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B cells expressing two different Igκ L chains (allotype included) have been occasionally observed. To determine frequency and function of these cells, we have analyzed gene-targeted mice that carry a human and a mouse Igκ C region genes. Using different methodologies, we found that cells expressing two distinct κ-chains were 1.4–3% of all B cells and that they were present in the follicular, marginal zone, and B1 mature B cell subsets. When stimulated in vitro with anti-IgM, dual κ surface-positive cells underwent activation that manifested with cell proliferation and/or up-regulation of activation markers and similar to single κ-expressing B cells. Yet, when activated by divalent reagents that bound only one of the two κ-chains, dual κ B cells responded suboptimally in vitro, most likely because of reduced Ag receptor cross-linking. Nonetheless, dual κ B cells participated in a SRBC-specific immune response in vivo. Finally, we found that Ig allotype-included B cells that coexpress autoreactive and nonautoreactive Abs, our studies suggest a potential role of allotype-included B cells in both physiological and pathological immune responses. The Journal of Immunology, 2007, 179: 1049–1057.

Despite the physiological importance attributed to haplotype exclusion, allelic- and isotypic-included B cells expressing either two VH or two VL chains exist in both wild-type mice and in humans (11–20). B cells expressing two VH chains are found at a frequency of 1 in 10,000 in mice (18), and cells that coexpress κ and λ account for 0.2–0.5% of total B cells in both mice and humans (15, 20). Although the frequency of haplotype-included B cells is low, their existence suggests that they could have a physiologic or pathologic role in immunity. Currently, our knowledge about the functional capacity of wild-type haplotype-included B cells is limited to the fact that κ/λ-expressing B cells can differentiate into all mature B cell types in mice (20).

IgL haplotype-included B cells in which both L chains oligomerize with the H chain can generate three types of Ab molecules, two containing a pairing of the same H and L chains in individual Abs, and a third type consisting of different chains assembled together to form one chimeric 7S Ab. In the absence of preferential pairing, and when both L chains are expressed at equivalent levels, half of the Abs expressed by haplotype-included B cells are expected to be chimeric. Chimeric Abs are monomeric with respect to Ag binding because they possess two separate specificities on a single Ab molecule. Therefore, haplotype inclusion is expected to reduce the amount of Ag-mediated BCR cross-linking and consequently the efficiency of an immune response. Little is known about the Ag specificity of haplotype-included B cells in wild-type mice. Nevertheless, the possibility exists that some of these cells express autoantibodies. This is because most primary Igk gene rearrangements encode autoreactive Abs (21, 22), and also because haplotype inclusion can mediate differentiation and survival of self-reactive B cells in mouse models (4–6). The functional capacity of haplotype-included B cells that coexpress autoreactive and nonautoreactive Abs is not yet clear. These cells may respond to antigenic stimuli via their nonautoreactive Ag receptors or, alternatively, they may be either anergic or ignorant as the result of expressing autoreactive Ag receptors. Thus, determining...
the functional response of dual BCR-expressing cells is relevant to our understanding of the development of autoantibodies.

The high ratio of Igk to Igλ gene rearrangements (~10–20:1) in wild-type mice predicts that dual κ-expressing B cells are the most abundant among haplotype-included B cells in these animals. However, the frequency of dual κ-expressing B cells is poorly characterized and their functional capacity remains unknown. Mice engineered to carry the gene segment encoding the human κ C region on one allele (Iκ8/m) have been previously described and are useful for discriminating κ-chains expressed by two distinct Igκ loci in one cell (23). In this study, using the Iκ8/m mouse model, we have characterized the frequency and function of dual κ-expressing B cells in a normal Ab repertoire. We found that dual κ-expressing B cells were ~1.4–3% of the total peripheral B cell population in these mice and that they were capable of responding to antigenic stimuli in vitro and in vivo. Moreover, using the previously established B1-8/3-83IgK-H11003 mouse model in which 40% of peripheral B cells coexpress autoreactive and nonautoreactive Abs, we found that autoreactive haplotype-included B cells were responsive to Ag receptor stimulation in vitro.

Materials and Methods

Mice

Iκ8/m and B1-8/3-83IgK mice have been previously described (5, 23). Wild-type mice were from either BALB/c or C57Bl strains. All mice were housed and bred in a specific pathogen-free environment at the Biologic Resource Center of the National Jewish Medical and Research Center. Experiments were done in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Flow cytometric analysis and Abs

Bone marrow and spleen cells were prepared and stained, as previously described (24). Abs used for staining were against B220 (RA3-6B2; BD Pharmingen), IgM (R33-24), IgD (1.3-5), CD5 (53-7.3; BD Pharmingen), CD21 (7G6), CD23 (B3B4; BD Pharmingen), CD1d (1B1; Biotest), CD68 (H1.2F3; Becton Dickinson), IgM (R33-24), IgD (1.3-5), CD5 (53-7.3; BD Pharmingen), CD21 (7G6), CD23 (B3B4; BD Pharmingen), CD1d (1B1; Biotest), CD68 (H1.2F3; Becton Dickinson), CD86 (GL1; Becton Dickinson), mouse Igκ C region (H139.52.1, Southern Biotechnology Associates; 187.1, BD Pharmingen), human Igκ C region (HP6062, BD Biosciences; mouse Fab’ Ab fragment generated by papain digestion, followed by permanent reduction of the H-H chain disulfide bonds of F(ab’2), Proteos Immunoresearch). Abs were used as FITC, PE, allophycocyanin, PerCP, and biotin conjugates. Biotinylated Abs were revealed with streptavidin-allophycocyanin (BD Pharmingen) or streptavidin-PerCP (BD Pharmingen). Propidium iodide (1.25 μg/ml) was added before three-color flow cytometric analysis to exclude dead cells. Stained cells were analyzed either on a FACSCalibur (BD Pharmingen) and with FlowJo 4.4.3 software or on a CYTOF (DakoCytomation) with FlowJo 6.4.3 software. All cell analyses were conducted on a live lymphoid gate based on propidium iodide incorporation and/or forward and size scatter. When analyzed on a CYTOF flow cytometer, cell aggregates were also excluded from the analysis by using the pulse width (doubler discriminator) channel. An anti-mouse Igκ Fab Ab (clone 187.1) was generated by fragmentation of biotinylated 187.1 Igκ using preactivated papain (25). In brief, 4 ml of 1 mg/ml 187.1 Igκ in PBS was mixed with 4 ml of 0.075 mg/ml papain (Acros Organics) in digestion buffer (PBS, 0.02 M EDTA, and 0.02 M cysteine) and incubated for 4 h at 37°C. The reaction was terminated by adding iodoacetamide (Sigma-Aldrich) to a final concentration of 0.03 M. The 187.1 Fab was dialyzed against PBS at 4°C for 12 h, purified over a Superdex 200 high load 26/60 size-exclusion column (GE Healthcare), and analyzed by SDS-PAGE.

