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Development of human B cells and antibodies following human hematopoietic stem cell transplantation to Rag2$^{-/-}\gamma c^{-/-}$ mice

Anne Tanner, Steven J. Hallam, Stanton J. Nielsen, German I. Cuadra, Bradford K. Berges *

Department of Microbiology and Molecular Biology, Brigham Young University, Provo, UT 84602, USA

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ABSTRACT

Humanized mice represent a valuable model system to study the development and functionality of the human immune system. In the RAG-hu mouse model highly immunodeficient Rag2$^{-/-}\gamma c^{-/-}$ mice are transplanted with human CD34^{+} hematopoietic stem cells, resulting in human hematopoiesis and a predominant production of B and T lymphocytes. Human adaptive immune responses have been detected towards a variety of antigens in humanized mice but both cellular and humoral immune responses tend to be weak and sporadically detected. The underlying mechanisms for inconsistent responses are poorly understood. Here, we analyzed the kinetics of human B cell development and antibody production in RAG-hu mice to better understand the lack of effective antibody responses. We found that T cell levels in blood did not significantly change from 8 to 28 weeks post-engraftment, while B cells reached a peak at 14 weeks. Concentrations of 3 antibody classes (IgM, IgG, IgA) were found to be at levels about 0.1% or less of normal human levels, but human antibodies were still detected up to 32 weeks after engraftment. Human IgM was detected in 92.5% of animals while IgG and IgA were detected in about half of animals. We performed flow cytometric analysis of human B cells in bone marrow, spleen, and blood to examine the presence of precursor B cells, immature B cells, naïve B cells, and plasma B cells. We detected high levels of surface IgM^{+} B cells (immature and naïve B cells) and low levels of plasma B cells in these organs, suggesting that B cells do not mature properly in this model. Low levels of human T cells in the spleen were observed, and we suggest that the lack of T cell help may explain poor B cell development and antibody responses. We conclude that human B cells that develop in humanized mice do not receive the signals necessary to undergo class-switching or to secrete antibody effectively, and we discuss strategies to potentially overcome these barriers.

1. Introduction

Human immune system mice are a useful tool to study the development and functionality of the human immune system. The most common current human immune system mouse models use human hematopoietic stem cells (HSCs) isolated from either cord blood or fetal liver and are then transferred into highly immunodeficient mice such as the Rag2$^{-/-}\gamma c^{-/-}$ and NOD/SCID$\gamma c^{-/-}$ strains [1]. These humanized mice produce a wide variety of human immune cell types, including B cells, T cells, monocytes/macrophage, and dendritic cells [2]. Production of other immune components such as granulocytes, erythrocytes, and platelets is typically weak, and the B and T lymphocyte population represents an unusually large proportion of blood, bone marrow, and spleen cells due to poor granulocyte production.

Humanized mouse models have been very useful for studies of viral pathogens of human immune cells such as Human Immunodeficiency Virus type 1 (HIV-1) [3], herpesviruses [4], Dengue virus [5], and other pathogens. Human antibody responses have been reported against a variety of pathogens in humanized mice, including HIV-1, Dengue virus, Epstein–Barr virus, Kaposi's Sarcoma-associated herpesvirus, Herpes simplex virus type 2, and other pathogens/antigens [5–10]. Neutralizing antibody responses that target similar viral proteins as seen in humans have been reported in the case of Dengue virus [5]. Although the current humanized mouse models are capable of producing human humoral and cellular immune responses to these pathogens, in general the results have been inconsistent and when they are detected the immune responses are typically weak [6,11,12]. The reasons for these findings are currently unclear, but previous studies have indicated that total human antibody concentrations are much lower in humanized mice than in humans and there may be a defect in class-switching, since IgM concentrations tend to be closer to human concentrations than for IgG [2,13]. One study attempted to immortalize human B cells after immunizing humanized mice and was only able to produce IgM monoclonal antibodies and not IgG producing cells, lending further support to the hypothesis that there is a lack of effective class-switching [14]. Interestingly, this same paper showed through immunoscope analysis that a broad diversity of human antibody sequences are derived in humanized mice, indicating that derivation of diverse B cell receptors functions very similarly in humanized mice as compared to humans. If

* Corresponding author. Tel.: +1 801 422 8112.
E-mail address: brad.berges@gmail.com (B.K. Berges).

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there is a defect in effective antibody class-switching in humanized mice the mechanisms are not understood.

Since immune responses are critical to controlling pathogens and for vaccine studies, a better understanding of the reasons for poor B cell responses in humanized mice would be useful to assist in developing better humanized mouse models that reproduce normal human antibody concentrations and more robust antigen-specific responses. Here, we describe the B cell compartment of HSC-transplanted Rag2$^{-/-}\gamma c^{-/-}$ mice. We have examined blood, bone marrow, and spleen for the presence of human B cells and to analyze the kinetics of B cell engraftment. We have also measured total human antibody levels in serum across a time course in order to determine when B cell development is complete in humanized mice. We found that immature and naive cells are found at a high frequency in spleen and bone marrow and that CD138^{+} plasma B cells are found in low levels in these organs. Few T cells were detected in the spleen, which is an important site for B cell maturation. Our results suggest that early steps in B cell development function properly in humanized mice, but that mechanisms governing class-switching and activation of B cells to become plasma cells are not very effective. Methods used to enhance T cell development and maturation in humanized mice may help to solve these issues.

2. Materials and methods

2.1. Cells

Human cord blood samples were obtained from the University of Colorado Cord Blood Bank. The Brigham Young University Institutional Review Board does not require a protocol for human cord blood samples because they lack patient identifiers. Human hematopoietic stem cells were purified from human cord blood using the CD34 marker with the EasySep human cord blood CD34-positive selection kit (StemCell Technologies). Cells were cultured for 2 days in Iscove's modified Dulbecco's medium (IMDM; Invitrogen) supplemented with 10% fetal calf serum (FCS) and 10 ng/mL each of human interleukin-3 (IL-3), IL-6, and stem cell factor (SCF) (R&D Systems) [15].

