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Studies of Lymphocyte Reconstitution in a Humanized Mouse Model Reveal a Requirement of T Cells for Human B Cell Maturation

Julie Lang,*† Margot Kelly,*† Brian M. Freed,*† Martin D. McCarter,§ Ross M. Kedl,*† Raul M. Torres,*† and Roberta Pelanda*†

The hematopoietic humanized mouse (hu-mouse) model is a powerful resource to study and manipulate the human immune system. However, a major and recurrent issue with this model has been the poor maturation of B cells that fail to progress beyond the transitional B cell stage. Of interest, a similar problem has been reported in transplant patients who receive cord blood stem cells. In this study, we characterize the development of human B and T cells in the lymph nodes (LNs) and spleen of BALB/c-Rag2null II2rynull hu-mice. We find a dominant population of immature B cells in the blood and spleen early, followed by a population of human T cells, coincident with the detection of LNs. Notably, in older mice we observe a major population of mature B cells in LNs and in the spleens of mice with higher T cell frequencies. Moreover, we demonstrate that T cells are necessary for B cell maturation, as introduction of autologous human T cells expedites the appearance of mature B cells, whereas in vivo depletion of T cells retards B cell maturation. The presence of the mature B cell population correlates with enhanced IgG and Ag-specific responses to both T cell–dependent and T cell–independent challenges, indicating their functionality. These findings enhance our understanding of human B cell development, provide increased details of the reconstitution dynamics of hu-mice, and validate the use of this animal model to study mechanisms and treatments for the similar delay of functional B cells associated with cord blood transplantations. *The Journal of Immunology, 2013, 190: 2090–2101.

The hematopoietic humanized mouse (hu-mouse), in which human hematopoietic stem cells (HSCs) drive the development of a human hematopoietic system within a mouse host, provides a unique in vivo model in which to perform mechanistic, genetic, and pharmacological studies of the human immune system. Current host models enable notable human engraftment owing to a lack of T, B, and NK cells as a result of null genetic mutations in the Rag or Prkdc genes (1–6). The genetic background of the mouse strain is an important factor in human engraftment, and multilineage engraftment has been demonstrated in both the NOD and the BALB/c mutant strains (1–8). However, the frequencies of distinct hematopoietic lineages in hu-mice differ from those in a human.

In the bone marrow (BM) of hu-mice, human HSCs differentiate into pro-B, pre-B, and immature B cells, suggesting that the mouse environment supports human B cell development (9–13). However, several studies have shown that human B cells are blocked in maturation at the transitional stage in the peripheral blood (PBL) and spleen: The majority of hu-mice are populated primarily with immature B cells (14–17) that are inferior to mature B cells in their ability to respond to Ag (18). Not surprisingly, immunization challenges have yielded only weak immune responses in hu-mice compared with those achieved in immunologically intact mice or humans (1, 2, 10, 14–16, 19). A major goal in the hu-mouse field is the generation of a high-affinity, mutated Ab response to antigenic challenge (20). One obvious requirement is the generation of a mature B cell population.

The transplantation of cord blood (CB) HSCs now account for >25% of all hematopoietic transplantations in humans owing to enhanced availability and a lower requirement for HLA matching, compared with that for BM. However, infection-associated mortality resulting from a delayed reconstitution of the human immune system following CB transplantation remains a current challenge in the field (21). Specifically, B cells are found to repopulate the recipient early after engraftment, yet have limited functionality for up to 6 mo, around the time when significant T cell reconstitution occurs. Thus, reconstitution of functional B cells appears to be limited not only in hu-mice but also in human CB recipients. Therefore, the hu-mouse has the potential to be a useful animal model to investigate and solve issues related to CB transplantation.

Unlike typical mouse BM chimeras, hu-mice have a dynamic and inconsistent engraftment of hematopoietic lineages over time (1, 4, 22). Thus, understanding the details of human lymphocyte reconstitution in the primary and secondary organs and the factors that shape the B cell population is vital for appropriate experimental design using this model. In this study, we characterize the
frequency, maturation, and activation patterns of human T and B lymphocytes in the BM, spleen, PBL, and LNs of BALB/c-Rag2null (BALB/c-DKO) hu-mice generated with a protocol that we have optimized to reproducibly promote high levels of human chimerism (23). More importantly, we define the kinetics and reconstitution pattern of mature B cells in these hu-mice and report a requirement of T cells for human B cell maturation. Furthermore, we compare the tissue organization of T and B cells and the immune responses to T cell–dependent (TD) and –independent (TI) Ags in hu-mice with mature B cells to those with mostly immature B cells. Our study provides not only a detailed characterization of lymphocytes in hu-mice but also insights into mechanisms of human B cell maturation. We propose that the hu-mouse is an informative in vivo model that can be used to study factors necessary for human lymphocyte development and function.

Materials and Methods

**CD34+ and CD34- cell preparation from human umbilical CB**

Human cell preparation was performed as described previously (23). CB mononuclear cells were isolated over Ficoll-density gradients, and CD34+ cells were enriched using autoMACS (Miltenyi Biotec) technology. The CD34+ cell fraction was further depleted of T cells with CD2- and CD3-specific beads and used immediately or frozen for later use as “support” cells. The CD34- cells were used immediately or cultured at 1 × 10^6 cells per milliliter in IMDM supplemented with 10% FBS, 50 µM 2-ME, 2 mM GlutaMAX, IL-6 (10 ng/ml), stem cell factor (20 ng/ml), and FLT3 (10 ng/ml). Human BM samples were obtained from optimization assays described above and limited to IgM samples with no significant T cell chimerism. Human adult PBL samples were collected from healthy donors in the Clinical Division of NJH. Human spleen and lymph node (LN) samples were obtained in accordance with protocols approved by the University of Colorado Hospital. The human BM sample was obtained from optimization assays described above and limited to IgM samples with no significant T cell chimerism. Hu-mice were euthanized for tissue collection and analysis when ≥80˚C for future use.

