

Receptor editing and genetic variability in human autoreactive B cells

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The mechanisms by which B cells undergo tolerance, such as receptor editing, clonal deletion, and anergy, have been established in mice. However, corroborating these mechanisms in humans remains challenging. To study how autoreactive human B cells undergo tolerance, we developed a novel humanized mouse model. Mice expressing an anti-human Ig κ membrane protein to serve as a ubiquitous neo self-antigen (Ag) were transplanted with a human immune system. By following the fate of self-reactive human κ^+ B cells relative to nonautoreactive λ^+ cells, we show that tolerance of human B cells occurs at the first site of self-Ag encounter, the bone marrow, via a combination of receptor editing and clonal deletion. Moreover, the amount of available self-Ag and the genetics of the cord blood donor dictate the levels of central tolerance and autoreactive B cells in the periphery. Thus, this model can be useful for studying specific mechanisms of human B cell tolerance and to reveal differences in the extent of this process among human populations.

B lymphocytes are essential cells in establishing immunity, yet are also known contributors to autoimmune diseases. At least half of newly generated B cells are self-reactive (Grandien et al., 1994; Wardemann et al., 2003), and various selection checkpoints are enforced along B cell development and maturation pathways to increase immune function in host defense while preserving self-integrity (Shlomchik, 2008; Goodnow et al., 2010). Over the past several decades, we have acquired a greater understanding of how this selection operates, but more so in mice than in humans. BCR transgenic (Tg) or knock-in mouse models, in which the majority of the B cells harbor a single specificity that can be traced, have greatly aided in elucidating mechanisms of murine B cell selection (reviewed in Goodnow et al., 1995, 2010; Ait-Azzouzene et al., 2004; Pelanda and Torres, 2006, 2012; Kumar and Mohan, 2008; Shlomchik, 2008). These studies have shown that developing, self-reactive mouse B cells have several potential fates: one is to ignore antigen (Ag) if it is either sequestered or at a concentration too low for reactivity, a second is to become anergic (i.e., nonfunctional), a third is to undergo receptor editing, and a fourth is to undergo apoptosis. A fifth fate is to undergo positive selection to low-avidity self-Ags, an outcome accompanied by the differentiation into marginal zone or B1 B cells

(Hayakawa et al., 1999; Martin and Kearney, 2000; Wen et al., 2005). Which particular mechanism is invoked depends on both the strength of the signal the self-reactive BCR receives and the developmental state of the cell (Goodnow et al., 1995; Kouskoff et al., 2000; Qian et al., 2001; Ait-Azzouzene et al., 2004; Hippen et al., 2005; Wen et al., 2005; Diz et al., 2008; Andrews et al., 2013). Moreover, depending on the location of the self-Ag, tolerance is defined as central (i.e., in the bone marrow) or peripheral (i.e., in other tissues).

A criticism of using BCR Tg or knock-in mice for studying B cell selection is that these models hasten B cell development, restrict the B cell repertoire, and, sometimes (e.g., in some conventional Ig Tgs), express nonphysiological levels of BCR. These issues have been addressed by creating mice that express an Ig κ reactive self-Ag, enabling studies of tolerance in B cells developing with a wild-type antibody (Ab) repertoire (Ait-Azzouzene et al., 2005). This and other similar Tg models have confirmed that even wild-type murine B cells use deletion, anergy, and receptor editing for the establishment of tolerance (Ait-Azzouzene et al., 2005; Ait-Azzouzene et al., 2006; Duong et al., 2010, 2011; Ota et al., 2011).

The mechanisms that operate in humans to implement B cell tolerance have been more difficult to dissect, as human bone marrow tissue is less readily accessible, and determining the fate of any particular B cell with its own unique specificity is quite challenging. Therefore, human B cell tolerance studies have focused on measuring frequencies of a panel of

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Abbreviations used: Ab, antibody; Ag, antigen; AP, alkaline phosphatase; CB, cord blood; EC, extracellular; HSC, hematopoietic stem cell; hu-mouse, humanized mouse; IC, intracellular; MFI, mean fluorescence intensity; qPCR, quantitative PCR; RT, room temperature; Tg, transgenic.

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defined autoreactive or polyreactive B cell specificities mainly in the blood and in few bone marrow samples of healthy individuals or patients with autoimmunity (reviewed in Meffre and Wardemann, 2008; Meffre, 2011). Although these studies confirm that selection processes occur during human B cell development and with checkpoints similar to those established in mice, they have done little to determine the exact mechanisms of tolerance induction. This is particularly true for mechanisms of central B cell tolerance.

Immunodeficient mice transplanted with human hematopoietic stem cells (HSCs) provide a tool to study the human immune system in greater depth (Manz and Di Santo, 2009; Ito et al., 2012; Shultz et al., 2012). By using immunodeficient mice of the BALB/c-Rag2^{null}IL2Rγ^{null} strain (BRG or BALB/c-DKO), we have previously established a robust humanized mouse (hu-mouse) model for the analysis of human B cells and their development (Lang et al., 2011, 2013). Aiming to investigate mechanisms of human B cell tolerance, in this study we modified the BRG strain by introducing a ubiquitous synthetic neo self-Ag reactive with the Igκ⁺ fraction of human B cells. We then followed the fate of the “self-reactive” human κ⁺ cells relative to the nonautoreactive λ⁺ cells and measured the level of tolerance induction in animals reconstituted with a human immune system.

The results reveal the phenotype of human B cells while they undergo central tolerance and the mode of tolerance induction. They also indicate that our model can be used to explore differences in tolerance sensitivity among the human population.

RESULTS

Generation of Hck Tg mice

To study mechanisms of tolerance induction in developing human B cells, we developed a novel Tg mouse model similar to one created to investigate tolerance of wild-type murine B cells (Ait-Azzouzene et al., 2005). The novel transgene, which we refer to as Hck, encodes a membrane-bound neo self-Ag in the form of the single chain Fv anti-human Ig-Cκ HP6053 Ab tethered to a rat IgG1 constant region that is in turn linked to an MHC class I transmembrane peptide (Fig. 1 A). A ubiquitin promoter was used to direct the expression of the transgene to all tissues. Two Tg lines (Hck4 and Hck7) were created in a (BALB/c × DBA/2)F₁ genetic background and backcrossed 10 generations onto BALB/c. These mice were then crossed to immunodeficient BRG mice to generate BRG Hck4 and Hck7 animals that lack mouse T, B, and NK cells and can be used as recipients of human immune systems (Traggiai et al., 2004; Legrand et al., 2008; Manz and Di Santo, 2009; Lang et al., 2011). In these Hck⁺ hu-mice, human B cells can detect the self-Ag only in trans as the transgene is solely expressed on mouse host cells.

We verified the presence of the transgene on mouse host cells by PCR (not depicted) and measured the extent of its expression by flow cytometric analyses. There was consistently higher expression of the Hck protein in the Hck4 relative to the Hck7 line (Fig. 1 B). Moreover, expression of

Hck varied between cell types, being the lowest on GR1⁺ cells, intermediate on nonhematopoietic (mCD45⁺) cells, and the highest on GR1⁺ hematopoietic (mCD45⁺) cells, which are exclusively of myeloid origin in BRG mice. Furthermore, we observed higher Hck Ag expression on liver and kidney cells relative to cells from spleen and bone marrow tissues (Fig. 1 B), with potential implications for the establishment of B cell tolerance. Differential expression of Hck in the two Tg lines was confirmed in bone marrow tissue by immunofluorescent histology (Fig. 1 C). Hck Tg mice of both lines also exhibited soluble Hck protein (cleaved or secreted from cells) in their sera (Fig. 1 D), at concentrations of ~25 ng/ml. These data demonstrate the establishment of two severely immunodeficient mouse strains expressing on the surface of their cells and in their serum an Ag specific for human κ⁺ B cells.

Igκ⁺ human B cells undergo central tolerance in Hck Tg hu-mice

To test whether Hck could function as a tolerogenic self-Ag, we initially bred Hck (BALB/c × DBA/2) Tg mice to mice that carry a human Cκ allele (Casellas et al., 2001) and produced animals in which roughly half of the murine B cell population expressed human Cκ, whereas the other half expressed mouse Cκ. Flow cytometric analyses indicated a selective absence of human Cκ⁺ B cells, but not of mouse Cκ⁺ and λ⁺ cells (Fig. 2 A), demonstrating that Hck is specific for the human Cκ protein and capable of inducing B cell tolerance.

To investigate the effect of the Hck self-Ag on human B cells, we bred heterozygous Hck Tg BRG mice with non-Tg BRG mice to generate litters in which ~50% of the pups expressed the neo self-Ag. Neonatal (1–3 d old) Hck⁺ or non-Tg littermates were transplanted with human CD34⁺ HSCs, and the fate of the developing human B cells in the lymphoid organs of adult hu-mice was followed as previously described (Lang et al., 2011, 2013). Human B cells were analyzed by flow cytometry based on the CD19 pan-B cell marker. Additionally, CD20 and CD10 were used to differentiate developing B cell subsets in the bone marrow (with CD20⁺ being mostly pre-B cells and CD20⁺ being mostly immature/transitional B cells) and in the spleen (CD10 is expressed on transitional B cells and not on mature B cells), respectively (Fig. 2 B; Gorantla et al., 2007; Choi et al., 2011; Lang et al., 2013; Villaudy et al., 2014). Initial studies of tolerance were performed with the Hck4 Tg line, which expresses higher levels of the neo self-Ag. We reasoned that if clonal deletion were the overwhelming mechanism of B cell tolerance, we would find a reduction of ~50% in the number of B cells in Hck⁺ mice relative to Hck[−] littermates, especially in the spleen. It is important to preface that the hu-mouse model suffers from extensive variability in the amount of human chimerism (Lang et al., 2011, 2013). In spite of this experimental variability, similar numbers of pre-B (CD19⁺CD20[−]) and immature/transitional (CD20⁺) B cells were observed, on average, in the bone marrow of Hck⁺ and Hck[−] hu-mice, suggesting there was no gross arrest in maturation or death of B cells developing in the presence

