Correction

MICROBIOLOGY

The authors note that the Acknowledgments section appeared incorrectly. It should instead appear as: “We thank Professor Young-Hyeh Ko at Samsung Medical Center, a board member of the Korean Society of Hematologic Pathology, for helpful comments. This study was supported by Grants HI09C1552 and A110637 from the Korea Health Technology Research and Development (R&D) Project through the Korea Health Industry Development Institute, funded by the Ministry for Health & Welfare, Republic of Korea; Grant 1120010 from the National R&D Program for Cancer Control, Ministry for Health and Welfare, Republic of Korea; and Grants NRF-2011-0012393, NRF-1997-D00214, and NRF-2015-R1A2A2A010064659 from the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology.”

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Effects of lymphocyte profile on development of EBV-induced lymphoma subtypes in humanized mice

Eun Kyung Lee,a,b,1 Eun Hye Joo,a,b,1 Kyung-A Song,a,b,1 Bongkum Choi,b,c, Miyoung Kim,b,c, Seok-Hyung Kim,a,b,d, Sung Joo Kim,b,c,1 and Myung-Soo Kanga,b,2

aSamsung Advanced Institute for Health Sciences and Technology, Samsung Medical Center and Sungkyunkwan University, Seoul 06351, Korea; bSamsung Biomedical Research Institute, Seoul 06351, Korea; cDepartment of Transplantation Surgery, Samsung Medical Center and Sungkyunkwan University School of Medicine, Seoul 06351, Korea; and dDepartment of Pathology, Samsung Medical Center and Sungkyunkwan University School of Medicine, Seoul 06351, Korea

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Epstein-Barr virus (EBV) infection causes both Hodgkin’s lymphoma (HL) and non-Hodgkin’s lymphoma (NHL). The present study reveals that EBV-induced HL and NHL are intriguingly associated with a repopulated immune cell profile in humanized mice. Newborn immunodeficient NSG mice were engrafted with human cord blood CD34+ hematopoietic stem cells (HSCs) for a 8- or 15-wk reconstitution period (denoted 8wHSC and 15wHSC, respectively), resulting in human B-cell and T-cell predominance in peripheral blood cells, respectively. Further, novel humanized mice were established via engraftment of hCD34+ HSCs together with nonautologous fetal liver-derived mesenchymal stem cells (MSCs) or MSCs expressing an active notch ligand DLL1, resulting in mice skewed with human B or T cells, respectively. After EBV infection, whereas NHL developed more frequently in B-cell–predominant humanized mice, HL was seen in T-cell–predominant mice (P = 0.0013). Whereas human splenocytes from NHL-bearing mice were positive for EBV-associated NHL markers (hBCL2+, hCD20+, hKi67+, hCD20+/EBNA1+, and EBER−) but negative for HL markers (LMP1−, EBNA2−, and hCD30−), most HL-like tumors were characterized by the presence of malignant Hodgkin’s Reed–Sterneberg (HRS)-like cells, lacunar RS (hCD30+, hCD15+, IgG1+, EBER/hCD30+, EBNA1+/hCD30+, LMP+/EBNA2+, hCD68+, hBCL2−, hCD20+/weak, Phospho STAT6+), and mumified RS cells. This study reveals that immune cell composition plays an important role in the development of EBV-induced B-cell lymphoma.

Epstein–Barr virus | humanized mice | Non-Hodgkin’s lymphoma | Hodgkin’s lymphoma | Reed–Sterneberg cell

Results

NHL in B-Cell–Predominant hCD34+–EBV Mice. In the first experimental trial, 13 newborn NSG mice were engrafted with hCD34+ HSC for 8 wk (denoted by 8wHSC), after which components of the human immune system (HIS) (e.g., hCD45, hCD3, hCD19) were present in nearly all mice. After infection, only a subset of mice exhibited those HIS markers. This article is a PNAS Direct Submission. The authors declare no conflict of interest.


Significance

The mechanism of how Epstein-Barr virus (EBV) contributes to the development of two distinct lymphomas remains unknown. Intriguingly, EBV-associated Hodgkin’s lymphoma was seen exclusively in mice with activated T-cell conditions, whereas EBV-associated non-Hodgkin’s lymphoma was developed in mice with suppressed T-cell conditions, in which immature B cells were predominant at the time of EBV infection. This distinct association provides new insight into the pathogenesis of specific types of EBV-induced lymphomas.
evaluated (Fig. L4). The 13 8w hN mice had an average of 6.6% hCD45 human leukocytes in peripheral blood mononuclear cells (PBMCs), among which a remarkably high percentage of hCD19+ B cells (77.9%) and low percentage of hCD3+ T-cells (4.9%) were repopulated (Fig. 2A). Individually, 12 of the 13 8w hN mice had predominant hCD19+ B-cells (Fig. 1).

