Regulation of B Cell Development by Variable Gene Complexity in Mice Reconstituted with Human Immunoglobulin Yeast Artificial Chromosomes

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Summary

The relationship between variable (V) gene complexity and the efficiency of B cell development was studied in strains of mice deficient in mouse antibody production and engineered with yeast artificial chromosomes (YACs) containing different sized fragments of the human heavy (H) and k light (L) chain loci. Each of the two H and the two k chain fragments encompasses, in germline configuration, the same core variable and constant regions but contains different numbers of unique V_H (5 versus 66) or V_K genes (3 versus 32). Although each of these YACs was able to substitute for its respective inactivated murine counterpart to induce B cell development and to support production of human immunoglobulins (Igs), major differences in the efficiency of B cell development were detected. Whereas the YACs with great V gene complexity restored efficient development throughout all the different recombination and expression stages, the YACs with limited V gene repertoire exhibited inefficient differentiation with significant blocks at critical stages of B cell development in the bone marrow and peripheral lymphoid tissues. Our analysis identified four key checkpoints regulated by V_H and V_K gene complexity: (a) production of functional m chains at the transition from the pre B-I to the pre B-II stage; (b) productive V_K J_K recombination at the small pre B-II stage; (c) formation of surface Ig molecules through pairing of m chains with L chains; and (d) maturation of B cells. These findings demonstrate that V gene complexity is essential not only for production of a diverse repertoire of antigen-specific antibodies but also for efficient development of the B cell lineage.

Key words: B cell development • human immunoglobulin • variable genes • transgenic mice • yeast artificial chromosomes

Generation of Igs by B cells is an ordered and highly controlled process of gene recombination and expression that plays a key role in the regulation of B cell development. This process is initiated in the bone marrow by a series of steps in which the genes that encode the different Ig variable segments (V, D, and J) are joined by sequential rearrangements, followed by expression of functional Ig molecules on the surface of B cells. Distinct developmental stages were identified based on the rearrangement and expression of H and L chain genes and the expression of characteristic sets of cell surface antigens (for a review, see references 1 and 2). The immature pro B/pre B-I cells are B220^lo CD43^1 HSA^1 c-kit^1 Ig^2 and are rearranging the H chain genes. The more mature pre B-II cells are B220^lo CD43^+ HSA^+ c-kit^+ Ig^- and in the process of rearranging H chain genes. The immature pro B/pre B-I cells are B220^lo CD43^+ HSA^+ c-kit^+ Ig^- and in the process of rearranging H chain genes. The more mature pre B-II cells are B220^lo CD43^+ HSA^+ c-kit^+ CD25^+, cytoplasmic m^+ and are rearranging the L chain genes. Finally, there are two surface (s)Ig^+ expressing B cell populations—the immature B220^hi m^hi d^- and the mature B220^hi m^lo d^+ cells.

The regulation of B cell differentiation by proper rearrangement and expression of the H and L chain genes is well documented, primarily as a result of studies of mutant mice in which mediators or cis control elements of antibody production were inactivated by gene targeting technology. Elucidation of structure–function relationships of the Ig loci focused primarily on cis-acting sequences 3' of the mouse V region. These studies demonstrated the im-

1Abbreviations used in this paper: BCR, B cell receptor; h, human; m, mouse; RAG, recombination activating gene; sIg, surface immunoglobulin; SLC, surrogate light chain(s); TG, rearranged m chain transgene; YAC, yeast artificial chromosome.
portance of coding (e.g., J, Cμ, Cκ) and noncoding (e.g., Eμ, Eκ, 3′E, 3′κ) sequences in regulation of antibody diversification, assembly, and selection (for a review, see reference 3). However, the role of the V genes, the largest and most diverse gene family in the Ig loci, in controlling Ig production and B cell differentiation is still not fully understood. Introduction of rearranged Ig transgenes with defined single specificities into wild-type or Ig-deficient mice demonstrated the importance of antibody surface expression and its specificity both for development and for positive and negative selection of B cells (4–8). However, these model systems did not permit study of the effects of the V gene pool on either successful rearrangement, expression, and assembly of functional Ig molecules, or selection and expansion of Ig-expressing B cells. The availability of mouse strains containing varying portions of the Vμ or Vκ germline repertoire could clarify the extent to which the number and complexity of the V gene repertoire influences development of B lineage cells.

We have engineered strains of mice, collectively designated XenoMouse, that contain both inactivated mouse Ig genes and different portions of the human H and κ L chain loci cloned on yeast artificial chromosomes (YACs; references 9 and 10). The two human H chain YACs used, yH1 and yH2, encompass in germline configuration the same core variable and constant sequences (D, JH, Cμ, Cδ), but contain different numbers of VH genes, either 5 or 66, respectively. The two human κ chain YACs used, yκ1 and yκ2, contain in germline configuration the same Jκ and Cκ regions, but different numbers of VK genes, either 3 or 32, respectively. Each of the combinations, yH1 and yκ1 or yH2 and yκ2, restored in Ig-inactivated mice a humoral immune system and produced fully human antibodies indicating the compatibility of these human Ig transgenes with the mouse machinery for antibody recombination and expression (9, 10). Evaluation of antigen-specific human antibodies produced by mice engineered with a limited number of human V genes on small YACs or minigenes (9, 11, 12) in relation to those generated by mice engineered with the large antibody gene repertoire suggested the importance of V gene complexity in supporting production of high-affinity antibodies to multiple human antigens (10). These XenoMouse strains, equipped with different portions of the human V gene repertoire, also provided a unique model system to determine the impact of the structure and content of V gene complexity on shaping differentiation and proliferation of B lineage cell populations. Our results reveal the critical role of V gene complexity in supporting efficient B cell differentiation, and demonstrate the different developmental checkpoints controlled by VH and VK gene repertoire.

Materials and Methods

Mice. All mice were born, bred, and kept in a barrier facility until 1 or 2 d before killing. TG: mJμ−/− mice were generated by breeding TG transgenic mice (with a rearranged human μ transgene; provided by M. Nussenzweig, The Rockefeller University, New York [4]) onto the mJμ−/− background.