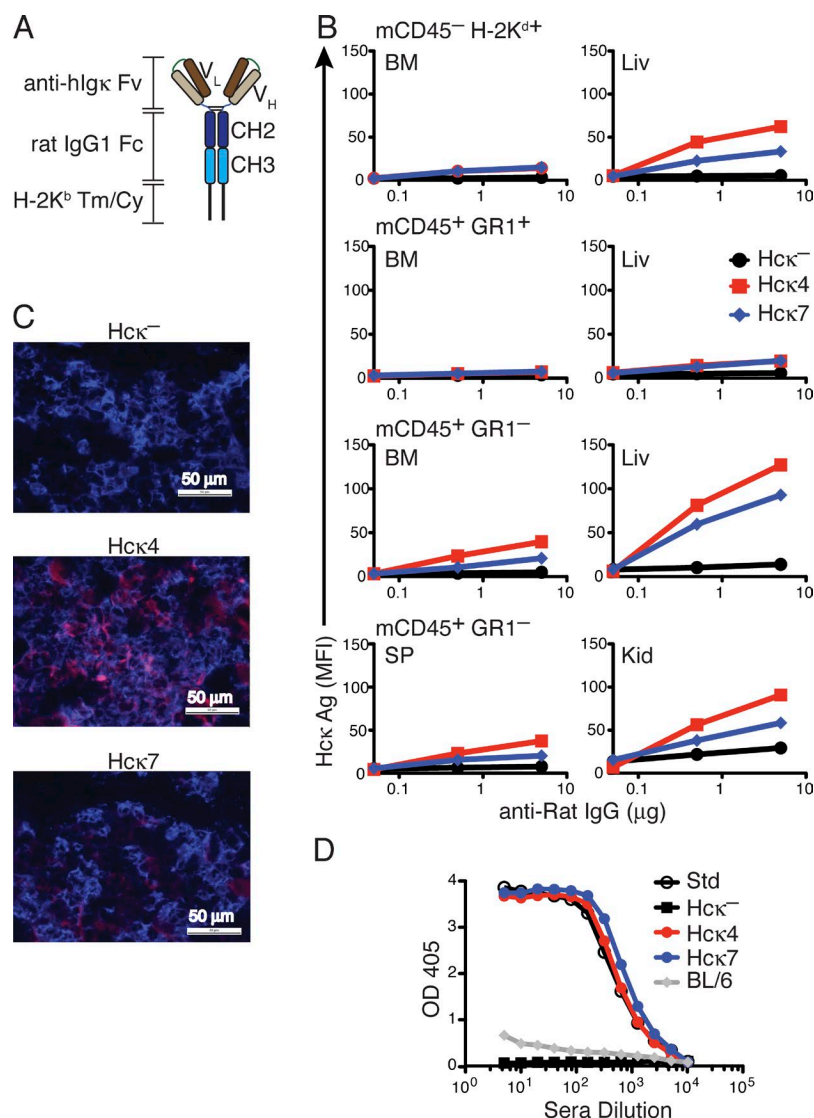


Figure 1. A novel Tg mouse model expressing a neo anti-human Igκ self-Ag. (A) Schematic diagram of the neo self-Ag: mouse anti-human Igκ Fv, from hybridoma HP6053, tethered to the CH2 and CH3 domains of the rat IgG1 constant region, linked to the transmembrane and cytoplasmic tail regions (Tm/Cy) of H-2K^b. (B) Expression of the Hck transgene in negative control (Hck^{-/-}) and two Tg lines (Hck4 and Hck7). Bone marrow (BM), spleen (SP), liver (Liv), and kidney (Kid) cells prepared from intact (nonhumanized) Hck^{-/-}, Hck4, and Hck7 BRG mice were analyzed for the presence of the neo self-Ag by staining with Ab to rat IgG1 at 10-fold serial dilutions. Abs to mCD45, H-2K^d (MHC class I), and GR1 were used to distinguish cell subsets. (C) Bone marrow tissue sections of Hck^{-/-}, Hck4, and Hck7 intact BRG mice were stained with Abs to rat IgG1 (in red) to detect Hck Ag and to GR1 (in blue) to detect murine granulocytes. (D) Sera collected from Hck^{-/-}, Hck4, and Hck7 intact BRG and negative control C57BL/6 mice analyzed by ELISA for soluble Hck protein with anti-rat IgG1 Abs. A rat IgG1 served as positive control to quantify Hck concentrations. Data in each panel are representative of a minimum of three independent experiments with *n* = 2.

of the neo self-Ag (Fig. 2 C). In contrast, the numbers of CD20⁺ B cells in the spleen of Hck⁺ hu-mice were decreased by 30%, on average, relative to those of Hck^{-/-} mice. A similar 30% reduction in cell numbers was observed in the transitional (CD10⁺) B cell population (Fig. 2 C), suggesting the difference in total B cell numbers was not caused by increased survival or proliferation of mature B cells in Hck^{-/-} hu-mice. Although this difference in transitional B cell numbers was not statistically significant, likely because of extensive variability in chimerism, the lower numbers of B cells in the spleen but not the bone marrow of Hck⁺ hu-mice relative to Hck^{-/-} controls suggests a subset of developing autoreactive B cells underwent clonal deletion between the immature and transitional B cell stages, at a checkpoint that has been previously reported in an equivalent model of murine B cell tolerance (Ait-Azzouzene et al., 2005).

To determine whether autoreactive κ⁺ B cells were regulated in Hck⁺ Tg mice, B cells were tracked by surface

staining for κ and λ IgL chains. In Hck^{-/-} control mice, κ to λ cells were in roughly similar proportions, and their ratio did not change with B cell development (Fig. 2, D and F). In the Hck⁺ mice, in contrast, κ IgL chain expression was minimally detected in B cell subsets of both bone marrow and spleen (Fig. 2, D and F). Nevertheless, the proportion of Igλ⁺ B cells was significantly increased only in the spleen of Hck⁺ hu-mice compared with Hck^{-/-} mice (Fig. 2, D and F). The fact that in the bone marrow the numbers of developing B cells (Fig. 2 C) and the proportion of Igλ⁺ cells (Fig. 2, D and F) was similar between Hck⁺ and Hck^{-/-} hu-mice suggested that Igκ⁺ cells were still present but not expressing the BCR on the surface. Indeed, intracellular (IC) staining with anti-human κ Abs revealed a significant population of κ⁺ B cells in the bone marrow of Hck⁺ mice (Fig. 2 E) that was only slightly smaller than the corresponding population in Hck^{-/-} mice (Fig. 2 F, third and fourth graphs). This was true for both the CD20⁺ cell subset, which comprises a small number of

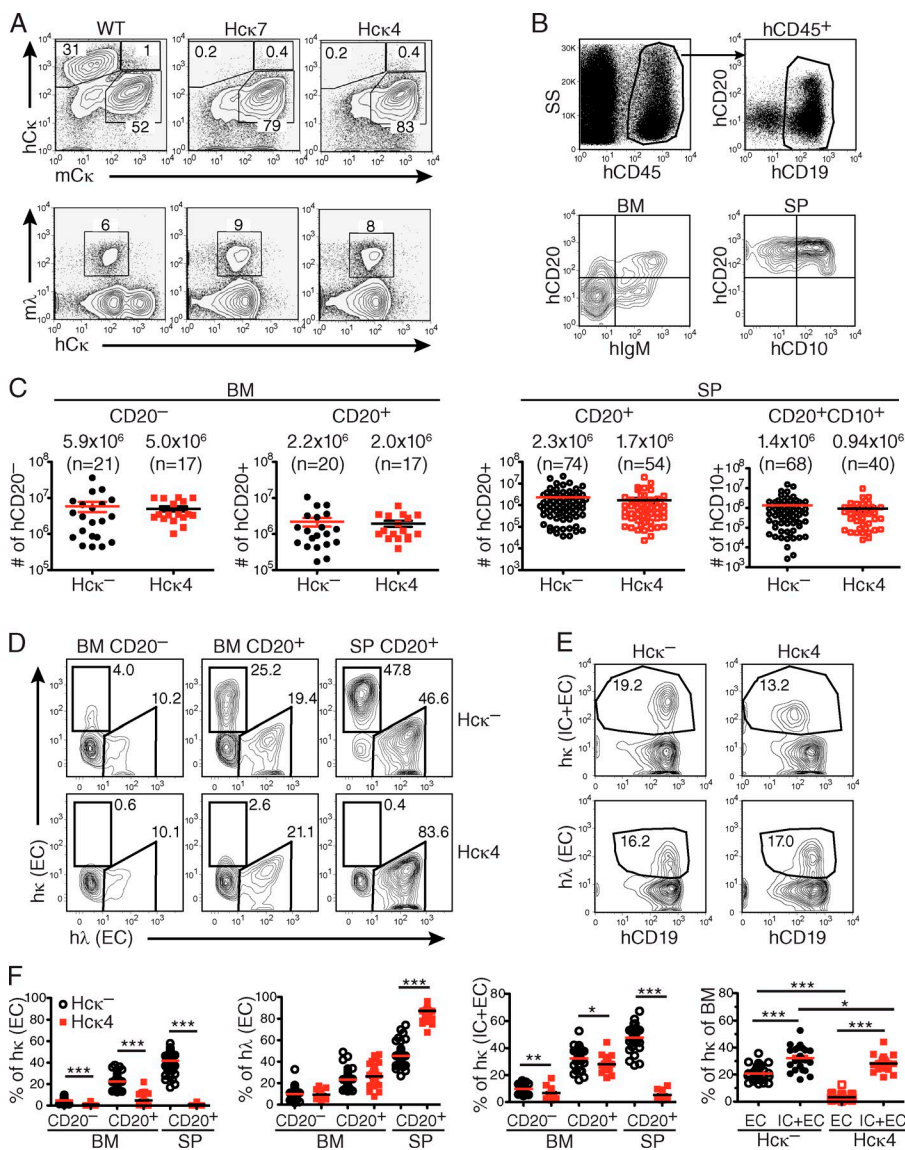


Figure 2. Hck⁺ hu-mice show a reduction of κ^+ and an increase of λ^+ B cells. (A) Mice were bred to carry one allele of human *Ck* and one of mouse *Ck* in the presence or absence of the human *Ck* reactive (Hck4 or Hck7) transgene. Spleen B220⁺ gated (mouse) cells were analyzed for the expression of mouse κ , human κ , or λ light chains. Data are representative of at least two independent experiments with $n = 1$. (B, top) Typical gating of human bone marrow B cells in BRG hu-mice, 12–16 wk after HSC transplant. (bottom) Representative analyses of CD20 and IgM expression or CD20 and CD19 expression on bone marrow (BM) and spleen (SP) B cells, respectively, from BRG hu-mice 12–16 wk after HSC transplant. Cells were gated as hCD45⁺hCD19⁺. (C) Absolute numbers of human B cells in the indicated B cell subsets of bone marrow and spleen from Hck⁻ and Hck4 hu-mice. Generally, bone marrow CD19⁺CD20⁻ cells represent pre-B and some immature B cells, bone marrow CD19⁺CD20⁺ cells are immature/transitional B cells, spleen CD20⁺ cells are total B cells, and spleen CD20⁺CD19⁺ cells are transitional B cells. Each symbol represents an individual hu-mouse; bars represent mean \pm SEM. (D) Flow cytometric analysis of EC κ and λ expression in bone marrow and spleen B cells from Hck⁻ and Hck4 hu-mice. (E) Flow analysis of CD19 and either IC + EC κ or EC λ in bone marrow B cells from Hck⁻ and Hck4 hu-mice. (F) Cumulative results from flow cytometric analyses shown in D and E. Data in each of B–F are representative of a minimum of 10 independent experiments with $n = 1$ –5. Each symbol in C and F represents an individual hu-mouse, and bars are arithmetic means. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$.

immature B cells, and the CD20⁺ B cell subset, which mainly consists of immature and transitional B cells. In contrast, IC κ^+ B cells were rare in the spleen of Hck⁺ mice (Fig. 2 F, third graph). These data indicate that κ^+ B cells undergo tolerance in response to the neo self-Ag and that most of the tolerance induction occurs centrally, in the bone marrow.

