Reproduction of Epstein-Barr Virus Infection and Pathogenesis in Humanized Mice

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Epstein-Barr virus (EBV) is etiologically associated with a variety of diseases including lymphoproliferative diseases, lymphomas, carcinomas, and autoimmune diseases. Humans are the only natural host of EBV and limited species of new-world monkeys can be infected with the virus in experimental conditions. Small animal models of EBV infection, required for evaluation of novel therapies and vaccines for EBV-associated diseases, have not been available. Recently the development of severely immunodeficient mouse strains enabled production of humanized mice in which human immune system components are reconstituted and express their normal functions. Humanized mice can serve as infection models for human-specific viruses such as EBV that target cells of the immune system. This review summarizes recent studies by the author’s group addressing reproduction of EBV infection and pathogenesis in humanized mice.

INTRODUCTION

Epstein-Barr virus (EBV) is the etiological agent of infectious mononucleosis and is associated with a variety of human diseases including lymphoproliferative diseases (LPDs), malignancies (e.g. Burkitt lymphoma, Hodgkin lymphoma, nasopharyngeal carcinoma, and gastric carcinoma), and autoimmune diseases (e.g. multiple sclerosis and rheumatoid arthritis (RA)) (1). EBV infection in the majority of hosts is however asymptomatic and the virus persists for life as latent infection. EBV has a unique biologic activity to transform B cells and establish immortalized lymphoblastoid cell lines. In immunocompetent hosts, these transformed cells are readily removed by EBV-specific CTLs, because they express highly antigenic viral proteins such as the EBV nuclear antigens 3 (EBNA3s) and EBNA2 (2). In immunocompromised hosts, however, EBV-transformed cells may continue to proliferate and cause LPDs such as post-transplant lymphoproliferative disease (PTLD) and AIDS-associated lymphomas.

Humans are the only natural host of EBV, while rabbits and limited species of new-world monkeys can be infected with the virus in experimental conditions (3-6). Small animal models of EBV infection and EBV-associated diseases have not been available, rendering studies of pathogenesis and therapies of EBV-associated diseases difficult. Recently, the development of severely immunodeficient mouse strains made it possible to transplant human hematopoietic stem cells (HSCs) and reconstitute human immune system components in mice (7-10). These ectopic human immune system components express their normal functions and these mice are called...
humanized mice (more accurately immune system humanized mice). Viruses that infect only humans and target cells of the immune system, including EBV, HIV-1, and human T-lymphotropic virus type 1 have been shown to infect humanized mice and reproduce cardinal features of their infection (11,12). In this review, recent studies by the author’s group addressing reproduction of EBV infection and pathogenesis in humanized mice are described. In addition, recent achievements in the field of humanized mouse models of EBV infection are briefly reviewed.

**EBV INFECTION AND PATHOGENESIS IN NOG MICE RECONSTITUTED WITH HUMAN IMMUNE SYSTEM COMPONENTS**

**Lymphoproliferative disease**

The immunodeficient mouse strain NOD/Shi-scid/IL-2R-γnull (NOG), that lacks B, T, and NK cells completely, was developed by Ito and his collaborators (7). Transplantation of human HSCs isolated from cord blood in NOG mice resulted in differentiation of human B cells, T cells, NK cells, macrophages, and dendritic cells (7,13-15). Inoculation with EBV of higher doses (>10^2 50% transforming dose (TD50)) resulted in B-cell LPD in the majority of humanized NOG mice (16). On autopsy, splenomegaly was observed in virtually all mice and tumors in the spleen, kidneys, and/or lymph nodes were seen in a fraction of them. In most cases, this LPD showed the histology of diffuse large B-cell lymphoma with the expression of the B-cell marker CD20, the B-cell activation marker CD23, and the germinal center marker Mum1. Expression of viral proteins such as EBNA1 and 2 and the latent membrane proteins (LMPs) 1 and 2 was detected, being consistent with the latency III type EBV gene expression, EBV-induced LPD in humanized NOG mice was thus remarkably similar to the most common type of PTLD and AIDS-associated lymphomas with respect to histology, marker expression, and EBV gene expression. Occasionally, Hodgkin-like cells with marked nucleoli and Reed-Sternberg-like cells with multiple nuclei were seen with Hodgkin lymphoma-like histology (16). Beside the NOG mouse-based model described above, models of EBV infection have been produced in other types of humanized mice (8-10) and similar EBV-induced LPD was observed in some of them (17-20).

