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B Cell Progenitors Are Arrested in Maturation but Have Intact VDJ Recombination in the Absence of Ig-α and Ig-β[1]

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Ig-α and Ig-β mediate surface expression and signaling of diverse B cell receptor complexes on precursor, immature, and mature B cells. Their expression begins before that of the Ig chains in early progenitor B cells. In this study, we describe the generation of Ig-α-deficient mice and their comparative analysis to mice deficient for Ig-β, the membrane-IgM, and recombination-activating gene 2 to determine the requirement of Ig-α and Ig-β in survival and differentiation of pro-B cells. We find that in the absence of Ig-α, B cell development does not progress beyond the progenitor stage, similar to what is observed in humans lacking this molecule. However, neither in Ig-α- nor in Ig-β-deficient mice are pro-B cells impaired in V(D)J recombination, in the expression of intracellular Ig μ-chains, or in surviving in the bone marrow microenvironment. Finally, Ig-α and Ig-β are not redundant in their putative function, as pro-B cells from Ig-α and Ig-β double-deficient mice are similar to those from single-deficient animals in every aspect analyzed. ***The Journal of Immunology,*** **2002, 169: 865–872.**

Immunoglobulin-α (CD79a) and Ig-β (CD79b) are transmembrane glycoproteins belonging to the Ig superfamily and encoded by the *mb-1* and *B29* genes, respectively. Both proteins are restricted to B lymphocytes, although *B29* transcripts have also been detected in early thymocytes (1). Both gene transcripts and their proteins have been detected at every B cell developmental stage except that of the plasma cell, which express only Ig-β (2–5). Ig-α and Ig-β form a disulfide-linked heterodimer that associates with membrane-bound Ig (mIg)[3] molecules of every Ig class to form the B cell Ag receptor (BCR) complex. The variable region of the H and L (IgH and IgL) chains of the mIg molecules constitute the Ag-binding portion of the BCR, whereas the Ig-α/Ig-β heterodimer is its signaling component (6–8).

The BCR is first expressed on immature B cells where it signals for IgH and IgL allelic and isotypic exclusion. Interaction of the BCR with an Ag at this stage of B cell development results in negative selection and consequent elimination of the receptor and/or the cell. On the surface of mature B cells, the BCR seem to give a ligand-independent signal that is required for the survival of B cells in the periphery (9). Stimulation of the BCR by Ag induces phosphorylation of Ig-α/Ig-β cytoplasmic tyrosine residues, thus increasing their affinity for intracellular signaling proteins. This initiates a signaling cascade that can result in proliferation, differentiation, or death of the mature B cells (10–13).

Progression through early stages of B cell development is also determined by Ig-α/Ig-β-mediated signaling. Before expression of conventional IgL (κ or λ) chains, the Ig-α/Ig-β heterodimer is part of the pre-BCR complex, which also includes the membrane IgM (mμ) H chain and the surrogate L chain components λ5 and VpreB (14). Natural and experimental mutations in the *mb-1* and *B29* genes of mice and/or humans have demonstrated the importance of Ig-α and Ig-β during B cell development. In the absence of either molecule, no pre-BCR can be expressed on the cell surface, and B cell development is blocked at the progenitor- (pro) B cell stage (15, 16). Mice bearing mutations in or deletions of the cytoplasmic portion of both Ig-α and Ig-β also show complete block at the pro-B cell stage of B cell development (17, 18). However, the same mutations in either Ig-α or Ig-β cause a partial block at the pre-B cell stage and a more severe arrest at the immature B cell stage, demonstrating that Ig-α and Ig-β have redundant signaling functions, at least during pre-BCR signaling (17–19).

Although the role of Ig-α/Ig-β in pre-B and B cell development has been progressively defined, it is still unclear whether these molecules are also required at the pro-B cell developmental stage. Pro-B cells are the most immature cells of the B lineage so far identified. They are characterized by the expression of the pan-B cell marker B220 and the capacity to differentiate into cells of later B cell developmental stages in vitro and in vivo. Cells defined as pro-B have been shown to be heterogeneous in the expression of surface markers like CD19, heat-stable Ag (HSA), BP-1, CD43, and the rearrangement status of the IgH locus (20, 21). Based on these differences, the pro-B cell population has been divided into fractions (A, B, and C) that are developmentally related (20).

To survive and proliferate, pro-B cells must be able to interact with intramarrow stromal cells and to react to soluble and membrane factors that these cells produce (22, 23). Moreover, to differentiate into pre-B cells, pro-B cells must initiate and successfully complete the V(D)J recombination program at the IgH locus (24–26), and to express an IgH chain capable of pairing with the surrogate L chains in a functional pre-BCR (27, 28). Signals driving the gradual commitment of pluripotent hematopietic stem cells to the B cell lineage, the final generation of pro-B cells, and the initiation of the Ig V(D)J gene recombination are not well defined.

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[3] Abbreviations used in this paper: mIg, membrane-bound Ig; BCR, B cell Ag receptor; mμ, membrane IgM; HSA, heat-stable Ag; pro, progenitor.

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Based on the observation that the surrogate L chains also assemble on the surface of IgH chain-negative pro-B cells, it was proposed that membrane protein complexes analogous to pre-BCR and BCR may regulate some of the survival and differentiation processes in early pro-B cells (29, 30). However, the signaling capacity and function of these latter surrogate L chains containing protein complexes is still unclear. More recently, Ig-α and Ig-β have also been found on the surface of IgH chain-negative pro-B cells in protein complexes that do not contain surrogate L chains, but four other proteins, one of which has been identified as calnexin (30, 31). The Ig-α/Ig-β-containing protein complexes of early pro-B cells appear to have some signaling capacity when engaged with anti-Ig-β Abs, resulting in tyrosine phosphorylation of several substrates, including extracellular signal-regulated kinase (30). The function of these signaling complexes in pro-B cells has not been established so far.