Human B cells down-regulate CD19 and internalize their BCR during central tolerance

To characterize the mechanisms of B cell tolerance, we next investigated the phenotype and developmental state of autoreactive human B cells by flow cytometry. We stained bone marrow B cells from Hck⁺ hu-mice for IC κ as well as for the maturation markers BAFFR, CD40, and IgD, to visualize the autoreactive cells and to determine their developmental stage, respectively. During human B cell development, acquisition of CD19 occurs before that of CD20 (Fig. 2 B), whereas

expression of BAFFR occurs concomitantly to that of IgM and is followed by the expression of CD40 and IgD, among other developmentally regulated markers (not depicted; Lang et al., 2013). The Ig κ^+ autoreactive B cells exhibited increased expression of CD40 and BAFFR similar to the expression of nonautoreactive (κ^- or λ^+) immature B cells (Fig. 3 A). In contrast, IgD, which is normally up-regulated on cells past the immature stage, was not expressed on autoreactive B cells (Fig. 3 A). As expected, because of BCR internalization, IgM was also minimally detected on IC κ^+ B cells of Hck⁺ mice relative to λ^+ cells in the same mice and all B cells in Hck⁻ mice (Fig. 3, B and C). In addition, there was a marked down-regulation of CD19 on autoreactive B cells (Fig. 3 D), which was confirmed at the cellular level by a significantly lower CD19 mean fluorescence intensity (MFI) on IC κ^+ cells (Fig. 3 E), whereas at a population level, it was represented by a higher percentage of CD19^{low} cells in Hck⁺

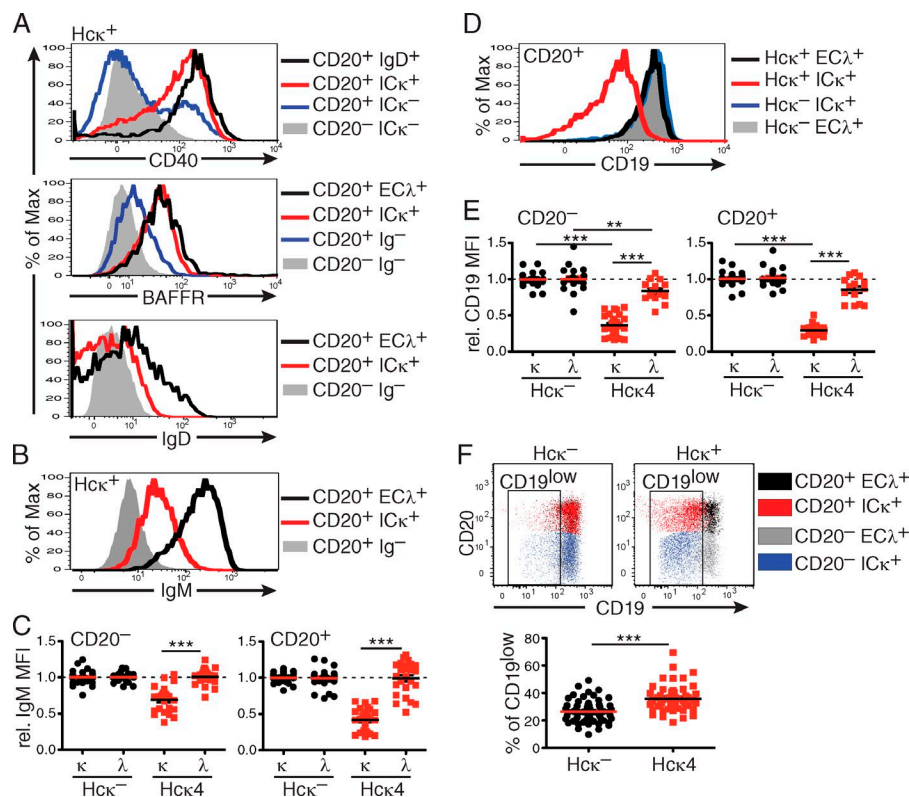


Figure 3. Phenotype of autoreactive B cells in the bone marrow of hu-mice. (A) Human B cells in bone marrow of Hck⁺ hu-mice were gated based on hCD45 and hCD19 expression and separated based on the expression of CD20, λ , and IC κ . Representative expression of CD40, BAFFR, and IgD are shown for the indicated B cell subsets. (B–E) Relative expression of IgM (B and C) and CD19 (D and E) on IC κ ⁺ or EC λ ⁺ cells from the indicated populations in the bone marrow of Hck⁺ and Hck⁺ hu-mice. The IgM and CD19 MFI values in Hck4 mice were normalized to those of the corresponding cell populations in Hck⁺ mice analyzed on the same day. (F) Plots show the gating strategy used to differentiate CD19^{low} from CD19^{hi} B cells in bone marrow. The graph shows the percentage of human B lineage cells with CD19^{low} expression in the bone marrow of Hck⁺ and Hck4 hu-mice. Data are representative of 3–12 independent experiments for each panel with $n = 1$ –5. Each symbol in graphs represents measurements from an individual hu-mouse, and bars show arithmetic means. **, $P < 0.001$; ***, $P < 0.0001$.

hu-mice relative to Hck⁺ hu-mice (Fig. 3 F). Thus, our data indicate that, upon encountering a membrane-bound self-Ag in the bone marrow tissue, human B cells internalize their BCR and down-regulate CD19.

Human autoreactive B cells undergo receptor editing in hu-mice

One of the mechanisms of central B cell tolerance in mice is receptor editing, which operates by secondary IgL chain gene recombination events in immature B cells. Characteristics of B cells undergoing editing are increased IgL gene rearrangement, higher expression of *Rag1* and *Rag2* genes, and lower levels of CD19 (Tiegs et al., 1993; Duong et al., 2011). Given the low CD19 expression on bone marrow κ ⁺ B cells, the negligible reduction of splenic B cell numbers and the increased λ ⁺ B cell frequency in Hck⁺ hu-mice, we predicted that developing B cells in these mice were actively undergoing receptor editing. We tested this prediction by first measuring *RAG* expression in sorted bone marrow B cell fractions of Hck⁺ hu-mice (Fig. 4 A). Indeed, we found that the CD19^{low} cells of Hck⁺ hu-mice, which are IC κ ⁺ and therefore carry an autoreactive Ag receptor, expressed significantly higher *RAG1/2* mRNA levels than IgM⁺CD19^{hi} cells in the same mice (Fig. 4 B), which are λ ⁺ and therefore generally nonautoreactive in our model. The *RAG1/2* levels of CD19^{low} cells were similar to those of IgM⁺CD19^{hi} cells, which are pro-B and pre-B cells actively rearranging Ig H and L chain genes for the first time and, thus, functioning as a positive control

for *RAG* expression. To determine whether differences in *RAG* expression were also present when B cells developed in Hck⁺ versus Hck⁺ hu-mice, we analyzed gene expression levels in total bone marrow B cells. Indeed, a significant difference in *RAG1/2* expression was observed when comparing total bone marrow B cells enriched from Hck⁺ and Hck⁺ hu-mice (Fig. 4 C). We further analyzed this cell population to assay IgL gene rearrangement events with a PCR assay that measures λ excision circles, circular DNA molecules excised from the IgL locus with each V λ rearrangement and that remain in B cells as nonreplicating episomes (Fig. 4 D). These circles, moreover, have been shown to correlate with receptor editing in mice (Tiegs et al., 1993; Ait-Azzouzene et al., 2005). Our analysis revealed significantly increased levels of V λ -J λ rearrangements in bone marrow B cells of Hck⁺ hu-mice compared with Hck⁺ hu-mice (Fig. 4 E).

