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Cutting Edge: Lack of Peripheral B Cells and Severe Agammaglobulinemia in Mice Simultaneously Lacking Bruton's Tyrosine Kinase and the B Cell-Specific Transcriptional Coactivator OBF-1

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OBF-1 is a B cell-restricted transcriptional coactivator that is recruited to octamer-containing promoters by interacting with the POU domain of Oct-1 or Oct-2. We have shown earlier that mice lacking OBF-1 were dramatically impaired in their ability to mount humoral immune responses and did not develop germinal centers in the spleen; however, they had a largely normal B cell development in the bone marrow. In this study, we demonstrate that OBF-1-deficient mice also have an early defect in B cell development and show that *OBF-1*$^{-/-}$ immature B cells are greatly impaired at the transition from the bone marrow to the spleen. In addition, when the OBF-1 mutation is combined to a mutation in the gene encoding Bruton's tyrosine kinase, a striking phenotype is observed. These double-deficient animals lack peripheral B cells and have virtually no serum Igs, thus closely resembling human X chromosome-linked agammaglobulinemia. *The Journal of Immunology*, 2000, 164: 18–22.

The highly conserved octamer motif, ATGCAAAT, has been tightly associated with the B cell-restricted activity of Ig gene promoters (1–3). Three transcriptional regulators have been implicated in mediating the activity associated with the octamer motif: the ubiquitously expressed Oct-1 transcription factor, the lymphoid and nervous system-restricted Oct-2, and the lymphoid-specific OBF-1 coactivator (also known as OCA-B or Bob1) (reviewed in Ref. 4). The latter protein forms ternary complexes with either Oct-1 or Oct-2, via their POU domain, on a subset of octamer sites and enhances octamer-mediated transcription in transfections and in vitro transcription assays (5–7).

Mice deficient in Oct-2 or OBF-1 have been generated (8–11), and surprisingly only small effects on Ig gene transcription or B cell development were observed. B cells derived from Oct-2-deficient mice were found to respond poorly to LPS stimulation and were blocked in the G_1 phase of the cell cycle; Ig gene transcription and Ig production, however, seemed largely normal (8, 12). OBF-1-deficient mice also had a largely normal early B cell development but showed a strong reduction in serum IgGs levels as well as an almost complete absence of humoral immune responses that correlated with a lack of germinal center formation (9, 13). Unexpectedly, expression of Igμ transcripts upon in vitro stimulation with a variety of stimuli was only marginally affected. This finding has been explained by a functional redundancy between Oct-2 and OBF-1 (reviewed in Ref. 4).

X chromosome-linked agammaglobulinemia (XLA)[3] in humans is an inherited humoral immunodeficiency disease in which mature B cells and circulating Igs are absent. Affected individuals, in particular children, have a high susceptibility to infections (14). A similar disease, yet displaying a much milder phenotype, is found in mice of the CBA/N strain carrying the Xid mutation (X-linked immunodeficiency) (15). Remarkably, both diseases are caused by mutations in the gene encoding the cytoplasmic tyrosine kinase Bruton's tyrosine kinase (Btk), and this has been confirmed by targeted inactivation of the *Btk* gene in mice (16–18).

In contrast to XLA patients, Xid mice have mature B cells in the periphery, although in reduced numbers; these mice do not respond to immunization with thymus-independent type 2 Ags such as polysaccharides (19) and also have reduced serum IgM and IgG3 levels. B cell development in the bone marrow is only minimally affected by the Xid mutation at the transition from pre-BII to immature cells (17, 20, 21); in the spleen however, the efficiency with which immature B cells, which are present at normal levels in these mice, enter the mature pool is severely reduced (22).

In this study, on the basis of FACS analysis, we have found that OBF-1 is critical for immature B cells at the transition from the bone marrow to the peripheral compartments: in the absence of OBF-1, the number of splenic immature B cells is greatly reduced, whereas the number of their precursors in the bone marrow is

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[3] Abbreviations used in this paper: XLA, X chromosome-linked agammaglobulinemia; Btk, Bruton's tyrosine kinase.

