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A Limited Number of Genes Are Involved in the Differentiation of Germinal Center B Cells

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Abstract Mature B cells, upon activation, progressively differentiate through centroblasts into centrocytes and finally to plasmacytes that express large amounts of selected immunoglobulins. A significant part of this maturation is thought to involve induction of the unfolded protein response (UPR). We have compared gene expression in normal germinal center centroblasts, centrocytes, lymphoblastoid cells undergoing induced UPR, and the CCL155 plasmacytoma cell line. In the centroblast to centrocyte transition there is a change in the expression of a relatively small number of genes. These include a limited subset of the genes upregulated by a fully activated UPR as well as a small number of other transcription factors, some disulfide isomerases, and other genes. This is consistent with a model in which this transition is mediated by changes in the levels of expression of transcription factor B-lymphocyte-induced maturation protein 1 (Blimp1) (PRDM1), BACH2, X-box binding protein 1 (XBP1), interferon regulatory factor 4 (IRF4), and possibly vitamin D receptor (VDR) expression, together with post-transcriptional changes and a limited induction of aspects of the UPR. J. Cell. Biochem. 99: 1308–1325, 2006.

Key words: gene expression; germinal center; centroblast; centrocyte; transcription factor

The molecular regulation of B-cell development is one of the most intensively studied mammalian cellular developmental systems and has provided insights for understanding mechanisms of mammalian development. B cells corresponding to several distinct stages of development can be obtained in substantial numbers from various B-cell lymphoma lines and Epstein–Barr virus (EBV) or Abelson virus transformed cell lines as well as by fluorescence-activated cell sorting (FACS) of normal B cells [Alt et al., 1981; Hardy et al., 1984; Sale and Neuberger, 1998; Nakayama et al., 2001; Kuppers, 2003]. There are significant correlations between surface antigen phenotype and intracellular events. This makes it possible to isolate untransformed cells at specific stages of development by use of surface markers.

Early B-cell lineage development takes place in the bone marrow [Osmond, 1990], and mature B cells migrate to secondary lymphoid organs. In the secondary lymphoid organs B cells encounter foreign antigens. Antigen-stimulated B cells in turn develop to form germinal centers [Jacob et al., 1993]. The micro

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environment of the secondary lymphoid organ provides not only antigen stimulation through the B-cell surface immunoglobulin receptor but also provides the environment in which B cells can interact with other cells, such as T cells and follicular dendritic cells [Callard et al., 1993].

Antigen stimulation triggers drastic cellular and genetic modifications in B cells. The antigen-triggered germinal center B cells become centroblasts that express CD77 [Liu et al., 1996] and proliferate rapidly [MacLennan et al., 1992] to form the B-cell dark zone. They progressively develop to form centrocytes that no longer express CD77 and constitute the B-cell light zone [Liu et al., 1996]. Immunoglobulin gene hypermutation and class switching, both mediated by activation-induced cytidine deaminase (AID) [Shinkura et al., 2004], take place mainly in centroblasts and centrocytes, respectively [Liu et al., 1992; Liu et al., 1996]. The CD77-positive centroblasts readily enter apoptosis but can be rescued by CD40–CD40L interactions [Mangeney et al., 1995]. Thus, death and survival signals [Guzman-Rojas et al., 2002] result in the selection of high-affinity antibody producing B cells, and eventually in the production of high-affinity antibody [Jacob et al., 1991; Jacob et al., 1993; Adams et al., 2003]. Finally, the antigen-triggered B cells develop into plasma cells that are exclusively engaged in the production of large amounts of antibodies.

Recent studies have identified a few key transcription factors that are important in plasma cell development from antigen-stimulated B cells. Metastasis-associated 1 family, member 3 (MTA3) controls the activity of B-cell chronic lymphocytic leukemia/lymphoma 6 (zinc finger protein 51) (BCL6). BCL6 represses various transcription factors in the germinal center [Fujita et al., 2004]. Release from BCL6 repression is required for differentiation into plasma cells. The B-lymphocyte-induced maturation protein 1 (Blimp1), also known as the PR domain containing protein 1 (PRDM1), plays important roles in promoting differentiation into plasma cells [Angelin-Duclos et al., 2000; Shapiro-Shelef et al., 2003]. The X-box binding protein 1 (XBP1) is involved in plasma cell development [Iwakoshi et al., 2003a,b]. In particular, a spliced form of XBP1 is a mediator of the unfolded protein response (UPR) [Ma and Hendershot, 2001]. It is likely that XBP1 in part mediates plasma cell development and also facilitates antibody production by inducing some of the components of the UPR in plasma cells [Iwakoshi et al., 2003b; Shaffer et al., 2004].

Pioneering work with yeast revealed that the UPR enables proper protein folding under conditions of stress in an endoplasmic reticulum (ER). The UPR prevents aggregation of immature or incorrectly folded proteins by increasing the components of the entire secretary pathway and by increasing proteasome activity to eliminate unfolded proteins [Patil and Walter, 2001]. In yeast, the UPR is dependent on the protein encoded by ER protein inositol-requiring 1 (IRE1p) creating a spliced form of the transcription factor HAC1 mRNA. XBP1 is the mammalian homologue of Hac1 [Yoshida et al., 2001]. IRE1p possesses endoribonuclease activity that is dependent on activation of its kinase activity in response to ER stress [Sidrauski and Walter, 1997]. Activated IRE1p splices HAC1 that leads to a translational reading frameshift that facilitates production of the active form of protein encoded by HAC1 (Hac1p). Hac1p induces transcription of various UPR genes. Recently, it has been shown that Hac1 mRNA is also regulated at the transcriptional level by an IRE1 independent pathway [Leber et al., 2004]. In addition, it has been also shown that a novel upstream element binding the transcriptional activator Gcn4p regulates UPR separately from the HAC1 pathway [Patil et al., 2004].

Recent studies in mammalian cells show that the UPR is regulated by three ER membrane localized proteins: IRE1/ER to nucleus signaling 1 (ERN1), activating transcription factor 6 (ATF6), and eukaryotic translation initiation factor 2-alpha kinase 3(EIF2AK3)/double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK) [Harding et al., 2002; Gass et al., 2004]. Under ER stress, these three proteins are activated by release from the ER-resident protein immunoglobulin heavy chain binding protein (BiP) [Prols et al., 2001; Gass et al., 2004]. Upon release of BiP, IRE1/ERN1 oligomerizes and gains endoribonuclease activity that induces production of the spliced form of XBP1 mRNA. The spliced form of XBP1 excises a 26-bp intron, causing a frameshift to produce a new C-terminus on XBP1 that confers transcription activating activity [Ma and Hendershot, 2001]. The spliced form of XBP1 binds to ER stress-response element (ERSE) and unfolded protein

response element (UPRE) motifs of UPR genes including various chaperones, folding proteins, and XBP1 [Yoshida et al., 1998; Ma and Hendershot, 2001]. ATF6 moves to the Golgi apparatus from the ER on release of BiP upon ER stress and in the Golgi ATF6 protein is cleaved by site-1 protease (S1P) and site-2 protease (S2P) to generate an N-terminal form of ATF6, which in turn acts as a transcription factor and induces XBP1 and ER chaperone and folding enzymes [Gass et al., 2004]. Activated PERK phosphorylates the EIF2 translation initiation factor to depress protein synthesis, presumably decreasing the load on the ER, and also permitting selective translation of some mRNAs.

In this report, we characterized gene expression in normal germinal center B cells at the centroblast and centrocyte stages and compared the results to those from B-cell lines using Affymetrix DNA microarray assays. We found that in the development from centroblasts to centrocytes only about 60 genes showed altered expression. We then compared these results to their expression status in plasma cells and to the expression levels resulting from induction of the UPR in a B-cell line. The results indicate that there is only a muted and limited UPR response during the centroblast–centrocyte transition. Only a small number of transcription factors is altered during the transition to centrocytes, including Blimp1, the spliced form of XBP1, interferon regulatory factor 4 (IRF4), broad complex-tramtrack-bric-a-brac (BTB), and Cap'n'collar (CNC) homology 1, basic leucine zipper transcription factor 2 (BACH2), and possibly Vitamin D receptor (VDR). The changes in these factors could account for a significant fraction of the total change in gene expression during this transition.