Finally, we reasoned that if receptor editing at the Ig κ locus was more extensive in Hck⁺ than Hck⁺ hu-mice, we should observe altered V κ and J κ usage among the V κ -J κ rearrangements detected in the λ ⁺ splenic B cell population. Sequencing of the V κ -J κ rearrangements from λ ⁺ B cells of Hck⁺ hu-mice revealed significantly increased frequencies of J κ 5 and decreased frequency of J κ 1 relative to those in λ ⁺ B cells from Hck⁺ hu-mice (Fig. 4 F). This bias usage of proximal and distal J κ segments is consistent with receptor editing. Moreover, sequences from Hck⁺ hu-mice displayed significantly increased usage of V κ 1 gene family members. Overall, results from these analyses indicate that

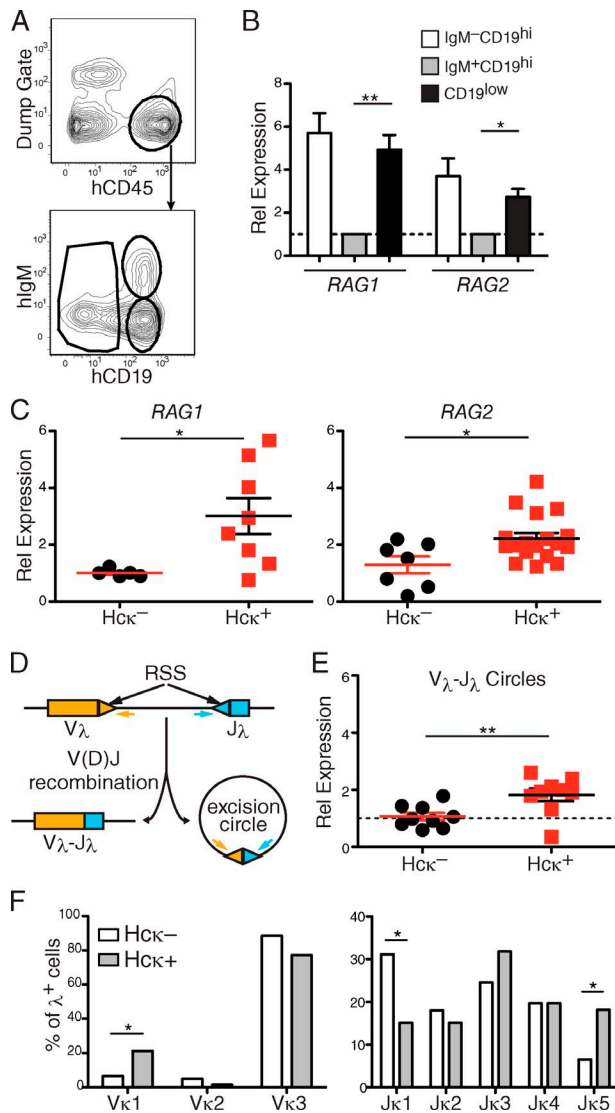


Figure 4. Receptor editing in human autoreactive B cells. (A) Representative gating of Hck⁺ bone marrow cell populations sorted for the analyses of *RAG1/2* gene expression. The dump gate represents mCD45⁺hCD3⁺hCD11c⁺ cells. (B) Real-time PCR analysis of *RAG1* and *RAG2* mRNA levels in the three cell populations of Hck⁺ mice (including both Hck4 and Hck7) shown in A: IgM⁺CD19^{hi} (immature, nonautoreactive), IgM⁺CD19^{hi} (pre/pro-B), and CD19^{low} (immature, autoreactive). *RAG1* and *RAG2* levels were first normalized to *GAPDH* and then to the corresponding *RAG1/2* levels of the IgM⁺CD19^{hi} population (set at 1). (C) *RAG1* and *RAG2* mRNA levels in B cells enriched from the bone marrow of Hck⁻ and Hck⁺ hu-mice. *RAG1* and *RAG2* levels in Hck⁺ samples were first normalized to *GAPDH* and then to mean *RAG1/2* levels in Hck⁻ samples. (D) Schematic of V_λ-J_λ excision circles and of primers designed to detect them. (E) Relative levels of V_λ-J_λ excision circles in genomic DNA from B cells enriched from the bone marrow of Hck⁺ and Hck⁻ hu-mice. V_λ-J_λ circles signals were normalized to *GAPDH* levels in each sample. Data in B–E are from three to four independent experiments for each panel and in which RNA or DNA was pooled from one to three hu-mice for each group. (F) Frequency of Vκs (Vκ1, Vκ2, and Vκ3 families) and Jκs in Vκ-Jκ rearrangements cloned from genomic DNA of CD20⁺λ⁺ spleen cells. Cells were sorted from the spleen of Hck⁻

receptor editing accompanies the development of autoreactive human B cells in hu-mice.

Break in central B cell tolerance is detected in peripheral tissue

Our data provide evidence for a robust central tolerance process in human B cells developing in hu-mice in the presence of a ubiquitous membrane-bound self-Ag. To determine whether this tolerance was complete, we next measured human Igκ in serum. We have previously shown that we can detect high levels of human IgM and IgG in the sera of hu-mice older than 12 wk (Lang et al., 2013). Upon similar analyses, we found comparable concentrations of human IgM and IgG in the sera of Hck⁻ and Hck⁺ animals (Fig. 5 A). However, there was a profound selective loss of Igκ (both IgM and IgG) in Hck⁺ mice (Fig. 5 B), supporting the notion of strict central tolerance induction. Nevertheless, small amounts of Igκ were detected in some Hck⁺ mice, suggesting that a fraction of autoreactive B cells were able to escape tolerance induction in the bone marrow. To further explore this matter we first questioned whether the measure of serum Igκ in Hck⁺ mice was underestimated by its binding to the neo self-Ag expressed on the surface of host mouse cells. This was experimentally addressed by injecting sera from Hck⁻ hu-mice, containing human Ig, into intact (i.e., nonhumanized) Hck⁺ and Hck⁻ animals and then testing for the presence of human Ig in the blood the next day. Although equivalent levels of IgG and Igλ were detected, there was a 22-fold reduction in the amount of Igκ in sera of Hck⁺ relative to Hck⁻ mice (Fig. 5 C), suggesting it was sequestered in tissue. To directly investigate whether Igκ was bound to cells in Hck⁺ hu-mice, we stained bone marrow cells with Abs to mCD45 and human Igκ and Igλ. In a small fraction of hu-mice (two of nine analyzed from different cohorts), hIgκ but not hIgλ was detected on mCD45⁺ cells (Fig. 5 D), indicating that a portion of the Igκ secreted by B cells in Hck⁺ hu-mice is removed from the circulation by binding mouse tissue. Thus, the total amount of Igκ measured in Hck⁺ hu-mice can be an underrepresentation of the actual Igκ present in the mouse. By using ELISPOT, Igκ-secreting plasmablasts were readily observed within the lymphoid tissue of some Hck⁺ hu-mice at lower or even comparable numbers to λ⁺ plasmablasts (Fig. 5 E). Overall, these data indicate that although a robust central tolerance process was established in the majority of hu-mice, a breach in tolerance could be detected in a fraction of these animals.

and Hck⁺ hu-mice in two independent experiments, with cells pooled from three mice for each sample. The data are from 61 Hck⁻ and 66 Hck⁺ individual sequences. Bars in B, C, and E represent mean ± SEM. *, P < 0.05; **, P < 0.001.

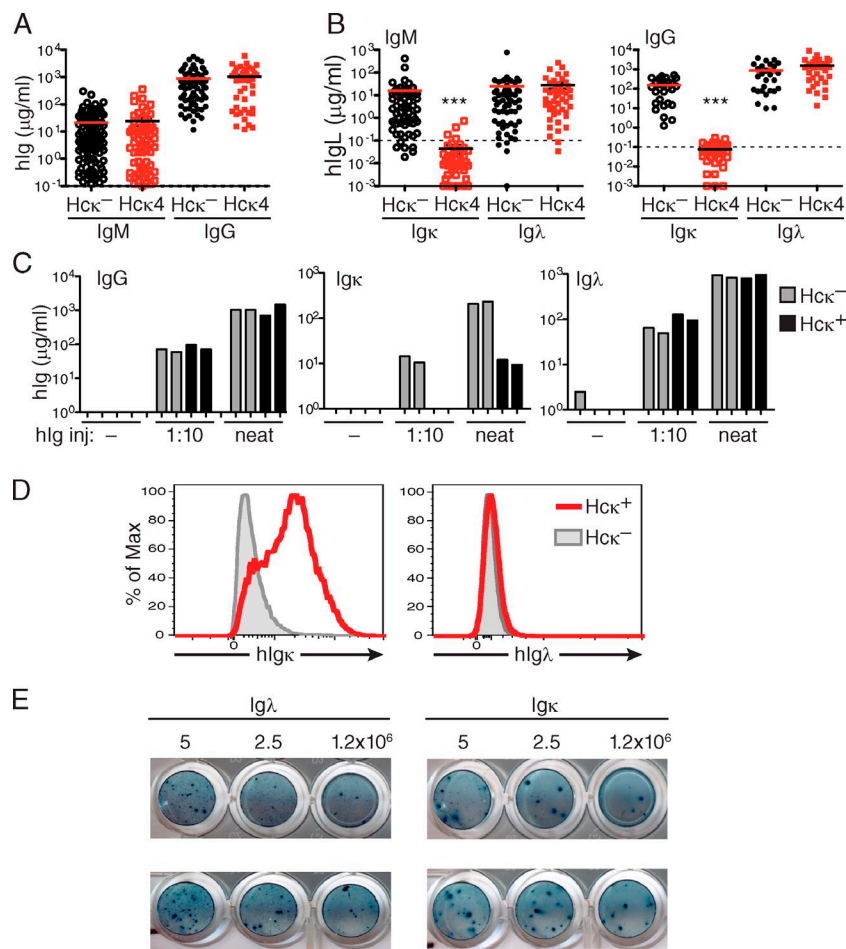


Figure 5. Production and detection of human Igκ and Igλ in hu-mice. (A and B) Total human IgM and IgG (A) and Igκ and Igλ isotypes of hIgM and hIgG (B) in the sera of Hck⁻ and Hck4 hu-mice. Each data point represents an individual hu-mouse, and bars show means. Data are from a minimum of 20 experiments with $n = 1-5$. ***, $P < 0.0001$. (C) Sera collected from Hck⁻ hu-mice were pooled and injected i.v. neat or at a 1:10 dilution ($\sim 200 \mu\text{l}$ per mouse) into intact (nonhumanized) Hck⁻ or Hck4 mice. The next day, the sera from the injected or uninjected control mice were collected and hIgG, hIgκ, and hIgλ levels measured by ELISA. Bars represent data from a single mouse and are the mean of four to five independent ELISA measurements. The data are representative of three independent experiments with $n = 1-4$. (D) Detection of human Igκ and Igλ on mouse CD45⁺ bone marrow cells from Hck⁻ and Hck4 hu-mice. Data are representative of two out of nine mice analyzed in three independent experiments. The other seven mice did not show detectable staining. (E) Spleen cells from Hck4 hu-mice were tested for the presence of Igκ and Igλ Ab secreting cells by ELISPOT. Data are from two 19-wk-old Hck4 littermates and are representative of two independent experiments.

