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

J Immunol published online 18 January 2013
http://www.jimmunol.org/content/early/2013/01/18/jimmunol.1202810

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/01/18/jimmunol.1202810.DC1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
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 Il2rgnull 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: 000–000.

Received for publication October 9, 2012. Accepted for publication December 18, 2012.

This work was supported by National Institutes of Health Grant R21-AI073629 (to R.P.), an Arthritis Foundation Innovative Research Grant (to R.P.), a National Jewish Health Translational Research Initiative Grant (to R.P.), and Department of Defense Grant W81XWH-07-01-0550 (to R.M.K.).

Address correspondence and reprint requests to Dr. Roberta Pelanda, Integrated Department of Immunology, National Jewish Health, 1400 Jackson Street, Room K814a, Denver, CO 80206. E-mail address: pelandar@njhealth.org

The online version of this article contains supplemental material.

Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; AP, alkaline phosphatase; BALB/c-DKO, BALB/c-Rag2nullIl2rgnull; BM, bone marrow; CB, cord blood; DT, diphtheria toxin; DTaP, diphtheria, tetanus, and pertussis; h, human; HSC, hematopoietic stem cell; hu-mouse, humanized mouse; LN, lymph node; m, mouse; OM, oncostatin M; PBL, peripheral blood; TD, T cell–dependent; TI, T cell–independent.

Copyright © 2013 by The American Association of Immunologists, Inc. 0022-1767/13/S16.00

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 LN s of BALB/c-Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> (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<sup>+</sup> and CD34<sup>−</sup> 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<sup>+</sup> cells were enriched using autoMACS (Miltenyi Biotec) technology. The CD34<sup>+</sup> 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<sup>+</sup> cells were used immediately or cultured at 1 × 10<sup>6</sup> per milliliter in IMDM supplemented with 10% FBS, 50 μM L-2-ME, 2 mM Glutathione, IL-6 (10 ng/ml), stem cell factor (20 ng/ml), and FLT3 (10 ng/ml). Biosafety Level 2 conditions at the Biological Resource Center at National Jewish Health (Denver, CO). Animal care and experiments were conducted in accordance with guidelines from the Institutional Animal Care and Use Committee. Each animal was monitored, and data were collected in accordance with the principles of humane treatment of experimental animals. The studies were performed in compliance with the National Jewish Health and University of Colorado Denver Institutional Review Board and were approved by the National Jewish Health Institutional Animal Care and Use Committee. Human CB17 mice (negative controls); and human CB and PBL (positive controls). Human spleen and lymph node (LN) samples were collected from cadavers from the International Institute for the Advancement of Medicine or from individuals who underwent surgery at the University of Colorado Hospital. The human BM sample was obtained from Lonza. The studies were performed in compliance with the National Jewish Health and University of Colorado Denver Institutional Review Boards. Mouse PBL samples were collected by either submandibular or tail vein bleed and processed over a Ficoll density gradient for leukocyte separation. Serum was prepared by centrifugation of naturally clotted blood. Single-cell suspensions of all tissues were prepared by standard cell preparation. Serum was prepared by centrifugation of naturally clotted blood. Single-cell suspensions of all tissues were prepared by standard methods. The hu-mice had serum Ig concentrations of 0–670 mg/ml (IgG). We tested coating the plates with 1% BSA or PBS–0.05% Tween 20; and detecting bound Igs with the appropriate secondary Abs. The plates were incubated at 37˚C for 1 h. After washing, the plates were assayed using an ELISA reader.

**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 cryostat 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 by incubating with avidin (Avidin/Biotin Blocking Kit, Vector Laboratories), and then for 15 min with a mixture of biotin, mlgG, mlgH, 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 (PacB) 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 Axiosvert 200 microscope (Carl Zeiss) outfitted with a Si Marians system and analyzed with SlideBook 4.0 software (Intelligent Imaging Innovations).