B cell hybridomas

B cell hybridomas were generated from LPS-stimulated spleen cells, as previously described (5), with the exception that SP2/0 (murine IL-6) myeloma cells (26) were used as the fusion partner and that LPS was used at 20–50 μg/ml. The first 96-well plate was seeded with cells at 0.5 × 106 cells/ml in complete RPMI 1640 medium with 10% FBS. Two-fold serial dilutions of this first plate were used to seed five more plates. Plates were incubated at 37°C in 5% CO2 for 48 h before starting with selection with 14.1 μg/ml of hygromycin (Sigma-Aldrich) and 0.5 μg/ml of azaserine (Sigma-Aldrich). Supernatants from wells with growing hybridomas were analyzed for the presence of chimeric human (h)Cκ+ mouse (m)Cκ+ Abs by ELISA. Hybridomas positive by ELISA were expanded and screened for hCκ and mCκ chain expression by intracellular staining with anti-human and anti-mouse Igκ C region Abs. For intracellular staining, cells were washed and resuspended in 0.5 ml of PBS, and fixed by adding 0.5 ml of a 4% paraformaldehyde solution in PBS for 20 min at room temperature in the dark. Cells were then washed with PBS and resuspended in 30 μl of anti-mouse and anti-human κ Abs diluted in saponin buffer (PBS, 0.5% BSA, 0.2% NaNo, 0.5% saponin; Sigma-Aldrich S-2149). Cells were incubated for 15 min at room temperature in the dark, then washed once with saponin buffer, and once with PBS/5% FBS. Cells were resuspended in PBS/5% FBS, and analyzed on a FACSCalibur (BD Pharmingen) with FlowJo 6.4.3 software.

ELISA detection of κ chimeric Abs

To detect chimeric hCκ/mCκ+ Abs in serum and cell supernatants, we developed the following ELISA. Briefly, 96-well Nunc-Immuno plates (Nalg Nunc International) were coated with 2 μg/ml goat F(ab’2), anti-human κ-chain polyclonal Ab (Southern Biotechnology Associates) in PBS containing 150 mM NaCl and 0.1% NaNo. Plates were washed once with PBS, 0.5% Tween 20, and blocked with PBS, 30% FBS, and 0.05% NaNa3 at 4°C overnight. Three-fold serial dilutions of mouse sera or cell supernatant in PBS, 1% BSA, and 0.05% NaNa3 were added to the wells. Standard Ab was cell culture supernatant of a dual κ hybridoma cell line (8G12) containing a mix of chimeric and nonchimeric hCκ/mCκ+ Abs at a starting total concentration of 1 μg/ml (as measured by ELISA for total IgM).

Plates were washed three times with PBS/0.5% Tween 20, and detected with an alkaline phosphatase (AP)-conjugated goat F(ab’2), anti-mouse κ-chain Ab (Southern Biotechnology Associates). ELISA plates were developed by addition of AP substrate (Sigma-Aldrich) in 0.1 M diethanolamine/0.02% NaNa3. Absorbance values at 405 nm were obtained by reading the plates on a Versamax ( Molecular Devices) ELISA reader, and the data was plotted in Microsoft Excel.

ELISPOT

To establish the presence of chimeric hCκ/mCκ+ Ab-secreting cells (ASCs), we developed the following ELISPOT assay. Spleen cells were isolated and depleted of CD43+ cells by magnetic cell sorting (Miltenyi Biotec) following manufacturer’s instructions. CD43-depleted purified B cells were cultured in complete Iscove’s medium supplemented with 5% PBS and 50 μg/ml LPS (Sigma-Aldrich) at 37°C in 5% CO2 for 48 h. Ninety-six well enzyme immunoassay plates (Costar, Corning Glass) were precoated with 2 μg/ml goat F(ab’2), anti-human κ-chain polyclonal Abs (Southern Biotechnology Associates) and subsequently blocked with 1% gelatin (Sigma-Aldrich) in PBS. Cultured cells were serially diluted and seeded in complete medium, and then incubated at 37°C for 6 h. Plates were washed with 0.05% Tween 20 in water, followed by three washes with PBS and 0.05% Tween 20. ASCs were detected by incubation with AP-conjugated goat F(ab’2), anti-mouse κ-chain Abs (Southern Biotechnology Associates). Plates were developed by adding 0.75 μg/ml ANTS (Sigma-Aldrich); human; h,

Analysis of B cell activation

CD43-depleted purified B cells (see above) were resuspended in complete Iscove’s cell culture medium at 2.5–5 × 106 cells/ml. Two milliliters of this cell suspension were cultured in 12-well plates at 37°C in 5% CO2 for 16 h with either 20 μg/ml LPS (Sigma-Aldrich); varying concentrations (1–10 μg/ml) of F(ab’2), rabbit anti-mouse IgM (Zymed Laboratories) polyclonal Abs; 15 μg/ml anti-CD40 mAb (FGK4.5) plus 50 ng/ml mouse rIL-4 (eBioscience); a combination of anti-CD40 (15 μg/ml), anti-IgM (10 μg/ml), and IL-4 (50 ng/ml), or 1 μg/ml goat F(ab’2), anti-human κ-chain polyclonal Abs (Southern Biotechnology Associates). Cultured cells were stained with anti-mouse Igκ, anti-human Igκ, and either anti-CD86 or anti-CD69 for flow cytometric analysis. For analysis of cell proliferation, spleen cells were isolated, washed in sterile PBS, and resuspended at 15 × 106 cells/ml in 37°C prewarmed PBS/0.1% BSA. CFSE (Carboxyfluorescein) was added at a final concentration of 2.5 μM, and the cell suspension was incubated in the dark for 10 min at 37°C. Cells were subsequently washed once with cold PBS/0.1% BSA, and once with cold