Dose of self-Ag affects modality and extent of human B cell tolerance

In mice, the mechanisms of B cell tolerance can vary depending on the amount of self-Ag. To test whether similar differences exist in human B cell tolerance, we compared tolerance induction in the Hck4 and Hck7 Tg lines, which express different levels of self-Ag (Fig. 1). Although we found similar numbers of immature/transitional B cells in the bone marrow and spleen of the two Tg lines, the numbers of CD20⁺ B cells in the spleen of Hck7 hu-mice tended to be lower, on average, than in Hck4, but this difference was not statistically significant (Fig. 6 A). However, Hck7 splenic B cell numbers were significantly different from those of Hck⁻ control mice (Hck⁻: $2.34 \times 10^6 \pm 0.43$, $n = 74$; Hck7: $1.15 \times 10^6 \pm 0.24$, $n = 39$; $P = 0.02$). In addition, although the proportion of B cells expressing κ on the surface of bone marrow B cells was similar in Hck4 and Hck7 hu-mice, there was a significant increase in B cells expressing λ in the Hck4 hu-mice or expressing IC κ in Hck7 hu-mice, both in bone marrow and spleen (Fig. 6 B). Furthermore, a greater and more consistent down-regulation of CD19 was observed in both CD20⁻ and CD20⁺ B cells from Hck4 hu-mice, and this was in spite of similar levels of Igκ internalization in the two

Tg lines (Fig. 6 C). In the sera of both Hck4 and Hck7 hu-mice, similar low levels of Igκ and high levels of Igλ were observed, with a trend of higher λ production in the Hck4 line (Fig. 6 D). The reduced frequency of λ⁺ cells and increased proportion of B cells expressing Igκ intracellularly in Hck7 hu-mice suggest a reduced prevalence of receptor editing and increased failure of central tolerance when B cells develop in the presence of lower self-Ag levels. Overall, these data provide evidence that human B cells undergo tolerance with different modalities in the presence of varying levels of self-Ag.

Genetic contribution to human B cell tolerance

Our data indicate that central B cell tolerance mechanisms in human B cells are similar to those established in mouse models (i.e., clonal deletion and receptor editing with associated CD19 and BCR down-regulation). However, through the course of these experiments, we observed considerable variability in tolerance induction among individual hu-mice. We reasoned that genetics of the cord blood (CB) donor might account for some of this variability. To assess the effect of the genetic variability of the donor, we analyzed several tolerance parameters in sets of Hck⁻, Hck4, and Hck7 hu-mice that were grouped according to their CB donor. We

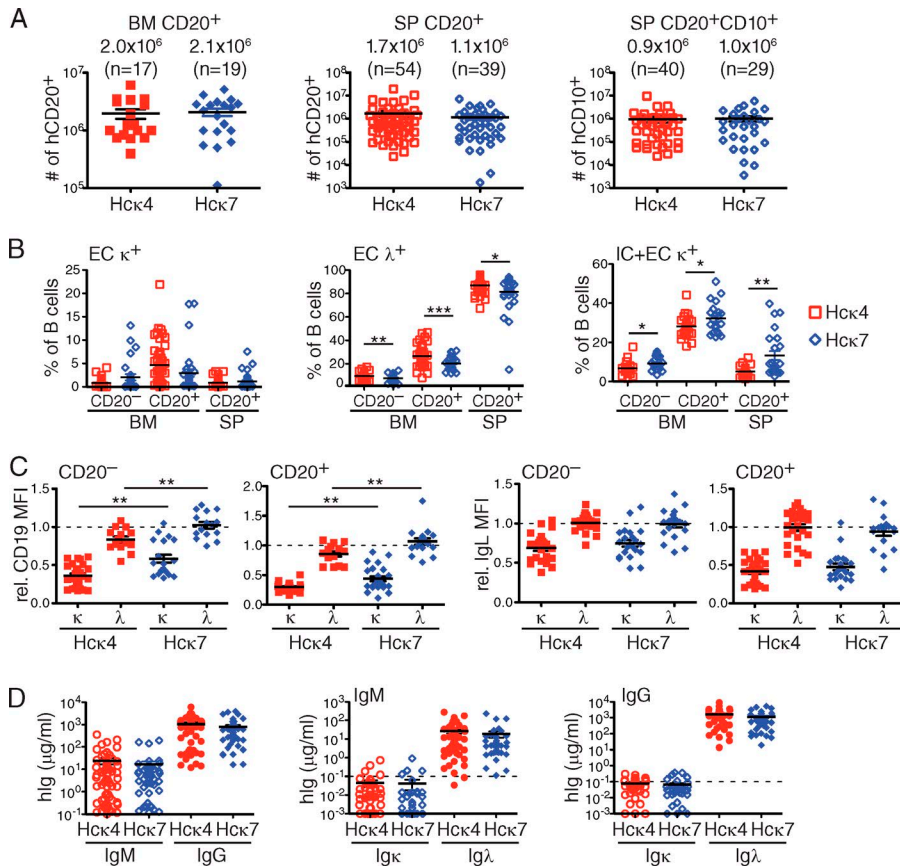


Figure 6. Tolerance differences in human B cells developing in Hck4 and Hck7 hu-mice. (A) Numbers of CD20⁺ in the bone marrow (BM) and of CD20⁺ (total B cells) and CD20⁺CD10⁺ (transitional B cells) in the spleen (SP) of Hck4 and Hck7 hu-mice. Numbers on top of each graph indicate the mean cell number and the size of the sample. (B) Frequencies of B cells expressing EC κ⁺ (left), EC λ⁺ (middle) or IC + EC κ⁺ (right) in the indicated populations of CD19⁺ gated human B cells from Hck4 or Hck7 hu-mice. (C) Relative expression of CD19 (left panels) and Igκ and λ light chains (right panels) on CD20⁻ or CD20⁺ B cells in the bone marrow of Hck4 and Hck7 hu-mice. Expression levels in Hck⁺ samples from different experiments were normalized by dividing the MFI of each sample by the mean MFI in Hck⁻ control mice in the same experiment. (D) Concentrations of total hIgM and hIgG (left) or of either κ or λ isotype of IgM (middle) and IgG (right) in the sera of Hck4 and Hck7 hu-mice. In all graphs, each symbol represents an individual hu-mouse, and bars represent the mean ± SEM. Data shown in each panel were obtained from a minimum of 10 independent experiments with $n = 1-5$. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$.

further reasoned that if the assay was stringent enough to detect differences in tolerance between CB donors, this difference would be more significant in Hck⁺ recipients than in Hck⁻ recipients because a large variation in Hck⁻ hu-mice would indicate an inherent assay variability or one less specific to tolerance induction. Using this approach, we found that the number of B cells, the frequency of surface κ⁺ and λ⁺ B cells, and the concentration of serum hIgκ had similar variability in Hck⁻ and Hck⁺ hu-mice generated with different CBs and, thus, these assays were not sensitive enough to detect differences with respect to tolerance (Fig. 7 A and not depicted). However, the frequencies of B cells displaying IC κ (Fig. 7 B) or low CD19 (Fig. 7 C) were less variable among Hck⁻ recipients and significantly more variable among Hck4 and Hck7 hu-mice generated with different CB donors. The proportion of CD19^{low} B cells, in particular, showed the largest variation (20–55%) among Hck7 hu-mice cohorts, suggesting that the reduced level of self-Ag in Hck7 animals provides a higher resolution in resolving individual differences in tolerance induction. To explore this possibility further, we analyzed Hck4 and Hck7 hu-mice in a scatter plot displaying the frequency of IC κ⁺ cells in relation to their CD19 MFI. As shown in Fig. 7 D, all Hck4 hu-mice fall within a narrow distribution in this scatter plot. In contrast, although some Hck7 hu-mice fall within the same Hck4 distribution, others are clear outliers, and this distinction depends

on the CB donor. Significant differences in tolerance parameters were also observed when comparing most Hck⁺ to Hck⁻ hu-mouse groups generated with the same CB, as shown for the percentage of CD19^{low} cells (Fig. 7 E), suggesting that our model can be used to assess tolerance at the individual level. Overall, our data indicate that Hck7 Tg hu-mice can be used to detect and explore differences in B cell tolerance induction in the human population.

DISCUSSION

We have used a novel hu-mouse model expressing a human-specific surrogate self-Ag to formally demonstrate that developing human B cells use receptor editing as a mechanism of central B cell tolerance. Central B cell tolerance in hu-mice is stringent but incomplete. Although the selection of autoreactive B cells into the periphery is rare, variations in the extent of tolerance were observed and shown to depend on the amount of self-Ag as well as the individual genetics of the source of CB.

To date, most studies of human B cell tolerance have focused on limited repertoire analyses of B cell subsets present in peripheral blood (Meffre and Wardemann, 2008; Meffre, 2011). These studies have been invaluable in establishing the presence of tolerance checkpoints, but they have been limited to a poorly defined set of self-Ags without a clear understanding of how these Ags directly affect B cells in vivo. In

