays a pro-apoptotic role in most of the systems tested. I extended my investigation on the functions of CHOP in B cells by examining the resistance to ER stress-induced apoptosis in wt and in *chop*$^{-/-}$ cells. Surprisingly, I observed that in B cells CHOP expression in the UPR plays an anti-apoptotic function. Altogether my data suggest a cell-type specific role for CHOP in B cells and add information on the multi-faceted role of this transcription factor.

Most plasma cells exhibit a short life span. The mechanisms at the basis of plasma cell apoptosis are still obscure. I propose that multiple forms of stress, linked to the massive antibody production, contribute to plasma cell death.

Frequently used abbreviations

Most of the abbreviations below and those less frequently used are stated in the text at the first encounter.

ASC	Antigen secreting cell
BCR	B cell receptor
CSR	Class switch recombination
DUOX	Dual oxidase
ER	Endoplasmic reticulum
ERAD	ER associated degradation
ERSE	ER stress response element
Gpx	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HC	Immunoglobulin heavy chain
Ig	Immunglobulin
LC	Immunoglobulin light chain
LPS	Bacterial lipopolysaccharide
NADP(H)	Nicotinamide adenine dinucleotide phophate (reduced)
$NADP^+$	Nicotinamide adenine dinucleotide phophate (oxidised)
NOX	NADPH oxidase
Prx	Peroxiredoxin
PTK	Protein tyrosin kinase
PTP	Protein tyrosin phosphatase
QC	Quality control
ROS	Reactive oxigen species
SOD	Superoxide dismutase
sXBP1	spliced form of X-Box binding protein-1
TLR	Toll-like receptor
UPR	Unfolded protein response
UPRE	UPR response element
uXBP1	un-spliced form of X-Box binding protein-1

1. Introduction

1.1 The plasma cell

To recognize and fight the wide range of pathogens that can infect the organism the lymphocytes of the adaptive immune system have evolved to recognize a great variety of different antigens from bacteria, viruses and other disease-causing organisms. The antigen-recognition molecules are the antibodies, or immunoglobulins (Ig). These proteins are produced by cells of the B lineage in a vast range of specificities, each B cell and its descendents producing Ig of a single specificity. Plasma cell mature from B lymphocytes which are small, resting cells patrolling the body for foreign agents. They recognize the antigen via the B cell receptor (BCR), which is a membrane bound form of the antibody that the B cell will secrete after activation and differentiation to plasma cell.

Hence, plasma cells are crucial for an effective immune response. Yet they can cause severe pathology in autoimmunity and multiple myeloma. So, activation of mature B cells, secretion of antibody and survival of plasma cells need to be tightly controlled.

1.1.1 The antibody

Antibodies have two separate functions: one is to bind specifically to molecules from the pathogen, the other is to recruit other cells and molecules to destroy the pathogen once it is bound to it. These functions are structurally separated in the antibody molecule, one part of which specifically recognizes and binds antigen whereas the other engages different effector mechanisms. The antigen-binding region varies extensively among Ig and thus is known as the variable region (V region). The region of the antibody that engages the effector function of the immune system does not vary in the same way and is thus defined constant region (C region). Many forms of it exist (isotypes), which are specialized for activating different effector

mechanisms and define the Ig classes. The membrane bound BCR does not have effector functions as the C region remains inserted in the plasma membrane of the B cell. Its function is to recognize and bind the antigens by the V regions exposed on the cell surface, thus transmitting the signals that eventually lead to B cell activation and plasma cell differentiation. An antibody is identical to the BCR of the cell that secretes it except for a small portion of the C-terminus of the heavy chain C region that in the BCR is a hydrophobic membrane-anchoring sequence and in the antibody is a hydrophilic sequence that allows secretion. The membrane and secreted forms are generated by alternative processing of the heavy chain transcripts.

The core structure of the antibody molecule is composed by two different kinds of polypeptide chains, one of higher and one of lower molecular weight, thus termed heavy (HC) and light chain (LC) respectively. Each HC is tightly bound to a LC (generally by disulphide bonds) in a structure that in the immunological jargon is defined "hemimer" (HL). Two hemimers are bound via disulphide bonds between the HC forming the immunoglobulin "monomer" (H_2L_2).

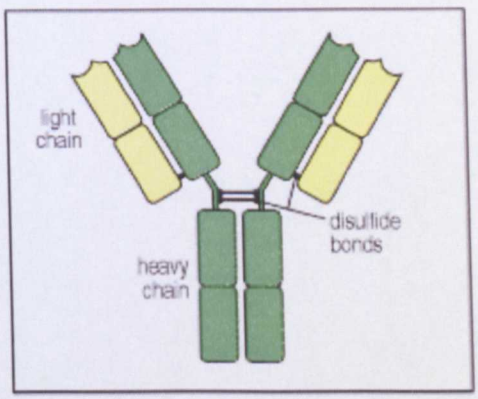

Fig. I 1 Antibodies are made up of four protein chains.

There are two types of chain in an antibody molecule: a larger chain called the heavy chain (green), and a smaller one called the light chain (yellow). Each chain has both a variable and a constant region, and there are two identical light chains and two identical heavy chains in each antibody molecule.

There are two different types of LC named lambda (λ) and kappa (κ) but, owing to the phenomenon of allelic exclusion, a single antibody molecule possesses either λ or κ

chains. No functional differences have been described between antibodies constituted by λ or κ chains, and either type of LC may be found in the different immunoglobulin classes. The ratio of the two types of LC varies from species to species. In the mouse, the average κ to λ ratio is 20:1 whereas in humans it is 2:1. By contrast, the class, and thus the effector function, of an antibody, is defined by the structure of its heavy chain. There are five main heavy-chain classes or isotypes, some of which have several subtypes, and these determine the functional properties of an antibody molecule. The five major classes are immunoglobulin M (IgM), D (IgD), G (IgG), A (IgA), and E (IgE). Their distinctive properties are conferred by the carboxy-terminal part of the heavy chain, where it is not associated with the light chain.

The amino acid sequences of many immunoglobulin heavy and light chains have been determined and reveal two important features of antibody molecules. First, each chain consists of a series of similar, although not identical, sequences, each about 110 amino acids long. Each of these repeats corresponds to a discrete domain. The light chain is made up of two such immunoglobulin domains, whereas the heavy chain of the IgG antibody contains four. This suggests that the immunoglobulin chains have evolved by repeated duplication of an ancestral gene corresponding to a single domain.

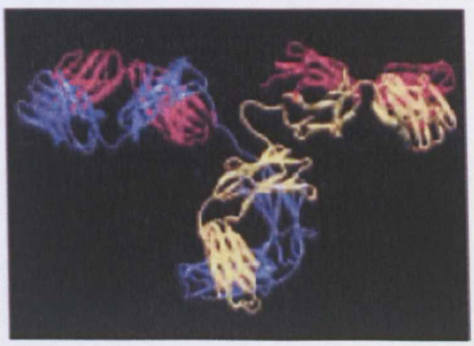

Fig. I 2 Structure of an antibody molecule.

The figure illustrates a ribbon diagram based on the X-ray crystallographic structure of an IgG antibody, showing the course of the backbones of the polypeptide chains. Three globular regions form a Y. The two antigen-binding sites are at the tips of the arms, which are tethered to the trunk of the Y by a flexible hinge region.

The second important feature revealed by comparisons of amino acid sequences is that the amino-terminal sequences of both the heavy and light chains vary greatly between different antibodies. The variability in sequence is limited to approximately the first 110 amino acids, corresponding to the first domain, whereas the remaining domains are constant between immunoglobulin chains of the same isotype. The amino-terminal variable or V domains of the heavy and light chains (VH and VL, respectively) together make up the V region of the antibody and confer on it the ability to bind specific antigen, while the constant domains CH and CL respectively, make up the C region. The multiple heavy-chain C domains are numbered from the amino-terminal end to the carboxy terminus, CH1, CH2, and so on.

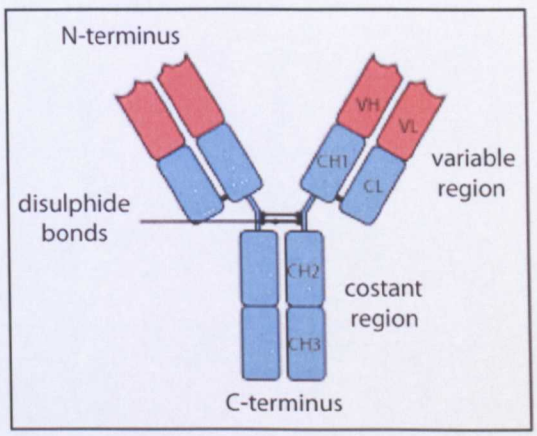

Fig. I 3 Schematic representation of the antibody structure.

The variable and constant domains of the light and heavy chains are labeled.

The V regions of any given antibody molecule differ from those of every other. Sequence variability is not, however, distributed evenly throughout the V regions but is concentrated in certain segments of the V region. Three segments of particular variability can be identified in both the VH and VL domains. They are designated hypervariable regions and are denoted HV1, HV2, and HV3. The most variable part of the domain is in the HV3 region. The regions between the hypervariable regions, which comprise the rest of the V domain, show less variability and are termed the framework regions. There are four such regions in each V domain, designated FR1,

FR2, FR3, and FR4. Not only is sequence diversity concentrated in particular parts of the V domain but it is localized to a particular region on the surface of the molecule. When the VH and VL domains are paired in the antibody molecule, the hypervariable loops from each domain are brought together, creating a single hypervariable site at the tip of each arm of the molecule. This is the binding site for antigen. The three hypervariable loops determine antigen specificity by forming a surface complementary to the antigen, and are more commonly termed the complementarity-determining regions, or CDRs (CDR1, CDR2, and CDR3). Because CDRs from both VH and VL domains contribute to the antigen-binding site, it is the combination of the H and the L chain, and not either alone, that determines the final antigen specificity. Thus, one way in which the immune system is able to generate antibodies of different specificities is by generating different combinations of H and L chain V regions. This means of producing variability is known as combinatorial diversity.

Virtually any substance can elicit an antibody response. Furthermore, the response even to a simple antigen bearing a single antigenic determinant is diverse, comprising many different antibody molecules each with a unique affinity, or binding strength, for the antigen and a subtly different specificity. The total number of antibody specificities available to an individual is known as the antibody repertoire, or immunoglobulin repertoire, and in humans is at least 10^{11}, perhaps more. The number of antibody specificities present at any one time is, however, limited by the total number of B cells in an individual, as well as by each individual's encounters with antigens. The antibody repertoire is generated by DNA rearrangements during B-cell development: a DNA sequence encoding a V region is assembled at each locus by selection from a relatively small group of inherited gene segments.

The V domain of an immunoglobulin H or L chain is encoded by more than one gene segment. For the light chain, the V domain is encoded by two separate DNA segments. The first segment encodes the first 95–101 amino acids of the light chain and is termed a V gene segment because it encodes most of the V domain. The second segment encodes the remainder of the V domain (up to 13 amino acids) and is termed a joining or J gene segment. The joining of a V and a J gene segment creates a continuous exon that encodes the whole of the light-chain V region. In the

un-rearranged DNA, the V gene segments are located relatively far away from the C region. The J gene segments are located close to the C region, however, and joining of a V segment to a J gene segment also brings the V gene close to a C-region sequence. The J gene segment of the rearranged V region is separated from a C-region sequence only by an intron. To make a complete Ig L messenger RNA, the V-region exon is joined to the C-region sequence by RNA splicing after transcription.

A heavy-chain V region is encoded in three gene segments. In addition to the V and J gene segments (denoted V_H and J_H to distinguish them from the light-chain V_L and J_L), there is a third gene segment called the diversity or D_H gene segment, which lies between the V_H and J_H gene segments. The process of recombination that generates a complete heavy-chain V region occurs in two separate stages. In the first, a D_H gene segment is joined to a J_H gene segment; then a V_H gene segment rearranges to DJ_H to make a complete V_H-region exon. As with the light-chain genes, RNA splicing joins the assembled V-region sequence to the neighboring C-region gene.

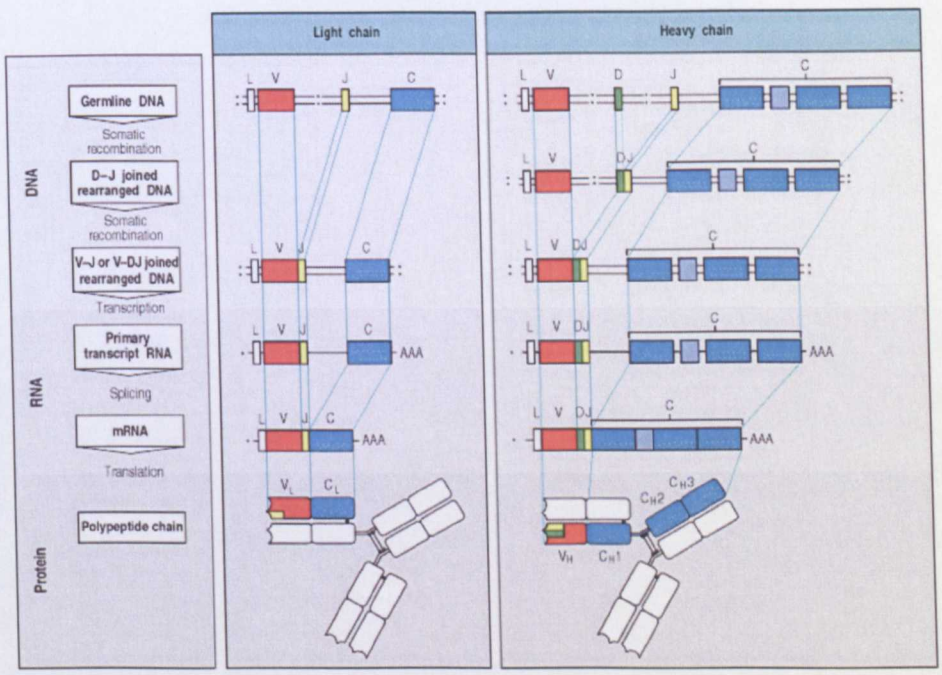

Fig. I 4 V-region genes are constructed from gene segments.

Light-chain V-region genes are constructed from two segments (center panel). A variable (V) and a joining (J) gene segment in the genomic DNA are joined to form a complete light-chain V-region exon. Immunoglobulin chains are extra-cellular proteins and the V gene segment is

preceded by an exon encoding a leader peptide (L), which directs the protein into the cell's secretory pathways and is then cleaved. The light-chain C region is encoded in a separate exon and is joined to the V-region exon by splicing of the light-chain RNA to remove the L-to-V and the J-to-C introns. Heavy-chain V regions are constructed from three gene segments (right panel). First, the diversity (D) and J gene segments join, then the V gene segment joins to the combined DJ sequence, forming a complete VH exon. A heavy-chain C-region gene is encoded by several exons. The C-region exons, together with the leader sequence, are spliced to the V-domain sequence during processing of the heavy-chain RNA transcript. The leader sequence is removed after translation and the disulfide bonds that link the polypeptide chains are formed. The hinge region is shown in purple.

There are multiple copies of all of the gene segments in germline DNA. It is the random selection of just one gene segment of each type to assemble a V region that makes possible the great diversity of V regions among immunoglobulins.

The immunoglobulin gene segments are organized into three clusters or genetic loci, the κ, λ, and heavy-chain loci. These are on different chromosomes and each is organized slightly differently for humans. At the λ IgL locus, located on chromosome 22, a cluster of V_λ gene segments is followed by four sets of J_λ gene segments each linked to a single C_λ gene. In the κ IgL locus, on chromosome 2, the cluster of V_κ gene segments is followed by a cluster of J_κ gene segments, and then by a single C_κ gene. The organization of the IgH locus, on chromosome 14, resembles that of the κ locus, with separate clusters of V_H, D_H, and J_H gene segments and of C_H genes. The IgH locus differs in one important way: instead of a single C-region, it contains a series of C regions arrayed one after the other, each of which corresponds to a different isotype. Generally, a cell expresses only one at a time, beginning with IgM. The expression of other isotypes, such as IgG, can occur through isotype switching, a process that will be discussed shortly below.

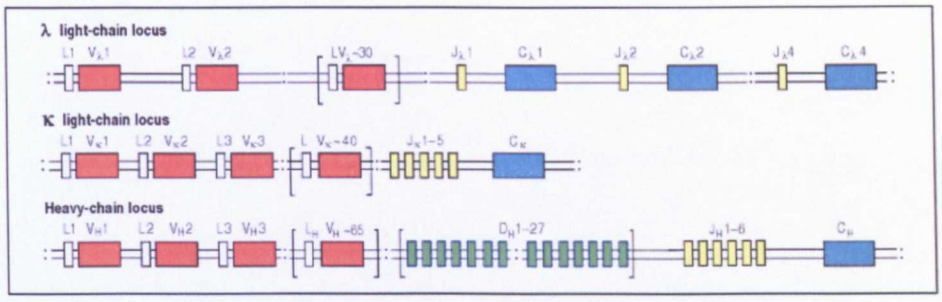

Fig. I 5 The germline organization of the immunoglobulin heavy- and light-chain loci in the human genome.

The genetic locus for the λ light chain (chromosome 22) has about 30 functional Vλ gene segments and four pairs of functional Jλ gene segments and Cλ genes. The κ locus (chromosome 2) is organized in a similar way, with about 40 functional Vκ gene segments accompanied by a cluster of five Jκ gene segments but with a single Cκ gene. In approximately 50% of individuals, the entire cluster of κ V gene segments has undergone an increase by duplication (not shown for simplicity). The heavy-chain locus (chromosome 14) has about 65 functional VH gene segments and a cluster of around 27 D segments lying between these VH gene segments and six JH gene segments. The heavy-chain locus also contains a large cluster of CH genes. For simplicity it is shown only a single CH gene in this diagram without illustrating its separate exons, pseudogenes have been omitted, and all V gene segments have been shown in the same orientation. L, leader sequence. This diagram is not to scale: the total length of the heavy-chain locus is over 2 megabases (2 million bases), whereas some of the D segments are only six bases long.

Although each B lymphocyte has multiple allelic loci for the different antibody chains, clonally derived mature B lymphocyte expresses a single species of antibody with a unique specificity via a process termed **allelic exclusion** (Fig.6). Despite some progress, the precise mechanism of allelic exclusion remains an enigma. In B cell development, IgH gene assembly generally precedes that of IgL genes. For IgH genes, D to J_H rearrangements occur on both allelic chromosomes before appendage of a V_H to DJ_H. In addition, rearrangement of Igλ occurs mostly in cells that have already rearranged or deleted both Igκ loci (isotype exclusion). The regulated model of allelic exclusion propose that IgL chain V gene assembly must proceed one chromosome at a time and that protein products from a functional IgL rearrangement mediates allelic exclusion through feedback inhibition of further IgL rearrangement (the same model applies to IgH).

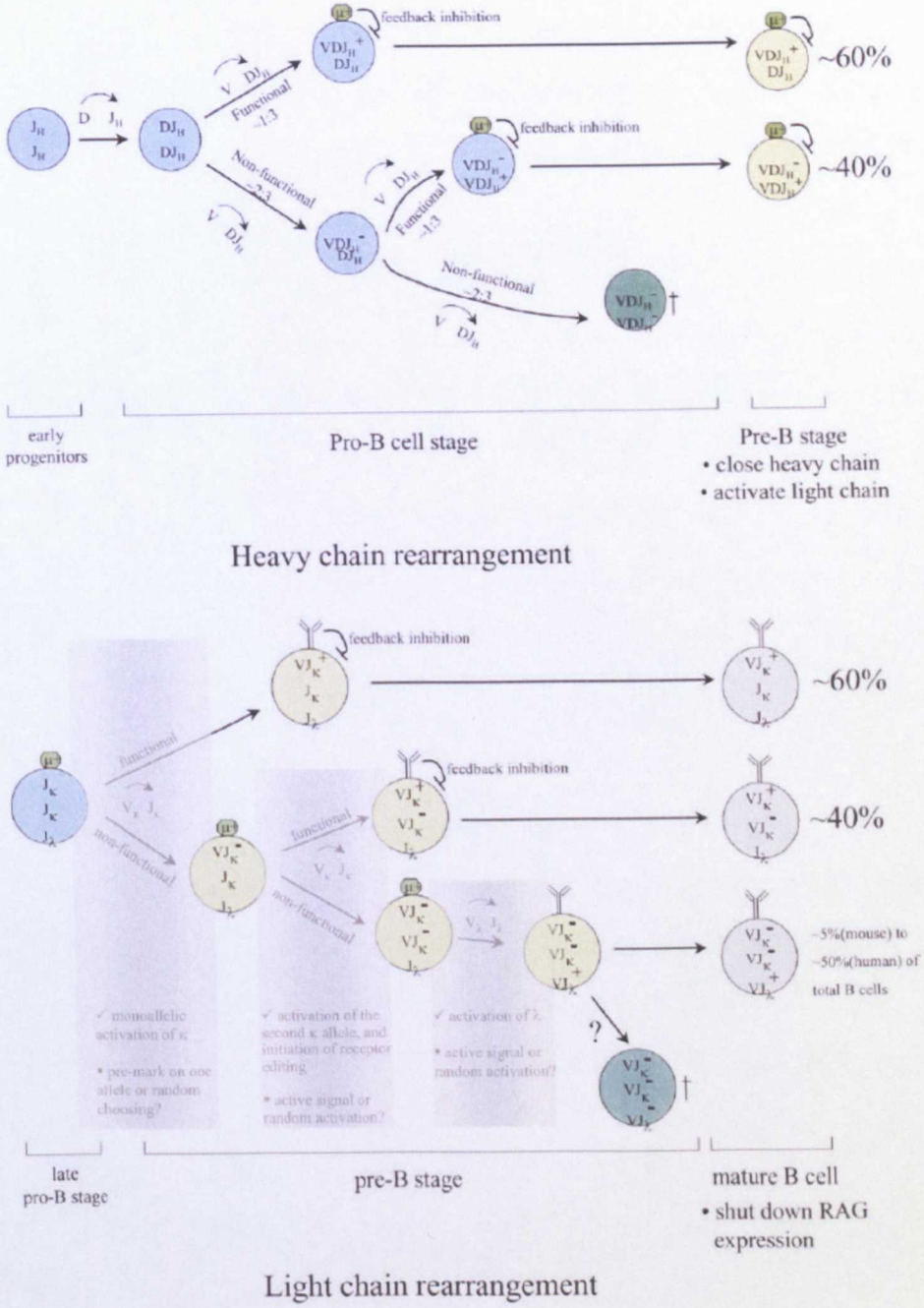

Fig. I 6 Allelic exclusion: heavy and light chain rearrangements.

See text for details

from (Mostoslavsky et al., 2004)

Two further processes add greatly to repertoire diversity, **imprecise joining** of V, D, and J gene segments and **somatic hypermutation**. Of the three hypervariable loops in the protein chains of immunoglobulins, two are encoded within the V gene

segment DNA. The third (HV3 or CDR3) falls at the joint between the V gene segment and the J gene segment, and in the heavy chain is partially encoded by the D gene segment. In both heavy and light chains, the diversity of CDR3 is significantly increased by the addition and deletion of nucleotides in the formation of the junctions between gene segments.

The mechanisms for generating diversity described so far all take place during the rearrangement of gene segments in the initial development of B cells in the central lymphoid organs. There is an additional mechanism that generates diversity throughout the V region and that operates on B cells in peripheral lymphoid organs after functional immunoglobulin genes have been assembled. This process, known as **somatic hypermutation**, introduces point mutations into the V regions of the rearranged heavy- and light-chain genes at a very high rate, giving rise to mutant B-cell receptors on the surface of the B cells. Some of the mutant immunoglobulin molecules bind antigen better than the original B-cell receptors, and B cells expressing them are preferentially selected to mature into antibody secreting cells. This gives rise to a phenomenon called affinity maturation of the antibody population. Somatic hypermutation occurs when B cells respond to antigen along with signals from activated T cells. The immunoglobulin C-region gene, and other genes expressed in the B cell, are not affected, whereas the rearranged V_H and V_L genes are mutated.

Where not indicated references and figures are from the textbook "Immunobiology" 5th edition, Janeway CA et al., Garland Sience Publishing.

1.1.2 The immunoglobulin isotypes

The five main isotypes of immunoglobulin are IgM, IgD, IgG, IgE, and IgA. Their heavy chains are denoted by the corresponding lower-case Greek letter (μ, δ, γ, ε, and α respectively). In humans, IgG antibodies can be further subdivided into four subclasses (IgG_1, IgG_2, IgG_3, and IgG_4), whereas IgA antibodies are found as two subclasses (IgA_1 and IgA_2). IgG is by far the most abundant immunoglobulin in the serum. IgM is present in the extracellular fluids as pentamers and hexamers, which

accounts for its high molecular weight. Secreted IgA can occur as either a monomer or as a dimer. Different isotypes and subtypes also differ in their ability to engage various effector functions.

	Immunoglobulin								
	IgG1	IgG2	IgG3	IgG4	IgM	IgA1	IgA2	IgD	IgE
Heavy chain	γ_1	γ_2	γ_3	γ_4	μ	α_1	α_2	δ	ε
Molecular weight (kDa)	146	146	165	146	970	160	160	184	188
Serum level (mean adult mg ml^{-1})	9	3	1	0.5	1.5	3.0	0.5	0.03	5×10^{-5}
Half-life in serum (days)	21	20	7	21	10	6	6	3	2
Classical pathway of complement activation	++	+	+++	−	+++	−	−	−	−
Alternative pathway of complement activation	−	−	−	−	−	+	−	−	−
Placental transfer	+++	+	++	−/+	−	−	−	−	−
Binding to macrophages and other phagocytes	+	−	+	−/+	−	+	+	−	+
High-affinity binding to mast cells and basophils	−	−	−	−	−	−	−	−	+++
Reactivity with staphylococcal Protein A	+	+	−/+	+	−	−	−	−	−

Fig. I 7 The properties of the human immunoglobulin isotypes.

Sequence differences between immunoglobulin heavy chains cause the various isotypes to differ in several characteristic respects. These include the number and location of interchain disulfide bonds, the number of attached oligosaccharide moieties, the number of C domains, and the length of the hinge region. IgM and IgE heavy chains contain an extra C domain that replaces the hinge region found in γ, δ, and α chains.

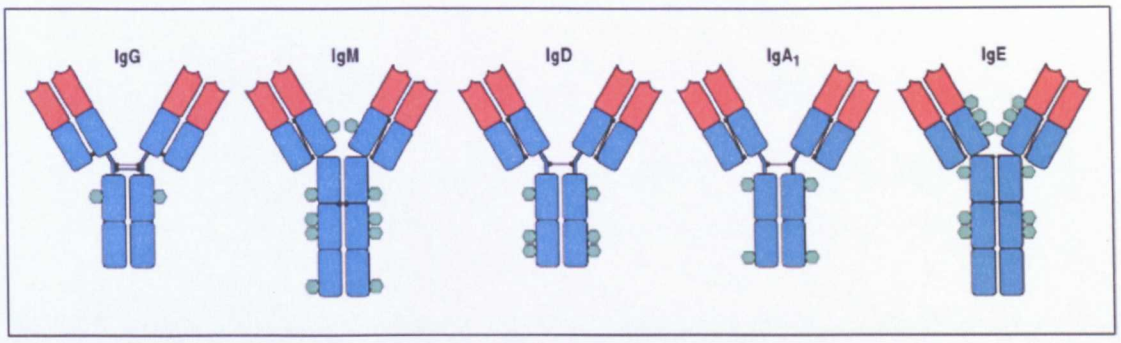

Fig. I 8 The structural organization of the main human immunoglobulin isotype monomers.

Both IgM and IgE lack a hinge region but each contains an extra heavy-chain domain. Note the differences in the numbers and locations of the disulfide bonds (black lines) linking the chains. The isotypes also differ in the distribution of N-linked carbohydrate groups, shown as turquoise hexagons.

The V-region exons expressed by any given B cell are determined during its early differentiation in the bone marrow. They may subsequently be modified by somatic hypermutation, and possibly by secondary V(D)J recombination outside the bone marrow (**receptor editing**). All the progeny of that B cell will therefore express the same assembled V genes. By contrast, several different C-region genes can be expressed in the B cell's progeny as the cells mature and proliferate in the course of an immune response. Every B cell begins by expressing IgM as its B-cell receptor, and the first antibody produced in an immune response is always IgM. Later in the immune response, however, the same assembled V region may be expressed in IgG, IgA, or IgE antibodies. This change is known as isotype switching. It is stimulated in the course of an immune response by external signals such as cytokines released by T cells or mitogenic signals delivered by pathogens.

The immunoglobulin C_H genes form a large cluster spanning about 200 kb to the 3' side of the J_H gene segments. Each CH gene is split into several exons, each corresponding to an individual immunoglobulin domain in the folded C region.

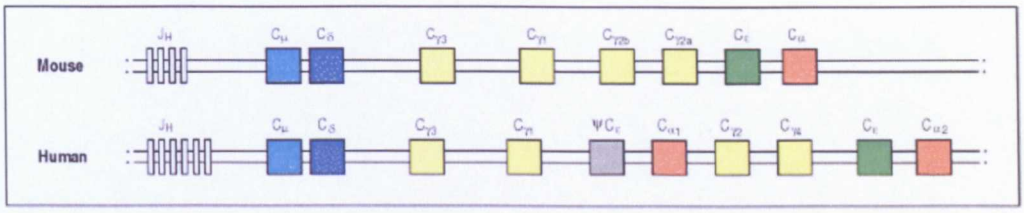

Fig. I 9 The organization of the immunoglobulin heavy-chain C-region genes in mice and humans.

(not to scale) In humans, the cluster shows evidence of evolutionary duplication of a unit consisting of two γ genes, an ε gene and an α gene. One of the ε genes has become inactivated and is now a pseudogene (ψ); hence only one subtype of IgE is expressed. For simplicity, other pseudogenes are not illustrated, and the exon details within each C gene are not shown. The classes of immunoglobulins found in mice are called IgM, IgD, IgG1, IgG2a, IgG2b, IgG3, IgA, and IgE.

The gene encoding the μ C region lies closest to the J_H gene segments, and therefore closest to the assembled V-region exon after DNA rearrangement. A complete μ heavy-chain transcript is produced from the newly rearranged gene. Any J_H gene segments remaining between the assembled V gene and the Cμ gene are removed during RNA processing to generate the mature mRNA. μ heavy chains are therefore the first to be expressed and IgM is the first immunoglobulin isotype to be expressed during B-cell development. Immediately 3' to the μ gene lies the δ gene, which encodes the C region of the IgD heavy chain. IgD is coexpressed with IgM on the surface of almost all mature B cells, although this isotype is secreted in only small amounts and its function is unknown. B cells expressing IgM and IgD have not undergone isotype switching, which entails an irreversible change in the DNA. Instead, these cells produce a long primary transcript that is differentially cleaved and spliced to yield one of two distinct mRNA molecules. In one of these, the VDJ exon is linked to the Cμ exons to encode a μ heavy chain, and in the other the VDJ exon is linked to the Cδ exons to encode a δ heavy chain. The differential processing of the long mRNA transcript is developmentally regulated, with immature B cells making mostly the μ transcript and mature B cells making mostly the δ form along with some of the μ transcript.

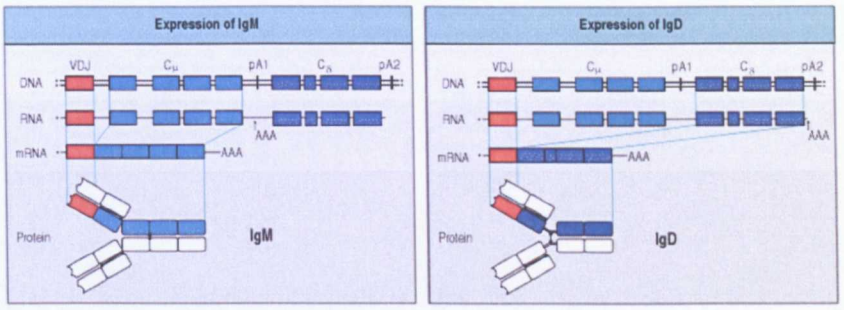

Fig. I 10 Co-expression of IgD and IgM is regulated by RNA processing.

In mature B cells, transcription initiated at the VH promoter extends through both Cμ and Cδ exons. This long primary transcript is then processed by cleavage and polyadenylation (AAA), and by splicing. Cleavage and polyadenylation at the μ site (pA1) and splicing between Cμ exons yields an mRNA encoding the μ heavy chain (left panel). Cleavage and polyadenylation at the δ site (pA2) and a different pattern of splicing that removes the Cμ exons yields mRNA encoding the δ heavy chain (right panel). For simplicity I have not shown all the individual C-region exons.

Switching to other isotypes occurs primarly after B cells have been stimulated by antigen. It occurs through a specialized non-homologous DNA recombination mechanism guided by stretches of repetitive DNA known as switch regions. Switch regions lie in the intron between the J_H gene segments and the Cμ gene, and at equivalent sites upstream of the genes for each of the other heavy-chain isotypes, with the exception of the δ gene. This recombination event is mediated by the enzyme activation-induced cytidine deaminase (AID) and involves germline transcription, DNA double strand breaks at switch regions and DNA repair. At the end of this process the Ig heavy chain constant region is rearranged and the intervening DNA is deleted.

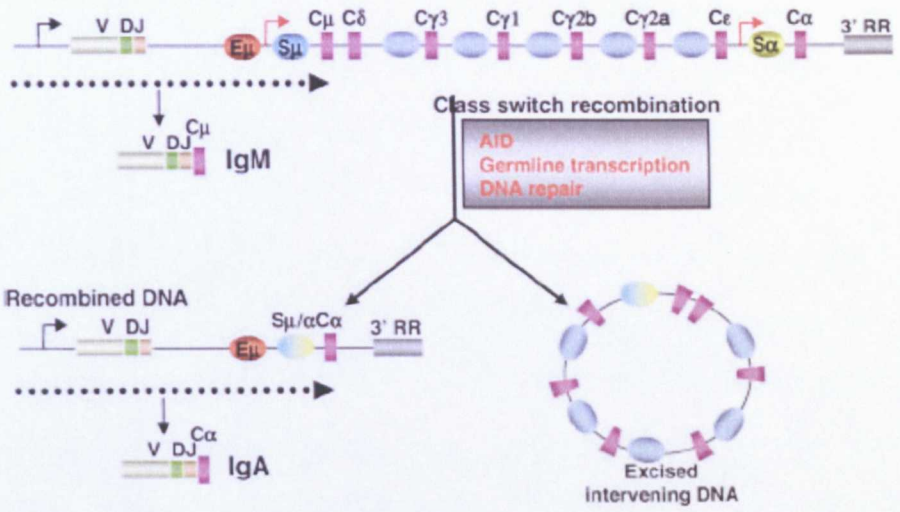

Fig. I 11 Class switch recombination.

Once the V(D)J exon is assembled a μ heavy chain is synthesized. Transcription across this region is mediated by a promoter located upstream of the V(D)J exon. Upon activation different effector heavy chains are produced by class switch recombination (CSR) to a down stream isotypes (CSR to IgA is shown). This recombination event is mediated by the enzyme activation-induced cytidine deaminase (AID) and involves germline transcription; DNA double strand breaks at switch regions and DNA repair. At the end of this process the Ig heavy chain constant region is rearranged and the intervening DNA is deleted.

from (Edry and Melamed, 2007)

Where not indicated references and figures are from the textbook "Immunobiology" 5th edition, Janeway CA et al., Garland Sience Publishing.

1.1.3 The distribution and functions of immunoglobulin isotypes

Pathogens most commonly enter the body across the epithelial barriers of the mucosa lining the respiratory, digestive, and urogenital tracts, or through damaged skin, and can then establish infections in the tissues. The body's mucosal surfaces, tissues, and blood are all protected by antibodies from such infections; these antibodies serve to neutralize the pathogen or promote its elimination before it can establish a significant infection. Antibodies of different isotypes are adapted to function in different compartments of the body. Because a given V region can become

associated with any C region through isotype switching, the progeny of a single B cell can produce antibodies, all specific for the same eliciting antigen, that provide all of the protective functions appropriate for each body compartment.

The first antibodies to be produced in a humoral immune response are always IgM, because IgM can be expressed without isotype switching. These early IgM antibodies are produced before B cells have undergone somatic hypermutation and therefore tend to be of low affinity. IgM molecules, however, form pentamers whose 10 antigen-binding sites can bind simultaneously to multivalent antigens such as bacterial capsular polysaccharides. This compensates for the relatively low affinity of the IgM monomers by multipoint binding that confers high overall avidity. As a result of the large size of the polymers, IgM is mainly found in the blood and, to a lesser extent, the lymph. The pentameric structure of IgM makes it especially effective in activating the complement system, as it will be discussed below. Infection of the bloodstream has serious consequences unless it is controlled quickly, and the rapid production of IgM and its efficient activation of the complement system are important in controlling such infections. Some IgM is also produced in secondary and subsequent responses, and after somatic hypermutation, although other isotypes dominate the later phases of the antibody response.