To analyze whether Ig-α and Ig-β are required for pro-B cell development, we generated mice lacking the expression of the Ig-α molecule and compared pro-B cells of these mice to those of Ig-β-, mμ- (μMT) and recombination-activating gene (RAG)2-deficient mice (15, 32, 33). We find that the IgH chain gene recombination is not affected in pro-B cells lacking Ig-α, Ig-β, or both molecules. Furthermore, the size and phenotype of the pro-B cell population in these mice is also not affected in vivo.

Materials and Methods

Generation of Ig-$\alpha^{-/-}$ mice

Two genomic library clones, kindly provided by Dr. N. Sakaguchi (Kumomoto, Japan), and containing the complete mouse *mb-1* locus from the BALB/c strain (34), were used for the generation of the targeting vector. The targeting construct consisted of two *mb-1* homologous DNA regions framing a heterologous fragment. These *mb-1* regions were a 3.8-kb DNA fragment 5′ of exon II, and a 9.6-kb *Eco*RI fragment starting within intron III. A short heterologous sequence (80 bp) containing a *loxP* site was cloned into the *Nhe*I restriction site of intron I, within the 3.8-kb *mb-1* fragment. A 4.8-kb heterologous DNA fragment containing, in the following order, *EGFP*, *mb-1* exons V-II cDNA cassette, and *loxP*-flanked *neo*^{r} was cloned between the two homologous *mb-1* regions. The targeting vector was initially constructed to generate at the same time a knockout and a Cre recombinase-dependent conditional allele of *mb-1* capable of expressing either EGFP or Ig-α depending on the orientation of the *loxP*-flanked DNA fragment. However, the *EGFP* and *mb-1* DNA cassettes were found not to be expressed in either cell lines or in mice, presumably due to the lack of a functional 3′ polyA site (35). Manipulation of BALB/c-derived embryonic stem cells (36) was performed as described (37). The Ig-$\alpha^{-/-}$ mutant mice were bred and maintained in a barrier mouse facility at the Max Planck Institute for Immunobiology (Freiburg, Germany).

Mice

RAG2$^{-/-}$ (C57BL/6) (33), Ig-$\beta^{-/-}$ (C57BL/6) (15), and μMT (C57BL/6) (32) mice were maintained in the specific pathogen-free facility of the Max Planck Institute. All animal studies were approved by the German Animal Rights Office.

Southern and Northern analyses

A 271-bp *Kpn*I genomic fragment spanning the end of intron I and two-thirds of exon II was used as a probe to discriminate between wild type (12 kb) and targeted allele (7.6 kb) when hybridized to *Sph*I-digested genomic DNA. For Northern blot analysis, total RNA was purified by TRIzol (Life Technologies, Rockville, MD) from CD19$^+$-sorted bone marrow cells. The 0.4-kb *Pvu*II cDNA fragment spanning exon II to exon V of *mb-1* and an actin 0.7-kb genomic fragment were used as probes.

Flow cytometry

Proteins expressed on the surface or intracellularly of isolated bone marrow and spleen cells were stained as previously described (38). The Abs for B220, CD19, CD2, CD22, BP-1, CD43, CD25, CD4, CD8, IL7Rα, and IL-7Rγ were purchased from BD PharMingen (San Diego, CA). FLUOS-conjugated (Boehringer Mannheim, Bergish Gladbach, Germany) anti-IgM Abs (39) were a kind gift of Dr. R. Torres and K. Hafen (Basel Institute for Immunology, Basel, Switzerland). FITC-conjugated polyclonal goat anti-mouse IgM (Southern Biotechnology Associates, Birmingham, AL) and monoclonal anti-Igκ (39) and anti-IgM (M41; Ref. 40) were used for the intracellular staining. The streptavidin-RED670 (Life Technologies) and streptavidin-Tri-color (Caltag Laboratories, Burlingame, CA) reagents were used for the detection of biotinylated Abs. Stained cells were analyzed on either FACS or FACSCalibur (BD Biosciences, Mountain View, CA) flow cytometers.

Semiquantitative PCR

Bone marrow cells were isolated from two mice for each strain. The cells were labeled with anti-CD19-beads (Miltenyi Biotec, Bergish Gladbach, Germany) and the CD19$^+$ cells were purified from the total bone marrow population by MACS (Miltenyi Biotec). Purity was between 75 and 93% in the different samples. In the case of wild-type mice, CD43$^+$CD19$^+$ cells were sorted to 89% purity using a MoFlo high speed sorter (Cytomation, Fort Collins, CO). Genomic DNA from the purified cell populations was quantified by GeneQuantII (Pharmacia Biotech, Uppsala, Sweden) or by Biophotometer (Brinkmann Instruments, Westbury, NJ) and then equilibrated between samples. Five-fold serial dilutions were prepared from each DNA sample. Primers for the PCR analysis of V_HDJ_H joints containing V_HJ558 and V_H7183 elements were as described (21, 41). The 738-bp actin fragment was amplified by a one-round PCR of 25 cycles using the GGTGTCATGGTAGGTATGGGT and CGCACAATCTCACGTTCAG oligonucleotides. V_HDJ_H joints belonging to the V_HJ558 family were amplified in a one round of 25–30 cycles. The V_H7183 containing V_HDJ_H rearrangements were amplified by nested PCR in two rounds of 25 and 23 cycles, respectively. The PCR products were hybridized to probes amplified by similar PCR and labeled with ^{32}P-dCTP by Megaprime DNA Labeling System (Amersham, Arlington Heights, IL). The amount of radioactivity of each band was quantified by Bio-Imaging Analyzer (FUJIX, Tokyo, Japan).

Results

Generation of Ig-α-deficient mice

The 34-kDa Ig-α protein is encoded by the *mb-1* gene on chromosome 7 in the mouse. The *mb-1* locus (34) is composed of five coding exons distributed over 5 kb of DNA sequence (Fig. 1*a*). Exons I, II, and III encode the leader peptide, extracellular, and transmembrane domains, respectively. Exons IV and V encode the intracellular domain (cytoplasmic tail), which contains the immunoreceptor tyrosine-based activation motif (42).