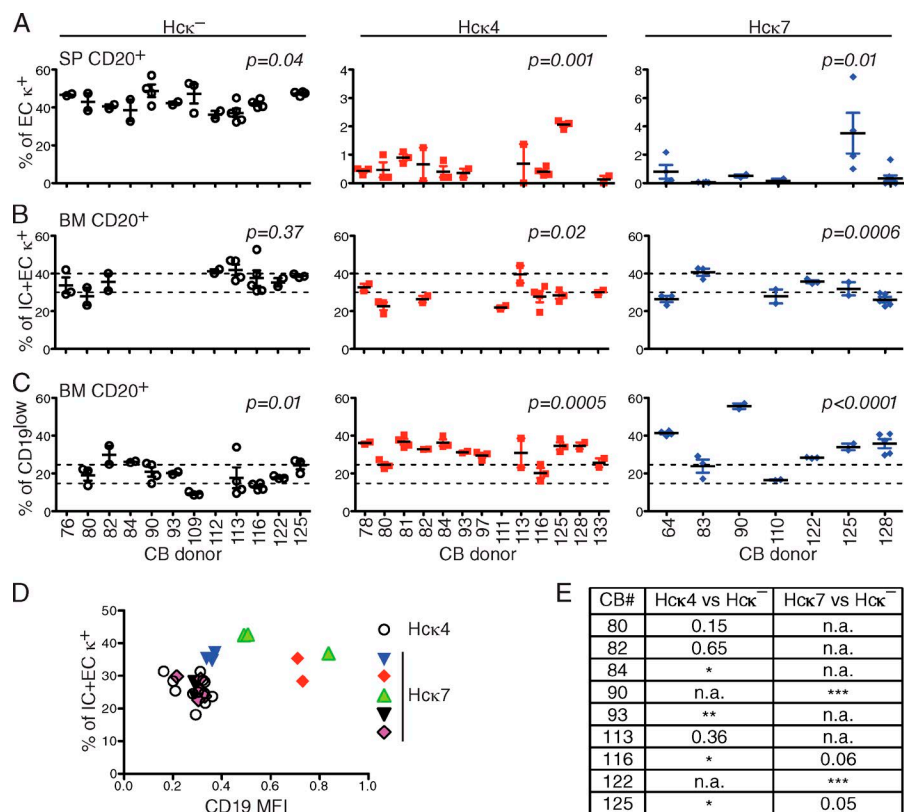


Figure 7. Differences in tolerance among hu-mice generated from different CB donors. (A–C) The percentage of spleen (CD20⁺) B cells with EC κ expression (A) and of bone marrow (CD20⁺) B cells with IC (+EC) κ expression (B) or CD19^{low} expression (C) in Hck⁻ (left), Hck4 (middle), and Hck7 (right) hu-mice. Each data point represents an individual hu-mouse, and hu-mice are grouped according to the CB donor. CB donors are identified by an ID number on the x axis. Bars represent mean \pm SEM. Not every parameter was measured in every CB group, as signified by the absence of symbols. The dotted lines (in B and C) are visual references for normal levels in Hck⁻ mice. The p-values indicate differences across CB samples within each type of mouse recipient (Hck⁻, Hck4, or Hck7). (D) Scatter plot analysis of the percentage of IC κ^+ B cells relative to their expression (MFI value) of CD19 in the bone marrow CD20⁺ B cell population of Hck4 and Hck7 hu-mice generated with different CB units. Each symbol represents an individual hu-mouse. Hck4 hu-mice generated with different CBs are represented by the same symbol (open circle). Hck7 hu-mice are represented with closed symbols and different colors correspond to distinct CB units. (E) Paired Student's *t* test analysis of differences in the percentage of CD19^{low} bone marrow cells between Hck⁻ and Hck⁺ hu-mice generated with individual CBs. Numbers in the second and third columns correspond to p-values: *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$. "n.a." indicates not available. Data in each panel were obtained over the course of at least seven independent experiments with $n = 1$ –4.

fact, although a significant reduction in the frequency of autoreactive clones in the human B cell population of the blood relative to the bone marrow has been clearly established, it has not been possible so far to determine whether this, at least in part, is caused by receptor editing, a tolerance mechanism that dominates in the mouse. This is mainly because of an inability to distinguish human autoreactive B cells within the total B cell population. Some studies investigating receptor editing in human B cells have only been able to assess this process indirectly (Wardemann et al., 2004; Schoettler et al., 2012; Kalinina et al., 2014). In other studies, differential levels of recombining sequence rearrangements and of distal J κ usage in peripheral human B cell populations (Lee et al., 2000; Ng et al., 2004; Panigrahi et al., 2008) have provided stronger support to the presence of receptor editing, but these findings have been highly correlative and did not exclude that a selective process was responsible for the observed repertoires.

Our study differs from those discussed in the previous paragraph by the ability to directly investigate human B cells while they undergo tolerance toward a defined mem-

brane-bound Ag. This feature has allowed us to examine whether human autoreactive B cells display the telltale signs of receptor editing. Indeed, we have observed higher *RAG* expression and V λ -J λ DNA excision circles in bone marrow B cells of hu-mice that express the neo self-Ag (Hck⁺ hu-mice) relative to cells in control animals. Moreover, bona fide autoreactive immature B cells, which we show display low levels of CD19 coreceptor and internalized Ig κ BCR, exhibited higher levels of *RAG1/2* mRNAs than nonautoreactive immature B cells in the same animals. The phenotype of autoreactive human B cells from Hck⁺ hu-mice is remarkably similar to that of murine immature B cells undergoing receptor editing (Tiegs et al., 1993; Pelanda et al., 1997; Ait-Azzouzene et al., 2005; Verkoczy et al., 2007; Duong et al., 2011). Expression of the Hck self-Ag was also associated with a skewing of the Ig κ repertoire in peripheral cells. Circulating λ^+ B cells normally bear V κ -J κ rearrangements that are not expressed (even when in-frame) as a result of recombining sequence-mediated inactivation of the Ig κ locus (Bräuninger et al., 2001), an event that takes place during B cell

development. Therefore, these V κ -J κ rearrangements are not subjected to peripheral selection events. The V κ -J κ rearrangements isolated from λ^+ B cells of H $\kappa\kappa^+$ hu-mice displayed a reduced use of the proximal J κ 1 and increased use of the distal J κ 5, a finding consistent with an increased frequency of secondary Ig κ rearrangements and receptor editing. In addition, these rearrangements displayed increased usage of V κ 1 gene family members relative to controls, suggesting a difference in the rearrangement process of developing autoreactive B cells. The V κ 1 genes (which are the most abundant among V κ s) are interspersed in the V κ locus, and a bias usage does not provide evidence of progressive secondary recombination events. We compared the usage of distal versus proximal V κ gene segments (not depicted) without finding a statistically significant difference between sequences isolated from H $\kappa\kappa^+$ and H $\kappa\kappa^-$ hu-mice. We argue, though, that the lack of difference is caused by a the small sample population (around 60 sequences per group) relative to the number of functional V κ elements (~20 in the V κ proximal cluster) and the likelihood that receptor editing is also frequent in H $\kappa\kappa^-$ hu-mice (indeed, CD19^{low} cells were also observed in the bone marrow of H $\kappa\kappa^-$ hu-mice). Furthermore, the finding that a fraction of the V κ -J κ rearrangements cloned from λ^+ B cells of H $\kappa\kappa^+$ hu-mice were in-frame (i.e., they expressed a κ chain during B cell development) does not appear to be compatible with “clonal deletion” being the only mechanism of central tolerance. Finally, numbers of transitional B cells were not statistically different in the spleen of H $\kappa\kappa^+$ and H $\kappa\kappa^-$ hu-mice. In their sum, these data demonstrate that human B cells that develop in the bone marrow of hu-mice activate receptor editing upon encountering a membrane-bound self-Ag.

Although we cannot exclude that our observations are valid only when human B cells develop in hu-mice, there are reasons to believe our findings can be generalized to B cells developing physiologically in humans. This is because human B cell development in hu-mice proceeds similarly to that of humans (Lang et al., 2013), and decades of murine studies have indicated that central B cell tolerance is a B cell-intrinsic process (Melamed et al., 1998; Verkoczy et al., 2005; Teodorovic et al., 2014) and, thus, does not require factors expressed by other cell types. Differences have been described in the development of B cells from fetal and adult precursors, with fetal precursors giving rise preferentially to B1a B cells (Yuan et al., 2012; Zhou et al., 2015). Thus, it is possible that our findings are restricted to newborns and hu-mice established with CB-derived HSCs and might not extend to adults and animals generated with HSCs from bone marrow. However, we find this possibility unlikely because of the following reasons: (a) human B cell precursors developing in our hu-mice did not express CD27 (Lang et al., 2013), a marker associated with fetal B cells (McWilliams et al., 2013), and did not prevalently differentiate into B1 cells (unpublished data), and (b) the mouse-specific version of the H $\kappa\kappa$ Ag causes negative selection in all B cell compartments, including B1 B cells (Ait-Azzouzene et al., 2005).

In spite of the presence of receptor editing during B cell development, there was a (nonstatistically significant) trend toward reduced transitional B cell numbers in the spleen of H $\kappa\kappa^+$ hu-mice. This difference likely reflects the death of those cells unable to successfully edit their receptors. Clonal deletion as a secondary event has been described in mice when the autoreactivity cannot be easily edited away (Halverson et al., 2004; Ait-Azzouzene et al., 2005; Ait-Azzouzene et al., 2006; Doyle et al., 2006). In our system, all κ^+ B cells are autoreactive independent of which V κ they express, and expression of a nonautoreactive BCR requires a productive V λ -J λ gene rearrangement, which normally occurs subsequent to the rearrangement of Ig κ (Siminovitch et al., 1985; Bräuninger et al., 2001; Langerak et al., 2004; Klein et al., 2005). Moreover, Ig gene rearrangements predictably result in out-of-frame V-J gene segments two thirds of the time. Thus, the chance to express a nonautoreactive BCR is unfavorable and likely requires multiple rearrangement attempts and a certain amount of time, which is probably limited (Hartley et al., 1993; Melamed and Nemazee, 1997). This reasoning suggests that a fraction of autoreactive Ig κ^+ cells will edit unsuccessfully and consequently undergo cell death, resulting in overall reduced B cell export to the spleen, a prediction consistent with our data.

In mice it has been shown that whether an individual B cell undergoes a particular tolerance mechanism or a positive selection event into the periphery depends on the BCR affinity for the Ag and the Ag concentration (i.e., the strength of BCR signaling), as well as the developmental state of the B cell when it encounters the self-Ag (Hayakawa et al., 1999; Kouskoff et al., 2000; Martin and Kearney, 2000; Tze et al., 2000; Qian et al., 2001; Hippen et al., 2005; Wen et al., 2005; Andrews et al., 2013). In our model, the affinity of the BCR for the neo self-Ag is equal for all Ig κ^+ B cells, but the self-Ag concentration varies among tissues, cell types, and the Tg lines themselves. The effect of self-Ag dose on human B cell tolerance was clearly demonstrated by the significant differences observed in B cells developing in H $\kappa\kappa$ 4 (high H $\kappa\kappa$ expression) compared with those developing in H $\kappa\kappa$ 7 (low H $\kappa\kappa$ expression) hu-mice. For instance, bone marrow B cells of H $\kappa\kappa$ 7 hu-mice had higher CD19 levels compared with B cells in H $\kappa\kappa$ 4, indicating that CD19 surface expression inversely correlates with self-Ag level and thus BCR avidity. H $\kappa\kappa$ 7 hu-mice also exhibited lower frequencies of λ^+ B cells and a higher proportion of cells with IC κ relative to H $\kappa\kappa$ 4, in both bone marrow and spleen. Overall, we interpret these data to indicate that receptor editing is less frequent in H $\kappa\kappa$ 7 hu-mice, resulting in a higher proportion of autoreactive (κ^+) B cells that escape the bone marrow and migrate to the spleen. Interestingly, although H $\kappa\kappa$ 4 and H $\kappa\kappa$ 7 hu-mice had, on average, similar numbers of transitional B cells in the spleen, H $\kappa\kappa$ 7 mice showed a reduction in the total B cell numbers and thus in mature B cells. This suggests that most of the peripheral autoreactive B cells in H $\kappa\kappa$ 7 hu-mice were then eliminated by mechanisms of peripheral tolerance between the transitional

and mature cell stages, a checkpoint that has been described in both mice and humans (Wardemann et al., 2003; Andrews et al., 2013). Because of these data and the fact that the great majority of κ^+ B cells undergo negative selection in all Hck⁺ hu-mice, we do not favor the possibility that the few κ^+ B cells in the periphery of Hck7 hu-mice have undergone a positive selection event. Collectively, these data indicate that, just as described for murine B cells, where and how human B cells undergo tolerance depends on the local concentration of self-Ag, the location of self-Ag, and the developmental state of the B cell when it encounters an effective dose of self-Ag.