**Mice and HSC transplantation**

BALB/c-Rag2null (BALB/c-DKO) mice were bred and maintained on a diet enriched with Septra every other week, under specific pathogen–free and Biosafety Level 2 conditions at the Biological Resource Center at the University of Colorado. Mice were treated with a [137Cs] irradiation (5C3), hHLA-DR (G46-6), hCD268 (11C1), hCD70 (Ki-24), hCD25 (M-52), and hCXCR5 (TG2) from BioLegend; hCD4 (RPA-T4), hCD8 (53-6.7), hCD5 (UCHT2), hCD27 (323), hCD11c (3.9), hIgD (IA6-2), hCXCR4 (JDC-12), hCD44 (G44-26, BD), hHLA-1 (G4-25B, BD), and hCD80 (L307.4, BD), hCD86 (2331), hCD197 (“CCR7,” 3D12), hCD2 (RPA-2.10), hCD28 (CD28.2), hCD49d (9F10), and hCD34 (581) from BD Biosciences; hCD5 (UCHT2), mouse (m)CD45 (30-F11), hCD38 (HT2), hCD21 (B32), hCD23 (EBVC32), IgM (SA-DA4), hCD62L (Deg56), and hCD196 (“CCR6,” R6H1) from Bioscience; and hCD122 (27302) from R&D Systems. Biotinylated Abs were revealed with streptavidin fluorescent conjugates (Invitrogen). To distinguish dead cells, 7-aminocinomycin D (7-AAD; BD) was added to some samples within 30 min of analysis. All cell samples were washed twice on an SPC-150 Analyzer (Beckman Coulter) and analyzed with FlowJo software (Tree Star). All flow cytometric analyses were made on a single-cell gate based on forward and side scatter and doublet discrimination.

**Histological examination**

Intact LN and spleen tissue samples were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek), frozen immediately on dry ice, and stored at −80˚C. Frozen samples were sliced into 4- to 7-μm-thick sections on a cryocut and transferred onto glass slides. Slides were dried at room temperature overnight and either frozen or directly fixed for 10 min in 4% formaldehyde. Each section was rehydrated for 15 min in PBS, followed by blocking for 15 min with avidin (Avidin/Biotin Blocking Kit, Vector Laboratories), and then for 15 min with a mixture of biotin, mlgG, hlgG, anti-mouse CD16 Abs (24G2), and goat serum. Tissue sections were stained with either anti-human CD20 (allophycocyanin) and CD3 (PE) Abs or anti-human CD45 (allophycocyanin) and anti-mouse CD45 (Pacific Blue) Abs for 1 h. After three washes, dried sections were mounted with a coverslip using Fluoromount G (SouthernBiotech) and sealed with nail polish. Sections were visualized on an Axiovert 200M microscope (Carl Zeiss) outfitted with a 3i Marianas system and analyzed with SlideBook 4.0 software (Intelligent Imaging Innovations).

**T cell adoptive transfer and T cell depletion**

Human T cells were isolated from CB preparations upon positive selection with anti-CD2 beads (Miltenyi Biotec) at the time of CD34+ cell purification and were frozen at −80˚C until use. T cells were thawed, washed, resuspended in PBS, and injected into the tail vein of 11- to 12-wk-old hu-mice (6 × 10^7 cells per mouse) that had been engrafted with HSC from the same CB prep. Control hu-mice were either left untreated or injected with a similar number of T cell–depleted CD34+ CB cells. For T cell depletion, hu-mice were injected twice weekly with 10 µg anti-CD3 Abs (UCHT1 or OKT3) starting at 9 wk of age. Control mice were left untreated. PBL samples were collected every 3–4 wk to assess human T and B cell chimerism. Hu-mice were euthanized for tissue collection and analysis when significant T cell chimerism was established in control untreated littersmates (generally between 18 and 24 wk).

**Immunizations**

Hu-mice were separated into groups according to their level of human chimerism established by flow cytometric analyses of leukocytes in PBL at 7–8 wk of age. The mice were injected either i.p. or s.c. under the scrub of the following vaccines: with the following Ags conjugated to keyhole limpet hemocyanin or diphtheria toxoid: DTAp (50 µl; Sanofi Pasteur). All mice were inoculated every other week for a total of three to four times beginning at 8–12 wk of age or 14–16 wk of age and their sera were analyzed 2 wk after the last inoculation. No differences were observed between i.p. and s.c. immunizations.