2 Abbreviations used in this paper: ASC, Ab-secreting cell; AP, alkaline phosphatase; FO, follicular; Igκ, human Igκ; int, intermediate; MZ, marginal zone; m, mouse; h, human.
complete cell culture medium. The cells were resuspended in cell culture medium at 2.5–5 × 10^6 cells/ml, and 2 ml was cultured in 12-well plates at 37°C in 5% CO₂ for 72 h with either 10 µg/ml Fab(IgG), rabbit anti-mouse IgM polyclonal Abs (Zymed Laboratories) or 3 µg/ml goat Fab(IgG), anti-human IgG polyclonal Ab (Southern Biotechnology Associates). Cultured cells were stained with anti-mouse IgG and anti-human IgG Abs for flow cytometric analysis.

**Generation of bone marrow chimera mice**

Bone marrow cells were isolated from Igkm/h and CB17 (Igkm/h) mice, mixed at equal ratios, and resuspended at 2 × 10^7 cells in sterile PBS. Recipient BALB/c or CB17 mice were irradiated with two doses of 400 rad, each given 3 h apart on the same day of cell transfer. A total of 2 × 10^7 cells in 100 µl of PBS was injected i.v. into each irradiated recipient, and mice were analyzed 9 wk later. At that time, flow cytometric analysis of PBLs stained with anti-human IgG and anti-mouse IgG Abs showed that animals were efficiently reconstituted with similar ratios of cells expressing the human and mouse κ C region.

**SRBC immunization**

SRBCs in Alsever’s solution (Colorado Serum) were washed three times with and resuspended in sterile PBS. Mice were injected i.p. with 200 µl of either 1 × 10^8 SRBCs or sterile PBS and sacrificed 6 days later when spleen and sera were collected for analysis of SRBC-specific Abs and ASCs by ELISA and ELISPOT, respectively, as previously described (27). In brief, 96-well immunoassay high-binding U-bottom plates (Greiner Bio-one) were coated with 150 µl/well 0.1% SRBCs in PBS for 1 h at room temperature. Glutaraldehyde, 0.25% in PBS, was added at 200 µl/well and incubated for 5 min to fix SRBCs to the plate. The plates were washed three times with PBS and blocked with PBS, 1% BSA/0.05% NaN₃ overnight at 4°C. Three-fold serial dilutions of mouse sera in PBS, 1% BSA/0.05% NaN₃ were added to the wells starting with a 1/20 dilution and incubated overnight at 4°C. Plates were washed three times with PBS and detected with either AP-conjugated goat anti-mouse IgG1 or AP-conjugated goat anti-mouse IgM Abs (Southern Biotechnology Associates). Detection of chimeric κ Ab was performed, as described above, in the ELISA section. Detection of dual κ Ab-secreting plasma cells was performed, as described above, in the ELISPOT section, with the exception that total spleen cells were directly plated without in vitro manipulation.

**Statistical analysis**

Statistical significance was assessed with a two-tailed Student’s t test with unequal variance; p < 0.05 was considered significant. Data are expressed as means ± SEM.

**Results**

**Dual κ-expressing B cells constitute 1.4–3% of the peripheral B cell population**

To determine the frequency and functional capacity of B cells expressing two κ-chains in mice with a wild-type Ig repertoire, we took advantage of the Igkm/h mouse strain engineered to carry the Igk gene exon encoding the C region of the human κ-chain in place of that of the mouse (23). These mice are hemizygous at the Igk locus, carrying both a mouse (mCK) and a human (hCK) Ck allele. Consequently, B cells of Igkm/h mice express Igk chains that possess either the mouse or the human C region, and these Ig L chains can be distinguished with specific Abs (23).

Previously flow cytometric studies showed that Igkm/h B cells expressing two types of κ-chains on the cell surface were ~6% of the total B cell population (23). Subsequent single-cell PCR and sequencing analyses, however, found that only 1.5% of B cells actually carried two productively rearranged Igκ genes revealing the presence of false-positive events in flow cytometry (23). Flow cytometric analysis may overestimate the actual frequency of dual-positive cells due to events binding to the C region of other Abs used in the same staining and/or to Fc receptors, and to the presence of cell doublets. In contrast, single-cell PCR analysis may under-estimate this frequency due to its low sensitivity and to the presence of false-negative events. Therefore, we revisited the frequency of dual κ B cells in Igkm/h mice using different approaches and included controls to account for the detection of false-positive events in each approach.

As a first approach, we performed flow cytometric analyses of single and dual κ-expressing B cells with combinations of different anti-human and anti-mouse κ Abs that were either whole molecules or Fab (or Fab’). It is important to note that because some H and κ-chains are unable to pair, there may exist Igk allelic-included B cells that are phenotypically excluded because they express an H chain that pairs with only one of the two κ-chains. Our analyses, however, were specific for κ-chains expressed on the cell surface, and κ surface expression occurs only upon κ- and H chain pairing. Therefore, these analyses detected only Igk allelic-included cells in which both κ-chains paired with the H chain. In addition, these analyses compared the frequency of dual κ-positive B cells in the spleen of Igkm/h mice with that of mixtures of wild-type Igkm/h (mκ/hκ') and homozygous Igkh/h (mκ/hκ') cells. These cell mixtures served as controls, because dual κ-positive events detected in these mixtures were true false positives. As shown in Fig. 1A (bottom two rows), the intact anti-mouse and anti-human κ mAbs cross-reacted with each other, causing a shift of the single κ-positive cell populations from their respective axes. Use of Fab Ab (top row in Fig. 1A) alleviated this artifact most likely because these reagents were monovalent and bound preferentially to the κ-chains expressed on the cells instead of those present in other staining Abs. Nevertheless, staining with either intact Abs or Fab identified a clear double-positive (mκ/hκ') cell population in Igkh/h mice. All Ab combinations also highlighted some false double-positive cell events, as demonstrated in the mix (m + h) of single-positive B cells. The number of false double-positive events increased when certain intact Abs were used, most likely due to the Abs binding to Fc receptors, but their frequency in Igkm/h + Igkh/h cell mixtures was always lower than that of dual-positive events observed in hemizygous mice (Fig. 1A). The frequencies of spleen dual κ B cells in hemizygous Igkm/h mice and Igkm/h + Igkh/h cell mixtures, determined by staining with Fab Abs, were 1.85 ± 0.2% (mean ± SE; n = 3) and 0.43 ± 0.07% (mean ± SE; n = 3), respectively (Fig. 1B), and the difference was statistically significant (p = 0.01). By subtracting the average frequency of false double-positive events obtained in the Igkm/h + Igkh/h cell mixtures from the mean frequency of dual-positive events in the Igkh/h cell populations, we found that ~1.4% of Igkh/h spleen B cells expressed two types of κ-chains on the cell surface when assessed by flow cytometry. This result is in strong agreement with the original single cell PCR analysis (23).

