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B- and T-cell Development Both Involve Activity of the Unfolded Protein Response Pathway*[S]

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Ryan Brunsing[‡], Sidne A. Omori[§], Frank Weber[‡1], Alicia Bicknell[‡], Leslie Friend[‡], Robert Rickert[§], and Maho Niwa[‡2]

From the [‡]Division of Biological Sciences, Section of Molecular Biology, University of California, San Diego , La Jolla, California, 92093-0377 and [§]The Burnham Institute, La Jolla, California 92037

The unfolded protein response (UPR) signaling pathway regulates the functional capacity of the endoplasmic reticulum for protein folding. Beyond a role for UPR signaling during terminal differentiation of mature B cells to antibody-secreting plasma cells, the status or importance of UPR signaling during hematopoiesis has not been explored, due in part to difficulties in isolating sufficient quantities of cells at developmentally intermediate stages required for biochemical analysis. Following reconstitution of irradiated mice with hematopoietic cells carrying a fluorescent UPR reporter construct, we found that IRE1 nuclease activity for XBP1 splicing is active at early stages of T- and B-lymphocyte differentiation: in bone marrow pro-B cells and in $CD4^+CD8^+$ double positive thymic T cells. IRE1 was not active in B cells at later stages. In T cells, IRE activity was not detected in the more mature $CD4^+$ T-cell population but was active in the $CD8^+$ cytotoxic T-cell population. Multiple signals are likely to be involved in activating IRE1 during lymphocyte differentiation, including rearrangement of antigen receptor genes. Our results show that reporter-transduced hematopoietic stem cells provide a quick and easy means to identify UPR signaling component activation in physiological settings.

Pluripotent stem cells are constantly faced with critical decisions between self-renewal and starting to differentiate into various cell types (1, 2). Commitment of differentiating stem cells toward the various lineages is influenced by many factors, including microenvironment and external cues that are integrated into signaling pathways regulating transcriptional programs and protein production. Hematopoietic stem cells (HSC)[3] that differentiate into the erythroid, myeloid, or lymphoid lineages, primarily in the bone marrow of vertebrate adults, undergo dramatic changes in cellular architecture during differentiation to functionally specialized cells. For cells that are specialized for protein secretion, such changes include the creation of extraordinary protein processing and secretory apparatus. The unfolded protein response (UPR) is a conserved signal transduction pathway that in response to endoplasmic reticulum (ER) stress enables cells to increase the protein folding capacity of the ER, the major cellular compartment for folding and maturation of secreted and membrane proteins, by increasing the transcription of genes involved in protein folding. In addition, UPR activation also increases expression of genes involved in ER membrane biosynthesis, presumably resulting in ER expansion. Thus, UPR signaling may play a role in the differentiation of HSC. In fact, an importance for the UPR signaling components IRE1 and XBP1 has been demonstrated during the terminal differentiation of activated B lymphocytes (B cells) to immunoglobulin-secreting plasma cells (3–6).

In mammalian cells, three ER transmembrane components, IRE1, PERK, and ATF6, serve to monitor ER protein folding needs and initiate UPR activation (7–10). In addition to its ER luminal sensor domain, IRE1 (inositol-requiring enzyme 1α), a type I ER transmembrane protein. is particularly unique for containing both kinase and sequence-specific endoribonuclease (RNase) activities (11–15). Upon sensing ER demand for protein folding capacity, often referred to as ER stress, initial steps of IRE1 activation include oligomerization and autophosphorylation. Despite efforts, IRE1 itself remains the only known substrate for IRE1 kinase activity. Rather, evidence suggests that the IRE1 kinase activity functions to regulate activation of the IRE1 RNase activity, which cleaves an intron (the UPR intron) from mRNA coding for a key UPR-specific transcription factor (XBP1 in mammalian cells, HAC1 in yeast) (5, 6, 16, 19). The subsequently spliced forms of *XBP1* and *HAC1* mRNA produce a potent UPR-specific transcription factor, and thus splicings are critical steps in UPR signaling and regulation.

For its part, recognition of ER stress by PERK (eukaryotic translation initiation factor 2α kinase) causes eIF2 α phosphorylation, producing a general attenuation of protein translation, presumably to reduce influx of new protein into the ER (20, 21). ER stress is also recognized by a second UPR-specific transcription, ATF6, a type II ER transmembrane protein (22–24). Stress recognition by ATF6 leads to proteolytic release of its transcription factor-containing cytosolic domain from the ER membrane. Together, the coupling of these three ER proximal stress sensors to downstream effectors produces an array of cellular effects that promote ER functional capacity.

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[1] Present address: Biochemie-Zentrum Heidelberg, Universität Heidelberg, Im Neuenheimer Feld 328, 69120 Heidelberg, Germany.

[2] To whom correspondence should be addressed. Tel.: 858-822-3451; Fax: 858-822-0317; E-mail: niwa@ucsd.edu.

[3] The abbreviations used are: HSC, hematopoietic stem cell; UPR, unfolded protein response; CHO, Chinese hamster ovary; GFP, green fluorescent protein; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; LPS, lipopolysaccharide; IL, interleukin; Syn-1, syndecan-1; ER, endoplasmic reticulum.

In addition to IRE1, activation of ATF6 during plasma cell differentiation has been reported, although the exact timing and duration of activation differs from that of IRE-dependent splicing of *XBP* (25). On the other hand, PERK-induced translation repression has not been observed during plasma cell differentiation (26, 27), although the status of PERK activation remains to be determined. The ability of UPR sensors to be activated independently from each other is supported by our recent findings that the activation profiles of IRE1, PERK, and ATF6 differ from each other both in response to a single form of ER stress and between different forms of stress (28). Thus, the ER sensor domains of each UPR initiator may respond preferentially to changes in the ER environment to provide a molecular basis for "best fit" responses in physiological settings through selective activation of the UPR signaling branches. Although the molecular mechanisms of the luminal domain recognition of certain ER luminal components have been proposed (29–32), little is known about the molecular basis for the recognition of specific forms of ER stress or about how the timing and magnitude of UPR signaling branch activation are regulated, highlighting a need for *in vivo* studies designed to gain insight into UPR regulation in physiological settings. Specifically, examination of UPR component activities during differentiation of pluripotent HSC poses significant challenges, as obtaining sufficient quantities of intermediate cell populations for conventional molecular analyses are problematic.

Commonly, UPR studies have involved the use of pharmacological agents such as dithiothreitol and the glycosylation inhibitor tunicamycin to generate massive ER stress. Although these agents have been invaluable for the discovery and characterization of UPR signaling fundamentals, similar conditions are unlikely to occur in physiological settings where the extent of UPR activation is likely to be more subtle. Furthermore, UPR signaling may have roles beyond regulation of protein processing capacity or may function in cells unrecognized as secretory cells. In addition, there may be cellular responses that do not require input from each UPR signaling branch. In this regard, the identification of cellular events dependent on UPR signaling is an important step to understanding how UPR signaling integrates into normal cell physiology. Toward this goal, we have developed a fluorescent reporter that allows the activation of IRE1 to be monitored in single cells. Here, in order to examine IRE1 activation in differentiating hematopoietic cells, we have performed bone marrow reconstitution experiments using hematopoietic stem cells carrying this reporter. Our analysis of mice reconstituted with reporter-carrying bone marrow cells reveals, for the first time, IRE1 activation in developmentally intermediate B and T lymphocytes. Demonstration and validation of this reporter-based approach will therefore facilitate the identification of UPR functions during HSC differentiation.