**ELISAs**

Total hlgM and hlgG concentrations were determined as described previously (23). During the optimization of Ag-specific ELISA, sera were collected from immunized (DTAp and NP-Ficoll) BALB/c-DKO hu-mice (“test” group); unimmunized hu-mice, nonhumanized BALB/c-DKO and CB17 mice (negative controls); and human CB and PBL (positive controls) by standard methods. The hu-mice had serum Ig concentrations of 0–670 µg/ml (IgM) and 0–3200 µg/ml (IgG). We tested coating the plates with the following Ags, all diluted in PBS: nicked diphtheria toxin (DT; 10 µg/ml; List Biological Laboratories), DTaP vaccine (1:333, Sanofi Pasteur). Biotinylated Abs were revealed with streptavidin–alkaline phosphatase (AP) conjugate (SouthernBiotech; NP36CGG (10 µg/ml), and NP16CGG (10 µg/ml), along with a PBS-only plate. In addition, we tested blocking plates with PBS–0.1% BSA, PBS–0.1% BSA, 20% FBS, or 1% gelatin; washing the plates with PBS–0.05% Tween 20, 0.1% Tween 20, or PBS 0.1% Brij solutions; diluting the serum in PBS–0.1% BSA or PBS–0.05% Tween 20; and detecting bound IgGs with the following secondary Abs conjugated to alkaline phosphatase (AP): monoclonal mouse-, and polyclonal goat-, human anti-IgM or IgG (SouthernBiotech). The ELISA protocol for measuring Ag-specific responses was adopted from optimization assays described above and limited to IgM samples with...
concentrations of 1–35 µg/ml to limit background effects. ELISA plates were coated overnight at 4°C with 10 µg/ml NP16-BSA or a 1:333 dilution of DTaP vaccine in PBS, and duplicate plates were left uncoated (PBS) to measure background values. Plates were washed in PBS–0.1% Tween 20 and blocked with PBS–1% BSA. Next, 2-fold serial dilutions of sera starting at 1:10 or 1:20 in PBS–1% BSA were incubated overnight at 4°C. Positive (human sera for DTaP) and negative (BALB/c-DKO sera) controls were added to most plates. The following day, plates were washed and incubated for 2–4 h at 37°C with AP-conjugated mouse anti-human (for NP) or goat anti-human (for DT) IgM or IgG Abs (SouthernBiotech). After a final wash, the AP substrate p-nitrophenyl phosphate (Sigma-Aldrich) was added to plates, and light absorbance was measured at OD 405 on a VersaMax Microplate Reader (Molecular Devices). Background values obtained from wells developed in the absence of sera and then values obtained from duplicate PBS-coated wells were subtracted from sample OD405 values. Relative Ab titers were defined as the serum dilution that generated an OD 405 equal to 0.5 for NP and 0.25 for DT. Comparable responses of identical samples prepared on different days were used to normalize the relative Ab titers. Data presented represent the average response of a serum measured one to four times.

**Statistical analysis**

Statistical significance was assessed using Prism software (GraphPad Software), with a two-tailed Student t test of equal variance or a Welch correction when appropriate.

**Results**

**Kinetics of human B cell, T cell, and LN development in BALB/c-DKO hu-mice**

To define the engraftment kinetics in our BALB/c-DKO hu-mouse model, we used FACS analysis to measure the percentage of human hematopoietic (hCD45+) cells in both primary (BM, thymus; Fig. 1A, left panel) and secondary (PBL, LN, and spleen; Fig. 1A, middle panel) lymphoid tissues of hu-mice over time. As in other reports (1, 3), human hematopoietic cell engraftment was observed as early as 6 wk but steadily declined thereafter in the BM and PBL (Fig. 1A). Notably, we found that the human engraftment

**FIGURE 1.** Kinetics of human hematopoietic cell engraftment in BALB/c-DKO hu-mice. (A) Humanized BALB/c-DKO mice were analyzed for human chimerism in the PBL at 3- to 4-wk intervals beginning at 7–8 wk of age, and in the BM, thymus, spleen, and LN upon harvest. The animals were euthanized at indicated ages (n = 4–51 for 6–30 wk and n = 1 for 37 wk). Human chimerism was determined by FACS analysis of cells stained with anti-human and anti-mouse CD45 Abs. In the BM, PBL, and spleen, the percentage of human CD45+ cells is calculated relative to total (mouse + human) hematopoietic cells, whereas in the LN and thymus, the percentage of hCD45+ cells is reported in the total live cell gate. The absolute cell numbers of hCD45+ cells in LN, thymus, and spleen of hu-mice at different time points are shown in the right panel. Graphs show the mean ± SEM. (B) Frequency of hu-mice at indicated age in weeks displaying detectable LNs (mesenteric, axillary, inguinal, cervical, or iliac), as defined by tissue exhibiting >1% hCD45+ cells by FACS analysis. Open symbols indicate hu-mice that were immunized as described in Fig. 4. (C) Iliac (i), cervical (ii), inguinal and axillary (iii), and mesenteric (iv) LNs were excised from a control CB17 mouse (left panels) and a 24-wk-old hu-mouse (right panels). All images shown are from the same photograph and, therefore, on equivalent scale. (D) Top panels. The percentage of CD20+ B cells (open square) and CD3+CD5+ T cells (closed circle) within the hCD45+ gate in the PBL (left), spleen (middle), and LNs (right) of hu-mice at indicated ages. Bottom panels. The absolute number of human B and T cells in the spleen (middle) and LNs (right). Data represent the mean ± SEM (n = 1 at 37 wk and n = 4–52 for all other points). (E) Human IgM and IgG concentrations (µg/ml) in the sera of hu-mice (n = 17) at the indicated age. Data are mean ± SEM. (F) Human IgM and IgG concentration in sera of hu-mice that either harbor LNs (n = 125–131) or not (n = 67–71) at the time of euthanasia. Each symbol represents an individual mouse and horizontal bars the median. ***p < 0.001.
kinetics were unique to distinct lymphoid organs. Human chimerism was detected early in the thymus and was sustained over time (Fig. 1A, left panel). Conversely, splenic engraftment was delayed relative to BM, was maximal between 15 and 24 wk, and then declined sharply as human hematopoietic production in the BM waned (Fig. 1A). Unlike in the spleen or BM, mCD45^+ hematopoietic cells were rare in the LN or thymi in hu-mice, so that >90% of the hematopoietic cells, if present at all, were human in these tissues (data not shown). Engraftment in the LN was detected in a fraction of hu-mice, which was a majority by 16 wk, and was not affected by immunization (Fig. 1B). Human cells were detected in mesenteric LNs prior to peripheral LNs, although in older mice, occupation of cervical, inguinal, axillary, and iliac LNs by human cells was common, and the size of LNs in these hu-mice was comparable to that in wild-type mice (Fig. 1C). The absolute numbers of human cells roughly mirrored their frequencies, with consistent numbers over time in the thymus, a decrease in the spleen at later time points, and a delayed appearance in the LNs (Fig. 1A, right panel). Thus, an optimal window of human hematopoietic chimerism generally exists in both primary and secondary lymphoid organs between 15 and 24 wk of age (or post transplantation), although this window can shift by several weeks in individual mice.