At 8 wk plus 1 d postgrafting, these 13 mice were infected with EBV (n = 9 mice) or PBS (n = 4) and further evaluated for immune cell profiles at 5 and 22 wk postinfection (wpi). Consistent with earlier reports (18, 21), in 11 out of 12 8w hN mice, the number of hCD19+ cells decreased sharply starting from 5 wpi and continuing until 22 wpi, whereas number of hCD3 cells increased at 5 wpi and then decreased thereafter up to 22 wpi (Figs. 1 B and C and 2A, and SI Appendix, Fig. S1). After EBV infection, eight of nine 8w hN-EBV mice that exhibited B-cell predominance at the time of infection developed NHL type B-cell lymphomas. These NHLs were characterized by BCL2+, hCD20+, hKi67+, EBNA1+, EBNA2+, and hCD30− cells. Seven of the eight mice showed no LMP1 expression (i.e., type I latency, EBNA2−/LMP1−) on immunohistochemical (IHC) analysis, and the remaining mouse showed LMP1 expression (i.e., type IIa latency, EBNA2−/LMP1+) (SI Appendix, Fig. S2). None of the four noninfected mice (8w hN-PBS) had a similar neoplasm (Table 1 and SI Appendix, Table S1).

**HL-Like Disorder in T-Cell–Predominant 15w hN-EBV Mice.** To establish humanized mice with T-cell dominance, NSG mice were housed for 15 wk after hCD34+ HSC grafting (denoted by 15w hN) (Fig. 1). This was done because the B-cell fraction decreased and the hCD3+ fraction increased concurrently up to at least 13 wk after transplantation in this study (Fig. 2A) as well as previous studies (18, 21). In this study, the 15-wk reconstitution period resulted in a better-balanced repopulation overall. The 15w hN mice (n = 15) had an average of 14.5% hCD45 cells, of which 61.4% were hCD3+ T cells and only 15.5% were hCD19+ B cells (Fig. 2B). Twelve of the 15 15w hN mice were T-cell predominant, whereas three remained B-cell predominant (Fig. 1B).

The mice were subjected to infection with PBS (n = 5) or EBV (n = 10). T-cell dominance remained unchanged in all 12 T-cell–predominant mice, and B-cell dominance was maintained in two of three mice. After EBV infection, 7 of 10 15w hN-EBV mice developed B-cell lymphomas, including NHL only in two mice, HL only in three mice, and both HL and NHL in two mice. None of the five noninfected mice (15w hN-PBS) had such a neoplasm. EBV infection and infection-initiated virus release into mouse serum were confirmed by real-time quantitative PCR (qPCR), Epstein–Barr early DNA (EBER) in situ hybridization, and EBNA1 IHC staining. Substantially more hCD20+ B cells and fewer hCD3+ T cells were noted in splenic follicles. EBV DNA was detected earlier and diminished more quickly in the sera of mice with HL compared with mice with NHL (Fig. 3). More frequent splenomegaly was observed in infected mice (Fig. 3).

**HL in EBV-Infected Humanized Mice Skewed with T-Cell Development.** Our findings of HL-like tumor development in T-cell–predominant mice are consistent with the fact that T cells are associated predominantly with classical HL (25). We next attempted to establish humanized mice skewed with B or T cells to directly demonstrate the role of T-cell predominance in HL development. For this, six NSG mice were engrafted with hCD34+ HSCs and nonautologous fetal liver (FL)-derived human mesenchymal stem cells (MSCs) expressing either vector or Notch ligand Delta-like-1 (DLK1) (Fig. 1 and SI Appendix). The mice were allowed 15 wk for reconstitution. This experimental design was based on the fact that expression of the Notch activator DLK1 is capable of skewing.
HSCs to develop preferentially into T cells, whereas MSCs alone skewed HSCs to develop preferentially into B cells (26, 27).

On evaluation, although the MSC and MSC-DLK1 methods reconstituted the total leukocyte population with comparable efficiency (hCD45+, 16.73% vs. 11.9%) (Fig. 2 C and D and SI Appendix, Fig. S1), the three MSC-engrafted humanized mice displayed B-cell dominance (average, hCD19+, 39.3% vs. hCD3+, 61.8%) vs. hCD3, and latent EBV infection markers EBER and EBNA2+ (Fig. S3 and S4). As a result, all three T-cell–predominant mice developed NHL. In contrast, all three MSC-DLK1-engrafted humanized mice displayed T-cell dominance (average, hCD3+, 61.8% vs. hCD19+, 3.3%) over T cells, which persisted for the next 5 wk after engraftment (Figs. 1B and 2D). As a result, all three T-cell–predominant mice developed HL (Table 1 and SI Appendix, Figs. S3 and S4).