MATERIALS AND METHODS

Cell Culture and UPR Induction—Chinese hamster ovary (CHO) cells were grown in Dulbecco's modified Eagle's medium/F12 (Cellgro) medium supplemented with 5% fetal calf serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2. The UPR was induced by treating cells with 1 μg/ml tunicamycin for 18 h (Calbiochem).

Transient and Stable Transfection—2.5×10^5 CHO cells were transfected with the reporter plasmid using Effectene transfection reagent (Invitrogen) following the manufacturer's instructions. To generate stable cell lines, transfected cells were transferred to selective medium containing 600 μg/ml G418.

RNA Isolation and RT-PCR—Upon harvesting cells, RNA was isolated using RNeasy isolation kit (Qiagen). After consecutive DNase I treatments of isolated RNA, cDNA was prepared using a mixture of random hexamers and poly(A) primers, followed by PCR using the primers described in Fig. 1. PCR fragments generated from unspliced and spliced forms of XBP1-GFP mRNA and the endogenous XBP1 mRNA were analyzed on 2% agarose gels. Sequences of primers used for PCR reactions were MN028, TTAGTTCATTAATGGCTTCCAGC; MN056, CACCTGAGCCCCGAGGAG; X3, GGAATGAAGTGAGGCCAGTGG; GFP reverse, CCATCGATCTTGTACAGCTCGTCCATGCCGAG.

Fluorescence-activated Cell Sorting (FACS)—Cells harvested for FACS analysis were washed with 3 ml of PBS containing 10% fetal bovine serum and stained with appropriate antibodies. Dilutions of individual antibodies were determined based on the manufacturer's instructions. Antibodies against mouse B220, IgM, IgD, CD5, CD43, CD11b, and hCD2 were purchased from eBioscience. Anti-mouse CD4 and CD8 antibodies were from BD Biosciences.

Isolation and Activation of Primary B Cells—Primary naive splenic B cells were isolated from three pooled spleens from C57B6 mice after osmotic lysis of erythrocytes by collecting flow-through from CD43 magnetic beads according to the manufacturer's instructions (Miltenyi Biotec). B cells were activated immediately after isolation by treatment with 25 μg/ml lipopolysaccharide (LPS) (Sigma) and 5 ng of IL-4 (Sigma). At indicated times after incubation with LPS and IL-4, B cells were harvested and protein lysates were prepared by lysing in Laemmli buffer containing 20 mM Na_2VO_4.

Mouse Bone Marrow Cell Collection and Infection—The bones were collected from the hind legs of two mice for every mouse to be reconstituted. After removing excess tissue, cells were flushed from bone marrow cavities with a 26-gauge needle. Upon lysis of red blood cells by addition of buffer containing 0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 M EDTA, cells were resuspended in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 10% IL-3 containing culture supernatant, 10 ng/ml IL-6, and 100 ng/ml SCF (Peprotech) and cultured at 37 °C in 5% CO_2. After a quick thaw at 37 °C, viral supernatants expressing the XBP1-GFP reporter gene were mixed with 10 μg/ml DOTAP (Roche Applied Science) and incubated on ice for 10 min. Cells were resuspended in DPTAP treated viral supernatants so that the final concentration was 10^6 cells/ml. After spinning at 2600 rpm for 90 min at 30 °C, cells were resuspended in fresh media plus cytokines and cultured overnight followed by a second spinfection prior to reconstitution of irradiated hosts.

Irradiation and Injection—The host mice were irradiated with 1000 rads (10 Gy) of γ irradiation. Approximately 1×10^6 cells were washed twice with PBS and injected through the mouse tail vein by standard procedures. Efficiencies of reconstitution were tested 4 weeks after injection.

Tissue Collection and Analysis—At 8 weeks after reconstitution, the mice were sacrificed using CO_2 asphyxiation. The