Mice harboring a null *mb-1* allele were generated by genetic manipulation of BALB/c-derived embryonic stem cells following standard procedures (37). Specifically, the targeted *mb-1* allele (Fig. 1*a*) carries the endogenous promoter, but is devoid of exons II and III. Transcription and splicing of the remaining exons would result in a frame shift that introduces a stop codon 15 bp after exon I and that directs the synthesis of just the Ig-α leader peptide. Therefore, it was predicted that the targeted *mb-1* allele would be unable to produce a functional Ig-α protein.

Germline transmission of the *mb-1* mutation in mice was detected by PCR (data not shown) and confirmed by Southern blot analysis of tail genomic DNA (Fig. 1*b*). The *mb-1*-targeted mice were generated and maintained on a BALB/c genetic background and intercrossed to generate homozygous mutants (Ig-$\alpha^{-/-}$). Northern analysis demonstrates that CD19$^+$ B cells from Ig-$\alpha^{-/-}$ mice do not express *mb-1* transcripts (Fig. 1*c*), possibly due to either RNA instability caused by the *neo*^{r} cassette or to nonsense-mediated RNA decay resulting from a premature stop codon (43). Therefore, we conclude that our targeting strategy was successful in preventing the expression of any Ig-α protein.

Ig-α expression is absolutely required for B cell maturation

The generation of B cell was examined in Ig-$\alpha^{-/-}$ mice in comparison to that of wild-type mice. To distinguish the different B

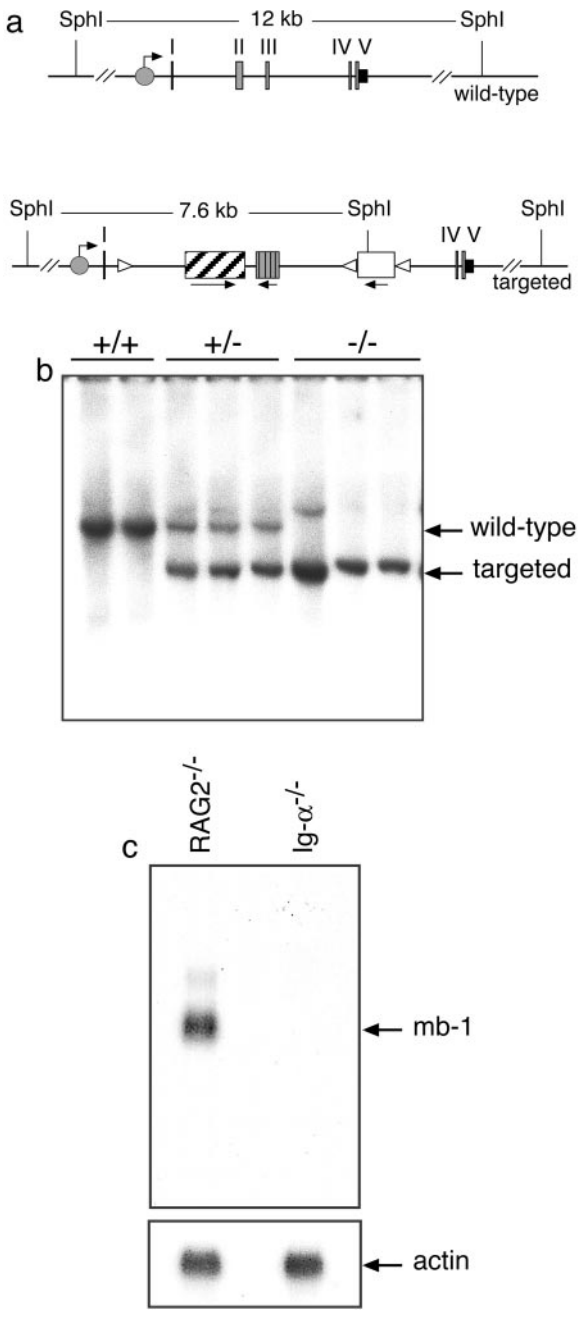

FIGURE 1. Strategy for the generation of Ig-α-deficient mice. *a*, Representation (not to scale) of wild-type and targeted *mb-1* alleles. The targeting vector was constructed to generate both a knockout and a Cre recombinase-dependent conditional allele of *mb-1* capable to express either EGFP or Ig-α depending on the orientation of the *loxP*-flanked DNA fragment. However, the *EGFP* and *mb-1* DNA cassettes were not expressed and the targeted *mb-1* allele carries the endogenous promoter, but is devoid of the endogenous *mb-1* exons II and III. The gray circles represent the *mb-1* promoter. The gray boxes represent the *mb-1* exons and the black box adjoined to exon V indicates the 3′ untranslated region that terminates with the *mb-1* polyA sequence. The triangles represent *loxP* sites, the rectangle with diagonal stripes corresponds to the *EGFP* gene cassette, and the rectangle following the *EGFP* represents *mb-1* exons V-II coding regions (these two gene cassette were not expressed, data not shown). An empty box flanked by two *loxP* sites represents the *neo*^{r} DNA cassette. Arrows indicate direction of transcription. The *Sph*I fragments relative to the wild type and the targeted alleles are indicated. *b*, Southern blot analysis of tail genomic DNA of littermate mice derived from the breeding of heterozygous mutant animals. The DNA was digested by *Sph*I and hybridized by a probe corresponding to the *Kpn*I 271 bp genomic DNA fragment spanning the end of intron I and two-thirds of exon II. The expected wild-type (12-kb) and targeted (7.6-kb) fragments are indicated by arrows. Mice carrying wild-type, heterozygous, and homozygous mutant alleles are represented by +/+, +/−, and −/−, respectively. The band running above the wild type in the sixth lane resulted from incomplete DNA digestion. *c*, Northern blot analysis of *mb-1* transcripts. Total RNA from RAG2$^{-/-}$ and Ig-$\alpha^{-/-}$ bone marrow CD19$^+$ cells was hybridized with a cDNA probe spanning exons II to V of *mb-1*. The same blot was stripped and rehybridized with an actin-specific probe for a loading control.