2.2. Animals

BALB/c-Rag2$^{-/-}\gamma c^{-/-}$ mice were humanized by engraftment with CD34^{+} human HSCs purified from human umbilical cord blood as described previously [15]. Mice were maintained in a specific pathogen-free (SPF) room at the Brigham Young University Central Animal Care Facility. Drinking water is supplemented with Trimethoprim-Sulfa antibiotics to prevent bacterial infection. These studies have been reviewed and approved by the Institutional Animal Use and Care Committee (protocols 120101 and 150108). Briefly, 1- to 5-day-old mice were conditioned by gamma irradiation with 350 rads and then injected intrahepatically with 2×10^5 to 7×10^5 human CD34^{+} cells. Mice were screened for human cell engraftment at 8–10 weeks post-engraftment, at which time plasma was also collected for antibody concentration analysis. Animals were additionally bled at numerous other time points in order to measure human cell types in blood and to collect additional plasma.

2.3. FACS analysis

70 μL of whole blood was obtained through tail bleed and treated with a mouse red blood cell lysis buffer for 15 min. Following this treatment the cells were centrifuged at 3300 rpm for 3 min and the supernatant was discarded. The pellet was then resuspended in 100 μL FACS staining buffer. 3 μL blocking buffer was then added and allowed to incubate in the dark at 4 °C for 15 min. This was followed by the addition of 3 μL of the desired fluorescent-linked antibodies and incubated in the dark at 4 °C for a period of 30 min. To determine percent human peripheral blood engraftment, peripheral blood was stained with human CD45 PECy-7 and mouse CD45 PE antibodies and FACS analysis was performed [16]. Antibodies used for B cell characterization were: hCD45 PECy-7, hCD19 APC eFluor 780 (eBioscience), hCD3 PE, hCD138 PECy5.5 (Invitrogen), and hIgM FITC (BioLegend). Immediately following this 30 min step 900 μL of 1% paraformaldehyde in 1× PBS was added to fix samples. Samples were then centrifuged at 3300 rpm for 3 min and the supernatant was discarded. The pellet was then resuspended in 150 μL 1× PBS and submitted for FACS analysis. An Attune Acoustic Focusing Cytometer (Applied Biosystems) was used to run samples, and Attune software v2.1 was used to analyze the results. All antibodies used in these studies do not cross-react with murine antigens, as assessed by staining of unengrafted Rag2$^{-/-}\gamma c^{-/-}$ blood and lymphoid organs.

2.4. Antibody concentrations

Mouse plasma samples were obtained by centrifuging whole blood at 3300 rpm for 3 min and then collecting the supernatant. ELISA tests were run using the Total Human IgM, Total Human IgG, and Total Human IgA kits (ALerCHEK) according to the manufacturer's instructions. Since human antibody concentrations are typically low in humanized mice, plasma was diluted 1:100 for IgM and IgG assays and 1:20 for IgA assays in order to obtain results in the linear range of the assay.

2.5. Statistics

Human T and B cell development in mouse peripheral blood was analyzed by Welch's ANOVA test (with Games–Howell post hoc test when applicable). To compare B cell levels in bone marrow, spleen, and blood, an ANOVA with Tukey–Kramer *post hoc* test was performed. For comparison of the different time points of plasma antibody concentrations an ANOVA with Tukey–Kramer post hoc test (where applicable) were performed. Standard error (SE) was calculated and indicated in all bar graphs. R^2 values were calculated to determine if a correlation exists between total antibody concentrations and peripheral blood engraftment levels in the humanized mice.

3. Results and discussion

3.1. Kinetics of human B and T lymphocyte development in peripheral blood of RAG-hu mice

In the RAG-hu model, purified human CD34^{+} hematopoietic stem cells are transplanted into neonatal Rag2$^{-/-}\gamma c^{-/-}$ mice following sub-lethal irradiation [15]. The kinetics of development of the human immune system following transplantation of human HSCs into mice is poorly understood. We hypothesized that one reason for weak adaptive immune responses in this model could be due to immaturity of the human immune system at the time of exposure to antigen. To examine the development of human lymphocytes, we used flow cytometry to monitor the relative frequency of human T cells (CD3^{+}) and B cells (CD19^{+}) in a time course in peripheral blood following human HSC engraftment (Fig. 1). Our findings confirmed previous reports that T and B lymphocytes are the major human cell types in humanized mouse peripheral blood, ranging from a combined sum of 54.6 ± 3.6% (SE) at 8 weeks post-engraftment (wpe) to 90.8 ± 1.2% at 14 wpe. We found that B cells outnumbered T cells at every time point except for 25–28 wpe, where levels were nearly identical. B cell levels ranged from 29.1 ± 5.0% to 76.7 ± 3.0% of human peripheral blood cells, while T cell levels ranged from 14.1 ± 2.6% to 28.3 ± 7.0%. The lowest levels of B cells were detected at 25–28 wpe, while a peak was detected at 14 wpe. The lowest levels of T cells were detected at 8 wpe and 14 wpe, although statistical analysis revealed that no significant differences in T cell populations were detected across the entire time course (Welch's ANOVA test, $p = .45$). At the 14 week time point there were significantly greater numbers of human B cells as compared

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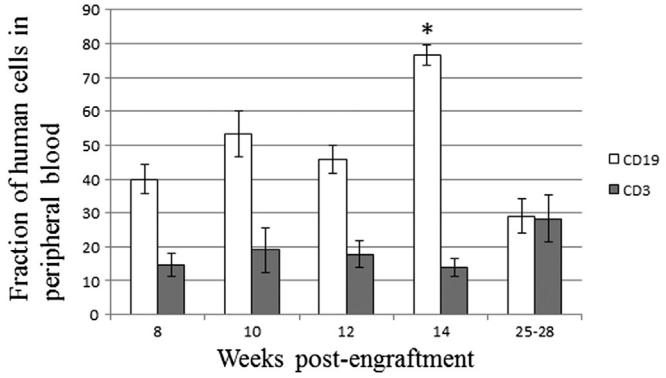

Fig. 1. Peripheral blood engraftment of human B and T cells in humanized mice. Flow cytometry was used to assess engraftment levels of human T cells ($CD3^+$) and human B cells ($CD19^+$) in peripheral blood. A human leukocyte antibody (anti-hCD45) was initially used for gating purposes to focus on human leukocyte populations. Mean levels in peripheral blood for each cell type were calculated and SE is indicated. (Welch's ANOVA test for B cell levels, $p < 0.000001$ with *post hoc* Games–Howell, $p < .01$ for all comparisons with week 14 except week 10 where $p < .05$). No significant differences in T cell populations were detected across the entire time course (Welch's ANOVA test, $p = .45$). Sample sizes per time point are $n = 11$ (8 w), $n = 14$ (10 w), $n = 7$ (12 w), $n = 6$ (14 w), and $n = 9$ (25–28 w). Unique cord blood samples used for engrafting animals at each time point: $n = 3$ (8 w), $n = 5$ (10 w), $n = 1$ (12 w), $n = 2$ (14 w), $n = 4$ (25–28 w).

to all other time points (Welch's ANOVA test, $p < 10^{-5}$ with *post hoc* Games–Howell test, $p < 0.01$). No significant differences were found between B cell levels at any other time points (*post hoc* Games–Howell test $p > 0.05$).