Antibodies of the other isotypes, IgG, IgA, and IgE, are smaller in size and diffuse easily out of the blood into the tissues. While IgA can form dimers and higher order assembly, IgG and IgE are always monomeric. The affinity of the individual antigen-binding sites for their antigen is therefore critical for the effectiveness of these antibodies, and most of the B cells expressing these isotypes have been selected for increased affinity of antigen binding in germinal centers. IgG is the principal isotype in the blood and extracellular fluid, whereas IgA is the principal isotype in secretions, the most important being those of the mucus epithelium of the intestinal and respiratory tracts. Whereas IgG efficiently opsonizes pathogens for engulfment by phagocytes and activates the complement system, IgA is a less potent opsonin and a weak activator of complement. This distinction is not surprising, as IgG operates mainly in the body tissues, where accessory cells and molecules are available, whereas IgA operates mainly on epithelial surfaces where complement and

phagocytes are not normally present, and therefore functions chiefly as a neutralizing antibody. Finally, IgE antibody is present only at very low levels in blood or extracellular fluid, but is bound avidly by receptors on mast cells that are found just beneath the skin and mucosa, and along blood vessels in connective tissue. Antigen binding to IgE triggers mast cells to release powerful chemical mediators that induce reactions, such as coughing, sneezing, and vomiting, that can expel infectious agents, and engage effector functions.

Many bacteria cause disease by secreting protein toxins, which damage or disrupt the function of the host's cells. To have an effect, a toxin must interact specifically with a receptor on the surface of the target cell. Antibodies that bind to the receptor-binding site preventing the toxin from binding to the cell are referred to as neutralizing antibodies. The ability of IgG antibodies to diffuse easily throughout the extracellular fluid and their high affinity make these the principal neutralizing antibodies for toxins found in tissues. IgA antibodies similarly neutralize toxins at the mucosal surfaces of the body. Many antibodies that neutralize viruses do so by directly blocking viral binding to surface receptors. Such antibodies are called virus-neutralizing antibodies and, as with the neutralization of toxins, high-affinity IgA and IgG antibodies are particularly important. Many bacteria have cell-surface molecules that enable them to bind to the surface of host cells. This adherence is critical to the ability of these bacteria to cause disease, whether they subsequently enter the cell, or remain attached to the cell surface as extracellular pathogens. IgA antibodies secreted onto the mucosal surfaces of the intestinal, respiratory, and reproductive tracts are particularly important in fighting infections by preventing the adhesion of bacteria, viruses, or other pathogens to the epithelial cells lining these surfaces. The adhesion of bacteria to cells within tissues can also contribute to pathogenesis, and IgG antibodies can protect from damage much as IgA antibodies protect at mucosal surfaces.

Another way in which antibodies can protect against infection is by activation of the cascade of complement proteins. Complement activation proceeds via a series of proteolytic cleavage reactions, in which inactive components, present in plasma, are cleaved to form proteolytic enzymes that attach covalently to the pathogen surface.

All known pathways of complement activation converge to generate the same set of effector actions: the pathogen surface or immune complex is coated with covalently attached fragments that act as opsonins to promote uptake and removal by phagocytes. At the same time, small peptides with inflammatory and chemotactic activity are released so that phagocytes are recruited to the site. In addition, the terminal complement components can form a membrane-attack complex that damages some bacteria

1.1.4 Polymeric immunoglobulins, IgA and IgM

The joining (J) chain is a small polypeptide, which regulates polymer formation of IgA and IgM. J chain incorporation into polymeric IgA (pIgA, mainly dimers) and pentameric IgM endows these antibodies with several salient features. First, a high valency of antigen-binding sites, which makes them suitable for agglutinating bacteria and viruses and, most importantly, only J chain containing polymers show high affinity for the polymeric Ig receptor (pIgR), also known as transmembrane secretory component (SC). This epithelial glycoprotein mediates active external transfer of pIgA and pentameric IgM to exocrine secretions. Thus, secretory IgA (SIgA) and SIgM, as well as free SC, are generated by endoproteolytic cleavage of the pIgR extracellular domain. The secretory antibodies form the first line of defence against pathogens and noxious substances that favor the mucosa as their portal of entry. The J chain is involved in creating the binding site for pIgR/SC in the Ig polymers, not only by determining the polymeric quaternary structure but apparently also by interacting directly with the receptor protein. Therefore, both the J chain and the pIgR/SC are key proteins in secretory immunity (Johansen et al., 2000). The J chain has been characterized in a range of species including mammals (human, mouse, bovine, rabbit), birds (chicken), reptiles (turtle), amphibians (*Xenopus laevis* and *Rana catesbeiana*), and cartilaginous fish (nurse shark and clearnose skate). A recent study demonstrated that J chain from several different tetrapod species is capable of inducing polymerization of human IgA and that the resulting pIgA binds human

pIgR/SC and amphibian pIgR pIg-binding domain is capable of binding human pIgA revealing a remarkable conservation of protein-protein interaction sites in secretory Ig from different species. These observations evidence the evolutionary significance of this first-line defense system (Braathen et al., 2007).

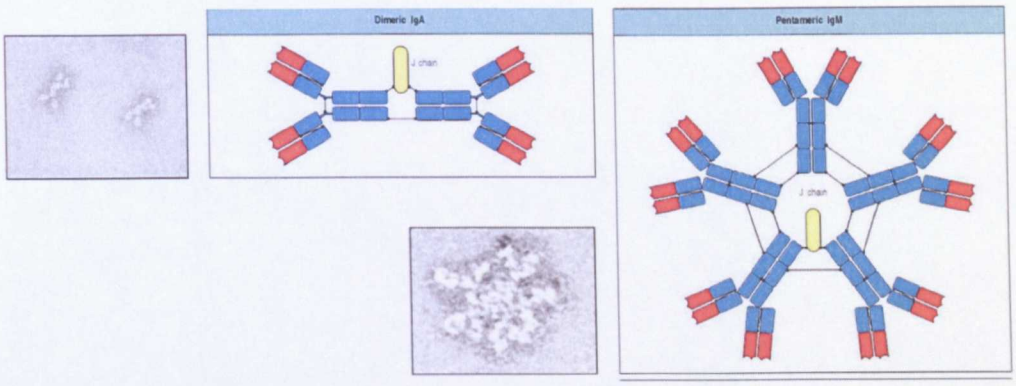

Fig. I 12 The IgM and IgA molecules can form multimers.

IgM and IgA are usually synthesized as multimers in association with an additional polypeptide chain, the J chain. In pentameric IgM, the monomers are cross-linked by disulfide bonds to each other and to the J chain. The bottom left panel shows an electron micrograph of an IgM pentamer, showing the arrangement of the monomers in a flat disc. IgM can also form hexamers that lack a J chain but are more efficient in complement activation. In dimeric IgA, the monomers have disulfide bonds to the J chain as well as to each other. The top left panel shows an electron micrograph of dimeric IgA.

IgA-secreting plasma cells are found predominantly in the connective tissue called the lamina propria, which lies immediately below the basement membrane of many surface epithelia. IgA is the predominant Ig class in the secretions on the mucosal surfaces and the second most prevalent antibody in the serum after IgG. However, since IgA are metabolized around five times faster than IgG the production rates of IgA and IgG must be similar and indeed the daily production of IgA exceeds that of all the other antibody classes combined (Woof and Kerr, 2006). Serum IgA are mainly monomeric while SIgA, as discussed above, are polymeric. SIgA are essential to protect the wide area occupied by the mucosal surface of the respiratory, gastrointestinal and genitourinary apparatuses. Furthermore IgA antibodies are secreted in breast milk and are thereby transferred to the gut of the newborn infant,

where they provide protection from newly encountered bacteria until the infant can synthesize its own protective antibody.

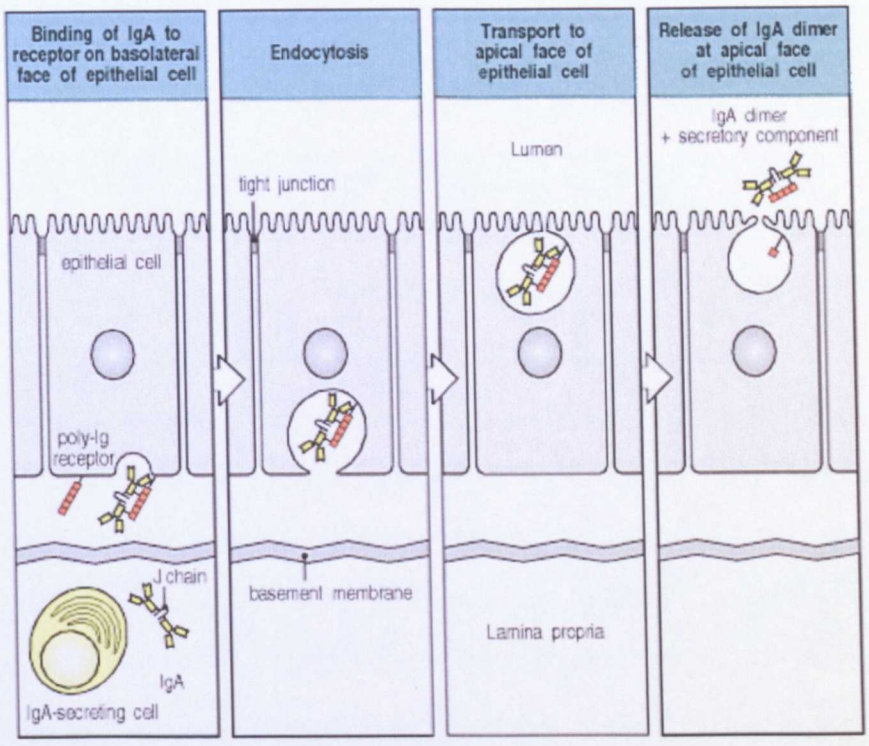

Fig. I 13 Transcytosis of IgA antibody across epithelia is mediated by the poly-Ig receptor, a specialized transport protein.

Most IgA antibody is synthesized in plasma cells lying just beneath epithelial basement membranes of the gut, the respiratory epithelia, the tear and salivary glands, and the lactating mammary gland. The IgA dimer bound to a J chain diffuses across the basement membrane and is bound by the poly-Ig receptor on the basolateral surface of the epithelial cell. The bound complex undergoes transcytosis in which it is transported in a vesicle across the cell to the apical surface, where the poly-Ig receptor is cleaved to leave the extracellular IgA-binding component bound to the IgA molecule as the so-called secretory component. The residual piece of the poly-Ig receptor is nonfunctional and is degraded. In this way, IgA is transported across epithelia into the lumens of several organs that are in contact with the external environment.

IgM are the first antibodies produced in primary immune responses and the predominant isotype secreted in T cell-independent immune responses. IgM are secreted by plasma cells only in the polymeric form, as will be discussed in greater details below. Secreted IgM is predominantly found as pentameric molecules $((\mu_2L_2)_5$-

J), but IgM can also be secreted as hexamers $(\mu_2L_2)_6$ by B cell lines. Murine hexamers activate the complement cascade 15-20 fold more efficiently than pentamers. Although hexameric IgM is not abundant *in vivo*, normal B cells are capable of producing significant amounts of IgM hexamers *in vitro*. IgM pentamers and hexamers exhibit very similar half-lives *in vivo*, suggesting that the predominance of circulating IgM pentamers in normal animals reflects the relative levels of assembly of the two types of polymers by IgM-secreting cells. However, hexameric IgM can be found at significant levels in the serum IgM autoantibodies of a number of patients with an IgM-mediated autoimmune condition (Hughey et al., 1998).

In plasma, the pentameric IgM molecule has a planar conformation that does not bind the initial factor for complement activation (C1q); however, binding to the surface of a pathogen deforms the IgM pentamer so that it looks like a staple, and this distortion exposes binding sites for the C1q heads. Although C1q binds with low affinity to some subclasses of IgG in solution, the binding energy required for C1q activation is achieved only when a single molecule of C1q can bind two or more IgG molecules that are held within 30–40 nm of each other as a result of binding antigen. This requires many molecules of IgG to be bound to a single pathogen. For this reason, IgM is much more efficient in activating complement than is IgG. The binding of C1q to a single bound IgM molecule, or to two or more bound IgG molecules, leads to the activation of an enzymatic activity in C1r, triggering the complement cascade translating antibody binding into the activation of the complement cascade.

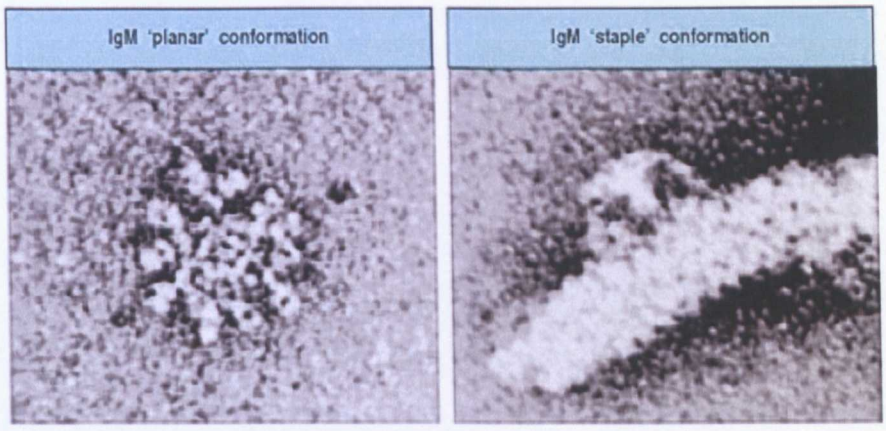

Fig. I 14 The two conformations of IgM.

The left panel shows the planar conformation of soluble IgM; the right panel shows the staple conformation of IgM bound to a bacterial flagellum. Photographs (× 760,000)

Where not indicated references and figures are from the textbook "Immunobiology" 5th edition, Janeway CA et al., Garland Sience Publishing.

1.1.5 The B cell receptor

The antigen-binding portion of the B-cell receptor complex is a cell-surface immunoglobulin that has the same antigen specificity as the secreted antibodies that the B cell will eventually produce. Indeed, it is identical to a secreted monomeric immunoglobulin, except that it is attached to the membrane through the carboxy termini of the paired heavy chains. The mRNA for the cell-surface heavy chain is spliced in such a way that the carboxy terminus of the protein is made up of a transmembrane domain and a very short cytoplasmic tail. One Igα chain and one Igβ chain associates with each surface immunoglobulin molecule. Thus the complete B-cell receptor is a complex of six chains, two identical light chains, two identical heavy chains, one Igα, and one Igβ.

The cytoplasmic tail of Igα and Igβ carries an immunoreceptor tyrosine–based activation motif (ITAM) characterized by a consensus sequence that includes two tyrosines that become phosphorylated upon BCR activation. Three PTKs (Lyn, Syk

and Btk) and one PTP (SHP-1) are involved in signal transduction from the BCR. The generation of a doubly phosphorylated ITAM allows the two amino-terminal SH2 domains of Syk to bind to the BCR. This activates the kinase to phosphorylate neighboring ITAM sequences, which results in more Syk recruitment and activation and thus in the amplification of the BCR signal. The cytosolic phosphatase SHP-1 is one of the PTPs counteracting Syk activity in B cells. In the past few years hydrogen peroxide has been identified as a second messenger molecule for BCR activation (Reth, 2002). In the presence of active SHP-1, the ITAM tyrosines are more rapidly dephosphorylated than they are phosphorylated by Syk. Therefore, no amplification of Syk activity can occur when the BCR is in a resting state. Upon exposure of B cells to H_2O_2 or upon stimulation of the H_2O_2-generating enzymes in B cells, the PTPs are inhibited and lose their negative regulatory power on the BCR. This results in rapid Syk activation and increased tyrosine phosphorylation.

An important aspect of ligand-dependent BCR activation may be rapid translocation of the receptor to a source of H_2O_2 production (like the NADPH oxidases, NOX) or, *viceversa*, translocation of the source to the receptor. Indeed in the reducing environment of the cytosol, H_2O_2 has only a short half-life and can act only close to its site of production.

BCR expression is indispensable for B cell survival even in the absence of antigen, implying that basal signals are generated and deciphered (Kraus et al., 2004; Meffre and Nussenzweig, 2002). Oxidants and Ca^{++} act in a cooperative manner to regulate the strength and duration of BCR signalling (Singh et al., 2005), crucial parameters for determining the subtype of B cell generated from transitional, immature B cells (Casola, 2007). It is still unclear what the source of the basal BCR signalling is and how the strength of the signals could be achieved and interpreted.

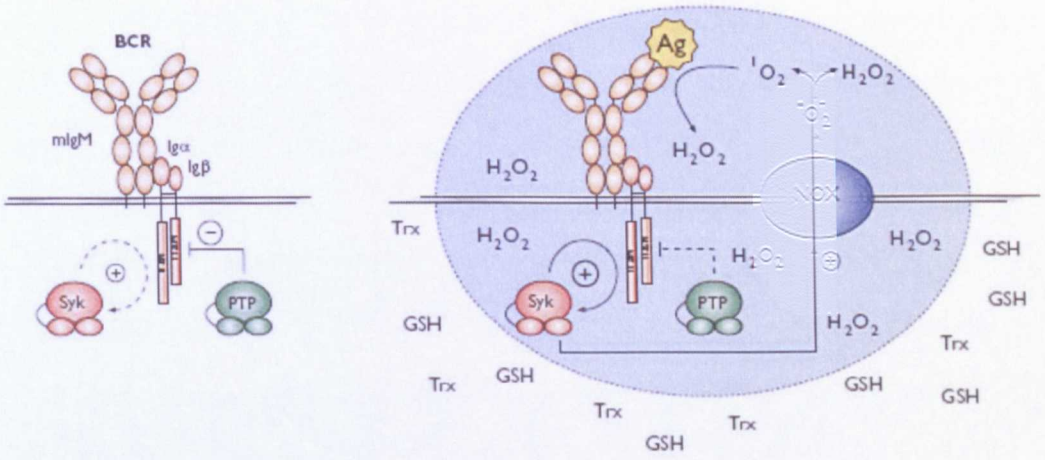

Fig. I 15 Model of the redox regulation of BCR signaling.

(Left panel) In the resting state of the BCR (a complex between membrane IgM (mIgM) and the Igαβ heterodimer), the signal-transducing kinase Syk cannot become activated at the ITAM, as any ITAM phosphorylation is prevented by dominant PTP activity. (Right panel) Upon antigen (Ag) binding, the BCR is localized closed to a ROS-producing NADPH oxidase. The increased H_2O_2 production generates around the BCR an oxidizing environment or domain (dashed circle) that inhibits PTP, thus allowing Syk to become active. Signals through Syk and Lyn can further activate the NADPH oxidase, resulting in increased H_2O_2production and spreading of the signal. During the conversion of •O2– into H_2O_2, singlet oxygen (1O2) is produced that is reduced by the catalytic activity of Ig (here, mIgM) into H_2O_2.This process may help increase the oxidizing domain range around the BCR.

from (Reth, 2002)

1.1.6 B lymphocyte maturation

B cells develop from haematopoietic stem cells in the bone marrow. Early development and commitment to the B-cell lineage depend on several transcription factors, including early B-cell factor, PU.1, E2A and paired box protein 5 (PAX5). Successful rearrangement of IgH gene segments in pro-B cells leads to precursor (pre)-B cells, which express μ_m; in the pre-B-cell receptor (pre-BCR), μ_m provides the first crucial check-point for B-cell development, which then allows the clonal expansion and rearrangement of IgL gene segments. The presence of surface IgM is required to pass the second check-point, which then allows negative selection to occur (in which tolerance mechanisms delete, anergize or edit autoreactive clones).

Naive B220^{+}IgM^{+} B cells that survive negative selection then exit the marrow. B cells continue their development in the spleen, where they pass through transitional stages T1 and T2 and are subjected to a further round of negative selection before becoming fully mature. A small proportion of transitional B cells home to the splenic marginal zone and remain there as naive non-circulating marginal zone B cells. However, most B cells in the T2 stage mature into naive long-lived follicular B cells, which continue circulating to the follicles in the spleen, to the lymph nodes and to the bone marrow until they either die or encounter cognate antigen and undergo further maturation (Shapiro-Shelef and Calame, 2005).

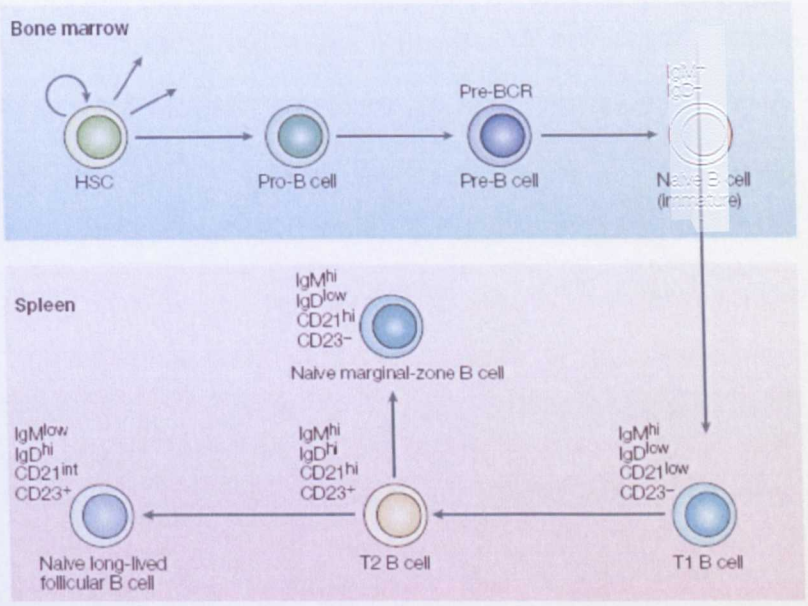

Fig. I 16 Antigen-independent development of B cells.

B cells develop from pluripotent stem cells in the bone marrow, where full commitment to the B-cell lineage requires the transcription factor paired box protein 5 (PAX5). Naive B cells that exit the bone marrow continue to undergo maturation in the spleen to form long-lived naive follicular B cells and, to a lesser extent, naïve marginal-zone B cells. BCR, B-cell receptor; HSC, haematopoietic stem cell; pre-B cell, precursor B cell; T1, transitional stage 1; T2, transitional stage 2.

from (Shapiro-Shelef and Calame, 2005)

1.1.7 B cell activation

After antigen encounter, plasma cells can develop from naive marginal-zone B cells and follicular B cells, from activated germinal-centre B cells and from memory B cells. Which B-cell subset(s) becomes terminally differentiated depends on the nature of the antigen, its dose and form, and the location of the encounter. The earliest antibody response to some pathogens is provided by pre-existing 'natural' antibodies that are secreted by B1 cells, which differs from conventional B cells (B2) in different ways (cell surface phenotype, localization, signal transduction pathways). Their BCR repertoire is skewed towards recognition of T-cell-independent type 2 (TI-2) antigens, i.e. antigens that contain multiple identical epitopes, which crosslink B-cell receptors, like bacterial lypopolysaccaride (LPS). They are responsible for the production of 'natural' IgM, which forms in response to self-antigens but often recognizes bacterial antigens and provides the first line of defense by antibodies against such pathogens. The first B cells to respond to a foreign antigen by differentiating into plasma cells are marginal-zone B cells. Consistent with this, marginal-zone B cells have a repertoire that is skewed towards recognition of TI-2 antigens, although some of these cells recognize T-cell-dependent antigens and therefore present antigen and provide co-stimulation to T cells. The location of naive marginal-zone B cells, which do not circulate, facilitates early encounter with blood-borne antigens. A crucial property of marginal-zone B cells is their inherent ability to respond rapidly to antigen. They have a lower threshold for antigen activation than follicular B cells and when stimulated with LPS, proliferate to a greater extent. Circulating mature follicular B cells that both encounter antigen and receive help from T cells also respond rapidly (albeit more slowly than marginal-zone B cells), undergoing proliferation and plasmacytic differentiation to form extrafollicular foci of plasmablasts and plasma cells. Plasma cells formed by either marginal-zone or follicular B cells in this early extrafollicular response do not have somatically mutated immunoglobulin genes and are short-lived, undergoing apoptosis *in situ*. The mechanisms leading to apoptosis are matter of particular interest in our laboratory and will be discussed in details below. However, these cells provide a

rapid initial response to pathogens. When follicular B cells both encounter antigen and receive T-cell help, a second developmental possibility is the establishment of a germinal centre. Germinal centers are specialized areas in the follicle where B cells undergo rounds of proliferation, which is accompanied by affinity maturation and class switch recombination (CSR) of immunoglobulin. Antigen-specific T helper cells and follicular dendritic cells (FDCs) are important for the germinal-centre response. The germinal-centre response peaks between day 10 and day 14 after immunization and then diminishes. Plasma cells and memory B cells, which mainly have somatically mutated, high-affinity BCRs and express switched immunoglobulin isotypes, exit the germinal centre. Memory B cells have the intrinsic ability to respond more rapidly than naive B cells, and they show a proliferative burst on secondary encounter with antigen (Shapiro-Shelef and Calame, 2005).

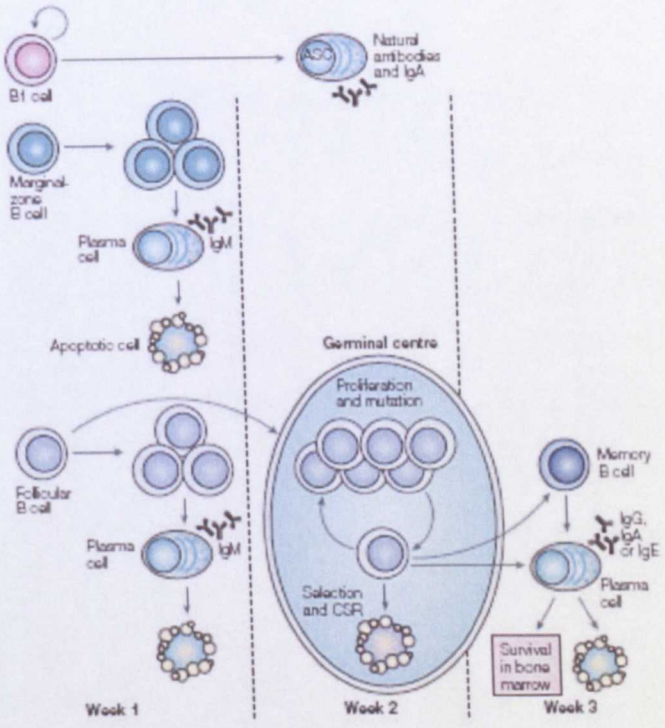

Fig. I 17 Formation of plasma cells.

Antibody-secreting cells (ASCs) formed from B1 cells secrete natural antibody in the absence of external antigen, and they also secrete IgA in the gut, in response to pathogens. On encounter with foreign antigen (indicated by week 1 in figure), naïve marginal-zone B cells differentiate into plasma cells, and subsequently, naive follicular B cells also differentiate into plasma cells. Most of the extrafollicular plasma cells that are formed in this early response are short-lived. Some activated follicular B cells form a germinal centre. Post-germinalcentre

plasma cells might progress through a memory B-cell stage in the primary response or might develop directly from germinal-centre B cells. Plasma cells that result from a germinal-centre reaction might become long-lived if they find survival niches, which are mainly located in the bone marrow. CSR, class-switch recombination.

from (Shapiro-Shelef and Calame, 2005)

Plasma cells mainly disappear from the spleen within 2 weeks of infection, but they are present in the bone marrow for up to 1 year, indicating that the bone marrow is a site of long-term antibody production. Bone-marrow plasma cells persist in the absence of DNA synthesis, and *in vivo* depletion of memory B cells and adoptive transfer of virus-specific plasma cells show that a substantial proportion of plasma cells survive and continue to secrete antibody for more than 1 year in the absence of detectable memory B cells. So, although there is evidence that some long-term immunity is derived from continuous differentiation of memory B cells, at least some persistent antibodies are derived from long-lived plasma cells, independent of memory B cells and antigen. Although there is evidence that some long-lived plasma cells are present in the spleen, it is generally agreed that the bone marrow is the primary site of long-lived plasma cells. The mechanism of survival of long lived plasma cells is based on competition for survival niches between newly generated plasmablasts and older plasma cells. Receptors expressed on the cell surface are important for homing of the plasma cells to the bone marrow. In particular plasmablasts deficient in the CXC-chemokine receptor CXCR4 are greatly impaired in their ability to home to the bone marrow, although there are probably alternative mechanisms of attraction to the bone marrow (Moser et al., 2006; Radbruch et al., 2006).

1.1.8 Plasma cell differentiation

The transcriptional repressor BLIMP1 is sufficient, when ectopically expressed by B cells at the appropriate developmental stage, to drive differentiation into plasma cells. BLIMP1 is expressed by splenic and bone-marrow plasma cells and is required for plasma cell formation and immunoglobulin secretion. BLIMP1 initiates cascades of

gene regulation leading to cessation of the cell cycle (by direct repression of genes such as *myc*), repression of genes that are required for the identity of mature and germinal centre B cells (including genes that regulate signaling through the BCR, T cell–B cell interactions, CSR and somatic hypermutation) and induction of the immunoglobulin secretory program. BLIMP1 represses the expression of two transcription factors that are required for germinal-centre reactions, BCL-6 and PAX5, thereby ensuring that, after plasma cell development is induced, B cells cannot return to an earlier developmental stage. Repression of PAX5 expression is important not only for inhibiting B-cell functions but also for allowing plasma cell development by de-repressing expression of the IgH, the IgL, the J chain and XBP1. BLIMP1 is also responsible for the induction of expression of many genes in the plasma-cell program, such as IRF4 and genes that are involved in immunoglobulin secretion.

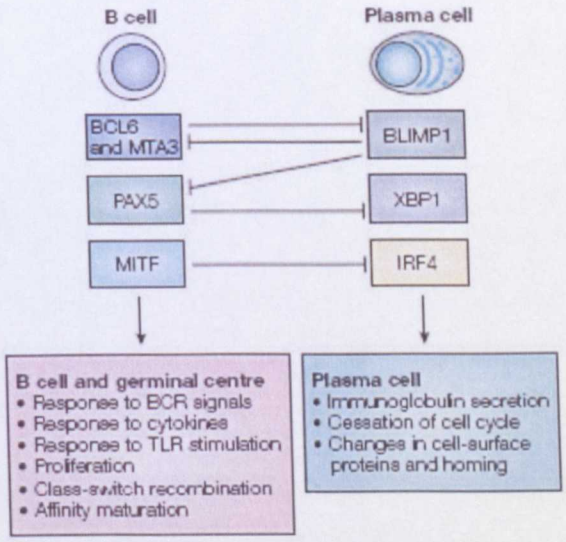

Fig. I 18 Transcriptional repression enforces mutually exclusive B-cell and plasma-cell gene-expression programmes.

Several transcription factors - BCL-6 (B-cell lymphoma 6), MTA3 (metastasis-associated 1 family, member 3), MITF (microphthalmia-associated transcription factor) and PAX5 (paired box protein 5) - repress plasmacytic development by repressing BLIMP1 (B-lymphocyte-induced maturation protein 1), XBP1 (X-box-binding protein 1) and IRF4 (interferon-regulatory factor 4). In plasma cells, BLIMP1 represses B-cell gene-expression programmes. This mutual repression prevents the unelicited formation of plasma cells in the germinal centre and

prevents the reversion of plasma cells to a B-cell stage. BCR, B-cell receptor; TLR, Toll-like receptor. BCL-6, MTA3, PAX5 and MITF also regulate the expression of genes that are required for B-cell and germinal-centre functions, which are outlined in the pink box. BLIMP1, XBP1 and IRF4 induce the expression of genes that are required for plasma cells, which are outlined in the blue box.

from (Shapiro-Shelef and Calame, 2005)

XBP1 was the first transcription factor that was shown to be uniquely required for plasmacytic differentiation. XBP1 acts downstream of BLIMP1 and seems to be the proximal regulator of the secretory phenotype in plasma cells, topic that will be discussed in much greater detail below. IRF4 is a transcriptional activator that seems to function early in plasma cell development because, in addition to being required for plasma-cell formation, it is required for the initial proliferative burst of activated B cells, which does not require BLIMP1 (Shapiro-Shelef and Calame, 2005). Recent findings demonstrated that the initial steps of plasma cell differentiation is independent of BLIMP-1, and requires BLIMP -1-independent down regulation of PAX5. This initial step of plasma-cell differentiation is represented by a newly identified pre-plasmablast phenotype that is characterized by low immunoglobulin secretion (Kallies et al., 2007; Klein and Dalla-Favera, 2007).

In addition to relief from repression, plasma cell factors such as BLIMP1 might require activating signals for induction of their expression. Antigen-mediated signaling through the BCR can result in B cell proliferation, death or differentiation, and the strength of signal and the presence of varying supplementary signals affect the outcome. Signals from the BCR lead to degradation of BCL-6, but there is no evidence of induction of BLIMP1 expression. In fact, IgM-specific antibodies inhibit the induction of BLIMP1 expression and the differentiation into plasma cells of mouse splenic B cells in the presence of LPS. In the presence of CD40-specific antibodies, IL-4 and IL-5, multivalent BCR signaling drives plasma cell formation much more efficiently than standard immunoglobulin-specific antibodies. Furthermore, the addition of IL-2 and IL-5 can overcome the effects of IgM-specific antibodies to drive induction of BLIMP1 expression. So, to induce BLIMP1 expression, a strong signal from the BCR or help from cytokine producing cells during signaling

through the BCR might be required. The cytokines could be derived from dendritic cells or T cells. In the absence of BCR ligation, the Toll-like receptor 4 (TLR4) ligand LPS is sufficient to induce the expression of BLIMP1 by mouse splenic B cells and their development into plasma cells (Shapiro-Shelef and Calame, 2005).

1.2 The secretory pathway

1.2.1 The ER

The ER is organized into a netlike labyrinth of branching tubules and flattened sacs extending throughout the cytosol. The tubules and sacs are all thought to interconnect, so that the ER membrane forms a continuous sheet enclosing a single internal space. This highly convoluted space is called the ER lumen. The ER membrane separates the ER lumen from the cytosol, and it mediates the selective transfer of molecules between these two compartments. The ER has a central role in lipid and protein biosynthesis. Its membrane is the site of production of all the transmembrane proteins and lipids for most of the cell's organelles, including the ER itself, the Golgi apparatus, lysosomes, endosomes, secretory vesicles, and the plasma membrane. Almost all of the proteins that will be secreted to the cell exterior, plus those destined for the lumen of the ER, Golgi apparatus, or lysosomes—are initially delivered to the ER lumen. Indeed specialized secretory cells like the exocrine pancreatic cells or the plasma cells share the common feature of a highly developed rough ER. The ionic and redox conditions found in the ER and downstream organelles resemble those encountered in the extracellular space (higher [Ca^{++}] and oxidized/reduced glutathione -GSSG/GSH- ratio). Therefore, the ER provides suitable conditions for secretory proteins to attain their three-dimensional structure. It also provides a test bench to ensure that ligands and receptors attain the very conformation that allows the proper signals to be transmitted and deciphered. Indeed, folding and assembly are intimately coupled to a stringent quality control schedule that restricts transport along the secretory route to native molecules.

Whilst the basic principles of folding are the same in all cell compartments, secretory proteins undergo unique covalent modifications, i.e. cleavage of the signal sequences, N-glycosylation, GPI additions and disulfide bond formation. These are catalyzed by a sophisticated network of specialized chaperones and enzymes spatially distributed along the various stations of the exocytic route, providing a most efficient assembly, quality control and transport line. Quality control (QC) mechanisms are active at the ER–Golgi interface to ensure that non-native molecules are retained in, or retrieved to, the ER, and eventually dispatched to the cytosol for proteasomal degradation (Anelli and Sitia, 2008; Ellgaard and Helenius, 2003; Sitia and Braakman, 2003).

1.2.2 Oxidative folding

Disulphide bonds are essential for the folding and assembly of many proteins synthesized in the ER. They increase stability, an important feature for Ig to recognize and neutralize foreign elements in the body fluids. Disulfide bond formation is based on redox reactions in which two cysteines are oxidized by removal of two electrons forming a covalent bond. Although direct oxidation could also take place, in living cells oxidative folding utilizes primarily disulfide interchange reactions in which an oxidized donor molecule (D) transfers the bond to a reduced acceptor (A) via formation of a transient mixed disulfide intermediate:

D-SS + HS-A-SH → HS-D-SS-A-SH → HS-D-SH + A-SS

Cargo proteins enter reduced in the ER and are rapidly oxidized by protein disulphide isomerase (PDI). PDI is then re-oxidized by Ero1 flavoproteins. Electrons are finally transferred to molecular oxygen and possibly to other electron acceptors, particularly in anaerobic conditions.