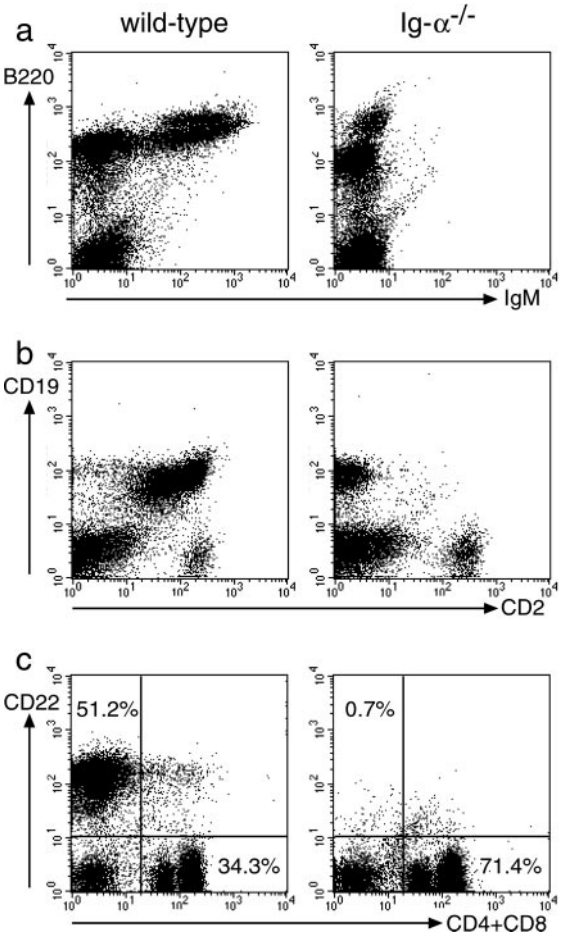

FIGURE 2. Ig-α-deficient B cells do not express surface Ig molecules and are blocked in development. Bone marrow and spleen cells of wild-type and Ig-$\alpha^{-/-}$ mice were analyzed by flow cytometry for the expression of surface markers. Cells falling in the lymphocyte gate are shown. *a*, Analysis of B220 and IgM expression on cells in the bone marrow. *b*, Analysis of CD19 and CD2 expression on cells in the bone marrow. *c*, Analysis of CD22, CD4, and CD8 expression on lymphocytes in the spleen. Numbers refer to the frequency of cells expressing the B cell marker CD22 or the T cell marker CD4 and CD8.

cell developmental stages, bone marrow cells were analyzed by flow cytometry for the expression of B220, CD19, IgM, and CD2. IgM-expressing immature and mature B cells were absent in the bone marrow of Ig-$\alpha^{-/-}$ mice (Fig. 2*a*). However, expression of the pan-B cell markers B220 and CD19 was observed, on average, in 14 and 8.5% of the total cells, respectively (Table I). While B220 is also expressed by cell progenitors of other lineages, CD19 expression is restricted to B cells and therefore indicates the presence of cells committed to the B cell lineage (44, 45). The CD19$^+$ cells of Ig-$\alpha^{-/-}$ bone marrows are phenotypically progenitor B cells as they express the CD43 Ag while they lack the expression of CD2 and CD25 (Fig. 2*b*, Table I, and data not shown) (20,

Table I. *Frequency of bone marrow B cells in wild-type and mutant mice*

	Total Cells $\times 10^7$	$B220^+ \times 10^6$ (% of total)	$CD19^+ \times 10^6$ (% of total)	$c\mu^+$ % of $B220^+$ Cells	$c\kappa^+$ % of $B220^+$ Cells
Wild-type BALB/c	2.4 ± 0.8 $n = 8$	6.6 ± 2.3 (35.8 ± 6.1) $n = 7$	5.9 ± 1.8 (32.0 ± 4.0) $n = 5$	61.5 ± 17.5 $n = 5$	42.6 ± 7.4 $n = 4$
Ig-$\alpha^{-/-}$ BALB/c	1.9 ± 0.5 $n = 10$	2.8 ± 1.8 (14.1 ± 5.9) $n = 8$	1.7 ± 1.1 (8.5 ± 4.2) $n = 6$	11.9 ± 1.5 $n = 5$	3.37 ± 1.07 $n = 3$
Ig-$\beta^{-/-}$ C57BL/6	2.5 ± 0.4 $n = 10$	4.0 ± 1.1 (16.4 ± 4.3) $n = 6$	1.9 ± 0.4 (8.1 ± 1.3) $n = 6$	9.3 ± 4.1 $n = 4$	1.85 ± 0.74 $n = 3$
μMT C57BL/6	2.7 ± 0.7 $n = 4$	3.1 ± 0.5 (13.0 ± 2.6) $n = 3$	1.7 ± 0.8 (7.5 ± 3.0) $n = 4$	8.2 ± 5.2 $n = 3$	2.9 $n = 2$
RAG2$^{-/-}$ C57BL/6	2.4 ± 0.7 $n = 7$	4.7 ± 2.0 (20.7 ± 6.2) $n = 4$	2.9 ± 0.8 (12.1 ± 3.4) $n = 6$	2.24 ± 1.27 $n = 3$	1.4 $n = 2$
Ig-$\alpha^{-/-}$; Ig-$\beta^{-/-}$ BALB/c × C57BL/6	2.4 ± 0.5 $n = 8$	2.1 ± 0.4 (9.9 ± 1.4) $n = 6$	1.0 ± 0.3 (4.5 ± 1.4) $n = 6$	11.8 ± 2.0 $n = 8$	ND

46–48). In addition, >80% of these cells express HSA and ~30% express BP-1 (data not shown); and therefore, belong to fractions B ($HSA^+/BP\text{-}1^-$) and C ($HSA^+/BP\text{-}1^+$) using Hardy's nomenclature (20). A population of $B220^{high}$ cells is observed in the marrow of Ig-$\alpha^{-/-}$ mice (Fig. 2*a*). This population, which is also found in other pro-B cell-blocked mutant animals such as RAG2$^{-/-}$, μMT, and Ig-$\beta^{-/-}$ (data not shown), does not express CD19 or intracellular Ig μ-chains (data not shown), and is likely composed of non-B lineage cells.