In spite of an efficient central B cell tolerance process, small amounts of Ig κ were measured in the serum of a minority of Hck⁺ hu-mice, and ELISPOT analyses confirmed the presence of Ig κ -producing plasmablasts, particularly in older animals. Together, the detection of a few IC κ^+ splenic B cells, of serum hIg κ , and of Ig κ -secreting plasmablasts demonstrate that central B cell tolerance was not absolute in some animals, possibly because of an insufficient concentration of self-Ag in the bone marrow or a defective receptor editing process, which is sometimes observed in mice (Liu et al., 2005; Fournier et al., 2012). In our hu-mouse model, human T cells develop in a mouse thymus and are thought of being educated mostly on mouse MHC (Legrand et al., 2009; Manz and Di Santo, 2009; Taneja and David, 2010; Ito et al., 2012; Shultz et al., 2012; Akkina, 2013). This likely leads to the development of peripheral T cells unable to provide cognate help to human B cells but possibly also to cells that can provide increased bystander help because of strong “allo” TCR response to human MHC. Moreover, many T cells display an activated state in older hu-mice (Lang et al., 2013). The presence of “alloreactive” and activated T cells might explain why autoreactive B cells that escaped central tolerance in Hck⁺ hu-mice did not remain tolerant in the periphery.

In measuring parameters of central B cell tolerance, we found that the expression level of CD19 and the frequency of cells that were CD19^{low} and IC κ^+ had limited variability in the non-Tg recipients and greatly increased variability in the presence of the Hck neo self-Ag, indicating this variability reflects differences in tolerance induction. Importantly, the variability in the frequency of CD19^{low} cells and IC κ^+ cells segregated significantly with the CB donor, suggesting an inherent individual distinction in tolerance induction based on heritable or epigenetic B cell qualities. The differences among CB cohorts were especially notable in Hck7 recipients, which was further demonstrated by plotting the frequency of IC κ^+ B cells against their CD19 MFI for both Hck4 and Hck7 hu-mice. In Hck4 animals, in which tolerance is more stringent, these parameters were distributed similarly among hu-mice. In contrast, although half of the Hck7 hu-mice had a distribution similar to that of Hck4, the other half had a markedly different distribution. This difference was inherent to the source of CB, suggesting individual variations in tolerance induction. These findings indicate that Hck7 animals, likely because of their lower self-Ag concentration, provide

a better resolution of the tolerance abilities of different immune systems. Because Hck7 hu-mice generally showed increased breaking of tolerance, we propose that CB samples displaying noncanonical frequencies and CD19 levels of IC κ^+ B cells might be less prone to induce receptor editing and more prone to break central B cell tolerance. A comparison of the percentage of CD19^{low} cells for individual CBs between Hck⁺ and Hck⁻ hu-mice additionally demonstrated individual variations in tolerance, with some samples displaying differences that were weakly or strongly significant and other samples that showed no difference.

In summary, using a novel hematopoietic hu-mouse model, we have been able to define certain characteristics of autoreactive human B cells undergoing tolerance. Similar to mouse autoreactive B cells, human B cells that engage membrane-bound self-Ag in the bone marrow withdraw the BCR from the surface and display lower surface CD19 levels. They also express higher levels of RAG and products of secondary Ig gene recombination, resulting in a skewed peripheral repertoire. These observations provide a formal and direct demonstration that human bone marrow B cells can use receptor editing during tolerance. In the future, we envision using this information to detect, isolate, and study autoreactive B cells from human bone marrow samples and to use our hu-mouse model system as a tool to investigate and compare the extent of tolerance induction among developing B cells with autoimmune-associated polymorphisms.

MATERIALS AND METHODS

Generation of Hck Tg mice. The construction of the Hck transgene and production of the two Tg mouse lines (Hck4 and Hck7) were as described previously for the similar anti-mouse κ Tg mice (Ait-Azzouzene et al., 2005) except that the Fv region of the transgene was from the mouse anti-human κ hybridoma HP6053. Pronuclear injection was performed at the Scripps Research Institute Mouse Genetics Core facility using BALB/c \times DBA/2 mice. At the Scripps Research Institute, Hck4 and Hck7 mice were bred to Ig $\kappa^{m/h}$ mice (donated by M. Nussenzweig, The Rockefeller University, New York, NY; Casellas et al., 2001) for a preliminary analysis. Hck4 and Hck7 mice were shipped to Denver, backcrossed 10 generations to BALB/c, and then backcrossed to BRG mice to generate Hck4 and Hck7 BRG animals. Hck4 and Hck7 BRG mice were bred as heterozygotes with BRG mice to produce litters with \sim 50% Tg⁺ pups. Genotyping was performed by PCR on genomic DNA isolated from a tail clip at weaning with primers specific for the Hck transgene (forward, 5'-GTGGAGGCTGAGGATCTGGGAGTT-3'; reverse, 5'-GGTTTCTGGGGGCTGTTGTTTCAG-3') using touchdown PCR: 94°C for 1 min, 72°C (-1°C) for 30 s, 72°C for 1 min for 15 cycles and 14 cycles at 94°C for 1 min, 59°C for 30 s, and 72°C for 1 min. Mice were also phenotyped using flow cytometry of blood, spleen, bone marrow, kidney, or liver single-cell suspensions stained with an Ab to rat IgG1 (RG11; BD), a component of the transgene

product. Mice were housed under specific pathogen-free and Biosafety Level 2 conditions at the Biological Resource Center at National Jewish Health (NJH) Denver, CO) or at the University of Colorado Denver (UCD) Anschutz Medical Center Vivarium (Aurora, CO). All animal procedures were approved by the NJH and UCD Institutional Animal Care and Use Committee. Animal studies presented in Fig. 2 A were approved by The Scripps Research Institute Institutional Animal Care and Use Committee.

Generation of hematopoietic hu-mice. Hck Tg-positive and -negative BRG neonates (1–3 d) were transplanted with “heathy” CB CD34⁺ HSCs to generate hu-mice using a method previously described (Lang et al., 2011, 2013). The CB units were obtained from the University of Colorado Cord Blood Bank at ClinImmune Labs (Aurora, CO) as samples that were rejected as the result of low volume. Investigators in this study were blinded from donor identities, and the experiments were performed in compliance with the NJH and University of Colorado Institutional Review Boards. Blood samples from mice at 7–9 wk of age were analyzed by flow cytometry with Abs to mouse CD45 (mCD45), human CD45 (hCD45), hCD3, hCD5, and hCD20 to determine the amount of human hematopoietic chimerism. Spleen and bone marrow single cell suspensions were prepared from hu-mice euthanized at 7–24 wk after engraftment. Only mice with >5% human chimerism were included in the study.

Abs and flow cytometry. Abs used in flow cytometric analyses were as described in Lang et al. (2011, 2013). Abs specific for mGR1 (RB6-8C5), mH-2D^d (34-2-12), mCD45 (30-F11), hCD3 (OKT3, HIT3a), hCD5 (UCHT1), hCD34 (581), hCD38 (HB-7), hCD40 (5C3), hCD45 (H130), hCD138 (ML15), hCD268 (11C1), hIgκ (MHK-49), hIgλ (MHL-38), and hIgM (MHM-88) were from BioLegend. Abs for rat IgG₁ (RG11/39.4), hCD3 (HIT3a), hCD19 (HIB19), and hIgM (G20-127) were from BD, and those for hIgκ (HP6062) and hIgM (SA-DA4) were from eBioscience. Fab’ goat anti-hIgM was from Protos Immunoresearch. Cells were stained in staining buffer (PBS, 1% BSA, 0.1% Na Azide) for 15 min at 4°C and washed twice with the same buffer. Samples were collected on CyAn analyzers (Beckman Coulter) at the flow cytometry cores at NJH or UCD Cancer Center, and analyses were performed with FlowJo software as described in Lang et al. (2013).

Cell sorting and enrichment. To sort CD19^{hi}IgM⁺, CD19^{hi}-IgM⁺, and CD19^{low} cell populations, bone marrow cells from one to three Hck⁺ mice were pooled and stained with Abs to mCD45, hCD3, and hCD11c (for the dump gate) and to hCD45, hCD19, and hIgM (with Fab’ anti-IgM Ab). Cells were sorted using a MoFlo XDP (Beckman Coulter) cell sorter. For the enrichment of total bone marrow B cells, pooled bone marrow cells from one to three mice for each strain were incubated with biotinylated Abs to mCD45 and

hCD3 for 15 min, washed three times in staining buffer, and then incubated with anti-biotin beads (Miltenyi Biotec) for 15 min in a refrigerator followed by three washes in MACS buffer (1× PBS, 2 mM EDTA, and 0.5% BSA). Cells were loaded on an autoMACS (Miltenyi Biotec) with a “depletes” program. The negative cell fractions were collected, and a sample was stained with Abs for hCD45, mCD45, hCD3, and hCD19 to check purity. All sorted populations were >85% pure.

IC staining. Cells were stained with Abs to extracellular (EC) proteins as described above in the section “Abs and flow cytometry” and then fixed with 2% formaldehyde for either 5 min at 37°C or 20 min at room temperature (RT). Fixed cells were washed twice in staining buffer (PBS, 1% BSA, and 0.1% Na Azide) and then incubated with either Triton permeabilization buffer (0.5% BSA, 2 mM EDTA, and 0.1% Triton-X 100 in PBS) for 1 h on ice or saponin permeabilization buffer (0.5% BSA and 0.5% saponin in PBS) for 15 min at RT (these methods were interchangeable). Cells were then stained with Abs for IgM, IgG, Igκ, or Igλ (described in “Abs and flow cytometry”) for 30 min in the dark at RT followed by five washes in staining or saponin buffer.