To verify the presence of dual κ-expressing B cells, we exploited the fact that these cells, upon differentiation by LPS into ASCs, could secrete chimeric κ Abs consisting of two H chains pairing with both distinct κ-chains in an individual dimeric 7S Ig molecule. Thus, we established a new method that detects chimeric κ-secreted Abs by using anti-human and anti-mouse Ck Abs in a sandwich ELISPOT assay. This assay detects dual κ B cells in which both κ-chains pair with the H chain. This is because two κ-chains are associated in the same secreted molecule only when each is bound to an H chain (28). This analysis, when performed on Igkh/h spleens, detected dual κ B cells expressing Ab molecules with human and mouse κ-chains (Fig. 1C). This result was significant because numbers of dual κ spots in Igkm/h + Igkh/h control cell mixtures were always lower than those found in Igkh/h cells (Fig. 1C). However, the low and variable frequency of LPS-responding B cells prevented the use of this method for quantification of dual κ-expressing B cells, although it was ideal for their identification.
As an alternative system to quantify dual $\kappa$-expressing B cells, $\text{Igkm}^{\text{m/h}}$ B cell hybridomas were generated and screened for the co-expression of human and mouse $\kappa$-chains. Two sets of hybridomas were independently generated from LPS-stimulated splenic $\text{Igkm}^{\text{m/h}}$ B cells and screened for secretion of $\kappa$ chimeric Abs by ELISA (data not shown). Clones identified by ELISA were subsequently analyzed for intracellular expression of human and mouse $\kappa$-chains by flow cytometry (Fig. 1D). This approach found 19 of a total 625 B cell hybridomas (3.0%) that clearly coexpressed distinct (human and mouse) $\kappa$-chains. Moreover, each of these hybridomas most likely expressed two $\kappa$-chain types that both paired with the corresponding H chain because these cells secreted chimeric $\kappa$ Abs (28).

In summary, depending on the type of analysis used, B cells allelically included at the $\text{Igk}$ loci and in which both $\kappa$-chains pair with the H chain were 1.4–3% of the total spleen B cell population in mice with a wild-type Ab repertoire.

FIGURE 1. Quantification of dual $\kappa$ B cells. A, Spleen cells from hemizygous $\text{Igkm}^{\text{m/h}}$ (m/h), wild-type $\text{Igkm}^{\text{m}}$ (m), and homozygous $\text{Igkm}^{\text{h}}$ (h) mice were analyzed for B220, hCk, and mCk expression by flow cytometry using the indicated Abs. A mixture of spleen cells from $\text{Igkm}^{\text{m}}$ and $\text{Igkm}^{\text{h}}$ mice (m+h) was analyzed in parallel to control for false double-positive events. Dot plots represent live B220$^+$ gated B cells. Numbers indicate frequencies of dual and single $\kappa$-expressing B cells. B, Average frequency of dual $\kappa$-expressing B cells (arithmetic mean ± SE, n = 3) obtained by flow cytometric analysis of three individual mice using anti-\kappa Fab/Fab’ Abs as shown in top row of A. C, ELISPOT analysis of dual $\kappa$ Ab-forming cells. CD43-depleted spleen B cells were cultured with 50 $\mu$g/ml LPS for 2 days and subsequently analyzed for expression of dual $\kappa$ Abs by ELISPOT. The graph represents the absolute number of dual $\kappa$-positive spots detected in 1 × 10$^6$ B cells from three mice. Each triangle represents an individual mouse, and the arithmetic mean is shown as a bar. D, Flow cytometric analysis of intracellular human and mouse $\kappa$-chain expression in two representative dual $\kappa$-expressing B cell hybridomas (m/h) and in control single $\kappa$-expressing hybridomas (m and h) from hemizygous $\text{Igkm}^{\text{m/h}}$ mice. B cell hybridomas were stained with anti-human and anti-mouse $\kappa$-chain intact Abs in saponin-containing buffer. Numbers indicate frequency of dual $\kappa$ cells.