Analysis of the spleen confirmed that Ig-$\alpha^{-/-}$ pro-B cells are unable to develop into mature B cells. Mature splenic B cells, which express the CD22 and CD19 Ags, were not observed (Fig. 2*c* and data not shown). Nevertheless, ~10% of Ig-$\alpha^{-/-}$ cells in the spleen were found to express low levels of B220 (data not shown). These latter cells are probably pro-B and/or non-B cells as they are also found in the spleen of RAG-deficient mice (33). The frequency of $CD4^+$ and $CD8^+$ T cells in the spleen of Ig-$\alpha^{-/-}$ mice was increased relative to wild-type controls (Fig. 2*c*). However, given the fact that the total number of cells in the spleen of Ig-$\alpha^{-/-}$ mice is only one-third of control animals (data not shown), the absolute number of T cells is actually reduced by >50% in the absence of Ig-α (absolute number of T cells in spleen is $11.1 \pm 6.1 \times 10^6$; $n = 4$ for Ig-$\alpha^{-/-}$ and $26.5 \pm 5.0 \times 10^6$; $n = 3$ for wild type). This result probably relates to the defect in T cell expansion and response described in μMT animals (49).

Thus, the results of the flow cytometric analyses fully support those from the Northern analysis and demonstrate that the targeted *mb-1* allele is not capable to direct the expression of a functional Ig-α protein. Commitment of hematopoietic stem cells to the B cell lineage does occur in the absence of Ig-α, but this molecule is absolutely required for further development to the pre-B, immature, and mature B cell stages. A similar phenotype has been observed in humans bearing a mutation that prevents Ig-α expression (16). Thus, Ig-α is similarly required for both human and mouse B cell development.

Ig gene rearrangement and expression is not altered in the absence of either Ig-α or Ig-β

V_HDJ_H-rearranged IgH chain genes are first found in pro-B cells of fractions B (50). However, the signal, if any, that induces the initiation of the V_HDJ_H recombination process in these cells has not yet been identified. It has been speculated that Ig-α/Ig-β-containing protein complexes on pro-B cells might signal for V_HDJ_H recombination and/or for the survival of cells that have undergone this process (51, 52).

To analyze whether Ig-α and Ig-β affect development and/or survival of pro-B cells that carry V_HDJ_H rearrangements, we analyzed the frequency of rearranged IgH genes in cells derived from the bone marrow of Ig-α- and Ig-β-deficient mice, in comparison to those derived from other mutant strains. RAG2$^{-/-}$ mice were used as a negative control, because they cannot undergo V(D)J recombination (33). The μMT mice, which carry a disruption of one of the membrane exons of the μ-chain gene, were used as a positive control. The B cell progenitors of these mice rearrange normally the IgH locus, but cannot express a μ-chain on the cell surface and are consequently blocked in development due to the inability to express a pre-BCR (32, 53). Nevertheless, the frequency of V_HDJ_H rearrangements found in μMT pro-B cells is equivalent, if not higher, of that found in wild-type pro-B cells (50). The $CD19^+CD43^+$ bone marrow fraction from wild-type mice, which comprises the pro-B cell population, was also used as a positive control in one analysis. However, ~30% of this population is composed of large, cycling pre-B cells that have developed based upon productive IgH chain gene rearrangements; and therefore, is expected to contain a higher frequency of V_HDJ_H products (50).

The frequency of V_HDJ_H joints using either V_H elements of the J_H-distal J558 or the J_H-proximal 7183 V_H families was assessed by semiquantitative PCR on genomic DNA isolated from purified $CD19^+(CD43^+)$ bone marrow cells (Fig. 3). In this analysis, four products of different size are dominantly amplified and these represent V_HDJ_H joints using one of four different J_H gene segments (21, 41). To normalize for the amount of template DNA in each sample, we used primers designed to specifically amplify a portion of the actin gene (Fig. 3, *b* and *c*). By comparing the degree of amplification of the V(D)J fragments between samples normalized for actin levels, we found that the frequency of V_HDJ_H joints in Ig-α- and Ig-β-deficient pro-B cells was similar to that found in μMT and wild-type pro-B cells. Thus, these data demonstrate that neither Ig-α nor Ig-β is required for V(D)J recombination at the IgH locus.

The expression of productively rearranged IgH alleles was examined by flow cytometric analysis. Bone marrow cells were stained intracellularly with anti-mouse μ H chain Abs to detect the