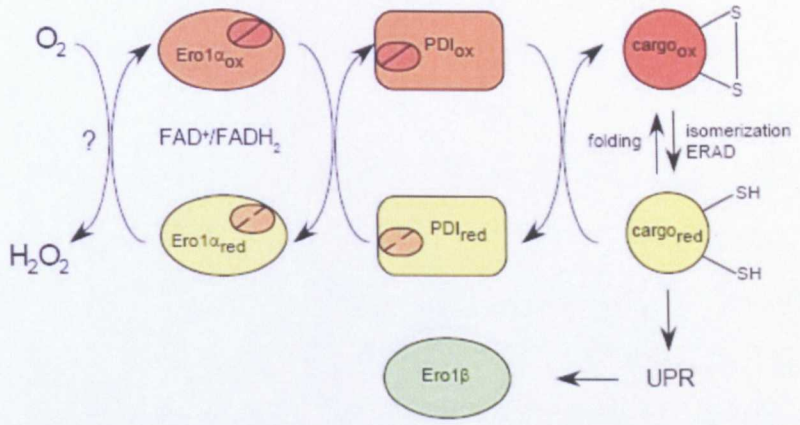

Fig. I 19 Oxidative folding and the generation of ROS.

In eukaryotic cells, oxidative folding of cargo proteins is based on serial disulfide interchange reactions involving PDI and Ero1 molecules. In vitro, yeast Ero1 is oxidised by molecular oxygen generating H_2O_2 in stoichiometric amounts to the disulphide bonds formed. Most mammalian cells express constitutively Ero1α. If cargo is produced in excess, Ero1β is induced via UPR pathways activated by inefficient oxidative folding.

In yeast, Ero1p is an essential gene, whose expression is induced in conditions of ER stress. Also PDI is essential in yeast, despite the presence of numerous other oxidoreductases in the ER. The forced expression of some of these can partially complement the absence of PDI.

This pathway is fundamentally conserved in higher eukaryotes, although some important differences exist. First, while a single Ero1 gene exists in *Saccharomyces cerevisiae*, two isoforms, Ero1α and Ero1β, are found in mammals. Both are able to complement yeast *ero1* mutants, underscoring the conservation of the oxidative folding pathways. Ero1α is induced by hypoxia and PPARγ and inhibited by SIRT1. In contrast, Ero1β increases during the unfolded protein response (UPR, an ER stress response that will be discussed shortly below), and is constitutively expressed at high levels in certain secretory tissues, confirming that ER stress responses are important for the acquisition of the secretory phenotype. The reasons underlying the functional differentiation between Ero1α and Ero1β in mammals are not yet clear.

Another remarkable difference between yeast and mammalian Ero1 molecules concerns the mechanisms of sub-cellular localization. A C-terminal tail is present in

yEro1p that mediates membrane association and is essential for function. Neither Ero1α nor Ero1β possess this tail. Both are secreted by mammalian cells when over-expressed, implying saturable retention mechanism(s). It was demonstrated that the localization of Ero1α and Ero1β depends on dynamic interactions with PDI or ERp44, two soluble proteins that despite the presence of ER localization motifs (KDEL and RDEL, respectively) are differentially distributed in the early secretory pathway. ERp44 accumulates in ERGIC and *cis*-Golgi, distally with respect to PDI. The dynamic retention of Ero1 may allow the oxidoreductin to distribute in distinct subcompartments, and provide the oxidative power needed in the sequential steps of protein folding like, as will be discussed below, in the case of IgM biogenesis.

References and figure from (Masciarelli and Sitia, 2008), Appendix III

1.2.3 *Regulating oxidative power in the ER*

As recalled above, Ero1β is expressed in conditions of ER stress, or in certain tissues specialised in protein secretion. A mechanism can be hence envisaged that adjusts oxidative power in the ER. When Ero1α becomes insufficient, reduced proteins would accumulate in the ER. The consequent response (UPR) increases Ero1β thus generating the required oxidative power. However, cells must also prevent over-oxidation. In the ER, disulphide bonds must be constantly isomerised for many proteins to attain their final three-dimensional structure. Furthermore, proteins that fail to fold or assemble are not allowed to proceed along the secretory pathway and inter-chain disulphide bonds are reduced prior to their retro-translocation to the cytosol for degradation during the process defined ER associated degradation (ERAD). Many PDI homologs reside in the ER of mammalian cells (e.g. ERp57, ERp72, P5, PDIR, PDIp and ERp27). Since Ero1 molecules selectively interact with a subset of them, some members could exert reductase activities. Recent results indicate that ERdj5, an ER resident protein containing a DnaJ domain and four thioredoxin-like domains, favours ER associated degradation of certain substrate proteins reducing

their inter-chain disulfide bonds (Nagata et al. personal communication). An exciting question that emerges from these findings is what maintains ERdj5 in the reduced state.

Despite the specificity of protein relays allows opposite redox reactions to coexist in the ER (disulfide bond formation in nascent proteins, reduction in terminally misfolded ones), Ero1 activity must be nonetheless tightly controlled. Its main substrate, PDI must remain in part reduced to act as an isomerase and as a redox sensitive chaperone, catalysing ERAD substrate unfolding before dislocation. Moreover, *in vitro* assays demonstrated that Ero1p generates H_2O_2 in equimolar amounts to the number of disulphide bonds formed. H_2O_2 can be converted to other ROS that can be highly detrimental for the cell. Suggestions of a significant contribution of Ero1 in cellular ROS production in metazoans come from the observation that down-regulation of Ero1 strongly reduces ROS levels in ER stressed *C. elegans*. Since human Ero1α and β can complement yeast defective cells, it would be extremely important to determine whether oxidative folding generates hydrogen peroxide also in mammalian cells, and if so in what molar ratio.

Recent findings revealed an in-built mechanism for regulating Ero1p activity. The oxidation of two non-catalytic cysteine pairs, leads to inactivation of the enzyme. This rapid feedback mechanism could be particularly important in physiological situations when the requirements for oxidative folding vary transiently and frequently, as in certain secretory cells. The translocation of reduced substrates into the ER lumen shifts the redox balance toward reducing conditions, and rapidly activates pre-existing Ero1 molecules, thus fulfilling the increased oxidative demand. If this pool proves insufficient, the transcription of Ero1 (Ero1β in mammalian cells) is induced. The two redox isoforms observed in human Ero1α (Ox1 and Ox2) might correspond to distinct functional states.

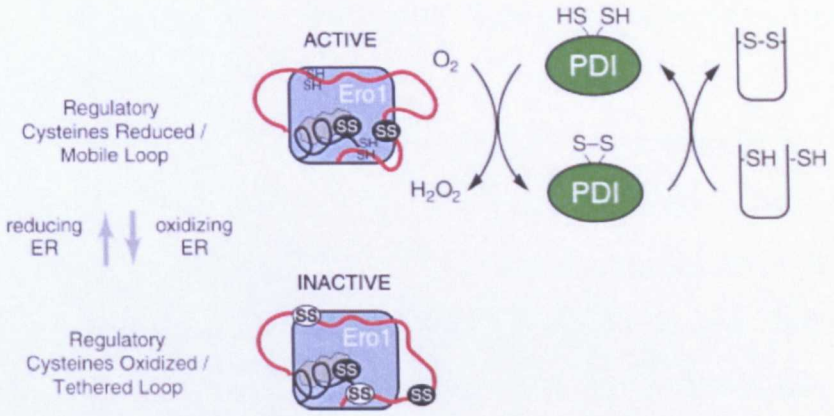

Fig. I 20 Post-translational Ero1 regulation.

The catalytic cysteines are shown as black ovals with white text and the extended non-helical polypeptide containing the shuttle cysteines is colored red. The regulatory cysteines are depicted as white "SS"-containing ovals when oxidized and black thiol "SH" groups when reduced. By tethering the non-helical loop domain to the protein core, formation of the regulatory disulfides may restrict mobility of the shuttle cysteines and decrease Ero1 activity under oxidizing ER conditions. When the regulatory disulfides are reduced, active Ero1 uses molecular oxygen to facilitate PDI and substrate protein oxidation.

from (Sevier and Kaiser, 2007)

Another redox-sensitive feedback mechanism could operate at the transcriptional level. Ero1α is regulated by the hypoxia-inducible factor 1 (HIF1). The O_2 sensing subunit of HIF1, HIF1α, has been found at the ER where it is kept silent by O_2-dependent generation of ·OH. The hydroxyl radical is produced by an ER localised Fenton reaction [$H_2O_2+Fe^{2+} \rightarrow Fe^{3+} + OH^- + \cdot OH$]. It could be speculated that Ero1 is the source of H_2O_2 production at the ER.

Mammalian Ero1α is generally expressed at rather low levels in cultured cell lines and the assumption is that its catalytic activity is enough to drive multiple PDI oxidation cycles and hence ensure the folding of many substrate molecules. Low expression would help avoid the risk of oxidative stress. However, data concerning Ero1β suggest that this model is maybe too simplistic. Ero1β is expressed at high levels in specific cell types of human stomach and pancreas. It can form homo- and hetero-dimers with Ero1α. *In vivo,* the ratio between Ero1β monomers and dimers differs in stomach and pancreas. Even though the biological meaning of Ero1β homo-

and hetero-dimers is not clear, their tissue specific regulation hints to a complex mode of control of Ero1 activity.

Besides the existence of Ero1-centered regulatory mechanisms, ER redox homeostasis heavily relies on a variety of cellular buffering systems, including glutathione (GSH) and $NADP^+$/NADPH. The cellular concentration of GSH ranges from 1 to 10mM but the ratio between oxidised (GSSG) and reduced (GSH) species is very different in the cytosol (1:30-100) and in the ER (1:1-3). This has been long considered the driving force for disulphide bond formation, but the discovery of the Ero1-PDI relays has changed this point of view. It seems now clear that GSH is crucial in balancing Ero1 function, avoiding ER hyper-oxidation and formation of non-native disulphide bonds.

Like the GSH/GSSG couple, NAD^+/NADH and $NADP^+$/NADPH have a pivotal role in protecting the cell against ROS damage, being involved in the main antioxidant defence systems comprising glutathione peroxidases (Gpx), the thioredoxin-thioredoxin reductase system (Trx), peroxiredoxins (Prx), glutaredoxins, superoxide dismutases (SOD), catalases and exogenous micronutrients and vitamins. Open questions concern the mechanisms that drive the import of GSH, NADH and NADPH into the ER lumen, and their regulation during differentiation or stress.

References from (Masciarelli and Sitia, 2008), Appendix III

1.2.4 IgM folding requires multiple quality control steps

Quality control (QC) mechanisms are active at the ER–Golgi interface to ensure that non-native molecules are retained in, or retrieved to, the ER, and eventually dispatched to the cytosol for proteasomal degradation (Anelli et al., 2007; Ellgaard and Helenius, 2003; Sitia and Braakman, 2003). QC recognizes different features, common to unfolded or not completely assembled molecules, such as immature N-glycans (Helenius and Aebi, 2004), hydrophobic patches (Blond-Elguindi et al., 1993) or exposed reactive cysteines by thiol-mediated retention (Fra et al., 1993; Sitia et al., 1990).

As discussed in paragraph 1.1.4, IgM polymers are planar, multimeric proteins. They consist of 21 or 24 subunits, depending on whether they are secreted as J-chain containing pentamers ($(\mu_2L_2)_5$-J) or hexamers ($(\mu_2L_2)_6$). In secreted polymers, individual monomers μ_2L_2 are assembled via disulfide bonds involving Cys^{575} in the C-terminal tailpiece of secretory μ (μ_s) chains (Sitia et al., 1990). Cys^{575} acts also as a retention and degradation signal for un-polymerized secretory IgM (thiol-mediated retention (Fra et al., 1993)). In membrane μ (μ_m) chains, this 20-residue tailpiece is replaced by a longer hydrophobic segment, essential for membrane insertion and assembly with B-cell receptor signaling components. The first assembly step (μ_2L_2 formation), common to both membrane and secreted IgM, is fast and efficient in both B and plasma cells: its fidelity is checked by BiP. BiP, also called GRP78, is an abundant chaperone of the hsp70 family, that was first isolated as a protein associating with unassembled IgH chains (Haas and Wabl, 1983). Most μ_s chains are degraded by proteasomes in B lymphocytes (Shachar et al., 1992), whereas μ_m chains that negotiated assembly into functional B-cell receptors are transported to the cell surface (Sitia et al., 1987).

Therefore, secretory IgM biogenesis occurs in at least two sequential and independently regulated steps, μ_2L_2 assembly preceding polymerization. We recently published a study suggesting that a post-ER QC mechanism plays a key role in the sequential assembly of IgM polymers (Anelli et al., 2007). An unexpected finding was that ERp44, a soluble member of the thioredoxin (trx) family, equipped with an RDEL localization motif (Anelli et al., 2002), accumulates in the ERGIC and cis-Golgi.

Of the three trx-like domains of ERp44, only the N-terminal one possesses a catalytic CRFS motif. It is via this cysteine, (Cys29), that ERp44 forms mixed disulfides with Ero1α, Ero1β, unpolymerized IgM subunits, adiponectin and other substrates of thiol-dependent protein quality control. ERp44 mediates the thiol-dependent retention of μ_2L_2, μL and other un-polymerized IgM subunits that have already passed the BiP-dependent checkpoints (Anelli et al., 2003). ERp44 localization partly depends on interactions with ERGIC-53, which also binds IgM subunits (Mattioli et al., 2006). As a hexameric membrane-embedded lectin, ERGIC-53 may provide a

platform for IgM polymerization (Anelli et al., 2007). In the cis-Golgi, ERp44 could capture un-polymerized IgM subunits and retrieve them via RDEL-dependent mechanisms. In view of its ability of binding Ero1 (Otsu et al., 2006), ERp44 could also provide oxidative power to the polymerization machinery. In this scenario, the compartmentalization of assembly and polymerization in the early secretory pathway of professional antibody secreting cells may couple QC and transport, thus achieving high production capacity.

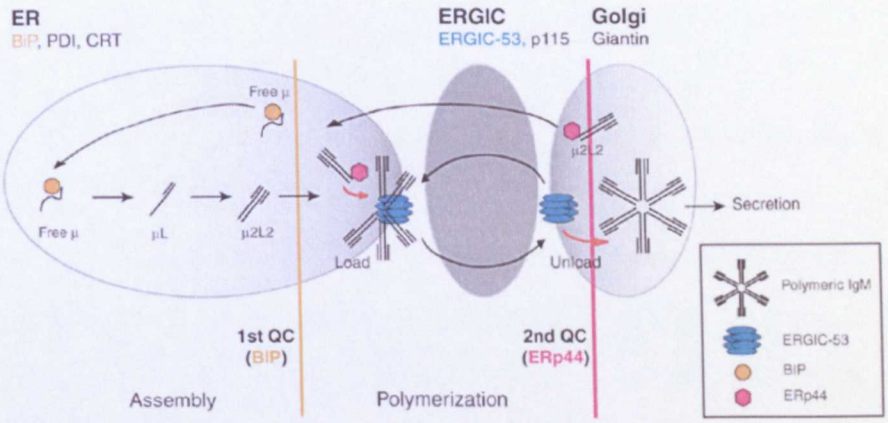

Fig. I 21 Schematic model of the IgM polymerization machinery.

The IgM assembly line is schematized in its sequential arrangement. The distribution of ERp44 in the early secretory pathway (primarily in the ERGIC) is depicted as a gradient of gray. BiP-dependent control ensures that m chains do not proceed to the polymerization machinery unless assembled with L chains (first QC step). ERGIC-53, a hexameric lectin acting in conjunction with MCFD2 (the latter is not shown) captures m2L2 subunits, likely aligning them in a planar conformation, suitable for polymerization. ERp44 ensures that unpolymerized subunits are not secreted, retrieving them into the assembly line (second QC step). Because of its ability to bind IgM subunits and ERGIC-53, ERp44 may recruit and locally concentrate m2L2, to stimulate further the polymerization machinery efficacy.

from (Anelli et al., 2007), Appendix II

1.2.5 The unfolded protein response

The flux of newly synthesized proteins into the ER is variable because it can change rapidly in response to programs of cell differentiation, environmental conditions and the physiological state of the cell. To handle this dynamic situation, cells adjust the

protein-folding capacity of the ER according to their requirements, thereby ensuring that the quality of cell-surface and secreted proteins can be maintained with high fidelity. Such homeostatic control is achieved through the action of signal transduction pathways that have sensors facing the ER lumen and effectors that convey the message to other compartments of the cell. The intracellular signalling pathway that mediates this regulation is named the unfolded protein response (UPR). An imbalance (defined ER stress) between the load of unfolded proteins that enter the ER and the capacity of the cellular machinery that handles this load sets three main responses in motion, the first two of which are rectifying. First, there is a reduction in the protein load that enters the ER, which is a transient adaptation that is achieved by lowering protein synthesis and translocation into the ER. Second, there is an increase in the capacity of the ER to handle unfolded proteins, which is a longer-term adaptation that entails transcriptional activation of UPR target genes, including those that function as part of the ER protein-folding machinery. If homeostasis cannot be re-established then a third mechanism, cell death, is triggered, presumably to protect the organism from rogue cells that display misfolded proteins.

Three different classes of ER stress transducers have been identified. Each class defines a distinct arm of the UPR that is mediated by inositol-requiring protein-1 (IRE1), activating transcription factor-6 (ATF6) or protein kinase RNA (PKR)-like ER kinase (PERK). In each case, these are integral membrane proteins that sense the protein-folding status in the ER lumen and transmit this information across the ER membrane to the cytosol and the nucleus. Indeed they are constituted by a lumenal portion that senses the protein-folding environment in the ER, and by a cytoplasmic effector portion that interacts with the transcriptional or translational apparatus. The present model to explain UPR activation involves the binding of the major ER chaperone, BiP, to the lumenal portions of the ER stress sensors, which represses their activation. In the presence of ER stress BiP releases the receptors to bind accumulating unfolded proteins, relieving repression (Bertolotti et al., 2000). However, this model must not be the only means of UPR signaling initiation since it does not completely explain the fine tuned modulation of UPR activation, as will be

discussed below.

The first stress transducer was identified by a screen for mutations that block the activation of a UPR-inducible reporter in yeast. The gene in question, IRE1, encodes a type 1 ER-resident transmembrane protein with a novel lumenal domain and a cytoplasmic portion that contains a protein kinase domain. In response to unfolded proteins, IRE1 oligomerizes in the plane of the membrane, allowing for trans-autophosphorylation of juxtaposed kinase domains. Oligomerization can be triggered directly by binding of unfolded proteins to the IRE1 lumenal domain (which bears an architectural resemblance to the peptide-binding domains of major histocompatibility complexes) (Zhou et al., 2006), or might involve the release of the oligomerization-repressing chaperones BiP, or both (Bertolotti et al., 2000). By a poorly understood mechanism, trans-autophosphorylation of the kinase domain of IRE1 activates its unusual effector function, which causes the endonucleolytic cleavage of the only known substrate: an mRNA that encodes a transcription factor named Hac1 (homologous to ATF/CREB1) in yeast or XBP1 (X-box binding protein-1) in metazoans. IRE1 cuts the precursor Hac1 or XBP1 mRNA twice, excising an intervening fragment or intron. The 5' and 3' mRNA fragments are then ligated, generating a spliced mRNA that encodes an activator of UPR target genes. Biochemical and genetic evidence indicates that in yeast, ligation of the two ends of the HAC1 mRNA is mediated by tRNA ligase (Trl1). The enzyme(s) responsible for this reaction in higher eukaryotes have not been identified, but recent evidence for a tight association of XBP1 mRNA with membranes indicates that, as in yeast, cleavage and ligation occur in association with the ER. The consequences of this IRE1-dependent splicing event differ in yeast and metazoans. In yeast, the HAC1 mRNA intron represses translation and relief of this repression is the key activating event of the yeast UPR. By contrast, in metazoans both the precursor and spliced form of XBP1 are translated. However, the encoded proteins, which differ markedly in sequence owing to a splicing-mediated frame shift, have different functional properties. The form of XBP1 encoded in the spliced mRNA (sXBP1) is more stable and works as a potent activator of UPR target genes, whereas the protein encoded by the precursor mRNA (uXBP1) is labile and represses UPR target genes. sXBP1 up-regulate

transcription of many genes involved in the secretory pathway (ER chaperones, members of the ERAD pathway, genes involved in membrane expansion) (Acosta-Alvear et al., 2007; Shaffer et al., 2004; Sriburi et al., 2007). In metazoan cells, levels of XBP1 mRNA also increase upon UPR induction and continue to rise as ER stress declines and IRE1 is inactivated. Therefore, the new XBP1 mRNA remains in its precursor, unspliced form, which encodes an inhibitor of XBP1 signalling. This might serve to terminate signaling by a combination of inhibitory heterodimerization with spliced XBP1 and competition for binding sites. Metazoan IRE1 probably has signaling functions beyond its nucleolytic activity. For example, mammalian IRE1 activates the stress-induced Jun N-terminal kinase (JNK) and interacts with components of the cell-death machinery, such as caspase-12, independently of its RNase activity. However, apart from indirect evidence for a contribution to the death of ER-stressed cells, the physiological significance of such pathway branches remains unclear. Analysis of mRNAs expressed in ER-stressed *Drosophila melanogaster* cells evidenced widespread IRE1-dependent degradation of ER-associated mRNAs. This process is likely to represent an XBP1-independent post-transcriptional mechanism for IRE1 control of gene expression that remodels the repertoire of proteins translated in ER-stressed cells. It is unknown whether the nuclease that initiates these events is IRE1 itself, functioning in a mode of relative specificity, or another, yet-to-be-identified nuclease that is recruited or locally activated by IRE1.

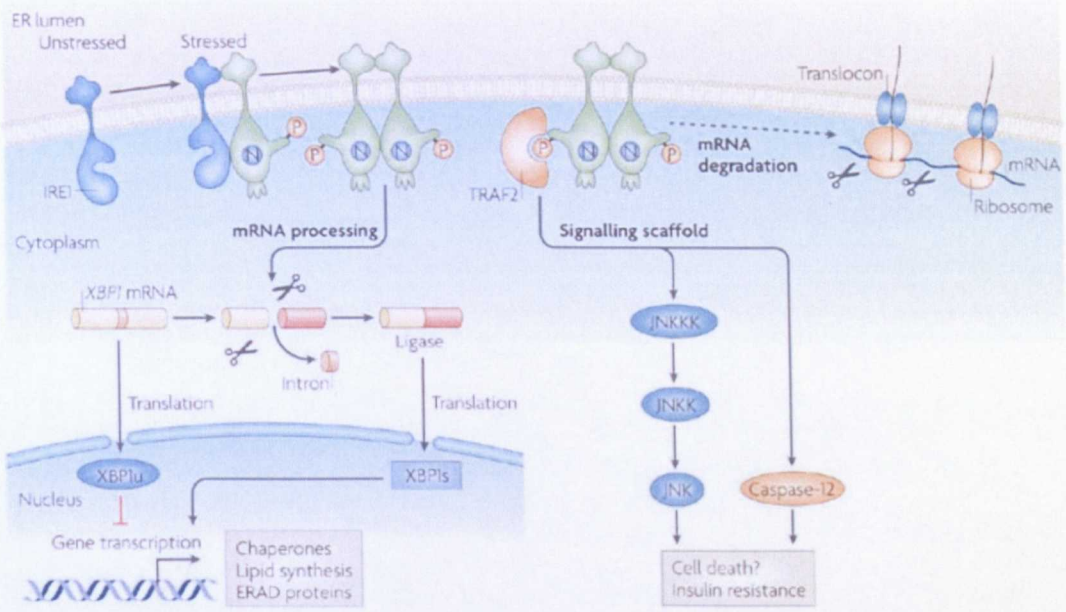

Fig. I 22 Signalling by IRE1.

Inositol-requiring protein-1 (IRE1) oligomerizes in the plane of the endoplasmic reticulum (ER) membrane in stressed cells. *Trans*-autophosphorylation in its cytosolic kinase domain increases the affinity for nucleotides (N), which allosterically activate IRE1 and unmask a dormant endoribonucleolytic activity. IRE1-mediated sequence-specific cleavage of a single known mRNA (X-box binding protein-1 (XBP1) in higher eukaryotes, HAC1 (homologous to ATF/CREB1) in yeast) excises a small RNA fragment (intron). The two ends of the mRNA are ligated (tRNA ligase (Trl1) has this role in yeast but the identity of the ligase is unknown in metazoans), which leads to a frame shift in the coding sequence (shown in the figure as a colour change from yellow to red after removal of the intron). Spliced XBP1 mRNA encodes a potent transcriptional activator (XBP1s), whereas the unspliced XBP1 mRNA encodes XBP1u, an inhibitor of the unfolded protein response (UPR). In yeast, the Hac1/XBP1 pathway activates most of the UPR, whereas in mammals, it appears that XBP1 regulates a subset of UPR genes that promote ER-associated degradation (ERAD) of misfolded proteins and ER biogenesis. IRE1 can also act by alternative means. In mammals, recruitment of TRAF2 (tumour necrosis factor receptor (TNFR)-associated factor-2) by phosphorylated IRE1 allows it to signal to Jun N-terminal kinase (JNK) and alter intracellular signalling (for example, resulting in insulin resistance). The IRE1–TRAF2 complex has also been linked to caspase-12 activation and cell death. In cultured Drosophila melanogaster cells, activated IRE1 can promote the cleavage of various ER-localized mRNAs, leading to their degradation. This reduces the load on the stressed ER and might facilitate reprogramming of the ER-associated protein synthesis and translocation machinery. It is unknown whether IRE1 cleaves these mRNAs directly or whether it promotes their degradation by activating or recruiting other RNases. JNKK, JNK kinase; JNKKK, JNKK kinase.

ATF6 is the founding member of a novel class of metazoan-specific ER stress transducers. These are synthesized as inactive precursors, tethered to the ER membrane by a transmembrane segment and have a stress-sensing portion into the ER lumen. Under conditions of ER stress, ATF6 is transported from the ER to the Golgi apparatus, where it is cleaved by Golgi-resident proteases, first by S1P (site 1 protease) and then in an intra-membrane region by S2P (site 2 protease) to release the cytosolic DNA-binding portion, ATF6f ('f' for fragment). From there, ATF6f moves to the nucleus to activate gene expression of a subset of UPR genes mostly involved in improving the secretory capacity of the cell (Haze et al., 1999; Hong et al., 2004; Nadanaka et al., 2007; Wu et al., 2007; Yamamoto et al., 2007). ATF6 trafficking from the ER to the Golgi is initiated by accumulation of unfolded proteins in the ER lumen. As discussed for Ire1 this could be sensed by release of the luminal portion of ATF6 by BiP inhibitory binding or directly. The predicted structures of several other proteins suggest that they are ER-anchored transcription factors that are related to ATF6, for example, LZIP (also known as luman or cyclic AMP-responsive element binding protein-3 (CREB3)), OASIS (also known as CREB3-like-1) and Tisp40 (transcript induced in spermiogenesis-40). One such protein, CREB-hepatocyte (CREBH), has recently been found to be activated by ER-stress-regulated proteolysis. However, CREBH does not activate genes that enhance the capacity of the secretory pathway but, rather, links ER stress in the liver to the secretion of serum proteins that are associated with inflammation (so-called acute-phase responsive proteins). These observations are an intriguing example of integration of the UPR with a range of physiological systems.

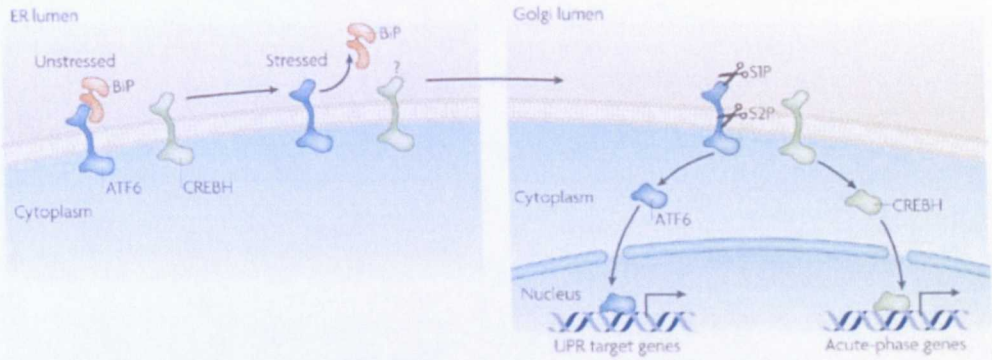

Fig. I 23 Signalling by ATF6.

Activating transcription factor-6 (ATF6) and cyclic AMP response element binding protein hepatocyte (CREBH) are transmembrane proteins with a cytoplasmic portion that, when liberated from its transmembrane tether, can bind to DNA and activate target genes. In unstressed cells, ATF6 and CREBH reside in the endoplasmic reticulum (ER) membrane. ATF6 trafficking appears to be hindered by binding of the ER chaperone immunoglobulinbinding protein (BiP) to its lumenal domain. ER stress disrupts BiP binding and ATF6 (and CREBH) are delivered to the Golgi apparatus. The details of this vesicular transport event remain unknown. In the Golgi apparatus, these proteins are subject to consecutive cleavage, first by the lumenal site 1 protease (S1P) and then the intra-membrane site 2 protease (S2P), which liberates the cytosolic effector portions of the proteins from the membrane and allows their import into the nucleus. ATF6 probably activates a subset of UPR target genes, although these remain to be characterized, whereas CREBH activates acute-phase response genes that encode secreted proteins involved in inflammation.

The third ER stress transducer, PERK, superficially resembles IRE1. Both are ER-localized type I transmembrane proteins with lumenal stress-sensing domains that are phylogenetically related, similar in structure and function, and experimentally interchangeable. The cytoplasmic portion of PERK also contains a protein kinase domain, which undergoes activating trans-autophosphorylation by oligomerization in ER-stressed cells; however, unlike IRE1, for which the only substrate is itself, PERK phosphorylates the α-subunit of eukaryotic translation initiation factor-2 (eIF2α) at Ser51. This phosphorylation inhibits the guanine nucleotide exchange factor eIF2B, a pentameric complex that recycles eIF2 to its active GTP-bound form. Lower levels of active eIF2 result in lower levels of translation initiation, globally reducing the load of newly synthesized proteins, many of which are destined to enter the already

stressed ER lumen. In addition to decreasing global protein synthesis to reduce the ER load, PERK-mediated eIF2α phosphorylation also contributes to transcriptional activation in the UPR. Expression profiling in ER-stressed PERK knockout cells showed the defective induction of numerous mRNAs that are responsible for the normal UPR. A similar defect in stress-induced gene expression was also observed in cells with a Ser51Ala mutation in eIF2α that prevents the regulatory phosphorylation event but does not otherwise affect eIF2 function. Furthermore, most genes that are down regulated in PERK-knockout cells were induced by the directed activation of PERK (in an experimental system that is uncoupled from ER stress). Most importantly, the entire range of the PERK-dependent gene expression program requires eIF2α phosphorylation as it was blocked by the Ser51Ala mutation. eIF2α phosphorylation leads to translational up regulation of the transcription factor ATF4. The 5'-untranslated region of ATF4 contains short, inhibitory upstream open reading frames (uORFs) that prevent translation of the downstream ATF4-encoding ORF in unstressed cells. Conditions that limit eIF2 activity (due to eIF2α phosphorylation) lead to ribosomes skipping the inhibitory uORFs so that the ATF4 ORF can be translated. As different types of stress converge on eIF2α phosphorylation, like starvation, the presence of double stranded RNA and heme deficiency, respectively mediated by the eIF2α kinases GCN2, PKR and HRI, this branch of the UPR is also termed integrated stress response (ISR) (Harding et al., 2003). General translation inhibition is quickly reversed by a negative feedback loop. Phosphorylation of eIF2α and the consequent decrease in initiation complexes availability that selectively promotes translation of the transcription factor ATF4 mRNA leads to up-regulation of genes involved in amino-acid transport, genes that protect against oxidative stress (Lu et al., 2004; Marciniak et al., 2004; Ron and Walter, 2007) and of *gadd34*, a regulatory subunit of the PP1 phosphatase which dephosphorylates eIF2α shutting down the signalling (Novoa et al., 2001).

Among the genes whose expression is induced by eIf2α phosphorylation there is the member of the C/EBP family of transcription factors CHOP. CHOP is mostly recognized as a pro-apoptotic factor, especially when induced by the UPR (Marciniak

et al., 2004; Oyadomari and Mori, 2004). CHOP role and mechanisms of up regulation, in the UPR, and elsewhere, will be the main topic throughout this work.

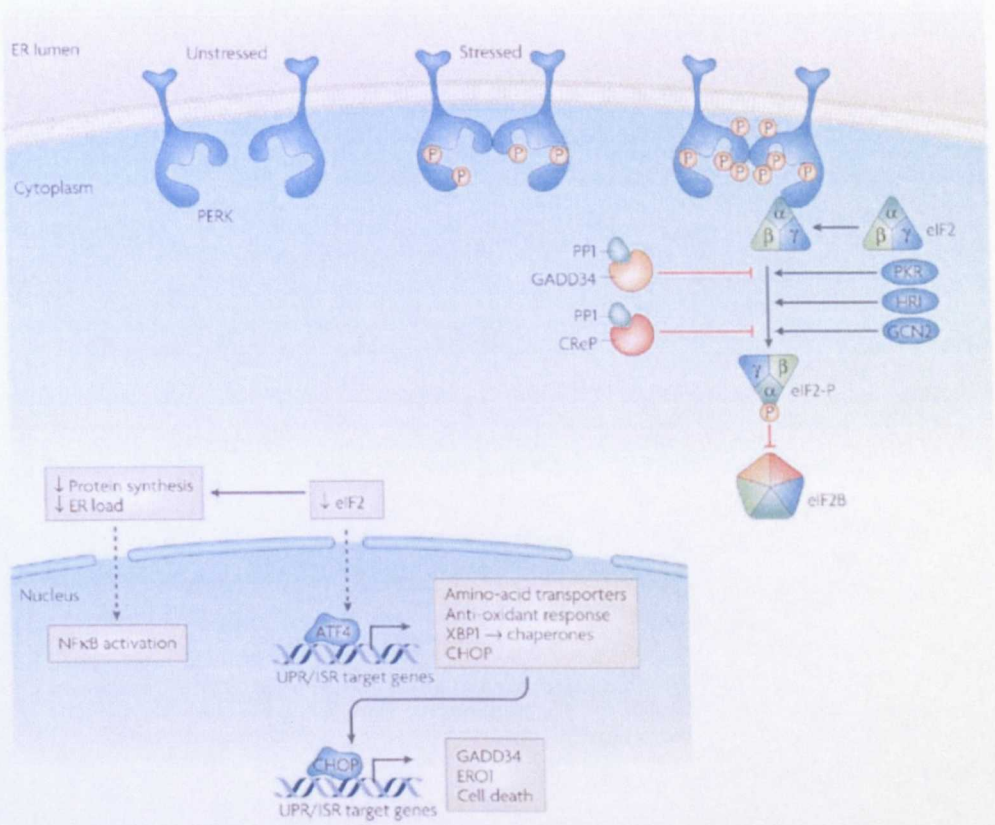

Fig. I 24 Signalling by PERK to the translational machinery.

In response to endoplasmic reticulum (ER) stress, protein kinase RNA (PKR)-like ER kinase (PERK), similar to inositol-requiring protein-1 (IRE1), oligomerizes in the plane of the membrane and is activated by trans-autophosphorylation of its activation loop. Extensive further phosphorylation of the large kinase insert loop facilitates substrate recruitment. Phosphorylation of a single known substrate, the α subunit of eukaryotic translation initiation factor-2 (eIF2) on Ser51, inhibits the pentameric guanine nucleotide exchange factor eIF2B from recycling eIF2 to its active GTP-bound form. The resulting reduced activities of eIF2B and the eIF2 complex account for all of the important consequences of PERK activity. Because other eIF2 kinases (PKR, haem-regulated inhibitor kinase (HRI) and general control non-derepressible-2 (GCN2)) can activate this pathway independently of ER stress, this portion of the unfolded protein response (UPR) is termed the integrated stress response (ISR). Lower global protein synthesis reduces ER unfolded protein load but also affects gene transcription. For example, translation of the activating transcription factor-4 (ATF4) is increased under conditions of limiting eIF2, whereas nuclear factor κB (NFκB) is activated post-translationally. The ISR activates genes that encode amino-acid transporters and genes that protect against oxidative stress, and it contributes to the transcriptional activation of XBP1. The transcription factor CHOP (C/EBPhomologous protein) is also activated transcriptionally by ATF4 and its target genes include GADD34 (growth arrest and DNA damage-inducible protein-34), a

regulatory subunit of phosphatase PP1 that dephosphorylates eIF2α and terminates signalling in the ISR, and ER oxidase-1 (ERO1), which is required for disulphide bond formation in protein folding. A constitutive phosphatase CReP (constitutive repressor of eIF2α phosphorylation) assists GADD34 in this task.

Although the three UPR pathways are distinct there is a considerable degree of cross-talking (Ron and Walter, 2007). Indeed ATF-6 increases transcription of *xbp-1* (Lee et al., 2002) and ATF-6 and sXBP-1 act cooperatively on various targets via the ER stress responsive element (ERSE) and the UPR element (UPRE). Whereas ATF6 activates transcription only via the ERSE, sXBP1 can recognize both ERSE and UPRE elemens (Wu et al., 2007; Yamamoto et al., 2007; Yamamoto et al., 2004). Also ATF4 (which activates transcription via the C/EBP-ATF composite site) and ATF6 cooperate in the activation of some UPR genes, as will be discussed in further details (Ma et al., 2002; Ma and Hendershot, 2004). sXBP-1 up-regulate the expression of $p58^{IPK}$ which inhibits PERK signalling (Lee et al., 2003; van Huizen et al., 2003; Yan et al., 2002). Depending on the duration and the strength of the stress the UPR can mediate recovery and adaptation or apoptosis (Lin et al., 2007; Rutkowski and Kaufman, 2007). In particular IRE1 and PERK are involved in activation of various pathways leading to apoptosis in a manner that is not fully elucidated (Meares et al., 2008; Puthalakath et al., 2007; Szegezdi et al., 2006).