FIGURE 2. Dual $\kappa$-expressing B cells are found in all mature B cell populations. A, Spleen B cells from $\text{Igkm}^{\text{m/h}}$ mice were analyzed for hCk and mCk, as well as CD21 and CD1d expression by four-color flow cytometry on a Cyan cytometer. Density plots represent the gating methodology used for the analysis of human $\kappa$ single-positive (hSP), human and mouse $\kappa$ double-positive (DP), and mouse $\kappa$ single-positive (mSP) B cell populations, in addition to the marginal (CD21$^{\text{low/}}$CD1d$^{\text{low}}$) and FO (CD21$^{\text{high}}$CD1d$^{\text{high}}$) B cell subsets. Numbers indicate relative frequencies of cells in gates for one representative experiment. B, Average frequency of spleen MZ B cells in single and dual $\kappa$-expressing B cell populations. Spleen cells from three individual $\text{Igkm}^{\text{m/h}}$ mice (m/h in figure) and three independent spleen cell mixtures from $\text{Igkm}^{\text{m}}$ and $\text{Igkm}^{\text{h}}$ mice (m+h in figure) were stained and analyzed as in A. The bar graph corresponds to the arithmetic mean and SE of the frequency of MZ B cells in $\text{Igkm}^{\text{m/h}}$ spleen B cell populations expressing human $\kappa$ (hSP), mouse $\kappa$ (mSP), or both human and mouse $\kappa$-chains (DP, m+h). The average frequency of MZ B cells in false dual $\kappa$ cell populations (DP, m+h) is also shown. C, Average frequency of MZ dual $\kappa$ events detected as in A and measured in $\text{Igkm}^{\text{m/h}}$ (m/h) and mixed $\text{Igkm}^{\text{m}}$ + $\text{Igkm}^{\text{h}}$ (m+h) spleen total lymphocyte populations. D, Peritoneal cavity cells from $\text{Igkm}^{\text{m/h}}$ mice were analyzed for hCk and mCk, as well as CD5 expression by flow cytometry. The average frequency (mean ± SE, n = 3) of B1a ($\text{CD5}^+$) cells in each individual single and dual $\kappa$-expressing B cell populations is represented in the graph.

**Dual $\kappa$ B cells differentiate into all mature B cell subsets**

Immature B cells can differentiate into three different mature B cell types, follicular (FO), marginal zone (MZ), and B1, that can be
distinguished by differential expression of cell surface markers as well as anatomical location. MZ and B1 B cells exhibit autoreactivity and polyreactivity (29, 30), and data from the 3H9/56R IgH knockin mouse model suggest that low-affinity autoreactive and haplotype-included B cells preferentially differentiate into MZ B cells (4). To determine the differentiation capability of dual \( B \) cells, we used flow cytometry to evaluate the distribution of these cells in the three mature B cell populations, and we compared this distribution with that of single \( B \) cells.

Four-color flow cytometric analyses were performed to identify dual and single \( B \) cells in the three mature B cell subsets (Fig. 2A). In the peritoneal cell population, FO and B1a IgH \( B \) cells were discriminated by three-color flow cytometry based on CD5 expression. By these analyses, dual \( B \) cells were found in the FO, MZ, and B1 mature B cell subsets (Fig. 2A and D). When the distribution of dual \( B \) cells in FO and MZ spleen B cell subsets was compared with that of single \( B \) cells (Fig. 2A and B), dual \( B \) cells appeared more enriched (up to 8-fold) in the MZ B cell population, supporting previous findings (4). However, because a similar enrichment in the MZ B cell subset was also observed with false dual \( B \)-positive events in Igkm/m Igh/h spleen cell mixtures (Fig. 2B) and among spleen cells from mixed Igkm/m Igh/h bone marrow chimeras (data not shown), these results were considered inconclusive. Nonetheless, the presence of dual \( B \) cells in the MZ B cell subset was supported by the fact that the frequencies of dual \( B \) events in the B cell population (Fig. 1B) and of dual \( B \) MZ

**FIGURE 3.** Dual \( B \) cells respond to antigenic and nonantigenic stimuli applied in vitro. A and B, CD43-depleted spleen \( B \) cells from Igkm/m mice were cultured with or without indicated concentrations of anti-IgM F(ab\(^\prime\))\(_2\) (αIgM in figure), anti-human \( \kappa \) L chain F(ab\(^\prime\))\(_2\) (αhL in figure), 20 μg/ml LPS, 15 μg/ml anti-CD40 plus 50 ng/ml IL-4, or a combination of anti-IgM and anti-CD40 plus IL-4 for 16–18 h and analyzed for hCc and mCc, as well as CD69 and CD86 expression by flow cytometry. The geometric mean of fluorescence intensity for both CD69 and CD86 in single human \( \kappa \) (hSP, ■, dual \( \kappa \) (DP, □), and single mouse \( \kappa \) (mSP, ▪) \( B \) cells are shown in this figure, which represents one of three independent experiments. C, Spleen \( B \) cells from Igkm/m mice were labeled with CFSE and subsequently cultured in either the presence or absence of 10 μg/ml anti-IgM F(ab\(^\prime\))\(_2\) (αIgM in figure) or of 3 μg/ml anti-human \( \kappa \) L chain F(ab\(^\prime\))\(_2\) (αhL in figure) for 3 days. At the end of culture, cells were stained for hCc and mCc, and analyzed for expression and CFSE levels by flow cytometry. CFSE levels are shown for human \( \kappa \) single-positive (hSP, thin line), dual \( \kappa \)-positive (DP, thick line), and mouse \( \kappa \) single-positive (mSP, dash line) gated cells. The shaded histogram indicates CFSE levels in mouse \( \kappa \) single-positive cells cultured in the absence of stimuli. Representative results from one of three independent experiments are shown. D, CD43-depleted spleen \( B \) cells from three different Igkm/m mice isolated and analyzed in three independent experiments were cultured with either 10 μg/ml anti-IgM F(ab\(^\prime\))\(_2\) or 1 μg/ml anti-human \( \kappa \) L chain F(ab\(^\prime\))\(_2\) (αIgM and αhL in figure) for 2 days and analyzed for the expression of CD69. The graph represents CD69 expression (geometric mean of fluorescent intensity) of dual \( \kappa \) (DP) \( B \) cells relative to that of single human \( \kappa \) (hSP) \( B \) cells. E, CD69 levels in DP and hSP \( B \) cells from one individual Igkm/m mouse prepared and treated, as described in D.
B cells in the total lymphocyte cell population (Fig. 2C) were significantly higher in Igk<sup>h</sup> than in Igk<sup>h</sup> + Igk<sup>l</sup> spleen cell preparations. In contrast to the MZ B cell population, the distribution of dual κ B cells in FO and B1 cell subsets was similar to that observed for single κ B cells (Fig. 2D).

In summary, these analyses indicate that dual κ-expressing B cells are present in all mature B cell populations without a specific bias.

**Dual κ B cells respond to antigenic stimuli in vitro**

Ig allelic exclusion is thought to be important for optimal B cell response to antigenic stimuli, implying that expression of more than one Ab specificity per B cell may impair the response to a particular Ag. To address this issue, we first compared the response of dual and single κ-expressing B cells to stimuli in vitro.