Flow Cytometry Analysis. Bone marrow, peripheral blood, and spleen were obtained from 8–10-wk-old mice, and the lymphocytes were separated from erythrocytes on Lympholyte M (ACL-5035; Accurate Chemical & Scientific Corp., Westbury, NY). Approximately 1×10^6 cells for each sample were treated with purified anti-mouse CD32/CD16 Fc receptor (01241D; Pharmingen, San Diego, CA) to block nonspecific binding to Fc receptors, stained with antibodies, and analyzed on a FACSCalibur® using CellQuest software (Becton Dickinson, San Jose, CA). Antibodies used except where indicated were from Pharmingen: FITC anti-mouse IgM (02084D); FITC anti-human IgM (08184D); FITC goat F(ab′)2, anti-human-IgD (2032-02; Southern Biotechnology Associates, Inc., Birmingham, AL); FITC anti-mouse IgD (05064D); FITC anti-mouse IgG2a (05074D); FITC anti-human λ (02174D); FITC anti-HSA (01754D); FITC anti-B7-1 (09604D); FITC anti-B7-2 (09274D); PE anti-HSA (01575A); PE anti-human κ (08175A); PE anti-mouse κ (02155A); PE anti-c-kit (0105B); PE anti-CD43 (01605B); CyChrome anti-B220 (01128A); allopurinol anti-B220 (01129A); biotin anti-human IgM (08072D); biotin anti–mouse IgM (02202D); biotin anti-CD25 (01092D); and biotin anti–6C3 (01282D). RED613 streptavidin (19541-010; GIBCO BRL, Gaithersburg, MD) was used to detect biotinylated antibodies.

In Vitro Proliferation Assays. Spleens from three to eight mice of each genotype were isolated and ground with a frosted glass microscope slide. The cell suspension in DME was then spun over a Lympholyte M step gradient. Lymphocytes from the gradient interface were isolated and washed twice in DME, then resuspended in a solution of PBS, 5 mM EDTA, and 0.5% FCS. T cells and macrophages were depleted from the spleen cell suspensions by magnetic cell sorting using anti-CD5 and anti-CD11b magnetic beads (493-01 and 496-01; Miltenyi Biotec Inc., Auburn, CA) and a Type B column according to the manufacturer’s instructions. Enrichment for live B cells was assayed by staining the cells for B220 and μ and with propidium iodide before and after depletion, followed by FACScan® analysis. This treatment resulted in a 70–90% B220−/− cell population. For each assay, 1.5 × 10^5 live B220−/− cells were grown in 96-well plates in DME supplemented with 10% FCS, 2 mM glutamine, and penicillin-streptomycin plus one of the following: LPS at a final concentration of 20 μg/ml, goat anti-mouse IgM F(ab′)2 (115-006-075; Jackson ImmunoResearch Labs, West Grove, PA) at a final concentration of 150 μg/ml, goat anti-human IgM F(ab′)2 (109-006-043; Jackson ImmunoResearch Labs) at a final concentration of 150 μg/ml, or medium alone as a negative control. After 2 d incubation at 37°C, 1 μCi [3H]thymidine (1 μCi/ml; Amersham Corp., Arlington Heights, IL) was added to each well, and after an additional 1 d of incubation, the samples were counted for incorporation of label into DNA. Each sample was assayed in triplicate.

Results

The effect of increasing V gene repertoire on antibody production and B cell development was evaluated in Ig-inactivated mice engineered with either of two pairs of human H and κ chain YACs—yH1 and yκ1, or yH2 and yκ2 (Fig. 1). The yH1 H chain YAC had a 220-kb insert of the human IgH locus, containing in germline configuration the Cμ and δ constant regions, the intronic enhancer, all six functional JH regions, the entire D complex, and the five most proximal variable genes from four Vμ families (Fig. 1; references 9 and 13). The yH2 H chain YAC contained the...
entire yH1 YAC as the core and 61 additional upstream VH genes (34 functional VH in total), all in germline configuration, plus the human γ2 constant region (Cγ2) and the murine 3' enhancer (m3'E) appended downstream of Cδ (10). The k chain YAC, yK1, had a 170-kb insert containing in germline configuration the k deleting element, the intronic and 3' enhancers, the Ck region, all five functional Jk regions, and the three most proximal Vk regions in the B cluster, two of which are functional (9, 13). The larger k chain YAC, yK2, had an 800-kb insert, with yK1 at the core and 29 additional Vk genes from the proximal Vk cluster, with 18 functional Vk genes in total (10).

Mice homozygous for both the inactivated mouse H and the inactivated k chain alleles (DI) were generated as described previously (9). Into this DI genetic background, we introduced either the yH1 and yK1, or the yH2 and yK2 transgenes to yield XenoMouse I or XenoMouse II strains, respectively. Using these strains, we evaluated the ability of individual H or k transgenes to yield XenoMouse I or XenoMouse II strains, introduced either the yH1 and yK1, or the yH2 and yK2 described previously (9). Into this DI genetic background, we described a large human V_H gene repertoire supporting efficient B cell development in mJH-deficient in antibody production (Fig. 2, A and B). By Hardy's convention, developing pro B/pre B-I cells at Fraction B (B220<sup>+</sup>C4D<sup>+</sup>HSA<sup>+</sup>BP-1<sup>+</sup>) accumulated to levels three- to fourfold over wild-type, and the bone marrow from mJH-deficient mice lacked B220<sup>+</sup>μ<sup>+</sup> cells due to terminal arrest of B cell development at the pro B/pre B-I stage (Fig. 2, A and B). By Hardy's convention, developing pro B/pre B-I cells at Fraction B (B220<sup>+</sup>C4D<sup>+</sup>HSA<sup>+</sup>BP-1<sup>+</sup>) accumulated to levels three- to fourfold over wild-type, and terminally arrested at Fraction C (B220<sup>+</sup>C4D<sup>+</sup>HSA<sup>+</sup>med BP-1<sup>+</sup>) (Fig. 2 D). The inability to develop beyond the pro B/pre B-I stage was consistent with a block in H chain rearrangement at the stage of D<sub>H</sub>-J<sub>H</sub> recombination (1–3).