Clarification of the UPR pathways was mainly obtained by heavy perturbation of the ER homeostasis obtained by drugs that led to the coordinate activation of all the branches of the UPR. For this reason the UPR was initially considered as an "all or nothing" response. It then became clear on the contrary that different types of secretory cells, which undergo physiological levels of ER stress, selectively exploit the various UPR branches (Brewer and Hendershot, 2005; Wu and Kaufman, 2006). For example whilst PERK pathway is essential for survival of insulin secreting β cells (Harding et al., 2001), IRE1/XBP-1 and ATF6 pathways are activated during plasma cell differentiation but PERK is not (Gass et al., 2002; Gass et al., 2008; Zhang et al., 2005). Indeed XBP-$1^{-/-}$ B lymphocytes cannot differentiate into plasma cells (Iwakoshi et al., 2003a; Reimold et al., 2001) but $PERK^{-/-}$ B cell differentiation is not impaired (Gass et al., 2008).

Unless stated otherwise references and figures are from (Ron and Walter, 2007)

1.3 Regulation of immunoglobulin secretion

1.3.1 The UPR in plasma cell differentiation

Ig are co-translationally translocated in the ER where they attain their proper quaternary structure, mature into the Golgi and proceed along the exocytotic pathway to be secreted. Indeed the most dramatic transformation when a B lymphocyte differentiates into a plasma cell concerns the ER expansion accompanied by an enlargement of the Golgi complex (Lewis et al., 1985; Rush et al., 1991; Shohat et al., 1973; Wiest et al., 1990) which are necessary to accommodate the increase in Ig synthesis. This transformation is accomplished by the interplay between B lineage-specific transcriptional programs that control plasma cell differentiation and the unfolded protein response.

The extensive secretory pathway in plasma cells is appropriately equipped for enhanced protein biosynthesis. In fact, it is specifically the ribosome-studded rough ER that is most drastically expanded, and this correlates well with the robust escalation of immunoglobulin translation. As differentiation proceeds, a large cohort of ER-resident proteins that constitute critical components of the protein folding machinery is up regulated. These proteins include molecular chaperones such as BiP that promote proper protein maturation and enzymes such as protein PDI and Ero1 that facilitate oxidative protein folding. Therefore, the enlarged ER is populated with the full complement of factors needed to efficiently accommodate the increased load of nascent immunoglobulin chains (Romijn et al., 2005; van Anken et al., 2003). The increased immunoglobulin synthesis is further managed by parallel enhancement of mechanisms that ensure fidelity in immunoglobulin secretion. For example, the amount of ERp44 and ERGIC-53 rise sharply during the differentiation process (Anelli et al., 2007). This observation fits well with the fact that plasma cells generally do not secrete incompletely assembled immunoglobulin molecules (Reddy

and Corley, 1999). Thus, quality is not sacrificed for quantity as B cells transition into high-rate antibody production.

The augmented flow of nascent immunoglobulin chains into the secretory pathway increases the demand placed on the protein folding capacity of the ER. Given its various roles in regulating ER homeostasis, it follows that the UPR would be intimately involved in the conversion of B cells into antibody factories. The essential connection between the UPR and plasma cell development was revealed by key studies from Glimcher and co-workers regarding the XBP1 transcription factor. Deletion of mouse *xbp1* induces an embryonic lethal phenotype owing to hypoplasia of the liver (Reimold et al., 2000). When *xbp1*-null ES cells were used to reconstitute recombination activating gene-2 (*rag-2*) deficient mice, the chimeric animals produced normal numbers of mature B cells in all compartments. The B cells were able to proliferate and form germinal centers in response to antigen but were unable to differentiate into plasma cells (Reimold et al., 2001). Plasma cell differentiation and the UPR then crossed when XBP1 mRNA was shown to be spliced during LPS-induced plasma cell differentiation (Calfon et al., 2002). Indeed, sXBP1 is capable of restoring differentiation potential to XBP-1 deficient B cells, whereas the uXBP1 is not (Iwakoshi et al., 2003b).

sXBP1 can orchestrate expansion of intracellular organelles. Enforced expression of sXBP1 in a B cell line was shown to be sufficient to amplify the abundance of ER, Golgi, mitochondria and lysosomes (Shaffer et al., 2004). Thus, sXBP1 can direct events that expand the compartments where antibodies are made while concomitantly increasing the quantities of the organelle that supplies the energy required for massive immunoglobulin biosynthesis. Organelle expansion in cells overexpressing sXBP1 is accompanied by elevated expression of many genes encoding secretory-pathway components. These include proteins that target and translocate nascent polypeptides into the ER, chaperones and their cofactors that promote protein folding and assembly, oxidoreductases that regulate oxidative protein folding, glycosylation enzymes, regulators of vesicular trafficking, and proteins implicated in ER associated degradation (ERAD). Notably, many of these same genes are up regulated in differentiating B cells in an sXBP1-dependent

fashion. These data strongly suggest that sXBP1 mediates a broad program of gene expression during differentiation that upgrades the entire secretory apparatus, thereby enhancing antibody production while ensuring retention of assembly intermediates and disposal of excess or misfolded immunoglobulin subunits by ERAD. Perturbation of UPR-mediated gene expression in differentiating B cells compromised quality control, resulting in improper secretion of IgM assembly intermediates (Gunn et al., 2004).

A growing body of data indicates that the UPR activation profile is likely to vary among different types of specialized secretory cells. For example, the IRE1-XBP-1 branch of the UPR is clearly active in differentiating B cells (Calfon et al., 2002; Iwakoshi et al., 2003b), and sXBP1 is essential for successful plasma cell development. ATF6 pathway is activated as well during plasma cell differentiation (Gass et al., 2008) even though a role for ATF6 in developing plasma cells has not been established. It is certainly possible that this factor contributes to the induction of XBP1 as well as many genes encoding ER chaperones and folding enzymes. PERK, in contrast, is not activated in the normal course of terminal B cell differentiation (Gass et al., 2008; Zhang et al., 2005). Notably, the PERK arm of the response is functional in B cells, as evidenced by potent induction of CHOP, a PERK-dependent UPR target gene, in response to ER stress agents, as will be shown in this work. This raises an interesting point, because PERK seems to be essential for maximal induction of ER chaperones during ER stress (Harding et al., 2000; Novoa et al., 2003), and chaperones are highly up regulated during plasma cell differentiation. In sharp contrast to differentiating B cells, pancreatic tissue shows high constitutive activity of both PERK and Ire1 (Harding et al., 2001). PERK is essential for proper control of insulin biosynthesis, β islet cell survival, and normal function of pancreatic acinar cells.

What is the mechanistic basis for UPR variation? One possibility is that the three transducers—IRE1, ATF6 and PERK—have different thresholds for activation. To date, there is no evidence supporting this idea, but it is conceivable that such differences may not have been apparent when strong ER stress-inducing agents were used to study the response. If such a hierarchy exists, it must vary by tissue given

the dissimilarity between PERK activity in B cells versus the pancreas. Another idea is that there are alternative mechanisms to activate the individual transducers independent of the load of unfolded proteins and the abundance of BiP. Although no other means of activating the transducers have been elucidated, this hypothesis remains an intriguing possibility. In the case of UPR activation in differentiating B cells, expression of immunoglobulin heavy chains is required for optimal synthesis of sXBP1 (Iwakoshi et al., 2003b). These data fit well with the idea that the UPR is triggered when BiP is needed to accommodate an increased flux of nascent polypeptides. It is worth noting, however, that μ-chain deficient B cells did show low, but detectable, amounts of sXBP1 when stimulated with LPS. Moreover, a kinetic analysis found that synthesis of sXBP1 precedes the massive increase in immunoglobulin translation during differentiation of the CH12 B cell lymphoma (Gass et al., 2002). Thus, the nature of the signals that can elicit the UPR in physiologic settings may not yet be fully understood.

A final possibility is that various arms of the UPR can be specifically suppressed or activated. Flexibility is likely to be a key feature of the physiologic UPR, allowing the response to appropriately fit the situation. For example, pancreatic cells must rapidly modulate protein synthesis according to sudden fluctuations in circulating glucose concentrations. As a result, the β islet and acinar cells must cope with episodic high-rate synthesis of secretory-pathway proteins. It follows, therefore, that these cell types might be especially reliant on the ability of PERK to rapidly regulate translation. However, as B cells terminally differentiate, they initiate and accelerate antibody secretion, presumably to an optimal level, and then continue in this capacity until death. Moreover, the differentiation process prepares the cell for high-rate secretion by up regulating a wide array of metabolic processes and initiating expansion of the secretory pathway before the point of greatest immunoglobulin synthesis (Romijn et al., 2005; van Anken et al., 2003). Thus, a UPR-initiated, PERK-mediated repression of protein synthesis would be counterproductive for cells that are designed to maximally synthesize and secrete immunoglobulins. One could regard the PERK-mediated attenuation of translation as an 'emergency brake' that, in certain cell types under specific conditions, slows the flow of nascent polypeptides into the

secretory pathway, thereby guarding against the potentially catastrophic effects of an overloaded ER. This translation 'brake' might be deliberately not in use when there is no need to balance high-rate protein production with long-term survival, as would be the case for short-lived plasma cells.

Interestingly we have previously reported that the UPR player CHOP (C/EBP homologous protein) is up regulated during differentiation of the B lymphoma cell line I.29μ+ (Cenci et al., 2006). CHOP belongs to the C/EBP family of transcription factors and is also called C/EBPζ, GADD153 (Growth Arrest and DNA Damage inducible gene 153) and *ddit3* (DNA-damage-inducible transcript 3) (Marciniak et al., 2004; Oyadomari and Mori, 2004). It heterodimerizes with other members of C/EBPs family (Ron and Habener, 1992; Ubeda et al., 1996) and also with different bZip transcription factors, like those belonging to the ATF/CREB family (Chen et al., 1996). However, CHOP contains proline and glycine substitutions in the basic region that disrupt its DNA-binding activity. Consequently, CHOP-C/EBP heterodimers cannot bind to canonical C/EBP sites. On the other hand, CHOP-C/EBP heterodimers can bind to another unique site (A/G) (A/G) (A/G)TGCAAT(A/C)CCC to activate target genes. Furthermore, CHOP can enhance the transcriptional activation of AP-1 by tethering to the AP-1 complex without direct binding of DNA. Thus, CHOP has a dual role both as an inhibitor of C/EBPs function and as an activator of other genes (Oyadomari and Mori, 2004).

ER stress is the strongest inducer of CHOP, downstream of ATF6, Ire1 and PERK pathways, where it mostly plays a proapototic role (Ma et al., 2002; Oyadomari and Mori, 2004). However, it is also involved in many different stress responses and physiological processes and it was originally described as a gene involved in the response to DNA damage (Eizirik et al., 1993; Fornace et al., 1988; Luethy and Holbrook, 1992; Oyadomari and Mori, 2004). CHOP is up regulated in response to accumulation of misfolded proteins in the mitochondria (mtUPR) (Horibe and Hoogenraad, 2007), upon amino-acid starvation (Averous et al., 2004; Bruhat et al., 2000) and upon exposure to oxidative stress (Guyton et al., 1996; Tang et al., 2002). Importantly CHOP plays a role in the differentiation of a variety of cell types, like adipocytes (Huang et al., 2005; Li et al., 2006; Tang and Lane, 2000), keratinocytes

(Maytin and Habener, 1998) and osteoblasts (Pereira et al., 2006; Shirakawa et al., 2006), together with different members of the C/EBP family of transcription factors. Expression of the XBP1 gene in B cells is controlled by the interplay of several transcriptional regulators. The BCL6 transcription factor is required for normal function of mature B cells and is a repressor of BLIMP1 (see paragraph 1.1.8). Signaling through the B cell antigen receptor (BCR) induces phosphorylation and ubiquitination of BCL6, thereby targeting it for degradation (Niu et al., 1998) and allowing induction of BLIMP1. Recent gene array studies have shown that BLIMP1 is upstream of a complex program of gene expression that involves both down and up regulation of transcription (Kallies et al., 2007; Klein and Dalla-Favera, 2007; Shaffer et al., 2004). In particular, Blimp-1 directly represses BCL6 and PAX5, a transcriptional regulator required for B lineage commitment and a repressor both of immunoglobulin and J chain genes and of XBP1 (Reimold et al., 1996). In keeping with these data, induction of XBP1 is dependent on BLIMP1 (Shaffer et al., 2004). XBP1 is not the only essential target downstream of BLIMP1, as enforced sXBP1 expression in *blimp*1-deficient cells is not sufficient to restore immunoglobulin secretion (Shapiro-Shelef et al., 2003). BLIMP1 regulates expression of several gene products that control cell cycle progression, thereby halting proliferation of terminally differentiating B cells. A number of cell surface proteins, such as major histocompatibility class II molecules, components required for BCR signaling and factors that control the germinal center reaction, are also downregulated by BLIMP1 (Brewer and Hendershot, 2005). These modulations in gene expression fit well with the fact that cells devoted to antibody secretion no longer need to communicate with T cells, respond to antigen or undergo affinity maturation. Furthermore, BLIMP1, perhaps via indirect mechanisms, augments the transcription of genes encoding the structural components of antibody molecules, including the immunoglobulin heavy and light chains and the J chain. It is also required for the switch to the production of mRNA encoding secretory rather than membrane-bound IgH chains (Shapiro-Shelef et al., 2003). Therefore, BLIMP1 functions as a master regulator of plasma cell differentiation by facilitating induction of XBP1, shutting down unnecessary cellular processes and enhancing immunoglobulin expression. Increased immunoglobulin

expression is, as discussed earlier, also linked to optimal induction of sXBP1.

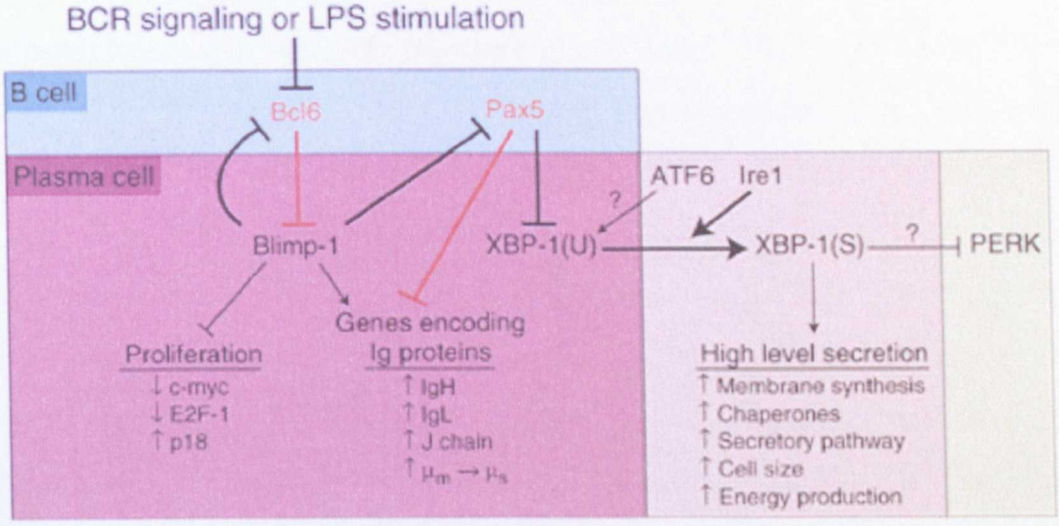

Fig. I 25 Interplay between transcriptional programs during plasma cell differentiation.

Signaling through the BCR or LPS stimulation leads to a loss of Bcl6 (blue box), which relieves the repression on *Blimp1*. In turn, Blimp-1 represses *Bcl6* transcription to maintain its own expression, blocks proliferation by repressing *Myc* and *E2f1* and inducing the cyclin-dependent kinase inhibitor *p18* (also known as *Cdkn2c*), and induces switching to the secretory form of immunoglobulin heavy chain. Additionally, Blimp-1 represses *Pax5*, which relieves the repression on the immunoglobulin genes, allowing their upregulation. *Bcl6* and *Pax5* thus directly repress genes involved in plasma cell differentiation (red lines). In addition, the Blimp-1-mediated loss of Pax5 relieves the repression on *Xbp1*, resulting in larger amounts of unspliced *XBP1*(U) mRNA, as does ATF6 activation via the UPR (purple box denotes potential overlap between these two pathways). Activation of Ire1 (via UPR; orange box) induces processing of the XBP-1 mRNA to produce the remodeled form of the XBP-1 protein, XBP-1(S), which drives many of the requirements for high-level secretion. Only the PERK component of the UPR seems to remain inactive (yellow box), and this regulation might also be linked to XBP-1(S).

from (Brewer and Hendershot, 2005)

1.3.2 Ending the antibody response

After few days of intense Ig secretion most plasma cells die (Moser et al., 2006; Radbruch et al., 2006). Their short lifespan is probably important to limit the immune response. If the antigen is not yet defeated, new specific B lymphocytes,

meanwhile expanded in numbers and equipped with better receptors by affinity maturation and isotype switching in germinal centers, will be activated. What causes apoptosis of plasma cells? Ig production itself could cause death via different mechanisms. One could be a prolonged UPR sustained by continuous Ig production. The UPR can lead to apoptosis through multiple pathways (Breckenridge et al., 2003; Scheuner et al., 2006; Szegezdi et al., 2006; Xu et al., 2005): activation of ER stress specific caspases (caspase 4 in human, 12 in mouse), mitochondrial activation by ER Ca^{++} release, activation of JNK via IRE1, and suppression of anti-apoptotic bcl-2 and sensitization to ROS via the transcription factor CHOP (downstream of the PERK branch of the UPR). However, recent data exclude a role for PERK and the downstream factor CHOP in plasma cell apoptosis ((Gass et al., 2008) and this work). A recent study supports a direct role of Bax, caspase 4 and the ER in plasma cell apoptosis (Pelletier et al., 2006), though autocrine or paracrine loops involving the death ligand TRAIL may also be important (Ursini-Siegel et al., 2002).

In all likelihood, different factors concur in rendering plasma cells susceptible to apoptosis. One predisposing factor could be the chaperone overload that might arise at the high rate of protein synthesis achieved by plasma cells (Soti et al., 2003; Sreedhar and Csermely, 2004). In addition, we recently showed that in the late phases of differentiation, when antibody production becomes maximal, proteasomal activity decreases. This correlates with parallel increased sensitivity to proteasome inhibitors, and stabilization of endogenous proteasomal substrates, including IK-Bα, and the proapoptotic factors Bim and Bax ((Cenci et al., 2006), appendix I). Aminoacid supply and energy production could also become limiting as Ig synthesis becomes preponderant, further lowering the threshold for apoptotic commitment (Cenci and Sitia, 2007).

Perturbation of the redox homeostasis could be yet another factor leading to plasma cell death. On the basis of the present knowledge about oxidative folding, the massive production and assembly of Ig could generate abundant ROS in differentiating plasma cells (paragraphs 1.2.2 and 1.2.3). The production of 10^3 IgM per second, each containing 10^2 disulfides, implies that 10^5 bonds be formed solely to satisfy the requirements of antibody production, explaining the increase in Ero1α

and β. If O_2 is the ultimate acceptor also in living mammalian cells, up to 10^5 H_2O_2 molecules per second could be generated as a byproduct of oxidative folding in IgM secreting cells.

Besides oxidative folding, additional processes could contribute to ROS production. It has been estimated that nearly 2% of the total oxygen consumed by mitochondria leaks from mitochondrial respiratory chain in a partially reduced form (Ames et al., 1993). It is reasonable to imagine that the higher metabolic requirements in plasma cells would cause a corresponding increase in mitochondrial ROS generation. Such mechanism has been proposed to explain the increase in ROS upon T lymphocytes activation (Hildeman et al., 2003).

A regulated source of ROS in differentiating B cells could be NADPH oxidases. These are complex enzymes known to generate superoxide in phagocytes during inflammatory processes (Babior, 2002). Different isoforms of NADPH oxidase (NOX) and dual oxidase (DUOX) have been described in non-phagocytic cells to generate ROS in a regulated manner, with important consequences upon signalling (Bedard and Krause, 2007; Lambeth, 2004). B lymphocytes express NOX2 and DUOX1 (Bedard and Krause, 2007; Reth, 2002; Singh et al., 2005). Intriguingly, I found increased expression of NOXA2 ($p67^{phox}$) transcripts in murine ASC with respect to un-stimulated B lymphocytes (transcriptional expression profiling described in this work). These preliminary findings deserve further analyses, especially since previous reports failed to detect NOXA2 in plasma cells (Inanami et al., 1998).

Although formal evidence of ROS generation during plasma cell differentiation remains to be gathered, many are the indications that plasma cells undergo oxidative stress. The up regulation of different enzymes involved in thiol redox balance upon stimulation of I.29μ^+ with LPS has been described in different proteomic analysis studies (Romijn et al., 2005; van Anken et al., 2003). Prx1 expression was detected by immunoistochemistry in human plasma cells but not in B lymphocytes infiltrating inflammatory tissue of the oral cavity, independently of the inflammation-inducing agent (Demasi et al., 2007). An ER resident oxidoreductase (plasma cell-Trx related protein, PC-TRP) is specifically expressed in murine primary plasma cells (Wrammert et al., 2004). Trx1 expression is higher in human cell lines with plasma cell-like

features than earlier stages of differentiation, even though immortalized lines could be selected for stronger anti-oxidant responses (Nilsson et al., 2004).

Further indication of ongoing oxidative stress in ASC comes from the observation that differentiating B lymphoma cells trigger a Nrf2-dependent anti-oxidant response (S. Nerini Molteni, S. Cozza and R.Sitia, unpublished data). Nrf-2 is a redox sensitive transcription factor that mediates the induction of glutathione S-transferase, glutamyl cysteine ligase (GCLM/GCLC, the limiting enzymes for GSH synthesis), heme oxigenase-1 (HO-1), phase II detoxifying enzymes and other anti-oxidant factors (Zhang, 2006).

The above observations indicate that oxidative stress could contribute to limit plasma cell lifespan, in a fashion possibly dependent on the rate and intensity of antibody secretion. Proteasomal insufficiency, a chronic UPR and ROS production could hence cooperate in inducing death when enough antibodies have been produced. It is worth recalling that the UPR and ROS share a common apoptotic pathway through the apoptosis signal-regulated kinase 1 (ASK1) (Bishopric and Webster, 2002; Kadowaki et al., 2005; Nagai et al., 2007). Activated IRE1 recruits the TNF receptor associated factor 2 (TRAF2), which in turn binds to ASK1 leading to apoptosis via JNK activation. ASK1 deficient cells are unable to activate JNK and are much more resistant to ER stress-dependent death (Nishitoh et al., 2002). ASK1 is negatively regulated by reduced Trx1 (Fujino et al., 2007; Nadeau et al., 2007). Ig synthesis could hence activate the ASK1/JNK apoptotic cascade, via the different mechanisms elicited by UPR/IRE1 pathway and ROS. Furthermore, the pro-apoptotic activity of JNK is executed via the bcl-2 family members, known to be involved in plasma cell death. JNK can inhibit bcl-2 (Yamamoto et al., 1999) and mcl-1 and activate Bax , Bim and Bmf (Inoshita et al., 2002; Lei and Davis, 2003; Putcha et al., 2003; Tsuruta et al., 2004). Recent studies point to a direct proapototic role for Bax in human plasma cells: Bax is recruited at the ER and contributes to caspase 4 cleavage before the release of mitochondrial apoptotic factors (Pelletier et al., 2006). Bax is stabilised and accumulates in differentiating B lymphoma cells whilst the level of bcl-2 expression remains constant (Cenci et al., 2006). An additional link among ER stress, ROS generation and Bax proaptotic activity comes

from studies regarding pharmacologically induced UPR and apoptosis. The ER resident protein Bax inhibitor 1 (BI-1) regulates ER stress associated ROS generation via up-regulation of HO-1 through Nrf-2 and protects cells from UPR induced apoptosis (Lee et al., 2007). Increased ROS could favour plasma cell apoptosis also perturbing Ca^{++} homeostasis. The activity of both sarcoplasmic/endoplasmic reticulum calcium ATPases (SERCA2b in particular) and IP3 Receptors type 1 are regulated in a redox dependent manner (Higo et al., 2005). In turn, ER Ca^{++} release potentiates mitochondria-dependent apoptosis (Gorlach et al., 2006).

2. Aims of the work

Plasma cell differentiation entails profound metabolic and morphological changes. In order to achieve the required levels of immunoglobulin production, assembly and secretion, B cells need to increase their secretory capacity by expanding the ER and the Golgi. Such transformation is obtained by the interplay between the B cell lineage specific transcriptional program and a stress response named Unfolded Protein Response (UPR). The UPR consists in a group of signaling pathways emanating from the ER when it undergoes stress, due to increased demands in the folding capacity or to disruption of its homeostasis. The UPR aims to re-establish ER homeostasis by augmenting the ER membrane and protein components. When ER stress is too strong or too prolonged the UPR switches from its adaptive nature to promoting apoptosis. For many years it has been considered as a uniform response, with the various branches activated altogether in the presence of ER stress. The main reason for this belief is that the elucidation of the response has been obtained mainly with drugs that dramatically disrupt the ER homeostasis, circumstances in which generally all the UPR arms are indeed set off. Studies on physiologically induced ER stress due to pathological conditions (like expression of mutant proteins) or to increased demand for secretory capacity (as in the case of secretory cells differentiation) showed on the contrary that different cell types selectively exploit various elements of the UPR. Indeed only certain components of the UPR are necessary for plasma cell differentiation.

My studies aimed mainly to the identification of elements of the UPR involved in plasma cell differentiation and to the characterization of their role. I indeed describe here the involvement of a UPR player, the transcription factor CHOP. Unexpectedly though, my data suggest that during this process CHOP is expressed independently of the UPR. The characterization of its role in plasma cell differentiation evidenced that CHOP expression is not essential but led to the identification of a novel role for this transcription factor in the ER homeostasis, likely cell-type specific. I furthermore identified a novel gene downstream of CHOP, the transcription factor ATF5 whose

role in plasma cell differentiation and in the UPR must be further analyzed.

Characterization of plasma cell molecular physiology being extensively studied in our laboratory, I collaborated with my colleagues in an effort to elucidate the mechanism regulating plasma cell life span (appendix I). In particular I utilized a murine lymphoma cell line and a mouse model to support our findings indicating that inefficient proteasomal capacity with respect to the increased load challenging ASC is one of the elements leading to plasma cell apoptosis. In the same context, in collaboration with a colleague, I recently set out to study the redox homeostasis during plasma cell differentiation. Our preliminary findings indicate that ASC undergo increased levels of oxidative stress when producing the maximum amount of IgM.

3. Results

3.1 Identification of a novel role for CHOP in the secretory pathway of antibody secreting cells

3.1.1 The transcription factor CHOP is expressed in I.29μ⁺, a model cell line to study antibody secreting cell differentiation

I.29μ⁺ is a murine lymphoma cell line that can be induced to differentiate to plasma cell-like antibody secreting cells (ASC) by exposure to bacterial lipopolysaccharide (LPS) (Romijn et al., 2005; van Anken et al., 2003). Differentiation can be assessed by analysing various parameters that I.29μ⁺ share with their primary counterpart, such as for instance the expression on their plasma membrane of the heparan sulphate proteoglycan syndecan-1 (CD138) (Fig.1 A). Upon LPS stimulation it is possible to observe the switch between the expression of the membrane isoform of μ chain (μ_m, part of the BCR) and the secretory one (μ_s), together with highly increased levels of IgM expression and secretion (Fig.1 B). The UPR transcription factor XBP-1 is essential for plasma cell differentiation and indeed differentiated I.29μ⁺ increase the expression of the active, spliced isoform sXBP1 (Fig.1 C). The life span of differentiating B cell is short, almost half of the cells die 4 days after LPS stimulation (Fig.1 C).

In a search for UPR players up regulated during I.29μ⁺ differentiation I found that these cells increase CHOP expression shortly after LPS stimulation (Fig.1 D). I then asked if CHOP induction could be observed also in differentiating primary B lymphocytes.

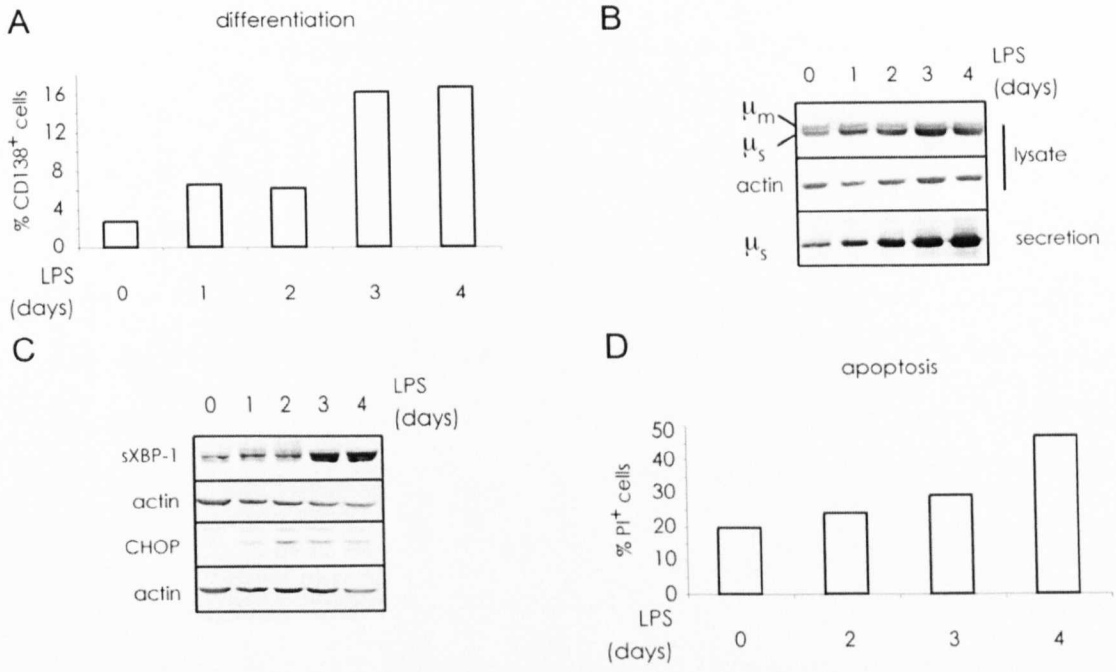

Fig. 1 I.29μ⁺ up regulate CHOP expression during differentiation to ASC

The murine lymphoma line I.29μ⁺ was induced to differentiate to ASC with 20 μg/ml LPS. At the indicated time points differentiation was followed by checking various parameters. **A** Surface expression of the plasma cell marker CD138/syndecan-1 was measured by flow cytometry, staining the cells with an anti-CD138 antibody PE-conjugated. **B** Protein extracts from differentiating cells (lysate) and culture media (secretion) were treated with the enzyme PNGase F to remove glycans and separated by SDS-PAGE. The blotted filters were probed with an anti-μ antibody to follow the expression levels of the membrane (μ_m) and secreted (μ_s) μ chain isoforms. **C** Filters prepared as in B were probed with antibodies specific to XBP-1 and CHOP. Only the region of the blot corresponding to molecular weight of the spliced form of XBP-1 is shown. Actin expression level was used as loading control. **D** Apoptosis was measured by propidium iodide (PI) staining followed by flow cytometry analysis.

3.1.2 CHOP is transiently induced during primary antibody secreting cell differentiation but its absence does not impede it

Mouse mature primary splenic B cells can be readily induced to undergo terminal differentiation *in vitro* by LPS treatment, providing an optimal tool to study differentiation of antibody secreting cells (Iwakoshi et al., 2003b; Shaffer et al., 2004). I found that the transcription factor CHOP is transiently up regulated early during ASC differentiation: in the experiment shown in figure 2A CHOP transcript expression level peaked at day 1 upon LPS stimulation and became barely detectable

thereafter. In other experiments, CHOP transcription and synthesis peaked at day 2, but I never observed CHOP in the nucleus at day 3 or after (Fig.2 B). As a control for CHOP induction I treated B lymphocytes and ASC with the ER stress inducing agent thapsigargin (Tg), which depletes ER Ca^{2+} stores heavily affecting protein folding. An increase in CHOP nuclear levels was observed in both lymphocytes and ASC indicating that CHOP silencing in the later phases of differentiation is not irreversible. Since CHOP is up regulated in response to various types of stress, including oxidative stress, and isolation and *in vitro* differentiation of primary B lymphocytes expose the cells to a much higher pO_2 with respect of that found *in vivo*, I checked if the same pattern of CHOP expression could be observed in *in vivo* differentiated plasma cells. To this aim wt mice were challenged with an intra-peritoneum injection of LPS and plasma cells were isolated at the indicated time points (Fig.2 C). Real time PCR analysis of mRNA purified from plasma cells confirmed that *chop* messenger is up-regulated at the early time points of B cell differentiation. On the basis of my experience, I believe that the diversity in the fold of increase between the experiments shown in figure 2A and 2C depends on the variability observed among mice and not on the different experimental settings.

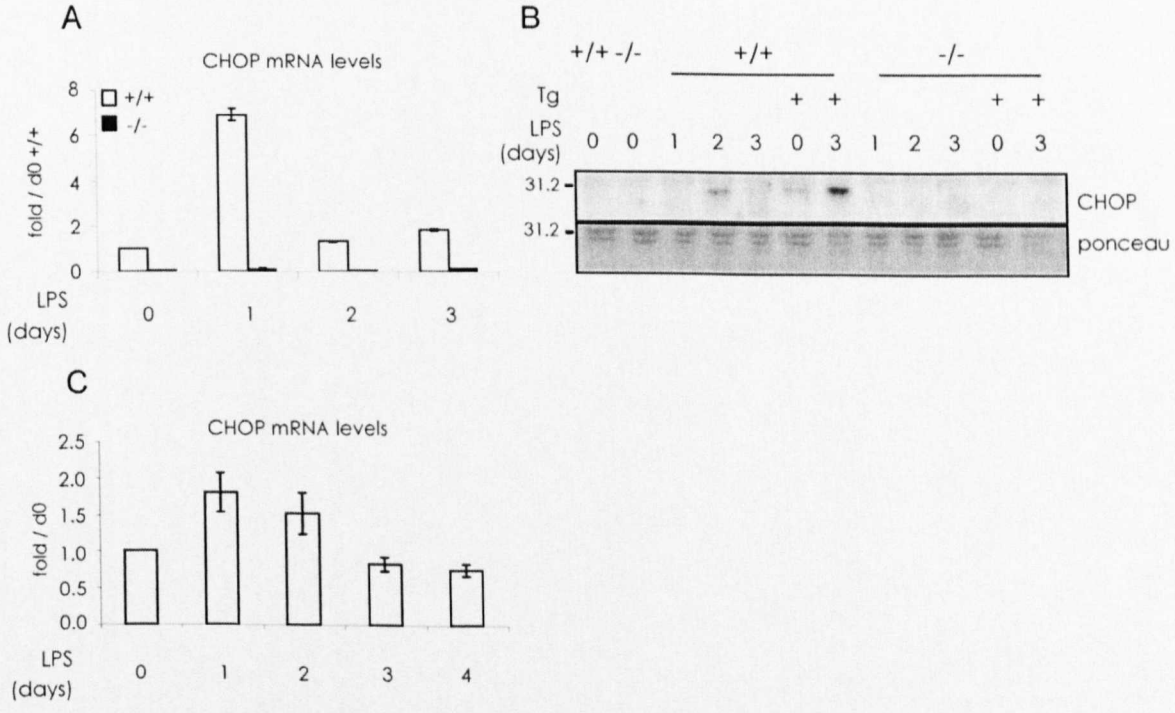

Fig. 2 Differentiating primary B lymphocytes transiently increase CHOP mRNA and protein

B lymphocytes ($CD19^+$) isolated from wt and *chop*$^{-/-}$ mouse spleens were induced to differentiate into antibody secreting cells (ASC) by incubation *in vitro* with 20 μg/ml LPS for the indicated time points. **A** CHOP mRNA expression pattern during differentiation was followed by relative real time PCR. The fold increase at each time point with respect to day 0 wt was calculated by the $\Delta\Delta C_t$ method, using histone 3 as reference gene. Error bars represent the SEM for the average of PCR triplicates of the same samples. **B** CHOP protein up regulation was detected by Western blot of nuclear extracts of differentiating B cells decorated with an anti-CHOP antibody. B cells at day 0 or day 3 after LPS stimulation were treated for 3hrs with 2 μg/ml of the ER stress inducer thapsigargin (Tg) and the nuclear protein extracts were used as positive controls for CHOP induction. Ponceau staining of the nitrocellulose filter is shown as loading control. **C** c57/BL6 mice were injected intra-peritoneum with LPS only at the starting point of the experiment (day0). After LPS injection 3 mice for each time point were sacrificed and differentiating B cells were purified and mixed to extract total RNA. CHOP transcript was detected as in A (error bars represent the SEM for the average of PCR triplicates of the same samples).