Since only a portion of humanized mice are good human antibody producers, we included data on peripheral blood T and B cell levels over time for individual animals (Table 1). Some animals followed the general trend seen in Fig. 1, while others did not. B cell levels in peripheral blood increased in all tested animals from week 8 to week 14, but then decreased in all tested animals from weeks 14 to 25. Interestingly, T cell levels in Fig. 1 showed insignificant changes over time, while individual mice show several different patterns. One animal steadily decreased from 8 to 25 weeks (mouse 2070), one animal showed a peak at 14 weeks (2071) and some animals increased across the time course (2072/2073). Mouse 2074 T cell levels increased from weeks 8 to 14, then appeared stable until week 25. These data suggest that T cell levels fluctuate more across a cohort of humanized mice whereas B cell levels follow a more similar pattern across groups of engrafted animals.

Harris et al. studied the duration of human immune system reconstitution in the NOD-Rag1-gammanull model engrafted with $CD34^+$ cells from cord blood [17]. They examined human B and T cell fractions in humanized mouse peripheral blood and found that B cells were predominant at early time points but that an inversion took place at about 15–20 wpe and T cells become the predominant lymphocyte in blood. Here, we showed that T cell levels do not significantly change between 8 and 28 wpe but that B cells had reached a peak at 14 wpe. We suggest that model-specific differences may explain our different results.

3.2. Proportions of human B cells in lymphoid organs and blood

Human B cell development in humanized mice has not been carefully studied and characterization of B cell development in primary and secondary lymphoid organs is also lacking. We sacrificed humanized mice and then used flow cytometry to detect different categories of B cells in bone marrow, spleen, and blood based upon phenotypic markers. We used CD19 as a marker for most human B cell populations, but also used CD138 as a marker for plasma B cells because CD19 expression is lost upon differentiation to this cell type. Some types of primitive B cells also express CD138, such as pro-B cells, pre-B cells, and immature B cells; however each of these populations also expresses CD19 which allows for differentiation from plasma B cells which are $CD19^-CD138^+$. We found that human B cells (sum of $CD19^+$ cells and $CD19^-IgM^-CD138^+$ cells) were the predominant human cell type (of the $hCD45^+$ population) in bone marrow (88.1 ± 4.3%; see Fig. 2), and to a lesser extent in spleen (60.1 ± 10.1%; see Fig. 3) and in blood (53.9 ± 5.3%; see Fig. 4).

3.3. Characterization of B cell types in bone marrow

In the bone marrow, precursor B cells ($CD19^+IgM^-CD138^+$) and immature B cells ($CD19^+IgM^+CD138^+$) represented the most common human B cells detected, at 42.2 ± 5.7% and 25.8 ± 7.6%, respectively (see Fig. 2C). Naïve B cells ($CD19^+IgM^+CD138^-$) and plasma B cells ($CD19^-IgM^-CD138^+$) were found in lower proportions, at 6.1 ± 1.8% and 1.0 ± 0.5%, respectively. Example FACS plots of stained bone marrow are shown in Fig. 2A, B. Statistical analyses were performed to determine if there were any significant differences between various B cell populations in bone marrow, spleen, and blood. The only significant difference in populations was that the $CD19^+IgM^-CD138^+$ population was larger in bone marrow than in spleen or blood (ANOVA $p = .003$ with *post hoc* Tukey–Kramer, $p < .05$).

Maturation of B cells to become plasma cells is dependent upon T cell help; specifically, CD40L on T cells interacts with CD40 on B cells to promote antibody secretion [18]. We detected only low numbers of human plasma B cells in the bone marrow of RAG-hu mice whereas in normal humans 4–5% of bone marrow cells are plasma cells [19,20]. One problem previously noted with most current humanized mice is that human T cells are largely selected based upon murine major histocompatibility complex (MHC) molecules in the thymus due to the lack of human stromal cells [21]. If human helper T cells are positively selected on murine MHC-II in the thymus, then their ability to interact with B cells expressing human MHC-II in the periphery is not expected to be effective and this would prevent effective plasma cell differentiation. Interestingly, many $CD5^+$ B cells have been reported to exist in humanized mice [22,23]. These cells are less prone to undergo class-switching

Table 1
Peripheral blood levels of human T and B lymphocytes tracked in individual mice over time. Human B and T lymphocyte populations were tracked in individual mice over a time course using flow cytometry analysis. Human leukocytes were gated based upon hCD45 expression, followed by analysis of $CD3^+$ cells (T cells) and $CD19^+$ cells (B cells).