Magnetically sorted total spleen B cells from Igk<sup>h</sup> mice were stimulated in vitro for 16–18 h with a range of anti-IgM Ab concentrations to compare the responsiveness of single and dual κ-expressing B cells to BCR aggregation. B cells were also stimulated with anti-CD40 plus IL-4 alone or in combination with anti-IgM to determine cell response to surrogate Th cell signals, whereas treatment with LPS was performed to determine B cell responsiveness to TLR-4 ligation. Treated and nontreated B cells were analyzed by flow cytometry to evaluate changes in expression of two common activation markers, CD69 and CD86, and to distinguish single from dual κ-expressing cells. We found that dual κ-expressing B cells increased expression of CD69 and CD86 to levels comparable to those of single κ-expressing (either mouse or human) B cells when stimulated with suboptimal (<10 μg/ml) and optimal (10 μg/ml) doses of anti-IgM (Fig. 3A). Single and dual κ B cells also showed similar responses when stimulated with anti-CD40/IL-4 in the presence or absence of anti-IgM, and with LPS (Fig. 3B). Proliferative responses of single and dual κ-expressing B cells were also assessed. Spleen cells from Igk<sup>h</sup> mice were loaded with CFSE and stimulated with 10 μg/ml anti-IgM. At the end of the culture, cells were stained for mouse and human C<sub>κ</sub>, and the dilution of CFSE on single and dual κ cells was measured by flow cytometry. We found that dual κ-expressing B cells underwent proliferation to the same extent as single mouse κ- and human κ-expressing B cells in response to anti-IgM stimulation (Fig. 3C, left histogram). The sum of these data indicates that dual κ-expressing B cells are not refractory to antigenic and nonantigenic stimuli and respond similar to single κ-expressing B cells when the response is measured by changes in relatively early and late parameters of B cell activation.

When assuming equal pairing of each κ with the H chain, only 75% of the BCRs on each dual κ-expressing B cells can react with a specific Ag. Moreover, 50% of the BCRs on each of these cells consist of a single H chain paired with two different κ-chains and are chimeric, hence monovalent, with respect to Ag binding. Thus, whereas anti-IgM Abs can aggregate all the BCR molecules on the cell surface, a divalent reagent specific for only one of the two H + L chain combinations would theoretically aggregate only a fraction of these receptors. As seen by stimulation with suboptimal amounts (1 and 3 μg/ml) of anti-IgM Ab (Fig. 3A), reduced BCR cross-linking results in reduced B cell activation. Consequently, we hypothesized that dual κ B cells stimulated with Abs reacting with only one of the two κ-chains would be activated to a lower extent than cells stimulated with anti-IgM. To this end, we evaluated the responsiveness of single and dual κ-expressing B cells to anti-human Ig<sub>k</sub> (hIg<sub>k</sub>) Abs that cross-linked BCRs containing human κ-chains, but not receptors containing mouse κ-chains. For these experiments, we chose a dose of anti-hIg<sub>k</sub> Ab (1 μg/ml) that elicited responses in single hκ<sup>+</sup> B cells similar to those caused by a dose of 10 μg/ml anti-IgM Ab (Fig. 3A). We found that stimulation with 1 μg/ml anti-hIg<sub>k</sub> induced activation of dual κ-expressing B cells and single human κ-expressing B cells, but not of single mouse κ-expressing cells, demonstrating the specificity of this reagent. Despite undergoing activation, the average levels of CD69 (and to a lesser extent of CD86; data not shown) were consistently and significantly (p = 0.05) lower in dual κ-expressing B cells stimulated with anti-hIg<sub>k</sub> than in cells stimulated with anti-IgM, and when normalized to the response of single human κ-expressing B cells (Fig. 3, A and D). The diminished CD69 and CD86 response to anti-hIg<sub>k</sub> was the combined result of a (20–25%) reduced frequency of cells responding to stimulation and a (20–25%) lower level of CD69/CD86 expression by each responding cell (Fig. 3E and data not shown). Moreover, when analyzing a cell proliferative response to the anti-hIg<sub>k</sub> stimulus (Fig. 3C, right histogram), we noted that more than half of dual κ B cells proliferated as much as single human κ B cells, but the rest did not cycle and behaved like nonstimulated B cells. These results suggest that allotypic inclusion may reduce antigenic responsiveness.

A fraction of wild-type haplotype-included B cells might coexpress autoreactive and nonautoreactive Abs (5, 31) and, consequently, may exhibit impaired response to certain stimuli. To evaluate how coexpression of autoreactive and nonautoreactive BCRs may influence B cell response to antigenic stimuli, we took advantage of a previously established mouse model, the B1−8/3-83Igi,H-2<sup>b</sup> strain. We previously showed that 40% of B1−8/3-83Igi,H-2<sup>b</sup> spleen B cells express both the autoreactive (anti-K<sup>b</sup>) 3-83 and the nonautoreactive B1−8 Abs (5). Because the 3-83 BCR is down-modulated from the cell surface following autoantigen binding (5), we could not directly evaluate the response of 3-83 Ig<sup>-</sup> autoreactive B cells, but we indirectly assessed their function following the response of the entire B cell population. Thus, using this system, we evaluated induction of CD69 and CD86 expression as a readout of B cell response, and we compared frequency of responding B cells to anti-BCR and LPS stimulations in cells in which either one (on H-2<sup>b</sup>) or none (on H-2<sup>d</sup>) of the two H + L chain combinations were autoreactive. Spleen B cells from B1−8/
IgM stimulations, as expected following reduced BCR cross-linking, mouse Ig Abs in sera were determined by ELISA. Relative titers of chimeric (human/A mouse) Ig Abs in sera were determined by ELISA. Relative titers of chimeric human/mouse Ig Abs in sera (C) were determined by ELISA, and numbers of chimeric Igk Ab-secreting plasma cells in the spleen (D) were determined by ELISPOT. Results from four PBS and four SRBC (A and B) or seven PBS and eight SRBC hemizygous mice (C and D), and six PBS and six SRBC bone marrow chimera mice (A–D) are shown. Each symbol represents an individual mouse, and bars represent arithmetic means.