In order to investigate the role of CHOP in plasma cells I turned to the analysis of *chop*-null mice. These are viable and fertile and show no obvious phenotype (Oyadomari et al., 2002; Zinszner et al., 1998). Accordingly, B and T lymphocytes were present in the same percentage in spleens and lymph nodes of wt or *chop*$^{-/-}$ mice (Fig.3 A). Spleens from *chop*$^{-/-}$ mice showed the same gross morphology as those harvested from wt littermates, and a comparable number of $CD19^+$ cells could be purified from spleens of either genotype (data not shown). These observations indicate that CHOP is not essential for B cell development. However, the transient appearance of CHOP in the nucleus might reflect a functional role for this transcription factor in ASC differentiation. Therefore, I stimulated B splenocytes from *chop*$^{-/-}$ mice or control littermates with LPS and analyzed numerous parameters of ASC differentiation (Figures 3 and 4). The percentage of cells expressing on their surface the plasma cell marker CD138 was alike in wt and *chop*$^{-/-}$ cultures (Fig.3 B). After an initial phase of intense proliferation ASC start dying and after 4-5 days of LPS stimulation around 40%-50% of cells undergo apoptosis. The patterns of proliferation and death were similar in wt and *chop*$^{-/-}$ differentiating B cells (Fig.3 C and results not shown) indicating that CHOP plays no essential role in

differentiation-related apoptosis. The strong increase in expression and synthesis of IgM subunits was unaffected in *chop*$^{-/-}$ ASC as well (Fig.3 D and E).

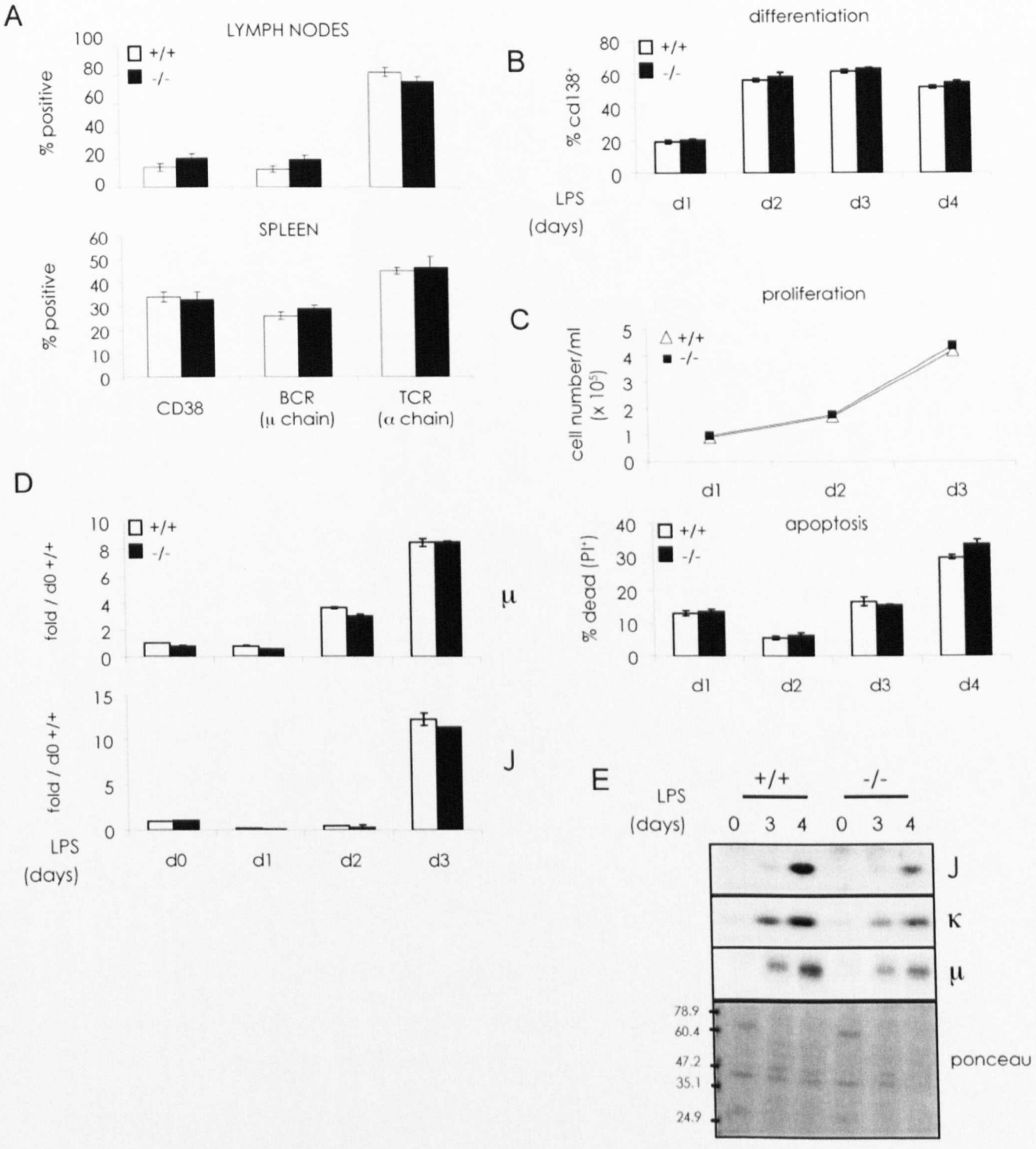

Fig. 3 CHOP deletion does not affect the normal pattern of B cell differentiation

A The totality of cells harvested from lymph nodes and spleens were stained with FITC-conjugated antibodies against the B lymphocyte markers CD38 and BCR μ_m chain and the T lymphocyte's one TCR α chain and analyzed by flow cytometry (n=3; error bars represent the SEM of the average). **B-E** B lymphocytes isolated from wt and *chop*$^{-/-}$ spleens were incubated in the presence of LPS and differentiation progression was analyzed by various parameters. **B** Surface expression of the plasma cell marker CD138/*syndecan-1* was measured by flow cytometry after staining the cells with an anti-CD138 antibody PE-conjugated (n=3; error bars represent the SEM of the average). **C** Growth curve *(top panel)* was obtained culturing the cells at the initial density of 10^5 cells/ml and measuring the cell density at the indicated time points. The percentage of dead cells *(bottom panel)* was measured by staining the cells with propidium iodide (PI) followed by flow cytometry analysis (n=3; error bars represent the SEM of the average) To confirm death by apoptosis the experiments were repeated performing the TUNEL assay, obtaining the same results (data not shown). **D** IgM subunits μ and J chains transcript levels were measured by relative real-time PCR performed as in Fig. 2 A **E** IgM subunits J, κ and μ chains protein expression was assessed by western blot, sequentially probing the filter with specific antibodies. Ponceau staining of the nitrocellulose filter is presented as loading control.

I then checked if components of the UPR, normally activated during plasma cell differentiation, were mis-regulated in *chop*$^{-/-}$ ASCs. Hence, I assessed the induction of ATF6/IRE1pathways and found that these were normal. Up regulation and splicing of *xbp1* mRNA and sXBP1 protein appearance showed the same dynamics in wt and in *chop*$^{-/-}$ cells (Fig.4 A, B and C). Consistently with the similar levels of sXBP1, the mRNA levels of its targets *edem* and *ERdj4* (Kanemoto et al., 2005; Lee et al., 2003; Yoshida et al., 2003), displayed indistinguishable kinetics of activation in wt and *chop*$^{-/-}$ cells (Fig.4 D). Also the levels of ERdj3, another target of sXBP1 during ER stress and PC differentiation (Shen and Hendershot, 2007) were the same after 3 and 4 days of LPS stimulation (Fig.4 C). Particularly important was to study the expression pattern of HERP in ASC differentiation, because together with CHOP is the only known UPR target dually regulated by the ATF6-XBP1 and by the PERK/ATF4 pathways, via ERSE and C/EBP-ATF composite element respectively (Ma and Hendershot, 2004). HERP is an ER localized protein involved in the ER associated degradation (ERAD) and protects against ER stress-induced apoptosis (Liang et al., 2006; Okuda-Shimizu and Hendershot, 2007). I found that HERP is

strongly up regulated in wt as well as in *chop*$^{-/-}$ ASC at the latest days of differentiation, as the other UPR players (Fig.4 C).

From the analysis of *chop*$^{-/-}$ B cell I reckon that CHOP is dispensable for plasma cell differentiation.

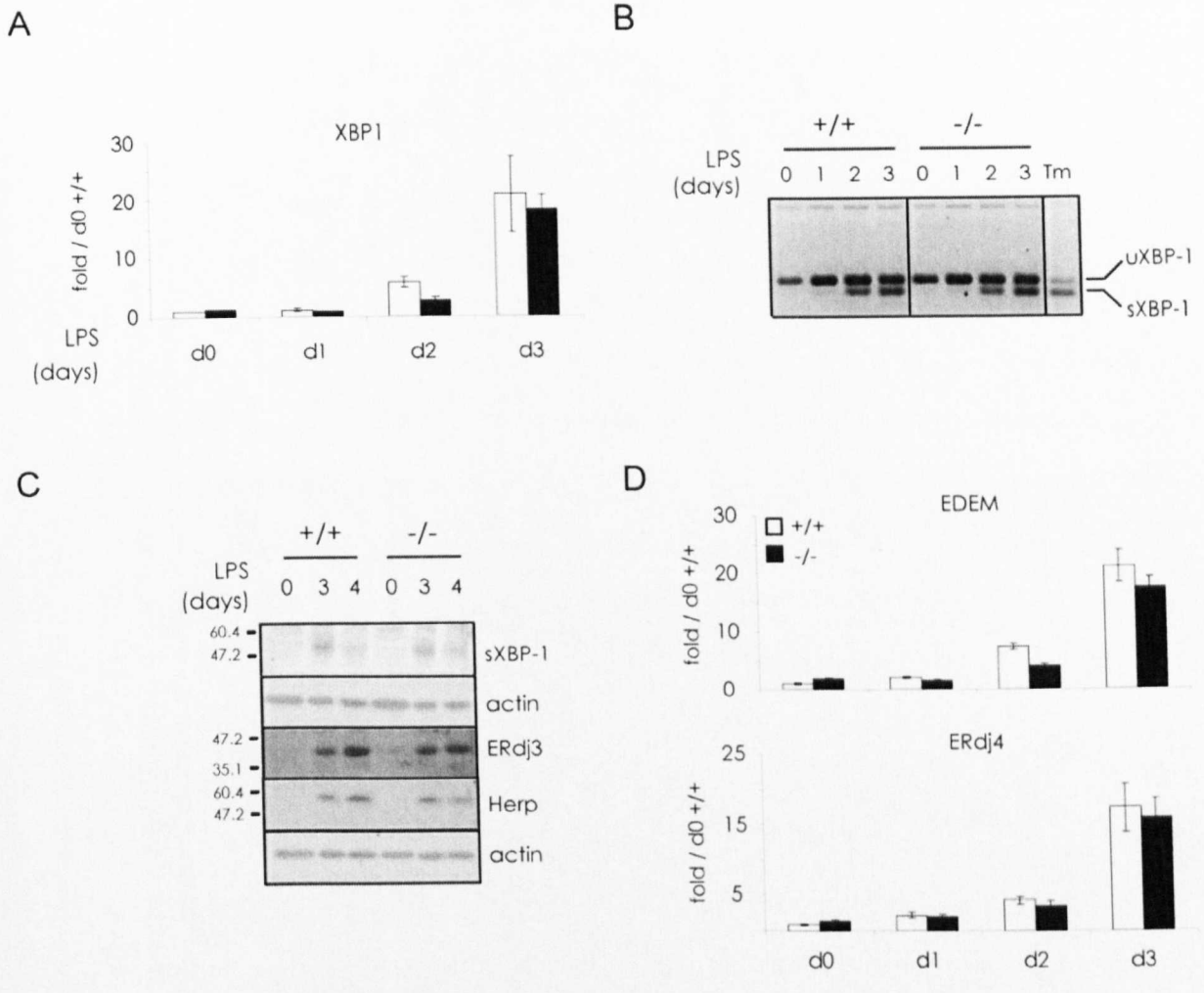

Fig. 4 UPR signaling is intact in chop$^{-/-}$ ASC

B lymphocytes isolated from wt and *chop*$^{-/-}$ spleens were incubated in the presence of LPS and at the indicated time points RNA and protein extracts were obtained to study the expression pattern of various components of the UPR **A** The amount of XBP1 transcript was measured by relative real-time PCR as in Fig.2 A. **B** Activation of XBP1 pathway was assessed by RT-PCR with primers specific for the un-spliced (uXBP1) and the spliced (sXBP1) forms of XBP1 mRNA. RNA purified from I.29μ^{+} cells treated with the ER stress inducer tunicamycin was used as positive control (Tm). **C** Protein levels of sXBP1, ERdj3 and HERP were measured by western blot with the corresponding antibodies. Actin was used as loading control. **D** EDEM and ERdj4 mRNA levels were quantified by relative real time PRC as in Fig.2 A.

3.1.3 CHOP expression is important for efficient IgM polymerization and secretion

The most important feature of plasma cells is their ability of efficiently producing, assembling and secreting very high amounts of Ig. As shown above, the increase in the synthesis of IgM subunit was similar in wt and *chop*$^{-/-}$ ASC cells (Fig.3 D and E). However, a subtle phenotype became evident when we compared IgM assembly by non-reducing western blotting. As shown in figure 5A, *chop*$^{-/-}$ ASC accumulated more high molecular weight (HMW) species than the wt cells at the latest days of differentiation, namely after three to five days upon LPS stimulation depending on the experiment (Fig.5A and not shown). These high molecular weight species contained J, κ and μ chains and consisted of disulphide-bonded aggregates, as they were no longer detectable after boiling in DTT (data not shown). Densitometric analyses confirmed that the *chop*$^{-/-}$ ASC accumulated significantly more HMW complexes while similarly assembling the other IgM species (Fig.5 B).

As these data suggested impairment of the secretory pathway and protein aggregation, I performed electron microscopy analysis to detect possible morphological alterations in the ER. Whilst wt ASC's cytoplasm was mostly filled by regular stacks of ER cisternae, many *chop*$^{-/-}$ cells presented deformed, very enlarged cisternae supporting the previous observations pointing to protein aggregation (Fig.5 C).

Since HMW aggregates are retained intracellularly, I reasoned that *chop*$^{-/-}$ ASC should secrete fewer IgM with respect to the wt cells. I thus quantified IgM secretion in wt and *chop*$^{-/-}$ ASC cultures by ELISA. The results indicated that *chop*$^{-/-}$ cells secrete less IgM polymers (Fig.5 D). Accordingly, different concentrations of the reducing agent 2ME were necessary to increase secretion of unpolymerized IgM (Alberini et al., 1990) in *chop*$^{-/-}$ rather than in wt ASC (Fig.5 E). At lower concentrations, 2ME promoted secretion in ko as well as in wt cultures. On the contrary, higher concentrations of 2ME allowed the release of the more abundant IgM aggregates in the *chop*$^{-/-}$ cells but negatively affected the wt cells, where IgM are assembled and secreted with higher efficiency and there is less aggregation. At high

concentrations, 2ME impairs IgM secretion and promotes μ chain degradation in hybridoma cells (Shachar et al., 1994), likely because it disturbs oxidative folding of nascent Igs (Valetti and Sitia, 1994). When I measured IgG concentrations in the culture media I found that secretion of this isotype was impaired as well in *chop*$^{-/-}$ ASC (Fig.5 D). However 2ME inhibited IgG secretion at the same rate in both genotypes pointing to a cause of reduced IgG secretion other than covalent aggregation (Fig.5 E).

Since CHOP is involved also in oxidative stress responses it was important to exclude that the phenotype observed in *chop*$^{-/-}$ ASC were related to altered redox equilibrium. Thus I investigated if *chop*$^{-/-}$ plasma cells exhibit the same defect at physiological pO_2. In order to verify if *chop*$^{-/-}$ plasma cells secrete sub-optimal amounts of IgM also *in vivo*, and if such defect extends to other Ig isotypes, wt and *chop*$^{-/-}$ mice were immunized sub-cutaneously with ovalbumin. Blood samples were taken at the indicated time points and sera levels of ovalbumin-specific IgM and IgG were assessed by ELISA. To normalize for the variability of Ig concentration in the pre-immune sera the data were analyzed as the ratio between the absorbance values obtained at a given time point and the earliest time point of the assay (i.e. day 0 for IgM and day 4 for IgG), as detailed in figure 5F. In keeping with the data obtained *in vitro*, I found that upon immunization the increase in IgM sera concentrations was lower in *chop*$^{-/-}$ than in wt mice. On the contrary there was no difference in IgG increase. This observation is in contrast with the results I obtained with ASC differentiated *in vitro*. Together with the data obtained with the 2ME treatment this fact supports the idea that the impairment of IgG secretion *in vitro* in *chop*$^{-/-}$ culture is due to a different mechanism than that affecting IgM folding. The *in vivo* analysis leads to the conclusion that IgG secretion is normal in *chop*$^{-/-}$ plasma cells.

In summary, I conclude that even though CHOP is not essential for plasma cell differentiation, it allows optimal functionality of the IgM assembly machinery and consequently higher levels of IgM secretion.

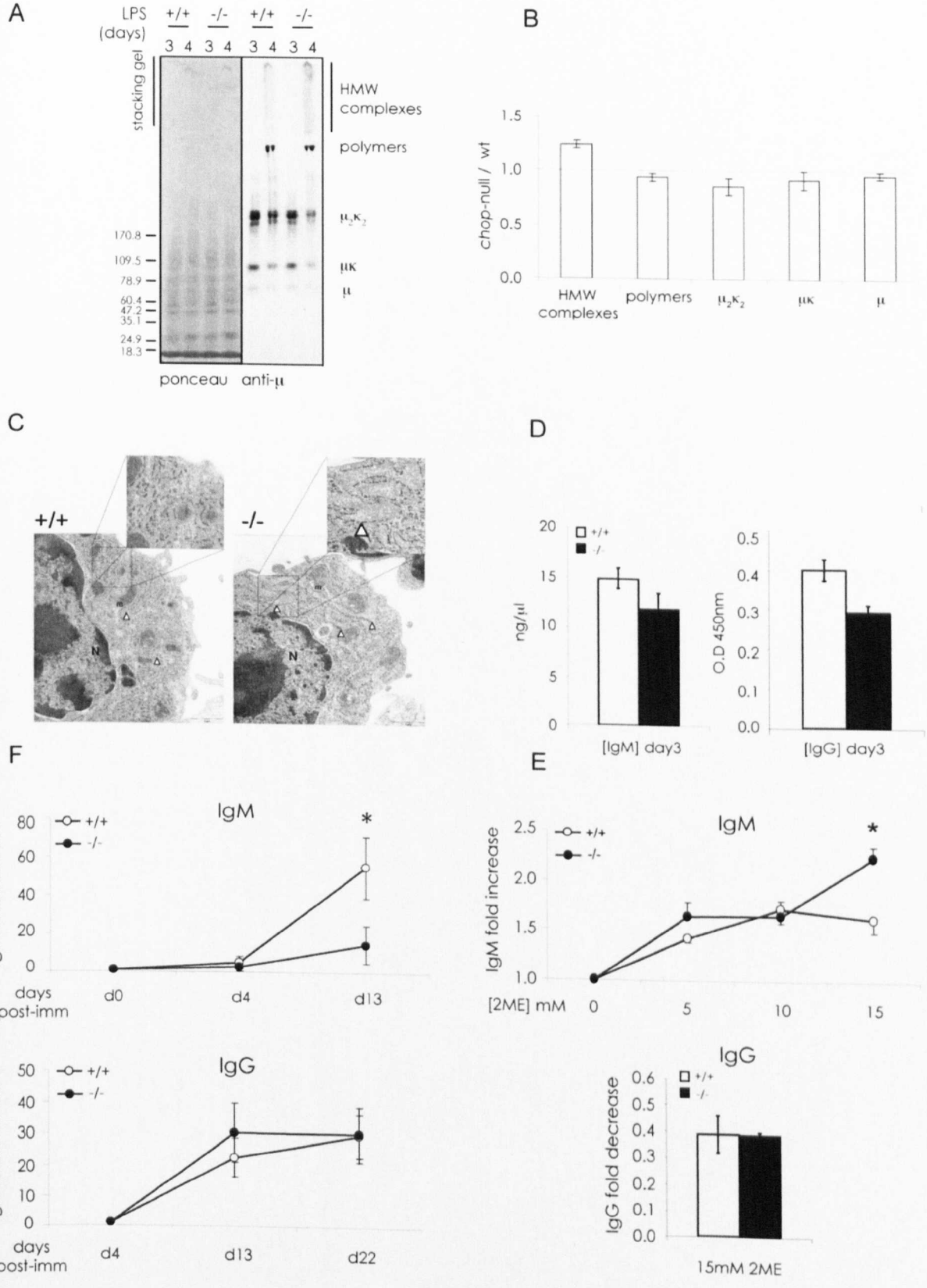
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Fig.5 CHOP promotes optimal assembly and secretion of IgM

A IgM polymerization was analyzed by non-reducing western blots. Total protein extracts from wt and *chop*$^{-/-}$ B cells after 3 and 4 days of LPS stimulation were subjected to SDS-PAGE in non-reducing conditions, in order to preserve disulphide bonds among the IgM subunits and blotted. The nitrocellulose filter was incubated with anti-μ chain antibodies and the main IgM assembly intermediates were identified. **B** Quantification of the relative amount of each IgM species in *chop*$^{-/-}$ ASC with respect to the wt. The percentage of each prevalent IgM species (HMW complexes, μ_2L_2, μL, μ) relative to the sum of all the species was calculated after densitometry analysis of non-reducing western blots as shown in A. The histogram reports the average of the ratio between the percentages of each IgM species in *chop*$^{-/-}$ samples to that found in the wt samples from four independent experiments (n=9; error bars represent the SEM of the average). Depending on the experiment the analysis was performed at 3 to 5 days after induction of differentiation. **C** Morphology of wt and *chop*$^{-/-}$ ASC at day 4 of differentiation was studied by transmission electron microscopy. N, nuclei; m, mitochondria; white arrowheads indicate rough ER tubules. wt: 77% (n=13); ko: 50% (n=14) of cells with regular ER. **D** Secretion efficiency of wt and *chop*$^{-/-}$ ASC 3 days after LPS stimulation was tested by ELISA measurement of IgM and IgG concentrations in medium of cultures at 10^6 cells/ml incubated for 4hrs. The histograms report the average concentrations obtained in two independent experiments (n=4; error bars represent the SEM of the average). **E** Effects of the reducing agent 2-mercaptoethanol (2-ME) on IgM secretion. Secretion assay was performed as described in D after 5 days upon LPS stimulation in the absence or in the presence of the indicated concentrations of 2-ME. IgM and IgG concentrations in the media were measured by ELISA. Data are reported as the ratio between the absorbance measured in the presence of the indicated concentrations of 2-ME and that measured in the absence (n=4, error bars represent the SEM of the average, * indicates a p value < 0.05 (Student's T test)). **F** Ovalbumin-specific IgM and IgG titers in mouse sera upon ovalbumin immunization. Mice were immunized with by sub-cutaneous injection of ovalbumin and sera were collected at 0, 4, 13 and 22 days post-immunization. IgM and IgG sera concentration were measured by ELISA. Data are reported as the ratio between the absorbance measured at the indicated time point and the earliest time point in each assay (d0 for IgM and d4 for IgG) (n=5; error bars represent the SEM of the average, * indicates a p value < 0.05 (Student's T test)).

3.1.4 CHOP absence lowers the expression of BiP and ERp44, ER chaperones involved in IgM polymerization

The observation that *chop*$^{-/-}$ ASC are more prone to generate IgM aggregates prompted me to investigate the levels of expression of the ER chaperones involved in IgM polymerization. IgM polymerization is a stepwise process under the control of a strict quality control system. BiP (originally known as GRP78), one of the main ER chaperones, is involved in the earliest step of IgM polymerization binding μ chain

thus avoiding its aggregation or secretion until it is joined to the light chain (Hendershot and Kearney, 1988; Vanhove et al., 2001). ERp44 has a main role in the thiol-mediated retention system, blocking secretion of un-polymerized IgM and recently we proposed that ERp44 collaborates with ERGICp53 favoring IgM polymerization (Anelli et al., 2007). Another player likely involved in IgM polymerization is the ER oxidoreductase Ero1α: Ig and in particular IgM, are rich in disulphide bonds and Ero1 proteins are essential for their formation. When I analyzed the levels of expression of this set of chaperones I found, as expected, that all of them were up regulated during ASC differentiation (Fig. 6). BiP up-regulation was slightly reduced in the differentiating *chop*$^{-/-}$ cells, both at the mRNA and at the protein level (Fig.6 A and B). ERGICp53 expression pattern was the same in wt and ko cells while I found a difference in the amount of ERp44 protein that was slightly less in the *chop*-null ASC compared to the wt (Fig.6 C). Immunofluorescence analysis of ASC indicates as well that ERp44 is less expressed and/or differentially localized in *chop*$^{-/-}$ ASC (Fig.6 D). Unfortunately, at this moment, variability among different experiments does not allow me to draw definitive conclusions about the transcriptional regulation of ERp44 in *chop*-null cells. Previous studies from Ron and colleagues (Marciniak et al., 2004) showed that *ero1α* expression is regulated by CHOP in mouse embryonic fibroblasts as *chop*-null MEF show strongly decreased up-regulation of Ero1α upon UPR induction. They also showed by ChIP assays that such regulation is directly exerted on Ero1α promoter. I found no differences between the two genotypes in the up-regulation of Ero1α protein during B cell differentiation (Fig.6 B). Analysis of the expression pattern of the mRNA however, indicates that at the time points in which CHOP is expressed during ASC differentiation Ero1α mRNA amount is less in the ko cells than in wt (Fig.6 A). A compensation mechanism seems to increase the amount of Ero1α messenger in the *chop*-null cells in the following hours, when CHOP expression is shut down. This observation could explain why I do not detect differences in the protein amounts between wt and *chop*$^{-/-}$ cells.

In summary, among the chaperones involved in IgM polymerization that I analyzed, I found that BiP and ERp44 proteins are still increased during differentiation of *chop*$^{-/-}$ B cells but to a lesser extent with respect to the wt ones, pointing to the presence of

alterations in the IgM polymerization machinery and quality control system. Indeed we recently showed that ERp44 down-regulation by RNAi in a reconstituted system of IgM polymerization in HeLa cells decreases polymers formation ((Anelli et al., 2007) appendix II). From my present data I are not able to assess how these observations are related with the tendency of *chop*$^{-/-}$ ASC to form IgM aggregates and this will be matter of further investigation. However, my results suggest that among CHOP targets during B cells differentiation there is one or more factor(s) whose expression allows full up-regulation of BiP and ERp44. It is indeed unlikely that CHOP directly regulates BiP and ERp44 expression since its transient up regulation terminates much before they reach the maximal levels of expression (compare Fig. 2 and 6)

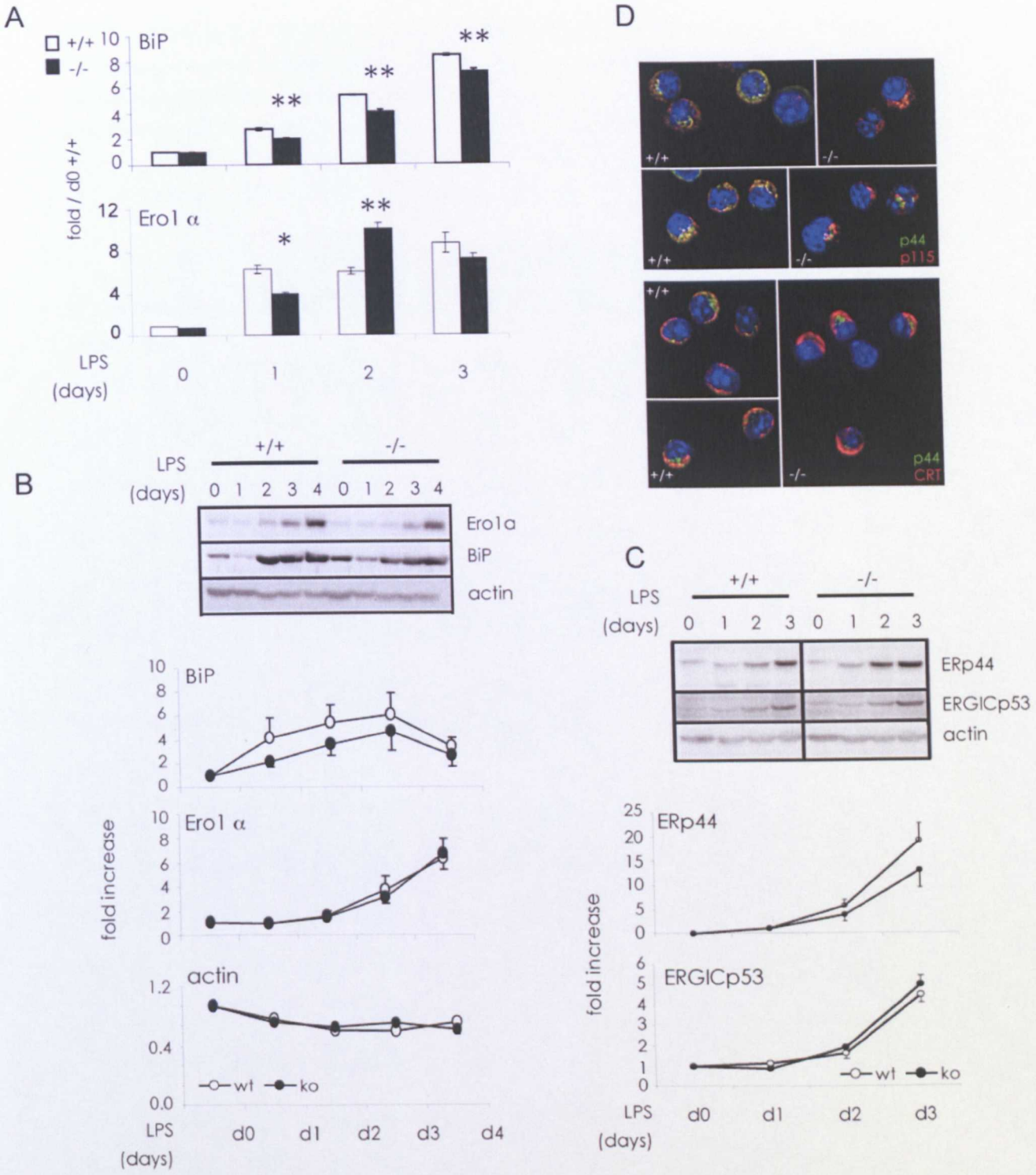


Fig.6 CHOP$^{-/-}$ ASC up regulate BiP and ERp44 to a lesser extent than wt cells

B splenocytes differentiated *in vitro* were analyzed for the expression of chaperones involved in IgM polymerization. **A** Relative real time PCR as in Fig.2 A was performed to quantify BiP and Ero1α transcript levels (error bars represent the SEM of the average among PCR triplicates of the same samples; ** p value < 0.005, * p value < 0.01 (Student's T test)). **B-C** Ero1α, BiP, ERp44 and ERGICp53 protein expression was analyzed by western blot with specific antibodies. Actin was used as loading control (*top panels*). Densitometric analysis of western

blots as shown in the top panels (*bottom panels).* Data are reported as fold increase relative to the protein amounts measured at day0, normalized to actin (error bars represent the SEM of the average fold increase in four independent samples for each genotype). **D** Expression level and localization of ERp44 (green) was analyzed by immunofluorescence staining of wt and *chop*$^{-/-}$ cells 3 days after LPS stimulation. ERGIC and ER compartments are labeled by the resident proteins p115 and calreticulin respectively (red).

3.1.5 CHOP$^{-/-}$ *ASC express lower levels of ERdj5, a newly identified ER resident reductase*

Very recently Prof. K. Nagata and his collaborators described for the first time the existence of an ER resident protein with a specific thiol-reductase activity, ERdj5 (personal communication, Ushioda et al. manuscript in preparation). ERdj5 was first identified in our laboratory as a chaperone expressed in secretory cells and induced during ER stress (Cunnea et al., 2003). It belongs to the ERdj family, which comprises ER proteins containing a DnaJ domain, by which they can interact with hsp70 family proteins like BiP. Among the five identified members of the ERdj family only ERdj5 possesses also a thioredoxin-like domain CXXC. Nagata and colleagues demonstrated that ERdj5 exerts reductase activity that requires its CXXC motifs and that it enhances ER associated degradation (ERAD) of misfolded proteins by preventing the formation of disulfide-bound aggregates. Furthermore they showed that it interacts with EDEM, a fundamental component of the ERAD machinery and that in order to promote degradation of misfolded proteins it must also have an intact DnaJ domain to associate with BiP.

These observations prompted me to investigate the expression pattern of ERdj5 during B cell differentiation, wondering if that could have a role in minimizing IgM aggregation.

I found strong up-regulation of ERdj5 protein during ASC differentiation, suggesting that it could play an important role. Surprisingly its levels of expression were significantly reduced in *chop*$^{-/-}$ cells (Fig.7 A). As discussed for BiP and ERp44, it is unlikely that CHOP directly activates ERdj5 expression: however it could induce the expression of one or more factor(s) that then enhance ERdj5 promoter activation. I

hypothesize that reduced amounts of ERdj5 in *chop*-null cells could generate over-oxidizing conditions in the ER and explain the increased tendency to generate IgM aggregates.

In order to prove this theory I would need on the one hand to knock-down Erdj5 in wt ASC and on the other to over-express it in *chop*$^{-/-}$ cells. If the hypothesis is correct then I should observe enhanced IgM aggregation in the first case and reduced aggregation in the second one. Since, for unclear reasons, transfection of B cells is not easily achieved, alternative strategies are being considered as discussed below.

While setting up the appropriate tools, I turned to other systems to start investigating the role of ERdj5 in IgM polymerization. Non-B cell types are able to assemble different IgM intermediates when transfected with cDNAs encoding for the various subunits. Diverse epithelial cell lines show differential efficiency in assembling higher order IgM intermediates. I exploited HEK293FT (293) to reconstitute IgM polymerization with or without simultaneous over-expression of ERdj5, in order to study the influence of this chaperone on IgM polymers formation (Fig.7 B). My results show that when 293 are simultaneously transfected with μ and λ they form various IgM intermediates species and I identified the main ones as $\mu\lambda$, $\mu_2\lambda_2$ and polymers. When also J is transfected together with μ and λ the polymers molecular weight is increased since they comprise the J chain, as expected. ERdj5 over-expression reduced the amount of polymers in both J$\mu\lambda$ and $\mu\lambda$ transfectants to the advantage of lower degree IgM assembly, $\mu_2\lambda_2$, $\mu\lambda$ and μ.

Taken together my data point to the involvement of ERdj5 in IgM polymerization during which it exerts reductase activity.