The presence of one allele of the yH1 transgene in mJ<sub>H</sub>-deficient mice partially alleviated the arrest at the pro B/pre B-I stage, resulting in incomplete reconstitution of B cell compartments with B220<sup>+</sup>μ<sup>+</sup> cells (Fig. 2 A) and formation of human μ chain–containing antibodies (9). Pro B/pre B-I cells in yH1; mJ<sub>H</sub>-deficient bone marrow successfully developed into large pre B-II (B220<sup>+</sup>c-kit<sup>-</sup>C2D<sup>+</sup>) or B220<sup>+</sup>C4D<sup>+</sup>HSA<sup>+</sup>BP-1<sup>+</sup> (Fraction C') cells, although at levels ~30% of wild-type mice (Fig. 2, B–D). The partial differentiation was consistent with the continued accumulation of pro B/pre B-I cells at the B220<sup>+</sup>μ<sup>+</sup>c-kit<sup>-</sup>C2D<sup>+</sup>-
stage or at Fraction B, similar to that observed in \( \text{m}_{\mu}^{-/-} \) mice. Cell accumulation was also detected in the Fraction C compartment, which was three- to fourfold larger than in both wild-type and \( \text{m}_{\mu}^{-/-} \) mice. A reconstitution level of \( \sim 50\% \) was detected at the small pre B-II stage (Fig. 2 C) and was sustained in the newly emerging \( B220^{\mu+} \) B cell population (Fig. 2 A). However, the levels of mature, recirculating \( B220^{\mu+} \) cells dropped to only 10% of wild-type. Consistent with the mature B cell population in bone marrow, spleens of \( yH1;\text{m}_{\mu}^{-/-} \) mice exhibited \( \sim 10\% \) B cell reconstitution (Fig. 2 E). The percentage of \( B220^{\mu+} \) cells that were \( \delta^{-} \text{HSA}^{6} \) in the spleen was similar to that of wild-type mice, demonstrating normal differentiation to mature B cells in \( yH1;\text{m}_{\mu}^{-/-} \) (reference 15, and data not shown). The proper pairing between human \( \mu \) protein and mouse \( \kappa \) and \( \lambda \) L chains was supported by the wild-type-like L chain distribution on the mouse B cells (\( \kappa/\lambda = 95:5 \); not shown).

These results indicate that \( yH1 \) can induce complete but inefficient B cell differentiation in H chain–inactivated mice, leading to a partial reconstitution of mature B cell populations in the different lymphoid organs. The stage at which the differentiation block is observed (Fractions B and C) suggests that a large fraction of pro B/pre B-I cells are unable to complete a productive VDJ rearrangement and/or to express a \( \mu \) protein capable of pairing with surrogate L chains (SLC) and forming a pre B cell receptor (pre-BCR), a prerequisite for differentiation to the pre-B-II cell stage (3).

The effect of a second \( yH1 \) allele on B cell development was evaluated in mice homozygous for the YAC (\( yH1/ yH1;\text{m}_{\mu}^{-/-} \)). This strain exhibited a twofold increase in B cell reconstitution over the hemizygous strain, starting at stages correlated with completion of a productive VDJ rearrangement and \( \mu \) chain expression. Accumulation of cells at the pro B/pre B-I stage in \( yH1/yH1;\text{m}_{\mu}^{-/-} \) mice was still observed (Fig. 2 B). However, the large pre B-II population was \( 50\% \) of wild-type, and the small pre B-II population (or Fraction D) reached wild-type levels (Fig. 2 C and D). The newly emerging \( B220^{\mu+} \) cells were \( \sim 80\% \) of wild-type, but the mature, recirculating \( B220^{\mu+} \) cells demonstrated a 20% reconstitution level, similar to that observed in the spleen and double the levels detected in \( yH1;\text{m}_{\mu}^{-/-} \) (Fig. 2 A and E). A proportional increase in the percentage of mature \( B220^{\mu+} \delta^{-} \text{HSA}^{6} \) cells in the spleen of \( yH1/yH1;\text{m}_{\mu}^{-/-} \) mice relative to \( yH1;\text{m}_{\mu}^{-/-} \) mice was detected as well (not shown). Human \( \mu \) in \( yH1/yH1;\text{m}_{\mu}^{-/-} \) serum, averaging 60 \( \mu \)g/ml, represented 10% of the mouse \( \mu \) serum levels in wild-type mice housed in the same pathogen-free facility. Thus, the second \( yH1 \) allele in \( \text{m}_{\mu}^{-/-} \) mice doubled the population of B cells that completed productive VDJ recombination, expressed surface \( \mu \) protein, and progressed to mature B cells.

To test the effect of increased \( \nu_{\kappa} \) repertoire on B cell development, the \( yH2 \) transgene was bred onto the \( \text{m}_{\mu}^{-/-} \) background. \( yH2 \) was able to improve significantly all stages of B cell development and reconstitution in bone marrow and in the peripheral lymphoid compartments. \( yH2 \) mice fully relieved the accumulation of developing B cells at the pro B/pre B-I stage observed in \( yH1 \)-bearing \( \text{m}_{\mu}^{-/-} \).
strains and restored fully the large and small pre B-II populations (Fig. 2, B-D). Reconstitution was also complete in the newly emerging B cell population, but it decreased to ~70% of wild-type in the mature, recirculating B cell population in both the bone marrow and spleen (Fig. 2, A and E). However, this decrease in reconstitution level upon maturation from B220<sup>+</sup>μ<sup>-</sup>- to B220<sup>+</sup>μ<sup>-</sup> was significantly lower than that observed in yK1-bearing m<sub>jH</sub>-<sup>-/−</sup> strains. Consistent with the improved B cell development, the levels of circulating human μ and γ2 chains in sera from yH2; m<sub>jH</sub>-<sup>-/−</sup> mice averaged 200 μg/ml for both, only twofold lower than the mouse μ levels in normal mice kept under pathogen-free conditions.