Taken together, the results of the three independent experimental trials suggest that B-cell predominance before EBV infection may have predisposed to NHL, whereas T-cell predominance was associated with, but not a prerequisite for, HL (P = 0.0013, Fisher’s exact test). In other words, NHL more frequently (but not exclusively) developed in B-cell–predominant mice, and all cases of HL were developed in mice with T-cell predominance at the time of EBV infection. Immune cell evolution appeared to not be associated with phenotypic neoplasm (SI Appendix, Table S1).

Characteristics of Experimental EBV-Associated Lymphoma. Of note, after EBV infection, although eight of the nine 15w hN mice had NHL, the 13 15w hN mice with mostly T-cell predominance before EBV infection developed HL-like tumors (n = 5). NHL (n = 4), or both NHL and H-like tumors (n = 3), indicating that T-cell predominance does not necessarily lead to the development of HL-like tumors (Table 1 and SI Appendix, Table S1 and Figs. S3–S5). HL-like splenocytes showed mostly type II latency (LMP1+ and EBNA2+ by IHC).

In neoplasms of T-cell–predominant 15N hN-EBV mice, atypical, transformed large HRS cells surrounded by abundant nonmalignant lymphocytes were consistently encountered (average 24

Table 1. Summary of EBV-infected humanized mice

<table>
<thead>
<tr>
<th>Phenotype*</th>
<th>EBV-noninfected in</th>
<th>EBV-infected in</th>
<th>P value5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B cells (n = 4)</td>
<td>T cells (n = 5)</td>
<td></td>
</tr>
<tr>
<td>NHL</td>
<td>0</td>
<td>0</td>
<td></td>
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<tr>
<td>HL-like</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>None§</td>
<td>4</td>
<td>5</td>
<td></td>
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<tr>
<td>Evolution††‡‡</td>
<td>T(4)</td>
<td>T (5**)</td>
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</tr>
<tr>
<td></td>
<td>B (5**, †††)</td>
<td>T (9**)</td>
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NA, not applicable.

* NHL, EBV-associated non-Hodgkin’s lymphomas (EBV+ BL, DLBCL, FBCL, and unspecified B-cell lymphoma), HL-like, EBV-associated Hodgkin’s lymphoma (HL), or HL-like [HRS cells with hCD30+/EBNA1+ (EBER+), HCD15+].

† B-cell predominant mice with T-cell development suppressed at the time of EBV infection.

‡ T-cell predominant mice with B-cell development suppressed at the time of EBV infection.

§ Two-sided Fisher’s exact t test.

†† Three mice showing both HL and NHL were included.

‡‡ No specific neoplasm.

†‡ Presumed dominance in one mouse included.

** Presumed dominance in one mouse included.

§§ Three of five mice maintaining B-cell predominance developed NHL.

††† Three of five mice showing both HL and NHL were included.

§§§ Eight of nine mice converted to T-cell predominance developed NHL.

¶¶¶ T-cell predominance remained unchanged in all 11 mice.
HRS cells per spleen). Most of these transformed cells were consistent with EBV-associated HL-like phenotypes (e.g., hCD30+, hCD15+, hCD20+/−, Ig−, hCD30+/EBNA1+, LMP1+, EBNA2+, phospho-STAT6+), but were negative for the NHL markers hBCL2− in the hCD20−/weak background cells (Figs. 3 and 4B and SI Appendix, Figs. S3–S13). In H&E and immunophenotyping analyses, each spleen best diagnosed as HL-like had phenotypic hallmarks of HL based on updated Revised European-American Lymphoma (REAL)/World Health Organization (WHO) criteria (28): numerous atypical malignant EBV-positive HRS-like cells, lacunar-type HRS cells, and mummified HRS cells in association with predominant T cells or hCD68+ histiocytes (29) (SI Appendix, Figs. S3 and S4). In addition, in accordance with the fact that STAT6 is constitutively phosphorylated in >80% of HRS cells of classical HL (30), phosphor-STAT6 was positive in all four HL-like tumors tested but negative in all three NHL tumors tested (SI Appendix, Fig. S12), supporting a diagnosis of HL-like tumors in this humanized setting. This activated the STAT6 signaling pathway, which most likely was activated by the cytokines IL-4 and IL-13, could induce EBV LMP1 even in the absence of EBNA-2, implicating type II EBV latent gene expression in EBVaHL (31) (SI Appendix, Fig. S3).