MATERIALS AND METHODS

Tonsillar B-Cell Subset Separation

Fresh tonsils were obtained from patients (2- to 7-year old) during routine tonsillectomy performed at the Yale-New Haven Hospital. Tonsils were finely minced with a razor blade in RPMI medium 1640 (Gibco/BRL, Gaithersburg, MD) without supplements and the cell suspension was passed through a 40-μm mesh cell strainer (Becton-Dickenson Immunocytometry Systems, San Jose, CA). Mononuclear cells (MNC) were recovered by Ficoll-Pacque Plus (Amersham Biosciences, Uppsala, Sweden) separation. MNC were kept overnight at 4°C. After thoroughly resuspending cells with a pipette, the cells were again passed through a 40-μm mesh cell strainer to remove aggregated cells. Tonsillar B cells were isolated by negative selection from the MNC with Dynabeads using a B-cell Negative isolation Kit (Dynal, Oslo, Norway) with the minor modification of adding anti-CD1a (Pharmingen, Los Angeles, CA) to an antibody mixture of anti-human CD2, CD3, CD7, CD14, CD16, and CD56 to deplete all non-B cells.

Immunoglobulin (Ig)D+/CD38− Naïve B cells were separated by binding to an anti-IgD monoclonal antibody (Pharmingen) using CELLectionTM Pan Mouse IgG Kit (Dynal) or by FACS sorting using anti-IgD monoclonal antibody and Cy-Chrome anti-human CD38 (Pharmingen).

IgD−/CD38+/CD77+ (centroblasts) and IgD−/CD38+/CD77− (centrocytes) were separated from the IgD-depleted B-cell fraction by FACS sorting. Phycoerythrin (PE)-conjugated anti-human IgD (Pharmingen), Cy-Chrome anti-human CD38 (Pharmingen), and rat anti-human CD77 IgM (ImmunoTech, Lyon, France) in combination with the secondary antibody fluorescein isothiocyanate (FITC)-conjugated goat anti-Rat IgM (Jackson ImmunoResearch, West Grove, PA) were used. Stained B cells were resuspended in RPMI1640 with 0.1% bovine serum albumin (BSA) at 1.7×10^7 cell/ml. IgD−/CD38+/CD77+ (centroblasts) and IgD−/CD38+/CD77− (centrocytes) were separated by FACS sorting.

Flow cytometric analysis of cell subpopulations was performed using a FACS Vantage flow cytometer (Becton-Dickenson Immunocytometry Systems). The cells were excited at 488 nm. The FITC, PE, and Cychrome fluorescence emissions were collected through 530/30 nm, 575/26 nm, and 660/20 nm band pass filters, respectively. The sorting was performed at 9,000 cells/s. FACS data analysis was performed using the CellQuest software (Becton-Dickenson Immunocytometry Systems) or Windows Multiple Document Interface for Flow Cytometry (WinMDI 2.8).

B-Cell Lines

An EBV-transformed peripheral blood B-cell line, the Pala cell line, was kindly provided by Dr. Peter Cresswell of Yale University. BL2, a Burkitt's lymphoma cell line, was kindly provided by Dr. George Miller of Yale University.

The Ramos cell line was obtained from Dr. Michael Neuberger of Cambridge University. CCL155, a plasmacytoma cell line was purchased from American Type Culture Collection (Manassas, VA). All cell lines were maintained in 10% fetal calf serum (FCS) supplemented RPMI1640 medium in a 5% CO_2 incubator.

RNA Sampling and Oligonucleotide Array Hybridization

Total RNA was extracted from B cells by RNAwiz (Ambion, Austin, TX) according to the company's protocol. RNA samples were cleaned by passage through RNeasy Mini Spin Columns (Qiagen, Valencia, CA) and kept at $-70^{\circ}C$ until use. RNA was converted to cDNA by SuperScript II Reverse Transcriptase (Life Technologies, Rockville, MD). Preparation of cRNA was performed by in vitro transcription with T7 RNA polymerase (Ambion) in a nucleotide triphosphate mixture containing the biotinylated reagents Bio-11-CTP and Bio-16-UTP (Enzo Life Sciences, Famingdale, NY). Labeled cRNA samples were fragmented and hybridized to HGU133A and B GeneChip arrays (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions.

For centroblasts and centrocytes analysis, three individual samples were analyzed by microarray. For B cells lines: CCL155 plasmacytoma, Pala control, and dithiothreitol (DTT)-treated samples were analyzed one time by microarray.

Affymetrix data analysis and normalization followed the standard Affymetrix processing procedures. Raw data output was managed by Affymetrix MicroArray Suite version 5.0, with average intensity (TGT) set as 150 and the presence or absence of expression was determined using the statistical algorithm of Microarray Suite 5.0.

Polymerase Chain Reaction (PCR)

Microarray data for some transcription factors as well as UPR-related genes were verified by reverse transcription and polymerase chain reaction (RT-PCR) and quantitated by using Quantity one (Bio-Rad, Hercules, CA) or by real time PCR. PCR primers were designed by using the MIT Primer3 program (http://www-genome.wi.mit.edu/cgibin/primer/primer3.cgi/results_from_primer3) to extend across 2 exons of each gene in order to detect if PCR products originated from mRNA or genomic DNA. Primers were designed to yield amplicons of about 100–250 bp. First-strand cDNA was synthesized from 1 μg of total RNA using SuperScript II Reverse Transcriptase.

Real time PCR was performed on a Smart Cycler (Cepheid, Sunnyvale, CA), using SYBR Green I fluorescent dye (QuantiTech SYBR Green PCR, Qiagen) or iCycler (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad).

A comparative threshold cycle (Ct) method was used to process the real time PCR data [Tsukahara et al., 2003]. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a reference gene for normalization. All samples were analyzed in triplicate. Results were calculated as the normalized difference in cycle threshold for a centrocyte or plasmacytoma relative to the centroblast (ddCt), calculated by subtracting the Ct of the GAPDH reference gene from the Ct of target gene (dCt) and then subtracting the dCt of centroblast from the dCt of centrocyte or plasmacytoma. Amplicon size and specificity were verified by 3% agarose gel electrophoresis in addition to amplicon melting curve analysis.

Western Blot

Western blotting analysis was carried out using conventional methods. Cell lysates were separated on 12,15, or 4–15% Tris HCl polyacrylamide gels. Separated proteins were transferred to Trans-Blot Transfer Medium Nitrocellulose Membrane (0.45 μm) (Bio-Rad) using a BioRad transferblot SD semidry transfer cell. Transferred membranes were stained with Ponceau Red to check the success of the transfer. After blocking the membrane with fat free milk, the first antibody was applied for 1 h. After extensive washing, the bands were visualized by the treatment with the second antibody conjugated with horse radish peroxidase (HRP) for 1 h and with Super Signal West Pico or West Femto Maximum sensitivity substrates (Pierce Chemical Co, Rockford, IL).

Immunohistochemistry

Giemsa staining and immunohistochemistry were performed with isolated centroblasts and centrocytes as well as B-cell lines. The XBP1 was stained with anti-XBP1 rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and Alexa Flour 594 donkey anti-rabbit IgG heavy chain + light chain (H + L) or Alexa Flour 488 goat anti-rabbit IgG (H + L) secondary

antibodies (Molecular Probes, Eugene, OR). VDR was stained with anti-VDR mouse monoclonal antibody (Santa Cruz Biotechnology) or anti-VDR rabbit polyclonal antibody (Active Motif, Carlsbad, CA) together with Alexa Flour 594 goat anti-mouse Ig (H + L) or Alexa Flour 594 donkey anti-rabbit IgG (H + L) (Molecular Probes). For immunohistochemical observations Z-sections were analyzed every 0.1 or 0.05 μm with an Olympus Provis fluorescent microscope and the program Openlab 3.1.5. For confocal analysis an Olympus IX70 dual-laser confocal microscope was used.

RESULTS

Isolation of Germinal Center B-Cell Subpopulations

Figure 1 shows a typical profile of FACS analysis of a negatively isolated B-cell population. Staining with IgD and CD38 distinguishes the developmental stages of B-cell subsets (Fig. 1a) [Liu et al., 1996]. The IgD-positive fraction represents resting B cells. The CD38-positive fraction consists of germinal center B cells. The CD38+ IgD− germinal center B cells were separated into centroblasts and centrocytes by the presence or absence of CD77, respectively [Liu et al., 1996]. Staining of the negatively isolated B cells with CD138 and CD3 antibodies revealed that in these samples there were less than 0.3% CD138 or CD3-positive cells (Fig. 1b,c), indicating lack of substantial contamination with plasma cells or T cells, respectively. Figure 1d,e shows the purity of separated centroblasts and centrocytes. We further confirmed by microscopic examination that isolated centroblasts and centrocytes indeed did not contain cells with plasma cell morphology.