yH2 differed from yH1 not only by its increased V<sub>H</sub> gene content but also by the presence of the human Cγ2 and the m3'E sequences. To study the contribution of these downstream sequences to the improved restoration of B cell development by the yH2 YAC, m<sub>jH</sub>-<sup>-/−</sup> mice bearing a yH2<sub>2w</sub> YAC transgene, containing all of yH2 except for the human Cγ2 and the m3'E sequences, were analyzed. Similar to the yH2 hemizygous strain, yH2<sub>2w</sub>;m<sub>jH</sub>-<sup>-/−</sup> mice exhibited complete reconstitution of all stages of B cell development, up to and including the newly emerging B cell compartment, and a mature B cell population ~70% of wild-type (not shown). These results indicate that the human Cγ2 and m3'E sequences are dispensable for the improved reconstitution of B cell development by yH2, and that the enhanced B cell development was the result of the increased number and complexity of the V<sub>H</sub> genes.

We compared reconstitution of B cell development in m<sub>jH</sub>-<sup>-/−</sup> mice by the H chain YACs to that obtained by a rearranged human μ transgene (TG). TG had previously been shown to be expressed at significant levels on mouse B cells resulting in a complete allelic exclusion of the murine H chains in wild-type mice (4), and to support B cell development in recombination activating gene (RAG)-deficient mice (7). Consistent with previous observations (7), TG; m<sub>jH</sub>-<sup>-/−</sup> mice had greatly reduced populations at the pro B/pre B-I, large and small pre B-II, and the newly emerging B cell stages, probably due to the acceleration of B cell development by early expression of the rearranged μ transgene (Fig. 2). Reconstitution of mature B220<sup>+</sup>μ<sup>-</sup> populations in the bone marrow and spleen was ~50 and 70% of wild-type mice, respectively, similar to that observed in yH2; m<sub>jH</sub>-<sup>-/−</sup> mice (Fig. 2 A). Thus, a partially reconstituted mature B cell population was also obtained with a rearranged human transgene, which is properly expressed and selected in wild-type mouse B cells. This observation may indicate that other components, in addition to a large V<sub>H</sub> repertoire, are required for complete maturation and/or expansion and survival of the recirculating B220<sup>+</sup>μ<sup>-</sup> cells (see Discussion).

The increased V<sub>K</sub> complexity on yK2 R esulted in functional replacement of the mouse λ locus. The ability of yK1 and yK2 YACs to substitute for the inactivated mouse κ chain locus was first evaluated in mice homozygous for the deletion of mouse Cκ (mCκ<sup>-/−</sup>). These mice displayed a complete absence of κ<sup>+</sup> B cells, and all B cell populations expressed the mouse λ L chain exclusively (Fig. 3). Analysis of the different B cell subpopulations in the bone marrow demonstrated a wild-type-like distribution in the developmental stages that precede L chain expression, the pro B/pre B-I and the large pre B-II populations (not shown). However, a twofold accumulation at the small pre B-II stage was detected (Fig. 3 B). As a result of the partial differentiation arrest, the newly emerging B cell compartment and the mature recirculating population reached only 50–60% of wild-type levels (Fig. 3 A), consistent with previous reports (16). In the serum of mCκ<sup>-/−</sup> mice, levels of circulating mouse L chains averaged 580 compared with 70 μg/ml in wild-type mice. These results indicate that the mouse λ locus can substitute for the inactivated κ locus only partially, resulting in a lower efficiency of differentiation to slg-expressing B cells.

The first human κ transgene tested, yK1, with its three V<sub>K</sub> genes, two of which are functional, could partially replace the inactivated mouse κ locus and compete with the mouse λ chain genes. mCκ<sup>-/−</sup> mice with either one or two yK1 alleles exhibited an accumulation at the small pre B-II stage (Fig. 3, A and B). The percentage of newly emerging B220<sup>+</sup>κ<sup>-</sup> B cells in yK1 hemizygous or homozygous mice, relative to the mCκ<sup>-/−</sup> mice, increased to ~70 and 100% of wild-type, respectively, whereas their mature bone marrow B220<sup>+</sup>μ<sup>-</sup> populations did not change significantly (Fig. 3 A). The B cell populations in the peripheral blood and lymph nodes of the yK1-bearing mCκ<sup>-/−</sup> strains increased by 20–25% over mCκ<sup>-/−</sup> mice, indicating improved B cell development by the yK1 YAC (Fig. 3, C and D).

Another manifestation of the ability of the yK1 YAC to compete effectively with the mouse λ chain and restore κ chain expression in mCκ<sup>-/−</sup> mice was the appearance of a significant human κ<sup>+</sup> B cell population, in particular in the presence of two yK1 alleles. In peripheral blood (Fig. 3 E), lymph nodes, and spleen (data not shown) of yK1/mCκ<sup>-/−</sup> mice, there were equivalent numbers of human κ<sup>+</sup> and mλ<sup>+</sup> B cells, whereas in yK1/yK1/mCκ<sup>-/−</sup> mice, human κ<sup>+</sup> B cells outnumbered mλ<sup>+</sup> B cells by a 2:1 ratio. Similar results were observed in the newly emerging B220<sup>+</sup>μ<sup>-</sup> population in the bone marrow, indicating that the preferential usage of human κ over mouse λ occurs at the stage of L chain rearrangement, as shown previously for wild-type mice (17). The circulating human Igκ levels in yK1 homozygotes were higher than those detected in the hemizygotes, averaging 720 and 250 μg/ml, respectively. The levels of circulating mouse λ chain were reduced in both strains to ~300 μg/ml.