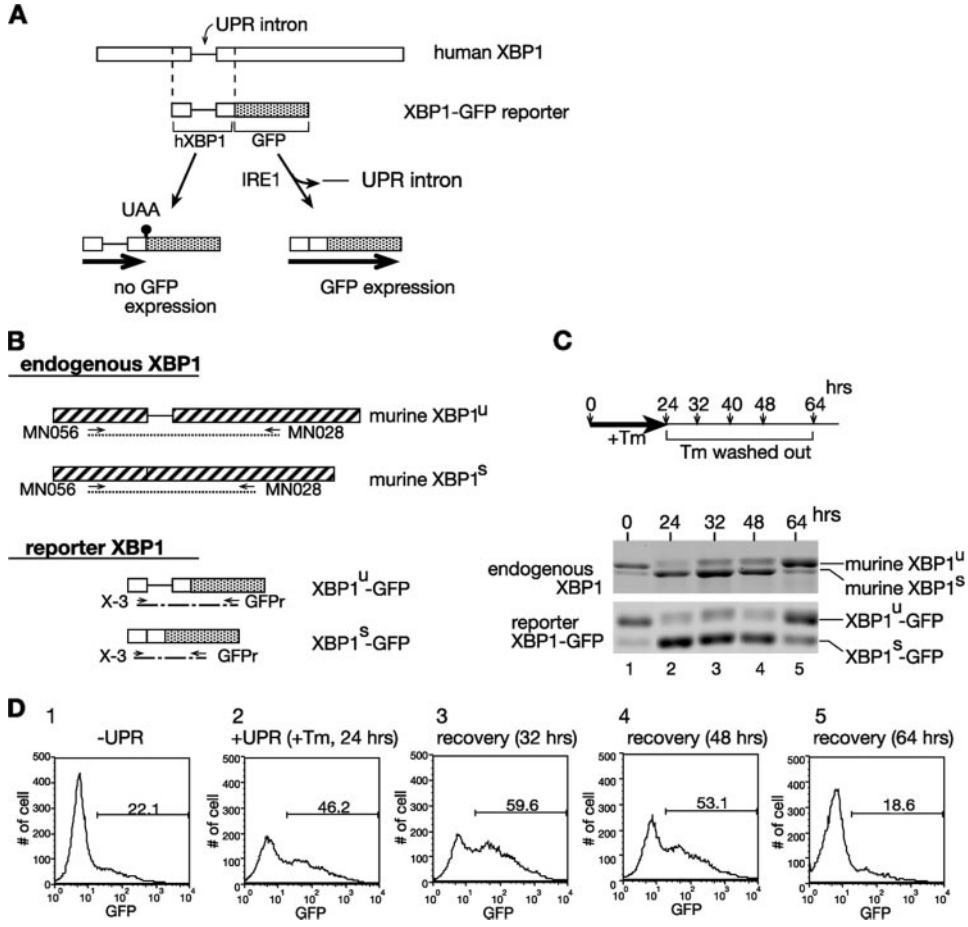

FIGURE 1. **The XBP1-GFP reporter construct for monitoring IRE1 activation.** *A*, design of the XBP1-GFP reporter. The human XBP1 (*hXBP1*) sequence flanking the UPR intron was fused to the coding sequence of GFP. A stop codon was inserted after the hXBP1 sequence such that the GFP coding sequence is in-frame with the translation initiation codon only when the UPR intron is spliced out. Because GFP expression occurs only from the spliced form of the XBP1-GFP mRNA, its presence signals the activation of IRE1. A truncated XBP1 fragment flanking the UPR intron containing only 163 bp of coding sequence (404–567) was used to minimize the possibility of constitutive UPR induction due to ectopic expression of UPR components and thus differed from the ERAI reporter described previously (18). *B*, primers used for PCR amplification of cDNA. For detection of endogenous XBP1 mRNA, two primers (*MN028* and *MN056*) specific to the mouse XBP1 sequence were used. The mouse XBP1 sequences chosen for primers are far away from the UPR intron and only present in the endogenous XBP1 sequence. For analysis of the XBP1-GFP mRNA, the X-3 and GFP reverse complementary to hXBP1 and GFP, respectively, were used for PCR. *C*, XBP1 splicing during UPR induction and recovery. Splicing of both the XBP1-GFP mRNA and endogenous XBP1 mRNA was analyzed by reverse transcription followed by PCR using primers described in *B*. The UPR was induced by incubating CHO cells carrying the XBP1-GFP reporter construct with 1 μg/ml tunicamycin for 24 h. At this point, tunicamycin was washed and replaced with normal medium. Samples were taken for analysis of GFP expression (*D*) and splicing of either XBP1-GFP or endogenous XBP1 mRNA (*C*) during the recovery phase of the UPR. Upon PCR, unspliced and spliced XBP1 mRNA from both endogenous and reporter genes were separated with 2% agarose gel electrophoresis and analyzed by staining with ethidium bromide. Positions of both spliced and unspliced murine XBP1 and XBP1-GFP are indicated. *D*, GFP expression levels in the cell samples analyzed in *C* were examined using FACS. The percent of cells with higher levels of GFP is indicated at each time point. *Tm*, tunicamycin.

bone marrow was collected as described above. The spleen, thymus, and lymph nodes were collected using common surgical techniques and homogenized in separate wells of a 6-well dish containing 4 ml of PBS. After a brief spin (1,200 rpm for 10 min at 4 °C), cells were resuspended in 1 ml of cold PBS on ice. After lysing the red blood cells as described above, cells were resuspended to 10^7 cells/ml in PBS for staining.

RESULTS

To monitor IRE1 activation status in single cells, we constructed a reporter gene by fusing human XBP1 sequence to green fluorescent protein (Fig. 1*A*, *GFP*). In contrast to full-length *XBP1* mRNA, the XBP1-GFP reporter only includes the sequence surrounding the XBP1 UPR intron to avoid potential effects from XBP1 protein fragment expression. However, the XBP1 sequence present in the reporter was sufficient for IRE1-dependent splicing of the XBP1 intron upon UPR induction. Without removal of the UPR intron, mRNA generated from the reporter stop its translation at the stop codon placed between XBP1 and the GFP sequence. Upon IRE1-mediated splicing of the XBP1 intron, the GFP sequence becomes in-frame, insuring GFP expression only upon correct splicing of the UPR intron mediated by activated IRE1 (Fig. 1*A*).

Validation of the XBP1-GFP reporter was carried out in a cultured cell line as well as in mouse primary splenic B cells. Upon stable transfection of the reporter in CHO cells, GFP expression during tunicamycin treatment was correlated with the extent of splicing of the UPR intron from XBP1/GFP mRNA in splicing assays. Tunicamycin is a glycosylation inhibitor and well characterized to induce UPR upon incubation with tissue culture cells. Splicing was assayed by reverse transcription of cellular RNA followed by PCR amplification with primers unique to the reporter sequence (Fig. 1, *B* and *C*). In addition, PCR primers complementary to the sequence unique to endogenous XBP1 showed that the extent of reporter splicing was similar to that of endogenous XBP1 mRNA (Fig. 1*C*). These results confirm that the XBP1 fragment of the reporter containing the UPR intron is sufficient to promote UPR-dependent splicing by IRE1. XBP1 splicing was further examined during recovery from UPR activation, following removal and washout of tunicamycin. In this case, splicing of endogenous and reporter XBP1 genes continued for an extended period before returning to basal levels (Fig. 1*C*). Because tunicamycin blocks an early step of protein glycosylation, continued splicing of XBP1 presumably reflects sustained levels of unfolded protein that remain high until oligosaccharides are replenished.

In addition, GFP expression was examined for fluorescence by FACS. Under normal growth conditions, XBP1/GFP-transfected CHO cells showed basal fluorescence similar to CHO