FIGURE 5. Dual κ B cells participate in a humoral immune response in vivo. Igkm/h mice (m/h, filled symbols) and control mixed bone marrow chimeras (m + h, open symbols) generated by mixing 1:1 wild-type Igkh/h and homozgyous Igkb/h bone marrow cells were immunized with either PBS (circles) or sheep RBC (squares) and analyzed 6 days postimmunization. The titers of SRBC-specific IgM (A) and SRBC-specific IgG1 (B) Abs in sera were determined by ELISA. Relative titers of chimeric human/mouse Igk Abs in sera (C) were determined by ELISA, and numbers of chimeric Igk Ab-secreting plasma cells in the spleen (D) were determined by ELISPOT. Results from four PBS and four SRBC (A and B) or seven PBS and eight SRBC hemizygous mice (C and D), and six PBS and six SRBC bone marrow chimera mice (A–D) are shown. Each symbol represents an individual mouse, and bars represent arithmetic means.

3-83Igk,H2d, B1-83/83Igk,H2d and wild-type mice were stimulated with a range of anti-IgM concentrations, or with LPS as a positive control. As shown in Fig. 4, 80–90% of B cells from all three strains responded equally to LPS and to the optimal 10 μg/ml anti-IgM BCR dose. In contrast, the frequencies of B cells that responded to the suboptimal amounts of 0.1 and 1 μg/ml anti-IgM Ab were lower than those responding to LPS and 10 μg/ml anti-IgM stimulations, as expected following reduced BCR cross-linking. Interestingly, however, >50% of the B1-83/83Igk,H2d B cells were activated by the suboptimal stimuli, a frequency that was even higher than those observed for wild-type and nonauto- reactive B1-83/83Igk,H-2d B cells. When looking at the response of dual BCR-expressing autoreactive B cells, we found that the frequency of B1-83/83Igk,H-2d B cells responding to BCR stimuli was >80% at 1 and 10 μg/ml anti-IgM Abs. Because 40% of these cells coexpress autoreactive and nonautoreactive H + L chain combinations, these results suggest that haplotype-included and autoreactive B cells are sensitive to BCR stimulation.

Dual κ B cells participate in an immune response in vivo

To determine whether dual κ-expressing B cells are able to participate in an immune response, we immunized Igkm/h mice with SRBCs and determined SRBC-specific and chimeric κ Ab production, as well as presence of ASCs 6 days after immunization. Bone marrow chimera mice generated with a 1:1 mixture of bone marrow cells from wild-type (Igkm/h) and homozgyous Igkb/h mice were similarly immunized and used as control. As shown in Fig. 5, A and B, intact Igkm/h (m/h) and Igkm/h + Igkb/h bone marrow chimera (m + h) mice responded similarly to SRBC immunization, as indicated by the presence of anti-SRBC IgM (Fig. 5A) and IgG1 (Fig. 5B) Abs. Detectable levels of chimeric κ Abs were measured in the sera of Igkm/h + Igkb/h bone marrow chimeras (Fig. 5C, m + h), most likely reflecting the presence of aggregates containing single mouse κ and human κ Ab molecules. Consistent with this hypothesis, these levels were similar in SRBC- and PBS-injected Igkm/h + Igkb/h bone marrow chimera mice. In contrast, chimeric κ Abs were significantly higher (p = 0.04) in increase in the number of dual κ-expressing plasma cells in Igkm/h SRBC-treated mice (Fig. 5D, m/h). This increase was not statistically significant due to the large variability in the number of ASCs in individual mice, but there was a clear trend with SRBC-immunized Igkm/h mice generating higher numbers of dual κ-expressing plasma cells than both PBS-injected Igkm/h mice and SRBC-immunized Igkm/min + Igkb/h bone marrow chimeras (Fig. 5D). These data strongly suggest that dual κ-expressing B cells can respond productively to Ag in vivo.

Discussion

In this study, we have quantified and analyzed the function of B cells that coexpress two κ-chains in mice carrying a wild-type V(D)J region repertoire under conditions that allowed us to distinguish dual from single κ-expressing B cells, i.e., in mice expressing an allotypic marker (Hck) at one of the Igk alleles. Although the existence of dual κ B cells has been previously disclosed using this same mouse strain, careful cell quantification and functional analyses were not performed at that time. Our study shows that replacement of one of the mouse Igk C region genes with the human counterpart does not affect VJ recombination at the Igk locus, confirming previous reports (33, 35). In this model, we found by flow cytometric analysis that 1.4% of Igkm/h splenic B cells coexpress two κ-chains on the cell surface. In contrast, by analyzing LPS-induced Igkm/h B cell hybridomas, we found that 3% coexpress two κ-chains. In both analyses, we detected dual κ cells in which both κ-chains paired with the H chain. This is because surface expression of L chains as well as secretion of oligomerized L chains are achieved only through pairing with H chains (28). Our hybridoma analysis, however, did not distinguish whether the original B cells expressed both κ-chains on the cell surface. Therefore, the difference in dual κ B cell frequency observed in flow cytometric and hybridoma analyses may be due to down-regulation of the cell surface of one of the two H + κ-chain combinations in vivo, a process that could be caused by autoregulatory binding in some dual κ B cells (5). In addition, there may also exist dual κ B cells that express lower levels of one of the two H + L chain combinations or in which one of the κ-chains does not pair with the H chain (genotypically included, but phenotypically excluded), but these cells would not be detectable in our assays. These cells would effectively produce only one type of Ab. Thus, our findings confirm that haplotype exclusion in B cells is quite stringent and contrasts the situation in T cells, in which up to 30% of cells carry two TCR α-chains (34). Yet despite their low numbers, haplotype-included B cells could still have physiologic and pathologic functions in immune responses, which warrants further analyses.