Costaining revealed colocalization of EBNA1 with hCD30 in HL-like cells. The lack of IHC-validated non-mouse hCD15 antibodies for double staining in HRS cells. Mouse monoclonal Ab hindered the double staining in HRS cells. The outcome should depend on different inputs (e.g., strain, age of recipient, donor cells, duration of infection with predominant T cells or hCD68+ histiocytes (29) (SI Appendix, Figs. S3 and S4)). In addition, in accordance with the fact that STAT6 is constitutively phosphorylated in >80% of HRS cells of classical HL (30), phosphor-STAT6 was positive in all four HL-like tumors tested but negative in all three NHL tumors tested (SI Appendix, Fig. S12), supporting a diagnosis of HL-like tumors in this humanized setting. This activated the STAT6 signaling pathway, which most likely was activated by the cytokines IL-4 and IL-13, could induce EBV LMP1 even in the absence of EBNA-2, implicating type II EBV latent gene expression in EBVaHL (31) (SI Appendix, Fig. S3).

Discussion

In the present study, humanized mice recapitulated many of the key characteristics of EBV infection-associated disease pathogenesis. EBV-associated disorders in humanized mice, including post-transplantation lymphoproliferative disorder, NHL (DLBCL, FBCL), HLH, arthritis, and chronic active EBV infection, have been reproduced. The outcome should depend on different inputs (e.g., strain, age of recipient, donor cells, duration of infection, and type of immune response).
reconstitution, dose of EBV, depth of analyses) (16–18, 20–23). IHC staining of spleen sections for EBV latent gene markers identified EBV infection in these humanized mice (SI Appendix, Figs. S8–S11). To our knowledge, this study is the first to describe EBVaHL-like disorder in multiple humanized mice with supportive evidence. The malignant HL-like tissues in this study were characterized by atypical EBV-infected HRS or HRS-like cells. Similar to typical HRS, where 90% HRS of human HL tissues in situ is hCD20+weak, the HL-like tissues in this study were clearly hCD20−weak, also consistent with the updated REAL/WHO criteria (28) and thus strongly suggestive of experimental HL-like neoplasms (12–14, 28, 32, 33). T-cell preexpansion likely does not prevent EBV lymphomagenesis, because T-cell–dominant mice developed lymphoma; rather, preconditioned mice with B-cell predominance (or T-cell suppression) developed NHL after EBV infection (Table 1 and SI Appendix, Table S1). Most of the NHL neoplasms in this study were characterized by hBCL2+, hKi67+, hCD30+, EBER+, EBNA2+, and LMP+ expression. Three mice were found to have both NHL and HL-like tumors.

Watanabe et al. (34) previously reported that a significant number of B-cell progenitors accumulate in the spleen in human cord blood-derived hCD34+ HSC-reconstituted humanized NSG (or NOG) mice. Numerous other groups also have reported obvious immature B-cell predominance in the periphery and spleen, especially in humanized mice reconstituted for short periods (<12 wk) (18, 34–38). Therefore, the predominant B cells before EBV infection in the h/hN mice in this study are believed to be immature B cells, which will differentiate to develop mature B-cell and T-cell fractions. In this regard, the development of NHL primarily in the h/hN mice after EBV infection (h/hN-EBV; eight of nine mice) suggests that a significant fraction of immature B cells at the time of EBV infection are likely associated with NHL development, although our study does not provide direct evidence of this. Despite apparent normal T-cell development, hCD3+ T cells could have functional abnormalities in central or peripheral lymphoid organs (34), related mainly to the lack of human thymic tissues normally required for thymic education during functional T-cell development. Inappropriate or incomplete immune function should affect malignant transformation after EBV infection. Nevertheless, many of the HL-like tumors in these mice displayed multiple HRS and lacunar-type cells with broken spleen architecture, the usual lack of germinal center, frequent—but not always (or entirely)—loss of lymphoid follicles (LFs) or LFs replaced with immature cells, frequent enlargement of white pulp or periarteriolar lymphoid sheaths (PALS), and often atrophy in red pulp. Therefore, the malignant HL-like tumor cells in this study were defined as atypical giant cells within or near abundant immature lymphocytes that replace follicles or disrupt splenic normal architecture such as follicles and germinal centers. Besides coexisting NHL or infectious mononucleosis (IM) in certain mice, almost all of the tumors in T-cell–dominant conditioned mice in this study certainly contained subsets of HL-like tumors, because they displayed multiple criteria of HL (i.e., frequent HRS cells with J chain+, CD30+, CD15+, EBER+, EBNA2+, hBCl2+, and phospho-STAT6+) (SI Appendix, Fig. S12) (30). The activated STAT6 signaling pathway could induce EBV LMP1 in absence of EBNA-2, implicating type II EBV latent gene expression in EBVaHL (30–31). The majority of HL-like tumors exhibited type 2 latency; five of eight HL-like tumors displayed type 2a latency (LMP1+/EBNA2−), whereas the remaining three showed apparent type 3 latency. Despite having multiple molecular characteristics of HL, the apparent type 3 latency in these three so-called “HL-like” tumors is not typically observed in human HL. This is suggestive of an atypical HL that may occur in certain conditions, such as an experimental animal model. On the other hand, given that the “markers” (such as CD30) used to define HL are not absolutely specific for HL, and that the EBV latency type is not that seen in human HL, it also is possible that the tumors in this T-cell–dominant model may be atypical HL-like tumors rather than true representatives of HL.