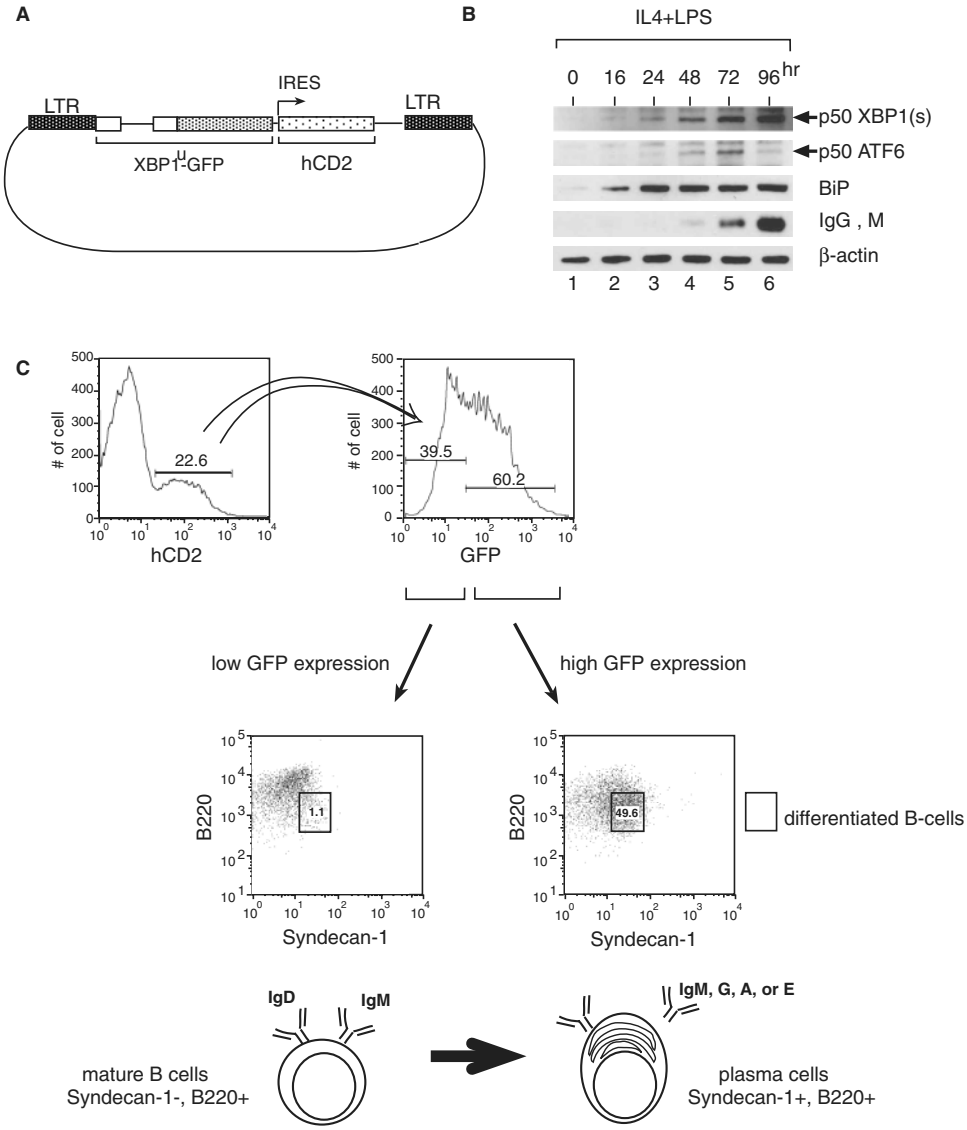

FIGURE 2. **Behavior of the XBP1-GFP reporter during terminal differentiation of mouse splenic primary B cells.** *A*, retroviral expression construct containing the XBP1-GFP reporter. The XBP1-GFP sequence was placed under a strong viral promoter in the long terminal repeats (*LTR*). For selection of cells carrying the reporter gene, the human CD2 (*hCD2*) coding sequence was inserted at the 3′-end of the XBP1-GFP sequence. mRNA transcripts produced from this construct contain bicistronic coding regions for XBP1-GFP and hCD2. Translation of hCD2 is mediated by an internal ribosome entry site (*IRES*) and is independent of upstream XBP1-GFP translation. *B*, activation states of multiple UPR components during primary B-cell terminal differentiation induced by treatment with IL-4 and LPS. The lysates prepared from each time point were immunoblotted with antibodies against p50 XBP1 (coded the spliced form of XBP1), p50ATF6 (the activated form of ATF6), the ER chaperone BiP, and IgG. Levels of β-actin are shown as a loading control. *C*, FACS analysis of B cells incubated with IL-4 and LPS for 72 h. hCD2-positive cells (22.6%) were analyzed for GFP expression. More than half the reporter-carrying cells expressed significant levels of GFP (60.2%). Among the GFP-expressing B cells, half the population (49.6%) were B220^{low} and Syn-1^{+}. Down-regulation of B220 and up-regulation of Syn-1 are well characterized markers of terminally differentiated plasma cells, and thus this population of cells represents terminally differentiated B cells. Cells with lower expression levels of GFP (39.5%) were mostly B220^{hi} but Syn-1$^{low/neg}$ B cells representing undifferentiated B cells.

cells without reporter (Fig. 1*D*, *panel 1*, *−UPR*). Treatment of XBP1/GFP-CHO cells with tunicamycin caused increased fluorescence in a significant proportion of cells (Fig. 1*D*, *panel 2*, *+UPR*). Similarly, increased fluorescence was observed in XBP1/GFP-CHO cells treated with thapsigargin, an inhibitor of ER-localized Ca^{2+}-dependent ATPase (data not shown). The observed heterogeneity in fluorescence levels presumably reflects cell populations carrying different copy numbers of reporter as well as differences due to reporter integration sites. Furthermore, GFP expression was in good correlation with splicing of the UPR intron from both endogenous and reporter XBP1 genes during the recovery phase and ultimately went back to the basal fluorescent level.

Next, XBP1-GFP reporter behavior was tested during the *in vitro* differentiation of activated splenic B cells to antibody-secreting plasma cells, a system in which XBP1 has been reported to undergo splicing (5, 33, 34). The XBP1-GFP reporter was introduced into mature B cells isolated from adult mouse spleens using a retroviral expression system (Fig. 2*A*). At 72 h after incubation with IL-4 and LPS, a majority of cultured B cells had differentiated into plasma cells, based on expression of the well established plasma cell surface markers B220, Syndecan-1 (Syn-1), CD69, and B7 (supplemental Fig. S1). Analyses of endogenous XBP1 activation and other UPR components during the 72-h incubation period correlated with increased cellular Ig heavy chain (Fig. 2*B*). Furthermore, both the kinetics and extent of XBP1 activation we observed are consistent with previous reports of splenic B-cell differentiation induced by either LPS, IL-4, or anti-CD40 alone or combination (26, 33, 34).

Based on cell surface markers, three distinct populations of cells were observed at 72 h after culture with IL-4 and LPS, fully differentiated, partially differentiated, and undifferentiated (Fig. 2*C*). In the retroviral transduction plasmid used for these experiments, the XBP1-GFP reporter sequence is followed by the coding sequence for the human cell surface protein CD2 (hCD2), such that reporter transcription produces a bicistronic mRNA containing both XBP1-GFP and hCD2. An internal ribosome entry site on this transcript assures expression of hCD2 protein regardless of the splicing state of XBP1-GFP. Thus, selection for hCD2^{+} cells allowed us to limit our subsequent analyses only to reporter-expressing cells.

Among the hCD2^{+} cell population (22.6%), ~60% of cells expressed significant levels of GFP resulting from XBP1-GFP mRNA splicing (Fig. 2*C*), and ~50% of these GFP-expressing cells expressed relatively high levels of Syn-1 and have down-