	Gender	Cord sample	Cells transplanted
Mouse			
2070	F	A	7.3×10^5
2071	F	A	7.3×10^5
2072	F	B	9.0×10^5
2073	M	B	9.0×10^5
2074	M	B	9.0×10^5
2077	M	C	2.8×10^5

T fraction	Week 8	Week 14	Week 25
Mouse			
2070	19.7	14.1	1.4
2071	5.4	10	1.5
2072	9.5	n/t	36.5
2073	6.6	25.2	53.9
2074	3.3	11.7	10
2077	7.1	6.8	n/t

B fraction	Week 8	Week 14	Week 25
Mouse			
2070	43.4	78.7	46.9
2071	39.4	79.7	46.6
2072	28.6	n/t	2.7
2073	33.7	65.6	20.4
2074	53.7	83.1	28.1
2077	55.5	83.3	n/t

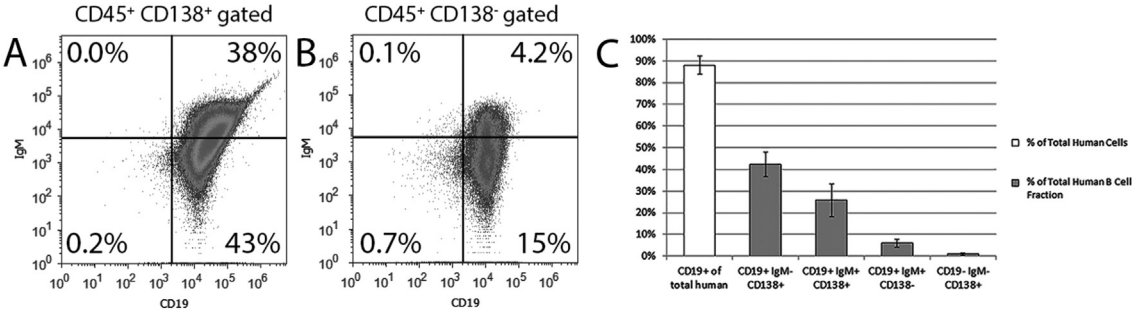

Fig. 2. Human B cell populations in humanized mouse bone marrow. Human B cells in bone marrow were immunophenotyped by flow cytometry. A human leukocyte antibody (anti-hCD45) was initially used for gating purposes to focus on human leukocyte populations. (A) FACS plot gated on $CD138^+$ cells to observe precursor B cell ($CD19^+IgM^-CD138^+$), immature B cell ($CD19^+IgM^+CD138^+$), and plasma B cell ($CD19^-IgM^-CD138^+$) populations. Percentages are out of total human B cells. (B) FACS plot gated on $CD138^-$ cells to observe naïve B cell population ($CD19^+IgM^+CD138^-$). Percentages are out of total human B cells. (C) Graph comparing averages of the different bone marrow cell populations in all mice tested. SE is indicated. The precursor B cell population in bone marrow is significantly different from this same population in spleen and blood (compare Fig. 3 and Fig. 4) (ANOVA $p = .003$ with *post hoc* Tukey–Kramer, $p < .05$). Sample sizes per population are $n = 7$ ($CD19^+IgM^-CD138^+$), and $n = 4$ for ($CD19^+IgM^+CD138^+$)($CD19^+IgM^-CD138^-$)($CD19^-IgM^-CD138^+$). Unique cord blood samples used for engrafting animals in each population: $n = 6$ ($CD19^+IgM^-CD138^+$), $n = 4$ for ($CD19^+IgM^+CD138^+$)($CD19^+IgM^-CD138^-$)($CD19^-IgM^-CD138^+$).

and are more prone to produce IgM antibodies independent of T cell help [24].

The lack of human MHC expression on thymic stromal cells likely has an impact on the ability to derive antigen-specific human T cell responses in humanized mice because humanized mouse T cells undergo selection on mostly murine MHC. Attempts to overcome this barrier to T cell development include the development of the bone marrow/liver/thymus (BLT) model which uses a human fetal thymic transplant to allow for selection on human MHC and various studies indicate that both cellular and humoral immune responses are more frequent and robust in the BLT model as compared to those that lack human thymic stromal cells [8,21,25] although careful studies to compare various humanized mouse models are still lacking. The BLT model is technically challenging because it requires human fetal tissues which can be challenging to obtain, and also requires survival surgery to implant the tissues. Another option is the use of human HLA-transgenic animals as another way to generate T cells that are selected on human MHC; this idea has been explored to some degree and appears to result in enhanced T cell responses [26,27]. No examination of human B cell responses in HLA-transgenic mice has been explored to our knowledge. Another hypothesis to explain the lack of human plasma B cells is that they are initially produced, but do not receive the survival signals necessary to persist longer than a few days after differentiation.

3.4. Characterization of B cell types in spleen

In the spleen, the predominant human B cell type was immature B cells (43.8 ± 7.5%), followed by naïve B cells at 27.7 ± 7.8% (see Fig. 3C). Precursor B cells were more rare in spleen (17.9 ± 3.1%) and plasma B cells were the least frequent (1.9 ± 1.6%). Example FACS plots of stained splenocytes are shown in Fig. 3A, B.

We also noted that human T cells are found in low numbers in the spleen of RAG-hu mice, whereas human B cells are found in much greater numbers. T cells represented an average of 36.6 ± 8.8% of total human leukocytes in the spleen, with a low of 12% and a high of 62%. This finding is similar to that reported in NOD/SCID gammanull mice engrafted with HSCs from cord blood [28]. Immature B cells are known to leave the bone marrow and to migrate to the spleen, where maturation to peripheral mature B cells and plasma cells occurs [18]. Thus, the paucity of T cells in the spleen of humanized mice may also explain the lack of plasma cells. A study conducted by Lang et al. confirms the necessity of T cells for B cell maturation; in their report they introduced autologous T cells and found increased numbers of mature B cells. Conversely, when T cells were depleted, in vivo B cell maturation was delayed [29]. In previous work with RAG-hu mice, we characterized levels of helper T cells ($CD3^+CD4^+$) and cytotoxic T cells ($CD3^+CD8^+$) in the blood of 28 humanized mice and found that the ratio of helper T cells to cytotoxic T cells is similar as compared to humans, with a mean ratio of 2.1:1 (±1.2 SD) for helper T cells compared to cytotoxic T cells [30]. There was clearly variability present between various engrafted animals, with a high of 6.1:1 and a low of 1.0:1. Poor development of helper T cells may contribute to poor B cell development.