FACS analysis of human B cell (CD19, CD20) and T cell (CD3, CD5) subsets revealed a consistent pattern of early B lineage and delayed T lineage reconstitution (Fig. 1D), similar to murine and human HSC transplantation (24, 25). T and B cell ratios varied greatly over time in the PBL and spleen, but once established in the LNs, they remained consistent for several weeks and were similar to LNs in humans and wild-type mice (~60% T, 20% B). In a subset of mice, notably those examined at later time points (>24 wk), the human T cell engraftment represented the majority of the human cells (Fig. 1D). Although variability in the timing and amount of chimerism existed among individual HSC recipients, the pattern of early B cell and later T cell reconstitution was a consistent finding. In contrast to the frequency, the absolute number of human B cells in the spleen remained fairly constant until 25 wk, whereas the number of human T cells increased sharply (Fig. 1D, bottom panels). In the LNs, the number of human T and B lymphocytes escalated between 10 and 20 wk of age (Fig. 1D). In all cases, LNs contained a majority of human T cells, suggesting B cells alone could not seed this tissue.

Both hIgM and hIgG were present in the sera of hu-mice at increasing concentrations, beginning at 9 wk post engraftment (Fig. 1E). Of note, concentrations of hIgG were higher than those of hIgM, suggesting that many human B cells in hu-mice undergo Ig class switch. The presence of engrafted LNs in individual mice significantly correlated with serum Ig (Fig. 1F).

**Mature B cells are present in all LNs and in the spleen of some hu-mice**

Several studies have reported an immature phenotype of B cells in hu-mouse models, warranting skepticism about their use for B cell studies (14–16). We investigated the state of B cell maturation in our hu-mice by analyzing the expression of CD10, CD24, and CD38 that are high on immature and low on mature B cells. In agreement with other studies (14, 15), we found mostly immature B cells in the spleen and PBL of many chimeric mice (Fig. 2A and data not shown). However, we also observed a significant fraction of mature B cells in all LNs and in the spleen of some hu-mice (Fig. 2A). These data were confirmed by additional analysis of the expression of CD21 and CD22 that are low on immature and high on mature B cells (data not shown).

Upon analysis of 215 chimeras, we categorized hu-mice into the following five distinct phenotypes based on the presence or absence of LNs and the frequency of mature (CD10^-) B cells in the spleen:

1. LN^-, <25% CD10^-;
2. LN^+, <25% CD10^-;
3. LN^-, >25% CD10^-;
4. LN^-, 25–60% CD10^-;
5. LN^+, >60% CD10^-.

Fig. 2A shows representative FACS analysis, and Fig. 2B reports the frequency of CD10^- mature B cells in the spleen of hu-mice assigned to different categories, relative to BM and LN. We found that the percentage of hu-mice with LNs and mature B cells in the spleen increases substantially with age, representing a majority by 18 wk (Fig. 2C). Moreover, we observed a positive correlation between mature B cell development in the spleen and both total human leukocytes and T cell frequencies (Fig. 2D), with the exception of mice in category III that do not develop LNs but still harbor a high frequency of mature B cells in the spleen (Fig. 2B, 2D).

These observations suggest that the human hematopoietic system evolves with time in hu-mice reaching conditions that foster the generation of mature B cells in most animals.