The second human Igκ transgene, yK2, with its increased Vκ repertoire, was able to substitute fully for the mouse κ chain locus and to dominate L chain use. Both hemizygous and homozygous yK2/mCκ<sup>-/−</sup> mice exhibited full restoration of B cell development in the bone marrow, with a complete relief of the cell accumulation at the small pre B-II stage detected in both mCκ<sup>-/−</sup> and yK1/mCκ<sup>-/−</sup>
mice and the appearance of wild-type-like newly emerging and mature B cell populations (Fig. 3, A and B). Complete reconstitution of B cell compartments was also detected in the peripheral blood, lymph nodes (Fig. 3, C and D), and spleen (not shown) of yK2/mC mice.

The apparent ability of yK2 to substitute for the mouse Igk locus was also observed at the level of hκ+ expressing B cells (Fig. 3 E). In hemizygous yK2/mCκ−/− mice, the majority of the peripheral blood lymphocytes (~75%) expressed human κ chain exclusively, whereas only a minority (15%) expressed mouse λ chain. A similar κ to λ chain distribution was detected in mice with only one functional mouse Igk locus (16). In yK2/yK2/mCκ−/− mice, the human κ− B cell population increased to >90%, and the

Table 1. The yK2 Transgene Competes Effectively with the Mm Igk Locus

<table>
<thead>
<tr>
<th>Genotype</th>
<th>yH2</th>
<th>yK2</th>
<th>mJH</th>
<th>mCκ</th>
<th>yK2/mlGκ+</th>
<th>% hκ+</th>
<th>% mκ+</th>
<th>% mλ+</th>
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<tbody>
<tr>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+/+</td>
<td>+/+</td>
<td>0:2</td>
<td>0.1 ± 0.1</td>
<td>90.0 ± 0.8</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+/+</td>
<td>−/−</td>
<td>1:1</td>
<td>40.2 ± 2.3</td>
<td>39.4 ± 2.9</td>
<td>4.6 ± 0.4</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−/−</td>
<td>+/−</td>
<td>1:1</td>
<td>34.8 ± 4.0</td>
<td>37.1 ± 1.6</td>
<td>11.2 ± 5.3</td>
</tr>
<tr>
<td>+</td>
<td>+/+</td>
<td>−</td>
<td>+/−</td>
<td>−/−</td>
<td>2:1</td>
<td>52.9 ± 2.3</td>
<td>25.0 ± 0.8</td>
<td>6.5 ± 1.3</td>
</tr>
</tbody>
</table>

B splenocytes were stained with antibodies against B220, human κ, and mouse κ, or B220, mouse κ, and mouse λ, then analyzed by three-color flow cytometry. The B220+ population was first gated, then analyzed for expression of other markers. The percentages of cells positive for human κ, mouse κ, or mouse λ were determined. Data are presented as the average ± SD. Three mice of each genotype were tested.

* The ratio of functional alleles for each locus.
mouse λ⁺ B cell population decreased to 5%, reaching a wild-type-like k to λ distribution ratio. Similar results were obtained in the spleen and lymph nodes, and in the newly emerging B220⁺μ⁺ and mature B220⁺μ⁺ cells in the bone marrow (not shown). The average levels of circulating hκ and mlκ in yK2:mCκ−/− mice were 1,400 and 100 μg/ml, respectively, equivalent to the levels of mouse κ and λ in wild-type mice kept under similar conditions. As the only known difference between the yK1 and yK2 YACs resides in the number and diversity of their V genes, these results provide evidence for the role of Vκ gene repertoire in supporting normal κ chain recombination and expression.

Equivalency of the human κ chain locus on yK2 with the mouse Iκκ locus was further demonstrated in mice with one functional mlκκ allele and either one or two alleles of yK2 (Table 1). In the peripheral blood of mice with functional H chain loci, one functional mlκκ allele, and one yK2 allele (yK2:mCκ−/−), the percentages of hκ⁺ and mlκ⁺ B cells were equivalent (Table 1). Similarly, equivalent usage of yK2 and mlκκ in a yH2;mJκ−/− background (yH2; yK2;mJκ−/−;mCκ−/− mice) was demonstrated. In mice with two yK2 alleles and one functional mlκκ allele (yH2; yK2/yK2;mJκ−/−;mCκ−/−), the ratio of hκ⁺ to mlκ⁺ B cells in the spleen increased to 2:1 (Table 1). Thus, yK2 competes effectively with the mouse κ chain locus. These data also demonstrated the lack of an apparent preference of the H chain for a κ chain of the same species.

Increased Vκ and Vκ Gene Repertoires Restore Efficient B Cell Development in XenoMouse Strains. The combined effects of yH1 and yK1 or yH2 and yK2 in replacing their inactivated mouse counterpart loci, thereby restoring B cell development and inducing human antibody production in DI (mJκ−/−; mCκ−/−) mice, was examined in the XenoMouse I (yH1; yK1; DI) and II (yH2; yK2; DI) strains. In the bone marrow of DI mice, the pattern of arrested B cell development was similar to that observed in mJκ−/− mice. Developing B cells accumulated at the pro B/pre B-I stage (Fraction B) and were terminally arrested at Fraction C, or the B220⁺c-kit⁺CD25⁺ stage (Fig. 4; references 9 and 10).

As demonstrated above (Figs. 2 and 3), a second yH1 allele affected B cell development in yH1;mJκ−/− mice significantly, whereas only a small effect was observed with a second yK1 allele in yK1;mCκ−/− mice. Moreover, no obvious differences in reconstitution of B220⁺μ⁺ compartments in XenoMouse I strains with one versus two alleles of either yK1 or yK2 were observed (not shown). Therefore, we concentrated on analyzing B cell development in XenoMouse I and II strains homozygous for the yK transgenes, in conjunction with either one or two alleles of the yH transgene.