Germinal centers are important locations for antibody class-switching, and structures that histologically resemble germinal centers are found in RAG-hu spleen [2]. To our knowledge, no studies have been carried out to analyze the functionality of these germinal centers in humanized mice. Follicular dendritic cells (FDCs) are critical components of germinal centers, but human FDCs are not detectable in

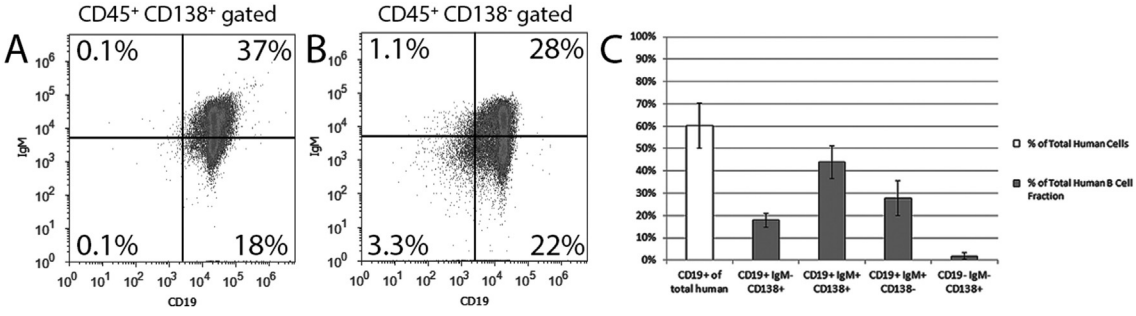

Fig. 3. Human B cell populations in humanized mouse spleen. Human B cells in spleen were immunophenotyped by flow cytometry. A human leukocyte antibody (anti-hCD45) was initially used for gating purposes to focus on human leukocyte populations. (A) FACS plot gated on $CD138^+$ cells to observe precursor B cell ($CD19^+IgM^-CD138^+$), immature B cell ($CD19^+IgM^+CD138^+$), and plasma B cell ($CD19^-IgM^-CD138^+$) populations. Percentages are out of total human B cells. (B) FACS plot gated on $CD138^-$ cells to observe naïve B cell population ($CD19^+IgM^+CD138^-$). Percentages are out of total human B cells. (C) Graph comparing averages of the different splenic cell populations in all mice tested. SE is indicated. Sample sizes per population are $n = 7$ ($CD19^+IgM^-CD138^+$), and $n = 4$ for ($CD19^+IgM^+CD138^+$)($CD19^+IgM^-CD138^-$)($CD19^-IgM^-CD138^+$). Unique cord blood samples used for engrafting animals in each population: $n = 6$ ($CD19^+IgM^-CD138^+$), $n = 4$ for ($CD19^+IgM^+CD138^+$)($CD19^+IgM^-CD138^-$)($CD19^-IgM^-CD138^+$).

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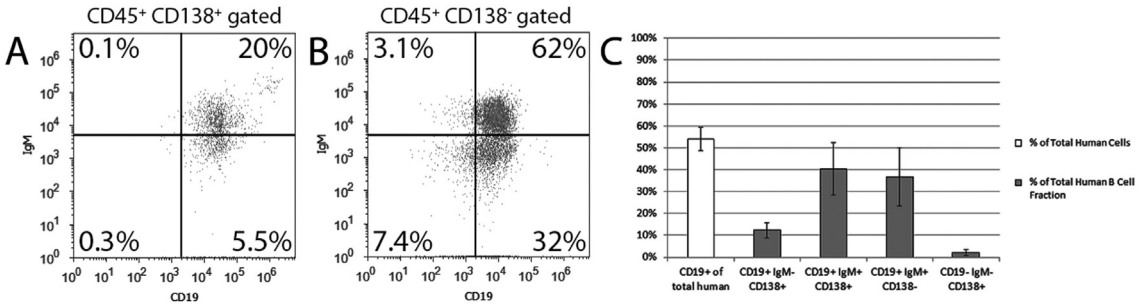

Fig. 4. Human B cell populations in humanized mouse peripheral blood. Human B cells in blood were immunophenotyped by flow cytometry. A human leukocyte antibody (anti-hCD45) was initially used for gating purposes to focus on human leukocyte populations. (A) FACS plot gated on $CD138^+$ cells to observe precursor B cell ($CD19^+IgM^-CD138^+$), immature B cell ($CD19^+IgM^+CD138^+$), and plasma B cell ($CD19^-IgM^-CD138^+$) populations. Percentages are out of total human B cells. (B) FACS plot gated on $CD138^-$ cells to observe naïve B cell population ($CD19^+IgM^+CD138^-$). Percentages are out of total human B cells. (C) Graph comparing averages of the different cell populations from blood in all mice tested. SE is indicated. Sample sizes per population are $n = 6$ ($CD19^+IgM^-CD138^+$), and $n = 3$ for ($CD19^+IgM^+CD138^+$)($CD19^+IgM^-CD138^-$)($CD19^-IgM^-CD138^+$). Unique cord blood samples used for engrafting animals in each population: $n = 5$ ($CD19^+IgM^-CD138^+$), $n = 3$ for ($CD19^+IgM^+CD138^+$)($CD19^+IgM^-CD138^-$)($CD19^-IgM^-CD138^+$).

humanized mice because these cells originate from a non-HSC source. Interestingly, cells bearing murine FDC markers are present in humanized mice germinal centers [2]. The lack of human FDCs in humanized mice may block efficient class-switching, development of memory B cells, and somatic hypermutation [31].

3.5. Characterization of B cell types in blood

In the blood, the most common human B cell types were immature and naïve B cells, at 40.4 ± 11.9% and 36.7 ± 13.2%, respectively (see Fig. 4C). Precursor B cells were found in lower levels (12.3 ± 3.4%) and plasma cells were even lower (2.2 ± 1.3%). Example FACS plots of stained blood cells are shown in Fig. 4A, B. We noted that the particular animal chosen for Fig. 4B had an unusually high level of plasma B cells ($CD19^-IgM^-CD138^+$). There is high variability in the levels of engraftment as well as in antibody production in individual humanized mice.

3.6. Measurement of total human plasma antibody concentrations

To examine the functionality of the human B cells, we measured human antibody concentrations in humanized mouse plasma. Total human IgM, IgG, and IgA concentrations were measured by ELISA. Plasma samples from unengrafted mice did not have detectable human antibody levels for any antibody class (data not shown).