Histology. Femurs from Hck⁺, Hck4, and Hck7 BRG mice were embedded in Tissue-Tek OCT Compound (Sakura Finetek), frozen immediately on dry ice, and stored at –80°C. Frozen samples were sliced into 4–7-μm sections using a CryoJane adaptation (Leica) on a cryostat and transferred onto glass slides. Slides were dried at RT for a minimum of 30 min, followed by fixation for 30 min in 50% acetone and then 3 min in 100% acetone. For staining, the slides were rehydrated in PBS for 20 min, blocked with a mixture of anti-mouse CD16 (24G2) Ab, mouse IgG2a (isotype control for the anti-rat IgG1 Ab), and goat serum in PBS, 2% BSA, and 0.05% Tween20 for 15 min at RT and then stained with DAPI, allophycocyanin-conjugated anti-mouse Gr1, and eFluor 615-conjugated anti-rat IgG1 Abs for 45 min at RT in the dark. After three washes in PBS, dried sections were mounted with a coverslip (# 1.5 thickness) using Fluoromount G (SouthernBiotech) and sealed with nail polish. Sections were visualized on an Eclipse TE 2000 microscope (Nikon) outfitted with a Plan Fluor ELWD Ph2 DM 40× objective dry with a 10× eyepiece for a total 400× magnification. Images were collected using a wide field lens and NIS Elements version 4.2 software and viewed with NIS Elements Viewer version 4.11.0 software (all from Nikon). No deconvolution or 3D reconstitutions were performed, and fluorochrome settings were set to equivalent intensities among all samples for comparison and analysis. In addition, all files were exported using equivalent settings and file formats.

ELISAs. Total, κ-containing, or λ-containing hIgM and hIgG were measured in the sera of hu-mice with monoclonal Abs purchased from SouthernBiotech. Plates were coated with mouse anti-hIgM (SA-DA4), hIgG (JDC-10), hIgκ (SB81a),

or hIg λ (JDC-12) at 10 μ g/ml in PBS and incubated overnight at 4°C. The next day, plates were washed five times in PBS/0.1% Tween followed by blocking for 1–2 h at RT with PBS, 1% BSA, and 0.1% Na Azide buffer, followed by three additional washes. Sera were added to plates with threefold serial dilutions starting at 1:10. Standard hIgM or hIgG was added to plates at 5 μ g/ml with threefold serial dilutions. Plates were then incubated overnight at 4°C and washed in the morning five times. This was followed by the addition of a 1:500 dilution of secondary alkaline phosphatase (AP)–conjugated Abs, mouse anti-hIgM (UHB) or hIgG (H2), and the plates were incubated for 2 h at 37°C. After washing five times, 100 μ l of developing buffer (AP substrate *p*-nitrophenyl phosphate; Sigma-Aldrich) was added per well, and light absorbance was measured at OD₄₀₅ multiple times with a VersaMax plate reader (Molecular Devices). To ensure consistency, total hIgM and hIgG were further measured using polyclonal Abs as previously described (Lang et al., 2011). They were also calculated by summing the κ - and λ -containing IgM and IgG concentrations. The total IgM and IgG concentrations in Fig. 5 A are the mean of these three independent measurements. Only samples with >0.5 μ g/ml hIgM or >10 μ g/ml hIgG were included in the respective analyses. For detection of rat IgG₁ (transgene product) in sera, an ELISA was performed similar to the hIgM or IgG polyclonal methods described previously (Lang et al., 2011) except using a goat anti-rat IgG₁ as the coating and secondary Abs.

ELISPOT. EIA/RIA 96-well plates (Corning) were coated with goat anti-human κ or λ Abs at 10 μ g/ml (2060-01, 2070-01; SouthernBiotech) in PBS and left overnight at 4°C, washed three times with PBS, and blocked with PBS/1% gelatin for 2 h at RT. 10×10^6 spleen cells from hu-mice were resuspended in 400 μ l RPMI media supplemented with 10% FBS, GlutaMAX, pen/strep, β -ME, and 10% protein G–absorbed FBS. 400- μ l cell suspensions were added in the first well of each plate followed by twofold serial dilutions, and plates were incubated overnight at 37°C. Plates were washed four times in PBS/0.1% Tween followed by the addition of biotinylated goat anti-human Ig(H+L) Abs (2010-08; SouthernBiotech) at 1 μ g/ml in staining buffer. Plates were incubated for 2 h at RT in the dark and washed three times with PBS/0.1% Tween, followed by incubation with a 1:2,000 dilution of Streptavidin-AP (SouthernBiotech) in staining buffer for 1.5 h in the dark at RT. Plates were washed four times with PBS/0.1% Tween, and 100 μ l of developing buffer (1M alkaline buffer [Sigma-Aldrich], 5 mM MgCl₂, 1 mM Triton X-405, 0.1% NaN₃, and BCID [1% wt/vol; Thermo Fisher Scientific]) was added per well, left to incubate overnight at 4°C, washed the next day with diH₂O three times, and allowed to dry before scanning. Spots were counted manually.

DNA, RNA, and cDNA preparation and quantitative PCR (qPCR). RNA was prepared from single-cell suspensions using TRIzol (Life Technologies) according to the manu-

facturer's instructions and then stored at –80°C. DNA was isolated using phenol/chloroform separation followed by ethanol precipitation. cDNA was prepared using the Superscript III Reverse Transcriptase kit (Invitrogen) according to the manufacturer's instructions. qPCR on cDNA was performed using TaqMan Universal PCR Master mix (Applied Biosystems) according to manufacturer's instructions with the following human-specific primers: RAG1 (Hs01920694_s1), RAG2 (Hs01851142_s1), and GAPDH (Hs02758991_g1) from Applied Biosystems. For the analysis of λ excision circles, rearrangements of V λ 1–40 and J λ 3 gene segments were chosen based on their relative high frequency (7% and 46%, respectively) among all rearranged V λ –J λ sequences deposited at IMGT (The International Immunogenetics Information System). The qPCR analysis for V λ 1–40–J λ 3 excision circles was performed on genomic DNA with the following custom-designed primers and probe: primer 1, 5'-AGTGGGTTCTTGCTCTCAGTTC-3'; primer 2, 5'-CCAGCTTCCTCCTTCACAG-3'; and probe, 56FAM/TGCACTGTG/ZEN/ACAAGGCTCATACA/3IABkFQ. GAPDH DNA levels were measured for normalization purposes with primers Hs.PT.58.589810.g purchased from Integrated DNA Technologies. 25- μ l samples were run on an ABI 7300 real-time PCR system (Applied Biosystems) using a standard protocol for RAG1 and RAG2 expression: 2 min at 50°C; 10 min at 95°C; 40 cycles of 15 s at 95°C and 1 min at 60°C. For the V λ excision circle, a lowered annealing temperature of 58°C and increased cycle number (50) was used because of decreased detection. Dilutions of DNA for each sample were tested to ensure a linear qPCR response. Differences in gene and gene expression levels between samples were calculated using the Comparative C_T Method ($\Delta\Delta C_T$; Applied Biosystems).

Analysis of Ig κ rearrangements. V κ –J κ rearrangements were amplified on genomic DNA isolated from CD20⁺ λ^+ cells sorted with a MoFlo XDP cell sorter from the spleen of H κ ⁺ and H κ ⁺ hu-mice (cells were pooled from three animals per group). The amplification of V κ –J κ rearrangements was performed with a semi-nested PCR protocol in two rounds. Round one was executed on genomic DNA with the mix of 5' primers LV κ 1/2, ATGAGGSTCCCYGCTCAGCTGCTGG; LV κ 3, CTCTTCCTCCTGCTACTCTGGCTCCCAG; and LV κ 4, ATTTCTCTGTTGCTCTGGATCTCTG (Tiller et al., 2008); and 3' primers J κ 1/2/4, ACTCACGTTTGATYTCCASCTTGGTCC; J κ 3, GTACTTACGTTTGATATCCACTTTGGTCC; and J κ 5, ACTTACGT TTAATCTCCAGTCGTGTCC (Küppers et al., 1993) for 35 cycles, each consisting of 30 s at 94°C, 45 s at 57°C, and 2.5 min at 70°C. Round two was performed on 2- μ l PCR reaction from round one and with the mix of 5' primers V κ 1, TGATGTCGACATCCRGWTGACCCAGTCTCCWTC; V κ 2, TGATGTCGACAGWCTCCACTCTCCCTGYCCGTCA; V κ 3, TGATGTCGACTCCAGSCACCCTGTCTKTGTCTC; V κ 4, TGATGTCGACTCCCTGGCTGTGTCTCTGGG; V κ 5, TGATGTCGACAGTCTCCA

GCATTCATGTCAGCGA; and V κ 6, TGATGTCGACTTY CTCTCTGTGACTCCARRGGAG (Küppers et al., 1993) and the 3' J κ primers listed above for 45 cycles, each consisting of 30 s at 94°C, 45 s at 57°C, and 30 s at 70°C. PCR products were purified from an agarose gel and cloned into the TOPOTA vector (Invitrogen). DNA inserts were sequenced, and sequences (both in frame and out of frame) were blasted against those deposited in IMGT database to assign V κ and J κ usage. Duplicate sequences (based on V κ , J κ , and CDR3) were removed for the analysis presented in Fig. 4.

Serum injections. Frozen serum samples from several H κ [−] hu-mice were thawed and pooled (mean concentration of 24 μ g/ml hIgM and 1,088 μ g/ml hIgG). The pooled sera was injected (~200 μ l per mouse) into the tail vein of intact (nonhumanized) H κ [−] control or H κ ⁺ mice either neat or at a 1:10 dilution. Uninjected mice served as controls. Sera were collected the next day and tested for the presence of hIg κ , hIg λ , hIgM, and hIgG by ELISA as described above in the “ELISAs” section.

Statistical analysis. Statistical significance was assessed using Prism software (GraphPad Software), with a two-tailed Student's *t* test of equal variance or a Welch correction when appropriate. Statistical analysis of V κ and J κ usage was performed by Z-test. To analyze differences among unique CB-derived hu-mice, data were analyzed using ANOVA with a Tukey post hoc test.

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