**Characterization of B cells in hu-mice**

To better understand the B cell populations that develop in hu-mice, we characterized the expression of multiple surface markers on B cells in the BM, LN, and spleens of hu-mice displaying immature (categories I and II) or mature (categories III–V) B cells. We compared the expression of these proteins with that on B cells not only of human cord and adult blood but also of human spleen, LN, and BM because tissue-specific differences in the B cell phenotype might exist. These data are shown in Table I, in which the markers are divided into distinct categories: B lineage (CD19, CD20, CD45RA, and CD45), immature B cell (CD5, CD10, CD24, and CD38), mature B cell (CD40, HLA-DR, CD21, CD22, CD268, and CD23), activation or memory (CD27, CD70, CD25, CD69, CD80, CD86, CD122, CD11c, CD95, and CD45RB), Ig isotypes (IgM, IgD, and IgG), and chemokine or adhesion receptors (CD62L, CD44, CD49d, LFA-1, CCR6, CCR7, CXCR4, and CXCR5). As shown previously, human B cells developing in the BM of hu-mice display characteristics very similar to those of B cells found in human BM (9, 15). Moreover, this analysis confirms that B cells in hu-mice of categories I and II are more immature than those of hu-mice classified as III–V. It also indicates that mature B cells of hu-mice are capable of activation and Ig class switch, as evidenced by the expression of CD27, CD11c, CD95, CD45RB, and IgG. In most cases, mature B cells in the LN and spleen of hu-mice express proteins at levels similar to those observed on B cells from human controls, with the exception of CD268 (BAFF receptor), which is expressed on B cells of hu-mice at only 10–20% of control B cells (Table I).

**Numbers and phenotype of T cells in hu-mice that harbor immature or mature B cells in the spleen**

Analysis of human T cell frequencies and numbers in the spleen of hu-mice revealed a larger T cell population among mice with mature splenic B cells (Fig. 3A). This correlation was confirmed by a linear regression analysis displaying the percentage of mature B cells, defined as CD10^- or CD22^- (Fig. 3B), as a function of T cell frequencies in the spleen. To extend these findings, we examined the expression of markers on T cells in peripheral organs of hu-mice to determine whether the presence of mature B cells correlated with a particular T cell phenotype. We found that LNs displayed a higher percentage of CD4 T cells, compared with the spleen, but observed no distinct correlation of CD4 (and conversely CD8) T cells with B cell maturation in the spleen (Fig. 3C). In mice with immature or mature splenic B cells, clear differences were not observed in the expression of numerous T cell proteins, including activation markers (CD69, CD25, CD44,
very little IgM or IgG was detectable in mice with only immature B cells (Fig. 4A, category I), whereas the presence of mature B cells in either the LN or the spleen (categories II–V) correlated with increased hIgM and hIgG (Fig. 4A).

The generation of a class-switched, Ag-specific Ab response upon immunization is currently a major challenge in the hu-mouse model. The predominant immature B cell population is considered partly responsible for this immunodeficiency (18). We reasoned that we should see improved Ag-specific responses in mice with mature B cells. To test this hypothesis, we immunized hu-mice with the TI-II Ag, NP-Ficoll, or the TD Ag, DTaP, and measured the respective Ab responses. Initial observations indicated that the determination of Ag-specific responses in hu-mice is challenging because of the extreme variation in hIg levels among individual mice (Fig. 1F). Comparing responses among hu-mice is complicated by two major factors, which both correlate with serum Ig concentrations: 1) a nonspecific background that is measured in sera even from plates that are not coated with Ag, and 2) a polyreactive response to Ag, most notably of the IgM isotype, that is detected in sera of

Improved Ab responses to TI and TD Ags in hu-mice with mature B cells

To evaluate whether increased numbers of mature B cells support higher Ig production, we measured over time the concentrations of hlgM and hlgG in sera of hu-mice classified into categories I–V.
both unimmunized and immunized mice. To limit the effect of these issues on our analyses, we compared Ab responses only in mice displaying a limited range of IgM concentration (1–35 μg/ml) and subtracted the background signal detected on plates in the absence of Ag from the signal measured in the Ag-coated plates for each sample.

No response was ever detected in sera from nonhumanized BALB/c-DKO mice, or from hu-mice without detectable Ig, indicating that a background response measured in naive hu-mice was due to Igs (Fig. 4B). Both the TI and TD Ag-specific Ab responses were increased in hu-mice that were immunized after 14 wk, when mature B cells are more prevalent (Fig. 4B). This increase was more pronounced for IgM responses, although IgG responses from mice immunized after 14 wk also showed modest increases over those from mice that were unimmunized or immunized before 14 wk (Fig. 4B). Even in mice immunized early, the presence of mature B cells in some hu-mice resulted in DT-reactive Ab levels above the background observed in unimmunized hu-mice (Fig. 4B). Nevertheless, IgG responses to the TD Ag were weak even when vaccinated after 14 wk, suggesting that other factors in addition to B cell maturation are required for an optimal Ig class-switched Ab response.

Increased colocalization, yet abnormal organization, of mature B and T cells in hu-mice

In humans and mice, T and B lymphocytes are organized into distinct zones within secondary lymphoid tissue. Previous histological analyses have shown a lack of organization of human T and B cells in spleens of hu-mice (8, 10, 26). We questioned whether lymphoid organization improves in LNs and spleen of hu-mice that develop mature B cells.

To address this question, we performed immunohistochemistry on spleen sections of hu-mice in categories I and II (immature B cells) and IV and V (mature B cells) and compared them with human spleen sections. LN tissue sections, in which the B cells are predominantly mature, were also analyzed. In most hu-mice with immature splenic B cells, the human and mouse hematopoietic cells were interspersed and randomly scattered throughout the spleen without any discernible organization (Fig. 5, I/II). Increased organization of human B cells into discrete follicle-like structures was observed only in the spleen of a category II hu-mouse that had a large B cell population (Fig. 5, SP II). In the LNs and spleens of mice with mature splenic B cells, human T and B cells colocalized, with some T cells also scattered outside these foci (Fig. 5, IV/V). However, none of the mice exhibited a T–B cellular organization similar to that seen in human spleens (Fig. 5) (27). Therefore, our data suggest that mature T cells likely form interactions with B cells in hu-mice with higher numbers of T cells and mature B cells, potentially supporting better immune responses despite abnormal lymphoid organization.