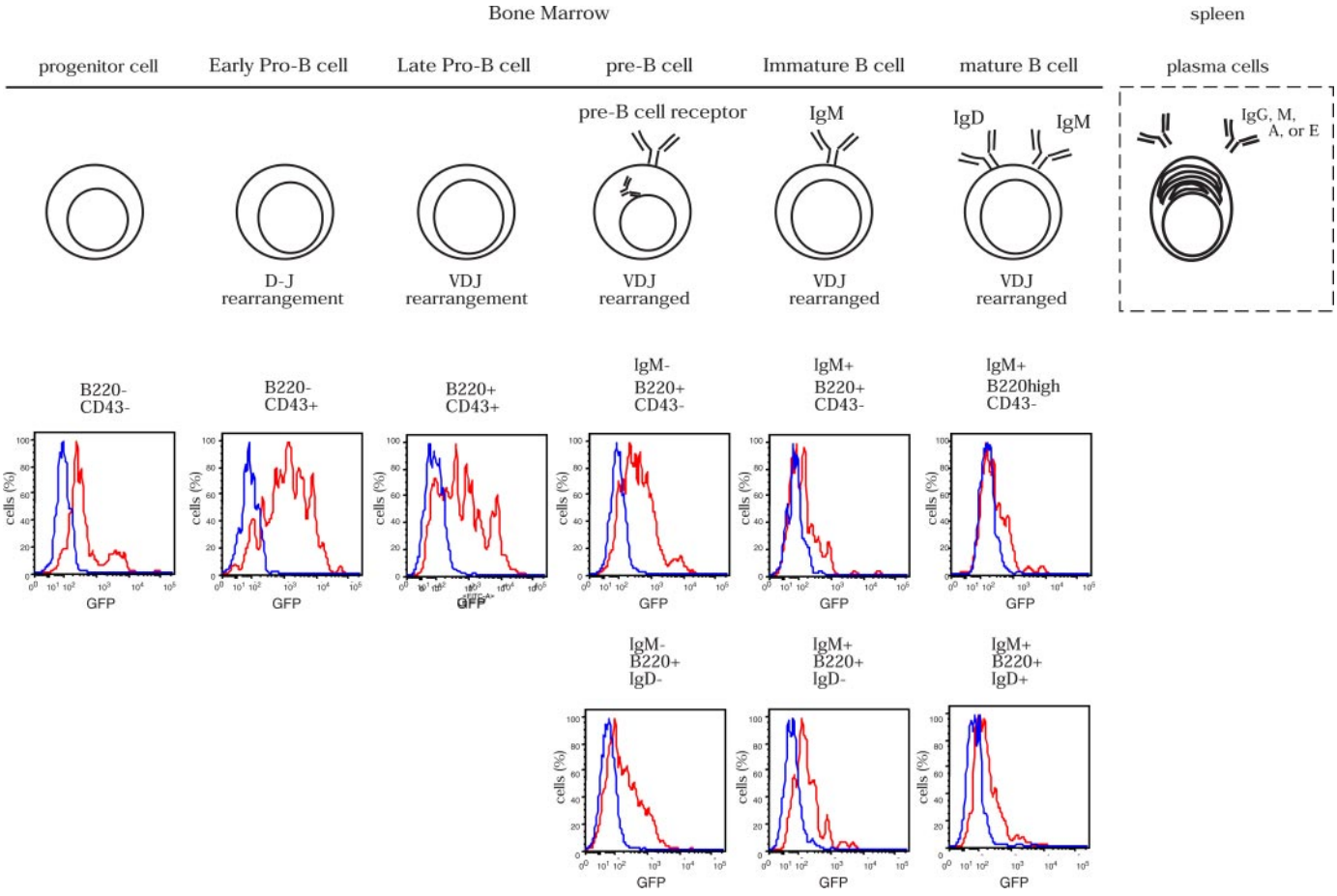

FIGURE 3. **The XBP1-GFP reporter becomes activated during early B-cell developmental stages in bone marrow.** Bone marrow B cells derived from donor hematopoietic stem cells were stained with B-cell surface markers, including B220, CD43, IgM, and IgD, to classify them into different developmental stages. After gating on hCD2-positive donor cells, expression of GFP in each developmental stage was analyzed and is shown in *red*. As a control, reconstitution experiments using donor bone marrow cells infected with a reporter containing only hCD2, but no XBP-GFP sequences, were carried out. Fluorescence detected in each cell type isolated from these control reconstitution experiments is shown in *blue*. Schematic representations of some developmental events characteristic to each of the B-cell developmental stages are shown.

regulated B220 (cells inside small boxes shown in FACS data). In contrast, GFP$^-$cells (~40% of hCD2$^+$ cells) were Syn-1$^{low\ negative}$, indicative of undifferentiated mature B cells. Thus, these results show that XBP1-GFP splicing correlates with B-cell differentiation and remains unspliced in undifferentiated cells. Furthermore, GFP expression in plasma cells corresponded to the appearance of the spliced form of endogenous p50 XBP1 protein (Fig. 2*B*).

Following these initial experiments to validate the utility of our XBP1-GFP reporter, we reasoned that our ability to detect IRE1 RNase activity in small cell populations would be of value in examining whether IRE1 is activated during earlier stages of hematopoiesis. Thus, we examined reporter expression in reporter-transduced murine HSCs during their development in irradiated hosts. HSCs from mouse bone marrow were infected with a retroviral vector carrying the XBP1-GFP reporter gene and transferred into recipient mice previously irradiated to eliminate endogenous HSC. Injected HSC home to bone marrow of recipient mice where they undergo differentiation into the various hematopoietic lineages and eventually populate the periphery. In control experiments, donor bone marrow stem cells were infected with an otherwise identical retroviral vector lacking the XBP1-GFP reporter sequence. Because of inherent differences between individual mice, each bone marrow transplant experiment was performed with three mice reconstituted with reporter-transduced HSC and three control reconstitution mice and all subsequent analyses were performed separately with each mouse. We repeated these procedures two additional times (total of three independent bone marrow reconstitution experiments), using freshly transduced donor HSC with the reporter each time. At 8 weeks post cell transfer, donor-derived hCD2$^+$ cells were isolated from recipient thymus, spleen, and bone marrow and analyzed for GFP expression. Although cells from any of the hematopoietic lineage could be examined for GFP expression at this point, we limited our analysis to cells of the B-cell and T-cell lineages, classified by developmental stage-specific surface markers, as our initial experiments demonstrating the usefulness of the reporter-carrying HSC. For the B-cell lineage, markers included B220, CD43, IgD, and IgM (Fig. 3). Selection for only hCD2$^+$ cells insured the donor origin of analyzed cells. GFP expression levels in each cell population (Fig. 3, *red line*) were compared with those from mice reconstituted only with control bone marrow cells (*blue*).

hCD2-positive cells were grouped based on their expression of B220 and CD43. GFP expression in B220$^-$/CD43$^+$ and B220$^+$ CD43$^+$ bone marrow populations, representing developmen-