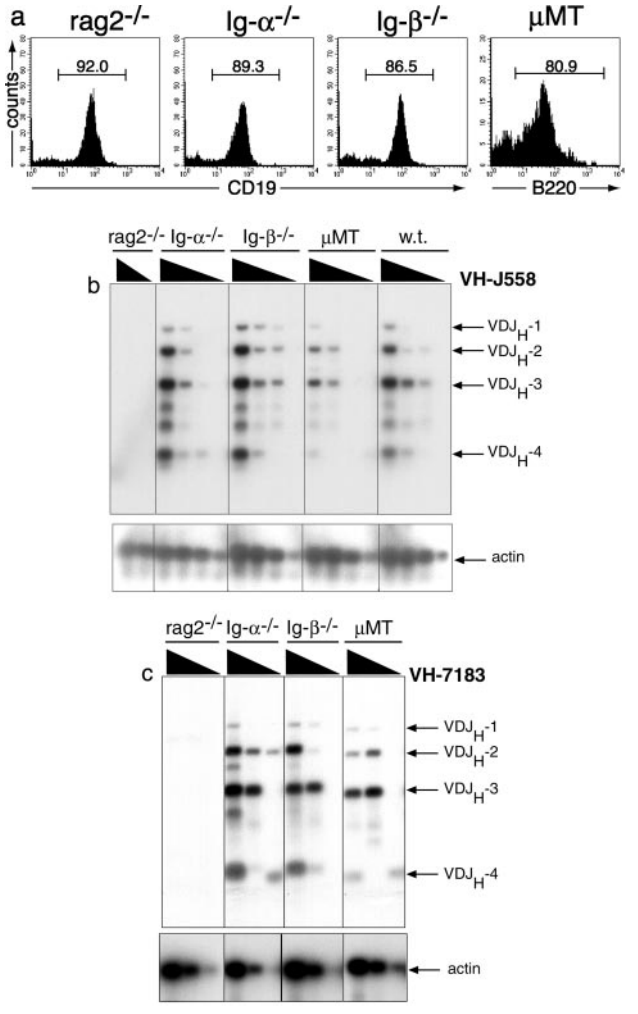

FIGURE 3. Ig V(D)J recombination is not impaired in the absence of either Ig-α or Ig-β. V_HDJ_H rearrangements in pro-B cells of the indicated mutant and wild-type mice were analyzed by semiquantitative PCR. Genomic DNA was extracted from either $CD19^+$ mutant or $CD19^+CD43^+$ wild-type bone marrow pro-B cells. *a*, Example of $CD19^+$ cell purification for the indicated mice. Cells were purified from bone marrow by magnetic sorting (as described in *Materials and Methods*) and restained for either CD19 or B220 (B220 is expressed on all $CD19^+$ cells) to assess purity. Numbers over histograms indicate percentage of positive cells in the total population. Wild-type $CD19^+CD43^+$ pro-B cells were purified by the MoFlo cell sorter to 89% purity (data not shown). Five-fold serial dilutions of genomic DNA from $CD19^+(CD43^+)$ bone marrow cells was subjected to PCR amplification specific for V(D)J rearrangements of the J558 (*b*) and 7183 (*c*) V_H families. For relative quantification, amplification of a genomic fragment of the *actin* gene was performed in parallel with the same DNA. Arrows indicate the expected bands for V_HDJ_H rearrangements and the *actin* gene.

production of μ H chains. This analysis is also likely to detect Dμ-chains that are the products of DJ_H rearrangements in the D reading frame 2 (54, 55). We found that an average of 15.2% (11.7% if we subtract the background staining obtained in RAG2-deficient cells) of the $B220^+$ cells in Ig-α-deficient mice express μ or Dμ-chains intracellularly (Fig. 4 and Table I). In line with the PCR results, the flow cytometric analysis shows that the frequencies of μ-expressing pro-B cells in Ig-α- and Ig-β-deficient mice are similar to those found in μMT and wild-type mice (Fig. 4 and Table I). In this analysis, we also observed that many of the mutant pro-B cells express lower levels of μ compared with wild type (notice the shoulder in the histogram of mutants vs the discrete population in that of the wild type in Fig. 4), probably reflecting the lower stability of μ-chains in the absence of one of the interacting protein partners (Ig-α, Ig-β, or both for soluble μ) in the mutant animals. In contrast to pro-B cells, the frequency of μ^+ cells in the wild-type pre-B cell population is 5-fold higher than that of the pro-B cell populations (Fig. 4). This difference is due to the fact that only μ-expressing cells can differentiate into pre-B cells and only when the μ-chains form a functional pre-BCR with Ig-α and Ig-β. A small number of the pro-B cells (1–3%) from the different mutant mice (except RAG2) express κ L chains (Table I).

In the bone marrow, early B cell progenitors can be divided into two fractions based on surface expression of the B220 and CD19 markers (44, 45, 56). It was shown that the development of pro-B cells progresses from the $B220^+CD19^-$ to the $B220^+CD19^+$ stage (56). The absolute number of cells belonging to these two populations was determined in the mutant mice to establish whether Ig-α and Ig-β might be necessary for this developmental step. Bone marrow $B220^+CD19^-$ and $B220^+CD19^+$ cell populations of Ig-α- and Ig-β-deficient mice were found in similar numbers to those of μMT and RAG-deficient mice (Table I and data not shown). In addition, similar frequencies of cells appeared to be in cycle, as judged by flow cytometric analysis of DNA stained by propidium iodide (data not shown). Finally, similar frequencies were also found to express IL-7R (α and γ) on the cell surface (data not shown), a molecule necessary for pro-B cell survival and proliferation in the bone marrow stroma microenvironment.

Thus, Ig-α and Ig-β have no apparent influence on either the onset of V_H to DJ_H recombination, or on the survival and proliferation of cells that carry V_HDJ_H rearrangements. Nevertheless, these molecules are essential for the development and, most likely, the expansion of pre-B cells that carry productively rearranged IgH genes.

Ig-α and Ig-β have no redundant function in pro-B cell differentiation

Studies in cell lines have shown that Ig-β molecules can also be expressed on the cell surface in the absence of Ig-α, suggesting that Ig-α and Ig-β may function independently of each other in