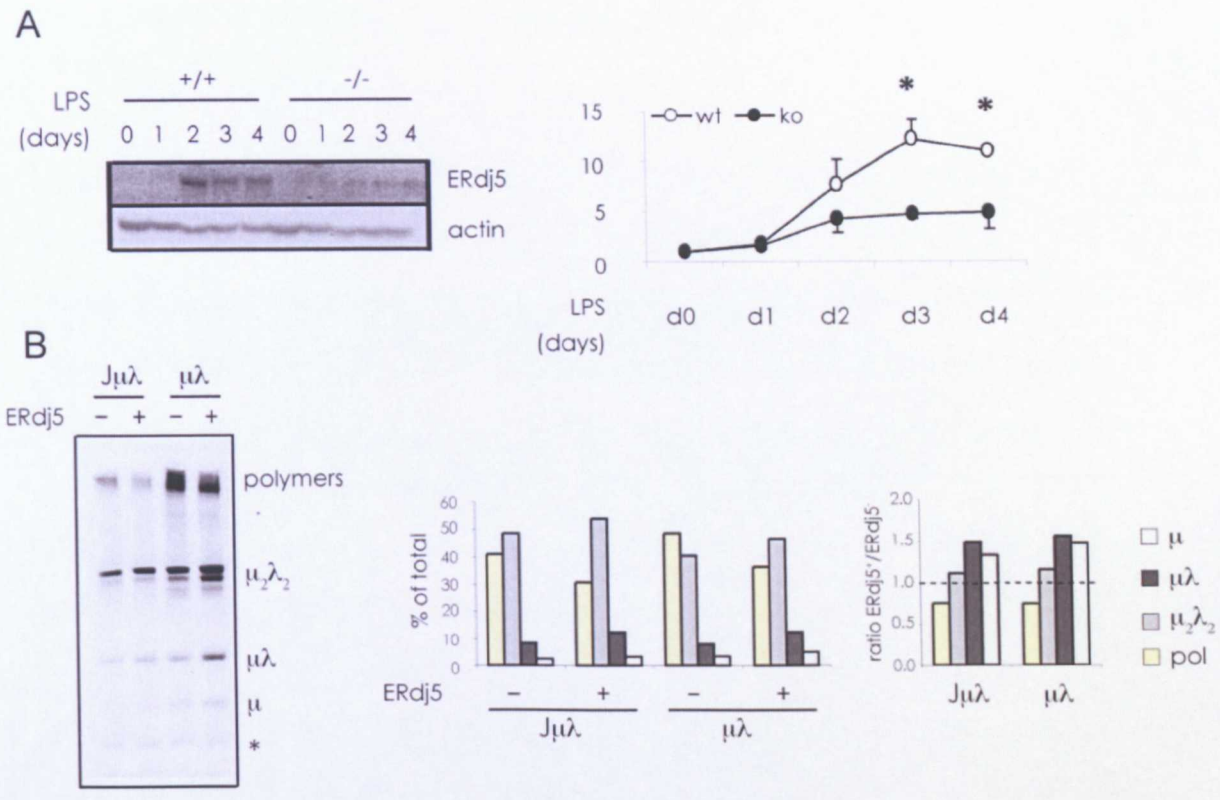


Fig.7 The ER resident reductase ERdj5 is involved in IgM polymerization and is expressed at lower levels in chop$^{-/-}$ ASC

A ERdj5 protein expression during *in vitro* differentiation of wt and *chop*$^{-/-}$ B cells was analyzed by western blot. Actin was used as loading control (*left panel*). Densitometric analysis of western blots as shown in the left panel (*right panel).* Data are reported as fold increase relative to the protein amounts measured at day0, normalized to actin (error bars represent the SEM of the average fold increase in four independent samples for each genotype; * p value <0.05 (Student's T test)). **B** HEK293FT cells were transfected with plasmids encoding for the IgM subunits μ, λ and J chains, as indicated, with or without an additional plasmid expressing ERdj5. 48 hrs after transfection protein extracts were separated by SDS-PAGE in non-reducing conditions to preserve disulphide bonds and blotted. The nitrocellulose filter was probed with anti-μ antibodies to individuate IgM polymerization intermediates (*left panel*). The asterisk indicate an aspecific band. Densitometric analysis of the western blot shown in the left panel (*right panel*). Data presented in the left side histograms report the percentage of each IgM intermediate relative to the total amount of μ in the lane. In the right side histograms are plotted the ratios between the percentage of each IgM intermediate in the presence of ERdj5 to that in the absence.

3.1.6 CHOP$^{-/-}$ B cells (but not T cells) are more sensitive to ER stress-induced death

It is well accepted that during the UPR CHOP mainly plays a pro-apoptotic role (Marciniak et al., 2004; Oyadomari et al., 2002; Oyadomari and Mori, 2004). The downstream pathways are not fully elucidated yet but *chop*$^{-/-}$ MEF are more resistant than wt to ER-stress induced death and *chop*-null mice show a lower degree of kidney damage relatively to wt siblings when systemically treated with the ER stress inducer tunicamycin (Marciniak et al., 2004). The same study indicated that CHOP deletion exerts a protective effect by reducing the load of client proteins in the ER and by altering the ER redox equilibrium toward reducing conditions. However, in one study on a physiological system of ER stress induction in oligodendrocytes CHOP was found to have an anti-apoptotic role, since oligodendrocytes from *chop*$^{-/-}$ mice were more resistant to death induced by over-expression of a mutant protein (Southwood et al., 2002). Furthermore a stably transfected clone of the myeloma line NS0, was protected from UPR-induced death when CHOP was down-regulated by siRNA (Cudna and Dickson, 2006). I thus wondered how CHOP deletion affected B cells sensitivity to ER stress-induced death. I incubated purified B and T cells with tunicamycin (Tm), which induces ER stress by inhibiting glycosylation of *de-novo* synthesized proteins impairing their folding. I found that *chop*$^{-/-}$ B cells were consistently more sensitive to ER stress when compared to wt cells, whereas T cells did not show a significantly different sensitivity (Fig.8 A). The same results were obtained when I tested the sensitivity of *chop*-null ASC compared to wt (Fig.8 B). Thus I show that even though B cells up regulate CHOP during the UPR (Fig.2 B) its deletion worsens the effects of ER stress, in contrast to observations in MEF or pancreatic β cells for example. T cells do not seem to be affected. My findings, together with the numerous published studies, suggest a cell type specific role in ER stress-induced apoptosis for this transcription factor.

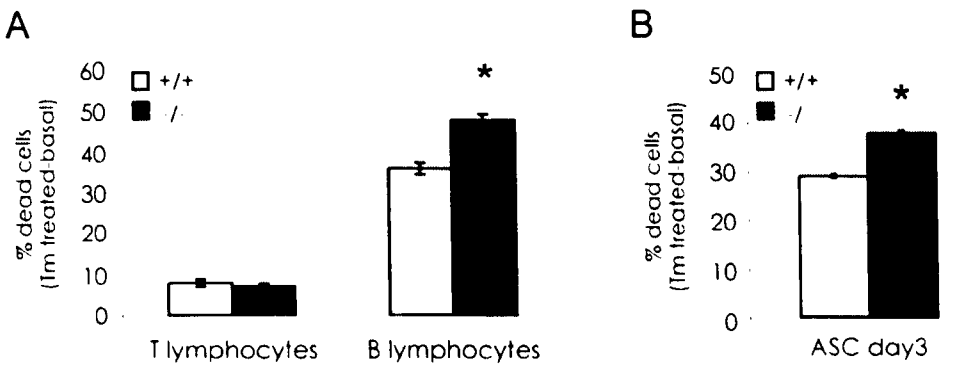


Fig.8 chop$^{-/-}$ B lymphoblasts and ASC are more sensitive to ER stress-induced apoptosis

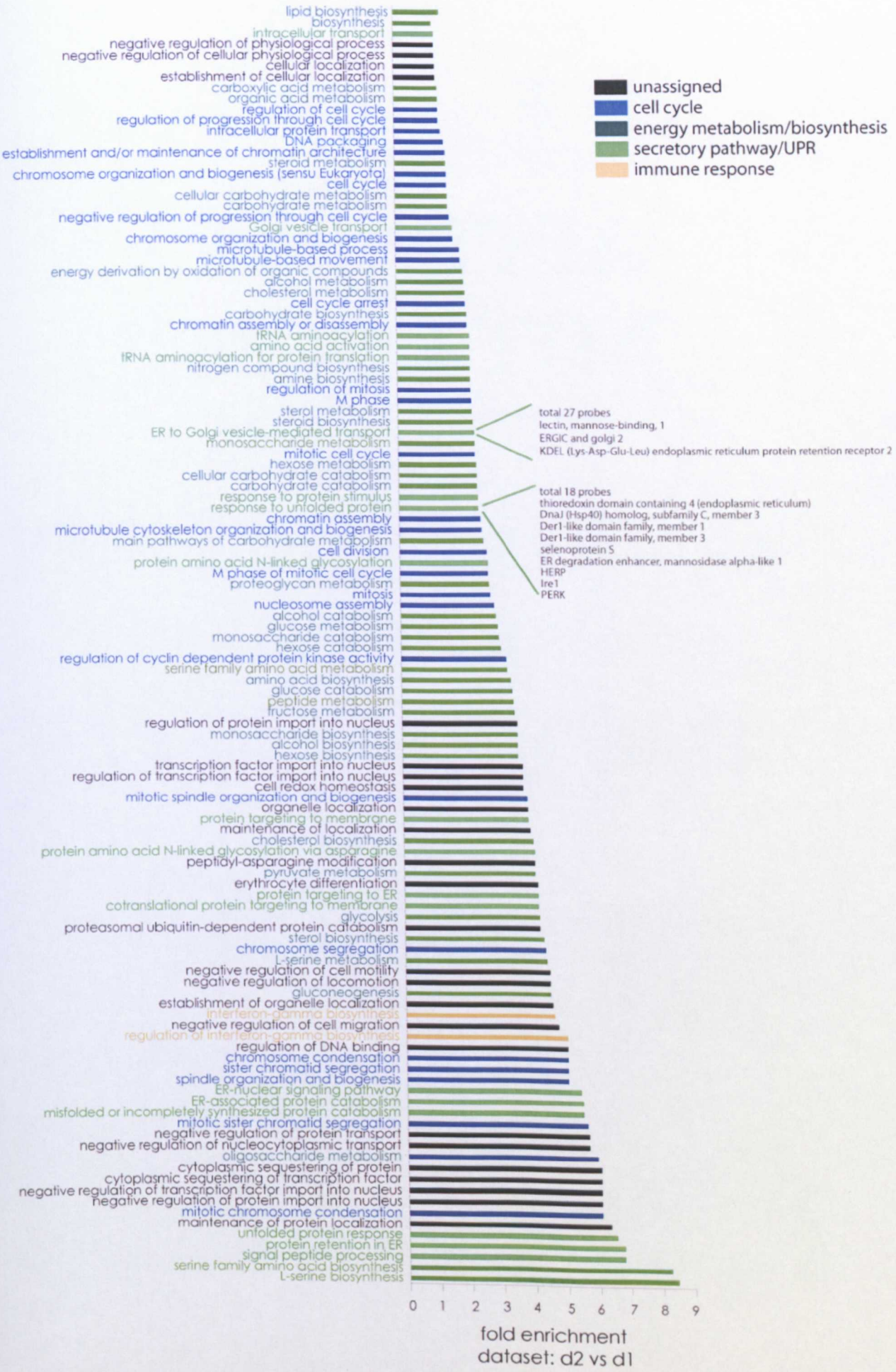
Sensivity to ER stress was assessed by measuring the percentage of propidium iodide positive cells by flow cytometry analysis after treatment with the ER stress inducer tunicamycin. **A** T and B lymphocytes were purified from the same preparation of wt and *chop*$^{-/-}$ splenocytes and cultured in the presence of LPS to reduce spontaneous apoptosis. 2 μg/ml Tm were added 8 hrs after cell purification. After additional 16hrs the cells were harvested, stained with PI and analyzed by flow cytometry (n=3; error bars represent the SEM of the average; p value < 0.005). **B** ASC at day3 of differentiation were treated and analyzed as in A. Tm concentration was reduced to 0.5 μg/ml to avoid excessive death rate (n=2; error bars represent the SEM of the average; p value < 0.005 (Student's T test)).

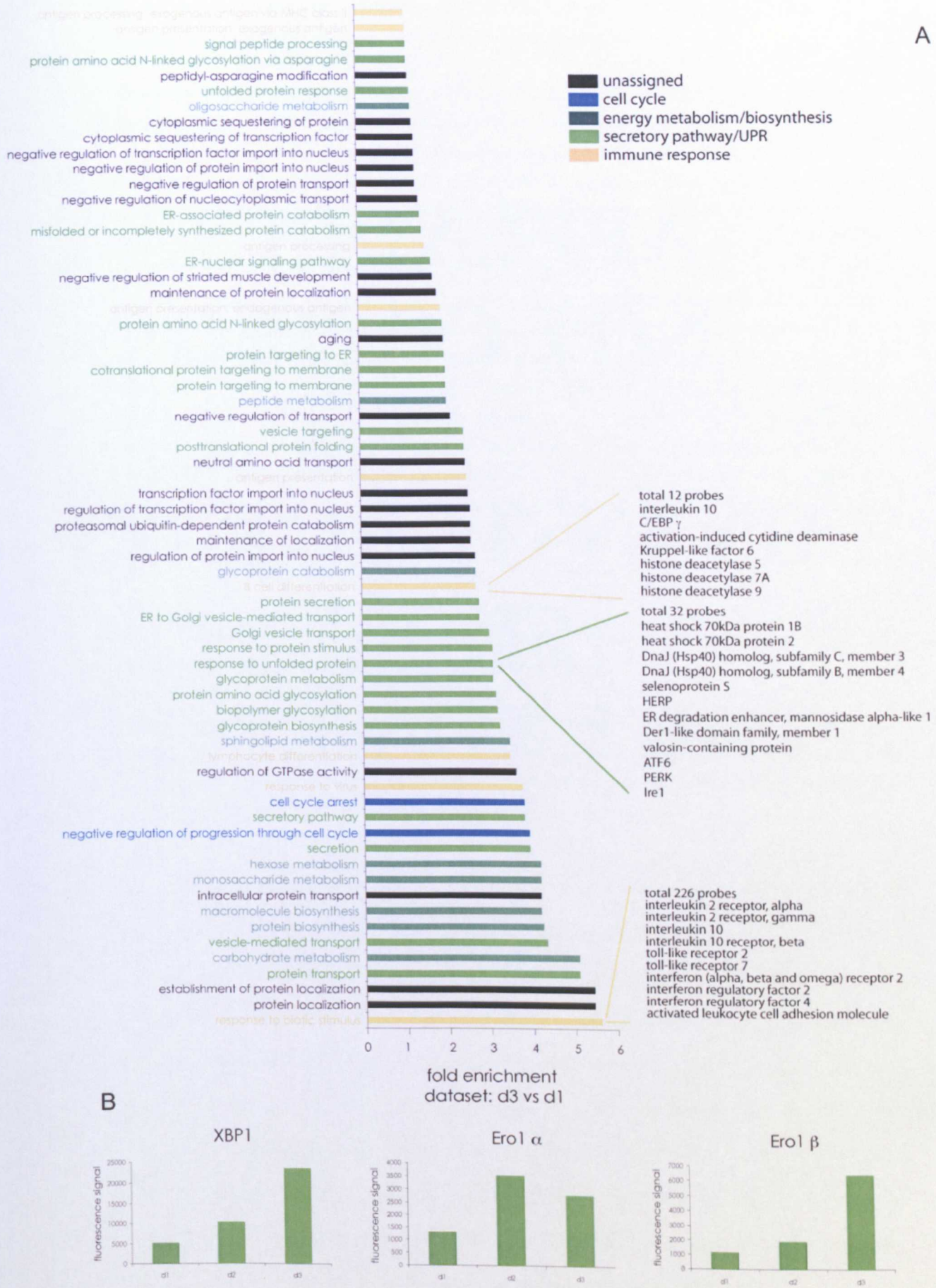
3.1.7 Transcriptional profiling analysis of wt and chop$^{-/-}$ differentiating B cells

In the attempt to identify CHOP target genes during B cell differentiation, I performed microarray transcriptional profiling analysis.

I analyzed the total mRNA expression profile in wt and *chop*$^{-/-}$ purified B splenocytes after 1, 2 and 3 days upon LPS exposure. I did not use the RNA purified from freshly isolated splenocytes before LPS stimulation and after 4 days since the quality of the RNA was not good enough; in the first case because magnetic separation is known to cause a stress from which the cells must recover before obtaining the RNA and in the second one because at day 4 of differentiation the percentage of dead cells is high (see Fig.3 C). Firstly I concentrated on expression profile changes in wt B cells. While in the literature it is possible to find various expression profiles of B cell lines and normal or myeloma plasma cell performed by lymphocyte-specific chips, as far as we

are aware no study has been published in which the entire mouse genome was probed (Peterson, 2007). I selected the genes whose levels significantly raised at day2 and day3 with respect to day1 and analyzed them with the microarray analysis tool L2L (Newman and Weiner, 2005). I thus identified the biological processes that were up regulated during B cell differentiation. As expected from the present knowledge about B cell differentiation I found profound differences between the two time points. It is clear, comparing figure 9 and 10, that after 2 days of LPS exposure most of the processes up regulated relative to day 1 belong to cell cycle control and metabolism/molecular biosynthesis groups, as well as to the secretory pathway/UPR one. After 3 days, most of these transcriptional groups are down-regulated to leave high rates of expression of genes related to the secretory/UPR pathway and to immunological functions (for example interferon and interleukin signaling). As already described by proteomic analysis of I.29μ^+ cells (Romijn et al., 2005; van Anken et al., 2003), I conclude as well that before starting massive Ig secretion primary B cells get ready by increasing their metabolic and secretory capacity. These findings add further consistence to the model I used to study plasma cell differentiation but especially provide a valuable set of data that we can exploit for whatever biological process, or single gene we want to follow during B cell differentiation (Fig.10 B).


lipid biosynthesis
biosynthesis
intracellular transport
negative regulation of physiological process
negative regulation of cellular physiological process
cellular localization
establishment of cellular localization
carboxylic acid metabolism
organic acid metabolism
regulation of cell cycle
regulation of progression through cell cycle
intracellular protein transport
DNA packaging
establishment and/or maintenance of chromatin architecture
steroid metabolism
chromosome organization and biogenesis (sensu Eukaryota)
cell cycle
cellular carbohydrate metabolism
carbohydrate metabolism
negative regulation of progression through cell cycle
Golgi vesicle transport
chromosome organization and biogenesis
microtubule-based process
microtubule-based movement
energy derivation by oxidation of organic compounds
alcohol metabolism
cholesterol metabolism
cell cycle arrest
carbohydrate biosynthesis
chromatin assembly or disassembly
tRNA aminoacylation
amino acid activation
tRNA aminoacylation for protein translation
nitrogen compound biosynthesis
amine biosynthesis
regulation of mitosis
M phase
sterol metabolism
steroid biosynthesis
ER to Golgi vesicle-mediated transport
monosaccharide metabolism
mitotic cell cycle
hexose metabolism
cellular carbohydrate catabolism
carbohydrate catabolism
response to protein stimulus
response to unfolded protein
chromatin assembly
microtubule cytoskeleton organization and biogenesis
main pathways of carbohydrate metabolism
cell division
protein amino acid N-linked glycosylation
M phase of mitotic cell cycle
proteoglycan metabolism
mitosis
nucleosome assembly
alcohol catabolism
glucose metabolism
monosaccharide catabolism
hexose catabolism
regulation of cyclin dependent protein kinase activity
serine family amino acid metabolism
amino acid biosynthesis
glucose catabolism
peptide metabolism
fructose metabolism
regulation of protein import into nucleus
monosaccharide biosynthesis
alcohol biosynthesis
hexose biosynthesis
transcription factor import into nucleus
regulation of transcription factor import into nucleus
cell redox homeostasis
mitotic spindle organization and biogenesis
organelle localization
protein targeting to membrane
maintenance of localization
cholesterol biosynthesis
protein amino acid N-linked glycosylation via asparagine
peptidyl-asparagine modification
pyruvate metabolism
erythrocyte differentiation
protein targeting to ER
cotranslational protein targeting to membrane
glycolysis
proteasomal ubiquitin-dependent protein catabolism
sterol biosynthesis
chromosome segregation
L-serine metabolism
negative regulation of cell motility
negative regulation of locomotion
gluconeogenesis
establishment of organelle localization
interferon-gamma biosynthesis
negative regulation of cell migration
regulation of interferon-gamma biosynthesis
regulation of DNA binding
chromosome condensation
sister chromatid segregation
spindle organization and biogenesis
ER-nuclear signaling pathway
ER-associated protein catabolism
misfolded or incompletely synthesized protein catabolism
mitotic sister chromatid segregation
negative regulation of protein transport
negative regulation of nucleocytoplasmic transport
oligosaccharide metabolism
cytoplasmic sequestering of protein
cytoplasmic sequestering of transcription factor
negative regulation of transcription factor import into nucleus
negative regulation of protein import into nucleus
mitotic chromosome condensation
maintenance of protein localization
unfolded protein response
protein retention in ER
signal peptide processing
serine family amino acid biosynthesis
L-serine biosynthesis
unassigned
cell cycle
energy metabolism/biosynthesis
secretory pathway/UPR
immune response
total 27 probes
lectin, mannose-binding, 1
ERGIC and golgi 2
KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 2
total 18 probes
thioredoxin domain containing 4 (endoplasmic reticulum)
DnaJ (Hsp40) homolog, subfamily C, member 3
Der1-like domain family, member 1
Der1-like domain family, member 3
selenoprotein S
ER degradation enhancer, mannosidase alpha-like 1
HERP
Ire1
PERK
0
1
2
3
4
5
6
7
8
9
fold enrichment
dataset: d2 vs d1


A
unassigned
cell cycle
energy metabolism/biosynthesis
secretory pathway/UPR
immune response
signal peptide processing
protein amino acid N-linked glycosylation via asparagine
peptidyl-asparagine modification
unfolded protein response
oligosaccharide metabolism
cytoplasmic sequestering of protein
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negative regulation of transcription factor import into nucleus
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negative regulation of nucleocytoplasmic transport
ER-associated protein catabolism
misfolded or incompletely synthesized protein catabolism
ER-nuclear signaling pathway
negative regulation of striated muscle development
maintenance of protein localization
protein amino acid N-linked glycosylation
aging
protein targeting to ER
cotranslational protein targeting to membrane
protein targeting to membrane
peptide metabolism
negative regulation of transport
vesicle targeting
posttranslational protein folding
neutral amino acid transport
transcription factor import into nucleus
regulation of transcription factor import into nucleus
proteasomal ubiquitin-dependent protein catabolism
maintenance of localization
regulation of protein import into nucleus
glycoprotein catabolism
protein secretion
ER to Golgi vesicle-mediated transport
Golgi vesicle transport
response to protein stimulus
response to unfolded protein
glycoprotein metabolism
protein amino acid glycosylation
biopolymer glycosylation
glycoprotein biosynthesis
sphingolipid metabolism
regulation of GTPase activity
cell cycle arrest
secretory pathway
negative regulation of progression through cell cycle
secretion
hexose metabolism
monosaccharide metabolism
intracellular protein transport
macromolecule biosynthesis
protein biosynthesis
vesicle-mediated transport
carbohydrate metabolism
protein transport
establishment of protein localization
protein localization
total 12 probes
interleukin 10
C/EBP γ
activation-induced cytidine deaminase
Kruppel-like factor 6
histone deacetylase 5
histone deacetylase 7A
histone deacetylase 9
total 32 probes
heat shock 70kDa protein 1B
heat shock 70kDa protein 2
DnaJ (Hsp40) homolog, subfamily C, member 3
DnaJ (Hsp40) homolog, subfamily B, member 4
selenoprotein S
HERP
ER degradation enhancer, mannosidase alpha-like 1
Der1-like domain family, member 1
valosin-containing protein
ATF6
PERK
Ire1
total 226 probes
interleukin 2 receptor, alpha
interleukin 2 receptor, gamma
interleukin 10
interleukin 10 receptor, beta
toll-like receptor 2
toll-like receptor 7
interferon (alpha, beta and omega) receptor 2
interferon regulatory factor 2
interferon regulatory factor 4
activated leukocyte cell adhesion molecule
0 1 2 3 4 5 6
fold enrichment
dataset: d3 vs d1
B
XBP1
Ero1 α
Ero1 β
fluorescence signal
d1 d2 d3

Fig.9 Transcriptional profiling of murine ASC: biological processes up regulated at differentiation day2 relatively to day1

Transcriptional profiles of wt B cells at day1 and day2 after LPS stimulation were analyzed by Affymetrix mouse 430 2.0 chip, containing probes recognizing the entire mouse genome. Significant changes in the expression level of each transcript at day2 relatively to day1 were identified by Affymetrix software. The probes whose complementary transcripts resulted significantly increased were selected and subjected to further analysis by the microarray analysis tool L2L (Newman and Weiner, 2005) which grouped them on the basis of the biological process they belong to as classified by the Gene Ontology (GO) consortium (Ashburner et al., 2000). Comparison of our data with the software databases indicated which biological process groups were "enriched" in our dataset, i.e. contained a higher number of probes than expected. The results of this comparison are reported in the figure as fold enrichment for each group. Since the set of probes analyzed were those increasing at day2 versus day1 such analysis reports which processes are up regulated at day2. The groups were further assigned to four main processes (cell cycle, energy metabolism/macromolecules biosynthesis, secretory pathway/UPR, immune response) evidenced by color code. The insets report the total number of probes belonging to a particular group and some of the most significant genes they corresponded to.

Fig.10 Transcriptional profiling of murine ASC: biological processes up regulated at differentiation day3 relatively to day1

A Transcriptional profiling of wt B cells at day1 and day3 after LPS stimulation, analyzed and reported as in Fig.9 **B** Examples of the expression pattern of single genes of interest. The fluorescence signals emitted by the different microarray probes corresponding to a single gene were averaged for each time point and plotted in histograms.

From the comparison of the expression profiles of wt versus *chop*$^{-/-}$ ASC at day 2 and day 3 of differentiation we selected 19 and 17 genes, respectively, either up- or down-regulated in the *chop*-null cells, which passed very strict statistical criteria (Table 1), obviously besides CHOP itself. We were not able to identify a particular biological process affected in the ko cells, if not maybe the mis-regulation of more than one gene involved in small GTPases signaling pathways. However, this was not un-expected considering the mildness of *chop*$^{-/-}$ ASC phenotype. Furthermore, the same kind of analysis, comparing wt and *chop*$^{-/-}$ MEF after ER stress stimuli identified only 26 genes that were never induced in the ko cells, despite a more evident phenotype (Marciniak et al., 2004). Microarray data regarding single genes always need to be

verified by real-time PCR analysis. We began the screening and then we focused on one gene that was definitively repressed in the *chop*-null cells, at all the time points analyzed: ATF5. The remaining data presented in table 1 still need proper validation.

Representative Public ID	Gene Title	Gene Symbol	fold change at d2	Gene Ontology Cellular Component	Gene Ontology Biological Process
				cAMP-dependent protein kinase	
BM240774	Rap guanine nucleotide exchange factor (GEF) 3	Rapgef3	-3.5	complex	regulation of small GTPase mediated signal transduction
NM_016720	neuraminidase 3	Neu3	-2.8	plasma membrane	carbohydrate metabolism
BM240674	transmembrane protein 135	Tmem135	-2.8	integral to membrane	proteolysis
BM211104	SMC6 structural maintenance of chromosomes 6-like 1 (yeast)	Smc6l1	-2.1	chromosome	chromosome organization and biogenesis
BM119522	Rho GTPase activating protein 5	Arhgap5	-2.0	membrane	Rho protein signal transduction; regulation of cell size
BC026842	GTPase activating RANGAP domain-like 1	Gaml1	-2.0	nucleus	regulation of transcription
BC020532	polymerase (RNA) II (DNA directed) polypeptide E	Polr2e	-2.0	nucleus	transcription
BB645629	Jumonji, AT rich interactive domain 1B (Rbp2 like)	Jarid1b	-2.0	nucleus	regulation of transcription, DNA-dependent
BB474913	integrin beta 4 binding protein	Itgb4bp	-2.0	nucleolus; cytoplasm	protein biosynthesis; translational initiation; integrin-mediated signaling pathway
BB209117	methionine-tRNA synthetase	Mars	-2.1	nucleolus; cytoplasm	protein biosynthesis; tRNA aminoacylation for protein translation
AV245241	RAP2B, member of RAS oncogene family	Rap2b	-2.1	intracellular, membrane	small GTPase mediated signal transduction
				nucleus,transcription factor	
AV225967	activating transcription factor 5	Atf5	-2.1	complex	regulation of transcription, DNA-dependent; anti-apoptosis; regulation of cell proliferation
AU022584	Tia1l cytotoxic granule-associated RNA binding protein-like 1	Tial1	-2.3	cytoplasm	apoptosis
		Ehd2 ///			
AK018313	EH-domain containing 2 /// similar to EH-domain containing 2	LOC673251	-2.3	plasma membrane	endocytosis; cortical actin cytoskeleton organization and biogenesis
AK017652	zinc finger protein 292	Zfp292	-2.3	nucleus	two-component signal transduction system (phosphorelay)
AK014601	protein tyrosine phosphatase 4a3	Ptp4a3	-2.6	membrane	protein amino acid dephosphorylation
AB506883	plexin B1	Plxnb1	-3.5	integral to membrane	development; positive regulation of axonogenesis
AF376476	UDP-glucose dehydrogenase	Ugdh	-3.5	---	electron transport
AA119513	membrane-associated ring finger (C3HC4) 5	3678	-3.0	---	ubiquitin cycle
			fold change at d3		
Representative Public ID	Gene Title	Gene Symbol		Gene Ontology Cellular Component	Gene Ontology Biological Process
AF376476	E2f transcription factor 5	E2f5	4	nucleus, cytoplasm	G1/S transition of mitotic cell cycle; regulation of transcription
AK004904	myosin, VIIb	Myo7b	3.2	cytoskeleton	cytoskeleton organization and biogenesis
AK017167	ral guanine nucleotide dissociation stimulator-like 1	Rgl1	2.5		small GTPase mediated signal transduction; guanyl-nucleotide exchange factor activity
AK020259	pregnancy-specific glycoprotein 23	Psg23	2.5	membrane	pregnancy
BB286270	histamine receptor H2	Hrh2	2.5	integral to membrane	G-protein coupled receptor protein signaling pathway
380963	interleukin 13 receptor, alpha 1	Il13ra1	2.5	integral to plasma membrane	cell surface receptor linked signal transduction
BB345680	aldehyde dehydrogenase 3 family, member B1	Aldh3b1	2.3		aldehyde dehydrogenase [NAD(P)+] activity; oxidoreductase activity
	valosin containing protein (p97)/p47 complex interacting				
BB532258	protein 1	Vcpip1	-2	endoplasmic reticulum, Golgi stack	ubiquitin cycle
BC003763	syndecan 4	Sdc4	-2	cell surface, integral to membrane	cytoskeletal protein binding
BC006679	phospholipase A2, activating protein	Plaa	-2		signal transduction, phospholipase A2 activator activity
BB413218	C-terminal binding protein 2 /// zinc finger, RAN-binding domain containing 1	Ctbp2 /// Zranb1	-2	nucleus nucleus, transcription factor complex	L-serine biosynthesis, negative regulation of transcription
BM240021	activating transcription factor 5	Atf5	-2		regulation of progression through cell cycle, transcription, transport
NM_007522	MON1 homolog b (yeast)	Mon1b	-2.6		
NM_007837	phosphatidylinositol-4-phosphate 5-kinase, type 1 beta	Pip5k1b	-3.2	membrane,Golgi stack	phosphatidylinositol metabolism
NM_016846	acyl-CoA synthetase long-chain family member 1	Acsl1	-3.7	mitochondrion	lipid metabolism, fatty acid metabolism
NM_020261	fucosyltransferase 11	Fut11	-4.3	integral to membrane,Golgi stack	protein amino acid glycosylation,
					induction of apoptosis, induction of apoptosis by extracellular signals, glucose homeostasis,
NM_032294	Bcl-associated death promoter	Bad	-4.6	mitochondrion	positive regulation of B cell differentiation, positive regulation of T cell differentiation

Table 1

Table 1 Genes differently expressed in chop$^{-/-}$ ASC

The table lists the genes that were either up (positive fold change) or down (negative fold change) regulated in *chop*$^{-/-}$ ASC at day2 and day3 after LPS stimulation relatively to wt cells at the corresponding time point. Analysis was performed by Affymetrix analysis software and only genes that showed at least two-fold change were considered.

3.1.8 Identification of the transcription factor ATF5 as a novel CHOP pathway target gene during B cell differentiation and in the UPR

ATF5 is a member of the Activating Transcription Factor family. Relative real-time PCR confirmed that ATF5 mRNA expression is down regulated in *chop*$^{-/-}$ B lymphocytes and ASC when compared to wt (Fig.11 A). Obviously, since there is still an increase in *chop*$^{-/-}$ ASC with respect to undifferentiated cells, ATF5 can be induced also by CHOP-independent pathways. CHOP control during B cell differentiation however seems to play a role (though in other experiments ATF5 increase in *chop*$^{-/-}$ cells is somewhat higher). The first question that I decided to answer to was if CHOP activates ATF5 expression also during the UPR. Treatment of unfractionated splenocytes with Tm increased the expression of ATF5 mRNA (Fig.11 B). However, *chop*$^{-/-}$ splenocytes had lower levels of ATF5 to start with and its up-regulation in response to the UPR was minor in comparison with wt cells. Therefore CHOP is required for full expression of ATF5 in B cell differentiation and in the UPR. To assess if CHOP directly binds the ATF5 regulatory sequences I will need to perform chromatin immunoprecipitation assays. As explained in the discussion below however, I do not think this is the case.

Since the above data suggest that the role of CHOP is quite specific in B cells, I next asked if CHOP-dependent ATF5 activation in the UPR was characteristic of this cell type only. To start answering this question I treated a mouse fibroblast (NIH3T3) and a B cell line (I.29μ^+), with Tm in order to obtain comparable levels of CHOP expression (Fig.11 C). ATF5 up-regulation by the UPR in 3T3 was the same, if not higher with respect to I.29μ^+. From this experiment I draw the important conclusion that ATF5 is generally up regulated by the UPR. I cannot however claim if also in

fibroblasts its up-regulation depends on CHOP. ER-stress induction in wt and *chop*$^{-/-}$ fibroblast will allow elucidating the last point.

The finding that ATF5 expression is activated by the UPR, likely downstream of CHOP raises very exciting questions. Despite the clear involvement of CHOP in ER stress-induced apoptosis, the exact pathways leading to cell death are still obscure. The literature regarding ATF5 is quite scarce. This transcription factor has been involved in neural cell differentiation, in response to starvation and very interestingly in apoptosis. The most intriguing point is that, depending on the cell type, ATF5 either promotes or prevents apoptosis induced by various means, a behavior reminiscent of CHOP. I wondered if ATF5 could be a member of the pathway(s) downstream of CHOP leading to ER stress-induced death. By generating ER stress in 3T3 cells previously transfected with siRNA against ATF5 or against the control gene luciferase, I obtained preliminary data supporting this hypothesis. In the experimental settings used, the cells transfected with ATF5 siRNA were resistant to ER stress-induced apoptosis, unlike the cells transfected with siRNA targeting the luciferase gene sequence (Fig.11 D). This experiment unfortunately lacked analysis of ATF5 mRNA, so even though the siRNA sequences used were published and tested by the authors (Angelastro et al., 2006), I cannot attribute with certainty the observed effects to ATF5 down regulation.

The role of ATF5 in the UPR clearly needs further investigations.