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Table I. *V family usage and N insertions in hybridomas derived from wild-type and OBF-1-deficient animals*[a]

V Family	Wild Type			*OBF-1*$^{-/-}$		
	No. of Clones	No. with N insertions between		No. of clones	No. with N insertions between	
		V and D	D and J		V and D	D and J
V_H 1	33	31	26	19	19	15
V_H 2	4	4	4	9	7	5
V_H 5	7	6	5	6	6	4
V_H 6	2	2	1			
V_H 7				1	1	1
V_H 9	2	2		2	2	1
V_H 10				2	2	2
V_H 12				1	1	1
V_H 14	2	2	2			

[a] Spleen cells were stimulated with LPS and hybridomas were generated. IgM-producing clones were expanded, RNA and cDNA were prepared and subjected to PCR analysis. In total, we obtained sequences from 50 wild-type and 40 *OBF-1*$^{-/-}$ clones; the number of clones in the different families are indicated. The raw sequence data are available at the following address: http://www.fmi.ch/groups/matthias/

normal. This observation is further strengthened by the analysis of animals deficient in both OBF-1 and Btk. These double mutant mice lack B cells in the periphery and have almost undetectable levels of circulating Igs, thereby resembling phenotypically human XLA.

Materials and Methods

Mice strains

CBA/N and *OBF-1*$^{-/-}$ mice (in 129SV-C57BL6 background) have been described (9, 19). To obtain double mutant mice, we bred OBF-1 and CBA/N mice. The female offspring (heterozygous for both mutations) were mated with *OBF-1*$^{-/-}$ males. Only the male progeny, Btk^{mut} or Btk^{wt} and heterozygous or homozygous for the OBF-1 deletion, were considered in the analysis. As controls, we always used littermates, mostly heterozygotes, since in our previous analysis we never observed differences between *OBF-1*$^{+/-}$and wild-type mice. Mice were analyzed between 6 and 12 wk of age. Btk^{wt} and Btk^{mut} loci were identified by PCR using forward primer 5′-ACA AGT TCC AGA GAG AGG-3′ and reverse primer 5′-CGG AAT CTG TCT TTC TGG-3′ (90°C, 5 min; 40 times: 50°C, 1 min; 70°C, 1 min; 94°C, 40 s). Half of the reaction was digested with *Hha*I and analyzed on a 2% gel. The Btk^{mut} resulting PCR product (813 bp) lacks a *Hha*I restriction site and can be distinguished from the Btk^{wt} product (768 bp).

Analysis of VDJ gene usage

For the production of hybridomas, splenic cells from two unchallenged mice were pooled, stimulated with LPS (20 μg/ml) for 3 days, and fused to the cell line SP2/0 (23). IgM-producing clones were expanded, and RNA was prepared and reverse transcribed. VDJ cDNAs were amplified with a set of V genes upstream primers and 1 downstream primer specific for the constant part of the μ gene (24). The sequence of the PCR products was analyzed with the dnaplot program (http://www.genetik.uni-koeln.de/dnaplot/).

Flow cytometric analysis

Single-cell suspensions of lymphoid tissues were prepared, stained, and analyzed on a FACScalibur (Becton Dickinson, Mountain View, CA) as described elsewhere (9). Thirty thousand events were counted per dot plot by gating on living cells and typical forward-side scatter appearance of lymphocytes.

ELISA

Serum Ig levels were determined using an ELISA in which Ig subclass-specific Abs were coated onto 96-well plates. Serial dilutions of serum samples were adsorbed, washed, and revealed with Ig subclass-specific Abs coupled to alkaline phosphatase. The serum Ig levels were determined by comparison with known standards.

Results and Discussion

Unchanged Ig gene repertoire in OBF-1-deficient mice

In the absence of OBF-1, transcription of some variable (V) IgH gene families might be impaired because their promoters are specifically dependent on this coactivator for activity. If this were the case, OBF-1-deficient mice would use only a limited repertoire of V genes and this could explain, at least in part, the impaired immune response observed in vivo.

To examine this, we generated hybridomas with wild-type or OBF-1-deficient cells and examined their VDJ usage. As shown in Table I from the sequences of 50 wild-type and 40 *OBF-1*$^{-/-}$ clones, nearly all genes of the V, D, and J IgH families that were found in wild-type hybridomas were also identified in the OBF-1-deficient cells; the differences in usage observed are marginal and not statistically significant. N insertions were found equally in cells of each genotype. Based on this, a critical role for OBF-1 in the selective activation of specific V region genes could not be established, and these results indicate that the immunodeficiency associated with the OBF-1 deletion is not due to a skewed Ig gene repertoire.