**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. with the mixtures of vaccines: NP-Ficoll (100 μg, Biosearch Technologies) or diphtheria, tetanus, and pertussis (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 hIgM and hIgG 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 mg/ml (IgM) and 0–3200 mg/ml (IgG). We tested coating the plates with the following Abs, all diluted in PBS: nickel-nitrotriacetate (AP): monoclonal mouse-, and polyclonal goat-, anti-human 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

Downloaded from http://www.jimmunol.org/ by guest on July 26, 2017
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...
kinetics were unique to distinct lymphoid organs. Human chimera
d dynamics was detected early in the thymus and was sustained over time
(Fig. 1 A, left panel). Conversely, splenic engraftment was delayed relative
to BM, was maximal between 15 and 24 wk, and then de
c lined sharply as human hematopoietic production in the BM waned
(Fig. 1 A). Unlike in the spleen or BM, mCD45<sup>+</sup> hematopoietic
cells were rare in the LN or thymus 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 im
umunization (Fig. 1 B). Human cells were detected in mesenteric LNs
prior to peripheral LNs, although in older mice, occupation of cer
vical, 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. 1 C). 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. 1 A, 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. 1 D), 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 constant 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. 1 D). Although variability in the timing
and amount of chimerism existed among individual HSC recipi
ents, 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. 1 D, bottom panels). In the LNs, the number of hu
man T and B lymphocytes escalated between 10 and 20 wk of age
(Fig. 1 D). In all cases, LNs contained a majority of human T cells,
suggesting B cells alone could not seed this tissue.

Both hlgM and hlgG were present in the sera of hu-mice at
increasing concentrations, beginning at 9 wk post engraftment (Fig.
1 E). Of note, concentrations of hlgG were higher than those of
hlgM, 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. 1 F).

**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. 2 A 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. 2 A). 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<sup>+</sup>) B cells in the spleen:
I) LN<sup>−</sup>, <25% CD10<sup>+</sup>; II) LN<sup>+</sup>, <25% CD10<sup>+</sup>; III) LN<sup>−</sup>, >25%
CD10<sup>+</sup>; IV) LN<sup>−</sup>, 25–60% CD10<sup>+</sup>; and V) LN<sup>+</sup>, >60% CD10<sup>+</sup>. The
absolute numbers of human cells roughly
and the size of LNs in these hu-mice was comparable to that in wild-
type mice (Fig. 1 C). 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.

**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 com
pared 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 pheno
type 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 in
dicates 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. 3 A). This correlation was confirmed
by a linear regression analysis displaying the percentage of mature
B cells, defined as CD10<sup>+</sup> or CD22<sup>+</sup> (Fig. 3 B), 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.
3 C). 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,
CD62L and CD122; Fig. 3C and data not shown), homing receptors (CCR7, CXCR4, CXCR5, and LFA-1; data not shown), or signaling receptors (CD2, CD3, CD5, and CD27; data not shown). However, T cells expressing the memory marker CD45RO and the activation markers HLA-DR and CD49d were more frequent in LNs and spleen of hu-mice displaying mature B cells than in the spleen of hu-mice with immature B cells (Fig. 3C, lower panels). We also noticed a relatively large proportion of 7-AAD + dying T cells in the LNs and spleen, but not in the BM, of hu-mice, but no correlation with the presence of mature B cells was found (Fig. 3C, upper right panel). These data suggest that T cells, and potentially their activation state, might be important for B cell maturation in hu-mice.

**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 hIgM and hIgG in sera of hu-mice classified into categories I–V. 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...
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 mice with mature splenic 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 separated for mice of categories I and II and categories III–V.