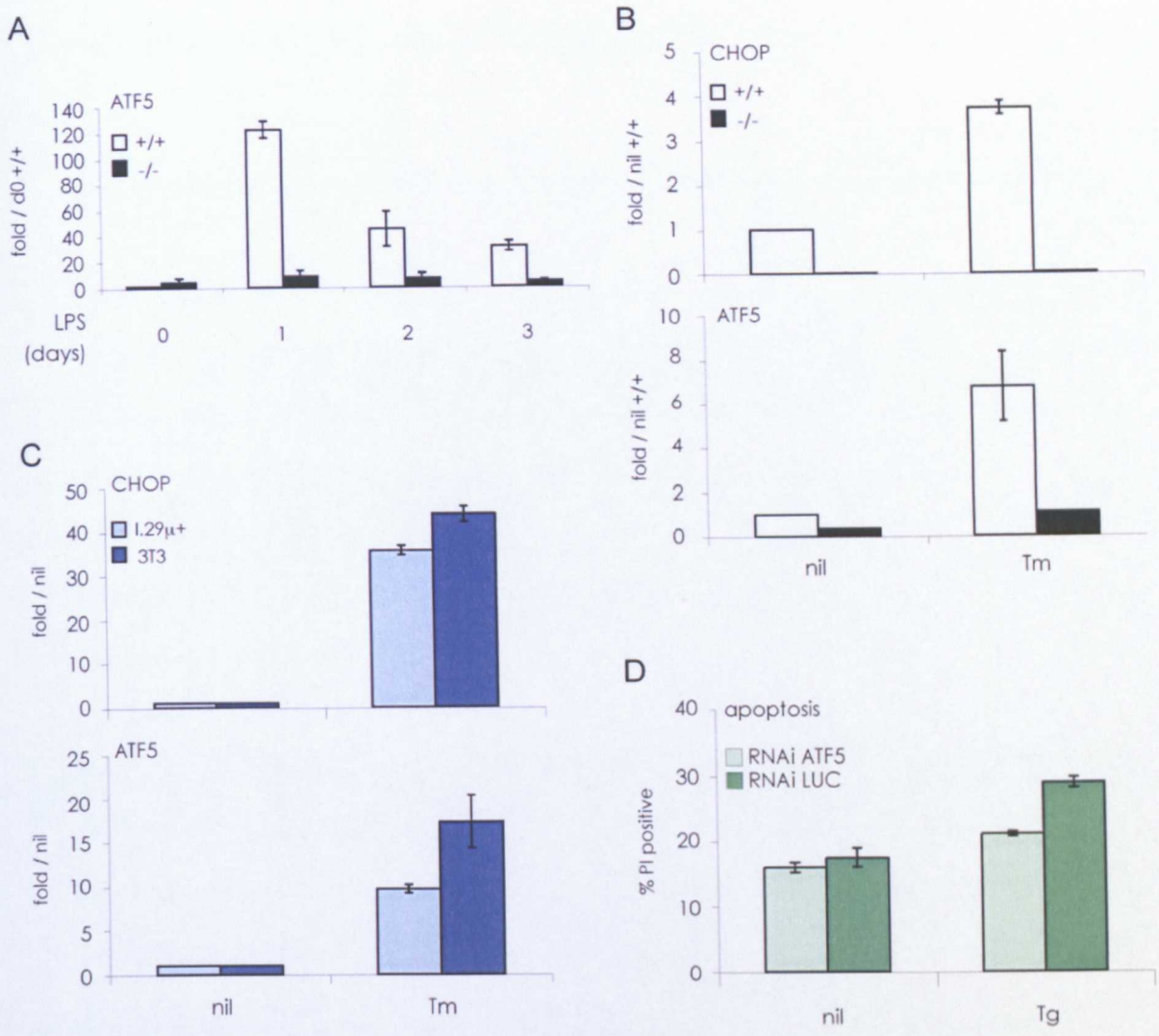

Fig.11 ATF5 expression is impaired in chop$^{-/-}$ cells

A B lymphocytes isolated from wt and *chop*$^{-/-}$ mouse spleens were induced to differentiate by incubation with LPS for the indicated time points. ATF5 mRNA expression pattern during differentiation was followed by relative real time PCR as in Fig.2 A (error bars represent the SEM of the average of PCR triplicates of the same samples). **B** The totality of cells harvested from wt and *chop*$^{-/-}$ spleens was cultured in the presence of 20 µg/ml Tm for 4hrs to induce UPR. After the incubation time total RNA was purified and analyzed by real time PCR as in A to follow the expression of CHOP and ATF5 transcripts (error bars represent the SEM of the average of PCR triplicates of the same samples). **C** ER stress was induced in I.29µ^{+} and 3T3 cells by 4hrs incubation with 10 µg/ml Tm. Then RNA was purified and analyzed by real time PCR as in A to follow the expression of CHOP and ATF5 transcripts (error bars represent the SEM of the average of PCR triplicates of the same samples). **D** 3T3 cells were transfected with 20 µM duplex RNA oligonucleotides targeting ATF5 or luciferase transcripts. 36hrs after transfection cells were incubated with 250nM thapsigargin (Tg) for 4hrs and then analyzed by flow cytometry. The percentage of apoptotic cells was identified by forward/side scatter physical parameters analysis (n=3; error bars represent the SEM of the average; p value= 0.082 (Student's T test))

3.2 Differentiating B cells suffer proteo-toxic and oxidative stress

3.2.1 Proteasomal capacity is impaired during plasma cell differentiation

While a minority of plasma cells home to the bone marrow and live longer, the vast majority, particularly those producing IgM, die by apoptosis after a few days of intense Ig secretion. Although extrinsic factors are crucial to shape the proper environment for long-lived plasma cells (Moser et al., 2006; Radbruch et al., 2006), little is known of the molecular events that cause apoptosis of short-lived Ig secretors. A considerable fraction of secretory μ-chains are degraded also in hybridoma cells (Fra et al., 1993; Sitia et al., 1990) via ERAD, probably owing to the inefficient assembly of IgM polymers. As discussed and shown above (Fig.4 C and D), some ERAD components are highly up regulated during plasma cell differentiation, presumably in order to cope with the increased load of proteins to be degraded. The terminal executers of ERAD are the cytosolic proteasomes. Since myeloma cells are particularly sensitive to treatments with proteasome inhibitors, we reasoned that the abundant production of IgM that characterizes activated B cells could represent a load on the protein degradation machinery. We thus asked if such load could be a source of stress rendering plasma cells particularly prone to apoptosis. Answering this question was the result of a collaborative effort of the laboratory in which I was involved. We demonstrated that in the late phases of plasma cell differentiation, when antibody production becomes maximal, proteasomal activity decreases ((Cenci et al., 2006), appendix I). In particular, I showed that I.29μ$^+$ cells accumulate poly-ubiquitinated proteins and consequently have decreased amounts of free ubiquitin in the later phases of differentiation (Fig.12 A) suggesting impairment of proteasomal activity. To provide further evidence of proteasomal insufficiency during B-cell differentiation, I then analyzed transgenic mice expressing a chimerical, un-cleavable ubiquitin-GFP ($Ub^{G76V}GFP$) that is rapidly degraded by proteasomes (Dantuma et al., 2000). A short exposure to the proteasome inhibitor MG132 caused a strong accumulation of the reporter in un-stimulated CD19$^+$ B cells (Fig.12 B, top). After 3

days of LPS stimulation in vitro, differentiating B cells accumulated the GFP reporter even in the absence of MG132 (Fig.12 B, bottom).

These data, together with many others produced and published in our laboratory demonstrate that a built-in plasma cell developmental program impairs the ubiquitin-proteasome disposal system and lead us to propose that the progressive impairment of proteasomal capacity is one of the mechanisms that contribute in limiting plasma cell lifespan, linking it to antibody production (Cascio et al., 2008; Cenci et al., 2006).

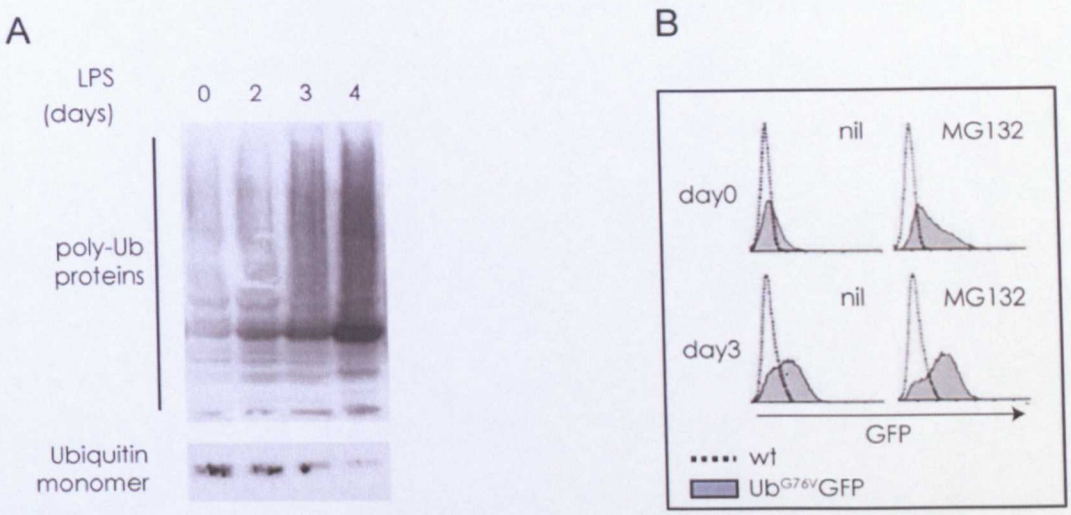

Fig.12 Differentiating B cells accumulate poly-ubiquitinated proteins

A The murine lymphoma line I.29μ+ was induced to differentiate to ASC by addition of 20 μg/ml LPS. At the indicated time points protein extracts were separated by SDS-PAGE and after blotting the filters were probed with anti-ubiquitin antibodies. The HMW poly-ubiquitinated proteins and the 7KD ubiquitin monomer were separated with 6% and 15% polyacrylamide gels respectively. **B** B lymphocytes were purified from spleens of $Ub^{G76V}GFP$ mice and wt littermates and cultured in the presence of LPS for 3 days. At the indicated time points cells were treated with the proteasome inhibitor MG132, 1.5 μM for 2.5hrs. $Ub^{G76V}GFP$ accumulation was followed by flow cytometry analysis.

3.2.2 Differentiating antibody secreting cells experience increasing oxidative stress

Perturbation of the redox homeostasis could be yet another factor leading to plasma cell death. Recent evidences prove that the process of oxidative folding can generate

H_2O_2 that can then be converted in other reactive oxygen species (ROS) (paragraphs 1.2.2 and 1.2.3). Owing to the reactivity of H_2O_2 and the existence of many buffering systems in living cells (catalase and peroxiredoxins for instance), a quantitative measurement of peroxides is likely to be a difficult task. Nonetheless the sharp increase in IgM biosynthesis might lead a related increase in H_2O_2, should O_2 be the electron acceptor during oxidative folding. The increased loads of H_2O_2 produced could represent an intracellular signal (Gross et al., 2006; Liu et al., 2005; Tu and Weissman, 2004). Hence I utilized an H_2O_2-specific dye (PG1) (Miller et al., 2007) to measure the concentration of hydrogen peroxide during ASC differentiation. If the hypothesis were correct I would expect an augmentation of H_2O_2 concentration in correspondence with increased IgM synthesis and polymerization. Using primary splenic B cells, I observed a strong increase in H_2O_2 concentration only at day 5 of differentiation, that is after the first wave of apoptosis, clearly detectable at days 3 and 4 of differentiation (Fig.13 A). I did not follow differentiation at later days yet, to understand if the increase in H_2O_2 observed at day 5 will coincide with a further peak of death at day 6. Interestingly however the raise in H_2O_2 concentration corresponded to increased accumulation of IgM HMW species (Fig.13 B). Furthermore *chop*$^{-/-}$ ASC, which experience higher degree of aggregation, present a slight, but statistically significant higher concentration of hydrogen peroxide at day 5. These observations suggest a correlation between oxidative folding and H_2O_2 accumulation. However, a significant increase in IgM synthesis and folding occurs already at earlier time points, after 3 or 4 days upon LPS stimulation. If it is true that oxidative folding generates H_2O_2, a possible explanation for the delayed increase in H_2O_2 concentration could be found in the regulation of scavenging systems. I thus began addressing this point by investigating the pattern of expression of the peroxiredoxins family members, thiol-specific antioxidant proteins also termed thioredoxin peroxidases. Six members of the family have been identified in mammalian cells that differ for the sub-cellular localization and for the recycling mechanisms. I found that PrxI, III and IV expression levels increase during differentiation (Fig.13 C). PrxI is localized in the cytosol, PrxIII is mitochondrial whereas PrxIV is an ER resident protein. It is worthy of note that, whereas PrxIV

continues increasing until day 4, PrxI and III reach a peak of expression at day 2 and 3 then decrease. Prxs are not the sole cellular peroxidases and these data are still preliminary. Nonetheless my findings open to the possibility that H_2O_2 levels could be controlled by up regulation of scavenging systems in the first days of differentiation and then reduction of the buffering capacity would determine increased oxidative stress. Continuous up-regulation of PrxIV in the ER could help maintaining the Ig folding compartment as efficient as possible until the cell dies.

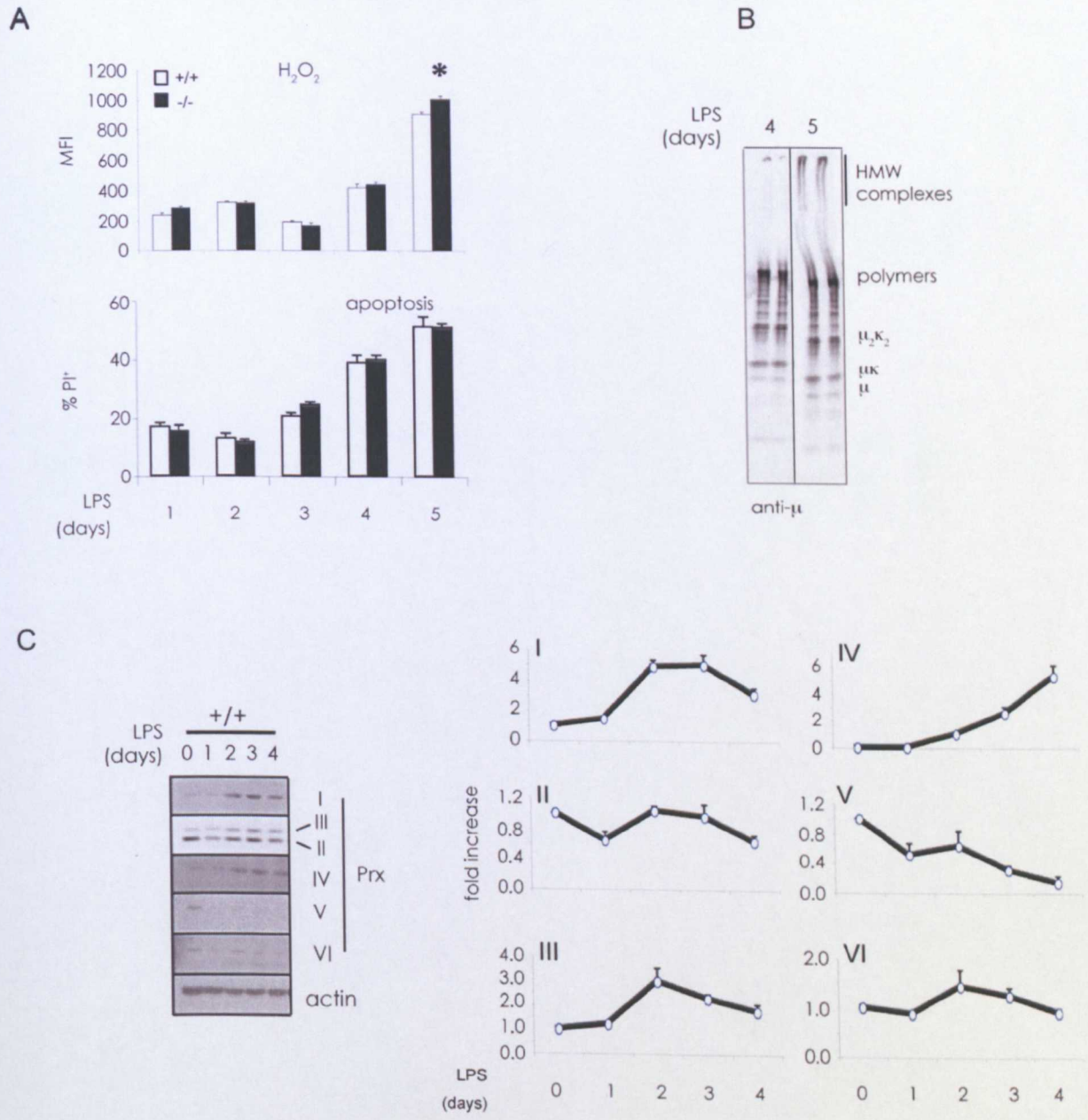

Fig.13 Differentiating B cells undergo oxidative stress

B lymphocytes isolated from wt and *chop*$^{-/-}$ mouse spleens were induced to differentiate by incubation with LPS. **A** At the indicated time points cells were analyzed by flow cytometry after staining with the H_2O_2 specific dye PG1 (Median Fluorescence Intensity) or with propidium iodide (percentage positive cells) to follow apoptosis. (n=4; error bars represent the SEM of the average; * p value < 0.02 (Student's T test)). **B** IgM polymerization of wt ASC at day4 and day5 of differentiation was analyzed by western blot as described in Fig.5 A. **C** The pattern of expression of the Peroxiredoxin family proteins (Prx I-VI) during B cell differentiation was followed by western blot analysis of protein extracts obtained by wt cells (*left panel).* Actin was used as loading control. Densitometric analysis of western blots as shown in the left panel (*right panel).* Data are reported as fold increase relative to the protein amounts measured at day0, normalized to actin. (error bars represent the SEM of the average fold increase in four independent samples).

4 Discussion

4.1 Plasma cell differentiation

4.1.1 Preparing for secretion

Plasma cells, specialists in antibody production, represent an ideal model to investigate the cellular dynamics of proteosynthetic stress. Their differentiation from small resting B lymphocytes into Ig secreting cells entails a series of genetic and functional changes, finalized to producing specific antibodies in adequate amounts at the proper time. Upon encounter with antigen, long-lived B lymphocytes activate a profound functional and structural metamorphosis that, in few days, leads to differentiation into a mature plasma cell, a terminally differentiated element capable of secreting enormous amounts of Ig. This process entails augmenting the secretory organelles and the proteins that populate them, up regulating their energy and translation potentials, and increasing the quality control systems. This transformation is accomplished by the interplay between B lineage-specific transcriptional programs that control plasma cell differentiation, the unfolded protein response and possibly redox homeostasis variations and other signaling pathways. The high rate of antibody production requires efficient folding and quality control machineries. Primary plasma cells produce large amounts of IgM (about 10^3/cell/s). It follows that up to 10^5 disulfides are formed per second in each plasma cell, solely to sustain IgM production. Eight $x \cdot 10^6$ aminoacids must be synthesized or imported each second to compensate for IgM secretion. IgM being planar disks 36 nm wide and 4 nm thick, an impressive number of vesicles must be produced to warrant their transport, and as many to recycle the intracellular membrane compartments. To satisfy the increased demand for oxidative folding, more Ero1α and β are produced. If the increased levels of Ero1α and β satisfies the redox demand, ASC differentiation needs to solve the problem of assembling thousands of IgM per second. These planar molecules are linked by disulphide bonds in their C-terminal

tails. Our recent work suggest that ERp44 and ERGIC-53 concur in IgM polymerization. Hexameric ERGIC-53 may provide a planar platform that concentrates μ_2L_2 subunits and favors their ordered assembly, avoiding formation of larger polymers (de Lalla et al., 1998). However, human patients with various deficiencies in the ERGIC-53 mediated transport machinery show impaired secretion only of the coagulation factors V and VIII, thus presenting bleeding problems (Neerman-Arbez et al., 1999; Nichols et al., 1998). Undergoing studies in our laboratory are investigating possible alterations of ERGIC-53 null patients plasma cell Ig secretion. Yet these patients do not show major immunological defects and have normal IgM titers though consistently in the lowest percentile. Preliminary work on *in vitro* PBL from FVFVIII patients suggests a delay in the onset of IgM polymers secretion. These observations point to the existence of redundant mechanisms for efficient IgM polymerization and secretion in cells of the B lineage, underscoring the importance of this process.

4.1.2 Dying

The vast majority of plasma cells are short-lived so as to limit the antibody response. The mechanisms leading to programmed death of plasma cells are still unclear. However, different stress factors, related to the high rate of protein synthesis concur in rendering plasma cells more susceptible to apoptosis. The system could be based on a "timer" or a "counter". In principle, the latter may result in better tuning of antibody responses, but how to count secreted molecules that are no longer there? An intracellular sensor of the work accomplished could transduce intrinsic stimuli and start apoptosis when a functional threshold is met. Proteasomal inhibition in the later stages of plasma cell differentiation could function as a bottleneck in an otherwise efficient quality control strategy dedicated to Ig folding and assembly. This could be a mechanism evolved by plasma cells to count the work accomplished, and predispose themselves to die on the basis of accumulated stress (proteo-toxic, oxidative and so forth). Selective accumulation of certain protein species, like the

pro-apoptotic Bcl-2 relative Bax, or the NF-kB counteractor IkBα, could play an important role in cells sensitized by the likely ensuing oxidative stress, UPR, ER calcium release, etc. Together, they may provide plasma cells with a powerful redundancy of apoptotic stimuli, and ensure apoptosis.

4.2 CHOP exhibits a B cell-specific role during ASC differentiation and in response to ER stress

My study revealed an unexpected role for CHOP in the secretory pathway of antibody secreting cells where it contributes to the full expression of elements of the folding and quality control machinery. Furthermore my findings point to a cell-type specific role for this transcription factor during the UPR. Indeed CHOP seems to protect B cells from ER stress induced apoptosis, unlike what is observed in other cell types.

4.2.1 CHOP is induced independently of the UPR during B cell differentiation

I found that CHOP is induced during B cell differentiation while looking for UPR players involved in this process. Surprisingly however, together with the existing literature discussed below, my data on the activation of elements of the UPR in ASC point to an UPR-independent regulation of CHOP during ASC differentiation. Zhang and colleagues concluded some time ago that the PERK pathway is not activated during ASC cell differentiation. Furthermore they excluded an essential role in plasma cell differentiation for the cascade downstream eIF2α phosphorylation by showing that knock-in mice expressing a mutant of eIF2α that cannot be phosphorylated ($eIF2\alpha^{Ser51Ala}$) achieved the same Ig titer as wt mice upon immunization (Zhang et al., 2005). Recently such conclusions were further demonstrated by Gass and colleagues who confirmed that PERK is not activated in *in vitro* B cell differentiation and showed that $PERK^{-/-}$ B cells can differentiate normally (Gass et al., 2008). Thus, if *chop* transcription were induced by activation of the UPR in ASC differentiation, then it should be via one of the ERSE (ER stress responsive

element) present in its promoter, recognized by ATF6 and/or XBP1. However, CHOP induction upon ER stress is completely abolished in PERK$^{-/-}$ and in eIF2$\alpha^{Ser51Ala}$ knock-in MEF and over-expression of ATF4 cannot restore CHOP induction in PERK$^{-/-}$ cells indicating the requirement of a signal downstream eIF2α phosphorylation, other than ATF4 (Harding et al., 2000; Scheuner et al., 2001). Although it cannot be excluded that, some other kinase(s) could phosphorylate eIF2α (e.g. GCN2 or PKR), since PERK pathway is disposable for ASC differentiation, it is reasonable to conclude that CHOP regulation in ASC cell is UPR independent. Another consideration suggesting that CHOP is expressed independently of PERK/eIF2α phosphorylation pathway consists in the fact that PERK$^{-/-}$ ASC seems to secrete the same amount of IgM as wt as assessed by *in vitro* secretion assay (Gass et al., 2008) and sera levels of IgM in eIF2$\alpha^{Ser51Ala}$ mice were the same if not higher, even though the analysis was performed at much longer time points with respect to my experiments (Zhang et al., 2005). The idea of an UPR-independent CHOP regulation in differentiating B cells is supported by the very different kinetics of CHOP expression with respect of all the other UPR elements that I studied. Particularly interesting is the difference between CHOP and HERP expression pattern in ASC. Both CHOP and HERP promoters have ERSE1, ERSE2 and ATF/C/EBP composite site regulatory elements and are dually regulated during the UPR by ATF6-XBP1 (via ERSE) and by PERK/ATF4 (via ATF/C/EBP composite element) pathways. Both can still be induced in the absence of ATF4 upon ER stress, although at a lower level, but not upon amino acid starvation, thus showing a very similar pattern of regulation (Ma and Hendershot, 2004). HERP expression, however, can be induced also by an ER resident transcription factor, LUMAN/CREB3, which is activated by proteolytic cleavage by SP1 protease as ATF6 and, like sXBP-1, can bind and activate transcription via UPRE and ERSE elements (Liang et al., 2006). LUMAN regulates *herp* transcription via the ERSE2; the c*hop* promoter contains an ERSE2 as well but I could not find published data about a possible regulation of *chop* by this transcription factor. Interestingly LUMAN is activated during the UPR only in response to certain ER stress-inducing agents maybe providing a fine tuned regulation of the response depending on the kind of ER stress. It can be speculated

that LUMAN, or another yet to be identified factor activation mediates the differential regulation of CHOP and HERP during ASC differentiation. These observations further underline the differences between a UPR pharmacologically induced and physiological responses to changing protein loads in which only certain elements are activated, likely depending on the nature of the cargo proteins and cell types.

4.2.2 What drives CHOP expression during B cell differentiation?

The pathway(s) upstream to CHOP induction in ASC remains to be elucidated. CHOP is up regulated in response to a variety of stimuli and examples of various cell- and stress-specific regulatory pathways have been described. For example, cell type specific control over *chop* transcription has been recently found in pancreatic β cells. MafA, a transcription factor expressed only in tissues involved in eye development and in β cells, inhibits or induces *chop* transcription downstream of the MAPKs ERK1/2, depending on glucose concentration in the culture medium (Lawrence et al., 2007). C*hop* transcription is activated during mitochondrial UPR through an AP1 element present in the promoter, possibly via the MEK/JNK2 pathway and the PI3K/PKCδ pathway seems to be involved in CHOP up regulation in response to oxidative stress (Oh-Hashi et al., 2004; Scott and Loo, 2007). In these studies eIF2α phophorylation was not analyzed so it is not clear if CHOP expression, promoted by different pathways, is dependent also on eIF2α phosphorylation. Nevertheless a recent study, demonstrated that insulin promoted CHOP expression in the absence of eIF2α phosphorylation in mature adipocytes (Miyata et al., 2008).

4.2.3 CHOP is involved in the differentiation of a variety of cell types

An important role for CHOP in cellular differentiation has been described in adipocytes, osteoblasts and keratinocytes. Different lines of evidence indicate that CHOP plays an inhibitory role in adipocyte differentiation. In the preadipocyte cell line 3T3L1 CHOP is up regulated in the early phases of differentiation while the cells

undergo mitotic clonal expansion. In this phase CHOP dimerizes with C/EBPβ inhibiting its DNA binding activity thus blocking the expression of the key transcription factors that drive adipocytes differentiation. When cells exit the mitotic clonal expansion, CHOP is down regulated and C/EBPβ activates transcription and the following steps of differentiation (Li et al., 2006). The inhibitory role of CHOP in differentiation of preadipocytes to adipocytes was confirmed in primary cultures: overexpression of CHOP inhibited differentiation. Furthermore the same study shows that the physiological fat decrease associated with aging correlates with higher levels of CHOP expression in older rat preadipocytes with respect to cells from middle-age or young animals and that such up regulation is due to increased secretion of TNFα (Tchkonia et al., 2007). *In vivo* no effects of *chop* deletion were observed in male mice either when fed by standard or by high fat diet whereas *chop*$^{-/-}$ females showed increased body weight already when fed by standard diet. Even though the authors do not draw definitive conclusions about the cause of the observed phenotype, they report mis-regulation of C/EBP family members in female *chop*-null fat cells and lower levels of estrogens in the sera, an hormonal alteration that can cause obesity (Ariyama et al., 2007). While inhibiting adipogenesis CHOP promotes osteoblastogenesis. Pereiera and colleagues showed that in ST-2 murine stromal cells, the levels of CHOP transcripts rise as the cells differentiate toward osteoblasts, and that overexpression of CHOP accelerates osteoblastic cell differentiation and suppresses adipogenesis. The effects of CHOP deletion *in vivo* are less clear: the same authors found that *chop*-null mice have impaired osteoblastic cell function but the skeleton revealed no abnormalities and bone mineral density was normal (Pereira et al., 2006). Indeed another study evidenced a very complex role for CHOP in osteoblast differentiation depending on the commitment of the cells analyzed and on the stimuli that induced differentiation. Shirakawa and others showed that CHOP is expressed in bone as well as in mesenchymal progenitors and primary osteoblasts and that *chop*-deficient osteoblasts differentiate more strongly than their wild-type counterparts, suggesting that endogenous CHOP plays an important role in the inhibition of osteoblast differentiation. At the same time though they found that endogenous CHOP induces differentiation of calvarial osteoblasts upon bone

morphogenetic protein (BMP) treatment. They also demonstrated that, as in adipocytes, also in osteoblasts CHOP forms heterodimers with C/EBPβ and inhibits its DNA-binding activity thus blocking transcription of osteoblast-specific genes. These findings indicate that CHOP acts as a dominant negative inhibitor of C/EBPβ and prevents osteoblast differentiation but promotes BMP signaling in a cell-type-dependent manner. Thus, they conclude that endogenous CHOP may have dual roles in regulating osteoblast differentiation and bone formation (Shirakawa et al., 2006). C/EBPs (α, β and CHOP) are involved also in keratinocytes differentiation. Keratinocyte differentiation follows a temporally and spatially well-defined program of stratification. As cells migrate upward from the proliferative basal layer, they first undergo growth-arrest, then sequentially express a series of unique gene products, among which secreted keratin, and finally die by apoptosis to form the dead barrier layer. This program of differentiation can be reproduced in vitro by culturing a keratinocyte cell line in high calcium conditions. In fact when induced to differentiate these cells undergo growth arrest within 24-48 hrs, start expressing keratinocyte specific genes beginning at 24 hrs and after 48 hrs start undergoing apoptosis, closely reminding B cell differentiation. In a keratinocyte model Maytin and Habener analyzed the pattern of expression of C/EBPα, β and CHOP. They described that C/EBPα increases at day 1 and 2 and remains elevated, C/EBPβ raises at day 2 and 4 and gradually falls and CHOP raises at day 1 and then returns rapidly to baseline (Maytin and Habener, 1998). I could not find more recent studies about C/EBPs, in particular CHOP, role in keratinocyte differentiation.

I did not investigate properly the pattern of expression of the C/EBP family members in plasma cell differentiation. However the transcriptional profiling analyses indicate that C/EBPβ and C/EBPγ transcripts rise at day2 and day3 relatively to day1, with different kinetics (Fig. *D*1), whereas CHOP mRNA and protein levels decrease from day1 to day3 (Fig. 2 and *D*1). Furthermore, a study conducted to follow the pattern of expression of C/EBP transcription factors in different stages of B cell differentiation showed that C/EBPβ and C/EBPγ were differentially regulated in splenic B cells differentiated *in vitro* by LPS and that transcriptional activity from a C/EBP minimal promoter was found only in cell line models of later stages of B cell differentiation

(Cooper et al., 1994). It is worth to point out that C/EBP binding sites have been shown to be functionally important in Ig heavy chain variable region promoters, the H chain intronic enhancer and the κ chain intronic enhancer . These considerations raise the hypothesis that CHOP's role during B cell differentiation could be via the interplay with C/EBPβ and C/EBPγ and that these transcription factors could play a role in activating a differentiation-related transcriptional program, as observed in other cell types.

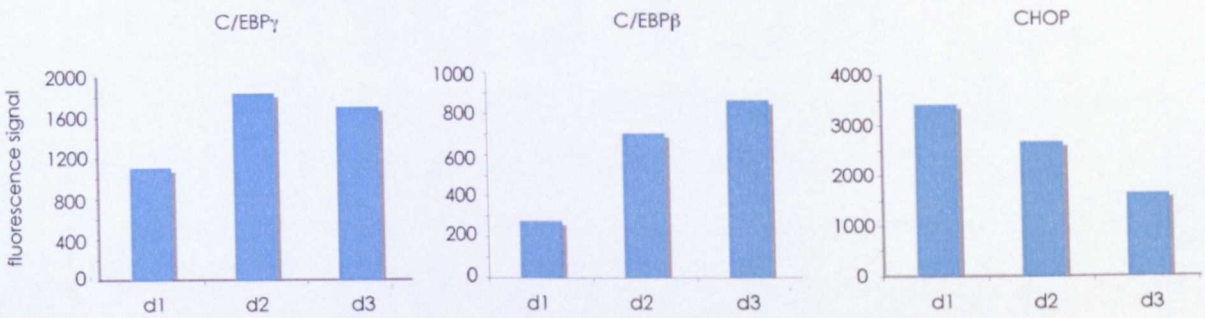

Fig. D1 Members of the C/EBP transcription factor family are up regulated during ASC differentiation

Expression pattern of C/EBPβ, C/EBPγ and CHOP as measured by microarray analysis. The fluorescence signals emitted by the different probes corresponding to a single gene were averaged for each time point and plotted in histograms.

4.2.4 How could CHOP deficiency during plasma cell differentiation affect the expression of chaperones involved in IgM polymerization?

A similar mechanism could explain why *chop*-null ASC phenotype consists in alterations of the folding machinery, whose components are part of the differentiation program. On the basis of this purely speculative hypothesis I analyzed about 1500 bp up-stream the transcriptional starting site of the chaperones whose expression is affected by *chop* deletion and indeed I could find various C/EBP binding sites (Table 2) (*http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3*) (Farre et al., 2003; Messeguer et al., 2002). It is worth pointing out that by the analysis performed I identified CHOP binding sites (Oyadomari and Mori, 2004) only in Ero1α regulatory region, but not in those of BiP, ERp44 or ERdj5. Anyway, other

considerations point to the idea that CHOP does not directly regulate these genes. In fact, CHOP is already back at basal level at the time points in which BiP, ERp44 and ERdj5 start rising and/or reach their maximal level of expression. The interplay with other members of the C/EBP family could justify such phenomenon. Among the many possible explanations, it could be imagined that CHOP/C/EBP complexes induce (or inhibit) the expression of some factor(s) that in turn activate the chaperones. Alternatively CHOP, alone or interacting with other factors could determine epi-genetic modifications that facilitate the expression of certain genes at later time points. ATF5 peak of expression on the contrary perfectly correlates with CHOP expression; however, when CHOP goes back to basal levels ATF5 remains highly expressed (compare figure 2 and 11 A). Furthermore, ATF5 mRNA level is lower in un-stimulated splenocytes suggesting that CHOP basal levels of expression are necessary to maintain normal ATF5 activation (Fig.11 B). When we analyzed the 2000 bp upstream the transcription initiation site we found many binding sites for the C/EBP family members but not for CHOP (Table 2).

gene	position	sequence	C/EBPα	C/EBPβ	C/EBPγ	CHOP	% dissimilarity
	-1075	TGCAAT				X	0
	-1541	TGCAAT				X	0
	-559	TTGCTG	X				0
	-777	GAGCAA	X				0
Ero1α	-834	TGTTGC	X				0
	-1312	TGTTGC	X				0
	-210	GCGCAA		X			0.58
	-559	TTGCTC		X			0.39
	-777	GAGCAA		X			0.39
	-559	TTGCTCAA			X		1.25
	-558	ATTGCTCAAT			X		6.05
	-155	AGTTGC	X				0.36
	-168	TAGCAA	X				0.86
	-1392	AGTTGC	X				0.86
BiP	**-153**	TTGCGG		X			2.3
	-168	TAGCAA		X			1.79
	-75	CTTCACCAAT			X		7.06
	-108	ATGGACCAAT			X		11.86
	-1392	TTGCTACA			X		3.64
	-860	TTGCTT	X				0.64
	-1301	GCAACA	X				0
	-860	TTGCTTCA		X			6.91
ERp44	-1032	TTGCAT		X			0.76
	-1058	TTGCACA		X			0
	-179	TTAAGCAA			X		0.11
	-860	TTGCTTCA			X		2.5
	-467	GCAACA	X				0
	-732	TTTCCTCAAT	X				10
ERdj5	-882	ATGCAA		X			0.76
	-1332	GTGCAA		X			0
	-471	TTTAGCAA			X		1.25
	-1473	TTAAGCAA			X		0.11
	-81	TTGCCT	X				2.96
	-279	TTGCCCA	X				3.2
	-359	GCAACC	X				0.36
	-279	TTGCCACC		X			13.4
ATF5	**-318**	TGGCGCAA		X			6.39
	-355	GGCAGCAA		X			12.95
	-81	TTGCCT			X		1.59
	-279	TTGCCACC			X		2.24
	-320	GCGCAA			X		0.58

Table 2

Table 2 C/EBP transcription factor binding sites are present in the promoters of Ero1α, BiP, ERp44, ERdj5 and ATF5

Putative binding sites for transcription factors belonging to the C/EBP family have been identified by PROMO analysis of about 1500-2000bp upstream the transcription initiation site (*http://alggen.lsi.upc.es/cgibin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3*). Only the sites with the highest similarity and closest to the transcription initiation site are listed in the table. Sites marked in bold are completely or partially overlapping for more than one transcription factor.

4.2.5 Chop$^{-/-}$ plasma cells do not exhibit increased levels of ER stress

These data led me to surmise that down regulation of components of the IgM folding machinery, in particular ERdj5, could explain the *chop*$^{-/-}$ ASC higher tendency to form HWM IgM aggregates. Such phenotype should give rise to intensified levels of ER stress in *chop*$^{-/-}$ ASC leading to an anticipated or stronger UPR that instead we

did not observe (Fig.4). There are two reasons to expect increased ER stress in the *chop*-null cells. The first is the 20% of IgM retained intracellularly in form of aggregates relatively to the wt (Fig.5 A and B), which likely determines the observed morphological alterations of the ER (Fig.5 C). Secondly, down regulation of BiP should reduce the amount of unfolded proteins accumulating in the ER required in order to free and thus activate, Ire1α and ATF6, especially in cells that retain higher amounts of secretory proteins than normal. However, cases of accumulation of aggregated misfolded proteins that do not induce UPR have already been described (Hidvegi et al., 2005; Mattioli et al., 2006). Regarding the BiP issue different considerations must be taken into account. Accordingly to many published studies and to the data presented in this work, it is clear that UPR activation is not easily confined into the model that described it until recently, based on the competition for BiP binding among the UPR sensors and the accumulating misfolded proteins (Bertolotti et al., 2000). As already discussed in the introduction, misfolded proteins could directly bind and activate Ire1α (Credle et al., 2005; Zhou et al., 2006) and physiological models of UPR, the one described in this work first of all, indicate that selected elements can be activated while others kept silent (Brewer and Hendershot, 2005; Gass et al., 2008; Zhang et al., 2005). Furthermore the analysis of BiP heterozygous mice (*grp78* $^{+/-}$) indicated that lower levels of BiP can be tolerated by the cells and by the entire organism. Whereas *grp78* $^{-/-}$ mice are lethal at the earliest stages of embryogenesis (E3.5) underscoring the essential role of this ER chaperone, *grp78* $^{+/-}$ mice, which express about half of the wt levels of BiP proteins, are viable and show no general defects. Moreover *grp78* $^{+/-}$ MEF show no basal up-regulation of the UPR, even though they express higher levels of PDI and grp94, and can still respond to ER stress with full expression of sXBP-1 and CHOP (Luo et al., 2006). Altogether these findings are compatible with the my observations showing that *chop*-null ASC, which express slightly lower levels of BiP, activate the UPR at the same extent as wt cells.