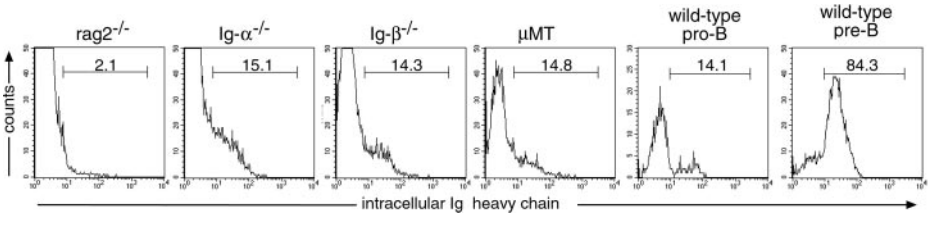

FIGURE 4. Intracellular expression of μ H chains is not altered in the absence of either Ig-α or Ig-β molecules. Bone marrow cells from $RAG2^{-/-}$, Ig-$\alpha^{-/-}$, Ig-$\beta^{-/-}$, and μMT mutant mice were stained for surface B220 (data not shown) and intracellular and surface μ H chains, and analyzed by flow cytometry. The histograms represent cells gated for B220 expression. Numbers in the histograms refer to the frequency of μ^+ cells in the $B220^+$ population. Bone marrow cells from wild-type mice were stained for surface B220, IgM, and CD25 and for intracellular μ H chain. The intracellular staining of μ H chains in the $B220^+IgM^-CD25^-$ (pro-B) and $B220^+IgM^-CD25^+$ (pre-B) cell populations is shown. The numbers refer to the frequency of μ^+ cells in these two populations.

pro-B cells (31). To exclude the possibility that Ig-α and Ig-β have a redundant signaling function in the initiation and/or completion of the V_HDJ_H recombination process, Ig-α- and Ig-β-double-deficient mice ($Ig\alpha^{-/-};Ig\beta^{-/-}$) were generated. In these latter animals, we analyzed the frequency of V_HJ558L to DJ_H rearrangements and intracellular Igμ expression in cells belonging to the pro-B cell population.

We found that pro-B cells from $Ig\alpha^{-/-};Ig\beta^{-/-}$ mice have a similar frequency of V_HDJ_H rearrangements to those isolated from Ig-α-only-deficient mice (Fig. 5*a*). Moreover, the frequency of pro-B cells that carry productive V_HDJ_H rearrangements and express μ-chains in the cytoplasm is also similar between mice that lack either Ig-α or Ig-β or both of these molecules (Fig. 5*b* and Table I). Bone marrow cells of $Ig\alpha^{-/-};Ig\beta^{-/-}$ mice were stained for B220 and CD19 to determine the frequency and absolute number of pro-B cells. We found that in the absence of both Ig-α and Ig-β molecules, the absolute number of $B220^+CD19^-$ and $B220^+CD19^+$ bone marrow cells is not significantly different from that observed in single-deficient animals (data not shown and Table I). However, these results would need to be confirmed with mutant animals on the same genetic backgrounds.

In summary, we can conclude that the V_HDJ_H recombination process at the IgH locus and the survival of pro-B cells that carry these rearrangements do not depend on the expression of Ig-α and Ig-β molecules.

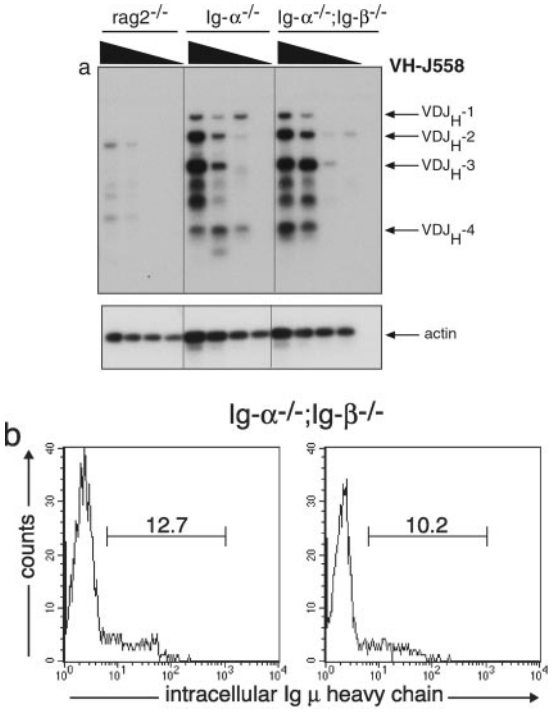

FIGURE 5. V(D)J recombination and intracellular μ-chain expression are not impaired in pro-B cells double-deficient for both Ig-α and Ig-β. *a*, V_HDJ_H rearrangements in sorted pro-B cells of Ig-α and Ig-β double-deficient mice ($Ig\alpha^{-/-};Ig\beta^{-/-}$) were compared with those from $Ig\alpha^{-/-}$ and $RAG2^{-/-}$ animals by semiquantitative PCR. Five-fold serial dilutions of genomic DNA from $CD19^+$ bone marrow cells was subjected to PCR amplification specific for V(D)J rearrangements of the J558 V_H family. Amplification of an actin-specific genomic fragment was performed in parallel for relative quantification. Arrows indicate bands of the sizes expected for the different V_HDJ_H rearrangements and for the actin fragment. *b*, Cells isolated from the bone marrow of two different $Ig\alpha^{-/-};Ig\beta^{-/-}$ double-mutant mice. Flow cytometry analysis of intracellular μ H chain expression in $B220^+$ gated cells. Numbers in the histograms refer to the frequency of μ^+ cells in the $B220^+$ population.

Discussion

Extensive studies in cell lines have demonstrated that the Ig-α/Ig-β heterodimer plays a role in the transport of the mIg molecules onto the cell surface and mediates pre-BCR and BCR signaling (13). Several in vivo studies have shown that expression and signaling of the pre-BCR and BCR are prerequisite to complete the development of B lymphocytes (15, 16, 18, 19, 32, 33, 57–60). We find that Ig-α-deficient mice have a block in B cell development that is similar to mice lacking Ig-β or mμ expression. In the absence of Ig-α, B cell development is arrested at the pro-B cell stage, as these cells are unable to express a pre-BCR.