Requirement of T cells for B cell maturation in hu-mice

The correlation observed between the number of T cells and the presence of mature B cells in the spleen of hu-mice (Figs. 2D, 3A, 3B) led us to hypothesize that T cells provide signals necessary for B cell maturation. To test this hypothesis, we performed experiments in which we either added exogenous T cells (Fig. 6A) or depleted developing human T cells in hu-mice (Fig. 6B). Enumeration of T and B cell populations indicated that both T cell treatments were effective, as T cell numbers were higher in hu-mice adoptively transferred with T cells and lower in those with anti-CD3 treatments, whereas total B cell numbers were similar in all animals (Fig. 6A, 6B). The adoptive transfer of autologous T cells to hu-mice led to a significant increase in the frequency of CD10+ and CD22+ mature B cells in the spleen (Figs. 6A, Supplemental Fig. 1A). In contrast, when human T cells were depleted

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Analysis of protein expression was performed by flow cytometry on gated human (Hu) and hu-mouse (Hu-Mo) B cells defined as hCD45+ and CD19+ and/or CD20+. In the BM of hu-mice, analysis was also performed on the developmental early fraction of CD19+CD20+ B cells. Analysis of spleen (SP) B cells from hu-mice is separated for mice of categories I and II and categories III–V.

The relative protein expression level is depicted in a grayscale format and with + and – symbols, with black and ++++ representing the highest expression.

The frequency of positive cells is depicted in a grayscale format and with + and – symbols, with black and ++++ representing the highest frequency.

with injections of anti-CD3 Abs, the percentage of mature B cells was significantly reduced compared with that in control hu-mice (Figs. 6B, Supplemental Fig. 1B). Thus, these data support our hypothesis that T cells are required for B cell maturation. In further support, a highly significant linear correlation was observed between the frequency of T cells and that of mature B cells in the spleen of these hu-mice (Fig. 6C). The effect of T cells was also clearly evident on LN development in these experiments, as only mice with significant T cells displayed obvious LNs that bore higher numbers of human lymphocytes (data not shown).

The results from these experiments indicate that human T cells play an important role in the generation of mature human B cells.

Discussion
This study provides a detailed description of human T and B lymphocyte engraftment in BALB/c-DKO hu-mice, with an extensive characterization of these cells over time. Importantly, we show that human T and B cell engraftment is a dynamic, yet predictable, process. We observed that immature B cells dominate the human population early after engraftment, and that T cells appear weeks later, coincident with LN occupation, B cell maturation, and Ig production. B cell production was not sustained over time, potentially owing to an insufficient stem cell niche in the mouse or engraftment of nonpluripotent, self-renewing HSCs (11, 15, 28).

Despite several previous studies describing the presence of only immature B cells in hu-mice (14–16), we report in this article that mature B cells develop in our model. Our observation of mature B cells is likely due to the timing of our experiments (we observe mature B cells in older mice) and the analysis of B cells in LNs, in which we always observe a dominant mature B cell population regardless of age. Analysis of multiple cell surface proteins demonstrates that the phenotype of mature B cells in our hu-mice is quite similar to that of B cells in human blood and tissue (Table I). This finding is true for markers of maturation and activation, as well as those related to cellular interaction or trafficking, with few exceptions. We also found that expression of CD44 on B cells of hu-mice is consistent with that of human B cell populations and clearly distinguishes mature B cells (high CD44) from immature B cells (low CD44) (29). On the basis of a previous study, we cannot exclude the possibility that the mature B cell population in our hu-mice includes a late transitional T3 population that is phenotypically indistinguishable from mature naive B cells by conventional markers (30). However, the fact that some B cells in our hu-mice class switch to IgG and upregulate activation markers highlights the fact that these cells are functional and suggest that they do not possess an inherent maturation block. Although most of the differentiation markers analyzed showed normal expression levels on mature B cells of hu-mice, one notable exception stood out. The expression of CD268 (BAFFR) was significantly reduced compared with that on B cells from human tissues (Table I). We suggest that CD268 might be downregulated in hu-mice following binding to mouse BAFF, which is at superphysiological concentrations relative to B cell numbers (31, 32). A similar phenomenon has been observed in human CB transplantation recipients (33).
In addition to characterizing human hematopoietic engraftment in hu-mice, we also used the model to study human B cell biology. The most notable aspect of this study was our unexpected finding that human T cells provide the resources for human B cell maturation. Our data predict that in patients and mice with T cell immunodeficiencies, the B cells might have a developmental defect. In fact, the few studies that addressed this issue in humans concluded that B cells in patients with SCID (34), X-linked lymphoproliferative disease, and common-variable immunodeficiency disease (18) are prevalently immature, as they are more similar to the B cells in CB than to those in adult peripheral blood.

Another study of patients with IL-7Rα deficiency, a defect that leads to low T cell and normal B cell numbers, observed barely detectable levels of serum Ig, suggesting the presence of immature and/or nonfunctional B cells (35). Importantly, the delayed T cell reconstitution as well as its association with impaired B cell function is common, and quite problematic, in the clinical CB transplantation setting (33). A recent finding that CB transplantation in the absence of T cell depletion rapidly recovers the B cell function is consistent with our data that T cells facilitate B cell maturation (21). Because most of the known T cell–specific genetic defects in mice (e.g., TCRβ−/−) and humans (e.g., DiGeorge syndrome) do not completely abolish T cell generation, validating our observation and defining the mechanism of TD B cell maturation will require future investigation with proper experimental models (e.g., TCRβ−/−/γδ−/− mice and manipulated hu-mice).