A developmental block of OBF-1-deficient B cells

One of the Abs used to characterize OBF-1-deficient mice showed a drastically different labeling of cells from wild-type and *OBF-1*$^{-/-}$origin. Fig. 1 shows representative FACS profiles of bone marrow cells (Fig. 1*A*) and spleen cells (Fig. 1*B*) doubly labeled with an anti-B220 Ab and the mAb mAb493 (25), which recognizes a protein of 130- to 140-kDa found on B cells representing early stages of development. This Ab distinguishes pro-/pre-BI, pre-BII, and immature B cells, which are all mAb493$^+$, from mature B cells which are mAb493$^-$. Therefore, in the bone marrow of a normal mouse practically all B cells are mAb493$^+$ and only the long-lived recirculating IgM^{low}IgD^{high}CD23$^+$ mature B cells are mAb493$^-$ (25). As shown in Fig. 1*A*, in the bone marrow, pro-/pre-BI, pre-BII, and immature B cells were labeled by mAb493 equally in wild-type (i) or *OBF-1*$^{-/-}$ mice (ii). In agreement with our previous findings, we observed that the number of mature recirculating B cells was reduced in bone marrow cells from OBF-1-deficient animals (boxed in Fig. 1*A*).

In the spleen of a normal adult mouse, 10–20% of all B cells are mAb493$^+$ (Fig. 1*Bi*) and represent immature B cells that have just

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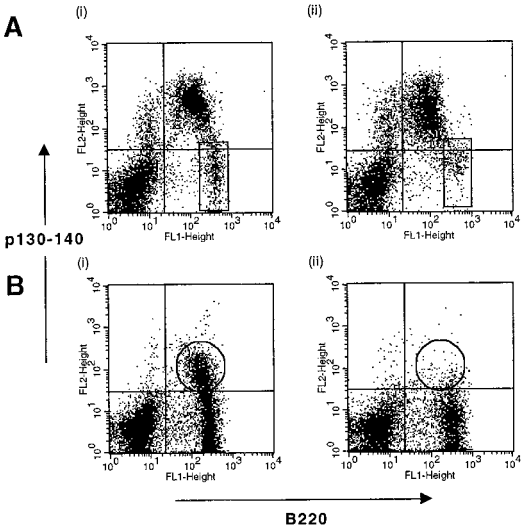

FIGURE 1. Lack of immature splenic B cells in OBF-1-deficient mice. Representative two-color flow cytometric analysis of B cells using the mAb493 Ab (seven *OBF-1*$^{-/-}$ and six control mice were analyzed). Bone marrow cells (*A*) and spleen cells (*B*) obtained from control (*OBF-1*$^{+/-}$, *i*) and OBF-1-deficient mice (*ii*) were analyzed using Abs directed against the pan B cell marker B220 (FITC-labeled) and p130–140 (mAb493, PE-labeled). *A*, Mature recirculating B cells (mAb493^{-}) are boxed. *B*, Immature B cells (mAb493^{+}) are surrounded by a circle.

entered the peripheral B cell pool; unlike their mature mAb493^{-} descendents, these cells have a short life span and are sensitive to anti-IgM-induced apoptosis (25). Strikingly, these mAb493^{+} B cells were almost completely absent from the spleen of OBF-1-deficient mice (circled in Fig. 1*Bii*). This finding indicates that, in the absence of OBF-1, immature B cells are severely impaired at the transition between the bone marrow and the spleen, and this may be the basis for the reduced peripheral B cell numbers present in these mice (9).

Several possibilities could be envisioned to explain this finding: it is conceivable, but seems unlikely, that expression of p130–140 is selectively down-regulated when OBF-1-deficient B cells reach the spleen, reflecting a requirement of the p130–140 gene for OBF-1 at this stage specifically. A small number of 493^{+} B cells can indeed be identified in the spleen of *OBF-1*$^{-/-}$ mice, and this argues against this possibility. Another, more likely possibility might be that splenic (but not bone marrow) *OBF-1*$^{-/-}$ immature B cells have a shorter life span than their wild-type counterparts, or else that their homing to the spleen is impaired. In this context it is noteworthy that expression of the BLR-1 gene, which encodes a chemokine receptor required for proper homing of mature B cells into splenic follicles (26), has recently been shown to depend on OBF-1 and Oct-2 (27).

OBF-1/Btk double mutant mice have an unaffected early B lymphoid development but lack B cells in peripheral lymphoid organs

Earlier studies have shown that Btk-deficient mice have a severely reduced mature B cell compartment although B cell development in the bone marrow is essentially normal (17, 20, 28). Recently, it has been demonstrated by 5-bromo-2′-deoxyuridine labeling that this reflects an impaired ability of the immature (i.e., mAb 493^{+}) splenic B cells to enter the mature B cell pool, which has a normal life span (22).