The complete absence of pre-B, immature B, and mature B cells in Ig-α-deficient mice demonstrates that, in vivo, Ig-β alone is not able to promote B cell development. In mice expressing chimeric IgM/Ig-β fusion proteins and in mice lacking two-thirds of the cytoplasmic tail of Ig-α including the immunoreceptor tyrosine-based activation motif, Ig-β reaches the cell surface and the signals transduced by the Ig-β molecule alone in this context are sufficient for the transition of pro-B to pre-B and the generation of immature B cells (19, 61, 62). Therefore, our data suggest that the absence of any further differentiation in Ig-α-deficient pro-B cells could be strictly related to the inability of the Ig-β molecules to be stably expressed on the cell surface in absence of Ig-α. This hypothesis would need to be tested biochemically, as the level of Ig-β on the surface of pro-B cells is too low to be detected by flow cytometric analysis (Ref. 51 and data not shown). A human patient carrying a mutation that prevents Ig-α expression was shown to lack pre-B, immature, and mature B cells as well (16). Thus, development of B cells is absolutely dependent on Ig-α expression in both mice and humans.

Ig-α- and Ig-β-deficient mice allow us to investigate the requirement of these proteins at early stages of B cell development. A surface protein complex (pro-BCR) has been speculated to exist and to signal in pro-B cells the initiation and completion of V_HDJ_H recombination (29, 30, 51, 52). This putative pro-BCR, in analogy to the pre-BCR, would also be envisioned to use Ig-α and Ig-β as signal transducers. Both proteins are indeed expressed on the surface of murine pro-B cells in signaling competent protein complexes that do not contain Ig chains (31, 51).

In this study, we have assessed the function of Ig-α and Ig-β in V_HDJ_H recombination by comparing the frequency of V_HDJ_H joints using elements of the V_HJ558 and V_H7183 families. In addition, pro-B cells of the mutant animals were tested for μ-chain production. These analyses indicate that V_HDJ_H recombination is independent of Ig-α or Ig-β expression. Indeed, a similar frequency of V_HDJ_H joints and of intracellular μ-chain-expressing cells was observed in Ig-α-deficient, Ig-β-deficient, μMT, and wild-type pro-B cell populations. These data agree with those obtained from pro-B cells of a human Ig-α-deficient patient that also showed to have normal frequency of V(D)J recombination at the IgH locus (16). A previous analysis indicated that $Ig\beta^{-/-}$ pro-B cells had decreased levels of V(D)J joints compared with wild-type pro-B cells and a block at the null pre-B or pre-BI stage of development (15). These earlier results had been interpreted to suggest that Ig-β-derived signals might be involved in the onset of V_H to DJ_H recombination or selection of successfully recombined IgH genes (15, 51, 52, 63). This early analysis was performed on the $B220^+CD43^+$ population that contains a large amount of V(D)J-selected pre-B cells in wild-type mice and, in proportion, a large amount of $B220^+CD19^-$ non-pro-B cell progenitors in the $Ig\beta^{-/-}$ mice, while the current analysis has been conducted on the $CD19^+CD43^+$ cell population and compared with μMT as well as wild type. The difference in cell sorting procedures used in this

work might explain why our results appear contradictory to those previously published. However, in a follow-up analysis, the frequency of intracellular μ-chain-positive pro-B cells of Ig-$\beta^{-/-}$ mice was found comparable to that of wild type, when pre-B cell contaminants were excluded from the pro-B cell population analyzed by sorting $B220^+CD43^+CD25^-$ cells (17). Thus, in summary these data demonstrate that Ig-β and Ig-α are not necessary for the initiation and completion of V(D)J recombination at the IgH locus, but they are for the selection and expansion of cells that express the product of a productively rearranged V_HDJ_H gene.

Positive selection and expansion of cells that carry productively rearranged IgH loci, an event that marks the pro-B to pre-B cell transition, is only observed in mice that express all the components of the pre-BCR complex. In lymphoid hematopoietic stem cells committed to the B cell lineage, the transcription of the *Rag1*, *Rag2*, *TdT*, *λ5*, *VpreB*, *B29*, and *mb-1* genes is up-regulated before rearrangement and expression of the Ig genes (64). Thus, expression of the proteins encoded by these genes, together with the accessibility of the germline IgH locus identified by its early transcription, may be sufficient for the initiation of D to J_H and, subsequently, V_H to DJ_H recombination, without the need of a specific signaling event.

We have also examined whether the Ig-α/Ig-β heterodimer influences the capacity of pro-B cells to proliferate, survive, and differentiate within the bone marrow environment and to express a functional IL-7R, which is necessary for these functions (65, 66). During the early stages of B cell development, $B220^+$ pro-B cells differentiate from $CD19^-$ (fraction A) to $CD19^+$ (fraction B). This differentiation is accompanied by an increased expression of the transcription factors E12, E47, and Pax5, and the up-regulation of *Rag1*, *Rag2*, *λ5*, *mb-1*, and *B29* gene transcription (45, 56). This differentiation also marks the final commitment of oligopotent stem cells to the B lineage (67). We have found that the absolute number of fraction A and fraction B bone marrow pro-B cells does not significantly differ in mice deficient for either Ig-α or Ig-β relative to that of μMT and RAG2-deficient animals. In addition, we found that lack of Ig-α or Ig-β expression does not influence entry into the cell cycle or expression of IL-7Rα and IL-7Rγ (data not shown). Thus, these data indicate that progressive differentiation of pro-B cells and their survival in the bone marrow environment do not require expression of Ig-α or Ig-β.

Finally, we have evaluated the possibility that Ig-α and Ig-β might be redundant in their signaling role in the context of pro-B cell development and differentiation, given that Ig-β has been found on the surface of these cells, even in the absence of Ig-α. We have found that pro-B cells lacking in the expression of both molecules (Ig-α and Ig-β) are still able to undergo V_HDJ_H recombination and produce intracellular μ-chain at frequencies similar to those observed for single-deficient pro-B cells. Moreover, double-deficient pro-B cells are also capable to differentiate into fraction B, judging by the coexpression of the CD19 and B220 surface markers.

In conclusion, our data demonstrate that commitment to the B cell lineage and survival of pro-B cells in the bone marrow microenvironment, as well initiation and completion of V(D)J recombination at the IgH locus, do not require expression of Ig-α, Ig-β, or both molecules.

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