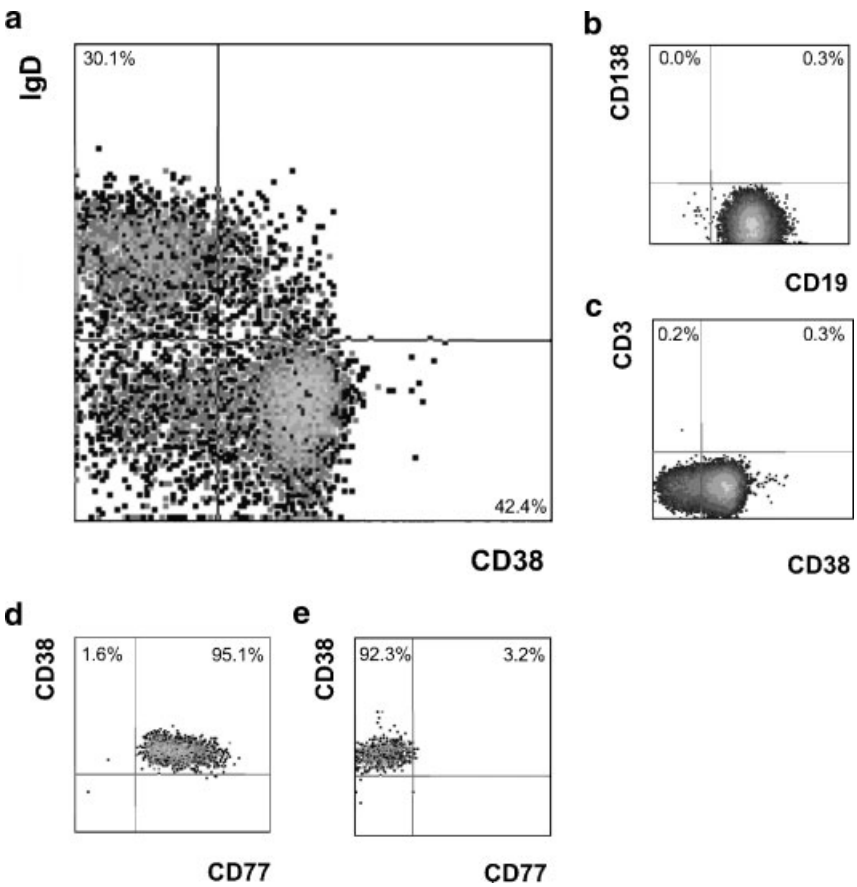

Fig. 1. FACS sorting of tonsil B-cell subsets. Preparation of B-cell subsets. **a**: Tonsil B cells consist of IgD+ naïve B cells, CD38+/IgD− germinal center B cells, and IgD−/CD38− memory B cells. FACS analysis showed that negatively isolated B cells were devoid of plasma cells (CD138) (**b**) or T cells (CD3) (**c**). The CD38+/IgD− germinal center B cells were separated into CD77+ centroblasts (**d**) and CD77− centrocytes (**e**) by FACS sorting.

Analysis of Gene Expression in Centrocytes Compared to Centroblasts

Three separate samples of purified cells were used to generate RNA for Affymetrix U133 chip analysis of gene expression. Table Ia shows that there were several genes expressed at higher levels in centrocytes compared with centroblasts. One significant group of upregulated mRNA consists of immunoglobulin genes, such as the heavy chains of IgM and IGHG3, and the light chains of kappa and lambda, immunoglobulin lambda-like polypeptide 3 (IGLL3) and immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides (IGJ). Several other receptors, two cytokines, signaling molecules, and cell cycle-related genes were also upregulated.

Interestingly, the mast cell-specific enzyme(s), tryptase beta 2 (TPSB2) and tryptase alpha/beta 1 (TPSAB1) were low in absolute levels but significantly upregulated in centrocytes as compared to centroblasts (Table Ia). Expression of the mast cell marker the high-affinity IgE receptor (FcER) was similar in centroblast and centrocytes suggesting that there was no mast cell contamination (data not shown).

Several chaperones and genes involved in intracellular traffic and protein modification, such as disulfide isomerase-related genes PDIA5 and TXNDC5 were upregulated. Only a few transcription factors showed higher level expression in centrocytes compared to centroblasts. The Blimp1, XBP1, ELL-related RNA polymerase II elongation factor (ELL2), and IRF4 were more than threefold increased in centrocytes. Other transcription factors that were less strongly induced include positive coactivator 4 (PC4), and VDR. BTB and CNC homology 1, basic leucine zipper transcription factor 2 (BACH2) expression was downregulated in the course of development from centroblasts to centrocytes (Table Ib).

To get more insight into the relationship of the changes between centroblasts and centrocytes to the process of plasma cell development, we compared genes that were upregulated in centrocytes with gene expression patterns in the CCL155 plasmacytoma cell line (Table Ia). Genes that were expressed at a higher level in plasmacytoma cells include the syndecan1 (SDC1)/CD138 plasma cell marker as well as chaperones and many intracellular trafficking-related genes, receptors, cytokines (adrenomedullin (ADM) and C19orf10/interleukin (IL)27w), transcription factors (IRF4, PC4), and others. Genes downregulated in centrocytes compared to centroblasts were expressed at yet lower levels in the plasmacytoma (Table Ib).

Expression of the Spliced Form of XBP1 in Centrocytes

A spliced form of XBP1 is known to mediate portions of the UPR and thus facilitates secretion of large amounts of immunoglobulin in plasma cells [Iwakoshi et al., 2003b]. Since total XBP1 expression was upregulated in centrocytes, we investigated by RT-PCR whether there is the spliced form of XBP1 in these cells. Among normal B-cell populations, the spliced form of XBP1 was detected only in the centrocytes (Fig. 2a,b). In these cells, the spliced form of XBP1 comprised 13–18% of total XBP1. The non-spliced form of XBP1 was observed in all B-cell subsets (Fig. 2a). We detected 17–22% of the spliced form of XBP1 in the Pala B-cell line, an EBV-transformed peripheral blood B-cell line, and the CCL155 plasmacytoma cell line (Fig. 2a,b).

B-Cell Responses to UPR Inducing Agents

We also wished to compare the genes differentially expressed in centrocytes to those induced by the UPR in B cells; treatment with drugs that interfere with production of mature forms of proteins can induce the UPR [Murray et al., 2004]. For that purpose we treated the Pala B-cell line with tunicamycin or DTT (Fig. 2c). DTT induced the spliced form of XBP1 in a time-dependent manner which represented 64–72% of XBP1. Effects of tunicamycin treatment on the XBP1 spliced form in Pala cells were minimal. In Table IIA, we determined that the transcriptional levels of known UPR inducible genes [Lee et al., 2003] are upregulated in DTT-treated Pala cells in addition to ER oxidoreductin 1-like alpha (ERO1La) which is hypoxia inducible but not UPR responsive [Pagani et al., 2000]. The microarray analysis of RNA isolated from the Pala B-cell line treated for 3 h with 2.5 mM DTT indicates that there was induction of UPR responsive genes (Table IIAc). However, expression of protein disulfide isomerase (PDI) and ERO1La was not significantly changed and double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK) was not detectable by Affymetrix analysis. The

TABLE I. Comparison of Gene Expression in Centroblast, Centrocyte B Cells, and Plasmacytoma