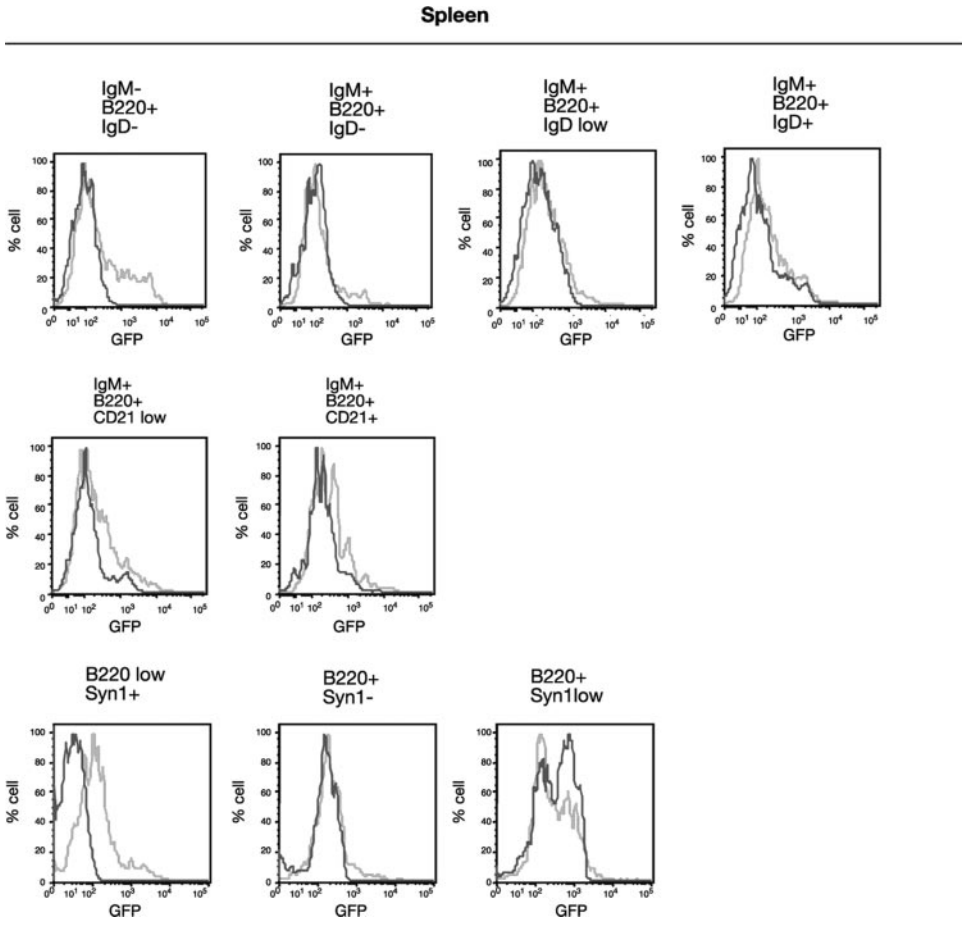

FIGURE 4. **The XBP1-GFP reporter was turned off at later stages of B-cell development in spleen.** GFP expression in reconstituted splenic B cells is shown at various developmental stages. Splenic B cells derived from donor stem cells carrying the reporter construct (hCD2-positive cells) were classified into various developmental stages based on the cell surface expression of IgM, B220, IgD, and CD21. In each developmental stage derived from the XBP1-GFP reporter-containing stem cells, the levels of fluorescence were analyzed by FACS (shown in *light gray*) and compared with similar analyses from the control reconstitution (shown in *black*).

tally early stages, was at least 10-fold higher than similar populations isolated from control mice (Fig. 3). $IgM^-B220^+CD43^-$ and $IgM^-B220^+IgD^-$ cell populations, indicative of early pre-B cells, also expressed GFP above control populations. In contrast, only minimal GFP expression was observed in more developed populations, including $IgM^+B220^+CD43^-$, $IgM^+B220^{high}CD43^-$, $IgM^+B220^+IgD^-$, and $IgM^+B220^+IgD^+$ B cells. Together, these results show that the XBP1-GFP reporter was spliced early in B-cell development.

Following early development in bone marrow, developing B cells migrate to populate the spleen, where they undergo further maturation. To examine reporter splicing past pre-B-cell stages, we analyzed splenic B cells in reconstituted mice (Fig. 4). In cells maturing to IgM or IgD surface expression, we observed no concomitant expression of GFP, confirming that XBP1 slicing was limited to earlier developmental stages. Furthermore, GFP expression was insignificant in mature B-cell populations. including $B220^+CD21^{low}$ follicular B cells and $B220^+CD21^+$ marginal zone B cells (Fig. 4).

Together, our analysis of bone marrow and splenic B cells showed that XBP1-GFP reporter splicing occurred early in lymphoid development at pro-B-cell stages. We performed three sets of experiments, each with three experimental and three control mice. Freshly transduced bone marrow cells were used as donors for each experimental set. Similar results were obtained both within each experimental set and between each experimental set. Our results therefore are consistent with IRE1 activation during two distinct phases of B-cell development: at the pro-B-cell stage during which precursors commit to the B-cell lineage and during the terminal differentiation of mature B cells to plasma cells. Although our classifications of B and T cells into developmental stages are somewhat coarse, they provide a foundation for future higher resolution analysis.

We next asked whether IRE1 activation occurs in developing cells of the T-lymphoid lineage by examining reporter activation in cells obtained from both thymus and spleen of recipient mice (Fig. 5). Immature T cells migrate from bone marrow to thymus, where as $CD4^-CD8^-$ cells (double negative) development continues through the $CD4^+CD8^+$ (double positive) stage and, later, to mature single positive $CD4^+CD8^-$ or $CD4^-CD8^+$ T cells. Thus, cells from each stage were characterized by expression of appropriate surface markers and analyzed for GFP expression (Fig. 5*A*). Cells at the earliest stage of development examined, the $CD4^-CD8^-$ population, showed levels of GFP expression only slightly higher than of double negative T cells from control hosts. On the other hand, GFP expression was significant in the $CD4^+CD8^+$ double positive population, indicating IRE1 activation had occurred in these cells. A role for UPR signaling during T-cell development has not been reported previously. These findings suggest that IRE1 activation may play a role during the "commitment stages" of T-cell development where both CD4 and CD8 appear on the cell surface. Cells may activate UPR (or, only IRE1) in anticipation of a pending need for protein-processing capacity. Later in T-cell development, we found that GFP expression remained significant in both $CD4^+CD8^-$ and $CD4^-CD8^+$ cells (Fig. 5*A*). Expression of GFP in normal mouse thymic T cells, which are ~80% double positive, confirmed endogenous splicing of XBP1 mRNA (data not shown).

Both $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells exit the thymus to populate peripheral lymphoid tissues, including spleen. Thus, mature T cells from spleen can be considered as being more "mature" than thymic T cells, and examination of splenic T cells would reveal the status of IRE1 in late stage T cells. We found minimal expression of GFP in splenic $CD4^+CD8^-$ cells, similar to

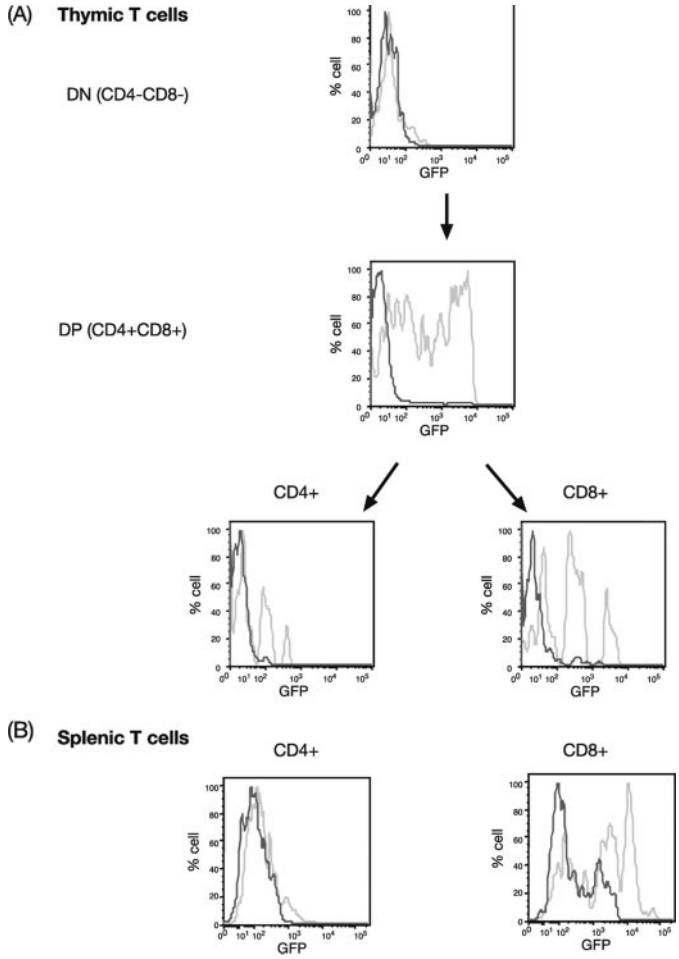

FIGURE 5. **GFP expression levels in reconstituted thymic and splenic T cells.** T cells isolated from the reconstituted thymus (*A*) and spleen (*B*) were stained with anti-CD4 and -CD8 antibodies. GFP levels in each T-cell type derived from the reconstitution experiments (shown in *light gray*) were compared with those derived from the control reconstitution experiments (shown in *black*) as described above.