This defect in the absence of Btk therefore appears to be just downstream of the impairment identified above in *OBF-1*$^{-/-}$ mice. Because of this, we hypothesized that a combination of these two mutations would maintain a relatively normal B cell development in the bone marrow, but would have a dramatic effect in peripheral lymphoid organs because it would affect both the size of the immature B cell pool in the spleen (OBF-1 mutation) as well

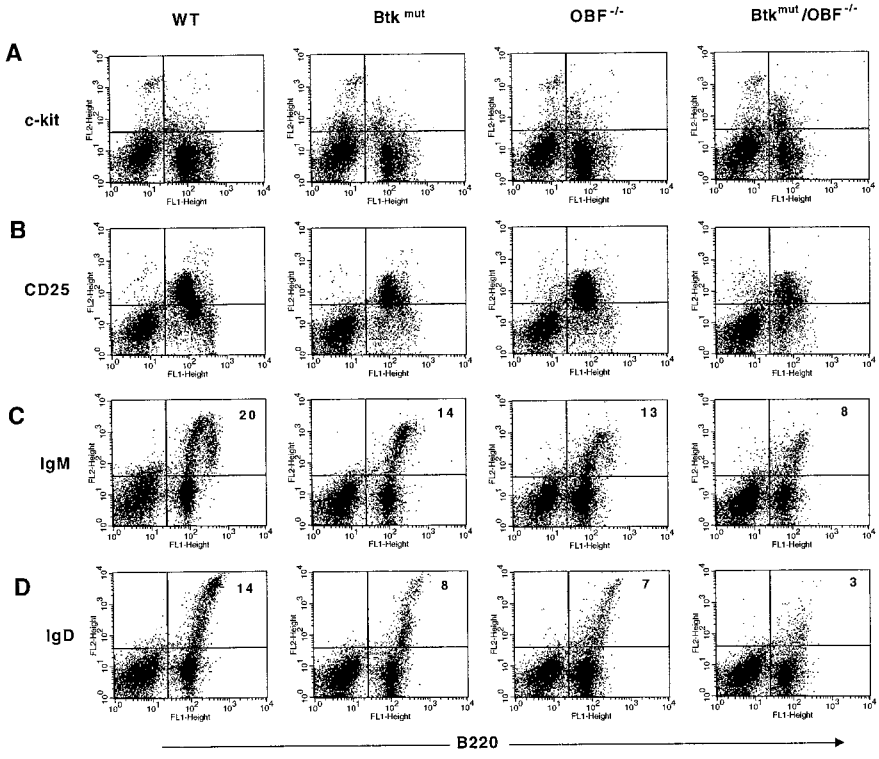

FIGURE 2. Normal early B cell development in OBF-1-, Btk-, or OBF-1/Btk- deficient mice. Representative two-color flow cytometric analysis of B lymphocyte development in the bone marrow of wild-type, Btk-deficient, OBF-1-deficient, and Btk/OBF-1 double mutant mice as indicated above the respective dot plots (four to six mice of each genotype). In all experiments, we used an anti-B220 Ab (FITC-labeled). Pro-B/pre-BI cells were identified with an anti-c-*kit* Ab (PE labeled; *A*) and pre-BII cells with an anti-IL-2Rα-chain Ab (TAC, CD25; *B*). Analysis of surface IgM (*C*) and surface IgD (*D*) expressing mature B cells. The percentage of double-positive cells is indicated in the *upper right corner* of the respective dot plot.

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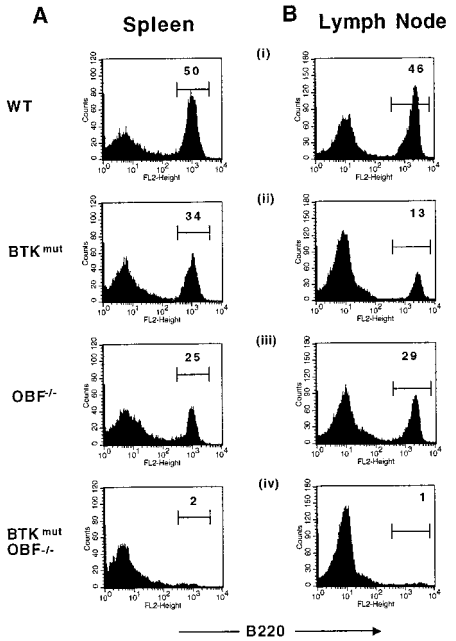

FIGURE 3. Lack of peripheral B cells in OBF-1/Btk double-deficient mice. Representative single-color flow cytometric analysis of cells isolated from spleen (*A*) and lymph node (*B*) from wild-type (*i*), Btk-deficient (*ii*), OBF-1-deficient (*iii*), and OBF-1/Btk double-deficient mice (iv; four to six mice of each genotype). An anti-B220 Ab (FITC labeled) was used to distinguish B cells from non-B cells. B220-positive B cells are boxed and their percentage is denoted. The genotype of the cells is indicated next to the respective histograms.

as the efficiency with which these B cells transit to the mature compartment (Btk mutation).