Gene symbols	Gene names	Gene ID	Centroblast (CB)	Centrocyte (CC)	*CC/CB*	*P*-value	Plasmacytoma (PC)	PC/CB	PC/CC
a. Upregulation of gene expressions in centrocytes									
Ig gene									
IGHG3	Immunoglobulin heavy constant gamma 3	3502	4,003.1	7,488.8	**1.9**	0.013	671.9	**0.2**	**0.1**
IGL@	Immunoglobulin lambda locus	3535	1,008.1	6,916.8	**6.8**	0.008	9086.9	**9.0**	**1.3**
IGHM	Immunoglobulin heavy constant mu	3507	1,398.0	2,337.0	**1.7**	0.006	37.4[a]	**0.0**	**0.0**
IGKC	Immunoglobulin kappa constant	3514	2,098.2	4,032.0	**1.9**	0.004			
IGJ	Immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	3512	579.8	1,404.7	**2.4**	0.001	3099.6	**5.3**	**2.2**
IGLL3	Immunoglobulin lambda-like polypeptide 3	3545	542.2	1,084.8	**2.0**	0.001	1639.9	**3**	**1.5**
IGLL1	Immunoglobulin lambda-like polypeptide 1	3543	77.1	161.1	**2.0**	0.209	19.2	**0.2**	**0.1**
Receptor									
ITGB1BP1	Integrin beta 1 binding protein 1	9270	134.6	1,481.0	**11.0**	0.043	490.6	**3.6**	**0.3**
ITM2C	Integral membrane protein 2C	81618	13.1[a]	123.2	**9.3**	0.002	648	**49.4**	**5.3**
SLAMF7	SLAM family member 7	57823	64.8	235.0	**3.6**	0.006	50.1[a]	**0.8**	**0.2**
SDC1(CD138)	Syndecan 1	6382	11.2[a]	37.9	**3.3**	0.044	730.7	**65.0**	**19.3**
MARCKS	Myristoylated alanine-rich protein kinase C substrate	4082	18.4	51.5	**2.8**	0.030	256.3	**13.9**	**5.0**
SSR4	Signal sequence receptor, delta (translocon-associated protein delta)	6748	655.2	1,765.5	**2.6**	0.012	3968.3	**6.1**	**2.2**
FNDC3B	Fibronectin type III domain containing 3B	64778	92.6	222.8	**2.4**	0.017	569.8	**6.2**	**2.6**
RARRES3	Retinoic acid receptor responder (tazarotene induced) 3	5920	24.5	49.1	**2.0**	0.017	42.4	**1.7**	**0.9**
Cytokine									
ADM	Adrenomedullin	133	21.4[a]	66.9	**3.1**	0.010	227.7	**10.6**	**3.4**
C19orf10/IL27W	Chromosome 19 open reading frame 10	56005	161.0	336.2	**2.0**	0.003	1856.1	**11.5**	**5.5**
Signaling									
LOC90701	Similar to signal peptidase complex (18 kD)	90701	1,036.8	2,273.8	**2.1**	0.004	9644.1	**9.3**	**4.2**
ENTPD1/CD39	Ectonucleoside triphosphate diphosphohydrolase 1	953	38.8	82.7	**2.1**	0.049	67.6	**1.7**	**0.8**
Cell cycle									
CCND2	Cyclin D2	894	20.9[a]	51.5	**2.4**	0.023	2168.6	**103.3**	**42.1**
CCPG1	Cell cycle progression 1	9236	32.6[a]	66.5	**2.0**	0.017	296.1	**9.1**	**4.5**
Tryptase									
TPSB2	Tryptase beta 2	64499	12.6[a]	89.0	**7.0**	0.021	4.1[a]	**0.3**	**0.0**
TPSAB1	Tryptase alpha/beta 1	7177	24.1[a]	136.0	**5.6**	0.051	26.2[a]	**1.1**	**0.2**

Chaperone									
FKBP11	FK506 binding protein 11, 19 kDa	51303	120.4	759.9	**6.3**	0.006	3184.1	**26.4**	**4.2**
TRA1	Tumor rejection antigen (gp96) 1	7184	302.3	1,287.3	**4.2**	0.000	2804.5	**9.3**	**2.2**
DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	4189	155.9	349.0	**2.2**	0.033	727.8	**4.7**	**2.1**
STCH	Stress 70 protein chaperone, microsome-associated, 60 kDa	6782	45.9	98.5	**2.1**	0.001	383.5	**8.3**	**3.9**
Intracellular trafficking and protein modifications									
SELM	Selenoprotein SelM	140606	14.5[a]	187.3	**12.8**	0.025	138.7	**9.5**	**0.7**
CHST12	Carbohydrate (chondroitin 4) sulfotransferase 12	55501	13.6[a]	74.9	**5.5**	0.034	540.1	**39.7**	**7.2**
CKAP4	Cytoskeleton-associated protein 4	10970	24.8[a]	105.8	**4.2**	0.009	187.7	**7.5**	**1.8**
HM13	Histocompatibility (minor) 13	81502	159.8	437.1	**2.7**	0.008	2177.4	**13.6**	**4.9**
SORT1	Sortilin 1	6272	20.3[a]	48.6	**2.3**	0.024	836.9	**41.2**	**17**
TXNDC5	Thioredoxin domain containing 5	81567	399.3	885.5	**2.2**	0.000	3427.8	**8.6**	**3.9**
MAN1A1	Mannosidase, alpha, class 1A, member 1	4121	85.9	177.5	**2.0**	0.023	269.9	**3.1**	**1.5**
SDF2L1	Stromal cell-derived factor 2-like 1	23753	117.5	239.6	**2.0**	0.002	352.2	**3.0**	**1.5**
PDIA5	Protein disulfide isomerase family A, member 5	10954	70.7	142.6	**2.0**	0.010	535.4	**7.6**	**3.8**
Transcription factor									
Blimp1(PRD-M1)	B lymphocyte-induced maturation protein-1								
	(PR domain containing 1, with ZNF domain)	639	144.5[a]	814.2	**5.6**	0.071	834	**5.8**	**1.0**
ELL2	ELL-related RNA polymerase II, elongation factor	22936	62.4	232.5	**3.7**	0.011	269	**4.3**	**1.2**
XBP1	X-box binding protein 1	7494	191.6	713.3	**3.7**	0.015	918.3	**4.8**	**1.3**
IRF4	Interferon regulatory factor 4	3662	65.7	227.6	**3.4**	0.005	546.8	**8.3**	**2.4**
PC4/SUB1	Positive coactivator 4/SUB1 homolog (*S. cerevisiae*)	10923	689.7	1508.8	**2.1**	0.020	6491	**9.4**	**4.3**
VDR	Vitamin D (1,25-dihydroxyvitamin D3) receptor	7421	57.0	120.5	**2.1**	0.019	100.7	**1.8**	**0.8**
ZMYND11	Zinc finger, MYND domain containing 11	10771	23.8	49.2	**2.0**	0.031	156.9	**6.6**	**3.2**
Others									
PPAPDC1B	Phosphatidic acid phosphatase type 2 domain containing 1B	84513	47.0	288.0	**6.1**	0.034	2634.6	**56.0**	**9.1**
PRDX4	Peroxiredoxin 4	10549	109.0	386.1	**3.5**	0.011	3793.2	**34.8**	**9.8**
TSPAN32	Tetraspanin 32	10077	43.6	120.6	**2.8**	0.029	62.5	**1.4**	**0.5**
ADCYAP1	Adenylate cyclase activating polypeptide 1 (pituitary)	51237	720.7	1,899.6	**2.6**	0.004	10418.5	**14.5**	**5.5**
SELS	Selenoprotein S	55829	503.7	1,102.9	**2.2**	0.014	4825	**9.6**	**4.4**
GUCA1A	Guanylate cyclase activator 1A (retina)	2978	19.1[a]	40.6	**2.1**	0.006	29.5[a]	**1.5**	**0.7**
C20orf31	Chromosome 20 open reading frame 31	55741	57.8	121.3	**2.1**	0.006	127.3	**2.2**	**1.0**
BTN3A3	Butyrophilin, subfamily 3, member A3	10384	105.8	217.0	**2.1**	0.002	1092.8	**10.3**	**5.0**
b. Downregulation of gene expressions in centrocytes									
BCA1	ATP-binding cassette, sub-family A (ABC1), member1	19	77.7	30.1	**0.4**	0.007	22.3	**0.3**	**0.7**

(*Continued*)

TABLE I. (*Continued*)