$CD4^+CD8^-$ splenic cells from control hosts, suggesting that activity of IRE1 in the less mature double positive cells is deactivated as cells undergo further maturation. In curious contrast, however, GFP expression in splenic $CD4^-CD8^+$ T cells was strong (Fig. 5*B*). Our findings suggest the exciting possibility that the UPR may play a role in such cell fate decisions.

DISCUSSION

In summary, reconstitution experiments with bone marrow stem cells carrying the XBP1-GFP reporter revealed IRE1 activation in differentiating cells of the T- and B-lymphoid lineages. In the B lineage, IRE1 was active in pro-B cells (and a small pre-B cell population) and, as previously reported, during the differentiation of activated mature B cells to antibody-secreting plasma cells. In the T lineage, IRE1 was activated in both thymic $CD4^+CD8^+$ double positive cells and in splenic $CD4^-CD8^+$ cells. In contrast to plasma cell differentiation, where facilitating ER expansion to accommodate massive antibody secretion provides a satisfying conceptual reason for UPR activation, reasons for IRE1 activation in earlier stages of B-cell development or during T-cell development are not immediately obvious. In this regard, it is interesting to note our recent findings describing a role for IRE1 in cytokinesis in the budding yeast *Saccharomyces cerevisiae* (35). As for early B or T cells, cytokinesis is not associated with a global increase in protein secretion or membrane protein expression, although localization of a specific cell cycle component(s) to the site of cytokinesis may require some increase in secretory capacity. Unlike pharmacological agents often used for experimental induction of UPR signaling, endogenous signals imposing protein-processing demand on the ER may be less obvious (3–6). It is noteworthy that our reporter has allowed us to identify IRE1 activation at discrete stages of lymphoid cell development without prior knowledge, setting the stage for further investigation. Similar analyses can therefore be applied to score IRE1 activation in other developing HSC.

Homozygous deletion of IRE1 is lethal to murine embryos (36, 37), but fetal liver cells isolated from *ire1*$^{-/-}$ embryos contain $CD43^+B220^+$ B-cell precursors. In these same experiments, very few $CD43^-B220^+$ and $B220^+IgM^+$ were found (27), suggesting a critical role for IRE1 at this stage of differentiation and that without IRE1, cells are unable to differentiate beyond the $CD43^+B200^+$ pro-B-cell stage. Thus, our results here showing that IRE1 is activated in $CD43^+B220^+$ are consistent with previous results and predict that conditional knock out of IRE1 in bone marrow hematopoietic stem cells would result in blockade of B-cell development at the pro-B stage and blockade of T-cell development at the $CD4^-CD8^-$ double negative stage.

Among the interesting questions these findings raise is the reason for IRE1 activation in developing T and B cells. For both early B and T cells, reporter activation does not appear to correlate with an unusual increase in secreted or membrane proteins. This is in contrast to terminal differentiation of mature B cells into antibody-secreting plasma cells where IRE1 activation appears to anticipate the massive increase in antibody production. In developing early B cells for example, there is constant expression of stage-specific cell surface markers (38–41). At first glance then, a simple increase in expression of secretory proteins or cell surface markers would seem unlikely to be the primary cause of IRE1 activation. For T cells, when double positive cells mature to single positive $CD4^+$ and $CD8^+$ cells, they acquire specific functional capabilities. $CD4^+$ "helper" cells can secrete cytokines upon antigen stimulation and begin to express accessory molecules such as CD40, which facilitates interaction with B cells (38–41). Single positive $CD8^+$ "cytotoxic " cells express proteins that lyse antigen-presenting cells. The mechanisms by which $CD4^+$ and $CD8^+$ cells acquire such specialized functions are not well understood. Because antigens are destroyed by direct lysis of antigen-presenting cells, timely release or secretion of lytic enzymes from $CD8^+$ cytotoxic T cells upon antigen binding may be critical. $CD8^+$ T cells may prepare for rapid enzyme release or secretion by inducing increases in ER protein-handling capacity.

In addition, interestingly, the transition of both pro-B to pre-B cells and $CD4^-CD8^-$ double negative to $CD4^+CD8^+$ double positive T cells involves similar molecular events, including the expression of stage-specific cell surface proteins and V(D)J antigen receptor gene rearrangements (38–41). In particular, for both types of cells expression of developmentally critical pre-B- and pre-T-cell receptors takes place. Both pre-B- and pre-T-cell receptors share similar functions, inhibiting further heavy chain (for B

cells) or β chain (for T cells) recombination and proliferation of pre-B or pre-T cells. Furthermore, stimulation of multiple κ light chain (B cells) or α chain (T cells) recombination events occurs, leading to both positive and negative selection of lymphocytes. During these selection periods, cell surface expression of different forms of κ light chains or α chains is generated by "trial and error" recombination. Lymphocytes expressing antigen receptor binding tightly to self-antigens are eliminated, whereas those with low avidity for major histocompatibility proteins are selected for further expansion. Thus, activation of IRE1 or the entire UPR pathway may facilitate these selections and could therefore be a part of the developmental program that insures proper maturation of B and T lymphocytes. An alternate possibility is that IRE1 signaling plays a role in V(D)J antigen receptor gene rearrangements themselves, for example, through association with the SAGA histone acetyltransferase complex. Curiously, IRE1 was found to be associated with the ADA5 transcription activator, part of the SAGA complexes (42).

In addition, throughout development, a variety of growth factors and cytokines are required for differentiation into mature cells. As shown in other systems, such factors could also induce Ire1 activation and other UPR components in certain cell types (17, 43–48). A further cause for IRE1 or UPR activation may be to support cell proliferation (clonal expansion) that follows antigen receptor gene rearrangement in B and T cells. Our recent identification of a role for IRE1 during cytokinesis in normal yeast may support this idea (35).

Our results suggest involvement of the IRE1 branch of the UPR signaling pathway during T- and B-cell development. It will be also informative to examine activation of additional UPR sensors, ATF6 and PERK, by similar methods. Such studies would likely provide insights into the functional similarities and differences between the UPR signaling branches and how ATF6, IRE1, and PERK coordinate their activities in physiological settings.