yH1 in conjunction with yK1 partially restored B cell development and B220⁺μ⁺;hκ⁺ compartments in XenoMouse I. Similar to yH1;mJκ−/− strains, XenoMouse I strains with one or two yH1 alleles still exhibited a threefold accumulation of developing B cells at the pro B/pre B-I stage (B220⁺μ⁺;c-kit⁺;CD25⁺ or Fraction B; Fig. 4, B and D). XenoMouse I with one yH1 allele had ~30% of wild-type levels at Fraction C⁺ (the large pre B-II stage), whereas the homozygous strain exhibited ~60% reconstitution level. The sizes of B cell populations in Fractions D and E in XenoMouse I were substantially lower than the respective ones in the yH1;mJκ−/− or yK1;mCκ−/− mouse strains (Figs. 2–5). Hemizygous and homozygous XenoMouse I strains represented reconstitution levels of 30 and 70% for the small pre B-II cells, and 10 and 30% for the newly emerging and mature B220⁺μ⁺;δ⁺ populations. These results illustrated a consistent twofold improvement.
in B cell development by the second yH1 allele, which was also manifested in the spleen (Fig. 6 A, and data not shown). In the B220<sup>+</sup> compartment of XenoMou se I, there was an overrepresentation of B220<sup>+</sup>Hi SA<sup>+</sup> cells and an underrepresentation of the mature B220<sup>+</sup>Hi SA<sup>lo</sup> cells compared with wild-type (Fig. 6 B). The B220<sup>+</sup> population had a twofold lower level (50% of wild-type) of h<sub>μ</sub>Hi<sup>+</sup> cells. The B220<sup>+</sup>Hi SA<sup>lo</sup> cells were CD5<sup>+</sup>-CD40<sup>+</sup> (not shown). These results indicate that yH1 and yK1 are capable of restoring the progression of precursor B cells through development and maturation, but with a limited efficiency that is likely to reflect the combined deficiencies associated with the small V<sub>H</sub> and V<sub>k</sub> gene repertoires (see Discussion).

We then evaluated the potency of yH2 and yK2, with their greatly increased V gene repertoires, for improving B cell development in XenoMou se II strains compared with the respective intermediate strains. Complete reconstitution of the pro B/pre B-I and the large and small pre B-II populations in the bone marrow of XenoMou se II was observed (Fig. 7), consistent with results from both yH2; m<sub>j1</sub>d<sub>1</sub>, yK2; mC<sub>k2</sub>d<sub>2</sub> strains. The newly emerging B220<sup>+</sup>Hi<sup>+</sup>δ<sup>+</sup> and the mature B220<sup>+</sup>μ<sup>+</sup>δ<sup>+</sup> populations both exhibited reconstitution of 50–70%. These reconstitution levels were similar to those detected in yH2; m<sub>j1</sub>d<sub>1</sub>, mice, whereas complete restoration of the B220<sup>+</sup>μ<sup>+</sup> population was demonstrated in yK2; mC<sub>k2</sub>d<sub>2</sub> mice (Figs. 2, 3, and 5).

Within this compartment, the ratio of H SA<sup>+</sup> (immature) to H SA<sup>lo</sup> (mature) B cells was reminiscent of that detected in wild-type mice, with both immature and mature populations ~70% of wild-type (Fig. 6 B). Furthermore, within the XenoMou se II population, the ratio of μ<sup>+</sup>δ<sup>+</sup> to μ<sup>+</sup>δ<sup>-</sup> cells was identical to wild-type mice (Fig. 6 C). The majority (80%) of the B220<sup>+</sup>Hi SA<sup>lo</sup> B cells in XenoMou se II spleen were h<sub>μ</sub>Hi<sup>+</sup> h<sub>δ</sub>lo<sup>+</sup> and CD5<sup>-</sup>CD40<sup>+</sup>, identical to the m<sub>μ</sub>Hi<sup>+</sup>δ<sup>+</sup> cells in the B220<sup>+</sup>Hi SA<sup>lo</sup> population in wild-type mice (not shown). Thus, the yH2 and yK2 YACs supported proper maturation of XenoMou se II B cells to H SA<sup>+</sup><sub>μ</sub><sup>+</sup>δ<sup>+</sup>. The percentages of h<sub>k</sub>- and m<sub>k</sub>-expressing B cells were identical to the L chain distribution in wild-type mice (Fig. 6 D). The restoration of normal B cell development in XenoMou se II was also manifested in circulating levels of human μ and k chains (300 and 530 μg/ml, respectively) that were higher than those detected in XenoMou se I (150 and 120 μg/ml, respectively). The average levels of hγ2 in XenoMou se II were 100–200 μg/ml. M<sub>ω</sub> λ levels were lower in XenoMou se II compared with XenoMou se I—10 versus 40 μg/ml.

A similar reconstitution pattern of the B cell lineage by yH2 and yK2 was observed in XenoMou se II,3 (Fig. 6), a mouse strain in which the two YACs were cointegrated on the same chromosome by fusing embryonic stem cells with a yeast strain containing both the yH2 and yK2 YACs. In transgenic mice, the two transgenes cosegregated through >1,000 progeny, strongly suggesting close genetic linkage. In the XenoMou se II,3 spleen, B cell reconstitution was

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The functionality of XenoMouse-derived B cells in vivo was confirmed by the ability of these mice to mount a strong human antibody response to multiple antigens and to produce high-affinity fully human mAbs against different antigens, including human IL-8, human TNF-α, and the human epidermal growth factor receptor (10).

Discussion

This report elucidates the role of the size and complexity of the native human antibody repertoire in shaping B cell differentiation and proliferation pathways by directly comparing B cell development in XenoMouse strains transgenic for segments of the human H and κ chain loci differing only in the spectrum of their V gene repertoire. As the V gene arrays on the integrated Ig YACs are in germline configuration and in a single copy, our study evaluated the human antibody response to multiple antigens and to produce high-affinity fully human mAbs against different antigens, including human IL-8, human TNF-α, and the human epidermal growth factor receptor (10).