Gene symbols	Gene names	Gene ID	Centroblast (CB)	Centrocyte (CC)	*CC/CB*	*P*-value	Plasmacytoma (PC)	PC/CB	PC/CC
NALP4	NACHT, leucine rich repeat, and PYD containing 4	147945	395.5	174.1	**0.4**	0.017	10.7[a]	**0.02**	**0.1**
WASF3	WAS protein family, member 3	10810	66.9	31.7	**0.5**	0.026	13.3[a]	**0.19**	**0.4**
DKFZP586-B0319	DKFZP586B0319 protein	26069	50.6	24.0[a]	**0.5**	0.001	3.9[a]	**0.1**	**0.2**
HTR3A	5-hydroxytryptamine (serotonin) receptor 3A	3359	188.2	91.4	**0.5**	0.002	4.9[a]	**0.0**	**0.1**
BACH2	BTB and CNC homology 1, basic leucine zipper transcription factor 2	60468	1,098.7	546.7	**0.5**	0.030	153.6	**0.1**	**0.3**
c. Actin control									
ACTB	Actin, beta	60	21,210.0	20,908.1	**1.0**	0.888	19902.6	**0.9**	**1.0**
ACTG1	Actin, gamma 1	71	16,636.7	16,151.1	**1.0**	0.641	15038.5	**0.9**	**0.9**

CB, centroblast; CC, centrocyte; PC, CCL155 plasmacytoma cell line.
(a) Upregulated genes in centrocytes versus centroblasts. (b) Downregulated genes in centrocytes compared to centroblasts. (c) Actin gene expression.
Gene symbols, gene names, Gene ID is shown followed by Centroblast (CB): average of triplicate Affymetrix analysis results of RNA expression in centroblasts, centrocyte (CC): average of triplicate results of centrocytes, CC/CB: proportion of centrocyte/centroblast and *P*-value. To compare gene expressions in plasma cell as a model experiment, plasmacytoma (PC): single Affymetrix analysis result of CCL155 plasmacytoma is shown together with the proportion of PC/CB: plasmacytoma /average centroblasts and PC/CC: plasmacytoma /average centrocytes.
[a]Indicates affymetrix analysis evaluates as absent (undetected).

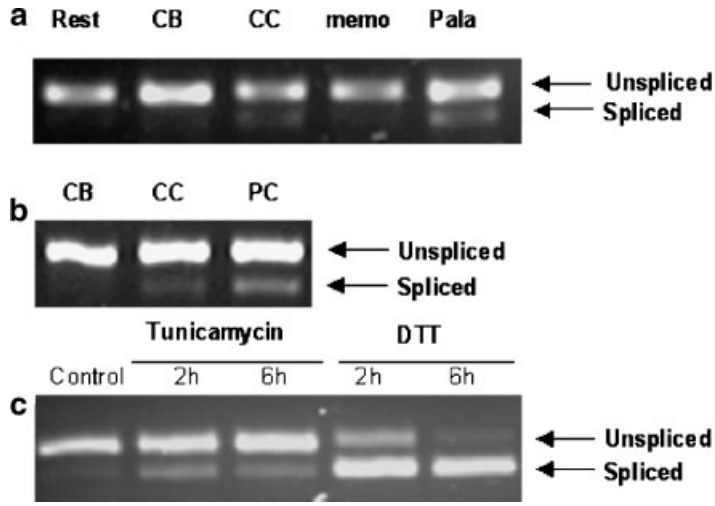

Fig. 2. RT-PCR of XBP1. **a**: XBP1 unspliced form is present in all B-cell stages. The spliced form of XBP1 is detected in centrocytes and Pala cells, as well as **b**: PC:CCL155 plasmacytoma cells. **c**: The spliced form of XBP1 is induced in Pala cells by treatment with tunicamycin or DTT. A 3% agarose gel was used to separate RT-PCR amplified forms of non-spliced and spliced XBP1. Rest: IgD+ resting B cells, CB: centroblasts, CC: centrocytes, memo: memory B cells, Pala: Pala B-cell line, PC: CCL155 plasmacytoma cell line.

levels of expression of the DTT-induced genes in Pala (Table IIAc) were compared to their expression in centroblasts, centrocytes, and CCL155 plasmacytoma cells (Table IIAa,b). Expression of immunoglobulin heavy chain binding protein (BiP), C/EBP-homologous protein (CHOP), endoplasmic reticulum oxidoreductin 1-like beta (ERO1Lb), and PDI was higher in centrocytes than centroblasts. In the plasmacytoma, Bip, PDI, and activating transcription factor 6 (ATF6) expression became higher but expression of CHOP, ER degradation enhancer, mannosidase alpha-like 1 (EDEM1), and ERO1Lb was reduced. Expression of ERO1La was not induced by DTT treatment in Pala B cells (Table IIAc) but became higher in centrocytes compared to centroblasts and further increased in plasmacytoma cells (Table IIAa,b).

The mRNA levels of a number of genes related to protein disulfide metabolism showed significant differences between the cell types we studied. Expression of two disulfide isomerases (thioredoxin domain containing (TXNDC)5 and PDI family A, member (PDIA)5) showed a two-fold increase in centrocytes compare to centroblasts (Table IIBa). Although we did not detect significant induction of disulfide isomerases and disulfide isomerase-related genes by DTT treatment in Pala cells (Table IIBc), there is increased expression in plasmacytoma cells compared to centroblasts (Table IIBb).

To further study the UPR in B cells, we treated the Pala cell line with 5 mM DTT for 0, 2, or 6 h and performed real time PCR to analyze the changes in certain UPR-related mRNAs. Induction of Bip, CHOP, ERO1Lb, and ATF6 was observed whereas ERO1La and PDI levels of expression in DTT-treated Pala and control cells were equivalent (Fig. 3a). Interestingly, ERO1Lb expression was significantly lower in plasmacytoma cells compared to centroblasts (Fig. 3b).

By real time PCR, chaperones, FK506 binding protein 11, 19 kDa (FKBP11), tumor rejection antigen 1 (TRA1), and DnaJ (Hsp40) homolog, subfamily B, member 9 (DNAJB9) (Fig. 3c), disulphide isomerase related genes TXNDC5, PDIA5, and PDI (Fig. 3e), and transcription factors Blimp1, XBP1, and IRF4 (Fig. 3d) were all expressed at higher levels in centrocytes and plasmacytoma cells compared to centroblasts. We detected higher VDR expression in centrocytes compared to centroblasts. Among transcription factors, only BACH2 expression was significantly reduced in centrocytes and plasmacytoma cells compared to centroblasts (Fig. 3d).

Immunohistochemical Observation of XBP1 and VDR

The subcellular distribution of XBP1 is a major factor regulating its transcriptional activity. We observed XBP1 staining in CD77

TABLE II. Affymetrix Analysis of Known UPR Related Genes and Disulfide Isomerase Related Genes in Centroblast, Centrocyte, Plasmacytoma, and DTT Treated Pala Cell

			(a) Germinal center B cells				(b) Plasmacytoma			(c) DTT-treated Pala		
Gene symbols	**Gene names**	**Gene ID**	**CB**	**CC**	**CC/CB**	***P*-value**	**PC**	**PC/CB**	**PC/CC**	**Pala cont**	**Pala DTT3h**	**Ratio DTT/cont**
A. Known UPR related genes												
BiP (HSPA5)	Immunoglobulin heavy chain-binding protein, (heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa))	3309	1,116.2	1,337.1	**1.2**	0.1011	2802.7	**2.5**	**2.1**	13463.3	21386.8	**1.6**
CHOP (DDIT3)	C/EBP homologous protein, (DNA-damage-inducible transcript 3)	1649	130.2	180.5	**1.4**	0.1105	90.3	**0.7**	**0.5**	380.3	668.8	**1.8**
EDEM1	ER degradation enhancer, mannosidase alpha-like 1	9695	445.9	365.9	**0.8**	0.2200	251.4	**0.6**	**0.7**	904.7	1638.1	**1.8**
PDI (P4HB)	Protein disulfide isomerase, (procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide (protein disulfide isomerase; thyroid hormone binding protein p55))	5034	440.7	806.5	**1.8**	0.0126	2984.9	**6.8**	**3.7**	4427.6	4942.5	**1.1**
ERO1Lb	ERO1-like beta (*S. cerevisiae*)	56605	71.9	94.9	**1.3**	0.0155	3.5[a]	**0.05**	**0.04**	621.5	1199.9	**1.9**
ERO1La	ERO1-like (*S. cerevisiae*)	30001	108.8	163.7	**1.5**	0.0328	663.2	**6.1**	**4.1**	896.4	768.9	**0.9**
PERK (EIF2AK3)	Double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase, (eukaryotic translation initiation factor 2-alpha kinase 3)	9451	253.6	266.2	**1.0**	0.4263	142	**0.6**	**0.5**	13[a]	70.8[a]	**5.4**
ATF6	Activating transcription factor 6	22926	30.9[a]	49.8[a]	**1.6**	0.1973	589.1	**19.1**	**11.8**	105.6	168.3	**1.6**
B. Disulfide isomerase related genes												
PDIA5	Protein disulfide isomerase family A, member 5	10954	70.7	142.6	**2.0**	0.0102	535.4	**7.6**	**3.8**	1395.5	1187.7	**0.9**
ERP70	Protein disulfide isomerase-related protein (calcium-binding protein, intestinal-related)	9601	422.6	627.7	**1.5**	0.0305	680.1	**1.6**	**1.1**	4233.0	5912.7	**1.4**
C12orf8	Chromosome 12 open reading frame 8	10961	808.4	1,004.1	**1.2**	0.0985	2861.2	**3.5**	**2.8**	8841.0	7538.5	**0.9**
TXNDC5 (ERP46)	Thioredoxin domain containing 5	81567	399.3	885.5	**2.2**	0.0005	3427.8	**8.6**	**3.9**	10189.8	5828.9	**0.6**
TXNDC7 (P5)	Thioredoxin domain containing 7	10130	705.3	943.2	**1.3**	0.1597	2568.0	**3.6**	**2.7**	9358.2	9330.8	**1.0**
TXNDC11	Thioredoxin domain containing 11	51061	386.9	502.1	**1.3**	0.0445	1183.2	**3.1**	**2.4**	1339.7	2516.5	**1.9**