We found that T cells expressing CD45RO, HLA-DR, CD49d, and CD95 (Fig. 3C and data not shown) were elevated in LNs and in spleens with mature B cells. Upregulation of these markers indicates an activated state and suggests that the activation of T cells might be required for B cell maturation. Whether the T cells developed de novo in hu-mice from HSCs or were transferred exogenously, the correlation of activated T cells with mature B cells in the spleens was consistent. Analysis of CD49d expression—a molecule shown to be upregulated on Ag-activated, but not lymphopenia-activated, T cells in mice (36)—suggests that both forms of activation occur in hu-mice, as >50% of the CD45RO+ T cells in spleen and LNs expressed CD49d. A similar activated phenotype was also observed on CB T cells recovered from BALB/c-DKO mice 3 wk after their adoptive transfer (data not shown), suggesting that T cell activation might also be mediated by reaction with xeno-Ags. Because hu-mice will always provide a lymphopenic and xenogeneic environment that drives

**FIGURE 4.** Improved Ab responses to TI and TD immunizations in hu-mice with mature B cells. (A) Concentration (μg/ml) of hlgM (left) and hlgG (right) in sera of hu-mice of categories I–V. The data represent the mean ± SEM of n = 3–17 samples per time point and per category, with the exception of category III, 12 wk (n = 1). (B) Hu-mice were immunized or not with either the TI-II Ag NP-Ficoll (top four panels) or the TD Ag DTAp (bottom four panels) between 8 and 14 (<14) or 14 and 22 (>14) wk of age, as described in Materials and Methods. Left panels show representative ELISA curves with OD405 on the y-axis and serum dilutions on the x-axis. Each graph includes hu-mice that were immunized (inert line, filled symbols) or not (dashed line, empty symbols) and that displayed mostly mature (black lines) or immature (red lines) B cells, and relative to an immunized intact BALB/c-DKO mouse (gray line). Right panels show relative Ab titers (calculated as described in Materials and Methods) in hu-mice that were nonimmunized (None) or immunized before (<14w) or after (>14w) 14 wk of age, and displaying mature B cells (closed circles) or immature B cells (open circles) in the spleen. *p < 0.05, ***p < 0.001.
T cell activation (37), it will be difficult to discriminate whether or not activation is required for B cell maturation. The activated state of T cells that correlated with B cell maturation was also associated with higher T cell numbers, and T cell numbers strongly correlated with mature B cells. Therefore, it is possible that T cell activation merely expands the T cell population to a size required to drive B cell maturation.

Similarly, our studies do not distinguish whether a CD4 or CD8 T cell subset is responsible for B cell maturation. We did not observe a correlation between frequencies of CD4 T cells (and conversely of CD8 T cells) and mature B cells in the spleen in both intact hu-mice (Fig. 3C) and those boosted with CB T cells (data not shown). Preliminary experiments to discern whether CD4 or CD8 subsets are required for B cell maturation were inconclusive because 3 wk after the injection of highly purified CD4 or CD8 T cells, a mixed (injected and endogenous CD4 and CD8) T cell population was observed in tissues (data not shown). Notably, the presence of mature B cells in the spleens of these hu-mice correlated with the frequency and number of total T cells, and not with a T cell subset, similar to what we observed in intact hu-mice.

The hu-mouse model is conceptually a very powerful resource for studies of the human hematopoietic system. However, the limitation of inadequate cellular development in these mice still warrants considerable skepticism regarding its usefulness. In this regard, what some may view as a limitation, others may consider a unique resource. An underappreciated advantage of the hu-mouse model is its utility in mapping cellular developmental pathways by adding or removing cellular or molecular components. Using this approach, future studies will explore the specific T cell factors that drive B cell maturation in hu-mice.
B cells in the spleen. We show that the human chimerism of hu-mice changes over time, including a decline in BM B cell output and an increase in the engraftment of LNs, the proportion of mature B cells, the production of IgM and IgG, and the thymic output and expansion of T cells. However, the specific timing of hematopoietic cell reconstitution in individual mice may vary by several weeks. For instance, in some hu-mice mature B cells are seen prior to 15 wk, whereas in others they appear later than 20 wk, and in some they never develop. The best indicator for the presence of mature B cells is not age but rather the frequency of T cells, which can be easily determined by PBL analysis.

The interpretation of Ag-specific ELISA data in hu-mice proved quite challenging. Unlike in mice and humans, the sera of hu-mice contain vastly differing Ig concentrations from animal to animal, and in the same animal over time, making it difficult to compare Ab responses in sera of immunized mice relative to preimmune sera (14, 16, 19, 22), or without proper controls (10, 17). Any hu-mouse sera, whether Ag reactive or not, generated a background signal that was dependent on the Ig concentration. This background was independent of Ag on the plate, it was present whether or not the plate was blocked with a variety of reagents, and it was absent only in sera of mice that did not contain any Ig. In addition, we observed inherent polyreactivity, particularly in the IgM responses, similar to polyreactive responses detected in both mice (38) and humans (39, 40), which also increased with Ig concentration. These issues emphasize the need for a well-controlled, standardized Ag-specific Ab assay to allow comparisons among laboratories using hu-mice.