4.2.6 Lower sera IgM titers do not affect the immunological response of chop$^{-/-}$ mice to a T dependent antigen

I showed that IgM titers after immunization with a T cell-dependent antigen are lower in *chop*$^{-/-}$ mice with respect to that of wt littermates. This finding is in accord to the results obtained testing *in vitro* differentiated ASC. This was not the case for IgG, whose sera levels were as high in *chop*-null as in wt mice, but that were secreted with lower efficiency in *in vitro* experiments. At the present I do not know how to explain the discrepancy between the data obtained *in vivo* and *in vitro*. I can hypothesize that *in vivo* the interactions with other cellular populations can compensate CHOP deficiency sufficiently to rescue the ability of properly assembling and secreting IgG but not IgM, a more complex molecule that requires further steps of assembly and must pass more quality control check points. Further investigation is required to address this point.

Chop-null mice show no obvious immunological phenotype. However, I did not perform experiments aimed at testing the mice's immunological competence nor could I find any published study. Ehrenstein and colleagues created mice that, although harboring a normal number of B cells expressing surface IgM, completely lacked serum IgM while retaining the other Ig isotypes, by targeting the μ_s polyadenylation site. These mice retained a generally normal B lymphocyte distribution but exhibited substantial delays in mounting affinity-matured IgG responses to T cell-dependent antigens. The T cell-independent response, however, was augmented. The authors concluded that the IgM present before antigen challenge (as well, possibly, as that elicited immediately after immunization) accelerates maturation of the primary response, presumably by complexing with the antigen and facilitating lymphocyte activation and or antigen trapping (Ehrenstein et al., 1998). The same authors also showed that while deficiency in serum IgM leads to diminished responsiveness to foreign antigens at the same time causes increased responsiveness to self-antigens (Ehrenstein et al., 2000). I did not observe a delayed or reduced primary response, in terms of total IgG titer, upon T cell-dependent immunization. Since only at later time points I observed a reduced IgM titer in *chop*-

null mice sera relatively to wt, it is likely that the amount of IgM produced by *chop*$^{-/-}$ mice is sufficient to guarantee enough natural, as well as affinity-matured, antibodies to promote an efficient primary response.

4.2.7 Chop$^{-/-}$ *B cells are more sensitive to ER stress*

Likely, the most striking phenotype I observed in *chop*-null B cells is their higher sensitivity to ER stress-induced apoptosis. To my knowledge only one study described CHOP expression during the UPR as protective from apoptosis (Southwood et al., 2002). Southwood and colleagues studied mice models of a pediatric neurodegenerative disease that exhibits different degrees of severity depending on which kind of mutation of the *plp1* gene causes it. They found that some mutants induced UPR in oligodendrocytes, leading to CHOP expression. In this context CHOP played a protective role, since the same mutation that caused a mild disease and normal life span in wt background led to development of severe symptoms and very precocious death, due to strongly increased oligodendrocyte apoptosis rate, in *chop*-null background. Target genes up regulated by CHOP in MEFs treated with Tm were not induced in this model of physiological UPR. As the authors point out such difference could be due either to a cell type-specific set of C/EBP and more generally bZIP transcription factors that CHOP associates with, or to the different stimuli that induced UPR, a physiological, persistent stress due to the expression of a mutant protein versus acute, strong, drug-induced UPR. However, CHOP manifested pro-apototic activity in many other mouse models of physiological UPR. The Akita mouse develops diabetes because of pancreatic β cell apoptosis induced by expression of misfolded mutant insulin. *Chop*-null mice harboring a single allele encoding for mutant insulin developed hyperglycemia at later age and had a minor number of apoptotic β cells relatively to those observed in a wt background. Yet, when both insulin alleles are mutated no difference was observed in the onset or in the severity of diabetes (Oyadomari et al., 2002). My data suggest that B cells, at different stages of differentiation, are another cell type in which CHOP induction during the UPR

exerts a protective role from ER stress induced apoptosis. Importantly my conclusions are based on acute, drug-induced UPR. I cannot exclude that LPS stimulation is part of the causes of the major sensitivity to ER stress of *chop*$^{-/-}$ cells. Indeed, in order to avoid spontaneous apoptosis due to culturing isolated lymphocytes (Souvannavong et al., 2004), I performed the experiments in the presence of LPS also when assessing the sensitivity of freshly isolated splenocytes. However, a study conducted in the myeloma cell line NS0 supports my findings. Stably transfected subclones of NS0, lacking or overexpressing CHOP, were generated and their sensitivity to ER stress-induced apoptosis was tested by induction of UPR by Tm. The clone with undetectable levels of CHOP expression exhibited increased sensitivity to Tm treatment relative to the parental cells; on the contrary the clone over-expressing CHOP showed a slightly increased resistance (Cudna and Dickson, 2006).

Altogether these considerations point to a B cell-specific role for CHOP during the UPR. It will be interesting to identify CHOP targets in B cell UPR and compare them with those already identified in MEFs (Marciniak et al., 2004). As well, it will be important to identify CHOP heterodimerization partners in the different cell types (MEFs, B cells, oligodendrocytes) to shed light on the eclectic role of CHOP in response to ER stress.

4.2.8 Identification of ATF5 as a new UPR player downstream of CHOP

My attempt to single out CHOP targets during B cell differentiation led to the recognition of the transcription factor ATF5 as one of these. I indeed found that ATF5 expression is partially dependent on CHOP both during B cell differentiation and upon UPR induction in B lymphocytes. Furthermore ATF5 transcript is up regulated also in the fibroblast cell line NIH3T3 upon UPR induction (Fig.11). While I obtained these results Zhou and colleagues published their work showing that ATF5 protein is up regulated upon induction of ER and oxidative stress and following proteasomal inhibition (Zhou et al., 2008). The responses to these different stresses lead, via

activation of diverse kinases, to phosphorylation of eIF2α and ATF4 and CHOP induction. They demonstrated that eIF2α phosphorylation is necessary to induce ATF5 protein in all these stress conditions since the phosphorylation-incompetent $eIF2\alpha^{Ser51Ala}$ MEFs failed to up regulate ATF5 protein. They describe a post-transcriptional mechanism of regulation for ATF5 mRNA very similar to that driving ATF4 translation upon eIF2α phosphorylation, based on the presence of inhibitory ORFs upstream the coding region. In contrast with what I observed, this study reported that whereas oxidative stress induced increased ATF5 transcription, blocked in *atf4*$^{-/-}$ MEFs, ER stress did not. They thus concluded that upon oxidative stress ATF5 is regulated both at the transcriptional and translational levels, depending on ATF4 and eIF2α phosphorylation respectively, whereas in ER stress conditions only post-transcriptional regulatory mechanisms dictate ATF5 induction. ATF5 RNA basal levels where in any case lower in *atf4*$^{-/-}$ MEFs suggesting that basal levels of ATF4 activation guarantee steady state transcription of ATF5, accordingly with my observations in *chop*$^{-/-}$ cells (Fig.11 B). I did not checked ATF5 protein expression levels yet, but I observed increased ATF5 mRNA expression upon induction of ER stress in primary splenocytes and in the cell lines I.29μ^{+} and NIH3T3 (Fig.11 B and C). A possible explanation for this discrepancy could be found in the different drugs used to induce ER stress. I used the inhibitor of protein glycosylation tunicamycin while Zhou and colleagues used the inhibitor of the SERCA Ca^{++} pump thapsigargin.

Anyway, my data add to those published by Zhou and colleagues and pose ATF5 up regulation during the UPR downstream of the PERK/eIF2α/ATF4/CHOP pathway.

What will be important to determine is the role played by ATF5 in the UPR. ATF5 has been involved in apoptosis mainly with an anti-apoptotic role. There are findings indicating that ATF5 is down regulated in a variety of cells undergoing apoptosis following growth factor deprivation. When stably expressed in an interleukin 3-dependent cell line, ATF5 suppressed apoptosis resulting from cytokine deprivation. Conversely, a dominant-negative ATF5 mutant induced apoptosis of cells cultured in the presence of growth factors suggesting an anti-apototic role for this transcription factor (Persengiev et al., 2002). Involvement of ATF5 as an anti-apoptotic factor has

been described also in cell lines subjected to heat shock. In fact ATF5 promoted cell survival during heat shock stress via heat shock protein 27 (Hsp27) up regulation, and ectopic expression of ATF5 increased cell tolerance to elevated temperature. ATF5 up regulated Hsp27 gene promoter activity and inhibition of Hsp27 expression by RNAi could increase apoptosis in cells ectopically expressing ATF5, indicating Hsp27 involvement in ATF5 mediated cell survival (Wang et al., 2007). Accordingly with the anti-apoptotic function of this transcription factor ATF5 is highly expressed in tumors, like glioblastoma multiforme and in rat and human glioma cell lines. Interference with ATF5 function or expression in glioma cell lines caused marked apoptotic cell death whereas such manipulations did not affect survival of ATF5-expressing cultured astrocytes or of several other cell types that express this protein. Moreover retroviral delivery of a function-blocking mutant form of ATF5 into a rat glioma model evoked death of the infected tumor cells, but not of infected brain cells outside the tumors (Angelastro et al., 2006). Finally, loss of ATF5 function caused significant apoptotic death of neoplastic breast cell lines, but not of non-neoplastic breast cell lines (Monaco et al., 2007). However, a case of pro-apototic function of ATF5 has also been reported. The ectopic expression of ATF5 in HeLa cells increased cisplatin-induced apoptosis and the cleavage of Caspase-3, and induced Cyclin D3 mRNA expression via cooperation with E2F1 transcription factor. Moreover, the interference of Cyclin D3 expression by transfection with Cyclin D3 RNAi protected cells from ATF5-mediated apoptosis induced by cisplatin, indicating the contribution of Cyclin D3 in ATF5-mediated apoptosis (Wei et al., 2006). Taking into account the fact that ATF5 is downstream of CHOP in the UPR and given its involvement in apoptosis, it is tempting to hypothesize that ATF5 could be one of the mediators of the apoptotic functions of CHOP. In contrast with this idea there is the observation that CHOP is mainly pro-apoptotic whereas ATF5 is mainly anti-apoptotic in the models that have been studied. However, I have already discussed that both transcription factors can exert opposite functions. In particular, chop-null MEFs are more resistant to ER stress-induced apoptosis, whereas *chop*-null lymphoblasts are more sensitive. Thus these two different cell types could provide suitable models to shed light not only on the function of ATF5 in the UPR but also on its relation with

CHOP. If indeed ATF5 is downstream of CHOP in the pathways leading to or protecting from apoptosis, then down regulation of ATF5 in MEFs could result in higher resistance to ER stress while interfering with ATF5 expression in B cells could lead to higher sensitivity. The preliminary data I obtained support this hypothesis (Fig.11 D): the use of RNA oligonucleotides targeted to ATF5 transcript exerted a protective role from ER stress-induced apoptosis in the fibroblast cell line NIH3T3.
A different issue concerns the role of ATF5 during plasma cell differentiation. ATF5 is a member of the ATF/CREB family of bZip proteins, often involved in cellular differentiation. Neural progenitor/stem cells in the developing brain express high levels of ATF5 and it must be downregulated to permit neural progenitor cells to differentiate into neurons and glia (Angelastro et al., 2006; Angelastro et al., 2005; Mason et al., 2005). The findings that ATF5 transcript is up regulated during ASC differentiation but no major phenotype is observed in *chop*$^{-/-}$ cells, in which ATF5 expression is strongly inhibited, point to the conclusion that, as CHOP, ATF5 play no essential role in B cell differentiation. Nonetheless, it must be considered that a certain increase in ATF5 transcript can still be detected in *chop*-null cells and it could be sufficient to perform the required activity; furthermore it cannot be excluded that full ATF5 depletion in wt background would lead to different conclusions.

4.2.9 CHOP role in B cells: concluding remarks

In conclusion, I showed that *chop*$^{-/-}$ B lymphocytes undergo broadly normal differentiation, exhibiting a mild phenotype consisting in increased IgM aggregation that leads to slightly decreased IgM secretion relatively to wt plasma cells. I propose an explanation to this phenotype with the observation that the expression level of important factors involved in IgM folding and assembly, BiP, ERp44 and ERdj5, is lower in *chop*-null ASC. Despite its mildness, the phenotype described in *chop*$^{-/-}$ ASC is open to interesting speculations, yet to be proven, that could add information regarding the multi-faceted role of this transcription factor. Indeed I propose that

CHOP expression during differentiation is not related to UPR induction but it is part of the differentiation program. Furthermore I speculate that the interaction between CHOP and other bZip transcription factors (particularly those belonging to the C/EBP family) could regulate the expression of differentiation-related genes, like those involved in the secretory pathway.

Very likely CHOP belongs to a group of redundant pathways. I described its involvement in plasma cell differentiation yet I found a very mild phenotype, both *in vitro* and *in vivo*. Furthermore, even though the analysis of the transcriptional profiling showed that the data obtained are in line with the knowledge about plasma cell differentiation and thus validates them, I found very few genes that where differentially regulated in *chop*$^{-/-}$ ASC relatively to the wt cells. My observations are in accordance with those obtained in the study of osteoblast, adipocyte and keratinocytes differentiation. Although a role for CHOP was defined *in vitro*, *chop*-null mice show no major phenotype.

In my opinion, one of the most interesting findings I show concerns the peculiar role of CHOP in ER stress-induced apoptosis in B cells, relatively to those described up to now in other cell types, like MEFs, pancreatic β cells and epithelial kidney cells. The most likely hypothesis to explain the cell type-specific role of CHOP in B cells is that these express a different set of CHOP dimerization partners, thus leading to activation of diverse target genes with respect to MEFs, for example.

Finally, the study of *chop*$^{-/-}$ B cell differentiation brought me to the discovery of the ATF/CREB family member ATF5 as a novel CHOP target in B cell differentiation and in the UPR.

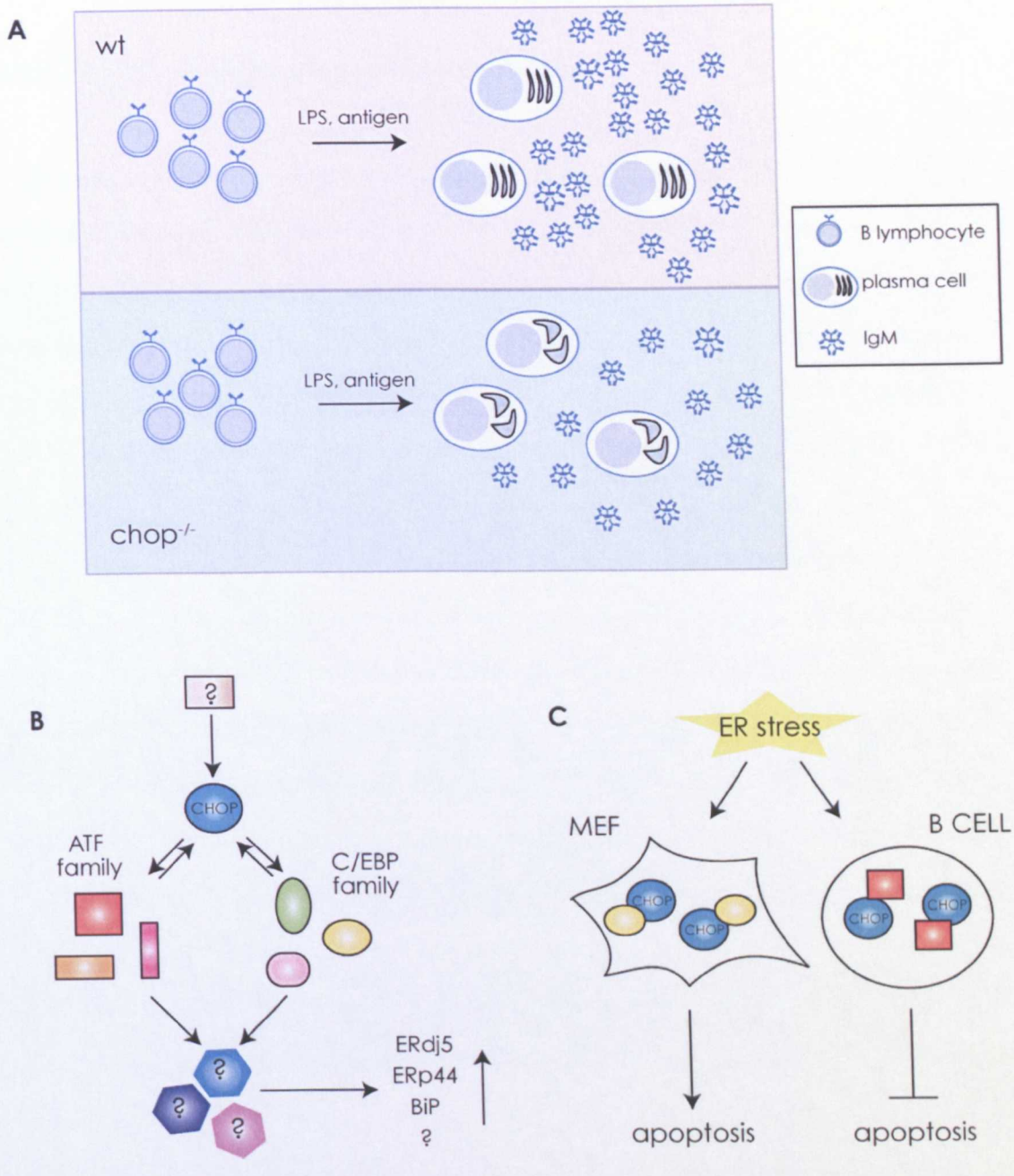

Fig. D2 CHOP role in B cells

A *chop-/-* B splenocytes undergo broadly normal differentiation, showing increased IgM aggregation, an altered ER morphology and decreased efficiency of IgM secretion. B During B cell differentiation CHOP is induced by pathways still to be identified. CHOP could interact with other members of the C/EBP family or with members of the ATF family of transcription factors. Such interaction could trigger pathways leading to full expression of genes related to plasma cell functions. C CHOP, induced by ER stress in different cell types, like MEF or B cell, could interact with diverse heterodimerization partners and depending on these interaction it could promote or antagonize apoptosis.

4.3 Manipulating gene expression in B cells

It is evident that some of the conclusions I draw are purely speculative and need further experimentation to be proven. For example it would be important to down regulate ERdj5 in wt ASC to undoubtedly assess a role for this chaperone in IgM assembly. Understanding the role of ATF5 during the UPR will require modulation of its expression in MEFs and in B cells, as discussed above. B cells are not transfected by traditional liposomal carriers but good enough levels of exogenous DNA expression can be achieved by the usage of viral vectors. At the moment we are working on the development of such vectors in order to be able to over express or down regulate by RNA interference whatever gene we are interested in and answer to some of the many questions we left open. Apart from viral vectors, we plan to use another tool to prove the role of the genes we are interested in, the cell line DT40. These are bursal-stage chicken B cells, expressing IgM on their surface. DT40 targeting system has been proven to be a valid model for plasma cell differentiation studies (Alinikula et al., 2006; Nera et al., 2006). The most obvious benefit of the DT40 cell line over other B cell lines is the ease of accomplishing a true knockout. Small interfering RNAs decrease the expression at best to a low level, whereas with conventional disruption methods in the DT40 cell line the expression of a functional protein is totally lost.

4.4 Limitation of plasma cell life span: a matter of multiple cellular processes failure?

The short lifespan of the majority of plasma cells is important to limit antibody responses. However, some of them home in the bone marrow where they find conditions for longer survival, which maintains protective Ig titers in the blood (Moser et al., 2006; Radbruch et al., 2006). While it is clear that extrinsic factors such as cytokines and contacts with stromal cells play important roles in shaping the environmental niche that promotes survival of long-lived plasma cells, little is

known about the mechanisms inducing the death of short-lived ones. In particular, the existence of intrinsic factors regulating plasma cell lifespan has been proposed, but never demonstrated.

4.4.1 Proteasomal capacity is impaired during plasma cell differentiation

Our findings reveal that in differentiating I.29μ$^+$ and primary spleen B cells, the proteasomal capacity decreases despite the higher demand imposed by the high rates of protein production (Fig.12 and appendix I). As an efficient ERAD is important for folding and secretion capacity, it was surprising to observe that the enzymatic activities and the relative abundance of proteasomes decreased during differentiation of both I.29μ$^+$ lymphoma cells and primary splenocytes. This could represent a distinctive feature of short-lived plasma cells, as dendritic cells upregulate proteasomal subunits in response to LPS (Macagno et al., 2001). No changes in proteasome activity were induced by LPS in a monocytic line, implying that TLR4 triggering *per se* is not sufficient to cause proteasome down regulation. Whilst polyubiquitinated proteins accumulated in activated B cells, the pool of free ubiquitin decreased. Either the latter or the relative number of proteasomes could limit degradation. As a consequence, endogenous proteasomal substrates were stabilized in differentiating I.29μ$^+$ cells, and the reporter of the ubiqutin-proteasome pathway GFP^{G76V} accumulated in LPS-activated GFP^{G76V}-transgenic splenocytes (Fig.12 B). We observed stabilization of endogenous proteasomal substrates like IκBα and Bax that might meet certain functional requirements of plasma cells. Accumulation of death factors may predispose to the apoptotic program. In this scenario, plasma cell death would be linked to Ig production, thus contributing to end humoral responses. Hence, we propose that the progressive impairment of proteasomal capacity is one of the mechanisms that contribute in limiting plasma cell lifespan, linking it to antibody production ((Cenci et al., 2006), appendix I).

4.4.2 Plasma cell differentiation entails oxidative stress and the activation of antioxidant responses

In all likelihood, different factors concur in rendering ASCs susceptible to apoptosis. Perturbation of the redox homeostasis could be one of these. Oxidative folding, i.e. intra- and inter-molecular disulphide bond formation, could be a source of reactive oxygen species (paragraphs 1.2.2 and 1.2.3). My preliminary findings exclude a determinant role for H_2O_2 accumulation in initiating the apoptotic program of ASC. I did measure highly increased concentration of H_2O_2 but only after the apoptotic program already began (Fig.13 A). Nonetheless, this does not exclude that the rise in hydrogen peroxide could contribute at later time points as a further apoptotic signal. Interestingly I could not detect accumulation of H_2O_2 in correspondence with the initiation of massive Ig production, after 3 and 4 days upon LPS stimulation, but only later, at day5. *In vitro* assays demonstrated that yeast Ero1 generates H_2O_2 in equimolar amounts to the number of disulphide bonds formed. Since the pathway is highly conserved in higher eukaryotes, it will be important to assess if H_2O_2 is generated in mammalian cells as well, and we are investigating the issue. Suggestions of a significant contribution of Ero1 in cellular ROS production in metazoans come from the observation that down-regulation of Ero1 strongly reduces ROS levels in ER stressed *C. elegans* (Harding et al., 2003). If we assume that H_2O_2 is indeed generated by oxidative folding, the missing correlation between the increase in Ig production and the rise in H_2O_2 concentration could be explained by the up regulation of the peroxiredoxins I, III and IV (Fig.13 C). Increased Prxs activity between day 2 and 3 could be sufficient to eliminate high amounts of hydrogen peroxide. Importantly, the cytosolic Prx I and the mitochondrial Prx III expression levels decrease at day 4, before the H_2O_2 rise observed at day 5. On the contrary Prx IV expression steadily increases during differentiation, at least until day 4, the last time point I analyzed. I hypothesize that Prx IV increase could reflect the need of keeping the Ig folding compartment as efficient as possible until cell death. As part of a collaboration in the laboratory, we are targeting an engineered YFP reporter of hydrogen peroxide concentration to the ER. Commercial versions of this reporter are

already available, targeted to cytosol or to mitochondria compartments (Belousov et al., 2006). We hope that such tools will allow discrimination of the site of H_2O_2 production and accumulation in differentiating B cells.

Lastly, it could be worth pointing out that in correspondence with increased levels of H_2O_2 HMW aggregates accumulate in higher measure (Fig.13 B) and *chop*$^{-/-}$ ASC, which exhibit increased aggregation of IgM relatively to wt cells, present slight, but statistically significant, higher amounts of hydrogen peroxide. The open question is who came first: when folding is impaired multiple cycles of misoxidation, reduction and reoxidation could increase the levels of oxidative stress; viceversa, unbalance toward oxidizing conditions in the ER could affect chaperones activity and impair the folding capacity of the ER.

5. Materials and Methods

5.1 Mouse strains, cell cultures and spleen B cell isolation

chop$^{-/-}$ mice were kindly provided by Dr. David Ron (Zinszner et al., 1998) while Ub^{G76V} were provided by Dr. Nico Dantuma (Dantuma et al., 2000).

I.29μ^{+} murine lymphoma cells and purified B splenocytes were cultured in RPMI with 10% FCS endotoxin-free, 100U/ml Pen/Strep, 1mM sodium-pyruvate, 2mM N-glutamine (GIBCO) and 50μM 2-mercaptoethanol (SIGMA) and induced to differentiate to ASC by addition of 20 μg/ml LPS (SIGMA). Purification of B lymphocytes from mouse spleen was performed by magnetic isolation with anti-CD19 beads (Miltenyi Biotec) and LS^{+} MACS separation columns following the manufacturer instructions. Primary B cells were seeded at an initial cell density of 10^6/ml. Cells were never allowed to reach a cell density higher than 2-3*10^6/ml to avoid risk of starvation. The cultures were diluted with complete medium. Treatments with the ER stressors tunicamycin and thapsigargin were performed as described in the figure legends. For the RT-PCR reported in Fig.4 B I.29 cells were treated with 1 μg/ml Tm O/N. The drugs were added at the indicated concentrations to the culture media.

For the LPS immunization experiment 3 mice/point were injected intra-peritoneum with 35μg/g LPS (L2637 SIGMA) and B cells were purified as described (Cascio et al., 2008). For the ovalbumin immunization experiment 5 mice for each genotype were injected sub-cutaneously with 100μl of PBS/0.5mg ovalbumin/75μg LPS as adjuvant.

5.2 Flow cytometry

2*10^5 to 5*10^5 I.29μ^{+} or differentiating B cells were washed in PBS/0.5% BSA and stained with each of the following reagents: 0.5μg/ml propidium iodide, 5mM PG1 (Peroxy-Green) 1:200 anti-CD138 PE-conjugated (BD Pharmingen), 1:200 anti-CD38

FITC-conjugated (BD Pharmingen), 1:10.000 anti-μ chain (Cappel) FITC-conjugated and 1:200 anti-α chain FITC-conjugated (BD Pharmingen).
Samples were read at the Cytomics FC500 flow cytometer (Beckman Coulter) and data analyzed by the FCS express analysis software.

5.3 Relative real time PCR

Total RNA was isolated from cells by the Trizol reagent (Invitrogen) following the manufacturer instructions and quality of the purified RNA was checked by OD 260-280 readings and by electrophoresis separation. 0.5-1 μg of total RNA was retro-transcribed by the Super-Script II kit (Invitrogen) in a final volume of 10μl. 0.5-1 μl of these were used in each PCR reaction. Real time PCR was performed with Sybr Green Master Mix (Applied Biosystems) in a final volume of 25μl by the ABI7900 HT thermal cycler (Applied Biosystems). Data were analyzed by the SDS 2.0 software. The increase of each transcript at a certain time point of differentiation relatively to day0 was calculated by the $\Delta\Delta C_t$ method using histone 3 and/or β-actin as reference genes. The following primers were used for PCR. The following primers were used: CHOP - FW: GCG ACA GAG CCA GAA TAA CA RW: ACC AGG TTC TGC TTT CAG GT; BiP - FW: TAT TGG AGG TGG GCA AAC CAA G RW: CGC TGG GCA TCA TTG AAG TAA G; Ero1α - FW: CCG AAA AAC TGA TCG CAA AT RW: CCG TCC TCC TCA GTG AAC AT; XBP1 splicing - FW: GGA GTG GAG TAA GGC TGG TG RW: CCA GAA TGC CCA AAA GGA TA; XBP1 total - FW: TTT GGG CAT TCT GGA CAA GT RW: AAA GGG AGG CTG GTA AGG AA; EDEM - FW: ACT GAT TCC AAA CAG CCC TT RW: GGA TCC CTG TCT TGG TGT TT; ERdj4 - FW: GCC ATG AAG TAC CAC CCT GA RW: CTT TCC GAC TAT TGG CAT CC; ATF5 – FW: GGCTGGCTCGTAGACTATGG RW: CAGCCTGGACCTGTACCCTA.

5.4 Western blot

Total cell lysates (to detect IgM assembly intermediates) were prepared by lysis of the

cells in a buffer containing 25mM Tris, 100 mM NaCl, 3mM EDTA, 2% SDS plus freshly added protease inhibitors (Roche Complete protease inhibitor cocktail, following manufacturer instructions) and 10mM NEM (N-ethyl maleimide) at room temperature, followed by sonication to remove DNA and boiling 10 to 15 mins. Post nuclear supernatants (to detect ERdj3, HERP, BiP, ERp44, ERGICp53, ERdj5, Ero1α, Prx I-VI) were prepared by lysis in 10mM Tris HCl pH 7.4, 150mM NaCl, 1% NP40 plus freshly added protease inhibitors (Roche Complete protease inhibitor cocktail, following manufacturer instructions) and 10mM NEM for 15 mins on ice, followed by centrifugation at 17.000xg for 15 mins at 4°C to remove nuclei. Nuclear extracts (to detect CHOP and sXBP1) were prepared by lysing the cells in harvest buffer (10mM HEPES, 50mM NaCl, 0.5M sucrose, 0.1mM EDTA, 0.5% Triton X-100 plus freshly added 1mM DTT and protease inhibitors – as above-), and lysing the nuclear pellet, obtained by centrifugation of the lysates at 17.000xg for 15 mins at 4°C, in nuclei buffer (10mM HEPES, 500mM NaCl, 0.1mM EDTA, 0.1mM EGTA, 0.1%NP40 plus freshly added 1mM DTT and protease inhibitors).

To detect IgM assembly intermediates total cell lysates were separated on gradient polyacrylamide running gels (2-14%) with 2.5% stacking gels. Stacking gels were blotted too since high molecular weight IgM aggregates were found mostly there. Total lysates of HEK293FT after transfection with μ, λ and J chains plus or minus ERdj5 were separated by pre-casted Bis-Tris 4-10% gels (Invitrogen) following manufacturer instructions.

To detect all the other proteins conventional SDS-PAGE was performed.

Transfer of the proteins on nitrocellulose filter was performed in wet conditions (Pharmacia Biotech transfer tank) with transfer buffer (1x tris-glycin buffer) containing 20% methanol overnight at 4°C. After transfer filters were blocked with 5% milk in PBS/0.1% tween 20 buffer for 1hr at r.t.

Anti-BiP and anti-HERP rabbit sera and anti-ERdj3 antibody were kindly provided by Dr. Linda Hendershot (St. Jude Children's research hospital, Memphis, TN, USA). Anti-ERp44 and anti-Ero1α monoclonal antibodies were produced by hybridoma cell cultures (anti-ERp44 (39E5) published in Anelli 2007, supplementary material appendix II; anti-Ero1 α Bergamelli et al. submitted). The anti-ERGICp53 rabbit

serum was kindly provided by Dr. HP Hauri (Basel, Switzerland). The monoclonal anti-ERdj5 was kindly provided by K. Nagata (Kyoto, Japan). XBP1 and CHOP antibodies were from Santa Cruz biotechnology. Anti-Prx I-VI polyclonal antibodies were from AB frontier. Anti-μ rabbit serum was from Zymed. Anti-κ antibody was from Southern Biotechnology and anti-J antibody was a polyclonal serum produced by PRIMM (Milan, Italy). Anti-actin was from SIGMA. Primary antibodies were diluted in PBS/0.1% tween buffer containing 5% milk, all at 1:1000 but XBP and CHOP that were diluted at 1:400. Incubation with all the primary antibodies was performed for 1hr at r.t, followed by 3 washes for 15' in PBS/0.1% Tween . Secondary antibodies, anti-mouse or anti-rabbit HRP conjugated (Southern Biotechnology) were diluted at 1:2000 in PBS/0.1% tween and filter incubated for 1hr at r.t. Filters were then washed 3x in PBS/0.1% Tween for 15 mins and signal was detected with Amersham ECL substrate. When indicated filters were stripped with 100mM glycin pH 2.5 for 30' at r.t. followed by 2 washes in PBS for 15' at r.t. and then blocked and re-probed as described above.

Western blot images were acquired with the Chemidoc-it Imaging System (UVP, Upland, CA) and processed with Adobe Photoshop 7.0 (Adobe Systems Inc.). Densitometric analysis was performed by the Image Quant 5.2 software (Molecular Dynamics).

5.5 Secretion assay and ELISA

Differentiating B cells were washed twice in PBS and cultured in Optimem (GIBCO) in the absence of FCS for 4 hrs at a cell density of 10^6/ml. Protease inhibitors and 10mM NEM were added to the media at the end of the incubation period and the cells were discarded by sequential centrifugation at 300xg first and at 17.000xg afterwards. 10μl of media or mice sera diluted 1:500 were analyzed by ELISA. Analysis of IgM secreted in the culture medium was performed as follow: the ELISA plate was coated with 1:200 anti-μ rabbit serum (Zymed) O/N at 4°C, blocked with 3% milk in PBS/0.05% Tween 20 for 1hr at r.t and media were incubated O/N at 4°C.

To detect IgG coating was performed with 1:200 anti-γ specific antibody (Southern Biotechnology) followed by the same steps as for IgM detection. After washing (3x with PBS/tween) a secondary anti-κ-HRP 1:1000 (Southern Biotechnology) was incubated for 1hr at r.t. After washing (3x with PBS/tween) the assay was developed with SIGMA OPD fast read and the plate read at 450nm. To detect IgM and IgG in the sera the plate was coated with ovalbumin conjugated to BSA (20 μg/ml) O/N at 4°C, blocked with 3% milk in PBS/0.05% Tween 20 for 1hr at r.t and sera were incubated O/N at 4°C. After washing (3x with PBS/tween) anti-γ-HRP 1:1000 (Southern Biotechnology) or anti-μ-HRP 1:1000 (Southern Biotechnology) was incubated for 1hr at r.t. After washing (3x with PBS/tween) the assay was developed with SIGMA OPD fast read and the plate read at 450nm. All the antibodies were diluted in PBS/0.05% tween 20.

5.6 Transfection

HEK293FT were cultured in DMEM with 10% FCS, 100U/ml Pen/Strep, 1mM sodium-pyruvate, 2mM N-glutamine (everything from GIBCO). $2x10^6$ cells were plated in 10cm Petri the day before transfection. Transfection was performed with a mix of 120 μg PEI plus 12μg DNA in PBS1x added to 6 ml of media O/N. Plasmid transfected were based on PCDNA 3.1 (-) where the cDNA sequences of J, λ and μ chains were cloned. ERdj5 plasmid was a kind gift of Prof. K. Nagata (Kyoto, Japan). The following day cells were cultured in normal medium and harvested for lysis 48hrs after transfection.

5.7 Immunofluorescence and electron microscopy

Lymphoid cells were incubated on poly-L-lysine-coated cover-slips for 20 min at 37°C on 10-mm^2 cover-slips. Cells were fixed in 3% paraformaldehyde and permeabilized with 0.1% TX100. Cells were then decorated with the indicated antibodies diluted in PBS 1:1, 1hr at r.t (anti-ERp44 monoclonal described in 5.4; rabbit anti-p115 was

kindly provided by Dr. M. A. De Matteis (Chieti, IT); rabbit anti-calreticulin was from Stressgen (Victoria, BC Canada)) and washed 3x with PBS. After staining with the appropriate secondary antibodies (Alexa Fluor 488 Goat anti-mouse IgG (H+L) or Alexa Fluor 564 Goat anti-rabbit IgG (H+L) from Molecular Probes (Oregon, USA)) diluted 1:200 cells and 3 washes in PBS cells were stained with Hoechst (1:1000 at r.t. for 5'), and then samples were mounted in Mowiol.

Slides were analyzed on an Olympus inverted fluorescence microscope (model IX70) with DeltaVision RT Deconvolution System (Alembic, HSR, Milano). After deconvolution, images were processed with Adobe Photoshop 7.0 (Adobe Systems Inc.).

B splenocyte pellets obtained by centrifugation at 300xg at r.t. for 5' were fixed for 1 hour at 4 °C with 4% paraformaldehyde and 2,5% glutaraldehyde in 125 mM phosphate buffer. The pellet was postfixed (1 hour) with 1% OsO4 in125 mM phosphate buffer, washed, dehydrated and embedded in Epon. Conventional thin sections parallel to pellet surface, were collected on uncoated grids, stained with uranil and lead citrate and examined in a Leo912 electron microscope.

5.8 Expression profiling analyses

Total RNA was isolated from cells by the Trizol reagent (Invitrogen) following the manufacturer instructions and quality of the purified RNA was checked by OD 260-280 readings and by electrophoresis separation. The RNA was analyzed by Dr. Geoffrey Neale, at the Hartwell Center for Bioinformatics and Biotechnology, St. Jude Children's Research Hospital, Memphis, Tennessee, USA. The analysis was performed using pairwise comparison in the Affymetrix GCOS software. The LogRatio was calculated, and a Change Call (Increase/Decrease, No Change) was provided in the data output. The probesets with >2-fold difference plus a change call at each of the three timepoints were selected. Further analysis of the data was performed by myself as described in chapter 3.1.7.

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