Figure 6. B cell maturation in spleen of XenoMouse I and XenoMouse II strains. Analysis of B splenocytes from wild-type (WT), DI (mJh1−/−; mCKκ−/−), XenoMouse I (hemizygous for yH1 and yK1), XenoMouse II (hemizygous for yH2; homozygous for yK2), and XenoMouse II.3 (homozygous yH2 and yK2) mice. Four-color flow cytometry was performed using antibodies against A) B220 versus mouse or human μ; (B) HSA and B220; (C) mouse δ and mouse μ or human δ and human μ in the gated B220+ population; (D) mouse λ and mouse κ or human κ in the gated B220+ population. The percentage of positive cells within a quadrant or region is indicated. The data shown are representative of multiple animals. The mean numbers of B220+ μ+ cells in the spleen were 68 ± 13 for wild-type (n = 3), 0 for DI (n = 3), 15 ± 3 for XenoMouse I (n = 4), and 24 ± 15 for XenoMouse II (n = 10).
Figure 7. Restoration of B cell development in bone marrow of X enoM ouse II. Analysis of bone marrow (A–E) of wild-type (WT), DI (mJH\textsuperscript{−/−}); mC\textsuperscript{κ−/−}), and X enoM ouse II (hemizygous for yH2 and homozygous for yK2). Four-color flow cytometry was performed using antibodies against (A) B220 and mouse or human \(\mu\) (B) c-kit and CD25 in the B220\(\mu\) population gated from A; (C) forward scatter size distribution of the B220\(\mu\) c-kit CD25\superscript{−} population gated from B; (D) HSA and BP-1 in the B220\textsuperscript{CD43−} gated population; and (E) mouse \(\delta\) and mouse \(\mu\) or human \(\delta\) and human \(\mu\) in the B220\textsuperscript{CD43−} gated population. The percentage of positive cells within a quadrant or region is indicated. The data shown are representative of multiple animals. The mean numbers of B220\(\mu\) cells in the bone marrow were 2.3 \(\pm\) 0.6 \(\times\) 10\(^6\) for wild-type (n = 3), 1.6 \(\times\) 10\(^6\) for DI (n = 1), and 1.8 \(\pm\) 1.0 \(\times\) 10\(^6\) for X enoM ouse II (n = 5). The mean numbers of B220\(\mu\) cells in the bone marrow were 0.8 \(\pm\) 0.2 \(\times\) 10\(^6\) for wild-type (n = 3), 0 for DI (n = 1), and 0.3 \(\pm\) 0.2 \(\times\) 10\(^6\) for X enoM ouse II (n = 5). The mean numbers of B220\(\mu\) cells in the bone marrow were 0.8 \(\pm\) 0.1 \(\times\) 10\(^6\) for wild-type (n = 3), 0 for DI (n = 1), and 0.3 \(\pm\) 0.1 \(\times\) 10\(^6\) for X enoM ouse II (n = 5).

Figure 8. Human \(\mu\) chains function to support B cell proliferation in vitro. B cells were enriched from pooled suspensions of splenocytes from wild-type, yH1; mJH\textsuperscript{−/−}, or yH2; mJH\textsuperscript{−/−} mice, then stimulated to proliferate in vitro using (A) LPS or (B) anti–mouse \(\mu\) or anti–human \(\mu\) F(ab′)\(_2\), as described in Materials and Methods. The data are presented as total cpm incorporated; bar indicates SEM. Background incorporation of [\(\text{H}\)] thymidine in nonstimulated cultures was 400–2,700 cpm.

and conventional L chains; and (d) maturation to recirculating B cells. All of these checkpoints are critical to successful B cell development. Therefore, any decline in the efficiency of cell progression at these stages can severely impair the entire humoral immune system, as demonstrated in this report.

The first developmental checkpoint regulated by \(V_H\) gene complexity is the production of a functional \(\mu\) protein, yH1, the human H chain YAC with core variable and constant sequences and only five \(V_H\) genes, was sufficient to relieve the block at H chain gene recombination in \(J_H\textsuperscript{−/−}\) mice and to induce B cell differentiation all the way to mature B cells, but only in a small fraction of the precursor B cells (Fig. 2). Accumulation of the majority of the yH1;mJH\textsuperscript{−/−} B cell population in Fractions B and C is indicative of a reduced efficiency in completing productive \(V_H\textsuperscript{++}\)D\(H\) recombination and/or an impaired ability of the generated \(\mu\) protein specificities to pair successfully with the SLC, the proposed function of which is to select for \(\mu\) chains capable of pairing with conventional L chains (19, 20). Inability to form a functional \(\mu\) chain–SLC complex triggers attempts to generate a compatible \(\mu\) protein by a recombination at the second H chain allele or by H chain replacement with an upstream \(V_H\) segment via cryptic recombination signals (21, 22). Indeed, the presence of a second yH1 allele in mJH\textsuperscript{−/−} mice doubled the number of pre B-II and mature B cells.

The ability of the yH2 and yH2\(\mu\) YACs, with their additional 61 \(V_H\) genes, to restore normal B cell development indicated that the limited \(V_H\) gene repertoire on the yH1 YAC is the cause for the inefficient B cell development in yH1;mJH\textsuperscript{−/−} mice. The large \(V_H\) gene complexity, even when presented on only one yH2 allele, restored a wild-type-like probability of productive H chain recombination events and ensured a wide range of \(V_H\) gene specificities that could form functional pre-BCR complexes.

The importance of \(V_H\) gene specificity for formation of a functional pre-BCR complex, and thus for the development of the B cells expressing it, was suggested by previous studies investigating the expression pattern of Ig transgenes or specific mouse endogenous \(V_H\) genes. For example, adult mouse pre B cells expressing \(V_H\text{81X}\) or Q52 genes were unable to progress efficiently to the CD43\superscript{−} stage due to the failure of these \(V_H\) genes to form functional pre-BCR complexes that deliver signals required for further differentiation (23–27).

The limited \(V_H\) diversity on the yH1 YAC reduced significantly the number of B cells that progressed from the immature B220\(\mu\) stage to the recirculating B220\(\mu\) stage.
stage (Fig. 5). The reduced mature B cell population could reflect elimination of B cells expressing autoreactive specificities, lack of specificities that trigger positive selection and expansion, or impaired cell survival in the periphery. This block at B cell maturation was relieved, although not fully, by the yH2 YAC, suggesting a necessity for diverse Vκ specificities to support efficient B cell maturation and expansion. The reduced mature B cell population in yH2 mice compared with wild-type mice may reveal possible deficiencies, such as a need for specificities of the human λ chain genes that comprise 40% of the repertoire presented on human B cells (28). The existing mature B cell population in yH1- and yH2-bearing mJk−/− mice indicates that human μ can be stably produced by the mouse B cells and can be assembled with the mouse Igα and Igβ to form a functional BCR capable of receiving and transmitting extracellular differentiation and proliferation signals (Fig. 8).