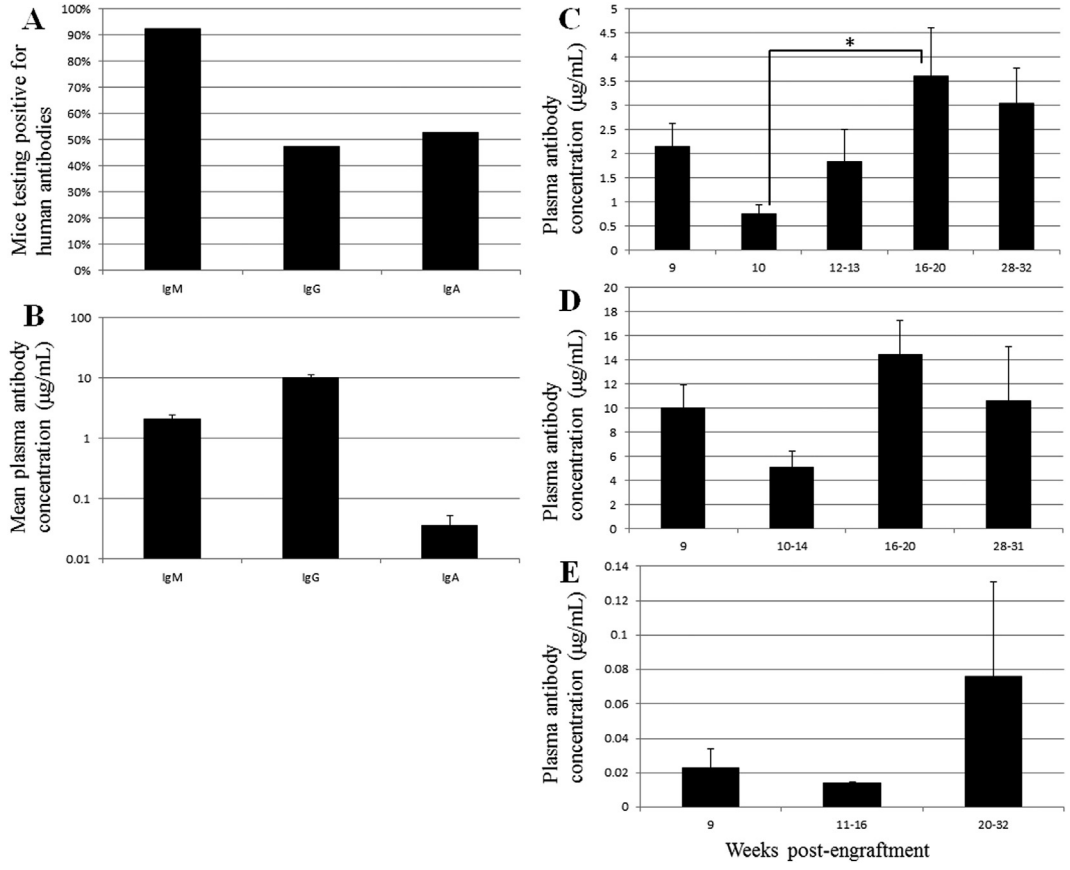

Fig. 5. Total human antibody concentrations in humanized mouse plasma. ELISA was used to measure total human antibody concentrations in plasma. (A) Fraction of animals testing positive for IgM, IgG, or IgA ($n = 53$ animals for each group). (B) Mean antibody concentrations for IgM, IgG, IgA; only including positive animals ($n = 49$, 25, and 28, respectively). A time course of (C) IgM, (D) IgG, and (E) IgA in plasma after human cell engraftment. (* ANOVA $p = .04$ with *post hoc* Tukey–Kramer, $p < .05$). Sample sizes per time point are: C) $n = 10$ (9 w), $n = 6$ (10 w), $n = 3$ (12–13 w), $n = 4$ (16–20 w), and $n = 3$ (28–32 w); D) $n = 4$ for all time points; and E) $n = 4$ (9 w), $n = 3$ (11–16 w), and $n = 3$ (20–32 w).

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Although all 3 antibody classes were detected in plasma, the frequency of detection differed amongst the classes. 49 of 53 samples tested positive for IgM (92.5%), while only 25 of 53 were positive for IgG (47.2%) and 28 of 53 were positive for IgA (52.8%) (Fig. 5A). The total average concentrations across all time points (only counting positive samples) were 2.1 μg/mL for IgM, 10 μg/mL for IgG, and 0.036 μg/mL for IgA (Fig. 5B). By way of comparison, the normal concentrations in human plasma are 1500 μg/mL for IgM, 14,000 μg/mL for IgG, and 3500 μg/mL for IgA. Thus, for all antibody classes we consistently measured approximately 0.1% or less of normal antibody concentrations in humanized mouse plasma as compared to normal humans. IgA concentrations were farthest from the normal human values (0.001% of normal), IgM levels were the closest (0.14%), and IgG levels were similar to IgM (0.07%).

Traggiai et al. monitored human B and T cell development over time in RAG-hu mice [2]. They showed that human antibody levels are immature at 8 wpe, with a rise in total IgM from 8 to 16 wpe and undetectable levels of IgG at 8 wpe with higher levels at 16 wpe. Our results somewhat agree in terms of the kinetics of the response, although we did detect IgG at 9 wpe in many animals. They showed total human antibody concentrations higher than what we have detected, but the defect in class-switching is similar. They reported a mean IgM concentration of about 15 μg/mL and a mean IgG of about 200 μg/mL [2] while Chen et al. reported mean IgM levels of 119 μg/mL and 1.1 μg/mL IgG in humanized NOD/SCID gammanull mice [32], while Wang et al. showed ~7 mg/mL IgM in humanized NOD/SCID mice with undetectable IgG [23]. Our results show a mean IgM titer of 2 μg/mL and a mean IgG titer of 10 μg/mL. The use of different mouse strains and sources of HSCs may explain the differing results.

Wang et al. examined IgG responses and T cell development in NOD/SCID mice transplanted with $CD34^+$ cells from cord blood [23]. They showed very little IgG responses, and also very few human T cells engrafted (appears to be much less than in RAG-hu mice). Since NOD/SCID mice are considered to have higher residual murine immunity than $Rag2^{-/-}\gamma c^{-/-}$ mice, the mouse strain might explain the differences in our results. B cells require T cell help for effective antibody responses, so we surmise that the increased numbers of human T cells might explain why the RAG-hu model has higher IgG production.