Table I. Characterization of B cells in tissues from humans and hu-mice

<table>
<thead>
<tr>
<th>Marker</th>
<th>Cond Blood</th>
<th>Hu PBMC</th>
<th>Hu SP</th>
<th>Hu LN</th>
<th>Hu BM</th>
<th>Hu-Mo BM</th>
<th>CD19*</th>
<th>CD20*</th>
<th>CD3*</th>
<th>CD4*</th>
<th>CD8*</th>
<th>CD19*</th>
<th>CD20*</th>
<th>CD4*</th>
<th>CD8*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Analysis of protein expression was performed by flow cytometry on gated human (Hu) and hu-mouse (Hu-Mo) B cells defined as hCD45+/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.

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...
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 individual hu-mice \( (n = 127–144) \). Each symbol represents an individual mouse.

**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).
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., TCRαβ−/−) and humans (e.g., DiGeorge syndrome) do not completely abolish T cell generation, validating our observation and defining the mechanism of T cell maturation will require future investigation with proper experimental models (e.g., TCRαβ−/−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
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 BM and LNs in control untreated hu-mice are shown as reference for tissues with a majority of immature B cells and mature B cells, respectively. Data represent mean ± SEM of eight cumulative mice per group from three independent experiments. Representative FACS data are shown in Supplemental Fig. 1A.

CD8 subsets are required for B cell maturation 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.

FIGURE 6. Human T cells are required for B cell maturation. (A) Maturation of B cells in hu-mice adoptively transferred with autologous T cells. Hu-mice were injected (+ T boost) or not (− T boost) at 11–12 wk of age with autologous T cells and harvested at 13–15 wk. Left panel, Numbers of T and B cells in the spleens. Middle and right panels, Frequencies of mature B cells (CD10−, middle panel; CD22+ right panel) in the spleens of mice injected or not injected with T cells. The frequencies of mature B cells in BM and LNs in control untreated hu-mice are shown as reference for tissues with a majority of immature B cells and mature B cells, respectively. Data represent mean ± SEM of eight cumulative mice per group from three independent experiments. Representative FACS data are shown in Supplemental Fig. 1A. (B) B cell maturation in hu-mice treated with CD3-depleting Abs. Left panel, Numbers of T and B cells in spleens of hu-mice injected (+) or not (−) semiweekly with CD3-depleting Abs from 9 wk of age and analyzed generally between 18 and 24 wk (n = 5–8). Middle and right panels, Frequencies of mature B cells (CD10−, middle panel; CD22+, right panel) in the spleens of mice injected or not with anti-CD3 Abs. The frequencies of mature B cells in BM and LNs in control untreated hu-mice are shown as reference. Data represent mean ± SEM of six to eight cumulative mice per group from three independent experiments. Representative FACS data are shown in Supplemental Fig. 1B. (C) Linear regression analysis for mature B cells (CD10−, left panel; CD22+, right panel) as a function of T cell frequency in the spleen of all mice described in (A). *p < 0.05, ***p < 0.001.
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 hlgM and hlgG, 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 human 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 corelative 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 extrathymic 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.
Acknowledgments
We thank Josh Loomis in the Flow Cytometry Core at National Jewish Health for technical help, Nick Weiss for technical assistance, Kim Jordan for providing human spleen cells, and Katie Haskins and Lenka Teodorovic (both of University of Colorado Denver and National Jewish Health) for critical reading of the manuscript.

The disclosures
The authors have no financial conflicts of interest.

References


Figure S1. FACS analysis of B cells in spleens of mice injected with or depleted of T cells.

(A) Expression of CD10 (top) and CD22 (bottom) on hCD45+CD20+ gated B cells from CB (right panels) or spleens of control hu-mice (left panels) or hu-mice injected with autologous CB cells (“T-boost”, middle panels) at 11-12 wks of age. Mice were euthanized and spleen cells analyzed by FACS at 14-15 wks of age. Data are representative of 8 mice per group and >25 CB samples.

(B) Same as panel A with the exception that the middle panels represent cells from T cell-depleted hu-mice (“Anti-CD3”). These hu-mice received anti-CD3 Ab injections twice weekly from 9 wks of age to time of euthanasia at 18-24 wks. Data are representative of 6-8 mice per group and >25 CB samples.