These humanized mouse models of EBV-associated LPD has been utilized to elucidate the role of individual EBV genes in oncogenesis, EBNA3B is one of the nine EBV proteins expressed in the virus-transformed lymphoblastoid cells. EBV recombinants with EBNA3B knocked out by homologous recombination, however, did not have any distinct phenotypes in *in vitro* infection of B cells (21), and the role of EBNA3B in EBV pathogenesis was not clear. White and others demonstrated that EBNA3B knock-out virus, as compared with the wild-type virus, has enhanced ability to generate lymphoma in humanized mice, and suggested a tumor-suppressing role for EBNA3B (20). BZLF1 is an EBV immediate-early gene and its role in lymphoma genesis was not expected because knocking-out BZLF1 did not affect *in vitro* transformation of B cells by EBV (22). Ma and others characterized various EBV recombinants in humanized mice and clearly showed that BZLF1 increases the efficiency of lymphomagenesis *in vivo* by inducing abortive lytic infection (18,19).

In similar characterization of EBV recombinants in humanized mice, Wahl and others demonstrated that a cluster of microRNAs encoded by the EBV BHRF1 gene locus facilitates the development of acute systemic infection but does not substantially enhance lymphomagenesis by the virus (23).

**Asymptomatic persistent infection**

In contrast to infection at high virus doses, most humanized NOG mice inoculated with <10^3 TD50 EBV survived without any signs of illness (16). EBV DNA load in the peripheral blood increased only transiently and soon returned to undetectable level in most mice (16). However, EBV was found to have persisted in these mice for long after infection (more than 30 weeks), because a few EBV-positive B cells were consistently found on autopsy in the liver and spleen (16). This situation might be similar to EBV latency in humans, although further characterization is required. In EBV latency in humans, the virus establishes persistent infection in memory B cells, Cocco and others utilized humanized mice to analyze the process by which EBV establishes persistent infection in memory B cells (24).

**Erosive arthritis resembling rheumatoid arthritis in EBV-infected humanized NOG mice**

RA is a common autoimmune disease associated with progressive disability and systemic complications; arthritis lesions in RA are characterized by synovial proliferation and destruction of bone and cartilage tissues (25). Evidence has accumulated suggesting a role for EBV in the pathogenesis of RA (26,27). Compared with normal controls, patients with RA have higher titers of anti-EBV antibodies and higher levels of
EBV DNA load in their peripheral blood. They have also high numbers of EBV-specific T cells in their affected joints. Furthermore, expression of EBV-encoded small RNAs (EBERs) and LMP1 has been demonstrated in synovial cells of RA lesions (28). Histological examination of 23 EBV-infected humanized NOG mice demonstrated erosive arthritis resembling RA in 15 of them but none in 9 uninfected counterparts (29). Arthritis of these mice was characterized by massive proliferation of synovial cells and marked infiltration of CD3+ cells of both CD4+ and CD8+ subsets, CD20+ B cells, and CD68+ macrophages. Most importantly, the pannus, a structure of inflammatory granulation tissue very characteristic to RA, was clearly demonstrated in these mice (29). Bone marrow tissue adjacent to affected joint showed the histology of edema, another finding reminiscent of RA. These results showed that EBV can trigger erosive arthritis similar to RA in humanized mice. The results obtained so far are however restricted to morphological observations and investigation on the mechanism of this arthritis is required.

**EBV-specific immune responses**

Following infection with EBV, the number of CD3+ cells increased dramatically in the peripheral blood of humanized NOG mice (16). This was mostly due to increase in CD8+ T cells and ELISPOT assay detected a large number of IFN-γ-producing cells when CD8+ T cells isolated from EBV-infected mice were cultured briefly (17h) with autologous EBV-transformed lymphoblastoid cells, ELISPOT counts suggested that 2~4% of CD8+ cells in the peripheral blood of infected mice were EBV-specific. An antibody specific to human MHC class I added to the culture efficiently blocked the production of IFN-γ, but that specific to murine MHC did not (16). No IFN-γ-producing cells were detected when the same CD8+ T cell population was cultured with control EBV-transformed cells with mismatch MHC. These results indicated that EBV-specific CD8+ T-cell responses restricted by human MHC class I were induced in humanized NOG mice. Since positive selection of thymic T cells is thought to occur on the surface of epithelial cells and thymic epithelial cells in humanized mice are murine in origin, it is a puzzling question how T-cell responses restricted by human MHC were induced in them. The finding by Watanabe and others that differentiation of human T cells is markedly suppressed in NOG I-A−/− mice transplanted with human HSCs suggests that murine MHC molecules are involved in positive selection of human T cells in humanized NOG mice (30). Differentiation of human T cells was not, however, blocked completely in humanized NOG I-A−/− mice, implying a participation of certain human HSC-derived cells, possible B cells or dendritic cells, in the positive selection of T cells in humanized NOG mice (30).