The explanation of the Table is the same as Table I. In addition, (a) germinal center B cells, (b) plasmacytoma, (c) Pala control, DTT 3 h treated Pala and ratio of DTT treated versus control of Pala cells are shown. CB and CC are average of triplicate Affymetrix analysis. Plasmacytoma, Pala control, and DTT 3 h treated Pala cells is a single analysis.
[a]Represents absence in Affymetrix analysis.

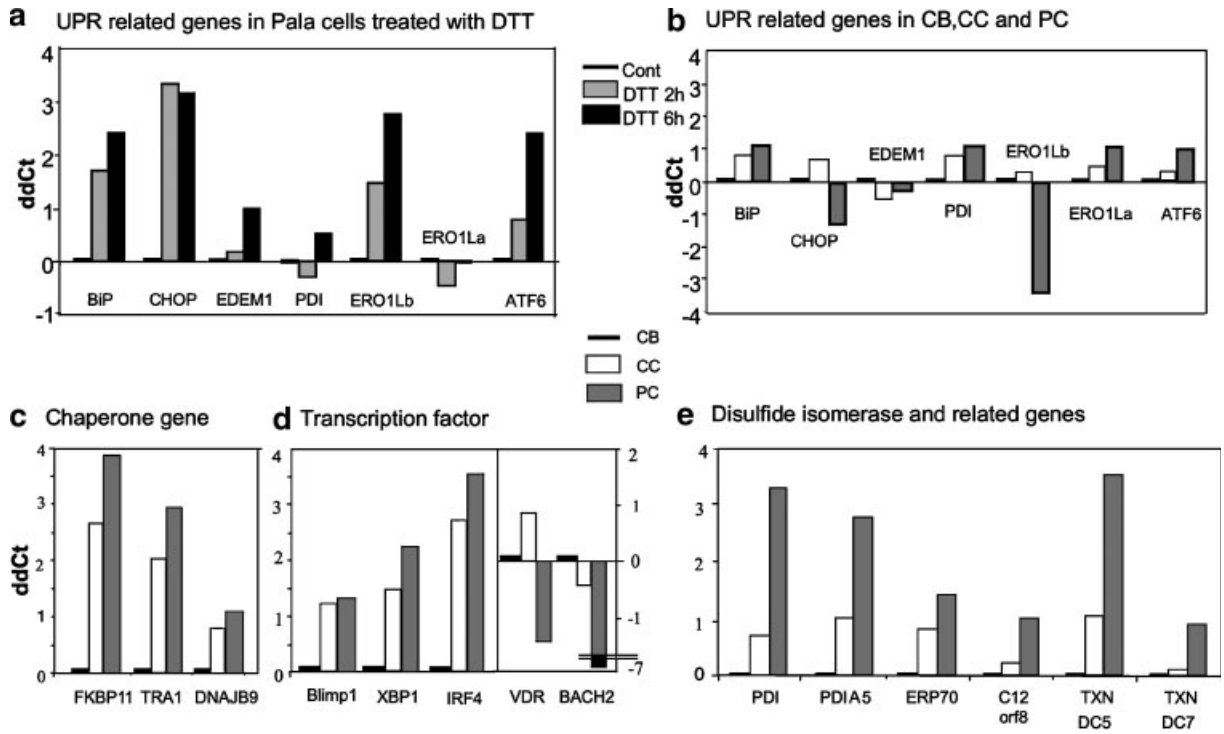

Fig. 3. Real time PCR analysis of gene expression in centrocytes and plasmacytoma cells compared to centroblasts. **a**: Time course of expression changes of UPR-related genes in DTT-treated Pala cells compared to non-treated Pala control cells. **b–e**: Comparison of gene expression in centrocytes and plasmacytoma cells with centroblasts. **b**: UPR-related genes, **c**: chaperones, **d**: transcription factors, **e**: disulphide isomerase and related genes. XBP1 expression includes both non-spliced and spliced forms. Results are shown as differences in the number of PCR cycles (ddCt) between control and test sample.

FITC-positive centroblasts and FITC-negative centrocytes by both Z-section deconvolution microscopy and confocal microscopy. With Z-section deconvolution microscopy we observed some XBP1 staining in the region of 4′,6′-diamidino-2-phenylindole (DAPI) DNA staining indicating XBP1 is present in the nucleus of both B-cell types. However, most of the XBP1 staining was in the area surrounding the area of DAPI staining indicating most of the XBP1 was located in the cytoplasm (Fig. 4a). With confocal microscopy XBP1 staining was observed in the whole cell (Fig. 4b) supporting the Z-section observation that XBP1 is present in cytoplasm as well as nucleus. There was no detectable difference in the pattern of XBP1 staining in centroblasts and centrocytes. Presumably the small amount of spliced XBP1 product in the centrocytes could not be seen above the background of unspliced and cytoplasmic products.

VDR is a transcription factor not extensively studied in germinal center B cells. We observed VDR in centroblasts and centrocytes. In both cases, VDR staining was observed in the region of DAPI DNA staining and the cytoplasm. Interestingly, VDR was on the cell surface in centrocytes with both Z-section deconvolution microscopy and confocal microscopy (Fig. 4f,h). This was seen with two different primary antibodies. In addition, there was some colocalization between caveolae and VDR in the Pala cell line (Fig. 4i).

DISCUSSION

In this study, we analyzed gene expression of endogenous centroblasts and centrocytes obtained from human tonsillar germinal centers as well as the Pala B-cell line and CCL155 plasmacytoma cell line using Affymetrix U133 DNA chips. Only about 60 genes were altered more than twofold in their transcript levels in centrocytes compared to centroblasts (Table I). These included: (1) immunoglobulin genes