3.7. Kinetics of production of human antibody classes

In order to study the kinetics of human antibody development in humanized mice, we measured human antibody concentrations in plasma across a time course that lasted up to 32 wpe. All 3 antibody classes (IgM, IgG, IgA) were detectable in plasma by 9 wpe, and only minor differences in antibody concentrations were noted at later time points. No significant differences in total antibody concentrations were detected across the time course for IgG (Welch's ANOVA test, $p = 0.11$) (Fig. 5D) or IgA (Welch's ANOVA test, $p = 0.53$) (Fig. 5E). A single significant difference was detected in IgM from 10 wpe to 16-20 wpe where an increase was detected (ANOVA $p = .04$ with *post hoc* Tukey–Kramer, $p < 0.05$) (Fig. 5C). However, this significant increase was not maintained to the 28–32 wpe time point. Our results show that human antibody production persists through at least 32 wpe, with no significant decreases at late time points.

3.8. No correlation exists between peripheral blood engraftment and plasma antibody concentration

Since only a fraction of humanized mice produce detectable antigen-specific responses to infection or immunization, it would be useful if a marker could be developed which would allow one to predict the likelihood of an animal producing a detectable response. However, such a marker has not yet been reported. We analyzed our data to see if a correlation exists between the percent peripheral blood engraftment (defined as $hCD45^+$ cells divided by the sum of ($hCD45^+$ plus $mCD45^+$ cells)) and the plasma concentrations of various human antibody classes. We failed to detect a correlation between animals with higher peripheral blood engraftment and those with higher levels of IgM, IgG, or IgA (Supplementary Fig. 1). These findings suggest that the level of chimerism in humanized mice is not the most critical aspect involved in antibody responses.

Improvements in human erythrocyte and platelet development have been made by eliminating murine macrophages which may engulf human immune system components [33–35]. In contrast, little research has been conducted to specifically improve B cell development and functionality. One interesting study analyzed the defect in class-switching from IgM to IgG responses in humanized mice. Chen et al. used NOD/SCID gammanull mice engrafted with either fetal liver or cord blood $CD34^+$ cells and found that better human antibody responses and enhanced class-switching were detected following administration of human Interleukin 4 (IL-4) and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) to humanized mice [32]. IgM responses were 42% higher in cytokine-treated animals and IgG responses were over 10.5-fold higher using this protocol. In addition, tetanus toxin-specific IgG went from undetectable to detectable levels in many animals administered IL-4 and GM-CSF. Thus, the production of humanized mice in a background strain that produces human cytokines may also be beneficial to achieve normal human antibody responses.

4. Conclusions

We have shown that RAG-hu mice produce human B cells which are dispersed in blood, bone marrow, and spleen. B cell engraftment in peripheral blood reached a peak in relative numbers of cells at 14 wpe, while T cell levels were stable from 8 to 28 wpe. The vast majority of mice had detectable levels of human IgM in plasma, while only about half had detectable IgG or IgA. The mean antibody concentrations were considerably lower than those seen humans, and in general antibody concentrations did not significantly change over time from 9 to ~30 wpe. We detected relatively few $CD138^+$ plasma cells in the bone marrow, despite the fact that most plasma cells reside in this site in humans. Taken together, our results indicate that in the RAG-hu model there are defects in class-switching from IgM to IgG or IgA production, and that naïve B cells are not effectively matured to become plasma B cells. Further work can now be done to test the hypothesis that improvements in T cell levels may enhance the reproducibility of human antibody responses in humanized mice.

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Abbreviations

FACS	Fluorescence activated cell sorting
Rag2	Recombinase activating gene 2
γc	Common gamma chain receptor
Wpe	Weeks post-engraftment
Ig	Immunoglobulin
NOD	Non-obese diabetic
SCID	Severe combined immunodeficiency
ELISA	Enzyme-linked immunosorbent assay
HSC	Hematopoietic stem cell
MHC	Major histocompability complex
BLT	Bone marrow/liver/thymus humanized mice
GM-CSF	Granulocyte macrophage colony stimulating factor