Nonetheless, using our optimized ELISA protocol, we were able to determine that immunizing hu-mice later (>14 wk), when T cells and consequently mature B cells reach significant numbers, results in improved Ag-specific responses. Even when hu-mice are immunized early, the Ab response to TD Ag is better in hu-mice with mature B cells in the spleen. These observations suggest that mature B cells support improved Ab responses to immunization in hu-mice. This Ag-specific IgG is particularly relevant in the context of a TD response, as it suggests the presence of both a population of competent B cells capable of Ig class-switch and a productive cognate B cell–T cell collaboration. Our histological studies noted an increased colocalization of human T and B cells in the spleen of mice with mature B cells (9, 26), suggesting the possibility of increased T cell–B cell cognate interactions. However, these analyses still indicate the presence of an abnormal lymphoid architecture even in those hu-mice with T cells and mature B cells, suggesting that the human cells do not correctly sense the chemokines driving lymphocyte localization. Other studies have shown that supplying a human MHC class II allele to hu-mice (41) or cotransplanting a human thymus (42) enhances TD Ab responses. It will be of interest to test whether these responses are further improved in the presence of mature B cells.

The large majority of B cells in all LNs analyzed in hu-mice were mature, clearly differentiating this tissue environment from those of the PBL and spleen. The LNs of hu-mice displayed other unique features: 1) LN anlagen appear to exist in the absence of hematopoietic chimerism (i.e., in intact BALB/c-DKO mice), as suggested by the existence of barely visible structures and the rapid infiltration of cells following human PBMC injection (data not shown); 2) mouse hematopoietic cells are not detected by flow cytometry within LNs in the absence of human hematopoietic cells; 3) only a subset of hu-mice harbor engrafted LNs—the engraftment appears to depend on time, absolute chimerism, and presence of T cells; 4) human hematopoietic engraftment of LNs is greatly delayed (>2 mo) compared with human engraftment of BM, spleen, and PBL; 5) LNs are differentially engrafted—mesenteric LNs are populated earlier and more commonly than peripheral LNs; however, even hematopoietic engraftment of peripheral LNs, which occurs typically in mice with dominant T cell chimerism, is irregular, with cervical and axillary LN engraftment more common but inconsistent and often unilateral; 6) the LNs contain a fraction of CD4/CD8 double-positive T cells (data not shown) that is normally found only in the thymus; and 7) the ratio of human T:B cells in the LNs is highly consistent and physiological (2:1) compared with that in the spleen. Our data overall suggest that the high frequency of mature B cells in the LN is due to the high proportion of T cells in this tissue. The presence of a minor population of immature B cells in the LN suggests that B cell maturation takes place within this tissue after entry and that it is not a requirement for entry into the LN.

The engraftment dynamics of LNs in hu-mice can potentially provide clues for understanding the mechanisms of murine and human LN development. We noticed that the spleen and BM of hu-mice with engrafted LNs display a correlative increase of human CD4⁺CD3⁺CD20⁻ cell numbers (data not shown). Cells with this phenotype have been implicated in the seeding of LNs in mice (43, 44), suggesting that they might be also promoting LN occupation in hu-mice. Alternatively, T cells might develop extrathymically, directly in the LN, a phenomenon that is amplified by oncostatin M (OM) and that is frequently observed in OM transgenic mice and, at times, in athymic mice (45). T cell development also occurs in the LNs of wild-type mice, although this process is inhibited or outcompeted by thymic-derived T cells (45). T cells in the LNs of hu-mice display a hyperproliferative and a hyperapoptotic phenotype (Fig. 3C and data not shown), which is consistent with T cells in LNs of OM transgenic mice. In addition, the more frequent development of mesenteric LNs over axillary and cervical LNs in hu-mice is similar to that observed in OM transgenic mice (45). Thus, whether the LNs in hu-mice are engrafted by specific CD4⁺CD3⁻ precursors or are the site of extrathyphic T cell development remains to be determined. The inconsistent frequency and site of LN engraftment among hu-mice suggest this is a relatively rare, stochastic event.

A final point of this study is the reliability of the BALB/c-DKO hu-mouse model as an experimental model of the human immune system. We believe that this model transplanted with CB HSCs offers certain advantages, including consistent engraftment of HSCs, longevity, productive breeding, significant thymic engraftment and T cell generation without the need for human thymus cotransplantation (16, 46), and robust IgG production. Furthermore, the susceptibility of the BALB/c embryonic stem cells to genetic manipulation makes it easier to introduce novel mutations. In this regard, other investigators have generated novel BALB/c-DKO strains that express human genes designed to enhance human hematopoietic engraftment, survival, and differentiation (47, 48).

Using this model, we were able to analyze human hematopoietic cells in hu-mice for >4 mo and detect mature B cells. This finding is important, considering the numerous reports of a B cell maturation block in hu-mice. We also determined that the generation of mature, naive B cells in hu-mice is dependent on the presence of human T cells. Notably, this B cell population is functional and produces improved Ag-specific TI and TD Ab responses over those measured in hu-mice with immature B cells. This finding suggests that experimental vaccine studies in hu-mice should be performed in older mice with higher frequencies of mature B cells, which can be estimated by the frequency of PBL T cells. Thus, this study enhances both the usefulness of the hu-mouse model and our understanding of human B cell development.
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Disclosures
The authors have no financial conflicts of interest.

References