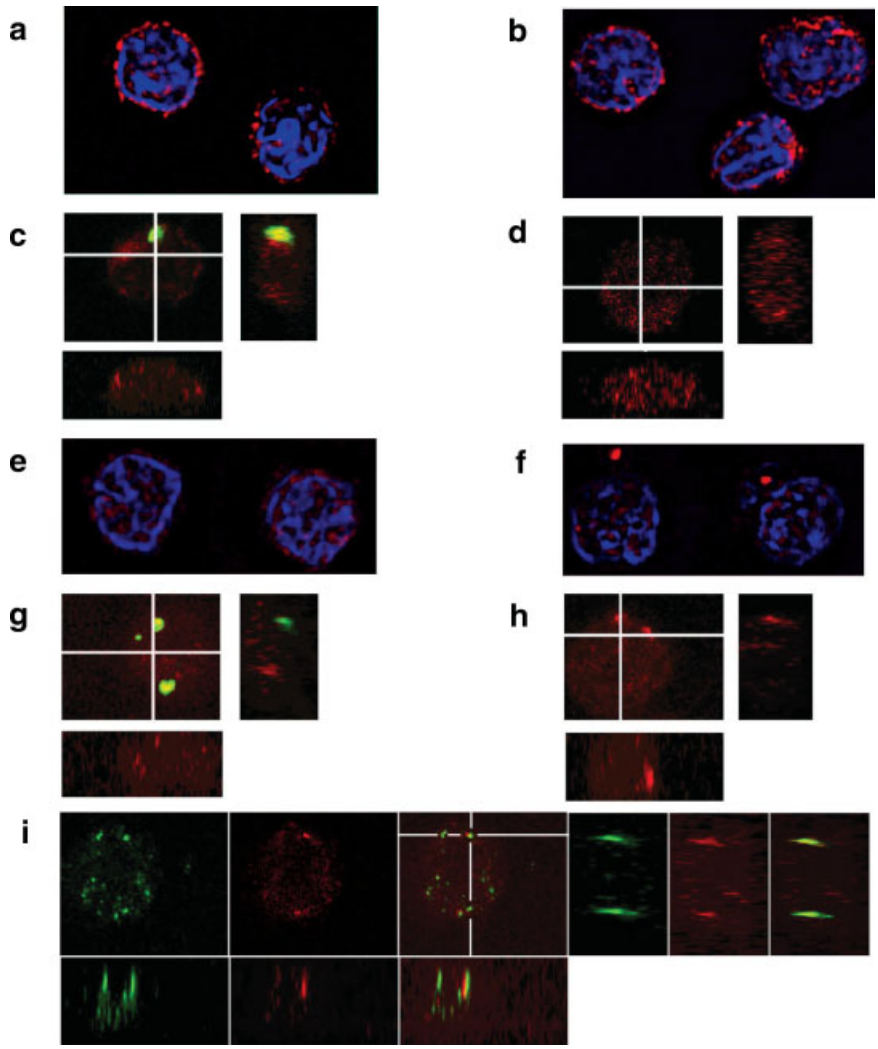

Fig. 4. Immunofluoresence microscopic observation of XBP1 and VDR in centroblasts, centrocytes, and Pala B cells. XBP1 was observed by immunofluorescence microscopy in centroblasts (**a** and **c**) and centrocytes (**b** and **d**). One layer of Z-sections at 1-μm intervals of centroblasts (**a**) and centrocytes (**b**) is shown. FACS-isolated cells were treated with anti-XBP1 antibody and colorized by dye-conjugated antibody. Red color indicates XBP1 and blue color Dapi-positive nucleus elements. Confocal microscopic observation of XBP1 in centroblasts (**c**) and centrocytes (**d**). Red color indicates XBP1, green color indicates CD77 which was used to distinguish centroblasts from centrocytes. VDR observation by Z-section (**e** and **f**) and confocal (**g** and **h**) immunofluorescence microscopy in centroblasts and centrocytes. Red color indicates VDR and blue color Dapi-positive nucleus elements. In the confocal microscopic observations, green color indicates CD77. Cells were fixed in 2.5% paraformaldehyde (**a–h**). Immunofluorescence confocal microscopic observation of VDR and caveolae in Pala B-cell (**i**). Cells were fixed in 4% paraformaldehyde. Red color indicates VDR. Green color caveolae. In merged photo, yellow color indicates colocalization of VDR and caveolae.

including B-cell receptor (BCR), (2) receptors including adhesion molecules, (3) the transcription factors Blimp1, XBP1, IRF4, VDR, and BACH2, (4) chaperones, (5) genes involved in intracellular traffic and protein modification, (6) cytokines, (7) cell cycle-related genes, and (8) tryptases.

In this study, we found significant expression changes of genes in the centrocyte stage that are involved in B-cell maturation toward plasma cells. Among about 60 genes we detected, none except IGLL1 was detected by Klein et al. [2003] who reported upregulation of deoxynucleotidyltransferase (TdT), recombination activating gene (RAG)1 and 17 other genes; the gene expression profile was totally different from this study. There are some conflicting reports of expressions of RAGs and TdT in germinal center of tonsils [Girschick et al., 2001; Meru et al., 2002]. Neither our array analysis with

centroblasts and centrocytes nor the analysis of another group with the whole germinal center B-cell population [Shen et al., 2004] detected RAGs and TdT.

We and others [Klein et al., 2003] observed that the centrocyte population consists of further subpopulations. The differences between ours and the other study could be due to the separation methods and sensitivity differences but the expression of RAG and TdT observed in the earlier studies suggests they were dealing with a less mature population of B cells. The genes we detected as upregulated in centrocytes match well with the expected B-cell maturation towards plasma cells.

Our findings of the relatively simple transition expression pattern of centroblasts to centrocytes provides a system in which it may be feasible to completely describe changes in transcriptional regulation during a developmental step in mammalian cells. Messenger RNA for only a few transcription factors was elevated in centrocytes compared to centroblasts and many of these have been previously implicated in plasma cell development (Fig. 5).

Previously Blimp1 protein was observed in tonsil tissue including epithelial cells, plasma cells, and germinal center B cells [Angelin-Duclos et al., 2000]. Our data specifically show that a substantial level of Blimp1 mRNA is detected by microarray analysis in centrocytes but not centroblasts.

Blimp1 knock out mice [Shapiro-Shelef et al., 2003] fail to develop plasma cells. Blimp1 is located upstream of XBP1 and has transcriptional target genes in plasma cell differentiation that are different from targets of XBP1 [Shaffer et al., 2004]. This factor is under intensive study by other groups. IRF4 is inducible by multiple stimuli, such as BCR, CD40, and IL4 stimulation. IRF4 binds the enhancers of Ig light chains [Pernis, 2002]. In IRF4−/− mice B-cell development is terminated at the germinal center stage resulting in an absence of serum immunoglobulin and lack of plasma cell production [Mittrucker et al., 1997]. IRF4 and Blimp1 DNA-binding motifs share similar DNA sequences and it is possible that IRF4 function could be modulated by Blimp1 [Gupta et al., 2001; Pernis, 2002]. Upregulation of IRF4 in developing B cells

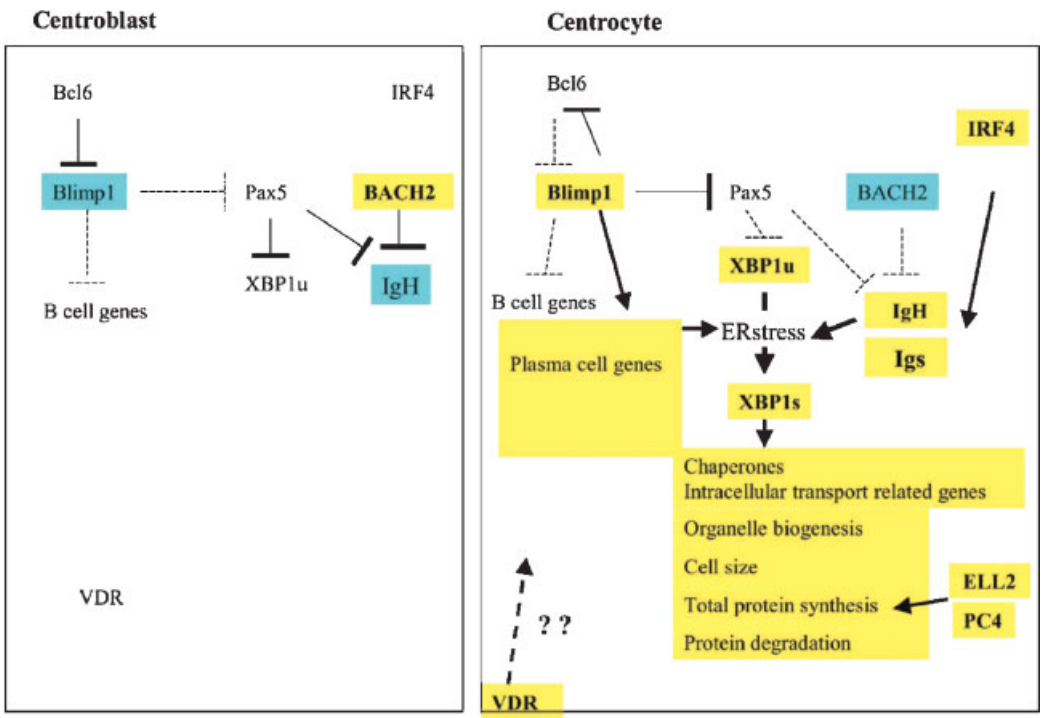

Fig. 5. Schematic of transcription factor networks in centroblasts and centrocytes. In centrocyte B cells *Blimp1* and *IRF4* genes are upregulated and BACH2 is downregulated leading to immunoglobulin upregulation and expression of plasma cell genes. Ig protein production in these cells leads to ER stress and production of XBP1s which induces the UPR including increased organelle biogenesis, and cell size increase. ELL2 and PC4 upregulation in centrocytes contributes to an increase in total protein synthesis. The contribution of VDR is unknown. XBP1u and XBP1s indicates unspliced and spliced form of XBP1, respectively. Dotted boxes indicate downregulated genes. Solid boxes indicate upregulated genes.

might be attributed to regulation by microphthalmia-associated transcription factor (MITF) [Lin et al., 2004]. However, in the present experiments, there were minimal changes in the level of MITF mRNA (data not shown).