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REFERENCES

1. Dzierzak, E. (2002) *Immunol. Rev.* **187,** 126–138
2. Giesen, K., Hoehn, J., Koegler, G., Wernet, P., and Giebel, B. (2001) *Blood* **98,** 115B–115B
3. Reimold, A. M., Iwakoshi, N. N., Manis, J., Vallabhajosyula, P., Szomolanyi-Tsuda, E., Gravallese, E. M., Friend, D., Grusby, M. J., Alt, F., and Glimcher, L. H. (2001) *Nature* **412,** 300–307
4. Yoshida, H., Okada, T., Haze, K., Yanagi, H., Yura, T., Negishi, M., and Mori, K. (2001) *Mol. Cell. Biol.* **21,** 1239–1248
5. Calfon, M., Zeng, H. Q., Urano, F., Till, J. H., Hubbard, S. R., Harding, H. P., Clark, S. G., and Ron, D. (2002) *Nature* **415,** 92–96
6. Shen, X. H., Ellis, R. E., Lee, K., Liu, C. Y., Yang, K., Solomon, A., Yoshida, H., Morimoto, R., Kurnit, D. M., Mori, K., and Kaufman, R. J. (2001) *Cell* **107,** 893–903
7. Mori, K. (2000) *Cell* **101,** 451–454
8. Kaufman, R. J. (1999) *Genes Dev.* **13,** 1211–1233
9. Patil, C., and Walter, P. (2001) *Curr. Opin. Cell Biol.* **13,** 349–355
10. Harding, H. P., Calfon, M., Urano, F., Novoa, I., and Ron, D. (2002) *Annu. Rev. Cell Dev. Biol.* **18,** 575–599
11. Tirasophon, W., Welihinda, A. A., and Kaufman, R. J. (1998) *Genes Dev.* **12,** 1812–1824
12. Wang, X. Z., Harding, H. P., Zhang, Y., Jolicoeur, E. M., Kuroda, M., and Ron, D. (1998) *EMBO J.* **17,** 5708–5717
13. Shamu, C. E., and Walter, P. (1996) *EMBO J.* **15,** 3028–3039
14. Cox, J. S., Shamu, C. E., and Walter, P. (1993) *Cell* **73,** 1197–1206
15. Mori, K., Ma, W. Z., Gething, M. J., and Sambrook, J. (1993) *Cell* **74,** 743–756
16. Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., and Mori, K. (2001) *Cell* **107,** 881–891
17. Fortunel, N., Batard, P., Hatzfeld, A., Monier, M. N., Panterne, B., Lebkowski, J., and Hatzfeld, J. (1998) *J. Cell Sci.* **111,** 1867–1875
18. Iwawaki, T., Akai, R., Kohno, K., and Miura, M. (2004) *Nat. Med.* **10,** 98–102
19. Cox, J. S., and Walter, P. (1996) *Cell* **87,** 391–404
20. Harding, H. P., Zhang, Y., and Ron, D. (1999) *Nature* **397,** 271–274
21. Shi, Y. G., Vattem, K. M., Sood, R., An, J., Liang, J. D., Stramm, L., and Wek, R. C. (1998) *Mol. Cell. Biol.* **18,** 7499–7509
22. Haze, K., Yoshida, H., Yanagi, H., Yura, T., and Mori, K. (1999) *Mol. Biol. Cell* **10,** 3787–3799
23. Shen, J. S., Chen, X., Hendershot, L., and Prywes, R. (2002) *Dev. Cell* **3,** 99–111
24. Ye, J., Rawson, R. B., Komuro, R., Chen, X., Dave, U. P., Prywes, R., Brown, M. S., and Goldstein, J. L. (2000) *Mol. Cell* **6,** 1355–1364
25. Gunn, K. E., Gifford, N. M., Mori, K., Brewer, J. W. (2004) *Mol. Immunol.* **41,** 919–927
26. Gass, J. N., Gifford, N. M., and Brewer, J. W. (2002) *J. of Biol. Chem.* **277,** 49047–49054
27. Zhang, K., Wong, H. N., Song, B., Miller, C. N., Scheuner, D., and Kaufman, R. J. (2005) *J. Clin. Investig.* **115,** 268–281
28. DuRose, J. B., Tam, A. B., and Niwa, M. (2006) *Mol. Biol. Cell* **17,** 3095–3107
29. Bertolotti, A., Zhang, Y. H., Hendershot, L. M., Harding, H. P., and Ron, D. (2000) *Nat. Cell Biol.* **2,** 326–332
30. Liu, C. Y., Schroder, M., and Kaufman, R. J. (2000) *J. Biol. Chem.* **275,** 24881–24885
31. Liu, C. Y., Wong, H. N., Schauerte, J. A., and Kaufman, R. J. (2002) *J. Biol. Chem.* **277,** 18346–18356
32. Credle, J. J., Finer-Moore, J. S., Papa, F. R., Stroud, R. M., and Walter, P. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 18773–18784
33. Iwakoshi, N. N., Lee, A. H., Vallabhajosyula, P., Otipoby, K. L., Rajewsky, K., and Glimcher, L. H. (2003) *Nat. Immunol.* **4,** 321–329
34. Gass, J. N., Gifford, N. M., and Brewer, J. W. (2002) *J. Biol. Chem.* **277,** 49047–49054
35. Bicknell, A. A., Babour, A., Federovitch, C. M., and Niwa, M. (2007) *J. Cell Biol.* **177,** 1017–1027
36. Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H. P., and Ron, D. (2000) *Science* **287,** 664–666
37. Bertolotti, A., Wang, X. Z., Novoa, I., Jungreis, R., Schlessinger, K., Cho, J. H., West, A. B., and Ron, D. (2001) *J. Clin. Investig.* **107,** 585–593
38. Rumfelt, L. L., Zhou, Y., Rowley, B. M., Shinton, S. A., and Hardy, R. R. (2006) *J. Exp. Med.* **203,** 675–687
39. Melchers, F., Haasner, D., Grawunder, U., Kalberer, C., Karasuyama, H., Winkler, T., and Rolink, A. G. (1994) *Annu. Rev. Immunol.* **12,** 209–225
40. Starr, T. K., Jameson, S. C., and Hogquist, K. A. (2003) *Annu. Rev. Immunol.* **21,** 139–176
41. Schmitt, T. M., de Pooter, R. F., Gronski, M. A., Cho, S. K., Ohashi, P. S., and Zuniga-Pflucker, J. C. (2004) *Nat. Immunol.* **5,** 410–417
42. Welihinda, A. K., Tirasophon, W., and Kaufman, R. J. (2000) *J. Biol. Chem.* **275,** 3377–3381
43. Chambers, K. T., Weber, S. M., and Corbett, J. A. (2006) *Diabetes* **55,** A354–A354
44. Chambers, K. T., Weber, S. M., and Corbett, J. A. (2007) *Am. J. Physiol.* **292,** E1052–E1061
45. Oyadomari, S., Takeda, K., Takiguchi, M., Gotoh, T., Matsumoto, M., Wada, I., Akira, S., Araki, E., and Mori, M. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 10845–10850
46. Batard, P., Monier, M. N., Fortunel, N., Ducos, K., Sansilvestri-Morel, P., Phan, T. H., Hatzfeld, A., and Hatzfeld, J. A. (2000) *J. Cell Sci.* **113,** 383–390
47. Fortunel, N. O., Hatzfeld, J. A., Monier, M. N., and Hatzfeld, A. (2003) *Oncol. Res.* **13,** 445–453
48. Fortunel, N., Hatzfeld, J., Kisselev, S., Monier, M. N., Ducos, K., Cardoso, A., Batard, P., and Hatzfeld, A. (2000) *Stem Cells* **18,** 102–111

B- and T-cell Development Both Involve Activity of the Unfolded Protein Response Pathway

Ryan Brunsing, Sidne A. Omori, Frank Weber, Alicia Bicknell, Leslie Friend, Robert Rickert and Maho Niwa

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