Studies with the two human κ chain YACs demonstrated the critical role of Vκ gene repertoire in efficient L chain recombination, in production of functional IgMκ protein, and thus in proper B cell differentiation (Fig. 5). yK1;mCκ−/− mouse strains, containing similar numbers of functional Vκ and Vλ genes, provided a unique tool to study the mechanisms underlying the regulation of L chain isotype use. One allele of yK1, with only two functional Vκ genes, competed effectively with the two λ chain alleles, each containing three functional V genes, and two yK1 alleles dominated L chain use. The dominance of yK1 can be attributed not to a larger repertoire but rather to intrinsic structural differences between the human κ and mouse λ chain loci, such as the inferior λ chain recombination signal sequences (29), thus favoring a stochastic model for κ/λ usage (30). In yK2;mCκ−/− strains, the κ to λ chain usage ratio equaled that of wild-type mice (16), indicating that the structural elements controlling L chain use are similar in humans and mice. The ability of yK2, with its increased number of Vκ genes, to restore a normal mouse κ to λ ratio indicated the importance of Vκ gene repertoire for wild-type-like L chain recombination.

yK1 supported modest improvements in B cell reconstitution in mCκ−/− mice (Figs 3 and 5). In contrast, yK2, with its 18 functional Vκ genes, restored normal B cell development. Therefore, the limited number of Vκ genes on yK1 is likely to be the reason for the low efficiency of productive Vκκκκ recombination events. Fewer Vκ genes could also potentially reduce the efficiency of κ chain editing, a valuable mechanism for rescuing incompatible κ chain specificities (31–34). Finally, the lower efficiency of recombination by inversion associated with the functional Vκ genes on yK1 (35) could also contribute to the reduced number of recombination products.

The importance of Vκ gene specificity for B cell development was suggested by previous studies with Ig transgenes. For example, a κ transgene paired with the TG transgene in a RAG-deficient background failed to generate a mature B cell compartment (7), in contrast to the substantial reconstitution we detected when the TG transgene could pair with the normal mouse L chain repertoire (Fig. 2). Spanopoulou et al. also demonstrated that a μκ transgene derived from a native antibody was able to support full reconstitution in RAG-deficient mice (7). In addition, differential abilities of two Vκκκκ constructs, in combination with endogenous mouse H chains, to generate a mature B cell compartment were proposed to originate from formation of incompatible specificities (36). The contribution of gene number or complexity to the ability of Vκ repertoire to support normal B cell development can be evaluated from comparison of our yK2;mCκ−/− strains, with 18 different Vκ genes, to mCκ−/− mice homozygous for a YAC containing 20 copies of the same 5 Vκ genes, i.e., 100 functional Vκ genes but with limited complexity. This transgene restored B cell development to a degree similar to that observed in yK1 homozygous strains, and clearly less efficiently than the yK2 YAC, as judged by the hκ+ B cell population and the human κ serum levels (37). Therefore, the diversity of the Vκ gene repertoire seems critical for proper L chain regulation.

The XenoMouse I (yH1;yK1;DI) strain exhibited the same developmental blocks at the pro B/pre B-I and small pre B-II stages, and twofold improvement in B cell development by the second yH1 allele, as observed in yH1;mJk−/− mice (Figs. 5 and 7). However, the impairment of B cell development at Fractions D–F, stages associated with generation of Ig+ B cells, was more severe in XenoMouse I than in either yH1;mJk−/− or yK2; mCκ−/− mice (Fig. 5), likely due to the combined limitations associated with the limited Vκ gene repertoire on both the H and the κ chain YACs. These limitations may have further reduced both the frequency of B cells with productive and compatible μ and κ chains (38), and the ability of the B cells to progress to mature HSAκ+ cells (Fig. 6). Consistent with our findings, mice engineered with multicity human Ig minigenes containing four Vκ and four Vκ genes exhibited low levels of B cell reconstitution compared with wild-type mice (11). In contrast to XenoMouse I, the XenoMouse II strains exhibited wild-type-like B cell development similar to that observed in yH2 or yK2 intermediate strains, demonstrating proper regulation of the H and κ chain recombination and expression (Fig. 5).

Significant but not fully restored mature B cell populations were detected in the bone marrow and in the periphery of XenoMouse II, exhibiting markers associated with B cell maturation (HSAκ+κ). As stated previously, the lack of complete reconstitution may stem from specific features of the human antibody repertoire. For example, some of the generated human antibody specificities may be recognized as autoreactive by the mouse and trigger B cell elimination in the bone marrow (39), or may have a decreased life-span due to lack of appropriate antigen stimulation (for a review, see references 20 and 40). Positive selection and expansion of the mature B cell population could also be impaired by the absence of specificities, such as those associated with the human Vλ genes. Nevertheless, the existing mature B cells respond properly to antigen stimulation in vivo, as demonstrated.
by efficient class switching, somatic hypermutation, and generation of high-affinity antigen-specific human mAbs (10). Our previous report demonstrated the critical role of a large V gene repertoire in providing the diverse specificities required for production of high-affinity human antibodies against a broad spectrum of antigens (10). This report proved that V gene complexity is also essential to support efficient B cell development, and thus to reconstitute a normal humoral immune system in Ig-inactivated mice. The findings reported here suggest the utility of Xeno-Mouse strains as a tool to elucidate the molecular mechanisms underlying the shaping of the human antibody gene repertoire during developmental and disease states that are not accessible for analysis in humans, including differential expression of V, D, and J genes, human H and k chain editing, and identification of autoreactive specificities.

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