The VDR is a nuclear hormone receptor that activates transcription upon binding to its specific ligands. Previously, a study proposed that the degree of cellular activation by IL4 determines the effect of Vitamin D hormone on B cells [Morgan et al., 2000]. We detected higher levels of VDR in centrocytes compared to centroblasts. As activated macrophages produce vitamin D and both B and T cells express the VDR [Hayes et al., 2003], it is intriguing to speculate that vitamin D may influence cells of the immune system in the microenvironment of the germinal center. Interestingly, we observed VDR localized on the cell surface in centrocytes (Fig. 4f,h) and in Pala cells. There was also some colocalization of VDR and caveolae (Fig. 4i). Recently, a VDR membrane form has been reported in caveolae-enriched plasma membranes in other cell types [Huhtakangas et al., 2004]. This may participate in signal transduction as do plasma membrane forms of the estrogen receptor [Li et al., 2003]. It will be interesting to know whether VDR acts as a cell surface signal transducer in addition to a nuclear transcription factor in B-cell physiology and development. An alternative possibility is that VDR on the cell surface may become concentrated at the site of cell–cell interaction and thereby reinforce the detection of and response to Vitamin D derived from other cells.

XBP1 mediates the UPR. XBP1 is expressed throughout B lineage development [Reimold et al., 1996] (Fig. 2). It localizes in cytoplasm as well as in nucleus (Fig. 4). XBP1 expression was upregulated in centrocytes (Table I), and expression of the spliced form of XBP1 was detected in centrocytes, and at a higher level in Pala and plasmacytoma cell lines (Fig. 2). This indicates that expression of the spliced forms of XBP1 and UPR begins as early as the centrocyte transitional stage before the cells mature to plasma cells.

Comparing the gene expressions in centroblasts and centrocytes, although there was not a full scale UPR, there is significant up regulation of UPR related genes in centrocytes that accord well to the finding of spliced from of XBP1 expression. XBP1 is required for the terminal differentiation to plasma cells [Reimold et al., 2001; Iwakoshi et al., 2003b]. In terminally differentiated plasma cells, the spliced form of XBP1 presumably enables proper folding of a large amount of proteins in the ER by upregulating the expressions of chaperones, folding enzymes, and repressing proteins, as well as initiating cell cycle arrest to allow secretion of antibody [Reimold et al., 2001; Gass et al., 2004]. The spliced form of XBP1 also induces organelle biosynthesis, cell size increase, and total protein synthesis [Iwakoshi et al., 2003b; Shaffer et al., 2004]. However, the amount of spliced XBP1 in centrocytes was much less than in cells treated with DTT. In addition there was a limited overlap between the genes up regulated in the centroblast to centrocyte transition and genes up-regulated during the UPR induced by DTT treatment of Pala cells (Fig. 6). There was also a limited overlap between genes expressed at higher levels in plasmacytoma cells or in Pala cells than in centroblasts, and the genes upregulated in the transition from centroblasts to centrocytes. Perhaps there is a graded response of various UPR genes to differing levels of XBP1 and this in part accounts for the limited aspects of the UPR seen in centrocytes. It seems likely that there are factors operative in one or another of these cell types that modulate the pattern of UPR response, and also that a large part of the centroblast to centrocyte transition is not mediated by the UPR.

The only transcription factor downregulated in centrocytes compared to centroblasts is the basic leucine zipper transcription factor 2, BACH2, a gene product which is a B-cell-specific partner for small v-maf musculoaponeurotic fibrosarcoma oncogene homolog (Maf) proteins and negatively regulates the immunoglobulin heavy chain gene 3′ enhancer [Muto et al., 1998]. Recent studies indicate BACH2 is critical for immunoglobulin gene hypermutation and class switching as well as germinal center formation [Muto et al., 2004]. However, BACH2 is not required for plasma cell differentiation. Hypermutation of BCR takes place in the Ig gene variable region in centroblasts followed by heavy chain class switching mainly in centrocytes. BACH2 expression pattern varies inversely with immunoglobulin gene expression during the centroblast to centrocyte transition and it is tempting to consider that this factor directly controls the relative levels of immunoglobulin expression in the two cell types (Table I) [Camacho et al., 1998].

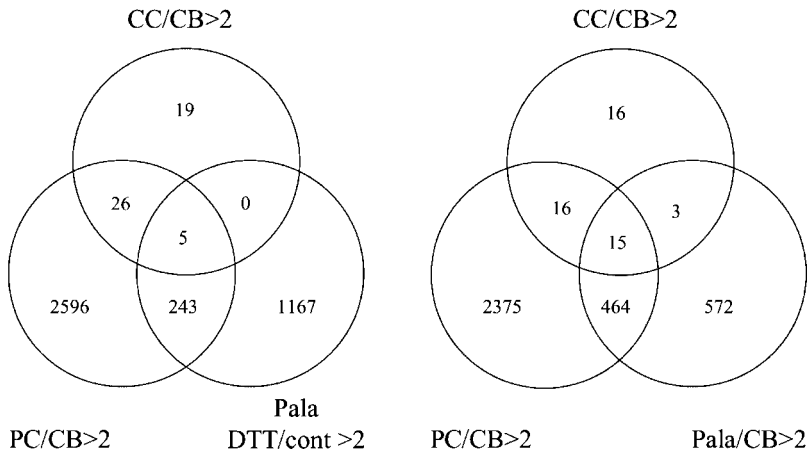

Fig. 6. Venn diagram comparison of the number of genes whose level of expression is more than twofold higher in centrocytes versus centroblasts, plasmacytoma cells versus centroblasts, and DTT-treated Pala B cells versus control or Pala control B cells versus centroblasts. Total number of genes expressed more than twofold higher in centrocytes versus centroblasts were 50, plasmacytoma versus centroblasts were 2,870, DTT-treated Pala versus Pala control were 1,415, Pala versus centroblasts were 1,054. Five of DTT-induced genes in Pala overlapped with genes expressed higher in centrocytes versus centroblasts.

Both ERO1La and ERO1Lb localize in the ER and maintain oxidizing conditions which can facilitate disulfide bond formation by oxidizing PDI. Only ERO1Lb but not ERO1La is induced by drugs that can induce UPR [Pagani et al., 2000]. However, ERO1Lb expression became lower in plasmacytoma cells. In centrocytes and plasma cells, not only ERO1Lb but also ERO1La might play a role in maintaining ER oxidizing conditions that allow disulfide binding and proper protein folding.

A group of disulfide isomerases and related genes were induced in centrocytes and further increased in the plasmacytoma (Table IIB, Fig. 3). Expression of TXNDC7 is dependent on the spliced form of XBP1 [Lee et al., 2003]. In a recent study with yeast, TXNDC5 could complement PDI activity and was proposed to be a new UPR gene [Knoblach et al., 2003]. The expression of TXNDC5 is responsive to oxygen tension [Sullivan et al., 2003; Wang et al., 2005]. However, except for PDI, the involvement of these gene products in UPR is yet to be characterized.

The limited changes in cytokine and cell surface receptor expression seen in the transition from centroblasts to centrocytes (Table I) also offer an opportunity for further dissection of interactions with the cellular microenvironment that distinguish these two types of cells.

In summary, the present work provides information that must be integrated into any model of B-cell maturation. The transition from centroblasts to centrocytes involves changes in the level of a relatively small number of mRNAs. As detailed above, several of these changes are consistent with previous studies of plasma cell development. However, centrocytes represent a distinct stage of maturation in which induction of only a limited and partial UPR occurs. The present results might offer an opportunity to study key elements regulating the transition from centroblasts as well as the further transition to plasma cells and also raise the possibility of previously unappreciated roles for Vitamin D and its receptor in the function of these cells. Currently further studies are underway to evaluate the role of specific transcription factors and other elements in the maturation of activated B cells.

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