The specificity of the BCR influences whether immature B cells differentiate into mature B cells and whether they become FO, MZ, or B1 B cells (29, 30, 35). Our results indicate that dual κ-expressing B cells differentiate into FO, MZ, and B1 cells without any
specific bias, a finding in agreement with the differentiation of κ/λ isotypic-included B cells (20). These findings suggest that dual κ B cells express a repertoire of Ag receptor specificities that is not skewed toward a specific Ag response. Previous studies using an anti-DNA Ig knockin mouse model showed that low avidity haplotype-included and autoreactive B cells differentiate primarily into MZ B cells (4), but this was not the case in an anti-MHC class I model in which the autoreactive B cells had high avidity for the autoantigen (5). In light of this, it will be important to determine the autoreactive potential of dual L chain-expressing B cells in the MZ and FO B cell subsets of wild-type mice, with the hypothesis being that the MZ cell population harbors an increased frequency of low avidity, and possibly anti-DNA, self-reactive cells.

It has been speculated that Ig haplotype exclusion is essential to generate functional B cells (7). In this study, we show that dual κ-expressing B cells are functional in vitro, because they respond to optimal and suboptimal doses of anti-IgM, LPS, and anti-CD40/IL-4, with increased expression of activation markers and proliferation comparable to single κ-expressing B cells. In contrast, we also provide some evidence indicating that dual κ B cell responsiveness to reagents that bind only one of the two H + L chain combinations is reduced relative to maximum BCR cross-linking. In fact, when B cells were stimulated with Abs that could bind only human κ-chains, activation of dual κ-expressing B cells did not reach the levels observed in single κ (human)-expressing cells, or in cells stimulated with anti-IgM Abs. The possibility exists that some of the nonresponsive cells were false dual κ events that expressed the mouse and not the human κ-chain. However, reduced levels of CD69 and CD86 expression in responding cells suggest that the responsiveness of dual κ B cells to reagents that bound to only one of the H + κ chain combinations was less than optimal. It is possible that dual κ B cells with impaired responsiveness to anti-human Igκ Abs were cells in which the H + κ chain combination was self-reactive. Presently, however, we favor the possibility that the diminished responsiveness to anti-human Igκ was due to a reduction in the number of BCR complexes that were aggregated by these Abs on the surface of dual κ B cells. Although the anti-human Igκ Abs used in our analysis were divalent, the spatial interspersion of H + κX and H + κκ combinations within BCRs on the surface of dual κ B cells may have caused reduced receptor aggregation and, therefore, stimulation.

Despite the fact that haplotype-included B cells demonstrated a diminished responsiveness to anti-human Igκ stimulation in vitro, dual κ-expressing B cells appeared capable of participating in an immune response to SRBC in vivo. We found a clear increase of κ chimeric serum Abs and dual κ-expressing plasma cells following immunization of Igκ<sup>neo</sup> hemizygous mice, which was not seen in bone marrow chimera mice carrying a mix of wild-type and homozygous Igκ<sup>neo</sup> cells. Moreover, in follow-up studies, spontaneous B cell hybridomas were generated from the spleen of SRBC-immunized Igκ<sup>neo</sup> mice and tested for the expression of anti-SRBC and of dual κ chimeric Abs by ELISA and flow cytometry. Five of these B cell clones expressed Abs that were both chimeric and SRBC reactive (data not shown). These results indicate that Igκ allelic-included B cells can participate in a SRBC-specific immune response. Thus, our findings strongly suggest that haplotype inclusion does not prevent functional Ab responses in vivo and are consistent with previous studies of haplotype-included B cells. In one of these studies, LK3 Igκ transgenic mice immunized with 4-hydroxy-3-nitrophenyl-acetyl generated Ag-specific chimeric Abs containing both the transgenic and an endogenous L chain and whose 4-hydroxy-3-nitrophenyl-acetyl-reactive portion was constituted by the endogenous and not the transgenic κ-chain (9). In another study, Giachino et al. (15) identified κ/λ dual L chain-expressing human B cells carrying somatic mutations in either or both IgL chain genes, a finding suggesting that these cells had participated in an Ag-driven immune response. The possibility still remains that haplotype-included B cell clones might have a reduced competitive capacity in the course of an immune response in vivo. This could result in the preferential expansion and differentiation (e.g., Ig class switch, affinity maturation) of haplotype-excluded B cell clones, as previously suggested (8). If so, haplotype-included B cells would be less represented in memory B and plasma cell populations, a possibility under investigation.

At present the origin of haplotype-included B cells has not been established. Haplotype inclusion may originate via random leakiness in the process of allelic/isotypic exclusion when, for example, both alleles or loci rearrange simultaneously (36). Alternatively, haplotype-included B cells may arise via the process of receptor editing (31, 37, 38). Studies with different Ig knockin mouse models have indicated that a fraction of editing autoreactive B cells acquires a novel specificity without disposing of the original autoreactive specificity, thus becoming haplotype included (4–6). There is also the possibility that haplotype-included B cells arise through receptor revision, a process that is associated with autoimmunity and by which novel Ig gene rearrangement events occur in mature B cells, most likely during the germinal center reaction (39). Because the majority of primary Ig gene rearrangements encode autoreactive specificities (21, 22), and because haplotype inclusion can support differentiation and survival of autoreactive B cells, there is a significant chance that haplotype-included B cells in mice with wild-type Ab repertoire coexpress autoreactive and nonautoantibody specificities regardless of which pathway has led to their development. Whether allotype-included B cells that carry autoreactive specificities exist in wild-type mice remains to be established, but their finding would solicit the question of whether these cells are capable of functional responses. Using the B1-NR383Ig, H2<sup>nu</sup>- 1g κ knockin mouse model, we have preliminarily assessed the in vitro response of B cells coexpressing autoreactive and nonautoantibody specificities to BCR aggregation. We found that these cells display normal in vitro responses to anti-IgM stimulation. At present it is unclear whether these cells express sufficient levels of autoreactive BCR to mediate an effective anergic signal, or are rather ignorant to the presence of the autoantigen due to dilution of the autoreactive BCRs with nonautoantibody receptors. Nevertheless, our results indicate that autoreactive haplotype-included B cells are responsive to BCR aggregation in vitro. It remains to be established whether autoreactive haplotype-included B cells can also be functionally activated in vivo to secrete autoantibodies, which is an area currently under investigation.

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