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References

[1] Tanner A, Taylor SE, Decottignies W, Berges BK. Humanized mice as a model to study human hematopoietic stem cell transplantation. Stem Cells Dev 2014;23:76–82.
[2] Traggiai E, Chicha L, Mazzucchelli L, Bronz L, Piffaretti JC, Lanzavecchia A, et al. Development of a human adaptive immune system in cord blood cell-transplanted mice. Science 2004;304:104–7.
[3] Berges BK, Rowan MR. The utility of the new generation of humanized mice to study HIV-1 infection: transmission, prevention, pathogenesis, and treatment. Retrovirology 2011;8:65.
[4] Berges BK, Tanner A. Modeling of human herpesvirus infections in humanized mice. J Gen Virol 2014;95:2106–17.
[5] Kuruvilla JG, Troyer RM, Devi S, Akkina R. Dengue virus infection and immune response in humanized Rag2$^{-/-}\gamma c^{-/-}$ (RAG-hu) mice. Virology 2007;369:143–52.
[6] Baenziger S, Tussiwand R, Schlaepfer E, Mazzucchelli L, Heikenwalder M, Kurrer MO, et al. Disseminated and sustained HIV infection in CD34+ cord blood cell-transplanted Rag2 −/−{gamma}c −/− mice. Proc Natl Acad Sci U S A 2006;103:15951–6.
[7] Kwant-Mitchell A, Ashkar AA, Rosenthal KL. Mucosal innate and adaptive immune responses against HSV-2 in a humanized mouse model. J Virol 2009;83:10664–76.
[8] Brainard DM, Seung E, Frahm N, Cariappa A, Bailey CC, Hart WK, et al. Induction of robust cellular and humoral virus-specific adaptive immune responses in HIV-infected humanized BLT mice. J Virol 2009;83:7305–21.
[9] Parsons CH, Adang LA, Overdevest J, O'Connor CM, Taylor JRJ, Camerini D, et al. KSHV targets multiple leukocyte lineages during long-term productive infection in NOD/SCID mice. J Clin Invest 2006;116:1963–73.
[10] Yajima M, Imadome K, Nakagawa A, Watanabe S, Terashima K, Nakamura H, et al. A new humanized mouse model of Epstein–Barr virus infection that reproduces persistent infection, lymphoproliferative disorder, and cell-mediated and humoral immune responses. J Infect Dis 2008;198:673–82.
[11] Watanabe S, Terashima K, Ohta S, Horibata S, Yajima M, Shiozawa Y, et al. Hematopoietic stem cell-engrafted NOD/SCID/IL2R{gamma}null mice develop human lymphoid system and induce long-lasting HIV-1 infection with specific humoral immune responses. Blood 2007;109:212–8.
[12] Sato K, Nie C, Misawa N, Tanaka Y, Ito M, Koyanagi Y. Dynamics of memory and naïve CD8(+) T lymphocytes in humanized NOD/SCID/IL-2Rgamma(null) mice infected with CCR5-tropic HIV-1. Vaccine 2010;28S2:B32–7.
[13] Akkina R, Berges BK, Palmer BE, Remling L, Neff CP, Kuruvilla J, et al. Humanized Rag1 −/− gamma-chain −/− mice support multilineage hematopoiesis and are susceptible to HIV-1 infection via systemic and vaginal routes. PLoS ONE 2011;6:e20169.
[14] Becker PD, Legrand N, van Geelen CM, Noerder M, Huntington ND, Lim A, et al. Generation of human antigen-specific monoclonal IgM antibodies using vaccinated "human immune system" mice. PLoS ONE 2010;5:e13137.
[15] Sanchez FM, Cuadra GI, Nielsen SJ, Tanner A, Berges BK. Production and characterization of humanized Rag2(−/−)γc (−/−) mice. Methods Mol Biol 2013;1031:19–26.
[16] Berges BK, Wheat WH, Palmer BE, Connick E, Akkina R. HIV-1 infection and CD4 T cell depletion in the humanized Rag2 −/− gc −/− (RAG-hu) mouse model. Retrovirology 2006;3:76.
[17] Harris DT, Badowski M. Long term human reconstitution and immune aging in NOD-Rag (−)-γ chain (−) mice. Immunobiology 2014;219:131–7.
[18] Nagasawa T. Microenvironmental niches in the bone marrow required for B-cell development. Nat Rev Immunol 2006;6:107–16.
[19] Bladé J, Samson D, Reece D, Apperley J, Björkstrand B, Gahrton G, et al. Criteria for evaluating disease response and progression in patients with multiple myeloma treated by high-dose therapy and haemopoietic stem cell transplantation. Myeloma Subcommittee of the EBMT. European Group for Blood and Marrow Transplant. Br J Haematol 1998;102:1115–23.
[20] Durie BG, Harousseau JL, Miguel JS, Bladé J, Barlogie B, Anderson K, et al. International uniform response criteria for multiple myeloma. Leukemia 2006;20:1467–73.
[21] Melkus MW, Estes JD, Padgett-Thomas A, Gatlin J, Denton PW, Othieno FA, et al. Humanized mice mount specific adaptive and innate immune responses to EBV and TSST-1. Nat Med 2006;12:1316–22.
[22] Biswas S, Chang H, Sarkis PT, Fikrig E, Zhu Q, Marasco WA. Humoral immune responses in humanized BLT mice immunized with West Nile virus and HIV-1 envelope proteins are largely mediated via human CD5(+) B cells. Immunology 2011;134:419–33.
[23] Wang X, Qi Z, Wei H, Tian Z, Sun R. Characterization of human B cells in umbilical cord blood-transplanted NOD/SCID mice. Transpl Immunol 2012;26:156–62.
[24] Baumgarth N, Tung J, Herzenberg L. Inherent specificities in natural 689 antibodies: a key to immune defense against pathogen invasion. Springer Semin Immunopathol 2005;26:347.
[25] Akkina R. New generation humanized mice for virus research: comparative aspects and future prospects. Virology 2013;435:14–28.
[26] Serra-Hassoun M, Bourgine M, Boniotto M, Berges J, Langa F, Michel ML, et al. Human hematopoietic reconstitution and HLA-restricted responses in nonpermissive alymphoid mice. J Immunol 2014;193:1504–11.
[27] Shultz LD, Saito Y, Najima Y, Tanaka S, Ochi T, Tomizawa M, et al. Generation of functional human T-cell subsets with HLA-restricted immune responses in HLA class I expressing NOD/SCID/IL2r{gamma}null humanized mice. Proc Natl Acad Sci U S A 2010;107:13022–7.
[28] Takahashi M, Tsujimura N, Otsuka K, Yoshino T, Mori T, Matsunaga T, et al. Comprehensive evaluation of leukocyte lineage derived from human hematopoietic cells in humanized mice. J Biosci Bioeng 2012;113:529–35.
[29] Lang J, Kelly M, Freed BM, McCarter MD, Kedl RM, Torres RM, et al. Studies of lymphocyte reconstitution in a humanized mouse model reveal a requirement of T cells for human B cell maturation. J Immunol 2013;190:2090–101.
[30] Berges BK, Akkina SR, Folkvord JM, Connick E, Akkina R. Mucosal transmission of R5 and X4 tropic HIV-1 via vaginal and rectal routes in humanized Rag2 −/− gc −/− (RAG-hu) mice. Virology 2008;373:342–51.
[31] Tew JG, Phipps RP, Mandel TE. The maintenance and regulation of the humoral immune response: persisting antigen and the role of follicular antigen-binding dendritic cells as accessory cells. Immunol Rev 1980;53:175–201.
[32] Chen Q, He F, Kwang J, Chan JK, Chen J. GM-CSF and IL-4 stimulate antibody responses in humanized mice by promoting T, B, and dendritic cell maturation. J Immunol 2012;189:5223–9.
[33] Pek EA, Chan T, Reid S, Ashkar AA. Characterization and IL-15 dependence of NK cells in humanized mice. Immunobiology 2011;216:218–24.
[34] Chen Q, Khoury M, Chen J. Expression of human cytokines dramatically improves reconstitution of specific human-blood lineage cells in humanized mice. Proc Natl Acad Sci U S A 2010;106:21783–8.
[35] Huntington ND, Legrand N, Alves NL, Jaron B, Weijer K, Plet A, et al. IL-15 trans-presentation promotes human NK cell development and differentiation in vivo. J Exp Med 2009;206:25–34.

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