When the possibility of using humanized mice for evaluation of vaccines and immunotherapies is considered, it is an important question whether anti-EBV immune responses mounted in humanized mice have protective effects. A clue to this question was first obtained when the fate of infected mice were compared among those infected at different stages of reconstitution of the immune system (31). T-cell development in humanized NOG mice occurred substantially later than that of B cells and became evident around six months following transplantation (14,15). Mice infected at three months after transplantation, when human cells in the peripheral blood were mostly B cells, had shorter lifespan compared with those infected at six months, when sufficient T-cell development was evident. When the OKT-3 antibody specific to the T-cell marker CD3 was given intravenously to humanized NOG mice that were infected at six months after transplantation of HSCs, the number of both CD4+ and CD8+ subsets of T cells were markedly reduced and EBV DNA load in the peripheral blood was significantly higher as compared with control mice. Lifespan following infection of these mice were significantly shorter than that of control mice (31). Importantly, OKT-3 antibody did not have any effect on the life span of un-infected mice. Administration of antibody specific to the CD8 molecule also shortened the life span of EBV-infected mice (31). These results indicated that T-cell responses to EBV, especially those by the CD8+ subset, have a protective role in humanized mice. In a regression assay, where EBV-infected B cells were cultured with autologous CD8+ T cells isolated from EBV-infected mice, transformation was inhibited in a dose-dependent manner as the number of effector CD8+ T cells increased. Similar protective roles for EBV-specific T-cell responses in humanized mice have been also reported by Strowig and others (17).

EBV-specific humoral immune responses in humanized NOG mice were less efficient than the cell-mediated counterpart. Only three out of forty EBV-infected mice produced IgM antibodies specific to p18\textsuperscript{capsid}, a major component of the viral capsid antigen of the virus (16). No IgG antibodies to EBV proteins were detected.
MOUSE XENOGRAFT MODELS FOR CHRONIC ACTIVE EBV INFECTION AND EBV-ASSOCIATED HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS

Although B cells and epithelial cells are its major targets, EBV can infect T and NK cells and induces their proliferation in a category of diseases with high morbidity and mortality, called EBV+ T/NK-cell lymphoproliferative diseases (EBV+ T/NK-LPDs) (32-37). Chronic active EBV infection (CAEBV) and EBV-associated hemophagocytic lymphohistiocytosis (EBV-HLH) are representatives of this category. Since proliferation of EBV-infected T or NK cells has not been observed in the virus-infected humanized NOG mice, patient proliferation of EBV-infected T or NK cells has not been observed when a cell fraction containing EBV-infected cells was isolated and transplanted to NOG mice. The only exception for this was the CD4+ T cells are re removed from PBMC; same results were obtained in transplantation from all four types of CAEBV.

Furthermore, when CD4+ T cells were depleted in vivo by administration of the OKT-4 antibody just following transplantation of PBMC, engraftment was completely blocked. These findings taken together indicate that CD4+ T cells play an essential role in in vivo proliferation of EBV-infected T and NK cells and suggest that novel therapies targeting CD4+ T cells or some factors produced by them might be possible. Indeed, OKT-4 was effective when administered after engraftment and reduced EBV DNA load in the peripheral blood and organs (unpublished results by Imadome and others).

Similar xenotransplantation experiments with PBMCs isolated from patients with EBV-HLH also resulted in systemic proliferation of EBV-infected T cells and splenomegaly. There were however unique finding in these EBV-HLH model mice, including hemorrhagic lesions in thoracic and/or abdominal cavities and extremely high human cytokine levels in the peripheral blood. Another unexpected finding in these mice was that although EBV-infected CD8+ cells were detected in the peripheral blood, only EBV-infected B cells were found in the spleen and liver. The reason for this discrepancy is not known, Sato and others reported that EBV-infected humanized NOG mice can develop hypercytokinemia and hemophagocytosis resembling HLH (39).

CONCLUDING REMARKS

The results described here indicate that humanized mice can reproduce cardinal features of EBV infection including pathogenesis and immune responses. Humanized mice are therefore promising tool for elucidation of EBV pathogenesis and evaluation of novel therapies for EBV-associated diseases. Although immune functions of current humanized mice are not satisfactory, various trials for their improvement are in progress. For example, introduction of a human MHC class I gene to humanized mice drastically improved their T-cell immune responses to EBV (40). These endeavors may finally enable evaluation of EBV vaccines in humanized mice.

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CONFLICTS OF INTEREST

The author has no financial conflict of interest.

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