Given that IM has no evidence of crippling SHMs in the VH gene (43), whereas HL has nonsense or deletion mutation in the VH gene, resulting in loss of the correct reading frame in ~30% of cases (44), the presence of SHMs in the HL-like tumors in this study further supports a pathological diagnosis of HL-like malignancy. SHMs were identified in all tumors examined, regardless of the presence of IM-like lesions. This is because malignant cells were present in all tissues examined. In addition to hCD30+/CD15+/EBNA1+/EBER+, the presence of phosphor-STAT6+ HRS-like cells and a V_{H} nonsense mutation strengthened the diagnosis of EBVaHL-like tumors (Fig. 4). The coexistence of occasional type III IM cells and type II HL-like cells accounts for the apparent type III latency in some of the HL-like tumor-bearing spleens. Along with the coexisting NHL or IM, almost all tumors in the T-cell–dominant conditioned mice in this study also contained subsets of HL-like tumors. Of note, the results showing apparently fewer numbers of EBER-positive cells than EBNA1-positive cells shown in Figs. 3 and 4 and SI Appendix, Figs. S8 and S9 were confirmed by additional staining (SI Appendix, Fig. S15). The discrepancy is likely due to decreased EBER promoter activity by unphosphorylated active retinoblastoma tumor-suppressor protein during cell cycle phases G0 and early G1 (45, 46).

Materials and Methods

Ethics Statement. Human protocol (IRB file no. 2010-08-159) for human material was approved by the Institutional Review Boards of Samsung Medical Center. Animal protocol (no. 20100210001) was approved by the Institutional Animal Care and Use Committee (IACUC) of Samsung Biomedical Research Institute (SBRi) (see SI Appendix for detail). Cell preparation for engraftment, infection with EBV, and characterization of humanized NSG (hNSG) mice are described in detail in SI Appendix.

Preparation of Humanized Mice. Nonobese diabetic/severe combined immunodeficient mice with IL2R knockout (NOD/LtSz-scid/IL2Rγ−/−), referred to herein as NSG, were purchased from The Jackson Laboratory (47, 48). Newborn progenies for transplantation experiments were obtained from inbred breeding and maintained under specific pathogen-free conditions. For reconstitution of the HIS in mice, 1-d-old newborn female NSG mice were injected i.p. with busulfan (Ben Eun Laboratories) at a dose of 15 mg/kg to ablate residual bone marrow (47, 48). At 24 h after busulfan injection, 2 × 107 hCD34+ HSCTs were injected intraperitoneally.

The mice were housed for 8 or 15 wk before characterization and EBV infection (Fig. 1A). NSG mice reconstituted with HIS components are referred to as hNSG mice. Reconstitution was evaluated as described previously (20, 24). Where necessary, humanized mice with skewed populations of B or T cells were generated as described above with the following modifications. B-cell–predominant NSG mice were coengranted with cord blood hCD34+ HSCs and human FL-MSCs, which enhanced HSC engraftment and suppressed T-cell proliferation (49–52). This treatment resulted in B-cell–predominant humanized

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mice. T-cell-predominant NSG mice were coengrafted with cord blood hCD34+ HSCs and NHL-MSC-DLK1 cells expressing an activated Notch ligand (DLK1) to suppress B-cell development (SB). This treatment resulted in T-cell-predominant humanized mice. After 15 wk, mice were infected with EBV as described in SI Appendix, followed by investigation for 13 wk.

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