To test this hypothesis, we crossed the OBF-1 deficiency into the CBA/N (Btk^{mut}) background. Wild-type, OBF-1-deficient, Btk^{mut}, and OBF-1/Btk double mutant mice were obtained at the expected frequency and lymphoid organs were analyzed by FACS using B cell stage-specific mAbs. Early B lymphopoiesis is characterized by the expression of c-*kit* and CD25 (TAC/IL-2Rα-chain) (29). As shown in Fig. 2*A* and *B*, pro-/pre-BI cells (c-*kit*$^+$/CD25$^-$) and pre-BII cells (c-*kit*$^-$/CD25$^+$) were found at equal levels in animals of all genotypes. As previously reported, the numbers of IgM$^+$ B cells were significantly reduced in the Btk mutant and also in OBF-1-deficient animals (Fig. 2*C*) (9–11, 17, 18). In the double mutant mice, the number of these B cells was further decreased but they were nevertheless present. In part, this observation can be explained by the strong reduction in mature recirculating B220^{high}/IgM^{int}IgD^{high} B cells (Fig. 2*D*).

Next, the spleen and lymph nodes of mice of the different genotypes were analyzed. As previously reported and shown in Fig. 3, the B cell numbers in Btk^{mut} or OBF-1-deficient mice were reduced compared with wild-type mice. Strikingly however, the organs derived from the double mutant mice almost entirely lacked a B cell compartment, as shown by staining with an anti-B220 Ab, and were filled mainly with T cells (data not shown). From these observations, we conclude that, in the absence of OBF-1, development of the B cells found in peripheral lymphoid organs of OBF-1-deficient animals is strictly Btk dependent.

OBF-1/Btk double mutant mice have strongly reduced serum Ig levels and a phenotype mimicking human XLA

We next measured serum Ab levels by ELISA. The Ig levels measured for the single mutations were consistent with previously published observations. Strikingly, the double mutant mice had serum Ig levels which were below the detection limit of the assay (double dashed line in Fig. 4). Only in one of the six double mutant mice tested did we find detectable levels of IgM, IgG2a, and IgA, which nevertheless were extremely low. Taken together, Btk/OBF-1 double mutant mice show a severe immunodeficiency that is manifested by a block in B cell differentiation, an absent B cell compartment in the periphery, and extremely low serum Ig levels. This phenotype is strikingly similar to XLA in humans (30).

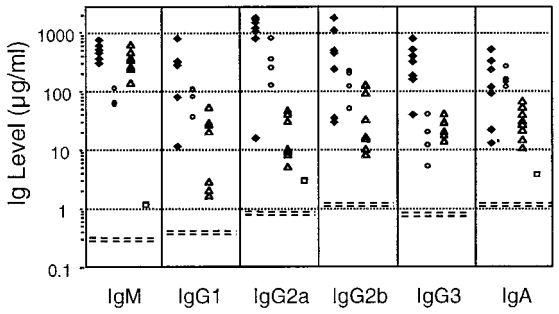

FIGURE 4. Serum Ig levels in wild-type, Btk-deficient, OBF-1-deficient, and Btk/OBF-1 double-deficient mice measured by ELISA. Each symbol represents an individual animal. We analyzed seven wild-type (♦), four Btk-deficient (○), eight OBF-1-deficient (△), and six double-deficient (□) animals. The Ig subclass is denoted below the corresponding part of the graph. The double dashed line represents the detection limit of the assay.

Analogous observations have been made while analyzing CD40/Btk double mutant animals (20, 31). In this case, combining the CD40 mutation to the Btk mutation also led to an increase in the severity of Xid, although less pronounced than in the case of the mutant mice described here. This finding of an increased severity of the Xid phenotype in CD40/Btk double mutant mice has been explained by a model whereby two independent pathways control mature B cell development (20). Similarly, OBF-1 and Btk also appear to be in two different pathways, both of which are critical for the mature B cell pool.

A better understanding of the molecular mechanisms underlying these observations will require the identification of OBF-1 target genes as well as of the signal transduction cascades impinging on this protein.

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